

Full Length Research Paper

Screening and identification of tannase-producing fungi isolated from Brazilian caves

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Tannase is an extracellular inducible enzyme of great biotechnological interest. The microorganisms such as species from the *Aspergillus* and *Penicillium* genus are the most important source of tannase. The objective of this study was to isolate, identify and select strains of filamentous fungi present in caves located in the Brazilian biome for tannase production. Five hundred and forty-four fungal strains were isolated and three hundred and eighty-six had the ability to grow in plates containing tannic acid medium as the sole carbon source. A total of 32 strains were considered to be good tannase producers. Morphological characterization indicated 20 *Aspergillus* and 12 *Penicillium* species. The highest tannase activity in submerged fermentation was obtained by *Aspergillus japonicus* 246A (16.45 U/mg) and *Aspergillus tamarii* 3 (12.95 U/mg).

Key words: *Aspergillus*, cave, filamentous fungi, screening, submerged fermentation, tannase.

INTRODUCTION

Tannase (E.C. 3.1.1.20) is an enzyme that hydrolyzes ester and depside linkages in galotannins to liberate gallic acid and glucose (Lekha and Lonsane, 1994). The main commercial applications of tannase are in the preparation of instant tea; in the production of gallic acid (Vardin and Fenercioglu, 2003); in the manufacturing of wine, beer, coffee and fruit juices (Aguilar et al., 2001); in cleaning up the leather industry effluents containing the pollutant tannin and in the reduction of antinutritional effects of tannins in animal feed (Mukherjee and Banerjee, 2006).

An important step in tannase production is the screening of strains able to produce this enzyme in great quantities and with desirable characteristics for industrial application (Macedo et al., 2005). Caves are an underexplored ecosystem that may reveal micro-

organisms of industrial and biotechnological application (Barton, 2006). Among South American countries, Brazil is known for its abundance of caves, which are scattered in several geologic formations (Auler et al., 2001). Besides the richness of cave habitats, the Brazilian subterranean biota are mostly unknown (Ferreira et al., 2009), though it is currently attracting the interest from many biologists. Many of the substrates found in caves are brought from the outside, so the selective pressure will benefit those microorganisms that have the ability to use the available substrates. Therefore, in the present study, we screened and identified filamentous fungal strains from the caves located in some Brazilian States for tannase production.

MATERIALS AND METHODS

Collection of caves fungi

The fungi were collected from the air in caves located in the Brazilian States Minas Gerais (MG), Bahia (BA) and Piauí (PI)

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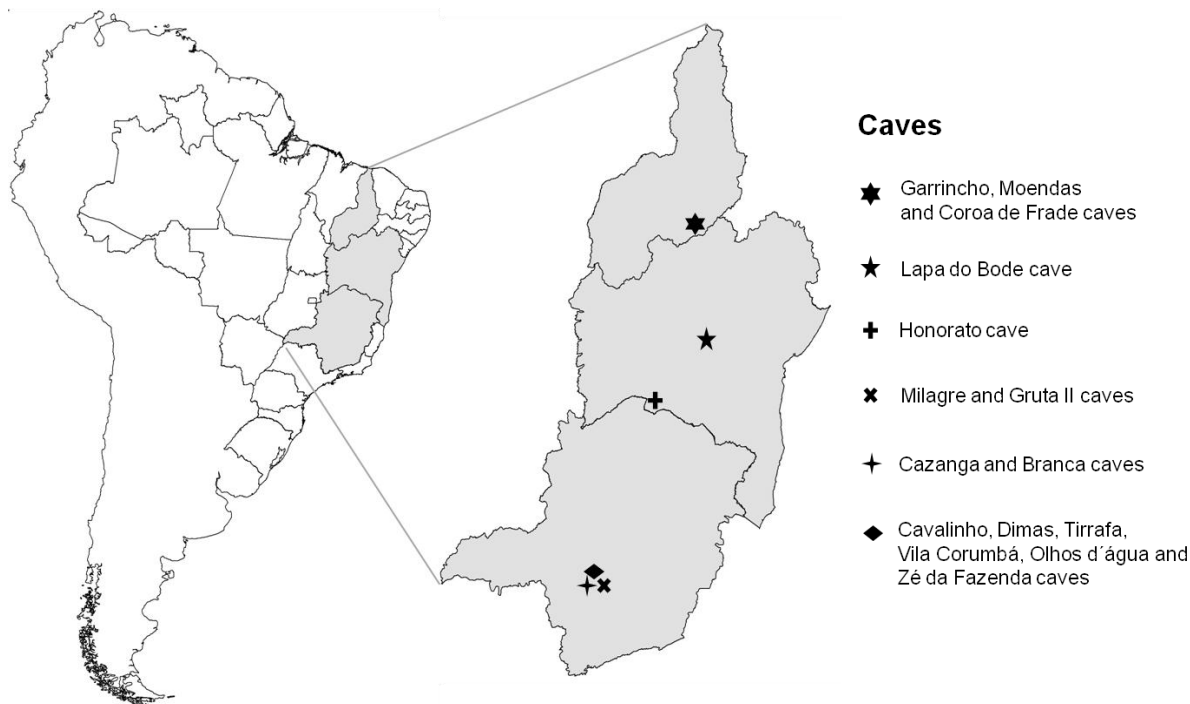


Figure 1. Location of the sampled caves.

(Figure 1). All those caves are located in Neotropics, although the external conditions were variable. Some caves (in Piauí and Bahia States) are located in the Brazilian “Caatinga”, a seasonally-dry forest formation (Mittermeier et al., 2003), while others are located in the “Cerrado” (Brazilian Savanna), as those situated in Minas Gerais State. All fungi were collected during dry seasons, in all states, due to the fact that visiting some caves in tropics can be dangerous in rainy seasons, since during strong rains or storms, they can eventually be flooded. All the caves are inserted in limestone, and their substrate (including soil) tend to be more alkaline due to the carbonatic nature of those rocks. However, one cave (Moendas cave) is associated to sandstone rocks, thus, possessing more acidic substrates, due to the silica. Unfortunately the temperature and moisture were not measured during collections in all caves. However, the collections were performed always in aphotic areas far from entrances, in very moist chambers. It is important to point out that the average temperature in those caves is, in general, between 21 and 25°C.

Isolation of caves fungi

The isolation of the air filamentous fungi was done in plates with PDA (Potato Dextrose Agar) media with chloramphenicol (25.0 µg/L). The plates were opened for five minutes inside the caves and incubated for 5 days at room temperature. Subsequently, isolate single colonies obtained on the PDA plates were transferred to medium containing tannic acid as sole carbon source (g/L): tannic acid, 10.0; NaNO₃, 3.0; KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.5; KCl, 0.5; FeSO₄·7H₂O, 0.01; agar, 30.0, pH 4.5.

Screening of fungal tannase producers

Screening was performed in plates with selection medium which

contained (g/L): tannic acid, 10.0; NaNO₃, 3.0; KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.5; KCl, 0.5; FeSO₄·7H₂O, 0.01; agar, 30.0, pH 4.5 (Pinto et al., 2001). The solution of tannic acid was sterilized separately by passing through a membrane filter (pore size 0.22 µm, Millipore) and was added to the medium at a final concentration of 1%. Point inoculations were carried out and plates were incubated at 25, 30 and 35°C and the diameters of the colonies were measured after 72, 120 and 168 h.

Selection by degradation halo

The best forty four filamentous fungi tannase producers were selected and cultured in tannic acid agar for 120 h at 30°C and an inoculum disc of each fungal culture was transferred to plates containing 1% tannic acid (in 0.01 M phosphate buffer, pH 6.0) and 3% agar, incubated at 30°C for 48 h (Bradoo et al., 1996). The enzyme activity was expressed by the diameter of the degradation halo observed as clear zones around the fungal colonies. The experiment was conducted with three replicates and the data were analyzed using analysis of variance and compared by the Tukey test ($p \leq 0.05$). The isolates that presented larger degradation halo were evaluated for the tannase production in submerged fermentation.

Fermentation process

For quantitative evaluation in the submerged fermentation, 6×10^5 spores of the fungal isolates were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of sterilized modified Czapek dox minimal medium (pH 6.0) (Batra and Saxena, 2005). Filtered-sterilized 1% tannic acid (Sigma Aldrich, USA) was added to the autoclaved medium. Cultures were grown for 120 h at 120 rpm and at 30°C in an incubator shaker. After the incubation period, the culture filtrate

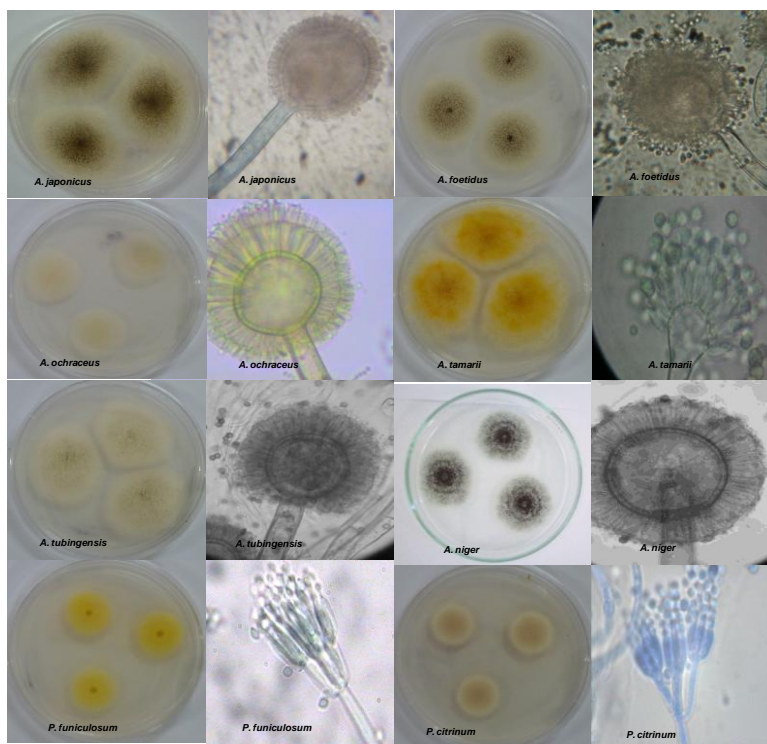


Figure 2. Colony appearance (7 days) and microscopy (in light microscope) of some species tannase producer isolated from Brazilian caves.

(through Whatman No.1 paper) was analyzed for tannase activity.

Enzyme assay

Tannase activity was estimated by a modified protein precipitation method (Deschamps et al., 1983). The reaction mixture (1 mL) contained 250 μ L 1% tannic acid (in phosphate buffer, pH 6.0), 500 μ L of phosphate buffer (pH 6.0) and 250 μ L of the culture filtrate. The mixture was incubated at 40°C for 30 min in a water bath. The reaction was stopped by adding 1mL 2% bovine serum albumin (BSA) solution. In the control, BSA was added in the mixture prior to incubation. All tubes were left for 20 min at room temperature to precipitate residual tannins and were centrifuged at 3000 g for 20 min. The tannase activity in the supernatant was estimated after appropriate dilution and reading absorbance at 260 nm (the optimal absorption of gallic acid) against the control in a UV spectrophotometer. One enzyme unit is the amount of enzyme that liberates 1 μ mol gallic acid per mL per min under standard assay conditions.

Protein assay

For the protein determination Bradford method (Bradford, 1976), with BSA as standard was used.

Morphological identification

For the morphological identification, *Aspergillus* and *Penicillium* isolates were inoculated in Malt Extract Agar (MEA) at 25°C and Czapek Yeast Agar (CYA) medium at 25 and 37°C for 7 days. In

addition, *Penicillium* isolates were inoculated in Creatine Sucrose Dichloram Agar (CREA) medium at 25°C for 7 days. After this period Petri dishes were examined to determine the colonial features and the morphological structures were observed under a light microscope. The *Aspergillus* isolates were identified according to Klich (2002), Samson et al. (2004) and Samson et al. (2007) and *Penicillium* species according to Pitt (2000).

RESULTS AND DISCUSSION

The fungi isolated in Brazilian caves were screened for tannase production using the plate assay method. From the 544 fungi isolated, 386 produced tannase, representing 70.96% of the isolates. This result shows that the caves represent environments with potential in the search for microorganism tannase producers and with biotechnological interest. The tannase-producing isolates were identified to the genera level. These isolates belong to seven different genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, *Epicoccum*, *Trichoderma* and *Cladosporium*. Morphological characterization of the best strain indicated 19 *Aspergillus* and 13 *Penicillium* species. The species were *Aspergillus japonicus* (6), *A. niger* (3), *A. tamarii* (3), *A. foetidus* (2), *A. tubingenensis* (1), *A. ochraceus* (1), *Aspergillus* sp. (3), *Penicillium funiculosum* (6), *P. oxalicum* (1), *P. corylophilum* (2), *P. citrinum* (1) and *Penicillium* sp. (3). Figure 2 shows colonies of some *Aspergillus* and *Penicillium* species

Table 1. Diameter of the degradation halos after 48 h at 30°C of thirty two isolates, and tannase activity of the eleven best isolates in submerged fermentation.

Fungal Strain	Hydrolytic zone (mm)	Tannase activity (U/mg)	Fungal Strain	Hydrolytic zone (mm)	Tannase activity (U/mg)
<i>A. japonicus</i> 238	38.33 ± 0.21	6.39 ± 1.66	<i>P. corylophilum</i> 493	18.67 ± 0.21	-
<i>Aspergillus</i> sp. 47	33.17 ± 0.36	5.98 ± 0.30	<i>Penicillium</i> sp. 273	18.50 ± 0.18	-
<i>Penicillium</i> sp. 330	32.67 ± 1.02	0.65 ± 0.15	<i>P. funiculosum</i> 718	17.50 ± 0.05	-
<i>P. funiculosum</i> 302	30.17 ± 0.43	0.98 ± 0.49	<i>A. ochraceus</i> 17	17.33 ± 0.12	-
<i>Aspergillus</i> sp. GM4	29.50 ± 0.18	5.60 ± 1.68	<i>P. funiculosum</i> 341	17.33 ± 0.08	-
<i>A. niger</i> 37	27.50 ± 0.30	4.92 ± 0.45	<i>P. funiculosum</i> 289	17.33 ± 0.25	-
<i>A. japonicus</i> 246A	26.33 ± 0.28	16.45 ± 1.05	<i>P. corylophilum</i> 92	15.33 ± 0.21	-
<i>A. foetidus</i> 35	25.67 ± 0.25	0.77 ± 0.04	<i>A. japonicus</i> 260	13.33 ± 0.00	-
<i>Penicillium</i> sp. 121	25.17 ± 0.34	1.41 ± 0.13	<i>P. citrinum</i> 185B	13.00 ± 0.07	-
<i>Aspergillus</i> sp. 9	24.33 ± 0.07	0.64 ± 0.17	<i>A. tamarii</i> 127	12.50 ± 0.18	-
<i>A. tamarii</i> 3	22.33 ± 0.03	12.95 ± 1.83	<i>A. japonicus</i> 111	12.33 ± 0.00	-
<i>P. oxilacum</i> 21	20.67 ± 0.06	-	<i>A. japonicus</i> 346A	11.67 ± 0.00	-
<i>A. tamarii</i> 53	20.67 ± 0.25	-	<i>P. funiculosum</i> 247	11.67 ± 0.00	-
<i>A. niger</i> 6	19.50 ± 0.25	-	<i>A. japonicus</i> 323	11.33 ± 0.00	-
<i>A. niger</i> 2A	19.33 ± 0.14	-	<i>A. tubingensis</i> 588	9.67 ± 0.00	-
<i>P. funiculosum</i> 340	19.00 ± 0.10	-	<i>A. foetidus</i> AT02	9.33 ± 0.00	-

isolated in this work. The species belonging to the genera *Aspergillus* and *Penicillium* existed in greater numbers and more frequently in the caves studied than the other genus or the isolation method may be favored this result.

The maximum colony diameter was found at 30°C after 120 h incubation in medium containing tannic acid as the sole carbon source. Tannase produced by most of the potent strains like *Aspergillus oryzae*, *Penicillium chrysogenum* and *Aspergillus niger* also showed optimum temperature at 30°C (Lekha and Lonsane, 1994). A total of 32 strains presented good growth and formed colonies with more than 40 mm of diameter. These were screened in plates containing 1% tannic acid and 3% agar at 30°C. After 48 h, clear zones appeared around the inoculum disc of each fungal culture due to hydrolysis of tannic acid. The largest zone was observed in *A. japonicus* strain 238 (Table 1).

Eleven fungi, eight *Aspergillus* species and three *Penicillium* species that presented tannic acid hydrolysis zones greater than 20 mm were selected for the quantitative production of tannase by submerged fermentation. The fungi tested could grow and produce the tannase enzyme in submerged fermentation within 120 h of incubation. Among *Aspergillus* species, the best tannase producers were *A. japonicus* 246A (16.45 U/mg), *A. tamarii* 3 (12.95 U/mg), *A. japonicus* 238 (6.39 U/mg) and *Aspergillus* sp. 47 (5.98 U/mg). Among *Penicillium* species only *Penicillium* sp. 121 produced moderate amounts of the enzyme (1.41 U/mg) (Table 1).

We observe that there was no direct relationship between the diameter of the halo and the tannase production in liquid media. Batra and Saxena (2005) also

obtained similar results. The authors observed that although tannase from *Aspergillus acolumaris* produced a good hydrolytic zone on tannic acid agar plates, the tannase activity in liquid medium was very low. The selection method in plates is fast and simple, but there is possibility of discard of strains that show good tannase production in submerged fermentation. Therefore, in the screening phase it is recommended that a larger number of microorganisms are assessed and a quantitative stage is associated to the process in subsequent steps.

The development of knowledge and studies about caves microbiota in Brazil can contribute to find new fungal species or even isolates of biotechnological interest. Such findings could strengthen conservational actions for this environment. This study showed the possibility of using filamentous fungi isolated from Brazilian caves for tannase production. Isolates like *Aspergillus japonicus* 246A and *A. tamarii* 3 showed high tannase yields in submerged fermentation, demonstrating that these strains are good producers of tannase in a medium containing tannic acid as sole carbon source. From the results, *Aspergillus* species were potent group of tannase producers and these species can be exploited for profitable production of this enzyme, which is very important in the food and pharmaceutical industries.

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