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Biosynthesis of Tannase and Simultaneous Determination of Phenolic Compounds in *Aspergillus niger* Fermented Paddy Straw by HPLC

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Abstract: The production of tannin acyl hydrolase by *Aspergillus niger* in solid state fermentation using paddy straw as a substrate was investigated. Maximum tannase production of 43 U/g/min was observed in 96 h of incubation at 30°C. Purification of Tannase by ammonium sulphate and Column chromatography which increase the enzyme activity. A phenolic compound in the fermented paddy straw was analyzed by HPLC. The contents in the fermented substrate were gallic acid 0. 434 mg/g, Rutin 0. 124/g and Quercetin 0. 202 mg/g of wet mass.

Key words: Paddy straw Tannase · Gallic acid · HPLC · Phenolic compounds

INTRODUCTION

A wide variety of biomass resources are available on our planet for conversion into bioproducts. These may include whole plants, plant parts (e. g. seeds, stalks), plant constituents (e.g. starch, lipids, protein and fibre), processing byproducts (distiller's grains, corn solubles), materials of marine origin and animal byproducts, municipal and industrial wastes [1] and also agricultural wastes (These resources can be used to create new biomaterials and this will require an intimate understanding of the composition of the raw material whether it is whole plant or constituents, so that the desired functional elements can be obtained for bioproduct production [2] Several naturally occurring agricultural byproducts were used for enzyme preparation in solid state fermentation such as wheat bran, coconut oil cake, groundnut oil cake, rice bran, wheat and paddy straw, sugar beet pulp, cobs, saw dust, maize bran, rice powder husk, soy hull, sago hampas, grape waste,coconut coir pith, banana waste, tea waste, cassava waste, aspen pulp, sweet sorghum pulp, apple pomace, peanut meal etc.

Tannase is an important enzyme. Tannase is utilized in a number of industrial applications including manufacture of instant tea, wine and gallic acid [3] and solubilization of tea cream in instant tea processing [4]. One of the major commercial applications of tannase is the hydrolysis of tannic acid to gallic acid, a key intermediate required for the synthesis of an antibiotic drug, trimethoprim [5]. The production of enzyme by using agricultural wastes in solid substrate fermentation (SSF) is cheaper, less technology oriented and also the enzyme extraction is easier with the release of negligible amount of liquid effluent and thereby produces less pollution as compared to other methods.

Gallic acid is found in almost all plants. Plants known for their high gallic acid content include gallnuts, grapes, tea, hops and oak bark. (1(Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic compound and finds application in various fields. The most important use is for manufacturing trimethoprim (TMP), an antibacterial agent used in combination with sulfonamide [5]. It is also used in the leather industry, in manufacturing gallic acid esters, eg, propyl gallate which is used as an antioxidant, in the manufacture of pyrogallol. Pyrogallol is used in staining fur, leather and hair and also as a photographic developer [6] Gallic acid production has been reported from myrabolan, tara [7] sumac [8] and Chinese tannins. Some tannin-rich sources and several microorganisms [9]: Kar, *et al*. [10] Mukherjee, Banerjee, [11] Belmares-Cerda, [12] have been used for gallic acid production and the hydrolytic enzyme responsible for its production is the tannase or tannin acylhydrolase.

In this study Paddy straw was used as a substrate for the production of Tannase by *Aspergillus niger* and determined the gallic acid and other phenolic compounds in fermented substrate by using HPLC.

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MATERIALS AND METHODS

Culture Used and Inoculum Preparation: The fungus *Aspergillus niger* used in this study was isolated from soil and maintained on Potato dextrose agar slants and subcultured for every 15 days. The inoculum was prepared by adding sterile distilled water to the culture slants and disperse the spores by using sterile loop and inoculated in to the medium.

Substrate and Fermentation Medium: The substrate used in this study was Paddy straw, which is agricultural by-product. The substrate was mixed with salt solution. The composition of the salt solution was NH_4NO_3 0. 5 %, NaCl 0. 1 %, $MgSO_4 \cdot 7H_2O$ 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 inoculum, mixed properly and incubated at 30°C for 120 h.

Extraction and Purification: After 96 h of incubation 0. 05 M citrate buffer, (pH 5.0) was added to the fermented substrate and homogenized with Mortar and Pestle. Crude enzyme was separated from the fermented matter by centrifugation at 8000 rpm at 4°C for 20 min. The crude extract was precipitated with solid Ammonium sulphate (80%) and collected the precipitate by centrifugation at 8000 rpm at 4°C for 20 min. The precipitate was dialyzed against citrate buffer (0. 05 M, pH=5) at 4°C. The dialyzed sample was subjected to column Chromatography (DEAE Sephadex A-50 chromatography) and collected the fractions. The tannase activity was estimated in each purification steps.

Tannase Assay: Tannase was assayed following Sharma *et al*. [13] method using gallic acid as standard. The pink color developed was read at 520 nm using a spectrophotometer (Shimadzu UV-160A). One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under defined reaction conditions. Enzyme yield was expressed as units/gram substrate (U/gms/min).

$$\Delta A520 = (\text{Atest-Ablank})-(\text{Acontrol-Ablank})$$

Determination of Phenolic Compounds by HPLC

Standard Preparation: Standard stock solutions of three phenolic compounds were prepared in methanol, at concentrations of 0.420, 0.434, 0.400, 0.402 and 0.402 mg. mL^{-1} for GA, RU and QU respectively. All standard solutions were filtered through 0. 45 mm membrane filter (Millipore) and injected by autosampler.

Sample Preparation: The sample was prepared according to the procedure of El Sohafy *et al*. [14]. The fermented paddy straw (0.5 g) was extracted by boiling for 5 min with 5 mL water, adjusting the volume to 5 mL and filtering. 5 mL of this filtrate was hydrolyzed by adding 0. 5 ml of 25% HCl and heating it in a boiling water bath for 25 min. The mixture was then extracted with four successive 4 ml portions of n-butanol. The combined n-butanol extracts were dried under reduced pressure and redissolved in 2 ml methanol.

HPLC Conditions: Flavonoids were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto,Japan) consisting of a LC-10AD pump, SCL 10A system controller and SPD-M 10A photodiode array detector. Chromatography of the phenolic acids was achieved using a prepacked LiChrospher 100 RP C-18 olumn (4´250 mm, 5 µm; Merck). The mobile phase comprised water-acetonitrile-acetic acid (88:10:2; v/v/v) [15] and was delivered at a rate of 1 mL/min. Detection was monitored at 280 nm. All results of this work are an average of two independent determinations.

The three phenolic compounds, GA, RU and QU, are polar molecules. 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.

RESULTS AND DISCUSSION

The production of tannase by *A. niger* on paddy straw was studied and the result was shown in Table 1. The maximum production of tannase was observed in 96 h of incubation at 30°C. Nisha K. Rana [16] reported, that the total tannase yield was maximum at 120 h for SmF and LSF, whereas it was at 96 h of growth for SSF process. Previously maximum extracellular tannase and gallic acid production was recorded in 96 h and 120 h by *A. niger* and *Rhizopus oryzae* [17-19]. The enzyme production started after 48 h of incubation and increased

Table 1: Production of Tannase in various incubation period

Paddy straw	Tannase activity U/g/min				
Incubation period	24 h	48 h	72 h	96 h	120 h
Tannase production	22	29	34	47	42

Table 2: Validation data from calibration curves of standard phenolic compounds

SPD10Avp (280nm)					
Retention Time	Area	Height	ESTD concentration	Units	Name
3. 325	1451317	239378	5. 0	ug/ul	Gallic Acid
5. 100	2945595	386194	5. 0	ug/ul	Rutin
5. 867	18720032	2030782	5. 0	ug/ul	Quercetin

Table 3: Validation data of phenolic compounds of Paddy Straw

SPD10Avp (280nm)					
Retention Time	Area	Height	ESTD concentration	Units	Name
3. 567	140352	16676	0. 217	ug/ul	Gallic Acid
4. 983	16332	3255	0. 062	ug/ul	Rutin
5. 867	188800	14825	0. 101	ug/ul	Querectin

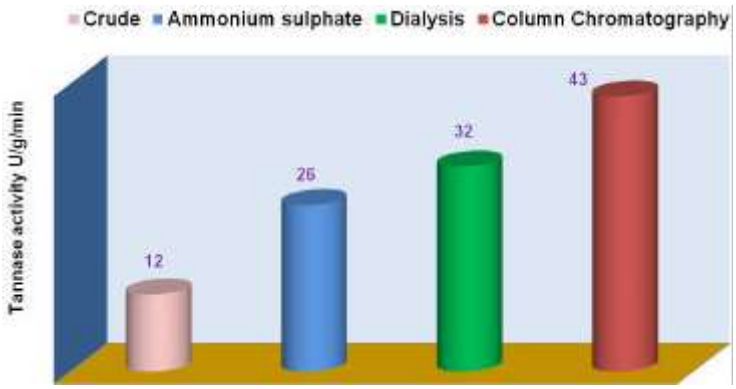

Fig. 1: Tannase production in solid state fermentation1

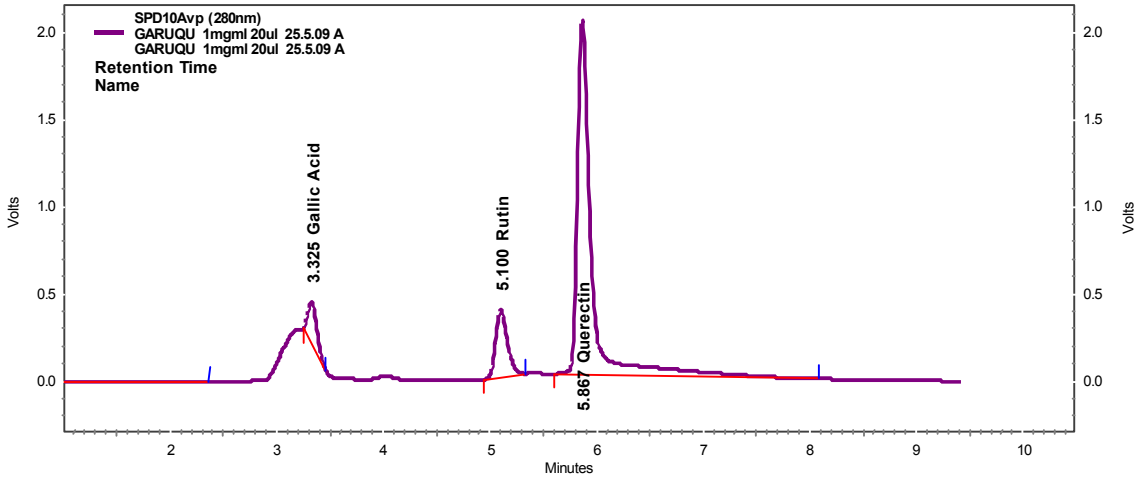

Fig. 2: Chromatographic profile using gradient elution. The peaks correspond to three reference standard phenolic compounds: 1 = gallic acid (*tR* = 3. 325 min); Rutin (*tR* = 5. 100 min); and 5 = quercetin (*tR* = 5. 867 min) measured at 280 nm

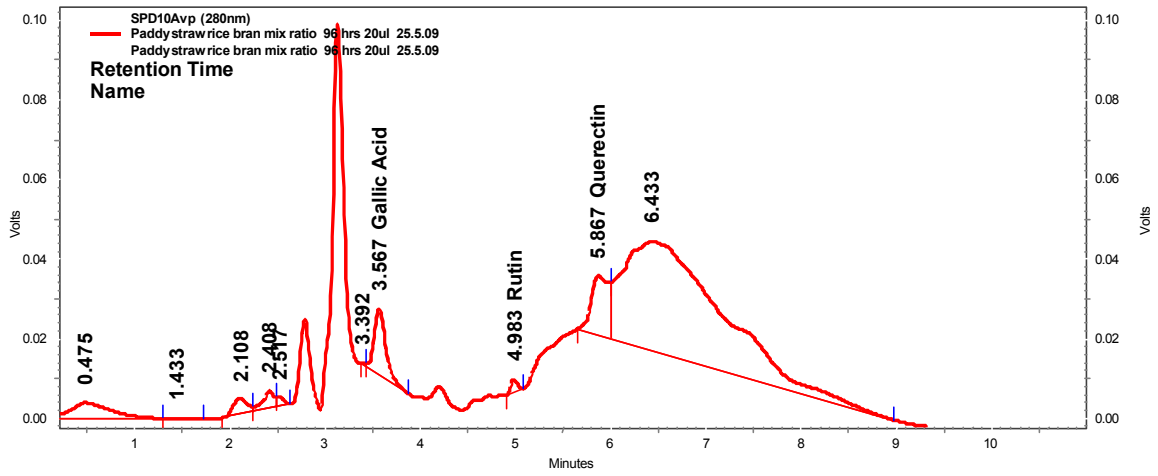

Fig. 3: HPLC chromatograms of Paddy Straw Peaks: 1 = gallic acid (*tR* = 3. 567 min);Rutin *tR* = 4. 983 and quercetin (*tR* = 5. 867 min)

with time reaching a maximum at 96 h. This might be the fungi entered in to its exponential phase. Thereafter, the enzyme production started decreasing.

Purification of Tannase: The crude tannase was precipitated by ammonium sulphate precipitation the activity was 26 U/g/min. After dialysis the specific activity activity of 32 U/g/min was obtained. The sample was further purifiedthrough DEAE-Sephadex A-50 chromatography and the eluted fractions, which showed 43 U/g/min. (Fig. 1).

Determination of Gallic Acid,rutin and Quercetin in Fermented Paddy Straw by HPLC:

Standard Chromatogram of Phenolic compounds

It can be seen from Figure 2 that a good separation can be achieved within 15 min using the condition described. Symmetrical, sharp and well-resolved peaks were observed for standards (GA, RU and QU). The elution order and the retention times for GA, RU and QU were 3. 325, 5. 100 and 5. 867 min respectively.

In this study Paddy straw was used as a substrate for Tannase production by using *Aspergillus niger*. Quantification of samples was compared with standards. The content of phenolic acids detected in the analysed samples is shown in Fig 3. Results are expressed as mg/g of fermented substrate. The experimental results indicated that Paddy straw fermented with *Aspergillus niger* extract contained gallic acid (0.43mg. /g of wet weight), Rutin (0.124 mg/ of wet weight) and Quercetin (0.202 mg/g wet weight). High yields of gallic acid recovery associated with high tannase activities were reported by Kar and Banerjee and Kar *et al.* during the fermentation of a tannin-rich forest residue (gilo seeds, *Caesalpinia digyna)* or powdered *Terminalia chebula* fruits using the fungal strains *Rhizopus oryzae* and *Aspergillus foetidus* (*G.* Mukherjee, R. Banerjee, 2006 and 2004).

CONCLUSION

Solid state fermentation was suitable for tannase production using agricultural by-products. In this study Paddy straw was used as a substrate for Tannase production. HPLC analysis of phenolic compounds 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. Several fungal strains and tannin-rich substrates have been used for the production of gallic acid.

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