HAYATI Journal of Biosciences, September 2009, p 95-99 Vol. 16, No. 3
ISSN: 1978-3019

Fractionation and Characterization of Tannin Acyl Hydrolase from *Aspergillus niger*

YUNITA ARIAN SANI ANWAR[1], I MADE ARTIKA[2]*, HASIM DANURI[2]

[1]Study Program of Chemistry Education, Faculty of Teacher Training and Education, University of Mataram, Jalan Majapahit No. 62, Mataram 83120, Indonesia

[2]Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia

Received April 20, 2009/Accepted August 7, 2009

We previously produced tannin acyl hydrolase (tannase) from *Aspergillus niger* isolated from cacao pod. In the present study the enzyme was subjected to fractionation by ammonium sulphate followed by dialysis process. The saturation level of ammonium sulphate used was 30-80% where the best enzyme activity was obtained at the saturation level of 60%. Compared to that of crude enzyme, specific activity of tannase after dialysis was four folds. Characterization results showed that optimum activity was at 35-50°C and pH 6. Tannase was activated by K^+ and Na^+ at concentration of 0.01 and 0.05 M respectively. Mg^{2+} was found activate tannase only at 0.01 M. Addition of metal ions like Zn^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+} and Fe^{2+} inhibited the enzyme activity. Kinetics analysis of various substrates tested showed that the K_m value of tannic acid and gallotannin was 0.401 and 6.611 mM respectively. V_{max} value of tannic acid was 10.804 U/ml and of gallotannin was 12.406 U/ml. Based on Michaelis-Menten constant (K_m), the tannase obtained in the present study was more active in hydrolysing depside bonds rather than ester bonds.

Key words: tannase, fermentation, fractionation, characterization, *Aspergillus niger*

INTRODUCTION

Tannin acyl hydrolase, commonly referred to as tannase (EC 3.1.1.20) is an enzyme that cleaves ester bonds of tannins such as tannic acid releasing glucose and gallic acid. Tannase is extensively used in the manufacture of instant tea and production of gallic acid, and substrate for chemical synthesis of propyl gallate and trimethoprim which have application in the food and pharmaceutical industries. Tannase is also used in fruit juice and wine making and in reduction of antinutritional effects of tannins in animal feed. In Brazil, tannase is used to reduce astringency of cashew apple juice (Pinto *et al*. 2001).

Tannase is an extracellular enzyme produced in the presence of tannic acid by various filamentous fungi, principally *Aspergillus* and *Penicillium*. Other microbes tannase producers are bacteria and yeast. Tannase production in plants has also been reported (Ramirez-Coronel *et al*. 2003; Battestin & Macedo 2007).

Purnama (2004) found that *Aspergillus niger* isolated from cacao pod is able to reduce tannin level up to 79.28%. This fungus was used by Anwar *et al*. (2007) to produce tannase in solid substrate using wheat flour as carbon source and tannic acid as an inducer.

Fungal tannase is glycoprotein with molecular mass between 165-310 kDa. The enzyme has optimum pH of 5-6 and stability pH of around 3.5-8. Optimum temperature of tannase is around 30-40 °C with stability temperature between 30 and 60 °C. There are some tannases with higher optimum temperature (50 to 70 °C). Tannase is inhibited by existence of metal ions like Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, and Mg^{2+}, but the enzyme is activated by K^+. Compounds like O-phenantroline, EDTA, 2-mercaptoethanol, sodium thioglycolate, magnesium sulphate, calcium chloride, and magnesium chloride inhibit the activity of tannase (Sabu *et al*. 2005; Wright 2005). Previous research reported that tannase has been purified from plant and microbial sources. Haslam and Stangroom (1966) purified tannase from *Aspergillus niger* by acetone precipitation followed by alumina chromatography. Sabu *et al*. (2005) reported that specific activity of tannase from ammonium sulphate precipitation at saturation level of 40-60% was higher than that of crude enzyme. Paranthaman *et al*. (2008) found that 60% of ammonium sulphate is suitable for tannase purification to maintain maximum tannase activity. By using ultrafiltration, anion-exchange chromatography, and gel filtration, Marco *et al*. (2009) produced pure tannase with a specific activity of 238 IU/mg protein. From this combination technique they achieved a purification fold of 46. The present study aimed to fractionate and characterize tannase produced by *Aspergillus niger* isolated from cacao pod.

MATERIALS AND METHODS

Maintenance of Culture. A strain of *A. niger* isolated from cacao pod was obtained from stock collection of Engineering and Bioprocess Laboratory, Bogor Agricultural University. Potato dextrose agar (PDA) slants were used for maintenance of *A. niger* with incubation at 28 °C for 6 days. Fully sporulated slants not in used were stored at 4 °C in a refrigerator. The slants were subcultured routinely once every three weeks.

*Corresponding author. Phone/Fax: +62-251-8423267,
E-mail: imart171@yahoo.com

Preparation of Spore Inoculum. Fungal spore inoculum was prepared by adding 10 ml of sterile distilled water containing 0.1% Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic condition and then vortexed with slant position. The volume of 1 ml of the prepared spore suspension was used as inoculum with concentration of 3.10^7 spores.

Production of Tannase under Solid State Fermentation. A mass of 5 g of wheat flour was taken in 125 ml Erlenmeyer flask and moistened with 5 ml Czapeck medium containing tannic acid. The composition of Czapeck medium was $NaNO_3$ g/l, KCl 0.5 g/l, $MgSO_4 \cdot 3H_2O$ 0.348 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $K_2HPO_4 \cdot 3H_2O$ 1.301 g/l, and tannic acid 50 g/l. The contents were sterilized by autoclaving at 121 °C for 15 min. After cooling the sterilized solid substrate was inoculated with 1 ml of the spore inoculum. The spore inoculum and medium were mixed properly and incubated at 28 °C for 72 h.

Extraction of Crude Enzyme. Tannase was extracted from the fermented substrate by adding 50 ml of distilled water containing 0.01% Tween 80. Content was mixed well using a magnetic stirrer. Crude enzyme was separated from fermented matter by centrifugation (Beckman J2-21 rotor) at 8,000 rpm, and 4 °C for 20 min. The supernatant was separated by filtration through Whatman no. 1 paper and the filtrate was collected in bottles for further studies.

Tannase assay was performed by the method of Rajakumar and Nandy (1983). Enzyme activity was expressed in international units (IU). One unit of tannase activity was defined as the amount of enzyme required to release 1 µmol gallic acid per minute under standard reaction conditions. Total soluble protein was determined by the method of Bradford (1976) and was expressed in mg/ml.

Ammonium Sulphate Fractionation and Dialysis. Crude enzyme was fractionated by ammonium sulphate at various saturation levels (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80%). The addition of ammonium sulphate to crude enzyme was done under constant stirring at 4 °C until well mixed. The mixture was kept for 3 h at 4 °C. Proteins were precipitated and then separated by centrifugation at 8,000 rpm, and 4 °C for 20 min. The separated proteins were then dissolved in 0.05 M citrate buffer pH 5. Each treatment was carried out in triplicate.

Enzyme resulted from ammonium sulphate fractionation was subjected to dialysis against citrate buffer 0.05 M pH 5. Before dialysis, strips of cellophanes were boiled twice in 10 g/l Na_2CO_3 containing 1 mM EDTA for 10 min. Cellophanes were stored in 0.05 M citrate buffer (pH 5) at 4 °C. Prior to use, the inside and outside parts of the cellophanes were rinsed with distilled water or buffer (Pingoud *et al*. 2002). Dialysis was conducted overnight and the buffer was changed several times until citrate buffer did not react with Nessler reagent.

Enzyme Characterization. Tannase characterization was performed by the method of Sabu *et al*. (2005). Effect of temperature, pH, substrate type, substrate concentration, and metal ions on dialyzed enzyme activity was studied. The K_m and V_{max} were also determined. Each treatment was conducted in triplicate.

Effect of Temperature. Enzyme, substrate solution (tannic acid) and citrate buffer were incubated at different temperature ranging from 30 to 85 °C. The enzyme assay was performed as described earlier to determine the optimal incubation temperature.

Effect of pH. The substrate tannic acid was dissolved in buffer solutions of varying pH. 0.05 M citrate buffer for pH 3-6, 0.05 M phosphate buffer for pH 7-8, and 0.05 M Tris-HCl for pH 9. Tannase activity assay was performed as described earlier to determined the optimal medium pH.

Effect of Substrate Type. Tannic acid and gallotannin were tested as tannase substrates for tannase activity. Each substrate was diluted in 0.05 M citrate buffer pH 6 to a final concentration of 0.01 M. Tannase assay was performed as described earlier.

Effect of Substrate Concentration. Substrates solution of different concentration (0.001 to 0.01 M) were prepared in 0.05 M pH 6 citrate buffer and the effect of substrate concentration on tannase activity were determined.

Effect of Metal Ions. To elucidate effect of metal ion on tannase activity, different metal ions like Mg^{2+}, Cu^{2+}, Fe^{2+}, K^+, Na^+, Ca^{2+}, Zn^{2+}, and Mn^{2+} were dissolved in 0.05 M citrate buffer (pH 6) at a concentration of 0.01 and 0.05 M. Tannase assay was performed using this buffer and the effect of metal ions on tannase activity was studied.

Determination of K_m and V_{max}. K_m and V_{max} were determined by plotting velocity against substrate concentration. K_m is equal to substrate concentration when initial velocity is equal to ½ V_{max}. K_m is a property of ES complex that does not depend on the concentration of enzyme or substrate.

RESULTS

Fractionation of Tannase. Crude tannase was fractionatied by ammonium sulphate at various saturation levels (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80%). The best tannase activity (4.110%) was obtained at ammonium sulphate saturation level of 60% (Figure 1).

Total soluble protein at ammonium sulphate saturation level of 55% was higher than saturation level of 60% (Figure 2). The maximum protein obtained at ammonium sulphate saturation level of 55 and 60% was 0.514 and 0.477 mg/ml, respectively.

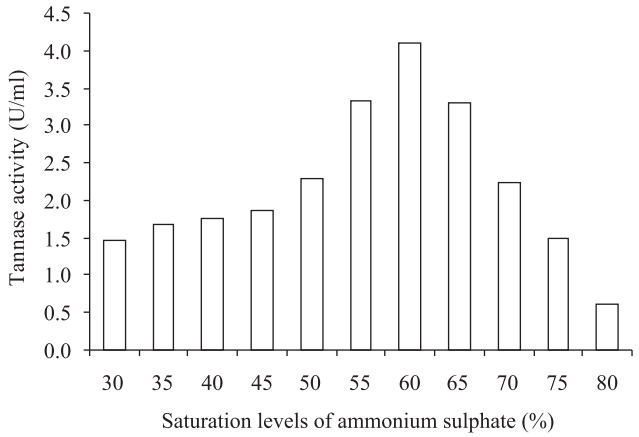

Figure 1. Tannase activity at different saturation levels of ammonium sulphate.

Tannase Characterization. The optimum temperature and pH for tannase activity were determined. Under our assay conditions the tannase showed activity at all the temperatures tested (30-85 °C), although activity was markedly reduced above 55 °C. Activity was highest at 35-50 °C (5.125 U/ml) (Figure 3). The tannase was active over a broad pH range (3-9) with an optimum at pH 6 (10.633 U/ml) (Figure 4).

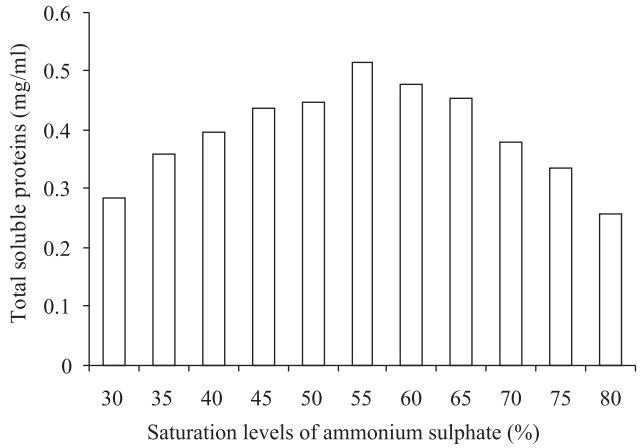

Figure 2. Total soluble protein at different saturation levels of ammonium sulphate.

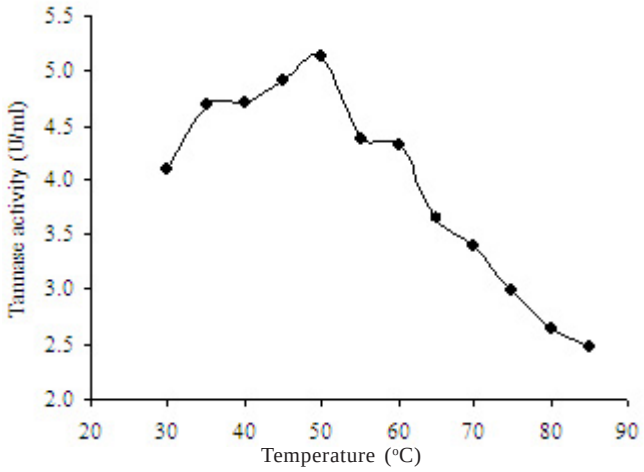

Figure 3. Effect of temperature on tannase activity.

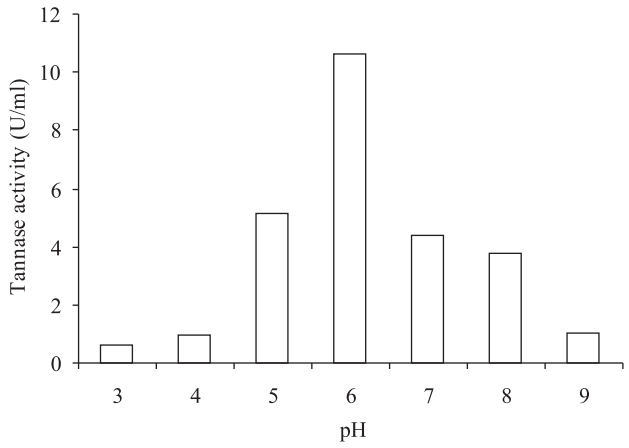

Figure 4. Effect of pH on tannase activity.

Kinetics analysis showed that K_m and V_{max} value of tannic acid was 0.401 mM and 10.804 U/ml respectively. Gallotannin yielded K_m and V_{max} value of 6.611 mM and 12.406 U/ml respectively (Figure 5).

Tannase was activated by K^+ and Na^+ with concentration of 0.01 and 0.05 M. Mg^{2+} ion only activated tannase at concentration of 0.01 M while at 0.05 M it inhibited tannase activity. Addition of metal ions like Cu^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, and Fe^{2+} inhibited the enzyme activity (Figure 6).

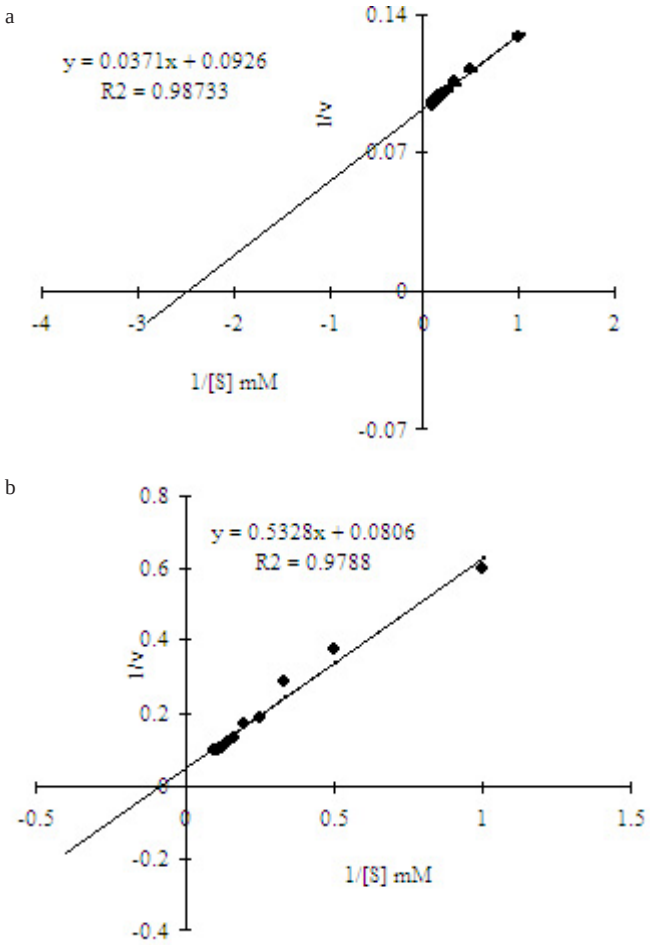

Figure 5. Lineweaver-Burk plot for (a) tannic acid, (b) gallotanin.

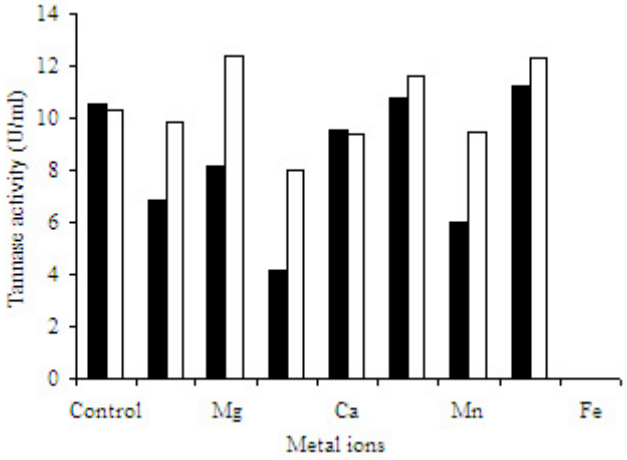

Figure 6. Effect of metal ions on tannase activity. ■ 0.01M, □ 0.05M

Table 1. Characteristics of microbial tannase from different sources

Microorganism	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	References
Aspergillus flavus	194	5.0-5.5	50-60	Adachi *et al*. 1968
Aspergillus flavus	80-85	6	70	Yamada *et al*. 1967
Aspergillus niger	-	5.5	60	Lekha and Lonsane 1994
Aspergillus niger	90, 180	6	60-70	Ramirez-Coronel *et al*. 2003
Aspergillus niger ATCC 16620	168	6	30-40	Sabu *et al*. 2005
Aspergillus niger LCF 8	186	6	35	Barthomeuf *et al*. 1994
Aspergillus niger van Tieghem	-	6	60	Sharma *et al*. 1999
Aspergillus niger	-	6	35-50	Present report

DISCUSSION

Tannase was produced extracellularly by *A. niger* isolated from cacao pod in a solid state fermentation using wheat flour as a substrate. The enzyme obtained was crude enzyme. In order to obtain pure enzyme, tannase was fractionatied by ammonium sulphate and then followed by dialysis process. The highest tannase activity (4.110 U/ml) was obtained at ammonium sulphate saturation level of 60%. However, the tannase activity between saturation level of 55, 60, and 65% was not significantly different.

After dialysis of the 60% fraction, a specific activity of 8.607 U/mg was obtained, which was a four folds enhancement in activity compared to the crude enzyme. Similar result was reported by Sabu *et al*. (2005) where maximum specific activity obtained was at 40-60% saturation of ammonium sulphate. Rajakumar and Nandy (1983) purified tannase from *Penicillium chrysogenum* using ammonium sulphate with saturation level of 100%. Difference in saturation level of ammonium sulphate used for precipitating proteins is related to the amount of the enzyme hydrophilic amino acids. Protein with relatively more hydrophilic amino acids require higher concentration of ammonium sulphate than protein with few hydrophilic amino acids (Scopes 1987).

The optimum temperature for tannase activity was found to be 35 to 50 °C. Similar data were reported by Sabu *et al*. (2005) and Mahendrar *et al*. (2006). The result of the present study, however, was different from that reported by Ramirez-Coronel *et al*. (2003) where the highest tannase activity was obtained at 60 to 70 °C. The tannase activity in the present study was markedly reduced above 55 °C. This indicated that the rate of protein enzyme denaturation increased at higher temperature leading to loss of tannase activity.

Tannase activity was found to increase with the increase of medium pH and the optimum activity was achieved at pH 6 (10.633 U/ml). Similar results were reported by Sharma *et al*. (1999) and Sabu *et al*. (2005) for tannase from *A. niger*. The effect of pH on enzyme activity is determined by the nature of amino acids at the active site, which undergoes protonation and deprotonation, and by the conformational changes induced by the ionization of other amino acids.

Based on the Michaelis-Menten constant (K_m), the tannase obtained was more active in degrading tannic acid rather than gallotannin. Tannic acid has one depside bond with no ester bond, and gallotannin have five depside bonds with five ester bonds. The K_m value indicated that the tannase obtained has relatively lower activity for ester bonds than depside bonds. Haslam and Stangroom (1966) suggested that tannase has independent esterase and depsidase activities with different affinities for substrates containing ester bonds and depside bonds. Growth of *A. niger* on depside-free media, gave tannase with marginal increase of the esterase/depsidase ratio compared with tannase of *A. niger* grown on ester-free media.

Metal ions like Zn^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, dan Fe^{2+} with concentration of 0.01 and 0.05 M inhibited the activity of tannase. Tannase was found to be activated by Na^{+} dan K^{+} with concentration of 0.01 and 0.05 M, while Mg^{2+} ion could activate only at 0.01 M. Similar results were reported previously by Sabu *et al*. (2005) and Rajakumar and Nandy (1983). Table 1 gives a comparative description of characteristics of tannase produced from different sources and that of tannase from the present study. Based on the present study, purification methods to obtain tannase of optimal activity can further be developed.

REFERENCES

Adachi O, Watanabe M, Yamada H. 1968. Studies on fungal tannase Part II physicochemical properties of tannase of *Aspergillus niger*. *Agr Biol Chem* 32:1079-1085.

Anwar YAS, Hasim, Artika IM. 2007. Production of tannin acyl hydrolase from *Aspergillus niger*. *Microbiol Indones* 1:91-94.

Barthomeuf C, Regerat F, Pourrat H. 1992. Production, purification, and characterization of a tannase from *Aspergillus niger* LCF 8. *J Ferment Bioeng* 77:320-323.

Battestin V, Macedo GA. 2007. Effects of temperature, pH and additives on the activity of tannase produced by *Paecilomyces variotii*. *Electro J Biotechnol* 10:191-199.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248-254.

Haslam E, Stangroom JE. 1966. The esterase and depsidase activities of tannase. *J Biochem* 99:28-31.

Lekha PK, Lonsane BK. 1994. Comparative titres, location and properties of tannin acyl hydrolase produced by *A. niger* PKL 104 in solid state, liquid surface and submerged fermentations. *Process Biochem* 29:497-503.

Mahendrar B, Raman N, Kim DJ. 2006. Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. *Appl Microbial Biotechnol* 70:444-450.

Marco M *et al*. 2009. A novel tannase from the xerophilic fungus *Aspergillus niger* GH1. *J Microbiol Biotechnol* 1:1-10.

Paranthaman R, Vidyalakshmi R, Murugesh S, Singaravadivel K. 2008. Optimisation of fermentation conditions for production of tannase enzyme by *Aspergillus oryzae* using sugarcane baggase and rice straw. *Global J Biotechnol Biochem* 3:105-110.

Pingoud A, Urbanke C, Hoggett J, Jeltsch A. 2002. *Biochemical Methods: A Concise Guide for Students and Researchers*. Weinheim: Willey-VCH.

Pinto GAS, Leite SGF, Terzi SC, Couri S. 2001. Selection of tannase-producing *Aspergillus niger* Strains. *Brazilian J Microbiol* 32:24-26.

Purnama IN. 2004. Studies on potency of fungal isolates to cleave tannin linkage in caccao pod (*Theobroma cacao* L) [Thesis]. Bogor: Bogor Agricultural University.

Rajakumar GS, Nandy SC. 1983. Isolation, purification, and some properties of *Penicillium chrysogenum* tannase. *Appl Environ Microbiol* 46:525-527.

Ramirez-Coronel A, Viniegra-Gonzalez G, Augur C. 2003. A novel tannase from *Aspergillus niger* with β-glucosidase activity. *Microbiol* 149:2941-2946.

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

Scopes RK. 1987. *Protein Purification Principles and Practice*. New York: Springger-Verlag.

Sharma S, Bhat TK, Dawra RK. 1999. Isolation, purification and properties of tannase from *Aspergillus niger* van Tieghem. *World Microbiol Biotechnol* 15:673-677.

Wright LP. 2005. *Biochemical Analysis for Identification of Quality in Black Tea (Camellia sinensis).* Pretoria: University of Pretoria.

Yamada K, Odachi O, Watanabe M, Ogata K. 1967. Tannase (tannin acyl hydrolase) a typical serine esterase. *Agr Biol Chem* 32:257-258.