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Production, purification and characterization of tannase from *Aspergillus tamarii*

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The production of tannases by *Aspergillus tamarii* was evaluated in submerged cultures using tannic acid and gallic acid as substrates. Two tannases, designated as TAH I and TAH II were produced in gallic acid submerged cultures. TAH I, responsible for 70% of the total tannase activity was purified to apparent electrophoretic homogeneity with 18.35% yield. The enzyme is a homodimeric protein with molecular mass of 180 kDa and 40.5% of its weight corresponds to carbohydrates. TAH I exhibited optimal activity at 30°C and pH 5.5 and was stable over a large pH range (3.0 to 9.0) and at temperatures up to 40°C. With methyl gallate as substrate, the enzyme presented a K_M of 0.77 mM and a V_{max} of 682.8 U/mg proteins. The enzyme was inhibited by metal ions but showed relative resistance to organic solvents and surfactants. Since the enzyme is active over a wide range of pH and temperature, it is potentially useful in food and pharmaceutical industries.

Key words: *Aspergillus tamarii*, enzyme purification, submerged culture, tannase.

INTRODUCTION

Enzymes involved in fungal degrading pathways of organic compounds have been the object of considerable attention due to their large industrial application spectra. Tannin acyl hydrolase (TAH, E.C.3.1.1.20), commonly called tannase, catalyzes the hydrolysis of ester and depside bonds into hydrolysable tannins, releasing glucose and gallic acid (Seth and Chand, 2000; Mahendran et al., 2006). These enzymes are extensively used in food, feed, beverage, brewing, pharmaceutical and chemical industries (Belmares et al., 2004; Chavez-Gonzalez et al., 2011). The major commercial applications of tannases consist in the elaboration of instantaneous tea and in the production of gallic acid (Belmares et al., 2004). Gallic acid is an important intermediary compound in the

synthesis of the antibacterial drug trimethoprim, used in the pharmaceutical industry (Sittig, 1988). It is also a substrate for the chemical or enzymatic synthesis of propyl gallate, a potent antioxidant (Aguilar and Gutiérrez-Sanchez, 2001; Banerjee et al., 2005; Sharma and Gupta, 2003). Tannases have also been used for the cleavage of polyphenolics present in the cell wall of plants which is essential for plant cell wall digestibility (Conesa et al., 2001; Mingshu et al., 2006). They might also find use in cosmetology to eliminate turbidity of plant extracts, and in the leather industry to homogenize tannin preparations for high grade leather tannins (Barthomeuf et al., 1994).

Tannases are produced in the presence of tannins, especially by microorganisms. Among filamentous fungi, the *Aspergillus* and *Penicillium* genera are the main tannase producers (Bajpai and Patil, 1997; Banerjee et al., 2001; Barthomeuf et al., 1994; Batra and Saxena, 2005;

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Bradoo et al., 1997; Chhokar et al., 2010; Enemuor and Odibo; 2010; Garcia-Conesa et al., 2001; Renovato et al., 2011; Sabu et al., 2005; Seth and Chand 2000; van Diepeningen et al., 2004). Other tannase producers include yeast (Aoki et al., 1976) and bacteria (Sivashanmugam and Jayaraman, 2011; Mondal et al., 2000, 2001; Sharma and John, 2011). Expression of a recombinant tannase has also been reported: a tannase from *Aspergillus oryzae* has been cloned and expressed in *Pichia pastoris* (Zhong et al., 2004).

Studies on tannase production by *Aspergillus* spp. can be done by various methods such as liquid surface, submerged, modified solid-state cultures and solid state cultures (Belmares et al., 2004). Submerged cultures have advantages in process control and recovery easiness of extracellular enzymes while the main advantages of solid state cultures include simplicity, lower production costs, high enzyme yield and low wastewater output. Recently we described a new tannase producer, *Aspergillus tamarii*, able to produce extracellular tannases in submerged cultures using tannic acid, methyl gallate and gallic acid as substrates (Costa et al., 2008). The objective of the present work was to purify and characterize the main tannase produced by this fungus in submerged culture.

MATERIALS AND METHODS

Microorganism and inoculum

Spores of *A. tamarii* were inoculated on potato dextrose agar using 250 mL Erlenmeyer flasks and incubated at 28°C for 5 days. The fungal spore inoculum was prepared by adding 20 mL of sterile distilled water containing 0.01% Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions and the number of viable spores in the suspension was determined using the plate count method.

Enzyme production in submerged conditions

Composition of the mineral medium was (in g/L): KH_2PO_4 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2.0 g; CaCl_2 1.0 g; NH_4Cl 3.0 g; yeast extract 1 g. The filter-sterilized substrates glucose, starch, sucrose, xylan, tannic acid or gallic acids were added to the medium after its sterilization for 15 min at 127°C. Spore suspensions (5×10^9 spores) were added to 50 mL of culture medium in 250 mL Erlenmeyer flasks. Cultures were grown for up to 144 h at 120 rpm in shaker at 28°C. Samples were withdrawn at regular intervals of 48 h. The mycelial biomass was separated by filtration through Whatman No. 1 filter paper. The cell free culture broth was assayed for extracellular tannase activity.

Enzyme assay

Tannase activity was estimated by the method of rhodanine (Sharma et al., 2000). The method is based on the formation of a chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2 thio-4-ketothiazolidine). The

pink color developed was read at 520 nm using a spectrophotometer (Shimadzu UV-160A, Japan). The tannase activity was expressed in international units. One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under defined reaction conditions.

Enzyme purification

All operations were carried out at 4°C. The crude extracellular extract was dialyzed 24 h against water and concentrated by freeze-drying. The lyophilized sample was reconstituted with a minimum amount of water. The soluble proteins were applied to a Sephadex G-150 column (2 x 60 cm), previously equilibrated with 10 mM acetate buffer, pH 5.0. The protein fractions (4.0 ml) were eluted at a flow rate of 2 ml/min. Active fractions were pooled, dialyzed against water and concentrated by freeze-drying. The concentrated fraction was then loaded onto a DEAE-Sephadex column (2 x 23 cm), pre-equilibrated with 20 mM phosphate buffer, pH 7.0. The column was washed with the same buffer to remove unbound proteins. The bound proteins were eluted by applying a linear gradient of NaCl (0 to 0.5 M). The protein fractions (10.0 mL) were eluted at a flow rate of 1 ml/min. The pooled active fractions were dialyzed against water, concentrated by freeze-drying and stored at -20°C. In both columns, each fraction was assayed for protein ($A_{280 \text{ nm}}$) and tannase activity.

SDS-PAGE and molecular weight determination

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the purity of the enzyme and its molecular weight. The electrophoresis was run in a vertical slab gel apparatus. Acrylamide concentration was 4% for stacking gel and 8% for separation gel (Laemmli, 1970). The gel was run at 150 V, 30 mA till the tracking dye bromophenol blue reached the other end of the gel. Staining of the gel was done by keeping it overnight at room temperature in a solution of Coomassie blue in ethanol: acetic acid: distilled water (5:1:5). The dye excess was removed by keeping it in a de-staining solution containing 10% acetic acid and 35% ethanol till the gel became transparent. The 10 to 120 kDa proteins produced by partial cleavage of a 120 kDa protein (GIBCO-BRL Cat. n° 10064-012) were used as molecular markers.

Molecular weight of native tannase

The molecular weight of native tannase was determined by gel-filtration chromatography using Sephadex G-200. The mixture of high non-denaturing molecular weight markers (2 mg) was loaded on Sephadex G-200 (2 x 70 cm) and eluted with 0.05 M acetate buffer plus 0.1 M NaCl at a flow rate of 1 ml/min. The void volume (V_0) of 65 ml was determined using Blue Dextran 2000. The samples containing proteins were collected at their elution volume (V_e) and V_e/V_0 against the log of molecular weight (MW) was plotted for estimating the native molecular weight of tannase.

Effect of pH and temperature on purified tannase activity and stability

The activity of purified tannase was assayed at temperatures ranging from 20 to 60°C under standard conditions. For the determination of the optimum pH, the enzyme was assayed with McIlvaine's buffer (pH 3.0 to 8.0). Thermal stability was investigated by incubating the enzyme at different temperatures for 30 and 60

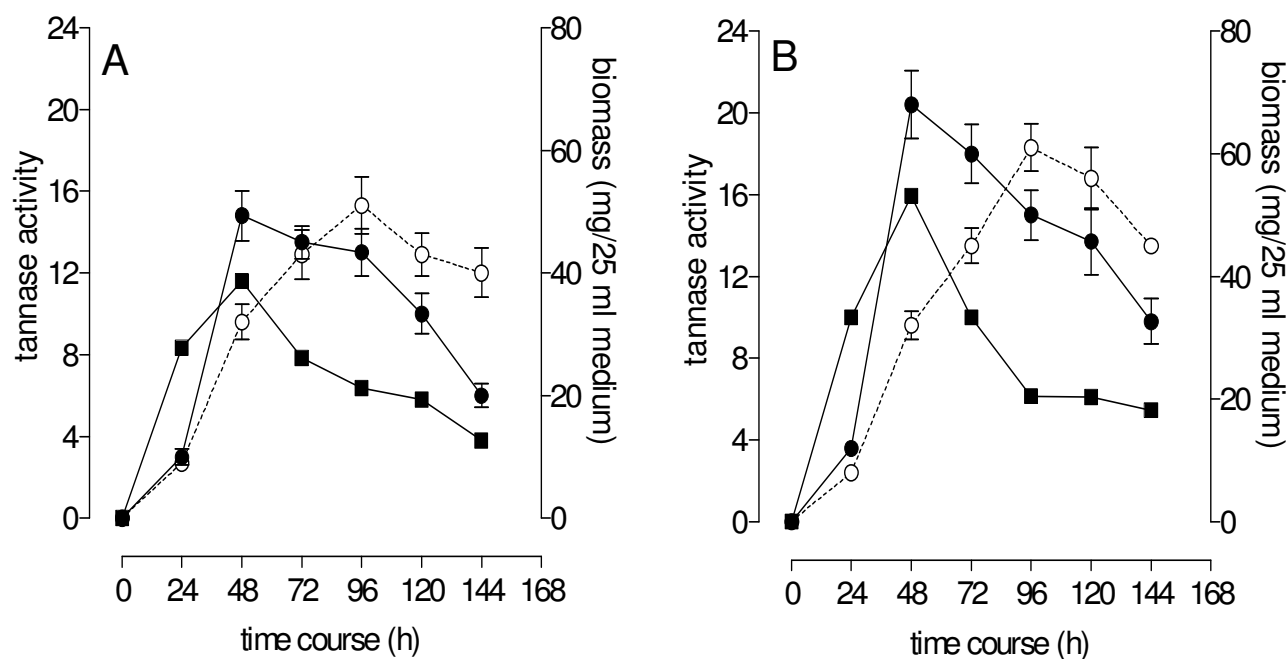


Figure 1. Production of tannase and mycelial biomass by *A. tamarii* in submerged cultures using 2% (w/v) tannic acid (A) and 2% gallic acid (B) as substrate. Cultures were grown for up to 144 h at 28°C and 130 rpm. (•) tannase activity in U/mL; (■) tannase activity in U/mg dry biomass; (○) dry mycelial biomass. Values are the mean \pm SD of triplicate cultures.

min. Immediately the enzyme was immersed in an ice bath and then the residual activity was tested under standard conditions. The pH stability was assayed by incubating the enzyme at different pH values (pH 3.0 to 8.0) for 2 h at 25°C. The remaining activities were measured under standard conditions.

Determination of protein and sugar content

Protein concentration was estimated using the Bradford method with crystalline bovine serum albumin (BSA) as standard (Bradford, 1976). Protein concentrations in the fractions from the chromatography were determined from the absorbance values at 280 nm. The carbohydrate content of purified tannase was estimated by the phenol-sulfuric acid method using D-mannose as the standard (Dubois et al., 1956).

Effect of different chemical compounds on tannase activity

To study the effects of different chemical compounds on the activity of purified tannase, the enzyme was incubated for 15 min at 4°C in solutions of the following compounds: 1 mM metal ions, 0.01% of the surfactants SDS and Triton X-100, 1 mM of the chelating agent ethylene diamine tetraacetic acid disodium salt (EDTA), and 1 mM β -mercaptoethanol. In all cases, the residual tannase activity was assayed by the standard procedure.

Data analysis

All experiments were carried out in triplicate. The results were analyzed by the Graph Pad Prism Program®

RESULTS AND DISCUSSION

Effect of substrate on the production of tannase by *A. tamarii* in submerged cultures

Good growth was observed but very low tannase activity (less than 1 U/ml) was found in the culture filtrates when glucose, starch, xylan and sucrose were used as carbon sources (data not shown). When 2% tannic acid or 2% gallic acid were used as substrates, Maximal tannase production was obtained at 48 h of cultivation, 14.8 U/ml (11.6 U/mg dry biomass) and 20.4 U/ml (15.9 U/mg dry biomass), respectively (Figure 1A and B). Analysis of the growth curves with both substrates suggests that the enzyme was produced during the exponential growth phase. The production of tannase during the primary phase of growth is found in several filamentous fungi, and has been reported for *Penicillium chrysogenum* (Rajakumar and Nandy, 1983), *Aspergillus aculeatus* (Banerjee et al., 2001) and *Aspergillus niger* (Darah et al., 2011). In general, tannase appears to be an induced enzyme, being produced at elevated amounts only when tannic acid is present (Aguilar and Gutiérrez-Sánchez, 2001). In the present study, gallic acid appears to be a more effective inducer than tannic acid. Due to the fact that tannase is an esterase and considering that gallic acid contains no ester linkage, it is surprising that gallic acid acts as a tannase inducer. There are indeed some

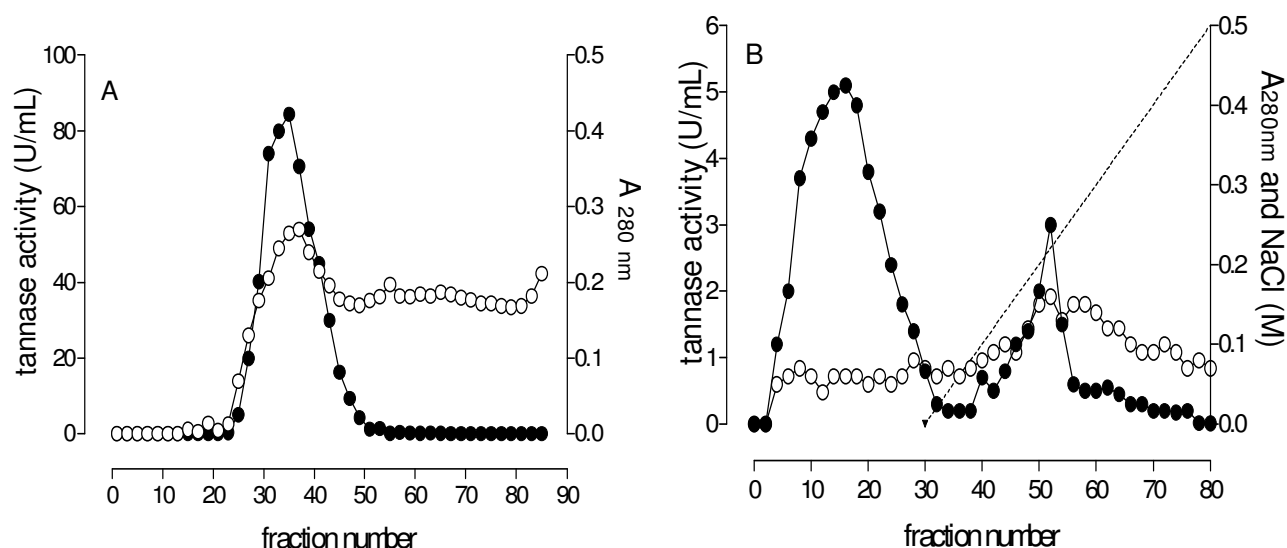


Figure 2. Chromatographic steps used in tannase purification; A: The crude filtrate was applied on Sephadex G-150; B: The fractions with high tannase activities eluted from Sephadex G-150 were pooled, dialyzed against water, concentrated by freeze-drying and applied on DEAE-Sephadex. (●) Tannase activity; (○) Absorbance at 280 nm.

reports that gallic acid represses the production of tannase when added to submerged cultures of *A. niger* Aa-20 (Aguilar and Gutiérrez-Sánchez, 2001) and *Aspergillus japonicus* (Bradoo et al., 1997). However, there are also reports that phenolic compounds in general, including gallic acid, can act as tannase inducers (Bajpai and Patil, 1997; Belmares et al., 2004). Certainly more studies are necessary to understand the role of gallic acid in the regulatory mechanisms of the tannase synthesis.

Enzyme purification and physico-chemical properties of purified tannase

The experiments to purify extracellular tannase were conducted using the culture broth supernatants obtained at 48 h of cultivation with 2% gallic acid as substrate. The extracellular tannase was purified by using two chromatographic techniques, filtration chromatography in a Sephadex G-150 column, followed by ion exchange chromatography in a DEAE-Sephadex column (Figure 2A and B). The filtration column allowed the separation of the tannases from minor proteins and fungal pigments (Figure 2A), while the chromatography in DEAE Sephadex allowed the separation of two isoforms of tannases, designated as TAH I and TAH II (Figure 2B). Multiple tannase forms are uncommon among filamentous fungi if one takes into account that not more than a single description of the phenomenon can be found (Kasieczka-Burnecka et al., 2007). TAH I was the predominant isoenzyme found in culture filtrates of *A. tamarii*

under the growth conditions used in this study, being responsible for more than 70% of the total tannase activity.

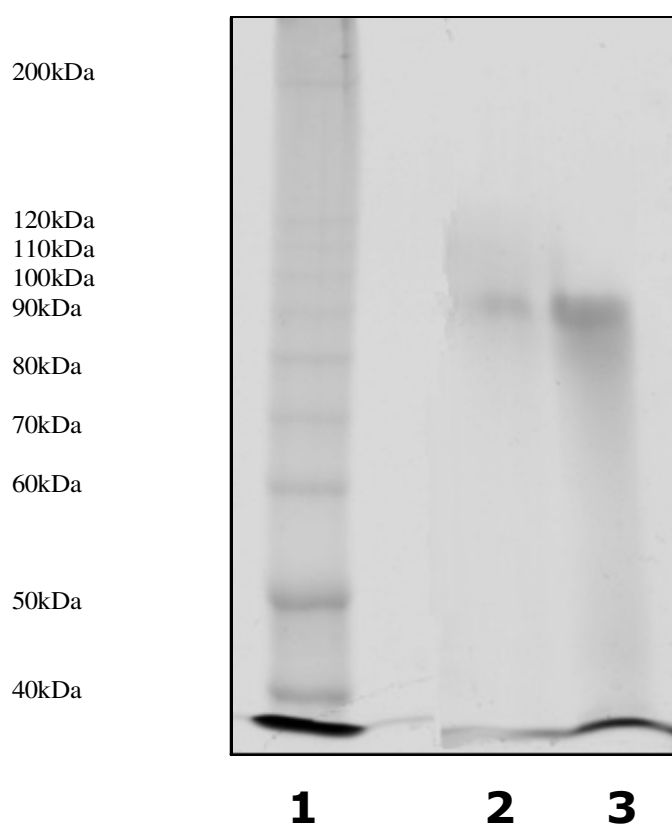
The fractions with high activity were pooled, dialyzed and concentrated by lyophilization. At the end of the process, TAH I was purified 7.17-fold with a specific activity of 1,350 U/mg protein and a yield of 18.35%, while TAH II was purified 1.11-fold with a specific activity of 209.26 U/mg protein and a yield of 3.84% (Table 1). TAH I appeared homogeneous in PAGE under non-denaturing conditions while a few protein contaminations were present in the TAH II preparation (data not shown). For this reason, only TAH I was characterized in this study.

The purified TAH I was eluted from gel filtration chromatography on a Sephadex-G-200 column with an apparent molecular mass of 180.0 kDa (data not shown). SDS-PAGE revealed the presence of one band with an apparent molecular mass of 90.0 kDa (Figure 3).

The carbohydrate content of tannase was estimated to be 40.5% by using the phenol-sulfuric acid method with D-mannose as the standard. These data allow us to conclude that the main tannase of *A. tamarii*, TAH I, is an oligomeric glycoprotein built of two sub-units with molecular masses of 90 kDa. Most of the purified tannase from filamentous fungi have a molecular mass in the range of 168 to 310 kDa (Ramirez-Coronel et al., 2003; Aguilar and Gutiérrez-Sánchez, 2001). According to Hatamoto et al. (1996) the tannase from *A. oryzae* has two subunits of 30 and 33 kDa, and the native tannase is a hetero-octamer with a molecular mass of 300 kDa. The molecular mass of *A. niger* MTCC 2425 tannase has been reported to be 185 kDa with two polypeptide chains

Table 1. Purification of tannase from *A. tamarii*.

Purification step	Volume (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell free extract	290	31.24	5,884.0	188.3	1.00	100.00
After concentration by freeze-drying	1	28.00	4,155.2	148.4	0.79	70.62
Sephadex G-150	36	4.12	2,976.0	722.3	3.84	50.58
DEAE Sephadex						
TAH I	150	0.98	1,080.00	1,350	7.17	18.35
TAH II	200	1.08	226.0	209.3	1.11	3.84

**Figure 3.** SDS-PAGE of *A. tamarii* TAH I. Lane 1: Molecular weight markers. Lanes 2 and 3: purified TAH I (10 and 25 µg protein).

of apparent molecular masses of 102 and 83 kDa (Bhardwaj et al., 2003). Glycosylation of tannases is also very common among the filamentous fungi (Aguilar and Guitierrez-Sánchez, 2001; Sharma et al., 2008). For example, the carbohydrate content of the *A. niger* tannase is 43% (Barthomeuf et al., 1994), while the enzyme from *A. awamarii* Nakazawa comprises 14.5% of its mass in carbohydrates (Mahapatra et al., 2005).

The kinetic properties of TAH I were investigated using methyl gallate as substrate. The graphical analysis of the

effect of the substrate concentration on the tannase activity yielded a K_M of 0.77 mM and a V_{max} of 682.8 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein at the optimum pH of 5.5 and at the temperature of 30°C. The K_M values for tannases from *A. flavus*, *Selenomonas ruminantium*, *Cryphonectria parasitica* and *A. niger* ATCC 16620 using methyl gallate as substrate have been found to be 0.86 mM (Yamada et al. 1968), 1.6 mM (Skene and Broker, 1995), 7.49 mM and 1.03 mM (Farias et al., 1994) respectively. This means that the tannase from *A. tamarii* has a higher affinity for methyl gallate than most of the hitherto reported enzymes.

Effect of temperature and pH on the activity and stability of TAH I

The effect of pH on the TAH I activity and stability was examined at pH values ranging from 3.0 to 8.0. The enzyme was stable over a large pH range and presented optimal activities at pH 5.0-6.0 (Figure 4A). Substantial activities were detected at pH values ranging from 4.0 to 7.0. Fungal tannases are generally acidic enzymes. There are reports of optimal pH values of 5.0 for the tannase from *Aspergillus awamori* (Mahapatra et al., 2005), pH of 5.5 for the tannase from *Aspergillus flavus* and *A. oryzae* (Batra and Saxena, 2005) and *Aspergillus heteromorphus* MTCC 8818 (Chhokar et al., 2010), pH of 6.0 for the enzyme from *A. niger* (Barthomeuf et al., 1994; Renovato et al., 2011; Sabu et al., 2005), and pH from 5.0 to 7.0 for the enzyme from *Paecilomyces variotii* (Mahendran et al., 2006).

The optimal temperature for the activity of TAH I was in the range between 30 and 35°C and the activity at 40°C was still above 90% of the maximal activity (Figure 4B). The enzyme was stable at low temperatures (5 to 25°C) for several hours (data not shown) and at temperatures up to 40°C for 1 h (Figure 4B). After 1 h at 45 and 50°C, the residual activities of the enzyme were 76 and 50%, respectively. Similar thermal stabilities were reported for tannases from several other fungal species including *A. oryzae* (Beverini and Metche, 1990), *A. awamorii* Nakazawa (Mahapatra et al., 2005), and *A. niger* (Barthomeuf

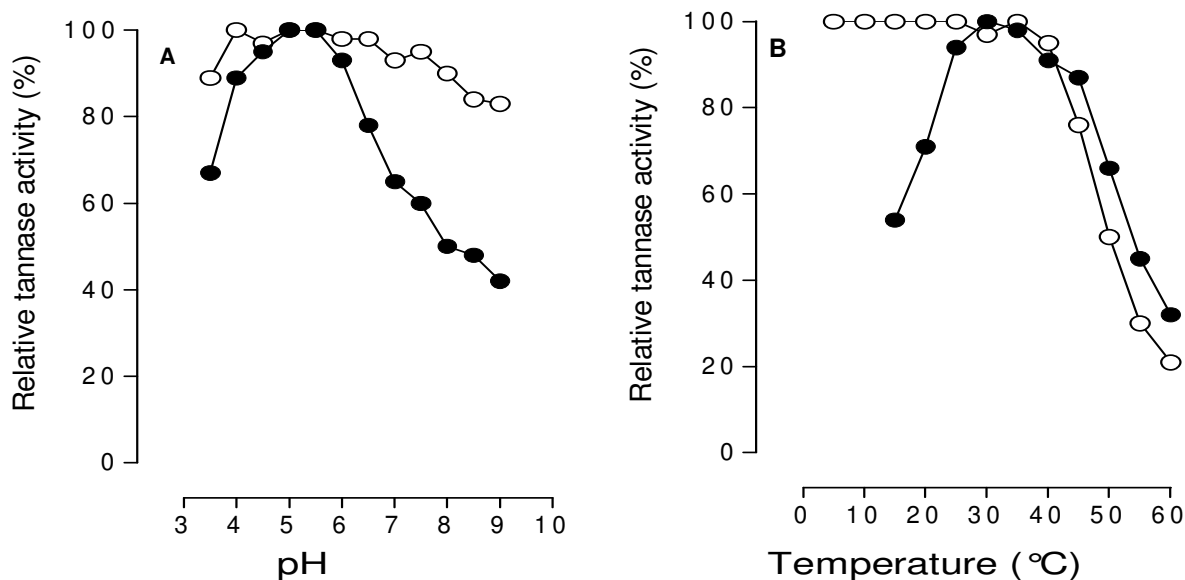


Figure 4. Effect of pH (A) and temperature (B) on the activity (●) and stability (○) of *A. tamarii* purified tannase I.

Table 2. Effects of metal ions and other chemicals on the activity of purified *A. tamarii* TAH I.

Compound	Residual activity (%)
None	100.0±4.3
1.0 mM MgCl ₂	104.0±4.3
1.0 mM CaCl ₂	53.5±5.0
1.0 mM CoCl ₂	63.6±3.7
1.0 mM FeSO ₄	43.8±1.7
1.0 mM CuSO ₄	31.5±2.7
1.0 mM HgCl ₂	13.8±1.8
1.0 mM ZnCl ₂	35.4±1.6
1 mM EDTA	73.5±4.8
1 mM β-mercaptoethanol	43.0±2.7
5.0% propanol	97.0±2.0
10.0% propanol	79.5±5.0
5.0% ethanol	98.5±1.5
10.0% ethanol	67.9±2.0
0.01% SDS	83.1±4.8
0.01% Triton X-100	76.4±5.1

et al., 1994). On the other hand, some fungal tannases are thermally more stable than the enzyme described in the present study, namely the tannases from *A. flavus* (Yamada et al., 1968; Pourrat et al., 1985), *A. niger* van Tieghem (Sharma et al., 1999) and *Penicillium variable* (Sharma et al., 2008). An extracellular tannase obtained by SSF from *A. niger* Aa20 using polyurethane foam presented an optimum temperature of 60 to 70 °C

(Ramirez-Coronel et al., 2003).

Effect of metal ions and other chemicals on TAH I activity

Except Mg²⁺, *A. tamarii* TAH I was inhibited by all metal ions tested (Table 2). Tannase was inhibited maximally by Hg²⁺, followed by Cu²⁺, Zn²⁺, Fe²⁺, Ca²⁺ and Co²⁺. Many enzymes require metal ion activators in order to achieve full catalytic efficiency. On the other hand, several enzymes are inhibited by metal ions and several other organic compounds. It seems thus that these metal ions are not necessary for the catalytic activity of tannase. The decrease in tannase activity in the presence of divalent cations could be due to nonspecific binding or aggregation of the enzyme. The inhibition of fungal tannases by metal ions is a common phenomenon. The activity of tannase from *P. chrysogenum* was inhibited by Cu²⁺ (53%), followed by Zn²⁺ and Fe³⁺ (45%) and Mg²⁺ (Rajakumar and Nandy, 1983). The purified tannase from *A. niger* ATCC 16620 was inhibited by Zn²⁺, Mn²⁺, Cu²⁺, Mg²⁺ and Fe²⁺, and only K⁺ enhanced its activity (Sabu et al., 2005). On the other hand, a recent purified tannase from *Aspergillus heteromorphus* MTCC 8818 was only marginally inhibited by several cations, including Hg²⁺, Ag⁺, Fe²⁺ and Cu²⁺ (Chhokar et al., 2010).

The effect of common chemicals on purified *A. tamarii* tannase was also tested. The enzyme was inhibited by β-mercaptoethanol but only partially inhibited by EDTA. These results suggest the presence of sulfur-containing amino acids at the enzyme active site. The enzyme was

resistant to the organic solvents propanol and ethanol and to the surfactants SDS and Triton X-100 (Table 2). Most fungal tannases are total or partially inhibited by these compounds (Belmares et al., 2004). Considering that the use of organic solvents can be advantageous in various industrial enzymatic processes, the resistance of enzymes to this kind of solvents is desirable.

In conclusion, the purified *A. tamari* tannase presents some qualities that are desirable for industrial applications. These qualities are low K_M value activities over a wide range of pH and at temperatures up to 40 °C, and resistance to denaturation in organic solvents and surfactant agents. These characteristics are considered to be especially favourable for applications in the food-processing industry.

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