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Research Article

Characterization of a multi-tolerant tannin acyl hydrolase II from *Aspergillus carbonarius* produced under solid-state fermentation

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ABSTRACT

Background: Tannases are enzymes with biotechnological potential produced mainly by microorganisms as filamentous fungi. In this context, the production and characterization of a multi-tolerant tannase from *Aspergillus carbonarius* is described.
Results: The filamentous fungus *A. carbonarius* produced high levels of tannase when cultivated under solid-state fermentation using green tea leaves as substrate/carbon source and tap water at a 1:1 ratio as the moisture agent for 72 h at 30°C. Two tannase activity peaks were obtained during the purification step using DEAE-Cellulose. The second peak (peak II) was purified 11-fold with 14% recovery from a Sepharose CL-6B chromatographic column. The tannase from peak II (tannase II) was characterized as a heterodimeric glycoprotein of 134.89 kDa, estimated through gel filtration, with subunits of 65 kDa and 100 kDa, estimated through SDS-PAGE, and 48% carbohydrate content. The optimal temperature and pH for tannase II activity was 60°C and 5.0, respectively. The enzyme was fully stable at temperatures ranging from 20–60°C for 120 min, and the half-life ($T_{1/2}$) at 75°C was 62 min. The activation energy was 28.93 kJ/mol. After incubation at pH 5.0 for 60 min, 75% of the enzyme activity was maintained. However, enzyme activity was increased in the presence of $AgNO_3$ and it was tolerant to solvents and detergents. Tannase II exhibited a better affinity for methyl gallate (Km = 1.42 mM) rather than for tannic acid (Km = 2.2 mM).
Conclusion: *A. carbonarius* tannase presented interesting properties as, for example, multi-tolerance, which highlight its potential for future application.

1. Introduction

Tannins are polyphenols found in different plant species. They are often found in the bark, root leaf, wood, seed and fruit of plants. These molecules are able to form complexes with proteins, digestive enzymes, starch and minerals. As a result, tannins are characterized as toxic, anti-nutritional agents that reduce digestibility and protein availability in ruminants. In general, tannins can be separated in two major groups: condensed tannins and completed tannins. The former is difficult to hydrolyze while the latter is not. Hydrolysable tannins are composed by a polyol (mainly glucose) as a central core esterified by gallic acid, digallic acid (gallotannins), or ellagic acid (ellagitannins) [1].

The enzymatic hydrolysis of these hydrolysable tannins is achieved by the action of the tannin acyl hydrolyze (EC 3.1.1.20), also known as tannase. This enzyme catalyzes the breakdown of the ester and depsidic bonds found in tannic acid to generate gallic acid and glucose as hydrolysis products [2]. Gallic acid is an important intermediary in the synthesis of the antibacterial drug trimethoprim, which is used in the pharmaceutical and food industries. Gallic acid is also used as a precursor in the chemical and enzymatic synthesis of the antioxidant propyl gallate. Tannases can also be used in the beverage industry in the clarification processes of beer, fruit juices, instant tea and wines, as well as in effluent treatment performed in the leather industry and for agro-industrial wastes [3]. Despite its applicability and importance, the practical use of tannases is limited.

The main sources of tannases are microorganisms such as bacteria, yeast and filamentous fungi. Among the filamentous fungi, *Aspergillus* and *Penicillium* are important tannase producers [4]. Recently, the production and characterization of fungal tannases have received significant scientific attention, aimed at understanding their biological function, mechanism of action, biotechnological potential and applicability. Fungal tannase can be produced by both submerged fermentation [5] and solid-state fermentation (SSF) methods using agro-industrial residues as carbon sources or substrates [4,6]. Thermo-tolerant and solvent tolerant tannases are considered important enzymes with biotechnological potential [5,7]. Although both the production of tannase from fungal sources

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Table 1
Influence of substrate/carbon source on tannin acyl hydrolase from *A. carbonarius* under SSF.

Substrate	Enzymatic activity (U/g of substrate)
Sugar cane bagasse	0.0
Wheat	0.56 ± 0.02
Leaves from *A. occidentale*	1.59 ± 0.01
Leaves from *Coffea arabica*	1.0 ± 0.01
Leaves from *Camellia sinensis*	3.60 ± 0.02
Leaves from *Eucalyptus* sp.	0.50 ± 0.01
Leaves from *M. indica*	0.1 ± 0.03
Leaves from *M. esculenta*	2.74 ± 0.04
Linseed	0.70 ± 0.01
Crushed corn	0.0
Sorghum	0.10 ± 0.01

and its characterization have been investigated, further studies are required to identify new tannase sources with novel attractive properties. The purpose of the present study was therefore to present *Aspergillus carbonarius* as a promising source of tannase with interesting properties for biotechnological application.

2. Material and methods

2.1. Microorganism and culture conditions

The filamentous fungus *A. carbonarius* was isolated from soil and identified by the Laboratory of Microbiology, Federal University of Pernambuco, Brazil, according to morphological characteristics, and deposited in the culture collection of the Laboratory of Microbiology from the Faculty of Philosophy, Science and Letters, University of São Paulo, Brazil. The microorganism was maintained in PDA slants stored at 4°C and new cultures were obtained in 30-d intervals.

SSF was achieved using 5 g of different plant leaves and agro-industrial residues as substrate/carbon sources in 25 mL Erlenmeyer flasks and humidification (1:1 w/v) with distilled water, tap water or salt solutions (SR salt solution [8], Khanna salt solution [9], Vogel salt solution [10]). The culture media was autoclaved at 120°C for 25 min and 1.5 atm. A spore suspension (2 mL of 10^5 spore/mL) was used to inoculate the media. Fungal growth was conducted at 30°C for different periods depending on the experiment.

2.2. Enzyme extraction

The crude extract containing tannin acyl hydrolase was obtained with the addition of 25 mL of cold distilled water. The mixture was agitated with a magnetic stirrer for 20 min at 4°C. Cultures were then harvested through vacuum filtration using gauze and Whatman filter paper No. 1. The free cell extract obtained was dialyzed in distilled water for 24 h at 4°C and used for determining enzymatic activity and for purification.

2.3. Analysis of enzymatic activity

Tannin acyl hydrolase activity was determined using methanolic rhodanine as described by Sharma et al. [11] with modification: 0.2%

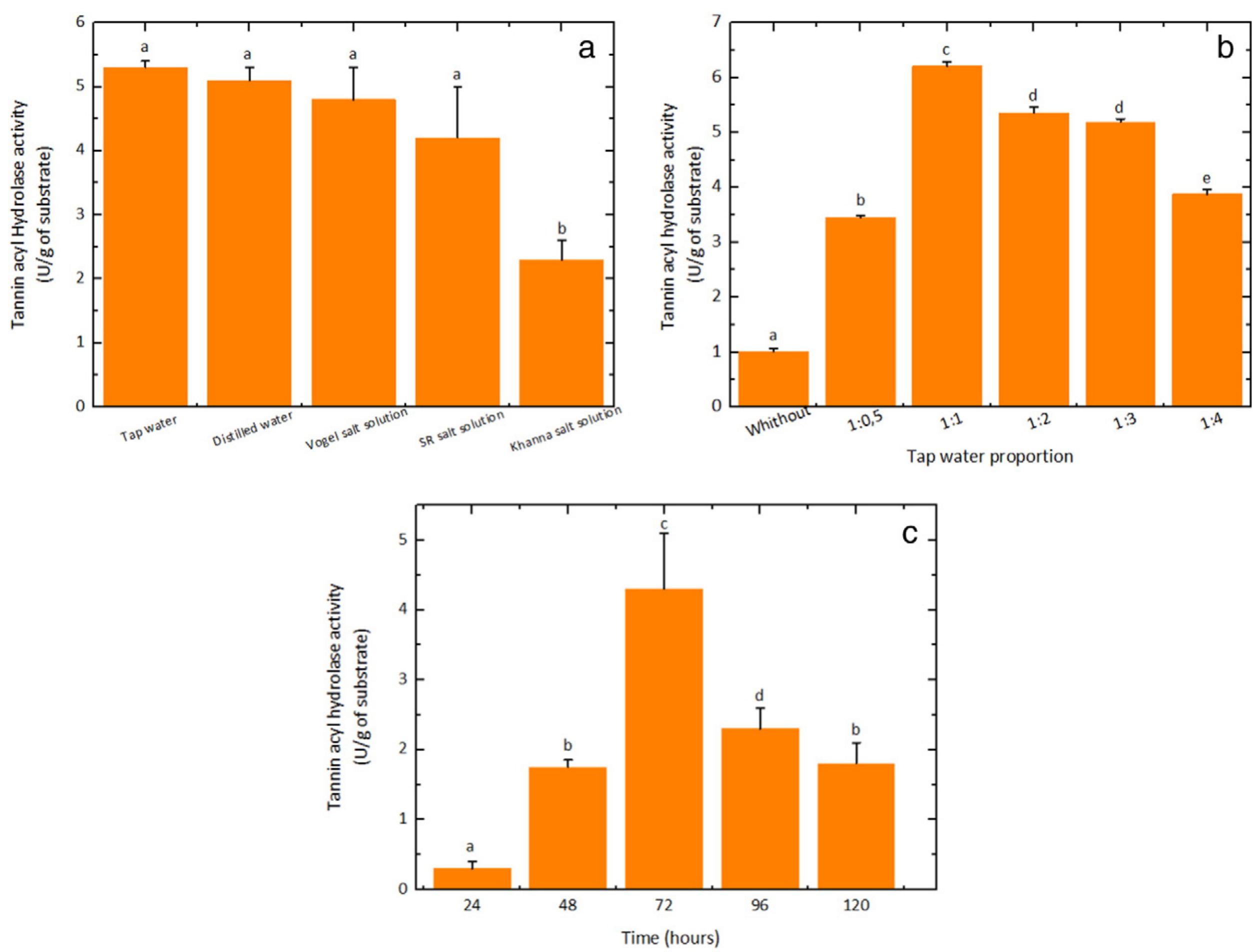

Fig. 1. (a) Tannase production by the fungus *A. carbonarius* under SSF using different moisture agents; (b) the effect of different proportions of tap water as the moisture agent on tannase production; (c) the influence of the incubation period on tannase production. The same case letter indicates that there is no significant statistical difference among the media ($p = 0.05$).

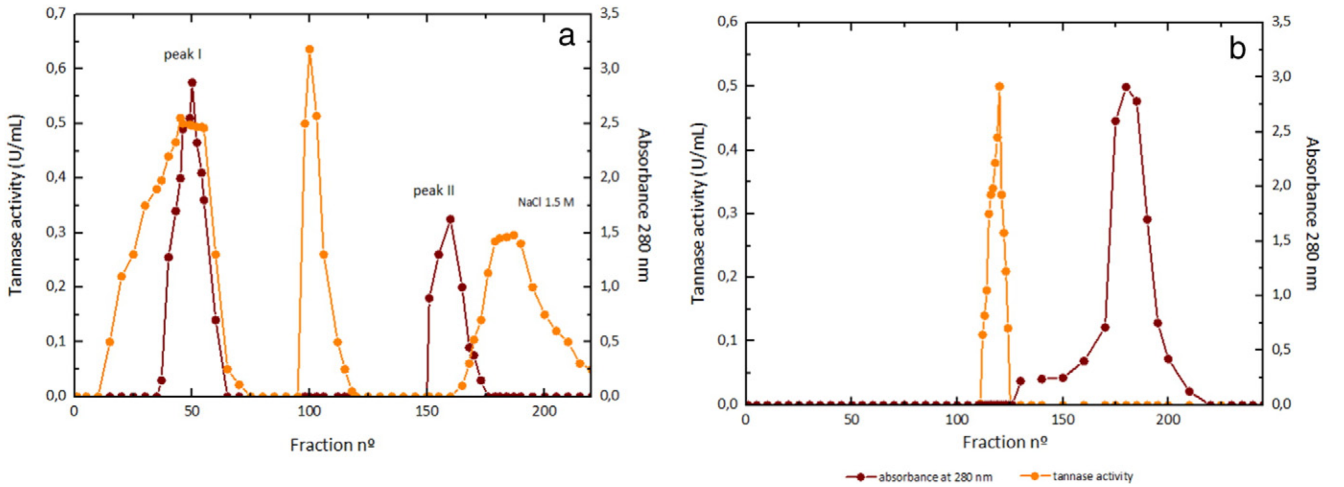

Fig. 2. (a) DEAE-Cellulose and (b) Sepharose CL-6B chromatographic profiles for tannase produced by the fungus *A. carbonarius.*

tannic acid, the natural substrate for tannases, in 100 mM sodium acetate buffer pH 5.0. The mixture of the reaction was constituted with 250 μL of substrate solution and 250 μL of enzymatic extract. The reaction was conducted at different temperatures and periods, depending on the experiment, and was finalized by adding of 300 μL of a methanolic rhodanine solution 0.667% (m/v). After 5 min, 200 μL of 0.5 N KOH were added, followed by 4 mL of distilled water. Absorbance was then measured at 520 nm. One unit of tannin acyl hydrolase activity (U) was defined as the amount of enzyme necessary to produce 1 μmol of gallic acid per min under the assay conditions. For SSF, the activity was expressed as U/mg of solid substrate.

2.4. Protein quantification and carbohydrate content

Protein quantification was performed according to the previously published Bradford method [12] using bovine serum albumin as the standard. Values are expressed as mg of protein per mL of sample. The carbohydrate content was estimated according to a previously published protocol [13] using mannose as the standard. Values are expressed as mg of carbohydrate per mL of sample.

2.5. Purification

The dialyzed crude extract containing tannin acyl hydrolase was clarified using aluminum oxide for 1 h under agitation at 4°C and centrifuged at 23,000 × g for 10 min. The supernatant was loaded onto a DEAE-Cellulose chromatographic column (1 × 12 cm) and equilibrated in 100 mM sodium acetate buffer pH 5.0. Fractions (3.0 mL) were collected at a flow rate of 1 mL/min. For elution, a continuous gradient of NaCl (0–1.5 M) was used in the same buffer. The fractions containing tannin acyl hydrolase activity (peak I and peak II) were pooled (one for each peak), dialyzed against distilled water for 24 h at 4°C, lyophilized, suspended in 50 mM Tris–HCl buffer pH 7.5 with 100 mM KCl, and loaded onto a Sepharose CL-6B chromatographic column (1 × 80 cm), which was previously equilibrated in 50 mM Tris–HCl buffer pH 7.5 with 100 mM KCl. Fractions (1.5 mL) were collected at a flow rate of 0.4 mL/min. Fractions showing enzymatic activity were pooled, dialyzed and used for enzymatic characterization and for electrophoresis analysis under denaturing (6% SDS-PAGE) and non-denaturing (6% PAGE) conditions.

2.6. Molecular mass determination

The native molecular mass for tannin acyl hydrolase II was determined using a Sepharose CL-6B gel filtration column as described above. The standards used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). The void of 86.3 mL was determined using blue dextran. The denatured molecular mass was determined by 6% SDS-PAGE [14]. The protein bands were stained as previously published [15] using Coomassie Blue Silver G-250. α-Macroglobulin (169 kDa), β-galactosidase (112.5 kDa), lactoferrin (92 kDa), pyruvate kinase (67 kDa), fumarase (60 kDa), lactic dehydrogenase (36.5 kDa) and triosephosphate isomerase (31.5 kDa) were used as molecular mass markers.

2.7. Influence of temperature and pH on enzyme activity

The enzymatic reaction was conducted at different temperatures (30–80°C). Thermal stability was determined at different temperatures (40–80°C) for different periods (5–120 min) using an aqueous solution containing the enzyme. After each time interval, samples were collected, maintained in an ice bath and then used to determine enzyme activity as presented previously. The activation energy (Ea) was estimated using the Arrhenius plot slope ($-Ea/R$). The thermal deactivation (K_d) constant at each temperature and the half-life ($T_{1/2}$) were calculated using [Equation 1] and [Equation 2], respectively:

$$\ln A = \ln Ao + K_d x\ t \qquad \text{[Equation 1]}$$

$$T_{1/2} = \ln 2/K_d \qquad \text{[Equation 2]}$$

where A is the enzyme activity at time t and Ao is the enzyme activity at time 0.

For the analysis of the pH influence on enzyme activity, the reaction was conducted at different pH values with their respective buffers (50 mM): sodium citrate pH 2.5 and 3.0, sodium acetate pH 3.5–5.5, MES pH 6.0 and 6.5, Tris–HCl pH 7.0–9.0, and glycine pH 9.5 and 10. The pH stability of the enzyme was determined by incubating the enzyme in the various buffers in an ice bath for different periods.

Table 2
Purification of tannin acyl hydrolase II from *A. carbonarius.*

Step	Activity (total U)	Protein (total mg)	Specific activity (U/mg of protein)	Yield (%)	Purification (folds)
Crude extract	75.0	64.5	1.2	100.0	1.0
Aluminum oxide	88.8	48.8	1.9	118.0	1.6
DEAE-Cellulose	5.4	1.2	4.5	7.2	3.7
Sepharose CL-6B	10.8	0.8	13.5	14.4	11.2

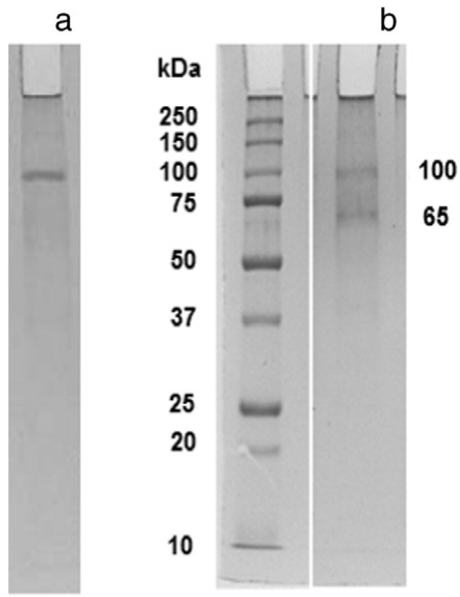

Fig. 3. Gel images for (b) 6% PAGE and (b) 6% SDS-PAGE of purified tannase from the fungus *A. carbonarius*.

Tannin acyl hydrolase activity was then determined as presented previously.

2.8. Influence of different compounds on enzyme activity

The effects of different salts at 1 mM, detergents at 0.01% (SDS and Tween-20), organic solvents at 1% (v/v) (methanol, ethanol, acetone, isopropanol, n-butanol and glycerol), as well as 1 mM β-mercaptoethanol, H_2O_2, and EDTA on tannin acyl hydrolase activity were analyzed.

2.9. Determination of kinetic parameters

The kinetic parameters (Km and Vmax) for the hydrolysis of tannic acid (0.1–10 mM), methyl gallate (0.1–60 mM), and propyl gallate (0.1–20 mM) were determined according to Lineweaver–Burk plots using the OriginPro 8 software. The Vmax/Km was also determined.

2.10. Statistical analysis

All experiments were conducted in triplicates and the results were expressed as media ± standard error. ANOVA was used for statistical comparisons with *p* value fixed at 0.05.

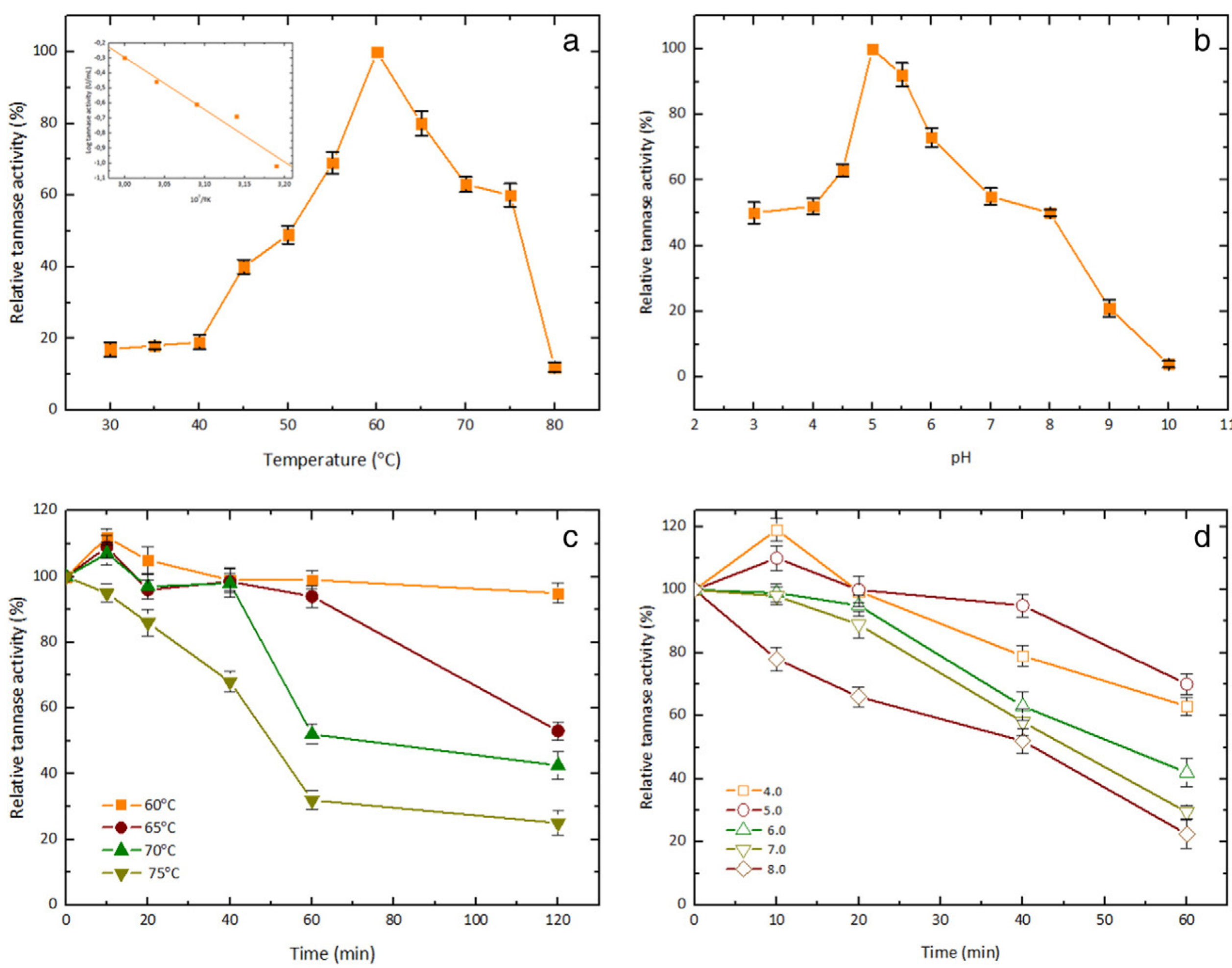

Fig. 4. (a) Optimal temperature and (b) pH, and (c) thermal stability at 60°C, 65°C, 70°C, and 75°C. (d) pH stability at 4.0, 5.0, 6.0, 7.0, and 8.0 for purified tannase from the fungus *A. carbonarius*. Inset: Arrhenius plot used for *Ea* calculation.

Table 3
Kinetic parameters demonstrating the thermal stability of tannin acyl hydrolase II from *A. carbonarius*.

Temperature (°C)	K_d (min^{-1})	Half life ($T_{1/2}$)
60	0.00058	20 h
65	0.006	115 min
70	0.007	98.6 min
75	0.011	62.7 min

Table 5
Kinetic parameters for the hydrolysis of tannic acid, methyl gallate and propyl gallate by tannin acyl hydrolase II from *A. carbonarius*.

Parameter	Tannic acid	Methyl gallate
Km (mM)	2.2	1.42
Vmax (U/mg of protein)	33.3	14.70
Vmax/Km (U/mg of protein/mM)	15.13	10.32

3. Results and discussion

3.1. Production of tannase under SSF

Enzymatic production was directly influenced by the carbon source used during SSF, with the highest level obtained using green tea leaves (3.6 U/g of substrate) (Table 1). The leaves of *Manihot esculenta* (2.74 U/g of substrate) and *Anacardium occidentale* (1.59 U/g of substrate) also promoted substantial tannase production compared to the other carbon sources used. Tannase production using green tea leaves was 36-fold higher than that obtained using *Mangifera indica* leaves. In addition, tannase production was 2-fold higher than that obtained with *Aspergillus niger* under SSF using tea residue as the substrate [16]. Green tea leaves are rich in polyphenols, mainly catechins (cathechin, epicatechin, epicatechin 3-gallate, epigallocatechin and epigallocatechin gallate), and also flavanols and their glycosides, and depsides as chlorogenic acid, coumarylquinic acid, and theogallin (3-galloylquinic acid) [17,18]. Tannase production by *A. carbonarius* in the presence of sugar cane bagasse and crushed corn was not observed.

Another aspect that should be considered in enzymatic production under SSF is the moisture agent used to humidify the carbon sources. There was no statistically significant difference in tannase production using tap and distilled water or Vogel and SR salt solutions (Fig. 1a). However, in the presence of Khanna salt solution, enzymatic production was 2-fold lower than that observed using tap water. Considering these results, the influence of different proportions of tap water, as the moisture agent, on tannase production, was analyzed (Fig. 1b). The highest production was achieved using a 1:1 (w/v) tap water ratio (6.21 U/g of substrate). Tap water proportions above and below this ratio resulted in reductions in enzyme production.

The period of cultivation is an important factor in enzyme production by a microorganism. Higher enzymatic levels were obtained with 72 h cultivation at 30°C (4.5 U/g of substrate) (Fig. 1c). Tannase production was reduced at time periods longer or shorter than 72 h, and differed from that reported for *Penicillium atramentosum* KM, which exhibited maximal production levels at 96 h using both jamum and keekar leaves as substrates [19] and from *A. niger* using tea residue as the substrate [16], under SSF.

Table 4
Effect of different compounds on the activity of tannin acyl hydrolase II from *A. carbonarius*.

Compound	Relative activity (%)	Compound	Relative activity (%)
Without	100	NH_4Cl	96.0 ± 1.5
NaCl	86.0 ± 5.0	$CuCl_2$	41.0 ± 3.8
$MgSO_4$	40.0 ± 3.2	Solvents	
Ag_2SO_4	112.0 ± 2.1	Methanol	95.0 ± 2.3
$FeCl_3$	15.0 ± 4.3	Ethanol	81.0 ± 1.2
$Zn(NO)_3$	34.0 ± 6.5	Acetone	94.0 ± 3.2
$AgNO_3$	96.0 ± 4.8	Isopropanol	90.0 ± 1.2
NaBr	104 ± 1.8	Butanol	71.0 ± 3.4
$BaCl_2$	100.0 ± 2.9	Glycerol	93.0 ± 1.0
KCl	96.0 ± 6.7	Detergents	
$ZnCl_2$	88.0 ± 5.3	SDS	94.0 ± 2.5
$CaCl_2$	73.0 ± 3.8	Tween 20	81.0 ± 1.3
$AlCl_3$	10.0 ± 4.3	Others	
$CuSO_4$	32.0 ± 2.8	EDTA	69.0 ± 1.8
KH_2PO_4	86.0 ± 3.7	β-mercaptoethanol	0.0

3.2. Purification

Extracellular tannase was purified using two chromatographic steps, DEAE-Cellulose and Sepharose CL-6B (Fig. 2). Two tannase peaks were obtained using the DEAE-Cellulose chromatographic column. One peak did not interact with the resin (peak I), whereas the other did interact (peak II). Peak II was eluted using 0.85 M NaCl, subjected to the Sepharose CL-6B step (Fig. 2b), and eluted as single form. The peak II was selected considering its reduced concentration of protein (at 280 nm), which facilitates the full purification of this enzymatic form. Using these procedures, the enzyme was purified 11.2-fold with a recovery of 14.4% (Table 2). Both the purification factor and the recovery were higher than those reported for purification of *Aspergillus ficuum* Gim 3.6 tannase [20]. The purity was confirmed by non-denaturing electrophoresis (6% PAGE) showing a single protein band (Fig. 3).

3.3. Molecular mass determination

The native molecular mass of extracellular tannase II from *A. carbonarius* was 134.89 kDa estimated through gel filtration with 47.83% carbohydrate content. Under denaturing conditions, two protein bands of 65 and 100 kDa were observed, indicating that the tannase produced is a heterodimeric structure (Fig. 3). Other fungal tannases containing two different subunits have been reported, such as that produced by *Aspergillus phoenicis* [7] and *Aspergillus oryzae* [21]. Tannases with identical subunits have also been described [22]. High carbohydrate content was also reported for tannases produced by *A. niger* (43%) [23] and *Emericella nivea* (50%) [5]. The importance of a high level of glycosylation for tannase is not completely understood but it is possible that the carbohydrate protects the enzyme under unfavorable conditions, such as high tannin concentration. High tannin concentrations can promote protein precipitation. In addition, the carbohydrate can direct the correct positioning of the substrate into the active site [2].

3.4. Influence of temperature and pH on tannase activity

The optimal temperature for extracellular tannase II activity from *A. carbonarius* was 60°C and the *Ea* was 28.93 kJ/mol (Fig. 4a). Temperatures of 50°C and 60°C have been reported as ideal for other tannases such as those produced by *Paecilomyces variotii* [24], *A. niger* [25] and *A. phoenicis* [7]. The *Ea* is an important aspect from an industrial point view, as it is relevant to know the *Ea* required for tannic acid hydrolysis by tannases for efficient reduction. The *Ea* for enzymes produced by *A. niger* GH1 and *Verticillium* sp. P9 were 21.38 [26], 28.04 (TAH I) and 33.68 (TAH II) kJ/mol [27], respectively, using methyl gallate as the substrate. The *A. carbonarius* tannase II was stable at a temperature range of 20–60°C for 120 min (Fig. 4c), with a $T_{1/2}$ of 98 and 62 min at 70°C and 75°C, respectively (Table 3). The enzyme produced by *A. niger* was also stable at 60°C [28]. The thermal stability observed in the present study was better than that reported for other tannases from *Aspergillus tamarii* [20], *A. niger* GH1 [29] and

A. phoenicis [7], among others. According to Yao et al. [1], tannases are stable at a temperature range of 30–60°C.

The optimal pH for enzyme activity was 5.0 (Fig. 4b), which correlates with the findings for other fungal tannases reported in the literature such as tannases produced by *Emericella nidulans* [5], *Aspergillus awamori* [30], *Aspergillus versicolor*, and *Penicillium charlesii* [31]. However, different optimal pH values for tannase activity have also been reported such as for tannases produced by *A. awamori* BTMFW032, which exhibits optimal activity at pH 2.0 and 8.0 [32]. The *A. carbonarius* tannase II was also able to function under alkaline conditions, and maintained approximately 45% and 20% of its activity at pH 8.0 and 9.0, respectively. Considering the enzyme stability at different pH values, the best results were obtained with acidic pH values. *A. carbonarius* tannase II maintained 75% of its activity when incubated at pH 5.0 for 60 min, with $T_{1/2}$ of 40 min at pH 7.0 and 8.0 (Fig. 4d). The tannase produced by *A. phoenicis* was stable at a wide pH range [7].

3.5. Influence of different compounds on tannase activity

Table 4 displays the results obtained for the influence of different compounds on purified tannase II activity. *A. carbonarius* tannase II activity increased 12% in the presence of 1 mM Ag_2SO_4. When the Ag_2SO_4 concentration was increased to 10 mM, the enzyme activity increased by 32%. To the best of our knowledge, this is the first time that tannase stimulation by silver has been reported. It is possible that the silver ion reacts with the thiol groups in the molecule, thus promoting structural modifications and, consequently, affecting the catalytic activity. It is also possible that modification of the overall charge of the enzyme molecule also affects its activity [1]. The tannase produced by *E. nidulans* was slightly affected by Ag^+ [5]. In contrast, it has been reported that several tannases are inhibited by silver [1]. The activity of the *A. carbonarius* tannase was severely reduced in the presence of Al^{3+} (-90%), Fe^{3+} (-85%), Cu^+ (-68%), Zn^+ (-66%), Mg^+ (-60%), and Cu^{2+} (-59%). Inhibition of tannase by different ions has been reported and is related to linkage in an unspecific site or with molecule aggregation [22]. The *A. niger* ATCC16620 tannase was inhibited by Cu^+, Zn^{2+}, Fe^{3+}, and Mg^{2+} [33]. The other salts tested did not significantly alter enzymatic activity.

Other compounds such as solvents, detergents and chelants can modify tannase activity. Extracellular tannase II activity from *A. carbonarius* was not severely influenced by organic solvents and detergents. In the presence of isopropanol, the enzyme maintained 90% of its activity, indicating its potential usefulness in the propyl gallate synthesis. Polar solvents such as glycerol, propanol, ethanol and methanol can increase enzyme activity by facilitating substrate dissolution and, consequently, binding to the enzyme active site. However, these solvents can remove the essential water molecule from the enzyme, thus reducing its activity [34]. Chhokar et al. [35] demonstrated that *A. awamori* MT9299 was stimulated by 60% butanol and benzene. The *A. carbonarius* tannase II maintained 94% of its activity in 1% SDS while the tannase produced by *Aspergillus aculeatus* was inhibited by 0.01% SDS [36]. Enzyme activity was reduced by 19% in the presence of Tween 20, which has also been reported to be an inhibitor of the tannase activity from *Verticillium* sp. P9 [27]. Non-ionic detergents promote conformational changes in protein structure, which affects enzymatic activity. However, ionic detergents promote unfavorable electrostatic interactions causing protein unfolding [37]. Reduction of 31% of enzyme activity was observed in the presence of 1 mM EDTA. In contrast, the tannase produced by *E. nivea* was fully inhibited under the same conditions [5]. EDTA is a potent chelating agent of divalent ions that can significantly impact on the catalytic activity. Enzyme activity was also drastically inhibited by β-mercaptoethanol, which acts on disulfide bridges, promoting the denaturation and, consequently, loss of activity.

3.6. Kinetic parameters

The Km value estimated for the hydrolysis of tannic acid was 2.2 mM, which was higher than that observed for methyl gallate (1.42 mM), indicating that methyl gallate displayed better affinity (Table 5). However, the Vmax and the efficiency (Vmax/Km) were higher for tannic acid. The affinity of the *A. carbonarius* tannase II for tannic acid was higher than that reported for *A. ficuum* Gim 3.6 tannase [20], *E. nidulans* tannase [11] and *Fusarium subglutinans* tannase [38], among others. Considering methyl gallate as a substrate, the affinity of the *A. carbonarius* tannase II was better than that reported for tannases from *A. awamori* BTMFW032 [32], *A. niger* GH1 [26] and *Penicillium variable* [39], among others.

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Conflict of interest

The authors declare that there is no conflict of interest.

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