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SCHOOL OF ARTS AND SCIENCE

MICROBIOLOGY

LABORATORY MANUAL

1. STUDY OF MICROBIOLOGY LABORATORY EQUIPMENT AND BIOSAFETY PRACTICES

AIM

To study the commonly used microbiology laboratory equipment and understand the basic biosafety practices followed in a microbiology laboratory.

PRINCIPLE

A microbiology laboratory is equipped with specialized instruments and equipment used for the cultivation, observation, identification, and preservation of microorganisms. Proper use of laboratory equipment ensures accurate experimental results, while adherence to biosafety practices minimizes the risk of contamination, laboratory-acquired infections, and environmental hazards. Biosafety refers to the safe handling, storage, and disposal of infectious materials to protect laboratory personnel, the community, and the environment. Most teaching microbiology laboratories follow Biosafety Level 1 (BSL-1) or Biosafety Level 2 (BSL-2) guidelines depending on the microorganisms handled. Strict aseptic techniques, proper personal protective equipment (PPE), and appropriate waste disposal are essential components of laboratory biosafety.

MATERIALS REQUIRED

Equipment: Compound microscope, Laminar Air Flow Chamber, Autoclave, Hot Air Oven, Incubator, BOD Incubator, Spectrophotometer/Colorimeter, Centrifuge, pH meter, Water bath, Colony counter, Analytical balance, Refrigerator/Deep freezer, Bunsen burner/Spirit lamp.

Glassware and Plasticware: Petri plates, Test tubes, Conical flasks, Beakers, Measuring cylinders, Pipettes, Micropipettes with sterile tips, Glass slides and cover slips, Durham tubes, 70% Ethanol, Sodium hypochlorite solution (Bleach).

PROCEDURE

A. Study of Laboratory Equipment

Observe the following laboratory instruments and record their function.

Equipment	Function
Compound Microscope	Observation of microorganisms
Laminar Air Flow Chamber	Provides aseptic working environment
Autoclave	Sterilization using steam under pressure (121°C, 15 psi)
Hot Air Oven	Sterilization of glassware by dry heat

Incubator	Maintains optimum temperature for microbial growth
Spectrophotometer	Measures optical density (OD) of microbial cultures
Centrifuge	Separates cells and particles by centrifugal force
pH Meter	Measures pH of culture media
Water Bath	Maintains constant temperature for reactions
Analytical Balance	Accurate weighing of chemicals
Colony Counter	Counts bacterial colonies on agar plates
Refrigerator/Freezer	Storage of media, reagents, and cultures

B. Biosafety Practices

1. Wear a clean laboratory coat, gloves, and closed footwear before entering the laboratory.
2. Wash hands thoroughly before and after practical work.
3. Disinfect the workbench with 70% ethanol before and after the experiment.
4. Sterilize inoculating loops and needles by flaming before and after use.
5. Perform culture transfers inside a Laminar Air Flow Chamber whenever possible.
6. Label all culture tubes, Petri plates, and reagents properly.
7. Avoid eating, drinking, or using mobile phones inside the laboratory.
8. Never pipette by mouth; always use micropipettes or pipette aids.
9. Dispose of contaminated materials in designated biohazard waste containers.
10. Autoclave infectious waste before disposal.
11. Report spills, accidents, or injuries immediately to the laboratory instructor.
12. Wash hands thoroughly after completing the experiment.

OBSERVATION

RESULT

2. DEMONSTRATION OF PHYSICAL AND CHEMICAL METHODS OF STERILIZATION

AIM

To demonstrate the physical and chemical methods of sterilization used in microbiology laboratories for the destruction of microorganisms and maintenance of aseptic conditions.

PRINCIPLE

Sterilization destroys microorganisms by denaturing proteins, damaging nucleic acids, disrupting cell membranes, or physically removing microorganisms. The method selected depends on the nature of the material and its resistance to heat or chemicals.

MATERIALS REQUIRED

Equipment: Autoclave, Hot Air Oven, Bunsen Burner/Spirit Lamp, Laminar Air Flow Chamber, Membrane Filtration Unit, UV Lamp, Incubator, **Reagents:** 70% Ethanol, Isopropyl alcohol, Sodium hypochlorite (Bleach), Phenol, Formaldehyde solution (Demonstration only), Glutaraldehyde solution, Hydrogen peroxide, Distilled water, Petri plates, Test tubes, Glass pipettes, Culture media, Syringe filter (0.22 µm), Cotton plugs, Aluminium foil.

PROCEDURE

A. Physical Methods of Sterilization

1. Moist Heat Sterilization (Autoclave)

- i. Arrange culture media and glassware inside the autoclave.
- ii. Close the autoclave lid securely.
- iii. Sterilize at **121°C, 15 psi pressure for 15–20 minutes**.
- iv. Allow the pressure to return to zero before opening the lid.
- v. Remove sterilized materials carefully.

2. Dry Heat Sterilization (Hot Air Oven)

- i. Wrap clean glassware in aluminium foil or kraft paper.
- ii. Arrange the glassware inside the hot air oven.
- iii. Sterilize at **160°C for 2 hours** or **170°C for 1 hour**.
- iv. Allow the oven to cool before removing the materials.

3. Flame Sterilization

- i. Hold the inoculating loop or needle in the blue flame of a Bunsen burner.
- ii. Heat until the wire becomes red hot.
- iii. Allow it to cool before use.

4. Filtration Sterilization

- i. Assemble a sterile membrane filtration unit fitted with a **0.22 µm membrane filter**.
- ii. Pass the heat-sensitive liquid through the filter using vacuum or syringe pressure.
- iii. Collect the sterile filtrate in a sterile container.

5. Radiation Sterilization (Demonstration)

- i. Expose laboratory work surfaces to **ultraviolet (UV) light** inside a laminar airflow cabinet for **15–30 minutes** before use.
- ii. Gamma irradiation is used industrially for sterilizing disposable medical equipment (demonstration only).

B. Chemical Methods of Sterilization

1. Alcohol Sterilization

- i. Clean the laboratory benches and instruments with **70% ethanol**.
- ii. Allow the alcohol to evaporate completely.

2. Sodium Hypochlorite Sterilization

- i. Prepare the recommended bleach solution.
- ii. Apply to contaminated laboratory surfaces.
- iii. Allow sufficient contact time before cleaning.

3. Glutaraldehyde Sterilization

- i. Immerse heat-sensitive instruments in glutaraldehyde solution.
- ii. Leave for the recommended exposure time.
- iii. Rinse thoroughly with sterile water before use.

4. Hydrogen Peroxide Sterilization

- i. Apply hydrogen peroxide to laboratory surfaces or instruments.
- ii. Allow adequate contact time before use.

5. Formaldehyde Sterilization (Demonstration)

- i. Formaldehyde gas is used for fumigation of laboratory rooms or biosafety cabinets by trained personnel under controlled conditions.

OBSERVATION

RESULT

3. HANDLING AND OPERATION OF COMPOUND MICROSCOPE

AIM

To study the parts, handling, and operation of a compound microscope for observing microorganisms.

PRINCIPLE

A compound microscope uses visible light and two sets of lenses to magnify microorganisms. Light passes through the specimen and is focused by the objective lens to produce a magnified image, which is further enlarged by the eyepiece lens.

Total Magnification = Eyepiece Magnification × Objective Magnification

MATERIALS REQUIRED

Compound microscope, Prepared slides (bacterial smear, fungal smear, or stained specimen), Lens cleaning paper, Immersion oil, Light source (built-in illuminator or mirror), Lens cleaning solution, Tissue paper,

PROCEDURE

A. Identification of Microscope Parts

- i. Observe the different parts of the compound microscope.
- ii. Identify the eyepiece, body tube, revolving nosepiece, objective lenses, stage, stage clips, condenser, iris diaphragm, coarse adjustment knob, fine adjustment knob, illuminator, arm, and base.

B. Handling the Microscope

- i. Carry the microscope with both hands, one holding the arm and the other supporting the base.
- ii. Place the microscope on a clean, flat, and stable laboratory bench.
- iii. Clean the eyepiece and objective lenses using lens paper only.

C. Focusing the Specimen

- i. Place the prepared slide on the stage and secure it with the stage clips.
- ii. Select the 10× objective lens using the revolving nosepiece.
- iii. Adjust the light using the condenser and iris diaphragm.
- iv. Looking from the side, raise the stage close to the objective lens.
- v. Look through the eyepiece and slowly lower the stage using the coarse adjustment knob until the image appears.
- vi. Sharpen the image using the fine adjustment knob.
- vii. Rotate the nosepiece to the 40× objective and refocus using only the fine adjustment knob.

viii. For the 100× oil immersion objective, place one drop of immersion oil on the stained smear.

ix. Rotate the oil immersion objective into position and focus using only the fine adjustment knob.

x. After observation, clean the oil immersion lens carefully using lens paper.

OBSERVATION

RESULT

4. PREPARATION AND STERILIZATION OF GLASSWARE AND CULTURE MEDIA

AIM

To prepare microbiological culture media and sterilize glassware and media using appropriate sterilization techniques.

PRINCIPLE

Culture media are prepared by dissolving dehydrated media in distilled water and adjusting the pH to the desired level. Sterilization destroys all microorganisms, including bacterial spores.

- Moist heat sterilization (Autoclave): 121°C, 15 psi, 15–20 minutes
- Dry heat sterilization (Hot Air Oven): 160°C for 2 hours or 170°C for 1 hour

MATERIALS REQUIRED

Nutrient Agar, Nutrient Broth, Distilled water, 1 N Hydrochloric acid (HCl), 1 N Sodium hydroxide (NaOH), Analytical balance, pH meter, Hot plate or magnetic stirrer, Autoclave, Hot air oven, Laminar Air Flow Chamber, Incubator, Conical flask, Beaker, Measuring cylinder, Test tubes, Petri plates, Glass rod, Cotton plugs, Aluminium foil.

PROCEDURE

A. Preparation of Nutrient Agar

- i. Weigh 28 g of Nutrient Agar powder and transfer it into a 1 L conical flask.
- ii. Add 1000 mL of distilled water.
- iii. Heat gently with continuous stirring until the medium dissolves completely.
- iv. Adjust the pH to 7.2 ± 0.2 using 1 N HCl or 1 N NaOH, if required.
- v. Dispense the medium into culture tubes or conical flasks.
- vi. Plug the tubes with cotton or close with screw caps.

B. Preparation of Nutrient Broth

- i. Weigh 13 g of Nutrient Broth powder.
- ii. Dissolve it in 1000 mL distilled water.
- iii. Adjust the pH to 7.2 ± 0.2 .
- iv. Dispense into culture tubes or conical flasks.

C. Sterilization of Glassware (Dry Heat Sterilization)

- i. Wash glassware thoroughly with detergent and rinse with tap water followed by distilled water.
- ii. Dry the glassware completely.

- iii. Wrap Petri plates, pipettes, flasks, and other glassware with aluminium foil or kraft paper.
- iv. Arrange the wrapped glassware inside the hot air oven.
- v. Sterilize at 160°C for 2 hours or 170°C for 1 hour.
- vi. Allow the oven to cool before removing the sterilized glassware.

D. Sterilization of Culture Media (Moist Heat Sterilization)

- i. Arrange the prepared media inside the autoclave.
- ii. Ensure that sufficient water is present in the autoclave.
- iii. Close the autoclave lid securely.
- iv. Sterilize at 121°C, 15 psi pressure for 15–20 minutes.
- v. Allow the pressure to return to zero before opening the lid.
- vi. Remove the sterilized media carefully.

E. Pouring Sterile Agar Plates

- i. Allow the sterilized nutrient agar to cool to 45–50°C.
- ii. Transfer the medium into a Laminar Air Flow Chamber.
- iii. Pour 15–20 mL of molten agar into each sterile Petri plate.
- iv. Allow the agar to solidify.
- v. Store the plates in an inverted position until use.

OBSERVATION

RESULT

5. SERIAL DILUTION TECHNIQUE, POUR PLATE, SPREAD PLATE AND STREAK PLATE METHODS

AIM

To perform serial dilution, pour plate, spread plate and streak plate methods for the isolation, enumeration and purification of bacteria.

PRINCIPLE

Isolation of microorganisms is one of the fundamental techniques in microbiology. Pure cultures are essential for studying the morphological, physiological and biochemical characteristics of microorganisms. Mixed microbial populations can be separated into individual colonies using various plating techniques. Serial dilution is used to reduce the concentration of microorganisms in a sample so that isolated colonies can be obtained after plating. In the pour plate method, a measured volume of diluted bacterial suspension is mixed with sterile molten nutrient agar and allowed to solidify. Colonies develop both within the agar medium and on its surface. This method is commonly used for viable bacterial counts (CFU/mL). In the spread plate method, a small volume of diluted bacterial suspension is spread evenly over the surface of solidified nutrient agar using a sterile L-shaped glass spreader. Since the inoculum remains on the surface, all colonies develop only on the agar surface, making them easy to observe and count. The streak plate method is a mechanical dilution technique used for obtaining pure cultures. By successively streaking the inoculum over different sectors of the agar plate with a sterilized inoculating loop, the bacterial cells become progressively separated, resulting in isolated colonies suitable for further study. These techniques are widely used in clinical, food, environmental and industrial microbiology laboratories for bacterial isolation, purification and enumeration.

MATERIALS REQUIRED

18–24-hour-old bacterial culture (*Escherichia coli* or any bacterial culture), Sterile Nutrient Agar, Sterile Normal Saline (0.85%), Distilled Water, 70% Ethanol, Sterile Petri plates, Test tubes (9 mL dilution blanks), Micropipettes and sterile tips, Sterile L-shaped glass spreader, Laminar Air Flow Chamber, Incubator (35–37°C), Water bath (45–50°C), Colony Counter.

PROCEDURE

A. Serial Dilution Technique

1. Label sterile dilution tubes as 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} .
2. Add 9 mL of sterile normal saline into each tube.

3. Transfer 1 mL of the bacterial culture into the first tube (10^{-1}) and mix thoroughly.
4. Transfer 1 mL from the 10^{-1} tube into the 10^{-2} tube and mix well.
5. Repeat the procedure sequentially until the desired dilution is obtained.

B. Pour Plate Method

1. Pipette 1 mL of the selected dilution into a sterile Petri plate.
2. Pour 15–20 mL of sterile molten nutrient agar cooled to 45–50°C into the plate.
3. Gently swirl the plate in clockwise and anticlockwise directions to mix the inoculum evenly.
4. Allow the agar to solidify.
5. Incubate the plates in an inverted position at 35–37°C for 24–48 hours.
6. Count colonies on plates containing 30–300 colonies and calculate CFU/mL

C. Spread Plate Method

1. Transfer 0.1 mL of the selected dilution onto the surface of a sterile nutrient agar plate.
2. Sterilize the L-shaped glass spreader by dipping it in alcohol and flaming.
3. Allow the spreader to cool.
4. Spread the inoculum evenly over the agar surface while rotating the plate.
5. Allow the inoculum to absorb into the medium.
6. Incubate the plate in an inverted position at 35–37°C for 24–48 hours.
7. Count colonies and calculate CFU/mL

D. Streak Plate Method

1. Sterilize the inoculating loop by flaming until red hot and allow it to cool.
2. Pick a loopful of bacterial culture.
3. Streak the first quadrant of the nutrient agar plate.
4. Flame and cool the loop.
5. Drag the loop through the edge of the first quadrant into the second quadrant.
6. Repeat the procedure for the third and fourth quadrants.
7. Incubate the plate in an inverted position at 35–37°C for 24 hours.
8. Observe isolated colonies in the final quadrants.

OBSERVATION

RESULT

6. AEROBIC AND ANAEROBIC CULTURE TECHNIQUES

AIM

To study the cultivation of microorganisms under aerobic and anaerobic conditions using appropriate culture techniques.

PRINCIPLE

Microorganisms differ in their oxygen requirements. Aerobic bacteria require atmospheric oxygen for growth and use oxygen as the terminal electron acceptor during respiration. Anaerobic bacteria grow only in the absence of oxygen because oxygen is toxic to them. Facultative anaerobes can grow in both aerobic and anaerobic conditions, whereas microaerophiles require reduced oxygen concentrations. Aerobic bacteria are cultured under normal atmospheric conditions, while anaerobic bacteria are cultivated in oxygen-free environments using methods such as the GasPak anaerobic jar or thioglycollate broth, which creates an oxygen gradient to differentiate bacterial oxygen requirements.

MATERIALS REQUIRED

Cultures – (*Escherichia coli*, *Bacillus subtilis*, *Clostridium perfringens* / *Clostridium sporogenes*), Nutrient Agar, Nutrient Broth, Thioglycollate Broth, Anaerobic GasPak sachet.

PROCEDURE

A. Aerobic Culture Technique

1. Prepare sterile nutrient agar plates.
2. Inoculate the agar surface with the test organism using the streak plate method.
3. Incubate the plates at **35–37°C for 24–48 hours** under normal atmospheric conditions.
4. Observe colony morphology and bacterial growth.

B. Anaerobic Culture Technique (GasPak Jar Method)

1. Prepare sterile nutrient agar plates.
2. Inoculate the plates with the test organism.
3. Place the inoculated plates inside the anaerobic jar.
4. Add a GasPak sachet according to the manufacturer's instructions.
5. Seal the anaerobic jar tightly.

6. Incubate at **35–37°C for 24–48 hours**.
7. Observe bacterial growth after incubation.

C. Thioglycollate Broth Method (Demonstration)

1. Inoculate sterile thioglycollate broth with the test organism.
2. Incubate at **35–37°C for 24–48 hours**.
3. Observe the location of bacterial growth in the broth.

OBSERVATION

RESULT

7. PRESERVATION OF MICROBIAL CULTURES

AIM

To study the various methods used for the preservation of microbial cultures for short-term and long-term maintenance.

PRINCIPLE

Microbial culture preservation is essential for maintaining the viability, purity, and genetic stability of microorganisms for future use. Preservation methods reduce the metabolic activity of microorganisms by lowering the temperature, reducing oxygen availability, removing moisture, or using cryoprotective agents such as glycerol. The selection of a preservation method depends on the storage duration and the type of microorganism. Agar slants and agar stabs are commonly used for short-term preservation, whereas glycerol stock preservation and lyophilization (freeze-drying) are suitable for long-term storage.

MATERIALS REQUIRED

Escherichia coli, Sterile Nutrient Agar, Sterile Nutrient Broth, Sterile 20% Glycerol, Distilled Water, 70% Ethanol, Sterile Cryovials, Agar Slant Tubes, Inoculating Loop, Inoculating Needle, Micropipettes.

PROCEDURE

A. Preservation by Agar Slant Method

1. Prepare sterile nutrient agar slants.
2. Sterilize the inoculating loop by flaming and allow it to cool.
3. Pick a loopful of pure bacterial culture.
4. Streak the culture gently over the surface of the agar slant in a zig-zag pattern.
5. Flame the mouth of the tube and close the cap.
6. Incubate at 35–37°C for 18–24 hours.
7. Store the culture at 4°C for short-term preservation.

B. Preservation by Agar Stab Method

1. Prepare sterile nutrient agar deep tubes.
2. Sterilize the inoculating needle by flaming.
3. Pick a small amount of bacterial culture.
4. Insert the needle vertically into the centre of the agar to about two-thirds of its depth.
5. Withdraw the needle carefully along the same path.

6. Flame the mouth of the tube and replace the cap.
7. Incubate at 35–37°C for 18–24 hours.
8. Store at 4°C.

C. Preservation by Glycerol Stock Method

1. Prepare sterile 20% glycerol solution.
2. Transfer 0.8 mL of overnight bacterial broth culture into a sterile cryovial.
3. Add 0.2 mL sterile glycerol to obtain a final glycerol concentration of approximately 20%.
4. Mix gently by inversion.
5. Label the cryovial with the organism's name and date.
6. Store at –20°C for medium-term storage or –80°C for long-term storage.

D. Demonstration of Lyophilization (Freeze-Drying)

1. Prepare a pure bacterial suspension.
2. Freeze the culture rapidly.
3. Subject the frozen culture to vacuum drying (lyophilization).
4. Seal the dried culture in sterile ampoules.
5. Store at room temperature or under refrigerated conditions.

OBSERVATION

RESULT

8. OBSERVATION OF MICROBIAL MORPHOLOGY

AIM

To observe the morphology of microorganisms using prepared slides under a compound microscope.

PRINCIPLE

Microbial morphology refers to the shape, size, arrangement, and structural characteristics of microorganisms. Prepared slides containing stained microorganisms are examined under a compound microscope to study these characteristics. Bacteria exhibit different shapes such as cocci, bacilli, vibrio, and spirilla with characteristic arrangements, while fungi possess structures such as hyphae, mycelium, conidia, sporangia, and spores. Bacterial morphology is best observed under the 100× oil immersion objective, whereas fungal structures are observed under 10× and 40× objectives.

MATERIALS REQUIRED

Bacterial slides, Fungal slides, Compound microscope, Immersion oil, Lens cleaning paper, Lens cleaning solution

PROCEDURE

A. Observation of Bacterial Morphology

1. Switch on the compound microscope and adjust the light.
2. Place the prepared bacterial slide on the microscope stage.
3. Focus the specimen under the 10× objective.
4. Observe the specimen under the 40× objective.
5. Place one drop of immersion oil on the stained smear.
6. Rotate the 100× oil immersion objective into position.
7. Focus using the fine adjustment knob.
8. Observe and record the bacterial shape and arrangement.

B. Observation of Fungal Morphology

1. Place the prepared fungal slide on the microscope stage.
2. Focus under the 10× objective.
3. Observe the specimen under the 40× objective.
4. Identify fungal structures such as hyphae, mycelium, conidia, spores, or sporangia.
5. Draw neat labelled diagrams of the observed structures.

OBSERVATION & RESULT

9. STAINING TECHNIQUES – SIMPLE STAINING, GRAM STAINING, SPORE STAINING AND NEGATIVE STAINING

AIM

To study and perform different bacterial staining techniques, namely Simple Staining, Gram Staining, Spore Staining, and Negative Staining, for observing bacterial morphology and cellular structures.

PRINCIPLE

Staining is an essential microbiological technique used to increase the contrast between microorganisms and the background, making them visible under a microscope. Basic dyes such as methylene blue, crystal violet, and safranin carry a positive charge and bind to negatively charged bacterial cells, whereas acidic dyes such as nigrosin or India ink stain the background without penetrating the cells. Different staining methods help in observing bacterial morphology, differentiating Gram-positive and Gram-negative bacteria, detecting endospores, and determining the true size and shape of bacterial cells.

MATERIALS REQUIRED

Cultures (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*), Crystal Violet, Gram's Iodine, 95% Ethanol, Safranin, Methylene Blue, Malachite Green, Nigrosin (or India Ink), Distilled Water, Inoculating Loop, Immersion Oil.

PROCEDURE

A. Simple Staining

1. Prepare a thin bacterial smear on a clean grease-free slide.
2. Air dry and heat-fix the smear.
3. Flood the smear with Methylene Blue for 1 minute.
4. Wash gently with water.
5. Blot dry using blotting paper.
6. Observe under the 100× oil immersion objective.

B. Gram Staining

1. Prepare a thin bacterial smear and heat-fix.
2. Flood with Crystal Violet for 1 minute.
3. Wash with water.
4. Add Gram's Iodine for 1 minute.

5. Wash with water.
6. Decolorize with 95% Ethanol for 30–60 seconds.
7. Wash immediately with water.
8. Counterstain with Safranin for 40–60 seconds.
9. Wash, blot dry and observe under the 100× oil immersion objective.

C. Spore Staining (Schaeffer–Fulton Method)

1. Prepare and heat-fix a bacterial smear.
2. Place the slide over steaming water.
3. Flood the smear with Malachite Green.
4. Steam for 5–10 minutes, keeping the stain moist.
5. Allow the slide to cool.
6. Wash with distilled water.
7. Counterstain with Safranin for 1 minute.
8. Wash, blot dry and observe under the 100× oil immersion objective.

D. Negative Staining

1. Place a drop of Nigrosin (or India Ink) near one end of a clean slide.
2. Mix a loopful of bacterial culture with the stain.
3. Using another clean slide held at 45°, spread the stain to form a thin smear.
4. Allow the smear to air dry only (do not heat-fix).
5. Observe under the 100× oil immersion objective.

OBSERVATION

RESULT

10. DEMONSTRATION OF BACTERIAL MOTILITY

AIM

To demonstrate bacterial motility using the Hanging Drop Method and Soft Agar Method.

PRINCIPLE

Bacterial motility is the ability of bacteria to move independently, usually by means of flagella. Motility is an important characteristic used in the identification and classification of bacteria. In the Hanging Drop Method, living bacterial cells are observed microscopically for true directional movement. In the Soft Agar Method, motile bacteria migrate away from the line of inoculation through semi-solid agar, whereas non-motile bacteria remain confined to the stab line.

MATERIALS REQUIRED

Cultures (*Escherichia coli*, *Klebsiella pneumoniae*), Soft Motility Agar (0.3–0.5% agar), Petroleum Jelly (Vaseline), Distilled Water, Cavity Slide, Cover Slip, Test Tubes containing Soft Agar, Inoculating Needle, Inoculating Loop.

PROCEDURE

A. Hanging Drop Method

1. Clean a cavity (depression) slide and cover slip.
2. Apply a small amount of petroleum jelly to the four corners of the cover slip.
3. Place a small drop of fresh bacterial broth culture at the centre of the cover slip.
4. Carefully place the cavity slide over the cover slip so that the depression surrounds the drop.
5. Invert the preparation so that the drop hangs freely into the cavity.
6. Observe under the 10× objective followed by the 40× objective.
7. Record the type of bacterial movement observed.

B. Soft Agar (Motility Agar) Method

1. Label sterile soft motility agar tubes.
2. Sterilize the inoculating needle by flaming and allow it to cool.
3. Pick a small amount of bacterial culture.
4. Stab the centre of the soft agar vertically to about two-thirds of its depth.
5. Incubate the tubes at 35–37°C for 24–48 hours.
6. Observe the bacterial growth pattern.

OBSERVATION & RESULT

11. EXAMINATION OF MITOSIS IN ONION ROOT TIP

AIM

To study the different stages of mitosis in onion (*Allium cepa*) root tip cells using the squash technique.

PRINCIPLE

Mitosis is an equational type of cell division in which a parent cell divides to produce two genetically identical daughter cells, maintaining the same chromosome number. It is responsible for growth, repair and replacement of worn-out cells in living organisms. The onion root tip is an ideal material for studying mitosis because its apical meristem contains actively dividing cells. The root tips are treated with dilute hydrochloric acid to soften the tissues and stained with Acetocarmine or Aceto-orcein stain to make the chromosomes clearly visible. The stained root tip is gently squashed on a glass slide so that individual cells spread into a single layer. Under the compound microscope, different stages of mitosis such as Interphase, Prophase, Metaphase, Anaphase and Telophase can be observed.

MATERIALS REQUIRED

Specimen: Fresh onion (*Allium cepa*) roots (1–2 cm long), **Reagents:** 1 N Hydrochloric acid (HCl), 2% Acetocarmine or Aceto-orcein stain, Distilled water, Forceps, Scalpel/Blade, Glass slides, Cover slips, Watch glass, Dropper, Needle, Filter paper and Tissue paper.

PROCEDURE:

1. Cut 1–2 cm long fresh onion root tips.
2. Place the root tips in 1 N HCl and warm at 60°C for 5 minutes to soften the tissues.
3. Wash the root tips thoroughly with distilled water.
4. Cut approximately 2–3 mm from the tip (meristematic region).
5. Place the root tip on a clean glass slide.
6. Add 1–2 drops of Acetocarmine (or Aceto-orcein) stain.
7. Allow the stain to act for 5–10 minutes.
8. Place a clean cover slip gently over the stained root tip.
9. Cover the slide with filter paper and gently press (squash) using the thumb or the blunt end of a pencil to spread the cells evenly.
10. Observe first under the 10× objective and then under the 40× objective of the compound microscope.

OBSERVATION & RESULT

12. EXAMINATION OF PLASMOLYSIS AND DEPLASMOLYSIS

AIM

To examine the process of plasmolysis and deplasmolysis in plant cells using the epidermal peel of *Rhoeo discolor* (*Tradescantia*) or onion (*Allium cepa*).

PRINCIPLE

Plasmolysis is the shrinkage of the protoplast away from the cell wall due to the loss of water when living plant cells are placed in a hypertonic solution. Water moves out of the cell by osmosis through the selectively permeable plasma membrane, causing the cytoplasm to contract. Deplasmolysis is the reverse process in which plasmolysed cells regain water and return to their normal turgid condition when transferred to distilled water (hypotonic solution). This experiment demonstrates the movement of water across the plasma membrane by osmosis.

MATERIALS REQUIRED

Specimen: Fresh onion bulb (*Allium cepa*) or *Rhoeo discolor* leaf, Reagents: 10% Sodium chloride (NaCl) solution (or 20% sucrose solution), Distilled water, Glass slides, Cover slips, Watch glass, Dropper, Forceps, Needle, Blade, Filter paper and Tissue paper.

PROCEDURE

A. Observation of Plasmolysis

1. Peel a thin epidermal layer from the inner surface of a fresh onion scale leaf (or use a peel from *Rhoeo discolor*).
2. Place the peel on a clean glass slide.
3. Add 2–3 drops of 10% sodium chloride solution.
4. Place a cover slip gently over the peel without trapping air bubbles.
5. Allow the preparation to stand for 5–10 minutes.
6. Observe the specimen under the light microscope.
7. Note the shrinkage of the protoplast away from the cell wall.

B. Observation of Deplasmolysis

1. Remove the sodium chloride solution from one side of the cover slip using filter paper.
2. Add 2–3 drops of distilled water from the opposite side while continuing to absorb the salt solution.
3. Allow the preparation to stand for 5–10 minutes.
4. Observe the slide under the microscope.

OBSERVATION & RESULT

13. EXAMINATION OF MITOSIS IN ONION ROOT TIP

AIM

To study the different stages of mitosis in onion (*Allium cepa*) root tip cells using the squash technique.

PRINCIPLE

Mitosis is an equational type of cell division in which one parent cell divides into two genetically identical daughter cells while maintaining the chromosome number. The apical meristem of onion root tips contains actively dividing cells, making it an ideal material for studying mitosis. The root tips are treated with dilute hydrochloric acid to soften the tissues, stained with acetocarmine (or aceto-orcein), and squashed gently on a glass slide. Under the compound microscope, chromosomes become clearly visible and the different stages of mitosis - Interphase, Prophase, Metaphase, Anaphase, and Telophase can be observed.

MATERIALS REQUIRED

Specimen: Fresh onion (*Allium cepa*) root tips (1–2 cm long), 1 N Hydrochloric acid, 2% Acetocarmine (or Aceto-orcein), Distilled water, Forceps, Scalpel/Blade, Glass slides, Cover slips, Dropper, Filter paper, Needle.

PROCEDURE

1. Cut 1–2 cm long fresh onion root tips.
2. Place the root tips in 1 N HCl and warm at 60°C for about 5 minutes.
3. Wash thoroughly with distilled water.
4. Cut 2–3 mm from the tip (meristematic region).
5. Place the root tip on a clean glass slide.
6. Add one or two drops of acetocarmine stain.
7. Leave for 5–10 minutes.
8. Place a cover slip carefully.
9. Cover with filter paper and gently squash the tissue.
10. Observe under 10× and then 40× objective.

OBSERVATION

RESULT

14. SEPARATION OF PHOTOSYNTHETIC PIGMENTS BY PAPER CHROMATOGRAPHY

AIM

To separate the photosynthetic pigments, present in green leaves using the paper chromatography technique.

PRINCIPLE

Photosynthetic pigments such as chlorophyll **a**, chlorophyll **b**, xanthophyll, and carotene differ in their solubility in the solvent and their adsorption to the chromatography paper. In paper chromatography, the solvent (mobile phase) moves upward through the chromatography paper (stationary phase) by capillary action, carrying the pigments at different rates. Highly soluble pigments move farther, whereas less soluble pigments remain closer to the origin.

The movement of each pigment is expressed by its Retention Factor (Rf).

Formula: $R_f = \text{Distance travelled by pigment} / \text{Distance travelled by solvent front}$

MATERIALS REQUIRED

Specimen: Fresh spinach leaves or other green leaves, Reagents: 80–90% Acetone, Petroleum ether, Chromatography solvent (Petroleum ether:Acetone = 9:1), Whatman No. 1 chromatography paper, Capillary tube, Beaker, Test tube, Measuring cylinder, Glass rod, Forceps, Ruler.

PROCEDURE

A. Preparation of Pigment Extract

1. Wash fresh spinach leaves thoroughly.
2. Cut the leaves into small pieces.
3. Grind the leaves with 80–90% acetone using a mortar and pestle until a dark green extract is obtained.
4. Filter the extract through filter paper into a clean test tube.

B. Preparation of Chromatography Paper

1. Cut a strip of Whatman No.1 chromatography paper (approximately 2 cm × 15 cm).
2. Draw a light pencil line about 2 cm from one end.
3. Using a capillary tube, apply a small drop of pigment extract on the pencil line.

4. Allow the spot to dry and repeat 3–4 times to obtain a concentrated spot.

C. Chromatographic Separation

1. Pour the chromatography solvent into the chamber to a depth of about 1 cm.
2. Suspend the chromatography paper vertically so that only the lower end touches the solvent.
3. Ensure the pigment spot remains above the solvent level.
4. Close the chamber tightly.
5. Allow the solvent to rise until it reaches about 2 cm below the top of the paper.
6. Remove the paper and immediately mark the solvent front with a pencil.
7. Dry the chromatogram.
8. Measure the distance travelled by each pigment and the solvent front.
9. Calculate the **Rf value** of each pigment.

CALCULATION

$R_f = \text{Distance travelled by pigment} / \text{Distance travelled by solvent front}$

OBSERVATION

RESULT

15. CELL COUNTING USING A HAEMOCYTOMETER

AIM

To determine the number of cells, present in a microbial or yeast suspension using a haemocytometer (Neubauer counting chamber).

PRINCIPLE

A haemocytometer is a specialized microscope slide with a precisely ruled counting chamber used to determine the number of cells present in a known volume of liquid. The Neubauer counting chamber has a depth of **0.1 mm** and contains engraved grids of known dimensions. A known volume of cell suspension is introduced into the chamber, and the cells present within selected squares are counted under a microscope. Since the chamber volume is known, the concentration of cells in the original sample can be calculated.

Formula: $\text{Cells/mL} = \text{Average cell count} \times \text{Dilution Factor} \times 10^4$

where 10^4 is the Neubauer chamber conversion factor.

MATERIALS REQUIRED

Specimen: Yeast suspension (*Saccharomyces cerevisiae*) or bacterial culture, Reagents: Sterile normal saline (0.85%), Methylene blue or Trypan blue, Micropipette with sterile tips, Test tubes, Lens cleaning paper, Tissue paper

PROCEDURE

A. Preparation of Cell Suspension

1. Mix the microbial culture thoroughly to obtain a uniform suspension.
2. Dilute the sample with sterile normal saline if necessary.
3. Mix the diluted suspension gently.

B. Loading the Haemocytometer

1. Clean the haemocytometer and coverslip with lens paper.
2. Place the special coverslip over the counting chamber.
3. Using a micropipette, place 10–20 μL of the cell suspension at the edge of the coverslip.
4. Allow the chamber to fill by capillary action without introducing air bubbles.
5. Leave the chamber undisturbed for 1–2 minutes to allow the cells to settle.

C. Cell Counting

1. Place the haemocytometer on the microscope stage.
2. Focus the counting grid under the **10× objective**.
3. Count the cells under the **40× objective**.
4. Count the cells in the **four corner squares and the central square**.
5. Include cells touching the **top and left boundary lines** and exclude cells touching the **bottom and right boundary lines**.
6. Calculate the average number of cells counted.
7. Determine the cell concentration using the formula.

CALCULATION

Formula: $\text{Cells/mL} = \text{Average Cell Count} \times \text{Dilution Factor} \times 10^4$

OBSERVATION

RESULT

16. PREPARATION OF CULTURE MEDIA (LIQUID & SOLID) FOR BACTERIAL CULTIVATION

AIM

To prepare general-purpose liquid (nutrient broth) and solid (nutrient agar) culture media for the cultivation of different bacterial species.

PRINCIPLE

Culture media are nutrient preparations used for the growth, isolation, cultivation, maintenance, and preservation of microorganisms under laboratory conditions. A suitable culture medium provides essential nutrients such as carbon, nitrogen, minerals, vitamins, and water required for microbial growth. Culture media may be liquid (broth) or solid. Liquid media do not contain a solidifying agent and are mainly used for propagating bacteria in large numbers. Solid media are prepared by adding agar (1.5–2.0%), an inert polysaccharide obtained from seaweed, which provides a firm surface for the growth of isolated bacterial colonies. Semi-solid media contain less than 1% agar and are commonly used for motility studies. Nutrient broth and nutrient agar are general-purpose media widely used for the cultivation of non-fastidious bacteria. They contain peptone, which supplies amino acids and nitrogen, beef extract, which provides vitamins and growth factors, and sodium chloride, which maintains osmotic balance. The pH of the medium is adjusted to 7.0–7.3, which is suitable for the growth of most bacteria. After preparation, the media are sterilized in an autoclave at 121°C (15 psi) for 15–20 minutes to destroy all microorganisms and spores. Sterile nutrient agar is poured into Petri plates or tubes to prepare agar plates, slants, and deep tubes. The prepared media can then be inoculated with bacterial cultures such as *Escherichia coli*, *Bacillus*, *Pseudomonas*, and *Staphylococcus*, and incubated to observe bacterial growth.

MATERIALS REQUIRED

Microorganisms - *Escherichia coli*, *Bacillus* spp., *Pseudomonas* spp., *Staphylococcus* spp.,

Reagents: Peptone, Beef extract, Sodium chloride, Agar, Distilled water, Conical flask, Measuring cylinder, Petri plates, Test tubes.

PROCEDURE

1. Weigh the required quantities of peptone, beef extract, and sodium chloride.
2. Dissolve the ingredients in approximately 500 mL of distilled water with continuous stirring.
3. Make up the volume to 1000 mL using distilled water and adjust the pH to 7.0–7.3.

4. Dispense the nutrient broth into suitable flasks or test tubes and sterilize by autoclaving at 121°C (15 psi) for 15–20 minutes.
5. For the preparation of nutrient agar, add 15 g agar to the nutrient broth and heat gently until the agar dissolves completely.
6. Dispense the medium into flasks and sterilize by autoclaving.
7. Cool the sterilized nutrient agar to about 45–50°C and pour aseptically into sterile Petri plates or test tubes to prepare agar plates, slants, and deep tubes.
8. Allow the medium to solidify. For agar slants, keep the tubes in an inclined position until the agar hardens.
9. Inoculate the prepared nutrient agar plates, agar slants, and nutrient broth with bacterial cultures using aseptic techniques.
10. Incubate all cultures at 37°C for 24 hours.
11. Observe the bacterial growth on solid media as colonies and in broth as turbidity.

OBSERVATION

RESULT

17. OBSERVATION OF FUNGAL STRUCTURES USING LACTOPHENOL COTTON BLUE (LPCB) STAINING

AIM

To observe the morphological structures of fungi using Lactophenol Cotton Blue (LPCB) staining under a compound microscope.

PRINCIPLE

Lactophenol Cotton Blue (LPCB) staining is a standard microbiological technique used for the microscopic examination and identification of fungi. The stain acts as both a mounting medium and a staining reagent, allowing clear visualization of fungal structures without significant distortion. The different components of LPCB perform specific functions:

- Lactic acid preserves and clears the fungal structures.
- Phenol kills the fungal cells, preventing contamination and ensuring safe handling.
- Cotton Blue stains the chitin present in the fungal cell wall, making fungal structures appear blue under the microscope.
- Glycerol acts as a hygroscopic agent, preventing the preparation from drying and maintaining the fungal morphology.

Using LPCB staining, important fungal structures such as hyphae, conidia, conidiophores, sporangia, sporangiophores, spores, rhizoids, and budding yeast cells can be observed. The morphology of these structures is useful for the identification of medically and industrially important fungi including *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, and *Candida* species.

MATERIALS REQUIRED

Fungal cultures: *Aspergillus niger*, *Penicillium* spp., *Rhizopus stolonifer*, *Mucor* spp., *Candida albicans*. Reagents: Lactophenol Cotton Blue (LPCB) stain, Glass slides, Cover slips, Inoculating needle or teasing needle, Forceps.

PROCEDURE

1. Place a drop of Lactophenol Cotton Blue (LPCB) stain in the center of a clean grease-free glass slide.
2. Using a sterile inoculating or teasing needle, pick a small portion of fungal mycelium from the edge of a young fungal colony.
3. Transfer the fungal material into the drop of LPCB stain.
4. Tease the fungal mycelium gently with sterile needles to separate the hyphae and reproductive structures.

5. Carefully place a clean cover slip over the preparation without trapping air bubbles.
6. Remove excess stain using tissue paper.
7. Observe the preparation first under the 10× objective and then under the 40× objective of the compound microscope.
8. Record the observed fungal structures such as hyphae, conidiophores, conidia, sporangiophores, sporangia, spores, and rhizoids.

OBSERVATION

RESULT

18. PRESERVATION OF MICROBIAL CULTURES

AIM

To study the preservation of microbial cultures using agar slant, agar stab, and glycerol stock methods for short-term and long-term maintenance.

PRINCIPLE

Microbial cultures must be preserved to maintain their viability, purity, and genetic stability for future laboratory use. Preservation prevents contamination, minimizes genetic variation, and retains the physiological and biochemical characteristics of microorganisms during storage. The choice of preservation method depends on the duration of storage and the type of microorganism. Agar slant cultures are commonly used for short-term preservation because they provide a large surface area for microbial growth and can be stored under refrigeration. Agar stab (deep) cultures reduce microbial metabolic activity by limiting oxygen availability and are suitable for preserving motile and anaerobic bacteria for short- to intermediate-term storage. For long-term preservation, glycerol stock cultures are widely used. Glycerol acts as a cryoprotectant, preventing the formation of intracellular ice crystals that can damage microbial cells during freezing. Glycerol stocks are stored at -20°C or -80°C , where microorganisms remain viable for several months to years while maintaining their genetic and physiological characteristics. Strict aseptic techniques must be followed during preservation to ensure that cultures remain pure and uncontaminated.

MATERIALS REQUIRED

Pure culture (18–24-hour-old) of *Escherichia coli* or any bacterial culture, Sterile Nutrient Agar, Sterile Nutrient Broth, Sterile Glycerol (80% stock or sterile 40% glycerol), 70% Ethanol, Cryovials, Test tubes, Inoculation loop, inoculating needle, Micropipettes & tips, etc.,

PROCEDURE

A. Preservation by Agar Slant Method

1. Prepare sterile nutrient agar slants and allow them to solidify in an inclined position.
2. Sterilize the inoculating loop by flaming until red hot and allow it to cool.
3. Transfer a loopful of pure microbial culture onto the agar slant.
4. Flame the mouth of the culture tube before and after inoculation.
5. Streak the culture gently in a zig-zag pattern over the surface of the slant.
6. Close the tube and incubate at $35\text{--}37^{\circ}\text{C}$ for 18–24 hours.
7. After adequate growth, store the culture at 4°C for short-term preservation.

B. Preservation by Agar Stab Method

1. Prepare sterile nutrient agar deep tubes.
2. Sterilize the inoculating needle by flaming and allow it to cool.
3. Pick a small amount of pure microbial culture.
4. Flame the mouth of the agar deep tube.
5. Insert the inoculating needle vertically into the center of the agar to about two-thirds of its depth.
6. Withdraw the needle carefully along the same stab line.
7. Flame the mouth of the tube and replace the cap.
8. Incubate at 35–37°C for 18–24 hours.
9. Store the stab culture at 4°C for preservation.

C. Preservation by Glycerol Stock Method

1. Prepare a sterile 20% glycerol solution or mix equal volumes of sterile 40% glycerol and bacterial culture to obtain a final concentration of 20% glycerol.
2. Transfer 0.8 mL of an overnight bacterial broth culture into a sterile cryovial.
3. Add 0.2 mL of sterile glycerol solution.
4. Mix gently by pipetting or gentle inversion. Do not vortex vigorously.
5. Label the cryovial with the organism name, culture number, and date.
6. Store the glycerol stock at –20°C for medium-term storage or –80°C for long-term preservation.

OBSERVATION

RESULT

19. ENUMERATION OF BACTERIA – VIABLE COUNT (PLATE COUNT) AND TOTAL COUNT (HAEMOCYTOMETER COUNT)

AIM

To determine the number of bacteria, present in a sample by the Viable Plate Count Method and Total Count using a Haemocytometer.

PRINCIPLE

Enumeration of microorganisms is an important microbiological technique used to determine the concentration of bacteria in a given sample. Accurate bacterial counting is essential in clinical microbiology, food microbiology, environmental studies, pharmaceutical industries, and research laboratories. The Viable Plate Count Method estimates the number of living bacteria capable of multiplying and forming visible colonies on a solid nutrient medium. Each colony is assumed to arise from a single viable bacterial cell or a group of cells and is expressed as Colony Forming Units per millilitre (CFU/mL). Since highly concentrated bacterial cultures cannot be counted directly, serial dilution is performed before plating to obtain countable colonies (30–300 colonies per plate). The Haemocytometer Count Method is a direct microscopic counting technique that determines the total number of bacterial cells present in a known volume of suspension. The haemocytometer consists of a counting chamber of fixed dimensions that allows calculation of the total number of cells per millilitre. This method counts both living and dead cells and therefore provides the total bacterial count rather than viable count. Comparison of these two methods helps distinguish between the total bacterial population and the viable population in a sample.

MATERIALS REQUIRED

18–24-hour-old bacterial broth culture, Normal Saline (0.85%), Nutrient Agar, Distilled Water, 70% Ethanol, Petri plates, Test tubes, Cover slip, Micropipettes & tips, L- rod, Inoculating loop.

PROCEDURE

A. Viable Count (Plate Count Method)

1. Prepare a series of 10-fold serial dilutions of the bacterial culture using sterile normal saline.
2. Label sterile nutrient agar plates according to the dilution.
3. Transfer 0.1 mL of the selected dilution onto the surface of a sterile nutrient agar plate.
4. Spread the inoculum evenly using a sterile L-shaped glass spreader.

5. Allow the inoculum to absorb completely into the agar.
6. Incubate the plates in an inverted position at 35–37°C for 24–48 hours.
7. Select plates containing 30–300 colonies.
8. Count the colonies using a colony counter or manually.
9. Calculate the viable bacterial count using the formula:

$$\text{CFU/mL} = \text{Number of colonies} \times \text{Dilution Factor} / \text{Volume plated}$$

B. Total Count (Haemocytometer Count Method)

1. Clean the haemocytometer and special cover slip with 70% ethanol.
2. Place the cover slip over the counting chamber.
3. Mix the bacterial suspension thoroughly.
4. Using a micropipette, introduce a small drop of the suspension into the haemocytometer chamber.
5. Avoid the formation of air bubbles.
6. Allow the cells to settle for 1–2 minutes.
7. Observe under the 40× objective (or oil immersion if required).
8. Count the bacterial cells present in five large squares.
9. Calculate the total bacterial count using the standard haemocytometer formula:

$$\text{CFU/mL} = \text{Average Count} \times \text{Dilution Factor} \times 10^4$$

OBSERVATION

RESULT

20. DETERMINATION OF BACTERIAL GROWTH CURVE

TURBIDOMETRIC METHOD

AIM

To study the various phases of the bacterial growth curve using the Turbidometric Method and to determine the generation time of bacteria.

PRINCIPLE

Bacterial growth is the increase in the number of bacterial cells through binary fission under favourable environmental conditions. When bacteria are inoculated into a fresh nutrient medium, they undergo a characteristic sequence of growth phases collectively known as the bacterial growth curve. This growth curve is obtained by plotting the increase in bacterial population or cell mass against time. The bacterial growth curve consists of four phases:

- **Lag Phase:** Bacteria adapt to the new environment, synthesize enzymes, and prepare for active cell division. There is little or no increase in cell number.
- **Log (Exponential) Phase:** Cells divide at a constant and maximum rate. The bacterial population doubles at regular intervals, and the generation time can be calculated during this phase.
- **Stationary Phase:** Nutrient depletion and accumulation of toxic metabolic products slow cell division. The rate of cell growth equals the rate of cell death, resulting in a constant population.
- **Death (Decline) Phase:** Cells die at a faster rate than new cells are produced because of nutrient exhaustion and toxic waste accumulation.

The turbidometric method estimates bacterial growth by measuring the turbidity (cloudiness) of a bacterial suspension using a colorimeter or spectrophotometer at 600 nm (OD600). As bacterial cells multiply, the culture becomes increasingly turbid, scattering more light and increasing the optical density (OD). Optical density is directly proportional to bacterial cell concentration within a certain range. The growth curve obtained from OD measurements allows the determination of the bacterial growth phases and the calculation of generation time, which is the time required for the bacterial population to double during the exponential phase.

MATERIALS REQUIRED

Escherichia coli, Nutrient Broth, 250 mL Erlenmeyer flask, Pipettes, Petri plates, inoculating loop, inoculating needle.

PROCEDURE

1. Dispense 100 mL of sterile nutrient broth into a 250 mL Erlenmeyer (side-arm) flask.
2. Sterilize the medium by autoclaving at 121°C for 15–20 minutes.
3. Allow the medium to cool and inoculate with 5–10% actively growing *E. coli* culture under aseptic conditions.
4. Mix the culture thoroughly.
5. Measure the initial Optical Density (OD600) using a colorimeter or spectrophotometer, using uninoculated nutrient broth as the blank.
6. Incubate the culture in a shaking incubator at 37°C and 150 rpm.
7. Measure the OD600 at 30-minute intervals for 24–48 hours or until the optical density becomes constant.
8. Record all OD readings.
9. Plot a graph of Optical Density (OD600) versus Time (hours).
10. Identify the lag, log, stationary, and death phases from the growth curve.
11. Calculate the generation time during the exponential phase.

CALCULATION OF GENERATION TIME

The **generation time (g)** is calculated during the logarithmic phase using the formula: $g = t/n$

Where,

- **g** = Generation time (minutes)
- **t** = Time interval during exponential growth
- **n** = Number of generations

The number of generations is calculated as: $n = \log N - \log N_0 / \log 2$

Where,

- **N₀** = Initial bacterial population (or OD)
- **N** = Final bacterial population (or OD)

Since **log 2 = 0.301**,

$$n = \log N - \log N_0 / 0.301$$

OBSERVATION

RESULT

21. ESTIMATION OF GENERATION TIME AND GROWTH RATE OF BACTERIA

AIM

To determine the generation time (doubling time) and growth rate constant of bacteria by measuring bacterial growth at regular time intervals.

PRINCIPLE

Bacteria reproduce by binary fission, in which one bacterial cell divides into two genetically identical daughter cells. Under favourable environmental conditions, bacterial cells multiply exponentially during the logarithmic (log) phase of growth. During this phase, the rate of increase in bacterial population is directly proportional to the number of cells present. The generation time (g) is the time required for one bacterial cell to divide into two daughter cells or for the bacterial population to double. The growth rate constant (k) represents the number of generations occurring per unit time. These parameters are important for comparing the growth characteristics of different microorganisms under standardized conditions. Bacterial growth can be monitored by measuring the optical density (OD₆₀₀) using a spectrophotometer or colorimeter, or by determining viable or total cell counts. A graph of optical density against time is plotted to identify the logarithmic phase, from which the number of generations, generation time, and growth rate constant are calculated.

MATERIALS REQUIRED

18–24-hour-old culture of *Escherichia coli*, Nutrient Broth, Normal Saline, Distilled Water, 250 mL conical flask, Test tubes, Cuvettes, Micropipettes & tips, Inoculating loop.

PROCEDURE

1. Prepare 100 mL of sterile nutrient broth in a sterile 250 mL conical flask.
2. Inoculate the broth with 1 mL of an overnight bacterial culture under aseptic conditions.
3. Incubate the culture at 37°C in a shaking incubator.
4. Measure the Optical Density (OD₆₀₀) at 30-minute intervals for 3–4 hours using a spectrophotometer or colorimeter.
5. Record the OD values at each time interval.
6. Plot a graph of Optical Density (Y-axis) against Time (X-axis).
7. Identify the logarithmic (log) phase of bacterial growth.
8. Calculate the number of generations (n).
9. Calculate the generation time (g).

10. Calculate the growth rate constant (k).

CALCULATIONS

Number of Generations (n) $n = \log N - \log N_0 / \log 2$

Generation Time (g) $g = t/n$

Growth Rate Constant (k) $k = n/t$

Where,

- N_0 = Initial number of bacterial cells
- N = Final number of bacterial cells
- t = Time (minutes or hours)
- n = Number of generations
- g = Generation time
- k = Growth rate constant

OBSERVATION

RESULT

22. EFFECT OF TEMPERATURE, pH AND SALINITY ON BACTERIAL GROWTH

AIM

To study the effect of temperature, pH, and salinity (NaCl concentration) on the growth of bacteria.

PRINCIPLE

The growth of bacteria is greatly influenced by environmental factors such as temperature, pH, and salinity. Each bacterial species has an optimum range for these factors at which growth is maximum. Deviation from these optimum conditions reduces metabolic activity, enzyme function, and cell multiplication, thereby affecting bacterial growth.

- **Temperature** influences enzyme activity, membrane fluidity, and metabolic reactions. Most pathogenic bacteria such as *Escherichia coli* grow best at **37°C**.
- **pH** affects enzyme activity, nutrient transport, and cellular metabolism. Most bacteria prefer a **neutral pH (around 7.0)** for optimum growth.
- **Salinity (NaCl concentration)** affects osmotic pressure and water availability. Excessive salt concentration causes plasmolysis and inhibits bacterial growth, whereas moderate salt concentrations may support optimum growth in some bacteria.

Bacterial growth under different environmental conditions can be assessed by observing turbidity in nutrient broth or by measuring Optical Density (OD₆₀₀) using a spectrophotometer.

MATERIALS REQUIRED

18–24-hour-old culture of *Escherichia coli* or *Bacillus subtilis*, Nutrient Broth, Hydrochloric Acid, Sodium Hydroxide, Sodium Chloride, Distilled Water, Test tubes, Conical flasks, Measuring cylinder, Micropipettes & tips, Inoculating loop.

PROCEDURE

A. Effect of Temperature

1. Label four sterile nutrient broth tubes.
2. Inoculate each tube with an equal volume of bacterial culture.
3. Incubate the tubes at different temperatures: 4°C, 25°C, 37°C, 50°C
4. Incubate for 24 hours.
5. Observe turbidity or measure OD₆₀₀ using a spectrophotometer.
6. Record the observations.

B. Effect of pH

1. Prepare nutrient broth adjusted to different pH values: pH 5, pH 7, pH 9
2. Sterilize the media.
3. Inoculate each tube with equal amounts of bacterial culture.
4. Incubate at 37°C for 24 hours.
5. Observe turbidity or measure OD₆₀₀.
6. Record the observations.

C. Effect of Salinity

1. Prepare nutrient broth containing different concentrations of sodium chloride: 0% NaCl, 2% NaCl, 5% NaCl, 10% NaCl
2. Sterilize the media.
3. Inoculate each tube with equal amounts of bacterial culture.
4. Incubate at 37°C for 24 hours.
5. Observe turbidity or measure OD₆₀₀.
6. Record the observations.

OBSERVATION

RESULT

23. CARBOHYDRATE FERMENTATION TESTS

AIM

To determine the ability of bacteria to ferment different carbohydrates by detecting acid and gas production.

PRINCIPLE

Carbohydrate fermentation tests are used to determine the ability of bacteria to ferment specific carbohydrates such as glucose, lactose, sucrose, mannitol, and maltose. During fermentation, bacteria produce organic acids, resulting in a decrease in the pH of the medium. The pH change is detected by the indicator phenol red, which changes from red to yellow under acidic conditions. Some bacteria also produce gas (CO₂ and H₂) during fermentation. Gas production is detected using a Durham tube, which collects gas released during fermentation. The carbohydrate fermentation test is widely used in the identification and differentiation of bacteria, especially members of the family Enterobacteriaceae.

MATERIALS REQUIRED

Escherichia coli, *Salmonella Typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, etc.,

Reagents: Phenol Red Carbohydrate Broths containing: Glucose, Lactose, Sucrose, Mannitol, Maltose, Distilled water, Test tubes, Durham tubes, Inoculating loop.

PROCEDURE

1. Label the carbohydrate fermentation tubes containing different sugars.
2. Ensure that each tube contains an inverted Durham tube.
3. Sterilize the inoculating loop by flaming and allow it to cool.
4. Inoculate each carbohydrate broth with a loopful of the test bacterial culture.
5. Incubate the tubes at 35–37°C for 24–48 hours.
6. Observe the colour of the medium and the presence or absence of gas in the Durham tube.
7. Record the observations.

OBSERVATION

RESULT

24. DETECTION OF ENZYMATIC ACTIVITIES RELATED TO MICROBIAL METABOLISM (CATALASE AND OXIDASE TESTS)

AIM

To detect the catalase and oxidase enzyme activities in bacteria for their identification and differentiation.

PRINCIPLE

Bacteria produce various enzymes that play important roles in cellular metabolism. Detection of these enzymes helps in the identification and differentiation of bacterial species. The Catalase Test detects the presence of the enzyme catalase, which breaks down hydrogen peroxide (H_2O_2), a toxic by-product of aerobic metabolism, into water and oxygen. The release of oxygen appears as immediate bubble formation, indicating a positive catalase reaction. The Oxidase Test detects the presence of the enzyme cytochrome c oxidase, an important component of the bacterial electron transport chain. The enzyme oxidizes the oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride), producing a dark purple colour within a few seconds. These tests are routinely used in clinical and diagnostic microbiology for the identification of Gram-positive and Gram-negative bacteria.

MATERIALS REQUIRED

Bacterial cultures - *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, 3% Hydrogen Peroxide, Oxidase reagent (1% Tetramethyl-p-phenylenediamine dihydrochloride), Glass slides, Filter paper, Petri plates, Inoculating loop, Wooden applicator stick.

PROCEDURE

A. Catalase Test

1. Place a clean, dry glass slide on the laboratory bench.
2. Using a sterile inoculating loop, transfer a small amount of the bacterial colony onto the slide.
3. Add 1–2 drops of 3% hydrogen peroxide (H_2O_2) onto the bacterial smear.
4. Observe immediately for bubble formation.

B. Oxidase Test

1. Place a piece of clean filter paper in a Petri plate.
2. Add 2–3 drops of oxidase reagent to moisten the filter paper.

3. Using a sterile wooden applicator stick or platinum loop, transfer a bacterial colony onto the reagent-soaked filter paper.
4. Observe for colour change within 10–30 seconds.

OBSERVATION

RESULT

25. CULTURE TECHNIQUES USED TO ENUMERATE MICROORGANISMS

AIM

To study the culture techniques used for the enumeration (counting) of microorganisms in a given sample.

PRINCIPLE

Enumeration of microorganisms is the process of determining the number of viable microorganisms present in a sample. This is based on the principle that each viable microbial cell, when cultured under suitable conditions, multiplies to form a visible colony known as a Colony Forming Unit (CFU). By using appropriate culture techniques, the number of microorganisms in a sample can be estimated accurately. The commonly used techniques include the Spread Plate Method, Pour Plate Method, and Serial Dilution Method.

MATERIALS REQUIRED

Sterile Petri plates, Nutrient agar, test tubes, pipettes/micropipette, Micropipette tips, L-shaped glass spreader, normal saline/distilled water.

PROCEDURE

A. Serial Dilution

1. Label sterile test tubes containing 9 mL of sterile saline as 10^{-1} , 10^{-2} , 10^{-3} , etc.
2. Transfer 1 mL of the sample into the first tube (10^{-1}) and mix well.
3. Transfer 1 mL from the first tube to the second tube (10^{-2}).
4. Repeat the process to obtain the required dilutions.

B. Spread Plate Method

1. Pipette 0.1 mL of the selected diluted sample onto the surface of agar plate.
2. Spread the inoculum evenly using a sterile L-shaped spreader.
3. Incubate the plates at 37°C for 24–48 hours.
4. Count the colonies formed using a colony counter.

C. Pour Plate Method

1. Transfer 1 mL of the diluted sample into a sterile Petri plate.
2. Pour about 15–20 mL of molten nutrient agar ($45\text{--}50^{\circ}\text{C}$) into the plate.
3. Mix gently by rotating the plate, Allow the agar to solidify.
4. Incubate at 37°C for 24–48 hours.
5. Count both surface and subsurface colonies.

OBSERVATION & RESULT

26. IDENTIFICATION OF BACTERIAL PATHOGENS BY THEIR BIOCHEMICAL REACTIONS

AIM

To identify bacterial pathogens based on their biochemical reactions using standard biochemical tests.

PRINCIPLE

Bacteria possess different enzymes that enable them to utilize carbohydrates, proteins, amino acids, and other substrates in different ways. These metabolic activities produce characteristic biochemical reactions that help distinguish one bacterial species from another. By performing a series of biochemical tests and comparing the results with standard identification charts, bacterial pathogens can be identified accurately.

MATERIALS REQUIRED

Pure bacterial culture, Nutrient agar culture, Inoculating loop, test tubes, Catalase reagent (3% H₂O₂), Oxidase reagent, SIM medium, Triple Sugar Iron (TSI) agar, Simmons Citrate agar, Urea agar, MR-VP broth, Peptone water, Kovac's reagent, Methyl Red reagent, Voges-Proskauer reagents A & B.

PROCEDURE

1. Catalase Test

- Place a drop of 3% hydrogen peroxide on a clean slide.
- Using a sterile loop, transfer a small amount of bacterial culture into the drop.
- Observe immediately for bubble formation.

2. Oxidase Test

- Place a drop of oxidase reagent on filter paper.
- Smear the bacterial colony onto the reagent.
- Observe for color change within 30 seconds.

3. Indole Test

- Inoculate peptone water with the bacterial culture.
- Incubate at 37°C for 24–48 hours.
- Add a few drops of Kovac's reagent.
- Observe for a red ring at the surface.

4. Methyl Red (MR) Test

- Inoculate MR-VP broth and incubate.

- Add Methyl Red reagent.
- Observe for red color formation.

5. Voges-Proskauer (VP) Test

- Use the same MR-VP broth after incubation.
- Add VP reagents A and B.
- Shake gently and allow to stand for 15–30 minutes.
- Observe for pink/red color.

6. Citrate Test

- Streak the bacterial culture on Simmons Citrate agar.
- Incubate at 37°C for 24–48 hours.
- Observe for color change from green to blue.

7. Urease Test

- Streak the organism on Urea agar slant.
- Incubate at 37°C.
- Observe for pink color.

8. Triple Sugar Iron (TSI) Test

- Stab the butt and streak the slant of the TSI agar.
- Incubate at 37°C for 24 hours.
- Observe sugar fermentation, gas production, and H₂S production.

OBSERVATION

RESULT

27. ANTIBACTERIAL SUSCEPTIBILITY TESTING BY DISC DIFFUSION TECHNIQUE AND DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

AIM

To determine the susceptibility of bacteria to antimicrobial agents by the Kirby–Bauer disc diffusion method and to determine the Minimum Inhibitory Concentration (MIC) of an antibiotic against a bacterial isolate.

PRINCIPLE

Antibacterial susceptibility testing is performed to determine the effectiveness of antibiotics against bacterial pathogens. In the Kirby–Bauer disc diffusion method, antibiotic-impregnated discs are placed on the surface of Mueller-Hinton agar inoculated with the test organism. During incubation, the antibiotic diffuses into the agar and inhibits bacterial growth around the disc. The diameter of the zone of inhibition is measured and interpreted as Sensitive (S), Intermediate (I), or Resistant (R) according to standard guidelines. The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that completely inhibits the visible growth of a microorganism after incubation. MIC helps clinicians select the appropriate antibiotic and dosage for effective treatment.

MATERIALS REQUIRED

Pure bacterial culture, Mueller-Hinton Agar, Mueller-Hinton Broth, Sterile cotton swabs, test tubes, Micropipette and sterile tips, Antibiotic discs, Antibiotic stock solution, Sterile saline (0.85%), McFarland turbidity standard (0.5), Forceps, etc.,

PROCEDURE

A. Disc Diffusion (Kirby–Bauer) Method

1. Prepare a bacterial suspension equivalent to 0.5 McFarland standard.
2. Dip a sterile cotton swab into the suspension.
3. Spread the inoculum evenly over the entire surface of a Mueller-Hinton agar plate.
4. Allow the surface to dry for 3–5 minutes.
5. Place antibiotic discs evenly on the agar using sterile forceps.
6. Gently press each disc to ensure complete contact with the agar.

7. Incubate the plate at 37°C for 18–24 hours.
8. Measure the diameter of the zone of inhibition around each antibiotic disc in millimeters.

B. Determination of MIC (Broth Dilution Method)

1. Prepare a series of two-fold dilutions of the antibiotic in Mueller-Hinton broth.
2. Add an equal volume of standardized bacterial suspension to each tube.
3. Include one positive control (broth + bacteria) and one negative control.
4. Incubate all tubes at 37°C for 18–24 hours.
5. Observe each tube for visible bacterial growth (turbidity).

OBSERVATION & RESULT

28. COLLECTION AND TRANSPORT OF CLINICAL SPECIMENS

AIM

To study the correct methods for the collection, labeling, storage, and transportation of clinical specimens for microbiological examination to ensure accurate laboratory diagnosis.

PRINCIPLE

Proper collection and transportation of clinical specimens are essential for the isolation and identification of disease-causing microorganisms. The specimen should be collected aseptically from the site of infection before antimicrobial therapy begins. Appropriate sterile containers and transport media should be used to preserve the viability of microorganisms and prevent contamination. Delays in transportation or improper storage may result in the death of pathogens or overgrowth of contaminants, leading to inaccurate laboratory results.

MATERIALS REQUIRED

Sterile cotton swabs, Sterile specimen containers, Blood collection tubes (EDTA, plain, citrate), Syringes and needles, Sterile urine collection container, Sputum collection container, Stool collection container, Transport media (Stuart's medium, Amies medium, Cary-Blair medium).

PROCEDURE

A. General Guidelines

1. Wash hands and wear appropriate PPE.
2. Explain the procedure to the patient.
3. Collect the specimen aseptically before antibiotic therapy whenever possible.
4. Collect an adequate quantity of specimen.
5. Avoid contamination with normal flora.
6. Label the specimen with: Patient name, Age and sex, Hospital/Registration number, Date and time of collection, Type of specimen
7. Complete the laboratory request form.
8. Transport the specimen immediately to the microbiology laboratory.

B. Collection of Common Clinical Specimens

1. Blood

- Clean the venipuncture site with 70% alcohol followed by povidone-iodine.
- Collect 5–10 mL of blood using sterile technique.

- Transfer into blood culture bottles.
- Send immediately to the laboratory.

2. Urine

- Collect midstream urine in a sterile container.
- Avoid touching the inside of the container.
- Transport within 2 hours or refrigerate at 4°C if delayed.

3. Sputum

- Instruct the patient to rinse the mouth with water.
- Collect an early morning deep cough specimen.
- Avoid saliva contamination.
- Send immediately.

4. Stool

- Collect freshly passed stool in a sterile container.
- Avoid contamination with urine.
- If delay exceeds 2 hours, use Cary-Blair transport medium.

5. Throat Swab

- Use a sterile swab.
- Swab the inflamed area and tonsils without touching the tongue or cheeks.
- Place the swab in Stuart's or Amies transport medium.

6. Wound Swab

- Remove surface debris.
- Collect pus or exudate using a sterile swab.
- Place in transport medium.

C. Transport of Specimens

- Use appropriate transport media when immediate processing is not possible.
- Maintain recommended temperature:
 - Most specimens: Room temperature
 - CSF: Immediately transport; do not refrigerate
 - Urine: Refrigerate at 4°C if delayed
 - Viral specimens: Cold chain (2–8°C) or frozen as recommended
- Deliver to the laboratory as soon as possible.

OBSERVATION

RESULT

29. ISOLATION OF BACTERIOPHAGES FROM SEWAGE AND OTHER NATURAL SOURCES

AIM

To isolate bacteriophages from sewage and other natural sources by enrichment and plaque assay techniques using a susceptible bacterial host.

PRINCIPLE

Bacteriophages are viruses that specifically infect bacterial cells and are widely distributed in environments where bacteria are abundant, such as sewage, river water, pond water, soil, and wastewater. Sewage is considered one of the richest sources of bacteriophages because it contains a high concentration of diverse bacterial populations that serve as natural hosts. The isolation of bacteriophages was based on the principle that phages can infect only specific bacterial hosts and multiply within susceptible bacterial cells. During the enrichment process, the environmental sample was mixed with a growing culture of the host bacterium and incubated under suitable conditions, allowing the phages present in the sample to infect, replicate, and increase in number. After incubation, bacterial cells and debris were removed by centrifugation and filtration through a 0.22 μm membrane filter, while the much smaller bacteriophage particles passed through the filter into the filtrate. The presence of bacteriophages was detected by the double-layer agar (plaque assay) technique, in which the filtrate was mixed with fresh host bacteria and soft agar before being poured onto nutrient agar plates. During incubation, each infectious phage particle infected a bacterial cell, multiplied, lysed the host cell, and spread to neighboring cells, producing clear circular zones known as plaques. Each plaque represented the progeny of a single infectious phage particle and was therefore considered a plaque-forming unit (PFU). The number, size, and morphology of plaques provided information about the concentration and characteristics of bacteriophages present in the sample. This method is simple, highly sensitive, and widely used for the isolation, enumeration, purification, and characterization of bacteriophages for microbiological and molecular biology studies.

MATERIALS REQUIRED

Sewage sample/pond water/river water/soil extract, Pure culture of host bacterium (*Escherichia coli*), Nutrient broth, Nutrient agar plates, Soft agar (0.7% agar), Sterile saline, Sterile centrifuge tubes, Sterile conical flask, Micropipettes and sterile tips, Sterile syringe, 0.22 μm membrane filter, Sterile test tubes.

PROCEDURE

1. A fresh sewage sample was collected in a sterile container.
2. The sample was centrifuged at 5,000 rpm for 10 minutes to remove suspended particles.
3. The supernatant was carefully collected and filtered through a sterile 0.22 µm membrane filter to remove bacterial cells.
4. Five millilitres of the filtered sample were mixed with 5 mL of nutrient broth containing a logarithmic-phase culture of *E. coli*.
5. The enrichment culture was incubated at 37°C for 18–24 hours with gentle shaking.
6. After incubation, the enriched culture was centrifuged to remove bacterial debris.
7. The supernatant was again filtered through a sterile 0.22 µm membrane filter to obtain the bacteriophage suspension.
8. One hundred microlitres of the phage filtrate were mixed with 100 µL of fresh host bacterial culture.
9. Approximately 3 mL of molten soft agar maintained at 45°C was added to the mixture.
10. The contents were mixed gently and poured over a nutrient agar plate.
11. The plates were allowed to solidify and incubated in an inverted position at 37°C for 18–24 hours.
12. The plates were examined for the appearance of clear plaques indicating the presence of bacteriophages.

OBSERVATION

RESULT

30. DIRECT EXAMINATION OF FAECES – WET MOUNT

AIM

To perform the direct wet mount examination of a faecal specimen for the detection of intestinal parasites, including trophozoites, cysts, ova, larvae, yeast cells, red blood cells, white blood cells, and other abnormal constituents.

PRINCIPLE

Direct wet mount examination is a rapid and simple microscopic technique used for the preliminary diagnosis of intestinal parasitic infections. A small quantity of fresh faeces is mixed with normal saline and Lugol's iodine on separate glass slides. The saline preparation preserves the natural morphology and motility of trophozoites, larvae, and helminth eggs, while the iodine preparation stains glycogen and nuclear structures of protozoan cysts, making them easier to identify. The method provides immediate information regarding the presence of parasites, inflammatory cells, mucus, and other abnormal elements. Fresh specimens should have been examined promptly to ensure accurate observation of motile organisms.

MATERIALS REQUIRED

Fresh faecal specimen, Clean glass slides, Cover slips, Applicator sticks, Normal saline (0.85%), Lugol's iodine solution, Dropper, etc.,

PROCEDURE

1. A clean glass slide was taken and labelled.
2. One drop of normal saline was placed on one end of the slide.
3. One drop of Lugol's iodine was placed on the other end of the same slide.
4. A small amount of fresh faeces was picked up using an applicator stick.
5. The specimen was mixed thoroughly with the saline to prepare a thin suspension.
6. Another small portion of the specimen was mixed with iodine to prepare a second thin smear.
7. Cover slips were carefully placed over both preparations without trapping air bubbles.
8. The preparations were examined under the low-power (10×) objective.
9. Suspicious structures were further examined under the high-power (40×) objective.
10. The presence of ova, cysts, trophozoites, larvae, yeast cells, red blood cells, white blood cells, mucus, and undigested food particles was observed and recorded.

OBSERVATION & RESULT:

31. DIRECT EXAMINATION OF FAECES – IODINE MOUNT

AIM

To perform the iodine wet mount examination of a faecal specimen for the detection and identification of intestinal protozoan cysts, helminth eggs, larvae, and other faecal constituents under the microscope.

PRINCIPLE

The iodine mount is a simple microscopic technique used for the rapid examination of faecal specimens. A small quantity of stool is mixed with Lugol's iodine solution, which stains glycogen, nuclei, and internal structures of protozoan cysts, thereby enhancing their visibility and facilitating identification. Although iodine kills motile trophozoites, it provides excellent contrast for observing cyst morphology and helminth ova. This method serves as a valuable preliminary diagnostic tool for intestinal parasitic infections and should have been performed using fresh specimens for optimal results.

MATERIALS REQUIRED

Fresh faecal specimen, Clean glass slides, Cover slips, Applicator sticks, Normal saline (0.85%), Lugol's iodine solution, Dropper, etc.,

PROCEDURE

1. A clean glass slide was taken and labelled.
2. One drop of Lugol's iodine solution was placed in the centre of the slide.
3. A small amount of fresh faecal specimen was collected using a sterile applicator stick.
4. The specimen was mixed thoroughly with the iodine solution to prepare a thin, uniform suspension.
5. A cover slip was carefully placed over the preparation without trapping air bubbles.
6. The slide was first examined under the low-power (10×) objective.
7. Areas containing suspicious structures were further examined under the high-power (40×) objective.
8. The morphology of protozoan cysts, helminth eggs, larvae, yeast cells, and other faecal elements was observed and recorded.

OBSERVATION & RESULT

32. DEMONSTRATION OF PROTOZOAN CYSTS AND HELMINTH EGGS

AIM

To demonstrate and identify the morphological characteristics of protozoan cysts and helminth eggs in faecal specimens using light microscopy.

PRINCIPLE

Protozoan cysts and helminth eggs are important diagnostic stages of intestinal parasites that can be detected by microscopic examination of faecal specimens. Protozoan cysts are the infective and resistant forms of protozoa, while helminth eggs are produced by adult worms inhabiting the intestinal tract. These parasitic forms possess characteristic shapes, sizes, nuclei, shells, and internal structures that allow species identification. Wet mount preparations using normal saline and Lugol's iodine, or concentrated stool preparations, enhance visualization of these structures. Microscopic identification plays a vital role in the laboratory diagnosis of parasitic infections and aids in appropriate treatment and epidemiological studies.

MATERIALS REQUIRED

Fresh faecal specimens, Normal saline, Lugol's iodine solution, Glass slides, Cover slips, Applicator sticks, Lens tissue, Gloves, Laboratory record sheet

PROCEDURE

1. Prepared slides containing protozoan cysts and helminth eggs were obtained.
2. The slides were placed on the microscope stage.
3. The preparations were first examined under the low-power (10×) objective.
4. The structures were further observed under the high-power (40×) objective.
5. The characteristic morphology of protozoan cysts and helminth eggs was carefully studied.
6. The size, shape, colour, shell, nuclei, and internal structures were observed.
7. The observed parasites were identified using standard laboratory identification keys.
8. The findings were recorded in the practical record.

OBSERVATION

RESULT

33. CONCENTRATION TECHNIQUES OF STOOL SPECIMEN – FLOTATION METHOD

AIM

To concentrate and demonstrate parasite eggs, larvae, and protozoan cysts present in a stool specimen using the flotation technique for microscopic examination.

PRINCIPLE

The flotation technique is a concentration method used to detect intestinal parasites that are present in small numbers in faecal specimens. It is based on the principle of specific gravity, where parasite eggs and protozoan cysts, having a lower specific gravity than the flotation solution, float to the surface, while heavier faecal debris settles at the bottom. Saturated sodium chloride, zinc sulphate, or saturated sugar solution is commonly used as the flotation medium. The parasites adhering to the cover slip are then transferred to a glass slide and examined microscopically. This technique increases the sensitivity of stool examination, particularly for detecting helminth eggs and protozoan cysts.

MATERIALS REQUIRED

Fresh faecal specimen, Flotation solution (Saturated sodium chloride/Zinc sulphate/Sheather's sugar solution), Test tube or flotation tube, Glass rod or applicator stick, Gauze or wire mesh filter, Funnel, Glass slides, Cover slips, Pasteur pipette or dropper, etc.,

PROCEDURE

1. Approximately 1–2 g of fresh stool specimen was collected in a clean container.
2. Specimen was mixed thoroughly with flotation solution to prepare a uniform suspension.
3. The suspension was filtered through gauze into a clean test tube to remove large debris.
4. Additional flotation solution was added until a convex meniscus was formed at the top of the tube.
5. A clean cover slip was carefully placed over the meniscus.
6. The preparation was allowed to stand for 10–15 minutes.
7. During this period, parasite eggs and cysts floated upward and adhered to the cover slip.
8. The cover slip was gently removed and placed on a clean glass slide.
9. The preparation was examined under the low-power (10×) objective and confirmed under the high-power (40×) objective.
10. The characteristic morphology of parasite eggs and cysts was observed and recorded.

OBSERVATION & RESULT:

34. SEDIMENTATION METHOD

AIM

To concentrate and demonstrate protozoan cysts, helminth eggs, and larvae present in a stool specimen using the sedimentation technique for microscopic examination.

PRINCIPLE

The sedimentation technique is a concentration method based on the principle that parasite eggs, larvae, and protozoan cysts possess a higher specific gravity than the suspending fluid. When the stool suspension is allowed to stand or is centrifuged, the parasitic elements settle at the bottom of the tube, while lighter faecal debris remains in the supernatant. The sediment obtained is then examined microscopically. Formalin–ether (or formalin–ethyl acetate) sedimentation is the most commonly used method because it effectively concentrates a wide range of intestinal parasites while removing fat and debris. This technique is particularly useful for detecting heavy helminth eggs that may not be recovered by flotation methods.

MATERIALS REQUIRED

Fresh faecal specimen, 10% Formalin solution, Ether or Ethyl acetate, Normal saline, Test tubes with stoppers,, Centrifuge, Gauze or wire mesh, Funnel, Glass slides, Cover slips, Pasteur pipette or dropper, Applicator sticks.

PROCEDURE

1. Approximately 1–2 g of fresh stool specimen was collected.
2. The specimen was mixed thoroughly with 10% formalin to prepare a uniform suspension.
3. The suspension was filtered through gauze into a centrifuge tube.
4. Additional formalin was added to make the required volume.
5. Ether (or ethyl acetate) was added to the tube.
6. The tube was tightly stoppered and shaken vigorously.
7. The mixture was centrifuged at 2000–3000 rpm for 2–3 minutes.
8. After centrifugation, four layers were formed.
9. The upper three layers were carefully discarded.
10. The sediment at the bottom of the tube was transferred onto a clean glass slide.
11. A cover slip was placed over the preparation.
12. The slide was examined under low-power (10×) and high-power (40×) objectives.
13. The morphology of parasite eggs, larvae, and protozoan cysts was observed and recorded.

OBSERVATION & RESULT:

35. EXAMINATION OF BLOOD FOR MALARIAL PARASITES THIN AND THICK SMEAR PREPARATIONS

AIM

To prepare, stain, and examine thin and thick blood smears for the detection and identification of malarial parasites under a microscope.

PRINCIPLE

Microscopic examination of stained peripheral blood smears is the gold standard for the laboratory diagnosis of malaria. Two types of blood smears are prepared: thin smear and thick smear. The thin smear preserves the morphology of red blood cells and malaria parasites, allowing accurate identification of *Plasmodium* species. The thick smear contains a larger volume of blood and is more sensitive for detecting low levels of parasitaemia because red blood cells are lysed during staining, leaving the parasites concentrated. Both smears are commonly stained using Giemsa stain, which clearly demonstrates the different developmental stages of *Plasmodium* species.

MATERIALS REQUIRED

Sterile lancet, Alcohol swab, Cotton, Clean glass slides, Giemsa stain, Buffered water (pH 7.2), Methanol, Staining rack, Wash bottle, Immersion oil

PROCEDURE

A. Preparation of Thin Blood Smear

1. The fingertip was cleaned with 70% alcohol and allowed to dry.
2. The finger was pricked using a sterile lancet.
3. A small drop of blood was placed near one end of a clean glass slide.
4. Another slide was used as a spreader at an angle of approximately 30–45°.
5. The blood was spread evenly to produce a thin feather-edged smear.
6. The smear was allowed to air dry.
7. The smear was fixed with methanol for 1–2 minutes.
8. The slide was stained with diluted Giemsa stain for 10–15 minutes.
9. The stained smear was washed with buffered water, air dried, and examined under the oil immersion objective (100×).

B. Preparation of Thick Blood Smear

1. A large drop of blood was placed at the centre of a clean glass slide.
2. The blood was spread gently in a circular motion to form a thick smear of approximately 1–2 cm diameter.
3. The smear was allowed to air dry completely without heat fixation.
4. The thick smear was not fixed with methanol.
5. The smear was stained with diluted Giemsa stain for 20–30 minutes.
6. The slide was washed gently with buffered water and allowed to air dry.
7. The smear was examined under the oil immersion objective for the presence of malarial parasites.

OBSERVATION

RESULT

36. CULTIVATION OF VIRUSES IN EMBRYONATED EGGS

AIM

To demonstrate the cultivation of viruses in embryonated chicken eggs by different inoculation routes such as the chorioallantoic membrane (CAM), allantoic cavity, amniotic cavity, and yolk sac.

PRINCIPLE

Embryonated chicken eggs have been widely used for the isolation, cultivation, and propagation of viruses because they provide sterile, living tissues suitable for viral multiplication. Fertilized eggs aged 5–12 days contain well-developed membranes and cavities that support the growth of different viruses. Each inoculation route offers specific host cells required for the replication of particular viruses. After inoculation, the eggs were incubated under controlled conditions, and viral growth was detected by observing embryo death, pock lesions, hemorrhages, edema, or by harvesting infected fluids. This method has been extensively used for vaccine production, virus isolation, and research.

MATERIALS REQUIRED

Fertilized embryonated chicken eggs (5–12 days old), Sterile syringes and needles, Alcohol (70% ethanol), Iodine solution, Candle lamp/egg candler, Pencil for marking inoculation site, Paraffin wax or adhesive tape, Forceps and scissors, Cotton swabs, etc.,

PROCEDURE

A. Preparation of Eggs

1. Healthy fertilized embryonated eggs were selected.
2. The eggs were candled to locate the embryo and air sac.
3. The inoculation site was marked with a pencil.
4. The shell surface was disinfected using 70% alcohol followed by iodine.
5. A small hole was drilled aseptically over the marked site.

B. Inoculation Routes

1. Chorioallantoic Membrane (CAM) Route

1. The virus suspension was inoculated onto the chorioallantoic membrane.
2. The opening was sealed with melted wax or adhesive tape.
3. The eggs were incubated at **37°C** for 2–5 days.
4. The CAM was examined for pock lesions or hemorrhagic lesions.

2. Allantoic Cavity Route

1. The needle was inserted into the allantoic cavity.
2. The virus suspension was injected carefully.
3. The hole was sealed and the eggs were incubated.
4. The allantoic fluid was harvested after incubation for virus detection.

3. Amniotic Cavity Route

1. The needle was introduced into the amniotic cavity using candling guidance.
2. The viral suspension was injected aseptically.
3. The eggs were sealed and incubated.
4. The embryo and amniotic fluid were examined after incubation.

4. Yolk Sac Route

1. The virus suspension was injected into the yolk sac of young embryos.
2. The inoculation site was sealed.
3. The eggs were incubated for the recommended period.
4. The yolk sac and embryo were examined for evidence of viral growth.

OBSERVATION

RESULT