Food Sci. Biotechnol. Vol. 16, No. 2, pp. 243 ~ 248 (2007)

Food Science and Biotechnology

© The Korean Society of Food Science and Technology

Biochemical Characterization of Tannases from *Paecilomyces variotii* and *Aspergillus niger*

Vania Battestin, Gustavo Adolfo Saavedra Pinto[1], and Gabriela Alves Macedo[1*]

Food Science Department, Faculty of Food Engineering, Campinas State University, P.O. Box 6121, 13083-862, SP, Brazil

[1]Embrapa Agroindustrial Tropical, 60511-110, Fortaleza, CE, Brazil

Abstract A biochemical characterization of the tannases from *Paecilomyces variotii* (produced at Unicamp), *Aspergillus niger* (purchased from Industrial Kerry Bio-Science) and *A. niger* cnpat 001 (purchased from Embrapa Agroindustrial Tropical-Brazil) was carried out. *P. variotii* is a new strain obtained from the screening of 500 fungi that were tested for their production of tannase. The biochemical properties of this new tannase from *P. variotii* were determined and compared with those of two other tannase preparations. The tannase produced from *P. variotii* showed optimum activity at pH 6.5. The functional temperature range of the tannases was from 20-70°C, with optima at 70°C for *P. variotii* and at 60°C for the commercially obtained tannase, whereas *A. niger* cnpat 001 showed optimum activity at 40°C. The effects of 1 mM preparations of cations and anions, inhibitors, surfactants, and chelators on the tannase activity from *P. variotii* were also studied.

Keywords: Biochemical characterization, tannase, *Paecilomyces variotii*, residue, fermentation

Introduction

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an inducible enzyme that catalyses the breakdown of ester linkages in hydrolysable tannins such as tannic acid, resulting in glucose and gallic acid. Tannase is an extra cellular enzyme produced in the presence of tannic acid by fungi, bacteria, and yeast (1-4). The first step in the development of microbial enzyme production is the lineage selection. Extra cellular enzymes were preferred because they are easily extracted and do not require expensive extraction methods. Studies on the production of tannase using solid, liquid, and submerged fermentation have been reported. Solid-state fermentation provides several advantages over conventional enzyme production processes (5). Tannic acid is a hydrolysable polyphenol which is found, along with other condensed tannins. It is most commonly found in the bark and fruits of many plants, in several beverages such as red wine and green tea, and has a structure consisting of many galloyl groups (6).

The fermentation broth can use by-products such as wheat bran, rice bran, sugar beet pulp, fruit pulps, banana waste, cassava waste and coffee residues, adding tannic acid. The use of by products or residues rich in sources of carbon for fermentation purposes is an alternative way of solving pollution problems that can be caused by incorrect disposal in the environment (7). In the present work, a *Paecilomyces variotii* lineage obtained by fungal isolation procedures was used for the production of tannase using coffee husk and wheat bran residues.

Tannases find widespread application in food and beverage processing. The enzyme is extensively used in wine, beer, and coffee-flavored soft drinks or as an additive for food detanification. Gallic acid is also used in the enzymatic synthesis of propyl gallate, which is mainly used as an antioxidant in fats and oils, as well as in beverages and trimethoprim in the pharmaceutical industry (8, 9).

There are several reports on the production of tannase but there are few reports on the characterization, i.e., the effect of additives such as metal ions, cations, anions, surfactants, and chelators on the enzyme tannase. This paper reports on the determinations of pH and temperature optima and stabilities of tannases from the newly isolated strain *P. variotii, Aspergillus niger*, and *A. niger* cnpat 001. The effects of cations, anions, inhibitors, chelators, and surfactants on the tannase activity of *P. variotii* were also determined.

*Corresponding author: Tel: 55-19-3788-2175; Fax: 55-19-3289-1513
E-mail: gmacedo@fea.unicamp.br
Received September 5, 2006; accepted January 29, 2007

Material and Methods

Screening and microorganism Five hundred fungal cultures were obtained from the departmental stock culture collection of the Food Science Department-Unicamp and were screened for their tannase producing ability. The best tannase producing fungus was identified as *P. variotii.* The tannin acyl hydrolases studied were obtained from the three strains, *P. variotii* (produced at Unicamp), *A. niger* (purchased tannase from Kerry Bio-Science Rochester, MN, USA – this is an enzyme system containing pectinase, tannase, and esterase activities), *A. niger* cnpat 001 (purchased tannase from Embrapa Tropical Agroindustry, Brazil).

Chemicals All the chemicals were of analytical grade. Tannic acid was from N.V. Ajinomoto OmniChem S.A. (Lourain-la Neuve, Belgium).

Microorganism preservation and preparation of the pre-inoculum A *P. variotii* strain obtained using fungal

isolation procedures was used for the production of tannase. The strain was maintained in potato dextrose agar (PDA, Acumedia Manufactures Inc., Lansing, MI, USA) slants, stored at 4°C. The lineage was replicated in PDA containing 0.2%(w/v) of tannic acid and incubated at 30°C for 72 hr. The pre-inoculum was prepared by adding 2.5 mL of distilled water to remove the spores, obtaining a suspension containing 5.0×10^7 spores/mL.

Fermentation media For the fermentation process, a 250 mL conical flask was used containing the following constituents: 5 g of wheat bran and 5 g of coffee husk, 10 mL of distilled water and 10%(w/w) of tannic acid. The culture medium (pH 5.7) was sterilized at 120°C for 20 min and the relative humidity of the medium after sterilization remained at 60%(w.b.). After sterilization, the flasks were inoculated with 2.5 mL of the pre-inoculum suspension and incubated at 30°C for 120 hr. After fermentation, 80 mL of 20 mM acetate buffer, pH 5.0 were added and shaken at 200 rpm for 1 hr. The solution was filtered and centrifuged at 9,650×g for 30 min at 4°C (Centrifuge Beckman J2-21; Beckman-Coulter Inc., Fullerton, CA, USA). The supernatant was then treated with solid ammonium sulphate (80% saturation) and allowed to stand overnight at 4°C. The precipitate was collected by centrifugation (9,650×g, 30 min), dissolved in distilled water and dialysed (using cellulose dialysis tubing - size 43×27 mm, D9527-30 m; Sigma-Aldrich, St. Louis, MO, USA) against distilled water. The dialysed preparation was used as crude tannase.

Determination of tannase activity A colorimetric assay was used to determine tannase activity, based on measuring the residual tannic acid content after the enzymatic reaction (10). The reaction mixture consisted of 0.3 mL of the substrate tannic acid (0.7%, w/v) in 0.2 M acetate buffer at pH 5.5) and 0.5 mL of the enzyme extract, incubating at 60°C for 10 min. The enzymatic reaction was paralysed by the addition of 3 mL of a bovine serum albumin solution (BSA, 1 mg/mL), leading to the precipitation of the remaining tannic acid. The tubes were then centrifuged at 9,650×g for 15 min at 4°C and the precipitate dissolved in 3 mL of SDS-triethanolamine, followed by the addition of 1 mL of $FeCl_3$ reagent and holding for 15 min for colour stabilization. The absorbance was measured at 530 nm and the enzyme activity calculated from the change in absorbance at 530 nm. One unit of tannase activity was defined as the amount of tannic acid hydrolysed by 1 mL of enzyme per min of reaction): $Abs_{530} = Abs_{control} - Abs_{test}$.

Biochemical characterisation of the tannases The determinations of the pH and temperature optima, and stabilities were used for the biochemical characterisations of the tannase from *P. variotii*, the commercially obtained tannase from *A. niger* and the tannase from *A. niger* cnpat 001. The effects of cations, anions, inhibitors, chelators, and surfactants on the tannase activity of *P. variotii* were also determined.

pH optimum The optimum and the pH range for tannase activity were determined by testing the enzyme activity in different buffers for a pH range from 3.5 to 9.0. Acetate buffer (0.2 M) was used for the range from 3.5 to 5.5, phosphate buffer (0.2 M) for pH 6.0 to 8.0 and Tris-HCl buffer (0.2 M) for pH 8.5 to 9.0 for 10 min.

Temperature optimum The optimum temperature and the temperature range for tannase activity were determined by carrying out the reaction at different temperatures ranging from 20 to 90°C (at regular intervals of 10°C).

pH stability The stability of the enzyme was examined at different pH values by incubating the enzyme in buffers at different pH values ranging from 3.5 to 9.0 for 24 hr at 30°C. The residual activity was estimated and expressed as a percentage of the relative tannase activity.

Temperature stability The heat stability of the enzymes was examined by incubating the test sample at different temperatures ranging from 20 to 100°C for 30 min. Residual activity was estimated and expressed as a percentage of the relative tannase activity. All the above tests were carried out in duplicate.

Results and Discussion

pH optima and stability The tannase produced by *P. variotii* showed optimum activity at pH 6.5, whereas *A.*

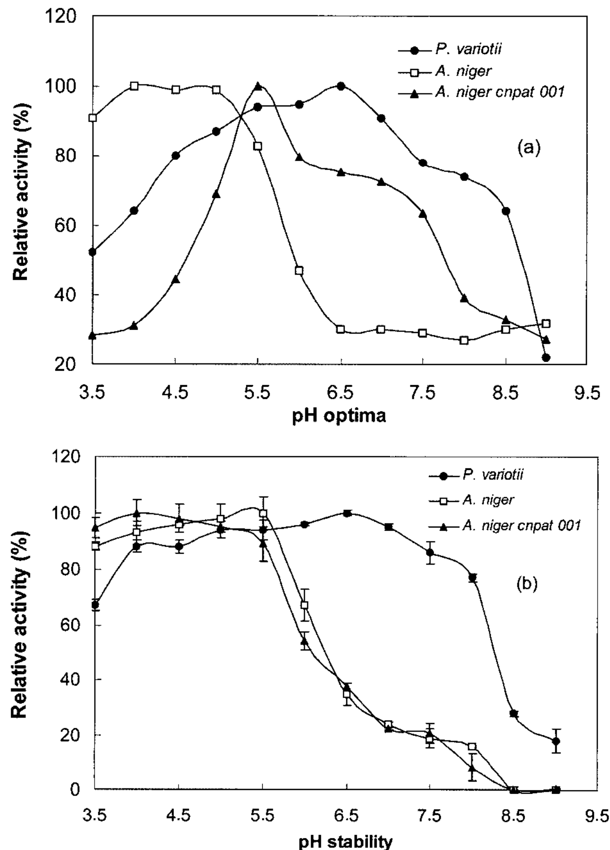

Fig. 1. pH optima (a) and pH stability (b) of tannases from *Paecilomyces variotii*, *Aspergillus niger*, and *Aspergillus niger* cnpat 001.

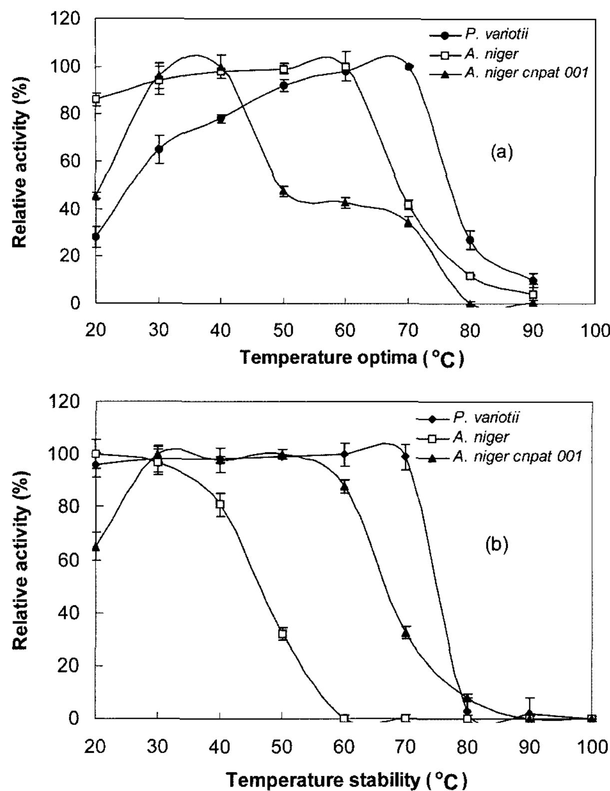

Fig. 2. Temperature optima (a) and temperature stability (b) of tannases from *Paecilomyces variotii*, *Aspergillus niger*, and *Aspergillus niger* cnpat 001.

niger, and *A. niger* cnpat 001 showed pH optima at 4.0 and 5.5 (Fig. 1). These results are in agreement with earlier reports in which a pH optimum of 5.0–6.0 was reported for *A. niger* (11), *Penicillium chrysogenum* (12), and *Aspergillus caespitosum*, *Penicillium crustosum*, and *Penicillium variable* (3). The tannases produced by *Aspergillus versicolor*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Penicillium restricticum* showed optimal tannase activity at pH 5.0 (3). Tannase from *P. variotii* showed 100% stability at pH 6.5 and 88 and 86% stability, respectively, at pH 4.0 and 7.5 after 24 hr of incubation. This enzyme showed a wide range of pH stability, was moderately active at an alkaline pH of 8.0 (retaining 77% activity) and at an acidic pH of 3.5 (retaining 67% activity). In contrast, the tannase produced by the commercially obtained *A. niger* and that produced by *A. niger* cnpat 001 showed 100% activity at pH 5.5 and 4.0 respectively. These enzymes were active at an acidic pH 3.5 (retaining 88 and 95% activity) and at an alkaline pH of 8.0 (retaining 16 and 8.0% activity respectively) (Fig. 1). Similar results were reported for *A. niger* LCF 8 (11), *Candida sp.* (13), *A. oryzae* (14), and *P. restricticum* (3), where the tannases were found to be stable in the pH range from 3.5 to 8.0 for 16 and 24 hr. On the other hand, tannases produced by *A. flavus* IFO5839 (15), *P. chrysogenum* (12), *A. flavus* and *Penicillium charlesii* (3) were only stable in a narrow pH range of from 5.0-5.5 and a wider pH range of 4.5-6.0, 3.0-6.0, and 4.0-5.0 respectively, for 30-60 min.

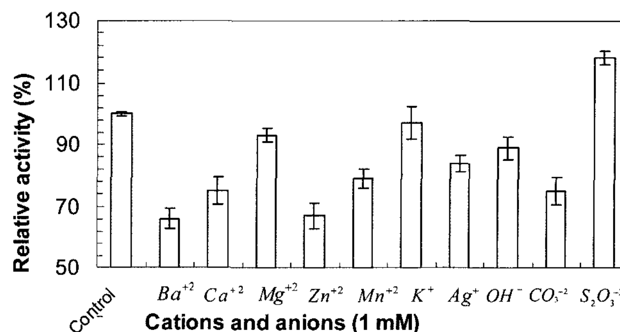

Fig. 3. Effect of cations and anions on tannase activity.

Temperature optima and stability The functional temperature range of the tannases produced was 20-70°C with optima at 70°C for *P. variotii*, 60°C for the tannase obtained commercially (produced from *A. niger*) and 40°C for that from *A. niger* cnpat 001 (Fig. 2). These results are also in agreement with previous reports concerning *A. flavus* (15), *A. niger* van Tieghem (16), and *A.* PKL 104 (5), where the temperature optima were 50, 60, and 70°C, respectively. However, lower temperature optima of 40°C have also been reported for *A. caespitosum*, *P. charlesii*, *P. crustosum*, and *P. restrictum* (3), and of 30°C for *A. oryzae* (14), *A. niger* (11), and *P. chrysogenum* (12).

The tannase from *P. variotii* was stable in a temperature range from 20-70°C where it retained 96 and 99% residual activity at 20 and 90°C respectively, with maximum stability at 60°C, retaining 100% activity after 30 min. The tannase obtained commercially (produced from *A. niger*) showed maximum stability at 20°C, but 81 and 32% residual activity was observed at 40 and 50°C. This tannase was relatively less stable at higher temperatures. The tannase from *A. niger* cnpat 001 was stable in a temperature range of 20-60°C, showed 100% stability at 30°C, and 65 and 88% residual activity was observed at 20 and 60°C respectively (Fig. 2). Similarly, the tannases from *A. niger* (15) and *A. fumigatus* (3) have been reported to be stable at 60°C for 30 and 60 min respectively. Tannases from *P. charlesii* and *P. crustosum* retained 100% activity at 40 and 50°C respectively. *A. caespitosum* tannase retained 100 and 92% residual activity at 40 and 50°C, but retained only 46 and 18% residual activity at 60 and 70°C, respectively (3).

The experimental properties of the commercial tannase preparation were in agreement with the manufacturer's specifications: pH stability between 4.0-5.0; temperature optima around 40-50°C and maximum stability at 30-50°C for 4 hr.

Effect of monovalent and divalent cations on tannase activity The influence of various compounds, including $BaCl_2$, $CaCl_2$, $MgSO_4$, $ZnSO_4$, $MnSO_4$, K_2SO_4, $AgNO_3$, NaOH, $CaCO_3$, and $Na_2S_2O_3$ on tannase activity was studied (Fig. 3). Samples of 0.12%(v/v) solutions of the enzyme containing 1 mM of the compounds were incubated at 60°C for 10 min. The tannase assays were then carried out following the procedure described by Mondal *et al.* (10).

Of the monovalent and divalent cations studied Ba^{+2},

Ca^{+2}, Zn^{+2}, Mn^{+2}, and Ag^{+} acted as inhibitors of the tannase activity produced by *P. variotii.* Kar (17) and Brado (18) reported similar results, where Ba^{+2}, Ca^{+2}, Zn^{+2}, Mn^{+2}, and Ag^{+} inhibited tannase activity at concentrations of 1 mM and 0.2% respectively. In contrast, Mg^{+2} stimulated the maximum tannase activity at concentrations of 1 and 5 mM (17, 19). The inhibitory effect of metal ions is well documented in the literature. It is known that mercury ions react with protein thiol groups (converting them to mercaptides) and also react with histidine and tryptophan residues. Moreover the disulphide bond can also be hydrolytically degraded by the action of silver and mercury. Furthermore, the decreased activity in the presence of divalent cations could be due to the non-specific binding or aggregation of the enzyme (17). The tannases from *A. oryzae* (14) and *P. chrysogenum* (12) were greatly inhibited by Zn^{+2}, Cu^{+2}, and Fe^{+2}.

Effect of monovalent and divalent anions on tannase activity Anions also play an important role in inducing enzyme activity. Figure 3 shows the effect of OH^{-}, CO_3^{-2}, and $S_2O_3^{-2}$ on tannase activity. Of these anions, only $S_2O_3^{-2}$ induced tannase activity at a concentration of 1 mM. Kar *et al.* (17) observed similar results, with $S_2O_3^{-2}$ enhancing tannase activity and CO_3^{-2} inhibiting tannase activity at a concentration of 1 mM. At a concentration of 10 mM, CO_3^{-2} inhibited about 70% of D-sorbitol dehydrogenase activity (20). Inhibition studies primarily provide an insight into the nature of the enzymes, their cofactor requirements and the nature of the active enzyme (17).

Effect of inhibitors on tannase activity In the enzyme industry, the main importance of inhibitors is that they reduce the efficiency of the enzyme reaction (17). The inhibitors evaluated for their effects on tannase activity were sodium bisulphite, iodoacetamide, 2-mercaptoethanol, 4-aminobenzoic acid, sodium azide, *n*-bromosuccinimide and cysteine. Tannase activity was inhibited by sodium bisulphite, 2-mercaptoethanol, 4-aminobenzoic acid, sodium azide, *n*-bromosuccinimide and cysteine at a concentration of 1 mM (Fig. 4). The tannase from *A. niger* was reported to be inactivated by 2-mercaptoethanol (11, 21). N-Bromosuccinimide strongly inhibited the enzyme activity of the 5-Oxo-L-prolinase from *Alcaligenes sp.* F-137 (19). Sodium azide completely inhibited tannase activity (17) but, in contrast, it increased the enzyme activity of the sorbitol dehydrogenase produced by *Candida boidinii* (20). When added to the reaction medium, cysteine inhibited the tannase activity of *P. variotii.* The inhibition of tannase activity by cysteine and 2-mercaptoethanol suggests the present of sulphur containing amino acids at the active site of the enzyme. Inhibition by *n*-bromosuccinimide indicated that tryptophan residues played an important role in maintaining the active conformation of the enzyme (22, 23). Inhibition studies primarily provide an insight into the nature of the enzyme, its cofactor requirements and the nature of the active enzyme (24).

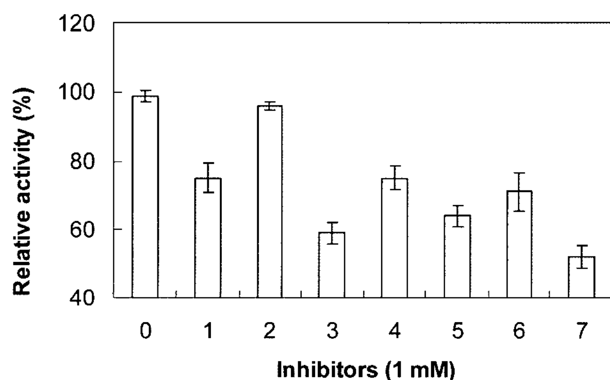

Fig. 4. Effect of inhibitors on tannase activity. 0) Control; 1) sodium bisulphite; 2) iodoacetamide; 3) 2-mercaptoethanol; 4) 4-aminobenzoic acid; 5) sodium azide; 6) *n*-bromosuccinimide; 7) cysteine.

Effect of chelator on tannase activity The chelator ethylene diamine tetra acetic acid disodium salt (EDTA disodium salt) at a concentration 1 mM, inhibited the tannase from *P. variotii* (Table 1). The tannase from *A. niger* was inactivated by EDTA (11, 17) and the tannase from *A. oryzae* was completely inactivated by EDTA (14), whereas no inhibition by EDTA was observed in the case of the tannase from *A. flavus* (15). Yeast tannase was also not inhibited by EDTA (13). On the other hand, this chelator has been shown to enhance the protopectinase activity from *Bacillus subtilis* at a concentration of 1 mM (25). The decrease detected in the presence of EDTA could be due to its influence on the interfacial area between the substrate and enzyme (26, 27).

Effect of surfactants on crude tannase activity The effects of chemical substances on the activity of an enzyme are often precise and specific. In the present study, surfactants and chelators were chosen for an evaluation of their effects on tannase activity. The effects of Tween 80, Tween 20, and Triton X-100 (0.25-1%, v/v) were studied, using enzyme solutions containing 0.12%(v/v) and the above chemical substances at the concentrations mentioned.

Tween 80 and Tween 20 caused a decrease in tannase activity at concentrations of 0.025, 0.5, and 1%(v/v) (Table 2). Tween 80 is predominantly composed of oleic acid (70 %). Tween 20 consists of lauric acid. Due to the predominance of oleic acid and lauric acid in Tween 80 and Tween 20, they cause a decrease in tannase activity. Similarly, Tween 80 (1%, v/v) caused an inhibition of the lipase activity from *Pseudomonas sp.* KWI-56 (28), and Tween 60 at 0.05-1.0%(v/v) and another anionic surfactant, SLS, at 0.05-0.7%, caused inhibition of tannase activity (17). This inhibition may be the result of a combined effect of factors such as the reduction in the hydrophobic interactions that play a crucial role in holding together the tertiary protein structure, and a direct interaction with the protein molecule (17).

Triton X-100 caused a decrease in tannase activity at

Table 1. Effect of chelator on tannase activity

Chelator	Concentration	Relative activity (%)
Control	-	100±2.67
EDTA	1 mM	62±3.59

Table 2. Effect of Tween 80, Tween 20, and Triton X-100 on tannase activity

Additives	Concentration (%, v/v)	Relative activity (%)
Control	-	100±0.64
Tween 80	0.25	85±1.83
	0.5	77±1.53
	1.0	70±1.84
Tween 20	0.25	81±2.12
	0.5	75±2.44
	1.0	66±0.91
Triton X-100	0.25	96±0.31
	0.5	93±0.61
	1.0	87±0.62

concentrations of 0.5 and 1%(v/v). These results are in agreement with those of Kar *et al.* (17), who used Triton X-100 at concentrations of 0.03-0.5%(v/v) and showed a reduction in tannase activity. Triton X-100 at a concentration of 0.1%(v/v) had a significant effect on the lipase activity of *Pseudomonas sp.* KWI-56 (28). In contrast, Triton X-100 did not significantly affect the α-amylase activity of the *Bacillus* strain GM 8901 (29). The extent of stimulation by surfactants varies for the different enzymes (30).

In most countries where the economy is largely based on agriculture and where farming practice is very intensive, accumulation of agricultural residues is a serious problem. The presence of tannins and their derivatives in agro residues is a major hurdle in their utilization as feed material. Solid-state fermentation technology using non-pathogenic microorganisms that can produce hydrolytic enzymes such as tannases would be advantageous for the proper utilization of these residues. Our isolate, identified as *P. variotii*, was able to grow in media containing a mixed substrate including coffee husk and wheat bran residues. Wheat bran is a good substrate for tannase production and coffee husk is a highly available, economically viable agro-industrial residue in Brazil.

The biotechnological use of agricultural residues is becoming more and more significant, with the dual goal of waste disposal and value addition. Since microbial activity, especially fungal activity, is the key aspect in this area, there is enormous opportunity for the cost effective production of tannase. So far, few reports are available on tannase production from *P. variotii*. Many organisms have been reported to be capable of degrading tannins, but their efficiency was low and they could only use low tannin concentrations.

This work allowed for a better understanding of the effects of temperature, pH, cations, anions, and inhibitors on the tannase activity of *P. variotii*, presenting important data from a newly-isolated fungus that produces an interesting tannase. The biochemical properties of this new tannase produced by *P. variotii* were determined and compared with those of two other tannase preparations. The tannase from *P. variotii* was shown to be more stable under extreme values of pH and temperature. Since the last decades many enzymes have gained great attention because they are capable of catalyzing reactions under extreme conditions of high temperature (31). The tannase produced was functional at a wide range of temperature and pH values. Our inhibitory reagent studies suggest the presence of sulphur containing amino acids at the active site and also tryptophan residues. These properties can be further exploited in developing tannase for a wider range of applications in the pharmaceutical, food, feed, and leather industries and thus their production at higher levels should be sought for. Therefore, *P. variotii* would provide a new source for the efficient production of tannase for industrial applications.

Acknowledgments

This work was supported by CAPES. The authors are grateful to Embrapa Agroindustrial Tropical and Kerry Bio-Science.

References

1. Saxena S, Saxena RK. Statistical optimization of tannase production from *Penicillium variable* using fruits (chebulic myrobalan) of *Terminalia chebula*. Biotechnol. Appl. Bio. 39: 99-106 (2004).
2. Zhong X, Peng L, Zheng S, Sun Zhizhi, Ren Y, Dong M, Xu A. Secretion, purification, and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*. Protein Expres. Purif. 36: 165-169 (2004).
3. Batra A, Saxena RK. Potential tannase producers from the genera *Aspergillus* and *Penicillium*. Process Biochem. 40: 1553-1557 (2005).
4. Van de Lagemaat J, Pyle DL. Modelling the uptake and growth kinetics of *Penicillium glabrum* in a tannic acid-containing solid-state fermentation for tannase production. Process Biochem. 40: 1773-1782 (2005).
5. Lekha PK, Lonsane BK. Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface, and submerged fermentations. Process Biochem. 29: 497-503 (1994).
6. Choi JM, Han J, Yoon BS, Chng JH, Shin DB, Lee SK, Hwang JK, Ryang R. Antioxidant properties of tannic acid its inhibitors effects on paraquat-induced oxidative stress in mice. Food Sci. Biotechnol. 15: 728-734 (2006).
7. Battestin V, Macedo G, Pastore P. Otimizing the fermentation broth for tannase production by a new isolated strain *Paecilomyces variotii*. abstract no. 118:S49. In: Abstracts: 12th European Congress in Biotechnology. J. Technol. Copenhagen, Denmark (2005)
8. Vaquero I, Marcobal A, Munos R. Tannase activity by lactic acid bacteria isolated from grape must and wine. Intern. J. Food Microbiol. 96: 199-204 (2004).
9. Belmares R, Contreras-Esquival JC, Rodriguez-Herrera R, Coronel AR, Aguilar CN. Microbial production of tannase: an enzyme with potential use in food industry. Lebensm.-Wiss. Technol. 37: 857-864 (2004).
10. Mondal KC, Banerjee D, Jana M, Pati BR. Colorimetric assay method for determination of the tannin acyl hydrolase activity. Anal. Biochem. 295: 168-171 (2001).
11. Barthomeuf C, Regerat F, Pouratt H. Production, purification, and characterization of tannase from *Aspergillus niger* LCF 8. J. Ferment. Technol. 77: 320-323 (1994).
12. Rajkumar S, Nandy SC. Isolation, purification, and some properties of *Penicilliun chrysogenum* tannase. Appl. Envirom. Microb. 46: 525-527 (1983).
13. Aoki K, Shinke H, Nishira H. Purification and some properties of yeast tannase. Agr. Biol. Chem. Tokyo 40: 79-85 (1976).
14. Iibuchi S, Minoda Y, Yamada S. Studies on tannin acyl hydrolase of microorganisms. Part III. Purification of the enzyme and some

properties of it. Agr. Biol. Chem. Tokyo 32: 803-809 (1968).
15. Yamada H, Adachi O, Watanbe M, Sato N. Studies on fungal tannase. Fomation, purification, and catalytic properties of tannase of *Aspergillus flavus*. Agr. Biol. Chem. Tokyo 32: 1070-1078 (1968).
16. Sharma S, Bhat TK, Dawra RK. Isolation, purification, and properties of tannase from *Aspergillus niger* van Thieghem. World J. Microb. Biot. 15: 673-677 (1999).
17. Kar B, Banerjee R, Bhattacharyya BM. Effect of additives on the behavioural properties of tannin acyl hydrolase. Process Biochem. 38: 1285-1293 (2003).
18. Bradoo S, Gupta R, Saxena RK. Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus*. Process Biochem. 32: 135-139 (1997).
19. Koyama H. Purification and characterization of 5-Oxo-L-prolinase (L-pyroglutamate hydrolase) from *Alcaligenes sp.* F-137. Agr. Biol. Chem. Tokyo 52: 735-741 (1988).
20. Vongsuvanlert V, Tani Y. Characterization of D-sorbitol dehydrogenase involved in D-sorbitol production of a methonol yeast, *Candida boidinii* (Kloeckera sp.) no. 2201. Agr. Biol. Chem. Tokyo 52: 419-426 (1988).
21. Aguilar CN, Gutiérrez-Sánchez G. Review: Sources, properties, applications and potential uses of tannin acyl hydrolase. Food Sci. Technol. Int. 7: 373-382 (2001).
22. Saxena RK, Davidson WS, Sheoran A, Giri B. Purification and characterization of an alkalibe thermostable lipase from *Aspergillus carneus*. Process Biochem. 39: 239-247 (2003).
23. Whitaker JR. Principles of enzymology for food sciences. pp. 255-282. In: Enzyme Inhibitors. Oxford University Press, New York, NY, USA (1972).
24. Sigma DS, Mooser G. Chemical studies of enzyme active sites. Annu. Rev. Biochem. 44: 889-931 (1975).
25. Sakai T, Sakamoto T. Purification and some properties of a protopectin-solubilizing enzyme that has potent activity on sugar beet protopectin. Agr. Biol. Chem. Tokyo 54: 879-809 (1990).
26. Lopes MFS, Leitao AL, Regalla M, Marques JJF, Carrondo MJT, Crespo MTB. Characterization of a highly thermostable extracellular lipase from *Lactobacillus plantarum*. Int. J. Food Microbiol. 76: 107-115 (2002).
27. Jinwal UK, Roy U, Chowdhury AR, Bhaduri AP, Roy PK. Purification and characterization of an alkaline lipase from a newly isolated *Pseudomonas mendocina* PK-12CS and chemoselective hydrolysis of fatty acid ester. Bioorg. Med. Chem. 11: 1041-1046 (2003).
28. Iizumi T, Nakamura K, Fukase T. Purification and characterization of a thermostable lipase from newly isolated *Pseudomonas sp.* KWI-56. Agr. Biol. Chem. Tokyo 54: 1253-1258 (1990).
29. Kim TU, Gu BG, Jeong JY, Byun SM, Shin YC. Purification and characterization of a maltotetraose-forming alkaline α-amylase from an alkalophilic Bacillus strain GM8901. Appl. Environ. Microb. 61: 3105-3112 (1995).
30. Reese ET, Maguire A. Surfactants as stimulants of enzymes production by microorganisms. Appl. Environ. Microb. 17: 242-245 (1969).
31. Njoroge RN, Li D, Park JT, Cha H, Kim MS, Kim JW. Characterization and application of a novel thermostable glucoamylase cloned from a hyperthermophilic Archaeon *Sulfolobus*. Food Sci. Biotechnol. 14: 860-865 (2005).