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Microbial production of gallic acid by modified solid state fermentation

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Bioconversion of tannin to gallic acid from powder of teri pod (*Caesalpinia digyna*) cover was achieved by the locally isolated fungus, *Rhizopus oryzae*, in a bioreactor with a perforated float for carrying solid substrate and induced inoculum. Modified Czapek-Dox medium, put beneath the perforated float, with 2% tannic acid at pH 4.5, temperature 32°C, 93% relative humidity, incubated for 3 days with 3-day-old inoculum was optimum for the synthesis of tannase vis-à-vis gallic acid production. Conversion of tannin to gallic acid was 90.9%. Diethyl ether was used as the solvent for extraction of gallic acid from the fermented biomass.

Keywords: teri pod; *Rhizopus oryzae*; tannase; modified solid state fermentation

Introduction

Tannase (tannin acyl hydrolase) is an extracellular, inducible enzyme which cleaves the ester linkages in hydrolysable tannins and gallic acid esters. It is an important enzyme used in the pharmaceutical industry and also for analytical and developmental purposes [9].

Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic compound and finds application in various fields. The most important use is for manufacturing trimethoprim (TMP), an antibacterial agent used in combination with sulfonamide [5]. It is also used in the leather industry, in manufacturing gallic acid esters, eg, propyl gallate which is used as an antioxidant, in the manufacture of pyrogallol. Pyrogallol is used in staining fur, leather and hair, and also as a photographic developer [6]. Gallic acid production has been reported from myrabolan, tara [12], sumac [13] and Chinese tannins.

In this study, teri pod (*Caesalpinia digyna*) cover powder containing tannin was selected as the substrate for microbial conversion to gallic acid. Moreover, modified solid state fermentation (MSSF) has been introduced. The purpose of using such a system was to improve the yield of gallic acid as the microorganism, *Rhizopus oryzae* is a filamentous fungus.

Materials and methods

Microorganism

Rhizopus oryzae (RO, IIT RB-13, NRRL 21498) was isolated from soil on the IIT campus and was maintained in 2% malt extract agar slants.

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Chemicals

All chemicals were of analytical grade. Tannic acid was from Merck (Darmstadt, Germany).

Raw material

Teri pod cover powder is readily available in the eastern part of India. The pod cover was obtained during processing of the pod for recovery of oil. The teri pod cover was powdered to 74–422 μm and varying amounts were studied in the bioreactor (Growtek, Calcutta, India) for the recovery of gallic acid.

Inoculum preparation

Preinduced inoculum was prepared using Czapek–Dox medium with 2% tannic acid as sole carbon source [3].

Experimental set-up

The bioreactor is a cylindrical vessel (Figure 1), having a height of 16.0 cm and diameter 11.3 cm. Near the base on the wall is a spout, inclined 15° to the vertical axis, having a diameter of 2.6 cm and length 8.5 cm. The body of the vessel and the spout are made of polycarbonate whereas the lids (body and spout) are made of polypropylene. Through the spout, medium can be removed without disturbing the fermentation. A float is provided inside the vessel, which consists of a base of glass wool cloth having an area of 72 cm^2 and a periphery made of polypropylene having a hollow structure. Fermentation of the solid substrate is carried out on the float by the induced inoculum. One advantage of this kind of float is, MSSF can be carried out, in which the solid substrate placed on the float comes in contact with the liquid medium in the vessel. The microorganism added to the substrate converts the substrate into the desired product, which leaches into the liquid medium. The maximum volume of liquid that can be placed in the vessel is 400 ml.

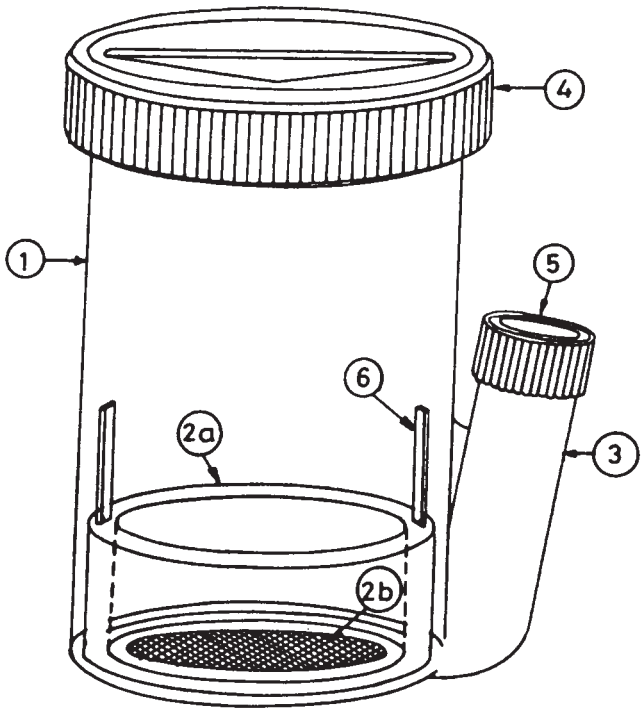

Figure 1 Bioreactor (Growtek) for modified solid state fermentation. (1) Transparent polycarbonate body of the bioreactor; (2a) float; (2b) perforated base of the float carrying solid substrate and inoculum; (3) side tube to the vessel to facilitate medium changes or sample collection; (4) threaded lid for sterile aeration; (5) threaded lid to side tube; (6) float holder.

Tannin estimation

The tannin content of the crude extract was determined by adding the sample to a standard solution of BSA, isolating the tannin protein complex, dissolving it in alkaline solution and measuring the absorbance at 510 nm after adding ferric chloride [7].

Tannase production

Varying quantities of powdered teri pod cover were placed in bioreactors to which Czapek–Dox medium ($NaNO_3$ 0.25%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, KCl 0.05%, teri pod cover powder 2.0%) was added. It was then autoclaved at 121°C for 15 min and after it had cooled, it was inoculated with a preinduced culture of *Rhizopus oryzae* and incubated for enzyme production under different conditions. After fermentation, the culture broth was centrifuged to separate the mycelial mass, and supernatant containing the enzyme was processed for assay.

Tannase assay

Tannase activity was determined spectrophotometrically [8]. Enzyme solution (ie, broth) 0.5 ml was added to 2.0 ml of 0.35% (w/v) tannic acid solution in 0.2 M acetate buffer (pH 5.0) placed in a test tube. The reaction mixture (20 μl) was taken out and 2.0 ml of 95% ethanol was added to it to stop the enzyme reaction. The absorbance at 310 nm was noted immediately (t_1). The test tube was then incubated in a water bath at 37°C for 5 min (t_2), after which ethanol was added to the reaction mixture to stop the enzyme reaction. The absorbance was noted for activity calculation. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 μmol of ester in 1 min. Enzyme activity is expressed as unit ml^{-1} min^{-1} (IU).

Gallic acid extraction and measurement

The enzyme broth was boiled for 5 min. After it had cooled, the broth was centrifuged to separate the mycelial mass. Diethyl ether was added to the supernatant. Gallic acid, being soluble in the organic solvent, extracted into the organic layer which was then separated using a rotary evaporator. The characterization of gallic acid was further carried out by thin layer chromatography (TLC) and melting point analysis. Purity of the product was determined by NMR studies.

Results

Gallic acid production depends on the synthesis of tannase. Therefore, for maximum tannase production the following physicochemical parameters were optimized.

Incubation period

This is the most important parameter for maximum tannase production. Initially a 120-h-old inoculum was used and the period of incubation was varied from 24–96 h. Up to 72 h there was a rise in tannase activity, after which it decreased (Figure 2).

Substrate variation

The amount of substrate was varied from 5–40 g, while keeping the liquid volume constant (50 ml). After incubation for 72 h, it was found that 20 g gave optimum tannase activity. There was an initial rise in the activity up to 20 g after which it decreased (Figure 3).

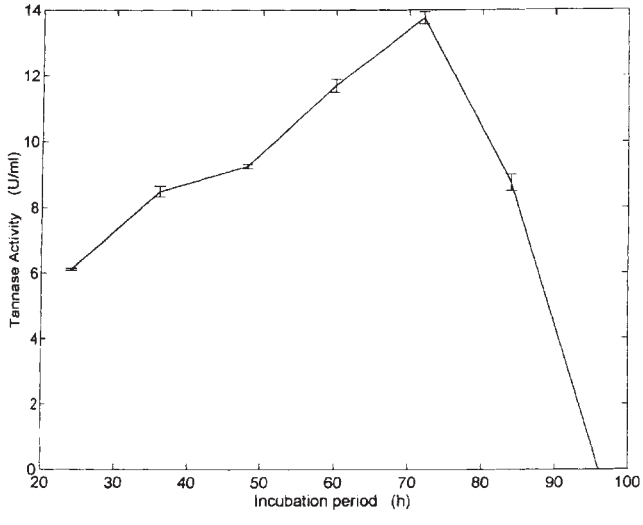

Figure 2 Effect of incubation period on tannase production at constant pH (4.5), temperature (32°C), relative humidity (93%), particle size (74 μm), inoculum age (120 h), substrate content (20 g) and solid to liquid ratio (0.4:1).

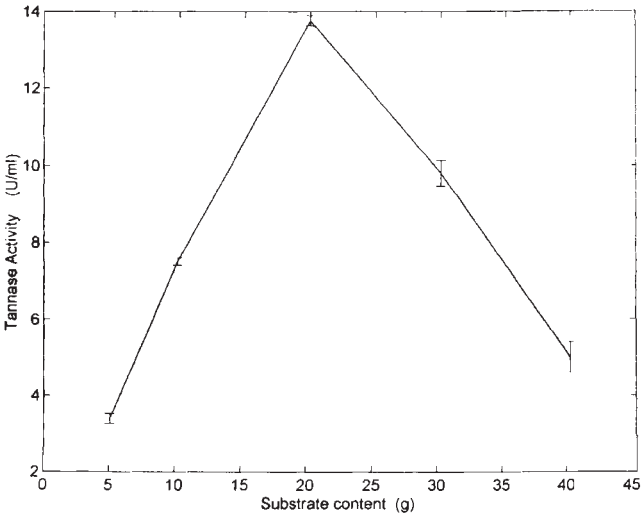

Figure 3 Effect of substrate content on tannase production at constant pH (4.5), temperature (32°C), relative humidity (93%), particle size (74 μm), inoculum age (120 h), solid to liquid ratio (0.4:1) and incubation period (72 h).

Variation of volume of medium

Keeping the substrate content constant (20 g), the solid to liquid ratio was varied from 0.067–0.8. It was found that 0.4:1 (solid-liquid ratio) gave optimum enzyme activity (Figure 4).

Inoculum age

Tannase is an inducible enzyme, therefore inoculum was cultured in the presence of 2% tannic acid, varying the incubation period from 24–96 h (Figure 5). A 72-h-old inoculum gave maximum tannase production with 72 h of incubation.

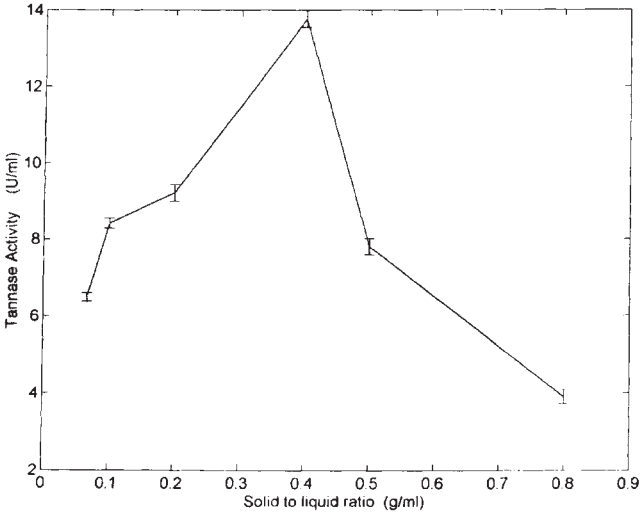

Figure 4 Effect of solid-liquid ratio on tannase production at constant pH (4.5), temperature (32°C), relative humidity (93%), particle size (74 μm), inoculum age (120 h), incubation period (72 h) and substrate content (20 g).

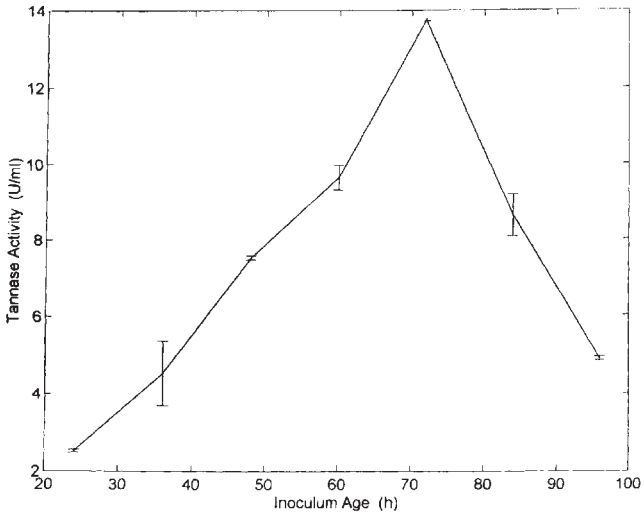

Figure 5 Effect of inoculum age on tannase production at constant pH (4.5), temperature (32°C), relative humidity (93%), particle size (74 μm), inoculum period (72 h), substrate content (20 g) and solid to liquid ratio (0.4:1).

pH

To observe the effect of initial pH on tannase production, the liquid medium under the float was adjusted to different pH levels ranging from 3.0–6.5. Maximum synthesis of tannase was observed at initial pH 4.5 (Figure 6).

Temperature

The optimum temperature for maximum tannase production was 32°C when the temperature was varied from 20–40°C (Figure 7).

Relative humidity

By keeping the above parameters optimum, the culture was subjected to humidity variation of 67–94% in a humidifier (Biotron LPH 200, Nippon Medical and Chemical Instru-

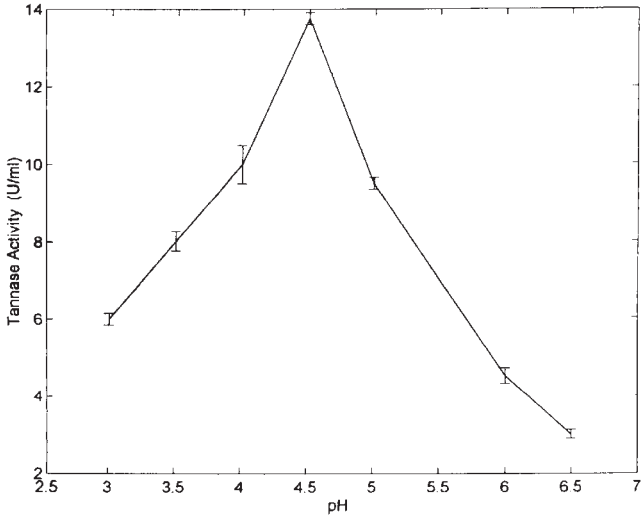

Figure 6 Effect of pH on tannase production at constant temperature (32°C), relative humidity (93%), particle size (74 μm), incubation period (72 h), substrate content (20 g), solid to liquid ratio (0.4:1) and inoculum age (72 h).

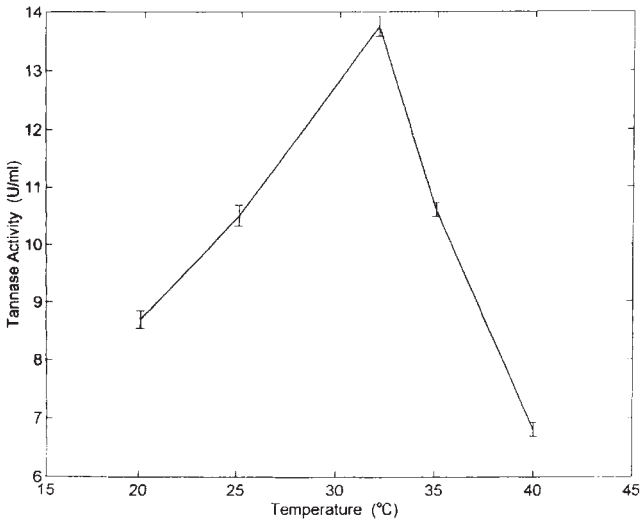

Figure 7 Effect of temperature on tannase production at constant pH (4.5), relative humidity (93%), particle size (74 μm), incubation period (72 h), substrate content (20 g), solid to liquid ratio (0.4:1) and inoculum age (72 h).

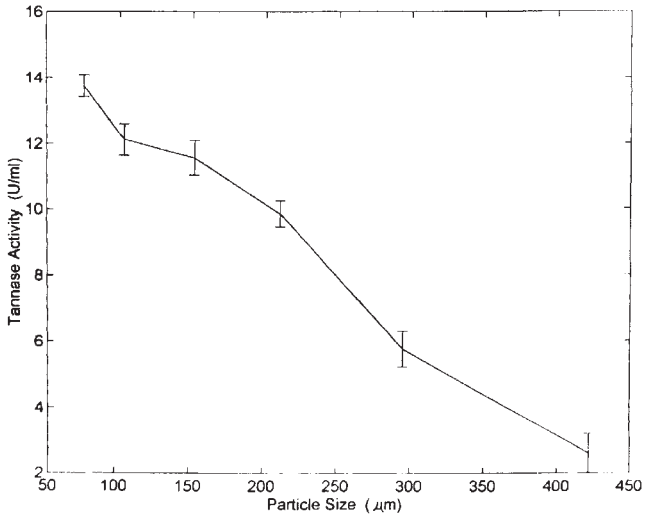

Figure 9 Effect of particle size on tannase production at constant pH (4.5), temperature (32°C), substrate content (20 g), solid to liquid ratio (0.4:1), incubation period (72 h) and inoculum age (72 h) and relative humidity (93%).

ments Co Ltd, Osaka, Japan) with controlled humidity and temperature. Maximum production of tannase was at 93% relative humidity (Figure 8).

Particle size

Particle size plays an important role in solid state fermentation. Under the experimental conditions, finer particles gave higher tannase production (Figure 9) when the study was carried out with particle size varying from 74–422 μm.

Yield of gallic acid

Fermentation was carried out using the above optimum conditions, and gallic acid was recovered using diethyl ether as mentioned in Materials and methods. Under these conditions the percentage yield of gallic acid based on tannin content of raw material was 90.9%.

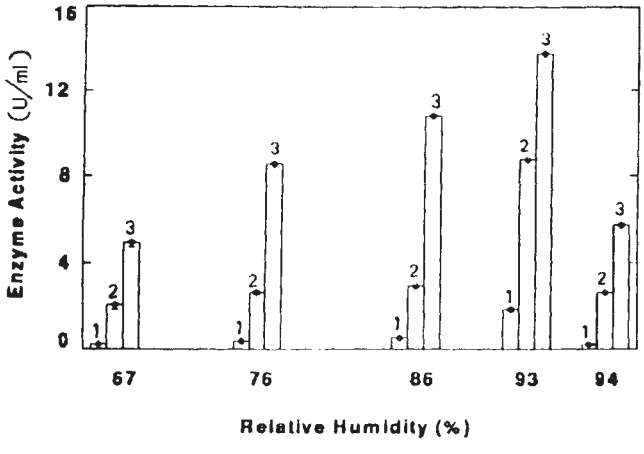

Figure 8 Effect of relative humidity on tannase production at constant pH (4.5), temperature (32°C), substrate content (20 g), solid to liquid ratio (0.4:1), particle size (74 μm), incubation period (72 h), and inoculum age (72 h). (1) 24 h of incubation; (2) 48 h of incubation; (3) 72 h of incubation.

Discussion

Enzyme activity (U ml^{-1}) was high when 20 g of substrate, and a 72-h-old inoculum (on float), was kept in contact with 50 ml of Czapek–Dox medium (in vessel) having a solid-liquid contact area of 72 cm^2, in the bioreactor for an incubation period of up to 96 h. After 72 h of incubation, the yield of gallic acid was maximum. The enzyme, tannase, hydrolyzes the ester bonds of tannin in the substrate to produce gallic acid and glucose [4]. The initial increase in enzyme activity up to 72 h and its subsequent decrease may be due to catabolite repression [2]. Another reason for the initial rise followed by a decrease in tannase is possibly due to the secretion of toxic substances, like catechuic acid, 2,6-dihydroxy benzoic acid and pyrogallol which can cause cell lysis. A third possibility is metabolite regulation which is related to the release of gallic acid. In MSSF conditions, the optimum incubation period was 72 h, whereas in both SSF (solid state fermentation) and SmF (submerged fermentation) conditions the optimum incubation period was 120 h.

Substrate quantity is an important parameter for gallic acid production. It was found that 20 g of powdered teri pod cover kept on the float of area 72 cm^2 gave optimum enzyme activity. The reason for the observed decline in tannase activity and also gallic acid production with increasing substrate quantity can be attributed to the formation of intermediate hydrolysates binding competitively or non-competitively with the active sites of the enzyme. Gallic acid itself can also act as a competitive inhibitor [8]. Further, as gallic acid contains hydroxyl groups, these could form hydrogen bonds with the amino acids present in the active sites of the enzyme leading to conformational changes. Another reason for the decrease in gallic acid production with the increase in substrate quantity on the float may be due to an increase in bed height causing insufficient diffusion of liquid through the entire bed, resulting in incomplete hydrolysis of substrate to gallic acid.

Solid-liquid ratio also influences enzyme activity. When

the solid-liquid ratio was varied from 0.067–0.8, keeping the solid content constant (20 g), there was an increase in activity of tannase up to 0.4:1 followed by a decrease when incubated for 72 h. Tannase is an extracellular enzyme which gets diluted with the increase in liquid volume, leading to a decrease in activity. Further, with higher solid content, the volume of liquid medium is insufficient for complete leaching of the product from the fermented biomass.

Up to pH 4.5 there was an increase in enzyme activity followed by a decrease. Tannase is an acidic glycoprotein having an isoelectric point at about pH 4.0 [1]. Thus, the acidic environment favors the transport of metal ions into the cells required for metabolic reactions of the organism [10]. From earlier reports on gallic acid production in SmF and SSF conditions, the optimum pH values were found to be 5.0 and 5.5, respectively [5,9].

Significant improvements in enzyme and gallic acid production were realized with finer particles. The reason for this could be that small particles provide a greater surface area, facilitating bioconversion due to the greater availability of substrate to the organism.

Relative humidity also plays an important role in the production of enzyme and gallic acid. There was an increase in enzyme activity up to 93% relative humidity. In SSF conditions, as the fermentation proceeds, the water content of the substrate is evaporated due to heat in the bed. The appropriate relative humidity level prevents undesired evaporation, and thus moisture content in the substrate is maintained.

Based on 58% tannin content in the powder of teri pod cover, gallic acid recovery in MSSF conditions was 90.9%. The yield of gallic acid from tannic acid in free cell culture was 83.5% and with immobilized cells it was 78.5% [11]. Gallic acid obtained from Sumac tannin containing 10% tannin was 9.75% under SmF conditions [13]. Gallic acid also has been produced from tara tannins using *Aspergillus niger* [12]. In conclusion, the higher yield of gallic acid under MSSF conditions can be attributed to the special features of the bioreactor. In the MSSF conditions, due to continuous contact of liquid in the vessel with the solid substrate on the float, heat accumulation in the bed is minimized. Again, in the MSSF conditions, due to the contact of liquid with the substrate, the product leaches into the liquid medium bringing about reduction in product accumulation in the substrate and thereby protecting the organism from feed-back inhibition [14].

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