## Practical Work N° 3

# Microscopic observation of bacteria after staining

### Introduction

Bacterial staining is a method to enhance their contrast, making it easier to observe them under a bright-field microscope. The following types of staining are distinguished:

- Simple staining (using a single dye)
- Differential staining, such as Gram staining (using two dyes)
- Special staining for bacterial structures (capsules, spores, flagella, etc.).

### Objective

To prepare a bacterial smear and apply simple staining (Methylene Blue) and differential staining (Gram staining) techniques to observe bacterial morphology and classify bacteria based on their cell wall composition.

### Materials

- Methylene Blue
- Gentian Violet (Crystal violet)
- Lugol's iodine
- Fuchsin
- Decolorizing agent (70-90% alcohol)
- Bunsen burner
- Wash bottle
- Sterile distilled water
- Glass slides + wooden forceps
- Optical microscope

### Methods

**1. Preparation of the bacterial smear**

A bacterial smear is a thin layer of bacteria spread onto a glass slide and fixed to ensure the cells adhere firmly during staining.

This step is essential for maintaining the integrity of the bacterial structure and preventing the sample from washing off during the staining process. To achieve this, follow these steps:

1.1. **Spreading on the glass slide**

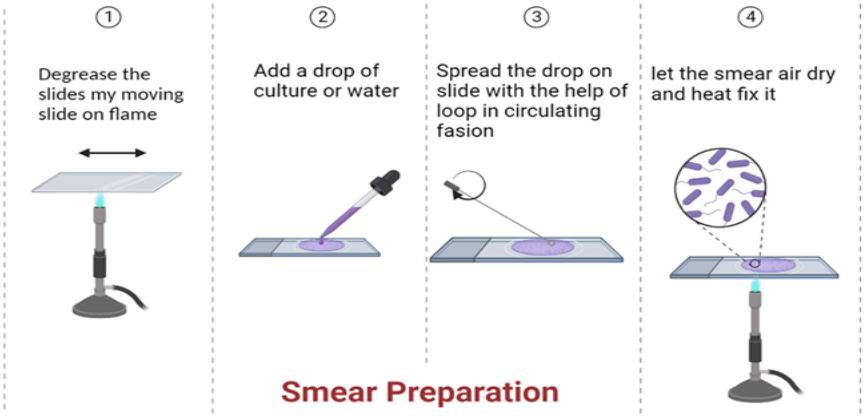
- Place a few drops of sterile distilled water on a clean, grease-free slide
- Aseptically pick up a small portion of a bacterial colony (or sample) using a platinum loop.
- Emulsify the colony in the water droplets and spread into a thin layer using circular motions with the loop.
- Air-dry the smear until it appears matte.

1.2. **Fixing the dried smear**:

- Hold the slide with forceps (smear side up) and pass it 3-4 times through the Bunsen burner flame.
- Allow it to cool before proceeding to staining.

This step aims to:

- ✓ Kill the bacteria, making their membranes more permeable to dyes.
- ✓ Preserve the cytological structure of the bacteria without altering their shape.
- ✓ Ensure the bacteria adhere to the slide surface.




**2. Simple staining using Methylene Blue dye**

Methylene blue staining is a quick, cost-effective, and commonly used staining method.

**2.1. Principle**

Methylene blue staining is a simple staining technique where a single dye is used to highlight specific structures in the sample, such as the shape (size and arrangement of bacteria). All organisms in the sample will appear the same colour, even if the sample contains more than one type of organism.

**2.2. Procedure for Methylene Blue staining**

After preparing the bacterial smear

- Place the slide on a staining rack and flood it with methylene blue. Let it sit for 1-3 minutes.
- Gently rinse the slide with distilled water, drain the excess water, and blot (do not rub) with absorbent paper. Allow the slide to air-dry completely.
- Examine under the 100x oil immersion objective.

**2.3. Result**

Stainable structures appear blue. The staining allows for the detection of the morphology of these structures, their grouping patterns (arrangement).

3. **Gram staining**

Gram staining differentiates bacteria into two main groups: Gram-positive and Gram-negative, based on their cell wall composition.

**3.1. Principle**

Gram-positive bacteria retain the crystal violet dye due to their thick peptidoglycan layer, while Gram-negative bacteria lose the dye and are counterstained pink with fuchsin. Gram staining also provides information about shapes of bacteria and their grouping patterns.

- Gentian Violet binds to cytoplasmic components; at this stage, all bacteria appear purple.
- Lugol's iodine reinforces this staining by forming a complex with Gentian Violet.
- Decolorization: Lipids are highly soluble in alcohol.

- ✓ In Gram-negative bacteria, whose cell walls are rich in lipids and low in peptidoglycan, alcohol dissolves these lipids, increasing wall permeability. Alcohol easily penetrates the cytoplasm and decolorizes it (dissolving the Gentian Violet).
- ✓ In Gram-positive bacteria, the cell wall is low in lipids and rich in peptidoglycan, which acts as an impermeable barrier to alcohol, leaving the cytoplasm stained purple.

- Fuchsin is used to visualize Gram-negative bacteria, which have become colourless. They are counterstained pink, while Gram-positive bacteria retain their purple colour.

**3.2. Procedure**

On the fixed and cooled smear:

- Cover the smear with Gentian Violet solution and let it sit for 1 minute.
- Rinse with tap water to remove excess dye.
- Cover the preparation with Lugol's iodine and let it sit for 1 minute.
- Rinse with water.
- Decolorize with 95% alcohol for 15 seconds.
- Rinse with running water.
- Cover the slide with Fuchsin solution and let it sit for 1 minute.
- Rinse thoroughly with water.
- Drain and gently dry between two sheets of clean, fine paper without rubbing.
- Observe under an optical microscope at 100x magnification with a drop of immersion oil.

**3.2. Results**

After staining, the following observation can be made:

- Bacteria stained dark purple; they retained the violet dye and are called **Gram-positive**.
- Bacteria stained pink or pale red; they lost the violet dye and are called **Gram-negative**.