Characterization of *Aspergillus fumigatus* CAS-21 tannase with potential for propyl gallate synthesis and treatment of tannery effluent from leather industry

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Abstract

One of the tannase isoforms produced by the fungus *Aspergillus fumigatus* CAS-21 under submerged fermentation (SbmF) was purified 4.9-fold with a 10.2% recovery. The glycoprotein (39.1% carbohydrate content) showed an estimated molecular mass of 60 kDa. Optimum temperature and pH for its activity were 30–40 °C and 5.0, respectively. It showed a half-life (t_{50}) of 60 min at 45 and 50 °C, and it was stable at pH 5.0 and 6.0 for 3 h. The tannase activity was insensitive to most salts used, but it reduced in the presence of $\text{Fe}_2(\text{SO}_4)_3$ and FeCl_3 . On contrary, in presence of SDS, Triton-X100, and urea the enzyme activity increased. The K_m value indicated high affinity for propyl gallate (3.61 mmol L^{-1}) when compared with tannic acid (6.38 mmol L^{-1}) and methyl gallate (6.28 mmol L^{-1}), but the best K_{cat} (362.24 s^{-1}) and K_{cat}/K_m ($56.78 \text{ s}^{-1} \text{ mmol}^{-1} \text{ L}$) were obtained for tannic acid. The purified tannase reduced 89 and 25% of tannin content of the leather tannery effluent generated by manual and mechanical processing, respectively, after 2-h treatment. The total phenolic content was also reduced. Additionally, the enzyme produced propyl gallate, indicating its ability to do the transesterification reaction. Thus, *A. fumigatus* CAS-21 tannase presents interesting properties, especially the ability to degrade tannery effluent, highlighting its potential in biotechnological applications.

Keywords *Aspergillus* · Propyl gallate · Submerged fermentation · Tannic acid · Tannin acyl hydrolase · Tannery effluent

Introduction

Tannase (tannin acyl hydrolase, EC 3.1.1.20), an enzyme with biotechnological importance, catalyzes the hydrolysis of ester and depsidic linkage in hydrolysable tannins such as tannic acid, releasing glucose and gallic acid (Yao et al. 2014). Tannins are one of the most important groups of polyphenols in plants. They are characterized as anti-nutritional agents owing to their property to complex with proteins, digestive enzymes, polysaccharides, and minerals (Goel et al. 2005; Santos and Mello 2001). Additional effects of tannins include the formation of tea cream in tea drink,

turbidity of alcohol and coffee, inhibition of the growth of many microorganisms, resistance to microbial attack, and recalcitrance to biodegradation (Rodríguez-Durán et al. 2011). Fortunately, various microbial tannases can be applied in different sectors such as food and feed, beverages, chemicals, and for the tannin-rich effluent treatment (Yao et al. 2014). The most important application of tannase is the production of gallic acid. Gallic acid is a substrate for the chemical or enzymatic synthesis of propyl gallate as well as an antioxidant used as additive in food, oils, and lipid-rich products (Fernandez-Lorente et al. 2011).

Despite their importance, the practical use of microbial tannases is still limited and advanced studies on their properties are necessary. Recently, special attention has been given to the production and characterization of fungal tannases, especially from the genus *Aspergillus* and *Penicillium* (Yao et al. 2014). Both submerged fermentation and solid-state fermentation have been used for tannase production and a majority of these enzymes is extracellular (Aguilar et al. 2007). Different factors influence the production of tannase by microorganisms. These include carbon and nitrogen

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sources, chemical composition of the culture medium, pH, incubation temperature, agitation, and incubation period (Yao et al. 2014).

Fungal tannase properties such as molecular mass (between 50 and 320 kDa) have been determined (Yao et al. 2014). Some of these enzymes are monomeric, while others have two or more subunits, such as the enzymes produced by *Aspergillus oryzae* (octamer) (Hatamoto et al. 1996), *Emericella nidulans* (hexamer) (Gonçalves et al. 2011), and *Arxula adenivorans* (tetramer) (Böer et al. 2011). In general, tannases are active in a broad temperature range (20–60 °C) under acidic conditions. Ions, organic solvents, and surfactants also influence tannase activity. Some authors have reported tannases as inducible enzymes, while others demonstrated their constitutive expression (Yao et al. 2014).

Despite the determination of the properties of some fungal tannases and their potential application in the removal of phenolic compounds from tea, juices, wines, beers, and soft drinks (Beniwal et al. 2013), their effective use for the treatment of tannin-rich effluents is still limited, especially for the tannery effluent from the leather industry. This industry is an important economical field for developing countries (Lofrano et al. 2013). It is responsible for consuming large volumes of water and producing significant volumes of wastewater with high concentration of chemicals such as vegetable tannins and organic matter, contributing to environmental pollution (Romero-Dondiz et al. 2015; Dixit et al. 2015). The methods used for the treatment of tannery wastewater can be biological, chemical, physical, or a combination of these (Lofrano et al. 2013; Durai and Rajasimman 2011). In this light, our aim was to characterize the tannase from *A. fumigatus* CAS-21 and to analyze its potential for the treatment of tannery effluent from the leather industry and for propyl gallate production.

Materials and methods

Microorganism and culture conditions

The endophytic fungus *Aspergillus fumigatus* CAS-21 was maintained on slants of PDA (Potato-Dextrose-Agar; Neogen® Corporation) medium at 4 °C. For obtaining new cultures, spores from 30-day-old cultures were collected with a loop, transferred to the PDA medium, incubated at 37 °C for 4 days, and stored at 4 °C.

Submerged cultures (SbmF) were obtained by inoculation of 1 mL spore suspension (10^5 spores mL⁻¹) in a mineral medium (Costa et al. 2008), containing 2% (m/v) tannic acid as carbon source, previously autoclaved at 120 °C and 1.5 atm for 20 min. The cultures were kept at 37 °C for 24 h under orbital agitation (120 rpm) using a shaker equipment (Tecnal—TE422). After cultivation, the medium was

harvested using a vacuum pump and Whatman filter paper no. 1. The filtrate was used as a source of extracellular tannase for enzyme activity determination and purification.

Determination of the enzyme activity

The tannase activity was determined using 0.2% (m/v) methyl gallate (C₈H₈O₅) (Sigma-Aldrich) as substrate in 100 mmol L⁻¹ sodium acetate buffer (pH 5.0) according to the methanolic rhodanine method (Sharma et al. 2000). Briefly, the reaction mixture containing 250 µL of substrate solution and 250 µL of enzyme sample was incubated at 30 °C for 5 min and added to 300 µL of methanolic rhodanine (0.667%; w/v) at 30 °C. After 5 min, 200 µL of 0.5 N potassium hydroxide was added and maintained at 30 °C. Finally, the mixture was diluted with 4 mL of distilled water and incubated at 30 °C for 10 min. The absorbance was recorded at 520 nm using a spectrophotometer (UV Mini-1240, Shimadzu). One unit (U) of enzymatic activity was defined as the quantity of enzyme necessary to produce 1 µmol of gallic acid per minute under the assay conditions.

Protein and carbohydrate quantification

The protein quantification was performed according to the Bradford method (Bradford 1976) using bovine serum albumin (BSA) (Sigma-Aldrich) as standard and expressed as milligrams of protein per mL of the sample. The carbohydrate content was determined according to Dubois et al. (1956), using mannose as standard (0–0.1 mg mL⁻¹).

Purification

The crude filtrate from SbmF was loaded in DEAE-Cellulose (Santa Cruz Biotechnology) chromatographic column (1 × 12 cm) equilibrated with 10 mmol L⁻¹ sodium acetate buffer (pH 5.0, buffer A) and eluted by a linear gradient of NaCl (0–1.5 mol L⁻¹) in the buffer (A). Three-milliliter fractions were collected at a flow rate of 1.5 mL min⁻¹. Fractions with tannase activity were pooled, dialyzed against distilled water for 24 h at 4 °C, lyophilized, suspended in 50 mmol L⁻¹ Tris-HCl buffer (pH 7.5) containing 100 mmol L⁻¹ KCl (buffer B) and loaded in Sepharose CL-6B (1 × 70 cm) (Pharmacia Fine Chemicals) chromatographic column previously equilibrated and eluted with the buffer (B). One milliliter fractions were collected at a flow rate of 0.4 mL min⁻¹. Fractions with tannase activity were pooled, dialyzed against distilled water for 24 h at 4 °C and used for enzyme characterization. β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and BSA (66 kDa) were used as molecular mass markers. The void of 56 mL was estimated using Blue Dextran 2000.

Denaturing electrophoresis—SDS—PAGE

The purified enzyme was subjected to 12% SDS-PAGE according to Laemmli (1970), using a power source (PowerPac™ Bio-Rad®) adjusted to 120 V and 40 mA. After the run, the gel was stained with Coomassie Blue Silver G-250 (Candiano et al. 2004). The Precision Plus Protein Kaleidoscope Standards (Bio-Rad®) was used as molecular mass marker at a range from 10 to 250 kDa.

Influence of different compounds on enzyme activity

The effect of different salts (1 mmol L⁻¹: AgNO₃, BaCl₂, CaCl₂, CoCl₂, CuCl₂, CuSO₄, Fe₂(SO₄)₃, FeCl₃, HgCl₂, KH₂PO₄, MgCl₂, MgSO₄, MnCl₂, MnSO₄, NaCl, NH₄Cl, Zn(NO₃)₂, and ZnSO₄), denaturing agents (1 mmol L⁻¹: β-mercaptoethanol and urea), 1 mmol L⁻¹ EDTA, detergents (0.01% v/v: SDS, Triton X-100, Tween-20) and organic solvents (1% v/v: methanol, acetonitrile, ethanol, acetone, isopropanol, *n*-butanol) was evaluated. The effect of different concentrations (0–50 mmol L⁻¹) of glucose and gallic acid (C₇H₆O₅) (Sigma-Aldrich) on the enzyme activity was also evaluated.

Hydrolysis of substrates and kinetic parameters

The ability of the enzyme to hydrolyze different substrates as tannic acid (C₇₆H₅₂O₄₆) (Sigma-Aldrich), methyl gallate (C₈H₈O₅) (Sigma-Aldrich) and their mixtures (1:1 v/v), as well as propyl gallate (C₁₀H₁₂O₅) (Sigma-Aldrich), was analyzed as described above. All substrates were prepared in 100 mmol L⁻¹ sodium acetate buffer (pH 5.0). The kinetic parameters K_m and V_{max} , K_{cat} and K_{cat}/K_m were determined for each substrate using the software SIGRAF (Leone et al. 1992).

Synthesis of propyl gallate

Propyl gallate was produced by transesterification of tannic acid in the presence of the purified *A. fumigatus* CAS-21 tannase. The reaction mixture containing 5 mmol L⁻¹ tannic acid in 100 mmol L⁻¹ MES buffer (pH 6.0), 1-propanol (1:1 v/v), and the tannase was maintained at 30 °C for different periods (48 h and 72 h). Five-microliter samples of each reaction were loaded in a column Ascentis® Express C18 (100×4.6 mm, 2.7 μm particle diameter) from Supelco in the System ACQUITY UPLC *H-Class* with the diodes arrangement detector (CLUE-DAD, Waters Corporation). An aqueous solution of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) were used as mobile phase. The separation was carried out using isocratic mode with 5% acetonitrile for 0–3 min followed

by a separation using a gradient of 5–60% acetonitrile from 3 min to 10 min. Then, a gradient of 60–90% acetonitrile was applied from 10 min to 15 min, followed by 90% acetonitrile for the next 5 min, and 5% acetonitrile for another 5 min. The flow rate was maintained at 400 μL min⁻¹ and the compounds present in the samples were monitored by DAD at 220–600 nm. Tannic acid, gallic acid, and propyl gallate were used as standards.

Treatment of leather effluent using tannase

The industrial effluent samples were obtained from the tanning process of he-goat leather in the Arteza cooperative, Paraíba, Brazil. Effluent samples obtained after mechanical and manual processing of the leather were treated with crude filtrate containing tannase (1:1 v/v) for 1 and 2 h at 30 °C, and the content of tannins and total phenols was determined.

The total phenol content was determined using a modified Folin–Ciocalteu method from Agostini-Costa et al. (1999). After the treatment, 50 μL of the effluent samples was added with 2.5 mL of the Folin–Ciocalteu (10% v/v) (Merck) reagent, 2 mL of sodium carbonate (25% m/v) and maintained in absence of light for 30 min. Thereafter, the tube content was analyzed at 760 nm. For both analyses, in the blank tube (control) the enzyme sample was replaced by distilled water.

The tannin levels of the treated and untreated effluent samples were determined according to the methodology described by Hagerman and Butler (1978). The reaction consisted of 1 mL of the sample and 3 mL of BSA prepared in 0.2 mol L⁻¹ sodium acetate buffer (pH 5.0) added with 0.17 mol L⁻¹ sodium chloride and maintained for 15 min at room temperature. The tubes were centrifuged at 5000×g for 10 min (Centrifuge 5415 C—Eppendorf). The supernatant was discarded and the pellet was dissolved in 1 mL of 1% (w/v) BSA solution, added with 3 mL of 1% (w/v) SDS in 5% (v/v) triethanolamine solution and 1 mL of 0.01 mol L⁻¹ FeCl₃. After 15 min at room temperature, the absorbance was recorded at 510 nm using a spectrophotometer (UV Mini-1240, Shimadzu).

The enzymatic hydrolysis of the effluent tannins was also analyzed by CLUE-DAD as described above. For this purpose, the reactions containing effluent or tannic acid samples were conducted for 2 h at 30 °C.

Results

Purification

Figure 1a depicts the DEAE-Cellulose chromatographic profile obtained for the *A. fumigatus* CAS-21 tannase. Three peaks with tannase activity were obtained. The first peak (FI) did not interact with the resin, while the peaks

Fig. 1 DEAE-Cellulose (a) and Sepharose CL-6B (b) chromatographic profiles for the tannase produced by *A. fumigatus* CAS-21 and 12% SDS-PAGE results (c) for proteins obtained from DEAE-Cellulose (I) and Sepharose CL-6B (II) column chromatography. 345 μ g of protein was applied in (I) and 390 μ g of protein in (II). In (III): Molecular Mass Markers (Bio-Rad). Symbols: (open circle) tannase activity; (closed circle) absorbance at 280 nm

FII and FIII interacted and they were eluted with 0.25 and 0.50 mol L⁻¹ of NaCl, respectively. Considering the higher activity obtained for the FII, it was selected for the next purification step. The FII fractions were pooled, dialyzed, lyophilized, and loaded in Sepharose CL-6B (Fig. 1b). A single peak with tannase activity was obtained. Using these procedures, one tannase isoform was purified 4.9-fold with a recovery of 10.2% (Table 1). The native molecular mass for the purified enzyme was estimated as 60.34 ± 0.84 kDa through the Sepharose CL-6B chromatographic column and 60.85 ± 5.80 kDa by SDS-PAGE (Fig. 1c), confirming that it is a monomeric glycoprotein with 39.1% carbohydrate content.

Effect of temperature and pH on the tannase activity

The maximal activity for *A. fumigatus* CAS-21 tannase was observed at a range from 30 to 40 °C (Fig. 2a), while the optimum pH was 5.0 (Fig. 2b). The *A. fumigatus* CAS-21 tannase maintained 80% of its initial activity when incubated at 30 °C for 60 min (Fig. 2c). Reduction of the enzyme activity to 50% of the initial value was observed upon incubation for 120 min at this temperature. At 45 and 50 °C, the half-life (t_{50}) for the enzyme was 60 min. The enzyme was fully stable at pH 5.0 and 6.0 after 3 h of incubation, while it retained 70% of its initial activity at these pH values after 24 h incubation (Fig. 2d).

Effect of salts and other compounds

The effect of different salts on tannase activity is presented in Table 2. The enzyme was slightly activated in the presence of AgNO₃, KH₂PO₄, and MnSO₄, but inhibited approximately 50% by 1 mmol L⁻¹ Fe₂(SO₄)₃ and FeCl₃. In case of detergents and denaturing agents, tannase activity was increased in the presence of SDS (+15%), Triton X-100 (+18%), methanol (+8%), and especially urea (Table 3). The activity of the *A. fumigatus* CAS-21 tannase was increased at urea concentrations from 1×10^{-3} mol L⁻¹ to 1 mol L⁻¹, with the highest activity at a concentration of 4×10^{-3} mol L⁻¹ (+90%). In addition, the enzyme activity was maintained at 80–90% at high urea concentrations (2 and 3 mol L⁻¹) (Fig. 2e).

Similarly, it was verified that the addition of glucose in the enzymatic reaction did not affect tannase activity under all concentrations analyzed (Fig. 2f). On the contrary, addition of gallic acid at a concentration higher than 5 mmol L⁻¹ reduced the enzyme activity, and at 10 mmol L⁻¹ the tannase activity was totally inhibited (Fig. 2f).

Table 1 Purification of the extracellular tannase produced by *A. fumigatus* CAS-21

Step	Volume (mL)	Activity (total U)	Protein (total mg)	SA (U/mg)	Yield (%)	Purification (fold)
Crude filtrate	200	8669.0	204.7	42.3	100	1
DEAE – cellulose (peak FII)	47	2105.3	10.8	194.6	24.3	4.6
Sepharose CL-6B	16	881.3	4.2	209.2	10.2	4.9

SA specific activity

Fig. 2 Influence of temperature (a), pH (b), thermal stability (c), pH stability (d), and concentration of urea (e), gallic acid and glucose (f) on tannase activity from *A. fumigatus* CAS-21. Symbols: in c—(open circle) 30 °C, (open square) 45 °C, (open upwards triangle) 50 °C and(open downwards triangle) 65 °C; in d: (closed upwards triangle) 3 h and (closed downwards triangle) 24 h. 100% activity corresponds to $48.74 \pm 0.90 \text{ U mL}^{-1}$ (d) and $31.61 \pm 0.82 \text{ U mL}^{-1}$ (e)

Table 2 Influence of different salts on the activity of tannase from *A. fumigatus* CAS-21

Salts (1 mmol L ⁻¹)	Relative activity (%) ^a
Control	100.0
AgNO ₃	110.7 ± 1.2
BaCl ₂	78.2 ± 5.7
CaCl ₂	95.0 ± 4.8
CoCl ₂	74.7 ± 5.4
CuCl ₂	100.3 ± 8.3
CuSO ₄	92.8 ± 5.9
Fe ₂ (SO ₄) ₃	58.2 ± 6.7
FeCl ₃	49.7 ± 3.2
HgCl ₂	96.3 ± 0.1
KH ₂ PO ₄	110.3 ± 1.0
MgCl ₂	75.6 ± 6.2
MgSO ₄	97.6 ± 3.9
MnCl ₂	87.1 ± 5.8
MnSO ₄	112.9 ± 2.1
NaCl	78.4 ± 2.2
NH ₄ Cl	93.2 ± 7.5
Zn(NO ₃) ₂	100.8 ± 7.0
ZnSO ₄	101.2 ± 0.2

^a100% relative activity corresponds to 31.61 ± 0.82 U mL⁻¹**Table 3** Influence of different compounds on the activity of tannase from *A. fumigatus* CAS-21

Compounds	Relative activity (%) ^a
Control	100.0
Acetone (1%)	90.0 ± 2.9
Acetonitrile (1%)	69.4 ± 0.5
Butanol (1%)	94.3 ± 2.7
EDTA (1 mmol L ⁻¹)	80.6 ± 7.8
Ethanol (1%)	88.1 ± 2.4
Isopropanol (1%)	87.6 ± 6.0
Methanol (1%)	108.8 ± 4.5
SDS (1 mmol L ⁻¹)	115.7 ± 1.7
Triton X-100 (0.01%)	118.4 ± 4.9
Tween-20 (0.01%)	89.2 ± 2.6
Urea (1 mmol L ⁻¹)	154.7 ± 4.0
β-Mercaptoethanol (1 mmol L ⁻¹)	82.8 ± 7.9

^a100% relative activity corresponds to 31.61 ± 0.82 U mL⁻¹

Hydrolysis of substrates and kinetic parameters

The purified *A. fumigatus* CAS-21 tannase was able to hydrolyze tannic acid and methyl gallate, as well as the mixture of these two substrates (Fig. 3). The K_m value (3.61 mmol L⁻¹) indicated high affinity for propyl gallate,

Fig. 3 Hydrolysis of the substrates methyl gallate (open circle), tannic acid (closed circle), and methyl gallate+tannic acid (1:1 v/v) (closed triangle) as a function of time of reaction by the tannase from *A. fumigatus* CAS-21**Table 4** Kinetic parameters for the extracellular tannase produced by *A. fumigatus* CAS-21

Substrate	K_m (mmol L ⁻¹)	V_{max} (U mg ⁻¹)	K_{cat} (s ⁻¹)	K_{cat}/K_m (s ⁻¹ mmol ⁻¹ L)
Tannic acid	6.38	360.20	362.24	56.78
Methyl gallate	6.28	152.00	152.86	24.34
Propyl gallate	3.61	111.30	111.93	31.00

but the best K_{cat} (362.24 s⁻¹) and K_{cat}/K_m (56.78 s⁻¹ mmol⁻¹ L) were obtained for tannic acid (Table 4).

Propyl gallate synthesis by transesterification

The *A. fumigatus* CAS-21 tannase was able to catalyze transesterification reaction in presence of 1-propanol to produce propyl gallate, evident from the CLUE-DAD profiles (Fig. 4). The peaks with retention times of 10.53 min and 10.56 min obtained after 48 h (Fig. 4d) and 72 h (Fig. 4e) reactions, respectively, were similar to that obtained for the propyl gallate standard (10.56 min) (Fig. 4c). Moreover, residual tannic acid could be observed (Fig. 4a), while gallic acid was not detected.

Effluent treatment

While analyzing the CLUE-DAD profiles for the effluent treatment (Fig. 4f, g), a single peak at 5.3 min that corresponded to the retention time for the gallic acid was observed, indicating that the *A. fumigatus* CAS-21 tannase was effective in hydrolyzing the tannic acid content in effluents generated by

Fig. 4 CLUE-DAD profiles for the propyl gallate synthesis after 48-h (**d**) and 72-h (**e**) reactions through esterification of tannic acid (5 mmol L^{-1}) in 50% of 1-propanol and for the products obtained

from the 2-h treatment of leather effluent generated by manual (**f**) and mechanical (**g**) processes using *A. fumigatus* CAS-21 tannase. Standards: tannic acid (**a**), gallic acid (**b**), and propyl gallate (**c**)

mechanical as well as manual processes. The tannase reduced 89 and 25% of the tannin content in manually and mechanically processed samples of the leather effluent, respectively, after 2 h of treatment (Fig. 5a). In addition, the filtrate containing tannase from *A. fumigatus* CAS-21 was also able to reduce 30% of the content of phenolic compounds in both the effluent samples (Fig. 5b).

Discussion

The fungus *A. fumigatus* CAS-21 can produce tannase isoforms when maintained under SbmF in presence of tannic acid as carbon source. One of the enzyme isoforms was effectively purified using DEAE-Cellulose and Sephacryl

Fig. 5 Treatment of the leather effluent after manual (closed square) and mechanical (open square) procedures, with tannase produced by *A. fumigatus* CAS-21, considering the tannin (a) and phenolic (b) content

S-200. Notably, the enzyme recuperation after these procedures was higher than that reported for the tannases produced by *Aspergillus ochraceus* (2.12%) (Gonçalves et al. 2012), *A. tamarii* (TAH II- 3.84%) (Costa et al. 2012), and *Verticillium* sp. (TAH I- 1.6% and TAH II- 0.9%) (Kasieczka-Burnecka et al. 2007).

Enzyme purity was confirmed by SDS-PAGE, observing a single protein band with similar molecular mass obtained using gel filtration, indicating the monomeric characteristic for this enzyme. Moreover, the analysis of the carbohydrate content indicates that the *A. fumigatus* CAS-21 is a glycoprotein. In general, tannases have a molecular mass ranging from 50 to 320 kDa (Yao et al. 2014) and carbohydrate content from 5 to 64% (Kasieczka-Burnecka et al. 2007; Costa et al. 2012). Monomeric tannases were reported for *Aspergillus melleus* (Liu et al. 2017), *A. niger* (Lal and Gardner 2012), *A. ochraceus* (Gonçalves et al. 2012), and *Penicillium herquei* (Qiu et al. 2011). The TAH I and TAH II tannases from *Verticillium* sp. were also characterized as glycoproteins with 11% and 26% of carbohydrate content, respectively (Kasieczka-Burnecka et al. 2007), whereas the enzyme of *A. awamori* BTMFW032 showed 8.02% carbohydrate content (Beena et al. 2010). Other fungal tannases were also characterized as glycoproteins such as those from *A. tamarii* (Costa et al. 2012), *A. phoenicis* (Riul et al. 2013), and *A. carbonarius* (Valera et al. 2015). Remarkably, glycosylation is an important characteristic to protect the tannases from the negative effects of tannin (Lekha and Lonsane 1997).

The optimal temperature (30–40 °C) for *A. fumigatus* CAS-21 tannase activity is in agreement with the data reported by other authors. For example, Gonçalves et al. (2012) found optimal activity at 30–45 °C for *A. ochraceus* tannase. According to Yao et al. (2014), tannases exhibit optimal activity between 30 and 60 °C. The optimal temperature of activity for the enzymes from *A. awamori*

(Kumar et al. 2015) and *A. awamori* MTCC9299 (Chhokar et al. 2010) was 30 °C, while for the isoforms TAH I and TAH II from *A. tamarii* tannases, it was 30 °C and 40 °C, respectively (Costa et al. 2012). For *A. fumigatus* CAS-21 tannase, temperatures above 65 °C promoted reduction in enzymatic activity because under high temperatures, the weak bonds in the three-dimensional structure of the protein break, causing its denaturation and loss of activity (Mukherjee and Banerjee 2006). Considering the fact that *A. fumigatus* CAS-21 was isolated from a plant found in the Caatinga biome, which presents high temperatures during the day and mild temperatures during the night, the presence of a tannase able to act at a wide temperature range is justified. The *A. fumigatus* CAS-21 tannase was stable for 1 h at 30 °C, which is also an optimal temperature for its activity, with a half-life of 30 min at 45 and 50 °C, in agreement with the literature. Interestingly, some tannases were previously reported to be stable at a temperature range from 10 to 60 °C (Costa et al. 2008; Sharma et al. 1999; Pinto et al. 2005).

Similar to our results, tannases from different *Aspergillus* species presented optimal activity at pH 5.0 as observed for *A. tamarii* (Costa et al. 2008), *A. ochraceus* (Gonçalves et al. 2012), *A. awamori nakazawa* (Mahapatra et al. 2005), and *A. oryzae* (Mizuno et al. 2014), among others. Moreover, most tannases are stable at pH 5.0–6.0 (Aguilar et al. 2007), such as those from *A. niger* GH1 (Mata-Gomez et al. 2009), *A. awamori* (Kumar et al. 2015), *A. oryzae* (Abdel-Nabey et al. 2016), and *A. fumigatus* CAS-21. Importantly, pH affects the ionization of amino acids in the protein, which can modify ionic interactions that determine the molecule structure, thereby affecting linkage to the substrate, inactivating the enzyme or even promoting its instability. In addition, the abundance of acidic amino acids in the enzyme can explain the stability of fungal tannases at an acidic pH (Abdel-Nabey et al. 2016).

Different classes of compounds can positively or negatively affect tannase activity. The most pronounced effect on the *A. fumigatus* CAS-21 tannase was observed in presence of FeCl_3 and $\text{Fe}_2(\text{SO}_4)_3$, which inhibited enzyme activity. Similarly, inhibition by FeCl_3 was also observed for the enzymes produced by *A. carbonarius* (Valera et al. 2015), *A. niger* ATCC 16620 (Sabu et al. 2005), *A. oryzae* (Abdel-Nabey et al. 2016), and *A. awamori* MTCC9299 (Chhokar et al. 2010). This effect can be justified by the linkage between iron ions and the thiol groups and/or by the interaction of ions with the carboxylic group of amino acids at the catalytic site of the protein (Abdel-Nabey et al. 2016). Furthermore, the presence of Mg^{2+} , Co^{2+} , and Ba^{2+} inhibited 25% of the enzyme activity. Interestingly, the presence of sulfate or chloride in the salts is an important factor regulating the enzyme activity. For example, the negative effects were mostly accentuated in the presence of MnCl_2 and MgCl_2 than in the presence of MnSO_4 and MgSO_4 . For the other salts analyzed, the activity was not significantly affected. For instance, the tannase produced by *Pestalotiopsis guepinii* URM 7114 was activated by Ca^{2+} (Sena et al. 2014), while the *A. fumigatus* CAS-21 tannase was not affected by Ca^{2+} . Consistent with our results, Valera et al. (2015) also observed activation by silver of the enzyme produced by *A. carbonarius*. However, for most proteins, silver acts as a precipitation agent inhibiting their activities.

Other compounds also affected the activity of the *A. fumigatus* CAS-21 tannase. Increase in tannase activity by urea, as observed by us, was also reported for the enzymes from *A. awamori nakazawa* and *Verticillium* sp. in presence of 1.5 mol L^{-1} urea (Kasieczka-Burnecka et al. 2007; Mahapatra et al. 2005). According to Mahapatra et al. (2005), tannase is denatured only at high urea concentrations, which can be explained by the conformational changes in the tertiary structure of the enzyme. On the contrary, in the presence of acetonitrile, the enzymatic activity was inhibited by 30%. For other compounds, tannase activity was slightly reduced, retaining 80–90% of the initial activity. Thus, the ability to remain stable in presence of organic solvents, surfactants, and denaturants are favorable characteristics for industrial applications of *A. fumigatus* CAS-21 tannase.

Glucose and gallic acid are the products of tannic acid hydrolysis under tannase action. The products obtained from the substrate hydrolysis inhibit many enzymes. The hydrolysis of substrates containing different molecules such as lignin, saccharides, and tannins, can be achieved by application of enzymatic cocktails (Riul et al. 2013). Considering the resistance of the *A. fumigatus* CAS-21 tannase to the inhibition by glucose, it can be exploited for the formulation of enzymatic cocktails to hydrolyze tannin substrates at a high saccharide concentration. The *A. fumigatus* CAS-21 tannase was inhibited by gallic acid, as also reported by Kar et al.

(1999) and El-Fouly et al. (2010), where it acts as a competitive inhibitor of the enzyme. Consistently, the tannase produced by *A. phoenicis* was not regulated by glucose, but it was drastically inhibited at high concentrations of gallic acid (Riul et al. 2013).

The *A. fumigatus* CAS-21 was able to hydrolyze different substrates, but the highest values of hydrolysis were obtained using tannic acid. The values of hydrolysis for the mixture (tannic acid + methyl gallate) were intermediary between the values obtained for the separate hydrolysis of each substrate, indicating that there is a single active site for hydrolysis of the substrates. If there are two different active sites, one for each substrate, then the expected values for the hydrolysis of the mixture of the two substrates should be approximately similar to the addition of the separate values obtained. Generally, tannases are isoenzymes with different specificity for each substrate according to its structure, exhibiting both esterase and depsidic activities (Rodríguez-Durán et al. 2011). Other tannases can also hydrolyze different substrates, for instance, the enzymes produced by *Aspergillus oryzae* (Mizuno et al. 2014) and *Emericella nidulans* (Gonçalves et al. 2011).

On the other hand, the best affinity was observed for propyl gallate, which is also observed for the tannases produced by *A. phoenicis* (Riul et al. 2013) and *Penicillium variable* (Sharma et al. 2008). The affinity of the *A. fumigatus* CAS-21 tannase for the tannic acid and methyl gallate substrates was similar to that of the *A. ochraceus* enzyme (Gonçalves et al. 2012). The K_{cat} for the *A. fumigatus* CAS-21 tannase was lower than that obtained for the enzyme from *A. oryzae* (Abdel-Nabey et al. 2016), indicating that the catalytic cycle performed by the CAS-21 tannase is faster than that performed by the *A. oryzae* enzyme.

The potential of *A. fumigatus* CAS-21 tannase for propyl gallate synthesis was analyzed, considering its favorable characteristics such as the stability over a wide range of pH and temperature, tolerance to surfactants, organic solvents and denaturing agents, and a high activity in the presence of glucose. The propyl gallate production continuously increased during the 72-h reaction, indicating that the enzyme can be applied for the production of this important antioxidant in food industry (Fernandez-Lorente et al. 2011). Tannases produced by *A. phoenicis* (Riul et al. 2013), *A. awamori* BTMFW032 (Beena et al. 2010), *A. niger*, and *Penicillium variable* (Sharma and Saxena 2012) were also described as potential enzymes to produce propyl gallate.

Another important application of tannases is the treatment of tannin-rich waste generated by leather industry. As tannin is an important recalcitrant agent found in the tannery effluent, special attention is required for its degradation prior to elimination in the environment. In Brazil, many leather industries use tannic acid to treat the leather. The use of *A. fumigatus* CAS-21 tannase to treat the leather effluent was

effective and a single gallic acid peak was observed after 2-h treatment of both mechanically and manually processed effluents. No tannic acid signal was detected, indicating the potential of *A. fumigatus* CAS-21 tannase for an eco-friendly treatment of tannin-rich effluents, especially in the leather industry. In addition, an alternative procedure for the synthesis of gallic acid is introduced using a low cost substrate, i.e., the effluent, thereby enhancing its commercial value. Gallic acid is an important compound for the pharmaceutical and food industries (Yao et al. 2014).

To date, no tannase application has been reported for the treatment of tannery effluents generated by he-goat leather industries. On the other hand, the treatment of tannery effluents from Mango industry using *Aspergillus candidus* MTTC 9628 tannase reduced the tannin content, and the effluent color (Murugan and Al-Sohaibani 2010). The use of *A. niger* and *Penicillium variable* tannases for the treatment of tannery effluent allowed 45 and 36% reduction in the tannin content, respectively (Sharma and Saxena 2012). Additionally, Salgado et al. (2016) demonstrated that the use of fungi in the bioremediation of olive mill and winery wastewaters resulted in higher reductions of phenolic compounds and color because of the production of enzymes such as tannase.

In conclusion, the fungus *A. fumigatus* CAS-21, isolated from the Caatinga biome, was found to be a good source of tannases. One of these tannases was characterized and it presented tremendous biotechnological potential for the treatment of tannery effluent and propyl gallate production using a transesterification reaction. In addition, the enzyme presented interesting properties such as thermal and pH stability, insensitivity to glucose, tolerance to solvents and detergents, thereby amplifying the possibilities of its biotechnological application.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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