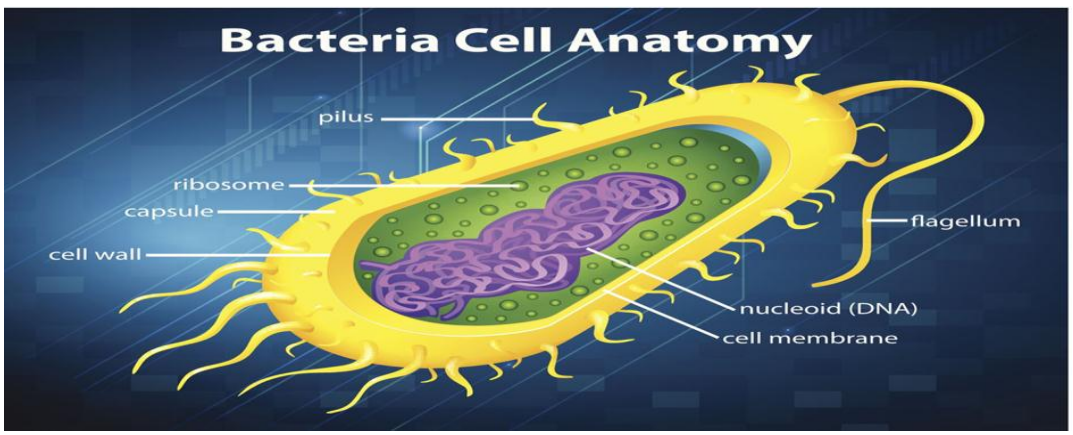
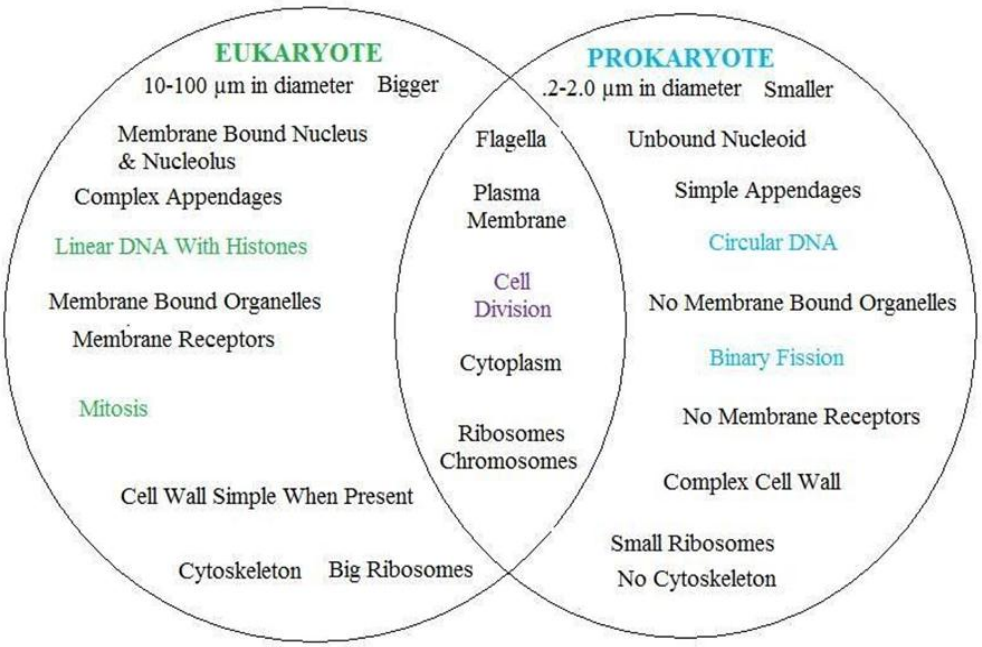
Practicle part of Bacteriology

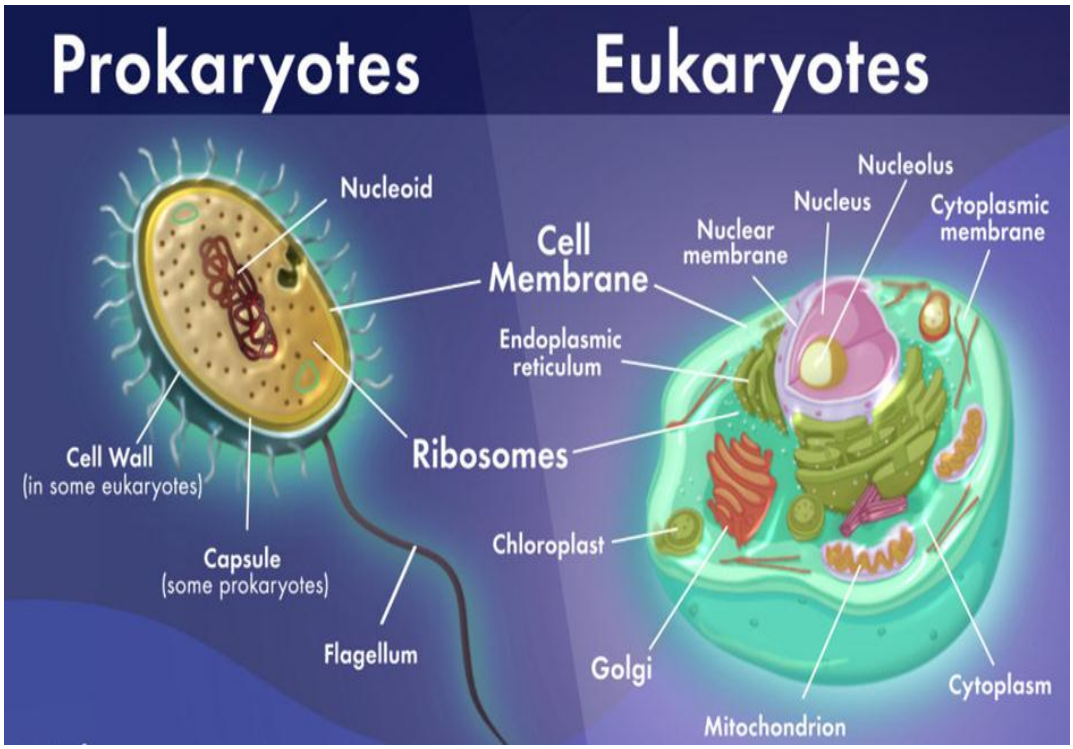
- Bacteriology is the branch and specialty of biology that studies the morphology, ecology, genetics and biochemistry of bacteria as well as many other aspects related to them. This subdivision of microbiology involves the identification, classification, and characterization of bacterial species.

Bacteria, singular bacterium, any of a group of microscopic single-celled organisms that live in enormous numbers in almost every environment on Earth, from deep-sea vents to deep below Earth's surface to the digestive tracts of humans.

- Some bacteria can cause diseases in humans, animals, or plants, but most are harmless and are beneficial ecological agents. Other bacteria carry out important functions for the host, such as nitrogen fixation and cellulose degradation.
- Bacteria lack a membrane-bound nucleus and other internal structures and are therefore ranked among the unicellular life-forms called prokaryotes.




EUKARYOTE
10-100 µm in diameter Bigger
Membrane Bound Nucleus & Nucleolus
Complex Appendages
Linear DNA With Histones
Membrane Bound Organelles
Membrane Receptors
Mitosis
Cell Wall Simple When Present
Cytoskeleton
Big Ribosomes
Flagella
Plasma Membrane
Cell Division
Cytoplasm
Ribosomes
Chromosomes
PROKARYOTE
.2-2.0 µm in diameter Smaller
Unbound Nucleoid
Simple Appendages
Circular DNA
No Membrane Bound Organelles
Binary Fission
No Membrane Receptors
Complex Cell Wall
Small Ribosomes
No Cytoskeleton


Prokaryotes
Eukaryotes
Nucleoid
Cell Membrane
Cell Wall
(in some eukaryotes)
Capsule
(some prokaryotes)
Flagellum
Ribosomes
Nucleolus
Nucleus
Nuclear membrane
Cytoplasmic membrane
Endoplasmic reticulum
Chloroplast
Golgi
Mitochondrion
Cytoplasm

- **The six main physical factors affecting the growth of microorganisms. The factors are:**
    1. Water acidity
    2. Temperature
    3. pH
    4. Oxygen requirements
    5. Pressure
    6. Radiation.

- **Control of microorganisms is essential in order to:**
    1. Prevent the transmission of diseases and infection.
    2. Stop decomposition and spoilage and prevent unwanted microbial contamination.
- **Microorganisms are controlled by means of physical agents and chemical agents.**

1. Control by physical agents include:
    - high or low temperature.
    - Desiccation.
    - Osmotic pressure.
    - Radiation.
    - Filtration.
2. Control by chemical agents refers to the use of:
    - Disinfectants.
    - Antiseptics.
    - Antibiotics.
    - Chemotherapeutic antimicrobial chemicals.

- **Basic terms used in discussing the control of microorganisms include:**

1. Sterilization:

Sterilization is the process of destroying all living organisms and viruses. A sterile object is one free of all life forms, including bacterial endospores, as well as viruses.

2. Disinfection:

Disinfection is the elimination of microorganisms, but not necessarily endospores, from inanimate objects or surfaces.

3. **Decontamination:**

Decontamination is the treatment of an object or inanimate surface to make it safe to handle.

Disinfectant:

A disinfectant is an agent used to disinfect inanimate objects but generally too toxic to use on human tissues.

Antiseptic:

An antiseptic is an agent that kills or inhibits growth of microbes but is safe to use on human tissue.

6. **Sanitizer:**

A sanitizer is an agent that reduces microbial numbers to a safe level.

7. **Antibiotic:**

An antibiotic is a metabolic product produced by one microorganism that inhibits or kills other microorganisms.

8. **Chemotherapeutic synthetic drugs:**

Synthetic chemicals that can be used therapeutically.

9**. Cidal:**

An agent that is cidal in action will kill microorganisms and viruses.

10. **Static:**

An agent that is static in action will inhibit the growth of microorganisms.

- **Sterilization processes:**

1. **Heat:**

A. **Dry heat:**

- **(hot air oven):**

Basically, the cooking oven. but dry heat is not as effective as moist heat (i.e., higher temperatures are needed for longer periods of time). For example, 160 °C /2hours or 170 °C /1hour is necessary for sterilization. The dry heat oven is used for glassware, metal, and objects that will not melt.

- **Incineration:**

Burns organisms and physically destroys them. Used for needles, inoculating wires, glassware, etc. and objects not destroyed in the incineration process.

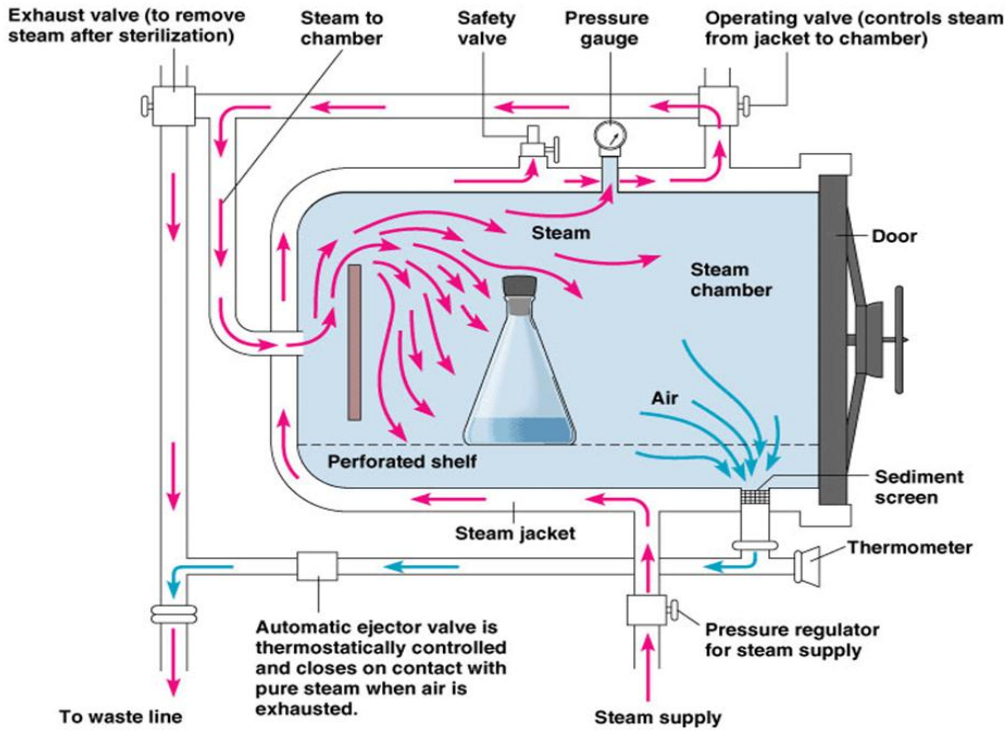
B. Wet heat:

- **Boiling:**

  100°C for 30 minutes. Kills everything except some endospores. To kill endospores, and therefore sterilize a solution, very long (>6 hours) boiling, or intermittent boiling is required.

- **Autoclaving (steam under pressure or pressure cooker):**

  Autoclaving is the most effective and most efficient means of sterilization. The usual standard temperature/pressure employed is 121°C /15 psi for 15 minutes. Autoclaving is ideal for sterilizing biohazardous waste, surgical dressings, glassware, many types of microbiologic media, liquids, and many other things.



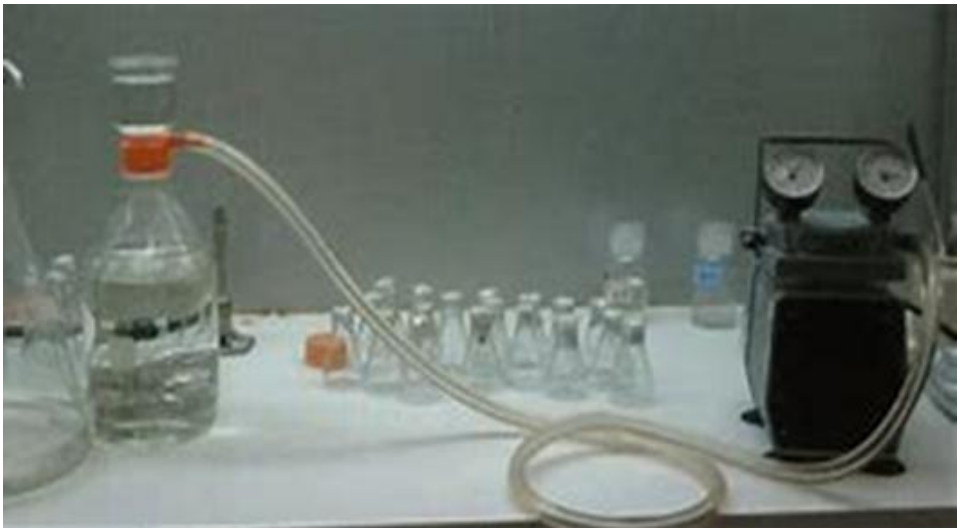
Ionizing radiation:

A: Gamma radiation:

Are forms of ionizing radiation used primarily in the health care industry, are similar in many ways to microwaves and x-rays. Gamma rays are highly effective in killing microorganisms and do not leave residues.

B: Electron beam radiation:

E-beam irradiation is like gamma radiation in that it alters various chemical and molecular bonds on contact.

- **3. Filtration:**
- Involves the physical removal (exclusion) of all cells in a liquid or gas. It is especially important for sterilization of solutions which would be denatured by heat (e.g. antibiotics, injectable drugs, amino acids, vitamins, etc.).
- Essentially, solutions or gases are passed through a filter of sufficient pore diameter (generally 0.22 micron) to remove the smallest known bacterial cells.
- 

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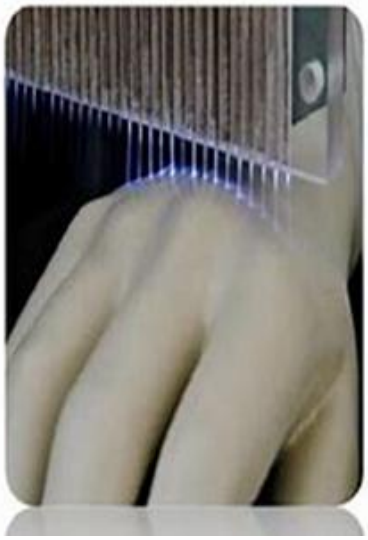
4. Chemical and gas sterilization:

A. Low temperature gas plasma (LTGP):

Gas plasmas are generated in an enclosed chamber under deep vacuum using RF or microwave energy to excite the gas molecules and produce charged particles, many of which are in the form of free radicals. Plasma treatment has been used to alter the surface properties of polymers without affecting their bulk properties.

What is Gas Plasma

- Plasma is a *fourth state of matter* which is distinguishable from liquid, solid, or gas. In nature, plasma is widespread in outer space.
- Gas plasma generated in an enclosed chamber under deep vacuum using Radio frequency or Microwave emery to excite gas molecules are produced charged particles
- **Can be used for hand sterilization**



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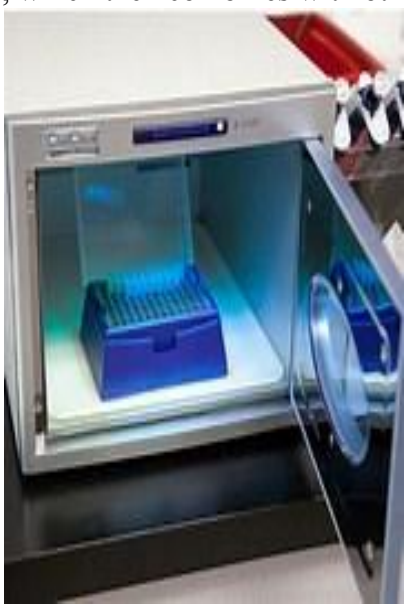
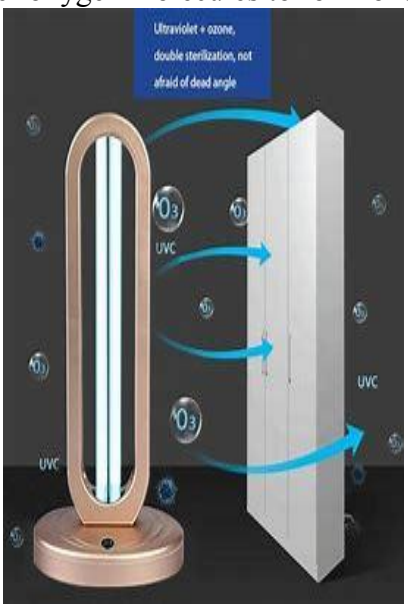
B: Ethylene oxide (ETO):

Is the most used form of chemical sterilization. Due to its low boiling point of 10.4◦C at atmospheric pressure, EtO behaves as a gas at room temperature. EtO chemically reacts with amino acids, proteins, and DNA to prevent microbial reproduction.

C: UV ozone sterilization:

Has been recently approved for use in the U.S. It uses oxygen that is subjected to an intense electrical field that separates oxygen molecules into atomic oxygen, which then combines with other oxygen molecules to form ozone.





D. common atiseptics and disinfectants

Chemical	Uses
Ethanol (50–70%)	Antiseptic used on skin
Isopropanol (50–70%)	Antiseptic used on skin
Formaldehyde (8%)	Disinfectant, kills endospores
Tincture of Iodine (2% I2 in 70% alcohol)	Antiseptic used on skin Disinfection of drinking water
Chlorine (Cl_2) gas	Disinfect drinking water; general disinfectant
Silver nitrate ($AgNO_3$)	General antiseptic and used in the eyes of newborns
Mercuric chloride	Disinfectant, although occasionally used as an antiseptic on skin
Detergents (e.g. quaternary ammonium compounds)	Skin antiseptics and disinfectants
Phenolic compounds (e.g. carbolic acid, lysol, hexylresorcinol, hexachlorophene)	Antiseptics at low concentrations; disinfectants at high concentrations

Culture media

contains nutrients and physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium.

1 Classification of bacterial culture media on the basis of consistency:

1.0.1 Solid medium

1.0.2 Semisolid medium

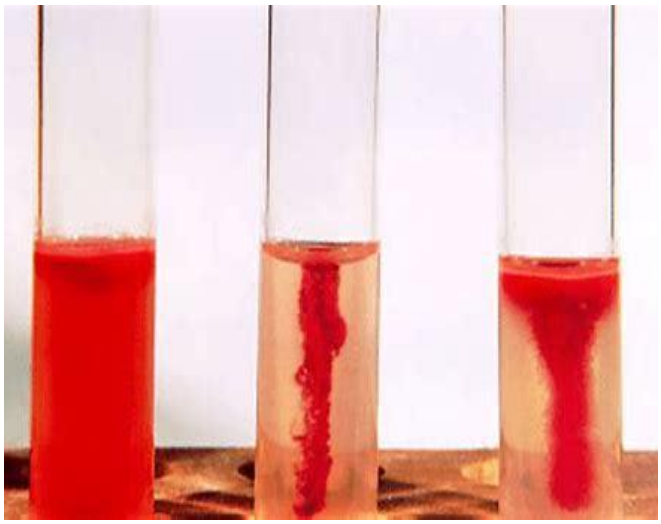
1.0.3 Liquid (Broth) medium

1.0.1 Solid medium:

Solid medium contains agar at a concentration of 1.5-2.0%. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful for isolating bacteria or for determining the colony characteristics of the isolate.

1.0.2 Semisolid medium

Semisolid medium is prepared with agar at concentrations of 0.5% or less. Semisolid medium has a soft custard-like consistency and is useful for the cultivation of microaerophilic bacteria or for the determination of bacterial motility.



1.0.3 Liquid (Broth) medium

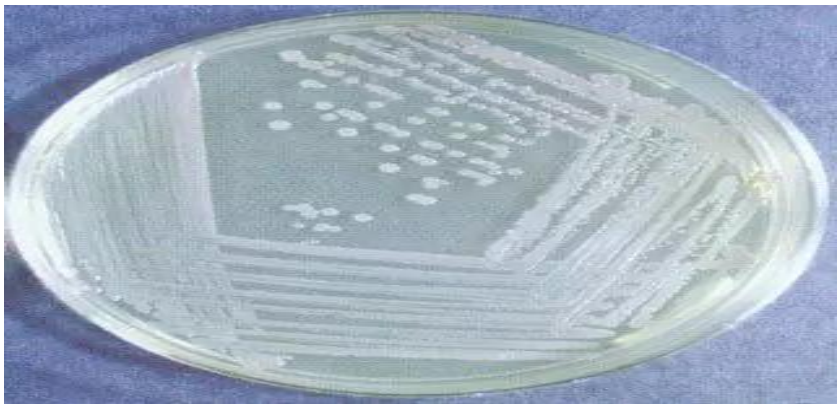
These media contain specific amounts of nutrients but don't have a trace of gelling agents such as gelatine or agar. Broth medium serves various purposes such as propagation of a large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, MR-VR broth.

2 Classification of Bacterial Culture media on the basis of purpose/ functional use/ application:

2.1 General-purpose media/ Basic media
2.2 Enriched medium (Added growth factors)
2.3 Selective and enrichment media
    2.3.1 Selective medium
    2.3.2 Enrichment culture medium
2.4 Differential/ indicator medium: differential appearance
2.5 Transport media
2.6 Anaerobic media
2.7 Assay media

2.1 General-purpose media/ Basic media:

Peptone-water, nutrient broth, and nutrient agar (NA) are considered as basal medium. These media are generally used for the primary isolation of microorganisms.



2.2 Enriched medium (Added growth factors):

Addition of extra nutrients in the form of blood, serum, egg yolk, etc, to basal medium makes enriched media.

2.3 Selective and enrichment media:

These media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogens from a mixture of bacteria.

2.3.1 Selective medium:

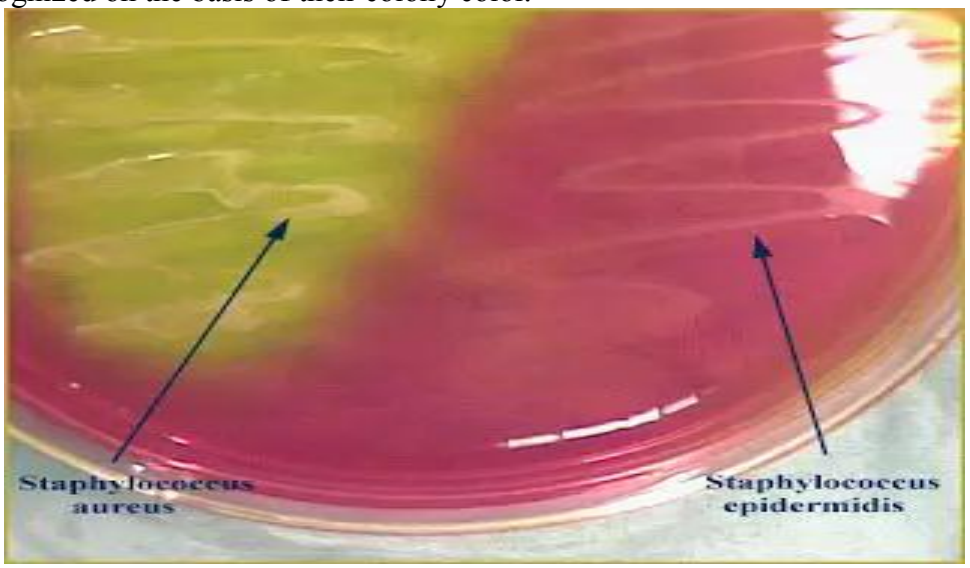
Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others. Selective medium is agar-based (solid) medium so that individual colonies may be isolated.

2.3.2 Enrichment culture

Enrichment medium is used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. Unlike selective media, enrichment culture is typically used as a broth medium.

2.4 Differential/ indicator medium: differential appearance:

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony color.



2.5 Transport media:

Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or

commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of a specimen, maintain the pathogen to commensal ratio, and inhibit the overgrowth of unwanted bacteria

2.6 Anaerobic media:

Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation-reduction potential and extra nutrients.

2.7 Assay media:

These media are used for the assay of vitamins, amino acids, and antibiotics. E.g. antibiotic assay media are used for determining antibiotic potency by the microbiological assay technique.

Bacterial staining

- **Staining is a biochemical technique of coloring specimens.**
- **Dyes are the chemical substances which commonly used to stain specimen.**

Why we stain Bacteria?

Bacteria are stained for better visual observation, to highlight differences, to enhance cell components, to help identify the bacterium, etc.

- **There are three types of staining protocol or procedures:**

1) Simple staining.
2) Differential staining.
3) Specialised staining

- **Stains are of 2 types:**
    - Acidic stains e.g., picric acid.
    - Basic stains e.g., methylene blue.
- **Why are basic stains attracted to the bacteria itself?**

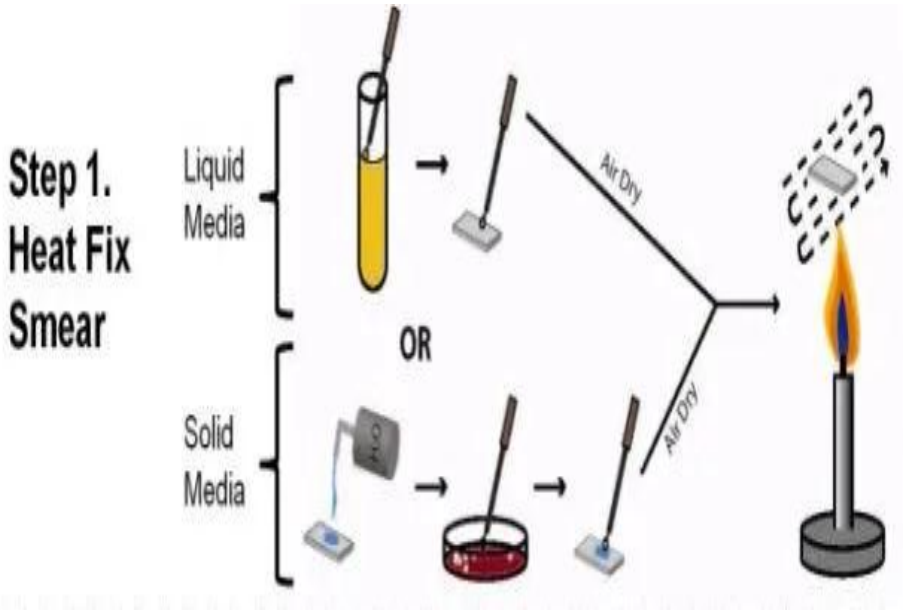
The bacterial cell wall has a negative charge. The basic stain has a positive charge. Since they have opposite charges, the bacterial cell wall and the basic stain are attracted to each other; hence the basic stain dyes the bacteria.

- **What is the difference between acidic dyes and basic dyes?**
- **Basic Dye:**
    - Basic dyes are positively charged and work with negatively charged tissue components,

- Basic dyes are constructed from cationic salts of colored bases, and acidic salts consist of salt of a sulfuric, phenolic or carboxylic organic acid.
- Basic dyes work better with staining synthetic materials like nylon, polyester, modacrylic and olefin.

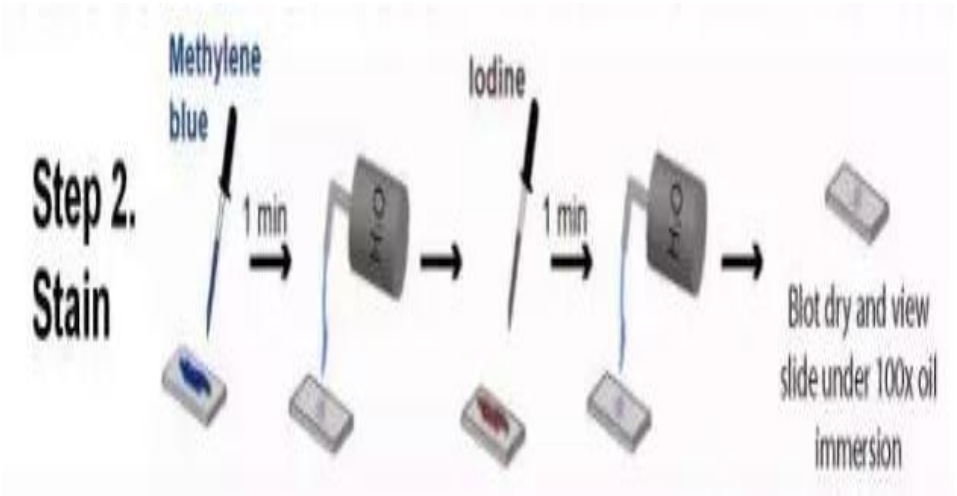
- **Acidic Dye:**
  - Acidic dyes are negatively charged and instead work with tissue components that are positively charged
  - Acidic dyes are more often used in labs as a dye bath and are effective in staining textiles because they are made up of protein fibers. These dyes bind best to animal hair such as wool or alpaca hair.
  - Use acidic dyes for mostly mycobacterium because of the presence of lipids in their cell walls.
- **Preparation of a smear and heat fixing:**
  - Using a sterilized inoculating loop, transfer loopful of liquid suspension containing bacteria to a slide (clean grease free microscopic slide) or transfer an isolated colony from a culture plate to a slide with a water drop.
  - Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of a dime. It should be a thin, even smear.

- Allow the smear to dry thoroughly.
- Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.



- **Simple Staining:**
  1) Cover the smear with methylene blue and allow the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).

2) Using distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.
3) Wash off any stain that got on the bottom of the slide as well.
4) Wipe the back of the slide and blot the stained surface with bibulous paper or with a paper towel.
5) Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.
6) Choose an area of the smear in which the cells are well spread in a monolayer. Centre the area to be studied, apply immersion oil directly to the smear, and focus the smear under oil with the 100X objective.

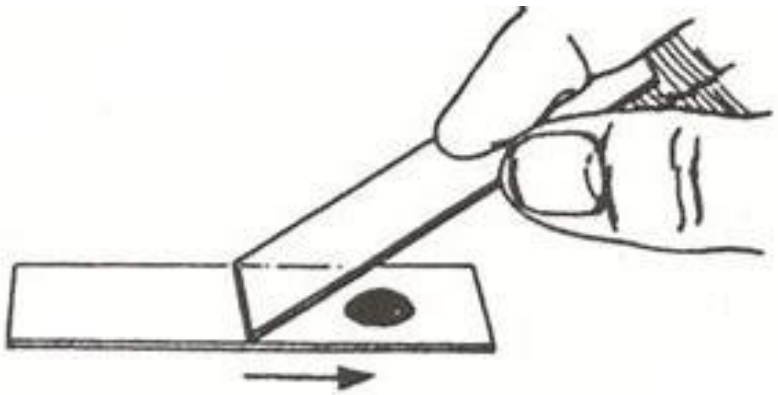


Indirect staining

1) Place a small drop of nigrosine on a clean slide.
2) Aseptically add a small amount of bacterial growth to the dye and mix gently with the loop.

3) Using the edge of another slide, spread the mixture with varying pressure across the slide so that there are alternating light and dark areas. Make sure the dye is not too thick or you will not see the bacteria.
4) Let the film of dyed bacteria air dry completely on the slide. Do not heat fix and do not wash off the dye.
5) Observe using oil immersion microscopy.

   Find an area that has neither too much nor too little dye (an area that appears light purple where the light comes through the slide). If the dye is too thick, not enough light will pass through; if the dye is too thin, the background will be too light for sufficient contrast.



- **Gram Staining:**

In this staining bacterial smear is of four different reagents:

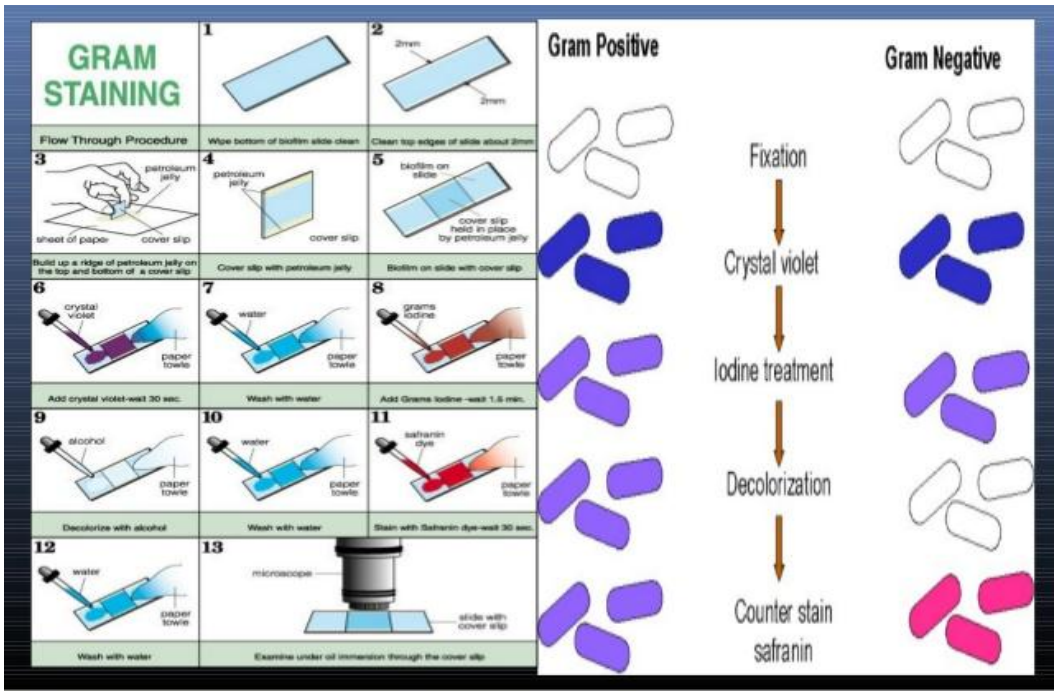
- Crystal Violet it is also known as primary stain.
- Iodine it is referred as mordant because it increases the affinity of dyes or a stain for smear.
- Alcohol or ethanol it is referred as de-colorizing agent that remove stain from the specimen.

- Safranin it is referred as counterstain.

- Procedures of Gram staining

1) Prepare a smear of bacteria culture and heat fix the smear.
2) Smear are first stained with Crystal Violet which is a basic dye it imparts purple colour to all cells.
3) Washed the slide with distilled water for seconds.
4) Now, smear is treated with iodine (mordant), this permit the stain to retained by forming an insoluble CV-I complex (Crystal Violet-iodine complex).
5) Wash the slide with ethyl alcohol (95%) this is a differential step. At this step some bacteria retain the primary stain i.e. Crystal Violet and appears purple colour while some bacteria not retain stain and loose purple colour and appears colourless.

Bacteria that retain Crystal Violet and appears purple are classified as gram positive bacteria, while that not retain stain and loose the colour after decolourisation are classified as Gram negative bacteria.



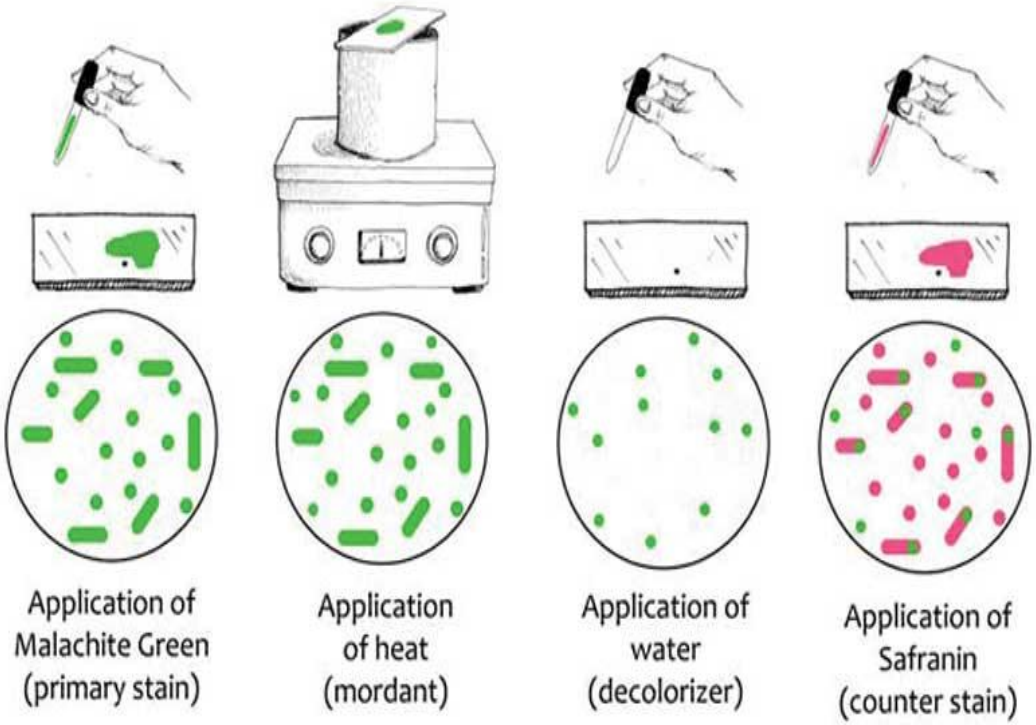
- **Endospore Staining:**

An endospore is a non-vegetative structure produced by a group of bacteria belonging to the Firmicute family. They have special characteristics that stabilize them to survive in adverse conditions for long periods of time.

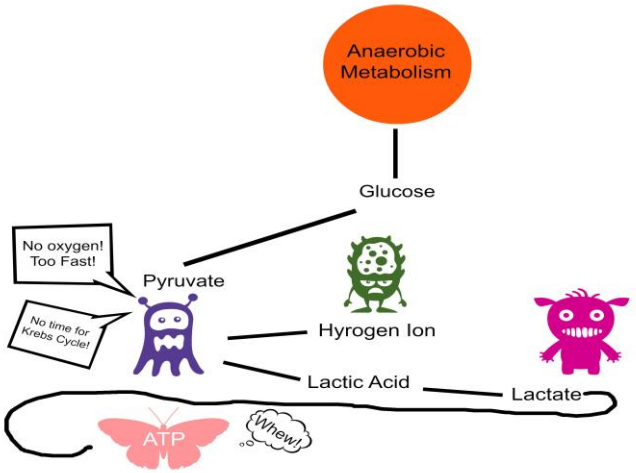
- **Endospore staining techniques are classified based on the types of reagents used:**
    1. Schaeffer Fulton Stain- used Malachite Green dye and safranin
    2. Dorner method of endospore staining –uses Carbolfuchsin stain, acid alcohol, and Nigrosin solution).
- **Staining procedure:**

1) Cover the smears with a piece of absorbent paper.

2) Place the slide over a staining rack, that has a beaker/water bath of steaming water.
3) Flood the absorbent paper with malachite green and let it steam for 3-5 minutes.
4) Remove the stained absorbent paper carefully and discard and allow it to cool for 1-2 minutes.
5) Gently rinse the slide with tap water by tilting the slide to allow the water to flow over the smeared stain. This is to remove the extra dye present on the slide on both sides and to also remove extra dye staining any vegetative forms in the heat-fixed smear.
6) Add the counterstain, safranin for 1 minute.
7) Rinse the slide with water, on both sides to remove the safranin reagent.
8) Ensure the bottom of the slide is dry before placing it on the stage of the microscope to view with the oil immersion lens, at 1000x for maximum magnification.


Application of Malachite Green (primary stain)
Application of heat (mordant)
Application of water (decolorizer)
Application of Safranin (counter stain)

Bacterial metabolism



Metabolism

- Sum up all the chemical processes that occur within a cell

1. Anabolism: Synthesis of more complex compounds and use of energy
2. Catabolism: Break down a substrate and capture energy

Microbial metabolism is the means by which a microbe obtains the energy and nutrients (e.g. carbon) it needs to live and reproduce. Also it is the set of life-sustaining chemical reactions in organisms.

The three main purposes of metabolism are:

1) **The conversion of food to energy to run cellular processes.**
2) **The conversion of food/fuel to building blocks for proteins, lipids, nucleic acids, and some carbohydrates.**
3) **The elimination of metabolic wastes.**

Enzymes

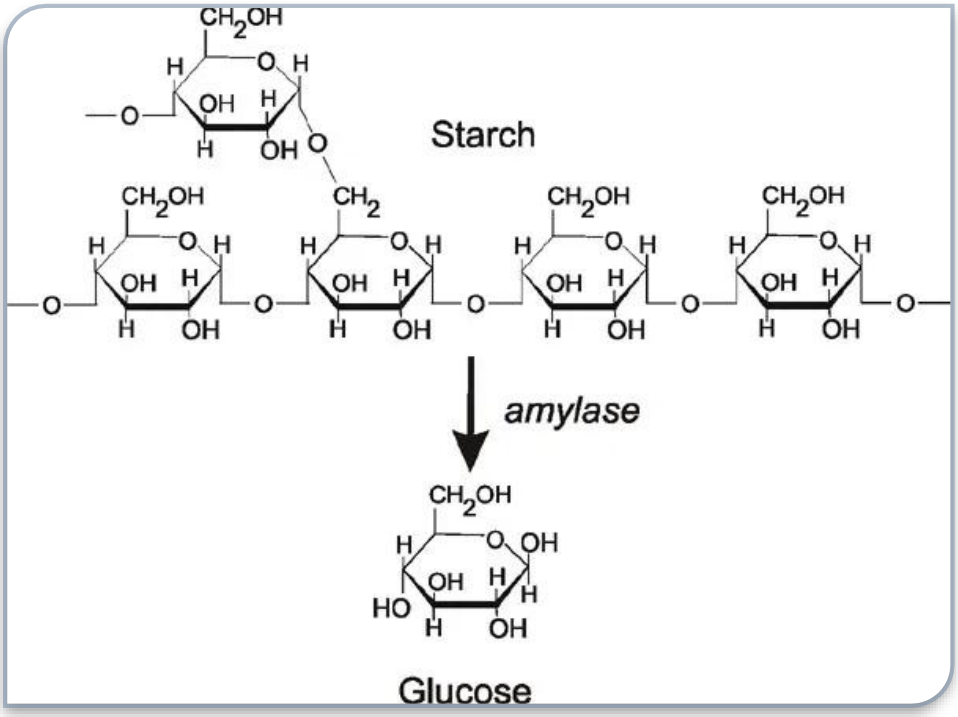
- **In the living world, each chemical reaction is catalyzed by its own enzyme.**
- **It is any of numerous compounds that are produced by living organisms and function as biochemical catalysts. Some enzymes are simple proteins, and others consist of a protein linked to one or more nonprotein groups.**

carbohydrates

- Are a biomolecule consisting of carbon (C), hydrogen (H) and oxygen (O) atoms, usually with a hydrogen–oxygen atom ratio of 2:1 (as in water) and thus with the empirical formula Cm(H2O)n.
- Carbohydrates perform numerous roles in living organisms. Polysaccharides serve for the storage of energy (e.g. starch and glycogen) and as structural components (e.g. cellulose in plants).

STARCH HYDROLYSIS TEST:

- Starch, the most important source of carbohydrate for human, is a polysaccharide mixture of two polymers.



- **Principle**:

Starch molecules are too large to enter the bacterial cell, so only bacteria that secrete exoenzymes (α -amylase and oligo-1,6-glucosidase) are able to hydrolyze starch into subunits (dextrin, maltose, or glucose). These molecules are readily transported into the bacterial cell to be used in metabolism.

- **Test Objective:**

To determine if the organism is capable of breaking down starch into maltose through the activity of the extra-cellular α-amylase enzyme.

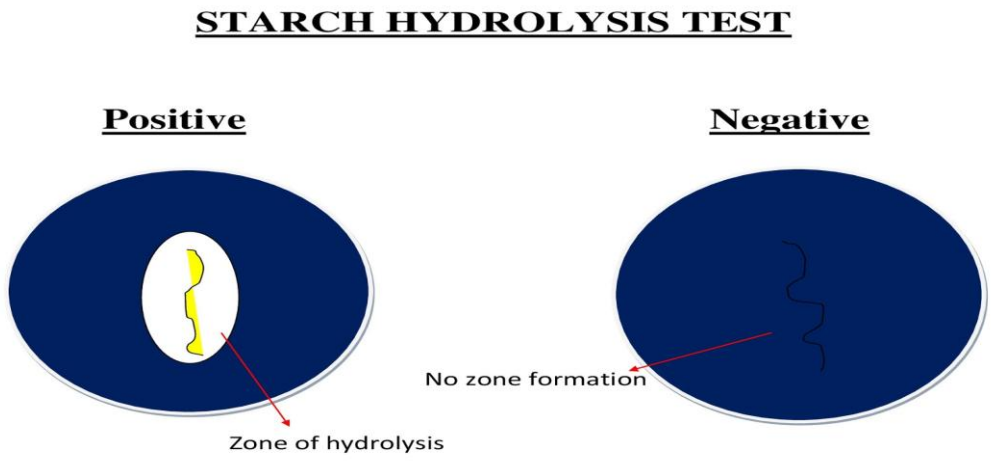
- **Test procedure:**

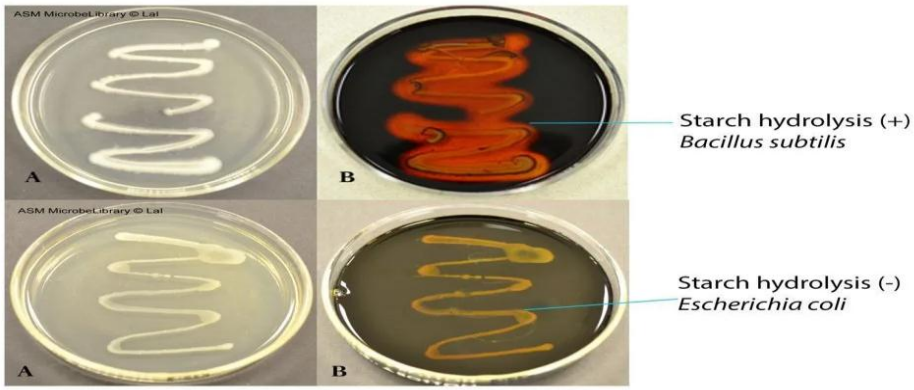
1) Pick a few colonies of test organism using a sterile swab or loop.

2) Streak a starch plate in the form of a line across the width of the plate. Several cultures can be tested on a single agar plate, each represented by a line or the plate may be divided into four quadrants for this purpose.
3) Incubate plate at 37 °C for 24-48 hours.

Add 2-3 drops of 10% iodine solution directly onto the edge of colonies. Wait 10-15 minutes and record the results.

Interpretation:
Positive test ("+"): Characteristic purple-black color will appear in the medium. However, a clear halo will appear around the colonies of amylase positive species.
Negative test ("-"): Characteristic purple-black color will appear in the medium, right up to the edge of isolated colonies of amylase negative species.





Oxidation and fermentation test

Whether an organism is oxidative or fermentative can be determined by using Hugh and Leifson's medium, commonly called as OF medium which contain tryptone and bromothymol blue (an indicator). One of the sugars, such as glucose, xylose, mannitol, lactose, sucrose, and maltose is added to the medium which serves as the fermentable carbohydrate.

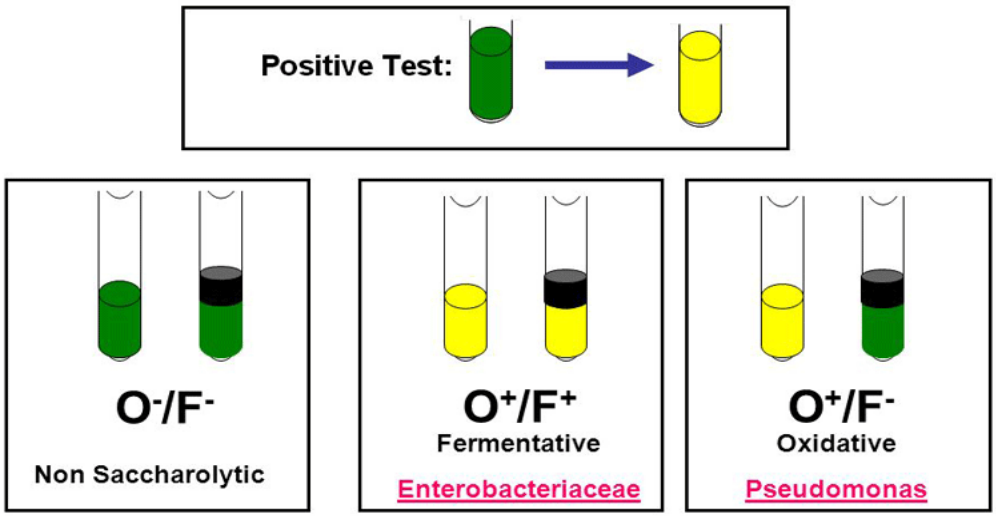
- An organism is inoculated to two tubes of each OF Medium. Once inoculated, one tube is overlaid with mineral oil or melted paraffin producing an anaerobic environment. The other tube is left open to the air. Growth of microorganisms in this medium is either by utilizing the tryptone which results in an alkaline reaction (dark blue colour) or by utilizing glucose, which results in the production of acid (turning bromothymol blue to yellow).

- **Media:**

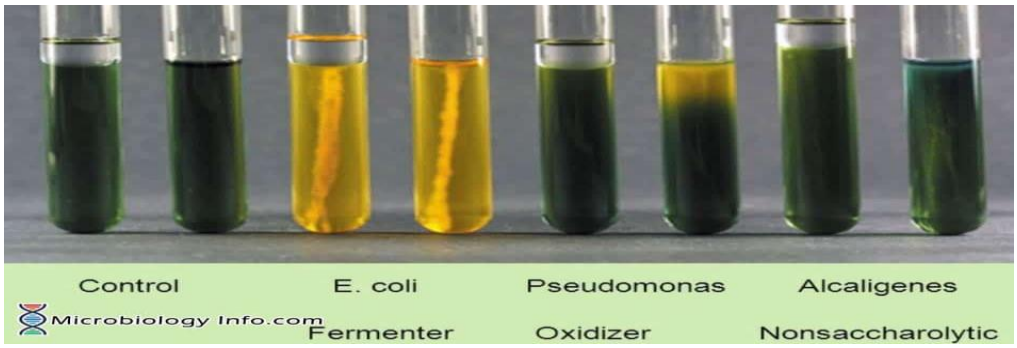
Hugh and Leifson's medium: Peptone 2.0gm/L, Sodium chloride 5.0gm/L, Dipotassium phosphate 0.30gm/L, Glucose (Dextrose) 10.0gm/L, Bromothymol blue 0.030gm/L, Agar 3.0gm/L, Final pH (at 25°C) 7.1±0.2.

- **Procedure of OF Test:**

1) Inoculate two tubes of OF medium with organism by stabbing with a straight wire.
2) Pour liquid paraffin over the medium to form a layer about one cm in depth into one of the tubes.
3) Incubate the tubes at 35-37°C for 24-48 hours.
4) Examine both open and closed tubes for the color change.



Oxidative: yellow colouration in open tube only
Fermentative: yellow colouration on both open and closed tubes



- It aids in the identification of gram-negative bacteria on the basis of their ability to oxidize or ferment a specific carbohydrate.
- It is used to determine whether an organism uses carbohydrate substrates to produce acid by-products.

Non fermentative bacteria are routinely tested for their ability to produce acid from six carbohydrates (glucose, xylose, mannitol, lactose, sucrose, and maltose).

METHYL RED-VOGES PROSKAUER (MR-VP) TEST:

- All enteric bacteria ferment glucose. Some species produce small amounts of organic acids via glucose fermentation, such species, typified by the genera *Enterobacter* and *Klebsiella.* In contrast, other enteric species, like *Escherichia coli,* liberate relatively high concentrations of acidic substances.
- Based upon the differences in fermentation products, these two groups of enterics can be differentiated by the Methyl Red-Voges Proskauer (MR-VP) test.
- Hence, the addition of methyl red to a culture of a mixed acid fermenter grown in MR-VP broth would make it appear red, that is, a positive MR reaction.
- In contrast, a non-mixed acid fermenter grown in MR-VP broth would cause the dye to appear yellow, a negative MR reaction.
- It is important to note that resulting color of a postive and negative MR test is opposite of tests that incorporate phenol red as a pH indicator

(phenol red is yellow under acidic conditions and red under alkaline conditions).

- Non-mixed acid fermenters can readily detectable using the Voges-Proskauer (VP) test. In this test, alpha-napthol (termed Barritt's A reagent) and potassium hydroxide (Barritt's B reagent) are added to a culture of a grown in MR-VP broth.
- Which in the presence of Barritt's A reagent forms a red complex .The latter is indicative of a positive VP reaction. The absence of a red color is considered a negative VP result.
- It is generally true that a MR positive enteric species is VP negative, and vice versa.
- **Media and Reagents:**
- Methyl Red-Voges Proskauer (MR-VP) broth [5 ml].
- Methyl Red Test Reagent [0.2% methyl red in 57% ethanol].
- Voges-Proskauer Reagent A [Barritt's Reagent A; 5% alpha-naphthol in absolute ethanol].
- Voges-Proskauer Reagent B [Barritt's Reagent B; 40% potassium hydroxide]
- **Initial Cultures (Day 1):**

1) Obtain four (4) MR-VP broth tubes and allow them to warm to room temperature before use.

2) Using a microbiological loop and aseptic technique, lightly inoculate the tube of medium with cells from the TSA slant culture matched to the appropriate bacterium.

4) Incubate all the tubes at 37°C for 18-24 hours.

- **Voges-Proskauer (VP) Test (Day 2):**

1) Remove the tubes from the incubator. Using a separate sterile, plastic bulb pipet for each MR-VP culture, aseptically transfer 1 ml to separate, clean, and appropriately labelled test tube. These tubes will be used in step 4 below.

2) Return the original cultures to 37°C for an additional 18-24 hours of incubation.

3) To each 1 ml aliquot prepared in step 5, add 15 drops of Voges-Proskauer Reagent A (Barritt's A) followed by 5 drops of Voges-Proskauer Reagent B (Barritt's B). Gently shake the tube to provide atmospheric oxygen.

4) Allow the tube to set at room temperature for 10-15 minutes undisturbed.

5) Observe the tube for the formation of a pink-red color.

- ❖ The formation of the pink-red color is taken as a positive result. The test may be read for up to, but not longer than, one hour following the addition of the VogesProskauer Reagents A and B.

- **Methyl Red (MR) Test (Day 3)**

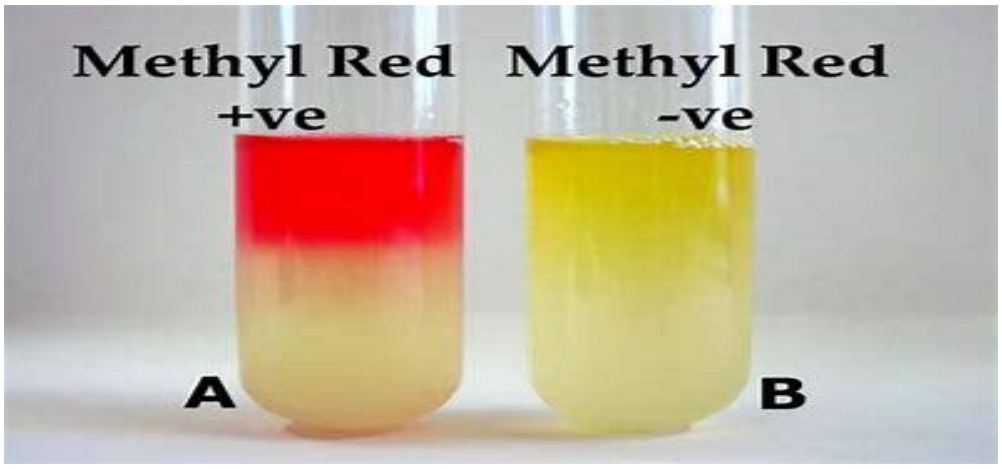
1) Remove the tubes (see step 3 above) from the incubator after 48 hours. Using a separate sterile, plastic bulb pipet for each MR-VP culture, aseptically transfer 2.5 ml to separate, clean, and appropriately labeled test tubes.

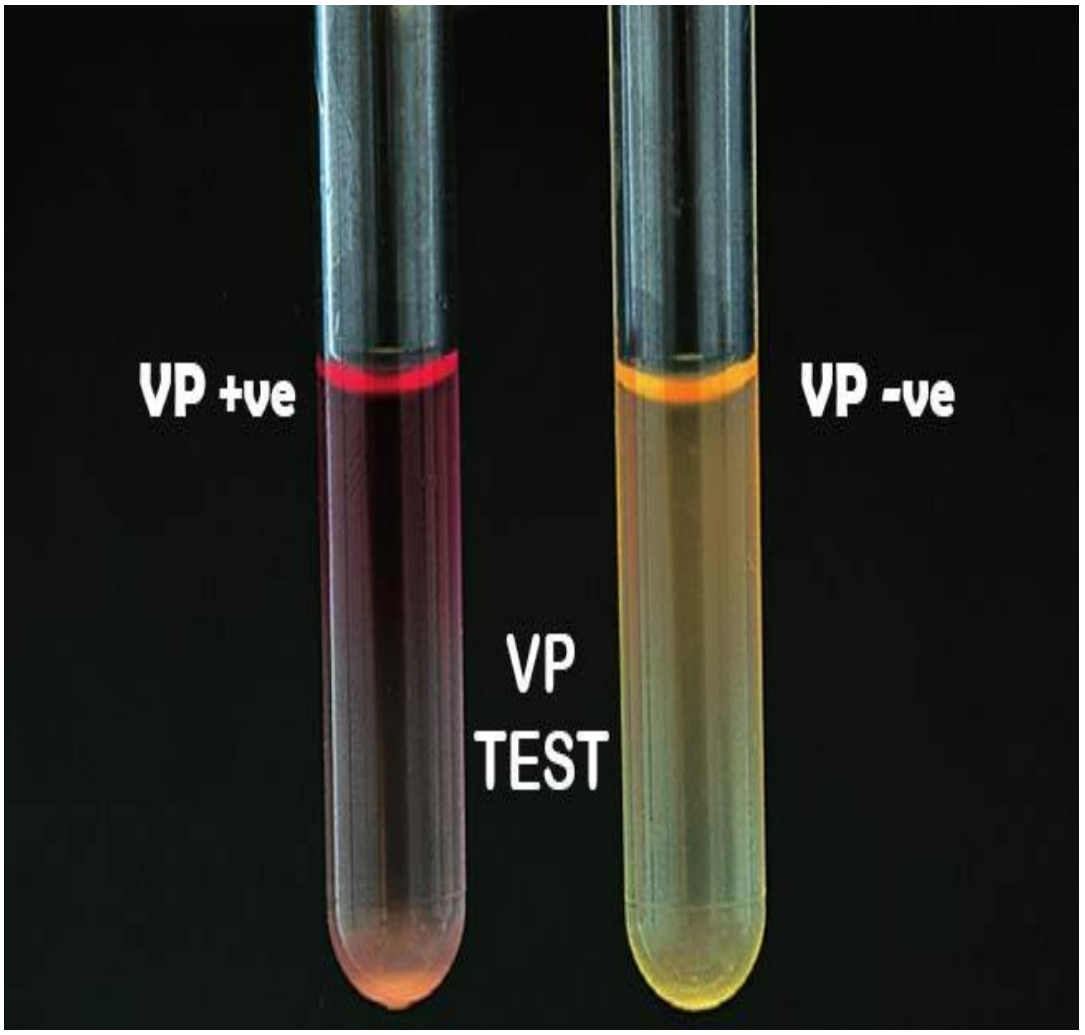
2) Discard each used bulb pipet in the appropriate waste bin.

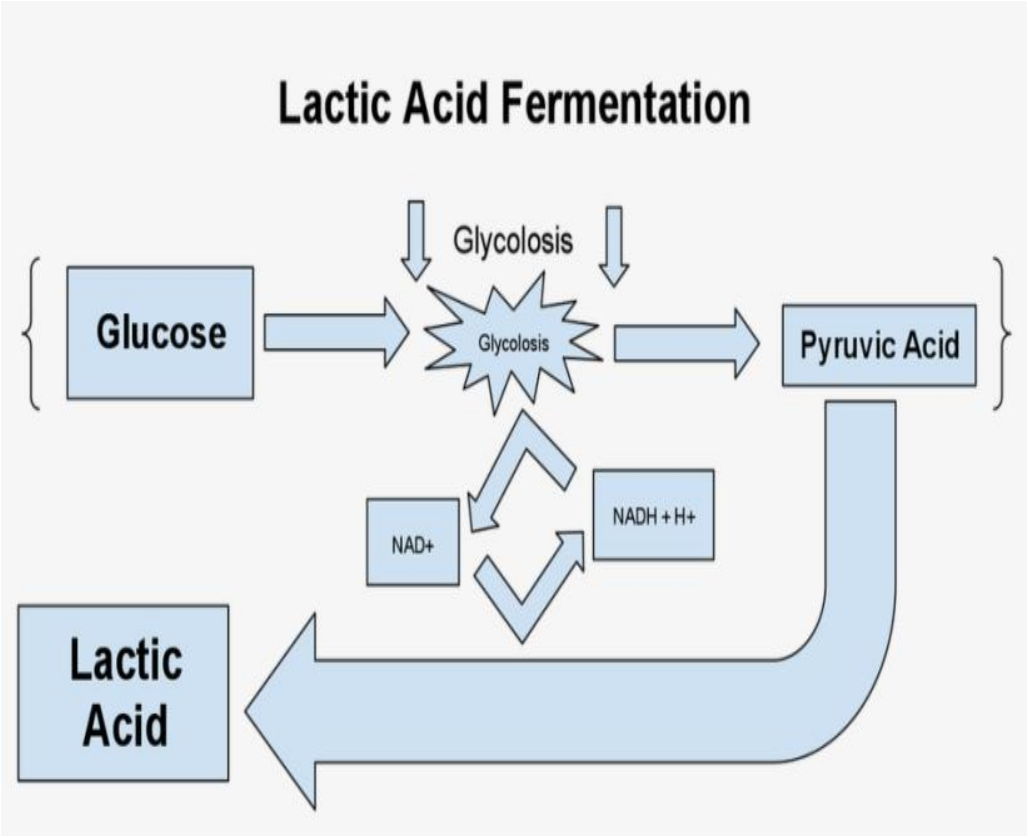
3) To each tube, add five drops of Methyl Red Reagent.

4) Observe the tubes for the immediate development of a red color.

- ❖ A positive MR test is indicated by the development of a stable red color on the broth surface, whereas negative test result is depicted by a yellow color on the broth surface.


Methyl Red
+ve
Methyl Red
-ve
A
B


VP +ve
VP -ve
VP
TEST



Gelatine hydrolysis test:

- The main purpose of this test is to detect the ability of organism to produce enzyme gelatinase. Gelatin is a globular protein that is produced by hydrolysis of collagen and used solidification substance. Collagen is the major component of connective tissues in humans and animals and extracted from animal protein.

Gelatin dissolves in water at 50°C and exist in liquid form above 25°C and solidifies as gel below 25°C. Once the gelatin is hydrolysed, even 4°C temperature cannot restore gel like characteristic.

Requirements:

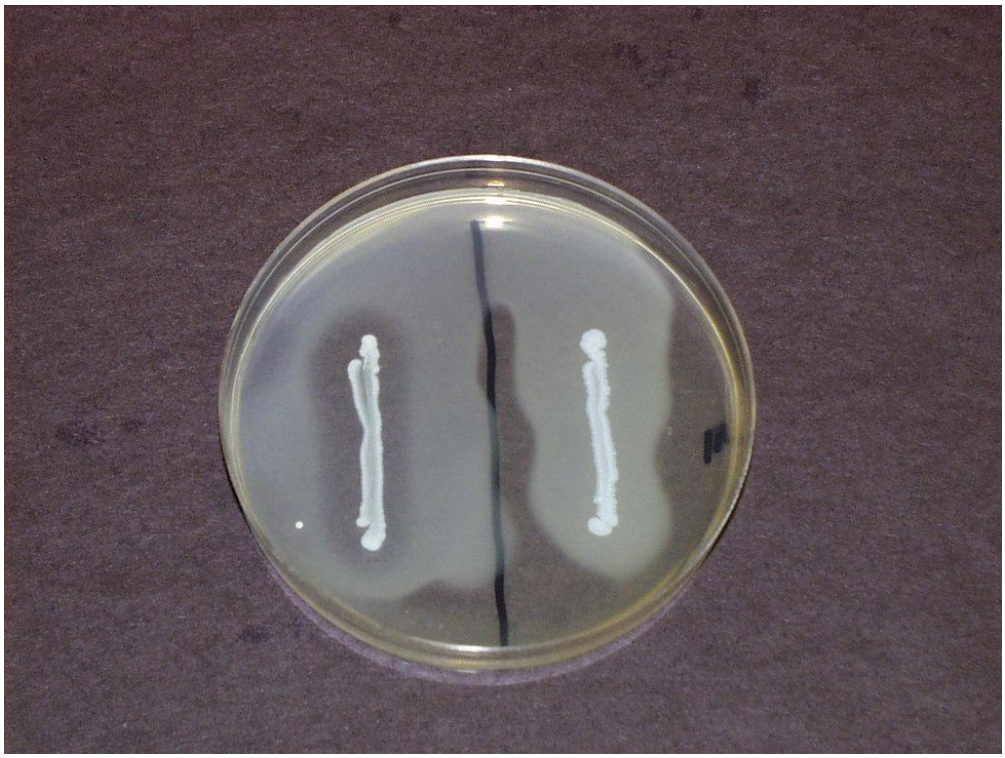
Media: 1% gelatin agar
Mercuric chloride ($HgCl_2$)
Culture: 24 hours culture of bacteria.

Procedures:

Gelatin hydrolysis test in agar plate.

1) Prepare agar media with 1% gelatin.
2) Inoculate the gelatin agar plate with the given organism, using inoculating loop making single central streak in the plate.
3) Incubate the plates at 37 °C for 24-48 hours.
4) Flood the plates with $HgCl_2$ solution (0.1 %).
5) Observe the plates after few minutes. A clear halo-zone around the inoculated area indicates gelatin hydrolysis; positive test.
6) Note in this experiment gelatin used as chemical agent not as solidification agent

- The presence of gelatin protein is detected by adding acidic HgCl2 (mercuric chloride). HgCl2 forms white precipitate with gelatin protein.
- The microorganism producing an extracellular gelatinase hydrolyses gelatin releasing constituent amino acids and short peptides. These amino acids are then utilized by microorganisms.
- HgCl2 does not give white precipitate with amino acids, so the microorganism which hydrolyses gelatin give no white precipitation with HgCl2.



Gelatine liquefaction Test

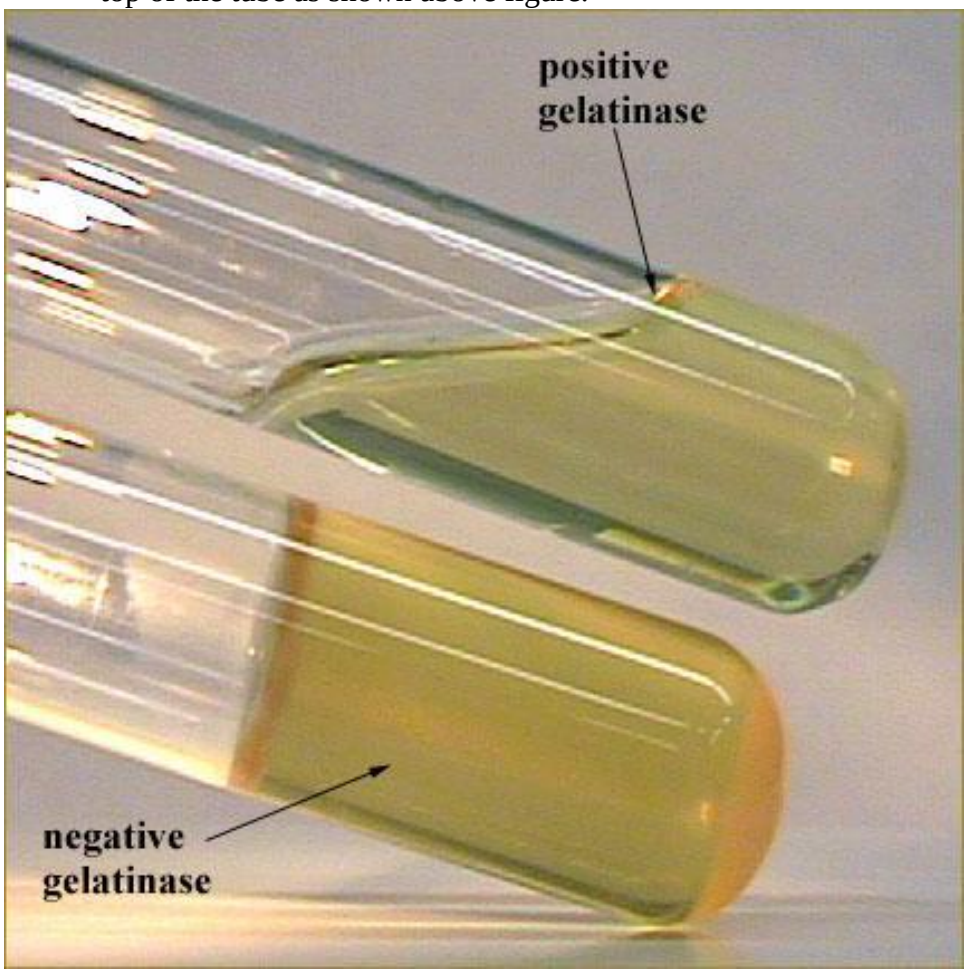
- Gelatine is a solidifying agent and used in microbiology for culture media before use of agar. It is nowadays replaced by agar due to having better solidifying properties than gelatine but even in use for gelatine liquefaction test to identify various organisms like *Bacillus*, *Clostridium*, *Proteus*, *Pseudomonas*, and *Serratia*. It is protein derived from the animal protein collagen – component of vertebrate connective tissue. Gelatine is a liquid at 28°C or higher.
- Gelatinase is proteolytic enzyme that hydrolyzes gelatin into polypeptides and individual amino acids. This enzyme destroys the structure of the gelatin, and it becomes liquid.

Requirement

- Test organism (pure or well isolated colonies, 18-24 hours old culture)
- Nutrient gelatine medium without agar
- Inoculating wire
- Bunsen burner
- Refrigerator (ice bath).
- Incubator
- **Test procedure:**

1) Incubation temperatures varies according to nature of organisms e.g for Enterobacteriaceae and fluorescent *Pseudomonas* 22°c and for non-fermenting, gram-negative rods 30°C
2) The medium should be solidified at room temperature. After touching several well-isolated colonies with a sterile needle, stab directly down the center of the tube to approximately 10 mm from the bottom.
3) Incubate the test and an un-inoculated control tube for 48 hours.
4) Gently remove the inoculated and uninoculated tubes from the incubator and refrigerate for at least 30 min or until the control tube solidifies.
5) Note: a. Do not shake or invert the tubes prior to refrigeration. b. Gently invert to detect liquefaction by the test organism after 30 min of refrigeration or 50 minutes in ice bath.
6) Re-incubate a negative test for up to 2 weeks if indicated by the nature of the organism, and examine at regular intervals.
7) **Interpretation:**
8) Gelatine Liquefaction Test Positive: at the end of the refrigeration period, the control tube will be resolidified and the test tube will remain liquid at least to the depth of the stab. Note: Some organisms only partially liquefy gelatin or liquefy just at the surface of the tube.

9) Gelatin Liquefaction test Negative: at the end of the refrigeration period, the control tube and the test tube will be resolidified, even at the top of the tube as shown above figure.



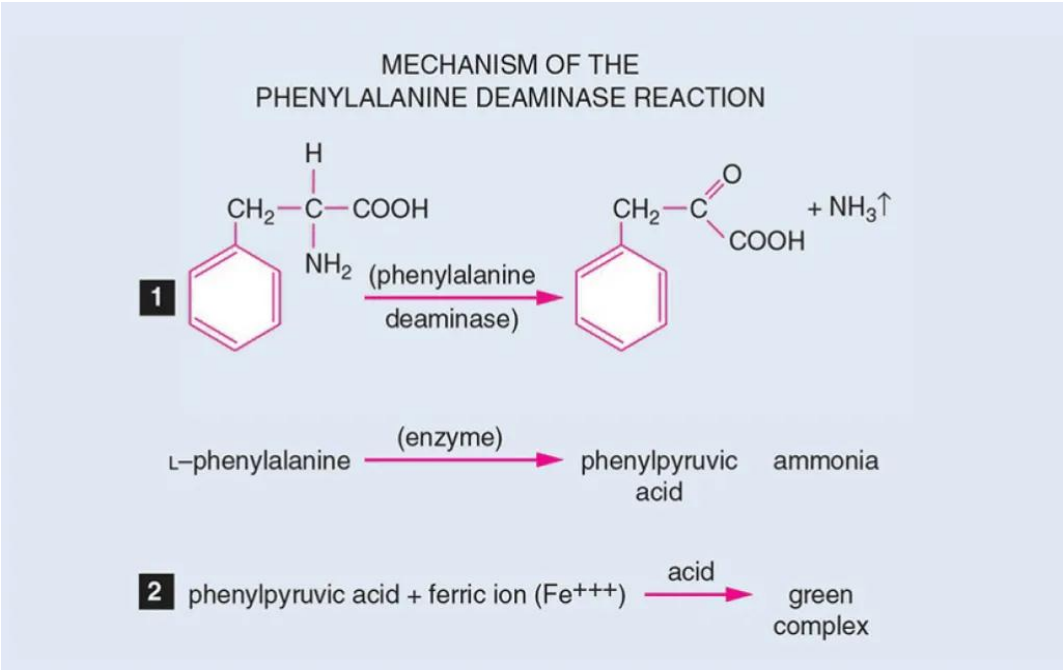
Amino acid catabolism

Amino acid is simple compound formed from amino group and carboxylic acid
1- Deamination and ammonia formation
This test is used to indicate the hydrolysis
(Phenylalanine Deaminase Test)

- Phenylalanine deaminase test also known as phenylpyruvic acid (PPA) test is used to test the ability of an organism to produce enzyme Phenylalanine deaminase. This enzyme removes the amine group from

the amino acid phenylalanine and produces phenylpyruvic acid (PPA) and ammonia i.e. oxidative deamination of phenylalanine. Phenylpyruvic acid reacts with ferric iron (10% ferric chloride is added in the medium) producing a visible green color.

- Phenylalanine agar, also known as phenylalanine deaminase medium which contains DL-phenylalanine and nutrients is used as a test medium.



Media and Reagents:

Phenylalanine agar medium is prepared and poured as a slant into a tube. The composition of the medium is as follows:

- DL-Phenylalanine: 2 gm
- Yeast extract: 3 gm

- Sodium chloride: 5 gm
- Disodium phosphate: 1 gm
- Agar: 12 gm
- Distilled water: 1 L
- pH: 7.3

Yeast extract serves as the carbon and nitrogen source. Meat extracts or protein hydrolysates cannot be used because of the varying natural content of phenylalanine.

Procedure:

1) Take or prepare a phenylalanine deaminase agar medium
2) Inoculate the phenylalanine slant (with a loop on the surface) with a test organism.
3) Note: If you are using the test medium i.e. phenylalanine agar for the first time use positive (Proteus vulgaris) and negative control (Escherichia coli) to check the efficacy of the test medium.
4) Incubate the test medium at 37°C for overnight.
5) Add 4-5 drops of 10% aqueous ferric chloride (FeCl3) solution to the slant. After addition of the reagent, rotate the tube to dislodge the surface colonies.
6) **Results:**
7) Positive test: Production of green colour (Phenylpyruvic acid thus formed reacts with ferric chloride producing a green colored compound thus turning the medium dark green).
8) Negative: No colour change (medium remains straw/yellow color; no PPA to react with ferric chloride).

Positive result
(Formation of dark green color)

Negative result
(No color change)

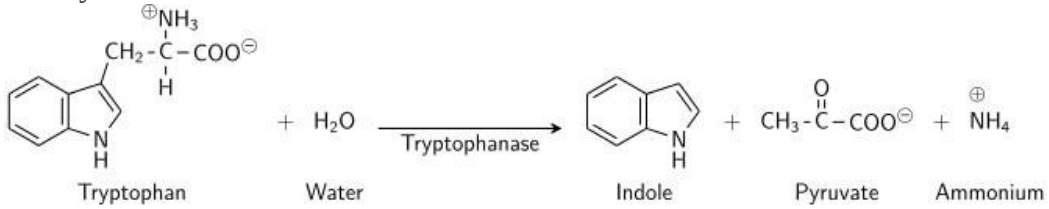
Indole Test- Principle, Reagents, Procedure, Result Interpretation and Limitations

This test demonstrate the ability of certain bacteria to decompose the amino acid **tryptophane** to **indole**, which accumulates in the medium. Indole production test is important in the identification of Enterobacteria. Most strains of *E. coli, P. vulgaris, P. rettgeri, M. morgani* and *Providencia* species break down the amino acid tryptophan with the release of indole. This is performed by a chain of a number of different intracellular enzymes, a system generally referred to as "tryptophanase." It is used as part of the IMViC procedures,a tests designed to distinguish among members of the family Enterobacteriaceae.

A variation on this test using **Ehrlich's reagent** (using ethyl alcohol in place of isoamyl alcohol, developed by Paul Ehrlich) is used when performing the test on **non-fermenters and anaerobes**.

Principle of Indole Test

Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme. **Indole** is generated by reductive **deamination** from **tryptophan** via the intermediate molecule **indolepyruvic acid**. **Tryptophanase** catalyzes the deamination reaction, during which the **amine (-NH2)** group of the **tryptophan** molecule is removed. Final products of the reaction are **indole, pyruvic acid, ammonium** (NH4+) and **energy**. **Pyridoxal phosphate** is required as a coenzyme.



When **indole** is combined with **Kovac's Reagent** (which contains hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol) the solution turns from yellow to **cherry red**. Because amyl alcohol is not water soluble, the red coloration will form in an **oily layer at the top of the broth**.

In the spot test, indole combines, in the filter paper matrix, at an acid pH with p-Dimethylaminocinnamaldehyde (DMACA) to produce a **blue to blue-green compound.** Indole Spot Reagent has been reported to be useful in detecting indole production by members of the family Enterobacteriaceae and certain anaerobic species.

Reagents Used in Indole Test

Ingredients per liter: *

Indole Spot Reagent:	
p-Dimethylaminocinnamaldehyde (DMACA)	10.0 gm
Hydrochloric Acid, 37%	100.0 ml
Deionized Water	900.0 ml

Indole Kovacs Reagent:	
p-Dimethylaminobenzaldehyde	50.0 gm
Hydrochloric Acid, 37%	250.0 ml
Amyl Alcohol	750.0 ml

* Adjusted and/or supplemented as required to meet performance criteria.

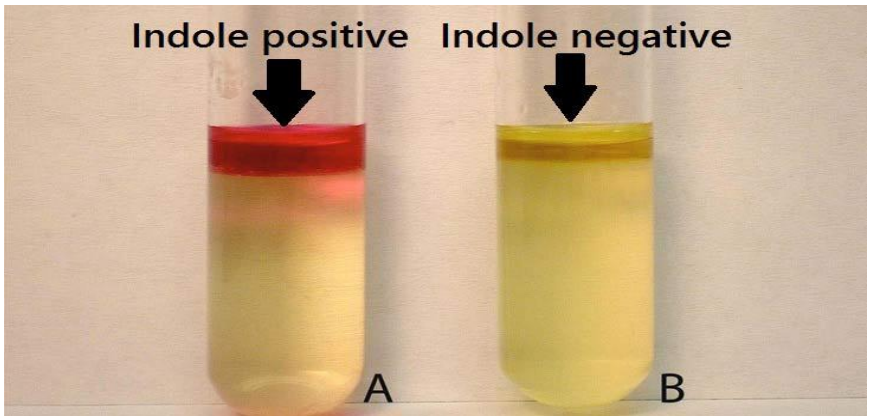
Procedure of Indole Test

1. Take a sterilized test tubes containing 4 ml of tryptophan broth.
2. Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture.
3. Incubate the tube at 37°C for 24-28 hours.
4. Add 0.5 ml of Kovac's reagent to the broth culture.
5. Observe for the presence or absence of ring.

Indole Spot Reagent (DMACA) Procedure

1. Place several drops of Indole Spot Reagent on a piece of filter paper.
2. With an inoculating loop or wooden applicator stick, pick a portion of an 18-24-hour isolated colony from a non-selective media and rub it onto the reagent saturated area of the filter paper.
3. Examine immediately

Result Interpretation of Indole Test



Positive: Formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent.

Mycological practical course

Kingdom: Mycophyta

Division (1): Myxomycophyta

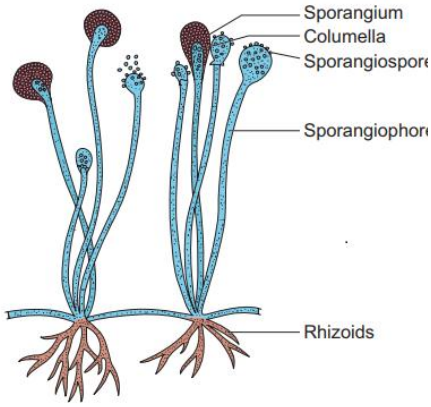
Division (2): Eumycophyta

Eumycophyta classified into four clases as follow:

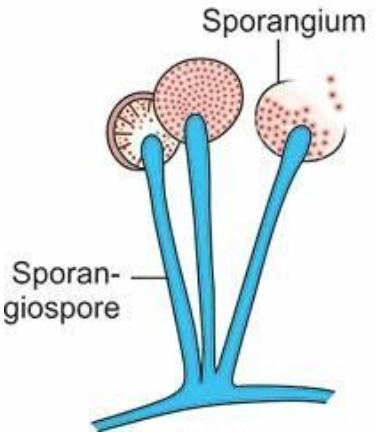
	Class(1) Phycomycetes Zygomycetes	**Class(2) Ascomycetes**	**Class(3) Basidiomycetes**	**Class(4) Deutromycetes**
Mycelium	Aseptate	Septate	Septate	Septate
Asexual spores	Zoospores Sporangiospores	Conidia	Conidia	Conidia
Sexual spores	zygospores	Ascospores	Basidiospores	Absent

Zygomycotina

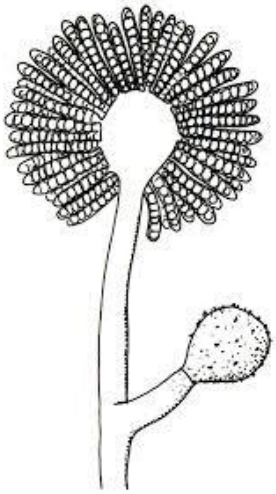
Kingdom : Mycophyta
Division : Eumycophyta
Class (1): Phycomycetes
Subclass: Zygomycetes
Order : Mucorales
Family(1): Mucoraceae
e.g : *Rhizopus* sp.
e.g: *Mucor* sp.
e.g : *Circinella* sp.
Family(2): Cephalidiaceae
e.g: *Syncephalastrum* sp.



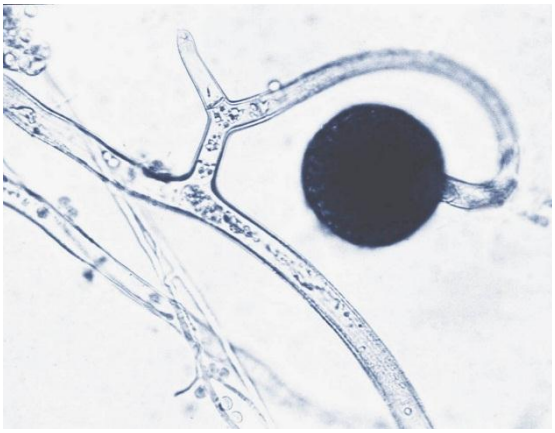
Rhizopus sp.



Mucor sp.



Syncephalastrum sp.



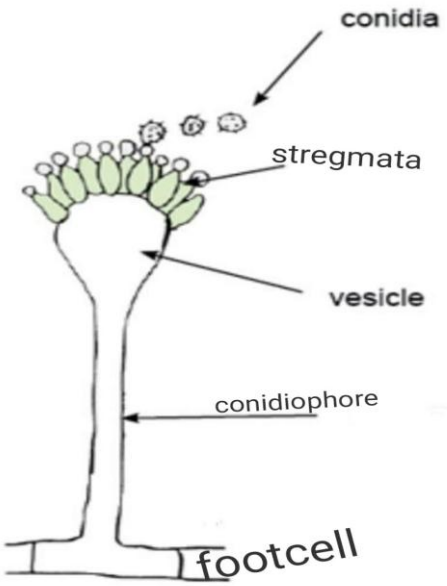
Circinella sp.

Ascomycotina

Kingdom: Mycophyta
Division: Eumycophyta
Class (2): Ascomycetes
Subclass: Euascomycetes
Order : Aspergillales
Family: Aspergillaceae
e.g : *Aspergillus* sp.
e.g: *Penicillium* sp.

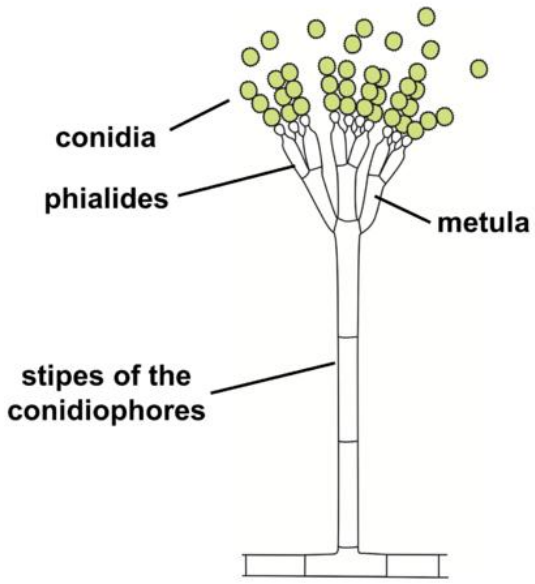
General character for ***Aspergillus*.**

1- Colony color
2- Colony reverse
3- Vesicle
4- Stregmata
5- Conidial head
6- Conidiophore
7- Conidia
8- Ascospore
9- Hull cell
10- Sclerotia



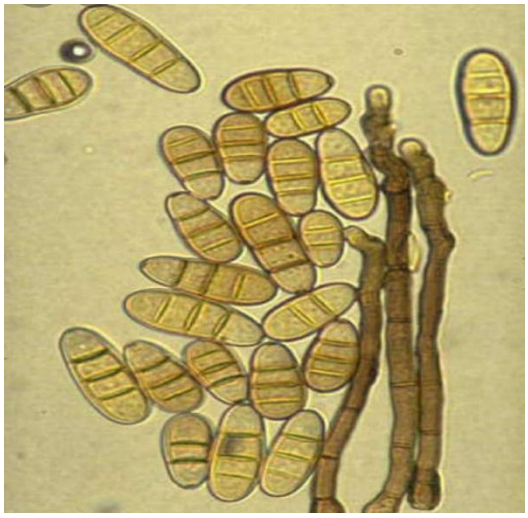
General character for *Penicillium*

1- Colony color
2- Colony reverse
3- Matullae: Absent or present
4- Pencilli: Monoverticillata or Biverticillata (Symmetric or Asymmetric)
5- Conidiophore: long or short/smooth or rough/ piment or hyaline/ branched or unbranched.
6- Conidia: Globose or sub or ovate/hyaline or pigment/smooth or rough
7- Ascospore: Present or absent
8- Hull cell: Present or absent
9- Sclerotia: Present or absent



Deuteromycotina

Kingdom: Mycophyta
Division : Eumycophyta
Class(3) : Deutromycetes
Order : Moniliales
Family (1): Dematiaceae
e.g. **:***Drechslera* sp.
:*Curvularia* sp.
:*Alternaria* sp.
:*Ulocladium* sp.
:*Cladosporium* sp.

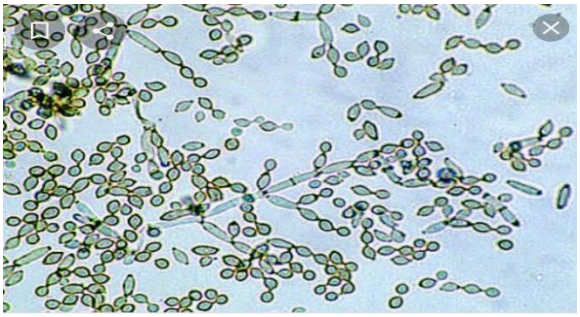


Drechslera sp.

Curvularia sp.

Alternaria sp.

Ulocladium sp.



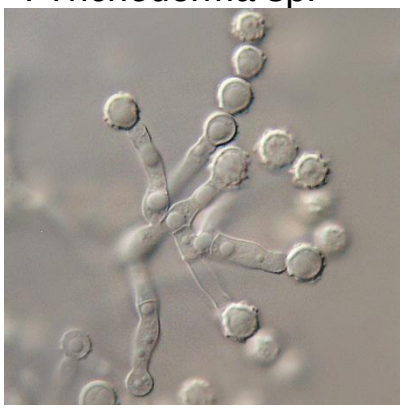
Cladosporium sp.

Order: Moniliales

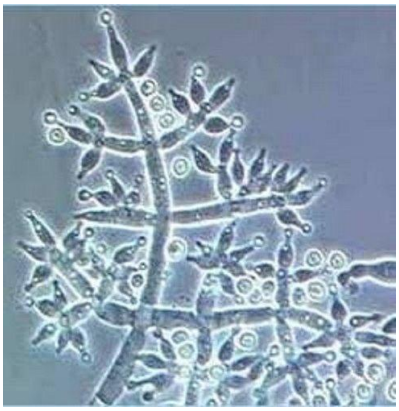
Family (2): Moniliaceae

e.g: *Scopulariopsis* sp.

: Trichoderma sp.



Scopulariopsis sp.



Trichoderma sp.

Family (3): Tuberculariaceae

e.g. : *Fusarium* sp.

Fusarium sp.

References

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