9. APPENDIX-I & II

APPENDIX – I

Composition of staining reagents and Culture media

I-Acid fast stain

(i) Carbol Fuchsin (Ziehl’s)

Solution A

- Basic Fuchsin (90% dye content) 0.3 g
- Ethyl alcohol (95%) 10 ml

Solution B

- Phenol crystals 5.0 g
- Distilled water 95 ml

(ii) Acid alcohol

- Ethyl alcohol (95%) 97 ml
- HCl (37%) 3.0 ml

Add the acid to the alcohol slowly.

(iii) Methylene blue

- Methylene blue (90% dye content) 0.3 g
- Distilled water 100 ml

II-Capsule stain

(i) Crystal violet (1%)

- Crystal violet (85% dye content) 1.0 g
- Distilled water 99 ml

(ii) Copper sulphate solution (20%)

- Copper sulphate (CuSO₄·5H₂O) 20 g
- Distilled water 80 ml

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**III-Gram stain**

(i) **Crystal violet**

**Solution A**

- Crystal violet (90% dye content) 2.0 g
- Ethyl alcohol (95%) 20 ml

**Solution B**

- Ammonium oxalate 0.8 g
- Distilled water 80 ml

Dissolve crystal violet in ethyl alcohol and the ammonium oxalate in Distilled water. Mix solutions A and B.

(ii) **Gram’s iodine**

- Iodine 1.0 g
- Potassium Iodide 2.0 g
- Distilled water 300 ml

(iii) **Ethyl alcohol (95%)**

- Ethyl alcohol (100%) 95 ml
- Distilled water 5.0 ml

(iv) **Safranin**

- Safranin (2.5% solution in 95% ethyl alcohol) 10 ml
- Distilled water 100 ml

**IV-Spore stain**

(i) **Malachite green (5%)**

- Malachite green 5.0 g
- Distilled water 100 ml

(ii) **Safranin**

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Safranin (2.5% solution in 95% ethyl alcohol) 10 ml
Distilled water 100 ml

**Composition of media for 1L**

**BSM (Basal salt media) pH-7.0**

Potassium dihydrogen phosphate 0.38 g
DiPotassium hydrogen phosphate 0.6 g
Magnesium chloride 0.2 g
Ammonium chloride 1.0 g
Ferric chloride 0.05 g

Dissolve in 500 ml Milli-Q water and finally volume was made up to 1L with Milli-Q water.

**BSMG (Basal salt media with glucose) pH-7.0**

Potassium dihydrogen phosphate 0.38 g
DiPotassium hydrogen phosphate 0.6 g
Magnesium chloride 0.2 g
Ammonium chloride 1.0 g
Ferric chloride 0.05 g
Glucose 10 g

Dissolve in 500 ml Milli-Q water and finally volume was made up to 1L with Milli-Q water.

**Carbohydrate fermentation medium (pH- 7.3)**

Trypticase/Peptone 10 g
Carbohydrate 5.0 g
Sodium chloride 15 g
Phenol red 0.018 g

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Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Litmus milk medium (pH 6.8)**

Skim milk powder 100 g  
Litmus 0.075 g  

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

Autoclave at 12 psi pressure for 15 min.

**LB (Luria-Bertani) medium (pH 7.5)**

Tryptone 10 g  
Yeast 5 g  
Sodium chloride 10 g  

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**LB-X-gal/IPTG/Amp plates**

Autoclave 1.5% agar in fresh LB medium for 20 min at 15 psi pressure. Left at room temperature till temperature of the medium is reached 50°C. Added ampicillin at final concentration of 50 µg/ml and poured on autoclaved petriplates. Plates were allowed to cool down to solidify the medium. Kept the plates at 37°C overnight to ensure that there was no bacterial growth. Plates were always kept in inverted position to avoid condensation of moisture on the surface of the solidified medium. Added 40 µg/ml of X-gal stock (20 mg/ml in dimethylformamide) solution and 4 µl of IPTG stock (200 mg/ml) solution to a prepared LB plate containing ampicillin; spreaded the solution over entire surface of the plate with the help of a glass spreader. Incubated the plate at 37°C until the added liquid dried.

**MR-VP broth (pH 6.9)**

Peptone 7.0 g  

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Potassium phosphate 5.0 g
Dextrose/Glucose 5.0 g

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Nutrient agar (pH 7.0)**

Peptone 5.0 g
Beef extract 3.0g
Sodium chloride 5.0 g

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Nutrient gelatin (pH 6.8)**

Peptone 5.0 g
Beef extract 3.0 g
Gelatin 120 g

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**SIM agar (pH 7.3)**

Peptone 30 g
Beef extract 3.0 g
Ferrous ammonium sulphate 0.2 g
Sodium thiosulphate 0.025 g
Agar 3.0 g

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Simmons’s citrate agar (pH 6.9)**

Ammonium dihydrogen phosphate 1.0 g

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<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Bromo thymol blue</td>
<td>0.08 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
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</tbody>
</table>

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Starch agar (pH 7.0)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch (soluble)</td>
<td>20 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Skim milk agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk powder</td>
<td>100.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Trypticase soy agar (pH 7.3)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase</td>
<td>15 g</td>
</tr>
<tr>
<td>Soy peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>
Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Trypticase nitrate broth (pH 7.2)**

- Trypticase: 20 g
- Disodium phosphate: 2.0 g
- Dextrose: 1.0 g
- Agar: 1.0 g
- Potassium nitrate: 1.0 g

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Tributyrin agar (pH 7.2)**

- Peptone: 5.0g
- Beef extract: 3.0g
- Agar: 15.0g
- Tributyrin: 10.0g

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Typtone broth (pH 7.2)**

- Peptone: 10 g

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**Urea broth (pH 6.8)**

- Peptone: 1.0 g
- Sodium chloride: 5.0 g
- Potassium dihydrogen phosphate: 2.0 g
- Glucose: 1.0 g
- Phenol red (0.02% solution): 6.0 ml

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Urea (20% aq. solution) 100 ml

Dissolve in 500 ml distilled water and finally volume was made up to 1L with distilled water.

**APPENDIX— II**

**Composition of chemical reagents**

**Alpha(α)-napthol solution (VP test reagent I)**
- α-naphthol 5.0 g
- Ethyl alcohol 95 ml

**VP test reagent II**
- Potassium Hydroxide 40 g
- Distilled water 60 ml

**Diphenylamine reagent**
- Diphenylamine 0.7 g
- Conc. sulphuric acid 60 ml
- Conc. Hydrochloric acid 11.3 ml
- Distilled water 28.8 ml

**Kovac’s reagent**
- p-Dimethylaminobenzaldehyde 5.0 g
- Amyl alcohol 75 ml
- Conc. Hydrochloric acid 25 ml

**Nitrate test solution**

**Solution-A**
- Sulfanilic acid 8.0g
- Acetic acid (5 N) 1000 ml

**Solution-B**

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Alpha-naphthylamine  5.0g
Acetic acid (5 N)   1000 ml

To prepare 5 N, take 287 ml of concentrated acetic acid and adjust with distilled water to 1000 ml.

**Grams iodine**

For detection of starch as in Grams stain.

**Hydrogen peroxide, 3% ,**

For detection of catalase activity

**Stock solutions**

Aridine orange (1 mg/ml in double distilled water)
Ampicillin (100 mg/ml in sterilized double distilled water)
DBT (100 mg/ml in methanol)
2-HBP (5 mg/ml in methanol)
5-Bromo uracil (1 mg/ml in double distilled water)
Ethidium bromide (10 mg/ml double distilled water)
Isopropylthio-β-D-galactoside (IPTG; 200 mg/ml in double distilled water)
5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-gal; 20 mg/ml in dimethylformamide)

Sterilize all the stock solutions by Millipore micro syringe filter assembly (0.45 µm pore size).

**Soil analysis reagents**

**Ammonium molydate solution** - Dissolve 31.4 g in about 200 ml distilled water. Add carefully 252 ml concentrated H₂SO₄ to 400 ml distilled water. Cool and add 3.4 ml concentrated HNO₃. To this sol. add ammonium molydate solution and dilute to 1000 ml.

**Stannous chloride solution** - Dissolve 2.5 g fresh SnCl₂.2 H₂O in 100 ml glycerol. Heat on water to ensure complete dissolution.

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**Aluminum hydroxide suspension** - Dissolve 125 g Potash alum in 1000 ml distilled water. Warm to 60°C & add 55-60 ml NH₄OH & allow to stand for 1 h. Decant the supernatant & wash the precipitate number of times till it is free from Cl⁻, NO₂⁻ & NO₃⁻. Finally, after settling decant off as much clean liquid as possible, leaving only the concentrated suspension.

**Diphenylamine indicator** - Dissolve 0.25 g of Diphenylamine in 10 ml of distilled water & add gradually 50 ml of concentrated H₂SO₄.

**Ferrous ammonium sulphate sol. (0.5 N)** – Add 20 ml of concentrated H₂SO₄ to 800 ml of distilled water & dissolve in it 196.1 g of ferrous ammonium sulphate. Further add distilled water to make the volume 1 L.