Characterization of an Extracellular lipase from *Yarrowia lipolytica*
Proceedings of European Congress of Chemical Engineering (ECCE-6)
Copenhagen, 16-20 September 2007

Characterization of an extracellular lipase from *Yarrowia lipolytica*

A. I. S. Brígida[a], P. F. Amaral[a], L. R. Gonçalves[b], M. A. Z. Coelho[a]

[a]*Department of Biochemical Engineering, School of Chemistry, Federal University of Rio de Janeiro, RJ 21949-900 Rio de Janeiro, Brazil*
[b]*Department of Chemical Engineering, Federal University of Ceará,CE 60455-760 Fortaleza,Brazil*

Abstract

Lipases from different sources have distinct properties, such as specificity, stability and optimal operational conditions. Moreover, different conditions of lipase production may alter these enzyme properties. Therefore, the aims of this study were related to the evaluation of lipase activity of Yarrowia lipolytica IMUFRJ 50682 (isolated from Baía de Guanabara, Brazil) produced under submerged fermentation, and its characteristics determination. Characterization studies under pH 3-10 (at 37 °C) and temperature 25-55 ºC (at pH 7) in the p-nitrophenyl laurate hydrolysis revealed that the enzyme is active in a pH range of 7-9, with a maximum lipase activity at pH 7, and between temperatures of 25-55 ºC with an optimum temperature for the lipase activity at 37 ºC. The *Y. lipolytica* enzyme was incubated in dry bath at 25ºC, 37ºC and 60ºC and the residual activities were measured under standard conditions. It became completely inactive after incubation for 15 minutes at 60ºC but was quite stable at 25ºC and 37ºC. The half-lives were 156.5 h, 106.8 h and 0.058 h at 25ºC, 37ºC and 60ºC, respectively. Regarding storage stability at -10ºC, *Y. lipolytica* lipase was very stable, keeping 100% of residual activity after seven months. Finally, initial rates of hydrolysis were obtained and the Michaelis-Menten constant and V_{max} were calculated as 0.234 mM and 0.033 μM/mL/min, respectively.

Keywords: *Yarrowia lipolytica*, lipase, characterization, p-nitrophenyl laurate, hydrolysis reaction.

1. Introduction

Lipase (triacylglycerol ester hydrolases, E.C. 3.1.1.3) constitutes a group of enzymes having as main biological function the hydrolytic catalysis of lipids. At low water content, theses enzymes are also able to catalyse synthesis reactions, achieving esterification, transesterification, aminolysis, oximolysis and thiotransesterification [1]. Somes studies have reported the yeast *Yarrowia lipolytica*, formerly known as *Candida, Endomycopsis* or *Saccharomycopsis lipolytica*, as a good lipase producer. This yeast is able to produce extracellular, membrane-bound and intracellular lipases encoded by approximating, eighteen genes [2].

The major level of lipase produced by *Y. lipolytica* is an extracellular lipase (*YlLip2*) encoded by *Lip*2 gene [3]. A mutant (LgX64.81) that possesses high level of *YlLip*2 expression, produced by chemical mutagenesis on *Y. lipolytica* CBS6303, is

able to produce 1,000 U/mL of lipase activity in a 15 L bioreactor and 1,118 U/mL in a 2,000 L bioreactor using an optimized medium [4]. Beyond *YlLip*2, other *Y. lipolytica* lipases were identified, purified and characterized. Some characteristics as optimum pH and temperature for *YlLip1*, *YlLip2* [5], *YlLip7* and *YlLip8* [6] were defined.

In Brazil, a *Yarrowia lipolytica* strain (IMUFRJ 50682) was selected from an estuary in the vicinity of Rio de Janeiro State [7]. This strain showed to be a good lipase producer both in shaking flasks [8, 9, 10] and in a 1.5 L bench bioreactor [11, 12]. However, the characteristics of these enzymatic extracts are hardly studied. Thus, in this study, lipase characterization was performed with the enzymatic extract produced under the previous conditions studied [12]. Characteristics as optimum pH and temperature was compared to data published by Pereira-Meirelles *et al.* [8] for lipase extract obtained by the same *Y. lipolytica* strain (IMUFRJ 50682) and to a commercial extract of *Candida antarctica* lipase type B.

2. Materials and Methods

2.1. Materials

Commercial *Candida antarctica* lipase type B (CALB) was kindly donated by Novozymes Latin America Ltd. Methyl butyrate and bovine serum albumin was purchased from Sigma-Aldrich Chemical Co. Perfluorodecalin was obtained from F2 Chemicals Ltd. (Preston, Lancashire, UK). Folin-Coicalteau's phenol and p-nitrophenyl laurate were purchased from Fluka. Peptone, yeast extract, and glucose were obtained from Merck, Oxoid, and Isofar, respectively. Other chemicals were of analytical grade.

2.2. Microorganism and maintenance of cultures

Yarrowia lipolytica IMUFRJ 50682 was used. It was stored at 4ºC on YPD-agar medium. For pre-inoculum, cells were cultivated at 28^{o}C in a rotary shaker at 160 rpm, in 500 mL flasks containing 200 mL YPD medium (w/v: Yeast Extract 1%; Peptone, 2%; Glucose, 2%).

2.3. Production of lipase

Cells from the pre-inoculum were harvest (26,000 *g*) from the exhausted medium and used to inoculate 1,200 mL YPD medium (w/v: Yeast Extract 1%; Peptone, 0.64%; Glucose, 2%) in sufficient amount to obtain 1 mg d.w. cells.mL^{-1}. This inoculated medium was used in the bioreactor along with 300 mL of Perfluorodecalin, as an oxygen carrier. Fermentation was carried out for up to 48 h at 25 ºC in a 2 L bioreactor using 1.5 L of total volume of working medium, maintaining 250 rpm and with a 1.5 $m^3 \cdot s^{-1}$ of oxygen outflow. After 48 h, the cells were separated by centrifugation (26,000 *g* x 15 min) at 10 ºC in a refrigerated centrifuge.

2.4. Assay of hydrolytic activity: p-nitrophenyl laurate

The hydrolysis of p-nitrophenyl laurate was defined as standard method to determine lipase activity from crude extract of *Y. lipolytica* (LYL) in this work. The reaction occurs at 37 ºC by the addition of 0.2 mL of enzyme solution to 1.8 mL of 560 µM p-nitrophenyl laurate (pNP-laurate) dissolved in 50mM potassium-phospate buffer (pH 7.0), containing 1% (v/v) of dimethyl sulfoxide (DMSO). The reaction was followed along 100 seconds in a spectrophotometer (HACH, DR/4000U) at λ= 410 nm. One lipase unit (pNPLU) is defined as the amount of enzyme which releases 1 µmol of p-nitrophenol per minute at pH 7.0 and 37ºC [9].

2.5. Assay of hydrolytic activity: methyl butirate

In this work, methyl butyrate hydrolysis was used to determine the LYL esterase activity. Experiments were performed using an automatic titrator (pHstat) and 50 mM NaOH as titrating agent. The pH was set at 7.0. The reaction was initiated by the addition of 0.1 mL of extract to 30 mL methyl butyrate solution dissolved in 25 mM phosphate buffer pH 7.0 [13]. One unit (MBU) of enzymatic activity was defined as the amount of enzyme that hydrolysis 1 µmol of methyl butyrate per minute at pH 7.0 and 28 ºC.

2.6. Determination of protein

Protein was estimated by the Folin-Ciocalteau's phenol reagent as outlined by Lowry *et al.* [14], at 660 nm, using bovine serum albumin (BSA) as standard.

2.7. Biochemical characterization of Yarrowia lipolytica lipase extract

2.7.1. Effect of pH and temperature on enzyme activity

The pH effects on lipase activity were measured using buffers of different pH's (3 – 11) at 37ºC. The employed buffers were as follows: 50 mM citrate buffer (pH 3 – 6); 50 mM Na-phosphate buffer (pH 6 – 8); 50 mM K-phosphate buffer (pH 7); 50 mM sodium bicarbonate buffer (pH 9.2 – 10.7). The pH stability was checked by incubating lipase extract at 37ºC at different pH values for 2 h and 24 h.

Temperature influence on lipase activity was determined by carrying out the enzyme assay at different temperatures (25 – 55ºC) and pH 7.

2.7.2. Thermal stability

Lipase thermal stability was determined by incubating the biocatalyst at 25, 37 or 60 ºC. Periodically, samples were withdrawn and its residual activities were assayed by pNPL hydrolysis. Residual activity is given as percentage of activity taken as 100% the enzyme activity before incubation. Thermal deactivation profiles have been explained following the first-order deactivation model [15], as described in Equation 1, and the respective coefficients (k_d) were estimated from the experimental data.

$$\ln(A) = \ln(A_0) - K_d t \quad (1)$$

where A_0 is the initial activity, A is the residual activity in time t and k_d is first-order deactivation rate coefficient.

Biocatalyst half-life ($t_{1/2}$) was estimated with the aid of Equation 2 using the previously estimated parameter k_d.

$$t_{1/2} = \frac{\ln(0.5)}{-k_d} \quad (2)$$

2.7.3. Storage stability

To evaluate the storage stability of the lipase crude extract, it was stored at -10ºC in a freezer and the residual hydrolytic activity was determined along time (every 24h).

2.7.4. Determination of kinetic parameters

The effect of pNP-laurate concentration (0.05 to 3.00 mM) on the initial hydrolysis rate was assayed under the standard conditions. The Michaelis-Menten constant (K_m) and respective maximum reaction velocity (V_{max}), with pNPL as substrate, were estimated by fitting the experimental data to Michaelis' Equation.

2.7.5. Effect of additives on lipase activity

To determine the effect of some chemicals on lipase activity, different compounds as metal ions (1 mM) and organic solvents (9%, v/v) were added to the reaction mixture and the lipase activity was assayed under standard conditions.

3. Results and discussion

3.1. Lipase production

Lipase production was followed by the measurement of enzyme activity along *Y. lipolytica* cultivation and lipase maximum activity was detected after 48 h of process [12]. The lipase extract showed a specific activity of 22.4 pNPLU/g protein. However, in methyl butirate hydrolysis, only 3 MBU/mL of extract was found, which is a low activity value for this method. In diluted solutions of lipase B from *C. antarctica* (1%, v/v), for example, there is 40 MBU/mL. It is known that lipase B from *C. antarctica* have esterase activity, thus accepting short acetyl moieties [16]. In parallel, previous characterization studies of *YlLip*2 available in literature, it showed that this lipase has higher specificity to triglycerides than hydrophilic esters (methyl fatty acid esters). In case of hydrophilic esters, it possesses maximum specificity to methyl mystirate, with lower activity toward methyl butyrate [17]. Although it has been reported that *Y. lipolytica* secrete not only lipase but esterase too [18], according to these results it is possible to assume that the amount of esterase in the produced extract is minimum or null. Therefore, the activity expressed for pNPL hydrolysis is essentially due to the lipase presence.

3.2. Effect of pH on lipase activity and stability

The pH effects on pPNL hydrolysis catalysed by lipase from *Yarrowia lipolytica* IMUFRJ 50682 (LYL) and from *Candida antarctica* lipase type B (CALB)

and on both enzyme stability are shown in Fig. 1 and 2, respectively. LYL was found to be active between pH 7 and 9, while CALB showed activity between pH 7 and 11. The optimum pH was found to be 7 and 9.2 for LYL and CALB, respectively. Such observed pH range for LYL is similar to *YlLip*2 purified, 5.5 – 9.0, in olive oil hydrolysis [17, 19]. Optimum pH values for lipases of *Y. lipolytica* produced from different strains and available in the literature were as follow: 7 for *Y. lipolytica* LgX64.81 [4], 8 for *Candida* sp 99-125 [17] and 6-7 for *Y. lipolytica* 681 [20]. Despite some similarities, these values are not comparable since the reactions used for enzyme activity assay are different. Ota et al. [21] studied the pH optimum of a lipase from *Saccharomycopsis lipolytica* strain (*Yarrowia lipolytica*) in three different substrates (olive oil; tributyrin and oleic acid; triolein and oleic acid) and obtained distinct values (7, 7.8 and 8, respectively).

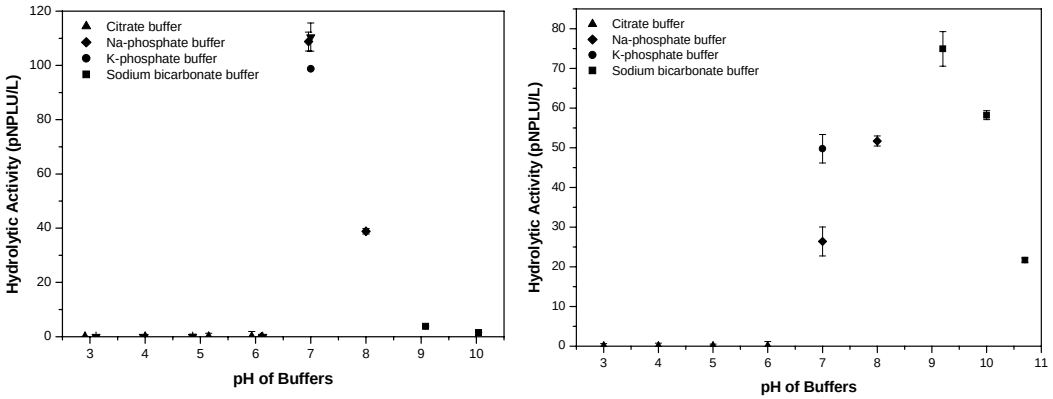

Figure 1: Effect of pH on lipase activity from *Y. lipolytica* (a) and *C. antarctica* (b).

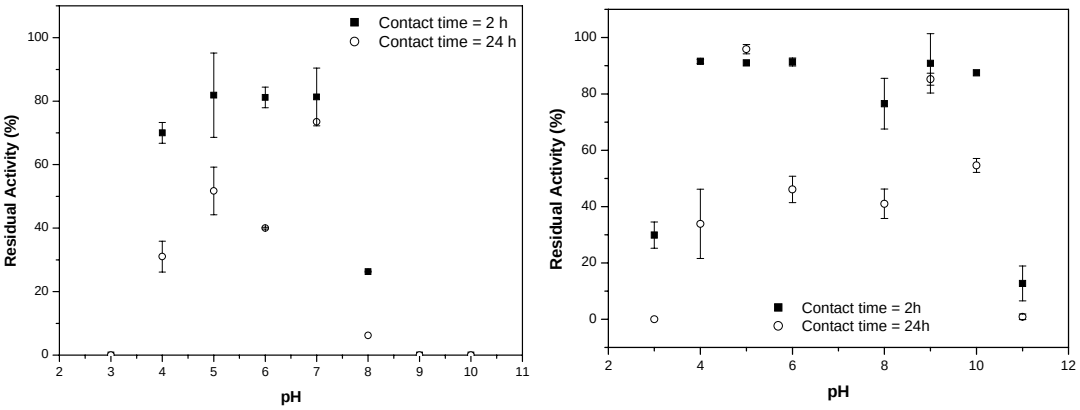

Figure 2: Effect of pH on the stability of lipase produced by *Y. lipolytica* (a) and *C. antarctica* (b).

After enzyme incubation for 2 hours in different pH's, LYL and CALB showed a good stability in pH range of 4-7 and 4-10, respectively. For pH 7, LYL had higher stability when incubated aong 24 h at 37ºC, retaining 75% of initial activity. CALB was stable at pH 5 and 9, where decreases of circa 10 and 15 % in its original

activity were observed after 24 h. Lipase I and II from *Saccharomycopsis lipolytica* (CBS 6303) were stable in pH range from 4.5 to 8 for 22 h at 5 ºC [21]. For lipase from *Y. lipolytica* 681, higher stability was obtained at pH 6 [20]. Moreover, a purified extract of *YlLip2* showed good stability in pH range of 4-7 for 1 h at 37 ºC, with similar profile to found to crude extract herein studied [19]. These results can be an indicative of the predominance of lipase *YlLip2* in the obtained extract.

3.3. Effect of temperature on activity of extract of lipase

The lipase activities of both LYL and CALB were assayed at different temperatures ranging from 25 to 65ºC at pH 7 (Fig. 3). For LYL, the enzyme activity increased with temperature in the range of 25 - 37ºC, presenting a decrease at temperatures above 37ºC, while the optimum temperature range observed for CALB was between 26 - 37ºC. The optimum temperature of *Y. lipolytica* IMUFRJ lipase extract was 37ºC, which is similar to that described for other lipases from different *Y. lipolytica* strains [4, 20]. However Pereira-Meirelles *et al.* [8], using this same strain but in different production conditions (with olive oil), determined 55ºC as the optimum temperature for the crude lipase extract of *Y. lipolytica* catalyzing the pNPL hydrolysis. Such difference for optimum temperature value using the same microorganism and reaction can be justified by the possible production of multiple lipase forms, depending on the cultivation conditions [5].

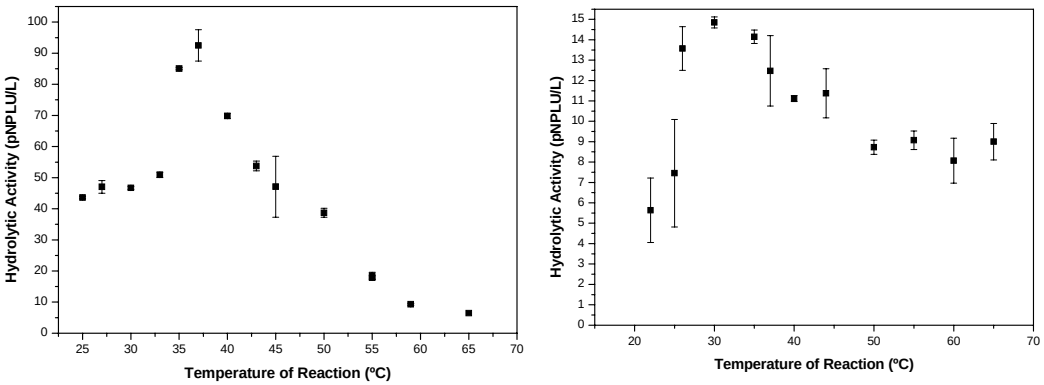

Figure 3: Effect of temperature on lipase activity from *Y. lipolytica* (a) and *C. antarctica* (b).

3.4. Thermal stability

The thermal stability were studied at room temperature (25ºC), at optimum activity temperature (37ºC) and at an extreme condition (60ºC) for both LYL (Fig. 4) and CALB (Fig. 5). The experimental profiles achieved for residual activity along incubation time were fitted to the decay model (Equation 1) and allowed to determine the respective half-lives ($t_{1/2}$) and deactivation constants (K_d) as shown in Table 1. At room temperature, CALB presents higher stability, retaining 85% of its original activity along 525 hours while LYL retained only 20% after 300 hours. Despite of such different behavior between CALB and LYL, at 37ºC showed similar deactivation profiles, presenting half-lives of 100.2 h and 106.8 h, respectively.

Nevertheless at an extreme condition (60ºC), CALB revealed to be more stable. Thus, comparing LYL performance with that one demonstrated by the commercial enzyme CALB, it is possible to say that both are similar when used at 37ºC.

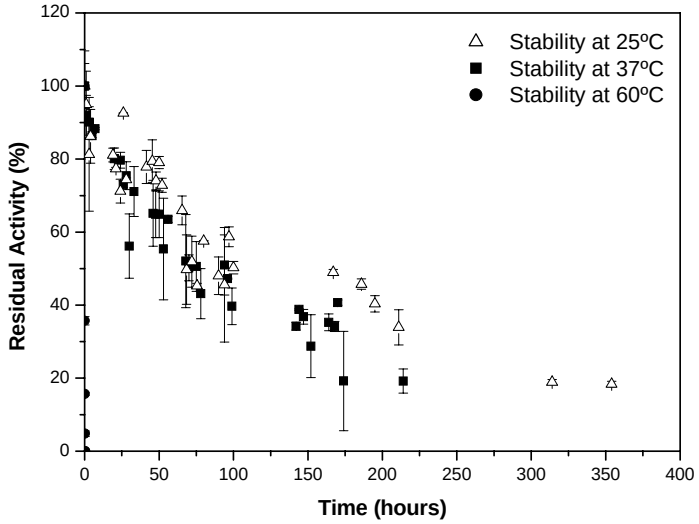

Figure 4: Thermal stability of lipase from *Y. lipolytica* at 25ºC, 37 ºC or 60 ºC.

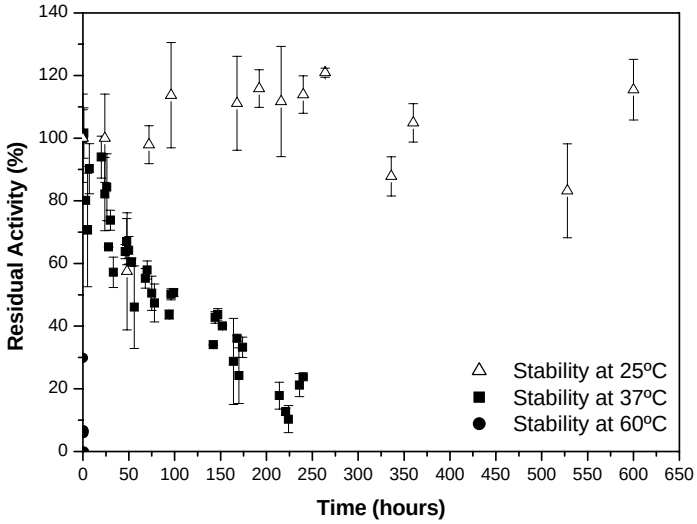

Figure 5: Thermal stability of lipase B from *C. antarctica* at 25ºC, 37 ºC or 60 ºC.

Face to these results, YL lipase extract cannot be rich in *YlLip7* or *YlLip8* since these heterologous lipases present higher thermostability and are capable to retain over 85% activity after incubation at 65ºC for 3 h [6]. On the other hand, lipase extract herein characterized revealed to be quite similar to the data described for *YlLip2* [17], since it is capable to retain 86% activity for 5 h at 37ºC while *YlLip2* retained 83% for 4 h at 35ºC.

Table 1: Kinetics parameters of thermal deactivation, at 25ºC, 50°C and 60 ºC of lipase from *Yarrowia lipolytica* (LYL) and from *Candida Antarctica* (CALB)

Enzyme	Temperature (ºC)	k_d (h^{-1})	$t_{1/2}$ (h)
LYL	25	0.00443	156.5
	37	0.00649	106.8
	60	11.9267	0.058
CALB	25	-	-
	37	0.00692	100.17
	60	3.2932	0.2105

3.5. Storage stability

Storage stability at -10°C of lipase extract was determined and the results are presented in Fig. 6. It can be observed that it presents a high stability at -10 ºC: after seven months, the enzyme retained 100% of its initial activity. This behavior is similar to that obtained by Pereira-Meirelles *et al.* [8] for storage stability at 5ºC during 370 days, retaining 100% activity.

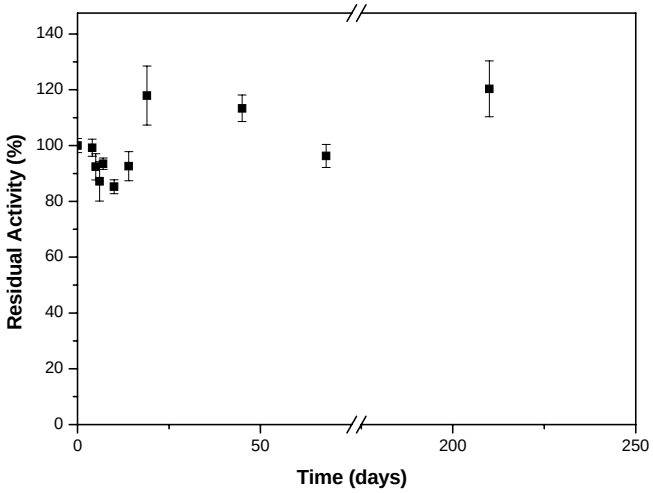

Figure 6: Storage stability, at -10ºC, of lipase extract produced by *Y. lipolytica*.

3.6. Effect of substrate concentration

The effect of pNP-laurate concentration (0.05 to 3.00 mM) in the initial hydrolysis rates was assayed. The experimental data obeyed classical Michaelis-Menten kinetics (Fig. 7). Michaelis-Menten constant and V_{max} were determined as 0.234 mM and 0.033 µM/mL/min for *Y. lipolytica* lipase and 0.192 mM and 0.042 µM/mL/min for *C. antarctica* lipase type B, respectively. K_m value obtained for LYL is slightly higher than that one described for *YlLip2* (0.19 mM) and lower than *YlLip1* K_m (0.5 mM) [5]. This observation implies that the lipase produced in the present work presents more affinity for the substrate than *YlLip1*, and almost similar affinity than *YlLip2* and CALB.

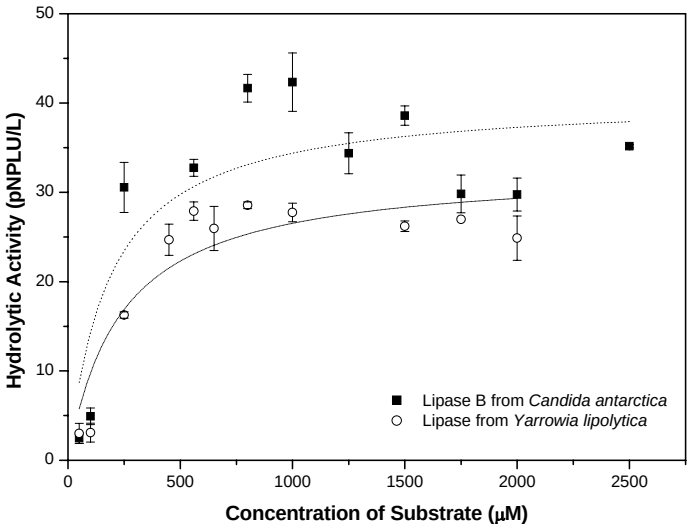

Figure 7: Initial hydrolysis rate of p-nitrophenyl laurate by soluble lipase from *Yarrowia lipolytica* and from *Candida antarctica*. The lines represent the Michaelis-Menten model adjustment to experimental data.

3.7. Effect of some additives on lipase activity

The effects of some metals salts and solvents on the activity of *Y. lipolytica* lipase and *C. antarctica* lipase type B were investigated (Fig. 8). For LYL, only ethanol and $CaCl_2$ had not caused a reduction in activity, whereas CALB activity was reduced only by ethanol, hexane and NaCl 100 mM. Since no activation profile was observed in both cases, these results suggest that both lipases do not require any of these ions for catalytic activity. Yu *et al.* [17] observed similar relative activity for *YlLip2* after contact with ethanol, 88.6 ± 2.00, but it did not present similar behavior for metals ions.

4. Conclusions

According to the data presented above, the crude lipase extract from *Y. lipolytica* IMUFRJ 50682 showed to have similar characteristics to other purified lipase extracts from *Y. lipolytica*, mainly for *YlLip2* producers. LYL, produced under conditions herein reported, demonstrate a hardly similar behaviour to that obtained by Pereira-Meirelles *et al.* [8], using the same strain. This can be an indicative that *Y. lipolytica* IMUFRJ 50682 strain is capable to produce lipase isoforms, concerning to the production conditions, denoting that further studies aiming to purify the lipase are necessary.

Acknowledgments
The authors would like to thank the Brazilian research-funding agencies CAPES and CNPq.

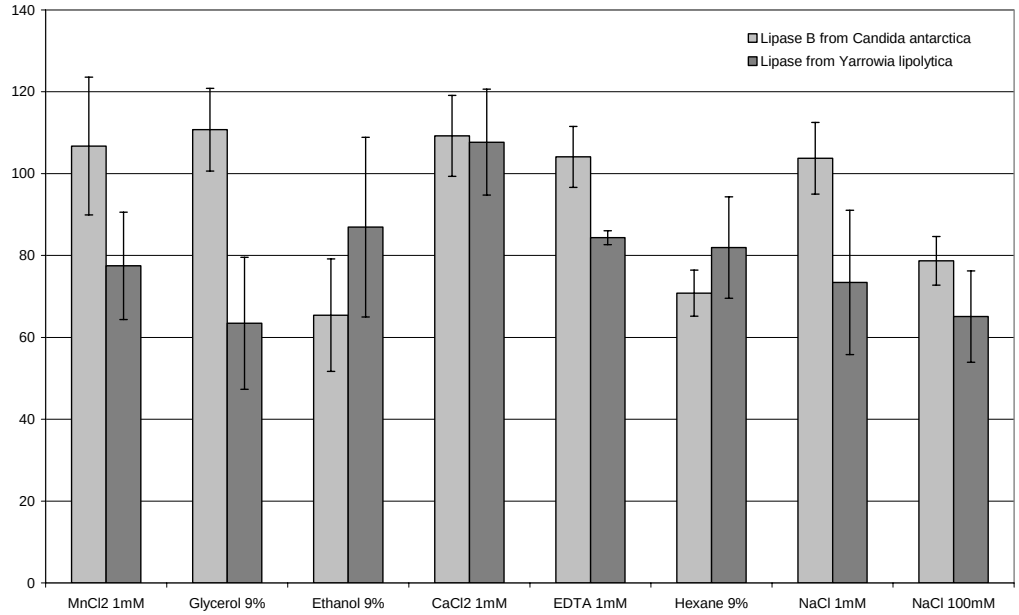

Figure 8: Effect of some chemical compounds on lipase activity: produced by *Yarrowia lipolytica* and lipase B from *Candida antarctica*.

References

[1] Villeneuve, P., Muderhwa, J. M., Graille, J., Haas, M. J., (2000) *J. Mol. Catal. B: Enzym.*, 9, 113-148.

[2] Fickers, P., Benetti, P.H., Waché, Y., Marty, A., Mauersberger, S., Smit, M. S., Nicaud, J.M., (2005) *FEMS Yeast Research*, 5, 527-543.

[3] Pignède, G., Wang, H., Fudalej, F., Gaillardin, C., Seman, M., Nicaud, J.-M., (2000) *J. Bacteriol.*, 182, 2802-2810.

[4] Fickers, P., Ongena, M., Destain, J., Weekers, F., Thonart, P., (2006) *Enz. Microb. Technol*., 38, 756-759.

[5] Nawani, N., Kaur, J., (2007) *Enz. Microb. Technol.*, 40, 881-887.

[6] Song, H.-T., Jiang, Z.-B., Ma, L.-X., (2006) *Protein Expression and Purification*, 47, 393-397.

[7] Haegler, A. N, Mendonça-Haegler, L. C., (1981) *Appl. Environ. Microbiol.*, 41, 173-178.

[8] Pereira-Meirelles, F. V., Rochão-Leão, M. H. M., Sant´Anna Jr., G. L., (1997) *Appl. Biochem. Biotechnol.*, 63-65, 73-85.

[9] Amaral, P. F. F., Rocha-Leão, M. H. M., Marrucho, I. M., C., J. A. P., Coelho, M. A. Z., (2005) *J. Chem. Technol. Biotechnol.*, 81, 1368-1374.

[10] Amaral, P. F. F., Almeida, A. P. R., Peixoto, T., Rocha-Leão, M. H. M., Coutinho, J. A. P., Coelho, M. A. Z., (2006) *World J. Microbiol. Biotechnol*., 23, 339-344.

[11] Alonso, F. O. M., Oliveira, E. B. L., Dellamora-Ortiz, G. M., Pereira-Meirelles, F. V., (2005) *Brazilian Journal of Chemical Engineering*, 22, 9-18.

[12] Amaral *et al.*, unpublished results.

[13] Brígida, A. I. S., Pinheiro, A. D. T., Ferreira, A. L. O., Pinto, G. A. S., Gonçalves, L. R. B. (2007), *Appl. Biochem. Biotechnol.* 136-140, 67-80.

[14] Lowry, U. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., (1951) *J. Biol. Chem*., 193, 265-275.

[15] Soares, C. M. F.; Castro, H. F.; Santana, M.H.A. and Zanin, G.M. (2002), *Appl. Biochem. Biotechnol.* 98-100, 863-874

[16] Pleiss, J.; Fischer, M.; Schmid, R. D., (1998) *Chemistry and Physics of Lipids*, 93, 67-80.

[17] Yu, M., Qin, S., Tan, T., (2007) *Process Biochemistry*, 42, 384-391.

[18] Vakhul, J., Kour, A., (2006) *Electronic Journal of Biotechnology*, 9, 69-85.

[19] Aloulou, A., Rodriguez, J. A., Puccinelli, D., Mouz, N., Leclaire, J., Leblond, Y., Carrière, F., (2007) *Biochimica et Biophysica Acta*, 1771, 228-237.

[20] Corzo, G., Revah, S., (1999) *Bioresource Technology*, 70, 173-180.

[21] Ota, Y., Gomi, K., Kato, S., Sugiura, T., (1982) *Agric. Biol. Chem.*, 46, 2885-2893.