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BIOPROSPECÇÃO DE LIPASES DE FUNGOS DE SEDIMENTOS MARINHOS DA COSTA CEARENSE (NORDESTE BRASILEIRO)

BIOPROSPECTION OF FUNGI LIPASE FROM MARINE SEDIMENT OF CEARÁ COAST (NORTHEASTERN, BRAZIL)

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RESUMO

Este trabalho reporta a bioprospecção de lipases de fungos de sedimentos marinhos da Costa Cearense (Nordeste Brasileiro). Cinco cepas fúngicas foram isoladas de sedimentos marinhos (SM1-SM5), coletados na Barra do Ceará, Fortaleza-CE. Foram submetidos ao teste de atividade lipolítica através do método de difusão em gel, utilizando-se o azeite de oliva como substrato específico de lipases e tributirina para esterases. Rodamina B foi utilizado como o reagente colorimétrico. Das cinco cepas fúngicas testadas, três (SM1, SM2 e SM3) se mostraram ativas especificamente para atividade de lipases (AL), apresentando de moderada a elevada AL (2,4 <AL ≤ 3,8). Dentre essas, um (SM2) apresentou elevada atividade lipolítica, com valor de AL igual a 3,8, significantemente semelhante ao controle positivo (*Aspergillus niger*). Os isolados foram identificados de acordo com suas características morfológicas e classificados como *Paecilomyces* sp. (SM1), *Aspergillus* sp. (SM2) e *Aspergillus flavus* (SM3).

Palavras-Chave: Atividade lipolítica, Fungos marinhos, *Paecilomyces* sp., *Aspergillus* sp.; *Aspergillus flavus*;

Abstract

This work reports the bioprospection of fungi lipase from marine sediments of Ceará Coast (Northeastern Brazil). Five fungi strains were isolated from marine sediments (SM1 - SM5), collected at Barra do Ceará, Fortaleza-CE. They were subjected to the test of lipolytic activity by gel diffusion method, using olive oil and tributyrin as lipase-specific and esterase-specific substrate respectively. Rhodamine B was employed as colorimetric reagent. Of the five tested fungi strains, three (SM1, SM2, and SM3) were specifically positive for the lipase activity (LA), with moderate to high LA (2.4 <LA ≤ 3.8). Among these, one (SM2) showed high lipase activity, with a LA value equal to 3.8, quite similar to the positive control (*Aspergillus niger*). Isolates were identified according to their morphological characteristics and classified as *Paecilomyces* sp. (SM1), *Aspergillus* sp. (SM2) and *Aspergillus flavus* (SM3).

***Keywords*:** Lipase activity; Marine Fungi; *Paecilomyces* sp.; *Aspergillus* sp.; *Aspergillus flavus*;

1. INTRODUCTION

Lipases (EC 3.1.1.1) are extracellular enzymes of animal, microbial or plant origin. They are often employed in synthetic organic chemistry for the hydrolysis of fats and oils as well as in ester synthesis and resolution of drugs chiral centers (SAID; PIETRO, 2004). They act in aqueous-organic interface catalyzing the hydrolysis of triglycerides, releasing fatty acids and glycerol. At low water concentrations they can also catalyze reactions of esterification, transesterification or interesterification. Besides the classical advantages of enzymes employment microbial lipases still have a number of additional advantages when compared with the lipase extracted from animal and vegetable sources. These advantages are related to some peculiar characteristics of lipases as its regioenantioselectivity and stability at high temperatures and at a wide pH range (VARGAS, 2004).

Lipases are used as catalysts in the synthesis of chiral pharmaceutical intermediates as precursors for antidiabetic, antivirals, anti-Alzheimer's drugs as well as in the synthesis of anti-cholesterol and anxiolytic drugs (PATEL, 2008).

The discovered methods of new enzymes for the development of industrial scale biocatalysis usually involve the exploitation of biodiversity through selected techniques of microorganisms, plant or animal cells in the search for new catalysts. In that case, microorganisms are of particular interest due to short generation times and the great diversity of strains as well as metabolic processes and involved enzymes. The number of quite different microorganisms in nature that can be tested is certainly not very limited. Microorganisms isolated in Brazil demonstrated excellent biocatalysis potential on different interesting organic reactions such as in the hydrolysis of marine oils and the synthesis of polyunsaturated fatty acids and aroma esters. It’s also worth to mention that fungi isolated from marine organisms of the Ceará coast have been reported as lipases producers (MAGALHÃES, 2011).

Considering the foregoing the present work aimed the bioprospection of lipase activity of fungi isolated from marine sediments of Ceará coast in northeastern Brazil.

2. MATERIALS AND METHODS

2.1 *Sample collection, isolation and identification of fungi*

Samples of marine sediments (pieces of wood) were collected at Praia da Barra do Ceará, in Fortaleza-CE (0.38°35'15'' W; 0.3°41'54'' S). They were placed in sterile plastic bags and then transferred to the laboratory for cleansing and for the isolation of fungi following the methodology proposed by Phipps et al. (2004) with adaptations. Once in the lab, the sediments were washed with tap water and then with distilled water. Further cleansing was done by soaking it in sodium hypochlorite solution 5% for 5 min. The outer layer of the sample was scraped off and discarded. The samples were still soaked in 70% alcohol solution for 5 min prior to the exposition of the inner layer of which small pieces were finally collected and placed in Petri dishes under aseptic conditions. Isolation of fungi was performed in triplicate and the culture medium used was Potato Dextrose Agar (PDA) supplemented with commercial solutions of antibiotics ampicillin and tetracycline (125 mg/mL) prepared with artificial seawater synthesized in the laboratory (ASW). The plates were sealed with a thin plastic film and incubated at room temperature (± 30 °C) for seven days. Thereafter the healthiest colonies were inoculated in Petri dishes containing PDA-ASW for the purification of the cultures. Subsequently the isolates were stored in ampoules (Mycology) containing PDA medium-AMS. Shortly thereafter identification of fungi was done by Prof. Dr. Francisco das Chagas de Oliveira Freire (Embrapa-CE) through morphological analysis using taxonomic keys (SINGH et al., 1991).

2.2 *Preparation of culture media for primary selection of lipolytic fungi*

The culture media for primary selection of lipolytic fungi were prepared according to methodology proposed by Magalhães (2011). The culture enzyme medium 1 (EM1) consisted of 20.0 g/L peptone, 0.5 g/L yeast extract, 5.0 g/L of ammonium sulfate [$(NH_4)_2SO_4$], 1.0 g/L of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 1.0 g/L sodium chloride (NaCl), 1.0 g/L anhydrous monobasic potassium phosphate (KH_2PO_4), 20.0 g/L agar, 10.0 mg/L of Rhodamine B, 20.0 mL/L of olive oil (Gallo®), 5.0 g/L arabic gum powder. One liter of phosphate buffer (PB) was previously prepared with Milli-Q water, 0.93 g of anhydrous monobasic sodium phosphate (NaH_2PO_4) and 1.74 g

dibasic sodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$). The pH was adjusted at 7.0 to 7.5. The olive oil emulsion (OOE) was subsequently prepared in a 250 mL erlenmeyer flask by mixing 5.0 g of arabic gum powder, 20.0 mL of olive oil and 100 mL of sodium phosphate buffer. Emulsification was achieved by stirring on a magnetic stirrer for 30 minutes. The emulsion was added to an erlenmeyer flask containing 880 mL of the buffer solution prior to the addition of all the other above mentioned reagents. The culture medium was sterilized in an autoclave for 15 minutes at 121 °C and 1 atm. The non-enzyme medium (NEM1) was prepared lacking the addition of enzyme substrate (Gallo® olive oil emulsion). At this stage previously sterilized Petri dishes (80 x 15 cm) were filled with 20 mL of enzymatic and non-enzymatic culture media in laminar flow and cooled to solidification. The plates were stored in refrigerator (5 °C) until use.

2.3 Preparation of culture media for secondary selection of lipolytic fungi

The culture media for secondary selection of lipolytic fungi were prepared by adapting the methods proposed by Colen, Junqueira and Moraes-Santos (2006) and Magalhães (2011). The culture media for enzymatic and non enzymatic secondary screening were prepared according to previously described methods without addition of agar and Rhodamine B for enzyme medium 2 (EM2) and without addition of agar, Rhodamine B and olive oil emulsion to the non-enzyme medium 2 (NEM2).

2.4 Preparation of gels for characterization of lipolytic activity

The gels for characterization of lipase activity were prepared by adapting the methods proposed by Colen, Junqueira and Moraes-Santos (2006) and Magalhães (2011). Initially three distinct media were prepared with the above described buffer solution. Gel 1 (G1) was composed of agar (20 g/L), Rhodamine B (0.001%) and the previously described olive oil emulsion (20 mL/L). Gel 2 (G2) was composed of agar (20 g/L), Rhodamine B (0.001%) and tributyrin emulsion (20 mL/L) prepared in similar way as the described olive oil emulsion but putting tributyrin in the place of olive oil. Gel 3 (G3) was prepared with agar (20 g/L) and Rhodamine B (0.001%). The pH of the gels was adjusted to 7.0 and then they were sterilized in an autoclave for 15 minutes at 121 °C and 1 atm. Previously sterilized Petri dishes (80 x 15 cm) were then filled with

20 mL of media in laminar flow cabinet and cooled to solidification. The plates were stored in refrigerator (5 ºC) until use.

2.5 *Primary selection of lipolytic fungi*

The primary selection of lipolytic fungi was performed by adapting the methods proposed by Ionita et al. (1997) and Magalhães (2011). The tests were performed by solid state fermentation in Petri dishes using fungi cells as enzymes sources. For the assays 5 mm discs of each isolate previously grown on PDA for 7 days were transferred to Petri dishes containing culture EM1 and incubated for 1-5 days at room temperature (± 30 °C) for vegetative growth. The tests were performed in triplicate and the fungus *Aspergillus niger* was used as positive control (IONITA et al., 1997). For the negative control the fungi were than transferred to NEM1.

2.6 *Secondary selection of lipolytic fungi*

The lipolytic fungi secondary selection was performed based on methods proposed by Kouker and Jaeger (1987), Ionita et al. (1997) and Colen, Junqueira and Moraes-Santos (2006) with adaptations. The assays were performed employing the filtrates of fungi pre-selected as sources of enzymes. Five millimeters discs of each fungal strain previously grown in EM1 (without Rhodamine B) for 7 days were inoculated in sterile eppendorf tubes containing 1 mL of enzyme culture medium 2 and left in vegetative growth for 72 h in orbital Shaker (125 rpm) at 30 °C. Biomass was then separated by centrifugation (at 9,000 g for 15 minutes) and discarded. The filtered material (enzyme extract) were then taken for tests. Fermentations were performed in triplicate and the fungus *Aspergillus niger* was used as positive control Ionita et al. (1997). For the negative control the fungi were inoculated in a non-enzyme culture medium 2. Subsequently the detection of lipolytic activity was performed in Petri dishes containing the media G1, G2 and G3. Therefore three wells (5 mm in diameter) were drilled in each one of triplicate plates with the help of a sterile stainless steel cylinder. In each well 20 mL of each filtrate were added and the plates were then incubated at room temperature (30 ± 2 °C) for 24 h.

2.7 Characterization of enzyme activity

The characterization of enzyme activity was performed by adapting the methods proposed by Ionita et al. (1997), Colen, Junqueira and Moraes-Santos (2006) and Magalhães (2011). The enzyme activity was detected by observing the fluorescent halos (FH) in the plates under irradiation with UV light (365 nm). The halos were measured with calipers. For primary screening the enzyme activity was expressed according to the formula LA = R/r. Where LA means lipolytic activity. R variables are the values of fluorescent halos radius and r the values of the non-fluorescent ones. The strains with lipolytic activity equal to or greater than the positive control were considered promising and are therefore subjected to secondary screening. For the secondary selection the enzyme activity was expressed only by measuring the FH and the isolates were classified as low lipase activity, LLA ($0<FH\leq1.5$), moderate lipase activity, MLA ($1.5<FH\leq 3.0$), and high lipase activity, EAL ($FH > 3.0$).

3. STATISTICAL ANALYSIS

Means and standard deviations were determined and subjected to analysis of variance (ANOVA) using the *GraphPadPrism* program (version 5.0). Significative differences ($p<0.05$) between means were determined by multiple comparison Tukey test.

4. RESULTS AND DISCUSSIONS

In this study five filamentous fungi were isolated from marine sediments (SM1-SM5). They were identified trough their morphological features and classified as *Paecilomyces* sp. (SM1), *Aspergillus* sp. (SM2) and *Aspergillus flavus* (SM3). It was not possible to identify two fungal strains coded as SM4 and SM5 based on the morphology.

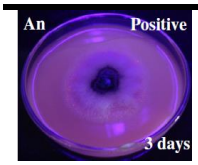
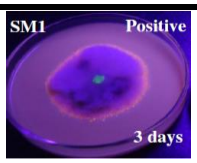
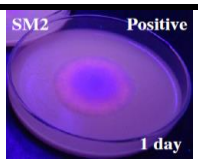
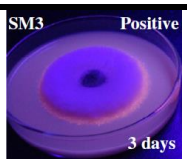
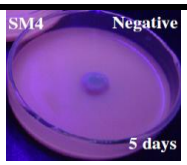
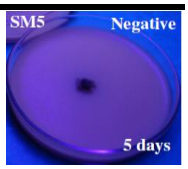
Numerous screenings of microorganism's lipase activity employ as standard methods the use of simple triglycerides and natural oils as enzyme inducing carbon sources. The employed colorimetric reagents are Nile Blue, Fast Blue B, Victoria Blue B, 1,2-o-dilauril-rac-glycero-3-glutaric acid resorufinil ester and Rhodamine B (JAEGER et

al., 1994; GUDELJ, 1998; CARDENAS et al., 2001; COLEN, JUNQUEIRA and MORAES-SANTOS, 2006).

In order to accomplish the primary selection of lipolytic fungi it was adopted the qualitative method using olive oil (Gallo®) as a carbon source and enzyme inducer and Rhodamine B as a colorimetric reagent.

Of the five tested marine isolates, three (60%) strains, *Paecilomyces* sp. (SM1), *Aspergillus* sp. (SM2) and *Aspergillus flavus* (SM3) were active once they formed fluorescent halos around the colonies on UV light (365 nm) irradiated plates just as did the positive control *Aspergillus niger* (Figure 1).

Figure 1: Results of the lipolytic activity in the primary selection trials*.

*A. niger***	*Paecilomyces* sp.	*Aspergillus* sp.	*A. flavus*	Isolate SM4	Isolate SM5
					
1,2±0,12[a]	1,2±0,12[a]	2,7±0,75[b]	1,2±0,064[a]	0,0±0,0[c]	0,0±0,0[c]

* average values and standard deviations of the halos of fungal strains (in triplicate) calculated in *Prism GraphPad* (version 5.0), ** positive control; Same small letters indicate no significant difference between average lipase activity ($p<0.05$);

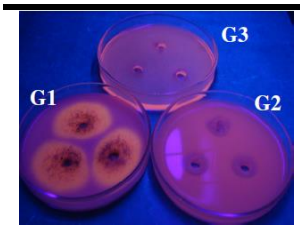
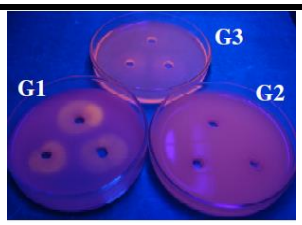
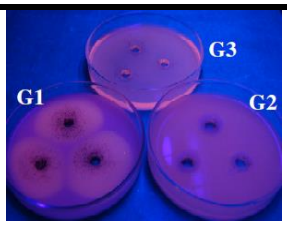
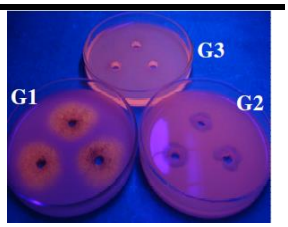
According to Jaeger et al. (1994) the formation of these fluorescent halos is due to complexation of Rhodamine B with the free fatty acids formed by the action of hydrolytic enzymes secreted by microorganisms.

The isolates SM1, SM2, and SM3 were considered promising because of their values of lipolytic activity (LA) varying from 1.2 to 2.7 (Figure 1). Therefore they were subjected to secondary screening. On the 3rd day of incubation the isolates *Paecilomyces* sp. (SM1) and *Aspergillus flavus* (SM3) showed LA value of 1.2, substantially similar to that of *A. niger* also featured on the 3rd day of incubation. The isolated *Aspergillus* sp. (SM2) showed more expressive lipolytic activity beeing active from the very first day of incubation with LA value equal to 2.7. The unidentified strains SM4 and SM5 showed no lipolytic activity even after 5 days of incubation.

To select specific lipase-producing fungi it was done a secondary selection of active fungi. It was adopted a qualitative-quantitative method by employing specific substrates of lipase (olive oil) and esterase (tributyrin) as recommended by Colen,

Junqueira and Moraes-Santos (2006). The three tested isolates displayed specific activity of the lipases since fluorescent halos formed only in the gel containing the specific substrate for lipases just like the positive control *Aspergillus niger* (Figure 2). The isolates showed moderate to high lipolytic activity with LA values ranging from 2.4 to 3.8. *Paecilomyces* sp. (strain SM1) and *Aspergillus flavus* (strain SM3) had moderate lipase activity with LA values of 2.4 and 2.8 respectively. *Aspergillus* sp. showed high lipase activity with an LA value of 3.8 quite similar to *A. niger*.

Figure 2: Results of lipase activity in secondary screening assays.

A. niger (An)**	*Paecilomyces* sp. (SM1)	*Aspergillus* sp. (SM2)	*A. flavus* (SM2)
			
*3,7±0,13^{a} (HLA)	*2,4±0,15^{b} (MLA)	*3,8±0,13^{a} (HLA)	*2,8±0,15^{c} (MLA)

* average values and standard deviations of the fluorescent halos of fungal strains on the Gel 1 (in triplicate) calculated in Prism GraphPad (version 5.0), ** positive control, G1 – gel 1, G2 - gel 2, G3 – gel 3; MLA - moderate lipolytic activity (1.5 <FH ≤ 3.0), HLA - high lipolytic activity (FH > 3.0); Same small letters indicate no significant difference between average lipase activity ($p < 0.05$);

Reports in the literature also cited *Aspergillus flavus* as specific producers of lipases many of which are already commercialized (SHARMA, CHRISTI and BANERJEE, 2001).

According to Nagy et al. (2006), a strain of *Paecilomyces varioti* IFO 4855 from a microorganism's culture collection was also selected as a lipase producer. The fungus was selected by employing the olive oil as enzyme inducer. Subsequently the strain was used in solid state fermentation with supplementation of olive oil. In that work the enzymatic extract showed significant lipolytic activity with an LA value of 16 U/g.

According to Contesini et al. (2010) *Aspergillus* lipases has shown remarkable importance in biotechnological applications and numerous studies have shown that many lipases from *Aspergillus* spp. from a variety of habitats have several important properties for foods, detergents and also for the kinetic resolution of chiral drugs and intermediates.

It is worth to mention that the present results are considerably original since there is no previous reported study on the bioprospection of lipases activity of fungi strains isolated from marine sediments.

5. CONCLUSIONS

The results have shown the importance of strains *Paecilomyces* sp. (SM1), *Aspergillus* sp. (SM2) and *Aspergillus flavus* (SM3) isolated from marine sediments of the Ceará coast in northeastern Brazil as promising sources of lipases. Furthermore *Aspergillus* sp. (SM2) showed the most promising lipolytic activity what suggests it to be a good candidate for lipases source for biotechnological purposes.

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