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Study on Tannase Producing *Bacillus megaterium* Isolated from Tannery Effluent

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Abstract

Tannery effluent containing soil samples were used for isolating best tannase producer. Bacteria were isolated from the soil samples after enrichment of the effluent using tannin as a carbon source. The best tannase producer was isolated and identified as *Bacillus megaterium.* The tannase activity of the *Bacillus megaterium* was analysed using modified spectrophotometric method of Sharma *et al*., 2000 and was found to have10.77 U/ml/ min enzyme activity. The effect of various parameters like inoculum size, pH, temperature, carbon and nitrogen source on tannase activity were also evaluated. The result shows that tannase activity was maximum at pH 6, temperature at 37˚C and inoculum size of 10%. Addition of glucose, peptone, yeast extract reduced the tannase activity. Tannase activity of *Bacillus megaterium* was further confirmed by detection of gallic acid using chromatographic method.

Keywords: Tannase, *Bacillus*, Gallic acid , Tannery Effluent

1. Introduction

Tannin is the phenolic compound with molecular weight 0.5-3.0 kDa. Its molecular weight depends on the bond possessed with protein and polysaccharide. Tannase is an extracellular hydrolytic enzyme that catalyzes the hydrolysis of tannin or gallic acid esters, liberating glucose and gallic acid as a final product. Tannase is commonly known as Tannin acyl hydrolase (E.C.3.1.1.20).

Tannase enzymes can be used as a hydrolysing agent in cleaning up the highly polluting tannin from the effluent of leather industry. Tannase enzyme also used in the prevention of phenol-induced turbidity in wine (Koichi and Tokuji, 1972). Coffee flavoured soft drinks manufacturing (Suzuki, 1973), clarification of beer and fruit juices (Massechelin and Batum, 1981), stabilization of polyphenol (Giovanelli, 1989) and as a sensitive analytical probe for determining the structure of naturally occurring gallic acid esters. Gallic acid has significance in food and pharmaceutical industry (Pourrat *et al*., 1985). It can be used as a precursor in production of antioxidant such as, propyl gallate. It is an important intermediary compound in the synthesis of the anti-bacterial drug, trimethoprim, used in pharmaceutical industry. In recent years, it is find out that 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).

Tannase is an inducible enzyme produced by variety of microorganisms such as fungi, bacteria, and yeast

(Aguilar and Gutie'rrez-Sanchez, 2001). Tannase can be extracted from different sources like; microbes, plants, and animals. In plants, tannase is extracted from leaves, branch, and bark and in animals tannase is extracted from bovine intestine and ruminal mucous. But, the most important source to obtain this enzyme is by microbial way, because the produced enzymes are more stable than similar ones obtain from other sources (Lekha and Lonsane, 1997). Various microbial sources for tannase production are, filamentous fungi (*Aspergillus, Mucor, Myrothecium, Neurospora, Rhizopus, Fusarium, Trichoderma* and *Penicillium*). Several bacterial sources such as; *Bacillus, Corynebacterium, Klebsiella, Streptococcus* and several *Lactobacilli* sp. have been studied for tannase production. The present investigation aims to study the role of *Bacillus megaterium*, isolated from tannery effluent in tannin degradation.

2. Materials and Methods

Chemicals:

The chemicals used throughout the study were of analytical reagent grade and obtained from Sigma, Aldrich and Merck.

Sample Collection:

Tannery effluent and tannery effluent soil samples were collected from Central Leather Research Institute (CLRI), GIDC, Ahmedabad in sterile polypropylene bottles and were stored in a refrigerator.

Enrichment of Samples for Isolating Tannase Producers:

One gram of soil samples, collected from different tannery effluent Deposition site of CLRI were suspended in 50 ml minimal medium ($NaNO_3$ 0.6 % w/v, KCl 0.5 % w/v, $MgSO_4$ 0.05 % w/v, K_2HPO_4 0.05 % w/v & KH_2PO_4 0.05 % w/v) containing 1 % (w/v) filtered sterilized tannic acid (designated as TA broth) and incubated at 37 ^{0}C for 2 weeks.

Isolation and Screening of Tannase Producing Bacteria:

Enriched samples were serially diluted up to 10^{-3} and plated on Tannic acid agar medium (TAA) and incubated at 37 ^{0}C for 96 hours. After incubation TAA plates were flooded with Gram's iodine (modified method, i.e, Gram's Iodine was used instead of $FeCl_3$). Gram's iodine formed a dark brown complex with tannic acid but not with hydrolysed tannic acid and giving a sharp distinct zone around the tannase producing microbial colonies even in cases of low levels of tannase production. Colonies exhibiting clear zone of tannic acid hydrolysis were selected as tannase producers. Secondary screening was carried out on the basis of study of zone of tannic acid hydrolysis activity of the different isolates. The colony showing highest zone was selected for further studies.

Cultural, Morphological and Biochemical Characteristics of Isolate:

The isolate was subcultured on nutrient agar medium. Colony characteristics such as size, shape, texture, consistency and transparency were noted down. Gram staining was carried out for the isolate. Biochemical tests like Indole test, Methyl red test, Voges-proskauer, Triple sugar iron (TSI) agar test and Carbohydrate utilization test were performed for the isolate.

Enzyme Spectrum and Various Carbohydrate Utilization of Selected Bacterial Isolate:

Selected bacterial isolate was analyzed for the production of different enzymes i.e amylase, catalase, lipase, gelatinase and protease. Various carbohydrate utilization test were also studied.

Thin Layer Chromatographically Analysis of Gallic Acid:

The degradation product gallic acid liberated by the action of tannase from bacterial isolate was detected by thin layer chromatography. The solvent system, ethyl acetate: chloroform: acetic acid in a ratio of 50:50:1 was used for the study. The results were visualized by using iodine crystal vapour. Retention factor (R_f) value was calculated according to the following equation from the chromatogram.

$$R_f = \frac{\text{Distance travelled by solute (cm)}}{\text{Distance travelled by solvent (cm)}}$$

Tannase production:

The isolate which produced maximum zone in TA agar plates was inoculated in to 50ml of tannic acid broth in a 250 ml Erlenmeyer flask and incubated at 37 ˚C for 24 hours .The culture was filtered and the filtrate was used as the crude enzyme for tannase assay.

Tannase assay:

The tannase activity was estimated by modified spectrophotometric method of Sharma *et al*., 2000 .Tannic Acid was used as substrate. The basic principle of this assay is based on the formation of chromogen between gallic acid (released by the action of tannase on tannic acid) and rhodanine (2-thio-4-ketothiazolidine). A standard curve was prepared using gallic acid.Crude enzyme was used for the assay. All the tests were performed in triplicates. One unit of tannase activity is defined as the amount of enzyme required to liberate 1µM of gallic acid /min under defined conditions. Enzyme activity was expressed as U/ml/min.

Effect of Various Factors on Tannase Production:

The effect of various physico-chemical factors like pH, temperature, inoculum size, carbon and nitrogen sources on Tannase activity were assayed. For evaluating the effect of the above factors on tannase activity, the bacterial culture was grown on TA medium.

Effect of Temperature and pH on Tannase Activity

The effect of temperature on the production of tannase was studied by assaying the enzyme after 24 hours of incubation period in the culture medium at varying temperatures (i.e., 5 °C, 27 °C, 30 °C, 37 °C and 45 °C) and for optimizing pH, the medium was prepared by varying the pH (i.e. 2,4,5,6 and 8).

Effect of Inoculum Size, Carbon and Nitrogen Sources on Tannase Activity

Effect of varying inoculum size (i.e. 5%, 10 %, 15% and 20%) on Tannase production was determined. Incorporation of carbohydrates and various nitrogen sources in the presence of tannic acid also was studied. Two nitrogen sources (yeast extract and peptone) and one carbon source (Glucose) were separately incorporated at concentration of 1% into the production medium in the presence of 1% tannic acid and their effect on tannase production were also analyzed.

3. Results and Discussion

Isolation and Screening of Tannase Producing Bacteria:

Ten morphologically different bacterial colonies were obtained from the tannery effluent containing waste samples which showed tannic acid hydrolysis on TAA plates. Out of ten, S3/1 isolate, exhibiting higher zone of tannic acid hydrolysis was selected for further studies. The isolate S3/1 showed maximum zone of tannic acid hydrolysis i.e., 25 mm. The zone formed by isolates on TAA plates was not very clear and was not differentiable between tannase-producing and non-tannase-producing bacteria using $FeCl_3$ (Osawa and Walsh, 1993). To overcome this, the TAA plates were flooded with Gram's iodine instead of $FeCl_3$ as reported by Kumar *et al.*, 2010. Gram's iodine formed a dark brown complex with tannic acid but not with hydrolysed tannic acid and giving a sharp distinct zone around the tannase producing microbial colonies even in cases of low levels of tannase production (Fig.1) within 3-5 minutes.

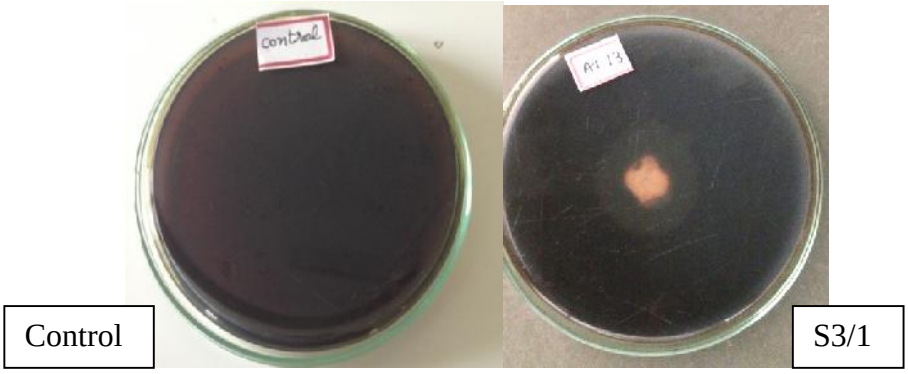


Figure-1.Tannic acid hydrolysis by S3/1 bacterial strain detected by addition of iodine compared with control plate.

Cultural, Morphological and Biochemical Characteristics of Isolate:

Cultural, morphological and biochemical characteristics of Bacterial strain S3/1 were studied and the results are represented in Figure-2 and Table-1.The colony of S3/1 was medium in size, round, smooth, opaque and off white in colour with moist consistency. The isolate was motile with gram positive cell wall. S3/ 1 showed M-R test positive, fermentation of sugars like glucose, sucrose and lactose were analysed by triple sugar iron test and was found to be positive. The isolated organism had the ability to utilize citrate. Other biochemical tests like formation of indole and production of acetylmethyl carbinol from glucose fermentation was negative.

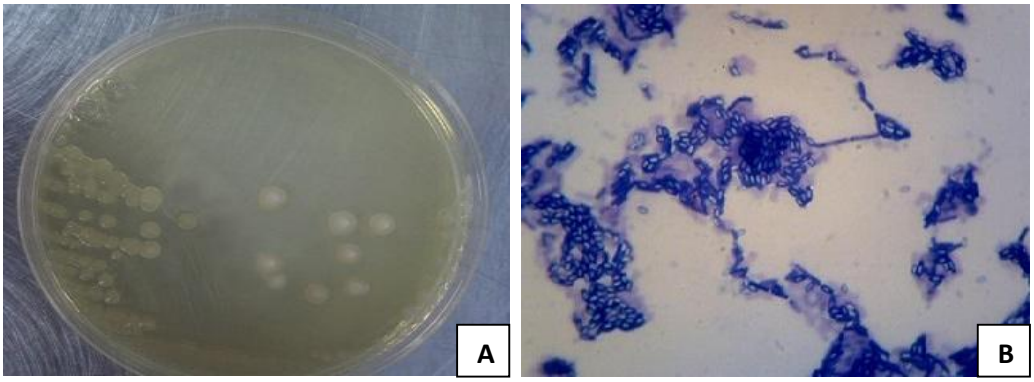


Figure -2. A. Growth of S3/ 1 on nutrient agar plate, B. Microscopically view of S3/ 1 after gram staining (1000X)

Table-1. Morphological and Biochemical Characteristics of Bacterial Strain S3 /1:

Isolate	Colony characters	Morphological characters						
		Gram reaction & cell shape	Motility	Biochemical tests				
				1	2	3	4	5
S3 /1	Medium, Round, Smooth, Flat Opaque , Moist, off white	+ve, Thick rod	Motile	-	+	-	+	+

1-Indole test, 2- Methyl Red test, 3- V-P test ,4- Triple Sugar Iron test ,5- Citrate utilization

Enzyme Spectrum and Various Carbohydrate Utilization of Selected Bacterial Isolate:

Total seven different enzymes activity and nine various carbohydrate sources were checked for S3/1 bacterial strain (Table-2). Among them S3/1 able to utilize lactose and maltose as a carbon source**.** The isolated culture showed positive for amylase, protease, lipase , gelatinase and catalase**.**

Table-2. Different Enzymatic Activity and Various Carbohydrates Utilization by S3/ 1.

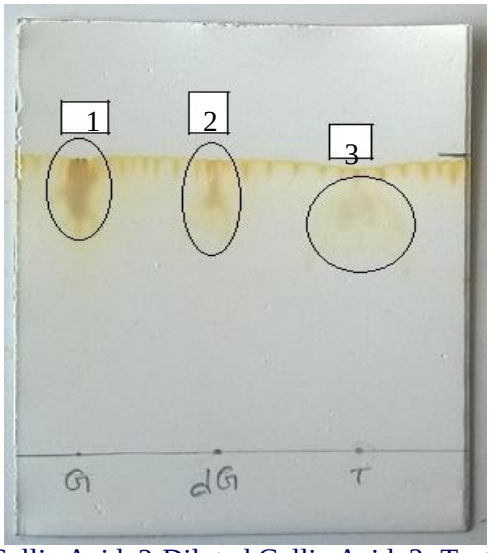
Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
S3/ 1	+	+	+	+	+	-	-	-	-	-	+	-	-	-	+	-

1-Amylase, 2-Protease, 3-Lipase, 4- gelatinase, 5-Catalase, 6-oxidase, 7-malonate utilization test , 8-Nitrate reducase, 9-mannitol ,10- Glucose, 11- Lactose, 12- Arabinose, 13- Sucrose, 14 -Trehalose, 15- Maltose, 16-xylose

The cultural characteristics of the isolated organism, its biochemical reactions and enzyme activity were compared with Bergey's Manual of Systematic Bacteriology and found similar to *Bacillus megaterium.*

Thin Layer Chromatographical Analysis of Gallic Acid:

Thin layer chromatography was used to detect, the presence of gallic acid as a product of tannin hydrolysis. Pure gallic acid was used as standard, and was compared with the test to detect the presence of gallic acid. R_f value of standard gallic acid was 1.0 and the Rf value of test sample was 0.90, which is nearer to the R_f value of standard gallic acid. This shows that tannic acid was hydrolysed by the tannase enzyme produced by *Bacillus megaterium*. The tannase produced by the microbe converted tannic acid in to gallic acid and glucose.



1-Gallic Acid, 2-Diluted Gallic Acid, 3- Test

Figure-3. Thin layer chromatography of gallic acid librated by S3/1

Tannase Assay:

Tannase assay was carried out by growing organism at static condition. Highest enzyme activity (10.77 U/ml/min) was observed after 72hr of inoculation and the results are represented in Table 3. After 72hrs of growth the tannase activity was decreased and remained stable thereafter. Wilson *et. al* (2009) reported *Citrobacter sp* isolated from water and soil samples, which showed highest activity of 0.5 U/ml after 48 hours. Study by Rodríguez *et al*., 2008 on *Bacillus sphaericus* showed higher tannase activity after a fermentation period of 96 h and Jana *et al*., 2013 report showed that *Bacillus subtilis* higher activity after 72 h , which is in correspondence with the present data .

Table -3.Tannase Activity (U/ml/min) of The Isolated Bacterium at Varying Time Interval

Hours	24	48	72	96	120
Tannase Activity (U/ml /min)	3.68	3.12	10.77	5.38	5.09

Effect of Various Factors on Tannase Production:

Effect of Temperature and pH on Tannase Production

The maximum tannase production (15.69 U/ml/min) was obtained at 37 ^{O}Cand the results are represented in Fig 4. This result is in accordance with Selwal *et al*., 2010, who reported that maximum tannase activity produced by *Pseudomonas aeruginosa*III B 8914 was observed at an incubation temperature of 37 °C.Kumar *et al*. (1999) reported maximum tannase activity at 30°C by *Citrobacter freundii.* Maximum tannase production at 35°C by *Bacillus lichiniformis* KBR6 was reported by Das Mohapatra *et al*. (2006). Mondal *et al*., 2001, reported an optimum temperature of 40°C for their strain *Bacillus cereus* KBR9, while tannase production was optimum at 30°C in case of *Lactobacillus* sp. ASR-S1 (Sabu *et al*., 2006).

Role of various environment factors on tannase activity of *Bacillus megaterium* were analysed. Maximum enzyme production was observed at pH 6 after 72 hrs and the enzyme activity was 13.06 U/ml/min. The activity decreased after 72 hrs and again increased at 96 hours.

The results are represented in Fig 5. Similar results were reported by Ayed and Hamadi (2002). In case of bacterial cultures, Selwal *et al*. (2010) has reported an optimum pH of 5.5 for *Pseudomonas aeruginosa* III B 8914, while Mondal *et al*. (2001) reported an optimum pH of 5.0 for *Bacillus cereus*KBR9. For *Bacillus lichiniformis* KBR6, Mondal and Pati (2000) have reported the optimum pH as 5.0. Belur *et al*. (2010) have found that initial medium pH of 6.0 was the optimum for their strain *Serratia ficaria* DTC.

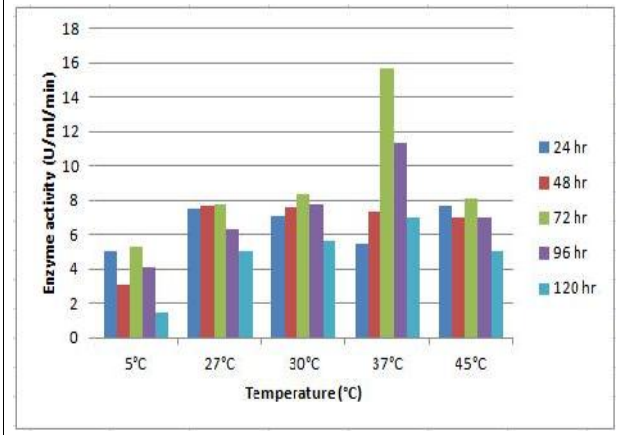


Fig 4. Effect of Temperature on tannase activity

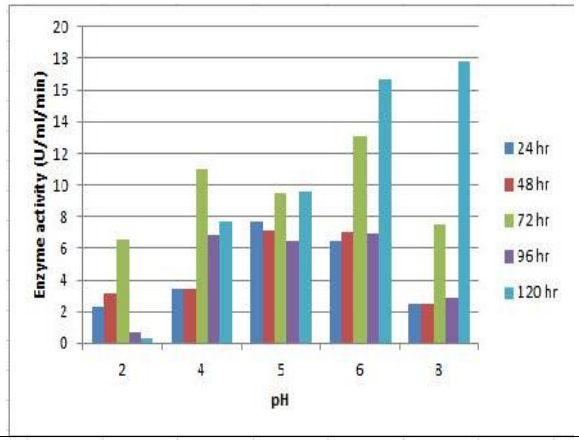


Fig 5. Effect of different pH on tannase activity

Effect of Inoculums Size, Carbon and Nitrogen Sources on Tannase Production:

Higher tannase activity of the isolated bacterium was observed at 10% innoculum size after 72 hr of incubation period. The enzyme activity of the bacterium at 72 hr was 10.08 U/ml/min. The activity decreased after 72 hrs and again increased after 120 hr. 10% inoculum size was considered for all further studies. The results are represented in Fig 6.

TAA media containing Glucose showed higher enzyme activity when compared to TAA medium supplemented with peptone and yeast extract. Here also the maximum activity was observed after 72 hr of incubation period. TAA media containing glucose showed 7.92 U/ml/min tannase activity and is represented in Fig 7. The enzyme activity was reduced when additional substrates like peptone, yeast extract and glucose were added. The reason behind the decrease of enzyme activity at 96 hrs might be due to decrease in tannic acid and increased availability of glucose produced as a by-product of tannic acid hydrolysis. As mentioned above the large availability of glucose in the medium made the organism to utilize more glucose for their growth and not tannic acid. The sharp increase of enzyme activity at 120 hrs might be depletion of glucose in the medium and the bacterium starts utilizing tannic acid in the medium. Further studies like the amount of glucose formed at varying interval and the biomass formed will confirm the results. Effect of different carbon and nitrogen source also was studied. Addition of glucose, peptone, yeast extract to the TAA medium reduced the activity. The reason behind, this might be the easy utilization of these carbon source by the microbes for their growth when compared to tannic acid. Kumar *et al.* used a combination of nutrient agar and tannic acid for the growth and selective isolation of tannase producing bacteria. Mondal and Pati (2000) observed that addition of low concentration of glucose, lactose and sucrose (0.1% w/v) were not repressive, but at high concentrations (0.3 and 0.5%), repressive effect was witnessed. Sabu *et al*. (2006) reported the inhibition of tannase production when the medium was supplemented with 1% (w/v) carbon sources in solid state fermentation of *Lactobacillus* sp.ASR-S1. Selwal *et al*. (2010) reported that carbon source supplementation suppressed the enzyme yield. These contradictions indicate that the regulatory mechanism may vary for different microorganisms and fermentation conditions.

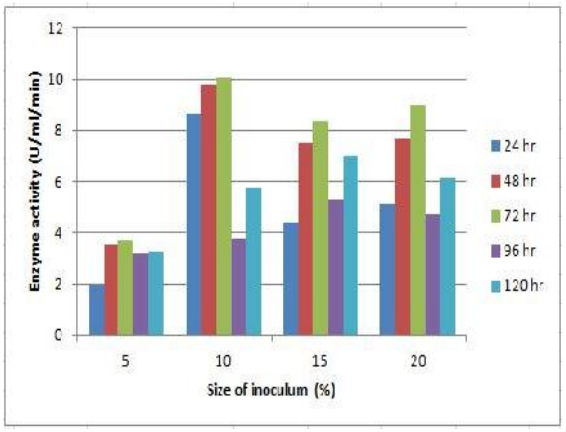


Fig 6. Effect of inoculum size on tannase activity

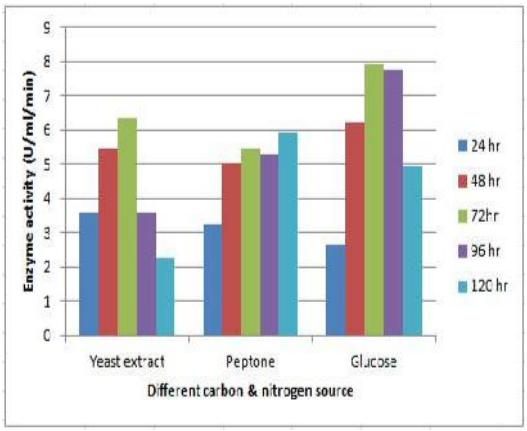


Fig 7. Effect of different carbon and nitrogen source on tannase activity

Conclusion

Tannery effluent was used to isolate the bacteria having natural ability to degrade hydrolysable tannins by producing tannase. Ten bacterial strains were isolated from various tannin rich soil samples. Among them one strain S3/ 1 was selected which could hydrolysis tannin. The isolate was identified by morphological and biochemical characteristics and was found to be *Bacillus megaterium.*

In the present study, bacterium, showed a tannase activity of 10.77 Units/ml/minute at 72 hours of incubation. The effect of various environmental factors on enzyme activity was carried out by growing the organism in varying conditions. The maximum tannase activity was observed in 10 % inoculum size (10.08 U/min/ml) after 72 hr of incubation period. pH also played a major role in tannase production. Maximum activity of enzyme was observed at 6 pH after 72 hr of incubation period (13.06 U/min/ml).

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