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TANNASE PRODUCTION FROM CASHEW HUSK BY SOLID-STATE FERMENTATION

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ABSTRACT
Tannase production under solid-state fermentation was investigated using isolated *Aspergillus oryzae*. Among all agro-industrial waste material evaluated, cashew husk supported maximum tannase production. The metabolic processes of microorganisms are influenced by changes in parameters like Temperature, pH, incubation time, humidity etc., which are very specific for a particular organism. Microbial synthesis of enzymes in a SSF process are also affected by factors like particle size of substrate, water content, relative humidity, type and size of inoculum, control of temperature, period of cultivation, etc. Biotransformation of cashew husk tannin to gallic acid by SSF is also influenced by all the factors affecting tannase production, since the synthesized enzyme causes the breakdown of tannin to gallic acid and glucose.

KEYWORDS: Tannins, Tannase, cashew husk, Enzyme extraction, solid-state Fermentation, Gallotannins.

INTRODUCTION

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. 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 by *Aspergillus oryzae*. Tannase production as well as the biodegradation of the substrate reached maximum within 24 to 36h 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. Microbial production of tannin acyl hydrolase (EC 3.1.1.20), commonly referred as tannase is well documented (Madhevan *et al.,* 1985). It is used widely in the manufacture of instant tea, acorn wine and gallic acid (Lekha and Lonsane, 1997). Gallic acid is an important substrate for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry (Hadi et al., 1994; Mukherjee and Banerjee, 2003). 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 (Aoki *et al*., 1976, Bhat *et al*., 1998, Mondal *et al*., 2001c; Ramirez-Coronel *et al.,* 2003) and only a few are bacteria (Deschamps *et al*., 1983; Mondal and Pati, 2000; Mondal *et al*., 2001a). Many authors studied tannase production by these organisms in the medium containing pure tannic acid acting as both inducer as well 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 (Pandey *et al*., 1999). Production of tannase by *Rhizopus oryzae* and *Aspergillus foetidus* from the powdered fruits of *Terminalia chebula* and *Caesalpinia digyna* has been reported (Mukherjee and Banerjee, 2004). 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.

MATERIALS AND METHODS

Microorganism: The non-pathogenic tannase – producing strain of *Aspergillus oryzae* 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 (Schalderi, 1970).

Detection of tannin by Thin layer chromatography

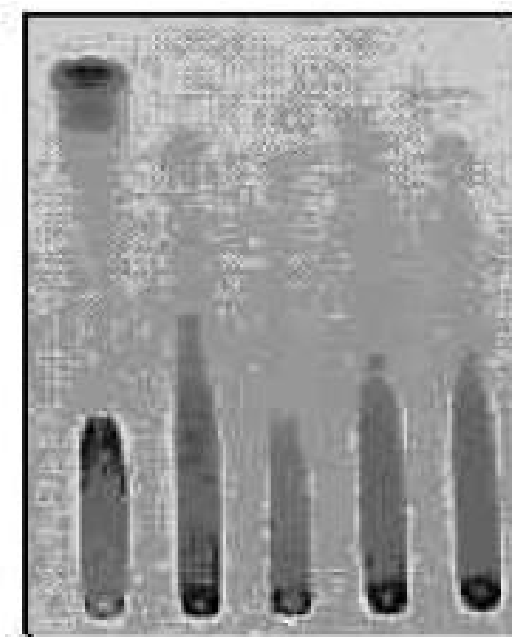

Figure 1- TLC ethanol extract from cashew husk (1-dry and milled: 2-dry and entire: 3-natural and milled: 4-natural and entire: A-tannic acid standard).

- Presence of tannin in the cashew testa extract was confirmed through thin layer chromatographic analysis.
- To obtain pure compounds, the appropriate chromatographic techniques are used.
- Followed by reverse-phase chromatography (in water and alcohol, or water, alcohol and acetone mixtures)

Measurement of tannin biodegradation

The tannin content of the crude cashew testa extract was measured before and after fermentation by Floin – Denis method (Schanderi, 1970). 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 spectro-photometrically 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 Mondal *et al*. (2001b). 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 (Mondal *et al*., 2001b). 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 & 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 (Pandey *et al*., 1999). Tannin contents of the bark of some commonly available plants were initially examined by paper chromatography and quantified by colorimetric method (Table 1).

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

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

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 (Mondal and Pati, 2000). 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.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

1. Effect of incubation period on tannase production

The production of tannase has increased with increase in incubation time up to 48 hrs, with further increase in the incubation time, decrease in the tannase production was observed. This may be due to the starting of the declining phase of the organism after 48 hrs. Fig. I shows that, an incubation period of 48 hrs was optimum for tannase production by *Aspergillus oryzae.* An optimum incubation period around 36 h has been reported for tannase activity in case of *A. aculeatus D B F 9*. Lekha and Lonsane (1994).

Table II. 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

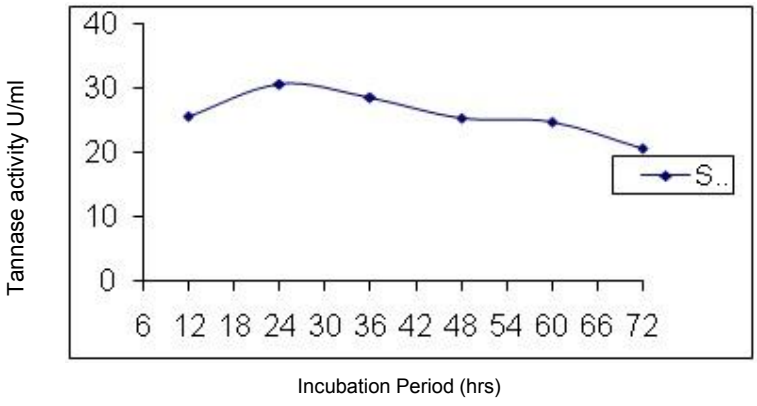

FIGURE1. Effect of Incubation period on Tannase production

2. Effect of P^H on tannase production

It could be concluded from the results that tannase from *Aspergillus oryzae* needed an acidic environment to be active. Fungal tannase is an acidic protein in general. 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,* (yamada *et al*. 1968) and 6.0 in case of tannase obtained from *P.chrysogenum* and *A. niger,* Barthonery *et al*. (1994).

TABLE III. 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

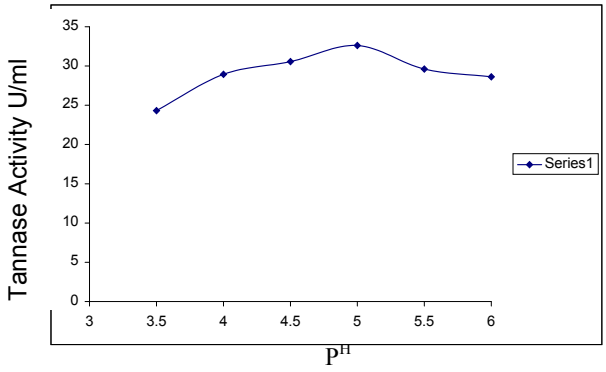

FIGURE 2. Effect of P^H on Tannase Production

3. 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* (Iibuchi's, *et al*. 1968) and *P. chysogenum*, around 35°C in case of *A. Niger* and 50°C in case of candida sp. (Aokoki *et al.,* 1976).

TABLE IV: 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

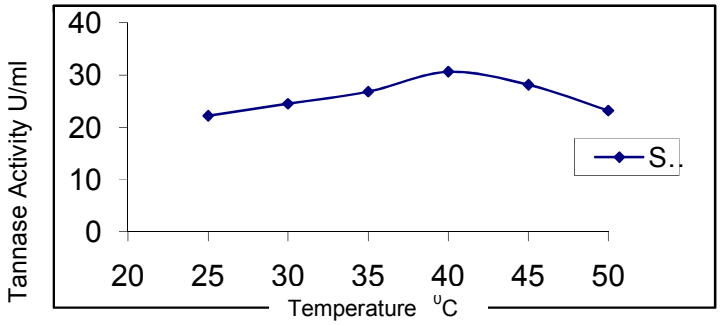

FIGURE 3. Effect of Temperature on Tannase production

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 (Mondal *et al*., 2000) 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 (Lekha and Lonsane, 1997; Mondal *et al*., 2000; Banerjee *et al*., 2001). The actual mode of tannase induction in a particular concentration of tannin has not been properly explained until now. Lewis and Starkey (1969) mentioned that 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 II). 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.

TABLE V- 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

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.

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