J Ind Microbiol Biotechnol (2008) 35:1053–1059
DOI 10.1007/s10295-008-0382-1

ORIGINAL PAPER

Investigation of the effect of different extracellular factors on the lipase production by *Yarrowia lipolityca* on the basis of a scale-down approach

T. Kar · F. Delvigne · M. Masson · J. Destain · P. Thonart

Received: 20 March 2008 / Accepted: 28 May 2008 / Published online: 11 June 2008
© Society for Industrial Microbiology 2008

Abstract The influence of three extracellular factors (namely, the methyl oleate dispersion in the broth, the dissolved oxygen variations, and the pH fluctuation) on the lipase production by *Y. lipolytica* in batch bioreactor has been investigated in different scale-down apparatus. These systems allow to reproduce the hydrodynamic phenomena encountered in large-scale equipments for the three specified factors. The effects of the extracellular factors have been observed at three distinct levels: the microbial growth, the extracellular lipase production, and the induction of the gene LIP2 encoding for the main lipase of *Y. lipolytica*. Among the set of environmental factors investigated, the dissolved oxygen fluctuations generated in a controlled scale-down reactor (C-SDR) have led to the more pronounced physiological effect by decreasing the LIP2 gene expression level. The other environmental factors observed in a partitioned scale-down reactor, i.e., the methyl oleate dispersion and the pH fluctuations, have led to a less severe stress traduced only by a decrease of the microbial yield and thus of the extracellular lipase specific production rate.

Keywords Methyl oleate · LIP2 expression · Oxygen transfer · Heterogeneities · Scale-up

Kar T. and Delvigne F. have contributed equally to this work.

T. Kar · F. Delvigne (✉) · M. Masson · J. Destain · P. Thonart
Centre Wallon de Biologie Industrielle, Unité de Bio-industries, Faculté Universitaire des Sciences Agronomiques de Gembloux, Passage des déportés, 2, 5030 Gembloux, Belgium
e-mail: delvigne.f@fsagx.ac.be

Introduction

Yarrowia lipolytica is a non-conventional yeast that has been widely studied for its lipase production capacity. The lipase production is affected by several extracellular factors such as the carbon source level, the pH, the dissolved oxygen level, the nitrogen source, etc., [1] At this time, some mechanisms at the level of the nutrient assimilation by *Y. lipolytica* cells remain incompletely understood [2]. However, there is also a lack at the level of understanding of the scale-up effect on microbial production of lipase. Indeed, the up-scaling procedure leads to an irremediable increase of the mixing time of the bioreactor. The direct consequence of this phenomenon is the appearance of gradient and related mixing imperfections at large-scale [3]. In this work, three extracellular parameters have been studied in a scaling-up perspective: the carbon source accessibility (methyl oleate has been used as a carbon source and must be efficiently dispersed in the aqueous culture media), the dissolved oxygen level, and the pH fluctuations. These extracellular factors have been observed in a small-scale bioreactor (20 L) and in two distinct scale-down apparatus designed to reproduce the hydrodynamic phenomena occurring in large-scale bioreactors. The methyl oleate dispersion and the pH gradients have been studied in a partitioned bioreactor comprising a classical 20-L bioreactor connected to a tubular part in which the mixing intensity is significantly altered compared to the stirred part. The oxygen level fluctuation has been studied in a controlled 20-L bioreactor in which an automatic air injection valve is closed or open according to a specific algorithm. Three physiological parameters have been followed in front of these extracellular conditions: the microbial growth, the extracellular lipase level, and the LIP2 gene expression level (coding for the main lipase

produced by *Y. lipolytica* and monitored in this work by a LIP2–lacZ reporter gene [4]).

Materials and methods

Strain and culture conditions

Yarrowia lipolytica JMY775 is stored at −80 °C in working seeds vials (cells in suspension in glycerol solution 40%). The JMY775 strain has been obtained by genetic manipulation from a lipase overproducing strain (LgX64.81 [5]) modified with a LIP2-LacZ reporter gene [6]. Cells are fist precultivated in a 250-mL baffled shake flask containing 100 mL of YPG medium at 30 °C for 24 h. A second precultivation step is then carried out during 24 h in 2-L baffled shaken flasks containing 750 mL of medium containing glucose (15 g/L), whey powder (30 g/L) (BHA, Belgium), corn steep (10 g/L) (Roquette, France), and $(NH_4)_2SO_4$ (8 g/L) (VWR, Belgium). After the precultivation steps, the culture is transferred to a 20-L bioreactor (Biolaffite-France; internal diameter 0.22 m) with a working volume of 12 L and equipped with two RDT6 rushton turbines ($d = 0.1$ m). The cultivation media for the bioreactor has been optimized previously for the lipase production by *Y. lipolytica* [5, 7] and contains per liter: methyl oleate 25 mL (Cognis, France), whey powder 30 g, corn steep 10 g, and $(NH_4)_2SO_4$ 8 g. The regulation of the culture parameters (pH, temperature, etc.,) is ensured by a direct control system (ABB). The culture in the bioreactor is carried out at 30 °C with a fixed stirring speed of 350 rpm and an air flow rate of 1 vvm (volume of air per volume of medium per minute. In our case 1 vvm corresponds to 12 L/min). The dissolved oxygen is continuously monitored with an oxygen probe (Mettler Toledo InPro 6800 series). The foam level in the reactor is monitored by an antifoam probe placed at 10 cm from top of the vessel. The foam level is then controlled by the addition of antifoam Tego KS911 (Goldschmidt, Germany). The pH of the broth is measured with a pH probe (Mettler Toledo InPro 2000/120/Pt100/9848) and is regulated at a value of 7 ± 0.1 by the addition of KOH 6 N or H_3PO_4 6 N.

Analytical methods

The microbial growth is monitored by direct counting method on a hemacytometer (Bürker type; 10 repetitions are performed for each estimation of the number of microbial cells per mL). The extracellular lipase activity is determined as follows: samples of the culture medium are withdrawn at various times of fermentation, centrifuged for 20 min at 10,000 *g*. The supernatant is then used for the extracellular lipase activity estimation using an olive oil emulsion as the enzyme substrate [olive oil 25%, 0.1 M NaOH 7.5%, polyvinylic alcohol (2%) 67.5%]. The enzymatic reaction is initiated by adding 1 mL of supernatant to 4 mL of emulsion with 5 mL of 0.1 M of phosphate buffer at pH 7. The enzymatic reaction is maintained for 15 min at 37 °C on a rotary shaker (150 rpm) and is subsequently stopped by the addition 20 mL of acetone–ethanol mix [1:1(v/v)]. The free fatty acids released during the reaction are then titrated with 0.05 M NaOH [8]. One unit of lipase activity is defined as the amount of lipase inducing the release of 1 mmol of fatty acid per minute at 37 °C and pH 7. The expression of the LIP2 gene is performed on the basis of the parallel synthesis β-galactosidase by the LIP2–LacZ reporter gene JMY775 strain. The β-galactosidase activity, expressed in Miller unit, is defined as the amount of enzyme releasing 1 µmol of *o*-nitrophenol (subsequent to the hydrolysis of the ortho-nitrophenyl-β-galactoside ONPG) per minute and per unit of optical density (600 nm) at 37 °C. The β-galactosidase is extracted from the microbial cells by a chloroform permeabilization procedure as described previously [4].

Scale-down reactor strategies

Three specific environmental factors having a potential impact at the level of lipase production in large-scale bioreactors have been investigated; the dispersion efficiency of the hydrophobic substrate used to cultivate *Y. lipolytica* (methyl oleate), the dissolved oxygen fluctuations and the pH gradients. In order to limit the scale of the experimental apparatus, several scale-down reactors have been designed to reproduce the environmental behavior of each factor in laboratory conditions. The different reactor configurations are briefly presented in Fig. 1. The P-SDR comprises a classical 20-L bioreactor connected to a glass bulb (length 0.25 m, internal diameter 85 mm). The connections between the two parts of the P-SDR are made of norprene tubing (masterflex). Norprene has been chosen for its low oxygen permeability and for its high mechanical resistance. The P-SDR has been used by numerous authors in order to study the influence of the scale-up procedure on the microbial physiology [3, 9–11]. In our case, the P-SDR has been used to observe the impact of the methyl oleate dispersion efficiency and pH gradient formation at different physiological levels. Indeed, the non-mixed part connected to the classical stirred reactor exhibits lower hydrodynamic efficiency, leading to an alteration of the methyl oleate dispersion (or in other words, an alteration of the substrate accessibility) and to the formation of concentration gradients. The intensity of the concentration gradient and/or the residence time of the cells inside the non-mixed part of the P-SDR can be

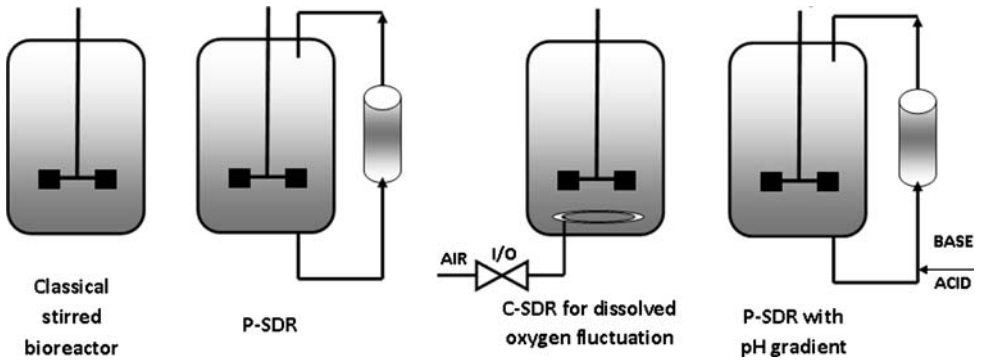

Fig. 1 Scheme of the different reactor configurations used in this work. From *left* to *right*; classical stirred bioreactor in which the reference cultures are performed, partitioned scale-down reactor (P-SDR) designed to observe the impact of the drop of the methyl oleate dispersion efficiency at the level of the non-mixed part, controlled scale-down reactor (C-SDR) with an electrical air admission valve used to reproduce the dissolved oxygen fluctuations encountered in large-scale bioreactors and P-SDR with the pH regulation performed at the level of the non-mixed part in order to promote pH gradients formation

modified by varying the recirculation flow rate of the pump. Three recirculation flow rates have been investigated, i.e., 480, 360, and 240 mL/min, leading, respectively, to a mean residence time inside the non-mixed part of 2.5, 3.3, and 5 min. In a second time, the effect of the dissolved oxygen depletion occurring in the non-mixed part of the P-SDR has been isolated using a fundamentally different scale-down strategy relying on the control of the air admission valve. This kind of C-SDR has been previously developed to specifically observe the impact of the dissolved oxygen fluctuations on the microbial physiology [12]. In our case, the on/off regulation scheme for the air admission valve has been computed firstly from the mean residence time inside the different parts of the P-SDR, and secondly from a stochastic model reproducing the circulation of the microbial cells inside the P-SDR. This model is used in the context of this work to simulate the displacement of several microbial cells from the mixed to the non-mixed part of a P-SDR. The two parts of the reactor are modeled as distinct compartments or states in the context of the model. The microbial cells are allowed to switch to a state to another according to the probability:

$$P = \frac{Q_{\text{recirc}}}{V}\,\Delta t.$$

With Q_{recirc} being the recirculation flow rate (mL/s), V being the volume of the considered state (part) of the P-SDR (mL) and Δt being the simulation time interval (s). In our case, we have performed discrete simulation with a constant time interval of 1 s. On the basis of the computed probabilities, a Monte Carlo simulation of the displacement of the microbial cells from a state to another has been performed. The simulated displacement profile allows the computation of the respective residence time of each microbial cell in different parts of the reactor. These residence time distributions are then used to control the air admission valve at the level of the C-SDR by considering that the 'on' state corresponds to the passage of the cells at the level of the mixed part of the reactor and the 'off" state corresponds to the passage of the cell at the level of the non-mixed part.

Results and discussion

Effect of the methyloleate and dissolved oxygen availability: involvement of a partitioned scale-down reactor (P-SDR)

The first set of experiments has been performed by cultivating *Y. lipolytica* in a 20 L bioreactor and in a partitioned scale-down reactor. The P-SDR has been designed to reproduce the passage of the microorganisms from a well-mixed zone to an imperfectly mixed zone, a phenomena occurring in large-scale bioreactors. The imperfectly mixed zone has been designed by positioning a tubular part at the outlet of the 20-L stirred bioreactor. Figure 2a shows a significant decrease of the cell growth correlated directly to a drop of extracellular lipase activity. However, the LIP2 gene expression monitored by measuring the β-galactosidase activity is not altered by the scale-down strategy (Fig. 2c), except in the case of the lower recirculation flow rate for which the mixing efficiency of the SDR is very low. This means that the intracellular lipase synthesis process is not affected by the modulation of the mixing efficiency of the P-SDR, with an exception to be mentioned for the lowest recirculation flow rate (corresponding to the higher mean residence time in the non-mixed part of the P-SDR). From these result, it can be concluded that modulating the recirculation flow rate of the P-SDR, i.e., modifying the residence

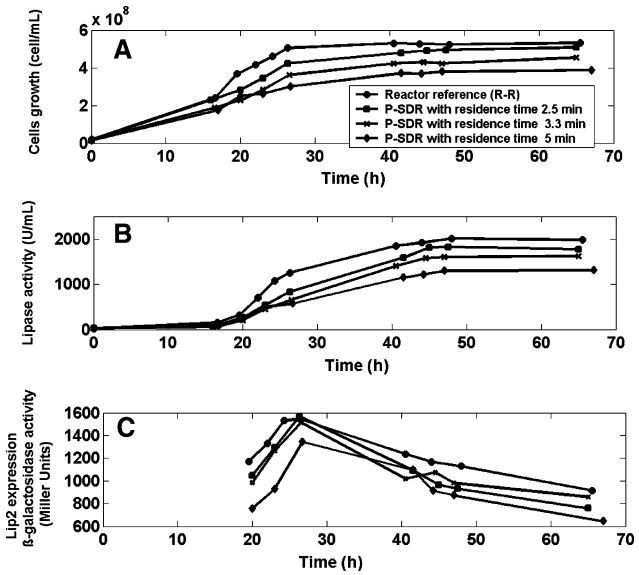

Fig. 2 Evolution of the amount of microbial cells (**a**), the extracellular lipase activity (**b**), and the LIP2 expression (**c**) in function of the reactor configuration

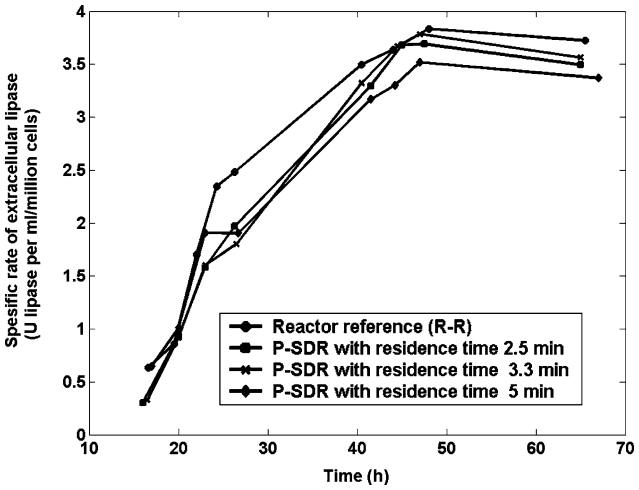

Fig. 3 Specific rate for the extracellular lipase production by *Y. lipolytica* cultivated in a classical 20-L bioreactor (reference reactor) and in a P-SDR under different recirculation flow rates (corresponding each to a mean residence time in the non-mixed part of the reactor)

time of the microorganisms in a badly mixed environment, has a direct impact on cell growth and on the total lipase content of the broth (the specific lipase production is not affected as shown in Fig. 3). In the case of the P-SDR with the lowest mixing efficiency, i.e., with the microorganisms being exposed more frequently to bad mixing conditions, the intracellular lipase synthesis is affected, as shown by the reporter gene analysis. Two environmental factors are potentially involved in the scale-down effect at the level of the P-SDR. The first one is the drop at the level of the shear forces in the non-mixed part of the P-SDR, by comparison with the mixed one where the rushton disk turbines ensure an efficient dispersion of the methyl oleate. This drop at the level of the methyl oleate dispersion efficiency limits the substrate accessibility and is potentially detrimental for the lipase production. The second environmental factor is the diminution of the oxygen transfer efficiency at the level of the non-mixed part of the P-SDR. Indeed, the dissolved oxygen consitutes another important substrate for the optimal cell growth and for the lipase production. However, at this stage, the respective effects of the methyl oleate dispersion and the dissolved oxygen availability cannot be distinguished. In the next section, a second scale-down reactor has been designed in order to isolate the effect of the dissolved oxygen fluctuations on the lipase production by *Y. lipolytica*.

Effect of the dissolved oxygen level fluctuations: involvement of a C-SDR

In order to isolate the respective effect of the dissolved oxygen fluctuation, a controlled SDR has been designed (Fig. 1). In the C-SDR, the air flow rate is sequentially closed and opened by an electric valve controlled by a specific algorithm in order to reproduce the oxygen transfer fluctuations occurring in the corresponding P-SDR when the cells are crossing the non-aerated non-mixed part of this reactor. Two kinds of control algorithm have been tested. The first control algorithm imposes regular on/off cycles to the air admission valve of the C-SDR. The time lengths of the on and off cycles are based on the respective residence time inside the mixed (where the broth is well aerated) and non-mixed (where the broth is badly aerated) parts of the reactor, the goal being to isolate the effect of the dissolved oxygen fluctuation by comparing with the results obtained in the previous section. However, previous hydrodynamic results have shown that the passage of the cells from mixed to non-mixed part of the P-SDR is strongly stochastic [13]. In order to take this important feature into account, a second air valve control algorithm has been elaborated on the basis of a two-compartment stochastic hydrodynamic model developed in our laboratory [14]. Samples of the two controlling schemes are shown at Fig. 4. Figure 4 shows that air admission valve on/off cycles based on the direct consideration of the mean residence time inside the different parts of the P-SDR (Fig. 4b) tends to overestimate the stress by comparing with the on/off scheme computed from the stochastic model (Fig. 4a). This fact is confirmed by the microbial growth curves shown in Fig. 5a, where the C-SDR with the cycling on/off scheme gives the lower biomass yield. On the other hand, the two C-SDRs show a significant drop at the level of the extracellular lipase production, this drop

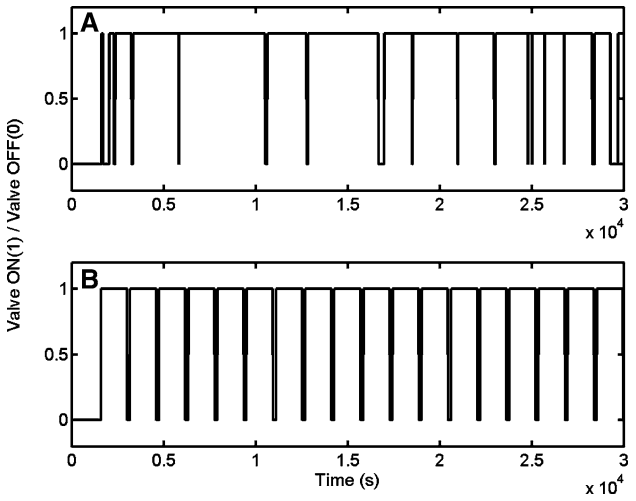

Fig. 4 On/off cycle scheme for C-SDR (**a** stochastic scheme and **b** constant cycles scheme)

being more pronounced in the case of the C-SDR with regular on/off scheme (Fig. 4b), where the stress induced by the dissolved oxygen fluctuations is higher. On the opposite of the results shown in the previous section for the effect of methyl oleate dispersion, the extracellular lipase yield drop cannot be exclusively attributed to the lower amount of biomass, as shown by the specific levels of lipase production (Fig. 6). Indeed, in the case of the dissolved oxygen fluctuations generated at the level of the C-SDRs, the stress has an impact at the level of the LIP2 expression (Fig. 5c).

Effect of the pH local variations: involvement of a partitioned scale-down reactor (part-SDR)

A third environmental factor potentially significant for the lipase production by *Y. lipolytica* in large-scale bioreactors is the pH gradient induced by the control systems. Indeed, the pH is typically regulated in bioreactor by adding base or acid at the top of the reactor and recording the pH value with a probe located at the lower part of the reactor. The response time of the probe and the local action of the controller, as well as their physical separation at the level of the reacting volume, induces the development of pH gradients. In order to observe the effect of such gradient at small-scale, the previous P-SDR has been used. The pH gradient is generated by injecting the base or acid solution at the inlet of the non-mixed part of the P-SDR. The experiments have been conducted by comparing a culture performed in a P-SDR with a classical pH regulation performed at the level of the mixed part (in this case the pH gradient is rapidly attenuated by the mixing efficiency

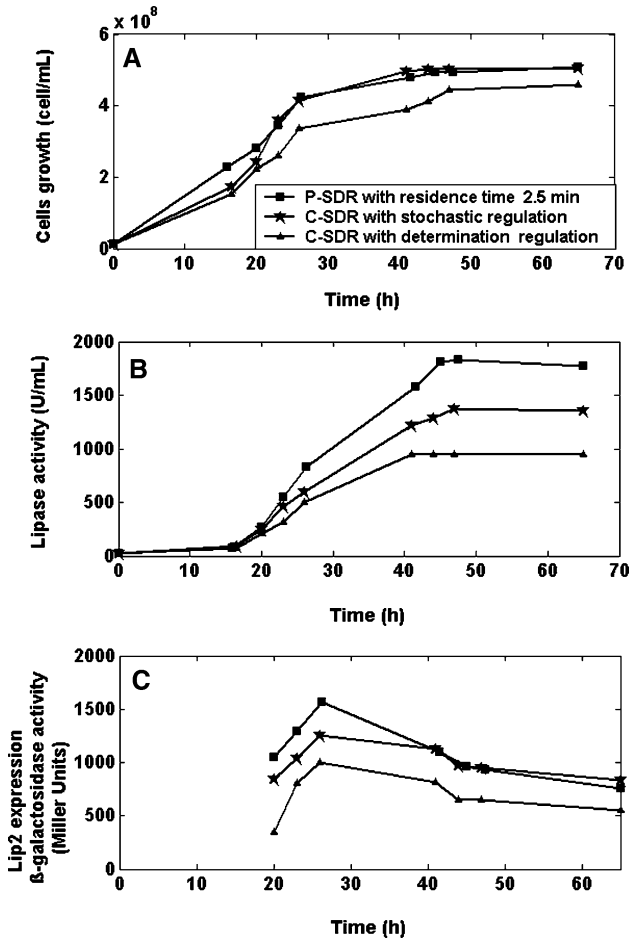

Fig. 5 Evolution of the amount of microbial cells (**a**), the extracellular lipase activity (**b**), and the LIP2 expression (**c**) in function of the reactor configuration

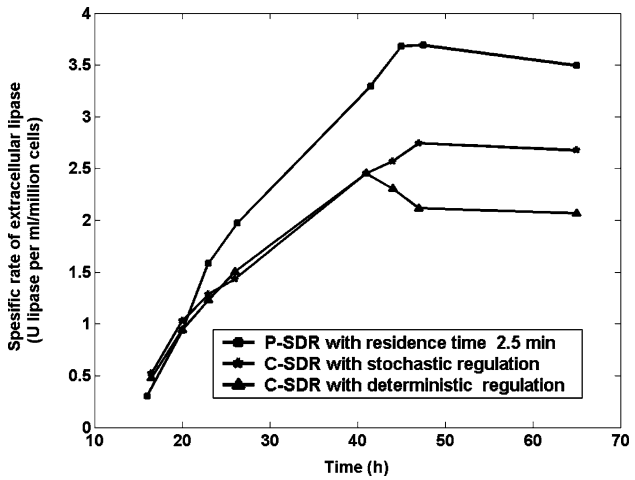

Fig. 6 Specific rate for the extracellular lipase production by *Y. lipolytica* cultivated in different reactor configuration (see Fig. 1 for more details about these configurations)

of the impeller) with a culture performed in a P-SDR with the pH regulation performed at the level of the non-mixed part of the reactor (in this case the pH gradient is promoted by the plug-flow effect occurring in this part of the reactor). As shown in Fig. 7b, the extracellular lipase yield is slightly lower for the P-SDR with pH regulation performed at the level of non-mixed part in order to promote the gradient formation. This effect is more pronounced in the case of P-SDR with higher recirculation flow rate (residence time of 2.5 min). For this reactor, the β-galactosidase assays (Figs. 7c, 8) clearly show a drop at the level of the LIP2 gene expression. No additional effect to the P-SDR has been observed when promoting pH gradients. However, a morphological modification of the cells has been observed when performing the pH regulation at the level of the non-mixed part of the reactor (Fig. 9). These results are in accordance with previously reported dimorphism manifestation of *Y. lipolytica* exposed to pH fluctuations [15, 16].

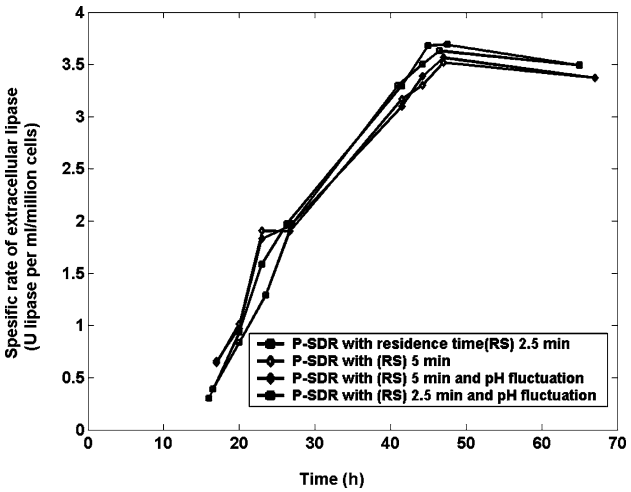

Fig. 8 Specific rate for the extracellular lipase production by *Y. lipolytica* cultivated in different reactor configuration (see Fig. 1 for more details about these configurations)

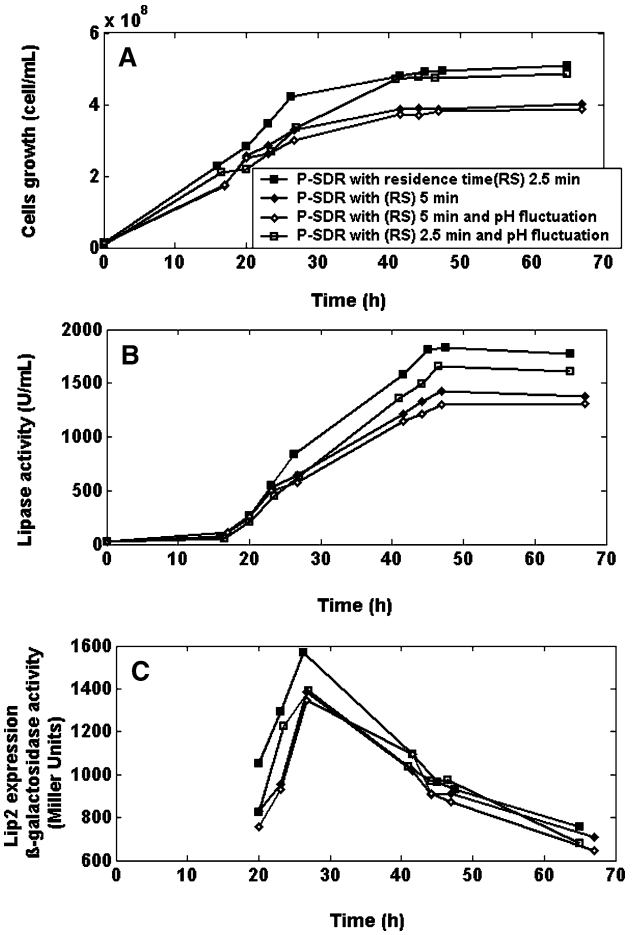

Fig. 7 Evolution of the amount of microbial cells (**a**), the extracellular lipase activity (**b**), and the LIP2 expression (**c**) in function of the reactor configuration

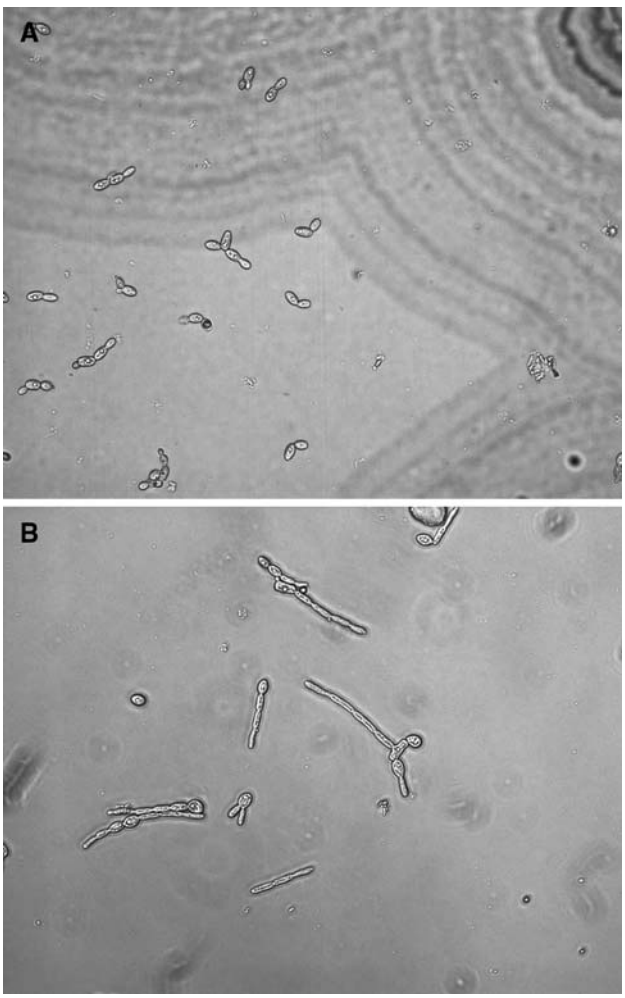

Fig. 9 Comparison of a culture conducted in a P-SDR with classical pH regulation (**a**) where the normal yeast cell shape is observed and in a P-SDR conducted with the pH regulation performed at the level of non-mixed part (**b**) where filamentation is observed

Conclusion

In this work, the effect of the environmental factors on *Y. lipolytica* has been observed at two distinct physiological levels. At a first physiological level, corresponding to mild stressful conditions, the impact of the considered environmental factor is observed at the level of the biomass yield and the specific rate of lipase production. In case of severe stressful conditions, a second physiological level is involved and the impact of the environmental condition is observed directly at the level of the lipase gene expression (in our case the LIP2 gene, encoding for the main lipase of *Y. lipolytica*). This second physiological level has been observed mainly for the experiments involving the dissolved oxygen fluctuations performed in a C-SDR. For two other environmental factors, i.e., the methyl oleate dispersion efficiency and the pH gradient, only an impact on the biomass growth and on the specific rate of lipase production has been noted (except for the pH gradient experiment conducted in a P-SDR with a residence time of 2.5 min for which a slight effect on the LIP2 gene expression has been observed). However, the impact of these two environmental factors have been studied in a partitioned scale-down reactor and are difficult to characterize in intensity, considering the special configuration of this reactor. Further studies will be conducted for a better characterization of the intensity and the frequency of the above mentioned environmental factors by the implementation of some hydrodynamic model. These experiments will be conducted in parallel with the numerical characterization of the local intensity of the environmental factors in industrial-scale bioreactors.

Acknowledgment Frank Delvigne is a postdoctoral researcher at the Belgian "Fond pour la recherche scientifique (FNRS-FRS)" and gratefully acknowledges the FNRS-FRS for financial supports.

References

1. Guerzoni ME, Lanciotti R, Vannini L, Galgano F, Favati F, Gardini F et al (2001) Variability of the lipolytic activity in *Yarrowia lipolytica* and its dependence on environmental conditions. Int J Food Microbiol 69:79–89. doi:10.1016/S0168-1605(01)00575-X
2. Fickers P, Benetti P-H, Wache Y, Marty A, Mauersberger S, Smit MS et al (2005) Hydrophobic substrate utilisation by the yeast Yarrowia lipolytica and its potential applications. FEMS Yeast Res 5:527–543. doi:10.1016/j.femsyr.2004.09.004
3. Enfors SO, Jahic M, Rozkov A, Xu B, Hecker M, Jürgen B et al (2001) Physiological responses to mixing in large scale bioreactors. J Biotechnol 85:175–185. doi:10.1016/S0168-1656(00)00365-5
4. Fickers P, Nicaud JM, Gaillardin C, Destain J, Thonart P (2004) Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica*. J Appl Microbiol 96:742–749. doi:10.1111/j.1365-2672.2004.02190.x
5. Destain J, Roblain D, Thonart P (1997) Improvement of lipase production from *Yarrowia lipolytica*. Biotechnol Lett 19:105–107. doi:10.1023/A:1018339709368
6. Fickers P, Destain J, Thonart P (2005) Methyl oleate modulates LIP2 expression in the lipolytic yeast Yarrowia lipolytica. Biotechnol Lett 27:1751–1754. doi:10.1007/s10529-005-3547-2
7. Destain J, Fickers P, Weeckers F, Moreau B, Thonart P (2005) Utilization of methyloelate in production of microbial lipase. Appl Biochem Biotechnol 121–124:269–278. doi:10.1385/ABAB:121:1-3:0269
8. Destain J, Fickers P, Weekers F, Moreau B, Thonart P (2005) Utilization of methyloleate in production of microbial lipase. Appl Biochem Biotechnol 121:269–277. doi:10.1385/ABAB:121:1-3:0269
9. Neubauer P, Häggström L, Enfors SO (1995) Influence of substrate oscillations on acetate formation and growth yield in *Escherichia coli* glucose limited fed-batch cultivations. Biotechnol Bioeng 47:139–146. doi:10.1002/bit.260470204
10. Hewitt CJ, Onyeaka H, Lewis G, Taylor IW, Nienow AW (2007) A comparison of high cell density fed-batch fermentations involving both induced and non-induced recombinant *Escherichia coli* under well-mixed small-scale and simulated poorly mixed large-scale conditions. Biotechnol Bioeng 96:495–505. doi:10.1002/bit.21127
11. Lara AR, Galindo E, Ramirez OT, Palomares LA (2006) Living with heterogeneities in bioreactors—Understanding the effects of environmental gradients on cells. Mol Biotechnol 34:355–381. doi:10.1385/MB:34:3:355
12. Namdev PK, Yegneswaran PK, Thompson BG, Gray MR (1991) Experimental simulation of large-scale bioreactor environments using a monte carlo method. Can J Chem Eng 69:513–519
13. Delvigne F, Destain J, Thonart P (2006) A methodology for the design of scale-down bioreactors by the use of mixing and circulation stochastic models. Biochem Eng J 28:256–268. doi:10.1016/j.bej.2005.11.009
14. Delvigne F, Lejeune A, Destain J, Thonart P (2006) Stochastic models to study the impact of mixing on a fed-batch culture of *Saccharomyces cerevisiae*. Biotechnol Prog 22:259–269. doi:10.1021/bp050255 m
15. Ruiz-Herrera J, Sentandreu R (2002) Different effectors of dimorphism in *Yarrowia lipolytica*. Arch Microbiol 178:477–483. doi:10.1007/s00203-002-0478-3
16. Szabo R, Stofanikova V (2002) Presence of organic sources of nitrogen is critical for filament formation and pH-dependent morphogenesis in *Yarrowia lipolytica*. FEMS Microbiol Lett 206:45–50. doi:10.1111/j.1574-6968.2002.tb10984.x