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# Production on Tannin Acyl Hydrolase from Pulse Milling By-Products Using Solid State Fermentation

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**Abstract:** The aim of this work was to utilize Red gram husk as a substrate for tannase production and optimize the Temperature and incubation period of tannase production in solid state fermentation. The maximum production of tannase was found to be 43 U/g/min at 35°C, incubated for 96 h. The tannin content in the substrate was decreased during incubation period. The tannase prepared from *Aspergillus niger* had a specific activity of 45 U/g/min in immobilized form.

**Key words:** Tannase · Redgram husk · Immobilization · Tannin

## INTRODUCTION

Red gram ranks sixth among pulses production in world and major legume crop. In India it is the second largest pulse crop accounting about 20 percent of total pulse production (Tab 1). India annually produces about 2.0-2.5 million tonnes and stagnant in the past 10 years. The shift in cultivation from pulses to commercial crops and lack of technological innovations to increase yields has hindered the rise in output. The area sown under crop was 34.02 lakh hectares during 2005-06 against 31.47 lakh hectares in previous year.

Tannase is an extremely important in various industries such as in the manufacturer of instant tea, beer, fruit juice, coffee-flavour soft drink and grape wine. In addition, gallic acid, the product of hydrolytic cleavage of tannic acid, is a chemical used in pharmaceuticals. Tannase catalyses the breakdown of hydrolysable tannins such as tannic acid,methylgallate, ethyl gallate, n-propylgallate and isoamylgallate.A typical reaction of tannase is the hydrolytic cleavage of(-) epigallocatechin-3-ol gallate. [1,2].

Table 1: World wide production of Rice gram in 2005

| Worldwide Area, Production and Productivity in 2005 | | | |
|---|---|---|---|
| Country | Area in Ha | Production Mt | Yield in Kg/Ha |
| World | 4,587,042 | 3,277,995 | 714 |
| India | 3,500,000 | 2,400,000 | 685 |
| Kenya | 200,000 | 105,000 | 525 |
| Malawi | 123,000 | 79,000 | 642 |
| Myanmar | 540,000 | 500,000 | 925 |
| Nepal | 29,000 | 26,000 | 896 |
| Tanzania | 68,000 | 50,000 | 735 |
| Uganda | 84,000 | 84,000 | 1000 |

The present day trend is the utilization of waste material for production of byproducts which boosts up high economic returns in many industries [3]. With the advent of biotechnology, attempts have increasingly been made globally to make potential use of agro-industrial residues for value addition by production of enzymes, organic acids, bioactive secondary metabolites, single-cell protein, etc [4-6].

Solid-state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites.

In this study, Solid state fermentation was carried out using Red gram husk as a substrate for tannase production and optimize its process parameters. No attempt has been made elsewhere to use Red gram waste as a substrate for Tannase enzyme production.

**Microorganisms and Screening of *Aspergillus niger* for Tannase Production:** The strain *Aspergillus niger* used in this study was isolated from the soil and maintained on Potato dextrose agar slants. The screening of *A.niger* for the production of tannase was carried out in agar plates following the method of Murugan *et al*. [7] with filter sterilized tannic acid as substrate.

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**Preparation of Spore Inoculum:** The inoculum was prepared by adding 10 ml of sterile distilled water to the sporulated slants and dispersed the spores; 1 ml of the spore suspension was inoculated in to the production medium.

**Substrate and Solid State Fermentation:** Five gram of Red gram husk was moistened with 10 ml of salt solution. The composition of the salt solution was $NH_4NO_3$ 0.5 %, NaCl 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.1 % and Tannic acid 4% at pH = 5.5. The contents were sterilized by autoclaving at 121°C; 15lbps for 20 min. The cooled sterilized solid substrate was inoculated with 1 ml of the spore inoculums, mixed properly and incubated at 30°C for 96 h.

**Extraction and Estimation of Crude Tannase:** Tannase was extracted from the fermented substrate by adding 0.05 M citrate buffer, pH 5.0 and crushed with Mortar and Pestle, the crude enzyme was centrifuged at 8000 rpm at 4°C for 20 min. The filtrate was collected and estimation of tannase was carried out. Tannase activity was estimated following the method of Sharma *et al*., [8].

**Purification of Tannase:** Purification of tannase was carried out by ammonium sulphate precipitation and column chromatography. To the crude extract 80% of solid ammonium sulphate was added, mixed well and kept it for over night at 4°C. The precipitate was collected by centrifugation at 8000 rpm for 20 minutes. The precipitate was suspended in 0.05 M citrate buffer (pH-5) and transferred to dialysis tube and dialyzed over night at 4° C against the same buffer. The dialyzed sample then subjected to DEAE Sephadex A-50 chromatography and collected the fractions.

**Effect of Temperature:** The solid state fermentation was carried out at different temperature such as 30°C, 35°C and 40°C.

**Effect of Incubation Period:** The effect of incubation period for solid state fermentation was carried out for 24 h, 48 h, 72 h, 96 h and 120 h.

**Extraction and Estimation of Tannin:** The tannin was extracted before and after fermentation from the substrate and estimated the tannin content following the method of Folin-Denis [9].

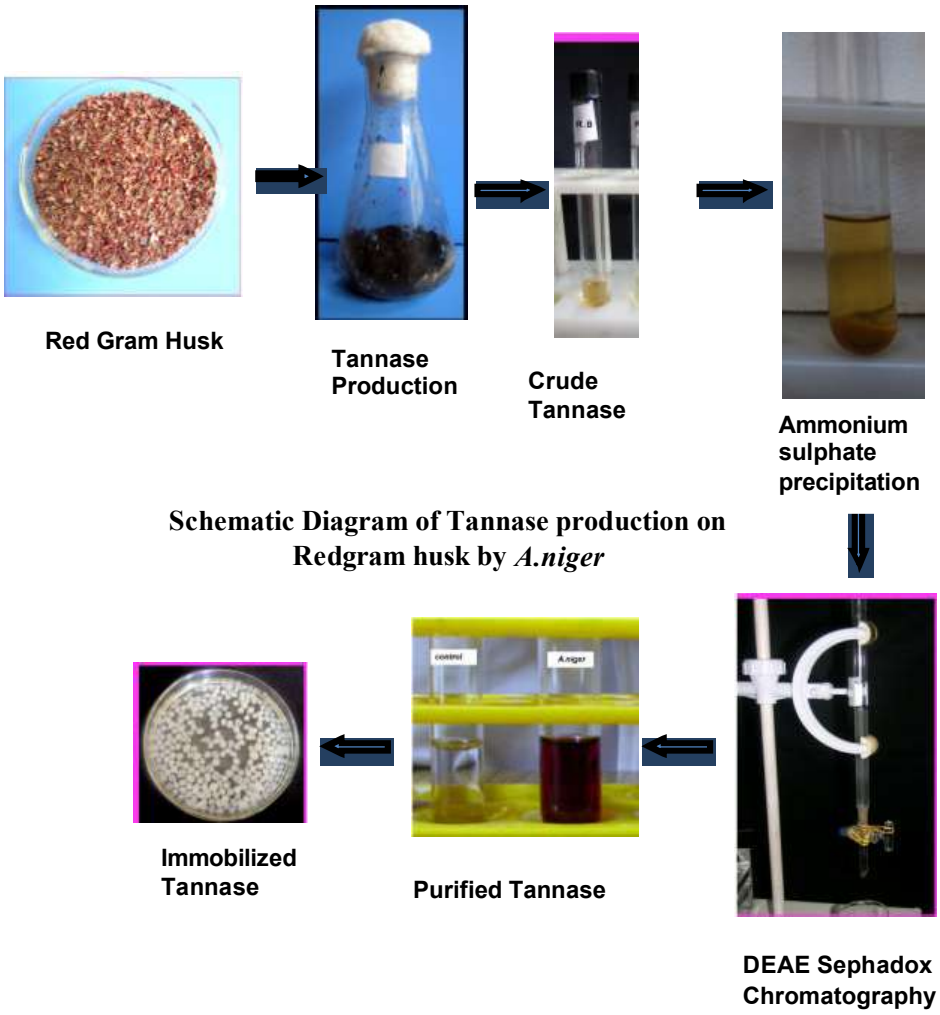
**Immobilization of Tannase:** The purified enzyme from *Aspergillus niger* fermented with Redgram husk was taken for immobilization. An equal volume of enzyme solution and sodium alginate solution was mixed to give a 4% (w/v) final concentration of sodium alginate solution in the mixture. The mixture obtained was extruded dropwise through a pastuer pipette (1mm diameter) into a gently stirred 2% (w/v) $CaCl_2.2H_2O$ solution for 2 h to give bead size of 3mm. The calcium alginate beads containing the enzyme were thoroughly washed with distilled water and used for further studies.

## RESULTS AND DISCUSSION

**Screening of *A. niger* for Tannase Production:** *Aspergillus niger* was screened for the production of tannase on solid media. After three days of incubation clear zones appeared around the colonies (Fig. 1). Zones formed due to hydrolysis of tannic acid to gallic acid and glucose [10], leading to a decrease in opacity of the media.


Fig. 1: Zone of hydrolysis produced by the fungus *A.niger*

**Tannase Production by SSF:** The extracellular tannase produced by the fungus *A.niger* on Redgram husk was showed in Fig.2.The maximum production of tannase was found at 96 hrs of incubation. The same results we have obtained in our previous study. In crude form the activity was 14 U/g/min, after ammonium sulphate precipitation the activity increased up to 25 U/g/min.It was further purified with column chromatography the activity was found to be 43 U/g/min. Lekha, [11] and Sabu *et a*l. [12] reported maximum extracellular tannase and gallic acid production was recorded in 96 h and 120 h by *Aspergillus niger* and *Rhizopus oryzae* [13].



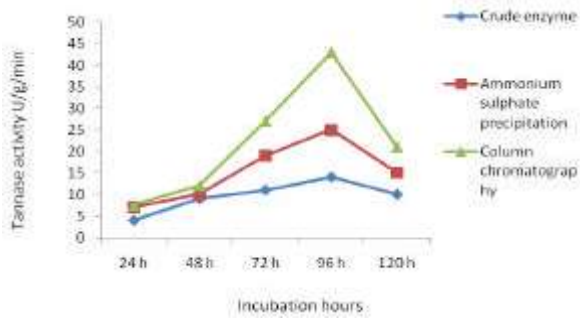




Fig. 2: Tannase activity *by A.niger* fermented with Redgram husk

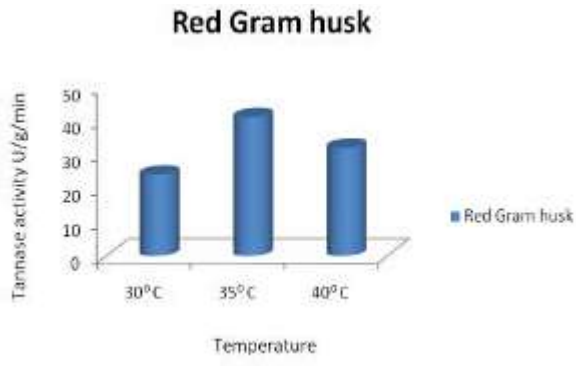



Fig. 3: Effect of temperature for tannase production

**Effect of Temperature:** The optimum temperature for the highest tannase production (41 U/g/min.) was found to be at 35°C. (Fig. 3). The temperature is one of the most critical parameters that have to be controlled in a bioprocess [14]. The process temperature should ensure the optimum growth of the organism.

**Estimation of Tannin:** Before fermentation the red gram husk had 2.6 mg/g but after fermentation the tannin content was reduced by the fungus *A.niger*. It might be the enzyme produced by the fungus, degrade tannin in the substrate. The final tannin content in the substrate was 0.3 mg/g. Fungal tannases have a better activity in degrading hydrolysable tannins, whereas yeast tannases degrade tannic acid better and has a lower affinity for naturally occurring tannins [14]. Filamentous fungi also have the ability to degrade tannins as a sole source of carbon [15,16].

**Immobilization of Tannase:** Immobilized tannase produced from *A.niger* fermented with Redgram huk showed the highest activity of 45 U/g/min. The immobilized preparation was quite stable to reuse, there was no loss of enzyme activity after three cycles and it retained 81% activityeven after the sixth cycle. Ester hydrolysis using the immobilized enzyme led to a 40% conversion into gallic acid as compared with 30% obtained with the free enzyme.

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