Lab 8+9+10: Identification of G+ and G- Bacteria.

❖ Identification of unknown bacteria

1. **Cultural characterise (as discussed above)**

2. Staining technique

The Gram stain is one of the most useful tools in the microbiology laboratory and is used universally. In the diagnostic laboratory, it is used not only to study microorganisms in cultures, but it is also applied to smears made directly from clinical specimens. Direct, Gram-stained smears are read promptly to determine the relative numbers and morphology of bacteria in the specimen, and it is one of important techniques used to differentiation the gram positive bacteria and gram negative bacteria. There are many different type of staining:

1. Simple stains: use of single stain **(methylene blue, crystal violet)**

2. Differential stains: using of two contrasting stain that divided in two types depended on their function:

a. For identification **(Gram stain, acid fact stain)**

b. Visualization of structure **(spore stain, capsular stain)**

3. Special stain (India ink stains, flagella stain)

1. Simple Stain: that used to learn the value of simple stains in studying basic microbial morphology

Materials

1. 24-hour agar culture of *Staphylococcus epidermidis*
2. 24-hour agar culture of *Bacillus subtilis*
3. 24-hour agar culture of *Escherichia coli*
4. Prepared stained smear of a spiraled organism Methylene blue
5. Absolute methanol (if bacterial incinerator used)
6. Toothpicks
7. Slides
8. China-marking pencil or permanent marking pen

Procedures

1. Slides for microscopic smears must always be sparkling clean. They may be stored or dipped in alcohol and polished clean (free of grease) with a tissue or soft cloth.

2. Take three clean slides and with your marking pencil or pen make a circle (about 1 1/2 cm in diameter) in the centre. At one end of the slide write the initials of one of the three assigned organisms (your three slides should read Se, Bs, and Ec, respectively).

3. Turn the slides over so that the unmarked side is up. (When slides are to be stained, pen or pencil markings should always be placed on the underside so that the mark will not smear, wash off, or run into the smear itself.)

4. With your inoculating loop, place a loopful of water in the ringed area of the slide. Using proper aseptic transfer techniques mix a **small** amount of bacteria in the water and spread it out. Repeat this step until smears of all three organisms have been made.

5. Allow the smears to air dry. You should be able to see a thin white film on each slide. If not, add another loopful of water and more bacteria as in step 4.

6. Heat-fix the smears by passing the slides rapidly through the Bunsen flame three times so that the smears will not wash off. If a Bunsen burner is not available, fix the smears by placing the slides on a staining rack and flooding them with absolute methanol. Allow the slides to sit for one minute, and then drain off the alcohol and air dries them completely.

7. Place the slides on a staining rack and flood them with methylene blue. Leave the stain on for three minutes.

8. Wash each slide gently with distilled water, drain off excess water, blot (do not rub) with bibulous paper, and let the slides dry completely in air.

9. While the slides are drying, take two more clean slides and draw a circle on the bottom with your wax pencil or marking pen.

10. Place a loopful of distilled water (or sterile saline) over the circle on each slide.

11. With the flat end of a toothpick, scrape some material from the surface of your teeth and around the gums. Emulsify the material in the drop of water on one slide. Repeat this procedure on the other slide.

12. Allow both slides to dry in air; then fix them with heat or methanol. Stain one slide with methylene blue for three minutes

13. Wash, drain, and dry the slides as in step 8.

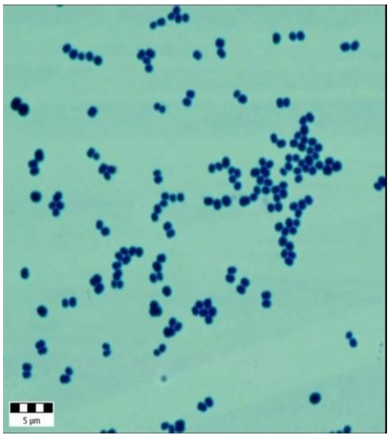
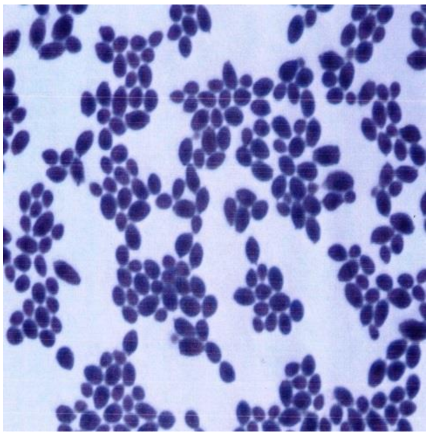
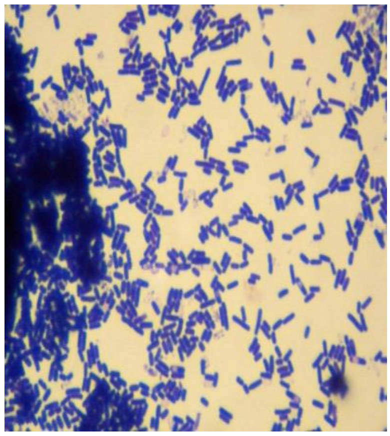
14. Examine all slides, including the prepared stained smear assigned to you, with all three microscope objectives. Record your results in the table.

Results

Organism in Coccus, Cell Broth Culture Stain Color Rod, or Spiral Grouping Diagram

No	*Microorganisms*	*Shapes of colonies*	*Color of colonies*
1.	*S. Epidermidis*		
2.	*B. subtilis*		
3.	*Saccharomyces Cervesia*		

Q.1 Draw the organisms you saw in the scraping from your teeth. And Describe the results you obtained with this stain used (morphology of bacteria). And write the type of bacteria for each the figures?

2. Gram stain

Gram staining method, the most important procedure in Microbiology, was developed by Danish physician Hans Christian Gram in 1884. Gram staining is still the cornerstone of bacterial identification and taxonomic division. This staining procedure separates most bacteria into two groups on the basis of cell wall composition:

1. Gram positive bacteria *(thick layer of peptidoglycan-90% of cell wall*)- **stains purple**

2. Gram negative bacteria *(thin layer of peptidoglycan-10% of cell wall and high lipid content)* –**stains red/pink**

❖ Classic Gram staining techniques involves following steps:

1. **Fixation of clinical materials** to the surface of the microscope slide either by heating or by using methanol. (# Methanol fixation preserves the morphology of host cells, as well as bacteria, and is especially useful for examining bloody specimen material). An easy way to remember the steps of the Gram stain
2. **Application of the primary stain** (crystal violet). Crystal violet 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. **Decolourization step:** The decolourization 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 counter stain (safranin):** The red dye safranin stains the decolorized gram-negative cells red/pink; the gram-positive bacteria remain blue.

❖ Principle of Gram Stain

The differences in cell wall composition of Gram positive and Gram negative bacteria accounts for the Gram staining differences. Gram positive cell wall contain thick layer of peptidoglycan with numerous teichoic acid cross linking which resists the decolourization.

In aqueous solutions crystal violet dissociates into CV+ and Cl – ions that penetrate through the wall and membrane of both Gram-positive and Gram-negative cells. The CV+ interacts with negatively charged components of bacterial cells, staining the cells purple. When added, iodine (I- or I3-) interacts with CV+ to form large crystal violet iodine (CV-I) complexes within the cytoplasm and outer layers of the cell.

The decolorizing agent, (ethanol or an ethanol and acetone solution), interacts with the lipids of the membranes of both gram-positive and gram negative bacteria.

The outer membrane of the Gram-negative cell (lipopolysaccharide layer) is lost from the cell, leaving the peptidoglycan layer exposed. Gram-negative cells have thin layers of peptidoglycan, one to three layers deep with a slightly different structure than the peptidoglycan of gram-positive cells. With ethanol treatment, gram-negative cell walls become leaky and allow the large CV-I complexes to be washed from the cell.

The highly cross-linked and multi-layered peptidoglycan of the gram-positive cell is dehydrated by the addition of ethanol. The multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the cell.

After decolourization, the gram-positive cell remains purple in color, whereas the gram-negative cell loses the purple color and is only revealed when the counterstain, the positively charged dye safranin, is added.

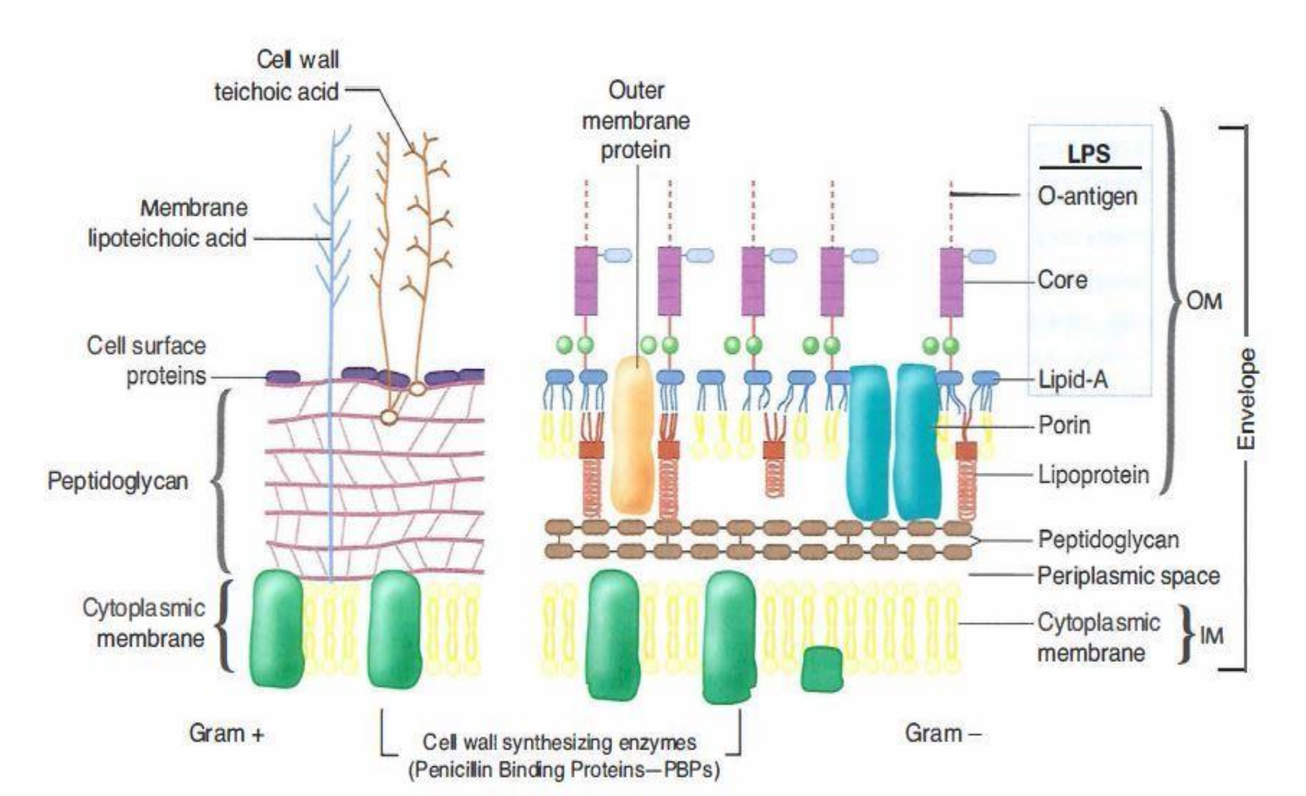


Figure 2: Cell wall of Gram Positive and Gram Negative Bacteria

Materials

1. 24-hour agar culture of:

   -*Staphylococcus Epidermis*

   -*Bacillus subtilis*

   -*Escherichia coli*

   -*Streptomyces sp.*

2. Crystal violet
3. Gram's iodine
4. Ethyl alcohol, 95%
5. Safranin
6. Slides
7. Marking pen or pencil and slide labels

Procedures

1. Prepare a fixed smear of each culture. You will have 4 smears labelled. On the underside of each slide, make a code mark so that you can identify the slides after staining.

2. Stain each smear by the following procedures (this is Hucker's modification of the Gram stain):

a. Flood slide with **crystal violet**. Allow to stand for **one minute** Please note that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.

b. **Wash** slide in a gentle and indirect stream **of tap water for 2 seconds.**

c. Flood with Gram's iodine (a mordant). Leave for one minute.

d. **Wash** slide in a gentle and indirect stream **of tap water for 2 seconds.**

e. Decolorize with alcohol (95%) until no more color washes off (usually 10–20 seconds). This is a most critical step or adds drop by drop to slide until decolorizing agent running from the slide runs clear. Be careful not to over decolorize, as many gram-positive organisms may lose the violet stain easily and thus appear to be gram negative after they are counterstained.

g. Apply **safranin** (the counterstain) for **30 second** to one minute.

1. h. Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent

i. Drain and blot gently with absorbent paper. Air dries the slide thoroughly before you examine the preparation under the microscope.

3. When slides are dry, label them as shown:

4. Examine all slides under oil immersion (100x) using a Bright field microscope.

5. Record observations in table under Results and noting which bacteria are grams positive or gram negative. And examines the Morphology of the bacteria, whether cocci, diplococci, streptococci, rods, or coccobacilli.

Results:

- Gram-negative bacteria will stain pink/red and
- Gram-positive bacteria will stain blue/purple.

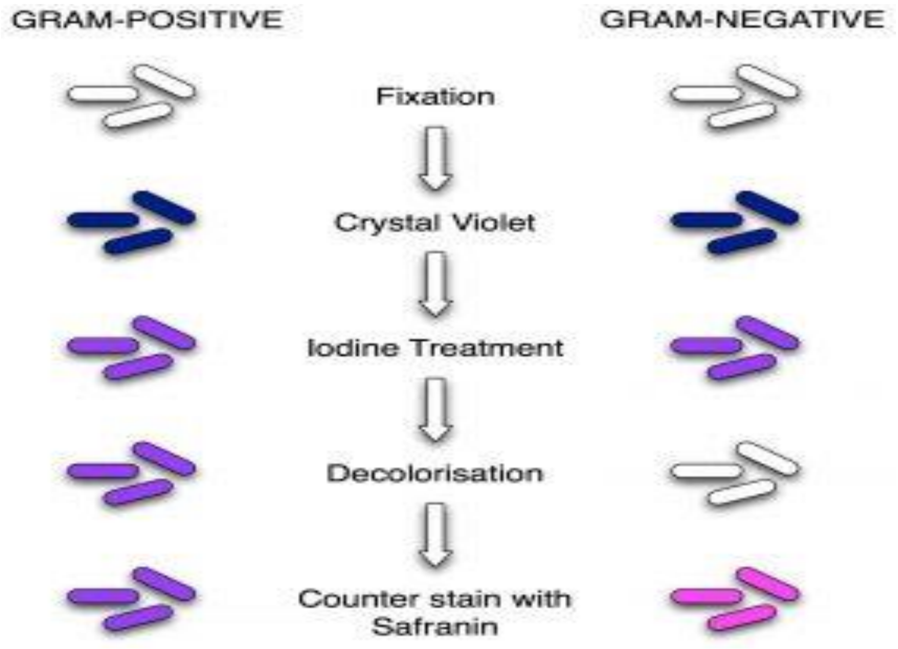


Figure: Procedure of Gram Staining; note the color change after each step

✓ **Note:**

- **Nearly all clinically important bacteria can be detected/visualized using Gram staining method the only exceptions being those organisms;**

1. That exists almost exclusively within host cells i.e. Intracellular bacteria (e.g., Chlamydia)

2. Those that lack a cell wall (e.g., Mycoplasma)

3. Those of insufficient dimensions to be resolved by light microscopy (e.g., Spirochetes)

- **Smear Preparation**

Fix material on slide with methanol or heat. If slide is heat fixed, allow it to cool to the touch before applying stain.

- **Always check new batches of stain and reagents for correct staining reactions using a smear containing known Gram positive and Gram negative organisms.**

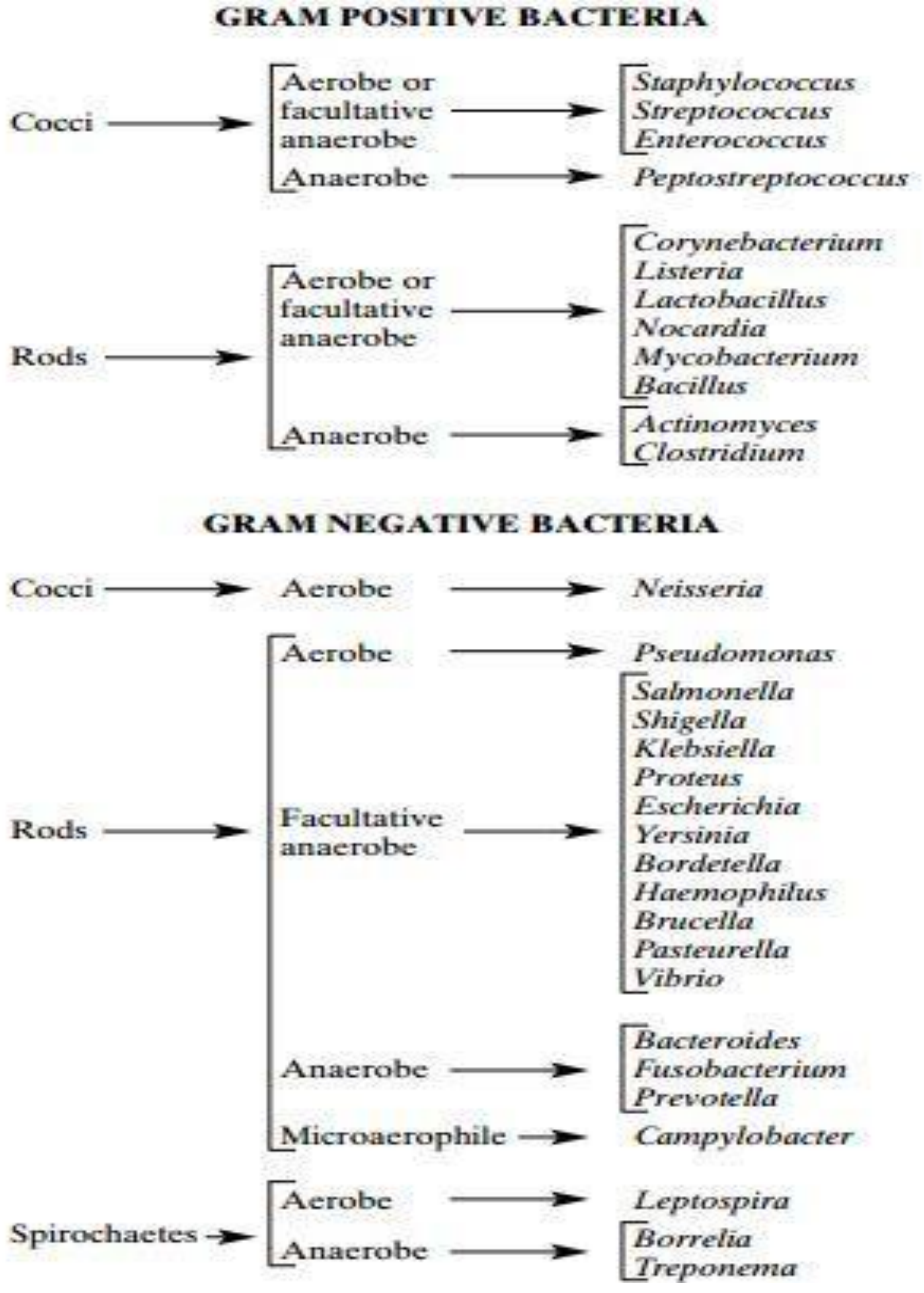


Figure 2: Basic classification of Medically Important Bacteria

Capsule stain: is a type of differential stain which uses acidic and basic dyes to stain background & bacterial cells respectively so that presence of capsule is easily visualized. Capsule is synthesized in the cytoplasm and secreted to the outside of the cell where it surrounds the bacterium. Most of the capsulated bacteria have a capsule made up of polysaccharide layer but some bacteria have capsule made up of polypeptide, or glycoprotein. Capsules are associated with virulence in several microorganisms, including *Streptococcus Pneumoniae* and *Neisseria meningitides*, *Haemophilus Influenza, Klebsiella pneumoniae* are common capsulated bacteria. Because capsules resist phagocytosis thus evading the host immune system.

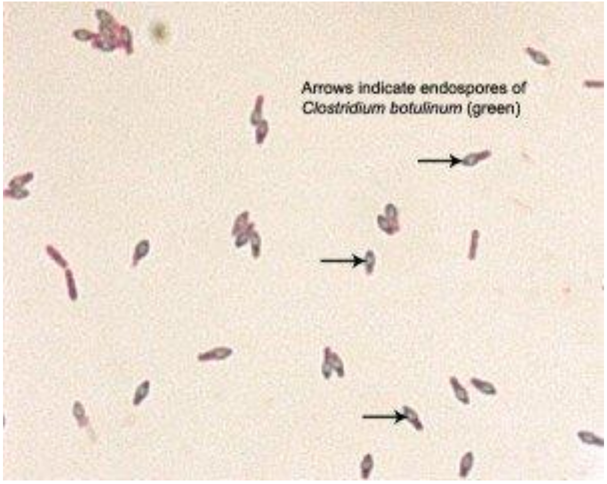


Figure: spore stain *Clostridium botulinum*

Principle of Capsule Stain

Bacterial capsules are non-ionic, so neither acidic nor basic stains will adhere to their surfaces. Therefore, the best way to visualize them is to **stain the background using an acidic stain** (e.g., Nigrosine, Congo red) and to **stain the cell itself using a basic stain** (e.g. Crystal violet, safranin, basic fuchsin and methylene blue).

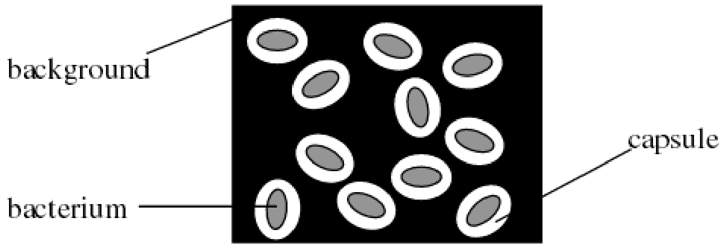


Figure: Capsule Staining

Various types of methods are available for the demonstration of the presence of

Capsule. The results (stain of the cells, background and capsule) depend on the type of the method used. Two commonly used methods are discussed here:

A. India ink method (special stains)

In this method two dyes, crystal violet and India ink are used. The capsule is seen as a clear halo around the microorganism against the black background. This method is used for demonstrating *Cryptococcus.*

- The background will be dark *(color of india ink).*
- The bacterial cells will be stained purple *(bacterial cells takes crystal violet-basic dyes as they are negatively charged).*
- The capsule (if present) will appear clear against the dark background *(capsule does not take any stain).*

Note: "I use Nigrosine instead of India ink as Nigrosin gives a more even background and spreads little easier". Read details in the comment section.

Procedure

1. Place a single drop of **India ink** on a clean microscope slide, adjacent to the frosted edge.
2. Using a flamed loop and sterile technique, remove some *Klebsiella pneumoniae* from culture tube or plate and mix it into the drop of India ink. Be sure there are no large clumps of organism, but try to avoid spreading the drop.
3. Place the end of another clean microscope slide at an angle to the end of the slide containing the organism. Spread out the drop out into a film. This is done by contacting the drop of India ink with the clean microscope slide and using the capillary action of the dye/ slide to spread the India ink across the smear.
4. Allow the film to air dry ***(will take 5-7 minutes).*** *DO NOT heat or blot dry! Heat will melt the capsule!*
5. Saturate the slide with crystal violet for 1 minute and rinse slightly & very gently with water. Be cautious w*ater may remove the capsule from the cell.*

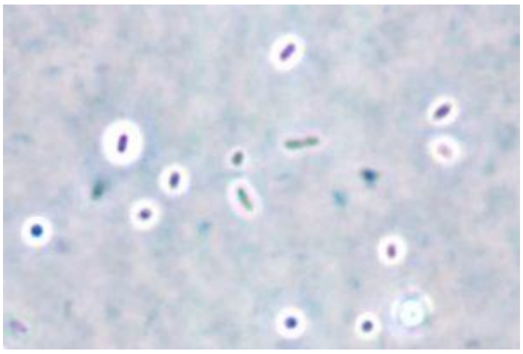


Figure: Capsule staining by India ink method (at 1000x magnification)

6. Let the slide air dry for a few minutes. *DO NOT blot the slide! Blotting will remove the bacteria from the slide and/or distort the capsule.*
7. Observe the slide under oil immersion.

Results: Look for purple cells surrounded by a clear halo on a dark background. The

Halo is the capsule. You may need to decrease the amount of light in order to make the capsule easier to see

India ink Preparation (Negative staining):

Negative stains are used when a specimen or a part of it, such as the capsule resists taking up the stain. India ink preparation is recommended for use in the identification of ***Cryptococcus Neoformans.***

Giemsa stain: Giemsa stain is a Romano sky stain. It is widely used in Microbiology laboratory for the staining of:

1. Malaria and other blood parasites
2. *Chlamydia trachomatis* inclusion bodies
3. *Borrelia* species
4. *Yersinia Pestis*
5. *Histoplasma* species
6. *Pneumocystis Jiroveci* cysts (formerly *Pneumocystis Carinii*)

***B. Anthony's* stain method**

In this type of capsule staining procedure, the **primary stain is crystal violet,** and all parts of the cell take up the purple crystal violet stain. There is no mordant in the capsule staining procedure. A **20% copper sulfate solution serves a dual role as both the decolorizing agent and counter stain.** It decolorizes the capsule by washing out the crystal violet, but will not decolorize the cell. As the copper sulfate decolorizes the capsule, it also counter stains the capsule. Thus, the **capsule appears as a faint blue halo around a purple cell.**

Materials and reagents required

- **Test bacteria:** 36-48 hour culture of capsulated bacteria e.g. *Klebsiella pneumoniae* growing on a slant of **EMB Agar** or culture of other capsulated bacteria and non-capsulated bacteria [Note: *Growing Klebsiella pneumoniae in* *milk based media (e.g. Skim milk) increase its capsule size, making it easier to visualize.*]

- Stain solutions: Depending on the types of method used (Crystal violet, India ink, Nigrosine, Copper Sulphate, Basic carbol fuchsin solution, Methylene blue solution etc.).
- Microscopic slides
- Inoculating loop
- Microscope with 100x objective lens (oil immersion)
- Immersion oil
- Gas burner
- Tissue paper

Procedure

1. Place a single drop of **crystal violet** on a clean microscope slide, adjacent to the frosted edge.
2. Using a flamed loop and sterile technique, add three loopful of test bacterium (any capsulated bacteria such as *Klebsiella pneumoniae, Streptococcus pneumoniae*) from broth culture. If you are adding bacteria from a culture plate make sure that there are no large clumps of organism, but try to avoid spreading the drop.
3. Place the end of another clean microscope slide at an angle to the end of the slide containing the organism. Spread out the drop out into a film. This is done by contacting the drop of crystal violet with the clean microscope slide and using the capillary action of the dye/ slide to spread the crystal violet across the smear.
4. Allow the film to air dry ***(will take 5-7 minutes). DO NOT*** *heat or blot dry! Heat will melt the capsule!*
5. Tilt the slide and rinse with 20% copper sulfate solution. ***DO NOT*** *RINSE WITH WATER! Water will remove the capsule from the cell.*
6. Let the slide air dry for a few minutes. *DO NOT blot the slide! Blotting will remove the bacteria from the slide and/or distort the capsule.*
7. Observe the slide under oil immersion.

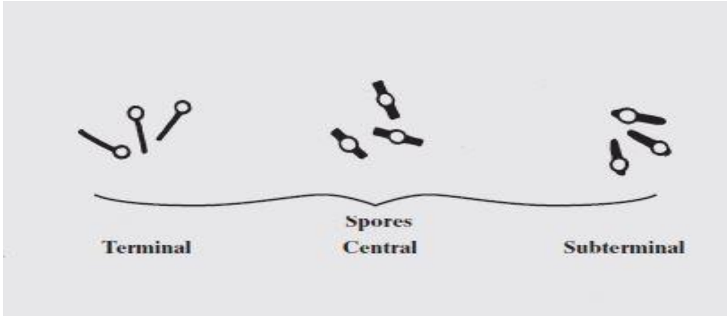
Results: Look for purple cells surrounded by a clear or faint blue halo on transparent Background. The halo is the capsule. *You may need to decrease the amount of light in Order to make the capsule easier to see.*

✓ **Note:**

- **Clean your microscope with lens cleaner, removing all oil from lenses.**
- **Dispose of staining waste and slides in designated waste containers.**
- **Be cautious while handling the slide, since the organisms have not been killed.**
- **In capsule staining procedure "we do not heat fix and rinse the smear with water" as heat and water may dislodge capsules from bacteria.**

Endospore stain: It demonstrates spore structure in bacteria as well as free spores. Relatively few species of bacteria produce **endospores**, so a positive result from endospore staining methods is an important clue in bacterial identification. *Bacillus* spp and *Clostridium* spp are main endospore producing bacterial genera.

When vegetative cells of certain bacteria such as *Bacillus* spp and *Clostridium* spp are subjected to environmental stresses such as nutrient deprivation, they produce metabolically inactive or dormant form-endospore. Formation of endospore circumvents the problems associated with environmental stress and helps them to survive. During unfavourable conditions (*especially when carbon and nitrogen become unavailable*) endospores can form within different areas of the vegetative cell. They can be central, sub-terminal, or terminal. Central endospores are located within the middle of the vegetative cell. Terminal endospores are located at the end of the vegetative cell. Sub-terminal endospores are located between the middle and the end of the cell.



Most endospore forming bacteria are found in soil or aquatic environments. However, some species of *Bacillus* and *Clostridium* have **medical significance**. *Clostridium perfringens, C. botulinum* (a potential agent of bioterrorism) and *C. tetani* are the causative agents of gas gangrene, botulism and tetanus, respectively. *Bacillus anthracis* and *Bacillus cereus* are the causative agents of anthrax and a self-limiting food poisoning, respectively.

Principle of Spore Staining:

A **differential staining** technique (the Schaeffer-Fulton method) is used to distinguish between the vegetative cells and the endospores. A **primary stain (malachite green)** is used to stain the endospores. Because endospores resist staining, the malachite green will be **forced** into *(i.e., malachite green permeate the spore wall)* the endospores by **heating**. In this technique heating acts as a **mordant**.

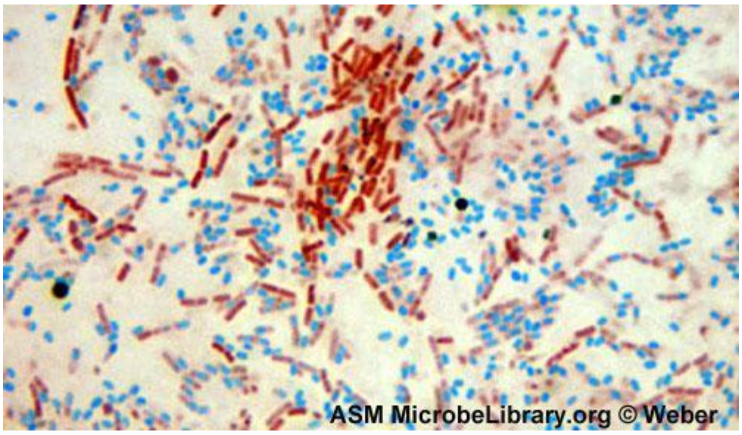
There is no need of using any decolourizer in this spore staining as the primary dye malachite green bind relatively weakly to the cell wall and spore wall .In fact If washed well with water the dye come right out of cell wall however not from spore wall once the dye is locked in. **Water is used to decolorize** the vegetative cells.

(Note**:** In **Gram Staining** and **AFB Staining** we use Alcohol or Acid Acohol or Acid as a decolorizer but in spore staining water is sufficient (to be used as decolorizer) because:

- malachite green dye is water-soluble and does not adhere well to the cell wall
- Vegetative cells have been disrupted by heat, because of these reasons, the malachite green rinses easily from the vegetative cells. **)**

As the endospores are resistant to staining, the endospores are equally resistant to de-

Staining and will retain the primary dye while the vegetative cells will lose the stain. The addition of a counterstain or **secondary stain (safranin) is used to stain the decolorized vegetative cells**.



When visualized under microscopy the cells should have three characteristics:

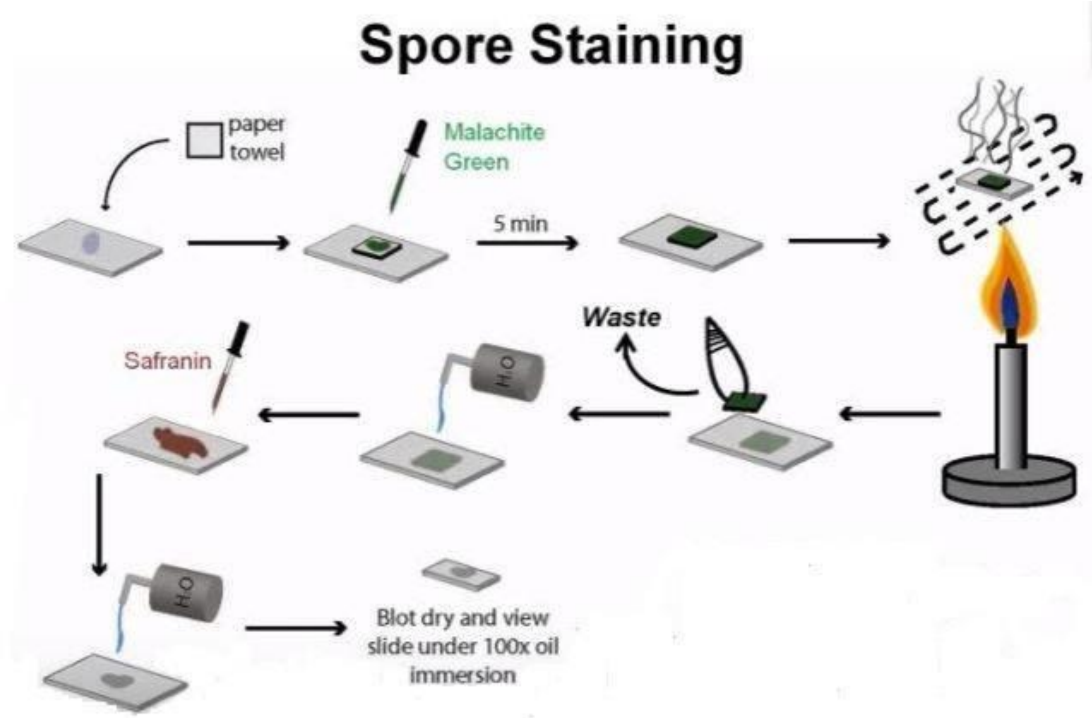
1. The vegetative cells should appear pink/red (i.e. color of counter stain),
2. The vegetative cells that contain endospores should stain pink while the **spores should be seen as green** ellipses within the cells.
3. Mature, free endospores should not be associated with the vegetative bacteria and should be seen as green ellipses.

❖ Procedure of endospore stain:

1. Prepare smears of organisms to be tested for presence of endospores on a clean microscope slide and air dries it.

2. Heat fixes the smear.
3. Place a small piece of blotting paper (absorbent paper) over the smear and place the slide (smear side up) on a wire gauze on a ring stand.

4. Heat the slide gently till it starts to evaporate (either by putting the slide on a staining rack that has been placed over a boiling water bath or via bunsen burner). After 5 minutes carefully remove the slide from the rack using a clothespin Remove the blotting paper and allow the slide to cool to room temperature for 2 minutes.



5. Rinse the slide thoroughly with tap water (to wash the malachite green from both sides of the microscope slide).

6. Stain the smear with **safranin** for 2 minutes.

7. Rinse both side of the slide to remove the secondary stain and blot the slide/ air dry. Observe the bacteria under 1000X (oil immersion) total magnification.

Results: The vegetative cells will appear pink/red and the spores will appear green.

Acid fast stain: It distinguishes acid fast bacteria such as *Mycobacterium spp* from no-acid fast bacteria; which do not stain well by the Gram Staining. It is used to stain Mycobacterium species (*Mycobacterium tuberculosis, M.ulcerans* and *M. leprae*)

3. Flagella stain: Demonstrate presence and arrangement of flagella. Flagellar stains are painstakingly prepared to coat the surface of the flagella with dye or a metal such as silver. The number and arrangements of flagella are critical in identifying species of motile bacteria

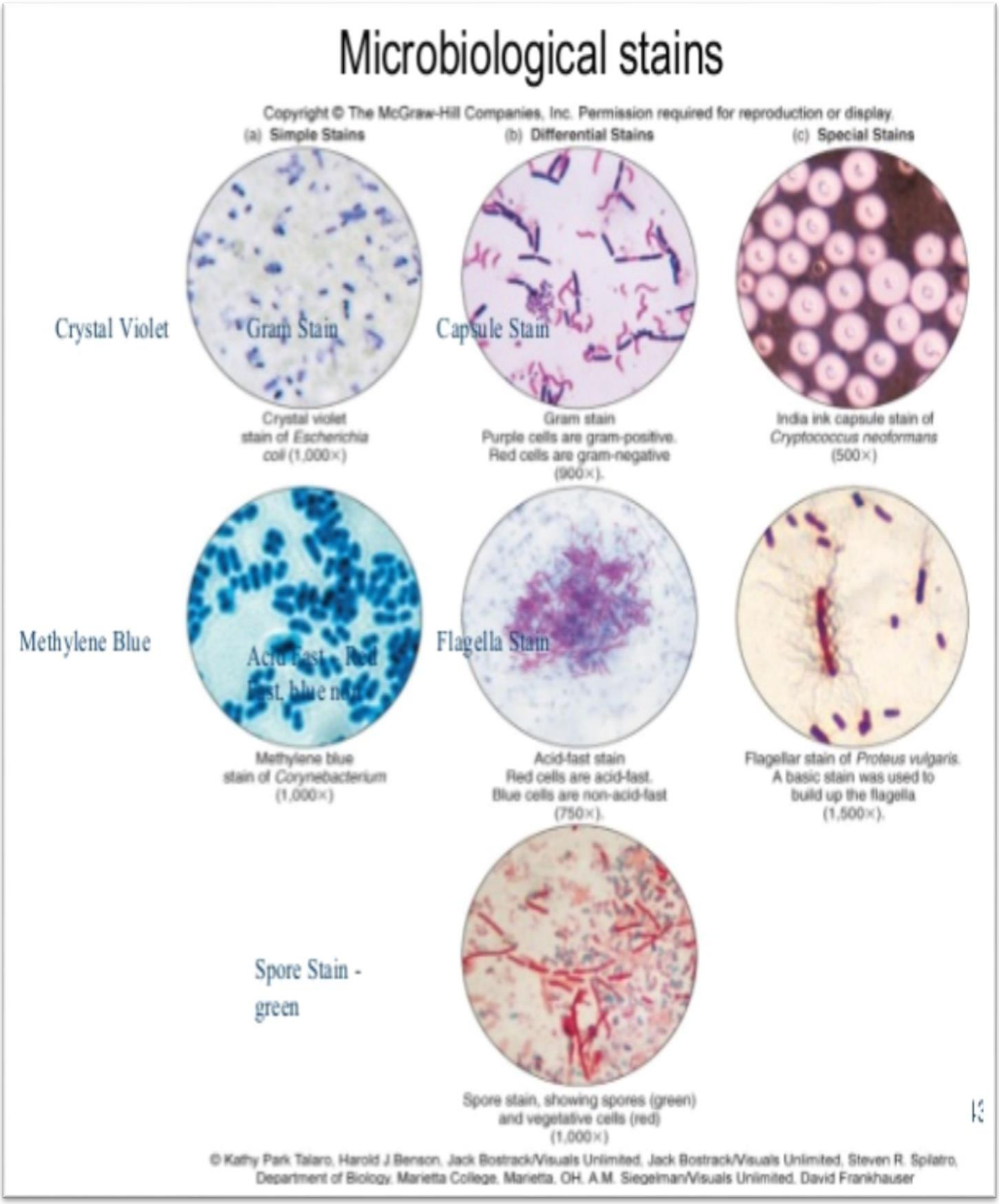
4. Acridine Orange Stain: This staining method is used to confirm the presence of bacteria in blood cultures when Gram stain results are difficult to interpret or when presence of bacteria is highly suspected but none are detected using light microscopy. Acridine orange binds to nucleic acid and stains them. It is also used for the detection of Mycoplasmas (cell wall deficient bacteria)

5. Auramine-Rhodamine technique: This fluorochrome staining method is used to enhance the detection of mycobacteria directly in patient specimens and initial characterization of cells grown in culture.

6. Calcofluor White Staining: It is commonly used to directly detect fungal element and to observe the subtle characteristics of fungi grown in culture. The cell walls of fungi will bind the stain calcofluor white, which greatly enhances visibility of fungal element in tissue or other specimens.

7. Cytoplasmic inclusion stains: Identifies intracellular deposits of starch, glycogen, polyphosphates, hydroxybutyrate, and other substances. E.g. **Albert staining** is used to stain the volutin or metachromatic granules of *C. diphtheria.*

8. **Lacto phenol cotton blue (LPCB) wet mount** is the most widely used method of staining and observing fungi.


Microbiological stains
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(a) Simple Stains
(b) Differential Stains
(c) Special Stains
Crystal Violet
Gram Stain
Capsule Stain
Crystal violet stain of Escherichia coli (1,000×)
Gram stain Purple cells are gram-positive. Red cells are gram-negative (900×).
India ink capsule stain of Cryptococcus neoformans (500×)
Methylene Blue
Acid fast – Red fast, blue non
Flagella Stain
Methylene blue stain of Corynebacterium (1,000×)
Acid-fast stain Red cells are acid-fast. Blue cells are non-acid-fast (750×).
Flagellar stain of Proteus vulgaris. A basic stain was used to build up the flagella (1,500×).
Spore Stain - green
Spore stain, showing spores (green) and vegetative cells (red) (1,000×)
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3. Biochemical characterises

3. What is Biochemical characterises?

1. Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical activities of different bacteria.
2. Bacterial physiology differs from one species to the other.

These differences in carbohydrate metabolism, protein metabolism, fat metabolism, production of certain enzymes, ability to utilize a particular compound etc. help them to be identified by the biochemical tests.

3. The structural differences with respect to shape, size and arrangement of bacteria only help in the process of identification, because there are many species of bacteria having similar shape, size and arrangement.
4. Therefore, ultimately, the identification of bacteria is mostly based on the differences in their biochemical activities. A list of some of the most commonly used biochemical tests performed for the identification of bacteria has been given in Figure 1.

- The bacterial enzymes can be grouped into two categories as follows:

1. Intracellular Enzymes (Endoenzymes):

These enzymes function inside the bacteria cells. They are mainly responsible for the synthesis of new protoplasmic substances and production of cellular energy from simple substances permeating into the cells through the cell membrane. It is necessary for cellular survival and function and is the basis of cellular metabolism.

2. Extracellular Enzymes (Exoenzymes):

These are the enzymes secreted by the bacteria cells to the surrounding to hydrolyse high molecular weight or complex substances, such as proteins, polysaccharides and fats, which cannot permeate the bacterial cell membrane, because of their large size (proteins and polysaccharides) or because of their complex nature (fats).The extracellular enzymes hydrolyse them into their respective simple building blocks. Proteins are hydrolysed to amino acids, polysaccharides like starch to glucose and fats like triglycerides to glycerol and fatty acids.

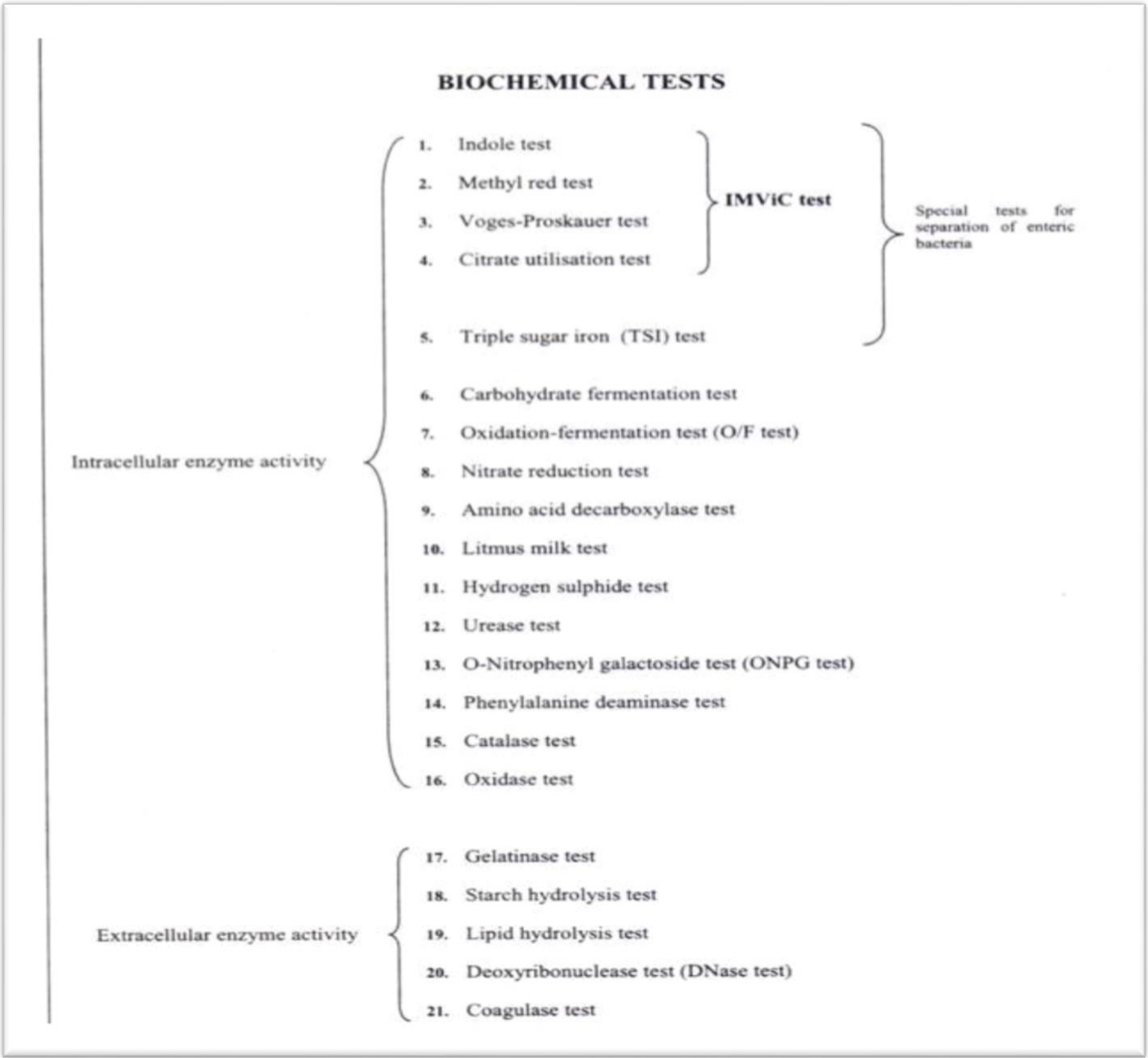


Figure 1: Some common biochemical test performed for identification of bacteria

1. **Intracellular enzymes** that involved many of enzymes that discussed in this tests

IMVC Tests: this test are employed in the identification/differentiation of members of family Entero-bacteriaceae, this name become from the first letters of this microbiologist known tests (tryptone broth (indole test), methyl red, Vogues Proskauer broth (MR-VP broth), citrate test).

❖ General procedure for performing IMVIC Tests

Requirement

Cultures of any members of **Entero-bacteriaceae** have to grow for 24 to 48 hours at 37°C and the respective tests can be performed:

1. Indole test SIM: Indole test, H2S, and motility

Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by **tryptophanans** to produce three possible end products – one of which is indole. Indole production is detected by **Kovac's or Ehrlich's** reagent which contains **4 (p)-dimethyl amino Benz aldehyde**, this reacts with indole to produce a red coloured compound.

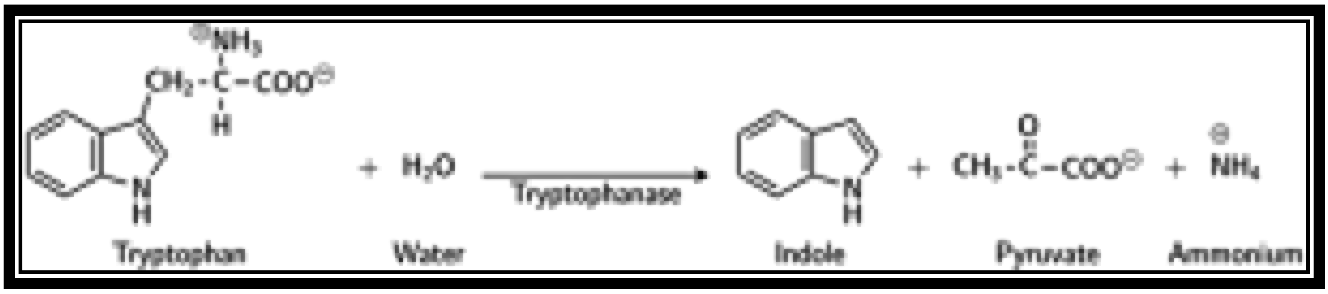


Figure 2: Indole Test Reaction

Two methods are in use: It is performed on **sulphide-indole-motility** (SIM) medium **or** in **tryptophan broth**, **or** in **motility urease indole** (MIU) medium. Result is read

After adding **Kovac's reagent**.

1. a conventional tube method requiring overnight incubation, which identifies weak indole producing organisms and
2. a spot indole test, which detects rapid indole producing organisms

❖ Procedure of Conventional Tube method for Indole Test

a. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.

b. incubates at 37°C for 24-28 hours in ambient air.

c. Add 0.5 ml of Kovac's reagent to the broth culture.

Expected results:

- **Positive**: Pink colour rink after addition of appropriate reagent *E.coli;*
- **Negative**: No color change even after the addition of appropriate reagent. e.g. *Klebsiella pneumoniae*

❖ Spot Indole Test

It is used to determine the presence of the enzyme tryptophanase that breaks down tryptophan to release indole, which when reacts with cinnam aldehyde produces a blue-green compound. The absence of enzyme results in no color production (i.e. indole negative).

❖ Procedure of Spot Indole Test

- Saturate a piece of filter paper with the 1% Para di methyl amino cinnam aldehyde reagent.
- Use a wooden stick or bacteriologic loop to remove a small portion of a bacterial colony from the agar surface and rub the sample on the filter paper.

Note: The bacterial inoculum should not be taken from MacConkey Agar because the color of lactose fermenting colonies on this medium can interfere with test interpretation.

Expected results:

1. **Positive:** Development of a blue color within 30 seconds. Most indole positive organism turn blue within 30 seconds.
2. **Negative:** No color development or slightly pink color.

- **Uses of spot indole test**

1. Spot indole test along with **gram stain** result and colony characteristics can assist in the rapid identification of isolates. For example A flat, dry lactose fermenting (pink) colony on **MacConkey agar** that is also spot indole positive and **oxidase** negative can be reported presumptively as *E.coli.*
2. Organisms that swarm on 5% sheep **blood agar**, exhibit a characteristics odour, and are oxidase negative can be presumptively identified as *Proteus spp*. With further testing by spot indole, the positive isolates may be presumptively reported as *Proteus vulgaris* and the negative ones as *Proteus mirabilis.*

Indole positive organisms: Most strains of *E.coli*, *P. vulgaris*, *M. morganii* and *Providenica* are indole positive

Note: Indole test can also aid in species differentiation.

1. *Klebsiella* species: *Klebsiella oxytoca* is indole positive whereas *Klebsiella pneumoniae* is indole negative.
2. *Citrobacter* species: *Citrobacter Koseri* is indole positive whereas *Citrobacter freundii* is indole negative
3. *Proteus* species: *Proteus Vulgaris* is indole positive whereas *Proteus mirabilis* is indole negative

Note: Indole test can is performed **using multi test agar**. Three most commonly used agar medium are:

1. **Sulphide-Indole-Motility (SIM)** medium: The SIM medium is a multi-test agar used to test for indole production while simultaneously **determining motility** and hydrogen sulphide producing abilities of the isolate.
2. **Motility-Indole-Urease (MIU)** medium: MIU medium is used to test for indole and **urease** producing characteristics of the organism along with test for motility.
3. **Motility-Indole-Ornithine (MIO)** medium: In addition to testing for indole production, MIO agar is used to **test for motility** and **ornithine decarboxylase.**

Expected results:

1. **Positive result** is indicated by the **red layer at the top of the tube** after the addition of Kovács reagent.

2. **Negative result** is indicated by the **lack of color** change at the top of the tube after the addition of Kovács reagent.

2. Methyl red test

Principle to test the ability of the organism to produce **acid end product** from **glucose fermentation**, this is a qualitative test for acid production (mainly lactic and acetic acid). And neutral fermentation product (ethanol), CO_2, H_2 per result from glucose fermentation .These large amounts of acid results significant decrease in the pH of the medium below 4.4. This is visualized by using pH indicator, **methyl red (p-di-**

methyl-amino-aeobenzene-O-carboxylic acid), which is yellow above pH 5.1 and red at pH 4.4.The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, to produce a color change, the test organism must produce large quantities of acid from carbohydrate substrate being used.

Requirement

1. Methyl red indicator

2. Culture of *Escherichia coli*, ***Klebsiella***

❖ Procedure for Methyl Red (MR) Test

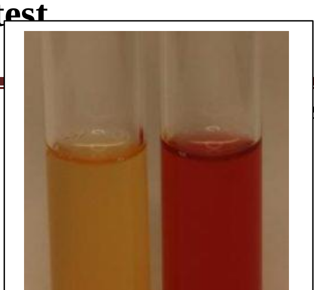
MR-VP broth is used for both MR Test and VP test. Only the addition of reagent differs, and both

Tests are carried out consecutively.

1. Inoculate two tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation.
2. Incubate at 35 °C for up to 4 days.
3. Add about 5 drops of the methyl red indicator solution to the first tube (**for Voges-Proskauer test**, **Barrit's reagent** is added to another tube).
4. A positive reaction is indicated, if the colour of the medium changes to red within a few minutes.

- **MR Positive**: When the culture medium turns red after addition of methyl red, because of a pH at or below 4.4 from the fermentation of glucose,
- **MR Negative**: When the culture medium remains yellow, which occurs when less acid is produced (pH is higher) from the fermentation of glucose.

Quality Control strains used in citrate utilization test



- Positive control: *Escherichia coli*
- Negative control: ***Klebsiella***

Note: Positive and negative controls should be run
After preparation of each lot of medium
And after making each batch of reagent.

3. Vogues-Proskauer test

Vogues-Proskauer is named after two microbiologists working at the beginning of the 20th century. They first observed the red color reaction produced by appropriate culture media after treatment with potassium hydroxide. Pyruvic acid, the pivotal compound in the **fermentative degradation of glucose**, is further metabolized through various metabolic pathways, depending on the enzyme systems possessed by different bacteria. One such pathways result in the **production of acetone** (acetyl methyl carbinol), a neutral-reacting end product. It used to determine the ability of the organisms to produce neutral end product (acetone) from glucose fermentation.

Organisms such as members of the *Klebsiella-Enterobacter-Hafnia-Serratia* group produce acetoin as the chief end product of glucose metabolism and form smaller quantities of mixed acids. In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and alpha-naphthol serves as a catalyst to bring out a red complex.

Requirements from Media and Reagents

1. Media: The medium is MR/VP broth
2. Reagents:
    1. Alpha-naphthol, 5% color intensifier
        a) Alpha Naphthol-5g

b) Absolute ethyl alcohol- 100 mL

2. Potassium Hydrooxide, 40%, oxidizing agent

a) Potassium hydroxide 40g

b) Distilled water to: 100 mL

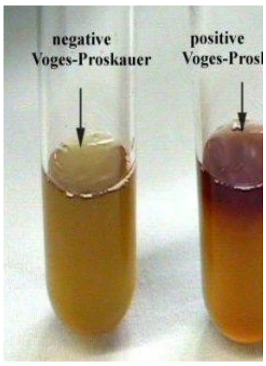
3. Culture of E. coli and *Klebsiella*

❖ Procedure of Vogues Proskauer Test

1. Inoculate a tube of MR/VP broth with a pure culture of the test organism.
2. Incubate for 24 hours at 35ºC
3. At the end of this time, aliquot 1 mL of broth to clean test tube.
4. Add 0.6mL of 5% alpha naphthol, followed by 0.2 mL of 40% KOH. *(Note: It is essential that the reagents be added in this order.)*
5. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.

Results and Interpretation

- A **positive Voges-Proskauer test** is indicated by the development of red-brown color after the addition of Barritt's A and Barritt's B reagents. indicating the presence of diacetyl, the oxidation product of acetoin .
- **Negative test** is indicated by lack of color change after the addition of Barritt's A and Barritt's B reagent



Quality Control strains used in citrate utilization test

- **Positive control**: *Klebsiella*
- **Negative control**: *Escherichia coli*

✓ Note: The test should not be read

After standing for over 1 hour because negative Vogues-Proskauer , Cultures may produce a copper like color, potentially resulting in a false positive interpretation.

4. Citrate utilization test

Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ($NH_4H_2PO_4$) is the sole fixed nitrogen source.

Simmons Citrate agar is a defined medium containing sodium citrate as the sole carbon source. **The pH indicator, bromthymol blue, will turn from green at neutral pH (6.9) to blue when a pH higher than 7.6 is reached (alkaline)**. If the citrate is utilized, the resulting growth will produce alkaline products changing the color of the medium from green to blue. (Blue color= positive reaction eg; *Klebsiella)* (green color=negative reaction eg; *E.coli*), Citrate utilization can be used to distinguish between coliforms such as *Enterobacter aerogenes* (+ve) which occur naturally in the soil and in aquatic environments and faecal coliforms such as *Escherichia coli (-ve)* whose presence would be indicative of faecal contamination.

Further metabolic breakdown is dependent upon the pH of the medium.

A. Under alkaline conditions, pyruvate is metabolized to acetate and formate. Pyruvate = acetate + format

B. At pH 7.0 and below, lactate and acetoin are also produced. The carbon dioxide that is released will subsequently react with water and the sodium ion in the medium to produce **sodium carbonate, an alkaline compound** that will raise the pH. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source.

Growth usually results in the **bromo-thymol blue indicator, turning from green to blue.** The bromo-thymol blue pH indicator is a deep forest green at neutral pH. With an increase in medium pH to above 7.6, bromo-thymol blue changes to blue

❖ Procedure of citrate utilization test:

1. Inoculate **Simmons citrate agar** lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.
2. Incubate at 35^{o}C to 37^{o}C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
3. Observe the development of blue color; denoting alkalinization.

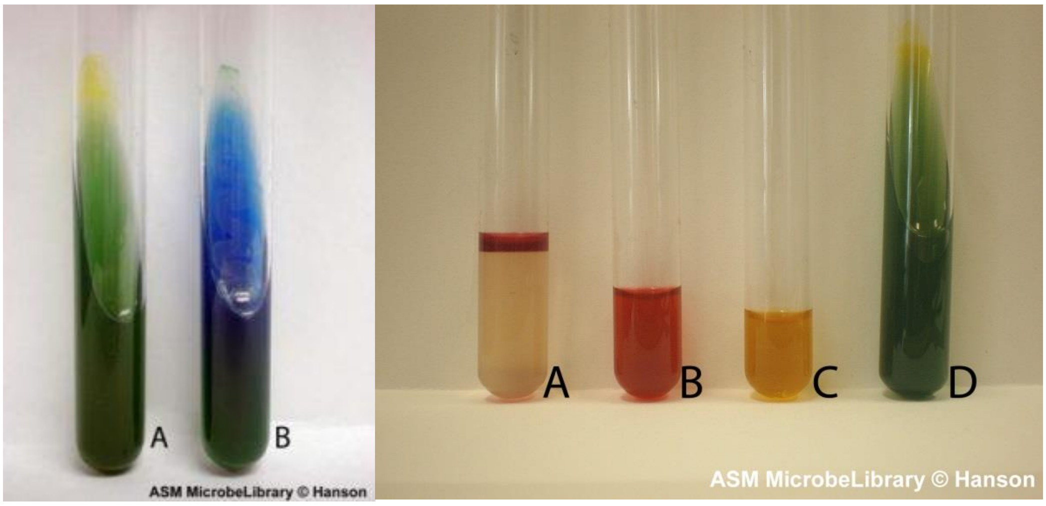
Expected results in citrate utilization test:

Citrate positive: growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromo-thymol blue to change from the original green color to blue.

Citrate negative: trace or no growth will be visible. No color change will occur; the medium will remain the deep forest green color of the inoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an inoculated slant

Quality Control strains used in citrate utilization test

- **Negative**: *Escherichia coli*
- **Positive**: *Klebsiella pneumoniae*



Another type of tests that include

5. Catalase test

Catalase test Enzymes that decompose hydrogen peroxide into water and oxygen.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. If this is allowed to accumulate in the bacterial cells it becomes lethal to the bacteria. This test determines bacterial production of catalase enzymes.

Requirement for Catalase test

1. **Reagent: 3% hydrogen peroxide** stored in dark brown bottle under refrigeration, 18 to 24 hrs.

2. **Culture of the organism** to be tested. ***Staphylococcus* aureus** and ***Streptococcus*** spp.

Procedure of Catalase test

1. Place a drop of hydrogen peroxides 3% h2o2 reagent grade on microscope slides or in concave surface of hanging drop slides.

2. With sterile loop, collect sample of 18-24 h old pure bacterial culture

3. Place the loop in hydrogen peroxide

4. Result of Catalase test.

- **Positive result**: The bubbles of O 2 gas, resulting from production of oxygen gas clearly indicate a catalase positive result.
- **Negative result**: no presence of bubbles of O 2 gas

Quality Control strains used in citrate utilization test

- **Positive result**. The *Staphylococcus* spp.
- **Negative result:** *Streptococcus* and *Enterococcus spp.*

5. Record result

6. Oxidase Test

Determine the presence of bacterial cytochrome enzyme oxidase. Cytochromes in aerobic respiration transfer electrons (H) to oxygen to form water. The reagent used is a dye p-phenylenediamide dihydrochloride acts as an artificial electron accepter substituting the oxygen. In the presence of enzyme cytochrome oxidase dye is oxidized to indophenol blue which is a dark purple collared end products. When the enzyme is not present, the reagent remains reduced and is colourless.

- ✓ **Note: All bacteria that are oxidase positive are aerobic, and can use oxygen as a terminal electron acceptor in respiration. This does NOT mean that they are strict aerobes. Bacteria that are oxidase-negative may be anaerobic, aerobic, or facultative; the oxidase negative result just means that these organisms do not have the cytochrome c oxidase that oxidizes the test reagent. They may respire using other oxidases in electron transport.**

Requirements for Oxidase test:

1. **Reagent:** filter paper with the substrate (1% tetramethyl-p-phenylenediamine dihydrochloride) **(PPDD)**, or commercially prepared paper disk, wooden wire or platinum wire.

2. **Culture of the organism** to be tested It is commonly used to distinguish between

Oxidase negative *Entero-bacteriaceae* and oxidase positive *Pseudomadaceae*.

Procedure of Oxidase test:

1. Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride
2. Moisten the paper with a sterile distilled water
3. Pick the colony to be tested with wooden or platinum loop and smear in the filter paper
4. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

- ## Result of Oxidase test:

- **Positive**: Development of **dark purple color** (indophenols) within 10 second,
- **Negative: Absence of color**.

Quality Control strains used in citrate utilization test

This test it is commonly used to distinguish between Oxidase negative *Entero-bacteriaceae* and oxidase positive *Pseudomadaceae*. In the Picture below the organism on the right (*Pseudomonas aeruginosa*) is oxidase Positive.

5. Record result

✓ **Notes**

- **Do not use Nickel-base alloy wires containing chromium and iron (nichrome) to pick the colony and make smear as this may give false positive results.**
- **Interpret the results within 10 seconds, timing is critical**

7. The carbohydrate fermentation test

This test used for determine whether or not bacteria can ferment a specific carbohydrate. Carbohydrate fermentation patterns are useful in differentiating among bacterial groups or species. Carbohydrate fermentation patterns can be used to differentiate among bacterial groups or species.

1. All members of **Entero-bacteriaceae** family are glucose fermenters (they can metabolize glucose anaerobically).
2. Maltose fermentation differentiates *Proteus vulgaris* (positive) from *Proteus mirabilis* (negative).
3. Both *Neisseria gonorrhoeae* (gonococci) and *Neisseria meningitides* (meningococci) ferments glucose, but only meningococci ferments maltose.
4. Rapid carbohydrate utilization test can be performed to identity *Corynebacterium diphtheriae* and other *Corynebacterium* species.

Principle of Carbohydrate Fermentation Test

It tests for the presence of **acid and/or gas** produced **from carbohydrate fermentation**, Depending up on the organisms involved and the substrate being fermented Basal medium containing a single carbohydrate source such as Glucose, Lactose, Sucrose or any other carbohydrate is used for this purpose. A pH indicator (such as **Andrade's solution**, **Bromcresol purple (BCP), Bromothymol blue (BTB)** or **Phenol red)** is also present in the medium; the end products may varies. Common end-products of bacterial fermentation include lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide and hydrogen. The production of the acid lowers the pH of the test medium, which is detected by the color change of the pH indicator. Color change only occurs when sufficient amount of acid is produced, as bacteria may utilize the peptone producing alkaline by products.

Phenol red is commonly used as a pH indicator in carbohydrate fermentation tests. This will detect the lowering of the pH of the medium due to acid production. Small inverted tubes called Durham tubes are inserted upside down in the test tubes to detect gas production (hydrogen or carbon dioxide). If the test organism produces gas, the gases displace the media present inside the tube and get trapped producing a visible air bubble. Based on the characteristics reactions observed, bacteria can be classified as:

- Fermenter with acid production only
- Fermenter with acid and gas production
- Non-fermenter

Test procedure

Phenol Red Carbohydrate Broth is commonly used in carbohydrate fermentation test. The carbohydrate source can varies based on your test requirements.

Common broth media are:

1. Phenol Red Glucose Broth
2. Phenol Red Lactose Broth
3. Phenol Red Maltose Broth
4. Phenol Red Manitol Broth
5. Phenol Red Sucrose Broth

Preparation and Composition of the media

Get specific Phenol Red Carbohydrate Test media from the commercial suppliers or Phenol Red Broth Base and add specific carbohydrate source based on your test requirements, or you can prepare media mixing the following ingredients.

Composition of Phenol Red Carbohydrate Broth

- Trypticase or protease peptone No. 3: 10 g
- Sodium Chloride (NaCL): 5 g
- Beef extract (optional): 1 g
- Phenol red (7.2 ml of 0.25% phenol red solution): 0.018 g
- Carbohydrate source: 10 g

A. Preparation of the media

- Prepare broth media by mixing all ingredients in 1000 mL of distilled/deionized water and heating gently to dissolve it *(Note: Use single carbohydrate source based on your requirements).*
- Fill 13 x 100 mm test tubes with 4-5 ml of phenol red carbohydrate broth.
- Insert a Durham tube to detect gas production.

- Autoclave the prepared test media (at 121°C for 15 minutes) to sterilize. The sterilization process will also drive the broth into the inverted Durham tube. (Note: When using arabinose, lactose, maltose, sucrose, trehalose, or xyloses, autoclave at 121°C for only 3 minutes as these carbohydrates are subject to breakdown by autoclaving). The prepared broth media will be a light red color and the final pH should be 7.4 ± 0.2.

- Alternatively, prepare Phenol Red Broth Base, heat sterilize and cool to 45°C.
- Prepare specific carbohydrate solution separately, filter the solution using membrane filter (pore size: 0.45 µm). Add carbohydrate solution to the broth base and mix it. **The preferred carbohydrate concentration is 1%.**

Inoculation and Incubation

- Aseptically inoculate each test tube with the test microorganism using an inoculating needle or loop. Alternatively, inoculate each test tube with 1-2 drops of an 18- to 24-hour brain-heart infusion broth culture of the desired organism.
- Incubate tubes at 35-37°C for 18-24 hours. Longer incubation periods may be required to confirm a negative result

C. Interpretation of the results

1. **Acid production:**
   - **Positive:** After incubation the liquid in the tube turns yellow (indicated by the change in the color of the phenol red indicator). It indicates that there is drop in the pH because of the production of the acid by the fermentation of the carbohydrate (sugar) present in the media. **NOTE**:*If you are using other pH indicators please refer for their corresponding colors in particular pH.

- **Negative:** The tube containing medium will remain red, indicating the bacteria cannot ferment that particular carbohydrate source present in the media.

2. **Gas Production**
   - **Positive:** A bubble (small or big depending up the amount of gas produced) will be seen in the inverted Durham tube.
   - **Negative:** There won't be any bubble in the inverted Durham tube i.e. bacteria does not produce gas from the fermentation of that particular carbohydrate present in the media i.e. anaerogenic organism.

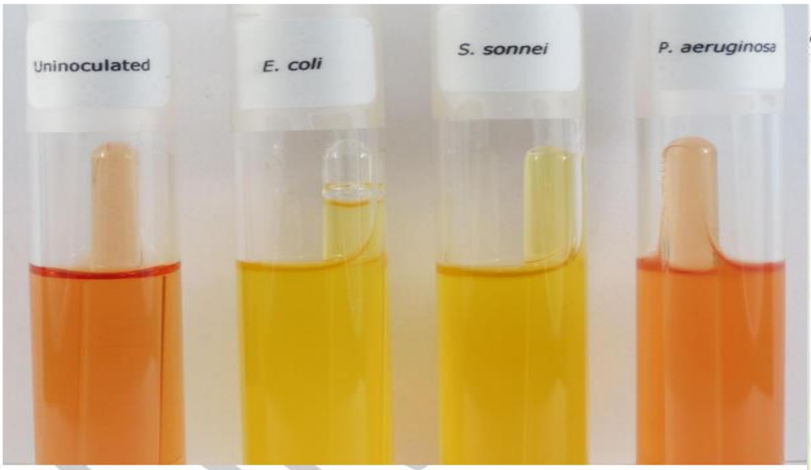
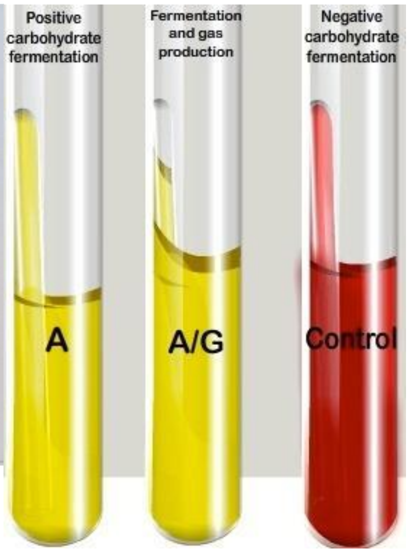


Figure 1. Peptone media with phenol red indicator. From left to right: Uninoculated tube; glucose fermenter with gas production (visible air bubble in the inverted Durham tube); glucose fermenter without gas production (no visible air bubble in the inverted Durham tube); non-fermenter.



8. Urease test:

Urea is a di-amide of carbonic acid. It is hydrolysed with the release of ammonia and carbon dioxide. Many organisms especially those that infect the urinary tract, have an urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright

$$\underset{\text{Urea}}{(NH_2)_2CO} + 2\,H_2O \xrightarrow{\text{Urease}} \underset{\text{Carbon dioxide}}{CO_2} + \underset{\text{Water}}{H_2O} + \underset{\text{Ammonia}}{2\,NH_3}$$

Medium used for urease test: Any urea medium, agar or broth (It can be a sole medium or part of panel like motility indole urease (MIU) test **Indicator used in urease test: Phenol red**

Color change:

- Original: orange yellow color
- Final color (in positive test): Bright pink

Use of Urease test

1. Urease test is used for the presumptive evidence of the presence of ***Helicobacter pylori*** in tissue biopsy material. This is done by placing a portion of crushed tissue biopsy material directly into urease broth. A positive urease test is considered presence of *Helicobacter pylori*. Commercially available urease agar kits are also available.
2. **Rapid Urease test** is can be used to differentiate between the yeasts, ***Candida albicans*** and ***Cryptococcus neoformans***. **A presumptive identification of *C. neoformans*** may be based on rapid urease production, whereas *Candida albicans* do not.
3. **Urea breath test:** A common noninvasive test to detect *Helicobacter pylori*also based on urease activity. This is highly sensitive and specific test.

Procedure for urease test

1. The broth medium is inoculated with a loopful of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism.

2. Leave the cap on loosely and incubate the test tube at 35 °C in ambient air for 18 to 24 hours; unless specified for longer incubation.

Result and Interpretation

Organisms that hydrolyse urea rapidly (e.g. *Proteus* spp) may produce positive reactions within 1 or 2 hours; less active species (e.g. *Klebsiella* spp) may require 3 or more days. In routine diagnostic laboratories the urease test result is read within 24 hours.

- **Positive result:** magenta color
- **Negative result**: light orange

9. Triple Sugar Iron Agar (TSI):

Whenever you see the name of this test i.e. **Triple Sugar Iron Agar** ,you have to remember that it's a test which has **three sugar (Lactose, Sucrose, and Glucose)** and also iron; and it contains Agar Agar as solidifying agent (TSI is a semi solid media having slant and butt).

Composition of Triple Sugar Iron Agar (TSI)

Lactose, Sucrose and Glucose in the concentration of 10:10:1 (i.e. 10 part Lactose (1%), 10 part Sucrose (1%) and 1 part Glucose (0.1%). TSI is similar to **Kligler's iron agar (KIA),** except that Kligler's iron agar contains only two carbohydrates: glucose (0.1%) and lactose (1%).

- **0.1% Glucose**: If only glucose is fermented, only enough acid is produced to turn the butt yellow. The slant will remain red

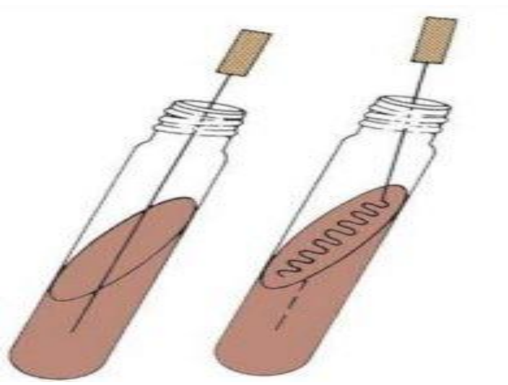
- **1.0 % lactose/1.0% sucrose:** a large amount of acid turns both butt and slant yellow, thus indicating the ability of the culture to ferment either lactose or sucrose.
- **Iron:** Ferrous sulfate: Indicator of H2S formation
- **Phenol red:** Indicator of acidification (It is **yellow in acidic condition** and red under alkaline conditions).
- It also contains **Peptone** which acts as source of nitrogen. (Remember that whenever peptone is utilized under aerobic condition ammonia is produced)

Why Sucrose is added in TSI?

Addition of sucrose in TSI Agar permits earlier detection of coliform bacteria that ferment sucrose more rapidly than lactose. Adding sucrose also aids the identification of certain gram-negative bacteria that could ferment sucrose but not lactose. Other basic understanding is TSI Tube contains **butt** (poorly oxygenated area on the bottom) **slant** (angled well oxygenated area on the top).

Procedure

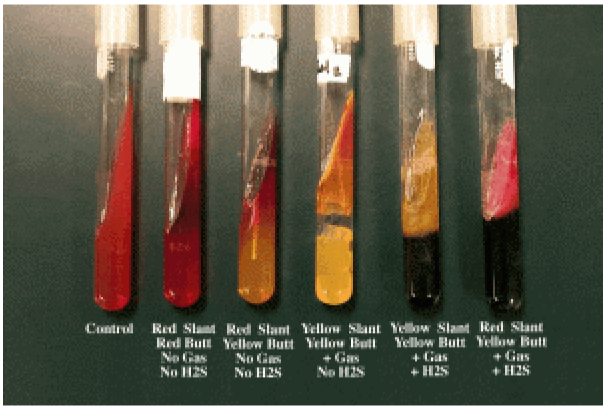
1. With a sterilized straight inoculation needle touch the top of a well-isolated colony
2. Inoculate TSI Agar by **first stabbing** through the center of the medium to the bottom of the tube and then **streaking on the surface** of the agar
3. slant
4. Leave the cap on loosely and incubate the tube at 35°C in ambient air for 18 to 24 hours.



Interpretation of Triple Sugar Iron Agar Test

1. If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator **yellow both in butt and in the slant.** Some organisms generate **gases**, which produces **bubbles/cracks** on the medium.
2. If **lactose is not fermented** but the small amount of glucose is, the oxygen deficient **butt will be yellow** (remember that butt comparatively have more glucose compared to slant i.e. more media more glucose), but on the slant the acid (less acid as media in slant is very less) will be oxidized to carbon dioxide and water by the organism and the **slant will be red** (alkaline or neutral pH).
3. If **neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red**. The slant can become a deeper red-purple (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids (remember peptone is a major constituents of TSI Agar).
4. If H2S is produced, the black color of ferrous sulphide is seen.

So the expected results of TSI Agar test are:



Triple Sugar Iron Agar Test Results

Alkaline slant/no change in butt (K/NC) i.e Red/Red = glucose, lactose and sucrose non-fermenter

1. Alkaline slant/Alkaline butt (K/K) i.e Red/Red = glucose, lactose and sucrose non-fermenter

2. Alkaline slant/acidic butt (K/A); Red/Yellow = glucose fermentation only, gas (+ or -), H2s (+ or -)
3. Acidic slant/acidic butt (A/A); Yellow/Yellow = glucose, lactose and/or sucrose fermenter gas (+ or -), H2s (+ or -).

❖ Some example of Triple Sugar Iron (TSI) Agar Reactions:

Name of organisms	**Slant**	**Butt**	**Gas**	**H2S**
Escherichia, Klebsiella, Enterobacter	Acid (A)	Acid (A)	Pos (+)	Neg (-)
Shigella, Serratia	Alkaline (K)	Acid (A)	Neg (-)	Neg (-)
Salmonella, Proteus	Alkaline (K)	Acid (A)	Pos (+)	Pos (+)
Pseudomonas	Alkaline (K)	Alkaline (K)	Neg (-)	Neg (-)

10. Nitratase test

This is a differential medium. It is used to determine if an organism is capable of reducing nitrate (NO_3^-) to nitrite (NO_2^-) or other nitrogenous compounds via the action of the enzyme nitratase (also called nitrate reductase). This test is important in the identification of both Gram-positive and Gram-negative species. After incubation, these tubes are first inspected for the presence of gas in the Durham tube. In the case of non-fermenters, this is indicative of reduction of nitrate to nitrogen gas. However, in many cases gas is produced by fermentation and further testing is necessary to determine if reduction of nitrate has occurred. This further testing includes the addition of **sulfanilic acid** (often called **nitrate I**) and d**imethyl-alpha-napthalamine** (**nitrate II**)

❖ Interpretation of Nitratase test

- ❖ If the nitrate broth **turns light red (no color change)** after nitrate I and nitrate II are added upon addition of zinc then this means that the NO_3^- was converted to NO_2^- and then was converted to some other undetectable form of nitrogen), this color indicates a **positive result** (tubes pictured in the centre).
- ❖ If instead, **the tube turns red after the addition of Zn (Zinc** will convert any remaining NO_3^- to NO_2^- thus allowing nitrate I and nitrate II to react with the NO_2^- and form the red pigment**)**, this indicates a **negative result**. (Tube pictured on the left).
- ❖ If there is **no color change** in the tube after the addition of nitrate I and nitrate II, the result is uncertain.

11. MacConkey agar fermentation test

This medium is both selective and differential. The selective ingredients are the bile salts and the dye, crystal violet which inhibit the growth of Gram-positive bacteria. The differential ingredient is lactose. Fermentation of this sugar results in an acidic pH and causes the pH indicator, neutral red, to turn a bright pinky-red color. Thus organisms capable of lactose fermentation such as *Escherichia coli*, form bright pinky-red colonies (plate pictured on the left here). MacConkey agar is commonly used to differentiate between the *Entero-bacteriaceae*.

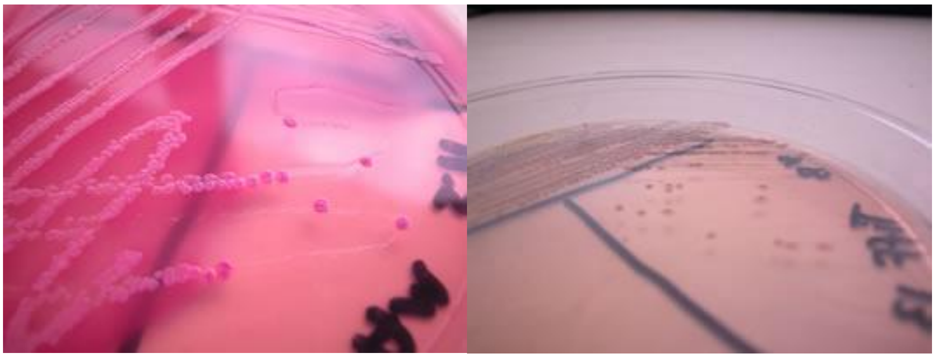


Figure: Organism on left is positive for lactose fermentation and that on the right is negative.

12. Motility agar test

This test used to determine whether an organism is equipped with flagella and thus capable of swimming away from a stab mark. The results of motility agar are often difficult to interpret. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (are motile).

The organisms in the two tubes pictured on the right are motile.

If, however, the stab mark is clearly visible and the rest of the tube is not turbid, the organism is likely non motile (tube pictured on the left).

2. Extracellular enzymes

1. Gelatinase test Gelatine hydrolysis test is used to detect the ability of an organism to produce gelatinase (photolytic enzyme) that liquefy gelatin. Hydrolysis of gelatin indicates the presence of gelatinase.

This process takes place in two sequential reactions. In the first reaction, gelatinases degrade gelatine to polypeptides. Then, the polypeptides are further converted into amino acids. The bacterial cells can then take up these amino acids and use them in their metabolic processes.

Uses of Gelatine Hydrolysis test

Gelatine hydrolysis test is helpful in identifying and differentiating species of *Bacillus, Clostridium, Proteus, Pseudomonas,* and *Serratia*. It distinguishes the gelatinase-positive, pathogenic *Staphylococcus aureus* from the gelatinase-negative, non-pathogenic *S. Epidermidis*. Gram-positive, spore-forming, rod shaped, aerobic or anaerobic bacteria such as *Bacillus Anthracis, Bacillus cereus, Bacillus subtilis, Clostridium Perfringens and Clostridium Tetani,* are also positive for gelatine hydrolysis. The test can also be used to differentiate genera of gelatinase-producing bacteria such *Serratia* and *Proteus* from other members of the family Entero-bacteriaceae.

Procedure of Gelatinase hydrolysis test

There are several methods for determining gelatinase production, all of which make use of gelatine as the substrate. The standard and most commonly employed method is the nutrient gelatine stab method.

1. Inoculate a heavy inoculum of test bacteria (18- to 24-hour-old) by stabbing 4-5 times (half inch) on the tube containing nutrient gelatine medium.
2. Incubate the inoculated tube along with an uninoculated medium at 35°C, or at the test bacterium's optimal growth temperature, for up to 2 weeks.
3. Remove the tubes daily from the incubator and place in ice bath or refrigerator (4°C) for 15-30 minutes (until control is gelled) every day to check for gelatin liquefaction.(Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath or kept in refrigerator at 4°C).
4. Tilt the tubes to observe if gelatine has been hydrolysed.

Result of Gelatine hydrolysis test

Positive: Partial or total liquefaction of the inoculated tube (uninoculated control

Medium must be completely solidified) even after exposure to cold temperature of ice bath or refrigerator (4°C)

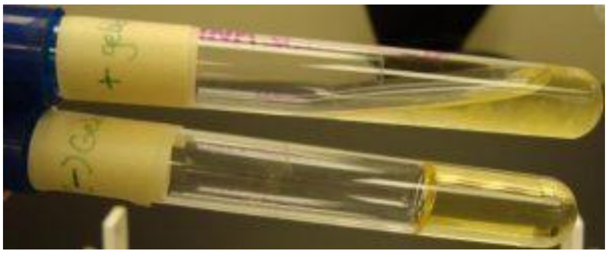


Figure Gelatine Hydrolysis Test: Above tube: Positive

Below Tube: Negative

Negative: Complete solidification of the inoculated tube even after exposure to cold

Temperature of ice bath or refrigerator (4°C), Common bacteria and their reactions to the gelatine hydrolysis test performed on nutrient gelatine.

Control organisms

- **Positive** Control: *Proteus vulgaris*
- **Negative** Control: *Enterobacter aerogenes*

2. Starch hydrolysis test

This test is used to identify bacteria that can hydrolyse starch (amylose and amylopectin) using the enzymes **amylase and oligo-1, 6-glucosidase**. Often used to differentiate species from the genera *Clostridium* and *Bacillus*. Because of the large size of amylose and amylopectin molecules, these organisms cannot pass through the bacterial cell wall. In order to use these starches as a carbon source, bacteria must secrete amylase and oligo-1, 6-glucosidase into the extracellular space.

These enzymes break the starch molecules into smaller glucose subunits which can then enter directly into the glycolytic pathway. In order to interpret the results of the starch hydrolysis test, iodine must be added to the agar. The iodine reacts with the starch to form a dark brown color. Thus, hydrolysis of the starch will create a clear zone around the bacterial growth. *Bacillus subtilis* is positive for starch hydrolysis (pictured below on the left). The organism shown on the right is negative for starch hydrolysis.

3. Lipase test

This test is used to identify organisms that are capable of producing the enzyme lipase. This enzyme is secreted and hydrolyses triglycerides to glycerol and three long chain fatty acids. These compounds are small enough to pass through the bacterial cell wall. Glycerol can be converted into a glycolysis intermediate. The fatty acids can be catabolized and their fragments can eventually enter the Kreb's cycle. The Gram-positive rod, *Bacillus subtilis* is lipase positive (pictured on the right) The plate pictured on the left is lipase negative.

Equipment

- One Tributyrin Agar plate
- Fresh cultures of: Escherichia coli Proteus ~al:filis

Procedure of lipase test

1. using a marking pen, divide the plate into three equal sectors. Be sure to mark on the bottom of the plate. Note: make sure the medium is opaque prior to inoculation. If it is not, obtain a plate that is.

2. Label the plate with the organisms' names, your name, and the date.

3. Spot inoculate two sectors with the test organisms leaving the

Third sector uninoculated as a control.

4. Invert the plate and incubate it aerobically at 35°C for 24 hours.

In Lab Two, Examine the plates for clearing around the bacterial

Growth and record your results in the table provided

4. Coagulase test

Coagulase is an enzyme that clots blood plasma. This test is performed on Gram-positive, catalase positive species to identify the coagulase positive *Staphylococcus aureus*. Coagulase is a virulence factor of *S. aureus*. The formation of clot around an infection caused by this bacterium likely protects it from phagocytosis. This test differentiates *Staphylococcus aureus* from other coagulase negative *Staphylococcus* species.