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Isolation Of Tannase Producing Fungi And Optimization Of Culture Conditions For Tannase Production By Fungus tws-3

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Abstract

The tannase also known as tannin acyl hydrolase (TAH, E.C 3.1.1.20)), is a hydrolytic enzyme that acts on tannins. Primary screening for tannase was carried out from different soil samples. Six fungal isolates were obtained on the basis of zone of clearance produced on plates containing tannic acid. Among the isolates, fungus TWS-3 produced the highest 0.96 U/ml of tannase in submerged condition at 30°C, 150 rpm on a rotary shaker after 48 h. As inoculum 2×10^7 numbers of spores were found optimum for enzyme production. 1.0 gm% tannic acid showed the maximum tannase production. Among the other supplementary carbon sources tested, glucose was found as the best and it produced 2.51 U/ml of tannase with amla juice as a source of tannins. Ammonium chloride at 0.3 gm% showed the highest 3.45 U/ml of enzyme production among the organic and inorganic nitrogen sources tested. The tannase produced was partially purified by ammonium sulphate precipitation and 20-70% fraction gave 43.53 U/mg specific activity with 6.8 fold purification.

Keywords: Screening, tannase, tannic acid, amla juice, ammonium sulphate precipitation

Introduction

The enzyme tannase (E.C 3.1.1.20) also known as tannin acyl hydrolase (TAH), is a hydrolytic enzyme that acts on tannin such as tannic acid, methyl gallate, ethyl gallate, n- propylgallate, and isoamyl gallate. Tannase is responsible for the hydrolysis of ester and depside linkages in tannins to liberate gallic acid and glucose (Belur and Mugeraya, 2011). Such enzymes are naturally produced by ruminant animals, plants and microorganisms such as filamentous fungi belonging to the genera *Aspergillus* and *Penicillium*. The genus *Aspergillus* is considered as the best producer, followed by *Penicillium*, both standing out as great decomposers of tannins and have better thermal and pH stability (Lekha and Lonsane, 1994, Sabu et al., 2005).

Tannins are naturally-occurring plant polyphenols with proteins and other polymers such as cellulose, hemicellulose and pectin to form stable complexes. Tannins are found in leaves, bark, galls and wood. Tannins have important role in plant immunity and protect them from microbial attacks (Aguilar et al., 2001). Plants are rich sources of gallic acid either in free form or as a part of tannin molecule. Industrial production of gallic acid (3,4,5-trihydroxybenzoic acid) is generally accomplished by the bioconversion of tannic acid by tannase. Gallic acid is mostly used in the pharmaceutical industry for production of antibacterial drug trimethoprim. It is also used in the manufacturing of gallic acid esters such as propyl gallate, which is widely used as food antioxidant in the manufacture of pyrogallol, in leather industry and as a photosensitive resin in semiconductor production. Pyrogallol is used in staining fur, leather and hair, and also as a photographic developer (Kar et al., 1999).

Tannase has potential applications especially in the beverage industry and instant teas and coffees, as well as in the production of gallic acid and clarification of fruit juice rich in tannins, aiming to reduce the astringency of such products (Selwal and Selwal, 2012). Tannase is used in the treatment of tannery effluents and pretreatment of tannin rich of animal feed (Aguilar et al. 2007).

In the present study, isolation of tannase producing fungi, optimization of culture conditions and partial purification of enzyme is reported.

Materials and Methods

Tannic acid, rhodanine, Potato dextrose agar medium and agar powder were purchased from Hi-Media laboratories Pvt. Ltd. Gallic acid was bought from Sloca Research Laboratories Pvt. Ltd. Folin-phenol reagent and ammonium sulfate were purchased from SRL. All other chemicals used were of analytical grade.

Isolation Of Tannase Producing Microorganisms

The tannase producing microorganisms were isolated from different soil samples like fertile soil, amla litter soil, jamun leaves litter soil, tea waste dump soil etc. Ten different soil samples collected from various places of Anand district, Gujarat were screened for tannase producing microorganisms. For primary screening of tannase producers, medium containing (% w/v) tannic acid-1.0, $NaNO_3$-0.3, KH_2PO_4-0.1, $MgSO_4 \cdot 7H_2O$-0.05, KCl-0.05, $FeSO_4.7H_2O$-0.001, Agar- 3.0, pH- 4.5 was used. Approximately 1.0 g of soil sample was added in sterile distilled water and shaked vigorously. Then, the suspended soil particles were allowed to settle down and supernatant was used as suspension. It was diluted appropriately and 0.1 ml was spreaded on the tannic acid

agar plates and incubated at 30°C for 48 h. Growth of colonies and zone of clearance produced upon tannic acid hydrolysis was checked for isolation of tannase producing microorganisms.

Tannase Assay

Tannase activity was measured by chromogen formation between gallic acid and rhodanine (Sharma et al., 2000). The reaction mixture containing 0.25 ml of 0.01 M, methyl gallate in 0.05 M citrate buffer, (pH 5.0) and 0.25 ml of appropriately diluted enzyme was incubated at 50 °C in waterbath for 10 min. After that 0.2 ml of 0.5 M potassium hydroxide (KOH) was added to stop the reaction. Then, 0.3 ml of methanolic rhodanine 0.667% (w/v) was added. In a control tube, KOH was added first with the substrate methyl gallate and after that enzyme was added. The reaction mixture was diluted by adding 4.0 ml distilled water and absorbance was measured at 520 nm. The amount of gallic acid produced was estimated from a standard calibration curve of 0 – 200 µg. One unit was defined as the amount of enzyme that produced one µmol of gallic acid per min under assay conditions.

Protein concentration was determined by Folin phenol reagent method (Lowry et al, 1951) with bovine serum albumin as a standard (0-100 µg/ml).

Tannic Acid Estimation

Tannic acid compounds were estimated using the Folin and Ciocalteu reagent. To 100 µl of appropriately diluted sample, 1.5 ml of 20% (w/v) sodium carbonate and 0.5 ml of Folin-phenol reagent were added. The mixture was kept at room temperature for 1 h and absorbance was measured at 725 nm. The amount of tannic acid was determined from a standard calibration curve of 0 – 100 µg.

Preparation Of Spore Suspension

For fungal tannase production, young spore suspension was prepared and directly used as inoculum. To prepare spore suspension, isolated fungal cultures were grown on potato dextrose agar slant at 30°C upto 4 days for sporulation. 10 ml sterile distilled water containing 0.1 (v/v %) Tween 80 was added to harvest the spores from slant. The slant cultures were vortexed properly to obtain spore suspension. Spore count was carried out using Neuber's Chamber after appropriate dilution of the prepared spore suspension. The prepared spore suspension was directly used as inoculum for submerged tannase production.

Screening Of Tannase Producing Isolates

Five fungal isolates were isolated on the basis of zone of clearance on the tannic acid agar plates and they were further screened for tannase production in shake flask cultures. Five fungal isolates viz. ALS-1, ALS-2, ALS-3, TWS-1 and TWS-3 were inoculated in sterile 50 ml tannic acid medium as mentioned above except ager in 250 ml Erlenmeyer flask and incubated at 30°C, 150 rpm on a rotary shaker. Extracellular tannase production was checked after 24 h up 96 h. To estimate tannase from the samples, fungal biomass was separated using Whatman No.1 filter paper and filtrate was analyzed. The isolated fungal cultures were maintained on potato dextrose agar slants at 4°C by periodic transfer.

Optimization Of Inoculum Size

Fungus TWS-3 showed the highest tannase production among the five isolates in shake flask

culture. Therefore, it was selected for further optimization studies. The inoculum size for TWS-3 was optimized for submerged tannase production. As the inoculum size 1×10^7, 2×10^7 and 1×10^8 number of spores were inoculated in 50 ml tannic acid medium and incubated at 30°C, 150 rpm for 48 h on a rotary shaker. After incubation, tannase production was checked from the filtrate.

Optimization Of Tannic Acid Concentration

As tannase production was induced in presence of tannic acid, its different concentrations were tested for enzyme production. Tannic acid (w/v/%) was added at 0.3%, 0.6%, 1.0%, 1.5% concentrations keeping other components same as tannic acid medium. Prepared sterile media flasks were inoculated by fresh spore suspension with final count of 2×10^7 spores/ml. The inoculated culture flasks were incubated at 30°C, 150 rpm on a rotary shaker for 48 h. After incubation, the fungal biomass was separated by the filtration and filtrate was analyzed for tannase production.

Preparation Of Amla Juice

100 gm of fresh amla (Indian gooseberry) was washed thoroughly and chopped with a clean knife to remove seeds. It was crushed in a jar of juicer-mixer. The crushed amla pulp was filtered through cheese cloth. The filtered amla juice obtained was stored at 0°C until further use.

Effect Of Supplementary Carbon Sources On Tannase Production

To check the effect of various carbon sources on tannase production, tannic acid medium was supplemented with carbon source. Fungus TWS-3 was tested for tannase production using different carbon sources viz. glucose, fructose, sucrose and starch at 0.3 gm%. In a separate experiment, 1 gm% tannic acid was replaced by amla juice (12.5 ml) supplemented with 0.3 gm% glucose in the production medium. Each 250 ml flask containing 50 ml media were inoculated by fresh spore suspension. The inoculated culture flasks were incubated at 30°C, 150 rpm for 48 h on a rotary shaker. After incubation, the fungal biomass was separated by the filtration and filtrate was analyzed for tannase production.

Effect of Nitrogen Sources On Tannase Production

Effect of different organic and inorganic nitrogen sources was tested on tannase production. In this set of experiments 1 gm% tannic acid was replaced by amla juice (12.5 ml) in the tannic acid medium. To check the effect of nitrogen sources, 0.2 gm% $NaNO_3$ was replaced by ammonium chloride, ammonium sulfate, ammonium nitrate as inorganic nitrogen sources and peptone and yeast extract as organic nitrogen sources. Prepared sterile media flasks were inoculated by fresh spore suspension. The inoculated culture flasks were incubated at 30°C, 150 rpm on a rotary shaker for 48 h. After incubation, the fungal biomass was separated by the filtration and analyzed for tannase production.

For tannase production, ammonium chloride was found as the best among the nitrogen sources tested. In subsequent experiment, different concentrations of ammonium chloride (w/v %) i.e. 0.1%, 0.2%, 0.3%, 0.4% and 0.5% were tested.

Enzyme Precipitation

The optimized medium containing amla juice - 12.5 ml, glucose - 0.2 gm%, ammonium chloride - 0.2 gm%, KH_2PO_4 - 0.1 gm%, $MgSO_4.7H_2O$ - 0.05 gm%, KCl - 0.05 gm%, pH 4.5 was inoculated with young spore suspension and incubated at 30°C, 150 rpm for 48 h for tannase production. After

incubation, the medium was filtered to remove the fungal biomass and filtrate obtained was subjected to ammonium sulphate precipitation. To 90 ml of crude enzyme 20 gm% ammonium sulphate was added slowly in an ice bath with gentle stirring and kept for 2 h. The precipitated proteins were separated at 8000rpm for 15 min in refrigerated centrifuge. The first protein pellet obtained was dissolved in 0.5 M acetate buffer, pH 5.0 and stored at 4°C until further use. The remaining supernatant was further precipitated to achieve 20-70 gm% ammonium sulphate saturation in an ice bath and kept overnight to facilitate protein precipitation. Then, it was centrifuged at 8000rpm for 15 min to separate the precipitated proteins. The second protein pellet was dissolved in 0.5 M acetate buffer, pH 5.0 and stored at 4°C. Both the precipitated protein fractions were carefully transferred to dialysis bags and dialyzed against acetate buffer, 0.5 M, pH-5 at 4°C. The buffer assembly with dialysis bags was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis was carried out overnight with three buffer changes.

Amount of enzyme tannase and protein was determined in crude as well as in both the fractions of ammonium sulphate precipitation. For concentrated tannase, specific activity and fold purification was calculated.

Results and Discussion

Isolation Of Tannase Producing Organisms

Screening is defined as the detection and isolation of desired microorganisms from a large microbial population by using highly selective methods. The plate screening method is a qualitative, simple and rapid screening procedure for tannase production. Fungi producing tannase showed zone of clearance surroundings the colonies (Fig. 1). These clear zones were formed due to the hydrolysis of tannic acid to gallic acid and glucose.

We got five fungal isolates producing clear zone on plate with tannic acid and named them according to their soil source like amla litter soil (ALS), tea waste dump soil (TWS). Among the isolates namely ALS-1, ALS-2, ALS-3, TWS-1 and TWS-3, zone of clearance produced by TWS-3 and ALS-2 is shown in Figure 1.

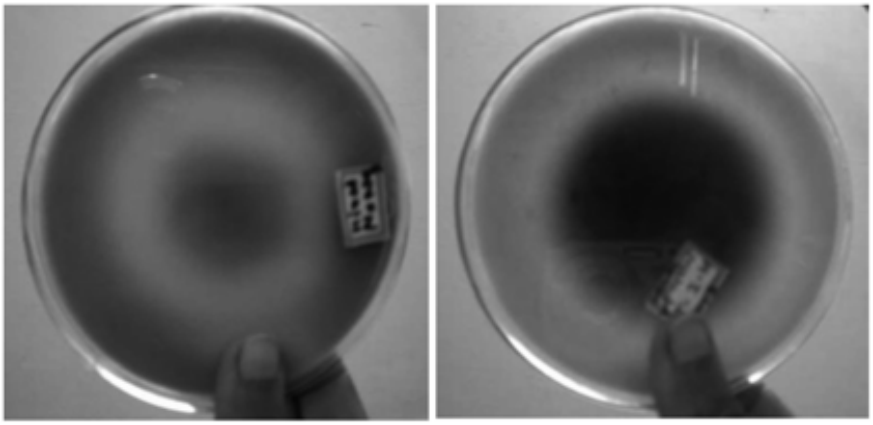

Figure 1 - Zone of clearance produced by fungi TWS-3 and ALS-2

Screening Of Tannase Producing Isolates

Five fungal cultures viz. TWS-1, TWS-3, ALS-1, ALS-2 and ALS-3 were selected for further tannase production on the basis of plate assay. All the cultures were inoculated with spore suspension (1×10^7 spores/ml) in 250 ml flask containing 50 ml of tannic acid medium. The inoculated flasks were incubated at 30°C, 150 rpm for 96 and were analysed for tannase production at 24 h time interval. Tannase production by these five isolates is shown in Table 1. Fungal culture TWS-3 produced the highest quantity of tannase (0.96 U/ml) after 48 h and hence it was selected for further studies.

Incubation	Tannase production (U/ml)				
time (h)	ALS-1	ALS-2	ALS-3	TWS-1	TWS-3
24	0.016	0.027	0.25	0.18	0.49
48	0.099	0.42	0.63	0.34	0.96
72	0.045	0.18	0.30	0.19	0.63
96	0.032	0.02	0.26	0.04	0.25

Table 1: Tannase production from five fungal isolates

Optimization Of Different Inoculum Size

The effect of inoculum size was studied for optimal tannase production using TWS-3. The different inoculum size was tested as number of spores 1×10^7, 2×10^7 and 1×10^8 inoculated for enzyme production in 250 ml flask containing 50 ml of tannic acid medium and incubated at 30°C, 150 rpm for 48 h on a rotary shaker. 2×107 no. of spores as inoculum showed the maximum tannase production of 1.09 U /ml (Table 2). It may be because of formation of very good small pellets of fungus which is different in other two cases.

Innoculam size	Tannase (U/ml)
1×10^7	0.52
2×10^7	1.09
1×10^8	0.86

Table 2: Tannase production with different inoculum size

Optimization Of Tannic Acid

As tannic acid is hydrolysable tannin and so it is the most suitable carbon source used for tannase production. The different concentrations of tannic acid were checked in the medium to get higher tannase production. The addition of 1gm% tannic acid was proved as the best for tannase production (Table 3) and the highest extracellular tannase of 1.07 U/ml was produced after 48 h.

Tannic acid (gm %)	Tannase (U/ml)
0.3	0.40
0.6	0.85
1.0	1.07
1.5	0.60

Table 3: Effect of Tannic Acid on enzyme production

Banerjee and Pati (2007) reported 1.0 gm% tannic acid as optimum for tannase produced by *Aureobasidium pullulans* DBS66. In other report, 2 gm% tannic acid showed maximum tannase production using *Asp. japonicus* in Czapeck's Dox medium (Bradoo et al. 1997). Sharma et al. (2007) reported 5 gm% tannic acid for maximum tannase production using *Asp. niger*.

Effect Of Supplementary Carbon Sources

Various carbon sources were tested at 0.3 gm% in addition to tannic acid in the medium to maximize tannase production. TWS-3 produced considerable amount of tannase with all the tested supplement any carbon sources viz. glucose, fructose, sucrose and starch (Table 4). Addition of glucose produced 1.88 U/ml of tannase showed about 75% increase in enzyme production. When glucose and amla juice (in replacement of 1 gm% tannic acid) combination was checked for tannase production, 2.51 U/ml of tannase was produced indicating 134% increase in enzyme. As addition of glucose may be beneficial to the initial growth of fungi and natural tannins and growth factors present in amla juice might have enhanced the tannase production.

Carbon sources	Tannase (U/ml)
Sucrose	0.47
Fructose	0.35
Starch	1.3
Glucose	1.88
Glucose with amla juice	2.51

Table 4: Effect of supplementary carbon sources on tannase production

Kar and Banerjee (2000) reported higher tannase production using *Caesalpina digyna* seed cover powder than that obtained with only tannic acid. Sabu et al. (2005) stated that glucose and other readily metabolized carbon source reduce the lag period required for tannase synthesis and production. Banerjee and Pati (2007) also reported stimulatory effect of glucose at 0.1 gm% glucose on tannase production. Aguilar et al. (2001) reported the increased tannase production at 0.06 to 0.25 gm% glucose but but observed strong catabolite repression at 0.5 gm% using *Aspergillus niger* Aa-20 in submerged fermentation.

Effect Of Different Nitrogen Sources

The effect of different nitrogen sources was tested in the medium to increase tannase production. TWS-3 produced tannase with all the tested inorganic nitrogen sources (ammonium chloride, ammonium sulphate, ammonium nitrate) and organic nitrogen sources (yeast extract and peptone) as shown in Table 5. The highest tannase production of 2.18 U/ml was observed with ammonium chloride.

Nitrogen sources	Tannase (U/ml)
Ammonium chloride	2.18
Ammonium sulphate	1.38
Yeast extract	1.20
Ammonium nitrate	0.58
Peptone	0.63

Table 5: Effect of inorganic and organic nitrogen sources on tannase production

In compare to organic nitrogen sources, inorganic nitrogen sources proved better for TWS-3. The effect of inorganic nitrogen source like $NaNO_3$ has been reported during the production of tannase by *Aspergillus janponicus* (Bradoo et al., 1997). Similarly, Paranthaman et al. (2009) reported tannase production by *Aspergillus flavus* in the medium containing $NaNO_3$. The *Aureobasidium pullulans* DBS66 showed maximum tannase production with $(NH_4)2HPO_4$ as nitrogen source (Banerjee and Pati, 2007).

In subsequent experiment tannase production was measured at different ammonium chloride concentrations (w/v %) like 0.1, 0.2, 0.3, 0.4, 0.5 and results are shown in Table 6. The ammonium chloride at 0.3 gm% concentration showed 3.45 U/ml tannase production indicated 222% increase.

Ammonium chloride (gm%)	Tannase (U/ml)
0.10	2.65
0.20	3.18
0.30	3.45
0.40	3.4
0.50	1.6

Table 6: The effect of ammonium chloride concentrations on tannase production

Ammonium Sulphate Precipitation

Crude tannase produced by TWS-3 fungal culture was concentrated and partially purified by ammonium sulphate precipitation. The tannase was partially purified in two fractions of 0-20% and 20-70% ammonium sulphate saturation. The result of specific activity and fold purification is shown in Table 7. The protein fraction of 20-70% ammonium sulphate saturation showed 43.53 U/mg of specific activity with 6.8 fold purification.

Procedure	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	5.97	2.83	2.10	1	100
Ammonium sulphate : 0-20%	5.6	0.73	7.67	4.1	22.19
Ammonium sulphate: 20-70%	7.41	0.17	43.53	6.8	

Table 7: The partial purification of tannase by ammonium sulphate precipitation

The partial purification of tannase from *Aspergillus ficuum* Gim 3.6 was carried out by aqueous two-phase extraction (ATPE) and 2.74 fold tannase was obtained (Ma et al. 2015). The tannase from *Penicillium notatum* NCIM 923 was purified by precipitation with ammonium sulphate at the saturation level of 75% and it was purified 5.96-fold with the specific activity was 6.74 U/mg (Gayen and Ghosh, 2013). The tannase from *Aspergillus niger* MTCC 2425 was purified to 1.4-fold with a yield of about 72.5% and the specific activity was 40.5 U/mg by

ammonium sulphate precipitation (Nandi and Chaterjee, 2016).

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