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Screening, Selection and Optimization of the Culture Conditions for Tannase Production by Endophytic Fungi Isolated from Caatinga

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ABSTRACT

Tannin acyl hydrolase (EC 3.1.1.20), called tannase, is an enzyme of great biotechnological interest for applications in food, chemical, beverage and pharmaceutical industries. Therefore, the objective of this study was to isolate, select and identify strains of endophytic fungi from rich tannin plants collected in the Caatinga, as well as, the optimization of culture conditions for the best producers. Sixteen endophyte fungi were isolated from the barks of mastic (*Myracrodruon urundeuva* Allemão), angico (*Anadenanthera colubrina* Vell.), barauna (*Schinopsis brasiliensis* Engl.), cajueiro (*Anacardium occidentale* L.) and catingueira (*Caesalpinia pyramidalis* Tul). All strains showed ability of using tannic acid as carbon source. The species *A. niger* and *A. fumigatus* isolated from angico and cajueiro, respectively, presented the highest enzyme production. The optimum conditions for the production of tannase by *A. niger* were 24 h cultivation in Khanna medium containing 2% tannic acid, in the absence of nitrogen source, at 37 ºC. *A. fumigatus* showed increased production of tannase when cultured in mineral medium for 24 h using 2% tannic acid as carbon source and peptone as additional nitrogen source, at 37 ºC. The optimum apparent temperature and pH of activity for the enzymes produced by both fungal species were 30 ºC and 4.0, respectively.

1. INTRODUCTION

Tannin acyl hydrolase (TAH; EC 3.1.1.20), also known as tannase, is an enzyme that hydrolyzes esters and lateral links of hydrolyzable tannins, such as tannic acid, releasing glucose and gallic acid or ellagic acid (in the case of ellagitannins). They have wide potential of application in the food, cosmetic and pharmaceutical industries. Noteworthy is that they can be used in the manufacture of instant teas with high tannin content and in the treatment of leather for removing of polyphenols [1]. However, the most important application of these enzymes is in the production of gallic acid. In the pharmaceutical industries, gallic acid is an important intermediate in the synthesis of antibacterial drug trimetroprim. In addition, gallic acid can be used as substrate for pyrogallol synthesis, as well as, preservative in food industries.

TAH can also be used to obtain propyl gallate that can be used as oxidizing agent [2, 3]. Tannases can be synthesized in the presence of high concentrations of tannic acid or gallic acid by bacteria, yeasts and filamentous fungi. Many studies concerning the production of tannases have been focused on the latter organisms because their versatility to degrade different types of tannins. These enzymes can exhibit different physicochemical properties according to the producer strain and the culture conditions. Under this aspect, many researchers have conducted studies aiming to isolate and characterize the enzyme produced by different fungal strains [4]. However, most fungi reported as TAH producers have been isolated from soil, such as *Aspergillus phoenicis* [5] and *Aspergillus ochraceus* [6], among others. On the other hand, endophytic fungi are still an untapped source. Different studies have indicated the presence of hydrolyzable tannins in diverse plant species, especially in their leaves, fruits, bark and branches. According to Sena *et al*. (2014) [7], this fact increases the chances to find endophytic microorganisms able to produce tannase, which is one of the important enzymes in the invasion process of plant tissues.

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In addition, tannase facilitates the adaptation of the microorganism to the new condition. Endophytic microorganisms are fungi and bacteria living inside the aerial parts of the plants without, causing any seemingly damage to the host [8].

As the only exclusively Brazilian biome and the least explored in biotechnological terms, Caatinga is a valuable source for the isolation of new species of organisms and for obtaining biological products. Characterized as a habitat of extreme conditions, such as low rainfall, high temperature, saline soils and low in nutrients, isolated species from Caatinga can present different metabolic pathways and, consequently, interesting adaptive responses with great interest for the scientific scope and for biotechnological application [9]. Therefore, due to the unknown microbial biodiversity in Brazil, especially from Caatinga, associated to their potential for the production of bioactive compounds, studies based on biotechnology for the search of enzymes of industrial interest, such as tannase, are attractive. According to this aspect, our aim was the isolation and selection of endophytic fungi associated to different plant species from Caatinga for high levels of tannase production under optimized culture conditions.

2. MATERIAL AND METHODS

2.1 Isolation of endophytic fungi

The endophytic fungi were obtained from plant barks collected in the municipality of Sumé (07º 40' 18" S and 36º 52' 48" W), state of Paraíba, Brazil, region that covers the Caatinga biome (Fig. 1). They were collected in May 2015 from mastic barks (*Myracrodruon urundeuva* Allemão), angico (*Anadenanthera colubrina* Vell.), baraúna (*Schinopsis brasiliensis* Engl.), cajueiro (*Anacardium occidentale* L.) and catingueira (*Caesalpinia pyramidalis* Tul), plants with high tannin content in their composition as described by Araujo (2008) [10]. Plant samples were placed in plastic bags, labeled and taken to the Microbiology Laboratory of the semi-arid Sustainable Development Center at the Federal University of Campina Grande, Paraíba, Brazil. Preparation of the plant barks and fungi isolation were done according to the methodology described by Araujo *et al*. (2002) [11]. Petri dishes containing PDA medium (Potato Dextrose Agar) were previously autoclaved at 121 °C for 15 min, and added with azithromycin (500 mg/L). The cultures were maintained at 37 °C for 96 h. The microorganisms recovered were considered endophytic and they were stored according to Castellani method (1967) [12]. Thereafter, the fungal strains were transported and deposited at the Laboratory of Microbiology from the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto at University of São Paulo (USP), São Paulo, Brazil. The best tannase producers were identified using morphological analysis at the Laboratory of Microbiology of the Federal University of Viçosa (UFV/MG), Minas Gerais, Brazil.

2.2 Screening of the isolates

2.2.1 Selection by degradation halo

In order to select tannase producing strains, microorganisms were subjected to plate assay following the methodology described by Bradoo *et al*. (1996) [13] with modifications in the mineral medium composition as follow (g/L): $NaNO_3$, 3.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1.0; $FeSO_4.7H_2O$, 0.01; and agar, 30.0. The medium was previously autoclaved at 121 °C for 15 min, and added with 10.0 g/L of tannic acid solution that was sterilized throughout a 0.22 µm membrane. Fungi were inoculated in Petri dishes containing medium and kept at 37 ºC for 144 h. Every 24 h the halo of hydrolysis of tannic acid was measured.

Fig. 1: Location of Sumé municipality (♦) in the state of Paraiba, Brazil where the plant materials from Caatinga were collected to isolate endophytic fungi.

2.2.2 Submerged fermentation

The endophytic fungi were cultured under Submerged Fermentation (SmF) using 25 mL of mineral medium (g/L): KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 2.0; $CaCl_2$, 1.0; NH_4Cl, 3.0; yeast extract, 1.0 and 1% (w/v) tannic acid (pH 5.0). 1 mL of the spore suspension (5 x 106 spores/mL) of each fungal strain was inoculated into the culture medium and maintained at 37 ºC for 72 h under orbital agitation (120 rpm). After the incubation period, cultures were harvested using a vacuum pump and Büchner funnel with filter paper Whatman No. 1.

The free cell filtrate was named as crude filtrate and it was used as source of extracellular tannase. The mycelium was macerated with pretreated sand in a mortar porcelain kept in ice bath and subsequently suspended in 100 mM sodium acetate buffer pH 5.0, centrifuged at 10 000 *g* for 10 min at 4 ºC, and the supernatant obtained was termed as intracellular crude extract. Both extracellular filtrate and intracellular crude extract were dialyzed against 4 L of distilled water *overnight* at 4 ºC and used for the determination of tannase activity.

2.3 Enzyme assay

Tannase activity was determined through the method of methanolic rhodanine, as described by Sharma *et al*. (2000) [14] using methyl gallate (0.2% w/v) as substrate in sodium acetate buffer (100 mM, pH 5.0).

The enzymatic reaction was conducted at 30 °C for 5 min and it was stopped by addition of methanolic rhodanine solution 0.667% (w/v). The gallic acid discharged was quantified in a spectrophotometer with a wavelength set to 520 nm. One unit of tannase activity (U) was defined as the amount of enzyme required to produce 1 μmol of gallic acid per minute under assay conditions.

2.4 Protein assay

Total soluble protein was determined by the Bradford method (1976) [15], using bovine serum albumin (BSA) as standard. The protein concentration was expressed as mg of protein per mL of sample used.

2.5 Optimization of the production of extracellular tannase under SmF

Based on studies of screening, fungi with the highest production of tannase were grown in SmF under different conditions to optimize enzyme production. For this purpose, it was considered the period and temperature of cultivation, culture medium, carbon and nitrogen sources, and concentration of tannic acid for maximal enzyme production as described below.

2.6 Cultivation time

The optimal period of culture for tannase production at high levels was determined using 1 mL of spore suspension ($5x10^6$ spores/mL) inoculated into 125 mL Erlenmeyer flasks containing 25 mL of mineral medium (pH 5.0) as described above, containing 1% (w/v) tannic acid, maintained at 37 ºC, under agitation at 120 rpm for different periods (12-144 h).

2.7 Effect of different culture media

The production of extracellular tannases by the selected fungal strains was analyzed using Czapeck [16], Khanna [17], M5 [18], Mineral Medium (MM) [19] and SR [20] media, containing 1% (w/v) tannic acid as additional or as the only carbon source, with initial pH adjusted to 5.0. Cultures were maintained under both orbital agitation at 120 rpm and stationary conditions, at 37°C, for different periods according to the best cultivation period for each specie previously determined.

2.8 Influence of temperature on the culture

In order to study the effect of different culture temperatures on the enzymes production by the selected fungal strains, cultivations at 30 ºC, 35 ºC, 37 ºC and 40 °C were performed.

2.9 Influence of carbon and nitrogen sources on tannase production

The influence of different carbon sources on the production of extracellular tannase was analyzed using carbohydrates (m/v) (glucose 2%, lactose 1%, maltose 1% and sucrose 1%), plant leaves (m/v) (green tea 1%, *Eucalyptus* sp. 1%, and *Jatropha curcas* 1%), tannins (m/v) (tannic acid 1%, methyl gallate 1%, *Acacia* sp. tannin 1%) and other carbon sources (m/v) (gallic acid 1%, cassava bark 1%, mint 1%, basil 1% and pomegranate 1%). The plant leaves were previously dried in a kiln at 40 °C for 3 to 5 days and mashed. Additionally, the effect of concentration tannic acid (0-3.5%; m/v) on enzyme production was analyzed. The effect of organic nitrogen sources (0.1%; m/v) such as casein, meat extract, yeast extract and peptone added to the culture medium was verified.

2.10 Determination of the optimum of temperature and pH for enzyme activity

The optimal temperature for tannase activity was determined as previously described at temperatures ranging from 30 ºC to 80 ºC and the optimal pH using 100 mM citric acid buffer (pH 3.0 to 4.0), 100 mM sodium acetate buffer (pH 5.0 to 6.0) and 100 mM Tris-HCl buffer (pH 7.0 to 8.0).

2.11 Analysis of experiments

Assays were performed in triplicate and the values obtained were expressed as mean ± standard deviation. The data analysis, graphing and the chi-square hypothesis test were performed in the softwares Microsoft Office Excel 2007 (Microsoft) and Origin 8.1 (OriginLab Corporation).

3. RESULTS AND DISCUSSION

Endophytic microorganisms represent new sources for obtaining biomolecules as, for example, enzymes, with

different properties. In this study, 16 endophytic fungi were isolated from different plant species of Caatinga. As can be observed in the Fig. 2, the major number of endophytic fungal strains were isolated of barks from angico and baraúna, 5 species in each one. Four strains were isolated from mastic and only 1 from cashew and catingueira. Initially, each fungal strain was named according to the plant species and the date of isolation.

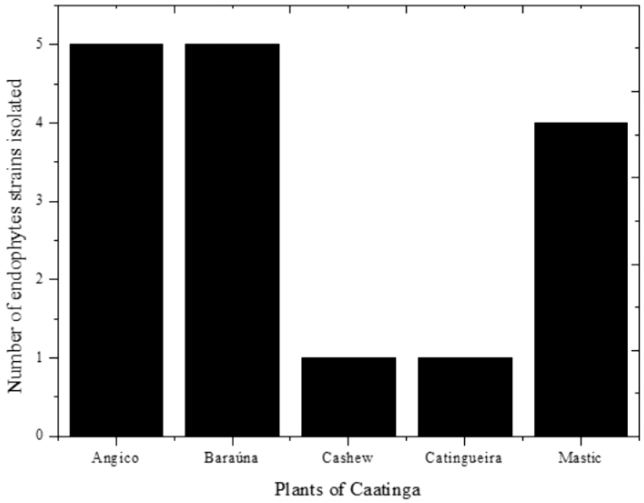

Fig. 2: Number of endophytic fungi isolated from different Caatinga plant barks.

According to Table 1, the 16 fungal strains isolated were able to grow in mineral medium agar containing tannic acid as the only carbon source. After 24 h of cultivation, it was possible to observe clear zones around the fungal colony with diameter of the halos of hydrolysis ranging from 6.0 mm to 8.5 mm. After 72 h of cultivation the halos of hydrolysis reached diameters between 25 mm and 35 mm, especially for the fungal strains isolated from angico (18/05) and baraúna 2 (21/05) (Table 1).

Table 1: Potential of tannase production using plate assay by the endophytic fungi isolated from different plants from Caatinga.

Strains	Tannic Acid Medium[1]	Halo 24 h (mm)	Halo 72 h (mm)
Angico 15/05	+	7.25 ± 0.25	*
Angico 18/05	+	6.38 ± 0.13	35.00 ± 1.63
Angico 1 19/05	+	8.17 ± 1.67	*
Angico 2 19/05	+	*	27.00 ± 1.40
Angico 1 21/05	+	7.08 ± 0.13	25.00 ± 0.50
Angico 2 21/05	+	*	*
Mastic 18/05	+	6.83 ± 0.63	27.00 ± 1.03
Mastic 1 19/05	+	7.33 ± 1.01	*
Mastic 2 19/05	+	7.08 ± 0.52	28.00 ± 1.68
Mastic 21/05	+	6.00 ± 0.90	25.00 ± 1.93
Baraúna 18/05	+	7.25 ± 1.15	28.00 ± 0.83
Baraúna 2 19/05	+	6.58 ± 0.52	25.00 ± 0.83
Baraúna 1 21/05	+	6.88 ± 0.63	*
Baraúna 2 21/05	+	7.75 ± 0.00	30.00 ± 0.62
Cashew 21/05	+	8.17 ± 0.72	28.00 ± 1.16
Catingueira 21/05	+	8.50 ± 1.30	28.00 ± 1.31

[1] Growth in culture medium;
* It was not possible to measure the halo.

At 96 h of cultivation, measurement of the diameter of the halos was impossible due to uncontrolled growth of the microorganisms in culture medium. Regarded as simple and rapid screening procedure, the plate assay is a good indicator of the microbial ability to use tannic acid as carbon source [21]. Considering that all fungal strains were able to grow in the plates with mineral medium agar containing tannic acid, the potential for enzyme production under SmF was also analyzed for mycelial and extracellular tannases.

According to Table 2, all fungal strains produced both mycelial and extracellular tannase in the presence of tannic acid as the only carbon source. The highest extracellular activities (10.14 U/mL and 10.05 U/mL) were obtained for the enzymes produced by fungi isolated from cashew (cashew 21/05) and from angico (angico 18/05), respectively, while for the mycelial tannase the best activity (2.83 U/mL) was observed for the fungus referred as angico 19/05. Other fungal strains showed low mycelial tannase activity.

Table 2: Enzymatic activity of endophytic fungi in mineral medium with 1% tannic acid at 37 ºC after 72 h of culture.

Fungal Strains	Enzymatic activity (U/mL)		
	Extracellular	Mycelial	Σ total (extra + mycelial)
Angico 15/05	9.41 ± 0.52	1.47 ± 0.52	10.89
Angico 18/05	10.05 ± 0.43	2.13 ± 0.92	12.18
Angico 1 19/05	6.97 ± 0.82	2.83 ± 0.94	9.80
Angico 2 19/05	2.82 ± 1.53	0.17 ± 0.22	2.99
Angico 1 21/05	1.09 ± 0.60	0.18 ± 0.22	1.27
Angico 2 21/05	8.44 ± 0.94	0.20 ± 0.11	8.64
Mastic 18/05	8.17 ± 0.86	1.59 ± 0.35	8.17
Mastic 1 19/05	4.74 ± 0.37	1.75 ± 0.35	4.74
Mastic 2 19/05	6.67 ± 0.39	2.33 ± 0.72	6.67
Mastic 21/05	5.98 ± 0.63	0.88 ± 0.34	5.86
Baraúna 18/05	7.42 ± 0.73	0.71 ± 0.82	8.13
Baraúna 2 19/05	5.98 ± 2.09	0.50 ± 0.46	6.47
Baraúna 1 21/05	6.36 ± 0.42	0.29 ± 0.23	6.64
Baraúna 2 21/05	5.82 ± 0.66	1.40 ± 0.63	7.21
Cashew 21/05	10.14 ± 0.74	1.15 ± 0.18	11.29
Catingueira 21/05	5.02 ± 2.05	0.90 ± 0.14	5.93

Taking in account the total production of tannases (mycelial + extracellular), the fungi referred as angico 18/05 and cashew 21/05 were the best producers. The relationship between plate assay tests and enzyme production by SmF was determined by statistical analyzes. Concerning the Chi-square test, it was found that X^2 calculated (9.11) was less than X^2 tabulated (18.31), indicating that the null hypothesis can be accepted. There was no relationship between the enzyme activity observed for SmF and the diameter of hydrolysis of tannic acid in the plate assay. Similar results were observed by Batra and Saxena (2005) [22], when analyzed the production of tannase by *Aspergillus acolumaris*. Melo *et al*. (2013) [23] reported the importance of checking the results obtained through the plate assay to minimize the loss of microorganisms which had good production of tannase under SmF and recommended the association of the quantitative selection with the plate assay. Under this view, the fungal strains angico 18/05 (halo = 35 mm; activity = 12.18 U/mL) and cashew 21/05 (halo = 28 mm; activity = 11.29 U/mL), selected as the best tannase producers, were submitted to identification. According to their

morphological characteristics, they were identified as *Aspergillus niger* ANG18 (angico 18/05) and *Aspergillus fumigatus* CAS21 (cashew 21/05).

Aspergillus species have been described as the best tannase producers. Batra and Saxena (2005) [22], studied the potential of this enzyme production by fungi belonging to the genera *Aspergillus* and *Penicillium.* The authors observed that *A. niger* produced hydrolysis halo of tannic acid with 21 mm of diameter and an enzymatic activity of 1.65 U/mL, while *A. fumigatus* presented halo of hydrolysis with 65 mm of diameter and activity of 8.65 U/mL after 24 h of cultivation. Murugan *et al*. (2007) [24], using fungi isolated from tannery effluents, observed halos of hydrolysis for tannic acid ranging from 12 mm to 14 mm of diameter and the total enzyme activity varied from 10 U/mL to 20.4 U/mL for *A. flavus* and *A. niger*, respectively, when they were submitted to SmF, conducted in a bioreactor. Melo *et al*. (2013) [23] found that *Aspergillus* species isolated from Brazilian caves produced halos of hydrolysis ranging from 24 mm to 33 mm, after 48 h of cultivation, and tannase activity of 6.0 U/mg of protein at 120 h.

These data are in accordance with the results obtained in this work, but the enzymatic activities obtained in this study were relatively higher than those reported by Batra and Saxena (2005) [22] and Melo *et al*. (2013) [23]. According to Aguilar *et al*. (2007) [4], tannases are induced and expressed at different levels, with patterns of production depending on the strain and cultivation conditions.

3.1 Optimization of the tannase production by *A. niger* ANG18 and *A. fumigatus* CAS21 under SmF

The production of tannases by the selected fungal strains, *A. niger* ANG18 and *A. fumigatus* CAS21, was evaluated taking in account some culture parameters such as period of cultivation, culture media, carbon and nitrogen sources and concentration of tannic acid. The Fig. 3 displays the tannase production by both fungal strains from 12 h to 144 h of cultivation. *A. niger* ANG18 and *A. fumigatus* CAS21 have similar behavior concerning the production and secretion of enzyme with high enzymatic levels (36.34 U/mL and 27.07 U/mL, respectively) obtained with 24 h of cultivation.

The same could be observed considering the specific activities. Then, the tannase were synthesized by both fungal strains during the early periods of SmF in agreement with the exponential phase of microbial growth, indicating their importance for nutritional metabolism of the microorganisms. Similar results were obtained for the production of tannase by *A. oryzae* with maximum activity of 30.62 U/mL [25].

According to Banerjee *et al*. (2001) [26], the optimal period of fermentation for tannase production can range from 24 h to 150 h. Most studies on the production of tannase by *Aspergillus* report maximal synthesis of the enzyme after 72 h under SmF, as observed for *A. foetidus* [27] and *A. niger* FBT1 [28]. There are also reports of maximum enzyme production after 48 h of cultivation by *A. niger* [13] and *A. tamarii* [19].

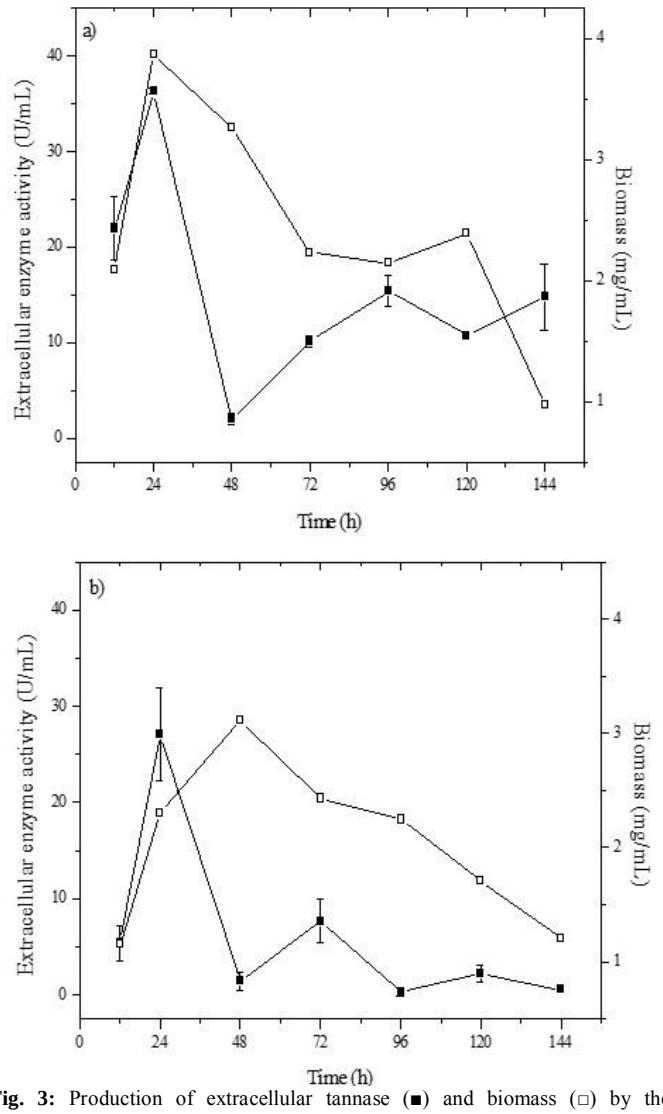

Fig. 3: Production of extracellular tannase (■) and biomass (□) by the endophytic fungi A. niger ANG18 (A) and *A. fumigatus* CAS21 (B) as function of the period of cultivation under SmF using Mineral Medium containing tannic acid as carbon source.

3.2 Effect of culture medium

The conditions and composition of the culture medium are some of the factors that influence the production of enzymes by microorganisms. Among the culture media tested for the production of extracellular tannase (Table 3), M5 medium maintained in a stationary condition provided the highest enzyme production by *A. niger* ANG18. However, the highest specific activity (414.81 U/mg of protein) was obtained using Khanna medium under agitation. SR medium, in stationary conditions, resulted in the lowest production of tannase (19.00 U/mL), followed by Czapeck under agitation (19.30 U/mL) and stationary condition (19.50 U/mL). For *A. fumigatus* CAS21 the highest enzymatic level was obtained in MM under orbital agitation (36.52 U/mL), as well as, the highest specific activity (965.12 U/mg of

protein). The synthesis of the enzyme was reduced in SR medium maintained under agitation (16.31 U/mL). Czapeck medium is one of the most cited for the production of tannase by fungi, such as *A. awamori* MTCC9299 [29], *A. niger* GH1 [30] and *A. oryzae* [25]. Adams medium was more favorable for the enzyme production by *A. japonicus* 246A (19.8 U/mg of protein) and *Aspergillus* sp. GM4 (12.0 U/mg of protein), followed by Khanna and Czapeck media [31]. MM was cited as the best for the cultivation of the fungus *A. aculeatus* DBF 9 [26] and *A. tamarii* [19] for enzymatic production.

Table 3: Influence of the culture media on the production of extracellular tannase by the endophytic fungi *A. niger* ANG18 and *A. fumigatus* CAS21 under SmF, underorbital agitation (120 rpm) and stationary condition for 24 h at 37 ºC.

Culture media	Growing conditions	Enzymatic activity (U/mL)	
		A. niger	*A. fumigatus*
Czapeck	Agitation	19.30 ± 4.85	29.16 ± 4.02
	Stationary	19.50 ± 1.38	25.43 ± 1.83
Khanna	Agitation	28.87 ± 0.43	30.14 ± 4.18
	Stationary	31.33 ± 3.01	25.45 ± 3.56
M5	Agitation	27.03 ± 4.51	21.31 ± 2.87
	Stationary	33.64 ± 3.59	23.66 ± 1.67
Mineral medium	Agitation	21.34 ± 2.42	36.52 ± 5.34
	Stationary	22.56 ± 1.10	27.31 ± 1.61
SR	Agitation	28.58 ± 4.78	16.31 ± 1.60
	Stationary	19.00 ± 1.99	22.85 ± 2.20

3.3 Effect of incubation temperature

The temperature of growth is another important factor that should be taking in account for fungal development and, consequently, enzyme production. The high enzymatic level was obtained at 37 ºC for both fungal strains while under other temperatures the synthesis of the extracellular enzyme was reduced (Fig. 4).

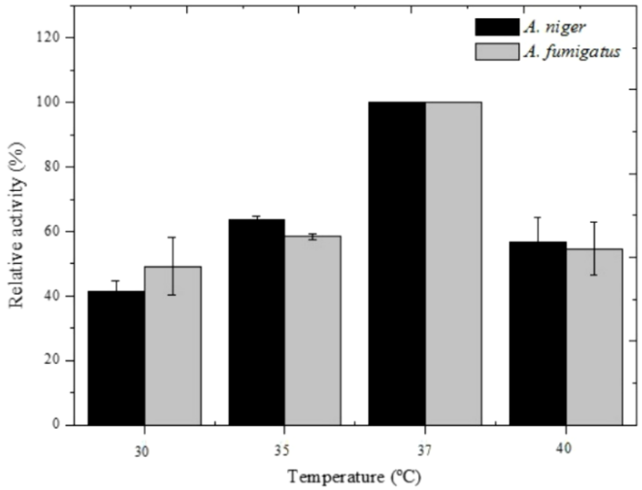

Fig. 4: Effect of growth temperature on the production of tannase by A. niger ANG18 cultured in Khanna medium and by *A. fumigatus* CAS21 cultured in Mineral Medium under SmF, both containing tannic acid as carbon source.

In general, *Aspergillus* species are described as tannase producers when maintained at 30 ºC, such as *A. niger* [32], *A. aculeatus* DBF9 [33], *A. tamarii* [19] and *A. niger* FBT1 [28]. However, similar to our results, the temperature of 37 ºC was described as optimal for the production of tannase by *A. awamori* [34] and by *A. niger* ITCC 6514.07 [35].

3.4 Influence of the carbon sources on enzyme production

The carbon source is an important factor for the production of tannase by microorganisms, since this enzyme is mostly synthesized in the presence of inducers such as tannic acid, as observed for *A. niger* ANG18 (21.94 U/mL) and *A. fumigatus* CAS21 (31.89 U/mL) despite the best fungal development in the presence of glucose, sucrose, lactose and maltose (Table 4). Phenolic compounds such as methyl gallate and gallic acid are also reported as possible inducers of tannase production [19, 36, 37]. It is important to observe that the fungus *A. niger* ANG18 was also able to produce tannase in the presence of the monosaccharide glucose and other carbohydrates (maltose and sucrose) indicating a constitutive production of tannase by this strain, what was not observed for *A. fumigatus* CAS21. Lower levels of enzyme production was also observed in the absence of carbon source for *A. niger* ANG18. Similar results with higher enzyme production using tannic acid were reported for *A. niger* [38], *A. tamarii* [19] and *Aspergillus niger* Van Tieghem [39]. Significant amounts of enzyme were also produced by both strains in the presence of gallic acid. In some cases it has been observed that the presence of gallic acid can suppress the production of tannase [19]. Tannase production in the presence of other carbon sources such as arabinose, fructose, glucose, lactose, maltose, mannitol, sucrose and xylose has been observed for other fungal strains [40]. Although the production of tannase in presence of other carbon sources, the concentrations of tannic or gallic acid is the main factor that should be considered aiming the maximal tannase production [41].

Table 4: Effect of carbon sources on the production of tannase by *A. niger* ANG18 in Khanna medium and *A. fumigatus* CAS21 in MM under SmF.

Carbon source		Enzymatic activity (U/mL)	
		niger	***fumigatus***
Carbohydrates			
➢	Glucose 2%	11.85 ± 0.3	0.0
➢	Lactose 1%	0.0	0.0
➢	Maltose 1%	4.92 ± 0.2	0.0
➢	Sucrose 1%	4.34 ± 1.9	1.19 ± 0.1
Tannins			
➢	Tannic acid 1%	21.94 ± 5.6	31.89 ± 3.3
➢	Methyl gallate 1%	10.16 ± 0.5	4.62 ± 0.1
➢	Gallic acid 1%	16.85 ± 3.1	12.18 ± 4.5
Plant leaves			
➢	Green tea 1%	11.45 ± 0.2	3.52 ± 0.6
➢	*Jatropha curcas* 1%	3.61 ± 0.5	0.0
➢	*Eucalyptus* sp 1%	9.56 ± 0.7	2.61 ± 0.8
➢	*Ocimum basilicum* 1%	1.65 ± 0.2	0.65 ± 0.0
➢	*Punica granatu* 1%	11.24 ± 4.0	0.65 ± 0.5
➢	Mint 1%	4.24 ± 0.8	1.16 ± 0.3
Other			
➢	Cassava peel 1%	1.10 ± 0.0	4.55 ± 2.0
Control			
➢	Without carbon source	1.94 ± 1.1	0.0

According to this aspect and considering the best tannase production in the presence of tannic acid, the influence of its

different concentrations (0 to 3.5%; m/v) on the enzyme production were evaluated. As can be observed in Fig. 5, 2.0% tannic acid into the culture medium promoted higher tannase levels for *A. fumigatus* CAS21, while for *A. niger* ANG18, the best production was observed in a range varying from 1.5% to 2.5% (m/v). Increases in tannic acid concentration up 2.0% and 2.5% promoted a reduction in enzyme production by *A. fumigatus* CAS21 and *A. niger* ANG18, respectively. Similar results were obtained for the enzymes produced by *A. niger* [38], *A. pullulans* DBS66 [42] and *A. awamori* [34] when high concentrations of tannic acid were used, a decrease in tannase levels was observed. Banerjee and Pati (2007) [42], showed that high concentrations of tannic acid leads to the formation of complexes with the membrane proteins of the microorganism, inhibiting their growth and the production of tannase.

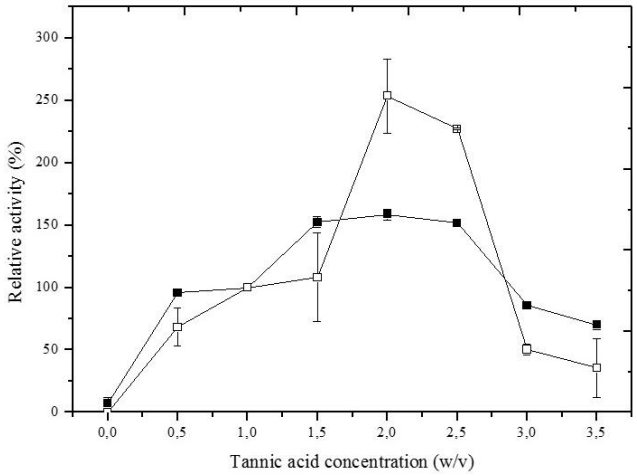

Fig. 5: Effect of tannic acid concentration on the production of tannase by A. niger ANG18 (■) cultured in Khanna medium and by *A. fumigatus* CAS21 (□) cultured in Mineral Medium under SmF.

3.5 Influence of nitrogen source

Tannase production was also influenced by nitrogen sources as casein, beef extract, peptone and yeast extract.

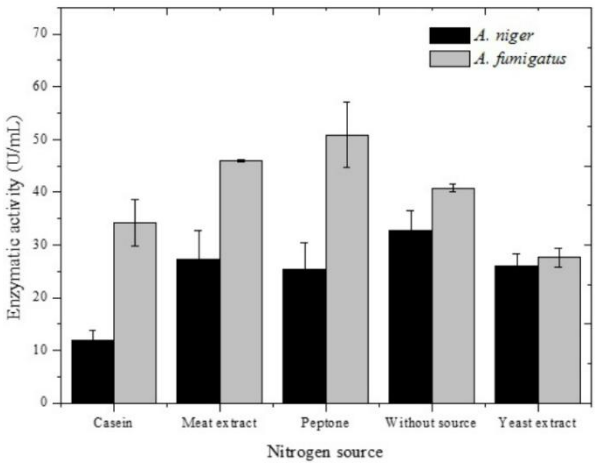

Fig. 6: Effect of different nitrogen sources on the production of tannase by A. niger ANG18 cultured in Khanna medium and by *A. fumigatus* CAS21 cultured in Mineral Medium, both containing tannic acid as carbon source.

A. niger ANG18 cultured in Khanna medium presented the maximal enzyme production (32.74 U/mL), in the absence of nitrogen source, while in the presence of casein lower enzyme levels were (11.85 U/mL) (Fig. 6). For the fungus *A. fumigatus* CAS21 grown in MM containing ammonium chloride in its formulation, the additional use of peptone provided the highest enzyme production (50.93 U/mL), while the addition of yeast extract reduced it to 27.62 U/mL (Fig. 6). Similar results were observed for the tannase produced by *Aspergillus* SH6, with increased enzymatic levels in the presence of peptone [43]. *A. niger* ITCC 6514.07 [35] and *A. niger* FBT1 [28] showed higher tannase production in the presence of $NaNO_3$.

3.6 Optimum of temperature and pH for extracellular tannase activity

The influence of the temperature and pH on extracellular tannase activity produced by *A. niger* ANG 18 and *A. fumigatus* CAS21 were evaluated. The two species produced tannase with optimum temperature of activity at 30 °C. Above this value the enzymatic activities gradually declined (Fig. 7A).

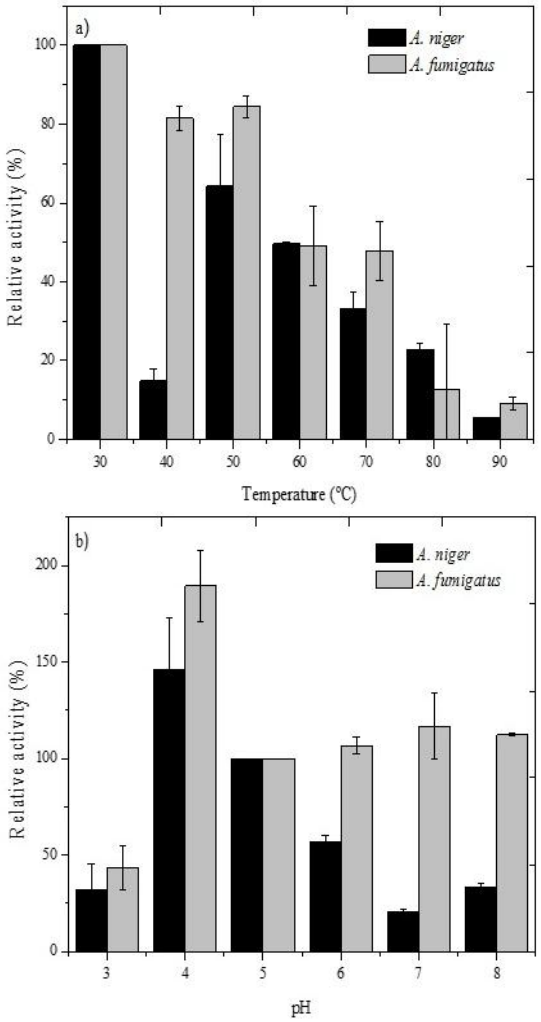

Fig. 7: Effect of the temperature (A) and pH (B) on the extracellular tannase activity from A. niger ANG18 and A. fumigatus CAS21.

Tannases obtained from microbial sources have optimum of temperature in the range varying from 20 ºC to 60 °C [40] but, in general, optimum of temperature is close to 35 °C. The optimum pH value for tannase activities for both fungal strains was reached at 4.0 (Fig. 7B). According to Costa *et al*. (2008) [19], fungal tannases are generally characterized as acidic enzymes with optimum of pH for their activities close to 5.5.

4.CONCLUSION

The knowledge and exploration of Caatinga microbiota can contribute to the discovery of new species of microorganisms or compounds of biotechnological interest. This study demonstrated the potential of endophytic fungi, especially *A. niger* ANG 18 and *A. fumigatus* CAS21, isolated from rich-tannin plants from Caatinga to produce high levels of tannases with potential for industrial application.

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