

A PRESENTATION ON BACTERIAL STAINING

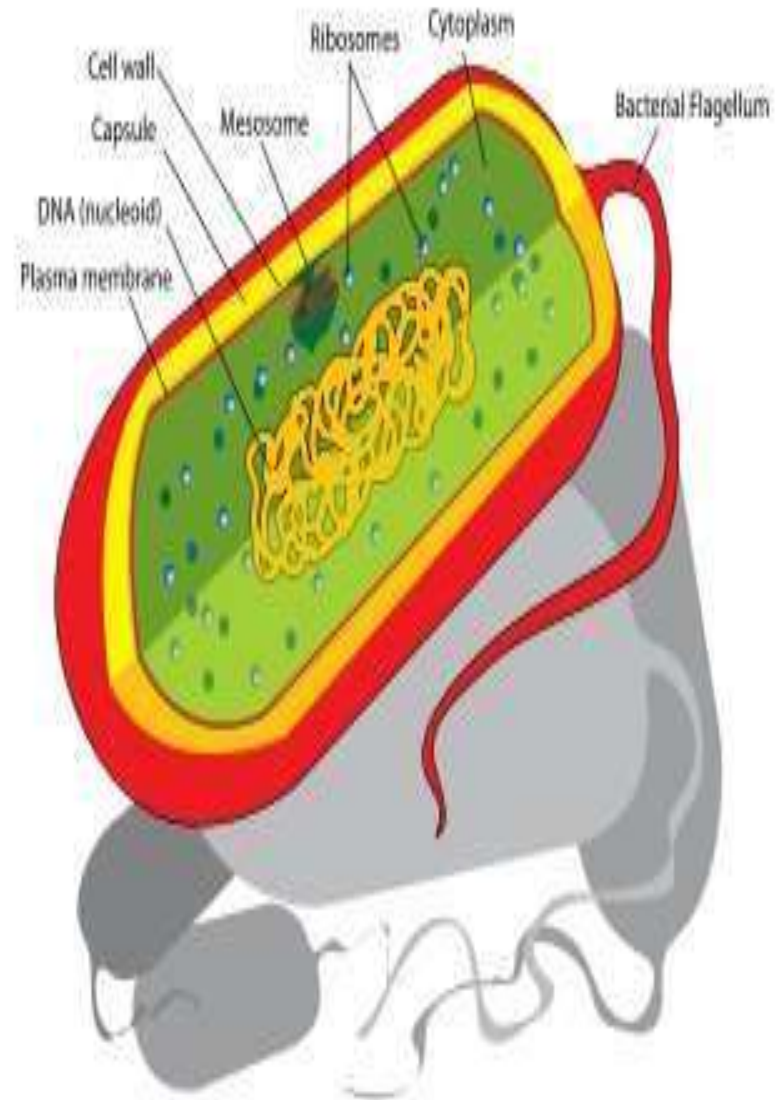


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INTRODUCTION

❑ *Bacterial Staining:*

- *Process in which bacteria are stained to give color to them.*
- *Because microbes are colorless and highly transparent structures.*
- *Bacteria have nearly the same refractive index as water, therefore, when they are observed under a microscope they are opaque or nearly invisible to the naked eye.*
- *So different types of staining methods are used to make the cells and their internal structures more visible under the light microscope.*



STAINS OR DYES

- ❑ *STAINS or DYES are organic compounds which carries either positive charges or negative charges or both.*
- ❑ *They adheres to a cell, giving the cell color as different stains have different affinities for different organisms, or different parts of organisms so they are used to differentiate different types of organisms.*

- ❑ *Based on the charges: the commonly used stains are salts.*
 - ***Basic stain/dyes:** stain with +ve charge, i.e., **colored cation** + colorless anion. E.g., methylene blue chloride*
 - ***Acidic stain/dyes:** stain with -ve charge, i.e., colored anion + colorless cation. E.g., Eosin- + Na+*
 - ***Neutral stain/dyes:** stain with both charges. E.g., Eosinate of Methylene blue*

- ❑ *Bacterial cells are slightly negatively charged at pH 7.0*
 - *So +ve charged Basic dye stains bacteria*
 - *And Acidic dye stains background*

PRINCIPLE OF STAINING:

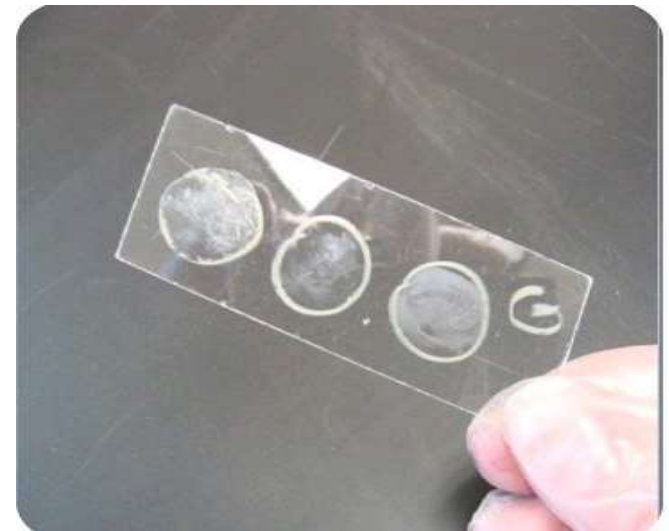
- *Each staining methods have own principles but the following steps may be common:*
 - ❑ *Stains* → combine *chemically* with the bacterial protoplasm.
 - ❑ *Basic stain(+ve charge) –*
To stain -ve charged molecules of bacteria
Mostly used because cell surface is –ve charge.
 - ❑ *Acidic Stain(-ve charge)*
To stain +ve charged molecules of bacteria.
Used to stain the bacterial capsules.
 - ❑ *As cell surface is –ve charged: Basic dyes are mostly used.*

BASIC REQUIREMENT AND INITIAL STEP

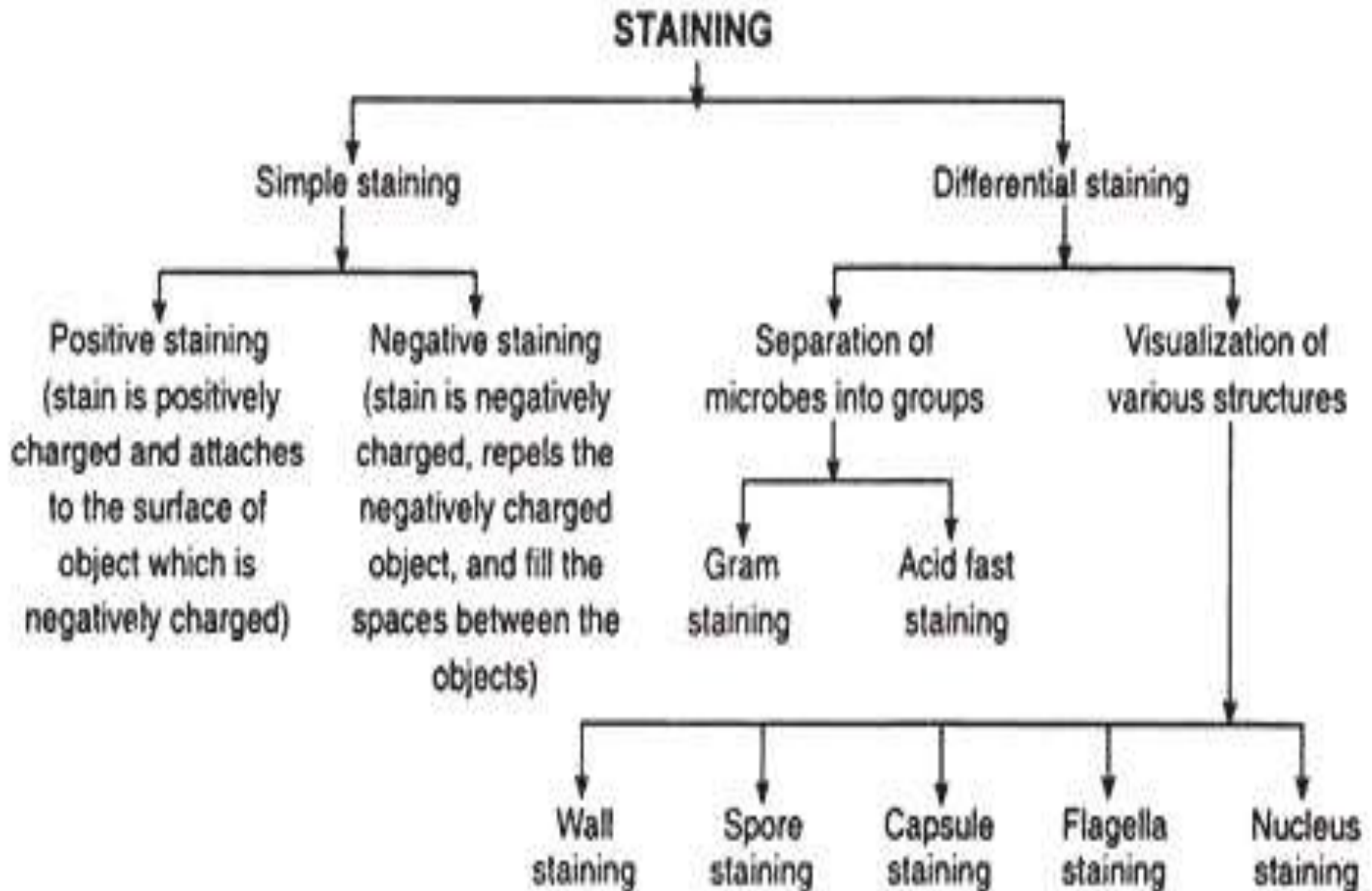
- **Clean grease-free slide.**
- **Bacteria to be stained.**
- **Inoculating loops- to transfer bacterial suspension to slide.**
- **Bunsen burner – to sterilize inoculating loops before and after smear preparation.**
- **Pencil marker – to mark (particularly central portion of slide) where bacterial smear is applied.**

➤ **SMEAR PREPARATION**

➤ **FIXATION**



CLASSIFICATION OF STAINING



1. Simple Staining Procedure:

When a single staining-reagent is used and all cells and their structures stain in the same manner, the procedure is called simple staining procedure.

This procedure is of two types – positive and negative

2. Differential Staining Procedure:

When more than one staining reagents are used and specific objects (e.g., specific microorganisms and/or particular structure of a microorganism) exhibit different staining reactions readily distinguishable, the procedure is called differential staining.

The most widely used differential staining in microbiology are Gram-staining and acid-fast staining. Acid-fast staining is especially useful in identifying *Mycobacterium tuberculosis*, the causative agent of tuberculosis.

SIMPLE STAINING:

1. POSITIVE STAINING:

- Simple to perform- only one basic stain used.

E.g. **Crystal violet, Methylene blue, Basic fuchsin etc.,**

- **Principle:**

- All bacteria in smear takes stain and appears in colour of stain.
- Basic stain more affinity towards bacterial surface & stains the bacteria.

- **Uses:**

To study morphology and arrangement of bacteria.

PROCEDURE:

- A bacterial smear is prepared, air-dried and heat fixed.
- A Heat-fixed smear is flooded with either one of the basic stain and allowed to react for 1-2 minutes and then washed under running tap water.
- Air dried and focused with 10x,45x & 100x.



DIFFERENTIAL STAINING

- **more than one dye is used- Differentiation among bacteria is possible- Eg. Gram's staining & Acid-fast staining**
- **It allows the observation of cell morphology, or shape, but usually provides more information about the characteristics of the cell wall (Thickness).**

GRAM STAINING:

Named after DANISH BACTERIOLOGIST HANS CHRISTIAN GRAM (1880),

It differentiates between Gram-positive (purple) and Gram-negative (pink) stains and is used to differentiate two Large groups of bacteria based on their Different cell wall constituents.



Gram staining - Principles

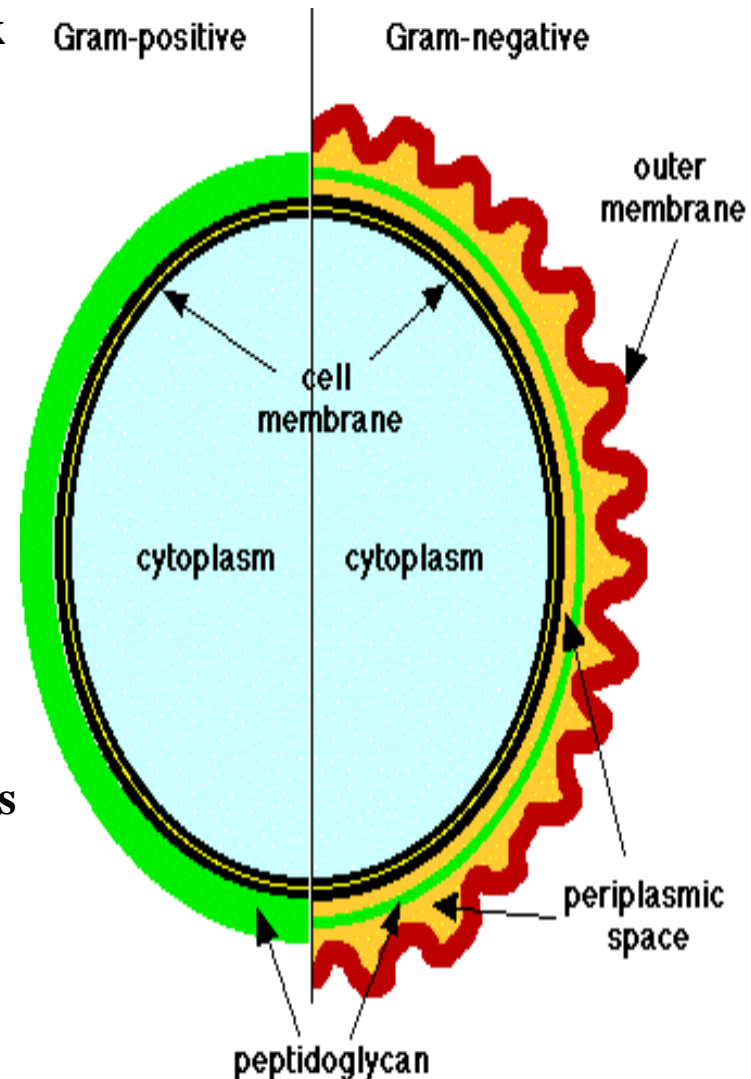
- It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin as counterstain to mark all bacteria.

Gram-positive bacteria

- Have a thick peptidoglycan layer surrounds the cell.
- Retain the color of the primary stain (crystal violet) after decolorization with alcohol

Gram-negative bacteria

- have a thin peptidoglycan layer that does not retain crystal violet stain. Instead, it has a thick lipid layer which dissolved easily upon decolorization with Acetone-Alcohol.
- Therefore, cells will be counterstained with safranin and turned red.



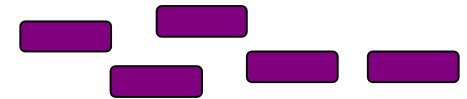
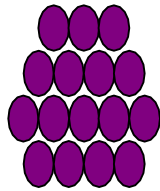
STEPS OF GRAM STAINING

- 1. Crystal violet acts as the primary stain. Crystal violet may also be used as a simple stain because it dyes the cell wall of any bacteria.**
- 2. Gram's iodine acts as a mordant (Helps to fix the primary dye to the cell wall).**
- 3. Decolorizer is used next to remove the primary stain (crystal violet) from Gram Negative bacteria (those with LPS imbedded in their cell walls). Decolorizer is composed of an organic solvent, such as, acetone or ethanol or a combination of both.)**
- 4. Finally, a counter stain (Safranin), is applied to stain those cells (Gram Negative) that have lost the primary stain as a result of decolorization.**

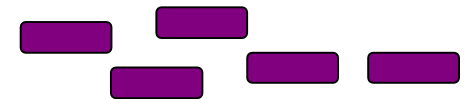
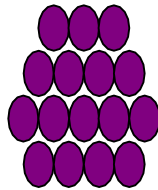
Gram +ve e.g.
S.aureus

Gram -ve
E.coli

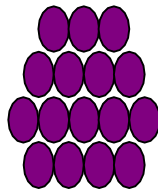
Step 1: Crystal Violet



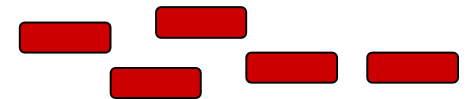
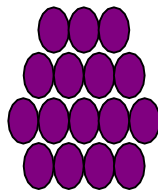
Step 2: Gram's Iodine



**Step 3: Decolorization
(Aceton-Alcohol)**



Step 4: Safranin Red



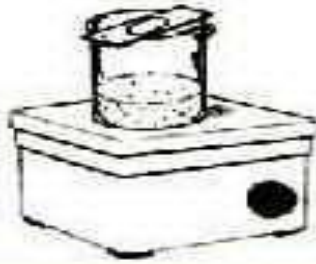
ACID-FAST STAINING

- **Acid-fast cells contain a large amount of lipids and waxes in their cell walls**
 - **primarily mycolic acid**
- **Acid fast bacteria are usually members of the genus *Mycobacterium* or *Nocardia* & are Gram-resistant (waxy cell walls)**
 - **Therefore, this stain is important to identify *Mycobacterium* or *Nocardia***

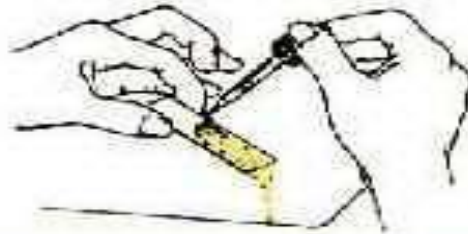


Ziehl-Neelsen staining

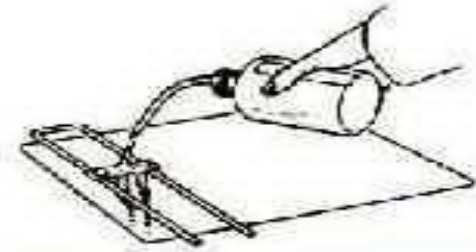
- **Ziehl-Neelsen staining is used to stain AFB [acid fast bacteria] that do not stain with the standard laboratory staining procedures like Gram staining.**
- **The stains used are the red colored Carbol fuchsin that stains the bacteria and a counter stain like Methylene blue or Malachite green.**
- **Primary stain binds to the cell wall mycolic acids so intense decolorization does not release primary stain from the cell wall of AFB.**
- **Color of AFB-based on primary stain, whereas Counterstain provides contrasting background**



1 Cover smear with carbolfuchsin. Steam over boiling water for 8 minutes. Add additional stain if stain boils off.



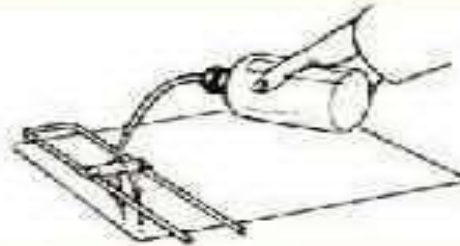
2 After slide has cooled decolorize with acid-alcohol for 15 to 20 seconds.



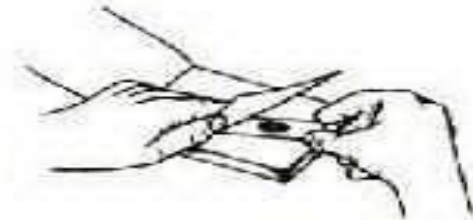
3 Stop decolorization action of acid-rinsing briefly with water.



4 Counterstain with methylene blue for 30 seconds.



5 Rinse briefly with water to remove excess methylene blue.



6 Blot dry with bibulous paper. Examine directly under oil immersion.

Ziehl-Neelsen acid-fast staining procedure

- **Pink bacilli – Acid fast bacteria/bacilli**
E.g., *M. tuberculosis* – long slender bacilli.
M. leprae – short thick bacilli.
- **Blue colored bacteria – Non-acid fast**
E.g., Epithelial cells, pus cells, other bacteria.

SPECIAL STAIN:

- Used to stain special structures of bacteria– capsule, spores, flagella, metachromatic granules etc.

CAPSULE STAIN:

Negative stain:

1. Drop of Nigrosin ink+ indian ink
2. Bacterial culture (1-2 colonies)
3. Spread evenly and air-dry.
4. Look for unstained structures against stained background

SPORE STAIN:

1. Malachite green- 2 min- heat stain till steam rises -2 min - wash.
2. Counterstain with safranin –1 min- wash.
3. Dry the slide and examine.

Spore forming bacteria:

Eg., Clostridium species.

Bacillus species – Eg.

B.anthraxis

THANK YOU