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Production, characterization and purification of tannase from *Aspergillus niger*

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ABSTRACT

Tannin acyl hydrolase (E.C.3.1.1.20) commonly referred to as tannase, is an industrially important enzyme that is mainly used in the food, chemical, beverage and pharmaceutical industry. In this study, tannase production was investigated using Aspergillus niger isolated from bark of tannin rich Acacia nilotica. Optimization of culture conditions for maximum tannase production included studying the effects of incubation period, incubation temperature, pH, carbon and nitrogen sources, inducers and metal ions on A. niger and enzyme activity. The optimum culture conditions determined were tannic acid as inducer, 7 days(168hrs) incubation period, 30°C incubation temperature, pH 5.0, 1%(w/v) tannic acid as carbon source and 1%(w/v) sodium nitrate as nitrogen source for maximum tannase activity. Tannase was purified 7.17 fold with a specific activity of 101.428U/ml protein and a yield of 18.35%. Molecular characterization included the zymography and purification of tannase enzyme.

Key words: Tannin acyl hydrolase, *Aspergillus niger*, enzyme production, characterization, purification.

INTRODUCTION

Tannin Acyl Hydrolase (E.C. 3.1.1.20) is an inducible enzyme commonly known as tannase. Tannase (TAH) catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate, n-propyl gallate and isoamyl gallate, releasing glucose and gallic acid. Gallic acid catalyzes the second step in the degradation of the polyphenol, tannic acid [17]. Tannase is known to be a membrane bound enzyme [22] and is also secreted outside the cell [13]. Tannase can be obtained from various sources such as animals, plants and microorganisms [1, 15, 30]. Tannase is produced extensively from fungi as they are fast growing and very diverse naturally. Their environmental and genetic manipulation is easier, needed for generation of novel producers in reduced time period. Tannase from fungal sources is reported to be highly active over a wide range of pH and temperature. Although tannase production by *Aspergillus niger* can occur in the absence of tannic acid, this fungus tolerates tannic acid concentrations as high as 20%(w/v), without having a deleterious effect on both the growth and enzyme production [24].

Fungal tannase is used in many industrial applications including clarification of fruit juices [4], de-tannification of food [10], preparation of food preservatives [24], high grade leather tanning [20], clarification of beer and wines [5], beer chill proofing [23], manufacture of coffee flavored drinks [33], manufacture of instant tea [20], production of

gallic acid which is used for the synthesis of trimethoprim [32], manufacture of pyrogallol and propyl gallate, dyes and inks [29], treatment of green tea to inhibit the carcinogenic and mutagenic effects of N-nitrosamines, stabilization of malt polyphenols [21], improved color stability and additional organoleptic properties. In animal feeding, tannase is used to reduce the anti-nutritional effects of tannins and improved animal digestibility [7]. Tannase is also utilized for bioremediation of effluents from tanneries [24]. In addition, tannase is used as a sensitive analytical probe for determining the structure of naturally occurring gallic acid ester [11]. Thus, *Aspergillus niger* isolated from tannin-rich bark of *Acacia nilotica* has been used as an important bioreactor in this study for maximum tannase production.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents were of analytical grade purchased from HI-MEDIA Pvt. Ltd., Mumbai, India.

Microorganism

One hundred and fifteen fungal isolates were collected from the bark of *Acacia nilotica* trees from AOC Centre, Secunderabad, Andhra Pradesh, India in the month of November, 2010. Fungal isolates that succeeded to grow in the presence of tannic acid-supplemented Potato Dextrose Agar media were tested for tannase production individually.

All fungal species were identified by microscopic analysis by using taxonomic guides and standard procedures [14]. The most active tannase producing fungus was identified as *Aspergillus niger* and was maintained on PDA plates containing tannic acid and stored at 4°C and sub-cultured at monthly intervals.

Qualitative screening for tannase producing *Aspergillus niger* culture was carried out on tannic acid agar plates (TAA) containing 1% tannic acid and 3% agar and recording the clear zones formed due to hydrolysis of tannic acid around the fungal colony [26].

Preparation of spore suspension

Fungal spore suspension was prepared by adding 10ml of sterile distilled water containing 0.1% (v/v) Tween 80 to a fully sporulated and induced culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions and then vortexed. The spores were counted using Neubauer chamber before inoculation. The volume of 1 ml of the prepared spore suspension containing 3×10^7 spores was used as inoculum.

Mode of fermentation

Cultivation of fungus was carried out by submerged fermentation (SmF) in 250ml Erlenmeyer flasks using the Czapek's Dox medium supplemented with filter sterilized tannic acid at 1% concentration as sole source of carbon, autoclaved at 121°C; 15 lbs. for 20 minutes and then inoculated with prepared inoculum (1%v/v) and incubated for 72 hours at room temperature (28±2ºC). Samples were withdrawn at regular intervals of 24 hours and observed for tannase activity.

Enzyme extraction

Tannase was extracted according to standard enzyme extraction method [26] and was either stored at -20°C or used immediately.

Tannase assay

Tannase activity produced by *Aspergillus niger* isolate was assessed using the Rhodanine method [28]. 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 the amount of enzyme required to liberate one micromole of gallic acid formed per minute under the defined reaction conditions.

Protein determination
The total soluble protein was determined by measuring optical density of developed color at 595 nm [16]. The µg of protein was estimated using µg standard of bovine serum albumin (BSA).

Optimization of factors affecting tannase activity:
Effect of tannic acid concentration: Tannic acid was used as an inducer for maximum tannase production varying from 0.5%-10% (w/v) concentrations. Flasks were maintained in triplicates and tannase activity was determined as mentioned earlier.

Effect of different incubation periods: To evaluate the effect of different incubation periods on tannase production, the flasks were incubated at 30°C for different time periods ranging from 3 days to 21 days intervals. Samples were withdrawn periodically at every 24 hours and assayed for tannase activity as described earlier.

Effect of different incubation temperatures: To study the effect of different incubation temperatures on tannase production, the flasks were kept at temperatures ranging from 15°C to 65°C for 5 days. The tannase activity was measured as described earlier.

Effect of different pH: The effect of initial pH on tannase production was studied by adjusting the production medium at various levels of pH by IN HCl and IN NaOH solutions ranging from 3.0 to 11.0. The tannase activity was measured as described earlier.

Effect of different carbon sources: Several carbon sources such as Mannose, Galactose, Glycerol, and Ribose at concentrations ranging from 0.5% to 2% were supplemented to the culture medium. The tannase activity was determined as described earlier.

Effect of different nitrogen sources: Several organic nitrogen sources such as Peptone, Yeast extract, Beef extract, Urea and Casein as well as inorganic nitrogen sources such as Ammonium sulfate, Ammonium chloride, Ammonium nitrate, Ammonium dihydrogen phosphate, Sodium nitrate and Potassium nitrate at concentrations ranging from 0.5% to 2% were supplemented to the culture medium. The tannase activity was determined as described earlier.

Effect if different metal ions: To evaluate the effect of metal ions on the production of tannase, different concentrations of metal ions (Zn^{+2}, Mo^{+6}, Al^{+3}, Mn^{+2}, Cu^{+3}, Li^{3+}) ranging from 1mM to 20mM were used as additives in the culture medium. The tannase activity was determined as described earlier.

Characterization and Purification of tannase:
Enzyme purification
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. In both columns, each fraction was assayed for protein (280nm) and tannase activity.

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 dye excess was removed by keeping it in a de-staining solution containing 10% acetic acid and 35% ethanol till the gel became transparent. The 10 to 120 kDa proteins produced by partial cleavage of a 120 kDa protein were used as molecular markers.

Molecular weight of native tannase

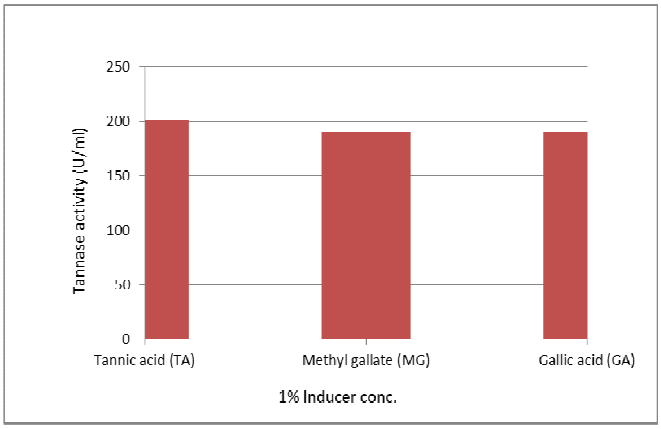
The molecular weight of native tannase was determined by gel filtration chromatography using Sephadex G-200. The mixture of high non-denaturing molecular weight markers (2mg) was loaded on Sephadex G-200 (2 x 70cm) and eluted with 0.05 M acetate buffer plus 0.1 M NaCl at a flow rate of 1 ml/min. The void volume of 65ml was determined using Blue Dextran 2000. The samples containing proteins were collected the native molecular weight of tannase was determined. All these protocols were conducted under 4°C conditions.

RESULTS AND DISCUSSION

Effect of tannic acid concentration: Tannase activity was affected by concentration of inducer in the medium. Maximum tannase activity was observed at a concentration of 1% (w/v) tannic acid as inducer (Fig.1). Similar results were reported previously using gallic acid and methyl gallate as inducers [6, 21].

Fig.1 Effect of Inducers on Tannase activity

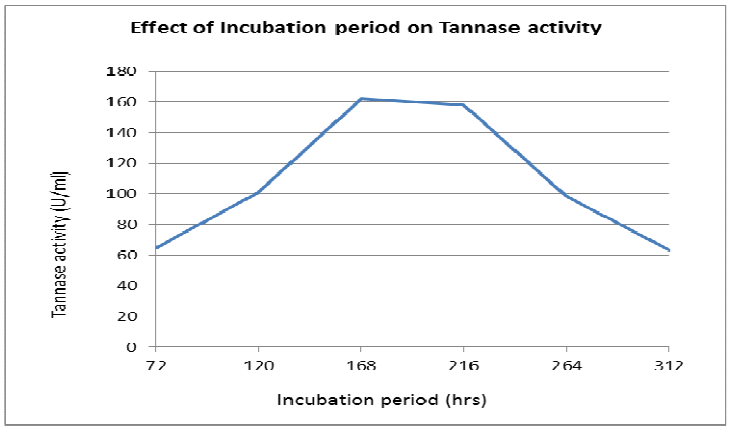
Inducer	Tannase activity (U/ml)
Tannic acid (TA)	201.234
Methyl gallate (MG)	189.505
Gallic acid (GA)	189.814



Effect of different incubation periods: Optimization of incubation periods (3-14 days) was carried out in order to determine the best harvesting time for tannase from production culture. Maximum tannase activity (162.3 U/ml) was observed on the 7th day (168 hrs) of incubation (Fig. 2). Different incubation periods of were reported previously too [9, 12, 27].

Fig. 2 Effect of Incubation period on Tannase activity

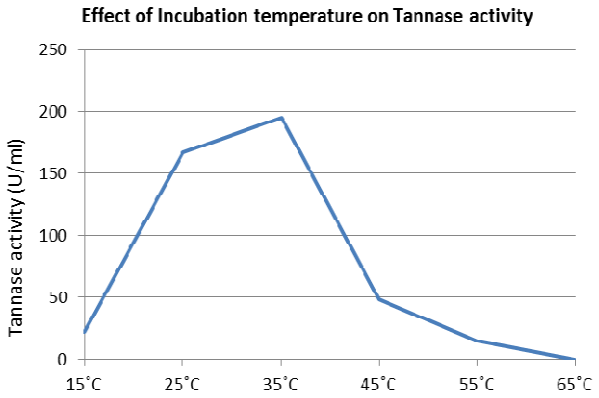
Hours	Tannase activity (U/ml)
72	64.814
120	100.822
168	162.345
216	158.23
264	98.559
312	63.374



Effect of different incubation temperatures: Maximum tannase activity (195.0 U/ml) was observed at an incubation temperature of 30°C although the organism had a capacity to grow over a wide temperature range (25°C to 35°C) (Fig. 3). Tannase production by *A. niger* can be evaluated at room temperature in our nation without additional efforts or costs. Similar optimum temperatures were reported for tannase from fungal isolates previously [3, 18**]**.

Fig. 3 Effect of Incubation temperature on Tannase activity

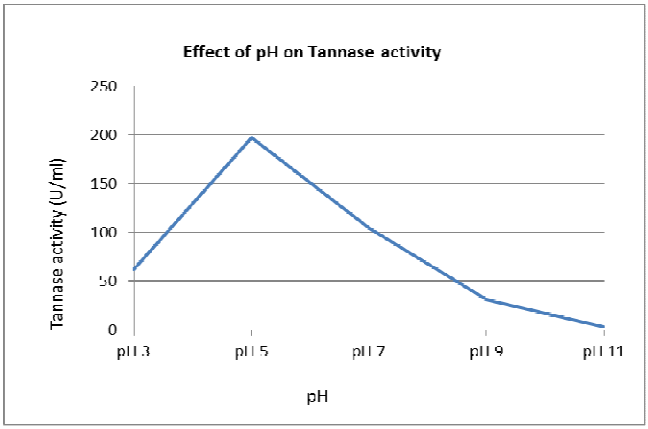
Temperature	Tannase activity (U/ml)
15˚C	22.221
25˚C	167.077
35˚C	195.061
45˚C	48.147
55˚C	15.02
65˚C	0



Effect of different pH: Different pH values (3.0, 5.0, 7.0, 9.0 and 11.0) were chosen to investigate the effect on tannase activity in the culture medium. Maximum tannase activity (196.9 U/ml) was observed at an acidic pH of 5.0 and gradual decrease in tannase activity was observed as the pH reached the alkaline range (Fig. 4). This pH optimum is similar to previously recorded reports for tannase [4, 8**]**.

Fig. 4 Effect if pH on Tannase activity

pH	Tannase activity (U/ml)
pH 3	62.345
pH 5	196.913
pH 7	104.732
pH 9	30.657
pH 11	2.647



Effect of different carbon sources: Since tannase is an inducible enzyme, different carbon sources (Mannose, Galactose, Glycerol and Ribose) were supplemented in the culture medium instead of tannic acid in the same ratio. All the carbon sources showed a negative effect on tannase production. Tannic acid (1% w/v) was the most suitable carbon source for tannase induction by the fungal isolate (Fig. 5). These results are in agreement with those reported for tannase production from different microorganisms earlier [2, 25].

Effect of different nitrogen sources: Sodium nitrate (1% w/v) present in the culture medium was replaced by equivalent amounts of different nitrogen sources (Peptone, Yeast extract, Beef extract, Urea, Casein, Ammonium sulfate, Ammonium chloride, Ammonium nitrate, Ammonium dihydrogen phosphate, Sodium nitrate and Potassium nitrate). Tannase activity increased slightly with Sodium nitrate but later on decrease gradually (Fig. 6). Similar results were obtained previously also [9, 13, 25**]**.

Fig. 5 Effect of Carbon sources on Tannase activity

Carbon source (1%)	Tannase activity (U/ml)
Tannic acid	189.814
Ribose	129.629
Mannose	43.827
Galactose	12.036
Glycerol	83.95

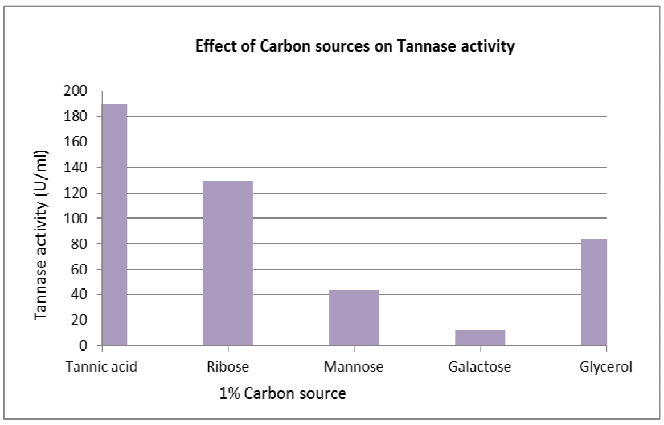
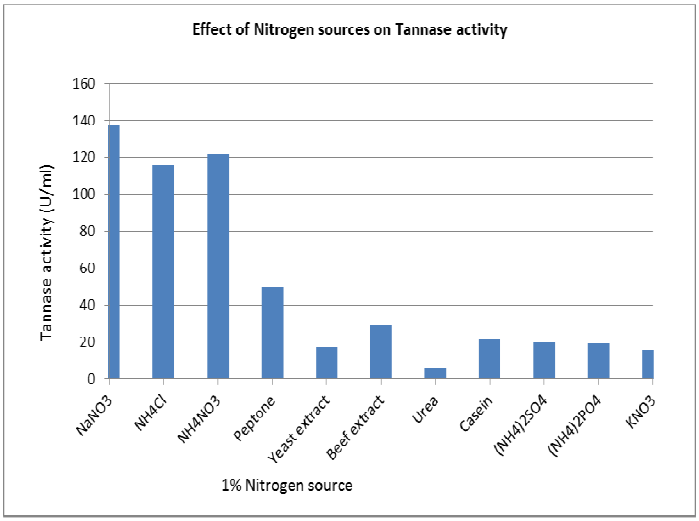


Fig. 6 Effect of Nitrogen sources on Tannase activity

Nitrogen source (1%)	Tannse activity (U/ml)
$NaNO_3$	137.036
NH_4Cl	115.74
NH_4NO_3	121.913
Peptone	49.69
Yeast extract	17.283
Beef extract	29.32
Urea	5.836
Casein	21.913
$(NH_4)_2SO_4$	19.752
$(NH_4)_2PO_4$	19.135
KNO_3	15.74



Effect of different metal ions: The metal ions (Zn^{+2}, Mo^{+6}, Al^{+3}, Mn^{+2}, Cu^{+3}, Li^{3+}) concentrations were used to replace KCl in the culture medium in concentrations ranging from 1mM to 20 Mm, with 10mM showing maximum tannase production. All the metal ions were inhibitory on tannase activity (Fig. 7). These results are in agreement with those reported earlier [13].

Enzyme purification and physico-chemical properties of purified tannase:
All the experiments were carried out under sterilized 4°C conditions. The protocol to purify extracellular tannase was conducted using the culture broth supernatants obtained at 48hrs of cultivation with 1% tannic acid as substrate. The extracellular tannase was purified by using filtration chromatography in a Sephadex G-150 column allowing the separation of the enzyme from minor proteins and fungal pigments. The fractions with high activity were pooled, dialyzed and concentrated by lyophilization (Fig. 8). At the end of the process, tannase was purified 7.17 fold with a specific activity of 101.428 U/mg protein and a yield of 18.35% (Table 8).

Fig. 7 Effect of Metal ions on Tannase activity

Metal ions (10mM)	Tannase activity (U/ml)
$ZnSO_4$	54.629
MoO_3	46.913
Al_2O_3	94.443
$MnSO_4$	46.604
$CuSO_4$	49.073
$LiCl_3$	50.925

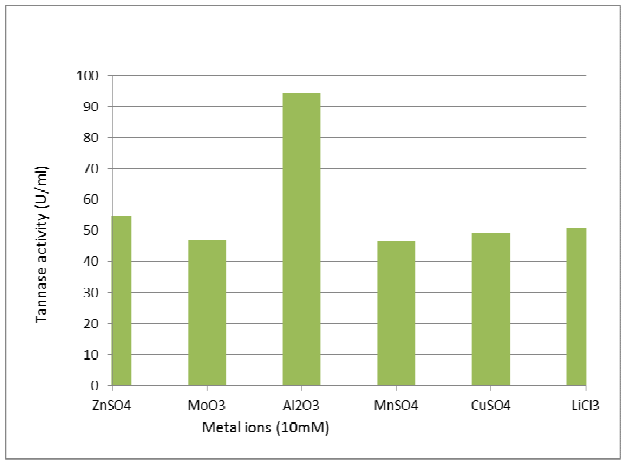
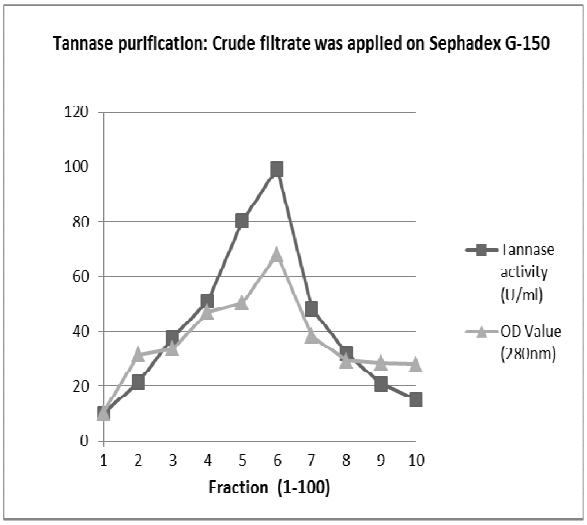


Table 8 Purification results of tannase during this study.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Sephadex G-150	4.12	58.3	14.150	3.84	50.58
DEAE Sephadex	0.98	99.4	**101.428**	7.17	18.35

Tannase appeared homogeneous in PAGE under non-denaturing conditions and was characterized in this study. SDS-PAGE revealed the presence of one band with an apparent molecular mass of 66 kDa (Fig. 9).

Fig. 8 Graphs depicting the results of two methods used for tannase purification.



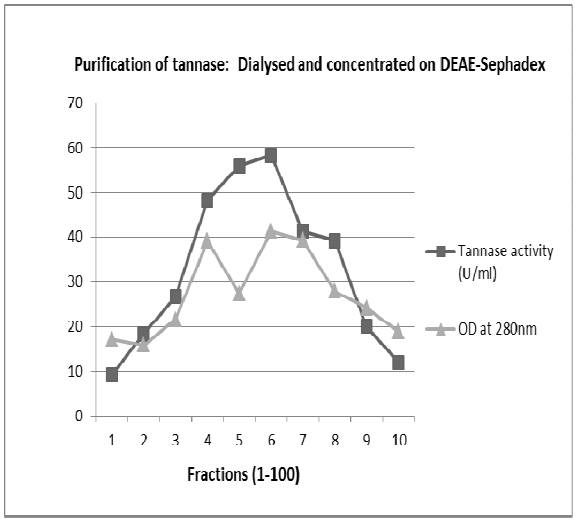
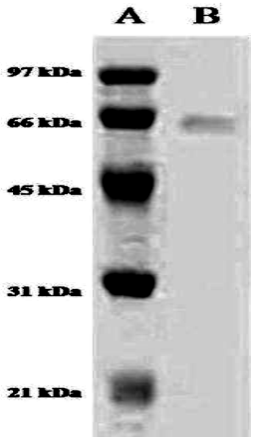


Fig. 9 SDS-PAGE of purified tannase. (A: Molecular weight marker, B: Purified tannase)



According to Hatamoto et al. (1996) 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.

CONCLUSION

The present work has been taken up with a view of using *A. niger* as a microbial source for the production of tannase which can hydrolyze tannic acid to gallic acid. Since fungal activity is the key aspect in this area, there is enormous opportunity for the cost effective production of tannase which is a very versatile enzyme. Tannase finds application in the food, beverage, industrial and pharmaceutical industry. In view of the results obtained, it is concluded that the isolate was induced by 1% tannic acid as a substrate. Tannase was purified 7.17 fold with a specific activity of 101.428 U/ml and a yield of 18.35%. In future, we are interested to scale up the production of tannase by using various recombinant engineering techniques so that the industrial demand for tannase is taken care of on a large scale.

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