3. MATERIALS AND METHODS

3.0.0 COLLECTION OF SAMPLE

The tannery effluent was collected from M/s Venketeswaran and Brothers Tannery, BB Agraharam, Erode where skins and hides are processed by vegetable tanning method. The effluent was collected freshly from treatment plant and it was stored in a brown coloured bottle, prior to the collection, the sample bottle was rinsed thoroughly with sample water. The samples were taken to the laboratory as early as possible and it was protected from direct sunlight. The samples were stored in refrigerated condition.

3.1.0 ANALYSIS OF PHYSICOCHEMICAL CHARACTERS (APHA,1995)

3.1.1 MEASUREMENT OF pH

Measurement of pH is one of the most important and frequently used tests in water chemistry. pH defined as the negative logarithm of hydrogen ion concentration or simply, the log of the reciprocal of the hydrogen ion concentration. pH indicates the degree of acidity or alkalinity of water and it is used to characterize a given water sample.

PROCEDURE

1. The electrodes of the pH meter were calibrated with standard buffer solutions of known pH
2. 50 mL effluent was taken in beaker.
3. The electrodes were washed with distilled water and wiped it dry with filter paper
4. The electrodes were immersed in to the beaker containing the effluent and the meter readings were recorded

3.1.2 ESTIMATION OF TOTAL DISSOLVED SOLIDS

Total dissolved solids denote mainly the various kinds of minerals present in water. It does not contain any gas or colloids. The maximum permissible limit of TDS for any effluent is 2100mg/mL.

The total dissolved solids can be determined by evaporating, drying the known volume of the filtered sample.

PROCEDURE

1. The evaporating dish of suitable size, were taken dried and weighted.
2. The sample was filtered through Whatman No.1 filter paper.
3. The clear filtrates in the evaporating dish were evaporated on a water bath until constant weight.
4. The dish was again weighed and the difference was noted.

CALCULATION

\[
\text{TDS (mgL}^{-1}\text{)} = \frac{(A - B) \times 1000}{\text{Sample volume, mL}}
\]

Where

A- Weight of the dried residue + dish, mg  and
B- Weight of the dish, mg
3.1.3 ESTIMATION OF TOTAL SUSPENDED SOLIDS (TSS)

Total Suspended Solids indicate a measure of the amount of all kinds of soluble solids. It is one of the valuable parameters in judging the pollution load of an effluent on the receiving stream and also to decide the efficiency of the treatment.

Filtering or centrifuging the sample, drying the residue and determining its weight by difference, determines the suspended matter.

PROCEDURE

1. 50 mL of effluent was filtered using predried and weighed Whatman No-1 filter paper.
2. The filter paper containing the residue was dried at 150°C for 8 hours or to constant weight and weight of the dried contents was taken.

CALCULATION

\[
\text{TDS (mgL}^{-1}\) = \frac{(A - B) \times 1000}{\text{Sample volume, mL}}
\]

Where

A - Weight of the filter paper + residue (dried) (mg)
B - Weight of the filter paper alone (mg)

3.1.4 ESTIMATION OF DISSOLVED OXYGEN

The amount of oxygen present in water is called the dissolved oxygen. The measurement of DO indicates the purity of water and is important for
maintaining aerobic conditions in the receiving waters and is the basis for BOD test which is used to evaluate strength of waters and rate of biochemical oxidation.

The "Winkler's method was followed to estimate the DO value. This method is based on the fact that when manganous sulphate is added to the sample containing alkaline potassium iodide, manganese hydroxide is formed, which is oxidized by the dissolved oxygen of the sample to basic manganic oxide. On addition of sulphuric acid, the basic manganic oxide liberates iodide equivalents to that of dissolved oxygen originally present in the sample. The liberated iodide is titrated with standard solution of sodium thiosulphate using starch as an indicator.

SAMPLE COLLECTION AND PRESERVATION

Samples have to be collected in BOD bottles. The bottles were filled without entrapment of any air. Sample collected in BOD bottles in the field and brought to the lab is likely to undergo a change in DO value because of changes in temperature and also occurrence of biological reaction with time. Therefore to obtain correct DO value the sample must be "fixed" immediately after collection fixing is done by adding 2mL of Manganese sulphate solution and alkali iodide oxide to the sample in the BOD bottles. Preservation of the fixed sample was done by keeping at 4°C in dark.

REAGENTS USED

Manganese Sulphate Solution

48.00g of MnSO₄. 4H₂O was dissolved in 100mL of distilled water; filtration of solution is necessary since it is not clear.
Alkali Iodide Azide Reagent

1. 175 KOH (or 125g NaOH) was dissolved in 100mL diluted to 250mL
2. 2.5g of sodium azide were dissolved in 10mL of distilled water.
3. The azide solution was poured in to the alkali iodide solution and mixed well.

Concentrated sulphuric acid
Starch indicator

1.0g starch dissolved was in one liter of water. A thin paste was prepared by stirring it with a glass rod. This paste was poured into 100mL boiling water, boiled for two minutes and cooled.

Sodium thiosulphate solution. 0.1 N

1.2257g potassium dichromate was dissolved (previously dried at 103°C for 1 hour) in distilled water and made up to 250mL in a volumetric flask. This is exactly 0.1 N solution 100mL of boiled cool distilled water was placed in a 500mL conical flask. 3g of KI, 2g NaHCO₃ were added and shaken until the salts dissolved. 6mL concentrated sulphuric acid was added. 25mL 0.1 N K₂Cr₂O₇ solutions was pipeted in to the flask and kept in the dark for 5 min. The watch glass was rinsed, and solution was diluted to 250mL with boiled cooled distilled water. The liberated iodide was titrated with the sodium thiosulphate solution in the burette. When the solution acquires yellowish green colour. 1.0mL starch indicator was added. Blue shade was obtained along the sides of the flask was rinsed the titration was continued until last one drop changes the color from greenish blue to light green. Normality of sodium thiosulphate solution was calculated.
PROCEDURE

The sample (3.2mL of the effluent +122.8mL of phosphate buffer) was pipetted out into a stopped bottle. Then 2mL of manganous sulphate solution followed 2mL of alkali-iodide azide solution were added. The bottle was stopped without the entrapment of air and mixed by inverting the bottle not less than 10 minutes. Then 2.0mL of concentrated sulphuric acid was added by the sides of bottle to dissolve the precipitate formed.

The liberated iodide was titrated with the standardized thiosulphate in the burette 2mL of starch solution was added when the color of the solution turns straw yellow. The titration was continued by adding the thiosulphate solution in drops until the disappearance of the color. The experiment was repeated to obtain concordant titer values.

CALCULATION

1000ML of 1 N thiosulphate = 8g oxygen

\[
\text{Dissolve oxygen (mg/l)} = \frac{(V_2 \times N \times 8 \times 1000)}{V_1}
\]

Where,

\[
V_1 = \text{volume of sample in mL}
\]

\[
V_2 = \text{Titer value (thiosulphate)in mL}
\]

\[
N = \text{Normality of thiosulphate.}
\]

3.1.5 ESTIMATION OF BIOCHEMICAL OXYGEN DEMAND (BOD)

This test measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material and the
oxygen used to oxidize inorganic material such as sulfides and ferrous ion. BOD is defined as the amount of oxygen required by bacteria for stabilizing decomposable organic matter under aerobic condition. BOD is the major criteria used in stream pollution control where organic loading must be restricted to maintain desired dissolved oxygen levels. It is highly useful in studies of measuring the purification capacity of streams and serves regulatory authorities as a means of checking the quality of effluent discharged in to such waters. It is the only test that gives a measure of the amount of biologically oxidisable organic matter.

Measuring the oxygen consumed by bacterial and chemical action in a closed sample of water maintained at 20°C for five days. A sample with low BOD can be straight away used for the BOD determination. However, a sample with high BOD must be diluted and pretreated before BOD determination.

REAGENTS USED

Phosphate Buffer solution

8.5g of KH₂PO₄ 21.75g K₂HPO₄, 33.4g Na₂ HPO₄ 7H₂O and 1.7 g NH₄Cl were dissolved 500mL distilled water. This solution was diluted to one liter.

Ferric chloride

0.25g FeCl₃ 6H₂O was dissolved in water and the solution was diluted to 1 liter.

Calcium chloride

27.5 gm CaCl₂ was dissolved in 1 liter distilled water.
Materials and methods

Magnesium sulphate solution

22.5g MgSO₄·7H₂O dissolved in 1 Liter of distilled water.

Water for dilution

2 liter of distilled water was taken in 3-liter bottle. This partially filled bottle was shaken for about ten minutes so that the distilled water was saturated with atmospheric oxygen. Then to it 2mL of phosphate buffer solution, 2mL of MgSO₄ solution, 2mL CaCl₂ solution and 2mL of ferric chloride solution were added.

PROCEDURE

BOD ESTIMATION AFTER DILUTION

This method was used since the BOD value exceeds five. DO of the diluted sample was determined using one of the bottles immediately. The other bottle was incubated for 5 days at 20°C. After 5 days DO was determined. The difference in DO was recorded.

CALCULATION

\[ \text{BOD}_5 (\text{mg/L}) = \frac{D_1 - D_2}{P} \]

Where,

\[ D_1 = \text{DO of diluted water sample immediately after preparation, mg/L} \]

\[ D_2 = \text{DO of diluted water sample after five days incubation, mg/L} \]

\[ P = \frac{\text{mL of sample} + \text{mL of dilution water added}}{\text{mL of sample}} \]
3.1.6 ESTIMATION OF CHEMICAL OXYGEN DEMAND (COD)

Chemical oxygen demand is the oxygen requirement of a sample for oxidation of organic and inorganic matter chemically under specified conditions. The major advantage of the COD test short time is required to carry out rather than the longer 5 days procedure for BOD. This test is recommended as a supplement to BOD test.

In COD test the sample is subject to a chemical oxidation induced by chemical reagents and it is an artificial oxidation by which both biologically oxidisable and biologically inert organic matters are oxidized.

The organic matter of the sample is oxidized to water, CO₂ and ammonia by refluxion with a known excess of potassium dichromate in 50% sulphate acid solution. The excess dichromate is titrated with a standard of ferrous ammonium sulphate solution.

Silver sulphate is added as catalyst to promote oxidation of certain classes of organic compounds such as straight chain aliphatic compounds like acetic acid. Amino acids etc Mercuric sulphate is added to eliminate the interference due to chlorides.

REAGENTS USED

Mercuric sulphate crystals.

Silver sulphate crystals.

Sulphuric acid-concentrated.
Potassium dichromate 0.25 N solution.

12.25g potassium dichromate was dissolved (previously dried at 103 °C for 2 hours) in distilled water and made up to 1000mL in a volumetric flask.

1.0mL of 0.025N $\text{K}_2\text{Cr}_2\text{O}_7 = 2.0$ mg oxygen.

Ferroin Indicator

695mg ferrous sulphate and 1.485g of 1,10 phenanthroline monohydrate together were dissolved in water and diluted to 100ml.

Ferrous Ammonium Sulphate 0.1N

39.29g ferrous ammonium sulphate was dissolved in distilled water, 20mL concentrated sulphuric acid were added cooled, and made up to 1000mL in a volumetric flask.

Sulphuric acid Reagent

5.5 g of AgSO$_4$ was added to 1 Kg of concentrated H$_2$SO$_4$. The mixture was allowed to stand for one day to dissolve AgSO$_4$.

STANDARDIZATION AGAINST 1.023 N POTASSIUM DICROMATE

10mL 0.25N potassium dichromate solution was pipetted out to a 250mL conical flask. Diluted to 100mL and 30mL concentrated sulphuric acid was added. The mixture was cooled to room temperature and 2 to 5 drops of ferroin indicator was added and titrated against ferrous ammonium sulphate solution. The end is the color changes from blue green to reddish brown.

PROCEDURE

1. 0.5mL sample was pipetted out and diluted with 19.5mL in distilled water in a 250mL conical flask.
2. 10mL of standardized dichromate solution was added.
3. 0.4g of mercuric sulphate crystals were added to the mixture.
4. 30mL of conc. sulphuric acid was carefully added with gentle mixing.
5. Two to 3 boiling chips were added in to the flask.
6. A pinch of silver sulphate crystals were added.
7. The reflux condenser was attached and the mixture was refluxed for 2 hours with a heating mantle.
8. After cooling the flask the inside of the condenser was rinsed with a small amount of distilled water allowing the washing to run into the flask.
9. The digested sample was diluted appropriately with distilled water. So that the final volume after dilution should be about 2.5 times the volume of sample + dichromate+ H₂SO₄
10. 2 drops of ferroin indicator were added and the unreacted dichromate was titrated with standardized ferrous ammonium sulphate in the burette. The end point is the sharp changes of color from blue green to reddish brown.

**CALCULATION**

\[
\text{COD (mg/L)} = \frac{(V2-V3) \times N \times 8 \times 1000}{V1}
\]

Where

\( V1 = \text{Volume of sample taken in mL} \)
\( V2 = \text{Blank titre value in mL} \)
\( V3 = \text{Sample titer value in mL} \)
\( N = \text{Normality of ferrous ammonium sulphate solution} \)
3.1.7 ESTIMATION OF CHLORIDE

Chloride is the common anion found in water and industrial effluents. It is estimated by Mohr’s method. Silver nitrate reacts with chloride ions to form silver chloride. The completion of the reaction is indicated by the red color produced by the reaction of silver chloride with potassium chromate solution, which is added as an indicator. For all titration, a definite volume of the indicator should be accurately pipetted in to the analyte.

Sulphate interference can be eliminated of 1mL of 30% H2O2 to each 100mL of water sample keeping the pH 7.0 Cyanide can be eliminated by acidifying the sample with 1N sulphuric acid to pH 4.0 and boiling for about 15min and then the cooled sample is neutralized with 1N sodium hydroxide before final titration.

REAGENT
Distilled Water

Double distilled water was used for the preparation of all solutions.

Silver nitrate (0.05N)

Dissolve 8.486g of AgNO₃ in distilled water and the solution was made up to 1000mL It was stored in a brown bottle.

Potassium chromate indicator

5g of potassium chromate were dissolved in 25mL of water. AgNO₃ is added until a definite red precipitate was formed. After 12 h, the preparation was filtered and diluted to 100 m.
Aluminum hydroxide

100g potassium aluminium sulphate was dissolved in 1000mL distilled water. Warmed up to 60°C and 55mL of concentrated ammonia solution was slowly added with constant stirring. The precipitate was allowed to settle for about 1 h. Washed by decantation with distilled water to make the precipitate free from chloride. A portion of the decante was checked every time with silver nitrate solution. Diluted to 100mL with distilled water.

PROCEDURE.
FOR CLEAR SAMPLE

1. 25mL of the sample was taken (p$^H$ adjusted to 7-8) in to 250mL conical flask.
2. 0.25mL potassium chromate indicator solution was added.
3. The solution was titrated with constant agitation against silver nitrate solution until the color appeared.
4. Blank value was carried out from the sample value.

FOR COLOURED AND TURBID SAMPLES

1. Since the raw effluent sample is highly turbid, 3mL of aluminium hydroxide was added to a measured volume of the sample in a beaker.
2. The mixture was stirred well and allowed to settle.
3. The precipitate was filtered and precipitate was washed with chloride free distilled water.
4. The filtrate and washings were combined.
5. The p$^H$ of the pooled precipitate and washings were adjusted to 7-8.
6. Titration against silver nitrate solution using potassium chromate, an indicator, was carried out the end point is appearance of the reddish brown precipitate.

**CALCULATION**

\[
100\text{ML of 1NagNO}_3 = 35.45 \text{ of C1}
\]

\[
\text{Chloride in water sample (mg/L)} = \frac{(A-B) \times N \times 35450}{\text{mL sample}}
\]

Where,

- \(A\) = mL titration for sample
- \(B\) = mL titration for blank and
- \(N\) = Normality of AgNO₃

**3.1.8 ESTIMATION OF SULPHATE**

The sulphate ions present in a water sample can be determined by turbidimetric method. In this method, white light is passed through a finely divided suspension of the substance to be estimated. The light transmitted by the suspension is compared with the light transmitted by the suspension of known concentration from which the concentration of the sample is determined. Color or suspended materials in large amounts will interfere. Some suspended matter may be removed by filtration. Sample color be corrected by running blanks to which barium chloride is not added.

**REAGENTS USED**

Standard Sulphate solution

0.1479g of anhydrous Na₂SO₄ was dissolved in distilled water and diluted to 1000ml.
Materials and methods

Barium Chloride salt

Conditioning agent

75g of NaCl were dissolved in 300mL of water and to this 30mL of concentrated HCl, 50mL of glycerol and 50mL of ethyl alcohol were added.

PROCEDURE

1. Standards of 200,400,600,800,1000g of sulphate in 25mL of volumetric flasks were prepared.
2. To each flask, 5mL of conditioning reagent was added and stirred.
3. A pinch of barium chloride salt was added and the flask was shaken exactly for 5mins.
4. The absorbance was read at 420nm and standard graph was drawn.
5. The procedure was repeated for unknown sample with 5mL of the effluent diluted to 25ml.
6. The standard graph was constructed taking the concentration in X-axis and absorbance in Y-axis and the concentration of unknown sample was deduced from the graph.

CALCULATION

Concentration of sulphates (g/mL) = C/V x 100

C = Concentration deduced from standard graph.
V = Volume of sample taken for analysis.

Sulphate content = \[
\frac{\text{Graph value X aliquot.vol}}{\text{Sample value}}
\]
3.1.9 ESTIMATION OF TOTAL ALKALINITY

Alkalinity of water may be defined as its capacity to neutralize a strong acid. It has been characterized by the presence of hydroxyl (OH⁻) ions capable of combining with hydrogen (H⁺) ions. A number of bases such as carbonates, bicarbonates, hydroxides have been regarded to the predominant bases. Thus, alkalinity could be expressed as total alkalinity due to individual bases.

The amount of CO₃²⁻/OH⁻ is determined by titration with acid to pH 8.3. The end point being detected with phenolphthalein. The amount of HCO₃ is determined by further titration with acid to an end pH between 4.2 and 5.4 with methyl orange or mixed indicator, an end point indicator.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Symbol</th>
<th>Compounds</th>
<th>End point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolphthalein alkalinity</td>
<td>PA</td>
<td>OHCO₃²⁻</td>
<td>8.3</td>
</tr>
<tr>
<td>Total alkalinity</td>
<td>TA</td>
<td>OHCO₃²⁻+HCO₃</td>
<td>4.2-5.4</td>
</tr>
</tbody>
</table>

REAGENTS USED

Sulphuric acid 1N

28mL of concentrated H₂SO₄ was taken in a 1000mL volumetric flask and made up to the mark with carbon dioxide free distilled water. It was standardized against 1N sodium carbonate solution using methyl orange as indicator and the exact normality was calculated:

1mL of 1N H₂SO₄ = 50mg CaCO₃

Sodium Carbonate solution 1N

13.25g of an anhydrous sodium carbonate was weighed transferred it in little distilled water first and finally made up to 250mL in a volumetric flask.
Phenolphthalein indicator

0.5g of phenolphthalein was dissolved in 50mL of 95% ethanol and 50mL water was added. Diluted (0.05N) NaOH Solution was added drop wise until the solution turns faintly pink

Methyl Orange indicator

0.05g of methyl orange was dissolved in about n100mL of water. This indicator is suitable for equivalent points below pH 4.6.

Mixed indicator

20mg of methyl red 80mg of Bromocresol green were dissolved in about 100mL of 95% ethanol. This indicator is suitable for pH range for 4.6 to 5.2 and gives better color changes and hence used in place of methyl orange indicator.

PROCEDURE

I FOR COLOURLESS AND SLIGHTLY COLOURED SAMPLES

(A) Phenolphthalein Alkalinity

• 25mL of sample was taken in a conical titration beaker and 6 drops of phenolphthalein indicator was added (B)

• The solution terms pink so it’s PA is not zero.

• It was titrated to pH 8.3 (disappearance of pink color)

(B) Total Alkalinity

• 4 drops of mixed indicator were added (or methyl orange) to the solution in which PA has been determined.
Materials and methods

Titrated against sulphuric acid to pH 4.5 (color change from emerald green to light pink when mixed indicator is used or yellow to orange red when methyl orange indicator is used.)

**CALCULATION**

\[
\text{Alkalinity, mg CaCO}_3/\text{L} = \frac{A \times N \times 50,000}{\text{mL sample}}
\]

Where

- \( A = \) mL Standard acid used and
- \( N = \) Normality of the standard acid.

**3.1.10 ESTIMATION OF TANNIN**

The tannin content of the tannery wastewater is estimated by colorimetric method. (APHA, 1995).

**REAGENTS USED**

Folin Phenol reagent

100 g sodium tungstate (\( \text{Na}_2 \text{WO}_4 \cdot 2\text{HO} \)) and 25g sodium molybdate(\( \text{Na}_2 \text{Mo O}_4 \cdot 2\text{H}_2\text{O} \)) and 700 mL distilled water were taken in a conical flask. 50 mL of 85% \( \text{H}_3\text{PO}_4 \) and 100 mL con HCl were added. The contents were boiled in a reflux container for 10h. 150g LiSO\(_4\), 50 mL distilled water and a few drops of liquid bromine were added. The excess bromine was removed by boiling for 15 min. After cooling it to 25\(^\circ\)c, diluted one lit and stored in a tightly stoppered bottle.
Materials and methods

Carbonate-tartrate reagent

200 g Na$_2$CO$_3$ and 12 g sodium tartrate (Na$_2$ C$_4$H$_4$O$_6$. 2H$_2$O) were dissolved in 750 mL hot distilled water. After cooling it to 20°C, it was diluted to 1 liter.

Tannic Acid Stock Solution

1.0 g tannic acid is dissolved in 100 mL distilled water diluted to 1 liter.

Tannic acid standard solution

50 mL of stock solution was diluted to 1000 mL with distilled water.

$1 \text{ mL} = 50 \mu g.$

PROCEDURE

1. 50 mL of the sample was taken in a conical flask.
2. 1 mL of Folin phenol reagent and 10 mL Carbonate tartrate reagent with succession.
3. The mixture was kept as such for 30 min for color development.
4. The different concentrations of the standard were prepared along with a blank without tannic acid.
5. The absorbance was measured at 700 nm using systronics u.v spectrophotometer.

From the standard graph, the amount of tannin present in the effluent was determined.
3.2.0 ISOLATION OF FUNGI

The tannery effluent was brought to room temperature. The fungal forms were isolated using serial dilution and spread plate method. 1 mL of effluent was taken and added to 9 mL of sterile distilled water. Making it $10^1$ dilution. 1 mL was taken and transferred to next tube making it $10^2$ dilution. Dilution was carried up to $10^{-1}$ and 1 mL was discarded from the last tube. From each dilution, 1 mL was taken out and it was spreaded properly using a glass rod and potato dextrose agar (PDA.). Duplicates were maintained for each dilution. All the plates were kept at $25^\circ$C for 48-72 hours. After incubation, the plates were observed for colonies (Graca et. al., 1997).

3.2.1 CULTURE IDENTIFICATION OF TANNASE PRODUCING FUNGI

The fungal isolates that have been screened by the above process are identified morphologically by the slide culture technique using lacto phenol cotton blue staining (Allexopholus, 1962)

Slide Culture of Isolates

1. The filter paper in the petriplates along with glass rod, clean glass slide were taken. The whole set was then sterilized.
2. 10 mm piece of PDA agar block was removed from the plate and placed on the center of the slide aseptically.
3. The screened fungal isolates were inoculated on all four corners of the agar block.
4. Flamed cover slip was placed over the inoculated agar block.
5. A piece of cotton soaked in sterile water was placed in the plate to maintain moisture inside.
6. The plate was inoculated at $25^\circ$C for four days.
Preparation of Wet Mount

1. The cover slip was gently lifted and placed on another clean slide with a drop lacto phenol cotton blue stain.
2. The agar was discarded and a drop lacto phenol cotton blue strain (LCB) was added to the slide and mounted with a cover slip.
3. Both the preparations were observed under the high power objective (40x).
4. The fungal specimen was studied and the morphology was noted and photographed.

3.3.0 SCREENING

Screening is of utmost importance in any industrial and research work. For the identification of organisms capable of producing desired product, screening procedure is carried out. There are specific media with a specific substrate for screening purpose. By the screening method unwanted organism are deleted out.

Screening of organisms capable of producing tannase was carried out, by a method described by Sapna Bradoo et. al., (1996) for the isolated fungi using tannic acid agar medium. In tannic acid agar medium tannic acid is the sole carbon source, so organism that can degrade tannic acid only can grow and produce a clear zone of hydrolysis by producing the enzyme tannase. It was a simple and rapid assay for the screening of extra cellular tannase producing fungi. There was a high correlation coefficient between halosize and quantitative productions of enzyme in broth.
PROCEDURE

Screening medium: Czapeks dox minimal medium supplemented with tannic acid and agar agar

Media composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrate</td>
<td>6 gm</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.52 gm</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.52 gm</td>
</tr>
<tr>
<td>Dihydrogen ortho phosphate</td>
<td>1.52 gm</td>
</tr>
<tr>
<td>Copper nitrate</td>
<td>traces</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>traces</td>
</tr>
<tr>
<td>Ferrous salt</td>
<td>traces</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>10 gms</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gms</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Preparation of medium

1. All the ingredients except tannic acid was weighed and dissolved properly in distilled water.
2. It was kept for sterilization at 121°C for 15 minutes.
3. Tannic acid was weighed and dissolved completely is distilled water.
4. Tannic acid solution was subjected to membrane filtration.
5. Filter sterilized tannic acid was added slowly to the sterile medium at an appropriate temperature.
6. It was poured in to sterile petri plate and allowed to cool.
Materials and methods

Screening method

1. Under sterile conditions, one loopful of isolated fungi was spot inoculated on sterile Tannic Acid Agar medium.
2. Same method was carried out for each and every isolated fungi.
3. The plates were then incubated at 25°C for 48 – 72 years.
4. After incubation period the plates were observed for the formation of zone of hydrolyzing surrounding the colonies.

3.4.0 ENZYME PRODUCTION

Erlenmeyer flasks containing 50mL of Czapek dox liquid medium (containing NaNO$_3$ 6 gm, KCl 0.52gm, MgSO$_4$ 0.52gm, KH$_2$PO$_4$ 1.52gm, Cu(NO$_3$)$_2$ trace, ZnSO$_4$ trace, FeSO$_4$ 7H$_2$O trace, distilled water 1000ml, tannic acid 1%) were inoculated with 5X10$^7$ Fungal spores and were incubated at 30°C for 48-72 hours (Lekha and Lonsane, 1994).

3.4.1 EXTRACTION OF ENZYME

Tannase in both extra cellular and membrane bound. Extra cellular tannase is released in to the medium. The medium itself can be tested for tannase activity. For the extraction of membrane bound enzyme the following procedure can be followed.

PROCEDURE

1. Mycelial mat was removed from the broth and it was washed properly to remove the tannic acid adhering to the mycelial mat.
2. The was homogenized with acid washed sand in chilled mortar using 0.01 M acetate buffer (pH - 5.0)
3. Homogenate centrifuged at 8000 rpm for 15 minutes.
4. This supernatant was collected and used as enzyme extract.
5. It was stored at 4°C for further studies.

3.5.0 DETERMINATION OF TANNASE ACTIVITY

The activity titer of the enzyme tannase was determined based on the decrease in the absorbance of the substrate tannic acid at 310nm. (Libuchi et al., 1967)

PROCEDURE

1. 0.5mL of enzyme solution was mixed with 2mL of 0.35% tannic acid in 0.05m-citrate buffer.
2. 0.1mL of above reaction mixture is taken out and 10mL of 95% ethanol was added to stop the reaction of enzyme.
3. The absorbance was measured at 310 nm (T₁)
4. After a specific period at 30°C, the absorbance was measured again (T₂)
5. Difference between T₁ and T₂ was calculated.

3.5.1 THE UNIT OF ENZYME ACTIVITY

One unit of enzyme activity is the amount of enzyme required to hydrolyze 1 mole of ester bond in one minute. The unit of enzyme activity was defined by following equation.

\[ U = C.V = \frac{\Delta E (t1-t2)}{\Delta E (0-T)} \cdot \frac{1}{t2-t1} \cdot \frac{1}{V} \]

Where C is the total concentration of total ester bonds contained in the substrate at first, which is the same as that of gallic acid contained in the
substrate tannic acid. Reaction time T means the time after which all the ester bonds of the substrate were hydrolyzed, and $\Delta E (0-T) = E_0 - E_T$.

With slight modification the equation can be as follows

$$U = 20.3 \times 4 \times 1/0.71 \frac{\Delta E}{\Delta T}$$

$$= 114 \frac{\Delta E}{\Delta T} \text{ unit/mL}$$

Where

$$\Delta E = E_{t_1} - E_{t_2}$$
$$\Delta T = t_2 - t_1$$

3.6.0 PURIFICATION OF ENZYME

3.6.1 AMMONIUM SULPHATE PRECIPITATION

The crude extract of enzyme is purified for further analysis. The first step used is the precipitation of the proteins by Ammonium sulfate precipitation method. The enzyme proteins present in the crude extract were precipitated by means of ammonium sulfate addition. The process called 'salting out' is carried out by addition of ammonium sulfate till 100% saturation to precipitate the proteins. This was then centrifuged at 8000g for 20 minutes.

3.6.2 DIALYSIS

Dialysis is a process used selectively to remove small molecule from a sample with both small and large molecules. This process was performed by using a semipermeable dialysis membrane.
PROCEDURE

1. The next step of purification is done by dialyzing the precipitated enzyme. (Suseela. G. Rajkumar et al., 1998)

2. The precipitated enzyme samples were then reconstituted in a small quantity of distilled water.

3. They were then covered in tubes covered with pre treated dialysis membrane and dialyzed against 0.01M acetate buffer (pH 5) for three days. The buffer was constantly replaced to avoid concentration of impurities.

4. This dialyzed extract was taken as the purified sample and were utilized for further processing like

   → Analysis of sugars & amino acids
   → Protein estimation
   → Estimation of specific activity

3.7.0 ANALYSIS OF AMINO ACIDS AND SUGARS OF TANNASE BY PAPER CHROMATOGRAPHY

Chromatography is the most powerful technique to separate chemically closely related substances in to the individual components based on their physicochemical properties. The compounds are separated based on their partition coefficients between two immiscible phases. The static phase may be a solid or liquid while the mobile phase may be a solid, liquid or gas. Depending upon the static and mobile phases, a variety of chromatographic techniques is available. These include chromatography on paper, thin layer gel, ion-exchange resin etc.
Although modern instrument facilities such as High Performance Liquid Chromatography (HPLC) are available for the separation of chemical substances, the classical techniques- paper chromatography and thin layer chromatography are still easy, can be set up even in an ordinary laboratory without much expenditure. It may be recalled that Calvin and his associates used paper chromatography to elucidate the pathway of carbon dioxide fixation in photosynthesis. The separation, identification and (semi) quantification of amino acids using paper Chromatography can also be used to separate other smaller molecules such as sugars, organic acids etc. by changing the mobile phase and detection (spray) agents.

The separation of the solutes (amino acids or sugars) is based on the liquid-liquid partitioning of amino acids or sugars in paper chromatography. The partitioning takes place between the water molecule (static phase) adsorbed to the cellulosic matter of the paper and the organic (mobile) phase.

3.7.1 SEPARATION OF AMINO ACIDS

Mobile phase (Solvent System)

Mix n-butanol, glacial acetic and water in the ratio 4:1:5 in a separating funnel and stand to equilibrate for 30 min. Drain off the lower aqueous phase into a beaker and place it inside to saturate the chromatography chamber. Save the upper organic phase and use it for developing the chromatogram.

Dissolve different individual amino acids in distilled water at a concentration of 1mg/mL. Use very dilute (0.05N) Hydrochloric acid to dissolve the free amino acids tyrosine and phenylalanine. Dissolve tryptophan in very dilute (0.05N) Sodium hydroxide.
Ninhydrin Reagent (spray reagent)

Dissolve 100mg ninhydrin in 100mL acetone.

Elution Mixture

Prepare 1% copper sulphate solution. Mix ethanol and copper sulphate solution in the ratio 80:20 (v/v).

Amino acids

Histidine, Phenylalanine, tryptophan, Isoleucine, Tyrosine.

Separation of sugars

Mobile phase (Solvent System)

1. Solvent 1- Water saturated Phenol + 1% Ammonia
2. Solvent 2- Butanol + Acetic acid + Water (4:1:5) (v/v)
3. Isopropanol-Pyridine-Water-Acetic acid (8:8:4:1) (v/v)

Sugars – Glucose, Fructose and Sucrose (Sadaaki Iibuchi et al., 1968)

Spray Reagents-

Ammonicle silver nitrate

Add equal volumes of Ammonium hydroxide to a saturated solution of silver nitrate and dilute with methanol to give a final concentration of 0.3M. After spraying the chromatograms, place them in an oven for 5 to 10 min, when the reducing sugars appear as brown spots.
Alkaline permanganate

Prepare an aqueous solution of Potassium permanganate containing 2 % Sodium carbonate. After spraying the mixture, the chromatograms are kept at 100° C for a few minutes, when the sugar spots appear as yellow in purple background.

Aniline diphenylamine reagent

Mix five volumes of 1 % aniline and five volumes of 1 % diphenylamine in acetone with one volume of 85 % Phosphoric acid. After spraying, the dried chromatograms with the solutions, the spots are visualized by heating the paper at 100° C for a few minutes.

Resorcinol reagent

Mix 1 % ethanolic solution of resorcinol and 0.2 N Hydrochloric acid (1:1 v/v). Spray the dried chromatograms and visualize spots by heating at 90° C.

PROCEDURE

1. The chromatography sheet was cut carefully to a convenient size (40x24 cm). A line was drawn with pencil across the sheet about 5 cm away from one end. A number of points were marked at intervals of 3 cm.
2. A small volume of sample (25μl) was applied as a separate small spot using a micropipette or micro syringe.
3. Different known aliquots of sample extract were placed on the spots.
4. After spotting, the sheet was rolled as a cylinder, tied together with fine thread and placed upright with the spots as the bottom in the chamber for upward movement of solvent. (Ascending chromatography).
5. The organic solvent was added to the chamber and the chamber was closed airtight. The chromatogram was developed, overnight or longer, until the solvent had moved almost to the other end.

6. The solvent front was noted and the chromatogram was dried to remove the solvent in a fume chamber.

7. The chromatogram was sprayed with ninhydrin (for amino acids)/Resorcinol (for sugars) reagent using an Atomizer. The paper was dried for about 5 min at room temperature followed by at 100°C in the oven for 2 to 3 mins. Amino acids appear as purple spots hydroxyproline and proline give yellow colored spots.

8. All the spots were marked and their Rf values (Relative front) were calculated by the following formula.

\[
Rf = \frac{\text{Distance (cm) traveled by solute form the origin}}{\text{Distance (cm) traveled by solvent form the origin}}
\]

9. Comparing the Rf values with that of the standard chart then identified the amino acids present in the sample.

3.7.2 SODIUM DODECYL SULFATE POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis is widely used to separate and characterize proteins by applying electric current. Electrophoresis procedures are rapid and relatively sensitive requiring only micro weight of proteins. Electrophoresis in the polyacrylamide gel is more convenient that any other medium such as paper and starch. Gel electrophoresis of proteins in polyacrylamide gels is carried out in buffer gels (non-denaturing) as well as in SDS containing gel (denaturing). Separation in buffer gels relies on both the charge and size of the proteins whereas it depends only upon the size in the SDS Gels. Analysis and comparison
of proteins in a large number of samples is easily made on polyacrylamide gel slabs.

Polyacrylamide gels are formed polymerizing acrylamide with a cross linking agent (bisacrylamide in the presence of a catalyst (per sulphate iron and chain initiator TEMED) N, N, N, N-tetramethy ethylene diamine) solution are normally degassed by evacuation prior to polymerization since O₂ inhibits polymerization. The porosity of the gel is determined by the relative proportion of acrylamide monomer to bis acrylamide gels which are usually referred to in terms of the total percentage of acrylamide and bis acrylamide present and most protein separation are performed using the gels in the range 7-15%. A low percentage gel (with large pore size) is used to separate high molecular weight protein and vice versa. At high concentration of persulphate and TEMED, the rate of polymerization is also high. Among a number of method commonly used, the sodium dodecyl sulphate (SDS) PAGE in slabs facilitating the characterization of polypeptides and determination of their molecular weight by co-electrophoresis is described below.

SDS is an anionic detergent, which binds strongly to and denatures proteins. The number of SDS molecule bound to a polypeptide chain is approximately half the number of amino acid residues in that chain. The protein-SDS complex carries net negative charges hence move towards the anode and the separation is based on the size of the protein.

REAGENTS USED

1. Separating gel buffer (pH 8.8)
   Tris HCL - 18.5gm
   Water - 75ml
2. Separating gel/Resolving gel

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamides</td>
<td>12.5ml</td>
</tr>
<tr>
<td>Separating gel buffer</td>
<td>6ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>0.3ml</td>
</tr>
<tr>
<td>Ammonium Per Sulfate (APS, 5%)</td>
<td>0.15ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.03ml</td>
</tr>
<tr>
<td>Water</td>
<td>11.53ml</td>
</tr>
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</table>

3. Stacking gel buffer (pH 6.8)

<table>
<thead>
<tr>
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<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCL</td>
<td>1.5 gm</td>
</tr>
<tr>
<td>Water</td>
<td>25ml</td>
</tr>
</tbody>
</table>

pH can be adjusted by adding 1N HCl

4. Stacking gel (4x)

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>1.66ml</td>
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<tr>
<td>Separating gel buffer</td>
<td>1.38ml</td>
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<tr>
<td>SDS (10%)</td>
<td>0.2ml</td>
</tr>
<tr>
<td>Ammonium Per Sulfate (APS, 5%)</td>
<td>0.2ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01ml</td>
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<tr>
<td>Water</td>
<td>6.86ml</td>
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5. Gel overlay solution

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>Separating gel buffer</td>
<td>12.5ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5ml</td>
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<tr>
<td>Water</td>
<td>37ml</td>
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6. Water saturated n-Butanol solution

<table>
<thead>
<tr>
<th>Material</th>
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</thead>
<tbody>
<tr>
<td>n-Butanol</td>
<td>9ml</td>
</tr>
<tr>
<td>Water</td>
<td>1ml</td>
</tr>
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7. **Tank buffer (pH 8.3)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Tris base</td>
<td>2.82gm</td>
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<tr>
<td>Glycine</td>
<td>14.4 gm</td>
</tr>
<tr>
<td>SDS</td>
<td>7gm</td>
</tr>
<tr>
<td>Water</td>
<td>1 Litre</td>
</tr>
</tbody>
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8. **Sample Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking gel buffer</td>
<td>1.25mL (pH 6.8)</td>
</tr>
<tr>
<td>Water</td>
<td>7.25ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1ml</td>
</tr>
<tr>
<td>Betamercaptoethanol</td>
<td>0.5ml</td>
</tr>
<tr>
<td>SDS</td>
<td>2ml</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

9. **Staining solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Brilliant Blue</td>
<td>0.025gm</td>
</tr>
<tr>
<td>Methanol</td>
<td>400ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>70ml</td>
</tr>
<tr>
<td>Water</td>
<td>530ml</td>
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</table>

10. **Destaining solution (1L)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
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<tr>
<td>Acetic acid</td>
<td>50ml</td>
</tr>
<tr>
<td>Water</td>
<td>880ml</td>
</tr>
</tbody>
</table>

**PROCEDURE**

1. Glass plates and spacers were thoroughly cleaned and dried, then assembled properly. The assembling was fitted together with bulldog clips clamped in an upright position. White petroleum jelly or 2% agar (melted in a boiling water bath) was then applied around the edges of the spacers to hold them in place and seal the chamber between the glass plates.
2. A sufficient volume of separating gel mixture (30mL) was prepared for a chamber of about (18 X 9 X 0.1 cm).

3. Immediately gel overlay solution was poured to form a layer over the separating gel.

4. A layer of distilled water or n-butanol solution was placed on the top of the gel overlay solution and let it set for 30-60 minutes.

5. Stacking gel (4%) was prepared (Total volume 10mL).

6. After the gel had set, the top layers (gel overlay solution and water saturated n-butane solution) were discarded and dried with blotting paper and washed with stacking buffer.

7. The stacking gel mixture was poured, and the comb was placed in the stacking gel and allowed the gel to set for 30-60 minutes.

8. After stacking gel has polymerized, the comb was removed without distorting the shape of the well and washed with stacking buffer. The gel was carefully installed after removing the clips, agar etc. in the electrophoresis apparatus. It was filled with electrode buffer and any trapped air bubbles were removed at the bottom of the gel and cathode was connected at the top and turned on the DC power briefly to check the electrical circuit.

9. The samples were prepared by dissolving in stacking buffer and heated (A general practice, heat sample solution in boiling water 2-3 minutes to ensure complete interaction between proteins and SDS).

10. The sample solution was cooled and a volume of 20μl was taken in a micro syringe and carefully injected into the sample well through the electrode buffer.

11. Turned on the current to 50mA for initial 10-15 minutes until the samples traveled through the stacking the stacking gel and continued the run at
100m A until the bromophenol blue reached the bottom of the gel (About 2 hours).

12. After the run was complete, the gel was carefully removed from the plates and immersed in staining solution for at least 3 hours or overnight with uniform shaking. The proteins adsorbed the Coomassie brilliant blue.

13. The gel was transferred to a suitable container with at least 200-300mL of destaining solution and shaken gently and continuously. Dye that was not bound to protein was thus removed. The destainer was changed frequently in the initial periods, until the background of the gel was colorless. The protein fractionated into a band was seen colored blue as the protein of minute quantities was stained faintly, destaining process was stopped at appropriate stage to visualize as many band as possible.

14. The gel was photographed and stored in polythene bag (may be dried in vacuum for a permanent record).

3.8.0 ESTIMATION OF PROTEIN BY LOWRY’S METHOD

Proteins can be estimated by different methods as described by Lowry and also by estimating the total Nitrogen content. No method is 100% sensitive, hydrolyzing the proteins and estimating the amino acids alone will give the exact quantification. The method developed by Lowry et. al., (1951) is sensitive enough it give a moderately constant value and hence largely followed, protein content of enzyme extracts is usually determined by this method. The blue color developed by the reduction of the phosphomolybdide and phosphotungstic components in the Folin- ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein and the color developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry’s methods.
Reagents

1. 2% Sodium carbonate in 0.1 N sodium hydroxide (Reagent A)
2. 0.5% Copper sulfate solution in 1% potassium sodium tartrate (Reagent B)
3. Alkaline Copper Solution – Mix 50 mL of A and 1 mL of B prior to use (Reagent C).
4. Folin-Ciocalteau Reagent (Reagent D)
5. Protein stock (Bovine Serum Albumin)

PROCEDURE

1. A stock protein solution (BSA, 10 mg/mL) was prepared.
2. From this stock, a series of standard solutions (50µg/mL to 500 µg/mL) was made in test tubes.
3. 5 mL of reagent C was added to test tubes, mixed well and allowed to stand for 10 min.
4. 0.5 mL of reagent D was added to all test tubes, mixed well and incubated at room temperature in the dark for 30 min.
5. Blue color was developed in all test tubes.
6. The tubes were removed from the dark room and the absorbance was read at 660 nm against the blank.
7. A blank was prepared using reagents only.
8. Similarly, the steps 3, 4, and 5 were repeated for unknown samples.
9. The known concentrations and OD (Optical Density) values were plotted on a graph.
10. The OD for unknown samples was noted on the graph.
11. From this, the concentrations in unknown samples were identified and the results recorded.
3.9.0 OPTIMIZATION OF ENZYME PRODUCTION

Optimization is of great importance in the production of any enzyme in industrial scale. Different parameter has to be optimized to get high yield and the economical feasibility is also to be considered. Various factors affecting microbial enzyme production are concentration of substrate, $p^H$, aeration, Temperature, complex medium, effect of co-substrate etc were optimized using surface culture response method (Lekha, et. al., 1961).

3.9.1 CONCENTRATION OF TANNIC ACID

Different concentration of tannic acid was examined for the optimization process. Concentrations such as 0.5%, 1.0%, 1.5%, 2.0%, 2%, 2.5%, 3.0%, 4.0%, of tannic acid were poured in to conical flask containing 50 mL of Czapeks Dox broth. It was then inoculated with respective fungal isolates and incubated at appropriate temperature for appropriate time. Control flasks were also kept without inoculation. The mycelial mat from different concentration was taken out, extracted and used as enzyme extract. It was tested for tannic activity and unit was measured.

3.9.2 TEMPERATURE

The effect of different temperature were examined for optimization; Fungal isolates in Czapeks Dox liquid medium were kept in temperatures such as $20^0$ C, $25^0$ C, $30^0$ C, $35^0$ C and $40^0$ C and incubated for 4-5 days. Control flasks were also kept without inoculation. After incubation enzyme was extracted and enzyme assay was carried out.
3.9.3 $p^H$

The $p^H$ of the medium was varied in order to find out the optimum $p^H$. Different $p^H$ was used such as 3, 3.5, 4, 4.5, 5, 5.5. The inoculated media were kept at appropriate temperature for 4-5 days. Control flasks were also kept without inoculation. After incubation enzyme was extracted and enzyme assay was carried out.

3.9.4 AERATION

Inoculated flasks were kept at both static and stirred condition (100 rpm) at appropriate temperature for 4-5 days. Control flasks were also kept without inoculation. Enzyme was then extracted and tannase assay was carried out.

3.9.5 EFFECT OF TIME OF INCUBATION

Inoculated fungal isolates were incubated for different incubation period such as 5 days, 7 days and 9 days. It was then examined for tannase activity.

3.9.6 CHI-SQUARE TEST

The $X^2$ test (pronounced as chi-square test) is one of the simplest and most widely used non-parametric tests in statistical work. The quantity $X^2$ describes the magnitude of the discrepancy between theory and observation. It is defined as:

$$X^2 = \sum \frac{(O-E)^2}{E}$$

Where $O$ refers to the observed frequency and $E$ refer to the expected frequencies.
Steps. To determine the value of $X^2$, the steps required are:

1. Calculate the expected frequencies. In general, the expected frequency $f$ from any cell can be calculated from the following equation:

$$E = \frac{RT \times CT}{N}$$

Where

- $E$ = Expected frequency
- $RT$ = The row total for the row containing the cell
- $CT$ = The column total for the column containing the cell
- $N$ = The total number of observations.

2. Take the difference between observed and expected frequencies and obtain the squares of these differences, i.e., obtain the values of $(O-E)^2$.

3. Divide the values of $(O-E)^2$ obtained in step (ii) by their respective expected frequency and obtain the total $E[O-E]^2/E$. This gives the value of $X^2$ which can range from zero to infinity. $X^2$ is zero means that the observed and expected frequencies completely coincide. The greater the discrepancy between the observed and expected frequencies, the greater shall be the value of $X^2$.

The calculated value of $X^2$ is compared with the table value of $X^2$ for given degrees of freedom at a certain specified level of significance. If at the stated level (generally 5% level is selected), the calculated value of $X^2$ is more than the table value of $X^2$, the difference between theory and observation is simple sampling. If, on the other hand, the calculated value of $X^2$ is less than the table value, the difference between theory and observation is not considered as
significant, i.e., it is regarded as due to fluctuations of simple sampling and hence ignored.

3.10.0 IMMOBILIZATION OF ENZYME

The immobilization of the enzyme was carried using sodium alginate by following the method of Kierstan and Bucke (1977).

PROCEDURE

1. 4.0 g sodium was dissolved in 100 mL 1% saline and heated till sodium alginate dissolves.
2. After the preparation of 4% sodium alginate solution was cooled to 37°C, the purified enzyme is added to it aseptically and it is mixed well.
3. The mixture/suspension was passed through a narrow tube or injector drop wise into 2% calcium chloride (50 mL) pH 6.0 solution.
4. The beads obtained are incubated in CaCl₂ at 20-22°C for 2 hours.
5. The beads are then transferred to a bead-stabilizing medium (5% glutaraldehyde) for a few hours and stored at 4°C and used whenever needed.

The activity of the immobilized enzyme was measured.

3.11.0 SUBMERGED FERMENTATION

Submerged fermentation involves the growth of the microorganism as a suspension in liquid medium in which various nutrients are either dissolved or suspended as particulate solids. Since the sterilization and process control are easier to engineer this system, it is the prepared method of production of most of
the commercially important enzymes. The submerged fermentation production was carried out according to Pourrat et. al., 1982

3.11.1 MEDIA COMPOSITION

The fermentation medium must meet the nutritional requirements of the organism. As tannase is an inducible enzyme, tannic acid itself was used as the role carbon source as well as an inducer. Tannic acid liquid medium. The tannic acid medium was prepared and used.

Composition of TAA medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.0g</td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>2.0g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2g</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>0.02g</td>
</tr>
<tr>
<td>MnCl$_2$.6H$_2$O</td>
<td>0.004g</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
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</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.0025g</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000mL</td>
</tr>
</tbody>
</table>

The medium was autoclaved at 121°C for 20 min and filter sterilized tannic acid was added.

3.11.2 INOCULUM PREPARATION

The spores of the cultures were inoculated into 250mL of sterilized TAA medium taken in a 500mL flask. The flask was inoculated at 30°C and left undisturbed so that a surface mar of mycelium formed. This layer was sporulating strongly by day 3.
After 7 days, the mycelium was harvested under sterile conditions, ground and homogenized in 50mL of sterile water working in a laminar flow ventilated box 50mL placed in a dean room. After addition of sterile glycerol, the suspension was stored in refrigerated and used whenever needed. A spare concentration of 2 X 10^7 spores/mL suspension was used for inoculation.

3.11.3 FERMENTATION CONDITION

Submerged cultures were grown in 3.5l Applikon B.V, Netherlands fermenter in 1.5 liter medium. 20mL of inoculums were used.

3.11.4 pH

The pH of the fermentation medium were maintained at 5.5 with automatic regulation using aqueous ammonia and orthophosphoric acid.

3.11.5 TEMPERATURE

The fermentation temperature was maintained at 30°C by the internal circulation of water.

3.11.6 DISSOLVED OXYGEN

The Fermentation is carried out by shirring at 400 rpm aeration was at constant dissolved oxygen regulated at 30%

The Variations in the temperature, pH, stirring, and aeration were recorded throughout the fermentation run. Samples were removed at 24 hr intervals.
3.12.7 TANNASE ASSAY

Biomass was separated by filtration through what man No.1 filter paper. The cell free culture broth was assayed for extra cellular tannase activity. The fungal cells were used for intra cellular tannase assay.

3.12.0 SOLID STATE FERMENTATION

The SSF process can be defined as a four-phase system. The continuous phase is air (or another gas mixture) that usually flows through a solid bed. This solid is composed of a water-insoluble support, which contains an aqueous solution of nutrients. This solution is highly absorbed within the matrix of the insoluble support and no drainage is observed. The fourth phase is the microorganism, which grows inside the support and/or on its support and/or in the interparticulate free space (Lehka and Lonsane, 1994).

3.12.1 INOCULUM PREPARATION

The isolate were maintained on tannic acid agar (TAA) slopes, stored at 40°C and sub cultured every alternate month.

The composition of TAA Medium

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1.0g</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>2.0g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.2g</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
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<tr>
<td>MnCl₂.6H₂O</td>
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<td>Na₂MoO₄.2H₂O</td>
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<tr>
<td>FeSO₄.7H₂O</td>
<td>0.0025g</td>
</tr>
</tbody>
</table>
Materials and methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

The medium was autoclaved at 121°C for 20 min and after filter sterilized tannic acid solution was added to provide a final concentration of 2% (W/o).

3.12.2 INOCULUM PREPARATION

The inoculum was prepared according to the method reported by (Lekha and Lonsane 1994). The culture was grown on Tannic acid agar medium (TAA) plates at 30°C for 7 days. The spores were scrapped into 10mL sterile 0.01% Tween 80 solution in distilled water. The number of spores was counted in a Neubauer chamber. A spore concentration of 2 X 10^7 spores/g dry weight of the substrate was used for inoculation.

3.12.3 SOLID STATE FERMENTATION PRODUCTION

The solid fermentation process involved the use of Sorghum straw, and Paddy stress the commonly available agricultural by-products of Tamil Nadu as an inert support for absorbing the liquid medium.

The SSF process was carried out according to the method described by Lekha and Lonsane (1994).

1. The support media were cut into small pieces about 1 cm length after removing the leaves. Only the stem portions were used for study.
2. The inert were washed and processed using the technique described by Saucedo-Castaneda et. al., (1992).
3. 10g of support material were transferred into 500mL capacity Erlenmeyer flasks along with 10mL liquid medium, without tannic acid mixed thoroughly.

4. The above preparation was sterilized by autoclaving it at 121°C for 20 minutes.

5. Filter sterilized medium (10mL) containing of appropriate amount of tannic acid was added to the sterilized moist solids and mixed thoroughly.

6. The medium was inoculated with 2.5mL of spore suspension (2 X 10^7 spores/mL suspension) and incubated at 30°C statically in an inclined position.

7. After seven days of incubation, the contents were removed and used for enzyme study.

### 3.12.4 ENZYME LEACHING

Solid-state fermentation process gives highly concentrated products many times higher product titers as compared to conventional SMF process. The enzyme leaching was carried according to the method described by Lekha and Lonsane (1994).

1. About 5g-fermented moist solid were removed from each flask and taken in a separate conical flask.

2. The fermented materials were added with 50mL of 1.5% aqueous solution of NaCl.

3. The conical flasks were agitated rotary shaker at 220 rpm for 2hrs.

4. After agitation, the solid particles with adhering fungal cells were separated using what man No1 filter paper.

5. The filtrate obtained was centrifuged at 5000g for 20minutes.

6. The clear upper supernatant was used for extra cellular tannase assay.
7. The remaining solids were subjected to recovery of intracellular enzyme as described above.

3.12.5 DETERMINATION OF MOISURE CONTENT

Microorganisms have an absolute demand for water, for without water no growth can occur. The moisture content of the moist solid medium in solid orate fermentation indicates the total amount of water present in the system. The weight of known quantity of the moist solid is taken in the beginning. Then the moist solids were dried at 90° C overnight to determine the moisture content.

3.13.0 ASSAY OF TANNASE PRODUCTION IN SmF and SSF

The new colorimetric assay method based on the specific substrate optical density developed by Mondal et. al., (2001) is followed for tannase assay.

REAGENTS USED

Substrate: Commercial tannic acid (C_{76}H_{50}O_{46}) E.Merck, India) with a concentration of 0.5g% (W/v) in 0.2m acetate buffer pH 5.5 was used as substrate.

Bovine Serum Albumin Solution

A 1mg/e bovine serum albumin was prepared with 0.17mm sodium chloride in 0.2m acetate buffer (pH 5.0)

DS - Triethanolamine Solution

A 1%(w/v) SDS (BDH, India) Solution containing 5% (v/v) of triethanolamine (E.Merck) was prepared in distilled water.

FeCl₃ reagent

FeCl₃ reagent was prepared with 0.01 M FeCl₃ 0.01 N Hydrochloric acid.
**PROCEDURE**

1. To 0.31mL of substrate tannic acid, 1mL of the crude enzyme was added and incubated at 30°C for a defined period.
2. To stop the enzymatic reaction, 3mL BSA solution was added. The BSA precipitates the remaining tannic acid.
3. The reference sample was prepared in the same way with heat-denatured enzyme.
4. Standard curve was prepared by using different concentrations of tannic acid and a constant volume of denatured enzyme preparation in the same way as described above.
5. The blank was prepared without tannic acid.
6. The tubes were centrifuged at 5000g for 5 minutes.
7. The resultant precipitate was dissolved in 3mL SDS triethanolamine solution.
8. Then 1mL of Fecl₃ reagent was added and kept for 15min for stabilization of co lour.
9. The absorbencies of all the tubes were measured at 530nm.

The specific extinction coefficient of tannic acid at 530nm 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 1mm of substrate tannic acid in 1 min under assay conditions.

**3.14.0 MICROBIAL TREATMENT OF TANNERY EFFLUENT**

The microbial treatment of tannery effluent was carried out by the methodology described by Abadulla et. al., (2000).
The different fungal isolates were grown in Czapek Dox's minimal both which contains tannic acid as a carbon source in a 500 mL conical flask 30°C for 3 days

1. The mycelium were collected by filtration under aseptic condition and washed thrice with 250 mL of sterile distilled water.
2. Samples of mycelium (2g) were inoculated to the effluent and incubated for 5 days under aerated condition.
3. Sterile controls without inoculum were also maintained under the same conditions

After the incubation, the samples were taken and analyzed for the changes in physicochemical characters like BOD, COD chloride, sulfate and tannin content. The treated and raw samples were also subjected to Ame's rest mutagenicity or carcinogenicity determination.

3.14.1 DETERMINATION OF METABOLIC ACTIVITY

The changes in physicochemical characters and mutagenicity of the treated effluent by the fungus were confirmed by the following experiment.

1. Two sets of experimental set up were maintained, one with the fungi and the effluent and another with the same along with the antibiotics, which inhibit the metabolism of fungi.
2. A mixture of 100 mg benylate liter⁻¹, 1000 mg cyclohexamide liter⁻¹ and 300 mg streptomycin antibiotic solution liter⁻¹ were prepared.
3. A volume of 10%(v/v) of this solution was added to the one set incubation mixtures.
4. After 5 days of incubation under the above said same condition, all the mixtures were taken and subjected to the physicochemical character and tannin analysis.

3.14.2 ENZYMATIC TREATMENT OF TANNERY EFFLUENT

The enzymatic treatment of the effluent is carried out by the following way.

1. 10 mL of the supernatant of the culture filtrate/mycelial extract was added to 250mL of the effluent taken in a conical flask.
2. The enzyme activity of the effluent was determined previously.
3. 10 mL of the enzyme supernatant was immobilized with alginate as described earlier.
4. The alginate beads were added to the of 250mL of effluent taken in a 500 mL conical flask.
5. All the flasks were incubated on a rotary shaker at 30°C for 3 days.
6. Controls were prepared by heat inactivating the enzyme at 80°C
7. After incubation, the physicochemical characters of the effluent were analyzed and recorded.