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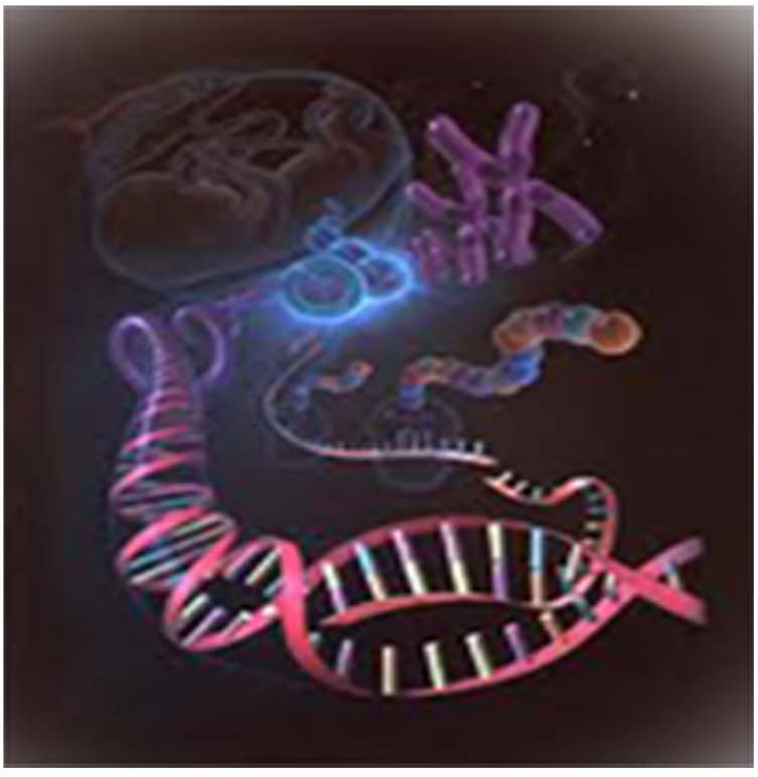

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Research Paper

TANNIN DEGRADATION EFFICIENCY OF TANNASE PRODUCED BY *TRICHODERMA HARZIANUM* MTCC 10841 AND ITS BIOCHEMICAL PROPERTIES

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The present study aims to utilize natural tannins as substrate by the tannase produced by *Trichoderma harzianum* MTCC10841 for gallic acid production. Tannin degradation efficiency of tannase was investigated using different tannin rich agro-residues as substrates. Amla fruit, tamarind seed, jamun leaves, mulberry leaves and keekar leaves proved to be better substrate than tannic acid. The result of enzymatic hydrolysis of these substrates was also observed by paper chromatography which showed the appearance of end product gallic acid. The optimum pH and temperature for the enzyme was 5.5 and 40°C respectively. The enzyme was found to be stable at 40°C for 2hr. Effect of various additives on tannase activity was also evaluated. Among the different metal salts tested, $HgCl_2$ strongly inhibited the enzyme followed with $ZnCl_2$ and $MnCl_2$. The detergents (SDS, Tween-20, -60 and -80) did not affect enzyme activity while β-mercaptoethanol and EDTA had inhibitory effect.

Keywords: Amla fruit, Jamun leaves, Gallic acid, Tannase, Tannin biodegradation, *Trichoderma harzianum*

INTRODUCTION

Tannins are water soluble polyphenolic compounds which are widespread in plants and found in bark, galls, leaves, fruits and wood. Tannins are the fourth most abundant plant constituents after cellulose, hemicelluloses and lignin (Rana and Bhat, 2005). Tannase (E.C. 3.1.1.20) also known as tannin acyl hydrolase, is the enzyme which catalyses decomposition of hydrolysable tannins especially gallo-tannins to glucose and gallic acid (Van de Lagemaat and Pyle, 2005). Gallic acid, the product of tannin hydrolysis finds application in many fields including dye-making, pharmaceutical, leather and chemical industries (Hota *et al*., 2007).

Gallic acid, a precursor for the antimalarial drug trimethoprim and the food preservative, propyl gallate, and some dyes is one of the many

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chemicals that are imported. Therefore, attempts have been made in India to find out some of the suitable tannin rich agroresidues for the enzymatic conversion of their tannin content to gallic acid. The conventional production of gallic acid through acid hydrolysis of tannic acid is not only costly but also the yields and purity is low. Of late, production of gallic acid through microbial tannase has been attracting the attention of researchers. It is interesting to note that though tannase production by microorganisms has been well documented (Belur and Mugeraya, 2011), attention leading towards tannin hydrolysis to yield gallic acid appears to be limited (Reddy and Rathod, 2012).

Other important application of tannase includes production of instant tea by solubilization of tea cream and manufacture of coffee-flavored soft drinks (Lu *et al*., 2009). Tannase also participates in the preparation of animal feeding (Nuero and Reyes, 2002) and in leather industry (Orlita, 2004). Tannase can be potentially used for the degradation of tannins present in the effluents of tanneries, which represent serious environmental problems (Van de-Lagemaat and Pyle, 2001). Tannase is also used for clarification and removal of unwanted bitterness from the untraditional fruit juices (pomegranate, cranberry, raspberry, etc.). The presence of high tannin content in these fruits is responsible for haze which results from protein–polyphenol interaction. Tannase applied to remove haze, improves color, bitterness and astringency of the juice upon storage (Rout and Banerjee, 2006; Srivastva and Kar, 2010). The present study evaluates the efficiency of tannase from *Trichoderma harzianum* MTCC10841 to degrade natural tannins, further exploring the possibilities of using these cheaper agro-residues as substrates for gallic acid production. This is the first report on efficient utilization of different natural tannins by the crude tannase. Some biochemical properties of the enzyme have also been studied.

MATERIALS AND METHODS

Micro-Organism

The tannase producing fungal strain *Trichoderma harzianum* MTCC 10841 used in the present investigation was isolated from soil sample collected around cassia tree (*Cassia fistula*), Meerut city, Uttar Pradesh, India. The culture was maintained on slants of malt extract medium which consisted of (g/L): malt extract 20.0g, KH_2PO_4 0.5g, NH_4CL and agar 20.0g.

Extraction of Tannase Enzyme

The fungal strain was inoculated in malt extract liquid medium containing 1% (w/v) tannic acid. A disc (0.8cm) diameter of freshly grown (48h) fungal culture was inoculated in malt extract liquid medium (50ml) and incubated at 30ºC for 96hr. After incubation, the culture was centrifuged at 8,000xg for 10 min at 4ºC. The supernatant was used as crude enzyme and extracellular tannase activity was estimated by performing tannase assay.

Tannase Assay

Tannase was assayed following the method of Mondal *et al*. (2001) using tannic acid as substrate at a concentration of 1% in 0.2M acetate buffer (pH 5.5). The reaction mixture was prepared by the addition of 0.5 ml substrate with 0.1 ml of the crude enzyme and incubated at 40ºC for 20 minutes. The enzymatic reaction was stopped by adding 3ml bovine serum albumin (BSA) (1 mg/ml). The tubes were centrifuged at 5000g for 10 min. The precipitate was dissolved in 2 ml SDS-triethanolamine solution followed by the

addition of 1ml of $FeCl_3$ reagent. The contents were kept for 15 min for stabilizing the colour formed and the absorbance was measured at 530nm against the blank. One unit of tannase activity can be defined as the amount of enzyme which is able to hydrolyze 1M of substrate tannic acid in 1min under assay conditions.

Tannin-rich Agro Residues

Various tannin-rich agro-residues, amla (*Phyllanthus amblica,* bark, leaves and fruit), amaltash (golden shower cassia) (*Cassia fistula*, leaves), ber (*Zyzipus maurtiana*, leaves), Eucalyptus (*Eucalyptus glogus*, bark and leaves), jamun (*Syzgium cumini*, bark and leaves), guava (*Psidium guazava,* bark and leaves), keekar (*Acacia nilotica*, leaves), mango (*Magnifera indica*, leaves), mulberry (*Morus macroura,* leaves), tamarind (*Tamarindus indica*, seed) and pomegranate (*Punica granatum,* rind) were collected from the gardens. They were shade dried and powdered with grinder. The coarse particles were sieved out and the finely grinded powder was used in the present study.

Extraction of Crude Tannins

The finely grinded powder of the agro-residues at 1% concentration was mixed with distilled water (100ml) and kept at room temperature for 3 days. After soaking, the mixture was boiled for 10 min. and filtered. The filtered extracts were used as source of crude natural tannin.

Estimation of tannin content

The tannin content in the crude extract of natural tannin substrates was measured following protein precipitation method (Hagerman and Butler, 1978).

Biodegradation of Natural Tannins

Different tannin rich agro-residues like amaltash leaves, amla leaves and fruit, ber leaves, guava leaves and bark, jamun leaves and pomegranate rind were used as substrate for enzymatic conversion of their tannin content to gallic acid. The crude tannin extract (2ml) of these substrates were treated with tannase (32.3U) produced by *Trichoderma harzianum* for 2 hrs and tannase activity was determined. The pure tannic acid was kept as control. The biodegradation product was also observed by paper chromatography.

Detection of Tannin Degradation Product

The degradation product gallic acid liberated by the action of tannase from *Trichoderma harzianum* was detected by ascending paper chromatography following the method of Katwa *et al.* (1981). The solvent system used was 6% acetic acid. The results were visualized by heating, after spraying with 2% ferric chloride in 30% methanol. Pure gallic acid was used as standard.

Biochemical Characterization of the Tannase

Effect of Reaction Time: The enzyme sample and the substrate were incubated for 10, 15, 20, 25 and 30 min. Standard protocol was used for tannase assay.

Effect of Substrate Concentration: Various concentrations of tannic acid as substrate (0.25-2.0% which corresponds to 1.47-10.31µmoles/ml) were used for studying the effect of substrate concentration under standard tannase assay conditions.

Temperature Optima and Thermal Stability: The optimum temperature for the crude enzyme was determined by subjecting the reaction mixture at different temperatures ranging from 25°-60°C and tannase activity was calculated. To

determine thermal stability, the crude enzyme was incubated at 40°C for 3hr and lipase activity was determined at regular intervals of 30min.

pH Optima: In order to find the optimum pH, tannase was assayed with substrates prepared in 0.1M citrate buffer of pH range 4.0-6.0.

Effect of Additives: The effect of various metal ions (NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, $ZnCl_2$, $MnCl_2$, and $HgCl_2$), detergents (SDS, Tween-20, -60 and -80), β-mercaptoethanol and EDTA on tannase activity was studied. The reaction mixture containing additive (1mM) was incubated at 40°C for 20 min and tannase activity was calculated.

RESULT ANALYSIS

All the experiments and their assays were carried out in triplicate and the mean value was presented.

RESULTS AND DISCUSSION

Biodegradation of Natural Tannins by Tannase from *Trichoderma harzianum* MTCC10841

To investigate the tannin degradation efficiency of tannase from *Trichoderma harzianum*, the enzyme was incubated at 40°C for 2hrs with crude tannins of different agro-residues as substrate. Tannic acid (1%) was kept as control. The tannin content of different agro residues and tannase activity towards different natural tannin substrates were listed in Table 1. The results showed that the tannase from *Trichoderma harzianum* utilized many tannin substrates efficiently after 2 hr incubation. Further incubation did not enhance the enzyme activity significantly. Some tannin sources proved to be better substrates than tannic acid which includes amla

Table 1: Degradation of Natural Tannins by Tannase

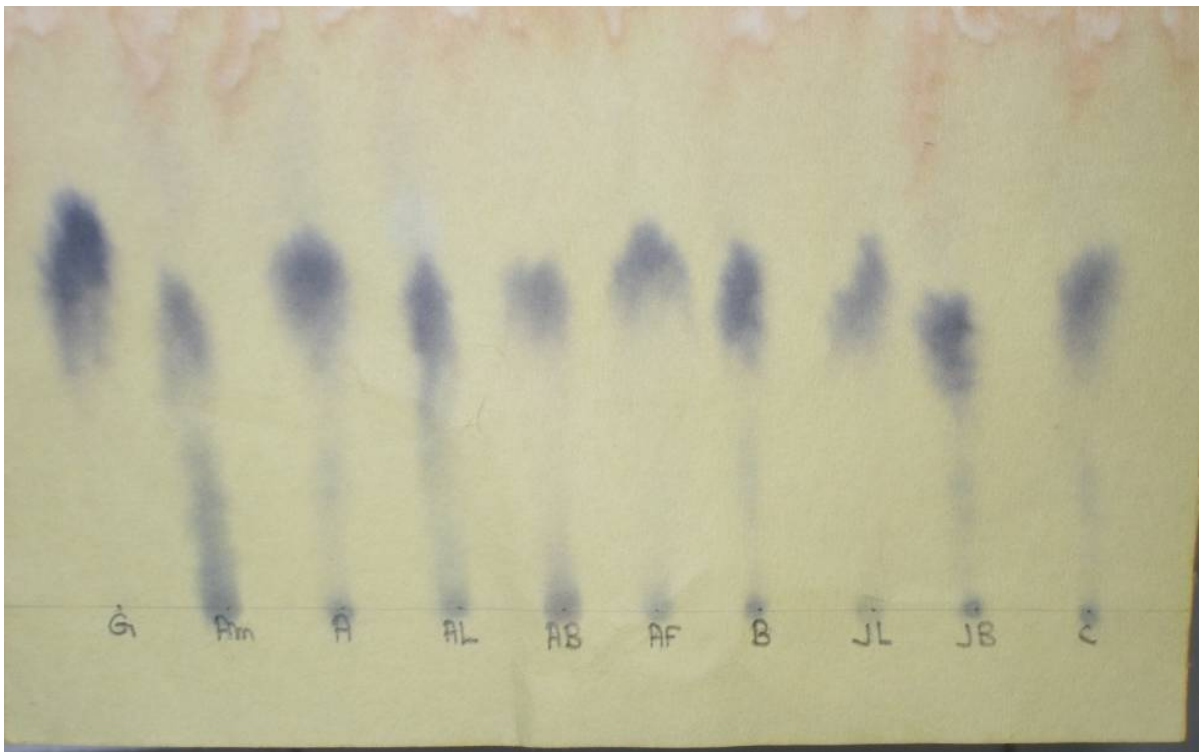
Tannin source	Tannin content (mg/ml)	Relative activity (%) after	
		1h	2h
Control (Tannic acid)	1.90	100	100
Amaltash (*Cassia fistula*) leaves	0.82	23	51
Amla (*Phyllanthus amblica*) bark	0.81	13	62
Amla (*Phyllanthus amblica*) fruit	1.83	72	123
Amla (*Phyllanthus amblica*) leaves	0.93	21	59
Ber (*Zyzyphus mauritiana*) leaves	0.51	20	56
Eucalyptus (*Eucalyptus glogus*) bark	0.22	56	88
Eucalyptus (*Eucalyptus glogus*) leaves	0.53	43	73
Guava (*Psidium guazava*) bark	0.66	61	74
Guava (*Psidium guazava*) leaves	0.61	24	55
Jamun (*Syzygium cumini*) bark	0.78	34	72
Jamun (*Syzygium cumini*) leaves	0.74	75	112
Keekar (*Acacia nilotica*) leaves	0.31	83	105
Mango (*Magnifera indica*) leaves	0.60	48	66
Mulberry (*Morus macroura*) leaves	0.67	64	109
Pomegranate (*Punica granatum*) rind	1.22	66	97
Tamarind (*Tmarindus indica*) seed	1.02	83	119

fruits, jamun leaves, tamarind seed, keekar leaves and mulberry leaves as substrate. The enzyme also showed appreciable activity with pomegranate rind (97%) and eucalyptus bark (88%) extract as substrate. These results were also observed in paper chromatogram showing appearance of gallic acid from the degradation of tannin from these substrates (Figures 1a and 1b). The rate of gallic acid release from tannins obtained from the other agro residues was lower relative to tannic acid.

The tannase from *Trichoderma harzianum* can be employed for gallic acid production using these cheaper agro residues. It makes the process of gallic acid production economic and ecofriendly, and also suggests a beneficial utilization of agro wastes. Further, the tannin biodegradation efficiency of this tannase can also be exploited for a number of industrial applications like treatment of tannery effluents, fruit juice debittering, wine clarification etc.

Many researchers have reported gallic acid production from plant tannins such as *Cassia siamea* (Banerjee *et al.,* 2007), sal seed (*Shorea robusta*), fruit of myrobalan (*Terminalia chebula*) and tea-leaf (*Camellia sinensis*) (Hota *et al.,* 2007), *Camellia sinensis* (Akroum *et al.,* 2009), jamun (*Syzygium cumini*) and keekar (*Acacia nilotica*) leaves (Selwal *et al.,* 2011) and *Acacia* pod substrate, redgram husk, sorghum husk and spent tea powder (Reddy and Rathod, 2012). Rout and Banerjee (2006) attempted enzymatic

Figure 1a: Paper Chromatogram Showing Gallic Acid Liberation by Tannase from Natural Tannins [G-Gallic Acid (standard), Am-Amaltash Leaves, A-Pomegranate Rind, AL-Amla Leaves, AB-Amla Bark, Af-Amla Fruit, B-Ber Leaves, JL-Jamun Leaves, JB-Jamun bark, C-Control (Tannic Acid)]



debittering of pomegranate juice using tannase. Tannase treatment resulted in 25% tannin degradation without affecting biochemical and quality attributes of the juice. Tannase from *Aspergillus candidus* MTCC9682 was found to degrade and thereby reduce the tannin content of the tannery effluent resulting in the decolourisation of the effluent (Murugan and Al-Sohaibani, 2010).

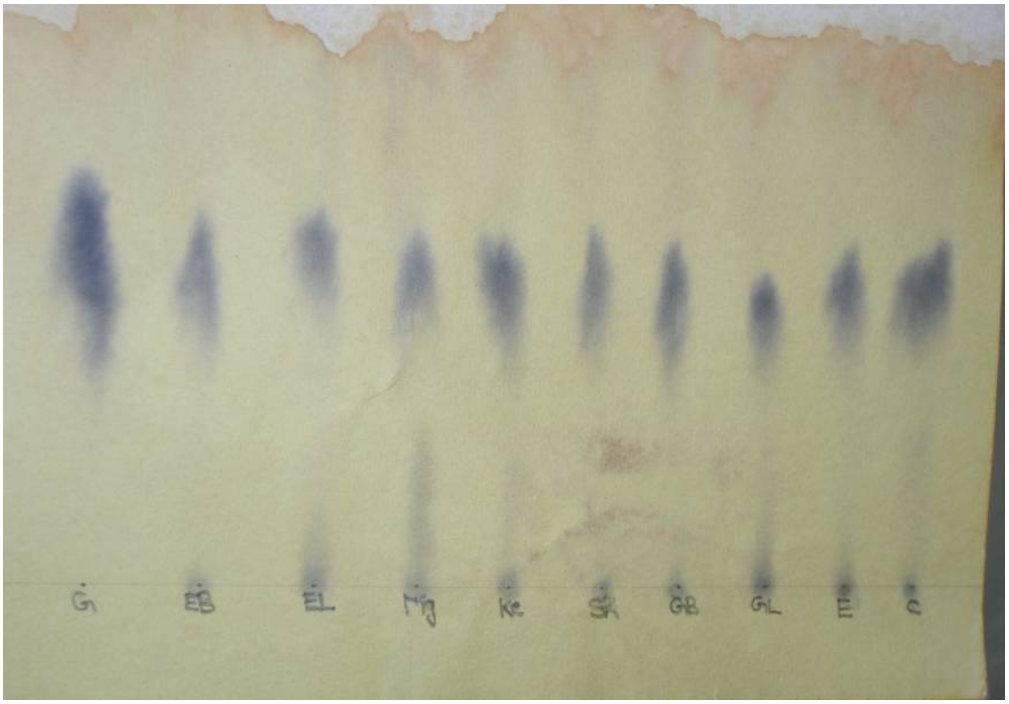
Biochemical Characterization of the Tannase

The hydrolytic efficiency of the enzyme is greatly influenced by assay parameters such as time of incubation, pH, temperature and substrate concentration. In order to obtain maximum liberation of gallic acid by tannase, some physicochemical properties of the crude tannase were studied.

Effect of Reaction Time on Tannase Activity

The incubation time of 20 min was found optimum for tannase activity (Figure 2) when treated the enzyme with substrate for 30 minutes. Rana and Bhat (2005) investigated the effect of reaction time on the activity of tannase produced by *Aspergillus niger* by liquid-surface (LSF), submerged (SmF) and solid-state (SSF) fermantations. The SmF- and LSF-produced enzyme was active at 10min of incubation, while SSF-produced enzyme had peak activity at 15 min. Crude tannase from *Aspergillus niger* AUMC 4301 had maximum activity at 20 min as a function of reaction time (El-Fouly *et al.,* 2010). EL-Tanash *et al.* (2012) obtained highest gallic acid production by tannase at 30.4 min of incubation.

Figure 1b: Paper Chromatogram Showing Gallic Acid Liberation by Tannase from Natural Tannins [G-Gallic Acid (Standard), Eb-Eucalyptus Bark, El-eucalyptus Leaves, Mg-Mango Leaves, Ke-Keekar Leaves, Sh-Mulberry, Gb-Guava Bark, Gl-Guava Leaves, E-tamarind Seed, C-control (Tannic Acid)]



Effect of Substrate Concentration

To determine the optimum concentration of the substrate for enzyme, the tannase was incubated with substrate of different concentrations ranging from 0.25-2.0% (1.47-10.31 µmoles/ml). The maximum activity was observed at 1.0% concentration (Figure 3). Tannic acid concentration of 25.3mg/mL was found to be optimum for maximum gallic acid production by tannase from *Aspergillus awamori* (EL-Tanash *et al.*, 2012).

Temperature Optima and thermal stability

The optimum temperature of crude tannase was found to be 40ºC (Figure 4). Similar results were reported for tannase from *Rhizopus oryzae* (Hota *et al*., 2007) and *Klebsiella pneumoniae* MTCC 7162 (Sivashanmugam and Jayaraman, 2011). The crude enzyme was found stable at 40ºC retaining about 71% of original activity for 2 hr (Figure 5). The tannase from *A. tamarii* TAH I was found to be stable at low temperatures (5 to 25°C) for several hours and at temperature up to 40°C for 1 h (Costa *et al.*, 2012).

Figure 2: Effect of Reaction Time on Tannase Activity

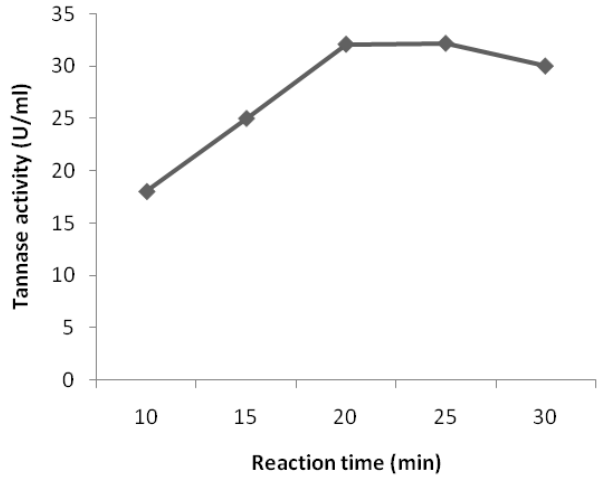


Figure 3: Effect of Substrate Concentration

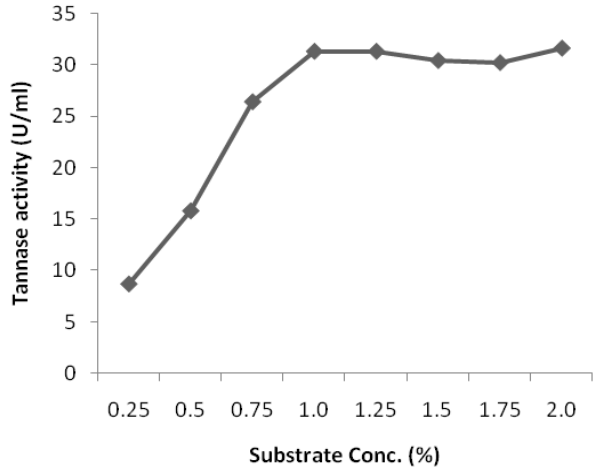


Figure 4: Temperature Optima of Tannase

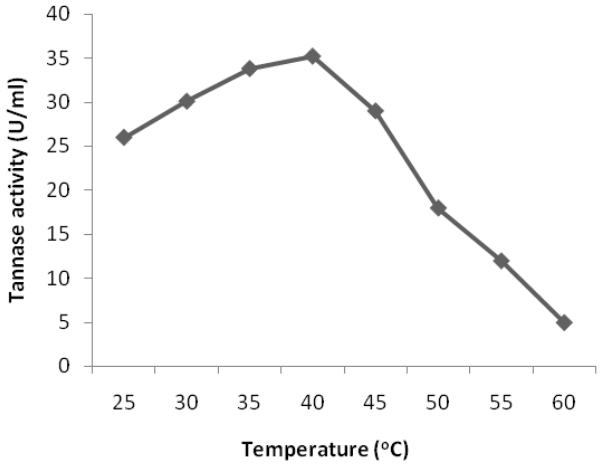
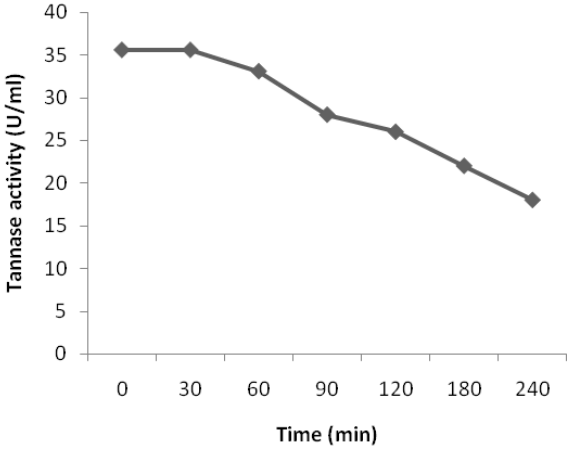


Figure 5: Thermal Stability of Tannase at 40°C



pH Optima

The crude tannase from *Trichoderma harzianum* showed maximum activity at pH 5.5 (Figure 6). The optimum pH for tannase is widely reported at pH 5.0-5.5 (Costa *et al*., 2008; Schons *et al.,* 2011; Reddy and Rathod, 2012). However, the tannase from *Rhizopus oryzae* was found to be highly acidic exhibiting pH optima at 4.5 (Hota *et al*., 2007*)*. Some fungus also produced tannase which were maximally active at pH 6.0-6.5 (Rana and Bhat, 2005; Battestin and Mecedo, 2007; Srivastva and Kar, 2009).

Effect of Additives

The effect of different additives such as metal salts, detergents, chelator and reducing agent on tannase activity was studied by supplementing the reaction mixture with the additive at 1mM concentration. The salts NaCl, KCl, $MgCl_2$, $CaCl_2$ and $BaCl_2$ did not affect the enzyme activity significantly; however $HgCl_2$ (75%) and $ZnCl_2$ (60%) followed with $MnCl_2$ (48%) caused strong inhibition of the enzyme (Table 2). The detergents SDS and Tween-80 had no effect on enzyme activity, while Tween-20 and Tween-60 had a mild

the surfactants SDS and Triton X-100.

Figure 6: pH Optima of tannase

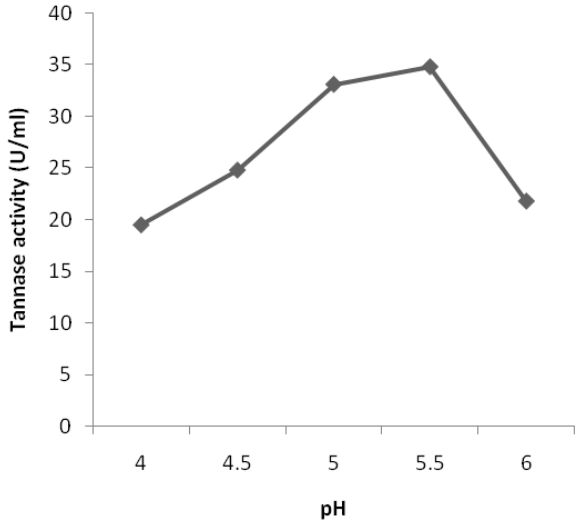


Table 2: Effect of Metal Salts on Tannase Activity

Metal Salts (1 mM)	Tannase Activity (U/ml)
Control	31.78
NaCl	30.45
KCl	26.88
$CaCl_2$	32.04
$MgCl_2$	30.22
$BaCl_2$	26.11
$MnCl_2$	20.15
$ZnCl_2$	12.66
$HgCl_2$	7.88

Table 3: Effect of Other Additives on Tannase Activity

Additives (1 mM)	Tannase Activity (U/ml)
Control	31.78
SDS	29.52
Tween-20	25.13
Tween-60	27.61
Tween-80	30.82
β-mercaptoethanol	11.56
EDTA	9.47

inhibitory effect. The presence of β-mercaptoethanol and EDTA strongly inhibited the enzyme (Table 3). Mata-Gomez *et al*. (2009) investigated effect of metal ions and surfactants on tannase producted by *Aspergillus* GH1, Ca^{2+} enhanced the tannase activity while Fe^{2+} strongly inhibited it. Cu^{2+} and Zn^{2+} had only a mild inhibitory effect. The enzyme showed good stability in (Tween-20, 80, Triton X-100 and SDS). El-Fouly *et al.* (2010) reported that tannase from *Aspergillus niger* AUMC 4301 was silightly inhibited by different metal ions tested (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}), however Fe^{2+} and Mn^{2+} caused maximum decrease in enzyme activity (90%). Costa *et al.* (2012) also investigated the effect of some metal ions and common chemicals on tannase activity and observed that except Mg^{2+}, the enzyme was inhibited by all metal ions tested. The enzyme was inhibited by β-mercaptoethanol but only partially inhibited by EDTA however, resistant to

CONCLUSION

Studies on natural tannin degradation by tannase from *Trichoderma harzianum* indicate its potential in economic production of commercially important gallic acid. It can be further exploited for other industrial applications such as fruit juice clarification and tannery effluent treatment. The crude tannase exhibits biochemical properties that are desirable for industrial applications. Thermal stability and optimum activity at 40°C and resistance to detergents makes this tannase suitable for industrial use.

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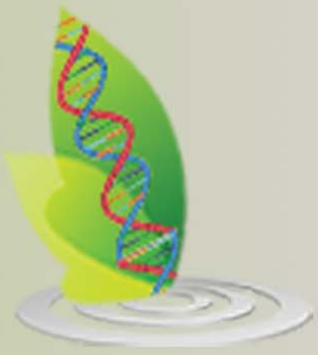

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