Global Journal of Biotechnology & Biochemistry 4 (1): 19-24, 2009
ISSN 2078-466X

Accelerated Bioconversion of Agricultural By-Products by Supplementation of Tannic Acid in Tannase Production by Aspergillus Oryzae

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Abstract: 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.The present work has been taken up with a view of exploring the possibilities of using Agriculture by products with Tannic acid as a source for the production of tannase and optimizing condition require to get maximum production. *Aspergillus oryzae* MTCC 1122 *was* selected and optimized for Tannase enzyme production in solid state fermentation using cheaper sources of Sugarcane Baggase and rice straw. Tannase production has been evaluated using solid-state fermentation (SSF) at different temperatures, tannic acid, glucose concentration and substrate concentration and incubation time. Addition of Tannic acid concentrations increased total activity of crude tannase (121.0U/ml/min). Optimum fermentation conditions of pH, temperature and Incubation period for Tannase production were found to be 5.5 and 30°C at 72hrs. In purification step, 60% ammonium sulphate saturation was found to be suitable giving maximum Tannase activity. Thus the present study proved that the fungal strain *Aspergillus oryzae* used is highly potential and useful for industrial productions of tannase.

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

INTRODUCTION

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 [1] and [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 like groundnut shells, rice husk, bagasse, cotton waste, coconut shell and coir pith are used for enzyme production. Several agro-industrial waste and by-products such as orange bagasse [6], sugar cane bagasse [7] wheat bran [8] and other food processing waste [9] 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 [10].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 [11]. Purification and evaluation of the enzyme require a sensitive, reproducible and convenient assay method. Tannase has found application in various domains, for example as inhibitor of foam in tea and as a clarifying agent in the production of beer and fruits juices [12-15]. Tannic acid is an important gallotannin belonging to the hydrolysable class and consists of esters of gallic acid and a polyol, usually glucose [16-18]. The strain Aspergillus niger HA37 was

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previously isolated from OMWW, a substrate containing an important amount of hydrolysable tannins acting as inducers for tannase production [19].

This study was taken up with the objective of Bioconversion of sugarcane bagasse and rice straw powder for tannase production influence of tannic acid concentration.

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 be used for the preservation of *Aspergillus oryzae*. Fungal spore inoculums were prearranged 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 number of viable spores in the suspension was determined using the plate count method. A volume of 1 mL with concentration of 36×10^9 spores was used as inoculum.

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 gram mixed substrate of rice straw powder with 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 NH_4NO3 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 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% mycelia suspension collection was prepared in 0.05 M citrate buffer, pH 5.0 and frozen during the night. 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 with the various concentration levels (0-40, 40-60 and 60-80%) of ammonium sulphate. 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 Activity Assay: Tannase was assayed following Sharma *et al.* [20] 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/gms)/min..

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

Determination of Soluble Protein in Fungal Biomass: Protein was estimated following the method of Lowry [21] using bovine serum albumin as a standard.

Optimization of Fermentation Process

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

Effect of pH: Solid-state fermentation was carried out using moistened salt solution with different pH ranging from 5.0 to 7.5. 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.

Effect of Glucose Concentrations Supplementation: The effect of different concentrations of glucose (0-02,0.04, 0.06, 0.08 and 0.1%) as additive was studied on tannase production

Effect of Substrate Concentration: The substrate was Mixed in different ratios Rice straw: Sugarcane baggasse (RS: SB) starting from 1:1,2:1, 3:1and 4:1 to find out the best ratio for enzyme production under SSF.

Effect of Tannic Acid Concentrations: Different concentrations of Tannic acid (0-02,0.04, 0.06, 0.08 and 0.1%) was added to substoreted and studied the best concentration for tannase production

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. The Crude tannase observed was 17.84 (U/ml/min) which on purification showed tannase activity of 55.5 U/ml/min by 40-60% of ammonium sulphate fractionation (Fig. 1). The maximum tannase activity of colum purified sample was found to be 121.0 U/ml/min. SSF offers a number of advantages over conventional submerged fermentation for enzyme production. 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.

Effect Incubation Period on Tannase Production: The results on the optimum incubation period requires for maximum tannase production showed in Fig. 2 that the enzyme production started after 24 hrs of incubation and progressively increased with time, the maximum production of 78.3 U/ml/min was observed after 72 hrs incubation. Thereafter, the enzyme production started decreasing. Decreased enzyme yield on prolonged incubation could also be due to inhibition and denaturation of the enzyme. 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.

Effect of Ph of Moisturizing Agent on Tannase Production: Among the various pH tested, maximum production of enzyme (60.24 U/ml/min) was observed at pH 5.5. The increase in pH over 6.0 drastically reduced the tannase activity showed in Fig. 3, Lekha and Lonsane, [22] also reparted that tannases are acidic proteins with an optimum pH around 5.5. Similarly, 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 30°C (121.0 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 show in Fig. 4. Similar observations were reported for tannase from A. oryzae [22], *Aspergillus* sp [1]. With further increase in temperature tannase activity was found to decrease.

Effect of Glucose Concentration: Among the different concentrations of glucose (0-02, 0.04, 0.06, 0.08 and 0.1%) tried as additive on tannase production, the maximum enzyme production was observed at 0.1% (106.55 U/ml/min) shown in Fig. 5.

Effect of Tannic Acid Concentration: Among the different concentrations of Tannic acid (0-02, 0.04, 0.06, 0.08 and 0.1%) tried as additive on tannase production shown in Fig. 6. the maximum enzyme production was observed at 0.06% (91.00 U/ml/min). Tannase has been reported to be an inducible enzyme having tannic acid as inducer as well as carbon source [23, 24].

Effect of Substrate Concentration: Among the different concentration of substrate (Rice straw: Sugarcane baggasse) starting from 1:1,2:1, 3:1and 4:1 tried on tannase production shown in Fig. 7. The maximum enzyme production was observed at RS:SB(1:1) (121.00 U/ml/min).

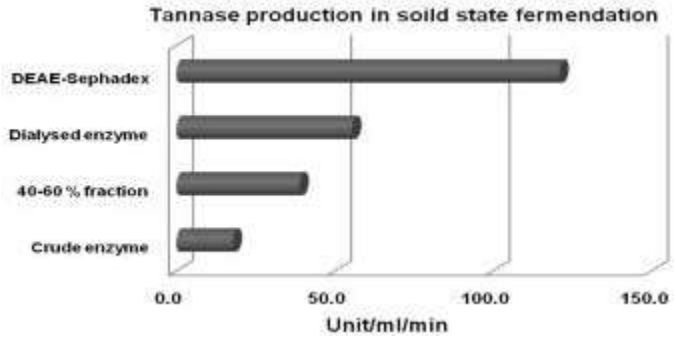

Fig. 1: Tannase production in soild state fermendation

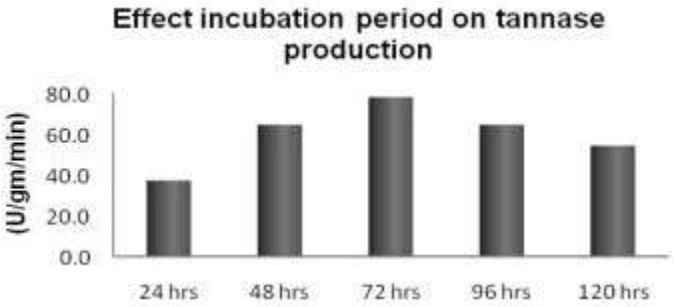

Fig. 2: Effect incubation period on tannase production

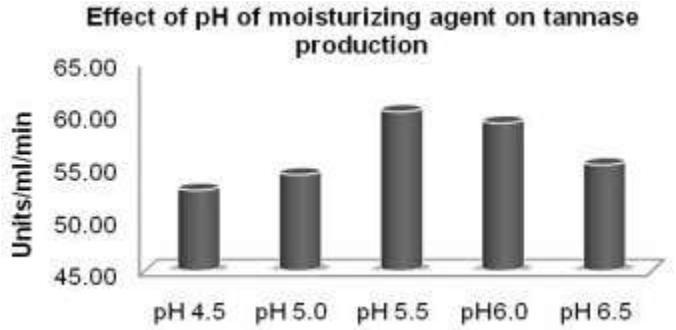

Fig. 3: Effect of pH of moisturizing agent on tannase production

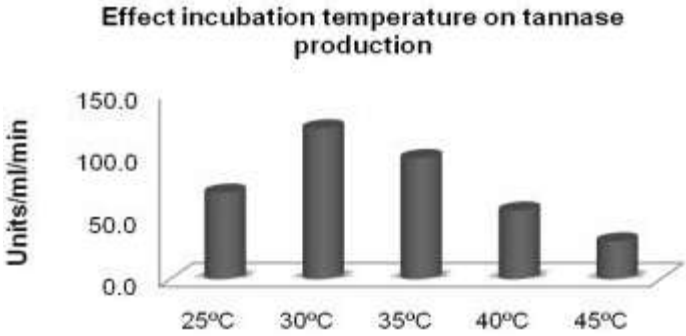

Fig. 4: Effect incubation temperature on tannase production

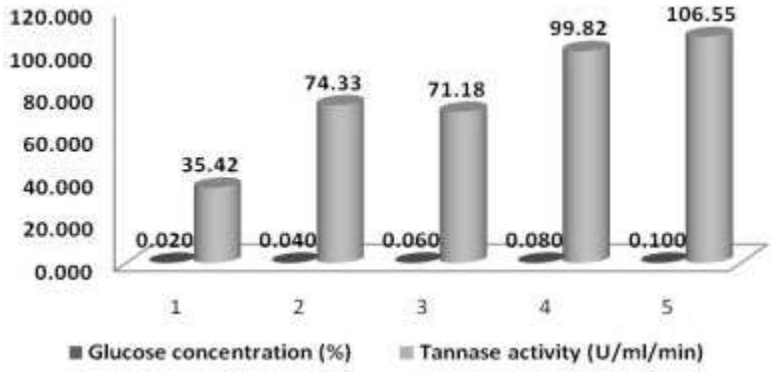

Fig. 5: Effect of Glucose concentration

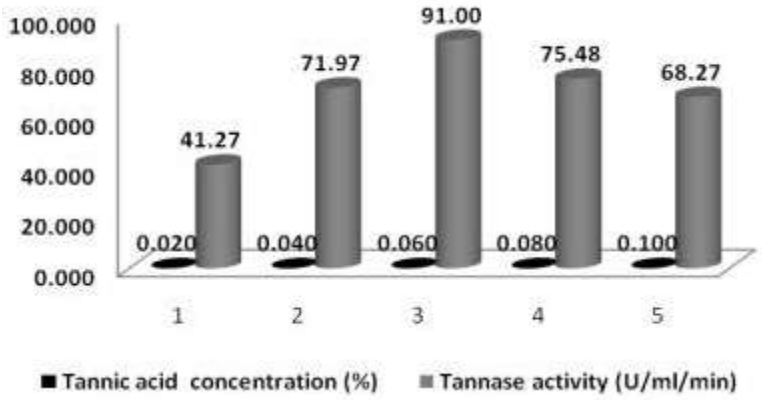

Fig. 6: Effect of Tannic acid Concentration

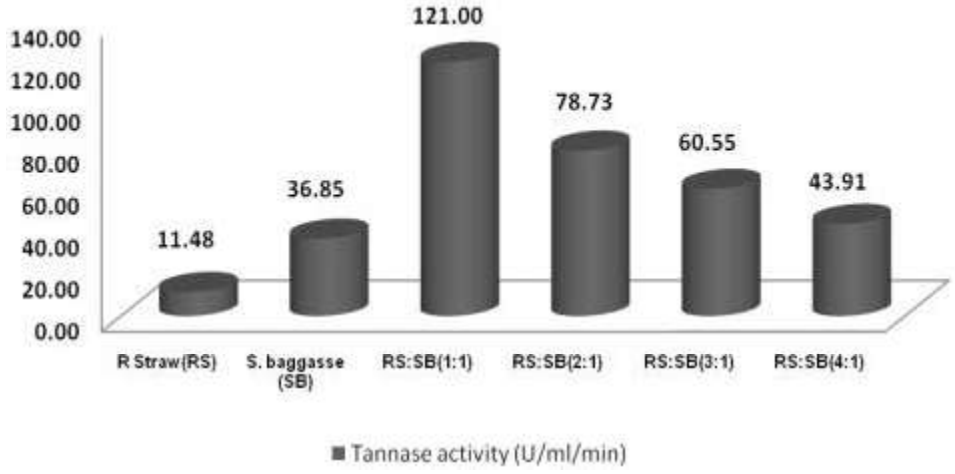

Fig. 7: Effect of Substrate concentration

CONCLUSIONS

The production of tannase from *Aspergillus oryzae,* were evaluated and standardized. These conditions were: solid-state fermentation with *Rice straw and Sugarcane baggasse* substrate *ratio (1:1)*, incubation temperature of 30°C, fermentation time of 72h and pH 5.5. This strain is able to produce tannase in the medium containing Tannic acid as the sole carbon source. These culture conditions can be used for further studies on the purification, Immobilization and applications of tannase. All these characteristics are considered favourable for industrial processing, especially in the food-processing industry.

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