

Full Length Research Paper

A method for the determination of tannase activity based on gallic acid measurement by high performance liquid chromatography (HPLC)

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A new high performance liquid chromatography (HPLC) method was developed based on an indirect determination of tannase activity through gallic acid measurement using paracetamol as an internal standard. The separation was performed on a C₁₈ column using a mobile phase consisting of aqueous formic acid solution (1%) and methanol (85:15; v/v) pumped at 1 ml min⁻¹. Samples (10 µl) were injected and signals were detected at 254 nm. Repeatability was 0.81 and 1.28 for peak normalization values of gallic acid (4.16 x 10⁻⁵ M) and internal standard (2.02 x 10⁻⁵ M). A good linearity was shown in the range of 1.04 x 10⁻⁵ to 8.32 x 10⁻⁵ M. Limit of detection (LOD) and limit of quantification (LOQ) values were calculated to be 2.2 x 10⁻⁶ M and 6.6 x 10⁻⁶ M, respectively. The applicability of the method was tested using *Penicillium spinulosum* which produces tannase. The method was highly sensitive, accurate, reliable, and repeatable and at the same time it is applicable to both pure and crude enzyme.

Key words: Tannase activity, gallic acid, tannic acid, high performance liquid chromatography, enzyme activity, validation.

INTRODUCTION

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an inducible enzyme that catalyzes the hydrolysis of ester and depside bonds in tannins such as gallotannin and ellagitannin, releasing gallic acid (GA) and glucose (Barthomeuf et al., 1994; Kar and Banerjee, 2000; Sharma et al., 2000; Aguilar et al., 2001a; Das Mohapatra et al., 2009). The enzyme has wide applications in the food, beverage, brewing, cosmetic, and chemical industries (Sharma et al., 1999; Mondal et al., 2000). It is mainly used in the manufacture of instant tea, acorn wine and in the production of GA (Mondal and Pati, 2000). It is also used in the clarification of beer, fruit juice and coffee-flavored soft drinks, detannification of food, high-grade leather tannin, and to clean-up highly

polluting tannin from the effluent of leather industry (Aguilar et al., 2001a).

The enzymatic product, GA (3,4,5-trihydroxybenzoic acid) is a phenolic compound and finds application in various fields. Its major use is in pharmaceutical industries for manufacture of trimethoprim (TMP), an antibacterial agent that is usually given along with sulfonamide; together they have a broad spectrum of action (Kar and Banerjee, 2000; Seth and Chand, 2000). GA is also used in leather industry, in manufacture of GA esters, e.g. propyl gallate that is widely used as food antioxidant, in the manufacture of pyrogallol, and photosensitive resin in semiconductor production (Mondal and Pati, 2000).

Certain studies were reported for the measurement of tannase activity. Colorimetric methods which have been based on the changing of light absorbance of colored tannic acid with FeCl₃ reagent (Mondal and Pati, 2000; Mondal et al., 2000, 2001; Nishitani and Osawa, 2003),

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UV-spectrophotometric methods variation of the absorbance of tannin at certain wavelengths (libuchi et al., 1967; Aoki et al., 1979; Suseela Rajakumar and Nandy, 1983; Chen and Matsumoto, 1995; Bajpai and Patil, 1997; Sharma et al., 1999, 2000, 2002; Kar and Banerjee, 2000; Seth and Chand, 2000; Aguilar et al., 2001a; Bhardwaj et al., 2003), chromatographic methods such as reverse phase HPLC and gas chromatography (GC) (Jean et al., 1981; Osawa and Walsh, 1993; Barthomeuf et al., 1994; Aguilar et al., 1999, 2001b; Ramirez-Coronel et al., 2003).

In the present study, a selective and repeatable HPLC method was developed for the measurement of tannase activity. This method is based on an indirect determination of tannase activity through GA measurement. The repeatability of the method was improved by addition of internal standard (IS). All of the data were evaluated statistically. The applicability of the method was examined with the tannase produced by *Penicillium spinulosum* isolated and identified in our laboratory.

MATERIALS AND METHODS

Apparatus

An HPLC Model of LC-10AT VP consisting of FCV-10AL VP pump, SIL-10AD VP auto injector, DGU-14A degasser, CTO-10AS VP column oven, SPD-M10A VP Photodiode Array Detector (PDA), SCL-10A VP system controller, FRC-10A fraction collector; Class-VP (Version 6.10) processor of the data (All Shimadzu, Kyoto, J); a model of RK 106 Bandelin Sonorex ultrasonic bath (Bandelin Electronics, Berlin, G); Nucleosil column 100-5 C₁₈; 250 mm length, 4.6 mm inner diameter, 5 μ particle diameter from (Machery-Nagel, Düren, G); a WB-10 model of adjustable water bath from (Mettler, Schwabach, G) were used during the studies.

Chemicals

Tannase as a standard, GA monohydrate and tannic acid from Fluka Chemie GmbH (Buchs, SW), methanol from J. T. Baker (Deventer, NL) were used. All other chemicals were of an analytical grade (Merck GmbH Darmstadt, G).

Preparation of the solutions

Standard chemicals such as GA and tannic acids were weighed and transferred to a flask and made up to 100 ml with double distilled water. Dilutions were prepared by using this stock solution and injected into HPLC. For reaction buffer, necessary amount of citric acid was weighed, transferred to a beaker, added some double distilled water and the pH of the solution was adjusted to 5.5 by adding 1 M NaOH and completed to volume. Mobile phase was prepared by mixing aqueous formic acid solution (1%) and methanol (85:15; v/v). It was passed through a G4 glass filter, degassed by ultra sonication before use.

Chromatographic procedure

The study was conducted by using 1 ml min⁻¹ flow-rate. Before injection, the column was washed as in the common and it was conditioned by passing through the mobile phase half an hour. Injections were always made and signals were detected at 254 nm throughout the study. The column was washed with methanol after each 8 or 10 injections prior to use.

Inoculation and growth of microorganism

Previously isolated tannase-producing fungal strain *P. spinulosum* was used in this study. Inoculums were prepared by transferring spores to potato dextrose agar (PDA) plates containing 1% tannic acid (filter sterilized) and 1.5% agar. The plates were point inoculated and incubated at room temperature for 7 to 10 days. Spores were then scraped into 0.1% Tween-80 and counted using a thoma slide.

Production of tannase

Tannase production was carried out in 250 ml Erlenmeyer flasks containing 50 ml sterilized basal medium which was consisting of composition (g L⁻¹) ammonium nitrate, 1.2; magnesium sulfate with 7H₂O, 0.5; potassium chloride, 0.1; potassium phosphate, monobasic, 1. Sterilized tannic acid by filtration was added that its final concentration will be 1% in the autoclaved medium (at 121°C for 15 min). The medium at an initial pH of 6 was inoculated with spore suspension (3.7 x 10⁶ spores/ml) and incubated at 30°C on a rotary shaker (120 rpm) for up to 48 h.

Preparation of the crude extract

The mycelia mass was harvested after 48 h of incubation by filtration using Whatman No.1 filter paper (Whatman Int. Ltd., Maidstone, GB.), and washed with distilled water till no pigments appeared in the filtrate. The final washing was done with citrate buffer (0.05 M, pH 5.5). The mycelial mass was suspended in citrate buffer and homogenized using a model of Ultra-Turrax T25 (Janke and Kunkel IKA- Labortechnik, Staufen, G.) homogenizer at 5 min keeping it in an ice bath. It was centrifuged using a model of Biofuge Stratos (Heraeus, Osterode am Harz, G.) at 4°C and 10,000 x g for 10 min. The supernatant was freeze dried using a model of Alpha 1-4 freeze drier (Christ, Osterode am Harz, G). The material was weighed, transferred to a tube and suspended in citrate buffer.

Examination of tannase activity

A 50 ml tannic acid 3.5 g L⁻¹ was prepared in an electrolyte solution of 50 mM citric acid at pH 5.5 as applied by some researchers (libuchi et al., 1967; Seth and Chand, 2000; Kikkoman Group, 2003). This solution (1 ml) transferred to a tube and incubated at least 5 min at 30°C in a water bath, 0.25 ml standard or tannase of *P. spinulosum* was added and it was incubated for 15 min. Then 1 ml of this solution was withdrawn, added to a 10-ml tube containing 5 ml ethanol and it was made up to volume. Injection solution was prepared as in the following; 1 ml of reaction solution, 2 ml of IS and 7 ml de-ionized water. This solution was injected into HPLC for measuring the concentration of GA hydrolyzed from tannic acid by the enzyme tannase. Similar procedure was repeated for blank sample, to subtract the amount of GA that naturally becomes in tannic acid.

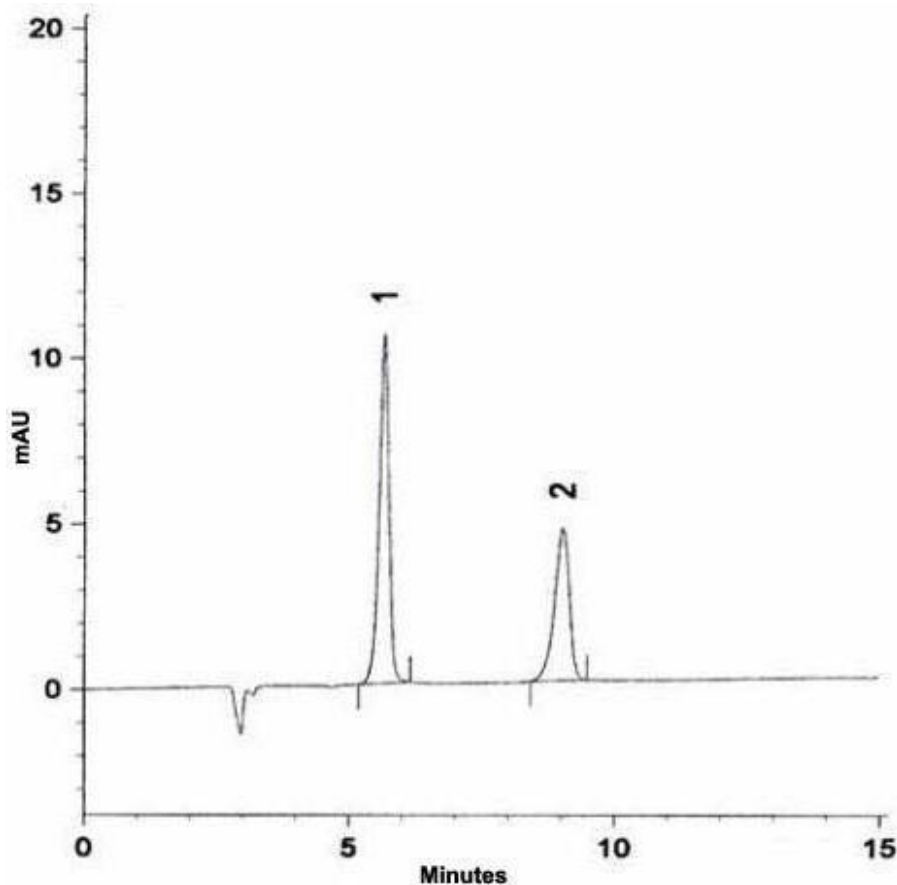


Figure 1. The chromatogram of GA (4.16×10^{-5} M) and paracetamol (2.02×10^{-5} M) as IS detected at 254 nm and injected to HPLC only 10 μ l using a mobile phase consist of aqueous solution 1% formic acid and methanol (85:15). Gallic acid ($R_t = 5.8$ min) and paracetamol ($R_t = 9.2$ min) are designated as 1 and 2, respectively.

RESULTS

Certain compositions of mobile phase systems were tried and it was found out that low amount of methanol and distilled water containing 1% formic acid system is the most suitable mixture to analyze GA. The composition of this mobile phase looks like those of the mobile phase which are used for the analysis of phenolic acids which include GA (Häkkinen et al., 1999; Robbins and Bean, 2004; Tsao and Deng, 2004). They were all gradient elution. In the employment of the mentioned mobile phase, it was observed that the retention time was increasing with the decrease of methanol and or *vice versa*. A mobile phase consisting of aqueous solution of formic acid (1%): methanol (85:15) was always used. Detection was made at 254 nm and 1 ml flow rate was conducted by using isocratic mode.

At the mentioned conditions, GA (4.16×10^{-5} M) appeared at 5.8 min. It was well-defined, symmetrical and sharp peak. As a second step for the efficacy, some

compounds were tested in order to find out an internal standard (IS). Paracetamol (IS, 2.02×10^{-5} M) was found appropriate for this purpose and it appeared at 9.2 min. The duration of analysis was very reasonable, as well. The chromatogram of GA (4.16×10^{-5} M) and IS (2.02×10^{-5} M) is demonstrated in Figure 1.

Repeatability

A mixture of GA (4.16×10^{-5} M) and IS (2.02×10^{-5} M) was prepared and it was consequently injected to the HPLC. The results were evaluated with regard to the area of the peaks and peak normalizations ($PN = \text{peak area}/\text{peak retention time}$) and the rate of the peak normalizations ($R = PN_{CTZ}/PN_{IS}$). It was observed that the precision of the experiments increased depending on that turn. This can be attributed to the fact that the employment of peak normalization and the processing of the internal standard become more repeatable by the use

Table 1. Repeatability studies of 4.16×10^{-5} M GA and 2.02×10^{-5} M IS using aqueous formic acid solution 1%: methanol (85:15) mobile phase solution, detecting at 254 nm with 1 ml min^{-1} of flow-rate (n = 6 injection).

	R _t GA	Area GA	PN GA	R _t IS	Area IS	PN IS	Rate of PN (R=PN _{GA} /PN _{IS})
Mean	5.74	144007	25077	9.30	88566	9528	2.63
SD	0.04	1974	203	0.06	651	121	0.06
RSD %	0.66	1.37	0.81	0.67	0.74	1.28	2.21

R_t retention time, PN peak normalizations, SD standard deviation, RSD relative standard deviation.

Table 2. Calibration results of GA for intra-day and inter-day studies.

	Intra-day (I = 3)			Inter-day (n = 24)
	First day (n=8)	Second day (n = 8)	Third day (n = 8)	
a	60199	60710	60691	60813
b	-0.0175	-0.0175	-0.0091	-0.020
S _x	2.55×10^{-5}	2.43×10^{-5}	2.46×10^{-5}	2.41×10^{-5}
S _y	1.53	1.48	1.49	1.47
r	0.9997	0.9995	0.9997	0.9995

n: number of experiments, I: number of days, a: slope, b: intercept; S_x and S_y: standard deviation of x and y values, r: correlation coefficient.

of the internal standard method. Certain injections were made, and the results were processed. The results of repeatability, considered as intra-day, are demonstrated in Table 1.

The RSD values for PN were calculated to be 0.81 and 1.28 for GA and IS, respectively. Evaluating the values in the table, it was decided that it would be more precise to use the PN values for the quantification of GA. It is resulted that the analysis of GA could be performed precisely.

Linearity

Three sets of GA, each has 8 dilutions with increasing concentration (1.04×10^{-5} M and 8.32×10^{-5} M) and fix concentration of IS (2.02×10^{-5} M) were prepared and each sets were injected consequent days, employing optimum conditions to examine and evaluate the intra-day and inter-day precision of linearity.

Internal standard technique was adapted to the analysis system to increase linearity. It is known that IS technique is more confidential way for the quantification of the compounds to provide repeatable results. Here, the rate of the PN values of the peaks was also employed as in the repeatability studies. The results related to the calibration equation as intra-day and inter-day are shown in Table 2.

It can be obviously seen from Table 2 that the intercept values are very low and correlation coefficients are

acceptable. The results indicate that the method developed here is quite reliable for the determination of GA and estimation of tannase activity. According to the results it was decided that the method could be applied.

Certain analytical parameters such as limit of detection (LOD) and limit of quantification (LOQ) values were calculated by computing the processed of integrated peak from electropherogram LOD and LOQ values were estimated as [standard deviation of regression equation, S_r /slope of the calibration equation, a] by multiplying with 3.3 and 10, respectively. They were found to be 2.2×10^{-6} M for LOD and 6.6×10^{-6} M for LOQ.

Application of the method to standard of tannase activity

Tannic acid which is used as substrate was injected to HPLC and it was found that it contains 4.03% GA. This shows that one should be careful and consider the free of GA in tannins because free GA affects the result of tannase activity measurement. The chromatogram of standard tannic acid before and after standard tannase addition is demonstrated in Figure 2.

The standard tannase has a definite weight activity that it should be equivalent to hydrolyzed GA. But, data show that the concentration of hydrolyzed GA is gradually decreases linearly that it deviates from the theoretical values. It might be necessary to use a correction factor. It has already been used such kind of a coefficient (1.04)

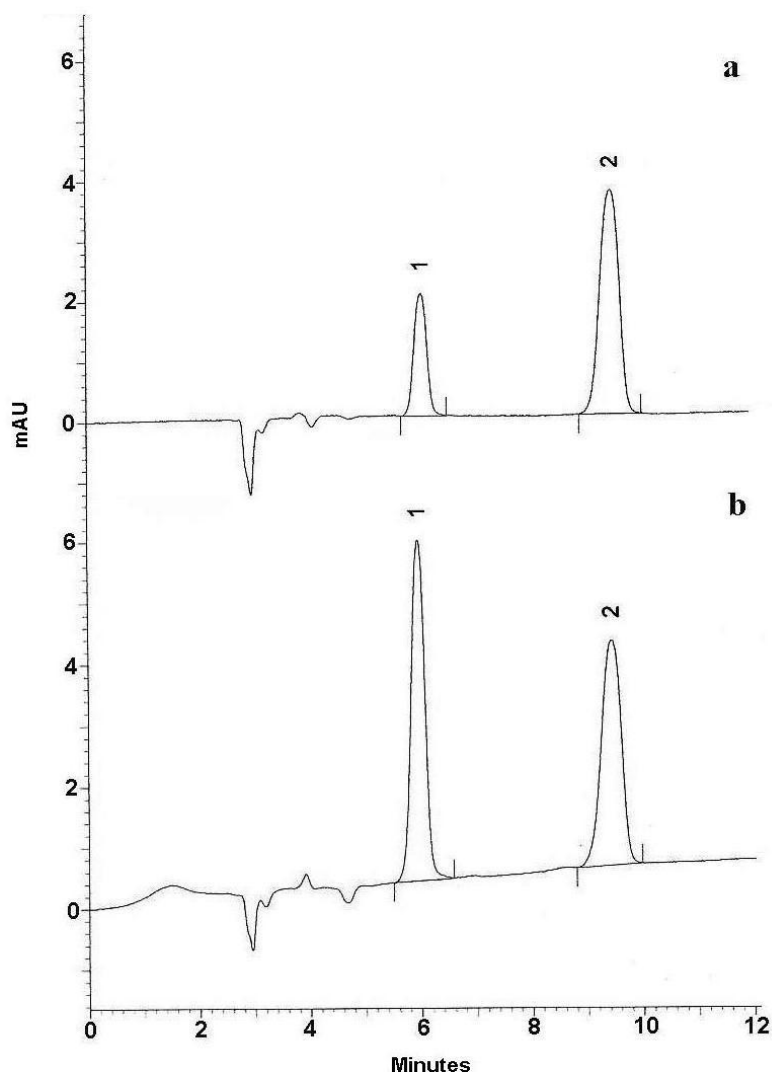


Figure 2. The chromatogram of standard tannic acid before (a) and after (b) addition of standard tannase. Gallic acid ($R_t = 5.9$ min) and paracetamol ($R_t = 9.4$ min) are designated as 1 and 2, respectively.

by elsewhere (Kikkoman Group, 2003). It was observed that the variation was not a constant value in the conditions, because it changed linearly. The GA which corresponds to tannase activity has to be equivalent to hydrolyzed GA from tannic acid and that amount should be observed or for real tannase activity.

The measurement of tannase activity was done as described under experimental section. Similar application was reported by libuchi et al. (1967). It is known that tannase activity that hydrolyzes $1 \mu\text{M}$ of ester bond in tannic acid per min under the described conditions is called one unit. To examine the tannase activity, standard solutions were prepared in the range of 0.25 to 5 mg ml^{-1} from a standard tannase (355 U g^{-1}). Produced GA from 1% tannic acid by tannase showed a linear dependence

in the range of 0.25 to 2 mg ml^{-1} . The variation of GA found as micro molar (μM) concentration versus tannase concentration as U ml^{-1} was computed as in the following:

$$Y(\mu\text{M}) = 26.28 \text{ VA}(\text{U mL}^{-1}) - 0.26 \quad (r = 0.9997; \text{Sy.x} = 0.21) \quad (1)$$

Where; VA is volume activity of tannase as U ml^{-1} , Y is concentration of GA as μM , r is correlation coefficient, Sy.x is the standard deviation of the vertical distances of the points from the linear line.

As it was mentioned above, VA is volume activity of tannase solution and its activity can be converted to several expressions. Common expressions is weight activity and it can be calculated from the data as in the

following

:

$$WA (U g^{-1}) = VA (U mL^{-1}) v (mL)/w (g)$$

Here, WA is weight activity as $U g^{-1}$; VA is volume activity as $U mL^{-1}$, v is volume of enzyme solution, w is the weight of tannase.

Application of the method for the tannase produced by *P. spinulosum*

The measurement of tannase activity was done as described in the experimental section. The method is very selective because it is based on the measurement of GA. The applicability of the method was tested using tannase produced in the basal medium by *P. spinulosum*. For five experiments, the average activity $11.88 U g^{-1}$, $\sigma = 0.86$ and $RSD\% = 7.23$ and confidence interval 11.88 ± 1.04 ($p < 0.05$) values were found. Since high tannin solution was employed in the experiments, a higher $RSD\%$ around 10 may be obtained, depending on the pipette procedure. $RSD\%$ value seems a little high but it is accepted because it exhibits the biological behavior and this value are usually higher in the mentioned systems (Shah et al., 1991). The results show the applicability of the method for the measurement of tannase activity. It is selective and highly precise, and thus significant in studies of analytical chemistry. Therefore, the method may be proposed for use in laboratories involved in tannase analysis.

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REFERENCES

- Aguilar C, Augur C, Viniestra-González G, Favela E (1999). A comparison of methods to determine tannin acyl hydrolase activity. Braz. Arch. Biol. Technol., 42: 355-361.
- Aguilar CN, Augur C, Favela-Torres E, Viniestra-González G (2001a). Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state fermentation: influence of glucose and tannic acid. J. Ind. Microbiol. Biotechnol., 26: 296-302.
- Aguilar CN, Augur C, Favela-Torres E, Viniestra-González G (2001b). Induction and repression patterns of fungal tannase in solid-state and submerged cultures. Process Biochem., 36: 565-574.
- Aoki K, Tanaka T, Shinke R, Nishira H (1979). Detection of tannase in polyacrylamide gels. J. Chromatogr., 170: 446-448.
- Bajpai B, Patil S (1997). Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of *fungi imperfecti*. Enzyme Microb. Tech., 20: 612-614.
- Barthomeuf C, Regerat F, Pourrat H (1994). Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. J. Ferment. Bioeng., 77: 320-323.

- Bhardwaj R, Singh B, Bhat TK (2003). Purification and characterization of tannin acyl hydrolase from *Aspergillus niger* MTCC 2425. J. Basic Microbiol., 43: 449-461.

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- Chen RCL, Matsumoto K (1995). Enzymatic determination of gallotannin in green tea infusions by a photometric flow-injection analytical method. Anal. Sci., 11: 777-780.

- Das Mohapatra PK, Maity C, Rao RS, Pati BR, Mondal KC (2009). Tannase production by *Bacillus licheniformis* KBR6: Optimization of submerged culture conditions by Taguchi DOE methodology. Food Res. Int., 42: 430-435.

- Häkkinen S, Heinonen M, Kärenlampi S, Mykkänen H, Ruuskanen J, Törrönen R (1999). Screening of selected flavonoids and phenolic acids in 19 berries. Food Res. Int., 32: 345-353.

- Iibuchi S, Minoda Y, Yamada K (1967). Studies on tannin acyl hydrolase of microorganisms Part II. A new method determining the enzyme activity using the change of ultra violet absorption. Agric. J. Biol. Chem., 31: 513-518.

- Jean D, Pourrat H, Pourrat A, Carnat A (1981). Assay of tannase (tannin acyl hydrolase EC 3.1.1.20) by gas chromatography. Anal. Biochem., 110: 369-372.

- Kar B, Banerjee R (2000). Biosynthesis of tannin acyl hydrolase from tannin-rich forest residue under different fermentation conditions. J. Ind. Microbiol. Biotechnol., 25: 29-38.

- Kikko Group, Tannase, <<http://www.kikkoman.co.jp/bio/e/common/skakou.html>>. Accessed on 06 June 2003.

- Mondal KC, Banerjee D, Jana M, Pati BR (2001). Colorimetric assay method for determination of the tannin acyl hydrolase (EC 3.1.1.20) activity. Anal. Biochem., 295: 168-171.

- Mondal KC, Banerjee R, Pati BR (2000). Tannase production by *Bacillus licheniformis*. Biotechnol. Lett., 0: 767-769.

- Mondal KC, Pati BR (2000). Studies on the extracellular tannase from newly isolated *Bacillus licheniformis* KBR 6. J. Basic Microbiol., 40: 223-232.

- Nishitani Y, Osawa R (2003). A novel colorimetric method to quantify tannase activity of viable bacteria. J. Microbiol. Meth., 54: 281-284.

- Osawa R, Walsh TP (1993). Visual reading method for detection of bacterial tannase. Appl. Environ. Microbiol., 59: 1251-1252.

- Ramirez-Coronel MA, Viniestra-Gonzalez G, Darvill A, Augur C (2003). A novel tannase from *Aspergillus niger* with β -glucosidase activity. Microbiol-SGM, 149: 2941-2946.

- Robbins RJ, Bean SR (2004). Development of a quantitative high-performance liquid chromatography-photodiode array detection measurement system for phenolic acids. J. Chromatogr. A, 1038: 97-105.

- Seth M, Chand S (2000). Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*- optimisation of process parameters. Process Biochem., 36: 39-44.

- Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE, McDowall RD (1991). Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Eur. J. Drug Metab. Pharmacokin., 16: 249-255.

- Sharma S, Bhat TK, Dawra RK (1999). Isolation, purification and properties of tannase from *Aspergillus niger* van Tieghem. World J. Microbiol.

Biotechnol., 15: 673-677.

Sharma S, Bhat TK, Dawra RK (2000). A spectrophotometric method for assay of tannase using rhodanine. *Anal. Biochem.*, 279: 85-89.

Sharma S, Bhat TK, Gupta MN (2002). Bioaffinity immobilization of tannase from *Aspergillus niger* on concanavalin A-Sepharose CL-4B. *Biotechnol. Appl. Biochem.*, 35: 165-169.

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

Tsao R, Deng Z (2004): Separation procedures for naturally occurring antioxidant phytochemicals. *J. Chromatogr. B*, 812: 85-99.