

APPENDICES

APPENDIX 1

BIOCHEMICAL TESTS FOR THE IDENTIFICATION OF BACTERIA

(Bergey's Manual of Determinative Bacteriology, 1984 and Aneja, 1996)

GRAM STAINING

Principle

In 1884, Christian Gram developed this method, to identify Gram positive and Gram negative bacteria. A smear was prepared on the slide, stained with crystal violet and then treated with iodine which acts as a mordant. The Crystal Violet – Iodine complex (CV-I) imparts purple colour to the cells. In Gram positive cells, this complex binds to the Mg-RNA component of the cell wall, which is difficult to remove. The intensely stained cells were then washed with ethanol. The ethanol dissolves the lipids and allows the leakage of CV-I complex. Due to the presence of less lipid content in Gram positive bacteria, the lipid is easily dissolved by ethanol. This makes minute pores in the cell wall that are closed by dehydration effect of ethanol. In Gram negative cells, large pores are formed that do not close appropriately, hence dehydration of cell wall protein does not occur completely. This facilitates the release of the unbound crystal violet complex leaving the cell colourless or unstained. If the smear is counter stained with safranin, the Gram negative cells are easily seen due to absorption of safranin and imparting the cells pink colour, while Gram positive cells retain the blue colour of the primary stain.

Reagents

1. Crystal violet

Solution A : Two grams of crystal violet was dissolved in
120 ml of ethyl alcohol.

Solution B : Ammonium oxalate - 0.8 g

Solution A and B were mixed and filtered.

2. Gram's Iodine

Potassium Iodide - 2 g

Iodine - 1 g

Distilled water - 290 ml

The solution was made up to 300 ml with distilled water.

3. Ethanol 95%

4. Safranin 1%

Safranin (2.5%wt/vol) in 95% (vol / vol) ethanol - 10ml

Distilled water - 90ml

Procedure

The bacterial smears were heat fixed and each smear was covered with crystal violet for 30 seconds and the slides were washed with distilled water for few seconds. These slides were then covered with Grams iodine solution for 30 seconds and washed off the iodine solution with 95% ethyl alcohol. Ethyl alcohol was added drop by drop, until no more colour flew from the smear (the Gram positive bacteria are not affected while all Gram negative bacteria are completely decolourised). The slides were washed with distilled water and drained. Safranin was applied to the smears for 30 seconds (counter stain), washed with distilled water and blotted with absorbent paper. Air dried slides were examined microscopically using oil immersion objective.

MOTILITY TEST

Principle

This technique is meant for microscopic observation of living bacteria. Due to their small size and close refractive index to that of water, they cannot be observed readily under unstained condition.

Procedure

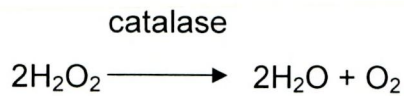
A drop of unknown bacteria was placed on the centre of the cover slip with the four corners covered by vaseline. The cavity slide was placed on the cover slip in such a way that the drop does not move or contact the sidewall of the well. The preparation was examined under low and high power objectives of compound microscope.

CATLASE TEST

Principle

During aerobic respiration, microorganisms produce hydrogen peroxide (H_2O_2) which is lethal to the cell. The enzyme catalase present in

some microorganisms breaks down hydrogen peroxide to water and oxygen and helps them in their survival. Release of free oxygen gas (O₂) bubbles indicates the presence of catalase.



Reagents

3% H₂O₂

Procedure

The nutrient broth was inoculated with the isolated bacterial cultures separately. The cultures were incubated at 35°C for 24-48 hours. The inoculated tubes were held at right angle and 3-4 drops of 3% H₂O₂ was added to the broth. Air bubbles were liberated immediately indicating the presence of catalase.

OXIDASE TEST

Principle

During aerobic respiration, oxidases play a vital role in the operation of electron transport system. Cytochrome oxidase catalyses the oxidation of a reduced cytochrome by molecular oxygen, resulting in the formation of water or hydrogen peroxide.

The test depends on the presence of certain oxidases in bacteria which catalyse the transport of electrons between electron donors in the bacteria and a redox dye tetramethyl - p - phenylenediamine dihydrochloride. The dye is reduced to a deep purple colour.

Reagents

Tetramethyl - p - phenylenediamine dihydrochloride

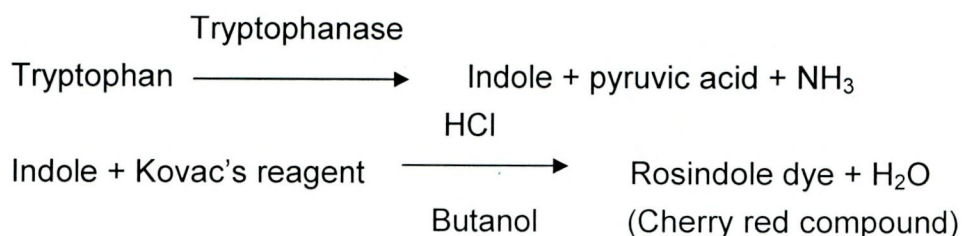
Procedure

A piece of filter paper was taken in a clean petri dish and 2 or 3 drops of freshly prepared oxidase reagent was added. A loopful of each bacterial culture was placed on the filter paper separately. The appearance of purple colour indicated that the organism was oxidase positive.

INDOLE PRODUCTION TEST

Principle

Tryptophan present in peptone of the culture media is acted upon by the enzyme tryptophanase and converted into indole, pyruvic acid and ammonia. Indole reacts with Kovac's reagent (para dimethyl aminobenzaldehyde) to produce a cherry red colour product.



Reagents

1. 1% Peptone broth

Peptone	– 10 g
NaCl	– 5 g
Distilled water	– 1000 ml
pH	– 7

2. KOVAC'S/ EHRILICH'S REAGENT

p-dimethyl aminobenzaldehyde	– 5 g
Amyl alcohol	– 75 ml
Concentrated HCl	– 25 ml

p-dimethyl aminobenzaldehyde was dissolved in amyl alcohol and concentrated HCl was added.

Procedure

Peptone broth was taken in a test tube, sterilized, cooled, inoculated with the isolated bacterial cultures separately and incubated at 37°C for 24 hours. After incubation period, 1 ml of Kovac's reagent was added to each tube including the control. The tubes were shaken gently after an interval of 10-15 minutes and were allowed to stand so that the reagent come to the top. The tubes were examined for the presence of cherry red ring.

METHYL RED TEST

Principle

Organisms belonging to enterobacteriaceae ferment glucose viz., pyruvate and produces mixed acids such as acetic acid, lactic acid, succinic acid, formic acid, ethanol, CO₂ and H₂. Because of the abundant acid production, the final pH of the broth drops to less than 4.5 which can be detected by pH indicators.

Reagents

1. MR - VP BROTH

Peptone	– 7 g
Dextrose	– 5 g
Potassium phosphate	– 5 g
Distilled water	– 1000 ml
pH	– 7

2. METHYL RED REAGENT

Methyl red	– 100 mg
Ethanol (95%)	– 300 ml
Distilled water	– 200 ml

Methyl red was dissolved in ethanol, distilled water was added and then filtered.

Procedure

MR-VP broth was inoculated with the isolated bacterial cultures separately and incubated at 37°C for 24 hrs. Five drops of methyl red indicator was added to all the tubes after incubation period. The change in colour of the broth from yellow to red indicated a positive result, while no change in the colour indicated negative result.

VOGES PROSKAUER TEST

Principle

Glucose undergoes fermentation by a group of bacteria called enterobacteriaceae to produce a non-acidic or neutral end product called acetyl methyl carbinol (CH₃ CO CHO. CH₃) or its reduction product

2, 3 - butylene glycol. Acetyl methyl carbinol undergoes oxidation, on reaction with the guanidine group present in peptone, in the presence of a catalyst α -naphthol and 40% KOH to form a diacetyl compound.

Reagents

1. MR - VP BROTH
2. BARRIT'S REAGENT

VP Reagent I

α -naphthol – 5 g

Ethanol (absolute) – 95 ml

α -naphthol was dissolved in ethanol with constant stirring.

VP Reagent II

KOH – 40 g

Creatine – 0.3 g

Distilled water – 100 ml

KOH was dissolved in 75 ml of distilled water and then creatine was added to the solution and made up to 100 ml with distilled water.

Procedure

MR-VP broth was sterilized, inoculated with the isolated bacterial cultures separately and incubated at 37°C for 24 hrs. Twelve drops of VP reagent I (alpha naphthol solution) and 2-3 drops of VP reagent II (40% KOH solution) was added to all the test tubes including control after incubation period. The tubes were gently shaken for 30 seconds with the caps off to expose the media to oxygen. The tubes were observed for the development of crimson ruby pink colour which indicated the positive result while no change in the colour indicated the negative result.

CITRATE UTILIZATION TEST

Principle

Certain organisms can utilize citrate as sole carbon source for its growth. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism that breaks down the citrate to

Procedure

The isolated bacterial cultures were streaked onto Simmon's citrate agar slants separately. One tube was kept as an uninoculated control for comparison. These tubes were incubated at 37°C for 24-48 hours. Following the incubation period, the change in green to deep prussian blue colour in the tubes were noted which indicated positive result. No colour change indicated negative result.

STARCH HYDROLYSIS TEST

Principle

Starch is an insoluble polymer of glucose, which acts as a source of carbon and nitrogen for microorganism, which has an ability to degrade them. Starch degrading microorganism transports the degraded form across the cytoplasmic membrane of the cell. Some bacteria possess the ability to produce amylase that breaks starch into maltose. The amylase is an extra cellular enzyme, which is released from the cells of microorganism.

Reagents

1. STARCH AGAR MEDIUM

Peptone	– 5 g
Beef extract	– 3 g
Starch (soluble)	– 2 g
Agar	– 15 g
Distilled water	– 1000 ml
pH	– 7

2. IODINE SOLUTION

Potassium iodide	– 2 g
Iodine	– 1 g
Distilled water	– 300 ml

Procedure

Sterile starch agar plates were prepared and the bacterial cultures were streaked onto the plates separately. The plates were incubated

at 37°C for 48 hours. The surface of the plates was flooded with iodine solution. A clear zone surrounding the organism indicated the positive result. Dark blue colouration of the medium with no clear zone formation indicated the negative result.

GELATIN HYDROLYSIS TEST

Principle

Gelatin is an incomplete protein present as liquid above 25°C and solidified when cooled below 25°C. Large protein molecules are hydrolyzed by exoenzymes and the smaller products of hydrolysis are transported into the entire cell. Hydrolysis (liquefaction) of gelatin is brought about by microbes producing proteolytic exoenzymes known as gelatinase, which acts on gelatin in the medium. It can be detected by observing liquefaction (i.e., even very low temperature at 4°C will not restore the gelatin characteristics) or testing with a protein precipitating material i.e., flooding the gelatin agar medium with mercuric chloride solution and observing the plates of clearing around the line of growth. Liquefaction of gelatin being the commonest proteolytic property is used as an index to determine the proteolytic activity of an organism.

Reagents

GELATIN AGAR MEDIUM

Peptone	– 25 g
Meat extract	– 7.5 g
NaCl	– 5 g
Gelatin	– 120 g
Ferrous chloride	– 0.5 g
Agar	– 15 g
Distilled water	– 1000 ml
pH	– 7

All the contents were dissolved in 1000 ml of distilled water and pH was adjusted to 7. The medium was poured in to the test tubes and sterilized by autoclaving at 15 lb pressure for 15 minutes.

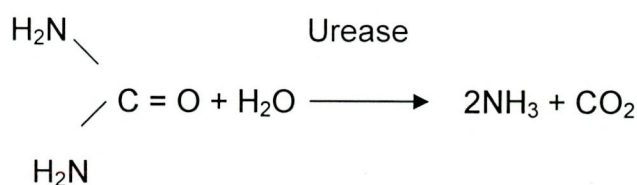
Procedure

Gelatin agar medium was prepared, melted, cooled to 45–50°C, poured into sterile petri dishes and allowed to solidify. Single streak inoculation was made using bacterial cultures in the petri dish separately and incubated the plates at 37°C for 4 - 7 days. The surface of the incubated plates was flooded with mercuric chloride solution and the plates were allowed to stand for 5 -10 minutes. A clear zone surrounding the organism indicated the positive result. No clear zone formation indicated the negative result.

UREASE TEST

Principle

Urea is a waste nitrogenous material excreted out by animals. Some bacteria degrade the nitrogen and carbon bond in urea to form carbondioxide and ammonia in the presence of a hydrolytic enzyme urease. The carbon dioxide and ammonia reacts to form ammonium carbonate, an alkaline end product, which increase the pH of the medium. This can be detected by incorporating a pH indicator in the medium, which changes the colour during alkaline conditions.



Reagents

CHRISTENSEN'S UREA AGAR MEDIUM

Peptone	- 2 g
Sodium chloride	- 0.2 g
KHPO ₄	- 0.1 g
Glucose	- 1 g
Phenol red (0.2% solution)	- 6 ml
Agar	- 15 g
Distilled water	- 1000 ml
pH	- 7

All the ingredients were dissolved by heating, adjusted the pH to 7 and autoclaved at 121°C for 15 minutes and cooled to 50°C.

Urea (20% aqueous solution) -100 ml

Urea was added to the sterile molten base, cooled, mixed well, distributed into sterile test tubes and allowed the medium to solidify in a slanting position to form slants.

Procedure

Sterile Christensen's urea agar tubes were prepared and the tubes were inoculated with the isolated bacterial cultures separately and incubated at 37°C for 24-48 hours. The slants were examined for deep pink colour which indicated the presence of urease and yellow colour indicated the negative result.

NITRATE REDUCTION TEST

Principle

Certain bacteria use nitrates in the place of oxygen as an external terminal electron acceptor. Nitrate can be easily reduced to nitrite by nitrate reductase. In case of aerobic bacteria, oxygen is first used to prevent nitrite reduction and then utilize nitrate. The nitrite may further give rise to nitrogen, ammonia and nitrogen oxide.



Reagents

1. Nitrite broth

KNO ₃ (nitrite free)	- 0.2 g
Peptone	- 5 g
Distilled water	- 100 ml

2. Test reagent

Solution A: Dissolved 8 g of sulphanilic acid in 1 litre acetic acid

Solution B: Dissolved 5 g of alpha naphtholamine in 1 litre acetic acid

Equal volumes of solution A and solution B were mixed (immediately before use).

Procedure

To 5 ml of sterile nitrate broth the isolated bacterial cultures were inoculated separately and incubated at 35°C for 96 hours. After incubation, 0.1 ml of test reagent was added to the culture tubes. Development of red colour in the tubes indicated the presence of nitrates and absence of red colour indicated a negative result.

CARBOHYDRATE FERMENTATION TEST

Principle

Microbes use carbohydrates as energy source depending on their enzyme components. Major products of carbohydrate catabolism are lactic, formic or acetic acid with the production of H₂ or CO₂ as gas. Fermentative degradation is carried out in a fermentation broth containing pH indicator and durham's tube for gas collection.

Reagents

CARBOHYDRATE FERMENTATION MEDIUM

Peptone	- 10 g
*Carbohydrate	- 5 g
Sodium chloride	- 5 g
Phenol red	- 0.018 g
Distilled water	- 1000 ml
pH	- 7.3

(* a specific carbohydrate namely glucose, lactose, sucrose and maltose was added separately).

Procedure

Four types of sugar fermentation broth containing durham's tube was inoculated with each of the isolated bacterium separately and one uninoculated tube of each fermentation broth was kept as a comparative control. The tubes were incubated at 37°C for 24 – 48 hours and after incubation period, the tubes showing acid and gas formations were recorded. Acid production is indicated by change in colour of the medium from red to yellow and the accumulation of gas in durham's tube indicated the positive result.

APPENDIX 2

LACTO PHENOL COTTON BLUE STAINING

(Cappuccino and Sherman, 1999)

Principle

Lactophenol cotton blue is a stain commonly used for making semi permanent microscopic preparation of fungi. It stains the fungal cytoplasm and provides a light blue background against which the walls of the hyphae can readily be seen. It contains four constituents, phenol which serves as a fungicide, lactic acid which acts as a cleaning agent, cotton blue which stains the cytoplasm of the fungus and glycerol which gives a semi permanent preparation.

Requirements

Reagents

20 ml of lactic acid and 40 ml of glycerol was dissolved in distilled water, 20 g of phenol crystals were added to the above contents and heated to dissolve thoroughly. Two ml of 1% aqueous cotton blue was added and mixed well.

Procedure

A drop of lacto phenol cotton blue was placed on a clean glass slide. Using a flamed needle, a small piece of young fungal culture (5 – 7 days old) with spores was added onto the stain separately. The mold was mixed gently with the stain using two teasing needles and a cover slip was placed on the preparation and examined under microscope.

APPENDIX 3

PHYSICOCHEMICAL CHARACTERISATION OF THE EFFLUENT

a. DETERMINATION OF pH

(APHA, 1998)

Principle

A glass surface in contact with hydrogen ions of the solution under test, acquires an electrical potential which depends on the concentration

of H⁺ ions. A measure of the electrical potential (emf), gives H⁺ ion concentration or pH of the solution.

Procedure

The pH meter was first standardized using buffer solutions of pH 7.0 and pH 9.2. The contents were stirred intermittently using glass rod and after 20 minutes the electrodes were washed with distilled water, wiped with filter paper and the electrodes were immersed in the effluent and readings were recorded.

b. DETERMINATION OF ELECTRICAL CONDUCTIVITY (APHA, 1998)

It was measured using conductivity bridge and expressed in (mmhos/cm).

c. DETERMINATION OF TOTAL SUSPENDED SOLIDS Filtration Method (APHA, 1998)

Principle

A well-mixed sample is filtered through a weighed standard glass-fibre filter and the residues retained on the filter are dried to a constant weight at 103°C – 105°C. The increase in weight of the filter represented the total suspended solids. If the suspended material clogs the filter and prolongs filtration, it may be necessary to increase the diameter of the filter or decrease the sample volume.

Procedure

250ml of the effluent was filtered through a tared filter paper by applying suction. The filter paper was washed with distilled water to remove the soluble salts and dried the filter paper for one hour in an oven at 103°C – 105°C. The increase in weight represents the amount of total suspended solids. The results were expressed as mg total suspended solids per litre of the sample. The suspended solids present in the sample were calculated by using the formula

$$\text{Total suspended solids in mg/l} = \frac{\text{Final wt.} - \text{Initial wt. of the crucible}}{\text{Volume of the sample}} \times 1000$$

d. DETERMINATION OF TOTAL DISSOLVED SOLIDS

Filtration Method (APHA, 1998)

Principle

A well-mixed sample is filtered through a standard glass fiber filter and the filtrate is evaporated to dryness in a weighed dish and dried to constant weight at 180°C. The increase in dish weight represents the total dissolved solids.

Procedure

250ml of the effluent was filtered through a glass microfiber filter paper and the filtrate was evaporated in a tared porcelain dish which was preheated at 105°C and then at 550°C for one hour in a muffle furnace. The porcelain dish was cooled and brought to constant weight. The dish was kept at 180°C for 1 hour, cooled and weighed. The increase in weight denoted the amount of total dissolved solids. The results were expressed as mg total dissolved solids per litre of the sample. The total dissolved solids present in the sample was calculated by using the following formula

$$\text{Total dissolved solids in mg/l} = \frac{\text{Final wt.} - \text{Initial wt. of the crucible}}{\text{Volume of the sample}} \times 1000$$

e. ESTIMATION OF BIOCHEMICAL OXYGEN DEMAND

Winkler's Iodometric Method (APHA, 1989)

Principle

BOD determination involves the measurement of dissolved oxygen content of the sample, before and after 5 days incubation at 20°C. The reduction in oxygen content is due to the demand exerted by the microbiological population and it is a measure of oxidisable organic matter in the sample.

When manganous sulphate is added to the sample containing potassium iodide, manganous hydroxide is formed, which is oxidized by the dissolved oxygen of the sample to basic manganic oxide. On addition of

sulphuric acid, the basic manganic oxide liberates iodine, equivalent to that of dissolved oxygen originally present in the sample. The liberated iodine is titrated with a standard solution of sodium thiosulphate using starch as indicator.

Reagents for the preparation of dilution water

1. Calcium chloride solution : 27.5 g was dissolved in one litre of distilled water.
2. Magnesium sulphate solution : 25 g was dissolved in one litre of distilled water.
3. Ferric chloride solution : 0.25 g was dissolved in one litre of distilled water.
4. Phosphate buffer (pH 7.2) : 21.75 g dipotassium hydrogen phosphate, 33.4 g disodium hydrogen phosphate heptahydrate and 1.7 g ammonium chloride were taken in one litre standard flask, dissolved in distilled water and made up to the mark. The buffer was stored in a refrigerator to prevent mold growth.

Reagents for the estimation of dissolved oxygen

1. Manganous sulfate solution : 91.9 manganous sulfate monohydrate was dissolved and diluted to 250 ml with distilled water.
2. Alkali-iodide-azide reagent : Reagent A – 175 g potassium hydroxide and 37.5 g potassium iodide were dissolved in 250 ml of water. Reagent B – 2.5 g sodium azide was dissolved in 10.0 ml of water. Reagent A and B were mixed.
3. Concentration sulphuric acid.
4. Phosphoric acid : 85 – 90%.
5. Sodium thiosulphate solution (0.1 N) : 24.82 g was dissolved in distilled water and made up to one litre.
6. Sodium thiosulphate solution, 0.025 N : 250 ml of 0.1 N sodium thiosulphate solution was diluted to 1000 ml with distilled water. 1 ml of 0.025 N sodium thiosulphate \equiv 0.2 mg dissolved oxygen.
7. Starch solution - 1%

Procedure

Preparation of dilution water : 1 ml each of calcium chloride, magnesium sulphate, ferric chloride and phosphate buffer solution was added 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 ml of raw sewage was added to one litre of dilution water.

Dilution of the samples : The test water samples were diluted with seeded dilution water sample (1%, 5% and 10%) in dilution mixture for the water sample. Each dilution sample was taken in a set of two BOD bottles.

Determination of dissolved oxygen (DO) before and after 5 days incubation : In one set of flasks DO was determined immediately while the other set was kept for incubation at 20° C for 5 days. After 5 days, DO for the incubated sample was determined. Determination of DO is as follows:

To the contents of the BOD bottle 2 ml of manganous sulphate solution and 2 ml of alkali - iodide - azide solution was added the bottle was stoppered and mixed thoroughly. A brown precipitate of basic manganic oxide was formed, which was allowed to settle completely leaving a clear supernatant liquid. Then 2 ml of conc. sulphuric acid was added along the sides of the bottle, stoppered and mixed for complete dissolution. The contents were transferred to a 500 ml conical flask and titrated immediately against 0.025 N sodium thiosulphate using starch as an indicator.

Calculation for DO

$$\begin{aligned} \text{Volume of 0.025 N thiosulphate used up in the titration} &= \text{DO in mg/l} \\ \text{DO at 0° C and 760 mm pressure} &= \text{DO} \times 0.07 \text{ mg/l} \end{aligned}$$

Calculation for BOD

$$\text{BOD (5 days at 20° C)} = \frac{(\text{DO}_0 - \text{DO}_5 - \text{BC}) \times 100}{\text{percent sample}}$$

DO_0 = Initial DO

DO_5 = DO after 20° C incubation for 5 days

BC = Blank Correction ie, Difference in DO of blank on the initial day and after 5 days incubation.

f. ESTIMATION OF CHEMICAL OXYGEN DEMAND

Titrimetric Method (APHA, 1998)

Principle

Chemical Oxygen Demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. Because of its unique chemical properties, the dichromate ion ($\text{Cr}_2\text{O}_7^{2-}$), the specified oxidant is reduced to the chromic ion (Cr^{3+}).

COD often is used as a measurement of pollutants in waste water and natural waters. Most types of organic matter are oxidized by boiling the mixture of chromic and sulfuric acids. A sample is refluxed in strongly acid solution with a known excess of potassium dichromate. After digestion, the remaining unreduced potassium dichromate is titrated with ferrous ammonium sulfate to determine the amount of potassium dichromate consumed and the oxidizable matter is calculated in terms of oxygen equivalent.

Reagents

1. Mercuric sulphate crystals.
2. Sulphuric acid – silver sulphate reagent: Dissolved 10.1 g of silver sulphate in 1 litre of concentrated sulphuric acid and allowed the solution to stand for two days for complete dissolution.
3. Potassium dichromate solution 0.125N: Dissolved 0.129 g of potassium dichromate in distilled water and made up to 1 litre. 1 ml of 0.125N potassium dichromate \equiv 1 mg of oxygen.
4. Ferroin indicator solution: Dissolved 95 mg of ferrous sulphate in 500ml of distilled water, added 1.485g of 1, 10 phenanthroline monohydrate and mixed thoroughly.
5. Ferrous ammonium sulphate solution 0.125 N : 40g of ferrous ammonium sulphate was dissolved in distilled water, 20ml of concentrated sulphuric acid was added and made up to one litre with water. The contents were standardized with 0.125 N potassium dichromate.

Procedure

A refluxing flask of 250 ml capacity was used with a ground glass 24/40 neck fitted with a 300 mm double surface condenser to which a glass cap was fitted. 50 ml of the sample was placed in the flask and a suitable quantity of mercuric sulphate was added so that the ratio of chloride content of the sample to mercuric sulphate was 1: 10 (For this, chloride content of the sample was estimated). Five ml of sulphuric acid – silver sulphate reagent was added to dissolve the mercuric sulphate and cooled in cold water while mixing. 25ml of 0.125 N potassium dichromate was pipetted into the flask and mixed well. 70 ml of sulphuric acid - silver sulphate reagent was added very carefully and mixed. A few porcelain bits were added and the condenser was attached to the refluxing flask. Water was circulated and refluxed for two hours. The flask was cooled after removing from the flask. The contents of the flask were transferred and diluted to 350ml with distilled water. About 2 to 3 drops of ferroin indicator was added 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

$$\text{COD in mg / l} = \frac{(\text{Blank titre value} - \text{sample titre value}) \times 0.125 \times 1000 \times 8}{\text{Volume of sample taken}}$$

g. ESTIMATION OF TOTAL HARDNESS

EDTA-Titrimetric Method (APHA, 1998)

Principle

Ethylene diamine tetra acetic acid and its sodium salts (EDTA) form a chelated soluble complex when added to a solution of certain metal cations. If a small amount of dye such as Eriochrome Black – T or calmagite is added to an aqueous solution containing calcium and magnesium ions at a pH of 10.0 ± 0.1 , the solution becomes wine red. If

EDTA is added as a titrant, the calcium and magnesium will be complexed and the solution turns from wine red to blue, marking the end point of the titration.

Reagents

1. Calcium standard solution: 1 g of pure calcium carbonate was dissolved in 1 litre of distilled water using 20.5 ml of HCl and the contents were warmed. 1 ml = 1 mg CaCO₃.
2. Standard EDTA titrant (0.02 N): 3.723 g disodium ethylene diamine tetra acetate dihydrate was dissolved in 1 litre of water. It was standardized against standard calcium solution. One ml of 0.02 N EDTA \equiv 1 mg of CaCO₃.
3. Eriochrome Black – T indicator: 0.5 g of the dye was mixed with 100g of sodium chloride to obtain a dry powder mixture.

Procedure

50 ml of the effluent was placed in a conical flask, diluted to 50 ml and added. One millilitre of buffer solution was added per 50 ml volume of the sample. The pH of the titre should be 10.0 ± 0.1 . A pinch of the indicator was added and titrated with standard EDTA, until a reddish tinge appeared. The end point was the appearance of blue colour.

Calculation

If EDTA titrant is exactly 0.02 N,

$$\text{mg / Total Hardness (as CaCO}_3\text{)} = \frac{\text{ml EDTA titant} \times 1 \times 1000}{\text{ml sample taken for estimation}}$$

h. ESTIMATION OF TOTAL ALKALINITY

Titrimetric Method (APHA, 1998)

Principle

When a sample containing carbonate and bicarbonate are titrated against the standard sulphuric acid, phenolphthalein loses its pink colour

when half of the carbonate is converted to bicarbonate. Twice this value is a measure of carbonates present in the sample.

To the colourless solution, a few drops of methyl orange is added and titrated against sulphuric acid till straw yellow colour changes to pinkish red colour. This value gives the amount of acid required to neutralize the bicarbonate originally present and that from the carbonates. By subtracting the first titre value from the second one, acid required to neutralise the bicarbonate originally present in the sample is obtained.

Reagents

1. Sodium carbonate solution 1N: 13.25 g was dissolved in 250 ml water.
2. Sulphuric acid 1N: 28 ml of concentrated sulphuric acid was made up to a litre with distilled water.
3. Sulphuric acid 0.02 N: Diluted approximate volumes of 1N sulphuric acid to prepare 0.02 N sulphuric acid \equiv 1.0 mg CaCO_3 .
4. Phenolphthalein indicator: 500 mg of phenolphthalein was dissolved in 50 ml of ethyl alcohol and 50 ml of distilled water. 0.02N sodium hydroxide solution was added dropwise until a faint pink colour appeared.
5. Mixed indicator solution: 20 mg of methyl red and 100 mg of bromocresol green were dissolved in 100 ml of 95% isopropyl alcohol.

Procedure

To 25 ml of the effluent equal volume of distilled water and a pinch of phenolphthalein indicator solution was added in a 250ml conical flask. Pink colour appeared which was then titrated with 0.02N sulphuric acid until the solution became colourless. Three drops of mixed indicator solution was added to the solution in which phenolphthalein alkalinity had been determined and titrated against 0.02N sulphuric acid. The colour was changed from emerald green to light pink. (If no pink colouration occurred, it indicated nil phenolphthalein alkalinity).

Calculation

$$\text{Total alkalinity as CaCO}_3(\text{mg/l}) = \frac{\text{ml of 0.02N H}_2\text{SO}_4 \text{ for total alkalinity end point} \times 50 \times 0.02 \times 1000}{\text{ml sample taken for titration}}$$

i. ESTIMATION OF CHLORIDES

Silver Nitrate Titrimetric Method (Vogel, 1964)

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 added as an indicator.

Reagents

Chloride free double distilled water was used for all reagents.

1. Standard silver nitrate titrant, 0.0282N: 4.791 g of silver nitrate was dissolved in 1 litre of distilled water. Standardized it against 0.0282 N sodium chloride solution. 1 ml of exactly 0.0282 N AgNO_3 \equiv 1.0 mg chloride.
2. Standard sodium chloride titrant, 0.0282 N : 1.648 g of sodium chloride was dissolved in 1 litre of distilled water. 1.0 ml \equiv mg chloride.
3. Potassium chromate indicator solution: 25 g of potassium chromate was dissolved in 100ml of distilled water and silver nitrate solution was added dropwise until a slight red precipitate was formed. The contents were allowed to stand for 12 hours, filtered and made up to 500ml with distilled water.
4. Aluminium hydroxide suspension: 100 g of aluminium ammonium sulphate was dissolved in 1000 ml distilled water and warmed to 60°C by stirring 55 ml of conc. ammonia solution. The precipitate was allowed to settle for an hour and washed with distilled water to make the precipitate free from chloride. The decantate was checked by treating a portion of it with silver nitrate solution until the precipitate was free from chloride and diluted it to 1000 ml with distilled water.

Procedure

To 50 ml of the effluent, 3 ml of aluminium hydroxide was added, stirred well, allowed to settle and filtered. The precipitate was washed with chloride free distilled water, the filtrate and washings were combined. 100ml of the samples were pipetted into a porcelain dish and the pH was adjusted in the range of 7 - 9.5. One ml of potassium chromate indicator solution was added and titrated it against standard silver nitrate solution with constant stirring until a slight precipitable reddish colouration persisted. A blank was measured by placing 100 ml chloride – free distilled water instead of sample.

Calculations

If the silver nitrate solution is exactly 0.0282 N,

$$\text{Chloride (mg per litre)} = \frac{(\text{ml AgNO}_3 \text{ for sample} - \text{ml AgNO}_3 \text{ for blank}) \times 1000}{\text{ml sample taken for estimation}}$$

If the silver nitrate solution is not exactly 0.0282N,

$$\text{Chloride (mg per litre)} = \frac{(\text{ml AgNO}_3 \text{ for sample} - \text{ml AgNO}_3 \text{ for blank}) \times \text{Normality of AgNO}_3 \times 35.45 \times 1000}{\text{ml sample taken for estimation}}$$

j. ESTIMATION OF SULPHATES

Turbidimetric Method (APHA, 1998)

Principle

Sulphate ions are precipitated as barium sulphate crystals of uniform size in acid medium. Light absorbed by the precipitate is measured using a spectrophotometer.

Reagents

1. Conditioning reagent: 75 g of sodium chloride was dissolved in 300 ml distilled water, added 30 ml conc. HCl and 100 ml of 95% ethyl alcohol (or isopropyl alcohol). To the above, 50 ml of glycerol was added and mixed well.
2. Barium chloride solution: 100 g of barium chloride was dissolved in 1000 ml of distilled water and was filtered through Whatman No.1 filter paper.

3. Standard sulphate solution: 147 mg anhydrous sodium sulphate was dissolved in distilled water and made up to 1000ml. 1 ml = 100 $\mu\text{g SO}_4^{2-}$

Procedure

Standards

In a 250 ml conical flask 5, 10, 15, 20, 25, 30, 35 and 40 ml of standard sulphate solution was measured separately and diluted to 100 ml. 5 ml of conditioning reagent was added and mixed well using magnetic stirrer. The speed of stirring should be the same for both standards and samples. While stirring, 0.5 g of barium chloride crystals was added and stirring was continued for one minute. Immediately after one minute, an aliquot of the solution was taken in an absorption cell and measured the optical density at a wavelength of 420 nm. A blank was carried out and a graph was plotted relating optical density to μg of SO_4 .

Samples

To a 250 ml conical flask, 50 ml of the sample was measured and diluted to 100 ml and preceded from steps as for standards. From the calibration graph, the mg of sulphate equivalent to the optical density was calculated.

Calculation

$$\text{mg/l sulphate as SO}_4^{2-} = \frac{\text{mg SO}_4 \times 1000}{\text{ml sample taken for estimation}}$$

K. ESTIMATION OF PHOSPHATES

Turbidimetric Method (APHA, 1998)

Principle

Ammonium molybdate reacts with phosphate to form molybdophosphoric acid which is reduced to a blue coloured complex (molybdenum blue) by the addition of stannous chloride.

Reagents

Phenolphthalein indicator solution

500 mg of phenolphthalein was dissolved in 50 ml of ethyl alcohol and to this 50 ml of distilled water was added.

Sulphuric acid – nitric acid solution

75 ml of conc. H_2SO_4 was added to 150 ml of distilled water and cooled. 1 ml of conc. HNO_3 was added and diluted to 250 ml with distilled water.

Ammonium molybdate solution

2.5 g of ammonium molybdate was dissolved in 200 ml of distilled water and 280 ml of conc. H_2SO_4 was added and made up to 400 ml with distilled water and cooled. Molybdate solution was added to the diluted acid and made up to 1000 ml.

Stannous chloride solution

2.5 g of stannous chloride was dissolved in 100 ml of glycerol and heated in a water bath.

Phosphate stock solution

439 mg of potassium dihydrogen phosphate was dissolved in distilled water and made up to 1000 ml in a volumetric flask. Two drops of toluene was added as a preservative.

Phosphate working standard solution

10 ml of phosphate stock solution was pipetted into a 1000 ml volumetric flask and made up to the mark with distilled water (freshly prepared). $1.0 \text{ ml} \equiv 1 \text{ mg P}$

Procedure

100 ml of the sample was taken in a Nessler tube and 1 drop of phenolphthalein indicator was added. The pink colour developed was destroyed by adding one or two drops of sulphuric – nitric acid solution. Phosphate working solution was pipetted into a series of 100 ml Nessler tubes covering the range up to 20 $\mu\text{g P}$ and made up to 100 ml with distilled water. Nessler tube containing 100 ml distilled water was kept as a blank. Four ml of ammonium molybdate solution and 0.5 ml of stannous chloride solution was added to the blank, standards and sample. The colour developed within 10 – 12 minutes was measured at 690nm against

the reagent blank using a spectrophotometer. The calibration curve was prepared and the amount of phosphate equivalent to the observed optical density was calculated and the result was expressed as mg phosphate per litre of sample.

I. ESTIMATION OF AMMONIACAL NITROGEN

Titrimetric Method (APHA, 1998)

Principle

The sample was adjusted to pH 7.4 with phosphate buffer and the distillate containing ammonia was treated with Nessler reagent and the yellow brown colour produced is compared with that of standards.

Procedure

Preliminary treatment

100 ml of sample was taken in a beaker and the sample was dechlorinated with disodium sulphate solution. The contents of the beaker were transferred to a distillation flask and made up to 250 ml with ammonia free distilled water.

Distillation

10 ml of phosphate buffer solution was added (if the sample contains calcium above 250 mg/l, additional 10 ml of buffer solution was added for each 250 mg in the sample), adjusted the pH to 7.4 and the distillation was started immediately. The rate of distillation was adjusted to 6-10 ml/minute and distillation continued until the distillate showed no ammonia when tested with Nessler's reagent. The water loss was compensated by adding free distilled water.

Titration

0.5 ml of mixed indicator solution was added to the distillate and titrated against 0.02 N sulphuric acid. The colour change was observed from pale green to lavender which was the end point. The volume of H₂SO₄ used in the titration was noted and a blank was also used along with the sample.

Calculation

$$\text{Ammonia nitrogen as N (mg/l)} = \frac{\text{ml. 0.02 N. H}_2\text{SO}_4 \text{ for sample} - \text{ml 0.02 N H}_2\text{SO}_4 \text{ for blank} \times 0.28 \times 1000}{\text{ml sample taken for determination}}$$

m. ESTIMATION OF CALCIUM

EDTA Titrimetric Method (APHA, 1998)

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: 40 g of sodium hydroxide was dissolved in 1 litre of distilled water.
2. Murexide indicator: 200 mg of the dye was ground with 100 g of sodium chloride.
3. Standard EDTA titrant, 0.02 N : 3.723 g disodium ethylene diamine tetra acetate dihydrate was dissolved in 1 litre of water. It was standardized against standard calcium solution. 1 ml of 0.02N EDTA \equiv 1 mg of CaCO₃.

Procedure

To 50 ml of the sample 2 ml of sodium hydroxide was added to produce a pH of 12 - 13 and mixed well. 0.1 – 0.2 g of the indicator was added and titrated immediately with EDTA. The end point was from pink to purple.

Calculation

$$\text{mg / 1 calcium (as CaCO}_3 \text{)} = \frac{\text{ml EDTA titrant} \times 1 \times 1000}{\text{ml sample taken for titration}}$$

n. ESTIMATION OF MAGNESIUM

By Calculation (APHA, 1998)

Calculation

$$\text{mg/l magnesium (as CaCO}_3\text{)} = \text{mg / l Total hardness as (CaCO}_3\text{)} - \text{mg/l Calcium (as CaCO}_3\text{)}.$$

o. ESTIMATION OF SODIUM AND POTASSIUM

Flame Photometric Method (Natarajan *et al.*, 1988)

Principle

In flame photometry, the test solution is passed carefully under controlled conditions as a very fine spray in the air supply to a burner. In the flame, the solution evaporates and the salt dissociates to give neutral atoms and a small proportion of this move into a high energy state. When these excited atoms fall back to the ground state, the light emitted of characteristic wavelength is measured. Sodium and potassium are estimated at 590 nm and 770 nm respectively.

Reagents

1. Sodium stock solution : 2.524g of sodium chloride was dissolved in deionised distilled water and made up to one litre. 1 ml = 1 mg sodium.
2. Potassium stock solution : 1.907 g of potassium chloride was dissolved in deionised distilled water and made up to 1 litre.
1 ml = 1 mg potassium.

Procedure

Estimation of sodium and potassium

The flame photometer was standardized before feeding the sample and the reading was set to zero using deionised water. Using the stock solutions of sodium and potassium the readings were adjusted to 100 at their specific wavelengths. Then the samples were fed in the flame photometer and the readings were noted to get the amount of sodium and potassium directly.

p. ESTIMATION OF NICKEL

Dimethyl Glyoxime Colorimetric Method (APHA, 1998)

Principle

Nickel reacts with dimethyl glyoxime in the presence of an alkaline oxidising agent to form a characteristic red colour complex which can either be measured visually or photometrically.

Interferences

Iron, manganese and copper interfere and if they are present in concentration thrice that of nickel, their interference is suppressed by the addition of sodium citrate. If they are present in excess concentrations, nickel dimethyl glyoxime complex alone is separated by extraction with chloroform and preceded further.

Reagents

1. Nickel stock solution : 447.9 mg nickel sulphate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$) was dissolved in distilled water and made up to 1000 ml in a volumetric flask (1 ml = $10\mu\text{g}$ Ni).
 2. Nickel working solution : 10 ml of nickel stock solution was pipetted into a 100 ml volumetric flask and made up to the mark with distilled water (1 ml = $10\mu\text{g}$ Ni).
 3. Hydrochloric acid, 0.5N : 43 ml of conc. HCl was diluted to 1000 ml with distilled water.
 4. Sodium citrate solution : 125g of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) was dissolved in 500 ml of distilled water.
 5. Iodine solution, 0.05 N : 20 g of potassium iodide was dissolved in 50 ml of distilled water and to this 6.4 g of iodine was added and made up to 1000 ml.
 6. Dimethyl glyoxime solution, 0.5 % : 1g of dimethyl glyoxime was dissolved in 100 ml of concentrated ammonia solution, 100 ml distilled water was added and filtered.
-

7. Additional reagents for the removal of interferences :

- (i) Dilute ammonia solution : 10 ml of concentrated ammonia solution was diluted to 450 ml with distilled water.
- (ii) Chloroform
- (iii) 4 ml of dimethyl glyoxime solution

Preparation of samples

i. Effluent, plant and pod (Krishna and Ranjan, 1991)

To 50 ml of the effluent / 1 g each of plant and pod of green gram, 25 ml of 3:2:1 triple acid mixture (conc. nitric acid - conc. perchloric acid - conc. sulphuric acid) was added separately and kept aside for 3 - 4 hours in a fume cupboard. The contents were heated for 30 minutes until vigorous reaction had subsided and heated more strongly for 4 hours until the nitrous fumes are removed and white fumes of perchloric acid are evolved. The contents were allowed to cool and transferred with 3 - 4 washings of deionized water to 50 ml volumetric flask and made up to the mark with water. Aliquot of the samples were taken for the estimation of heavy metals.

ii. Soil (Khopkar, 1993)

10 g of the sample was ground to fine powder and digested in a mixture of nitric acid: perchloric acid (3 : 1). The resulting residue was evaporated to dryness and then extracted with water and made up to 25ml.

iii. Fish (Riley and Segan, 1970)

The tissues namely muscle, liver, gill and kidney of 1g each were subjected to acid digestion [Nitric acid : Perchloric acid (3 : 1)], cooled and made up to 100 ml in a volumetric flask and the level of nickel was determined spectrophotometrically.

Procedure

Nickel working solution covering the range up to 100 μ g was taken in a series of 50ml Nessler tubes and distilled water was kept as the blank. One millilitre of the neutralised (acid digested sample containing not more

than 100 µg nickel) sample was taken in a 50ml Nessler tube. To the blank, standards and samples, 20 ml of 0.5 N HCl was added. Then the following reagents were added in order with mixing after each addition:

- (i) 10 ml sodium citrate solution
- (ii) 2 ml iodine solution
- (iii) 4 ml dimethyl glyoxime solution

The volume in all the flasks were made up to 50 ml with distilled water and allowed to stand for 20 minutes. Optical density was measured in a spectrophotometer at 470 nm against the reagent blank. A calibration curve was prepared and the microgram of nickel equivalent to the observed optical density was determined and the results were expressed.

q. OIL AND GREASE

Soxhlet extraction method, APHA (1998)

Principle

Oil and grease are dissolved in a suitable solvent and extracted from the aqueous phase. The solvent layer is then evaporated and the residue is weighed as oil and grease.

Reagents

1. Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) – 1% aqueous solution
2. Milk of lime - 2% aqueous solution
3. Dilute HCl –25 ml of conc. HCl was added to 75 ml of distilled water and mixed well
4. Petroleum ether - Boiling range 40°C to 60°C
5. Anhydrous sodium sulphate

Procedure

250 ml of well mixed sample was taken in a beaker and 5 ml of magnesium sulphate solution was added. Small amount of milk of lime was added with continuous stirring until flocculation occurred. The precipitate was dissolved in dilute hydrochloric acid and transferred to the separating

funnel. The beaker was washed with 50 ml of petroleum ether and poured into the separating funnel. The aqueous layer was transferred to another separating funnel and again extracted with 50 ml of petroleum ether. Two ether extracts were taken in a 200 ml beaker and 2 g of anhydrous sodium sulphate was added. The beaker was covered with a watch glass and the contents were mixed at frequent intervals for about 30 minutes. The contents were filtered into an evaporating dish using Whatman No. 42 filter paper containing sodium sulphate in its cone, moistened with the solvent. The beaker was washed with two 20 ml portions of petroleum ether and filtered through the same filter paper. Petroleum ether was evaporated by keeping it on a water bath. The dish was weighed and the difference in weight corresponded to the amount of oil and grease present in the aliquot of the sample was noted.

APPENDIX 4

BIOCHEMICAL PARAMETERS OF PLANT AND FISH SAMPLES

a. ESTIMATION OF TOTAL PROTEINS

Spectrophotometric Method (Lowry *et al.*, 1951)

Principle

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin – Ciocalteu reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry's method.

Reagents

1. 2 % sodium carbonate in 0.1N sodium hydroxide (Reagent A)
2. 0.5 % copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in 1 % potassium sodium tartarate (Reagent B)
3. Alkaline copper solution : 50 ml of A and 1 ml of B was mixed prior to use (Reagent C)
4. Folin – Ciocalteu reagent : Folin phenol reagent was diluted with equal volume of distilled water (Reagent D)

5. Protein solution (Stock standard)

50 mg of bovine serum albumin was weighed, dissolved in distilled water and made up to 50 ml in a standard flask.

6. Working standard

10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg of proteins.

Procedure

Extraction of protein from plant sample

500 mg of the sample was weighed and ground well with mortar and pestle using 5 -10 ml of the buffer. The contents were centrifuged and the supernatant was used for protein estimation.

Extraction of protein from fish sample

100 mg of different organs (muscle, liver, gills and kidney) of the fish were homogenised separately with 1 ml of 0.9 per cent sodium chloride solution. One ml of 5 percent trichloroacetic acid was added and then centrifuged at 8000 rpm for 20 minutes. The precipitate was dissolved in 1 ml of 0.1N sodium hydroxide and 0.1 ml of the aliquot was taken and made up to a final volume of 1 ml.

Estimation of protein

The working standard ranging from 0.1 to 1ml was pipetted out into a series of test tubes. 0.2 ml of the extracted sample was pipetted out in a separate test tube and the solution was made up to 1 ml in all the test tubes. A tube with 1 ml of water served as the blank. Five millilitre of Reagent C was added to each tube including the blank, mixed well and allowed to stand for 10 minutes. 0.5 ml of reagent D was added, mixed well and incubated at room temperature in dark for 30 minutes and the blue colour developed was observed at 660 nm. A standard graph was drawn and the amount of protein in the samples was calculated. The amount of protein was expressed in mg/g of sample.

b. ESTIMATION OF TOTAL CARBOHYDRATES

Colorimetric Method (Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

Reagents

1. 2.5 N hydrochloric acid
2. Anthrone reagent: 200 mg of anthrone was dissolved in 100 ml of ice cold 95% sulphuric acid. Prepared freshly before use.
3. Stock standard glucose : 100 mg of glucose was dissolved in 100 ml of distilled water.
4. Working standard glucose : 10 ml of stock was diluted to 100 ml with distilled water.

Extraction of carbohydrate from plant sample

100 mg of the sample was weighed in a boiling test tube and hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N hydrochloric acid and cooled to room temperature. It was neutralized with solid sodium carbonate until the effervescence was ceased and made up the volume to 100 ml. The contents were centrifuged and the supernatant was collected. From this, 1 ml of the aliquot was used for analysis.

Extraction of carbohydrates from fish sample

100 mg of different organs (muscle, liver, gills and kidney) of the fish were homogenized separately in 1 ml of 0.9 per cent sodium chloride solution. One ml of 5 per cent trichloroacetic acid was added to 1 ml of each extract. The homogenate was centrifuged at 8000 rpm for 20 minutes and the supernatant was analysed for the presence of carbohydrates.

Estimation of carbohydrate

The working standard ranging from 0.2 to 1 ml was prepared and a blank was maintained. 0.2 ml of the extracted sample was pipetted in a test tube and made up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water. Then 4 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath. The contents were cooled rapidly and read the green to dark green colour at 630 nm. A standard graph was drawn by plotting concentration of the standard on the X axis versus absorbance on the Y axis. From the graph the amount of carbohydrates present in the samples were calculated.

Calculation

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

c. ESTIMATION OF TOTAL CHLOROPHYLL

Spectrophotometric Method (Yoshida *et al.*, 1971)

Principle

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

Reagents

Acetone (80%, prechilled)

Procedure

Chlorophyll was extracted from 1 g of green gram leaves using 20ml of 80% acetone. The supernatant was transferred to a volumetric flask after centrifugation at 5000 rpm for 5 minutes. The extraction was repeated until the residue was colourless. The mortar and pestle was washed thoroughly with 80% acetone and the clear washings were collected in the volumetric flask. The volume in the flask was made up to 100ml with 80% acetone. The absorbance of the green coloured solution was read at 645 nm and

663 nm against the solvent (80% acetone) blank. The total chlorophyll content in the leaf was calculated using the formula,

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

where

A = absorbance at specific wavelength

V = final volume of chlorophyll extract in 80% acetone

W = fresh weight of the leaves taken for extraction

d. ESTIMATION OF LIPIDS (Folch *et al.*, 1957)

Principle

Lipids form a homogenous group of compounds which have the common property of not dissolving in water but dissolving in organic substances like alcohol, chloroform, ether and hexane. For the extraction of lipids, either a mixture of ethanol and ethyl ether or a mixture of chloroform and methanol were used. The inclusion of methanol or ethanol helps in breaking the bonds between the lipids and proteins.

Reagents

Chloroform – methanol mixture (3 : 1)

Procedure

100 mg of different organs (muscle, liver, gills and kidney) of fish sample was weighed separately and ground well with 5 ml of chloroform methanol mixture. The homogenate was centrifuged, taken in a small weighed beaker and this beaker was placed inside a large beaker filled with water along the sides and kept overnight in hot air oven without any disturbance. In between the methanol (with dissolved protein layer) and chloroform (with dissolved fat), a white precipitate was formed. The methanol layer was removed without disturbing the chloroform layer. The chloroform was evaporated in oven at about 60°C. The beaker was weighed and the difference between the final and initial weight of the beaker gives the lipid content of the tissue.

APPENDIX - 5

BIOCHEMICAL ANALYSIS IN THE SOIL SAMPLES

DETERMINATION OF SOIL pH

(Jackson, 1973)

Principle

A glass surface in contact with hydrogen ions of the solution under test, acquires an electrical potential which depends on the concentration of H^+ ions. A measure of the electrical potential (emf), gives H^+ ion concentration or pH of the solution.

Procedure

pH (1 : 2 soil water suspension) was determined by using pH meter and the standard buffers such as pH 4.0, 7.0 and 9.2 were used. 20 g of the air dried soil samples were passed through 2 mm sieve, transferred to a clean 100 ml beaker and 50 ml of distilled water was added. The contents were stirred intermittently using glass rod and after 20 minutes the electrodes were washed with distilled water, wiped with filter paper and the electrodes were immersed in soil water suspension and the readings were recorded.

DETERMINATION OF SOIL ELECTRICAL CONDUCTIVITY

(Jackson, 1973)

Principle

Electrical conductivity represents the total amount of soluble salts present in the soil.

Procedure

The electrical conductivity was measured in terms of the resistance offered to the flow of current using a conductivity bridge and expressed in mmhos / cm and the EC of the sample was measured.

ESTIMATION OF ORGANIC CARBON

Titrimetry (Walkley and Black, 1934)

Principle

Organic carbon present in organic matter is oxidized by chromic acid ($K_2Cr_2O_7$) in the presence of concentrated H_2SO_4 . This provides nascent

oxygen which combines with carbon and form CO₂. The H₂SO₄ enables easy digestion of organic matter by rendering heat of dilution. Only a certain quantity of chromic acid is used for oxidation. The excess chromic acid left by organic matter is determined by titration with 0.5 N ferrous sulphate or ferrous ammonium using diphenylamine indicator.

Reagents

Potassium dichromate (1 N), diphenylamine indicator (aqueous), ferrous sulphate solution (0.5 N), concentrated sulphuric acid, orthophosphoric acid (85 per cent).

Procedure

0.2 g of sieved soil samples were transferred into a 500 ml conical flask separately. 10 ml of 1 N potassium dichromate and 10 ml of concentrated sulphuric acid solution was added, stirred well and kept for 30 minutes. To this, 200 ml of distilled water, 10 ml of phosphoric acid and 1 ml of diphenylamine indicator were added. The solution in the conical flask was titrated against 0.5 N ferrous ammonium sulphate and the end point was brilliant bright green.

The organic carbon content in the soil sample was determined using the formula,

$$\text{Organic carbon (per cent)} = \frac{(\text{BV} \times \text{SV}) \times 10 \times 0.003 \times 100}{\text{BV} \times \text{W}}$$

where,

BV – Titre value of blank (ml)

SV – Titre value of sample (ml)

10 – Quantity of 1 N potassium dichromate used (ml)

0.003 – 1 ml of 1 N potassium dichromate used (conversion factor)

W – Weight of soil sample (on dry weight basis)

Organic matter (in per cent) = Organic x 1.724 carbon

ESTIMATION OF AVAILABLE NITROGEN IN SOIL

Alkaline Potassium Permanganate Method (Subbaih and Asija, 1956)

Principle

The amount of soil nitrogen released by the potassium permanganate oxidation of part of soil organic matter is estimated by distillation with sodium hydroxide. The distillate is collected in boric acid containing double (mixed) indicator and titrated against standard sulphuric acid.

Reagent

1. 0.32 % potassium permanganate
2. 2.5 % sodium hydroxide
3. Double (mixed) indicator: Bromocresol green (0.5 g) and methyl red (0.01 g) were dissolved in 100 ml of ethyl alcohol
4. Standard sulphuric acid (N/50)
5. 2 % boric acid

Procedure

Twenty grams of soil was weighed and transferred to a distillation flask. To this, 20 ml of distilled water and 1 ml of liquid paraffin (to control frothing) were added. A few glass beads (with holes) were added to prevent bumping. 100 ml of 2.32 % potassium permanganate and 2.5% sodium hydroxide solutions were added to the distillation flask. The contents were distilled and the liberated ammonia was collected in 500 ml ice tumbler containing 20 ml of boric acid with double mixed indicator. The distillation was continued, for 30 minutes until 100 ml of distillate was collected in the beaker. The ammonia collected was titrated against the standard acid (N/50) and from the titre value (a) available nitrogen content of the soil was calculated.

Calculation

$$\text{Amount of available nitrogen present in 100 g of soil} = \frac{0.00028 a}{20} \times 100 \text{ g}$$

ESTIMATION OF AVAILABLE PHOSPHORUS IN SOIL

Flame photometric Method (Olsen *et al.*, 1954)

Principle

Phosphorus is extracted from the soil with 0.5M NaHCO₃ and adjusted to pH 8.5. The CO₃²⁻ ions from NaHCO₃ will react with Ca²⁺ and forms CaCO₃ in the solution which develops blue colour by ascorbic acid method. The amount of phosphorus extracted was measured colorimetrically.

Reagents

1. 0.5 N sodium bicarbonate (pH adjusted to 8.5) - 42 g of NaHCO₃ was dissolved in one litre of distilled water.
2. Activated carbon (free of phosphorus by washing with 0.5 M sodium bicarbonate)
3. Reagent A: 12 g of ammonium molybdate was dissolved in 250 ml of distilled water. 0.291g of antimony potassium tartarate was dissolved in 100 ml distilled water. Both the solutions were added to 1000 ml of 5 N sulphuric acid (13ml of sulphuric acid added to 100ml of distilled water). The above contents were mixed thoroughly and made up to 2 litres with distilled water.
4. Reagent B : 1.056 g of ascorbic acid was dissolved in 200 ml of reagent A. This solution was prepared freshly as and when required.

Procedure

Five gram of soil was weighed and transferred to a 100 ml polyethylene shaking bottle (carried out a blank side by side) to which 50 ml of 0.05 M NaHCO₃ (pH - 8.5) and a pinch of activated carbon (to make the extracted solution colourless) was added. The above contents were mixed in a reciprocating mechanical shaker for 30 minutes, filtered through Whatman number 4 filter paper and the filtrate was collected in a clean dry beaker (The filtrate should be clean and colourless). Five ml of the filtrate was pipetted in a 25 ml volumetric flask, 4 ml of reagent B was added and made up the volume to 25 ml. The contents were allowed for 10 minutes

and the colour development was measured. The intensity of the blue colour developed was read in a photoelectric colorimeter using a red filter (660 nm wave length) by adjusting the meter to 100 per cent transmittance with the blank. From the standard curve, the concentrations of phosphorus (ppm) were calculated in the solution against the per cent transmittance observed in the above step.

Calculation

Weight of the soil taken	- 50 g
Volume of NaHCO ₃	- 50 ml
Volume of extracted solution	
used for P estimation (aliquot)	- 5 ml
Colorimeter reading	- T

Concentration of P read from the standard graph for the reading T - X ppm
 - X µg /ml
 - X / 10⁶ g/ml

Therefore, in 25 ml of the solution contains = $\frac{X}{10^6} \times 25$ g

Therefore in 50ml of the extracted solution and 5g of soil = $\frac{X}{10^6} \times \frac{25}{5} \times \frac{50}{5}$

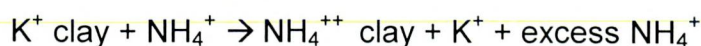
Therefore available P in kg/ha = $\frac{X}{10^6} \times \frac{25}{5} \times \frac{50}{5} \times 2 \times 10^6$

ESTIMATION OF AVAILABLE POTASSIUM AND SODIUM IN SOIL

Flame photometric method (Olsen *et al.*, 1954)

Principle

The potassium (K⁺) and sodium (Na⁺) ions in the exchange sites are replaced with ammonium (NH₄⁺) ions leached from the soil. The reaction may be illustrated as follows:



The reaction goes to the right because of excess ammonium ions as shown by NH_4^+ also being present on the right side of the equation. The K^+ and Na^+ ions in solution were determined with the flame photometer.

Reagent

1. Neutral normal ammonium acetate
2. Standard potassium solution

Procedure

Five gram of soil samples were taken in a 100 ml polythene shaking bottle separately. 25 ml of neutral normal ammonium acetate was added and kept in a mechanical reciprocating shaker for 5 minutes. The contents were filtered through dry Whatman No. 40 filter paper and the filtrates were collected in a dry test tube. The amount of potassium and sodium in the filtrate was measured in the flame photometer.

From the concentration of potassium and sodium read in the standard curve corresponding to the flame photometer reading, the amount of available potassium and sodium in 100 g of soil was calculated.

ESTIMATION OF AVAILABLE CALCIUM AND MAGNESIUM IN SOIL

EDTA Method (Subbaih and Asija, 1956)

Principle

Ca and Mg ions form stable complex by EDTA at different pH. Calcium is estimated by murexide indicator at pH 12 in the presence of NaOH. Then Ca+Mg is estimated using eriochrome black T indicator at pH 10 in the presence of NH_4Cl and NH_4OH buffer solution and titrated against EDTA (versenate).

Reagents

1. Ammonium chloride, ammonium hydroxide buffer solution :
67.5 g of ammonium chloride was dissolved in 570 ml of concentrated ammonium hydroxide and made up to 1 litre.
2. 4 N sodium hydroxide
160 g of NaOH was dissolved in 1 litre of water.

3. Standard calcium chloride solution (0.01 N)

To 0.5 g of pure calcium carbonate, 10 ml of 3 N HCl was added. The contents were boiled to expel carbondioxide and then made up to 1 litre with distilled water.

4. Eriochrome black T indicator

0.5 g of Erichrome black T indicator and 4.5 g of hydroxylamine hydrochloride was dissolved in 100 ml of 95% ethanol.

5. Ammonium purpurate (Murexide) indicator

0.5 g of ammonium purpurate was mixed with 100 g of powdered potassium sulphate (This is not stored in the form of solution as it gets oxidised).

6. Ethylene diamine tetra acetic acid (Versenate) solution (0.01 N)

Two grams of disodium hydrogen ethylene diamine tetraacetate and 0.05 g of magnesium chloride hexahydrate was dissolved in distilled water and made up to 1 litre. The solutions were titrated against calcium chloride using the indicators separately for calcium and magnesium.

Procedure

To 5 g of air dried soil, 25 ml of neutral normal ammonium acetate was added, placed it on a shaker for 5 minutes and filtered it through Whatman filter paper No.1. Calcium and magnesium concentration was determined after pretreatment.

Pretreatment of soil sample

Ammonium acetate extract was transferred to 250ml conical flask and evaporated to dryness on a hot plate. One millilitre of nitric acid and 3ml of hydrochloric acid was added and evaporated again. The residue was dissolved after evaporation in 20ml of 0.1N acetic acid, filtered and diluted to 100ml.

Determination of Calcium

Five millilitre of the above aliquot was pipetted and diluted to 25 ml. To this 0.25 ml of 4 N sodium hydroxide and 50 mg of ammonium

purpurate indicator was added and titrated with 0.01 N EDTA until the colour change was observed from orange red to purple.

Determination of Calcium and Magnesium

Five millilitre of the above aliquot was made up to 25 ml and to this 0.5 ml of ammonium chloride - ammonium hydroxide buffer and 3 to 4 drops of Erichrome black T indicator was added. Titrated with 0.01 N EDTA and the end point was observed from wine red to blue.

Calculation

Weight of soil sample taken - W g
Volume of acid extract prepared - V ml
Volume of acid extract pipetted out for titration - 5 ml
Volume of 0.01 N EDTA used for Ca + Mg - A ml
Volume of 0.01 N EDTA used for Ca alone - B ml
Volume of 0.01 N EDTA used for Mg alone - A - B ml
1 ml of 0.01 N EDTA \equiv 0.0002 g of Ca

$$\text{Percentage of calcium in the given sample} = \frac{0.0002 \times B \times V \times 100 \times 100}{5 \quad W}$$

1 ml of 0.01 N EDTA \equiv 0.00012 g of Mg

$$\text{Percentage of magnesium in the given sample} = \frac{0.00012 \times (A-B) \times V \times 100 \times 100}{5 \quad W}$$

ESTIMATION OF AVAILABLE MICRONUTRIENTS

Lindsay and Norvell (1978)

DTPA (Diethylene triamine pentaacetic acid) was used as an extractant which has been widely accepted for the simultaneous extraction of micronutrient cations viz. Zinc (Zn), Copper (Cu), Iron (Fe) and Manganese (Mn) in neutral and alkaline soil. The content of this cation in the extract was determined on an Atomic Absorption Spectrophotometer (AAS).

Principle

DTPA, a chelating agent combines with free metal ions in the solution to form soluble complexes. The stability constants for simultaneous

complexing of Zn, Cu, Mn and Fe show DTPA as a most suitable extractants. The DTPA has a capacity to complex each of the micronutrient cations as 10 times of its atomic weight.

Reagents

1. Extracting solution : 0.005 M DTPA, 0.01 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.1 M TEA (Triethanolamine) was adjusted to pH 7.3. 1.967 g DTPA and 13.3 ml TEA was dissolved in 100 ml of deionized distilled water. 1.47 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was added and the volume was made up to 500 ml with distilled water. DTPA – TEA mixture was added to it and the final volume was made up to 900 ml. The pH was adjusted to 7.3 using 1 N HCl and made the final volume to one litre and mixed thoroughly.

2. Working standard solutions

Zinc : 0.439 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 5 ml of 1:5 sulphuric acid and made up to 1000 ml with distilled water (This gives 100 ppm of zinc). 2, 4, 6 and 8 ml of stock solution (10 μg Zn/ml) was taken and diluted to 100 ml using DTPA extracting solution, which gives the concentration of 0.2, 0.4, 0.6 and 0.8 μg / ml (ppm) of zinc.

Iron : 0.702 g of ferrous ammonium sulphate hexahydrate was dissolved in 5 ml of 1:5 sulphuric acid and made up to 1 litre with distilled water. 1, 2, 4, 6 and 8 ml of stock solution (100 μg Fe/ml or 100 ppm Fe) was taken and diluted to 100 ml with DTPA extracting solution which gives the concentration of 2, 4, 6 and 8 μg /ml (ppm) of iron.

Copper : 0.392 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was dissolved in 5 ml of 1:5 sulphuric acid and made up to 1000 ml with distilled water. 2, 4, 6 and 8 ml of stock solution containing 100 μg Cu /ml (100 ppm) was made up to 100 ml with DTPA extracting solution which gives the concentration of 2, 4, 6 and 8 μg /ml (ppm) of Cu.

Manganese : 0.288 g of KMnO_4 was dissolved in 300 ml of distilled water. To this, 20 ml of concentrated sulphuric acid was added and made up to 1000 ml with distilled water. 2, 4, 6 and 8 ml of stock solution containing 100 μg Mn/ml was made up to 100 ml with DTPA extracting solution which gives a concentration of 2, 4, 6 and 8 μg /ml (ppm) of Mn.

Procedure

10g of air dried soil sample was weighed in a polythene bottle and 20 ml of the DTPA – extracting solution was added. The bottles were closed and kept on an electric shaker for two hours, filtered and the filtrate was analysed for Zn, Cu, Fe and Mn with an atomic absorption spectrophotometer. From the standard curve the concentrations of micronutrients (ppm) were calculated and the result was expressed in ppm.