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Bioprocessing of Paddy Straw for the Production and Purification of Gallic acid using *Penicillium chrysogenum*

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Tannin acyl hydrolase is an industrially important enzyme that is mainly used in the food and pharmaceutical industry. The present work has been taken up with a view of exploring the possibilities of using Agriculture by products for the production of tannase and optimizing condition require to get maximum production using *Aspergillus flavus, Trichoderma viride* and *Penicillium chrysogenum was* selected and optimized for Tannase enzyme production in solid state fermentation using cheaper sources of paddy straw. Tannase production has been evaluated using solid-state fermentation (SSF) at different temperatures, pH, and incubation time. Optimum fermentation conditions of pH, temperature and Incubation period for Tannase production were found to be 5.5 and 30°C at 96 hrs. Thus the present study proved that the *Penicillium chrysogenum* produced *t*annase shown in crude activity 17.0 (U/g/min), Ammonium sulphate precipitation 27.0 (U/g/min), Dialysis 34.0 (U/g/min), Column Chromatography 43.0(U/g/min) and gallic acid content (0.18 mg/gm)was determined by using HPLC method. The molecular structure of Gallic acid (Rt.6.55 min) was confirmed by using Gas Chromatograpgy with Mass Spectrophotometer (GC-MS) method.

Key words: Tannase, gallic acid, , Paddy straw, HPLC, GC-MS.

Paddy or rice straw is one such lignocellulosic material that is produced abundantly as a byproduct of rice crop with an annual worldwide production of 800 MT[1]. Rice straw contains bound sugars in the form of cellulose and hemicellulose meshed with lignin, it needs to be promoted as a fermentation raw material for enzyme production. The utilization of rice straw for the purpose of carbon source for production of tannase.

The present day trend is the utilization of waste material for production of byproducts which boosts up high economic returns in many industries. With the advent of biotechnology, attempts have increasingly been made globally to make potential use of agro-industrial residues for value addition by production of enzymes, organic acids, bioactive secondary metabolites, single-cell protein etc[2-4].

Solid-State Fermentation (SSF) involves growth of microorganisms on moist solid substrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites.

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Tannin acyl hydrolase commonly called tannase is produced by a number of microorganisms including, Fungi *Aspergillus, Penicillium, Rhizopus* sp, Yeast - *Candida* sp. and Bacteria - *Bacillus* sp. Several agro-industrial waste and byproducts such as orange bagasse[3], sugar cane bagasse[5] ,wheat bran[6] and other food processing waste[7] are effective substrates for depolymerizing enzyme production by solid-state fermentation, which proved to be highly efficient technique in the production of tannase. The major commercial application of this enzyme is in the hydrolysis of gallotannin to gallic acid, which is an intermediate required for the synthesis of an antifolic antibacterial drug trimethoprim[8].

Tannase is extensively used in the preparation of instant tea, wine, beer, and coffee-flavored soft drinks and also as additive for detannification of food[9] .

Tannase hydrolyses ester bonds of tannins such as tannic acid to produce gallic acid and glucose[10]. Gallic acid, the hydrolytic product of tannin hydrolysis finds application in many fields including pharmaceutical, dye making, leather and chemical industries[11,12]. Besides this, gallic acid possesses wide range of biological activities, such as antioxidant, antibacterial, antiviral, analgesic etc. As antioxidant gallic acid acts as an antiapoptotic agent and helps to protect human cells against oxidative damage.

In this study, paddy straw, a locally available industrial waste, was used as a substrate for the production of Tannase and analysis of the gallic acid in fermented paddy straw by using HPLC and molecular structure confirmation by using GC-MS.

MATERIAL AND METHODS

Raw material

Paddy straw was collected from local farmers and grinded to obtain 0.5 mm particle size using a standard sieve. This study was conducted on November 2010 at Indian Institute of Crop Processing Technogy, Thanjavur, Tamilnadu.

Microorganisms

Aspergillus flavus, Penicillium chrysogenum and *Trichoderma viride* were isolated and identified by their morphology and colony characteristics. The isolated cultures were maintained on PDA slants.

Preparation of spore inoculums

The spore suspension was prepared by adding adding 2.5mL of sterile distilled water containing 0.1 % Tween 80 to a fully sporulated culture and dislodged using a sterile inoculation loop under strict aseptic conditions. A volume of 1 ml with concentration of $36x10^9$ spores was used as inoculums.

Production of tannase under SSF

A five gram of paddy straw powder was taken in 250-mL Erlenmeyer flask and moistened with 5 mL of salt solution. The composition of the salt solution was NH_4NO_3 0.5 %, NaCl 0.1 %, $MgSO_4.7H_2O$ 0.1 % and Tannic acid 4% at pH =5.5. The contents were sterilized by autoclaving at 121ºC; 15lbps for 20 min. The cooled sterilized solid substrate was inoculated with 1 ml of the spore suspension and incubated at 30 °C for 96 h.

Extraction, Purification and estimation of Tannase enzyme

The enzyme tannase was extracted from the fermented paddy straw by adding 0.05 M citrate buffer, pH 5.0. The fermented substrate was crushed with Mortar & pestle. The homogenized substrate was subjected to centrifugation at 8000 rpm at 4°C for 20 min and collected the supernatant.

The supernatant was precipitated with solid ammonium sulphate (80%) and the precipitate was dialyzed against same buffer for overnight at 4 °C. The dialyzed sample was then subjected to DEAE Sephadex A-50 column chromatography and collected the fractions. The tannase activity was estimated following the method of Sharma *et al.,* [13].

Optimization of Fermentation process

Effect of temperature

The Solid-state fermentation was carried out at different temperatures such as 25, 30, and 35, 40 and 45ºC for 96 h.

Effect of pH

The pH of the salt solution was tried at different pH 5.0 to 7.5.

Effect of incubation Period

The solidstate fermentation was carried out for different time periods ranging from 24 to 120 h.

Estimation of Tannin:

The tannin content was estimated following the method of Haggerman *et al.*[14].

HPLC analysis of Gallic acid

Standard preparation

Standard stock solution of gallic acid (Sigma) was prepared in methanol, at concentrations of 5mg/ml. Standard solution were filtered through 0.45 mm membrane filter and injected by autosampler.

Sample Preparation

In this study we have taken the sample from paddy straw fermented with *Penicillium chrysogenum*. The sample was prepared according to the procedure of El Sohafy [15]. 5 ml of water was added into the 0.5 g of the sample and extracted by boiling the sample for 5 min then adjust the volume to 5 ml. After filtering the sample 0.5 ml of 25% HCl was added and kept in boiling water bath for 25 min for hydrolysis. Then the mixture was extracted with 4 successive 4 ml portions of n-butanol. Then the mixture was dried under reduced pressure and redissolved in 2 ml ethanol**.**

HPLC conditions

Gallic acid was analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a LC-10AD pump, SCL 10A system controller and SPD-M 10A UV detector. Chromatography of the phenolic acids was achieved using a prepacked LiChrospher 100 RP C-18 column (4´250 mm, 5 ìm; Merck). The mobile phase comprised water-acetonitrile-acetic acid (88:10:2; v/v/v)[2] and was delivered at a rate of 1 ml/min. Detection was monitored at 280 nm. All the results of this work are an average of two independent determinations.

The gradient elution of solvent A [water-acetic acid (25:1 v/v)] and solvent B (methanol) had a significant effect on the resolution of compounds. As a result, solvent gradients were formed, using dual pumping system, by varying the proportion of solvent A [water-acetic acid (25:1, v/v)] to solvent B (methanol). Solvent B was increased to 50% in 4 min and subsequently increased to 80% in 10 min at a flow rate of 1.0 mL/min. Detection wavelength was 280 nm.

Determination of Molecular structure by Gas chromatography –Mass Spectrphotometer (GC-MS)

The analysis of Gallic acid was performed with GC Perkin-Elmer-Clarus-500 plus MS Perkin-Elmer-Clarus-500 (Column: Elite-1 (100% Dimethyl poly siloxane), 30 x 0.25mm x 1mmdf, Carrier gas: 1ml per min, Split: 10:1, Detector: Mass detector: Turbo mass gold-Perkin Elmer Software: Turbomass 5.4.2, Sample injected: 2ml Oven temperature programme: -110 deg C-2 min hold, Up to 200 deg C at the rate of 10 deg C / min-no hold, Up to 280 deg C at the rate of 5 deg C / min-9 min hold, Injector temperature: 250 deg C, Total GC running time: 36 min: MS Programme

Library used: NIST Version-Year 2005,Inlet line temperature: 200 deg C, Source temperature: 200 deg C,Electron energy: 70 eV, Mass scan: (m/z): 45-450,Total MS running time: 36 min. The individual constituents showed by GC were identified by comparing their MS with standard compounds of Nist library.

RESULTS AND DISCUSSION

Tannase production in Solid State fermentation using Paddy straw

Production of Tannase by *Penicillium chrysogenum*, *Aspergillus flavus* and *Trichoderma viride* were reported in Table 1. In the present study *Penicillum chrysogenum* possess higher

Table 1. Tannase production in paddy straw

S. No	Organism	Tannase activity (U/g/min)			
		Crude	Ammonium sulphate	Dialysis	Column Chromatography
1	*Aspergillus flavus*	16.0	18.0	21.0	27.0
2	*Penicillum chrysogenum*	17.0	27.0	34.0	43.0
3	*Trichoderma viride*	19.0	23.0	26.0	31.0

tannase activity of 43.0 U/g/min in purified form (Fig. 1). Previously maximum extracellular tannase and gallic acid production was recorded in 96h and 120h by *A. niger* and *R. oryzae*[16,17] (Cavalitto *et al*., 1996; Iibuchi *et al*., 1967).

Effect of incubation period on tannase production

The results on the optimum incubation period requires for maximum tannase production showed in Fig. 1 that the enzyme production started after 24 h of incubation and progressively increased with time, the maximum production of 39.8 U/gm/min was observed at 96 h incubation. Thereafter, the enzyme production started decreasing. Decreased enzyme yield on prolonged incubation could also be due to inhibition and denaturation of the enzyme[18] (Gautam *et al*., 2002). It has been reported before that tannase was produced during the primary phase of growth and thereafter the activity decreases either due to the decrease in production or due to enzyme degradation[19] (Suseela and Nandy, 1985).

Effect of pH on tannase production

Among the various pH tested, maximum production of tannase(38.60 U/gm/min) was observed at pH 5.5. The increase in pH over 6.0 drastically reduced the tannase activity, which was shown in Fig. 2, Lekha and Lonsane, [20] also reported that tannases are acidic proteins with an optimum pH around 5.5. Similarly, Sabu *et al*., [21] reported optimum pH of 5.5 for tannase production by *Aspergillus niger* ATCC 16620.

Effect of incubation temperature on tannase production

Among the different temperatures such as 25, 30, 35, 40 and 45ºC tried, the maximum enzyme production was observed at 30ºC (41.60 U/g/min).

Estimation of tannin

The tannin content in the fermented substrate was degraded by the enzyme tannase, which was produced by the fungi. In the present study, the tannin content was initially high in level. (Table 2) afterwards it was reduced. Generally tannins are toxic as well as bacteriostatic compounds and have non-reversible reaction to protein[22] (Scalbert, 1991). The enzyme degrades the tannic acid into gallic acid and glucose, which are ultimately utilized by the organism for growth[23] (Lekha & Lonsane, 1997).

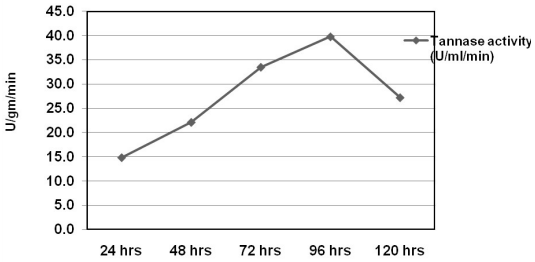

Fig. 1. Effect incubation period on tannase production

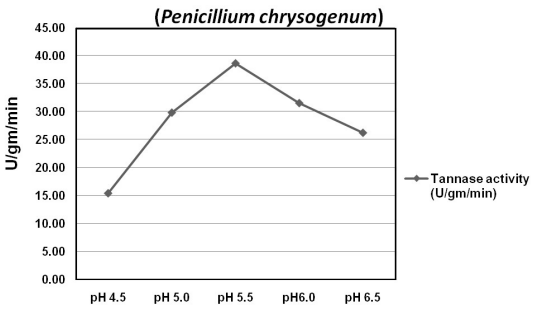

Fig. 2. Effect pH of moisturizing agent on tannase production

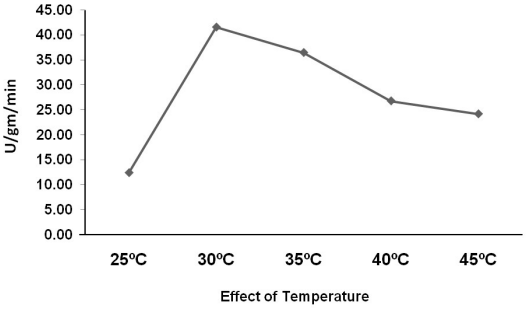

Fig. 3. Effect of incubation temperature tannase production

Table 2. Determination of tannin content in fermented biomass

S. No.	Sample	Tannin content (%)
1.	Uninoculated	10.0
2.	Fermented paddy straw with *P. chrysogenum*	6.2

HPLC analysis of Gallic acid

The gradient elution of solvent A [water-acetic acid (25:1 v/v)] and solvent B (methanol) had a significant effect on the resolution of compounds. As a result, solvent gradients were formed, using dual pumping system, by varying the proportion of solvent A [water-acetic acid (25:1, v/v)] to solvent B (methanol). Solvent B was increased to 50% in 4 min and subsequently increased to 80% in 10 min at a flow rate of 1.0 mL/min. Detection wavelength was 280 nm.

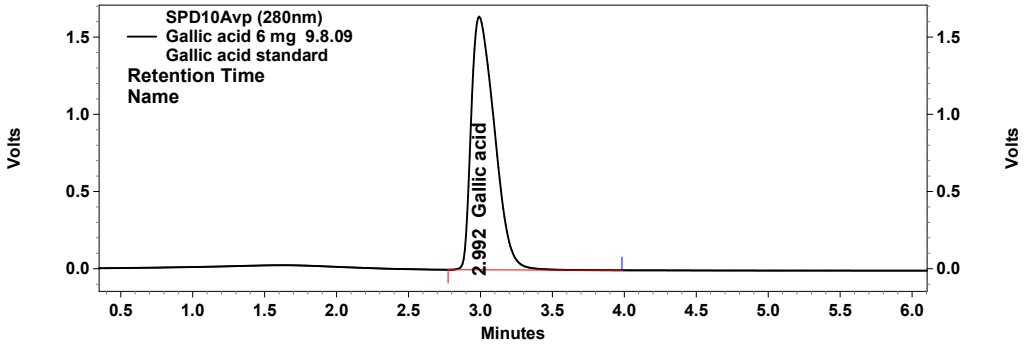

Fig. 4. Chromatographic profile using gradient elution. The peaks correspond to five reference standard gallic acid (*tR* = 2.992) min measured at 280 nm

Table 3. SPD10Avp (280nm)

Pk #	Retention Time	Area	Height	ESTD concentration	Units
1	2.992	18441272	1639767	6.000	mg

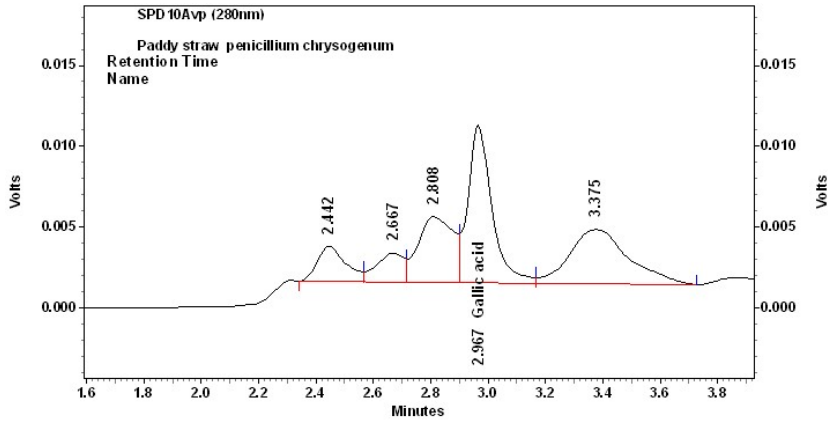

Fig. 5. HPLC chromatograms of Fermented Paddy straw Peaks: 1 = gallic acid (*tR* = 2.967) min)

Table 4. SPD10Avp (280nm)

Pk #	Retention Time	Area	Height	Concentration mg/gm (wet basis)
1	2.967	56231	9756	0.18

Sample analysis and recovery

From the fig. 5 we have found that the ethanol extracts of fermented paddy straw contained 0.18mg of gallic acid compared with standard chromatogram of gallic acid.

Determination of Molecular structure by Gas chromatography –Mass Spectrphotometer (GS)

In the GC-MS analysis, 17 compounds were identified in the extract of *Pennicillium chrysogenum* fermented paddy straw .The identification of compounds is based on the peak area, molecular weight and molecular formula. Glucose with RT 2.68 has peak area 0.44% , Gallic acid with RT (6.55) respectively given in Table 5. The Fig. 6 shows the GC-MS spectrum of gallic acid produced by *Pennicillium chrysogenum*. Fermented paddy straw and Identified molecular structure of gallic acid are given in Fig. 7.

Table 5. Phyto-constituents identified in the Fermented Paddy Straw – *Penicillium chrysogenum* [GC MS study]

No	RT	Name of the compound	Molecular Formula	MW	Peak Area %
1	2.68	L-Glucose	$C_6H_{12}O_6$	180	0.44
2	5.75	Acetic acid, 2-ethylhexyl ester	$C_{10}H_{20}O_2$	172	0.04
3	6.55	Benzoic acid, 3,4,5-trihydroxy- (Synonym: Gallic acid)	$C_7H_6O_5$	170	0.25
4	9.21	Tetradecane	$C_{14}H_{30}$	198	0.10
5	11.65	Hexadecane	$C_{16}H_{34}$	226	0.12
6	12.71	Dodecyl acrylate	$C_{15}H_{28}O_2$	240	0.15
7	13.42	Azulene, 1,4-dimethyl-7-(1-methylethyl)-	$C_{15}H_{18}$	198	0.30
8	13.80	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.25
9	14.29	Octadecane	$C_{18}H_{38}$	254	0.12
10	15.14	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278	7.51
11	16.67	n-Hexadecanoic acid (Synonym:Palmitic acid)	$C_{16}H_{32}O_2$	256	4.49
12	16.97	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	0.32
13	19.40	Oleic Acid	$C_{18}H_{34}O_2$	282	11.51
14	23.07	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	370	1.20
15	24.72	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy- (Synonyms: Quercetin)	$C_{15}H_{10}O_7$	302	1.19
16	25.43	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	66.50
17	29.72	Squalene	$C_{30}H_{50}$	410	5.51

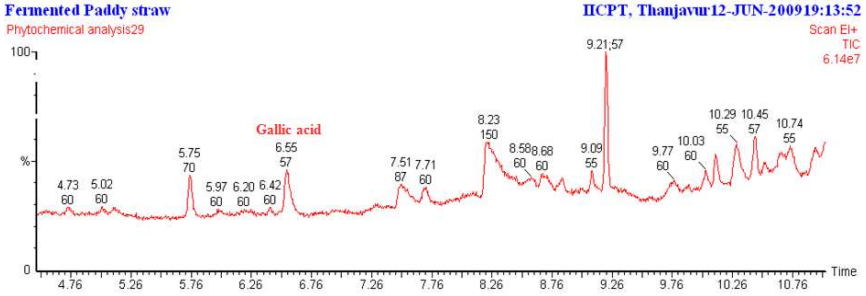

Fig. 6. GC-MS spectrum of gallic acid produced by *Pennicillium chrysogenum*

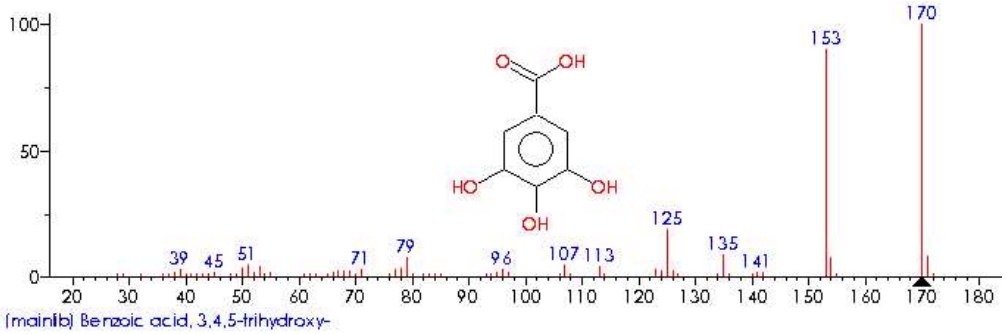

Fig. 7. Fermented paddy straw and Identified molecular structure of gallic acid.

Name: Benzoic acid, 3,4,5-trihydroxy-
Formula: $C_7H_6O_5$

MW: 170 CAS#: 149-91-7 NIST#: 76442 ID#: 98269 DB: mainlib
Other DBs: None
Contributor: RADIAN CORP
10 largest peaks:

170 999 |	153 898 |	125 183 |	135	87 |	171
77 |					
154	73 |	79	73 |	107	45 |
51	41 |	113	39 |		

Synonyms:
1.Gallic acid
2.3,4,5-Trihydroxybenzoic acid
3.Kyselina gallova
4.Kyselina 3,4,5-trihydroxybenzoova

CONCLUSION

Solid state fermentation was suitable for tannase production using agricultural byproducts. In this study Paddy straw was used as a substrate for Tannase production. From this study it is concluded that *Pennicillium chrysogenum* is found to be the most suitable for bioconversion of paddy straw to obtain increased quantities of tannase. HPLC analysis of Gallic acid obtained from fermented paddy straw contained high amount of gallic acid compared with other compounds. Gallic acid is used in the manufacture of trimethoprim (TMP), an antibacterial agent, in leather industry, and as an antioxidant.

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