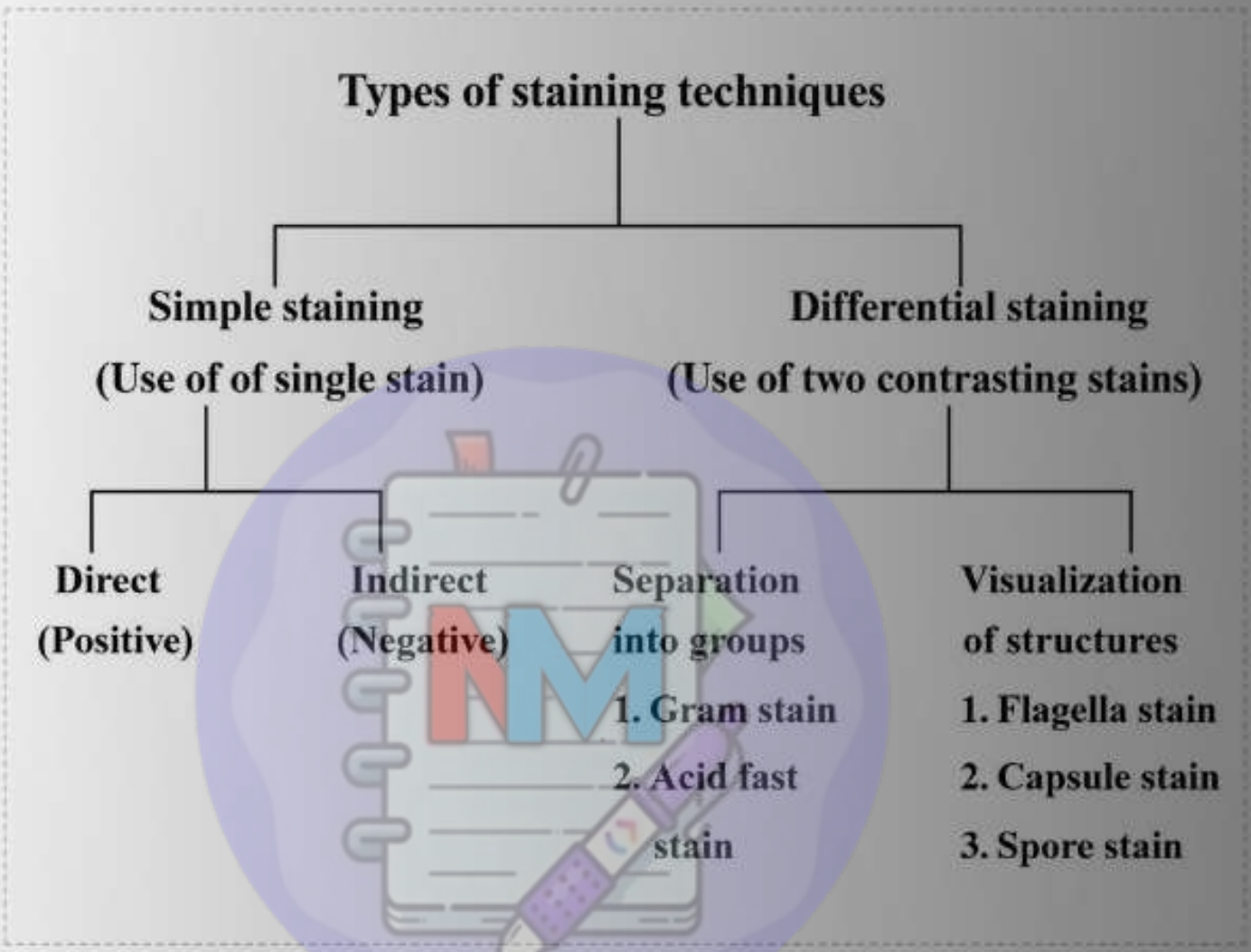
STAINING

Staining is the process of applying dyes to microorganisms to make them visible under a microscope. Most bacteria are colorless and transparent, so staining helps in identifying their shape, size, arrangement, and structure.

The terms stain and dye are not the same. A coloring agent that is used for general purposes is called a dye. The one that is used for biological purposes is called a stain.

Objectives of Staining

- To visualize microorganisms clearly.
- To differentiate between different types of bacteria.
- To study bacterial morphology and arrangement.
- To identify specific cellular structures.



SIMPLE STAINING

Simple staining is a microbiological technique in which only one basic dye is used to stain bacterial cells. It helps in observing the shape, size, arrangement, and morphology of bacteria under a microscope.

Principle

Bacterial cells carry a negative charge on their surface. Basic dyes have positively charged (cationic) chromophores, which are attracted to the bacterial cell and stain it.

As a result :-

- Bacterial cells become colored.
- Background remains colorless.

objectives

- To observe the morphology of bacteria.
- To determine the size and shape of bacterial cells.
- To study the arrangement of bacteria.
- To make bacteria clearly visible under a microscope.

Dyes used in Simple Staining

Common basic dyes include :-

- Methylene Blue
- Crystal Violet
- Safranin

Materials Required

- Clean glass slide
- Bacterial culture
- Inoculating loop
- Bunsen burner/spirit lamp
- Staining dye
- Distilled water
- Microscope
- Immersion oil

Procedure of Simple Staining

1. Prepare a thin bacterial smear on a clean

glass slide.

2. Allow the smear to air dry.

3. Heat-fix the smear by passing the slide through a flame a few times.

4. Cover the smear with a basic stain (Methylene Blue, Crystal Violet, or Safranin).

5. Leave the stain for 30-60 seconds.

6. Wash the slide gently with water to remove excess stain.

7. Dry the slide using blotting paper.

8. Place a drop of immersion oil on the smear.

9. Observe under the oil immersion objective (100×) of the microscope.

Negative (Indirect) Staining

Definition

Negative staining is a technique in which the background is stained while the bacterial cells remain unstained.

Principle

Acidic dyes possess negatively charged chromophores. Since bacterial cells are also negatively charged, both repel each other.

As a result :-

- Dye does not enter bacterial cells.
- Background becomes stained.
- Bacteria appear clear against a dark background.

Dyes Used

- Nigrosin
- India Ink
- Congo Red
- Eosin
- Rose Bengal

Procedure

- Clean a glass slide.
- Place a drop of nigrosin or India ink near one end.
- Add a loopful of bacterial culture.
- Mix gently.
- Spread into a thin film using another slide held at 45°.
- Air dry only (do not heat-fix).
- Observe under microscope.

Result

- Background appears dark grey or black.
- Bacterial cells appear colourless, bright, or

white.

Positive (Monochrome) Staining

Definition

Monochrome staining is a staining technique that uses a single basic dye to stain bacterial cells.

Principle

Negatively charged bacterial cells attract positively charged basic dyes.

Examples :-

Crystal Violet
Methylene Blue
Carbol Fuchsin

The bacterial cell takes up the stain while the background remains colourless.

Procedure

- Clean a glass slide thoroughly to remove dirt and grease.
- Prepare a thin bacterial smear on the slide.
- Allow the smear to air dry.
- Heat-fix the smear by passing the slide through a flame 2-3 times.
- Flood the smear with a basic stain such as methylene blue, crystal violet, or carbol fuchsin.
- Leave the stain for the required time:
  - # Crystal violet :- 2-6 seconds
  - # Methylene blue :- 60-120 seconds
  - # Carbol fuchsin :- 15-30 seconds
- Gently wash the slide with tap water to remove excess stain.
- Allow the slide to air dry or blot dry carefully.
- Examine the stained smear under the oil immersion objective (100×) of a microscope.

Result

The bacterial cells appear uniformly coloured (blue,

violet, or red depending on the stain used), while the background remains clear.

DIFFERENTIAL STAINING

Definition

Differential staining uses two or more stains to differentiate microorganisms into different groups.

Importance

- Distinguishes bacterial species.
- Assists in diagnosis.
- Helps in selection of antibiotics.
- Identifies structural differences.

Types

- Gram Staining
- Acid-Fast Staining

Gram Staining

Gram staining is a differential staining technique

used to differentiate bacteria into Gram-positive and Gram-negative groups based on the structure of their cell wall.

Principle

Gram staining is based on the ability of bacterial cell walls to retain the crystal violet-iodine complex during decolorization.

- Gram-positive bacteria have a thick peptidoglycan layer and retain the crystal violet-iodine complex, appearing purple/violet.
- Gram-negative bacteria have a thin peptidoglycan layer and lose the crystal violet stain during decolorization. They take up the counterstain (safranin) and appear pink/red.

Reagents Used

1. Crystal Violet - Primary stain
2. Gram's Iodine - Mordant
3. 95% Ethyl Alcohol/Acetone -

Decolorizer

- Safranin - Counterstain

Procedure

- Prepare a thin bacterial smear on a clean slide.
- Air dry and heat-fix the smear.
- Flood with crystal violet for 1 minute and wash with water.
- Add Gram's iodine for 1 minute and wash with water.
- Decolorize with 95% ethyl alcohol for 20-30 seconds and wash immediately.
- Counterstain with safranin for 30-60 seconds.
- Wash with water, blot dry, and observe under the oil immersion objective.

Result

Type of Bacteria	Colour
Gram-positive	Purple/Violet
Gram-negative	Pink/Red

Examples

- Gram-positive :- Staphylococcus, Streptococcus, Bacillus
- Gram-negative :- Escherichia coli, Salmonella, Pseudomonas

Acid Fast Staining

Acid-fast staining (Ziehl-Neelsen stain) is a special staining technique used to identify bacteria that have waxy cell walls rich in mycolic acid, particularly Mycobacterium species such as Tuberculosis.

Principle

Some bacteria contain a high amount of mycolic acid (waxy lipid) in their cell wall. These bacteria resist decolorization by acid-alcohol after staining with carbol fuchsin and are called acid-fast bacteria.

- Acid-fast bacteria retain the primary stain and appear red/pink.

- Non-acid-fast bacteria lose the primary stain and take up the counterstain, appearing blue.

Reagents used

Reagent	Function
Carbol Fuchsin	Primary stain
Acid-Alcohol	Decolorizer
Methylene Blue	Counterstain

Procedure (Ziehl-Neelsen Method)

- Prepare a thin bacterial smear on a clean glass slide.
- Air dry and heat-fix the smear.
- Flood the smear with carbol fuchsin.
- Gently heat the slide until steam appears (do not boil) for about 3-5

minutes.

- Wash with water.
- Decolorize with acid-alcohol for 1-2 minutes.
- Wash with water.
- Counterstain with methylene blue for 1 minute.
- Wash, air dry, and observe under the oil immersion objective.

Result

organism	Colour
Acid-fast bacteria	Red/Pink
Non-acid-fast bacteria	Blue

Examples

Acid-Fast Bacteria

- Mycobacterium tuberculosis
- Mycobacterium leprae

Non- Acid- Fast Bacteria

- Escherichia coli
- Staphylococcus aureus
- Streptococcus species

SPECIAL STAINING

Special staining is a staining technique used to demonstrate and identify specific structures of bacterial cells that cannot be seen clearly with simple or differential staining.

These structures include :-

1. Capsules (Capsule staining)
2. Endospores (Spore staining)
3. Flagella (Flagella staining)
4. Metachromatic granules (Albert staining)

Purpose

Special staining helps in the identification of

bacteria by highlighting their unique cellular structures.

Examples of Special Stains

- Capsule Staining - Demonstrates bacterial capsules.
- Endospore Staining - Demonstrates bacterial spores.
- Flagella Staining - Demonstrates bacterial flagella.
- Albert Staining - Demonstrates metachromatic granules of Corynebacterium diphtheriae.

Capsule Staining

Capsule staining is a special staining method used to demonstrate the presence of capsules surrounding certain bacterial cells. Since capsules do not readily absorb ordinary stains, a combination of negative and positive staining techniques is used to visualize

them.

Principle

The bacterial capsule is a non-ionic, gelatinous layer composed mainly of polysaccharides. It does not readily absorb most stains.

Therefore :-

- The background is stained with an acidic dye (e.g., India ink or nigrosin).
- The bacterial cell is stained with a basic dye.
- The capsule remains unstained and appears as a clear halo around the stained cell.

Reagents used

Reagent	Function
India Ink/Nigrosin	Negative stain (background stain)
Crystal Violet/Safranin	Positive stain (cell stain)

Distilled water washing

Procedure of Capsule Staining

1. Take a clean, grease-free glass slide.
2. Place a drop of India ink or Nigrosin near one end of the slide.
3. Add a small amount of bacterial culture to the drop and mix gently.
4. Using another slide held at a 45° angle, spread the mixture into a thin smear.
5. Allow the smear to air dry completely.
6. Do not heat-fix the smear, as heat may destroy or shrink the capsule.
7. Flood the smear with crystal violet for about 1 minute.
8. Gently wash the slide with water.
9. Allow the slide to dry.
10. Examine under the oil immersion objective (100×) of the microscope.

Result

- Background → Dark/Black
- Bacterial cell → Purple/Violet
- Capsule → Clear, unstained halo surrounding the bacterial cell

Examples of Encapsulated Bacteria

- Klebsiella pneumoniae
- Streptococcus pneumoniae
- Bacillus anthracis
- Neisseria meningitidis
- Haemophilus influenzae

Endospores Staining

Endospore staining is a special staining technique used to demonstrate the presence of endospores within bacterial cells. Endospores are highly resistant structures produced by certain bacteria under unfavorable environmental conditions.

Principle

Endospores have a thick, impermeable wall that resists ordinary stains. Therefore, heat is used to drive the primary stain (malachite green) into the spore.

- Malachite Green stains the endospore.
- water removes the stain from vegetative cells but not from spores.
- Safranin counterstains the vegetative cells.

As a result :-

- Endospores appear green.
- Vegetative cells appear red/pink.

Reagents Used

Reagent	Function
Malachite Green	Primary stain
water	Decolorizer
Safranin	

Counterstain

Procedure (Schaeffer-Fulton Method)

- Prepare a thin bacterial smear on a clean glass slide.
- Allow it to air dry and heat-fix.
- Place the slide over steaming water.
- Flood the smear with malachite green.
- Heat gently for 5-10 minutes, keeping the stain moist.
- Remove the slide and allow it to cool.
- Wash with water to remove excess stain.
- Counterstain with safranin for 30-60 seconds.
- Wash with water and blot dry.
- Observe under the oil immersion objective (100×).

Result

Structure	Colour
Endospore	Green
Vegetative Cell	Red/Pink

Examples of Spore-Forming Bacteria

- Bacillus anthracis
- Bacillus subtilis
- Clostridium tetani
- Clostridium botulinum

Flagella Staining

Flagella are extremely thin (fragile appendages) and cannot be seen under an ordinary light microscope.

- A mordant is used to form a colloidal precipitate on the surface of flagella.
- This precipitate increases thickness of flagella.
- Then a basic dye (basic fuchsin) stains the coated structure.
- Thus, flagella become visible as stainable thread-like structures.

This staining gives taxonomically important

information such as :-

- Presence of flagella
- Number of flagella
- Pattern of arrangement

Materials Required

Solution A (Mordant)

- Tannic acid (20% aqueous solution) - 2 ml
- Potassium alum (saturated solution) - 5 ml
- Mercuric chloride (saturated solution) - 2 ml

Solution B (Stain)

- Basic fuchsin (3%) in 95% alcohol - 0.4 ml

Note :-

- Both solutions are prepared fresh (<24 hours before

use)

- Stored in dark/coloured bottles.

Procedure (Gray Method)

1. Grow bacteria in a suitable broth culture.
   # If grown on solid media → add 1% peptone water to allow motility

2. Centrifuge the culture to obtain a bacterial pellet.

3. Suspend the pellet in 10% formalin to make a light suspension (faint turbidity).

4. Place a loopful of suspension on a clean grease-free slide and prepare a thin smear.

5. Flood the smear with Solution A (mordant).
   # Leave for 8-10 minutes

6. Wash gently with distilled water.

7. Flood with Solution B (basic fuchsin stain).
   # Leave for 5 minutes (no

heating)

8. Wash gently with distilled water.

9. Air dry the slide.

10. Observe under oil immersion lens (100× objective).

Result

- Flagella appear as thin, red, hair-like structures extending from bacterial cells.
- Arrangement can be identified:
  - # Monotrichous (single)
  - # Lophotrichous (tuft at one end)
  - # Amphitrichous (both ends)
  - # Peritrichous (all around)objective).

Albert staining

Albert staining is a special staining technique used to demonstrate metachromatic granules (volutin

granules) in bacteria such as Corynebacterium diphtheriae.

These granules are important for identifying diphtheria-causing bacteria.

Principle

- Bacterial cells contain metachromatic (volutin) granules made of polyphosphate.
- Albert stain contains:
  - # Toluidine blue / malachite green (basic dye)
  - # Iodine (mordant-like effect)
- The granules take a different color (deep bluish-black/black) than the cytoplasm.
- Cytoplasm stains greenish, while granules appear dark blue/black.

This color difference is due to metachromasia (color change property of

granules).

Reagents Used

Albert Solution I

- Toluidine blue
- Malachite green
- Acetic acid
- Ethanol
- Distilled water

Albert Solution II

- Iodine solution
- Potassium iodide

Procedure

1. Prepare a thin smear of bacterial culture on a clean slide.
2. Air dry and heat fix the smear.
3. Flood the smear with Albert Solution

1.

- Leave for 3-5 minutes.
- Drain (do not wash).
- Flood with Albert Solution II.
- Leave for 1-2 minutes.
- Rinse gently with water.
- Air dry the slide.
- Observe under oil immersion lens (100× objective).

Result

- Cytoplasm → green color
- Metachromatic granules → bluish-black / dark blue
- Cell shape → rod-shaped bacteria (Corynebacterium)