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STUDIES ON TANNASE PRODUCING BACTERIA FROM SOIL

SUNITA HANAMANT PATIL[1], BHALERAO KIRAN[2], GHUMARE GAYATRI[2] AND MUTHAL DAKSHATA[2]

Department of Microbiology, K.R.T. Arts, B. H. Commerce & A.M. Science College, Savitribai Phule Pune University, Nasik, India

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ABSTRACT

The enzyme tannase hydrolyses the ester bonds of tannic acid to release gallic acid and glucose. Tannic acid is known to be bacteriostatic, but a few organisms are resistant to it by producing tannase enzyme. Frequently used assay for tannase is the isolation of tannase producers in the presence of tannic acid on a minimal agar plate with tannic acid. Tannic acid is an integral and important plant constituent. Soil samples would logically be an excellent source for the isolation of tannase producing bacteria. Soil samples were collected from various regions such as dump yard, market waste and agrowaste. Tannase producing bacteria were isolated from the soil samples after enrichment of the sample using tannin as a carbon and energy source. The best tannase producers was isolated and identified. The effect of various parameters like pH, temperature, substrate concentration, carbon sources on growth of isolates were also evaluated. The results showed that the growth of tannase producing bacteria was maximum at pH of 7 to 7.5, temperature at 35 °C to 37 °C and agitation rate of 200 rpm. Amongst the cultures obtained, five bacterial tannase producers were showed relatively high enzyme activity. The bacterial isolates were identified and characterized using various biochemical tests. The highest tannase activity was showed by two isolates such as T6 (*Bacillus* species) and T11 (unidentified) after 96 hours of incubation whereas the lowest tannase activity shown by isolate T8 (*Micrococcus* species). Dye decolourization and tea powder degradation was observed by tannase enzyme produced by these isolates.

KEY WORDS : Tannic acid, Tannase, Gallic acid, Microbial degradation

INTRODUCTION

Tannins are naturally occurring polyphenolic compounds with varying molecular weight in the plant kingdom. Tannins are considered to be plants secondary metabolic products because they play no direct role in plant metabolism. After lignin, tannins are the second most abundant group of plant phenolics. One of the major characteristics of tannins is their ability to form strong complexes with protein and other macromolecules such as starch, cellulose and minerals (Lekha and Lonsane, 1997; Aguilar *et al.*, 2001) hence they are nutritionally undesirable (Zarate *et al.*, 2014). The term tannins includes tannic acid as well as hydrolysable gallotannins, i.e., those which yield gallic acid on hydrolysis. Tannin Acyl Hydrolase (E.C. 3.1.1.20) is commonly referred to as tannase. The enzyme was discovered in 1867 by Teighem. He reported the formation of gallic acid when two fungal species were exposed to an aqueous solution of tannins (Teighem, 1867). The fungal species were later identified as *Penicillium glaucum* and *Aspergillus niger* (Lekha and Lonsane, 1997). It is an inducible enzyme that catalyses the breakdown of ester linkage in hydrolysable tannins such as tannic acid resulting in the production of gallic acid and glucose (Batra and Saxena, 2005). Majority of tannase producing microorganisms are fungi and bacteria (Zarate *et al.*, 2014). Fungal tannase is an inducible enzyme induced by phenolic compounds such as gallic acid, pyrogallol, methyl gallate and hydrolyzable tannins as tannic acid (Belmares *et al.*, 2004). Filamentous fungi such as *Aspergillus* and *Penicillium* have been widely used for tannase production. Among bacterial isolates *Lactobacillus*

apodemi, *Klebisella pneumonia*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Bacillus licheniformis* are tannase producers. Tannase production at industrial level is in a microbial way using tannic acid, which is quite expensive, implying additional costs in its production. Therefore agricultural wastes containing tannin could be considered to be an alternative source of tannic acid for producing this enzyme. The potential of agricultural residues such as jamun leaves (Kumar *et al.,* 2007), palm kernel cake, tamarind seed powder (Sabu *et al.,* 2005), olive mill waste (Aissam *et al.,* 2005) and coffee husk (Battestin and Macedo, 2007) could be used as substrates for the production of the high value industrial enzyme tannase. Tannase can be produced under liquid state fermentation and solid state fermentation (Paranthaman *et al.,* 2008).

In this study, culturable bacterial populations fromthe soil samples collected from different places were characterized. Bacterial cultures able to degrade tannin were enriched and isolated. Bacterial isolateswere subsequently characterized, identified and their ability for tannin degradation was assessed.

MATERIALS AND METHODS

Enrichment of Soil Samples for Isolation of Tannase Producing Bacteria

Soil samples were collected from the different locations such as Botanical garden of college, dump yard and market yard, wet poultry and dry poultry, hostel compost, soil compost and farm soil from agriculture land. First enrichment culture was established in which soil samples was inoculated in minimal salts medium ($NaNO_3$ 0.6 %w/v, KCl 0.5 % w/v, $MgSO_4$ 0.05 % w/v, K_2HPO4 0.05 % w/v & KH_2PO_4 0.05 % w/v) containing 1 %(w/v) filtered sterilized tannic acid with an antibiotic amphotericin B (Mondal *et al.,* 2001). One gram of the soil was inoculated in medium containing 1%(w/v) tannic acid as the sole carbon and energy source and incubated at 37 °C for 72hrs. After incubation turbidity of growth was observed and then the enrichment culture was transfered into minimal salt medium using 2% inoculum and incubation continued for another at 37 °C for 72 hrs.

Isolation and Screening of Tannase Producing Bacteria

After incubation the culture was serially diluted and inoculated on minimal agar supplemented with 1%(w/v) tannic acid. The plates were incubated at 37 °C for 72 hrs. Bacterial colonies developed on minimal agar containing tannin was purified and coded appropriately.

Cultural, Morphological and Biochemical Characteristics of Isolates

The bacterial isolates were subcultured on minimal agar medium containing tannic acid. Bacterial isolates were then characterized according to Bergey's manual of Systematic Bacteriology, by Gram staining and biochemical tests. Colony characteristics such as size, shape, colour, consistency and transparency were recorded. Biochemical tests like Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Starch hydrolysis test, Urease test, catalase test, nitrate reduction test and Carbohydrate test such as mannitol utilization test were performed for the isolates.

Effect of various physio-chemical factors on bacterial growth

The effect of various physico-chemical factors like pH, temperature, substrate concentration, agitation rate, carbon and nitrogen sources on bacterial growth. Bacterial growth was monitoredby measuring the optical density (OD) at 510 nm.

Effect of substrate concentration (tannic acid concentration) on bacterial growth

Using different tannic acid concentrations (1% to 10 %) supplemented into minimal medium, the growth of selected bacteria was determined by incubating at 37 °C for 72 hours on incubator shaker. Odenyo *et al*. also isolated tannin tolerant ruminal bacteria from East African ruminants with the maximum tolerant concentration of 50- 70 g/L (Odenyo *et al.,* 2001).

Effect of pH and temperature onbacterial growth

The effect of pH (4 to 9.5) and temperature (25° to 50°C) on growth of tannase producing bacteria was studied.

Effect of agitation rate on bacterial growth

The effect of different agitation rates in the range of 50 to 300 rpm at regular intervals of 50 rpm was evaluated on bacterial growth.

Effect of different carbon sources on bacterial growth

Different carbon sources such as glucose, maltose, lactose and sucrose were added individually in the

medium as replacement of D-glucose in the production medium at 1 to 5% (w/v) concentration along with the tannic acid and incubated at 37 °C for 72 hours onincubator shaker. A negative control devoid of any carbon source was also set up. The addition of carbon and nitrogensources favoured the production of tannase for the subsequent cleavage ofthe tannin molecules to liberate a supply of carbon for growth.

Tannase Assay by UV spectrophotometric Method

This method is based on change in ultraviolet absorption. The enzyme activityis determined by the hydrolysis of the ester bonds of tannic acid. The assay method commences by adding 0.5 mL of crude enzyme to 2 mL of 0.35% (w/v) tannic acid dissolved in 0.05M citrate buffer (pH 5.5) solution. 2 mL of ethanol solution was used to stop enzyme reaction. Absorbance on UV spectrophotometer was noted as t1 at 310 nm immediately after adding ethanol and as t2 after 10 min of incubation at 37 °C. Formula for the calculation of enzyme activity by this method is:

Enzyme activity (U/ mL) = 114 x Change in absorbance/ Difference in time (t2-t1).

All the tests were performed in triplicates. One unit of tannase activity is defined as amount of enzyme required to hydrolyse 1µmol of ester in 1 min per mL under assay conditions.

RESULTS AND DISCUSSION

Screening and isolation of tannase producing bacteria

Out of fifteen tannin degrading bacteria, five bacterial isolates were selected (which showed growth up to 10% of tannic acid) and coded as T1, T6, T8, T11 and T15. The bacterial cultures werepreserved on Nutrient agar slants and were further characterized.

Characterization and Identification of bacterial isolates

Colony Characteristics and biochemical characteristics of tannic acid degrading bacteria were recorded (Table 1 and 2). Based on the morphology, carbohydrate utilization capacity and biochemical tests, they were identified. Out of five isolates, only four isolates were identified while one isolate remains unidentified. The isolates T1, T6, T8 and T15 were identified as *Pseudomonas aeruginosa*, *Bacillus*, *Micrococcus* and *Serriatia marcescens*, respectively. Deschamps *et al.* (1983) isolated fifteen bacterial strains belonging to the genera *Bacillus*, *Staphylococcus* and *Klebsiella* by enrichment culture technique, using tannic acid as the sole source of carbon. Usingtannin-extract from chestnut (HT) or quebracho (CT) as the only carbon source in a culture technique, certain microbes, including *Bacillus pumilus*, *B. polymyxia*, *Klebsiella planticola*, *Cellulomonas*, *Arthrobacter*, *Micrococcus*, *Corynebacterium* and *Pseudomonas* were shown to produce enzymes that degradetannins (Deschamps *et al.*, 1980).

Table 1. Colony characteristics

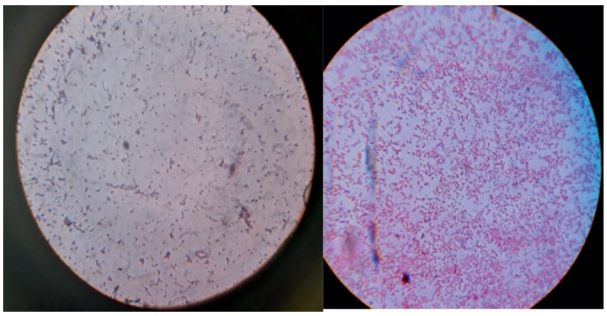
Isolates	Size	Shape	Color	Margin	Elevation	Opacity	Consistency	Gram character	Motility
T1	1mm	Circular	Bluish green	regular	convex	opaque	smooth	Gram negative short rod	Sluggish motile
T6	2mm	Circular	white	regular	convex	opaque	sticky	Gram positive rod	Highly motile
T8	1mm	Circular	Lemon yellow	Entire	convex	opaque	smooth	Gram positive cocci	Non-motile
T11	1mm	Circular	yellow	regular	convex	opaque	sticky	Gram positive cocci	Non-motile
T15	2mm	Circular	Orange	regular	convex	opaque	smooth	Gram negative short rod	motile

Table 2. Biochemical Characteristics

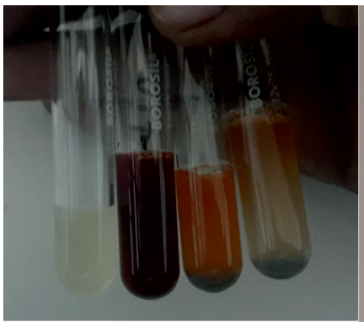
Biochemical Tests	Bacterial isolates				
	T1	T6	T8	T11	T15
Indole Production	Negative	Positive	Negative	Positive	Negative
Methyl red test	Negative	Negative	Negative	Negative	Negative
Voges- Proskauer test	Negative	Negative	Negative	Negative	Positive
Citrate utilization test	Positive	Negative	Negative	Positive	Positive
Hydrogen Sulphide production	Negative	Positive	Negative	Negative	Negative
Mannitol fermentation	Positive	Positive	Positive	Negative	Positive
Starch hydrolysis test	Negative	Positive	Positive	Negative	Negative
Gelatin hydrolysis test	Positive	Positive	Positive	Negative	Positive
Urea hydrolysis test	Negative	Negative	Positive	Positive	Positive
Nitrate reduction test	Positive	Positive	Positive	Negative	Positive
Catalase test	Positive	Positive	Positive	Positive	Positive
Oxidase test	Positive	Positive	Positive	Positive	Negative

PHOTOS

1. Gram Staining

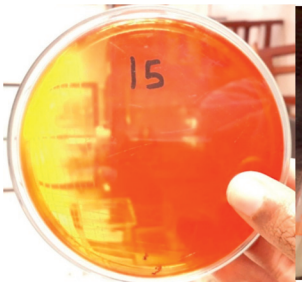


2. Biochemical tests

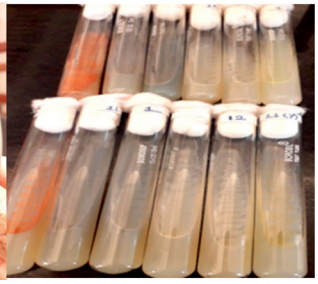


Nitrate Reduction Test

Catalase test



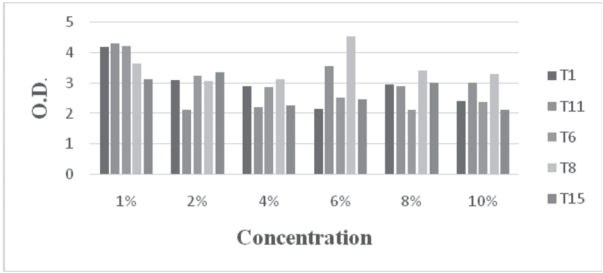
Growth on Mannitol Agar



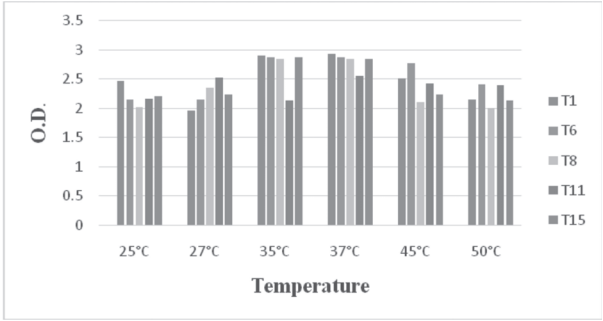
Bacterial isolates T1 to T15

Effect of various physio-chemical factors on bacterial growth

The effect of various concentration of tannic acid on growth of the bacterium was studied. The various concentrations of tannic acid used were 1% to 10%. The maximum growth of the bacteria was observed at 1% concentration while maximum growth of T8 was observed at 6% concentration (Graph 1). The isolates were incubated in different temperature ranging from 25 °C to 50 °C from which maximum growth was observed from 35 °C to 37 °C (Graph 2). T8, T11 and T15 showed maximum growth at pH 7 while remaining two isolates showed maximum

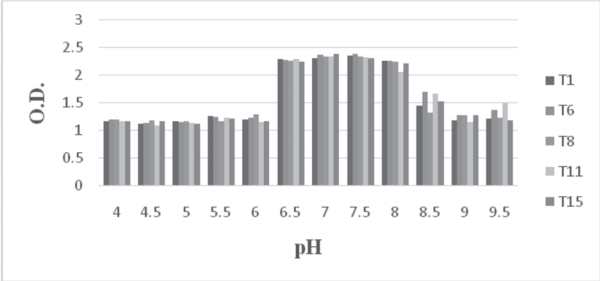


Graph 1. Effect of various concentration of tannic acid on growth

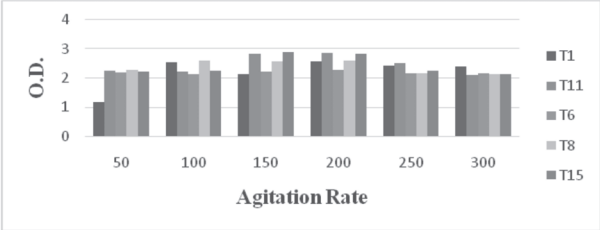


Graph 2. Effect of temperature on bacterial growth

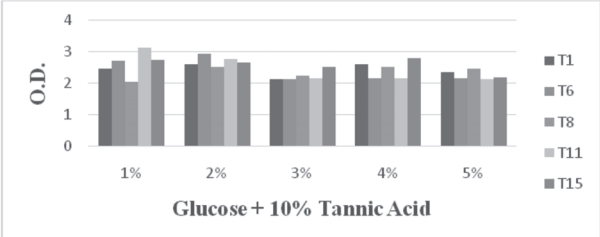
growth at pH 7.5 (Graph 3). Maximum growth of all five isolates was observed at 200 rpm agitation rate (Graph 4). Minimal medium containing 10% tannic acid and 1% to 5% sugar (glucose, sucrose, maltose, lactose) solution were prepared. T1, T11 and T15 showed maximum growth at 1 concentration of glucose and tannic acid while T6 and T8 showed maximum growth at 2 concentration of glucose and tannic acid. T11 and T15 Showed maximum growth at 1% sucrose and tannic acid. T1, T6, T8 and T15 Showed maximum growth at 4% maltose and tannic acid while T11 showed maximum growth at 5%. T1 and T6 Showed maximum growth at 2% lactose and tannic acid while T8 and T11 showed maximum growth at 3% lactose and tannic acid (Graph 5, 6, 7 & 8).



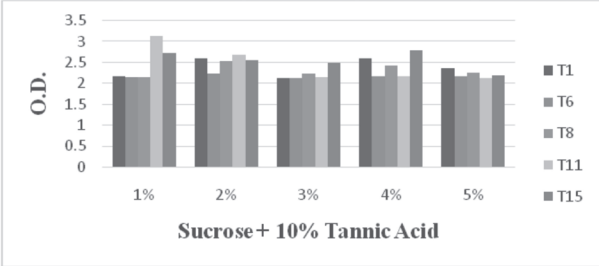
Graph 3. Effect of pH on bacterial growth



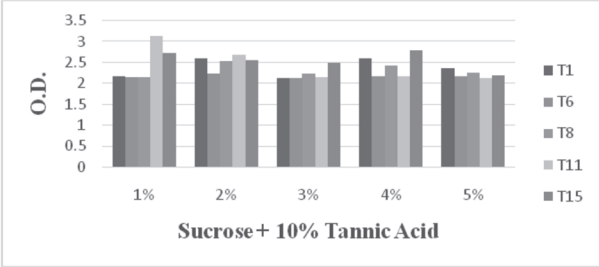
Graph 4. Effect of agitation rate on bacterial growth



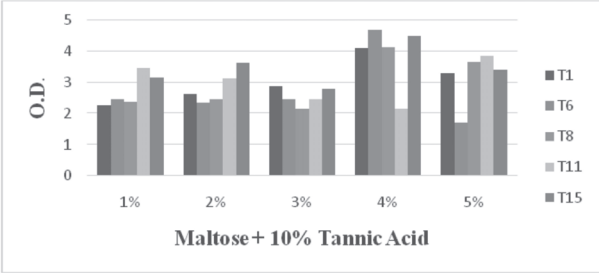
Graph 5. Effect of Glucose and tannic acid on bacterial growth



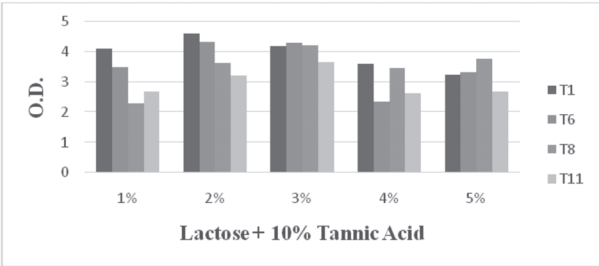
Graph 6. Effect of Sucrose and tannic acid on bacterial growth



Graph 7. Effect of Maltose and tannic acid on bacterial growth



Graph 8. Effect of Lactose and tannic acid on bacterial growth



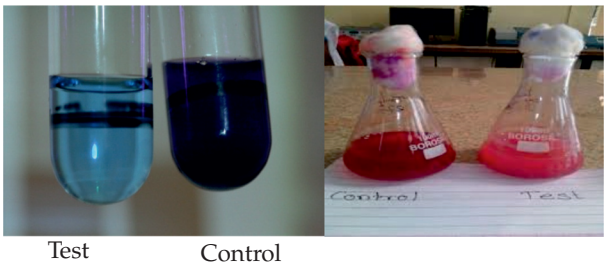
Graph 9. UV spectrophotometric Method

Tannase assay

Tannase activity of the five isolates was determined by UV Spectrophotometric method (Graph 9). Out of five isolate T1 showed maximum activity after 48 hours while remaining isolates (T6, T8, T11 and T15) showed maximum activity after 96 hours of incubation period. UV spectrophotometric method estimates the esterase activity of tannase by detecting the hydrolysis of ester bonds.

Applications of Tannase

Dye Decolorization: Crystal violet, safranine dyes



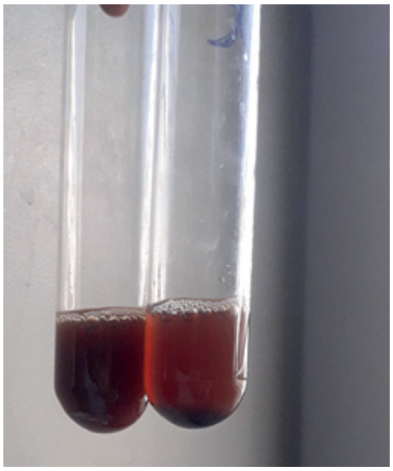
Test Control

Decolorization of dyes by enzyme

were treated individually with crude enzyme in the ratio 1:1 (v/v). A control was run simultaneously with water in the ratio of 1:1 (v/v). After 48 h of incubation there was removal of colour.

Degradation of Tea

Tea solution was treated with crude tannase in the ratio of 1:1 (v/v) and incubated for at room temperature and change in colour was observed.A control was run simultaneously with same amount of water instead of enzyme. The change incolour of enzyme treated teafrom dark to lighter as compared to control (without enzyme) was observed.



Control Test

Degradation of Tea by enzyme

CONCLUSION

In the present study, tannase producing bacteria were isolated from soil samples. On the basis of their morphological and biochemical characteristics, bacterial isolates T1, T6, T8 and T15 identified as *Pseudomonas aeruginosa*, *Bacillus*, *Micrococcus* and *Serriatia marcescens* respectively while one isolate T11 remains unidentified. The highest tannase activity was shown by T6 (*Bacillus* species) and T11 (Unidentified) after 96 hours of incubation whereas the lowest tannase activity showed byT8 (*Micrococcus* species). Tannase enzyme from these isolates was used for decolorization of two dyes such as crystal violet and safranine as well as for tea powder degradation.

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