APPENDICES

APPENDIX A-Medium Preparation

A.1 Nutrient Agar

Preparation was according to direction as given on the label of content.

A.2 Nutrient Rich Medium (Doi, *et al.*, 1989)

Yeast Extract	10 g
Polypeptone	10 g
Beef Extract	10 g
Distilled water	1000 ml

A.3 E2 medium (Lageveen *et al.*, 1988)

$K_2HPO_4.3H_2O$	7.5 g
KH_2PO_4	3.7 g
$NaNH_4HPO_4.H_2O$	3.5 g
100 mM $MgSO_4.7H_2O$	1 ml
Distilled water	1000 ml
MT microelement solution	1 ml

MT microelements composition

$FeSO_4.7H_2O$	7.5 g
$MnCl_2.4H_2O$	0.99 g
$CoSO_4.7H_2O$	1.41 g
$CaCl_2.2H_2O$	0.742 g
$CuCl_2.2H_2O$	0.08 g
$ZnSO_4.7H_2O$	0.14 g
1N HCl	500 ml

A.4 Isolation Medium A (Demain and Soloman, 1986)

POME	10% v/v
Beef extract	0.5% w/v
Yeast extract	0.5% w/v

Antifungal agent	30 µg/ml
Agar	2.5% w/v

A.5 Isolation Medium B (Aaronson,1970)

NH_4Cl	0.05 g
$NaH_2PO_4.H_2O$	0.05 g
KH_2PO_4	0.05 g
$MgSO_4.7H_2O$	0.05 g
NaCl	0.4 g
Distilled water	100 ml

APPENDIX B-Stain Preparation

B.1 Sudan Black B (Burdon,1946)

B.1.1 Material

• Sudan Black B (Sigma Chemical Co.)	0.3 g
70% ethanol	100 ml

(Counterstain)

• Safranin stain (sigma Chemical Co.)	0.5 g
Distilled water	100 ml

- Xylol

B.1.2 Procedure

1. Flood the glass slide contains heat-fixed bacterial cells with Sudan Black B stain for 5 to 10 minutes.
2. Wash thoroughly through running tap water and blot-dry.
3. Destain the smear with Xylol.

4. Counterstain with safranin by flooding the smear for 5 to 10 seconds and wash through running tap water.
5. Blot-dry the slide and viewed under light microscope (Olympus BH-2) with 1000 times magnification.
6. Lipidic inclusions appear as black spots in pinkish cells.

B.2 Nile Blue A (Ostle and Holt, 1982)

B.2.1 Material

- Nile Blue A stain (Sigma Chemical Co.) 1 g

Distilled water 100 ml

- 1% (v/v) acetic acid 100 ml

B.2.2 Procedure

1. Prepare Nile Blue A stain by dissolving the powder in distilled water. Heat mildly in 55°C water bath for complete dissolvement. Filter the solution into amber bottle for storage.
2. Flood the Nile Blue A stain on slide contains heat-fixed bacterial cells for 10 to 15 minutes.
3. Wash through running tap water.
4. Flood the stained slide with 1% acetic acid for 1 minutes for final removal of non-absorbed stain.
5. Wash again through running tap water.
6. View the slide under epiflourescence microscope (Olympus BH-2) at 1000 times magnification.
7. PHA granules exhibit strong orange flourescence when observed at wavelength 460 nm.

B.3 Gram Staining (Sirockin and Cullimore, 1969)

B.3.1 Material

- Ammonium Oxalate crystal violet solution

Crystal violet	2 g
Methylated spirit	20 ml
1% aq ammonium oxalate solution	80 ml

- Lugol's iodine

Iodine	0.1 g
Potassium Iodide	0.2 g
Distilled water	100 ml

- Liquor iodine fortis for iodine-acetone solution

Iodine	10 g
Potassium Iodide	6 g
Methylated spirit	90 ml
Distilled water	10 ml

- Iodine/acetone

Liqour iodine fortis	3.5 ml
Acetone	96.5 ml

- Counterstain

0.5% safranin solution

B.3.2 Procedure

1. Flood the heat fixed smear of bacterial cells with ammonium oxalate crystal violet solution for 30 seconds and wash through running tap water.
2. Blot-dry the slide and flood with Lugol's iodine for 30 seconds and wash through running tap water.

3. Add dropwise of iodine-acetone solution onto the smear to remove non-absorbed crystal violet. The reaction is stopped by immediate washing through tap water.
4. Blot dry and counterstain the slide with 0.5% safranin solution for 10 seconds, wash through tap water and blot dry.
5. The cells are observed under the immersion-oil objective (Nikon HFX-DX). Gram positive cells appear blue while gram negative cells appear pink.

APPENDIX C-Analytical Standard

C.1 Determination of Ammonium Content by Phenolhypochlorite Method. (Solorzono,1969)

C.1.1 Material

- 0.5% Sodium nitroprusside

Sodium nitropruside	1 g
Distilled water	200 ml

- Alkaline solution

Trisodium citrate	100 g
Sodium hydroxide	5 g
Distilled water	500 ml

- Sodium hypoclorite solution

Commercial hypoclorite (chlorox)

- Oxidising solution

Sodium citrate solution	100 ml
Hypoclorite solution	25 ml

C.1.2 Ammonium Standard Graph

Concentrations of 0.08, 0.16, 0.24, 0.32, 0.4, 0.48, and 0.5 µg/ml were prepared in triplicates from stock of NH_4Cl solution (20mg/ml). About 25 ml of each concentration was measured into a 50 ml conical flask. 1 ml of phenol solution, 1 ml of sodium nitroprusside solution and 2.5 ml of oxidising reagent were added successively. The colour was allowed to develop at room temperature for 1 hour and read at 640 nm in a spectrophotometer. The data and standard graph are shown on Table 10 and Figure 22, respectively.

C.2 Determination of Fatty Acids Content by Titration (Cocks and Rede, 1966)

C.2.1 Material

- Solvent

Equal volumes of 96% ethanol and diethyl ether. Neutralised shortly before use with 0.1N sodium hydroxide using phenophthalein as indicator.

- Sodium hydroxide

0.1N sodium Hydroxide.

- Phenophthalein indicator

10 g/l solution in 95% ethanol.

C.2.2 Fatty Acids Standard Graph

Palmitic acids with quantities of 20, 40, 60, 80 and 100 mg were dissolved into 50 ml solvent. The solution was titrated with 0.1N NaOH solution by using 25 ml Biurette. The amount of NaOH required to change the colour to pink was noted. The data and standard graph are shown on Table 11 and Figure 23, respectively.

Table 10

Ammonium concentrations vs optical density (OD) 640 nm. Data for standard graph.

Ammonium Concentration (mg/l)	Optical Density (640nm)			
	R1	R2	R3	Average
0.08	0.0634	0.0520	0.0141	0.0523
0.16	0.1143	0.0854	0.1029	0.1009
0.24	0.1573	0.1667	0.1680	0.1640
0.32	0.1653	0.2157	0.2072	0.1961
0.40	0.2542	0.2688	0.2328	0.2516
0.48	0.2848	0.3077	0.3036	0.2987
0.50	0.3462	0.3394	0.3434	0.3430

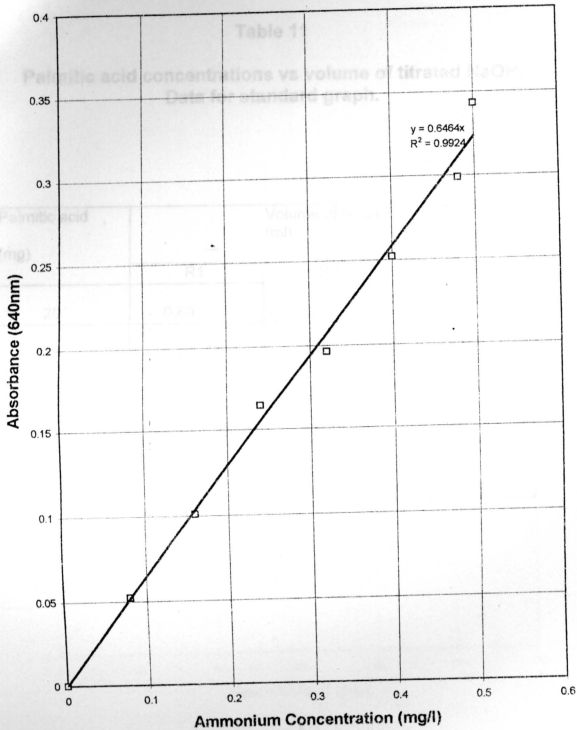

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Figure 22

Standard graph for ammonium nitrogen.

Table 11

Palmitic acid concentrations vs volume of titrated NaOH. Data for standard graph.

Palmitic acid (mg)	Volume of NaOH (ml)		
	R1	R2	Average
20	0.80	0.75	0.75
40	1.70	1.70	1.65
60	2.50	2.60	2.60
80	3.30	3.45	3.40
100	4.10	4.20	4.30

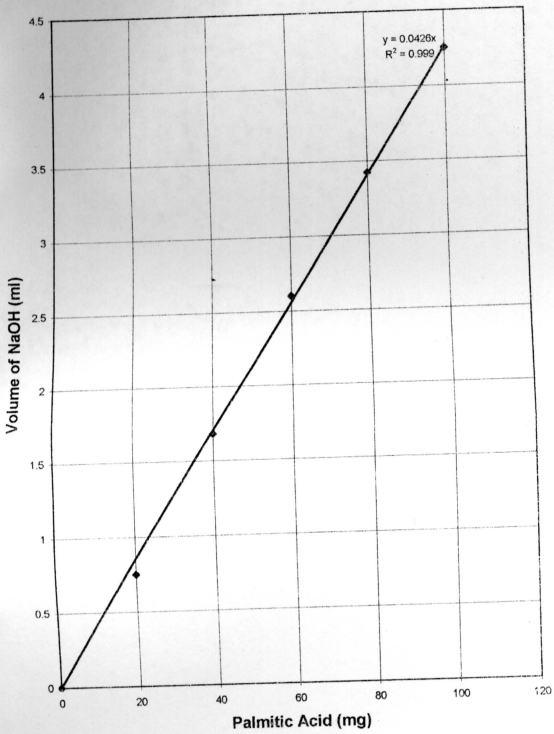

Figure 23

Standard graph for fatty acid.