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Effect of Additives on the Activity of Tannase from *Aspergillus awamori* MTCC9299

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Abstract Tannase from *Aspergillus awamori* MTCC 9299 was purified using ammonium sulfate precipitation followed by ion-exchange chromatography. A purification fold of 19.5 with 13.5% yield was obtained. Temperature of 30 °C and pH of 5.5 were found optimum for tannase activity. The effects of metals and organic solvents on the activity of tannase were also studied. Metal ions Mg^{+2}, Mn^{+2}, Ca^{+2}, Na^{+}, and K^{+} stimulated the tannase activity, while Cu^{+2}, Fe^{+3}, and Co^{+2} acted as inhibitors of the enzyme. The addition of organic solvents like acetic acid, isoamylalcohol, chloroform, isopropyl alcohol, and ethanol completely inhibited the enzyme activity. However, butanol and benzene increased the enzyme activity.

Keywords Tannase · DEAE-cellulose · Organic solvents · Metal ions · *Aspergillus awamori* MTCC 9299

Introduction

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an inducible enzyme that catalyzes the hydrolysis of ester and depside bonds in hydrolyzable tannins by releasing glucose and gallic acid [1, 2]. Gallic acid finds application in many fields like dye making, pharmaceuticals, leather, and chemical industries. Besides gallic acid production, the enzyme is extensively used in the preparation of instant tea, wine, beer, and coffee-flavored soft drinks and also as additive for detannification of food. A potential use of tannase is in

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the treatment of waste water contaimination with polyphenolic compounds such as tannic acids and as an analytical probe for determining the structures of naturally occurring gallic acid esters [3, 4].

The *Aspergillus* species produces a large variety of extracellular enzymes, of which tannases are of significant industrial importance. As each industrial application may require specific properties of the biocatalysts, there is still an interest in finding new tannases that could find novel applications. There are several reports on the production of tannase [5], but there are only a few reports on the detailed characterization, i.e., the effect of additives such as metal ions, cations, anions, surfactants, reducing agents, chelators, organic solvents, etc. on the enzyme activity [6]. Organic solvents can be advantageous in various industrial enzymatic processes, e.g., the reaction media used in biocatalytic esterification and transesterification contains less than 1% water. The use of organic solvents can increase the solubility of nonpolar substrates, increase the thermal stability of enzymes, decrease water-dependent side reactions, or eliminate microbial contamination [7]. Keeping this in view, the effect of additives such as metal ions, organic solvents, etc. on the activity of tannase from *Aspergillus awamori* MTCC 9299 was investigated.

Materials and Methods

Microorganism and Maintenance of Culture

A tannase-producing fungus was isolated from the soil sample collected from Guru Jambheshwar University of Science and Technology campus. Soil sample (1 g) was dissolved in 10 ml sterile distilled water. Of this, 1 ml was inoculated into potato dextrose broth containing 0.5% tannic acid and incubated at 30 °C for 72 h. Aliquots from this were plated on agar plates containing 0.2% tannic acid. Fungus colony capable of forming clear zone around the mycelium due to the hydrolysis of tannic acid was selected and purified. Selected strain was then morphologically identified as *A. awamori* MTCC9299 by Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The strain was maintained on potato dextrose agar slants in a refrigerator at 4 °C by regular transfers.

Preparation of Spore Inoculum

Fungal spore inoculum was prepared by adding 2.5 ml of sterile distilled water containing 0.1% Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions, and the number of spores in the suspension was determined using the Neubauer chamber. The volume of 1 ml of the prepared spore suspension was used as the inoculum with concentration of 5×10^9 spores.

Fermentation Medium

For the fermentation process, a 250-ml Erlenmeyer flask with 50 ml of Czapek Dox minimal medium containing (gram per liter): $NaNO_3$, 6; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4.7H_2O$, 0.52; $FeSO_4.7H_2O$, 0.01; and $ZnSO_4.7H_2O$, 0.01 was employed [8]. The medium was adjusted to pH 5.0 and then sterilized at 121°C for 15 min. Tannic acid solution was prepared separately, and the solution was adjusted to pH 5.0 with 0.1 M NaOH, then sterilized by filtering through a sterile millipore membrane (pore size 0.2 μm) and added to the medium to have a final tannic acid concentration of 1%.

Tannase Assay

Tannase activity was determined colorimetrically using the method of Mondal [9]. The reaction mixture contained 0.3 ml of tannic acid (0.5% in 0.2 M sodium acetate buffer, pH 5.5) and 0.1 ml of enzyme and was incubated at 30 °C for 20 min. The enzymatic reaction was stopped by addition of 3 ml of BSA solution, which precipitates the remaining tannic acid. The tubes were centrifuged (5,000×g 10 min), and the resultant precipitate was dissolved in a 3-ml SDS-triethanolamine solution. A 1 ml of $FeCl_3$ reagent was added to each tube and was kept for 15 min at room temperature for stabilization of the color. The absorbance was read at 530 nm against the blank. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 mM of tannic acid in 1 min under assay conditions.

Assay of Protein Concentration

The protein concentration was determined by the Bradford method [10] using bovine serum albumin as the standard.

Purification of Tannase

Supernatants from batch cultures were concentrated using ammonium sulfate fractionate and dialyzed against acetate buffer (0.02 M and pH 5.5) at 4 °C. The resultant enzyme solution was loaded onto 2.5×10 cm DEAE-cellulose column equilibrated with 0.02 M acetate buffer (pH 5.5), and the proteins were eluted with a linear gradient of 0.0–0.5 M NaCl at a flow rate of 5 ml/h. The fractions of 2 ml each were collected and analyzed for enzyme activity. Fractions with high enzyme activity (fraction number 4 and 5) were pooled together and used for further experiments.

SDS-PAGE and Molecular Weight (Mr) Determination

The comparative mobility of partially purified and purified tannase was carried out on 7.5% SDS-PAGE. Molecular weight markers were purchased from Sigma and were run parallel to the samples.

Enzyme Characterization

The effect of different temperatures, pH, organic solvent, and metal ions on the enzyme fractions obtained after DEAE-cellulose chromatography was studied.

Optimum pH and Temperature for Tannase Activity

The optimum pH for tannase activity was determined at 30 °C by incubating the enzyme with substrate at different pH ranges from 3 to 6. The pH of the reaction mixture was varied using different buffers (acetate buffer for pH 3.5–5.5 and phosphate buffer for pH 6). The optimum temperature was determined by incubating the reaction mixture for 20 min at different temperatures ranging from 20 to 70 °C.

Effect of Organic Solvents and Metals Ions

The enzyme solution containing different concentrations (20%, 40%, and 60%) of various organic solvents (acetone, toluene, benzene, ethanol, acetic acid, isoamylalcohol,

chloroform, phenol, butanol, and glycerol) and 1 mM concentration of various metal ions like Ca^{+2}, Na^{+}, K^{+}, Mn^{+2}, Fe^{+3}, Zn^{+1}, Co^{+2}, Cu^{+2}, and Mg^{+2} were incubated (acetate buffer 0.2 M, pH 5.5) at 30 °C for 20 min, and the effect of organic solvents and metal ions on tannase activity was studied.

Results and Discussion

Purification of Tannase

Ammonium sulfate fractionation was done at various concentrations (50–90%). Recovery of enzyme was maximum at 80% fractionation. The elution profile of tannase from the DEAE-cellulose column filtration is shown in Fig. 1. The elution profile of the enzyme showed two peaks. Maximum tannase activity was found at the first peak. The active fraction (fraction number 4 and 5) were pooled. DEAE-cellulose column chromatography led to an overall purification of 19.5-fold with a yield of 13.5% (Table 1). Mahendran et.al. [11] and Kasieczka-Burnecka et.al. [12] also obtained similar values after DEAE-cellulose column chromatography of tannase from *Paecilomyces variotii* and *Verticillium* sp. P9, respectively. However, a purification fold of 135 with 91% yield of tannase from *Penicillium variable* has also been reported using gel-filtration chromatography [13].

SDS-PAGE and Molecular Weight (Mr) Determination

Figure 2 shows the molecular mass of purified tannase obtained from SDS-PAGE analysis, with a single band of 101±2 kDa indicating the homogeneity of the enzyme. Tannases reported so far are generally of high molecular weight ranging from 80 to 310 kDa [11–13]. Tannase from *Aspergillus niger* ATTC 16620 have been reported as a single monomer unit of 149 kDa [14]. Tannase from *Aspergillus flavus and A. niger* N 888 has also been reported as single peptide of 80–85 and 165 kDa, respectively [14]. However, the molecular weight of *P. variable* tannase has been reported to be 310 kDa with a dimer of two subunit of 158 kDa [13].

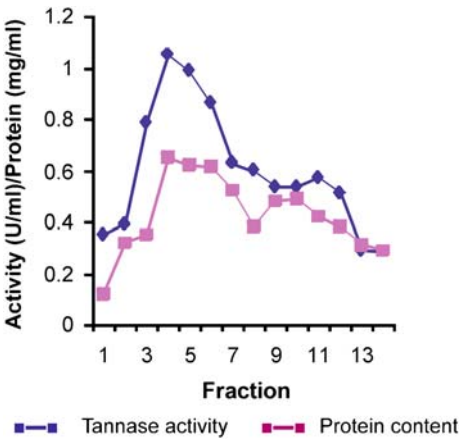

Fig. 1 Elution graph of ion-exchange chromatography

Table 1 Purification table of tannase from *A. awamori* MTCC 9299.

Source	Total activity	Total protein (mg)	Specific activity (μ/mg)	Purification (fold)	Yield (%)
Crude	128	261.0	0.489	1	100
Ammonium sulfate purification	37.12	41.7	0.89	1.82	29
Ion exchange Chromatography	17.28	3.76	9.55	19.5	13.5

Effect of pH and Temperature on the Activity of Enzyme

Effect of initial pH (Fig. 3) of the reaction mixture on the tannase activity showed that the activity is extremely low at pH 3.0 (1.63 U/ml). The activity of enzyme increased gradually with increase in pH peaking at pH 5.5 (2.88 U/ml). Further increase in pH resulted in decrease in the activity of tannase. To evaluate the effect of temperature on the activity of purified tannase, the temperature was varied from 25 to 70 °C. With an increase in temperature, the tannase activity increased, and optimum activity was recorded at 30 °C (Fig. 4). These results are in agreement with the previous reports of pH 5.5 for *Aspergillus ruber* [15] and a pH range of 5–7 for *P. variotii* [11]. The optimum temperature for tannase activity was 30 °C, which was similar to those obtained for *Lactobacillus plantarum* CECT748 [16]. However, optimum temperature and pH of tannase from *A. awamori* Nakazawa have been reported to be 35 °C and pH 5.0, respectively [2].

Effect of Organic Solvents on Enzyme Activity

In nature, enzymes function in aqueous solutions. Therefore, it is not surprising that virtually all studies in enzymology so far have used water as the reaction medium. However, from the biotechnological standpoint, there are numerous advantages of conducting enzymatic

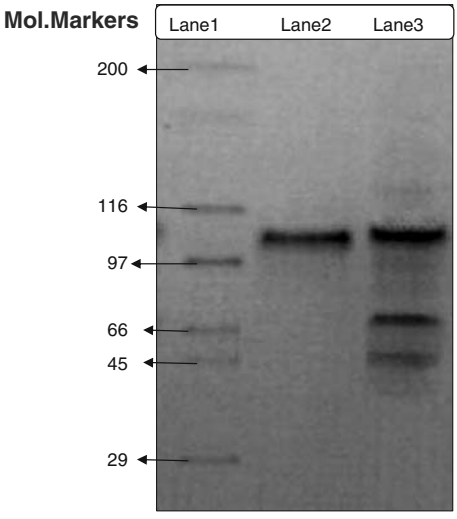

Fig. 2 Molecular mass of purified *A. awamori* MTCC 9299 tannase estimated by electrophoresis. *Lane 1* shows marker proteins (kilodalton); *lane 2* shows purified tannase (101±2 kDa molecular mass); and *lane 3* shows partially purified tannase (ammonium sulfate precipitation)

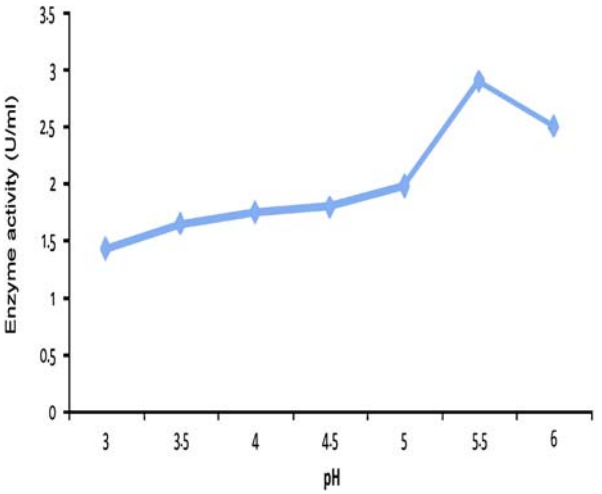

Fig. 3 Effect of pH on tannase activity

conversions in organic solvents as opposed to water: (1) high solubility of most organic compounds in nonaqueous media; (2) ability to carry out new reactions impossible in water because of kinetic or thermodynamic restrictions; (3) greater stability of enzymes; (4) relative ease of product recovery from organic solvents as compared to water; and (5) the insolubility of enzymes in organic media, which permits their easy recovery and reuse and, thus, eliminates the need for immobilization [17]. To determine the effect of organic solvents on the activity of tannase, various organic solvents (Table 2) were used at different concentrations (20%, 40%, and 60%). It was found that acetic acid, isoamylalcohol, chloroform, and isopropyl alcohol completely inhibited the activity of tannase at all concentrations. However, ethanol inhibited the tannase activity by 48.84% initially; thereafter, complete loss in the enzyme activity was observed at 40% and 60%. A gradual decrease in the activity of tannase was observed with the increasing concentration of acetone and toluene, and finally, at 60%, the activity was reduced to 55.01% and 28.49%, respectively. With the initial inhibitory effect at 20% and 40% methanol, the original activity was regained at 60%. It was also observed that the

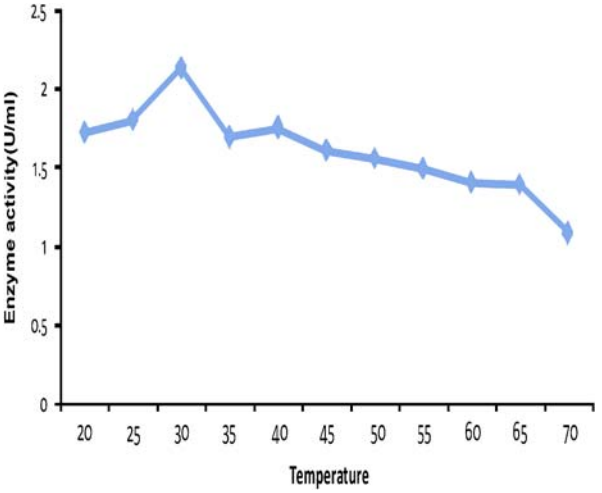

Fig. 4 Effect of temperature on tannase activity

Table 2 Effect of organic solvents on tannase activity.

Control	100%		
Concentration (*v*/*v*; %)	20%	40%	60%
Acetone	78.51±0.94	63.92±0.94	55.01±0.84
Butanol	163.86±1.14	203.74±2.07	199.44±3.16
Benzene	138.76±0.48	127.53±1.12	199.44±1.21
Toluene	86.66±2.69	82.67±1.52	28.49±1.21
Acetic acid	–	–	–
Methanol	54.62±2.60	76.68±5.94	100.31±0.54
Ethanol	51.16±1.14	–	–
Chloroform	–	–	–
Isopropyl alcohol	–	–	–
Isoamyl alcohol	–	–	–

presence of butanol and benzene increased the enzyme activity by twofold at 60% *v*/*v* concentration. Sharma et.al. [13] have also studied the effect of organic solvents on tannase from *P. variable* and reported more than 60% residual activity in 20% *v*/*v* of carbon tetrachloride, heptane, petroleum ether, and toluene after 60 min. They also observed that the enzyme was stable more than 50% in 60% *v*/*v* of carbon tetrachloride, heptane, petroleum ether, and toluene for 5 min. Saborowski et.al [7] studied the effect of organic solvents on endopeptidases and found that the chymotrypsin and protease activity were slightly elevated at 5% and 10% concentration of acetone, 2-propanol, methanol, and ethanol, respectively. Trypsin activity, in contrast, was strongly elevated by organic solvents; the activity rose concomitantly to eightfold of initial value at a concentration of 40% of 2-propanol. Fridovichi [18] reported that in most of the cases, the organic solvents acted as competitive inhibitors at low concentrations and became increasingly noncompetitive as the concentration of the solvent was raised.

Effect of Metal Ions on Enzyme Activity

Various metal ions like $ZnSO_4$, $MgSO_4$, $CaCl_2$, $CuSO_4$, $MnSO_4$, $Fe_2(SO_4)_3$, $CoCl_2$, NaCl, and KCl at 1 mM concentration each were tested for their effect on tannase activity. Table 3

Table 3 Effect of metal ions on tannase activity.

Additives	Relative activity (%)
Control	100
$ZnSO_4$	96.52±0.2
$MgSO_4$	123.99±0.22
CoCl	28.86±0.85
Cu SO_4	48.79±1.33
MnSO4	115.23±1.07
Fe SO_4	23.11±0.44
CaCl2	116±0.57
NaCl	111.45±0.89
KCl	109.89±1.13

shows that among all the metal ions studied Mg^{+2}, Mn^{+2}, Ca^{+2}, Na^{+}, and K^{+} elevated the tannase activity by 23.9%, 15.23%, 16%, 11%, and 9%, respectively. Zn^{+2} did not show any significant effect on tannase. Cu^{+2}, Fe^{+3}, and Co^{+2} were found to be strongly inhibiting the tannase activity by 51.21%, 76.89%, and 71.14%, respectively. The effect of metal ions on tannase activity was studied by Kar et.al. [6]. Mg^{+2} or Hg^{+} (1.0 mM) activated tannase activity; on the other hand, Ba^{+2}, Ca^{+2}, Zn^{+2}, Hg^{+2}, Ag^{+}, Fe^{+3}, and Co^{+2} inhibited tannase activity at 1.0-mM concentration. Mukherjee and Banerjee [4] found that the presence of the divalent ion Mg^{+2} at low concentration increases tannase activity, whereas, it was inhibited maximally by Hg^{++} followed by Fe^{+3}, Zn^{+2}, and Ba^{+2}. Sabu et.al. [14] also studied effect of metal ions on tannase from *A. niger* ATCC 16620 and found that the addition of metal ions like Zn^{+2}, Mn^{+2}, Cu^{+2}, Ca^{+2}, Mg^{+2}, and Fe^{+2} inhibited the enzyme activity. Kasieczka-Burnecka et.al. [12] have recently reported inhibitory effect of Zn^{+2}, Cu^{+2}, K^{+}, Cd^{+2}, Ag^{+}, Fe^{+3}, Mn^{+2}, Co^{+2}, Hg^{+2}, Pb^{+2}, and Sn^{+2} on tannase from *Verticillium* sp.

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