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IMMOBILIZATION OF TANNIN ACYL HYDROLASE FROM *ASPERGILLUS NIGER*

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ABSTRACT: Tannin acyl hydrolase, commonly referred to as tannase (E.C. 3.1.1.20), an inducible extra-cellular enzyme produced by a number of animals, plants and microbes. In this investigation, tannase production under solid-state fermentation by using *Aspergillus niger* and the waste residue of cashew husk was used as substrate for obtaining the desired fermented product. After termination of fermentation gallic acid was isolated from the tannase, and it was used in the production of an antibacterial drug Trimethoprim by using chemical and bioconversions. Microbial tannase is more stable than tannase from other sources like plants or animals. Tannase from fungal sources are reported to be active in a wide range of pH and temperature. *Aspergillus niger* was used in this study for the immobilization of tannase enzyme. Tannase production was reached maximum within 24 to 36 h against crude tannin extract obtained from *Anacardium occidentale* by using *Aspergillus niger*. Tannase assay was calculated. Activation energy of the immobilized enzyme was lower than that of the free enzyme. Optimum pH and temperature was evaluated for the free and immobilized enzyme.

INTRODUCTION: Tannase (tannin-acyl-hydrolase, E.C. 3.1.1.20) catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins, as tannic acid, releasing glucose and gallic acid. Bacteria [6], yeast [2] and filamentous fungi [3, 5, 11] are known tannase producers. Species of *Aspergilli* were reported as the best tannase producers in submerged and solid state fermentations [4]. The major contribution to gallotannin fermentation was that of Knudson [10], he demonstrated the toxic nature of tannic acid to most of the fungi at low concentration except various species of *Aspergillus* and *Penicillium*.

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Production of tannins by using suitable tannase producing microorganisms was preferred today. Present work describes the immobilization of *Aspergillus niger* tannase and optimum conditions of the parameters pH and temperature of immobilized tannase.

MATERIALS AND METHODS:

Screening and culture conditions: A strain *Aspergillus niger* was obtained from NCIM Pune, India. For maintenance of *A. niger*, Potato dextrose agar (PDA) slants were used with 28ºC for six days of incubation period. The slants were sub cultured routinely once every three weeks [14].

Fermentation and isolation of tannase: *A. niger* was grown of modified Czapek's Dox medium for one week at thirty degree centigrade. Spores were collected under aseptic condition using Tween

eighty. The prepared spore suspension was adjusted to 10^7 spores/ml. Three milliliters of prepared spore suspension were inoculated into 250ml Erlenmeyer flasks containing 50g of cashew testa, 3g tannic acid and KH_2PO_4 5g. NH_4NO_3 10g, $MgSO_4 7H_2O$ 1g, $CaCl_2 6H_2O$ 0.1g, $MnCl_2 6H_2O$ 0.02g, NaMo O_4 $2H_2O$ 0.01g, $FeSO_4 7H_2O$ 0.125g, was adjusted to pH 5.5 with 100mM NaOH and incubated at thirty degree centigrade for two days. After incubation, the fermented biomass mixed thoroughly followed by centrifugation at ten thousand revolutions per minute to remove the mycelia mass. The supernatant was used for enzyme purification.

Tannase assay: Tannase activity was determined by the method of Mondal and Pati [12]. Enzyme solution (0.1 ml) was incubated with 0.3 ml of 1.0% (w/v) tannic acid, in 0.2 M citrate buffer (pH 5.0) at 40°C for thirty minutes and then the reaction was terminated at 0°C by the addition of 2ml BSA (1 mg/ml), which precipitates the remaining tannic acid. A control reaction was also done side by side with heat denatured enzyme.

The tubes were then centrifuged (5,000 x g, 10min) and the precipitate was dissolved in 2ml of SDS-triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1ml of $FeCl_3$ (0.13 M). One unit of the tannase was defined as the amount of enzyme, which is able to hydrolyze 1µ mole of ester linkage of tannic acid in one minute at specific condition. Activity calculated by using the following formulae

Volume Activity (U/ml) =

$$\frac{(AO^* \_ AS^*)20.3^*1.0(ml)81.04^*df}{0.71^*0.25(ml)^*15(\text{min})}$$

= A* 7.93* df

where, 20.3* = micromoles of tannic acid in 1.0 ml of substrate solution, 0.71* = change in absorbance after complete hydrolegies of 20.3 micromoles of tanninc acid under the assay conditions.

1.04 = correction factor; df = dilution factor; AO* = Test; AS* = Blank; A= (AO-AS)

Immobilization methods:

1. **Sodium alginate entrapment method:** 1ml of dialyzed enzyme solution will be added to 20ml of 30% sodium alginate (w/v) solution and will be mixed thoroughly. Then the solution will be added drop wise to 2% calcium chloride solution(w/v) using a hypodermic syringe. After curing, the beads will be washed with sterile water, drained and used for further studies[7].

2. **Cross linking method:** The dialyzed enzyme solution (8.5 ml) will be transferred into a beaker containing one percentage glutaraldehyde (v/v) and silica gel. After subjecting to shaking for twenty minutes the above mixture will be filtered so as to separate excess of glutaral-dehyde from the enzyme solution. The cross linked enzyme thus formed will be used for hydrolysis of castor oil.

3. **Optimization conditions:**

a. **pH**: The effect of pH on the free and immobilized tannase was studied using citrate buffer (0·1 mol l^{-1}, pH 3·5–5·5), citrate-phosphate buffer (0·1 mol l^{-1} pH 6–7·5) and phosphate buffer (0·1 mol l^{-1}, pH 7·0–8·0).

b. **Temperature**: The enzyme samples were incubated with citrate-phosphate buffer (0·1 mol l^{-1}) at the optimum pH value (5·5 for the free enzyme and 4·5 for the immobilized enzyme) at a designated temperature (50-70°C) for five to sixty minutes.

RESULTS & DISCUSSION: Immobilization of the tannase enzyme was carried out in two different methods: sodium alginate entrapment method and cross linking method. The experimental results of the tannase production with free and immobilized enzymes were shown in figures one and two. *A. niger* cells in immobilized form are useful for repeated use of tannase production. The immobilized tannase activity was evaluated, highest immobilized activity 106U/ml and the highest immobilization yield is 25.6%. The decrease in specific activity after enzyme immobilization has been previously reported [13, 8]. The optimum pH of the immobilized enzyme was shifted to pH 4.5 – 5.5, which was the optimum for the free enzyme (**Fig. 1**).

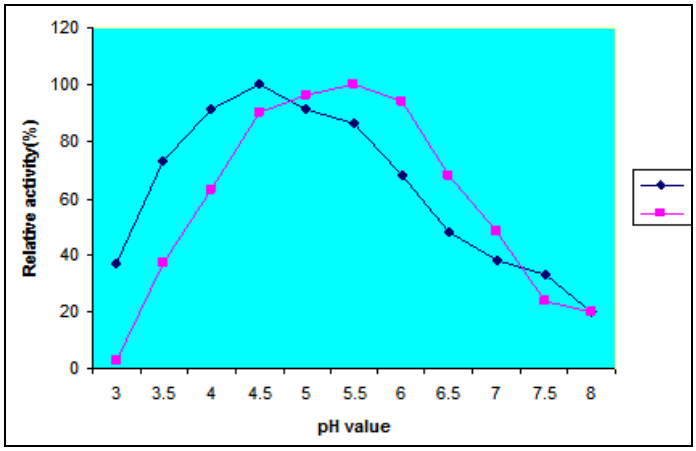

FIG. 1: Effect of pH on the activity of free and immobilized tannase. (♦) Free enzyme; (▄) Immobilized enzyme

The free enzyme had an optimum temperature of about 40°C, whereas that of the immobilized enzyme was shifted to 55°C (**Fig. 2**). The increase in the optimum temperature is probably a consequence of enhanced thermal stability.

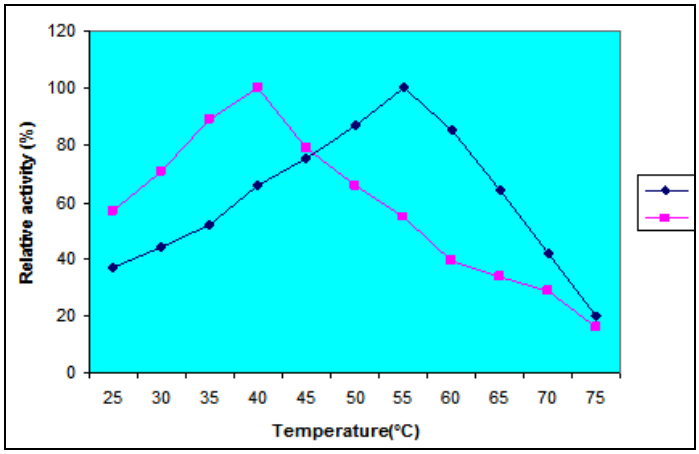

Fig. 2: Effect of temperature on the activity of free and immobilized tannase. (♦) free enzyme; (▄) immobilized enzyme

The activation energy for the immobilized enzyme was 5·77 kcal mol_1, which is lower than that of the free enzyme (6·75 kcal mol_1).[9, 1] reported that the activation energy of the immobilized enzyme was lower than that of the free enzyme because the internal diffusion of the substrate into the carrier-enzyme system was the rate-limiting step.

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