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Production and Purification of Tannase from *Aspergillus aculeatus* Using Plant Derived Raw Tannin

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Abstract:Tannase, an enzyme with immense potential has lot of applications in food, beverage, pharmaceutical and chemical industries. During the present study, tannin content of different plant materials was determined. Tannase has been produced from Aspergillus aculeatus through fermentation of tannin rich plant materials. Among the plant materials used, Cassia ciamia gives better result. Maximum oxygen is utilized by the organism after 24 h of growth. pH of the fermented broth falls to 4.1 after 28 h of growth. Organism utilizes wheat bran as a solid substrate to produce maximum tannase. Maximum enzyme production through solid state fermentation was achieved after 72 h of growth. Purified enzyme has pH & temperature optima at 5.0 and 60^{0}c respectively.

Key Words: Tannase, *Aspergillus aculeatus*, plant material, optimum pH, optimum temperature.

INTRODUCTION

Tannin acyl hydrolase (E.C.3.1.1.20), commonly called tannase hydrolyses ester and depside bonds of hydrolysable tannins (tannic acid) & thereby releases glucose, gallic acid and galloyl esters (Mehta *et al*., 2013). There are two types of tannin (a) Hydrolyzable tannin and (b) Condensed tannin. Hydrolyzable tannin consist of a polyhydric alcohol esterified with gallic acid or derivatives of gallic acid whereas Condensed tannin is made up of phenols of flavan type & are often called flavolans because they are polymers of such flavan 3-ols. According to the product obtained by its hydrolysis, Hydrolyzable tannins is classified into i) Gallatannin & ii) Ellagitannin. Tannase cleaves ester & depside linkage in tannic acid & chebulinic acid, both Hydrolyzable tannins. The enzyme tannase is extensively used in different industries such as food, beverage, brewing, pharmaceutical (Pourrat *et al*.,1987), chemical, cosmetics etc.

(Mehta *et al*., 2013). Instant tea preparation is one of the most promising application of this enzyme. The tea cream contains polyphenolic complex which are insoluble in cold water. Tannase make this insoluble tea cream soluble in cold water. The enzyme hydrolyses the ester linkage between galloyl groups & various compounds present in unconverted tea leaves. A continous process for solubilizing tea cream by passing a hot water extract of black tea through a column containing immobilized tannase was also described by Coggon et al. (1975). Besides, tannase has been found to be of immense importance in Beer chillproofing (Massechelin & Batum, 1981), Wine making etc. (Koichi & Tokuji, 1972). In animal feed additives, the use of tannin containing feed may prove beneficial in removal of antinutritional effects of tannin and improve their digestibility. Tannase may also find use in cosmetology to eliminate the turbidity of plant extracts and in the leather industry to homogenize tannin preparation for high-grade leather tannin. The enzymatic product gallic acid also has various industrial uses, such as- manufacture of gallic acid ester (propyll gallate), which in turn is an antioxidant, preparation of trimethoprim (a broad-spectrum anti-bacterial agent used as photosensitive resin in semiconductor production) and manufacture of pyrogallol.

In view of the above mentioned immense importance of the enzyme tannase and its products, various workers (Hadi *et al*.,1994; Lekha & Lonsane 1994; Bajpai & Pati 1996; Bradoo *et al*., 1997; Bhat *et a*l., 1998; Kar *et al*., 1999; Mondal& Pati 2000; Aboubakr *et al*., 2013) have studied the different aspects of its production. However, tannase is still considered a costly industrial enzyme owing to its low yield and long fermentation time of the processes (Kumar *et al*., 2007; Aboubakr *et al*., 2013; Mehta *et al*., 2013). Therefore, research is still needed to develop a cost effective protocol for tannase production. In this paper, we present our study carried on the production of tannase using raw tannin from different plant materials. Purification and some properties of tannase have also been studied. Proper exploitation of these techniques would enable a significant reduction in the cost of enzyme production.

MATERIALS & METHODS

Chemical: Following chemicals are used for experiment : Tannic acid (Qualigens, India), Gallic acid (Himedia, India), BSA [Bovine Serum Albumin, (Loba, India)] $MgSO_4$ (Qualigen), $(NH_4)_2HPO_4$,KH_2 Po_4, $CaCl_2$,Glucose, $(NH_4)_2So_4$, DEAE[diethyl amino ethyl] cellulose, Acetic acid, sodium acetate, sephadex G-100, NaOH, sodium tungstate, phosphomolybic acid, phosphoric acid, Na_2co_3. Otherwise stated all the chemicals are of analytical grade.

Microorganism: Aspergillus aculeatus .

Inoculum: Organism was grown on tannic a agar media containing (g/1) $(NH_4)_2HPO_4$, 3,0; $MgSO_4$, 1.0 KH_2 PO_4 0.05; $CaCl_2$, 0.3; tannic acid 10.0 & Agar, 25.0. The pH of the medium was adjust to 5.5 After growth at 30.^{0}c for 72 hrs spores are collected & spore suspension ($5X10^7$ spores/ m1) was used as source of inoculum.

Measurement of tannin content of some plants : Tannin content of different plant specimens were measured by Folin-Denis method. 10 gm of sample was taken with 50ml of distilled water & boiled for 30min. Then 0.2 ml of filtrate was taken & 8.3 ml, 0.5ml, 1ml of DH_2O, Folin-Denis reagent, 15% Na_2 CO_3 respectively was added. After retaining the mixture for 30 min. optical density was measured at 700 nm.

Production of tannase using raw tannin :

Different tannin rich plant materials ware inoculated with Aspergillus aculeatus I spores to find out their suitability for tannase production.

Fermentation in EYELA 5-L Fermentation : Organism was grown in a EYELA 5-L fermentor in 2.5 L of medium 20ml of spore suspension was used as inoculum. Composition of medium was same as inoculum medium except agar. Fermentation took place at 30^0c with pH 5.5 & the pH of the medium was adjust using 1 (N) NaOH or 1(N)HCL.

Solid State fermentation: Solid state fermentation (SSF) for tannase production was carried out in 500ml Erlenmeyer flask containing 20 gm of wheat bran with 5% (W/V) tannic acid & 50ml distilled water. Medium was inoculated with $5x10^8$ spores/ml & incubated at 30^0c with 80% humidity for 72hrs. After fermentation enzyme was extracted from the fermented solid by addition of 100 ml of 1% (W/V) aqueous solution of NaCl through agitation for 3hrs. in a rotary shaker (200rpm). Filtration was done using Whatman No.1 filter paper. Filtrate was centrifuged at 6000xg for 15min & the supernatant was assayed for tannase activity.

Preparation of enzyme: Fermented broth was filtered & filtrate was used as the source of extracellular enzyme. Harvested mycelium was homogenized & centrifuged & supernatant was used as the source of intracellular enzyme.

Purification of tannase: Intracellular & extracellular enzyme suspension was brought to 70% saturation with solid enzyme grade $(NH_4)_2$ So_4 & kept overnight at 4 ºC. The supernatant was separated by centrifugation (10,000 for 15 min) & the precipitate was taken & dissolved in 0.02(M) acetate buffer (pH 5.5). The remaining salt in the protein was removed by dialysis of the same. In a DEAE- cellulose packed column (2.5 x 30cm.) dialysis enzyme solution was applied & eluted through the acetate buffer (pH 5.5) containing linear gradient of NaCl [0.01-1.0(M)]. After elution, the active fractions containing tannase activity were collected & concentrated in lyophilizer & stored at 0ºC for further purification. The enzyme solution was now put on sephadex G-100 column (1.5 x100 cm) that is pre-equilibrated with 0.02 (M) acetate buffer & then eluted with same buffer (pH 5.5). The collected protein fractions were assayed for enzyme activity.

Properties of tannase :

(a) **Effect of temperature on tannase activity & stability :** To determine optimum temperature, tannase activity was measured at different temperature (30-80 ºC) in pH 5.5. Thermal stability of the enzymes was determined by incubating it at different temperatures for 60 min & then residual activity was measured at optimum pH & temperature.

(b) **Effect of pH on tannase activity & stability :** To find out optimum pH, tannase activity was measured at different pH values (3-8). For pH stability, the enzyme solution was kept at different pH values at 4ºC for 24 hrs & then residual activity was measured at optimum pH temperature.

Assay of tannase: Tannase activity was determined by the method of MONDAL et al. (2001). Enzyme solution (0.1 ml) was incubated with 0.3 ml of 1.0% (W/V) tannic acid, in 0.2 M citrate buffer (pH 5.0) at 400ºC for 30 min and then the reaction was terminated by the addition of 2 BSA (1 mg/ml), which precipitates the remaining centrifuged (5,000 x g, 10 min). The precipitate was dissolved in 2 ml of SDS triethanolamine (1% W/V, SDS in 5% V/V, triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1 ml of $FeCl_3$ (0.13 M) (systronics spectrophotometer 105). One unit of the tannase was defined as the amount of enzyme, which is able to produce 1 µg of gallic acid from tannic acid in 1 min at specific condition.

RESULTS & DISCUSSION

(A) Determination of tannin content of some plant material: Tannin content of different plant material was determined (Fig. I). It has been found that Acacia gives maximum tannin among the different plants tested. Eucalyptus, Psidium, Anacardium, Jam , Terminalia , Delonix are the moderate tannin producer.

(B) Production of tannase using raw tannin : Among the different plant sources tested Cassia ciamia gave better result (Fig. II a,b), where production of tannase is about 6 unit/ml. Fermentation was carried out in EYELA 5 L fermentor. This fermentation process gives maximum tannase and biomass after 30 hrs and 36 hrs respectively.

(C) Fermentation : Enzyme production by the organism was made in fermentor by using raw tannin.

(i) Fermentation at constant pH :- During submerged fermentation if pH retains constant, O_2 consumption varies with time (Fig : III a,b,c). During fermentation dissolved oxygen level decrease from 30% to 5% after 20 hrs of growth and again increased to 25% after 42 hrs. It has been found that oxygen consumption increased during active growth of the organism.

(ii) Fermentation at constant dissolved oxygen :- When D.O. become constant, initial pH 6.2, gradually falls to 4.1 after 28 hrs. of growth and then slowly increases upto 4.7 after 36 hrs. of growth (Fig : IV a,b,c). Actually gallic acid production takes place at initial growth phase when pH falls, but when organism utilizes gallic acid as carbon source, then pH slightly increase. Product analysis from the fermentation broth at different fermentation period confirms our prediction.

(D) Solid State fermentation : Solid state fermentation was carried out in presence of different solid substance & the result is represented in the table-1.The amount of enzyme production in relation to the substrates can be arranged in following order: wheat bran> rice bran> sawdust> rice straw dust> sugar cane pith. Earlier Chatterjee et al., (1996) showed that bran could enhance tannase production in Rhizopus oryzae. Enzyme synthesis was also studied in relation to tannic acid concentration (Fig :V a,b). It has been observed that higher concentration is inhibitory to tannase synthesis whereas lower concentrations are not repressive. Actually, tannic acid at higher concentration produces complex with membrane protein. Enzyme as well as gallic acid production were studied simultaneously in relation to time (Fig : Vi a,b). Organism produce maximum enzyme and gallic acid with in 72-84 hrs & in 60hrs respectively. Previously maximum extracellular tannase and gallic acid production in 96 hrs and 120 hrs has been

recorded in Aspergillus niger (Lekha and Lonsane, 1997) and Rhizopus oryzae (Chatterjee et al., 1996) respectively.

(E) Purification of extracellular and intracellular tannase : Various steps of purification of both extracellular and intracellular tannase was represented in Table-2. In DEAE-Cellulose column about six protein peaks are found of which 34-36th tubes posses tannase activity (Fig VII). In Sephadex G-100 Column single protein peak with maximum tannase activity at tube no 48-49 was found (Fig : VIII). The specific activity of purified enzyme was 79.7 unit/ mg protein. Earlier Banerjee et al. (2001) have reported the production and characterization of extracellular and intracellular tannase from newly isolated Aspergillus aculeatus. Costa et al. (2012) purified the extracellular tannase by using two chromatography techniques, filteration chromatography using G-150 sephadex column followed by ion exchange chromatography in a DEAE Sephadex column.

(F) Properties of purified tannase : The optimum pH of the enzyme was 5.0 and the enzyme retained more than 85% of its original activity between pH 5.0-6.0. The enzyme is stable in pH 4.0-7.0 (Fig : X a,b). Batra & Saxena (2005) reported that tannase from A. fumigatus and A. flavus is stable at pH 4.0. & doesn't show tannase activity at alkaline pH of 8.0. In contrast, A. versicolor tannase showed less stability at pH 3.0. with a maximum (100%) activity at pH 6.0.

Optimum temperature of the enzyme is 60ºC. Enzyme is stable upto 50 ºC and looses only 5% activity at 60ºC (Fig: IX a,b). Similar optimum temperature of the enzyme has been found in different spp. of Aspergillus by Batra & Saxena (2005). Banerjee et al. (2005) reported that maintenance of an optimal fermentation temperature is important as it affects the fungal growth, spore formation, germination, microbial physiology, and thus product formation.

Fig- I

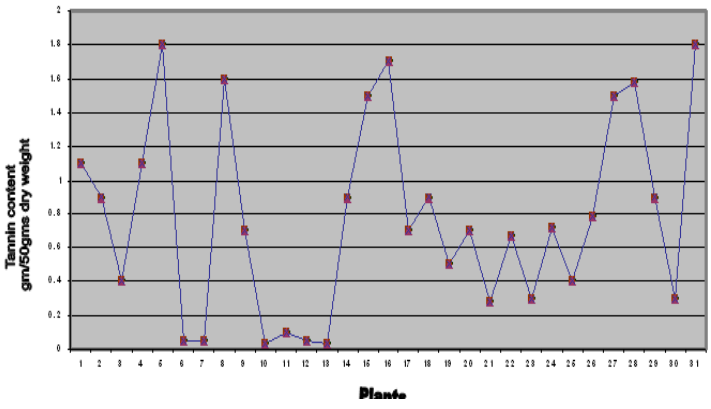

Fig –I : Tannin content of some plants (1. *Bruguiera* 2. *Soneratia* 3. *Rhizophora* 4. *Terminalia* 5. *Jam*; 6. *Curcuma* 7. *Tectona* 8. *Delonix* 9. *Shorea* 10. *Mimosa pudica* 11. *Zizyphus* 12.*Cassia* 13.*Alstonia* 14. *Casuarina* 15. *Anacardium* 16. *Acacia* 17. *Eupatorium* 18. *Trewia* 19.*Mimosa* sp 20. *Sida* 21.*Parthenium* 22. *Azadiracta* 23. *Crotolaria* 24.*Lantana* 25. *Carthamus* 26. *Cyperus* 27. *Ficus* 28. *Eucalyptus* 29.*Polyalthia* 30. *Calotropis* 31. *Psidium*.)

Fig-II(a)

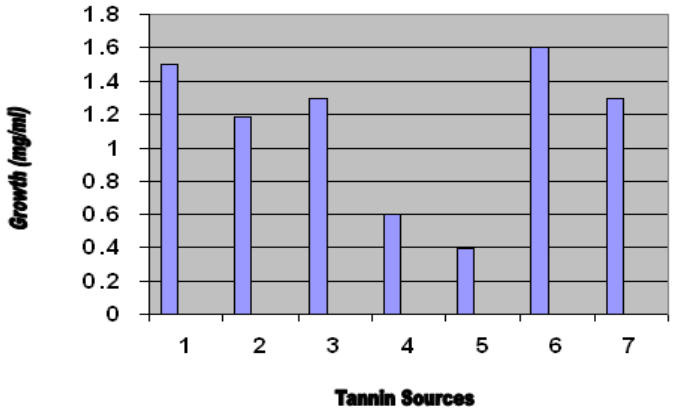

Fig-II (b)

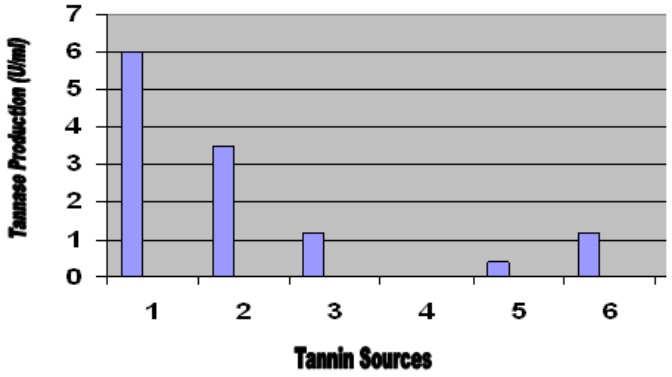

Fig-II (a,b) : Production of tannase using different plant tannin (1. *Cassia ciamia*; 2. *Delonix*; 3. *Anacardium*; 4. *Bruguiera*; 5. *Acacia*; 6. *Mimosa*; 7.*Casuarina*).

Fig-III(a)

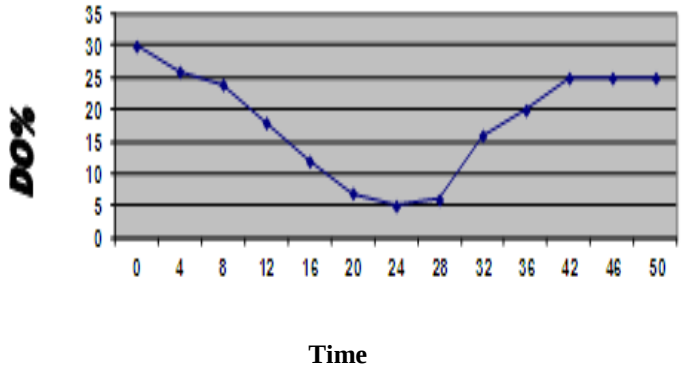

Fig-III(b)

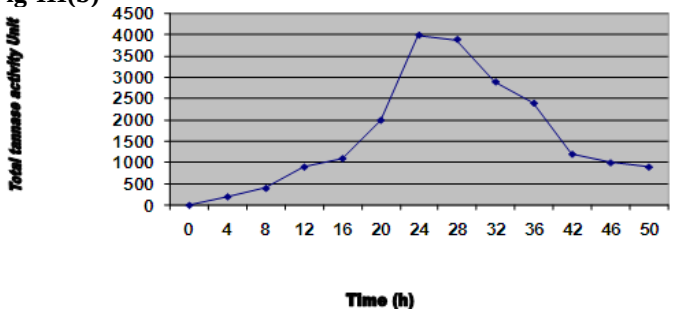

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Fig-III(c)

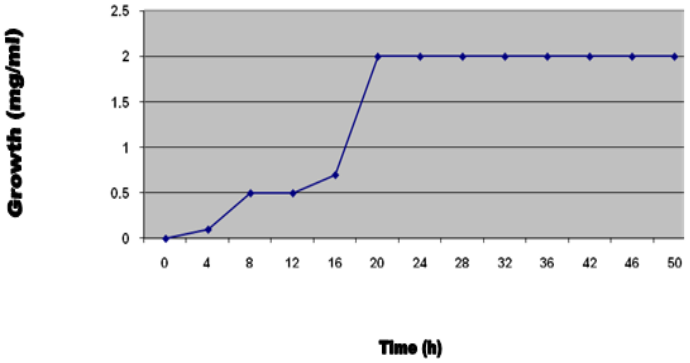

Fig-III(a,b,c) : Fermentation profile of *Aspergillus aculeatus* tannase fermentation where pH is fixed.

Fig-IV (a)

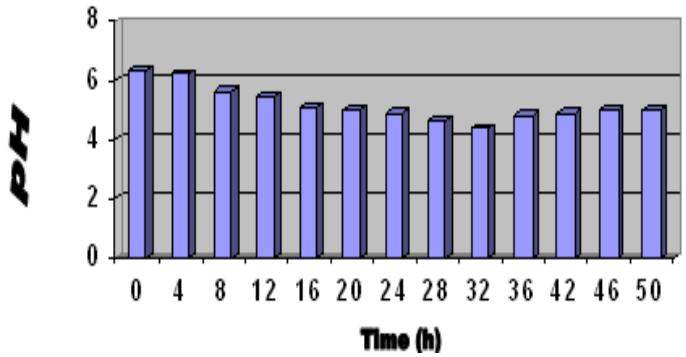

Fig-IV(b)

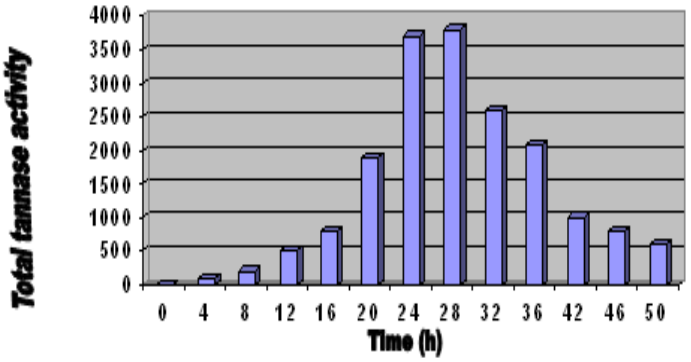

Fig-IV(c)

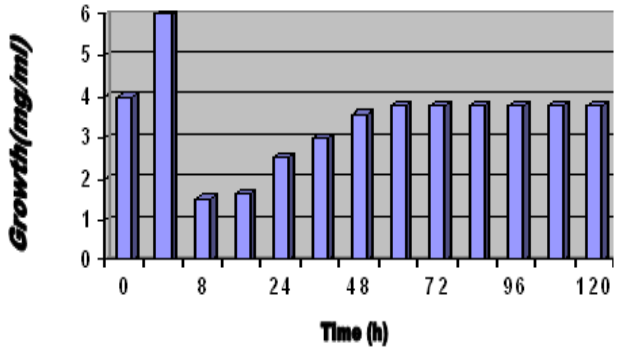

Fig –IV a-c : Fermentation profile of *Aspergillus aculeatus* tannase fermentation when dissolved oxygen is fixed

Fig –V(a)

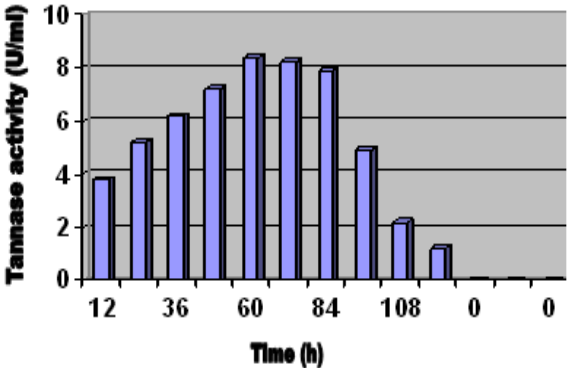

Fig-V(b)

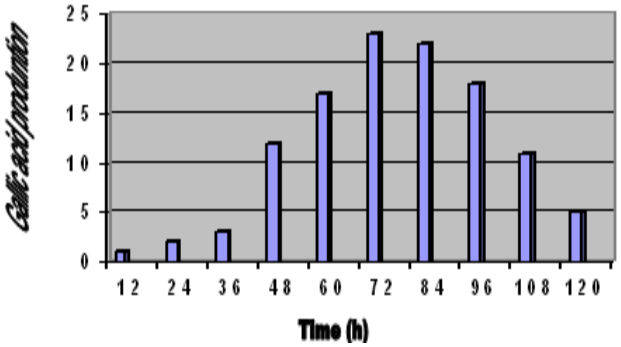

Fig-V (a,b) Effect of tannin acid concentration on tannase

Fig-VI(a)

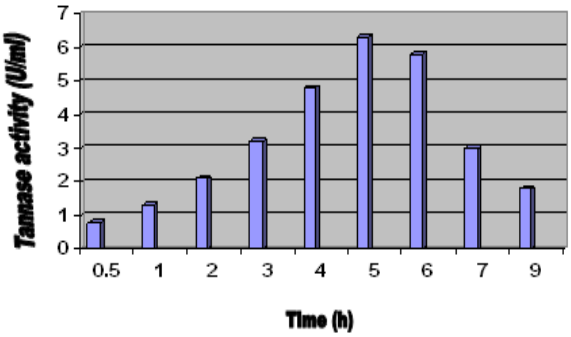

Fig-VI(b)

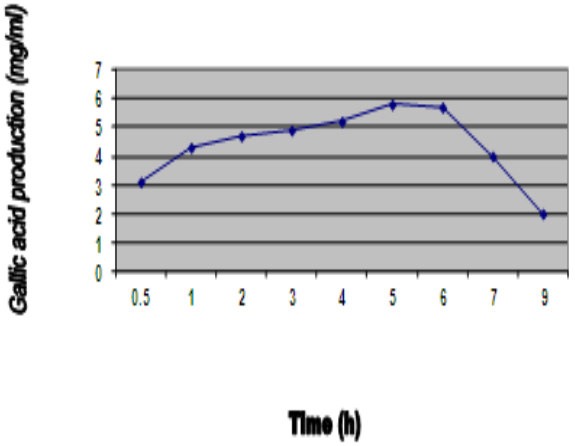

Fig-VI(a,b) Effect of incubation time on tannase activity

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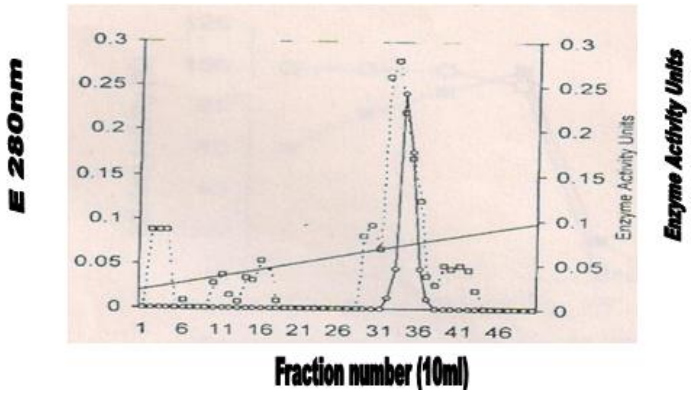

Fig-VII: Column chromatography of *Aspergillus aculeatus* tannase on DEAE-cellulose. The enzyme was applied to the column (2X25m) of DEAE- cellulose equilibrated with 0.02 M acetate, pH5.5.

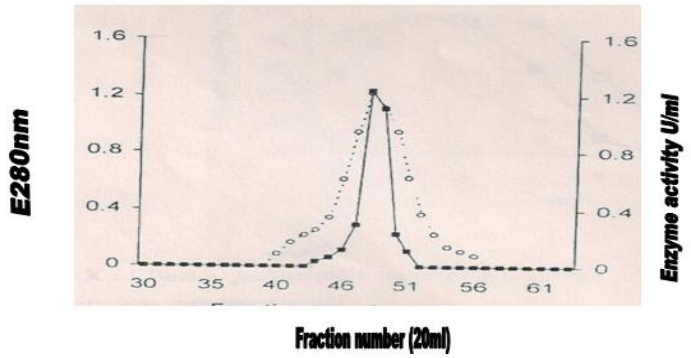

Fig –VIII: Column chromatography of *Aspergillus aculeatus* tannase on Sephadex G-100.

Fig-IX(a)

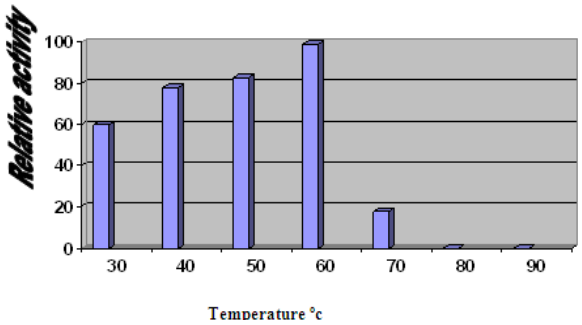

Fig-IX(b)

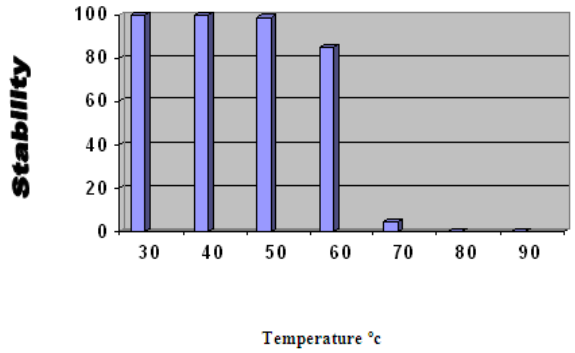

Fig- IX (a, b) : Effect of temperature on tannase activity

Fig-X(a)

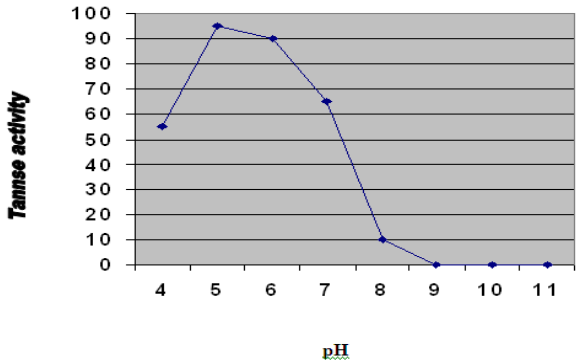

Fig-X(b)

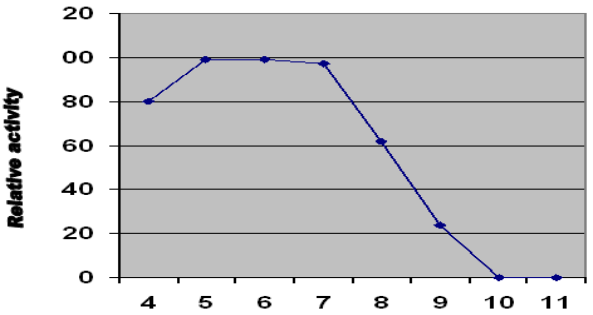

Fig –X(a,b) : Effect of pH on tannase activity

Step	Total Activity (U)	Specific activity (U/mg protein)	% recovery	Fold purificatication
Fermented Liquid	2,207	3.4	100	1
Ammounium Sulphate Precipitation	1,706	8.5	77.3	2.5
DEAE-cellusose Chromatography	1,162	22.9	52.6	6.7
Sephadex G-200 Gel Filtration	562	79.7	25.5	23.4

Table -1: Purification of tannase of *Aspergillus aculeatus*

Solid Substrate	Enzyme activity (U/ml)	Gallic acid production (mg/ml)
Saw dust	3.8	13.5
Wheat bran	8.5	16.3
Rice bran	4.7	14.7
Sugarcane pith	3.1	11.2
Rice Straw Dust	3.5	11.9

Table-2 : Effect of solid substrate on tannase and gallic acid production by *Aspergillus aculeatus* grown on 5% tannic acid at 300ºC for 72 h & pH 5.0.

CONCLUSION

The enzyme tannase has wider application in different chemical, pharmaceutical and food industries. Thus, it is important to reduce the cost and scale up tannase production as well as determine the factors affecting enzyme activity. This would enable the utilization of enzyme in the most efficient manner. We have prepared tannase from raw tannin sources, which is cheaper, so it is industrially useful.

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