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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF TANNASE FROM NATIVE *ASPERGILLUS SP* USING *SYZYGIUM CUMINI* (L) SKEELS (EUGENIA JAMBOLANA) SEED POWDER

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Abstract:-Tannin acyl hydrolase (E.C.3.1.1.20) commonly referred as tannase, is an industrially important enzyme that is mainly used in the food, chemical, beverage and pharmaceutical industry. In this study, tannase yielding native fungal isolate were identified as *Aspergillus niger* and *Aspergillus flavus.* The fungal culture produced high yields of extracellular tannase (30.05±1.32 U/ml and 23.43±1.43 U/ml) under solid-state fermentation (SSF) using *Syzygium cumini* seed powder. To enhance the production level of the enzyme, different culture conditions were optimized with the native isolates and observed that 30°C incubation temperature, pH 6.0, 1%(w/v) tannic acid as carbon source and 1%(w/v) Ammonium nitrate as a nitrogen source for maximum tannase activity. Tannase was purified from both isolates showed 2.29 & 1.43 purification fold with a specific activity of 10.24mg/ml & 12.36 mg/ml protein. This is the first report on production of tannase by *A. niger & A.flavus*, giving a higher yield of enzyme under SSF with Jamun seed powder as the substrate.

Keywords: *Aspergillus niger , Aspergillus flavus*,Jamun Seed Powder, Solid State Fermentation, Tannase

INTRODUCTION:

Tannins are naturally occurring secondary metabolites found in plants, and have been considered as the fourth most abundant plant constituents after cellulose, hemicelluloses and lignin. They are water-soluble polyphenols with a molecular mass ranging from 0.3 to 5 kDa [8, 41]. Tannase (tannin acyl hydrolase, E.C.3.1.1.20) specifically breaks the galloyl ester bond of tannins and produce gallic acid and glucose. Due to this property, the enzyme has been used extensively in foods, beverages, pharmaceuticals and chemical industries. Microorganisms have been reported to produce tannase capable of hydrolyzing tannins to Gallic acid during fermentation [30, 36]. The enzyme is also used in the pre-treatment of animal feed additives, to cleanup highly polluting tannin from the effluent of leather industry, pharmaceutical and chemical industries [1, 30, 32].

Tannase can be obtained from plant, animal and microbial sources. It is produced by a number of microorganisms like fungi *Aspergillus niger, Aspergillus oryzae, Aspergillus japonicus, Aspergillus gallonyce*s [10,26,32]. Yeast Candida sp., *Mycotororula japonica, Pichia sp.* [3,13] and bacteria *Bacillus pumilus, Bacillus polymyxa, Klebsiella pneumonia, Citrobacter freundii* [13,28].

A number of reports given by different workers showed the use of the liquid surface, submerged (SmF) or solid state fermentation (SSF) for the production of tannase. The submerged fermentation is mostly preferred for sterilization and process-control [30]. But this technique is not only expensive but also energy intensive, hence solid state fermentation (SSF) is the alternative method, since obtained levels of tannase are higher on solid substrates.

Solid-state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites. Agro-industrial residues are generally considered the best substrates for the process, including enzyme production, based on SSF [15]. Solid state fermentation involves non-aseptic conditions with the use of cheap, simple and easily available raw materials as substrates, along with several economical and engineering advantages including low capital cost, low energy expenditure, less expensive downstream processing, less water usage and

lower wastewater output, potential higher volumetric productivity, higher concentration of the products, high reproducibility, lesser fermentation space., easier control of contamination and generally simpler fermentation media [25].

Fungal tannase are very diverse and efficiently degrade different types of hydrolyzable tannins [13]. Filamentous fungi are ideal and the best adapted for SSF. The hyphal mode of fungal growth and their good tolerance to low water activity and high osmotic pressure conditions give fungi major advantages over unicellular microorganisms in the colonization of solid substrates and proper utilization of available nutrients [40].However, substrates used for tannase production under SSF had been like palm kernel cake (PKC), tamarind seed powder (TSP) [25] wheat bran [26,27] plant leaves like jamun leaves [28] etc. So keeping this in view, the present study, for the first time, reporting the production of high yields of tannase by a Native fungal isolate *A. niger and A. flavus* through solid state fermentation utilizing Jamun Seed powder, a cheap and easily available substrate.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade purchased from Sigma and Hi-media.

Microorganism and inoculum preparation

The fungal strain used in the present investigation was isolated from the ground under *Syzygium cumini* tree at Namakkal by making a 'V' shaped pit and identified as *Aspergillus niger* and *Aspergillus flavus* by the Department of Plant Biotechnology, Presidency college (Aut), Chennai. The fungal culture was grown separately on Potato Dextrose Agar (PDA) medium for 7 days at room temperature. The slants were then flooded with sterile distilled water and scrapped smoothly without disturbing the mycelial growth and filtered through sterile filter. The concentration of the filtrate was adjusted to 108 spores / ml and used as inoculum for further studies [17].

Preparation of SSF medium and effect of substrates on tannase production

The *Syzygium cumini* fruits were obtained from local markets, Coimbatore. Seeds were separated and shade dried. The dried seeds were ground into fine powder and then sieved. The substrate was stored in polyethylene bags at room temperature. They were autoclaved at 15 lbs for 20 minutes before use[9,39]. Ten grams of untreated *Syzygium cumini* seed powder were added to 250 ml Erlenmeyer conical flasks, moistened with 25 ml of Mineral salt solution [45] containing Ammonium nitrate - 0.5%, Magnesium sulphate - 0.1% Sodium chloride - 0.1% of pH 5.5. The SSF medium were autoclaved at 121°C for 20 mins, cooled to room temperature and inoculated with one ml of spore suspension (108 spores/ ml). The inoculated flask was slowly agitated for few seconds for complete spreading of spore suspension throughout the medium surface. The flask was incubated for a desired period of time.

Extraction of tannase enzyme

After 96 h of incubation, the fermented substrates were mixed properly by adding 50 ml of 0.2 M acetate buffer (pH 5.5) to the flasks [18, 42, 46]. Then, the flasks were kept on the rotary shaker at 150 rev min-1 for one hour. The crude enzyme was extracted by passing the extract through muslin cloth and centrifuged at 10,000 X g for 20 min. The filtrate was collected in test tubes and stored at 4°C for further analysis.

Enzyme assay

The estimation of tannase activity was carried out by colorimetric method of [34]. The tannic acid was used as a substrate at a concentration of 0.5% (w/v) in 0.2 M acetate buffer (pH 5.5). The reaction mixture was prepared by the addition of one ml substrate with 1 ml of the crude enzyme and incubated at 30°C for 30 minutes. The enzymatic reaction was stopped by adding 3 ml bovine serum albumin (1mg/ml) prepared with 0.17M sodium chloride in 0.2M acetate buffer (pH 5.0). The reference tube also prepared parallel using heat-denatured enzyme. The tubes were centrifuged at 5000g for 5 minutes. The resulted precipitate was dissolved in SDS triethanolamine solution followed by the addition of 1 ml of ferric chloride reagent. The contents were kept for 15 minutes for stabilizing the color formed and the absorbency was measured at 530nm against the blank. The specific extinction co-efficient of tannic acid at 530 nm was found to be 0.577 [34]. Using this co-efficient, one unit of tannase activity is defined as the amount of enzyme required to hydrolyze 1 mM of substrate (tannic acid) in 1 min under the assay conditions.

Determination of optimum parameters for production of tannase

The SSF medium containing the solid substrates and moistening media were taken under study to determine the

optimum condition with different pH, incubation temperature, concentration of substrate (Syzygium cumini seed powder), carbon sources, and nitrogen sources [4,45].

Effect of pH

The fungal culture was inoculated in the autoclaved SSF flasks containing moistening agent maintained at different pH ranging from 2.0 to 10.0. The contents were mixed thoroughly and then the cultured flasks were incubated at 25°C for 7 days and the maximal tannase production was determined [45].

Effect of different temperatures on tannase production

The SSF was carried out at different temperatures such as a range from 20-600C for 7 days and then biomass and tannase yield in the medium was assayed [4,45].

Effect of Substrate Concentration (*Syzygium cumini*)

The fungal culture was inoculated in the autoclaved SSF flasks containing different concentrations of *Syzygium cumini* seed powder from 1% - 5.0% w/v. The biomass and tannase yield in the media was assayed. The effective *Syzygium cumini* seed powder concentration in the tannase production was determined [4, 45].

Effect of different carbon and Nitrogen Sources

To study the effect of different carbon source on tannase production the medium was supplemented with different carbon sources (starch, glucose, tannic acid, glycerol and sucrose at 1% w/v) and nitrogen sources (peptone, yeast extract, ammonium nitrate, ammonium chloride, sodium nitrate at 1% w/v) of the fermentation media. The biomass and tannase yield in the medium was assayed. The effective carbon and nitrogen source for the tannase production was determined [4,45].

Characterization and Purification of tannase

The crude extracellular extract was dialyzed 24hrs against water and concentrated by freeze-drying. The lyophilized sample was reconstituted with a minimum amount of water. The soluble proteins were applied to a Sephadex G-150 column (2x60cm), previously equilibrated with 10 mM acetate buffer, pH 5.0. The protein fractions (4ml) were eluted at a flow rate of 2ml/min. Active fractions were pooled, dialyzed against water and concentrated by freeze drying. The concentrated fraction was then loaded onto a DEAE-Sephadex column (2x23cm), pre-equilibrated with 20 mM phosphate buffer, pH 7.0. The column was washed with the same buffer to remove unbound proteins. The bound proteins were eluted by applying a linear gradient of NaCl (0 to 0.5 M). The protein fractions (10ml) were eluted at a flow rate of 1 ml/min. The pooled active fractions were dialyzed against water, concentrated by freeze drying and stored at -20°C.

Protein estimation

Protein estimation was done by the Lowry method using bovine serum albumin as standard [31].

SDS-PAGE and molecular weight determination

SDS-PAGE was carried out to determine the purity of the enzyme and its molecular weight. Acrylamide concentration was 4% for stacking gel and 8% for separation gel [31]. The gel was run at 150V; 30mA till the tracking dye bromophenol blue reached the other end of the gel. Staining of the gel was done by keeping it overnight at room temperature in a solution of Coomassie blue in ethanol:acetic acid:distilled water (5:1:5). The excess dye was removed by keeping it in a de-staining solution containing 10% acetic acid and 35% ethanol till the gel became transparent. The 14 to 191 kDa proteins produced by partial cleavage of a 200 kDa protein were used as molecular markers.

Effect of pH and temperature on enzyme activity

The substrate solutions were prepared in 0.05M citrate buffer (pH 2.0-6.0) and 0.05M phosphate buffer (pH 8-10). The activity profile of the enzyme was determined by incubating the enzyme in buffer of varying pH, simultaneously the enzyme activity of tannase was assayed at different temperatures incubated at 20-60ºC for 5 minutes [38].

Effect of substrate concentration on enzyme activity

The effect of substrate concentration was studied by adding the substrate of different concentration (0.5gm to

2.5gm/100ml) to the enzyme and incubated under standard assay conditions [38]. The substrate concentration for maximum enzyme activity was determined in terms of maximum reaction velocity (V_{max}) and Michalies constant (Km at which reaction velocity is half-maximum). For this various concentration of tannic acid (0.5-2.5) in 0.05 M citrate buffer (pH-5) was incubated with purified enzyme preparations. The enzyme activity was measured at all concentration. The the V_{max} and K_m were estimated graphically by plotting substrate concentration on X axis against enzyme activity (U/ml) on the Y axis. The accurate values of V_{max} and K_m obtained from the double reciprocal line Weaver Burk plot (Line Weaver Burk in 1934). The double reciprocal plot was obtained from Line Weaver Burk equation, which states that $1/Vo = K_m/V_{max}$ [1/[s]] + 1/Vmax When 1/Vo plotted against 1/[s], a straight line was obtained. This line had a slope of K_m/V_{max} on the 1/Vo and an intercept of $1/K_m$ on the 1/[s] axis. Such a double reciprocal plot had the advantage of allowing much more accurate determination of the Vmax.

Effect of metal ions on enzyme activity

The effect of metal ions on the activity of the enzyme was examined with various metal ions of concentration 0.05M at pH 6 and temperature 30^0C for 10 minutes. The enzyme activity after the incubation time was determined under standard assay conditions [38].

RESULTS

Production of Tannase by *A. niger* and *A. flavus* was determined using *Syzygium cumini* seed powder as the carbon source through Solid substrate fermentation (SSF) and the best concentration was optimized. In addition, the enzyme was purified and characterized.

Effect of pH on enzyme production

The optimum pH for the enzyme activity was found to be 6.0 in both *A. niger* (28.2 U/ml) and *A. flavus* (23.50 U/ml) upon varying the pH from 2.0 to 10.0 (Table 1). The maximum production was observed in *A. niger* than in the *A. flavus* (Fig 1,2). It was shown that the wild isolate *A. niger* and *A. flavus* needs an acidic pH for the growth and production.

Effect of Temperature

The effect of incubation temperature on tannase production from *A. niger* and *A. flavus* was studied in the temperature range of 20º- 60ºC under solid substrate fermentation conditions. The optimum temperature for growth and tannase production from *A. niger* 30.0 U/ml (Fig 3) and *A. flavus* 25.42 U/ml (Fig 4) was found to be 30ºC (Table 1). A further rise in temperature decreased the production of tannase and the minimum tannase activity was observed.

Effect of Syzygium cumini seed powder Concentration

The ability of *A. niger* and *A. flavus* to grow and to produce tannase on different *syzygium cumini* seed powder concentration on the fermentation medium (1 – 5%) were studied (Table 1). The maximum tannase production from *A. niger* (30.05 U/ml) and *A. flavus* (23.43 U/ml) were obtained in 3% concentration (Fig.5,6). Further increase in concentration, decreased the production of tannase and the minimum tannase activity was observed

Effect of Carbon and Nitrogen source

Since tannase is an inducible enzyme, the production medium used in the SSF for the enzyme production were supplemented with different carbon (starch, glucose, tannic acid, glycerol and sucrose at 1% w/v) and Nitrogen sources (peptone, yeast extract, amm onium nitrate, ammonium chloride, sodium nitrate) separately. There was an increase in enzyme production with Carbon source and Nitrogen source (Table 1). Among this Tannic acid (Fig.7, 8) and Ammonium nitrate (Fig.9, 10) were the most suitable carbon and Nitrogen source for tannase induction of the fungal isolates.

Purification of Tannase

The purification and the activity of extracted tannase from both *A. niger* and *A. flavus* at different stages of purification is given in (Table 2). It has been noted that the DEAE cellulose column chromatography was giving the best yield of the enzyme in both the test organism. The specific activities of Tannase were measured about 2.96 U/mg of protein in *A.niger* and1.87 U/mg in *A.flavus* using *S. cumini* seed powder as substrate. SDS/PAGE analysis revealed the presence of one band with an apparent molecular mass of 51 kDa (Fig 11).

CHARACTERIZATION OF TANNASE

Effect of pH and Temperature on enzyme activity

The purified enzyme of *A. niger* and *A. flavus* incubated separately with acetate buffer with varying pH 2 – 10 and temperature increase from 20ºC to 60ºC. The optimum pH of the enzyme activity from both test organisms were observed in this study, pH 6.0, and the temperature optimum for tannase activity was observed at 40°C. This value was considerably better than those of tannases produced in liquid-state fermentation [7, 47].

Effect of Substrate Concentration on enzyme activity

The purified enzymes of *A. niger* and *A. flavus* were incubated with different concentrations of tannic acid ranging between 0.5gm/100ml to 2.5gm/100 ml under standard assay conditions. The purified enzyme of A. niger shows maximum activity supplemented with 2% w/v concentration whereas *A. flavus* reached maximum activity with 1.5% w/v concentration at room temperature for 5 minutes. Substrate concentrations for maximum enzyme activity in terms of V_{max} and K_m against tannic acid were determined. V_{max} and K_m Values were obtained from MM equation and Line Weaver Burk plot. Tannase enzyme from *A. nig*er had Vmax of 17.14 U/ml and Km of 3.35(%) (Fig 12).and *A. flavus* had V_{max} of 13.76 U/ml and K_m of 0.421(%) (Fig 13).

Effect of different Metal ions

The purified enzymes of *A. niger* and *A. flavus* were incubated with different cations. Tannase activity was stimulated by $Mgcl_2$ and NaCl. The elevated concentrations of Zn^{2+}, $Ca^{2+}$and Cu^{2+} drastically inhibited the tannase activity.

Statistical analysis

All the parameters were analyzed using T- test shows the significant differences (* < 0.05) in A. niger when compared to the A. flavus (Table 2).

DISCUSSION

Only a few fungi such as *Aspergillus niger*[30], *Aspergillus ruber* [27] and *Aspergillus aculeatus* [6] have been reported to produce tannase through SSF. Our study compared the yield of tannase between A. niger and A. flavus between these native isolates the best strain was identified based on the tannase production and its well characterized. A previous study showed that a pH around 5.5 [29] is the right pH for the production of fungal tannase from *Aspergillus strains* Similarly [44] had reported that the optimum pH of 5.5 was best for tannase production by *A. niger* ATCC 16620.620 4 had reported that the optimum pH of 5 was best for tannase production by A. niger. From earlier reports on Gallic acid production in SmF and SSF conditions, the optimum pH values were found to be 5.0 and 6.0, respectively [20,22 ,29] However it was confirmed the pH 6.0 was the optimum for the production of tannase in this study.

The Rise in temperature decreased the enzyme production in our experiment this may be due to the fact that with an increase in temperature, sporulation is induced thereby hampering mycelial growth. An optimum temperature of around 30ºC has been reported by many workers in various fungi [5,6, 11, 22,45] except [24] who reported optimum temperature of 16ºC for the maximum tannase production by Verticillium sp. This filamentous fungus produced two types of tannase which were optimally active at 25ºC and 20ºC. In our investigation, the selected fungal strains were found to produce tannase at 30ºC which may account for difference in optimum temperature from other workers.

Increase in the substrate concentration of this study showed decrease the activity of tannase, this may be due to the fact that tannic acid at higher concentration produces complexes with membrane protein of the organism and inhibits the growth and enzyme production [5]. In the present investigation, to the best of our knowledge we are first time reporting the use of Jamun (S. Cumini) seed powder as a solid substrate for the production of tannase by A. niger and A. flavus. There are no such reports of production of tannase through SSF using Jamun seed powder . Only a few reports of tannase production through SSF using different substrates like palm kernel cake, tamarind seed powder, wheat bran [19,45] are available in the previous studies.

The carbon source amendment studies result revealed that tannic acid was the best carbon source for the production of tannase by A. niger and A. flavus. In PKC (Palm Kernal Cake) medium tannic acid induced enhanced synthesis and secretion of tannase. The sugars can stimulate tannase production less but they mostly inhibit tannase production [1, 27]. [45] reported the decrease in tannase activity in the presence of glucose. A number of workers studied fungal tannase production in the presence of different inorganic nitrogen sources such as sodium nitrate, ammonium oxalate, ammonium sulfate and ammonium chloride [14,30,48, 50] all of these sources were found to be stimulatory. As told in the previous reports in our investigation Ammonium nitrate an inorganic nitrogen source stimulate the higher tannase production, Probably the presence

of additional nitrogen sources along with nitrogenous compounds present in the substrate promotes enhanced growth and consequent enzyme production [12].

In Present study SDS/PAGE analysis revealed the presence of one band with an apparent molecular mass of 51 kDa According to [21] the tannase from A. oryzae has two subunits of 30 and 33 kDa, and the native tannase is a hetero-octamer with a molecular mass of 300 kDa.[35] reported the production of two extracellular tannase but cold adapted from Verticillum sp. each consisting of approximately 40 and 60 kDa subunits.

DEAE-cellulose column chromatography led to an overall purification of 3 fold with a yield 10- 12 mg/ml of total protein in both isolates in agreement with those of previous study. However, the purification factor was similar to that of the purified tannase obtained from various different fungi, as reported by other workers [7,16,47]. [16] working on a Cryphonectria tannase, detected activity between pH 2.5 and 8.5, and high and stable activity was observed between pH 4 and pH 7. We determined the Km values for tannic acid of tannases produced by other fungal strains ranged from 0.20 to 1.03 mM [37, 43,45] Similar to those reported previously [23].The metal ions towards the enzyme activity reveled Mgcl2 and NaCl was the potential metal ion to stimulate the production of tannase whereas Zn2+ was found to be the potent inhibitor of tannase.

CONCLUSION

In conclusion, the tannase production by microorganism is extensively studied with respect to the food and pharmaceutical industries. However we are reporting for the first time the Native fungal culture A. niger capable of producing high yields of tannase under SSF conditions than A. flavus with Jamun seed powder as substrate which is cheap and easily available in abundance as agro forest residue. It makes the process of gallic acid production economic and ecofriendly, and also suggests a beneficial utilization of agro wastes. In future, It is very important to scale up the production of tannase by using various substrates and recombinant engineering techniques so that the industrial demand for tannase is taken care of on a large scale.

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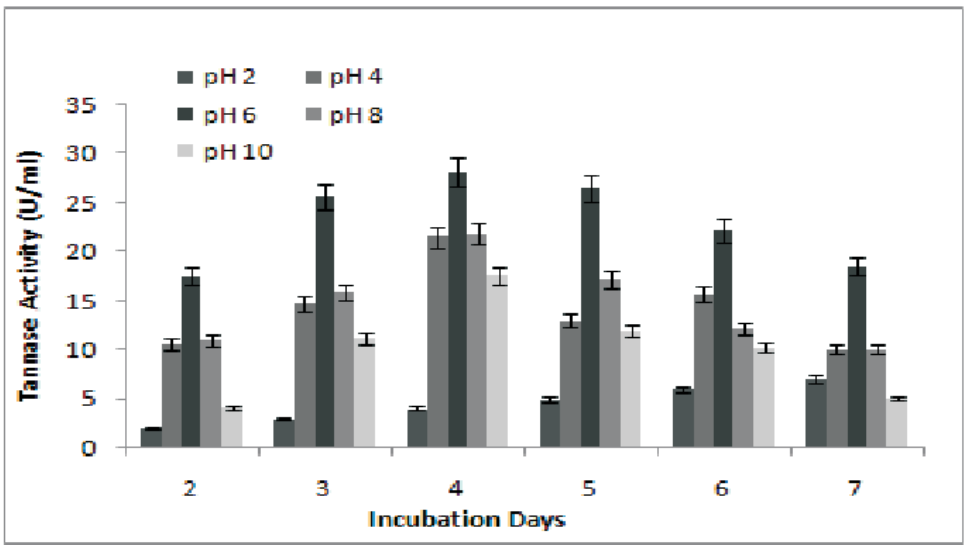


Fig 1 : Effect of pH on tannase production by Aspergillus niger

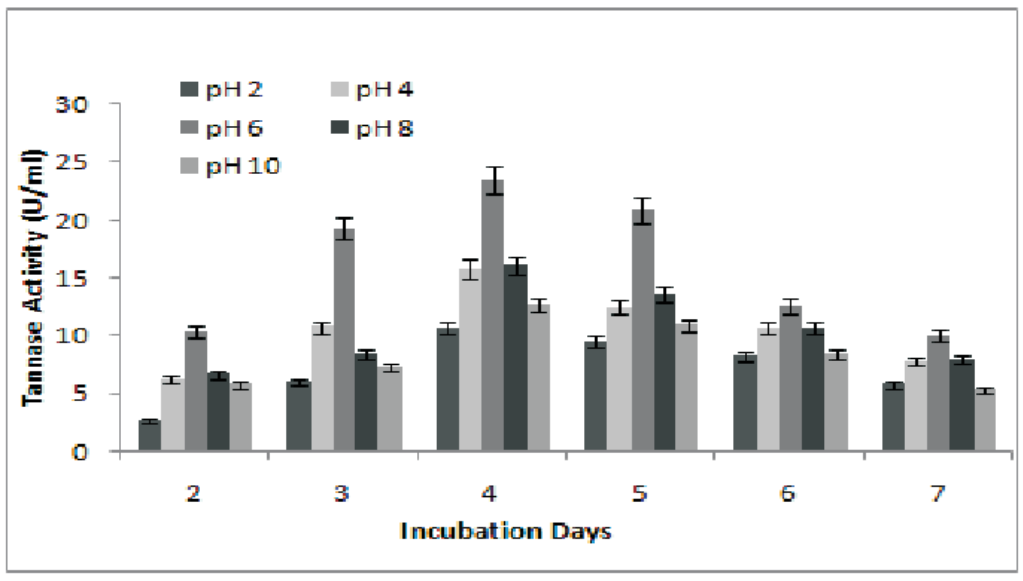


Fig 2 : Effect of pH on tannase production by Aspergillus flavus

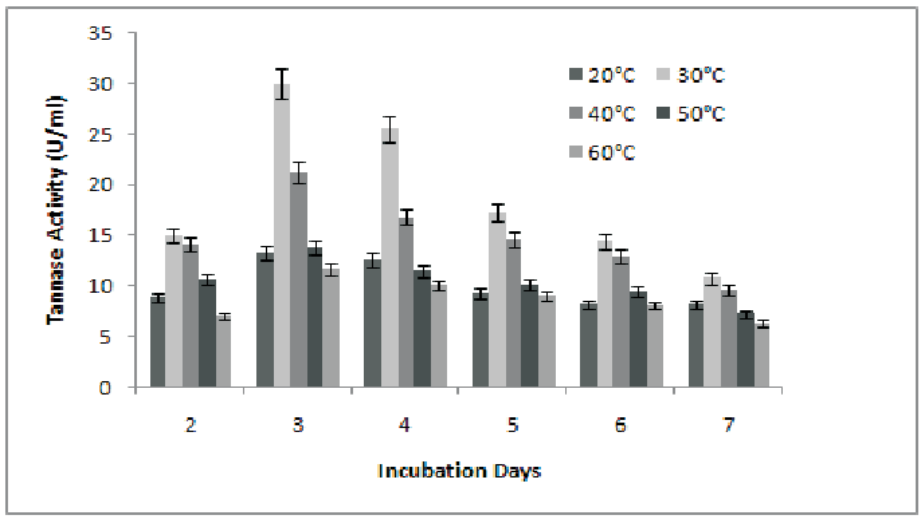


Fig 3 : Effect of Temperature on tannase production by Aspergillus niger

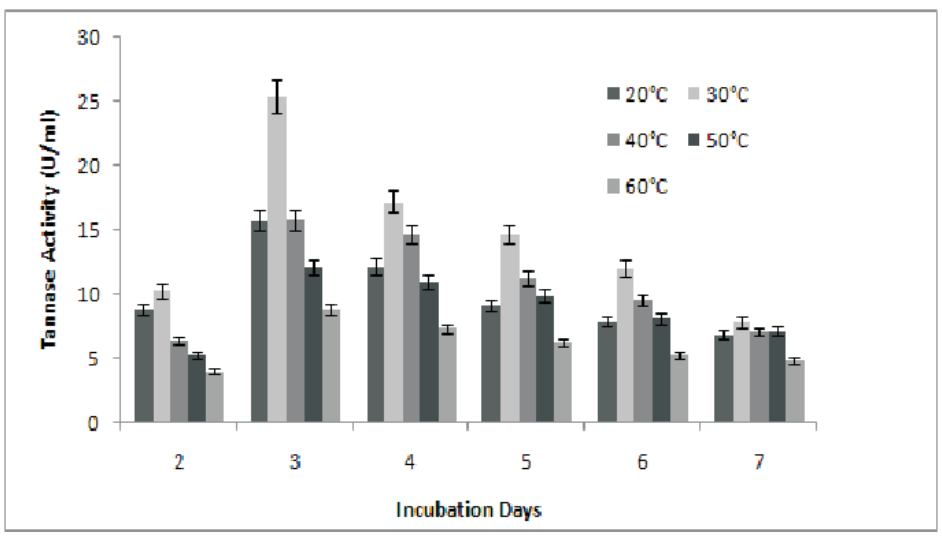


Fig 4 : Effect of Temperature on tannase production by Aspergillus flavus

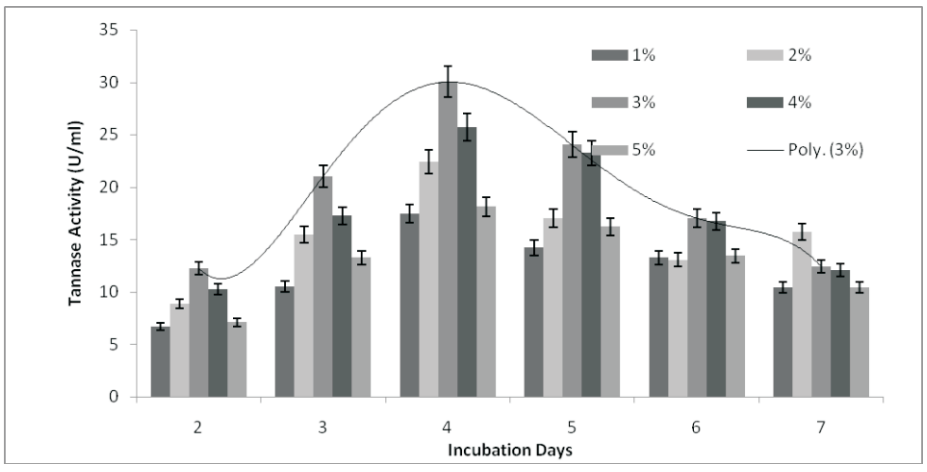


Fig 5 : Effect of Substrate on tannase production by Aspergillus flavus

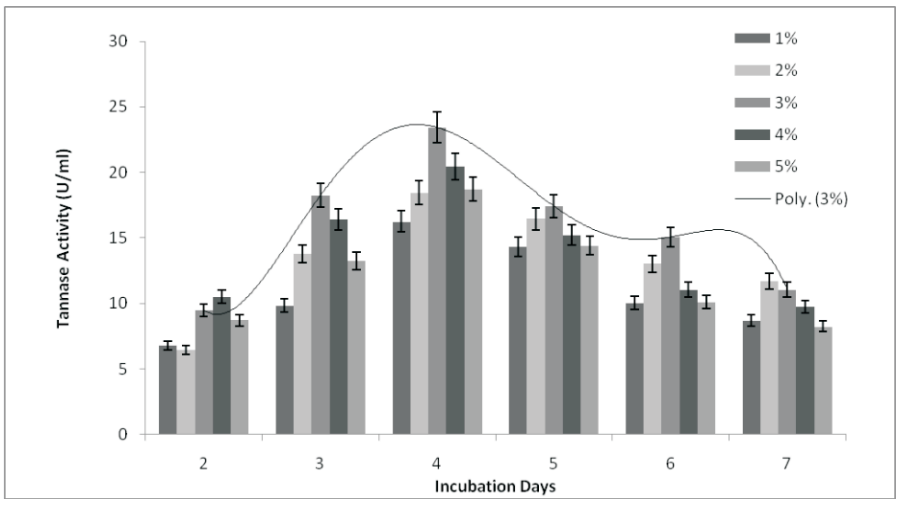
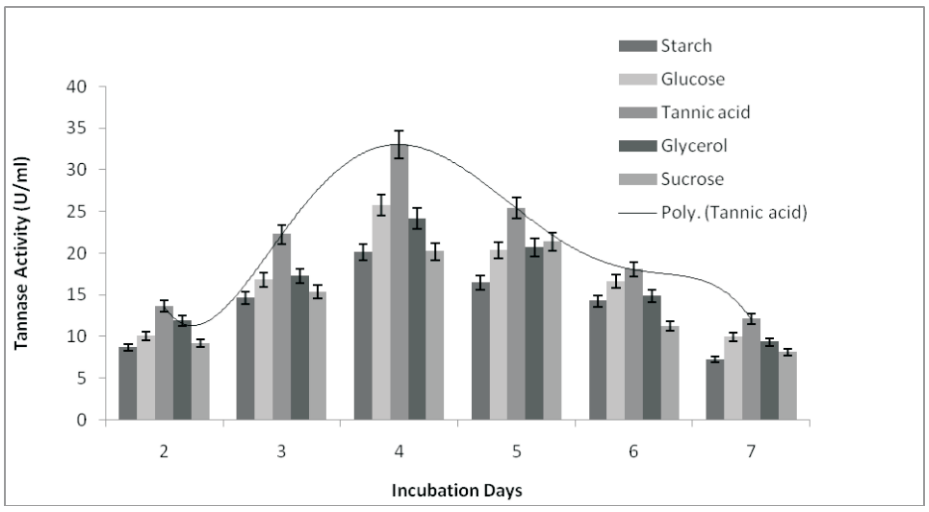
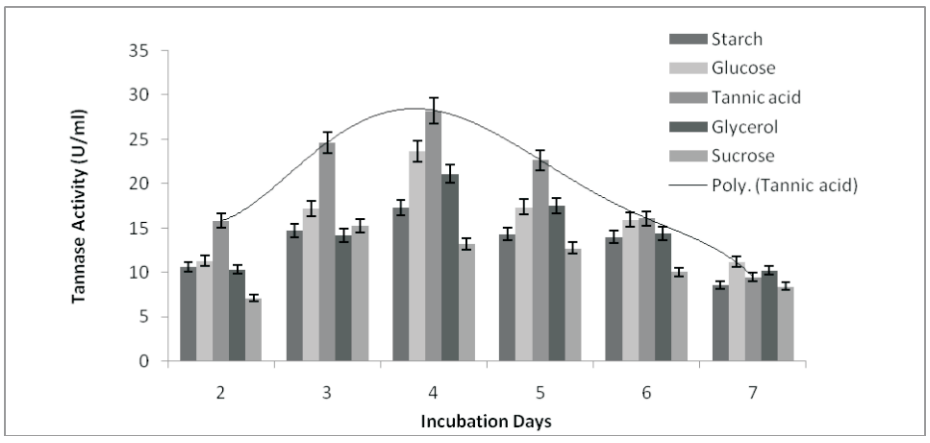
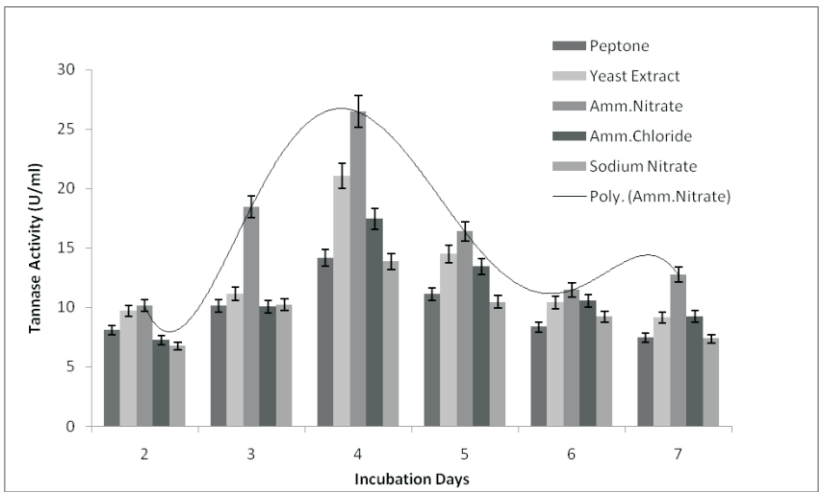


Fig 6 : Effect of Substrate on tannase production by Aspergillus niger

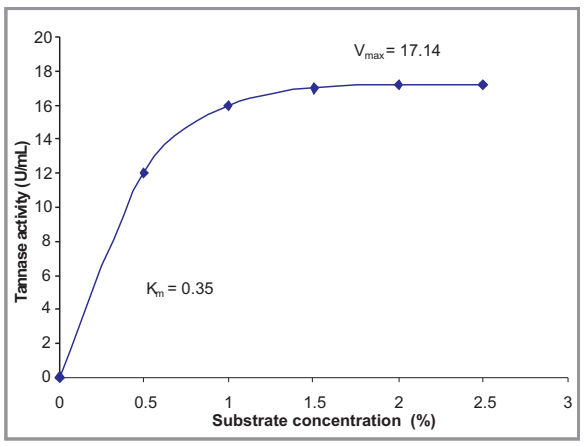




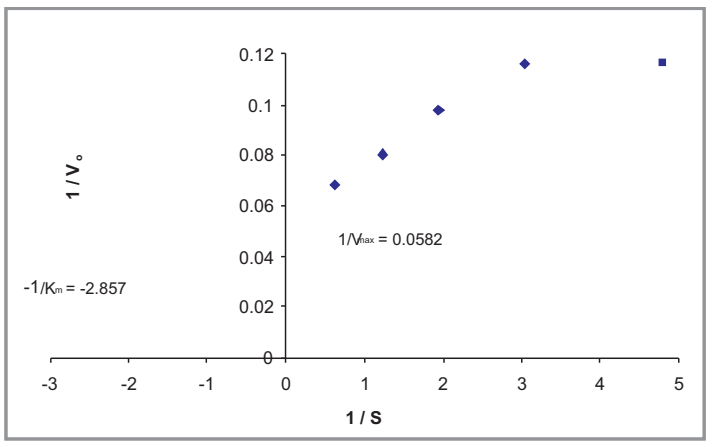


Effect of Substrate concentration on Tannase Activity by *Aspergillus niger*

MM plot

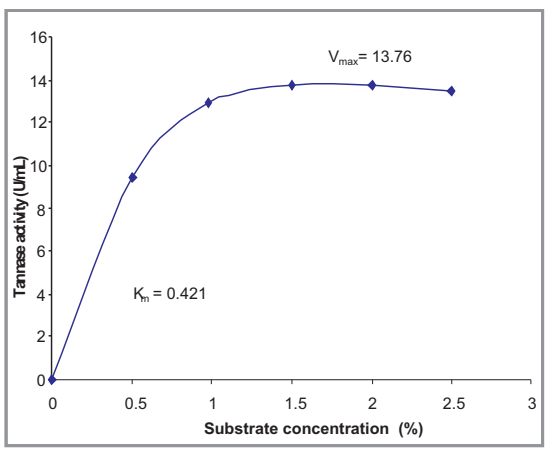


Line Weaver burk plot

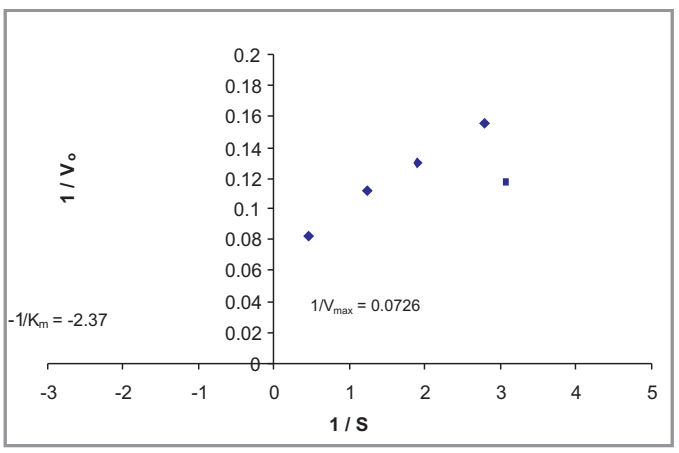


Substrate concentration on tannase Activity by Aspergillus niger

MM plot



Line Weaver Burk Plot



S.NO	Parameters	SSF Range	Optimum	*Aspergillus niger*	*Aspergillus flavus*	' t ' test
1	**pH**	2-10	6.0	28.25±1.97	23.50±1.38	2.784*
2	**Temperature**	20-60°C	30°C	30.0±1.48	25.42±0.99	3.626*
3	**Substrate of *Syzygium* *cumini* seed powder**	1-5%	3%	30.05±1.32	23.43±1.43	4.81*
4	**Carbon source (Starch, Glucose, Tannic acid, Glycerol, Sucrose)**	Tannic Acid		33.04±1.13	28.25±1.28	3.955*
5	**Nitrogen source (Peptone, Yeast extract, Ammonium nitrate, Ammonium chloride, Sodium nitrate)**	Ammonium nitrate		26.47±1.38	24.42±0.976	1.715^{ns}

Data represent the mean values ± Standard deviation. (* - t < 0.05); ns – not significant.

S.NO	Enzyme	Tannase Activity (Units)		Total protein (mg\ml)		Specific Activity (Units/mg)		Purification fold	
		A niger	*A flavus*	*A niger*	*A flavus*	*A niger*	*A flavus*	*A niger*	*A flavus*
1	**Crude extract**	85.23	76.42	66.10	58.42	1.29	1.31	1	1
2	**70% Ammonium sulphate saturation**	76.47	61.23	54.24	41.32	1.41	1.48	1.093	1.130
3	**Dialysis**	45.5	32.74	15.72	20.74	2.89	1.58	2.240	1.206
4	**DEAE Cellulose Column Chromatography**	30.40	23.14	10.24	12.36	2.96	1.87	2.294	1.427

All the parameters analysed using't' test shows the significant different (* < 0.05)

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