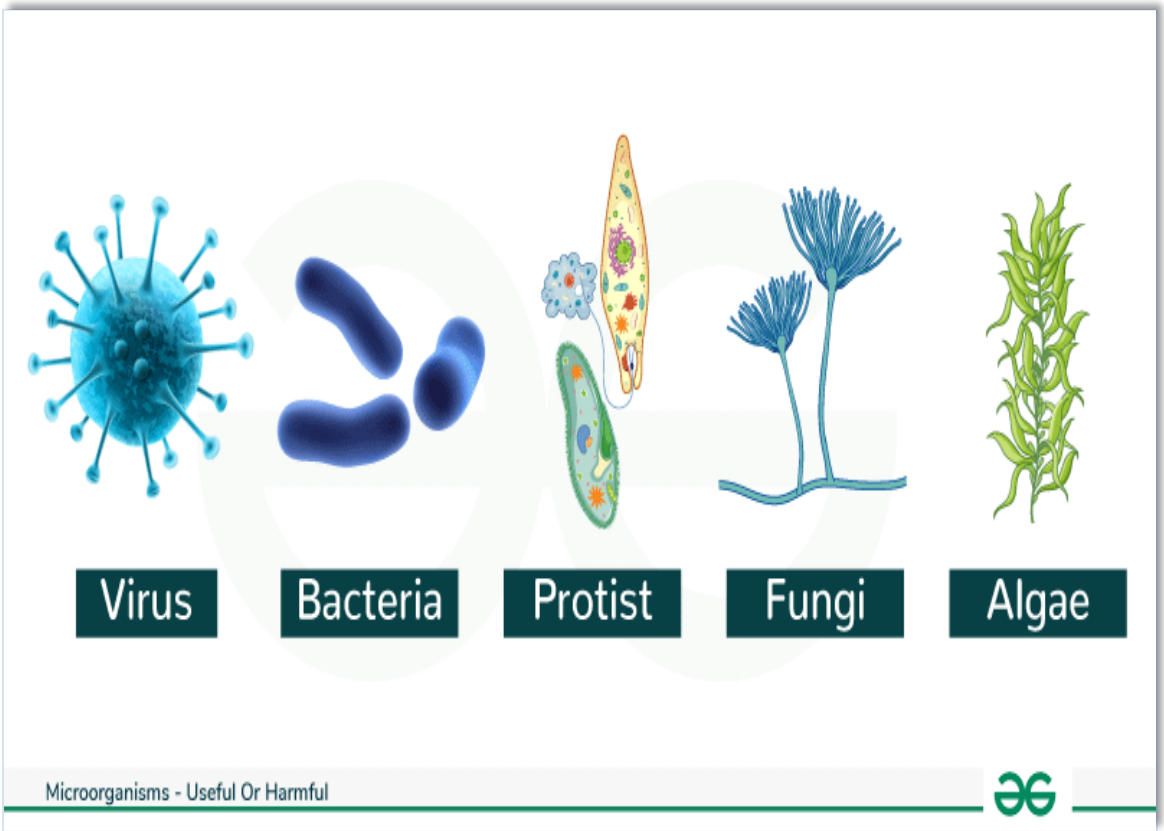
KING SAUD UNIVERSITY
COLLEGE OF SCIENCE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

Microbial Fine Structure

MBIO 222

Practical



Dr. Mohamed A. El-Tayeb

MBIO 222

Microbial Fine structures

Lab No# 1

Smear Preparation

- ## Aim of the Experiment:

  Prepare microorganisms for microscopic observation

- ## Introduction:

  The first step in most bacterial staining procedures is smear preparation. In a smear preparation, cells from a culture are spread in a thin film over a small area of a microscope slide, dried, and then fixed to the slide by heating or other chemical fixatives. A good smear preparation is the key to a good stain.

- ## Materials:

  1. Clean microscope slides
  2. Bunsen burner flame
  3. Staining trays
  4. Water bottle (for rinsing)
  5. Bacterial cultures

- ## Methods:

  1. Assemble the materials necessary for making the smears: a rack with the stock cultures to be used (these will be shared with other students working at your table), glass slides that have been soaked in acid alcohol or cleansed with a nonabrasive cleanser, an inoculating loop, a Bunsen burner, and a glass-marking pencil.

  2. Mark the first glass slide with a glass-marking pencil on the back of the slide.

  3. Follow the proper steps below depending on the source of your culture.

  <u>Preparing a smear from a plate culture</u>

  a. Sterilize your loop (from the hub of the handle along the entire length) in the Bunsen burner flame by holding it almost perpendicular to the flame until it glows. Remove the loop and air-cool.

b. Place a drop of water in the middle of each circle with your loop or a dropper. The water need not be sterile.

c. Sterilize your loop again and after it has cooled, remove a very small amount of inoculum from a single representative colony on the plate.

NOTE: If too much inoculum is taken, you will not obtain a good smear due to the flaking of cell aggregates upon drying. You are taking too much if you see a clump of cells on your loop.

d. Spread the inoculum in the first circle, filling the circle and mixing it with the water.

e. Flame the loop by placing it in the flame at the hub of the holder where the wire is attached and then gradually move the wire through the flame, until the wire glows. Finally, the loop itself is flamed until it is glowing. (If the wet loop is put immediately in the flame, it may splash out and contaminate the area.)

f. Let the slide air dry. You may speed up the drying process by placing the slide in a warm spot near a lamp or a Bunsen burner.

NOTE: When the slide is dry, the specimen may be hardly visible especially if the culture was taken from a broth; however, the surface of the slide will be dull and not shiny.

g. Fix the bacteria onto the slide by passing the slide, smear side up, and quickly through the flame of the burner two or three times. Avoid getting the slide too hot. This fixation process coagulates the proteins and fixes the bacteria onto the slide so they will not get washed off.

h. Your smear is now ready to be stained

- **Preparing a smear from a broth culture**

a. Sterilize your loop (from the hub of the handle along the entire length) in the Bunsen burner flame by holding it almost perpendicular to the flame until it glows. Remove the loop and air-cool.

b. Choose two of the cultures for the first slide. Holding one culture tube in your left hand at an angle (in the right hand if you are left-handed), remove the cap with your right hand. Flame the mouth of the slanted broth tube.

c. Insert the sterile, cooled loop into the culture and remove a loopful from the tube carefully to avoid touching the sides of the tube.

d. Flame the mouth of the tube, and quickly return the cap to the tube. Replace the tube in the rack.

e. Spread the inoculum in the first circle, filling the circle. Do not spread out the inoculum too thinly, as the bacterial cells will be widely separated in the broth medium to begin with, making it difficult for you to locate and focus on the cells during microscopic examination.

f. Flame the loop by placing it in the flame at the hub of the holder where the wire is attached and then gradually moving the wire through the flame, until the wire glows. Finally, the loop itself is flamed until it is glowing. In this way, the broth dries out gradually before incineration. If the wet loop is put immediately in the flame, it may splash out and contaminate the area.

g. Repeat steps c and d several times, each time waiting for the inoculum to dry on the slide. This procedure is especially needed if your sample is dilute.

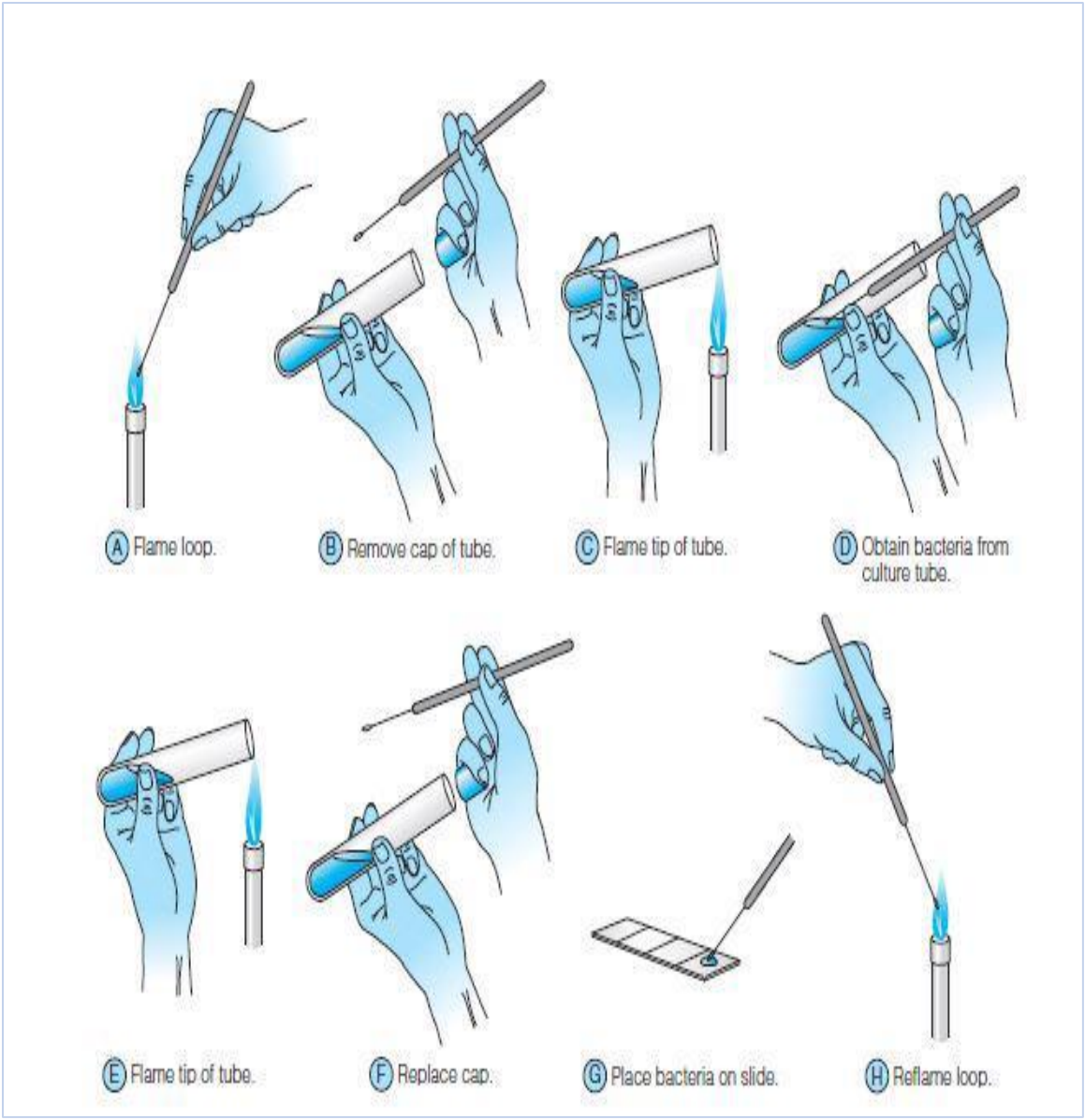
h. Repeat steps a to g for the second sample.

i. Let the slide air dry. You may speed up the drying process by placing the slide in a warm spot near a lamp or a Bunsen burner.

NOTE: When the slide is dry, the specimen may be hardly visible especially if the culture was taken from a broth; however, the surface of the slide will be dull and not shiny.

j. Fix the bacteria onto the slide by passing the slide, smear side up, and quickly through the flame of the burner two or three times. Avoid getting the slide too hot. This fixation process coagulates the proteins and fixes the bacteria onto the slide so they will not get washed off.

k. Your smear is now ready to be stained


A Flame loop.
B Remove cap of tube.
C Flame tip of tube.
D Obtain bacteria from culture tube.
E Flame tip of tube.
F Replace cap.
G Place bacteria on slide.
H Reflame loop.

MBIO 222

Microbial Fine structures

Lab No# 2

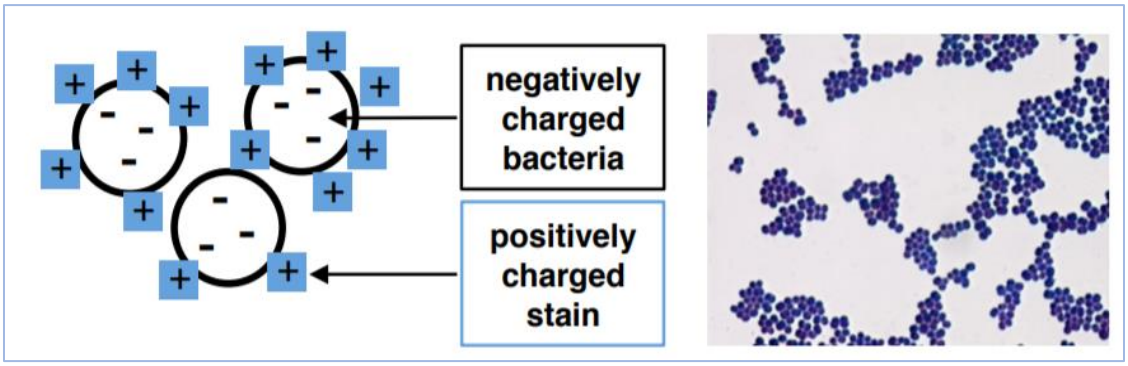
Simple Staining

- ## Aim of the Experiment:

  Observe stained microorganisms and identify their size, shape, and arrangement.

- ## Introduction:

One type of staining procedure that can be used is the **simple stain**, in which only one stain is used, and all types of bacteria appear as the color of that stain when viewed under the microscope. Some stains commonly used for simple staining include crystal violet, safranin, and methylene blue. Simple stains can be used to determine a bacterial species' morphology (cell shape) and arrangement (single, chains, clusters, etc.), but they do not give any additional information.

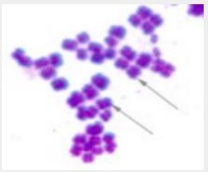
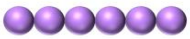
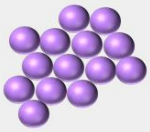
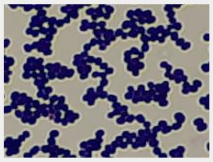
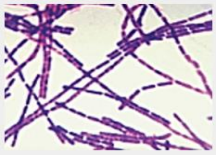
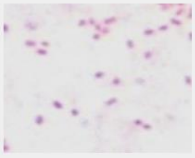


- ## Materials:

1. Clean microscope slides
2. Bunsen burner flame
3. Staining trays
4. Water bottle (for rinsing)
5. Bacterial cultures
6. Simple stains (crystal violet, safranin, and methylene blue)

- **Methods:**
  1. Use the slide(s) that you already prepared when creating bacterial smears and heat fixing (see above).
  2. Grip the microscope slide in wood clip over a waste container bucket.
  3. Add methylene blue stain to the heat-fixed smear. There is no reason to cover the entire slide with stain. Just make sure to cover the smear with stain.
  4. Set a timer for 1.5 or 2 minutes. The stain will remain on the smear during this time.
  5. Angle the slide and gently rinse the slide with DI or distilled water using a squirt bottle. Water is not applied directly to the smear. Instead, apply the water to the area of the slide just above the smear so that water will rinse across the smear and into the waste bucket. Continue rinsing until water running off the slide appears clear.
  6. Blot gently with bibulous paper. Do not wipe. Just gently blot with bibulous paper to get rid of excess water.
  7. Place the stained slide on a microscope and examine. Be sure to begin at the lowest power (look for the blue color of the methylene blue dye). Focus at lowest power and then increase one objective magnification at a time.

Cell Shape (singular / plural)	**Cell Arrangement** (singular / plural)	**Illustration**	**Description**	**Image** (color of cells is based on the type of stain used)
coccus / cocci	single or solitary		Cells are alone and round or spherical.	
coccus / cocci	diplococcus / diplococci		Cells are in pairs and round or spherical.	
coccus / cocci	tetrad		Cells are grouped in fours and are round or spherical.	

coccus / cocci	sarcina		Cells are group in eights as two groups of four on top of each other. Cells are round.	
coccus / cocci	streptococcus / streptococci		Cells are in a chain and are round or spherical.	
coccus / cocci	staphylococcus / staphylococci		Cells are in bunches or clusters and are round or spherical.	
spirochete / spirochetes	single or solitary		Cells are alone and long and thin with a three-dimensional corkscrew forming a helical shape.	
spirillum / spirilla	single or solitary		Cells are alone and long and thin with a wavy appearance but are not helical in shape.	
vibrio / vibrios	single or solitary		Cells are alone and have a rod shape with an arch or curve.	
bacillus / bacilli	single or solitary		Cells are alone and rod shaped.	
bacillus / bacilli	diplobacillus / diplobacilli		Cells are in pairs and are rod shaped.	
bacillus / bacilli	streptobacillus / streptobacilli		Cells are in chains and are rod shaped.	
bacillus / bacilli	palisades		Cells are rod shaped and lined up with each other long-ways resembling the pickets in a picket fence.	
coccobacillus / coccobacilli	single or solitary		Cells are alone and are short rods.	

MBIO 222

Microbial Fine structures

Lab No# 3

Capsule stain

- ## Aim of the Experiment:

  The purpose of the capsule stain is to reveal the presence of the bacterial capsule

- ## Introduction:

  Many bacteria, including both gram-positive and gram-negative, may be surrounded by an outer polysaccharide-containing layer termed the glycocalyx (Madigan, et al., 2000). Capsules are characterized by poor staining with standard dyes. Capsule staining methods thus depend upon revealing the presence of the capsule indirectly. Often capsule staining methods are accomplished using a combination of the following: (i) a basic dye that interacts with the negative ions of the bacterial cell, (ii) a mordant that causes the precipitation of the capsular material, e.g., metal ions, alcohol, and acetic acid, and (iii) an acidic stain used to color the background.

Anthony's capsule stain

In Anthony's capsule stain, crystal violet is used as the primary stain, interacting with the protein material in the culture broth or added during the staining, and copper sulfate serves as the mordant. There is no additional negative stain. At the completion of the stain, the bacterial cells and the background will be stained by crystal violet while the unstained capsule will appear white.

- ## Materials:

A. General materials ·

Staining tray ·

Staining rack ·

Slide holder ·

Disposable gloves

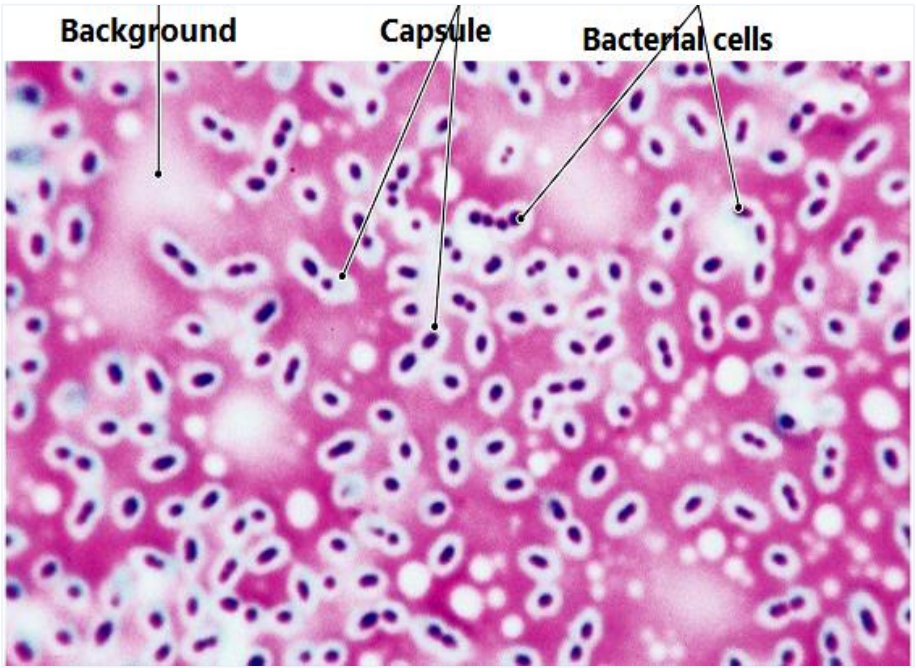
B. **Staining reagents** ·

Crystal violet 1% solution (primary stain) ·

Copper sulfate 20% (decolorizer agent)

- **Methods:**

1. Prepare a smear from a 12- to 18-hour culture grown in milk broth or litmus milk. (Serum protein may be used to prepare the smear if the organism was not grown in milk broth or litmus milk.) This is to provide a proteinaceous background for contrast.
2. Allow the smear to air dry. DO NOT HEAT FIX (to avoid destroying or distorting the capsule or causing shrinkage).
3. Cover the slide with 1% crystal violet for 2 minutes.
4. Rinse gently with a 20% solution of copper sulfate.
5. Air dry the slide. DO NOT BLOT. (Blotting will remove the unheated-fixed bacteria from the slide and/or cause disruption of the capsule.)
6. Examine the slide under an oil immersion lens. Bacterial cells and the proteinaceous background will appear purplish while the capsules will appear transparent.



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Microbial Fine structures

Lab No# 4

Flagella Stain

- **Aim of the Experiment:**

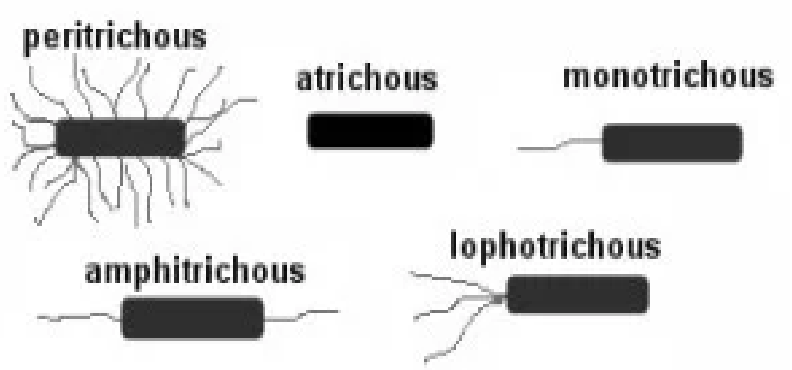
The purpose of the flagella stain is to reveal the presence of the bacterial flagella

- **Introduction:**

Flagella are complex filamentous cytoplasmic structures protruding through the cell wall. These are unbranched, long, thread-like structures, mostly composed of the protein *flagellin*, intricately embedded in the cell envelope. They are about 12-30 nm in diameter and 5-16 µm in length. They are responsible for the bacterial motility. Motility plays an important role in survival and the ability of certain bacteria to cause disease.

Types and Examples of Flagella

There are 4 types of flagellar distribution on bacteria:



1. Monotrichous

– Single polar flagellum

– Example: *Vibrio cholerae*

2. Amphitrichous

– Single flagellum on both sides

– Example: *Alkaligens faecalis*

3. Lophotrichous

– Tufts of flagella at one or both sides

– Example: *Spirillum*

4. Peritrichous

– Numerous flagella all over the bacterial body

– Example: *Salmonella* Typhi

- **Materials:**

- Clean microscope slides
- Bunsen burner flame
- Staining trays
- Water bottle (for rinsing)
- Bacterial cultures
- RYU flagella stain

Principle of Flagella Staining

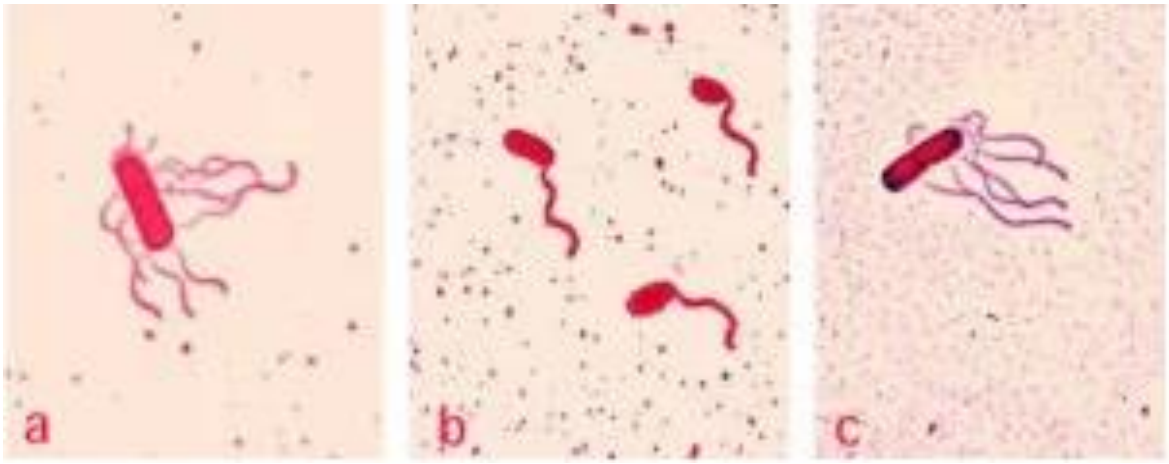
A wet mount technique for staining bacterial flagella is simple and is useful when the number and arrangement of flagella are critical in identifying species of motile bacteria.

Procedure of Flagella Staining

1. Grow the organisms to be stained at room temperature on blood agar for 16 to 24 hours.
2. Add a small drop of water to a microscope slide.
3. Dip a sterile inoculating loop into sterile water
4. Touch the loopful of water to the colony margin briefly (this allows motile cells to swim into the droplet of water).
5. Touch the loopful of motile cells to the drop of water on the slide.
6. Cover the faintly turbid drop of water on the slide with a cover slip. A proper wet mount has barely enough liquid to fill the space under a cover slip. Small air spaces around the edge are preferable.
7. Examine the slide immediately under 40x for motile cells.
8. If motile cells are seen, leave the slide at room temperature for 5 to 10 minutes.

9. Apply 2 drops of RYU flagella stain gently on the edge of the coverslip. The stain will flow by capillary action and mix with the cell suspension.
10. After 5 to 10 minutes at room temperature, examine the cells for flagella.
11. Cells with flagella may be observed at 100x.

Staining



Observe the slide and note the following:

1. Presence or absence of flagella
2. Number of flagella per cell
3. Location of flagella per cell

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Microbial Fine structures

Lab No# 5

Motility tests

- **Aim of the Experiment:**
  - To determine the motility of the bacterium.
  - To differentiate between motile and nonmotile bacteria.
- **Introduction:**

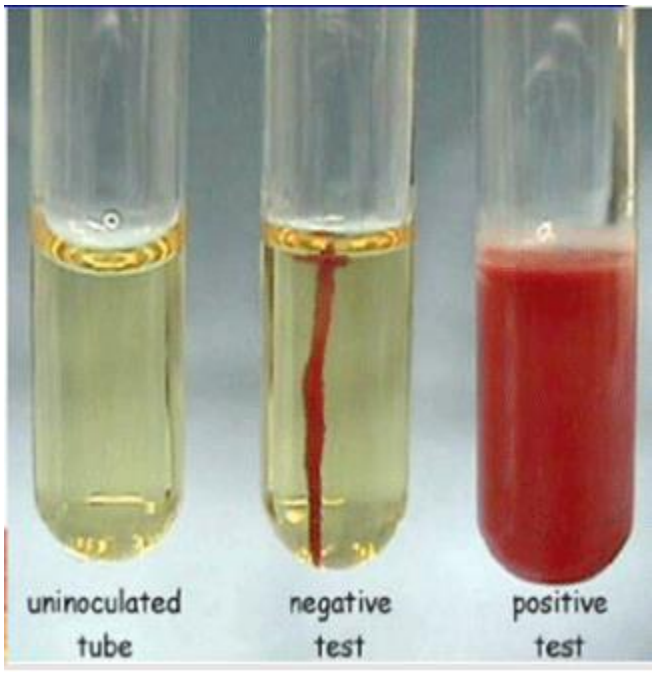
Motility is the ability of an organism to move by itself using propeller-like flagella unique to bacteria or by special fibrils that produce a gliding form of motility.

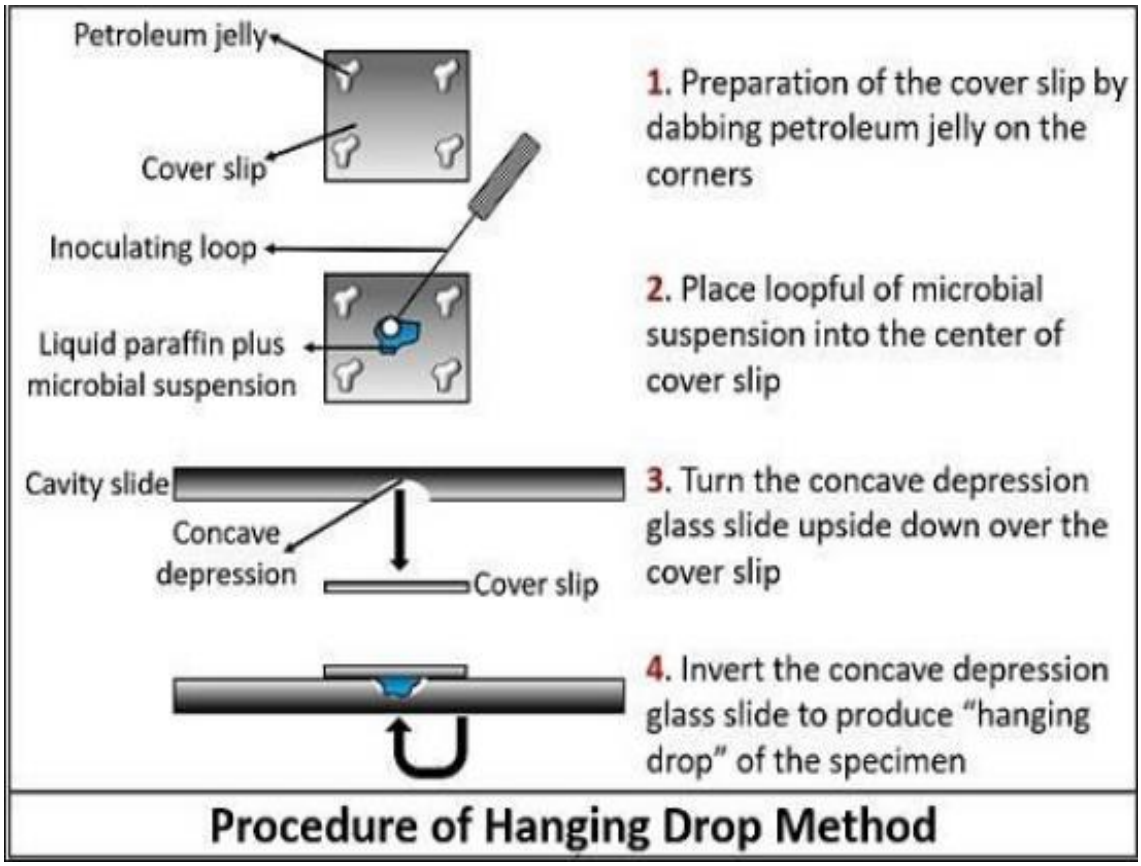
1. Motility by the bacterium is demonstrated in a semi-solid agar medium. The medium mainly used for this purpose is SIM medium (Sulphide Indole Motility medium) which is a combination differential medium that tests three different parameters, Sulfur Reduction, Indole Production, and Motility. This media has a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness. The inoculum is stabbed into the center of a semisolid agar deep using a sterile inoculating needle. Bacterial motility is evident by a diffuse zone of growth extending out from the line of inoculation. Some organisms grow throughout the entire medium, whereas others show small areas or nodules that grow out from the line of inoculation. The non-motile bacteria will only grow in the soft agar tube and only the area where they are inoculated.
2. The hanging drop method involves observing the tested organism in a drop suspended under a cover slip in a cavity slide

- **Methods:**

1. With a sterile straight needle, touch a colony of a young (18 to 24 hour) culture growing on agar medium.
2. Single stab down the center of the tube to about half the depth of the medium.

3. Incubate at 35°-37°C and examine daily for up to 7 days.





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Microbial Fine structures

Lab No# 6

Gram Staining

- ## Aim of the Experiment:

A method of staining is used to classify bacterial species into two large groups: gram-positive bacteria and gram-negative.

- ## Introduction:

Gram staining differentiates bacteria by the chemical and physical properties of their cell walls. Gram-positive cells have a thick layer of peptidoglycan in the cell wall that retains the primary stain, crystal violet. Gram-negative cells have a thinner peptidoglycan layer that allows the crystal violet to wash out on addition of ethanol. They are stained pink or red by the counterstain.

The first step in gram staining is the use of crystal violet dye for the slide's initial staining. The next step, also known as fixing the dye, involves using iodine to form a crystal violet-iodine complex to prevent easy removal of dye. Subsequently, a decolorizer, often a solvent of ethanol and acetone, is used to remove the dye. The basic principle of gram staining involves the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment. Gram-positive microorganisms have higher peptidoglycan content, whereas gram-negative organisms have higher lipid content. Initially, all bacteria take up crystal violet dye; however, with the use of solvent, the lipid layer from gram-negative organisms is dissolved. With the dissolution of the lipid layer, gram negatives lose the primary stain. In contrast, solvent dehydrates the gram-positive cell walls with the closure of pores preventing diffusion of violet-iodine complex, and thus, bacteria remain stained. The length of decolorization is a critical step in gram staining as prolonged exposure to a decolorizing agent can remove all the stains from both types of bacteria. The final step in gram staining is to use a basic fuchsin stain to give decolorized gram-negative bacteria a pink color for easier identification. It is also known as counterstain. Some laboratories use safranin as a counterstain; however, basic fuchsin stains gram-negative organisms more

intensely than safranin. Similarly, *Hemophilus* spp., *Legionella* app, and some anaerobic bacteria stain poorly with safranin.

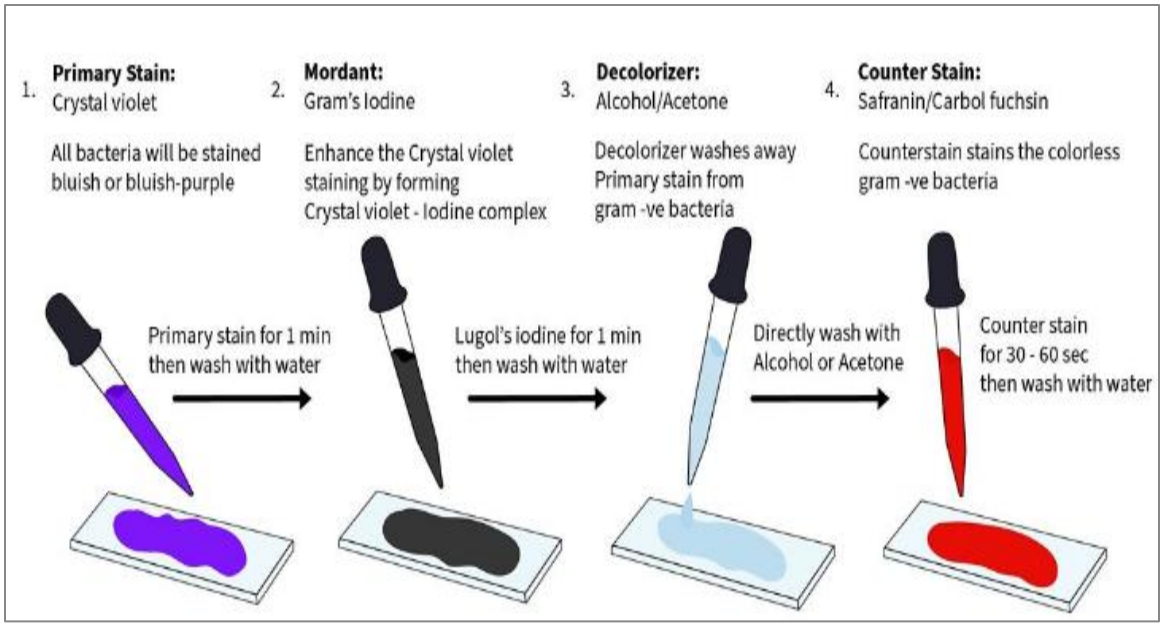
- **Materials:**

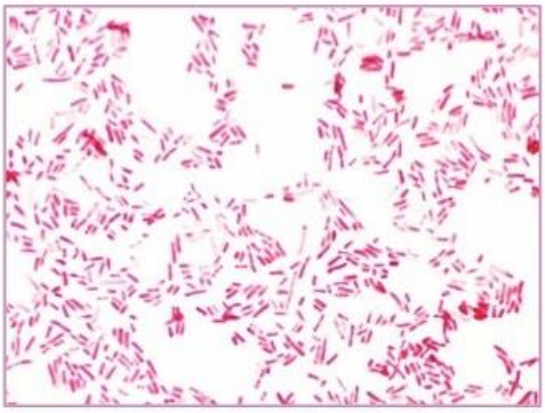
- Clean microscope slides
- Bunsen burner flame
- Staining trays
- Water bottle (for rinsing)
- Bacterial cultures
- Microscope
- Crystal violet (primary stain)
- Iodine solution/Gram's Iodine (mordant that fixes crystal violet to cell wall)
- Decolorizer (e.g. ethanol)
- Safranin (secondary stain)

- **Methods:**

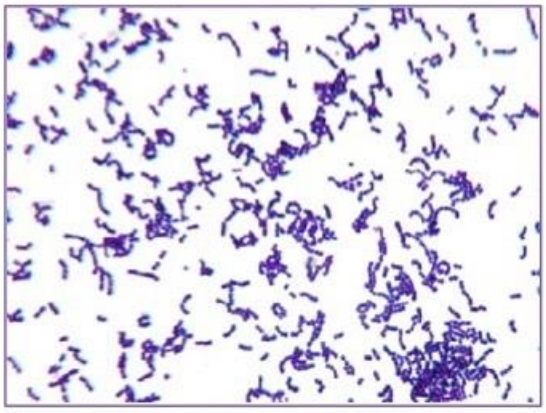
- Crystal violet stain is added over the fixed culture.
- After 10 to 60 seconds, the stain is poured off, and the excess stain is rinsed with water. The goal is to wash off the stain without losing the fixed culture.
- Iodine solution is used to cover the smear for 10 to 60 seconds. This step is known as "fixing the dye." The iodine solution is poured off, and the slide is rinsed with running water. Excess water from the surface is shaken off.
- A few drops of decolorizer are added to the slide. Decolorizers are often the mixed solvents of ethanol and acetone. This step is known as "solvent treatment." The slide is rinsed with water for 5 seconds. To prevent excess decolorization in the gram-positive cells, stop adding decolorizer as soon as the solvent is not colored as it flows over the slide.

- The smear is counterstained with Safranin solution for 40 to 60 seconds. The Safranin is washed off with water, and excess water is blotted with the bibulous paper. The slide can also be air-dried after shaking off excess water.
- The slide should undergo an examination under a microscope under oil immersion.
- The initial slide examination should use the X40 objective to evaluate the smear distribution, and then they should be examined using the X100 oil immersion objective.
- All areas of the slide require an initial examination. Areas that are only one cell thick should be examined. Thick areas in slides often give variable and incorrect results.
- White blood cells and macrophages stain Gram-negative.
- Squamous epithelial cells stain Gram-positive.





Gram-Negative Bacteria



Gram-Positive Bacteria

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Microbial Fine structures

Lab No# 7

Gram Staining (2)

Specimen Collection

Various clinical specimens can be used to perform Gram staining. Some of the commonly used specimens are sputum, blood, cerebrospinal fluid, ascitic fluid, synovial fluid, pleural fluid, and urine, etc. Swabs from nostrils, throat, rectum, wound, and cervix, etc. can also be used. The collection of specimens should always be in sterile containers.

Indications

Gram staining is indicated whenever a bacterial infection is suspected for easy and early diagnosis.

Potential Diagnosis

Gram staining aids in the diagnosis of a disease or a pathologic condition.

Examples of gram-positive organisms are:

- Cocci: *Staphylococcus* species, and *Streptococcus* species
- Bacilli: *Corynebacterium* species, *Clostridium* species, and *Listeria* species
- Examples of gram-negative organisms are
- Cocci: *Neisseria gonorrheae, Neisseria meningitidis,* and *Moraxella* species
- Bacilli: *Escherichia coil, Pseudomonas* species, *Proteus* species, and *Klebsiella* species
- Examples of gram variable organisms include:
- *Actinomyces* species

Interfering Factors

If the specimen collection is not sterile, multiple organisms can contaminate the specimen. Similarly, improper specimen collection and prior use of antibiotics can interfere with the growth of organisms. During the interpretation of the Gram stain, as described by the World Health Organization in 2003, the following steps should be followed:

1. General nature of the smear requires analysis under low power magnification (10X)

- The background of the slide should generally be gram-negative or clear
- White blood cells when present should stain gram-negative
- Thin crystal violet or gentian violent precipitates should not be confused with gram-positive bacillus bacteria
- The smear should be one cell thick with no overlapping of cells

2. Low power magnification should be utilized to note the following:

- Relative numbers of polymorphonuclear neutrophils (PMNs), mononuclear cells and red blood cells (RBCs)
- Relative numbers of squamous epithelial cells, and normal microbiota bacteria
- Location, arrangement, and shape of the organisms

3. Oil immersion examination of multiple fields is necessary to note the following:

- Micro-organisms: If identified, please note numbers and morphology
- Shapes: coccus, bacillus, coccobacillus, filaments, and yeast-like
- The appearance of ends: rounded, tapered, concave, clubbed and flattened
- The appearance of sides: parallel, ovoid, irregular or concave
- The axis of the organism: straight, curved or spiral
- Pleomorphism (variation in shape)
- Branching or cellular extensions

Complications

The interpretation of slides can be difficult if the microscopic smear is thick and clumped. Decolorization time should have very close monitoring to avoid under-decolorization or over-decolorization. Thicker smears require longer decolorizing time. Similarly, cultures should undergo evaluation while they are still fresh. Old cultures tend to lose the peptidoglycan cell walls, which predisposes gram-positive cells to be gram-negative or gram variable. Gram stain is not useful for organisms without a cell wall like *Mycoplasma* species, and for smaller bacteria like *Chlamydia* and *Rickettsia* species.

Gram stain may not falsely reveal organisms in the following scenario:

- Use of antibiotics before collecting a specimen
- Inappropriate age of culture: too young or too old
- Fixing the smear before it dry
- The smear is too thick
- Low concentration of crystal violet
- Excessive heat fixation
- Excessive washing between steps
- Insufficient exposure to iodine
- Prolonged decolorization
- Excessive counterstaining
- Lack of experience in preparing the slide, and reviewing the slide

Sometimes results of Gram-stain may not match the final results of cultures and could potentially lead to inappropriate use of antibiotics.

Clinical Significance

Gram stain is often the initial diagnostic test for the evaluation of infections. The use of Gram stain facilitates the rapid use of appropriate antibiotics. However, genetic sequences and molecular techniques are more specific than classic gram stain.

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Microbial Fine structures

Lab No# 8

Spore stain

- ## Aim of the Experiment:

  To differentiate the spores within the bacterial cell

- ## Introduction

Bacterial endospores are differentiated cells formed within the vegetative cells. In bacteria, the endospores serve as a protective structure for the survival of the organisms; they do not have a role in reproduction. Some endospores are visible within vegetative cells; others appear outside of vegetative cells when the cellular content that surrounds an endospore disintegrates (lyses). The endospores located outside of cells are called free spores. Bacteria in genera such as Bacillus and Clostridium produce quite a resistant structure capable of surviving for long periods in an unfavorable environment and then giving rise to a new bacterial cell.

This structure is called an Endospore since it develops within the bacterial cell.

Endospores may be located: - In the middle of the bacterium (Central). -At the end of the bacterium (Terminal). -Near the end of the bacteria (Subterminal) and may be spherical or elliptical.

Endospores do not stain easily, but, once stained, they strongly resist decolorization. This property is the basis of the Schaeffer-Fulton or Wirtz-Conklin method of staining endospores.

- ## Materials
  - Ethanol
  - Malachite green
  - Safranin
  - Slides
  - covers

- ## Method

1. Air dry and heat fix the organism on a glass slide and cover it with a square of blotting paper or toweling cut to fit the slide.

2. Saturate the blotting paper with malachite green stain solution and steam for 5 minutes, keeping the paper moist and adding more dye as required.

Alternatively, the slide may be steamed over a container of boiling water.

3. Wash the slide in tap water.

4. Counterstain with safranin for 30 seconds. Wash with tap water; blot dry.

5. Examine the slide under the oil immersion lens (1,000X) for the presence of endospores.

Endospores are **bright green** and vegetative cells are **brownish red to pink**.

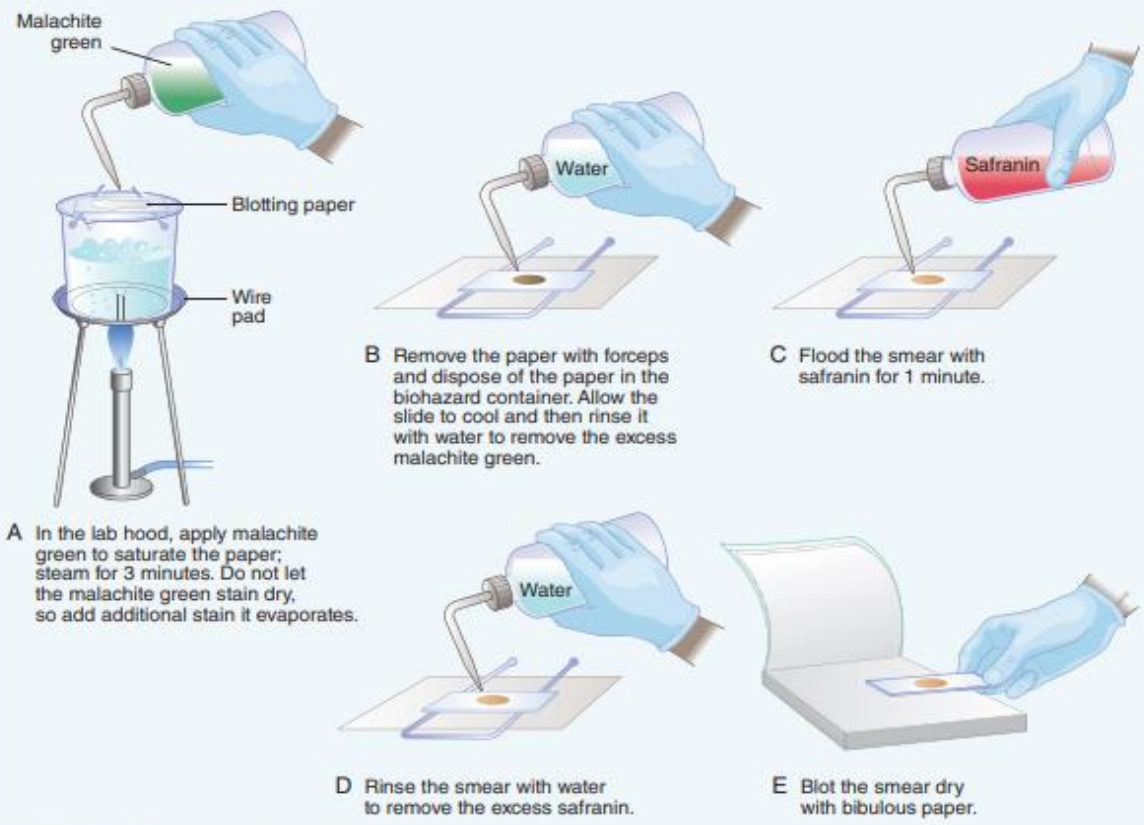


FIGURE 16.2 The spore stain technique.

FIGURE 16.3 Counterstain with safranin for approximately 1 minute.

MBIO 222

Microbial Fine structures

Lab No# 9

Fungi Staining

- ## Aim of the Experiment:

  Detection of fungal cells

- ## Introduction

Lactophenol Cotton Blue (LPCB) Staining is a simple histological staining method used for the microscopic examination and identification of fungi.

- ## Materials
  - Lactophenol Cotton Blue
  - Fungal culture
  - Coverslip
  - Slides
- ## Method

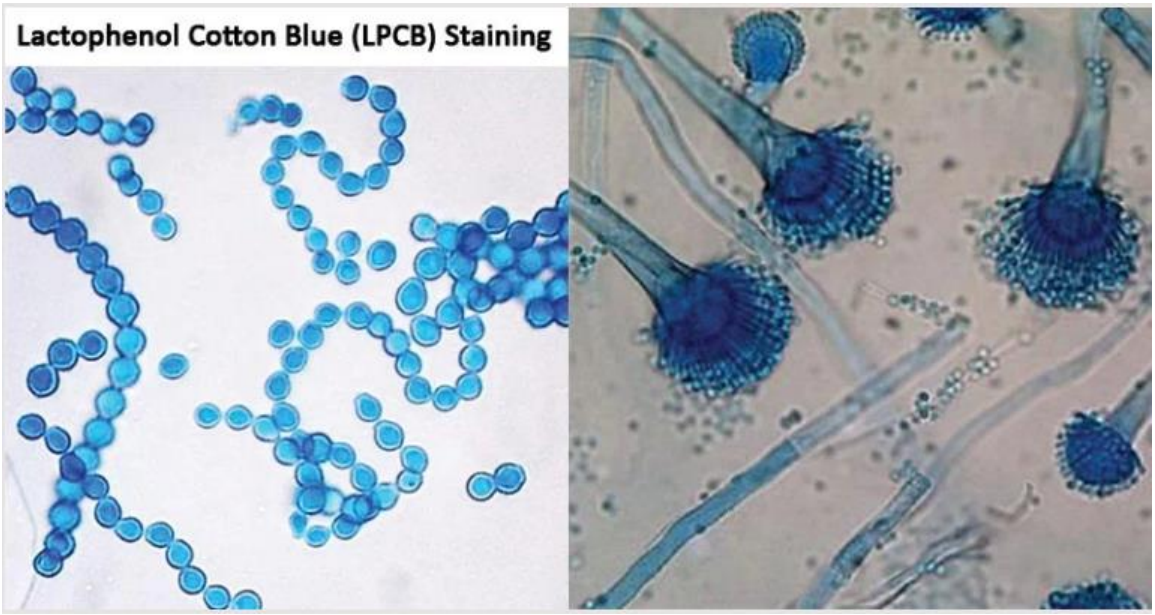
1. On a clean microscopic glass slide, add a drop of 70% ethanol
2. Add the fungal specimen to the drop of alcohol using a sterile mounter such as an inoculation loop (from solid medium), depending on the sample of use.
3. Tease the fungal sample of the alcohol using a needle mounter, to ensure the sample mixes well with the alcohol.
4. Using a dropper or pipette, add one or two drops of Lactophenol Cotton Blue Solution (prepared above) before the ethanol dries off.
5. Carefully cover the stain with a clean sterile coverslip without making air bubbles to the stain.
6. Examine the stain microscopically at 40X, to observe for fungal spores and other fungal structures.

- **Result**

Fungal spores, hyphae, and fruiting structures stain blue while the background stains pale blue.

For example,

- *Aspergillus niger* stains the hyphae and fruiting structures a delicate blue with a pale blue background.
- *Trichophyton mentagrophytes* also stains the hyphae and fruiting structures a delicate blue with a pale blue background.



MBIO 222

Microbial Fine structures

Lab No# 10

Electronic Microscopes