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# PRODUCTION OF TANNASE THROUGH SUBMERGED FERMENTATION OF TANNIN-CONTAINING CASHEW HUSK BY *ASPERGILLUS ORYZAE*

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**ABSTRACT**

Tannins are water soluble polyphenolic compounds found in plants as secondary metabolites which can be grouped as hydrolysable and non-hydrolysable tannins. The presence of these substances in the husk of cashew was initially extracted and their crude extract was used separately as a substrate for the production of tannase through submerged fermentation by *Aspergillus oryzae*. Tannase production as well as the biodegradation of the substrate reached maximum within 24 to 36 h against crude tannin extract obtained from *Anacardium occidentale*. Tannse production was higher by almost two fold in the presence of crude tannin compared to pure tannic acid used as a substrate. It seems that the industrial production of tannase, using husk extract of *Anacardium occidentale* can be a very simple and suitable alternative to presently used procedures. This method appears to be much more accurate than those reported earlier.

**Keywords**: Tannase, Tannic acid, Submerged fermentation, *Aspergillus oryzae.*

## INTRODUCTION

Microbial production of tannin acyl hydrolase (EC 3.1.1.20), commonly referred as tannase is well documented[12]. It is used widely in the manufacture of instant tea, acorn wine and gallic acid [10]. Gallic acid is an important substrate for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry [4]. Tannase also has potential applications in the clarification of bear and fruit juices, manufacture of coffee flavored soft drinks, improvement in the flavor of grape wine and as an analytical probe for determining the structures of naturally occurring gallic acid esters.

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 enzyme is synthesized by a number of fungi. Based on a preliminary screening of various isolates and other available fungal cultures, one of these organisms, *Aspergillus oryzae*, was selected for further studies. The present studies were aimed at identifying key environmental parameters that play important roles in enzyme synthesis by *Aspergillus oryzae* and their optimization by submerged fermentation.

Most of the reported tannase-producing organisms are fungi [1] and only a few are bacteria. Many authors studied tannase production by these organisms in the medium containing purre tannic acid acting as both inducer as weel as available carbon source. Pure tannic acid is a very costly substrate and is not suitable for large-scale production of the enzyme. In this respect crude tannin could be cost effective and suitable for the commercial production of the enzyme. Agro-residues and forest products are generally considered the best source of tannin-rich substrate [14]. Production of tannase by *Rhizopus oryzae* and *Aspergillus foetidus* from the powdered fruits of Terminalia chebula and Caesalpinia digyna has been reported[11]. In this regard there are no reports on bacterial tannase production using tannin containing any agro-based substrates.

In the present publication we are reporting for the first time the production of tannase by *Asperigllus oryzae* through submerged fermentation of crude tannin extracted from the cashew husk.

## EXPERIMENTAL

### Materials and methods

Microorganism: The non-pathogenic tannase – producing strain of *Aspergillus oryzae* 643 ATTC 93692 (*Aspergillus sajae*) obtained from the NCIM Pune, was used in the present study.

### Preparation of Inoculums

Tannase being an adaptive enzyme, pre-Induced inoculum is required to be prepared. Inoculum was prepared by growing a loopful amount of stock culture of the Fungi in 50 ml sterile modified Czapek's dox medium and slightly modified mildew test (MT) medium is used. Medium will be autoclaved (21°C) for 20 min and allowed to cool at room temperature. The filter sterilized, tannic acid (2%) will be added to each sample. Composition of alternative medium was (g %, w/v): $KH_2PO_4$, 5; $NH_4NO_3$,10; $MgSO_4$ $7H_2O$, 1: $CaCl_2$ $6H_2O$, 0.1: $MnCl_2$ $6H_2O$, 0.02: NaMO $O_4$ $2H_2O$, 0.01: $FeSO_4$ $7H_2O$, 0.125 glucose, 25.

### Extraction of crude tannins

Collected cashew husk from cashew industries near to Visakhapatnam, were grained into small particles, and dried in hot air oven at 60°C for 24 hours. The testa (50 g) were then mixed with distilled water (200 ml) and kept at room temperature overnight. After soaking, the mixture was autoclaved under pressure autoclaving for 30 min. The filtered solutions were used as source of crude tannin.

### Detection of tannin by paper chromatography

Presence of tannin in the cashew testa extract was confirmed through paper chromatographic analysis. A descending mode of solvent system containing n – butanol, acetic acid and water (4:1:5) was used for the study. Detection of the spot was made by $FeCl_3$ (0.1 g% in 30% methanol) as coloring spray reagent and confirmed after comparing it with standard tannic acid.

### Measurement of tannin biodegradation

The tannin content of the crude cashew testa extract was measured before and after fermentation by Floin – Denis method[13] . The crude extract (0.2 ml) was initially diluted with 8.3 ml of distilled water and then mixed with 0.5 ml of Folin – Denis reagent. After proper mixing, 1 ml of 15% (w/v) $Na_2CO_3$ was added to it and kept in the dark for 30 min at room temperature. The absorbency of tannin was measured spectrophotometrically at 700 nm and its concentration was calculated using pure tannic acid as standard.

### Fermentation process and extraction of tannase enzyme

Tannase production by *Aspergillus oryzae* was achieved through submerged fermentation of crude tannin at 35°C in a rotary shaker (160 rpm). Different concentrations of tannin were prepared by diluting the measured crude tannin with distilled water. The pH of the medium was adjusted to 5.5 after sterilization. Fermentations were carried out separately in individual 250 ml Erlenmeyer flasks containing 50 ml modified Czapek's dox medium with (1% v/v) fresh inoculum.The cell – free fermented broth was used as the source of the enzyme. The growth of organism in culture media was monitored by measuring dry weight of the biomass (mg/ml).

### Assay of tannase

Tannase activity in the fermented medium was determined by the colorimetric method of[12]. For assay, 0.1 ml of enzyme was incubated with 0.3 ml of substrate tannic acid (1.0% w/v in 0.2 M citrate buffer, pH 5.0) at 50°C for 30 min. The reactin ws terminated by the addition of 3 ml BSA solution (1 mg/ml), which also precipitated the residual tannic acid. A control reaction was done side by using heat-denatured enzyme. The tubes were then centrifuged (5000 x g, 10 min) and precipitate was dissolved in 2 ml of

SDS-triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine) solution. Absorbency was measured at 530 nm after addition of 1 ml of $FeCl_3$ (0.13 M).

The specific extinction co-efficient of tannic acid at 530 nm was 0.577 . Using this co-efficient, one unit of tannase activity is defined as the amount of enzyme required to hydrolyze 1 mm substrate (tannic acid) in 1 min at 50°C and pH 5.0.

## RESULTS AND DISCUSSION

The selection of a substrate for large – scale enzyme production by fermentation depends upon its availability and cost. In this regard the waste residue of cashew husk was used as substrate for obtaining the desired fermented product[11-14].

Tannin contents of the bark of some commonly available plants were initially examined by paper chromatography and quantified by colorimetric method (Table 1). Among the eight plant species tested, the maximum amount of tannin was found in the extract of the husk of cahew. Tannase production by A. Oryzae was studied using cashew testa tannins as subnmerged fermentation media. It has been found that extract of A. occidentale was the best for induction of tannase production. A similar type of timer related enzyme production by the same organism was also reported with pure tannic acid as substrate . Tannase production by the organism was found to be maximal in the extract of A. occidentale. It is not clear to us why the production of enzyme in the extract of A. occidentale is high, but we assume that there may be some inducing factors that accelerate enzyme synthesis [8-9].

In the present work, studies on the tannase production from *Aspergillus oryzae* using cashew husk was carried out and the results were given in the tables and figs. The effect of some parameters at different ranges was studied and their influence on the production was discussed in this paper.

### Optimization of some parameters for maximum tannase production

#### *Effect of incubation period on tannase production*

To evaluate the effect of different incubation period on tannase production, the incubation period of the medium range was varied from 12 h to 72 h. With a rise in incubation period, the tannase production increased and optimum activity was recorded at 24 h (Fig. 1). With a further increase in incubation period, there was a decrease in activity. Maximum Tannase activity was 30.6 U/ml by the *Aspergillus oryzae* after the optimum incubation period of 24 h. The obtained results were tabulated in Table - 2 and also shown in Fig.1. An optimum incubation period around 36 h has been reported for tannase activity in case of A. aculeatus D B F 9[10].

#### *Effect of pH on tannase production*

To Study the effect of initial pH on tannase production, the pH of the medium was varied from 3.5 – 6.0 using 1 N HCl and 1 N NaoH, and fermentation was done as usual. The enzyme was active at acidic pH and activity decreased as the pH 5.0 (Fig.2). Maximum tannase activity was 32.62 U/ml by the *Aspergillus oryzae* after the optimum pH of 5.0. It could be concluded from the results that tannse from the *Aspergillus Oryzae* needed an acidic environment to be active. Fungal tannase is an acidic protein in general. The obtained results were tabulated in Table - 3 and also shown in Fig.2. There are reports describing of the optimum pH as 5.5 in case of tannase obtained from *A. Oryzae,* and 6.0 in case of tannase obtained from *P.chrysogenum* and *A. niger*[2-5] .

#### *Effect of temperature on tannase production*

To Study the effect of different temperatures on tannase production, the flasks containing medium kept at temperature range was varied from 25°C – 50°C. With a rise in temperature, the tannase production increased and optimum activity 30.62U/ml was recorded at 40°C (Fig.3). With a further increae in temperature, there was a decrease in activity. The optimum temperature for tannase production was 40°C. The obtained results were tabulated in Table- 4 and also shown in Fig. 3. An optimum temperature around 30°C has been reported for tannase activity in case of *A. oryrae*[7] and *P. chysogenum,* around 35°C in case of *A. Niger* and 50°C in case of candida sp[1].

Enzyme production was also studied at different concentration (0.5 – 2.0%, w/v) of crude tannins. It was observed that a specific concentration of crude tannin from a plant influenced enzyme production the strongest (table 11). In our experiments a maximal amount of enzyme was produced in medium containing 0.5% (w/v) of crude tannin of A. occidentale, but the highest enzyme production was observed when 1.5% (w/v) of pure tannic acid was used as substrate in the medium the concentration of tannin is thus a very important determining factor for tannase biosynthesis for most fungi and bacteria[10] . The actual mode of tannase induction in a particular concentration of tannin has not been properly explained until now. The higher concentrations of tannin lead to non-reversible bonds with surface proteins and impair the metabolism as well as growth of the organism. One of the most striking observations in this experiment is that enzyme production was increased about four-fold in the medium containing basal salt with crude tannin (0.5%) compared to medium containing crude tannin extract (0.5%) of *A. occidentale* alone (Table-2). This result revealed that some specific microelements (salts and ions) are probably essential for growth as well as enzyme synthesis by A. Oryzae. Both growth of the organism and enzyme production increased two-fold when it was grown in salt containing crude tannin extract rather than enriched pure tannic acid medium. All these beneficial effects of the plant extract of A. occidentale make it promising as one of the best as well as cheaper substrates for the large scale production of microbial tannase[3].

In conclusion, tannase has now been extensively used in different biochemical industries. The selected bacterium used in this study is able to synthesize high amounts of tannase through fermentation of crude tannin of *A. Occidentale*. Exploitation of these plant extracts could be a source of cheaper substrate for industrial production of microbial tannase.

Table-1:Measurement of tannin content in crude plant extract, tannin biodegradation and tannase production by *Aspergillus oryzae* 643 ATTC93692.

| Plant source | Tannin content in the crude extract (g% w/v) | Biodegradation (%) of tannins through fermentation | Tannase production (U/ml) |
|---|---|---|---|
| Acacia auriculiformis | 1.33 ± 0.18 | 27 ± 3.60 | 0.32 ± 0.09 |
| Anacardium occidentale | 0.65 ± 0.10 | 73 ± 4.65 | 0.62 ± 0.04 |
| Casuarina equisetifolia | 0.85 ± 0.09 | 34.5 ± 5.33 | 0.06 ± 0.10 |
| Cassia fistula | 0.43 ± 0.16 | 64.4 ± 1.88 | 0.52 ± 0.01 |
| Delonix regia | 0.54 ± 0.09 | 21 ± 3.38 | 0.12 ± 0.07 |
| Eucalyptus tereticornis | 0.45 ± 0.12 | 58 ± 6.10 | 0.17 ± 0.02 |
| Ficus benghalensis | 0.39 ± 0.20 | 53 ± 1.58 | 0.40 ± 0.11 |
| Psidium guazava | 0.73 ± 0.03 | 0.08 ± 0.01 | 0.32 ± 0.06 |

Table-2:Comparative study of tannase production in pure tannic acid and crude tannin extract as substrates by *Aspergillus Oryzae*

| Substrates in fermentation medium | Growth (mg/ml) | Tannase (U/ml) |
|---|---|---|
| Crude tannin (0.5 g/100 ml) | 0.82 ± 0.18 | 0.17 ± 0.06 |
| Crude tannin (0.5 g/100 ml) + Basal salts* | 1.12 ± 0.22 | 0.66 ± 0.12 |
| Tannic acid (1.5 g/100 ml) containing enriched medium** | 0.58 ± 0.12 | 0.31 ± 0.13 |

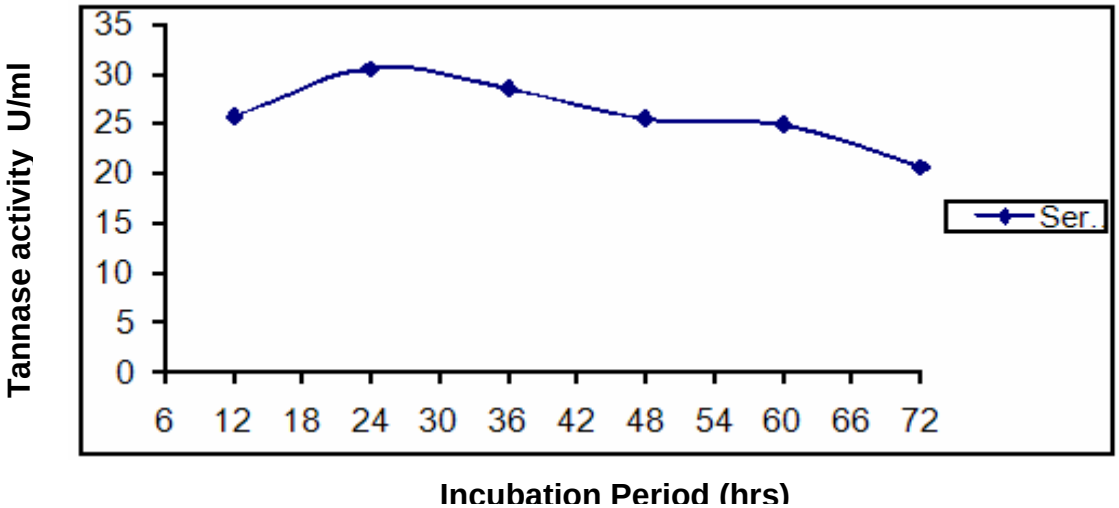


Fig.-1: Effect of Incubation period on Tannase production

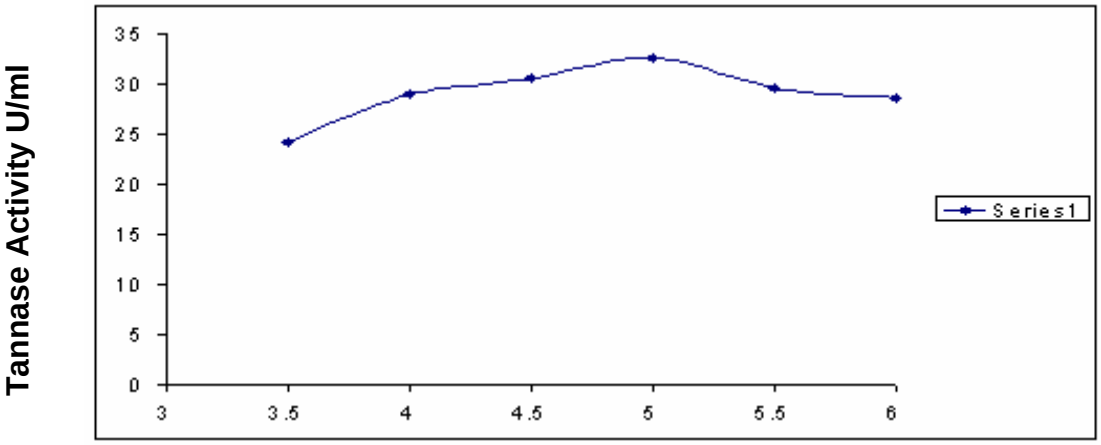


Fig.-2: Effect of pH on Tannase Production

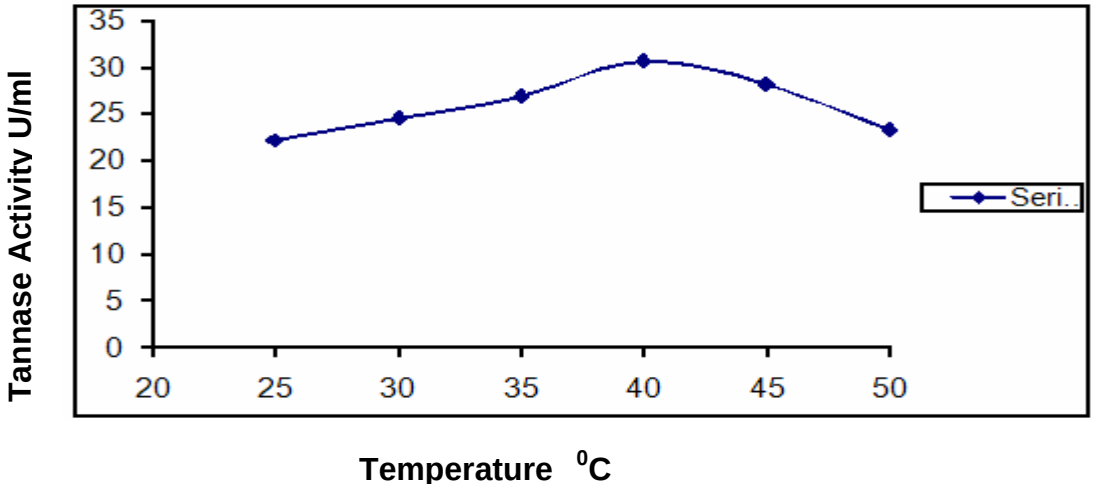


Fig.-3: Effect of Temperature on Tannase production

Table-3- Effect of Incubation period on Tannase production

| S.No. | Incubation period (h) | Tannase activity U / ml |
|---|---|---|
| 1. | 12 | 22.62 |
| 2. | 24 | 30.62 |
| 3. | 36 | 28.54 |
| 4. | 48 | 25.42 |
| 5. | 60 | 24.83 |
| 6. | 72 | 20.62 |

Table-4: Effect of pH on Tannase production:

| S.No | pH | Enzyme activity (U/ml) |
|---|---|---|
| 1 | 3.5 | 24.32 |
| 2 | 4 | 28.94 |
| 3 | 4.5 | 30.56 |
| 4 | 5 | 32.62 |
| 5 | 5.5 | 29.62 |
| 6 | 6 | 28.62 |

Table-5: Effect of Temperature on Tannase production:

| S.No. | Temperature °C | Tannase activity U / ml |
|---|---|---|
| 1. | 25 | 22.20 |
| 2. | 30 | 24.52 |
| 3. | 35 | 26.85 |
| 4. | 40 | 30.6 |
| 5. | 45 | 28.12 |
| 6. | 50 | 23.23 |

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