Bachelor of Science in Medical Laboratory Technology (BMLT)

Microbiology Lab - II

Semester: IV

PRACTICAL LABORATORY MANUAL

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EXPERIMENT- 1

Title: Isolation of micro-organism from various sources like Air, water, and soil

Aim
To isolate microorganisms from different environmental sources such as air, water, and soil using suitable culture techniques.

Principle
Microorganisms are widely present in the environment. When samples from air, water, or soil are inoculated onto nutrient agar medium and incubated under favorable conditions, microorganisms grow and form visible colonies. Each colony represents growth from a single or a group of microorganisms, allowing their isolation.

Requirements
- Nutrient agar plates
- Sterile Petri dishes
- Sterile cotton swabs
- Sterile test tubes
- Sterile distilled water
- Inoculating loop
- Bunsen burner / spirit lamp
- Incubator (37°C)
- Soil sample
- Water sample
- Marker pen

Procedure
A. Isolation of Microorganisms from Air
1. Take a sterile nutrient agar plate.
2. Open the lid slightly and expose the plate to air for 10–15 minutes.
3. Close the lid and label the plate.
4. Incubate the plate at 37°C for 24–48 hours.

B. Isolation of Microorganisms from Water
1. Collect the water sample in a sterile container.
2. Using a sterile inoculating loop, transfer a loopful of water onto a nutrient agar plate.
3. Spread evenly using streaking technique.
4. Label the plate and incubate at 37°C for 24–48 hours.

C. Isolation of Microorganisms from Soil
1. Take 1 gram of soil and add it to 9 ml of sterile distilled water.
2. Mix well to prepare a soil suspension.
3. Using a sterile loop, inoculate the suspension onto a nutrient agar plate.
4. Spread evenly and incubate at 37°C for 24–48 hours.

Observation

After incubation, visible colonies appear on the agar plates:

- Air: Fewer colonies, mostly bacteria and fungal spores.
- Water: Moderate number of bacterial colonies.
- Soil: Maximum number and diversity of colonies.

Colonies vary in size, shape, color, elevation, and margin.

Result

Microorganisms were successfully isolated from air, water, and soil, showing that these sources contain a wide variety of microorganisms.

Precautions

- Maintain strict aseptic conditions.
- Sterilize inoculating loop before and after use.
- Do not open plates unnecessarily.
- Label plates properly.
- Dispose of used materials safely.

Applications

- Environmental microbiology studies
- Water quality analysis
- Understanding microbial distribution
- Basis for further identification of microorganisms

Conclusion

The experiment confirms that air, water, and soil are rich sources of microorganisms, and nutrient agar is suitable for their isolation under laboratory conditions.

EXPERIMENT- 2

Title: Preparation of Pure Culture of Various Gram-Positive and Gram-Negative Bacteria

Aim

To obtain pure cultures of selected Gram-positive and Gram-negative bacteria using the streak plate method.

Principle

A pure culture contains only one type of microorganism. When a mixed culture is streaked over the surface of a solid agar medium, individual bacterial cells get separated and multiply to form isolated colonies. Each isolated colony represents a pure culture. These can be further sub-cultured for identification and study.

Requirements

- Nutrient agar plates
- Sterile Petri dishes
- Inoculating loop
- Bunsen burner / spirit lamp
- Mixed bacterial culture
- Incubator (37°C)
- Marker pen
- Sterile normal saline

Examples of Organisms Used

- Gram-positive bacteria:
    - *Staphylococcus aureus*
    - *Bacillus spp.*
- Gram-negative bacteria:
    - *Escherichia coli*
    - *Pseudomonas aeruginosa*

Procedure

1. Inoculation:

- Take a sterile nutrient agar plate and label it.
- Sterilize the inoculating loop by flaming and allow it to cool.
- Pick a small amount of mixed bacterial culture using the loop.

2. Streak Plate Method:

- Gently streak the culture over one quadrant of the agar plate.
- Sterilize the loop again and streak from the first quadrant into the second.
- Repeat flaming and streaking for the third and fourth quadrants.
- Close the lid and label the plate.

3. Incubation:

- Incubate the plate at 37°C for 24 hours in inverted position.

4. Preparation of Pure Culture:

- After incubation, observe isolated colonies.
- Select a well-isolated colony.
- Transfer it to a fresh nutrient agar slant using a sterile loop.
- Incubate at 37°C for 24 hours to obtain a pure culture.

Observation

- Isolated colonies are seen on the agar plate.
- Gram-positive and Gram-negative bacteria show differences in:
  - Colony size
  - Shape
  - Color
  - Margin
  - Elevation

Result

Pure cultures of Gram-positive and Gram-negative bacteria were successfully prepared using the streak plate method.

Precautions

- Follow strict aseptic techniques.
- Flame inoculating loop before and after use.
- Avoid touching the agar surface unnecessarily.
- Incubate plates in inverted position.
- Handle bacterial cultures carefully.

Applications

- Identification of bacteria
- Gram staining
- Biochemical tests
- Antibiotic sensitivity testing
- Research and teaching purposes

Conclusion

The streak plate method is a simple and effective technique for obtaining pure cultures of both Gram-positive and Gram-negative bacteria, which is essential for microbiological analysis.

EXPERIMENT- 3

Title: To differentiate gram positive and gram-negative bacteria by Gram staining

Aim

To differentiate Gram-positive and Gram-negative bacteria using the Gram staining technique.

Principle

Gram staining is a differential staining technique based on differences in the cell wall structure of bacteria.

- Gram-positive bacteria have a thick peptidoglycan layer, which retains the crystal violet–iodine complex and appears purple/violet.
- Gram-negative bacteria have a thin peptidoglycan layer and an outer lipid membrane. During decolorization, the primary stain is removed, and these bacteria take up the counterstain (safranin), appearing pink/red.

Requirements

- Clean glass slides
- Bacterial culture (Gram-positive and Gram-negative)
- Inoculating loop
- Bunsen burner / spirit lamp
- Crystal violet
- Gram's iodine
- Decolorizer (95% ethanol or acetone-alcohol)
- Safranin
- Distilled water
- Microscope
- Immersion oil

Procedure

➢ Preparation of Smear

1. Take a clean glass slide.
2. Place a drop of sterile water on the slide.
3. Using a sterile loop, pick a small amount of bacterial culture and mix with water.
4. Spread to make a thin smear.
5. Air dry and heat fix the smear gently.

➢ Staining Procedure

1. Flood the smear with crystal violet for 1 minute → rinse with water.
2. Add Gram's iodine for 1 minute → rinse with water.
3. Decolorize with alcohol for 10–20 seconds → rinse immediately.
4. Counterstain with safranin for 30–60 seconds → rinse and air dry.

➢ Microscopic Examination

1. Add a drop of immersion oil.
2. Observe under oil immersion objective (100×).

Observation

Type of Bacteria	Color Observed	Example
Gram-positive	Purple / Violet	*Staphylococcus*, *Bacillus*
Gram-negative	Pink / Red	*E. coli*, *Pseudomonas*

Result

- Gram-positive bacteria appeared purple/violet.
- Gram-negative bacteria appeared pink/red.

Thus, the bacteria were successfully differentiated by Gram staining.

Precautions

- Prepare a thin smear.
- Avoid over-decolorization or under-decolorization.
- Use fresh cultures (18–24 hours old).
- Wash gently to avoid washing off the smear.
- Use clean slides and reagents.

Applications

- Preliminary identification of bacteria
- Selection of appropriate culture media
- Choice of antibiotics
- Routine diagnostic microbiology

Conclusion

Gram staining is a simple and reliable differential staining technique that helps in the identification and classification of bacteria into Gram-positive and Gram-negative groups based on cell wall structure.

EXPERIMENT- 4

Title: Differentiation of Bacteria by Acid-Fast Staining (Ziehl–Neelsen Method)

AIM

To differentiate acid-fast bacteria from non-acid-fast bacteria using the Ziehl–Neelsen (ZN) staining technique.

Principle

Some bacteria (e.g. *Mycobacterium*) possess a waxy cell wall rich in mycolic acid. These bacteria retain the primary stain (carbol fuchsin) even after decolorization with acid-alcohol and are called acid-fast.

Non-acid-fast bacteria lack mycolic acid, lose the primary stain during decolorization, and take up the counterstain (methylene blue).

Requirements

- Clean glass slides
- Bacterial culture / sputum smear
- Inoculating loop
- Bunsen burner
- Carbol fuchsin
- Acid-alcohol (decolorizer)
- Methylene blue
- Distilled water
- Microscope
- Immersion oil

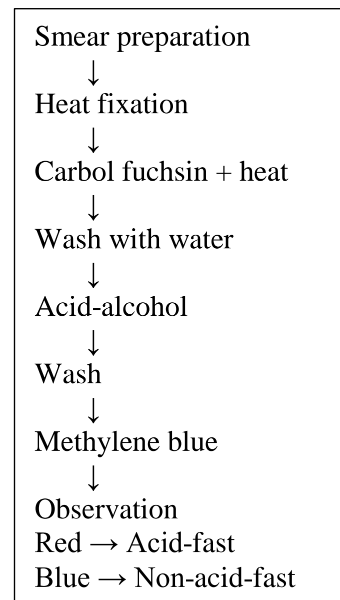
Procedure

➢ Smear Preparation

1. Prepare a thin smear on a clean slide.
2. Air dry and heat fix the smear.

➢ Staining Procedure

1. Flood the smear with carbol fuchsin.
2. Gently heat until steam appears (do not boil).
3. Keep for 5 minutes, adding stain if needed.
4. Wash with water.
5. Decolorize with acid-alcohol for 2–3 minutes.
6. Wash with water.
7. Counterstain with methylene blue for 1 minute.
8. Wash, air dry, and examine under oil immersion (100×).



Observation

Type of Bacteria	Color Observed	Example
Acid-fast	Bright red / pink	*Mycobacterium tuberculosis*
Non-acid-fast	Blue	*E. coli*, *Staphylococcus*

Result

- Acid-fast bacteria appeared red.
- Non-acid-fast bacteria appeared blue.

Thus, bacteria were successfully differentiated using acid-fast staining.

Precautions

- Prepare thin smears.
- Do not overheat the stain.
- Do not over-decolorize.
- Use fresh reagents.
- Handle slides carefully.

Applications

- Diagnosis of tuberculosis and leprosy
- Identification of acid-fast organisms
- Routine clinical microbiology

Conclusion

Acid-fast staining is a specific differential staining technique used to identify bacteria with mycolic acid-rich cell walls, especially *Mycobacterium* species.

EXPERIMENT- 5

Title: Identification of Unknown Fungus by Lactophenol Cotton Blue (LPCB) Staining Method

Aim

To identify an unknown fungus by studying its microscopic morphology using the Lactophenol Cotton Blue staining method.

Principle

Fungi consist of hyphae, mycelium, and spores, whose arrangement is characteristic for each species. Lactophenol Cotton Blue (LPCB) kills the fungus, preserves its structure, clears the background, and stains the chitinous cell wall blue, enabling detailed microscopic observation.
Identification is based on:

- Type of hyphae (septate / aseptate)
- Type of spores
- Arrangement of conidiophores or sporangiophores

Requirements

- Unknown fungal culture
- Clean glass slides
- Inoculating needle / teasing needle
- Coverslip
- Lactophenol cotton blue (LPCB)
- Microscope

Procedure

1. Place one drop of LPCB on a clean glass slide.
2. Using a sterile teasing needle, take a small portion of the fungal colony from the culture.
3. Gently tease the fungal material in the LPCB drop.
4. Place a coverslip carefully without air bubbles.
5. Allow the preparation to stand for 2–3 minutes.
6. Examine under low power (10×) and then high power (40×) objectives.

Observation

The following fungal structures were observed:

- Hyphae: septate / aseptate
- Mycelium
- Conidiophores / Sporangiophores
- Conidia / Sporangia
- Arrangement and shape of spores

Identification (For an Examples)

Fungus	Microscopic Features
Aspergillus	Septate hyphae, conidiophore with vesicle and chains of conidia
Penicillium	Septate hyphae, brush-like conidiophores
Rhizopus	Broad aseptate hyphae, sporangium with sporangiophores
Candida	Budding yeast cells with pseudohyphae

Result

Based on LPCB staining and microscopic morphology, the unknown fungus was identified as ____________. **OR**

"Based on Lactophenol Cotton Blue staining, the fungus isolated from bread was identified as Rhizopus species, showing broad aseptate hyphae with sporangiophores and sporangia." **OR**

"Based on Lactophenol Cotton Blue staining, the fungus isolated from lemon was identified as Penicillium species, showing septate hyphae with brush-like conidiophores and chains of conidia."

Precautions

- Handle fungal cultures carefully to prevent spore spread.
- Take minimal fungal material.
- Avoid air bubbles.
- Use clean slides and coverslips.
- Dispose materials safely.

Applications

- Identification of fungi
- Diagnostic mycology
- Teaching and research
- Study of fungal morphology

Conclusion

Lactophenol Cotton Blue staining is a simple and reliable method for identifying fungi based on microscopic morphology.

EXPERIMENT- 6

Title: Scotch tape preparation for studying morphology of fungi.

Aim

To study the morphology of fungi using the Scotch tape (cellophane tape) preparation technique.

Principle

Fungi grow as mycelia consisting of hyphae and spores on culture media. The Scotch tape technique allows direct transfer of fungal structures from the culture surface onto a slide without disturbing their natural arrangement. Lactophenol cotton blue (LPCB) stains fungal cell walls and clears cytoplasmic material, making fungal structures clearly visible under the microscope.

Requirements

- Fungal culture (e.g. *Aspergillus*, *Penicillium*, *Rhizopus*)
- Clean glass slides
- Scotch tape (cellophane tape)
- Lactophenol cotton blue (LPCB)
- Forceps
- Microscope

LPCB – Lactophenol Cotton Blue

Composition and Function

Component	Composition	Function
Lactic acid	~20%	Preserves fungal structures and clears cytoplasmic material
Phenol	~20%	Kills fungi (fungicidal action) and prevents autolysis
Glycerol	~40%	Acts as a mounting medium and prevents drying
Cotton blue (Aniline blue)	~0.05%	Stains chitin in fungal cell wall, giving blue color

Functions of LPCB

- Kills fungi safely
- Preserves fungal morphology
- Clears background for better visibility
- Stains fungal cell wall blue
- Acts as a mounting medium

Procedure

1. Take a clean glass slide and place one drop of LPCB stain on it.
2. Cut a small piece of Scotch tape using forceps.
3. Gently press the sticky side of the tape onto the surface of the fungal colony.
4. Place the tape carefully over the LPCB drop on the slide, sticky side down.
5. Avoid air bubbles and press gently.

6. Examine the preparation under low power (10×) and then high power (40×) objective.

Observation

Fungal structures are clearly seen:

- Hyphae (septate or aseptate)
- Mycelium
- Conidiophores / Sporangiophores
- Conidia / Sporangia
- Arrangement of spores characteristic of each fungus

Examples of Morphology

- *Aspergillus*: Septate hyphae, conidiophore with vesicle and chains of conidia
- *Penicillium*: Brush-like conidiophores
- *Rhizopus*: Broad aseptate hyphae with sporangium

Result

The morphology of the given fungal culture was successfully studied using the Scotch tape preparation method.

Precautions

- Handle fungal cultures carefully to avoid spore dispersal.
- Do not press tape too hard.
- Avoid air bubbles.
- Use fresh fungal growth.
- Dispose of used materials properly.

Application

- Identification of fungi
- Study of fungal morphology
- Teaching and diagnostic microbiology

Conclusion

The Scotch tape preparation is a simple, rapid, and effective technique for studying the microscopic morphology of fungi while preserving their natural structure.

EXPERIMENT- 7

Title: Effect of Different pH on Microbial Growth

Aim

To study the effect of different pH values on the growth of microorganisms.

Principle

pH is an important environmental factor that influences enzyme activity, membrane permeability, and metabolic reactions of microorganisms.

Each microorganism has an optimum pH range for growth:

- Acidophiles grow best at acidic pH
- Neutrophiles grow best at neutral pH
- Alkaliphiles grow best at alkaline pH

Deviation from optimum pH results in reduced or no growth.

Requirements

- Nutrient broth / Nutrient agar
- Test tubes / Petri plates
- Microbial culture (bacteria / yeast / fungi)
- Buffer solutions of different pH (pH 4, 6, 7, 8, 9)
- Inoculating loop
- Incubator (37°C)
- Marker pen

Procedure

1. Prepare nutrient broth/agar media adjusted to different pH values (4, 6, 7, 8, and 9).
2. Label each tube/plate with the respective pH.
3. Inoculate each medium with equal quantity of microbial culture using aseptic technique.
4. Incubate all tubes/plates at 37°C for 24–48 hours.
5. Observe growth in terms of turbidity (broth) or colony formation (agar).

Observation

pH	Growth Observed
4	Growth in acidophiles / fungi
6	Moderate growth
7	Maximum growth in neutrophilic bacteria
8	Moderate growth
9	Growth in alkaliphilic bacteria

NOTE: (Growth pattern varies depending on microorganism used)

Result

Microbial growth was affected by pH. Neutrophilic bacteria showed maximum growth at neutral pH, acidophilic microorganisms showed better growth at acidic pH, and alkaliphilic bacteria showed growth at alkaline pH.

Precautions

- Maintain accurate pH of media.
- Use equal inoculum size in all media.
- Follow strict aseptic techniques.
- Label tubes/plates correctly.
- Avoid contamination.

Applications

- Food preservation and fermentation
- Industrial microbiology
- Clinical microbiology
- Environmental microbiology

Conclusion

Different microorganisms have different optimum pH requirements for growth. Therefore, pH plays a crucial role in regulating microbial growth and survival.

EXPERIMENT- 8

Title: Effect of Different Incubation Temperature on Microbial Growth

Aim

To study the effect of different incubation temperatures on the growth of microorganisms.

Principle

Temperature is a key environmental factor affecting microbial growth. It influences enzyme activity, membrane fluidity, and metabolism. Microorganisms are classified based on their preferred temperature range:

- Psychrophiles → cold-loving, grow at 0–20°C
- Mesophiles → moderate temperature, grow at 20–45°C (most human pathogens)
- Thermophiles → heat-loving, grow at 45–80°C
- Hyperthermophiles → extreme heat, grow above 80°C

Growth decreases if the temperature deviates from the optimum.

Requirements

- Nutrient broth / Nutrient agar
- Test tubes / Petri plates
- Microbial culture (e.g., *E. coli*, *Staphylococcus aureus*, yeast)
- Inoculating loop
- Incubators set at different temperatures (e.g., 10°C, 25°C, 37°C, 45°C, 55°C)
- Marker pen

Procedure

1. Prepare nutrient broth or nutrient agar plates.
2. Label the petri plates according to incubation temperatures.
3. Inoculate equal amounts of microbial culture into each tube/plate using aseptic technique.
4. Incubate at different temperatures for 24–48 hours.
5. Observe microbial growth:
    - Broth → turbidity
    - Agar plates → number and size of colonies

Observation

Temperature (°C)	Growth Observed	Type of Microorganism Favored
10	Poor / slow growth	Psychrophiles
25	Moderate growth	Mesophiles
37	Maximum growth	Mesophiles / human pathogens
45	Moderate growth	Thermophiles
55	Poor / limited growth	Thermophiles (selective)

NOTE: *(Observation may vary depending on the microorganism used)*

Result

Microbial growth varied with incubation temperature. Maximum growth for mesophilic bacteria was observed at 37°C, while psychrophiles grew best at low temperatures and thermophiles at high temperatures.

Precautions

- Use fresh cultures.
- Maintain aseptic techniques.
- Avoid repeated opening of incubators.
- Label tubes/plates properly.
- Ensure correct incubation temperature for each set.

Applications

- Food preservation and spoilage studies
- Clinical microbiology (pathogen isolation)
- Industrial microbiology (fermentation processes)
- Environmental microbiology

Conclusion

Temperature strongly affects microbial growth. Each microorganism has an optimum temperature range: psychrophiles (cold), mesophiles (moderate), thermophiles (hot), and hyperthermophiles (extreme heat).

EXPERIMENT- 9

Title: Effect of Different Salt Concentrations on Microbial Growth.

Aim

To study the effect of different NaCl (salt) concentrations on the growth of microorganisms.

Principle

Salt (NaCl) affects microbial growth by influencing osmotic pressure. Microorganisms are classified based on their ability to grow in salt:

- Non-halophiles: Cannot tolerate high salt, grow best at 0–1% NaCl.
- Halotolerant: Can tolerate moderate salt, grow at 0–5% NaCl.
- Halophiles: Require high salt, grow optimally at 5–20% NaCl.

High salt concentrations can cause plasmolysis, inhibiting growth, while low to moderate concentrations may allow normal growth.

Requirements

- Nutrient broth / Nutrient agar
- Test tubes / Petri plates
- Microbial culture (e.g., *E. coli*, *Staphylococcus aureus*, *Halobacterium*)
- NaCl solutions of different concentrations (0%, 2%, 5%, 10%, 15%, 20%)
- Inoculating loop
- Incubator (37°C)
- Marker pen

2% NaCl solution → 2 g of NaCl dissolved in 100 mL of distilled water.
Similarly:

- 5% → 5 g in 100 mL
- 10% → 10 g in 100 mL
- 15% → 15 g in 100 mL
- 20% → 20 g in 100 mL

Procedure

1. Prepare nutrient broth/agar with different NaCl concentrations.
2. Label each tube/plate according to salt concentration.
3. Inoculate equal amounts of microbial culture in each tube/plate using aseptic technique.
4. Incubate at 37°C for 24–48 hours.
5. Observe growth:
    - Broth: turbidity
    - Agar: number and size of colonies

Observation

NaCl Concentration (%)	Growth Observed	Type of Microorganism Favored
0	Good growth	Non-halophiles
2	Good growth	Non-halophiles / Halotolerant
5	Moderate growth	Halotolerant
10	Poor growth	Halophiles (some growth)
15	Very poor	Halophiles (required)
20	Minimal / none	Extreme halophiles only

NOTE: *(Observation may vary depending on the microorganism used)*

Result:

Microbial growth varied with salt concentration.

- Non-halophiles grew best at low salt (0–2%).
- Halotolerant organisms tolerated moderate salt (2–5%).
- Halophiles grew well at high salt concentrations (10–20%).

Precautions

- Use aseptic technique.
- Prepare accurate NaCl concentrations.
- Use equal inoculum size.
- Label tubes/plates properly.
- Avoid contamination.

Applications

- Food preservation (high salt inhibits bacterial growth)
- Isolation of halophilic microorganisms
- Industrial microbiology (fermentation)
- Environmental microbiology (salt marsh, saline water)

Conclusion

Microbial growth is strongly influenced by salt concentration. Each microorganism has an optimum NaCl range, classifying them as non-halophiles, halotolerant, or halophiles.

EXPERIMENT- 10

Title: Evaluation of Alcohol Effectiveness as an Antiseptic

Aim

To evaluate the effectiveness of alcohol (ethanol / isopropyl alcohol) as an antiseptic against microbial growth.

Principle

Alcohol is a commonly used antiseptic that kills or inhibits microorganisms by:

- Denaturing proteins
- Disrupting cell membranes
- Dehydrating microbial cells

The effectiveness can be assessed by observing the zone of inhibition on agar or by comparing growth on treated vs untreated surfaces.

Requirements

- Nutrient agar plates
- Bacterial culture (*E. coli*, *Staphylococcus aureus*, or mixed culture from skin swab)
- Sterile filter paper discs or cotton swabs
- Ethanol (70%) or Isopropyl alcohol (70%)
- Sterile forceps
- Incubator (37°C)
- Marker pen

Procedure (Disc Diffusion Method)

1. Prepare agar plates: Pour nutrient agar into Petri dishes and allow to solidify.
2. Inoculate bacteria: Using a sterile swab, spread bacterial culture evenly over the agar surface.
3. Prepare discs: Sterile filter paper discs are soaked in 70% alcohol and allowed to drain slightly.
4. Place discs: Using sterile forceps, place the discs on the inoculated agar surface.
5. Control: Place one disc soaked in sterile water as a control.
6. Incubate: Keep plates at 37°C for 24 hours.
7. Observe: Check for zones of inhibition (clear area around the disc where bacteria did not grow).

Observation

Sample	Zone of Inhibition (mm)	Interpretation
70% Alcohol	15–20 mm (example)	Effective in inhibiting bacterial growth
Control (Water)	0 mm	No effect

Note: The actual size of the zone may vary depending on bacterial species and alcohol type.

Result

Alcohol (70%) showed clear inhibition of microbial growth around the disc, indicating it is an effective antiseptic. The control disc showed no inhibition, confirming the effect is due to alcohol.

Precautions

- Use sterile technique to avoid contamination.
- Do not touch the agar surface with fingers.
- Alcohol is flammable — keep away from open flames.
- Use fresh cultures for accurate results.

Applications

- Disinfection of skin before injections
- Hand sanitizers and surface sterilization
- Clinical and laboratory antiseptic procedures

Conclusion

Alcohol is an effective antiseptic that inhibits microbial growth by denaturing proteins and disrupting membranes. 70% alcohol is more effective than absolute alcohol because it penetrates cells better.

Experiment No. 11

Title: Evaluation of Antiseptic by Filter Paper Disc Method

Aim
To evaluate the effectiveness of an antiseptic in inhibiting microbial growth using the filter paper disc (Kirby-Bauer) method.

Principle
Antiseptics inhibit or kill microorganisms by affecting cell membrane, proteins, or enzymes.
The filter paper disc method is based on the principle that an effective antiseptic diffuses from a filter paper disc into the surrounding agar medium and inhibits the growth of microorganisms.

When a filter paper disc soaked with an antiseptic is placed on an agar plate inoculated with a test organism, the antiseptic diffuses radially into the agar. If the antiseptic is effective, it prevents microbial growth around the disc, producing a clear circular area called the zone of inhibition.

The size of the zone of inhibition is directly proportional to the antimicrobial effectiveness of the antiseptic.

- Larger zone of inhibition → more effective
- No zone → ineffective (Not effective).

Requirements
- Nutrient agar plates
- Bacterial culture (*E. coli*, *Staphylococcus aureus*, or mixed skin flora)
- Sterile filter paper discs (6 mm)
- Antiseptics to be tested (alcohol, iodine, mercurial solutions, Dettol, etc.)
- Sterile forceps
- Marker pen
- Incubator (37°C)

Procedure

1. Prepare agar plate: Pour nutrient agar into Petri dishes and allow to solidify.
2. Inoculate bacteria: Using a sterile swab, spread the bacterial culture evenly over the agar surface to form a lawn.
3. Prepare discs: Soak sterile filter paper discs in the antiseptic to be tested. Allow excess liquid to drain.
4. Place discs: Using sterile forceps, place the discs on the inoculated agar surface at equal distances.
5. Control: Include a disc soaked in sterile water as a negative control.
6. Incubate: Keep plates at 37°C for 24 hours.
7. Observe: Measure the diameter of clear zones around each disc in millimeters.

Observation

Antiseptic	Zone of Inhibition (mm)	Interpretation
Alcohol (70%)	15	Effective
Iodine	18	Very effective
Dettol	12	Moderately effective
Water (Control)	0	No effect

Note: Zone size may vary with organism and antiseptic used.

Result

The antiseptics produced clear zones of inhibition of varying sizes. Alcohol, iodine, and Dettol were effective against the tested bacteria, while the control (water) showed no inhibition, confirming the effect was due to the antiseptics.

Precautions

- Use sterile technique to avoid contamination.
- Do not overlap discs.
- Do not touch agar surface.
- Measure zones carefully using a ruler.
- Handle antiseptics carefully; some may be corrosive or irritant.

Applications

- Testing effectiveness of antiseptics in hospitals and labs
- Selection of proper antiseptic for clinical use
- Educational and research purposes

Conclusion

The filter paper disc method is a simple and effective way to evaluate antiseptic activity. The size of the inhibition zone reflects the relative effectiveness of each antiseptic.

Experiment No. 12

Title: Phenol-Coefficient Test (Rideal-Walker Test)

Aim

To determine the efficiency of an antiseptic or disinfectant relative to phenol using the phenol-coefficient method (Rideal-Walker Test).

Principle

The **Phenol-Coefficient Test (Rideal–Walker test)** is based on the principle of **comparing the disinfectant power of a test disinfectant with that of phenol under standard conditions**.

In this test, different dilutions of **phenol** and the **test disinfectant** are prepared and exposed to a standard test organism (usually *Salmonella typhi*) for fixed time intervals (5, 10, and 15 minutes) at a constant temperature.
The highest dilution of the disinfectant that **kills the organism in 10 minutes but not in 5 minutes** is noted and compared with the corresponding dilution of phenol.

The **phenol coefficient** is then calculated as:

Phenol Coefficient = (Highest dilution of disinfectant killing the organism in 10 min but not in 5 min) ÷ (Highest dilution of phenol killing the organism in 10 min but not in 5 min)

Interpretation:

Phenol coefficient > 1 → Disinfectant is **more effective than phenol**

Phenol coefficient = 1 → Equal effectiveness

Phenol coefficient < 1 → Less effective than phenol

$$\text{Phenol coefficient} = \frac{\text{dilution ratio of disinfectant}}{\text{dilution ratio of phenol}}$$

Requirements

- Test organism (*Salmonella typhi*, *Staphylococcus aureus*)
- Phenol solution (standard reference)
- Test disinfectant / antiseptic
- Sterile test tubes
- Nutrient broth / nutrient agar
- Incubator (37°C)
- Timer / stopwatch

- Sterile pipettes / inoculating loops

Procedure

1. Prepare serial dilutions:
   - Dilute phenol (reference) in sterile water to make a series of concentrations.
   - Dilute the test disinfectant similarly.
2. Inoculate bacteria:
   - Add a measured quantity of bacterial culture to each dilution.
3. Expose bacteria:
   - Incubate tubes with disinfectant or phenol for 5 minutes and 10 minutes separately.
4. Transfer to growth medium:
   - After exposure, transfer a loopful to fresh nutrient broth or agar plates.
   - Incubate at 37°C for 24 hours.
5. Check for growth:
   - No growth after 10 min but growth after 5 min → suitable dilution.
6. Determine phenol coefficient:

Observation Table

Dilution	Phenol 5 min	Phenol 10 min	Test Disinfectant 5 min	Test Disinfectant 10 min
1:100	Growth	No growth	Growth	No growth
1:200	Growth	Growth	Growth	No growth
1:400	Growth	Growth	Growth	Growth

Calculation Example:

Highest dilution killing bacteria in 10 min:

- Phenol → 1:100
- Disinfectant → 1:200

Phenol Coefficient (PC)= 100/200=2

Interpretation: Disinfectant is twice as effective as phenol.

Result

The phenol coefficient of the tested disinfectant was 2, indicating it is more effective than phenol.

Precautions

- Use fresh cultures and sterile equipment.
- Avoid contamination.
- Maintain accurate timing (5 and 10 minutes).

- Use correct dilutions.
- Handle disinfectants carefully; some are corrosive.

Applications

- Comparing efficiency of disinfectants
- Selecting disinfectants for hospital and laboratory use
- Standardization of antiseptics

Conclusion

The phenol-coefficient test (Rideal-Walker) is a standard method to quantitatively evaluate the effectiveness of a disinfectant relative to phenol.