Research Article

Production and Optimization of Solid State Fermentation Media for Tannase Enzyme by Aspergillus heteromorphus

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ABSTRACT
Tannase is an industrially important enzyme. When produced through solid state fermentation by *A. heteromorphus* of different substrates like, Mango kernel and Ragi flour were found to be ideal for tannase production. It can be attributed to their narrow C:N ratio (34:1) and high phosphorous content (0.71%) than other solid substrates. The tannase enzyme production by *A. heteromorphus* is enhanced by supplementation of Ragi flour and Mango kernel powder with 1% tannic acid while biomass of *A. heteromorphus* is increased at 2% tannic acid.

Keywords: Tannase, Industrial enzyme, *A. heteromorphus*, SSF.

INTRODUCTION

Tannase (Tannin acyl hydrolase, E.C.3.1.1.20) is an industrially important inducible enzyme produced by large number of fungi and a few bacteria which hydrolyze the ester and depside bonds of tannin and gallic acid esters. This enzyme is used in the industrial processing and clarification of beer, fruit juice and corn wine, detannification of food, high grade leather tannin and coffee-flavored soft drinks but the major commercial applications of the tannase are in the manufacture of instant tea and in the production of gallic acid (Co'rdova *et al*., 1996). Tannase is also used in the pre-treatment of animal feed additives, to clean-up highly polluting tannin from the effluent of leather industry, pharmaceutical and chemical industries (Lekha and Lonsane, 1997; Aguilar *et al*., 2001; Mahendran *et al*., 2006). Gallic acid, a hydrolytic product of tannin, finds uses in preparation of trimethoprim, pyrogallol, propyl gallate, dyes and inks etc. (Hadi *et al*., 1994).

A number of reports given by different workers have shown the use of liquid surface, submerged (SmF) or solid state fermentation (SSF) for tannase production by fungi. 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 (Pandey and Radhakrishnan, 1993). Recently the Indian Institute of Horticultural Research, Bangalore, has developed a technology for the production of coco-peat using the fungus *A.heteromorphus*, based on the principle of reducing the tannin content of the raw material viz., coir pith, through a simple solid state fermentation process. Hence this investigation was undertaken with the objective of standardizing the media for the solid state fermentation for the mass production of the fungal species *A.heteromorphus* and optimizing tannase production by the fungus under SSF conditions.

MATERIALS AND METHODS

Stock Culture Maintenance

The pure stock culture of *A.heteromorphus* was maintained in Potato Dextrose Agar (Potato extract 20%, Dextrose 2%, Agar 2% w/v)(PDA) slants under liquid paraffin. It was further sub-cultured at periodic intervals on PDA and incubated for 3 days at 29° C for various studies.

Substrate Collection

Mango leaf litter, Jamun leaf litter,and Simarouba leaf liter collected from IIHR campus. The Mango kernel powder was obtained from variety Totapuri.

Effect of different solid substrates on biomass production by *A. heteromorphus*

Four different types of solid substrates were initially screened for the mass production of *A. heteromorphus*. They were powdered mango leaf litter, mango kernel powder, powdered jamun leaf litter and powdered Simarouba leaf litter. Twenty five grams of each substrate was taken in 250ml Erlenmeyer flasks and sterilized at 15psi for 20mins in an autoclave.

The moisture level was maintained at 50 %, throughout the experimental period by using sterile distilled water. A mycelial disc (8mm dm) was cut with the help of sterile cork borer and used to inoculate the individual substrates. The cultures were incubated at 29°C for 7days. After 7 days of incubation, the biomass production by the fungus was estimated by (a) determining the number of colony forming units/ gram of substrate (b) determining the spore count / gram of substrate

a. Determining the number of colony forming units per gram of substrate

Ten gram of substrate was serially diluted in 100 ml water. The dilutions of 10^{-2} to 10^{-7} were plated out in Potato Dextrose Agar (PDA) plates. The plates were incubated at 29°C for 72 h. After the incubation period, the number of colonies in the plates was counted, and the population was expressed as number of colony forming units per gram of substrate.

b. Determining the spore count per gram of substrate

The serially diluted samples from the 10^{-4} to 10^{-6} dilutions were used for spore counting. An aliquot of 0.1ml was taken in a cavity slide and the number of spores present in the aliquot was counted under a microscope. The population was expressed as number of spores per gram of substrate.

Effect of different grains flours on biomass production by *A. heteromorphus*

Three different types of grain flours were initially screened for the mass production of *A. heteromorphus*. Rice flour, ragi flour and maize flour (25gram) were taken in 250ml Erlenmeyer flasks and sterilized at 15psi for 20 minutes in an autoclave. A mycelia disc of *A. heteromorphus* (8mm diameter) was used to inoculate the individual substrates. The moisture level was maintained at 50 %, throughout the experimental period by using sterile distilled water. The cultures were incubated at 29°C for 7days, after 7 days of incubation the biomass production by the fungus was estimated.

Effect of tannic acid supplementation of mango kernel, ragi grain on biomass production by *A.heteromorphus*

Mango kernel (25 g) with various concentrations of tannic acid (0.5%, 1%, 1.5, and 2%) were taken in 250ml Erlenmeyer flasks and sterilized at 15psi for 20 minutes in an autoclave. A mycelial disc (8mm dm) was cut with the help of sterile cork borer and used to inoculate the individual substrates. The moisture level was maintained at 50 %, throughout the experimental period by using sterile distilled water. The spore count of *A. heteromorphus* was determined. The same manner ragi grain also tested.

Estimation of total nitrogen content of the substrates

Individual samples (0.3g) were weighed in 100ml flasks. To this a pinch of the digestion mixture containing K_2SO_4, CuSO4 and Se in the ratio of 40:20:1 and 10ml of H_2SO_4 were added. The flasks were then heated to 300-350°C, in a hot plate until the solution became clear. After digestion, the samples were made-up to 100ml using distilled water. The digested aliquot (5ml) and 40% NaOH (8ml) were subjected to distillation in a distillation unit during which the ammonia liberated was collected in 10ml of 4% boric acid, until the boric acid turned to a deep blue colour. This was titrated against 0.02M H_2SO_4, until the appearance of a deep red colour. The total nitrogen content was calculated using the formula

$$N\ (\%) = \frac{TV \times N \text{ of acid} \times 0.014 \times \text{volume of digested sample} \times 100}{\text{Weight of sample} \times \text{aliquot taken}}$$

Where TV – is the titer value in ml

Estimation of phosphorus content of the substrates by Diacid digestion

Diacid digestion was done to estimate phosphorus content of the substrates. One gram of sample was digested with a diacid mixture containing nitric acid and perchloric acid. The digested volume was made-up to 100ml using distilled water. To estimate phosphorus, 5 ml of sample and 5ml of vanadomolybdate solution was taken in a 25ml volumetric flask and the colour was allowed to develop. The volume was then made up to 25ml and the absorbance was read at 470nm in a spectrophotometer. The phosphorus content was calculated using the formula

$$P\ (\%) = \frac{\text{graph ppm (y) x volume of digested sample x volume made up x 100}}{10^6 \text{ x weight of sample x aliquot taken}}$$

Determination of the Carbon: Nitrogen (C: N) ratio of the substrates

The C:N ratio of the substrates was determined in a CHNS analyzer (Elementar Microcube, Germany) by following the principle of complete combustion of the substrate and detecting the oxides of the individual elements using a Thermal Conductivity Detector.

Estimation of Tannase activity of *A.heteromorphus*

A seven day old culture of *A.heteromorphus* grown on mango kernel and ragi flour containing various levels of tannic acid (0.5, 1.0 and 2.0 %) were used for the estimation of tannase activity. The enzyme was extracted by using phosphate buffer (50 ml), and this served as the crude enzyme extract. One ml of enzyme extract and 4ml of 0.35% tannic acid in citrate phosphate buffer (pH 5.5) were added in a test tube and incubated at 37°C for 30mins. After incubation, 10ml of 95% ethanol was added to arrest the reaction. The reaction mixture was mixed thoroughly and absorbance was measured at 254nm and 290nm. The concentration of gallic acid produced by the hydrolysis of tannic acid substrate was calculated by the formula of Aguilar et al. (1999).

Concentration of Gallic acid (µg/ml) = 42.5 (abs_{254}) – 27.0 (abs_{290})

Gallic acid concentration (µg/ml) = Gallic acid concentration of test sample – Gallic acid concentration of control

One unit (U) 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/g of substrate /minute (U/g/min).

Statistical Design and Analysis

All the experiments were laid out in the Completely Randomized Design (CRD) and replicated adequately so that the degrees of freedom were greater than 12. The formula used for this purpose was $t(r-1) \geq 12$, where t is the number of treatments and r is the number of replicates.

RESULTS AND DISCUSSION

Analysis of the nutrient contents (Table 1) of the substrates used in the study revealed that Simarouba leaf litter had the highest nitrogen content (1.52 %) followed by mango kernel powder (1.31 %). Jamun leaf litter possessed the highest carbon content (66.88 %) while mango kernel possessed the highest phosphorus content (0.71 %). Mango kernel powder had the narrowest C:N ratio while other substrates had the widest C:N ratio. Tannase producing *A.heteromorphus* was grown on different solid media like powdered mango leaf litter, powdered mango kernel, powdered jamun leaf litter, and powdered simarouba leaf litter. It was observed that mango kernel powder supported the highest colony growth and number of spores per gram of substrate (Table 2). It can be attributed to the narrow C:N ratio and high phosphorous content of the mango kernel than other solid substrates. Species of *Aspergillus* are highly aerobic and are found in almost all oxygen-rich environments, where they grow as molds on the surface of substrates, as a result of the high oxygen tension. They are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments. Earlier investigations on tannase production indicated very clearly that almost all species of *Aspergillus* are capable of synthesizing tannase on induction.

Solid state fermentation involves non-aseptic conditions with the use of cheap, simple and easily available raw materials as substrates, along with several economical and engineering advantages including low capital cost, low energy expenditure, less expensive downstream processing, less water usage and lower wastewater output, potential higher volumetric productivity, higher concentration of the products, high reproducibility, lesser fermentation space, easier control of contamination and generally simpler fermentation media. As the moisture level is low, the volume of medium per unit weight of substrate is low. Therefore, the enzyme activity is usually very high (Deschamps and Huet, 1985). In this experiment evaluation of biomass production by *A. heteromorphus* on different grain flours revealed that ragi grain supported the maximum growth of the fungus, followed by rice and maize grains (Table 3). Evaluation of different concentration of tannic acid on biomass production by *A.heteromorphus* on ragi flour (Table 4), revealed that the fungus produced maximum

biomass (15 x 10^8 cfu/g and 10.8 spores / g of substrate), when ragi flour was supplemented with 2 % tannic acid. Evaluation of different concentration of tannic acid on biomass production by *A.heteromorphus* on powdered mango kernel (Table 5), revealed that the fungus produced maximum biomass (31.5 x 10^8 cfu/g and 10.8 spores / g of substrate), when mango kernel was supplemented with 2 % tannic acid. A comparison of different types of fermentation conditions optimized by Kumar et al (1999) was found to be the tab water is best moistening agent, with pH 5.5 in the ratio of 1:2 (w/v) with substrate. Addition of carbon and nitrogen sources to the medium did not increase tannase production (Kumar *et al*.,1999). Tannase production by *A. niger* Aa-20 was studied in submerged (SmF) and solid-state (SSF) fermentation systems with different tannic acid and glucose concentrations. Tannase activity and productivity were at least 2.5 times higher in SSF than in SmF. Addition of high tannic acid concentrations increased total tannase activity in SSF, while in SmF it was decreased. SSF when compared to SmF techniques; SSF has advantages for tannase production in terms of an increment in purity and stability. It was observed that the tannase produced by SSF was more stable and total proteins were lower; moreover, undesirable proteolytic enzymes production was not observed during SSF (Co´rdoba- Salgado *et al*., 1998). Many workers reported that tannase production by SSF is more advantageous over submerged or liquid surface fermentation (Mudgett, 1986; Lekha and Lonsane, 1994; Aguilar *et al*., 2001). Determination of the tannase activity of *A. heteromorphus* grown on ragi and mango kernel substrates revealed that the highest tannase activity of tannase enzyme was recorded when the substrates *viz.,* mango kernel powder and ragi flour were ammended with 1 % tannic acid, while a marginal decrease was observed when 2 % tannic acid was used (Table 6).

The industrial applications of tannase have not been fully exploited because of its high cost, although there are a large number of reports on the production of tannase by submerged fermentation. Most of these do not involve the identification of critical parameters for enzyme biosynthesis and their optimization. This study thus provides a basis for a cheaper alternate for production of tannase enzyme thereby extending its chance of industrial use.

Table 1: Nutrient composition of different plant residues used as substrates

Residues	N%	C%	P%	C:N ratio
Mango leaf litter powder	0.66	30.64	0.02	46.2:1
Mango kernel powder	1.31	45.07	0.71	34.4 :1
Jamun leaf litter powder	0.99	66.88	0.01	67.5: 1
Simarouba leaf litter powder	1.52	54.19	0.08	35.5:1
CD (0.05 %)	0.14	6.12	0.04	-

Table 2: Biomass production by *A. heteromorphus* on different plant residues

Residues	10^7 cfu/g of substrate	10^8 spores/g of substrate
Mango leaf litter powder	9	0.5
Mango kernel powder	10	1.4
Jamun leaf litter powder	8	0.4
Simarouba leaf litter powder	10	0.6
CD (0.05 %)	NS	0.57

Table 3: Biomass production by *A. heteromorphus* on different grain flours

Grain flour	10^8 cfu/g of substrate	10^7 spores/g of substrate
Maize	2.5	1.2
Ragi	10.6	9.0
Rice	5.8	4.4
CD (0.05 %)	0.37	0.30

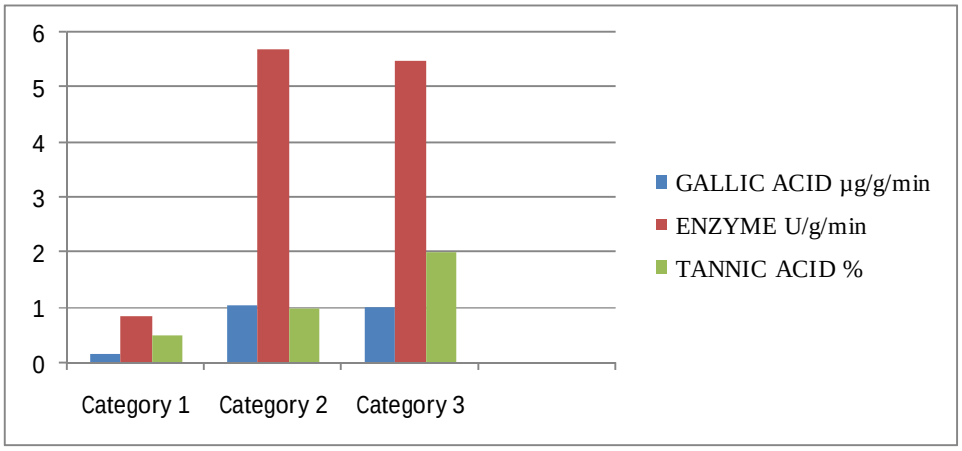
Table 4: Effect of tannic acid on biomass production by *A. heteromorphus* on ragi flour

Tannic acid concentration	10^8 cfu/g of substrate	10^8 spores/g of substrate
0.5%	2.1	4
1%	7.2	6
1.5%	6.9	7.2
2%	15	10.8
CD (0.05 %)	1.05	0.87

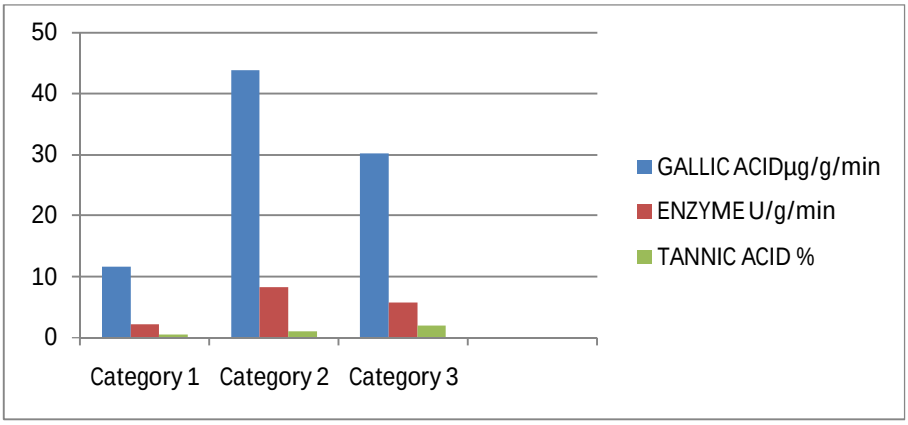
Table 5: Effect of tannic acid on growth of *A. heteromorphus* on mango kernel

Tannic acid concentration	10^8 cfu/g of substrate	10^8 spores/g of substrate
0.5%	2.3	3.9
1%	4.6	5.7
1.5%	7.3	6.9
2%	13.4	10.8
CD (0.05 %)	0.93	0.42

Table 6: Effect of tannic acid on of tannase activity of *A. heteromorphus* grown on ragi and mango kernel substrates



Mango Kernelµ



Ragi flour

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