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A Novel Tannase from the Xerophilic Fungus *Aspergillus niger* GH1

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Aspergillus niger **GH1 previously isolated and identified by our group as a wild tannase producer was grown under solid-state (SSC) and submerged culture (SmC) conditions to select the enzyme production system. For tannase purification, extracellular tannase was produced under SSC using polyurethane foam as the inert support. Tannase was purified to apparent homogeneity by ultrafiltration, anion-exchange chromatography, and gel filtration that led to a purified enzyme with a specific activity of 238.14 IU/mg protein with a final yield of 0.3% and a purification fold of 46. Three bands were found on the SDS–PAG with molecular masses of 50, 75, and 100 kDa. PI of 3.5 and 7.1% N-glycosylation were noted. Temperature and pH optima were 60°C and 6.0 [methyl 3,4,5-trihydroxybenzoate (MTB) as substrate], respectively. Tannase was found with a K_M value of 0.41×10^{-4} M and the value of V_{max} was 11.03 μmoL/min at 60°C for MTB. Effects of several metal salts, solvents, surfactants, and typical enzyme inhibitors on tannase activity were evaluated to establish the novelty of the enzyme. Finally, the tannase from *A. niger* GH1 was significantly inhibited by PMSF (phenylmethylsulfonyl fluoride), and therefore, it is possible to consider the presence of a serine or cysteine residue in the catalytic site.**

Keywords: *Aspergillus niger* GH1 tannase, properties, production, purification, solid-state culture

Tannins are polyphenolic compounds with varying molecular weights that occur naturally in the plant kingdom. These phenolic compounds differ from others by having the ability to precipitate proteins from solutions. Tannins are considered to be secondary metabolic products from plants because they play no direct role in the plant metabolism. After lignin, tannins are the second most abundant group of plant phenolics. One of the major characteristics of tannins is their ability to form strong complexes with protein and other macromolecules such as starch, cellulose, and minerals [2, 29]. It is widely accepted that tannins are divided into four major groups: gallotannins, ellagitannins, condensed tannins, and complex tannins. Tannins have a toxic effect on animals, being growth inhibitory to microorganisms. Some microbes are, however, resistant to tannins, and have developed various mechanisms and pathways for tannin degradation in their natural habitat [5, 14]. Tannase is a special protein with the capacity to prevent the formation of a complex with tannins.

Tannase catalyzes the hydrolysis reaction of the ester bonds present in gallotannins, complex tannins, and gallic acid esters [37]. Tannase is used in food and beverage processing; however, the practical use of this enzyme is at present limited owing to insufficient information on its properties, optimal expression, and large-scale application [5, 10]. The major commercial applications of the tannases reside in the manufacture of instant tea and for the elaboration of acorn wine, to produce the antioxidants gallic acid [6, 12, 21, 31, 32, 39, 47, 48], catechin gallates [41], and propyl gallate [52, 53]. Moreover, tannase is used as a clarifying agent in some wines, beers, fruit juices, and in refreshing drinks with coffee flavor [12, 28, 30]. Moreover, innovative applications of tannase have been reported in enhancing of antioxidant activity and *in vitro* inhibitory activity against *N*-nitrosation of dimethylamine in green tea [33, 34], in the production of derivatives from prunioside-A with anti-inflammatory activity [23], in the hydrolysis of epigallocatechin

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gallates [11], and in the enzymatic treatment for nutritive utilization of protein and carbohydrates from pea [50]. In this respect, an efficient use of tannase and the development of tailor-made applications require intensive characterization of the same.

Tannase production is mainly from microorganisms using the submerged culture (SmC) technique, where the enzyme is intracellularly expressed, implying additional costs for the downstream processing [12, 28]. Tannase production by solid-state culture (SSC) has demonstrated attractive advantages over the SmC, due to higher yields and the complete expression of the enzyme in an extracellular form [5, 6, 26, 48].

The xerophilic fungus *Aspergillus niger* GH1 was found to be an efficient producer of tannase [16, 17]. In addition, it proved to efficiently degrade and detoxify tannin-rich plant extracts for the production of high-value antioxidants [3, 51]. This prompted us to purify a major tannase isoenzyme and to characterize its main physicochemical and biochemical properties in order to gain further information on this promising strain for possible future applications. These properties are discussed comparatively with those reported for other microbial sources [7, 13, 19, 20, 35, 36, 38, 42–44, 46, 54].

Materials and Methods

Microorganism and Inoculum Preparation

Aspergillus niger (strain GH1) was obtained from the DIA-UAdeC culture collection (Saltillo, Coahuila, Mexico). The strain was maintained on potato dextrose agar plates at 4°C. Inocula were prepared by growing the fungus for 96 h at 30°C. Spores were harvested in a 0.01% (v/v) solution of sterile Tween 80 in distilled water and were counted in a Neubauer chamber.

Selection of Culture System

A tannic acid (5%) culture medium (TACM) previously reported [49] was used for tannase production under SmC and SSC systems. TACM was sterilized by filtration through sterile nylon membranes, (0.02 μm) to prevent the hydrolysis of the thermolabile substrate (tannic acid). Erlenmeyer flasks (250 ml) were used as reactors, and for SmC, 50 ml of sterile TACM was inoculated with the fungal spore inoculum (5×10^7 spores per reactor). Flasks were incubated at 30°C in a rotary shaker at 250 rpm. For SSC, polyurethane foam was used as the inert support (3 g per reactor) and the same was impregnated with TACM (7 ml) inoculated with fungal spores (2×10^7 spores per reactor) at an initial moisture content of 70% (v/w). Another set of experiment was conducted in tray reactors, where 50 g of wet inert support was used for SSC. The SSC systems were also incubated at 30°C. Kinetics of extra/intracellular tannase production was monitored for 96 h and samples were taken in triplicates for analysis.

Tannase Production

SSC was selected for the production of tannase and further experiments were carried out in a tray reactor having a capacity of 2 kg (made out of aluminum with a height of 10 cm and length of 40 cm) and filled with 1 kg of wet solid support. Experiments were performed under the above-mentioned conditions. After 30 h of incubation, the fermented wet support was removed and pressed to obtain the crude enzyme extract which was expressed extracellularly under SSC.

Tannase and Protein Assays

Tannase activity was evaluated according to the HPLC method reported by Aguilar [4] using tannic acid or methyl gallate as substrate. Tannase activity was expressed in international units, and one unit of tannase activity was defined as the amount of enzyme required for the release of 1 μmol of gallic acid per minute under standard assay conditions. Protein estimation was done according to the method of Bradford [15].

Purification of Tannase

Enzyme concentration by ultrafiltration (UF): Crude enzyme extract was filtrated using Whatman filter paper (No. 1041) and 0.45 μm nylon membrane. It was further concentrated by ultrafiltration using a Watson Marlow and UF Quix Stand Bench top system using a 1 kDa (MWCO) membrane at 60 rpm.

Purification by ion-exchange chromatography (IEC): Crude enzyme extract was desalinized on a Hi Trap G25 Sephadex column (Marshal Biosciences). The desalinized extract was placed on a Sartobind D 100X DEAE adsorbent membrane (Sartorius) and equilibrated with 100 ml of 20 mM acetate buffer (pH 5) with 0.02% sodium azide 0.02% (w/v). The enzyme was eluted with a linear salt gradient (NaCl, 0–1 M) in the same buffer at a flow rate of 60 ml/min, and 103 fractions of 5 ml each were collected. The fractions with tannase activity were pooled, desalinized, and lyophilized. Lyophilized enzyme was resuspended in 7 ml of the same buffer.

Purification by gel filtration chromatography: 1.5 ml of the tannase enzyme solution was subjected to gel filtration chromatography using a Sephacryl S-300 gel filtration column equilibrated with 200 ml of 20 mM acetate buffer (pH 5) containing 0.02% (w/v) sodium azide. Elution was carried out with the same buffer at a flow rate of 1 ml/min and fractions (1.5 ml) were collected. The column was maintained at 4°C throughout the experiment.

SDS–PAGE analysis: SDS–PAGE analysis was carried out according to the method of Laemmli [27]. A 12% separating gel was used for this study. Protein bands were detected by silver staining. A standard protein mixture was used to estimate the molecular mass of tannase. The presence of carbohydrate in the lyophilized-resuspended tannase enzyme was estimated using the spectrophotometric method of Dubois *et al.* [18].

Effects of pH and Temperature on Enzyme Activity and Stability

Tannase activity as a function of pH was measured at 30°C using methyl 3,4,5-trihydroxybenzoate (MTB) as substrate, using citrate–phosphate buffer in the pH range 3–9. The temperature activity profile was determined in 0.1 M citrate buffer (pH 5.5) in the temperature range 10–70°C. To test the pH stability, 10 IU of tannase was incubated at 25°C for 300 h in 5 ml of 0.1 M citrate buffer over the pH range 3.5–5.5. Incubations in the same buffer at pH 5.5 and 6.0 were prolonged to 600 h. Aliquots (10 ml) were assayed for residual tannase activity using MTB as a substrate. Thermal stability experiments were conducted by incubating 40 IU of tannase in 4 ml of 0.1 M citrate buffer (pH 5.5) at 50, 60, and

70°C for 360, 90, and 12 min, respectively. Aliquots (50 µl) were chilled in ice and assayed for residual activity as described above.

Effect of Metal Salts, Solvents, Surfactants, and Typical Enzyme Inhibitors on Tannase Activity

Effects of many metals salts on tannase activity were determined. Twenty mM of each metal salts ($ZnCl_2$, $MgSO_4$, $CuSO_4$, $FeCl_3$, $CaCl_2$, $MnCl_2$, $CoCl_2$) solutions were mixed with tannase extract (1:1) and incubated at 30°C. Tannase activity was assayed after 5 and 60 min of incubation as described before.

Solvents tested in this study were acetone, heptane, ethanol, petroleum ether, tetrahydrofurane (THF), and formaldehyde, using two different concentrations, 20% and 60% (v/v, solvent: tannase extract), at an incubation temperature of 30°C. Tannase activity was assayed after 5 and 60 min of incubation.

Surfactants like Tween 80, Tween 20, Triton X-100, SDS, and EDTA were used to study their effect on tannase activity at two concentrations, 0.1% and 0.01% (w/v, surfactant:tannase extract). Residual tannase activity was assayed as described before.

Finally, the effects of enzyme inhibitors like hydroxyquinone, phenylmethylsulfonyl fluoride (PMSF), mercaptoethanol, and 1,10 *o*-fenanthroline on the tannase activity were evaluated. Each inhibitor solution was prepared at 2 mM level and mixed at 1:1 (v/v) ratio with the tannase extract. Residual tannase activity was assayed as mentioned above.

Kinetic Constants of Tannase

Values of K_M and V_{max} were calculated at 60°C using MTB and tannic acid as substrates at initial concentration in the range 0.001–0.1 M. Enzyme kinetic studies were carried out for 30 min and the initial enzyme activity rates were calculated. Kinetic constants were then determined.

Tannase activity rate was estimated as V (IU) following the Michaelis–Menten equation. Estimation of different parameters for the equation was obtained through the minimization of the sum of squared errors between experimental and calculated values for V, VS, and S, using a program called Solver Routine from Microsoft Excel, avoiding the use of the linearization method.

RESULTS

Selection of Culture System

Fig. 1 shows the results of tannase production kinetics, where the main graph presents the comparative kinetics of extra/intracellular activities in SmC and SSC. The highest value of tannase activity was extracellularly reached in SSC (390 IU/l) after 30 h of culture. In this culture system, intracellular tannase activity was not detected. From the results obtained with the tannase production profile, it was easy to select the solid-state culture system for enhanced tannase production. The inset graph in Fig. 1 shows the extracellular tannase production kinetics, including the volumetric and specific activities. The highest value of specific tannase activity was reached just when the system was stopped (5.19 IU per mg of protein). A total of 0.27 l of crude extract was recovered by pressing the fermented wet material per one SSC, which was used to purify the tannase.

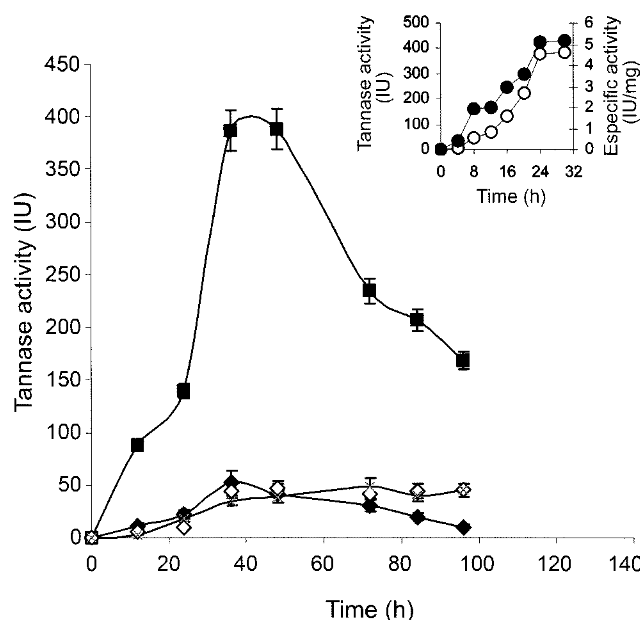

Fig. 1. Time course of extracellular tannase production by *A. niger* GH1 in SmC (◆) and SSC (■) using tannic acid (5%) as sole carbon source and inductor at 30°C, pH 5.5, during 96 h; intracellular tannase activity in SmC (◇); extracellular specific activity (●) and volumetric activity (○).

Tannase Purification

Extracellular tannase from *A. niger* GH1 was eluted through DEAE–cellulose adsorbent membrane with a linear salt gradient (0–1 M NaCl). The elution profile is shown in Fig. 2. The elution profile showed eight protein peaks, and tannase activity was found at the fourth, fifth,

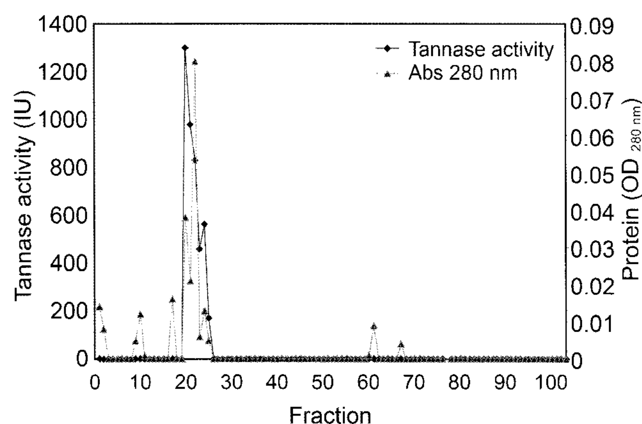

Fig. 2. Elution profile of *A. niger* GH1 extracellular tannase in Sartobind D 100X DEAE–cellulose adsorbent membrane.
The enzyme was eluted with a linear salt gradient (NaCl, 0–1 M). Fractions of 5 ml were collected and assayed for protein and tannase activity. Protein concentration is indicated as absorbance at 280 nm and tannase activity as U/l.

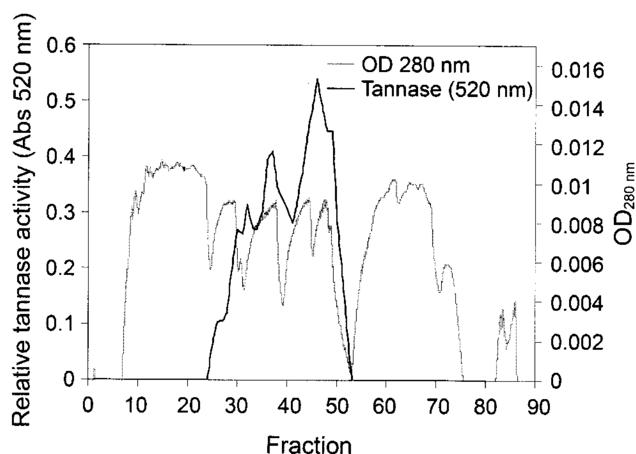

Fig. 3. Elution profile of *A. niger* GH1 extracellular tannase on Sephacryl S-300 gel filtration column chromatography.
The enzyme was eluted with 20 mM acetate buffer (pH 5). The column was maintained at 4°C throughout the experiment. Fractions of 1.5 ml were collected and assayed for protein and tannase activity. Protein and tannase activity were indicated as absorbance at 280 and 520 nm, respectively.

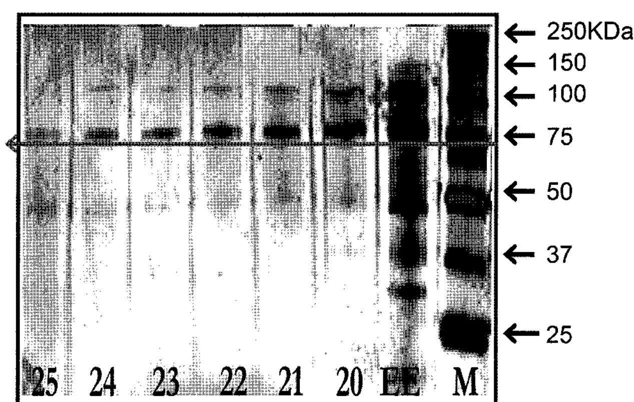

Fig. 4. SDS-PAGE of *A. niger* GH1 tannase.
D-100 X (DEAE) fractions (20, 21, 22, 23, 24, 25), (EEC), molecular marker (M).

and sixth peaks that were eluted with 200 mM NaCl, with a maximum tannase activity of 130 IU/l at the fourth peak. Fractions with enzyme activity were pooled, dialyzed, and lyophilized. The lyophilized enzyme was resuspended on acetate buffer and was applied onto a Sephacryl S-300 gel filtration column (Fig. 3). The protein elution profile showed four peaks with tannase activity. Maximum tannase activity was found at the third peak. A summary of the purity levels obtained after different steps of purification is shown in Table 1. The crude extract initially had an enzymatic activity of 387.81 IU/l and a specific activity of 5.2 IU/mg. The enzyme preparation obtained from DEAE−cellulose absorbent membrane led to an overall purification achievement of 35-fold with a yield of 113.2% and specific activity of 181.5 IU/mg (Table 1). In the last step, 46-fold purity was obtained with a yield of 0.3%. SDS−PAGE analysis of tannase (Fig. 4) gave three bands, indicating that the enzyme may be composed of three subunits of molecular masses of 50, 75, and 100 kDa.

Effects of pH and Temperature on Tannase Activity and Stability

The bell-shaped pH activity profile shows that the optimal pH for tannase activity was at 6.0 (with MTB as substrate), whereas the enzyme exhibited a relative activity of about 45% at pH 2.0 (Fig. 5B). Fig. 5A shows that the optimal temperature was at 60°C and that a relative activity of 10% was observed at 20°C.

Tannase stability as a function of pH was determined at values lower than or equal to 6.0, due to the negligible activity exhibited by the enzyme above pH 6. Fig. 6A shows that the enzyme was maximally stable at pH 6.0 and 5.5 with half-lives of 632 h and 607 h. However, in the pH range 3.0−5.0, tannase proved to be significantly stable, showing half-lives ranging from 131 to 372 h. Thermal stability profiles of *A. niger* tannase at 50, 60, and 70°C are shown in Fig. 6B. Half-lives of the enzyme at these temperatures were 281, 25, and 4 min, respectively.

Effects of Metal Salts, Solvents, Surfactants, and Typical Enzyme Inhibitors on Tannase Activity

Enzyme inhibition study of tannase is important, as it will generate significant information on the general structure of the enzyme and its active site, which can be utilized for finding special applications of the enzyme. Nonspecific inhibitors also give information for a better utilization of *A. niger* GH1 tannase. In this section, we report the modulator effect of different additives.

More than 75% of enzymes require metallic ions to express their maximal catalytic capacities. At low concentrations, some metals act as cofactors enhancing the enzymatic activity, but at high concentrations the effect is inhibitory. Compared with the effect of surfactants and organic solvents, a better documentation about the effect of metals on tannase activity is available. Fig. 7 shows the results obtained from the study on the effect of some metallic ions

Table 1. Purification scheme of extracellular tannase from *A. niger* GH1.

Purification step	Volume (l)	Activity (U/l)	Protein (mg/l)	Specific activity (U/mg)	Yield (%)	Purity (fold)	U
Crude extract	0.27	387.81	74.78	5.2	100	1	104.7
DEAE−cellulose	0.13	911.52	5.02	181.5	113.2	35	118.5
Sephacryl S-300	0.0015	238.14	1.00	238.14	0.30	46.0	0.35

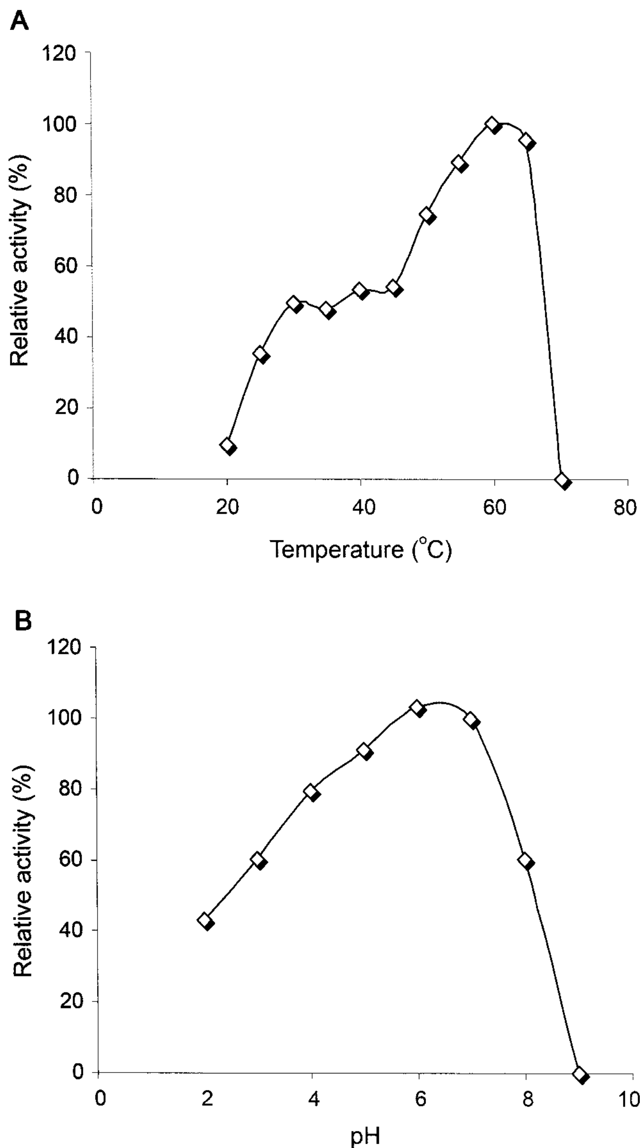

Fig. 5. A. Temperature activity profile of *A. niger* GH1 tannase at pH 6. **B**. pH activity profile of *A. niger* GH1 tannase. Mean±standard deviation of three replicates.

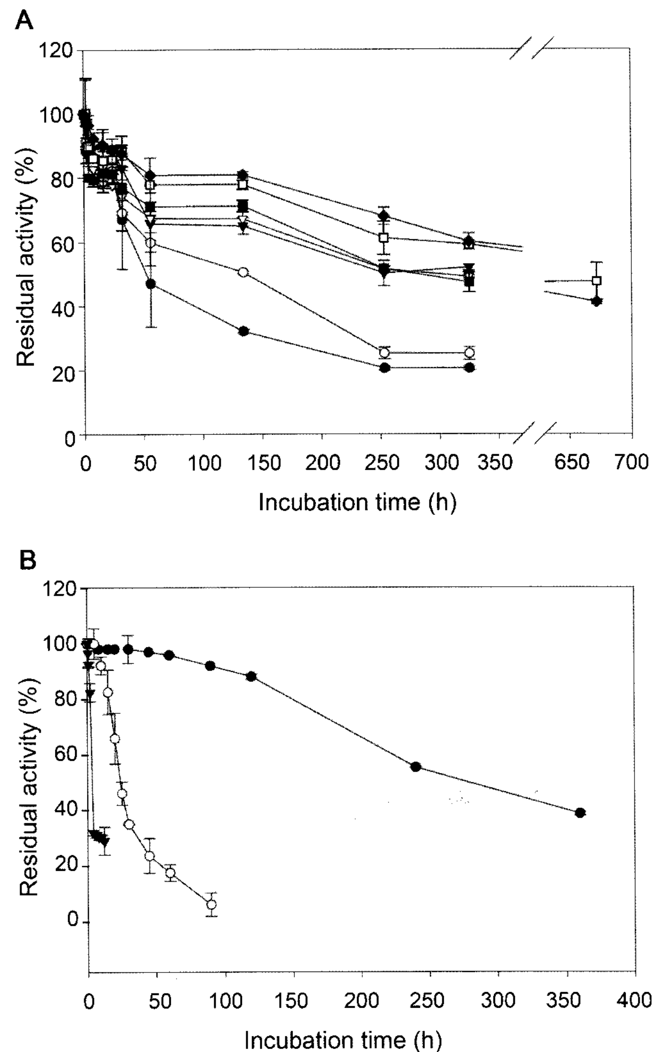

Fig. 6. A. Residual activity of tannase from *A. niger* GH1 throughout 325 h incubation at 30°C in 0.1 M citrate phosphate buffer at pH 3.0 (●), 3.5 (○), 4.0 (▼), 4.5 (▽), 5.0 (■), 5.5 (□), and 6.0 (◆). The incubation of the tannase at pH 5.5 and 6.0 was prolonged to 670 h. **B**. Residual activity of *A. niger* GH1 tannase throughout 360, 90, and 12 min incubation in 0.1 M citrate phosphate buffer pH 6.0 at 50 (●), 60 (○), and 70 (▼)°C, respectively. Data are the mean±standard deviation of three replicates

on tannase from *A. niger* GH1. The enzyme was highly inhibited by Fe^{3+}, whereas Cu^{2+} and Zn^{2+} had only a mild inhibitory effect. At the same time, Co^{2+} enhanced the enzyme activity. Information on the effect of organic solvents on tannase activity is very limited. In the present study, the effect of different solvents at two levels (20% and 60%) were tested (Table 2). At 60% of concentration, ethanol and acetone completely inhibited the enzyme activity, whereas THF and formaldehyde exhibited inhibitory effect only at 20%. Tannase activity was apparently enhanced with petroleum ether. *P. variable* tannase was similarly inhibited by acetone and formaldehyde, but it was not seriously affected by ethanol and heptane; also, an inhibitory effect was reported with petroleum ether [45].

Surfactants are substances that can alter the conditions prevailing at interfaces, and because of their amphiphilic property, they have a tendency to accumulate at interfaces and to adsorb onto surfaces. They can modify the surface tension by dispersing the proteins to the hydrophobic extreme of the peptide and interacting with the aqueous medium by another extreme. Surfactants can denature the enzyme protein, and because of this reason, it is very important to describe the effect of surfactants on the enzyme (Table 3). Surprisingly, *A. niger* GH1 tannase showed good stability in all the detergents tested. In this study, the role of some specific enzyme inhibitors were

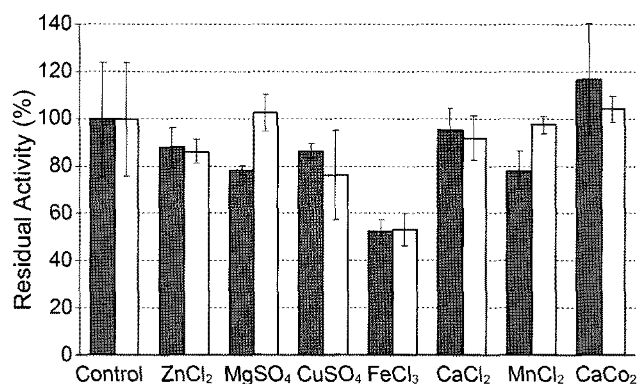

Fig. 7. Effects of metal ions (20 mM ionic strength) on *A. niger* GH1 tannase activity.
Residual activity was measured after 5 min (■) and 60 min (□) of incubation at 30°C and pH 5.

also experimented to see their effect on tannase activity (Fig. 8). PMSF considerably inhibited tannase activity (38%), which suggests the presence of a serine or cysteine residue in the catalytic site of *A. niger* GH1 tannase.

Kinetic Constants of Tannase

Kinetic constants of *A. niger* GH1 extracellular tannase were estimated using Michaelis–Menten plot (data not shown). Results presented in Table 4 report the values of kinetic constants for the tannase-catalyzed hydrolysis of naturally occurring phenolic compounds. The K_M and V_{max} values were found to be 0.41×10^{-4} M and 11.03 μmol/min (using MTB as substrate), respectively. When tannic acid (TA) was used as substrate, K_M and V_{max} values were found to be 2.3×10^{-5} M and 13.10 μmol/min, respectively.

DISCUSSION

SSC was a better option to produce the fungal tannase than in SmC, due to high levels of enzyme activity reached during the first hours of the culture. Conditions of tannase production by *A. niger* GH1 in SSC were previously reported by Cruz-Hernandez and co-workers [17].

Table 2. Effects of organic solvents on *A. niger* GH1 tannase activity.

Organic solvent	Residual activity (%)			
	20%, v/v		60%, v/v	
	5 min	60 min	5 min	60 min
Control	100.00	100.00	100.00	100.00
Acetone	70.16	84.40	0.00	0.00
Heptane	115.49	119.26	196.74	5.06
Ethanol	75.19	87.13	0.00	0.00
Petroleum ether	94.49	118.67	173.90	113.84
Tetrahydrofurane	36.39	31.90	0.00	0.00
Formaldehyde	50.21	48.68	ND	ND

Table 3. Effects of surfactants and EDTA on *A. niger* GH1 tannase activity.

Surfactant	Residual activity (%)			
	0.01%, v/v		0.1%, v/v	
	5 min	60 min	5 min	60 min
Control	100.00	100.00	100.00	100.00
Tween 80	65.05	76.93	105.48	125.41
Tween 20	97.79	93.53	103.76	101.25
Triton X-100	102.15	98.06	92.93	107.13
SDS	90.68	85.10	108.89	100.39
EDTA	77.97	84.17	86.67	80.92

The tannase yield of 0.3% obtained in this work was lower as compared to 2–20% recovery reported by others [13, 36, 46]. However, purification factor reported in this paper is higher as compared to 24–31 fold reported previously by several other authors [36, 42, 46]. Most of the available literature on tannase purification also reveals that the enzyme is composed of several subunits. Farías *et al.* [19] found that the molecular mass of tannase from *Cryophonectrica parasitica* was 240 kDa and they reported that the enzyme comprised four subunits of 58 kDa. Similarly, Ramírez-Coronel *et al.* [43] reported that the tannase from *A. niger* was composed of two subunits of molecular masses 90 and 180 kDa.

A higher purification factor was reached with the protocol employed in this study. This protocol employs only minimal steps for purification of *A. niger* tannase and hence it is having many advantages over the purification procedures already reported in terms of cost and time [9, 25, 36, 42, 46], and it is as fast as that reported by Sharma *et al.* [45]. Most of the available protocols for

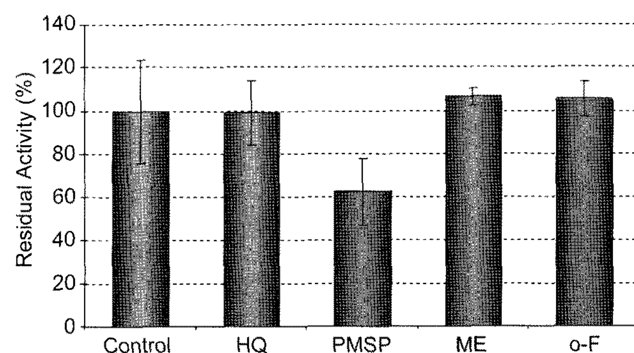

Fig. 8. Effects of classical enzyme inhibitors on the *A. niger* GH1 tannase activity.
Hydroxyquinone (HQ), phenylmethylsulfonyl fluoride (PMSP), mercaptoethanol (ME), and *o*-fenantroline (o-F). Each inhibitor solution was prepared at 2 mM level and mixed at 1:1 (v/v) ratio with the tannase extract. Residual activity was measured after 5 min of incubation at 30°C and pH 5.

Table 4. K_M and V_{max} values for microbial tannases using tannic acid (TA) and methyl 3,4,5-trihydroxybenzoate (MTB) as substrates.

Author	Year	Microorganism	Substrate	K_M (M)	V_{max} (μmol/min)
Rajakumar and Nandy	1983	*Penicillium chrysogenum*	TA	0.48×10^{-4}	–
Sharma *et al.*	1999	*A. niger* van Tiehgem	MTB	2×10^{-4}	5.00
Bhardwaj *et al.*	2003	*A. niger* MTCC 2425	MTB	9.5×10^{-4}	1.54
Sabu *et al.*	2005	*A. niger* ATCC 16620	MTB	1.03×10^{-3}	4.25
This study	2008	*A. niger* GH1	MTB	0.41×10^{-4}	11.03
This study	2008	*A. niger* GH1	TA	2.3×10^{-5}	13.10

purification of tannase are with three or more steps, increasing costs and time, besides the use of conventional chromatographic methods. The use of membrane technology makes the present protocol most attractive besides the other characteristics mentioned above. A comparative summary of tannase purification protocols previously reported is shown in Table 5.

Among all tannases previously described [8, 36, 37, 43, 44, 46], A. *niger* GH1 tannase showed better activity at extreme conditions of pH and temperature. Recently, Sharma *et al.* [45] reported that *Penicillium variable* tannase has temperature and pH optima of 50°C and 5, respectively. However, the functional temperature range was from 25 to 80°C and functional pH range from 3.0 to 8.0. In the present study, *A. niger* GH1 tannase was more active at lower pH values (2.0) with better thermostability. In contrast, Kasieczka-Burnecka *et al.* [25] reported two *Verticillium* sp. tannases with optimum activity at a pH of 5.5 at 20–25°C.

The inhibitory effects of Fe^{3+}, Cu^{2+}, and Zn^{2+} ions have been previously reported on tannases [7, 24, 25, 44]. Tannase from other strains of *A. niger* have been strongly inhibited by Mg^{2+} and Mn^{2+} [13, 44], whereas *Verticillium* sp. tannase was inhibited only by Mn^{2+} and activated by Mg^{2+} [25].

Significant loss of activity in the presence of Tween 20 was reported for tannase produced by *Verticillium* sp. [25] and *Paecilomyces variotii* [9]. *Aspergillus foetidus* tannase was completely inhibited by Tween 80 and SDS [40]. EDTA is a potent inhibitor of metal-dependent enzymes, which is generally used as commercial inhibitor of proteases, where it acts by chelating metals like lead and zinc. A 20% of inhibition of tannase activity was observed under standard assay conditions. A strong inhibitory effect of EDTA on *A. oryzae* tannase was reported by Iibuchi et al. [22]. Nonmodulatory effect of EDTA was reported on tannase from other *Aspergillus* species [1, 7, 13].

A strong negative effect of diisopropyl-fluorophosphate on tannase activity was previously described [1, 7, 40]. In this study, no other tested inhibitor had a considerable effect on the *A. niger* GH1 tannase. Mercaptoethanol was a potent inhibitor of other tannases [9, 25, 45].

The present study reports a tannase enzyme with properties distinct from those previously reported from this species and provides further important knowledge on the strain GH1. The use of *A. niger* GH1 has been shown to be very promising in tannin-rich wastes treatment, to enhance the biological activity of tea, to produce important potent phenolic antioxidants, *etc.* Comparatively high stability of *A. niger* GH1 tannase to pH, temperature, and other additives, which are generally encountered in tannin-rich systems, and the high values of the specificity constants for several polyphenolic compounds that are commonly found in those systems, clearly indicate the multiple advantages of this enzyme for industrial applications.

The values of kinetic constants (K_M and V_{max}) depend on the particular substrate used and the enzyme source. A wide range of values (2×10^{-5}–1.03×10^{-3} M) for K_M and V_{max} have been reported for tannases from several microorganisms [13, 25, 42, 44–46]. The K_M value of 2.3×10^{-5} M obtained in this work was lower as compared with the K_M value of 2.0×10^{-4} M for *A. niger* van Tieghem tannase [46]; in both cases, MTB was used as substrate. In this work, V_{max} values of 11.03 and 13.10 μmol/min using MTB and TA were higher than those reported by several authors [13, 44, 46]. Recently, Kasieczka-Burnecka *et al.* [25] reported two cold-adapted extracelullar tannases isolated from antarctic strain *Verticillium* sp. p9. Both tannases had K_M values of 3.65×10^{-3} and 2.43×10^{-3} M using MTB as substrate; when TA was used as the substrate, the values were 5×10^{-4} and 3.88×10^{-3} M. Moreover, Sharma *et al.* [46] reported that the tannase from *Penicillium variable* has a K_M value of 3.2×10^{-2} M (using TA as substrate), higher than the values of 2.3×10^{-5} M (reported in this work) and 0.48×10^{-4} M reported by Rajakumar and Nandy [42]. A K_M value of 1.03×10^{-3} M was obtained for *A. niger* ATCC 16620 tannase. Bhardwaj *et al.* [13] reported a K_M value of 2.8^{-4} M (using TA as substrate) for A. *niger* MTCC 2425 tannase, higher than reported in this work. A. *niger* GH1 tannase exhibits kinetic constants similar to those reported for other tannases from microbial sources; however, its affinity to tannic acid is high. Although a significant amount of information is available on the tannin-degrading system of *A. niger*, the fact that the fungus *A. niger* GH1 was isolated from the Mexican desert (a place of extreme conditions of salinity, humidity, and temperature) makes the study of this enzyme attractive. The results presented in this work shows that *A. niger* GH1 tannase has interesting and attractive properties for industrial use.

Table 5. Summary of purification protocols for microbial tannases.

Author	Purification step	Vol. (ml)	Protein (mg)	Activity (U)	Sp. act. (U/mg)	Fold	Yield (%)
Rajakumar and Nandy (1983)	Crude extract	800	2981.6	10,800	3.6	1	100
	Ammonium sulfate precipitation	125	810	7,500	9.2	2.5	69
	DEAE–cellulose column chromatography	90	120	3,300.00	27	7.5	30
	Sephadex G 200 gel filtration	35	23	2,000.00	86	24	18.5
Sharma *et al.* (1999)	Crude extract	400	200	148	0.7	1	100
	DEAE–Sephadex A-50	25	1.3	12	9	13	8
	Sephadex G-150	15	0.2	4	20	29	2.7
Bhardwaj *et al.* (2003)	Crude extract	1000	23	160	7	1	100
	Sephadex G-150	35	2.9	83	28.6	4	52
	DEAE–Sephadex A-50	15	0.09	32	355.6	51	20
Zhong *et al.* (2004)	Crude extract	1000		7,000			100
	Ultrafiltration	104		6,580			94
	DEAE Sepharose	16		3,600			51
Sabu *et al.* (2005)	Crude extract				0.27		
	40–60% fraction				0.916		
	DEAE–Sephadex A-50 chromatography				1.6		
Mahendran *et al.* (2006)	Crude extract	1000	260	420,000	1,615	1	100
	Activated charcoal treatment	975	253	409,500	1,615	1	97.5
	Ammonium sulfate precipitation	15	15	330,750	22,050	13.6	78.7
	DEAE–cellulose column chromatography	15	7.5	235,820	31,442	19.4	56.1
	Sephadex G 200 column chromatography	5	1.5	74,000	49,333	30.5	17.6
Mahapatra *et al.* (2005)	Crude extract						
	Acetone precipitation						
	HPLC (GF-250 column)						
Ramírez-Coronel (2003)	Crude extract						
	Isoelectric focusing						
	Mono-Q column chromatography						
	Sephadex column G-100 gel-filtration column						
Mukherjee and Banerjee (2005)	Crude extract						
	Cetona precipitation						
	DEAE–Sephadex column						
Farias *et al.* (2004)	Crude extract						
	Ion-exchange chromatography						
	Gel-filtration chromatography					142	10
Barthomeuf *et al.* (1994)	Crude extract						
	Ultrafiltration						
	High-pressure size exclusion chromatography						
Gupta (1997)	Extract crude						
	Precipitation by pH variation						
	Polyethylene glycol–tannic acid complex precipitation						

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