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Appendix

Appendix 1

Instruments

Analytical balance H 6 T (E. Metter, Switzerland)

Autoclave model HA-3D (Hirayama Manufacturing Corporation, Japan)

Colony counter (New Brunswick Scientific, U.S.A)

Critical point dryer (Samdri-780)

Deep freeze refrigerator (Continental)

Incubator, Precision model 6 (Precision Scientific Co., U.S.A.)

Light microscope (Olympus, Japan)

Maxthermo(R) (Bonnchoo Engineering, Thailand)

Oven, Precision model 27 (Pricision Scientific Co., U.S.A.)

pH meter ø 43 (Beckman, U.S.A)

Refrigerator (Hitachi, Japan)

Sartorious GMBH Göttingen (Satorius, Geramany)

Scanning electron microscope JSM-T20, JSM-35CF(Jeol LTD., Japan)

Shaker G10 gyrotory(R) (New Brunswick Scientific Co., U.S.A.)

Shaker Julabo SW1 (Julabo Labortechnik GMBH, West germany)

Spectronic 710(Baush & Lomb, U.S.A.)

Sputter coater (JFC-1100)

Vortex cyclomixer (Clay Adams, U.S.A.)

Water bath (Precision Scientific Co., U.S.A.)

Appendix II

1. Preparation of media

1.1 Antibiotic medium no. 1 (Difco)

Bacto-Beef Extract	1.5 gm
Bacto-Yeast Extract	3.0 gm
Bacto-Casitone	4.0 gm
Bacto-Peptone	6.0 gm
Bacto-Dextrose	1.0 gm
Bacto-Agar	15.0 gm

To rehydrate the medium, suspend 30.5 gm in 1000 ml of distilled water and heat to boiling to dissolve the medium completely. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure ($121^{o}C$). The pH would be obtained to 6.6 ± 0.1.

1.2 Casein agar

1.2.1 Nutrient agar (see in 1.17)

1.2.2 Sterile fresh skim milk

Fresh Skim Milk	75.0 gm
Demineralized water	1000.0 ml

Add the milk to 1000 ml of water, a little at a time, stirring constantly, do not leave lumps. Dispense and sterilize in the autoclave for 15 min at 10 pounds pressure.

1.3 Gelatin (0.4%) agar

Tryptose	20.0 gm
Yeast Extract	0.3 gm

$MgSO_4$	0.1	gm
Gelatin	4.0	gm
Agar	15.0	gm

Add the above ingredients to 1000 ml of distilled water and heat until completely dissolved. Dispense and autoclave for 15 min at 10 pounds pressure.

1.4 Glucose peptone medium

Glucose	10.0	gm
Peptone	5.0	gm
Beef Extract	5.0	gm
NaCl	5.0	gm

Mix the ingredients in 1000 ml of distilled water and heat to boiling to completely dissolved.Adjust the pH to 7.2-7.4. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure ($121^{o}C$).

1.5 Glucose soybean medium

Glucose	20.0	gm
Soybean Powder	20.0	gm
$(NH_4)_2SO_4$	3.0	gm
K_2HPO_4	0.05	gm
NaCl	4.0	gm
$MgSO_4.7H_2O$	0.5	gm
$CaCO_3$	5.0	gm

Mix the ingredients in 1000 ml of distilled water and heat to boiling to complete dissolved. Adjust the pH to 7.0. Dispense and

sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.6 Litmus milk

Litmus Milk	105.0	gm
Distilled water	1000.0	ml

Mix to obtain a homogenous suspension. Dispense and autoclave for 10 min at 15 pounds pressure (121°C).

1.7 Maltose soybean medium

Maltose	20.0	gm
Soybean Powder	20.0	gm
$(NH_4)_2SO_4$	3.0	gm
K_2HPO_4	0.05	gm
NaCl	4.0	gm
$MgSO_4.7H_2O$	0.5	gm
$CaCO_3$	5.0	gm

Mix the ingredients in 1000 ml of distilled water and heat to boiling to complete dissolved. Adjust the pH to 7.0. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.8 Medium 1 : Tryptone-yeast extract broth

Bacto-Tryptone	5.0	gm
Bacto-Yeast Extract	3.0	gm

Mix the ingredients in 1000 ml of distilled water and heat to boiling to dissolve the medium completely. Adjust the pH to 7.0-7.2. Dispense and sterilize in the autoclave fot 15 min at 15 pounds pressure (121°C).

1.9 Medium 2 : Yeast extract-malt extract agar

Bacto-Yeast Extract	4.0	gm
Bacto-Malt Extract	10.0	gm
Bacto-Dextrose	4.0	gm
Bacto-Agar	20.0	gm

Mix the ingredients to 1000 ml of distilled water and heat to boiling until completely dissolved. Adjust the pH to 7.0-7.2. Dispense and sterilize in the autoclave for 15 min at pounds pressure ($121^{o}C$).

1.10 Medium 3 : Oatmeal agar

Oatmeal	60.0	gm
Bacto-Agar	12.5	gm

To rehydrate the medium suspend 72.5 gm in 1000 ml of distilled water and heat to boiling with constant stirring. Add trace salt solution 1.0 ml. Adjust to pH 7.2.

Trace salt solution

$FeSO_4.7H_2O$	0.1	gm
$MnCl_2.4H_2O$	0.1	gm
$ZnSO_4.7H_2O$	0.1	gm
Distilled water	100.0	ml

1.11 Medium 4 : Inorganic salts-starch agar

Solution I : Difco soluble starch 10.0 g. Make a paste of the starch with a small amount of cold distilled water and bring to

a volume of 500 ml.

Solution II :

K_2HPO_4 (anhydrous basis)	1.0	gm
$MgSO_4.7H_2O$	1.0	gm
NaCl	1.0	gm
$(NH_4)_2SO_4$	2.0	gm
$CaCO_3$	2.0	gm
Distilled water	500.0	ml
Trace salts solution (from 1.10)	1.0	ml

pH should be between 7.0 and 7.4

Mix starch suspension and salts solution. Add Bacto-Agar 20.0 gm and heat to boiling until completely dissolved. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.12 Medium 5 : Glycerol-asparagine agar

L-asparagine (anhydrous basis)	1.0	gm
Glycerol	10.0	gm
K_2HPO_4 (anhydrous basis)	1.0	gm
Distilled water	1000.0	ml
Trace salts solution (from 1.10)	1.0	ml

The pH of this solution is about 7.0-7.4

Add Bacto-Agar 20.0 gm to the solution and heat to boiling until completely dissolved. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.13. Medium 6 : Peptone-yeast extract iron agar

Bacto-Peptone Iron Agar, dehydrated	36.0	gm
Bacto-Yeast Extract	1.0	gm
Distilled water	1000.0	ml

Mix the ingredients in 1000 ml of distilled water and heat to boiling to dissolve the medium completely. Adjust the pH to 7.0-7.2. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.14 Medium 7 : Tyrosine agar

Glycerol	15.0	gm
L-tyrosine	0.5	gm
L-asparagine	1.0	gm
K_2HPO_4 (anhydrous basis)	0.5	gm
$MgSO_4.7H_2O$	0.5	gm
NaCl	0.5	gm
$FeSO_4.7H_2O$	0.01	gm
Distilled water	1000.0	ml
Trace salts solution (from 1.10)	1.0	ml

Adjust to pH 7.2-7.4

Add Bacto-Agar 20.0 gm to the solution and heat to boiling until completely dissolved. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.14 Medium 8 : Carbon utilization medium

A. Sterile carbon source

Use chemically pure carbon source certified to be free of admixture with other carbohydrates or contaminating material.

Carbon soures for this test are :

D-glucose	D-mannose
L-arabinose	Sucrose
D-xylose	Raffinose
D-fructose	D-mannitol
Inositol	Salicin
L-rhamnose	Cellulose
D-galactose	

Sterilize without heat by ether sterilization. Allow ether to evaporate at room temperature under a ventilated fume hood overnight. Add sterile distilled water aseptically to make a 10% w/v solution.

B. Trace salts

$CuSO_4.5H_2O$	0.64	gm
$FeSO_4.7H_2O$	0.11	gm
$MnCl_2.4H_2O$	0.79	gm
$ZnSO_4.7H_2O$	0.15	ml
Distilled water	100.0	ml

Store at 3-5^{o}C until required for use. Bring to room temperature before using. Prepare fresh solution each months. Disregard any precipitate or scale.

C. Basal mineral salts agar (use analytical reagent grade chemicals)

$(NH_4)_2SO_4$	2.64	gm
KH_2PO_4.anhydrous	2.38	gm

$K_2HPO_4.3H_2O$	5.65	gm
$MgSO_4.7H_2O$	1.00	gm
Trace salts (B)	1.00	ml
Distilled water	1000.0	ml
Adjust to pH 6.8-7.0		

Add Bacto-Agar 15.0 gm to the solution and heat to boiling to dissolve the medium completely.

D. Complete medium

Dispense and sterilize basal agar medium (C); cool it to 60°C and add sterile carbon source (A) aseptically to give a concentration of approximately 1%.

1.15 Nitrate Broth

KNO_3	1.0	gm
NaCl	0.5	gm
Peptone	2.0	gm

Mix the ingredients to 1000 ml of distilled water. Stir to completely dissolve. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.16 Mueller Hinton medium

Beef, Infusion form	300.0	gm
Bacto-Casamino Acids, Technical	17.5	gm
Starch	1.5	gm
Bacto-Agar	17.0	gm

To rehydrate the medium, suspend 38 gm in 1000 ml of distilled water and heat to boiling to dissolve the medium completely. Dispense and sterilize in the autoclave for 15 min at 15 pounds (121°C). The final reaction of the medium will be pH 7.4.

1.17 Nutrient agar

Bacto-Beef Extract	3.0	gm
Bacto-Peptone	5.0	gm
Bacto-Agar	15.0	gm

Mix the ingredients in 1000 ml distilled water and heat to boiling to completely dissolved. Adjust the pH to 7.0. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.18 Potato dextrose agar (Difco)

Potatoes, Infusion form	200.0	gm
Bacto-Dextrose	20.0	gm
Bacto-Agar	15.0	gm

To rehydrate the medium, suspend 39 gm in 1000 ml of distilled water and heat to boiling to dissolve the medium completely. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C). The medium will have a final reaction of pH 5.6.

1.19 Sabouraud dextrose agar (Difco)

Neopeptone, Difco	10.0	gm
Bacto-Dextrose	40.0	gm
Bacto-Agar	15.0	gm

To rehydrate the medium, suspend 65 gm in 1000 ml of distilled water and heat to boiling to dissolve the medium completely. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C). The final reaction of the medium will be pH 5.6.

1.20 Soybean-casein digest broth (USP)

Pancreatic digest of casein USP	17.0 gm
Papaic digest of soy meal USP	3.0 gm
NaCl	5.0 gm
K_2HPO_4	2.5 gm
Dextrose	2.5 gm

Mix the ingredients in 1000 ml of distilled water and heat to boiling to completely dissolved. Adjust the pH to 7.3. Dispense and sterilize in the autoclave for 15 min at 15 pounds pressure (121°C).

1.21 Starch agar

Nutrient Agar	1000.0 ml
Starch (soluble)	2.0 gm

When making up the nutrient agar, add 2.0 gm of starch to the dry ingredients. Heat to boiling to completely dissolved. Adjust the pH 6.8-7.0 and dispense. Autoclave for 15 min at 15 pounds pressure (121°C).

1.22 Tyrosine or Xanthine agar

Nutrient Agar	23.0 gm
Tyrosine or	5.0 gm

Xanthine	4.0	gm
Demineralized water	1000.0	ml

Dissolve the nutrient agar in 500 ml of water. Add tyrosine or xanthine and mix to distribute the crystals evenly. Adjust to pH 7.0 and autoclave for 15 min 15 pounds pressure (121^{o}C).

2. Preparation of solutions

2.1 Dimethyl-α-napthylamine solution

Dimethyl-α-napthylamine	6.0	ml
Acetic acid, 5N	1000.0	ml

Add dimethyl-α-napthylamine to acetic acid. Warm in a water bath to completely dissolve.

2.2 Gram's iodine solution

I_2 (crystal form)	1.0	gm
KI	2.0	gm
Distilled water	300.0	ml

Mix the iodine and the potassium iodine in a mortar and then grind with a pestle until finely divided. Add water in a small portions to wash out the contents. Add the rest of the water and mix well.

2.3 Nessler's reagent

Dissolve 50.0 gm of potassium iodine in 35 ml of cold distilled water. Add a saturated solution of mercuric chloride until a slight precipitate persists. Add 400 ml of a 50% solution of potassium hydroxide. Dilute to one liter, allow to settle, and decant the supernatant for use.

2.4 Normal saline solution

NaCl	8.5	gm
Distilled water to	1000.0	ml

Dissolve 8.5 gm of sodium chloride in 800 ml of cold distilled water and adjust to 1000 ml.

2.5 Osmium tetroxide (1.0%) solution

Solution I 4% OsO_4 aqueous stock solution

OsO_4	1.0	gm
Distilled water	25.0	ml

Solution II 0.2 M phosphate buffer pH 7.4 (see in 2.7)

0.2 M Na_2HPO_4	40.5	ml
0.2 M NaH_2PO_4	9.5	ml

To prepare 1.0% solution of osmium tetroxide, mixed solution I 1 part to 3 parts of 0.2 M phosphate buffer.

2.6 4% Paraformaldehyde in 0.1 M phosphate buffer (pH 7.2)

Solution I 40% paraformaldehyde

Paraformaldehyde (powder)	40.0	gm
Distilled water to	100.0	ml

Dissolve paraformaldehyde in 100.0 ml of distilled water at 60-65^{o}C. Add 40% NaOH to the solution until it clear.

Solution II 0.2 M phosphate buffer pH 7.2 (see in 2.7)

0.2 M Na_2HPO_4	36.0	ml
0.2 M NaH_2PO_4	14.0	ml

To prepare 4% solution of paraformaldehyde, dilivered the solution I and solution II as follow.

0.2 M phosphate buffer	50.0 ml
40% paraformaldehyde	10.0 ml
Distilled water	40.0 ml

2.7 0.1 M phosphate buffer (pH 7.2)

Solution A 0.2 M dibasic sodium phosphate

$Na_2HPO_4.2H_2O$	35.61 gm
Distilled water to	1000.0 ml

Solution B 0.2 M monobasic sodium phosphate

$NaH_2PO_4.H_2O$	27.60 gm
Distilled water to	1000.0 ml

Dissolve each stock solution seperately and store at room temperature. To prepare 100 ml of 0.1 M phosphate buffer, deliver the stock solution in the following amounts :

Solution A	36.0 ml
Solution B	14.0 ml
Distilled water	50.0 ml

2.8 Saturated ammonium sulfate solution

$(NH_4)_2SO_4$	1000.0 gm
Distilled water	1000.0 ml

Dissolve 1000 gm ammonium sulfate in 1000 ml of distilled water at 50°C, allow to stand over night at room temperature.

2.9 Sulfanilic acid reagent

Sulfanilic acid	8.0	gm
Acetic acid, 5N	1000.0	ml

Dissolve 8.0 gm of sulfanilic acid in acetic acid and mix well.

Vita

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