

Bacterial taxonomy Lab:1

Staining & Differential stains

Bacterial taxonomy

Bacterial taxonomy includes three main components:

1-Classification: the arrangement of bacteria into groups based on similarities in their characteristics such as morphology, physiology, biochemical properties, and genetic relationships.

2-Nomenclature: the system of naming bacteria according to internationally accepted rules and standards.

2-Identification: the process of determining the identity of bacterial isolates through laboratory methods such as staining techniques, biochemical tests, and molecular analysis.

Bacterial shapes

Bacteria have three main shapes: cocci (spherical), bacilli (rod-shaped), and spirals (helical or curved), with variations like comma-shaped vibrio, flexible spirochetes, and different arrangements like chains (strepto-) or clusters (staphylo). These shapes help in identification, with subgroups like diplococci (pairs of spheres) or streptobacilli (chains of rods) emerging from how they divide and group.

1-Basic Shapes

- Coccus (plural: Cocci): Spherical or round, sometimes oval or bean-shaped.
- Bacillus (plural: Bacilli): Rod-shaped.
- Spiral: Curved or twisted shapes.
 - Vibrio: Comma-shaped.
 - Spirillum (plural: Spirilla): Rigid, corkscrew-like spirals.
 - Spirochete: Long, flexible, and slender spirals.

2-Common Arrangements (for Cocci & Bacilli)

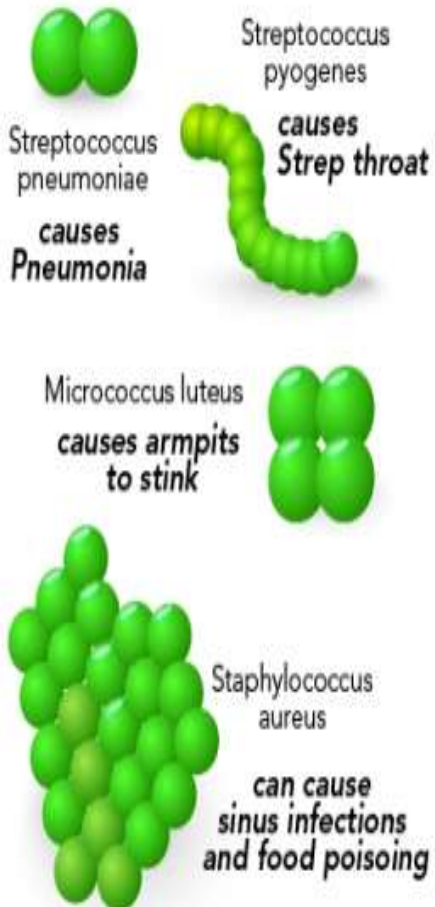
- **Diplo-**: In pairs (e.g., Diplococci, Diplobacilli).
- **Strepto-**: In chains (e.g., Streptococci, Streptobacilli).
- **Staphylo-**: In grape-like clusters (e.g., Staphylococci).
- **Tetrad**: Groups of four cocci.
- **Sarcina**: Cubical packets of eight cocci.

3-Other Forms

- **Pleomorphic**: Bacteria that can vary in shape.
- **Coccobacillus**: An intermediate shape, between cocci and bacilli.

SHAPES OF BACTERIA

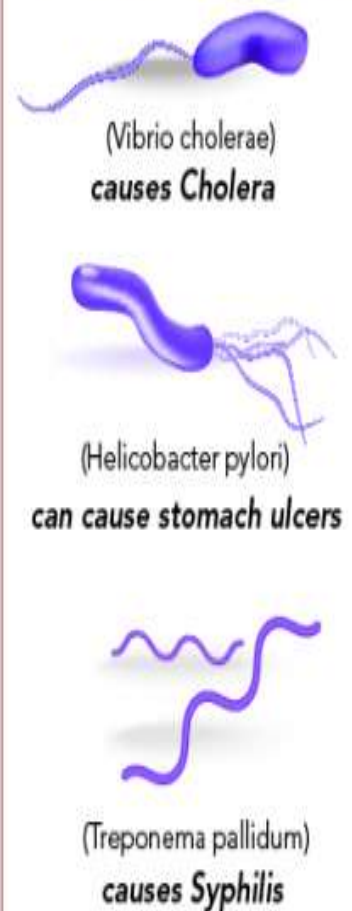
SPHERICAL (COCCI)



ROD-SHAPED (BACILLI)



SPIRAL-SHAPED (AND OTHERS)



Staining

Bacterial staining techniques are essential microbiology methods used to enhance contrast, observe morphology (shape/arrangement), and differentiate bacteria. The most critical techniques include Gram staining (dividing bacteria into Gram-positive/purple & Gram-negative/red), acid-fast staining for *Mycobacterium* and special staining.

The types of dyes

Dyes used in microbiological staining are classified according to their chemical properties and charge into three main types:

1-Basic dyes (cationic dyes):

These dyes carry a positive charge and bind to negatively charged components of bacterial cells, resulting in direct staining of the cells. Examples include crystal violet, methylene blue, and safranin.

2-Acidic dyes (anionic dyes):

These dyes carry a negative charge and are repelled by the negatively charged bacterial surface; therefore, they stain the background rather than the bacterial cells (negative staining). Examples include Eosin, Congo red and Nigrosin.

3-Neutral dyes:

These dyes are formed by combining acidic and basic dyes and are used in certain differential staining techniques.

Common Staining Categories

- **Simple Stain:** Uses one dye (e.g., methylene blue, safranin) to stain all cells the same color.
- **Differential Stain:** Uses multiple dyes to differentiate types (e.g., Gram stain, Acid-fast).
- **Special Stain:** Special stains are staining techniques used to visualize specific structures or components of bacterial cells that cannot be clearly observed using simple or Gram staining methods. These stains include structural stains such as capsule, endospore, and flagella stains.

1-Gram staining

In Biology, specifically in Microbiology Gram Staining is an inevitable technique. It was introduced in the year 1884 by Hans Christian Gram. This staining process can classify a bacteria into Gram Positive and Gram Negative. It can be used as a step in various bacterial diagnosis.

Purpose

Gram staining is used to identify the bacteria which cause certain infections. This can be done using blood, body fluids, etc. This helps to give clear information to start medication. This is not the final or only way of diagnosis. This is only used to classify the bacteria. All the bacteria cannot be classified by this method.

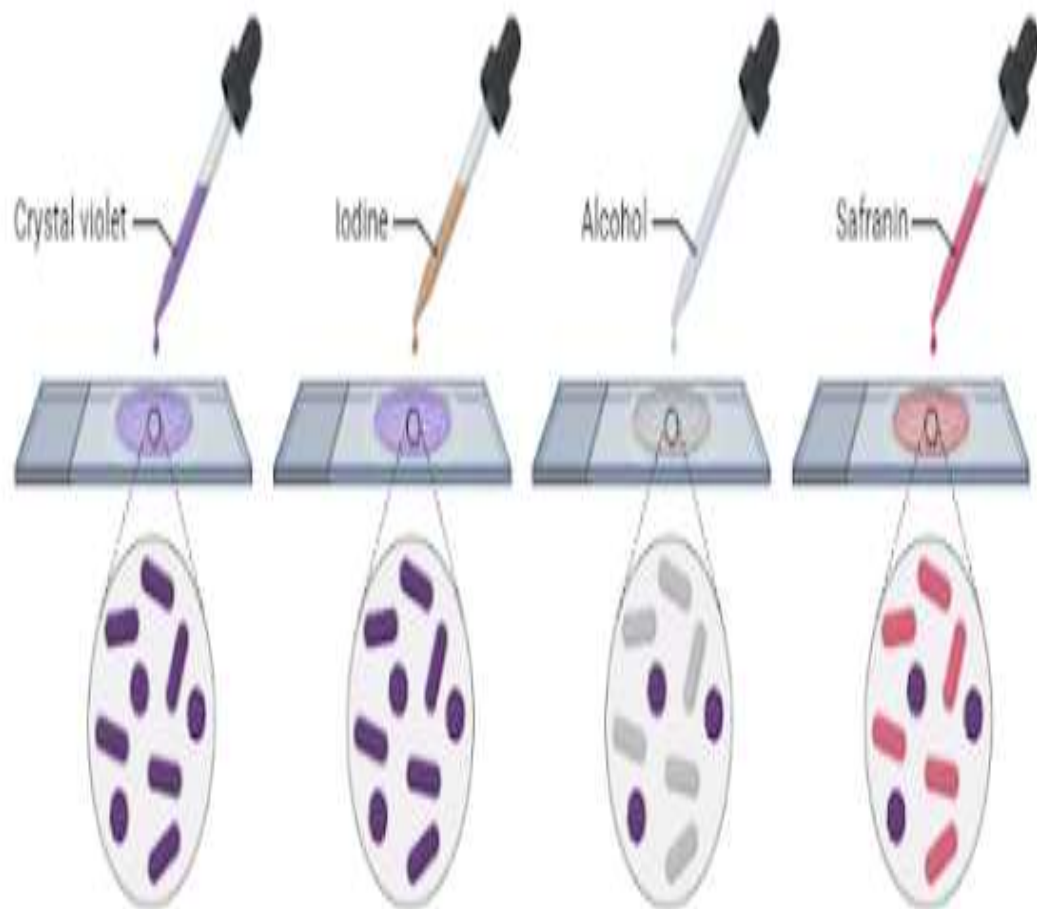
Reagents Used

1. Primary stain – Crystal Violet
2. Secondary stain or counter stain – Safranin
3. Gram's Iodine (Mordant – Used along with Crystal Violet)
4. Acetone and Alcohol based decolorizing agent.

Principle

It is the process in which the bacteria is stained with a series of reagents used for gram staining. It is initially stained with the primary stain which is Crystal Violet. The primary stain is fixed by iodine. Then it is treated with alcohol so that decolorization will take place. Some bacteria can retain the stain but some cannot. So the bacteria which retains the Crystal Violet – Iodine mixture is called Gram-Positive Bacteria. They will appear in blue or purple color. The retaining of the stain complex is due to the cell wall of the bacteria. During decolorization, the pores in the cell wall containing the stain complex dehydrate and shrink. So the stain cannot move out and decolorization will not take place.

The cell wall of the Gram-Negative bacteria is very thin. It cannot retain the Crystal Violet – Iodine mixture. So during destaining, the stain mixture will be washed off. For identification, it is again stained with safranin. This bacteria take up the safranin and look red/pink in color.



GRAM-POSITIVE



Fixation



Crystal Violet



Iodine Treatment



Decolorisation



Counter stain with
Safranin

GRAM-NEGATIVE



Preparation of the smear

- Take a clean grease free slide.
- Transfer a loop of the sample (for example, sputum, CSF, or pus) to the microscope slide. If performing a Gram stain from a bacterial colony, first put a drop or a few loopful of water and emulsify the bacterial colony in the water drop.
- Spread the sample to an even-thin film over a circle of 15 mm diameter.
- Air dry the sample, and once the sample gets air dried, heat fix the smear by passing it through a bunsen burner three times. Heat application helps the cell adhesion (fixation) to the glass slide and prevents its loss during rinsing.

Allow the slide to cool to the touch before applying the stain. Alternatively, the smear can be fixed using methanol.

Methanol fixation

Place or hold the slide over a paper towel and flood the slide with absolute methanol for two minutes. Alternatively, dip the slide into a Coplin jar filled with methanol.

Once two minutes have passed, tilt the slide and drain off the excess methanol and let the slide air dry. Do not wipe or blot the slide, as this can remove cells.

Procedure

- 1. Take a freshly cleaned glass slide.**
- 2. Smear the sample into the slide with the appropriate smearing procedure.**
- 3. Dry the sample at room temperature and then gently heat-fix it.**
- 4. The primary dye Crystal Violet must be poured over the slide containing the sample and kept for about 60 seconds and rinsed with water.**
- 5. Add the mordant (Gram's Iodine), keep it for 60 seconds, and wash it with water.**
- 6. Add 95% alcohol or acetone or a mixture of both used for rapid decolorization (20-30 sec).**
- 7. Add safranin for 60 seconds (Counter staining).**
- 8. Dry the slide and observe under the microscope.**
 - Gram Negative: Red/Pink Color**
 - Gram Positive: Blue Color**

Explain the Steps of Gram Staining

- 1. Fixation of clinical materials to the surface of the microscope slide either by heating or by using methanol. (Methanol fixation is recommended rather than heat fixation. Methanol fixation preserves the morphology of host cells and bacteria. Heating the slide causes cell distortion, could increase cell debris, and may cause erroneous Gram stain results.**
- 2. Application of the primary stain (crystal violet). Crystal violet is a dark blue to purple dye. It stains all cells blue/purple.**
- 3. Application of mordant: The iodine solution (mordant) is added to form a crystal violet-iodine (CV-I) complex; all cells continue to appear blue.**
- 4. Decolorization step: The decolorization step distinguishes gram-positive from gram-negative cells. The organic solvent such as acetone or ethanol extracts the blue dye complex from the lipid-rich, thin-walled gram-negative bacteria to a greater degree than from the lipid-poor, thick-walled, gram-positive bacteria. The gram-negative bacteria appear colorless, and gram-positive bacteria remain blue.**
- 5. Application of counterstain (safranin): The red dye safranin stains the decolorized gram-negative cells red/pink; the gram-positive bacteria remain purple.**

Gram-Positive Bacteria

It is a type of bacteria which have a thick peptidoglycan outer cell wall. This peptidoglycan layer causes Gram-Positive bacteria to look Blue by Gram Staining.

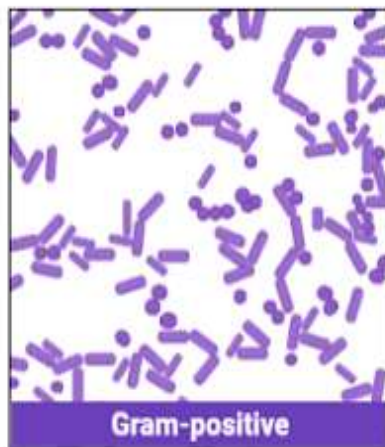
Examples: *Streptococcus* spp, *Clostridium* spp

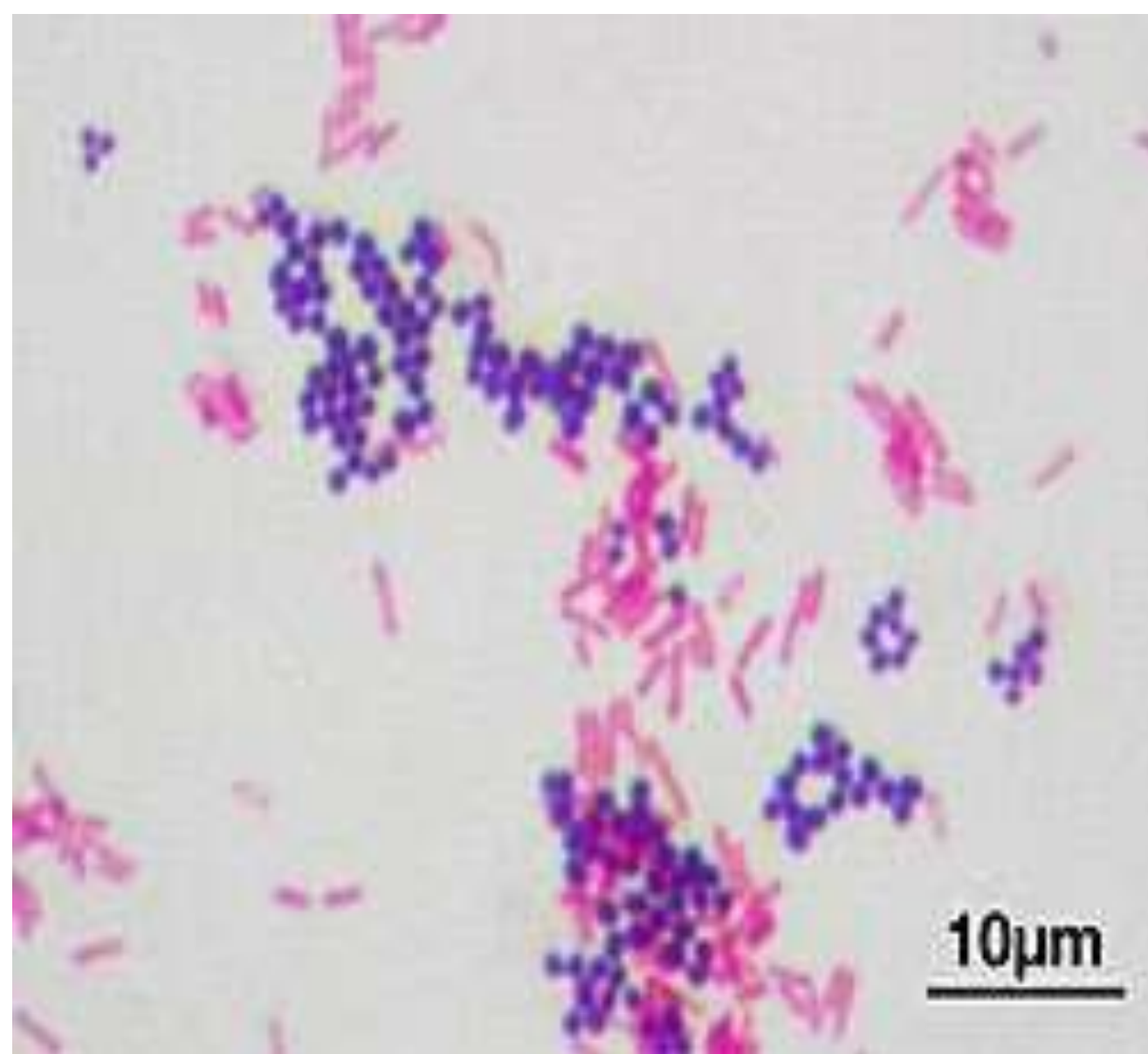
Gram-Negative Bacteria

It is a type of bacteria having thin layers of a peptidoglycan layer. This causes the Gram stain to get washed away and retains the counter stain safranin to retain and appear Red.

Examples:

Escherichia Coli, *Pseudomonas* species





Bacterial taxonomy

Lab: Two

Staining & Differential stains

Acid fast Stain (Ziehl-Neelsen stain)

Acid-fast staining is a crucial differential laboratory technique used to identify waxy-walled bacteria, primarily *Mycobacterium tuberculosis*, by staining them with red

carbol-fuchsin that resists acid-alcohol decolorization. This method separates organisms into acid-fast (**red/pink**) and non-acid-fast (**blue**) groups, aiding in the diagnosis of TB, leprosy, and Nocardia infections.

Acid fast Staining (Ziehl-Neelsen staining)

Technique

Procedure Steps

1. Prepare Smear: Air-dry and heat-fix a thin smear of the specimen onto a clean slide.

2. Primary Stain (Carbolfuchsin):

- Cover the smear with carbolfuchsin.
- Place a piece of filter paper over the stain and steam the slide over a water bath (or using a Bunsen burner) for 3-5 minutes, ensuring it doesn't boil dry and adding more stain if needed.
- Rinse thoroughly with water.

3. Decolorization (Acid-Alcohol):

- Apply acid-alcohol for about 15-30 seconds, or until the red color runs pale pink. (Careful: it's flammable).
- Rinse well with water.

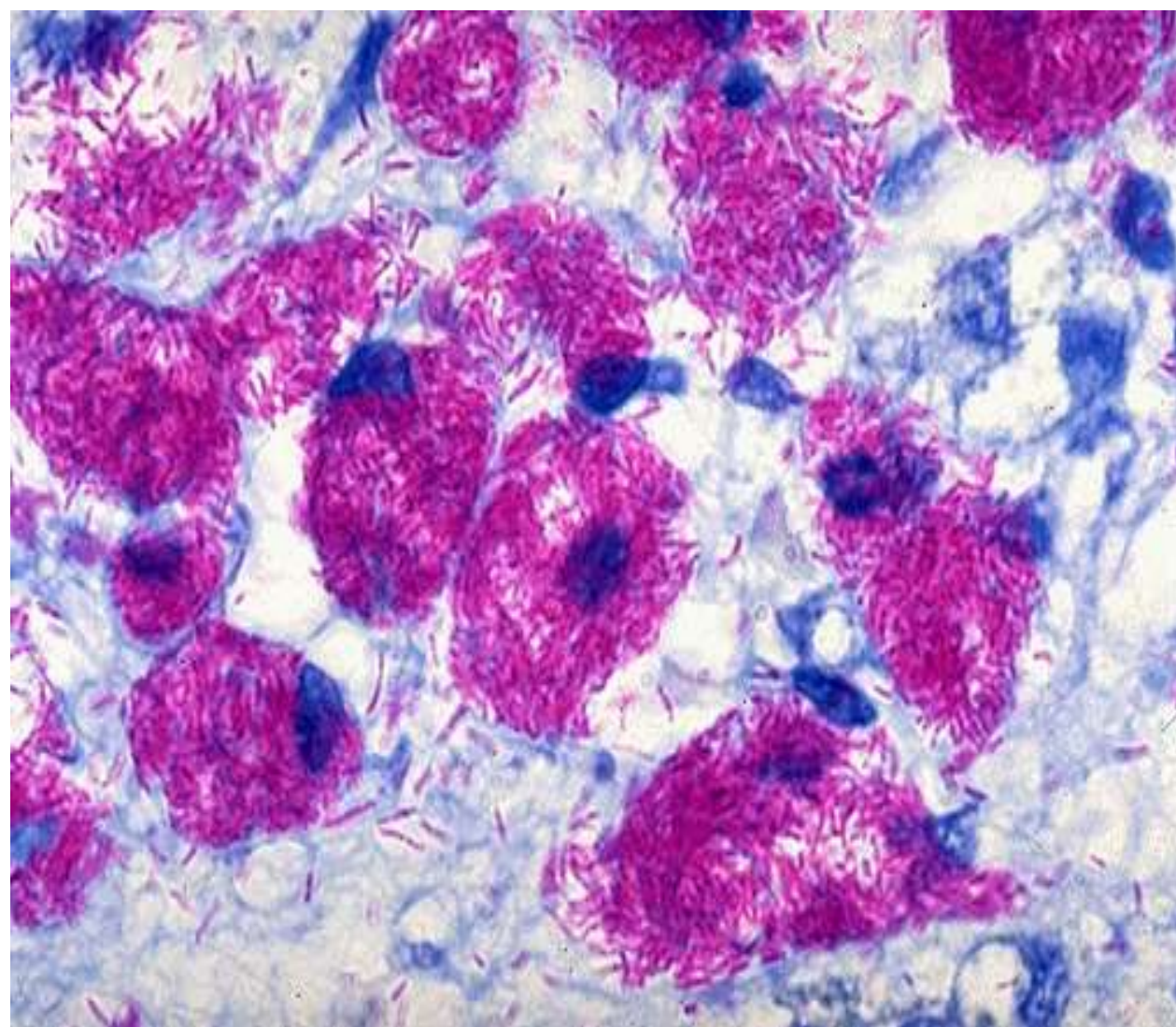
4. Counterstain (Methylene Blue):

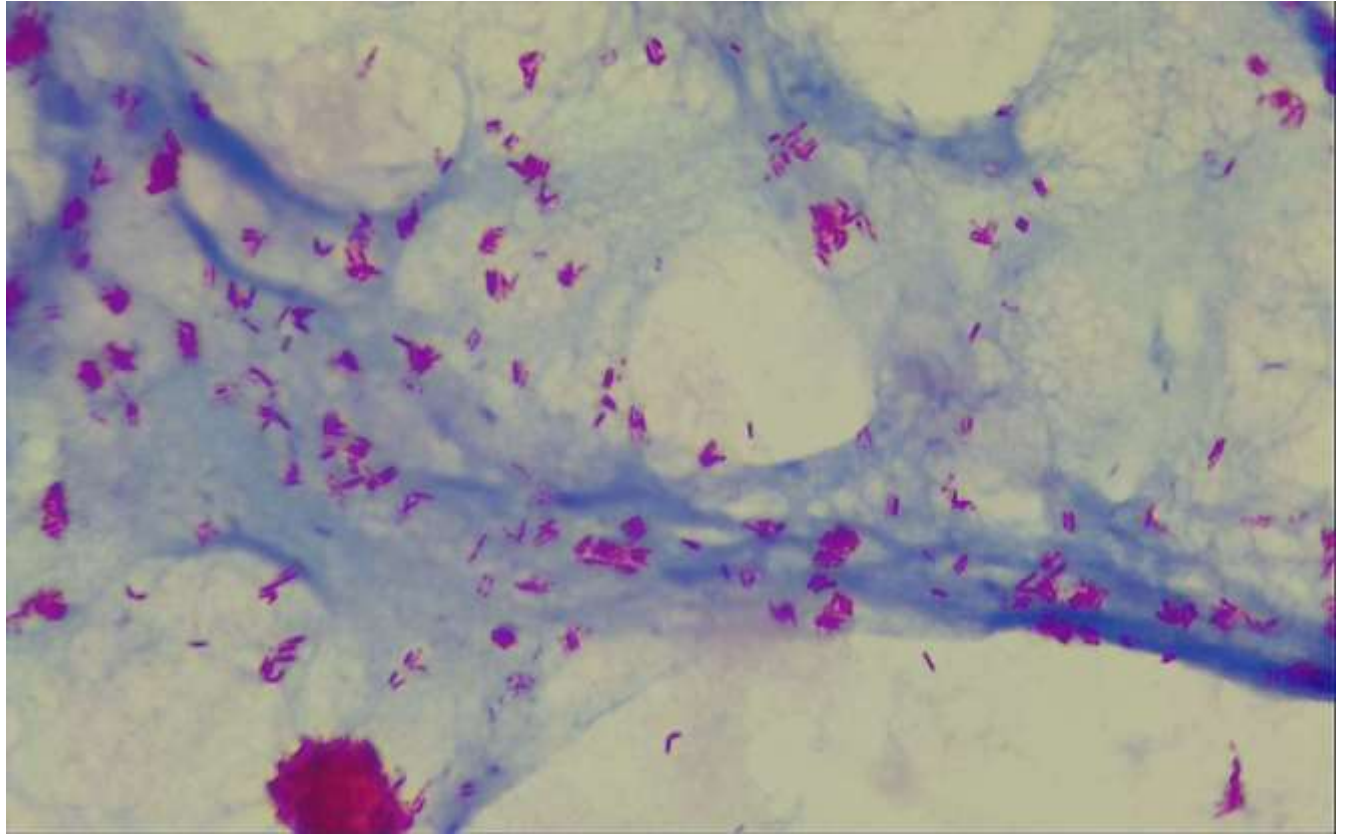
- Flood the slide with methylene blue for 1-2 minutes.
- Rinse with water.

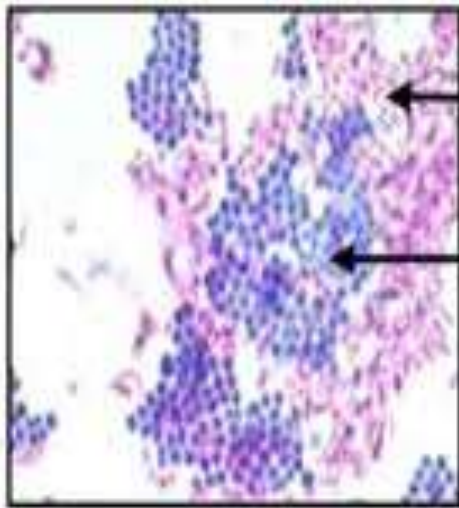
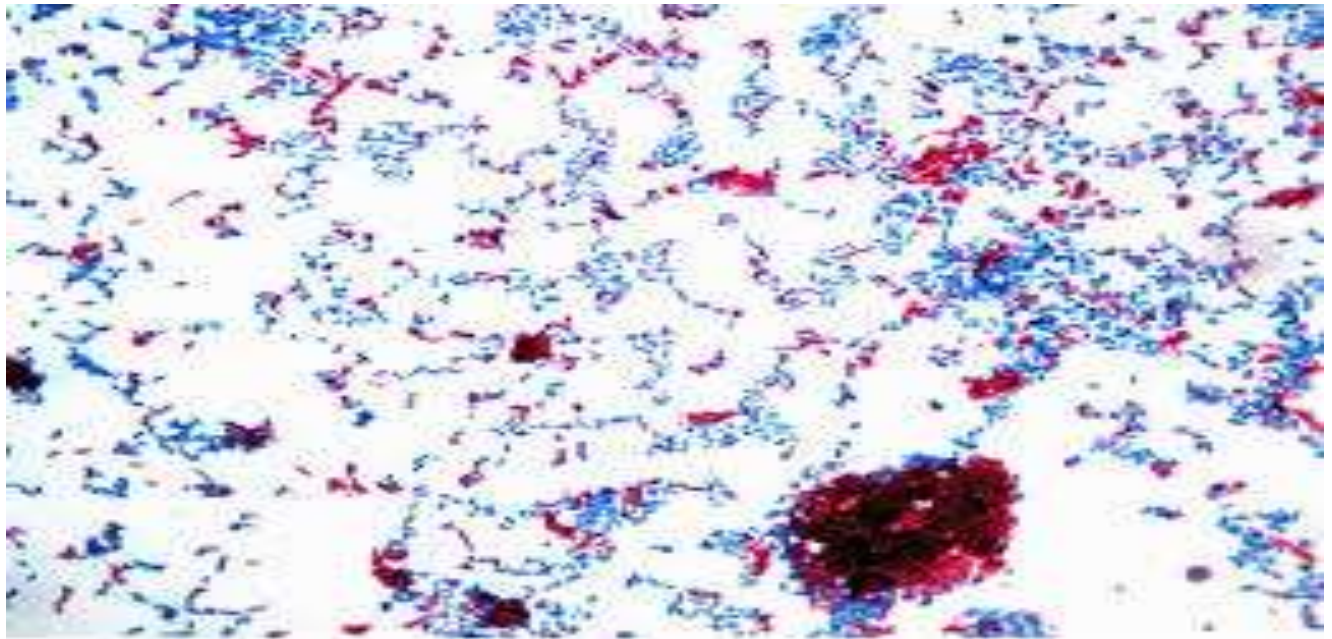
5. Dry & Observe: Gently blot dry with bibulous paper and examine under an oil immersion objective.

Expected Results

- **Acid-Fast Bacteria:** Retain the red carbolfuchsin and appear bright pink/red.
- **Non-Acid-Fast Bacteria & Background:** Lose the red stain and take up the blue counterstain, appearing blue.

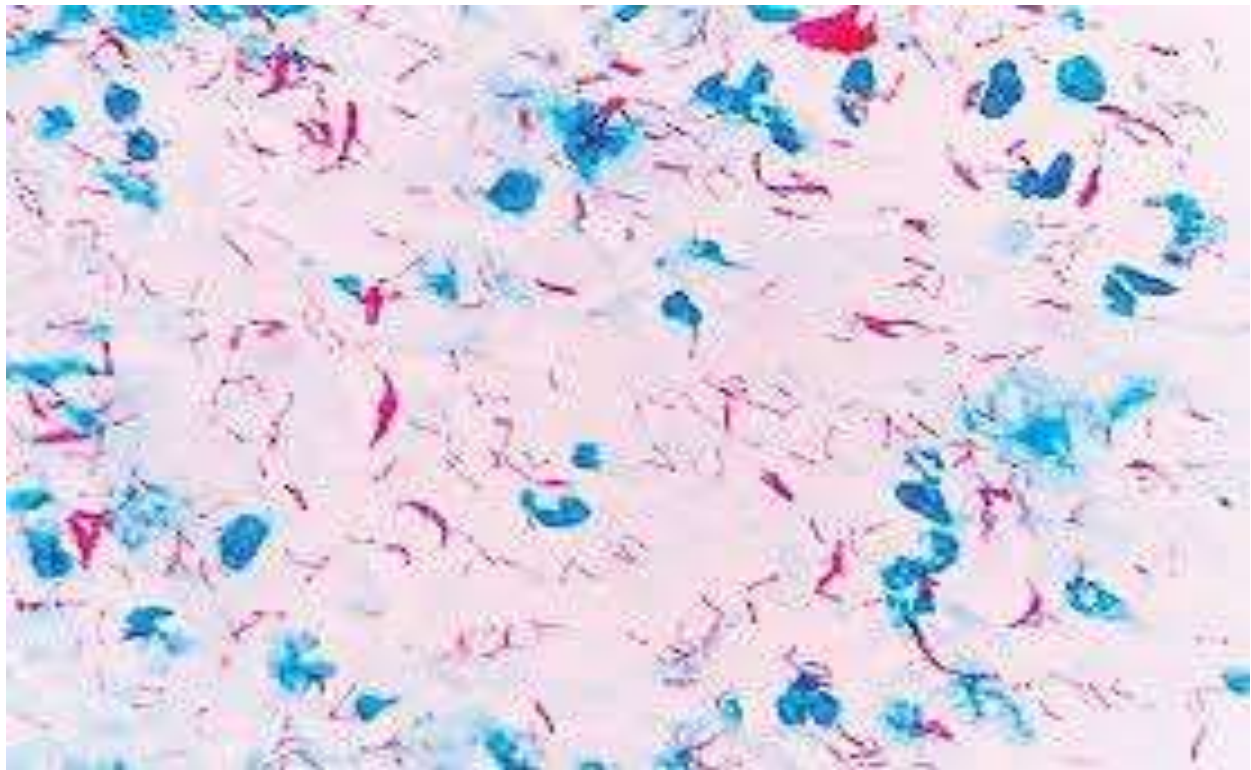






← acid fast positive (pink)

← acid fast negative (dark blue/purple)



Acid-Fast

Non-AF



Heat + Carbol Fuchsin

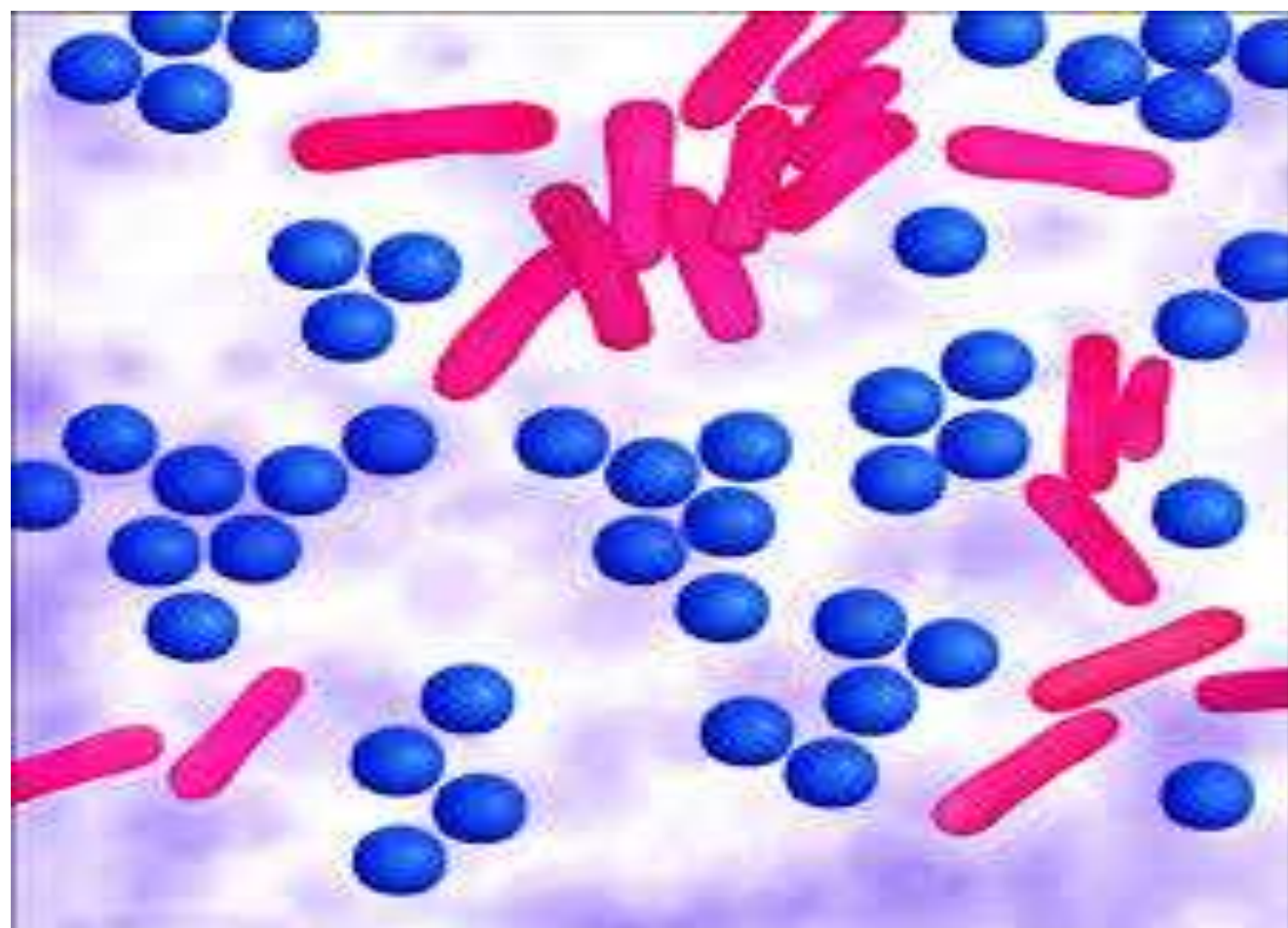


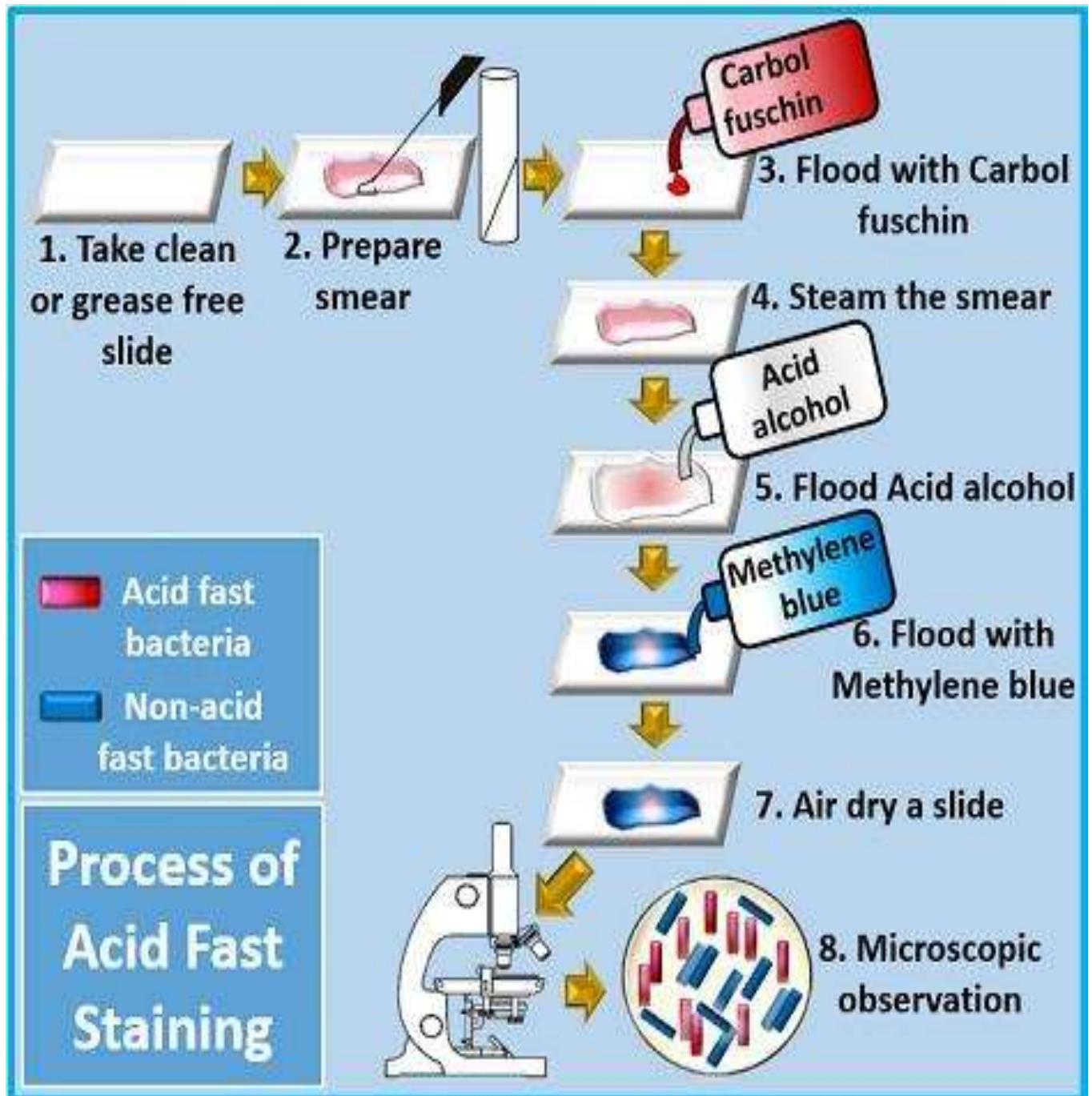
Acid alcohol



Methylene blue







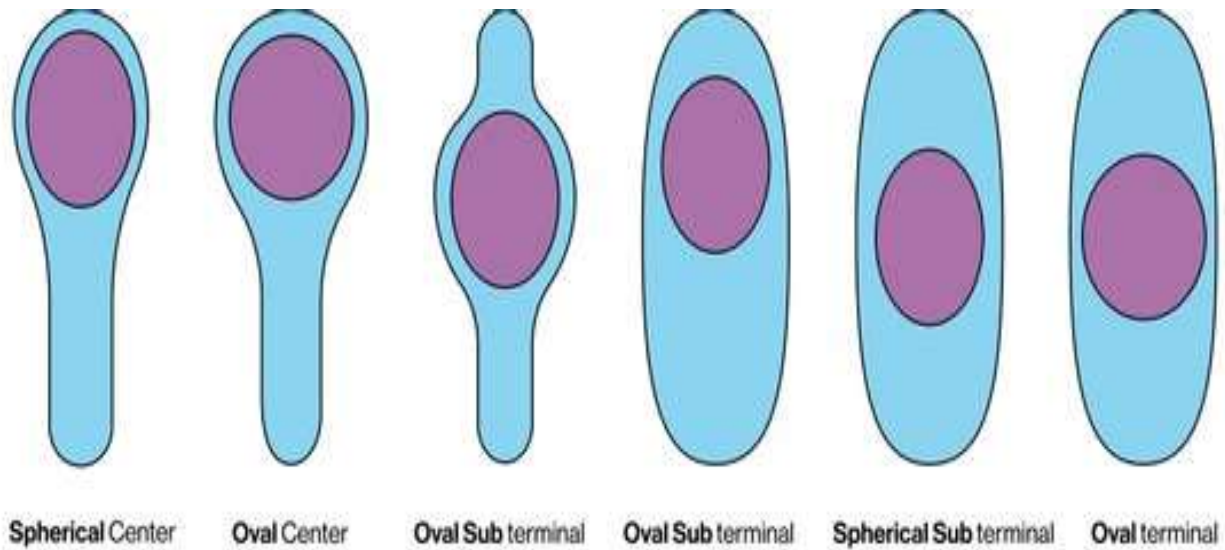
Bacterial taxonomy

Lab:3

Special Stains

Endospore stain

Endospore staining is a differential staining technique, most commonly the Schaeffer-Fulton method, used to identify and visualize bacterial endospores (**Bacillus or Clostridium**) by staining them green with heat-fixed malachite green and vegetative cells red/pink with safranin. It distinguishes tough, dormant, keratin-coated endospores from metabolically active vegetative cells.



Endospore Types

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The Principles and Procedures:

- **Purpose:** Differentiates endospores from vegetative cells and detects spore-formers.
- **Mechanism:** Endospores are highly resistant to chemicals; heat (**steaming**) is required to drive the primary stain, malachite green, into the spore wall.

. Steps:

- . Prepare a smear and heat-fix it.
 - . Apply malachite green while steaming for 5-10 minutes.
 - . Rinse with water to decolorize the vegetative cells.
 - . Counterstain with safranin for 1-2 minutes.
- . **Results:** Endospores appear green, while vegetative cells appear red or pink.

Vegetative cell

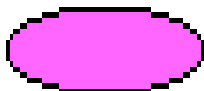
Cell with Endospore



Heat + Malachite Green

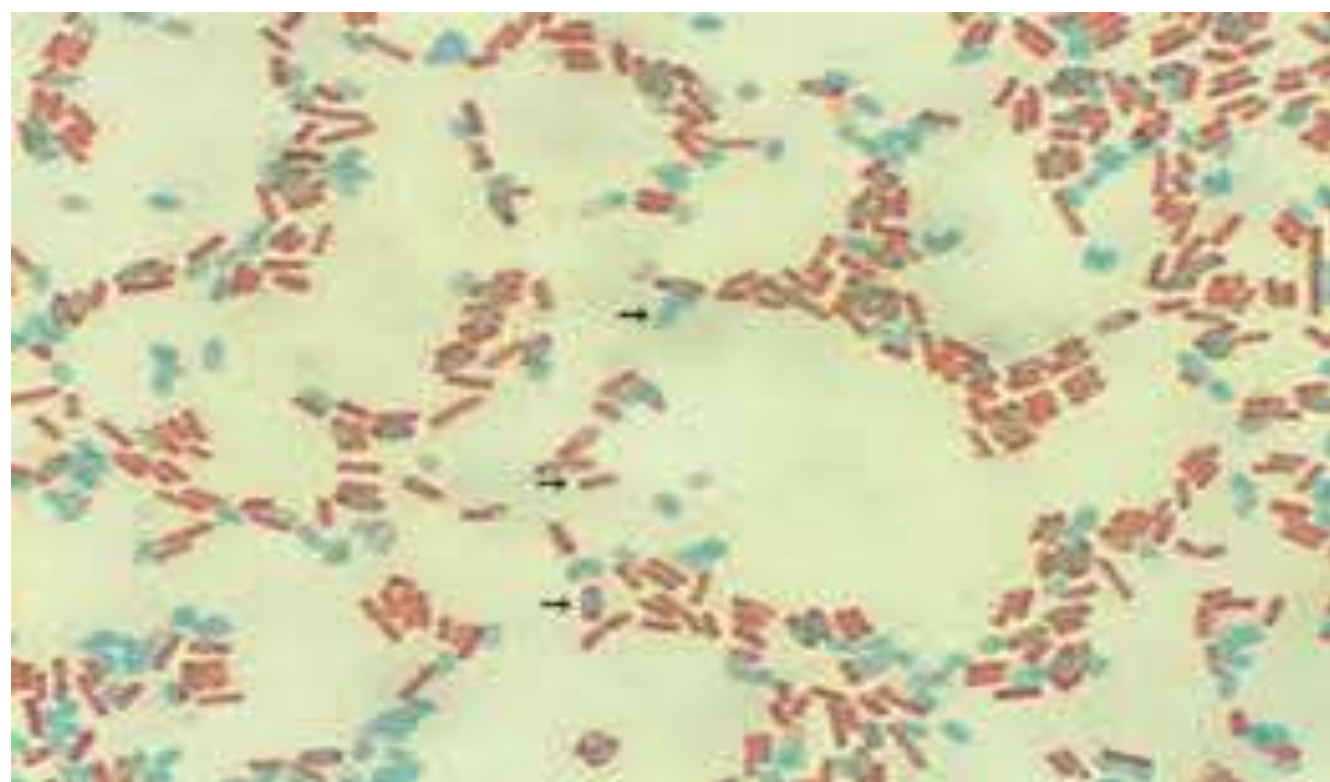
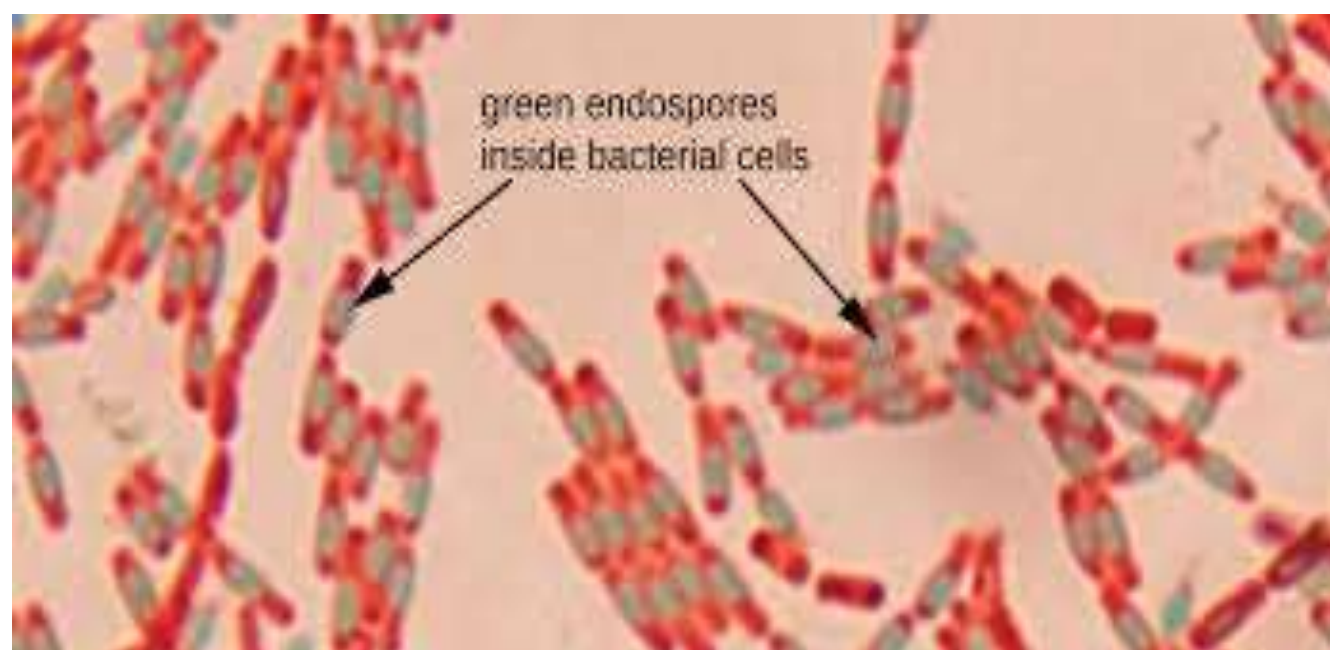


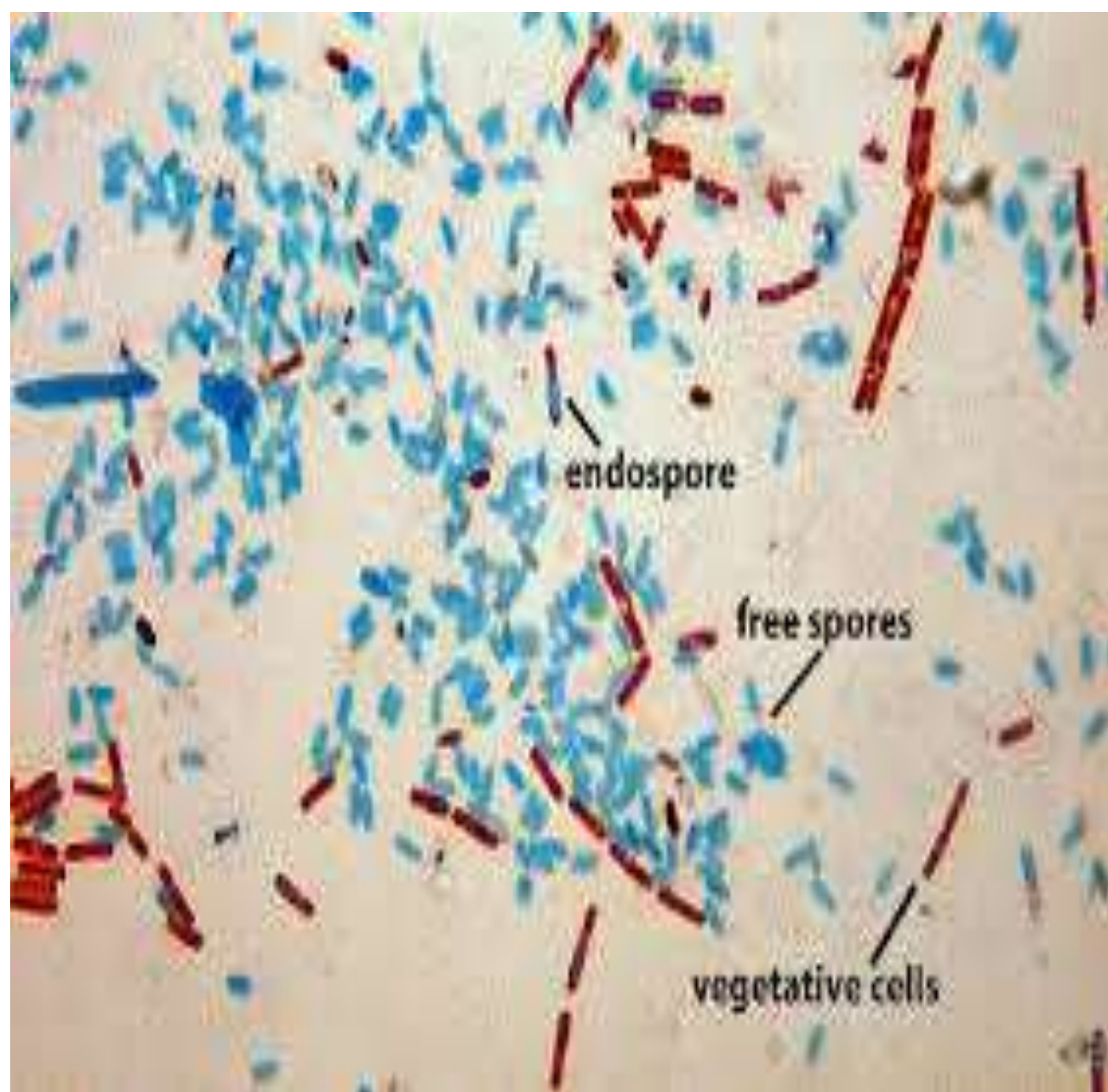
Water



Safranin









Bacterial taxonomy

Lab-4

Special stain

Capsule stain

A capsule staining is a special staining method, which uses differential capsule stains that either highlight the capsule or stains the bacterial cell along with the background. It is an important staining method because some bacteria like *Bacillus anthracis*, *Streptococcus pneumoniae* etc., have a capsule, which can cause pathogenicity in humans and animals. Thus, it becomes necessary to identify the presence of an extracellular capsule. Capsule staining can be performed by employing India ink, Anthony's, Maneval's and Hiss method.

Bacterial Capsule

A bacterial capsule refers to the mucilaginous coating that surrounds the cell wall of bacteria. The capsule or glycocalyx is composed of glycoproteins. The cytoplasm of bacteria

partially forms a capsule, which then migrates to the cell wall, and there it exists as a mucous or slime covering.

A bacterial capsule protects a cell from desiccation because of its mucous content. A mucus layer also protects a cell from phagocytosis. The capsule also acts as a virulence factor and is responsible for the pathogenicity of many microorganisms like *Streptococcus pneumoniae*, *Escherichia coli*, *Neisseria meningitis* etc.

In the majority, a capsule consists of polysaccharides, but a few may possess polypeptides or glycoproteins. The formation of a capsule is a process controlled genetically. Capsules are the structures clearly visible under the light microscope after they get stained via differential capsule stain.

Bacteria may possess a capsule (capsulated bacteria) or lack a capsule (non-capsulated bacteria). Capsule are of two types, namely

micro and macro capsule.

The microcapsule has a size of less than 0.2 μ , whereas the macrocapsule has a size of more than 0.2 μ .

The bacterial capsule is non-ionic in nature, i.e. it will neither take up the colour of acidic stains nor basic stains. The basic dye will stain the negative bacterial cell, and an acidic stain will stain the positive background. To stain a capsule, we need a special capsule stain that can focus on the capsule.

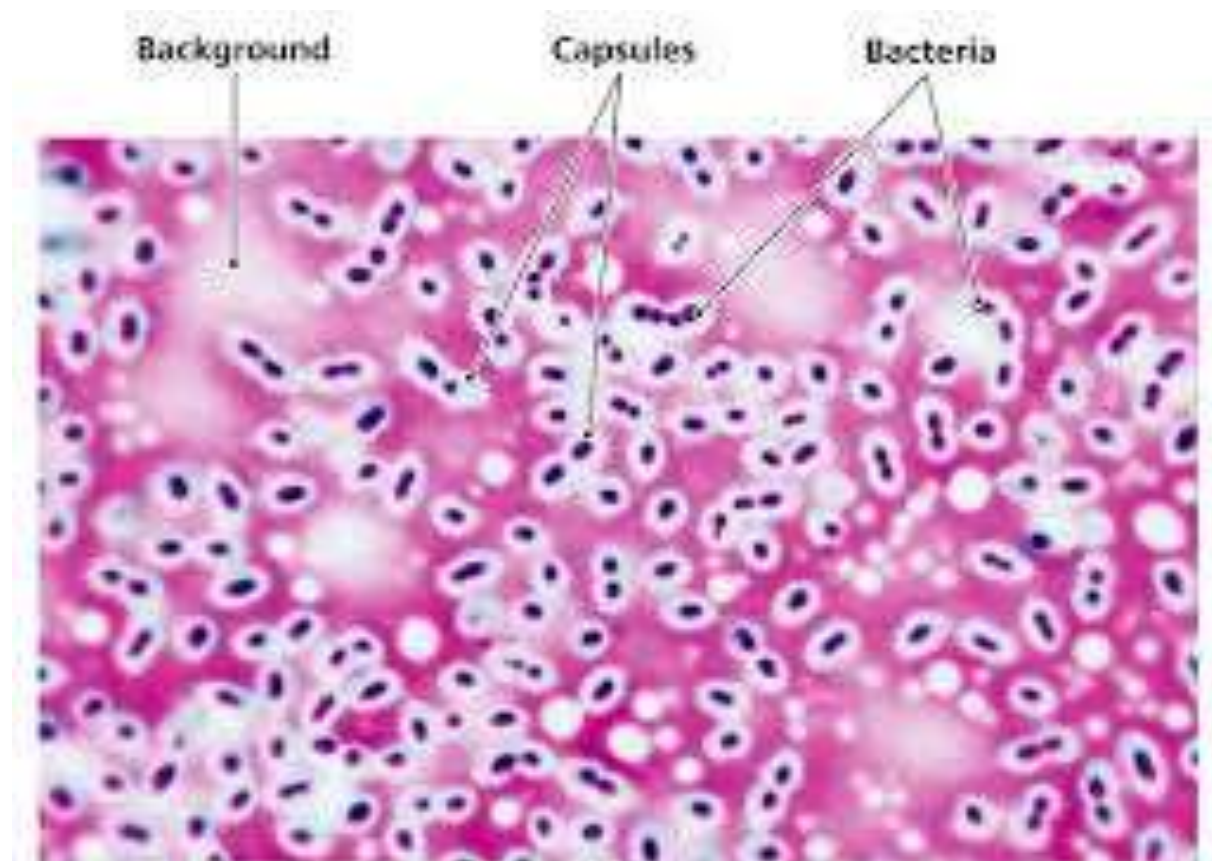
A capsule can be easily destroyed by heat treatment, due to which a step of heat fixing is skipped in capsule staining. In addition to this, avoid steps like washing or rinsing because it can dislodge the bacterial cell capsule. To enhance the capsule's size or increase its visibility, we can also add a drop of serum. The addition of serum provides a more unobstructed view of a capsule under a light microscope.

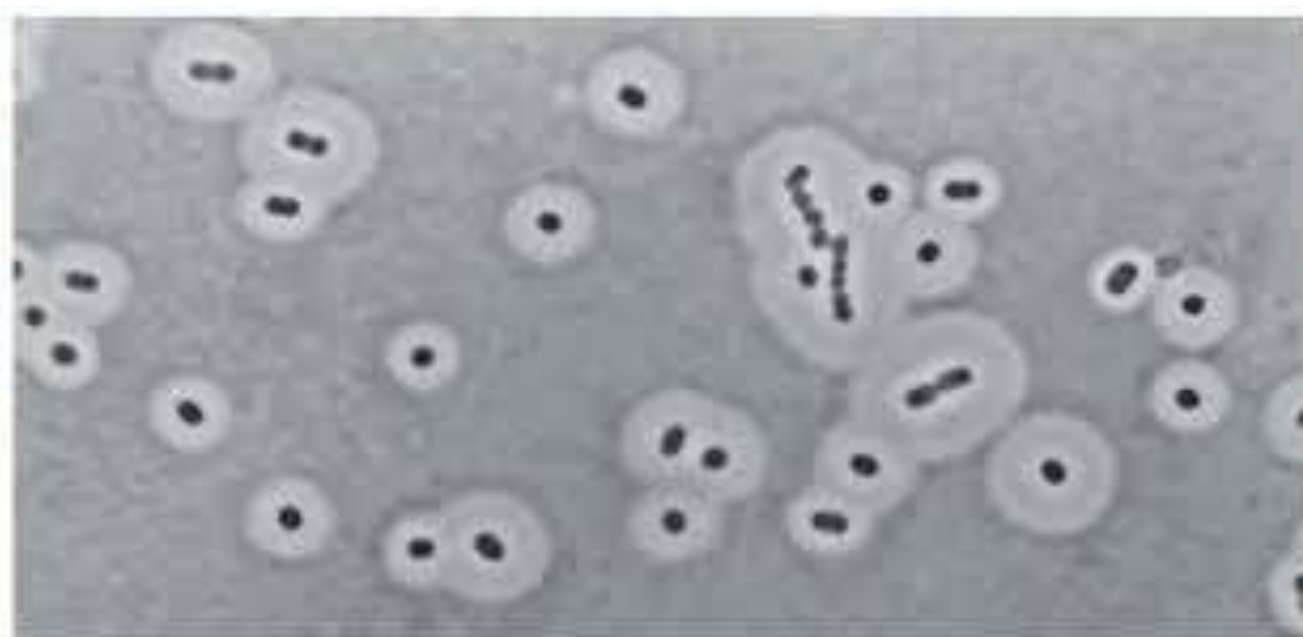
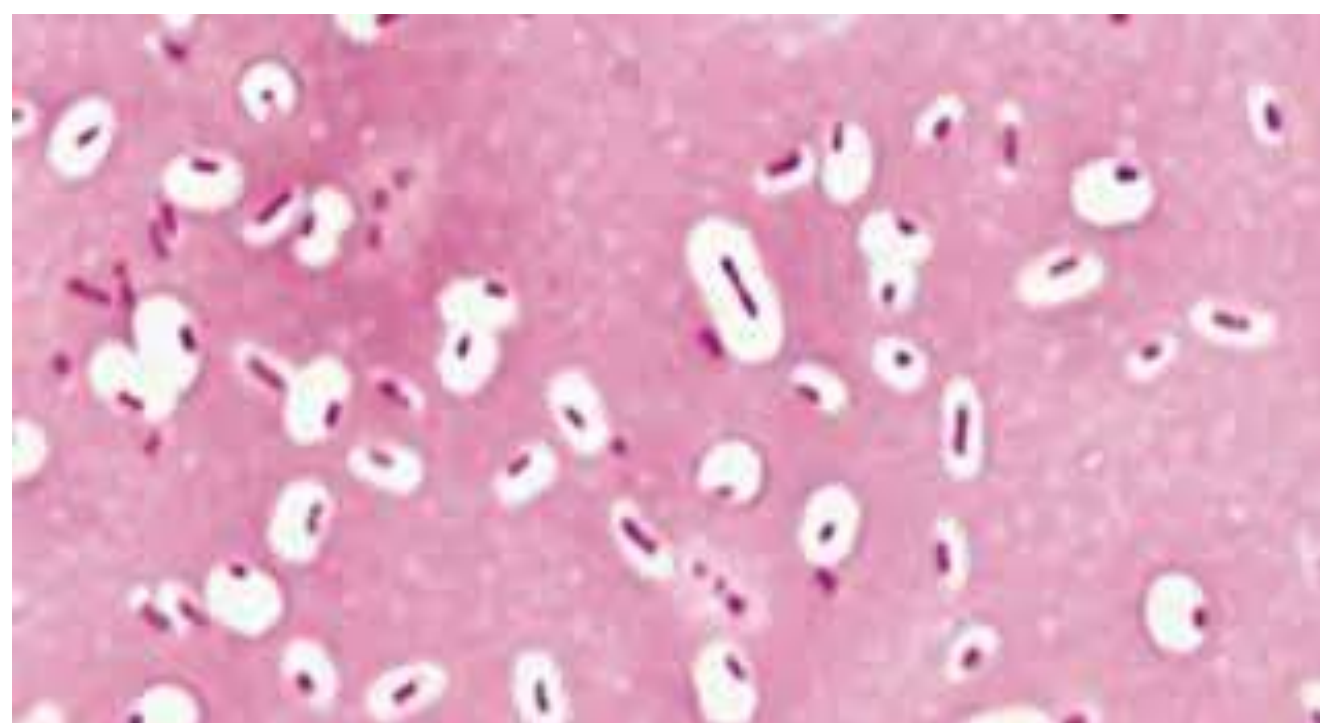
Principle

The principle of capsule staining is based on staining of background with an acidic stain and staining of bacterial cell with a basic stain. A capsule being non-ionic will not stain by either of the two dyes. Thus, capsule staining creates contrast by staining a bacterial cell along with its background and leaving a capsule as a colourless halo. The other approach of capsule staining is to stain the capsule by leaving a bacterial cell and background colourless. Differential capsule stains are available to highlight the specific structure (like capsule) in a bacterial cell.

Note:

The capsule enhances bacterial virulence by protecting against phagocytosis by host immune cells. Identifying the capsule assists in identifying pathogenic bacteria such as *Streptococcus pneumoniae* or *Klebsiella pneumoniae*.



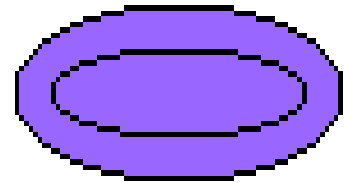


No Capsule

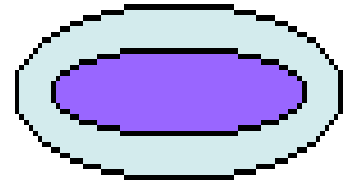


Crystal Violet

Capsule



20% Copper Sulfate



Methods of Capsule Staining

The capsule staining employs various techniques, among which the most common methods are:

1. India ink method
2. Anthony's method
3. Maneval's method
4. Hiss method

1-India ink Method

India ink method uses two types of stain, i.e. a basic stain (Crystal violet) and an acidic stain (India ink). Crystal violet is a positive stain that will stain the negatively charged bacterial cell. India ink is a negative stain that will stain the positively charged background. After staining:

- . The background seems darker or black.**
- . A bacterial cell appears violet.**
- . The capsule seems like a clear halo.**

Procedure

India ink method involves the following steps:

- 1.Take a clean, sterilized or grease-free slide.**
- 2.Add a drop of India ink to the center of the glass slide.**
- 3.Prepare a smear by taking an inoculum from the bacterial culture and mix it with a drop of India ink.**

4. Then, allow the smear to air dry (do not heat fix the smear, as it may result in cell shrinkage that in turn can distort the bacterial capsule).

5. Flood a smear with the crystal violet stain and leave it for 30 seconds. Later, remove the extra stain by tilting a glass slide.

6. Add oil immersion to the stained area and observe it under the microscope using a 100X objective.

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2-Anthony's Method

This method makes the use of two reagents, namely crystal violet as a primary stain, 20% of CuSO_4 solution as a decolouring agent and counterstain. Crystal violet stains the bacterial cell and background. The CuSO_4 solution stains the non-ionic capsule. After staining:

- . A bacterial cell appears violet.**
- . The background appears light violet.**
- . The capsule appears as a faint blue halo.**

Procedure

Anthony's method involves the following steps:

- 1.Take a clean, sterilized or grease-free slide.**
- 2.Add a drop of crystal violet to the centre of the glass slide.**
- 3.Prepare a smear by taking an inoculum from the bacterial culture and mix it with a drop of crystal violet.**

4. Then, allow the smear to air dry (do not heat fix the smear).

5. Flood a smear with 20% of CuSO_4 solution and leave it for at least 30 seconds. Later, remove the extra stain by tilting a glass slide.

6. Add oil immersion to the stained area and observe it under the microscope using a 100X objective.



Anthony's method is a type of positive staining method, which stains the capsule along with the bacterial cell (not the background). As a result, a capsule appears as a faint blue halo between the violet bacterial cell and purple background.

3-Maneval's Method

This method makes the use of two reagents, i.e. acidic stain (Congo red) and a special stain (Maneval's stain). The composition of Maneval's dye includes:

- **10% Ferric chloride (acts as a mordant).**
- **5% phenol (increases the penetration of the stain between the smear).**
- **Acid fuschin (stains the bacterial cell being a basic dye).**
- **Acetic acid (decreases the pH of the smear to the acidic side).**

- **Congo red appears red (at neutral pH) and appears blue in colour (at acidic pH).**

After staining, we could see the specimen with the following microscopic observation:

- **A bacterial cell appears bright red-pink.**
- **The background appears dark blue in colour.**
- **The capsule appears as a clear halo.**

Procedure

Maneval's method involves the following steps:

- 1.Take a clean, sterilized or grease-free slide.**
- 2.Add a drop of 1% Congo red to the centre of the glass slide.**
- 3.Prepare a smear by taking an inoculum from the bacterial culture and mix it with a drop of Congo red.**
- 4.Then, allow the smear to air dry (do not heat fix the smear).**

5. Flood a smear with Maneval's stain and leave it for at least 1 minute. Later, remove the excess stain by tilting a glass slide.

6. Add oil immersion to the stained area and observe it under the microscope, having a 100X objective.



Maneval's method is also a type of negative staining method, which stains the bacterial cell and its background, but not a capsule. As a result, a capsule appears as a clear halo between the pink bacterial cell and blue background.

4- Hiss Method

This method uses two reagents, namely crystal violet and copper sulphate solution. After staining:

- A Bacterial cell appears dark violet.
- The background seems brighter in colour.
- The capsule appears as a light violet colour.

Procedure

Hiss method involves the following steps:

1. Take a clean, sterilized or grease-free slide.
2. Add a drop of crystal violet to the center of the glass slide.

- 3.Prepare a smear by taking an inoculum from the bacterial culture and mix it with a drop of crystal violet.**
- 4.Then, allow the smear to air dry (do not heat fix the smear).**
- 5.Flood a smear with copper sulphate solution and remove the excess stain by tilting a glass slide.**
- 6.Add oil immersion to the stained area and observe it under the microscope, having a 100X objective.**

The Hiss method is also a type of positive staining method that stains the capsule and the bacterial cell with a brighter background. As a result, a capsule appears as a light violet colour between a dark violet coloured bacterial cell and colourless background.

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1. Take clean or grease free slide



2. Add a drop of crystal violet



3. Prepare smear



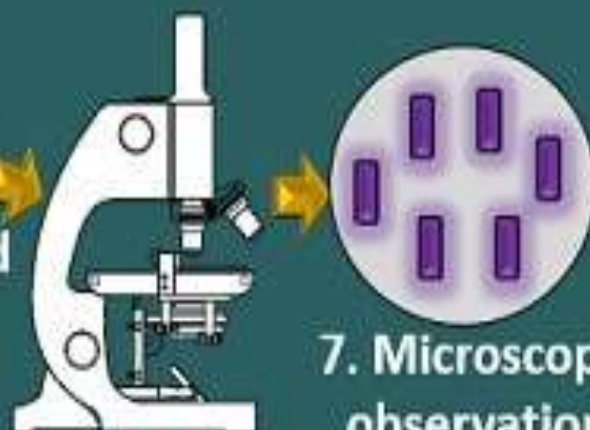
4. Air dry the smear



5. Flood copper sulphate



6. Air dry and add oil immersion



7. Microscopic observation

Flagella Stain



Flagella (singular flagellum) are threadlike, long, thin helical filaments measuring 0.01-0.02nm in diameter. These appendages extend outward from the plasma membrane and cell wall. Flagella are so thin that they cannot be observed directly with a bright field microscope, but must be stained with special techniques (example: Fontana's silver staining technique) that increase their

thickness. The detailed structure of a flagellum can only be seen in the electron microscope

The bacterial flagellum is composed of three parts: a basal body (associated with the cytoplasmic membrane and cell wall), a short hook and a helical filament (which is usually several times as long as the cell). Filament is external to cell wall and is connected to the hook at cell surface; the hook and basal body are embedded in the cell envelope (Figure 7.4). Hook and filament are composed of protein subunits called as flagellin.

One can generalize that all spirilla, about half of the bacilli and a small number of cocci are flagellated. Some bacteria do not have flagella. Flagella vary both in number and arrangement on the cell surface. Flagella are arranged generally in two patterns.

1. In polar arrangement, the flagella are attached at one or both ends of the cell. Bacteria with polar flagellar arrangement are

further classified into monotrichous, lophotrichous, and amphitrichous.

2. In lateral arrangement, flagella are arranged randomly all over the surface of the cell. Bacteria with lateral flagellar arrangement are called peritrichous.

Various types of mobility are observed based on the arrangement of the flagella. Serpentine motility is seen with *Salmonella*, darting motility with *Vibrio* and tumbling motility with *Listeria monocytogenes*. Some bacteria like *Cytophaga* exhibit a gliding motility, which is slow sinuous flexing motion. This occurs when the cells come in contact with solid surface.

Some bacteria have the ability to move toward or away from chemical substance. This movement is called chemotaxis. Positive chemotaxis is the movement of a cell in the direction of a favorable chemical stimulus (usually a nutrient). Negative chemotaxis is the

movement away from a chemical substance (usually harmful compound). Some photosynthetic bacteria exhibit phototaxis, movement in response to light rather than chemicals.

The presence of motility is one piece of information used to identify a pathogen in the laboratory. One way to detect motility is to stab a tiny mass of cells into soft (semi solid) medium in a test tube. Growth spreading rapidly through the entire medium is indicative of motility. Alternatively, cells can be observed microscopically by a hanging drop method. As shown in the table (7.1).

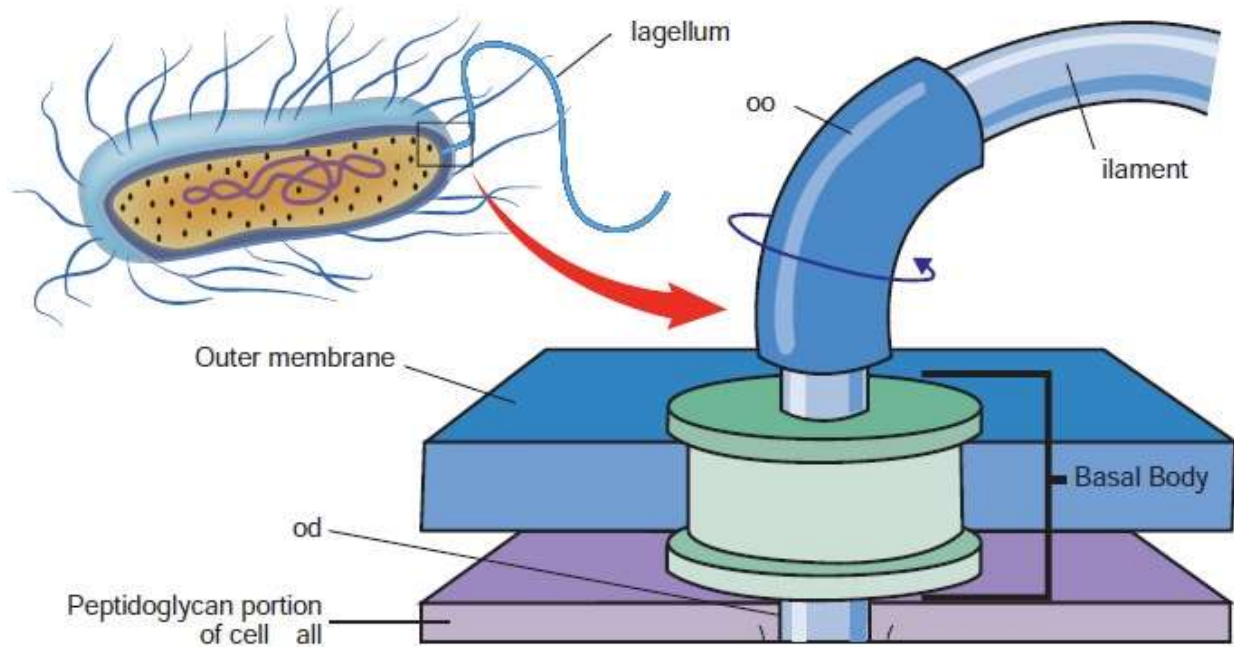


Figure 7.4: Structure of bacterial flagella

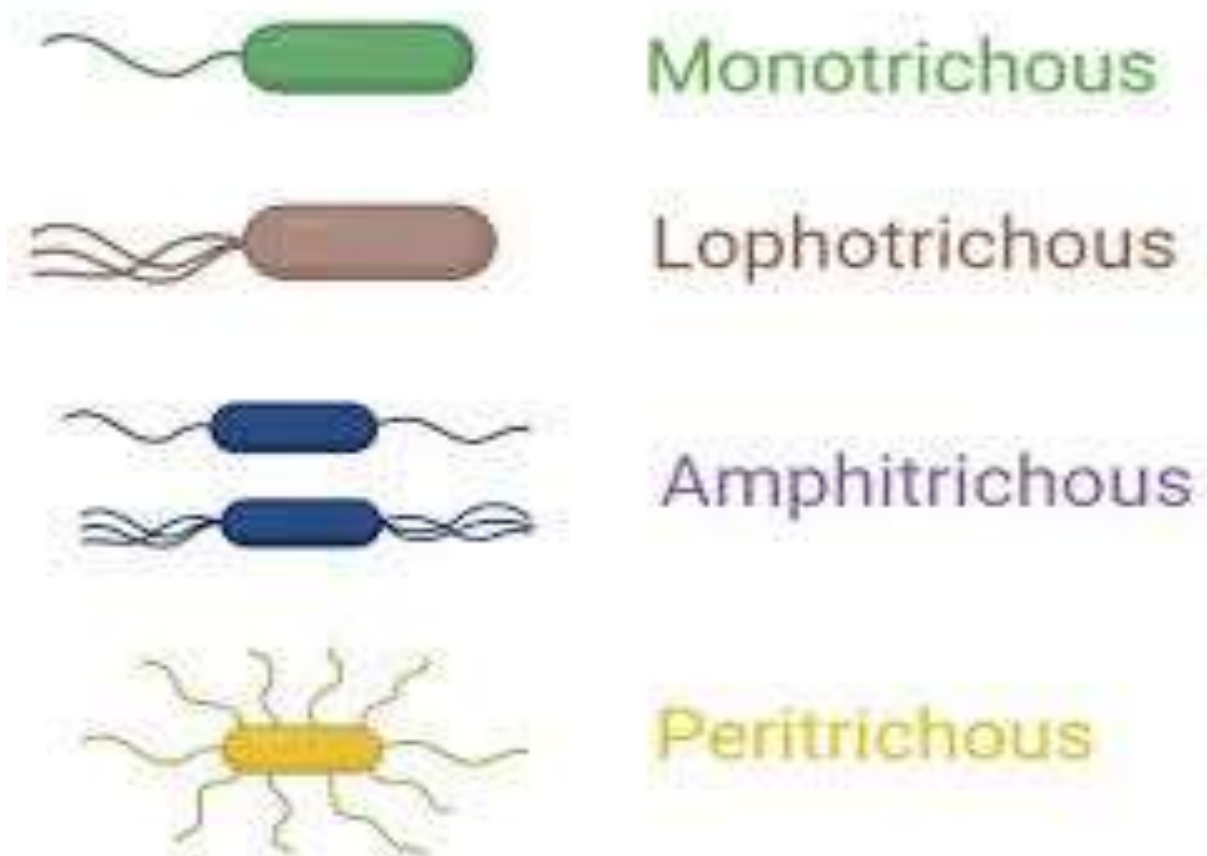
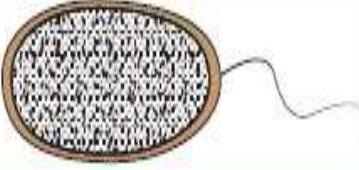
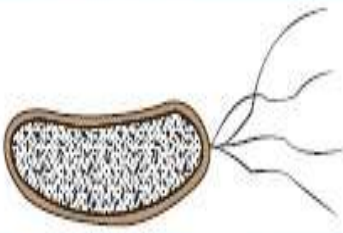
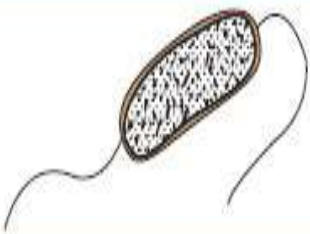
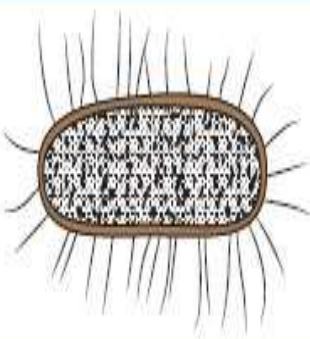


Table 7.1: Arrangement of bacterial flagella

Structure	Flagella type	Example
	Monotrichous(single flagella on one side)	<i>Vibrio cholera</i>
	Lophotrichous(tuft of flagella on one end)	<i>Pseudomonas fluorescens</i>
	Amphitrichous(single or tuft on both ends)	<i>Aquaspirillum serpens</i>
	Peritrichous(flagella throughout the cells)	<i>Salmonella typhi</i>

Flagella stain

Flagella stain is a specialized microbiology technique used to visualize delicate bacterial flagella (10–30 nm) under a light microscope by coating them with mordants (like tannic acid or potassium alum) and staining with dyes (e.g., crystal violet or basic fuchsin). The stain increases the apparent diameter of the flagella, allowing for identification of their presence, number, and arrangement.

Principles and Techniques

Purpose:

To determine the motility and arrangement of flagella (e.g., peritrichous, monotrichous, lophotrichous) for bacterial identification.

Mechanism:

The method relies on a mordant to thicken the slender flagella, as they are too thin to be seen with simple staining alone.

Common Methods:

Leifson method: Uses tannic acid and a dye in an alcoholic solution to form a precipitate.

Ryu method: Uses crystal violet, tannic acid, and aluminum potassium sulfate to stain the flagella.

Note: Samples must be handled carefully (no harsh heat fixing or blotting) to avoid breaking the delicate, hair-like flagella.

The Leifson Method:

The Leifson Method is a specific staining technique used in microbiology to visualize bacterial flagella, which are generally too thin (10–30 nm) to be seen with a standard light microscope. This method involves using a mordant (tannic acid) to coat the flagella, increasing their thickness, followed by staining with basic fuchsin.

Principle

Flagella are below the visual limit of a light microscope (roughly 0.1 to 0.3 μ m in diameter). The Leifson stain uses an alcoholic solution containing basic fuchsin (dye), tannic acid (mordant), and NaCl.

- . Mordant (Tannic acid):** Adsorbs onto the flagella, causing heavy precipitation, which increases the thickness of the flagella.
- . Dye (Basic Fuchsin):** Colors the thickened flagella.
- . Alcohol:** Facilitates the process as it evaporates, creating a deposit of stain around the flagella.

Staining Solution

The working solution is usually prepared by mixing equal parts of three stock solutions, which should be prepared fresh or stored properly (4°C for 1 month or -20°C for 1 year):

- . Solution I: 1.2% Basic Fuchsin in 95% Ethanol.**
- . Solution II: 3% Tannic Acid in Distilled Water.**
- . Solution III: 1.5% Sodium Chloride (NaCl) in Distilled Water.**

Procedure

- 1. Slide Preparation:** Use a young (18–24 hour) broth culture or a light suspension of colony growth in distilled water.
- 2. Smear & Fixation:** Place the suspension on a clean, preferably grease-free slide. Allow it to air-dry. Do not heat fix, as this destroys the delicate proteinaceous structure of the flagella.

3. **Staining:** Flood the smear with the Leifson stain solution for 7–15 minutes. A shiny film indicates proper reaction.
4. **Rinsing:** Gently wash the slide with water to remove excess stain.
5. **Observation:** Air-dry and examine under oil immersion.

Appearance

- . **Flagella:** Appear red, pink, or sometimes dark brown.
- . **Bacteria:** Appear red or blue-black.

Factors Affecting Results

- . **Culture Age:** Young cultures (18-24 hours) are essential for active motility and presence of flagella.
- . **Slide Cleanliness:** Slides must be perfectly clean to prevent high background debris.
- . **Suspension Medium:** Use distilled water; avoid media with excessive proteins.

- **Temperature & pH:** The pH should be adjusted (e.g., pH 5.0) for optimal results, as the stain is sensitive to temperature and time.

Applications

- Identifying the presence, number, and arrangement of flagella (e.g., polar, peritrichous) for bacterial classification.
- Diagnosis of *Helicobacter pylori* in gastric biopsy specimens.

The Ryu method

The Ryu method is a rapid, wet-mount flagella staining technique that uses a stable, pre-mixed solution of tannic acid (mordant) and crystal violet (stain) to coat and thicken bacterial flagella, making them visible under a light microscope. It involves

adding the stain to a wet mount of motile bacteria, allowing 5–15 minutes of incubation, and observing with oil immersion.

Procedure for Ryu Method (Wet Mount):

- 1. Sample Prep:** Place a small drop of sterile water on a clean slide. Using a sterile loop, gently transfer bacteria from a 16-24 hour culture into the water.
- 2. Coverslip:** Place a coverslip over the suspension; it should barely reach the edges.
- 3. Attachment:** Allow the slide to sit for 5-10 minutes to allow the bacteria to attach to the glass.
- 4. Stain Application:** Apply 1-2 drops of Ryu stain (1 part crystal violet, 10 parts mordant) to the edge of the coverslip, letting it flow underneath via capillary action.

5. Incubation: Incubate for 5 to 15 minutes at room temperature.

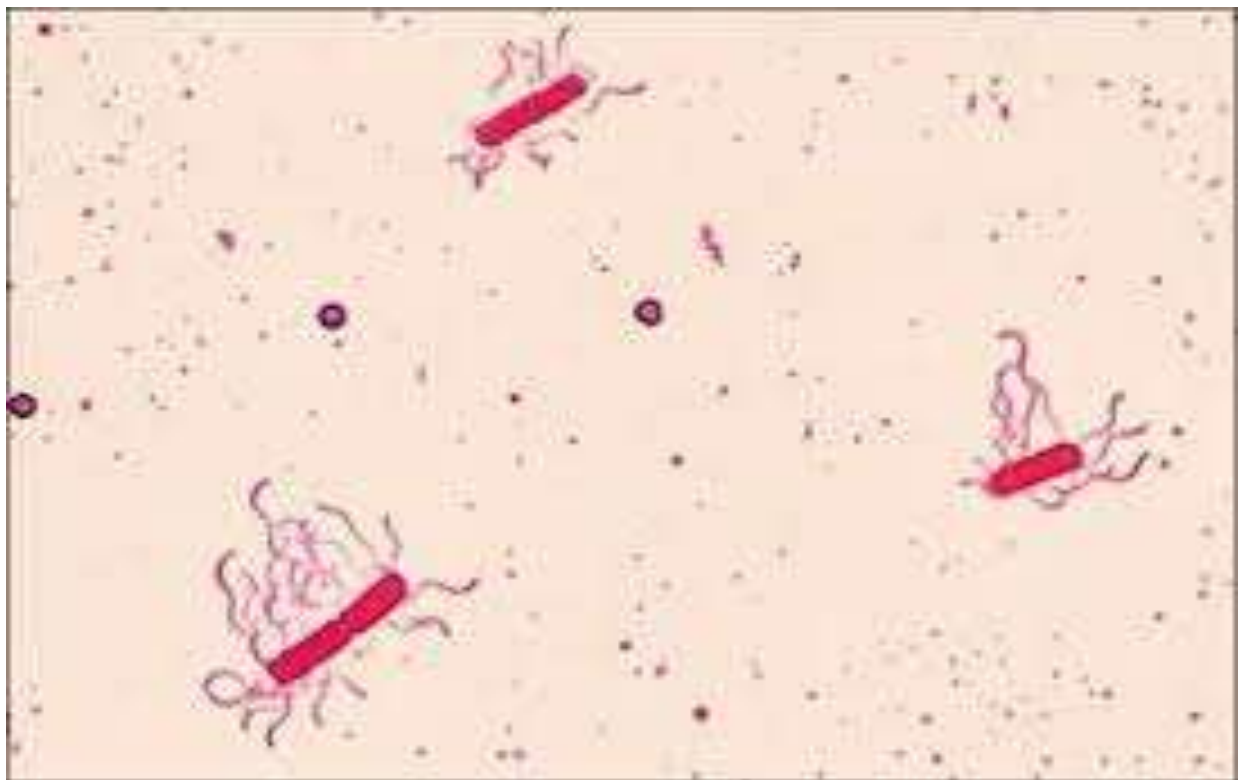
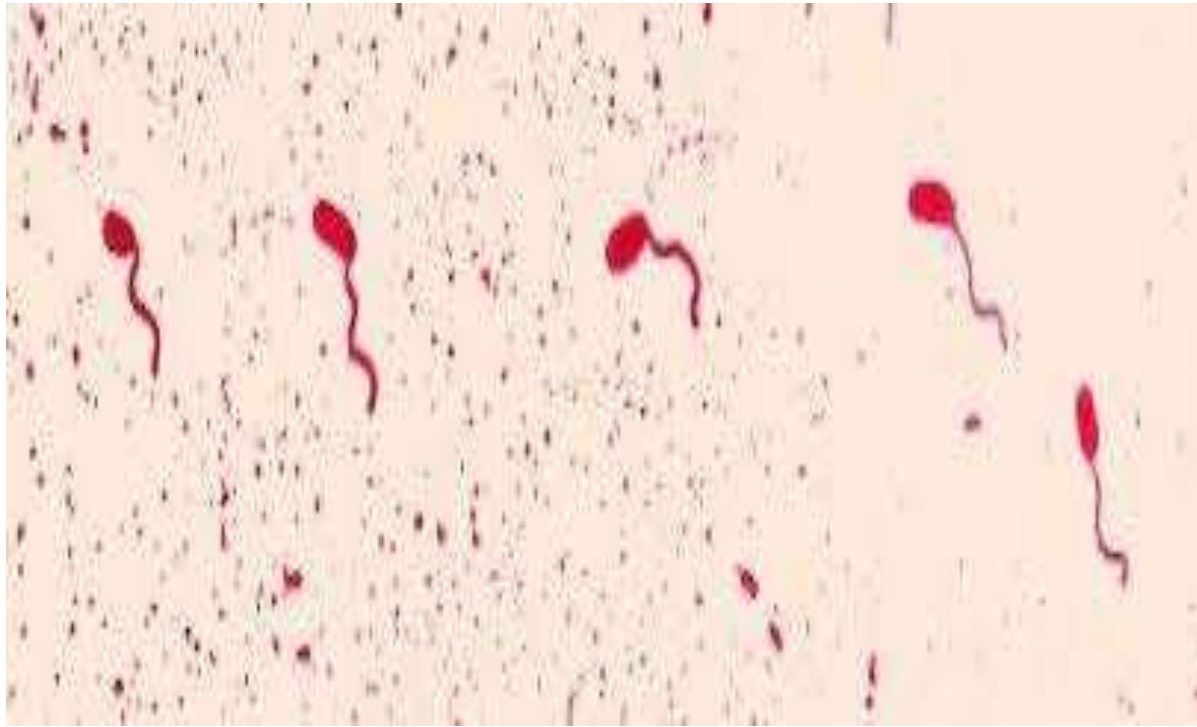
6. Observation: Examine under 100x oil immersion; flagella will appear purple.

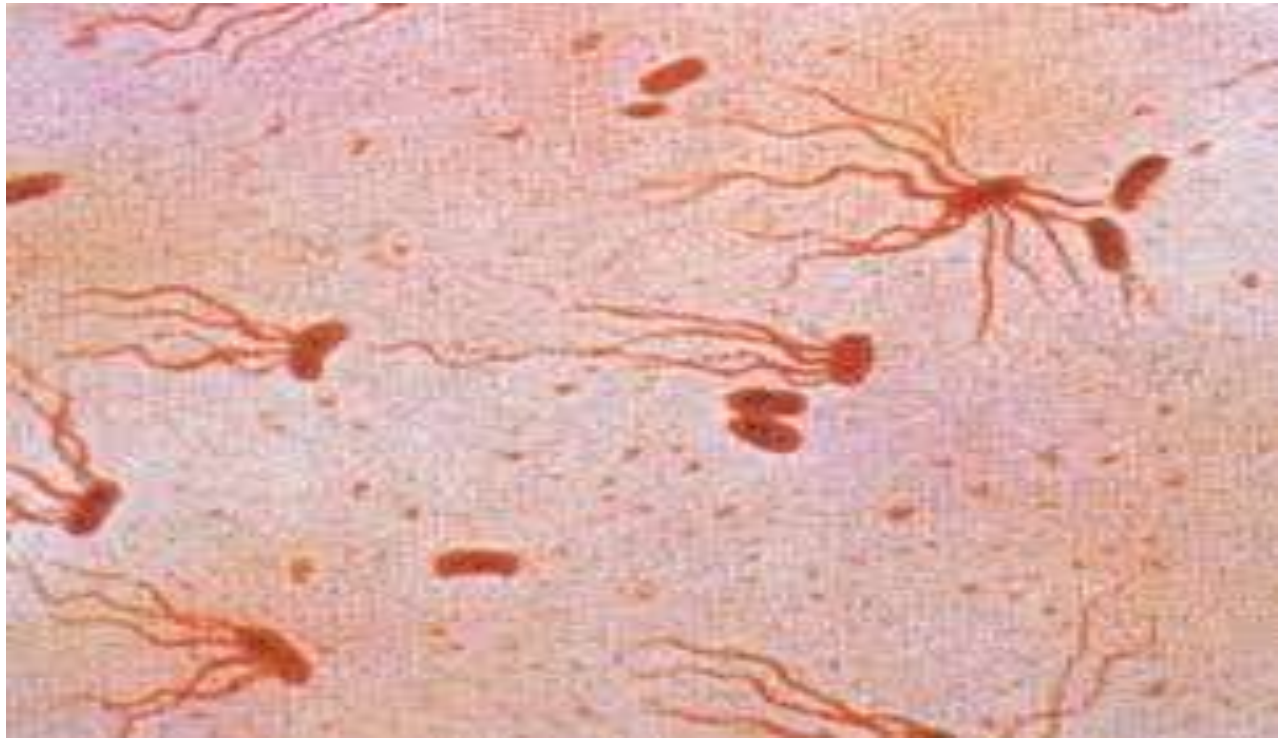
Key Aspects of the Ryu Method:

- **Ready-to-Use:** The stain is stable at room temperature indefinitely.
- **No Heat Fixing:** The wet-mount technique avoids the delicate, heat-sensitive nature of traditional, dried flagella smears.

Mechanism:

The tannic acid acts as a mordant to precipitate onto the flagella, while crystal violet stains the thickened appendage, allowing visualization of the number and arrangement of flagella.



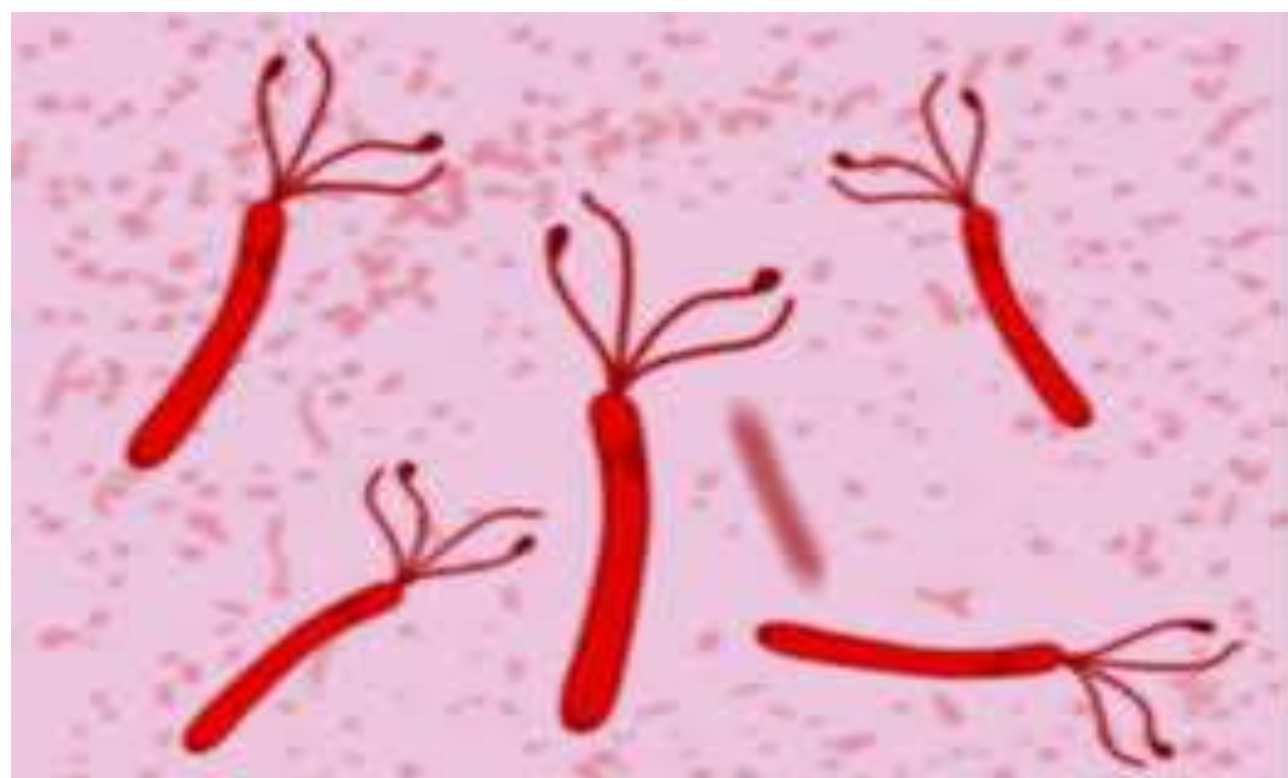


monotrichous flagellation
bacillus shapes

100X

10 μm





Bacterial taxonomy

Lab:6

Differential biochemical tests

Indole , Methyl Red , Voges-Proskauer and Citrate utilization tests (IMViC).

Indole Test

The indole test determines a bacterium's ability to produce the enzyme tryptophanase, which breaks down the amino acid tryptophan into indole. It is a key test in identifying Gram-negative bacteria, particularly in the IMViC series (Indole, Methyl red, Voges-Proskauer, Citrate) to differentiate **Enterobacteriaceae**. A positive result is indicated by a **red ring** after adding **Kovac's reagent**, while a negative result remains **yellow**.

Principle and Procedure

- **Principle:** Bacteria possessing the enzyme **tryptophanase** break down tryptophan into **indole**, **ammonia**, and **pyruvic acid**.
- **Reagent:** **Kovac's reagent** (containing p-dimethyl amino benzaldehyde) is added, which reacts with indole to form a red dye (rosin dole).

• Procedure (Tube Method):

1. Inoculate tryptophan or peptone broth with the bacteria.
 2. Incubate for 24–48 hours at 35–37°C.
 3. Add 5 drops of Kovac's reagent.
 4. Observe for a red layer at the top of the broth within minutes.
- **Rapid/Spot Method:** Rub a colony onto filter paper saturated with dimethylamino-cinnamaldehyde (DMACA) reagent. A blue-green color indicates a positive result.

Results

- **Positive (+):** Formation of a red (Kovac's) or blue (spot test) color.
- **Negative (-):** The reagent layer remains yellow or colorless.

Common Bacteria Tested

Indole Positive:

Escherichia coli,

Klebsiella oxytoca, Proteus vulgaris,

Morganella morganii, Vibrio cholerae.

Indole Negative:

Klebsiella pneumoniae

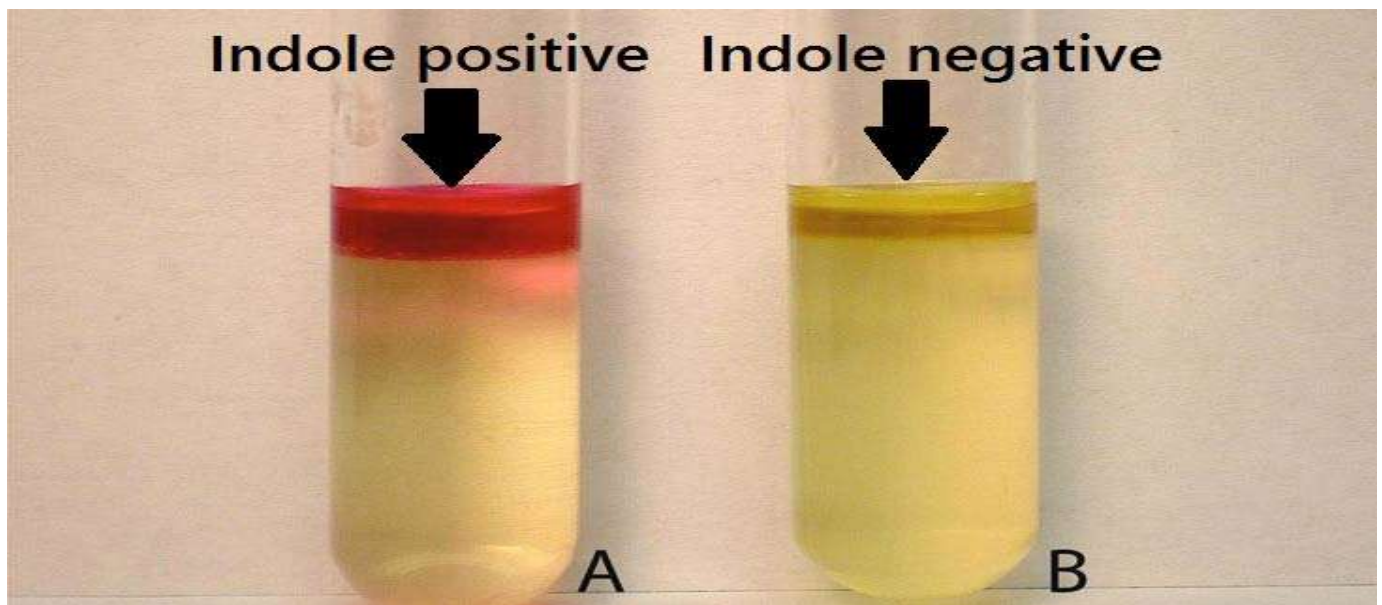
Proteus mirabilis

Salmonella spp

Pseudomonas aeruginosa.

Limitations

- **Media Requirements:** The medium must be rich in tryptophan (e.g., tryptone or peptone broth).
- **Medium Interference:** Do not use indole tests on colonies from MacConkey agar as the medium color can interfere with results.
- **Anaerobes:** For anaerobes, Ehrlich's reagent is preferred over Kovac's reagent



Spot Indole Test

-ve

+ve



A

B

Methyl Red test

The Methyl Red test (MR test) identifies bacteria that perform mixed acid fermentation of glucose, producing stable, acidic end-products, by using the pH indicator methyl red which turns red in acidic conditions (pH below 4.4) and yellow at higher pH. Used with the Voges-Proskauer test (VP) and Indole test (part of IMViC), it helps **differentiate Enterobacteriaceae**, like *E. coli* (positive) from *Enterobacter* (negative). A red color indicates a positive result, while yellow signifies a negative-result.

Principle

- **Bacteria ferment glucose, producing pyruvic acid.**
- **Positive (Mixed Acid Pathway):**

Some bacteria further convert pyruvic acid into significant amounts of stable acids (lactic, acetic, formic, succinic), drastically lowering the pH below 4.4.

- **Negative (Butylene Glycol Pathway):**

Other bacteria convert pyruvic acid to neutral products (like acetoin), resulting in less acidic conditions.

- **Methyl red indicator is added; red means positive (low pH), yellow means negative (higher pH).**

Procedure

- 1. Inoculate MR-VP broth (containing glucose, peptone, buffer) with the test organism.**
- 2. Incubate aerobically at 37°C for 24-48 hours.**
- 3. Add 2-3 drops of methyl red indicator to a portion of the broth.**
- 4. Observe the color change immediately.**

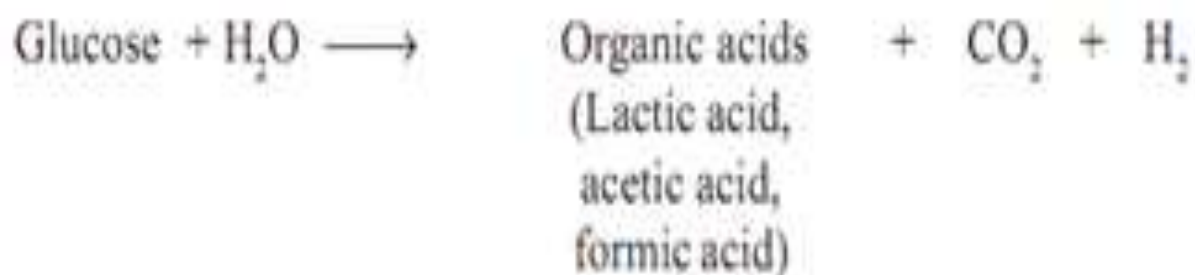
Results

- **Positive: A stable red color.**
- **Negative: A yellow color (or no change from the initial yellow/orange of the broth).**

Common Uses

- **Differentiating bacteria within the Enterobacteriaceae family, such as Escherichia coli (MR positive) from Enterobacter aerogenes (MR negative).**
- **Microbiology identification schemes (IMViC tests).**





Methyl red

Red
+ve MR
(pH approx. 4)

Yellow
-ve MR
(pH approx. 6)

Glucose

Acidic pathway

Neutral pathway

Mixed acids
↓ pH less than 4.4

Acety methyl carbinol
(ACETOIN)

Methyl Red
indicator

Barrit's A
Barrit's B

Red color

MR positive
E. coli

VP positive
Klebsiella

Pink color



Voges Proskauer test

The **Voges-Proskauer** (VP) test is a biochemical test used to determine if a bacterium produces acetoin (acetyl methyl carbinol) as a neutral end product of glucose fermentation. It is a key test in the IMViC series.

primarily used to differentiate members of the family *Enterobacteriaceae*, such as *Escherichia coli*(VP= -)

from *Klebsiella* and *Enterobacter* species VP=+).

Principle

Bacteria that use the butanediol fermentation pathway metabolize pyruvic acid (a product of glucose metabolism) into acetoin, a neutral intermediate product. The VP test works by detecting this intermediate. When the VP reagents (**alpha-naphthol and potassium hydroxide**) are added, the acetoin in the medium is oxidized to diacetyl in the presence of atmospheric oxygen. Diacetyl then reacts with the guanidine compounds in the peptone broth to form a **pinkish-red polymer**, indicating a positive result. Alpha-naphthol serves as a color intensifier in this reaction.

Procedure

- 1. Inoculation:** A pure culture of the bacterium is aseptically inoculated into a tube containing MR-VP broth (Methyl Red-Voges-Proskauer broth), a buffered peptone-glucose medium.
- 2. Incubation:** The inoculated tube is incubated at 35-37°C for 24 to 48 hours.
- 3. Reagent Addition:** After incubation, the following reagents are added to an aliquot of the culture:
 - 1. 5% alpha-naphthol (VP Reagent A)** is added first.
 - 2. 40% potassium hydroxide (KOH) (VP Reagent B)** is then added.
- 3. The order of reagent addition is critical to prevent false results.**
- 4. Observation:** The tube is gently shaken to expose the medium to atmospheric oxygen and observed for color development for up to **one hour.**

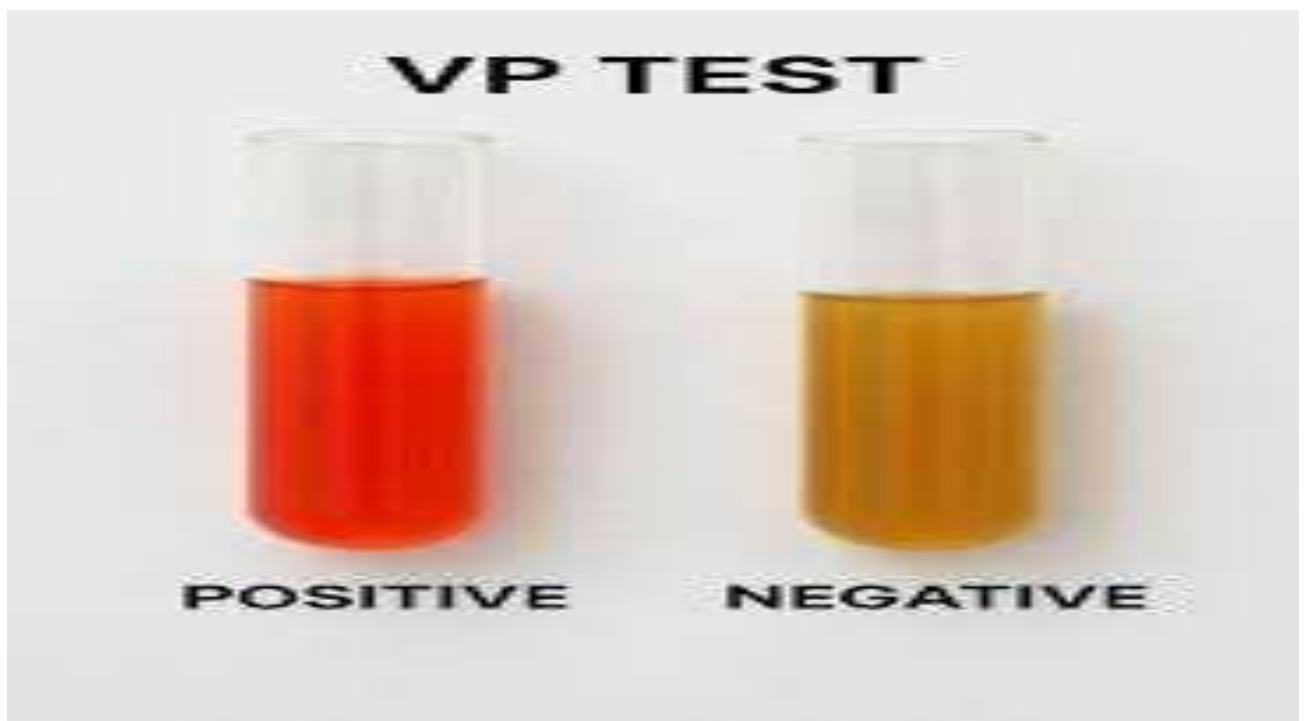
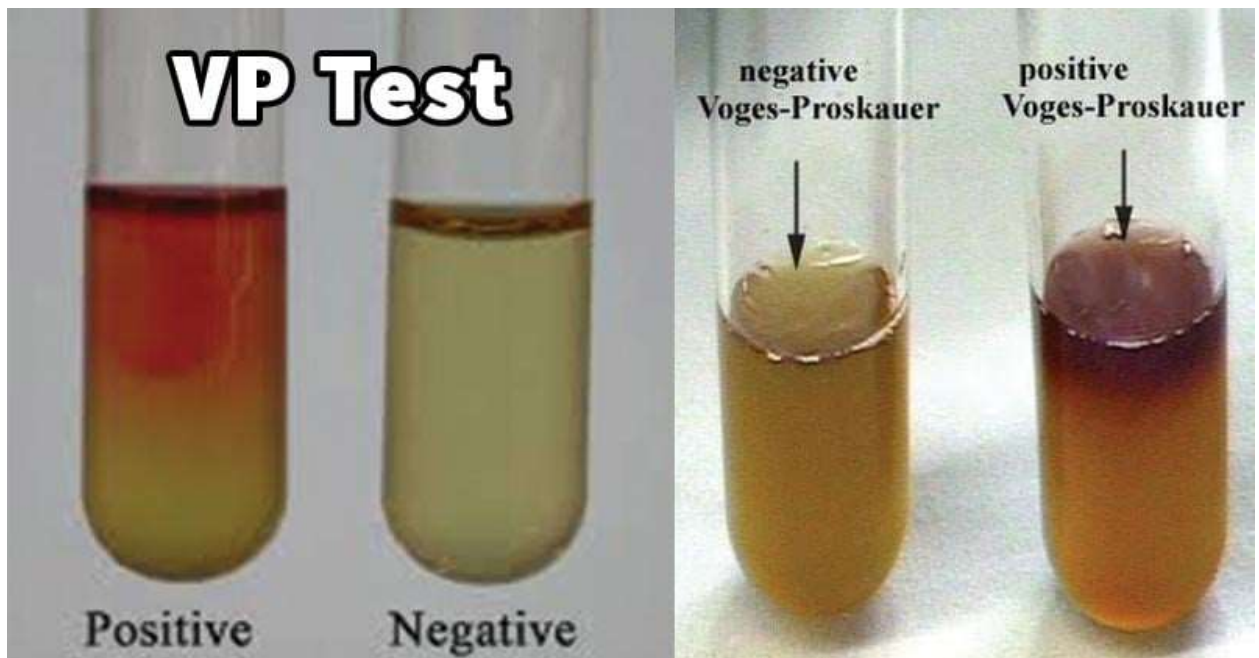
Results and Interpretation

- **VP Positive:** A pink-red or cherry red color develops on the surface of the medium within 15 minutes to an hour, indicating the presence of acetoin.
- **VP Negative:** The medium remains yellow or yellow-brown (or may develop a copper color) after the addition of reagents, indicating the absence of acetoin.

VP Positive Organisms

Specific bacteria are known to be VP positive, including:

- *Klebsiella* species
- *Enterobacter* species
- *Hafnia* species
- *Serratia* species
- *Staphylococcus aureus*
- Most Viridans streptococci (except for some species like *S. mitis*)



Citrate Utilization Test

The citrate utilization test determines if an organism can use sodium citrate as its sole carbon source and ammonium salts as a nitrogen source, typically using Simmons Citrate Agar. A positive result, indicating the enzyme citrase is present, turns the green medium blue ($\text{pH} > 7.6$) due to alkaline byproducts.

common for identifying *Enterobacteriaceae*.

Purpose:

Differentiates gram-negative bacteria based on their ability to metabolize citrate.

Medium: Simmons Citrate Agar

(contains sodium citrate, ammonium salt, and bromothymol blue).

Procedure: A streak-stab technique is used to inoculate a slant, which is incubated at $35\text{--}37^{\circ}\text{C}$ for 24–48 hours.

Interpretation:

- **Positive (+):** Growth on the slant and a color change from green to deep blue.
- **Negative (-):** No growth, medium remains green.

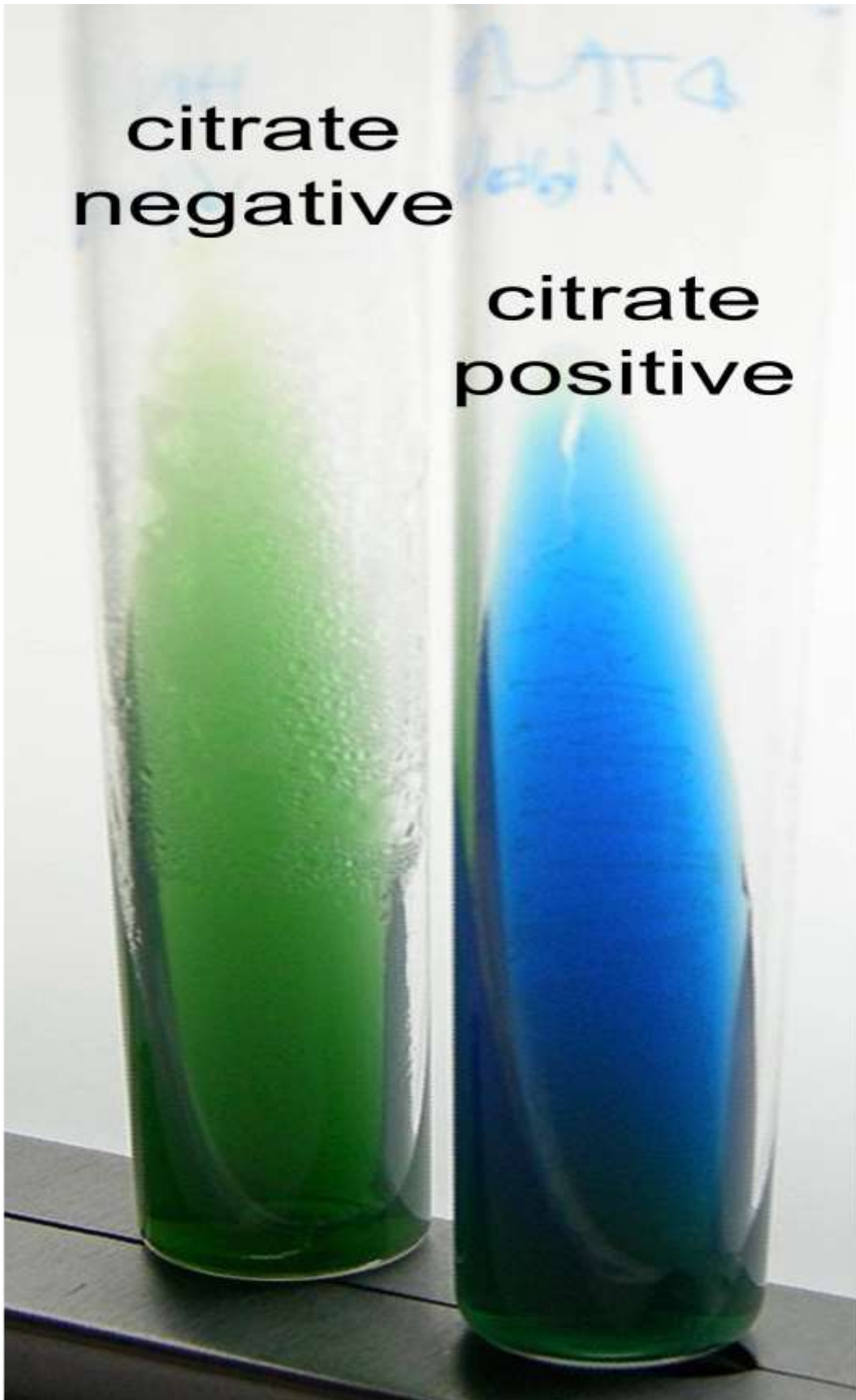
Examples:

***Klebsiella pneumoniae* and *Enterobacter aerogenes* are positive while *Escherichia coli* is negative.**



**citrate
negative**

**citrate
positive**



Differential Media

Differential media (or indicator media) are specialized culture media designed to allow multiple types of microorganisms to grow while visually distinguishing them based on metabolic or biochemical differences. These media often contain specific substrates (e.g., sugars) and pH indicators (neutral red, phenol red , Bromothymol blue) that cause color changes in colonies or the agar, allowing for the differentiation of closely related species.

Purpose:

To differentiate species of microorganisms on a single plate by distinguishing between, for example, lactose fermenters and non-fermenters.

Mechanism:

Typically utilizes pH indicators to show metabolic changes (such as acid production) or reagents that produce color changes.

Examples:

Blood Agar:

Differentiates bacteria based on hemolysis (destruction of red blood cells), such as alpha, beta, or gamma hemolytic activity.

MacConkey Agar (MAC):

Differentiates based on lactose fermentation; lactose fermenters turn pink/red.

Mannitol Salt Agar (MSA):

Differentiates mannitol fermenters (e.g., *Staphylococcus aureus*, which turns the medium yellow) from non-fermenters such as *Staphylococcus epidermidis* which turns the medium red.

Eosin Methylene Blue (EMB) Agar:

Differentiates lactose fermentation *Escherichia coli* produces a green metallic sheen. These media are essential in clinical laboratories for identifying pathogens. such as separating *E. coli* from other Enterobacteriaceae.

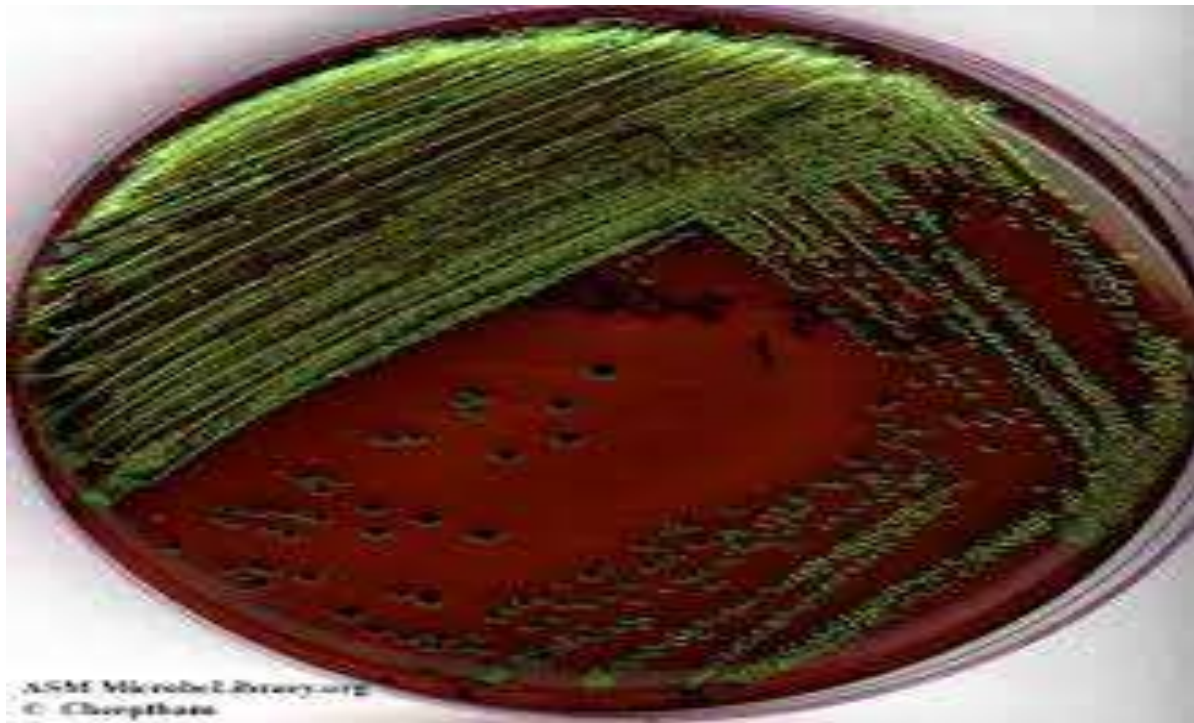




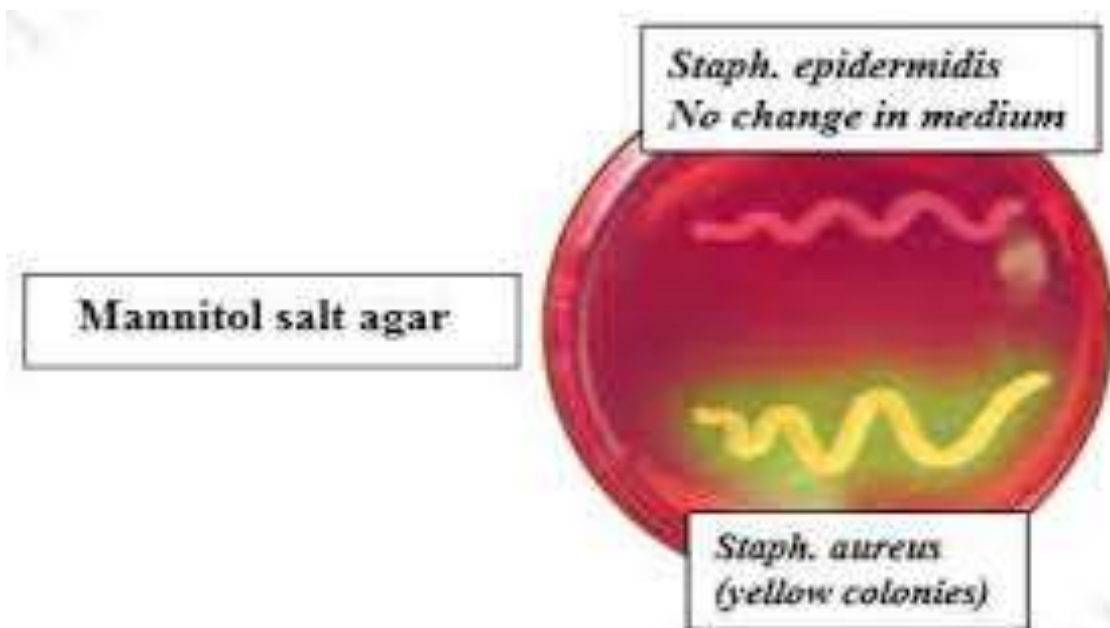


MacConkey agar





EMB agar



Mannitol salt agar

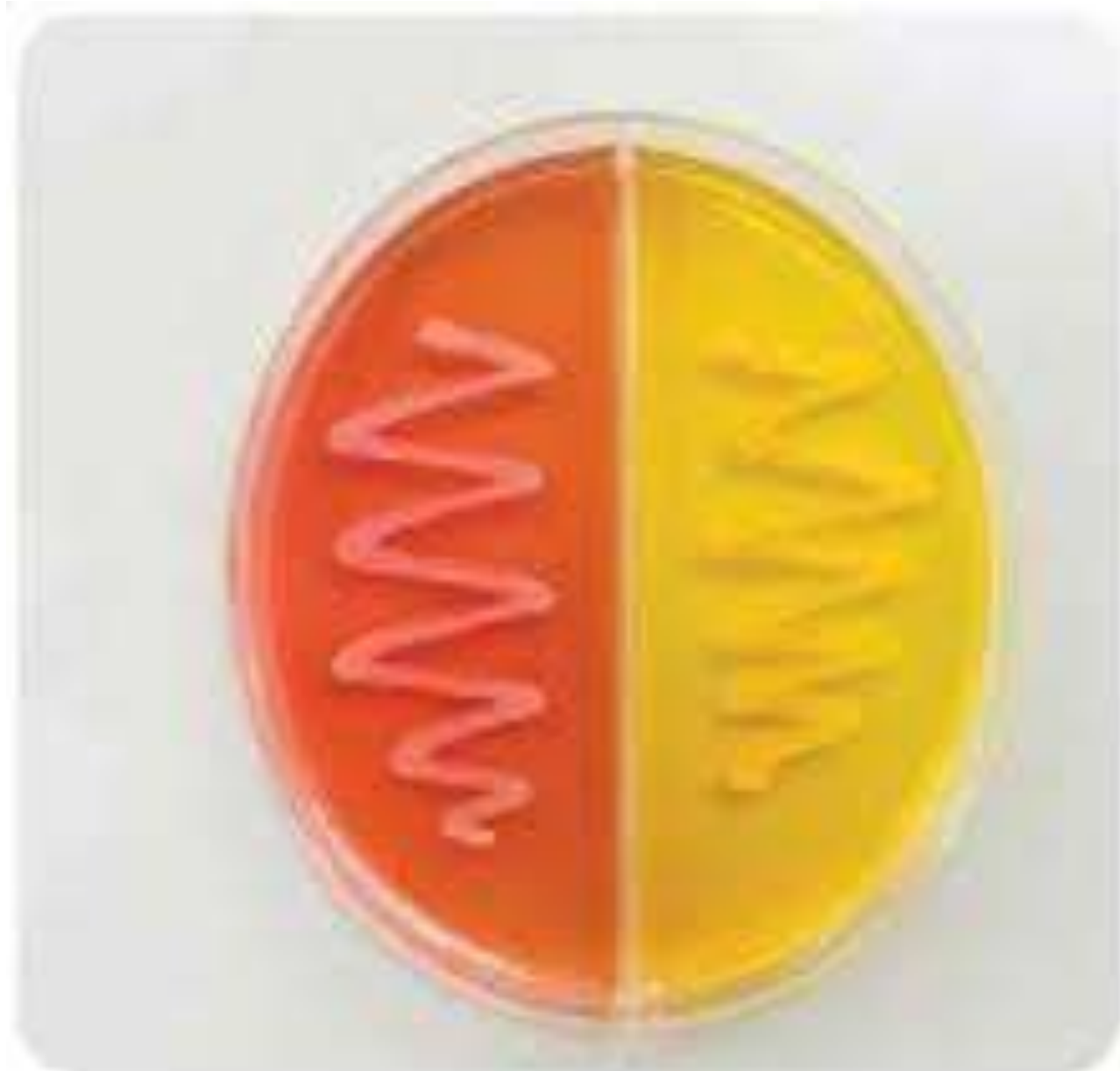
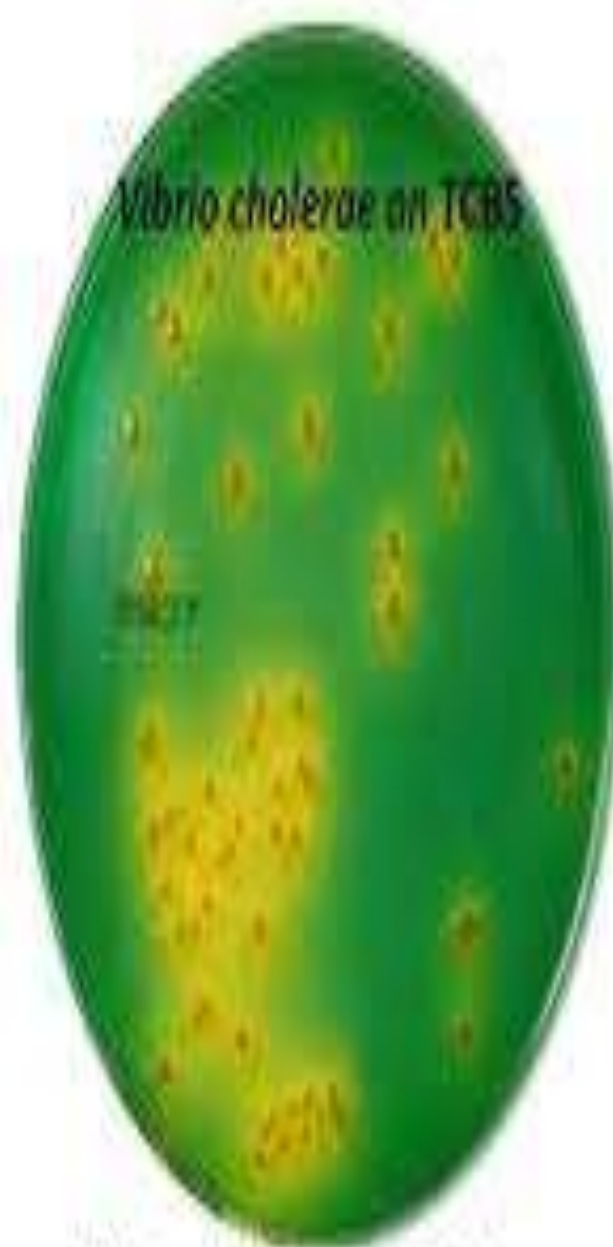


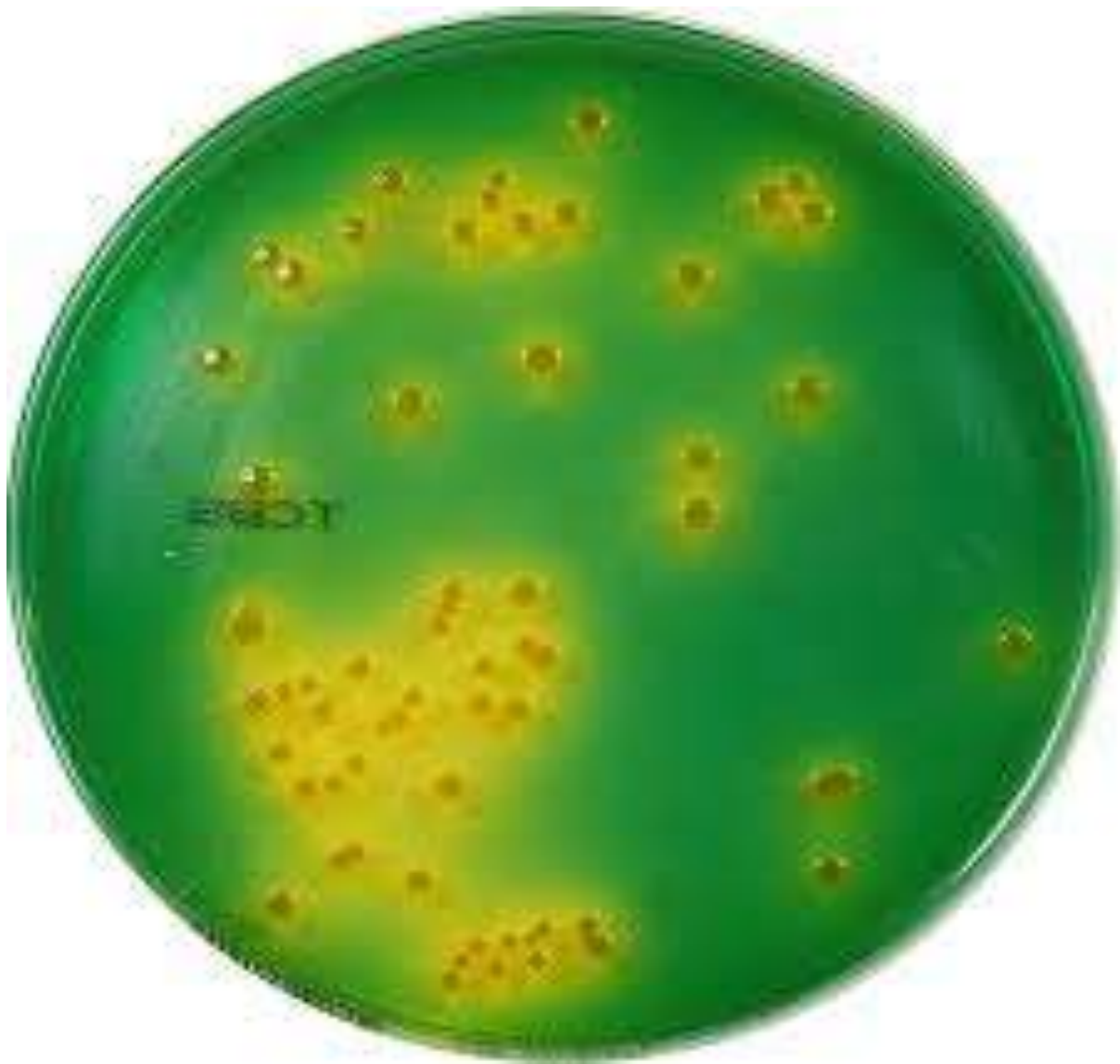
Figure 5.5: Growth of *Staphylococcus aureus* on Mannitol salt agar



Vibrio cholerae on TCBS Agar

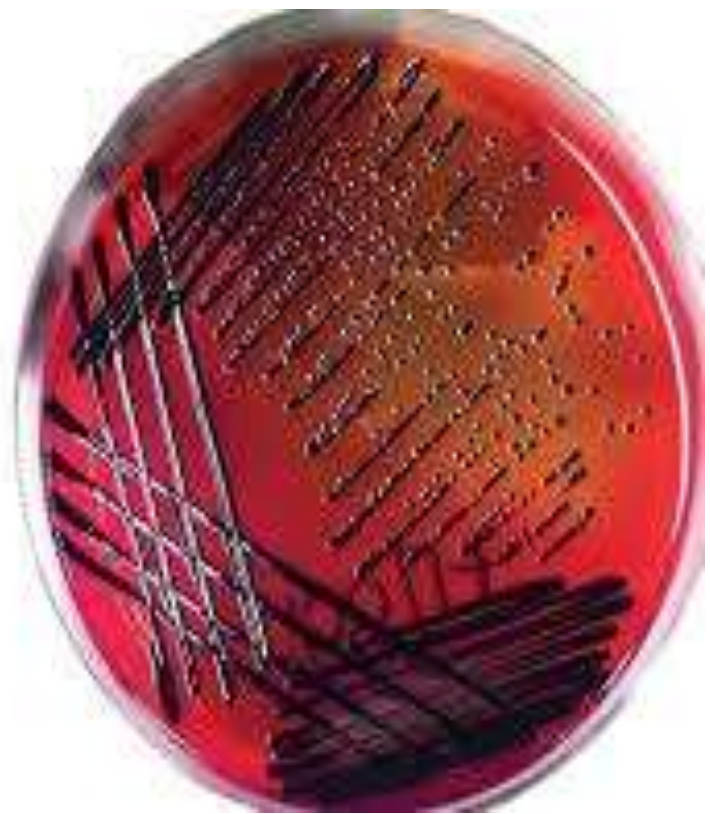


Vibrio parahaemolyticus on TCBS Agar



TCBS agar

Salmonella
Typhimurium
on
XLD agar.



XLD Agar media

Salmonella

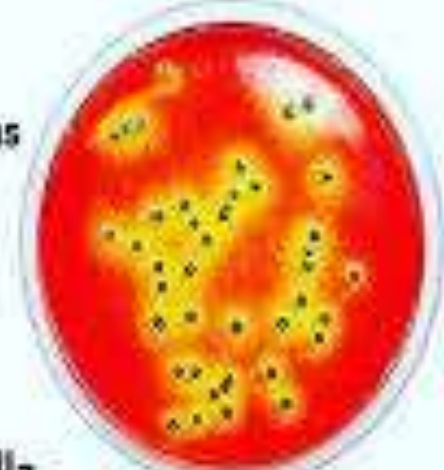


Coliforms



Shigella

Proteus
Citrobacter





Blood agar

Selective media

Selective media are specialized culture media designed to isolate specific microorganisms from mixed populations by promoting the growth of desired organisms while inhibiting the growth of unwanted, contaminating microbes. This selectivity is achieved by adding specific inhibitors like antibiotics, dyes, high salt concentrations, or adjusting pH to inhibit unwanted bacteria.

Mannitol Salt Agar (MSA):

Selects for *Staphylococcus* species due to high sodium chloride concentration.

Eosin Methylene Blue (EMB) Agar:

Selects for Gram-negative bacteria by inhibiting Gram-positive bacteria with dyes

MacConkey Agar:

Selects for Gram-negative organisms, particularly Enterobacteriaceae.

Phenyl Ethyl Alcohol (PEA) Agar:

Inhibits most Gram-negative bacteria, selecting for Gram-positive organisms.

Thayer-Martin Agar:

Used to isolate *Neisseria gonorrhoeae*.

Lowenstein-Jensen Medium:

Selects for *Mycobacterium tuberculosis*.

Mechanisms of Selective Media

- **Antibiotics:** PEA agar inhibits Gram-negative DNA synthesis.
- **Dyes:** Crystal violet inhibits Gram-positive bacteria.
- **Salts:** High NaCl concentrations (e.g., 7.5% in MSA) inhibit most bacteria except *Staphylococcus*.

- **pH:** Low pH (acidic) environments in Media like **Sabouraud Dextrose Agar (SDA)** are used to isolate fungi/yeast.

Selective media are essential in clinical, environmental, and food laboratories to quickly identify pathogens.

such as isolating *Salmonella* from food samples.





Thayer Martin Agar



Lowenstein-Jensen Medium:

Colonies of *Mycobacterium tuberculosis* on
Lowenstein-Jensen (LJ) Medium





Phenyl Ethyl Alcohol (PEA) Agar:

Bacterial taxonomy

Lab:8

Differential Media

Differentiation between aerobic and anaerobic bacteria

oxygen requirement

According to oxygen requirement, bacteria can be divided into five types:

1-Obligate aerobes:

Definition: Bacteria that require oxygen to grow; they cannot survive without it.

Examples: *Mycobacterium tuberculosis*,
Pseudomonas aeruginosa

2-Obligate anaerobes:

Definition: Bacteria that cannot tolerate oxygen; they die in its presence.

Examples: *Clostridium botulinum*, *Bacteroides fragilis*

3-Facultative anaerobes:

Definition: Bacteria that can grow with or without oxygen, but grow better in the presence of oxygen.

Examples: *Escherichia coli*, *Staphylococcus aureus*

4-Microaerophiles:

Definition: Bacteria that require a small amount of oxygen for growth, but high concentrations are toxic.

Examples: *Helicobacter pylori*, *Campylobacter jejuni*

5-Aerotolerant anaerobes:

Definition: Bacteria that do not use oxygen but can tolerate its presence.

Examples: *Lactobacillus* spp, *Streptococcus pyogenes*

obligate
aerobes



A

obligate
anaerobes



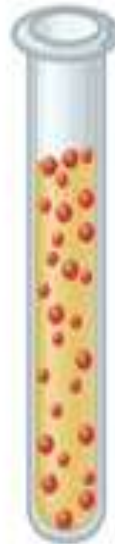
B

facultative
anaerobes



C

aerotolerant
anaerobes

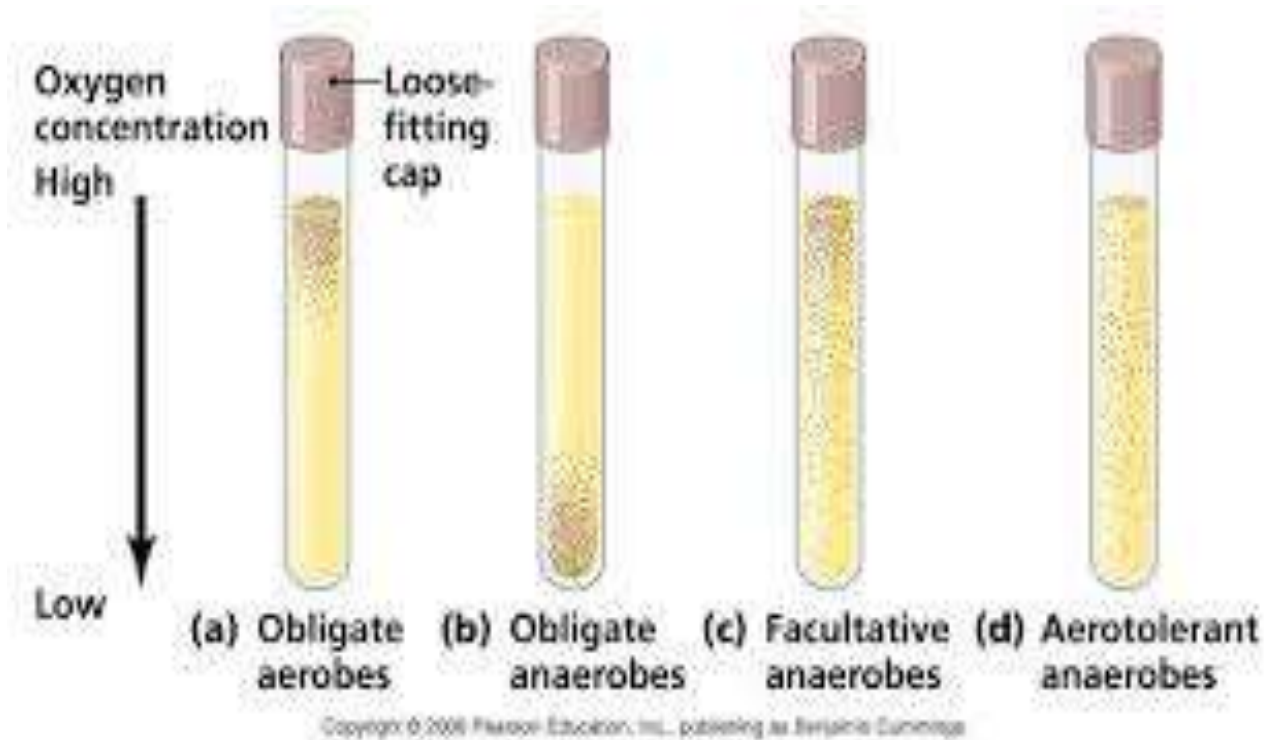


D

microaerophiles



E



Culture media

Culture media can be divided according to oxygen requirement into two main types:

1-Aerobic culture media:

These media are used for the growth of microorganisms that require oxygen for their metabolism and growth. Examples include **nutrient agar, **blood agar**, and **tryptic soy agar** incubated under aerobic conditions.**

2-Anaerobic culture media:

These media are designed for the cultivation of microorganisms that grow in the absence of oxygen or are inhibited by its presence. Examples include **thioglycolate broth, **Robertson's cooked meat medium**, and **anaerobic blood agar**.**



Media used in anaerobic bacteriology can be freshly prepared or purchased from commercial suppliers. Media for anaerobic culture prepared in the laboratory should be used within two weeks of preparation as long storage degrades the quality of the media due to peroxide accumulation and dehydration. Anaerobic culture media contains reducing agents such as cysteine.

Pre-reduced, anaerobically sterilized media are produced by different commercial suppliers, which have extended shelf life up to six months. The primary plating media for inoculating anaerobic specimens includes a non-selective **blood agar and one or all of the following mentioned selective media.**



Clostridium perfringens in
anaerobic blood agar



Clostridium perfringens in
egg yolk agar



Bacteroides bile esculin agar

Anaerobic Culture Media



Robertson's cooked meat
broth



Anaerobic phenylethyl
alcohol blood agar



C. difficile in cycloserine
cefoxitin fructose agar

1-Nonselective media used in anaerobic bacteriology:

- 1.Cooked meat broth (e.g. Robertson's Cooked Meat Medium):** Non-selective for the cultivation of anaerobic organisms; with the addition of glucose, can be used for gas-liquid chromatography.
- 2.Anaerobic blood agar:** It is a non-selective medium for the isolation of anaerobes and facultative anaerobes.
- 3.Egg-yolk agar (EYA):** Non-selective for determination of lecithinase and lipase production by Clostridia and Fusobacteria.
- 4.Peptone-yeast extract glucose broth (PYG):** Non-selective for the cultivation of anaerobic bacteria for gas-liquid chromatography.

2-Selective and differential media used in anaerobic bacteriology:

1. **Bacteroides bile esculin agar (BBE):** It is selective and differential for *Bacteroides fragilis* group and good for presumptive identification.
2. **Laked Kanamycin-vancomycin blood agar (LKV):** It is selective for isolation of *Prevotella* and *Bacteroides* spp.
3. **Anaerobic phenylethyl alcohol agar (PEA):** Selective for inhibition of gram-negative rods and swarming by some *Clostridia*.
4. **Cycloserine cefoxitin fructose agar (CCFA):** Selective for *Clostridium difficile*.
5. **Thioglycollate broth:** Nonselective for the cultivation of anaerobes; as well as facultative anaerobes and aerobes.



bottles of aerobic and anaerobic media that can be used to detect microbes using dilution and timing Captions are provided by our contributors.

Anaerobic Blood Agar

- Composition
- Principle
- Preparation
- Method of Use
- Result Interpretation
- Uses
- Limitations



Anaerobic Blood Agar- Composition, Principle, Preparation, Results, Uses

Anaerobic Blood Agar is a solid media recommended for use in qualitative procedures for primary isolation and cultivation of anaerobic organisms, including fastidious strains. Anaerobic Blood Agar supports good growth and typical pigmentation of fastidious and slow-growing anaerobes, as well as other anaerobes of significant clinical importance.

Composition of Anaerobic Blood Agar

Ingredients	Gm/liter
Casein enzymic hydrolysate	15.000
Papaic digest of soybean meal	5.000
Yeast extract	5.000
Sodium chloride	5.000
L-Cysteine	0.500
Hemin	0.005
Agar	13.50

Principle of Anaerobic Blood Agar

- . Anaerobic Blood Agar base serves as a nutritious, nonselective medium allowing the cultivation of not only fastidious anaerobes but also of aerobic and microaerophilic microorganisms.**
- . It contains peptones which supply nitrogenous substances and amino acids necessary for the growth of anaerobic bacteria.**
- . Yeast extract provides B-complex vitamins and serves as a growth enhancer.**
- . Hemin, vitamin K, and sheep blood stimulate the growth of anaerobes like Bacteroides species and gram-positive spore bearers like Clostridium species.**
- . Sodium chloride is a source of essential electrolytes and maintains osmotic equilibrium.**

- . Addition of blood provides nutrients and helps to differentiate hemolytic organisms.**

Preparation and Method of Use of Anaerobic Blood Agar

- 1.Suspend 44.0 grams in 1000 ml distilled water.**
- 2.Heat to boiling to dissolve the medium completely.**
- 3.Add the rehydrated contents of 1 vial of Vitamin K1 solution.**
- 4.Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.**
- 5.Cool to 45-50°C.**
- 6.Aseptically add 5% v/v sterile defibrinated sheep blood.**
- 7.Mix well and pour into sterile Petri plates.**

8. Prior to use, reduce the plates for a minimum of 24 hours by placing them in an anaerobic environment at room temperature.

9. Inoculate specimens for anaerobic culture on both selective and non-selective media as soon as possible after receipt in the laboratory; streak plates for isolation.

10. Incubate anaerobically at 33-37°C for 48-72 hours.

11. Confirm anaerobic growth by subculture to anaerobic blood agar plate.

Luxuriant Growth: Growth of microorganisms that is very abundant, dense, or vigorous on a culture medium.

Result Interpretation of Anaerobic Blood Agar

Organisms	Growth
<i>Bacteroides fragilis</i>	Luxuriant Growth
<i>Bacteroides melaninogenicus</i>	Luxuriant Growth
<i>Peptostreptococcus anaerobius</i>	Luxuriant Growth
<i>Clostridium perfringens</i>	Growth, double zone β -hemolysis
<i>Fusobacterium nucleatum</i>	Growth
<i>Prevotella melaninogenica</i>	Growth; pigment (tan to brown)
<i>Escherichia coli</i>	Growth
<i>Staphylococcus aureus</i>	Growth

Uses of Anaerobic Blood Agar

1-Anaerobic Blood Agar Base is recommended for the cultivation of anaerobic microorganisms, including very fastidious organisms from clinical specimens.

2-It promotes both typical pigment formation in *Bacteroides melaninogenicus* and displays double hemolytic reaction in *Clostridium perfringens* with added blood to the medium base.

3-The media supports typical pigment production by pigmented *Prevotella* and *Porphyromonas*

Limitations of Anaerobic Blood Agar

1-The media must be prepared, dispensed, and packaged under oxygen-free conditions to prevent the formation of oxidized products prior to use.

2-This Agar will not provide complete information for identification of bacterial isolates. Additional test procedures and media are required for complete identification.

3-It is recommended that a selective media such as Anaerobic Brucella Laked Blood Agar with Kanamycin and Vancomycin and/or Anaerobic Brucella Blood Agar with Phenylethyl Alcohol also be inoculated with clinical specimens to assure growth of all species present.