Appendix

APPENDIX – I

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 residue retained on the filter is dried to a constant weight at 103°C–105°C. The increase in weight of the filter represents the total suspended solids. If the suspended material clogs the filter and prolongs filtration, it may be necessary the diameter of the filter or decrease the sample volume.

PROCEDURE

Filtered 250ml of the effluent through a tared filter paper by applying suction. Washed the filter paper with distilled water to remove the soluble salts. Dried the filter paper for at least one hour in an oven at 103°C –105°C.The increase in weight was the total suspended solids. Expressed the result as mg total suspended solids per liter of the sample.

APPENDIX – II

DETERMINATION OF TOTAL DISSOLVED SOLIDS FILTRATON METHOD (APHA, 1998)

PRINCIPLE

Filtered 250ml of the effluent through a glass micro fiber filter paper. Evaporated the filtrate in a tarred porcelain dish which was preheated at 105°C and then at 550°C for one hour in a muffle furnace and cooled and brought to constant weight. Kept the dish at 180°C. The increase in dish weight represents the total dissolved solids.

PROCEDURE

Filtered 250ml of the effluent through a glass micro fiber filter paper. Evaporated the filtrate in a tared porcelain dish which was preheated at 105°C and then at 550°C for one hour in a muffle furnace and cooled and brought to constant weight. Kept the dish at 180°C for about one hour, cooled and weighed. The increase in weight denoted the total dissolved solid content. Expressed the result as mg total dissolved solids per liter of the sample.

APPENDIX- III
GRAM STAINING
(Sundarajan, 1995)

PRINCIPLE

In 1884, Christian Gram developed this method, to identify gram positive and gram negative bacteria. A smear is 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, forming a complex, which is difficult to remove. The intensely stained cells are then washed with ethanol. This serves as a lipid solvent and a dehydrating agent for protein. The gram positive bacteria contain low lipid content which is easily dissolved by alcohol. This makes minute pores in the cell wall that are closed by dehydration effect of alcohol. In Gram negative cells, large pores are formed that do not close properly; hence dehydration of cell wall protein does not occur completely. This facilitates the release of the unbound crystal violet complex leaving the cell colorless or unstained. If the smear is counter stained with safranine, gram negative cells appear pink due to the

absorption of safranine while gram positive cells retain the blue colour of the primary stain.

REAGENTS

1. Crystal Violet

Solution A

Crystal Violet	-	2g
Ethylalcohol	-	120ml

Dissolved the dye completely

Solution B

Ammonium Oxalate	–	0.8g

Mixed solution A and B and filtered

2. Grams Iodine

Potassium Iodide	–	2g
Distilled water	–	10ml
Iodine	–	1g
Distilled water	–	290ml

Made up the solution to 300 ml

3. Ethanol 95%
4. Safranine (2.5% w/v) in 95% (v/v)

Ethanol	–	10ml
Distilled water	–	90ml.

PROCEDURE

Heat fixed bacterial smears of all the bacterial cultures were prepared. Each smear was covered with crystal violet for 30 seconds and washed slides with distilled water for a few seconds. Then the smears were covered with Iodine solution. After 30 seconds and washed off the iodine solution with 95% Ethyl alcohol. Added ethyl alcohol drop by drop until no more colour flows from the smear. The slides were then washed with distilled water and drained. Applied safranine to the smears for 30 seconds (counter staining), washed with distilled water and blot dried with absorbent

paper. Air dried the slides and examined microscopically using oil immersion objective.

BIOCHEMICAL TESTS FOR THE DETERMINATION OF BACTERIA

(Dubey and Maheshwari, 2002)

APPENDIX - IV

CABOHYDRATE FERMENTATION TEST

(Glucose)

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 H_2 or CO_2 as gas. Fermentative degradation is carried out in a fermentation broth containing pH indicator under Durham's tube for gas collection.

REAGENTS

1. Carbohydrate fermentation media

Peptone	-	10g
Nacl	-	5g
Bromothymolblue	-	0.025g
Distilled water	-	1000 ml
pH	-	7.3

2. Sugars

Glucose	-	10g

PROCEDURE

The bacterial culture was inoculated into different sugar broth tubes. The sugars used were glucose. The tubes were incubated at 37°C for 24-48

hours. Following incubation period the tubes showing acid formation was recorded.

APPENDIX - V

STARCH HYDROLYSIS TEST

PRINCIPLE

Starch is an insoluble polymer of glucose, 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 extracellular enzyme which is released from the microorganism.

REAGENTS

1. Starch agar medium

Peptone	-	5g
Beef extract	-	3g
Starch (Soluble)	-	2g
Agar	-	15g
Distilled Water	-	1000ml
pH	-	7.0

2. Iodine Solution

PROCEDURE

Sterile starch agar plates were prepared and the bacterial culture was streaked onto the plates. The plates were incubated at 37°C for 48 hours. The surfaces of the plates were flooded with Iodine solution.

APPENDIX - VI

INDOLE PRODUCTION TEST

PRINCIPLE

Tryptophan present in peptones 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.

$$\text{Tryptophan} \xrightarrow{\text{Trytophanase}} \text{Indole} + \text{Pyruvic acid} + NH_3$$

$$\text{Indole} + \text{Kovac's reagent} \xrightarrow[\text{Butanol}]{\text{HCl}} \underset{\text{(cherry Red Compound)}}{\text{Rosindole dye}} + H_2O$$

REAGENTS

1. Peptone broth (1%)

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

2. Kovac's reagent

P-Dimethyl aminobenzaldehyde	-	5g
Amyl alcohol	-	75ml
Concentrated HCl	-	25ml

p-Dimethyl aminobenzaldehyde was dissolved in amyl alcohol and to that concentrated HCl was added.

PROCEDURE

Peptone broth was taken in a test tube, sterilized, cooled, inoculated with the bacterial culture and incubated at 37°C for 24 hours. After incubation period, Kovac's reagent was added.

APPENDIX- VII

METHYL RED TEST

PRINCIPLE

Organisms belonging to Enterobacteriaceae ferment glucose via pyurvate and produce mixed acids such as acetic acid, lactic acid, succinic acid, formic acid, ethanol, Co_2 and H_2. 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	-	7g
Dextrose	-	5g
Potassium Phosphate	-	5g
Distilled water	-	1000ml
pH	-	7

2. **Methyl Red**

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

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

PROCEDURE

MR-VP broth was inoculated with the bacterial culture and incubated at 37°C for 24 hrs. Methyl red solution was added after incubation period and the change in colour was noted.

APPENDIX -VIII

VOGES PROSKAUER TEST

PRINCIPLE

A group of bacteria belonging to Enterobacteriaceae ferment glucose to produce butylene glycol, and acetone which are neutral in nature. The end products are detected by VP reagent.

REAGENTS

1. MR-VP broth

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

2. BARRITS REAGENT

Solution A

α- Napthol	-	5g
Ethanol (absolute)	-	95ml

α-Napthol was dissolved in ethanol with constant stirring.

Solution B

KOH	-	40 g
Creatine	-	0.3g
Distilled water	-	100 ml

Dissolved Potassium hydroxide in 75ml of distilled water, added creatine to the solution and made up to 100 ml with distilled water.

PROCEDURE

MR-VP broth was sterilized, inoculated with bacterial cultural and incubated at 37°C for 24 hrs. 40% KOH solution (VP reagent 1) and Barrit's alpha naphthol solution (VP reagent 2) were added after incubation

period. Gently shook the tubes for 30 seconds with the caps off to expose the media to oxygen.

APPENDIX -IX

CITRATE UTILISATION TEST

PRINCIPLE

Certain organisms can utilize citrate as sole carbon source and grow. During growth acetate and other alkaline carbonates are produced. This reaction is shown by the change in colour of the indicator (Bromothymol blue).

$Co_2 + 2Na^+ + H_2O \longrightarrow Na_2Co_3$

Produced during citric acid metabolism (alkalinepH) (Blue colour)

Bromothymol blue is green when acidic (pH 6.9 and below) and blue when alkaline (pH 7.6 and higher).

REAGENTS

Simmon's citrate Agar medium

Sodium citrate	-	2g
$MgSo_4$	-	0.2g
$(NH_4)H_2PO_4$	-	0.1g
K_2HPO_4	-	1g
Nacl	-	5g
Bromothymol blue	-	0.089g
Agar	-	15g
Distilled water	-	1000 ml
pH	-	7

PROCEDURE

The culture was streaked on Simmon's citrate agar slants. The tubes were incubated at 37°C for 24-48 hours and change in colour was noted.

APPENDIX – X
UREASE TEST

PRINCIPLE

Urea is a diamide of carbonic acid. Urease, present in organism acts upon urea and hydrolyses it to ammonia and carbon dioxide, which in turn results in the formation of an alkaline end product callend ammonium carbonate. This leads to the increase in the pH of the medium, which can be detected using phenol red as an indicator.

REAGENTS

1.Christensen's urea agar

Peptone – 1g

Sodium chloride-5g

Dipotassium hydrogen phosphate-2g

Agar-20g

Phenol red-0.012g

Glucose- 10ml

Urea – 100ml

PROCEDURE

1. To the three tubes add 5ml of CUA medium was added and sterilized

2. Inoculate the tubes with organism

3. The tubes were incubated at 37^0c for 24-48 hrs and colour changes of the pH indicator was observed

APPENDIX – XI
OXIDASE TEST

PRINCIPLE

To determine the presence of oxidase enzymes, the reagent which contains tetramethyl-p-Phenylenediammine, serves as an alternate substrate for the cytochrome oxidase reaction. In the reduced state the reagent is colourless but when oxidized it becomes purple.

REAGENTS

1% Tetramethyl-p-Phenylenediamine dihydrochloride.

PROCEDURE

1.The filter paper disc impregnated with oxidase reagent was placed aseptically on a clean sterile slide.

2. With the help of sterile glass rod, a small amount of culture was transferred to one disc

3.The colour change of the disc was examined

APPENDIX - XII

CATALASE TEST

PRINCIPLE

Some organisms possess the enzyme catalase that splits hydrogen peroxide into oxygen and water.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O_2 + O_2$$

Presence of catalase is indicated by bubbles of free oxygen gas.

REAGENTS

3% H_2O_2

PROCEDURE

To 1ml of bacterial culture 0.5ml of 3% Hydrogen peroxide was added. Culture was noted for liberation of air bubbles.

APPENDIX – XIII
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 $(Cr_2O_7)_2$ the specified oxidant is reduced to the chromate ion(Cr^{3+}).

COD often is used as a measurement of pollutants in wastewater and natural waters. Most types of organic matter are oxidized by boiling mixture of chromic and sulphuric 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 features ammonium sulphate 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 10.1g 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 (For this, chloride content of the sample was estimated as given in appendix X).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 –XIV

ESTIMATION OF BIOCHEMICAL OXYGEN DEMAND AND DISSOLVED OXYGEN WINKLERS IODOMETRIC METHOD (APHA, 1998)

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 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 oxide,magnesium 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 liberated iodine equivalent to the 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 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 (pH7.2):** 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. Mixed reagent A and reagent B.

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 thio sulphate 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 were diluted with seeded dilution water sample (1%,5%,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 20°C 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 conc.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 0°C 760 mm pressure = DO x 0.07 mg/l

CALCULATION FOR BOD:

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

DO_0 = Initial DO

DO_5 = DO after 20°C incubation for 5 days

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