

Full Length Research Paper

Manipulation of fermentation conditions on production of Tannase from agricultural by-products with *Aspergillus oryzae*

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Accepted 8 June, 2016

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 agricultural by products as a source for the production of Tannase and optimizing conditions required to get maximum production. *Aspergillus oryzae* MTCC 634 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 (27.8 U/gm/min). Optimum fermentation conditions of pH, temperature and incubation period for Tannase production were found to be 5.5 and 30°C at 72 h. In purification step, 60% ammonium sulphate fractionation 51.6 U/gm/min was found and maximum tannase activity of DEAE Sephadex colum chromatography purified sample was found to be 116.4 U/gm/min. 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 and 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 (Iibuchi et al., 1967; Rajakumar et al., 1983). Agro-industrial residues are generally considered the best substrates for the process of enzyme production, (Ellaiah et al., 2002). Compared with submerged fermentation, the use of SSF presents advantages such as lower power requirements, smaller reactor volume and high productivity (Bertolin et al., 2001), low capital investment, low waste water output, higher concentration of metabolites obtained and low downstream processing cost (Kumaran, 1997). 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 (Martins et al., 2002), sugar cane bagasse (Silva et al., 2002) wheat bran (Cavalitto et al., 1996) and other food processing waste (Zheng and Shetty, 2000) 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 (Sitting et al., 1988). Tannase is extensively used in the preparation of instant tea, wine, beer and coffee - flavored soft drinks and also as additive for detanification of food (Lekha et

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al., 1993). 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 (Masschelein and Batum, 1981; Cantarelli et al., 1989; Lane et al., 1997; Boadi and Neufeld, 2001). Tannic acid is an important gallotannin belonging to the hydrolysable class and consists of esters of gallic acid and a polyol, usually glucose (Spencer et al., 1988; Kumar et al., 1999; Mondal et al., 2000). The strain *Aspergillus niger* HA37 was previously isolated from OMWW, a substrate containing an important amount of hydrolysable Tannins acting as inducers for Tannase production (Aissam et al., 2002). This study aimed to investigate the bioconversion of sugarcane bagasse and rice straw powder for Tannase production and optimization of fermentation processing condition requires for maximum Tannase production.

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 inoculate

A strain of *Aspergillus oryzae* MTCC 634 was used for the study. Potato dextrose agar slants be used for the preservation of *A. oryzae*. Fungal spore inoculums were prearranged by adding 2.5 mL 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 solid-state fermentation

A five gram mixed substrate of rice straw powder with sugarcane bagasse 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_4NO_3 0.5%, NaCl 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1% and Tannic acid 4% at pH = 5.5. The contents were sterilized by autoclaving at 121°C , 15 lbs 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 assay

Tannase was assayed following Sharma et al. (2000) 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. $A_{520} = (\text{Absorbance test} - \text{Absorbance blank}) - (\text{Absorbance control} - \text{Absorbance blank})$

Determination of soluble protein in fungal biomass

Protein was estimated following the method of Lowry (1962) using bovine serum albumin as a standard.

Optimization of fermentation process

Effect of temperature

The solid-state fermentation was carried out at different temperatures

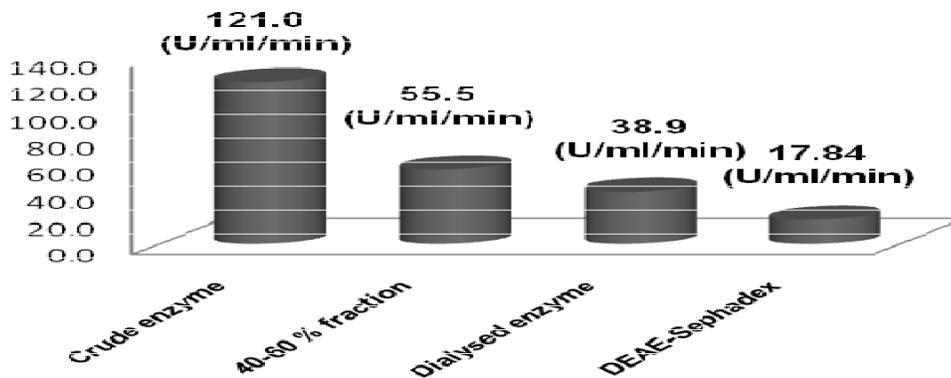


Figure 1. Tannase production in solid state fermentation.

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 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 to 120 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 bagasse) starting from 1:1, 2:1, 3:1 and 4:1 to find out the best ratio for enzyme production under solid-state fermentation (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 substrates 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 bagasse and rice straw as substitute. The crude Tannase observed was 27.8 (U/gm/min) which on purification

showed Tannase activity of 51.6 U/gm/min by 40 - 60% of ammonium sulphate fractionation and after dialysis membrane purification shows 66.8 U/gm/min (Figure 1). The Tannase activity of column purified sample was found to be 116.4 U/gm/min. SSF offers a number of advantages over conventional submerged fermentation for enzyme production (Mudgett, 1986). Mitchell and Lonsane (1992) reported 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 (Deschamps and Huet, 1985).

Effect incubation period on tannase production

The results on the optimum incubation period require for maximum Tannase production showed in Figure 2 that the enzyme production started after 24 h of incubation and progressively increased with time, the maximum production of 77.3 U/ml/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 (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 (Suseela and Nandy, 1985).

Effect of pH of moisturizing agent on tannase production

Among the various pH tested, maximum production of enzyme (62.24 U/ml/min) was observed at pH 5.5. The increase in pH over 6.0 drastically reduced the Tannase activity showed in Figure 3, Lekha and Lonsane (1997) also reported that Tannases are acidic proteins with an optimum pH around 5.5. Similarly, Sabu and coworkers

Effect incubation period on tannase production

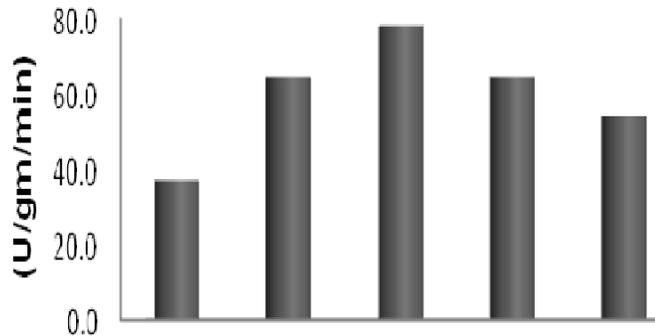


Figure 2. Effect incubation period on tannase production.

Effect incubation temperature on tannase production

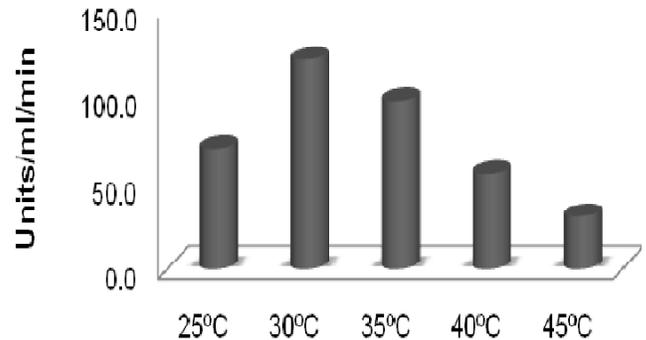


Figure 4. Effect incubation temperature on tannase production.

Effect of pH of moisturizing agent on tannase production

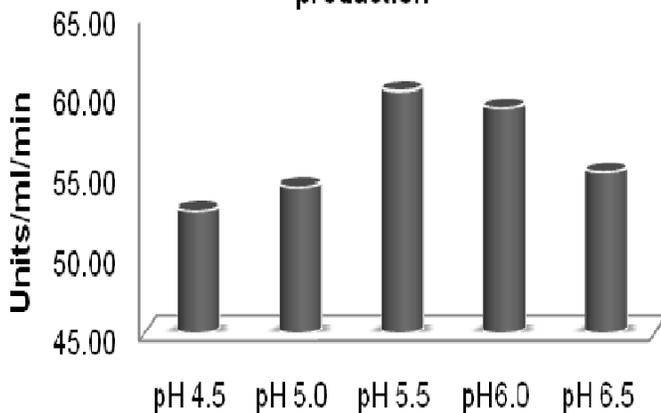


Figure 3. Effect of pH of moisturizing agent on tannase production.

(2005) reported optimum pH of 5.5 for Tannase production by *A. 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 (114.0 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 shown in Figure 4. Similar observations were reported for Tannase from *A. oryzae* (Lekha et al., 1997), *Aspergillus* sp (Rajakumar et al., 1983) and *P. chrysogenum* (Libuchi et al., 1968). 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 /ml/min) shown in Figure 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 Figure 6. The maximum enzyme production was observed at 0.06% (91.00 /ml/min). Tannase has been reported to be an inducible enzyme having tannic acid as inducer as well as carbon source (Aoki et al., 1976; Lekha et al., 1994; Yamada et al., 1968).

Effect of substrate concentration

Among the different concentration of substrate (rice straw: sugarcane bagasse) starting from 1:1, 2:1, 3:1 and 4:1 tried on Tannase production shown in Figure 7. The maximum enzyme production was observed at RS: SB (1:1) (121.00 /ml/min).

Conclusions

The production of Tannase from *A. oryzae*, were evaluated and standardized. These conditions were: solid-state fermentation with rice straw and sugarcane bagasse substrate ratio (1:1), incubation temperature of 30°C, fermentation time of 72 h 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

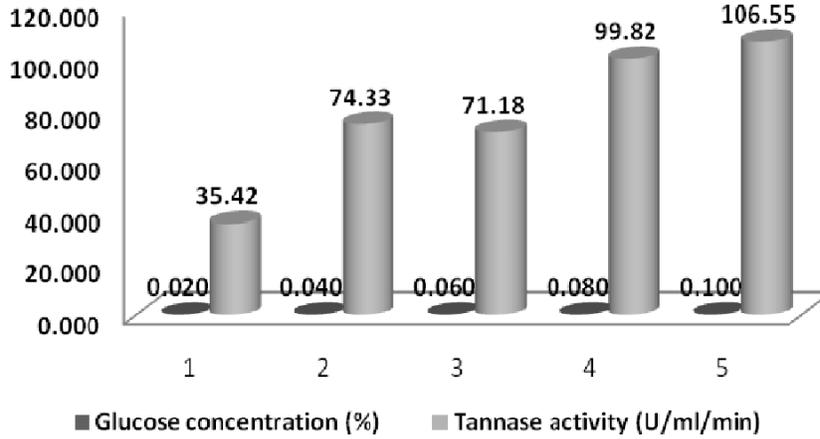


Figure 5. Effect of Glucose concentration

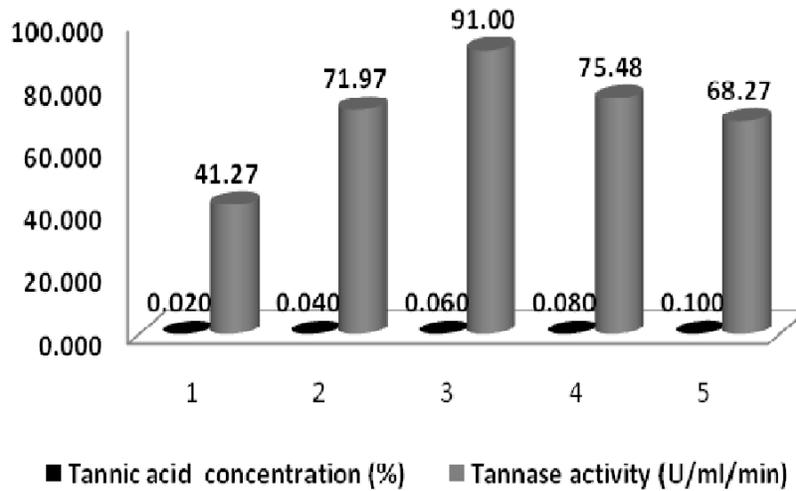


Figure 6. Effect of tannic acid concentration.

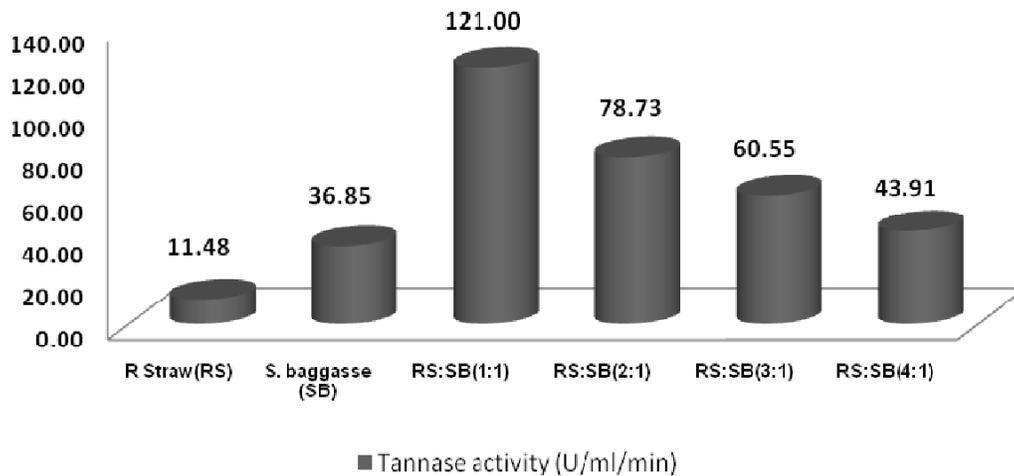


Figure 7. Effect of substrate concentration.

can be used for further studies on the purification, immobilization and applications of Tannase. All these characteristics are considered favorable for industrial processing, especially in the food-processing industry.

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