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Tannin Biodegradation by Tannase Produced from *Aspergillus terreus* ITCC 8413.11 and its Culture conditions

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ABSTRACT

The tannase producing fungal strain Aspergillus terreus ITCC 8413.11 was isolated from soil producing 5.8 U/ml tannase initially. To enhance the production level of the enzyme different culture conditions were optimized and observed that optimum temperature and pH for tannase production was 37°C and 5.5 respectively. Maximum tannase was produced by the fungus after 144 hrs of incubation period in the medium containing 1% tannic acid while growth was found maximum at 192 hrs. Among the different natural substrates tested as carbon source, cassia leaves and jamun leaves supported tannase production appreciably. However, pure tannic acid as carbon source as well as inducer gave comparatively higher tannase yield. The optimization studies enhanced the production of tannase to 1.41-fold. The enzyme was immobilized by entrapment method using alginate and polyacrylamide and had highest activity yield (80%) in calcium alginate beads. The immobilized tannase showed slight shift in optimum temperature and pH from that of free enzyme and exhibited appreciable reusability and storage stability. Natural tannin degradation efficiency of both free and immobilized tannase was investigated using different tannin-rich agro-residues as substrates. Maximum tannase activity was estimated when amla fruits, amla leaves and cassia leaves were used as substrate. Hence, the tannase from Aspergillus terreus can be employed for gallic acid production using these cheaper natural substrates which also suggests a beneficial utilization of agro-wastes. The tannin biodegradation efficiency of tannase can also be exploited for a number of industrial applications like treatment of tannery effluents, fruit juice debittering etc.
Key words: *Aspergillus terreus*, amla fruits, immobilization, tannin biodegradation, tannase production.

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INTRODUCTION

Tannins are naturally occurring plant phenolics compounds that have wide ranging effects on animals and microbes [1]. They are polyphenolic secondary metabolites of plants which form hydrogen bonds in solutions, resulting in the formation of tannin-protein complexes[2]. Tannins are present in large number of feed and forages. The formation of complexes of tannins with nutrients, such as carbohydrates, proteins and minerals, has negative effects on their utilization. High concentrations of tannins depress voluntary feed intake and digestive efficiency. The nutrient value of tanniferous feed may be enhanced by various detannification procedures viz., physical, chemical and biological. In biological treatment, various tannase producing microbial strains have been tried for reduction of tannin content and nutritive enhancement of treated material [3].

In this respect, tannase find potential applications in feed, food and beverage industry. Tannase is used as clarifying agent in some wines, juices of fruits and refreshing drinks with coffee flavour. The use of tannase helps in overcoming the problem of undesirable turbidity in these drinks which poses the quality problem. Enzymatic treatment of fruit juices reduces bitterness, haze and sediment formation, hence are acclaimed for health benefits and industrial use. The enzyme has potential uses in treatment of tannery effluents and pretreatment of tannin containing animal feed [4]. One of the major applications of tannase is the production of gallic acid. It is used for the manufacture of an anti-malarial drug Trimethoprim. Gallic acid is a substrate for the chemical and enzymatic synthesis of propyl gallate, used as anti-oxidants in fats and oils. Gallic acid, being used extensively in pharmaceutical manufacturing and analytical industries, is one of the many chemicals that are imported. Therefore, attempts have been made in India

to find out some of the suitable tannin rich agro residues for the enzymatic conversion of their tannin content to gallic acid [5].

The enzyme tannase (E.C. 3.1.1.20) also known as tannin acyl hydrolase, is a hydrolytic enzyme that acts on tannin. It catalyses the hydrolysis of bonds present in the molecules of hydrolysable tannins and gallic acid esters producing gallic acid and glucose[6]. Though, tannase has been isolated from number of micro-organisms like fungi, bacteria and yeast [7, 8], still, enzymes of industrial interest are routinely being explored from variety of microbial sources to isolate the enzyme with more desirable properties for commercial applications. For industrial application, the immobilized form of enzyme offers several advantages, including repeated use of the enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bed reactors.

The present paper reports the production of tannase from newly isolated *Aspergillus terreus* ITCC 8413.11 by liquid-surface fermentation technique. Studies on immobilization and biodegradation efficiency of tannase are also presented.

MATERIAL AND METHODS

Micro-organism: The tannase producing fungal strain used in the present study was isolated from garden soil collected from MIET campus, Meerut (UP), India and was identified as *Aspergillus terreus* (ITCC 8413.11) by Indian Type Culture Collection, New Delhi. The strain was maintained on malt extract medium slants at 4^{o}C. The constituents of the medium were (g/L): malt extract, 20.0g; K_2HPO_4, 1.0g; NH_4Cl, 1.0g and agar 20.0g (pH 5.5).

Tannin-rich agro-residues: Various tannin-rich agro-residues, amla (*Phyllanthus amblica*) leaves and fruits, ber (*Zyzipus maurtiana*) leaves, cassia (*Cassia fistula*) leaves, guava (*Psidium guazava*) leaves and bark, jamun (*Syzgium cumini*) leaves and pomegranate (*Punica granatum*) rind were collected from gardens. They were shade dried and the finely grinded powder was used in the present study.

Tannase production: For extracellular tannase production, the culture was grown in malt extract liquid medium containing 1% (w/v) tannic acid and incubated at 37ºC for 96 hrs. After incubation, the culture was filtered through Whatman no.1 filter paper and the filtrate was used as enzyme. The mycelial biomass was separated and was dried at 60^{o}C for 24 hr and expressed as g dry weight/50 ml medium.

Tannase assay: Tannase was assayed following the method of Mondal *et al*.[9] 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 0.5 ml of the crude enzyme and incubated at 40ºC for 20 minutes. The enzymatic reaction was stopped by adding 3ml bovine serum albumin (BSA) (1mg/ml). The tubes were centrifuged at 5000g for 10 min. The precipitate was dissolved in 2ml SDS triethanolamine solution followed by the addition of 1ml of $FeCl_3$ reagent. The contents were kept at 15 min. and the absorbance was measured at 530nm against the blank.

Assay for immobilized tannase: The reaction mixture consisted of 1ml substrate (1% tannic acid in 0.2 M acetate buffer of pH 5.5) and tannase entrapped alginate beads were incubated at 40°C for 30 min. After incubation, beads were removed from the tubes for its reuse and 3ml BSA solution was added to stop the reaction. Further tannase assay procedure was similar as that of soluble tannase assay.

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.

Optimization of culture conditions for tannase production:

Effect of medium pH and temperature on enzyme production: The optimum pH of the culture medium and incubation temperature for tannase production was determined in the pH range of (4.5-6.5) and (25^{o}-50^{o}C) respectively.

Effect of incubation period:To evaluate the effect of incubation period, different flasks containing production medium were inoculated with freshly grown (96h) culture of *Aspergillus terreus* and incubated at 37ºC. The biomass and tannase activity was estimated up to 8 days at regular intervals of 24 hrs by harvesting each flask at a time.

Effect of carbon source: The effect of carbon source on tannase production from *Aspergillus terreus* was investigated using different natural tannin-rich substrates namely dried ber leaves, cassia leaves, guava leaves, guava bark, jamun leaves and pomegranate rind. The production medium was supplemented with tannin substrates (1%) and after incubation the amount of tannase produced was estimated. The production medium containing commercial tannic acid powder (1%) was kept as control.

Effect of nitrogen source: Effect of nitrogen supplements like organic sources (2%) such as peptone, malt extract, yeast extract, beef extract and inorganic sources (0.2%) such as $(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl on tannase production from *Aspergillus terreus* was examined.

Immobilization of tannase by entrapment method

Entrapment in alginate: The crude enzyme was immobilized by entrapment method[6] using 2% sodium alginate solution initially. A suspension (10ml) of sodium alginate containing tannase (16.5U) was extruded drop-wise through a 2 ml syringe into cold 0.2M $CaCl_2$ solution and 0.2M $CuSO_4$ which resulted into the formation of calcium and copper alginate beads respectively. After 2 hours the beads (4 mm diameter) containing enzyme were washed with water and used for further studies or stored at 4°C.

Entrapment in polyacrylamide:

Entrapment of tannase in polyacrylamide gel lattice was done by the modified method of Skrylabin and Kosheenko[10]. For gel polymerization, a 10% concentration of acrylamide and bisacrylamide monomers in the ratio 9:1 was used. The gelation mixture for immobilization consisted of 11ml solution containing 0.9 g acrylamide and 0.1 g methylene bisacrylamide, 2ml cold crude enzyme (16.4U), 0.3 ml of 0.5% ammonium per sulphate (APS) solution and 0.2 ml of 50% TEMED solution. The mixture was quickly poured in a petriplate and kept at 4°C for about one hour. After polymerization, blocks of size 0.5×0.5 cm^2 were cut. The immobilized enzyme prepared was washed with distilled water thrice and used for further studies.

Properties of free and immobilized tannase

Effect of temperature and thermal stability: The effect of temperature on free and immobilized tannase was evaluated by incubating the reaction mixture at temperatures 30°-50°C and assayed for tannase activity. Thermal stability of immobilized tannase was determined at different temperatures 30° to 45°C for 2 hours and residual activity was measured at regular intervals of 30 min.

Optimum pH and pH stability: The optimum pH for free and immobilized tannase was determined in the pH range 4.0-6.5. The reaction mixtures consisting of enzyme and substrate solution prepared in buffers of different pH were incubated at 40°C for 40 min. and tannase activity was calculated. To determine pH stability, the alginate entrapped enzyme was incubated in the buffers of pH 4.0 to 6.5 for one hour at 10°C before the addition of substrate. The residual enzyme activity was assayed under the standard conditions.

Operational stability and storage stability of immobilized tannase:The reusability of alginate entrapped tannase was evaluated at the optimized assay conditions in a repeat batch process. At the end of the reaction, the beads were collected, washed with water and were used again for another reaction (by adding 1ml fresh substrate solution). The suspended fluids of each run were assayed for tannase activity. To determine the storage stability, the immobilized enzyme (alginate entrapped tannase beads) suspended in water was stored at 4°C and tannase activity was measured at different storage periods (every 48 hr) upto two weeks.

Biodegradation of natural tannins

Effects of substrates on tannase activity:Different tannin rich agro-residues mentioned above were used as substrate for enzymatic conversion of their tannin content to gallic acid. The finely grind powder of the agro-residues (1%) were mixed with distilled water and kept at room temperature for 3 days. After soaking, the mixture was boiled for 10 min. The filtered extracts were used as crude natural tannin. Bioconversion of the natural tannins from these agro-residues was carried out with free as well as immobilized tannase produced by *Aspergillus terreus*. The tannic acid (control) and crude tannin extract were treated with enzyme and tannase activity was determined after 2 hrs.

Estimation of tannin content:The tannin content in the natural substrates was measured following protein precipitation method[11].

All the fermentation and assays were carried out in triplicates and the mean value was presented.

RESULTS AND DISCUSSION

Tannase production from *Aspergillus terreus*

The fungal strain *Aspergillus terreus* ITCC 8413.11 was isolated from soil sample on malt extract agar medium (supplemented with 1% tannic acid). The zone of tannic acid hydrolysis around its growth, confirm it as a tannase producer. The culture initially produced 5.8 U/ml tannase in malt extract liquid medium at 37ºC after 96 hrs under stationary fermentation conditions.

Optimization of culture conditions for maximum tannase production.

The different physico-chemical parameters were optimized by the classical one-at-a-time optimization method in order to achieve maximum tannase production by *Aspergillus terreus*.

Effect of pH of the culture medium:Tannic acid, being an acidic substrate, makes the pH of the medium acidic. Hence, the pH of the medium was adjusted at different pH of acidic range (4.5-6.5). Maximum tannase production from *Aspergillus terreus* was observed at pH 5.5. The growth and enzyme production was however, considerable in pH range of 5.5-6.5. Decrease in pH of the medium reduced tannase production sharply (Table 1). There are reports describing of the optimum pH as 5.5 for tannase

production from *A. tamarii* [9], *A. awamori* [12] and *A. flavus* [13]. Reddy and Rathod [14] also observed optimum tannase activity at pH 5.5 from the isolate *Penicillium purpurogenum* BVG7.

Table 1: Effect of pH on tannase production

pH	Tannase activity (U/ml)
4.5	3.5 ± (0.65)
5.0	3.9 ± (0.70)
5.5	5.8 ± (0.98)
6.0	5.7 ± (0.87)
6.5	5.0 ± (0.75)

Values in parenthesis represent biomass (g dry wt/50ml)

Effect of incubation temperature: The effect of incubation temperature on tannase production from *Aspergillus terreus* was studied in the temperature range of 25º-50ºC under stationary fermentation conditions. With the rise in temperature the tannase production increased and reached maximum at 37ºC with appreciable activity up to 40ºC. However, the optimum temperature for growth and tannase production from *Aspergillus terreus* was 37ºC (Table2). Above 40°C, tannase production decreased drastically, however, the culture could grow upto 45°C. Tannase production from *Aspergillus niger*[6], *Aspergillus* flavus[15] and *Aspergillus* awamori [16] has also been reported at 37ºC. However, the fermentation temperature for optimum tannase production from *Aspergillus* sp. is mostly reported to be 30°C[17,18].

Table 2. Effect of incubation temperature on tannase production

Temperature (°C)	Tannase activity (U/ml)
25°	3.3 ± (0.60)
30°	5.0 ± (0.76)
37°	5.8 ± (0.90)
40°	5.6± (0.88)
45°	3.2 ± (0.71)
50°	1.3 ± (0.38)

Effect of incubation period:The maximum production of 8.3 U/ml was observed after 144 hrs (6 days) of incubation (Fig. 1). Thereafter, the enzyme production slightly decreased. Decreased enzyme yield on prolonged incubation could also be due to reduced nutrient level of medium. It has been reported that tannase activity decreases after reaching maximum level, due to inhibition or degradation of enzyme. Similar observations have been reported for tannase production from *Aspergillus* sp.[3, 16, 19]. However, 3-4 days was found to be optimum incubation time for enzyme production by various tannase producing *Aspergillus* sp. strains [18, 20].

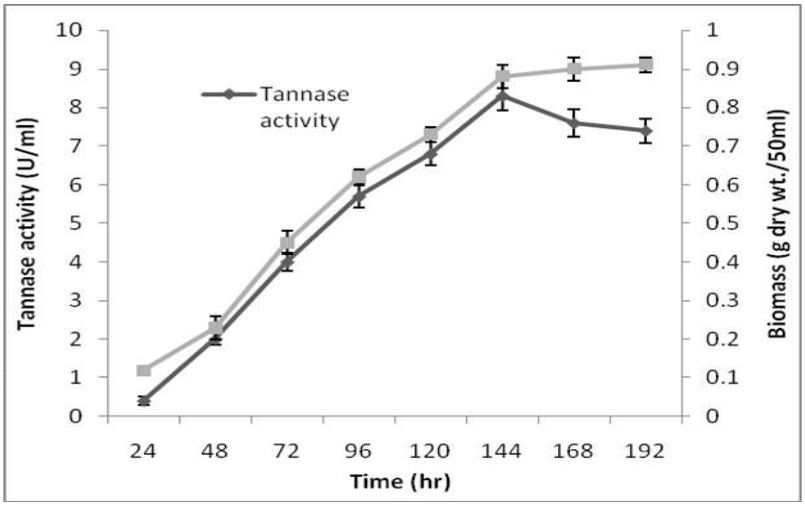

Fig. 1 Effect of incubation period on tannase production

Effect of carbon source:Tannins are the natural substrates of tannase. These tannins in the production medium acts as inducer as well as carbon source for the microbial tannase producers. Additionally, these tannins are easily available in plenty as agro-forestry wastes. With this view, different natural tannins (1%) such as ber leaves, cassia leaves, jamun leaves, guava bark, guava leaves and pomegranate rind were tested as carbon source. The commercial tannic acid powder was used as control. The enzyme production was highest with tannic acid (1%) as carbon source in the medium. However, among the natural substrates cassia leaves and jamun leaves supported considerable tannase production (Fig. 2). These agro-residues as substrates can be substituted for costly tannic acid to make the process of industrial tannase production more economical.

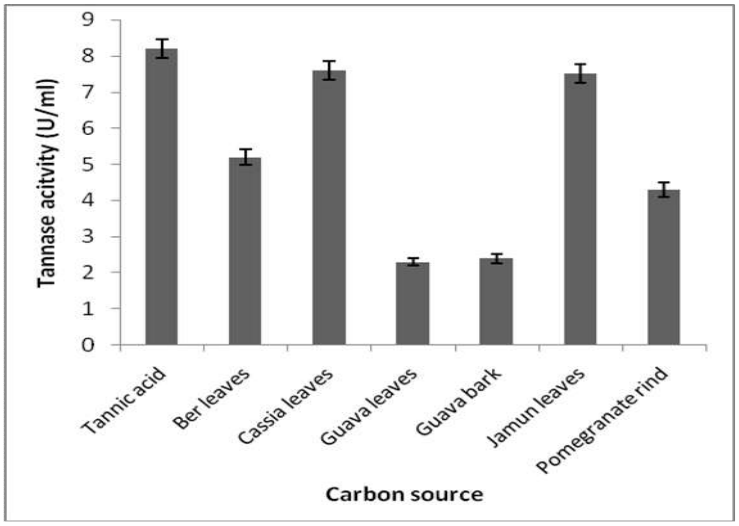

Fig 2. Effect of carbon source

Tannic acid at 1% concentration was maximally used as substrate for tannase production[16,21]. Many workers have attempted tannase production using natural tannin substrates. Kumar *et al*.[22] studied tannase production under SSF using different tannin-rich substrates and found maximum enzyme production with jamun leaves. Tannase production from *A. niger* ITCC 6514.07 using pomegranate rind as the sole carbon source in the medium was also reported [6]. Tannin-rich plant residues were used as substrate and sole carbon source for tannase production by *Penicillium purpurogenum* PAF6 and among them, tamarind seed was found to be the most favorable substrate than haritaki, pomegranate, tea leaf waste and arjun fruit[23].

Effect of nitrogen source:Among the various nitrogen sources tested alone, none of the nitrogen source supported enzyme production effectively (Fig.3). On the contrary, the medium containing the combination of malt extract and NH_4Cl (control) resulted in maximum production of tannase (8.2 U/ml). Ammonium nitrate[24,25], ammonium chloride [26] and sodium nitrate [16] supported tannase production better than the organic nitrogen sources.

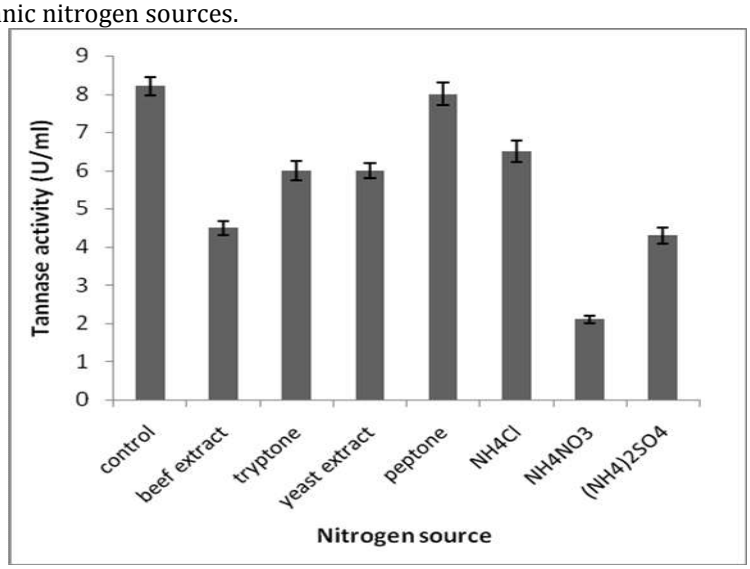

Fig 3. Effect of nitrogen source

The optimization studies enhanced the tannase production to 1.41-fold with 8.2U/ml tannase activity produced under optimized culture conditions.

Immobilization of tannase by entrapment method

Tannase enzyme from *A. terreus* was immobilized by entrapment method in calcium alginate beads, copper alginate beads and polyacrylamide gel blocks and the immobilized tannase activity was evaluated (Table 3).

Table. 3 Effect of different matrices used for immobilization of tannase

S. no.	Matrix	Enzyme activity (U/ml)	Activity yield (%)
1.	Free enzyme	8.2	100
2.	Ca-alginate beads	6.6	80
3.	Cu-alginate beads	6.0	73
4.	Polyacrylamide blocks	5.2	61

The immobilized enzyme prepared by calcium alginate beads had the highest immobilized activity (6.6 U/ml) and the highest activity yield (80.4%). Therefore, tannase entrapped in calcium alginate beads was used for immobilization studies.

Effects of different concentrations of sodium alginate solution:The effect of concentration of sodium alginate solution on bead formation was studied in the range of 2.0-4.0%. As shown in the Table 4, tannase activity (7.0 U/ml) slightly increased when 3% alginate solution was used to immobilize the enzyme as compared to enzyme activity (6.6 U/ml) obtained with 2% solution used initially. Sodium alginate solution of 2-3% concentration was found to be the most suitable for entrapment of tannase[27,28] by most of the researchers, however, highest concentration of 5% alginate solution for tannase immobilization has also been reported [29].

Table 4. Effect of concentration of alginate on tannase activity

Concentration of alginate (%)	Tannase activity (U/ml)
2.0	6.6
2.5	6.6
3.0	7.0
3.5	7.0
4.0	5.8

Since the rate of enzyme catalyzed reaction is dependent on concentration of enzyme, the effects of different amount of beads ranging from 50-350 mg (which corresponds to 01 to 07 beads in a reaction mixture) on enzyme activity was studied. The rate of reaction increased up to 250 mg (five) beads in the reaction mixture, after which no further increase was observed (Data not shown).

Properties of free and immobilized tannase

Effect of temperature and thermal stability: The free enzyme had optimum activity at 40°- 45°C, while, immobilized enzyme had maximum activity at 40°C (Fig. 4). The immobilized tannase could not withstand the temperature above 40°C.

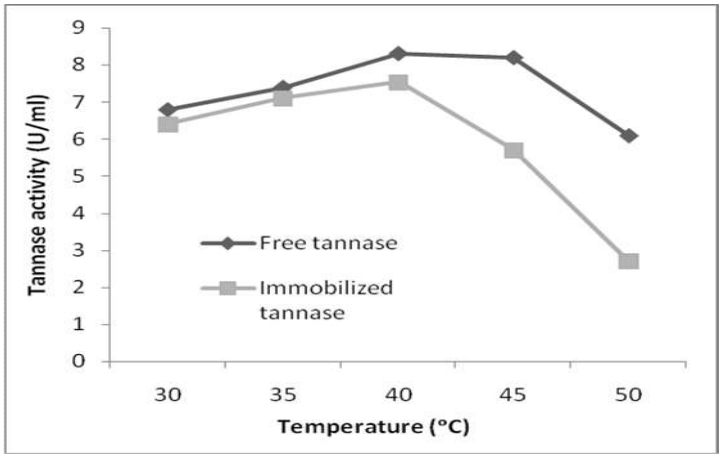

Fig 4. Temperature optima of free and immobilized tannase

The thermal stability of immobilized tannase was studied at the temperature range of 30°- 45°C for 2 hrs. Immobilization of tannase in alginate beads improved the stability of enzyme at temperature range 30°C to 35°C retaining 80% of original activity for 120 min. At 40°C the enzyme was stable up to 90 min only (Fig. 5).

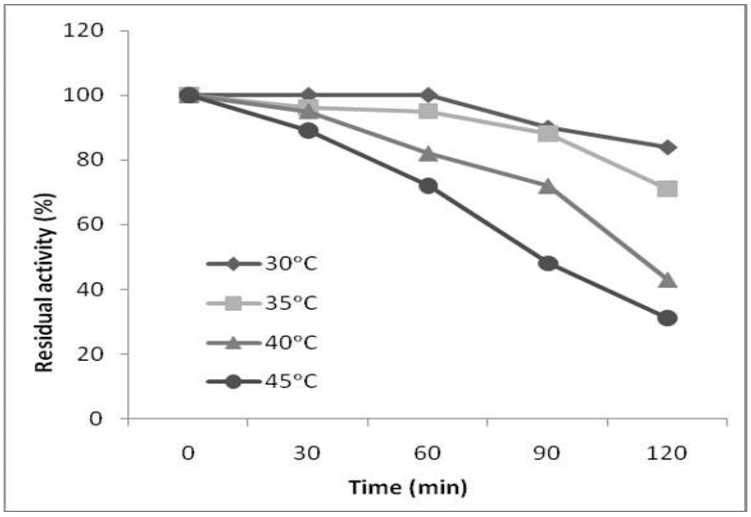

Fig 5. Thermal stability of immobilized tannase at different temperatures

Optimum pH optima and pH stability:The optimum pH for immobilized tannase was determined at 40°C by incubating the reaction mixture in buffers of different pH (ranging from 4.0 to 6.5) and compared with that of soluble tannase. The pH optima of the free tannase were 5.0 to 5.5, which was shifted to pH 5.5 on immobilization of enzyme. Therefore, for further studies, acetate buffer of pH 5.5 was used (Fig. 6). The shift in pH and temperature optima of immobilized enzyme relative to soluble enzyme is the common observation. Decrease in pH optima for immobilized tannase as compared to soluble enzyme was observed by Abdel-Naby *et al.*[30]and Srivastava and Kar[6] while some investigators reported no change in optimum pH for free as well as encapsulated enzyme [29, 31].

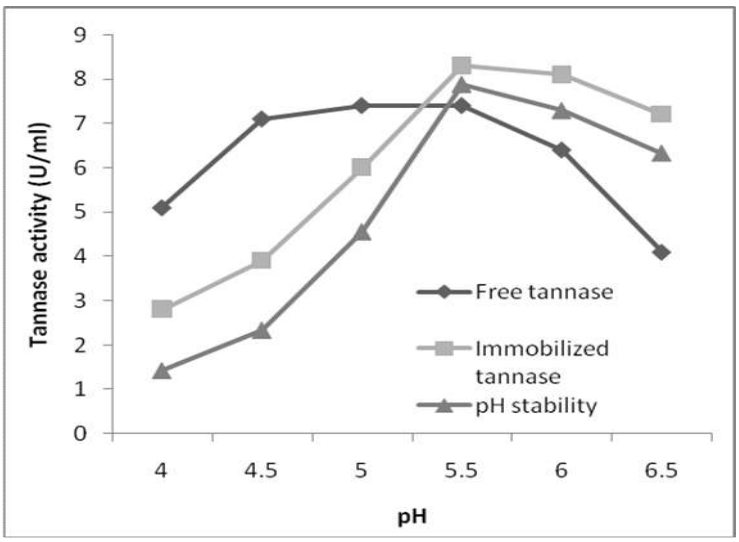

Fig 6. pH optima of free and immobilized tannase and pH stability of immobilized tannase

The pH stability of the enzyme was examined by incubating the enzyme in buffer of different pH values (4.0 to 6.5) at 10° C. The immobilized tannase showed 100% stability at pH range of 5.5 to 6.0 after 60 min of incubation and 92% essential activity at pH 5.5 (Fig. 6).

Operational stability and storage stability:The most important attribute of immobilized enzyme is its reusability. Therefore, the operational stability of tannase entrapped in alginate beads was assessed by reusing the immobilized enzyme for total of nine cycles (Fig. 7). The alginate entrapped enzyme retained approximately 100% activity in the first four rounds of successive use, while retained upto 76% in sixth cycle, with gradual decrease in activity. After this, activity decreased sharply and was negligible in ninth cycle. Hence, the alginate entrapped tannase from *Aspergillus terreus* could be reused up to six cycles.

Immobilization of *Paecilomyces variotii* tannase in alginate beads retained about 60% original activity in fifth round of repeated use[29].

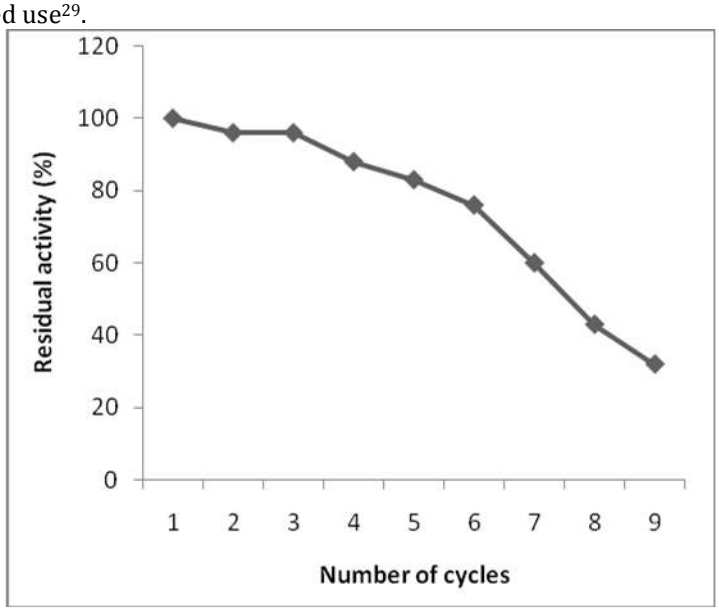

Fig 7. Operational stability of immobilized tannase

The immobilized enzyme preparation was stable upto two weeks retaining about 100% activity in the first week, while 60% upto next week, thereafter activity declined very sharply in the third week (Fig 8).

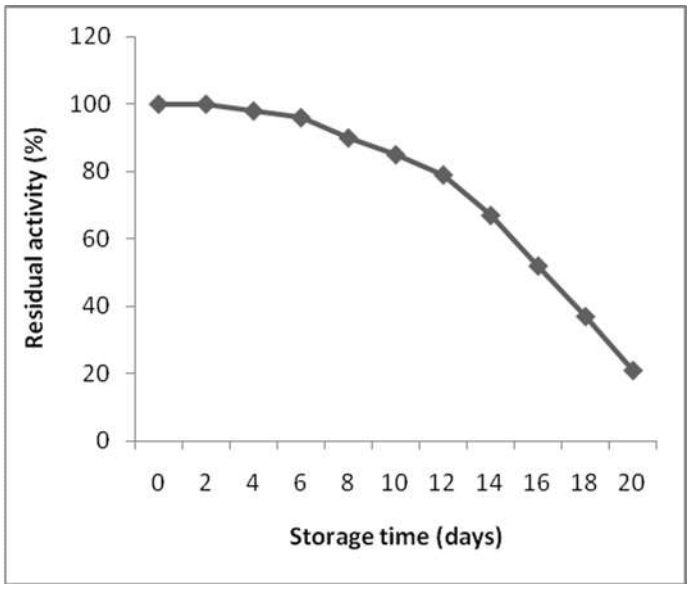

Fig 8. Storage stability of immobilized tannase

Biodegradation of natural tannins by tannase

To investigate the tannin degradation efficiency of tannase from *Aspergillus terreus*, both free and immobilized tannase were incubated at 40°C for 2 hrs with crude tannins (1%) of different agro residues like amla leaves and fruits, cassia leaves, guava leaves and bark, jamun leaves and pomegranate rind. Tannic acid (1%) was kept as control. The tannin content of different agro residues and tannase activity towards different natural tannin substrates are presented in Table 5. The free enzyme as well as immobilized tannase from *Aspergillus terreus* showed maximum activity with amla fruits as substrate followed with amla leaves, which was comparable to that of tannic acid. The free enzyme also showed appreciable activity (72%) with guava leaves and cassia leaves extract as substrate.

Table 5. Tannin degradation efficiency of tannase from *Aspergillus terreus*

Tannin source	Tannin content(mg/ml)	Residual activity (%)	
		Free tannase	Immobilized tannase
Tannic acid (control)	10.0	100	100
Amla leaves	8.52	83	75
Amla fruit	11.8	88	76
Ber leaves	6.1	55	33
Cassia leaves	7.8	72	51
Guava leaves	6.8	72	42
Guava bark	4.1	56	23
Jamun leaves	5.3	30	25
Pomegranate rind	4.4	52	45

*Tannin content estimated in 1% crude tannin extract

The reusability of immobilized tannase was investigated for tannin removal from amla fruits extract at 40°C for 2 hr for four successive runs. The maximum hydrolysis of 76% took place in the first run. The beads were reused which resulted in 53% tannin hydrolysis upto third cycle (Fig.9).

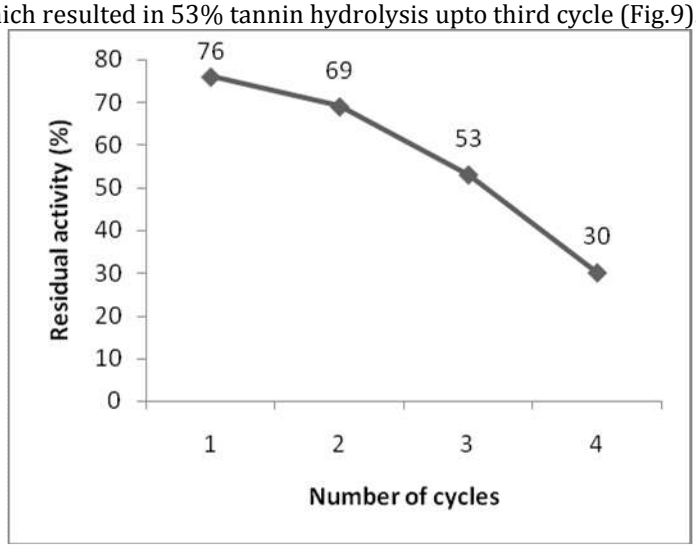

Fig 9. Reusability of immobilized tannase in tannin removal from amla leaves extract

CONCLUSION

The fungal strain *Aspergillus terreus* ITCC 8413.11 can be used as a source of industrially potent tannase. The results of the present study revealed that optimization of various nutritional and cultural conditions enhanced the level of tannase production to 1.4 fold as compared to initial fermentation conditions. The efficiency of this tannase in degradation of natural tannins provides an economic alternative for the production of commercially important chemical- Gallic acid. This characteristic is also considered useful for its application in food-processing industry. A wide variety of waste bioresources are available on our planet for conversion into bioproducts. The utilization of agro residues for tannase production could be a novel, cost-effective and valuable approach in tannase production and solid waste management.

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