APPENDICES

APPENDIX - 1

Preparation of Cultivated medium

Yeast mannitol Medium (Cultivation of *Rhizobium sp.)*		Azospirillum Medium (Cultivation of *Azospirillum sp.)*	
K_2HPO_4	- 0.5g	Malic acid	5.000g
$MgSO_4.7H_2O$	- 0.1g	Dipotassium hydrogen phosphate	0.500g
NaCl	- 0.2g	Ferrous sulphate	0.500g
Mannitol	- 10.0g	Manganese sulphate	0.010g
Yeast extract	- 0.5g	Magnesium sulphate	0.200g
Agar	- 1.5g	Sodium chloride	0.100g
Distilled water	- 1 litre	Bromo thymol blue	0.002g
		Sodium molybdate	0.002g
		Calcium chloride	0.020g
		Agar	1.750g

King's B medium (Cultivation of *Pseudomonas fluorescens*)

Proteose peptone - 10 g

Anhydrous K_2HPO_4 -1.5 g

Glycerol -15 g

$MgSO_4$ (1 M; sterile) - 5 mL

Agar - 1.5 g

Antibiotics (as needed)

Part B

Potassium hydroxide 4.000g

Final pH (at 25°C) - 6.8±0.2

**Formula adjusted, standardized to suit performance parameters

Add distilled water to first three ingredients to bring volume to 1 litre. Adjust the pH to 7.0 with HCl. Autoclave and then add 5 mL of sterile 1 M $MgSO_4$. If $MgSO_4$ is added before autoclaving, the medium becomes cloudy.

APPENDIX - 2

EVALUVATION OF TURBIDITY UNITS
(By Nephelometric method)
(APHA, 1995)

Apparatus

a. Nephelometric turbidity meter with sample cells

Reagents

a. **Solution I.** Dissolve 1.000g hydrazine sulphate, $(NH_2)_2.H_2SO_4$ in distilled water and dilute to 100 mL in a volumetric flask.

b. **Solution II**. Dissolve 10.00g hexamethylenetetramine, $(CH_2).6N_4$, in distilled water and dilute to 100 mL in a volumetric flask.
c. **4000 NTU suspension**. In a flask mix 5.0 mL of Solution I and 5.0 mL of Solution II. Let it stand for 24 h at 25 ±3°C. This results in a 4000 NTU suspension. Store in an amber glass bottle. The suspension is stable for up to 1 year.
d. **Dilute 4000 NTU** stock solution with distilled water to prepare dilute standards just before use and discard after use.
Procedure
a. **Calibrate nephelometer** according to manufacturer's operating instructions. Run at least one standard in each instrument range to be used.
b. **Gently agitate sample**. Wait until air bubbles disappear and pour sample into cell. Read turbidity directly from instrument display.

APPENDIX - 3

ANALYSIS OF TOTAL DISSOLVED SOLIDS (APHA, 1995)

Apparatus

a. ***Evaporating dishes***, 100 mL capacity of porcelain, platinum or high-silica glass made

b. ***Drying oven*****,** 104 ± 1°C

c. ***Desiccator***

d. ***Magnetic stirrer***

e. Glass-fibre filter disk, Whatmann grade 934 AH, Gelman type A/E, Millipore type AP4O or equivalent, diameter 2.2 to 12.5 cm.

f. Filtration apparatus, Membrane filter funnel or Gooch crucible with adapter and suction flask of sufficient capacity for sample size selected

Procedure

a. Wash filter paper by inserting it in the filtration assembly and filtering 3 successive 20 mL portions of distilled water. Continue suction to remove all traces of water. Discard washings.

b. Dry evaporating dish at 104 ± 1ºC for 1 h, cool and store in desiccator. Weigh immediately before use.

c. Stir sample with a magnetic stirrer and while stirring pipette a measured volume on to the filter using a wide bore pipette. Choose sample volume to yield between 10 and 200 mg dried residue. Wash with three successive 10 mL volumes of distilled water. Continue suction for about 3 min after filtration is complete.

d. Transfer total filtrate with washings to a weighed evaporating dish and evaporate to dryness in an oven at 104 ±1ºC. If necessary add successive portions to the same dish after evaporation in order to yield between 10 and 200 mg dried residue. To prevent splattering oven temperature may be lowered initially by 20°C below boiling point and raised to 104ºC after evaporation for 1h. Cool in a desiccator and weigh.

Calculate

$$\text{mL sample} = \frac{(A - B) * 1000}{\text{mg Dissolved Solids/L}}$$

where

A = weight of dried residue + dish, mg

B = weight of dish, mg.

APPENDIX - 4
ESTIMATION OF BIOCHEMICAL OXYGEN DEMAND
Winkler's iodometric method (APHA, 2005)

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Reagents for the Preparation of dilution water

1. Calcium chloride solution: 27.5g was dissolved in one litre of distilled water.
2. Magnesium sulphate solution: 0.25g was dissolved in one litre of distilled water.
3. Ferric chloride solution: 0.25g was dissolved in one litre of distilled water.
4. Phosphate buffer (pH 7.2): 21.75g dipotassium hydrogen phosphate, 23.4g of disodium hydrogen phosphate, 8.5g of potassium hydrogen phosphate and 1.7g of ammonium chloride, dissolved in 500ml of distilled water and make upto one litre with water.

Reagents for the estimation of dissolved oxygen

1. Manganous Sulphate solution: Dissolved 91.0g of manganous sulphate in 250ml of distilled water.
2. Alkali-iodide-azide reagent:
   Reagent A: 175g of potassium hydroxide and 37.5g of potassium iodide were dissolved in 250ml water.
   Reagent B: 2.5g sodium azide was dissolved in 10ml of water. Both reagent A and reagent B were mixed.
3. Concentrated sulphuric acid
4. Phosphoric acid: 80-90%
5. Sodium thiosulphate solution (0.1N): 24.82g was dissolved in one litre of distilled water.
6. Sodium thiosulphate solution (0.025N): Diluted 250ml of sodium thiosulphate solution (0.1N) to 1000ml of distilled water.1.0ml of 0.025N sodium thiosulphate is equivalent to 0.2mg dissolved oxygen.
7. 1% starch solution.

Procedure

Preparation of dilution water: Added 1.0ml of calcium chloride, magnesium sulphate, ferric chloride and phosphate buffer solutions to one litre of aerated distilled water and mixed thoroughly. This is the standard dilution water, prepared freshly every time. Seeding of the dilution water: It is essential to seed the dilution water. The seeding material generally used is freshly settled raw sewage. 2.0ml of raw sewage was added to one litre of dilution water. Dilution of the sample: The test water sample was diluted with seeded dilution water sample (1%, 5% and 10%) in dilution mixture for the water sample. Each dilution sample was taken in a two set of BOD bottles.

Determination of dissolved oxygen (DO) before and after five days incubation: In one set of flasks DO was determined immediately while other set was kept for incubation at 20ºC for five days. DO of the incubated sample was determined.

Determination of DO is as follows: To the contents of the BOD bottle added 2.0ml of manganous sulphate solution and 2.0ml of alkali-iodide-azide solution. Stoppered the bottle and mixed thoroughly. A brown precipitate of basic manganic oxide was formed, which was allowed to settle completely leaving a clear supernatant liquid. Then added 2.0ml of concentrated sulphuric acid along the sides of the bottle. Stoppered and mixed for complete dissolution.Transferred the contents to a 500ml conical flask and titrated immediately against 0.025N sodium thiosulphate using starch as an indicator.

Calculation for DO:

Volume of 0.025N sodium thiosulphate used in the titration = DO in mg/l DO at 0ºC 760 mm pressure = DO x 0.07 mg/l

Calculation for Bod:

BOD (5 days at 20ºC) = (DO_0 - DO_5 - BC) x100\percent sample.

DO_0 = Initial DO

DO_5 = DO after 20ºC incubation for 5 days

BC = Blank correction i.e., Difference in DO of blank on the initial day and after 5 days incubation.

APPENDIX- 5
ESTIMATION OF CHEMICAL OXYGEN DEMAND
Titrimetric method (APHA, 2005)

Reagents

1. Mercuric sulphate crystals

2. Sulphuric acid- silver sulphate reagent: Dissolved 10.1g of silver sulphate in one litre of concentrated sulphuric acid. Allowed the solution to stand for two days for complete dissolution.

3. Potassium dichromate solution 0.125N: Dissolved 0.129g in distilled water and made upto one litre. 1.0ml of 0.125N potassium dichromate = 1.0mg of oxygen.

4. Ferroin Indicator solution: Dissolved 95mg of ferrous sulphate in 500ml of distilled water. Added 1.48g of 1.10g phenanthroline monohydrate and mixed thoroughly.

5. Ferrous Ammonium Sulphate solution 0.125N: 40g of ferrous ammonium sulphate was dissolved in distilled water. Added 20ml of concentrated sulphuric acid. Made upto one litre with water. Standardized of it with 0.125N potassium dichromate.

Procedure

A refluxing flask of 250ml capacity was used with a ground glass 24/40 neck fitted with a 300mm double surface condenser to which, a glass cap was fitted. Placed 50ml of the sample in the flask. Added mercuric sulphate of suitable quantity such that the ratio of chloride content of the sample to mercuric sulphate was 1:10. Then added 5ml of sulphuric acid-silver sulphate reagent and dissolved the mercuric sulphate. Cooled in cold water while mixing. Pipetted 25ml of 0.125N potassium dichromate into the flask and mixed. Added a few porcelain bits and attached the condenser. Started water circulation and refluxed for two hours. Removed the flame, allowed the flask to cool. Transferred the contents of the flask and diluted to about 350ml with distilled water. Added 2 to 3 drops of ferroin indicator and titrated against 0.125N ferrous ammonium sulphate solution. The end point was the sharp colour change from blue- green to reddish brown. A blank was conducted using 50ml of distilled water instead of the sample.

Calculation

COD in mg/l = (blank titre value-sample titre value) x 0.125 x 1000 x 8 volume of the sample taken.

APPENDIX- 6
ELECTRICAL CONDUCTIVITY
Conductivity method (APHA, 2005)

Reagents

1. Conductivity water: Passed distilled water through a mixed bed deionize and discarded the first litre.

2. Standard potassium chloride solution 0.0100M: Dissolved 745.6mg of anhydrous potassium chloride (KCl) in conductivity water and diluted to 1000ml at 25ºC.

Procedure

1. Determination of cell constant: rinsed conductivity cell with at least three portions of 0.01M KCl solution. Adjusted temperature to 25.0 ± 0.1ºC.

Complete cell constant, C = (0.001413) (RkCl) (1+0.0191 (t-25)

Where Rkcl=measured resistance ohms, t = observed temperature tºc.

2. Conductivity measurement: Rinsed cell with one or more portions of sample. Adjusted the temperature to 25.0 ± 0.1ºC.

Calculation

K= (km) (c)/1+0.0019 (t-25), km=measured conductance mhos at tºC.

APPENDIX- 7
ESTIMATION OF CARBONATE AND BICARBONATE (ALKALINITY)
Titrimetric method (Natarajan *et al.*, 1988)

Reagents

1. Sodium carbonate solution 1N: 1 3.25g was dissolved in 250ml water.
2. Sulphuric acid 1N: 28ml of concentrated sulphuric acid was made upto a liter with water.
3. Sulphuric acid 0.02N: Prepared from 1N sulphuric acid
4. Phenolphthalein indicator: 500mg was dissolved in 50ml of alcohol and 50ml of distilled water. Added 0.02N sodium carbonate solution until a faint pink colour appeared.
5. Mixed indicator solution: 20 mg of methyl red and 100mg of bromocresol green were dissolved in100ml of 95% isopropyl alcohol.

Procedure

Added 25ml of the sample and 25ml of distilled water in a 250ml conical flask. Added phenolphthalein indicator solution. If no pink colouration, it indicated phenolphthalein alkalinity. If pink colour appeared then titrated with sulphuric acid (0.02N), until the solution became colourless. Added 3 drops of mixed indicator solution in which phenolphthalein alkalinity had been determined and titrated against sulphuric acid (0.02N) to light pink colour.

Calculation

Volume of the sample taken = 25ml

Volume of 0.1N sulphuric acid used up to phenolphthalein end point = A ml

Volume of 0.1N sulphuric acid used up to methyl orange end point =B ml

Volume of 0.1N sulphuric acid required up to neutralize bicarbonate alone = (B-A) ml.

Carbonate

1.0ml of 0.1N sulphuric acid=0.003g of CO_3

2 x A ml of 0.1N sulphuric acid =0.003 x 2 x Ag

Amount of carbonate per litre of sample=0.003 x 2 x 1000 x 1000/25mg

Bicarbonate

1.0ml of 0.1N sulphuric acid = 0.0061g of HCO_3

(B-A) ml of 0.1N sulphuric acid = 0.0061 x (B-A) g of HCO_3

Amount of bicarbonate per litre of sample = 0.0061 x (B-A) x 1000 x 1000/25mg

APPENDIX- 8
ESTIMATION OF TOTAL ALKALINITY (pH 4.5)
(By Titrimetric method) (APHA, 1995)

Apparatus

a. Standard laboratory glassware such as burettes, volumetric flasks and beakers.

Reagents

a. Standard sodium carbonate, approximately 0.05N. Dry 3 to 5g sodium carbonate, Na_2CO_3, at 250°C for 4h and cool in a desiccator. Accurately weigh 2.5±0.2g to the nearest mg, dissolve in distilled water and make to 1L.

b. Standard H_2SO_4, approximately 0.1N. Dilute 2.8 mL conc. sulphuric acid to 1L.Standardise against 40.00 mL 0.05*N* Na2CO3 with about 60 mL distilled water, in a beaker by titrating potentiometrically to pH 5. Lift out electrodes, rinse into the same beaker and boil gently for 3 to 5 min under a watch glass cover. Cool to room temperature, rinse cover glass into beaker and finish titration to pH 4.3. Calculate normality of sulphuric acid.

Normality, N = A*B/ 53.00*C

where:

A = g Na_2CO_3 weighed into the 1L-flask for the Na_2CO_3 standard (see *a.*)

B = mL Na_2CO_3 solution taken for standardisation titration

C = mL acid used in standardisation titration

c. In case potentiometric titration is not possible use bromcresol green indicator to complete the titration.

d. Standard sulphuric acid, 0.02N. Dilute the approximate 0.1*N* solution to 1L.

Calculate volume to be diluted as: mL volume = *N/* 20

where:

N = exact normality of the approximate 0.1*N* solution.

e. Bromcresol green indicator, pH 4.5: Dissolve 100mg bromcresol green sodium salt in 100 mL distilled water

Procedure

a. Add 2 to 3 drops of bromcresol green indicator. Titrate until change in colour (blue to yellow, pH 4.9 to 4.3) is observed. Record total mL titrant used.

Calculation

Total alkalinity, mg CaCO /L = B*N*5000/mL sample

where B = total mL of titrant used to bromcresol green end point

N= normality of titrant

Note:

For turbid/coloured samples, titration can be performed using a pH meter to end point pH value of 4.5.

APPENDIX- 9
TOTAL HARDNESS
EDTA titrimetric method (APHA, 2005)

Reagents

1. Buffer solution: Dissolved 16.9g of ammonium chloride in 143ml ammonium hydroxide. Added 1.25g magnesium salt of EDTA and diluted to 250ml with distilled water.

2. Indicator: Ferrochrome black T: Dissolved 0.5g of dye in 100g of 2, 2, 2- nitrilotriethanol added 2 drops per 50ml solution to be titrated.

3. Calmagite: Dissolved 0.1g of Calmagite in 100ml-distilled water. Used 1ml per 50 ml solution to be titrated.

4. Standard EDTA titrant, 0.01M: Weighed 3.723g of analytical reagent grade EDTA, dissolved in distilled water and diluted to 1000ml. Standardized against standard calcium solution.

5. Standard calcium solution: weighed 1.000g of anhydrous $CaCO_3$ powder and diluted to 1000ml with distilled water.1ml=1.00mg $CaCO_3$.

Procedure

Diluted 25ml sample to about 50ml with distilled water in a suitable vessel. Added 1 to 2ml buffer solution. Usually 1ml will be sufficient to give a pH 10 to 10.1. Added 1 to 2 drops of indicator solution. Added EDTA titrant slowly, with continuous stirring, until the last reddish tinge disappears. Added the last few drops at 3 to 5 seconds intervals. At the end point the solution was normally blue.

Calculation

Hardness (EDTA) as mg $CaCO_3$/ L= AxBx1000/ml sample.

Where

A= ml titration for sample

B=mg $CaCO_3$ equivalent to 1.00ml EDTA titrant.

APPENDIX- 10

ESTIMATION OF CALCIUM

EDTA titrimetric method (APHA, 2005)

Principle

The pH of the sample is made sufficiently high (12-13) to precipitate magnesium as hydroxide and calcium only is allowed to react with EDTA in the presence of a selective indicator.

Reagents

1. Sodium hydroxide 1N: 40g of sodium hydroxide was dissolved in one liter of distilled water.
2. Murexide indicator: 200mg of the dye was ground with 100mg of sodium chloride.
3. Standard EDTA titrant, 0.02N.

Procedure

Pipetted out 50ml of the sample. Added 2.0ml of sodium hydroxide to it to produce a pH of 12- 13 and mixed well. Added 0.1-0.2g of the indicator and titrated immediately with EDTA. The end point is from pink to purple.

Calculation

If the EDTA titrant is exactly 0.02N mg/l calcium (as $CaCO_3$) = ml EDTA titrant x 1x 1000ml sample taken for the titration.

APPENDIX- 11

ESTIMATION OF MAGNESIUM

Calculation method (APHA, 2005)

Calculation

Mg/l magnesium (as $CaCO_3$) = mg/l total hardness (as $CaCO_3$) - mg/l calcium (as $CaCO_3$) mg/l.

APPENDIX- 12

ESTIMATION OF SODIUM AND POTASSIUM

Flame photometric method (Natarajan *et al.,* 1988)

Reagents

1. Sodium stock solution: 2.524g of sodium chloride was dissolved in deionised water and made upto one liter. 1.0ml = 1.0mg of sodium.
2. Potassium stock solution: 1.907g of potassium chloride was dissolved in deionised water and made upto one liter. 1.0ml = 1.0mg of Potassium.

Procedure

Standardized the flame photometer before feeding the sample. Set the reading to zero using deionised water. Using the stock solutions of sodium and potassium, adjusted the reading to 100 at their specific wavelengths. Then feed the sample in the flame photometer and noted the readings to get the amounts of sodium and potassium directly on in milligrams per litre, by referring to the appropriate calibration curve.

APPENDIX- 13

ESTIMATION OF IRON

DTPA method (Shanmugam *et al.*, 1994)

Reagents:

1. The diethylene triamine penta acetic acid (DTPA) extracting solution was prepared to contain 0.005M DTPA, 0.01M $CaCl_2$ 0.1M TEA and pH adjusted to 7.3. To prepare 1 litre of this solution, dissolved 1.967g of DTPA, 1.47g $CaCl_2.2H_2O$, 13.3ml of reagent grade TEA in approximately 20ml of water. Allowed sufficient time for DTPA to dissolve and diluted to one litre. Adjusted the pH to 7.3+0.05 with 1:1 HCl.

2. Preparation of standard solution: 1.0g of Fe pure metal in 20ml of 1:1 HCl and diluted to 1litre to 1000ppm or μg/ml. If $FeSO_4 .7H_2O$ was used. 4.4g of $FeSO_4 .7H_2O$ was dissolved in 1 liter to give 1000ppm of Fe.

Procedure:

10g of sample was placed in a 50ml polyethylene bottle and 20 ml of DTPA extracting solution was added. The bottle was tightly closed with a stopper and shaken on a horizontal shaker for 2 hours. Then the suspension was filtered through Whatmann No.42 filter paper. The filtrate was analysed for iron using atomic absorption spectrophotometer. Used the standard solution to prepare a calibration curve. Obtained the observation in pap of element in the sample solution or on direct read out.

Calculation:

Weight of sample taken = 10g
Volume of DTPA extract added = 20ml
Dilution = 2 times
Concentration of the element in the sample =Sppm solution as read for km the standard curve
Concentration of element in the blank solution = Tppm
Concentration of element in the soil = (S-T) x 2ppm.

APPENDIX- 14
ESTIMATION OF MANGANESE
(APHA, 1995)

Apparatus

a. Spectrophotometer, for use at 525 nm, providing a light path of 1 cm or greater.

Reagents

a. Special reagent: Dissolve 75 g $HgSO_4$ in 400 mL conc HNO_3 and 200 mL distilled water. Add 200 mL 85% phosphoric acid and 35 mg silver nitrate. Dilute the cooled solution to 1L.

b. Ammonium persulphate, $(NH_4)2S_2O_8$, solid.

c. Standard manganese solution, 1.00 mL = 50.0 μg Mn: Dissolve 3.2 g $KMnO_4$ in distilled water and make upto 1 L. Heat for several hours near the boiling point, cool and filter. Standardise against sodium oxalate, as follows:

− Weigh accurately to 0.1 mg, several 100 to 200 mg samples of $Na_2C_2O_4$ and transfer to 400 mL beakers. To each beaker, add 100 mL distilled water and stir to dissolve.

− Add 10 mL of 1 + 1 H_2SO_4 and rapidly heat to 90 to 95 °C.

− Titrate rapidly with $KMnO_4$ to slight pink end point. Do not let temperature fall below 85ºC. If necessary, warm during titration. Run a blank on distilled water and H_2SO_4.

Calculate normality:

$$\text{Normality of } KMnO_4 = \frac{g\ Na_2\ C_2\ O_4}{(A-B)*0.06701}$$

where:
A = mL titrant for sample
B = mL titrant for blank

− Average the results of several titrations and calculate volume of this solution necessary to prepare 1 L of standard manganese solution as follows:

$$\text{mL } KMnO_4 = \frac{4.55}{\text{normality of } KMnO_4}$$

− To this volume add 2 to 3 mL conc. H_2SO_4 and $NaHSO_3$ solution drop wise, until the permanganate colour disappears. Boil to remove excess SO_2, cool and dilute to 1000 mL. Dilute this solution further with distilled water to measure small amounts of Mn.

d. Sodium oxalate: $Na_2C_2O_4$, primary standard, solid.

e. Sodium bisulphite: Dissolve 10 g $NaHSO_3$ in 100mL distilled water.

f. Hydrogen peroxide, H_2O_2, 30%.

Procedure

a. Take a suitable volume of sample, containing 0.05 to 2.0 mg Mn, in a 250 mL conical flask. Add 5 mL special reagent and one drop H_2O_2. Concentrate to 90 mL by boiling or dilute to 90 mL

b. Add 1g $(NH_4)2S_2O_8$ and boil for 1 min then cool under the tap. Dilute to 100 mL with distilled water.

c. Prepare standards in the range of the sample concentration by treating various amounts of standard Mn solution in the same manner as in *a* and *b* above.

d. Make photometric measurements of standards and sample at 525 nm against a distilled water blank. Use light path of 1 cm for Mn range of 100 – 1500 µg/100mL final reaction volume. Plot standard calibration curve and read Mn concentration in the final 100 mL reaction volume from the standard curve.

Calculation

$$\text{mg Mn/L} = \frac{\mu\text{g Mn/100mL final volume}}{\text{mL sample}}$$

APPENDIX- 15

ESTIMATION OF FREE AMMONIA

(Phenate Spectrophotometric Method)

(APHA, 1995)

Apparatus

a. Spectrophotometer for use at 640nm with a cell of 1cm or longer light path.

Reagents

a. Phenol solution: Mix 11.1 mL liquified phenol (>89%) with 95% V/V ethylalcohol to a final volume of 100 mL. Toxic, avoid personal exposure, discard after a week.

b. Sodium nitroprusside, 0.5%: dissolve 0.5g sodium nitroprusside in 100 mL de-ionised water, store in amber bottle, discard after a month.

c. Alkaline citrate: Dissolve 200 g trisodium citrate and 10g sodium hydroxide in de-ionised water, dilute to 1L.

d. Sodium hypochlorite solution, 5%: Commercial, replace every 2 months.

e. Oxidizing solution: Take 100 mL alkaline citrate solution and mix with 25 mL sodium hypochlorite, prepare daily.

f. Stock ammonium solution: Weigh 3.819g anhydrous, NH_4Cl, earlier dried at 100°C and cooled in desiccator, in ammonia free water and dilute to 1L; 1 mL = 1mgN = 1.22 mg NH_3.

g. Standard ammonium solution: Prepare dilutions from the stock ammonium solution, in a range appropriate for the concentration of the samples; prepare a calibration curve.

Procedure

a. Take 25 mL sample in a 50 mL conical flask, and add with mixing, 1 mL phenol solution, 1 mL sodium nitroprusside solution, and 2.5 mL oxidising solution. Avoid light exposure by suitably covering the flasks at room temperature.

b. Prepare a blank and 2 other ammonia standards in the range, treating in the same way as sample, measure absorbance after 1h at 640nm.

Calculation

Prepare calibration curve by plotting absorbance readings against ammonia concentration of standards, compute sample concentration from the standard curve.

APPENDIX- 16

ESTIMATION OF NITRITE

Colorimetric method (APHA, 2005)

Principle

Nitrite is determined through formation of a reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized sulfanilamide with N- 1- naphthyl ethylenediamine dihydrochloride.

Reagents

1. Color reagent: To 800ml water, added 100ml 85% phosphoric acid and 10g sulfanilamide. After dissolving sulfanilamide completely, add 1g N- 1 - naphthyl ethylenediamine dihydrichloride. Mixed to dissolve and dilute to one litre with water.
2. Stock nitrite solution: Dissolved 1.232g sodium nitrite in 1000ml of distilled water.
3. Standard nitrite solution: Diluted 10.0ml nitrite solution to 1000ml with water, 1.00ml=0.500µg N.

Procedure

Sample pH which was not between 5 and 9 was adjusted to that range. To 50.0ml of sample added 2.0ml of color reagent and mixed. After adding color reagent to standard and sample, the absorbance was measured at 543nm.

Calculation

Prepare a standard curve by plotting absorbance of standard against NO_2-N concentration.

APPENDIX- 17

ESTIMATION OF NITRATE

Nitrate Electrode Method

(APHA, 2005)

Reagents

1. Nitrate free water
2. Stock nitrate solution: Dissolved 0.7218g of potassium nitrate in 1000ml of distilled water.
3. Standard nitrate solution: Diluted 1.0, 10 and 50ml stock nitrate solution to 100ml of water to obtain standard solutions of 1.0, 10, and 50mg nitrate/L.
4. Buffer solution: Dissolved 17.32g aluminium sulfate, 3.43g silver sulfate. Adjust the pH to 3.0 and diluted to 1000ml with distilled water.
5. Reference electrode filling solution: Dissolved 0.53g (NH_4) $2SO_4$ in water and diluted to 100ml.

Procedure

Transferred 10ml sample to a 50ml beaker, added 10ml buffer solution and stirred with a magnetic stirrer. Measured standards and samples at about the same temperature. Read concentration from calibration curve.

APPENDIX- 18

ESTIMATION OF CHLORIDE

Silver nitrate titrimetric method

(Vogel, 1978)

Principle

Silver nitrate reacts with chloride ions to form silver chloride. The completion of reaction is indicated by the red colour produced by the reaction of silver nitrate with potassium chromate solution, which is as added an indicator.

Reagents

Chloride free double distilled water was used for all the reagents

1. Standard silver nitrate titrant, 0.0282N: 4.791g of silver nitrate was dissolved in one litre of distilled water. Standardized it against 0.02N sodium chloride solution. 1.0ml of exactly 0.02N $AgNO_3$ = 1.0mg of chloride.
2. Standard sodium chloride titrant, 0.0282N: 1.648g of sodium chloride was dissolved in one litre of distilled water 1.0ml=1.0mg of chloride.
3. Potassium chromate indicator solution: Dissolved 25g in 100ml of distilled water. Added silver nitrate solution drop wise until a slight red precipitate was formed. Allowed to stand for twelve hours. Filtered and made upto 500ml with distilled water.
4. Aluminium hydroxide suspension: Dissolved 100g of Aluminium ammonium sulphate in 1000ml distilled water. Warmed to 60ºC and added 55ml concentrated ammonia solution.

Allowed the precipitate to settle for about an hour. Washed by decantation with distilled water to make the precipitate from chloride. Checked it by treating portion of the decanting every time with silver nitrate solution. After the precipitate was free from chloride, diluted it to 1000ml with distilled water.

Procedure

Added 3.0ml of Aluminum hydroxide to a measured volume of the sample in a beaker. Stirred well and allowed to settle. Filtered, washed the precipitate with chloride free distilled water. Pipetted out 100ml of the sample into a porcelain dish. Adjusted the pH to be in the range of 7-9.5. Added 1ml of potassium chromate indicator solution. Titrated it against Standard silver nitrate solution with constant stirring until a slight precipitate reddish colouration persisted. Conducted a blank by placing 100ml chloride –free distilled water instead of sample.

Calculation

If the silver nitrate solution is exactly 0.0282N,

Chloride mg/l = (ml $AgNO_3$ for sample- ml $AgNO_3$ for blank x1000 ml sample taken for estimation.

If the silver nitrate solution is not exactly 0.0282N = (ml for $AgNO_3$ sample- ml $AgNO_3$ for blank)

Chloride mg/l = Normality of $AgNO_3$ x 35.45 x 1000ml sample taken for estimation.

APPENDIX- 19

ESTIMATION OF FLUORIDE

Ion selective electrode method

(APHA, 2005)

Principle

The fluoride electrode is an ion-selective sensor. The fluoride electrode is the laser type doped lanthanum fluoride crystal across which a potential is established by fluoride solutions of different concentrations.

Reagents

1. Stock fluoride solution: Dissolved 221.0mg anhydrous sodium fluoride in 1000ml distilled water. 100ml=100 µg fluoride.
2. Standard fluoride solution: Diluted 100ml of stock solution to 1000ml with distilled water.100ml =10.0µg fluoride.
3. Fluoride buffer: Placed approximately 500ml distilled water in a one liter beaker and added 57ml glacial acetic acid, 58g Nail and 4.0g 1, 2 EDTA. Placed the beaker in cool water bath and added slowly 6N NaOH, until the pH is between 5.3 and 5.5 make upto one litre with distilled water.

Procedure

Prepared a series of standards by diluting standard fluoride solution 5.0, 10.0 and 20.0ml with 100ml distilled water, these standards are equivalent to 0.5, 1.0 and 2.0 mg fluoride/l. Added equal volume of fluoride buffer to samples were, standardized and immerse the electrode are measured.

Calculation

mg fluoride/l= μg fluoride/ml sample.

APPENDIX- 20

ESTIMATION OF SULPHATE

Turbidometric method

(APHA, 2005)

Principle

Sulfate ion is precipitated in an acetic acid medium with barium chloride so as to form barium sulfate crystals. The sulfate concentration is determined by comparison of the reading with the standard curve.

Reagents

1. Buffer solution: Dissolved 30g magnesium chloride, sodium acetate, 1.0g potassium nitrate and 20ml acetic acid in 500ml distilled water and made upto 1000ml.
2. Barium chloride, standard sulfate solution: Dissolved 0.1479g anhydrous sodium sulfate in distilled water and diluted to 1000ml.

Procedure

Measured 100ml sample into an Erlenmeyer flask. Added 20ml buffer solution and mixed by stirring apparatus. Added a spoonful of barium chloride crystals. After stirring the readings are taken in a spectrophotometer at 420nm and measured turbidity at 5 ± 0.5 min.

APPENDIX- 21

ESTIMATION OF PHOSPHATE

(APHA, 1995)

Apparatus

a. Spectrophotometer with infrared phototube for use at 880nm or filter photometer, equipped with a red filter.

b. Acid washed glassware, use dilute HCl and rinse with distilled water.

Reagents

a. Sulphuric acid, H_2SO_4, 5N: Dilute 70 mL conc. H_2SO_4 to 500 mL with distilled water.

b. Potassium antimony tartarate solution: Dissolve 1.3715g $K(Sob)C_4H_4O_6.1/2\ H_2O$ in 400 mL distilled water and dilute to 500 mL, store in glass-stoppered bottle.

c. Ammonium molybdate solution: Dissolve 20g $(NH_4)6\ Mo7O_{24}.4H_2O$ in 500 mL distilled water, store in a glass stoppered bottle.

d. Ascorbic acid, 0.1M: Dissolve 1.76g ascorbic acid in 100 mL distilled water, keep at 4°C, use within a week.

e. Combined reagents: Mix 50 mL 5N, H_2SO_4, 5 mL potassium antimony tartarate, 15 mL ammonium molybdate solution, and 30 mL ascorbic acid solution, in the order given and at room temperature. Stable for 4 hours.

f. Stock phosphate solution, Dissolve 219.5 mg anhydrous KH_2PO_4 in distilled water and dilute to 1 L; 1 mL = 50μg PO_4.

g. Standard phosphate solution: Dilute 50 mL stock solution to 1L with distilled water; 1 Ml = 2.5μg P.

Procedure

a. Treatment of sample: Take 50 mL sample into a 125 mL conical flask, add 1 drop of phenolphthalein indicator. Discharge any red colour by adding $5NH_2SO_4$. Add 8 ml combined reagent and mix.

b. Wait for 10 minutes, but no more than 30 minutes and measure absorbance of each sample at 880nm. Use reagent blank as reference.

c. Correction for turbid or coloured samples. Prepare a sample blank by adding all reagents except ascorbic acid and potassium antimony tartarate to the sample. Subtract blank absorbance from sample absorbance reading.

d. Preparation of calibration curve: Prepare calibration from a series of standards between 0.15-1.30 mL range (for a 1 cm light path). Use distilled water blank with the combined reagent. Plot a graph with absorbance versus phosphate concentration to give a straight line. Test at least one phosphate standard with each set of samples.

Calculation

$$o\text{-}PO_4 \text{ as mg P/L} = \frac{\text{mg P from the calibration curve} \times 1000}{\text{mL Sample}}$$

APPENDIX- 22
CHROMIUM CELL TEST
(Kit method)

Digestion with HNO_3 and HCl

5 ml of conc. HNO_3 was added to the silk dyeing effluent and evaporated, then it was transferred to a conical flask. Then 5 ml of conc. HNO_3 and 10 ml HCl (70%) were added. Then it was heated gently till white dense fumes of HCl appeared. The digested samples were cooled at room temperature, filtered through Whatmann No. 41 or sintered glass crucible and finally the volume was made upto 100 ml with distilled water. The digested samples were subjected to Photometer SQ 118 to analyse the heavy metals.

Reagents

Package kit contains:

1. 1 bottle of Cr-1
2. 1 bottle of reagent Cr-2A
3. Blank cell

Sample preparation

The silk dyeing effluent (untreated silk dyeing effluent, biotreated effluent I with *Azospirillum sp.,* and biotreated effluent II with *Pseudomonas fluorescens*) was subjected to acid digestion with HNO_3 and HCl. The pH must be in the range of 1-8. Adjust, if necessary, with the sodium hydroxide solution or sulphuric acid. Filter the turbid samples.

Procedure

5 ml of the pretreated samples was added using the pipette and mixed well. One level of green microspoon of the reagent Cr-1 was added and shaken vigorously until the reagent was completely dissolved. Add 6 drops of the reagent Cr-2A was pipetted into the reaction cell and mixed well. It was left to stand for 1 minute (reaction time). The blank solution given in the kit were measured initially followed by the sample in the photometer.

APPENDIX- 23
LEAD CELL TEST
(Kit method)

Reagents

Package kit contains:

1. 1 bottle of reagent Pb-1K
2. 1 bottle of Pb-2K
3. Blank cell

Sample preparation

The silk dyeing effluent (untreated silk dyeing effluent, biotreated effluent I with *Azospirillum sp.,* and biotreated effluent II with *Pseudomonas fluorescens*) was subjected to acid digestion with HNO_3 and HCl mentioned above. The pH must be in the range of 3-6. Adjust, if necessary, with the sodium hydroxide solution or sulphuric acid. Filter the turbid samples.

Procedure

5 ml of the pretreated samples was added using the pipette and mixed well. Add five drops of Pb-1K into the reaction cell and mixed well. The blank solution given in the kit were measured initially followed by the sample in the photometer. The results were recorded as Result A.

Open reaction cell of value A. One 1 grey microspoonful of Pb-2K was added and shaken vigorously until the reagent was completely dissolved. The samples were recorded as Result B.

Calculation of Lead content (mg/l) = Result A-Result B.

APPENDIX- 24
NICKEL CELL TEST
(Kit method)

Reagents

Package kit contains:

1. 1 bottle of Ni-1K
2. 1 bottle of reagent Ni-2K
3. Blank cell

Sample preparation

The silk dyeing effluent (untreated silk dyeing effluent, biotreated effluent I with *Azospirillum sp.,* and biotreated effluent II with *Pseudomonas fluorescens*) was subjected to acid digestion with HNO_3 and HCl mentioned above. The pH must be in the range of 4-8. Adjust, if necessary, with the sodium hydroxide solution or sulphuric acid. Filter the turbid samples.

Procedure

4 ml of the predigested samples was added using the pipette and mixed well. Two drops of reagent Ni-1K was added and mixed well. Then add two drops of the reagent Ni-2K was pipetted into the reaction cell and mixed well. It was left to stand for 2 minutes (reaction time). The blank solution given in the kit were measured initially followed by the sample in the photometer.

APPENDIX- 25
ZINC CELL TEST
(Kit method)

Principle

In alkaline solution, zinc ions react with pyridylazoresorcinol (PAR) to form a red complex that is determined photometrically at 494nm.

Reagents

Package kit contains:

1. 1 bottle of Zn-1K
2. 1 bottle of reagent Zn-2K
3. 1 bottle of reagent Zn-3K
4. Blank cell

Sample preparation

The silk dyeing effluent (untreated silk dyeing effluent, biotreated effluent I with *Azospirillum sp.,* and biotreated effluent II with *Pseudomonas fluorescens*) was subjected to acid digestion with HNO_3 and HCl

mentioned above. The pH must be in the range of 1-7.Adjust, if necessary, with the sodium hydroxide solution or sulphuric acid. Filter the turbid samples.

Procedure

10 ml of the pretreated samples was added using the pipette and mixed well. One level of green microspoon of the Zn-1K was added and shaken vigorously until the reagent was completely dissolved. 0.5 ml of the reagent Zn-2K was pipetted into the reaction cell and mixed well. Then 5 drops of reagent Zn-3K was added and mixed well. It was left to stand for 15 minutes (reaction time). The blank solution given in the kit were measured initially followed by the sample in the photometer.

APPENDIX- 26
CADMIUM CELL TEST
(Kit method)

Principle

In alkaline solution, cadmium ions react with a cadion derivative (cadion=trivial name for 1-(4-nitrophenyl)-3-(4-phenylazophenyl) to form a red complex that is determined photometrically at 526nm.

Reagents

Package kit contains:

1. 1 bottle of reagent Cd-1
2. 1 bottle of reagent Cd-2
3. 1 bottle of Cd-3
4. Blank cell

Sample preparation

The sample (untreated silk dyeing effluent, biotreated effluent I with *Azospirillum sp.,* and biotreated effluent II with *Pseudomonas fluorescens)* was subjected to acid digestion with HNO_3 and HCl mentioned above. The pH must be in the range of 3-11.Adjust, if necessary, with the sodium hydroxide solution or sulphuric acid. Filter the turbid samples.

Procedure

1 ml of the reagent Cd-1 was pipetted into the test tube. 5 ml of the pretreated samples was added using the pipette and mixed well. Then 0.2 ml of reagent Cd-2 was added and mixed well. One level of green microspoon of reagent Cd-3 was added and shaken vigorously until the reagent was completely dissolved. It was left to stand for 2 minutes (reaction time). The blank solution given in the kit were measured initially followed by the sample in the photometer.

APPENDIX- 27
ESTIMATION OF INDOLE ACETIC ACID (IAA)
(Gordon and Paleg, 1957)

One ml broth of each isolates of *Azospirillum sp.,* and *Pseudomonas fluorescens* were added separately in the silk dyeing effluent and incubated at 37°C for seven days. After seven days of incubation, Fifteen ml of the acidified supernatant was taken in 100 ml conical flask and to it equal volume of diethyl ether was added and incubated in dark for 4 h. IAA extraction was done at 4°C in a separating funnel using diethyl ether. The organic phase was discarded and the solvent phase was pooled and evaporated to dryness. To the dried material, 2 ml of methanol was added and pooled. Take 0.5 ml of the methanol extract, 1.5 ml of distilled water and four ml of Salper's reagent (1.0 ml of 0.5 M $FeCl_3$ in 50 ml of 35 per cent perchloric acid) were added and incubated in dark for one hour. The intensity of pink color developed was read at 535 nm in a spectrophotometer. From a standard curve prepared with known concentrations of IAA, the quantity in the culture filtrate was determined and expressed as μg 25 ml^{-1} of culture medium. The standard curves of IAA were prepared by using graded concentrations of IAA.

APPENDIX 28
ESTIMATION OF GIBERRELLIC ACID
(Borrow *et al.,* 1955)

Principle

The *in vitro* production of phytohormones gibberellic acid (GA_3) by plant growth promoting rhizobacterial isolates was estimated.

Materials required

Zinc acetate solution

A quantity of 21.9 g of zinc acetate was dissolved in 80 ml of distilled water and one ml of glacial acetic acid was added and the volume was made up to 100 ml with distilled water.

Potassium ferrocyanide solution

A quantity of 10.6 g of potassium ferrocyanide was dissolved in 100 ml of distilled water.

Procedure

One ml broth of each isolates of *Azospirillum sp.* and *Pseudomonas fluorescens* were added separately in the silk dyeing effluent and incubated at 37°C for seven days. After seven days of incubation, the culture was centrifuged at 8000 rpm for 10 min to remove the bacterial cells. Fifteen ml of the culture was pipetted out separately into the test tubes and two ml of zinc acetate solution was added. After two minutes, two ml of potassium ferrocyanide solution was added and centrifuged at 8000 rpm for 10 minutes. Five ml of supernatant was added to five ml of 30 per cent hydrochloric acid and the mixture was incubated at 27°C for 75 minutes. The blank was prepared with five percent hydrochloric acid. Absorbance was measured at 254 nm in a UV VIS spectrophotometer. From the standard graph prepared by using gibberellic acid solutions of known quantities, the amount of GA_3 produced by the culture was calculated and expressed as µg 25 ml^{-1} broth.

APPENDIX 29
PHYTOCHEMICAL ANALYSIS

Preparation of aqueous extract

5 g of the plant material was covered with 5 times its weight of water and its suspension was heated in a water bath for 30 minutes to 1 hour, cooled and filtered. The filtrate was used for the phytochemical analysis.

Preparation of acid extract

The plant material (5 g) was covered with 5 times its weight of 5% HCl and suspension was heated in a water bath for 1 hour at 60°C, stirred at intervals. Cooled and filtered and the occurrence of phytochemicals were tested on the filtrate.

Preparation of alkaline extract

A mixture of ethyl ether – chloroform – ethanol – ammonium hydroxide (25:8:2.5:1 in volume) the so called "Liquor of Prolius" was used for the extraction. A portion of the plant material (5 g) was covered with 5 times the volume of that mixture and was allowed to stand for 24 hours. It was then filtered and the filtrate was evaporated to dryness and the residue was treated with 1% HCl favoring the dissolution of the bases with the magnetic stirring. It was further filtered and phytochemicals were tested using the filtrate.

Preparation of reagents

1. Molisch's reagent – 20% α-naphthol methyl alcohol.
2. Fehling's reagent – Copper sulphate in alkaline conditions.
3. Iodine solution – Few crystals of iodine were added to 2% KI till the solution becomes deep yellow.
4. Millon's reagent – 10% Mercuric sulphate in 10% H_2SO_4.

5. Biuret's reagent – 10% NaOH and 0.1% copper sulphate were mixed.
6. Ninhydrin reagent – 0.1 g of ninhydrin in 100 ml of water.
7. Bradford's reagent – Coomassive Brilliant Blue G-250.
8. Raymond's reagent – 1% solution of m-dinitrobenzene in ethanol or methanol.
9. Xanthydrol reagent – A solution of 0.125% xanthydrol in glacial acetic acid containing 1% HCl.
10. Kadde's reagent – Mixed equal volume of 2% solution of 3, 5- dinitrobenzoic acid in methanol and 7.5% KOH solution.
11. Mayer's reagent – A solution of mercuric chloride (13.6 g) in water (600 ml) and potassium iodide (50 g) in 100ml of water were prepared. Both solutions were mixed under stirring and the volume was made up to 1000ml with water.
12. Dragendroff's reagent
    a. Solution A – Glacial acetic acid (10 ml) was added to a suspension of bismuth subnitrate (0.8 g) in water (40 ml).
    b. Solution B – Solution of Potassium Iodide (20 g) in water (50 ml) was prepared. Both solutions were mixed together. To the mixture 100 ml of glacial acetic acid was added and the volume was made up to 1000ml with water.
13. Hager's reagent – 20 g of picric acid was dissolved in warm water (1000 ml). The solution was allowed to cool down.
14. Wager's reagent – Dissolved 2 g of Iodine and 6 g of Potassium Iodide in 100 ml of water.
15. Phloroglucinol reagent – 1 g of phloroglucinol, 50 ml of absolute ethanol, 50 ml of concentrated HCl and 50 ml of distilled water were mixed.

PHYTOCHEMICAL ANALYSIS

IDENTIFICATION OF CARBOHYDRATES

Molisch' test

To 2 ml of the extract, few drops of 20% α-naphthol in ethyl alcohol were added. Then about 1 ml of concentrated sulphuric acid was added along the side of the tube. Reddish violet colour appeared at the junction of the two layers indicated the presence of carbohydrates.

Fehling's test

1ml of the Fehling's reagent (copper sulphate in alkaline condition) was added to the filtrate of the extract in distilled water and heated in a steam bath. Formation of brick red precipitate indicated the presence of carbohydrates.

The **Hofmann isocyanide synthesis** is a chemical test for primary amines based on their reaction with potassium hydroxide and chloroform as dichlorocarbene precursors to foul smelling isocyanides.

Test for starch

To 1 ml of the extract, few drops of iodine solution were added. Blue color product was obtained and indicated the presence of starch.

Test for cellulose

To 1 ml of the extract, 2-3 drops of iodine solution was added followed by 2 drops of sulphuric acid. Appearance of dark/deep brown/cherry red color indicated the presence of cellulose.

IDENTIFICATION OF AMINO ACIDS AND PROTEINS

Millon's test

To 1 ml of the extract, 1 ml of 10% mercuric sulphate in 10% sulphuric acid was added and boiled. Yellow coloration was obtained. Cooled and a drop of 1% sodium nitrite was added and warmed. Appearance of red color indicated the presence of proteins and free amino acids.

Biuret test

To 2 ml of the alkaline extract, 2 ml of 10% NaOH was added followed by 2 drops of 0.1% copper sulphate. Appearance of violet or pink color indicated the presence of proteins and free amino acids.

Ninhydrin test

To 4 ml of the extract (neutral pH), 1 ml of 0.1% freshly prepared Ninhydrin reagent was added, boiled and cooled. Appearance of violet or pink color indicated the presence of proteins and free amino acids.

Bradford's test

To 1 ml of the extract, few drops of Bradford's reagent (Coomassive Brilliant Blue G 250) was added. Formation of blue color product indicated the presence of proteins and free amino acids.

IDENTIFICATION OF PHENOLS

Ferric chloride test

2 ml of the extract was added to 2 ml of ferric chloride solution. Formation of deep bluish green solution indicated the presence of phenols.

Phosphomolybdic test

To the ethanolic extract, a few ml of phosphomolybdic acid reagent and ammonia vapours were added. Appearance of blue color indicated the presence of phenols.

IDENTIFICATION OF CATECHOLS

To 2 ml of the test solution, 1 ml of Ehrlich's reagent and few drops of concentrated HCl were added. Appearance of greenish blue solution indicated the presence of catechols.

IDENTIFICATION OF STEROLS AND STEROIDS

Libermann-Buchard test

To the ethanolic extract added 2 ml of chloroform followed by 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. Rosy red color developed which quickly changed through blue to green, indicated the presence of cholesterol.

Salkowski test

The ethanolic extract was dissolved in chloroform and shaken with an equal volume of concentrated sulphuric acid. Red color in the chloroform layer and green fluorescence in the acid layer indicated the presence of cholesterol.

IDENTIFICATION OF GLYCOSIDES

Kellar-Killani test

The ethanolic extract was dissolved in glacial acetic acid containing a trace of ferric chloride and the same amount of ferric chloride dissolved in concentrated sulphuric acid was added along the sides of the test tube to settle at the bottom. Reddish brown color changing to bluish green color appeared at the junction of the two reagents within 2-5 minutes spreading slowly into the acetic acid layer confirmed the presence of cardiac glycosides.

Legal's test

Few ml of ethanolic extract was dissolved in a few drops of pyridine. To this added a drop of 2% w/v sodium nitroprusside solution and a drop of 20% NaOH solution. Appearance of pink or deep red color indicated the presence of glycosides.

Raymond's test

Dissolved a small quantity of ethanolic extract in 1 ml of 50% ethanol. Added to it 0.1 ml of Raymond's reagent and 2-3 drops of 20% NaOH. Appearance of violet color slowly changing to blue indicated the presence of glycosides.

Xanthydrol test

Added to the ethanolic extract 0.5 ml of Xanthydrol solution (for deoxysugars only). Development of red color indicated the presence of glycosides.

Antimony Trichloride test

To the ethanolic extract added a solution of Antimony Trichloride and trichloroacetic acid and then heated the mixture. Appearance of blue or violet color indicated the presence of cardiac glycosides.

Kadde's test

Ethanolic extract was treated with a small amount of Kadde's reagent and development of blue or violet color that fades out in 1-2 hours showed the presence of cardiac glycosides.

IDENTIFICATION OF SAPONINS/SAPONIN-GLYCOSIDES

Froth test

1 g of the sample was weighed into a conical flask in which 10 ml of sterile distilled water was added and boiled for 5 minutes. The mixture was filtered and 2.5ml of the filtrate was added to 10 ml of the sterile distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. Honeycomb froth formation indicated the presence of saponins. The froth was mixed with 3 drops of olive oil and shaken vigorously. Formation of emulsion confirmed the presence of saponins.

Sodium Bicarbonate test

To few ml of ethanolic extract, few drops of sodium bicarbonate was added and shaken well. Formation of honeycomb indicated the presence of saponins.

IDENTIFICATION OF QUINONES/ANTHRAQUINONES

Chloroform-ammonia test

0.5 gm of the plant sample was boiled with 10ml of 5% sulphuric acid and filtered. To the hot filtrate added 5 ml of chloroform and heated in a boiling water bath. To 2 ml of the chloroform, extract 1 ml of diluted 10% ammonia was added and the mixture was shaken. Appearance of pink red color in the ammoniacal layer indicated the presence of anthracene derivatives.

Borntrager's test

About 50 ml of the extract was heated with 10% ferric chloride solution and 1 ml of concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicated the presence of anthraquinone.

Hydrogen peroxide test

The ethanolic extract was filtered. To 1 ml of the filtrate added 10 ml of dichloromethane. The aqueous and organic layers were separated. To 5 ml of the aqueous layer added 1 ml of 20% H_2O_2 and 1 ml of 50% H_2SO_4 and heated in a boiling water bath. Then added 5 ml of toluene and 1 ml of 5% NaOH. Separated the aqueous and toluene phase. Red color toluene phase indicated the presence of quinones.

IDENTIFICATION OF CYANOGENIC GLYCOSIDES

Fresh plant material was cut into small pieces and placed in a test tube with 1.5 ml of distilled water, 6 drops of chloroform followed by briefly crushing the material with a glass rod. The tube was stoppered with a cork containing a strip of picrate-impregnated paper hanging down from the stopper and incubated at ambient temperature for 2 hours. A color change of the paper from yellow to brown red indicated the release of HCN. When there was no release of HCN within 2 hours, the tube was left at ambient temperature for 24 to 48 hours it could be re-examined. Appearance of brown red coloration within 2 hours indicated the presence of cyanogenic glycosides. A brown red color within 48 hours indicated that the cyanogenic glycoside spontaneously released HCN without the action of the enzyme. No color change after 48 hours indicated that the test was negative for cyanogenic glycoside.

IDENTIFICATION OF ALKALOIDS

To 5 g of the sample added 50 ml of 5% HCl and heated in a boiling water bath for 1 hour and filtered. To the filtrate added 5 ml of dilute ammonia and 5 ml of chloroform. The aqueous layer was taken for the following tests.

Mayer's test

Added a few drops of Mayer's reagent to the aqueous layer. Formation of creamy layer indicated the presence of alkaloids.

Dragondroff's test

To 1 ml of the aqueous layer added 1 ml of Dragondroff's reagent. Formation of orange precipitate indicated the presence of alkaloids.

Hager's test

1 ml of the aqueous layer was treated with 1 ml of Hager's reagent. Formation of orange precipitate indicated the presence of alkaloids.

Wagner's test

1 ml of the aqueous layer was treated with few ml of Wagner's reagent. Formation of reddish brown precipitate indicated the presence of alkaloids.

IDENTIFICATION OF FLAVONOIDS

Decolorization test

Water extract of the sample was reduced to dryness in the boiling water bath. The residue was treated with dilute NaOH followed by addition of dilute HCl. Yellow solution with NaOH which turns colorless with dilute HCl indicated the presence of flavonoids.

Shinoda test

To 5 g of the sample added 50 ml of ethanol and heated in a boiling water bath. To ethanolic extract, 8-9 drops of concentrated HCl and some magnesium fillings were added. After 10-15 minutes at room temperature red color formation indicated the presence of flavonoids.

Ammonia test

Filter paper strips were dipped in the ethanolic and aqueous extract and ammoniated. The filter paper changed its color to yellow indicated the presence of flavonoids. Added 10 ml of concentrated H_2SO_4 to the above yellow colored filter paper. Disappearance of the yellow color confirmed the presence of flavonoids.

Pew test

To 1 ml of extract, a piece of metallic magnesium/zinc was added followed by addition of two drops of concentrated HCl. Brown color indicated the presence of flavonoids.

Lead acetate test

To the ethanolic extract added equal volume of 0.5% acetic acid and filtered. To the filtrate added 1 ml of 1% lead acetate. Flocculant white precipitate indicated the presence of flavonoids.

Aluminium chloride test

To the aqueous extract added 2 drops of 1 % aluminium chloride. Yellow coloration indicated the presence of flavonoids.

IDENTIFICATION OF LEUCOANTHOCYANIDINES

Concentrated HCl was added to the extract and then heated until boiling. Appearance of reddish color indicated the presence of leucoanthocyanidines.

IDENTIFICATION OF TANNINS

Preparation of the extract

The plant material was suspended in ethanol and suspension was kept in rest overnight. Then it was heated under reflux for 4 hours. Then filtered, warmed and the residue was washed with methanol. The filtrate was allowed to cool down, the aliquot was taken in order to assay tannins.

Braemer's test

To the methanolic extract added 10 ml of water, boiled and filtered. To the filtrate few drops of 10% of ferric chloride was added. A dark green, blue or brown color indicated the presence of tannin.

Test for hydrolysable tannins

4 ml of the extract was shaken in a test tube, after which 4 ml of 10% ammonia solution was added. Formation of an emulsion on shaking indicated the presence of hydrolysable tannins.

IDENTIFICATION OF ANTHOCYANIN

The aqueous extract was boiled for 5 minutes and filtered. To 2 ml of the filtrate added 1 ml of NaOH. Formation of yellow colour indicated the presence of primary amine. To another 2 ml of the filtrate added 1 ml of HCl. Different coloration indicated the presence of secondary amine. Change in colour for both primary and secondary amine confirmed the presence of anthocyanin.

IDENTIFICATION OF VOLATILE OILS

2 ml of the extract solution was shaken with 0.1 ml of dilute NaOH and small quantity of dilute HCl. White precipitate indicated the presence of volatile oils.

IDENTIFICATION OF LIGNIN

Spot test

1 drop of phloroglucinol reagent was placed on a filter paper dipped in aqueous, ethanolic and acidic extract. Red or purple spots on the filter paper indicated the presence of lignin.

IDENTIFICATION OF TERPENOIDS

To 0.5 g of the sample added 2 ml of chloroform. To the chloroform extract added 5 ml of concentrated H_2SO_4 along the sides of the test tube. Reddish brown coloration in the interphase indicated the presence of terpenoids.

APPENDIX 30
ESTIMATION OF CRUDE FIBRE
(Maynard, 1970)

Materials:

- Sulphuric acid solution (0.255 ± 0.005N): 1.25g concentrated sulphuric acid diluted to 100mL (concentration must be checked by titration).
- Sodium hydroxide solution (0.313 ± 0.005N): 1.25g sodium hydroxide in 100ml distilled water concentration must be checked by titration.

Procedure

- Extract 2g of ground material with ether or petroleum ether to remove fat (initial boiling temperature 35-38°C and final temperature 52°C). If fat content is below 1%, extraction may be omitted.
- After extraction with ether boil 2g of dried material with 200ml of sulphuric acid for 30 min with bumping chips.
- Filter through muslin and wash with boiling water until washing are no longer acidic.
- Boil with 200mL of sodium hydroxide solution for 30 minutes.
- Filter through muslin cloth again and wash with 25ml of boiling 1.25% H_2SO_4, three 50 ml portions of water and 25ml alcohol.
- Remove the residue and transfer to ashing dish (reweighed dish W_1).
- Dry the residue for 2hrs at 130 ± 2°C. Cool the dish in desiccators and weigh (W_2).
- Ignite for 30 min at 600 ± 15°C.
- Cool in a dedicator and reweigh (W_3).

Calculation

% crude fibre in ground sample

$$= \frac{\text{Loss in weight on ignition } (W_2 - W_1)-(W_3 - W_1) \text{ (g)}}{\text{Weight of the sample (g)}} \times 100$$

APPENDIX 31

ESTIMATION OF TOTAL CARBOHYDRATE

(Hedge and Hofreiter, 1962)

Reagents

1. 2.5N HCl
2. Anthrone reagent: Dissolved 200 mg anthrone in 100 ml of ice cold 95% H_2SO_4 (Prepared fresh before use)
3. Stock standard: Dissolved 100mg of glucose in 100 ml distilled water
4. Working standard: 10 ml of stock solution is made up to 100 ml of distilled water

Procedure

Weighed 100 mg of the sample in a boiling tube. Hydrolyzed by keeping it in a water bath for 3 hours with 5 ml of 2.5N HCl and cooled to room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the aliquots for analysis. Prepared the standards by making 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standards. '0' served as the blank. Made up the volume to 1 min all the test tubes including the sample tubes by adding distilled water. Then added 4.0 ml of anthrone reagent. Heated for eight minutes in boiling water bath. Cooled rapidly and read the green to dark green colour at 630nm.

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

NOTE:

Cool the contents of all the tubes on ice before adding ice-cold anthrone reagent.

APPENDIX 32

ESTIMATION OF PROTEIN

(Lowry *et al*., 1951)

Reagents

1) **Reagent A:** 2% sodium carbonate in 0.1N sodium hydroxide.

2) **Reagent B:** 0.5% copper sulphate ($CuSo_4.5H_2O$) in 1% potassium sodium tartarate.

3) **Reagent C:** Alkaline copper sulphate solution: Mixed 50ml of A and 1ml of B prior to use.

4) **Reagent D:** Folin-Ciocalteau Reagent - Refluxed gently for 10 hours a mixture consisting of 100g sodium tungstate, 25gm sodium molybdate, 700ml water, 50ml of 85% phosphoric acid and 100ml of concentrated hydrochloric acid in a 1.5 litre flask. Added 150gm of lithium sulphate, 50ml water and a few drops of bromine water. Boiled the mixture for 15min without condenser to remove excess bromine. Cooled, diluted to 1 litre and filtered.

5) **Stock standard protein solution:** Weighed accurately 50mg of bovine serum albumin and dissolved in 0.1N NaOH and made up to 50ml in a standard flask.

6) **Working standard:** Diluted 10ml of the stock solution to 50ml with distilled water in a standard flask. 1ml of the solution contains 200 µg of proteins.

Procedure

Extraction of protein from sample

Weighed 500mg of the sample and ground well with a pestle and mortar in 5-10ml of the phosphate buffer. Centrifuged and used the supernatant for the protein estimation.

Estimation of protein

Pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tubes. Pipetted out 0.1ml of the sample extract in another test tube. The volume was made up to 1ml in all the test tubes. A tube with 1ml of water served as a blank. Added 5ml of reagent C to all the test tubes including the blank. Mixed well and allowed to stand for 10 minutes and then 0.5ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes. Blue colour was developed. Readings were taken in spectrophotometer at 660nm. Standard graph was drawn and the amount of protein in the sample was calculated.

APPENDIX – 33
DETERMINATION OF CELLULOSE
(Elena, 2012)

Materials

Acetic/Nitric reagent: Mix 150 mL of 80% acetic acid and 15 mL of concentrated nitric acid.

Anthrone reagent: Dissolve 200 mg anthrone in 100 mL concentrated sulphuric acid. Prepare fresh and chill for 2 h before use.

67% sulphuric acid.

Procedure

Add 3 mL acetic/nitric reagent to a known amount (0.5 g or 1 g) of the sample in a test tube and mix in a vortex mixer. Place the tube in a water-bath at 100°C for 30 min. Cool and then centrifuge the contents for 15–20 min. Discard the supernatant. Wash the residue with distilled water. Add 10 mL of 67% sulphuric acid and allow it to stand for 1 h. Dilute 1 mL of the above solution to 100 mL. To 1 mL of this diluted solution, add 10 mL of anthrone reagent and mix well. Heat the tubes in a boiling water-bath for 10 min. Cool and measure the colour at 630 nm. Set a blank with anthrone reagent and distilled water. Take 100 mg cellulose in a test tube and proceed from Step No. 6 for standard. Instead of just taking 1 mL of the diluted solution (Step 7) take a series of volumes (say 0.4–2 mL corresponding to 40–200 µg of cellulose) and develop the colour.

Calculation

Draw the standard graph and calculate the amount of cellulose in the sample.

APPENDIX 34
ESTIMATION OF IRON
(Wong, 1928)

Principle

Iron is determined colorimetrically with ferric iron which gives a blood red colour with potassium thiocyanate which is measured at 540nm.

Reagents

1. 30% H_2SO_4
2. 7% Potassium per sulphate solution
3. 40% Potassium thiocyanate solution: 40 g KCNS is dissolved in 90 ml distilled water, 4.0 ml acetone is added and the volume is made up to 100 ml.
4. Standard Iron solution: 70.22 mg ferrous ammonium sulphate is dissolved in 10 ml distilled water and after of 0.5 ml of 1:1 HCL the solution is made up to 100 ml in standard flask (0.1 mg Fe/ml).

5. **Working standard**: Diluted 10 ml of stock standard solution to 100 ml. The concentration of working standard is 10µg Fe/ml.
6. **Sample preparation**: About 5.0 g of the sample is weighed accurately into a tared platinum (or porcelain) crucible (which has been previously heated to about 600˚C and cooled). The crucible is placed over a low flame till all the material is completely charred followed by heating in a muffle furnace for about 3.5 hr at about 600˚C. It is then cooled in a dessicator and weighed. To ensure completion of aching, the crucible is again heated in the muffle furnace for 1 hr, cooled and weighed. This is repeated till two consecutive weight are the same and the ash is almost white or grayish white in colour.

Ash content (g/ 100g sample) = Wt. of the ash/ Wt. of the sample taken X 100

Ash solution

The ash is moistened with a small amount of distilled water (0.5 – 1.0 ml) and 5.0 ml of distilled HCL is added to it. The mixture is evaporated to dryness in a boiling water bath. Another 5.0 ml of HCL is added again and the solution is evaporated to dryness as before. 4.0 ml of HCL and a few ml of water are then added and the solution is warmed over a boiling water bath and filtered into a 50 ml volumetric flask using Whatmann No: 40 filter paper. After cooling, the volume is made up to 50 ml and suitable aliquots are used for the estimation of iron.

Procedure

Pipetted out 1.0 – 5.0 ml of working standard solution into the series of test tubes corresponding to µg values 10 – 50. 2.0 ml of the sample solution was taken in separate test tubes. The volume in all the tubes was made up to 6.5 ml with distilled water. Set up a blank with 6.5 ml of distilled water. Added 1.0 ml of 30% H_2SO_4, 1.0 ml of 7% Potassium per sulphate and 1.5 ml of 40 % Potassium thiocyanate solution were added. The red colour developed was measured within 20 min at 540nm in a spectrophotometer. A standard graph was drawn by plotting the concentration of iron on X- axis and the optical density on Y – axis. From this the concentration of iron in the sample solution was calculated.

APPENDIX 35
ESTIMATION OF PHOSPHORUS
(Fiske and Subbarow, 1925)

Principle

Acid molybdate reagents react with inorganic phosphate to form phosphomolybdic acid. The hexavalent molybdenum of phosphomolybdic acid is reduced to give a blue compound, which is estimated colorimetrically.

Reagents

1. Molybdate I solution: 2.5% ammonium molybdate in 5N sulphuric acid.
2. Amino Naphthol Sulphonic Acid (ANSA):Added 0.5g of 1, 2, 4 – amino naphthol sulphonic acid in 195ml of 15% sodium bi sulphite and added 5 ml of 20% sodium sulphite and shaken until it is dissolved.
3. **Stock standard phosphate solution:** 35.1mg of potassium dihydrogen phosphate was dissolved in 20ml of water. 1.0 ml of 10N sulphuric acid was added and volume was made up to 100ml with water.
4. **Working standard**: 10 ml of stock standard phosphate solution was made upto 100ml with distilled water.

Procedure

2g of vegetable samples was taken and were ashed. Dissolved in 2 to 3 drops of concentrated hydrochloric acid and made up to 25ml with distilled water. Into a series of test tubes pipetted out 0.5, 1.0, 1.5, 2.0 and 2.5ml of working standard solution and made up the volume to 4.7ml with water and added 0.5 ml of molybdate I solution and 0.2 ml of ANSA. 0.1 ml of the ash solution was taken and treated similarly. The colour developed was read after 20 minutes in a colorimeter using red filter against a reagent blank.

APPENDIX 36
ESTIMATION OF CALCIUM AND MAGNESIUM
(A.O.A.C, 1990)

One gram of the selected five GLVs grown in fresh water, crude effluent (75%) and biotreated effluent were pulverized, placed in a crucible and ignited in a muffle furnace at 550°C for 6 hours. The resulting ash was dissolved in 10 ml of 10 % HNO_3 and heated slowly for 20 minutes. After heating, it was filtered and the filtrate was used for the determination of Calcium and Magnesium in Atomic absorption spectrophotometer (AAS).

APPENDIX 37
ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY
(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating systems and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can de assayed spectrophotometrically.

Reagents

1. 50mM potassium phosphate buffer, (pH 7.8)
2. 45µM Methionine
3. 5.3µM Riboflavin
4. 84µM Nitro blue tetrozolium (NBT)
5. 20mM potassium cyanide

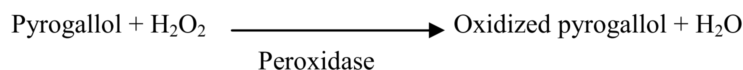
Procedure

The incubation medium contained a final volume of 3ml, 50mM potassium phosphate buffer (pH 7.8), 45µM Methionine, 5.3µM Riboflavin, 84µM Nitro blue tetrozolium (NBT), and 20mM potassium cyanide. The tubes were placed in aluminum foil – lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophtometrically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the amount of enzyme giving 50% inhibition of the reduction of NBT.The values are calculated as units /mg protein.

APPENDIX 38
ESTIMATION OF PEROXIDASE ACTIVITY
(Reddy *et al.*, 1995)

Principle

Peroxidase converts H_2O_2 to water and oxygen in the presence of hydrogen donor (pyrogallol or dianisidine) the oxidation of pyrogallol or dianisidine to colored product called purpurogalli is measured colorimetically.



Pyrogallol + H_2O_2 $\xrightarrow{\text{Peroxidase}}$ Oxidized pyrogallol + H_2O

Reagents

1. Pyrogallol – 0.05M phosphate buffer (pH 6.5)
2. 1 % H_2O_2

Enzyme extract

Measured one gram of the sample with 5ml (w/v) 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuged the homogenate at 300g for 15 minutes. Used the supernatant as the enzyme source. All procedures were carried out at 0-5°C.

Procedure

Pipetted out 3ml of 0.05 M- pyrogallol solution and 0.5 to 0.1ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400nm.Added 0.5ml of 1% H_2O_2 in the test cuvette. Recorded the change in the absorbance every 30 seconds up to 3 minutes.

Calculations

Change in absorbance / min	=	X
Weight of the plant material taken	=	250mg
Volume of the extract taken for the assay	=	0.1ml
Change in absorbance for 1.5ml extract	=	(X/0.1) × 1.50
(i.e) Peroxidase activity in 250mg plant tissue	=	Y
Peroxidase activity / gram of plant tissue	=	Y × (1000/250) Units.

APPENDIX 39
ESTIMATION OF TOTAL PHENOL
(Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin - ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrometer at 650nm.

Reagents

1. 80%ethanol
2. Diluted Folin - Ciocalteau reagent
3. 20% sodium carbonate
4. **Stock solution**: Dissolved 100mg of catechol is made up with 100ml distilled water
5. **Working standard**: 10ml of stock standard was diluted to 100ml. 1.0ml of this contains 100µg of catechol.

Procedure

1g of sample was homogenized using 20ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The supernatant was saved. The residue was reextracted with 10ml of 80% ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (50ml) and 2.0ml was taken for the experiment. A working standard of 0.5 – 2.5ml catechol solution corresponding to 50 - 250µg of catechol were pipetted out into a series of test tubes. The volume was made upto 2.5ml with water. To all the tubes added 0.5ml of diluted Folin – Ciocalteau reagent. After 3 minutes, added 2.0ml of 20% Na_2CO_3 solution to each tube and mixed thoroughly. The tubes were placed in a boiling waterbath for exactly one minute. Cooled and measured at 650nm against a reagent blank. Constructed a standard graph by plotting the concentration of catechol on X-axis and absorbance on Y-axis. From the graph, the amount of total phenol present in the sample was estimated and expressed as mg of total phenols per gram of the sample.

APPENDIX 40
ESTIMATION OF ASCORBIC ACID
(Roe and Kuether, 1953)

Reagents

1. 1% TCA
2. 9 N H_2SO_4
3. 2% 2, 4-dinitrophenyl hydrazine: dissolved 2g of DNPH in 100ml of 9N H_2SO_4
4. 10% theorem
5. 80% sulfuric acid

6. **Stock standard solution**: Dissolved 100mg of ascorbic acid in 100ml 4%TCA
7. **Working standard**: Diluted 10ml of the stock solution to 100ml with 4%TCA

Procedure

About 1g of the sample was homogenized in 4% TCA up to 10ml. Centrifuged at 2000rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. Centrifuged once again and removed the charcoal residue. The volume of the clear supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made up 2.0ml with 4%TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0ml with 4%TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by 2 drops of 10% theorem solution. Incubated at 37°C for 3 hours. The osazones formed were dissolved in 2.5ml of 85% sulfuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and theorem were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophtometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX 41
ESTIMATION OF TOCOPHEROL
(Rosenberg, 1992)

Reagents

1. Absolute alcohol
2. Xylene
3. 2, 2'- dipyridyl

4. **Standard solution**: Dissolved 10mg/ 10ml of œ-tocopherol in absolute alcohol 91mg of α- tocopherol is equivalent to 100mg of tocopherol acetate.

Extraction

The sample was homogenized with water in a blender. Weighed accurately 2.5g of the homogenized sample into a conical flask. Added 50ml of 0.1N H_2SO_4 slowly without shaking. Stoppered and allowed to stand overnight. The next day contents of the flask were shaken vigorously and filtered through whatmann No.1 filter paper, discarding the initial 10-15ml of filtrate. Aliquots of the filtrate were used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5ml of extract, 1.5ml of standard, 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard, added 1.5ml of water. Added 1.5ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0ml of 2, 2'- dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula,

$$\text{Amount of Tocopherol } \mu g = \frac{\text{Reading at 520nm - Reading at 420nm}}{\text{Reading of Standard at 520}} * 0.29*15$$

APPENDIX 42
HYDROGEN PEROXIDE SCAVENGING ASSAY
(Ruch *et al.*, 1989)

Principle

Ruch and coworkers proposed an assay for the determination of antioxidant activity of compounds by their ability to scavenge the oxidant hydrogen peroxide.

Reagents

1. Phosphate buffer (pH-7.4)
2. Hydrogen peroxide in phosphate buffer (40mM)

Procedure

Plant extracts at the concentration of 10µg/10µl was added to a Hydrogen peroxide solution (0.6ml,40mM) was prepared in 3.4ml of 0.1M phosphate buffer. The total volume was made upto 3ml.The absorbance of the reaction mixture was recorded at 0 min and then at every 10 min up to 40 min at 230nm.The blank solution containing phosphate buffer without Hydrogen peroxide. The percentage of Hydrogen peroxide scavenged by the plant extract was calculated as

$$\text{Percentage of scavenged } H_2O_2 = \frac{A_0 - A_1}{A_0} \times 100$$

A_0- Absorbance of control
A_1- Absorbance in the presence of plant extract.

APPENDIX 43
DPPH ASSAY
(Mensor *et al.*, 2001)

Principle

Antioxidant activity of the phenolic compounds depends on their ability to decrease the stable free radical.

Reagents

1. Methanol-50 ml
2. DPPH (Diphenyl-2-picryl hydrazyl radical)-1mM

Procedure

3 ml of 1mM DPPH in methanol was added to 100µl of PBS containing 10-100µg of the protein fraction. DPPH solution with methanol was used as a positive control and methanol acted as a control. When DPPH reacts with antioxidant, the sample of DPPH was reduced and the color changed from deep violet to light yellow. This was measured at 518 nm. The percentage antioxidant activity was calculated by the following formula.

$$\text{Scavenging activity (\%)} = \frac{A_{518}\text{ (control)} - A_{518}\text{ (sample) x100}}{A_{518}\text{ (control)}}$$

APPENDIX 44
ESTIMATION OF CHLOROPHYLL
(Witham *et al.*, 1971)

Principle

Chlorophyll was extracted in 80% acetone and the absorption at 663 nm and 645 nm was read in a spectrophotometer. Using the absorption coefficients, the amount of chlorophyll was calculated.

Reagents

80% Acetone

Procedure

Weighed 1 g of finely cut and well mixed representative sample of leaf tissue into a clean mortar. Ground the tissue into a fine pulp with the addition of 20 ml of 80% acetone. Centrifuged (5000 rpm for 5 minutes) and transferred the supernatant to 100 ml volumetric flask. Ground the residue with 20 ml of 80% acetone, centrifuged and transferred the supernatant to the same volumetric flask. Repeated this procedure until the residue was colorless. Washed the mortar and pestle thoroughly with 80% acetone and collected the clear washings in the volumetric flask. Made up the volume to 100 ml with 80% acetone. Read the absorbance value of the solution at 645 and 663 nm against the solvent (80% acetone) blank.

Calculation

Calculated the amount of chlorophyll present in the extract as mg chlorophyll per g tissue using the following equations.

$$\text{mg chlorophyll a/g tissue} = 12.7 (A_{663}) - 2.69 (A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{mg chlorophyll b/g tissue} = 22.9 (A_{645}) - 2.69 (A_{663}) \times \frac{V}{1000 \times W}$$

$$\text{mg total chlorophyll /g tissue} = 20.2 (A_{645}) + 8.02 (A_{663}) \times \frac{V}{1000 \times W}$$

Where,

A – Absorbance at specific wavelengths,

V – Final volume of chlorophyll extract in 80% acetone

W – Fresh weight of tissue extract

APPENDIX- 45
ESTIMATION OF CAROTENOIDS
(Zakaria *et al.*, 1979)

Principle

The total carotenoids in the sample were extracted in petroleum ether. The total carotenoids were estimated in a UV/Visible spectrophotometer at 450nm.

Reagents

1. Petroleum ether (40°C - 60°C)
2. Anhydrous Sodium sulphate
3. Calcium carbonate
4. 12% alcoholic potassium hydroxide

Procedure

Weighed 5-10 g of the sample. Saponified for about 30 minutes in a shaking water bath at 37°C after extracting the sample in 12% alcoholic KOH. Transferred the saponified extract into a separating funnel packed with glass wool and calcium carbonate containing 10 to 15 ml of petroleum ether layer. Transferred the lower aqueous phase to another separating funnel, and the petroleum ether extract containing the cartenoids pigments to amber colored bottle. Repeated the extraction of the aqueous phase. To the petroleum ether extract added a small quantity of anhydrous sodium sulphate to remove the turbidity. Noted the final volume of the petroleum ether extract and diluted, if needed, by a known dilution factor. The absorbance of the extracts at 450nm and 503 nm was noted in spectrophotometer.

Amount of total carotenoids present $= \frac{P \times 4 \times V \times 100}{W}$

Where,

P – Optical density of the sample V – Volume of the sample W – Weight of the sample

APPENDIX- 46
IDENTIFICATION OF SOIL pH
(Jackson, 1962)

Apparatus

• pH meter with a range of 0-14 pH
• Pipette/dispenser
• Beaker
• Glass rod

Reagent

• Buffer solutions of pH 4, 7 and 9
• Calcium chloride solution (0.01M): Dissolve 14.7 g $CaCl_2.2H_2O$ in 10 litre of water to obtain 0.01M solution.

Procedure

1. Calibrate the pH meter, using 2 buffer solutions, one should be the buffer with neutral pH (7.0) and the other should be chosen based on the range of pH in the soil. Take the buffer solution in the beaker. Insert the electrode alternately in the beakers containing 2 buffer solutions and adjust the pH. The instrument indicating pH as per the buffers is ready to test the samples
2. Weigh 10.0g of soil sample into 50 or 100 ml beaker; add 20ml of $CaCl_2$ solution (use water instead of $CaCl_2$ solution throughout the procedure if water is used as a suspension medium).
3. Allow the soil to absorb $CaCl_2$ solution without stirring and then thoroughly stir for 10 second using a glass rod.
4. Stir the suspension for 30 minutes and record the pH on the calibrated pH meter.

Table: Based on soil pH values, following types of soil reactions are distinguished.

PH Range	Soil Reaction Rating
<4.6	Extremely acid
4.6-5.5	Strongly acid
5.6-6.5	Moderately acid
6.6-6.9	Slightly acid
7.0	Neutral
7.1-8.5	Moderately alkaline
>8.5	Strongly alkaline

The acidic soil need to be limed before they can be put to normal agricultural production. The alkali soil need to be treated with gypsum to remove the excessive content of sodium.

APPENDIX- 47
IDENTIFICATION OF ELECTRICAL CONDUCTIVITY (EC)
(Jackson, 1962)

Apparatus

• EC meter
• Beakers (25 ml), erlenmeyer flasks (250 ml) and pipettes
• Filter paper

Reagent

0.01M Potassium chloride solution: Dry a small quantity of AR grade potassium chloride at 600°C for two hours. Weigh 0.7456 g of it and dissolve in freshly prepared distilled water and make the volume to one litre. This solution gives an electrical conductivity of 1411.8×10^{-3} i.e. 1.412 mS/cm at 250°C. For best result, select a conductivity standard (KCl solution) close to the sample value.

Procedure

1. Take 40 g soil into 250 ml Erlenmeyer flask, add 80 ml of distilled water, stopper the flask and shake on reciprocating shaker for one hour. Filter through Whatmann No.1 filter paper. The filtrate is ready for measurement of conductivity.
2. Wash the conductivity electrode with distilled water and rinse with standard KCl solution.
3. Pour some KCl solution into a 25 ml beaker and dip the electrode in the solution. Adjust the conductivity meter to read 1.412 mS/cm, corrected to 250°C.
4. Wash the electrode and dip it in the soil extract.
5. Record the digital display corrected to 250°C. The reading in mS/cm of electrical conductivity is a measure of the soluble salt content in the extract and an indication of salinity status of this soil (Table). The conductivity can also be expressed as mmhos/cm.

APPENDIX- 48
ESTIMATION OF CALCIUM BY VERSENATE
EDTA method, (Cheng and Bray, 1951)

Apparatus

- Shaker
- Porcelain dish
- Beakers
- Volumetric/conical flask.

Reagents

- **Ammonium chloride** – ammonium hydroxide buffer solution: Dissolve 67.5 g ammonium chloride in 570 ml of conc. ammonium hydroxide and make to 1 litre.
- **Standard 0.01N calcium solution**: Take accurately 0.5 g of pure calcium carbonate and dissolve it in 10 ml of 3N HCl. Boil to expel CO_2 and then make the volume to 1 litre with distilled water.
- **EDTA solution (0.01N):** Take 2.0 g of versenate, dissolve in distilled water and make the volume to 1 litre. Titrate it with 0.01N calcium solution and make necessary dilution so that its normality is exactly equal to 0.01N.
- **Muroxide indicator powder**: Take 0.2 g of muroxide (also known as ammonium purpurate) and mix it with 40 g of powdered potassium sulphate. This indicator should not be stored in the form of solution, otherwise it gets oxidized.
- **Sodium diethyl dithiocarbamate crystals:** It is used to remove the interference of other metal ions.
- **Sodium hydroxide 4N**: Prepare 16% soda solution by dissolving 160 g of pure sodium hydroxide in water and make the volume to 1 litre. This will give pH 12.

Procedure

1. Take 5 g air dried soil sample in 150 ml conical flask and add 25 ml of neutral normal ammonium acetate. Shake on mechanical shaker for 5 minutes and filter through Whatmann filter paper No.1.
2. Take a suitable aliquot (5 or 10 ml) and add 2-3 crystals of carbamate and 5 ml of 16% NaOH solution.

3. Add 40-50 mg of the indicator powder. Titrate it with 0.01N EDTA solution till the colour gradually changes from orange red to reddish violet (purple). It is advised to add a drop of EDTA solution at an interval of 5 to 10 seconds, as the change of colour is not instantaneous.
4. The end point must be compared with a blank reading. If the solution is over titrated, it should be back titrated with standard calcium solution and exact volume used is thus found.
5. Note the volume of EDTA used for titration.

Calculation

If N_1 is normality of Ca++ and V_1 is volume of aliquot taken and N_2V_2 are the normality and volume of EDTA used, respectively, then,

$N_1V_1 = N_2V_2$

$$N_2 = \frac{N_1 V_1}{V_2} = \frac{\text{Normality of EDTA x Vol. of EDTA}}{\text{ml of aliquot taken}}$$

Here N_1 (Normality) = equivalent of Ca^{2+} present in one litre of aliquot.

$$\text{Hence, } Ca^{2+} \text{ mg/litre} = \frac{\text{Normality of EDTA x Vol. of EDTA x1000}}{\text{ml of aliquot taken}}$$

When expressed on soil weight basis,

$$Ca^{2+} \text{ mg/100 g soil} = \frac{100}{\text{Wt of soil}} \times \frac{\text{Extract volume}}{1000} \times \text{Ca as mg/litre}$$

APPENDIX - 49

ESTIMATION OF AVAILABLE NITROGEN BY KJELDAHL METHOD

(Johan Kjeldahl, 1883)

Apparatus

• Kjeldahl digestion and distillation unit
• Conical flasks
• Burettes
• Pipettes

Reagents

• Sulphuric acid – H_2SO_4 (93-98%)
• Copper sulphate – $CuSO_4H_2O$ (AR grade)
• Potassium sulphate or anhydrous sodium sulphate (AR grade)
• 35% sodium hydroxide solution: Dissolve 350 g solid NaOH in water and dilute to one litre
• 0.1M NaOH: Prepare 0.1M NaOH by dissolving 4.0 g NaOH in water and make volume to 1 litre. Standardize against 0.1N potassium hydrogen phthalate or standard H_2SO_4
• 0.1M HCl or 0.1M H_2SO_4: Prepare approximately 0.1M acid solution and standardize against 0.1M sodium carbonate
• Methyl red indicator
• Salicyclic acid for reducing NO_3 to NH_4, if present in the sample
• Devarda's alloy for reducing NO_3 to NH_4, if present in the sample.

Procedure

1. Weigh 1 g sample of soil. Place in Kjeldahl flask.
2. Add 0.7 g copper sulphate, 1.5 g K_2SO_4 and 30 ml H_2SO_4.

3. Heat gently until frothing ceases. If necessary, add small amount of paraffin or glass beads to reduce frothing.
4. Boil briskly until solution is clear and then continue digestion for at least 30 minutes.
5. Remove the flask from the heater and cool, add 50 ml water and transfer to distilling flask.
6. Take accurately 20-25 ml standard acid (0.1M HCl or 0.1M H_2SO_4) in the receiving conical flask. So that there will be an excess of at least 5 ml of the acid. Add 2-3 drops of methyl red indicator. Add enough water to cover the end of the condenser outlet tubes.
7. Add 30 ml of 35% NaOH in the distilling flask in such a way that the contents do not mix.
8. Heat the contents to distil the ammonia for about 30-40 minutes.
9. Remove receiving flask and rinse outlet tube into receiving flask with a small amount of distilled water.
10. Titrate excess acid in the distillate with 0.1M NaOH.
11. Determine blank on reagents using same quantity of standard acid in a receiving conical flask.

Calculation

$$\text{Percent N} = \frac{1.401\ (V_1M_1 - V_2M_2) - (V_3M_1 - V_4M_2)}{W} \times df$$

Where,

V_1 - ml of standard acid taken in receiving flask for samples
V_2 - ml of standard NaOH used in titration
V_3 - ml of standard acid taken to receiving flask for blank
V_4 - ml of standard NaOH used in titrating blank
M_1 - Molarity of standard acid
M_2 - Molarity of standard NaOH
W - Weight of sample taken (1 g)
df - Dilution factor of sample (if 1 g was taken for estimation, the dilution factor will be 100).
Note: 1000 ml of 0.1 M HCl or 0.1 M H_2SO_4 = 1.401 g Nitrogen

Precautions

- The material after digestion should not solidify.
- No NH_4 should be lost during distillation.
- If the indicator changes colour during distillation, determination must be repeated using either a smaller sample weight or a larger volume of standard acid.

APPENDIX- 50
ESTIMATION OF AVAILABLE PHOSPHORUS

(Olsen *et al.*, 1954)

Apparatus

• Spectrophotometer
• Pipette - 2 ml, 5 ml, 10 ml and 20 ml
• Bearkers/flasks - 25 ml, 50 ml, 100 ml and 500 ml

Reagents

• Bicarbonate extractant: Dissolve 42 g Sodium bicarbonate in 1 litre of distilled water and adjust the pH to 8.5 by addition of dilute NaOH or HCl. Filter, if necessary.
• Activated carbon – Darco G 60.
• Molybdate reagent: Dissolve 1.50 g $(NH_4)_2MoO_4$ in 300 ml distilled water. Add the solution to 350 ml of 10M HCl solution gradually with stirring. Dilute to 1 litre with distilled water.

• Stannous chloride solution: (Stock Solution): Dissolve 10 g $SnCl_2.2H_2O$ in 25 ml of concentrated HCl. Add a piece of pure metallic tin and store the solution in a glass stoppered bottle.

• Stannous chloride solution (Working Solution): Dilute 1 ml of the stock solution of stannous chloride to 66.0 ml with distilled water just before use. Prepare fresh dilute solution every working day.

Procedure

1. Preparation of the standard curve: Dissolve 0.1916 g of pure dry KH_2PO_4 in 1 litre of distilled water. This solution contains 0.10 mg P_2O_5/ml. Preserve this as a stock standard solution of phosphate. Take 10 ml of this solution and dilute it to 1 litre with distilled water. This solution contains 1 µg P_2O_5/ml (0.001 mg P_2O_5/ml). Take 1, 2, 4, 6 and 10 ml of this solution in separate 25 ml flasks. Add to each, 5 ml of the extractant solution, 5 ml of the molybdate reagent and dilute with distilled water to about 20 ml. Add 1 ml dilute $SnCl_2$ solution, shake again and dilute to the 25 ml mark. After 10 minutes, read the blue colour of the solution on the spectrophotometer at 660 nm wavelength. Plot the absorbance reading against µg P_2O_5 and join the points.

2. Extraction: Add 50 ml of the bicarbonate extractant to 100 ml conical flask, containing 2.5 g soil sample. Add 1 g activated carbon. Shake for 30 minutes on the mechanical shaker and filter.

3. Development of Colour: Take 5 ml of the filtered soil extract with a bulb pipette in a 25 ml measuring flask; deliver 5 ml of the molybdate reagent with an automatic pipette, dilute to about 20 ml with distilled water, shake and add 1 ml of the dilute $SnCl_2$ solution with a bulb pipette. Fill to the 25 ml mark and shake thoroughly. Read the blue colour after 10 minutes on the spectrophotometer at 660 nm wavelengths after setting the instrument to zero with the blank prepared similarly but without the soil.

Calculation

P(kg / ha) = (A/ 1000 000) X (50/5) X (2000 000/5)=4A

Where,

Weight of the soil taken = 5 g

Volume of the extract = 50 ml

Volume of the extract taken for estimation = 5 ml

Volume made for estimation (dilution = 5 times) = 25 ml

Amount of P observed in the sample on the standard curve = A (µg).

Wt. of 1 ha of soil upto a depth of 22 cm is taken as 2 million kg.

Caution:

In spite of all precautions, intensity of blue colour changes slightly with every batch of molybdate reagent. It is imperative to check standard curve every day by using 2 or 3 dilutions of the standard phosphate solution. If the standard curve does not tally, draw a new standard curve with fresh molybdate reagent.

APPENDIX- 51

ESTIMATION OF AVAILABLE POTASSIUM

Flame Photometeric Method, (Toth and Prince, 1949)

Potassium present in the soil is extracted with neutral ammonium acetate of 1 molarity. This is considered as plant available K in the soils. It is estimated with the help of flame photometer. This is a well-accepted method.

Apparatus

• Multiple Dispenser or automatic pipette – 25 ml
• Flasks and beakers - 100 ml
• Flame Photometer

Reagents

• Molar neutral ammonium acetate solution: Dissolve 77 g of ammonium acetate ($NH_4C_2H_3O_2$) in 1 litre of water. Check the pH with bromothymol blue or with a pH meter. If not neutral, add either ammonium hydroxide or acetic acid as per the need to neutralize it to pH 7.0.

• Standard potassium solution: Dissolve 1.908 g pure KCl in 1 litre of distilled water. This solution contains 1 mg K/ml. Take 100 ml of this solution and dilute to 1 litre with ammonium acetate solution. This gives 0.1 mg K/ml as stock solution.

• Working potassium standard solutions: Take 0, 5, 10, 15 and 20 ml of the stock solution separately and dilute each to 100 ml with the ammonium acetate solution. These solutions contain 0, 5, 10, 15 and 20 µg K/ml, respectively.

Procedure

1. Preparation of the Standard Curve: Set up the flame photometer by atomizing 0 and 20 µg K/ml solutions alternatively to 0 and 100 reading. Atomize intermediate working standard solutions and record the readings. Plot these readings against the respective potassium contents and connect the points with a straight line to obtain a standard curve.

2. Extraction: Add 25 ml of the ammonium acetate extractant to conical flask fixed in a wooden rack containing 5 g soil sample. Shake for 5 minutes and filter.

3. Determine potash in the filtrate with the flame photometer.

Calculation

K (kg / ha) = A X (25/5) X (2000 000/1000 000) =10 A

Where,

A = content of K (µg) in the sample, as read from the standard curve:

Weight of 1 ha of soil upto a plough depth of 22 cm is approx. 2 million kg.

APPENDIX- 52

ESTIMATION OF IRON, MANGANESE, ZINC AND COPPER, (Jackson, 1962)

Preparation of standard solutions

Readymade standard solutions 1000 µg/ml or 1 mg/ml of dependable accuracy are supplied with the AAS and are also available with the suppliers of chemical reagents. If the standard solutions are to be prepared in the laboratory, either metal element foils of 100% purity or the standard chemical salts can be used. The quantities of chemical required to make 1 litre standard solution of 100 µg/ml for different elements are given below.

Specifications for preparing micronutrient standard solutions

- Zinc Sulphate ($ZnSO_4.7H_2O$) for element (Zn) - 100(µg/ml) Conc. of stock solution- 0.4398 quantity of salt required/litre
- Copper Sulphate ($CuSO_4.5H_2O$) for element (Cu) - 100(µg/ml) Conc. of stock solution- 0.4398 quantity of salt required/litre
- Ferrous Sulphate ($FeSO_4.7H_2O$) for element (Fe) - 100(µg/ml) Conc. of stock solution- 0.3928 quantity of salt required/litre
- Manganese Sulphate ($MnSO_4.H_2O$) for element (Mn) - 100(µg/ml) Conc. of stock solution- 0.3075 quantity of salt required/litre

In case of Zn, Cu and Fe, 1000 µg/ml (1000 ppm) standard solution are preferably prepared by dissolving 1.0 g pure metal wire and volume made to 1 litre as per the method described under each element. It is diluted to obtain the required concentration. In case of Mn, Mn $SO_4.H_2O$ is preferred.

Preparation of standard curves

i. Zinc

Reagents

• Standard Zinc Solution: Weigh 1.0 g of pure zinc metal in a beaker. Add 20 ml HCl (1:1). Keep for few hours allowing the metal to dissolve completely. Transfer the solution to 1 litre volumetric flask. Make up the volume with glass-distilled water. This is 1000 µg/ml zinc solution. For preparation of standard curve, refer 1000 µg/ml solution as solution A. Dilute 1 ml of standard A to 100 ml to get 10 µg/ml solution to be designated as standard B.

• Glass-distilled or demineralized acidified water of pH 2.5 + 0.5: Dilute 1 ml of 10% sulphuric acid to one litre with glass-distilled or mineralized water and adjust the pH to 2.5 with a pH meter using 10% H_2SO_4 or NaOH. This solution is called acidified water.

• Working Zn standard solutions: Pipette 1, 2, 4, 6, 8 and 10 ml of standard B solution in 50 ml numbered volumetric flask and make the volume with DTPA solution to obtain 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 µg/ml zinc. Stopper the flasks and shake them well. Fresh standards should be prepared every time when a fresh lot of acidified water is prepared.

Procedure

1. Flaming the solutions: Atomise the standards on atomic absorption spectrophotometer at a wavelength of 213.8 nm (Zn line of the instrument).

2. Prepare a standard curve of known concentrations of zinc solution by plotting the absorbance values on Y–axis against their respective zinc concentration on X– axis.

Precautions

• Weighing must be done on an electronic balance.

• All the glass apparatus to be used should be washed first with dilute hydrochloric acid (1:4) and then with distilled water.

• The pipette should be rinsed with the same solution to be measured.

• The outer surface of the pipette should be wiped with filter paper after use.

• After using the pipette, place them on a clean dry filter paper in order to prevent contamination out.

ii. Copper

Reagents

• Standard copper solution: Weigh 1 g of pure copper wire on a clean watch glass and transfer it to one litre flask. Add 30 ml of HNO_3 (1:1) and make up the mark. Stopper the flask and shake the solution well. This is 1000 µg/ml Cu solution and should be stored in a clean bottle for further use. Dilute 1 ml of 1000 µg/ml solution of copper to 100 ml to get 10 µg/ml standard copper solution.

• Glass-distilled or demineralized acidified water of pH 2.5 + 0.5: Same as that done for Zn.

• Working Cu standard solutions: Pipette 2, 3, 4, 5, 6 and 7 ml of 10 µg/ml standard Cu solution in 50 ml numbered volumetric flasks and make the volume with DTPA solution to get 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 µg/ml copper. Stopper the flasks and shake them well. Prepare fresh standards every fortnight.

Procedure

1. Flame the standards on an atomic absorption spectrophotometer at a wavelength of 324.8 nm (Cu line of the instrument).

2. Prepare the standard curve with the known concentration of copper on X-axis by plotting against absorbance value on Y-axis.

iii. **Iron**

Reagents

• Standard iron solution: Weigh accurately 1 g pure iron wire and put it in a beaker and add approximately 30 ml of 6M HCl and boil. Transfer it to one litre volumetric flask through the funnel giving several washings to the beaker and funnel with glass-distilled water. Make the volume up to the mark. Stopper the flask and shake the solution well. This is 1000 µg/ml iron solution.

• Glass-distilled or demineralized acidified water of pH 2.5 ± 0.5: Same as that done for Zn.

• Working Fe standard solutions: Pipette 10 ml of iron stock solution in 100 ml volumetric flask and dilute to volume with DTPA solution. This is 100 µg/ml iron solution. Take 2, 4, 8, 12 and 16 ml of 100 µg/ml solution and dilute each to 100 ml to obtain 2, 3, 8, 12 and 16 µg/ml of Fe solution.

Procedure

1. Flame the standards on an atomic absorption spectrophotometer at a wavelength of 248.3 nm (Fe line of the instrument).

2. Prepare the standard curve with the known concentration of copper on X-axis by plotting against absorbance value on Y-axis.

iv. **Manganese**

Reagents

• Standard Mn solution: Weigh 3.0751 g of AR grade manganese sulphate ($MnSO_4$ H_2O) on a clean watch glass and transfer it to one litre flask through the funnel giving several washings to watch glass and funnel with acidified water and make the volume up to the mark. This solution will be 1000 µg/ml Mn. A secondary dilution of 5 ml to 100 ml with acidified water gives a 50 µg/ml solution.

• Glass-distilled or de-mineralized acidified water of pH 2.5 + 0.2: Same as that for Zn.

• Working Mn standard solutions: Standard curve is prepared by taking lower concentrations of Mn in the range of 0-10 µg/ml Take 1, 2, 4, 6 and 8 ml of 50 µg/ml solution and make up the volume with DTPA solution to 50 ml to obtain 1, 2, 4, 6 and 8 µg/ml working standards.

Procedure

1. Flame the standards on an atomic absorption spectrophotometer at a wavelength of 279.5 nm (Mn line of the instrument).

2. Prepare the standard curve with the known concentration of Mn on X-axis by plotting against absorbance value on Y-axis.

APPENDIX- 53

SAFFRANIN O STAINING

(Jong Kwiton 1970)

Reagents required

50% alcohol

Hydrochloric acid in 100 ml

0.01% aqueous safranin O

15%, 30%, 50%, 70%, 95%, 100% alcohol

Xylene III and IV.

Procedure

A thoroughly ripened solution should always be used.

1. Transfer the sections to the stain from either water or 50% alcohol. The length of the time in stain varies from 15 to 30 minutes depending on the material. Try a trial slide for 10 minutes.

2. Wash in running tap water a few minutes to remove completely all excess stain. The washing should be as thorough as possible to avoid the formation of troublesome precipitates.

3. Treat briefly (5-10 secs) with acidulated water (2-3 drops of hydrochloric acid in 100 ml water) until the sections turn to a pale pinkish purple color. Care must be taken not to extract too much stain; then transfer quickly to water and wash in slowly running tap water for 20 minutes at least until the sections acquire a rich purple color.

4. Counter stain directly in 0.01% aqueous safranin 0 for 10-15 minutes. (Note: prepare a 2% stock solution by shaking up 2 g of safranin 0 in 100 ml water. A 0.01% solution is made by diluting 1 ml stock with 200 ml water).

5. Remove excess stain be rinsing in water then pass sections in 15%, 30%, 50%, 70% alcohol leaving in each strength for 30-60 secs. Destain further in 95% and 100% alcohol until sections appear purple with only a slight tinge of red. Rinse in 100% alcohol and then in xylene/ethanol, xylene III, IV.

APPENDIX 54
GIEMSA STAINING
(Giemsa, 1904)

Reagents required

Methanol

Giemsa stock solution

Giemsa's solution is a mixture of methylene blue, eosin, and azure B. It is made from 7.6 g Giemsa powder, 500 ml Glycerol, and 500 ml Methyl alcohol.

The stock solution is stable for several years.

Giemsa working solution:

Dilute stock solution with distilled water at an approximate ratio of 1: 25.

Differentiator

0.5% acetic acid in 95% ethyl alcohol.

Procedure

- A thin film of the specimen on a microscope slide is fixed in pure Methanol for 30 seconds.
- By immersing it or by putting a few drops of methanol on the slide.
- The slide is immersed in a freshly prepared Giemsa working solution for 20–30 minutes (in emergencies 5–10 minutes in 10% solution can be used). Rinse sections in distilled water, drain well or blot).
- Differentiate in acetified alcohol, checking progress microscopically.
- Arrest differentiation by washing in absolute alcohol.
- Examine the stained sections mounted and photograph at magnifications up to X250 in the microscope.

For examination with high-dry and oil immersion objectives reinove as much of the wash water as possible from around the section on the slide.

Publications

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2. Sumayya, A. R., Sivagami Srinivasan, Nabeelah Amatullah, (2012), “Screening and biochemical quantification of Phytochemical in Fenugreek *(Trigonella foenum*)”, Research Journal of Pharmaceutical, Biological and Chemical Sciences, RJPBCS, 3(1): 165-169, ISSN-0975-8585. Impact factor-0.35.

3. Sumayya, A. R., Sivagami Srinivasan, (2013), “Quantification of biochemical parameters in Araikeerai (*Amaranthus tristis*) and Agati (*Sesbania grandiflora*)”, International Journal of Pharmaceutical Science Review and Research, Int. J. Pharma. Sci. Rev. Res., 19(1): 58-59, ISSN 0967-044X. Impact factor-2.19.

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5. Sumayya, A. R., Sivagami Srinivasan, (2014), “Analysis of Chemical characteristics in untreated and biotreated Silk dyeing effluent by *Azospirillum sp*. and *Pseudomonas fluorescens”,* International Journal Applied Biological and Chemical Sciences, IJAPBC, 3(1): 176-179, ISSN: 2277 - 4688.

6. Sumayya, A. R., Sivagami Srinivasan, (2014), “Biotoxicity analysis of fish in fresh water, untreated and biotreated Silk dyeing effluent”, Journal of Chemical, Biological and physical sciences, JCBPS, 4(3): 2258-2264. Impact factor-0.7

7. Sumayya, A.R., Sivagami Srinivasan, (2014), “Study of physical parameters of untreated and treated Silk dyeing effluent by *Azospirillum sp*., and *Pseudomonas fluorescens*”, Book published in the title Global opportunities for recent developments in science and technology, pp D-277 –D-279.