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Efficiency of Tannase Produced by *Trichoderma Harzianum* MTCC 10841 in Pomegranate Juice Clarification and Natural Tannin Degradation

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Abstract

Tannases (Tannin Acyl Hydrolase, E.C. 3.1.1.20) are industrially important enzymes which find applications mainly in production of Gallic acid, in beer, wine and fruit juice clarification and detannification of poultry feed. Hence, the present investigation was carried out with a view to obtain industrially potent extracellular tannase from the fungal source. Among the total eighty four fungal strains isolated, the isolate Trichoderma harzianum (MTCC no. 10841) was selected for tannase production and optimization studies. The crude tannase had maximum activity of 31.36 U/ml with 1.0% tannic acid as substrate. The industrial potential of tannase from Trichoderma harzianum in fruit juice clarification and gallic acid production was explored. On treatment of varying amount of tannase with pomegranate juice at 40^{o}C tannin reduction of 57% was observed without the loss of its biochemical attributes such as pH, viscosity and sugar content and protein content. The natural tannin degradation efficiency of the enzyme was also investigated for gallic acid production using different tannin-rich agro residues as substrate. The enzyme showed maximum activity with amla fruits as substrate which was higher than tannic acid. The tannase from Trichoderma harzianum can therefore be employed for gallic acid production using cheaper agro residues.

Keywords: Tannase, natural tannins, gallic acid.

1. Introduction

In the last three decades, with the rapid advancement in the field of genetic and protein engineering enzymes have found their way into many new industrial processes. Enzymes offers biological alternative to the chemical processes at industrial scale. Biological methods are adjudged as an important mechanism of organic chemical removal in the natural systems (Illori *et al.*, 2007). Microorganisms have been the most important source for the production of industrial enzymes due to their biochemical diversity and their technical and economic advantages. Tannases are therefore produced and characterized from number of micro-organisms including fungi, bacteria and yeast (Belur and Mugreya, 2011).

Microbial tannases, tannin acyl hydrolases (E.C.3.1.1.20), are amongst important industrial enzymes with immense applications in food, feed, leather and pharmaceutical industries (Lekha and Lonsane, 1997). Tannase (Tannin Acyl Hydrolase, E.C. 3.1.1.20) is an important inducible 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, serves as a precursor for the commercial production of an anti-microbial drug trimethoprim, a food –preservative propyl gallate (Hota *et al.*, 2007). Conventionally gallic acid is produced by acid hydrolysis of tannic acid but it has cost, yield and low purity disadvantages. Alternatively, gallic acid can be produced by the microbial hydrolysis of tannic acid by tannase. The use of tannin-rich agricultural and forest residues as a substitute of costly tannic acid substrate is an economic alternative for gallic acid production which also suggest the beneficial utilization of agro wastes.

Other applications of tannases include production of instant tea and coffee-flavoured soft drinks (Lu *et al.*, 2009), detannification of poultry feed to improve the feed efficiency (Nuero and Reyes, 2002) and in leather industry (Orlita, 2004). 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 (Srivastva and Kar, 2010; Hamdy and Fawzy, 2012).

Thus, the aim of this study was to investigate the industrial potential of the tannase produced by newly isolated *Trichoderma harzianum.*

2. Material and Methods

2.1 Micro-organism

The tannase producing fungal strain *Trichoderma harzianum* used in the present investigation was isolated from soil sample collected around cassia tree, Meerut city (UP) (Hina Iqbal and Ashima Kapoor, 2012).

2.2 Fermentation conditions

The culture was grown in malt extract liquid medium (malt extract, 2%; K_2HPO_4, 0.05%; NH_4Cl, 0.1%) containing 1% (w/v) tannic acid. The medium was inoculated with disc of 0.8 cm diameter of freshly grown (48h) fungal culture and incubated at 30ºC for 3-4 days. After incubation, the culture was filtered and the supernatant was used as crude enzyme.

2.3 Tannase Assay

Tannase was assayed following the colorimetric method of Mondal *et al*. (2001) using tannic acid as substrate at a concentration of 1% in 0.2M acetate buffer (pH 5.5) (Hina Iqbal and Ashima Kapoor, 2012).

2.4 Enzymatic Pomegranate Juice Clarification

The pomegranate juice was extracted from fresh fruits. To the 8ml of juice different amount (1ml, 2ml, 3ml and 4ml) of tannase was added and incubated at 40^{o}C for upto 120 min. 1ml of aliquots of fruit juice was taken from each test tube at different time intervals and their tannin content was measured.

2.5 Biochemical Characterization of Enzyme-treated Juice

The quantitative estimation of protein was done by following the method of Lowry *et al.* (1951). The total carbohydrate content was determined by phenol-sulphuric acid method (Dubois *et al.*, 1956).

Titratable acidity was measured by titrating 5ml of juice (diluted with 20ml water) with 0.05 N NaOH using phenolphthalein as indicator. Viscosity of fresh and enzyme treated juice was determined by viscometer at 30^{o}C. pH of the fresh and enzyme treated juice was determined by pH meter.

2.6 Extraction of Crude Tannins

The finely grinded powder of the various 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.

2.7 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).

2.8 Biodegradation of Natural Tannins

Different tannin rich agro-residues 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 (31.3 U) 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.

2.9 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 after spraying with ferric chloride reagent. Pure gallic acid was used as standard.

2.10 Result Analysis

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

3. Results

3.1 Tannase Production

The tannase producing fungal strains were isolated from soil and screened by plate assay method. The fungal isolates exhibiting the zone of hydrolysis on the malt extract tannic acid agar plates were selected and subjected to tannase production in malt extract liquid medium for quantitative estimation of tannase activity. Among the eighty four fungal strains, the isolate 'T16' identified as *Trichoderma harzianum* (MTCC no. 10841) produced highest tannase activity of 16.31 U/ml. The liberation of end product gallic acid and glucose from tannic acid by the action of tannase was also observed by paper chromatograpy (Fig. 1).

Fig. 1: Analysis of hydrolytic product of tannic acid by tannase from Trichoderma harzianum (Lane GA- Gallic acid (standard), Lane a- Hydrolytic product released after 40 min., Lane b- after 20 min.)

To improve the production of tannase, various culture conditions were optimized including incubation temperature, initial pH of the medium, incubation time, carbon source and nitrogen source (Hina Iqbal and Ashima, 2012). Under optimized culture conditions such as malt extract medium with 1% amla fruit powder as carbon source, 4

days incubation at 30°C and pH 5.5, the production level of enzyme increased to 1.96-fold as compared to initial unoptimized conditions (malt extract medium with 1% tannic acid as carbon source, 5 days incubation at 37°C and pH 5.5) (Fig. 2).

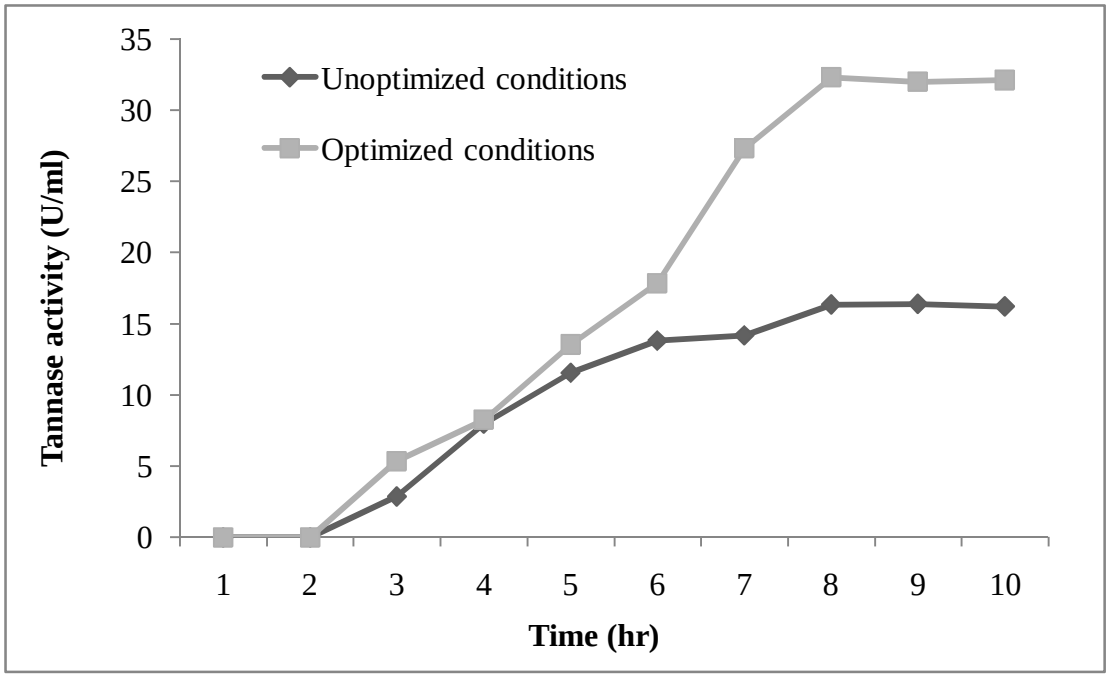

Fig. 2: Time course of tannase production from Trichoderma harzianum under optimized and unoptimized culture conditions.

3.2 Applications of Tannase from Trichoderma harzianum.

The application of tannase produced by *Trichoderma harzianum* in pomegranate juice clarification and natural tannin degradation for gallic acid production was investigated.

3.2.1 Pomegranate Juice Clarification

The efficiency of tannase in fruit juice clarification was examined by incubating the pomegranate juice with varying amount of enzyme (32.0 U/ml) at 40°C. The tannin content of the tannase-treated juice was estimated at regular intervals of 30min. for 2hrs and compared with the tannin content of fresh untreated juice. As shown in the Fig. 3, Maximum tannin reduction of 57% was obtained when 8ml of juice was treated with 4ml tannase for 90 min.

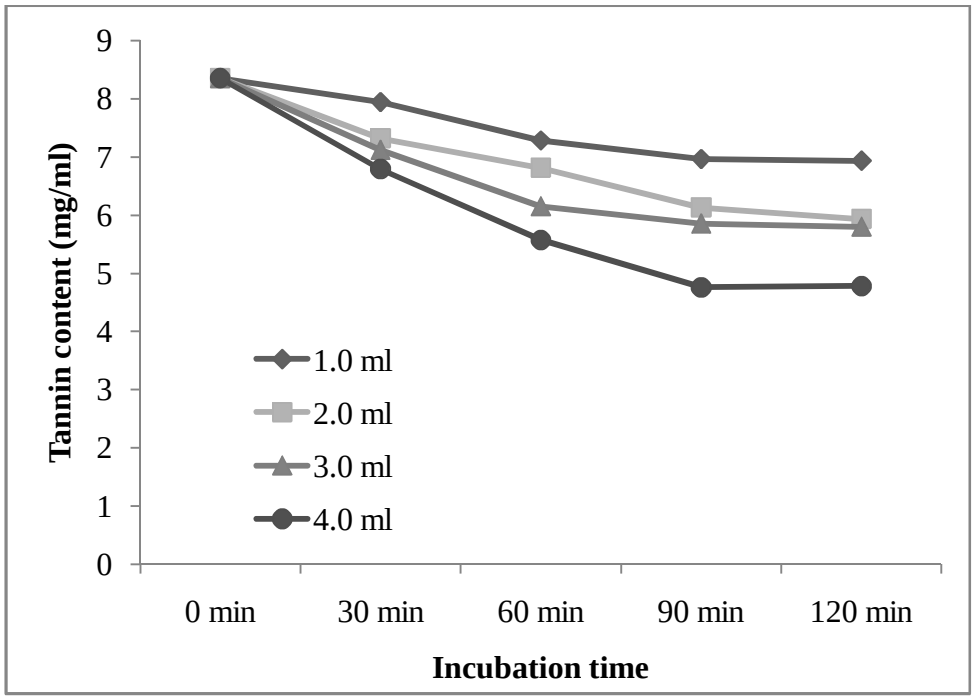

Fig. 3: Effect of incubation time on enzymatic treatment of juice.

The effect of enzymatic treatment of juice on its quality was determined by subjecting the treated and untreated juice for analysis of total sugars, protein content, pH, viscosity and acidity. The results indicate that pre-treatment of pomegranate juice with tannase did not affect its biochemical attributes (Table 1).

Table 1: Effect of enzyme pretreatment on properties of pomegranate juice.

Parameters	Fresh Juice	Treated Juice
Total sugar (µg/ml)	937	917
Total protein (µg/ml)	557	545
pH	4.45	4.35
Viscosity (centipoises)	3.2	3.1
Titratable acidity (ml of 0.05N NaOH)	3.8	3.4

Srivastva and Kar (2010) reported 73.6% tannin removal from fresh aonla (*Phyllanthus emblica*) juice by immobilized tannase from *Aspergillus niger*, while soluble tannase removed 45.2% of tannin in the same time period. Reduction in its vitamin C content was only 2% during the treatment. Tannase from *Penicillium atramentosum* has been shown to reduce 38% tannin content of jamum wine, 43.5% of grape wine and 74% of tea extract after 3hrs (Selwal *et al.*, 2011).

3.2.2 Natural Tannin Degradation - Gallic acid production

Tannase is industrially used as a catalyst in the manufacture of commercially important compound gallic acid by tannin degradation. Tannins are widespread in the plant kingdom, and are found in the leaves, fruits, bark and wood. Hydrolysable tannins are readily hydrolyzed chemically by acidification or biologically by tannase. The price of tannase and gallic acid is quite high at present to consider for many industrial applications and hence, it is necessary to search for the strategies to produce it at cheaper rates. The use of agro-residues and forest products as source of tannin for tannase and gallic acid production is a cost effective and environment friendly approach.

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

Table 2: Natural tannin degradation by tannase from Trichoderma harzianum.

S. No.	Tannin source	Tannin content (mg/ml)	Relative activity (%)	
			1h	2h
1	Control (Tannic acid)	1.90	100	100
2	Amaltash (Cassia fistula) leaves	0.82	23	51
3	Amla (Phyllanthus amblica) bark	0.81	13	62
4	Amla (Phyllanthus amblica) fruit	1.83	72	123
5	Amla (Phyllanthus amblica) leaves	0.93	21	59
6	Ber (Zyzyphus mauritiana) leaves	0.51	20	56
7	Eucalyptus (Eucalyptus glogus) bark	0.22	56	88
8	Eucalyptus (Eucalyptus glogus) leaves	0.53	43	73
9	Guava (Psidium guazava) bark	0.66	61	74
10	Guava (Psidium guazav) leaves	0.61	24	55
11	Jamun (Syzygium cumini) bark	0.78	34	72
12	Jamun (Syzygium cumini) leaves	0.74	75	112
13	Keekar (Acacia nilotica) leaves	0.31	83	105
14	Mango (Magnifera indica) leaves	0.60	48	66
15	Mulberry (Morus macroura) leaves	0.67	64	109
16	Pomegranate (Punica granatum) rind	1.22	66	97
17	Tamarind (Tamarindus indica) seed	1.02	83	119

These results were also observed in paper chromatogram showing release of gallic acid from the degradation of tannin from these substrates (Fig. 4a and 4b).

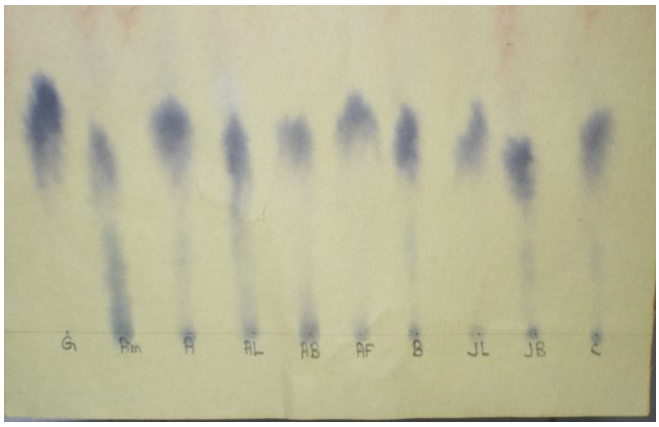

Fig. 4a: Paper chromatogram showing gallic acid liberation by tannase from natural tannins as substrate. (G- Gallic acid (standard), Am-Amaltash leaves, A- Pomegranate rind, AL- Amla leaves, AB- Amla bark, Af- Amla fruit, B- Ber leaves, JL- Jamun leaves, JB- Jamun bark, C- Control –pure tannic acid as substrate)

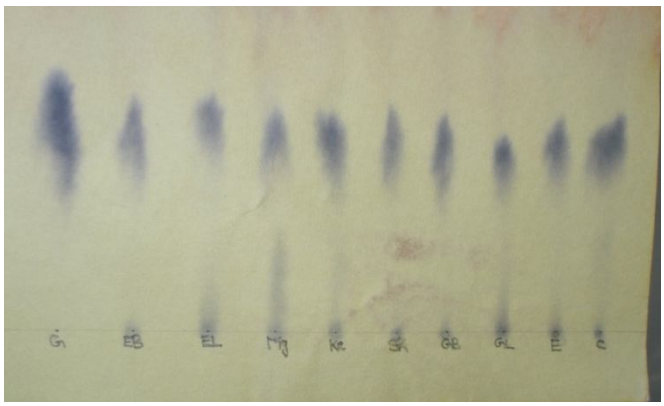

Fig. 4b: Paper chromatogram showing gallic acid liberation by tannase from natural tannins as substrate. (G- Gallic acid (standard), EB- Eucalyptus bark, EL- Eucalyptus leaves, Mg- Mango leaves, Ke- Keekar leaves, Sh- Shatoot (mulberry), GB- Guava bark, GL- Guava leaves, E- Tamarind seed, C- Control- pure tannic acid as substrate)

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), jamun (*Syzygium cumini*) and keekar leaves (*Acacia nilotica*) (Selwal *et al.*, 2011)

4. Conclusion

These results showed that tannase from *Trichoderma harzianum* have the potential to be used for commercial gallic acid production using cheaper agro residues. The tannin degradation efficiency of this tannase can be used as powerful tool for a number of industrial applications like treatment of tannery effluents, fruit juice debittering, wine clarification etc.

References

[1] A E Hagerman and L G Butler (1978), Protein precipitation method for the quantitative determination of tannins, *J. Agric. Food Chem.,* **26**, pp. 809-812.

[2] A Orlita (2004), Microbial biodeterioration of leather and its control: A Review, *Int. Biodet. Biodegd.,* **53**, 3, pp. 157-163.

[3] A Srivastava and R Kar (2010), Application of immobilized tannase from *Aspergillus niger* for the removal of tannin from myrobalan juice, *Indian J. Microbio.*, **50**, pp. 46-51.

[4] D Banerjee, S Mahapatra and B R Pati (2007), Gallic acid production by submerged fermentation of *Aspergillus aculeatus* DBF9. *Res. J. Microbio.,* **2**, pp. 462-468.

[5] H S Hamdy and E M Fawzy (2012), Economic production of tannase by *Aspergillus niger van* Tiegh adopting different fermentation protocols and possible applications, *Romanian Biotechnol. Lett.*, **17,** 4, pp. 7442-7457.

[6] Hina Iqbal and Ashima Kapoor (2012), Culture conditions for the production of tannase from Trichoderma harzianum MTCC 10841, *Int. J. Sci. Technol.*, **1**, 11, pp. 584-595.

[7] J Van de-Lagemaat and D L Pyle (2005), Modelling the uptake and growth kinetics of *Penicillium glabrum* in a tannic acid-containing solid-state fermentation for tannase production, *Proc Biochem.*, **40**, 5, pp. 1773-1782.

[8] M Dubois, K A Gilles and J K Hamilton (1956), Colorimetric method of determination of sugar and related compounds. *Anal. Chem.,* **26**, pp. 350.

[9] M J Lu, S C Chu, L Yan and C Chen (2009), Effect of tannase treatment on protein–tannin aggregation and sensory attributes of green tea infusion, *Food Sci. Technol.*, **42**, 1, pp. 338-342.

[10] M K Selwal, A Yadav, K K Selwal, N K Aggarwal, R Gupta and S K Gautam (2011), Tannase production by *Penicillium atramentosum* KM under SSF and its applications in wine clarification and tea cream solubilization. *Brazilian J. Microbio.,* **42**, pp. 374-387.

[11] M O Illori, S O Adebusoye, O O Amund and B O Oyetoran (2007), A study of tannic acid degradation by soil bacteria. *Pakistan J. Biol. Sci.,* **10**, 18, pp. 3224-3227.

[12] O H Lowry, N J Rosenbrough, A L Farr and R J Randall (1951), Protein measurement with Folin-phenol reagent. *J. Biol. Chem.*, **193**, pp. 265-275.

[13] O M Nuero and F Reyes (2002), Enzymes for animal feeding from *Penicillium chrysogenum m*ycelial wastes from penicillin manufacture, *Lett. Appl. Microbio.*, **34**, 6, pp. 413-416.

[14] P D Belur and G Mugeraya (2011), Microbial production of tannase. *Res. J. Microbio.*, **6**, 1, pp. 25-40.

[15] P K Lekha and B K Lonsane (1997), Production and application of tannin acyl hydrolase. State of the art. *Adv. Appl. Microbiol.* **44**, pp. 215-260.

[16] S K Hota, J R Dutta and R Banerjee (2007), Immobilization of tannase from *Rhizopus oryzae* and its efficiency to produce gallic acid from tannin rich agro residues, *Indian J. Biotechnol.*, **6**, pp. 200-204.