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# Optimisation of Fermentation Conditions for Production of Tannase Enzyme by *Aspergillus oryzae* Using Sugarcane Baggasse and Rice Straw

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**Abstract:** The production of enzymes by bioprocesses is a good alternative to add value to agro industry residues. Tannaase enzyme production was attempted using *Aspergillus* oryzae from Sugarcane baggasse and Rice straw under Solid state fermentation. The condition requires for Maximum enzyme production optimized. The maximum crude enzyme activity of 60.5 U/gm/min was offered in sugarcane baggasse and rice straw substrate used in 1:1 ratio. The Optimum fermentation conditions like pH, temperature and a Incubation period for Tannase production were found to be 5.5, 35°C at 72 h, respectively. Purification by 60% ammonium sulphate treatment was found to be suitable for maximum Tannase activity. The present study showed that the fungal strain *Aspergillus oryzae* has high potential for industrial productions of tannase.

**Key words:** Tannase · Fermentation · Purification · Rice straw powder · Sugarcane Baggase

## INTRODUCTION

Solid-state fermentation (SSF) involves the 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 [1,2]. Agro-industrial residues are generally considered the best substrates for the process of enzyme production, [3]. Compared with submerged fermentation, the use of SSF presents advantages such as lower power requirements, smaller reactor volume and high productivity [4], low capital investment, low waste water output, higher concentration of metabolites obtained and low downstream processing cost [5]. The major crop residues produced in India are straws of paddy, wheat, millet, sorghum, pulses, oilseed crops, maize stalks and cobs, cotton stalks, jute sticks, sugar cane trash, mustard stalks, etc. The agro-industrial residues are groundnut shells, rice husk, bagasse, cotton waste, coconut shell and coir pith used enzyme production.

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 and starch. could be used in one or the other industrial bioprocess for the production of value added products through SSF [6]. Several agro-industrial waste and by-products such as orange bagasse [7], sugar cane bagasse [8] wheat bran [9] and other food processing waste [10] are effective substrates for depolymerizing enzyme production by solid-state fermentation. which proved to be highly efficient technique in the production of tannase.

Tannin acyl hydrolase commonly called tannase is produced by a number of microorganisms like fungi - *Aspergillus, Penicillium, Rhizopus* sp, yeast - *Candida* sp and bacteria - *Bacillus* sp [11,12]. 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 [13]. 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 [14]. Purification and evaluation of the enzyme require a sensitive, reproducible and convenient assay method.

This study was taken up with the objective of Bioconversion of sugarcane bagasse and rice straw powder for tannase production and Optimization of fermentation process in condition requires for maximum for Tannase production.

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## MATERIALS AND METHODS

**Chemicals:** Tannase, Rhodanine, gallic acid and bovine serum albumin were purchased from Sigma Chemical, India.Tannic acid (analytical grade), Dialysis tubing (12±14 kDa cut off, pore size 2.4 nm) and DEAE-Sephadex A-50 was obtained from HiMedia Laboratories, Mumbai, India. Folin-Cio- calteu reagent was purchased from Sisco Research Laboratory, Mumbai, India. All other chemicals were of analytical grade.

**Preparation of Spore Inoculums:** A strain of *Aspergillus oryzae* MTCC, was used for the study. Potato dextrose agar slants were used for the maintenance of *A. oryzae*. 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 and the number of viable spores in the suspension was determined using the plate count method. The volume of 1 mL of the prepared spore suspension was used as the inoculum, with concentration of $36x10^9$ spores.

**Substrates:** Natural lignocelluloses (agro-industrial wastes), namely sugarcane bagasse and rice straw were procured locally, air dried, pulverized to 40-mesh size and utilized as substrates enzyme production under Solid-state fermentation.

**Production of Tannase Under SSF:** A five g mixed substrate of rice straw powder +sugarcane baggase powder (1:1 ratio) was taken in 250-mL Erlenmeyer flask and moistened with 5 mL of salt solution. The composition of the salt solution was NH4NO3 0.5 %, NaCl 0.1 %, MgSO4 •7H2O 0.1 % and Gallic acid 4% at pH=5.5. The contents were sterilized by autoclaving at 121°C, 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. A 10% mycelial suspension mass was prepared in 0.05 M citrate buffer, pH 5.0 and frozen overnight. Acid washed sand, four times the weight of the mycelium, was added and the mixture was ground in a chilled pestle–mortar kept in an ice bath. Crude enzyme was separated from the fermented matter by centrifugation at 8000 rpm at 4°C for 20 min. The filtrate was collected in bottles and preserved for further studies. The supernatant (mycelia extract) was used for tannase assay.

**Purification and Characterization:** A volume each of 100 mL of crude tannase was taken, added slowly the required quantity of ammonium sulphate obtain various saturation levels (0–40, 40–60 and 60–80 %). The addition of ammonium sulphate 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. 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.

**DEAE Sephadex A-50 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.* [15] 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/gds)/min..

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

**Determination of Soluble Protein in Fungal Biomass:** Protein was estimated following the method of Lowry [16] using bovine serum albumin as a standard

**Optimization of Fermentation Process**

**Effect of Incubation Temperature:** The Solid-state fermentation was carried out at different temperatures such as 25, 30, 35 40 and 45°C for 72 h and the enzyme was assayed.

**Effect of pH:** Solid-state fermentation was carried out using moistened salt solution with different pH viz., 4.5, 5.0, 5.5, 6.0 and 6.5. The flasks were incubated at 35°C for 72 h and the enzyme production was measured as described earlier

**Effect of Incubation Period:** After inoculation, the flasks were incubated at 35°C for different time periods ranging from 24 to120 h. All the experiments were carried out in triplicate and average reproduced

## RESULTS AND DISCUSSION

**Production of Tannase under Solid-State Fermentation:** *A. oryzae* MTCC produced Tannase was produced extracellularly under solid state fermentation using Sugarcane Baggase and rice straw as substitute (Table 1 and Fig. 1). The Crude tannase observed was 60.5 (U/gm/min) which on purification showed tannase activity of 33.5 U/gm/min by 40–60% of ammonium sulphate fractionation. The tannase activity of colum purified sample was found to be 8.68 U/gm/min,. SSF offers a number of advantages over conventional submerged fermentation for enzyme production [17]. Mitchell and Lonsane, [18] repeated that the Production enzyme is often simple, when agro-industrial by-products like wheat bran, rice bran or wheat straw are used as substrate. Because the moisture level is low, the volume of medium per unit weight of substrate is low. Hence, enzyme activity is usually very high [19].

**Effect Incubation Period on Tannase Production:** The results on the optimum incubation period requires for maximum tannase production showed (Table 2 and Fig. 2) that the enzyme production started after 24 h of incubation and progressively increased with time, the maximum production of 60.5 U/g/min was observed after 72 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 [20]. 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 [21].

**Effect of Ph of Moisturizing Agent on Tannase Production:** Among the various pH tested, maximum production of enzyme (43.41 U/g/min) was observed at pH 5.5 (Table 3 and Fig. 3). The increase in pH over 6.0 drastically reduced the tannase activity Lekha and Lonsane; [22] also repeated that tannases are acidic proteins with an optimum pH around 5.5. Similarly, Sabu and coworkers [23] reported optimum pH of 5.5 for tannase production by Aspergillus niger ATCC 16620.

**Effect of Temperature on Tannase Production:** Among the different temperatures such as 25, 30, 35, 40 and 45°C tried, the maximum enzyme production was observed at 35°C (48 U/g/min). The optimum temperature for the enzyme activity was found to be 30–40°C, at which the enzyme activity was the highest (Table 4 and Fig. 4). Similar observations were reported for tannase from *A. oryzae* [14], *Aspergillus* sp [12] and *Penicillium chrysogenum* [24]. With further increase in temperature tannase activity was found to decrease.

Table 1: Tannase activity after purification steps

| S.No | Purification step | Tannase activity/(U/gm/min) |
|---|---|---|
| 1 | Crude enzyme | 60.5 |
| 2 | 40-60 % fraction | 33.5 |
| 3 | Dialysed enzyme | 14.6 |
| 4 | DEAE-Sephadex chromatography | 8.68 |

Table 2: Effect incubation period on tannase production

| Effect of Incubation | Tannase activity (U/gm/min) |
|---|---|
| 24 h | 14.8 |
| 48 h | 54.6 |
| 72 h | 60.5 |
| 96 h | 39.8 |
| 120 h | 28.3 |

Table 3: Effect of pH of moisturizing agent on tannase production

| Effect of PH | Tannase activity (U/gm/min) |
|---|---|
| pH 4.5 | 36.59 |
| pH 5.0 | 38.27 |
| pH 5.5 | 43.41 |
| pH 6.0 | 41.82 |
| pH 6.5 | 27.74 |

Table 4: Effect of temperature on tannase production

| Effect of Temperature | Tannase activity (U/gm/min) |
|---|---|
| 25°C | 24.5 |
| 30°C | 43.4 |
| 35°C | 48.6 |
| 40°C | 38.6 |
| 45°C | 26.3 |

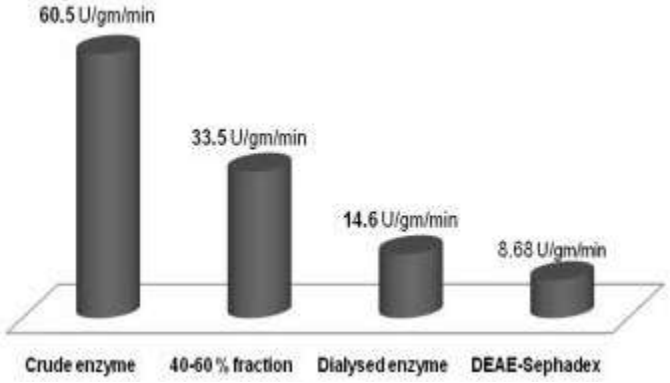


Fig. 1: Tannase activity after different purification steps

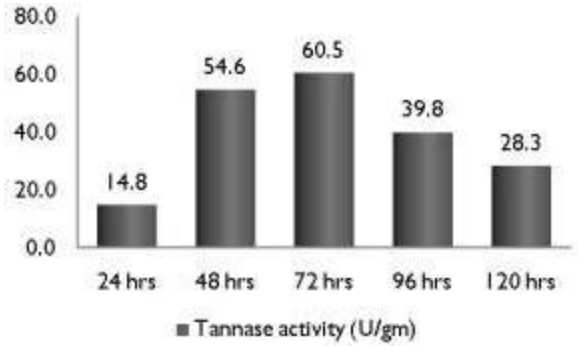


Fig. 2:

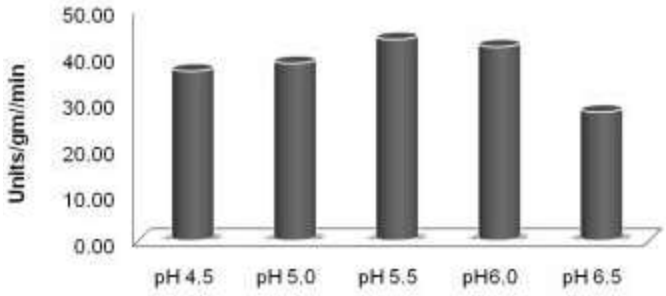


Fig. 3: Effect of pH of moisturizing agent on tannase production

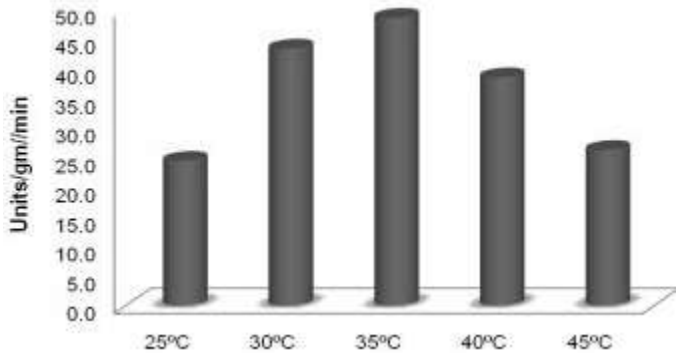


Fig. 4: Effect incubation temperature on tannase production

## CONCLUSION

The present work has been taken up with a view of exploring the possibilities of using *Rice straw and Sugarcane as* a substrate and *Aspergillus oryzae* as a microbial source for the production of tannase which can hydrolyse Tannic acid to gallic acid. This strain is able to produce tannase in the medium containing Tannic acid as the sole carbon source. Tannin acyl hydrolase is an industrially important enzyme that is mainly used in the food and pharmaceutical industry. As the range of applications of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suitable in the diverse fields of applications. Solid state fermentation technology using non pathogenic microorganisms which can produce hydrolytic enzymes such as tannase will be advantageous for the proper utilization of these residues. Since microbial activity especially fungal activity is the key aspect in this area, there is enormous opportunity for the cost effective production of tannase, which is an important enzyme in the food and pharmaceutical industry.

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