Culture Media

Much of the study of microbiology depends on the ability to grow and maintain microorganisms in the laboratory, and this is possible only if suitable culture media are available. A culture medium is a solid or liquid preparation used to grow, transport,and store microorganisms. To be effective, the medium must

contain all the nutrients the microorganism requires for growth.Specialized media are essential in the isolation and identification of microorganisms, the testing of antibiotic sensitivities,water and food analysis, industrial microbiology, and other activities. a medium is used to select and grow specific microorganisms or to help identify a particular species. In such cases the function of the medium also will determine its composition.

Synthetic or Defined Media

Some microorganisms, particularly photolithotrophic autotrophs such as cyanobacteria and eucaryotic algae, can be grown on relatively simple media containing CO2 as a carbon source (often added as sodium carbonate or bicarbonate), nitrate or ammonia as a nitrogen source, sulfate, phosphate, and a variety of

Such a medium in which all components are known is a **defined medium** or **synthetic medium.** Many chemoorganotrophic heterotrophs also can be grown in defined media with glucose as a carbon source and an ammonium salt as a nitrogen source.

Complex Media

Media that contain some ingredients of unknown chemical composition are **complex media.** Such media are very useful, as a single complex medium may be sufficiently rich and complete to meet the nutritional requirements of many different microorganisms. In addition, complex media often are needed because the

nutritional requirements of a particular microorganism are unknown, and thus a defined medium cannot be constructed. This is the situation with many fastidious bacteria, some of which may even require a medium containing blood or serum. Complex media contain undefined components like peptones, meat extract, and yeast extract. **Peptones** are protein hydrolysates prepared by partial proteolytic digestion of meat, casein, soya meal, gelatin, and other protein sources. They serve as sources of carbon, energy, and nitrogen. Beef extract and yeast extract are aqueous extracts of lean beef and brewer's yeast, respectively.Beef extract contains amino acids, peptides, nucleotides, organic acids, vitamins, and minerals. Yeast extract is an excellent source of B vitamins as well as nitrogen and carbon compounds. Three commonly used complex media are (1) nutrient broth, (2) tryptic soy broth, and (3) MacConkey agar If a solid medium is needed for surface cultivation of microorganisms, liquid media can be solidified with the addition of 1.0 to 2.0% agar; most commonly 1.5% is used. **Agar** is a sulfated polymer composed mainly of D-galactose, 3,6-anhydro-L-galactose, and D-glucuronic acid. It usually is extracted from red algae. Agar is well suited as a solidifying agent because after it has been melted in boiling water, it can be cooled to about

40 to 42°C before hardening and will not melt again until the temperature rises to about 80 to 90°C. Agar is also an excellent hardening agent because most microorganisms cannot degrade it. Other solidifying agents are sometimes employed. For example,silica gel is used to grow autotrophic bacteria on solid media in the absence of organic substances and to determine carbon sources for heterotrophic bacteria by supplementing the medium with various organic compounds.

Types of Media

Media such as tryptic soy broth and tryptic soy agar are called **general purpose media** because they support the growth of many microorganisms. Blood and other special nutrients may be added to general purpose media to encourage the growth of fastidious heterotrophs. These specially fortified media (e.g., blood agar) are called **enriched media**.

Selective media favor the growth of particular microorganisms.Bile salts or dyes like basic fuchsin and crystal violet favor the growth of gram-negative bacteria by inhibiting the growth of gram-positive bacteria without affecting gram-negative organisms.Endo agar, eosin methylene blue agar, and MacConkey agar three media widely used for the detection of *E. coli* and related bacteria in water supplies and elsewhere, contain dyes that suppress gra m-positive bacterial growth. MacConkey agar also contains bile salts. Bacteria also may be selected by incubation with nutrients that they specifically can use. A medium containing only cellulose as a carbon and energy source is quite effective in the isolation of cellulose-digesting bacteria. The possibilities for selection are endless, and there are dozens of special

selective media in use.

Differential media are media that distinguish between different groups of bacteria and even permit tentative identification of microorganisms based on their biological characteristics. Blood agar is both a differential medium and an enriched one. It distinguishes between hemolytic and nonhemolytic bacteria. Hemolytic bacteria (e.g., many streptococci and staphylococci isolated from throats) produce clear zones around their colonies because of red blood cell destruction. MacConkey agar is both differential and selective. Since it contains lactose and neutral red dye, lactose-fermenting colonies appear pink to red in color and are easily distinguished from colonies of nonfermenters.

Isolation of Pure Cultures

In natural habitats microorganisms usually grow in complex,mixed populations containing several species. This presents a problem for the microbiologist because a single type of microorganism cannot be studied adequately in a mixed culture. One needs a **pure culture,** a population of cells arising from a single cell, to characterize an individual species. Pure cultures are so important that the development of pure culture techniques by the German bacteriologist Robert Koch transformed microbiology. Within about 20 years after the development of pure culture techniques most pathogens responsible for the major human

bacterial diseases had been isolated. There are several ways to prepare pure cultures; a few of the more common approaches are reviewed here.

SERIAL DILUTION

Definition

Serial dilution, as the name suggests, is a series of sequential dilutions that are performed to convert a dense solution into a more usable concentration.

- In simple words, serial dilution is the process of stepwise dilution of a solution with an associated dilution factor.
- In biology, serial dilution is often associated with reducing the concentration of cells in a culture to simplify the operation.

Objectives of Serial dilution

- The objective of the serial dilution method is to estimate the concentration (number of organisms, bacteria, viruses, or colonies) of an unknown sample by enumeration of the number of colonies cultured from serial dilutions of the sample.
- In serial dilution, the density of cells is reduced in each step so that it is easier to calculate the concentration of the cells in the original solution by calculating the total dilution over the entire series.
- Serial dilutions are commonly performed to avoid having to pipette very small volumes (1-10 µl) to make a dilution of a solution.
- By diluting a sample in a controlled way, it is possible to obtain incubated culture plates with an easily countable number of colonies (around 30–100) and calculate the number of microbes present in the sample.

Serial dilution formula/calculations

- Serial dilution involves the process of taking a sample and diluting it through a series of standard volumes of sterile diluent, which can either be distilled water or 0.9 % saline.
- Then, a small measured volume of each dilution is used to make a series of pour or spread plates.
- Depending on the estimated concentration of cells/organisms in a sample, the extent of dilution is determined. For e.g., if a water sample is taken from an extremely polluted environment, the dilution factor is increased. In contrast, for a less contaminated sample, a low dilution factor might be sufficient.
- Serial two-fold and ten-fold dilutions are commonly used to titer antibodies or prepare diluted analytes in the laboratory.
- The dilution factor in a serial dilution can be determined either for an individual test tube or can be calculated as a total dilution factor in the entire series.
- The dilution factor of each tube in a set:

$$\frac{volume\ of\ sample}{volume\ of\ sample + volume\ of\ diluent}$$

- For a ten-fold dilution, 1 ml of sample is added to 9 ml of diluent. In this case, the dilution factor for that test tube will be:

$$\text{Dilution factor} = \frac{1\ ml}{1\ ml + 9\ ml} = \frac{1}{10} = 10^{-1}$$

- After the first tube, each tube is the dilution of the previous dilution tube.

Now, for total dilution factor,

- Total dilution factor for the second tube = dilution of first tube × dilution of the second tube.

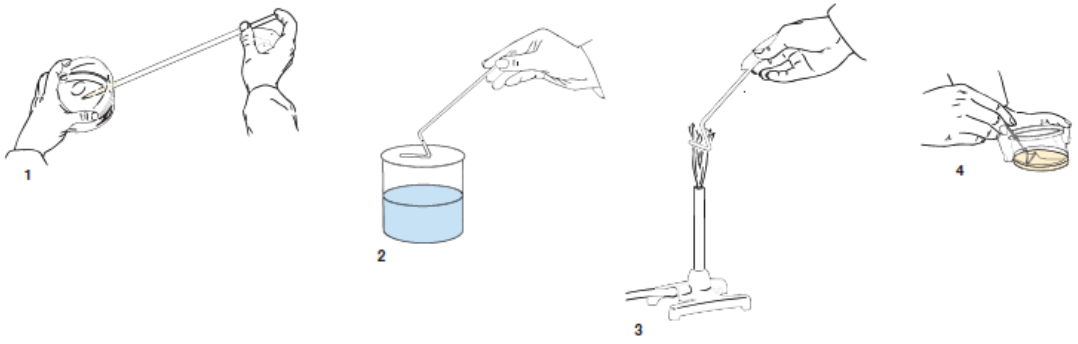
Example:

For the first tube, dilution factor = 10^{-1} (1 ml added to 9 ml)
For the second tube, dilution factor = 10^{-1} (1ml added to 9 ml)
Total dilution factor = previous dilution × dilution of next tube

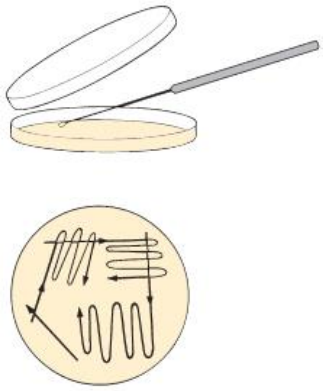
= total dilution of $10^{-1} \times 10^{-1} = 10^{-2}$

The Spread Plate and Streak Plate

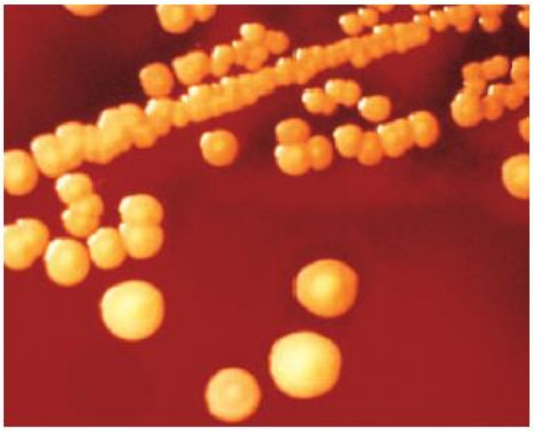
If a mixture of cells is spread out on an agar surface so that every cell grows into a completely separate **colony,** a macroscopically visible growth or cluster of microorganisms on a solid medium,each colony represents a pure culture. The **spread plate** is an easy, direct way of achieving this result. A small volume of dilute microbial mixture containing around 30 to 300 cells is transferred to the center of an agar plate and spread evenly over the surface with a sterile bent-glass rod . The dispersed cells develop into isolated colonies. Because the number of colonies should equal the number of viable organisms in the sample, spread plates can be used to count the microbial population.Pure colonies also can be obtained from **streak plates.** The microbial mixture is transferred to the edge of an agar plate with an inoculating loop or swab and then streaked out over the surface in one of several patterns . At some point in the process, single cells drop from the loop as it is rubbed along the agar surface and develop into separate colonies . In both spread-plate and streak-plate techniques, successful isolation depends on spatial separation of single cells.



Spread-Plate Technique. The preparation of a spread plate. (*1*) Pipette a small sample onto the center of an agar medium plate. (*2*) Dip a glass spreader into a beaker of ethanol. (3) Briefly flame the ethanol-soaked spreader and allow it to cool. (*4*) Spread the sample evenly over the agar surface with the sterilized spreader. Incubate.



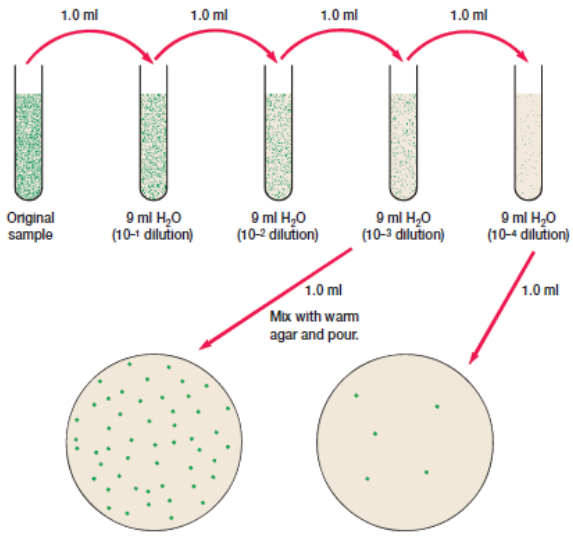
Streak-Plate Technique. Preparation of streak plates. The upper illustration shows a petri dish of agar being streaked with an inoculating loop. A commonly used streaking pattern is pictured at the bottom.



Bacterial Colonies on Agar. Colonies growing on a streak plate. A blood-agar plate has been inoculated with *Staphylococcus aureus*. After incubation, large, golden colonies have formed on the agar.

The Pour Plate

Extensively used with bacteria and fungi, a **pour plate** also can yield isolated colonies. The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies when plating. Then small volumes of several diluted samples are mixed with liquid agar that has been cooled to about 45°C, and the mixtures are poured immediately into sterile culture dishes. Most bacteria and fungi are not killed by a brief exposure to the warm agar. After the agar has hardened, each cell is fixed in place and forms an individual colony. Plates containing between 30 and 300 colonies are counted. The total number of colonies equals the number of viable microorganisms in the diluted sample. Colonies growing on the surface also can be used to inoculate fresh medium and prepare pure cultures .The preceding techniques require the use of special culture dishes named **petri dishes** or plates after their inventor Julius Richard Petri, a member of Robert Koch's laboratory; Petri developed these dishes around 1887 and they immediately replaced agar-coated glass plates. They consist of two round halves, the top half overlapping the bottom. Petri dishes are very easy to use, may be stacked on each other to save space, and are one of the most common items in microbiology laboratories.



The Pour-Plate Technique. The original sample is diluted several times to thin out the population sufficiently. The most diluted samples are then mixed with warm agar and poured into petri dishes. Isolated cells grow into colonies and can be used to establish pure cultures. The surface colonies are circular; subsurface colonies would be lenticular or lens shaped.

Maintenance and Preservation of pure cultures

- Biopreservation is the process of preserving the integrity and functionality of cells.
- Most bacteriological laboratories maintain stock cultures of microorganisms for educational, research, bioassay, industrial, or other purposes.
- A wide variety of techniques are available for the preservation of bacteria and it may be difficult to choose a method for a particular strain, which not only assures survival, but which also makes certain that the genotype and hence the unique characteristics do not change.
- The primary aim of culture preservation is to maintain the organism alive, uncontaminated, and without variation or mutation, that is, to preserve the culture in a condition that is as close as possible to the original isolate.

- **Sub culturing:** All microbiology laboratories preserve micro-organisms on agar slant.
- The agar slants are inoculated and incubated until good growth appears.
- They are then covered with sterile mineral oil to a depth of 1 cm above the tip of slant surface.
- The slants are incubated for 24hr or more and are then stored in a refrigerator.
- These cultures are periodically transferred to fresh media.
- Transfers are made by removing a loop full of the growth, touching the loop to the glass surface to drain off excess oil, inoculating a fresh medium and then preserving the initial stock culture.
- Time intervals at which the transfers are made which varies with the origin and condition of growth.
- This is a simple and most economical method of preserving bacteria and fungi where they remain viable for several years at room temperature.

Mineral oil overlay method

This technique (method) is very simple,economical and proved to be very successful for storing cultures of bacteria and fungi. The microbes remain viable for several years at room

temperature. During revival or transfer from oiled cultures, a loopful of culture is removed, inoculated on an agar slant(in case of fungi) or on a broth tube(in case of bacteria)

Requirements:Slant cultures of microorganisms, liquid paraffin, Hot air oven

Procedure:

1. Sterilize the liduid paraffin at 180˚C in hot air oven for one hour
2. Pour sufficient amount of sterized paraffin over a slant culture of microorganism so that paraffin should form a layer about half an inch above the agar surface
3. Store the agar slant upright at room temperature
4. During transfer from a paraffin preserved culture, draw a loopful of the culture and inoculate into an agar slant (for fungi) or into a tube of broth(for bacteria)

Precautions:

1. While sterilizing the paraffin the flasks should be kept in a metal box before placing them in the oven to avoid the pollution due to the production of offensive odour when heat
2. Sufficient amount of the paraffin should be added to the slant culture, so that it completely covers the agar surface. Otherwise the tube will be dehydrated in due course of time

Refrigeration

- Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms.
- This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped.
- Thus their growth continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

Cryopreservation

- Cryopreservation (i.e., freezing in liquid nitrogen at-196°C) helps survival of pure cultures for long storage times.
- In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol, that prevent the formation of ice crystals and promote cell survival.

Lyophilisation (Freeze-Drying)

- In this method, the culture is rapidly frozen at a very low temperature (around -70°C) and then dehydrated by vacuum.
- Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years.
- Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators.
- Freeze- drying method is the most frequently used technique by culture collection centres.

The Lyophilisation Process

- In this process the microbial suspension is placed in small vials.
- A thin film is frozen over the inside surface of the vial by rotating it in mixture of dry ice (solid carbon dioxide) and alcohol, or acetone at a temperature of −78ºC .
- The vials are immediately connected to a high vacuum line. This dries the organism while still frozen.
- Finally, the ampules are sealed off in a vacuum with small flame.
- These cultures can be stored for several years at 40°C.
- This method is also employed for preservation of toxins, sera, enzymes and other biological material.
- To revive microbial cultures it is merely necessary to break open the vial aseptically, add a suitable stale medium, and after incubation make further transfers.
- The process permits the maintenance of longer number of culture without variation in characteristics of the culture and greatly reduces the danger of contamination.

STAINS AND STAINING TECHNIQUES

Although living microorganisms can be directly examined with the light microscope, they often must be fixed and stained to increase visibility, accentuate specific morphological features, and preserve them for future study.

Fixation

Fixation is the process by which the internal and external structures of cells and microorganisms are preserved and fixed in position. It inactivates enzymes that might disrupt cell morphology and toughens cell structures so that they do not change during staining and observation. A microorganism usually is killed and attached firmly to the microscope slide during fixation.

There are two fundamentally different types of fixation.

(1) Bacteriologists heat-fix bacterial smears by gently flame heating an air-dried film of bacteria. This adequately preserves overall morphology but not structures within cells.

(2) Chemical fixation must be used to protect fine cellular substructure and the morphology of larger, more delicate microorganisms. Chemical fixatives penetrate cells and react with cellular components, usually proteins and lipids, to render them inactive, insoluble, and immobile. Common fixative mixtures contain such components as ethanol, aceticacid, mercuric chloride, formaldehyde, and glutaraldehyde.

Stains:

The many types of dyes used to stain microorganisms have two features in common. (1) They have **chromophore groups,** groups with conjugated double bonds that give the dye its colour.(2) They can bind with cells by ionic, covalent, or hydrophobic bonding. For example, a positively charged dye binds to negatively charged structures on the cell. Ionizable dyes may be divided into two general classes based on the nature of their charged group.

1. **Basic dyes**—methylene blue, basic fuchsin, crystal violet, safranin, malachite green—have positively charged groups (usually some form of pentavalent nitrogen) and are generally sold as chloride salts. Basic dyes bind to negatively charged molecules like nucleic acids and many proteins. Because the surfaces of bacterial cells also are negatively charged, basic dyes are most often used in bacteriology.

2. **Acid dyes**—eosin, rose bengal, and acid fuchsin—possess negatively charged groups such as carboxyls (—COOH) and phenolic hydroxyls (—OH). Acid dyes, because of their negative charge, bind to positively charged cell structures. The pH may alter staining

effectiveness since the nature and degree of the charge on cell components change with pH. Thus anionic dyes stain best under acidic conditions when proteins and many other molecules carry a positive charge; basic dyes are most effective at higher pHs.

PREPARATION OF STAINS :

Most of the microorganisms are colourless and can be studied only when they are stained before observation under the microscope. The main purpose of staining is to make organisms visible by creating a contrast between the cell and the surrounding medium so that their morphology and some cellular structures can be studied. Chemically a stain can be defined as an organic compound containing a benzene ring and a chromophore and auxochrome group. Stains can be classified into basic,acidic and neutral stains depending upon the charge on the chromophore.Acidic stains are anionic ,that is, they have a negatively charged chromophore with strong affinity for positively charged components of the cell e.g., Picric acid,Nigrosine or Indian ink. Basic stains are cationic and have positively charged chromophores which have a strong affinity for negatively charged components of the cell e.g., Methylene blue, crystal violet, safranin.

PREPARATION OF METHYLENE BLUE :

Requirements : Methylene powder,distilled water.
Procedure: 0.3g of methylene blue powder is dissolved in 100ml of distilled water taken in a beaker. The solution is stirred well , filtered and preserved in a bottle.
Uses: It is a basic dye used for simple direct staining of bacteria. It imparts blue color to the cells.

PREPARATION OF CRYSTAL VIOLET

Requirements: Solution A: Crystal violet 2g, Ethanol (95%) – 20ml. Solution B: Ammonium oxalate 0.8g, distilled water 80ml.
Procedure: Solution A is prepared by dissolving 2g of crystal violet in 20ml of 95% ethanol and solution B is prepared by dissolving 0.8g of ammonium oxalate in 80ml of distilled water. Solutions A and B are mixed, filtered and stored in a bottle.
Uses: Crystal violet is a basic dye generally used for simple direct staining of bacteria and also as a primary stain in Gram's staining technique. Bacteria get stained violet or purple.

PREPARATION OF SAFRANIN:

Requirements: Safranin powder, 95% ethanol, distilled water.
Procedure: 2.5g of saffranin is weighed and dissolved in 100ml of 95% ethanol . the solution is warmed on a water bath to hasten dissolution. 10ml of this stock solution is added to 90ml of distilled water to get the working solution (0.25%).

Uses: It is a basic dye used for simple direct staining of bacteria and as a counter stain in the Gram's staining technique. It is also used for staining of algae. It colors the cell wall and cytoplasm pink or red.

PREPARATION OF CARBOL FUCHSIN:

Requirements: Solution A-1g Basic fuchsin, 10 ml absolute ethanol ; Solution B- 5g phenol crystals , 95ml distilled water

Procedure : 1g of basic fuchsin is dissolved in 10ml of ethanol to get solution A, 5g of phenol is dissolved in 95ml distilled water to get solution B. Solution A and B are mixed and allowed to stand over night. The solution is then filtered and stored in a glass stoppered bottle.

Uses: Carbol fuchsin is a basic dye used as a primary stain in acid-fast (Ziehl-Neelsen or ZN) staining technique.It also used to stain endospores of bacteria by Dorner's method .It stains the cells or endospores pink .

PREPARATION OF MALACHITE GREEN :

Requirements: Malachite green,distilled water
Procedure : 5 gm of malachite green is dissolved in 100ml of distilled water to get 5 % aqueous solution.
Uses : Malachite green is used to stain endospores of the bacteria by the Schaeffer- Fulton method. The spores stain green in colour.

PREPARATION OF COTTON BLUE :
Requirements :Cotton blue powder, 90% ethanol,1N HCl
Procedure : Cotton blue is prepared by dissolving 1 g of cotton blue powder in 100 ml of 90% ethanol. For better results it is acidified with a drop of 1N HCl.
Uses : It is generally used for staining fungal specimens.

PREPARATION OF NIGROSIN :
Requirements : Nigrosine powder,distilled water, formaline,filter paper
Procedure : 10 g of nigrosine is dissolved in 100 ml of distilled water taken in a beaker .The beaker is immersed in a boiling water bath for 30 minutes .The solution is cooled and 0.5 ml of formalin is added as a preservative.The solution is filtered twice through double filter paper and stored in a brown bottle.
Uses: It is an acidic dye used for indirect or negative staining of bacteria where organisms appear colourless while the background is coloured dark.

PREPARATION OF MORDANT (GRAM'S IODINE):
Requirements : Iodine, Potassium iodide,distilled water
Procedure : 1gm of iodine crystals and 2 gm of potassium iodide are dissolved in 300 ml of distilled water taken in a beaker. The solution is then stored in a brown bottle.
Uses: It is used as a mordant in Gram's staining technique where it increases the affinity between crystal violet and bacterial cells by forming an insoluble crystal violet- iodine (CV-I) complex.

PREPARATION OF LACTOPHENOL :
Requirements : Lactic acid,phenol crystals, glycerol or glycerine,distilled water.
Procedure : Lactophenol is prepared by mixing 20 ml of lactic acid and 40 ml of glycerol with 40 ml of distilled water . To this mixture 20 gms of phenol crystals are added and heated gently on a hot water bath with frequent stirring until the crystals dissolve completely. To this solution 5 ml of 1 % aqueous cotton blue solution is added and mixed thoroughly to get cotton blue in lactophenol. The stain is stored in a brown bottle.
Uses : Lactophenol is used as a mounting medium for staining fungal specimens . Cotton blue in lactophenol is a stain as well as a mounting medium.

Simple Staining- Principle, Procedure and Result Interpretation

Objective

1. To perform a simple staining procedure.
2. To compare the morphological shapes and arrangements of bacterial cells.

Principle of Simple Staining: In simple staining, the bacterial smear is stained with a single reagent, which produces a distinctive contrast between the organism and its background. Basic stains with a positively charged chromogen are preferred because bacterial nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue, crystal violet, and carbol fuchsin. Basic dyes such as **crystal violet** (20 to 30

seconds staining time), **carbolfuchsin** (5 to 10 seconds staining time), or **methylene blue** (1 minute staining time) are often used. Once bacteria have been properly stained, it is usually an easy matter to discern their overall shape. Bacterial morphology is usually uncomplicated and limited to one of a few variations.

Materials required: 24- to 48-hour tryptic soy broth or agar slants of *Bacillus subtilis* and *E.coli*

Microscope, clean microscope slides, bibulous paper, inoculating loop and needle, sterile distilled water, Bunsen burner, crystal violet (1% aqueous solution), wax pencil,immersion oil, lens paper and lens cleaner, slide holder or clothespin and slide warmer

Procedure of Simple Staining

1. Place a slide on the staining tray and flood the smear with one of the indicated stains, using the appropriate exposure time for each: carbol fuchsin, 15 to 30 seconds; crystal violet, 20 to 60 seconds; methylene blue, 1 to 2 minutes.
2. Gently wash the smear with tap water to remove excess stain. During this step, hold the slide parallel to the stream of water; in this way you can reduce the loss of organisms from the preparation.
3. Using bibulous paper, blot dry, but do not wipe the slide.
4. Examine all stained slides under oil immersion.

Result Interpretation of Simple Staining:

Bacilli and diplobacilli: Rod-shaped bacteria, purple
Spirilla: spiral-shaped bacteria, purple
Cocci: spherical-shaped, bacteria, purple

Negative Staining- Principle, Procedure and Result Interpretation

Objectives of Negative Staining

1. To perform a negative staining procedure.
2. To understand the benefit obtained from visualizing unstained microorganisms.

Principle of Negative Staining

Negative staining requires the use of an acidic stain such as India ink or nigrosin. The acidic stain, with its negatively charged chromogen, will not penetrate the cells because of the negative charge on the surface of bacteria. Therefore, the unstained cells are easily discernible against the colored background. The practical application of negative staining is twofold.First, since heat fixation is not required and the cells are not subjected to the distorting effects of chemicals and heat, their natural size and shape can be seen.Second, it is possible to observe bacteria that are difficult to stain, such as some spirilla. Because heat fixation is not done during the staining process, keep in mind that the organisms are not killed and slides should be handled with care.

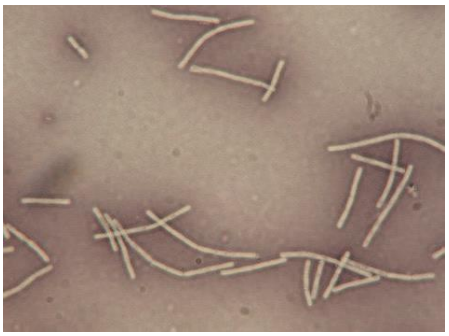
Reagent and Equipment's for Negative Staining: Nigrosin, Microincinerator or Bunsen burner, inoculating loop, staining tray, glass slides, lens paper, and microscope.

Procedure of Negative Staining

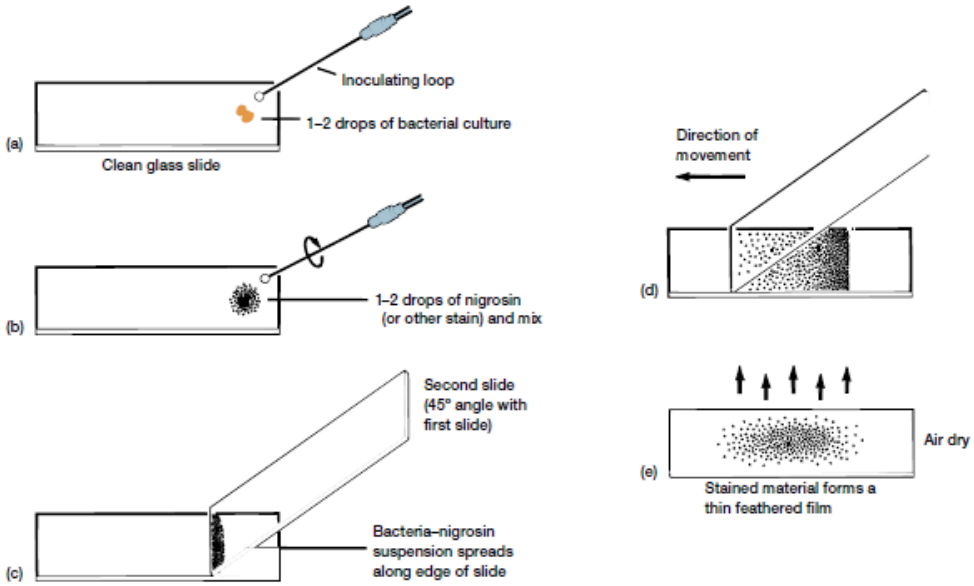
1. Place a small drop of nigrosin close to one end of a clean slide.

2. Using aseptic technique, place a loopful of inoculum from the bacterial culture in the drop of nigrosin and mix.
3. Place a slide against the drop of suspended organisms at a 45° angle and allow the drop to spread along the edge of the applied slide.
4. Push the slide away from the drop of suspended organisms to form a thin smear. Air-dry.
   Note: Do not heat fix the slide.
5. Examine the slides under oil immersion.

Result Interpretation of Negative Staining



Negative Staining Procedure and Thin Smear Preparation.



Gram staining:

The Gram stain was developed by Christian Gram in 1884 and modified by Hucker in 1921.

The objective of Gram Stain

This test differentiates the bacteria into Gram-Positive and Gram-Negative Bacteria, which helps in the classification and differentiation of microorganisms. The Gram stain separates bacteria into two groups: (1) Gram-positive microorganisms that retain the primary dye (Crystal violet) and (2) Gram-negative microorganisms that take the color of the counterstain (usually Safranin O).

GRAM'S STAINING

The Gram stain was developed by Christian Gram in 1884 and modified by Hucker in 1921. The Gram stain separates bacteria into two groups: (1) Gram-positive microorganisms that retain the primary dye (Crystal violet) and (2) Gram-negative microorganisms that take the color of the counterstain (usually Safranin O). These results are due to differences in the structure of the cell wall. Crystal violet is attracted to both Gram-positive and Gram-negative microorganisms. The second step (Gram's Iodine, a mordant) stabilizes the Crystal violet into the peptidoglycan layer of the cell wall. The peptidoglycan layer is much thicker in Gram-positive bacteria than in Gram-negative bacteria; hence, the Crystal violet is more extensively entrapped in the peptidoglycan of Gram positive bacteria. The third step (alcohol decolorization) dissolves lipids in the outer membrane of Gram-negative bacteria and removes the Crystal violet from the peptidoglycan layer. In contrast, the Crystal violet is relatively inaccessible in Gram-positive microorganisms and cannot readily be removed by alcohol in Gram-positive microorganisms. After the alcohol step, only the colorless Gram-negative microorganisms can accept the Safranin (counterstain). Carbol-fuchsin and Basic fuchsin are sometimes employed in the counterstain to stain anaerobes and other weakly staining
Gram-negative bacteria.

The most frequent errors in the Gram stain often are associated with slide preparation. Thick slide preparations, excessive heat fixing that distorts the bacteria, and improper decolorizations are common problems encountered with the stain. Inexperience can lead to over-decolorization (too many Gramnegative bacteria) or under-decolorization (too many Gram-positive bacteria). The decolorization step is the most problematic part of the procedure. Bacteria are often called Gram variable if half are stained violet and the other half are pink.

The Gram Stain Reagents

1. Primary stain: 2 g Crystal violet, 20 mL 95% ethyl alcohol, 0.8 g ammonium oxalate, and 100 mL distilled water.
2. Gram's iodine: 2 g potassium iodide, 1 g iodine crystals, and 100 mL distilled water.
3. Decolorizer: 50 mL acetone and 50 mL ethanol.
4. Counterstain: 4.0 g Safranin, 200 mL 95% ethanol, and 800 mL distilled water.

Procedure:

1. Prepare and fix the specimen to the microscope slide before staining.
2. Cover the smear with crystal violet, the primary stain, for 20 seconds.
3. Gently rinse off the stain with water.
4. Cover the smear with Gram's iodine, the mordant, for 1 minute.
5. Pour off the excess Gram's iodine.
6. Run the acid-alcohol decolorizer over the smear until the solution appears clear.
7. Gently rinse with water.
8. Cover the smear with safranin, the secondary or counterstain, for 20 seconds.
9. Gently rinse the stain with water.
10. Blot dry with bibulous paper.

Result Interpretation of Gram Stain

Gram-positive: Blue/Purple Color
Gram-Negative: Red/Pink Color

Acid fast staining

Acid-fast staining is another important differential staining procedure. A few species, particularly those in the genus *Mycobacterium* do not bind simple stains readily and must be stained by a harsher treatment: heating with a mixture of basic fuchsin and phenol (the Ziehl-Neelsen method).Once basic fuchsin has penetrated with the aid of heat and phenol,acid-fast cells are not easily decolorized by an acid-alcohol wash and hence remain red. This is due to the quite high lipid content of acid-fast cell walls; in particular, mycolic acid—a group of branched chain hydroxy lipids—appears responsible for acidfastness.Non-acid-fast bacteria are decolorized by acid-alcohol and thus are stained blue by methylene blue counterstain. This method is used to identify *Mycobacterium tuberculosis* and *M.leprae*, the pathogens responsible for tuberculosis and leprosy, respectively.

Ziehl Neelsen Staining Procedure

1. Fix the prepared slide with gentle heat.
2. Flood the slide with Carbol-fuchsin and heat to steaming only once.
3. Leave for 10 min.
4. Rinse with distilled water.
5. Decolorize with acid alcohol for 3 min.
6. Rinse with distilled water.
7. Counterstain with methylene blue for 1 min.
8. Rinse with distilled water, drain, and air dry.

Ziehl Neelsen Staining Reagents

1. **Primary Stain**: 0.3% Carbol-fuchsin. Dissolve 50 g phenol in 100 mL ethanol (95%) or methanol (95%). Dissolve 3 g Basic fuchsin in the mixture and add distilled water to bring the volume to 1 L.
2. **Decolorization Solution**: Add 30 mL hydrochloric acid to 1 L of 95% denatured alcohol. Cool and mix well before use. **Alternate decolorizing reagent** (without alcohol): Slowly add 250 mL sulfuric acid (at least 95%) to 750 mL distilled water. Cool and mix well before using.
3. **Counterstain:** 0.3% nethylene blue. Dissolve 3 g nethylene blue in 1 L distilled water.

Result Interpretation of Acid Fast Stain

Acid fast: Bright red to intensive purple, Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded
Non-acid fast: Blue color; In addition, background material should stain blue.

STRUCTURAL STAINING

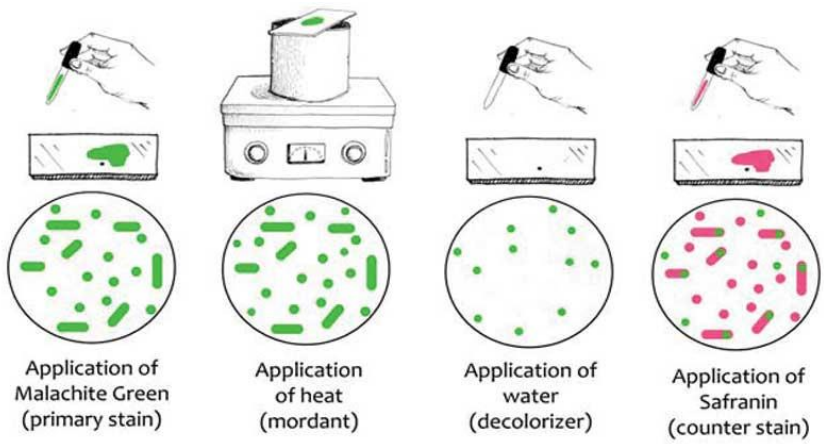
Endospore staining:

I**ntroduction** : A normal bacterial cell is known as a vegetative form of a bacterial cell. It can multiply freely when provided with conducive environmental conditions like the provision of nutrients, temperature, water, and oxygen. But some groups of bacteria, when exposed to unfavorable conditions such us, lack of nutrients, an insufficient supply of oxygen or CO2, lack of water and moisture, they have the ability to form a protective covering to protect themselves when they are exposed to harsh environmental conditions adapting comfortably to these conditions. This special structure is known as an **endospore**.

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.

These include; a phase of dormancy, a tough outer covering and they are non-reproductive forms called spores, which offer great resistance to high temperature, radiations, and chemicals like disinfectants and acids. This enables them to survive thus they are difficult to stain with basic dyes and hence a special stain is applied that uses a special dye, along with heat-steam. This staining technique is known as the **Endospore stain,** also known as the **spore stain.** It is used majorly to detect and identify the presence of a bacterial endospore and bacterial vegetative forms in a cell. Examples of these endospore-forming bacteria include *Clostridium spp* and *Bacillus spp*. These bacteria naturally grow in soil, but they have great clinical implications by causing human bacterial infections eg *Clostridium tetani* causes tetanus, *Clostridium botulinum* produces botulin toxin which causes paralysis, *Bacillus cereus* causing food poisoning and *Bacillus anthracis* causes anthrax in cattle and humans.
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)



Objectives of endospore staining

1. To detect for the presence of an endospore.
2. To identify endospore producing bacteria
3. To differentiate between the vegetative forms and the endospore

Principle:

Endospore staining is a differential stain that aims at detecting, identifying and differentiating an endospore from the vegetative cell (an underdeveloped endospore). The principle of the role is to detect the presence or absence of the endospore, but some procedures have modified the technique by increasing the concentrations of the dyes, increasing the duration of heat fixing, application of ultraviolet radiation.
With the improved technology in microscopy, some use phase-contrast microscopy which is fast and it produces more detailed morphologies of the bacterial endospore.

1. Schaeffer Fulton Stain

In basic Laboratories, the simplest endospore staining technique is the Schaeffer-Fulton technique because of its easy and it is rapid to identify the bacteria. It applies the use of **Malachite green dye** (alkaline solution with a pH of 11-11.2 and its a water-soluble dye)

along with the use of steamed-heat which softens the endospore covering allowing penetration of the dye into the spore. The malachite green dye binds to the spore mildly and if washed with water, without fixing, it easily washes away, and that's why the application of steamed heat is important to allow the dye to penetrate the endospore. Water is used as a decolorizing agent, to was away from the malachite dye from vegetative forms. Lastly, the use of a **counterstain, Safranin reagent,** also knows as the secondary stain, is to stain the vegetative forms of the underdeveloped Furmigates vegetative forms after the malachite dye has been washed away by the decolorizing agent (Water).
Malachite Green dye and Safranin work well in bacteria because of the alkaline nature of the Malachite Green reagents which are charged positively while the cytoplasm of the bacterial cell is basophilic hence there is an attraction between the malachite green dye with the bacterial cell, making it easier to absorb the dye. Visualization of the cells under the microscope will show the appearance of pink-red stain for the vegetative cell forms, which take up the counterstain while the endospores will appear as green dotted particles (ellipses), having taken up the Malachite green dye.

Reagents

1. Malachite green dye
2. Water (Decoloriser)
3. Safranin

Procedure

Equipment: *Glass slide, Inoculation loop, Bunsen burner*

Preparation of microscope slide (adapted for all other endospore staining techniques)

1. Clean the glass slide (with visible circles), with alcohol to remove any stains.
2. Using a sterile inoculation loop, put two **small** drops of water in each circle.
3. Aseptically, open the tube with a bacteria culture and flame it at the top and collect a loopful of the bacterial culture from the tube, Flame the tube again and close.
4. Smear the bacterial culture in the drop of water on the slide.
5. Air dry till its completely dry.
6. Heat-fix the slide with smear facing up, by running it over the blue flame 3-4 times **NB**: Do not flame the side with the bacteria
7. Leave to cool and then start to stain.

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.

Result

The vegetative forms will take up the pink/red stain from safranin while the endospores will stain green, from the malachite green dye.

Interpretation of results

The vegetative forms stain pink/red because they take up the counterstain (Safranin) while the endospores take up the green from the Malachite green.

This is because, during smearing and heat fixing, the malachite green penetrates into the endospore with the help of the heat from the steam, and during the water-rinse, the dye is not easily washed away.

And for the vegetative forms, the dye is easily washed away because of their fragile outer covering, hence they take up the last stain which is the counterstain, hence they appear pink-red.

2. Dorner method for staining

Reagents:

Carbolfuchsin stain,
Decolorizing
(Preparation of solvent (acid-alcohol)
Counterstain (Nigrosin solution)

Procedure

microscopic slide is adapted from the above procedure)

Staining procedure

1. cover the smear with an absorbent paper.
2. Saturate it with carbol-fuschin and heat fix by steaming over a boiling water bath or beaker for 5-10 minutes while adding more dye to the smear.
3. Remove the absorbent paper and decolorize it with acid-alcohol for 1 minute; rinse with tap water and tap dry.
4. Add a thin film of nigrosin reagent as a counterstain.
5. Visualize the slide under the oil immersion lens (1,000X) for the presence of endospores.

Result

Vegetative cells appear colorless, while endospores are red.

.**Applications of the Endospore stain**

1. For detection of Firmicute groups of bacteria i.e *Clostridium* spp and *Bacillus* spp
2. For identification of endospore producing bacteria in samples
3. for differentiation of spore-producing bacterial from vegetative forms of bacteria

Advantages of endospore staining

- Being a Differential stain, allows you to identify specific bacteria that have an endospore
- It also allows the detection and presence of vegetative forms in bacterial culture, besides identifying the presence of endospore producing bacteria.

Disadvantages

- It can only specifically identify the presence of endospore-forming bacteria

NOTE

- Another endospore staining technique that is not commonly used is known as the Klein method of endospore staining.

- The difference between these Shcauffer Fulton and Klein staining techniques is the application of dyes i.e in Schahuffer Fulton stain, Malachite Green dye is used while in the Klein methodology, Methylene blue solution is used.

FLAGELLA STAINING
Objective of Flagella Stain

This technique is used to visualize the presence and arrangement of flagella for the presumptive identification of motile bacterial species.

Flagella are fine thread like appendages arising from cytoplasm of motile bacteria. Most motile bacteria possess flagella but other forms of motility are also seen in bacteria. *Myxobacteria* exhibit gliding motion and spirochetes exhibit screw like motion using axial filament. Flagella are protein in nature and project out from cell wall. They are very fragile and break on mere shaking, heating and on treating with acid or detergent.

Flagella are not visible with light microscope being very thin much below the resolving power of bright field microscope. Hence, special staining methods are employed to increase the thickness of the flagella by depositing coats of mordant that increases their diameter. Presence and location of flagella is also helpful in the identification and classification of bacteria. Bascd on flagellation the bacteria have been grouped as : Peritrichous: flagella all around the surface; Amphitrichous: two or more flagella on both the ends; Lophotrichous: a tuft of flagella at one end and Monotrichous: single flagellum at one end.

- **Requirements**
- a. Bacterial culture: *Proteus vulgaris* (8-16 h old), *Pseudomonas aeruginosa* (8 h old), and
- *Escherichia coli* (8-16 hold).
- d. Stain: Ziehl-Neelsen carbol fuchsin
- e. Mordant (tannic acid 10% in 5% NaCI solution)
- **Procedure**
- I. Take a clean grease free slide and pass it through Bunsen burner blue flame.
- 2. Add 2-3 ml sterile saline to the slant and keep it for an hour. Now with sterile pipette or sterile loop transfer a drop of culture on one end of the slide, tilt the slide, and allow the drop to trickle down slowly.
- 3. Air-dry the film. Do not heat fix.
- 4. Cover the smear with mordant for 10-30 min. Then rinse it gently with water.
- 5. Now, add stain over the smear and let it remain for 5-15 min. Rinse off the stain with water, air dry and observe under microscope using oil immersion objective

Result Interpretation of Flagella Stain

- Presence or absence of flagella
- Number of flagella per cell
- Location of flagella per cell
    - Peritrichous
    - Lophotrichous
    - Polar
- The amplitude of wavelength
    - Short

    - Long
- Whether or not “tufted”

Limitation

Even with a specific stain, visualization of flagella requires an experienced laboratory scientist and is not considered an entry-level technique.

CAPSULE STAINING

Capsule Stain- Principle, Procedure and Result Interpretation

A capsule is a gelatinous outer layer that is secreted by the cell and that surrounds and adheres to the cell wall. It is not common to all organisms. Cells that have a heavy capsule are generally virulent and capable of producing disease, since the structure protects bacteria against the normal phagocytic activities of host cells. Chemically, the capsular material is composed mainly of complex polysaccharides such as levans, dextrans, and celluloses.

Capsule staining is more difficult than other types of differential staining procedures because the capsular materials are water-soluble and may be dislodged and removed with vigorous washing. Smears should not be heated because the resultant cell shrinkage may create a clear zone around the organism that is an artifact that can be mistaken for the capsule.

Objectives of Capsule Stain

1. To prepare a smear of an encapsulated bacterium and stain its capsule using the Anthony capsule stain.
2. To visualize the capsule and differentiate it from the cell body.

Principle of Capsule Stain

Primary Stain: Crystal Violet (1% aqueous)

A violet stain is applied to a non–heat-fixed smear. At this point, the cell and the capsular material will take on the dark color.

Decolorizing Agent: Copper Sulfate (20%)

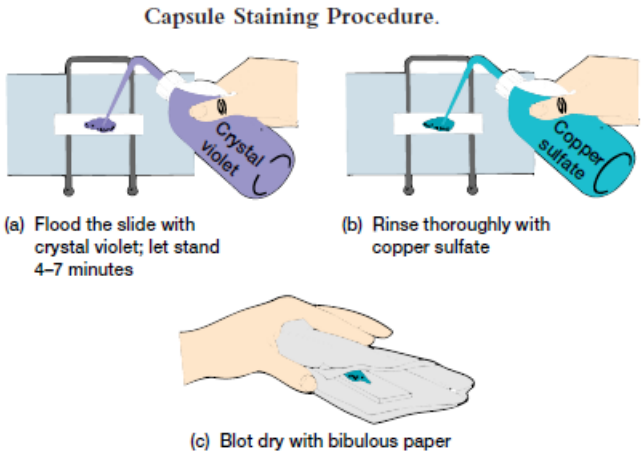
Because the capsule is nonionic, unlike the bacterial cell, the primary stain adheres to the capsule but does not bind to it. In the capsule staining method, copper sulfate is used as a decolorizing agent rather than water. The copper sulfate washes the purple primary stain out of the capsular material without removing the stain bound to the cell wall. At the same time, the decolorized capsule absorbs the copper sulfate, and the capsule will now appear blue in contrast to the deep purple color of the cell.

Procedure of Capsule Stain

1. Prepare thin smears of bacterial culture on a microscope slide.
2. Allow the smear to only air-dry. *Do not heat-fix as this will cause the capsule to shrink or be destroyed.*
3. Apply 1% crystal violet and allow it to remain on the slide for 2 minutes.
4. With the slide over the proper waste container provided, gently wash off the crystal violet with 20% copper sulfate. *Caution: Do not wash the copper sulfate and stain directly into the sink.*
5. Blot the slide dry with bibulous paper.
6. Observe with the oil immersion lens.

Result Interpretation of Capsule Stain

Capsule: Clear halos zone against dark background
No Capsule: No Clear halos zone



CELL WALL STAINING

Bacterial cell wall encasing the bacterial cytoplasm is rigid in nature with little plasticity. Besides protecting the bacterial internal structure, it assigns cell shape, size and integrity to bacteria. Even rod shaped bacteria deprived of cell wall often assume spherical shapes in isotonic solutions. Bacteria devoid of complete cell wall are called protoplasts and the bacteria with incomplete cell wall are known as spheroplasts. Though the bacterial cell wall structure varies from one cell to another but in general the basic structure is made up of peptidoglycan. Cell wall is thinner in Gram-negative bacteria as compared to Gram-positive bacteria. It is not visible in bacteria stained with simple stain as cell \\/all is very thin and is not within the resolving power of ordinary microscope. Therefore, cell wall demonstration technique makes use of mordant like tannic acid that makes the cell wall thicker thus making it visible after staining under microscope.

Requirements

a. Racterial cultures: *Bacilllls lIIega/erilllll, S/aphylococcus aureus* and *Proteus vulgaris*

Rainbow method

(i) Bouin's fixative (ii) 0.2% crystal violet in ethanol (iii) 1 % congo red.

Ringer's method

(r) Houin's fixative

(iii)0.5% crystal violet in ethanol

Cetylpyridinium chloride method

(i) 0.34% cetylpyridinium chloride

(iii) Loeffler methylene blue.

Procedure:

Rainbow method

(ii) 10% tannic acid

(iv) 0.5% congo red .

(ii) Saturated congo red solution

I. Prepare the smear and air dry. Cover it with Bouin’s fixative for 30 min .

Drain off the fixative by tilting the slide and add tannic acid and let remain for 30 min .

Wash gently with water and stain with crystal violet for 5-10 seconds.

Wash with water, blot dry. and examine under oil immersion objective. Record the observations.
Results: Cell wall appears as violet colored around light blue colored cytoplasm.

Ringer's method

1. Prepare the smear on clean grease free slide and air dry .
2. Cover the smear with Bouin's fixative for 30 min . for fixation of smear.
3. Pour off the fixative and cover it with tannic acid for another 30m in.
4. Drain off the tannic acid and stain with crystal violet for 1-2 min.
S. Wash of the stain and treat the smear for 2-3 min with congo red.
6. Decant off congo red, blot dry the smear and wash with distilled water.
7. Air-dry and examine under oil immersion objective.
8. Cell is stained violet in contrast to pinkish cytoplasm.

Cetylpyridinium chloride method

I. Prepare the smear on clean grease free slide and air dry.
2. Add three drops of cetylpyridinium chloride and one drop of congo red to the smear and mix the drops well witl) inoculating needle takirig care not to scratch the smear. Let it stain for 5 min.
3. Rinse the smear with tap water and blot dry or air dry.
4. Stain the smear with methylene blue for 10 sec. Rinse off the dye with water.
5. Air dry and examine under oil immersion objective and record the observations.

FLAGELLA STAINING

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Flagella are not visible with light microscope being very thin much below the resolving power of bright field microscope. Hence, special staining methods are employed to increase the thickness of the flagella by depositing coats of mordant that increases their diameter. Presence and location of flagella is also helpful in the identification and classification of bacteria. Bascd on flagellation the bacteria have been grouped as : Peritrichous: flagella all around the surface; Amphitrichous: two or more flagella on both the ends; Lophotrichous: a tuft of flagella at one end and Monotrichous: single flagellum at one end.

Requirements

a. Bacterial culture: *Proteus vulgaris* (8-16 h old), *Pseudomonas aeruginosa* (8 h old), and *Escherichia coli* (8-16 hold).
d. Stain: Ziehl-Neelsen carbol fuchsin
e. Mordant (tannic acid 10% in 5% NaCI solution)

Procedure

I. Take a clean grease free slide and pass it through Bunsen burner blue flame.
2. Add 2-3 ml sterile saline to the slant and keep it for an hour. Now with sterile pipette or sterile loop transfer a drop of culture on one end of the slide, tilt the slide, and allow the drop to trickle down slowly.
3. Air-dry the film. Do not heat fix.
4. Cover the smear with mordant for 10-30 min. Then rinse it gently with water.
5. Now, add stain over the smear and let it remain for 5-15 min. Rinse off the stain with water, air dry and observe under microscope using oil immersion objective.

NUCLEIC ACID STAINING

In a typical eucarkotic cell. the nucleus encased in a thin nuclear membrane and positioned centrally inside the cytoplasm. In contrast. the prokaryotes, including bacteria, lack a cell-demarcated nucleus. Hence the term nuclear material is used instead of nucleus. The nuclear material being rich in chromatin has great affinity for coal tar dyes that stain it intensly) It is thehub of all inheritable properties and phenotypic activities of bacterial cdl. Nuclear stains color the whole bacterial cell cytoplasm suggesting it being distributed in the cytoplasm though at specific points it may be more localized. In most stained preparations, the basophilic nature of bacterial cytoplasm masks the chromatin material staining.

Requirements:

Bacterial cultures: *Bacillus suhtilis. Staphylucoccus aureus* and *Ecoli.*

Feulgens method

a. Bouin's fixative

b. IN HCI

c. Schiffs fuchsin sulphate (Schiffs base)

Giemsa's method

a. Bouin's fixative

b. INllel

c. Giemsa stain

Procedure

Feulgen's method

I Prepare bacterial smear on ckan grease free slide and air dry.

2. Cover the smear with Bouins fixative for 30 min.

3. Keep the slide on a beaker containing boiling water and add a few drops of IN Hcl. Let it remain for 10 min .

4. Wash it with Schiffs reagent for 20 min.

5. Wash with water.

6. Immerse the slides in sodium bisulphate solution for 10 min.

7. Wash with water. Air dry. examine under oil immersion objective, and record the observation with illustration.

8.Nuclear material appears pinkish in a colorless cytoplasm.

Giemsa's Method

I. Prepare bacterial smear on clean glass slide and air dry .

2. cover the smear with Bouins fixative for 30 min.

3. Keep the slide on a beaker containing boiling Water and add a few drops of IN Hcl. Let it remain for 10 min

4. Rinse with water and stain with Giemsa's stain for 1-2 min.

5. Rinse with water and air dry. Examine under oil immersion objective

6. Nuclear material appears pinkish in a colorless

HANGING DROP

Many bacteria show no motion and are termed **nonmotile.** However, in an aqueous environment, these same bacteria appear to be moving erratically. This erratic movement is due to **Brownian movement.**Brownian movement results from the random motion of the water molecules bombarding the bacteria and causing them to move. True **motility** (self-propulsion) has been recognized in other bacteria and involves several different mechanisms. Bacteria that possess flagella exhibit **flagellar motion.** Helical-shaped spirochetes have axial

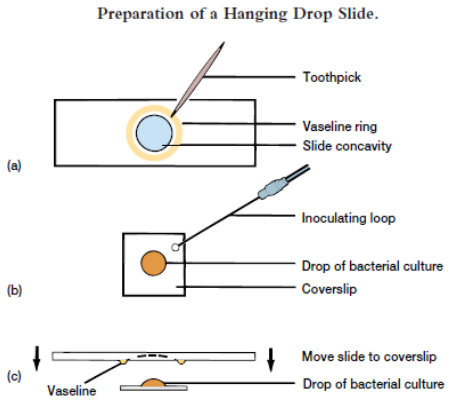
fibrils (modified flagella that wrap around the bacterium) that form axial filaments. These spirochetes
move in a **corkscrew-** and **bending-type motion.** Other bacteria simply slide over moist surfaces in a form of **gliding motion.** The above types of motility or nonmotility can be
observed over a long period in a hanging drop slide. Hanging drop slides are also useful in observing the general shape of living bacteria and the arrangement of bacterial cells when they associate .A ring of Vaseline around the edge of the coverslip keeps the slide from drying out.

Procedure

1. With a toothpick, spread a small ring of Vaseline around the concavity of a depression slide . Do not use too much Vaseline.
2. After thoroughly mixing one of the cultures, use the inoculating loop to aseptically place a small drop of one of the bacterial suspensions in the center of a coverslip.
3. Lower the depression slide, with the concavity facing down, onto the coverslip so that the drop protrudes into the center of the concavity of the slide. Press gently to form a seal.
4. Turn the hanging drop slide over and place on the stage of the microscope so that the drop is over the light hole.
5. Examine the drop by first locating its edge under low power and focusing on the drop. Switch to the high-dry objective and then, using immersion oil, to the 90 to 100× objective. In order to see the bacteria clearly, close the diaphragm as much as possible for increased contrast. Note bacteria2

SAFETY PRECAUTIONS

Be careful with the Bunsen burner flame. Slides and coverslips are glass. Do not cut yourself when using them. Dispose of any broken glass in the appropriately labeled container. Discard contaminated depression slides in a container with disinfectant.



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