ISSN Online: 2349-7785

Hyperproduction of Tannin Acylhydrolase in Submerged Fermentation from *Aspergillus fumigatus*

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Received 24 June 2017; accepted in revised form 27 July 2017

Abstract: Tannin acyl hydrolase (E.C.3.1.1.20) is commonly referred as tannase, hydrolyses ester and depside bonds of hydrolysable tannins to produce gallic acid, glucose and galloyl esters. Tannase finds application in many industrial sectors which includes pharmaceutical, food, chemical and beverages industry. The enzyme has potential uses in the treatment of tannery effluents and pre-treatment of tannin containing animal feed. *Aspergillus fumigaus*-10561 produced maximum extracellular tannase activity (0.430 U/ml) grown in medium supplemented with 0.05 % each of K_2HPO_4, KH_2PO_4, $MgSO_4$, 0.3 % NH_4NO_3, and 0.5 % tannic acid as substrate inducer at 40°C, pH 7.0 for 48 h. Maximum tannase activity was reached in 50 mM citrate buffer (pH 5.5) with substrate concentration of 100 mM methyl gallate at 35°C in 5 min. All the metal ions inhibited the enzyme activity, but maximum inhibition (45 %) was recorded in presence of β-mercaptoethanol. The thermal stability of this enzyme was studied and the enzyme was found to be fairly stable at 50°C up to 120 min. On performing the time course of enzyme reaction at different temperatures, maximum product yield was detected at 35°C after 40 minutes of reaction. Only 7 % loss of activity was observed on storage at 4°C, whereas at room temperature 66.50 % residual activity was retained when stored for 26 days. The present study showed that the fungal strain *Aspergillus fumigatus*-10561 has high potential for industrial production of tannase.

Keywords: *Aspergillus fumigatus*-10561; tannase; tannic acid; β-mercaptoethanol

Introduction

The Tannase (tannin acyl hydrolase E.C: 3.1.1.20), discovered by Scheele, (1786), has been known to hydrolyze the ester linkages of tannic acid into gallic acid and glucose [5,14].Tannase is as extracellular inducible enzyme produced in the presence of tannic acid by plant, fungi, bacteria and yeast [1,3,5,7,12]. Tannic acid is polyphenolic mixture of polyhydric alcohols that can easily binds with any proteins, and generally referred to as an anti-nutrient and anti-microbial agent [5]. Tannase on the other hand, is an industrially important enzyme that extensively used in manufacturing of instant tea [2] and Wine [16] and also act as a sensitive analytical probe for determining the structure of naturally occurring gallic acid esters [10]. Besides these, the hydrolytic product of tannic acid i.e. gallic acid (3, 4, 5 tri-hydroxy benzoic acid) is used as the precursor substrate for the synthesis of food antioxidant, propyl gallate and pharmaceutical antibacterial agent, trimethoprim. Gallic acid also shows cytotoxic activity against cancer

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cells, without harming normal cells [8]. Therefore, considering the industrial applications of tannase, we reported the isolation and screening of the tannase producing microorganisms in different media under different experimental conditions, and optimized the culture conditions for maximum yield of enzyme product in terms of enzyme activity under submerged fermentation.

Materials and methods

Chemicals

Tannic acid, gallic acid and rhodanine were procured from Sigma-Aldric (St. Louis, Mo). Media components were purchased from HiMedia (India). All the other chemicals were of analytical grade and were obtained from Sigma and Hi Media, unless otherwise specified.

Sample collection

Soil sample were collected in the polythene bags from various naturally tannins rich sites of Himachal Pradesh, India and brought to the laboratory for further processing.

Enrichment of tannase producing microbes

Soil samples and tannic acid was added to physiological saline (pH 7.0) at 2:1, ratio and incubated for 24 to 48 h at 30°C and 55°C in a BOD incubator.

Isolation of microbes

A number of tannase producing fungal and bacterial strains were isolated from 1 ml of enriched sample serially diluted to 10^{-4} to 10^{-6} times with physiological saline of (pH 7.4). Than 100 µl of diluted sample were spread over 60 mm nutrient agar 2 % plate containing (0.05 %) each of K_2HPO_4, KH_2PO_4, $MgSO_4$, 0.3 % NH_4NO_3, 0.01 % $CaCl_2$ and 0.1 % tannic acid, and incubated at 30°C and 55°C for 48 h. Pure colonies were picked up depending upon terure and morphology. Pure line cultures were established by repeatedly streaking single colonies on same nutrient agar plates, and pure cultures were maintained on slants at 4°C.

Screening of tannase producing microbes

One hundred and twenty microbes were isolated from different soil samples and each isolate was assigned separate code number. Out of these isolate only 20 showed good tannase activity according to the method described by Sharma, *et. al*.[22]. On the basis of which, one fungal isolate designated as TPF-13 from the soil of Kheer Ganga, District Kulu, Himachal Pradesh, India was selected and preserved at 4°C on agar slant for further work.

Production and assay of enzyme

The seed culture was prepared by growing the isolates in 50 ml of nutrient broth (pH=7.0) containing 0.5 % Peptone, 0.3 % Beef extract, 0.3 %, NaCl and 0.3 % Yeast extract (pH 7.0) at 30°C in incubator shaker with continuous agitation using 160 rpm. After 24 hours, 8 % of the seed culture was inoculated into 50 ml of production medium containing 0.05 % each of K_2HPO_4, KH_2PO_4, $MgSO_4$, 0.3 % NH_4NO_3 as nitrogen source and 0.5 % tannic acid as carbon source and incubated as mentioned above. The culture contents were centrifuged at 10,000 g for 20 min at 4°C, and filtrate was assayed the tannase activity at 520 nm as per the standard laboratory protocol [22] using 0.1 M methyl gallate in 0.05 M citrate buffer (pH 5.0) as substrate. One unit of the tannase activity was defined as the amount of enzyme required to produce 1 µmole of gallic acid per min in standard assay conditions.

The protein estimation in fungal biomass estimated by method described by Bradford [6].

Optimization of culture conditions for tannase production

Optimization of production media

Seventeen different media were used to test the production of tannase at 30°C for 48 hr. (Table I).

Effect of inorganic salts and nitrogen sources

The concentration of K_2HPO_4, KH_2PO_4 and $MgSO_4$ was varied in the selected production medium 13 from 0.01 % to 1 % w/v, to get the optimum concentration of inorganic ions for maximum production of enzyme. Nitrogen is the most important limiting factor in the microbial enzyme production. Hence, various inorganic (NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, Ca $(NO_3)_2$, $(NH_4)_2HPO_4$,

Table I. Composition of various media used for the production of tannase in terms of enzyme activity from fungal isolate TPF-13. Parenthesis indicated media constituents in g/l

No.	Media Used	Media Constituents (g/l)	pH	Enzyme activity (U/mL)	Reference
1	Medium 1	K_2HPO_4(0.5),KH_2PO_4 (0.5), $MgSO_4.7H_20$ (0.5),NH_4Cl (3), $CaCl_2$ (1), Tannic acid (10).	5	0.089	Enemuor, S.C. and Odibo, F.J.C. 2009
2	Medium 2	$(NH_4)_2HPO_4$(2),KH_2PO_4(1),$MgSO_4$. $7H_20$ (0.5),NH_4Cl (1), Yeast extract (0.5),Tannic acid (10).	5.9	0.079	Mitsuhiro, Z. 1998
3	Medium 3	K_2HPO_4(1), $NaNO_3$ (3),$MgSO_4$. $7H_20$ (0.5), KCl (0.5) Tannic acid (2)	5.5	0.012	Paranthaman, R. et. al. 2009
4	Medium 4	KH_2PO_4(1),$MgSO_4.7H_2O$ (0.2),NH_4NO (3),$CaCl_2$(20mg), $MnSO_4$(4),$Na_2MoO_4.2H_2O$ (2),$FeSO_4$. $7H_2$(2.5), Tannic acid (2).	5.5	0.02	Paranthaman, R. *et. al*. 2009
5	Medium 5	KH_2PO_4(2),NaH_2PO_4. $2H_2O$ (1),$MnSO_4.7H_2O$ (1.5), $FeSO_4.7H_2O$ (5),$CaCl_2$(2.5),$ZnSO_4$. $7H_2O$ (0.03),$(NH_4)_2SO_4$(1), Fructose (1),Tannic acid (1.0).	5.5	0.07	Paranthaman, R. *et. al.* 2009
6	Medium 6	KH_2PO_4(2), $MgSO_4.7H_2O$ (2), NH_4Cl (3),$CaCl_2$(1),Yeast extract (2),Methyl gallate (1.5).	5.5	0.043	Self design
7	Medium 7	KH_2PO_4(0.1),$MgSO_4.7H_20$(0.5), NH_4NO (0.25),KCl (0.5), Tannic acid (20).	6	0.069	Purwanto, L.A. 2009
8	Medium 8	K_2HPO_4(1.5),$NaNO_3$(4),$MgSO_4$. $7H_20$ (0.7), KCl (0.3), Tannic acid (1.5.0).	5	0.026	Self design
9	Medium 9	KH_2PO_4(2.19),$(NH_4)_2SO_4$(4.38), $MgSO_4.7H_2O$ (0.44), $CaCl_2$(0.044), $MnCl_2.6H_2O$ (0.009), Na_2MoO_2(0.004), $FeSO_4.7H_2O$ (0.06),Tannic acid (12.5).	5.5	0.058	Hernandez, M.C. *et. al.* 2006
10	Medium 10	KH_2PO_4(5),$MgSO_4$. $7H_2O$ (1), NH_4NO3 (10),$CaCl_2$(0.1),$MnCl_2.6H_2O$ (0.02),$Na_2MoO_2.2H_2O$ (0.01),$FeSO_4$. $7H_2O$ (0.125),Glucose (2.5), Tannic acid (10).	5.5	0.024	Aguilar, C.N. *et al,* 2004
11	Medium 11	K_2HPO_4(0.5),KH_2PO_4(0.5),$MgSO_4$. $7H_20$ (0.5),NH_4NO_3(3.0), Tannic acid (10).	5	0.107	Mondal, K.C. and Pati BR, 2000
12	Medium 12	K_2HPO_4(0.5),KH_2PO_4(0.5), $MgSO_4.7H_20$ (0.5),NH_4Cl (3.0), Tannic acid (1).	5	0.095	Mohaptra, *et. al.* 2007

table 1. (continued).

No.	Media Used	Media Constituents (g/l)	pH	Enzyme activity (U/mL)	Reference
13	Medium 13	K_2HPO_4(0.5),KH_2PO_4(0.5), $MgSO_4.7H_20$(0.5),NH_4NO_3 (3.0),Tannic acid (5.0).	7	0.191	Self-designed
14	Medium 14	Peptone (5), Beef Extract (3), Yeast Extract (3), NaCl (5), Glucose (10), Tannic acid (1).	7	0.091	Self-designed
15	Medium 15	Na_2HPO_4(0.5),KH_2PO_4(0.5), $MgSO_4.7H_20$ (0.5),NH_4NO_3(3), Tannic acid (5.0).	7	0.08	Self-designed
16	Medium 16	K_2HPO_4(0.5),NaH_2PO_4(0.5), $MgSO_4.7H_20$ (0.5),NH_4NO_3 (3), Tannic acid (5.0).	7	0.077	Self-designed
17	Medium 17	Na_2HPO_4(0.5),NaH_2PO_4(0.5),. $MgSO_4.7H_20$ (0.5),NH_4NO_3 (3.0), Tannic acid (5).	7	0.059	Self-designed

$NH_4H_2PO_4$, $NaNO_3$, KNO_3 KNO_2) and organic (peptone, beef extract, yeast extract, malt extract, meat extract, urea, tryptone casein) nitrogen sources were added to the production medium at a concentration of 37.5 Mm and 0.01 % to 1 % w/v respectively. Maximum tannase activity was reached with NH_4NO_3 and further tested its varied concentration from 0.1 % to 1 % w/v in the production medium as mentioned above.

Optimization of tannic acid concentration as an inducer

Tannic acid act as the sole carbon source as well as inducer for tannase in the production medium, so in order to get the maximum tannase induction, the concentration of tannic acid was varied from 0.25 % to 2 % (w/v) in the production medium.

Optimization of production pH and temperature

The medium supplemented with tannic acid was adjusted with different pH ranging from 3 to 8 and temperature (20°C to 60°C) respectively.

Optimization of inoculum size

Inoculum size was optimized by seeding the production broth with varying density of seed culture from 3.25 x 10^8 to 29.5 x 10^8 % spores.

Growth and enzyme production profile

The growth and enzyme production profile of fungal isolate was studied by growing isolate in the production medium (pH 7.0) at 40°C for 72. The aliquots were taken with an interval of 6 h and assayed for enzyme activity. The final pH at the time of harvesting was also recorded to study the change in the pH with the growth of fungus. The wet weight of the harvested biomass was used for determining the growth of fungus.

Optimization of reaction conditions for tannase production

The optimization of reaction conditions was described below, where supernatant obtained after harvesting the fungal isolate *Aspergillus fumigatus*-10561 in culture was assayed for tannase activity in every case.

Effect of various buffer system and pH on enzyme reaction

Five different buffers of pH (4.0 to 10.5) were optimized for enzyme reaction (Table 2). The effect of selected buffer system for tannase activity was tested by adding it in various concentrations (25-300 mM).

Table 2. pH range of various buffer system used for the enzyme reaction of tannase

No.	Buffer name	pH range
1	Citrate buffer	4.0 to 6.0
2	Sodium phosphate buffer	6.0 to 8.0
3	Potassium phosphate buffer	6.0 to 8.0
4.	Glycine-NaOH buffer	7.5 to 10.5
5	Sodium carbonate-bicarbonate buffer	9.0 to 10.5

Effect of incubation temperature and time for enzyme reaction

The optimum temperature for the enzyme reaction was evaluated by measuring the tannase activity at different temperature (30°C to 60°C). Further, the optimum time for enzyme reaction was evaluated by incubating the enzyme for different time intervals (2 to 30 min) in the reaction mixture at 35°C.

Effect of substrate and enzyme concentration

Substrate is the most important factor in the microbial enzyme reaction process. Hence, different concentrations of methyl gallate were used (25 mM to 350 mM) to study the effect of substrate concentration on enzyme activity. Further, Varied amount of enzyme (10 µg-50 µg) was used to study the optimum concentration of enzyme.

Effect of metal ions and inhibitors

To work out the effect of various metal ions, $ZnCl_2$, $CaCl_2$, $MnCl_2$, $AgNO_3$, $MgCl_2$, $MgSO_4.7H_2O$, $BaCl_2$, $HgCl_2$, EDTA and β-Mercaptoethanolto on the tannase activity, these were added to the reaction mixture at 1 mM final concentration to get maximum activity of enzyme.

Thermo-stability of tannase

To evaluate the thermo-stability of enzyme the supernatant was pre-incubated (for 120 min) at different temperatures ranging from 30°C to 70°C. The enzyme activity was assayed after every 30 min.

Optimization of time course of enzyme reaction at different temperatures

To achieve the maximum gallic acid production, time course of enzyme reaction was studied by terminating the reaction after different time intervals (5 to 120 min) at different temperature ranging from 30°C to 50°C.

Storage stability of tannase

Storage stability of enzyme was investigated at 4°C and 30°C for 25 days. Enzyme activity was assayed daily by the method described above.

Results

Isolation of tannase producing microbes

Among 120 microbes screened from various naturally tannins rich site of Himachal Pradesh only 20 isolates showed the tannase activity (Table 3). The fungal isolate TPF-13 was the most potent isolate that revealed maximum activity (0.191 U/ml). TPF-13 was also identified as *Aspergillus fumigatus* and deposited at microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh, India with the accession number of MTCC-10561.

Characterization of fungal isolate TPF-13

The Isolate TPF-13 was aerobic, zone forming and spore former as shown in the Fig. 1 (a,b). The colony was pale greenish or yellowish in color, mycelium was Coenocytic (Aseptate) and Sporangium (Columella present) like fruiting body was present as shown in the Fig. 1 (d,c). On the basis of tannase activity, one of the most potent isolate was selected for further studies. This isolate was designated as TPF-13. TPF-13 showed optimum enzyme activity when alloweded to grow in tannic acid agar medium (pH 7.0) at 30°C for 24 to 48 hr with a moderate growth to fast growth rate as shown in the Fig. 1 (e).

Optimization of culture conditions for tannase production

Effect of media for tannase production

Among seventeen media tested as shown in Table I, the maximum activity (0.191 U/ml) of

Table 3. Fungal isolate with tannase activity in U/ml at (40^{0}C) and 72 hr in culture (pH 7.0)

Strain no.	Tannase activity (U/mL)	Strain no.	Tannase activity (U/ml)
TPF-1	0.011	TPF-11	0.030
TPF-2	0.020	TPF-12	0.035
TPF-3	0.024	TPF-13	0.191
TPF-4	0.109	TPF-14	0.105
TPF-5	0.047	TPF-15	0.036
TPF-6	0.023	TPF-16	0.016
TPF-7	0.105	TPF-17	0.047
TPF-8	0.091	TPF-18	0.056
TPF-9	0.080	TPF-19	0.039
TPF-10	0.055	TPF-20	0.065

Fig. 1 Screening and characterization of fungal strain TPF-13 (a) Red arrows representing colonies of fungal strain on Tannic acid-agar plate at 30^{0}C for 24-48 hr.(b) Black arrow representing clear round shaped zone formation by *Aspergillus fumigatus*-10561(c) White arrow representing pure line culture of *Aspergillus fumigatus*-10561 on tannic acid-agar slant (d) Optical microscopic image of fungal strain *Aspergillus fumigatus*-10561 with mycelium(blue arrow) and fruiting body(green arrow) (e) Fungal isolate growth in submerged fermentation.

tannase was observed in medium 13, supplemented with 0.5 % (w/v) tannic acid as an inducer. However, lowest tannase activity, 0.012 U/ml was detected in medium 3.

Effect of inorganic salts and nitrogen sources

Maximum tannase production was observed in the above mentioned media, supplemented with 0.5 % (w/v) K_2HPO_4, KH_2PO_4 and $MgSO^4$ as shown in Fig. 2 (a, b, c).

Among various inorganic and organic nitrogen sources tested as shown in Figure 3 (a,b,c) the maximum production of tannase was observed in ammonium nitrate, supplemented with (37.5 mM (w/v) [Fig. 3 (a)]. Further maximum tannase production was observed in above mentioned media, supple-mented with 0.3 % (w/v) NH_4NO_3 as a best inorganic nitrogen source [Fig. 3 (b)].

Tannase activity in the above optimized media was more as compared to organic nitrogen sources, and hence they did not increase enzyme production.

Effect of tannic acid concentration as an inducer

Tannic acid concentrations ranged from 0 to 2 % (w/v) and were found to substantially affect tannase productivity. The maximum production of tannase (0.205 U/ml) was observed, supplemented with 1 % tannic acid as an inducer as shown in Fig. 4 (a).

Effect of incubation temperature and time for enzyme reaction

Fungal isolate produced appreciably higher level of tannase at pH 7.0 and temperature 40^{o}C as shown in Fig. 4 (b, c) respectively.

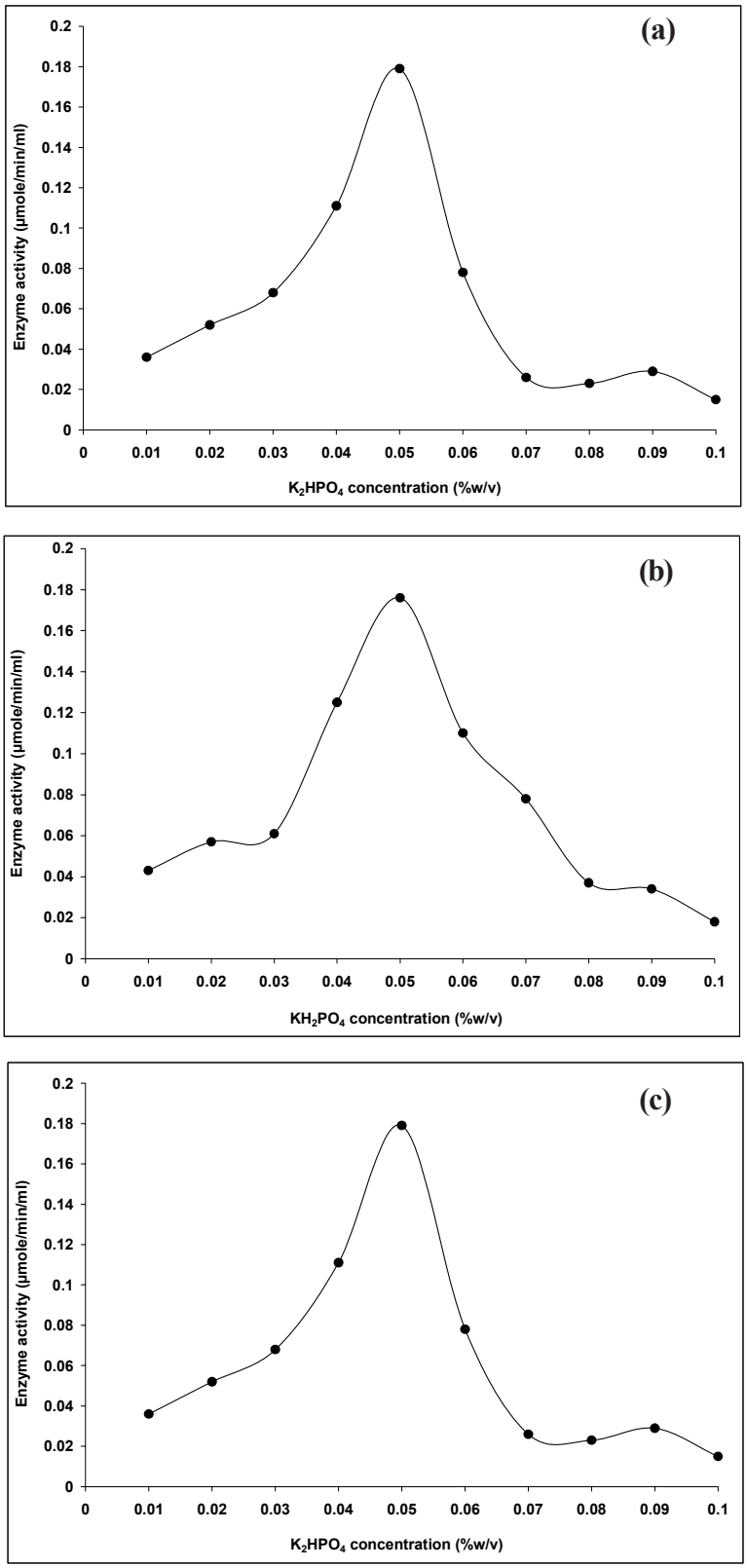


Fig. 2. The fungal isolate TPF-13 showed maximum production at the optimum concentration of (a) K_2HPO_4 (b) KH_2PO_4 (c) $MgSO_4$ was 0.05% (w/v)

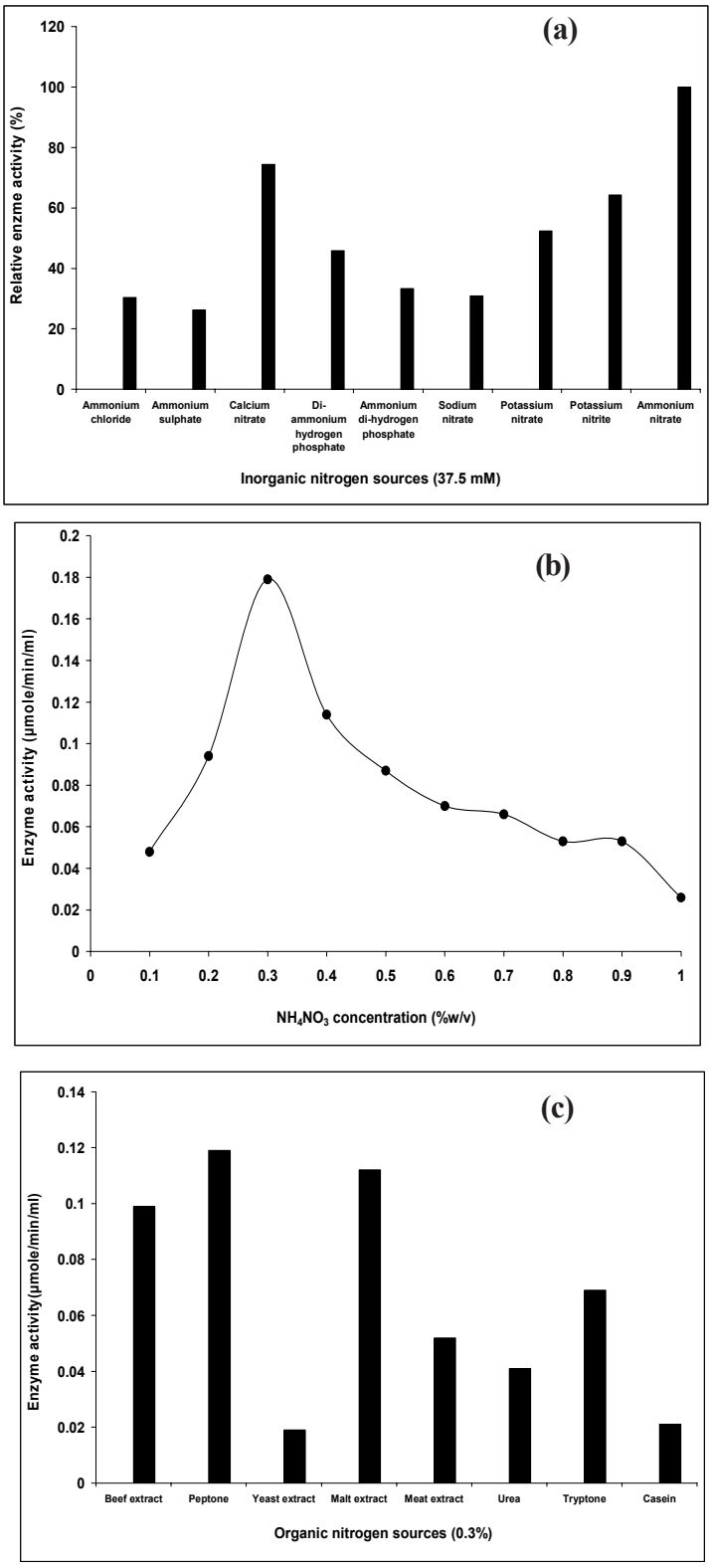
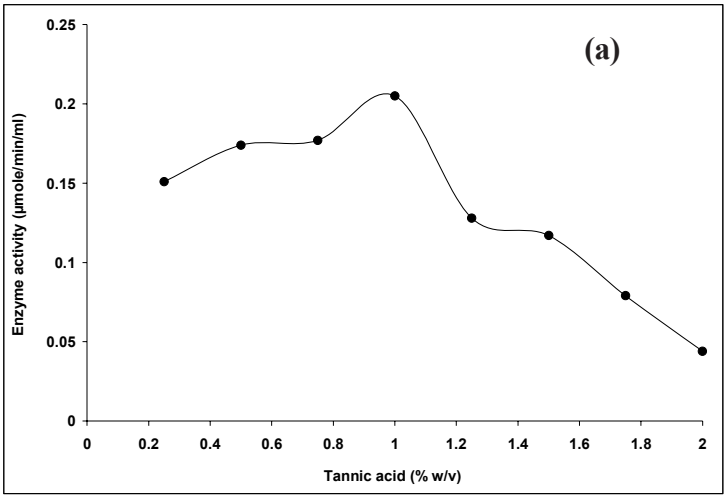
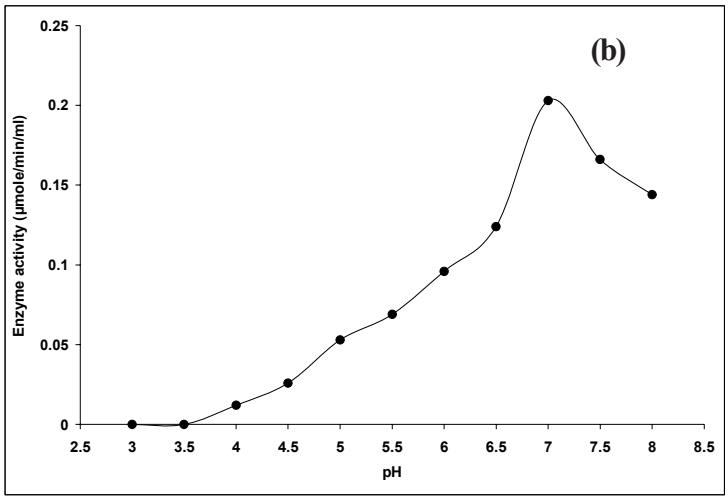


Fig. 3. The fungal isolate TPF-13 showed maximum production at the optimum concentration of (a) inorganic nitrogen sources were 37.5mM (b) NH_4NO_3 was (0.3%) (c) organic nitrogen sources were 0.3% (w/v)





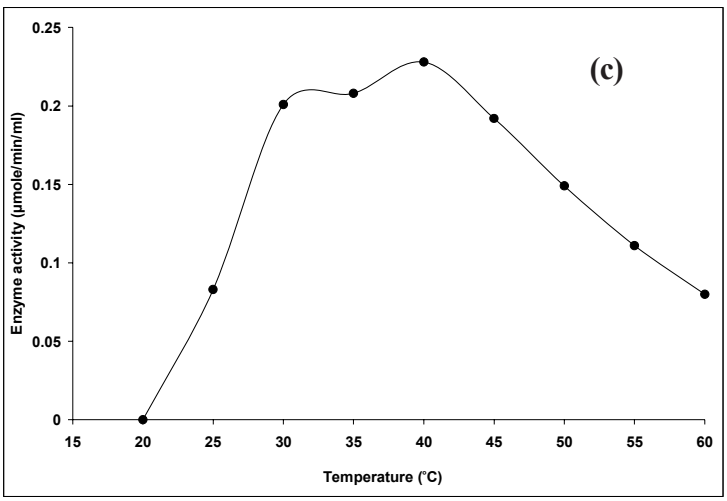


Fig. 4. The fungal isolate TPF-13 showed maximum production at the concentration of (a) tannic acid concentration was 1% (b) optimum pH was 7.0 (c) optimum production temperature was 40^0C

Optimization of inoculum size

Aspergillus fumigatus-10561 showed maximum enzyme yield at the concentration of 15.25 × 10^8 spore as shown in Fig. 5 (a).

Growth and Enzyme production profile

Highest activity of tannase and biomass was recorded after 48h of incubation as shown in Fig. 5 (b).Variation in pH with the time of production was also recorded; results are shown in Fig. 5 (c). There was a continuous increase in the pH of the medium with the passage of time. It can degrade tannic acid and produce maximum tannase (0.22 U/ml) at stationary phases of growth (24 h). The increase in pH over 6.0 drastically reduced the tannase activity.

Optimization of reaction conditions for tannase production

Optimization of buffer system and pH for tannase assay

Among five different buffers tested as Shown in Table 3, the maximum activity was recorded in citrate buffer of (0.05 M) concentration at pH 5.5 [Fig 6 (a)]. Further, optimum buffer concentration was recorded to be 50 mM [Fig. 6 (b)]. Effect of incubation temperature and time for enzyme reaction

Tannase produced appreciably higher level of enzyme activity at 35 ºC as shown in the Fig. 6 (c). The optimum time for enzyme reaction was 5 minute [Fig.7 (a)].

Effect of substrate and enzyme concentration

Maximum tannase activity was recorded in the presence of 100mM of substrate as shown in Fig. 7 (b). Higher concentration beyond tends to suppress the enzyme activity. A fall in tannase activity is suggestive of a possible tannase inhibition probably by repression. Varied amount of enzyme (10 µg-50 µg) was used to study the optimum concentration of enzyme. Maximum activity was observed by preparing the enzyme sample of (200 µg protein per ml) in the reaction method [Fig.7 (c)].

Effect of metal ions and inhibitors

In the enzyme industry, the main importance of inhibitors is that they reduce the efficiency of the enzyme reaction. The effect of metal ions, EDTA and compounds containing thiol protecting groups (β-mercaptoethanol) on tannase activity was studied and results are summarized in Fig. 8(a). All the metal ions inhibited tannase activity at 1 mM concentration. Maximum inhibition occurred in a-mercaptoethanol (45 %).

Thermostability of tannase

The enzyme was pre-incubated at different temperatures and its activity was assayed after every 30 min until 120 min. The enzyme was fairly stable at 30ºC, 40ºC and 50ºC up to 2 h [Fig.8 (b)].

Time course of enzyme reaction at different temperatures

Tannase reactions were followed up to 120 min at different temperature (30°C-50°C) and the results recorded are shown in Fig. 8(c). Product formation was detected by estimation of gallic acid formed at different interval of time viz. 5, 10, 15, 20, 40, 60, 80, 100 and 120 min. Maximum product yield was detected at 35 ^{0}C after 40 minutes of reaction. The product concentration remained almost constant after 40 minutes of enzyme reaction that might be due to denaturation of the enzyme or product inhibition.

Storage stability of tannase

Shelf life of tannase from *Aspergillus fumigatus*-10561 was studied at both 4°C and 30°C. The results obtained have been shown in Fig. 8(d). Only 7 % loss of activity was observed on storage at 4°C, whereas at room temperature 66.50 % residual activity was retained even after 25 days.

Discussion

A fungal strain capable of producing tannase was isolated from tannin rich soil and identified as *Aspergillus fumigatus* by microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh, India with the accession number of MTCC-10561 was chosen for the study because it grew faster than other isolate on a basal

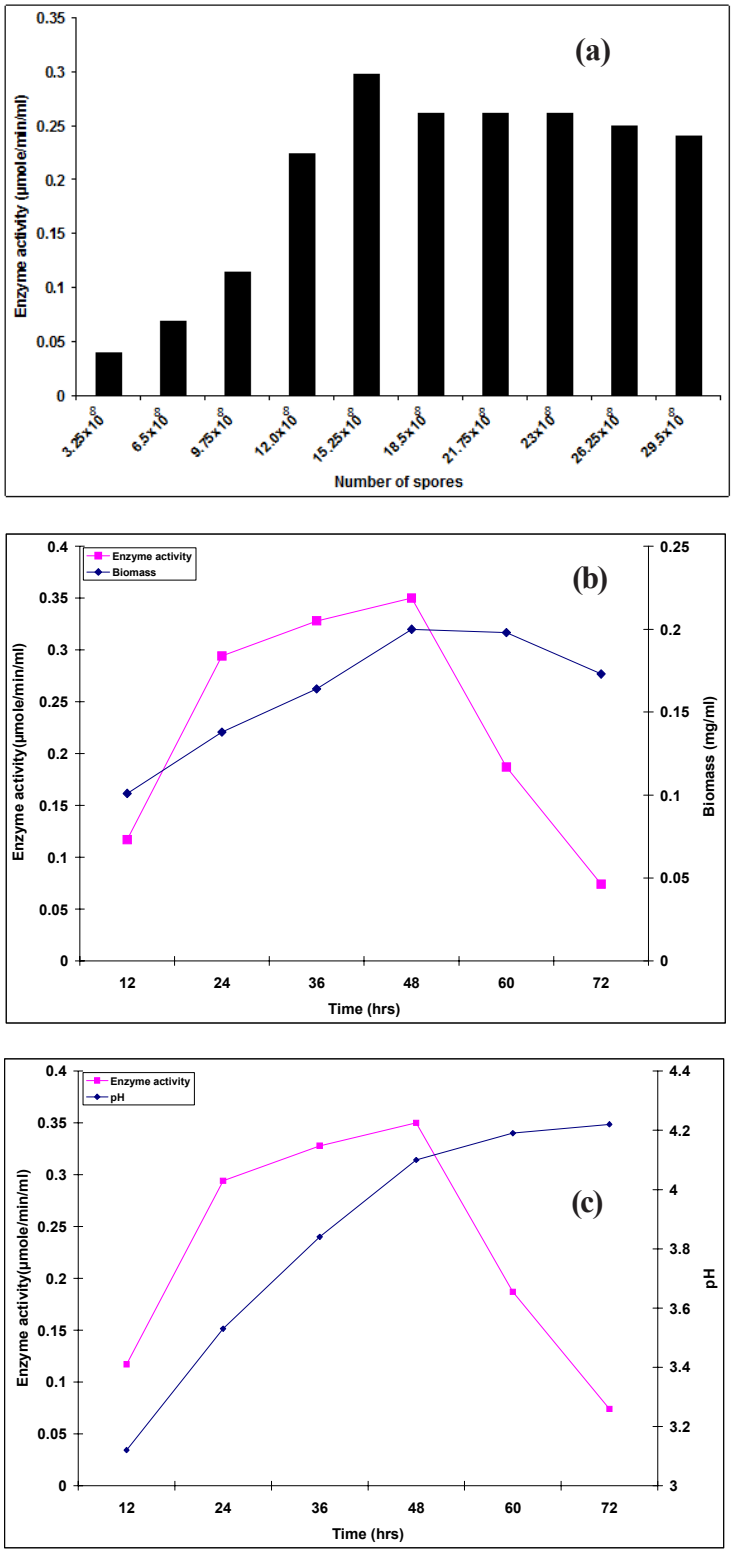


Fig. 5. The fungal isolate TPF-13 showed maximum production when the inoculum size was 15.25 $\times 10^8$ (a), time course of tannase production in relation to biomass production (b), and in relation to the pH changes in the medium.

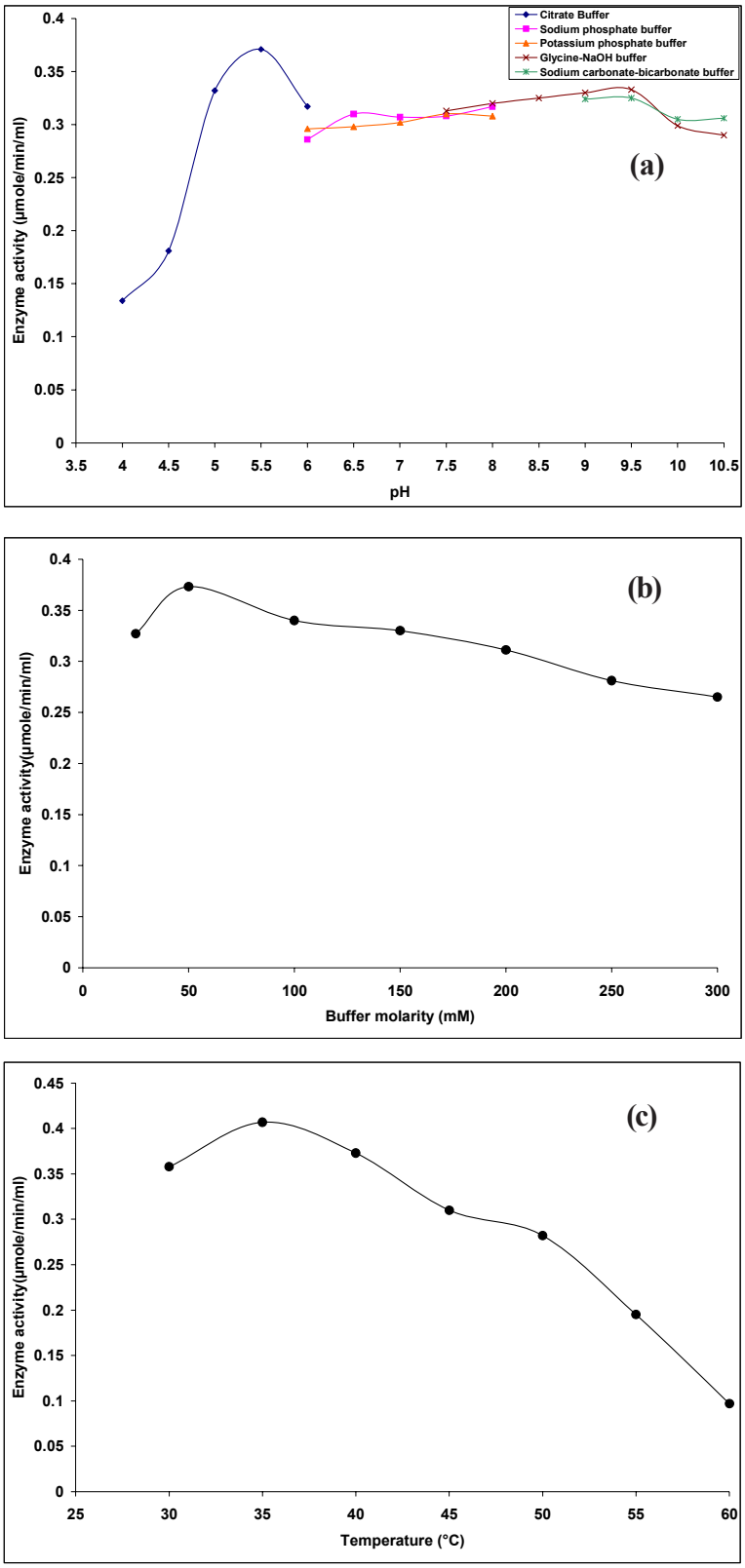


Fig. 6. (a) Optimization of buffer system and pH for tannase assay. (b) Optimization of buffer molarity (c) Effect of reaction temperature on tannase activity

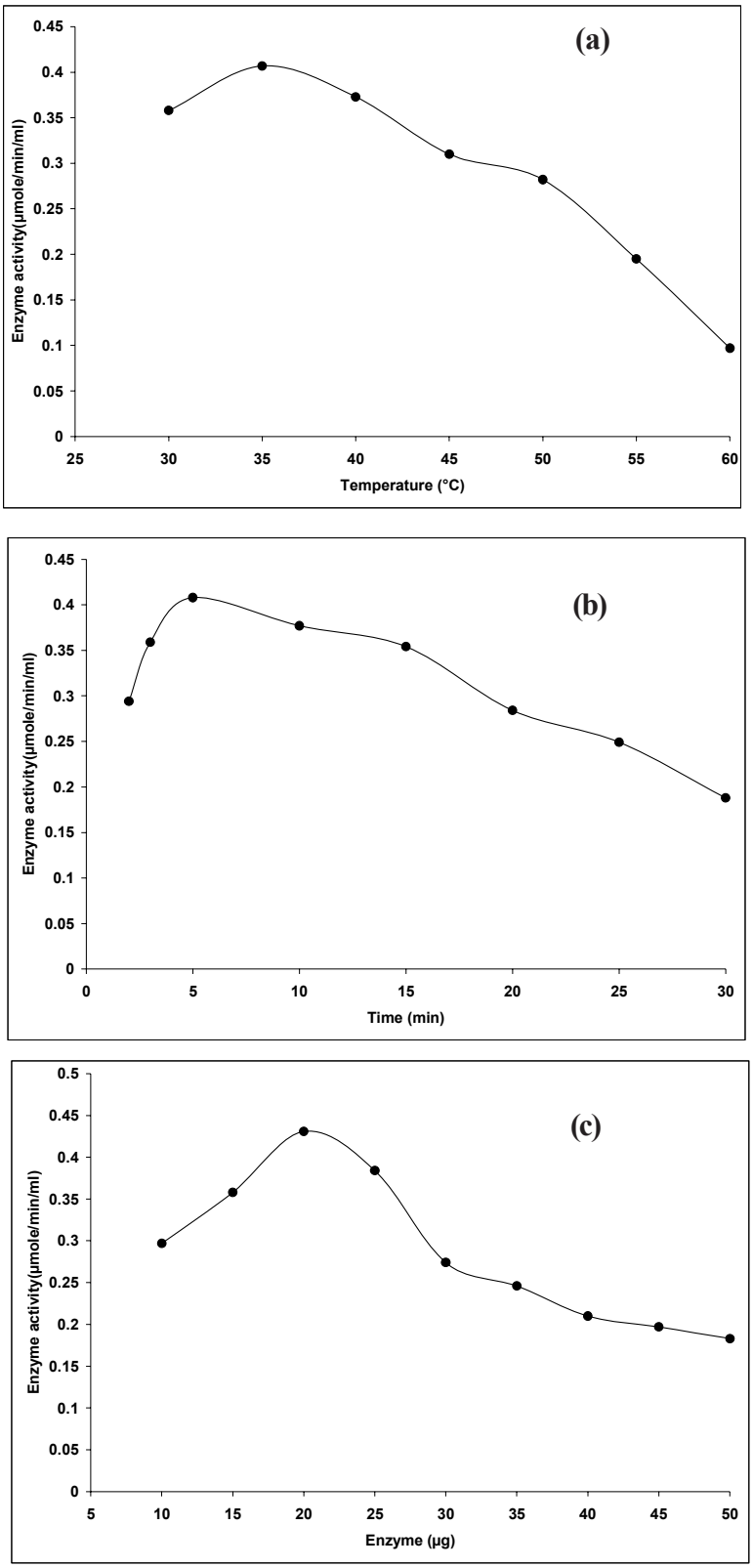


Fig.7. (a) Optimization of substrate concentration (b) Optimization of reaction time (c) Optimization of enzyme concentration

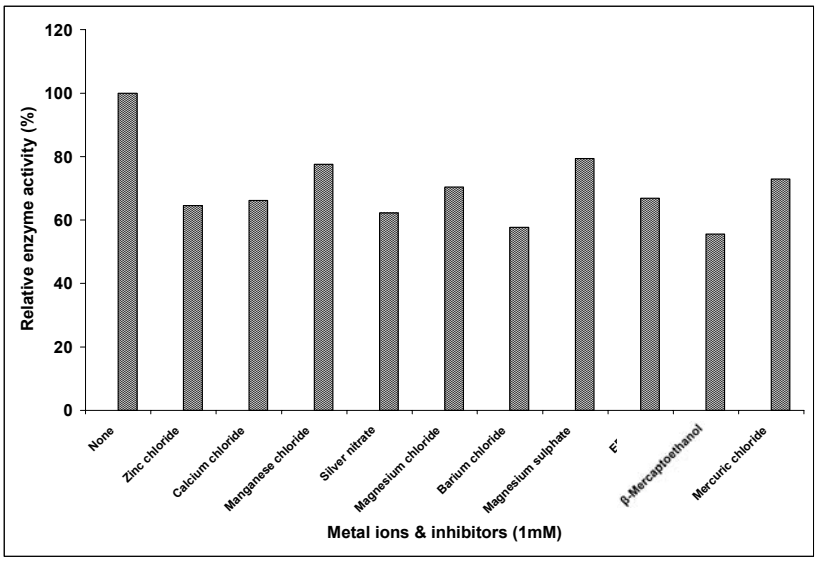


Fig. 8(a). Effect of metal ions and inhibitors

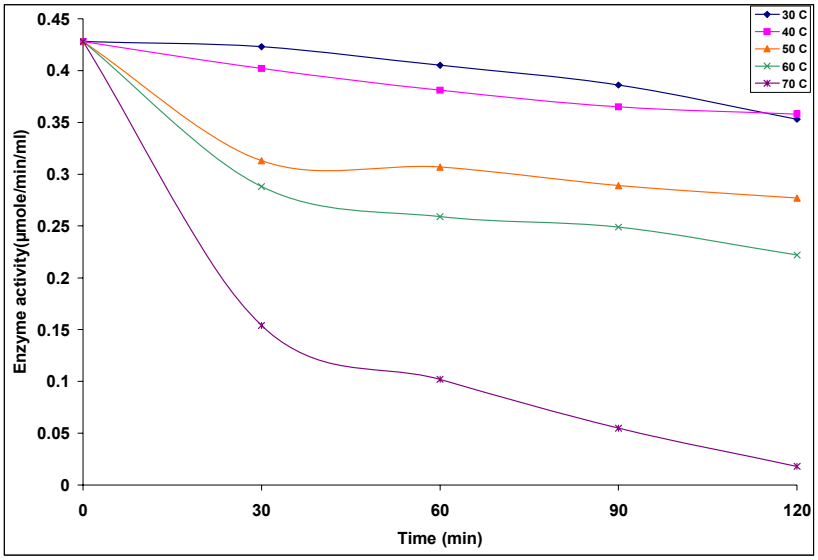


Fig. 8(b). Thermo stability of tannase

medium containing tannic acid as the only source of carbon (Table 1). Similarly, Enemuor and Odibo [9], demonstrated the production of tannase in cultivated strain of *Aspergillus tamari* using tannic acid as carbon source. As per of knowledge this may be the first observation that *Aspergillus fumigatus* is also a tannase producer. It was found that enzyme production is directly proportional to the growth [Fig. 5 (b)] of the organism. This seems to indicate that such extracellular enzyme accumulation occurred with the rapid increasement of cell mass. Similar finding was observed by Enemuor and Odibo [9], during tannase production in *Aspergillus tamari* IMI388810 (B).

Aspergillus fumigatus produced maximum enzyme at its logarithmic phase of growth (24-48 h) of incubation after which it decreases. The tannic acid content in fermented broth decreases sharply and is completely altered within 48 h of growth. Generally tannins are toxic compound and have non-reversible reaction to protein. This analysis of the fermented broth reveals that tannase hydrolyzed the tannic acid into gallic acid, which

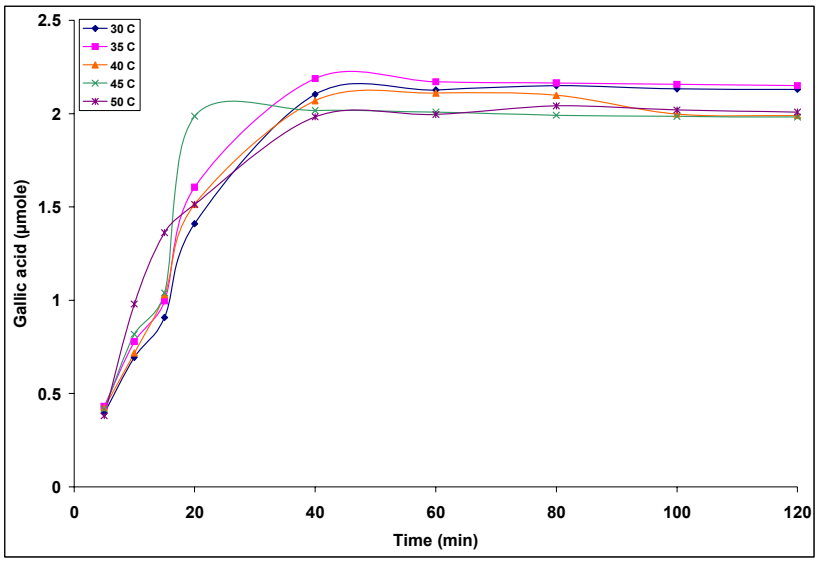


Fig. 8(c). Time course of enzyme reaction at different temperatures

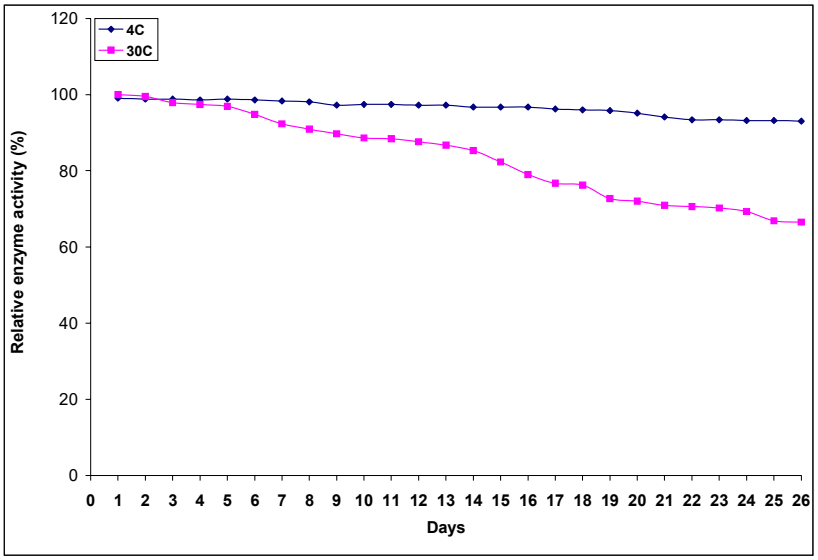


Fig. 8(d). Storage stability of tannase from *Aspergillus fumigatus*-10561

remain persist in the medium up to 48 hrs and ultimately being utilized by the organism. Actually the organism degrades tannic acid by tannase into gallic acid and glucose. Variation in pH with the time of production was also recorded and the results obtained are illustrated in Fig. 5 (b, c). There was a continuous increase in the pH of the medium with the passage of time. It can degrade tannic acid and produce maximum tannase at stationary phases of growth (48 h). A decline in tannase activity is suggestive of a possible tannase inhibition probably by repression. The increase in pH over 7.0 drastically reduced the tannase activity. Mondal, *et. al.* [12] and Paranthaman, *et. al.* [19] has also reported showed maximum enzyme yield in *Aspergillus flavus* when harvested after 48 hrs of incubation. In the present studies the production rate of enzyme was enhanced when the medium was supplemented with inorganic salts like K_2HPO_4, KH_2PO_4, $MgSO_4$ as shown in Fig. 2 (a, b, c,) and inorganic and organic nitrogen sources as shown in Fig. 3 (a,b). Similar results were shown by Bhardwaj *et al.* [7] that producing tannase from *Aspergillus niger*. Addition of these

salts enhances the growth rate of the organism thereby increasing the production of enzyme. The production reaches its peak when concentration of tannic acid was enhanced in addition to basal salts [Fig. 4 (a)]. This may be due to catabolic induction of tannic acid for tannase production. Earlier Purwanto, *et. al.* [20] also mentioned that tannic acid act as a catabolic inducer for fungal tannase production. An interesting feature of the strain is that, it is capable of producing tannase also in the presence of tannic acid in the medium. This is an additional advantage of the strain in large-scale of enzyme production because this media constituents is cost effective. From the result it was observed that the optimum pH for growth and enzyme production is 7.0 [Fig. 4 (b)] and temperature is 40°C [Fig. 4 (c)]. These properties of the enzyme are likely to be similar with other fungal tannase [20]. It is probably the first report of *Aspergillus fumigatus*-10561 that is capable of producing tannase in the medium containing tannic acid. In this study inoculum size of 15.25 x 10^8 spores showed maximum tannase activity [Fig. 5 (a)]. Although several fungal and bacterial species have been reported as potent producer of this enzyme but in the most of the cases the production time were quite lengthy. Maximum activity was recorded in citrate buffer (0.05 M) concentration at pH 5.5 [Fig. 6 (a)]. Hernandez, (2006) has also reported the same buffer of (pH 5) for the assay of tannase in *Aspergillus niger* GH. In contrast, *Bacillus pumilus* HY1 exhibited maximum enzyme activity in 50 mM sodium phosphate buffer (pH 7.0). Optimum buffer concentration was recorded to be 50 mM [Fig. 7 (b)]. *Bacillus cereus* KBR9 exhibited maximum enzyme activity in (0.02 M) acetate buffer [13]. Where, *Aspergillus oryzae* shows maximum activity in (0.05 M) citrate buffer [18]

Tannase from *Aspergillus fumigatus* exhibited appreciably higher level of enzyme activity at 35 ºC as shown in the Fig. 6 (c). Similarly, tannase from *Aspergillus niger* showed maximum activity at 35 °C [19]. Maximum turnover of substrate was recorded in the presence of 100 mM of methyl gallate as shown in Fig. 7 (a). Higher concentration beyond tends to suppress the enzyme activity because of the substrate inhibition. A fall in tannase activity is suggestive of a possible tannase inhibition probably by repression. Enemuor and Odibo [9], have reported 0.10 M methyl gallate in 0.05 M citrate buffer as the optimized substrate concentration from the tannase from *Aspergillus tamarrii*. Varied amount of enzyme (10 µg-50 µg) was used to work out the optimum concentration of enzyme. Maximum activity was observed by preparing the enzyme sample of (20 ug protein per ml) in the reaction sample [Fig. 7 (c)]. All the metal ions inhibited tannase activity at 1 mM concentration with maximum inhibition in a-mercaptoethanol (45 %) [Fig.7 (a)]. Metals ions such as Ag^{2+}, Ba^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+},Co^{2+},Ca^{2+} and Hg^{2+} have also been found inhibitory [2]. The time course of the enzyme reaction was studied at 30 ºC, 40 ºC and 50 ºC up to 2 h [Fig. 8 (b)] and the maximum product yield was detected at 35°C after 40 minutes of reaction. Enzyme was fairly stable from temperature range of 4.0-30°C [Fig. 8 (d)]. Tannases from *Aspergillus* strains were stable at temperature range from 4.0 to 40°C [3] The product concentration remained almost constant after 40 minutes of enzyme reaction that might be due to denaturation of the enzyme or product inhibition. The present fungal strain will be very suitable for tannase production because it can grow easily and produce fairly appreciable amount of enzyme within short period.

Conclusion

The culture conditions for the production of tannase enzyme from *Aspergillus fumigatus*-10561 strain were evaluated and standardized. The results obtained in this investigation show that *Aspergillus fumigatus*-10561 efficiently expresses tannase activity that has been successfully used for the bench scale molar conversion tannic acid to gallic acid and glucose. This strain is able to produce tannase in the medium containing Tannic acid as the sole carbon source. Tannin acyl hydrolase is an industrially important enzyme that is mainly used in the food and pharmaceutical industry. As the range of applications of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suitable in the diverse fields of applica-

tions. Since microbial activity especially fungal activity is the key aspect in this area, there is enormous opportunity for the cost effective production of tannase. Enzyme can be immobilized to make it more stable to stringent conditions like high temperature, pH value and high tolerance to substrate concentration Hence, activity *Aspergillus fumigatus*-10561 can be enhanced by further purification, molecular characterization and protein engineering of this potent enzyme and will open new possibilities in the field of pharmaceuticals and as a flavouring enhancing commodity or clarifying agent and very efficient neutraceutical in food industry.

Acknowledgements

The authors are thankful to Department of Biotechnology, Himachal Pradesh University Shimla, India for the laboratory facility. The computational facility availed at Bioinformatics Centre, H.P University Shimla is also duly acknowledged.

Conflict of interest

Authors declare that there is no conflict of interest.

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