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Original Research Article

Preliminary isolation and screening of tannase producing bacteria and fungi

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Tannins, Tannase, Screening, Tannase production, Tannase activity

A B S T R A C T

Even though tannase has wide applications it is still considered as novel and one of the costly enzymes. Recognizing the need of the hour it becomes important to isolate and develop potent tannase producing strains that can be used for economical and feasible tannase production. The aims of the present study are to isolate, identify and screen the tannase producing microorganisms from various tea waste dump sites, agro-residue waste sites and tannery effluents. These soil samples and tannery effluents are rich in tannins and its derivatives. Seven fungal isolates and thirteen bacterial isolates were obtained as tannase producers from tannic acid agar plates. Their morphology, microscopic and biochemical characteristics were studied. Zone of hydrolysis formed by them revealed their tannin degrading ability. Enzyme activity of these cultures producing tannase was studied under static and shaking condition. The enzyme activity of fungal cultures FT 2 (6.35 U/mL) and FT 6 (6.09 U/mL) and bacterial cultures BT 6 (20.9 U/mL) and BT 11 (20.9 U/mL) were maximum in static condition while fungal isolates FT 1 (5.58 U/mL) and FT 4 (7.5 U/mL) and bacterial isolates BT 3 (25.73 U/mL) and BT 8 (25.71 U/mL) showed optimum activity in shaking condition. However, FT 5(6.20 U/mL and 4.24 U/mL) and BT 7 (19.55 U/mL and 25.35 U/mL) showed consistent activity under both the conditions. Thus the present study helps in screening the potent tannase producers from waste dump sites and tannery effluents.

Introduction

Tannins are naturally occurring water-soluble polyphenolic compounds found in various parts of plant bodies like leaves, barks, stem and fruits. Tannins are classified into two major classes: (a) Hydrolysable tannins and (b) Condensed tannins. Mostly they occur in condensed form (Bhat *et al.*, 1998). Tannins are abundantly present in natural plants like monocots, tea, coffee, sorghum, berries, nuts, pomegranates, legumes, some herbs and spices like cloves and cinnamon, palm kernel, *Phyllanthus emblica* (amla) and other different species of plants or plant products which are used for human consumption (Bhat *et al.*, 1998). Tannin in form of Catechin (Flavan-3-ols) is present in tea, cocoa, acacia and catechu plants. Catechins are present in all types of

tea; almost 25–30% of catechin constitutes dry weight of fresh tea leaf, although the content may vary according to season, altitude, environment and species (Balentine *et al.*, 1998). Tannins are known for their antimicrobial property and are resistant against microbes to protect plant bodies. They are toxic and release bacteriostatic compounds making non-reversible action with proteins (Bhat *et al.*, 1998). Although having antimicrobial activity, tannins serve as a nutrient compound or substrate for some microbes that utilise it with the help of the hydrolytic enzyme named Tannase. Tannic acid is a heteropolymer composed of glucose and gallic acid in 1:9 ratios and has various commercial applications. Industrial bioconversion of tannic acid is achieved with Tannase (Mondal *et al.*, 2001). Tannase is also known as tannin acyl hydrolyase (E.C. 3.1.1.20) that catalyses the hydrolysis of ester and depside linkages in hydrolysable tannins yielding glucose and gallic acid as products. Tannase is an extracellular inducible enzyme that can be obtained from fungi, bacteria, some yeasts, higher plants and animal sources (Belur and Mugeraya, 2011). Tannase has wide industrial applications, in different food and feed, beverage, cosmetics, chemicals and brewing industries, in preparation of gallic acid, in instant tea, acron wine, coffee flavoured soft drinks, clarification of beer and fruit juices, detanification of food and increasing the nutritive property of feed provided to cattle and also in bioremediation by cleaning up the tannins from effluents of industries specially from leather industries. Recently, bacteria producing tannase have been associated with colon cancer allocating the possibility of bacterial tannase as biomarker for colon cancer (Lekha and Lonsane, 1997; Das Mohapatra *et al.*, 2012). Many reports regarding fungal and bacterial tannase have been studied and it is evident that fungal tannase are exploited and studied extensively as compared to the bacterial tannase, however reports on bacterial tannase are also very interesting and show fascinating results. Most of the studies on bacterial tannase reveal that the organisms were known and they exploited their tannin utilising property through production of tannase. Studies on optimization of the enzyme production, its characterization and its applications in industrial sectors are also being carried out. Nevertheless there are also scientists working on isolation, identification and screening the novel species of bacteria from different samples or ecosystems and developing techniques which are more sensitive and easily reproducible for identifying the activity of tannase produced from them.

Because of the varied applications of tannase it becomes necessary to isolate and identify the potent tannase producing microorganisms. Therefore in the present study, we have screened tannase producing bacteria as well as fungi from different samples. The characteristics of the organisms and the activity of tannase produced by them were studied.

Materials and Methods

Screening and isolating tannase producers

Soil samples of various tea waste dump sites and agro-residue waste sites were collected and treated with calcium carbonate in appropriate amount and buried in soil again, for maintaining the alkalinity of soil and enhancing the bacterial growth. The tannery effluent waste was collected from Central Leather Research Institute (CLRI), GIDC, Ahmedabad. The soil samples and the effluent waste were diluted up to 10^{-5} dilution. The suspensions were subjected to nutrient agar (HiMedia, India) plates

containing 0.5% tannic acid (Merck, India) as the substrate and sole source of carbon having pH 7.0. These plates were incubated at 37°C for 4 days and the colonies which showed dark zone surrounding them were to be observed (Osawa and T. P. Walsh, 1993). Fungi as well as bacteria were obtained after incubation and to isolate them, fungi were plated on rose bengal agar medium (HiMedia, India) and bacteria in the same medium but with addition of an antifungal compound and these plates were further incubated and their morphological characters were observed.

Finally the selected organisms were plated again on Tannic acid containing agar plates (TAA) to check the dark zone formation by them as a confirmation. Fungi were preserved on Rose Bengal agar slants and bacteria on Nutrient agar slants, regular sub-culturing were done after every 30 days.

Characteristic studies of microorganisms

Morphological features were noted from the plates according to their growth pattern and zone formations by fungi and bacterial cultures were measured. Microscopic examination of fungi was done with lactophenol whereas Gram's staining was performed for bacteria. Bacterial cell growth pattern was checked from absorbance 600-670 nm at regular interval using spectrophotometer (Visiscan 167, Systronics). Biochemical tests mainly Citrate utilization, Lysine decarboxylase, Ornithine decarboxylase, Urease production, H_2S production, Phenylalanine deamination test was detected by adding 2-3 drops of TDA reagent next day and Nitrate reduction was detected by addition of zinc dust and utilization of sugars like glucose, adonitol, arabinose, lactose and sorbitol were tested for all the bacterial isolates.

Tannase Production

Enzyme production was performed in 250 mL Erlenmeyer flask containing 100 mL Nutrient broth media(HiMedia, India) supplemented with 0.5%Tannic acid (5.0 gL^{-1}) (Merck, India)as substrate and the sole source of carbon. The media was sterilised at 121°C at 15 lbs for 15 min and pH was adjusted to 7.0. In case of Fungi, spores from the plates were scrapped off into 10 mL sterile 0.01 % Tween 80 solution prepared in distilled water. Suspension was mixed using cyclomixer to break cell aggregates. The spore suspension was serially diluted and spore count was determined using 1mL of that suspension. 2% v/v spore suspension containing 2 x 10^7 spores per mL was used as inoculum in 100 mL media (Murugan *et al.,* 2007). In case of bacteria, 2% v/v suspension (i.e. 2mL of culture added to 100mL medium) prepared in sterile nutrient broth after 48 hours of incubation was used as inoculum. These flasks were then incubated at 37°C till 96 hours to study and compare the effect of static as well as shaking condition on enzyme production. For shaking condition, flasks were incubated in orbital shaker (REMI, India) at 100 rpm. Biomass of fungi was measured under static and shaking conditions. Cells were removed by centrifugation and filtration through Whatman filter paper No. 1. Cell-free broth containing crude enzyme i.e. supernatant or filtrate was used for checking the tannase activity.

Tannase Activity

Tannase cleaves tannic acid into gallic acid and liberates glucose. Tannase activity was determined by estimating the reduced glucose liberated using 3,5-dinitrosalicylic acid following the Miller method. Standard curve was studied by preparing 100-1000

µg/mL of glucose solution. The tannase activity was determined by using the supernatant or the filtered cell-free broth and pre-incubating it with 0.1M acetate buffer (pH 5.0) containing 0.5% tannic acid as substrate. 1mL crude enzyme and 1mL substrate dissolved in the buffer were reacted for 30 min at 50°C followed by the incubation in boiling water bath for another 15 min to deactivate the enzyme-substrate activity. From this 2mL system, 1mL was withdrawn and reacted with 3, 5-dinitrosalicylic acid and finally the system was made up to 10mL by adding distilled water and absorbance was measured at 540nm.

The activity was calculated using following formula: Enzyme activity (U/mL) = Microgram of glucose produced/ V x T, Where microgram of glucose can be obtained from standard graph and V is the aliquot of enzyme sample (here 1mL) and T is the time of hydrolysis (here 30 min). All the tests were performed in triplicates. *One unit of tannase activity was defined as the amount of enzyme releasing 1µmol min^{-1}of glucose under assay conditions.*

Results and Discussion

Screening and isolating tannase producers

From the different soil samples and tannery effluent sample that were collected, we were able to isolate fungi as well as bacteria producing tannase from the tannic acid agar plate. Seven isolates of fungi (FT1 to FT7) and thirteen isolates of bacteria (BT1 to BT13) were obtained which showed dark zone of hydrolysis on the plates (As shown in Fig. 1), thus confirming their ability to degrade tannin. The fungal cultures were preserved on RBA slants and the bacterial cultures on Nutrient agar slants.

Characteristic studies of microorganisms

Morphological features (colony characteristics) based on size, shape, margin, texture, opacity, elevation and pigment (Table 1 and Table 2) were noted of bacteria and fungi. Zone formations were measured of fungi and bacterial cultures from plates after 48hoursincubation (As shown in Table 3). Microscopic examination of fungi showed us that 3 *Aspergillus*, 2 *Mucor*, 1 *Penicillium* and1 *Rhizopus* species were present whereas from Gram's staining of bacteria it was observed that all of them were Gram negative short rods. Bacterial cell growth pattern was checked after24 and 48 hours of incubation at absorbance range of 600-670 nm and it was observed that after 48 hours and at absorbance of 660 nm bacterial growth showed maximum peak. Wet and Dry biomass of fungal cultures were measured in grams and basic dry biomass was calculated for both static as well as shaking condition. The biomass was found to be optimum between 48-72 hours. Biochemical characteristics through following tests: citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease production, phenylalanine deamination, nitrate reduction, H_2S production and sugar utilization of following sugars: glucose, adonitol, arabinose, lactose and sorbitol were checked on bacteria (Table 4).

Tannase activity

Tannase production was carried out by both Static as well as Shaking condition. The activity was calculated based on the given formula and represented here in form of graphs along with standard error. FT2 (6.35 U/mL), FT 5 (6.20 U/mL) and FT 6 (6.09 U/mL) were showing good tannase activity in static condition whereas FT 1 (5.58 U/mL), FT 3 (5.46 U/mL) and FT 4 (7.5

U/mL) showed better activity in shaking condition. Bacterial cultures BT 6 (20.9 U/mL), BT 7 (19.5 U/mL) and BT 11 (20.9 U/mL) were having better activity in static condition while BT 3 (25.73 U/mL), BT 6 (25.46 U/mL) and BT 8 (25.71 U/mL) showed activity in shaking condition (Fig. 2, 3, 4 and 5).

Screening and isolation of tannase producers

There were seven fungal isolates and thirteen bacterial cultures that were screened for their tannase producing ability. Results show us that more bacterial strains are obtained than fungal species. Reports on tannin degradation of soil by bacteria, isolation from tannery effluent and tannase production by microorganisms have been carried out previously and known (Osawa and Walsh, 1993; Matthew Ilori *et al.,* 2007, Dave *et al.,* 2011, Ingole *et al.,* 2012, Gilna *et al.,* 2007).

Characteristic studies of microorganisms

Morphological features of fungi and bacteria are included in identification scheme which helps in identifying genus and their extracellular characters. Zone formed by hydrolysis of the tannic acid present in the medium were measured which helped in identifying and screening the tannase producers. Fungal cultures showed better zone formations than the bacteria. The results in the present study were in relevance to the work done by Abou-Bakrand *et al.,* 2013 on fungi and some fungi in the present study showed better zones, indicating better tannase producing species.

Microscopic examination of fungi revealed 3 *Aspergillus* sp., 2 *Mucor* sp., 1 *Penicillium* sp. and 1 *Rhizopus* sp. whereas Gram's staining revealed all bacterial isolates are Gram negative and short rods which is similar as reported by Dave *et al.,* 2011. Bacterial cell biomass was found to be highest after 48 hours at absorbance 660 nm. Fungal biomass was measured under static and shaking conditions and it was found that consistent biomass was produced under static as well as shaking condition and it was optimum between 48–72 hours. Biochemical characters help in species identification of bacteria and their biochemistry. Thirteen tannase producing bacteria were subjected to different biochemical tests and sugar utilization tests which revealed that almost all were able to utilize glucose and lactose sugars.

Tannase assay

From all the fungal and bacterial isolates which were checked for their tannase activity as secondary screening under static and shaking condition only few of them yield good results. Fungal cultures FT 2 and FT 6 and bacterial cultures BT 6 and BT 11 showed maximum activity in static condition while fungal isolates FT 1 and FT 4 and bacterial isolates BT 3 and BT 8 showed optimum activity in shaking condition.

However, cultures FT 5 and BT 7 showed consistent and good activity under both the conditions. It is revealed from the study that some fungal tannase producers perform well in static condition and some bacterial producers perform well in shaking condition. The report here also shows that tannase produced from bacterial source is quantitatively more than that from fungal source.

Thus in the present study, various fungal and bacterial tannase producers were isolated, their morphological, microscopic and biochemical characters were studied.

Table.1 Cultural (Colony) characters of bacteria

Bacteria Isolate code	Size	Shape	Margin	Texture	Opacity	Elevation	Pigment
BT1	Big	Circular	Even	Mucoid	Opaque	Flat	Off-White
BT2	Small	Circular	Even	Smooth	Translucent	Raised	Off-White
BT3	Small	Circular	Even	Smooth	Translucent	Sligtly raised	Nil
BT4	Small	Circular	Even	Smooth	Translucent	Sligtly raised	Nil
BT5	Big	Circular	Even	Smooth	Translucent	Raised	Off-White
BT6	Small	Circular	Even	Smooth	Translucent	Flat	Nil
BT7	Small	Circular	Even	Smooth	Translucent	Flat	Nil
BT8	Small	Irregular	Uneven	Rough	Translucent	Flat	Off-White
BT9	Small	Irregular	Uneven	Rough	Translucent	Flat	Off-White
BT10	Big	Circular	Even	Mucoid	Translucent	Raised	Off-White
BT11	Small	Circular	Even	Smooth	Translucent	Flat	Off-White
BT12	Small	Circular	Even	Rough	Translucent	Flat	Off-White
BT13	Small	Circular	Even	Smooth	Translucent	Flat	Off-White

Table.2 Cultural (Colony) characters of fungi

Fungal Isolate code	Size	Form	Margin	Texture	Opacity	Elevation	Pigment
FT1	Big	Irregular	Entire	Rough and dry	Opaque	Raised	White
FT2	Big	Circular	Entire	Rough and dry	Opaque	Rasied	Yellow
FT3	Big	Rhizoidal	Undulate	Rough and dry	Opaque	Umbonate	White
FT4	Big	Rhizoidal	Undulate	Rough and dry	Opaque	Umbonate	White
FT5	Big	Filamentous	Filiform	Rough and dry	Opaque	Umbonate	Greenish
FT6	Big	Filamentous	Filiform	Rough and dry	Opaque	Umbonate	Greenish
FT7	Small	Circular	Lobate	Rough and dry	Opaque	Umbonate	Blackish

Table.3 Measurements of zone size (mm) formed by hydrolysis of tannic acid by fungi and bacteria from plates

Fungi	Zone size (mm)	Bacteria	Zone Size (mm)
FT1	34	BT1	20
FT2	48	BT2	22
FT3	33	BT3	26
FT4	40	BT4	28
FT5	45	BT5	30
FT6	45	BT6	18
FT7	32	BT7	15
		BT8	16
		BT9	20
		BT10	28
		BT11	22
		BT12	19
		BT13	20

Table.4 Biochemical characteristics and sugar utilization tests of all bacterial isolates

Tests	BT 1	BT 2	BT 3	BT 4	BT 5	BT 6	BT 7	BT 8	BT 9	BT 10	BT 11	BT 12	BT 13
Citrate Utilization	-	-	-	+	-	-	+	+	-	-	+	-	-
Lysine Decarboxylase	-	-	+	-	+	+	-	-	-	-	-	+	-
Ornithine Decarboxlyase	-	+	-	-	+	+	-	+	-	+	+	-	+
Urease Production	-	+	+	-	-	-	-	-	-	-	+	-	-
Phenylalanine Deamination	-	-	-	+	-	-	-	-	-	+	-	-	+
Nitrate Reduction	-	-	+	-	-	-	+	-	+	-	+	-	+
H_2S Production	+	-	-	+	-	+	-	-	-	-	-	-	-
Glucose Utilization	-	+	+	+	+	+	+	+	+	+	+	+	+
Adonitol Utilization	+	-	-	-	+	+	+	+	-	+	-	+	+
Arabinose Utilization	-	-	-	-	-	-	+	+	-	+	-	-	+
Lactose Utilization	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol Utilization	+	+	-	+	+	+	+	+	+	+	-	-	+

Legends: "+" Positive result; "-" Negative result

Figure.1 Bacteria showing zone of hydrolysis on tannic acid containing nutrient plate

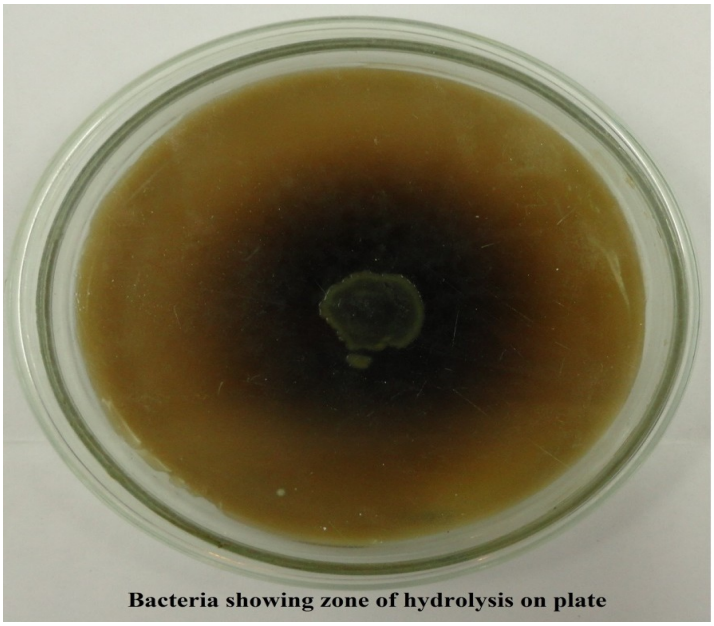


Figure.2 Quantitative tannase profile (U/mL) of fungal isolates in static condition

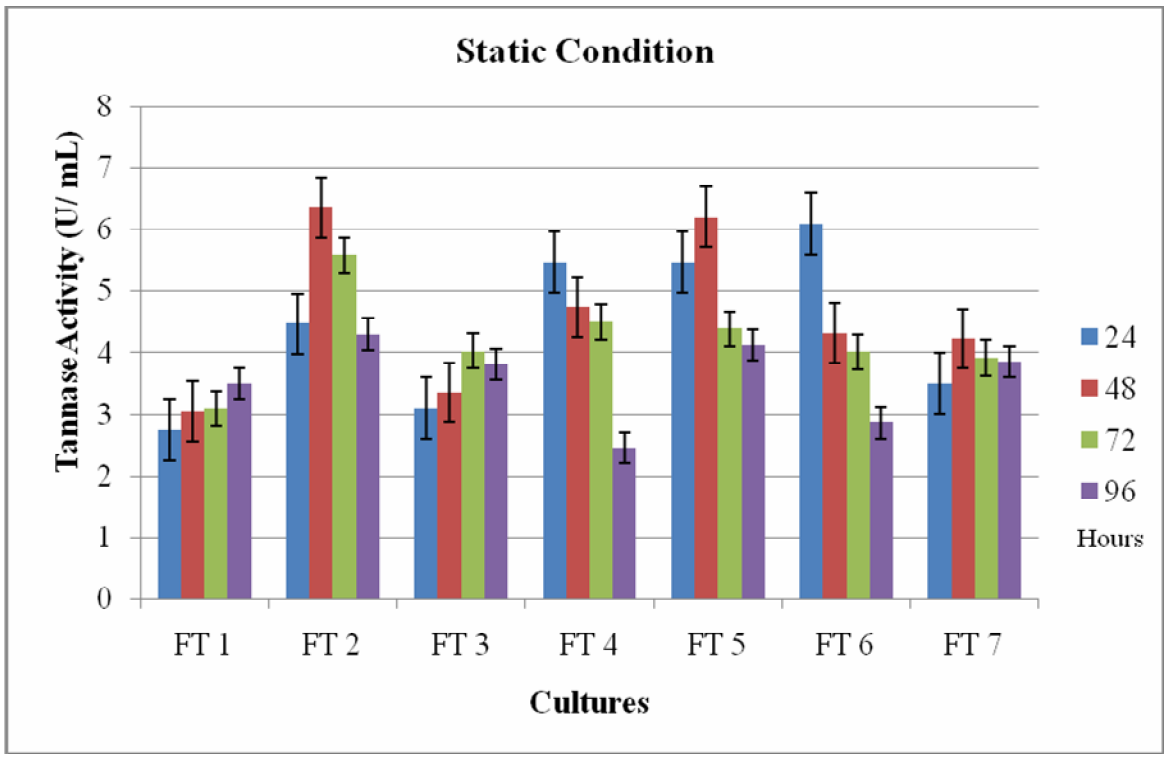


Figure.3 Quantitative Tannase profile (U/mL) of fungal isolates in shaking condition

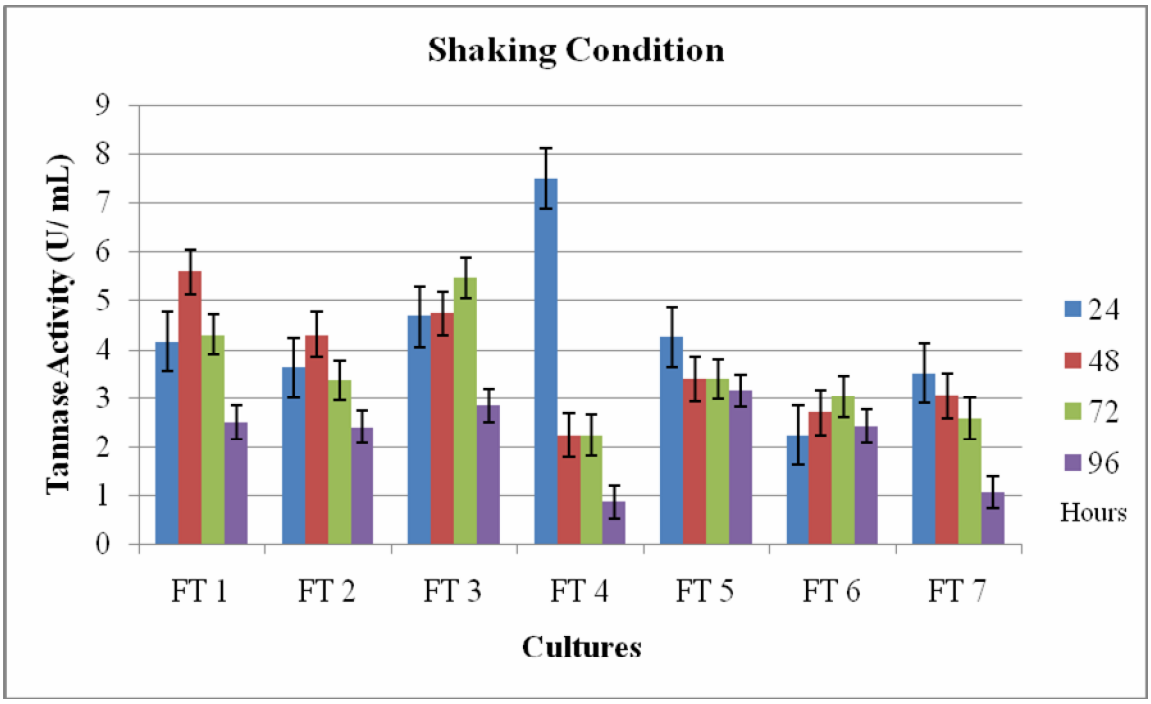


Figure.4 Quantitative Tannase profile (U/mL) of bacterial isolates in static condition

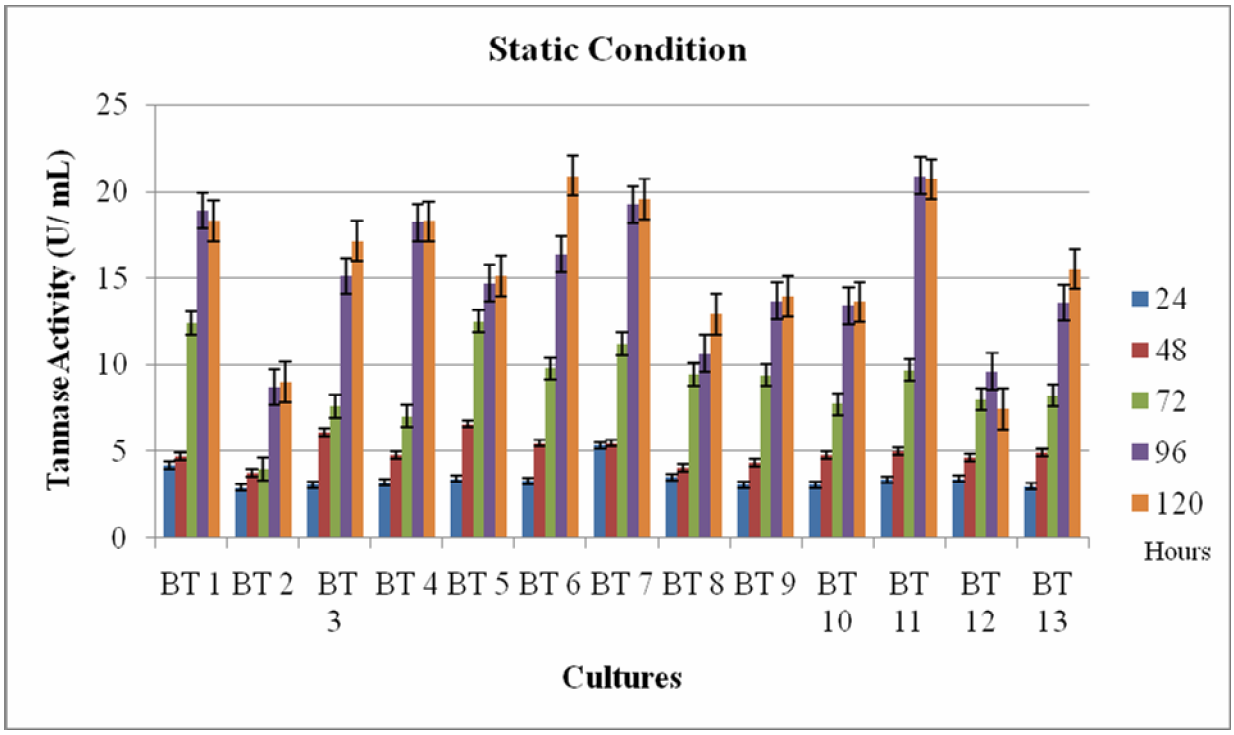
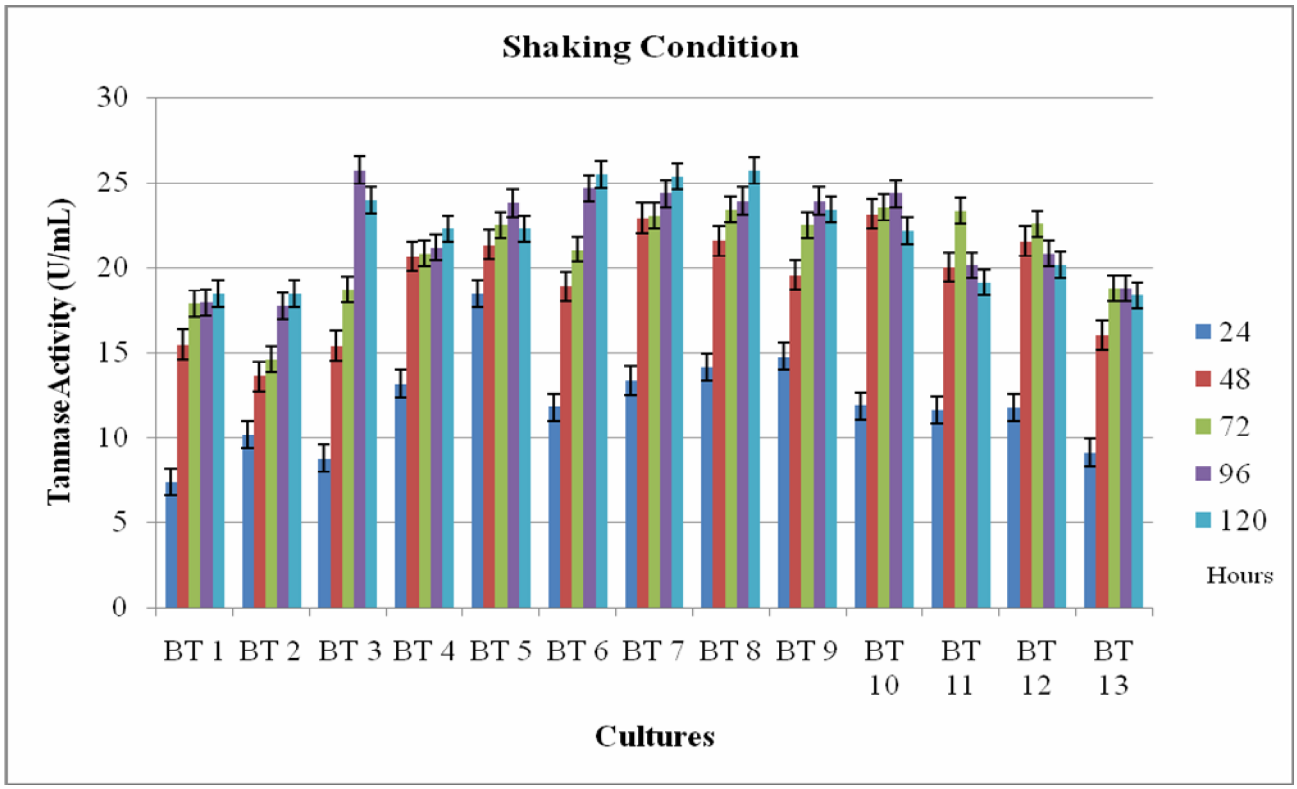


Figure.5 Quantitative tannase profile (U/mL) of bacterial isolates in static condition



The tannase activity of these isolates helped in screening the microorganisms which can be further exploited to study the optimization and characterization of tannase and in quantitative analysis. The present study is the beginner's step towards understanding and identifying the potent tannase producing microorganisms from natural environment.

References

Abou-Bakr, H.A., El-Sahn, M.A., El-Banna, A.A. 2013. Screening of tannase producing fungi isolated from tannin rich sources. *Int. J. Agric. Food Res.*, 2(3): 1–12.

Balentine, D.A., Harbowry, M.E., Graham, H.N. 1998. Tea: the plant and its manufacture; Chemistry and consumption of the beverage. In: Spiller, G.A. (Ed.), Caffeine, CRC press, Boca Raton. Pp. 2647–2648.

Belur, P.D., Mugeraya, G. 2011. Microbial production of tannase: State of the art. *Res. J. Microbiol.*, 6(1): 25–40.

Bhat, T.K., Singh, B., Sharma, O.P. 1998. Microbial degradation of tannins-A current perspective. *Biodegradation*, 25: 343–357.

Das Mohapatra, P.K., Mondal, K.C., Pati, B.R., Halder, S.K., Maity, C., Jana, A. 2012. Rapid screening of tannase producing microbes by using natural tannin. *Braz. J. Microbiol.*, 43(3): 1080–1083.

Dave, A., Modi., H.A., Chavada, N. 2011. Study and isolation of tannase enzyme production bacteria from tea waste dump soil site. *Int. J. Pharm. Appl. Sci.*, 2(1): 20–22.

Gilna, V.V., Khaleel, K.M., Sabu, A. 2007. Isolation of novel tannase producing microorganisms from the mangrove forests of North Malabar. Proceedings of the 19th Kerala Science Congress, January 29–31, 2007, Kannur, Pp. 3–17.

Ilori, M.O., Adebusoye, S.A., Amund, O.O., Oyetoran, B. 2007. A study of tannic acid degradation by soil bacteria. *Pak. J. Biol. Sci.*, 10(18): 3224–3227.

Ingole, S.P., Kakde, A.U., Maggiwat, R.C. 2012. Biodegradation of tannery effluent by using tannery effluent isolate. *Int. Multidiscip. Res. J.*, 2(3): 43–44.

Lekha, P.K., Lonsane, B.K. 1997. Production and application of tannin acyl hydrolase: state of art. *Adv. Appl. Microbiol.*, 44: 215–260.

Mondal, K.C., Banerjee, D., Banerjee, R., Pati, B.R. 2001. Production and characterization of tannase from *Bacillus cereus* KBR 9. *J. Gen. Appl. Microbiol.*, 47: 263–267.

Murugan, K., Saravanababu, S., Arnachalam, M. 2007. Screening of tannin acyl hydrolase (E. C. 3.1.1.20) producing tannery effluent fungal isolates using simple agar plate and smF process. *Bioresour. Technol.*, 98: 946–949.

Osawa, R., Walsh, T.P. 1993. Visual reading method for detection of bacterial tannase. *Appl. Environ. Microbiol.*, 59: 1251–1252.