3.0. MATERIAL AND METHODS

3.1. CHEMICALS

Acrylamide, Bis-acrylamide, Phenyl methyl sulfonyl fluoride (PMSF), Tetramethyl ethylene diamine (TEMED), Ammonium per sulphate (APS), Tris base, Trichloroacetic acid (TCA), Poly thiourea, Ethylene-diamine-tetraacetic acid (EDTA), Glycine, Sodium Dodecyl Sulphate (SDS), Coomassie brilliant blue R-250, Coomassie brilliant blue G-250, were purchased from Sigma, India. Sodium hydroxide, Glycerol, Ampicillin, β-Mercaptoethanol, Nutrient broth were purchased from Himedia Mumbai, India. India. Trypsin, Ammonium bicarbonate, Acetonitrile, Trifluoroacetic acid (TFA), Dithiothreitol (DTT), 3-3 Cholamidopropyl dimethyl ammonio-1-propane sulfonate (CHAPS), Iodoacetamide, Urea, Thiourea, Ethanol, Nitrocellulose membrane (0.4µm) and bovine serum albumin, Non-Linear IPG strip (pH: 3-10), Bio-lyte (pH: 3-10), Bromophenol blue were purchased from Bio-Rad, Germany. Methanol, chloroform, hydrochloric acid, Glacial acetic acid, Orthophosphoric acid, Acetone, Sodium chloride, Sodium monobasic and Sodium dibasic and other analytical chemicals were purchased from Qualigens Fine Chemicals, India. Protein molecular weight standard markers (prestained) and unstained were purchased from Fermentas, Canada. Ni-NTA sepharose was purchased from Wipro, India.

3.2. INSTRUMENTS

3.2.1. Electronic Balance

All weighing were carried out in a top loading mono-pan electronic balance (Sartorius Basic, Germany) with an accuracy of 0.1 mg.
3.2.2. Refrigerated Centrifuge

Refrigerated Centrifuge Remi (German make) was used for centrifugation of samples.

3.2.3. Ultra flow Freezer

Panasonic -20°C ultra-flow freezer was used to store the protein and DNA samples.

3.2.4. Ice Making Machine

F90 Compact Electronic ICematic machine was used to make ice for sample collection and processing.

3.2.5. Electrophoretic Apparatus

Designed for research, our horizontal electrophoresis apparatus can be incorporated into many classroom activities to teach topics in molecular biology, such as DNA separation and size analysis. Widely used for routine nucleic acid work, these horizontal electrophoresis systems for agarose gels are available in mini and wide formats (BIO-RAD, USA).

3.2.6. Power Pack

The Bio-Rad models 50-200 constant voltage/ constant current was used for all separation techniques.

3.2.7. Milli-Q Water

Millipore Academic Bench – integrated water purification system (USA) was used for obtaining pyrogen free ultra-pure water.
3.2.8. Spectrophotometer

UV-Visible spectrophotometer (UV-1601-SHIMADZU, Japan) was used for quantification of DNA and protein.

3.2.9. First Dimension apparatus

Ettan IPG phor3, GE Healthcare, Germany was used for Isoelectric focusing of protein.

3.2.10. Second Dimension gel apparatus

Protean II xi Cell (BIO-RAD, USA) was used for SDS-PAGE.

3.2.11. Sonicator

Branson Scientific, Germany was used for gut lysis.

3.2.12. MALDI-MS

MALDI-MS, Brucker Daltonic, Germany was used to analyze the peptides of SDS-PAGE/2-D PAGE at Sandor Life Science, Hyderabad, Telangana.

3.2.13. Gel Shaker

Rivotek Gel Shaker is used for shaking during staining and destaining of gels.

3.2.14. Gel Documentation

LARK, Germany Gel Documentation system was used to observe and photograph the agarose gels.
3.2.15. Thermal Cycler

ABI - Applied Bio Systems, USA was used to run the PCR analysis.

3.2.16. Micropipettes

Thermo Scientific micropipettes in the range of 0.2 μl, 10 μl, 100 μl, 200 μl and 1000 μl were used in the study.

3.2.17. Microwave Oven

LG – MS – 2049UW was used for melting agarose.

3.2.18. Double distillation unit

Distillation unit 3365 -1.5L (Borosil, India) is used for obtaining distilled water.

3.3. Isolation of insect gut microbes

3.3.1. Culture Media

[a] Luria Bertani (LB) broth (g/L)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>-</td>
<td>10.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>-</td>
<td>10.0</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.0 with 10 N NaOH and made upto 1 liter with distilled water. Medium was sterilized by autoclaving at 15 LBS at 121°C for 15 min.

[b] Luria Bertani (LB) agar medium- LB medium containing 1.5% agar.
[c] Nutrient agar (NA) medium (g/L)

- Peptone: 5.0
- Sodium chloride: 5.0
- Beef extract: 3.0
- Agar: 2.0

pH was adjusted to 7.2 with 10 N NaOH and made up to 1 litre with distilled water.

Sterilized by autoclaving at 15 LBS at 121°C for 15 min.

[g] Nitrate broth (g/L)

- Peptone: 5.0
- Beef extract: 3.0
- Potassium nitrate: 5.0

pH was adjusted to 7.2 with 10 N NaOH and made up to 1 litre with distilled water.

Sterilized by autoclaving at 15 LBS at 121°C for 15 min.

[h] Semi solid motility medium (g/L)

- Peptone: 5.0
- Beef extract: 3.0
- Sodium chloride: 5.0
- Agar: 3.0

pH was adjusted to 7.2 with 10 N NaOH and made up to 1 litre with distilled water.

Sterilized by autoclaving at 15 LBS at 121°C for 15 min.

[i] Tryptone Soya Agar (g/L)

- Tryptone: 15.0
- Soya peptone: 5.0
- Sodium chloride: 5.0
- Agar: 15.0

Final pH (at 25°C): 7.3±0.2
Suspend 40 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 LBS pressure 121°C for 15 min.

3.3.2. Sample collection insect *Gryllotalpa krishnani*

The insect was collected in wet soil near to a kitchen waste, Salem district, Tamil Nadu, India. The soil was buried up to 10-15 cm depth by a digger and a colony of the insect species was found beneath the soil (Fig.7). The insect was collected dissection in our laboratory conditions (latitude: 11.6500°N, longitude: 78.1600°E; elevation: 154 ft (46.7 m).

**Taxonomic details of *Gryllotalpa krishnani***

Kingdom : Animalia  
Phylum : Arthropoda  
Class : Insecta  
Order : Orthoptera  
Suborder : Ensifera  
Family : Gryllotalpidae  
Genus : *Gryllotalpa*  
Species : *krishnani*  

**Binominal name** : *Gryllotalpa krishnani*

![Fig. 7. Insect *Gryllotalpa krishnani*](image)
3.3.3. Dissection of insect gut

The gut was isolated from of the Orthoptera insect, *G. krishnani* (i.e., during the active feeding stage). Prior to dissection, sterilized by wiping with 70% ethanol on the cuticle for 5 seconds (Pandiarajan et al., 2014; Arun Prasanna et al., 2014). Dissection scissors were used to cut laterally behind the head capsule and the gut was removed and washed in 1× PBS to remove the leaf litter. Then, it was transferred into an autoclaved 1.5 mL micro centrifuge tube for further processing.

3.3.4. Culturing of the gut microbiota and tannase plate assay

The dissected gut was suspended in 10mM sterile phosphate-buffered saline (PBS) Sambrook et al. (1989). The guts were sonicated (50/60 Hz,117 V, 1.0 Amps; Branson Ultrasonics, Danbury, CT) for 30 Sec, macerated with a plastic pestle, and vortexed at medium speed for 10 sec to separate bacterial cells from the gut suspension were cultured immediately on tryptone soya agar plates and plates were incubated for 24 h at 37°C. Screening of the potent tannase producing bacterial isolates were screened for tannase activity by hydrolysis of tannin. After incubation plate was flooded with thereafter, the isolation of tannase-producing bacteria was carried out on nutrient agar plates supplemented with tannic acid (2%; filter sterilized). Addition of tannic acid to nutrient agar forms tannin–protein complex; cleavage of this complex by bacteria producing tannase forms a zone around the colonies.

3.3.5. Microorganism and tannase enzyme production

Enzyme production was carryout in a 250 ml Erlenmeyer flask containing 100 ml of basal medium [(g/l) KH$_2$PO$_4$, 0.5; CaCl$_2$, 0.5; MgCl$_2$, 2.0; NaNO$_3$, 2.0] supplemented with 12 g/l tannic acid as a sole carbon source. The medium was inoculated with 1% of 18 h old inoculums cultures were incubated at 37°C for 24 h.
3.3.6. Morphological and biochemical characterization of the isolates

The isolated colonies were observed under microscope to obtain the colony morphology i.e. colour, shape, size, nature of colony and pigmentation (Lu et al., 2010). The bacterial isolate were gram stained and observed under a high power magnifying lens in light microscope. The bacterial isolates were biochemically characterized by Indole, Methyl red, Vogesproskauer, Citrate utilization, Catalase, Urease, Oxidase test and Triple sugar iron agar test etc., (Bossis et al., 2000).

3.3.7. Extraction of Genomic DNA

The genomic DNA was extracted from the isolated bacteria colonies using the slightly modified protocol described by Broderick et al. (2004). The 12 h cultures of bacterial isolates were taken in the micro centrifuge tube and the tubes were centrifuged at 10,000 rpm for 10 min. Supernatant was discarded the pellet was collected and 90 µl of 10% SDS was added. Vortexed and incubated at 37°C for 1 h. After incubation, addition 150 µl of 5M NaCl prior to the addition of 100 µl of 10% cetyltrimethyl ammonium bromide (CTAB). The sample was mixed thoroughly and incubated at 65°C kept in a water bath for 30 min, after incubation, add phenol, chloroform and isoamylalcohol in the ratio of 25:24:1 (Vol/Vol/Vol). The tube was centrifuged at 13,000 rpm for 15 min and the aqueous layer was separated and added into a fresh tube. Then it was precipitated with 70% ethanol and centrifuged at 7000 rpm for 5 min. Pellets were suspended in 30 µl of TE buffer. The DNA sample was separated according to their molecular weights under electrophoresis system. Finally the DNA band was visualized under gel documentation system (Lark, Germany). The DNA sample was stored -20°C it is used for further analysis.
3.3.8. Yield, purity and integrity of DNA

The isolated DNA was subjected to spectrophotometric analysis (Ultrospec 2100, Amersham Bioscience, Hong Kong) to determine the quality and quantity. The DNA purity was determined from the ratios 260/280 nm (indicator of protein contamination) and 260/230 nm (indicator of organic solvent residues). The size and intactness of the isolated DNA was checked by agarose gel electrophoresis. The isolated DNA was loaded on 1% agarose gel stained with ethidium bromide (1 µg/µl) and run for 30 min at 60 V, for image acquisition, a LARK India gel documentation system was used. The isolated genomic DNA size was determined by using a 1kb DNA ladder (Bangalore Genei).

3.3.9. PCR amplification of 16S rDNA gene

The selected bacterium was identified on the basis of its 16S rDNA sequence. DNA from the bacterial cells was isolated using QIAamp DNA Purification Kit (Qiagen, Japan) and electrophoresed in agarose gel. Fragment of 16S rDNA gene was amplified by PCR upto 30 cycles (using the following profile: initial denaturation, 95°C for 1min; denaturation, 94°C for 30; annealing, 52°C for 30; extension,72°C for 90; final extension,72°C for 10 min). Amplified PCR product was purified using Qiagen Mini elute gel extraction kit (Qiagen, Japan). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27 F’ AGAGTTTGATCCTGGCTCAG and 1492 R’ GGTACCTTGTTACGACTT primers using BDTv3.1 Cycle sequencing kit on (ABI3730 × l) Genetic Analyzer (Maity et al., 2011). A single discrete PCR amplicon band of 1500 bp was observed when resolved on 1.0% agarose gel.
3.3.10. Phylogenetic analysis

The reference sequences required for comparison were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank). The aligned sequences were then manually checked for gaps in each row and saved in molecular evolutionary genetics analysis (MEGA) format using MEGA v.2.1 software. Pair-wise evolutionary distances were computed using the Kimura 2-parameter model (Kimura, 1980). To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method. The bootstrapped dataset was used directly for constructing the phylogenetic tree with the MEGA program or for calculating multiple distance matrixes. The multiple distance matrix obtained was then used to construct phylogenetic trees using the neighbour-joining method of Saitou and Nei (1987). All of these analyses were performed using MEGA v.2.1 (Kumar et al., 2004).

3.4. Degradation of tannic acid by cell-free extract

The experiment was performed in 2 ml-Eppendorf tubes in a final volume of 1.1 ml containing 1 mM tannic acid final concentration. This involved the addition of 45 μl of 25 mM stock solution of tannic acid (Sigma, Germany) to the cell-free extract. The mixture was incubated in the dark at 37°C for 18 h. The reaction products were extracted twice with one third of the reaction volume of ethyl acetate (Lab-Scan, Ireland). The solvent fractions were filtered through a 0.45 μm PVDF filter (Teknokroma, Spain) and analysed by HPLC.

3.4.1. Tannase assay unit

The reaction mixture consisting of substrate tannic acid 0.3 ml and 0.05 ml enzyme was incubated at 60°C for a defined period. The enzymatic reaction was
stopped by the addition of 3 ml BSA solution, which also precipitated the remaining tannic acid. In the same way a reference tube was prepared with heat denatured enzyme. The tubes were centrifuged (5000gx5 min) and the resultant precipitate was dissolved in 3 ml SDS–triethanolamine solution. Then 1 ml of FeCl₃ reagent was added and kept for 15 min for stabilization of the color. The absorbencies of both the tubes were measured at 530 nm, against the blank (i.e., without tannic acid). The specific extinction coefficient of tannic acid at 530 nm was found to be 0.577. Using this coefficient one unit of tannase activity can be defined as the amount of enzyme which is able to hydrolyze 1 mM of substrate tannic acid in 1 min under assay conditions.

3.4.2. Protein estimation

Protein content was determined according to Bradford (1976) using Bradford kit (Biogene, USA). The samples were diluted to 100 µl with 0.15 N NaCl. One ml of Bradford reagent was added, vortexed, allowed to stand for 2 min and the absorbance was read at 595 nm. Protein was quantified in comparison with a standard curve plotted with Bovine Serum Albumin (BSA) as standard and was expressed as mg/ml.

3.4.3. Specific activity

Specific activity was calculated by dividing the enzyme units with protein content and was expressed as U/mg protein.

\[
\text{Specific activity} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein (mg/ml)}}
\]
3.4.4. Culture conditions of tannase pH and temperature

The different pH optimum culture conditions of tannase enzyme preparation were studied in terms of the pH profile of carrying out the reaction at different pH ranging from 5.0 to 9.0. Optimum temperatures of the tannase enzyme from *E. cloacae* were studied in different temperature ranging from 28 to 80°C for 24 h, incubation, under standard assay conditions.

3.4.5. Effect of carbon and nitrogen sources

Various simple and complex compounds including glucose, fructose, sucrose, mannitol and maltose were used as a carbon source (0.5% w v⁻¹) in medium with tannic acid broth. Similarly, the effect of nitrogen sources was studied using urea, ammonium nitrate, ammonium chloride, potassium nitrate, yeast extract and corn steep liquor. The respective nitrogen sources (0.5% w v⁻¹) were used instead of nitrogen source medium tannic acid broth. The amount of extracellular tannase produced was monitored after 24 h growth at 37°C and with the agitation speed of 100 rpm.

3.4.6. Substrate concentration of tannase enzyme production

Culture optimized conditions of different substrate concentration of tannic acid range from 0.25%, 0.5%, 0.75% and 1% at 37°C, 24 h incubation. These contents were autoclaved at 121°C for 15 min. After cooling the flasks to room temperature, the contents were inoculated with 5% of bacterial fresh culture inoculums. The flasks were then incubated at 37°C for 24 h under shaking conditions.
3.4.7. HPLC analysis

The water soluble polyphenolic compound tannic acid treated with tannase enzyme was analyzed with high performance liquid chromatography (HPLC). The analysis was carried out on a Shimadzu chromatography equipment SPD-20A model with a UV visible detector and C18 (4.60 mm X 250 mm) column with LC-8A pump followed by solvent system using acetonitrile, water and ethanol (3:5:2) as eluent at a flow rate of 1.4 ml/min with a total running time of 20 min. Detection was performed at 254 nm.

3.4.8. Tannin acid degradation confirm to FT-IR analysis

In determining the possible functional groups FT-IR analysis was performed using Perkin Elmer’s most power, which is used to detect the characteristic peaks and their functional groups. The vibration pattern that appears in the infrared spectra provides information about the chemical functional group of the sample. Tannase enzymes prepared and 500 µl of 0.5% tannin acid was mixed together and incubated at 37°C for 30 min. A fraction of sample was encased directly in sample holder and spectra were scanned from 500-4000 cm⁻¹.

3.4.9. SDS-PAGE and Zymogram analysis

The culture filtrate with tannase enzyme an aliquot of 2 to 5 mg was used for separation of the extracellular proteins by sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS–PAGE–12% gel) (Laemmli, 1971). The sample was mixed with SDS buffer without b-mercaptoethanol and heated for 5 min at 40°C, and then loaded and run in Mini Protein II Electrophoresis unit (Bio Red) at 4°C. The location of tannase activity within the gel was determined as described by Aoki et al.
(1979). After electrophoresis, the gel was removed and washed for 1 h in 100 ml of 1% (v/v) triton 100-X followed by 30 min washes (twice) with 100 ml of 0.5 mol acetate buffer (pH 5.5) with constant shaking. The gel was incubated with 0.5% (w/v) tannic acid in 50 ml of acetate buffer (pH 5.5) for 15 min at 25°C. Tannic acid solution was discarded and subsequently the gel was rinsed with the same buffer and replaced with 0.5% (w/v) quinine hydrochloride and incubated at 25°C to visualize the tannase enzyme activity on the gel.

3.5. Purification and characterization of tannase enzyme from Enterobacter cloacae

3.5.1. Purification of tannase enzyme

Tannase produced by Enterobacter cloacae was purified employing standard protein purification protocols, which included ammonium sulphate precipitation; followed by dialysis, gel filtration and concentration by ultra filtration. All the experiments were done at 4°C unless otherwise specified.

3.5.2. Ammonium sulphate precipitation

Ammonium sulphate precipitation was done according to England and Seifter (1990). Ammonium sulphate (Sisco Research Laboratories Pvt. Ltd., India) required to precipitate tannase was optimized by its addition, at varying levels of concentrations (20, 40, 60, 80 and 100 % saturation) to the crude extracts. To precipitate the protein, ammonium sulphate was slowly added initially at 60 % saturation to the culture supernatant while keeping in ice with gentle stirring. After complete dissolution of ammonium sulphate, the solution was kept at 4°C for overnight. Precipitated protein was collected by centrifugation at 10000 rpm for 15
min at 4°C. The precipitate was resuspended in minimum quantity of 0.2 M phosphate buffer (pH 6.0).

3.5.3. Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against the phosphate buffer 0.2 M (pH 6.0) in order to remove the ammonium sulphate from the precipitate. Dialysis tube (Sigma-Aldrich) was first treated to remove the protectants like glycerin and sulphur compounds present in it and to make the pores of the tube more clear. The pretreatment involved washing of the tube in running water for 3-4 h, dipping in 0.3 % (w/v) sodium sulfide at 80°C for 1 min, further washing with hot water (60°C) for 2 min followed by acid wash in 0.2 % (v/v) sulphuric acid. Finally the tube was rinsed with distilled water. The precipitated protein was dialyzed in the pretreated dialysis tube for 48 h at 4°C with several changes of buffer and assayed for tannase activity, protein content, specific activity and yield was calculated. The treated tube retained most of the proteins of molecular weight ~ 50 kDa.

3.5.4. Characterization of purified enzyme

Purified protein was further characterized for their biophysical and biochemical properties by subjecting the purified protein to molecular mass determination, zymogram analysis, enzyme kinetics etc as described in the following sections.

3.5.5. Ion exchange chromatography (DEAE cellulose)

The concentrated enzyme preparation was carefully layered over the top of the glass column (18 cm 2.5 cm) equilibrated with 0.2 M phosphate buffer (pH 6.0)
and eluted with a linear gradient (0–0.5 M NaCl) of 0.2 M phosphate buffer (pH 6.0) at a flow rate of 1 ml/2 min. To fractions showing high tannase activity were pooled, concentrated and stored at 4°C.

3.5.6. Gel filtration chromatography (Sephadex G 100)

Purification of enzyme protein by Gel filtration chromatography was performed using sephadex G100 (Sigma-Aldrich) column.

3.5.7. Preparation of column

A fraction of 3.5 g of Sephadex G-100 (Sigma-Aldrich) was suspended in distilled water, allowed to hydrate for 3 h at 100ºC in a water bath, and fine particles were removed by decantation. Hydrated gel suspension was degassed under vacuum to remove the air bubbles. Filled the glass column with eluent (HCl-KCl buffer, pH-2.0) without air bubble. Prepared Gel suspension was carefully poured into the column without trapping air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column. Column was stabilized by allowing two times the bed volumes of eluent to pass through the column bed in descending eluent flow.

3.5.8. Reagents for Polyacrylamide Gel Electrophoresis

(1) Stock acrylamide solution (30: 0.8)

<table>
<thead>
<tr>
<th>Acrylamide (30 %)</th>
<th>-</th>
<th>60.0 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis-acrylamide (0.8 %)</td>
<td>-</td>
<td>1.6 g</td>
</tr>
<tr>
<td>Distilled water (DW)</td>
<td>-</td>
<td>200.0 ml</td>
</tr>
</tbody>
</table>

Stored at a temperature of 4ºC in amber colored bottle.
(2) **Stacking gel buffer stock (0.5 M Tris-HCl, pH 6.8)**

Tris buffer - 6 g in 40 ml of distilled water titrated to pH 6.8 with 1 M HCl and made up to 100 ml with distilled water. Filtered with Whatman No.1 filter paper and stored at 4°C.

(3) **Resolving gel buffer stock (3 M Tris-HCl, pH 8.8)**

Tris buffer - 36.3 g titrated to pH 8.8 with 1 M HCl and made up to 100 ml with distilled water. Filtered with Whatman No.1 filter paper and stored at 4°C.

(4) **Reservoir buffer for SDS-PAGE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
<tr>
<td>SDS</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Dissolved and made up to 1 L with distilled water. Prepared in 10X concentration and stored at 4°C.

(5) **Sample buffers**

(a) Sample buffer for non-reductive SDS-PAGE Tris-HCl (pH 6.8) - 0.0625 M

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>10 % (v/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>2 %</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.01 %</td>
</tr>
</tbody>
</table>

(b) Sample buffer for reductive SDS-PAGE

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>0.0625 M</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 % (v/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>2 %</td>
</tr>
</tbody>
</table>
Dithiothreitol - 0.1 M
Bromophenol blue - 0.01 %

Prepared as 2× concentrations and stored at 4 °C.

(6) SDS (10 %) - 1gm in 10 ml distilled water

(7) Protein staining solution Coomassie Brilliant blue (0.1 %) - 100 mg Methanol (40 %) - 40 ml Glacial acetic acid - 10 ml distilled water - 50 ml.

(8) Destaining Solution Methanol (40 %) - 40 ml Glacial acetic acid (10 %) - 10 ml, distilled water - 50 ml

(9) Protein marker for SDS-PAGE Myosin, rabbit muscle - 205 kDa

Phosphorylase b - 97.4 kDa
Bovine serum albumin - 66 kDa
Ovalbumin - 43 kDa
Carbonic anhydrase - 29 kDa
Soya bean trypsin inhibitor - 20.1 kDa

**Gel preparation resolving Gel (10 %)**

Acrylamide: bis-acrylamide - 5.00 ml
Resolving gel buffer stock - 1.875 ml
SDS (10%) - 0.15 ml
Distilled water - 7.975 ml
TEMED - 15 µl
Ammonium per sulphate (APS) - 0.05 ml

**Stacking gel (2.5 %)**

Acrylamide: bis-acrylamide - 1.25 ml
Stacking gel buffer stock - 2.5 ml
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS (10%)</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.15 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 µl</td>
</tr>
<tr>
<td>Ammonium per sulphate (APS)</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

3.5.9. Sample preparation for reductive SDS-PAGE

Added 100 µl of 1X sample buffer to the concentrated sample, mixed well, boiled for 5 min in a water bath, cooled to room temperature. A mixture containing 25 µl of this sample and 5 µl of low molecular weight marker was loaded onto the gel.

Cleaned and assembled the gel plates, Resolving gel - Added all the components except APS in to a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and a layer of water over the gel, and allowed to solidify at least for half an hour. Stacking gel - Added the components of stacking gel, except APS, into a beaker, mixed gently, and finally added APS. Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed it to solidify at least for 30 min. Gel was placed in the electrophoresis apparatus, and then the upper and lower reservoirs were filled with reservoir buffer for SDS-PAGE. The gel was pre run for 1 h at 80V. Loaded the gel with the protein sample. The gel was run at 50V till the sample entered the resolving gel. When the dye front entered the resolving gel, increased the current to 100V Stopped the current when the dye front reached 1cm above the lower end of the glass plate. Removed the gel from the cast and stained for at least 1 h in the staining solution Destained till the bands became clear and observed under a transilluminator.
3.5.10. Electrophoresis methods

All the samples obtained at various stages of purification namely ultrafiltration, and gel filtration chromatography were run on Native-PAGE gel and SDS-PAGE gel prepared using 10 % polyacrylamide gel according to the method of Laemmlli (1971). SDS page was performed under reductive and non reductive conditions i.e. with and without β mercaptoethanol.

3.5.11. Crystallization structure purified tannase enzyme

For crystallization, the purified tannase fraction was maintained at 4°C for overnight in hanging drop preparation. The purified tannase enzyme crystal structures of gallic acid were observed microscopically (Nikon Eclipse LV100POL, Tokyo, Japan).

3.5.12. Thin layer Chromatography (TLC)

The gallic acid extracted was also analyzed by thin layer chromatography with silica gel G-60 F254 (E. Merck, Mumbai, India). The solvent system consisted of ethyl acetate, chloroform and formic acid (4:4:1). After drying the plates were developed by spraying a solution of FeCl₃ (Naidu et al., 2008). Standard gallic acid and sample were run in same plate. Retention factor ( Rf ) value was calculated according to the following equation from the chromatogram.

\[ R_f = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}} \]

3.5.13. Circular Dichroism (CD) spectroscopy

The secondary tannase structure was studied and in 10 mM phosphate buffer pH 6.0 were carried out using a CD spectrometer (Jasco spectropolarimeter, model
Ellipticity values [ø] were obtained in millidegree (mdeg) directly from the instrument. A sample volume of 0.7 µL, 0.1 g/L tannase solution was added into a 2-mm path length quartz cuvette and scanned at the far UV region (260–190nm) with a step resolution of 1.0 nm and a speed of 500 nm/min, bandwidth was 1.0 nm. In all cases, CD spectra were recorded by averaging three scans, converted to molar ellipticity, and applied to calculate secondary structure. The calculation of secondary structure was conducted using CDNN software. The effect of pH 6.0 to 8.0 range tannase enzyme was also measured after incubating tannase at different pH for 1h.

3.5.14. Atomic force microscopy (AFM)

To study the morphology of tannase by AFM, purified tannase solution from *E. cloacae* was concentrated by ultrafiltration to remove buffer salt and then diluted with milli-Q water. Each sample (10 µL) was deposited onto a freshly cleaved mica sheet and embedded into the mica surface after the sheet was left to dry at room temperature for 10 min in a clean petri dish. All mica sheets were laid in air and scanned at room temperature with an AFM instrument (SPI 4000 Probe Station, SPA 400 SPM Unit, Seiko Instruments Inc., Chiba, Japan) under the tapping mode. Images of the mica surface were taken utilizing an Olympus Si-DF20 Cantilever with a tip radius of 10 nm, spring constant of 12 N/m, and vibration frequency of 127 kHz. Furthermore, topographic and phase images were recorded at a scan speed of 1 Hz and resolution set at 512 X 512 pixels.
3.6. Characterization of purified tannase enzyme

The *E. cloaca* tannase enzyme characterized with respect to pH, temperature, substrate inhibitions, reaction time intervals, inhibitors, chelators, and surfactant and metal ions.

3.6.1. Stability of pH purified tannase enzyme from *E. cloaca*

The stability of pH for tannase activity was determined at various pH levels between pH 3.0 and 10.0 using acetate buffers (pH 3.0–5.0), phosphate buffers (6.0–7.0) Tris HCl buffer (pH 8.0) glycine NaOH buffer (pH 9.0 to 10.0) were used for the assay. Effect of agitation were also studied with speed of 100 rpm, residual tannase activity was measured after 1 h of incubation at 50°C in different pH buffers.

3.6.2. Thermal stability of the purified tannase enzyme from *E. cloaca*

Stability of tannase activity was tested at different temperatures ranging from 30 to 100°C in 0.2 M phosphate buffer (pH 6.0). The enzyme solution was incubated for 1 h at the tested temperature and the tannase enzyme unit activity was measured at the stability of temperature.

3.6.3. Effect of substrate specificity purified tannase enzyme

Tannic acid solution of different concentrations (0.5 to 5.0 mM) was prepared in a 0.2 M phosphate buffer (pH 6.0) and the effect of substrate concentration on purified tannase activity was determined. Michaelis constant $K_m$ and $V_{max}$ values were determined from Line weaver–Burke plot calculated for the substrate.
3.6.4. Effect on reaction time purified tannase enzyme

The tannase enzyme sample and the substrate tannic acid were incubated for 15, 30, 45, 60, 75 and 90 min. Standard protocol was used for tannase assay.

3.6.5. Salt tolerance test purified tannase enzyme

Effect of NaCl on purified tannase was determined at different concentrations of NaCl ranging from (1–5 M) Salt tolerance of the purified tannase was determined by incubating the enzyme at different concentrations of NaCl, up to 5 M for 1 h incubation at 50°C. The enzyme unit activity was calculated under the standard assay conditions.

3.6.6. Effect of inhibitors purified tannase enzyme

The enzyme solution containing 1mM concentration of various inhibitors like Gallic acid, Methyl gallate, Pyrogallol, n-Propyl gallate, Sodium bisulfate and Sodium thioglycolate was incubated at 50°C for duration of time 5 and 60 min. The enzyme samples were then assayed for tannase enzyme activity.

3.6.7. Effect of chelators and surfactants on tannase activity

The chelators, namely ethylene diamine tetra acetic acid disodium salt (EDTA disodium salt 1 mM) and the surfactants such as sodium lauryl sulfate (SLS), Tween 20, Tween 80 and Triton X-100, at a concentration of 0.5% were also studied for their influence on tannase activity.

3.6.8. Effect of different metal ions

The enzyme solution containing 1 and 5 mM concentration of various metal ions like Mg²⁺, Fe²⁺, Cu²⁺, Zn²⁺, Fe³⁺, Ba²⁺, Hg²⁺, Ag⁺, Na⁺ and K⁺ was
incubated at 50°C for 60 min. The blank containing enzyme and buffer were incubated at 100°C. Tannase enzymes were then assayed for tannase enzyme activity.

3.7. Cell culture

The green monkey kidney cells (vero) were purchased from the National Center for Cell Sciences (NCCS), Pune, India. The cells were maintained in Dulbecco’s modified eagles medium (DMEM) supplemented with 2 mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na₂CO₃, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100µg) were adjusted to 1 mL/L. The cells were maintained at 37°C with 5% CO₂ in a humidified CO₂ incubator.

3.7.1. Evaluation of cytotoxicity

The inhibitory concentration (IC₅₀) value was evaluated using an MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were grown (1x10⁴ cells/well) in a 96 well plate for 48 h in to 80 % confluence. The medium was replaced with fresh medium containing serially diluted tannase and the cells were further incubated for 48 h. The culture medium was removed, and 100 µL of the MTT [3-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well and incubated at 37°C for 4 h. After removal of the supernatant, 50 µL of DMSO was added to each of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was
measured at 620 nm in an ELISA multi well plate reader (Thermo Multiskan EX, USA). The OD value was used to calculate the percentage of viability using the following formula.

\[
\% \text{ of viability} = \frac{\text{OD value of experimental sample}}{\text{OD value of experimental control}} \times 100
\]

3.8. Cloning of the tannase gene fragments from \textit{Enterobacter cloacae}

3.8.1. Primer used for this study

Designed four set of degenerative primers for amplifying and sequencing of the tannase gene from \textit{E. cloacae} (Table. 2).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' to 3'</th>
<th>Length (bp)</th>
<th>Tm value (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Cgg HTg Cgg Ygg NYT gTg Cgg SA</td>
<td>23</td>
<td>68.5</td>
</tr>
<tr>
<td>R1</td>
<td>CCR TgC CAS Agg ATM AAK YTT gCC gCC</td>
<td>27</td>
<td>66.3</td>
</tr>
<tr>
<td>F2</td>
<td>ggY TgC TCN AAY ggN ggN CgC VAR gCg</td>
<td>27</td>
<td>72.4</td>
</tr>
<tr>
<td>R2</td>
<td>AAg ARS CgC gCR AAN gNC STS NgC</td>
<td>24</td>
<td>66.5</td>
</tr>
<tr>
<td>F3</td>
<td>gAN TTC gAC ggC ATY NTC gCN ggC</td>
<td>24</td>
<td>65.4</td>
</tr>
<tr>
<td>R3</td>
<td>ATC ATY TTg CCR CCN CgN T</td>
<td>19</td>
<td>54.4</td>
</tr>
<tr>
<td>F4</td>
<td>CBg AYg ANT TgC ACg gYA TCN TgC NC</td>
<td>26</td>
<td>66.4</td>
</tr>
<tr>
<td>R4</td>
<td>CCg CCN CgC CAR TgN BDC ATN CCN ggC</td>
<td>27</td>
<td>73.5</td>
</tr>
</tbody>
</table>

3.8.2. Gene amplification

A PCR was performed in a total reaction mix of 50 µl of the isolated genomic DNA from \textit{E. cloacae} to amplify the tannase gene. The PCR was performed with the primer pairs F1-R1, F2-R2, F3-R3, F4-R4 combinations of the forward and reverse primers. The PCR mixture contained 1µl of isolated genomic DNA (200 ng) from \textit{E. cloacae}, 1 µl of each primer (100 pmol/µl), 2 µl 10 mM deoxyribonucleoside triphoshate, 5 µl 10X PCR buffer containing MgCl₂, and 1µ l
of 5 U/µl Taq DNA Polymerase. The PCR reaction was conducted with the initial
denaturation at 94°C for 2 min followed by denaturation at 94°C for 45 sec,
annealing at 60°C for 60 seconds and elongation at 72°C for 2 min. These cycles
were then followed by 34 cycles of denaturation, annealing and elongation was
followed by an extended final elongation step at 72°C for 10 min. The PCR product
was electrophoresed in a 1 % (w/v) agarose gel stained with ethidium bromide and
observed on an UV transilluminator. The amplicon was excised from the gel and the
DNA was eluted from the gel slice by using the EZ-10 Spin column DNA Gel
extraction kit obtained from Biogene, USA, according to the manufacturer’s
 specifications. The purified amplicon after gel extraction was cloned into pGEM®-T
vector of pGEM®-T Easy vector system II-Promega as per manufacturer’s
 specifications. The ligated plasmids were transformed in to CaCl₂ competent cells
(E. coli DH5α) as described below.

3.8.3. Ligation

The amplicon obtained was ligated to pGEM®-T vector (pGEM®-T Easy
 vector system II-Promega as per manufacturer’s specifications). Ligation mix was
prepared with 5 µl of 1X ligation buffer, 3 µl of insert, 1 µl of pGEM®-T vector and
1 µl of T4 ligase enzyme. All the components were mixed by pipetting and kept at
4°C overnight.

3.8.4. Competent cell preparation

A single colony of E. coli host cell (DH5α) was inoculated in 5 ml of Luria
Bertani (LB) broth and incubated overnight with a constant shaking of 150 rpm at
37°C. 1 % (v/v) of overnight culture was inoculated to 50 ml of LB broth and
incubated at 37°C in a shaker at 150 rpm until the OD was 0.4 - 0.6. The cells were harvested at 10,000 rpm for 10 min at 4°C. The pellet was suspended in 10 ml of ice cold 0.1 M CaCl₂ and incubated in ice for 30 min. The cells were harvested again by centrifugation at 7,000 rpm for 5 min at 4°C. The pellet was resuspended in 1ml of 0.1 M CaCl₂. This was aliquoted as 80 µl fractions and added with chilled glycerol (20 µl) and stored at -80°C until use (Sambrook et al., 1989).

3.8.5. Transformation

Ten µl of ligation mix was added to 100 µl of stored competent cells and incubated in ice for 40 min. Then the cells were given a heat shock at 42°C for 90 sec in a water bath followed by quick chilling on ice for 5 min. A volume of 250 µl LB was added to the transformed cells and incubated at 37°C for 1 h. 50 µl of the cells were plated on LB agar plates containing 50 µg/ml ampicillin, 100 µg/ml IPTG and 40 µg/ml X-gal employing spread plate technique and incubated at 37°C. The plates were checked for the transformants after overnight incubation. Colonies containing plasmids with inserts were identified by blue/white colony selection. Isolated colonies were grown in 5 ml LB containing 50 µg/ml concentration of ampicillin at 37°C for 16 h, after which the plasmid DNA was isolated from the bacterial cells by using the alkaline lysis plasmid isolation procedure (Maniatis et al., 1982).

3.8.6. Plasmid isolation

Reagents

Solution I

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>50 mM</td>
</tr>
<tr>
<td>Tris-HCl (pH 8)</td>
<td>25 mM</td>
</tr>
<tr>
<td>EDTA (pH 8)</td>
<td>10 mM</td>
</tr>
</tbody>
</table>
Solution II

NaOH - 0.2 N SDS - 1 % (w/v)

Solution III

Potassium acetate 5M (pH 7.5) - 60.0 ml
Glacial acetic acid - 11.5 ml
Deionizer water - 28.5 ml, pH - 5.4

The cultures were centrifuged at 12,000 rpm for 2 min at 4°C. The pellets were suspended in 100µl each of ice cold solution I for 5 min. 200 µl of freshly prepared solution II were added, mixed well by inversion and incubated for 10 min. This was followed by addition of 150 µl (- 20°C) of ice cold solution III to each tube and then incubated in ice for 15 min. Centrifuged at 12000 rpm for 15 min and the clear supernatants were transferred to new tubes. 0.6 volumes of ice cold isopropanol was added to each tube, mixed well and incubated for 10 min. The plasmid DNA was pelleted by centrifugation and washed with 70 % ethanol; air dried and dissolved each in 20 µl TE buffer (pH 8.0). The plasmid DNA samples were stored at -20°C. The prepared plasmid DNA was subjected to agarose gel electrophoresis on a 0.8 % (w/v) agarose gel.

3.8.7. Bioinformatics analysis

Softwares and World Wide Web servers used in this study are summarized in Table 3.
Table 3. Online tools and other software’s used in this study

<table>
<thead>
<tr>
<th><a href="http://www.interface/software">www.interface/software</a></th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bio Edit</strong> (version 7.0.4.1)*</td>
<td>Sequence editing and analysis</td>
<td><a href="http://www.mbio.ncsu.edu/BioEdit">www.mbio.ncsu.edu/BioEdit</a></td>
</tr>
<tr>
<td><strong>BLAST</strong></td>
<td>Sequence homology search</td>
<td><a href="http://www.ncbi.nlm.nih.gov/BLAST">www.ncbi.nlm.nih.gov/BLAST</a></td>
</tr>
<tr>
<td><strong>Net Primer</strong></td>
<td>Primer designing</td>
<td><a href="http://www.premierbiosoft.com">www.premierbiosoft.com</a></td>
</tr>
<tr>
<td><strong>ClustalW (version 1.8)</strong></td>
<td>Multiple sequence alignment.</td>
<td><a href="http://www.ebi.ac.uk/clustalW/">www.ebi.ac.uk/clustalW/</a></td>
</tr>
<tr>
<td><strong>Quantity one (version 1.1)</strong>*</td>
<td>Gel documentation</td>
<td><a href="http://www.biorad.com">www.biorad.com</a></td>
</tr>
<tr>
<td><strong>CDD</strong></td>
<td>Protein domain similarity search</td>
<td><a href="http://www.ncbi.nlm.nih.gov/Structure/cdd">www.ncbi.nlm.nih.gov/Structure/cdd</a></td>
</tr>
<tr>
<td><strong>Signal IP site</strong></td>
<td>Prediction of signal peptidase cleavage</td>
<td><a href="http://www.cbs.dtu.dk.Services/SignalP">www.cbs.dtu.dk.Services/SignalP</a></td>
</tr>
<tr>
<td><strong>MEGA 3.1</strong></td>
<td>Molecular evolutionary genetics analysis</td>
<td><a href="http://www.megasoftware.net">www.megasoftware.net</a></td>
</tr>
</tbody>
</table>

3.9. Application of tannase enzyme

3.9.1. Gallic acid production

Production of gallic acid was carried out by following procedures Mondal et al. (2001).

3.9.2. Gallic acid extraction

The crude extract obtained after fermentation was equilibrated with different matrices like diatomaceous earth, silica gel and activated charcoal. Sodium chloride was used for eluting the gallic acid from the equilibrated culture filtrate. Initially the concentration of sodium chloride was standardized for the maximal recovery of gallic acid. Elute obtained was mixed well with two volumes of ethyl acetate and separated using a separating funnel. Later ethyl acetate was evaporated to recover gallic acid in powder form. The reaction mixture was maintained at 4°C for 24 h for gallic acid crystallization production. The contents were filtered using Whatman filter paper (No. 1) and were analyzed by HPLC for gallic acid produced. For
HPLC, Shimadzu Shimpack C\textsubscript{18} column (150 X 6.0 mm, I.D. = 5 \textmu m) and UV detector at 254 nm were used. The optimized chromatographic conditions were water and methanol (70:30 v/v) as the mobile phase at a flow rate of 1.0 ml min\textsuperscript{-1}, a temperature of 50°C and an injection volume of 20.0 \textmu l.

3.9.3. Enzymatic treatment of black tea to improve the quality of tea

3.9.3.1. Preparation of tea infusion

For this, CTC tea and Kangra orthodox black tea (5.0 g each) were mixed with 100.0 ml of boiling water (reverse osmosis) in 250 ml beaker separately. Both the samples were incubated at 85°C for 20 min in a water bath (Lu and Chen, 2007). The tea infusions obtained in each case were filtered through Whatman filter paper No. 1 and the filtrate was analyzed to determine contents of the individual catechins. In all further experimental studies, both tea infusions will act as control, i.e., without enzyme. The pH of the tea infusion was 5.0 ± 0.2.

3.9.3.2. Enzymatic biotransformation

The filtrate obtained from tea infusion of black tea, i.e., CTC tea and Kangra orthodox tea samples was used as substrates (100 ml each) and each sample was incubated with 100 mg of tannase at 30°C for 1 h. The hydrolysis process was stopped by placing the reaction in an ice bath for 15 min.

3.9.3.3. Evaluation of tea cream formation

Each of the black tea infusions, i.e., CTC and Kangra orthodox tea, was treated with different concentrations of tannase (0.05–0.2%) as against control. The treated and untreated tea infusion samples obtained from both the tea were centrifuged in a refrigerated centrifuge (Sigma centrifuge, 4X15, Germany) at 8°C,
10,000 rpm for 25 min. The precipitate obtained was dried in a hot-air oven at 100°C till a constant weight is obtained. The tea cream (g/100 ml) was expressed as weight of precipitate obtained per 100 ml of tea infusion.

3.9.3.4. Evaluation of antioxidant activity (DPPH assay)

The biotransformed tea samples (test samples) obtained after enzymatic reaction were subjected to DPPH assay using the method of Turkmen et al. (2006) with slight modification. The appropriate dilution of test samples was prepared with 70% ethanol. Fifty microliter (50 µl) of test sample and 1,950 µl of DPPH radical in ethanol (100 µM) were mixed by vortexing and the reaction mixture was incubated at 25°C in dark for 30 min. The process of decolorization was recorded at 520 nm using Shimadzu UV-2450 UV-VIS spectrophotometer (Kyoto, Japan). Similar set of reaction was carried out with tea infusion sample, which served as control. The antioxidant activity was determined as percentage inhibition of the DPPH radical using the following equation (Yen and Duh, 1994).

\[
AA \, (\%) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100
\]

3.9.4. Collected tannery effluent

The study area is Ranipet (Ranipettai) sub urban town is an major industrial area located in Wallajah taluk, Vellore district, Tamil Nadu at 79°19′–79°22′ E longitude and 12°53′–12°57′ N latitude and is 114 Km west of Chennai. It is situated at a distance of 3.5 km from river Palar and adjoining Chennai- Bangalore-Chittoor Highway (NH-4). It is one of the biggest exporting centers of tanned leather in India and discharging their effluents on the open land and surrounding water bodies (Govil et al., 2004). The effluent samples were collected from the all stages of
tanning processing viz., soaking, liming, deliming, pickling, chrome tanning and retanning. The effluent was collected in polythene containers of 2 to 10 liters capacity and were brought to the laboratory with due care and was stored at 20°C for further analysis. Chemicals used for the analysis of spent liquor were analytical grade reagents. The physical and chemical characteristics of tannery effluents parameters viz. pH, total alkalinity, total acidity, COD, BOD, total solids (TS), total dissolved (TDS), total suspended solids (TSS), chlorides and sulfides were analyses as per standard procedures.

3.9.4.1. Determination of pH

The pH is determined by measurement of the electromotive force (emf) of a cell comprising of an indicator electrode (an electrode responsive to hydrogen ions such as glass electrode) immersed in the test solution and a reference electrode (usually a calomel electrode). Contact is achieved by means of a liquid junction, which forms a part of the reference electrode. The emf of this cell is measured with pH meter.

3.9.4.2. Determination of total alkalinity and acidity

The alkalinity of sample can be determined by titrating the sample with sulphuric acid or hydrochloric acid of known value of pH, volume and concentrations. Based on stoichiometry of the reaction and number of moles of sulphuric acid or hydrochloric acid needed to reach the end point, the concentration of alkalinity in sample is calculated. A known volume of the sample (50 ml) is taken in a beaker and a pH probe was immersed in the sample. HCl or H₂SO₄ acid (0.1 N e.g 8.3 ml concentration of HCl in 1000 ml distilled water) added drop by drop until
the pH of the sample reached. The volume of the acid added was noted (Clesceri et al., 1989).

**Calculation for alkalinity**

Alkalinity as mg/l of CaCO₃ = (50000x N of HCl x ml acid titrated value)/volume of sample taken

**Calculation for Acidity**

Acidity as mg/l of CaCO₃ = (50000X N of NaOH X ml NaOH titrated value)/volume of sample taken

### 3.9.4.3. Determination of chemical oxygen demand

Determination of chemical oxygen demand (COD) The chemical oxygen demand of an effluent means the quantity of oxygen, in milligram, required to oxidize or stabilize the oxidizable chemicals present in one liters of effluent under specific condition. 2.5 ml of the sample was taken in tube, 1.5 ml of 0.25 N K₂Cr₂O₇ (potassium dichromate), spatula of mercuric sulphate HgSO₄ and 3.5 ml of COD acid were added and kept in COD reactor for 2 h at 150°C. After cooling the sample titrated against FAS (standard ferrous ammonium sulfate 0.1N) and used ferrion as indicator. The end point is reddish brown color. In the blank tube 2.5 ml of distilled water was taken and then follow the same procedure in the sample (Clesceri et al., 1989).

**Calculation**

COD (mg/l) = (blank value-titrated value) XN of FASX8000/ volume of sample 8000 = mill equivalent wt of O₂x1000 ml
3.9.4.4. Determination of biochemical oxygen demand

Determination of biochemical oxygen demand (BOD) Biochemical oxygen demand (BOD) of an effluent is the milligram of oxygen required to biologically stabilize one liter of that effluent (by bio-degradation of organic compounds with the help of micro-organisms) in 5 days at 20°C. If the BOD value of an effluent is high, then that effluent contains too much of bio-degradable organic compounds and so will pollute the receiving water highly.

Aerated for 3.5 h, Added nutrients 1 ml nutrient for 1 liter aerated distilled water (FeCl₃, CaCl₂, PO₄, MgSO₄, domestic water) aeration for 30 min. BOD bottle (300 ml) add sample fill the bottle with aerated water put the lid (avoid air bubbles) keeping BOD incubator at 20°C for 5 days. After 5 days take the bottle and add 2 ml MnSO₄, 2 ml alkali azide iodide and 2 ml concentration of H₂SO₄, shake the bottle well (yellow colour) take 200 ml sample add starch solution as indicator (purple colour) titrated with 0.025 N sodium thiosulphate end point colour change from purple to colorless. In blank filled the bottle with aerated water without the sample and follow the procedure (Clesceri et al., 1989).

Calculation

\[ \text{BOD}_5 = (\text{blank value}-\text{titrated value}) \times 300/\text{volume of sample}. \]

3.9.4.5. Determination of total solid

The term ‘solid’ refers to the matter either filtrable or non-filtrable that remains as residue upon evaporation and subsequent drying at a defined temperature. Residue left after the evaporation and subsequent drying in oven at specific temperature 103-105°C of a known volume of sample are total solids. Total solids include “Total suspended solids” (TSS).
3.9.4.6. **Total dissolved solids (TDS)**

Dry weight of empty dish or crucible (initial weight) add 50 ml sample keep it in water bath until dry keep it in oven (103 to 105°C) for at least 1 h desiccator take final weight of dish (Clesceri et al., 1989).

**Calculation**

Total solid (mg/l) = (final weight-initial weight) x1000x1000 / volume of sample

3.9.4.7. **Determination of total suspended solid**

The difference between the total solids and total dissolved solids is suspended solids. TSS = TS-TDS

Determination of chloride is determined in a natural or slightly alkaline solution by titration with standard silver nitrate, using potassium chromate as an indicator. Silver chloride is quantitatively precipitated before red silver chromate is formed. Take sample (10 ml to 50 ml) add 2 ml of hydrogen peroxide (H₂O₂) add 2 ml K₂CrO₄ (potassium chromate indicator) titrate with silver nitrate (0.0141N) end point formation of water instead of sample and follow the same procedure above (Clesceri et al., 1989).

**Calculation**

Chloride (mg/l) = (A-B) xN. of silver nitrate x 35.45 x 1000/ volume of sample

A = Titration for sample ml

B = Titration for blank ml

N = Normality of AgNO₃
3.9.4.8. Determination of sulfide

The sulfides in the solution are oxidized with an excess of a standard iodine solution and the excess back titrated with a standard thiosulfate solution.

Take sample (10 ml) in conical flask add 5 ml zinc acetate (5%) filter through filter paper take the filter paper and put it in the same conical flask add 100 ml distilled water. Then add 20 ml iodine solution and 4 ml 6 N HCl add 2 drops of starch as indicator (purple colour will form) titrate against sodium thiosulphate (0.025N) end point the colour change from blue colour to colorless. In the blank test take 100 ml distilled water instead of sample and follow the same procedure above for the sample (Clesceri et al., 1989).

Calculation

\[
\text{Sulfide (mg/l) } = \frac{\text{BV}}{\text{TV}}
\]

BV = Blank value

TV = Titrated value

3.9.5. Removal of tannin stains

The potential of tannase produced by the selected organism for removing the tannin stains was evaluated. Cotton cloth pieces of the size (5x5 cm) were stained with concentrated black tea and betel juice extract separately. The stained pieces were then subjected to the following treatments:

A. Tap water
B. Hot tap water
C. Detergent
D. Detergent and hot tap water
E. Detergent and tannase
F. Tannase
The stained cloth, which was not subjected to any treatment, was taken as the control. The efficiency of the removal of tea stain was compared on the basis of percent increase in reflectance, using a reflectometer (model No. Universal 1700).