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# Extraction, partial purification and application of tannase from *Aspergillus niger* MTCC 2425

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**Abstract**
Tannin acyl hydrolase produced extra-cellularly by *Aspergillus niger* in solid state fermentation of Rice husk. The enzyme was purified from the cell-free extract by ammonium sulphate precipitation followed by diethylaminoethyl-cellulose column chromatography. The protein content of crude and purified was found to be 7·6 and 0·45 mg/ml respectively. The specific activity of crude tannase was found to be 28·5 U/mg of protein while that of purified tannase was 173·0 U/mg of protein. The optimum temperature and pH of the crude sample was analysed and found to be 35 to 40 ºC and pH 5 respectively. The enzyme obtained was applied for declarification of pomegranate juice. In the declarification of fruit juice, a 56% decrease in tannin content was observed after 2 h of incubation at 37 ºC with 1 ml of purified tannase (173 U/mg).

**Keywords:** Rice husk, Tannase, *Asperigillus niger*, Pomegranate Debittering.

## 1. Introduction
Tannase is a hydrolytic enzyme, catalyses the hydrolysis of ester bonds in hydrolysable tannins such as tannic acid, thereby releasing glucose and gallic acid. Tannase production can be achieved by various methods such as liquid surface; submerged (SmF) and solid state fermentation (SSF). Filamentous fungi are ideally suited for successful solid state fermentation since they grow on solid substrate in the absence of free water [1].

Agro-industrial waste materials are generally considered as the substrate for the process of enzyme production. Studies have shown that production of tannase was laid down using waste materials like apple bagasse [2]. Red gram husk [3]. sugar cane bagasse [4] and also using different microbial strains like bacteria [5,6] and other fungal strains [7, 8, 9]. The major crop waste produced in India are straws of paddy, wheat, millet, sorghum, pulses, oil seed crops, maize stalks, jute sticks, sugar cane trash, mustard stalks, etc. Several agro-industrial waste and by-products such as orange bagasse, sugar cane bagasse [4]. Wheat bran [10] and other food processing waste [11]. are effective substrates for depolymerising enzyme production by solid state fermentation, which proved to be highly efficient technique in the production of tannase.

Till today there are no reports regarding usage of rice husk as substrate for production of tannase by *Asperigillus niger*. The substrate employed in this study is an agro-industrial waste generated by paddy industries, which are very common in our country that are discarded as dump and often become environmental pollutants. An alternative solution to this problem would be the use of such agro-industrial wastes as sources of carbon, nitrogen, and tannins for the production of tannase by microorganisms through SSF. However, both the optimization of the growth parameters in a bioreactor and the purification of the enzyme are necessary to determine the commercial viability of the enzyme. Thus this study was aimed to investigate the bioconversion of rice husk for tannase production and optimization of fermentation processing condition required for maximum production and possible application in the de-clarification of juices like pomegranate.

## 2. Materials & Methods
### 2.1. Microorganism and Maintenance of Culture
*Aspergillus niger* strain no. MTCC 2425 was collected from Institute of Microbial Technology, Chandigarh, India and was maintained on Czapek Dox agar media, acquired from Hi-media laboratories. The fungal strains were cultured, grown at 30 ºC for 6 days, and were stored at 4 ºC.

### 2.2. Production of tannase under SSF
Fermentation medium used for tannase production contained 5 g of rice husk moistened with 10 ml of prepared salt solution ($NH_4NO_3$-0.5%, NaCl-0.1%, $MgSO_4$, 7 $H_2O$-0.1% and Tannic acid-4% at pH-5.0). The media was autoclaved and the solid substrate was inoculated with spore suspension (1 x $10^7$ spores/ml). The contents were mixed properly and incubated at 30 ºC for 96 h in a BOD incubator (Sambros, India) [4].

### 2.3. Extraction and Analysis of Crude Enzyme
The fermented substrate produced by SSF was mixed with 0.05 M citrate buffer and agitated for 2 h at 90 rpm in a BOD incubator shaker (Sambros, India) and filtered through Whatman No. 1 filter paper followed by centrifugation at 8000 rpm at 4 °C for 20 min (Cooling Centrifuge, REMI, India). The clear supernatant was used as crude enzyme.

The enzyme activity was estimated following protein precipitation method [12]. using gallic acid as standard. The absorbance was read at 260 nm using a spectrophotometer (Perkin Elmer Lambda 25, UV-Vis Spectrophotometer). One unit of enzyme activity was defined as the number of micromoles of gallic acid formed per min. Protein was estimated by the method using bovine serum albumin as a standard and was expressed in mg/ml [13].

### 2.4. Purification and Characterization of tannase

#### 2.4.1 Ammonium Sulphate precipitation and Dialysis

The crude enzyme was precipitated employing ammonium sulphate at the saturation level of 75% concentration. Precipitated protein was collected by centrifugation at 10,000 rpm for 15 min at 4 ºC. The precipitate was re-suspended in 0.1 M citrate phosphate buffer (pH-5). The enzyme activity and protein content of the fractions were measured. The crude enzyme extract was further dialyzed against 0.1 M citrate phosphate buffer (pH-5) using Dialysis Membrane-50 (Hi Media Laboratories, India) for 24 h with regular changes in 2 hrs interval.

#### 2.4.2 DEAE-Cellulose Chromatography

The column was packed with DEAE-Cellulose 23 (Sisco Research Laboratories, India) and was equilibrated with 0.1 M citrate phosphate buffer (pH-5). 10 ml of the dialysed enzyme was applied to the equilibrated DEAE-cellulose column and the enzyme was eluted with the buffer at a flow rate of 20 ml/h. Eluted fractions were pooled and analyzed for protein content and enzyme activity [10].

### 2.5. Temperature Activity Profile

To determine the temperature activity profile of crude enzyme, the enzyme substrate reaction was carried out by protein precipitation assay at various temperatures (10–80) ºC, and enzyme activity was measured.

### 2.6. pH Activity Profile

To determine the pH activity profile of crude enzyme, the enzyme substrate reaction by protein precipitation assay at different pH range from 3 to 7 was carried out and the enzyme activity was measured.

### 2.7. Declarification of Fruit Juice

#### 2.7.1 Preparation of Juice

100 g of fresh pomegranate seeds were washed with water to remove any adhering substance. Juice was extracted from the seeds by homogenizing using an electric blender followed by filtration through a fresh cotton cloth.

#### 2.7.2 Treatment of Fruit Juice with enzyme

10 ml of fruit juice was incubated with enzyme at 37 ºC under shaking conditions (150 rpm). The tannin content of the enzyme treated juice was determined and compared with non-treated juice, using different amount of tannase and at different time interval of incubation by protein precipitation method [14].

## 3. Results & Discussion

**Table 1:** Purification of tannase from *Aspergillus niger*

| Purification stage | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 215 | 7·55 | 28·5 | 1 | 100 |
| 75% ammonium sulphate fractionation & Dialysis | 156 | 3·85 | 40·5 | 1·4 | 72·5 |
| DEAE cellulose column chromatography | 78 | 0·45 | 173·0 | 6·1 | 36·3 |

### 3.1 Purification of Tannase

Tannase was produced by solid state fermentation of rice husk by *A. niger*. Enzyme purification was achieved by ammonium sulphate precipitation and dialysis followed by chromatographic resolution using DEAE-cellulose column. Initially the crude enzyme was purified by precipitation with ammonium sulphate at the saturation level of 75%. In this step, the enzyme was purified 1·4-fold with a yield of about 72·5% and the specific activity was 40·5 U/mg protein. Comparative studies with *Aspergillus niger* showed purification by ammonium sulphate at the saturation level of 70%, with 1·9-fold purity followed by a yield of 68·75% and specific activity of 45·8 U/mg [15].

The dialyzed sample was further purified by chromatographic method using DEAE-cellulose column equilibrated with 0.1(M) citrate phosphate buffer. By DEAE-cellulose column chromatography, 6·1-fold purification was achieved and the specific activity was found to be 173·0 U/mg protein with a yield of 36·3% (Table 1). Studies also showed that after DEAE-Cellulose column Chromatography, the final purified enzyme was achieved with a specific activity of 60 U/mg with a purification fold of 2·5 and yield of about 24% [15]. Similar studies reported that the crude tannase showed specific activity of 27·8 U/mg, further with purification by 40-60% ammonium sulphate fractionation followed by dialysis and column chromatography, there was an increase in the specific activity of 66·8 U/mg and 116·4 U/mg respectively [4].

### 3.2 Optimum Temperature for Tannase Activity

The temperature optimum of crude tannase was represented in the figure 1. The enzyme was active in the temperature range of 20º–60 ºC with an optimal activity (23·3 U/mg) at 35 ºC. Earlier studies reported that optimal activity of tannase from *A. niger* ATCC 16620 was in the range 30º–40 ºC [5]. Further increase in temperature, the crude enzyme activity was found to decrease. This may be due to the fact that increase in temperature increases the rate of denaturation of the enzyme, with the loss of its structure [5]. The purified enzyme was fairly active even at 70 ºC (3·88U/mg) and this can be considered as an additional advantage.

### 3.3 Optimum pH for Tannase Activity

The pH optimum for crude tannase was shown in the figure 1. The enzymes were found active in the pH range of 4–7 with an optimal activity at pH 5 (31·1 U/mg). Below or above pH 5, the enzyme activity decreased for crude sample. Similar studies reported that optimal activity of tannase from *A. niger* was 22·6 U/mL after the optimum pH of 5·5 [1]. Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum [5].

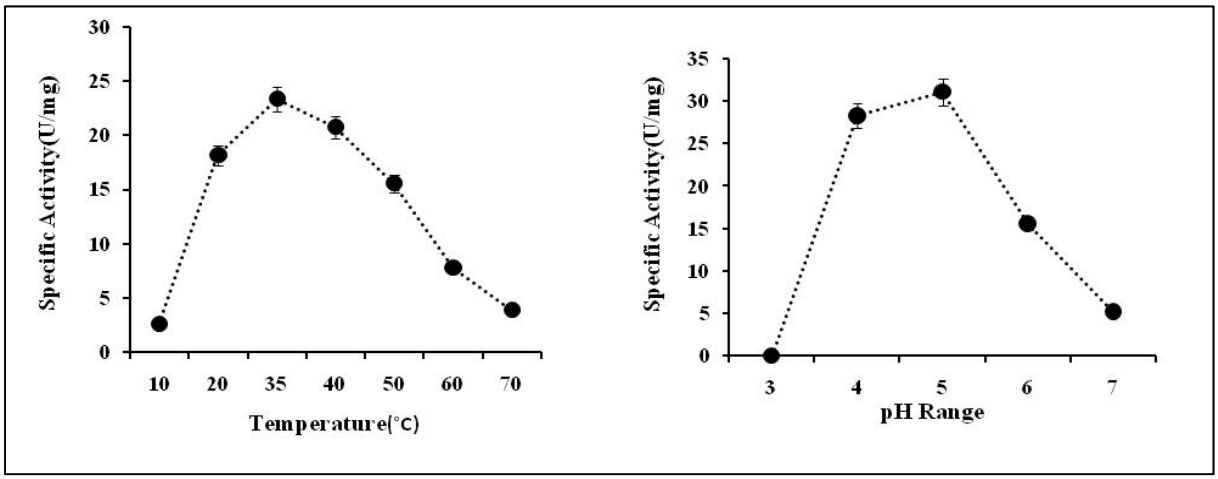


**Fig 1:** Effect of temperature and pH on crude tannase activity

### 3.4 Treatment of Fruit Juice with Tannase

The effect of tannase on the clarification of Pomegranate juice is shown in the figure 2. 1 ml of purified tannase (173·0 U/mg) was used to clarify 10 ml of fruit juice with a maximum reduction of 56% in tannin content. A further increase in the tannase concentration did not improve the extent of clarification. Similar observation reported that 1 ml of tannase (35·6 U/mg) from fungal strain *Aspergillus foetidus* was used to clarify 10 ml of Pomegranate juice with a maximum reduction of 25% in tannin content [14]. Studies also reported that 1 ml of tannase (19·75 U/mg) from fungal strain *Aspergillus niger* was used to clarify 15 ml of fruit juice with a maximum reduction of 45% in tannin content [16]. Similarly, the effect of incubation of the fruit juice was also tested with 1 ml of enzyme at various intervals shown in figure 2. A 56% decrease in tannin content was observed after 2 hrs of incubation with 1 ml of enzyme (173·0 U/mg) at 37 ºC. This indicated that the debittering efficiency depends on the concentration of enzyme used and the incubation period. Earlier studies also reported that a 25% decrease in the tannin content was observed after 2 hrs of incubation with 1 ml enzyme (35·6 U/mg) at 37 ºC using fungal strain *Aspergillus foetidus* [14]. Previous studies reported that a 45% decrease in the tannin content was observed after 2 hrs incubation with 1 ml enzyme (19·75 U/mg) using fungal strain *Aspergillus niger* [16].

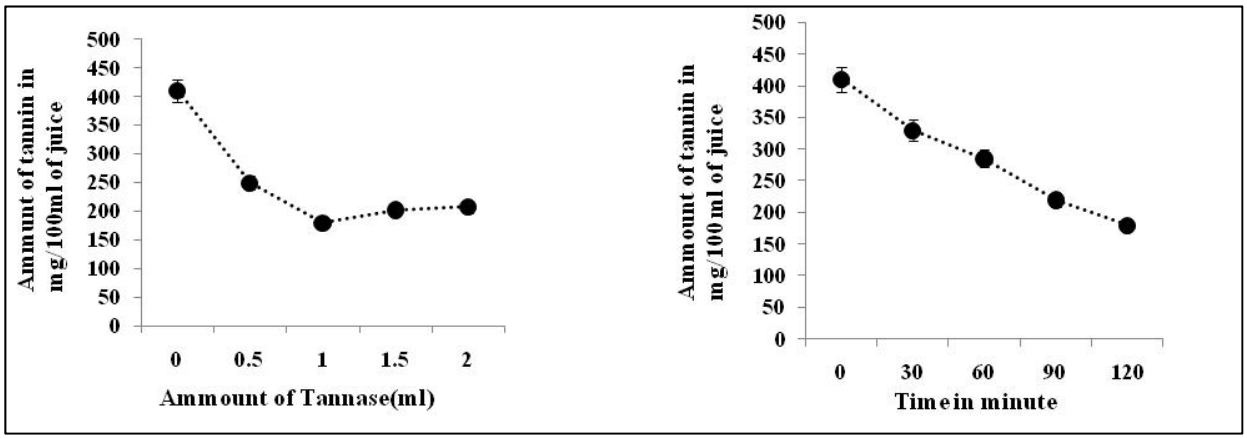


**Fig 2:** Effect of concentration and incubation on tannin degradation in pomegranate juice

### 4. Conclusion

This experimental piece of work deals with tannase, which was extracted using fungal strain *Aspergillus niger* and the substrate rice husk which is a waste material of the paddy industry. In the study crude tannase was extracted by solid state fermentation of rice husk employing *A. Niger*. From this study, it can be concluded that tannase produced by *Aspergillus niger* by SSF on rice husk as substrate showed a specific activity which is much greater than other fungal strains as well as other substrates. The crude enzyme obtained from this method is active over a wide range of pH and temperature. The application of this enzyme on juice declarification also states that it can be employed in fruit juice processing for removal of tannin efficiently.

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