ISOLATION OF TANNASE PRODUCING FUNGI AND PARTIAL CHARACTERIZATION OF TANNASE

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Abstract: Tannase also referred as tannin acyl hydrolase (EC 3.1.1.20), is mainly used for the bioconversion of tannic acid to gallic acid and sugar moiety.Tannase is an extracellular, enzyme active over a wide range of pH and temperature. Fungal tannase has many industrial applications including clarification of fruit juices, de-tannification of food, and preparation of food preservatives. Tree bark scrapings from in and around Hubli were screened for isolation of tannase producing fungi. Fungi were cultivated on Czapek Dox plates containing indicator compounds namely tannic acid. It resulted in isolation of five fungal strains, among which one of the strains was presumed to be potent depending on its growth characteristics and was used for further experiments. It was identified as belonging to *Aspergillus* genus. Higher level of tannase activity (5.97mg/ml min) was observed on third day of inoculation. Enzyme was precipitated at 80% saturation of Ammonium sulphate salt. It was also found out that the tannase was active over broad range of temperature and pH, with optimum temperature of 60^{o}C and pH 5.5. The isolated strain can be further worked for other characteristics and it could prove to be a potent enzyme for many industrial applications.

Keywords: tannase, tannic acid, temperature, *Apergillus*

Introduction: Tannase also referred to as tannin acyl hydrolase (EC 3.1.1.20), is mainly used for the bioconversion of tannic acid (hydrolysable) to nine parts of gallic acid and one molecule of sugar moiety.the molecular weight of native tannase is 300kDa [1] Tannase is an extracellular, inducible enzyme produced in the presence of tannic acid by fungi, bacteria and yeast. Tannase is produced extensively from fungi, as they are fast growing, it iss easy for their environmental and genetic manipulation which may be required for generation of novel producers in reduced time period. Tannase from fungal sources is reported to be highly active over a wide range of pH and temperature. Fungal tannase is used in many industrial applications including clarification of fruit juices, de-tannification of food, preparation of food preservatives, high grade leather tanning, clarification of beer and wines, beer chill proofing, manufacture of coffee flavoured drinks, manufacture of instant tea, production of gallic acid which is used for the synthesis of propyl gallate, which is mainly used as antioxidant in fats and oils, as well as in beverages and also being an important intermediary compound in the synthesis of the antibacterial drug, trimethroprim, used in the pharmaceutical industry, dyes and inks, treatment of green tea to inhibit the carcinogenic and mutagenic effects of N-nitrosamines, stabilization of malt polyphenols, improved colour stability and additional organoleptic properties. In animal feeding, tannase is used to reduce the anti-nutritional effects of tannins and improved animal digestibility [2].Tannase is also utilized for bioremediation of effluents from tanneries. In addition, tannase is used as a sensitive analytical probe for determining the structure of naturally occurring gallic acid ester. There is clearly much scope for the industrially important enzyme tannase for the removal of undesirable waste materials (tannins) present in the beverages and food materials and has the application of medical research in products like anti-oxidants, anti-bacterial drug [3]. Since it has large Hence, the present research work aimed at isolation of potent tannase producing fungi, establishment of time for maximum tannase production and partial characterization of the enzyme.

Materials And Methods

Isolation of tannase producing microorganisms: Tree bark scrapings, collected from in and around Hubli were used for isolation by employing standard serial dilution method. The tannase producing fungi were screened based on the growth on Czapek Dox`s media(g/100ml: 0.6 g of $NaNO_3$, 0.052 g of KCl, 0.052 g of $MgSO_4.7H_2O$, 0.152 g of KH_2PO_4 and traces of $Cu(NO_3)_2.3H_2O$, $ZnSO_4.7H_2O$ & $FeSO_4$) media containing 1% tannic acid. The plates were observed for growth and development zone of clearance around the organism on these plates. The organism showing faster growth was selected as potent strain. The strain was identified by Lactophenol dye method and observed under microscope [4]. [Cappucino and Sherman 2007].

Residual Tannic acid method of Enzyme assay: A spectrophotometric assay [5] was used to determine tannase activity, based on measuring the residual tannic acid content after the enzymatic reaction. The reaction mixture consisted of 0.3 mL of the substrate tannic acid (0.7% (w/v) in 0.2 M acetate buffer at pH

5.5) and 0.5 mL of the enzyme extract, incubating at 60ºC for 10 min. The enzymatic reaction was paralyzed by the addition of 3 ml of a bovine serum albumin solution - BSA (1 mg/mL), leading to the precipitation of the remaining tannic acid. The tubes were then centrifuged at 10,000 rpm for 15 min at 4ºC and the precipitate dissolved in 3 mL of SDS-triethanolamine, followed by the addition of 1 mL of $FeCl_3$ reagent and holding for 15 min for colour stabilization. The absorbance was measured at 530 nm and the enzyme activity calculated from the change in absorbance at 530 nm. One unit of tannase activity was defined as the amount of tannic acid hydrolysed by 1 mL of enzyme per minute of reaction [2]. Enzyme assay was carried for 96 hours to analyze maximum enzyme production.

Ammonium sulphate precipitation: Enzyme was precipitated by saturating the broth to 80%. The mixture was kept overnight to settle at 4 °C. Precipitated enzyme was separated by centrifugation at 10,000 rpm at 4 °C for 15min. The separated proteins were then dissolved in minimum amount of 0.2 M Acetate (pH=5) and used for further analysis [6].

Enzyme Characterization: The effect of temperature on tannase activity was determined by recording the absorbance of enzyme catalyzed reaction using tannin acid as substrate dissolved in appropriate buffer and incubated at 30-80ºC. Temperature at which enzyme had maximum activity, was noted as optimum. The influence of pH on tannase activity was studied by recording the absorbance of enzyme catalyzed reaction at optimum temperature, in buffers of different pH (acetate buffer pH 3.5, 4.5 and pH .55, phosphate buffer pH 6, pH 7) and incubated at 60ºC for 15min and absorbance were recorded.

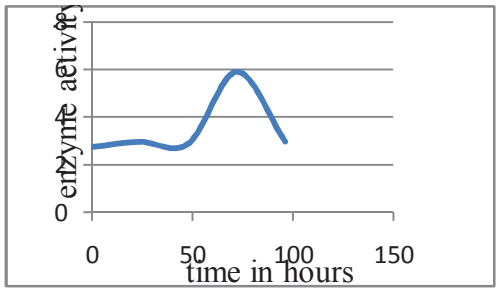

Fig.2: Profile of tannase production with time.

The effect of substrate concentration on enzyme activity was studied and K_m and V_{max} was observed using Line-Weaver Burk plot (LB Plot). The effects of inhibitors, chelators and surfactants on the tannase activity of the isolate were also determined. The inhibitor evaluated for its effects on tannase activity was sodium bisulphate at a concentration of 1 mM.The effect of surfactant, Tween 20 (1% (v/v)) and chelator, ethylene diamine tetra acetic acid disodium salt (EDTA disodium salt) at a concentration of 1 mM were also studied.

Results And Discussion

Screening of tannase producing fungi: Of 5 different organisms (TS121, TS122, TS131, TS134 and TS215) obtained from the bark

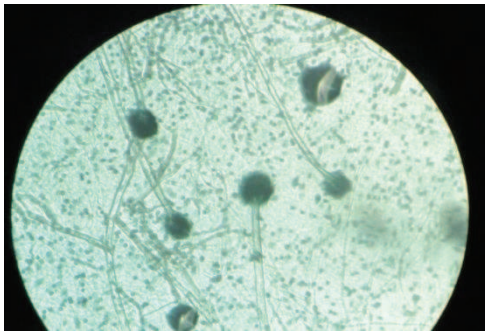

Fig 1: Microscopy of TSS-5

Of tree and screened, only one (TS215) showed zone of clearance (Table No.1) which indicated the presence of tannase enzyme. The selected strain (TS215) was identified based on its colony morphology as well as microscopic visualizations (spore and hyphae). The microscopic observations (Figure 1) showed the arrangements of conidia in balls at the tips of filaments. Paranthaman [7] obtained isolates of *Aspergillus sp.*

Table 1: Screening tests of samples collected

Plates	Isolates	Screening Test
1	TS111 (S1)	Negative
2	TS222 (S2)	Negative
3	TS333 (S3)	Negative
4	TS444 (S4)	Negative
5	TS555 (S5)	Positive

Enzyme production rate: The enzyme production rate of the organism TS215 was studied for every 24 hours and it was observed that at 72 hours of inoculation, the tannase production was maximum (5.97mg/ml. min) (Fig. 2).

Ammonium sulphate precipitation: The flask

containing production media and fungal sample was harvested after 72 hours since maximum enzyme activity was maximum.The cell free supernatant was subjected to ammonium sulphate precipitation (ASP) at 80% saturation [1] and a activity of 3.89 mg/(ml.min) was obtained.

Table 2: Enzyme activity of ASP sample and crude sample

Purification step	Concentration of tannic acid hydrolyzed in mg	Enzyme activity in mg/(ml.min)
Crude enzyme	9.7836	3.9
ASP	9.737	3.89

Characterization of enzyme: The functional temperature range of the tannase produced was 30-80ºC with optima at 50°C for the fungal sample TS215 (Fig. 3). These results were in agreement with previous reports Battestin and Macedo [1].

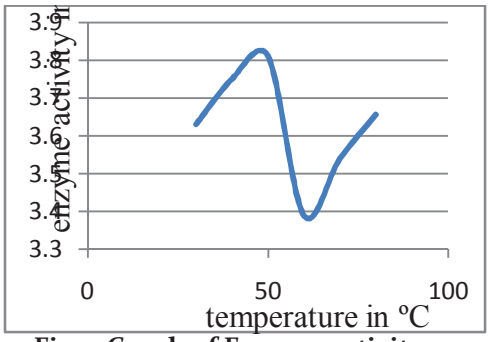

Fig.3 :Graph of Enzyme activity vs temperature

The crude tannase produced by TS215 showed optimum activity at pH 5.5(Fig.4).

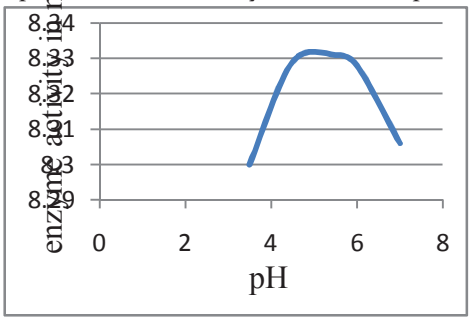

Fig.4: Graph of Enzyme activity vs pH. similar results were reported by Roopali *et al* [8]. The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range. It could be concluded from the results that tannase from the new fungal sample needed an acidic protein environment to be active. fungal tannase are geneally acidic protein [9].

The inhibitor evaluated for its effect on tannase activity was sodium bisulphite, Tannase activity was inhibited at a concentration of 1 mM (fig.5). It is an inhibitor of tannase as reported by Battestin and Macedo[1]. The chelator EDTA at a concentration of 1mM showed no inhibition (Fig.5) in the case of the tannase from TS215. The results are in line with Nadaf and Ghosh [10],Sabu *et al* 2005 [11]. The effect of chemical substances on the activity of an Enzyme is often precise and specific. In the present study, Tween 20 surfactant was chosen for an evaluation of its effect on tannase activity at a concentration of 1% and showed no effective inhibition in the enzyme activity for TS555 isolate. Similar kind of study on *Paecilomyces variotii* showed decrease in enzyme activity as described by Battestin and Macedo [1].

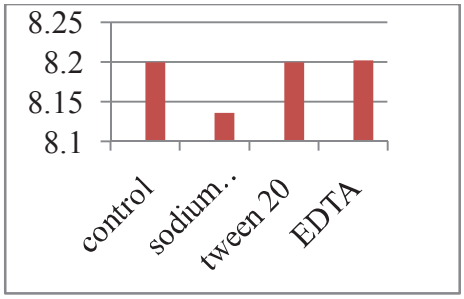

Fig.5: Graph of effect of additives on enzyme activity

To study the kinetics of tannase, varying concentration of tannic acid on the activity was studied. Line-Weaver Burk (LB) plot was plotted .Accordingly the K_m value for tannase is 0.033mg and V_{max}= 8.196 mg/ (ml.min) as seen in Fig.6.

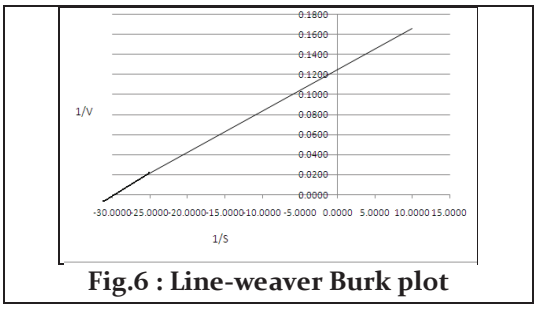

Fig.6 : Line-weaver Burk plot

Conclusion: Screening for tannase producing fungi on plates containing tannic acid resulted in isolation of five different fungal strains. One of the strains was identified as potent due to its fast growth rate and greater zone of clearance medium and microscopically was identified as belonging to *Aspegillus* genus. Tannase production by isolated strain was carried out in production media. On third

day of inoculation, enzyme activity was maximum . Ammonium sulphate precipitation was carried out and the partially purified tannase was found to have maximum activity at 60ºC and at pH 5.5. This potent organism can be used for large scale tannase production and its use in bioremediation.

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