APPENDIX 2

CHEMICALS USED:

1 AMINOCYCLOPROPANE 1- CARBOXYLIC ACID (ACC)
2,3 DIHYDROXY BENZOIC ACID
2,4 DINITROPHENYL HYDRAZINE
8 HYDROXYQUINOLINE
ACETONE 80%
AMMONIA
AMMONIUM SULPHATE
BENZYL ALCOHOL
BROMOPHENYLE BLUE
BROMOTHYMOL BLUE
CHLOROFORM
CONGO RED
DIETHYLE ETHER
HYDROXYAMINE HYDROCHLORIDE.
METHANOL
RIFAMPICIN (2000μg/mL-1)
TOLUENE
XAD 4 COLUMN
α-KETOButRYIC ACID
REAGENTS

1. **SALKOVVYSKY'S REAGENT**
   Add 1 mL of 0.5M FeCl₃ in 50mL of 35% HClO₄ with continuous stirring.

2. **1% PICRIC ACID**
   Add 1 g of Picric acid in 100mL of D.W.

3. **CHROME AZUROL S DYE**
   Dissolve 60.5 mg CAS in 50 mL MQ water. Add 72.9 mg of HDTMA on 40 mL of MQ water. Mix CAS solution and HDTMA solution. Add 10mL of 1mM FeCl₃ solution slowly by stirring without foaming till the color turns brown to blue.

4. **CAS ASSAY SOLUTION**
   Add 6mL of 10mM HDTMA in 100mL volumetric flask and dilute with water. Add 1.5 mL of 1mM FeCl₃. 6H₂O prepared in 10mM HCl. Slowly add 7.5 mL of 2mM aqueous CAS dye solution by stirring. Separately prepare 4.307 g anhydrous piperazine solution in water and make its pH up to 5.6. add 6.25mL of HCL (12M) to get a buffer solution. Add this to above volumetric flask and make volume upto 100mL with double distilled water to give CAS assay solution

5. **NITRITE MOLYBDATE REAGENT**
   Add 10 g of sodium nitrite and 10 g of sodium molybdate in 100 mL of MQ to give 100 mL of nitrite molybdate reagent

6. **1 % SULPHANILIC ACID**
   Add 1 gm of sulphanilic acid in 100 mL of 30% acetic acid.

7. **2 % NA₂HASO₄**
   Add 2 gm of sodium arsenate in 100 mL of 30 % acetic acid.

8. **0.3 % 1-NAPHTHYLAMINE**
   Add 0.3 g of 1-Naphtylamine in 100 mL of 30 % acetic acid.
9. 1.3 % IODINE

Add 1.3 gm of sublimed iodine crystals with KI and add a little of 30 % acetic acid. Stir thoroughly till crystals dissolve. Add rest of the acetic acid to make 100 mL solution.

10. STANNOUS CHLORIDE (SnCl₂₂H₂O)

Dissolve 2.5 g SnCl₂₂H₂O in 10 mL of concentrated HCl by heating till solution turns clear. Transfer this solution to 100 mL of volumetric flask and make the volume up to 100 mL by adding double distilled water.

11. CHLOROMOLYBDIC ACID

Dissolve 15.0 g of Ammonium molybdate in about 400 mL of warm D. W. filter if necessary and add 400 mL of 10 N HCl or 342 mL of 12 N HCl slowly with rapid stirring. Cool and make the volume up to one litre with distilled water and store in amber glass bottle.

12. 25 % CITRIC ACID.

25 g OF citric acid I N 100 mL of D.W.

13. DIMETHYLE GLYOXIME

Take 0.5 gm of Dimethyle glyoxime in 250 mL ammonia. Dilute to 500 mL by adding D.W.
STANDARD METHODS:

1. ESTIMATION OF SOLUBLE PHOSPHATES IN PIKOVSKYAYA'S MEDIUM (Gaur, 1990)

REQUIREMENTS:

1. Chloromolybdic Acid
2. Chlorostannous Acid
3. Std Phosphate solution:
   Dissolve 0.4390 g of dried $\text{K}_2\text{HPO}_4$ in 400 ml distilled water. Add 25 mL of 7 N $\text{H}_2\text{SO}_4$. Make up to 1 L to give a standard stock of (100 ppm).
   Working solution: Dilute 2 mL of the stock solution and make up to 100 mL with distilled water.
   Transfer different aliquots of the working solution in 50 mL volumetric flasks and make the total volume up to 10 mL with double distilled water.
   Add 10 mL of Chloromolybdic acid. Dilute the contents in flasks up to 40 mL by adding double distilled water.
   Add 5 drops of Chlorostannous acid reagent and mix well till blue colour develops. Make the final volume up to 50 mL with double distilled water as quickly as possible.
   Take OD at 600 nm and plot a standard graph so as to get a straight line.
   To check the amount of phosphate solubilized in the medium, collect 1 mL of supernatant and add 9 mL of double distilled water. Perform the whole procedure mentioned above till blue colour develops and take OD at 600 nm. Calculate the total amount of phosphate solubilized and present in 1 mL of supernatant from the standard graph.
2. ESTIMATION OF INDOLE ACETIC ACID.
(SARWER AND KRAMER, 1995)

REQUIREMENTS:
1. Salkowsky’s reagent
2. Standard IAA: Stock: (1000 μg ml⁻¹)
   Working Solution (10-100 μg ml⁻¹)

Prepare different aliquots of Standard IAA solution so as to get final concentration in the range of 10 - 100 μg mL⁻¹. Make final concentration up to 1 mL by adding MQW.

Add 1 mL of Salkowsky’s reagent and keep in dark for incubation for 30 min for pink colour to develop.

Measure the optical density at 536 nm and plot standard graph.

Take 1 mL of supernatant and add equal amount of Salkowsky’s reagent.

Incubate for 30 min and take OD at 536 nm.

Estimate the amount of IAA produced from the standard graph.
3. ESTIMATION OF CATECHOLATE TYPE OF SIDEROPHORES BY ARNOW'S METHOD (ARNOW, 1937)

REQUIREMENTS:
1. Nitrite Molybdate reagent
2. 0.5 N HCL
3. 1 N NaOH
4. STANDARD Dihydroxybenzoic acid (DHBA):
   a. Stock : 1mg mL⁻¹     b. Working Solution: 100 μg mL⁻¹
   → Prepare different aliquots of DHBA solution so as to get final concentration in the range of 10 - 100 μg mL⁻¹. Make final concentration up to 1 mL by adding MQW.
   → Add 1 mL of 0.5 N HCl and then add 1 mL of sodium molybdate reagent.
   → Incubate the tubes for 5 min and then add 1 mL of 1 N NaOH and allow the pink colour to develop.
   → Take OD at 536 nm within 30 min and plot a standard graph so as to get a straight line.
   → Collect 1 mL of supernatant of actively growing cultures from deferrated medium and follow the steps mentioned above. Take OD at 536 nm and calculate the catecholate siderophores from standard graph.

4. DEFFERATION OF MEDIUM:

REQUIREMENTS:
1. 8 hydroxyquinoline
2. Chloroform
   → Take 100 mL of medium and add 100 mL of 0.25 % 8 - Hydroxyquinoline added chloroform.
   → Mix vigorously in a separating funnel and give 100 strokes. Allow the mixture to stand till chloroform separates. Whatever iron is present in the medium will form iron-quinolates and will be collected in the chloroform.
   → Collect the chloroform layer and wash the medium twice with pure chloroform so as to give deferrated medium.
5. ESTIMATION OF HYDROXAMATE TYPE OF SIDEROPHORES BY CSAKY'S METHOD (CSAKY'S, 1948)

REQUIREMENTS:
1. 1 % Sulphanilic acid in 30 % Acetic acid.
2. 1.3 % Iodine solution in 30 % Acetic acid.
3. 2 % Sodium Arsenate in 30 % Acetic acid.
4. 0.3 % α-napthylamine solution in 30 % Acetic acid.

Standard: Hydroxylamine hydrochloride (1000 µg mL⁻¹)

→ Collect 0.5 ml supernatant and add 0.5 mL H₂SO₄ and autoclave it. Allow to cool.

→ Add 1 mL of 1 % sulphanilic acid and 0.5 ml of 1.3 % Iodine solution. Allow to stand for 5 min.

→ Destroy excess of iodine by adding 1 mL of 2 % sodium arsenate solution. Wait till yellow colour disappears.

→ Add 1 mL of 0.3 % α-napthylamine solution and incubate for 30 min till pink colour develops. Take OD at 536 nm.

→ Prepare standard graph of hydroxylamine hydrochloride (100 - 1000 µg mL⁻¹) and calculate the amount of hydroxamate type of siderophore from the standard graph.
6. ESTIMATION OF RESIDUAL NICKEL IN THE SUPERNATANT

(REQUIREMENTS:
1. 25 % Citric acid
2. Dimethyle glyoxime
3. Dilute Ammonia

- Stock Nickel chloride solution: 0.1 M, working solution: 1 mM
- Prepare different aliquots of nickel chloride so as to get a concentration range of 0.1 to 1.0 mM of Nickel and make the final volume 2 mL with double distilled water or MQW.
- Add 5 mL of 25 % citric acid and adjust the pH upto 7.5 with dilute ammonia. Shake well and allow to cool.
- Add 20 mL of Dimethyle Glyoxime (DMG) solution so as to get pink colour and allow to stand of 2-3 min for colour to develop fully.
- Add 12 ml of chloroform, shakewell and all to separate in separating funnel.
- Collect solvent layer and take OD at 540 nm and plot standard graph.
- For estimation of residual nickel in the supernatant, take one mL of supernatant and mix 1 mL of MQW. Follow the procedure as mentioned and take OD at 540 nm. Estimate the concentration in 1 mL of supernatant from the standard graph of nickel chloride.)
7. ESTIMATION OF CHLOROPHYLL A AND B FROM LEAF TISSUE (HISCOX AND ISRAETUM, 1978)

REQUIREMENTS:
1. 100 mg Leaf tissue
2. 80% Acetone.
   → Take 100 mg leaf tissue and crush with the help of pestle and mortar.
   → Mix 15 mL of acetone and incubate in water bath at 60 °C for 3 h.
   → Take OD at 645 nm for estimating chlorophyll a and 663 nm for estimating chlorophyll b.
   → Calculate chlorophyll a and b as well as total chlorophyll by following formula.

Chlorophyll a: \[ 12.7 \times \text{O.D. (663)} - 2.69 \times \text{OD (645)} \times \text{Vol (15 mL)} \]
\[ \frac{1000 \times \text{Weight of the sample (100 mg)}}{1} \]

Chlorophyll b: \[ 22.9 \times \text{OD (645)} - 4.68 \times \text{OD (663)} \times \text{Vol (15 mL)} \]
\[ \frac{1000 \times \text{Weight of the sample (100 mg)}}{1} \]

Total Chlorophyll: \[ 20.2 \times \text{OD (645)} + 8.02 \times \text{OD (663)} \times \text{Vol (15 mL)} \]
\[ \frac{1000 \times \text{Weight of the sample (100 mg)}}{1} \]