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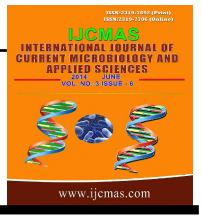


**Original Research Article**

# Production of Taninolytic Enzyme Under different Fermentation Process with reference to substrate consumption profile

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**A B S T R A C T**

Tannins are defined as naturally occurring water-soluble polyphenolic secondary metabolites of higher plants with varying molecular weight. They are the fourth most abundant plant constituents following cellulose, hemicellulose and lignin; and together with the latter are the most abundant and widely distributed phenolic polymers of higher plants. Biological degradation is adjudged as an important mechanism of organic chemical removal if natural system owing to its environmental capability and plasticity. Consequently, microbial metabolic potentials are widely exploited for a number of industrial applications including decommissioning of environmental pollutants. The present study was undertaken with a prime objective to degrade such organic chemicals under different fermentation process. The result obtained was quite striking with respect to degradation of tannin by *Rhizopus sp*. Highest Taninolytic activity was observed by solid state fermentation(SSF) process i.e. 2449.22 U/g dw, whereas the submerged fermentation (SMF) also favors tannase production, with enzymatic activity 97.96 U/ml, for flask B (1% Tannic Acid) and 48.98 U/ml & 146.95 U/ml for flask A (0.5 % Tannic Acid) and (1.5 % Tannic Acid).The progressive increase in enzyme activity was observed with cumulative increase in substrate concentration.

**Keywords**

Solid State fermentation, submerged fermentation, *Rhizopus sp*, Tannic Acid.

## Introduction

Tannin Acyl Hydrolase (E.C. 3.1.1.20) is an inducible enzyme commonly known as tannase. Tannase (TAH)catalyzes the hydrolysis of galloyl ester bond of hydrolysable tannins such as tannic acid, methyl gallate, ethylgallate, n-propyl gallate and iso-amyl gallate, releasing glucose and gallic acid (Bhat et al, 1998).

Tannase is known to be a membrane bound enzyme and is also secreted extra-cellularly. Tannase can be obtained from various sources such as animals, plants and microorganisms; their environmental and genetic manipulation is easier, for generation of novel producers in short time span. Tannase is produced extensively from fungi as they are fast growing and very diverse in nature. Tannase from fungal sources is reported to be highly active over a wide range of pH and temperature. Although tannase production by *Rhizopus sp.* can occur in the absence of tannic acid, this fungi tolerates tannic acid concentrations as high as 20%(w/v), without

having a deleterious effect on both the growth and enzyme production (R. Belmares, et al 2004).

Tannins are widely distributed in common foodstuffs such as tea, straw berry, grapes, mango, walnut, and cashew nuts etc. On the basis of their structural characteristics, Tannin is divided into four major groups namely Gallo tannins, Ellagitannins, complex tannins and condensed Tannins. The most famous source of Gallo tannins is tannic acid, which is in the form of a yellowish white or pale brown powder usually obtained from twig galls of *Rhussemialata.*

Tannic acid, a hetero-polymer of glucose and gallic acid (1: 9), is one of the most abundant reserve materials of plants (Bhat et al., 1998). Industrial bioconversion of tannic acid is generally accomplished by enzyme tannase for the production of gallic acid (3,4,5-trihydroxy benzoic acid). Gallic acid is mostly utilized in the food as antioxidant and pharmaceutical industry for manufacture of trimethoxy benzaldehyde, which is used in the production of a broad-spectrum antibiotic, trimethoprim: an antimalarial drug (Bajpai and Patil, 1996).

Other potential uses of tannase are in manufacture of instant tea, stabilization of malt polyphenols, clarification of beer and fruit juices to reduce bitterness, and prevention of phenol-induced madeirization in wine, fruit juices and reduction of antinutritional effects of tannins in animal feed.

In animals, especially ruminants, tannins have a toxic nutritional effect, which can reduce feed intake and lower nutritional digestibility and protein availability. The reason being that tannin can form complex with protein, starch and digestive enzymes and reduced the nutritional qualities of feeds. The bacterial species with ability to degrade such poly phenolic includes *citrobacter, streptococcus and coryebacterium.* Along with many fungal sp. Like *Rhizopus sp., Aspergillus. Sp. ( A.niger, A tamarii, A.kawachii) Penicillium sp.(Penicillium charlesii, Penicillium crustosum, Penicillium restrictum) etc.*

Studies of tannase production by fungi *Rhizopus* can be proceeded by various fermentation methods like liquid, surface, submerged and solid state fermentation (Bradoo et.al 1997). But the use of SSF is advantageous because of the ease of sterilization and process control.

*Rhizopus* is a filamentous, cosmopolitan and ubiquitous fungus found in nature it is commonly isolated from soil, plant debris and indoor air environment. It is also called as saprophytic fungi, which is widely distributed in nature.

Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns. However substrate used for tannase production under SSF was Palm Karnel cake, Wheat bran (Gustavo et.al 2001).

## Materials and Methods

All the chemicals used for experiments were of pure grade, procured from Hi-Media Pvt. Ltd. Mumbai. The glassware used were of Borosil, all the glassware used for practical purpose was first clean with acid and rinsed with double distilled water.

### Isolation, Identification &Enrichment of Tannase producing Microorganism

### Isolation of Tannase producers

The humus soil was collected from under dense old tree *Tamrindus Indica* and the sample was diluted decimally. One gram of soil was inoculated to the 100 ml of Potato

Dextrose Broth (PDB) and incubated on orbital shaker incubator for 24 hrs.

After 24 hrs of incubation 0.1 ml of culture was spraded on Potato Dextrose Agar (PDA), and incubated at room temperature for 48 hrs. The fungal and bacterial isolates were examined and identified by microscopic and morphological characters.

**Screening of Tannase producer**

Screening was performed on plates of selection medium i.e. tannic acid Potato dextrose agar medium.

Point inoculations were carried out and plates were incubated at 37ºC for 72 hours. After incubation fungal mycelium producing higher zone of clearance were selected for further studies (Bradoo et.al 1996).

**Maintenance of Culture**

The screened of microorganism was used for further studies. The fungi was grown and maintained on Potato Dextrose Agar (PDA. Hi-Media, India) slants by culturing at 30°C and cultures were further preserved at 4ºC.

**Inoculum preparation**

The spore suspension was prepared by scrapping the spores from 1 week old culture and mixing it with 10 ml of 0.1% sterile Tween-80 prepared using distilled water (Sabu et al., 2005).

**Enrichment & Optimization of Culture condition**

The isolates were grown on the fresh media with constant sub-culturing with gradual increase in tannic acid concentration viz. 0.25 % tannic acid up to 1.5 % tannic acid.

**Pilot Scale Fermentation**

**Comparative analysis between Submerged & Solid State fermentation processes & Substrate consumption Profile**

The SMF & SSF was compared for their capacities to produce the Extracellular tannase, the analysis was done by culturing the organism under SSF with 1:1 ration of *Tamrindus Indica* seed powder along with Bran of *Triticum arariticum*, and SMF in flask containing variable concentration of pure grade tannin as substrate (Table: 3 & 4).The enzyme activity was also determined by extracting & Purifying the enzyme by ammonium sulfate precipitation method (Table 4).

**Preparation of Submerged (SMF) Production medium**

The production media for submerged fermentation is prepared from commercially available PDB media and in addition to that 0.5 %, 1% and 1.5 % of Tannic acid was added in corresponding flask i.e. SMF flask "A", SMF flask "B" and SMF flask "C" .Flasks were autoclaved at 110ºC for 30 min and allowed to cool at room temperature. After sterilization cycle the SMF flasks were inoculated with 0.1 ml of Inoculum, the inoculated flask was slowly agitated for few seconds for complete spreading of spore suspension in the medium and then incubated for 72 hrs at 37ºC.

**Preparation of Solid State (SSF) production medium**

The main constituent of SSF medium was Natural Tanin Powder comprising of equimixture of *Tamrindus Indica* seed powder and *Triticum arariticum* Bran(Sabu.*et al*, 2005).

The SSF flask was autoclaved at 121ºC for 15 min and allowed to cool at room temperature. After sterilization the content of flask was distributed in Petri dish aseptically and a uniform lawn was prepared, Then SSF medium was inoculated with one ml of Inoculum and incubated at 37ºC for 72 hrs, Moisture was maintained by spraying sterile saline.

**Enzyme Extraction & Purification**

**Enzyme extraction**

The enzyme extraction was carried out in cold condition using sterile 1% (w/v) sodium chloride solution. 25 ml of 1% (w/v) NaCl was added to pre inoculated & pre incubated flasks, petri dishes after the completion of incubation period and the enzyme was extracted from solid substrate by shaking at 150 rpm for 1 h. The extract was passed through Whatman filter paper No.1 and centrifuged at 10,000g for 5 min. The supernatant was considered as crude enzyme solution and stored at $4^{O}$C.

**Partial purification by Salt Precipitation Method**
**(Ammonium sulphate Fractionation)**

The crude enzyme obtained after fermentation process was purified by Ammonium Sulfate precipitation method.

**Dialysis**

Ammonium sulphate was gradually added to the crude extract to obtain a final concentration of 80% saturation. The ammonium sulphate was added with constant stirring at 4ºC and the mixture stood overnight at 4ºC. The precipitated proteins were separated by centrifugation at 10000 rpm at 5ºC for 30 min. The separated proteins were then re-suspended in distilled water and the solution dialyzed (using cellulose dialysis tubing – Hi-Media) for 24 hrs against distilled water.

**Estimation of Protein by Folin-Ciocalteu Method**

The total protein concentration was determined by folin-ciocalteu method, considering bovine serum albumin as standard protein, 100µg/ml.

**Enzyme assay**

The estimation of tannase activity was carried out by colorimetric assay method of Mondal et al., (2001). The tannic acid was used as a substrate at a concentration of 0.5% (w/v) in 0.2M acetate buffer (pH 5.5). The reaction mixture was prepared by the addition of one ml substrate with 1ml of the crude enzyme and incubated at 30°C for 30 minutes. The enzymatic reaction was stopped by adding 3ml bovine serum albumin (1mg/ml) prepared with 0.17M sodium chloride in 0.2M acetate buffer (pH 5.0). The reference tube also was prepared parallelly using heat-denatured enzyme. The tubes were centrifuged at 5000g for 5min. The resulted precipitate was dissolved in SDS Tri-ethanolamine solution followed by the addition of 1ml of $FeCl_3$ reagent. The contents were kept for 15min for stabilizing the color formed and the absorbency was measured at 530nm against the blank. One unit of tannase activity can be defined as the amount of enzyme, which is able to hydrolyze 1M of substrate tannic acid in 1min under assay conditions.

**Parameter optimization studies**

**Effect of Incubation period**

The point inoculated plates were examined for Taninolytic activity by incubating the inoculated plated for various time intervals like (24, 48, 72, 96 hrs.) To analyze the growth of fungal isolate by utilizing tannin,

the zone of clearance form was considered as a demarcation point for Taninolytic activity and enzyme production (Table 2).

## Results and Discussion

The present study was undertaken in order to isolate tannase producing organism. And to screen their taninolytic potential under different fermentation process (SSF & SMF).

### Isolation and Identification of fungal Isolates

The organism was isolated from humus under the tamarind tree. Characterization and identification of Rhizpus isolates were done on the basis of morphological characteristics in the light of standard literature available. (Practical Microbiology by Dubey & Maheshwari).

### Screening of Tannase producers

Screening was performed on tannic acid agar medium Point inoculations were carried out and after incubations mycelium Producing zone of clearance was selected for further studies.

The study on optimization of incubation time required for the production of tannase by *Rhizopus sp.* and *Penicillium sp*. was carried out and it was found that an incubation time of 72 hours was optimum for maximal production of tannase where zone of clearance was 4.3 cm & 2.1 cm respectively, whereas the further incubation of organism shows no any increase in zone. This may be due to inhibition of enzyme or due to substrate scarcity (Table No.2).

The fungal biomass produced after the fermentation process was measured by taking wet Weight & dry weight, the values obtained were quite striking and mentioned in Table 3, The maximum amount of fungal cake was produced by SSF (7.21 gm) followed by SMF "B" (6.4 gm), SMF "C" (5.49 gm) and least biomass was of SMF "A" (4.42 gm) in terms of wet weight.

The comparison between these two fermentation processes indicates the maximum tannase production takes place by SSF process, the total protein concentration was found to be 96 µg/ml with enzyme activity 2449.22 U/gdw, whereas the SMF retards the growth of fungi and as compare to SSF the SMF does not provides quite feasible conditions for growth of *Rhizopus sp*.

The submerged fermentation shows the degradation of pure grade tannic acid was slow initially among the three sets of SMF, flask "B" produces 93µg/ml of protein with enzyme activity 97.96U/ml. Similarly the 82µg/ml and 86 µg/ml protein was produced by SMF flask "A" & flask "C". The flask "A" & "C" exhibits enzyme activity 48.98 U/ml and 146.95 U/ml respectively. The progressive increase in enzyme activity was observed with cumulative increase in substrate concentration.

The fungi *Rhizopus* was isolated from the humus under the *Tamrindus indica* tree because most of the fungal strains growing under the tree uses the tamarind tree litters as nutrient. So the strains which are growing under the tree soil have the ability to produce different enzymes by utilizing different substrates of the litters of Tamarind tree. Since the Tamarind seed powder contain considerable amount of tannin, it was found that the *Rhizopus* sp. isolated from humus under the *Tamrindus indica* tree possess capability to produce tannase.

During the study four isolates were examined for their tannase production (Table 1), Among these four isolates (B) & (C) were found to be producer of tannase as they exhibits.

**Table.1** Screening of Tannase producers

| Isolates | Taninolytic Activity | Zone of Clearance (after 24 hrs) |
|---|---|---|
| A (*Aspergillus sp.*) | - | No zone of clearance |
| B (Rhizpous *sp.*) | + | 1.6 cm |
| C (Penicillium *sp.*) | + | 1.2cm |
| D (Bacterial colony*) | - | No zone of clearance |

(*Screening was negative for isolate D, therefore only fungal isolates with Taninolytic Activity were selected for further studies), (+ Present & - Absent).

**Table.2** Effect of Incubation period over Taninolytic Activity

| Time Interval | 24 hrs. | 48hrs. | 72 hrs. | 96 hrs. |
|---|---|---|---|---|
| Zone of Clearance in centimeters. | | | | |
| Isolate B (Rhizpus sp.) | 1.6 | 1.8 | 4.3 | 4.3 |
| Isolate C (Penicillium sp.) | 1.2 | 1.6 | 2.1 | 2.1 |

**Table.3** Determination of Fungal Biomass

| Fungal Biomass | SMF (Chemically Defined Medium) | | | S.S.F *Natural Media |
|---|---|---|---|---|
| | **A (0.5 %)** | **B (1 %)** | **C (1.5%)** | |
| Wet Weight (gm.) | 52.70 | 39.35 | 49.29 | 86.25 |
| Net wet Weight (o.1 ml of Inoculum in gm) | **4.42** | **6.4** | **5.49** | **7.21** |
| Dry Weight (gm) | 49.33 | 37.41 | 45.39 | 79.19 |
| Net Dry Weight (o.1 ml of Inoculum in gm) | **2.48** | **3.03** | **1.59** | **4.13** |

**(***equimixture of *Tamrindus Indica* seed powder and *Triticum araritícum* Bran**).**

**Table.4** Comparative analysis between Submerged & Solid State fermentation processes With reference to Substrate consumption Profile

| Type of Fermentation | Substrate Concentration | O.D at 670nm | Concentration of Protein µg/ml | Enzyme Activity U/ml |
|---|---|---|---|---|
| Submerged | "A" 0.5% | 0.14 | 82 | 48.98 |
| | **"B" 1%** | **0.16** | **93** | **97.96** |
| | "C" 1.5% | 0.15 | 86 | 146.95 |
| Solid State | **1:1** | **0.17** | **96** | **2449.22 U/gdw*** |

(*Units per gram of dry weight).

Taninolytic activity their potency was also examined by observing increase in zone of clearance with respect to time (Table 2), the maximum activity was found on and after 72 hrs of incubation in terms of tannin degradation.

Among the isolates B & C, Isolate B (*Rhizopus sp.*) shows maximum zone of clearance at all elected time intervals in comparison to isolate C (*Penicillium sp.*), Hence the isolate B (*Rhizopus sp.*) was selected for production. In different

incubations studies, higher production had occurred at 72 hours and further incubation showed decrease in enzyme production. Decreased enzyme yield on prolonged incubation was due to inhibition and denaturation of the enzyme or depletion of substrate. It has been reported earlier that tannase was produced during the primary phase of growth and there after the activity decreases either due to the decrease in production or due to enzyme degradation.

During fermentation processes, the initial slow growth phase was observed in all three SMF flasks as contrary to rapid growth in SSF plates. The fungal biomass produced after the fermentation process was measured by taking wet weight& dry weight, the values obtained were quite striking and mentioned in Table 3, The maximum amount of fungal cake was produced by SSF (7.21 gm) followed by SMF "B" (6.4 gm), SMF "C" (5.49 gm) and least biomass was of SMF "A" (4.42 gm) in terms of wet weight.

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Tannase are tannin degrading enzyme and hence significant and crucial enzyme for industrial biotechnology. It has wide application in manufacture of instant tea, production of gallic acid, various processes of pharmaceutical industry, and in food & beverages processes viz. clarification of beer and juices to reduce bitterness, reduction of anti-nutritional effect of tannin in animal feeds and in Tannery industries.

The production of tannase by Rhizopus was studied in solid state fermentation & compared with SMF, The fungus produced an extra cellular tannase in two-three days of growth on media containing Tamrind seed powder and Wheat bran. The present study was planned with objective to isolate tannase producing microorganism from soil, Production of tannase, study of incubation period, and comparison of Taninolytic activity of fungal origin on Natural substrate against chemical grade substrate.

During the work tannase production was carried out by two different fermentation process, and it was found notice that the SSF process is favorable for fungi to sustain and grow by utilizing natural tannic acid in the form of Tamrind seed powder, Whereas the SMF which was supplemented with pure grade Tannic acid the growth is similar to SSF but the enzyme activity was lagging as compare to SSF.

The maximum yield of enzyme was achieved on incubation at 72 hrs. And latter incubation shows inhibition and slows down in enzyme activity. From the data obtained it can be interpreted that the obtained fungal isolate is potent producer of Tannase and SSF process may be a method of choice for large scale production. One most striking outcome of the study is that the little amount of tannic acid either pure grade or raw source is sufficient to induce the enzyme production, and remaining fermentation cycle can be done from brans of staple food or Agro residues.

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