CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438071

Intracellular Lipase Production by *Yarrowia lipolytica* Using Different Carbon Sources

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Yarrowia lipolytica is capable of producing lipase, an enzyme widely used in industry for, under conditions microaquosas, catalyzing the synthesis of esters. The extracellular lipase produced by this yeast has been fully characterized and has wide range of application, but the intracellular and cell-bound fractions are still little reported production and characterization. The application of intracellular and cell-bound lipases in industrial processes can reduce the purification cost, facilitating the use of this enzyme. Therefore, the objective of this study was to evaluate the production of intracellular lipases induced by different carbon sources. We investigated the intracellular lipase production by *Yarrowia lipolytica* strain IMUFRJ 50682 using glucose, glycerol, crude glycerol, olive oil and frying oil. The methodology for the intracellular and cell-bound lipase extraction was standardized. Cell growth, concentration of glucose and glycerol was accompanied. Measures of enzymatic activity were carried out using spectrophotometric method. *Y. lipolytica* cells presenting in the form of hyphae were more sensitive to the two extraction processes used and the extraction with ultrasound possible to obtain activity values to cell bound fraction up to 9 times higher than those obtained with the mechanical extraction. Glycerol and glycerin were not effective for induction of enzymatic fractions. Glucose induced activity in extracellular fraction. Olive oil and waste frying oil were the best inducers for intracellular fractions. Frying oil was a good inductor to obtain intracellular lipases and the values of hydrolytic activity were similar to values obtained with the olive oil. In presence of frying oil, the hydrolytic activity was 12,19 U/g, in 24h of growth and when olive oil was used like inductor, the enzyme activity to the cell bound fraction reaching activity of 12,14 U/g in 24h of the experiment. Therefore, the intracellular fractions of *Yarrowia lipolytica* lipase can be induced efficiently and represent an interesting alternative for developing technology to the use of biomass in industrial processes.

1. Introduction

Lipases (glycerol ester hydrolases EC3.1.1.3.) are enzymes that catalyze the hydrolysis of ester bonds of triacylglycerols, forming fatty acids, glycerol, diacylglycerol and monoacylglycerol. In microaquous conditions, these enzymes catalyze the synthesis of esters via transesterification reactions. Thus, feature a wide use in industry and the interest in application of these enzymes in industrial bioprocesses is growing, because of its versatility (Ribeiro *et al*., 2011). New technologies are being developed to circumvent the economic limitation and lipases with more specific properties are being investigated (Ha *et al*., 2007; Wang *et al*., 2007). Many studies directed towards in the changing of production medium to optimize the production and excretion of these enzymes. The application of immobilized lipases thus reducing costs due the reusability of enzymes in various cycles and their use in continuous processes

Please cite this article as: Nunes P., Martins A.B., Santa Brigida A.I., Miguez Da Rocha Leao M.H., Amaral P., 2014, Intracellular lipase production by yarrowia lipolytica using different carbon sources, Chemical Engineering Transactions, 38, 421-426
DOI: 10.3303/CET1438071

(Ellaiah *et al*., 2004). Some microorganisms have, in addition to the extracellular lipase, fractions that remain bound to cell (Ota *et al.*, 1982). *Yarrowia lipolytica* has attracted great interest in the biotechnological area by having the ability to excrete various metabolites in large quantities (Barth & Gaillardin, 1997). Many works show intracellular lipase production by use medium containing olive oil as inducer. The frying oil used repeatedly in homes and restaurants is usually discarded improperly sewer system and is a residue that accumulates and can clog the sewer tubes. Researches for use of waste in lipases production are already being carried out. Good results were obtained by use of bacteria growing in waste frying of olive and sunflower oil (Haba *et al*., 2000) and yeasts grown in the presence of colza oil, after being used for frying potatoes (Rywińska *et al*., 2008). In this context, this work aims to contribute to advance the use of cells in biocatalysts, providing insight into the potential application of the intracellular enzyme of *Yarrowia lipolytica*, creating the possibility of carrying out further research to industrial application.

2. Materials and Methods

2.1 Microorganism producing Lipase

The strain *Yarrowia lipolytica* 50682 was obtained from the Microbiological Institute, Federal University of Rio de Janeiro, Brazil. The strain was maintained on a solid medium containing 2 % Glucose, 1 % Yeast extract, 2 % Peptone and 2 % Agar (p/v), cultivated for 48 h at 28 °C, stored at 4 °C.

2.2 Production of Intracellular Lipases

The inoculum medium consisted of 20 g glucose, 20 g peptone, and 10 g yeast extract per liter of distillate water. The yeast was inoculated into a 500mL flask with 200mL the medium. Then, the flasks were shaken at 160 rpm under 28°C, for 48 h for cell growing culture. The production medium consisted of: 6,4 g peptone, 10 g yeast extract, 20 g of carbon source (Glucose or Glycerol) per liter of distillate water. When the inductors were utilized, olive oil and frying oil were added at the concentration of 1%. Yeast cell was added to this medium for lipase production at 250 rpm under 28°C conditions for a certain time. Samples were taken at 15, 24, 30 and 48 h, where a 20 mL aliquot was subjected to extraction of lipase fractions. The medium supernatant was subjected to substrate and extracellular lipase analysis. The experiments were performed in triplicate and the characteristic profiles are presented.

2.3 Analytical methods

Culture samples were collected for analysis of cell concentration (optical density at 570 nm and cell number converted to g cell dry per liter). HPLC analysis of glucose and glycerol was performed on Aminex HPX-87H column, at 60ºC, with H_2SO_4 0,005 M as the mobile phase (0.8 ml/min). The fatty acid composition of frying oil was determined by gas chromatography of methyl esters of fatty acids (FAME) obtained after acid transesterification. The FAMEs analysis was performed with gun-type split /splitless and flame ionization detector (FID). The injector and detector are operated at temperatures of 260 °C and 280 °C, respectively. The hydrolysis of p-nitrophenyl laurate was defined as standard method to determine hydrolitic activity from lipase fractions. The reaction occurs at 25 ºC by addition of 1 mL of enzyme to 19 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 10 minutes 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 25ºC.

2.4 Enzyme extraction

Mechanic extraction: Initially, to obtain intracellular and cell-bound fractions glass beads and vortex were used. An aliquot of cultivation of 100 mg ps cells was collected by centrifugation at 4 °C, 4630 x *g* for 5 minutes. The supernatant (extracellular lipase fraction) was separated and frozen at -4 °C. Next, the cells (sediment obtained) were washed with distilled water and pH 7.0 MOPS buffer and centrifuged under the same conditions. Cells were resuspended in 1 ml of MOPS buffer and 3 g of glass beads 425-600µm - CO Sigma-Aldrich (MO, USA) were added. The following was the process of cell disruption with 3 cycles of vortex for 1 minute, intercalated with ice bath (Pereira-Meirelles *et al*., 1997). After, the cellular extract was centrifugated at 12850 x *g* for 5 minutes at 4 °C. The supernatant obtained (intracellular lipase) and the pellet resuspended in 1 ml of MOPS buffer pH 7.0 (cell-bound lipase) were frozen (-4 °C) until determination of enzyme activity.

Extraction by ultrasound waves: 20 mL from culture were centrifuged at 4 °C, 4630 x *g* for 5 minutes. The cells were washed with distilled water and MOPS buffer pH 7.0 and centrifuged under the same conditions. The cells were suspended in 20 ml of MOPS buffer and brought of Sonicator Ultrasonic Cleaner, in ice bath, subjected to two cycles of extraction with a constant acoustic power of 150 W and frequency of 20 kHz, for 9 minutes. The fractions were frozen for measurement of enzyme activity.

2.5 Cell viability

After subjecting cells to extraction process, a solution of Methylene Blue 0,25 g/L was mixed with an equal volume of sediment suspension. The percentage of viable cells is the ratio of the number of cells not stained and total number of cells and was determined in a Neubauer chamber under optical microscope (Pierce, 1970).

3. Results and Discussion

3.1 Effect of the carbon source and inducers on cellular growth

The lipase production by *Y. lipolytica* was investigated at different compositions of culture medium, the main components carbohydrates and triglycerides. *Y. lipolytica* is capable of to use tryglicerides as carbon source and the first step of this metabolism involves hydrolysis of this substrate by means of lipases to produce fatty acids and glycerol (Fickers *et al.*, 2005). However, triglycerides need to be degraded into smaller glycerides and fatty acids outside the cell, to then be absorbed. This occurs through lipase secreted in the medium, which will hydrolyze triglycerides (Najjar *et al*., 2011). *Y. lipolytica* grew in presence of olive oil and waste frying oil until 15 h of culture (Figure 1), and this fact reinforces the idea that the triglycerides present in these oils are hydrolyzed to obtain energy to support growth cell, as proposed by Pereira-Meirelles *et al* (1997). Some studies report that glycerol and the fatty acids from triglycerides hydrolysis presents in oil would be consumed by yeast sequentially (Del Rio *et al.*, 1990). It was observed that the cell concentration increased slightly after 48 hours of cultivation. And about 70 h, the growth of the yeast returnes to stabilize. The constitutive lipases present in this yeast, at the beginning of culture, are probably responsible for degradation of oil and fatty acid generated would be incorporated into the cell and induce the production of various enzymes. In the presence of glucose, this yeast grown for approximately 48 hours, and when the concentration of substrate is less than 30% of the initial concentration (Figure 1) a decline of cell growth was observed. When glucose/olive oil combination was used, it could be noted slightly slower carbohydrate consumption in the presence of oil and after 48 h of cultivation, cell growth still was observed. Only in the presence of glucose, at 70 h of cultivation, cell growth was in decline and the concentration of substrate is minimal in medium. However, when this carbon source was associated to olive oil, in 70 h, despite glucose was already exhausted in medium, the cell concentration reaches its maximum. From this moment, the cells are probably using glycerol and fatty acids, from olive oil hydrolysis for growth and at the end of 96 hours the biomass formed is slightly higher than that obtained in the presence only of inductors.

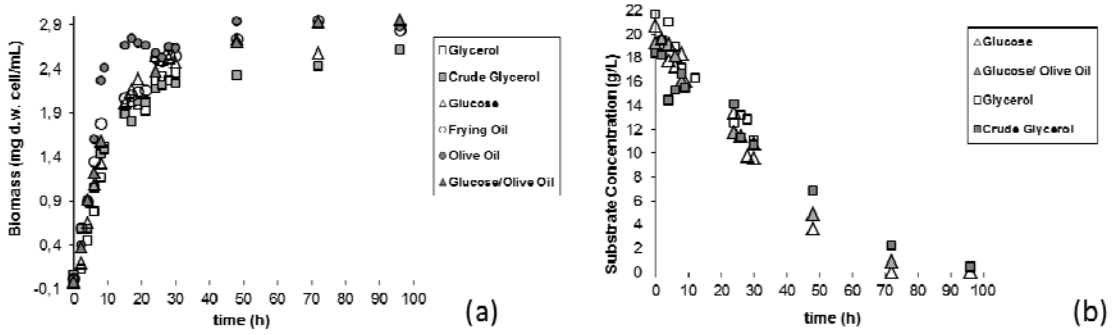

Figure 1. (a) Influence of inducers in cell growth of Y. lipolytica; (b) substrate consumption by Yarrowia lipolytica cultivated in media with glucose, glycerol and crude glycerol and in glucose associated to olive oil, cultivated in 1000 ml flasks with 500 ml of medium agitated at 250 rev/ min.

In medium containing glycerol or crude glycerol, *Y. lipolytica* showed similar growth until 96 h of cultivation, though this growth was slower (Table 1) than that observed in the presence of glucose and inductors. The specific cell growth rate (μ) calculated in the exponential growth phase in the presence of different carbon sources and inducers are shown in Table 1. It's observed that the growths with higher μ were those containing olive oil and residual frying oil without adding any additional carbon source. The residual frying oil appears not have inhibitory action of compounds on cell growth of this strain, with μ and biomass production (Δx) very close to those observed in the presence of olive oil. Knowing that olive oil has composition about 70 % oleic acid, it is likely that once lipase production begins, the yeast is hydrolyzing triglyceride and oleic acid and glycerol are releasing in the culture medium. Del Rio *et al.* (1990) found that *Candida rugosa*, in the presence of olive oil as the sole carbon source, also used this substrate by

sequential form. The triglycerides present in the oil were hydrolyzed by a small amount of lipases from cells, producing free fatty acids and glycerol. Glycerol is reversed in biomass without lipase production and represses the uptake of fatty acids. Finally, the free fatty acids are consumed and promote the induction of a significant amount of lipases. Analyzing the results shown in Figures 3 and 4, where the crude glycerol was used as carbon source in the absence and presence of inducer, it can be seen that exist cell growth after exhaustion of glycerol in the culture medium. Therefore, between 100 and 168 h of cultivation, it is likely that this yeast are using fatty acids for cell growth.

Table 1. Influence of different carbon sources and inducers in the specific growth rates, biomass production and activity of different lipase fractions of Yarrowia lipolytica, obtained by extraction with glass beads.

Carbon Sources/ Inducers	μ (h^{-1})	Δ_x 24h (mg d.w.cell/mL)	Lipase		
			Extracellular (U/L)	Intracellular (U/g)	Cell-bound (U/g)
Glucose	0,235	10,3	312, 87	0	0
Glucose/Olive Oil	0,220	9,79	299,86	0,40	0
Glycerol	0,180	8,65	0	0	0
Crude Glycerol	0,206	7,77	0	0	0
Olive Oil	0,310	11,95	152,89	1,55	1,97
Frying Oil	0,228	11,42	33,66	1,33	1,3

3.2 Study of waste frying from soybean oil

The frying oil used in this study was obtained through thermal processing of commercial soy oil. According to several studies in the literature, soybean oil is constituted about 80 % of unsaturated fatty acid, approximately 55 % linoleic acid (C 18:2) and 23 % oleic acid (C 18:1). The composition of waste frying oil used in this study is quite similar to the commercial soy oil (date not shown). There was a slight increase in the content of saturated fatty acids, but the proportions of oleic and linoleic acids remained. Although the frying process degrade the vegetable oil by the hydrolysis, oxidation and thermal change (Choe & Min, 2007), appear these reactions do not seem to have generated toxic products for yeast cells. Haba *et al*. (2000) observed differences in the levels of short chain fatty acids and a significant loss in oleic acid after the frying process of olive oil. However, these changes did not prevent cell growth and the residual oil was an effective carbon source for different genera of microorganisms, such as *Pseudomonas, Candida, Staphylococcus and Acinetobacter*.

3.3 Standardization of extraction methodology

To optimize the intracellular lipases extraction, another method was used through the use of ultrasound. High intensity ultrasonic energy is a useful tool for the permeabilization of cellular membrane and rupture of cellular wall, releasing intracellular enzymes (Chisti, 2003). Upon the separation of enzymatic fractions in the two methodologies (vortex and ultrasound), part of the cells still had intact (Figure 2).

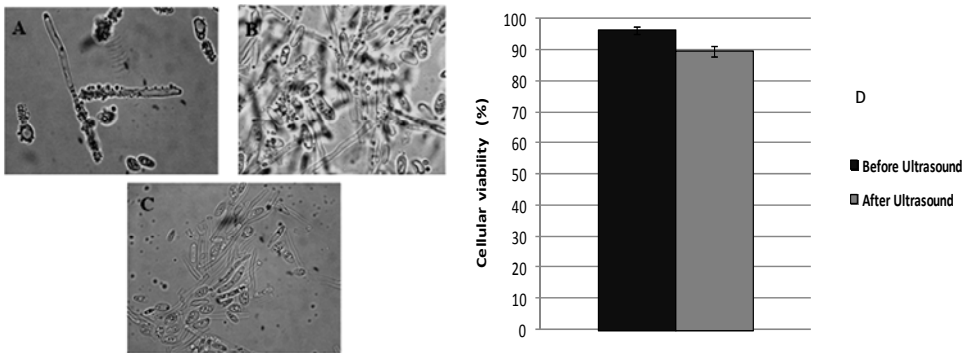

Figure 2: Images obtained by optical microscopy attached of Yarrowia lipolytica cells (magnification of 400x). In (A) cells in the presence of olive oil before extraction; (B) cells after extraction with glass beads; (C) cells after extraction by ultrasound waves; (D) cellular viability after extraction by ultrasound waves.

Its observed that the cells of *Y. lipolytica* that were presented in the form of hyphae were more sensitive to the two extraction processes used. Kapturowska *et al*. (2012) observed that the extraction using ultrasound promoted the formation of " pores " in the cell membrane through which the intracellular lipase is extracted. Cell viability was evaluated before and after the extraction process (Figure 2). About 93 % of the cells remained intact after extraction with ultrasound , confirming the results obtained by Kapturowska *et al.* (2012).The hydrolytic activity obtained for each fraction is shown in Table 2. Thus, ultrasound does not cause cellular disruption other than the mechanical method by glass beads, the enzyme extraction increases efficiency and the intracellular fractions had higher activity. The hydrolytic activity showed a significant increase in intracellular and cell bound fractions and this result was observed in lipases in the presence of two inductors studied. Increased intracellular activity should be due greater enzyme extraction efficiency, however, was also observed significant increase in the fraction bound to cell. Whereas most of cells remained intact, it is possible that the increased activity of the bound occur for greater interaction between substrate and enzyme, since occurs changes on cellular permeability, the substrate has facilitating entry into cell.

Table 2. Hydrolitic activity of intracellular and cell bound lipase fractions of Y. lipolytica obtained in olive oil anda frying oil, determinated by hydrolysis of p-nitrophenyl laurate, after mechanical and ultrasound (20 kHz) extractions in 24 h of culture.

Extraction	Inducer	Activity	
		Intracellular (U/g)	Linked (U/g)
Mechanic	Olive Oil	1,55	1,97
	Frying Oil	1,33	1,30
Ultrasound	Olive Oil	9,93	12,14
	Frying Oil	4,51	12,19

Due to increased hydrolytic activity obtained in extraction method by ultrasounds waves, under the conditions tested, this method was considered the most suitable for obtaining intracellular and cell bound lipases of *Y. lipolytica*. Fractions present in the cell represent over 80% of activity present in culture medium (Figure 3).

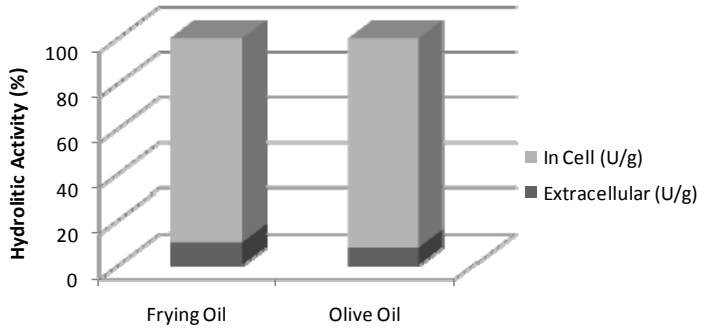

Figure 3: Percentage production of extracellular and intracellular fractions in olive oil and frying oil, in 24 h de culture.

3.4 Effect of the carbon source and inducers on intracellular lipase production

Comparing the values of hydrolytic activity of fractions obtained at 24 h of cultivation (Table 3), it was observed that the media containing olive oil and residue of soybean oil presented the best results for the production of intracellular lipases. Residual frying oil showed a good induction of intracellular lipases and their values of hydrolytic activity approached to that obtained in olive oil presence. Although intracellular lipase was detected in the presence of glycerin, this carbon source was not good inducer, even in the presence of oils. Similar results were observed by Corzo & Revah (1999), which did not have detectable extracellular lipase at 36h of cultivation, when used this carbon source. This probably occurs due glycerol is a product of triglycerides hydrolysis and when it is available at beginning of cultivation, there is no immediate need for lipase production by cell.

Table 3: Hydrolitic activity of intracellular and cell bound lipase fractions of Y. lipolytica obtained in differents carbon sources and inducers, determinated by hydrolysis of p-nitrophenyl laurate, after ultrasound extraction in 24 h of culture.

Carbon Source/ Inducer	Activity	
	Intracellular (U/g)	Linked (U/g)
Glucose	0	0
Glycerol	2,78	0
Crude Glycerol	0	0
Olive Oil	11,84	12,10
Frying Oil	4,34	13,59

4. Conclusion

Frying oil showed a high potential to be used for the production of intracellular lipases by *Y. lipolytica*. The use of this residue can contribute to costs reduction in lipase production and, moreover, has the benefit to reuse this industrial and commercial waste.

Acknowledgments

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support.

References

Barth, G. and Gaillardin, C. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*, 1977. FEMS Microbiol. Rev., v. 19: 219-237.

Chisti, Y. Sonobioreactors: using ultrasound for enhanced microbial productivity, 2003. Trends in Biotechnology 21.2: 89-93.

Choe, E. and Min D. B. Chemistry of deep-fat frying oils, 2007. Journal of Food Science 72.5: R77-R86.

Corzo, G. and Revah, S. Production and characteristics of the lipase from *Yarrowia lipolytica* 681, 1999. Bioresource Technol., v. 70: 173-180.

Del Rio, J.L.; Serra, P.; Valero, F., Pooh, M.; Sola, C. Reaction scheme of lipase production by *Candida rugosa* growing on olive oil, 1990. Biotechnology *Letters*, v. 12 (II): 835-838.

Ellaiah, P.; Prabhakar, T.; Ramakrishna, B.; Thaertaleb, A.; Eadinarayana, K. Production of lipase by immobilized cells of *Aspergillus nige,* 2004*.* Proc. Biochem*.,* v. 39: 525–528.

Fickers, P., Fudalej, F., Nicaud, J., Destain, J. E Thonart, P. Selection of new over-producing derivates for the improvement of extracellular lipase production by the non-conventional yeast *Yarrowia lipolytica*, 2005. Journal of Appl. Microb., v. 115,p. 379–386.

Ha, S.H.; Lan, M.N.; Lee, S.H.; Hwang, S.M.; Koo, Y.M. Lipase catalyzed biodiesel production from soybean oil in ionic liquids, 2007. Enzyme and Microbial Technology, v. 41: 480-483..

Haba, E., Brescoa, O., Ferrera, C., Marque´S A, A., Busquets B, M., Manresa a, A. Isolation of lipase-secreting bacteria by deploying used frying oil as selective substrate, 2000. Enz. and Microbial Technol., v. 26, p. 40–44.

Kapturowska, A.U.; Stolarzewicz, I.A.; Krzyczkowska, J.; Białecka-Florjanczyk, E. Studies on the lipolytic activity of sonicated enzymes from *Yarrowia lipolytica,* 2012. Ultrasonics Sonochemistry, v. 19: 186–191.

Najjar, A.; Robert, S.; Guérin, C.; Violet-Asther, M.; Carrière, F. Quantitative study of lipase secretion, extracellular lipolysis, and lipid storage in the yeast *Yarrowia lipolytica* grown in the presence of olive oil: analogies with lipolysis in humans, 2011. Appl. Microbiol. Biotechnol., v. 89: 1947–1962.

Ota, Y., Gomi, K., Kato, S., Sugiura, T., Minoda, Y. Purification and some properties of cell-bond lipase from Saccharomycopsis lipolytica; Agr. Biol. Chem., ,v.46, n.12, p.2885-2893, 1982.

Pereira-Meirelles, F.V.; Rocha-Leão, M.H.; Sant'anna, G.L. A Stable Lipase from *Candida lipolytica* cells, 1997. Biotechnology for Fuels and Chemicals. Humana Press, 73-85.

Pierce, J.S. Institute of Brewing: analysis committee measurement of yeast viability, 1970. *J.* Inst. Brew, v. 76: 442-443.

Ribeiro, B.D.; De Castro, A.M.; Coelho, M.A.Z.; Freire, D.M.G. Production and Use of Lipases in Bioenergy: A Review from the Feedstocks to Biodiesel Production, 2011. Enzyme Researche.

Rywińska, A., Witkowska, D., Juszczyk, P., Rymowicz, W., Kita, A. Waste Frying Oil as Substrate for Lipase Production by *Geotrichum candidum* strains, 2008. Polish J. of Environ. Stud. V. 17, n. 6, p. 925-931.

Wang, D.; Xu, H.; Teng, Y. Synthetic activity enhancement of membrane-bound lipase from *Rhizopus chinensis* by pretreatment with isooctane, 2007. Bioprocess Biosyst. Eng., v. 30: 147–155.