3.1 CHEMICALS:

The synthetic lignin buys up from Sigma. The entire solutions were ready in double distilled water.

3.2 CULTURE MEDIA AND GLASSWARES:

All the culture media used in the work were procured from Hi-Media (Mumbai), Qualigens (Mumbai were), Sd-fine Chemicals (Mumbai) or abroad [Sigma-Aldrich (USA), E. Merck (Germany)]. All the glassware’s used in the study were purchased either from prominent dealers of Duran (Germany) or Borosil (India).

3.3 MINERAL SALT MEDIUM:

Mineral salts medium (MSM) was used throughout the study with the following composition (g/l deionised water): Di-potassium phosphate 1.8; Disodium phosphate 2.0; Ammonium nitrate 0.2; Magnesium sulfate 0.05; Glucose, 1%; Calcium chloride 0.05; peptone, 0.5%; and pH 8.0.

3.4. SAMPLING SITE AND SAMPLE COLLECTION:

The wastewater was collected from M/s. Century Pulp Paper Mill, Uttrakhand, India and M/s Anand paper mill (APM), located at Meerut, Uttar
Pradesh, India in June 2011. The collection was taken into container. For bacterial isolation the sludge sample was collected in sterilized (121°C and 15 minute) cotton plugged cans.

3.5. PHYSICO-CHEMICAL ANALYSIS OF COLLECTED EFFLUENTS:

The effluent sample were analyzed in laboratory for various parameter i.e. Total Solid, Total Suspended Solid, Total Nitrogen, phosphate, pH, heavy metals, Total Dissolve Solid, phenol sulphate, color, COD, BOD and lignin (APHA, 1998), chlorophenols, nitrate, potassium, chloride, sodium and carbon dioxide by following methods:

3.5.1. pH:

Prior to pH measurement the electrode was calibrated in different buffer solution. The pH of wastewater samples were calculated by a pH electrode subsequent the manufacturer”s lessons.

3.5.2. Measurement of color (CPPA, 1974):

(1) **Color estimation (CAPA standard method)**

Take sample (pH-7.6) centrifuge it. Take O.D. by spectrophotometer at 465 nm using D.W. as a blank control.

**Calculation:**

\[ \text{CU} = 500 \times \text{Optical Density at 465 nm} \]

\[ \text{Optical Density of 500 Copt at 465 nm} \]
Note: for standard preparation of 500 Copt: dissolve 1.246 gm K$_2$PtCl$_2$ and 1 gm CoCl$_2$ x6H$_2$O in 100 ml in concentrated HCl & dilute up to 1 lit.

(2) Visual comparison method (APHA, 1998):

This method was followed for the measurement of color intensity in the different sample of paper mill effluent during the whole experiment.

Methodology:

A 50 ml Samples were filled in Nessler tube. The Observed sample color was capering with a series of standard which are also filling in Nessler tubes.

Since treated paper mill effluent after final treatment has very high color so it was diluted 100 times to bring the color within range of the standards. The pH of effluent was noted 7.5.

Calculation:

Calculated color units by the equation:

\[
\text{Color units} = \frac{D \times 50}{O}
\]

Where, \(D\) = estimated color of a diluted sample

\(O\) = ml sample taken for dilution
Figure 3.1: View of effluent discharge of M/S Anand Paper Mill (A); effluent collection from near the disposal site (B); effluent collection from Century Paper Mill (C) and showing apparent color demarcation after mixing of effluent to river water (D).

3.5.3. Estimation of lignin (APHA, 1998, 5550 B):

Principle:

Lignin contain aromatic hydroxyl group that counter with folin phenol and form colored products (blue color) which are apt in the measurement of lignin.
Apparatus:

Spectrophotometer and glassware’s.

Reagents:

- Folin phenol regent: commercially prepared folin phenol reagent of Merck was used before the recommended expiration date.
- Carbonate tartrate reagent: dissolved 200g Na$_2$CO$_3$ and 12 g sodium tartrate, 1 750ml distilled water, cool to 20°C, and dilute up to 1000 ml.
- Stock solution: weighed 1 gm lignin in one liter distilled water and used it as the stock solution.
- Standard solution: Different standard solutions were also prepared using lignin stock solution.

Procedure:

5 ml portion of effluent sample and standard solution was taken in to 10 ml test tubes and brought it to a temperature above 20°C and maintain within a ±2°C range. Added rapidly .1 ml folin phenol reagent and 1 ml carbonate reagent. Allowed 30 min for color development and measured O.D. (optical density) at 700 nm.

Calculation:

- Plotted standard curve of lignin with known values at 700 nm.
• From the standard curve of lignin at 700 nm calculated the value of unknown samples.

3.5.4. **Total solid (TS) (APHA, 1998, 2540 B):**

**Apparatus:**

Evaporating dish: - Dish of porcelain (90 mm diameter) 100 ml capacity.

Dessicator

Drying oven

Analytical balance

**Procedure:**

• Taken a dry pre wt. beaker.

• Taken 25ml sample in a beaker and dry it in an oven.

After drying wt. the beaker then

\[ \text{After wt. - Pre wt.} = \text{T.S.} \]

Total Suspended Solids = Total solid – Total dissolve solid.

\[ \text{Total solid mg/l} = (a-b) \times 1000 \]

Sample vol (ml)
3.5.5. *Total dissolved solid (TDS) (APHA, 1998, 2540 C):*

**Procedure:**

Filtrated sample i.e., supernatant was taken in a dry and pre-weighed crucible. Kept the samples in an oven for evaporation of liquid content at 180°C. After drying, cooled it in desiccators to balance temperature and weight.

**Calculation:** Total dissolves solid (T.D.S) estimation:

\[
\text{Total dissolve solid mg/l} = \frac{(a-b) \times 1000}{\text{Sample vol (ml)}}
\]

Note: Total suspended solid = TS-TDS

3.5.6. *Total suspended solid (TS) (APHA, 1998, 2540 D):*

**Procedure:**

Measured total solid (TS) and total dissolved solid (TDS) as mentioned above.

**Calculation:**

\[
\text{TSS} = \text{TS-TDS}
\]
3.5.7. *Biological oxygen demand (5-day test, APHA, 1998, 5210 B)*:

Biochemical oxygen demand (BOD) of the effluent was determined by 5 days test using azide modification method for measuring dissolved oxygen as per standard method of water and waste water analysis (APHA, 1992).

**Requirements:**

- **Alkali azide:** dissolve 500gm NaOH (or 700 gm KOH) & 135 gm NaI (150 gm KI) in D.W. & dilute up to 1 lit. add 10 g Sodium azide in forty ml of water.
- **Starch solution (2 %)**
- **Sodium thio-sulphate:** Dissolve 6.205 g sodium thio-sulfate in D.W. add one and half (1.5) ml 6 N sodium hydroxide or 5 g sodium hydroxide solid and dilute up to one liter.
- **MnSO4:** dissolve 480gm / 400gm/ 364gm MnSO4·4H2O/ MnSO4·2H2O/ MnSO4·H2O in D.W., filter & dilute to 1 lit. The MnSO4 should not give a color with starch when added to an acidified potassium iodide solution.

**Methodology:**

The different diluted sample was divided into three bottles each containing 300 ml in triplicate.

The initial D.O. for sample and control as follows.
In a 300ml BOD bottles filled with sample/control water

Add 1.0ml MnSO4 solution then Add 1ml alkali iodide-azide solution.

The content was mixed by inverting the bottles several times.

When the precipitate settled sufficiently, 1ml conc. H2SO4 was added to dissolve the flock by inverting the bottles several times. It was titrated against

0.025M Na2S2O3 solutions till a pale straw color was developed.

The few drops of starch were added and the titration continued till the Disappearance of blue color.

Both the control and samples were kept at 20 °C for 5 day incubation
Period and dissolve oxygen measured after incubation.

**Calculation:**

\[
\text{BOD of sample} = \frac{(A-B)-(C-D) \times \text{D.F}}{P}
\]

Where, \(A\) = D.O. of 0 day sample; \(B\) = D.O. after 5 day incubation; \(C\) = D.O. of 0 day sample; \(D\) = D.O. after 5 day incubation; \(\text{D.F}\) = Dilution factor, \(P\) = Decimal volumetric fraction of sample used.

### 3.5.8. Chemical oxygen demand (Open reflex method, APHA, 1998, 5220 B):

**Reagents:**

- Potassium dichromate solution 0.1N
- Ferrous ammonium sulphate (FAS) solution 0.1N
- Sulfuric acid
- Indicator solution
- Mercuric sulphate: A big pinch

**Procedure:**

Took 20 ml sample in a 250 ml refluxing flask.
Added 10ml K$_2$Cr$_2$O$_7$, 0.4 g HgSO$_4$, 0.4 g AgSO$_4$ and 30 ml conc. H$_2$SO$_4$ slowly and cooled the mixture.

Refluxed the sample mixture for 2 hours.

Disconnected reflux condenser and diluted mixture by adding 100 ml Distilled water.

This refluxed mixture left for one hour for cool down up to 30 °C.

Add 2-3 drops Ferro in indicator titrate

Titrate the extra K$_2$Cr$_2$O$_7$ with FAS.

Color was changes from blue to reddish brown.
The radish brown was taken as end point of the titration.

Exactly in same manner, refluxed and titrated a blank containing the reagent and a volume of distilled water which are similar to that sample.

**Calculation:**

\[
\text{COD (mgO}_2/\text{l}) = (A-B) \times M \times 8000
\]

Sample (ml)

Where,

- **A** = amount of FAS in ml used for blank,
- **B** = amount of FAS in ml used for sample,
- **M** = Morality of FAS

3.5.9. *Sulphate estimation (Gravimetric method, APHA, 1998, 4500-SO}_4^{2-}):

The sulphate content in effluent was determined by drying of residues
which are based on gravity separation technique.

**Principle:**

In acidic solution sulphate is participated by adding a conditioning reagent named \( \text{BaCl}_2 \). Barium sulphate is precipitated from which concentration of barium is determined.

**Reagents:**

- Activated charcoal powder.
- Conditioning reagents: 50 ml concentrated HCl added with three times more distilled water and 75 g sodium chloride after that 1000 ml 95% isopropyl alcohol is added.
- Barium chloride (\( \text{BaCl}_2 \)) crystal: 1 to 2 grams.

**Procedures:**

Firstly take 100ml samples pass it through a column of activated charcoal for removing its color.

After 24 hour filtered the sample and added 4ml-conditioning reagent. Shake well.
Add 1 or 2 g of barium chloride, precipitated of barium sulphate is formed.

Centrifuged at 5000 rpm for 30 minutes.

Pellet is formed, dried it in an air dried oven and weighed it.

Calculations:

Sulphate (mg/l) = \( \text{wt. G ppt} \times 411.6 \)

Sample taken (ml)


Phosphate present in wastewater released by paper industry was analyzed by stannous chloride method.

Principle:

Molybdophosphoric acid formed is reduced by stannous chloride to intensity colored molybdenum blue and the intensity of color formation is directly proportional to the amount of phosphate.
Reagents:

- Phenolphthalein indicator solution
- Strong acid solution: 300ml conc. H$_2$SO$_4$ was slowly added to about 600ml distilled water. Then 4.0 ml conc. HNO$_3$ added.
- Ammonium molybdate (AM) reagent

  1. Dissolve 25 g AM in 175 ml distilled water
  2. Add 280 ml con H$_2$SO$_4$ to 400 distilled water and cool it.

  Add 1 and 2 and dilute up to one lit.

- Stannous chloride reagent: 2.5 g SnCl$_2$ H$_2$O added to 1000 ml glycerol headed with constant stirring in a water bath.

**Note: solution should be transparent**

Methodology:

Take 100 ml colorless sample and add 4 ml ammonium molybdate reagent and 10 drop stannous chloride reagent.

After 10 minutes and before 12 minutes the intensity of color was read.
at 690 nm at specific interval for all determination.

Standard calibration curve was plotted using 10, 20, 30, 40, 50 mg/L of phosphates and developed color was measured.

**Preparation of standards:**

Standard calibration curve was plotted using 10, 20, 30, 40, 50 mg/l of phosphates and colored developed measured.

**3.5.11. Total nitrogen (APHA, 1998, macro-kjeldahl method 4500 B):**

**Principle:**

Sulphuric acid and potassium sulphate in presence of FeSO$_4$ converts into amino nitrogen to organic material to materials to ammonium sulphate [(NH$_4$)$_2$SO]. Free ammonia and ammonium nitrogen is also converted to (NH$_4$)$_2$. During sample digestion a mercury ammonium complex is formed and then decomposed by sodium thiosulphate. The ammonia is measured colorimetrically or by titration with standard mineral acid by the classic kjeldahl method.
Reagents:

- Mercuric sulphate solution; 8gm red mercuric oxide HgO in 100 ml 6N H$_2$SO$_4$.

- Digestion reagent; dissolved 134 gm K$_2$SO$_4$ in 650 ml water and 200 ml conc. H$_2$SO$_4$. Added string 25ml mercuric sulphate solution. Dilute the combined solution to 1 liter water keep at temperature close to 20$^0$C to prevent.

- Sodium hydroxide sodium thiosulphate reagent; dissolved 0.5 kg of sodium hydroxide and 25 gm of Na$_2$S$_2$O$_3$.

- Standard sulphuric acid solution; 0.02 N.

- Phenolphthalein indicator; dissolved 5gm phenolphthalein in 500 ml isopropyl alcohol in 500ml-distilled water.

- Mixed indicator solution;

Procedure:

Took 5 ml sample and added to 10 ml of digestion mixture.

Digested the sample on the hot plate under a fume exhaust apparatus.

After digestion made up the sample to 200 ml with water.
After that additional 25 ml sodium thiosulphate solution

Add 1 ml phenolphthalein in indicator.

Distilled the sample and collected the distillate in another conical flask containing 50 ml boric acid solution as indicator.

After distillation titrated the sample with 0.02N $\text{H}_2\text{SO}_4$ until indicator turns pale lavender.

Calculation:

Total Nitrogen mg/l = \( \frac{A-Z \times 280}{\text{Sample (ml)}} \)

Where,

\( S = \) sulfuric acid titrated for sample in ml

\( B = \) sulfuric acid titrated for the blank
3.5.12. Chloride estimation:

The presence of chloride in the effluent releases from pulp-paper mill was measured by auto ion analyzer (Thermo Orion-960) using respective ion electrodes by following methods.

Methodology:

Taken 50 ml distilled water into 150 ml beaker and added 1 ml I.S.A (Ionic Strength Adjuster). Calibrated the system through continuous stirring, until stable displayed of standard on meter, if reading was slope = 56 MV/dec, will indicated the calibration of system. Saved the data in memory, for use now and at any time in future similarly effluent sample was prepared and analyzed.

3.5.13. Potassium estimation:

Methodology:

Calibrated the system by adding 50 ml distilled water and 1 ml I.S.A. in 150 ml beaker. After calibration the sample was prepared same and analyzed.

3.5.14. Sodium estimation:

Methodology:

We take 50 ml distilled water and mixed with 5 ml I.S.A. Adjusted the pH to between 9-10 using sodium ISA (Orion Cat no 8411111). Placed the electrode in the given standard solution. Similarly the sample of effluent was prepared and analyzed.
3.5.15. **Nitrate estimation:**

**Methodology:**

Taken 20 ml distilled water and equal volume of nitrate meddling suppressor solution. Stirred thoroughly, rinse the electrode and placed in the solution and hang around for a steady interpretation to demonstrate the value of standard. Similarly the effluent sample was prepared and analyzed.

3.5.16. **Carbon dioxide estimation:**

**Methodology:**

Take 100 ml beaker and filled with 45 ml of de-ionized water. Add 5 ml buffer (carbon dioxide buffer) and adjusted the to the mV mode in meter. The electrodes washed with de-ionized water and placed in the solution. Pipette out 0.5 ml calcium carbonate from the stock solution 1000 mg/l and kept in a beaker and mixed properly. If the reading is stable on display unit than it record the reading this reading is express in millivolts.

3.5.17. **Metal analysis (plasma emission spectroscopy-inductively coupled plasma method, APHA, 1998, 3120 B):**

The sample was digested using digestion mixture (6:1; nitric acid and per chloride acid) at 60°C. The procedure is repeated until dark brown fumes are formed and solution becomes dry. The residue is cooled and dissolves with distilled water and is then analyzed for metals. The sample was read on inductively coupled atomic spectrophotometer at specific wavelength for
copper, chromium, cadmium, lead, nickel, zinc manganese and iron.

**Reagents:**

1. Nitric acid, HNO$_3$
2. Perchloric acid, HClO$_4$

**Procedure:**

A measured volume of sample (5 ml) was digested, in a beaker,

Add 5 ml digestion mixture (6:1 mixture of HNO$_3$:HClO$_4$).

This solution was then cooled, diluted with distilled water up to 200 ml volume and mixed thoroughly.

Further, took portions of the digested sample for required metal determinations through inductively coupled plasma (ICP) emission spectroscopy.
3.5.18. Total phenol (Chloroform extraction method, APHA, 1998, 5530):

Reagents:

- Standard phenol solution; 10, 20, 30, 40, 40, 50, 60-microgram/l phenol solutions were prepared in distilled water.
- Stock phenol solution; dissolved 100mg phenol in 100ml.
- Bromate bromide solution; dissolved 1.784g anhydrogen KBrO3, 10 g KBr crystal in 1000ml-distilled water.
- Standard sodium thiosulphate titrant; dissolved 6.205g Na₂S₂O₃, 5H₂O, 0.4 g solid NaOH in 1000 ml distilled water.
- Standard potassium iodate solution;
- 0.2 % Starch solution.
- Ammonium hydroxide; freshly prepared 0.5N solution.
- Phosphate buffer solution; dissolved 104.5g K₂HPO₄ and 72.3 g KH₂PO₄ in 500 ml distilled water and make up to one liter at pH 6.8.
- 4 amino anti-pyrene solution; dissolved 2 g 4-amino antipyrene solution in 1000 ml distilled water.
- Potassium ferricyanide solution; Dissolved 8 g K₃Fe (CN)₆ in 50 ml of de-ionized water and then make up to 100 ml.

Procedure:

Adjusted the pH of effluent sample to 2.3 with 11 NH₄Cl.
Took 250 ml sample in a separating funnel add 25 ml chloroform, shake vigorously and removed oil and grease.

Added 4 drops orthophosphoric acid + two to four drops of indicator and distilled the sample.

After distillation 3 ml 4-amino antipyrene + 3 ml K$_3$Fe(CN)$_6$, Wait 15 minutes

Added 25 ml CHCl$_3$ in separating funnel extracted phenol which is soluble in chloroform (Yellow color).

Took OD at 460 nm.

**Calculation:**

Plotted standard curve of phenol were plotted with known standard value of phenol at 460 nm. From the standard curve of phenol at 460 nm
Calculated the value of unknown sample of phenol.

3.5.19. Chlorphenols analysis:

Extraction of chlorphenols from wastewater released by Kraft and pulp industry were performed by a simple modification of the procedure used by Chandra et al. (2011) where 200 ml effluent was taken and acidified up to pH 1-2 with sulfuric acid. Further, chlorophenols was extracted with 60 ml ethyl acetate thrice in separating funnel. The upper layer was collected and purged through flow on Na₂SO₄ to remove H₂O and dry at 50 °C. The deposit was dissolved in 1 ml acetonitrile and fractionated with HPLC (Water make-515). A 20 ml samples was introduced followed by accomplishment of 70:30 v/v solvent and acetonitrile at 1ml/min. Reverse phase C-18 column of particle size 5μm were used to separate the component at 320 nm absorbance. Identification of the compound was performed by assessment of Retention value.

3.6. ISOLATION OF BACTERIAL STRAINS:

The sludge in which bacteria were isolated was analyzed for various physico-chemical parameters. The effluent generated from paper industry contains a vast number of organic contaminants, which are afterward going through activated sludge process for the number of process. That type of sludge remains rich with organic contents. That why we used this type of sludge for the bacterial isolation.
Enrichment method is one of the most powerful techniques available to the microbiologist to isolate the bacteria from mixed culture from nature. An almost infinite number of combinations of the different environment factors for nutritional and physical can be developed for the specific isolation of microorganisms from nature. Nutrient enrichment technique specifies the isolation of specified bacteria from nature, by taking advantages of their specific nutrient requirements.

In this method, carve out a portion of activated sludge, transferred aseptically to sterilized flasks containing lignin and chlorophenols as growth medium. This flask was kept in incubator at 30°C at 140 rpm for seven days.

Figure 3.2: Flow chart of serial dilution of sludge sample for isolation and purification of bacterial strains.
After completion of incubation period sample analyzed and if samples showing a significant reduction of color and other pollution parameters than we diluted and spread on fresh media and incubated in dark at 30 °C for 7 days as shown in Fig. 2.2. Eleven bacterial strains with different colony morphology were pulled out and purified by frequent sub-culturing on agar plate. Further the purity of individual stain was checked by microscope.

3.7. SCREENING OF POTENTIAL BACTERIAL STRAINS FOR LIGNIN DEGRADATION.

a) On MSM-agar plates:

To select the potential lignin-degrading bacterial strains, screenings were carried out on lignin and PCP amended agar plate;

- Medium where lignin was the only source of energy (Nitrogen and carbon)

- Medium containing increasing the level of lignin along with PCP supplemented with simpler carbon and nitrogen sources like glucose and peptone.

b) On MSM-broth:

In MSM broth medium bacterial strains may not be able to develop by using lignin as sole source of energy. They need an additional nutritional source in a simpler form which accelerates their growth. Therefore, bacterial screened in different nutrient in order to get lignin degrading bacterial strains. The mineral salt medium broth containing 600 ppm of lignin was
Supplemented with either 0.5% peptone as nitrogen or 1% glucose as a carbon source. In an additional set of experiment both 1 % glucose as carbon and 0.5 % peptone as nitrogen source were added at the same time. The growth and color reduction were measured after 6 days of incubation period.

3.8 CHARACTERIZATION OF BACTERIAL STRAINS:

Three bacterial strains isolated and screened by nutrient enrichment method from sludge which are affected by pulp wastewater. These three bacteria were subjected into biochemically characterization in the laboratory (Barrow and Feltham, 1993). The biochemical test methodologies are as follows:

3.8.1. Gram’s staining (Barrow and Feltham (1993) :

Gram’s staining method was discovered over 100 years ago by Hans Christian Gram. This method is most commonly used for direct microscopic examination of specimens and subculture.

Principle:

The Gram-negative bacteria are in general thinner cell wall than Gram positive bacterial strains. Experimental evidences suggested that during destaining of gram-negative bacteria with alcohol extract the lipid, which results increased porosity or permeability of the cell walls. Thus the crystal violet complex can be extracted and the Gram-negative organism is decolorized by alcohol.
**Procedures:**

- Made a thin smear of the material for study and allow to air dry.

- Fix the material to the slide by passing the slide 3 or 4 times through the flame of a Bunsen burner so that the material does not wash off during the staining procedure.

- Placed the smear on a staining rack and overlaid the surface with crystal violet solution.

- After a minute less time of exposure to the crystal violet cleaned with de-ionized water.

- Overlaid smear with iodine (Gram’s iodine) for 1 minute. Wash again with de-ionized water.
Hold the smear between the thumb and forefinger and flooded the surface with a few drops of the acetone alcohol decolorize until no violet color wash off. This usually takes 10 seconds or less.

Washed with running water and again place the smear on the staining rack. Overlaid the surface with safranin counter stain for 60 second and washed

Placed the smear in an upright position in staining rank, allowing the excess water to drain off and the smear to dry.

Examined the stained smear under the 100 X (oil immersion) objective of the microscopes, gram positive bacteria appear dark blue, while gram negative bacteria appear pink or red.

**Interpretation:**

Bluish violet colored cells – Gram Positive
Red or pinkish colored cells – Gram negative

3.8.2. Motility test: (Barrow and Feltham (1993))

Bacteria motility is another important determinant in deciding final genus identification.

Principle:

Flagella are the motile organ of Bacteria. Motility was tested in motility media, which is semi-solid in nature. If bacteria are motile it moves in semi-solid agar, which is visualized as bulky mass of growth.

Composition is as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>80 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>4 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Procedure:

Pure young broth cultures of the organism incubated at and below the optimum temperature (37°C and 22°C) prepare the test media. The test culture was stabbed with straight platinum wire into semi solid motility medium up to depth of 5 mm and incubated at 30°C.

Interpretation:

The cloudy appearance around the stab after overnight incubation indicated motility of organism.

3.8.3. Anaerobic growth: (Barrow and Feltham (1993))

Principle:

Microorganisms, which are grown in the presence of oxygen is called aerobic microorganisms and in absence of oxygen microorganisms have capability to grow are anaerobic microorganisms.

We prepared anaerobic agar slant, streaked the bacteria and immediately kept it under anaerobic conditions in an anaerobic jar (Hi-media).

Interpretation:

Growth - anaerobic strain

No growth - aerobic strain
3.8.4. Growth at 10% NaCl: (Barrow and Feltham (1993))

Inoculated nutrient broth of the required 10% salt concentration with the organism to be tested and incubated at the optimal temperature (37 °C) for growth.

Interpretation:

Growth indicated the tolerance of respective NaCl concentration (10%).

3.8.5. Growth at 50 °C: (Barrow and Feltham (1993))

Inoculated nutrient broth with the organism to be tested the temperature tolerance hence; it is incubated at 50 °C.

3.8.6. Catalase test: (Barrow and Feltham (1993))

The bacteria produce the enzyme catalase, but they vary in the quantity produced.

Principle:

The enzyme catalase, act on hydrogen peroxide and broken into oxygen ad water. If an organism tested for the production of catalase by
bringing it into touch with hydrogen peroxide. The oxygen bubbles are released which is the gaseous product of the enzyme’s activity is produced which indicates the liberation of oxygen and the presence of bacterial catalase. H₂O₂ is lethal to bacterial cells.

\[
\text{Catalase}
\]

\[
\begin{align*}
2\text{H}_2\text{O}_2 & \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\end{align*}
\]

The test for bacterial catalase differs from that used to detect catalase in other types of bacteria by using 3% hydrogen peroxide solution.

Procedure:

The culture was grown on an angle of nutrient agar plates and added 1 to 2 drops (1 ml) of freshly prepared 3%. H₂O₂ down the slope and examine immediately for the development of gas bubbles.

Interpretation:

Gagnon Hunting and Esselen described a simple method in which some of the growth of the organism in slant and dropped 3% H₂O₂, when catalase was present the evolution of gas bubbles quickly in the tube otherwise show the negative observation.

3.8.7. Oxidase test: (Barrow and Feltham 1993)
Principle:

The p-phenylenediamine dihydrochloride dye is used for oxidase test. It is the replacement for oxygen as synthetic acceptors of electron. This dye generally colorless if it is in reduced state, on the other hand, in the presence of oxygen; this dye is oxidized and forming a blue colored compound named by indophenol.

Reagents:

Tetramethyl p-phenylenediamine dihydrochloride, 1% (Kovac’s reagent).

Procedure:

In the direct paper procedure, a small amount of drops of the freshly prepared reagent are added on a paper strip.

Interpretation:

The bacteria having oxidase it build up a deep blue color at inoculation site within 10 seconds.

Positive – Colony deposits turned purple

Negative – No purple color develop

3.8.8. Oxidative fermentative test (OF): (Barrow and Feltham (1993))
Introduction:

Saccharolytic microorganisms degrade glucose either fermentatively or oxidatively. The end products of fermentation are relatively strong mixed acids that can be detected in a conventional fermentation test medium. However, the acids formed in oxidative degradation of glucose are extremely weak, and the more sensitive oxidation fermentation medium of Hugh and Leifson’s OF medium is required for the detection.

Principle:

- The OF medium of Hugh and Leifson differs carbohydrate fermentation media as follows:
  - The concentration of agar is decreased to 2% from 3%, making it semisolid in consistency.
  - The concentration of peptone is decreased from 11% to 2%.
  - Carbohydrate concentration is increased by 0.5% to 1.0%.

Media:

For the test; the constituents of O.F medium is as follows:

Sodium chloride : 5.0 g
Di-potassium phosphate : 0.3 g
Peptone : 2.0 g
Water : 1000 ml
Bromthymol blue (0.2%) : 15 ml
Agar : 1.0 g
Glucose : 1 %

**Procedure:**

Two tubes are required for the OF test, each inoculated with the organism using a straight needle, stabbing the medium 1 to 2 times halfway to the bottom of the tube. One tube of each pair is covered with a 1 cm layer of sterile mineral oil or liquid paraffin, leaving the other tube open to the air.

**Incubated both tubes at 35°C and examined daily for seven days.**

**Interpretation:**

Acid production is detected in the medium by the appearance of a yellow color in the case of oxidative organisms; color production may be first noted near the surface of the medium. Following are the reaction patterns.

<table>
<thead>
<tr>
<th>Open tube</th>
<th>Covered tube</th>
<th>Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid (yellow)</td>
<td>Alkaline (Green)</td>
<td>Oxidation</td>
</tr>
<tr>
<td>Acid (yellow)</td>
<td>Acid (yellow)</td>
<td>Fermentative</td>
</tr>
</tbody>
</table>


3.8.9. Acid from carbohydrates: (Barrow and Feltham (1993)

**Principle:**

Carbohydrates collectively called „sugars“ are usually used to enrich media to promote growth or pigmentation and to determine whether organisms can produce acid or acid and gas from them. The carbohydrates generally used are which also includes with concentrations of aqueous solutions suitable for addition to media. The concentration of carbohydrates in oxidation and fermentation studies is usually 0.5-1%, 1% carbohydrate is preferable as reversion of the reaction is then less likely. Some carbohydrate solutions may be sterilized by autoclaving whereas with others decomposition may occur.

**Composition of ammonium salt sugars (ASS):**

- Yeast extracts powder : 0.2 g
- Agar : 0.2 g
- (NH₂)₂ HPO₄ : 1 g
- Magnesium sulfate : 0.2 g
- Potassium chloride : 0.2 g
- Bromothymol blue (0.2%) : 0.04 ml
- Distilled water : 1000 ml
Add the solids to the water and dissolve by steaming. Added the indicator and sterilize at 115 °C for 15 min. After cooling, add the appropriate carbohydrates (cellobiose, fructose, lactose, mannose, raffinose and xylose) as a sterile solution (0.5-1%). Mix and distribute aseptically into sterile tubes that are inclined so that the medium sets as slopes.

Procedure:

Inoculated ASS or peptone water and examine daily for 7 days for acid or acid and gas production. Reversion to alkalinity should also be noted. Negative tests should be examined at regular intervals for up to 30 days.

The method of preparation will depend on the indicator:

Composition of peptone water:

- Peptone : 10g
- NaCl : 5g
- Distilled Water : 1 liter

pH adjusted at 7.2-7.4 and sterilize at 115 °C for 20 min.

Other indicators:

To 900 ml peptone water add 10 ml indicator solution (bromocresol purple, bromthymol blue or phenol red). Dissolve 5-10 g of the appropriate
Sugars in 90 ml water and steam for 30 min or sterilize by filtration. Added this solution to the sterile peptone water with indicator, distribute into sterile tubes with inverted inner (Durham) tubes and steam for 30 min. Where the solubility of a carbohydrate is low the required amount of solid material may be added to the base and when dissolved the complete medium is sterilized. With some organisms the ability to produce visible gas depends on the temperature of incubation and if equivocal or suspect results are obtained the tests should be repeated at a lower temperature. As some anaerobes can produce gas from proteins, gas production is not reliable as an indicator of fermentation.

When incubated anaerobically, some indicator may be „bleached” (reduced to a colorless state), in such case, test for acid production by the addition of fresh indicator solution.

3.8.10. Casein hydrolysis: (Barrow and Feltham (1993))

Principle:

Casein is present in the milk is hydrolyzed by some bacterial strains. Hydrolysis part is shown present around growth of organisms.

Casein agar:

Milk, skim : 500 ml

Nutrient agar, double strength : 500 ml

Prepared the skim-milk and sterilized by heating at 115 °C for ten min. after sterilization media cool down up to 50 °C than affix to the double strength nutrient agar melted and cooled to 50-55 °C. Mix and distribute in Petridis.
Acid mercuric chloride:

HgCl₂ : 12 g
Distilled water : 80 ml
Conc. HCl : 16 ml

Add water and acid in mercuric chloride by mild shaking until the solute is complete dissolve.

A clearing zone around the bacterial growth on casein agar pates showed the positive results.

3.8.11. Starch hydrolysis: (Barrow and Feltham (1993)

Starch agar:-

Potato starch : 10 g
Distilled water : 50 ml
Nutrient agar : 1000 ml

Triturate the starch with the water to a smooth cream and added to the molten agar medium. Mixed and sterilized at 115 °C for 10 min. Poured into Petri dishes. Overheating may hydrolyze the starch.

Distilled water
Lugol's iodine : 5 g
Potassium : 10 g

First take 5 g Iodine and dissolved in water (small volume) and then up to 100 ml. For use diluted 1/5 with distilled water. Inoculate lightly 0.2% of soluble starch and plates kept on 30 °C for 5 days incubate period. After completion of bacterial growth added a few drops of lugol's iodine solution.

Interpretation:

The medium having clear colorless zones than starch is hydrolyzed by bacterium and turns blue than starch has not been hydrolyzed,

3.8.12. Citrate utilization: (Barrow and Feltham (1993))

In Citrate test bromothymol blue is used as an indicator. Mainly this test is used to make different in the middle of enteric bacteria on the basis of ferment citrate as the sole carbon source. In this method carbon dioxide is generated when the citric acid is metabolized.

\[
\text{CO}_2 + 2\text{Na}^+ + \text{H}_2\text{O} \rightarrow \text{Na}_2\text{CO}_3
\]

(Producing during citric acid metabolism) (blue color at alkaline pH)

Note: In acidic (pH 6.8 & below) medium the dye Bromothymol blue is green and it turns in blue color when the medium is alkaline (pH 7.6 & higher).
Procedure:

Prepared Simmon’s citrate agar (pH 6.9) and heated to dissolve medium. Poured the medium in test tubes and sterilized it. Prepare the slants after sterilization and inoculated with culture by means of a stab and streak method and kept in incubator for 48 hrs at 37°C.

Interpretation:

If the medium color on inoculated slant, is blue and growth is visible on the surface (i.e. citrate test is positive) if there is no growth the color remains green (i.e. citrate test is negative).


Principle:

The H₂S test is one that can be made as sensitive as required with an adequate sulphur source (cysteine) and a delicate indicator (lead acetate papers) almost all the enteric bacteria can be shown to be able to produce H₂S. In this test, an accurate estimate can be obtained of an organism’s catabolic power in relation to sulphur compounds, but it is not possible to distinguish readily between those organisms with much and those with little ability to produce H₂S. With a poor medium or a less sensitive (ferrous chloride or lead acetate in the medium) only the strong H₂S producer are detected.

Composition of Triple sugar iron agar (TSI):

NaCl : 5 g
Agar : 20 g

Na$_2$S$_2$O$_3$.5H$_2$O : 0.3 g

Phenol red, 0.2% aq. Soln 12 ml

Ferric citrate : 0.3 g

Distilled water : 1000 ml

Procedure:

After properly dissolve the ingredient in distilled water, add an indicator solution and dispense into test-tubes.

After autoclaving about 3 cm long slopes with deep butts slant was prepared.

Take a whatman paper strips and saturate with the appropriate solution, dry at 50 to 60 °C. Stored in screw-capped containers.

For the detection of H$_2$S:
Lead acetate hot saturated aqueous solution (10 g lead acetate in 100 ml hot water).

Inoculated a tube of TSI agar by stabbing the butt.

After 144 hours of incubation in if only is butt is converted into dark black then we say that this blackening is due to H$_2$S production. Some organisms produce a dark pigment on the slope only and this is a positive result.

**3.8.14. Indole production test:** (Barrow and Feltham (1993))

Some bacterial sp. utilized the amino acid named Tryptophan, which is an type of essential amino acid, through their enzymatic system and the formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into tryptone broth.

**Procedure:**

- Prepared (1%) tryptone broth and Sterilize by autoclaving
- Tryptone broth inoculated with bacterial culture. Another as an uninoculated comparative control. Both were left for incubation at 35°C.
- After the completion of incubation period, 1 ml volume of Kovac's reagent was added in each tube including control and mixing with softly.
**Interpretation:**

If indol is produced in the liquid medium a cherry red color has been develop in the upper layer of the tube showed positive indication and vice versa.

**3.8.15. Urease test (urea hydrolysis):** (Barrow and Feltham (1993))

Principle: urease enzyme having the potential to break urea by the process of hydrolysis and released ammonia and carbon dioxide. If the tested strains utilized the urea in the culture medium it released ammonia that make the medium will be alkaline in nature. This change of pH is visualized by adding a ph indicator phenol red (change in color of the indicator to red-pink).

**Composition of Christensen’s medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>20% aqueous solution (10 ml)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2 g</td>
</tr>
<tr>
<td>0.2% Phenol red</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Dissolved all the ingredients and pH is adjusted through 6.8.

**Interpretation:**

Development of red color indicated urea hydrolysis

**3.8.16. Nitrate reduction:** (Barrow and Feltham (1993))

**Principle:**

Nitrate reduction may be shown either by detecting the presence of one of the breakdown products, or by showing the disappearance of nitrate from the medium. The products of reduction may include nitrite, hyponitrite, hydroxylamine, ammonia, nitrous oxide or gaseous nitrogen. The first test to be applied aims at showing the presence of nitrite. When this test is negative (i.e. nitrite is not detected) the medium is tested to see whether there is residual nitrate, if this test is negative it confirms that the first stage of the breakdown has been completed and the nitrite further broken down.

In un inoculated nitrate broth and with cultures of organisms that do not reduce nitrate, the test for nitrite is negative until zinc dust or other reducing agent is added to the culture medium to reduce the nitrate contained in it. To detect small amounts of residual nitrate the amount of zinc added may be critical. The tests are very sensitive and it is important to check the uninoculated medium for nitrite, which should not be present.

**Composition of nitrate broth**

\[\text{KNO}_3: \quad 1 \text{ g}\]
Nutrient broth : 1000 ml

Nitrite test reagents:

Solution A: 0.33% sulphanilic acid in 5N-acetic acid. All the content dissolves by gentle heating.

Solution B: dimethyl-α-naphthylamine (0.6%) in acetic acids (5N) dissolved the solids by mild heating and shaking.

10% zinc dust suspended in 1% methyl cellulose solution

Interpretation:

A deep red color, which shows the presence of nitrite and thus shows that nitrate has been reduced, indicates a positive reaction.

3.8.17. Acid Fuchsin Solution:

Composition per 50.0mL:

Acid Fuchsin : 0.25g

Aquesou dioxan, 5% : 50.0mL

Preparation of Acid Fuchsin Solution:

Add Acid Fuchsin to 50.0mL of 5% aqueous dioxan. Mix well to dissolve.

Caution:
Acid Fuchsin is a potential carcinogen and care must be taken to avoid inhalation of the powdered dye and contamination of the skin.

**Preparation of Medium:**

Add components to distilled/deionized water and bring volume to 1.0L. Mix thoroughly. Gently heat while stirring and bring to boiling. Distribute into tubes or flasks. Autoclave for 15 min at 15 psi pressure–121°C. Pour into sterile Petri dishes or leave in tubes.

For the isolation and differentiation of *Aeromonas* species from other Gram negative rods such as *Pseudomonas* and *Enterobacteriaceae*. Specimens with low numbers of *Aeromonas* may first be enriched by growth in starch broth for 4–9 days. After 24 hours of growth on this agar, colonies are sprayed with Nadi reagent (1% solution of *N, N, N’, N’*-tetramethyl-<p>-phenylene-diammonium dichloride). A positive Nadi reaction (dextrin degradation) is indicated by a purple color at the periphery of the colony. Dextrin fermentation is also indicated by red colonies. *Aeromonas* species appear as large, convex, dark red colonies with a purple periphery.

### 3.8.18. Alkaline Nutrient Agar

**Composition** per liter:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0g</td>
</tr>
</tbody>
</table>
Beef extract : 1.0g

Sodium sesquicarbonate solution 100.0mL

pH 9.7 ± 0.2 at 25°C

**Sodium Sesquicarbonate Solution:**

**Composition** per 100.0mL:

Na₂CO₃, anhydrous : 10.6g

NaHCO₃ : 8.42g

**Preparation of Sodium Sesquicarbonate Solution:**

Add components to distilled/deionized water and bring volume to 100.0mL. Mix thoroughly. Filter sterilizes. Warm to 50°–55°C.

**Preparation of Medium:**

Add components, except sodium sesquicarbonate solution, to distilled/deionized water and bring volume to 900.0mL. Mix thoroughly. Gently heat and bring to boiling. Autoclave for 15 min at 15 psi pressure–121°C. Cool to 50°–55°C. Aseptically add sterile sodium sesquicarbonate solution. Mix thoroughly. Adjust pH to 9.7. Pour into sterile Petri dishes or distribute into sterile tubes.

For the cultivation of alkalinophilic bacteria, including *Bacillus alcalophilus*, *Bacillus cohnii*, and other *Bacillus* species.
3.8.19. Alkaline Peptone Water

**Composition** per liter:

- Peptone : 10.0g
- NaCl : 5.0g

pH 8.4 ± 0.2 at 25°C

**Preparation of Medium:**

Add components to distilled/deionized water and bring volume to 1.0L. Mix thoroughly. Adjust pH to 8.4. Distribute into tubes or flasks. Autoclave for 20 minutes at 15 psi pressure–121°C.

For the cultivation of a variety of alkalophilic microorganisms.

3.8.20. Bismuth Sulfite Broth

**Composition** per liter:

- Bi₂ (SO₃)₃ : 16.0g
- Pancreatic digest of casein : 10.0g
- Peptic digest of animal tissue : 10.0g
- Beef extract : 10.0g
- Glucose : 10.0g
- Na₂HPO : 48.0g
**Preparation of Medium:**

Add components to distilled/deionized water and bring volume to 1.0 liter. Mix thoroughly and heat it with frequent agitation until boiling properly. Let it be boiling for 1 minute. Do not do autoclave. Let it be cool up to 45°–50°C. Mix it properly to disperse the precipitate and use the aseptically method to distribute into sterile tubes or flasks. Use approx. 2.0–2.2mL of medium in each tube or flask.

This selective broth used for the isolation of *Salmonella typhi* and other enteric bacilli and as well for the detection of *Salmonella* by the membrane filter method.

---

**3.8.21. Brilliant Green Bile Agar**

**Composition** per liter:

- Noble agar : 10.15g
- Pancreatic digest of gelatin : 8.25g
- Lactose : 1.9g
- Na2SO3 : 0.205g
- Basic Fuchsin : 0.078g
- Erioglaucine : 0.065g
- FeCl3 : 0.0295g
KH2PO4 : 0.015g
Oxgall, dehydrated : 2.95mg
Brilliant Green : 0.03mg
pH 6.9 ± 0.2 at 25°C

Source:
This medium is available as in premixed powder form by the BD Diagnostic Systems.

Caution:
Basic Fuchsin medium is a potential carcinogen and extra care must be taken to just avoid inhalation of this powdered dye and contamination of the skin.

Preparation of Medium:
Add components to deionized water and bring it up to volume 1.0 Liter. For plating 10.0 mL samples, prepare the medium double strength. Mix it thoroughly and gently heat and bring it to boiling. Distribute into glass tubes or flasks. Put it in autoclave for 15 minutes at 15 psi pressure–121°C. Pour into sterile Petri dishes each 5 to 6 ml. Care should be taken to avoid exposure of the prepared medium to light and keep it in dark place.

This medium using for the detection and enumeration of coliform bacteria in materials of sanitary importance such as water and sewage. In this
medium *Escherichia coli* appear as dark red colonies with a pink halo and *Enterobacter* species appear as pink colonies.

### 3.8.22. Citrate Medium, Koser’s modified

**Composition** per liter:

- **NaCl**: 5.0g
- **Citric acid**: 2.0g
- **(NH4)H2PO**: 4.1.0g
- **K2HPO4**: 1.0g
- **MgSO4·7H2O**: 0.2g

pH 6.8 ± 0.2 at 25°C

**Preparation of Medium:**

Add components to distilled/ deionized water and bring volume to 1.0L. Mix thoroughly. Adjust pH to 6.8. Distribute into tubes in 5.0mL volumes. Autoclave for 15 min at 15 psi pressure – 121°C.

### 3.8.23. Nutrient Agar with 3% NaCl

**Composition** per liter:

- **NaCl**: 30.0g
Agar : 15.0g
Pancreatic digest of gelatin : 5.0g
Beef extract : 3.0g

pH 6.8 ± 0.2 at 25°C

**Source:**

Nutrient agar is available as a premixed powder from BD Diagnostic Systems.

**Preparation of Medium:**

Add components to distilled/deionized water and bring volume to 1.0L. Mix thoroughly. Gently heat and bring to boiling. Distribute into tubes or flasks. Autoclave for 15 min at 15 psi pressure–121°C. Pour into sterile Petri dishes or leave in tubes.

### 3.8.24. Blood Agar Base with 2.5% NaCl

**Composition** per liter:

Beef heart, infusion from : 500.0g
NaCl : 30.0g
Agar : 15.0g
Tryptose : 10.0g
pH 6.8 ± 0.2 at 25°C

Preparation of Medium:

Add components to distilled/deionized water and bring volume to 1.0L. Mix thoroughly. Heat with frequent agitation and boil for 1 min to completely dissolve. Autoclave for 15 minutes at 15 psi pressure–121°C. Cool the basal medium to 45°–50°C. Aseptically add sterile, defibrinated blood to a final concentration of 5%. Mix thoroughly and pour into sterile Petri dishes.

3.8.25. MacConkey Agar

Composition per liter:

- Pancreatic digest of gelatin : 17.0g
- Agar : 13.5g
- Lactose : 10.0g
- NaCl : 5.0g
- Bile salts : 1.5g
- Pancreatic digest of casein : 1.5g
- Peptic digest of animal tissue : 1.5g
- Neutral Red : 0.03g
- Crystal Violet : 1.0mg

pH 7.1 ± 0.2 at 25°C
Source:

This medium is available in market as a premixed powder form by the BD Diagnostic Systems.

Preparation of Medium:

Add components to deionized water and bring it up to volume to 1.0 Liter. Mix it thoroughly and gently heat while stirring until boiling. Autoclave it for 15 minutes at 15 psi pressure–121°C. Pour it into sterile Petri dishes or distribute into sterile tubes each 5-6 ml.

This medium using for the selective isolation, cultivation, and differentiation of coliforms and enteric pathogens based on the ability to ferment lactose. Lactose fermenting organisms appear in this medium as red to pink colonies. Non lactose fermenting organisms appear as colorless or transparent colonies.

3.8.26. Muller Hinton Medium

Composition per liter:

- NaCl : 60.7g
- MgCl2·6H2O : 15.0g
- Yeast extract : 10.0g
- MgSO4·7H2O : 7.4g
- Proteose peptone No. 3 : 5.0g
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>1.5g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.27g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.045g</td>
</tr>
<tr>
<td>NaBr</td>
<td>0.019g</td>
</tr>
</tbody>
</table>

pH 7.2 ± 0.2 at 25°C

**Preparation of Medium:**

Add components to distilled/deionized water and bring volume to 1.0L. Mix thoroughly. Adjust pH to 7.2. Distribute into tubes or flasks. Autoclave for 15 min at 15 psi pressure—121°C.

**3.9. LIGNIN BIODEGRADATION:**

**3.9.1 Inoculums preparation:** The screened strains maintained at -4 °C on agar plates. Bacterial culture were introduced into 100 ml broth and incubated for 24 to 48 hours. This seed culture or broth medium was used as inoculums throughout the degradation experiment (Chandra et al., 2011).

**3.9.2 Inoculums size:**

The inoculums size used throughout study were (CFU/ml) 9.5x10⁵ for PPS-S6, 10.5x10⁵ for PPS-S7 and 10.5x10⁶ for PPS-S8.
3.9.3 **Removal of lignin by single and combined bacterial culture:**

One percent of liquid suspension from each bacterial strain having an inoculums size of $9.5 \times 10^5$ for PPS-S6, $10.5 \times 10^5$ for PPS-S7 and $10.5 \times 10^6$ for PPS-S8 per ml respectively, were used as seed culture. These cultures were introduced in the axenic and mixed condition in lignin amended medium with an extra supplement of 0.5% peptone and 1% glucose in 250 ml. The adjustment of pH (7.6) was done prior to sterilization (Chandra et al., 2011).

3.9.4 **Lignin degradation at different pH:**

Since the color of lignin is pH dependant therefore decolorization of lignin was carried out at differed pH values. Mineral salt medium amended with 500 mg/l lignin was adjusted for pH 6-9 with pH meter by adding 1N HCl or NaOH. Flask containing 100 ml culture medium of respective pH was inoculated with each bacterial strain. After six days of incubation, culture growth and reduction of color was measured. The three replicates of all the sets of experiments were carried out in the same time.

3.9.5 **ANALYTICAL TECHNIQUES:**

3.9.5.1 **Growth:**

At different time interval samples were removed aseptically from flask. The development of bacterial culture in medium was checked by spectrophotometric method at 620nm. At higher cells absorbance the sample and control were diluted with same factor to obtain desired absorbance.

3.9.6.2 **Color measurement:**
During degradation studies reduction of color was measured by Visual comparison method (APHA, 1998). Samples were centrifuged at 5000 rpm and filter it. The sample color compared with standard stock solution. Since treated paper mill effluent after final treatment has very high color so it was diluted 100 times to bring the color within range of the standards. The effluent pH was noted 7.5.

**Calculation:**

\[
\text{CU (Color units) = } \frac{E \times 50}{D}
\]

Where, \(E\) = calculated color of samples (diluted samples)

\(D\) = ml sample taken for dilution

### 3.9.5.3 Lignin estimation:

The degradation of lignin during course of incubation was monitored by spectrophotometer according to Ulmer et al. (1983). In this method, take 0.5 ml sample and diluted up to 10 folds volume of 0.55% (w/v) NaOH. All the centrifuged samples (at 12000 rpm for 10 min) were filter with 0.2 mm filter paper and after that take optical density at 280nm on a UV-visible spectrophotometer. The percent degradation of lignin is calculated by following formula:

\[
\text{Degradation (%)} = \left( \frac{\text{initial OD}_{280} - \text{final OD}_{280}}{\text{initial OD}_{280}} \right) \times 100
\]

### 3.9.5.4 Total substrate los: (Chandra et al., 2007)
For the estimation of total substrate loss during the course of biodegradation, acidified the sample supernatant collected after centrifugation. The lignin is precipitated in acidic medium that residual lignin was calm by centrifugation and dried overnight at 50°C (Anthony et al., 1986). The percent of total substrate loss in the bacterial decolorized sample was resolute in the form of dry weight by using this formula:

\[
\text{Total substrate loss (\%) = (IDW – FDW \times 100)}
\]

IDW

Hear, IDW: initial dry weight in mg and FDW – final dry weight in mg

3.9.5.5 Dissolved oxygen (DO):

DO of medium during course of biodegradation was actually monitored by the measurement of oxygen pressure using DO probe.

3.9.6.6 Glucose estimation:

During course of bacterial degradation, concentration of glucose (as total carbohydrate) was measured spectrophotometrically at 490 nm (Dubois et al. 1956). In this method, 2.0 ml centrifuged sample was drawn from control and bacterial degraded samples and added 2 ml of 5.0% phenol followed by 5.0 ml concentrated H₂SO₄ and the absorbance of samples was measure at 490 nm. The quantity of glucose present in the sample was calculated by comparing the sample absorbance with standard curve of D-glucose. The three replicates of all the experiments were carried out in the same time.

3.9.5.7 HPLC analysis:

The ethyl acetate-extractable compound of control and bacterial
degraded sample were analyzed by HPLC analysis. At the end of incubation, sample (50 ml) were taken and centrifuged (at 8000xg for 15 min) for elimination of undesirable solids which are in the form of cell biomass. The clear samples were again filtered through a 0.22 Millipore filter. These filtered samples were acidified up to pH 1-2 and carefully extracted with the organic solvent (ethyl acetate). Collect the organic layer and evaporated in the presence of liquid nitrogen and dissolve in 1 ml of acetonitrile (Chandra et al., 2007). A volume of 20 µl sample injected with injection loop. The resultant peaks were monitored at wavelength 280 nm.

3.10. OPTIMIZATION OF BACTERIAL TREATMENT

The pulp-paper mill effluent by the screened bacterial strains was subjected into optimization process. The bacterial consortium is developed and then adapted in the chemostate continuous enrichment mineral salt media (MSM) along with Kraft lignin (1000 mg/l) and pentachlorophenol (500 mg/l).

The stability of consortium (mixture of Paenibacillus sp., Aneurinibacillus aneurinilyticus, and Bacillus sp.) was maintained in same media. For decolorization study, pulp paper mill effluent having COD 23,700±707; BOD 8,250±102 and initial color 6700 CU was used throughout the experiment. Two percent (v/v) overnight grown suspension of constructed consortium (inoculum size 31 x 10^4, 28x10^4 and 33 x 10^4 for Aneurinibacillus aneurinilyticus, Paenibacillus sp., and Bacillus sp., respectively) was aseptically transferred to containing 98 ml pulp paper mill effluent.

The time course of detoxification and decolorization, bacterial decolorized and undecolorized (from control sample) samples were periodically taken and check for the reduction of color, carbon oxygen demand (COD) and toxicity evaluation.
3.10.1 Reduction of pollution parameters

The reduction of parameters like color, lignin, carbon oxygen demand (COD) etc. were done as described in APHA, 1998 and Kreetachat et al., 2007.

3.10.2 Experimental design

These designs were constructed for selecting the optimum simpler source of carbon (glucose, galactose, dextrose and fructose), Agitation speed; pH; temperature and incubation time. After selecting the three most influencing factor, RSM inbuilt with CCD were employed. The three parameters (factors) i.e. pH ($X_{pH}$), Agitation ($X_{Ag}$) and Carbon source ($X_c$) were selected at their different levels and every parameters to optimize the dependent variables i.e. % decolorization ($Y_1$).

There are fifteen experimented were conducted with five central replicates. A statistical model was developed, and outcomes were analyzed. The responses ($Y_1$ and $Y_2$) were related to chosen variables by full second-order quadratic model. This model express in terms of coded variables can be expressed as follows:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3 + \beta_7 x_1^2 + \beta_8 x_2^2 + \beta_9 x_3^2$$

(1)

Where $\beta_1$, $\beta_2$ and $\beta_3$ are first order linear coefficients; $\beta_4$, $\beta_5$ and $\beta_6$ are the interaction coefficients; $\beta_0$ is intercept term, $\beta_7$, $\beta_8$ and $\beta_9$ are the quadratic coefficients and $Y$ is the predicted response. The results were also analyzed by analysis of variance (ANOVA). Finally, the three dimensional plots of variable and their levels are constructed (Singh et al., 2008).

3.10.3 Metabolite characterization
The bacterial treated and untreated samples finally subjected into metabolite characterization by GC-MS. For this all the samples were extracted by adding an organic solvent (ethyl acetate) with equal volume. The organic extract was dried in the presence of liquid nitrogen. This dry residue was further dissolved in acetonitrile (HPLC grade) and used for characterization of metabolites (Chandra et al., 2011).

For this analysis ethyl acetate extracts were derivatized with trimethyl silyl. A fraction of 1 µl of derivatized samples was injected into the gas chromatography. The NIST library available with instrument which was helpful for identifying the compound by comparing their mass spectra.

3.11. ENVIRONMENTAL assessment of treated effluent

3.11.1 Toxicity evaluation using Tubifix-tubifix worm

Tubificid worms, T. tubifex were procured from fish aquarium from Meerut city. In laboratory, the culture was maintained and prepared by OECD Guideline 1994. The grayish, lethargic or stringly-looking (unhealthy) worms were discarded and fast coiling and dark color worm (healthy) were isolated and several times washed under water followed with double distilled water. The worms were maintained in aerated 20 cm x 20 cm plastic beaker with continuous flow of water at laboratory condition (Temperature 20°±1°C) and acclimatized for 7 days prior starting to experiments. Prior to test, the worms were fed on a diet of fish food.

3.11.1.1 Optimization of toxicity bioassay test with Tubificid worm for bacterial degraded and undegraded wastewater released by pulp & paper industry:
The undegraded wastewater collected from industrial site was filtered through filter paper (whatman-1) and adjusted to pH 7.6 before toxicity test. While bacterial degraded samples were prepared by removing all the cell debris by centrifugation at five thousand rpm for 1 hrs. All the samples were left for overnight for observation of re-growth of bacteria. If it was so, the effluents were again centrifuged to remove cells. Since, the pH of bacterial treated effluent was changed during bacterial treatment, therefore pH adjusted for 7.6 and kept in screw-cap glass bottle (1lit) until toxicity assessment. Effluent (75 ml) from undegraded (100%) was taken into 100 ml test vessels. Randomly 10 worms placed in vessels and mortality of worm was recorded at every two hrs for six hrs and then at 24 hrs intervals. There was no mortality recorded within six hrs bioassay. However, after 24 hrs, 100% mortality was observed in 100% undegraded effluent. Therefore the effluent was diluted to a concentration of 10-80% v/v with tap water.

3.11.1.2 Test conditions and procedures:

Acute toxicity test (96 hrs) procedure followed in the present study was based upon recommendation of APHA (1998). The brief summary of test conditions is summarized in Table 4.10. Six different concentrations viz; 0, 10, 20, 40, 60 and 80%, (v/v) of the effluent were ready using tap water. All studies were conducted in 50 ml Petri dishes and kept in environmental growth chamber, maintained at a constant temperature 20±1°C. Tubificid worms are exposed to above concentrations in two replicates.

3.11.1.3. Microscopic studies:

For microscopic studies, worms were carefully removed from test vessels. These worms were cleaned with de-ionized water thrice to remove contaminant and debris. These organisms is carefully placed on a slide and
examined at 400x of the light microscope (Nikon, Japan) for morphological changes.

3.11.1.4 Statistical analysis:

All the required Statistical analysis was carried out to calculate median lethal concentration for undegraded effluent. Their LC50 values were estimated by graphical interpolation based on % mortalities. Further their 95% confidence limits were calculated using following equation:

\[
x \pm 1.96 \left( \frac{\sigma}{\sqrt{N}} \right)
\]

Where, \(x = \text{Mean, } \sigma = \text{standard deviation}

\(N = \text{No. of observations, } 1.96 = \text{Constant}

3.11.2 Toxicity evaluation by Geno-toxicity tests

The genotoxicity test was performed for the evaluation of bacterial decolorized sample of pulp-paper mill effluent on *Saccharomyces cerevisiae* (yeast cell) by using comet assay. *Saccharomyces cerevisiae* was grown in malt-yeast extract media at pH 5.0. The test cells are treated with the bacterial decolorized and undecolorized sample for 3 hours. Culture medium (CM) was used as a control. After completion of treatment time, the exposed mixed with agarose and spread on slides. Electrophoresis of these cells was performed for 35 min at 20 V. Furthermore, slides were stained with EtBr (20 µg ml\(^{-1}\)) for 5 min and cover slip placed in dark humidified box to prevent drying of the gel (Miyamae et al., 1998).