World J Microbiol Biotechnol (2011) 27:2325–2333
DOI 10.1007/s11274-011-0699-1

ORIGINAL PAPER

# Production of tannase from wood-degrading fungus using as substrate plant residues: purification and characterization

**R. M. Ordoñez · I. Colombo · M. R. Alberto · M. I. Isla**

Received: 7 November 2010 / Accepted: 17 February 2011 / Published online: 2 March 2011


**Abstract** In the present study *Lenzites elegans*, *Schizophyllum commune*, *Ganoderma applanatum* and *Pycnoporus sanguineus* (wood-degrading fungi) were assayed for their tannase producing potential in culture media containing plant residues or/and tannic acid as carbon source. *Aspergillus niger* was used as positive control for tannase production. We also carried out the isolation, purification and characterization of the enzyme from the fungi selected as the major productor. The highest fungal growth was observed in *A. niger* and *L. elegans* in the media containing tannic acid + glucose + plant residues (*Fabiana densa*). *A. niger* and *L. elegans* reached the highest extracellular tannase production in a medium containing tannic acid + *F. densa* and in a medium supplemented with glucose + tannic acid + *F. densa*. The produced enzyme by *L. elegans* was purified by DEAE-Sepharose. Km value was 5.5 mM and relative molecular mass was about 163,000. Tannase was stable at a pH range 3.0–6.0 and its optimum pH was 5.5. The enzyme showed an optimum temperature of 60°C and was stable between 40 and 60°C. This paper is the first communication of tannase production by wood-degrading fungi. Fermentation technology to produce tannase using plant residues and xylophagous fungi could be very important in order to take advantage of plant industrial waste.

**Keywords** Tannase · Wood-degrading fungi · Plant substrates · *Lenzites elegans*

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## Introduction

Hydrolyzable tannins such as gallotannin and ellagitannin are widely distributed in the plant kingdom (Salunkhe et al. 1989). These tannins bind readily with proteins and other macromolecules to form indigestible or insoluble complexes. Tannase (tannin-acyl-hydrolase, E.C. 3.1.1.20) catalyzes the hydrolysis of tannins by breaking their esters and depside bonds releasing glucose and gallic acid. At present, tannase is mostly used to manufacture instant tea, acorn liquor and gallic acid (Coggon et al. 1975; Pourrat et al. 1985). This product of tannin hydrolysis finds application in many fields like the dye-making, pharmaceutical, leather and chemical industries (Hadi et al. 1994; Mukharjee and Banerjee 2003). It is an important intermediary compound in the synthesis of the antibacterial drug, trimetroprim, used in the pharmaceutical and food industries; gallic acid is a substrate for the chemical or

enzymatic synthesis of propylgallate, a potent antioxidant (Banerjee et al. 2005). It is used to manufacture ordinary writing inks and dyes, pyrogallol and gallic acid esters among other compounds and as a photographic developer. As tannery effluents contain high amounts of tannins, mainly polyphenols, which are pollutants, tannase represents a cheap treatment and cash for the removal of these compounds. Tannase is also used as a clarifying agent in some wines, beer, fruit juices and in refreshing drinks with coffee flavour (Lekha and Lonsane 1997).

There is a constant search for new sources of tannase with more desirable properties for commercial application such as higher catalytic stability and low production cost (Batra and Saxena 2005). *Lenzites elegans, Ganoderma applanatum* and *Pycnoporus sanguineus* isolated from Tucuman, Argentina have been used as the source of enzymes mixture with hydrolytic capacity on plant cell walls (Leal et al. 1994). The potential of these fungi to produce hydrolitic enzyme by a simple method and its low cost might suggest the possibility of potential commercial applications. For this reason, the present paper deals with the selection of wood-degrading fungi for their capacity to produce tannase using plant residues as carbon sources. Then, we proposed the production, purification and characterization of the enzyme from fungi that exhibit the highest activity.

## Materials and methods

### Chemicals

All chemicals and reagents were of analytical or microbiological grade. Sephadex G-100 and DEAE-Sepharose were provided by Amersham Biosciences (Uppsala, Sweden). All other chemicals and reagents were purchased from Sigma (St. Louis, Mo, USA) or Merck (Darmstad, Germany).

### Culture

#### *Microorganisms*

The fungal isolates were provided by Lic. A. Ladki from Instituto Miguel Lillo (Tucumán). *Ganoderma applanatum* per, ex Walls Pat. (IEV017), *Pycnoporus sanguineus* (L. Ex.Fr.) Murr. (IEV006), and *Schizophyllum commune* Fr. (IEV009) were isolated from *Quercus robur, Prosopis nigra* and *Nerium oleander,* respectively. *Lenzites elegans* Spreng ex Fr. (IEV012) were found on unidentified decaying wood. *Aspergillus niger* Tiegh. (IEV 100), a known tannase producer, was used as positive control for enzyme production.

#### *Culture conditions*

The fungi were maintained in Sabouraud agar medium (Britania, Argentina). The media were inoculated with each fungus and they were allowed to grow at 28°C for 15 days.

In order to identify tannase producing microorganisms, fungi were cultured in a basal medium (BM) containing: $NaNO_3$ (2.5 g/l), $KH_2PO_4$ (1 g/l), $MgSO_4$ (0.5 g/l) and KCl (0.5 g/l) in presence and absence of glucose (1%) and agar (3%). This BM was autoclaved at 121°C for 20 min. Tannic acid was added to the basal medium at final concentrations of 0.12, 0.25, 0.5 and 1%. Media without tannic acid in presence and absence of glucose were also prepared as controls. The pH was adjusted to 5.6 with 1 N NaOH. Plates containing 10 ml of each medium were added 5 µl of inocula ($4 \times 10^6$ spores/ml) and incubated for 2 days at 30°C. Radial mycelia growth of the fungi in the solid media was determined by measuring two right-angled diameters of the colonies. Clear zones due to tannic acid hydrolysis around the colonies after incubation confirmed tannase production.

To determine enzyme production, fungi were cultured in a basal liquid medium containing $NaNO_3$ (2.5 g/l), $KH_2PO_4$ (1 g/l), $MgSO_4$ (0.5 g/l) and KCl (0.5 g/l) and different carbon sources as inducers: glucose (1% w/v), tannic acid (0.5% w/v) or insoluble vegetable material (powdered aerial parts of *Fabiana densa*) (5% w/v). Media were inoculated at 10% (v/v).

The fungi were grown at 28°C in a static culture in erlenmeyer flasks containing 500 ml of the medium.

#### *Inocula preparation*

Inocula were prepared by growing each strain on Sabouraud medium at 30°C for 5 days to obtain heavily sporulating cultures. Sterile distilled water containing 0.1% Tween-80 (10 ml) was added in a fully sporulated agar culture. Spores were scraped using an inoculation needle under strict aseptic conditions. After homogenizing, the suspension was counted using a Neubauer chamber and adjusted to $4 \times 10^6$ spores/ml. The spore suspension thus obtained was used as the inoculum.

#### *Solid substrate*

*Fabiana densa* J. Rémy, of the Solanaceae family, was collected in the montainous area (Puna and high Andean phytogeographic provinces) (Cabrera 1978) of Antofagasta de la Sierra, Catamarca at 3,500 m above sea level (masl). The botanical identification of the plants was carried out by botanist Ana Soledad Cuello and the voucher specimens are preserved in the Fundación Miguel Lillo Herbarium (607770/LIL) and the Instituto de Estudios Vegetales

(IEV). The plant aerial parts were dried at room temperature, ground to a fine powder and then added to the fermentation media. *F. densa* powder was used as a substrate in the fermentation studies without any pre-treatment.

## Extraction and tannase purification

After 13 days, cultures were filtered and centrifuged at 10,000×*g* for 10 min to remove mycelial debris. The proteins from the supernatant were concentrated by solid ammonium sulphate until 100% saturation. After centrifugation at 10,000×*g* for 10 min, the precipitate was resuspended in 10 mM sodium acetate buffer (pH 5.0) containing 1 mM 2-mercaptoethanol and dialysed against the same buffer for 12 h at 4°C.

Tannase from *Lenzites elegans* was partially purified by anion-exchange chromatography using a fast protein liquid chromatography system, FPLC (AKTA, Amershan-Bioscience). The column (DEAE-Sepharose) was equilibrated in 10 mM sodium phosphate pH 7 (buffer A) at 1 ml/min. The enzymatic extract (800 µg of protein) was applied to a DEAE-Sepharose, 0.7 × 2.5 cm (HiTrap 1 ml, Amersham Pharmacia) column. The column was washed with buffer A and eluted at 1 ml/min by a linear gradient from 0 to 1 M of NaC1 in buffer A. The eluted fractions (2 ml) were collected by an automatic collector (Pharmacia Biotech) and absorbances at 280 nm and tannase activity were measured. The most active fractions were combined, dialysed for 12 h at 4°C against 10 mM sodium acetate buffer (pH 5.5) and used for tannase characterization. All procedures were carried out at 4°C.

## Analytical methods

### *Tannase activity*

Tannase activity was determined spectrophotometrically according to Sharma's method (Sharma et al. 2000). It is based on the formation of a chromogen between gallic acid (released by the esterase activity of tannase) and rhodanine (2-thio-4-ketothiazolidine). To determine gallic acid, 100 µl of enzymatic extracts were incubated with tannic acid (0.3 mM) in sodium phosphate buffer (10 mM, pH 5.5) for 30 min at 30°C. Next, 300 µl of a methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol) and 100 µl of 500 mM KOH were added to the mixture which was diluted with 900 µl of distilled water. After a 10 min additional incubation at 30°C, absorbance at 520 nm was measured on a Beckman-Coulter DU-650 spectrophotometer. A standard curve using gallic acid concentrations ranging was prepared. One unit of tannase activity (U) was defined as the amount of enzyme required to release 1 µmol of gallic acid per minute under defined reaction conditions. Enzyme yield was expressed as U/l. The specific activity was expressed as U/mg protein.

Also, we confirm the product release and the substrate purity by HPLC methods using Waters 510 with UV detector. A 20-µl sample was injected (250 mm × 4.6 mm i.d., 5 µm, C-18 column) and eluted with 5% acetic acid in distilled water until the gallic acid was eluted. The flow rate was maintained at 1.80 ml/min and the acid was monitored at 280 nm. Commercial gallic acid was used as standard (Weetall 1985).

### *Protein determination*

Protein concentration was determined by Lowry's method (1951) and expressed in mg/ml. Bovine serum albumin (BSA) was used as a standard.

### *Tannin content determination*

Tannin extraction from the plant material followed the method of Hagerman et al. (1998). Dried (finely ground) plant material (200 mg) was mixed thoroughly with acetone 70% (10 ml), subjected to ultrasonic treatment (Ultrasonic water bath, 80w potence, Testlab) for 30 min at 30°C and then centrifuged at 3,000×*g* for 10 min at 4°C. The supernatant was removed and the pellet was re-extracted twice with acetone 70% and the contents were again subjected to ultrasonic treatment. All supernatants were collected and tannin was then recovered by using a rotary vacuum evaporator. Proanthocyanidin content was quantified according to Porter et al. (1986). Briefly: 1.2 ml of n-butanol–HCl solution (95:5, v/v) and 40 µl of iron reagent (2% ferric ammonium sulphate in 2 N HCl) were added to 200–400 µl of extract. The test tubes were covered with glass marbles and heated at 100°C for 50 min. Absorbance was measured at 550 nm. Tannin content was expressed as µg of quebracho tannin equivalent/g dry weight, (µg QTE/g DW).

### *Sugar determination*

Total sugars were measured by the method of Dubois et al. (1956) and reducing sugars were analized with the method of Somogyi-Nelson (Nelson 1944; Somogyi 1945).

### *Phenolic compound determination*

Total phenolic compound content was determined according to Singleton et al. (1999).

## Molecular mass measurement

Tannase Mr was determined by the method of Andrews (1964). Tannase was filtered through a 45 × 0.5 cm

column of Sephadex G-150 equilibrated and eluted with 0.2 mM sodium acetate buffer pH 5.5. The Mr markers used were alkaline phosphatase (100,000), BSA (66,000), ovoalbumin (45,000) and carbonic anhydrase (29,000) (Sigma–Aldrich Co.). Fractions were collected and analysed for tannase activity and $A_{280}$.

### Mr and purity by electrophoresis techniques

Mr (SDS–PAGE) and purity of enzyme (native PAGE-SDS–PAGE) were determined by vertical gel electrophoresis of proteins. Briefly: 2 μg protein/lane were heated in 20 μl of loading buffer for 5 min according to Laemmli (1970) or 5 μg of protein/lane for activity determinations. Proteins were stained by the silver nitrate method. BSA (66,000), ovoalbumin (44,000), pepsin (34,000), β-lactoglobulin (18,400) and lysozyme (14,300) were used as standards. Enzyme activity was determined using tannic acid 0.5% (w/v) as a substrate and quinine hydrochloride 0.5% (w/v) was used as a developer (Aoki et al. 1976).

### Catalytical properties

Tannase activity profile at various pH values (3.5–9.0) was determined at 37°C. Stability was studied with the enzyme pre-incubated at 30°C for 30 min at different pH (3.5–9.0). Thereafter, enzyme activity was found to be at its optimal pH (5.5) at 37°C. Thermal optimum activity was measured by incubating the enzyme at the optimum pH (5.5) at different temperatures (10–80°C). Temperature stability profile was evaluated by measuring residual enzymatic activity after 30 min preincubation of the enzyme at temperatures ranging from 10 to 80°C.

The Michaelis–Menten parameter (Km) and Vm were calculated from a double reciprocal plot of tannase activity versus substrate concentration (Lineweaver and Burk 1934).

### Statistical analysis

Results are mean values obtained from at least three independent experiments. The values were evaluated using GraphPad Prism 5.0 software. The results of the enzymatic activities in different substrate conditions were analyzed using the media values and the relative standard deviation (RSD).

## Results and discussion

Tannase is known to be an ubiquitous enzyme of the microbial world and has a widespread occurrence in various fungi (Banerjee and Mondal 2001; Batra and Saxena 2005; Sabu et al. 2005) and bacteria (Curiel et al. 2010). Several fungi species such as *Aspergillus spp.* (Deschamps 1989) and *Penicillum spp*. (Rajakumar and Nandy 1983) have long been known to be capable of producing a large amount of tannase. Yeasts belonging to the *Candida* and *Saccharomyces* genus have also been reported to produce this enzyme (Belmares et al. 2004). No reports about tannase production by wood-decaying fungus have been published so far. In the present study five fungi were selected and assayed for their tannase producing potential. The xylophagous fungi were subjected to screening on agar plates containing a basal medium added with different tannic acid concentrations in presence and absence of glucose. Clear zones of hydrolysis surrounding the colony indicate utilization of tannic acid by producing tannase enzyme. This procedure has been reported to be quick and convenient for screening a large number of microorganisms as tannase producers (Bradoo et al. 1996).

Fungal growth was tested in media containing tannic acid in presence and absence of glucose and clear zones of hydrolysis surrounding the colonies appeared in all cases. Mycelial growth and tannase production followed this order: *A niger* > *L. elegans* > *P. sanguineus* > *S. commune* and *G. applanatum*. Tannic acid optimal concentration was 0.5% (Table 1).

Belmares et al. (2004) reported that the addition of carbon sources such as glucose, fructose, sucrose, maltose and arabinose to the culture medium at initial concentrations from 10 to 30 g/l improved tannase production by *Aspergillus niger*. We found a better growth for *A. niger* and *L. elegans* in presence of glucose than in its absence, but no diference was observed in tannase production. The other fungi species did not show any diference in growth and hydrolytic zones in these conditions. *L. elegans* was selected by its capacity to tannase produce. Spores of each tannase-producing fungus were inoculated in erlenmeyer flasks containing sterilized minimal medium added with different carbon sources and tannase production inducers: tannic acid (0.5% w/v); glucose (1% w/v) and/or *Fabiana densa* (5% w/v) powder.

Utilization of agricultural residues through biotechnology is becoming more and more significant with the dual goal of waste disposal and value addition. Hydrolysable tannins are present in most higher plant residues. They are polyphenolic compounds formed by the association of sugars, gallic and ellagic acids via ester linkages (Kumar and Sing 1984). The presence of tannins and their derivatives in agro residues is a major hurdle in their utilization as feed material because of their antifeedant effect. Sabu et al. (2005) utilized agricultural residues for tannase production from *Aspergillus niger* ATCC 16620. Treviño-Cueto et al. (2007) used *Larrea tridentata* powder as sole carbon source and inducer of tannase activity from *Aspergillus niger*.

**Table 1** Screening of tannase producing microorganisms

| Microorganism | Tannic acid (%) | Basal medium + 1% glucose | | Basal medium | |
|---|---|---|---|---|---|
| | | Mycelia growth[a] | Hydrolytic zone[a] | Mycelia growth[a] | Hydrolytic zone[a] |
| *L. elegans* | 0.00 | ++++ | – | +++ | – |
| | 0.12 | ++++ | + | +++ | + |
| | 0.25 | ++++ | ++ | +++ | ++ |
| | 0.50 | ++++ | ++++ | ++++ | +++ |
| | 1.00 | +++ | +++ | ++ | ++ |
| *A. niger* | 0.00 | ++++ | – | + | – |
| | 0.12 | ++++ | + | +++ | + |
| | 0.25 | ++++ | ++ | +++ | ++ |
| | 0.50 | ++++ | ++++ | ++++ | +++ |
| | 1.00 | +++ | +++ | ++ | ++ |
| *G. applanatum* | 0.00 | ++ | – | ++ | – |
| | 0.12 | + | + | + | + |
| | 0.25 | + | + | + | + |
| | 0.50 | + | + | + | + |
| | 1.00 | + | + | + | + |
| *S. commune* | 0.00 | ++ | – | ++ | – |
| | 0.12 | + | + | + | + |
| | 0.25 | + | + | + | + |
| | 0.50 | + | + | + | + |
| | 1.00 | + | + | + | + |
| *P. sanguineous* | 0.00 | + | – | + | – |
| | 0.12 | ++ | ++ | ++ | ++ |
| | 0.25 | +++ | ++ | +++ | ++ |
| | 0.50 | +++ | ++ | +++ | ++ |
| | 1.00 | ++ | ++ | ++ | ++ |

The microorganisms were grown on agar plates containing different tannic acid concentrations with and without glucose for 48 h incubation at 38°C

[a] Mycelia growth and hydrolytic zone: (+) 10 mm, (++) 10–15 mm, (+++) 15–20 mm, (++++) >20 mm

In these paper, *F. densa* was selected as tannase inducer because of its phenolic compounds, tannins and sugar content (3, 5 and 3%, respectively). *F. densa* tannin values were higher than those found in other vegetal residues used as tannase inducers such as coffee and grapes (0.073 and 0.941%, respectively) (Macedo et al. 2005). At the moment, *F. densa* is used in Tucumán, Argentina as a phytoterapeutic product in small scale (Zampini et al. 2009), but its scaling-up is probable.

The growth of *A. niger* and *L. elegans* in different culture media at different incubation times are shown in Fig. 1. *A. niger* reached the greatest biomass in media supplemented with tannic acid + *F. densa* + glucose, followed by the media supplemented with glucose + *F. densa*. The growth in the media containing only glucose or *F. densa* was lower, which suggests that the microorganism needs both glucose and plant residues as carbon sources. The addition of tannic acid to the media containing glucose and *F. densa* increased growth rate, but the biomass at 6 days of incubation was similar to that of the media without tannic acid (Fig. 1a).

*L. elegans* reached the largest biomass in media supplemented with tannic acid + *F. densa* + glucose and in media supplemented with glucose + *F. densa.* These results are similar to those found with *A. niger,* showing that vegetal residue stimulates fungal growth. The addition of tannic acid to the media containing glucose and *F. densa* did not modify the growth parameters of *L. elegans* (Fig. 1b). The final biomass (at the end of the exponential growth phase) was similar to that of *A. niger* (5 g/l). However, differences in growth rate were observed in both fungi. In *A. niger* cultures, the mycelium formation began on the second day, reaching the maximum between 4 and 6 days of incubation, whereas *L. elegans* presented a slower growth: mycelium growth was observed at 10 days and maximum biomass occurred between 13 and 16 days of incubation (Fig. 1a, b).

Figure 2 shows the results of extracellular tannase activity obtained by *A. niger* (Fig. 2a) and *L. elegans*

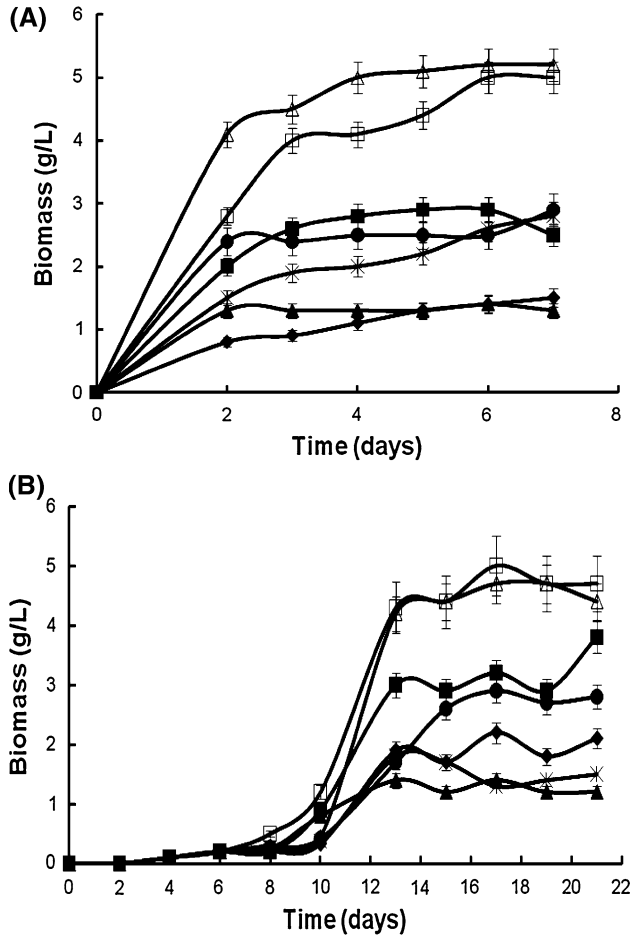


**Fig. 1** Growth of *A. niger* (**a**) and *L. elegans* (**b**) in different culture media supplemented with tannic acid (T); glucose (G) and/or *Fabiana densa* (F) during different incubation times at 30°C. Relative standard deviation (RSD) <10%. Symbols: (*filled diamond*) G, (*filled square*) F, (*filled triangle*) T, (*open square*) G F, (*asterisk*) G T, (*filled circle*) F T, (*open triangle*) F G T

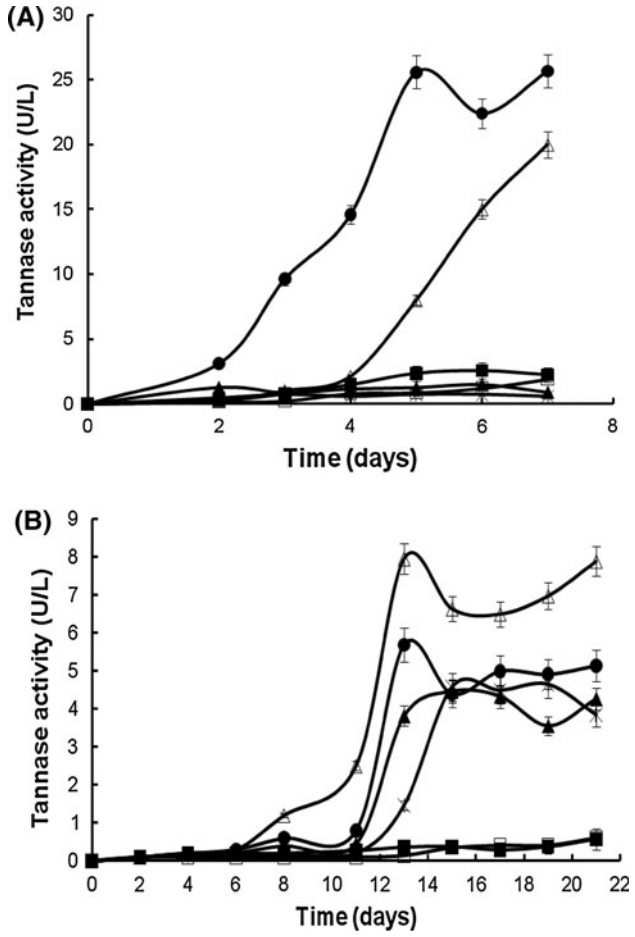


**Fig. 2** Extracellular tannase activity of *A. niger* (**a**) and *L. elegans* (**b**) in different culture media supplemented with tannic acid (T); glucose (G) and/or *Fabiana densa* (F) during different incubation times at 30°C. Relative standard deviation (RSD) <10%. Symbols: (*filled square*) F, (*filled triangle*) T, (*open square*) G F, (*asterisk*) G T, (*filled circle*) F T, (*open triangle*) F G T

(Fig. 2b) in different culture media supplemented with tannic acid (T), glucose (G) and/or *Fabiana densa* (F) at different incubation times.

*A. niger* reached the highest tannase activity in presence of tannic acid and *F. densa* as carbon sources while little activity was observed in the media containing only tannic acid or *F. densa*. Hence, both nutrients are neccesary for tannase production. Tannic acid has been considered one of the natural enzyme inducers. The tannins present in *F. densa* could act as enzyme inducers in a similar way. Our results indicate that *A. niger* is able to produce tannase in presence of tannic acid and tannin rich substrates. The results obtained with glucose as a carbon source show the absence of tannase activity. Similar results were reported by other authors (Aguilar and Gutiérrez-Sánchez 2001; Belmares et al. 2003). In this work we found little enzymatic activity in the media supplemented with tannic acid or glucose and its combinations.

Nevertheless, in our assay conditions, tannase production was not growth associated. Studies in liquid media indicated that at an incubation time of 2 days *A. niger* reached the maximal biomass while the optimum production of tannase was reached at 5 days of incubation in presence of tannic acid and *F. densa* (Fig. 2a). These results are not coincident with those reported by Sabu et al. (2005) that show that tannase yield was associated with fungal growth.

*L. elegans* reached the highest tannase activity at 13 days of growth in media supplemented with glucose, tannic acid and *F. densa* followed by the media supplemented with *F. densa* and tannic acid (Fig. 2b). Tannase production and secretion in these media were associated with biomass accumulation.

According to our results, fermentation technology using *F. densa* and non pathogenic microorganisms which can produce hydrolytic enzymes such as tannase will be

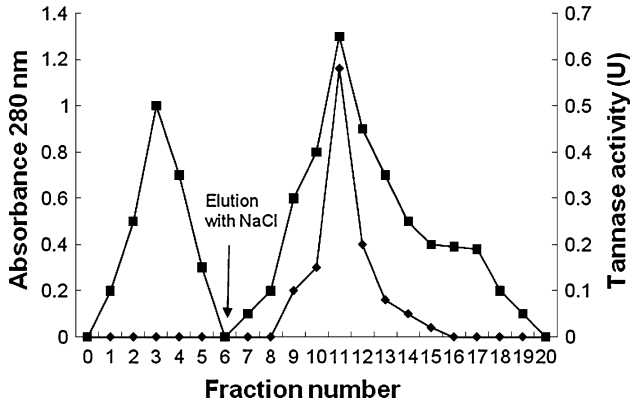


**Fig. 3** Elution profile of *L. elegans* tannase on DEAE-Sepharose column chromatography. Absorbance at 280 nm (*filled square*) and Tannase activity (*filled diamond*)

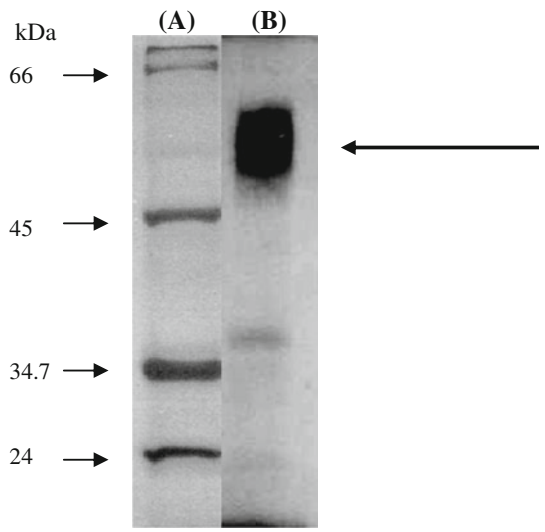


**Fig. 4** SDS-Polyacrylamide gel electrophoresis (SDS–PAGE) of *L. elegans* tannase. The partially purified enzyme was applied to a 15% gel and separated at a constant current. The gel was stained with $AgNO_3$ reagent. *Lane A* molecular weight markers (Sigma Co.): *Lane B* tannase (5 µg)

advantageous for the proper utilization of these and other vegetal substrates.

This is the first paper that reports on tannase production by xylophagous fungi. Hence, tannase isolation and purification from a thirteen-day culture of *L. elegans* were carried out.

Extracellular tannase was isolated from the culture media and the proteins were precipitated with $(NH_4)_2SO_4$ (100% stn) and separated by FPLC anion exchange chromatography (Fig. 3). Only one peak with tannase activity was obtained. We purified this enzyme 20 times with a 62.5% recovery. The specific activity increased until 6.41 U/mg protein during the purification process as shown in Table 2.

The enzyme was partially purified by anion-exchange chromatography (Fig. 4). The peak corresponding to tannase activity was assessed by native-PAGE, revealing a mayoritary protein by $AgNO_3$, which correspond with a single band revealed by tannase activity (data not shown). The relative molecular mass of the enzyme was estimated to be approximately 163,000 by gel filtration. The subunit molecular weight, determined by SDS–PAGE, showed a band at around 50,000 Da, thus suggesting that the enzyme should be a homo-trimer (Fig. 4).

The fungal tannase generally have acidic pH optimum (Belmares et al. 2004; Aguilar et al. 2007). The *L. elegans* tannase was stable over a pH range of 3.0–6.0 and the optimum pH was found to be 5.5. Minimal activity was recorded at strongly acidic and basic pHs (Figs. 5, 6). The enzyme showed an optimum temperature of 60°C and was

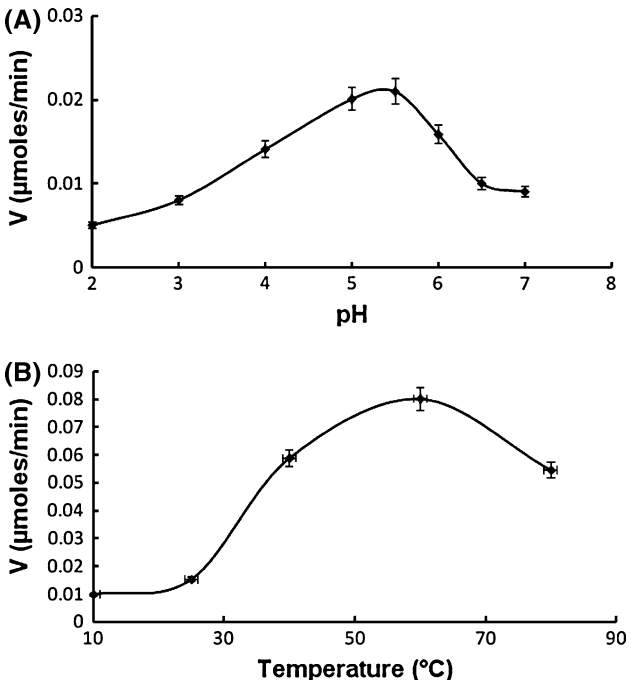


**Fig. 5** Effect of pH (**a**) and temperature (**b**) on *L. elegans* tannase activity. Relative standard deviation (RSD) <10%

**Table 2** Purification of *L. elegans* tannase

| Step | Volume (ml) | Enzyme unit (U) | Protein (mg) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Crude preparation | 15.0 | 0.8 | 2.52 | 0.31 | 1 | 100 |
| Ammonium sulphate precipitation | 7.0 | 0.6 | 0.11 | 5.35 | 17 | 75.0 |
| DEAE-Sepharose | 2.5 | 0.5 | 0.08 | 6.49 | 21 | 62.5 |

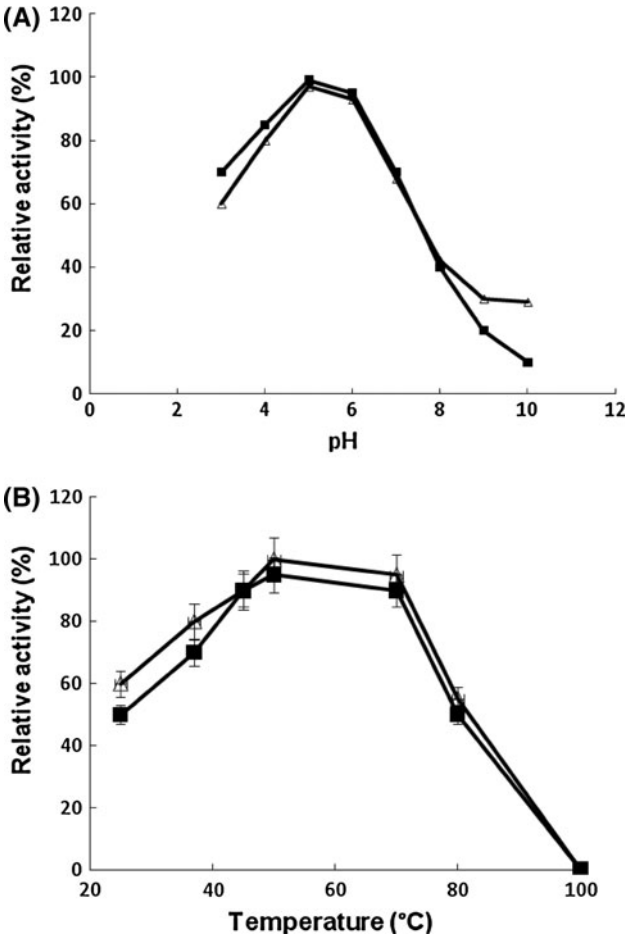


**Fig. 6** pH (**a**) and temperature (**b**) stability of *L. elegans* tannase. Relative standard deviation (RSD) <10%. Crude tannase (*open triangle*) and partially purified tannase (*filled square*)

stable at temperatures between 40 and 60°C but lost all activity at 90–100°C (Figs. 5, 6). The enzyme was routinely stored at 4°C in 10 mM acetate buffer, pH 5.5; under these conditions the tannase was stable for 3 months without loss of activity. The temperature stability was higher than that reported for other fungal tannase isolated from *Aspergillus* and *Penicillium* which demonstrates its great potential biotechnological (Belmares et al. 2004). When tannic acid was used as substrate, Km values of 11.25 and 0.048 mM were obtained with *Aspergillus* and *Penicillium*, respectively (Aguilar et al. 2007). The apparent Km value of *L. elegans* tannase was 5.5 mM (using tannic acid as a substrate) and the value of Vmax was 8.9 µmol/min.

## Conclusions

Our results indicate that a culture medium containing glucose, tannic acid and *F. densa* was the most suitable for fungal growth and enzyme production for *L. elegans* and *A. niger*. We report inducible tannase activity in *L. elegans* and the use of industrial plant residues in order to improve tannase production by *L. elegans*. The high thermal stability of *L. elegans* tannase is a useful characteristic for biotechnological applications.

The use of *L. elegans* tannase may have benefits for the food, beverage, cosmetic and pharmaceutical industries because this fungus is regarded as a non pathogenic microorganism.

**Acknowledgments** The authors acknowledge the cooperation from the inhabitants of the areas of study and the financial support from Consejo de Investigación de la Universidad Nacional de Tucumán (CIUNT), Argentina and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

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