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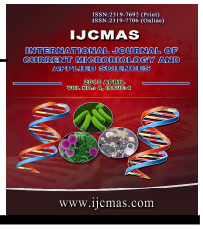

Original Research Article

Parametric Optimization for Extracellular Tannase Production in Submerged Fermentation by Isolated A*spergillus* Species

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Keywords

Tannase, Submerged fermentation, Tannin, *Aspergillus* sp., *Emblica officinalis* (amla)

A B S T R A C T

Tannin acyl hydrolase (E.C.3.1.1.20) commonly referred as tannase, hydrolyses ester and depside bonds of hydrolysable tannins to produce gallic acid, glucose and galloyl esters. Tannase finds application in many industrial sectors which includes pharmaceutical, food, chemical and beverages industry. The enzyme has potential uses in the treatment of tannery effluents and pre-treatment of tannin containing animal feed. The purpose of the present research was to investigate tannase producing microorganism isolated from soils obtained from different localities. Tannase was produced by submerged fermentation of tannin rich substrates by a culture of isolated *Aspergillus sp*. and production was enhanced by using *Emblica officinalis (amla*) as plant extract. The isolates were grown for different optimized parameters, incubated and the enzyme activity measured after optimization of different parameters. The enzyme production started after 24 hr of incubation and increased with time and at 168 hrs maximum enzyme activity recorded was as 35.6 U/ml thereafter, the enzyme production started decreasing. The highest enzyme production was achieved with 1% Tannic acid (20U/ml), sucrose (40.6 U/ml) as a carbon source and Yeast extract (39.6U/ml) as a nitrogen source at pH 5.6. The maximum biosynthesis of tannase enzyme was found to be optimal at incubation temperature as 32 ^{0}C at 150rpm.

Introduction

Tannins are polyphenolic secondary metabolites of plants, which form hydrogen bonds in solution, resulting in the formation of tannin-protein complexes. Tannins are water-soluble molecules with different molecular weights (500-3000 Da) and high content of polyphenols, occurring in certain parts of plants such as bark, wood, leaf, fruit, root and seed (Aguilar *et al.*,2007; Rodríguez *et al.*, 2008). This enzyme belongs to the family of hydrolases, specifically those acting on carboxylic ester bonds. The systematic name of this enzyme class is tannin acyl hydrolase. In addition to catalyzing the hydrolysis of the central ester bond between the two aromatic rings of digallate (depsidase activity), tannase may also have

an esterase activity. Tannase is utilized in a number of industrial applications including manufacture of instant tea, wine and gallic acid (Ascension Ramirez-Coronel *et al., 2003)* and solubilization of tea cream in instant tea processing (Nagalakshmi *et al., 1985*). Tannases have been used as clarifying agents in industrial processing of fruit juices and coffee flavoured soft drinks, in the manufacture of instant teas and in the production of gallic acid. 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 and used to produce propyl gallate, mainly used as an antioxidant in fats, oils and beverages.

Production of tannase by various bacterial strains *(*Banerjee *et al., 2007)* was reported by a number of researchers. As well as the filamentous fungi of genus *Aspergillus* (Paranthaman *et al.,* 2008) and genus *Penicillium* produce the enzyme. Submerged fermentation process is mostly preferred because the sterilization and process-control methods are easier in this system. The production of tannase by *Aspergillus sp*. is enhanced by Tannic acid concentration but these fungi can tolerate Tannic acid concentration as high as 20% and above that have a deleterious effect on both the growth and enzyme production (Mahapatra *et al., 2009).* Recently, agriculture residues have been utilized in enzyme production with the goal of minimizing production cost. *Emblica officinalis*, a plant widely distributed in tropical areas in the world and its fruits are commonly known as amla. They have been reported to have high content of phenolic compounds, the major one being gallic acid (GA) and ellagic acid (EA), which possess strong anti-oxidant properties.

In this study, plant extract from *Emblica officinalis* (with high tannin content) were investigated for the production of tannase by isolated fungal strain i.e. *Aspergillus sp*. This study includes the isolation and identification of the organism and production and characterization of tannase on the basis of optimization of various parameters (Incubation temperature, incubation period, tannic acid concentration, different carbon sources and nitrogen sources).

Materials and Methods

Preparation of Raw material

Emblica officinalis fruits were procured from the college campus. The fruits were dried at 60°C for 96 hrs and finally ground in a blender. A solution containing tannic acid (1%, w/v) and *Emblica officinalis* powder (3%, w/v) was prepared separately and its solution was sterilized by filtering it through a sterile membrane (0.2µ). (Fig 1)

Microorganims and Culture maintenance

A tannase producing fungus was isolated from a soil sample from the campus. Serially dilute the soil sample for inoculating the media plates. One ml aliquot appropriate dilutions were spreaded on Saboroud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) and plates were incubated at 30°C. Keep the plates for incubation at 30ºC for 5 days. The resulting colonies of *Aspergillus* were observed on the medium after 5 days of incubation and were transferred aseptically to SDA slants for future use. The colonies thus obtained were studied under microscope by Lactophenol-cotton blue staining and were identified as *Aspergillus* sp. The isolated *Aspergillus s*pecies was subcultured on SDA-TA medium containing 0.5% Tannic acid. The fungal colonies forming a clear zone round the mycelium due to tannic acid hydrolysis

were selected and further isolated for tannase production.

The fungal spores 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 inoculum. A 72 hrs old, 5% (v/v) seed culture in the sterilized Czapek Dox minimal medium was used. The spore suspension was inoculated into 250 ml Ehrlenmeyer flasks containing 100 ml sterilized medium. *Emblica officinalis* powder (EOP) was used for the induction of tannase in the fermentation medium. The sterilized solution of raw material was added. Flasks were incubated in (32°C, 150 rpm) in rotary shaker and further optimization of different parameters was done. Samples were withdrawn asceptically at regular time intervals and different culture conditions and analyzed for extracellular tannase activity.

Optimization of parameters for tannase activity

Effect of incubation temperature

The SmF was carried out in different temperatures such as 26°C, 28°C, 30°C, 32°C, 34°C and 36°C.

Effect of incubation time

Enzyme production under SmF was carried out and studied at for different periods of incubation (24hr, 48hr, 72hr, 96hr, 120hr, 144hr, 168hr, 192hr and 216hr.

Effect of Tannic Acid Concentration

Various concentrations of tannic acid was added to the production medium (0.5%, 1%, 2% and 3%) and incubated at 32°C for 96 hr.

Effect of different carbon sources

Different carbon sources such as Glucose, Galactose, Sucrose and Lactose were supplemented to fermentation medium at 1% level. The flasks with the production medium were inoculated and incubated at various volumes of carbon source at 32°C temperature. The general procedure mentioned earlier was followed for tannase assay.

Effect of different nitrogen sources

Different nitrogen sources such as Peptone, Yeast extract, Beef extract and Urea with 1% concentration was supplemented to the culture medium.

Enzymatic assay of Tannase

Tannase activity in the fermented media was determined by colorimetric method of (Mondal *et al.*, 2001b). For assay, 0.1ml of enzyme was incubated with 0.5ml of substrate tannic acid (1.0% w/v in 0.2Macetate buffer, pH 5.0) at 50°C for 30 minutes. The reaction was terminated by the addition of 3ml Bovine Serum Albumin (1mg/ml), which also precipitates the residual tannic acid.

A control reaction was done side by side using heat denatured enzyme. The tubes were then centrifuged (5000rpm for 10 minutes) and the precipitate was dissolved in 2ml of SDS-triethanolamine (1% w/v, SDS in 5%v/v, triethanolamine) solution. Absorbance was measured at 530nm after addition of 1ml of $Fecl_3$ (0.13M).

The specific extinction co-efficient of tannic acid at 530nm was 0.577 (Mondal *et al.*,

2001b). Using co-efficient, one unit of tannase activity is defined as the amount of enzyme required to hydrolyze 1mM substrate (tannic acid) in 1 minute at 50°C and pH 5.0.

Result and Discussion

Screening for tannase production on solid media

The fungus used for the production of tannase enzyme was screened by using plate assay method. *Aspergillus sp* produced zone of hydrolysis surroundings the colonies. Zones formed due to hydrolysis of tannic acid to gallic acid and glucose (Bradoo *et al.*, 1996) leading to a decrease in opacity of the media. (fig 2, 3)

Tannase Production under Submerged Fermentation

Aspergillus sp were cultivated on the minimal medium which was supplemented with *Emblica officinalis Powder* and tannic acid under submerged fermentation at 30ºC, pH 5.0. As the result indicated that *Aspergillus sp.* can utilize Tannic acid and EOP as a substrate for tannase production under Submerged fermentation.

Submerged culture fermentation is generally used for commercial production of microbial enzymes (Pandey *et al.*, 1999).The maximum activity was found in minimal medium at 180 hrs.

Effect of Incubation temperature

In the present study the maximum activity of (29.68 U/ml) was found at 32°C although the organism had a capacity to grow over a wide temperature range (25°C to 35°C). With a rise in temperature the tannase production was decreased.

Effect of Incubation period

Effect of different incubation periods (24hrs-216 hrs) was carried out in order to determine the best harvesting time for tannase from production medium. Maximum tannase activity (35.6U/ml) was observed at 168hrs of incubation. Thereafter, the enzyme production started decreasing. These results are consistent with Sabu *et al., (2005)* who demonstrated that tannase yield was associated with fungal growth. Enzyme activity decreased on prolonged incubation. According to (Gautam *et al*., 2002) this could be due to inhibition and denaturation of the enzyme.

Effect of Tannic acid concentration

Tannase activity was affected by the presence of tannic acid as an inducer in the production medium. Maximum tannase activity (20U/ml) was observed at a concentration of 1% (w/v) tannic acid as inducer.

Effect of different Carbon Sources

The effect of different carbon sources (Glucose, galactose, sucrose, lactose) on the enzyme activity of tannase was studied. The maximum enzyme production was observed with sucrose (40.6 U/ml).

Effect of different Nitrogen Sources

To study the effect of nitrogen sources on the growth and tannase production, different nitrogen sources (peptone, beef extract, yeast extract and urea) were used. The maximum enzyme production was observed with yeast extract (39.6U/ml).

The present work has been taken up with a view of using *A. niger* as a microbial source for the production of tannase which can

hydrolyze tannic acid to gallic acid. Tannase finds application in the food, beverage, industrial and pharmaceutical industry. This research is based on the *Emblica officinalis* powder assisted production, characterization and assay of tannase from *Aspergillus sp.* by optimization of different parameters. The effect of various culture conditions studied on the tannase activity. The highest enzyme activity (35.6 U/ml) was recorded at 168 hrs of incubation period and 32°C as the incubation temperature (29.68 U/ml). Along with this our results indicate that a culture medium containing plant extract (*Emblica officinalis* powder) assisted with 1% tannic acid (20 U/ml) for enzyme induction is the most suitable for fungal growth and enzyme production. In view of the results obtained, it is concluded that the isolate was able to show highest enzyme production with sucrose (40.6 U/ml) as a carbon source and yeast extract (39.6U/ml) as the nitrogen source. Further these optimized parameters can be used for large scale production of enzyme tannase.

Table.1 Effect of incubation temperature

	Incubation temperature	Tannase Activity(u/ml)
1	26°C	22.54
2	28°C	25.6
3	30°C	27.9
4	32°C	29.68
5	34°C	28.2
6	36°C	23.43

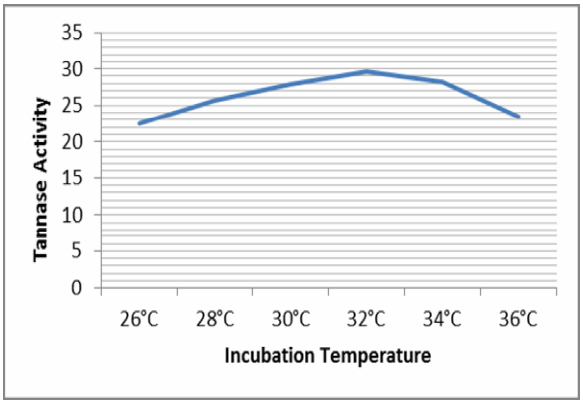


Table.2 Effect of incubation period

	Duration of Incubation (hrs)	Tannase Activity(u/ml)
1	24 hrs	20.7
2	48 hrs	21.56
3	72 hrs	23.34
4	96 hrs	26.09
5	120 hrs	28.004
6	144hrs	32.45
7	168 hrs	35.6
8	192 hrs	33.69
9	216 hrs	31.47

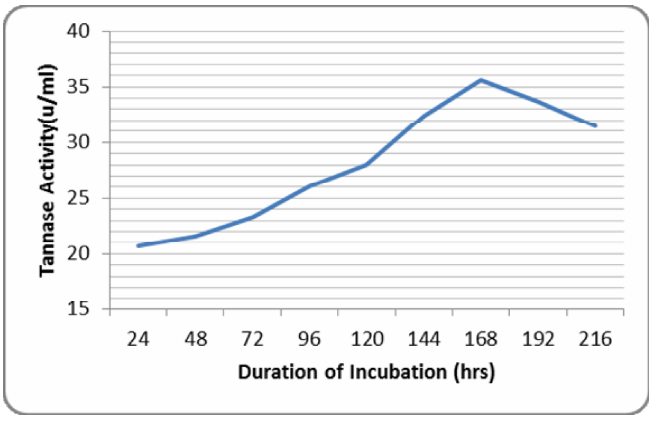


Table.3 Effect of tannic acid concentration

S.No.	Tannic acid concentration (%)	Tannase Activity(u/ml)
1	0.5	15
2	1	20
3	2	19.6
4	3	18.26

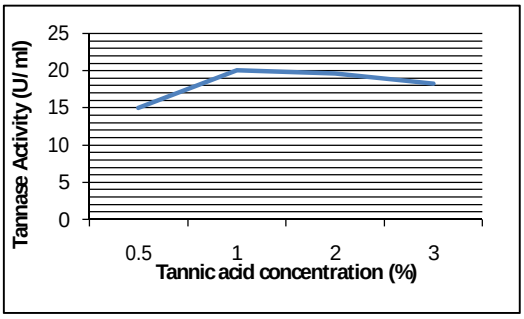


Table.4 Effect of Different carbon sources

S.No.	Carbon sources	Tannase Activity(U/ml)
1	Glucose	38.4
2	Galactose	32.7
3	Sucrose	40.6
4	Lactose	35.2

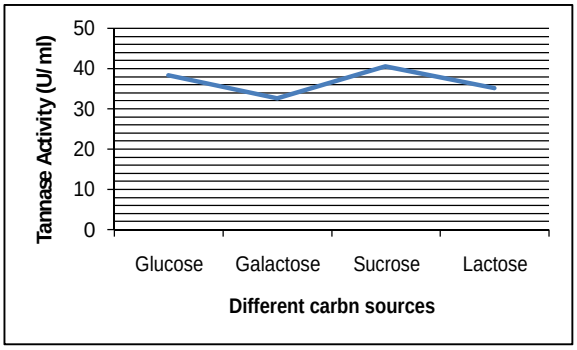


Table.5 Effect of Different Nitrogen sources

S.No.	Nitrogen sources	Tannase Activity(U/ml)
1	Peptone	32.6
2	Beef extract	34.0
3	Yeast extract	39.6
4	Urea	25.06

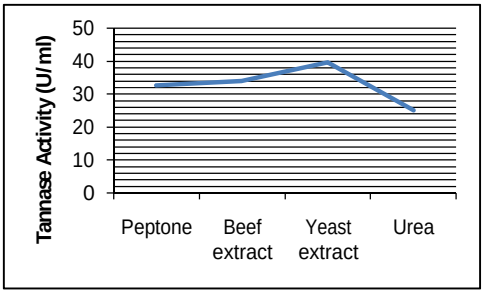
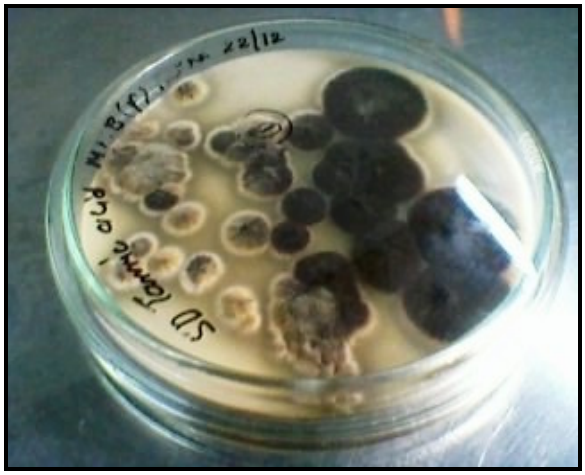


Fig.1 Preparation of inoculum

Fig.2 Isolated *Aspergillus* sp.

Fig.3 Fungal species showing zone of hydrolysis



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