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Comparative study on the suitability of different substrates for Tannin Acyl Hydrolase production using *Aspergillus oryzae*

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Abstract:
An extracellular tannase produced by the fungus *Aspergillus oryzae* in solid state fermentation using various agricultural by-products. Among different substrates used the maximum activity of 14.40 U/g/min was found in rice bran. Other substrates produced in low level. The activity was increased when purified by ammonium sulphate (60-80%), dialysis and DEAE Sephadex A-50 column chromatography and the yield was (62.57 U/g/min). The optimal conditions for culture fermentation were 30º C for 96 hrs at pH 5.5 Carbon and nitrogen sources were found to enhance tannase production when added as culture supplementations.
Key words: Solid state fermentation, tannase, Bioprocessing, *Aspergillus oryzae*

Introduction

Tannin acyl hydrolase commonly known as tannase, catalyses the hydrolysis of the ester and depside bonds in hydrolysable tannins such as tannic acid to release glucose and Gallic acid. [1]. Tannase (tannin acyl hydrolase) hydrolyses the ester bond between sugar and gallic acid. Tannase is known to be a membrane bound enzyme [2] and also secreted outside the cell [3]. The production of this enzyme has been carried out in semi liquid, high solids conditions. Microbial attachment to the solids, whether inert or degradable, and the low water conditions make SSF rather different from the more common submerged fermentation. Applications of SSF to other than purely profit-driven objectives, such as environmental control, include the production of compost and animal feed from solid waste [4].

Bacteria, yeast, filamentous fungi are known as tannase producers [5, 6] SSF mainly deals with the utilization of agro industrial residues as its substrates. Application of agro industrial residues as substrates is certainly economical and it also reduces environmental pollution. Several naturally occurring agricultural byproducts such as wheat bran, coconut oil cake, groundnut oil cake, rice bran, wheat and paddy straw, sugar beet pulp, fruit pulps and peels, corn cobs, saw dust, maize bran, rice husk, soy hull, sago hampas, grape marc, coconut coir pith, banana waste, tea waste, cassava waste, aspen pulp, sweet sorghum pulp, apple pomace, peanut meal, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch etc. could be used in one or the other industrial bioprocess for the production of value added products through Solid state fermentation.

Tannase has many potential applications in the food, pharmaceuticals and chemical industries but due to the shortage and high cost of the enzyme, the use of tannase in large-scale applications is limited at present. It is hoped therefore that the economic benefits of tannase production can help improve the economic benefits of tannase production can help improve the overall viability of the process.

In the present study we describe the screening of *Aspergillus oryzae*, standardize the culture conditions for the production of Tannase enzyme by using different substrates and also describe the immobilization of tannase enzyme.

Materials and Methods

Substrate: The substrate used in this study were Paddy husk, Rice bran, Millet husk and Groundnut shell, collected from Indian Institute of Crop Processing Technology, Thanjavur.

Preparation of spore inoculums: A strain of *Aspergillus oryzae* MTCC used in this study was maintained on Potato dextrose agar slants and subcultured for every month. Fungal spore inoculum was prepared by adding 2.5ml of sterile distilled water containing 0.1 % tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions. The

volume of 1 ml of the prepared spore suspension was used as inoculums at concentration of $34x10^8$ spores.

Screening of Tannin acyl Hydrolase by Plate Assay Method: Screening was performed in plates of selected medium which contains (g/L): tannic acid, 10.0, $NaN0_3$, 3.0; KH_2P0_4, 1.0; $MgS0_4$. $7H_20$, 0.5; KCI 0.5; $FeS0_4$. $7H_2O$, 0.01; agar, 30.0, pH 4.5. The fungal culture was inoculated on the center of the plates and incubated at 32ºC for 72 hours. The diameters of the clear zone were measured in 24, 48&72 hours of incubation.

Production of tannase under Solid State Fermentation: A five gram substrate of paddy husk, Rice bran and Millet husk and Ground nut shell was taken separately 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 %, $MgSO_4 \cdot 7H_2O$ 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 inoculums, mixed properly and incubated at 30 °C for 96 h.

Extraction and analysis of crude enzyme: Tannase was extracted from the fermented substrate. To the fermented substrate 0.05 M citrate buffer pH 5.0, was added and frozen overnight. Acid washed sand, four times the weight of the mycelium, was added and the mixture was ground in a pestle–mortar kept in an ice bath. Crude enzyme was separated from the fermented biomass by centrifugation at 8000 rpm at 4 °C for 20 min.

Purification and characterization

Ammonium Sulphate Preciptation: The addition of solid ammonium sulphate (40-80%) to the crude extract was done under constant stirring at 4 °C for 30 min and then stirring was continued for another 30 min, and then allowed for settlement for 3 h at 4 °C. The precipitated proteins were separated by centrifugation at 8000 rpm at 4 °C for 20 min. The separated proteins were then dissolved in minimum amount of 0.05 M citrate buffer (pH=5) and refrigerated for further analysis**.**

Dialysis: Precipitated proteins were transferred into a dialysis tube using a micropipette and dialyzed against citrate buffer (0.05 M, pH=5) at 4 °C. The buffer was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis was conducted over night and the buffer was changed several times to increase the efficiency of the dialysis.

Purification by Colum Chromatography: A glass column was packed with DEAE Sephadex A-50 and was equilibrated with 0.05 M citrate buffer (pH 5.0). One ml of the dialyzed sample was applied on the column and the elution was done using 0.05 M citrate buffer (pH 5.0). The fractions were monitored and collected. The fractions corresponding to tannase activity were pooled and used for estimation.

Tannase Assay: Tannase was assayed following Sharma *et al.* [7] method using gallic acid as standard. The pink color developed was read at 520 nm using a spectrophotometer (Shimadzu UV-160A). The enzyme activity was calculated from the change in absorbance. 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 dry substrate (U/g/min).

$\Delta A520$ = (Atest – Ablank) – (Acontrol – Ablank)

Estimation of tannin: The Folin-Denis method was used for Tannin estimation [8] using Tannic acid (SIGMA) as a standard.

Optimization of Fermentation process

Effect of temperature: The Solid-state fermentation was carried out at different temperatures of 30°C,35°C,40°C and 45°C for 96 hrs and the enzyme was assayed.

Effect of pH: Solid-state fermentation was carried out using moistened salt solution with different pH ranging from 4.5, 5.0, 5.5, 6.0 and 6.5.

Effect of incubation Period: Tannase assay was performed using the sample for various incubation times i.e 1,2,3,4 and 5 days (24 hours to 120 hrs) and the tannase activity was determined.

Effect of Nutrient Supplement

***Effect of nitrogen supplements*:** The effect of nitrogen sources was studied on enzyme production. Ammonium nitrate and Ammonium chloride (NH4NO3, NH_4Cl) were added to fermentation media at different concentrations of 0.5%, 1%, 1.5%.

Effect of Carbon Supplements: Influence of various carbon supplements on enzyme production was studied by adding different sugars (Glucose and Mannitol) at 1%, 2% and 3% (w/w) to the fermentation media.

RESULTS AND DISSCUSION

The present study was carried out to evalulate the tannase production by using various substrates. The selection of a substrate for enzyme production by fermentation depends on several factors, i.e. cost, availability and suitability of the substrate for obtaining the desired product of fermentation and thus requires screening of several agro industrial residues.

Screening for tannase production on solid media: After 72 hrs of incubation the fungus *A.oryzae* produced clear zone around the colony on the surface of the medium. Bradoo *et al*.,[9] reported formation of a clear zone around the mycelium, suggesting tannase activity. However, the observation of this clear zone was very difficult. After 48 hours of incubation, *Aspergillus niger, A. awamori and A. japonicus,* presented diameters between 14 and 16 mm [9]. Yamada *et al*.,[10] tested eighty strains of filamentous fungi for tannase production, and selected two colonies, identified as *Aspergillus oryzae,* which presented diameters of 20-22 mm after 72 hours. Direct measurement of the colony diameter was a good indicator of the ability of tannic acid utilization as a carbon source due to the tannase activity in the medium.

Tannase production by various substrates: In the present study Rice bran fermented with *A.oryzae* had a highest activity of 14.40 U/g/min in crude form. The crude tannase when precipitated using Ammonium sulphate 60- 80% saturation showed 39.3 U/g/min. After dialysis the enzyme activity which was enhanced when compared to the crude enzyme. The dialyzed enzyme was further purified through DEAE-Sephadex A-50 and the eluted fractions showed 62.57 U/g/min. (Table-1). Lower activity of 40.85 U/g/min was found in Paddy husk. SSF offers a number of advantages over conventional submerged fermentation for enzyme production [11]. Mitchell and Lonsane, [12] reported that the Production of enzyme is often simple, when agro-industrial by-products like wheat bran, rice bran or wheat straw are used as substrate, weight of substrate is low. Hence, enzyme activity is usually very high [13].

Estimation of Tannin: The tannin content of the fermented samples is given in Table-2. The tannin content was high in ground nut shell when compared to other substrates.

Standardizations of fermentation conditions:

Effect of incubation period: SSF was carried using agro- residues to optimize the time- course of incubation. In our studies the maximum enzyme production was found in Rice bran which yields 61.43 U/g/min at 96 h of incubation (4th day) followed by Ground nut shell, Paddy husk and Millet husk produced 44.40 U/g/min, 41.4 U/g/min and 37.41 U/g/min respectively.(Fig-1). Lekha and Lonsane [14] and Sabu *et al.* [15] reported maximum extra-cellular tannase production by *A. niger* in 96 h as reported by us also whereas Chatterjee *et al*. [16] reported maximum extra-cellular production obtained in 120 h by *Rhizopus oryzae* also Banerjee *et al*. [17] found

Table: 1 Tannase activity on various fermented substrates:

S.No	Tannase activity (U/g/min)				
	Substrate	Crude	Ammonium sulphate	Dialysis	Column Chromatography
1	Paddy Husk	4.14	15.2	26.89	40.85
2	Rice Bran	14.40	39.3	44.60	62.57
3	Millet Husk	7.41	22.5	26.34	49.68
4	Groundnut Shell	11.43	31.35	38.45	56.47

Table 2: Proximal analysis of substrate

Sample	Tannin (mg/g)	Sugar (mg/g)
Paddy Husk	0.018	5.08
Rice Bran	0.096	11.88
Millet Husk	0.028	9.65
Groundnut Shell	0.172	10.96

Table-3 Effect of different nitrogen supplements on Tannase activity

Substrate	Tannase activity (Unit/ml)					
	NH_4NO_3			NH_4Cl		
	0.5%	1%	1.5%	0.5%	1%	1.5%
Paddy Husk	2.12	5.46	6.76	0.94	1.22	2.19
Rice Bran	4.77	9.22	26.38	6.64	12.28	13.56
Millet Husk	6.64	10.15	13.78	0.35	2.70	7.12
Groundnut shell	4.93	9.20	18.44	3.57	7.40	9.48

Table-4 Effect of different carbon supplements on Tannase activity

Substrate	Tannase activity (Unit/ml)					
	GLUCOSE			MANNITOL		
	1%	2%	3%	1%	2%	3%
Paddy Husk	3.12	5.46	6.76	0.94	1.22	2.19
Rice Bran	8.93	9.20	18.44	6.64	12.28	13.56
Millet Husk	9.64	10.15	11.78	0.35	2.70	7.12
Groundnut shell	6.77	7.22	14.38	3.57	7.40	9.48

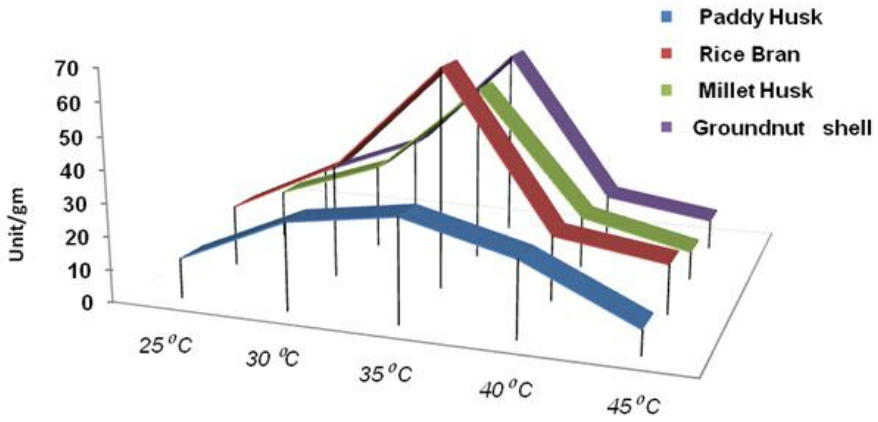

Figure: 2 Effect of Temperature for the production of Tannase by using various substrates

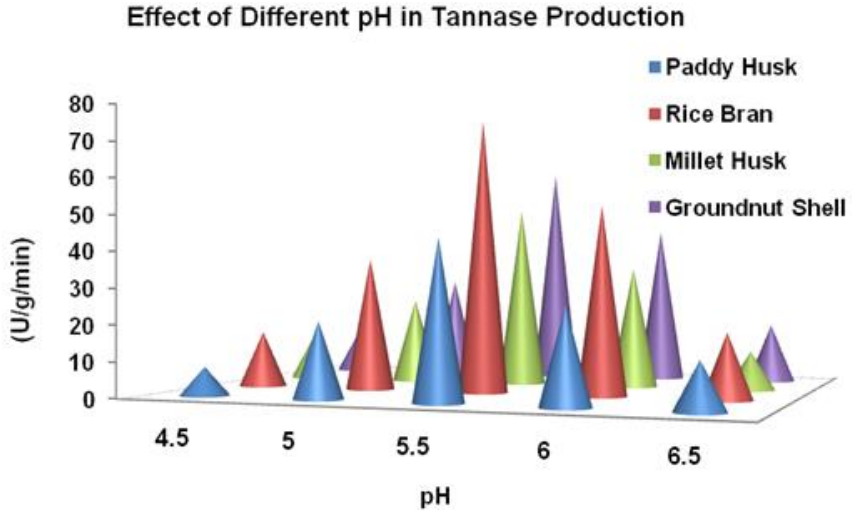

Figure. 3 Effect of Different pH onTannase Production by using various substrates

maximum production of extracellular tannase by *Aspergillus aculaetus* after 72h.

Effect of temperature: SSF was carried out at different temperatures ranging from 30- 45°C.. The organism exhibited its best performance for enzyme production in the mesophilic range; it yielded a maximum production of (67.91 U/g/min) in Rice bran at 35ºC. This was followed by Ground nut shell (60.11 U/g/min) and Millet husk (55.03 U/g/min). The lowest enzyme production of (31.78 U/g/min) (Fig-2) was found in Paddy husk. The significance of temperature in the development of a biological process is such that it could determine the effects of protein denaturation, enzyme inhibition, promotion or suppression of the production of a particular metabolite, cell viability and death.

Effect of pH: The suitable pH for the production of tannase was found to be 5.5. In the present study maximum production of (76.70 U/g/min) (Fig-3) was recorded by Rice bran.

***Effect of nutrient supplements*:**

Effect of nitrogen supplements: The effect of nitrogen sources on tannase production at different concentrations were shown in (Table-3). A number of workers studied fungal tannase production in the presence of different nitrogen source such as sodium nitrate, ammonium oxalate, ammonium sulphate and ammonium chloride [10,14,18 and 19].In our studies we found the maximum activity in all substrates was observed when used ammonium nitrate and ammonium chloride(1.5%) as a nitrogen source. The presence of additional nitrogen sources along with nitrogenous compounds present in the substrate promotes enhanced growth and consequent enzyme production [20].

Effect of carbon sources: The SSF production medium was supplemented with glucose and mannitol at different concentrations. In our studies maximum enzyme production occured at 3% (Table-4). Aguilar (2001) reported the role of carbon sources on the extracellular secretion of tannase is contradictory. Seiji *et al*. [21] mentioned that tannase activity is expressed only when the organism is grown in the presence of glucose. Van de Lagemaat and Pyle [22] reported that the glucose if present in the media will be exhausted rapidly and this may lead to the partial induction of tannase.

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