APPENDIX
A. Media composition

A.1 Mineral Salt media (single strength) for 1L:
(Seubert, 1960; Mahtani and Mavinkurve, 1979)

Ferrous sulphate (green crystals) 0.06 gm
Dipotassium hydrogen orthophosphate(12.6%) 100 mL
Potassium dihydrogen ortho phosphate (18.2%) 20 mL
Ammonium nitrate (10%) 20 mL
Magnesium sulphate (1%) 20 mL
Manganese sulphate (0.6%) 0.2 mL
Sodium molybdate (0.6%) 0.2 mL
Calcium chloride (dihydrate) (1%) 15 mL
Double Distilled Water 1000 mL
pH adjusted to 7.5 with 0.1 N NaOH

For use: 10 mL of double-strength media made to 20 mL with Double Distilled Water and sterilized for 10 min at 120°C temperature and 15 lbs pressure. To prepare MSM agar, MSM broth is mixed with agar (1.5%) and autoclaved accordingly.

A.2 Nutrient Broth

Peptone 10.0 gm
Beef extract 3.0 gm
Sodium-chloride 5.0 gm
Double Distilled Water 1000 mL
pH was adjusted to 7.5 with 0.1 N NaOH

For nutrient agar, 1.5 gm of agar added to 100 mL nutrient broth. Digested in water-bath and sterilized accordingly.

A. 3 Luria Bertani (L.B) Broth (Gerhardt et al., 1994)

Tryptone 10.0 gm
Yeast extract 5.0 gm
Sodium Chloride 10.0 gm
Double Distilled Water 1000 mL
Adjusted to pH 7.5 with 0.1 N NaOH

For L.B agar, 1.5 gm of agar is added to 100 ml L.B broth. Digested in water-bath and sterilized accordingly.
### A.4 Zobell marine Broth (Zobell, C.E. 1941)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>5 gm</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1 gm</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>19.45 gm</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>8.8 gm</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>1.8 gm</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.55 gm</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>3.24 gm</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.16 gm</td>
</tr>
<tr>
<td>Potassium bromide</td>
<td>0.08 gm</td>
</tr>
<tr>
<td>Strontium chloride</td>
<td>0.034 gm</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.22 gm</td>
</tr>
<tr>
<td>Sodium silicate</td>
<td>0.004 gm</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>0.0024 gm</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>0.0016 gm</td>
</tr>
<tr>
<td>Disodium Phosphate</td>
<td>0.008 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>15 gm</td>
</tr>
<tr>
<td>Double Distilled Water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

Adjusted to pH 7.5 with 0.1 N NaOH

For agar plates add 1.5% agar to Zobell marine broth and sterilized (autoclaved).

### A5. Thiosulfate Citrate Bile Sucrose Agar (TCBS)

Selective agar for *Vibrio* sp. *(Kobayashi et al, 1963)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone from casein</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Peptone from meat</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Sodium thiosulfate</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Ox bile</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>3.0 gm</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Iron (III) citrate</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Thymol blue</td>
<td>0.04 gm</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.04 gm</td>
</tr>
</tbody>
</table>
Agar-agar 14.0 gm

Preparation: Suspend 88 g/liter and pour plates, Do not autoclave. pH: 8. ± 0.2 at 25 °C.
The plates are clear and green-blue.

A6. Hugh Leifson's medium
Peptone 2 gm
Sodium Chloride 4 gm
DiPotassium Hydrogen Phosphate 0.3 gm
Bromothymol Blue 2 mL
Agar 4 gm
Glucose (10%) 20 mL
Distilled Water 1 Lt
pH 7.5
5 mL glucose (stock) was sterilized separately and 0.1 mL added to sterilized medium.

A7. Peptone sugar medium for sugar utilization.
Peptone 10 gm
NaCl 5 gm
Phenol red 0.2 gm
Distilled Water 1 Lt
pH 7.2
5 mL of medium was dispensed in test tubes along with Durhams’s tubes and sterilized sugars (10% stock) were sterilized separately at 15 psi for 10 min and 0.5 mL of sugar solution was added to sterilized medium.

A8. Muller's Hinton Agar (For antibiotic assay).
Meat infusion 2.0 gm
Casein hydrolysate 17.5 gm
Starch 1.5 gm
Agar 13.0 gm
pH 7.4±0.2 at 25°C

B Gram staining reagents:
1. Primary stain
Crystal violet (8.5%) dye 20 gm
C Reagents for Estimation of Salinity, Alkalinity, Organic content (Dissolved oxygen), Nitrite, Nitrate and Phosphate contents.

C.1. Reagents for estimation of Salinity of environmental samples:

1. AgNO₃ solution:
   Pure crystallized Silver nitrate (27.25 gm) was dissolved in 1L of double distilled water. This solution was mixed well and stored in amber colored bottle.

2. Potassium chromate indicator solution:
   Pure potassium chromate (10 gm) was dissolved in 100 mL of double distilled water.

3. Harvey correction table

<table>
<thead>
<tr>
<th>Burette Readling (mL)</th>
<th>Correction</th>
<th>Burette reading (mL)</th>
<th>Correction</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>-0.15</td>
<td>24</td>
<td>+0.20</td>
</tr>
<tr>
<td>38</td>
<td>-0.08</td>
<td>22</td>
<td>+0.22</td>
</tr>
<tr>
<td>36</td>
<td>-0.03</td>
<td>20</td>
<td>+0.23</td>
</tr>
<tr>
<td>34</td>
<td>+0.03</td>
<td>18</td>
<td>+0.23</td>
</tr>
<tr>
<td>32</td>
<td>+0.07</td>
<td>16</td>
<td>+0.23</td>
</tr>
<tr>
<td>30</td>
<td>+0.11</td>
<td>14</td>
<td>+0.20</td>
</tr>
<tr>
<td>28</td>
<td>+0.15</td>
<td>12</td>
<td>+0.19</td>
</tr>
<tr>
<td>26</td>
<td>+0.17</td>
<td>10</td>
<td>+0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>+0.15</td>
</tr>
</tbody>
</table>
C.2. Reagents for estimation of Alkalinity of environmental samples:

1. 0.1N HCl:

This solution is prepared by dissolving about 9.0 ml of Concentrated HCl in one litre of distilled water.

2. Methyl orange indicator (0.05%):

Dissolve 0.5 gm of methyl orange in 100 mL of distilled water.

C.3. Reagents for Organic content (Dissolved oxygen)

1. Winklers A reagent:

40 gm of manganous chloride is dissolved and made up to 100 mL distilled water.

2. Winklers B reagent:

60 gm of potassium iodide and 30 gm of potassium hydroxide are dissolved separately in minimum amount of distilled water and combined. The solution is made up to 100 mL with distilled water.

3. H₂SO₄ solution:

50 ml of concentrated Sulphuric acid is added carefully to 50 mL of distilled water.

4. Sodium thiosulphate method:

0.25 gm of Na₂S₂O₃ is dissolved in distilled water and volume is made up to 100 mL.

5. Starch solution:

1 gm of starch is dissolved in distilled water by boiling and the starch solution is made up to 100 mL with distilled water after cooling.
6. Potassium iodide solution:

0.3567 gm of potassium iodide is dissolved carefully and made up to 1000 mL with distilled water.

C4. Reagents for Nitrite estimation

1. Sulphanilamine:
   Sulphanilamine (1 gm) was dissolved in 10 mL concentrated HCl and the solution was made up to 100 ml with double distilled water.

2. N-(1-napthyl) ethylene di-amine di-hydrochloride:
   In 100 mL of double distilled water, 0.1 gm of reagent was dissolved.

3. Standard NaNO₂ solution:
   Pure analytical grade NaNO₂ (0.1725 gms) was dissolved in 250 mL of double distilled water (1 mL contains 10 μg atom of NO₂-N).

4. Working solution A:
   Above mentioned standard NaNO₂ (2.5 mL) solution was diluted to 250 mL with double distilled water (1 mL contains 0.1 μg atom of NO₂-N).

5. Working solution B:
   Working solution A (50 mL) was diluted to 500 mL with distilled water. (1 mL contains 0.01-μg atom of NO₂-N).

C5. Reagents for Nitrate estimation

1. Concentrated Ammonium Chloride (NH₄Cl):
   NH₄Cl (62.5 gm) was dissolved in a 250 mL volumetric flask with distilled water.

2. Diluted Ammonium Chloride (NH₄Cl):
   The above concentrated NH₄Cl (5 mL) was diluted to 200 mL with distilled water.

3. Amalgamated Cadmium granules:
   This reagent (100 gm) is treated with 500 mL of 2 % solution of CuSO₄. Then
the amalgamated Cadmium granules are washed several times with distilled water and stored in dilute NH₄Cl.

4. Sulphanilamide:

The reagent (1 gm) was dissolved in 10 mL of concentrated HCl and the volume was made to 100 mL with distilled water.

5. N-(1-napthyl)- ethylene diamine dihydrochloride :

The reagent (0.1 gm) was dissolved in 100 mL of distilled water to prepare the stock solution of the reagent.

6. Standard KNO₃ solution:

KNO₃ (0.1 gm) was dissolved in 100 mL of distilled water.

7. Working solution of KNO₃ (standard):

The above solution (2.5 mL) was diluted to 25 mL with distilled water.

C6. Reagents for Phosphate estimation:

1. 9 N H₂SO₄:

Concentrated H₂SO₄ (25 mL) was added to 75 mL double distilled water.

2. Ammonium molbydate solution:

Ammonium molbydate (9.5 gm) was dissolved in 100 mL of double distilled water.

3. Ascorbic acid:

Ascorbic acid (7 gm) was dissolved in 100 mL double distilled water.

4. Potassium antimonyl tartarate:

Potassium antimonyl tartarate (3.25 gms) was dissolved in 100 mL Double distilled water.

5. Mixed Reagent:

Ammonium molbydate (22.5 mL) solution, 100 mL of H₂SO₄ and 2.5 mL of Potassium antimonyl tartarate solution was mixed together.

6. Standard phosphate solution:

Potassium dihydrogen ortho phosphate (KH₂PO₄) 0.1361 gms was dissolved in 100 mL of double distilled water.
D. Scanning Electron Microscopy (S.E.M Sample preparation)

<table>
<thead>
<tr>
<th>Concentration Acetone %</th>
<th>Volume Acetone</th>
<th>Volume Distilled water</th>
<th>Total Volume</th>
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<td>10</td>
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<td>10</td>
<td>50</td>
</tr>
<tr>
<td>90</td>
<td>45</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>95</td>
<td>47.5</td>
<td>2.5</td>
<td>50</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

E Preparation of organotin stock solutions

E.1. Tributyltin chloride (TBTC) (C_{12}H_{27}ClSn) (F.W-325.49 g/mol), obtained from MERCK, Germany.

Preparation of 1M stock of TBTC

Absolute ethanol (filter sterilized) (72.2 mL) + TBTC (27.8 mL) (3.7 M) was mixed and the final volume was made up to 100 mL. The solution was kept in amber coloured bottle in cold and dark condition.

E.2. Dibutyltin chloride (DBT)(C_{8}H_{18}Cl_{2}Sn)(F.W-303.83 g/mol) from MERCK, Germany.

Stock solution of DBTC (1 M).

Absolute ethanol (Filter sterilized) (50 mL) + DBTC (15.1989 gm) (1 M) was mixed to make up the final volume of 50 mL. The solution was preserved in amber coloured bottle in cold dark condition. The fresh stock solutions were prepared when required.

F. Preparation of stock solutions (Heavy metals)

The stock solutions for Heavy metal ions HgCl₂ (10 mM), CdCl₂ (10 mM) (Merck), As₂O₃ (10 mM) (Qualigens). All stock metal solutions, with the exception of Sn²⁺,
were made up in double-distilled water, syringe-filtered (0.22 μm, Millipore) into sterile glass vials. Sn\(^{2+}\) was dissolved in 50% ethanol and stored in a sterile polypropylene tubes

**F.1. Mercuric chloride (HgCl\(_2\))(F.W-271.50)**

Stock solution (10 mM) - HgCl\(_2\) (0.2715 gm) was dissolved in 100mL of De-ionized Double Distilled Water. The solution was filter sterilized and stored at 4°C in a dark place.

**F.2. Cadmium chloride (CdCl\(_2\))(F.W-183.31)**

Stock solution (10 mM) - CdCl\(_2\) (0.1834 gm) was dissolved in 100 mL of De-ionized Double Distilled Water. The solution was filter sterilized and stored at 4°C in a dark place.

**F.3. Arsenic oxide (As\(_2\)O\(_3\))**

Stock solution (10 mM) - As\(_2\)O\(_3\) (0.1978gm) was dissolved in 100 mL of De-ionized Double Distilled Water. The solution was filter sterilized and stored at 4°C in a dark place.

**G. Antibiotics**

Antibiotic stock (10 mg) was weighed and dissolved in 1mL of sterile De-ionized Double Distilled Water sterilized by filtration through 0.22 μm filter (Millipore, GS). Antibiotic used were Chloramphenicol and Rifampicin (Methanol), Nalidixic acid (1N NaOH), Tetracyclin (70% ethanol), Erythromycin, Gentamycin, Streptomycin, Neomycin, Kanamycin, Ampicillin Penicillin, Antimycin, Amikacin, Novobiocin and Antimycin (Double Distilled Water). All solutions were stored in light-safe glass vials at 4 °C, except tetracycline, which was stored at –20 °C and working solutions were freshly made prior to prescribed.

**H. Glycerol**

Sterile glycerol (autoclaved) was added to the medium (v/v) to obtain the required concentration.

**I. Succinate**

\[
\begin{align*}
\text{Succinate} & \quad 10 \text{ gm} \\
\text{Double Distilled Water} & \quad 100 \text{ mL}
\end{align*}
\]

The stock solution was autoclaved and used for experimental purpose.
J. Glucose

D-Glucose 20 gm
De-ionized Double Distilled Water 100 mL
The stock solution was filter sterilized and used for experimental purpose.

K. Acridine orange

Acridine orange (50 mg) was dissolved in 100 mL of double distilled water and filter sterilized. The solution was kept in amber coloured bottle at 4°C in a dark place. (The final stock concentration was 500 μg/mL).

L. Thiols and Chelating agents:

L.1. 2-mercaptoethanol -(MW- 78.13, Specific gravity —1.11.5) (SIGMA).

β mercapto ethanol was used v/v in medium to obtain the required concentration. 1mM stock solution - 2-mercaptoethanol 714 μL in 10 mL Double distilled water.

L.2. Disodium EDTA - (MW-372.34 g/L)

EDTA 20 gm
Double Distilled Water 50 mL

Required amount of concentrated NaOH was added to dissolve EDTA and final volume was made up to 50 mL.

1mM stock solution -Disodium EDTA 0.0372 gm in 10 mL Double distilled water.

M. Reagents for chemical estimation of Exopolysaccharides

M.1. Reagents for sugar estimation (Phenol sulphuric acid method) (Dubois et al. 1956) Phenol sulphuric acid method for (Total Carbohydrates).

1. 5 % phenol: - 5 gm of redistilled phenol (reagent grade) dissolved in Double distilled water and diluted to 100 mL

2. Sulphuric acid: - 96 %, analytical grade

3. Standard Glucose solution:-0. 1 mg of glucose dissolved in 1mL of Double distilled water.
Procedure

To the known amount of sample, 1 mL of Conc. H₂SO₄ was added followed by the addition of 1 mL of 2.5% aqueous phenol and 4 mL of Conc. H₂SO₄. The tubes were rapidly kept in ice bath and held for 10 min(s) at room temperature. The yellow brown color obtained was measured at 490 nm against distilled water blank. Standard curve was plotted using glucose (0-100 µg/mL as the standard). Factor F was calculated and the concentration of the total sugars in the samples was determined.

M.2. Reagents for Uronic acid estimation (Carbazole method).

1. Sulphuric acid reagent: (H₂SO₄)
   0.025 M Na—tetraborate in Concentrated H₂SO₄ (0.5485 gm in 100 mL)

2. Carbazole reagent:
   (0.125 % carbazole in 20 mL of ethanol).

3. Standard Galactouronic acid:
   0.1 mg of galactouronic acid dissolved in 1mL of double distilled water.

Procedure

5 mL of H₂SO₄ reagent in tube was cooled to 4°C using ice bath. To this a known amount of test sample was added and the tube was closed with a stopper and shaken gently and later on vigorously on a vortex mixer with constant cooling. The tube was heated for 10 min(s) in a boiling water bath and cooled at room temperature. 0.2 mL of carbazole reagent was then added and after mixing, the samples were heated in a boiling water bath for 15 min(s), cooled to room temperature and the absorbance was measured against reagent blank at 530 nm. Concentration of uronic acid was determined using Galactouronic acid (0-100 µg/mL) as standard.

M.3. Barium chloride-Gelatin method for sulphate estimation:-

1. BaCl₂-Gelatin reagent:
   Dissolved 2 gm of gelatin in 400 mL of hot Double distilled water (60-70°C) and allowed to stand at 4°C overnight. Further dissolved in 2 gm of BaCl₂ in the semi
gelatinous fluid and the resultant cloudy solution allowed standing for 2-3 mins before use.

2. 3% w/v Tricholoroacetic acid:

Dissolved 3 gm of TCA in 100 mL of Double distilled water.

3. Standard K₂SO₄ solution:

Dissolved 1.1814 gm of K₂SO₄ in 10 mL of 1N corresponds to 100 μg/mL of sulphate.

Procedure:

To the known amount of sample, 1 mL of 1 N HCL was added in a glass ampoule. The ampoule was flushed with N₂ gas and sealed. The sample was hydrolyzed in an oven for 105°C for 16 hrs. The content of the tube was cooled and mixed before opening the tube and the content added to another tube containing 3.8 mL of 3% TCA. 0.1 mL of BaCl₂-Gelatin reagent was then added. Mixed thoroughly and kept at room temperature for 20 mins. Absorbance of the white sulphate precipitate was measured at 360 nm against a blank containing 1 mL of 1 N HCL. Factor F was calculated using K₂SO₄ (0-100 μg/mL) as the standard and the concentration of sulphates in the sample was determined.

M.4 Cysteine Hydrogen chloride method for Methyl pentoses

(Dische et al. 1948)

Reagents for Methyl pentoses

1. H₂SO₄: H₂O mixture:

Added 1:6 (v/v) of H₂O and H₂SO₄

2. 3 % Cysteine hydrogen chloride:

0.3 gm in 10 mL of Double distilled water

3. Standard Arabinose solution:

0.1 mg of arabinose dissolved in 1mL of Double distilled water.

Procedure

To the known amount of sample, 4.5 mL of chilled 1:6 (v/v) of H₂O and H₂SO₄ was added and the mixture was then held at room temperature for few minutes.
and then in a boiling water for 3-10 min(s) and cooled under running tap water 0.1 mL of the 3 % Cysteine hydrogen chloride was added and the absorbance of greenish yellow color was read at 396 nm and 430 nm; Rhamnose 0-100 µg/mL) was used as a standard and the readings of 430 nm were subtracted from 396 nm in order to calculate the F factor.

N. Reagents for Protein Estimation

1. Reagent A: Sodium Carbonate reagent
   \[ \text{Na}_2\text{CO}_3 \quad 2 \text{ gm} \]
   \[ \text{NaOH (0.1N)} \quad 100 \text{ mL} \]

2. Reagent B: Copper sulphate solution
   - Sodium potassium tartarate \(1\) gm
   - Copper sulphate \(0.5 \) gm
   - Double distilled water \(100 \text{ mL}\)

3. Reagent C: Alkaline Copper sulphate solution
   - Reagent A: \(50 \text{ mL}\)
   - Reagent B: \(1 \text{ mL}\)
   Reagent C was prepared fresh at the time of estimation

4. Reagent D – Folin Ciocalteau (FC reagent)
   - FC reagent commercial grade \(10 \text{ mL}\)
   - Double distilled water \(20 \text{ mL}\)
   - Freshly prepared at the time of estimation.

Procedure

To the known amount of sample, 5mL of the Copper sulfate solution was added and kept at room temperature in the dark for 10 min(s). 0.25 mL of Folin-ciocalteau phenol reagent was added and kept in the dark for 20 min(s). Absorbance was measured at 660 nm against reagent blank and the concentrations of the unknown samples were determined from standard graph
and factor F was calculated using Bovine serum albumin as the standard (0-100 μg/mL).

O.1. **Reagents for protein separation by SDS-PAGE** (Laemelli. 1970)

i. Monomer solution (30 % T, 27 % C)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>29.2 gm</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>0.8 gm</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

Stored at 4°C in dark conditions

TEMED (use as purchased)

ii. Ammonium persulfate (prepare freshly)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulfate</td>
<td>10 mg</td>
</tr>
</tbody>
</table>

Dissolve in 1.0 mL Double distilled water

iii. Buffers

**Resolving Gel buffer**

1.5 M Tris,

45.4 gm of Tris : Dissolve in 150 mL distilled water

Adjust pH to 6.8 with 6 N HCl

Fill to 250 mL with distilled water

iv. **Stacking gel buffer (0.5 M, pH 6.8)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>3 gm</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

pH was adjusted to 6.8 with 6 N HCl, stored at 4°C

v. **10 % SDS**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>1 gm</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

vi. **10 X Electrophoresis Running Buffer**

(250 mM Tris, 1.92 M glycine, 1 % SDS)
Tris 30.3 gm
Glycine 144.0 gm
SDS 10.0 gm

Fill to 1 liter with distilled water

To make 1 liter of 1 X running buffer, add 900 mL of distilled water to 100 mL of 10 X running buffer.

vii. Sample Buffer with SDS

(60 mM Tris, 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol, 0.025 % Bromophenol blue)

Distilled water 25.0 mL
0.5 M Tris, 6.58 mL
Glycerol 5.26 mL
10 % SDS 10.5 mL
0.5 % Bromophenol blue 2.63 mL
pH 8.8

Fill to 50 mL with water in a volumetric flask

To prepare fresh working sample buffer, add 50 μL of β-mercaptoethanol to 950 μL of the stock sample buffer before adding to the sample.

O.2. Protein Staining and Developing Solutions

i. Staining Dye (Coomassie Brilliant Blue)

(0.2 % Coomassie Brilliant Blue-R250, 20 % methanol, 10 % acetic acid)

Coomassie Blue-R-250 0.4 gm
Methanol 40 mL
concentrated acetic acid 20 mL

Fill up to 200 mL with distilled water

ii. Destaining Solution (10 % acetic acid, 20 % methanol)

Concentrated acetic acid 100 mL
Methanol 200 mL

Fill to 1 liter with distilled water
Guide for the Preparation of Gels of Different Acrylamide Concentrations

<table>
<thead>
<tr>
<th></th>
<th>7.0% Acrylamide concentration</th>
<th>10% Acrylamide concentration</th>
<th>12% Acrylamide concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>2.5 ml.</td>
<td>5.0 ml.</td>
<td>10.0 ml.</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>1.3 ml.</td>
<td>2.5 ml.</td>
<td>5.0 ml.</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>50 ml.</td>
<td>100 ml.</td>
<td>200 ml.</td>
</tr>
<tr>
<td>Acrylamide (30%)</td>
<td>1.2 ml.</td>
<td>2.3 ml.</td>
<td>4.7 ml.</td>
</tr>
<tr>
<td>Ammonium persulfate* (10%) fresh</td>
<td>25 ml.</td>
<td>50 ml.</td>
<td>100 ml.</td>
</tr>
<tr>
<td>TEMED*</td>
<td>3 ml.</td>
<td>5 ml.</td>
<td>10 ml.</td>
</tr>
<tr>
<td>Total volume</td>
<td>5 ml.</td>
<td>10 ml.</td>
<td>20 ml.</td>
</tr>
</tbody>
</table>

P. Reagents for plasmid DNA isolation:

P.1.a. Alkaline Lysis Method (Birnboim & Doly, 1979)

i). Solution I (pH 8.0)
   - Glucose: 0.9 gm
   - Tris- chloride: 0.394 gm
   - EDTA: 0.292 gm
   - Double distilled water 100 mL

ii). Solution II
   - SDS: 1.0 gm
   - 0.2 NaOH: 100 mL

iii). Solution III (pH 5.0)
   - 5M Potassium acetate: 60.0 mL
   - Glacial acetic acid: 11.5 mL
   - Double distilled water: 28.5 mL

iv). TE buffer:
   - Tris HCl: 0.156 gm
   - EDTA: 0.029 gm
   - Double distilled water: 100 mL
   - pH: 7.6-8.0
v). **Tris acetate EDTA (TAE) buffer (pH 8.0)**

50X: Tris Base 2.42 gm  
0.5 M EDTA 1 mL

Tris base and 0.5 M EDTA was dissolved in 10 mL of Double distilled water and pH was adjusted to 8.0 with glacial acetic acid (0.57 mL) and the final volume was made up to 500 mL.

P.1.b. **Boll Prep method (Holmes and Quigley, 1979).**

i). **STET buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>8 gm</td>
</tr>
<tr>
<td>Triton X 100</td>
<td>5 mL</td>
</tr>
<tr>
<td>50 mM EDTA</td>
<td>5 mL</td>
</tr>
<tr>
<td>50 mM Tris</td>
<td>5 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

ii). **Lysozyme soln:**

15 mg lysozyme is dissolved in 1 mL of Tris HCl (pH-8), vortexed and stored at -20°C.

iii). **2.5 M Sodium acetate**

P.1.c. **Kado and Liu. (Kado and Liu 1981, Modified)**

i). **Tris acetate 0.04 M**

6.304 gm of Tris acetate was dissolved in 1000 mL of Double Distilled water.

ii). **EDTA 0.02 M**

Dissolve 7.444 gm of EDTA in 1000 mL of Double Distilled water.

P.2. **Reagents for Genomic DNA isolation**

**TE buffer: (10: 0.1); 10 mM Tris HCl) 0.1 mM EDTA, pH 7.6-8.0**

10 % w/v Sodium dodecyl sulphate

20 mg/mL Proteinase K
Phenol chloroform (1:1)  
Isopropanol  
70 % ethanol  
3 M Sodium acetate (pH -5.2) (40.82 gm in 100 mL of Double distilled water)

P.3. Electrophoresis

i). Agarose gel

Agarose - 0.8 gm  
1X TAE buffer - 100 mL

ii). Ethidium bromide solution (intercalating dye)

Ethidium bromide - 10 mg  
Distilled water - 1 mL  

Stock solutions was prepared and kept in cool and dark place. The final concentration used for agarose gel was 5 µg/mL.

iii). Bromophenol blue (tracking dye)

Bromophenol blue - 0.25 gm  
Sucrose - 40 gm  
0.1 M EDTA - 10 mL  
1 % SDS - 10 mL  
Distilled water - 100 mL  

Tracking dye was stored at 4°C

P.4. Plasmid Curing agents

Sodium Dodecylsulphate 10 % (1 gm in 10 mL)  
Acridine Orange - 10 mg/mL  
Ethidium Bromide - 10 mg/mL
**P. 5. PCR Reaction conditions:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 °C</td>
<td>10 mins</td>
</tr>
<tr>
<td>94 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>55 °C</td>
<td>1 min 30 cycles</td>
</tr>
<tr>
<td>72 °C</td>
<td>3 mins</td>
</tr>
<tr>
<td>72 °C</td>
<td>7 mins</td>
</tr>
<tr>
<td>15 °C</td>
<td>infinity</td>
</tr>
</tbody>
</table>

Reaction volume: 50 µL

Amplitaq DNA Polymerase along with 10 X Amplitaq buffer and also MgCl₂ (250mM) stored at -20°C the concentration was checked.

<table>
<thead>
<tr>
<th>Step</th>
<th>Component</th>
<th>Volume 1</th>
<th>Volume 2</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Milli Q DDW</td>
<td>31.5 µL</td>
<td>66.5 µL X2</td>
<td>133 µL</td>
</tr>
<tr>
<td>2</td>
<td>10X Amplitag cold buffer</td>
<td>5 µL</td>
<td>10 µL X2</td>
<td>20 µL</td>
</tr>
<tr>
<td>3</td>
<td>MgCl₂ (25mM)</td>
<td>5 µL</td>
<td>10 µL X2</td>
<td>20 µL</td>
</tr>
<tr>
<td>4</td>
<td>dNTP’s (2mM each)</td>
<td>5 µL</td>
<td>10 µL X2</td>
<td>20 µL</td>
</tr>
<tr>
<td>5</td>
<td>Forward Primer (EuB f-341)</td>
<td>1 µL</td>
<td>1 µL X2</td>
<td>2 µL</td>
</tr>
<tr>
<td>6</td>
<td>Reverse Primer (EuB r-1387)</td>
<td>1 µL</td>
<td>1 µL X2</td>
<td>2 µL</td>
</tr>
<tr>
<td>7</td>
<td>Amplitaq Gold DNA Polymerase</td>
<td>0.5 µL</td>
<td>0.5 µL X2</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>8</td>
<td>Total Volume</td>
<td>49 µL</td>
<td>99 µL X2</td>
<td>198 µL</td>
</tr>
</tbody>
</table>

1µL DNA template of strain S3 was mixed with 99 µL of the reaction mixture and then run PCR Reaction.

**Q. Analytical techniques**

**Q.1 UV-Visible spectrophotometry**

UV-Visible scans of the culture supernatants and other samples were done using UV-1601 Shimadzu spectrophotometer.

**Q.2 Thin layer chromatography (TLC) (Hamilton and Hamilton, 1987)**

The slurry, prepared by mixing 6 to 8g of Silica Gel G (Acme’s synthetic chemicals) in 15 mL of distilled water, was poured on glass plates (20 x 20cm) and drawn into thin layer of 0.2 mm. The plates were air-dried and then activated at 110°C for 30 min. Samples were spotted on the activated plates using thin glass capillaries. After drying the spots, plates were developed in solvent
chambers, which were previously saturated overnight. Solvent was allowed to run up to 3/4th of the plate, the solvent front was marked and coloured spots, if any, were noted. The plates were then placed in iodine chamber. Rf values were calculated using the formula: Rf = Distance travelled by solute /Distance travelled by solvent.

Q.3 Protein and sugar estimation

a. Protein estimation by Folin-Lowry’s method (Lowry, et al. 1951):
The protein concentration in samples was quantitated by Folin Lowry’s method. To 1 mL of sample; 5 mL of alkaline copper sulphate reagent was added. Mixture was thoroughly mixed and incubated at 28°C in dark for 10 minutes. To this solution, 0.5 mL of diluted Folin-ciocalteau reagent was added and incubated in dark for 30 minutes at 28°C. Absorbance of this solution was taken in dual beam UV-Visual Spectrophotometer (Shimadzu UV 1610) against protein blank at 660 nm.

b. Sugar estimation by phenol sulphuric acid method:
Samples were analyzed for their sugar content by the phenol-sulphuric acid method (Dubois, et al. 1956). To 1 mL of aqueous sample containing polysaccharides, 1 mL of 5 % aqueous phenol was added. The tubes were placed in ice and 5 ml of concentrated sulphuric acid was added carefully into the tubes. Tubes were incubated in ice for 10 min and subsequently kept at 25-30°C for 10 min. The absorbance was measured at 490 nm. Standard curve was plotted using glucose (0-100 mg/mL) as standard.
R. Standard graphs

Standard graph of Phosphate

a. Standard graph of phosphate

Standard graph of nitrate

b. Standard graph of nitrate
b. Standard curve of nitrite

d. Standard graph of sugar
Standard curve of protein (Bovine Serum Albumin)

O.D. at 660nm

Protein (µg/ml)

e. Standard graph of protein