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Yarrowia lipolytica lipase production enhanced by increased air pressure

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Abstract

Aims: To study the cellular growth and morphology of *Yarrowia lipolytica* W29 and its lipase and protease production under increased air pressures.
Methods and Results: Batch cultures of the yeast were conducted in a pressurized bioreactor at 4 and 8 bar of air pressure and the cellular behaviour was compared with cultures at atmospheric pressure. No inhibition of cellular growth was observed by the increase of pressure. Moreover, the improvement of the oxygen transfer rate (OTR) from the gas to the culture medium by pressurization enhanced the extracellular lipase activity from 96·6 U l^{-1} at 1 bar to 533·5 U l^{-1} at 8 bar. The extracellular protease activity was reduced by the air pressure increase, thereby eliciting further lipase productivity. Cell morphology was slightly affected by pressure, particularly at 8 bar, where cells kept the predominant oval form but decreased in size.
Conclusions: OTR improvement by total air pressure rise up to 8 bar in a bioreactor can be applied to the enhancement of lipase production by *Y. lipolytica*.
Significance and Impact of the Study: Hyperbaric bioreactors can be successfully applied for yeast cells cultivation, particularly in high-density cultures used for enzymes production, preventing oxygen limitation and consequently increasing overall productivity.

Introduction

Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes finding vast applications in food, dairy, detergent and pharmaceutical industries. They are generally produced from a lipidic carbon source, such as oils, fatty acids, glycerol or tweens in the presence of an organic nitrogen source (Gupta *et al.* 2004).

Yarrowia lipolytica was proposed as an alternative host organism for lipase production because it combines the facility of single cell use, high secretion ability and tools for post-translational modifications (Pignede *et al.* 1998).

The extracellular lipase production is affected by different environmental factors, namely, temperature, pH, medium composition, agitation, aeration and proteases. Many authors refer that the presence of air is essential for lipase production (Chen *et al.* 1999; Gupta *et al.* 2004; Alonso *et al.* 2005).

In high-density cultures, the 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. Special aeration systems and aeration using pure oxygen are most commonly applied (Chen *et al.* 1999; Zhao *et al.* 2001; Puthli *et al.* 2006). Previous work demonstrated that hyperbaric air could be successfully applied to yeast cultivation, as a way of improving the oxygen transfer rate (OTR) to aerobic cultures (Belo *et al.* 2003; Pinheiro *et al.* 2003; Aguedo *et al.* 2005). However, above certain limits the increased air pressure and the consequent increase in oxygen partial pressure may have detrimental effects on yeast cell activity

and product formation (Pinheiro *et al.* 2002; Belo *et al.* 2005).

In the present work, *Y. lipolytica* was grown in a pressurized bioreactor in order to obtain a further insight on the influence of moderate total air pressure both on yeast growth and on lipase production. Simultaneously, the effect of hyperbaric air on morphology of this dimorphic yeast was investigated, using an automatic image analysis technique.

Materials and methods

Strain and media

Yarrowia lipolytica W29 (ATCC 20460) was grown in glucose medium as described in Aguedo *et al.* (2005). The production medium was composed of 6·7 g l^{-1} yeast nitrogen base (Pronadisa, Madrid, Spain, 1545·1), 7 g l^{-1} olive oil, 5 g l^{-1} arabic gum and Tris–HCl 400 mmol l^{-1} buffer, pH 7·2.

Operating conditions

Yeast cells were pregrown in 500 ml Erlenmeyer flasks filled with 150 ml of the glucose medium. Batch cultivations were carried out using a 300-ml cylindrical stainless steel bioreactor previously described (Pinheiro *et al.* 2003). The reactor was operated with 150 ml of medium, an initial cell concentration of 10^{11} cells l^{-1}, at 180 rev min^{-1} of shaking rate, 0·4 l min^{-1} of aeration rate (2·7 vvm) and 27°C of temperature. The values of air pressure studied were 4 and 8 bar. An experiment in an Erlenmeyer flask (500 ml) with 150 ml of medium, under atmospheric pressure (1 bar) and at the same conditions of the reactor was used as a control.

Estimation of OTR

OTR in the hyperbaric reactor and in the Erlenmeyer flask was estimated in blank assays using the sulfite oxidation method (Maier *et al.* 2001). A sodium sulfite solution of 0·2 mol l^{-1} was used in the presence of a catalyst ($CuCl_2$ 0·001 mol l^{-1}). In fact, this method measures the rate of O_2 absorption by a Na_2SO_3 solution and enables to predict the effect of pressure increase on the oxygen mass transfer capacity of the system.

Analytical methods

At appropriate intervals, culture samples were collected for analysis of cell concentration (cell number and dry weight), lipase and protease activities, total soluble protein and organic acids concentration. Total soluble protein was obtained by Bradford's method. Organic acids were quantified by HPLC with a Metacarb 67H column (Varian, Palo Alto, CA) and an UV detector. The eluent was H_2SO_4 0·05 mol l^{-1} at 0·5 ml min^{-1} and the column temperature was 65°C.

Protease in cell-free samples was quantified using 0·5% (w/v) azocasein in acetate buffer as substrate at pH 5·0, 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 relatively to the blank per minute under assay conditions.

Extracellular and total lipase activity was measured in the samples supernatant and in the sample with cells, respectively, using *p*-nitrophenyl-butirate (pNPB) as substrate. Cell-bounded lipase activity was calculated by the difference between total and extracellular lipase activities. The enzymatic reaction was followed by the absorbance measurement at 400 nm during 3 min. Activities were calculated by linear regression of absorbance *vs* time, using the molar extinction coefficient of pNP of 8 mmol l^{-1}. One unit of activity was defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per minute under assay conditions. Lipase productivity (U h^{-1} l^{-1}) was determined by the ratio between the maximum lipase activity obtained in each experiment and respective time.

Image analysis procedure

The images were acquired in a Zeiss Axioscop microscope (Zeiss, Hamburg, Germany) coupled with a AxioCam (Zeiss) colour video camera with 1300 × 1030 pixels in 24 bits (8 bits per channel) linked to a personal computer by a frame grabber (DT3155; Data Translation, Inc., Marlboro, USA). Cells were observed immediately after sampling (24 h of growth) and at least 30 images per sample were obtained for the evaluation of cell size and morphology. The procedure followed was previously described (Coelho *et al.* 2004).

Results

Effect of air pressure on OTR

OTR enhancement, for a specific bioreactor and culture medium, can be achieved by the increase of airflow rate, stirring rate and oxygen solubility in the medium. The OTR could be improved by air pressure rise, which, according with Henry's law, increases the oxygen solubility in the medium. The OTR increased to 1248 mg O_2 l^{-1} h^{-1} at 4 bar and to 2924 mg O_2 l^{-1} h^{-1} at 8 bar. In the flask (atmospheric pressure) the OTR value was 12 mg O_2 l^{-1} h^{-1}.

The variation of OTR with pressure (from 2 to 8 bar) found for the bioreactor used fits well with following power function:

$$\mathrm{OTR} = 254{\cdot}89P^{1{\cdot}17} \quad (1)$$

This equation is in agreement with the results of Sato *et al.* (1981) and Maier *et al.* (2001).

Effect of air pressure on cell growth

Despite the OTR increase of 2·3-fold when the pressure varied from 4 to 8 bar, no improvement in the cellular growth was observed. In fact, for all the pressure conditions used, a same value of specific cellular growth rate of 0·1 h^{-1} was obtained and the cellular density increased 1 – log after 24 h. Probably, the highest value of OTR at 8 bar exceeded the demand for the cellular density present in the culture. Nevertheless no inhibitory effects were observed in the cellular activity under high air pressures of 4 and 8 bar as compared to the control.

Effect of air pressure on lipase activity

Figure 1 shows the extracellular lipase activity profiles during batch experiments. The kinetic profiles demonstrate that the variation of the enzymatic activity along time is similar in all experiments. In all cases, the activity increases up to a maximum, after what it decays. The maximum lipase activity was attained between 20 and 30 h of production. These profiles agree with the ones obtained by other authors (Corzo and Revah 1999; Domínguez *et al.* 2003; Alonso *et al.* 2005). In the assay under 8 bar 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.

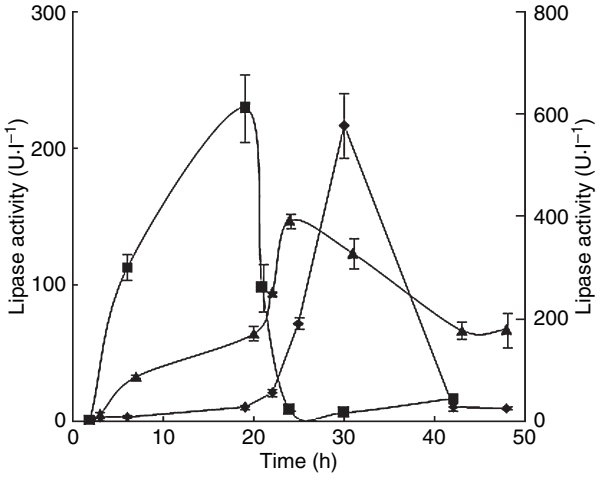

Figure 1 Extracellular lipase activity profiles by *Yarrowia lipolytica* during batch experiments made with: (▲) atmospheric pressure and (■) 4 bar and (♦; secondary axis) 8 bar air pressure. Data are the mean and SD values of two independent experiments.

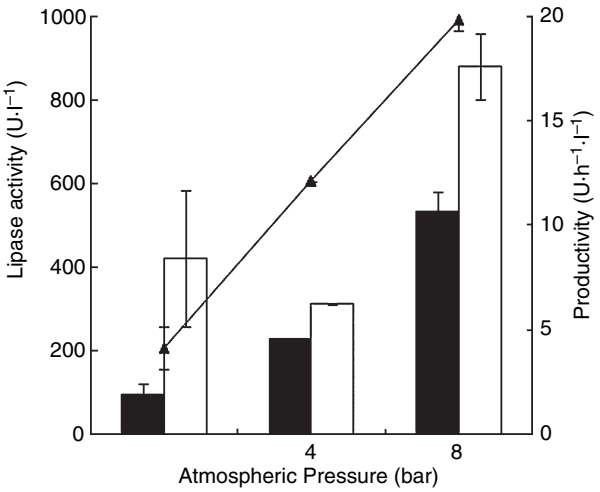

Figure 2 Effect of air pressure on: the extracellular (black bar) and total (white bar) lipase activity and the lipase productivity (▲). Lipase productivity was determined by the ratio between the maximum lipase activity obtained in each experiment and the time that value was obtained. Data are the mean and SD values of two independent experiments.

Figure 2 puts together the maximum values of lipase activity and productivity obtained in experiments under atmospheric pressure, 4 and 8 bar of air. The extracellular and total lipase activities increased with the rise of total air pressure. However, no significant effect of pressure on the proportion of cell-bound lipase fraction was observed. An increase of the lipase activity at 8 bar of 5·5- and 2·3-fold was obtained compared with the experiments under atmospheric pressure and 4 bar, respectively. Those differences on extracellular activities obtained under 1 and 8 bar are statistically significant ($P = 0{\cdot}007$).

The maximum lipase productivity also increased with the increase of the total air pressure, as can be observed in Fig. 2. The rise of the total air pressure from 1 to 8 bar led to a 4·8-fold improvement in the lipase productivity ($P = 0{\cdot}0004$).

As can be seen in Table 1 the specific lipase activities were enhanced by the increase of air pressure. A 10-fold improvement of lipase activity per unit dry weight was found when air pressure varies from 1 to 8 bar. Also, the lipase activity per soluble total protein increased 29% when the air pressure increased up to 8 bar.

Table 1 Effect of air pressure on specific activities of extracellular lipase from *Yarrowia lipolytica*

Air pressure (bar)	1·0	4·0	8·0
Activity per dry cell weight (U g^{-1})	8·8 ± 1·7	35·8 ± 4·3	86·4 ± 10·3
Activity per total protein (U mg^{-1})	5·8 ± 2·4	9·0 ± 0·8	20·1 ± 0·5

Data are mean ± SD of two independent experiments.

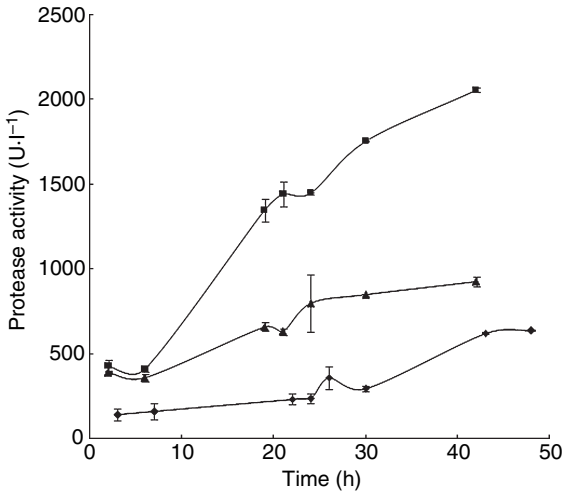

Figure 3 Protease activity profiles by *Yarrowia lipolytica* in the flask (▲) and in the pressurized reactor at different pressures: (■) 4 bar and (♦) 8 bar.

Figure 3 shows the results of monitoring protease secretion along time, using azocasein as substrate. During the first hours of culture the protease activity was low, increasing gradually until the end of the cultivation time, suggesting that the decrease of the medium pH (data not shown) favours the production of an acid protease by *Y. lipolytica*.

The highest decrease in pH was observed in the 8 bar experiment. This pH value decrease was due to the increased secretion of acetic acid. Around 1 g l^{-1} acetic acid concentration was accumulated in the 8 bar culture whereas only 0·03 g l^{-1} was detected under atmospheric pressure.

In all the experiments, the biggest increase in protease activity took place after the peak of lipase activity was reached. Therefore, it is possible to suggest that the inhibition of lipolytic activity with time is likely to be due to the degradation of the lipase by the secreted proteases. The highest value of protease production was found for the 4 bar assay, whereas in the experiments carried out under atmospheric pressure and 8 bar its concentration in the medium was lower.

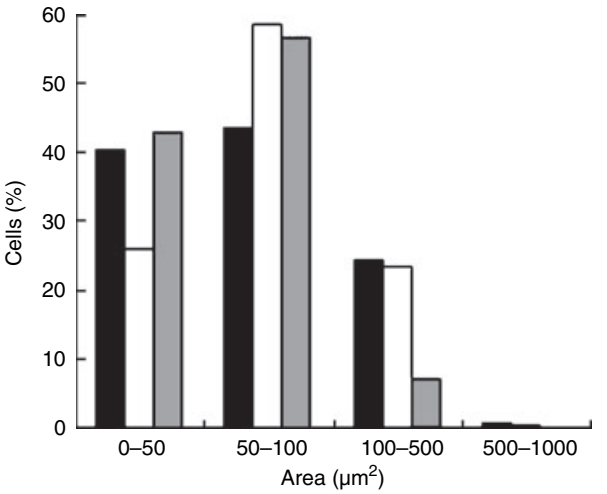

Figure 4 Cell-size distribution for *Yarrowia lipolytica* cells grown at: atmospheric pressure (black bar), 4 bar (white bar) and 8 bar (grey bar).

Effect of air pressure on cell morphology

Yeast cells were observed by optical microscopy and no differences were found between the morphology of *Y. lipolytica* cells under 4, 8 bar and at atmospheric pressure. Cells displayed a typical oval form in all the assays, which was confirmed by the application of an automatic image-processing methodology for yeast morphology analysis as described elsewhere (Coelho *et al.* 2004), using an elongation factor as a discriminatory parameter. The results demonstrated that cell exposure to increased air pressure did not induce hyphae formation. Cells remained oval under pressures up to 8 bar with elongation factors below 2·0 for the majority of cells (>85% of cells).

The cell-size distribution of yeast cells exposed to hyperbaric pressures of air and atmosphere pressure is depicted in Fig. 4. A cell size decrease was found for the 8 bar culture, since a decrease of the percentage of cells with a projected area higher than 100 μm^2 was obtained at 8 bar (7%) compared with the 25% of cells with this size obtained at 4 bar and at atmospheric pressure.

Discussion

The increase of OTR by total air pressure rise have been reported and applied in microbial cultures by some authors (Zhao *et al.* 2001; Pinheiro *et al.* 2003; Knoll *et al.* 2005). For the bioreactor used in the present work and according to eqn (1), pressure increase caused an increase in k_La besides the increase on the driving force for mass transfer. These results are in accordance with Sato *et al.* (1981).

In aerobic yeast cultures, as is the case of *Y. lipolytica*, the total air pressure is an important factor, as it alters the dissolved gas partial pressure, in particular the dissolved oxygen concentration, which can be a limiting factor for cell growth when low, but, on the other hand, might cause oxidative stress to cells when in excess.

The results found in the cultures of *Y. lipolytica* W29 are in accordance with the previous work of Belo *et al.* (2005), in which no cellular activity inhibition was detected in semi-continuous cultures of *Saccharomyces cerevisiae* for air pressures up to 10 bar (i.e. 2·1 bar of oxygen partial pressure). Also, Coelho *et al.* (2004) observed for batch cultures that an increase of pressure up to 6 bar had no metabolic impact in the cells whenever nitrogen or air was applied. Aguedo *et al.* (2005) reported that the application of 5 bar air pressure stimulated the cell growth of *Y. lipolytica* W29 comparatively to atmospheric growth conditions. Other nonconventional yeasts were cultivated under increased air pressures with no inhibitory effects observed (Pinheiro *et al.* 2003).

The effect of pressure on the yeast growth depends on the gas composition and on the pressurization mode, as well on the micro-organism and the strain. For the pressures used in this study it may be concluded that the total air pressure does not inhibit cell growth.

Moreover, air pressure rise did not inflict oxidative stress to the cells, as the morphological analysis has proven since cells kept the predominant oval form. Kawasse *et al.* (2003) reported a 25% increase in the elongation factor when the cells were exposed to a thermal and oxidative stress. The slight cell-size reduction due to air pressure increase was also reported for *S. cerevisiae* cells (Coelho *et al.* 2004; Belo *et al.* 2005) and was explained by cell membrane changes caused by pressure (e.g. permeability changes) leading to mass transfer from the interior of the cell to the exterior with the concomitant cell compression (Perrier-Cornet *et al.* 1995).

The increase of total air pressure influences enzymatic activity, as demonstrated by Pinheiro *et al.* (2003). The authors observed that an increase of OTR by raising air pressure up to 6 bar resulted in an increase of the specific productivity of β-galactosidase by *Kluyveromyces marxianus*. Also Charoenrat *et al.* (2006) reported that a slight increase of air pressure to 1·9 bar improved recombinant β-glucosidase production by *Pichia pastoris* in a fed-batch process, while biomass yield was low.

Previous work with the strain used in the present study also showed that oxygen and total pressure have an important role in the regulation of intracellular enzymes such as the ones involved in the biotransformation of ricinoleic acid into aroma (Aguedo *et al.* 2005).

Lipase productivity was enhanced by OTR improvement at increased pressure, contrarily to what happened with cellular growth, which is indirect evidence that oxygen demand is higher for lipase production than for cellular growth. These results corroborate those of Chen *et al.* (1999), who demonstrated that the increase of OTR, either by aeration or agitation, caused an increase of the lipase yield.

Besides lipase production, the production of other enzymes, such as proteases, by *Y. lipolytica* strains has been reported (Puthli *et al.* 2006). This can influence the production kinetics of lipases since the prolonged time of fermentation can lead to the loss of product due to its decomposition, from the moment when the secretion of proteases to the medium is started (Rozkov and Enfors 2004).

Results show that 8 bar of air pressure retards the production of both types of enzymes, lipase and protease, with a strong inhibiting effect on protease production, what could explain the increase in lipase productivity obtained for this pressure. Thus, the air pressure rise had different effects on proteases and lipases secretion by the yeast, 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.

The accumulation of acetic acid obtained under hyperbaric conditions also anticipated the influence of air pressure on *Y. lipolytica* metabolism, which will be investigated in future work.

References

Aguedo, M., Gomes, N., Garcia, E.E., Waché, Y., Mota, M., Teixeira, J.A. and Belo, I. (2005) Decalactone production by *Yarrowia lipolytica* under increased O_2 transfer rates. *Biotechnol Lett* **27**, 1617–1621.

Alonso, F.O.M., Oliveira, E.B.L., Dellamora-Ortiz, G.M. and Pereira-Meirelles, F.V. (2005) Improvement of lipase production at different stirring speeds and oxygen levels. *Braz J Chem Eng* **22**, 9–18.

Belo, I., Pinheiro, R. and Mota, M. (2003) Fed-batch cultivation of *Saccharomyces cerevisiae* in a hyperbaric bioreactor. *Biotechnol Prog* **19**, 665–671.

Belo, I., Pinheiro, R. and Mota, M. (2005) Morphological and physiological changes in *Saccharomyces cerevisiae* by oxidative stress from hyperbaric air. *J Biotechnol* **115**, 397–404.

Charoenrat, T., Ketudat-Cairns, M., Jahic, M., Veide, A. and Enfors, S-O. (2006) Increased total air pressure versus oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. *Biochem Eng J* **30**, 205–211.

Chen, J-Y., Wen, C-M. and Chen, T-L. (1999) Effect of oxygen transfer on lipase production by *Acinetobacter radioresistens*. *Biotechnol Bioeng* **62**, 311–316.

Coelho, M.A.Z., Belo, I., Pinheiro, R., Amaral, A.L., Mota, M., Coutinho, J.A.P. and Ferreira, E.C. (2004) Effect of hyperbaric stress on yeast morphology: study by automated image analysis. *Appl Microbiol Biotechnol* **66**, 318–324.

Corzo, G. and Revah, S. (1999) Production and characteristics of the lipase from *Yarrowia lipolytica* 681. *Bioresour Technol* **70**, 173–180.

Domínguez, A., Deive, F.J., Sanromán, M.A. and Longo, M.A. (2003) Effect of lipids and surfactants on extracellular lipase production by *Yarrowia lipolytica*. *J Chem Technol Biotechnol* **78**, 1166–1170.

Gupta, R., Gupta, N. and Rathi, P. (2004) Bacterial lipases: an overview of production, purification and biochemical properties. *Appl Microbiol Biotechnol* **64**, 763–781.

Kawasse, F.M, Amaral, P.F., Rocha-Leão, M.H.M., Amaral, A.L., Ferreira, E.C. and Coelho, M.A.Z. (2003) Morphological analysis of *Yarrowia lipolytica* under stress conditions through image processing. *Bioprocess Biosyst Eng* **25**, 371–375.

Knoll, A., Maier, B., Tscherrig, H. and Büchs, J. (2005) The oxygen mass transfer, carbon dioxide inhibition, heat removal, and the energy and cost efficiencies of high pressure fermentation. *Adv Biochem Eng Biotechnol* **92**, 77–99.

Maier, B., Dietrich, C. and Büchs, J. (2001) Correct application of the sulphite oxidation methodology of measuring the volumetric mass transfer coefficient K_La under non-pressurized and pressurized conditions. *Trans IChemE* **79**, 107–113.

Perrier-Cornet, J.M., Marechal, P.A. and Gervais, P. (1995) A new design intended to relate high pressure treatment to yeast cell mass transfer. *J Biotechnol* **41**, 49–58.

Pignede, G., Fudalej, F., Nicaud, J.M., Gaillardin, C. and Seman, M.(1998) *French patent* **98**, 899.

Pinheiro, R., Belo, I. and Mota, M. (2002) Oxidative stress response of *Kluyveromyces marxianus* to hydrogen peroxide, paraquat and pressure. *Appl Microbiol Biotechnol* **58**, 842–847.

Pinheiro, R., Belo, I. and Mota, M. (2003) Growth and β-galactosidase activity in cultures of *Kluyveromyces marxianus* under increased air pressure. *Lett Appl Microbiol* **37**, 438–442.

Puthli, M.S., Rathod, V.K. and Pandit, A.B. (2006) Optimization of lipase production in a triple impeller bioreactor. *Biochem Eng J* **27**, 287–294.

Rozkov, A. and Enfors, S.O. (2004) Analysis and control of proteolysis of recombinant proteins in *Escherichia coli*. *Adv Biochem Eng Biotechnol* **89**, 183–195.

Sato, S., Mukataka, S., Kataoka, H. and Takahashi, J. (1981) Oxygen absorption rate in an aerated stirred tank under increasing pressure. *J Ferment Technol* **59**, 221–225.

Zhao, H., Zhang, X., Zhou, X. and Li, Z. (2001) Effects of air pressure oscillation amplitude on oxygen transfer rate and biomass productivity in a solid-state fermenter. *Biotechnol Lett* **23**, 1197–1200.