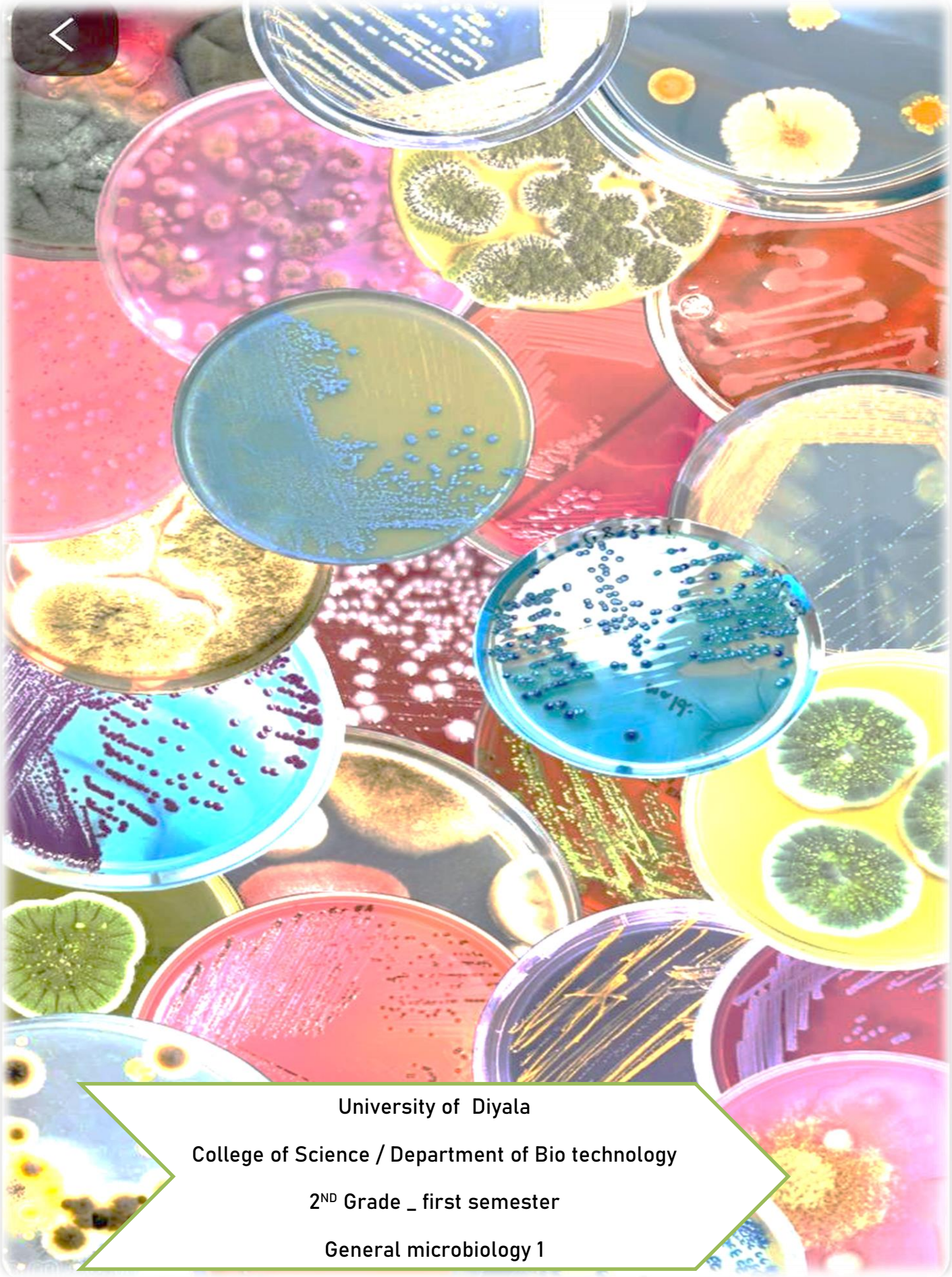


University of Diyala

College of Science / Department of Bio technology

2ND Grade _ first semester

General microbiology 1

Lab 1: Biosafety procedure, precautions & microscope

Microbiology: A science that specializes in the study of microorganism in terms of form and ways in which it combines and binds together and with other organisms, some of which may be useful, toxic or satisfactory. Micro-organisms are the oldest inhabitants of earth. They are masters in versality and adaptability to the changing environment. The diversity of their activities varies from causing diseases in human and other animals and plants to the production of various useful products. Microbiology comprises several organisms such as **Bacteria, Fungi, Algae, Protozoa and Viruses.**

Safety: in a microbiology laboratory is important in the prevention of infection that might be caused by the microorganisms being studied. In addition, many of the reagents, equipment, and procedures used are potentially hazardous. Attention to proper procedures and prudent laboratory practices are required for your safety and protection.

General Laboratory Safety Practices and Procedures:

1. Wash your hands well with disinfectants before and after working in lab .
2. Wear medical cloves, mask and coat in the lab.
3. If you are taking immune-suppressants, are pregnant, or have a known medical condition that would prevent full participation in the laboratory, please contact the course instructor before the first day of lab.
4. Read and understand each laboratory exercise before you come to class.
5. Do not eat, drink, smoke, or chew pens in the laboratory.
6. You must wear close-toed shoes while in the laboratory and long pants.
7. Long hair should be pulled back to keep it away from bacterial cultures, bacticinerator or open flames.
8. Personal electronic devices will be turned off and stored while in this laboratory. *The unauthorized use of any electronic device (phone, tablet, computer) in lab will result in a loss of course points.
9. Keep everything other than the cultures and tools you need OFF the lab bench. Only necessary work material should be at or on the laboratory bench. Coats, backpacks, and other personal belongings will not be allowed on the laboratory bench top.
10. Never, under any circumstances, remove equipment, media, or microbial cultures from the laboratory.
11. At the start and end of each laboratory session, students should clean their bench-top area with a disinfectant solution provided. Disinfect work areas before and after use with 70% alcohol or fresh 10% bleach. Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant on a routine basis and especially after spills, splashes or other contamination.
12. Turn off Bunsen burner when not in use ,Sterilize equipment and materials.
13. Never place contaminated pipettes on the bench top.
14. Discard all cultures and used glassware into the container labeled contaminated . (This container will later be sterilized.) Plastic or other disposable items should be discarded separately from glassware in containers to be sterilized.
15. Before leaving the laboratory, see that all the equipment are in the proper location and gas and water turned off.
16. Treat all cultures as potentially pathogenic, i.e., flood areas with disinfectant if cultures are spilled, wash hands after contact and notify your instructor at once.
17. Flame the inoculating loop or needle immediately before and after use.

18. Work either using laminar air flow chamber or light the burner at least five minutes prior to making any inoculations and work near the burner.
19. Aerosols should be avoided by the use of proper technique for sterilizing the inoculating loops and by performing any mixing of cultures and reagents in such a way as to avoid splashing.
20. Immediately report all accidents such as spills, cuts, burns, or other injuries to the instructor.



Personal protective instruments (biosafety protocol)



Laminar air flow

Any material which contains microorganisms should be treated first and thereafter, with the proper treatment should be thrown properly. The treatment is necessary due to the reasons:

- If it contains pathogenic microorganisms, the disease may transmit or spread to the healthy persons.
- It may contaminate soil and causes soil, water and air-pollution. Hence, to check from such hazards, proper treatment is required to kill microorganisms. The infected material is generally the solid or liquid culture media used for cultivation of microorganisms, or it may also contain cotton plugs, paper, cotton or cotton swabs, gloves, pins, PCR tubes, gel material etc. Some of the materials such as cotton plugs, paper, napkins, swabs etc. should be autoclaved first and then it is incinerated. But the microbial contaminants

containing materials should be treated with some disinfectant and thereafter autoclaved by putting them in suitable containers.

- **Microscope:** an essential tool in all microbiology labs There are many kinds of microscopes but the type most useful in diagnostic work is the compound microscope. By means of a series of lenses and a source of bright light, it magnifies and illuminates minute objects such as bacteria and other microorganisms that would otherwise be invisible to the eye.

- **Parts of microscope :**

- **Eyepiece Lens:** the lens at the top that you look through, usually 10x or 15x power.
- Tube: Connects the eyepiece to the objective lenses.
- Arm: Supports the tube and connects it to the base.
- Base: The bottom of the microscope, used for support.
- illuminator: A steady light source (110 volts) used in place of a mirror. If your microscope has a mirror, it is used to reflect light from an external light source up through the bottom of the stage.
- Stage with Stage Clips: The flat platform where you place your slides. Stage clips hold the slides in place. If your microscope has a mechanical stage, you will be able to move the slide around by turning two knobs. One moves it left and right, the other moves it up and down.
- Revolving Nosepiece or Turret: This is the part of the microscope that holds two or more objective lenses and can be rotated to easily change power.
- Objective Lenses: Usually you will find 3 or 4 objective lenses on a microscope. They almost always consist of 4x, 10x, 40x and 100x powers. When coupled with a 10x (most common) eyepiece lens, total magnification is 40x (4x times 10x), 100x , 400x and 1000x.
- condenser Lens: The purpose of the condenser lens is to focus the light onto the specimen.
- Diaphragm or Iris: Many microscopes have a rotating disk under the stage. This diaphragm has different sized holes and is used to vary the intensity and size of the cone of light that is projected upward into the slide.

- **Care and Handling of the Microscope**

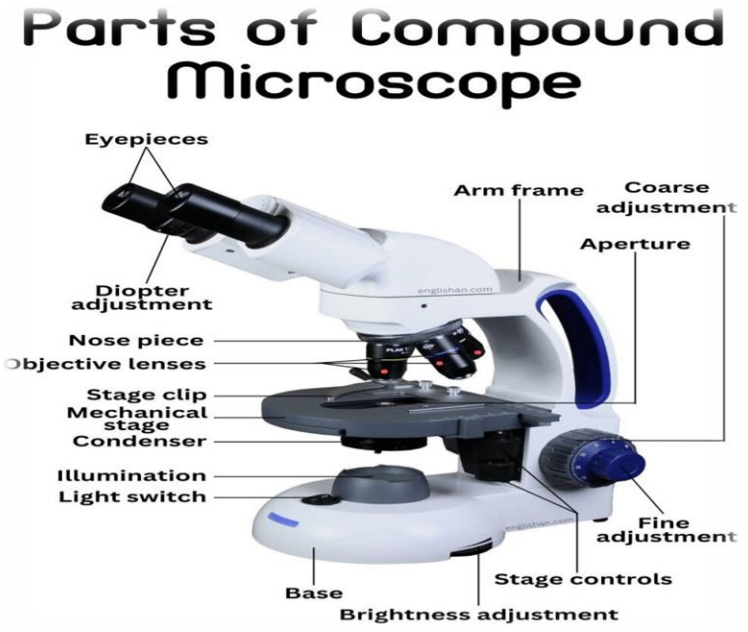
1-Always use both hands to carry the microscope, one holding the arm, other under the base.

2- Before each use, examine the microscope carefully and report any unusual condition or damage.

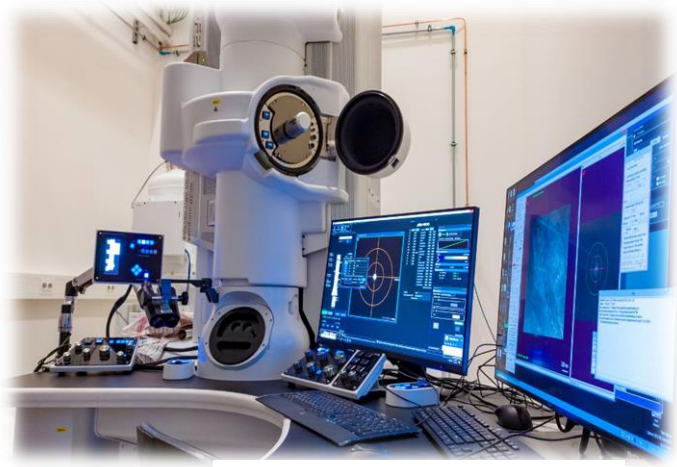
3-Keep the oculars, objectives, and condenser lens clean. Use dry lens paper only.

4-At the end of each laboratory period in which the microscope is used, remove the slide from the stage, wipe away the oil on the oil-immersion objective, and place the low-power objective in vertical position.

5- Replace the dust cover, if available, and return the microscope to its box.



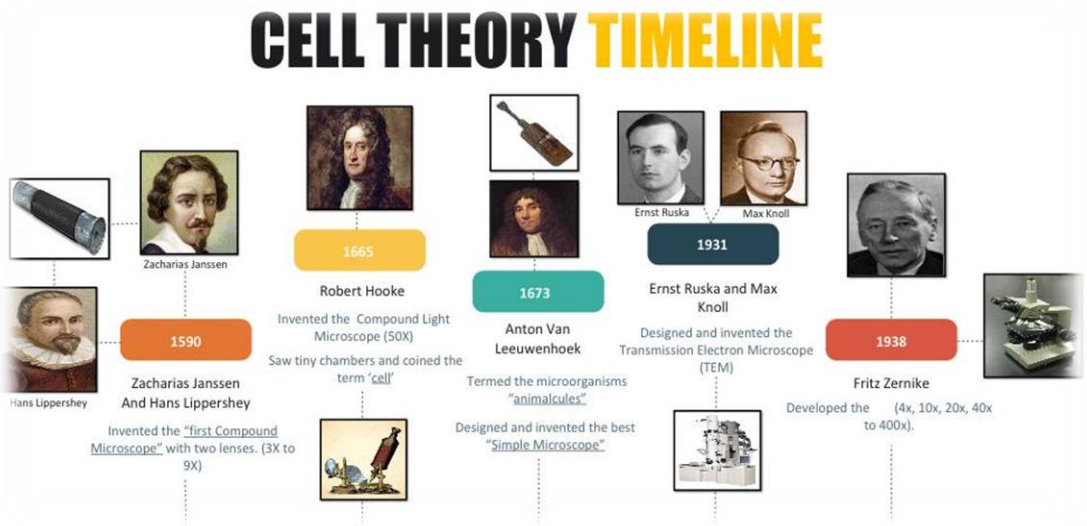




Electronic microscope



Figure 1
Development of microscope during time



Lab 2: Tools, instruments and Equipment in microbiology lab :



- **Autoclave :** most important equipment in lab we can't work without it , use in sterilization (make us sure every thinks enter inside autoclave exit sterile from all life forms bacteria, bacterial and fungal spores , parasites and viruses), many factories around world competing to supply the laboratories with best types of autoclaves



- **Incubator :** Very important equipment used to provide suitable conditions to bacterial growth ad reproduction (temperature, moist and oxygen requirement), optimum temperature for bacterial activity 25_37C ad ph 7 .



- **Oven :** Used to sterile classes and metallic tools , wood sticks and other objects in lab By dry heat and hot airs for 2_3 hours we can gate a tools ready to works.



Water bath : A water bath is a laboratory equipment that is used to incubate samples at a constant temperature over a long period of time.Water bath is a preferred heat source for heating flammable chemicals instead of an open flame to prevent ignition .



Sensitive balance : Analytical balances are highly sensitive instrument designed to accurately measure mass, Analytical balances have a draft shield or weighing chamber to prevent the very small samples from being affected by air currents .



Centrifuge : Centrifugation is used to collect cells , precipitate DNA, purify virus particles, and to distinguish subtle differences in the conformation of molecules. Most laboratories undertaking active research will have more than one type of centrifuge, each capable of using a variety of rotors Centrifugation .



Hot plate stirrer: The primary use of magnetic stirrer or hot plate with magnetic stirrer is and chemical experiments by mixing two components. It is equally suitable for solids or liquid mixture. Examples include media for bacterial to conduct biological liquid samples to obtain a consistent growth and chemical synthesis.

Tools/ glass wares:-



Flasks: hold solids or liquids that may release gases during reactions or that are like lit splatter if stirred or heated. other types of flasks (Florence flasks, volumetric flasks, round bottom flasks)

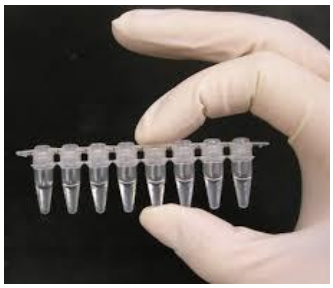


Beakers : Hold solids or liquids that will not release gases when react or are unlikely to splatter if stirred or heated .sterilized by oven.



Graduated cylinders: this tool useful for measuring liquid volumes; they are for general purpose use, but not for quantitive analysis, if greater accuracy is needed, use pipette or volumetric flasks.





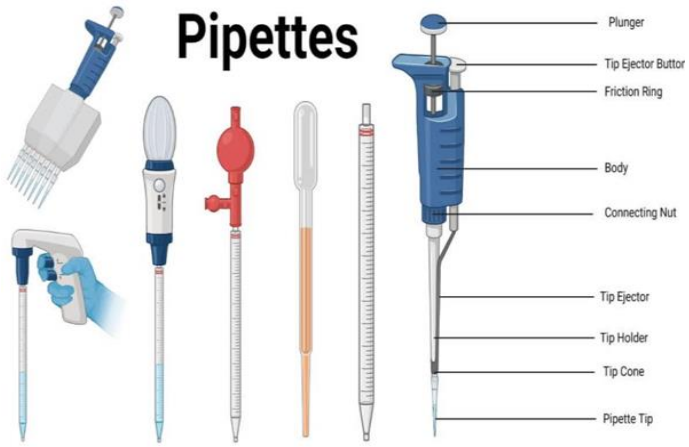
Test tubes: a small glass or plastic container used to view chemical reactions, or to heat small amounts of substances, it also used to place broth, solid or semisolid medium for stabbing technique or place aslant for bacterial culture. (Other types of tubes screw cap test tubes).



Test tube rack: use to hold test tubes in vertical position, comes in many style, and types made of metal or plastic, sometimes from woods.



Glass plates(slides): slides used for provide a surface for semi-micro scale experiments, such as drop reactions and testing of acids and bases.



Pipettes : are glass or plastic tubes, usually open at both ends, which are used for transfer specific amounts of liquids from one container to another, They come in different types, including micropipettes, volumetric pipettes, and electronic pipettes, usually used for volumes between 1 _ 100 millimeters .From medical research to chemical testing, the versatility of pipettes is evident in their various forms and functionalities.

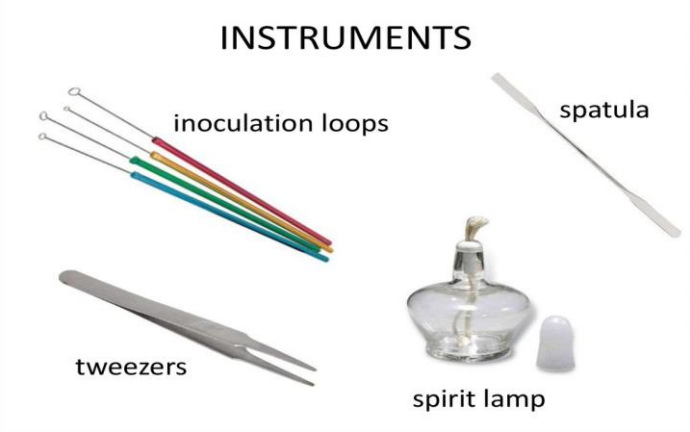
Tools / metals, woody & plastics :-



Bunsen burner: common piece of laboratory equipment used for heating, and sterilization, safely burns a continuous stream of flammable gas such as natural gas.



Loops& needles: used transfer bacterial cells from medium to another(as colony or 0.01ml), sterilized by direct flame of burner (before and after using) .the different between loops and needles , is that needles use to transfer bacterial cells to a solid medium or semisolid medium by stabbing method.



- **Spatulas:** it come in many sizes and shapes, used for transferring solids, powders like culture media, salts and others.
- **Forceps(tweezers):** this tool used for any situation where you have to grab a small item and cannot do it with your fingers.



- **Petri plates or Petri dishes:** used for putting the culture media in it (for solid medium only) glass Petri plates used for many tines & sterilized by oven and autoclave , while sterilized plastic Petri plates used for one time only (disposable).



- **Washing bottle & Funnels :** used fill with liquids(especially distilled water D.W) for washing and homogenized the glassware and washing the slides during staining, a funnel is used to aid in the transfer of liquid from one vessel to another (different in volumes) both of them don't need sterilization.



- **Swab:** used for swabbing the bacterial cells on the surface of solid medium in Petri plate, must be disinfectant before disposal. Ex. Cotton swabs& swabs with transport media.

Lab 3 : Culture media

Culture media are mediums that provide essential nutrients and minerals to support the growth of microorganisms in the laboratory. Microorganisms have varying nature, characteristics, habitat, and even nutritional requirements, thus it is impossible to culture them with one type of culture media. However, there are also microorganisms that can't grow on a culture media at all in any condition – these are called obligate parasites.

Importance of culture media :

- Essential for isolating pure cultures, storing culture stock.
- Studying biochemical reactions, testing microbial contamination.
- Checking antimicrobial agents and testing antibiotic sensitivity.
- Essential for diagnosing infectious diseases
- Obtaining antigens, developing serological assays for vaccines.
- For genetic studies, and identification of microbial species.

♣ **The survival and growth of microorganisms depend on available nutrients and a favorable growth environment.**

Nutritional Requirements of Bacteria:

Before one can construct a medium that will achieve a desired result in the growth of organisms, one must understand their basic needs. Any medium for cultivation of bacteria must provide certain basic nutritional requirements, which include:

1. A carbon source that may also serve as an energy source.
2. Water
3. A nitrogen source
4. A phosphate source
5. Various mineral nutrients, such as iron and magnesium
6. Growth factors.

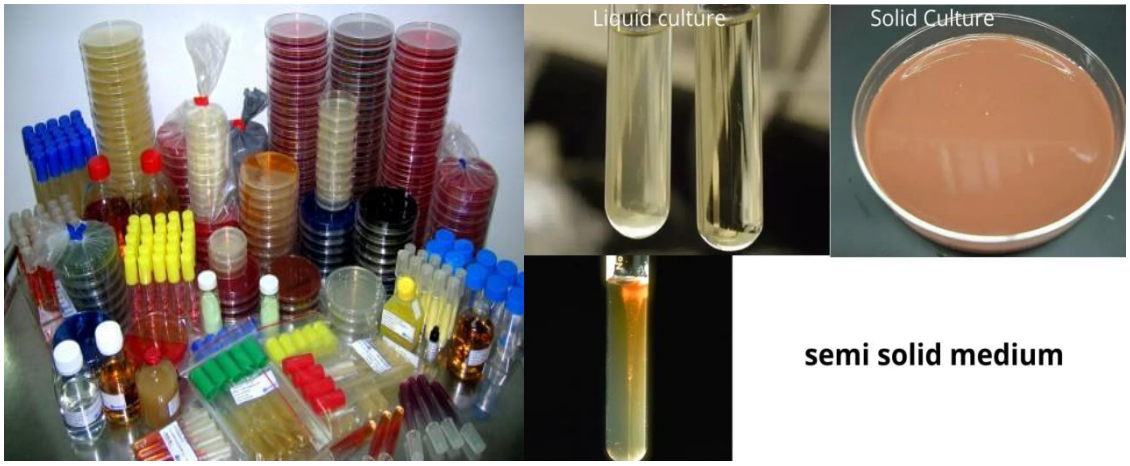
Classification and Types of Culture Media:

A. Based on consistency: three types

1. **Liquid media or broth:** such as nutrient broth, tryptic soy broth, or brain-heart infusion broth. These media are used for the propagation of large numbers of organisms, fermentation studies, and various other biochemical tests.
2. **Solid media:** are made by adding a solidifying agent, such as agar, gelatin, or silica gel, to a liquid medium. A good solidifying agent is one that is not utilized by microorganisms, does not inhibit bacterial growth, and does not liquefy at room temperature. The development of colonies on the surface of a medium is essential when trying to isolate organisms from mixed cultures. Solid media, such as nutrient agar or blood agar, are used :

- For the surface growth of microorganisms in order to observe colony appearance.
- For pure culture isolations,
- For storage of cultures.
- To observe specific biochemical reactions.

3. **Semisolid media:** fall in between liquid and solid media. Although they are similar to solid media in that they contain solidifying agents such as agar, they are more jellylike due to lower percentages of these solidifiers. These media can be used in fermentation studies, in determining bacterial motility, and in promoting anaerobic growth.



Types of culture media based on consistency

B. Classification based on the nutritional component:

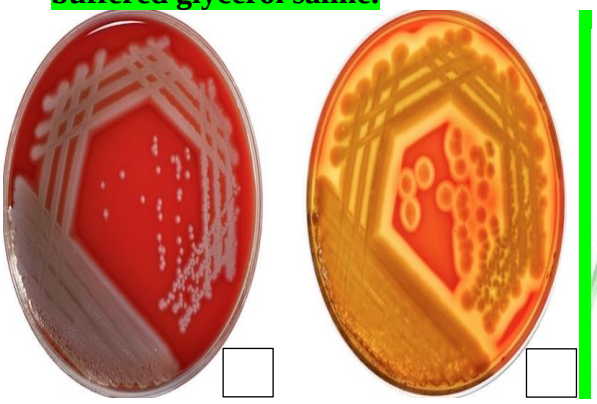
1. **Simple media: It's a general-purpose media that supports the growth of non-fastidious microbes, and it is primarily used for the isolation of microorganisms. Examples are nutrient broth, peptone water, and nutrient agar.**
2. **Complex media:** These are media containing nutrients in unknown quantities that are added to bring about a particular characteristic of a microbial strain. Examples are tryptic soy broth, blood agar, and nutrient broth.
3. **Synthetic media:** Synthetic media is a type of chemically defined media and is produced from pure chemical substances. A defined media refers to a medium having a known concentration of ingredients, like sugar (glucose or glycerol) and nitrogen source (such as ammonium salt or nitrate as inorganic nitrogen). It is generally used in scientific research, and an example is Czapek Dox Medium.

C. Classification of culture media based on application/chemical composition:

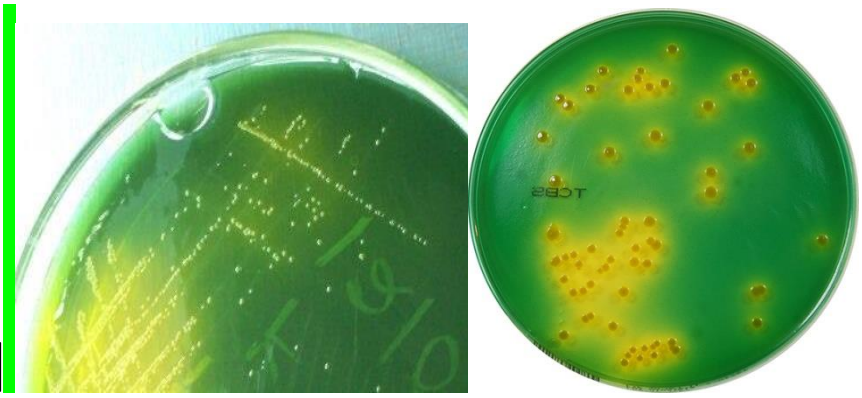
1. **Basal media:** These are routinely used simple media having carbon and nitrogen sources that boost the growth of many microorganisms. They are also known as general-purpose media and are considered non-selective media. They are generally used to isolate microorganisms in labs or in sub-culturing processes. Examples are nutrient broth, nutrient agar, and peptone water .
2. **Enriched media:** This media is prepared by adding additional substances like blood, serum, or egg yolk in the basal medium. It's used to grow fastidious microorganisms as they require additional nutrients and growth-promoting substances. Examples are chocolate agar, blood agar, and Loeffler's serum slope. Chocolate media is used to grow *N. gonorrhea* while blood agar (which is prepared by adding 5-10% blood by volume to a blood agar base) is used to identify hemolytic bacteria .
3. **Selective media:** This media allows the growth of certain microbes while inhibiting the growth of others. It's an agar-based medium that is used to isolate microorganisms in labs. The selective growth of microbes is decided by adding substances like antibiotics, dyes, bile salts, or by pH adjustments.
4. **Enrichment media:** It's a liquid medium, used to increase the relative concentration of certain microbes before culturing them on a solid medium plate. It's used as a broth medium and inhibits the growth of

commensal species of microorganisms (those who live in close association with each other) in the clinical specimen. It's also used in isolating fecal and soil microorganisms. Examples are selenite F broth which is used to isolate *Salmonella typhi* from a fecal sample, tetrathionate broth, and alkaline peptone water.

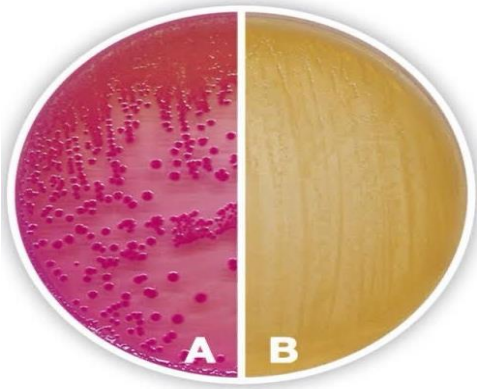
5. **Differential or indicator media:** It contains certain indicators like dyes or metabolic substrates in the medium composition which gives different colors to colonies of different microbial species when they utilize or react with these components. Ex. **MacConkey agar, Mannitol salt agar , Thiosulfate citrate bile salts sucrose (TCBS) agar and Salmonella, shegilla agar(SS agar), Eosin methylene blue (EMB agar).**
6. **Transport media:** Transport media are useful for clinical specimens which are required to be transferred immediately to labs to maintain the viability of potential pathogens and to prevent overgrowth of commensals or contaminating microorganisms. Some of them are semi-solid in consistency ex. **Sach's buffered glycerol saline.**



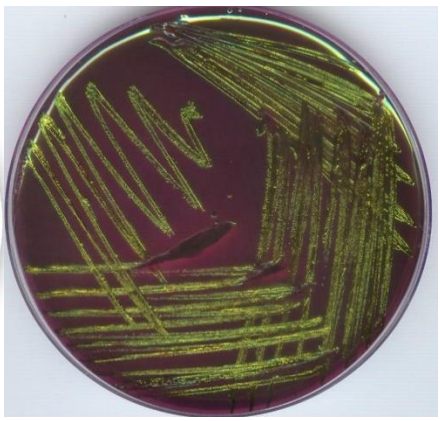
Blood agar (A: alpha hemolysis B: beta hemolysis)



TCBS agar (yellow colonies of *vibrio cholera*)



MacConkey agar
A: lactose fermenter *E.coli*
B: lactose non fermenter



***E. coli* on EMB agar with green metallic sheen**



SS agar (black colonies of *Salmonella SPP.*)



MSA (Mannitol Salt Agar)

Lab 4: Preparing and pouring culture media

Preparation of culture media:

1-Weightining the medium ingredients according to the direction written on its container.

2-Dissolve with little amount of D.W. then complete the volume to the volume you want and may be need using heating and stirrer for complete dissolving.

3-Check pH .

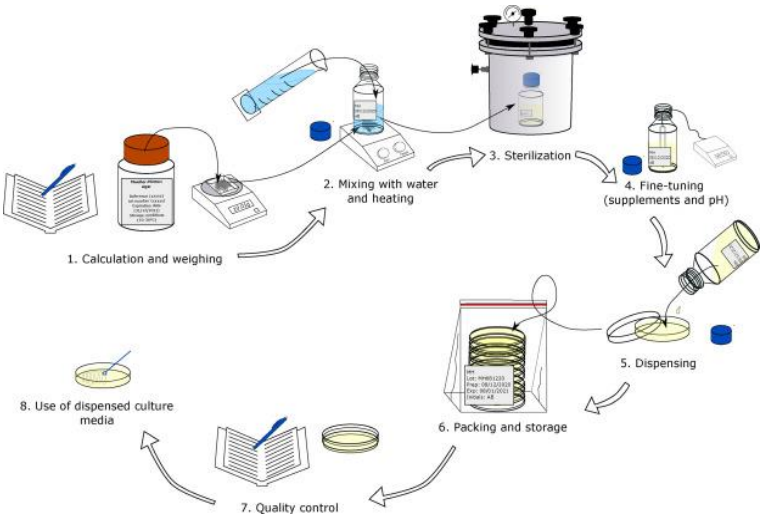
4-Dispensing the medium in to test tube by pipette. 5-Sterilization by autoclave.

6-Dispensed agar medium into petri dish when the heat reach to 45. EX :prepare 500ml of N.A. medium if the direction on container wrote 8gm/liter gm ml 8 1000 x 500 x=8 * 500/1000 = 4 gm of media dissolve in little amount of D.W. then complete the volume to 500 ml then autoclaved and poured in plates.

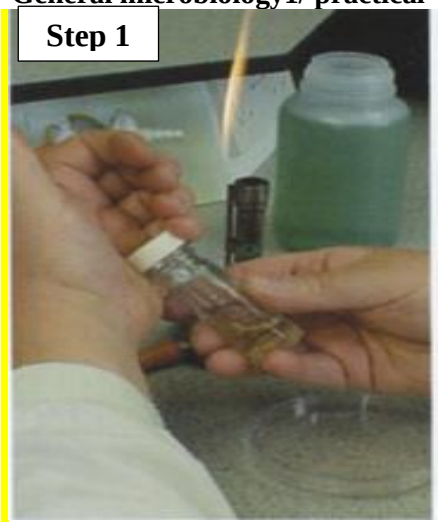


- **pouring the media in plate:** The sterile plates should be on the table near the burner then Cooling the solid medium to 45C˚ to avoid solidify it and to avoid forming of drop on the cover of plates Remove the cover (or cotton plug) and sterile the upper part by burner Remove the cover of plate near the burner and pouring the medium and close the cover of plate Moving the plate on table 5 times in two direction to distribute the media equally in plate.

- **Sterility test:** This test mean putting the flasks tubes and plates which contain sterile media before using in incubator at 37C for 24 hr. to ensure that there is no contamination while preparing and pouring the media.



Step 1



Step 2



Step 3



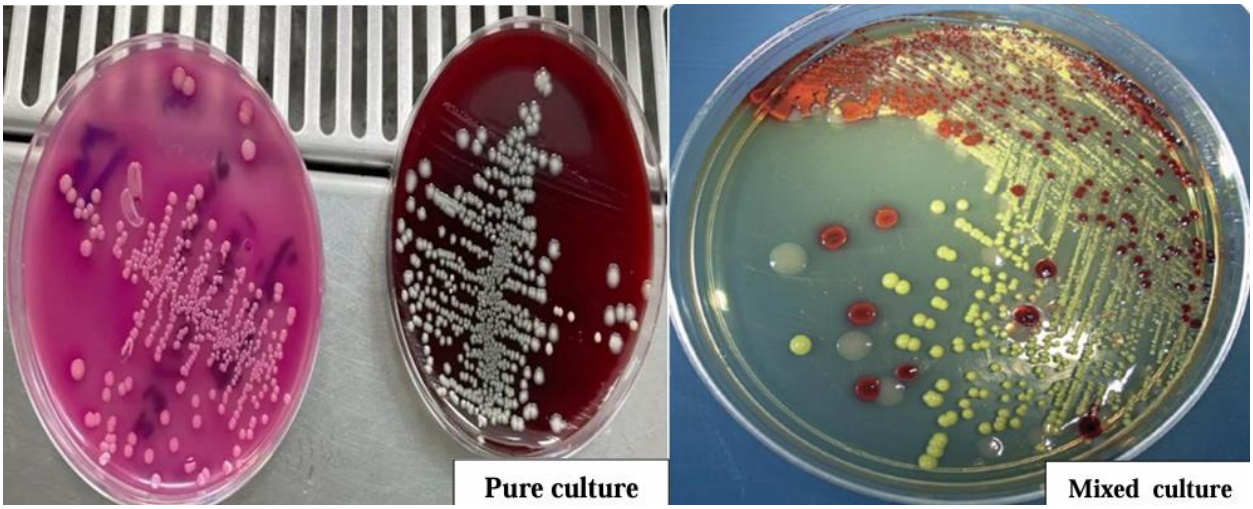
Lab5: Growing and cultivation of bacterial species in the lab (Pure culture technique):

- **Cultivation:** is the process of propagating organisms by providing the proper environmental conditions. Organisms require metabolic energy to synthesize macromolecules and to maintain essential chemical gradients across their membranes. Microorganisms vary widely in their nutritional demands and their source of metabolic energy. Factors that must be controlled during growth include the nutrients, PH, temperature, aeration, salt concentration, & ionic strength of the medium.
- **Microbial culturing:** is a method of multiplying microorganism by letting them reproduce in pre-determined culture media under controlled laboratory conditions.
- **Bacterial cultivation purposes:**

1. Isolation of a pure strain.
2. Culturing bacteria is the initial step for studying it's morphology and identification .
3. Testing the antibiotic sensitivity.
4. Genetic and molecular study (DNA extraction) .
5. Maintenance and storage stock culture.

♣ **Pure culture :** is a laboratory culture containing a single species of organism(all colonies on solid medium have a same color , size texture and colony edges) . A pure culture is usually derived from a mixed culture (one containing many species) by transferring a small sample into new, sterile growth medium.

♣ **A mixed culture :** contains two or more bacterial species or types of microorganisms coexisting in the same environment (all colonies on solid medium vary in color , size texture and colony edge , often used in microbiology to study their interactions and growth dynamics.



- **Culturing Bacteria:**

Culturing methods used for :

- Isolation of M.O. from sample.
- For culturing M.O. in the sample.
- Obtain pure culture.



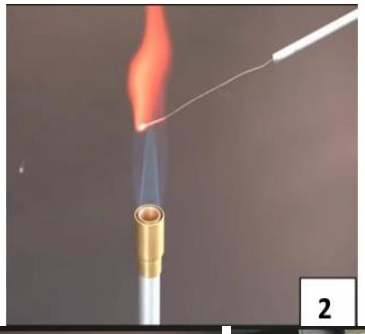
Culturing methods:

1. Pipetting by pipette (in broth medium).
2. Inoculation by loop (from solid into broth medium).
3. Streaking by loop(on solid medium).
4. Stabbing by needle (on solid or semi-solid medium).
5. Swabbing by swab (on solid medium).
6. Pouring plate method (on solid medium).
7. Spreading by spreader (on solid medium).

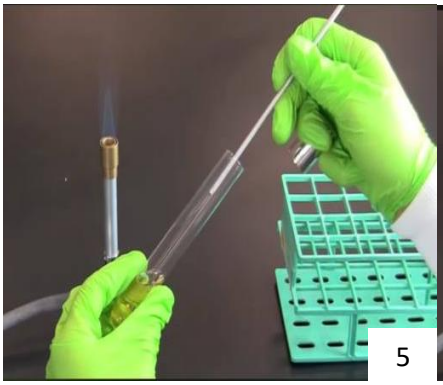
1- Pipetting: by pipette or micropipette This method was suitable in the dilution to extinction laboratory, the purpose from this method is to count the M.O, (viable plate count).

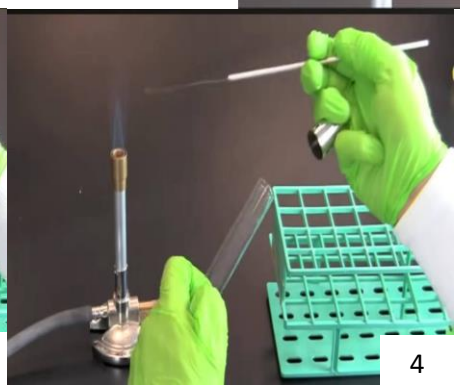


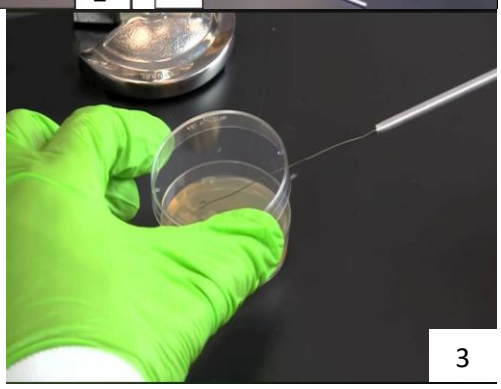
2- Inoculation : This method performed by using loop (from solid into broth medium) in order to increase the number of M.O. in liquid media(for activation of M.O ,for biochemical tests or preservation for long time


2


1


5

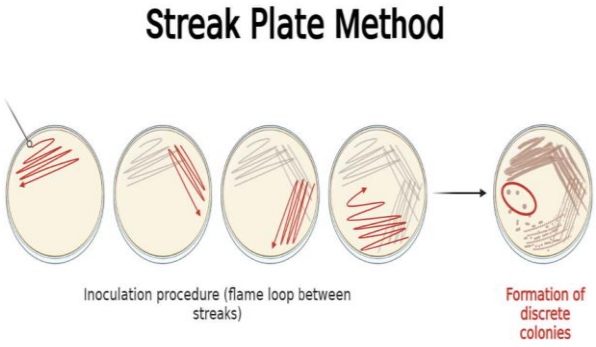

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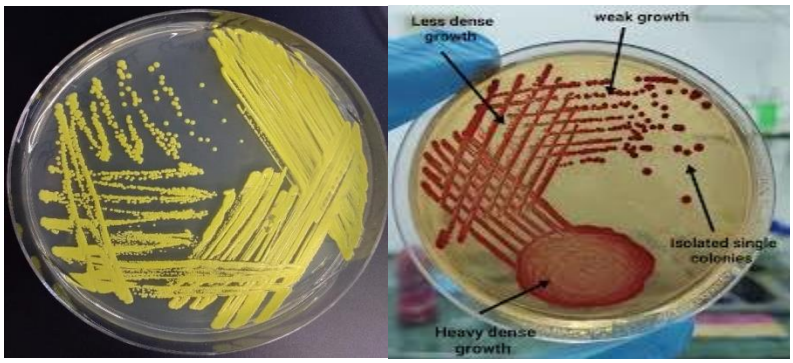

3

3-Streaking: by loop Streaking on agar plate in order to isolate bacteria , this will enable you to isolate pure cultures of bacteria by obtaining isolated pure colony.

A-Streaking on plates -ABCD method:

- Transfer by sterile loop colony from solid medium or drop from broth medium to solid medium in petri dish near the edge , spread it to become point A.
- Sterile the loop and cool it in the edge of solid medium.
- Rotate the dish , then touch the A area by sterile loop ,vertically draw several parallel lines to form in their end point B .
- Repeat step 2.
- Rotate the dish in the same direction, and touch the B point by sterile loop , vertically draw several parallel lines to form in their end point C .
- Repeat step 2. 7- Rotate the dish in the same direction, and touch the C point by sterile loop , vertically draw several parallel lines to form in their end point D .
- From D draw one zigzag line to the middle of dish.
- Repeat step 2. 10-Put petri dish in an incubator for 24hr at 37C°.

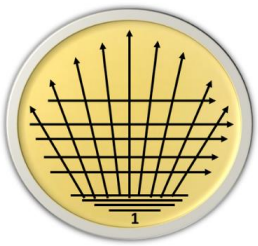




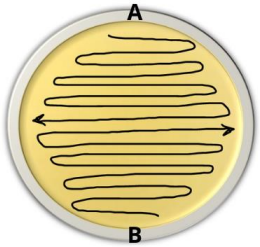
B- streaking Continuous:

1- Sterile the loop and cool it in the edge of solid medium.
2- Transfer the culture to the solid media
3- Spread the culture as continuous parallel lines to the end of the dish.
4- Sterile the loop.
5- Incubate at 37C˚ for 24 hr as inverted dishes

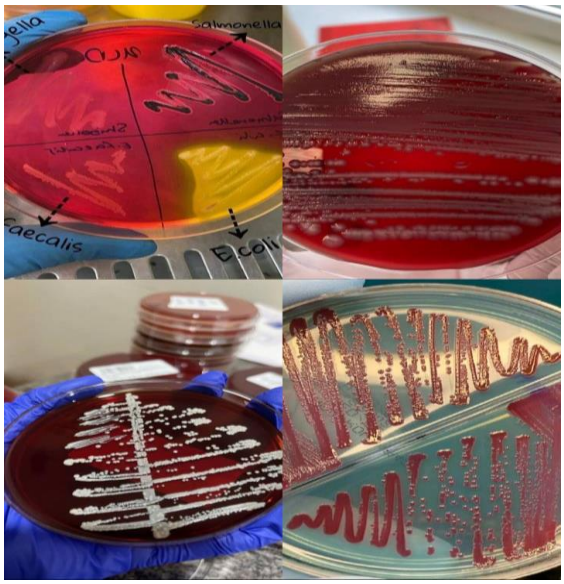

Zigzag Streak



Radiant Streak

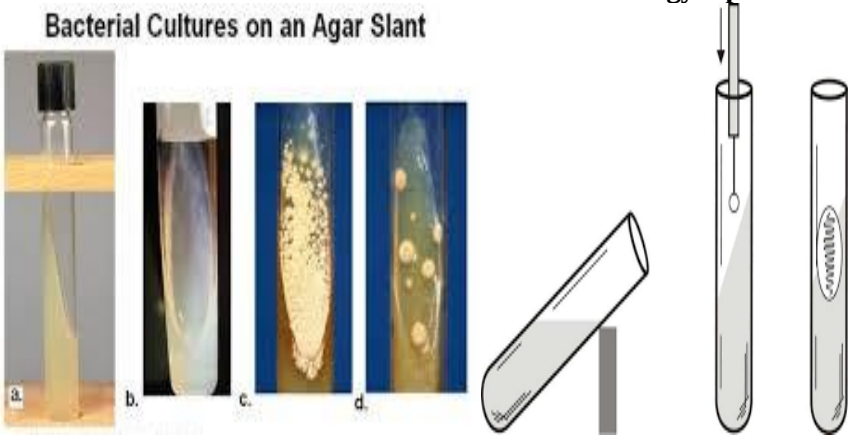


Continuous Streak



Types of continuous streaking plates

C-Streaking on slant : By sterile loop , take a colony from solid medium or drop from cultured broth medium, Remove the cover of slant tube and sterile the upper part, cultivate on the slant medium by streaking , then sterile the tube and close it. sterile the loop by flame and then put the slant in the incubator at 37C˚ for 24 hr. This technique used for keep microbial strains for few weeks to month .



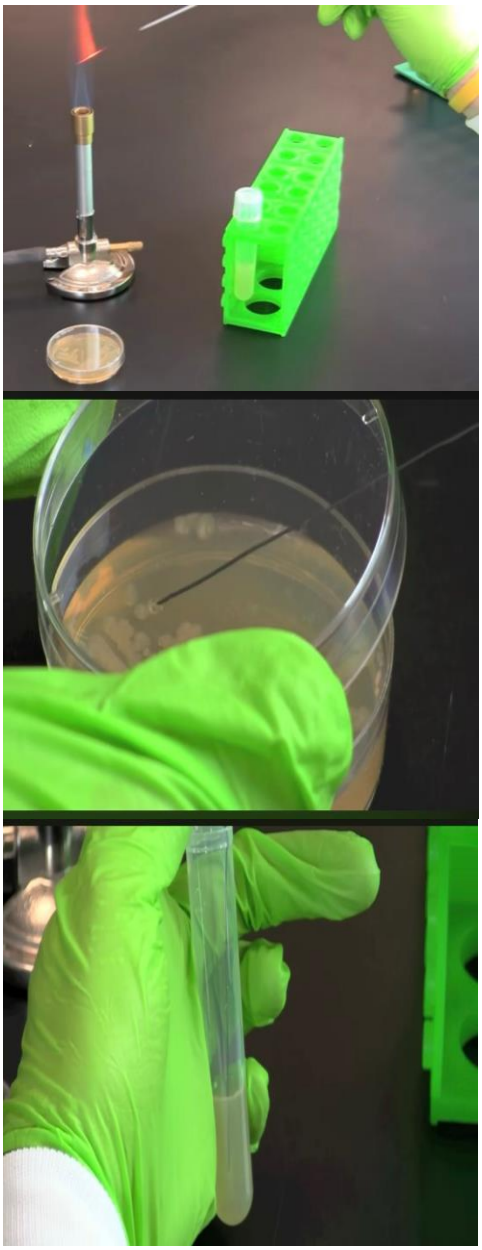
4-Stabbing : by using needle This method is used to study the ability of bacteria to:-

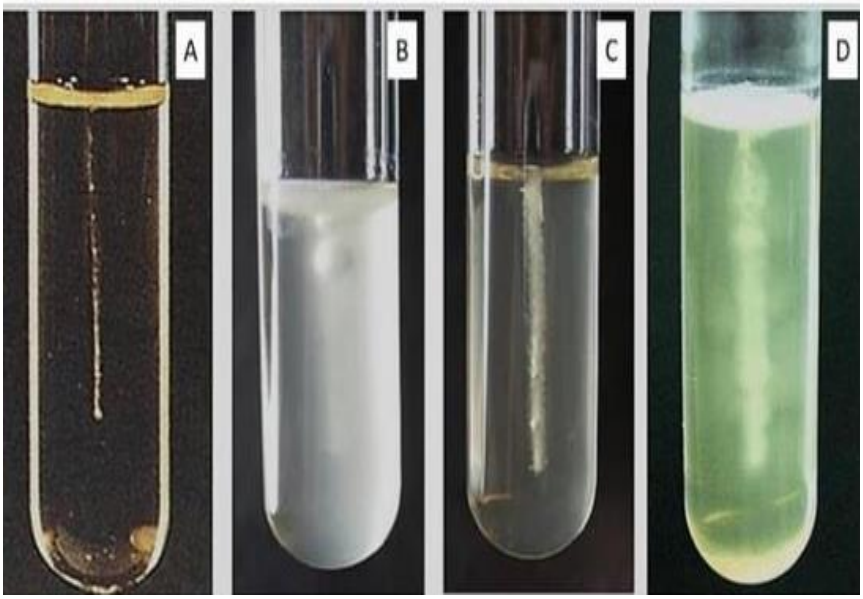
a. Grow with presence O2 or not.

b. -Motility.

c. Production of some chemical compounds during the metabolism. **Method:**

1-Sterile the loop by flame .
2-Hold one colony or part from bacterial growth from solid medium by needle.
3-Open the tube that contain sterile semisolid medium and sterile the upper of tube.
4-Stab the needle that contain bacteria in the medium vertically ,then hold it directly.
5-Sterile the upper of the tube again and close it.
6-Sterile the needle by flame.
7-Incubate the tube at 37C˚ for 24 hr.





Stabbing method





5-Swabbing : by swab Used to obtain heavy growth for some biochemical tests such as sensitivity test and for colony counting.

Method:

a. Submerge the sterile cotton swab in bacterial broth culture.

b. Swab on the sterile solid medium parallel lines over the medium.

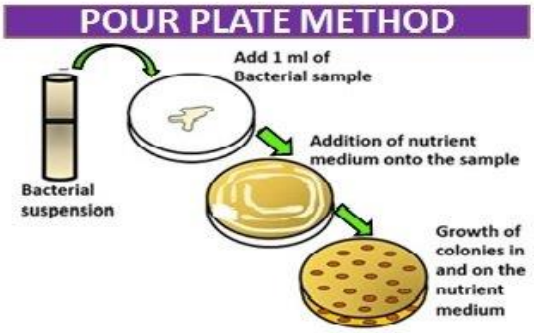
c. Rotate the plate and repeat step 2. d. Put the swab on alcohol (used for one time).

e. Incubate the tube at 37C˚ for 24 hr .

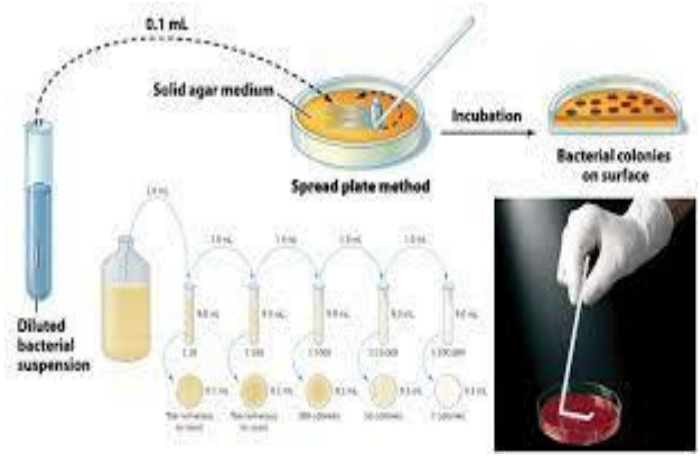
6-Pouring plate

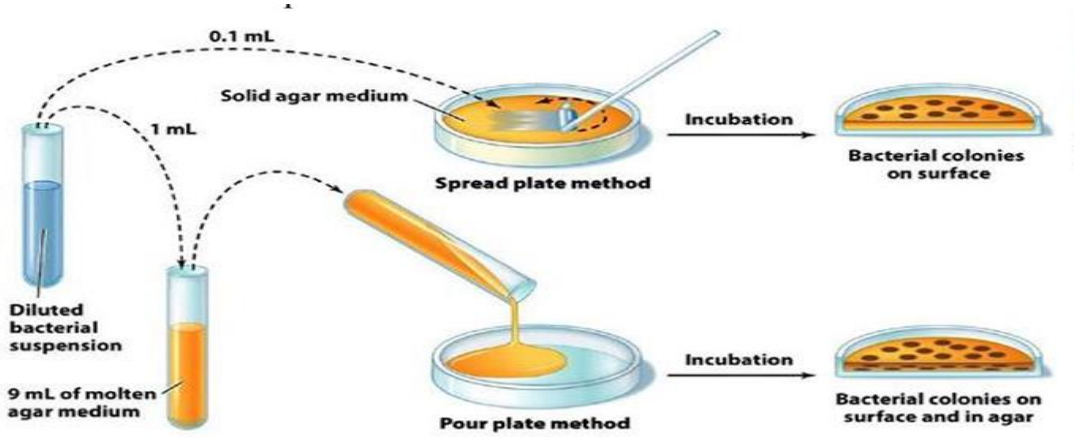
Method:

This method is used for counting the colonies . Method: Prepare serial dilutions from 10-1 to 10-5 of sample , Transfer by sterile pipette 0.1 ml of each dilution to sterile petri-plate . Prepare sterile solid culture medium and cooled it to 45-50 C˚ in water bath. Aseptically pour agar into each petri-plate , swirl the plate to mix the sample with the agar, Allow the agar to cool and solidify, then Incubate the inverted plate at 37C˚ for 24 hr.



7-Spreading : by spreader Used to spread the bacteria on the surface of the solid medium for colony counting .Sterilize the glass spreader by putting it in alcohol and burn it by burner , after the cooling of spreader , spread the sample over the surface of the plate then Incubate inverted plates at 37C˚ for 24 hr





Lab 6: Microbial staining techniques :

- **Staining,** in microbiology refers to technique which is used to enhance and contrast a biological specimen at the microscopic level. Stains and dyes are used to highlight the specimen at the microscopic level to study it at higher magnification for histopathological studies and diagnostic purposes.

- **Why do we have to stain microorganisms ?**

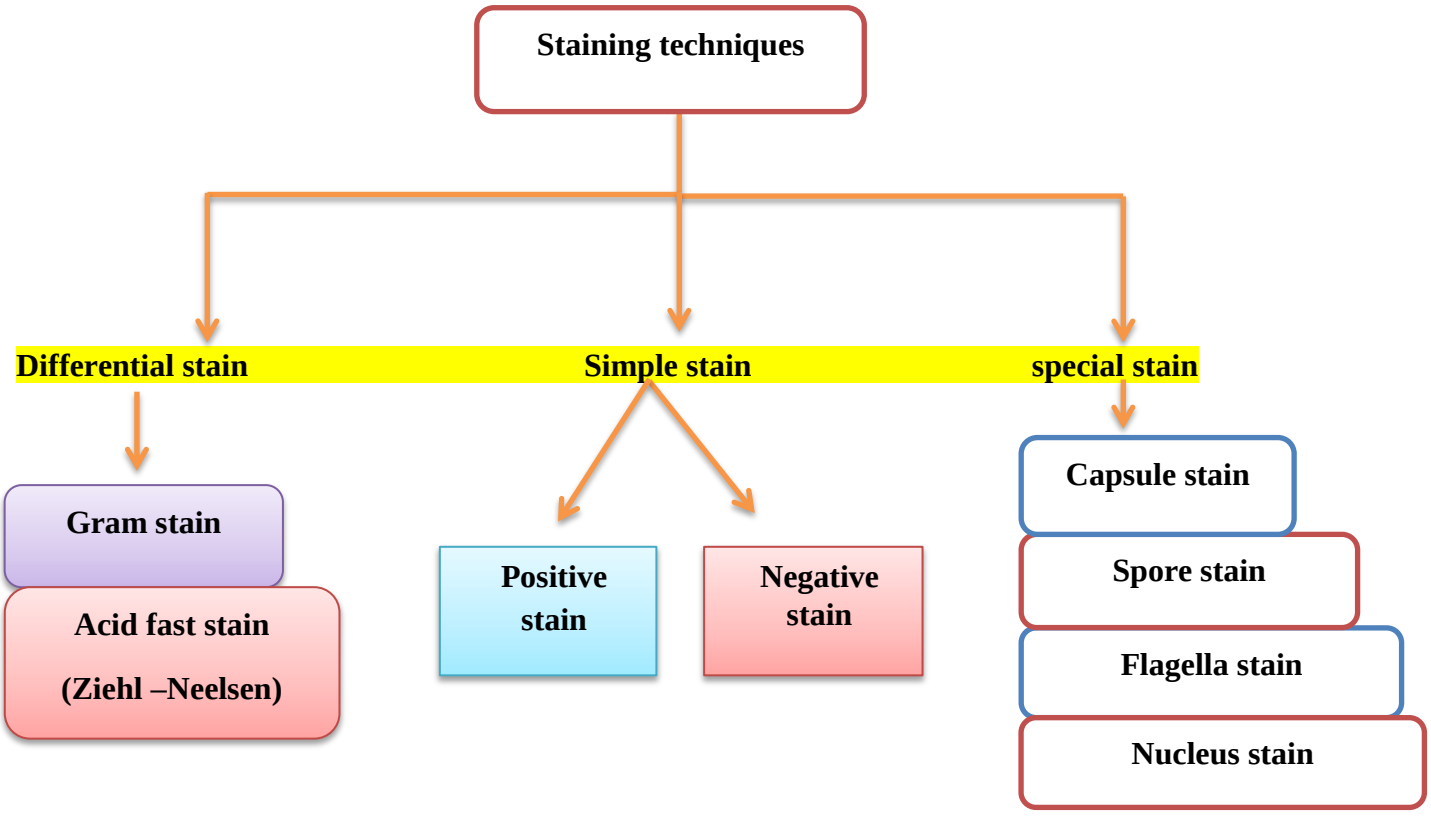
Most types of cells do not have much natural pigment and are therefore difficult to see under the light microscope unless they are stained. Several types of stains are used to:

- To study their shape, Size and aggregations
- To differentiate the bacterial species by using differential stain.
- To study the internal components of the bacterial cell.

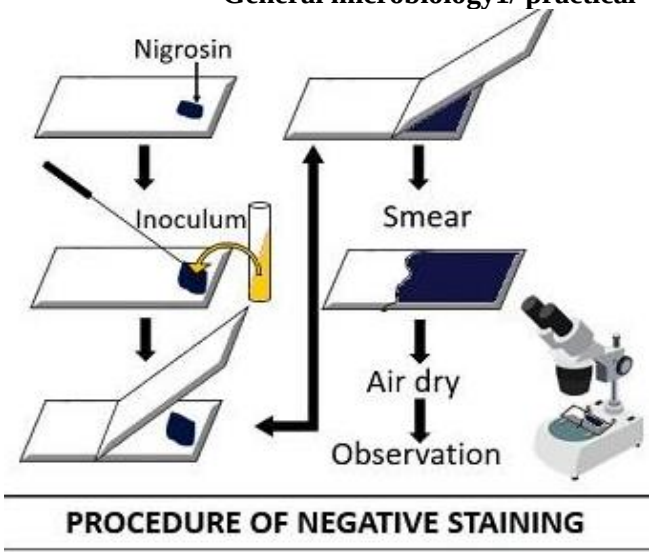
The stains divide into 2 groups (according to their affinity to cell components):

A. Indirect stain (Negative stain): It is can penetrate the cell envelope therefore the cell become very obvious by making counter dark (stain the slide but not the cell):India ink, Congo red.
B. Direct stain(Positive stain): It has strong affinity to one or more of cell components

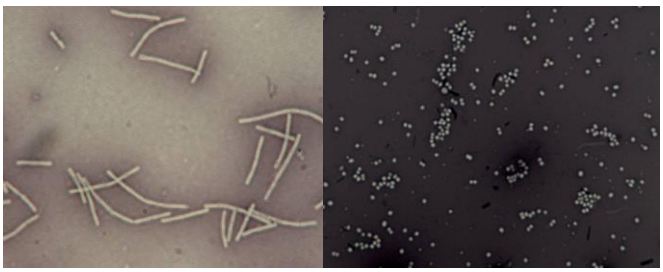
1. **Simple stain: Acidic (Safranine) , Basic (Methylene blue, Crystal violet)**
2. **Differential stain: Gram stain , Acid fast stain(Ziehl Neelson stain)**
3. **Selective stain: Spore stain , Capsule stain , Flagella stain , Cell wall stain , Nucleic acid stain.**



- **Indirect stain(Negative stain):** The main purpose of Negative staining is to study the morphological shape, size and arrangement of the bacteria cells that is difficult to stain. Negative staining requires an acidic dye such as India Ink or Nigrosin.
- **India Ink or Nigrosin :** is an acidic stain. Since the surface of most bacterial cells is negatively charged, the cell surface repels the stain. The glass of the slide will stain, but the bacterial cells will not. The bacteria will show up as clear spots against a dark background.
- **Procedure:** Mix one drop of India ink with culture by sterile loop on slide Used another slide for dispensing the mixture Air dry the smear(do not use heat fixation).



PROCEDURE OF NEGATIVE STAINING



- **The Simple Stain:** One type of staining procedure that can be used, in which only one stain is used, and all types of bacteria appear as the color of that stain when viewed under the microscope, there are two groups of simple stain

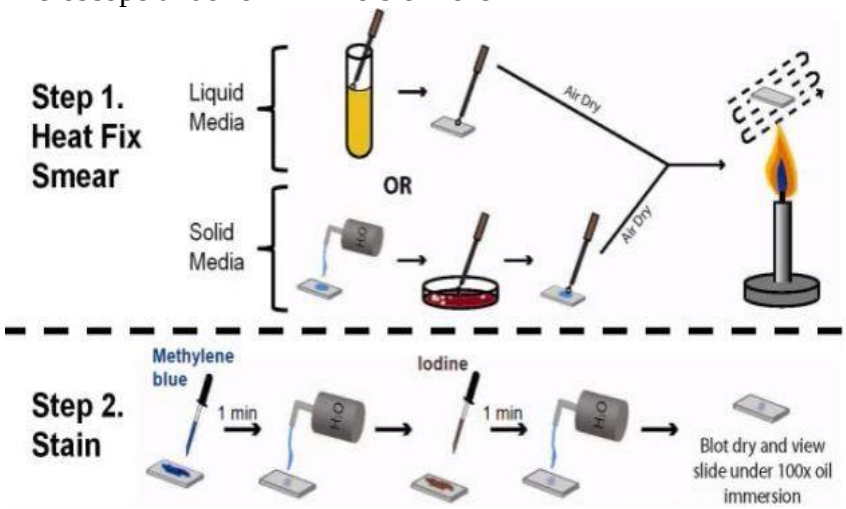
1-Acidic stain: has negative charge it is stain the basic compounds (protein)of the cell with a positive charge(safranine , acid fuchsin).

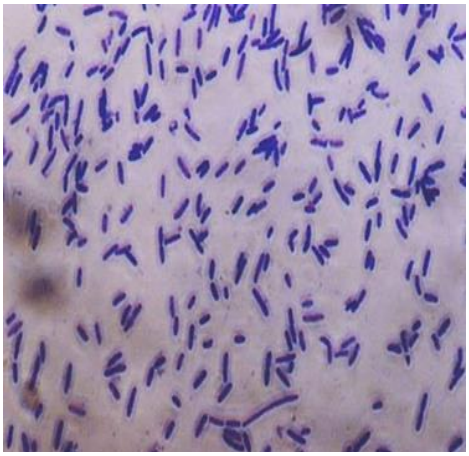
2-Basic or Alkaline stain: has positive charge it is stain the acidic compounds(RNA,DNA) of the cell with a negative charge (Methylene blue , Crystal violet) Simple stains can be used to determine a bacterial species' morphology and arrangement, but they do not give any additional information. Living bacteria are almost colorless, and do not present sufficient contrast with the water in which they are suspended to be clearly visible. The purpose of staining is to increase the contrast between the organisms and the background so that they are more readily seen in the light microscope.

Steps of work in general 1-Smear preparation 2-Fixation 3-Staining with stain

1-Smear preparation From broth: one loop ful directly from broth on the slide. From solid culture: put one drop of water in the center of the slide the take one touch from colony and mix well with the drop. Let smear until drying . 2-The fixation will done by Passing the slide rapidly across the flame three times the slide should be warm not hot , why? hot will change the shape of bacterial cell.

♣ Fixation will:- 1-Kill the bacteria so the stain will penetrate without serious destruction of cell structure. 2-Fix the cell on the slid (by coagulation of their protein) So they are not removed during staining. 3-Staining with dye: Put the slide on the staining rack and flood the smear with simple stain (Crystal violet or Safranin) for 1 min. Wash the slide with tap water gently and drain off excess water Air dry Examine with microscope under oil –immersion lens.





Lab7: Deferential staining / A. Gram stain

- **Gram staining:** is a differential bacterial staining technique used to differentiate bacteria into Gram Positive and Gram Negative types according to their cell wall composition.

It is the most widely used and the most important staining technique in bacteriology, especially in medical bacteriology. It is generally the first test performed on bacteria during their identification and observation process.

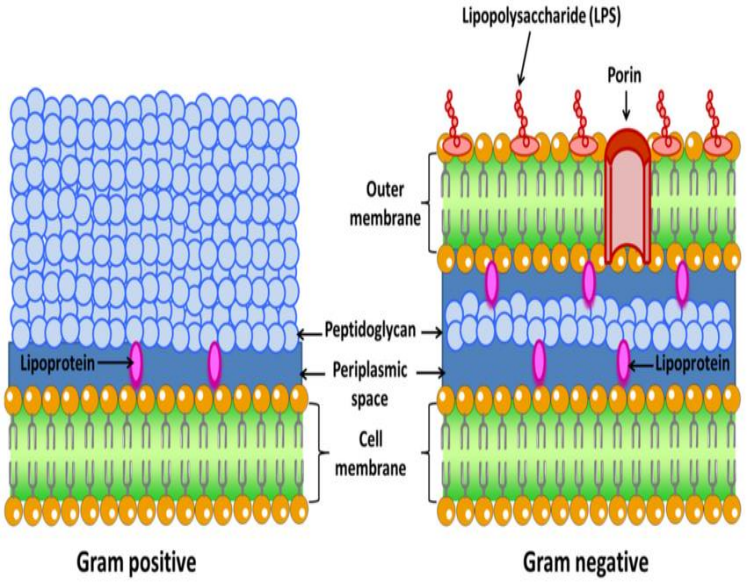
- The Gram stain was first used in 1884 by Hans Christian Gram (Gram,1884).
- We now know that those organisms that stained blue/violet with Gram's stain are gram positive bacteria like ***Staphylococcus , streptococcus*** , while those that were decolorized are gram negative bacteria such as the ***Salmonella Typhi*** that is associated with Typhoid fever.

- **Purpose:**

The Gram stain is fundamental to the phenotypic characterization of bacteria. The staining procedure differentiates organisms of the domain Bacteria according to cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. Gram-negative cells have a thin peptidoglycan layer and stain red to pink, also we can observed shapes and aggregation of bacterial cells by gram staining technique.

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Department of Biotechnology

- **Theory:** The Gram stain, the most widely used staining procedure in bacteriology, is a complex and differential staining procedure. Through a series of staining and decolorization steps, organisms in the Domain Bacteria are differentiated according to cell wall composition. Gram-positive bacteria have cell walls that contain thick layers of peptidoglycan (90% of cell wall). These stain purple. Gram-negative bacteria have walls with thin layers of peptidoglycan (10% of wall), and high lipid content. These stain pink. This staining procedure is not used for Archeae or Eukaryotes as both lack peptidoglycan.



Gram stain solution:

- **crystal** violet : primary stain
- Iodine
- Alcohol
- Safranin : final or counter stain



► **Gram staining procedure:** A. Make a smear from bacterial culture (culture must be young overnight incubation 18_ 24hr)

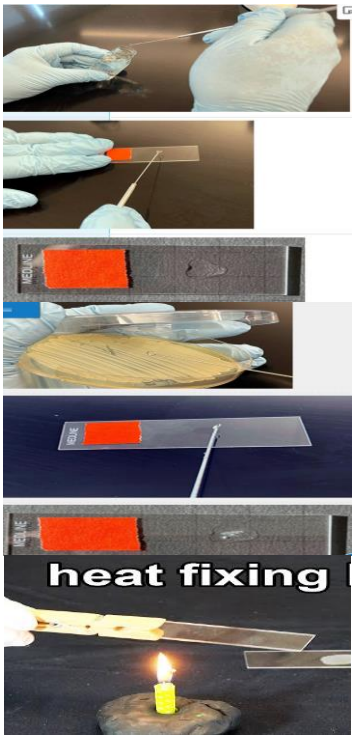
♣ If culture was on solid media must use water drop to dilute bacterial colonies in order to get thin smear .

1. first place a small piece of tape at one end of the slide and label it with the name of the bacterium you will be placing on that slide.

2. Sterilize your inoculating loop and place one loop full of deionized water from your dropper bottle on your slide.

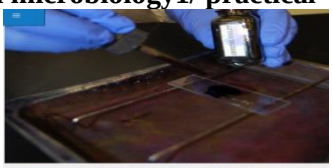
3. Using the edge of your sterile inoculating loop, aseptically remove a small amount of the culture from the agar surface and gently touch it several times to the drop of water until the water becomes visibly cloudy.

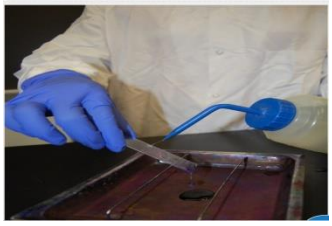
4. After the inoculating loop cools, spread the suspension over the slide to form a thin film.



5. spread the Mixture over the Slide. Using the loop, spread the mixture over the entire slide to form a thin film , Allow this thin suspension to completely air dry (The smear must be completely dry before the slide is fixed).

6. Pick up the air-dried slide with provided close pin and hold the bottom of the slide opposite the smear, then pass the slide quickly on the flame





B. staining procedure:

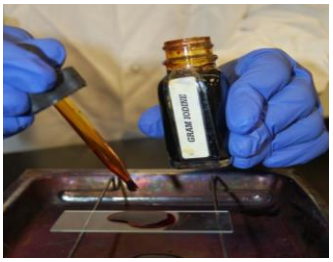
1. flood air-dried, heat-fixed smear of cells for 1 minute with crystal violet staining reagent, please note that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.

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

3. Flood slide with mordant: Grams' iodine. Wait 1 minute.

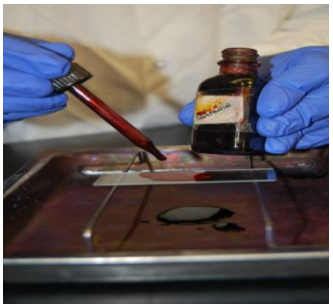
4. Wash slide in a gentle and indirect stream of tap water for 2 seconds.

5. Flood slide with decolorizing agent (Acetone-alcohol decolorizer). Wait 10-15 seconds or add drop by drop slide until decolorizing agent running from the slide runs clear.



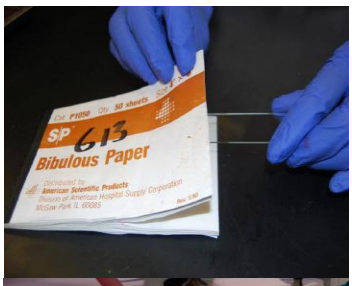
6. Flood slide with counterstain, Safranin. Wait 30 seconds to 1 minute.

7. Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.

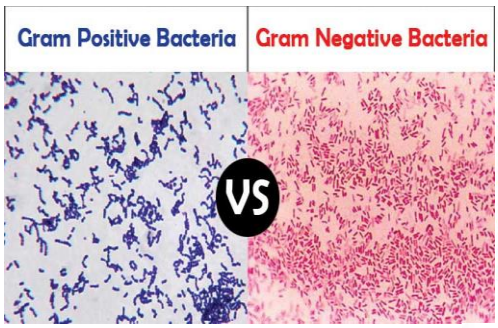


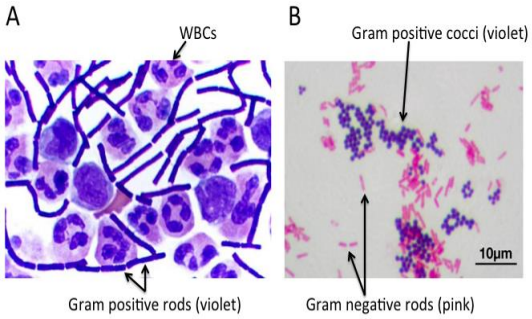
8. Observe the results of the staining procedure under microscope.

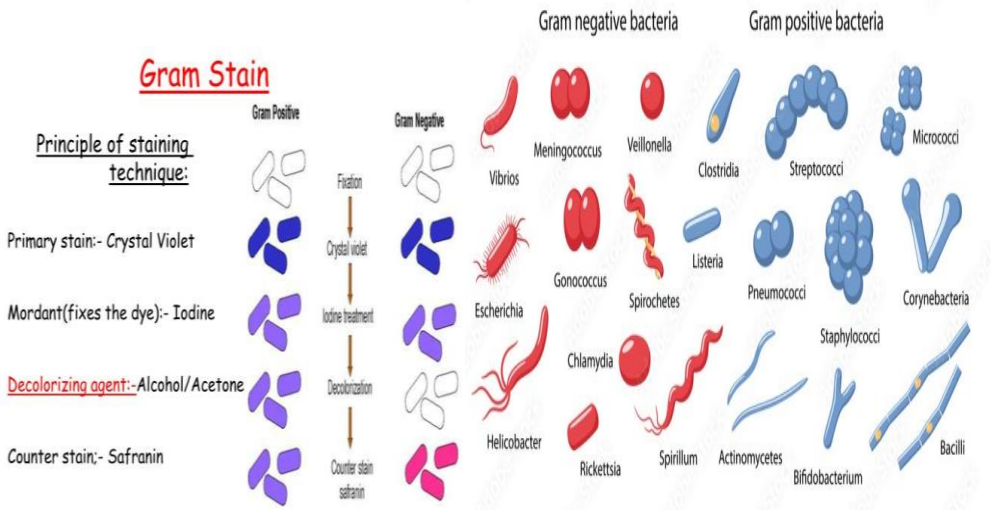
9. Gram-negative bacteria will stain pink/red and Gram-positive bacteria will stain blue/purple.











LAB 8: Defferintial stain / B.Acid fast stain (Ziehl–Neelsen) :

- **Ziehl–Neelsen stain**, also known as the acid-fast stain, It is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria. Mycobacterium tuberculosis (tuberculosis) (TB). Other important Mycobacterium species involved in human disease are Mycobacterium leprae, (leprosy). Acid fast organisms like Mycobacterium contain large amounts of lipid substances within their cell walls called mycolic acids. These acids resist staining by ordinary methods such as a Gram stain. it can also be used to stain a few other bacteria, such as Nocardia.
- **Principle of Acid-Fast Stain:** The presence of higher alcohol, glycerol, fatty acid, and especially mycolic acid in the cell wall has been found responsible for keeping the acid-fast property of bacteria. Therefore, AFB stain is useful for Mycobacterium tuberculosis, an etiological agent of tuberculosis.
- **Acid fast Reagents:**

-Carbol fuchsin - 3% Acid alcohol or 20% Sulphuric acid
- Methylene blue .

Specimens: • Sputum •Bronchial or laryngeal washing
• Gastric lavage when sputum is swallowed as in children.

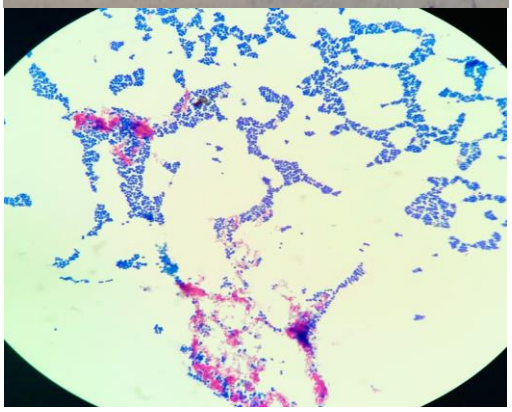
- **Staining procedure :**

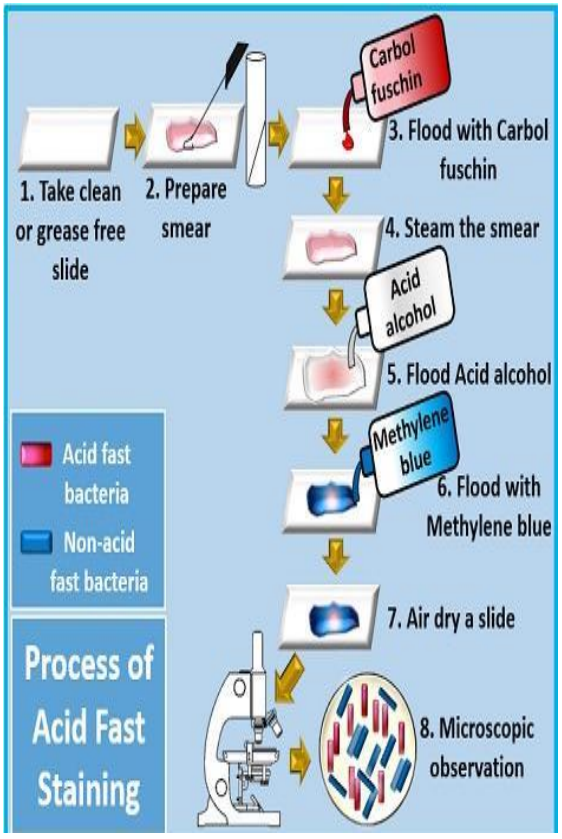
1.Make smear on a clean glass slide. 2. Dry and fix the smear. 3. Cover the smear with a strong carbol fuchsin solution. 4. The heat from underneath the slide until just steam comes from the stain. Do not boil. 5. Wait for five minutes. 6. Rinse with water. 7. Decolorize by 20% sulphuric acid or 3% acid alcohol until the smear becomes pale pink in color. (wait for nearly five minutes) 8. Rinse with water. 9. Counterstain with methylene blue for one minute. 10. Rinse with water. 11. Drain and dry. 12. Observe the smear first under the low power (10X) objective, and then under the oil immersion (100X) objective.

- **Result and Interpretation of Acid-Fast Stain**

AFB: pink or red bacillus Background:
Blue (as counterstain used) .







Lab 9: Special stains (structural stains)

capsule stain (Negative staining)

Capsule, an outer layer of polysaccharides that covers the cells of many different bacterial species. Capsules act as a sort of magic cloak, protecting bacteria from toxic compounds and desiccation and allowing them to adhere to surfaces and to escape the immune system of the host.

Principle of Capsule Staining: The main purpose of capsule stain is to distinguish capsular material from the bacterial cell. Negative staining methods contrast a translucent, darker colored, background with stained cells but an unstained capsule. The background is formed with india ink or nigrosin or congo red. India ink is difficult to obtain nowadays; however, nigrosin is easily acquired. A positive capsule stain requires a mordant that precipitates the capsule. By counterstaining with dyes like crystal violet or methylene blue, bacterial cell wall takes up the dye. Capsules appear colorless with stained cells against dark background. Capsules are fragile and can be diminished, desiccated, distorted, or destroyed by heating. A drop of serum can be used during smearing to enhance the size of the capsule and make it more easily observed with a typical compound light microscope. A drop of serum can be used during smearing to enhance the size of the capsule and make it more easily observed with a typical compound light microscope.



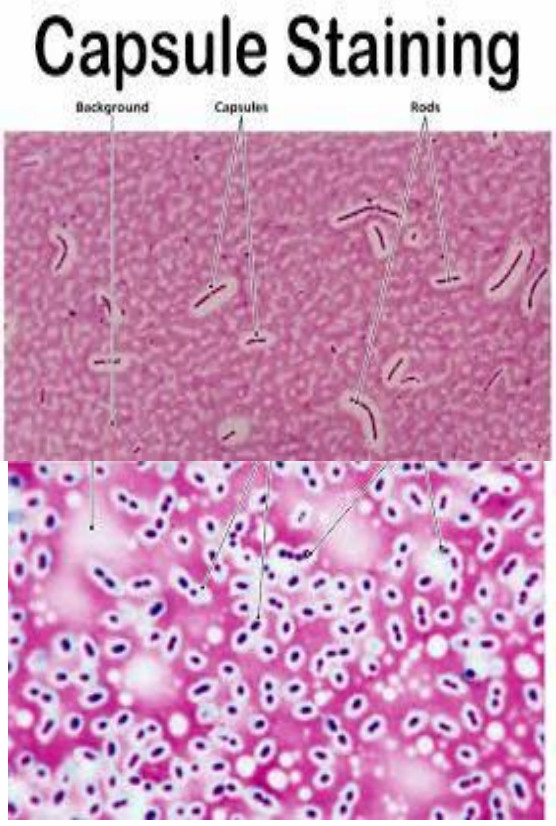
► Procedure of Capsule Staining

1. Place a small drop of a negative stain (India Ink, Congo Red, Nigrosin) on the slide.
2. Using sterile technique, add a loop-full of bacterial culture to slide, smearing it in the dye.

3. Use the other slide to drag the ink-cell mixture into a thin film along the first slide and let stand for 5-7 min.

4.Allow to air dry (do not heat fix).

5. Flood the smear with crystal violet stain (this will stain the cells but not the capsules) for about 1 min. Drain the crystal violet by tilting the slide at 45-degree angle and let stain run off until it air dries.
6. Examine the smear microscopically (100X) for the presence, add a loop-full of bacterial culture to slide, smearing it in the dye. 3. Use the other slide to drag the ink-cell mixture into a thin film along of encapsulated cells as indicated by clear zone surrounding the cells.



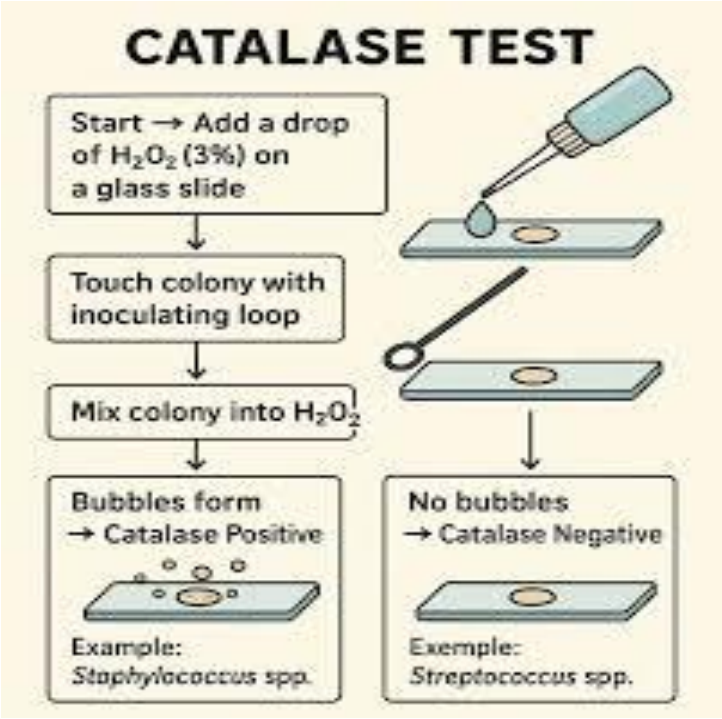
Lab10: Biochemical tests for bacterial identification:

Biochemical tests : are tests that identify the bacteria on the basis of the presence of certain enzymes and differences in the biochemical activities of different bacteria . They are among the most important methods for microbial identification ,and regards basic step in the identification of bacterial isolates and in the identification of different bacterial species.

Catalase test : Some bacteria contain flavoproteins that reduce O_2 resulting in the production of hydrogen peroxide (H_2O_2) or superoxide (O_2). These are toxic for obligate aerobes and facultative anaerobes. Those bacteria produce enzymes to protect them self against superoxide (O_2), these enzymes are catalase, peroxidase or superoxide dismutase , which catalyze the destruction of H_2O_2 or O2 as follows:

$$2O_2 + 2H \xrightarrow[\text{dismutase}]{\text{Super oxide}} O_2 + H_2O_2$$

$$2H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{Catalase or}} 2H_2O + O_2$$



Procedure :

Substrate of test :hydrogen peroxide (H_2O_2).

1.Slide Method:- -Pure growth of the organisms will transfer to the clean slide by using inoculation loop or glass rod.

-Immediately add a drop of **3%** hydrogen peroxide on bacterial culture.

- Observe the bubble formation.

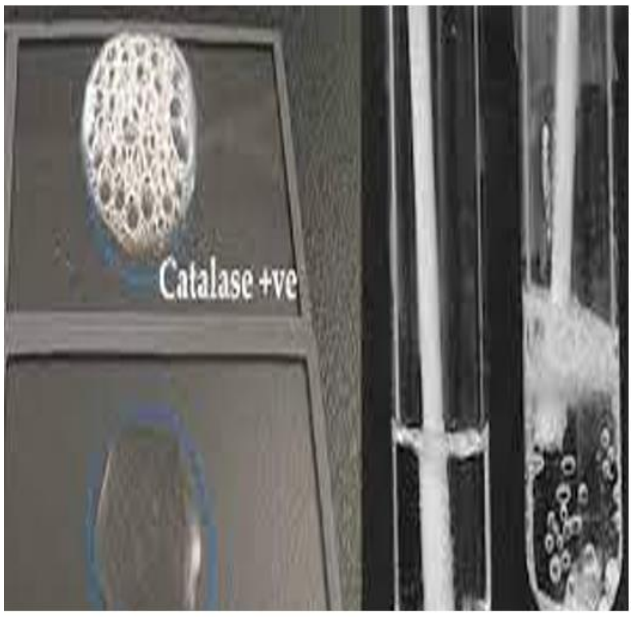
-Positive results : Bubbles formation

-Negative results : no bubbles appear

2. Tube Test:-

-Take one ml of **3 %** hydrogen peroxide in test tube.

- Small amount of bacterial culture introduce into the solution- Immediately observe the effervescence .



Oxidase test: Oxidase test is a key test to differentiate between the families of ***Pseudomonadaceae*** (ox+) and ***Enterobacteriaceae*** (ox -), and is useful for speciation and identification of many other bacteria. Test substrate: (Kovacs' oxidase reagent) tetramethyl-p phenylenediamine dihydrochloride (or dimethyl can be used).

♣ **Note** :

-We keep the oxidase reagent either frozen or unopened in dropper bottles until needed

-Use a young culture, preferably less than 24 hrs old. -Use a culture growing on an agar plate or agar slant.

-Use Fresh reagent, less than a couple of hours old.

-Pick your inoculum, not with a metal loop (reagent may react with the metal), but with a wooden stick.

-Read the reaction within 20 seconds (NOT after), usually it will change in less than 15 seconds. The oxygen will change the reagent color as time passes, so it must be read quickly.

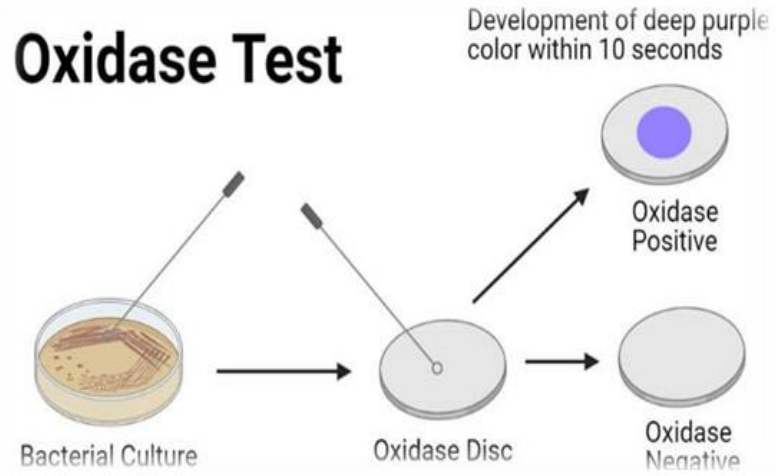
Procedure (Filter Paper Method):

1. In a sterile petri plate, place a strip/disc of Whatman no. 1 filter paper. Soak the filter paper with 1% Kovacs' oxidase reagent and let it dry.

2. Using a sterile inoculating loop, pick up a well-isolated colony of test bacteria from a fresh (18 to 24 hours old) culture and make a smear on the reagent-soaked filter paper piece.

3. Observe for color change.

4. TIME the reaction: a positive reaction will occur within 20 seconds. Do not read the reaction after 30 seconds.



Result:

- **Positive Test**

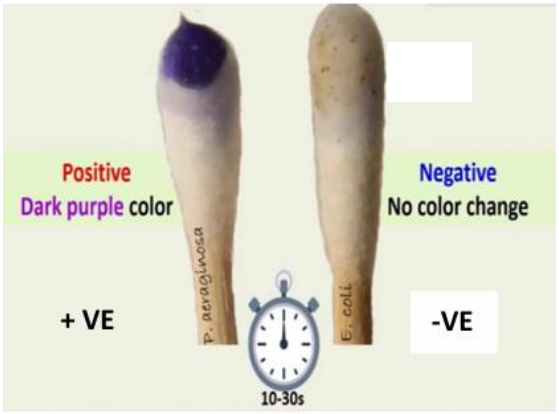
1. Development of purple to deep blue color within 10 to 30 seconds indicates a positive oxidase test.

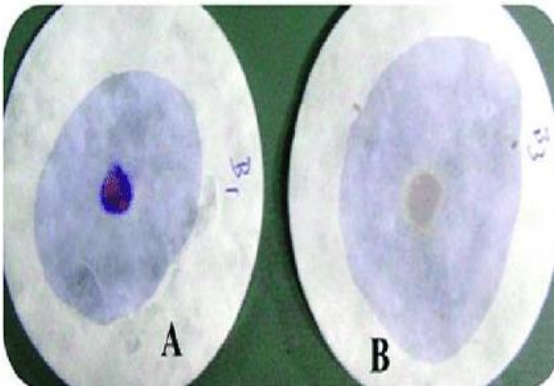
2. Development of purple to deep blue color within 30 to 60 seconds indicates a weak oxidase positive reaction or delayed oxidase positive.

-**Negative Test**

1. No development of purple to deep blue color within30 seconds.

2. Development of purple to deep blue color after 60 seconds.

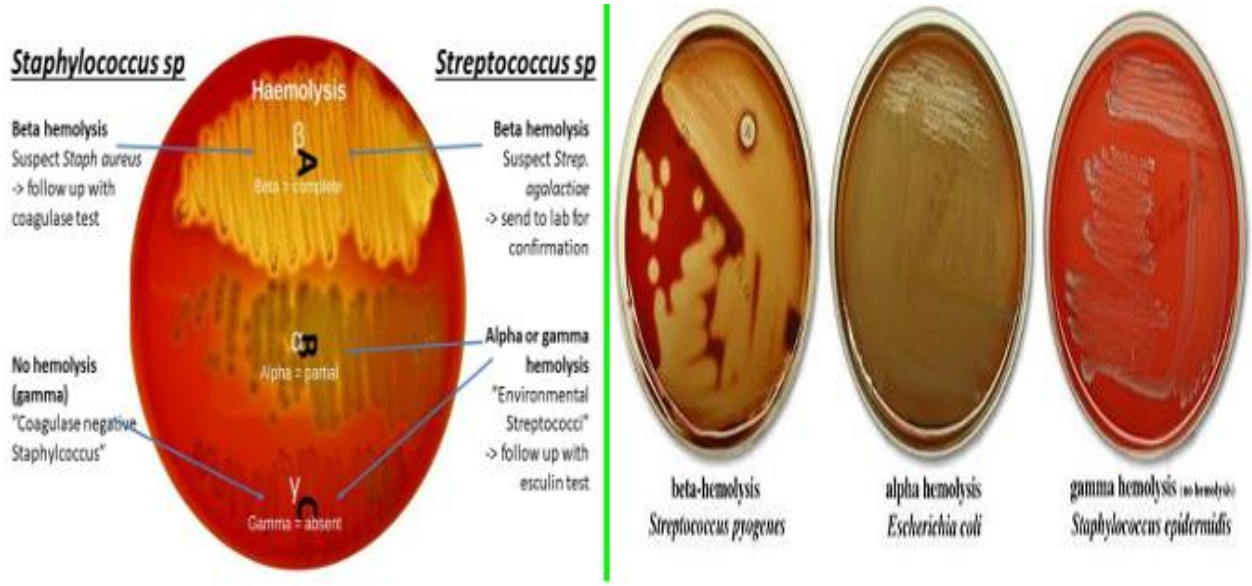




- **Hemolysis test :** This test provides information on what hemolytic enzymes a bacterium possesses. By providing a culture medium enriched with red blood cells, it is possible to determine whether a bacterium can destroy the cells and whether it can digest the hemoglobin inside. This test provides information on what hemolytic enzymes a bacterium possesses. By providing a culture medium enriched with red blood cells, it is possible to determine whether a bacterium can destroy the cells and whether it can digest the hemoglobin inside.
- **Substrate:** None
- **Medium : blood agar** (A nutrient medium augmented with the addition of 5% sheep blood routinely is used.
- **Procedure : -** Select the called blood agar plate medium.

- Using an inoculating loop, complete the process of a tube-to-plate aseptic transfer to inoculate the medium. -Incubation of the Inoculated Medium -Place the inoculated tube into the 35-37 C incubator.
-Press the New Day button to move forward 24 hours.
-Determination of Test Results -Incubate this test for only 24 hours.
- Retrieve the incubated culture from the incubator.
-Observe the medium surrounding colonies in the plate. The culture showing a darkening or discoloration of the medium in the vicinity of growth demonstrates alpha-hemolysis. Cultures showing clear halos around colonies and under growth is exhibiting beta-hemolysis.

- **Record test result.**
  - Un inoculated Blood Agar Plate
  - Positive for Alpha Hemolysis
  - Positive for Beta Hemolysis
  - Positive for Gamma Hemolysis.



TSI test (Triple Sugar Iron test) : (TSI) test is a biochemical test used to differentiate bacteria based on their ability to ferment these three sugars and release acid and hydrogen sulfide gas.

TSIA medium: is a selective and differential medium to differentiate bacteria, especially of the enterobacteraceae family.

Importance of TSI test:

- To differentiate Gram-negative enteric bacilli.
- To test the bacteria's ability to utilize glucose, lactose, and/or sucrose and produce H_2S gas.
- To differentiate lactose fermenters from non-lactose fermenters.

Procedure of TSI test : Media preparing according to manufacturing company and poured in test tubes in slant form , then :

1. Touch a well-isolated colony from a fresh culture of the test bacterium that is 18 to 24 hours old using a sterile inoculating wire.

2. Stab the butt up to 3 to 5 mm above the base of the test tube using the inoculating wire and while withdrawing, streak the slant.

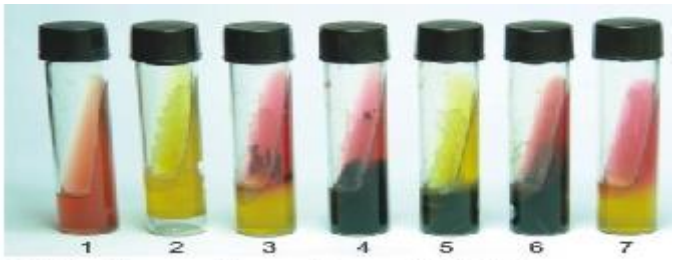
3. Incubate the tube aerobically (with a loose cap) at 35±22°C for about 24 hours. 4. Examine for color change of the slant and butt and report the color within 24 hours of incubation.

(If you want to read the H_2S production, incubate it for another 24 to 48 hours, but read sugar fermentation and color change within the first 24 hours of inoculation and incubation).

►Results of TSI test :

1. The red slant and yellow butt (Red/Yellow or Alkaline (K)/Acidic (A) indicate only glucose is fermented.
2. Yellow slant and yellow butt (Yellow/Yellow or Acidic (A)/Acidic (A) indicate lactose and/or sucrose fermentation or fermentation of all three sugars.
3. Red slant and red butt (Red/Red or Alkaline (K)/Alkaline (K) indicate none of the three sugars are fermented.
4. Blackening of the media or formation of the black-colored spots indicates H_2S production.
5. Cracking of the media, gas bubbles of the media, or forming a gap in the media indicates gas production.



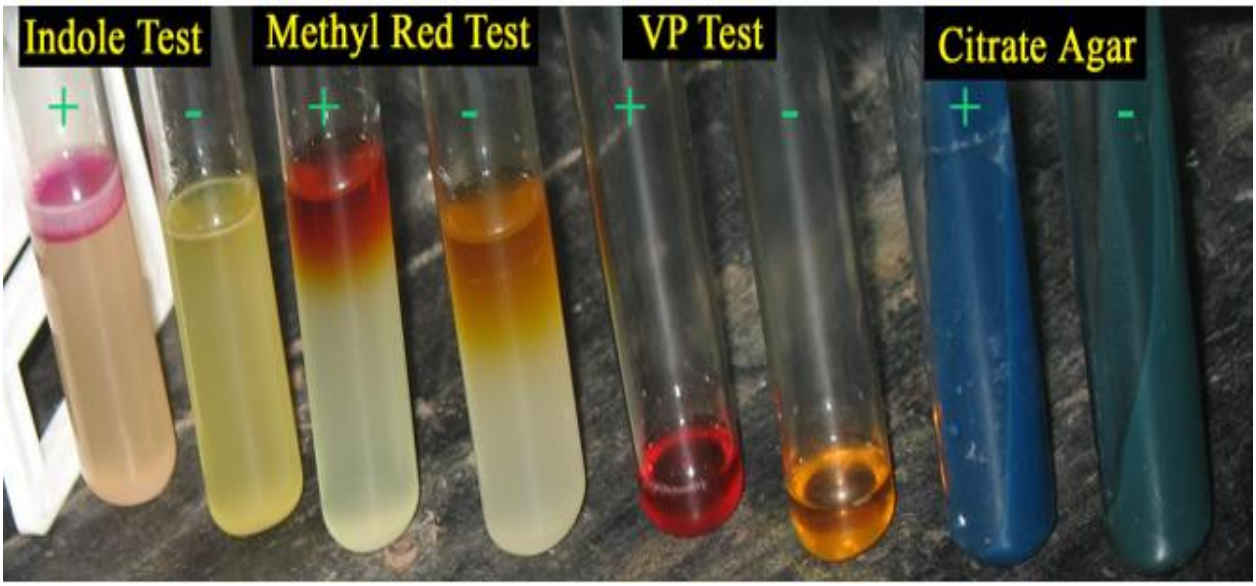


Triple Sugar Iron Agar (M021)
1. Control
2. Escherichia coli ATCC 25922
3. Salmonella Typhi ATCC 6539
4. Proteus vulgaris ATCC 13315
5. Citrobacter freundii ATCC 8090
6. Salmonella Typhimurium ATCC 1402

IMViC test: is a series of four different biochemical tests used in identifying and differentiating bacteria, especially the members of ***Enterobacteriaceae.***

►Each of the letters in "IMViC" stands for one of these tests. "I" is for Indole; "M" is for Methyl red; "V" is for Voges-Proskauer, and "C" is for Citrate, lowercase "i" is added for ease of pronunciation. IMViC tests are employed in the identification - differentiation of members of family ***Enterobacteriaceae.***

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

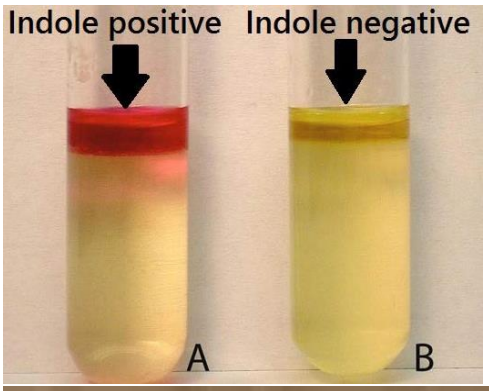


A. Indole test :It is performed on sulfide-indole-motility (SIM) medium or in tryptophan broth.

-Result is read after adding **Kovac's reagent.**

1. **Positive result :** indicated by the red layer at the top of the tube after the addition of Kovac's reagent.

2. Negative result : indicated by the lack of color change at the top of the tube after the addition of Kovac's reagent.

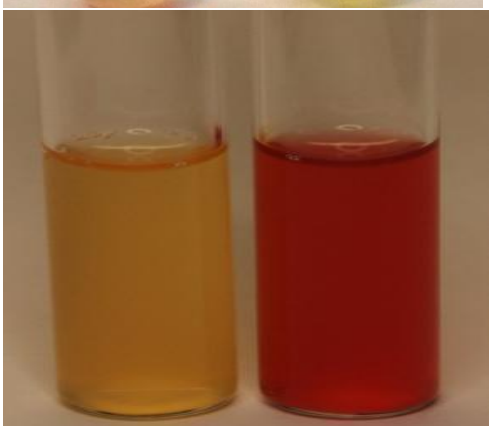


B: Methyl red test and Voges-Proskauer test both are done in methyl red – -Voges-Proskauer (MR-VP) broth, but the reagents that we added differs

*** Methyl Red (MR) test:**

● **Positive methyl red** test are indicated by the development of the red color after the addition of methyl red reagent.

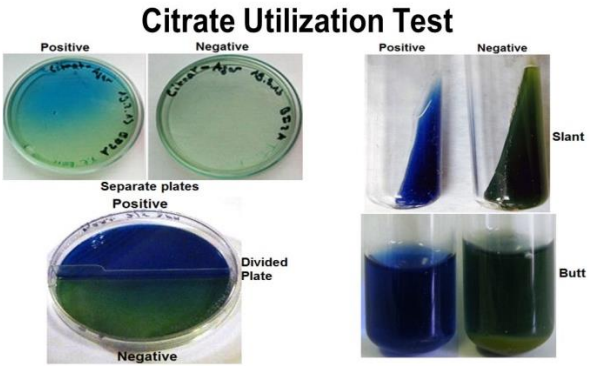
● A negative methyl red test is indicated by no color change after the addition of methyl red reagent.



C. Citrate utilization test: test performed on Simmons citrate agar:

● **Positive citrate result :**Is indicated by growth and a blue color change (Original color of media is green , positive result turned color to blue.

● **Negative citrate** utilization test is indicated by the lack of growth and color change in the tube (media color don't change) .



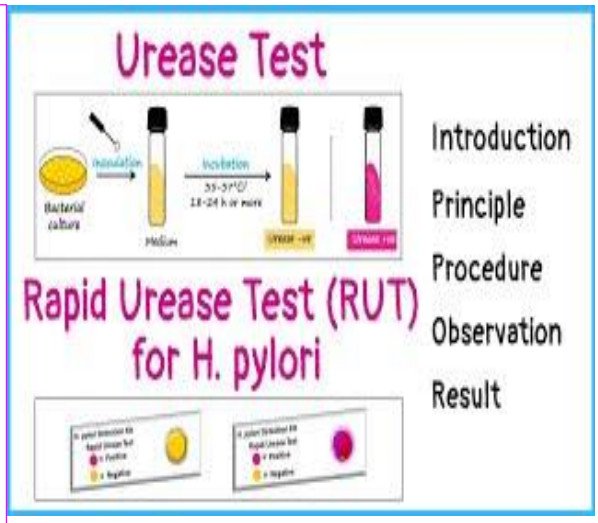
► **IMViC Test results of some Genera of *Enterobacteriaceae:* (IMViC tests of Escherichia coli) - Indol positive**
-Methyl-Red: positive
-Voges-Proskauer test: Negative
-Citrate test: Negative



Urease test: is used to differentiate urease-positive organisms (eg ***Proteus)*** from other organisms. It can also be used to detect the presence of ***Helicobacter pylori.*** The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease. Two units of ammonia are formed with resulting alkalinity in the presence of the enzyme, and the increased pH is detected by a pH indicator. This is shown in the reaction below:

$$(NH_2)_2CO + 2H_2O \xrightarrow{\text{Urase}} CO_2 + H_2O + 2NH_3$$

Urea — Carbon Dioxide — water — Ammonia

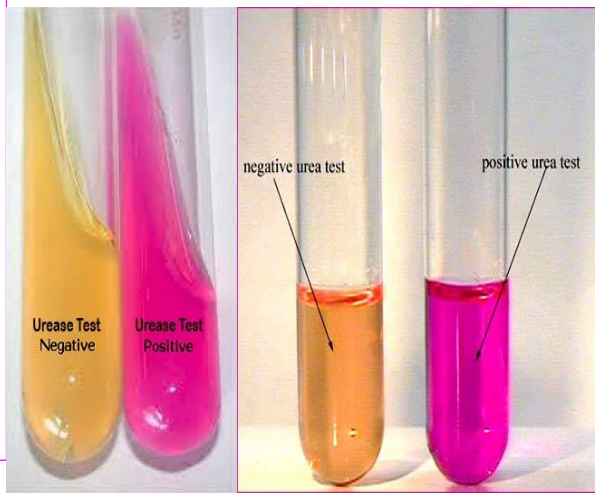


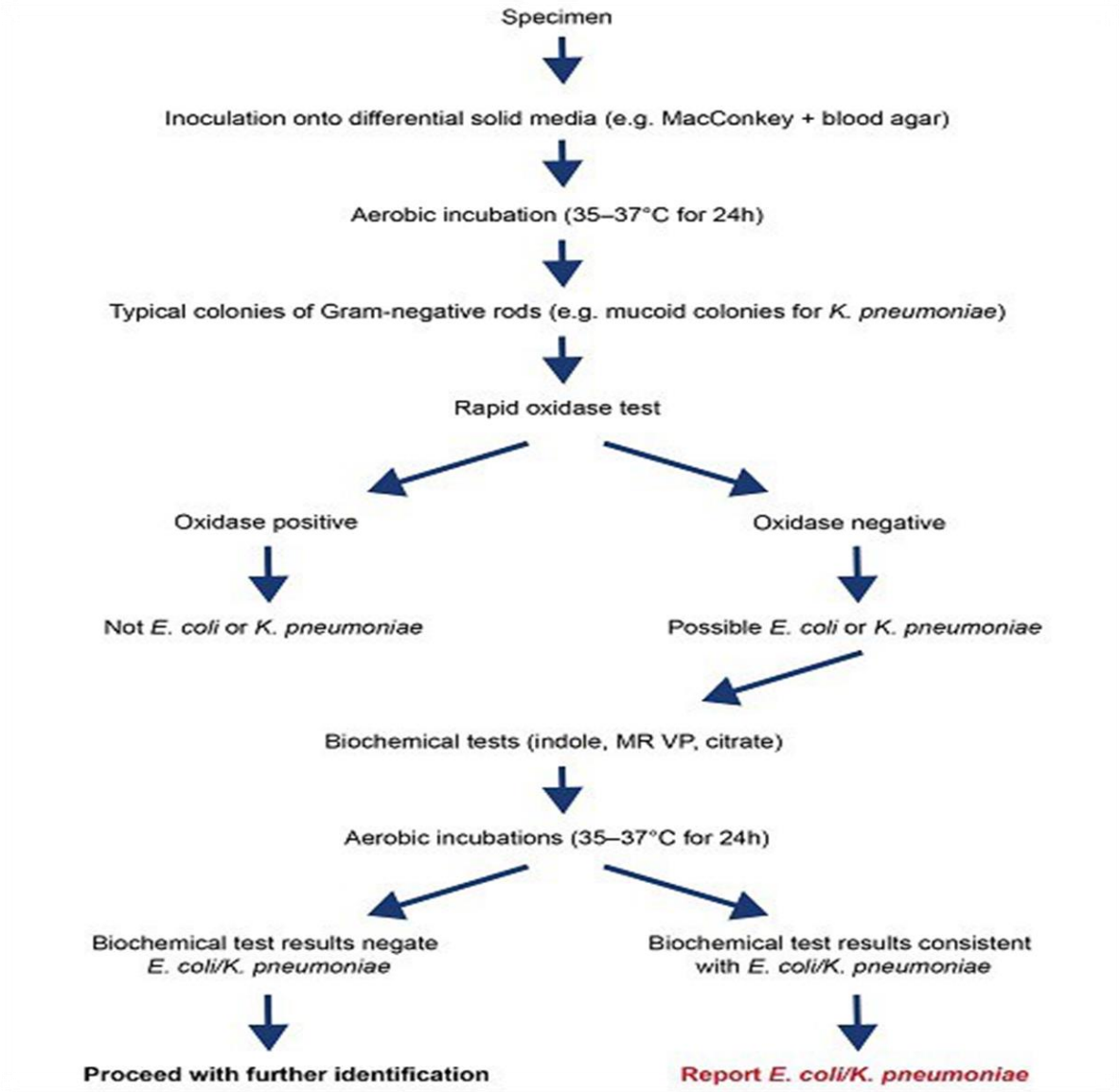
► **Procedure :**

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. incubate the test tube at 35 °C for 18 to 24 hours .

► **Positive:** If organism produces urease enzyme, the color of the slant changes from light orange to pink. Examples: ***Proteus spp***, Cryptococcus spp, Corynebacterium spp, Helicobacter pylori, Yersinia spp, Brucella spp .

► **Negative:** If organism do not produce urease the agar slant and butt remain light orange (medium retains original color). Examples: ***Escherichia coli* .**





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3	**Connie,R. Mahon; Donald, C. Leham and George Manguselis. (2011): Text book of DiagnosDc Microbiology. Fourth ediDon.**
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