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Partial purification and characterization of *Aspergillus tamarii* IMI388810 (B) tannin acyl hydrolase

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ABSTRACT

Aspergillus tamarii IMI388810 (B) a tannic acid degrading fungus produced two major tannases in a fermentation medium M, containing tannic acid as the only carbon source. The culture broth was filtered through filter paper and the crude enzyme filtrate was concentrated by dialysis against different changes of 6M sucrose solution. The tannases were separated by Ion-exchange chromatography on Q-Sepharose Fast Flow column and Phenyl Sepharose 6 Fast Flow (High Sub) hydrophobic interaction chromatography and were identified as Tannase I and II. The optimum temperature for enzyme activity was 35^{o}C for Tannase I and 50^{o}C for Tannase II. Both tannases retained over 70% of its activity between 35^{o} and 50^{o}C, for 30 minutes. The optimum pH for the enzyme activity was 5.0 for Tannase I and 3.0 for Tannase II and with their maximum stability at pH 3.0 and 6.0, respectively. Their K_m values for methyl gallate were 0.08 mM, for Tannase I, and 0.02mM, for Tannase II. Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, SDS, EDTA, EGTA and Bromosuccinamide were inhibitory to the Tannases.

Keywords: Aspergillus tamarii IMI388810 (B), tannin acyl hydrolase, partial purification and characterization.

INTRODUCTION

Tannase (tannin acyl hydrolase, EC 3.1.20) catalyzes the hydrolysis of ester and depside linkages in hydrolyzable tannins like tannic acid to give gallic acid and glucose [1]. Tannase is extensively used in food, beverage, brewing, pharmaceutical, and chemical industries [1-8]. The enzymatic product, gallic acid, is also used in dye making, pharmaceutical and leather industries [1].

Tannase has been isolated and characterized from strains of *Aspergillus* [9-11; *Penicillium* [12, 13]; *Paecilomyces varioti* [14]; *Candida sp.* [15] and bacterial species such as *Bacillus licheniformins* [16], *Serratia ficaria* DTC [17], *Lactobacillus plantarum* [18-21] and *Citrobacter freundii* [22].

MATERIALS AND METHODS

Fungal strain: The *Aspergillus tamarii* IMI388810 (B) used in this study was isolated from soil inundated by effluent from a tannery at Oji River L.G.A. of Enugu State, Nigeria, and identified by CABI Bioscience, United Kingdom [23].

Reagents and media:
Tannic acid (E. Merck), Methyl gallate (Sigma), Gallic acid (Sigma), Rhodanine (Aldrich), Bradford reagent (Serva), Dipotassium hydrogen phosphate (M & B), Potassium dihydrogen phosphate (M & B), Magnesium sulphate (BDH), Calcium chloride (Hopkin and William), Ammonium chloride (Hopkin & William), Agar (Oxoid), Potassium hydroxide (Arondole Lab. Ltd, England), Methanol (BDH)) Citric acid (Fisher Chemical/Scientific), Sodium citrate (BDH), Phenyl Sepharose 6 FF-High Sub- (Pharmacia, Sweden), Q-Sepharose Fast Flow (Pharmacia, Sweden), Ethanol (BDH), Sabouraud Dextrose Agar (Lab.M), Phenylmethylsulfonyl fluoride (Sigma), Sodium chloride (M & B), Cobalt chloride (BDH), Ammonium sulphate (BDH), Iron (II) sulphate (Analar), Copper sulphate (BDH), Mercuric sulphate (BDH), Manganese sulphate (BDH), Sodium dodecyl sulphate (M & B), EDTA (BDH), Bromosuccinamide (Aldrich), Disodium hydrogen orthophosphate anhydrose (BDH), Sodium hydrogen carbonate (M & B), Sodium Hydroxide (BDH), Serva Blue G (Coomassie Brilliant Blue G) (Serva), Phosphoric acid (BDH), Filter paper (Whatman). Spectronic21 (Milton Roy Company) and Biophotometer (Eppendorf)

Production of tannase:
Erlenmeyer flasks (250ml) each containing 50ml of medium M were inoculated with two agar plugs (1.4cm, diameter) luxuriant growth of *A. tamarii* IMI388810 (B) on SDA medium plates. Fermentation was carried out in a shaker for 6 days at 28^{o}C (160 r. p .m.). Following the termination of the fermentation the culture broth was filtered through filter paper (Whatman No.1) and the filtrate recovered. The volume of the filtrate was measured and to it was added a protease inhibitor, phenyl-methylsulfonyl fluoride to the strength of 0.005mM. Tannase activity of the filtrate was determined spectrophotometrically [24] and protein content determined using Bradford reagent [25].

Tannase assay:
Tannin hydrolysis was determined spectrophotometrically using rhodanine [24]. The substrate solution (0.10M methyl gallate in 0.05M citrate buffer, pH 5.0), 10.0ml; enzyme sample, 1.0ml; and buffer (0.05M citrate buffer, pH 5.0), 1.0ml; were separately preincubated at 40^{o}C for 10 minutes before the enzyme reaction was started. The reaction mixture in the blank, test and control tubes contained 0.5ml of substrate solution to which 0.5ml of the buffer and 0.5ml of the enzyme sample were added to the blank and test, respectively. The tubes were incubated at 40^{o}C for 5 minutes and 0.6ml of methanolic rhodanine (0.667%, w/v) was added to all the tubes and further incubated at 40^{o}C for 5minutes. Thereafter, 0.4ml of 0.5M potassium hydroxide was added to each tube followed by further incubation at 40^{o}C for 5 minutes. This was followed by addition of the enzyme sample (0.5ml) to the reaction mixture in the control tube only. Finally, each tube was diluted with 8ml deionized water and incubated at 40^{o}C for 10 minutes and the absorbance was recorded against water at 520nm using a Spectronic21 (Milton Roy Company) spectrophotometer.

The enzyme activity was calculated from the change in absorbance;
$\Delta_{A520} = (A_{test} - A_{blank}) - (A_{control} - A_{blank})$

One unit (U) of the enzyme activity was defined as micromole of gallic acid formed per minute.

Determination of protein:
Estimation of protein was carried out with Bradford reagent [25]. To the enzyme solution (0.1ml) was added 5ml of Bradford reagent prepared by dissolving 100mg of Coomassie Blue G250 in ethanol (12.5ml). This was mixed with 25ml of phosphoric acid and the mixture was made up to 250ml with distilled water and then filtered through filter paper. The optical density of the mixture was measured at 595 nm in Spectronic21, spectrophotometer.

Purification of tannase:
(a) Concentration of enzyme solution:
At the end of the fermentation the recovered filtrate (346ml) was concentrated by dialysis against different changes of 6M sucrose solution. The protein content and tannase activity were determined as earlier described.

(b) Ion-exchange chromatography on Q-Sepharose Fast Flow (FF):
The crude enzyme concentrate (15ml) was subjected to ion-exchange chromatography on Q-Sepharose FF resin. The resin was packed into a glass column (2 x 14.5cm) and was equilibrated with 0.05M citrate buffer (pH 5.0). The concentrated enzyme solution was then applied on the resin followed by elution with 150ml of 0.05M citrate buffer (pH 5.0). Thereafter, further elution was carried out with 0.05M citrate buffer (pH 5.0) with a 0.5M NaCl gradient at a flow rate of 1.4ml per minute. Fractions (10ml) were collected and were assayed for protein by measuring the absorbance at 280nm in a Biophotometer (Eppendorf) while tannase activity was detected as earlier described. Fifty fractions were collected in all. Fractions with high enzyme activity were pooled and concentrated by dialysis against 6M sucrose solution. The protein content [25] and the tannase activity of the concentrate were determined

(c) Hydrophobic interaction chromatography on Phenyl Sepharose 6 Fast Flow (High Sub):
The enzyme concentrate (7.2ml) from the ion-exchange chromatography was subjected to hydrophobic interaction chromatography on a Phenyl Sepharose 6 FF (High Sub) column (2 x 9.5cm) previously equilibrated with 0.05M citrate buffer (pH 5.0) containing 1M $(NH_4)_2\ SO_4$. Prior to loading, the enzyme solution was made to the ionic strength of 1M $(NH_4)_2\ SO_4$. Elution of proteins was done in a stepwise manner using 0.05M citrate buffer (pH 5.0) containing 1M $(NH_4)_2\ SO_4$ followed by 0.5M buffered salt solution and finally 0.05M citrate buffer (pH 5.0). Fractions (10ml) were collected at a flow rate of 1.4ml per minute. Seventy fractions were collected in all. The fractions were assayed for protein by measuring the absorbance at 280nm and tannase activity was determined. Fractions with high enzyme activity peaks were pooled in two separate volumes (designated as Tannase I and Tannase II) and concentrated by dialysis against 6M sucrose solution to 3.2ml and 4.5ml, respectively. Both enzyme activity and protein content of the concentrate were determined by methods earlier described.

Characterization of the tannases:
(a) Effect of temperature on enzyme activity and stability:
The activity of the tannase I and tannase II was studied at the following temperatures: 35^o, 40^o, 50^o, 60^o, 70^o and 80^oC following the method as earlier described [24]. Temperature stability was also determined by incubating 0.1ml each of the tannase I and tannase II solution alone in a test tube at the following temperatures 35^o, 40^o, 50^o, 60^o, 70^o, and 80^oC for 30 minutes. After incubation, the enzyme was immediately chilled and the residual activity assayed at 40^oC as earlier described [24].

(b) Effect of pH on enzyme activity and stability:
The effect of pH on tannase I and tannase II activity and stability was studied at different pH values, between pH 3.0 and 10.0. Different buffers were used: 0.50M citrate buffer (pH 3.0, 4.0, 5.0 and 6.0); 0.2 M phosphate buffer (pH 7.0 and 8.0) and 0.2M Carbonate-Bicarbonate buffer (pH 9.2 and 10.0). Each buffer at the respective pH values above was used to prepare the substrate solution, 0.1M methyl gallate. Thereafter, the effect of pH on the activity of tannase I and tannase II was determined as earlier described [24]. The effect of pH on enzyme stability was determined by separately incubating 0.1ml each of tannase I and tannase II solution in 0.2 ml of each of the buffers of different pH values at room temperature (28^{o}C) for 1 hour. After the incubation period the enzyme activity was determined at 40^{o}C following the method described earlier [24].

(c) Effect of metal ions and enzyme poisons on tannase activity:
The effect of 1mM concentration of Fe^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, EDTA (Ethylene diaminetetraacetic acid), EGTA (Ethylenebis (oxyethlenenitro) tetraacetic acid), SDS (Sodium dodecyl sulphate) and bromosuccinamide on tannase activity was studied. The substrate solution (0.01M methyl gallate in 0.05M citrate buffer, pH 5.0), 1ml; 0.1ml each of solution of tannase I and tannase II to which was added 0.2ml of solution of each of the metal ions and enzyme poisons above in test tubes were separately preincubated at 40^{o}C for 10 minutes before the enzyme reaction was started. The enzyme assay was carried out by method described earlier [24].

(d) Effect of substrate concentration on tannase activity:
Different concentrations of methyl gallate in 0.05M citrate buffer (pH 5.0) were prepared. The same concentrations: 0.05M, 0.01M, 0.015M, 0.02M, 0.025M and 0.03M of methyl gallate were used for tannase I and tannase II. Each concentration of the substrate solution, solutions of tannase I and tannase II were preincubated at 40^{o}C for 10 minutes before the enzyme reaction was started. Following the method [24] as earlier described the enzyme activity was determined for each substrate concentration above. From the values obtained the K_m values of the tannases were determined by the linear transformation of the Michaelis–Menten equation [26].

RESULTS

Tannase purification:
Phenyl Sepharose 6 Fast Flow (FF):
The elution pattern of protein and tannase on Phenyl Sepharose 6 FF are shown in Figure 1. Tannase elution pattern showed two major peaks corresponding to two different kinds of tannase produced by *A. tamarii* IMI388810 (B). These were identified as tannase I (Fraction numbers 22 - 25) and tannase II (Fraction numbers 42 - 46), respectively. Table 1, shows the summary of the tannases purification. The tannases of *A. tamarii* IMI388810 (B) were purified as follows: Tannase I was purified 0.215 fold with a yield of 0.061% and specific activity of 0.117U/mg protein. Tannase II was purified 0.265 fold with a yield of 0.053% and specific activity of 0.144U/mg protein.

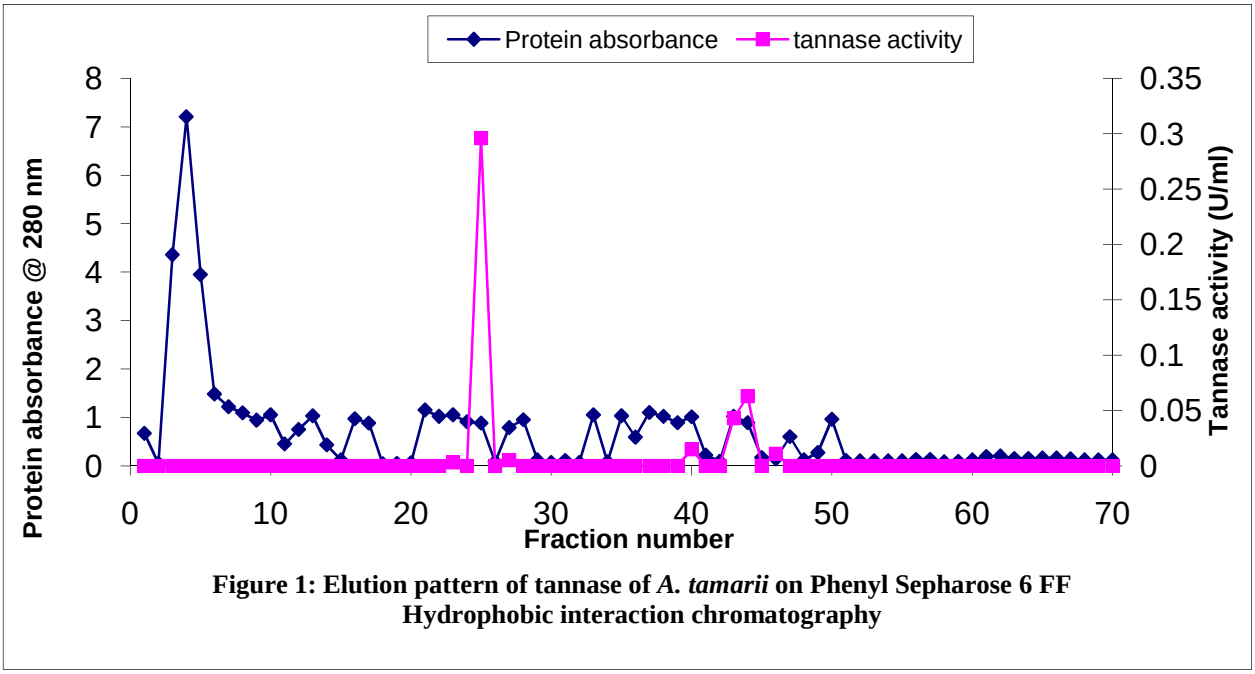

Figure 1: Elution pattern of tannase of *A. tamarii* on Phenyl Sepharose 6 FF Hydrophobic interaction chromatography

Table 1: Purification summary of tannase

Step	VOLUME (ML)	TOTAL ACTIVITY (Unit/ml)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg Protein)	YIELD (%)	PURIFICATION
Culture filtrate	346	24.409	44.98	0.543	100	1.0
Dialysis against 6m sucrose	15	0.467	2.7	0.173	1.913	0.319
Q-sepharose FF	7.2	0.178	0.648	0.278	0.729	0.506
Phenyl sepharose 6 FF	3.2	0.015	0.128	0.117	0.061	0.215
Tannase I Tannase II	4.5	0.013	0.09	0.144	0.053	0.265

Characterization of the tannases:

(a) Effect of temperature on tannase activity and stability:

Figure 2 shows the result of effect of temperature on tannase activity. Optimum activity of tannase I was at 35^{o}C while that of tannase II was at 50^{o}C. Figure 3, shows the effect of temperature on tannase stability. Between the temperature range of 35^{o}C and 60^{o}C tannases I and II retained over 70% and 80% of their activity, respectively for 30 minutes. Tannase II also showed better temperature stability than tannase I between 40^{o}C and 60^{o}C.

(b) Effect of pH on tannase activity and stability:

The result of the effect of pH on tannase activity is shown in Figure 4. The optimum pH for tannase I was 5.0 and 3.0 for tannase II. Tannase II showed another minor peak at pH 6.0 and activity between pH 5.0 and 7.0. Figure 5, presents the pH effect on enzyme stability. Tannase I had its maximum stability at pH 3.0 while tannase II had at pH 6.0 for 1h. Furthermore, tannase I showed other minor pH stability peaks at pH 6.0 and pH 9.2 while tannase II showed broad pH stability between pH 4.0 and 6.0. Tannase I retained over 65% of its activity between pH 3.0 and 10.0. Tannase II retained over 65% of its activity between pH 3.0 and 8.0.

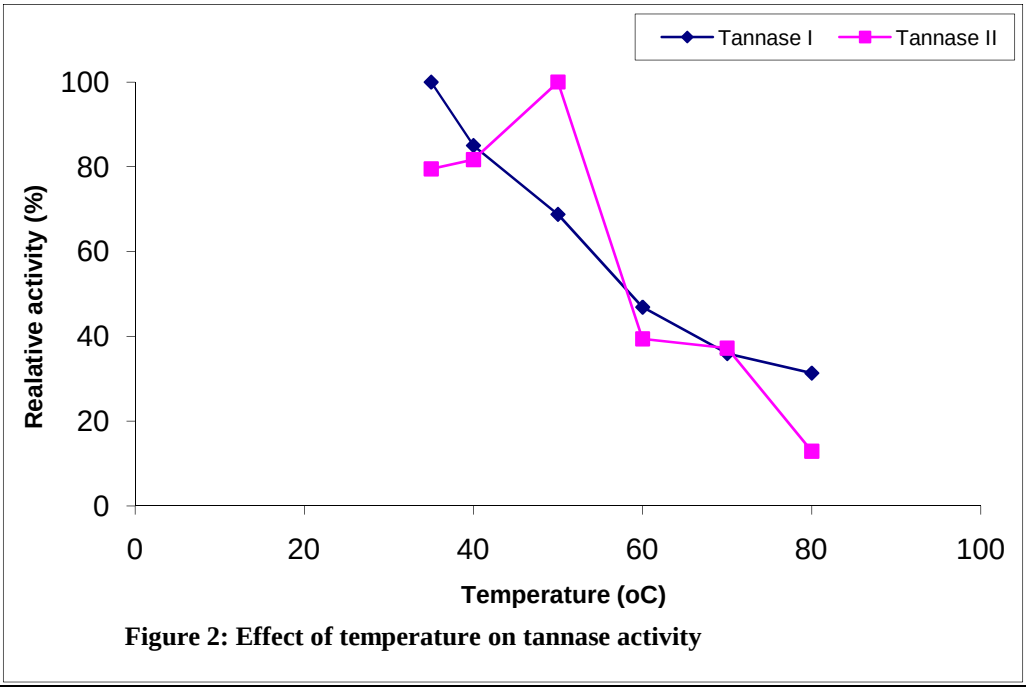

Figure 2: Effect of temperature on tannase activity

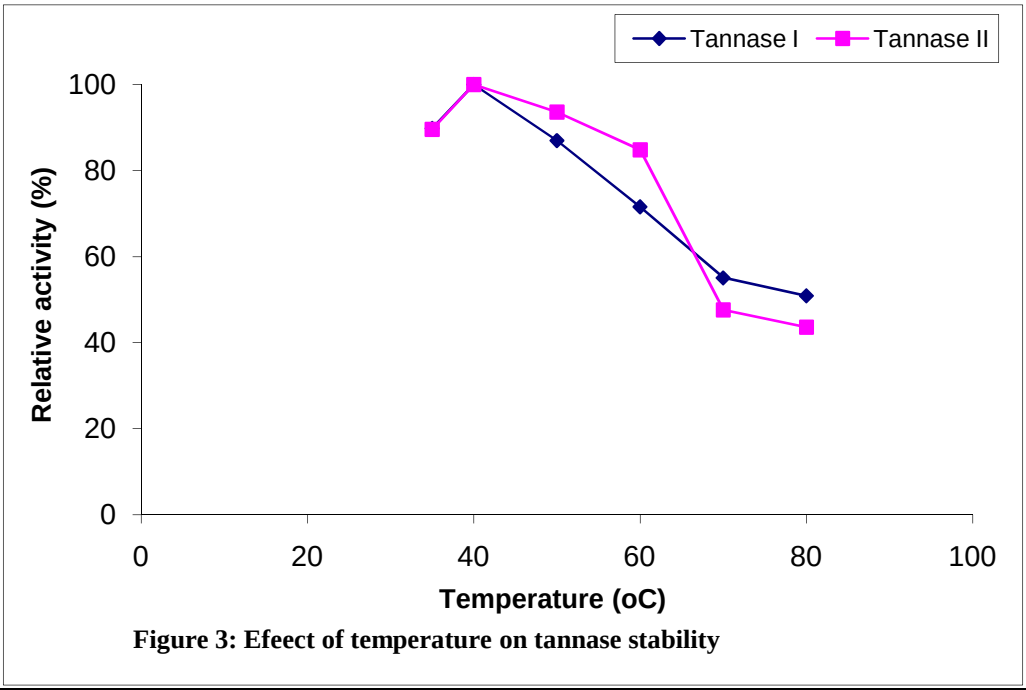

Figure 3: Efeect of temperature on tannase stability

(c) Effect of metal ions and enzyme poisons on tannase activity:

The result of the effect of metal ions and enzyme poisons on tannase activity is presented on Table 2. All the tested metal ions were inhibitory to the tannases. Maximum inhibition was achieved for both enzymes with EDTA.

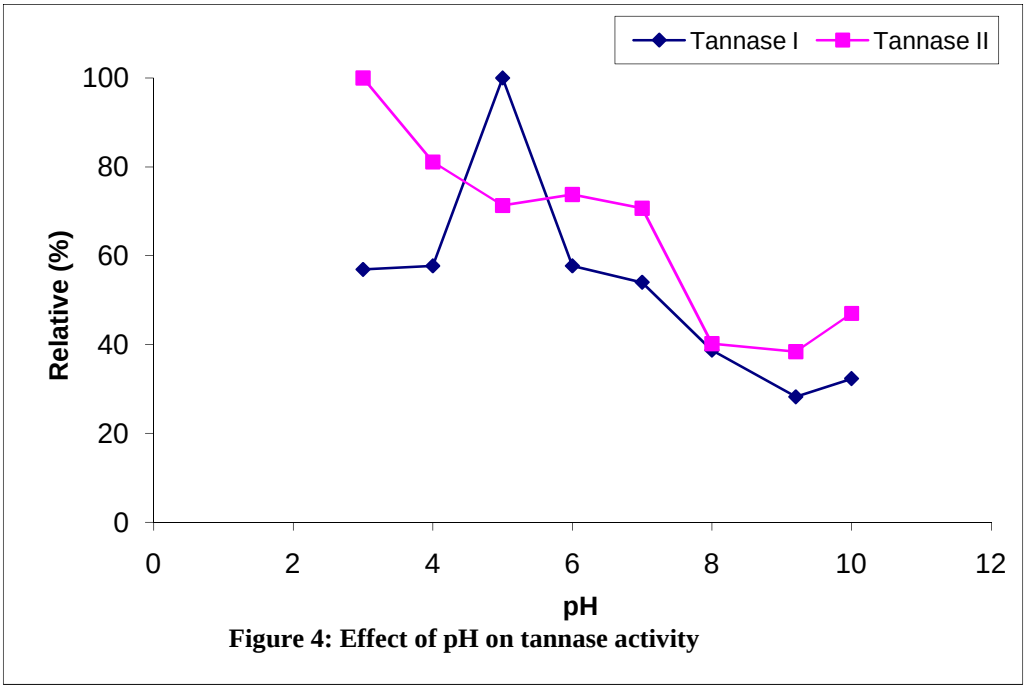

Figure 4: Effect of pH on tannase activity

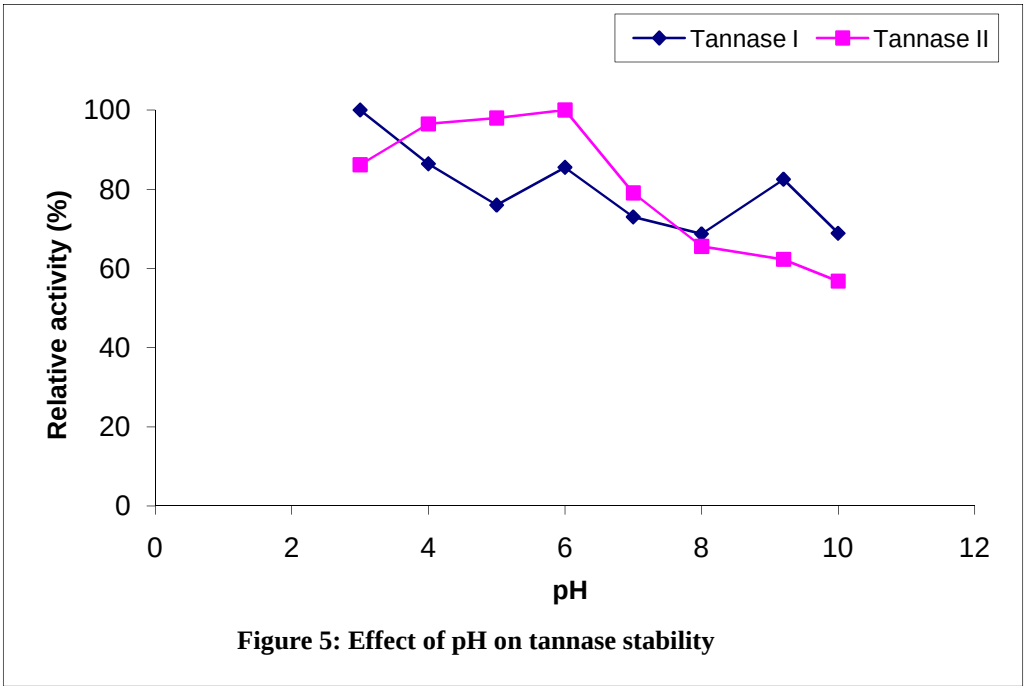

Figure 5: Effect of pH on tannase stability

(d) Effect of substrate concentration on tannase activity

The plot of the rate of hydrolysis of methyl gallate catalyzed by the two kinds of tannase against substrate concentration showed saturation kinetics, thus obeying Michaelis-Menten Kinetics. Although the two kinds of tannase can hydrolyze methyl gallate, tannase I (K_m, 0.008mM) had higher affinity for the substrate than tannase II (K_m, 0.02mM) (Figure 6).

Table 2: Effect of metal ions and enzyme poisons on tannase activity

METAL SALT (1mM)	RELATIVE ACTIVITY (%)	
	TANNASE I	TANNASEII
NONE	100.00	100.0
$CaCl_2$	.43.0	39.6
$CoCl_2$	80.4	78.2
$CuSO_4$	40.6	42.6
$FeS0_4$	84.2	71.2
$HgS0_4$	43.6	55.6
$MgS0_4$	41.8	41.6
$MnS0_4$	64.2	62.8
SDS	37.4	28.8
EDTA	9.6	10.0
EGTA	29.6	22.4
Bromoscuccinamide	35.4	41.6

SDS = Sodium Dodecyl Sulphate; EDTA = Ethylenediaminetetraacetic acid
EGTA = Ethylenebis(oxyethlenenitrolo) Tetraacetic acid

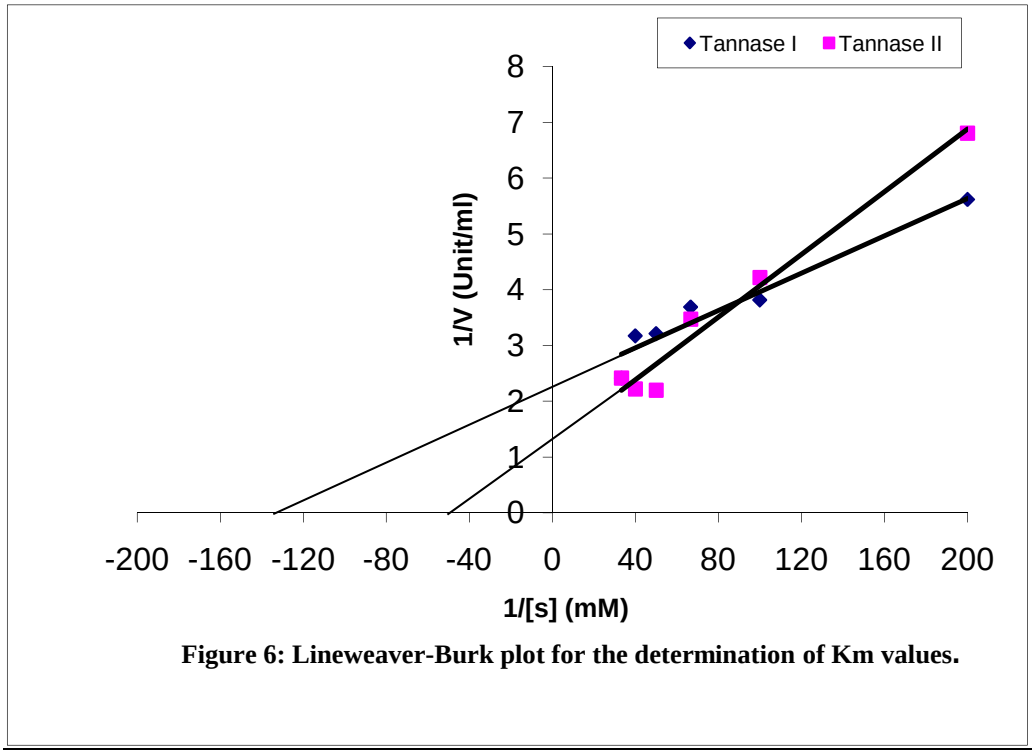

Figure 6: Lineweaver-Burk plot for the determination of Km values.

DISCUSSION

Aspergillus tamarii IMI388810 (B) produced two distinct extracellular tannases when grown in medium M (Figure 1). There are several reports on multiple forms of tannase produced by microorganisms. This multiplicity has been attributed in *Aspergillus*, to its ability to produce several tannase isoenzymes [27]. The two tannase forms separated in this work were bound to Phenyl Sepharose 6 FF (High Sub) column pre-equilibrated with 0.05M citrate buffer containing 1M $(NH_3)_2\ S0_4$. Both tannases I and II were eluted from the column with the equilibrating buffer only. They were both hydrophobic with tannase II being less weakly bound to the column.

The two tannases of *A. tamarii* IMI388810 (B) showed close biochemical properties with regard to temperature and pH activity optima. Tannase II had a higher optimal reaction temperature of

50°C while tannase I had at 35°C (Figure 3). The optimal temperature for activity of tannase of *A. niger* was reported to be 35°C [11]. This optimal temperature was the same for tannase I of *A. tamarii* IMI388810 (B). But the tannase isolated from *A. niger* van Tieghem and purified 29-fold had a temperature optimum of 60°C [28]. However, other workers [28, 19] reported that the optimum temperature of tannases from *A. oryzae*, and *Lactobacillus plantarum,* respectively was determined to be 40°C for the free enzyme. Other reports showed that *A. oryzae*, Asp. *sp.* AN11 and *P. chrysogenum* tannases have their optimal activity at 30°–40°C [1, 12] and *A. niger* LCF8 tannase had an optimum of 35°C [11]. This variation in biochemical properties of tannases could be attributed to differences between strains of species of the producing organism or mixture with other iso-enzymes produced by the organism.

Tannase I and tannase II had different optimal pH of 5.0 and 3.0, respectively (Figure 4). The pH optimum of tannase I agree with the reports [30, 31] that the pH optimum of tannase of *A. oryzae* is 5.0 to 6.0. In addition, a pH optimum of 6.0 was reported for both tannases of *A. niger* [11] and *A. niger* van Tieghem [27]. Therefore, tannase I of *A. tamarii* IMI388810 (B) compared closely with that of the *A. niger* in pH tolerance. Furthermore, from Figure 4 it is shown that tannase II had another minor peak at pH 6.0 and almost retained the same activity between pH 5.0 and 7.0. *A. niger* van Tieghem also had a second peak at pH 4.5 [27]. From Figure 5, Tannase II had optimal pH stability at pH 6.0 and was more stable between pH 4.0 and 6.0 than tannase I. It also retained over 65% of its activity between pH 3.0 and 8.0, while tannase I retained over 65% of its activity between pH 3.0 and 10.0 but had optimal pH stability at pH 3.0 and other two peaks at pH 6.0 and 9.2. This implies that tannase I can be stable in both acidic and alkaline conditions. This was similar to the tannase of *A. niger* being stable between pH 3.5 and 8.0 at temperatures below 50°C [11].

All the metal ions tested for their effect on tannase activity were inhibitory to tannases I and II (Table 2). The implication of this is that tannases of *A. tamarii* IMI388810 (B) will not be employed in detanification process containing any of these metal ions and enzyme poisons because they will inhibit or retard their activity.

The graphical analysis of the effect of substrate concentration on tannase activity yielded K_m of 0.008mM for tannase I and 0.02mM for tannase II (Figures 6). The K_m value for tannase from *A. niger* van Tieghem using methyl gallate as substrate were found to be 0.20mM [27]. This implies that tannases of *A. tamarii* IMI388810 (B) have higher affinity for methyl gallate. Tannase I had the higher affinity for methyl gallate than tannase II.

Enzymes with high temperature optimum and thermo-stability are preferred for industrial applications. From their substantial (comparative) activity and stability at 35°-50°C, the tannases of *A. tamarii* IMI388810 (B) should, therefore, be suitable for detanification processes which may be carried out at temperatures in this range, depending upon the nature of the material to be treated.

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