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Sequential Production of Amylolytic and Lipolytic Enzymes by Bacterium Strain Isolated from Petroleum Contaminated Soil

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Abstract Amylases and lipases are highly demanded industrial enzymes in various sectors such as food, pharmaceuticals, textiles, and detergents. Amylases are of ubiquitous occurrence and hold the maximum market share of enzyme sales. Lipases are the most versatile biocatalyst and bring about a range of bioconversion reactions such as hydrolysis, inter-esterification, esterification, alcoholysis, acidolysis, and aminolysis. The objective of this work was to study the feasibility for amylolitic and lipolytic production using a bacterium strain isolated from petroleum contaminated soil in the same submerged fermentation. This was a sequential process based on starch and vegetable oils feedstocks. Run were performed in batchwise using 2% starch supplemented with suitable nutrients and different vegetable oils as a lipase inducers. Fermentation conditions were pH 5.0; 30°C, and stirred speed (200 rpm). Maxima activities for amyloglucosidase and lipase were, respectively, 0.18 and 1,150 U/ml. These results showed a promising methodology to obtain both enzymes using industrial waste resources containing vegetable oils.

Keywords Amylolytic enzymes · Lipolytic enzymes · Submerged fermentation · Petroleum

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Introduction

The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe. At least 75% of all industrial enzymes (including lipases) are hydrolytic in action. Proteases dominate the market, accounting for approximately 40% of all enzyme sales [1].

Recycling perhaps is going to be one of the most essential waste management strategies in the contemporary world. Recently, with the increasing number of environmental legislations and public concern on environmental pollution, industrial commitment on waste treatment and disposal has become imperative. Bioconversion can be carried out using extracellular or intracellular enzymes as a whole cell biocatalyst; therefore, microorganisms from the petroleum contaminated soil and others sources can be used to produce these enzymes.

Lipases catalyze the bioconversion (synthesis as well as hydrolysis) of lipids within the organisms [2–5]. To react with water-insoluble lipids, lipases possess the unique feature of acting at an interface between an aqueous and a non-aqueous phase. Lipases also possess the stereoselectivity and the reaction capability in a non-aqueous phase. In addition to ester hydrolysis and inter-esterification, they catalyze alcoholysis, acidolysis, and amidolysis. By biochemical and three-dimensional structural studies, they were shown to act as serine hydrolases with a serine–histidine–aspartate triad at the active site, like serine proteases. The sequence around the reactive serine residues in the lipases from human pancreas and *Mucor miehei* is conserved. Homologous sequences have been found in all of the lipases sequenced. However, the mechanism of activation at interfaces and the way the bulky substrate gains access to the buried active site is not understood. The three-dimensional structures of other lipases may provide further insight into these questions. Hence, lipases have tremendous potentials in areas such as food technology, biomedical sciences, and chemical industries [6].

The major factor of expression of lipase activity has been the carbon source, as lipases are by and large inducible enzymes, thus are produced in the presence of oil or any other inducers, such as fatty acids, bile salts, triacyl glycerols [4, 5]. Majority of lipases exhibit high activity toward lipids with fatty acid residues of C_8 to C_{18} chain length [6]. Lipases are selected for each application based on its substrate specificity such as fatty acid alcohol, position and stereospecificity, as well as temperature and pH stability [2–5].

Amyloglucosidase and amylases are also produced by microorganisms, including bacteria, fungi, and yeast, but a single strain can produce both these enzymes as well. Digestion of starch is effected by hydrolyzing enzymes in a complex process which depends on many factors; these include the botanical origin of starch, whether the starch is amorphous or crystalline, the source of enzymes, substrate and enzyme concentration, temperature and time, as well as the presence of other substances in the multicomponent matrix in which starch occurs naturally, e.g., cereal grains [7, 8]. Native starch is digested (i.e., hydrolyzed) slowly compared with processed (gelatinized) starch whose crystallinity has been lost and where the accessibility of substrate to enzymes is greater and not restricted by α-glucan associations such as double helices (especially in crystallites) or amylose–lipid complexes (in cereal starches). Starch hydrolysis by specific amylolitic in model (in vitro) and more broadly in vivo systems, two enzymes carry out the conversion of starch to glucose, namely: (1) α-amylase, that cuts the large α-1,4-linked glucose polymers into smaller oligomers and (2) glucoamylase (synonym amyloglucosidase—also referred to as glucogenic enzyme, starch glucogenase, γ-amylase; exo-1,4-a-D-glucan glucanohydrolase, EC-3.2.1.3) that hydrolyzes oligomers to glucose [8–10].

Benjamin and Pandey [11] cultivated *Candida rugosa* on coconut oil cake for lipase production using solid state fermentation and submerge fermentation systems. Enzyme yields were higher in the former. Several carbon sources—individually and combinations—were tested for their efficiency to produce both lipolytic and amylolitic enzymes [12]. In this work, the production of lipolytic and amylolitic enzymes were studied by a bacterium strain isolated from petroleum-contaminated soil under submerged fermentation using three inducers (olive, castor, and coconut oils).

Experimental Procedures

Microorganism

A bacterium strain is isolated from petroleum-contaminated soil and codified as Biopetro-4. The bacteria was maintained on slants of nutrient agar at 30 °C, stored at 4 °C, and subcultured once a month.

Substrates and Chemicals

Olive, castor, and coconut oils (low acidity) were used as inducers (lipase production) and carbon sources and were purchased at a local market. Potato corn starch was purchased from Synth (Diadema-SP, Brazil). The other reagents were standard laboratory grade and purchased either from Aldrich Chemical Co. (Milwaukee, WI, USA) or Sigma Chemical Co. (St. Louis, MO, USA).

Microorganism Cultivation

A bacterium strain was cultivated in Erlenmeyer flasks containing 200 ml medium with the following composition (%, *w*/*v*): KH_2PO_4 (0.1), $MgSO_4{\cdot}7H_2O$ (0.05), $NaNO_3$ (0.3), yeast extract (0.6), peptone (0.13), and starch (2%) as carbon source. The fermentation conditions were: initial pH 5.0; incubation temperature 30°C; and stirred speed (200 rpm). After 72-h cultivation, olive oil was added as lipolytic enzyme inductor. Castor and coconut oils were also tested as inducers. At the end of fermentation, the cells were harvested by centrifugation. Recovered cells were assayed for protein content by Lowry's method [13]; afterwards, cells were dried at 100 °C up to constant dry weight [12]. Enzyme extractions were stored at −5°C for further determination of both amyloglucosidade and lipase activities.

Amyloglucosidase Activity

Amyloglucosidase activities were assayed according to a modified method proposed by Soccol et al. [14]. One unit (U) of amyloglucosidase activity was defined as the amount of enzyme that liberated 1 μmol of reduction sugar per minute (μmol min^{-1}).

Lipase Activity

Lipolytic activities were assayed by the oil emulsion method according to a modification used by Soares et al. [15]. The substrate was prepared by mixing 50 ml of the olive oil with 50 ml of gum Arabic solution (7% *w*/*v*). The reaction mixture containing 5 ml of the oil

emulsion, 2 ml of 100 mM sodium phosphate buffer (pH 7.0), and enzyme extract (1 ml) was incubated in a thermostated batch reactor for 60 min at 37°C. Samples were taken at 5, 10, 15, 30, and 60 min. A blank titration was done with a sample where the enzyme was replaced with distilled water. The reaction was stopped by the addition of 10 ml of acetone–ethanol solution (1:1). The liberated fatty acids were titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per minute (μmol min^{-1}) under the assay conditions (37 °C, pH 7.0, 150 rpm).

Results and Discussion

The objective of this work was to study alternatives for the sequential production of amylolitic and lipolytic enzymes by a bacterium strain isolated from petroleum contaminated soil under submerged fermentation using different inducers.

A typical curve showing the production of both enzymes is displayed in Fig. 1. The maximum amyloglucosidade activity (0.18 U/ml) occurred at 72 h of fermentation when the starch consumption reached approximately 50% from the initial value (Figs. 2 and 3). After this, olive oil was added as an inducer for lipase production, and high lipolytic activity (1,050 U/ml) was observed after an additional 24h cultivation. The biomass concentration also increased, probably due to the suitability of this carbon source for the bacterium strain, which also explained the slow down on the starch consumption.

In this context, the influence of the addition of different vegetable oils (olive, coconut, or castor oils) on the lipase production was further investigated.

Runs carried out with coconut oil gave the highest activity for lipase and lowest amyloglucosidase activity at pH≅5, with exception of initial and final time, pH≅7 (Table 1, Figs. 4, 5, 6, and 7). The maxima activity for amyloglucosidase was 0.18 U/ml and lipase was 1,675 U/ml (Figs. 4 and 5); these values indicated that the highest substrate affinity was obtained for coconut oil. As can be seen in Figs. 1, 2, 3, 4, 5, 6, 7, maxima enzyme activities were found after the addition of vegetables oils, with exception of amyloglucosidase in olive oil. A slight increase in the pH value was also observed after the addition of

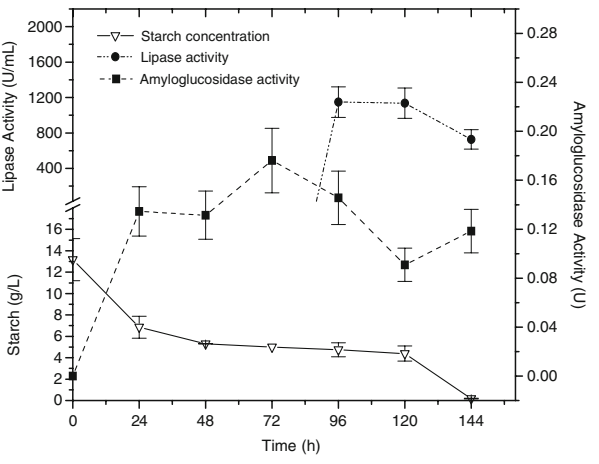

Fig. 1 Lipase and amyloglucosidase activities produced by a bacterium strain isolated from petroleum-contaminated soil under submerged fermentation using starch as carbon source and olive oil as lipase inducer

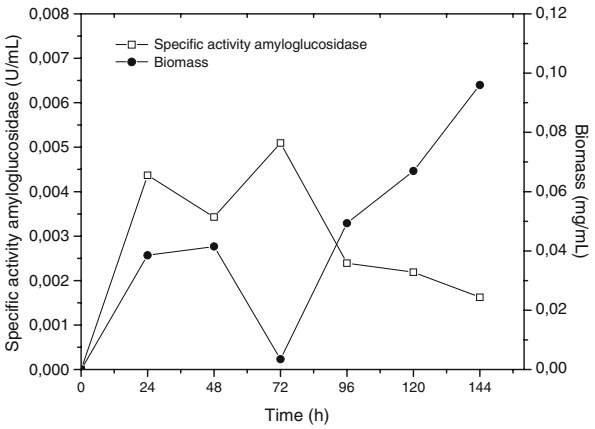

Fig. 2 Biomass of selection of amyloglucosidase in olive oil, activity specific enzymatic

olive oil, 7<pH<8 (Table 1, Figs. 1, 2, and 3). In this case, the lower amyloglucosidase activity was due possibly to the microorganisms' adaptation to the vegetables oils, the low starch, and biomass concentration. These systems also suggest protease production, which degraded the enzymes (Figs. 2, 3, 6, and 7).

Experiments reported by Bascaran et al. [15] using *S. clav* for protease production after nutritional shift-down indicated that initiation of protease formation is observed with the decrease in nutrients availability; it possibly attacks others enzymes, as lipase and amyloglucosidase. The mechanisms by which control of protease production is achieved in many prokaryotes systems are still unknown under alkaline or acid conditions [16].

The stability studies on lipase focused mainly on the conformational changes in relation to the topology of protein, caused also by pH change, reducing or imposing strain on the "lid" overarching the active centre, thereby opening or shutting down the catalytic center for substrate binding [10]. To improve the lipase and amyloglucosidase activities, strategies that function in enhancing the substrate availability should be exploited, and a common strategy to achieve these goals is to use emulsifiers such as *n*-hexadecane and gum Arabic [16].

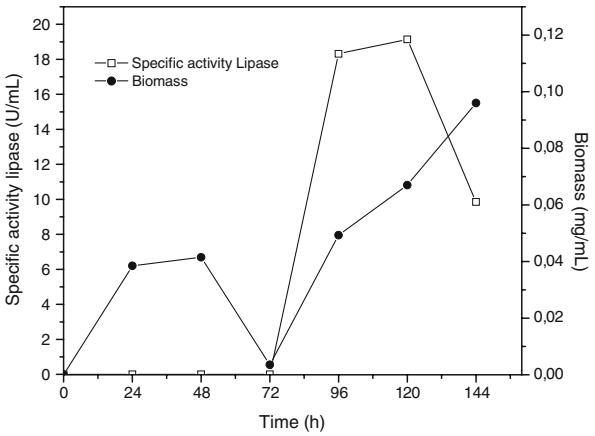

Fig. 3 Biomass of selection of lipase in olive oil, activity specific enzymatic

Table 1 pH changes during cultivation of bacterium strain using different vegetable oils as lipase inducer.

Cultivation time (h)	pH values		
	Coconut oil	Castor oil	Olive oil
0	5.00	5.00	5.00
24	5.02	5.03	7.65
48	5.06	5.14	8.02
72	5.00	5.00	7.28
96	5.06	5.05	7.65
120	5.18	5.06	7.82
144	7.00	5.44	7.03

Three-dimensional structures of enzymes are easily affected from the reaction parameters, and the changes in the structure cause a decrease or increase in the catalytic power of the enzyme. Due to the uncontrolled reaction parameters and nature of the enzymes (source and production procedure of enzyme), different kinetic models or constants can be obtained from the reaction of the same enzyme. In this study, lipase activity showed promising results.

Conclusions

This study verified that the submerged fermentation using a bacterium strain isolated from petroleum-contaminated soil was a suitable method to obtain both amylotic and lipolytic enzymes. The lipase formation was growth-associated when cells were cultivated in all tested vegetable oils tested. Although olive and castor oils show to be good inducers for the lipase production, maximum activity was attained when coconut oil was used. Maxima activities for amyloglucosidase and lipase were 0.18 and 1,675 U/ml, respectively.

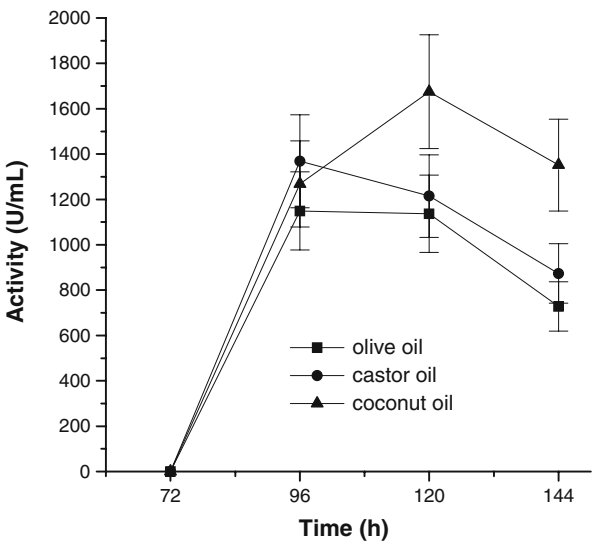

Fig. 4 Selection of lipase in different vegetable oils, activity enzymatic

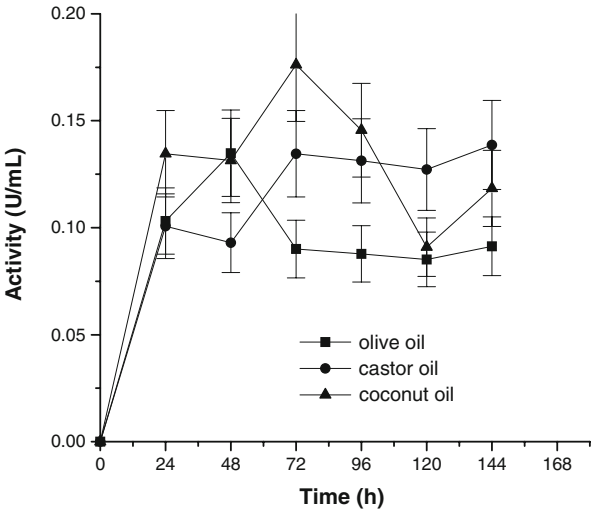

Fig. 5 Selection of amyloglucosidase in oils different, activity enzymatic

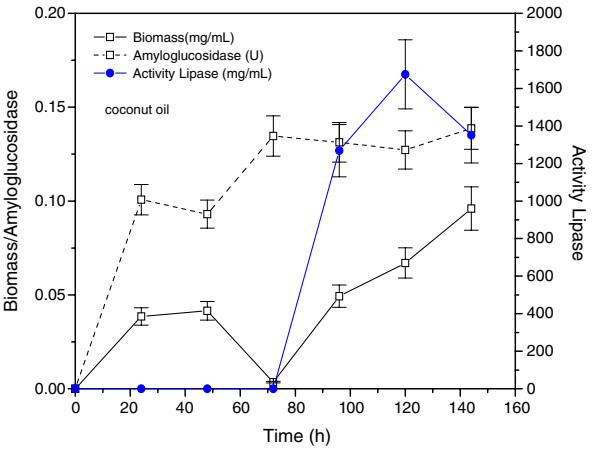

Fig. 6 Biomass of selection of enzymes tested in coconut oil, activity enzymatic

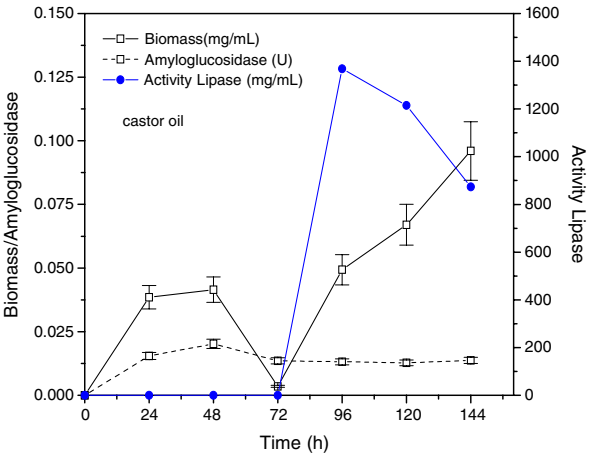

Fig. 7 Biomass of selection of enzymes tested in castor oil, activity enzymatic

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References

1. Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology Advances*, *19*, 627–662.
2. Lara, P. V., & Park, E. Y. (2004). Potential application of waste activated bleaching earth on the production of fatty acid alkyl esters using *Candida cylindracea* lipase in organic system. *Enzyme and Microbial Technology*, *34*, 270–277.
3. Koufodimos, G., & Samaras, Z. (2002). Waste management options in southern Europe using field and experimental data. *Waste Management*, *22*, 47–59.
4. Lara, A. V., & Park, E. Y. (2003). Lipase-catalyzed production of biodiesel fuel from vegetable oils contained in waste activated bleaching earth. *Process Biochemistry*, *38*, 1077–1082.
5. Mittelbach, M. (1990). Lipase catalyzed alcoholysis of sunflower oil. *Journal American Oil Chemistry Society*, *61*, 168–170.
6. Kordel, M., Hofmann, B., Schomburg, D., & Schmid, R. D. (1991). Extracellular lipase of *Pseudomonas* sp. Strain ATCC 21808: Purification, characterization, crystallization, and preliminary X-ray diffraction data. *Journal of Bacteriology*, *173*, 4836–4841.
7. Sayikaya, E., Higasa, T., Adachi, M., & Mikami, B. (2000). Comparison of degradation abilities of α- and β-amylases on raw starch granules. *Process Biochemistry*, *35*, 711–715.
8. Tester, R. F., Karkalas, J., & Qi, X. (2004). Starch structure and digestibility enzyme-substrate relationship. *World's Poultry Science Journal*, *60*, 186–195.
9. Guimarães, L. H. S., Nogueira, S. C. P., Michelin, M., Rizzatti, A. C. S., Sandrin, V. C., Zanoelo, F. F., et al. (2006). Screening of filamentous fungi for production of enzymes of biotechnological interest. *Journal Microbiology*, *37*, 474–480.
10. Pandey, A., Selvakumar, P., Soccol, C. R., & Nigam, P. (2007). Solid state fermentation for the production of industrial enzymes. Retrieved 16 March 2007 from http://www.ias.ac.in/currsci/ju110/articles23.htm.
11. Benjamin, S., & Pandey, A. (2001). Isolation and characterization of three distinct forms of lipases from *Candida rugosa* produced in solid state fermentation. *Brazilian Archives of Biology and Technology*, *44* (2), 213–221.
12. Chi, Z., Ma, C., Wang, P., & Li, H. F. (2001). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. *Bioresource Technology*, *98*, 534–538.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randal, R. J. (1997). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *33*, 206–221.
14. Soccol, C. R., Marin, B., Raimbault, M., & Lebeault, J. M. (1994). Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation. *Applied Microbiology and Biotechnology*, *41*, 330–336.
15. Rooney, D., & Weatherley, L. R. (2001). The effect of reaction conditions upon lipase catalysed hydrolysis of high oleate sunflower oil in a stirred liquid–liquid reactor. *Process Biochemistry*, *36*, 947–953.
16. Bascaran, V., Hardisson, C., & Brana, A. F. (2001). Regulation of extracellular protease production in *Streptomyces clavuligerus*. *Applied Microbiology and Biotechnology*, *34*, 208–213.