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# Extracellular tannase from *Emericella nidulans* showing hypertolerance to temperature and organic solvents

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ABSTRACT

The filamentous fungus *Emericella nidulans* (=*Aspergillus nidulans*) produced high levels of extracellular tannase when grown at 30 °C, under agitation (100 rpm), for 24 h in Khanna medium supplemented with tannic acid as carbon source. The enzyme was purified 61-fold, with 30% yield. The molecular mass of the native protein was estimated to be 302 kDa by gel filtration, with a carbohydrate content of 50%. Two protein bands (45.8 and 52 kDa) were observed after 12% SDS–PAGE, suggesting a glycoprotein constituted by three copies of each subunit. The extracellular tannase showed temperature and pH optima of 45 °C and 5.0, respectively, and was fully stable in the temperature range of 22–50 °C, with a half-life ($t_{50}$) of about 72 h at 90 °C. The enzyme retained around 80% of control activity when maintained for 60 h at pH 4.0 or 5.0. Tannase activity was stimulated by $Zn^{2+}$, $Hg^{2+}$, $Co^{2+}$, and the detergents SDS and Triton X-100. Organic solvents (about 50%, v/v) also increased enzyme activity, particularly isopropanol, acetonitrile, and ethanol. The $K_m$ and $V_{max}$ values were 14.01 mM and 2.63 U mg$^{-1}$ protein in the presence of tannic acid; and 4.78 mM and 0.29 U mg$^{-1}$ protein in the presence of methyl gallate. For propyl gallate, the $V_{max}$ was 0.05 U mg$^{-1}$ protein, with $K_m$ of 7.69 mM; for pyrogallol, the $V_{max}$ was 7.40 U mg$^{-1}$ protein and the $K_m$ was 16.94 mM.



## 1. Introduction

Microorganisms contribute in different ways to the decomposition of organic materials and plant tissues that contain high concentrations of phenolic compounds such as tannins [1]. The hydrolysis of tannins is mediated by two types of enzymatic activities, one acting on the ester linkages between the aromatic ring and the glucose residue, and the other on the depsidic linkage between the two aromatic rings [2]. According to Haslam and Stragroom [3], both esterase and depsidase activities may be attributed to tannases (tannin acyl hydrolase; EC 3.1.1.20), which act on hydrolysable tannins to produce glucose and gallic acid [4,5]. From a biotechnological perspective, tannases have been applied in different industrial sectors, such as those of instantaneous tea [6], beverages (wine, beer, and juice), food and feed [4,7] production. In the pharmaceutical industry, gallic acid obtained by the action of tannases can be used as an intermediate for the synthesis of the antibacterial drug Trimethoprim [8]. Additionally, gallic acid can be used as a substrate for the synthesis of propyl gallate, which is used as an important antioxidant in the food industry [9]. Tannases may be also applied for the treatment of tannin-rich effluents, such as wastewater from the leather industry [4]. In spite of the publication of many studies that deal with the technological potential of tannases, the real use of these enzymes remains limited. In fact, there is a deficiency in terms of studies and knowledge regarding their properties and large-scale applications [1,10,11]. Among microorganisms, filamentous fungi such as *Aspergillus niger* (MTCC 2425) [12], *A. oryzae* [13], *Paecilomyces variotti* [11,14], *Aspergillus awamori* [15], and *Penicillium chrysogenum* [16] may be highlighted as good producers of versatile tannases that act efficiently on different types of tannins [3,17]. Tannase production by fungi has been reported using both submerged and solid-state fermentations in the presence and absence of an inducer [18,19]. Additionally, tannases have been purified by different methods and the influence of several compounds such as salts and organic solvents on enzymatic activity has been analyzed. Generally, most of the solvents cause inhibition of tannase activity, as verified by Beena et al. [10] and Chokkar et al. [20] for the enzymes isolated from *A. awamori* strains BTMFW032 and MTCC9299, respectively. Consequently, the search for novel tannases, followed by characterization of their innovative properties, has assumed great importance. The aim of this work was to characterize a hypertolerant extracellular tannase that can withstand temperatures and is resistant to organic solvents, which was isolated from the

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filamentous fungus *E. nidulans* (=*Aspergillus nidulans*); this enzyme has a high potential for biotechnological applications.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

*E. nidulans* was isolated from the soil in Ribeirão Preto, SP, Brazil (21°12′42″ South and 47°48′24″ West) and identified by André Tosello Foundation, Campinas, SP, Brazil according to morphological, physiological, and biochemical analyses by comparison with the reference standard. The microorganism was maintained in potato dextrose agar (PDA) slants at 4 °C; after every 30 days, spores were inoculated on fresh PDA slants, incubated at 30 °C for 5 days and stored at 4 °C.

Submerged fermentation was carried out by the addition of 1 mL of aqueous spore solution ($10^5$ spores/mL), obtained from scraping the surface of 5-day-old PDA slants, in 25 mL of Khanna medium [21] (0.1%, w/v yeast extract; 5%, v/v Khanna salt solution) in 125 mL Erlenmeyer flasks with tannic acid 2% (w/v) as carbon source, initial pH 6.0, previously autoclaved at 120 °C and 1.5 atm for 30 min. The composition of Khanna salt solution was (w/v): 2% $NH_4NO_3$, 1.3% $KH_2PO_4$, 0.36% $MgSO_4$, 0.1% KCl, 0.01% $ZnSO_4$, 0.01% $MnSO_4$, 0.007% $Fe_2(SO_4)_3$, and 0.006% $CuSO_4$. The cultures were maintained at 30 °C for different periods (24–96 h) under agitation (100 rpm).

### 2.2. Extracellular crude extract

After incubation, the cultures were harvested by vacuum filtration using Whatman No.1 filter paper. The filtrate, named extracellular crude enzyme, was dialyzed overnight against distilled water at 4 °C and used for enzymatic assays.

### 2.3. Quantification of tannase activity and the concentrations of proteins and carbohydrates

The enzymatic assay was carried out using methyl gallate as substrate in 100 mM sodium acetate buffer, pH 5.0. The gallic acid produced was quantified according to the procedure described by Sharma et al. [22] using 0.66% (w/v) methanolic rhodanine; the absorbance of the solution was read at 520 nm. The reaction was conducted for different time intervals at various temperatures. One activity unit was defined as the amount of enzyme necessary to produce 1 μmol of gallic acid per minute under the assay conditions.

Protein was quantified according to the procedure described by Lowry et al. [23], using BSA as the standard; and the carbohydrate content of the purified enzyme was estimated based on the method of Dubois et al. [24], with mannose as the standard.

### 2.4. Influence of additional sources of carbon and nitrogen on production of tannase

Different concentrations of additional glucose (0.1–5.0%, w/v), yeast extract (0.1–2.0%, w/v), and $KH_2PO_4$ (0.1–2.0%, w/v), as sources of carbon, nitrogen, and phosphate, respectively, were added to the culture medium containing 2% tannic acid as the main carbon source.

### 2.5. Enzyme purification

The extracellular crude enzyme obtained from *E. nidulans* culture was treated with 40 mg/mL kaolinite at 4 °C for 2 h under agitation and centrifuged at 23,000 × *g*. Subsequently, the extracellular tannase was applied to a diethylaminoethyl (DEAE)-cellulose column (1 cm × 12 cm), which was previously equilibrated with 10 mM sodium acetate buffer, pH 5.0, and eluted with a NaCl linear gradient (0–1.5 M NaCl) in the same buffer. Fractions of 3.0 mL were collected at a flow rate of 1 mL/min and analyzed for protein content ($A_{280}$) and tannase activity. The most active fractions were pooled, dialyzed overnight against distilled water, lyophilized, and applied to a Sephacryl S-200 column (1 cm × 80 cm) equilibrated and eluted with 50 mM Tris–HCl buffer, pH 7.5, containing 50 mM NaCl. Fractions of 1.0 mL were collected at a flow rate of 0.33 mL/min. Active fractions were pooled, dialyzed overnight against distilled water at 4 °C, lyophilized, and used for electrophoretic analysis.

### 2.6. Electrophoresis and determination of molecular mass

The purified extracellular enzyme was subjected to nondenaturing electrophoresis (7% polyacrylamide gel electrophoresis, PAGE) [25] and stained for protein with silver, according to the procedure of Blum et al. [26]. The tannase activity was determined based on the method of Aoki et al. [27]. The polyacrylamide gel was washed in 2.5% Triton X-100 for 1 h, then washed with 10 mM sodium acetate buffer (pH 5.5) for 45 min, and placed in a solution of 0.5% (w/v) tannic acid in 100 mM sodium acetate buffer, at the same pH, for 24 h. Thereafter, the tannic acid solution was replaced by a solution of 0.5% quinine hydrochloride (w/v) in 50 mM sodium acetate buffer, pH 5.5, at room temperature. Denaturing electrophoresis (12% sodium dodecyl sulfate (SDS)–PAGE) was carried out according to the method described by Laemmli [28], and the protein bands were stained with Coomassie Brilliant Blue R-250. Phosphorylase-b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (33 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as molecular mass standards.

The molecular mass of the purified native enzyme was determined by gel filtration through a Sepharose CL-6B column (1 cm × 80 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.5, containing 100 mM KCl. Fractions of 1.0 mL were collected at a flow rate of 1.73 mL/min. The proteins β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as molecular mass standards.

### 2.7. Influence of temperature and pH on tannase activity

Optimum temperature for tannase activity was determined by carrying out the reaction at temperatures ranging from 25 to 90 °C. Thermal stability was determined by incubating an aqueous solution of the enzyme for 96 h at different temperatures (22, 30, 50, 70, and 90 °C). Optimum pH for tannase activity was determined using 100 mM citric acid (pH 3.0–4.0), 100 mM sodium acetate buffer (pH 4.5–6.0), 100 mM Tris–HCl buffer (pH 7.0–8.0). The stability at different pH values was determined using the same buffers used for optimum pH assay and, additionally, 100 mM N-cyclohexyl-3-aminopropanesulfonic acid or CAPS buffer (pH 10.0–11.0). The enzyme samples were incubated in the respective buffers for 60 h in an ice bath. For determination of thermal and pH stability, aliquots were obtained at different times and assayed for enzymatic activity as described above.

### 2.8. Influence of additives on tannase activity

The influence of 1 mM of different salts; 1–20 mM β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA); 0.01–0.2% (v/v) SDS and Triton X-100; and 1–20% (v/v) organic solvents (methanol, acetonitrile, ethanol, acetone, isopropanol, n-butanol, and glycerol) on tannase activity was tested. The stability of the enzyme in organic solvents was tested by incubating the aqueous enzyme solution containing one of the following: 50% (v/v)

methanol, acetonitrile, ethanol, acetone, isopropanol, n-butanol, and glycerol, for different periods in an ice bath. After durations of 10 min, 4 h, and 8 h of treatment, the samples were withdrawn and assayed for enzymatic activity as described before.

### 2.9. Hydrolysis of different substrates and estimation of kinetic parameters

The hydrolytic activity of the extracellular tannase produced by *E. nidulans* on tannic acid, methyl gallate, and tannic acid plus methyl gallate (1:1; v/v) was analyzed for different reaction times (0–120 min). The hydrolysis products were analyzed by thin-layer chromatography (TLC) as described by Mahendran et al. [29].

The kinetic parameters $K_m$ (Michaelis–Menten constant), $V_{max}$ (maximal velocity), and $V_{max}/K_m$ (catalytic efficiency) were determined using tannic acid (0.25–10 mM), methyl gallate (0.25–10 mM), propyl gallate (0.25–20 mM), and pyrogallol (0.25–4 mM) as substrates. The kinetic parameters were calculated based on Lineweaver–Burk [30] plots using the Enzyplot software [31].

### 2.10. Statistics

All experiments were conducted in triplicate, and the values were represented as mean values ± standard deviation.

## 3. Results and discussion

### 3.1. Production of tannases by E. nidulans

*E. nidulans* produced high levels of an extracellular tannase when grown for 24 h at 30 °C, under agitation (100 rpm), in Khanna medium supplemented with tannic acid as carbon source. Enzymatic activity was two fold higher in the extracellular medium, compared to the cell lysate, and maximal intracellular activity occurred after 3 days. The influence of other carbon sources on enzyme production was investigated; however, all of them resulted in lower levels of total activity. Most tannases are inducible enzymes, produced only in the presence of a source of tannin, such as tannic acid [32]. Bajpai and Patil [33] showed that methyl gallate, gallic acid, and pyrogallol can induce the production of tannases. On the contrary, according to Bradoo et al. [34], enzyme production may also occur in the absence of tannic acid, using other carbon sources, such as saccharides, peptone, or casein. However, the mechanisms involved in tannase induction have not been completely elucidated [35].

Addition of gallic acid at a final concentration of 0.25% to the culture medium supplemented with tannic acid promoted a small increase in extracellular activity, but a decrease occurred at higher concentrations ranging from 0.5 to 5.0% (Fig. 1). Intracellular levels increased two fold in the presence of 0.75% gallic acid, whereas the addition of low concentrations of glucose (0.1%) increased both extra- and intracellular activities. Enzyme production was drastically inhibited at higher concentrations of glucose (above 0.5%), characterizing catabolite repression, in spite of the presence of an inducer (Fig. 1). Addition of yeast extract (0.75%) or $KH_2PO_4$ (1.0%), as nitrogen or phosphate sources, increased the production of the intracellular form of the enzyme by approximately 14- and 11-fold, respectively; however, secretion of the enzyme was severely inhibited (data not shown).

### 3.2. Enzyme purification

After treatment with kaolinite, tannase-rich crude extracellular extract was loaded onto a DEAE-cellulose column, and a single peak of enzymatic activity was eluted with 0.58 M NaCl. Active fractions were pooled, dialyzed for 24 h against distilled water at 4 °C, lyophilized, and applied onto a Sephacryl S-200 column. A single peak of tannase activity was eluted. After this step, the enzyme was purified 61 times to electrophoretic homogeneity, with 30% yield (Table 1).

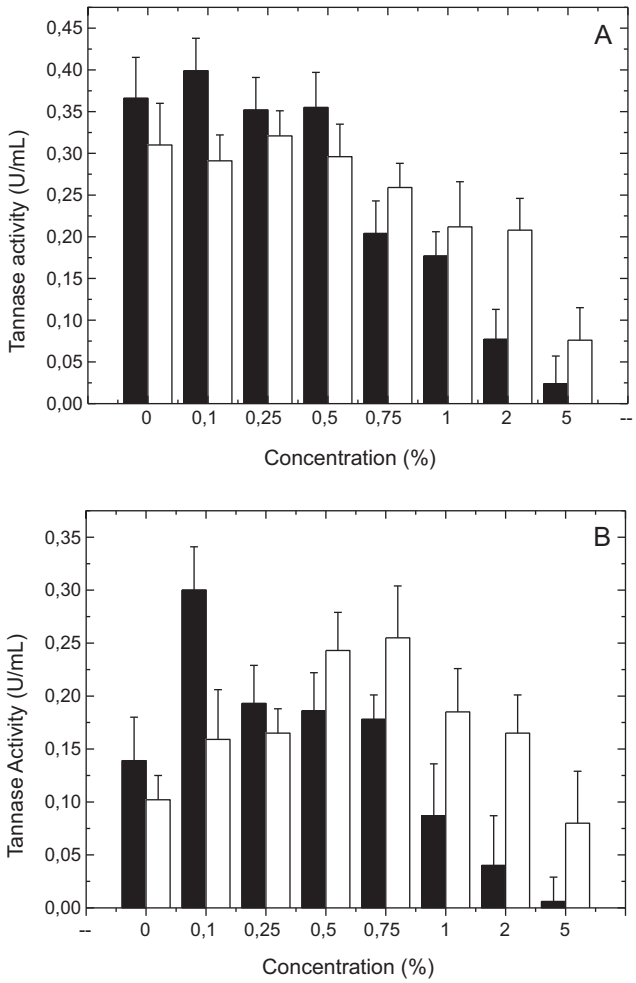


**Fig. 1.** Influence of addition of glucose (■) and gallic acid (□) to the culture medium on the production of extracellular (A) and intracellular (B) tannases by *E. nidulans*.

### 3.3. Molecular mass and carbohydrate content

The purified enzyme was subjected to nondenaturing electrophoresis (7% PAGE) and stained for both protein content and activity. In both cases, a single protein band was observed, and the relative mobilities were similar (Fig. 2, Lanes A and B). The molecular mass of the native extracellular tannase produced by *E. nidulans* was estimated to be 302 kDa by gel filtration using Sepharose CL6B, and the enzyme contained approximately 50% (w/w) carbohydrate. Two polypeptidic bands, corresponding to 45.8 and 52 kDa, were

**Table 1**
Purification of the extracellular tannase produced by *E. nidulans*.

| Step | Activity (total U) | Protein (total mg) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 12.25 | 395.50 | 0.031 | 1 | 100 |
| Kaolinite | 10.99 | 365.64 | 0.030 | 0.97 | 89.71 |
| DEAE-cellulose | 7.62 | 56.73 | 0.13 | 4.32 | 62.20 |
| Sephacryl S-200 | 3.67 | 1.92 | 1.91 | 61.61 | 29.95 |

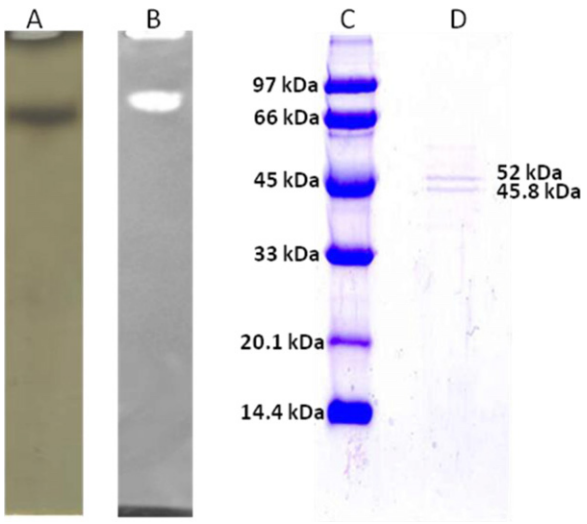


**Fig. 2.** A 7% polyacrylamide electrophoresis (PAGE) gel stained with silver (A), for tannase activity (B), and a 12% sodium dodecyl sulfate (SDS)–PAGE gel stained with Comassie Brilliant Blue R-250 showing molecular mass markers (C) and extracellular tannase (D).

observed after 12% SDS–PAGE (Fig. 2, Lane D), suggesting that the extracellular tannase was a heteromeric glycoprotein with three copies of each polypeptide. Most tannases produced by filamentous fungi show molecular weights ranging between 168 and 310 kDa [4,36]. Costa [37] described a homodimeric tannase from *Aspergillus tamarii* with 90 kDa subunits, a native molecular mass of 180 kDa and a carbohydrate content of 40.5%. In contrast, a dimeric tannase produced by *A. niger* MTCC 2425 showed two different subunits (102 and 83 kDa) [38]. A multimeric tannase was also reported by Hatamoto et al. [39] in *A. oryzae*, possessing eight subunits organized as four pairs of 30 and 33 kDa subunits.

### 3.4. Effects of temperature and pH

The purified tannase showed an optimum temperature of 45 °C (Fig. 3A), higher than those reported for the enzymes produced by *A. niger* [40] and *A. tamari* [41], corresponding to 35 and 30 °C, respectively. However, other authors reported optimum temperatures for tannase activity between 50 and 70 °C [11,19,42]. The extracellular tannase from *E. nidulans* was very stable in the temperature range of 22–50 °C for up to 70 h (Fig. 3C). In addition, the enzyme was stable at 90 °C for 24 h, with a half-life ($t_{50}$) of 72 h. According to some authors, tannases are thermostable between 10 and 60 °C [5,7,12].

Enzymes with high thermal stability are attractive for diverse industrial applications, as for instance food processing, in which high temperatures (80–90 °C) are used in pelleting procedures. Indeed, low thermal stability is frequently a problem while incorporating enzymes into foods, because they may be denatured at these high temperatures. In particular, tannases may be used as additives in food to increase digestibility and thus aid in animal growth [35]. The outstanding thermal stability of the extracellular tannase from *E. nidulans*, the highest ever described for tannases, suggests a great potential for its application in food processing, due to its resistance to heat-induced denaturation.

The extracellular tannase from *E. nidulans* showed an optimum pH of 5.0 (Fig. 3B), retaining approximately 80% of its activity when maintained at pH 4.0 or 5.0 for 60 h (Fig. 3D). Increasing pH values (6.0–10.0) resulted in decreased stability of the enzyme, with $t_{50}$ of 20 h at pH 7.0. These results are completely different from those reported for the tannases from *A. flavus* IFO 5839 (pH 5.0–5.5) [43], *P. chrysogenum* (pH 4.5–6.0) [16] and others, with lower $t_{50}$ and limited range of pH stability. The stability to high temperature and under a wide range of pH values can be justified by the high carbohydrate content in the *E. nidulans* tannase, which protects the enzyme. However, the specific function of the glycosylation in tannases is still unclear [9]. To our knowledge, this is the first report of a tannase being simultaneously stable at high temperatures and over a wide range of pH values for long periods, features that are very interesting for industrial applications.

**Table 2**
Influence of different salts on the activity of extracellular tannase produced by *E. nidulans*.

| Salts (1 mM) | Relative activity (%) |
|---|---|
| $AgNO_3$ | 98.77 ± 3.13 |
| $AlCl_3$ | 75.15 ± 3.97 |
| $BaCl_2$ | 117.94 ± 2.99 |
| $CaCl_2$ | 109.51 ± 3.00 |
| $CoCl_2$ | 133.59 ± 3.96 |
| $CuCl_2$ | 108.44 ± 4.93 |
| $FeCl_3$ | 85.74 ± 4.28 |
| $HgCl_2$ | 137.73 ± 3.96 |
| KCl | 115.18 ± 5.46 |
| $MgCl_2$ | 118.25 ± 3.96 |
| $MnCl_2$ | 115.80 ± 2.94 |
| NaCl | 124.69 ± 5.97 |
| $NH_4Cl$ | 110.74 ± 3.87 |
| $ZnCl_2$ | 142.09 ± 3.96 |
| Control | 100 |

### 3.5. Influence of different compounds on tannase activity

The activity of the extracellular tannase from *E. nidulans* was enhanced by approximately 40% in the presence of $Zn^{2+}$, $Hg^{2+}$, or $Co^{2+}$; and inhibited by $Fe^{3+}$, $Al^{3+}$, and $Ag^{+}$ (Table 2). The ionic radii of zinc and cobalt are very similar (0.74 Å and 0.745 Å, respectively), suggesting a specific binding site for both ions. However, the radius of the mercury ion is 1.02, suggesting a different binding site. Further, $Hg^{2+}$ may interact with the –SH and –S groups of proteins, promoting conformational changes in the protein structure. In this case, probably, these changes are favorable to the enzyme activity. Up to date, activation by $Zn^{2+}$ has not been reported for tannases. Indeed, the enzymes produced by *P. chrysogenum* [16], *A. niger* [19,40], and *A. awamori* BTMFW032 [10] were reported to be inhibited by $Zn^{2+}$, among other ions, such as $Cu^{2+}$ and $Mg^{2+}$. The addition of $Fe^{3+}$ inhibited the activity of *E. nidulans* tannase by approximately 15%, in contrast to that reported for the enzyme from *A. awamori* BTMFW032, which was stimulated by $Fe^{3+}$ [10]. The inhibition of tannase activity by metal ions is common and may be related to nonspecific binding or enzyme aggregation [10,37]. Tannase activity was reduced in the presence of high concentrations of EDTA and β-mercaptoethanol. EDTA has a chelating action and perhaps removes inhibitory ions. Additionally, β-mercapthoethanol acts on disulfide bonds, interfering with the structure of the enzyme and consequently with its activity.

Addition of low concentrations (1%, v/v) of organic solvents, especially isopropanol (50%), acetonitrile (49%), and ethanol (48%), to the reaction medium increased the tannase activity. Enzyme activity was also stimulated by the detergents SDS and Triton X-100 (Table 3). In contrast, the enzyme produced by *A. aculeatus* was inhibited by these detergents and β-mercaptoethanol [44]. According to Belmares et al. [35], most fungal tannases are totally or partially inhibited by organic solvents and detergents, in contrast to the activation of the *E. nidulans* tannase activity at low concentrations of these compounds. Chhokar et al. [20] reported that the activity of the tannase from *A. awamori* MT9299 increased in the presence of butanol and benzene. Although increasing of these compounds in the reaction medium reduced the tannase activity, approximately 70% activity was retained in the presence of 20%

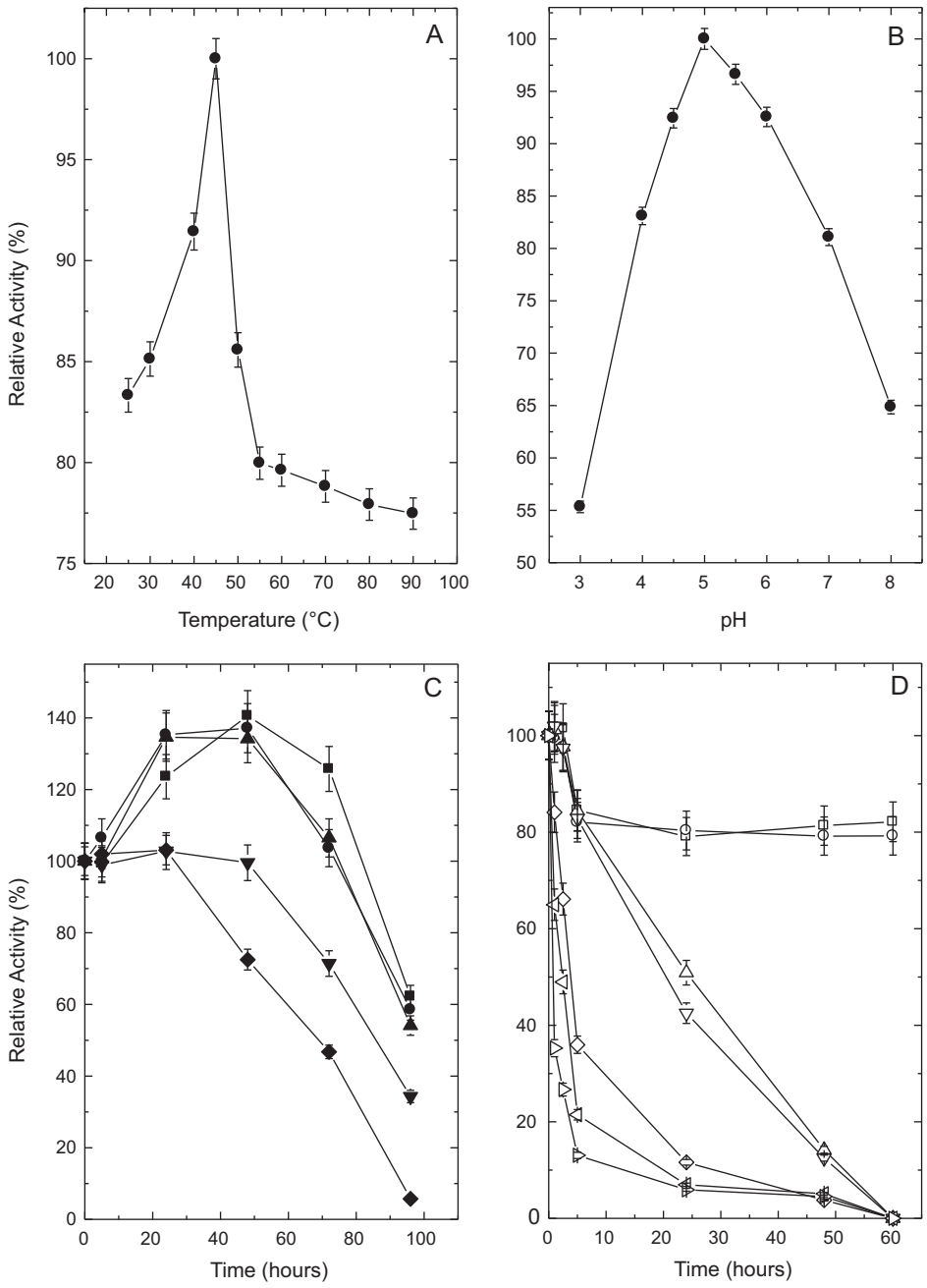


**Fig. 3.** Optima of temperature (A) and pH (B) for extracellular tannase activity; thermal stability (C) at 22 °C (■), 30 °C (●), 50 °C (▲), 70 °C (▼) and 90 °C (♦); and pH stability (D) at pH 4.0 (□), pH 5.0 (○), pH 6.0 (△); pH 7.0 (▽), pH 8.0 (◊), pH 9.0 (<), and pH 10.0 (>).

(v/v) of acetonitrile, ethanol, and isopropanol, and 80% activity was retained with the nonionic detergent Triton X-100, showing a good tolerance to these solvents and detergent. The tolerance of tannase to the presence of organic solvents is very attractive for industrial purposes, suggesting a potential application in the transesterification reactions used for various syntheses, for instance, of propyl gallate, an important antioxidant that is extensively used in the food industry. Additionally, the extracellular tannase from *E. nidulans* was stable in organic solvents such as methanol and ethanol for 8 h, while even undergoing a small activation (5–10%); it also retained approximately 85% of its activity after 8 h in the presence of isopropanol. It is possible that methanol and ethanol act by improving the solubility of the substrate, thus facilitating the catalysis. In contrast, acetone, butanol, and glycerol promoted the inhibition of tannase activity after incubation for all durations analyzed (Fig. 4). Organic solvents can promote protein denaturation by interference with hydrophobic areas in the molecule. Nonpolar solvents are often less harmful to the enzyme, and solvents with high polarity can strip the water around the enzyme and, consequently, promote inactivation [45]. Solvents with log *P* value <2.0 are considered toxic, interfering with the water layer around the enzyme. In spite of the low log *P* values of methanol (−0.76), ethanol (−0.24), and isopropanol (0.05), the tannase activity was maintained even after 8 h of incubation in each solvent. Reduced tannase activity was also observed with butanol (log *P* = 0.8), acetone (log *P* = −0.23), and glycerol (log *P* = −1.76). These facts suggest that the carbohydrate cover around the *E. nidulans* tannase may protect it from the actions of the organic solvent during incubation. Based on the observations in this work, we suggest using the terms "hypertolerant" and "hyperstable" to justify the maintenance

**Table 3**
Influence of different compounds on the extracellular tannase activity from *E. nidulans*.

| Compounds | Relative activity (%) | | | |
|---|---|---|---|---|
| | 1% | 5% | 10% | 20% |
| Acetone | 130.37 ± 2.47 | 61.57 ± 2.39 | 37.96 ± 2.53 | 29.96 ± 2.35 |
| Acetonitrile | 149.85 ± 2.04 | 83.14 ± 4.97 | 78.65 ± 2.13 | 68.53 ± 3.15 |
| Ethanol | 148.47 ± 2.59 | 76.40 ± 2.97 | 70.78 ± 3.66 | 65.16 ± 2.26 |
| Glycerol | 138.50 ± 2.94 | 79.43 ± 3.14 | 47.19 ± 2.13 | 35.95 ± 3.52 |
| Isopropanol | 150.00 ± 6.87 | 79.66 ± 3.65 | 73.90 ± 3.94 | 70.03 ± 1.96 |
| Methanol | 139.57 ± 1.13 | 82.89 ± 3.52 | 80.89 ± 3.97 | 73.03 ± 3.85 |
| n-Butanol | 140.95 ± 1.96 | 97.75 ± 2.96 | 79.77 ± 2.96 | 62.75 ± 2.98 |

| Compounds | Relative activity (%) | | | |
|---|---|---|---|---|
| | 0.01% | 0.05% | 0.1% | 0.2% |
| SDS | 138.96 ± 2.55 | 84.15 ± 2.39 | 75.26 ± 5.12 | 52.80 ± 2.69 |
| Triton X-100 | 121.63 ± 2.59 | 93.25 ± 4.78 | 90.02 ± 4.35 | 80.89 ± 2.78 |

| Compounds | Relative activity (%) | | | |
|---|---|---|---|---|
| | 1 mM | 5 mM | 10 mM | 20 mM |
| β-Mercaptoethanol | 110.12 ± 0.56 | 91.01 ± 6.01 | 60.66 ± 3.21 | 37.07 ± 2.68 |
| EDTA | 109.51 ± 0.98 | 75.26 ± 1.23 | 57.30 ± 2.34 | 25.89 ± 3.54 |
| Control | 100 | 100 | 100 | 100 |

of *E. nidulans* tannase activity under drastic conditions, such as high temperatures and presence of high concentrations of organic solvents (≥50%), for long periods.

### 3.6. Hydrolysis of substrates and kinetic parameters

Both tannic acid and methyl gallate were hydrolyzed by the extracellular tannase from *E. nidulans*. The hydrolysis of a mixture of the two substrates suggested the existence of a single active site for both, because intermediate values of activity were obtained for the mixture, compared to the values determined for each substrate alone (Fig. 5). TLC analysis of the hydrolysis products of the natural substrate, tannic acid, showed increasing accumulation of gallic acid with reaction time (data not shown).

The kinetic parameters $K_m$ and $V_{max}$ obtained for the hydrolysis of tannic acid and the synthetic substrate methyl gallate are shown in Table 4. The $K_m$ value for tannic acid was 14.01 mM, with a $V_{max}$ of 2.63 U mg$^{-1}$ protein; and for methyl gallate, the $K_m$ was 4.78 mM and the $V_{max}$ was 0.29 U mg$^{-1}$ protein. In addition, the kinetic parameters were also determined for propyl gallate ($V_{max}$ = 0.05 U mg$^{-1}$ protein and $K_m$ = 7.69 mM) and for pyrogallol ($V_{max}$ = 7.40 U mg$^{-1}$ protein and $K_m$ = 16.94 mM). According to

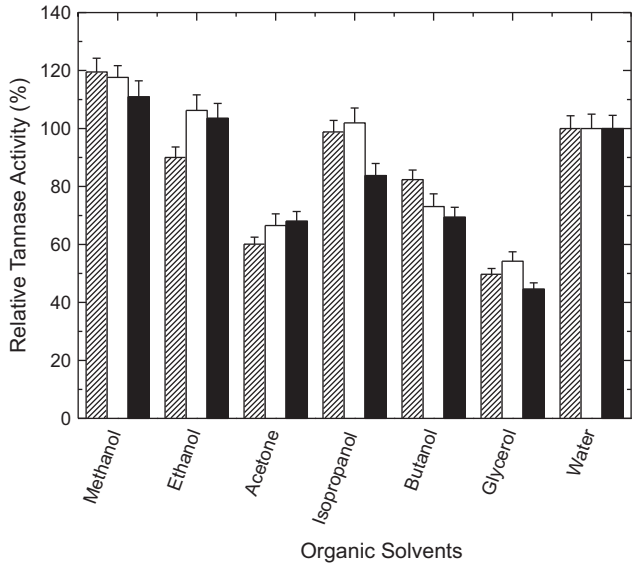


**Fig. 4.** Stability of the extracellular tannase in the presence of different organic solvents for 10 min (▨), 4 h (□), and 10 h (■).

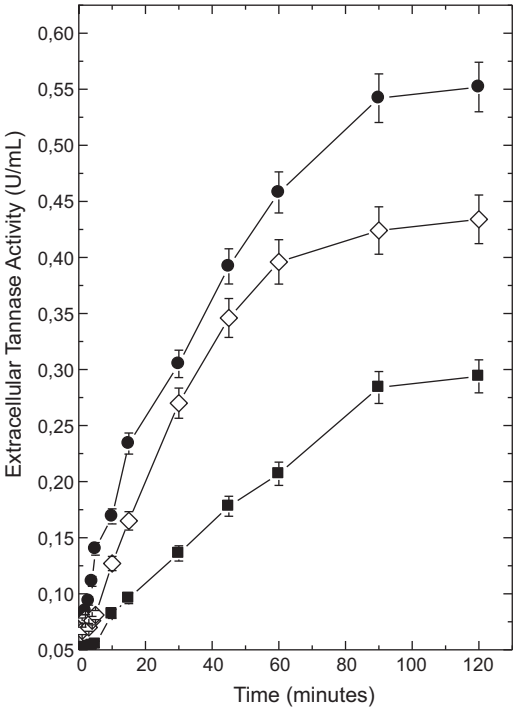


**Fig. 5.** Hydrolytic activity of the extracellular tannase produced by *E. nidulans* on the substrates methylgallate (●), tannic acid (■), and methylgallate + tannic acid (1:1) (◊).

**Table 4**
Kinetic parameters for the extracellular tannase produced by *E. nidulans*.

| Substrate | $K_m$ (mM) | $V_{max}$ (U/mg) | $V_{max}/K_m$ (U/mg mM) |
|---|---|---|---|
| Tannic acid | 14.01 | 2.63 | $18 \times 10^{-2}$ |
| Methyl gallate | 4.78 | 0.29 | $6 \times 10^{-2}$ |
| Propyl gallate | 7.69 | 0.05 | $0.6 \times 10^{-2}$ |
| Pyrogallol | 16.94 | 7.40 | $43 \times 10^{-2}$ |

these data, an apparently better affinity was observed for the synthetic substrate methyl gallate. The enzyme produced by *E. nidulans* showed higher affinity for methyl gallate than those reported for the enzymes from *A. oryzae* [46], *P. variable* [47], and *Cryphonectria parasitica* [48], among others. However, a higher catalytic efficiency was estimated for pyrogallol ($0.43\ U\ mg^{-1}\ mM^{-1}$), which is higher than that for tannic acid ($0.18\ U\ mg^{-1}\ mM^{-1}$). The affinity of the *E. nidulans* tannase for tannic acid was lower than that observed for the enzymes produced by *C. parasitica* (0.94 mM) [48], *A. oryzae* (1.37 mM) [46], and *A. niger* GH1 (0.05 mM) [49], but better than that observed for the enzyme from *P. variable* with a $K_m$ of 32 mM [47]. The *P. variable* enzyme was also able to hydrolyze propyl gallate, as reported in the present study.

## 4. Conclusion

In conclusion, the extracellular tannase produced by *E. nidulans* is a heteromeric glycoprotein that possesses unique, different, and interesting characteristics compared to other tannases reported in literature; it is especially very resistant to high temperatures and organic solvents, properties that suggest the identification of a new class of tannases with high biotechnological potential.

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## References

[1] C.N. Aguilar, R. Rodriguez, G. Gutiérrez-Sánchez, C. Augur, E. Favela-Torres, L.A. Prado-Barragan, A. Ramírez-Cornel, J.C. Contreas-Esquível, Appl. Microbiol. Biotechnol. 76 (2007) 47.
[2] Y. Nakamura, S. Tsuji, Y. Tonogai, J. Agric. Food Chem. 51 (2003) 331.
[3] E. Haslam, J.E. Stangroom, Biochem. J. 99 (1966) 28.
[4] C.N. Aguilar, G. Gutiérrez-Sánchez, Food Sci. Technol. Int. 7 (2001) 373.
[5] G.A.S. Pinto, S. Gouri, S.G.F. Leite, E.S. Brito, Bol. Centro Pesqui. Process. Aliment 23 (2005) 435.
[6] G. Sanderson, N. Englewood, P. Coggon, N. Orangeburg, U.S Patent 3,812,266 (1974).
[7] P.K. Lekha, B.K. Lonsane, Process Biochem. 29 (1994) 497.
[8] M. Sittig, Trimethoprim, Pharmaceutical Manufacturing Encyclopedia, Noyes Publication, New Jersey, USA, 1988.
[9] P.K. Lekha, B.K. Lonsane, Adv. Appl. Microbiol. 44 (1997) 215.
[10] P.S. Beena, M.B. Soorej, K.K. Elyas, G.B. Sarita, M. Chandrasekaran, J. Microbiol. Biotechnol. 20 (2010) 1403.
[11] V. Battestin, G.A. Pinto, G.A. Macedo, Food Sci. Biotechnol. 16 (2007) 243.
[12] S. Sharma, T.K. Bhat, R.K. Dawra, World J. Microbiol. Biotechnol. 15 (1999) 673.
[13] S. Ilbuchi, Y. Minoda, K. Yamada, Agric. Biol. Chem. 32 (1968) 803.
[14] N. Raaman, B. Mahendran, C. Jaganathan, S. Sukumar, V. Chandrasekaran, World J. Microbiol. Biotechnol. 26 (2010) 1033.
[15] P.S. Beena, S.M. Basheer, S.G. Bhat, A.H. Bahkali, M. Chandrasekaran, Appl. Biochem. Biotechnol. (2011), doi:10.1007/s12010-011-9162-x.
[16] G.S. Rajakumar, S.C. Nandy, Appl. Environ. Microbiol. 46 (1983) 525.
[17] T.K. Bhat, B. Singh, O.P. Sharma, Biodegradation 9 (1998) 343.
[18] V. Battestin, G.A. Macedo, Bioresour. Technol. 98 (2007) 1832.
[19] A. Sabu, A. Pandey, M.J. Daud, G. Szakacs, Bioresour. Technol. 96 (2005) 1223.
[20] V. Chhokar, M. Sangwan, V. Beniwal, K. Nehra, K.S. Nehra, Appl. Biochem. Biotechnol. 160 (2010) 2256.
[21] P. Khanna, S.S. Sundaril, N.J. Kumar, World J. Microbiol. Biotechnol. 11 (1995) 242.
[22] S. Sharma, T.K. Bhat, R.K. Dawra, Anal. Biochem. 279 (2000) 85.
[23] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[24] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350.
[25] B.J. Davis, Ann. N. Y. Acad. Sci. 121 (1964) 404.
[26] H. Blum, H. Beier, H.L. Gross, Electrophoresis 8 (1987) 93.
[27] K. Aoki, T. Tanaka, R. Shinke, H. Nishira, J. Chromatogr. 170 (1979) 446.
[28] U.K. Laemmli, Nature 227 (1970) 680.
[29] B. Mahendran, N. Raman, D.J. Kim, Appl. Microbiol. Biotechnol. 70 (2006) 444.
[30] H. Lineweaver, D. Burk, J. Am. Chem. Soc. 56 (1934) 658.
[31] F.A. Leone, J.A. Baranauskas, P. Ciancaglini, Biochem. Educ. 23 (1995) 35.
[32] K. Aoki, R. Shinke, H. Nishira, Agric. Biol. Chem. 40 (1976) 79.
[33] B. Bajpai, S. Patil, Enzyme Microb. Technol. 20 (1997) 612.
[34] S. Bradoo, R. Gupta, R.K. Saxena, Process Biochem. 32 (1997) 135.
[35] R. Belmares, J.C. Contreras-Esquivel, R. Rodrígues-Herrera, A.R. Coronel, C.N. Aguilar, Lebensm. Wiss. Technol. 37 (2004) 857.
[36] M.A. Ramírez-Coronel, G. Viniegra-González, A. Darvill, C. Augur, Microbiology 149 (2003) 2941.
[37] A.M. Costa, Produção, purificação e caracterização de tanases de Aspergillus tamarri, Doctoral Thesis, Universidade Estadual de Maringá, Maringá, Brasil, 2007.
[38] R. Bhardwaj, B. Singh, T.K. Bhat, J. Basic Microbiol. 43 (2003) 449.
[39] O. Hatamoto, T. Watarai, M. Kikuchi, K. Mizusawa, H. Sekine, Gene 175 (1996) 215.
[40] C. Barthomeuf, F. Regerat, H. Pourrat, J. Ferment. Bioeng. 77 (1994) 320.
[41] A.M. Costa, W.X. Ribeiro, E. Kato, A.R.G. Monteiro, R.M. Peralta, Braz. Arch. Biol. Technol. 51 (2008) 399.
[42] K. Mahapatra, R.K. Nanda, S.S. Bag, R. Banerjee, A. Pandey, G. Szakacs, Process Biochem. 40 (2005) 3251.
[43] H. Yamada, O. Adachi, M. Watanabe, N. Sato, Agric. Biol. Chem. 32 (1968) 1070.
[44] B. Kar, R. Banerjee, B.C. Bhattacharyya, Process Biochem. 38 (2003) 1285.
[45] M.A. Eltaweel, R.N.Z.R.A. Rahman, A.B. Salleh, M. Basri, Ann. Microbiol. 55 (2005) 187.
[46] F. Chang, P. Chen, R.L.C. Chen, F. Lu, T. Cheng, Bioelectrochemistry 69 (2006) 113.
[47] S. Sharma, L. Agarwal, R.K. Saxena, Bioresour. Technol. 99 (2008) 2544.
[48] G.M. Farias, C. Gorbea, J.R. Elkins, G.J. Griffin, Physiol. Mol. Plant Pathol. 44 (1994) 51.
[49] M. Mata-Gómez, L.V. Rodríguez, E.L. Ramos, J. Renovato, M.A. Cruz-Hernández, R. Rodríguez, J. Contreras, C.N. Aguilar, J. Microbiol. Biotechnol. 19 (2009) 987.