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RESEARCH ARTICLE

Effects of temperature, pH and additives on the activity of tannase produced by *Paecilomyces variotii*

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Abbreviations: DEAE: Diethylaminoethyl
EDTA: Ethylene diamine tetra acetic acid
PDA: Potato dextrose agar
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

A biochemical characterization of the tannase from a *Paecilomyces variotii* strain isolated in São Paulo, Brazil was carried out. *Paecilomyces variotii* is a strain obtained from the screening of five hundred fungi that were tested for their production of tannase. The enzyme produced was partially purified using ammonium sulfate precipitation followed by ion exchange chromatography, diethylaminoethyl (DEAE)-Sepharose. Effects of temperature and pH on the activity of crude tannase crude and purified tannase was studied. K_m was found to be 0.61 µmol and V_{max} = 0.55 U/mL. Temperature of 40 to 65ºC and pH 4.5 to 6.5 were optimum for tannase activity and stability; it could find potential use in the food-processing industry. The effects of different inhibitors, surfactants and chelators on the enzyme activity were also studied.

Tannin acyl hydrolase, commonly referred to as tannase (E.C: 3.1.1.20), is an enzyme that cleaves ester linkages in hydrolysable tannins (Banerjee et al. 2001; Belmares et al. 2004), producing glucose and gallic acid (Banerjee et al. 2005). Tannase is an extracellular, inducible enzyme produced in the presence of tannic acid by fungi, bacteria and yeast (Aguilar and Gutiérrez-Sanchez, 2001). The first step in the development of microbial enzyme production is the lineage selection. Extracellular 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 submersed fermentation have been reported (Lekha and Lonsane, 1997) duction processes (Van de Lagemaat and Pyle, 2001). 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 (Battestin et al. 2005). 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.

Tannase is extensively used in wine, beer and coffee-flavoured soft drinks or as an additive in 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 (Belmares et al. 2004; Vaquero et al. 2004; Yu et al. 2004). Usually the end products of a fermentation process contains some unwanted components, which have to be eliminated as far as possible by

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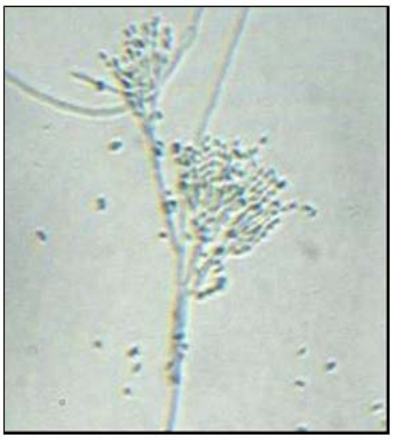

Figure 1. Optical microscopic image of *Paecilomyces variotii* at 1000x.

downstream processing (Mukherjee and Banerjee, 2006). Purification and characterization of tannase has been attempted earlier owing to its wide applications in various food, feed, leather and pharmaceutical industries. Various media preparations can be used with tannic acid as the sole carbon source for production of microbial tannase but biotransformation of tannin rich agro residue is cost-effective (Mukherjee and Banerjee, 2006).

This paper reports on the determinations of pH, temperature optima and stabilities of crude and partially purified tannase from the isolated strain *Paecilomyces variotii*. The effects of inhibitors, chelators and surfactants on the crude tannase activity were also determined.

MATERIALS AND METHODS

Screening and microorganism

Paecilomyces variotii is a lineage obtained by means of fungal isolation procedures and was used for the tannase production in coffee husk and wheat bran residues. Five hundred fungal cultures were obtained from the departmental stock culture collection (from Food Science Department-Unicamp), collected from different places in Sao Paulo State (Brazil) and screened for their tannase producing ability under induction by tannic acid. The best tannase producing fungus was identified as *Paecilomyces variotii*.

Chemicals

All the chemicals were of analytical grade. Tannic acid was from Ajinomoto OmniChem Division.

Microorganism preservation and preparation of the pre-inoculum

The strain was maintained in potato dextrose agar (PDA) slants, stored at 4ºC. The lineage was replicated in PDA containing 0.2% (w/v) of tannic acid and incubated at 30ºC for 72 hrs. The pre-inoculum was prepared by adding 2.5 mL of distilled water to remove the spores, obtaining a suspension containing 5.0 x 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% of tannic acid (w/w). The culture medium (pH 5.7) was sterilized at 120ºC for 20 min and the relative humidity of the medium after sterilization remained at 60% (WB). After sterilization, the flasks were inoculated with 2.5 mL of the pre-inoculum suspension and incubated at 30ºC for 120 hrs. 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 10000 rpm for 30 min at 4ºC (Centrifuge Beckman J2-21, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was than treated with solid ammonium sulphate (80% saturation) and allowed to stand overnight at 4ºC. The precipitate was collected by centrifugation (10000 rpm - 30 min), dissolved in distilled water and dialysed against

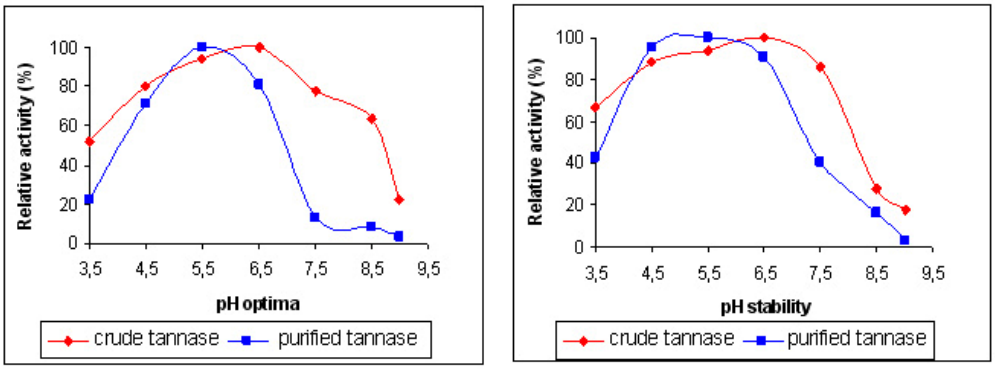

Figure 2. pH optima and pH stability of tannase from *Paecilomyces variotii.*

distilled water. The dialysed preparation was used as crude tannase.

Partial purification procedure

Ammonium sulphate Fractionation and dialysis. Ammonium sulphate was added to the supernatant to give a final concentration of 80% saturation. The ammonium sulphate was added with constant stirring at 4ºC and the mixture stood overnight at 4ºC. The precipitated proteins were separated by centrifugation at 10000 rpm at 5ºC for 30 min. The separated proteins were then re-suspended in a minimum amount of distilled water and the solution dialyzed (using cellulose dialysis tubing - Sigma) for 24 hrs against distilled water and concentrated by freeze-drying.

Anion-exchange chromatography (FPLC) on a DEAE sepharose column. The partially purified enzyme was dissolved in acetate buffer (20 mM - pH 6.0) and passed through a diethylaminoethyl (DEAE) Sepharose column (0.7 x 2.5 cm) equilibrated with the same buffer. The solution was passed through the column at a flow rate of 1 $mL.mim^{-1}$ with acetate buffer (20 mM - pH 6.0), followed by a linear gradient from 0-1M NaCl in the acetate buffer. The eluted fractions were collected in an automated fraction collector (Pharmacia Biotech) and the absorbance of the fractions was measured at 280 nm. The major peak fractions were then assayed for tannase activity, and only the fractions possessing tannase activity were pooled.

Molecular mass determination by SDS-PAGE. The properties of the purified tannase were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were detected by Coomassie blue staining and then de-stained using a mixture of methanol, glacial acetic acid and distilled water. The molecular weights of the proteins were determined using the standard protein mixture of 94, 67, 43, 30, 20 and 14 kDa.

Determination of K_m and V_{max}. K_m and V_{max} were determined by plotting velocity against substrate concentration (0.17 - 1.76 µmol-tannic acid [S]). To calculate kinetic constants, data were plotted and fitted directly to the Michaelis-Menten equation. Calculations were also performed by using the linear transform method of Lineweaver and Burk (1934).

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 (Mondal et al. 2001). 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 60ºC 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 than centrifuged at 10000 rpm for 15 min at 4ºC 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 minute of reaction:

$$Abs_{530} = Abs_{control} - Abs_{test}$$

Optimum pH and temperature for crude and purified tannase activity

The optimum pH for tannase activity was determined at 60ºC by incubating the enzyme at different pH (3.5 to 9.0) for 20 min. 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. The optimum temperature was determined by incubating the reaction mixture for 10 min at different temperatures from 20 to 100ºC (at regular intervals of 10ºC).

Optimum pH and temperature for crude and purified tannase stability

The stability of the enzyme was examined at different pH values by incubating the enzyme in buffers at different pH

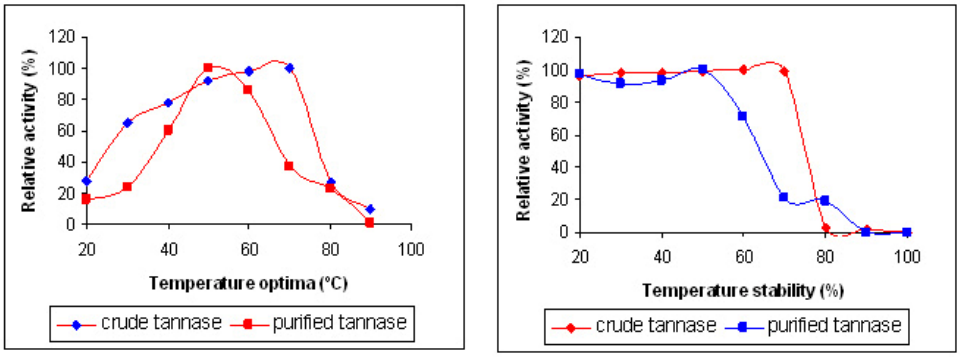

Figure 3. Temperature optima and temperature stability of tannase from *Paecilomyces variotii.*

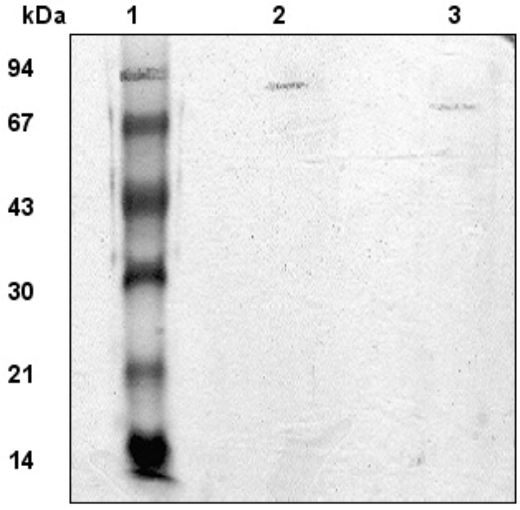

Figure 4. SDS-PAGE of purified tannase from *Paecilomyces variotii*. The lanes contain (1) molecular weight markers, (2) band corresponding to a molecular mass purified sample of 87.3 kDa (major peak) and (3) band corresponding to a molecular mass purified sample of 71.5 kDa (minor peak).

values ranging from 3.5 to 9.0 for 12 hrs at 30ºC. The thermal stability was examined by incubating the test sample at different temperatures ranging from 20 to 90ºC for 30 min. Residual activity was estimated and expressed as percentage of the relative tannase activity.

Effect of inhibitors, chelators and surfactants on crude tannase activity

The effects of inhibitors, chelators and surfactants on the tannase activity of *Paecilomyces variotii* were also determined. The inhibitors evaluated for their effects on tannase activity were sodium bisulphite, iodoacetamide, 2-mercaptoethanol, 4-aminobenzoic acid, sodium azide, n-bromosuccinimide and cysteine at a concentration of 1 mM. The effects of Tween 80, Tween 20 and Triton X-100 (0.025-1% (v/v)) and chelator ethylene diamine tetra acetic acid disodium salt (EDTA disodium salt) at a concentration 1 mM were studied.

RESULTS AND DISCUSSION

Screening

Among the 500 tested strains, 6.75% of the fungi produced the enzyme. The strains that showed the best activities were: LAB345G, LAB53G, and LAB153G. These strains were tested in agro-industrial residues and the best result was obtained using LA153G strain. The best tannase producing fungus was identified as *Paecilomyces variotii* (Figure 1).

pH optima and stability

The crude tannase produced by *Paecilomyces variotii* showed optimum activity at pH 6.5, whereas purified tannase showed pH optima at 5.5 (Figure 2). These results are in agreement with earlier reports by Batra and Saxena (2005) and Mahendran et al. (2006). The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range. Any change in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability. It could be concluded from the results that tannase from the new isolate needed an acidic protein environment to be active, fungal tannase is an acidic protein in general (Mahapatra et al. 2005). The effect of pH on the enzyme activity is determined by the nature of the aminoacids at the active site, which undergoes protonation and deprotonation, and by the conformational changes induced by the ionization of the amino acids. Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum (Sabu et al. 2005).

Crude tannase from *Paecilomyces variotii* showed 100% stability at pH 6.5 and 88% and 86% stability, respectively, at pH 4.0 and 7.5 after 24 hrs of incubation (Figure 2). This enzyme showed a wide range of pH stability. Similar results were reported for *Candida sp* (Aoki et al. 1976) and *Penicilliun restricticum* (Batra and Saxena, 2005).

Temperature optima and stability

The functional temperature range of the tannase produced was 30-70ºC with optima at 50 and 70ºC respectively for purified and crude tannase (Figure 3). These results are also in agreement with previous reports concerning *Aspergillus flavus* (Yamada et al. 1968), *Aspergillus niger* van Tieghem (Sharma et al. 1999) and *Paecilomyces variotii* (Mahendran et al. 2006). However, lower temperature optima of 40ºC have also been reported for *Aspergillus caespitosum*, *Peniccilium charlesii*, *Penicillium crustosum* and *Penicillium restrictum* (Batra and Saxena, 2005).

The crude tannase from *Paecilomyces variotii* was stable in a temperature range from 20-70ºC where it retained 96% and 99% residual activity at 20 and 90ºC respectively. The

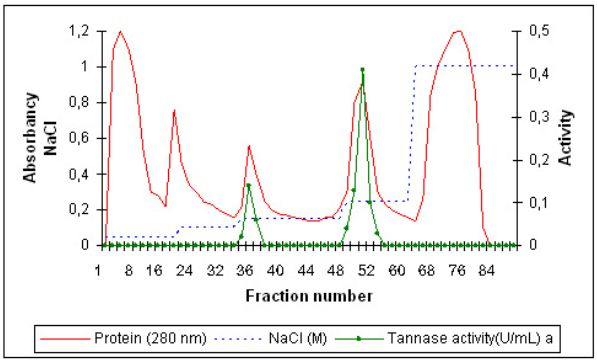

Figure 5. Elution profiles of tannase from *Paecilomyces variotii* using DEAE - Sepharose column chromatography.

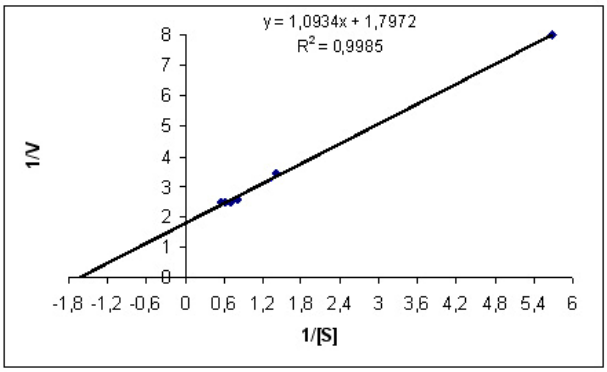

Figure 6. K_m and V_{max} the tannase with tannic acid with substrate.

purified tannase was stable in a temperature range from 20-55ºC (Figure 3). Tannases from *Aspergillus niger* (Yamada et al. 1968) and *Aspergillus fumigatus* (Batra and Saxena, 2005) have been reported to be stable at 60ºC.

The temperature for optimum activity of *Paecilomyces variotii* was 55 and 70ºC for purified and crude tannase respectively. Enzymes with high temperature optimum and thermo stability are preferred for industrial applications. Thus, tannase activity did not increase continuously with a rise in temperature. When the temperature increases, the kinetic energy of the substrate and enzyme molecules also increase which affects the reaction rate. With rise in temperature, the number of collisions per unit time of tannase and its substrate, tannic acid increases, resulting in a higher activity. Beyond the optimum level of temperature, the energy of the molecules increased further. But when the chemical potential energy increases enough, some of the weak bonds determining the three-dimensional shape of the active proteins break leading to thermal denaturation of the tannase protein causing its inactivation. Thus, an increase in temperature beyond the optimum value caused a decrease in the catalytic rate of tannase as either the enzyme or substrate became denatured and inactive. Temperatures above the optimum value also affect the protein ionization state, and the solubility of species in solution, which thus resulted in a reduction in enzyme activity (Mukherjee and Banerjee, 2006).

Purification of tannase

Tannase was produced extracellularly by isolated strain of *Pecilomyces variotii* using solid-state fermentation on wheat bran and coffee husk residues. A typical purification is shown in Table 2. The substrate tannic acid was used to monitor tannase activity throughout the purification procedure. A fractional precipitation with 80% ammonium sulphate removed some of the non-enzymatic proteins and about 34% of the total tannase was recovered (Table 2). The elution profile of the tannase extract obtained from the DEAE sepharose column showed five protein peaks, tannase activity being found in 2 of the peaks (Figure 5). These results agree with those of Beverini and Metche (1990), where a commercial tannase from *Aspergillus oryzae* was purified by affinity chromatography on Con A-Ultrogel and resulted in the separation of two fractions (tannase I and tannase II). The active fractions referring to the fourth peak were pooled and used for studying the biochemical properties of the tannase. DEAE sepharose column chromatography led to an overall purification of 10 fold with a yield of 3% (Table 2), results in agreement with those of Sharma et al. (1999), who purified a tannase from *Aspergillus niger* van Tieghem. The yield of 3% was lower than the value of 7-19% recovery reported by other authors (Rajakumar and Nandy, 1983; Farias et al. 1994). However, the purification factor was similar to that of the purified tannase obtained from various different fungi, as reported by other workers (Rajakumar and Nandy, 1983; Sharma et al. 1999). The molecular mass of the purified enzyme was determined by SDS-PAGE (Figure 4). The purified enzyme migrated as a single protein band corresponding to molecular masses of 87.3 kDa (major peak) and 71.5 kDa (minor peak).

Kinetic constants

To see the effect of substrate concentration on tannase activity, assay was performed at various concentrations of tannic acid. The graphical analysis of the effect of substrate concentration on tannase activity yielded K_m of 0.61 µmol and V_{max} of 0.55 $U.mL^{-1}$ protein (Figure 6). The K_m values for tannase from *Cryphonectria parasitica* using tannic acid as substrate have been found to be 0.94 mM (Farias et al. 1994). The K_m values for tannases from *Selenomonas ruminantium* and *Cryphonectria parasitica* using methyl gallate as substrate have been found to be 1.6 mM (Skene and Brooker, 1995) and 7.49 mM (Farias et al. 1994). This implies that tannase of *Paecilomyces variotii* has higher affinity for tannic acid.

Effect of inhibitors on crude tannase activity

In the enzyme industry, the main importance of inhibitors is that they reduce the efficiency of the enzyme reaction (Kar et al. 2003). 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 (Table 1). The tannase from *Aspergillus niger* was reported to be inactivated by 2-mercaptoethanol (Aguilar and Gutiérrez-Sánchez, 2001). Sodium azide completely inhibited tannase activity (Kar et al. 2003). When added to the reaction medium, cysteine inhibited the tannase activity of *Paecilomyces variotii*. The inhibition of tannase activity bycysteine 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

Table 1. Effect of Tween 80, Tween 20 and Triton X-100, chelator and inhibitors on crude 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
Control	-	100 ± 2.67
EDTA	1 mM	62 ± 3.59
Sodium bisulphite	1 mM	75 ± 4.40
Iodoacetamide	1 mM	96 ± 1.26
2-mercaptoethanol	1 mM	59 ± 3.14
4-aminobenzoic acid	1 mM	75 ± 3.45
Sodium azide	1 mM	64 ± 3.14
N- bromosuccinimide	1 mM	71 ± 5.60
Cysteine	1 mM	52 ± 3.14

played an important role in maintaining the active conformation of the enzyme. Inhibition studies primarily provide an insight into the nature of the enzyme, its cofactor requirements and the nature of the active enzyme (Whitaker, 1972; Saxena et al. 2003).

Effect of chelator on crude tannase activity

The chelator EDTA disodium salt at a concentration 1 mM, inhibited the tannase from *Paecilomyces variotii* (Table 1). The tannase from *Aspergillus niger* was inactivated by EDTA (Kar et al. 2003), whereas no inhibition by EDTA was observed in the case of the tannase from *Aspergillus flavus* (Yamada et al. 1968). Yeast tannase was also not inhibited by EDTA (Aoki et al. 1976). The decrease detected in the presence of EDTA could be due to its influence on the interfacial area between the substrate and enzyme (Silva Lopes et al. 2002; Jinwal et al. 2003).

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 (0.025-1% (v/v)) were studied, using enzyme solutions containing 0.12% (v/v) and the above chemical substances at the concentrations mentioned.

Table 2. Purification of tannase isolated from *Paecilomyces variotii.*

Purification step	Total activity (U)	Total protein (mg)	Specific activity ($U.mg^{-1}$)	Purification (fold)	Yield (%)
Crude enzyme	90	307,60	0,29	1	100
Ammonium sulphate	30,77	54,74	0,56	1,93	34
DEAE-Sepharose	2,7	0,48	5,6	10	3

Tween 80 and Tween 20 caused a decrease in tannase activity at concentrations of 0.025, 0.5% and 1% (v/v) (Table 1). 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 (Kar et al. 2003). 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 (Kar et al. 2003).

Triton X-100 caused a decrease in tannase activity at concentrations of 0.5 and 1% (v/v). These results are in agreement with those of Kar et al. (2003), who used Triton X-100 at concentrations of 0.03 - 0.5% (v/v) and showed a reduction in tannase activity. In contrast, Triton X-100 did not significantly affect the α-amylase activity of the *Bacillus* strain GM 8901. The extent of stimulation by surfactants varies for the different enzymes (Kim et al. 1995).

CONCLUDING REMARKS

In most countries where the economy is largely depend on agriculture and farming practice is 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 *Paecilomyces 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.

This work allowed for a better understanding of the effects of temperature, pH and inhibitors on the tannase activity of *Paecilomyces variotii*, presenting important data from a newly-isolated fungus that produces an interesting tannase. 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. The partial purified tannase from *Paecilomyces variotii* is a unique one with low K_m. 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, *Paecilomyces variotii* would provide a new source for the efficient production of tannase for industrial applications.

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