

## Isolation and Characterization of Tannic Acid Hydrolysing Bacteria from Soil

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### Abstract

Soil around slaughter house is continuously inoculated with aerobic bacteria from rumen of animals. Ruminant microflora, is able to degrade phenolic compounds such as tannins. Soil containing ruminant micro flora near slaughter house is cultured on Bushnell and Hans's medium containing tannic acid. Three bacterial colonies were isolated, cultivated for identification which are then analysed for biochemical characterization and physiological characterization. Furthermore tannase activity and tannin protein degrading activity of each isolate were determined qualitatively.

**Keywords:** Tannic acid; Degradation; Slaughter house; Aerobic bacteria

### Introduction

Tannins are polyphenolic compounds commonly found in a variety of plants (monocots, dicots, ferns etc.), and are abundant plant constituents after cellulose, hemicelluloses and lignin. Tannins are secondary metabolite of higher plant which is either galloyl ester or their derivatives [1]. Tannins occur naturally in common foodstuff such as tea, strawberry, grape, mango, cashew nut etc. Considering their structure and properties they are distributed into hydrolysable and condensed tannins. Hydrolysable tannins are important economically and are extracted from plant sources like *Terminalia chebula* [2]. Esters of gallic acid or ellagic acid with a sugar core (usually glucose) are the components of hydrolysable tannins. Tannins may reduce feed intake and lower nutrient digestibility and protein availability [2,3]. Tannins are toxic to micro-organisms due to enzyme inhibition and substrate and metal ion deprivation.

### Uses of Tannins

Tannins are used for tanning animal hides and skins for producing leather. The cross- links developed between proteins of the skin and phenolic hydroxylic groups present in tannins increase its stability to water, bacteria, heat and abrasion [4]. Tannic acid is used in treating tonsillitis, laryngitis, haemorrhoids and skin problems. It is also used to increase quality or improve of wine and beer by precipitating out unstable proteins, which produce haze upon chilling [3,4]. In addition gallic acid, the product of hydrolytic cleavage of tannic acid used in the pharmaceutical industry [5].

### Mechanism of action

The ester and depside bonds of hydrolysable tannins are broken down by enzyme tannase (tanninacyl hydrolase) to give product gallic acid and glucose [6]. The gallic acid monomers are utilised as substrates by oxidative breakdown to simple aliphatic acids, which then enter the citric acid cycle. Gallic acid is converted to pyrogallol by gallate decarboxylase before ring cleavage.

The anaerobic decomposition of gallic acid occurs by different mechanisms (Figure 1). The first step is decarboxylation of gallic acid to form pyrogallol which is then isomerized to phloroglucinol by pyrogallol-phloroglucinol isomerase, and finally to 3-hydroxy-5-oxohexanoate (HOHN) product. HOHN is degraded by different pathways in anaerobic and in ruminal systems. In the anaerobic system, it is converted to 3, 5- dioxohexanoate (triacetate) by HOHN dehydrogenase and ultimately to three molecules of acetyl-CoA via triacetyl-CoA by the sequential enzymatic action of triacetyl-CoA transferase, triacetateketothiolase, acetoacetyl-CoA-ketothiolase, phosphotransacetylase and acetate kinase.

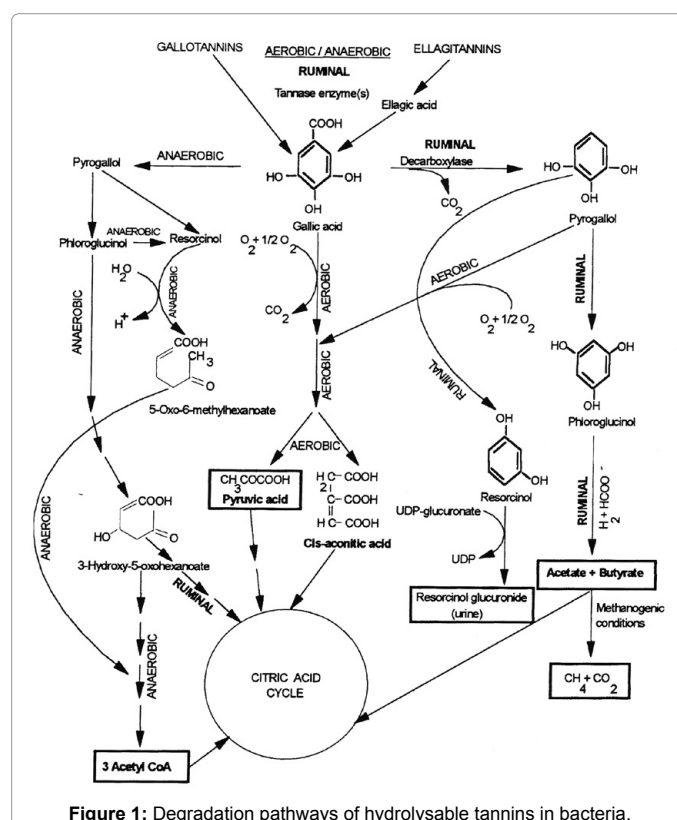


Figure 1: Degradation pathways of hydrolysable tannins in bacteria.

In the ruminal system HOHN-CoA which is derived by the enzymatic action of HOHN-CoA transferase, and it is transformed to acetate and butyrate by the rumen bacteria by the sequential enzymatic action of hydroxybutyryl-CoA dehydrogenase, butyryl-CoA dehydrogenase, acetyl-CoA acetyl transferase, enoyl-CoA hydratase, phosphate acetyl transferase and acetate kinase. Resorcinol,

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is metabolized anaerobically to acetyl-CoA first by reduction and then hydrolysis to a six-carbon keto acid, is however not degraded further by ruminal bacteria and excreted as a urinary phenolic conjugate.

### Tannase

Tannase enzyme fall under class of hydrolases having B.C. number 3.1.120. The enzyme tannase finds widespread applications in food processing, brewing, pharmaceuticals, medicine, textiles, detergents and tea industry [7]. The main commercial applications of tannase are in the preparation of instant tea and manufacture of acorn liquor. Tannase is also used in the production of gallic acid which is an important intermediary compound in the synthesis of antibacterial drugs. Tannase enzyme produced by different fungus like *Ascochyta*, *Aspergillus*, *Mucor*, *Neurospora*, *Rhizopus*, *Trichothecium*, *Fusarium*, *Trichoderma* and *Penicillium* and by different bacteria such as *Bacillus*, *Corynebacterium*, *Klebsiella*, *Streptococcus bovis* and *Selenomonas ruminantium*. However, utilization of fungal strain for industrial application restricted because production by fungi is relatively slow. In addition to that fungal strains are difficult to manipulate genetically because of their complexity. Tannase produced by bacteria can degrade tannic acid and also natural tannins like chestnut, tara, oak and myrobalan tannins [6-9]. Although a number of tannin-degrading micro-organisms which are potential sources of tannase have been reported [10], the search continues for organisms which are more prolific degraders of tannins and better sources of tannase.

Most of the organisms capable of degrading tannins isolated till date are either anaerobic or facultative anaerobic bacteria from the alimentary canal of ruminating animals or fungal strains associated with the degradation of wood and forest litter. Several tannin-degrading anaerobic bacteria have been isolated [9-12] but the processes based on anaerobic bacteria are slow, and the study of tannin degradation pathway is relatively difficult. Despite adverse effects of tannins on water and soil, there are very few reports on the pathway and genes involved in aerobic degradation of tannins, which constitute up to 20% of the leaf tissue [13] The problem of pollution of water and soil from the tannery effluent is a serious environmental threat, especially in the developing countries [12,13]

Phenolic compounds cause harmful effects on environment in two ways; first tannins are vulnerable to polymerization and oxidation in air [8,13] and secondly phenolic compounds are toxic to acquit organisms and microorganism [12] Tannins can cause several changes in properties of acquit system such as color, reactivity [13]. Tannin retard rate of decomposition of soil organic matter via inhibition of biodegradative enzyme of attacking organism [3,10].

Soil around slaughter house is continuously inoculated with anaerobic bacteria from rumen of animals. Ruminant micro flora is able to degrade phenolic compounds such as tannins. By keeping in the mind all these points, soil containing ruminant micro flora near slaughterhouse was cultured on Bushnell and Haas's medium containing tannic acid. The aim of the present study was to isolate cultivate and characterize bacteria from slaughter house soil that are able to degrade tannins aerobically.

## Materials and Methods

### Screening and isolation of tannic acid degrading bacteria

Soil around the slaughter-house was collected for isolation of tannic acid degrading bacteria.

Isolation was carried out on Bushnell and Hans's medium

containing  $\text{KH}_2\text{PO}_4$  - 1 gm/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  - 0.2 gm/L,  $\text{NH}_4\text{NO}_3$  - 1 gm/L,  $\text{K}_2\text{HPO}_4$  - 0.5 gm/L. One-gram soil was diluted and streaked onto petriplates containing Bushnell and Hans's agar medium with tannic acid (0.1%, 0.3%, 0.5% and 01%) and incubated at 37°C for 24-48 hrs. Different visible colonies on medium was transferred and subcultured until pure culture is obtained for identification. Culture is maintained at 4°C. Visible colonies obtained are used to characterize further.

### Microscopy

Wet preparations were performed as usual for visualization of cells under 100 x magnifications.

Gram staining of isolates was performed by heat fixation.

Motility test of the isolates was performed by "Hanging Drop Technique".

### Morphological and biochemical characterization

The cultures for morphology were prepared by incubating the isolates on Bushnell Hans's medium at 37°C and cultural properties of colonies were noted after incubation. All media supplemented with different concentration of tannic acid (0.1% 0.3%, 0.5%, 1%). Biochemical tests such as sugar utilization, catalase production, urease production, HaS production, Starch hydrolysis were performed according to Bergy's manual of systematic bacteriology. Fermentation reactions were tested using phenol red basal medium supplemented with appropriate carbohydrate and optimum concentration of tannic acid for growth.

### Physiological tests

Physiological tests were performed in order to find out the isolates at various pH and temperature conditions. To perform physiological tests the isolates were inoculated in Bushnell Hans's medium with different concentrations of tannic acid (0.1%, 0.2%, 0.3%, 0.4%, and 0.5%). The temperature range for growth was determined by incubating at room temperature and at 37°C. The pH ranges for optimum growth was determined at different pH values as 2, 4, 6, 7, 8 and 10. The pH was adjusted using 1M HCL and 1M NaOH.

### Sugar fermentation tests

1. Tube containing glucose broth with phenol red was taken for acid detection.
2. Inverted Durham's tube was placed in a test tube containing sugar basal medium
3. That tubes were inoculated with loopful bacterial culture and incubated at 37°C for 24 hrs.
4. After 24 hrs. The tubes were examined for results.
5. The colour change was observed and recorded.

### IMViC Test

The IMViC test includes four different types of tests such as,

1. Indole production
2. Methyl red
3. Voges-Proskauer test
4. Citrate utilization.

### Indole production test

1. Tryptone broth was prepared and inoculated with loopful bacterial culture.
2. Incubated the culture at 37°C for 48 hrs.
3. 0.5 ml of Kovacs reagent was added after incubation.
4. After two minutes tubes were observed for development of red colour band at the junction of medium.

### Methyl red (MR) and Voges-Proskauer (VP) tests

1. Two MRVP broth tubes were inoculated with bacterial suspension and one control was kept.
2. The tubes were incubated at 37°C for 48 hrs.
3. 5 drops of methyl red indicator were added into only one tube and observed for the change in colour.
4. To the rest tube of bacterial suspension and control, 10 drops of VP1 reagent and 2- 3 Drops of VP 2 reagent were added.
5. Shaken the tubes gently, remove caps/plugs and wait for 15-20 minutes to complete the reaction. Observed the tubes for color change.

### Catalase test

1. Picked up a colony of bacteria from a plate and transferred on a glass slide.
2. Few drops of 3% H<sub>2</sub>O<sub>2</sub> were added over it.
3. Observed the slide for effervesces of oxygen.

### Urease test

1. Urea Broth (pH 6.9) tubes were inoculated with bacterial suspension
2. Using aseptic conditions each tube was inoculated with loopful suspension of the appropriate isolate.
3. Incubated the tubes for 24 to 48 hours at 37°C.
4. Examined all of the urea broth tubes for color change.

### Tannin-protein complex degrading activity

Degradation of tannin-protein complex (T-PC) by the isolates was determined according to modified method of Osawa (1990). Ten microlitre of a freshly prepared, filter-sterilized solution of tannic acid (0.1% w/v) was poured on nutrient agar plates and allowed to stand

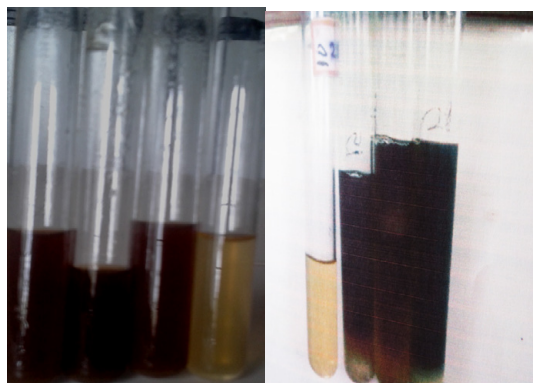


Figure 2: Tannase activity of the isolates.

Isolate	Size (mm)	Shape	Color	Margin	Opacity	Consistency
US1	<1	Circular	Yellowish White	Entire	Opaque	Moist
US 2	>1	Irregular	White	Entire	Opaque	Moist
US 3	>1	Irregular	White	Entire	Opaque	Moist

Morphological characters of the isolates are given in Table 2. All the three isolates were Gram negative cocci. Isolates US-1 and US-3 were non-motile while US-2 was motile organism.

**Table: 1** The colony characteristics of the isolates on Bushnell & Hans's agar plates, incubated at room temperature for 24-48 hrs.

at room temperature for 20 min. The excess tannic acid was then discarded with a sterile micro-pipette and the plates were washed three times with sterile Ringers solution (g/L: NaCl-6.5, KCL-0.14, CaCl<sub>2</sub>-0.12, NaHCO<sub>3</sub>-0.32, Na<sub>2</sub>HPO<sub>4</sub>-0.2 Tannase activity of the three representative isolates was demonstrated by a visual reading method by Osawa and Walsh and all the three representative isolates showed positive results for tannase activity confirmed by the change in color of the alkalized medium to green on exposure to atmosphere (Figure 2).

### Demonstration of tannase activity

To demonstrate the tannase activity of the isolates, a visual reading method by Osawa and Walsh was adopted with some modifications. Fresh cultures on nutrient agar plates were harvested with sterile cotton swabs and suspended in 5 mL of substrate medium (pH 5) containing NaH<sub>2</sub>PO<sub>4</sub> (33 mmol<sup>-1</sup>) and tannic acid (20 mmol<sup>-1</sup>) (Sigma Chemical Co.) to prepare a dense suspension. The substrate medium was incubated aerobically at 37°C for 24 hrs. After incubation, the sample was alkalized with equal amount of saturated NaH<sub>2</sub>CO<sub>3</sub> solution (pH 8.6) and exposed to the atmosphere at room temperature for 1 hr. Brown to Green coloration of the medium was judged as positive indicator of tannase activity.

### Results and Discussion

The research study was initiated to isolate, cultivate and identify tannic acid degrading bacteria from soil around slaughter house. Slaughter house soil was inoculated on Bushnell and Hins's medium to isolate tannic acid degrading microorganisms. Their presence was informed by growing them in increasing concentration of tannic acid (0.1-0.5%). Three isolates (US-1, US-2, US-3) showed growth on medium containing 0.1-0.5% tannic acid. The strains isolated in present research work bring about utilization of tannic acid as sole carbon source can be used for bioremediation of tannin from tannin contaminated soil.

These three isolates were further cultivated for colony, physiological and biochemical characterization. The colony characteristics of three isolates are given in Table 1. Only isolate US-1 showed colony size <1 mm and entire margin, but remaining isolates (US-2 and US-3) showed colony size >1 mm and entire margin. Isolate US-1 shows yellowish white colored colonies while US-2 and US-3 shows white colored colonies. All three isolates have opaque and moist colonies.

Morphological characters of the isolates are given in Table 2. All the three isolates were Gram negative cocci. Isolates US-1 and US-3 were non-motile while US-2 was motile organism.

Our result indicates that all the isolates show optimum growth in the pH range of 6-7 (Figures 9 and 10). Substrate optimization confirmed that all the isolate shows optimum growth at 0.1% Tannic acid (Table 3) (Figures 11 and 12). Isolates US-1 and US-2 showed optimum growth at

room temperature at 0.1% tannic acid while only isolate US-3 showed optimum growth at 37°C and 0.1% substrate concentration (Table 3) (Figures 11 and 12)

The results of biochemical test are shown in observation Table 4. These results indicate that all the isolates could ferment several sugars such as glucose, sucrose, maltose and lactose. None of the isolate

Characteristics	US1	US 2	US 3
Gram nature	Negative	Negative	Negative
Shape	Cocci	Cocci	Cocci
Motility	Non-Motile	Motile	Non-Motile

Table: 2 Morphological characteristics of isolates.

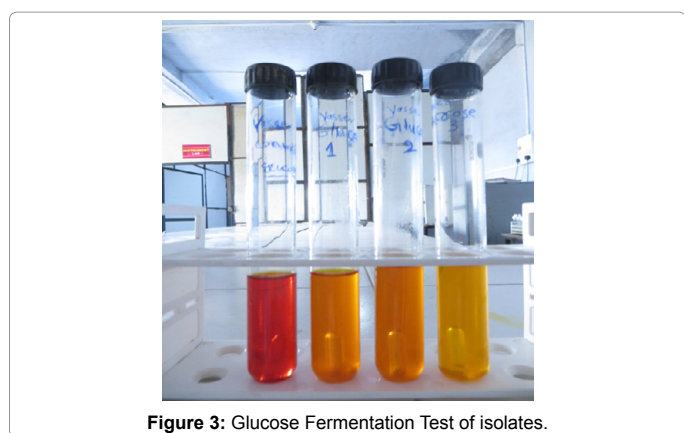


Figure 3: Glucose Fermentation Test of isolates.

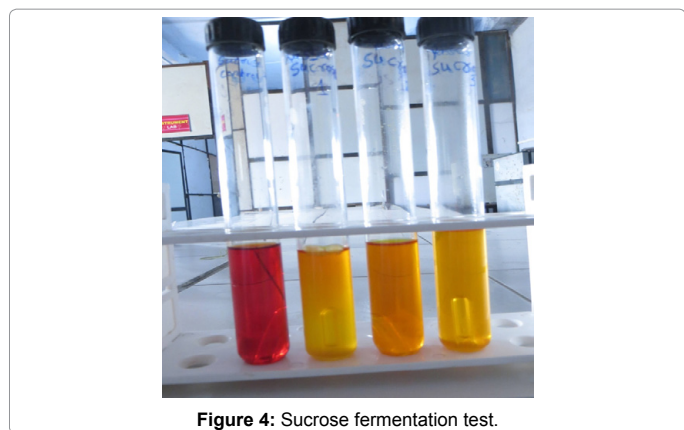


Figure 4: Sucrose fermentation test.

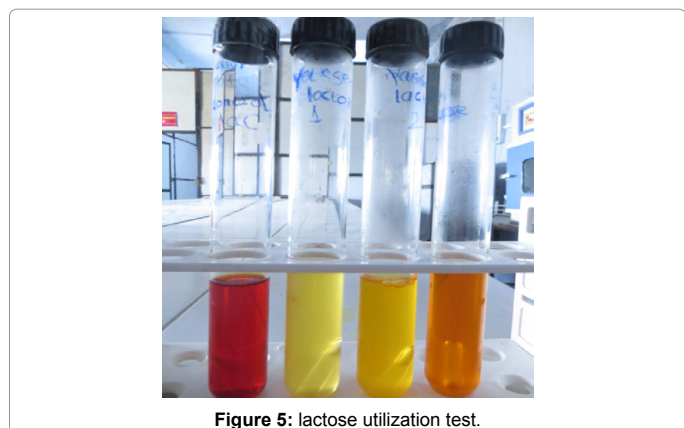


Figure 5: lactose utilization test.

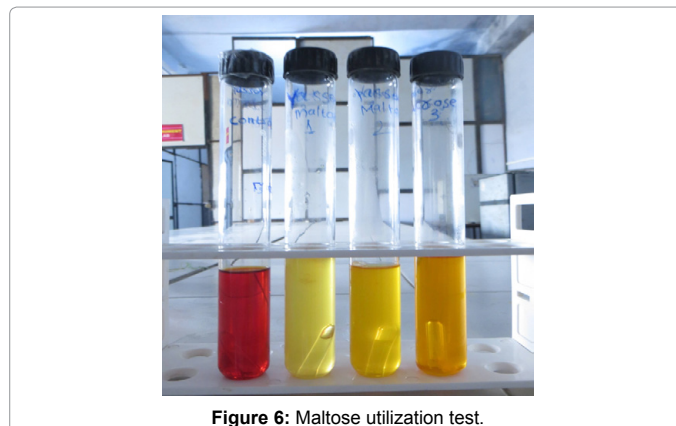


Figure 6: Maltose utilization test.

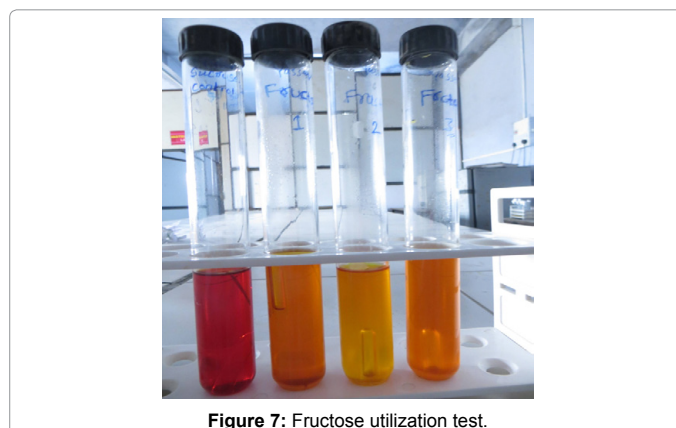


Figure 7: Fructose utilization test.

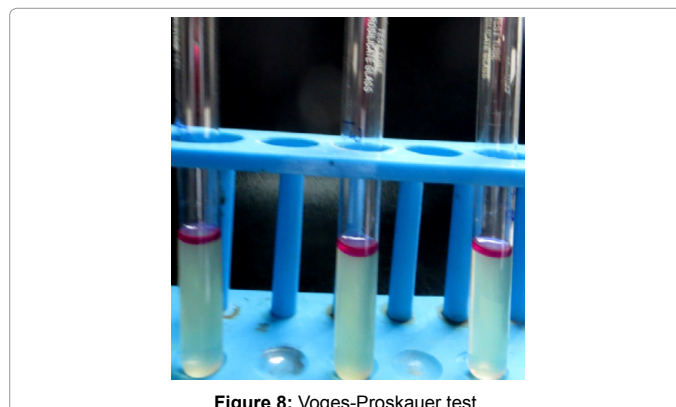


Figure 8: Voges-Proskauer test.

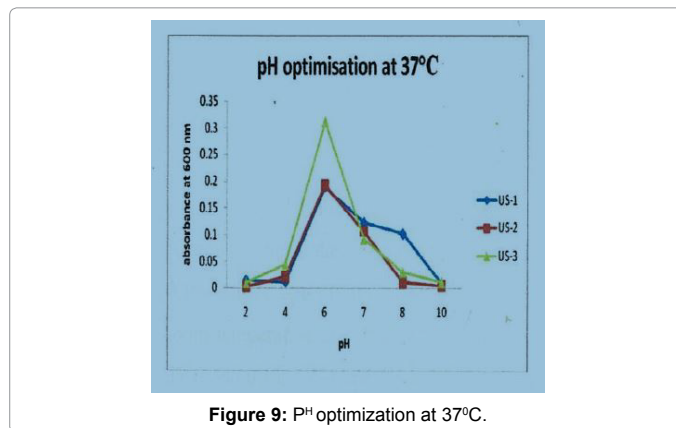


Figure 9: P<sup>H</sup> optimization at 37°C.

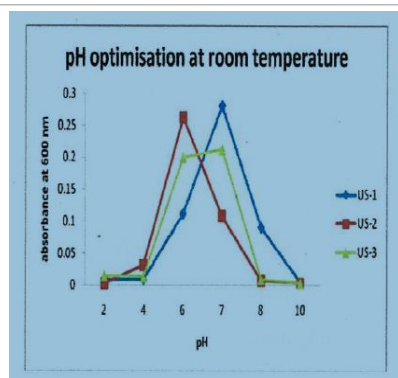


Figure 10: pH optimization at room temperature.

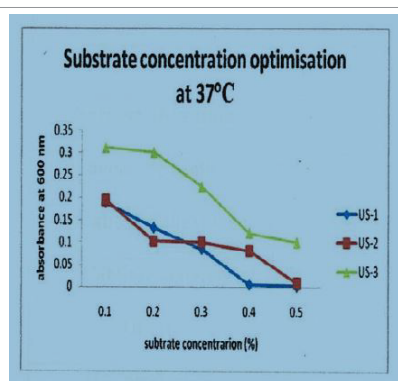


Figure 11: Substrate concentration optimization at 37°C.

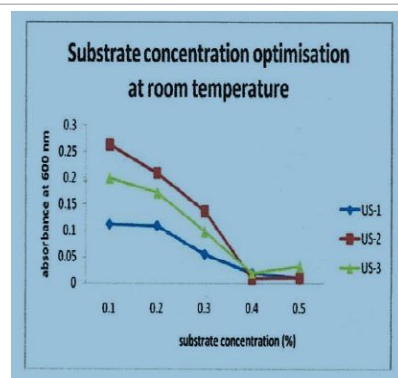


Figure 12: Substrate concentration optimization at room temperature.

Characteristics	US1	US 2	US 3
Optimum pH	7	6	6
Optimum substrate (Tannic acid) concentration for growth	0.1%	0.1%	0.1%
Optimum temperature.	Room temperature	Room Temperature	37°C

Table 3: Physiological characteristics of isolates.

produces an enzyme urease. It was also observed that all the isolates are able to produce hydrogen sulfide gas. All the isolates are unable to hydrolyze starch by producing enzyme amylase. Also all isolates produces enzyme catalase as in Figures 3-8.

Tannin-protein complex (T-PC) degrading activity of the isolates was checked on plates containing nutrient agar coated with tannic acid,

forming T-PC, which shows an opaque shiny color of the medium. A zone of clearing because of reduction of opacity around the colonies of the three isolates was taken as indication of the hydrolysis of the T-PC. Acidic pH around the colonies, tested by touching pH indicator strip near the colonies was indicative of the release of tannic acid. The clearing zone turned brown after 24 hr, presumably because of the oxidation of tannic acid released after the hydrolysis of T-PC (Figure 13).

Tannase activity of the three representative isolates was demonstrated by a visual reading method by Osawa and Walsh, and all the three representative isolates showed positive results for tannase activity confirmed by the change in color of the alkalized medium to green on exposure to atmosphere (Figure 2).

## Conclusion

Soil around slaughter house used to isolate tannic acid degrading bacteria. Three isolates were able to grow in medium containing 0.1-0.5% tannic acid. Results of present study clearly indicate that the isolates are able to hydrolyze tannic acid in considerable amount. The physiological characterization of the isolate shows that the all the isolates are able to multiply and grow optimally in pH range of 6-7. Optimum temperature for the maximum growth of the one isolates was 37°C while that of two isolate was grow well a room temperature. The optimum substrate concentration for the growth of the isolate is varied according to the isolates. It was found to be 0.1% for all the isolates after growing isolates in different concentrations of tannic acid. Tannin-protein complex degrading activity was present in all the three isolates. All the isolates have potential to produce enzyme tannase. On these results of the project work it can be concluded that all the isolated bacterial strains keeps potential for degradation of tannic acid.

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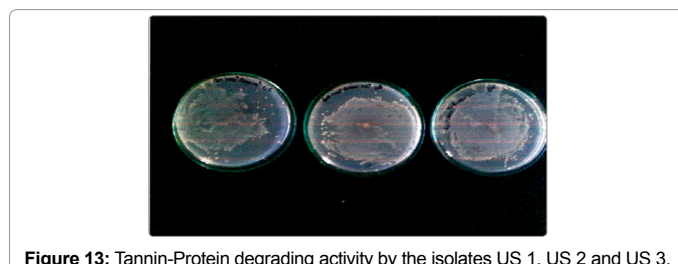


Figure 13: Tannin-Protein degrading activity by the isolates US 1, US 2 and US 3.

Characteristics	US1	US 2	US 3
Glucose utilization	+	+	+
Sucrose utilization	+	+	+
Lactose utilization	+	+	+
Maltose utilization	+	+	+
Fructose utilization	+	+	+
Catalase activity	+	+	+
Urease	-	-	-
H <sub>2</sub> S production	+	+	+
Indole production	-	-	-
Methyl Red	-	-	-
Voges- Proskauer	-	+	+
Starch hydrolysis	-	-	-

Key: +: positive reaction and -: Negative reaction.

Table 4: Biochemical characteristics of isolates.

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