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Culture Conditions for the Production of Tannase from *Trichoderma harzianum* MTCC 10841

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ABSTRACT

A total of 36 fungal strains were isolated from the different soil samples collected around the tannin-rich plants. The isolated cultures were screened for their tannase producing capability by observing the zone of hydrolysis on tannic acid agar plates. Among the twenty two fungal strains selected as tannase producers, the strain T16 produced highest (16.36 U/ml) tannase activity. Hence, it was selected for further study and identified as *Trichoderma harzianum* MTCC 10841. To enhance the production level of the enzyme different culture conditions were optimized and observed that optimum temperature and pH for tannase production was 30^{o}C and 5.5 respectively. Maximum growth and enzyme production was recorded after 96 hrs of incubation period in the medium containing 1% tannic acid. Among the different natural substrates tested as carbon source, amla fruit enhanced the tannase production by almost two fold compared to pure tannic acid used as a substrate. Pomegranate rind, jamun bark and amaltash leaves also supported tannase production appreciably. Malt extract (2%) with NH_4Cl (0.2%) was found to the best nitrogen source. Among the additives, metal ions Ca^{++}, Na^{+}, Mg^{++}, Mn^{++} and Zn^{++} did not affect enzyme production. However, metal ions like K^{+}, Cu^{++}, Co^{++}, Fe^{+++} and the various detergents tested, inhibited the production of tannase. The optimization of culture conditions enhanced the production level of tannase (32.2 U/ml) by two-fold. The tannase production was successfully scaled up to 1.5L at laboratory level.

Keywords: *Amla fruits, Amaltash leaves, tannase production, Trichoderma harzianum*

1. INTRODUCTION

Tannins are naturally occurring plant phenolics compounds that have wide ranging effects on animals and microbes [1]. They are polyphenolic secondary metabolites of plants which form hydrogen bonds in solutions, resulting in the formation of tannin-protein complexes [2]. Tannins are present in large number of feed and forages. The formation of complexes of tannins with nutrients, such as carbohydrates, proteins and minerals, has negative effects on their utilization. High concentrations of tannins depress voluntary feed intake and digestive efficiency. The nutrient value of tanniferous feed may be enhanced by various detannification procedures viz., physical, chemical and biological. In biological treatment, various tannase producing microbial strains have been tried for reduction of tannin content and nutritive enhancement of treated material [3].

In this respect, tannase find potential applications in feed, food and beverage industry. Tannase is used as clarifying agent in some wines, juices of fruits and refreshing drinks with coffee flavour. The use of tannase helps in overcoming the problem of undesirable turbidity in these drinks which poses the quality problem. Enzymatic treatment of fruit juices reduces bitterness, haze and sediment formation, hence are acclaimed for health benefits and industrial use. Tannase is also being used for production of instant tea preparations. The enzyme has potential uses in treatment of tannery effluents and pretreatment of tannin containing animal feed [4]. One of the major application of tannase is the production of gallic acid. Gallic acid is used for the manufacture of an anti-malarial drug Trimethoprim. Gallic acid is a substrate for the chemical and enzymatic synthesis of propyl gallate, used as anti-oxidants in fats and oils [5].

The enzyme tannase (E.C. 3.1.1.20) also known as tannin acyl hydrolase, is a hydrolytic enzyme that acts on tannin. It catalyses the hydrolysis of bonds present in the molecules of hydrolysable tannins and gallic acid esters producing gallic acid and glucose [6]. Tannase has been isolated from number of micro-organisms like fungi, bacteria and yeast. The fungal species *Aspergillus* and *Penicillium* are the most active micro-organisms capable of producing tannase through submerged and solid state fermentation [7]. But there is a constant search for tannase with more desirable properties for commercial applications. With this view studies on isolation, screening and production of tannase from *Trichoderma harzianum* MTCC 10841 by submerged fermentation technique was carried out. This is the first report on the production of tannase from *Trichoderma harzianum.*

2. MATERIAL AND METHODS

Micro-organism: The tannase producing fungal strain used in the present investigation was isolated from soil sample collected around amaltash tree from Meerut city. The culture was maintained on malt extract medium which consists of malt extract (2%), K_2HPO_4 (0.1%), NH_4Cl (0.1%) and agar (2%). For enzyme production, this medium was supplemented with 1% tannic acid.
The strain was identified as *Trichoderma harzianum* (MTCC no. 10841) by Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Tannin-rich agro residues: Various tannin-rich agro-residues, amla (*Phyllanthus amblica*, bark, leaves and fruit), amaltash (golden shower cassia) (*Cassia fistula*, leaves), ber (*Zyzipus maurtiana*, leaves), Eucalyptus (*Eucalyptus glogus*, bark and leaves), jamun (*Syzgium cumini*, bark and leaves), guava (*Psidium guazava*, bark and leaves), keekar (*Acacia nilotica*, leaves), mango (*Magnifera indica*, leaves), mulberry (*Morus macroura*, leaves), tamarind (*Tamarindus indica*, seed) and pomegranate (*Punica granatum*, rind) were collected from the gardens. They were shade dried and powdered with grinder. The coarse particles were sieved out and the finely grinded powder was used in the present study.

2.1 Isolation and Screening of Tannase producing Fungi

The four different soil samples collected around the tannin-rich plants viz. amaltash (cassia), jamun, pomegranate and tamarind tree. Each soil sample (0.2g) was spreaded on the tannic acid agar plates (malt extract medium supplemented with 1% tannic acid) in triplicates. The plates were incubated at 30ºC for 3-5 days. The fungal colonies appeared on the plates after 24h and were isolated and purified. The fungal isolates producing zone of hydrolysis around its growth were selected as tannase producers and maintained on malt extract slants.

2.2 Tannase Production

The fungal isolates positive for plate assay were further screened for extracellular tannase production in malt extract liquid medium containing 1% (w/v) tannic acid. The medium was inoculated with disc of 0.8 cm diameter of freshly grown (48h) fungal culture and incubated at 30ºC for 3-4 days. After incubation, the cultures were centrifuged at 8,000xg for 10 min at 4°C. The supernatant was used as crude enzyme and extracellular tannase activity was estimated by performing tannase assay.

2.3 Tannase Assay

Tannase was assayed following the method of Mondal *et al*. [8] using tannic acid as substrate at a concentration of 1% in 0.2M acetate buffer (pH 5.5). The reaction mixture was prepared by the addition of 0.5 ml substrate with 0.1 ml of the crude enzyme and incubated at 40ºC for 20 minutes. The enzymatic reaction was stopped by adding 3ml bovine serum albumin (BSA) (1mg/ml). The tubes were centrifuged at 5000g for 10 min. The precipitate was dissolved in 2ml SDS-triethanolamine solution followed by the addition of 1ml of $FeCl_3$ reagent. The contents were kept for 15 min for stabilizing the colour formed and the absorbance was measured at 530nm against the blank. One unit of tannase activity can be defined as the amount of enzyme which is able to hydrolyze 1M of substrate tannic acid in 1min under assay conditions.

2.4 Biomass Determination

To determine the fungal biomass the culture was centrifuged at 8,000xg for 10 min at 4°C and the pellet was washed twice with distilled water. The washed mycelium was dried at 60°C for 24 hr in a pre-weighed dry petridish to constant weight and expressed as g dry weight/50 ml medium.

2.5 Optimization of Culture Conditions for Tannase Production

The following culture conditions were optimized to enhance tannase production from *Trichoderma harzianum.*

Screening of different medium for enzyme production: To screen the medium for maximum production of tannase, different production medium viz. medium A, B, C, D and E were tested. The composition of different medium was as follows (g/L): Medium A: Malt extract medium supplemented with 1% tannic acid. Medium B [3]: K_2HPO_4, 0.5g; KH_2PO_4, 0.5 g; $MgSO_4$, 2.0g; $CaCl_2$, 1.0g; NH_4Cl, 3.0g; Tannic acid, 10.0g. Medium C [9]: $NaNO_3$, 3.0g; KH_2PO_4, 1.0g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; Tannic acid, 10.0 g. Medium D: (Czapek Dox's medium): sucrose, 2.0g; K_2HPO_4, 0.1g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4.7H_2O$, 0.001 g; Tannic acid, 10.0 g. Medium E: (Potato Dextrose Medium): Potato (peeled), 200g; Dextrose, 20.0g; Tannic acid, 10.0g.

Effect of Incubation Period

To study the effect of incubation period, the medium A was inoculated with disc of 0.8 cm diameter of freshly grown (48h) culture of *Trichoderma harzianum* and incubated at 30ºC. The biomass and tannase activity was estimated up to 5 days at regular intervals of 12 hrs.

Effect of Medium pH and Temperature on Enzyme Production

The optimum pH of the culture medium and incubation temperature for tannase production was determined in the pH range of (4.5-6.5) and (30°-55°C) respectively.

Effect of Carbon Source

To study the effect of different carbon source on tannase production the medium was supplemented with different sugars (glucose, fructose, cellulose, maltose, lactose, pectin and sucrose) and natural tannin-rich substrates namely dried amaltash leaves, amla bark, fruits and leaves, ber leaves, eucalyptus bark and leaves, guava bark and leaves, keekar leaves, jamun bark and leaves, mango leaves, mulberry leaves and pomegranate rind. The medium with 1% tannic acid was kept as control. These flasks were inoculated with *Trichoderma harzianum* and incubated at 30^{o}C for 96 hrs and tannase activity was estimated.

Effect of Nitrogen Source

Effect of nitrogen supplements like organic sources (2%) such as peptone, malt extract, yeast extract, beef extract, tryptone and inorganic sources (0.2%) such as $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, KNO_3 and NH_4Cl on tannase production from *Trichoderma harzianum* was studied.

Effect of Additives

The effect of additives (metal ions and detergents) (0.1% w/v) on enzyme production was studied by adding different salts viz. NaCl, KCl, $CaCl_2$, $CuSO_4$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $ZnSO_4$ and $FeCl_3$ and detergents like Tween-20, Tween-40, Tween-60, Tween-80, Triton X-100 and SDS in the production medium. After incubation of the culture in these conditions, tannase activity was determined as described earlier.

2.6 Laboratory Level Scale up Production of Tannase

Laboratory level scale up of tannase production by *Trichoderma harzianum* under submerged fermentation conditions was attempted by growing the fungus in different size flasks ranging from 250ml Erlenmeyer flask (containing 50 ml of the production medium) to 5000ml flask (containing 1500 ml of the production medium) under optimized conditions. The medium in each flask was inoculated with 48h grown culture and incubated at 30^{o}C for 6 days. Tannase activity was determined from each flask at regular intervals of 24hr after 3 days and compared.

2.7 Result Analysis

All the fermentations and assays were carried out in triplicate and the mean value was presented.

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Tannase Producing Fungal Strains

Thirty six fungal strains were isolated from the four soil samples on tannic acid agar plates. On the basis of the clear zone formation around the colonies, twenty two fungal isolates were selected as tannase producers. Further extracellular tannase production from these strains was studied in liquid medium. The isolate T16 produced highest tannase activity (16.36 U/ml) and hence selected for further studies. It was identified as *Trichoderma harzianum* (MTCC no. 10841) by MTCC, IMTECH, Chandigarh, INDIA.

3.2 Optimization of culture conditions for maximum tannase production

The proper combination of various cultural conditions was established in order to achieve maximum tannase production by *Trichoderma harzianum*. The different parameters optimized were as follows:

Screening of Different Medium for Tannase Production

Five production media viz. medium A, B, C, D and E were tested in an attempt to improve the tannase production from *Trichoderma harzianum*. Among the different medium tested, growth as well as enzyme production (16.36 U/ml) was found to be maximum in medium A (malt extract medium supplemented with 1% tannic acid), followed with medium C and B (Table 1). The minimum enzyme production was observed in medium D and E however, growth was appreciable in both the media.

Time Course of Tannase Production

Time course of tannase production was studied to determine the optimum incubation time required for maximum tannase production. The tannase activity was initially detected at 36hr and progressively increased with time. Maximum growth as well as tannase production (18.33 U/ml) was observed at 96 hrs of incubation (Fig.1). Thereafter, the enzyme production slightly decreased. Decreased enzyme yield on prolonged incubation could also be due to reduced nutrient level of medium. It has been reported that tannase activity decreases after reaching maximum level, due to inhibition or degradation of enzyme [10]. Similar observations have been reported for tannase production by many researchers [11,12,13,14]. However, maximum tannase production from *Trichoderma viride* was reported at 48hr by Lokeshwari *et al.* [15].

Effect of pH of the Culture Medium

Tannic acid, being an acidic substrate, makes the pH of the medium acidic. Hence, the pH of the medium was adjusted at different pH of acidic range (4.5-6.5). Maximum tannase production from *Trichoderma harzianum* was observed at pH 5.5. The growth and enzyme production was however, considerable in pH

range of 5.0-6.0. Decrease in pH of the medium reduced tannase production sharply (Table 2). There are reports describing of the optimum pH as 5.5 for tannase production [3,16,17,18].

Effect of Incubation Temperature

The effect of incubation temperature on tannase production from *Trichoderma harzianum* was studied in the temperature range of 30º-55ºC under submerged stationary fermentation conditions. The optimum temperature for growth and tannase production from *Trichoderma harzianum* was found to be 30ºC (Table 3). Further rise in temperature, decreased the production of tannase and the minimum tannase activity was observed at temperature 45ºC. Above 45ºC, the culture could not grow. The fermentation temperature for optimum production of tannase is mostly reported to be 30°C [5,12,18,19]. Some reports also mentioned tannase production at temperature 35°-40°C [9,20,21,22].

Effect of Carbon Source

To obtain the maximum tannase production from *Trichoderma harzianum* different carbon source were tested. It includes commercial tannic acid powder (control), different sugars and natural tannin-rich substrates. Natural tannins proved to be better carbon source than pure tannic acid and sugars. Amla fruit enhanced the level of enzyme production to two-fold, followed with pomegranate rind, jamun bark and amaltash (cassia) leaves (Table 4). These agro-residues substrates can be substituted for costly tannic acid, also suggesting beneficial utilization of agro-wastes. Among the sugars tested as carbon and energy source, except glucose none was found to support enzyme production (Table 5).

Many workers have also attempted tannase production using natural tannin substrates. Kumar *et al*. [23] studied tannase production under SSF using different tannin-rich substrates like ber leaves, amla leaves, jamun leaves and jowar leaves and found maximum enzyme production with jamun leaves. Srivastava and Kar [6] carried out tannase production from *A. niger* ITCC 6514.07 using pomegranate rind as the sole carbon source in the medium. Tannin-rich plant residues were used as substrate and sole carbon source for tannase production by *Penicillium purpurogenum* PAF6 among them, tamarind seed was found to be the most favorable substrate than haritaki, pomegranate, tea leaf waste and arjun fruit [24].

Effect of Nitrogen Source

The effect of nitrogen source on tannase production from *Trichoderma harzianum* was studied by supplementing the production medium with various organic (2%) and inorganic (0.2%) nitrogenous sources. The maximum production of tannase was obtained in control (malt extract and ammonium chloride) as compared to other organic and inorganic sources used alone (Table 6). However, enzyme production in the presence of ammonium chloride followed with peptone was found considerable. Arulpandi *et al.* [25] also studied the effect of supplementation of different inorganic and organic nitrogen sources on tannase production. The organic nitrogen sources such as casein and peptone gave considerable enzyme production.

Effect of Additives

The effect of additives on tannase production was studied by adding the different salts and detergents in the production medium. The metal ions Ca^{++}, Na^{+}, Mg^{++}, Mn^{++} and Zn^{++} did not affect enzyme production. However, metal ions like K^{+}, Cu^{++}, Co^{++}, Fe^{+++} and detergents (Tween-20, Tween-40, Tween-60, Tween-80, Triton X-100 and SDS) inhibited the production of tannase.

The optimization of various nutritional and cultural conditions enhanced the level of tannase production to 1.97-fold as compared to initial conditions.

3.3 Laboratory Level Scale up Production of Tannase

Almost same titre of tannase from *Trichoderma harzianum* was achieved in the medium ranging from 100ml to 1500ml as compared to 50ml (Table 8). However, more fermentation time (120 hr) was required to achieve same production level of tannase for the culture grown in 2L to 5L flasks.

4. CONCLUSION

The tannase from *Trichoderma harzianum* can be employed for gallic acid production using cheaper agro residues. It makes the process of gallic acid production economic and ecofriendly, and also suggests a beneficial utilization of agro wastes. Further, the tannin utilization efficiency of this tannase can also be exploited for a number of industrial applications like treatment of tannery effluents, fruit juice debittering, wine clarification etc.

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Table 1: Screening of different medium for maximum tannase production from *Trichoderma harzianum*

Medium	Tannase activity (U/ml)
A	16.36 (0.51)
B	11.11 (0.48)
C	13.67 (0.46)
D	7.88 (0.41)
E	8.61 (0.40)

Values in parenthesis represent Biomass (g dry wt/50 ml)

Table 2: Effect of pH on tannase production

pH	Tannase activity (U/ml)
4.5	8.42 (0.36)
5.0	14.17 (0.40)
5.5	16.86 (0.46)

6.0	13.05 (0.46)
6.5	10.75 (0.48)

Values in parenthesis represent Biomass (g dry wt/50 ml)

Table 3: Effect of incubation temperature on tannase production

Temperature (^oC)	Tannase activity (U/ml)
30^o	18.24 (0.49)
35^o	16.36 (0.46)
40^o	09.72 (0.34)
45^o	06.67 (0.30)
50^o	N.D.
55^o	N.D.

N.D.=Not Detected

Values in parenthesis represent Biomass (g dry wt/50 ml)

Table 4: Effect of natural tannins as carbon source on tannase production

Carbon source (1%)	Tannase activity (U/ml)
Control (Tannic acid)	18.19
Amaltash (*Cassia fistula*) leaves	24.82
Amla (*Phyllanthus amblica*) bark	19.87
Amla (*Phyllanthus amblica*) fruit	31.56

Amla (*Phyllanthus amblica*) leaves	23.71
Ber (*Zyzyphus mauritiana*) leaves	12.39
Eucalyptus (*Eucalyptus glogus*) bark	4.37
Eucalyptus (*Eucalyptus glogus*) leaves	11.80
Guava (*Psidium guazava*) bark	14.96
Guava (*Psidium guazava*) leaves	14.66
Jamun (*Syzygium cumini*) bark	25.19
Jamun (*Syzygium cumini*) leaves	16.01
Keekar (*Acacia nilotica*) leaves	7.58
Mango (*Magnifera indica*) leaves	14.58
Mulberry (*Morus macroura*) leaves	20.39
Pomegranate (*Punica granatum*) rind	26.63
Tamarind (*Tmarindus indica*) seed	22.01

Table 5: Effect of carbon source on production of tannase

Carbon source (1%)	Tannase activity (U/ml)
Control	18.29 (0.51)
Cellulose	5.13 (0.34)
Fructose	8.34 (0.33)

Glucose	14.88 (0.47)
Lactose	2.29 (0.29)
Maltose	3.93 (0.31)
Pectin	4.14 (0.28)
Sucrose	8.16 (0.41)

Values in parenthesis represent Biomass (g dry wt/50 ml)

Table 6: Effect of nitrogen source on tannase production

Nitrogen sources (2%)	Tannase activity (U/ml)
Control (Malt extract)	32.04 (0.54)
Beef extract	23.59 (0.34)
Peptone	26.07 (0.38)
Tryptone	22.35 (0.46)
Yeast extract	21.48 (0.41)
NH_4Cl	27.26 (0.47)
NH_4NO_3	21.74 (0.36)
$(NH_4)_2SO_4$	14.11 (0.38)
$NaNO_3$	9.66 (0.32)

KNO_3	13.53 (0.34)

Values in parenthesis represent Biomass (g dry wt/50 ml)

Table 7: Effect of metabolites on tannase production

Metabolites	Tannase activity (U/ml)
Control	31.78
NaCl	25.86
KCl	19.73
$CaCl_2$	31.08
$MgCl_2$	25.64
$CuSO_4$	10.67
$CoCl_2$	19.75
$MnCl_2$	26.25
$FeCl_3$	15.68
$ZnSO_4$	23.14
Tween-20	3.63
Tween-40	8.22
Tween-60	11.53
Tween-80	12.08
Triton X-100	4.05
SDS	9.33

Table 8: Laboratory-level scale-up of tannase production

Volume of the medium (ml)	Volume of the flask (ml)	Incubation period (days)	Tannase activity (U/ml)
50	250	4	32.26
100	500	4	31.08
150	500	4	30.69
200	1000	4	32.01
500	2000	5	29.91
750	2000	5	29.02
1000	5000	5	28.96
1500	5000	5	27.72

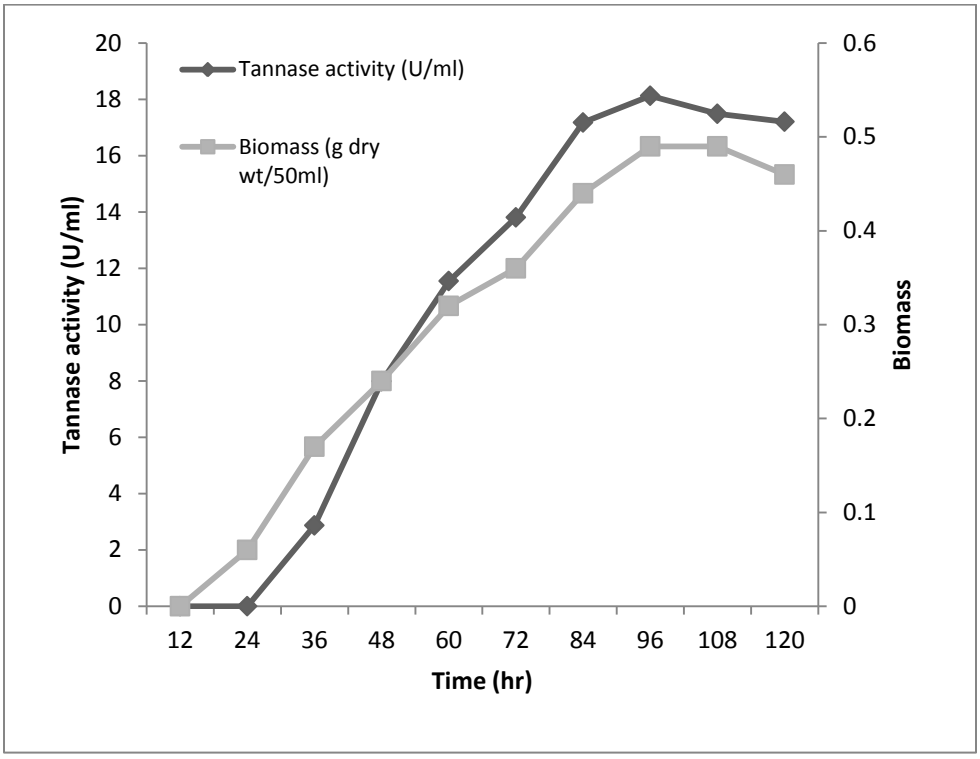

Fig.1 Time course of tannase production from *Trichoderma harzianum*