APPENDIX

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Preparation of Giemsa stain:

About 0.6 gm of certified powder of Giemsa stain was dissolved in 50ml glycerin in a flask and the flask was heated in a water bath at 55 °C – 60 °C for 2hrs or longer with occasional shaking till all the stain was dissolved. Now the flask was allowed to cool to room temperature and 50ml of methyl alcohol was added to it. The mouth of the flask was then closed tightly and allowed to stand in a dark place for 10-15 days for maturation. Finally, the stain was filtered and stored in small black colour bottles in dark place.

Winklers method reagents

i) Manganese sulphate: 120gm $MnSO_4$ was mixed in 250 ml distilled water.

ii) Sodium iodide: 125 gm Sodium hydroxide and 33.75 gm Sodium iodide was mixed in 250 ml distilled water.

Or Potassium iodide: 350 gm Potassium hydroxide and 150 gm Potassium iodide was mixed in 500 ml distilled water.

iii) Sodium thiosulphate (0.025N): 1.551gm sodium hydroxide, 0.1 gm NaOH in 250 ml was mixed distilled water.

iv) Starch: 1gm starch was mixed in 100 ml hot distilled water.

Gram staining

Reagents

The reagents listed below can be made or purchased commercially

- ***Primary stain: Crystal Violet staining reagent.***

Solution A for crystal violet staining reagent Crystal violet (certified 90% dye content): 2g

Ethanol, 95% (vol/vol): 20 ml

Solution B for crystal violet staining reagent Ammonium oxalate: 0.8 g,

Distilled water: 80 ml A and B was mixed in to obtain crystal violet staining reagent and stored for 24 h and filter prior to use.

- ***Mordant: Gram's Iodine***

Iodine: 1.0 g,

Potassium iodide, 2.0 g,

Distilled water: 300 ml

Iodine and potassium iodide was grinded in a mortar and water was added slowly with continuous grinding until the iodine is dissolved. The solution was store in amber bottle.

- ***Decolorizing agent***

Ethanol, 95% (vol/vol) Or 1:1 Acetone and Ethanol (95%) mixture.

- ***Counterstain: Safranin***

Stock solution: 2.5g Safranin O 100 ml 95% Ethanol Working Solution: 10 ml Stock Solution 90 ml Distilled water.

Catalase test

Reagent

3% Hydrogen peroxide

Reagents

Dimethyl-p-phenylenediamine/ N,N-dimethyl-p-phenylenediamine oxalate

α-naphthol.

Reagents

Kovac's reagent i.e. a mixture of Isoamyl alcohol,
p-Dimethylaminobenzaldehyde, concentrated hydrochloric acid.
Or, Ehrlich's reagent using ethyl alcohol in place of isoamyl alcohol, the other components remaining the same.

Citrate utilization test

Reagents:

Simmon's citrate agar medium containing sodium citrate and indicator bromothymol blue.

Magnesium Sulfate ..... 0.20 g

Monoammonium Phosphate 1.00

Dipotassium Phosphate 1.00

Sodium Citrate 2.00

Sodium Chloride 5.00

Agar 25.00

Bromthymol Blue 0.08

Reagents

1. Glucose phosphate broth

Composition:

Ingredients Grams/Litre

Buffered Peptone 7.0

Dextrose 5.0

Dipotassium Phosphate 5.0

Final pH 6.9 +/- 0.2 at 25°C. Prepared media was stored below 8°C, protected from direct light. Dehydrated powder was stored in a dry place, in tightly-sealed containers at 2-25°C.

2. Methyl Red Indicator: 5- 6 drops

Reagents

3. Glucose phosphate broth

Composition : as mentioned in MR test.

4. 40% KOH

Alpha-naphthol

Decarboxylase test

Reagents

1. Decarboxylase broth

Composition:

Ingredients Grams/Litre

Peptic Digest of Animal Tissue 5.0

Beef Extract 5.0

Dextrose 0.5

Bromo Cresol Purple 0.01

Cresol Red 0.005

Pyridoxal 0.005

Final pH 6.0 +/- 0.2 at 25°C

Prepared media was stored below 8°C, protected from direct light. Dehydrated powder was stored in a dry place in tightly-sealed containers at 2-25°C.

2. Amino acids arginine, lysine, and ornithine.

Reagents

1. Semi solid culture medium containing fermentable carbohydrate.

2. pH indicator

Reagents

1. **Phenol-Red carbohydrate fermentation broths** (PR-Carb broth) are useful for helping to characterize bacteria based on their fermentation abilities. Each PR-Carb broth contains the following:

i) Composition

Ingredients

Proteose peptone 10.000

Beef extract 1.000

Sodium chloride 5.000

Phenol red 0.018

Final pH (at 25°C) 7.4±0.2

ii) Carbohydrate Each broth contains a single fermentable carbohydrate. We use glucose, D- mannitol, L- arabinose and sucrose in our test.

2. Phenol-Red This is a pH indicator that is RED at pH 7 or higher (alkaline) but turns yellow at low pH (acidic).

Reagents

i) Phosphate buffer: 8 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na2HPO4 and 0.24 gm of KH2PO4 were dissolved in 800 ml of distilled water. pH was adjusted to 7.4 . Final volume was made upto 1 liter with distilled water.

ii) Alchohol: 30%, 50%, 70%, 90%, 100%.

iii) 0.25% Gluteraldehyde: 1 ml commercial or 25 % Gluteraldehyde was mixed in 100 ml phosphate buffer solution.

iv) Isoamyl alchohol.

v) Sput gold.

DNA extraction

Reagents

i) TE buffer: 10 mM Tris HCl, 1mM EDTA Final pH: 8.0

ii) 10% Sodium dodecyl sulphate: 10 ml SDS in 100 ml distilled water.

iii) Proteinase K: 20 mg in 1ml distilled water.

iv) Choroform:isoamyl alchohol: 24:1

v) Phenol- chloroform- isoamyl alchohol: 25:24:1

Phosphate buffer saline (PBS)

8 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na2HPO4 and 0.24 gm of KH2PO4 were dissolved in 800 ml of Milli-Q water. pH was adjusted to 7.4 with HCl . Final volume was made upto one liter with Milli-Q water.

Total protein

Reagents

- Stock standard protein solution

Fifty miligram of bovine serum albumin (BSA) (Sigma, USA) was dissolved in Milli-Q water to a final volume of 50 ml in a standard flask. (1mg/ml)

- Working standard solution

10 ml of the stock solution was diluted with Milli-Q water to make a total volume of 50 ml. Final concentration of working standard becomes 200µg/ml.

- 0.1(N) Sodium hydroxide (NaOH) solution

1gm of NaOH dissolved in 250ml of Milli-Q water.

- 0.2(N) Alkaline copper sulphate solution
  - Reagent A: 2% Sodium carbonate (Na2CO3) was dissolved in 0.1 (N) NaOH solution.
  - Reagent B: 0.5% Copper sulphate (CuSO4. 5H2O) was dissolved in 1% Sodium potassium tartarate.

50 ml of reagent A was mixed with 1 ml of reagent B to make the alkaline copper solution. The solution was freshly prepared every time before use.

- Folin-Ciocalteu reagent

Commercial Folin-Ciocalteu reagent was diluted with Milli-Q water (1:1) prior to addition.

Serum Glutamic Oxaloacetic Transaminase

Reagents

- *0.1(M) potassium phosphate buffer (pH 7. 4)*

14.2 gm of anhydrous di-sodium hydrogen phosphate was dissolved in Milli-Q water and the volume made up to 100 ml. 3.4 gm of potassium di-hydrogen phosphate was dissolved separately and made upto 250 ml. The two solutions were mixed and the pH was adjusted to 7.4.

- Buffered substrate

2.66 gm of L-aspartic acid and 0.0292 gm of □-ketoglutaric acid were dissolved in 18 ml of 1(N) sodium hydroxide solution. This step was accompanied by mild heating to dissolve the L-aspartic acid in 1(N) sodium hydroxide. The final volume of the solution was made upto 100 ml by adding phosphate buffer and the pH was adjusted to 7.4 by adding more sodium hydroxide, if necessary.

- 0.4 (N) Sodium hydroxide solution

16 grams of sodium hydroxide was dissolved in 1 litre of Milli-Q water to make 0.4 (N) solution.

- Dinitrophenylhydrazine (DNPH) solution

19.8 mg of dinitrophenylhydrazine was dissolved in 10 ml of concentrated hydrochloric acid and made up to 100 ml with Milli-Q water.

Serum Glutamate Pyruvate Transferase

Reagents

- Substrate solution

1.8 gm of alanine and 0.0584 gm of □-ketoglutaric acid were dissolved in 18 ml of 1(N) sodium hydroxide solution. The final volume of the solution was made up to 100 ml by adding phosphate buffer and the pH was adjusted to 7.4 by adding more sodium hydroxide, if necessary.

Acid and Alkaline phosphatase

Reagents

- Preparation of standard solution

  p-nitro phenol – 6.955 mg

  Milli-Q water- 5ml

The concentration of the final solution was 103 nM.

- Preparation of 0.085 (N) NaOH solution

  NaOH - 0.85 gm

  Milli-Q water- 250ml

- Preparation of acid buffer (pH- 4.8)

  Citric acid – 0.41 gm

  Sodium citrate - 1.125gm

p-nitrophenol phosphate – 0.203 gm

These constituents were dissolved in Milli-Q water to make the final volume 100 ml. The pH of the buffer was adjusted to 4.8 and kept at 4oC. The buffer solution was brought to 37oC before use.

- Preparation of 0.1(N) NaOH solution

  NaOH – 400mg

  Milli-Q water – 100ml.

- Preparation of alkaline buffer solution (pH-10.5)

  Glycine – 375 mg

  MgCl2 -10 mg

  p-nitrophenol phosphate – 165 mg

The constituents were dissolved in 42 ml of 0.1N NaOH and made upto a final volume of 100 ml with Milli-W water. The pH of the buffer was adjusted to 10.5 and kept at 4oC.

The buffer was brought to 37oC before use.

- Preparation of 0.05 (N) NaOH solutions

  NaOH -200mg

  Milli-Q water-100ml

RBC diluting fluid

NaCl - 1.0 gm

NaSO4

- 5.0 gm

HgCl2

- 0.5 gm

Milli-Q water - 200 ml

WBC diluting fluid

Glacial acetic acid - 1.5 ml

1% Gentian Violet sol. - 1.0 ml

Milli-Q water - 97.5 ml