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Regular Article

Evaluation of the versatility of the tannases produced from *Aspergillus niger* and *Penicillium variable* with respect to gallic acid production, gallate ester synthesis, animal feed improvement, tannery effluent degradation and tannin stain removal

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The potentiality of tannases produced from *A. niger* and *P. variable* were investigated with respect to gallic acid production, synthesis of gallate esters, animal feed improvement, tannery effluent degradation and tannin stain removal. Titrimetry results showed significant gallic acid production with 92 % conversion of 8 % tannic acid catalyzed by lyophilized *A. niger* tannase and 60 % conversion of 3 % tannic acid by lyophilized *P. variable* tannase in 8h. In gallate ester synthesis, esterfication rate was observed to be affected by the substrate molar ratio and temperature with maximum at 50°C. Amongst the various organic solvents evaluated, n-hexane (log P 2.0) promoted maximum synthesis of both methyl gallate (MG) and propyl gallate (PG). Also low initial water activity (a_w) of 0.11 – 0.33 obtained with $LiCl_2$ and $MgCl_2.2H_2O$ gave better yields for both MG and PG and by both tannases. Furthermore, addition of molecular sieves at 0h in esterification reaction resulted in enhanced conversion of gallic acid and methanol into methyl gallate (90.7 % and 83 %) by *A. niger* and *P. variable* tannase respectively. Similarly, 94.8 % and 89.6 % conversion of gallic acid and propanol into propyl gallate was achieved by *A. niger* and *P. variable* tannase after addition of molecular sieves at 0h. In the hydrolysis of xylan and pectin, *A. niger* tannase showed outstanding result by efficiently degrading them and producing ferulic acid at retention time of 75.90 min. Besides this, both *A. niger* and *P. variable* tannases showed considerable degradation of 15 X diluted tannery effluent i.e. 45 and 36 % in 48 h respectively. In the removal of tea / tannin stains, the increase in temperature from 37°C to 50°C showed 15 % increase in reflectance for *A. niger* tannase and 21 % for *P. variable* tannase indicating its potential use in tannin stain remover (Tannosol).

Key words: Tannase, Gallic acid (GA), Methyl gallate (MG), Propyl gallate (PG), Ferulic acid (FA)

Tannin acyl hydrolase (EC 3.1.1.20), commonly called tannase is a hydrolytic enzyme that catalyzes the hydrolysis of ester bonds in hydrolysable tannins such as tannic acid, thereby releasing glucose and gallic acid. Tannins are naturally occurring water-soluble polyphenols of varying molecular weight depending on the bonds possessed with proteins and polysaccharides. They occur in many edible fruits and vegetables and are often considered nutritionally undesirable because they form complexes with protein, starch and digestive enzymes and cause a reduction in nutritional value of food [Sariozlu and Kivane, 2009]. The major enzymatic product gallic acid (3,4,5-

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trihydroxybenzoic acid) is a phenolic compound and finds applications in various fields. Its major application area is to manufacture trimethoprim (TMP) which is an antibacterial agent used in combination with sulphonamide [Hadi et al., 1994; Kar et al., 1999; Kar and Banerjee, 2000]. It is also used in leather industry, in manufacturing gallic acid esters, such as propyl gallate, which is widely used as food antioxidant, in the manufacture of pyrogallol and as a photosensitive resin in semiconductor production [Kar et al., 1999; Mondal et al., 2000; Banerjee et al., 2001; Mondal et al., 2001]. Pyrogallol is used in staining fur, leather and hair and also as a photographic developer [Kar et al., 1999]. Besides this, tannase is extensively used in food, beverage and feed industries. In the food industry, it is used in the manufacture of instant tea as a clarifying agent for haze reduction in wine and beer, in reduction of astringency of fruit juices and in reducing anti-nutritional effects of tannins in animal feed. Its enzymatic application also involves the reduction of hyrolysable tannin levels in poultry feed for facilitating the use of tannin containing cheaper components in the animal feed and improving the feed efficiency [Conesa et al., 2001]. A potential use of tannase is the treatment of wastewater contaminated with polyphenolic compounds such as tannins [Aguilar et al., 2001].

Thus, tannases are known to have their potential as industrial catalysts with the ability to carry out different chemical reactions under mild or harsh conditions in both aqueous and non-aqueous media. Realizing the importance of this enzyme in the present scenario, investigations were carried out to explore the versatility of the tannases produced from *A. niger* using tannic acid as substrate [Sharma et al., 2007] and *P. variable* using *Chebulina myrobalan* as tannin substrate [Saxena & Saxena, 2004] respectively for various industrially important applications as - the production of gallic acid, synthesis of esters- methyl gallate and propyl gallate, animal feed improvement, degradation of tannery effluent and as tannin (tea) stain digestor.

MATERIALS AND METHODS

A. PRODUCTION OF GALLIC ACID

The production of gallic acid from tannic acid was monitored by adding 500 mg of lyophilized tannase of *A. niger* (4605 U) produced using 8% tannic acid as substrate [Sharma et al., 2007] and *P. variable* (7000 U) produced using fruits of *Chebulina myrobalan* (11.5% crude tannin) as tannin substrate [Saxena & Saxena, 2004]. Initially, approx. 500 mg of these respective enzymes were added to 2.0 ml volume of tannic acid (Sigma) of different concentrations of i.e. 1, 3, 5, 8, 10 and 12 % (w/v) in water. The reaction mixture was incubated at 37 °C for 120 h and analyzed for gallic acid production at regular intervals of 2h by thin layer chromatography (TLC), titrimetry and finally confirmed by high performance liquid chromatography (HPLC).

1. Thin layer chromatography: The reaction mixtures were concentrated time to time and spotted on silica gel plate (Silica gel 60 F_{254}, E.Merck Ltd., Germany) along with the controls of tannic acid and gallic acid. The thin layer chromatographic plates were run in a solvent system comprising of ethyl acetate, chloroform and formic acid in the ratio of 4:4:1. After the run, the plates were air-dried and developed with ferric chloride reagent (0.81 g of $FeCl_3$ dissolved in 50 ml of distilled water).

2. Titrimetry: The product of the reaction mixture samples were estimated for gallic acid by titrating them in a pH STAT Metrohm titrator (718 state Tritino) with 0.5 mM NaOH. The volume of NaOH used to neutralize the gallic acid formed in the reaction mixture was recorded. Zero hour sample served as the control (representing no conversion). At different time intervals, the percentage conversion of tannic acid to gallic acid was calculated.

3. High Performance Liquid chromatography: To further confirm that, gallic acid is the product, 5 ppm of the sample was dissolved in HPLC water and analyzed by HPLC using BioRad Aminex

HPX – 87H column with 5mM sulphuric acid as mobile phase at a flow rate of 0.6ml/min using UV detector (210 nm) at 50°C column temperature.

B. SYNTHESIS OF GALLIC AICD ESTERS - METHYL GALLATE (MG) AND PROPYL GALLATE (PG)

Tannases are known to carry out esterification between gallic acid and methanol / propanol. Henceforth, detailed investigations were carried out for these reactions. For a typical reaction, 0.1M gallic acid was mixed with 2.0 ml of alcoholic donor (methanol / propanol) in 10.0 ml of n-hexane in 15.0 ml screw – capped vials. Five hundred milligrams each of *A. niger* or *P. variable* crude tannase was added separately in vials containing the reaction mixture. These vials were incubated in a water bath shaker at 150 rpm for 48h at 37°C. The aliquots were withdrawn at regular intervals of 6 h.

The samples along with the standards, were analyzed qualitatively for product formation by thin – layer chromatography using silica gel 60 F_{254} plates (E. Merck Ltd., Germany). A solvent system comprising of ethyl acetate, chloroform and formic acid in the ratio of 4:4:1 was used and the plate was developed with ferric chloride reagent.

Subsequently, these samples were quantitatively estimated for gallic acid consumption by titrating them with 0.5 mM NaOH using pH STAT Titrator (718 STAT Tritinio). Zero hour sample served as the control. Finally the production of methyl gallate and propyl gallate was confirmed by HPLC using C-18 column with 95% methanol and 5% water as mobile phase.

Having confirmed the synthesis of gallic acid esters – methyl gallate and propyl gallate by *A. niger* and *P. variable* tannases, the conditions were optimized to obtain maximum conversion. The following factors were studied.

1. Substrate molar ratio

Gallic acid (0.1M) was mixed with different amounts of methanol / propanol *viz.* 0.1, 0.2, 0.3, 0.4, 0.5 M in n-hexane to obtain a substrate molar ratio of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. The reaction mixture was equilibrated at a_w of 0.33 (using saturated solution of $MgCl_2.6H_2O$). The reaction was carried out for 48h at 37°C and 150 rpm. Samples were withdrawn at regular intervals of 6h upto 48h. Zero hour sample served as the control. Samples withdrawn every 6h were titrated against 0.5mM NaOH. Percentage conversion of gallic acid into methyl gallate and propyl gallate for each molar ratio was calculated.

2. Effect of temperature

The effect of different temperatures on the esterification rate was determined at optimal molar ratio of 1:4 (in case of *A. niger*) and 1:3 (in case of *P. variable*) of gallic acid and methanol / propanol by incubating the reaction mixture in temperature range of 25 - 60°C for 24h.

3. Effect of organic solvents

Esterification reactions are affected by the type of solvent used in the reaction system. Normally, hydrophobic solvents having higher log P value (> 2) are favorable, as these are known to favor greater enzyme stability. On the other hand, hydrophilic solvents can cause inactivation of the enzyme. Thus, the effect of various hydrophobic (n-hexane, Diethyl ether and pyridine) and hydrophilic solvents (ethyl acetate and acetonitrile) were examined on production of methyl gallate and propyl gallate.

4. Effect of water activity (a_w) of the reaction system

Water in an experimental system is an important factor in the esterification reactions as it controls the reaction. Hence, the effect of different initial water activities on synthesis of methyl and propyl gallate was examined. The esterification was carried out using the optimal ratio of gallic acid and methanol / propanol in n-hexane in different initial a_w values of 0.11, 0.33, 0.53, 0.75 and 0.97 achieved using salt solutions of lithium chloride ($LiCl_2$), magnesium chloride

($MgCl_2.6H_2O$), magnesium nitrate ($MgNO_3$), sodium chloride (NaCl) and Potassium sulphate (K_2SO_4) respectively at 150 rpm and 50°C.

5. Effect of addition of molecular sieves

In order to further control the release of water during the reaction, the molecular sieves (type 4A°) were added at a concentration of 10% w/v to the reaction mixture at 0h. The effect was compared with a system without any sieves that served as control.

C. ANIMAL FEED IMPROVEMENT

Plant fibres form a major part of animal feed. The plant cell wall mainly comprises of polymers as arabinoxylans and pectins. In these polymers, diferulic acid forms cross-links which is a major obstacle that limits the accessibility of main chain - degrading enzymes thereby reducing the digestibility of cell wall [Bunzel et al., 2001]. The breakage of these dehydrodimer cross-links between plant cell wall polymers is essential for degradation of plant cell wall resulting in the improvement of its digestibility. Hence, the ability of tannases to carry out this reaction was estimated by performing preliminary experiments of hydrolysis of xylan and pectin using *A. niger* and *P. variable* tannase separately. To 0.5g of the substrate (xylan / pectin) in 20% (v/v) dimethyl sulphoxide (DMSO) in Mops buffer (100mM Mops, final pH 6.1), 1.0 g each of crude lyophilized tannase (9210 U of *A. niger* tannase and 14000 U of *P. variable* tannase) was added and the reaction mixture was incubated at 50°C in a water bath. The reaction was terminated by the addition of acetic acid (final pH < 2.0) after 6h and the samples were filtered using 0.2µm filters prior to incubation. All assays were performed in duplicate. Blanks contained substrate in 20% DMSO (in buffer), enzyme and acetic acid.

The reaction was monitored by spotting the samples on silica gel plates (Silica gel 60 F_{254}, E.Merck Ltd., Germany), along with the standard ferulic acid. The plate was run in a solvent system – choloroform: ethyl acetate: acetic acid in the ratio of 50: 50: 1. The plate was air dried and observed under UV light. It was later exposed to iodine vapours. The presence of ferulic acid in the samples was further confirmed by HPLC.

D. TANNERY EFFLUENT DEGRADATION

The degradation of tannery effluent was examined by incubating different dilutions of tannery effluent made in tap water with crude tannases of *A. niger* and *P. variable* in the ratio of 1:5 (Tannery effluent : crude enzyme) for 72h at room temperature.

The samples were withdrawn after every 24h and were titrated in a pH stat titrator against 0.5 mM NaOH. Zero hour samples served as control. The volume of NaOH consumed to neutralize gallic acid released by the hydrolysis of tannins in tannery effluent was recorded to calculate the % conversion.

E. REMOVAL OF TANNIN STAINS (TEA)

Removal of tea stains was studied by staining small square pieces (5 x 5 cm) of clean cloth with tea extract. The stained pieces were then subjected to the following treatments:

1. Washing with tap water.
2. Washing with tap water and *A. niger* tannase.
3. Washing with detergent and *A. niger* tannase.
4. Washing with tap water and *P. variable* tannase.
5. Washing with detergent and *P. variable* tannase.
6. Washing with detergent

In case of control, the cloth with the stain was not subjected to any treatment. The removal of tea stain was compared on the basis of % increase in reflectance using reflectometer model No. Universal 710.

RESULTS AND DISCUSSION

A. PRODUCTION OF GALLIC ACID

It is evident from **Fig 1a** that, distinct blue colored spots corresponding to the standard gallic acid with R_f value of 0.69 ±

0.02 were observed indicating the production of gallic acid by these organisms due to the hydrolysis of tannic acid and crude tannins. Titremetry results showed significant production of gallic acid catalyzed by *A. niger* tannase with 92 % conversion of 8 % tannic acid in 8h and 60 % conversion of 3 % tannic acid by *P. variable* tannase in 8h. This was further confirmed by analyzing the enzymatic product in HPLC (**Fig 1b**), when a distinct peak of gallic acid at a retention time of 19.28 min was obtained.

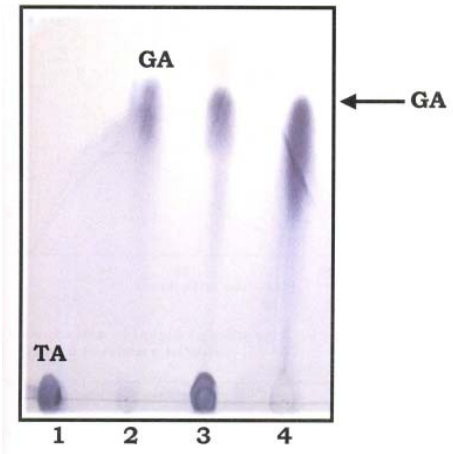

Fig 1a: TLC analyses of conversion of tannic acid to gallic acid by *A. niger* and *P. variable* tannase; Lane 1: Standard Tannic acid; Lane 2: Standard Gallic acid; Lane 3: Tannic acid converted to Gallic acid by action of *A. niger* tannase; Lane 4: Tannic acid converted to Gallic acid by action of *P. variable* tannase;

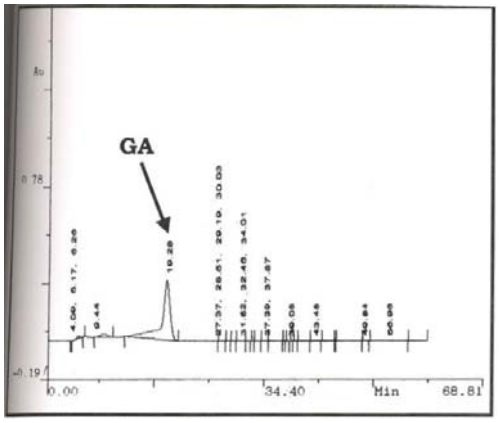

Fig 1b: HPLC chromatogram of gallic acid produced

Similar results were obtained when the tannases produced by these organisms were used to hydrolyse tannic acid. Seth and Chand, [2000] reported 40.3 g/L gallic acid production in 24 h from 45 g/L tannic acid by *A. awamori* tannase.

B. SYNTHESIS OF METHYL GALLATE AND PROPYL GALLATE (SYNTHETIC REACTION)

Distinct blue colored spots of methyl gallate and propyl gallate were detected on TLC plates with the R_f value of 0.75 and 0.78 which also corresponded with the R_f value of standard methyl and propyl gallate (**Fig 2a**). These were further confirmed by HPLC (**Fig 2b -1; 2b -2**)

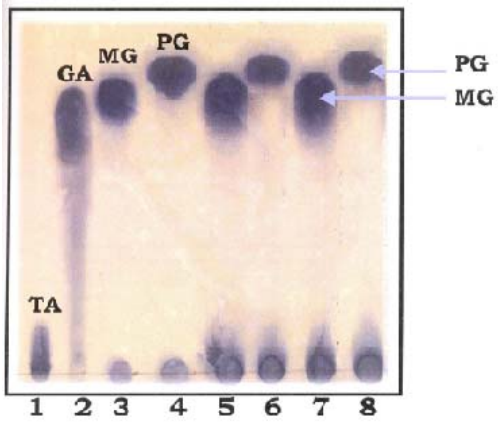

Fig 2a: TLC analyses of the esterification of Gallic acid (GA) and Methanol / Propanol to Methyl gallate (MG) and Propyl gallate (PG) catalyzed by *A. niger* and *P. variable* tannase; Lane 1: TA -Standard Tannic acid; Lane 2: GA - Standard Gallic acid; Lane 3: MG - Standard methyl Gallate; Lane 4: PG - Standard Propyl gallate; Lane 5 : MG produced by *A. niger* tannase; Lane 6 : PG produced by *A. niger* tannase; Lane 7 : MG produced by *P. variable* tannase; Lane 8: PG produced by *P. variable* tannase

1. Effect of substrate molar ratio

It is evident from **Table I & II** that, maximum synthesis of methyl and propyl gallate is achieved at a molar ratio of 1:4 by tannase from *A. niger* within 24 h with 72 % and 80 % synthesis for methyl gallate and

propyl gallate whereas Yu and Li [2005] reported maximum molar conversion (36. 2 %) with 9.1 % (v/v) 1- propanol and 8mM gallic acid and this decreased with increasing amounts of substrate concentration.

On the other hand, methyl gallate (54 %) and propyl gallate (75 %) were synthesized in 24 h by *P. variable* tannase at molar ratio of 1: 3.

Fig 2b -1 **Fig 2b -2**

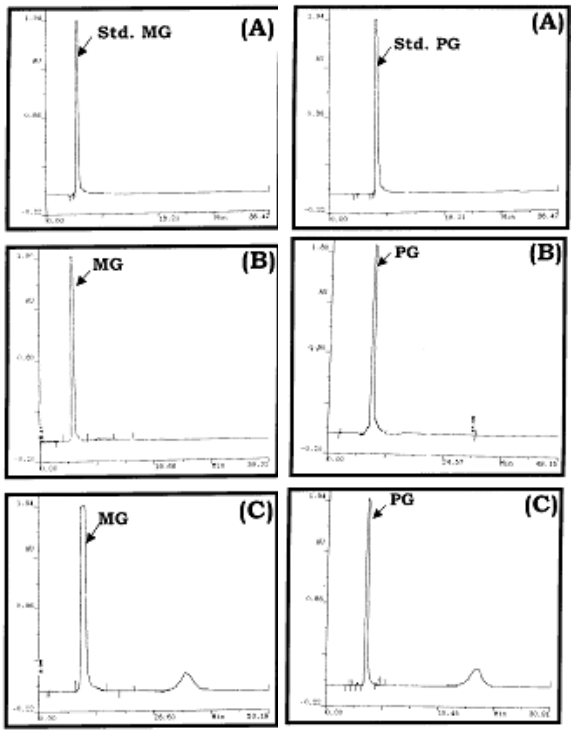

Fig 2b-1: HPLC chromatogram of methyl gallate (MG) produced by esterification reaction between methanol and gallic acid (GA) catalyzed by *A. niger* (B) and *P. variable* (C) tannase; **Fig 2b-2:** HPLC chromatogram of propyl gallate (PG) produced by esterification reaction between propanol and gallic acid (GA) catalyzed by *A. niger* (B) and *P. variable* (C) tannase

2. Effect of temperature

It is evident from **Fig 3** that, the rate of esterification between gallic acid and methanol / propanol is efficiently achieved in the temperature range from 37°C to 60°C with maximum at 50°C. At this temperature approx., 87 % synthesis of methyl and propyl gallate by *A. niger* tannase and 73% and 75 % synthesis of methyl and propyl gallate by *P. variable* tannase was achieved in 18h. Similarly, Weetal [1985a] reported optimal temperature of 35°C for maximum synthesis of amyl gallate. However, for propyl gallate they reported maximum synthesis of 60% at 4°C in 6h than at 23°C with only 25% synthesis in case of A. *oryzae* tannase.

3. Effect of organic solvents

It is evident from **Fig 4** that, among different solvents tried, n-hexane with log P value >2 i.e. 3.50, resulted in maximum synthesis (86.4 and 71.9 %) of methyl gallate from gallic acid and methanol catalyzed by *A. niger* and *P. variable* tannase. Similarly, 85.2 % and 76.4 % synthesis of propyl gallate from gallic acid and propanol was obtained by *A. niger* and *P. variable* tannase in n-hexane. Weetal [1985b] also reported 85 % yield of propyl gallate in system containing hexane, propanol and gallic acid after 120h. In 2004, Yu et al [2007], reported the highest yield of 44.3 % in benzene (log P 2.0) catalyzed by microencapsulated tannase from *A. niger*. Yu and Li [2005] reported 53 % propyl gallate yield by tannase produced from recombinant *P. pastoris* in 24 hrs using benzene as organic solvent.

Table 1: Production of methyl / propyl gallate from gallic acid and methanol / propanol (%) at different molar ratios by *A. niger* tannase at 37°C in relation to time

Molar ratio	Production of methyl/propyl gallate (%)											
	0h		6h		12 h		24 h		36 h		48 h	
	MG	PG	MG	PG	MG	PG	MG	PG	MG	PG	MG	PG
1: 1	-	-	10.2	23.7	20.2	41.4	33.2	50.2	25.2	45.2	9.3	39.9
1: 2	-	-	24.8	38.2	32.4	50.6	48.3	61.7	36.8	53.7	21.5	43.4
1: 3	-	-	38.3	54.3	51.7	65.4	63.4	73.4	61.6	70.3	52.4	67.1
1: 4	**-**	**-**	53.7	60.3	66.7	71.4	**72.4**	**80.4**	67.1	78.2	55.5	75.9
1: 5	-	-	55.8	65.1	68.3	70.3	71.9	81.4	68.2	80.1	50.2	77.9

MG : Methyl gallate ; PG : Propyl gallate

Table II: Production of methyl / propyl gallate from gallic acid and methanol / propanol (%) at different molar ratios by *P. variable* tannase at 37°C in relation to time

Molar ratio	Production of methyl/propyl gallate (%)											
	0h		6h		12 h		24 h		36 h		48 h	
	MG	PG	MG	PG	MG	PG	MG	PG	MG	PG	MG	PG
1: 1	-	-	8.8	18.5	15.3	34.7	29.4	48.9	26.6	37.5	18.2	20.1
1: 2	-	-	13.7	29.6	24.7	51.8	38.9	61.3	30.1	50.5	20.9	42.3
1: 3	**-**	**-**	20.3	48.5	33.7	63.4	**54.3**	**75.4**	48.6	71.4	36.1	65.6
1: 4	-	-	22.4	42.3	34.8	61.9	48.8	72.4	50.7	68.2	46.4	57.7
1: 5	-	-	19.2	43.9	27.6	62.3	38.9	73.9	31.4	69.2	26.6	52.7

MG : Methyl gallate ; PG : Propyl gallate

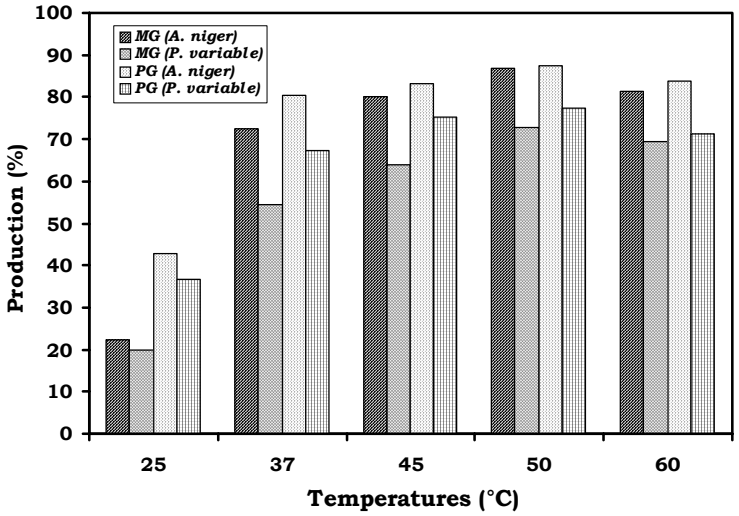

Fig. 3: Effect of temperature on the production of methyl and propyl gallate (MG & PG) by *A. niger* and *P. variable* tannase after 18h

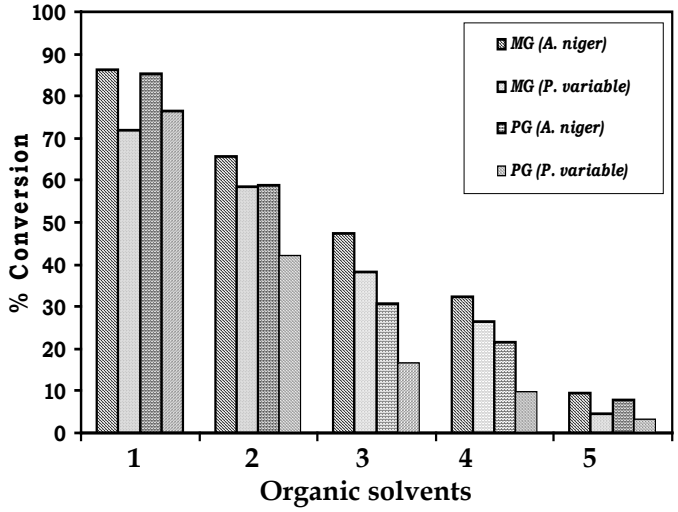

Fig. 4: Effect of organic solvents (hydrophobic and hydrophilic) on production of methyl and propyl gallate (MG & PG) at 50°C catalyzed by *A . niger* and *P. variable* tannases; 1. n-hexane; 2. Diethyl ether; 3. Pyridine; 4. Ethyl acetate; 5. Acetonitrile

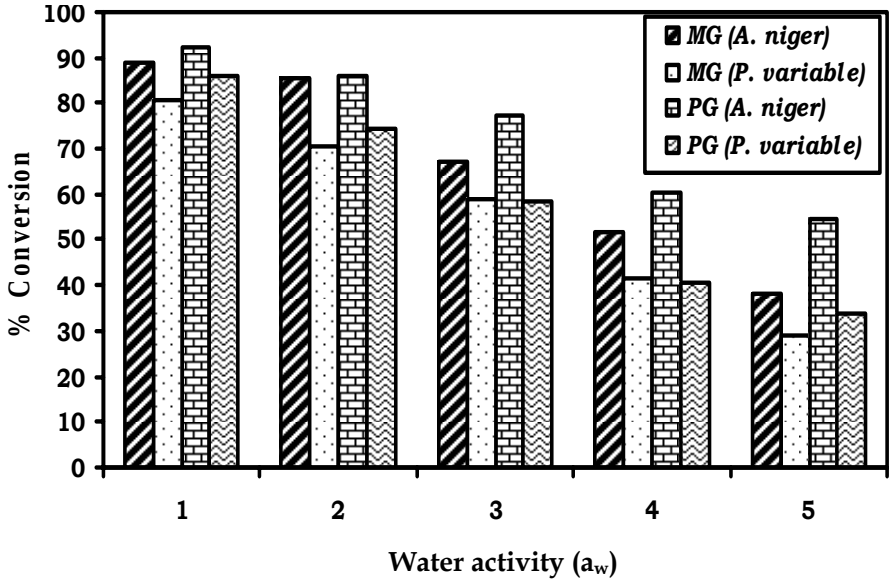

Fig. 5: **Effect of water activity (a_w) on production of methyl and propyl gallate (MG & PG) after 18 h at 50°C; 1. $LiCl_2$; 2. $MgCl_2.6H_2O$; 3. $MgNO_3$; 4. NaCl; 5. K_2SO_4**

4. Effect of water activity (a_w)

It is evident from **Fig 5** that, low initial water activity (a_w) of 0.11 – 0.33 obtained with $LiCl_2$ and $MgCl_2.6H_2O$ gave better yields. However, high initial water activity of 0.97 using K_2SO_4 gave low yields. This observation is also supported by Weetal [1985b] and Gaathon et al. [1989] for maximum synthesis of propyl gallate. Gaathon et al. [1989] reported a 50% yield of propyl gallate in low water activity by entrapping tannase in reverse micelles. Sharma and Gupta [2003] also reported the optimized yield of 86 % using simultaneously pH tuned *A. niger* van *Tiegham* tannase immobilized on celite and using the right amount of water in non-aqueous media. Yu and Li [2005] reported microencapsulated mycelium – bound tannase and water concentration optimum ratio of 25 capsules per 10.0 ml of benzene (log P 2.0) and 0.04 g of water / capsule for maxiumum propyl gallate synthesis.

5. Effect of addition of molecular sieves

In tannase mediated reaction systems, ours is the first attempt to control water activity for the production of esters using molecular sieves. Addition of sieves at 0h resulted in 90.7 and 83 % conversion of gallic acid and methanol into methyl gallate by *A. niger* and *P. variable* tannase. Similarly, 94.8 and 89.6 % conversion of gallic acid and propanol into propyl gallate was catalyzed by *A. niger* and *P. variable* tannase by addition of molecular sieves at 0h (**Table III & IV**). This also stabilized the products, methyl gallate and propyl gallate preventing a backward reaction as observed in the system without any molecular sieves. The addition of molecular sieves for controlling water activity has been reported in reactions catalyzed by lipases [Kim et al.,1995, 1998]. After optimization of different parameters for methyl gallate and propyl gallate esters using *A. niger* and *P. variable* tannase, a yield of 92.5 and 85 % of methyl gallate and 97 and 92 % of propyl gallate was obtained in 8h. Toth and Hensler [1952] reported the synthesis of methyl, ethyl, propyl gallate, digallic acid and glucogallin using freeze-dried tannases of *A. oryzae*. They reported 0.33% synthesis for glucogallin and 33 % for methyl gallate. Weetal [1985 a,b] reported maximum synthesis of amyl gallate than propyl gallate. A maximum synthesis of 40 % methyl gallate was obtained with freeze-dried tannase while 85 % synthesis of amyl gallate was obtained with immobilized *A. oryzae* tannase. The use of hexane as a solvent for maximum synthesis of propyl gallate thereby showing the stability of enzyme in hexane was also

reported. Gaathon et al [1989] reported a maximum yield of 50 % of propyl gallate in a reverse micellar system comprising of 0.2M sodium 1,2 tris (2-ethylhexylcarbonyl) 1-ethane sulfonate (AOT) in heptane, propanol, water, tannic acid and buffering ions in 120h. Similarly, Sharma and Gupta [2003] reported 86.4 % yield of propyl gallate using simultaneously pH tuned tannase from *Aspergillus niger* van *Tieghem* immobilized on celite with right amount of water in non-aqueous media. Yu et al [2007] reported maximum molar conversion of 65 % achieved with 7. 3 % (v/v) 1-propanol and 5.56 molar gallic acid in benzene at stirring speed of 200 rev / min, 40°C in presence of sodium sulphate and PEG 10,000.

Table III: Effect of molecular sieve addition at zero hour on the production of methyl gallate (%) by *A. niger* and *P. variable* tannase

Incubation period (h)	Production of methyl gallate (%)			
	A. niger tannase		*P. variable* tannase	
	a	b	a	b
4	43.9	55.6	40.2	52.3
8	87.7	90.7	80.4	83.4
10	78.6	89.7	70.3	84.2
20	64.3	86.5	56.8	84.1

a: without molecular sieve ; b: with molecular sieve

Table IV: Effect of molecular sieve addition at zero hour on the production of propyl gallate (%) by *A. niger* and *P. variable* tannase

Incubation period (h)	Production of methyl gallate (%)			
	A. niger tannase		*P. variable* tannase	
	a	b	A	b
4	57.1	69.3	59.8	65.4
8	91.6	94.8	84.8	89.6
10	82.1	93.7	73.4	88.5
20	**73.9**	**90.4**	**66.8**	**86.4**

a: without molecular sieve ; b: with molecular sieve

C. ANIMAL FEED IMPROVEMENT

It is evident from **Fig 6a** that, *A. niger* and *P. variable* tannase were able to hydrolyze xylan and pectin producing ferulic acid as is seen as a distinct spot corresponding to the standard ferulic acid (Rf 0.98) on the TLC plate when exposed to iodine vapours or when observed under UV light. The intensity of the spot of ferulic acid was higher in the hyrolysate samples of xylan and pectin obtained by the hydrolysis of *A. niger* tannase. **Fig 6b** shows that, a peak with a retention time of 75.90 min is more or less observed at the same retention time as the standard ferulic acid (74.73 min), further confirming the production of ferulic acid. This result is significant, as these tannases could produce ferulic acid as a result of hydrolysis of xylan and pectin. It shall be a worthwhile attempt to explore the use of these tannases to develop better quality animal feed as these will hydrolyse xylan and pectin components present in fodder. Conesa et al [2001] used tannase produced from *A. oryzae* to hydrolyse the synthetic ethyl esterified substrates such as diethyl 8-5-benzofuran diferulate, ethyl 8-5-benzofuran diferulate, diethyl 5-5-diferulate, diethyl 8-O-4-diferulate as model substrates. They reported that tannase was most efficient in hydrolyzing the first ester bond of the 5-5-types of dimer and also both the ester bonds of the 8-5-benzofuran dimer, thus forming the corresponding free acid product i.e. ferulic acid. Their results suggested that tannases may contribute to plant cell wall degradation by cleaving some of the cross-

links existing between cell wall polymers, increasing the digestibility of animal feed.

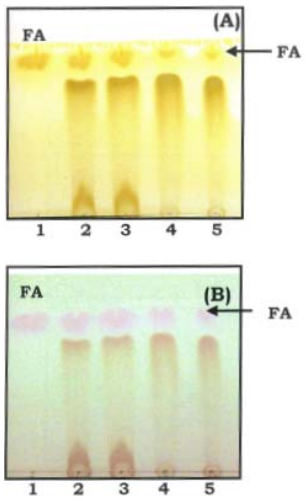

Fig 6a: TLC analysis of the hydrolysis of xylan and pectin by tannase from *A. niger* resulting in the production of Ferulic acid (FA). A: Developing the TLC plate in iodine vapours; B: Examining the plate under UV light (FA gives a pink spot); Lane1: TA – Standard Tannic acid; Lane 2: GA- Standard Gallic acid; Lane 3 : FA – Standard Ferulic acid; Lane 4: FA produced by hydrolysis of xylan by *A. niger* tannase; Lane 5 : FA produced by hydrolysis of pectin by *A. niger* tannase

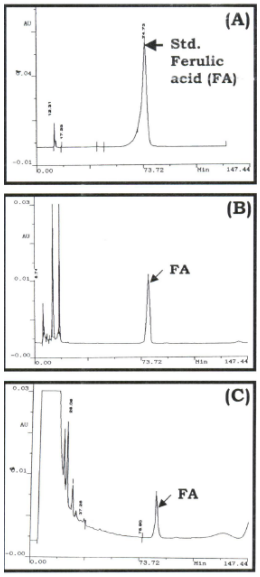

Fig 6b: HPLC chromatogram of the Ferulic acid produced by the hydrolysis of xylan and pectin catalyzed by *A. niger* tannase (A) Standard Ferulic acid (FA); (B) FA produced by hydrolysis of xylan; (C) FA produced by hydrolysis of pectin

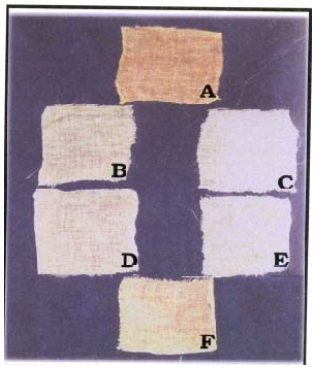

Fig7: Removal of tea stains by tannases from *A. niger* and *P. variable* at 50°C A: Washed with tap water; B: Tap water + *A. niger* tannase; C: Detergent + *A. niger* tannase; D: Tap water + *P. variable* tannase; E: Detergent + *P. variable* tannase; F: Detergent

D. DEGRADATION OF TANNERY EFFLUENT

It is evident from **Table V** that, both *A. niger* and *P. variable* tannase could degrade 45 and 36 % of 15X diluted tannery effluent after 48h. This is the first investigation wherein the monitoring of tannase in degradation of tannery effluent has been carried out. The results have shown its potentiality in treating effluent containing tannins as in leather industries.

E. REMOVAL OF TANNIN STAINS (TEA)

It is evident from **Table VI** and **Fig 7** that, cloth pieces having tea stains when washed in solutions containing detergent and tannase shows better brightness as compared to other solutions as estimated by the increase in percent reflectance. It was noticed that stain removal was better at 50°C (80% reflectance) when compared with at 37°C (65% reflectance). Similarly, *P. variable* tannase when used along with detergent also increased the reflectance by 75% at 50°C as compared with at 37°C (54% reflectance). Even the visible observation of cloth pieces after washing, showed better removal of tea stains when tannases along with detergent were used (**Fig 7**). This shows that, tannases from *A. niger* and *P. variable* can be efficiently used as formulation of tannin stain remover.

Table V: Degradation of tannery effluent by *A. niger* and *P. variable* tannase

Tannery Effluent (% dilution)	Degradation of tannery effluent (%)					
	A. niger tannase			*P. variable* tannase		
	24 h	48 h	72 h	24 h	48 h	72 h
Crude (X)	-	-	-	-	-	-
2X	-	-	-	-	-	-
5X	-	-	-	-	10.5	9.8
10X	25.5	38.4	32.5	-	36.7	32.3
15X	44.3	45.7	42.6	-	36.4	34.6
20X	43.7	41.2	39.8	-	32.1	33.8

Table VI: Removal of tea stains by *A. niger* and *P. variable* tannase

Samples	% Increase in reflectance after treatment	
	37°C	50°C
A. Tap water	10.0	16.7
B. Tap Water + *A. niger* tannase	15.0	22.9
C. Detergent + *A. niger* tannase	65	80
D. Tap Water + *P. variable* tannase	11.7	18.6
E. Detergent + *P. variable* tannase	54	75
F. Detergent	33.7	45.0

Conclusion

The results have shown that the tannases produced from *A. niger* and *P. variable* are very versatile as these could be successfully used for various industrial applications – pharmaceutical, feed, waste water treatment contaminated with tannery effluent and in detergents. Here, the use of molecular sieves at 0 hr for the synthesis of methyl gallate and propyl gallate during the esterification reaction catalyzed by lyophilized *A. niger* and *P. variable* tannases have proven to be outstanding as compared to that without the molecular sieves. Moreover, the use of these tannases have shown a significant result in the hydrolysis of xylan and pectin by cleaving the cross links between cell wall polymers thereby increasing the digestibility of animal feed. Besides this, their usage in degrading the tannery effluent and in the increase in the % reflectance for the removal of tea stains have shown their marvelous potentiality in the field of tannery effluent treatment and wash performance. These enzymes could be engineered through biocatalyst engineering such as rational design or directed evolution to further enhance their activity and specific properties.

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References

Aguilar, C.N., Augur, C., Favela-Torres, E., and Viniegra-Gonzalez, G. (2001). Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid state fermentation: influence of gallic acid and tannic acid. J. Ind. Microbiol. Biotechnol. 26: 296-302.

Bunzel, M., Ralph, J., Marita, J.M., Hatfield, R.D. and Steinhart, H. (2001) Diferulates as structural components in soluble and insoluble cereal dietary fibre. J. Sci. Food Agric. 81: 653-660.

Banerjee, D., Mondal, K.C and Pati, B.R. (2001). Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus acuteatus* DBF 9. J. Basic Microbiol. 41(6): 313- 318.

Conesa, M.T., Ostergaard, P., Kauppineu, S., Williamson, G. (2001). Hydrolysis of diethyl diferulates by a tannase from *Aspergillus oryzae*. Carbohydrate Polymers. 44, 319-324.

Gaathon, A., Gross, Z., and Rozhanski, M. (1989) Propyl gallate: enzymatic synthesis in a reverse micelle system. Enzyme Microb. Technol. 11, 604-609.

Hadi T.A., Banerjee, R., and Bhattacharya, B.C. (1994). Optimization of tannase biosynthesis by a newly isolate *Rhizopus oryzae*. Bioprocess Engg. 11(6): 239-243.

Kim, T.U., Gu, B.G., Jeong J.Y., Byun, S.M., Shin, Y.C. (1995) Purification and characterization of a maltotetrose forming alkaline α- amylase from an alkaliphilic *Bacillus* strain GM 8901. Appl. Environ Microbiol, 61, 3105-12.

Kim, J.E., Han, J.J., Yoon, J.H. and Rhee, J.S. (1998) Effect of salt hydrate pair on lipase – catalyzed regioselective monoacylation of sucrose. Biotechnol. Bioengg. 57, 121–125.

Kar, B., Banerjee, R and Bhattacharya, B. (1999). Microbial production of gallic acid by modified solid - state fermentation. J. Ind. Microbiol. Biotechnol. 23: 173-177.

Kar, B and Banerjee, R. (2000). Biosynthesis of tannin acyl hydrolase from tannin - rich forest residue under different fermentation conditions. J. Ind. Microbiol. Biotechnol. 23:173-177.

Mondal, K.C., Banerjee, R and Pati, B.R. (2000). Tannase production by *Bacillus licheniformis*. Biotechnol. Lett. 22:767-769.

Mondal, K.C., Banerjee, D., Banerjee, R. and Pati, B.R. (2001). Production and characterization of tannase from *Bacillus cereus* KBR 9. J. Gen. Appl. Microbiol. 47(5): 263-267.

Seth, M and Chand, S. (2000). Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori* – optimization of process parameters. Process Biochem. 36: 39-44.

Sharma, S. and Gupta, M.N. (2003) Synthesis of Antioxidant propyl gallate using tannase from *Aspergillus niger* van *Tieghem* in non-aqueous media. Bioorg. Med. Chem. Lett. 13, 395-397.

Saxena S and Saxena RK. (2004). Statitical optimization of tannase production from *Penicillium variable* using fruits (chebulic myrobalan) of *Terminalia chebula*. Biotechnol. Appl. Bichem. 39, 99-106.

Sharma S, Agarwal L and Saxena RK. (2007). Statitical optimization of tannase production from *Aspergillus niger* under submerged fermentation. Ind. J. Microbiol. 47 (2), 132-138.

Sariozlu NY and Kivanc M (2009). Isolation of gallic acid –producing microorganism and their use in the production of gallic acid from gall nuts and sumac. African J Biotechnol. 8 (6), 1110 -1115.

Toth G and Hensler D (1952). The enzymatic synthesis of gallic acid derivatives. Acta Chim II 10:209 - 212.

Weetal, H.H. (1985a) Enzymatic synthesis of gallic acid esters. Appl. Biochem. Biotechnol. 11, 25-28.

Weetal H.H. (1985b). Enzymatic gallic acid esterification. Biotechnol. Bioengg. 27(2):124-127.

Yu X; Li Y and Wu D (2004). Enzymatic synthesis of gallic acid esters using microencapsulated tannase: effect of organic solvents and enzyme specificity. J Molecular Catalysis B: Enzymatic 30 (2), 69-73.

Yu XW and Li YQ (2005). Microencapsulated mycelium-bound tannase from *Aspergillus niger* : an efficient catalyst for esterification of propyl gallate in organic solvents. Appl. Biochem. Biotechnol. 126: 177-188.

Yu XW; Li YQ; Zheu SM and Zheng YY (2007) Synthesis of propyl gallate by mycelium-bound tannase from *Aspergillus niger* in organic solvent. World J Microbiol Biotechnol. 23: 1091-1098.

Yu XW and Li YQ. (2008). Expression of *Aspergillus oryzae* tannase in *Pichia pastoris* and its application in the synthesis of propyl gallate in organic solvent. Food Technol. Biotechnol. 46(1) 80-85.