Production of Tannase by *Aspergillus tamarii* in Submerged Cultures

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ABSTRACT

The production of tannase by Aspergillus tamarii was studied in submerged cultures. The fungus produced an extracellular tannase after two days of growth in mineral medium containing tannic acid, gallic acid and methyl gallate as carbon source. The best result was obtained using gallic acid as inducer (20.6 U/ml). The production of enzyme was strongly repressed by the presence of glucose. Crude enzyme was optimally active at pH 5.0 and 30º C. The enzyme was stable in a large range of pH and up to the temperature of 45º C.

Key words: *Aspergillus tamarii*, enzyme, fungal biomass, submerged cultures, tannase, tannins

INTRODUCTION

Tannin acyl hydrolase (EC 3.1.1.20), commonly called tannase, catalyzes the hydrolysis of ester bonds in hydrolysable tannins such as tannic acid, thereby releasing glucose and gallic acid (Bhat et al., 1998, Lekha and Lonsane, 1997). Tannins are defined as naturally occurring water-soluble polyphenols of varying molecular weight depending on the bonds possessed with proteins and polysaccharides (cellulose and pectin). Tannins are widespread in the plant kingdom, are found in the leaves, fruits, bark and wood. They occur in many edible fruits and vegetables and are often considered nutritionally undesirable because they form complexes with protein, starch and digestive enzymes and cause a reduction in nutritional value of food (Chung et al., 1998).

Tannase finds widespread application in the food and beverage processing. At the moment, most of the commercial applications of tannase are in the manufacturing of instant tea, where it is used to eliminate the water insoluble precipitates, wine, beer and coffee-flavored soft drinks (Aguilar and Sanches, 2001). Other important application of tannase in the food industry is its use as substrate for the chemical synthesis of pyrogallol or ester gallates, which are used as preservatives (Sharma and Gupta, 2003, Kar and Banerjee, 2000). Gallic acid is also used in the enzymatic synthesis of propyl gallate, which is mainly used as antioxidant in fats and oils, as well as in beverages (Banerjee et al., 2005).

Although tannase is present in the plants, animals and microorganisms, it is mainly produced by the microorganisms (Ayed and Hamdi, 2002, Belmares et al., 2004). The filamentous fungi of the *Aspergillus* genus have been widely used for tannase production (Bajpai and Patil, 1996, Banerjee et al., 2001, Pinto et al., 2001). The production of tannase by *Aspergillus* sp can occur in the absence of tannic acid, but these fungi

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tolerate tannic acid concentrations as high as 20% without having a deleterious effect on both the growth and enzyme production. Studies on tannase production by *Aspergillus* sp can be done by various methods such as the liquid surface, submerged, modified solid-state cultures and solid state cultures (Bradoo et al., 1997, Belmares et al., 2004). The use of the submerged culture is advantageous because of the ease of sterilization, and process control is easier to engineer in these systems. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns. Phenolic compounds such as gallic acid, pyrogallol, methyl gallate and tannic acid are normally considered as tannase inducers, although repression in the production of tannase by gallic acid has also been described (Bradoo et al., 1997). Based on a preliminary screening of various soil isolates and other available fungal cultures, one of these organisms, *Aspergillus tamarii,* was selected for further studies. Therefore, the purpose of this work was to study the production of tannase by *A tamarii* in submerged cultures as well as the effect of pH and temperature on the enzyme activity and stability.

MATERIALS AND METHODS

Microorganism and maintenance of culture
The strain of *Aspergillus tamarii* used in this study was isolated from the soil during a screening program for hydrolytic enzyme-producing microorganisms (Kadowaki et al., 1997, Souza et al. 1997). It was deposited in the Fundação Tropical de Pesquisas e Tecnologia André Tosello Collection, Campinas, Brazil. In the lab, it was routinely maintained on potato dextrose agar at 4° C.

Preparation of spore inoculum.
The fungal spore inoculum was prepared by adding 2.5 ml of the sterile distilled water containing Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under the strict aseptic conditions and the number of viable spores in the suspension was determined using the plate count method. The volume of 1 ml of spore suspension was used as the inoculum, with concentration of 5 x 10^9 spores.

Production of extracellular tannase in submerged cultures
The spore suspension was inoculated in 125 ml Erlenmeyer flasks containing 25 ml of sterilized mineral medium containing per liter: KH_2PO_4 1.0 g; $MgSO_4.7H_2O$ 2.0 g, $CaCl_2$, 1.0 g; NH_4Cl, 3.0 g; yeast extract, 1 g. Filter-sterilized tannic acid, methyl gallate, gallic acid at 1% concentration was added to the autoclaved medium. Different additional carbon source were added separately to the above-mentioned medium for studying their effect on the enzyme production. The cultures were grown for up six days at 140 rpm in an incubator shaker at 28° C. The samples were withdrawn at regular intervals of one day. The biomass were separated by the filtration through Whatman n° 1 filter paper. The cell-free culture broth was assayed for the extracellular tannase activity. The mycelium was washed three times with water. To determine the dry weight of the mycelium (biomass production), it was dried overnight at 60° C.

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). 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 the defined reaction conditions.

Enzyme precipitation
The enzyme from the clear gallic acid culture filtrate was precipitated by the drop-wise addition of cooled acetone at 4° C to a saturation level of 75% (v/v). After 1 h contact time, the precipitate was removed by centrifugation at 5,000 rpm (4° C) for 30 min, freed of acetone by vacuum drying and dissolved in a minimal amount of the distilled water. The acetone-precipitated enzyme was used in the following experiments.

Effect of pH and temperature on the activity and stability of tannase

The optimum pH was determined using the substrate in McIlvaine's buffer (pH 3.5 to 8.5). The stability of the crude enzyme was examined at different pH by incubating the enzyme with the buffers of different pH ranging from 3.5 to 8.5 for 24 h. The residual activity was estimated after incubation under the standard assay conditions and expressed as the percentage of the initial activity. For the determination of the temperature optimum, the enzyme assays were carried out at temperature ranging from 25 to 70° C. The thermal stability was investigated by incubating the enzyme at 30, 40, 50 and 60° C for 2 h. Immediately afterwards the reaction mixtures were immersed in an ice bath and then the residual activities were tested under standard conditions and it is expressed as the percentage of the initial activity.

Chemicals

The enzymatic substrates and carbon sources were obtained from Sigma Chemical Corp. (St. Louis, Mo). All other reagents were of analytical grade.

RESULTS AND DISCUSSION

The ability of *A. tamarii* to grow and to produce tannase on different carbon sources was studied (Fig. 1). Tannase activity and biomass were determined in the early stationary growth phase. Among the carbon source assayed, good growth was obtained with glucose, starch, xylan and sucrose, but the highest tannase activities were observed when the organism was grown on gallic acid (13.1 U/ml), which was followed by methyl gallate (9.7 U/ml) and tannic acid (9.3 U/ml). These results showed the potential of *A. tamarii* as a tannase producer. In a recent work where *Aspergillus* sp and *Penicillium* sp were screened to produce tannase, the highest levels of the activities produced by *A. fumigatus* and *A. versicolor* in submerged cultures with tannic acid as substrate were 8.3 and 7.0 U/ml, respectively (Batra and Saxena, 2005).
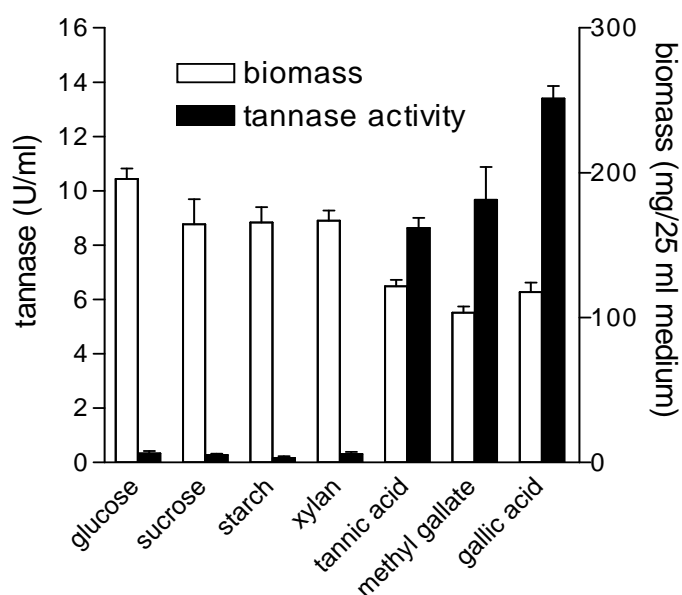

Figure 1 - Production of extracellular tannase and biomass by *A. tamarii* in submerged cultures. The cultures were developed for 4 days at 140 rpm and 28° C. Values are the mean ± SD of triplicate cultures

A typical time course of the extracellular tannase production by *A. tamarii* in submerged cultures is shown in Fig. 2A. Optimum enzyme production occurred after two days of cultivation, though the

maximum growth was not attained until four days. The cultures where gallic acid was used as the substrate produced the highest tannase level (20.6 U/ml). High levels of tannase activity were also obtained in the cultures with tannic acid and methyl gallate (12.4 and 10.1 U/ml, respectively). Phenolic compounds such as gallic acid, pyrogallol, methyl gallate and tannic acid have been described as tannase inducers (Bajpai and Patil, 1997). However, the induction mechanism

has not been demonstrated and there is some controversy about the role of some of the hydrolysable tannins constituents on the synthesis of tannase (Belmares et al., 2004). For example, the addition of gallic acid in the submerged cultures of *A. niger* Aa-20 (Aguilar et al., 2001) and *A. japonicus* (Bradoo et al., 1997) repressed the production of tannase.
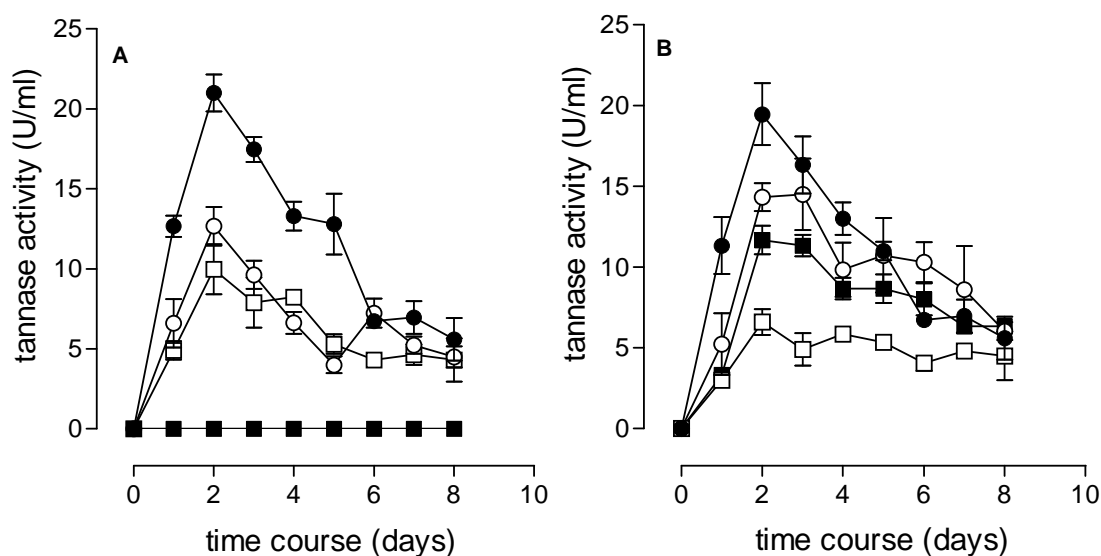

Figure 2 - Time course of extracellular tannase production by *A. tamarii* in submerged cultures. In A: The following carbon sources at 1.0% (w/v) were used: starch, glucose or sucrose (■), tannic acid (○), gallic acid (□), and methyl gallate (●). In B: The cultures were developed using 1.0% gallic acid as substrate in association with different amounts of glucose. Without glucose (●); plus glucose 0.2% (○); plus glucose 0.5% (■); plus glucose 1.0% (□).Values are the mean ± SD of triplicate cultures

Tannase repression experiments were carried out using gallic acid as inducer and several glucose concentrations as a potential repressor (Fig. 2B). Even the addition of small amounts of glucose (0.2 %) caused a decrease in the level of tannase. The addition of higher amounts of glucose (1.0%) drastically reduced the production of the enzyme. It has been reported that the synthesis of tannase is not under catabolic repression in the presence of glucose or sucrose (Aguilar and Sanches, 2001). In fact, 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 the production of tannase by *A. niger* (Belmares et al., 2004). The addition of

glucose in the submerged cultures of *A. japonicus* using tannic acid as substrate, did not decreased the production of tannase (Bradoo and Gupta, 1997). On the other hand, the addition of 2.0 % glucose in the submerged cultures of *Aspergillus niger* Aa-20, resulted in strong catabolite repression (Aguilar et al., 2001).
Some properties of *A. tamarii* extracellular tannase were studied. The enzyme was precipitated with cold acetone with more than 70% of recovery. The acetone-precipitated tannase was active at the acidic pH and the activity decreased as the pH approached the alkaline range. The optimum activity was recorded at pH 5.0 (Fig. 3A), but substantial activities were detected at pH values ranging

from 4.0 to 8.0, which could be a positive aspect regarding its use in the brewing industry. At 30° C, the enzyme was totally stable between pH 3.0 and 8.0 for 2 h (Fig 3A). Fungal tannases are acidic enzymes in general. There are reports describing the optimum pH as 5.0 in case of tannase from *A. awamori* (Mahapatra et al., 2005), pH 5.5 in case of tannase from *A. flavus* and *A. oryzae* (Batra and Saxena, 2005) and pH

6.0 in case of tannase from *A. niger* (Sabu et al., 2005). The enzyme was optimally active between 30-35° C (Fig 3B). Similar observations were related for tannase from several *Aspergillus sp* (Mahapatra et al., 2005). The enzyme was stable at 40° C for more than 2 h, but it was not stable at temperature higher than 45° C (Fig 3B).
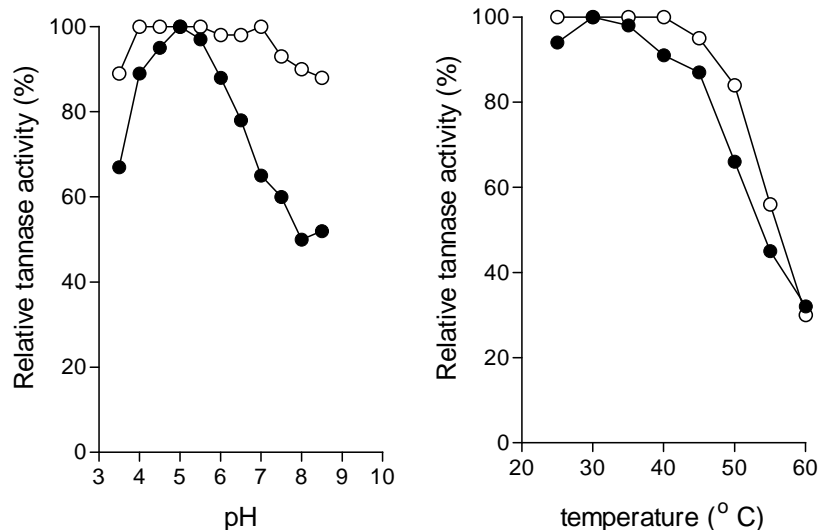

Figure 3 - Effect of pH (A) and temperature (B) on tannase activity (●) and stability (○). The stability of the crude enzyme was examined at different pH by incubating the enzyme with the buffers of different pH ranging from 3.5 to 8.5 for 24 h. For the determination of the temperature optimum, the enzyme assays were carried out at temperature ranging from 25 to 70° C. The thermal stability was investigated by incubating the enzyme at 30, 40, 50 and 60° C for 2 h

CONCLUSION

This is the first report of tannase production by *A. tamarii*. From the above results it is evident that this fungus could be a potential tannase producer. The strain was able to produce tannase in the medium containing tannic acid as the sole carbon source. However, gallic acid was the best inducer for tannase activity. One advantage of the use of this fungus to produce tannase is that it can be produce the maximum enzyme within a short period of cultivation. Thus, its production at higher levels should be attempted.

RESUMO

A produção de tanase por um novo potencial produtor, o fungo filamentoso *Aspergillus tamarii,* foi parcialmente caracterizada neste estudo. O fungo produziu uma tanase

extracelular em culturas submersas após 2 dias de crescimento em meio mineral contendo ácido tânico, ácido gálico ou metil galato como fonts de carbono. Os melhores resultados foram obtidos em culturas com ácido gálico (20,6 U/ml). A produção da enzima foi fortemente inibida por glicose. A enzima bruta foi otimamente ativa em pH 5,0 e a 30° C e estável em ampla faixa de pH e em temperaturas inferiores a 45°C.

REFERENCES

Aguilar, C.N.; Augur, C.; Favela-Torres, E. and Viniegra-González G. (2001), Induction and repression patterns of fungal tannase in solid-state and submerged cultures. *Proc Biochem.,* **36,** 565-570.

Aguilar, C.N. and Sanches, G. (2001), Review: sources, properties, applications and potential use of tannin acyl hydrolase. *Food Sci Technol. Int.,* **7**, 373-382.

Ayed,L.; Hamdi, M. (2002), Culture conditions of tannase production by *Lactobacillus plantarum. Biotechnol. Let.,* **24**, 1763-1765.

Bajpai, B. and Patil S. (1997), Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of fungi imperfecti. *Enz Microb Technol.,* **20**, 612 – 614.

Bajpai, B. and Patil S. (1996), Tannin acyl hydrolase (EC 3.1.1.20) activity of *Aspergillus, Penicillium, Fusarium* and *Trichoderma. World J. Microbiol. Biotechnol.,* **12,** 217-220.

Banerjee, R.; Mondal, K.C. and Pati, B.R. (2001), Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF 9. J. *Bas. Microbiol.,* **41**, 313-318.

Banerjee, R.; Mukherjee, G. and Patra, K.C. (2005), Microbial transformation of tannin-rich substrate to gallic acid through co-culture method. *Biores. Technol.,* **96**, 949-953.

Batra, A. and Saxena, R.K. (2005), Potential tannase producers from the genera *Aspergillus* and *Penicillium. Proc. Biochem.,* **40**, 1553-1557.

Belmares, R.; Contreras-Esquivel, J.C.; Rodriguez-Herrera, R.; Coronel, A.R. and Aguilar, C.N. (2004), Microbial production of tannase: an enzyme with potential use in food industry. *Lebensm-Wiss U-Techno,* **37**, 857-864.

Bhat, T.K.; Singh,, B. and Sharma, O.P. (1998), Microbial degradation of tannins–a current perspective. *Biodegradation,* **9**, 343-357.

Boadi, D.K. and Neufeld, R.J. (2001), Encapsulation of tannase for the hydrolysis of tea tannins. *Enz. Microb. Technol.,* **28**,590-595.

Bradoo, S.; Gupta, R. and Saxena, R.K. (1997), Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus. Proc. Biochem.* **32**,135-139.

Chung, K.T.; Wong, T.Y.; Wei, C.I.; Huang, Y.W. and Lin, Y. (1998), Tannins and human health: a review. *CRC Crit. Rev. Food Sci. Nutr.,* **38**, 4241-4264 .

Kadowaki, M.K.; Souza, C.G.M.; Simão, R.C.G. and Peralta, R.M. 1997, Xylanase production by *Aspergillus tamarii. Appl. Biochem. Biotechnol.,* **66**, 97-106.

Kar, B.; Banerjee, R. and Bhattacharyya, B.C. (2000), Modeling gallic acid production rate by empirical and statistical analysis. *Braz. Arch. Biol. Technol.,* **43**, 509-513.

Lekha, P.K. and Lonsane, B.K. (1997), Production and application of tannin acyl hydrolase: state of art. *Adv. Appl. Microbiol.,* **44**, 215-260.

Mahapatra, K.; Nanda, R.K., Bag, S.S.; Banerjee, R.; Pandey, A. and Szakacs, G. (2005) Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori* Nakazawa. *Proc. Biochem.,* **40**, 3251-3254 .

Pinto, G.A.S.; Leite, S.G.F.; Terzi, S.C. and Couri, S. (2001), Selection of tannase-producing *Aspergillus* strains. *Braz. J. Microbiol.,* **32**, 24-24.

Sabu, A.; Kiran, G. and Pandey, A. (2005), Purification and characterization of tannin acyl hydrolase from *Aspergillus niger* ATCC 16620. *Food Technol. Biotechnol.,* **43**, 133-138.

Sharma, S.; Bhat, T.K. and Dawra, R.K. (2000), A spectrophotometric method for assay of tannase using rhodanine. *Anal. Biochem.,* **279**, 85-89.

Sharma, S. and Gupta, M.N. (2003), Synthesis of antioxidant propyl gallate using tannase from *Aspergilus niger* van Teighem in nonaqueous media. *Bioorganic and Medicinal Chemistry Letters,* **13**, 395-397.

Souza, C.G.M.; Simão, R.C. and Peralta, R.M. (1997), Effect of pH on the production, activity and stability of xylanases by *Aspergillus tamarii. Braz. Arch. Biol. Technol.,* **44**, 809-815.