JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2012. Vol. **6**(1), p. 185-194

Plant Extract Assisted Extracellular Tannase Production by *Aspergillus* Sp MIK23

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(Received: 18 February 2011; accepted: 21 April 2011)

An extracellular tannase (E.C. 3.1.1.20) producing fungal strain was isolated from soil and identified as *Aspergillus* sp MIK23. Out of various plant extracts, *Terminalia chebula* powder (TCP) in the optimized medium enhanced enzyme production. Maximum yield of tannase (3 IU ml^{-1}) was obtained with glucose (10 g/L), urea (2 g/L), and yeast extract (2.5 g/L) when inoculated with 10% inoculum in 48 h. An initial medium at pH 6.0 and a cultivation temperature of 37°C was found to be optimum for enzyme production. Metal ions Mg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+} and Cd^{2+} did not improve enzyme activity, whereas, Ca^{2+}, Fe^{2+} and Hg^{2+} repressed enzyme activity. The enzyme was purified using ammonium sulfate precipitation followed by Q-sepharose ion-exchange chromatography. The enzyme was purified to 42-fold with an overall recovery of 20. The pH and temperature optima of the purified tannase were found to be 7.0 and 37°C, respectively.

Key words: Plant extracts, Tannins, Optimization, *Terminalia chebula*.

Tannins are a group of water-soluble phenolic compounds, which are widely distributed in the plant kingdom[1]. The presence of tannins and their derivatives in agro residues is a major hurdle in their utilization as feed material; perhaps same may be used as an inducer for the industrial production of enzymes[2]. Tannase is an extracellular (Tannin acyl hydrolase, EC 3.1.1.20) enzyme that catalyzes the hydrolysis of tannins by breaking their esters and depside bonds releasing glucose and gallic acid[3]. Although tannase is present in the plants and animals, it is mainly produced by microorganisms[4]. This enzyme has found extensive usage in the food, pharmaceutical and chemical industries[3]. Tannase is an industrially important enzyme and used in the manufacture of wine[5], gallic acid[6], and treatment of tannery effluents[7]. Gallic acid is a key intermediate required for the synthesis of the antibacterial drug trimethoprim[8] and propyl gallate which is widely used as a food antioxidant[9]. Other potential uses of tannase are stabilization of malt polyphenols, clarification of beer and fruit juices[10], and improvement of black tea[11].

Microbial tannase, especially fungal tannase has been used for tannin removal from animal feeds[12]. The filamentous fungus of the *Aspergillus* genus has been widely used for

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tannase production[13,14]. A variety of fungi and bacteria with the tannase producing ability have already been reported and most of them are isolated from soil. The production of tannase by *Aspergillus* sp can occur in the absence of tannic acid but these fungi tolerate tannic acid concentrations as high as 20% without having a deleterious effect on both the growth and enzyme production[15]. Various medium preparations can be used with tannic acid as the sole carbon source for production of microbial tannase.

Recently, agriculture residue has been utilized in enzyme production with the goal of minimizing production cost[12,14]. *Terminalia chebula*, a plant widely distributed in tropical areas in the world and its fruits are commonly known as black myroblans[16]. They have been reported to have high content of phenolic compounds, the major one being gallic acid (GA) and ellagic acid (EA), which possess strong anti-oxidant properties. In this study, plant extracts from *T. chebula*, *Emblica officinalis* and *Zizyphus mauritiana* (with high tannin content) were investigated for the production of tannase by a newly isolated fungal strain i.e. *Aspergillus* sp MIK23. This study includes the isolation and identification of the organism, optimization of process parameters, purification and characterization of tannase.

MATERIALS AND METHODS

Raw material

Terminalia cheubula (black myrobalan or harad), *Emblica officinalis* (myrobalan or amla) and *Zizyphus mauritiana* (Jujube or ber) fruits were procured from the local market in North India. The fruits were dried at 60°C for 96 h and finely ground in a blender. The powdered sample i.e TCP (*Terminalia cheubula* powder) and EOP (*Emblica officinalis* powder) were sieved through a screen (mesh size of 500 µm).

Microorganism and maintenance of culture

A tannase-producing fungus was isolated from a soil sample collected from a local village Amargarh (known for olive cultivation, District Sangrur, Punjab), India. The soil sample (1 g) dissolved in 100 ml sterile distilled water was used to inoculate potato dextrose broth containing 1% tannic acid and incubated at 37°C for 72 h. The resulting inoculum was used for inoculating nutrient agar plates containing 0.5 % tannic acid. The fungal colonies forming a clear zone around the mycelium due to tannic acid hydrolysis were selected and further isolated for tannase production. The isolate was maintained on nutrient-tannic acid agar (TAA) slopes, stored at 4°C and sub cultured after every 15 days. The microorganism was initially grown at 37°C for 72 h in a medium with following composition (g/L): glucose, 2; and sodium nitrate, 6 by inoculating the medium with a loopful of culture and incubated in a temperature controlled orbital shaker (150 rpm). A 72 h old, 5% (v/v) seed culture was used to inoculate sterilized Czapek Dox minimal medium (50 ml in 250 ml Erlenmeyer flask) with the following composition (g/L): sodium nitrate, 6; KH_2PO_4, 1.5; KCl, 0.45; $MgSO_4.7H_2O$, 0.55; $FeSO_4.7H_2O$, 0.52; and Zn $SO_4.7H_2O$, 0.01. The medium was sterilized for 15 min at 121°C, 15 psi pressure, and the initial pH of the medium was adjusted to 6.0. *Terminalia chebula* powder (TCP) was used for the induction of tannase in the fermentation medium. TC powder (3.0%, w/v) containing maximum concentration of tannic acid (0.34%, w/v) was prepared separately and its solution was sterilized by filtering it through a sterile membrane (pore size 0.2 um, Sartorius,US). Flasks were incubated (30°C, 150 rpm) in a rotary shaker for 48 h. Samples were withdrawn aseptically at regular time intervals and analyzed for extracellular tannase activity.

Optimization of process parameters

Using *Aspergillus* sp MIK23 for tannase production, a range of medium constituents was optimized in a shake flask for enzyme production. To study the effect on enzyme production, different carbon sources and their concentrations (0.5%, w/v) were added to the medium before sterilization. Different organic (0.5%, w/v) and inorganic (0.5%, w/v) nitrogen sources and their concentrations were added to the medium to determine their effect on tannase production. The chloride salts of different metal ions, including Mg^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+} were used in the culture medium. The concentration of TCP (0.25-3.5%) was optimized to test its effect on enzyme production. The effect of incubation time (0, 24, 48 and 66 h), pH (3.5, 4.0, 4.5, 5.0. 5.5, 6.0. 6.5, and 7.0), and temperature (25, 30, 37 and 45°C) on enzyme production was tested in 250 ml Erlenmeyer flasks containing 50 ml of the medium. The effect of

inoculum age (6, 42 h), and inoculum volume (5-20%, v/v) was tested at the shake flask level.

Tannin estimation

Total tannin content of the raw material was estimated as described by Hagerman and Butler[17] with BSA as protein standard. Black myrobalian was found to contain 34% tannins.

Enzyme Assay

Tannase activity was measured using a modification of the Deschamp's *et al.* [18]. A typical assay mixture comprised of 1ml of tannic acid (1%, w/v), dissolved in a phosphate buffer (10 mM, pH 6.0) and 100 µl of culture filtrate was incubated at 37°C for 45 min. The residual tannic acid from the enzymatic reaction was precipitated with 2 ml bovine serum albumin (BSA) solution, and was centrifuged at 3000×*g* for 20 min. To the resultant supernatant, 2.4 ml of 10 mM phosphate buffer (pH 6.0) and 2.5 ml saturated $NaHCO_3$ (pH 8.6) was added. Samples were kept at room temperature and the resultant green color was determined at 440 nm. One unit of tannase activity was defined as mount of enzyme that liberates one micromole of gallic acid per minute under the above assay conditions. Extracellular protein was measured by the method of Lowry *et al*. using BSA as the standard[19].

Purification and Characterization

Ammonium sulfate fractionation

A volume of 500 ml of crude tannase was taken, and then the required quantity of ammonium sulfate (50-90%) was added slowly following constant stirring at 4°C. The precipitated proteins were separated by centrifugation at 3,000 x *g* for 20 min at 4°C. The separated proteins were then re-suspended in a minimum amount of phosphate buffer (10 mM , pH 6.0) and refrigerated for further analysis. Precipitated proteins were dialyzed against 10 mM phosphate buffer (10 mM, pH 6.0) for 24 h at 4°C.

Anion-exchange chromatography

The chromatography column (2 x 10 cm; Pharmacia) was packed with Q-sepharose resin (GE Healthcare, US). The pre-packed column was equilibrated with 10 mM phosphate buffer (pH 6.0). The concentrated enzyme sample obtained after dialysis was loaded onto the column. Elution from the column was performed with a linear gradient of 0-1 M NaCl in the same buffer at a flow rate of 1ml/min. Fractions (1ml) were collected and analyzed for tannase activity. The fractions possessing high tannase activity were pooled and further used for characterization.

Effect of pH

To study the effect of pH on tannase activity, the enzyme activity was measured in buffers of pH ranging from 5.0 to 7.0 using acetate buffer for pH 5.0 and phosphate buffer for pH 5.5-7.0.

Optimum temperature

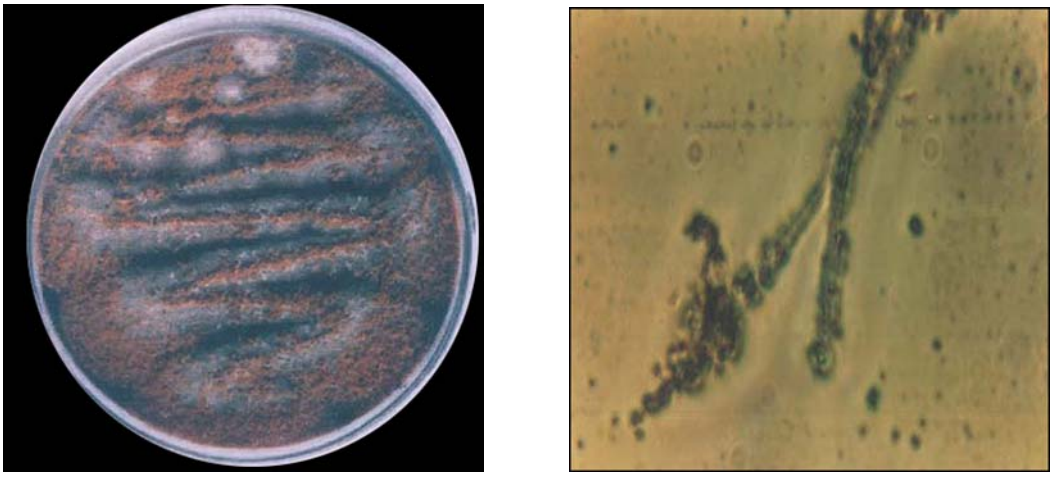
To study the effect of temperature on enzyme activity, the purified enzyme was incubated at different temperature ranging 25°C, 30°C, 37°C and 40°C. The activity was assayed as described earlier.

Effect of plant extracts

Different substrates known to possess high tannic content (1% w/v) like *Emblica officinalis* (common name Amla) and *Terminalia chebula* (common name Harad) were added into the fermentation medium to enhance tannase activity.

RESULTS AND DISCUSSION

In this study six isolates were studied for their tannase producing potential. Three fungal cultures were checked for their ability to grow on an inducer (TCP, 3.5%, w/v), present in the growth medium. Only one isolate showed growth on a medium containing TCP (tannin 0.34%, w/v) after 48-72 h of incubation at 37°C. This isolate named MIK23 was identified based on its colony morphology; presence of spores and its ability to hydrolyze tannins (data not shown). The isolated strain was finally deposited with International depository Microbial Type Culture Collection and Gene Bank (MTCC, India) and identified as *Aspergillus flavus* (Fig. 1). The strain was assigned accession # MTCC 9639. Growth studies in the shake flasks indicated that the tannase production is growth associated. Further, increase in incubation time up to 60 h did not improve enzyme activity (Fig. 2). *Aspergillus* MIK23 reached the highest activity of 0.126 IU/ml at 30°C at an incubation time of 48 h. These results are consistent with Sabu *et al*. [20] who demonstrated that tannase yield was associated with fungal growth. However, tannase production by *Penicillum variable* was observed in 72 h[21]. Enzyme activity decreased on



A **B**

Fig. 1. Growth of *Aspergillus* sp MIK23 on (A) tannic acid-agar plate and (B) photomicrograph of an isolate.

prolonged incubation. This could be due to inhibition and denaturation of the enzyme[22].

Effect of Carbon sources

Various carbon compounds were added at a concentration of 5 g/L to the fermentation medium containing TCP. Glucose exhibited the highest tannase production (0.14 IU/ml, Fig. 3) after 48 h of fermentation. Other carbon sources, fructose, maltose and sucrose too supported tannase production but yield was lower when compared to that obtained from glucose. The growth in the medium containing only glucose was lower, which suggests that fungi needs both glucose and plant residues (TCP) as carbon sources for higher tannase production. For all subsequent experiments, glucose was used as the carbon source. In order to determine the optimum concentration of the glucose for tannase production, different concentrations (2-20 g/L) of glucose was used in the medium. With increasing concentrations of glucose, tannase activity increased up to 10 g/L in the medium and thereafter declined. The maximum enzyme activity (0.142 IU/ml) was achieved with 10 g/L glucose (data not shown). With increase in further glucose concentration in the medium, the enzyme activity

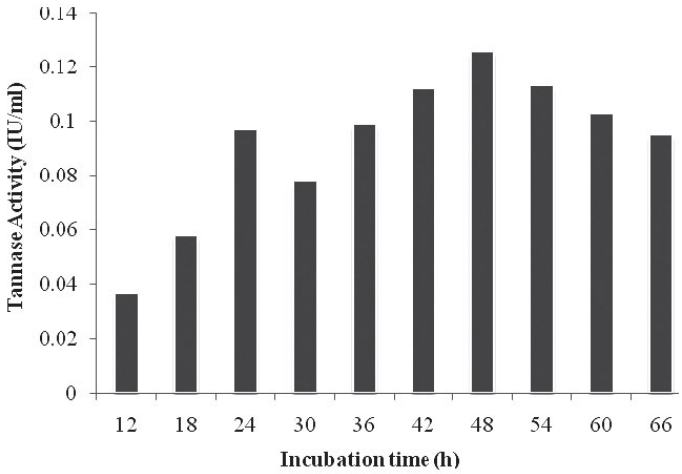


Fig. 2. Effect of incubation period on the production of tannase by *A. flavus* MIK23.

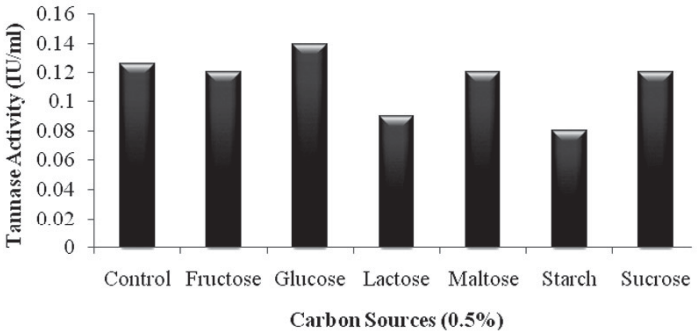


Fig. 3. Effect of carbon sources on the production of tannase by *Aspergillus* sp MIK23.

decreased thus indicating product inhibition. Similar results were reported by other authors[3,6]. *A. niger* reached the highest enzyme activity in the presence of tannic acid as carbon sources while little activity was observed in the medium containing glucose as carbon source[23].

Effect of nitrogen sources

Six different nitrogen sources were used at a concentration of 5 g/L into the fermentation medium containing glucose (10 g/L). Among all the inorganic nitrogen sources tested, urea was the most effective for tannase (0.130 IU/ml) production (Fig. 4a). Therefore, urea at different concentrations was considered for further medium optimization. On increasing the concentration of urea in fermentation medium, pH was also increased, which interferes during enzyme activity. For further experiment urea (2 g/L) was used in the production of tannase from *A. flavus* MIK23. Mukherjee and Banerjee[24] found urea at low concentration increases tannase activity by a co-culture of *R. oryzae* and *A. foetidus*. At higher concentration, urea caused inhibitory effect on enzyme production. Urea at higher concentration might cause denaturation by causing conformational change in the tertiary structure of the enzyme[24].

Five organic nitrogen sources were used at the concentration of 5 g/L in a medium containing glucose (10 g/L). Among all the organic nitrogen sources tested, yeast extract was the most effective for tannase production (Fig. 4b). In order to determine the optimum concentration of yeast extract for tannase production, different concentrations (2-20 g/L) of yeast extract was used in the medium containing glucose and urea. Tannase production was found to be highest (0.20 IU/ml) with optimum concentration of yeast extract being 2 g/L. Tannase production depends on the availability of nitrogen source in the medium and has regulatory effects on enzyme synthesis[25]. Nitrogen can be an important limiting factor in the microbial production of enzymes.

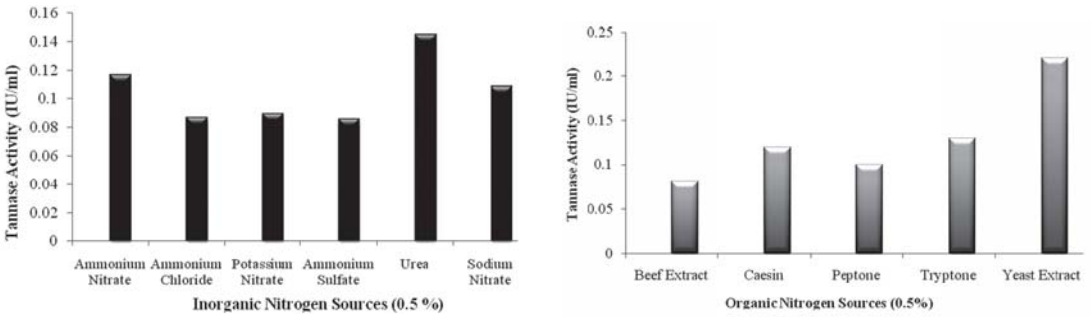


Fig. 4. Effect of (A) inorganic and (B) organic nitrogen sources on the production of tannase by *A. flavus* MIK23.

Effect of environmental factors

The pH of the production medium plays a significant role in the production of metabolites. The tannase production increased with increase in pH up to 6.0. On further increasing the pH of the medium, tannase production decreased as shown in (Fig. 5a). Maximum enzyme activity (0.21 IU/ml) was observed at pH 6.0. It may be concluded that enzyme was active at slightly acidic pH value, whereas the enzyme activity start decreasing as the pH value approached to the alkaline range. Generally in fungal tannase, optimum pH for tannase production has been found to vary from 4.5-6.6 [26,27].

On increasing temperature of the fermentation by 7°C, no improvement in tannase production was observed. With further increase in temperature above 37°C, there was a decrease in enzyme activity (Fig. 5b). There are reports that described optimum temperature ranges from 30 to 35°C in case of tannase production by fungi[3]. Kar *et al.*[28] observed optimum enzyme activity for tannase production at 32°C.

Effect of inoculum age and size

A 24-h-old seed culture when used as inoculum, gave maximum tannase production in the fermentation medium (data not shown). Gupta *et al.*[29] obtained best results for the production of

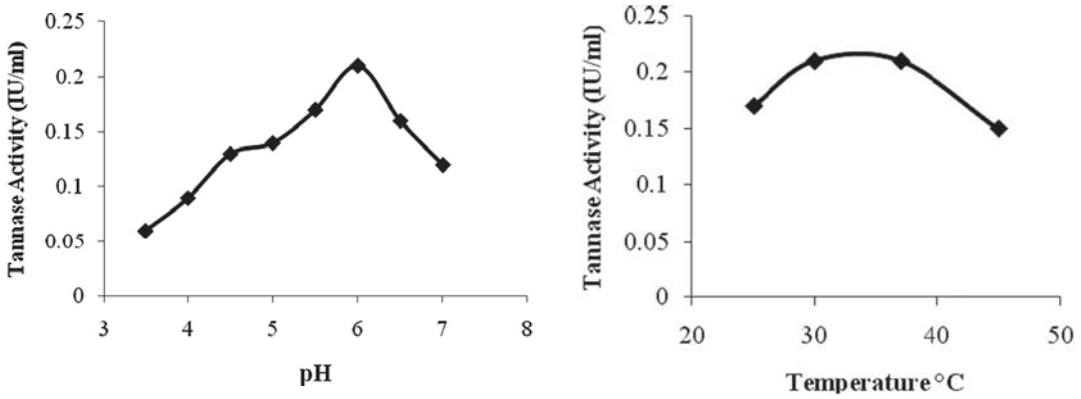


Fig. 5. Effect of (A) pH and (B) temperature on the production of tannase by *Aspergillus* sp MIK23.

tannase from *Aspergillus japonicus* with inoculum age of 24 h. The inoculum level of 5.0 to 20% (v/v) was used in the cultivation medium to establish the effect of inoculum on the tannase production by *Aspergillus* sp MIK23. A 10% (v/v) inoculum was optimal for the growth and tannase production (0.22 IU/ml) and the lag phase was also minimal (Fig. 6b). Lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis.

Effect of metal ions

Different metal ions were used in the cultivation medium to determine the effects of metal ions on growth and tannase production by *Aspergillus* sp MIK23. Mg^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+} and Cd^{2+} did not improve enzyme activity, whereas some of the metal ions Ca^{2+}, Fe^{2+} and Hg^{2+} were found to be inhibitory (Table 1). No additional metal ions were required to support the tannase production from *Aspergillus* sp MIK23. The inhibition of enzyme activity by Hg^{2+} ions may have been due to its interaction with sulfhydryl

Table 1. Effect of metal ions on the production of tannase by *Aspergillus* sp MIK23.

Metal	Percentage activity retained at concentration (mM)			
	1	5	10	20
Mg^{2+}	62	59	58	50
Zn^{2+}	34	39	45	37
Ca^{2+}	30	29	24	20
Fe^{2+}	26	19	17	16
Cu^{2+}	62	48	44	39
Cd^{2+}	48	59	49	41
Hg^{2+}	28	15	12	11

Table 2. Summary of the purification of tannase from *Aspergillus* sp MIK23.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Fold Purification	Yield (%)
Crude extract Ammonium	216	47.8	0.22	1	100
Sulphate precipitation	28	11.98	0.42	2	25
Q-Sepharose	0.88	8.07	9.17	41	18

groups, suggesting that an important cysteine residue is in or close to the enzyme active site. Addition of metal ions like Mg^{2+} and Ca^{2+} in fermentation medium found to decrease in tannase activity in *Aspergillus niger*[22].

Effect of plant extracts

Various concentrations of TCP were used separately in the medium to find out the optimum concentration for enzyme production. It has been observed that 25 g/L tannic acid was most suitable for maximum activity (0.30 IU/ml) production with *Aspergillus* sp MIK23 (Fig. 7). With further increase in TCP concentration, the enzyme activity decreased. Tannase is an inducible enzyme in fungi therefore; inducer concentration plays an important role in the production of tannase. Misro *et al.* [30] demonstrated 20 g/L as suitable concentration by *Rhizopus oryzae*. Three plant extracts (known to possess high concentrations of tannins) were further used to enhance the production of tannase activity. Maximum enzyme activity was obtained with TCP tannic acid (relative activity 100%); however, *Emblica officinalis* and *Zizyphus mauritiana* recorded decrease in activity *i.e.* 33 and 38%, respectively. Different substrates rich in tannins like *Zizyphus mauritiana*, *Syzygium cumini*, *Phyllanthus emblica* and *Sorghum vulgaris* leaves were used for tannase production[31].

Utilization of agriculture residue in enzyme production through biotechnology is significantly increasing with the dual goal of waste management and value addition[32]. *Larrea tridentate* powder was used as a sole carbon source and inducer for the production of tannase activity from *Aspergillus niger*[33]. Agricultural residue for tannase production from *A. niger* ATCC 16620 was reported elsewhere[20]. TCP was selected as tannase inducer because of its phenolic compounds, tannins and sugar content (3, 5 and 3%).

Purification and characterization of Tannase

The purification experiment is summarized in Table 2. Protein content and enzyme activity were determined after each purification step, and extracellular tannase was isolated from the culture medium of *Aspergillus* sp MIK23 by ammonium sulfate fractionation (80% saturation). Fractional precipitation resulted in 25% recovery of tannase

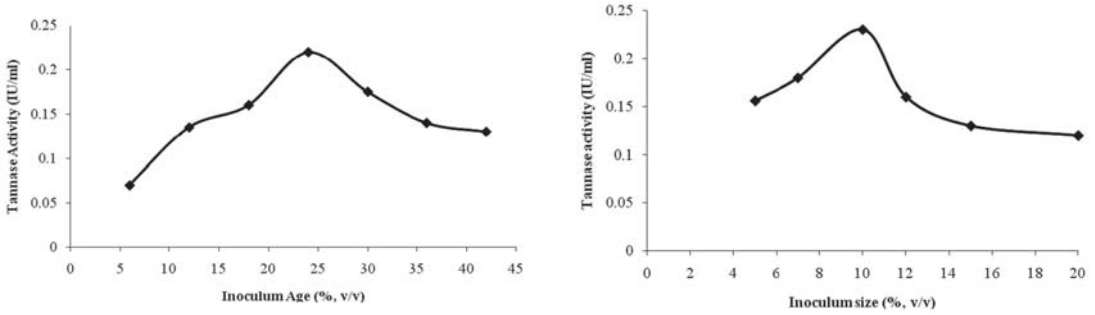


Fig. 6. Effect of (A) inoculum age and (B) size on the production of tannase by *Aspergillus* sp.

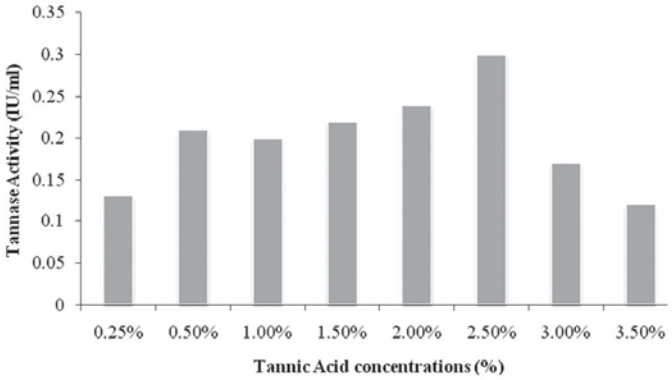


Fig. 7. Effect of addition of different plant extracts as a source of tannic acid on the production of tannase by *Aspergillus* sp MIK23

activity. The protein was further purified on a Q-sepharose column, pre-equilibrated with buffer A (10 mM phosphate buffer, pH 6.0). The column was eluted with a linear gradient buffer B (10 mM phosphate buffer and 0.1-1 M NaCl). Fractions from Q-sepharose column with high tannase activity were pooled and concentrated. The Q-sepharose step yielded a purification of 41 fold with a specific activity of 9.17 units per mg while the percentage yield 18-fold (Table 2). Tannase from *A. heteromorphus* and *P. variotii* were purified using same techniques with yields of 3 and 13.5 % respectively[34,35]. However, tannase from *A. niger* GH1 purified to homogeneity by anion exchange and gel filtration chromatography led to a yield of 46-fold.

Characterization of Tannase

Optimum pH

The optimum pH for purified tannase from *A. flavus* was found to be 7.0. The enzyme activity showed a sharp increase at pH 7.0 (data not shown). Generally, the fungal tannase have acidic pH optimum[3]. However, the optimum pH for tannase isolated from *A. niger* was shown to be between 5.0-6.0 with instability occurring at a pH above pH 6.0[22]. Optimum pH values of enzyme from other sources like *Candida* sp. K 16 [3], *A. niger* LCF 8 [26], *Penicillium chrysogenum*[36] had been reported to be 6.0, 6.0 and 5.0-6.0, respectively.

Optimum temperature

To evaluate the effect of temperature on purified tannase activity, the temperature was varied from 25 to 40°C. The maximum enzyme activity of tannase was observed at 37°C. Further, increase in temperature (40°C) resulted in a decrease in enzyme activity (data not shown). An optimum temperature between 30-40°C was reported for tannase activity in case of *A. oryzae*[7]. The optimum temperature for tannase activity was 50°C, which was similar to that obtained for *P. variable* tannase[37].

CONCLUSION

This is the first report on the TCP assisted production, purification and characterization of tannase from *Aspergillus* sp MIK23 which has accession number MTCC 9639. Our results indicate that a culture medium containing plant extract (TC powder), glucose and urea were the most suitable for fungal growth and enzyme production. Using the optimized process, maximum production of tannse was achieved within a short duration (48 h) of cultivation as compared to other fungal strains. The enzyme was partially purified and exhibited optimum activity at pH 7.0 and 37°C. The tannase produced was functional at a wide range of temperature and pH values which is a useful feature for biotechnological applications.

ACKNOWLEDGEMENTS

The authors thank CBIS, Deakin University, Australia and Department of Biotechnology, Punjabi University, India for supporting collaborative research.

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