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Optimization of Various Culture Media for Tannase Production in Submerged Fermentation by *Aspergillus flavus*

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Abstract: Tannase, produced from the fungus *Aspergillus flavus* by submerged fermentation on different medium was studied. Tannic acid 1 had a higher activity of 70 U/g/min in purified form. The process parameter was optimized and higher production of tannase was found at 35°C and 96 hours of incubation with 2% tannic acid.

Key words: Tannase · Submerged fermentation · Immobilization · Purification · Screening

INTRODUCTION

Tannase or tannin acyl hydrolase catalyzes the hydrolysis of the ester bonds present in the hydrolysable tannins and gallic acid esters. Tannases reported in the literature vary greatly in molecular mass, ranging from 186 to 300 kDa [1, 2]. Tannase is utilized in a number of industrial applications including manufacture of instant tea, wine and gallic acid [3] and solubilization of tea cream in instant tea processing [4]. One of the major commercial applications of tannase is the hydrolysis of tannic acid to gallic acid, a key intermediate required for the synthesis of an antibiotic drug, trimethoprim [5].

Production of tannase by various bacterial [6-8] and fungal strains Banerjee *et al.* [9], was reported by a number of workers. Different workers used liquid surface, submerged or solid-state fermentation for tannase production. Among these, submerged fermentation process is mostly preferred because the sterilization and process-control methods are easier in this system [10]. Tannase has been produced essentially by submerged fermentation (SmF). At industrial level, tannase is produced by microbial means using SmF, where the activity is expressed mainly in intracellular form, implying additional costs in its production [11]. Unlike SmF, studies on tannase produced by solid-state fermentation (SSF) are recent. The enzyme produced in SSF is extracellular, facilitating its recovery. Lekha and Lonsane [12] reported the production of an extracellular thermostable tannase, with higher titres in SSF as compared to SmF. During SSF, most of the supports are also substrates. Sugarcane pith bagasse has been used as solid support, absorbing the liquid medium used in the SmF process for extracellular tannase production [12].

Some of the moulds and fungi belonging to the species *Aspergillus* and *Penicillium* produce the enzyme [13]. According to the work done by Yamada *et al.* [14] the enzyme was mainly found intracellularly although the culture broth also contained the enzyme. *Aspergillus niger*, *A. flavus* and *A. oryzae* were found to be the best tannase producers on tannic acid as a sole source of carbon. From these growth studies it became evident that the tannase enzyme was an inducible enzyme [15-17].

The present study deals with the production of Tannase in different medium and optimized the culture conditions for maximum enzyme production under solid state fermentation.

MATERIALS AND METHODS

Microorganisms: The strain of *Aspergillu flavus* used in this study was isolated from soil and maintained on Potato dextrose agar slants and subcultured for every month.

Inoculum Preparation: The fungal spore inoculum was prepared by adding 10ml of the sterile distilled water containing Tween 80 to the PDA slants. The spores were dislodged using a sterile inoculation loop under aseptic conditions. The volume of 1 ml of spore suspension was used as the inoculums.

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Table 1: Various medium for the production of Tannase in submerged culture

Tannic acid Media 1		Tannic acid Media 2		Dimitri's Media	
Sodium nitrate	-3.0	KH_2PO_4	-1	$NaH_2PO_4.2H_2O$	-1
Dipotassium		NH_4NO_3	-2	KH_2PO_4	-2
Hydrogen Phosphate	-1.0g	$MgSo_4.7H_2O$	-0.2	$Cacl_2$	-25mg
Magnesium Sulphate	-0.5g	$CaCl_2.2H_2O$	-20mg	$FeSo_4.7H_2O$	-5mg
Potassium Chloride	-0.5	$MnSO_4$	-4mg	$MnSo_4.7H_20$	-15mg
Tannic acid	-20	$Na_2 MoO_4.2H_2O$	-2mg	$ZnSo_4.7H_20$	-0.03
Agar	-20	$FeSo_4.7H_2O$	-2.5mg	$(NH_4)_2 SO_4$	-1
pH	-5.5	Tannic acid	-20	Fructose	-1
		pH-5.5		Tannic acid	-10
				pH	-5.5

Screening of Tannase Producing Organisms: Screening was performed in plates of selection medium which contained (g/L): Tannic acid, 10.0, $NaN0_3$, 3.0; KH_2P0_4, 1.0; $MgS0_4.7H_20$, 0.5; KCI 0.5; $FeS0_4.7H_2O$, 0.01; Agar, 30.0, pH 4.5. Point inoculations were carried out and plates were incubated at 32°C for 72 hours. The diameters of the colonies were measured in 24 hours periods [18].

Production of Extra Cellular Tannase in Submerged Fermentation: The spore suspension was inoculated in to 250 ml Ehrlenmeyer flasks containing 100 ml sterilized medium.There are three types of medium were used in this study.(Table: 1). The composition of the medium are as follows.(g/l).

The cultures were grown for 96 hrs at 35°C. The samples were withdrawn at regular intervals of one day. The biomass was separated by the filtration through Whatman No 1filter paper. The cell-free culture broth was assayed for the extracellular tannase activity.

Enzyme Purification: Enzyme obtained from the culture filtrate was precipitated with solid ammonium sulphate(60-80%) at 4°C for overnight. The precipitate was collected by centrifugation (8000 rpm, 20 min), dissolved in citrate buffer (0.05 M, pH 5.0) and dialyzed against the same buffer for overnight

DEAE Sephadex A-50 Chromatography: A Glass column was packed with DEAE Sephadex A-50 and was equilibrated with 0.05 M citrate buffer (pH 5.0). One ml of the dialyzed sample was applied on the column and the elution was done using 0.05 M citrate buffer (pH 5.0). The fractions were monitored and collected. The fractions corresponding to tannase activity were pooled and used for estimation.

Assay of Tannase: Tannase was assayed following Sharma *et al.* [19] method using gallic acid as standard. The pink color developed was read at 520 nm using a spectrophotometer (Shimadzu UV-160A). The enzyme activity was calculated from the change in absorbance. One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under defined reaction conditions. Enzyme yield was expressed as units/gram dry substrate (U/g/min).

$$\Delta A520 = (Atest\text{-}Ablank)\text{-}(Acontrol\text{-}Ablank)$$

Estimation of Protein: The total soluble protein during incubation was determined following the method of Lowry *et al*.

Effect of Incubation Temperature: The SmF was carried out in different temperatures such as 25°C, 30°C, 35°C and40°C.

Effect of Incubation Period: Enzyme production in SmF was studied for different period of incubation (24 h,48 h,72 h,96 h and 120 h)

Effect of Tannic Acid Concentration: Various concentrations of tannic acid was added to the production medium (0.5, 1, 1.5, 2, 2.5 and 3%) and incubated for 35°C for 96 h.

Immobilization of Tannase Enzyme at Different Storage Period: Enzyme was prepared from *Aspergillus flavus* cultivated at 35°C for 96hrs in Tannic acid medium 1. 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 for Tannase Production on Solid Media: The plate method is a qualitative, simple and rapid screening procedure for tannase production. The fungus used for the production of tannase enzyme were screened by using plate assay method. *A. flavus* was produced zone of hydrolysis surroundings the colonies showed in Fig.1. Zones formed due to hydrolysis of tannic acid to gallic acid and glucose [20], leading to a decrease in opacity of the media. Diameter of clear zone at different dilution was represented in Table 2. The highest zone was recorded in 10^{-1} dilution (18 mm). The least zone of diameter was recorded in 10^{-3} dilution (12 mm). Yamada *et al.* [21] tested eighty strains of filamentous fungi for tannase production and selected two colonies, identified as *Aspergillus oryzae,* which presented diameters of 20-22 mm after 72 hours. Direct measurement of the colony diameter was a good indicator of the ability of tannic acid utilization as a carbon source due to the tannase activity in the medium.

Tannase Production on Different Medium under Submerged Fermentation: *Aspergillus niger* was grown on different medium under submerged fermentation. The maximum activity was found in Tannic acid 1 medium.The obtained results were tabulated in Tab-3. From these results Tannic acid 1 had higher activity of 30.12 (U/g/min) in the crude form. The crude tannase when precipitated using Ammonium sulphate 60-80% saturation showed 42 U/g/min of Tannase activity. It was shown earlier that when tannase from *Paecilomyces variotii was* precipitated using 50% saturation of ammonium sulphate, some of the nonenzymatic proteins were shown to be removed and at 70% saturation of ammonium sulphate, a yield of 78.7% was reported by Mahendran *et al.* [22]. After dialysis the enzyme activity was enhanced when compared to the crude enzyme. The dialyzed enzyme was further purified through DEAE-Sephadex A-50 and the eluted fractions showed 70 U/g/min.

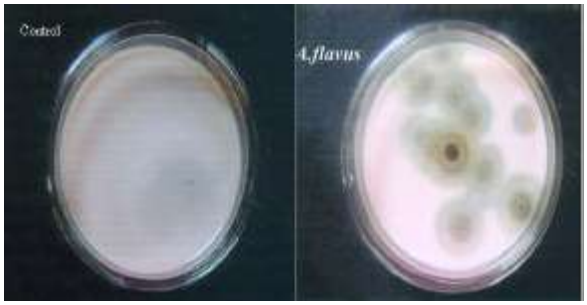

Fig. 1: Zone formation by A.flavus

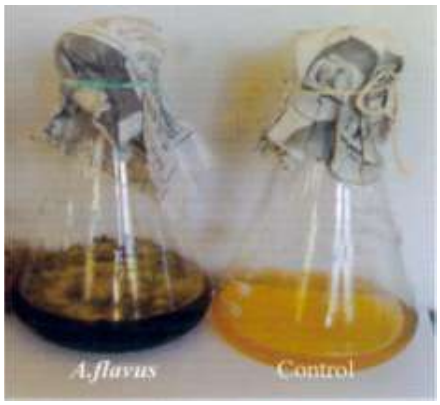

Fig. 2: Tannase production on submerged fermentation

Table 2: Screening of *Aspergillus flavus* on solid media

Microorganism	Diameter (average) of clearance zone at different dilutions		
Dilutions	10^{-1}	10^{-2}	10^{-3}
A.flavus	18 mm	14 mm	12 mm

Tannic acid medium 2 and Dimitri,s medium produced lower amount when compared to Tannic acid medium 1. (Table: 3) These findings also showed that the SmF system can be used for the exclusive production of extracellular TAH at 96 h of incubation. A similar type of observation has been made by Bradoo *et al*. [20] for TAH production by *Aspergillus japonicus.* Presently, SmF is a preferred method for production of most of the commercial enzymes like TAH, principally because sterilization and process control are easier to handle in this system [11].

Table 3: Tannase productions on submerged fermentation

		Tannase activity (U/g/min)			
S.No	Organism	Crude	Ammonium sulphate	Dialysis	Column Chromatography
1	Tannic acid medium 1	30.12	42	55.92	70
2	Tannic acid medium 1	22	35	47	61
3	Dimitri's Media	19	31	42	59

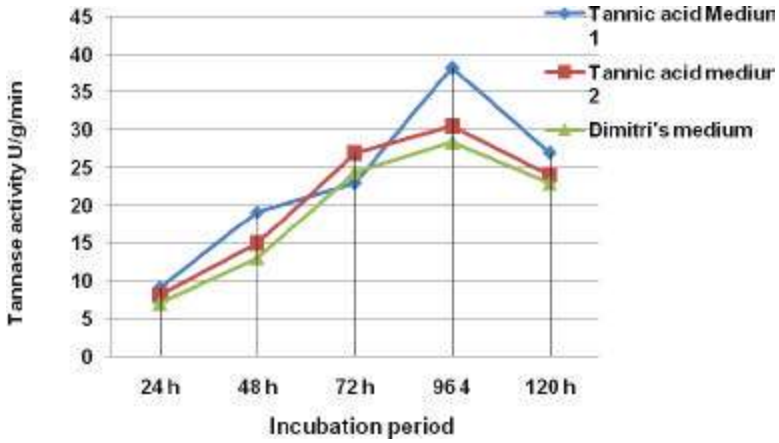

Fig. 3: Extracellular protein production during incubation

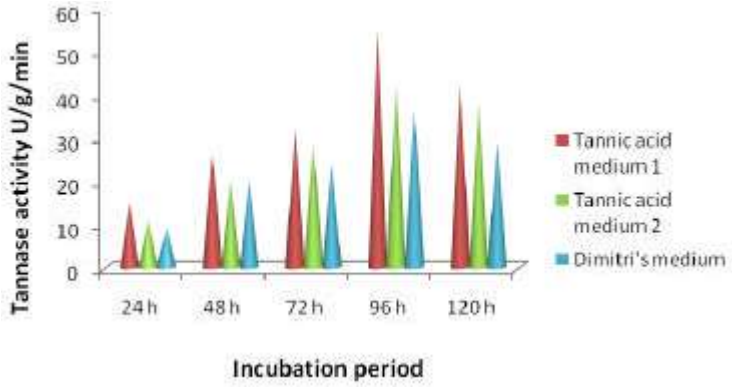

Fig. 4: Effect of incubation period for tannase production in different medium

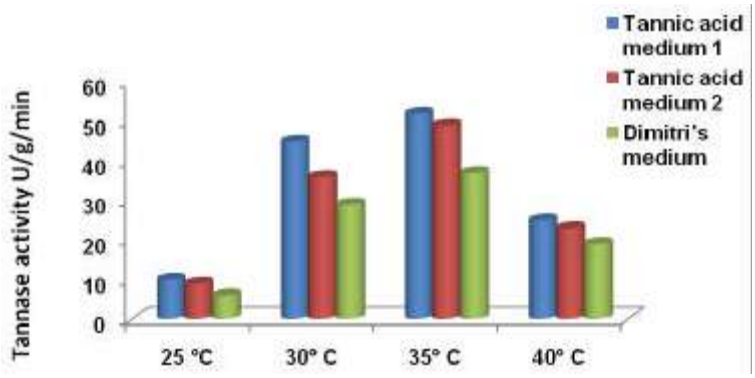

Fig. 5: Effect of temperature for tannase production in different medium

Submerged culture fermentation is generally used for commercial production of microbial enzymes [23].

Estimation of Protein: The protein content of the three medium showed maximum at 96 hrs (Fig.3) after that it was decreased.It might be the production of tannase was more at 96 hrs of incubation so the organisms produced more protein in that time. When the enzyme production started to decreased like wise the protein content also decreased. After studying the extracellular protein content it was found that the organism produced maximum tannase in its exponential phase of growth [9].

Effect of Incubation Period: In our present study the maximum tannase production was found in 96 h (42 U/g/min, 38 U/g/min and 29 U/g/min) in all the medium. This might be the fungi would have

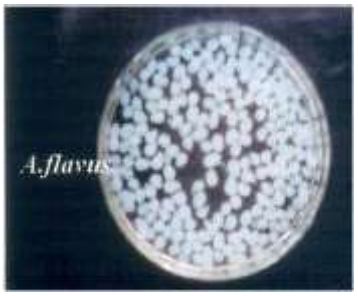

Fig. 6: Immobilized tannase on different storage period

entered in to its exponential phase. Thereafter, the enzyme production started decreasing (Fig. 4).There is various reports of different incubation period for maximal extracellular production of tannase production by fungi. Lekha [11] and Sabu *et al*. [24] reported maximum extra-cellular tannase production by *A. niger and Rhizopus oryzae* [25] in 96 and 120 h of incubation. Banerjee *et al.* [26] found maximum production of extracellular tannase by *A. aculaetus* after 72 h.

Effect of Temperature: In our study the maximum activity of (52 U/g/min) was found at 35°C in Tannic acid 1 medium.(Fig. 5). With a rise in temperature the tannase production was decreased. This was in good agreement with the results obtained earlier for tannase from *Bacillus cereus* [27]. Tannase produced by most of the potent strains like *Aspergillus oryzae, Penicillium chrysogenum* and *Aspergillus niger* also showed temperature optima at 30°C [11]. An optimum temperature of 35°C was reported for tannase from *Aspergillus awamori nakazawa* [28] and in case of *Penicillium variable* [19], the optimum temperature was at 50°C.

Effect of Tannic Acid Concentration: Various concentration of tannic acid was used in medium to find out the optimum concentration for tannase production. It was observed that 2% tannic acid was suitable for tannase production.(Tab-4) Banerjee *et al*. [9] found maximum extracellular tannase and gallic acid [29] after 36 h in liquid submerged fermentation containing 2% tannic acid. At higher tannic acid concentration tannase activity was higher in SSF whereas it was repressed in submerged fermentation [10] Actually, tannic acid at higher concentration produces complexes with membrane protein of the organism thereby both growth and enzyme production may be inhibited [26].

Table 4: Tannase activity in different concentrations of Tannic acid

Tannic acid concentration (%)	Tannase activity U/g/min
0.5	9
1.0	18
1.5	26
2.0	44
2.5	28
3.0	23

Table 5: Immobilized tannase activity of different storage period.

Duration of Incubation	Tannase activity U/g/min
24 hrs	18
48 hrs	29
72 hrs	32
96 hrs	39
120 hrs	38

Immobilized Tannase Enzyme on Different Storage Period: For industrial application, the immobilized form of enzyme shown in Fig.6, offers several advantages, including repeated use of the enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bed reactors. However, there are few reports on immobilized tannases [30-32]. The activity of immobilized tannase on different storage period was tabulated in Tab 5. Based on these results up to 120 hrs the activity was stable. (Table: 5)

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