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Production of Lipolytic Enzymes by Endophytic Fungi and its Usein the Hydrolysis of Crude Oil.

Science

KEYWORDS :- Endophytic fungus, Penicilliumcitrinum, submerged culture, lipase production, enzymatic crude oil hydrolysis

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ABSTRACT

Endophytic fungi were isolated from the seeds of oil palm (ElaeisguineensisJacq) by biomonitoring lipase activity on semi-solid media with chromogenic substrate. Molecular and classical methods were used to identify the species, and the selected fungus was confirmed as Penicilliumcitrinum. Method for thermal stability of the enzyme carried on crude oil hydrolysis. The fermentation was carried out in shake flasks and results were compared with control at 30°C and pH 6.8 Induction of lipase activity promoted a concentrated fraction of extra-cell lipase (FOL) with 137mg/L and activity of 12000 U/L in submerged culture. The hydrolysis yielded YP/So = 0.4623 of free fatty acids. It was not observed that crude oil inhibit lipase activity, only products as known, whereas refined oil enhanced lipase activity. The micro-organism showed a production of a robust enzyme with prospects for technological application.

INTRODUCTION

In this work, systematic investigation of naturally occurring fungi producing lipase was carried out to select fungus with potential production of lipase for technological application. Lipases have been intensively investigated in micro-organisms, plants and animals sources [1], although the majority of the industrial enzymes are of microbial origin, and the most used microbial biocatalyst is lipase. In this way, yeast has been currently used for industrial production, so that CAL (*Candida antarctica* Lipase) and CRL (*Candida rugosa* Lipase) are the most used commercial lipases [2].

It is well known that fungal lipases have been deeply studied since 1950s due its physicochemical characteristics as biocatalysts: high thermal stability, mild pH conditions, activity in organic solvent and substrate specificity; that lead to find out several species as main producers of commercial lipases, like as *Aspergillusniger*[3], *Thermomyceslanuginosus* (*Humicolalanuginosa*) [4], *Mucormiehei (Rhizomucormiehei)* [5], *R. oryzae* [6], *R. niveus* and *R. japonicus* [7]. Fungi are preferable lipase sources because fungal enzymes are usually extracellularly presenting high mass amount, high stability, high activity and facility of extraction from culture media, and also triglycerides with unsaturated fatty acids or tween 80 are good inducer for lipase production in submerged culture. These characteristics are very important because lipase used in each application is selected based on its substrate and enantiospecificity, pH and solvent stability, as well as its temperature stability. As described by Gutarra et al. [8] an important requirement for industrial application of enzymes is thermal stability; that´s why the search for new lipases with improved thermal stability is still an important field of research for the application in industrial processes for fat and oil hydrolysis, modifications of triacylglycerol, and synthesis of ester with commercial importance [9]. Biotechnological processes using lipases from filamentous fungi are widespread, as these fungi are recognized for producing extracellular lipases, a characteristic that favors the recovery of the enzyme in a production process. Lipases are classified as triacylglycerol acylhydrolases, (E.C. 3.1.1.3) and are ubiquitous enzymes of considerable physiological and industrial significance being produced by bacteria, yeast and filamentous fungi from genera *Candida, Yarrowia, Pseudomonas*, *Geotrichum, Rhizopus, Mucor, Rhizomucor, Aspergillus*and*Penicillium* [10], [11], [12]. However, even with a wide variety of microbial lipases, the use of these enzymes on an industrial scale is still scarce due to high cost production [13], although there are many commercial lipases available from different origins and still are the most used enzymes in synthetic organic chemistry [14].

An intensive industrial application of these enzymes in the future depends on production facilities, cost reduction and biochemical characteristics what are reflections of the selection of highly productive strains, high-level expression of extracellular lipases in adequate hosts, development and optimization of production processes [15]. The great interest in finding new alternatives for obtaining biocatalysts with low costs and having the identical efficiency or greater than the existing one in the market is the using of this biocatalyst in the hydrolysis of triacylglycerol, which took us to explore the natural Amazonian resources to produce available products from enzymatic hydrolysis. Within this perspective, this paper describes the strategies of the usage of seed palm (*Elaeisguineensis*) to isolate endophytic microorganisms naturally adapted to the environment of oleaginous seeds, with potential to produce concentrated of lipase ready to

be applied in the hydrolysis of crude vegetable oils. In this work, effort was made to induce the production of a fraction of crude protein produced by filamentous fungi reach in lipase activity with the perspective to generate inputs to the cosmetic and fine chemistry industries.

MATERIALS AND METHODS

Fungi strains isolation, lipolytic activity detection and identifications

The endophytic fungi were isolated from pieces of palm seeds (1 cm^2) cultivated on semi-solid medium for five days at 30ºC according to the method describe [16]. Microorganisms were grown on plates with Sabouroud agar medium [17]. The isolated microorganisms were grown in specific media for the detection of lipolytic activity by Rhodamine B reactive [18]; [19]. The strains which showed maximum lipolytic activity were identified by classical methods (the macro and micro-morphology of vegetative and reproductive structures) in the Laboratoty of Fungal Culture Collection (Fiocruz-RJ-Brazil) according to usual methodology of this laboratory, and subsequently preserved in water : glycerol (95:5 v/v) and kept at 5ºC. These strains were also identified by molecular biology through the amplification of ITS regions specifics. The genomic cDNA was isolated and ITS region of rDNA was amplified, and the purified rDNA product was sequenced using primers forward (F) and reverse (R). Amplification of the ITS region was achieved with fungi-specific primers that amplify the ITS region from ascomycetes and basidiomycetes. The primers used were ITS2F (5'-GCATGCATGAAGAACGCAGC-3') and ITS2R (5'-TCCTCCGCTTATTGATATGC-3'). The conditions for reaction were as follows: Taq polymerase buffer 2.0 µL, dNTPs 200 mM, each primer 3.2 pM, Taq polymerase 5 U in a reaction mix of 20 µL. The amplification was done in a System 9700 Applied Biosystems with the following reaction conditions: initial denaturation at 95 ºC for 3 min followed by 14 cycles of denaturation at 95ºC for 30 s, annealing at 54.3ºC for 25 s, and extension at 60ºC for 4.0 min. There was a final extension step of 4.0 min at 60ºC with a 4ºC soak at the end. The capillary sequencing was performed on ABI 3130 equipment brand (Applied Biosystems) using a BigDye Kit V3.1 Cycle Sequencing Kit. The deduced sequence was subject to BLAST search tool for the close match in the database. The visualization of amplified fragments was performed by electrophoresis on 1% agarose gel.

Investigation of lipolytic activity of fungi strain in submerged culture.

The isolated filamentous fungi strains were investigated for potential lipolytic activity. 0.2 mL of spores of selected fungi strains in a concentration of 108 conidia/mL were inoculated in BOB medium containing 3% v/v of olive oil as inducer and the system was incubated at 30ºC with constant agitation of 120 rpm for 5 days. Aliquots of 2.0 mL were withdrawn every 24 hours for lipolytic activity and total protein quantification during time cultivation [20].

Preparation of lipolytic enzyme fraction concentrated

The culture supernatant was separated from the mycelium by filtration through sterilized Whatman® No. 1 filter paper, centrifuged at 8.000 rpm for 5 min, separated the supernatant from debris and the supernatant was lyophilized to reduce 70% of initial volume. Total enzyme precipitation was done by addition of ethanol (-20ºC), centrifuged at 10.000 rpm for 10 min. Enzyme precipitated (concentrated fraction of lipase (FOL) was resuspended by addition of buffer solution 0.1 M citrate/phosphate pH 6.5. Protein concentration measurements were determined by Bradford method employing bovine serum albumin as standard [21].

Lipolytic activity assay

The quantification of lipase activity was assayed using p-nitrophenylpalmitate (pNPP, Sigma-Aldrich®) as substrate. The reaction mixture was prepared by addition of 300 µL of sample (FOL) to 2.7 mL of pNPP solution, incubated at 30°C for 30 min, in the same buffer and pH as described above .The chromogenic product formation (pNP, para-nitrophenol) was monitored after pNPP hydrolysis by produced lipase [22], [23], [24]. Liberated p-nitrophenol was determined at 410 nm ($\varepsilon_{410\ nm\ =\ 0.0169\ \mu M}$-1.cm-1) using a UV-VIS spectrophotometer. A control was used as a blank replacing FOL by deionized water. In this work, one international unit (U) was defined as the amount of enzyme needed to liberate 1 µmol of p-nitrophenol per minute under the assay conditions, according to [25, 26].

Assay to determine the optimal temperature for lipase activity

The effect of different temperature on the lipase activity was evaluated by incubating 0,3 mL of FOL with 2.7 mL of pNPP reactive solution in different temperatures: 30, 40, 50, 60°C and 70ºC, using citrate-phosphate buffer pH 6.8 (50 mM) at 60 min.

Assay to determine the maximum time of thermal stability for lipase activity

After optimal temperature lipase activity had been determined, the thermal lipase stability was assayed. The FOL was incubated at optimal temperature for a period of 12 hour to evaluate the thermal stability of lipase activity. An aliquot of 0.3mL were withdrawn at each our and incubated in the presence of 2.7 mL of pNPP solution at optimal temperature for 30 min to evaluate the lipase activity. The tendency of lipase activity decrease was analyzed by graphical plotting.

Gel electrophoresis analysis

The sample (FOL) (16 µL) were treated with citrate-phosphate buffer, 2% SDS (Amresco®), 50 mMTris–HCl pH 6.8 (Amresco®), 10% glycerol (USB Corporation), and 0.1% bromophenol blue (Sigma-Aldrich®), in absence and presence of 100 mM DTT (Amresco®), by heating in a boiling water bath for 5 min. Polyacrylamide gel electrophoresis was carried out [27] using 12% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulphate at 150 V, 30 mA and 4 W. Protein were detected according colloidal method of Coomassie Brilliant Blue (Sigma-Aldrich®) [28]. Relative molecular mass were calculated using standards of Low MW Marker (GE Healthcare).

Hydrolysis of palm crude oil by fraction concentrated of lipase (FOL)

Amount of 250 mg of FOL, 3 mL (2.67 g) of palm oil and 20 mL of 50 mMTris-HCl pH 8.0 were added into 125 mL cylindrical glass flask. The reactant mixture was homogenized and subjected to agitation on magnetic stirrer at 120 rpm for 12 hours at 25 °C. Hydrolysis reaction was carried out and after this period of time, the free fatty acids released were titrated with 0,1 N sodium hydroxide by a factor equal to 0.9980. The samples were made in triplicate, followed by reference sample as a blank under the same conditions, except by FOL addition.

RESULTS AND DISCUSSION

Detection, selection, Isolation and identification of lipolytic microorganisms

Four strains of endophytic fungi detected and isolated from palm seeds were studied. Investigation with Rhodamine B resulted in a detection of lipolytic activity by halo formation with diameter of (1.5 mm; 1.8 mm; 2.0 mm; 2.1 mm: subtracted 0,5mm from inoculum), and posterior selection and isolation of only two strains, named D5-1B and D12-1A, which showed high activity hydrolysis by halo formation on the agar plate. D5-1.B showed better behave and activity than D12-1A, the result was confirmed by enzyme assay using pNPP as substrate. The two isolated strains were subjected of identification by molecular method and classical taxonomy for the certification of the mi-

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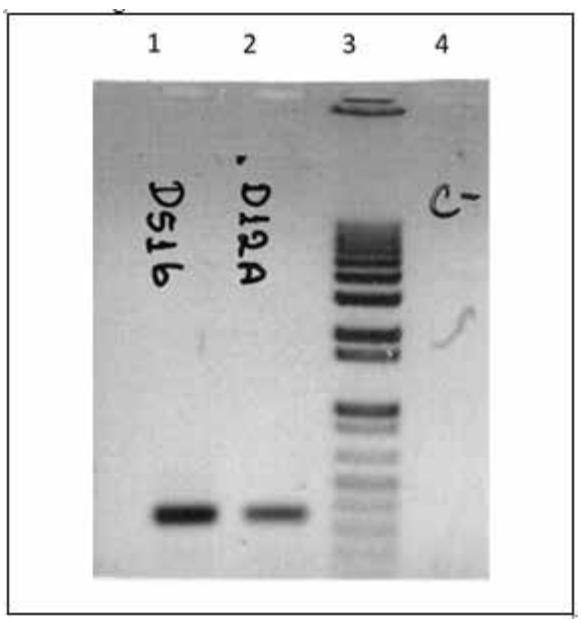
croorganisms. Molecular identification made from genetic material of hyphae and pellets from the two strains showed a similar electrophoretic profile, which shows the result of amplification fragments of 300 bp, and indicated that the two strains were the same microorganism, Figure 1. The results of sequencing and comparative analysis with databases by BLAST showed that the isolated microorganisms are *Penicilliumcitrinum*. The identification by classical taxonomy was done by Dr Maria Inez de M. Sarquis, from Fundação Oswaldo Cruz-FIOCRUZ (Brazil), and the result confirmed the identification obtained by molecular biology. In this way the certification of the microorganisms in question were *Penicilliumcitrinum*strains MIBA-D5-1B.

Figure 1. PCR- agarose gel electrophoresis: Column1 represent D5.1B, column 2 represents D12.1A. Column 3 is the standard marker of DNA and column 4 is the negative reference.



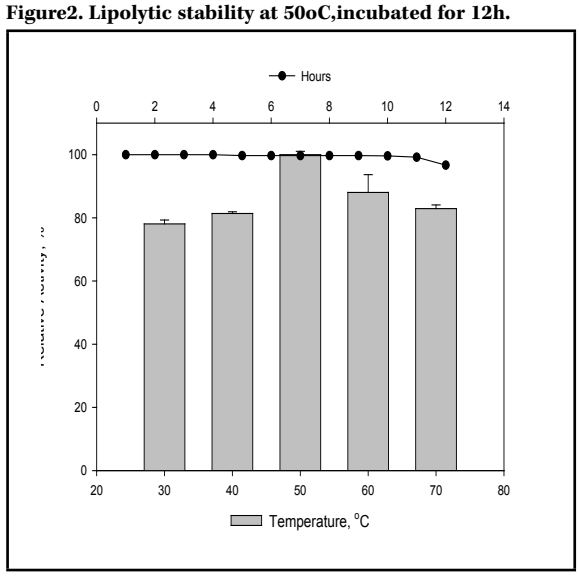
Biomonitoring of lipolytic enzyme production in submerged culture.

After microorganisms isolation and identification, it was proceeded the investigation of lipase production in submerged cultures of *P. citrinum*(strain D5-1B) by monitoring the production of lipase every 24 hours, using the adopted methodology of the hydrolysis of pNPP to release pNP [20], promoted by the biocatalyst (concentrated fraction of lipase - FOL) produced by identified microorganism. This methodology was initially used to obtain a general result for the lipase activity. Although it`s well known that this kind of substrate does not assure the real potentiality for the enzyme activity and in most cases it is not a final substrate to select enzymes to act as biocatalyst in the hydrolyze of crude vegetable oils, being necessary the investigation with crude oil to evaluate the potential for triacylglycerol hydrolysis by titrimetric methodology to quantify free fatty acids released into the medium. Chromogenic substrate such as pNPP is not a specific substrate and does behave as a general substrate. It is not adequate to select potential lipase for a specific fatty acid length chain [19], [8], even though in the presence of certain secondary metabolites that can act as inhibiter; although this methodology has been used in many experiments as a preliminary way of selecting and quantifying lipase activity [29].

Effect of temperature on lipase activity

The optimum temperature for lipase activity produced by *P. citrinum* in submerged culture was 50°C on pH 6.5. High values of lipase activity reached 12000 U/L, and from 60 oC to on the lipase enzyme lost parts of it activity (Figure 2). This result seems to be very interesting for technological application, due optimal lipolytic activity value be well defined at 50 oC. This result tends to direct lead the design of an experiment to use the FOL to be used as biocatalyst in the oil hydrolysis. A huge number of micro-organism has been evaluated as lipase producer and it is well known that enzyme from bacteria present high thermal stability [1], although fungi lipases present more stability and facility in the obtaining process.

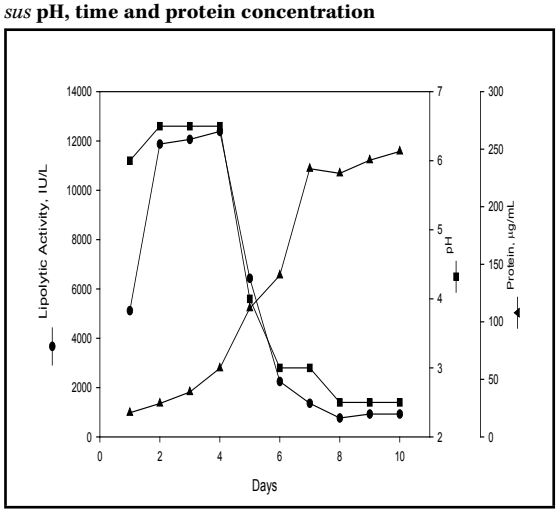
Figure2. Lipolytic stability at 50oC,incubated for 12h.



Effect of pH on the microorganism growth and lipase production

The submerged cultivation in BOB culture medium showed high activity on the fourth day reaching the value of 12,387.20 U/L. In comparison with the result obtained by Pimentel et al. [30], that studied a strain of *P.citrinum* isolated as a contaminant from olive oil presented an activity of 409 U/L after nine days of cultivation, this result obtained was higher than the value obtained by the contaminant and presented shorter time of cultivation than that one. In the same way result obtained with *Metarhiziumanisopliae* that produced extracellular lipase presented value of activity of 4,540 U/L with detergent induction [31]. This aspect is very important in an enzyme process production, which follows a tendency of technological application [32]. In Figure 3, was shown the profile of enzyme activity during 10 days of fermentation. It was observed that the values of enzyme activity is increased until the fourth day of fermentation, the maximum activity is kept from 2nd to 4th day. The optimum activity reach a value of 12.39 U/mL, and from the fifth day a decrease in the activity was observed (Figure 3). Decreasing in activity may be related to variation in pH and products released in the medium, as shown, where the highest values of enzymatic activity during the cultivation were observed at pH 6.5. The values of the activity variation during the time curse of cultivation were dependent of pH values and products (glycerol and free fatty acids). Based on these results could be inferred that the values of enzyme activity during the cultivation time were totally dependent on the values of pH change in the culture medium because the activity values decreased with the decrease of pH. These values were measured at 50 oC, after optimum temperature activity had been evaluated.

Figure 3. Lipase activity at 50oC 12.387,2 U/L: Activity *versus* pH, time and protein concentration



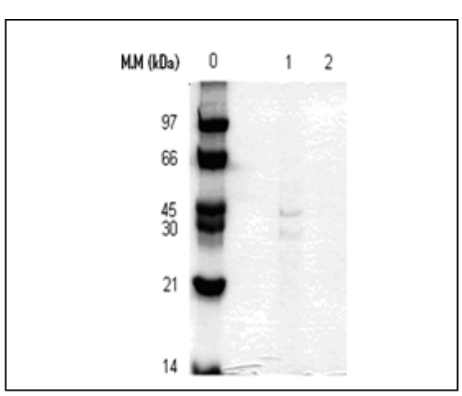
Thermal stability of lipase activity

After the incubation and monitoring the lipase activity at 50 °C, it was showed that the lipase concentrated remained its activity unchanged until 10 hours of incubation. Residual activity was measured to determine lipase stability after eleven hour of incubation, after this period of time a decreasing began and confirmation occurred after 12 hours in which time the experiment was terminated (Figure 2). We observed that thermal stability was strongly affected by pH variation of culture. This fraction FOL was more stable at pH 6.5 in culture. These results permitted to evaluate the thermal resistance of this enzyme, and to confirm the potential of this microorganism in producing a high stability lipase activity with perspective to be applied at crude oil hydrolysis. As described in the literature many enzymes produced by bacteria, fungi and yeast presented optimum activity above 60 oC, although neither of them presented a result with these characteristics of application.

Gel electrophoresis

The SDS-PAGE of the concentrated, with and without reducing agent (dithiothreitol, Sigma-Aldrich®), showed presence of only two bands with relative molecular mass of 41.7 kDa and 35.1 kDa (Figure 4). The molecular masses (MM) were calculated by linear regression of the log MM standards versus Rf (relative mobility), the equation of the line found was Y = 2.0717-0.9931X with the regression coefficient of 0.9825. The masses are found within the range reported in the literature, where most lipases have a molecular mass from 40 kDa to 50 kDa.

Figure 4. SDS-PAGE: column 0 molecular weight standard (Low marker Invitrogen), column 1 lipase fraction concentrated produced by D5.1B strain.



Hydrolysis of crude palm oil.

The hydrolysis reaction of crude palm oil catalyzed by FOL, showed high yield production of free fatty acids, the yield reached a value of 4,011.2 mmol fatty acids in 12 hours of reaction in the presence of 250 mg of FOL. This result indicates that the hydrolysis reaction released 334.27 mmol / hour of fatty acid or 5.57 mmol / min. The product yield coefficient ($Y_{P/So,}$ relates the micromoles of product formed by micromoles of initial substrate referred as linoleic acid molecular weight), found from this reaction was 46.74, a significant result justified by the value calculated for the apparent molecular weight of initial substrate (So) and apparent specific activity of FOL 8.06 U/mg [33]. It is important to know that free fatty acids based on linoleic acids presents 90.13% and the glycerol represents only 9.87% of total molecular weight of a generic triacylglycerol.

This work is the base investigation for endophytic producer lipolytic enzyme with technological perspective to be applied in the hydrolysis of crude palm oil to produce inputs for commercial products. As a complementary result, the half-life time of this enzyme activity seem to be high, even though it was not determined, and the resistance of this enzyme shows that it is very robust for using in a future scale up process.

CASE STUDY

The present study investigated if the enzymehas great potential to hydrolyze crude oil without pre-treatment. In this sense it is a fact that such kind of activity is required by the industry due the robustness in hydrolyze oil with many molecules dissolved that act as inhibiters. In this case FOL can be applied on raw material in a hydrolysis process to produce concentrate of free fatty acids to be used as inputs for cosmetic industry or as fatty acid patterns of commercial interest.

CONCLUSIONS

Several methods are been used for the screening of lipases from fungi with chromogenic lipid substrates, and sometimes the success of the assay depends on the fatty acids chain linked to the p-nitro-phenol; even the assay be positive, it is not a guarantee that the enzyme will hydrolyze crude oil. That's why is preferred to use titration as a complementary assay to investigate such real activity and possible application of the studied enzyme. In this work is described the investigation of endophytics fungus with potential to hydrolyze crude palm oil.

The Investigation of lipase production pointed that olive oil is one of the best inducers for this type of lipase producer. The submerged fermentation was carried out to obtain the enzyme fraction concentrated and was decisive for the determination of important parameters such as optimum time, temperature and pH of cultivation.

It is noteworthy that, based on the results of the literature, this concentrated enzyme showed great potential to hydrolyze of such crude oil. In this sense it is a fact that such kind of activity is required by the industry due the robustness in hydrolyze oil with many molecules dissolved that act as inhibiters of many enzymes with lipase activity. In this case FOL can be applied on raw material in a hydrolysis process to produce concentrate free fatty acids or isolated free fatty acid to be used as inputs for cosmetic industry or as fatty acid patterns of commercial interest, respectively. This amount of free fatty acid released is enough to initiate a biotechnological process for crude oil hydrolysis.

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REFERENCE

[1] Hasan F, Shah A A, Hameed A (2006), Industrial applications of microbial lipases. Enzyme and Microbial Technology, 39, 235-251. | [2] BussamaraR, Fuentefria A M, De Oliveira ES, Broetto L, Simcikova M, Valente P, Schrank A, Vainstein M H (2010), Isolation of a lipase-secreting yeast for enzyme production in a pilot-plant scale batch fermentation. Bioresource Technology, 101,268-275. | [3] Rai, B; Shrestha, A; Sharma, S; Joshi, J (2014), Screening, optimization and proves scale up for pilot scale production of lipase by Aspergillusniger. Biomedicine and Biotechnology, 2(3),54-59. | [4] Cruz-Ortiz, BR; Rios-Gonzales, LJ; Garci, YG; La Garza, JAR, Rodriguez-Martinez, J(2011), ImmobilizationofThermomyceslanuginosus Lipase in PVA-alginateBeads. J. Mex. ChemSoc, 55(3), 176-180. | [5] Krishna, S.H, Sattur, A.P; Karanth, N.G (2001), Lipase-catalysed synthesis of isoamylisobutyrate-optimization using a central composite rotabledesing. Process Biochemistry, 37, 9-16. | [6] Fernandez-Lafuente, R (2010), Lipase from Rhizomucormiehei as industrial biocatalyst in chemical process. Journal of Molecular Catalysis B: Enzymatic, 64, 1-22. | [7] Godfredson, S. E., in Microbial Enzymes and Biotechnology (eds Fogarty, W. M. and Kelly, E. T.), Elsevier Applied Sciences, The Netherlands, 1990, 255–273. | [8] Gutarra, MLE; Godoy, MG; Maugeri, F; Rodrigues, MI; Freire, DMG; Castilh, LR (2009), Production of an acidic and thermostable lipase of the mesophilic fungus Penicilliumsimplicissimum by solid-state fermentation. Bioresource Technology, 100. 5249-5254. | [9] Bosley, J.A; Peilow, A.D (1997), immobilization of lípases on porous poplypropylene: Reduction in esterification efficiency at low loading. Jour. Amer Chem Soc, 74(2),107-111. | [10] Sharma R, Chisti Y, Banerjee U C (2001), Production, purification, characterization, and applications of lipases. Biotechnology Advances, 19, 627–662. | [11] Mhetras NC, Bastawde KB, Gokhale DV. (2009), Purification and characterization of acidic lipase from Aspergillusniger NCIM 1207. Bioresource Technology, 100, 1486–1490. | [12] Hasan F, Shah AA, and Hameed A. (2009), Methods for detection and characterization of lipases: a comprehensive review. Biotechnology Advances, 27(6), 782-798. | [13] Paques FW, Macedo GA. (2006), Lipases de látex vegetais: Propriedades e aplicações industriais. Química Nova, 29(1) ,93-99. | [14] Reetz, M T (2002), Lipases as practical biocatalysts. Current Opinion in Chemical Biology, 6,145–150. | [15] Cammarota, MC and Freire DMG (2006), A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. Bioresource Technology, 97, 2195-2210. | [16] Souza AQL, Souza ADL, Filho AS, Pinheiro MLB, Sarquis MIM, Pereira, JO. (2004), Atividade antimicrobiana de fungos endofíticos isolados de plantas tóxicas da amazônia:Palicourealongiflora(aubl.) rich e Strychnoscogensbentham. Acta amazônica, Manaus, 34(2), 185 – 195. | [17] Keller FA, Hamilton JE and Nguyen QA. (2003), Microbial Pretreatment of Biomass. ApplBiochem Biotech, 105, 27–41. | [18] Shelley, A. W.; Deeth, H. C. e Macrae, I. C. (1987), Review of methods of enumeration, detection and isolation of lipolytic microorganisms with special reference to dairy applications. Journal of Microbiology Methods, 6, 123-137. | [19] Moraes, J.L.C. (2010), Biomonitoramento molecular para isolamento de fungos naturalmente ocorrentes (endofíticos) de sementes do dendezeiro (Elaeisguianensis) e de seu concentrado enzimático. Tese de Doutorado. UFPA. ICEN/PPGQ. 57p. | [20] Savitha J, Srividya S, Jagat R, Payal P, Priyanki S, Rashmi G. W., Roshini K. T. and Shantala Y. M. (2007), Identification of.potential fungal strain(s) for the production of inducible, extracellular and alkalophilic lipase. AfricanJournalofBiotechnology , 6(5), 564-568. | [21] Bradford, MM. (1976), A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding.AnalyticalBiochemistry,72, 248-254. | [22] Mahadik ND, Puntambekar US, Bastawde KB, Khire JM, Gokhale DV. (2003), Process for the Preparation of Acidic Lipase US Patent No. 06,534,303. | [23] Mahadik ND, Puntambekar US, Bastawde KB, Khire JM, Gokhale DV. (2002), Production of acidic lipase by Aspergillusniger in solid state fermentation. Process Biochem, 38, 715–721. | [24] Gupta N, Rathi P and Gupta R. (2002) Simplified para-nitrophenylpalmitate assay for lipases and esterases. Analytical Biochemistry, 311(1), 98-99. | [25] Dheeman DS, Antony-Babu S, Frias JM, Henehan GTM (2011), Purification and characterization of an extracellular lipase from a novel strain Penicillium sp. DS-39 (DSM 23773). J MolCatal B-Enzym, 72,256-262. | [26] Dheeman D.S., Frias J.M., Henehan G.T.M. (2010), Influence of cultivation conditions on the production of a thermostable extracellular lipase from Amycolatopsismediterranei DSM J. Ind. Microbiol. Biotechnol, 37, 1–17. | [27] Laemmli, UK. (1970), Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature,227, 680-685. | [28] Westermeier R. (2001) Electrophoresis in practice: a guide to methods and applications of DNA and protein separations. Third edition. Wiley-vchverlag-gmbh, Weinheim, Germany. p. 183-200. | [29] Margesin R, Feller, G, Hämmerle,M, Stegner U & Schinner F. (2002), A colorimetric method for the determination of lipase activity in soil. Biotechnology Letters, 24, 27–33. | [30] Pimentel, M. C.B; Melo, EHM, Filho JLM, Ledingham WM, Durán N. (1996), Lipase from a BrazilianstrainPenicilliumcitrinumcultured in simpleandinexpensivemédium .Heat-denaturation, kinetics and pH stability. Applied Biochemistry and Biotecnology, 66,186-195. | [31] Silva, WOB, Mitidieri S, Schrank A, Vainstein MH (2005), Production and extraction of an extracellular lipase from the ebthomopathogenic fungus Metarhiziumanisopliae. ProcesBiochemistry, 40(1), 321-326. | [32] Sebrão, D. et al. (2007), Imobilizção de lípases em filme de caseinato de sódio/glicerol: aplicação na síntese de ésteres. Quím. Nova, 30(5) ,1182-1187. | [33] Carvalho PO, Campos PRB, Noffs MA, Oliveira JP, Shimizu MT, Silva DM. (2003), Aplicação de lipases microbianas na obtenção de concentrados de ácidos. Quím. Nova, 26(1), 75-80. |