APPENDICES
APPENDIX A: COMPOSITION OF MEDIA

1. **Nutrient media (g/L)**
   - Peptone 5.0
   - Beef extract 3.0
   - NaCl 5.0
   - Distilled water 1000ml

   All the ingredients (except agar) were dissolved in deionized water. The pH of the medium was adjusted to 7.0 with 0.1 N HCl. Agar 15g was added if solid medium is required. Medium was sterilized by autoclaving at 121° C for 15 min.

2. **LB (Luria and Bertani) Medium**
   - Tryptone 10.0
   - Yeast extract 5.0
   - NaCl 10.0
   - Distilled water 1000ml

   All the ingredients (except agar) were dissolved in deionized water. The pH of the medium was adjusted to 7.0 with 0.1 N HCl. Agar 15g was added if solid medium is required. Medium was sterilized by autoclaving at 121° C for 15 min.

3. **Czapex Dox Agar medium (g/L)**
   - NaNO₃ 2.0
   - K₂HPO₄ 1.0
   - MgSO₄ 7H₂O 0.5
   - FeSO₄ 7H₂O 0.01
   - Sucrose 30.0
   - Agar 15.0
   - Antibiotics (Streptomycin) 0.03
   - Distilled water 1000ml
   - pH 6.8
4. Rose Bengal Agar medium (g/L):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>Rose Bengal</td>
<td>0.05</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.03</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

All ingredients (except agar) were dissolved in deionized water. The pH of the medium was adjusted to 6.8 with 1 M HCL. Agar was added. Medium was sterilized by autoclaving at 121° C for 15 min.

5. Simmon Citrate Medium (g/L)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH$_4$)H$_2$PO$_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Bromothymol Blue</td>
<td>0.8</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

All the ingredients except phosphate were dissolved in deionized water. Then K$_2$HPO$_4$ is dissolved separately and add to the rest. The total volume was made up to 1 liter. The pH of the medium was adjusted to 6.8 with 0.1 N HCl/ NaOH. The medium was sterilized by autoclaving at 121° C for 15 min.
6. **Tryptone Broth (g/L)**

- Tryptone: 10.0
- NaCl: 0.5
- DDW: 1000ml

The ingredients were dissolved in DDW, adjusted the pH to 7.0 and sterilized by autoclaving at 121°C for 15 min.

7. **MR- VP Broth (g/L)**

- Peptone: 7.0
- Dextrose: 5.0
- K$_2$HPO$_4$: 5.0
- DDW: 1000ml

All the ingredients were dissolved in DDW, adjusted the pH to 6.9 and sterilized by autoclaving at 121°C for 15 min.

8. **Motility test medium (g/L)**

- Gelatin: 80.0
- Peptone: 10.0
- Beef extract: 3.0
- NaCl: 5.0
- DDW: 1000ml

The above ingredients were dissolved in DDW, adjusted the pH to 7.0 and sterilized by autoclaving at 121°C for 15 min.

9. **SIM agar medium (g/L)**

- Peptone: 30.0
- Beef extract: 3.0
- Fe$_2$ (NH4)$_6$ (SO4)$_3$: 0.2
- Sodium thiosulfate: 0.025
- Agar: 3.0

The above ingredients (except agar,) were dissolved in DDW adjusted the required pH, agar was added and sterilized by autoclaving at 121°C for 15 min.
10. **Starch media (g/L)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>10.0</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.60</td>
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<tr>
<td>K$_2$HPO$_4$</td>
<td>0.15</td>
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<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.12</td>
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<tr>
<td>NaCl</td>
<td>0.25</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The above ingredients (except agar,) were dissolved in DDW adjusted the required pH, agar was added and sterilized by autoclaving at 121°C for 15 min.

11. **M9 mineral medium (g/L):**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.065</td>
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<tr>
<td>KH$_2$PO$_4$</td>
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<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.12</td>
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<tr>
<td>NaCl</td>
<td>0.25</td>
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<tr>
<td>NH$_4$Cl</td>
<td>0.5</td>
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<tr>
<td>CaCl$_2$</td>
<td>0.05</td>
</tr>
<tr>
<td>C-source (cmc/cellulose)</td>
<td>10.0</td>
</tr>
</tbody>
</table>

The above ingredients were dissolved in DDW, adjusted the required pH and sterilized by autoclaving at 121°C for 15 min.

12. **Plate screening medium (g/L)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO$_3$</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>0.01</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>CMC</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>17.0</td>
</tr>
<tr>
<td>DDW</td>
<td>1000ml</td>
</tr>
</tbody>
</table>
Table 1: Modified Biebl and Pfennig (g/L) medium for cultivation of anaerobic bacteria

<table>
<thead>
<tr>
<th>Contents</th>
<th>PNSB</th>
<th>PSB</th>
<th>GSB</th>
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<tbody>
<tr>
<td>KH2PO4</td>
<td>0.25g</td>
<td>0.25g</td>
<td>0.25g</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>0.34g</td>
<td>0.34g</td>
<td>0.34g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.48g</td>
<td>0.48g</td>
<td>0.48g</td>
</tr>
<tr>
<td>CaCl2. 2H2O</td>
<td>0.05g</td>
<td>0.05g</td>
<td>0.05g</td>
</tr>
<tr>
<td>MgSO4. 7H2O</td>
<td>0.5g</td>
<td>0.5g</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.4g</td>
<td>0.3g</td>
<td>-</td>
</tr>
<tr>
<td>Malate or Pyruvate</td>
<td>3g</td>
<td>3g</td>
<td>-</td>
</tr>
<tr>
<td>Ferric Citrate (0.1%)</td>
<td>5ml</td>
<td>5ml</td>
<td>5ml</td>
</tr>
<tr>
<td>SL-7(trace element)</td>
<td>1ml</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Agar (if needed)</td>
<td>20g</td>
<td>20g</td>
<td>20g</td>
</tr>
<tr>
<td>*Vitamin B12</td>
<td>1ml</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>*NaHCO3</td>
<td>-</td>
<td>10ml</td>
<td>10ml</td>
</tr>
<tr>
<td>*Na2S. 9H2O</td>
<td>-</td>
<td>0.5mM</td>
<td>3mM</td>
</tr>
<tr>
<td>pH</td>
<td>5.0, 7.0</td>
<td>5.0, 7.0</td>
<td>5.0, 7.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contents</th>
<th>GNSB</th>
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</thead>
<tbody>
<tr>
<td>Sodium glutamate</td>
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</tr>
<tr>
<td>Sodium succinate</td>
<td>0.5g</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>0.5g</td>
</tr>
<tr>
<td>Na2S2O3</td>
<td>2mM</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.38g</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
<td>0.5g</td>
</tr>
<tr>
<td>Vitamin Solution</td>
<td>1ml</td>
</tr>
<tr>
<td>Basal salt solution</td>
<td>5ml</td>
</tr>
<tr>
<td>Agar (if required)</td>
<td>20g</td>
</tr>
<tr>
<td>pH</td>
<td>5.0, 7.0</td>
</tr>
</tbody>
</table>
APPENDIX B: SOLUTIONS AND REAGENTS

1. **1N Potassium dichromate solution:**
   
   Dissolved 49.04g of K2Cr2O7 and the volume was made up to 1 liter with DDW.

2. **Diphenylamine indicator**
   
   To prepare the indicator, 0.5g of Diphenylamine was dissolved in 100ml of conc. H2SO4 and about 20ml of water is added to it.

3. **Anthrone Reagent:** 0.2% anthrone in conc. H2SO4

4. **1N Ferrous Ammonium Sulphate solution**
   
   The solution was prepared by dissolving the 98.28g of Fe2(NH4)6(SO4)3 in 3.75 ml of conc. H2SO4. The volume was made up to 250 ml and used immediately.

5. **0.1 M HCl solution:** (37% HCl= 12M)

   \[ M_1V_1 = M_2V_2 \]

   \[ V_1 = \frac{0.1 \times 1000}{12} = 8.33 \text{ml/L} \]

   To prepare 1000ml of 0.1M HCl solution, 8.33 ml of concentrated HCl and make up the volume with DDW.

6. **Phosphate buffer saline(PBS) pH 7.4**

   - NaCl (137mM) 8g/L
   - KCl (2.7mM) 0.2g/L
   - Na2HPO4 (10mM) 1.44g/L
   - KH2PO4 (2mM) 0.24g/L
7. **Citrate buffer (pH 4.0 -6.0)**

Citric acid (MW=210.14 g/mol)

Sodium citrate (MW= 294.19 g/mol)

Stock solutions:

A. 0.1 M solution of citric acid (21.01g in 1L)

B. 0.1 M solution of sodium citrate (29.41g in 1L)

X ml of A + Y ml of B diluted to a total of 100ml to get the required pH.

8. **Phosphate buffer (pH 6.0-8.0)**

NaH$_2$PO$_4$ .7 H$_2$O (MW= 138.09 g/mol)

Na$_2$HPO$_4$ .7H$_2$O (MW= 268.1 g/mol)

Stock solutions:

A. 0.2M solution of monobasic sodium phosphate (27.8g in 1L)

B. 0.2M solution of dibasic sodium phosphate (53.65 g of Na$_2$HPO$_4$. 7H$_2$O in 1L)

X ml of A + Y ml of B diluted to a total of 100ml to get the required pH.

9. **Glycine- NaOH buffer (pH 8.5-10.0)**

Glycine (MW= 75.07 g/mol)

NaOH (MW= 40.0 g/mol)

Stock solutions:

A. 0.2 M solution of Glycine (15.01g/L)

B. 0.2 M NaOH

X ml of A + Y ml of B diluted to a total of 100ml to get the required pH.
10. **50X TAE**

Two hundred and forty-two grams Tris base was dissolved in deionized water. 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

11. **1X TAE**

Twenty ml of 50X TAE buffer was taken and the volume was adjusted to 1 liter with deionized water to obtain 1 liter 1X TAE buffer.

12. **1M Tris-HCl pH 8**

Tris base (121.1 g) was dissolved in 800 ml of deionized water. pH was adjusted to 8 with concentrated HCl. Volume was brought to 1L with deionized water. Solution stored at 4°C.

13. **Ethidium Bromide Stock Solution (10 mg/ml)**

Ethidium bromide (0.5g) was dissolved in 50 ml of deionized water. The prepared solution is stored at 4°C.

14. **0.5 M EDTA pH 8.0**

EDTA (186.12g) was dissolved in 800 ml of deionized water and pH was adjusted to 8.0 with 10N NaOH. Volume was brought to 1000 ml with deionized water.

15. **Lysis solution:**

Tris HCl – 10mM (pH 8.0)

SDS - 2%

EDTA – 10mM (pH 8.0)
All the solutions were prepared separately, adjusted the pH and mix to get the required lysis buffer.

16. **DNS-reagent** (Miller, 1959)

- DNS 10 g
- Phenol 2 g
- Na2SO3 0.5 g
- NaOH 20 g
- Potassium sodium tartarate 400 g

Components were dissolved in 1 liter of distilled water under stirring and heating at 50°C and stored in brown glass bottles at room temperature.

17. **Solutions for Gram Staining**

17.1 **Crystal Violet Staining Reagent**

- **Solution A**
  - Crystal violet 2g
  - Ethanol (95%) 20ml

- **Solution B**
  - Ammonium oxalate 0.8g
  - Distilled water 80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

17.2 **Iodine Solution**

- Iodine 1g
- Potassium iodide 2g
- Distilled water 300ml
Iodine and potassium iodide were grinded. Water was added slowly and the solution was stirred until the iodine was dissolved. The solution was stored in amber bottle.

17.3 Saffranin Solution

Saffranin (2.5% in 95% alcohol) 10ml / Distilled water 100ml.

18. Kovac Reagent:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoamyl alcohol</td>
<td>15ml</td>
</tr>
<tr>
<td>p-Dimethylaminobenzaldehyde</td>
<td>1.0 g</td>
</tr>
<tr>
<td>HCl concentrated</td>
<td>5ml</td>
</tr>
</tbody>
</table>

The aldehyde is dissolved in isoamyl alcohol and then acid is slowly added to this mixture.

19. Voges-Proskauer (VP) reagents:

a) VP reagent I

α-Napthol (5%)

Ethyl alcohol

Dissolve α-Napthol in absolute ethyl alcohol and make up the volume required.

a) VP reagent II

KOH (40%)

DDW

The VP reagent I and II were mixed and the test performed.
20. Methyl red indicator:

Dissolve 0.01 g of methyl red in 30 ml of 95% ethyl alcohol. To the alcohol-indicator mixture, 20 ml of distilled water was added.

21. Phenolphthalein indicator:

The indicator was prepared by dissolving 1 g of phenolphthalein in 100 ml of ethanol.

22. Alkaline Copper sulphate solution (Lowry’s reagent):

22.1 Solution A: 2% Na$_2$CO$_3$ in 0.1 M NaOH solution.

22.2 Solution B: The solution has two components:

   a) 1% sodium potassium tartarate

   b) 0.5% Copper Sulphate

To prepare solution B, components b is added to component a

Both the solutions were prepared separately. Alkaline copper sulphate solution is prepared by mixing solution A and B in the ratio of 49:1

23. Folin Ciocalteau (FC) reagent: The FC reagent was diluted with equal volume of water on the day of use.

24. BSA standard (1 mg/ml): The protein standard solution was prepared by dissolving 50 mg of BSA in 50 ml of DDW giving the concentration of 1 mg/ml.

25. EMS working solution:

Stock from bottle (10 g/10 ml or 1 g/ml or 10$^6$ µg/ml)

Working stock (1 mg/ml or 10$^3$ µg/ml)

Using the equation, $S_1 V_1 = S_2 V_2$ we prepare working stock solution by taking 10 µl of EMS ($V_1$) from stock and made up the total volume to 10 ml ($V_2$). This stock solution is further diluted to get the required concentration of EMS used in the study.
26. **Stock solutions of chlorinated organic compounds:**

26.1 Phenol (MW = 94.11 g/mol)

26.2 Catecol or 1,2-dihydroxybenzene (MW = 110.11 g/mol)

26.3 p-Nitro phenol (MW = 139.11 g/mol)

26.4 DCP (MW = 163.00 g/mol)

26.5 TNP or picric acid (MW = 229.11 g/mol)

27. **Congo red solution (0.1%)**

   To prepare the staining solution, 0.1 gram of Congo red dye is dissolved in 100 ml of DDW.

28. **1 M NaCl solution (MW = 58.44 g/mol):**

   To prepare this destaining solution, 5.84 g of sodium chloride is dissolved in water and the volume made up to 100 ml with DDW.
### APPENDIX C: LISTS OF EQUIPMENTS

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of the Instruments</th>
<th>Company</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Anaerobic jars</td>
<td>Himedia</td>
</tr>
<tr>
<td>2.</td>
<td>Autoclave</td>
<td>MAC</td>
</tr>
<tr>
<td>3.</td>
<td>Centrifuge(s), Capacity 2ml, 15ml</td>
<td>Eppendorf,</td>
</tr>
<tr>
<td>4.</td>
<td>Compound Microscopes</td>
<td>Olympus</td>
</tr>
<tr>
<td>5.</td>
<td>Deep Freezer, -20°C and -80°C</td>
<td>MAC,</td>
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<tr>
<td>6.</td>
<td>Digital Colony Counter</td>
<td>MAC</td>
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<tr>
<td>7.</td>
<td>Electronic balance</td>
<td>Sartorius</td>
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<td>8.</td>
<td>Electrophoresis apparatus (Horizontal)</td>
<td>Genei</td>
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<tr>
<td>9.</td>
<td>Freeze, 4°C</td>
<td>LG</td>
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<td>10.</td>
<td>Gamma Chamber</td>
<td>BRIT, Mumbai</td>
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<tr>
<td>11.</td>
<td>Hot plate with magnetic stirrer</td>
<td>Genei</td>
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<tr>
<td>12.</td>
<td>Incubators</td>
<td>OrbiteK</td>
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<tr>
<td>13.</td>
<td>Laminar flow hood</td>
<td>Klenz Flo</td>
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<td>14.</td>
<td>Lyophilizers</td>
<td>NSW</td>
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<tr>
<td>15.</td>
<td>Microscope</td>
<td>Olympus</td>
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<tr>
<td>16.</td>
<td>Orbital Shaking Incubator</td>
<td>MAC</td>
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<tr>
<td>17.</td>
<td>pH meter</td>
<td>Systronics</td>
</tr>
<tr>
<td>18.</td>
<td>Pipette(s), Capacity 1-10 μl, 10-100μl, 20-200 μl, 200-1000 μl</td>
<td>Gilson, Eppendorf</td>
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<td>19.</td>
<td>Spectrophotometer</td>
<td>Thermo, Fisher Scientific</td>
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<td>20.</td>
<td>Sterilization Oven, Temp up to 250°C</td>
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<td>21.</td>
<td>Thermal Cycler(PCR machine)</td>
<td>Eppendorf</td>
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<tr>
<td>22.</td>
<td>Vortex Mixer</td>
<td>Genei</td>
</tr>
<tr>
<td>23.</td>
<td>Water Bath</td>
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<tr>
<td>24.</td>
<td>Water Distillation Unit</td>
<td>NSW, Sartorius</td>
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# APPENDIX D: LISTS OF CHEMICALS

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<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1-Chloro 2,4-Dinitrobenzene</td>
<td>SRL</td>
</tr>
<tr>
<td>2.</td>
<td>2,6-Dichloro nitro benzene</td>
<td>SRL</td>
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<tr>
<td>3.</td>
<td>Absolute Alcohol</td>
<td>SRL</td>
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<tr>
<td>4.</td>
<td>Acetic acid (Glacial)</td>
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</tr>
<tr>
<td>5.</td>
<td>Agar- Agar</td>
<td>Himedia</td>
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<td>6.</td>
<td>Ammonium Chloride</td>
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<td>7.</td>
<td>Ammonium Ferrous Sulfate (Hexahydrate)</td>
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<td>Ammonium Nitrate</td>
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<tr>
<td>9.</td>
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<td>10.</td>
<td>Ampicillin</td>
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<td>11.</td>
<td>Anaerobic Agar</td>
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<td>12.</td>
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<td>Benzene</td>
<td>SRL</td>
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<tr>
<td>15.</td>
<td>Bovine Serum Albumin</td>
<td>Himedia</td>
</tr>
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<td>16.</td>
<td>Bromophenol Blue</td>
<td>SRL</td>
</tr>
<tr>
<td>17.</td>
<td>Carboxy Methyl Cellulose</td>
<td>Himedia</td>
</tr>
<tr>
<td>18.</td>
<td>Calcium Chloride</td>
<td>Himedia</td>
</tr>
<tr>
<td>19.</td>
<td>Catecol</td>
<td>Himedia</td>
</tr>
<tr>
<td>20.</td>
<td>Celllobiose</td>
<td>Himedia</td>
</tr>
<tr>
<td>21.</td>
<td>Cellulose Powder</td>
<td>Himedia</td>
</tr>
<tr>
<td>22.</td>
<td>Chitin</td>
<td>Himedia</td>
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<tr>
<td>23.</td>
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</tr>
<tr>
<td>24.</td>
<td>Congo Red (Powder)</td>
<td>Merck</td>
</tr>
<tr>
<td>25.</td>
<td>Congo Red Solution</td>
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</tr>
<tr>
<td>26.</td>
<td>Crystal Violet</td>
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<tr>
<td>27.</td>
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<tr>
<td>28.</td>
<td>Dextrose</td>
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</tr>
<tr>
<td></td>
<td>Chemical Name</td>
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<tr>
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<tr>
<td>29.</td>
<td>Diammonium hydrogen phosphate</td>
<td>Himedia</td>
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<td>30.</td>
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<tr>
<td>31.</td>
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<td>32.</td>
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<td>45.</td>
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<td>Hydrogen Peroxide</td>
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<td>61.</td>
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<td>62.</td>
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<td>77.</td>
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<tr>
<td>78.</td>
<td>α-Naphthyl amine</td>
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APPENDIX E: LISTS OF PUBLICATIONS


4. Poster presentation in Recent Trends in Biodiversity Researches, Organized by Department of Life Sciences, Assam University, Silchar.

Microbial Diversity from Solid Wastes Disposal of Paper Industry, Panchgram, South Assam

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Microbial diversity constitutes the most extraordinary reservoir of life in the biosphere that we have only just begun to explore and understand. A preliminary investigation on the microbial diversity of paper mill effluents organic wastes from the landfill site of Cachar Paper mill, Panchgram, South Assam, the isolation and characterization of native microbes on different media may generate information on the nature, characteristics, and degrading efficiency of various hazardous wastes by the micro-organisms. Sludge samples were collected randomly from paper mill solid wastes dumping sites and their physico-chemical characteristics such as pH, moisture content, NPK and Cellulose content were determined. The qualitative analysis of species in microbial community was undertaken. The isolated bacteria and fungi were screened for their cellulase activity, characterized and identified. The study revealed that bacterial isolates showing higher zone of cellulolytic activity belong to Bacillus, Pseudomonas and Serratia spp, while fungal isolates mostly belong to Aspergillus and Penicillium spp. The isolates have also shown a wide range of pH and temperature tolerance. The study has suggested that the paper mill waste site harbors various microorganisms that are active in cellulose breakdown.

Key words: Microbial diversity, organic wastes, biological degradation, bacterial isolation, characterization, cellulose degradation.

Microorganisms constitute a huge and almost unexplained reservoir of resources likely to provide innovative applications useful to man and are capable of exploiting a vast range of energy sources and thriving in almost every habitat.

Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. Thus there is a general interest in studying the diversity of indigenous microorganisms capable of degrading different pollutants because of their varied effects on the environment. Efforts to convert waste into useful products can be achieved and maintained by biotechnological measures that include action of microorganisms, enzymes and technologies. Paper industry is one among various industries that produces large amount of cellulosic (cellulose, hemicelluloses and lignin) along with other toxic organic wastes generally disposed off as landfills that accumulate in nature leads to significant environmental impacts. Cellulosic biomass has...
attracted worldwide attention as renewable resource and now several federal agencies including National Science Foundation, the US department of Energy (DOE) and US department of agriculture (USDA) are strongly expanding the role of biomass (all plants and plants derived materials) as an alternative energy source that can be converted into bio based products and bioenergy1. The microbial communities of the site are responsible for pollutant degradation and transformation.

Glucose, an appropriate hydrolysis product of cellulosic biomass can be used in different applications such as production of fuel, ethanol, single cell protein, feed stock, industrially important chemicals and so on4. The role of fungi, bacteria and actinomycetes in the natural biodegradation process of cellulosic wastes from various environments has well been documented7-11. Lignin degrading fungi and their enzymes also have their ability to degrade highly toxic organic compounds such as dioxins and polychlorinated biphenyls and could have an important role to play in the remediation of contaminated soils. Bacteria also have their ability to detoxify the heavy metals (Lead, chromium, cadmium) along with degrading organic wastes of the contaminated site11.

Microbial communities however are subjected to various perturbations, such as variation of pH, temperature, organic loading rates, the toxicant level, and seasonal variations11. Ecological studies on microbial communities may provide useful information on their capability of degradation of wastes by native microbes.

The Cachar paper mill (CPM), Panchgram is the only major industrial undertaking in south Assam and the adjoining states of Mizoram, Meghalaya and Tripura. This pulp and paper mill has an annual capacity of 1,00,000 tonnes of products and is utilizing bamboo as a raw material for paper production. The production process produced large amount of effluents, both liquid wastes generally discharged into water bodies and solid wastes such as wastewater treatment sludge, unused bamboo chips, Lime sludge and coal ash generally disposed as landfills. Land fillings are relatively cheap, so the industry takes little efforts for making more efficient use of its materials. The paper mill sludge consumes large percentage of local landfill space each year and also cause several hazards including - increased alkalinity of the soil, fires in waste materials, increase in the population of disease vectors, offensive odors, methane leakage, leaching of toxic and corrosive compounds to surface and ground waters etc. Moreover due to slow degradation of the wastes the aesthetic value of that area is lost16. Worst yet, burning of sludge in incinerators, contribute to serious air pollution problems. Sludge from pulp and paper mills mainly contains cellulose fiber and are recyclable organic solids.

Microorganisms in the site use the waste constituents as nutrients, thus detoxifying the materials as their digestive processes breakdown complex organic molecules into simpler less toxic molecules15.

The present work aims to study the diversity of bacteria and fungi and their relative occurrence from waste mixed soil of paper mill, South Assam. Isolation and characterization of cellulose degrading microorganisms i.e. bacteria and fungi were studied on different media. Cellulolytic microorganisms from this site are not explored to a large extent and there is scanty information available on cellulolytic enzymes associated with it.

**MATERIAL AND METHODS**

**Description of the site**

The solid wastes dumping sites of Cachar Paper mill, Panchgram, located in Barak valley, South Assam, India is selected for study purpose. This zone is geographically located between 24° 15' and 25° 92' N latitude and between 90° 16' and 93° 15' E longitude.

The climate of the area is subtropical, warm and humid. The average annual rainfall is 3180mm with an average of 146 rainy days per annum. The period from Dec- Feb is dry while the period May-Sept is usually featured by heavy rainfall with occasionally floods. The minimum and maximum annual temperature varies from 12.2°C-24°C in Dec-Jan to 28°C- 38°C in June-July. The relative humidity varies from 92 to 98 % in the morning and 43 to 78% in the evening. The texture of the soil varies from sandy to clay type with pH of 4.7 to 5.7 (acidic range).

Collection of samples

Samples (sludge mixed with soil) were collected randomly from four different locations of the paper mill solid wastes dumping sites at bimonthly interval. At each sites, the soil dug to a 20cm was scooped into sterilized polythene bag, labeled and brought to laboratory for analysis. The study comprises of the following:

Study of the Physiological characteristics of wastes

The physico-chemical characteristics of the samples such as moisture content, pH, NPK and organic % carbon content, cellulose content were determined. Microbial respiration rates were also determined to estimate the microbial activity in the site.

Total microbial community study of the Waste

For enumeration of total bacterial and fungal load in the samples, 100µl of each dilution was spread on pre-sterilized agar plates. For bacterial isolation, the aliquots were plated on nutrient agar (NA) and fungi plated on Rose Bengal Agar (RBA) media and Czapex Dox Agar (CDA) media with added antibiotics. The mixed colonies that appeared on the plates are noted and counted.

Identification of the isolates

The isolated bacterial colonies showing different morphological features were picked, restreaked several times on NA plates to get pure cultures of isolates. The isolates were identified following various morphological and biochemical methods. The parameters for biochemical investigation included colony morphology, color, size, nature of the growth, Gram staining, catalase test, citrate utilization test, motility test, methyl red test, voges-proskauer test, starch hydrolysis test, indole test, gelatinase production test, and growth in different pH, different mediums, temperatures and NaCl concentrations.

The isolated fungi from the mixed culture plates were subcultured repeatedly to get pure cultures. The isolated pure cultures of fungi were subjected to taxonomic studies by comparing with the ‘A manual of soil fungi’ by Gilman (1971). All the isolates were maintained at 4°C for future use.

Screening of isolates for cellulase production

To screen for cellulolytic organisms, the isolated bacterial and fungal isolates were grown on NA and RBA supplemented with 1% (w/v) carboxymethylcellulose. For primary screening, the isolates that grow on the above media were inoculated on fresh CMC agar plates, containing KH₂PO₄ (0.1%), (NH₄)₂SO₄ (0.4%), NaCl (0.6%), MgSO₄ (0.05%), CaCl₂ (0.01%), and Carboxy Methyl Cellulose (0.5%) as carbon source, incubated at 28-30°C for 2-3 days. The plates were observed for clear zone formation around the colonies produced by cellulose degraders after staining with 1% Congo red dye and destaining with 1M NaCl²⁰. The diameter of the clear zone and the colony was measured and the ratio of clear zone was calculated. Bacterial and fungal isolates showing clear zones were taken for further characterization.

Effect of Temperature and pH on the growth of the isolates

The bacterial and fungal isolates showing cellulolytic activity on plate screening were tested for their ability to tolerate and grow at different pH and temperature.

Antibiotic sensitivity tests of the bacterial isolates

The bacterial isolates were grown overnight in nutrient broth at 30°C and following disc diffusion method of Bauer et.al (1966), the test organisms were spreaded over the surface on Muller Hinton agar plates to make a uniform lawn culture of the isolates. Selective antibiotics discs were put on the plates and incubated at 30°C for 24hrs. The antibiotics were selected depending on their use and their mode of action. The test antibiotics used were Ampicillin, Tetracycline, Penicillin, Vancomycin, and Erythromycin. The zone of inhibition was measured with the help of scale and categorized as sensitive and resistant.

Statistical analysis of data

The correlation coefficient study between microbial population and soil physico-chemical properties was undertaken to study the effects of those factors on the growth of microbes.

RESULTS AND DISCUSSIONS

The physico-chemical characteristics of waste soil and their microbial diversity are presented (Table 1) and their correlation coefficients are also presented (Table 2). The present study revealed that moisture content and
soil pH played significant role on the microbial population of the waste dumping site. The microbial community and their activity in the waste soil increased with decreased in pH and increased in moisture content. The negative correlation between soil organic nitrogen content and microbial population is that the nitrogen is utilized by the microbes during their growth. Fungal population seemed to decrease when bacterial population increased, thereby showing that they respond differently to seasonal influence. Bacteria and fungi compete for simple plant-derived substrates and might have developed antagonistic relationship.

However, for more recalcitrant organic substrates, e.g. cellulose and lignin, both competitive and mutualistic strategies between bacteria and fungi appear to have evolved. A total of 33 (designated as D1-D33) bacterial colonies were isolated from the samples at different period of time. The study of morphological, physical and biochemical characteristics of bacteria on pseudo selective media showed that 61% were gram -ve, 23% hydrolyzed starch, 92% produced the enzyme catalase, 30% produced gelatinase, 61% utilized citrate, 15% were indole positive and 23% were methyl red positive. The isolated bacteria are most commonly aerobes or facultative anaerobes. The bacterial species belong to genera Bacillus, Streptococcus, Klebsiella, Staphylococcus, Pseudomonas, Serratia, Proteus, E. coli, Corynebacterium and Salmonella spp. The isolated fungal species were identified as Mucor, Rhizopus, Aspergillus, Penicillium, Fusarium, Gliocladium, Alternaria, Geotrichium spp, etc. (Oilman, 1957). Relative occurrence and isolation of the more species of genus Bacillus (39.3 %), Pseudomonas (33.3%), Streptomyces (18.1 %) among the bacteria and of genus Aspergillus (36.3%), Penicillium (22.7%) are probably due to their diverse and extensive enzyme system that protect them from other soil organisms. (Fig. 1 A and B). Based on the preliminary screening studies, 14 (42.4%) bacterial isolates shows signs of growth on CMC-NA agar, of which 6 isolates shows higher clear zone on CMC agar plates by Congo red test (Table 3). Of the 22 fungal species isolated, 13 (59.09%) fungal isolates grows on the CMC-RBA, of which only 4 responds to Congo red test (Table 3). Based
on the screening results on CMCase plates, these isolates with higher zone of activity were selected for further morphological, biochemical and physiological identification and characterization studies (Table 4 & 5). Studies revealed that the isolated bacterial strain D2 and D3 are rod shaped, gram negative, non spore forming bacteria which imparts green coloration to the medium characteristic of P.auruginosa. Moreover, the morphological characteristics having circular, raised, entire, creamish, opaque colony formation is in agreement with Carson et al. (1972) who also described the good growth of P. cepacia on citrate agar slants along with other positive tests for catalase, and negative tests for indole production and starch hydrolysis. Also no hydrogen sulfide production and rapid gelatinase activity which is also true in case of these two strain. Like Pseudomonas spp. D2 and D3 also shows the optimum growth around 40°C and no tolerance to acidic pH. From table 4, the D4, D6 and D11 was found to be rod shaped, gram positive bacteria, optimal growth temperature found to be around 40°C and pH tolerance at pH 5, like Bacillus strains. Like Bacillus it showed non-pigmented circular colony showing no growth at pH 4. The characteristic biochemical tests for those bacteria were found to be in agreement with Bacillus cereus and Bacillus licheniformis. e.g., positive results for catalase, gelatin, citrate utilization, hydrogen sulfide production and starch utilization and the isolates D7 produced pink-red pigment, Gram –ve rods, positive for catalase, indole, methyl red and motile, thus showed the characteristics with Serratia sp. Moreover, the fungal isolates showing higher zone of CMCase activity belong to genus Aspergillus spp and Penicillium sp. (Table 5). The fungal

<table>
<thead>
<tr>
<th>Table 2. Correlation coefficient between microbial populations with soil Physico-chemical characteristics</th>
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<tbody>
<tr>
<td>Parameter</td>
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<td></td>
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<td>pH</td>
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<tr>
<td>Moisture content (%)</td>
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<td>Nitrogen %</td>
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<td>(P₂O₅)</td>
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<td>K₂O</td>
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*Significant at P>0.05, ** significant at P>0.01

<table>
<thead>
<tr>
<th>Table 3. Screening of bacterial and fungal isolates by Congo red test for cellulolytic activity</th>
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<tbody>
<tr>
<td>Designated bacterial strain no.</td>
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<tr>
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</tr>
<tr>
<td>D11</td>
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<td>D6</td>
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<td>D3</td>
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<td>F14</td>
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<tr>
<td>F3</td>
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<td>F2</td>
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<tr>
<td>F8</td>
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</table>

*D11, D6, D3, D2, D4, and D7—referred to the designated bacterial isolates and
*F14, F3, F2 and F8 are referred to designated fungal isolates

genera of *Aspergillus* sp. and *Penicillium* sp. have been extensively studied due to their ability to secrete cellulose-degrading enzymes which help in industrial applications. They had the ability to produce cellulolytic enzymes which act synergistically in the conversion of cellulose to glucose. Also the characterized bacterial and fungal isolates showed extra cellular endoglucanase activity, having a wide range of tolerance to pH and temperature. Moreover the

Table 4. Biochemical and physiological characterization for identification of cellulose degrading bacteria (CDB)

<table>
<thead>
<tr>
<th>Characterization parameters</th>
<th>D11</th>
<th>D3</th>
<th>D6</th>
<th>D2</th>
<th>D4</th>
<th>D7</th>
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<td>6</td>
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<td>_ ve</td>
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<td>+ ve</td>
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<td>Gram's reaction</td>
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- no growth, ± slight growth, + moderate growth, ++ abundant growth
- _ve negative, +ve positive, for the biochemical tests

Table 5. Characterization of isolated cellulose degrading fungi

<table>
<thead>
<tr>
<th>Fungal Isolates</th>
<th>Identification</th>
<th>Physiological parameters</th>
</tr>
</thead>
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<tr>
<td>F8</td>
<td><em>Aspergillus niger</em></td>
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</tr>
<tr>
<td>F3</td>
<td><em>Pencillium funiculosum</em></td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td><em>Fusarium oxysporium</em></td>
<td>-</td>
</tr>
<tr>
<td>F14</td>
<td><em>Aspergillus fumigatus</em></td>
<td>-</td>
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</table>

* - means no growth, 2+ means moderate growth, 3+ means abundant growth

antibiotic sensitivity tests pattern showed that nearly 68% of the isolated bacteria were resistant to most commonly used antibiotics which are of major concern regarding to the use of antibiotics. The present study also revealed that the bacteria produced larger clear zone than that of fungi indicating the active role of bacteria in cellulosic waste degradation. Though fungal

cellulases have been widely studied but cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to high bacterial rate compared to fungi. Among bacteria *Pseudomonas aeruginosa*, *P. fluorescens*, *Cellulomonas spp.*, *Clostridium spp.*, *Bacillus spp.* etc. are found to have cellulosylic activity and well documented. Therefore the solid waste disposals sites of paper industry harbored the rich diversity of microbes which may prove to be the source of different enzymes of industrial importance.

**ACKNOWLEDGMENTS**

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**REFERENCES**


Screening of Fungi for Cellulose Degradation

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Department of Biotechnology, Assam University, Silchar

Abstract
Cellulolytic microorganisms are commonly distributed in soil, the chief cellulose utilizing species includes aerobic and anaerobic mesophilic bacteria, filamentous fungi etc. In the present study soil samples were collected from paper mill waste zone at Panchgram, Halakandi District of Assam. 22 fungal isolates were obtained from the soil samples by using selective media for cellulose degraders and screened for their cellulase activity. Four isolates were found positive for cellulase activity on primary plate screening by Congo red dye. The results revealed that the four isolates showing cellulolytic activity belong to Aspergillus spp. and Penicillium spp. The study suggests that the paper mill effluent zone harbors various fungi that are active in cellulose breakdown.

Key words: Cellulolytic microorganism, selective media, cellulase enzyme, antimicrobial activity.

Introduction
During the last decade, fungi have been used in the treatment of a wide variety of wastes, wastewaters and the role of fungi in the bioremediation of various hazardous and toxic compounds in soils and sediments has been established (Wang and Chen, 2009). Fungi have also demonstrated the ability to remove heavy metals and to degrade, in some cases mineralize, phenols, halogenated phenolic compounds, petroleum hydrocarbons, polycyclic aromatic compounds, and polychlorinated biphenyls (Duarte and Costa-Ferreira, 1994). The environmental implications of fungi were mainly focused on the recent developments in the paper industry effluent area (Paciulyte Dale, 2007). There is a definite need for new approaches to protect wood in use from fungal degradation, because increasing environmental concerns are limiting use of hazardous chemicals (Mai et al., 2004). The ability of most fungi to produce extracellular enzymes for the assimilation of complex carbohydrates without prior hydrolysis makes possible the degradation of a wide range of pollutants.

Fungi have world wide distribution and grow in a wide range of habitats including deserts, hypersaline environments, deep seas, rocks and in extremely low or high temperature.

A great deal of research activity has been directed towards increasing our understanding of the mechanism by which fungi affect cellulose in the natural environment. In Industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol, organic acids, detergents and other chemicals. They have also been used in the pulp and paper industry. Study concentrates mainly on fungi isolated from waste paper raw materials and substrates collected at the different stages of waste paper recycling to cardboard, on fungal ability to utilize cellulose as a carbon source and on the screening of hyper-producers of cellulase. Cellulases have enormous potential in industrial applications (Adeny et al., 1991). Glucose produced from cellulolic substrate could be further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as...
ethanol, butanol, methane, amino acids, single cell protein etc. Cellulases have been used for several years in food processing, feed preparation, wastewater treatment, detergent formulation, textile production and in other areas (Kapoor et al., 1978). Additional potential applications include the production of wine, beer and fruit juices. Nevertheless, all these uses are of rather small magnitude compared with cellulose requirement for bioconversion of lignocellulosic biomass to fuel ethanol.

In the present investigation, an attempt is made to screen the cellulase enzyme from cellulose degrading fungi and simultaneously characterization of those isolated fungi species.

Methodology

Soil samples were collected from Panchgram paper mill effluent disposal site. The pH (7.93) and moisture content (33.5%) of the soil was recorded. The isolation of fungi was done using enrichment method. Pure cultures were obtained on Carboxy methyl cellulose (CMC) agar plates. Biochemical test and morphological characterization were carried out. The plate screening on Congo red dye was carried out and also secondary screening too was done to analyze cellulase production.

The cellulase production the fungal isolates which show a positive cellulase activity during primary screening were cultured in a liquid Carboxy methyl cellulose (CMC) broth medium, pH 7.0 and autoclaved. 40 ml of the broth was taken in a sterilized flask and was inoculated with the fungal culture. The cultures were incubated at 37°C on a rotary shaker at 200 rpm for 7 days. After 7 days of incubation about 5ml of culture filtrate was taken aseptically and centrifuged at 10,000 rpm for 15 minutes at 4°C. The culture filtrate was analyzed for cellulase activities as protein content, carbohydrate content, enzyme assay and for antimicrobial activity for 3-4 consecutive day. Antimicrobial activity, protein assay (by Bradfords method), Anthrone carbohydrate assay and enzyme assay by Dinitrosalicylic (DNS) method were carried out.

Results

Twenty two species of fungi were isolated in a mixed cultured colony. They are as follows:

Table 1: Different types of isolated fungal strains

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>FUNGI SPECIES</th>
<th>STRAIN</th>
<th>FUNGI SPECIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Aspergillus spp.</td>
<td>F12</td>
<td>Dark white, isolates Unidentified</td>
</tr>
<tr>
<td>F2</td>
<td>Aspergillus spp.</td>
<td>F13</td>
<td>Aspergillus spp.</td>
</tr>
<tr>
<td>F3</td>
<td>Spongy white, isolates Unidentified</td>
<td>F14</td>
<td>Yellow spores, isolates Unidentified</td>
</tr>
<tr>
<td>F4</td>
<td>Aspergillus spp.</td>
<td>F15</td>
<td>White thread like colony, isolates Unidentified</td>
</tr>
<tr>
<td>F5</td>
<td>Aspergillus spp.</td>
<td>F16</td>
<td>Aspergillus spp.</td>
</tr>
<tr>
<td>F6</td>
<td>Aspergillus spp.</td>
<td>F17</td>
<td>Aspergillus spp.</td>
</tr>
<tr>
<td>F7</td>
<td>Brown spores, isolates Unidentified</td>
<td>F18</td>
<td>Aspergillus spp.</td>
</tr>
<tr>
<td>F8</td>
<td>Aspergillus spp.</td>
<td>F19</td>
<td>Broom shaped, white colony, isolates Unidentified</td>
</tr>
<tr>
<td>F9</td>
<td>Spongy white, isolates Unidentified</td>
<td>M2</td>
<td>Aspergillus spp.</td>
</tr>
<tr>
<td>F10</td>
<td>Spongy green, spores brown in colour, isolates Unidentified</td>
<td>M3</td>
<td>Penicillium spp.</td>
</tr>
<tr>
<td>F11</td>
<td>Spongy white, isolates Unidentified</td>
<td>M4</td>
<td>Aspergillus spp.</td>
</tr>
</tbody>
</table>
Table 2: Identification and characterization of the fungi isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony Characterisation</th>
<th>Morphological Characterisation</th>
<th>Identifie Disolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Greyish white, velvety in structure</td>
<td>Conidia round, septate</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td>F8</td>
<td>Blackish white, velvety in appearance</td>
<td>Conidia round, septate</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td>F14</td>
<td>Dark brown, velvety in appearance</td>
<td>Conidia round, non-septate</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td>M2</td>
<td>Spongy black, thread like appearance</td>
<td>Conidia elliptical, non-septate</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td>M3</td>
<td>Greenish white, velvety in appearance</td>
<td>No conidia were found. non-septate</td>
<td>Penicillium sp.</td>
</tr>
<tr>
<td>M4</td>
<td>Yellowish white, velvety in appearance</td>
<td>Conidia round, non-septate</td>
<td>Aspergillus sp.</td>
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</tbody>
</table>

Out of 22 isolates, 13 isolates were identified and characterized. 6 isolates were taken after identification, characterization and comparing of one isolates with another. All the 6 fungal isolates were then transferred to pure culture media.

Table 3: Primary screening for the fungal isolates

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>CLEAR ZONE DIAMETER(cm)</th>
<th>COLONY DIAMETER(cm)</th>
<th>CLEAR ZONE VALUE(cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>2.2</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>M3</td>
<td>2.2</td>
<td>1.5</td>
<td>1.46</td>
</tr>
<tr>
<td>M2</td>
<td>2.1</td>
<td>1.6</td>
<td>1.31</td>
</tr>
<tr>
<td>F14</td>
<td>2.7</td>
<td>2.2</td>
<td>1.22</td>
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*Clear zone ratio = Clear zone diameter / Colony diameter
Table 4: Effect of pH on the fungal isolates

<table>
<thead>
<tr>
<th>STRAIN</th>
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<th>pH 7.0</th>
<th>pH 8.0</th>
<th>pH 9.0</th>
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</thead>
<tbody>
<tr>
<td>F8</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>M3</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>F14</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

+++ shows good growth, ++ shows moderate growth, - shows no growth.

Table 5: Effect of temperature on the fungal isolates

<table>
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<th>Strain</th>
<th>Temperature 20°C</th>
<th>Temperature 25°C</th>
<th>Temperature 37°C</th>
<th>Temperature 40°C</th>
<th>Temperature 50°C</th>
<th>Temperature 60°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>++</td>
<td>+++++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>F14</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

+++ shows good growth, ++ shows moderate growth, - shows no growth.

Discussion

In most investigations, members of the fungal genus *Aspergillus* sp. and *Penicillium* sp. have been extensively studied due to their ability to secrete cellulose degrading enzymes which help in industrial applications (Begum & Aubert, 1994). Their ability to produce cellulolytic enzymes, which acts synergistically in the conversion of cellulose to glucose. Fungi reveal many impacting factors of fungal number and its diversity in the paper recycling factories (Duncun et al., 2008). During our investigation, weight of the collected soil was taken to analyzed the moisture content in the soil. This is required to get an idea about the microorganism which can grow at particular moisture content (Jain, 2006). The pH of the collected soil was also measured to analyse the soil environment as acidic or alkaline (Jain, 2006). Among the 22 fungal strains isolated,
13 strains were identified and characterized. From that, 6 strains were grown on a pure culture plate and thus their complete identification and characterizations was done. These 6 strains are only taken because, they have the most distinguish colonies among the 13 strains. Among the 6 isolates identified 4 isolates shown positive response towards primary screening by Congo red dye. Observing the clear zones it may be said that the 4 isolates may have the enzyme system to convert cellulose to glucose. After that, the 4 isolates were analysed for any variation in their physiological conditions (i.e. pH, temperature). This study was carried out to analyse the optimum conditions (i.e. pH and temperature) (Table 4 & Table 5) needed for the growth of those 4 fungal isolates. This study also refers that the 4 fungal isolates may be mesophilic in nature (Jain, 2006). As the results shows no formation of clear zone in the plates which is mainly done by the release of enzymes which degrade the substrate in the plate. So it refers that the 4 fungal strains may not have pharmaceutical use. After that, protein concentration (Fig 1) and carbohydrate concentration was measured to study the amount of protein and carbohydrate in the particular culture broth. After studying the graphs it was found that protein content decreases day by day and carbohydrate content increases vice-versa. Thus it gives an idea that the culture broth may contain carbohydrate degrading enzymes which helps to convert cellulose to glucose. Further investigation is carried out by calculating the concentration of enzyme (i.e. cellulase) and finally its activity by dinitroslicyclic (DNS) method (Duncan, 2008). The microorganism have the capability to release some enzymes which helps in economical purposes. From the graph it is concluded that there is a very moderate amount of enzyme in the culture broth which helps in the process of degradation of cellulose. It can be said that the 4 fungal strains which were isolated, identified, and screened may be used as an important component for industrial purposes (i.e. in the process of fermentation, in the treatment of effluents, in the process of biodegradation). After investigation, it can be said that the 4 fungal strains may have an efficient enzyme system which have the capacity to degrade cellulose more in the culture broth if they were kept for 14 to 20 days in an incubator. So, from this study we can conclude that, optimum pH, temperature, incubation period and carbon sources are important limiting factors for the maximum cellulase production. Further works with the split of each factor and interactions of factors may provide clear picture about maximum cellulose production and optimum cellulose activity in the procedure of our isolates, which is variable for species to species.

References


Microbial Diversity in Solid Wastes Disposal from Paper Industry, South Assam

Durga Sharma and G.D. Sharma*

Abstract

Microbial diversity constitutes the most extraordinary reservoir of life in the biosphere that we have only just begun to explore and understand. A preliminary investigation on the microbial diversity of paper mill effluents organic wastes from the landfill site of Hindustan Paper Corporation, South Assam and their isolation, characterization by the use of different media may generate information on the nature characteristics, and further degrading efficiency of these hazardous wastes by the native micro-organisms. Samples (sludge mixed with soil) were collected randomly from paper mill solid wastes dumping sites and their physico-chemical characteristics such as pH, moisture content, NPK and Cellulose content were determined. The total microbial community study was also undertaken. The study indicated that increased moisture content of soil during rainy season lowers soil pH and favors the growth of microbial population both bacteri... and fungi. The negative correlation between the soil available nitrogen and microbial population is that the soil nitrogen is utilized by the microbes during their growth.

Key words: Soil, Organic wastes, Bacterial diversity, Bacterial isolation, Identification.

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* Department of Life Sciences, Assam University, Silchar-788011, Assam
Introduction

Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. Thus there is a general interest in studying the diversity of indigenous microorganisms capable of degrading different pollutants because of their varied effects on the environment. Paper industry produces large amount of solid wastes (both organic and inorganic), and are disposed as landfills that may have significant environmental impacts. The present paper deals with microbial diversity of paper industry effluents with specific emphasis on microorganisms degrading organic wastes. Microorganisms in waste dump use constituents as nutrients, thus detoxifying the materials as their digestive processes breakdown complex organic molecules into simpler less toxic molecules.

Materials and Methods

Selection of site

The solid wastes dumping sites of Hindustan Paper Corporation Limited, Panchgram, a paper industry located in Barak valley, South Assam, India has been selected for study purpose. This zone is geographically located between 24° 15'2" and 25° 9'2" N latitude and between 90° 16'2" and 93° 15'2" E longitude. The climate of the area is subtropical, warm and humid. The average annual rainfall is 3180mm with an average of 146 rainy days per annum. The period from Dec- Feb is dry while the period May-Sept is usually featured by heavy rainfall with occasionally floods. The minimum and maximum annual temperature varies from 12.2°C-24°C in Dec-Jan to 28°C- 38°C in June-July. The relative humidity varies from 92 to 98 % in the morning and 43 to 78% in the evening. The texture of the soil varies from sandy to clay type with pH of 4.7 to 5.7 (acidic range).

Collection of samples

Samples (sludge mixed with soil) were collected randomly
from four different locations of the paper mill solid wastes dumping sites at bimonthly interval. At each sites, the soil dug to a 20cm were scooped into sterilized polythene bags, labeled and bought to laboratory for analysis. The study comprises of the following-

A. Study of the Physiological characteristics of wastes

The physico-chemical characteristics of the samples such as moisture content, pH, texture, NPK and Organic % Carbon Content, cellulose content were determined (Rowel, 1995). Microbial respiration rate was also determined to estimate the percentage of microbial activity in the site.

B. Total microbial community study in the Wastes

For enumeration of total bacterial and fungal load in the samples, 0.1 ml or 100 µl of 10⁻⁵ for bacteria and 10⁻³ for fung each dilution was spread on pre-sterilized agar plates. For bacterial isolation, the aliquots were plated on nutrient agar (NA) and fungi plated on Rose Bengal Agar (RBA) were and Czapex Dox Agar (CDA) media with added antibiotics. The mixed colonies that appeared on the plates were noted and counted.

C. Effect of soil physico-chemical characteristics

The correlation coefficient study between microbial population and soil physico-chemical properties was undertaken to study the effects of these factors on the growth of microbes.

D. Biochemical characterization of bacterial isolate

The parameters for investigation included colony morphology, color, size, nature of the growth, Gram staining, catalase test, citrate utilization test, motility test, methyl red test, voges-proskauer test, starch hydrolysis test, indole test, gelatinase production test, and growth in different pH, temperatures and NaCl concentrations (Cappuchino and Sherman, 2001).

The isolated fungi from the culture plates were mounted on a glass slide stained with lacto phenol cotton blue dye and observed under microscope.
All the isolated bacterial and fungal strains were grown in selective media containing cellulose as a carbon source and their growth was measured spectrophotometrically.

Results and Discussion

Total microbial count and physico-chemical characteristics of waste soil are presented in Table 1. The present study revealed that moisture content and soil pH played a significant role on the microbial population of the waste dumping site. Fungi population seemed to decrease when bacterial population increased, thereby showing that they respond differently to seasonal influence. Bacteria and fungi compete for simple plant-derived substrates and might have developed antagonistic relationship. However, for more recalcitrant organic substrates, e.g. cellulose and lignin, both competitive and mutualistic strategies between bacteria and fungi appear to have evolved (Wietse et al., 2005).

**TABLE 1: Total microbial count and physico-chemical characteristics of waste soil.**

<table>
<thead>
<tr>
<th>Sampling period</th>
<th>Bacterial population/g of soil ($\times 10^2$)</th>
<th>Fungal population/g of soil ($\times 10^5$)</th>
<th>pH of soil</th>
<th>Moisture (%)</th>
<th>Nitrogen (%)</th>
<th>Phosphorus Kg/ha</th>
<th>Potassium Kg/ha</th>
<th>Microbial respiration rate $10^{-10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>June, 07</td>
<td>76.5</td>
<td>12.35</td>
<td>5.6</td>
<td>66.0</td>
<td>1.15</td>
<td>35</td>
<td>320</td>
<td>57.0</td>
</tr>
<tr>
<td>Sept, 07</td>
<td>17.4</td>
<td>22.23</td>
<td>6.2</td>
<td>48.4</td>
<td>1.7</td>
<td>23.8</td>
<td>245</td>
<td>45.0</td>
</tr>
<tr>
<td>Dec. 07</td>
<td>34.3</td>
<td>26.8</td>
<td>5.9</td>
<td>25.5</td>
<td>2.25</td>
<td>22.5</td>
<td>275</td>
<td>22.0</td>
</tr>
<tr>
<td>Mar. 08</td>
<td>501</td>
<td>6.4</td>
<td>5.7</td>
<td>40.8</td>
<td>1.88</td>
<td>33.6</td>
<td>572.0</td>
<td>34.0</td>
</tr>
<tr>
<td>June, 08</td>
<td>65</td>
<td>14.7</td>
<td>5.6</td>
<td>54.0</td>
<td>3.13</td>
<td>28.3</td>
<td>330</td>
<td>55.0</td>
</tr>
<tr>
<td>Sept. 08</td>
<td>26.28</td>
<td>17.2</td>
<td>6.1</td>
<td>42.3</td>
<td>2.75</td>
<td>32.5</td>
<td>352.5</td>
<td>49.0</td>
</tr>
</tbody>
</table>

The correlation study indicated that soil fungal population showed significant and negative correlation with soil pH but highly significant and positive correlation with available phosphorus in
soil while bacterial population shows positive correlation with soil moisture content. (Fig. 1 and 2)

![Correlation coefficients between bacterial population and soil physico-chemical properties](image)

**FIGURE 1**: Correlation coefficient between bacterial population and soil physico-chemical properties

The study of morphological, physical and biochemical characteristics of bacteria on selective media showed that 61% were gram−ve, 23% hydrolyzed starch, 92% produced the enzyme catalase, 30% produced gelatinase, 61% utilized citrate, 15% were indole positive and 23% were methyl red positive (fig.3). The isolated bacteria are most commonly aerobic or facultative anaerobes.

The solid waste disposals of paper industry harbored the rich diversity of microbes. Based on the above cultural and biochemical characterization, the isolated pure cultures of bacteria have been partially identified. The bacterial species belong to *Bacillus, Klebsiella, Staphylococcus, Pseudomonas, Serratia, Corynebacterium*, and *Salmonella*. Some of the fungal species were identified as *Mucor*. 
Correlation coefficients between Fungal population and soil physicochemical properties:

![Correlation Coefficient Chart](chart.png)

**FIGURE 2:** Correlation coefficient between fungal population and soil physico-chemical properties

![Biochemical Analysis Chart](chart2.png)

**FIGURE 3:** Biochemical Characterization of Bacterial Isolates
Rhizopus, Aspergillus spp, Penicillium spp, Fusarium spp, Trichoderma spp, etc by comparing with Manual of soil Fungi (Gilman, 1957).

Moreover, the growth of majority of the bacterial and fungal isolates on cellulose enrichment media, i.e. both on CMC agar plates and broth indicates the utilization of these compounds by the native microorganisms present in the wastes.

REFERENCES


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Bacillus sp. enrichment culture clone AUBT-11 16S ribosomal RNA gene, partial sequence

GenBank: GU449103.1

FASTA Graphics

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| Sharma,D., Sharma,G.D., Ghosh,S.K. and Podille,A.R.
| Isolation and characterization of cellulose degrading bacteria from wastes of paper industry, South Assam, India
JOURNAL Unpublished
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**GenBank**: JN416563.1

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          Bacteria; Firmicutes; Bacillales; Bacillaceae; Lysinibacillus.
REFERENCE 1 (bases 1 to 1475)
AUTHORS  Sharma, D., Sharma, G.D., Ghosh, S.K. and Joshi, S.R.
TITLE     Isolation of organic wastes degrading bacteria from paper industry wastes
JOURNAL   Unpublished
REFERENCE 2 (bases 1 to 1475)
AUTHORS  Sharma, D., Sharma, G.D., Ghosh, S.K. and Joshi, S.R.
TITLE     Direct Submission
JOURNAL   Submitted (01-AUG-2011) Biotechnology, Assam University, Dargakona, Silchar, Assam 788011, India
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GenBank: GU449104.1

**FASTA Graphics**

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VERSION   GU449104.1  G1:294486200
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REFERENCE 1 (bases 1 to 702)
          Sharma, D., Sharma, G.D., Ghosh, S.K. and Podille, A.R.
          Isolation and characterization of cellulose degrading bacteria from wastes of paper industry, South Assam, India
TITLE    Direct Submission
JOURNAL  Submitted (07-JAN-2010) Biotechnology, Assam University, DargaKona, Silchar, Assam 788011, India
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          Submitted (07-JAN-2010) Biotechnology, Assam University, DargaKona, Silchar, Assam 788011, India
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GenBank: GU390655.1

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**ACCESSION** GU390655

**VERSION** GU390655.1 GI:294486197

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**ORGANISM** Bacillus sp. enrichment culture clone AUBT-24

Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; environmental samples.

**REFERENCE** 1 (bases 1 to 762)

**AUTHORS** Sharma,D., Sharma,G.D., Ghosh,S.K. and Podille,A.R.

**TITLE** Isolation and characterization of cellulose degrading bacteria from wastes of paper industry, South Assam, India

**REFERENCE** 2 (bases 1 to 762)

**AUTHORS** Sharma,D., Sharma,G.D., Podille,A.R. and Ghosh,S.K.

**JOURNAL** Direct Submission

Submitted (06-JAN-2010) Biotechnology, Assam University, DargaKona, Silchar, Assam 788011, India

**FEATURES**

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**GenBank: JN416564.1**

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Achromobacter sp. AUBT 13

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REFERENCE 1 (bases 1 to 1343):
       AUTHORS Sharma, D., Sharma, G.D., Ghosh, S.K. and Joshi, S.R.
       TITLE Isolation of bacteria degrading organic wastes of paper industry, south Assam, India
       JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1345):
       AUTHORS Sharma, D., Sharma, G.D., Ghosh, S.K. and Joshi, S.R.
       TITLE Direct Submission
       JOURNAL Submitted (01-AUG-2011) Biotechnology, Assam University, Dargakona, Silchar, Assam 788011, India

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TITLE Isolation and characterization of bacteria degrading organic wastes from paper industry, Assam
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1458):
TITLE Direct Submission
JOURNAL Submitted (01-AUG-2011) Biotechnology, Assam University, Dargakona, Silchar, Assam 788011, India
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Pseudomonas aeruginosa strain AUBT-7 16S ribosomal RNA gene

Pseudomonas aeruginosa strain AUBT-7 16S ribosomal RNA gene, partial sequence

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Serratia sp. enrichment culture clone AUBT-17 16S ribosomal RNA gene, partial sequence

GenBank: GU449102.1

FASTA Graphics

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827 bp DNA  linear  ENV 15-APR-2010

Serratia sp. enrichment culture clone AUBT-17 16S ribosomal RNA gene, partial sequence.

GU449102

GU449102.1  GI:294486198

Serratia sp. enrichment culture clone AUBT-17

Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Serratia; environmental samples.

Sharma, D., Sharma, G.D., Ghosh, S.K. and Podille, A.R.

Isolation and characterization of cellulose degrading bacteria from wastes of paper industry, South Assam, India

Unpublished


Direct Submission

Submitted (07-JAN-2010) Biotechnology, Assam University, Dargakona, Silchar, Assam 788011, India

Location/Qualifiers

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Enterobacter sp. AUBT-sd 16S ribosomal RNA gene, partial sequence

GenBank: HQ316919.1

FASTA Graphics

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REFERENCE 1 (bases 1 to 890)
    Sharma, D., Sharma, G.D., Ghosh, S.K. and Podille, A.R.
    Isolation and molecular characterization of facultative anaerobic cellulose degrading bacteria from solid wastes of paper industry, south of Assam, India
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 890)
    Direct Submission
    Submitted (26-SEP-2010) Biotechnology, Assam University, DargaKona, Silchar, Assam 788011, India
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#### GenBank

**Klebsiella sp. AUBT-bd 16S ribosomal RNA gene, partial sequence**

**GenBank:** HQ316920.1

**FASTA Graphics**

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**DEFINITION** Klebsiella sp. AUBT-bd 16S ribosomal RNA gene, partial sequence.

**ACCESSION** HQ316920

**VERSION** HQ316920.1 G1:327178017

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**ORGANISM** Klebsiella sp. AUBT-bd

Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Klebsiella.

**REFERENCE** 1 (bases 1 to 882)

**AUTHORS** Sharma,D., Sharma,G.D., Ghosh,S.K. and Podille,A.R.

**TITLE** Isolation and molecular characterization of anaerobic cellulose degrading bacteria from wastes of paper industry, south of Assam, India

**JOURNAL** Unpublished

**REFERENCE** 2 (bases 1 to 882)

**AUTHORS** Sharma,D., Sharjna,G.D., Podille,A.R. and Ghosh,S.K.

**TITLE** Direct Submission

**JOURNAL** Submitted (26-SEP-2010) Biotechnology, Assam University, DargaKona, Silchar, Assam 788011, India

**FEATURES** Location/Qualifiers

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841 gcgatcaagat gggtgagatg atgtctggga tggaggggga taactgcctga tggaggggga taactactgg aaacggtagc

//
Bacterium AUBT-3 16S ribosomal RNA gene, partial sequence

GenBank: HM017976.1

FASTA Graphics

**DEFINITION**
Bacterium AUBT-3 16S ribosomal RNA gene, partial sequence.

**ACCESSION**
HM017976

**VERSION**
HM017976.1

**KEYWORDS**
Bacterium AUBT-3

**SOURCE**
Bacterium AUBT-3

**ORGANISM**
Bacterium AUBT-3

**REFERENCE**
1. (bases 1 to 735)

**AUTHORS**

**TITLE**
Isolation and characterization of cellulose degrading bacteria from paper industry wastes disposal sites

**REFERENCE**
2. (bases 1 to 735)

**AUTHORS**

**TITLE**
Direct Submission

**JOURNAL**
Submitted (21-MAR-2010) Biotechnology, Assam University, Dargakona, Silchar, Assam 788011, India

**FEATURES**
Location/Qualifiers

- **source**
  - (bases 1 to 735)
  - /organism="bacterium AUBT-3"
  - /mol_type="genomic DNA"
  - /strain="AUBT-3"
  - /isolation_source="paper industry wastes disposal site"
  - /db_xref="taxon:2i^ia2"
  - /country="India: South Assam"
  - /note="PCR_primers=fwd_name: F-27, rev_name: R-1489"

- **rRNA**
  - (bases 1 to 735)
  - /product="16S ribosomal RNA"

**ORIGIN**

1. ctaataccggt atacaattttg qaaccgcctag gttcuaatagq gaaagcgccc cttgctgctca
2. cttatatgatg gtacgcgtcc cattacgta tgcctgaagg taaccgcctta ccaagggacac
3. gatacgtagc cgacctgaga gggtgatcgg ccacactgga actgagacac ggtccagact
4. ccctacggggt gcaccaaatg gcctagcctc gcaaatctct gcgaatggcgc gggcccgaccc
5. cgccttggga gttacggaagc...
CALCULATION OF CELLULASE ASSAY

Derivation of the CMC Unit:

The unit of CMC is based on the International Unit (IU) and its calculation is analogous to that of the unit of FPU. It is a non-linear assay, and the results are expressed simply as units per milliliter.

\[ 1 \text{ IU} = 1 \mu \text{mol min}^{-1} \text{ of liberated hydrolysis product} \]
\[ = 0.18 \text{ mg min}^{-1} \text{ when the product is glucose} \]

The critical amount of glucose in the CMC assay is 0.5 mg:

\[ 0.5 \text{ mg glucose} = 0.5/0.18 \mu\text{mol} \]

This amount of glucose was produced by 0.5 ml in 30 mm, i.e., in the CMC reaction. Therefore,

\[ 0.5 \]

\[ 0.5 \text{ mg glucose} = \frac{0.5}{1.8 \times 0.5 \times 30} \mu\text{mol min}^{-1} \text{ ml}^{-1} \]

\[ \text{CMC} = 0.185 \mu\text{mol min}^{-1} \text{ ml}^{-1} \text{ (IU ml}^{-1}) \]
<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Mills</th>
<th>Capacity</th>
<th>Investment (Rs. in crores)</th>
<th>Raw Material</th>
<th>Commencement of Commercial Production</th>
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<td>1,00,000 (Writing &amp; Printing paper including 20,000 MT Newsprint)</td>
<td>287</td>
<td>Bamboo</td>
<td>October, 1985</td>
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<td>1,00,000 (Newsprint)</td>
<td>158</td>
<td>Eucalyptus Grandis &amp; Hybrids Reed &amp; Bamboo</td>
<td>November, 1982</td>
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<td>Bamboo &amp; Reed</td>
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