APPENDICES

Appendix:-I

Media used for the culture and enumeration of microorganisms:-

Different type of culture media such as enrichment media, selective media, and differential media were used. Composition and preparation of different type of culture media are given below:-

1. Total plate count agar (TPCA)

The total plate count agar is used for the enumeration of bacteria in foods and water.

Composition:-

Ingredients	gm/liter
Tryptone	5.0
Yeast	2.5
Dextrose	1.0
Agar	15.0
Final pH	7.00±.2

Direction for preparation:-

23.5 grams of TPCA was dissolved in 1000 ml of distill water, boiled to dissolve the medium completely and sterilized at 15 lbs pressure (at 1210C) for 15 minutes. 2. 2.

2. Nutrient Agar (NA)

The nutrient agar is basic culture medium used for the cultivation of pure culture for gram staining and various biochemical tests. It is also used for the preparation of blood agar and other medium.

Composition:-

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

Direction for preparation:-

28 grams of NA was dissolved in 1000 ml distilled water, boiled to dissolve the media completely. The media was sterilized by autoclaving at 15 lbs pressure at 121^0C for 15 minutes.

3. Mac-Conkey agar (MA)

Mac Conkey Agar is a differential and low selective medium used to distinguished lactose fermenting from non lactose fermenting bacteria. MA without salt and crystal violet and sodium taurocholate or bile salts are used for the detection and isolation of coliform.

Composition :-

Ingredients	gm/litre
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0

Agar	20.0
Neutral red	0.04
Final pH	7.4±0.2

Direction for preparation:-

55 gram of MA was suspended in 1000ml distilled water, boiled to dissolve completely. The media was sterilized by autoclaving at 15 lbs pressure at 121^{0}C for 15 minutes.

4. Violet Red Bile Agar (VRBA)

Violet Red Bile Agar is a selective medium used for the detection and enumeration of coli form organisms. The VRBA is recommended for the direct plate count of coli form bacteria in water, milk, dairy and other food products. Coli form organism produce dark pink colonies, which are surrounded by red zone.

Composition:-

Ingredients	gm/litre
Yeast extracts	3.0
Bile salts No 3	1.5
Peptone	7.0
Lactose	10.0
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.002
Agar	15.0
Final pH	7.4±0.2

Direction for preparation:-

41.5 grams of VRBA was suspended in 1000ml of distilled water, heated to boiling to dissolve the medium completely. The media was sterilized by autoclaving at 15 lbs pressure at 121^{0}C for 15 minutes.

5. Selenite F enrichment Broth

Selenite F Broth is used as an enrichment medium for the isolation of Salmonella groups and few species of Shigella groups, when isolating these organism from foods, dairy products etc.

Composition:-

Ingredients	gm/litre
Part A	
Tryptone	5.0
Lactose	4.0
Sodium phosphate	10.0
Part B	
Sodium acid selenite	4.0
Final pH (at 25^{0}C)	7.4±0.2

Direction for preparation:-

19 grams of part A and 4 grams of part B was suspended in1000ml of distilled water. It was warmed to dissolve the media and mixed well. It was then dispensed or sterilized in a boiling water bath or in free floating steam for 10 minutes. DO NOT AUTOCLAVE, EXCESSIVE heating is detrimental.

6. Xylose-lysin deoxycholate agar (XLD Agar)

Xylose lysine deoxycholate agar is a selective or differential medium used to isolate Salmonella and shigella. Based on xylose fermentation, lysine decarboxylation and hydrogen sulphide production, it is possible to differentiate Salmonella and Shigella from most non pathogenic enterobacteria.

Composition:-

Ingredrients	gm/ litre
Yeast extract	3.0
L-lysine	5.0
Lactose	7.5
Sucrose	7.5
Xylose	3.5
Sodium chloride	5.0
Sodium deoxycholate	2.5
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	15.0
Final pH(at 25^0C)	7.4±0.2

Direction for preparation:-

56.68gm of XLD was suspended in 1000ml distilled water. It was heated with frequent agitation until the medium boils. It was then autoclaved at 15 lbs pressure at 121^0C for 15 minutes.

7. Mannitol salt agar (MSA):-

It is a selective medium for the isolation of presumptive pathogenic Staphylococcus. Most other organisms were inhibited by the salt concentration.

Composition:-

Ingredrients	gm/ litre
Beef extract	1.0
Protease peptone	10.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

Direction for preparation:-

111gms of the mediums was suspended in 1000ml of distilled water and boiled to dissolve completely.

8. Nutrient broth

Composition:-

Ingredrients	gm/ litre
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH(at 25^0C)	7.4±0.2

Direction for preparation:-
28 grams of medium was suspended in 1000ml of distilled water and boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

7. Mueller Hinton Agar (MHA)

Composition	gm/liter
Beef Infusion Broth	300.0
Casein Acid Hydrolysate	17.0
Starch	1.0
Agar	17.0
Final pH	7.0±0.2

Procedure
3.8 gm of media was suspended in 100 ml distilled water, boiled to dissolve and sterilized by autoclaving at 121^0C for 15 minutes. It was poured while at $45\text{-}55^0$C in sterile 9 cm diameter plates in 25 ml quantities. To ensure the uniformity in depth of medium, the plates were placed over level surface and the medium was poured into it.

8. Eosine Methylene Blue Agar (EMB)

Composition	gm/liter
Peptone	10.0
Lactose	10.0
Dipottasium hydrogen phosphate	2.0
Eosine yellowish	0.4
Methylene blue	0.065
Agar-agar	13.5
pH	7.0±0.2 at 25°C

Procedure
36 grams of media was dissolved in 1000ml of distilled water and heated to dissolve the media. The media was autoclaved at 121°C for 15 minutes.

Appendix II

Composition Gram's staining reagents and gram's staining procedure

1. For Gram's staining

i. Crystal violet

Solution A

Crystal Violet	2.0gm
95% ethyl alcohol	20.0ml

Solution B

Ammonium oxalate	0.8gm
Distilled water	30.0ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water. Then solution A and B are mixed.

ii. Gram's Iodine

Iodine	1.0gm
Potassium iodide	2.0ml
Distilled water	300.0ml

Iodine and potassium iodide were dissolved in distilled water.

iii. Ethyl Alcohol (95%)

Absolute alcohol	95.0ml
Distilled water	5.0ml

iv. Safranin

Safranin (2.5 % solution in 95% ethyl alcohol)	10.0ml
Distilled water	100.0ml

Procedure

Heat fixed smear of bacterial culture was flooded with crystal violet for one minute and excess stain was washed out. The slide was treated with Gram's Iodine for 1 minute and washed. It was flooded with decolorize alcohol and immediately washed with water. Then smear was treated with safranin for 1 minute and washed with water. It was dried and observed under microscope.

Appendix III

Biochemical Tests

1. Catalase Test

Catalase test is done to test the presence of enzyme catalase. The enzyme catalase splits hydrogen peroxide to water and oxygen.

Reagents: (3 % Hydrogen peroxides).

Composition	
Concentration hydrogen peroxide	3ml
Distilled water	97ml

Procedure

3ml of 3 % hydrogen peroxide was taken in a test tube and colony of bacteria to be tested was picked up from nutrient agar with the help of glass rod and inserted into the tube-containing reagent. The production of gas bubbles immediately indicates positive catalase test.

2. Oxidase Test

Oxidase test is done to determine the presence of the oxidase enzyme which catalyses the transport of electron between electron donors in the bacteria and a redox dye teramethylene-p-phenylene diamine dihydrochloride. Oxidase reaction is due to the presence of a cytochrome oxidase system.

Oxidase reagent:-

1% solution of tetramethyl-p-phenylene diamine dihydrochloride

Preparation of dry filter paper:-

Whatman No. 1 filter paper was cut into strips of 6-8 cm in diameter. It was soaked in a freshly prepared 1% solution of tetramethyl-p-phenylene diamine dihydrochloride. The paper strips were drained and freeze dried and stored in a dark tightly sealed bottle.

Procedure

The Oxidase test paper was moistened with distilled water. A colony was picked up with clean sterile glass rod and smeared over the moist area. Development of violet colour within 10 seconds is an indicative of positive test.

3. Oxidative and Fermentative test (OF test):-

Medium :-Hugh and Leifson Medium

Composition	gm/liter
Peptone	20.0
Sodium Chloride	50.0
Dipotassium Phosphate	3.0
Agar	20.0
Bromo Thymol Blue	0.5
Glucose	100.0
Final pH	7.1±0.2

Preparation of medium

193 grams of media was dispensed in 1000ml of distilled water. It was boiled to dissolve the medium completely and distributed into tubes in duplicate. The medium was then sterilized by autoclaving at 15 lbs at 121^{0}C for 15 minutes.

4. Sulfide-Indole-Motility Medium (SIM)

Sulfide-Indole-Motility is a semi solid medium used for the determination of sulfide production, Indole formation and motility of enteric bacteria.

Composition	gm/liter
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium thiosulfate	0.025
Agar	3.0
Final pH (at 25^{0}C)	7.3±0.2

Procedure

36 grams was suspended in 100ml-distilled water. It was heated to boil to dissolve the medium completely. It was dispense in tubes and sterilized by autoclaving at 15 lbs pressure (121^{0}C) for 15 minutes. The medium was allowed to solidify in vertical position.

Reagent: Kovac's reagent

Composition	gm/liter
P-Dimethyl- aminobenzaldehyde	5.0
Amyl or Isoamyl alcohol	75.0 ml
Conc. Hydrochloric acid	25.0 ml

Procedure

The test organisms was stabbed into the medium and incubated at 37^{0}C for 24 hours. Motile organism show diffuse growth or turbidity away from the line of inoculation and non-motile grows only along the line of inoculation. Blackening along the line of inoculation indicates H2S positive test. 0.2ml of Kovac's reagent was added to the tube and allowed to stand for 10 minutes. A dark red colour in the reagent indicates a positive indole test.

5. Methyl Red test

The methyl red test is employed to detect the production of stable acid end products from glucose fermentation and to overcome the buffering capacity of the system. The pH decreases below 4.5, which can be shown by a change in the color of the methyl red indicator, which is added at the end of the period of incubation.

MR-VP medium (glucose-phosphate broth).

Composition	gm/liter
Buffered peptone	7.0
Dextrose	5.0
Tripotassim phosphate	5.0

Final pH (at 25^0C)	7.4±0.2

Procedure
Seventeen grams was dissolved in 1000ml-distilled water. It was distributed in test tubes in 10 ml amount and sterilized by autoclaving at 15 lbs pressure for 15 minutes.
Reagent – Methyl Red

Composition	gm/liter
Methyl red	0.04gm
Ethyl alcohol	40.0ml
Distilled water	60.0ml

Preparation
Methyl red was dissolved in ethyl alcohol and water was added.
Procedure
The glucose phosphate broth was inoculated with culture to be tested and incubated at 37^0C for 48 hours. Methyl red indicator was added to the culture and development of red colour indicates positive test while yellow colour indicates negative test.

6. Voges – Proskauer Test
Voges – Proskauer test is done to determine the ability of organism to produce a neutral end product, acetyl methyl carbinol (acetoin) or its end product 2,3-butylene glycol from glucose formation.
Medium -MR-VP medium (Glucose – phosphate broth)
Reagents:-

Solution A	
-naphthol	5.0gm
Ethyl alcohol (95%)	100.0ml
Solution B	
Potassium hydroxide	40.0gm
Distilled water	100.0ml

Procedure
Sterile MR-VP broth was inoculated with fresh culture medium and incubated at 37^0C for 48 hours. Development of red colour within 30 minutes after adding of 3ml of -naphthol and 1ml of 40% potassium hydroxide was recorded as positive test.

7. Citrate Utilization Test
Citrate utilization test is performed to determine if an organism is capable of utilizing citrate as the sole source of Carbon for metabolism and energy source for the growth,
Medium: – Simmon's Citrate Agar
Composition:-

Ingredients	gm/liter
Monoammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium Chloride	5.0
Sodium Citrate	2.0
Magnesium Sulphate	0.2
Bromothymol blue	0.08

Agar	15.0
Final pH (at 25^0C)	6.8±0.2

Preparation

24.2 grams was suspended in 1000ml-distilled water. It was heated to boil to dissolve the medium completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. The mediums in tubes were solidified in slanted position.

Procedure

The slant was streaked with test organism and incubated at 37^0C for 48 hours. Growth of organism with an intense blue colour on slant is the indicative of positive test. No growth no change in colour (green) is the negative test.

8. Triple Sugar Iron Agar Test

The test is done to determine the ability of an organism to utilize specific Carbohydrate incorporated in the medium, with or without the production of gas, along with determination of possible hydrogen sulfide production

Composition	gm/liter
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulfate	0.3
Phenol- red	0.024
Agar	12
Final pH (at 25^0C)	7.4±0.2

Preparation

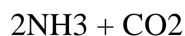
6.5 grams was suspended in 1000ml-distilled water. It was boiled to dissolve completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. The medium was allowed to set in slopped from with a butt about 1 inch long.

Procedure

The test organism was stabbed in the butt and streaked on the slant. The tubes were incubated at 37^0C for 24 hours. Black coloration of butt was indicative of H_2S formation. The change in colour of butt, slant and gas formation was also noted and recorded as alkali/alkali, alkali/acid and acid/acid for the growth of fermenters and all sugar fermenters.

9. Urease Test

Urease test is carried out to determine the ability of certain microorganism to produce the enzyme urease. The enzyme urease catalyse the breakdown of urea into ammonia and carbondioxide.



$NH_2.CO.NH_2 + H_2O$ $2NH_3 + CO_2$

Medium :– Urea agar base.

Composition:-

Ingredients	gm/liter
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Disodium hydrogen phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH (at 25^0C)	6.8±0.2.

Preparation

Twenty-four grams urea agar base was suspended in 950ml of distilled water. It was boiled to dissolve completely. It was distributed in tubes and sterilized by autoclaving at 10 Lbs pressure (115^0C) for 20 minutes. It was cooled down to 55^0C and aseptically introduced 1-2 drops of sterile 40% urea solution and mixed well. It was then allowed to solidify in slanted position.

Procedure

Fresh culture of test organism was streaked heavily on the slant and incubated at 37^0C for overnight. Change in colour of medium to pink indicates positive test and no change in colour indicate negative test.