Robert Koch was a German physician and microbiologist.
He is the main founder of modern bacteriology.
He received Nobel Prize in Physiology or Medicine in 1905 for his work on tuberculosis.
He also discovered new laboratory technologies and techniques in the field of microbiology.
He is well known for identifies the causative agents of tuberculosis, cholera, and anthrax.
He is also well known for his establishment of Koch's postulates.

Contributions:

1. **Isolating pure bacterial cultures:** Robert Koch utilized Agar for isolation and cultivation of bacterial pure culture. There were several benefits of this agar medium, such as; it easily solidify in 37-degree centigrade, it was undegradable to the most of bacteria, and produces a transparent medium.

2. **Koch's Postulates:** Robert Published four postulates, which shows us the importance of pure culture in isolation of disease-causing organisms and described each necessary step to achieve this. His four Postulates were;

A specific organism can always be found in Association with a given disease.
The organism can be isolated and grown in pure culture in the laboratory. The pure culture will produce the disease when inoculated into a respectable animal.
It is possible to recover the organism in pure culture from the experimentally infected animal.

3.**Tuberculosis:** In the earliest time, people have believed tuberculosis is an inherited disease. But later Koch experimentally proved that Tuberculosis is an infectious disease and caused by the bacteria *Mycobacterium tuberculosis.* In his experiment, the bacteria satisfied all four of his postulates.

4. **Causative Agent of Anthrax:** Robert Koch identifies the causative agent of the fatal disease Anthrax, which is *Bacillus anthracis*. He found that the spores of anthrax bacteria are remains in the dormant stage in most of the time. In the presence of optimal conditions, these spores are activated and cause disease.

For the isolation of this causative agent he dry-fixed bacterial cultures onto glass slides and then stain the culture with the help of dyes after that he observed the slides through the microscope.

5. **Causative Agent of Cholera:** In 1884, Robert Koch isolate the causative agent of Cholera diseases, called *Vibrio cholerae.*

6. **Acquired immunity:** Robert Koch observed the phenomenon of acquired immunity when he was examined the Papuan people. He found the *Plasmodium* parasite in their blood, but their symptoms of malaria were mild or could not even be noticed.

Koch's Postulates There are four postulates such as;
a. A specific organism can always be found in Association with a given disease. b. The organism can be isolated and grown in pure culture in the laboratory.
c. The pure culture will produce the disease when inoculated into a respectable animal.
d. It is possible to recover the organism in pure culture from the experimentally infected animal.

Louis Pasteur
Louis Pasteur was a French scientist who made significant contributions to the fields of microbiology and chemistry. He is best known for his work on the germ theory of disease, which states that many diseases are caused by microorganisms, and for his development of pasteurization, a process that involves heating liquids to kill harmful bacteria.

1. **Spontaneous generation:** At the earlier people were believed in spontaneous generation theory, that living cells were generated from nonliving cells. Pasteur believed in biogenesis theory means, living cells were generated from living

material rather than nonliving materials. Pasteur disproved the spontaneous generation theory by his Swan Necked Flask experiment.

2. **Crystallography:** In earlier Pasteur was a chemist. During his doctoral dissertation, he work as a laboratory assistant at the École Normale. During this time he investigates that the ability of certain crystals or solutions to rotate plane-polarized light clockwise or counterclockwise, that is, to exhibit "optical activity." Pasteur was able to exhibit that in some cases this activity related to the shape of the crystals of a compound.
3. **Fermentation:** In the earliest time people believe that the fermentation process is conducted by a series of chemical reactions. In 1854, while he was working at Lille,
4. **Pasteurization:** To prevent the souring of wine or to fight against the "diseases" of wine he discovered the Pasteurization Processes. He noticed that the spoiling beverages, such as beer, wine, and milk is caused by some unwanted microorganisms. So, he invented a process in which the microorganisms could be destroyed by heating the wine between 60° and 100°C temperature.
5. **Germ Theory of Diseases:** Pasteur and a minority of other scientists believed that diseases were caused by microorganisms—germ theory. Later he proved this theory by investigating the causes of silkworm disease.
6. **Anthrax vaccine:** In 1881, Louis Pasteurpulically announced that he successfully discovered the vaccine of anthrax
7. **Rabies:** Louis Pasteur first produced the vaccine of rabies virus. He produced it by growing it in rabbits, and then weakening it by drying the affected nerve tissue.
8. **Chicken cholera:** In 1879, Pasteur inoculate the culture of chicken cholera with a healthy chicken to infect them, but the chickens were survived. Later he inoculated this chicken with a virulent strain of the same culture, this time he noticed that they were immune to it. From this experiment he discovered that he could not infect them, even with fresh bacteria; the weakened bacteria had caused the chickens to become immune to the disease, though they had caused only mild symptoms.

The Lytic cycle consists of five steps:

1.
    1. **Attachment or adsorption**
    2. **Penetration or injection**
    3. **Synthesis or transcription**
    4. **Assembly**
    5. **Release or lysis**

ADSORPTION OR ATTACHMENT

- The first step of lytic cycle is adsorption which involves the attachment of tip of the **virus** tail at the bacterial cell wall surface via specific receptor site.
- The specific receptor of the bacterium is lipopolysaccharide although any surface structure functions as phage receptor including Flagella, pilli and carbohydrates.

PENETRATION OR INJECTION

- The actual penetration of phage into the bacterial (host cell) is mechanical.
- But it may be facilitated by digestion of cell surface structure either by phage enzymes E.g. Lysozyme or by viral activation of host degradative enzymes.
- Penetration is achieved when:

1. The tail fibers of virion attached to the cell and hold the tail firmly against the cell wall.
2. The tail sheath, contract and driving the tail core into the cell through cell wall.
3. The virus injects its genetic material into cytoplasm.

SYNTHESIS

- Once the bacteriophage genome enters the cytoplasm, the phage DNA initiate synthesis of early proteins.
- Some early proteins break the bacterial DNA and take the control of the bacterial (host) cell machinery.
- The other early proteins serve as an enzyme for replication of phage DNA.
- The newly synthesized phage DNAs produce
- Late proteins, that protein is used for phage capsid (head, tail and tail fibers).

ASSEMBLY

- After the synthesis, capsid protein assembles to form empty head, and viral DNA is packed inside it, results in the formation of numerous intact phage particles within cell.

RELEASE OR LYSIS

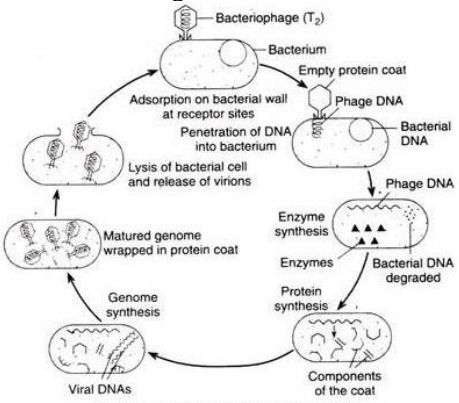
- After the assembly step is completed, viral proteins caused the lysis of host cell, and all the viral progeny are released into environment.
- The number of phage particles produces by every infected cell, known as the Burst size, and it is characteristics for each virus, ranges from 100 or more.
- 

Fig. 2.45 : Lytic life cycle of bacteriophage (T_2)

LYSOGENIC CYCLE

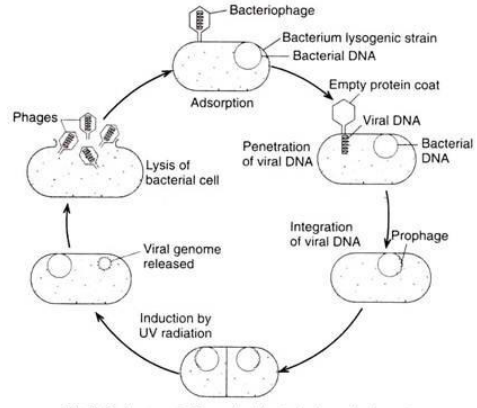
- In lysogeny, the viral DNA of the temperate phage, instead of taking control over the function of host cell machinery, it is incorporated into the host DNA and become prophage.
- A Cell that contain prophage called Lysogen.
- In this situation, the bacteria metabolize and reproduces normally, the viral DNA(genome) being transmitted to each daughter cell through all successive generation.
- Sometimes, when lysogenic bacteria are exposed to UV light or chemical, the viral DNA is removed from the host's chromosomes and the lytic cycle occurs, this process is called spontaneous induction.
- **Example** of avirulent phages are lambda phages.
- 

Fig. 2.46 : Lysogenic life-cycle of bacteriophage (λ-phage)

At first the phage is adsorbed on the wall of the host bacterium and its DNA becomes injected into the host cell. Here the phage DNA, like the lytic cycle, does not take over the protein synthesis machinery of the host cell, instead, it becomes integrated with the nucleoid of the host genome.

His integrated phage DNA is called a prophage. Thus the new composite genome replicates as one unit. The composite genome then multiplies for indefinite number of times and produces daughter lysogenic bacteria.

After a number of generations the viral genome gets detached from the composite genome and releases in the cytoplasm. This dissociation is called induction. The viral genome then enters the lytic cycle and forms temperate phages that are released by lysis of wall of the host bacterium.

Reproduction in fungi

Mainly there are three types of reproduction in fungi such as

1. Vegetative Reproduction
2. Asexual Reproduction
3. Sexual Reproduction

Vegetative Reproduction of Fungi

The vegetative reproduction of fungi is accomplished in four distinct modes such as;

1. Binary Fission
2. Budding
3. Fragmentation
4. Spore formation (asexual reproduction0

Budding

- In the budding process, a small protuberance occurs at a portion of the mother cell.
- The protuberance started to grow and increases its size
  - At the same time, the parent nucleus is started to divide and one portion of the nucleus is passed into the bud and another one remains at the mother cell, that's how the bud gets the nucleus of its parent cell.
  - Now a septum is formed between the mother cell and bud, finally, the bud get separated from the parent cell and developed into a new individual daughter cell.
  - Sometimes buds are remain attached with the mother cell and keep reproduce and form a branching appearance.

Binary Fission

- In this method first, the cell started to elongate and the nucleus divides into two new daughter nuclei.

Now two daughter nuclei are separated and during this time the cytoplasmic cleavage occurs centripetally in the middle until the mother protoplasm is formed two new daughter protoplasm.

- A double cross wall is formed between the two daughter cells and they get separated.
- *Saccharomyces pobbe* and *Psygosaccharomyces* reproduced by binary fission method.

Fragmentation

- In this method, the hyphae of fungi are fragmented and each of these fragments gives rise to a new daughter cell.

Asexual Reproduction of Fungi

In this method, algae reproduce by the formation of different types of asexual spores, which are formed in large numbers. The main function of these spores is to disseminate the species.

Types of asexual reproduction in fungi

There are mainly five types of asexual spores that are produced by fungi such as;

Sporangiosopres: These are the single-celled, formed within a specialized structure called sporangia, which is formed at the end of hyphae known as sporangiophores. There are two types of sporangiospores as motile and nonmotile. The motile spores contain flagella such as Zoospores. The nonmotile spores lack flagella such as aplanospores. **Example:** Rhizopus.

1. **Conidiospores or Conidia:** These are formed at the tip or side of hyphae. There are two types of conidiospores such as microconidia and macroconidia. The microconidia are small and single-celled conidia. While the macroconidia are large and multicelled onidia. **Example**: Penicillium, Apergillus
2. **Oidia or arthrospores:** These are single-celled spores. Oidia are developed by the disjoining of hyphal cells. **Example**: *Trichosporium, Geotrichum, Coccididious imitis.*

3. **Blastospores:** Blastospores are mainly developed by the budding process. These are formed at the tip of hyphae. Some **examples** of Blastospores are ascomycetes, basidiomycetes, zygomycetes.
4. **Chlamydospores:** These are thicked walled, single-celled spores which are mainly formed durign the adverse condition. These spores can resist adverse environmental conditions. Chlamydospores are developed when a hyphal cell or a portion of hyphae contracts, loses water, round up and gives rise to a thick-walled chalmydospore. In returning favourable consition the chlamydospores again form a new fungal cell. Some **example** of chlamydosores are ascomycetes, basidiomycetes, zygomycetes.

Sexual Reproduction of Fungi

The sexual mode of reproduction in fungi is accomplished by the fusion of two nuclei from two different parent cells. Sexual reproduction is accomplished in three distinct steps such as;

1. **Plasmogamy:** It is the first step of sexual reproduction in fungi. This step begins with the joining of two cells and fusion of protoplasm.
2. **Karyogamy:** In this step, two haploid nuclei of previously joined cells are fused and formed a diploid nucleus.
3. **Meiosis:** Now, the diploid nucleus goes through the meiosis process and again reduces the number of chromosomes to the haploid number.

Compound microscope A compound microscope is a type of microscope that uses lenses and light to magnify objects. It consists of two main parts: the objective lens, which is located near the object being viewed, and the eyepiece, which is located near the viewer's eye. The objective lens is typically composed of multiple lenses that work together to magnify the image, while the eyepiece contains a single lens that further magnifies the image for the viewer. Compound microscopes are used in a variety of settings, including scientific research, education, and industry, to observe and study small objects or organisms that are too small to be seen with the naked eye.

Principle:

Compound microscopes have a combination of lenses that enhances both magnifying powers as well as the resolving power.

- The specimen or object, to be examined is usually mounted on a transparent glass slide and positioned on the specimen stage between the condenser lens and objective lens.
- A beam of visible light from the base is focused by a condenser lens onto the specimen.
- The objective lens picks up the light transmitted by the specimen and creates a magnified image of the specimen called the primary image inside the body tube. This image is again magnified by the ocular lens or eyepiece.
- When higher magnification is required, the nose piece is rotated after low power focusing to bring the objective of a higher power (generally 45X) in line with the illuminated part of the slide.
- Occasionally very high magnification it required (e.g. for observing bacterial cell). In that case, an oil immersion objective lens (usually 100X) is employed.
- The common light microscope is also called a bright-field microscope because the image is produced amidst a brightly illuminated field. The image appears darker because the specimen or object is denser and somewhat opaque than the surroundings. Part of the light passing through or object is absorbed.

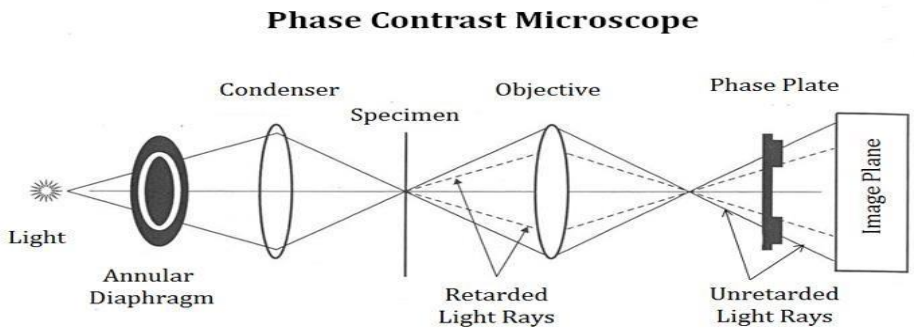
Applications: Compound microscopes are widely used in various fields for a range of applications due to their ability to magnify small samples for close observation. Some of the most common applications of a compound microscope are:

1. **Biology and Medicine:** Compound microscopes are extensively used in the fields of biology and medicine for studying the structure and function of cells, tissues, and organisms. They are used to observe microorganisms, blood cells, bacteria, viruses, and other small biological samples.
2. **Materials Science:** Compound microscopes are used to examine the microstructure of various materials, such as metals, ceramics, polymers, and composites. They are used to study the properties of materials, including their morphology, crystal structure, and defects.
3. **Quality Control:** Compound microscopes are used in manufacturing and quality control to examine products and components for defects or imperfections. They are used to inspect printed circuit boards, electronic components, and other products for quality control purposes.
4. **Education**: Compound microscopes are widely used in educational institutions to teach students about the structure and function of cells, tissues, and other biological samples. They are used to conduct experiments and demonstrations in biology and other science courses.

Phase-contrast microscopy:

Principle of Phase contrast Microscopy

When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.



The Working of Phase contrast Microscopy

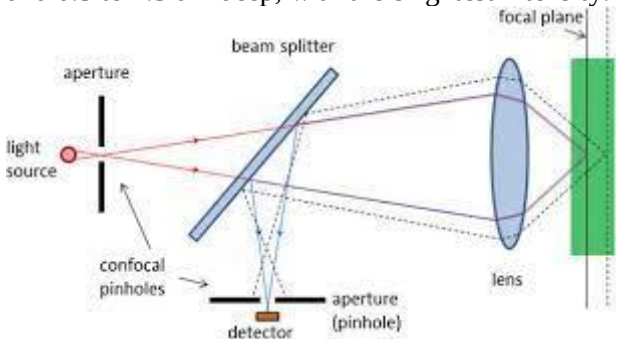
1. Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lensand focused on a specialized annulus (labeled condenser annulus) positioned in the substage condenser front focal plane.
2. Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.
3. Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phaseplate and focused at the intermediate image plane to form the final phase-contrast image observed in theeyepieces.

Confocal microscope

A confocal microscope is a type of microscope that uses laser light to produce high-resolution images of samples at different depths within the sample. It is based on the principle of confocal laser scanning microscopy (CLSM), which uses a focused laser beam to scan the sample and detect the fluorescence emitted by the sample at different depths.

Principle:

- A specimen is stained with fluorochrome is examined. When a beam of light is focused at a particular point of the fluoro-chromatic specimen, it produces an illumination that is focused by the objective lens to a plane above the objectives. The objective has an aperture on the focal plane located above it, which primarily functions to block any stray light from reaching the specimen.
- A measure of the illumination point is about 0.25 to 0.8 um in diameter, determined by the objective numerical aperture and 0.5 to 1.5 um deep, with the brightest intensity.



- The specimen normally lies between the camera lens and the perfect point of focus, known as the **plane of focus.** Using the laser from the microscope, the laser scans over a plane on the specimen (beam scanning0 or by moving the stage (stage scanning). A detector then will measure the illumination producing an image of the optical section. scanning several optical

sections, they are collected in a computerized system as data, forming a 3D image. The image can be measured and quantified.

- Its outcome is also favored by the aperture found above the objective which blocks stray light.
- Images produced by the confocal microscope have a very good contract and resolution capacity despite the thickness of the specimen. Images are stored in the high-resolution 3D image of the cell complexes including their structures.
- The main characteristic of the Confocal Microscope is that it only detects what is focused and anything outside the focus point, appears black.

The image of the specimen is formed when the microscope scanner, scans the focused beam across a selected area with the control of two high-speed oscillating mirrors. Their movement is facilitated by galvanometer motors. One mirror moves the beam from left to right on the lateral **X-axis** while the second mirror translates the beam along the **Y-axis.** After a scan on the X-axis, the beam moves rapidly back to the starting point to start a new scan, a process known as flyback. No information is collected during the flyback process, therefore the point of focus, which is the area of interest is what is illuminated by the laser scanner.

Transmission electron microscope:

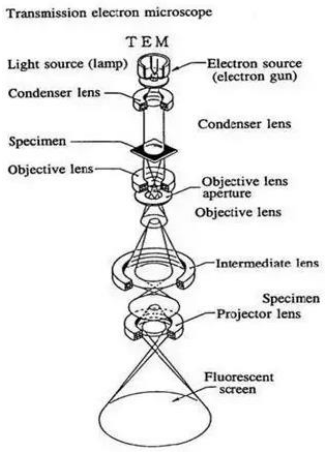
An electron cannon in a transmission electron microscope fires a beam of electrons. Electrons are accelerated to extraordinarily high speeds by electromagnetic coils and voltages of up to several million volts in the gun.

A condenser lens with a large aperture that excludes high-angle electrons focuses the electron beam into a narrow, tiny beam. Having attained their maximum velocity, the electrons rush through the ultrathin specimen, with the amount of electron transmission dependent on the sample's transparency.

The objective lens forms an image from the fraction of the beam emitted by the sample. Another component of the TEM is the vacuum system, which is necessary to prevent collisions between electrons and gas atoms.

Using either a rotary pump or diaphragm pump, a low vacuum is first created, allowing a low enough pressure for the operation of a diffusion pump, which eventually achieves a vacuum level sufficient for operations. High voltage TEMS necessitate exceptionally high vacuum levels, thus a third vacuum system may be employed.

The image created by the TEM, known as a micrograph, is projected onto a phosphorescent screen for viewing. This screen emits photons when bombarded with the electron beam. A film camera placed beneath the screen can be used to capture the image, or a charge-coupled device (CCD) camera can be used for digital capture.

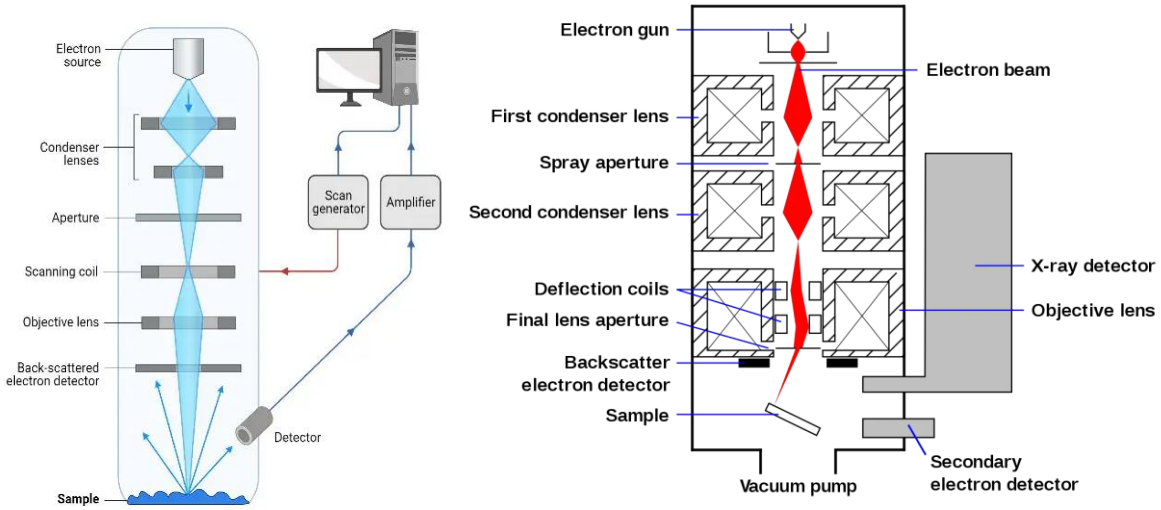


- First of all, a tungsten filament is heated, which is also called an electron gun.
- The heated tungsten filament or electron gun will start to release electron beams.
- An electromagnetic coil and high voltage(up to several million volts) applied to these electron beams to accelerate their speed (extremely high speeds).
- A condenser lens with a high aperture eliminates all the high angle electrons and focused all the electron beams into a thin, small beam.
- The high-speed electron beams are now transmitted through the specimen.
- The transmitted electron beams are focused into an image with the help of an objective lens.

- The vaccine chamber of TEM prevents the collide of electrons with the gas atoms.
- The electron beams are projected on to a phosphorescent screen, which creates an image of the specimen, also called a micrograph.
- All the images are captured by a charge-coupled device (CCD) camera, which is located underneath the screen.

Scanning Electron Microscope uses electrons that are emitted. Utilizing kinetic energy, the scanning electron microscope generates data based on the interaction between electrons. These electrons, which are secondary electrons, backscattered electrons, and diffracted backscattered electrons, are utilised to observe crystalline elements and photons. To create a picture, secondary and backscattered electrons are utilised. The secondary electrons released by the specimen are primarily responsible for detecting the specimen's morphology and topography, whilst the backscattered electrons reveal differences in the specimen's elemental makeup.

Fundamental Principles of Scanning Electron Microscopy (SEM)



Significant amounts of kinetic energy are carried by accelerated electrons in a SEM; this energy is released as a variety of signals created by electron-sample interactions when the incident electrons decelerate in the solid sample. These signals consist of secondary electrons (which produce SEM images), backscattered electrons (BSE), diffracted backscattered electrons (EBSD, used to determine crystal structures and orientations of minerals), photons (characteristic X-rays used for elemental analysis and continuum X-rays), visible light (cathodoluminescence—CL), and heat. Secondary electrons and backscattered electrons are frequently employed for imaging samples: secondary electrons are most useful for displaying morphology and topography on samples, whilst backscattered electrons are most useful for displaying compositional contrasts in multiphase samples (i.e. for rapid phase discrimination). Inelastic collisions of incoming electrons with electrons in discrete ortitals (shells) of atoms in the sample generate X-rays. As the electrons return to their lower energy states, they emit X-rays with a constant wavelength (that is related to the difference in energy levels of electrons in different shells for a given element). Thus, X-rays are produced for each element in a mineral "stimulated" by an electron beam. SEM examination is called "non-destructive" because x-rays generated by electron interactions do not result in sample volume loss, allowing repeated study of the same materials.

Spectrophotometer:

- A spectrophotometer is a crucial instrument used in the field of spectroscopy to quantitatively measure the reflection or transmission properties of a material as a function of its wavelength. It employs photometers, commonly known as spectrophotometers, which are capable of measuring the intensity of a light beam at various wavelengths. While spectrophotometry is commonly employed in analyzing ultraviolet, visible, and infrared radiation, modern spectrophotometers can also investigate a wide range of the electromagnetic spectrum, including x-ray, ultraviolet, visible, infrared, and microwave wavelengths.

- The primary purpose of a spectrophotometer is to measure the amount of light absorbed by a sample. It achieves this by passing a light beam through the sample and then measuring the intensity of the light after it has interacted with the sample. This information can be used to determine various characteristics of the sample, such as its concentration or the extent to which it absorbs specific wavelengths of light.

Principle:

- The principle of a spectrophotometer is based on the measurement of light intensity as a function of wavelength. It achieves this by utilizing a prism or grating to disperse the incident beam into different wavelengths. The device then manipulates waves of specific wavelengths to pass through the test solution. The range of wavelengths used can vary, typically ranging from 1 to 2 nanometers.

- The spectrophotometer is particularly useful for measuring the absorption spectrum of a compound, which refers to the absorption of light by a solution at each specific wavelength.

- The operation of a spectrophotometer follows the Beer-Lambert law, which establishes a linear relationship between the absorbance (the amount of light absorbed) of the solution and the product of the concentration of the sample and the path length of the light through the solution.

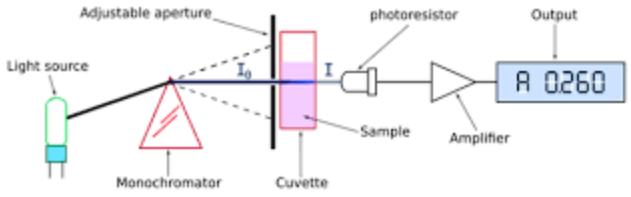
- Mathematically, this relationship can be represented as $A \propto CL$, where A represents the absorbance, C denotes the concentration of the sample, and L represents the path length.

- Alternatively, the equation can be expressed as $A = \varepsilon CL$, where ε is the molar extinction coefficient, a constant value specific to each molecule.

- The absorbance (A) is related to the transmittance (T) of light through the sample by the equation $A = -\log(T)$, where T represents the fraction of light passing through the sample, given as $T = It/Io$.

- Here, It represents the transmitted light, and Io represents the incident light.

- By utilizing a standard cuvette with a path length of 1 cm in a spectrophotometer, and knowing the values for path length (L), absorbance (A), and the molar extinction coefficient (ε), the concentration of the solution (C) can be calculated. This allows for the quantitative determination of the concentration of a sample based on the observed absorbance of light.



Principle of gel filtration

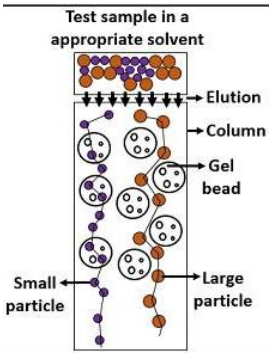
The separation of molecules based on their molecular weight or size differences is the basis of gel filtration chromatography, also known as size exclusion chromatography. A packed bed of porous gel beads is used as the stationary phase within a column in this approach.

The gel filtration medium is placed into the column to generate a bed of spherical particles in order to achieve a separation. These particles have been chosen because of their stability, inertness, lack of reactivity, and adsorptive qualities. After that, the packed bed is equilibrated with a buffer solution that fills the pores of the matrix as well as the spaces between the particles. The fixed phase is the liquid inside the pores, whereas the mobile phase is the liquid outside the particles.

The stationary phase in gel filtration chromatography is a porous polymer matrix with pores that are entirely filled with the mobile phase solvent. The molecules in the sample are pumped through specialised columns containing this microporous packing material, also known as gel beads.

The separation is based on the premise that molecules larger than a particular size are fully barred from entering the pores of the gel beads, whilst smaller molecules can enter the pores partially or completely. Larger molecules are unable to enter the gel matrix and pass through the column freely, eluting earlier as the mobile phase runs through the column. Smaller molecules, on the other hand, can enter the gel pores to variable degrees, causing them to be delayed or slowed in their elution from the column.

The size exclusion chromatography principle is based on the separation of biomolecules based on differences in molecular weight or size. As the packing material in the chromatography column, spherical gel beads with appropriate porosity are used in the gel filtration procedure. The components of a liquid combination flow through this column, and depending on the elution limit, some molecules elute sooner or later.



The elution limit is a parameter that affects whether molecules are retained or excluded within the packing material. Those with a larger molecular weight than the elution limit will elute first, while those with a lower molecular weight or size will elute later. In gel filtration chromatography, particles are separated based on their size in this manner.

Ion exchange chromatography:

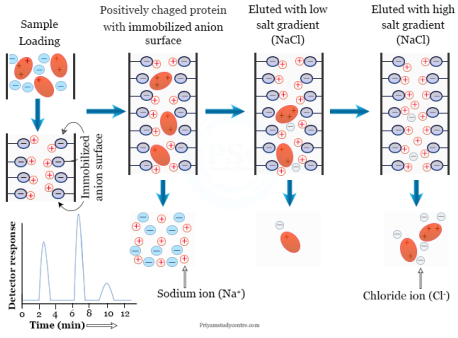
The working principle of ion exchange chromatography is based on the attraction between the oppositely charged stationary phase, known as an ion exchanger, and the analyte molecules.

The ion exchangers consist of charged groups that are covalently linked to an insoluble matrix. These charged groups can be either positively or negatively charged. When the ion exchanger is suspended in an aqueous solution, the charged groups on the matrix become surrounded by ions of the opposite charge, creating an “ion cloud.” Within this ion cloud, ions can be exchanged reversibly without altering the nature and properties of the matrix.

Ion exchange chromatography involves both a mobile phase and a stationary phase, similar to other column-based liquid chromatography techniques. The mobile phase is an aqueous buffer system into which the mixture to be separated is introduced. The stationary phase is made up of an inert organic matrix that is chemically modified with ionizable functional groups, also known as fixed ions. These fixed ions carry oppositely charged ions that can be displaced during the chromatographic process.

The equilibrium between the mobile phase and the stationary phase gives rise to two types of ion exchange chromatography: anion exchange and cation exchange. Anion exchange chromatography separates analytes based on their binding to positively charged groups on the stationary phase, while cation exchange chromatography separates analytes based on their binding to negatively charged groups on the stationary phase.

The separation occurs by binding analyte molecules to the charged groups fixed on the stationary phase, which are in equilibrium with free counter ions in the mobile phase. The differences in net surface charge among the analytes result in their differential binding and subsequent separation. Cations are separated on a cation-exchange resin column, while anions are separated on an anion exchange resin column.



Overall, the working principle of ion exchange chromatography involves the selective binding of analyte molecules to charged groups on the stationary phase, driven by the attraction between opposite charges. This allows for the separation of differently charged or ionizable compounds based on their interactions with the ion exchanger.

High-Performance Liquid Chromatography (HPLC)

The principle of High-Performance Liquid Chromatography (HPLC) is based on the separation of components in a mixture using a stationary phase and a mobile phase in a high-pressure system. HPLC offers high resolution and sensitivity, making it a valuable technique for analyzing complex mixtures.

The separation process in HPLC occurs in a separation column, which contains a stationary phase. The stationary phase consists of small porous particles, typically made of a granular material. These particles provide a large surface area for interactions with the sample components.

The mobile phase, often a solvent or solvent mixture, is forced through the separation column at high pressure. This is achieved using a pump that generates the necessary pressure to drive the mobile phase through the system. The mobile phase is responsible for carrying the sample through the column, allowing the components to separate.

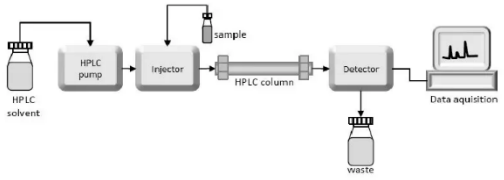
To introduce the sample into the mobile phase flow, an injection system is used. This typically involves a valve connected to a sample loop, which is a small tube or capillary made of stainless steel. The sample is injected into the mobile phase flow using a syringe, allowing it to mix with the mobile phase before entering the separation column.

Once inside the column, the individual components of the sample migrate through the column at different rates. This differential migration occurs because each component interacts with the stationary phase to varying degrees. Components that have stronger interactions with the stationary phase will be retained longer and will migrate more slowly through the column, while those with weaker interactions will move through the column more quickly.

After passing through the separation column, the individual substances are detected by a suitable detector. Various types of detectors can be used in HPLC, including UV/Visible light detectors, refractive index detectors, fluorescence detectors, and mass spectrometers. The detector generates a signal proportional to the concentration of the separated components.

The detector signal is then sent to the HPLC software on a computer, where it is processed and analyzed. The software generates a chromatogram, which is a graphical representation of the detector signal as a function of time. The chromatogram displays peaks corresponding to the separated components, allowing for their identification and quantification.

By comparing the retention times of the peaks in the chromatogram with those of known standards or reference compounds, the individual substances in the sample can be identified. The peak areas or heights can be used to quantify the amount of each component present in the mixture.



In conclusion, the principle of High-Performance Liquid Chromatography (HPLC) involves the separation of components in a mixture using a stationary phase and a mobile phase in a high-pressure system. Through interactions with the stationary phase, the components migrate through the separation column at different rates. The separated components are detected and analyzed using suitable detectors, and the resulting chromatogram allows for the identification and quantification of the substances present in the sample.

NATURAL MEDIA

These media are prepared from natural ingredients and extract, which can provide natural environment to the organisms aimed for isolation and cultivation.

These media were first employed by **Winogradsky** to isolate organisms from soil. The type of natural extracts used depends on the type of organisms to be Isolated.

e.g. Soil extract for isolation of soil organisms. Potato extract for isolation of plant pathogens etc.

These media are usually preferred for primary isolation of organisms from their ***natural habitats***.

SYNTHETIC MEDIA

These media are commonly known as chemically defined media. These are the media where the exact chemical nature and composition of all the ingredients used in the preparation of media is known. These media are prepared by adding

- Known concentration of ***sugar*** or other source of carbon and energy, whose chemical composition is known.
- Nitrogen source, usually as ***ammonium*** or ***nitrate salt*** or ***Liquor ammonia***.
- Salt solutions to provide ***mineral nutrients*** requirements.
- Amino acids and growth factors, If required, in known concentration.

These media are widely used to study

1. Influence of various chemicals on the growth
2. Bioassay etc.

• COMPLEX MEDIA

The media, where, the ***exact chemical*** composition of all the Ingredients of the medium is ***not known***, are called complex media.

These media are widely used for growth and cultivation of microorganisms. These media are relatively easy to prepare, comparatively cheaper than synthetic media and allow better growth of organisms. Media containing peptone, beef extract etc. fall in this category.

. Indicator or differential media

This media shows visible changes due to the presence of an indicator. It differentiates bacteria based on colony color growing on the same plate; biochemical characteristics show organism's growth with chemical indicators like neutral red, phenol red, methylene blue.

Certain media are designed to recognize different bacteria based on their colony color. Various approaches include incorporating dyes, metabolic substrates, etc., so those bacteria that utilize them appear as differently colored colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

Examples of Indicator or differential media

Mannitol salt agar (mannitol fermentation shows yellow color colonies); blood agar is used to differentiate between hemolytic and non-hemolytic. MacConkey agar produces pink colonies due to lactose utilization and, non-lactose shows pale color colonies.

Serial dilution

Definition: It 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.

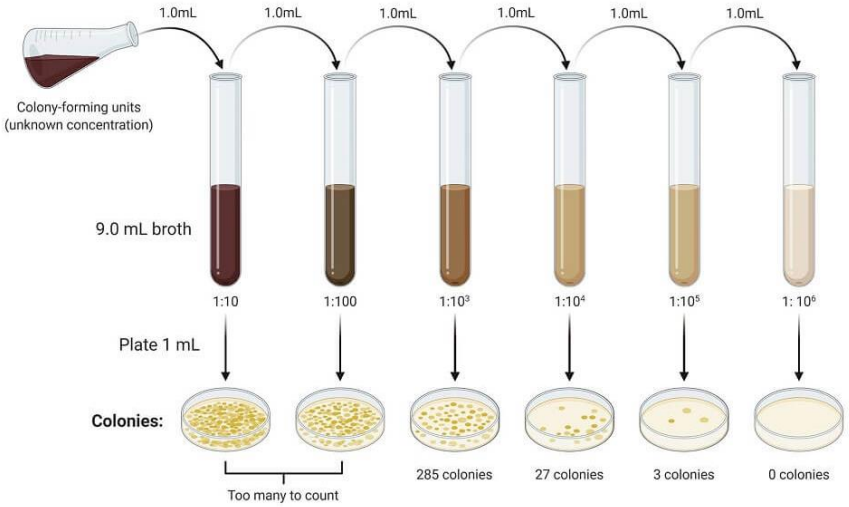
Objective of the serial dilution method

- The objective of the serial dilution method is to estimate the concentration (number of organisms, bacteria, viruses, or colonies) of an unknown sample by the 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.

Procedure of serial dilution:

The following is the procedure for a ten-fold dilution of a sample to a dilution factor of 10^{-6}:

1. The sample/culture is taken in a test tube and six test tubes, each with 9 ml of sterile diluents, which can either be distilled water or 0.9% saline, are taken.
2. A sterile pipette is taken.
3. 1 ml of properly mixed sample/culture is drawn into the pipette.
4. The sample is then added to the first tube to make the total volume of 10 ml. This provides an initial dilution of 10^{-1}.
5. The dilution is thoroughly mixed by emptying and filling the pipette several times.
6. The pipette tip is discarded, and a new pipette tip is attached to the pipette.
7. Now, 1 ml of mixture is taken from the 10^{-1} dilution and is emptied into the second tube. The second tube now has a total dilution factor of 10^{-2}.
8. The same process is then repeated for the remaining tube, taking 1 ml from the previous tube and adding it to the next 9 ml diluents.
9. As six tubes are used, the final dilution for the bacteria/cells will be 10^{-6} (1 in 1,000,000).



CFU/mL = (no. of colonies x dilution factor) / volume of culture plate

Example: (285 colonies x 10^3) / 1= 2.85 x 10^5 CFU/mL in sample

Serial dilution applications:

Serial dilution is performed in a number of experimental sciences like biochemistry, pharmacology, physics, and homeopathy.

1. Serial dilution is used in microbiology to estimate the concentration or number of cells/organisms in a sample to obtain an incubated plate with an easily countable number of colonies.
2. In biochemistry, serial dilution is used to obtain the desired concentration of reagents and chemicals from a higher concentration.
3. In pharmaceutical laboratories, serial dilution is performed to receive the necessary concentration of chemicals and compounds, as this method is more effective than individual dilutions.
4. In homeopathy, homeopathic dilutions are used where a substance is diluted in distilled water or alcohol. It is believed that dilution increases the potency of the diluted substance by activating its vital energy.

Limitation:

1. An error might occur during the propagation of the sample, and the transfer inaccuracies lead to less accurate and less precise transfer. This results in the highest dilution to have the most inaccuracies and the least accuracy.
2. Because serial dilution is performed in a stepwise manner, it requires a more extended period of time which limits the efficiency of the method.
3. Serial dilution only allows the reduction of bacteria/cells but not the separation of bacteria/cells like in other techniques like flow cytometry.

4. This technique also requires highly trained microbiologists and experts in aseptic techniques.

Plating methods (pour, spread, streak)

The pour plate method is a microbiological laboratory technique for isolating and counting the viable microorganisms present in a liquid sample, which is added along with or before molten agar medium prior to its solidification. This technique is generally used to count viable microorganisms in the given sample by enumerating the total number of colony-forming units (CFUs) within and/or on the surface of the solid medium. It is mostly used for enumerating bacteria; however, Actinobacteria, molds, and yeasts can also be isolated and enumerated.

Prior to performing the pour plate technique, the sample must be serially diluted to make the microbial load in the sample between 20 – 300 CFU/mL (suitable colony counting range is 20 – 200, some consider it to 30 – 300, and in average it is taken as 25 – 250). If the sample is liquid, then it can be serially diluted with sterile distilled water or sterile broth. If the sample is solid or semisolid, it must be first emulsified and then serially diluted to reduce microbial load up to the permitted range.

The sample is either added to the Petri plate and then the molten agar medium is poured over it, or the sample is mixed with the molten agar medium prior to pouring. After pouring in the Petri plate, the plate must be swirled quickly to properly mix the sample with the medium. The mixed medium is allowed to solidify and is incubated under the suitable condition to grow the microorganisms present in the sample.

Following the incubation, the numbers of isolated colonies are counted. If the colonies are uncountable or fused or more than 300 CFU/mL or less than 20 CFU/mL, it is recommended to repeat the process for getting the optimum count.

Objective of pour plate technique

1. To isolate the microorganisms from the liquid specimen (or suspension)
2. To calculate viable microbial load by counting colony formation unit (CFU) per mL
3. To isolate the pure culture of microorganisms from a mixed population
4. To isolate microorganisms in discrete colonies in order to study colony characters

Principle of pour plate technique

The pour Plate Method is based on the fact that when an agar medium mixed with microorganisms is incubated, each of the viable microorganisms will multiply forming a separate colony. In this method, a certain volume, usually 1 mL, of the serially diluted liquid sample is mixed properly with approximately 15 mL of specific molten agar medium of about 40 – 45°C (less than 50°C) in a Petri plate. The medium is allowed to solidify and is incubated, usually at 37°C for 24 – 48 hours. Following the incubation, the viable microorganisms in the sample will grow into visible colonies on the surface of and within the medium. The visible colonies can be counted and CFU/mL can be calculated using the following formula;

$$CFU/mL = \frac{Total\ number\ of\ colonies\ obtained \times dilution\ factor}{volume\ of\ specimen\ used\ (aliquot)}$$

Requirements for pour plate technique

1. **Liquid Specimen (or suspension of the solid sample)**
   For the pour plate, the sample must be either liquid or in suspension form. The solid sample must be suspended in a suitable solvent that doesn't influence the growth of any microorganisms or react with any material of the media.
2. **Suitable Solid Culture Media**
   Specific culture media is used for the isolation and differentiation of suspected (or specific) bacteria. The culture medium is a solid agar medium that is melted and is at a temperature of 40 – 45°C.
3. Petri Plates
4. Test-tubes
5. Sterile Distilled Water (or Sterile Broth)
6. Micropipette (or Graduated Pipette)
7. Other Laboratory Facilities

Procedure for pour plate technique

The general procedure for performing the pour plate method can be summarized as follows:

Sample preparation:

- If the sample is in liquid form, serially dilute it to make the microbial load to the range of 20 – 300 CFU/mL. (Prior pilot test may give exact value. You can prepare serial dilution up to 10^{-10} and use different dilutions.)
- If the sample is in solid or semisolid form, dissolve it in sterile distilled water or sterile broth, or any other solvent. (Generally, 1 gm sample is mixed with 9 ml of solvent to get the concentration of 10^{-1} gm/mL.)

Media preparation:

- Suitable media (general-purpose media like Nutrient Agar and Plate Count Agar for bacteria, and Potato Dextrose Agar or Sabouraud Dextrose Agar for fungi) are prepared and autoclaved. The media is allowed to cool to about 40 – 45°C (maximum up to 55°C), but don't let it solidify.
- If the media is prepared already and solidified, melt it by placing it over a water bath or other heat source.
- If you want to mix the sample in media prior to pouring it into the Petri plate, you can either add approximately 15 mL of media in one test tube or beaker and autoclave it. Alternatively, a fixed volume of media can be prepared in a large beaker or bottle and a sample can be added later by calculating the volume which will be equivalent to 1 mL sample per about 15 mL of media.

Arrange sterile Petri plates. Label at the edge of the bottom of the plate with the dilution factor, date, name, sample ID, and other required information.

Inoculation:

Method – I

Dispense 1 ml of diluted sample in the center of the Petri plate using a sterile micropipette or calibrated pipette.

Open the lid of the bottle and flame its mouth. Pour about 15 mL of sterilized molten media at the appropriate temperature above the sample.

Close the lid of the plate then mix the sample in the media properly by gently swirling the plate. The plate is generally swirled in an "S" or "8" shape.

(Put sample at specific dilution in the plate labeled with the specific dilution factor.)

Method – II

In a tube with about 15 mL of molten media at a suitable temperature, add 1 mL of sample. Mix the sample properly in the media. Pour the media into a sterile Petri plate.

Close the lid of the Petri plate. Allow the media to completely solidify.

Incubate the plate in an inverted position under suitable incubation conditions (mostly for 24 hours at 37°C).

Applications:

1. Used to isolate and enumerate viable bacteria and fungi (calculate CFU/ml) from suspensions or liquid samples.
2. Used in food and pharmaceutical industries to isolate microorganisms and calculate CFUs from raw materials and products, like water, beverages, foodstuff, tissue samples, etc. This will help in quality control to ensure whether the product is safe to consume or not.
3. Used to isolate and enumerate microorganisms from soil to study soil microflora.
4. Used to generate growth curves while studying microbial metabolisms and biochemical features, and the effects of environmental factors on microbial growth.
5. Used in getting discretely isolated colonies for obtaining pure culture and studying biochemical characters.
6. Used in separating pure culture from mixed cultures.

Spread Plate Method is one of the widely used culture techniques in microbiology laboratories due to its ease and simplicity. This method is suitable for aerobic and facultative aerobic microorganisms. It is an easy, simple, and economical method; however, it requires the sample to be in liquid or suspension.

Definition: The spread plate method is a technique for isolating and counting the viable microorganisms present in a liquid sample by spreading a certain volume of the sample over an appropriate solidified culture media.

Following the incubation, in a successful spread plate, there will be the formation of evenly distributed discrete colonies all over the surface of the culture media.

This technique is used for isolating and counting the total number of viable microorganisms (i.e. calculating the colony-forming units per mL (CFU/mL) in the given sample. It is also for propagating the old culture and mass producing them. It can be used for all of the culturable bacteria and fungi.

Objective of spread plate method

1. To isolate the microorganisms from the liquid specimen (or suspension)
2. To calculate viable microbial load by counting colony formation unit (CFU) per mL
3. To isolate the pure culture of microorganisms from a mixed population
4. To isolate microorganisms in discrete colonies in order to study their colony characters
5. To obtain sufficient growth for conducting antimicrobial sensitivity testing and biochemical studies

Principle of spread plate method

When a diluted liquid specimen containing one or more microorganisms, same or different species, is spread over a suitable solid agar media, each of the viable microorganisms will multiply forming a separate colony. These colonies can be counted and expressed in terms of the CFU/mL which can be used to calculate the microbial load in the sample.

A certain volume of the diluted sample, mostly 0.1 mL (0 to 1 mL can be used), is dispensed over the surface of a pre-sterilized solid medium. Usually, a bent glass rod or swab or glass beads are used to spread the sample. Following the incubation for usually 24 – 48 hours at 37°C, the viable microorganisms in the sample will grow into discrete visible colonies on the surface of the medium. The visible colonies can be counted and CFU/mL can be calculated using the following formula;

$$CFU/mL = \frac{Total\ number\ of\ colonies\ obtained \times dilution\ factor}{volume\ of\ specimen\ used\ (aliquot)}$$

Requirement:

1. **Liquid Specimen (or suspension of the solid sample)**
The sample must be either in liquid form or in suspension form. The solid sample must be dissolved in a suitable solvent.

The sample must be diluted to an appropriate concentration so that we can get a well-isolated 20 – 300 CFU/mL per plate after the incubation.

2. **Pre-solidified Suitable Solid Culture Media Plates**
Appropriate culture media which supports the growth of all desired or probable microorganisms in the specimen must be used. The media must be solidified properly before spreading the diluted sample. The media can be prepared and stored at 4°C for future use. If freshly prepared, make sure the media is completely solidified.

3. Test Tubes
4. Sterile Distilled Media (or Sterile Broth)
5. Micropipette
6. Spreaders
7. Ethanol (70%)
8. Bunsen Burner
9. Other Laboratory Facilities

The general procedure of the spread plate method can be summarized as:

1. Arrange all the requirements, put on the PPE, sterilize the work surface, and allow all the samples and media to come to room temperature if were refrigerated.
2. **Sample preparation**

- Liquid samples must be serially diluted to reduce the microbial load to the range of 20 – 300 CFU/mL. (If the sample is assumed to be sterile or the expected microbial load is very low, we can escape the dilution. The prior pilot test may give an exact value. You can prepare serial dilution up to 10^{-10} and use different dilutions.)
- If the sample is solid or semisolid, dissolve it with a suitable solvent to prepare its suspension. The suspension then should be serially diluted to reduce the microbial load at the desirable range. (Generally, 1 gm sample is mixed with 9 ml of solvent to get the concentration of 10^{-1} gm/mL.)
- Arrange the spreader. The glass rod must be sterilized. For this dip the rod in 70% ethanol solution and flame it over a Bunsen burner. Let the rod cool. (You can check if the rod is cold enough or not by touching a corner of solidified media. If you heard a sizzling sound or if the media melts, then cool the rod further.)
Likewise, the beads can be put in a bottle or beaker and then autoclaved to sterilize them.

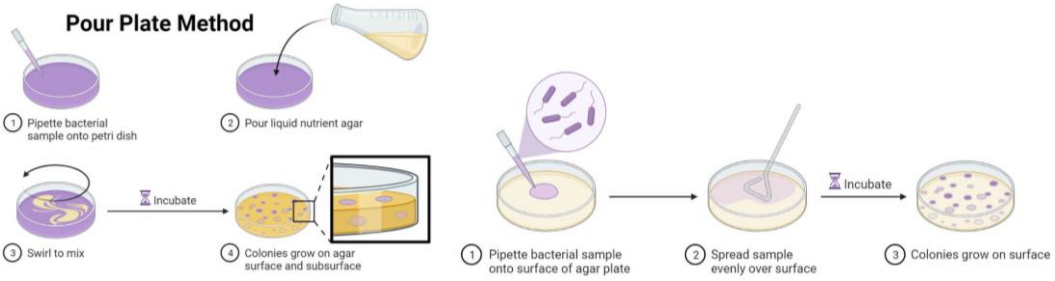
4. Label the plate at its bottom edge with the dilution factor, date, name, sample ID, and other required information.
5. The spreading can be done by either of the following two methods, viz.:

Spreading with a bent glass or metal rod

1. Open the lid of the plate and dispense 0.1 mL of the diluted sample in the center of the Petri plate using a calibrated pipette or micro-pipette.
2. Using a sterile bent glass rod uniformly spread the sample all over the plate.

- If you are using a turntable, spin it slowly and then hold your spreader gently on the surface of the media touching the sample, and gradually spread the sample uniformly all over the surface of the media. Moving the rod back and forth will allow you to spread the sample.
- If you are performing it manually, hold the plate in your left hand (or in your right if you are left-handed) or you can also put it still on the bench. You must move the spreader in either a circular path or in a back and forth motion to spread the sample evenly. At last, move the spreader in a circular motion around the edge of the plate to make sure that the sample is spread even at the corner.

3. Put on the lid, leave the plate in an upright position and allow the sample to be absorbed for around 5 minutes. Then incubate the plate in an inverted position.



Spread Plate Method

Streak plate method

Definition: It is a microbiological laboratory technique of isolating pure cultures, and/or getting well-isolated colonies of bacteria from a mixed population. It is mostly used to get pure cultures of bacteria; however, yeasts can also be isolated by this method. It is one of the most commonly used aseptic techniques in microbiology to isolate and propagate bacteria. It is a mechanical isolation technique used in microbiology, commonly known as the "**streaking method**".

This method dilutes the bacterial load, over the surface of agar medium, successively as streaking proceeds, and ultimately only a few bacterial cells will be inoculated at the end giving well-isolated colonies in the final streaks. Thus, this method mechanically isolated the bacteria from a mixed population of either the same or different species. After inoculation, the same types of colonies are seen in the terminal streaks if the specimen contained single species, whereas, different types of colonies may be seen if the specimen contained different species.

Objectives:

1. To obtain a pure culture of bacteria from a mixed culture
2. To obtain well-isolated colonies
3. To propagate bacteria

Principle: The streak plate method is based on dilution during the process of mechanical spreading of inoculum over the surface of solidified culture media in order to obtain well-isolated colonies of the sample at the terminal streaks. Sample can be either colony on solid media or suspension in broth. The sample is picked by using different tools, mostly using a sterile inoculating loop or swab. The sample is placed over a surface of sterile solid media at one edge of the petri dish and a smear is prepared. Using the tool, the smear is successively streaked over the agar medium on different patterns. As the streaking proceeds, the inoculum is gradually diluted to the point where bacterial cells are separated as individual cells or as a colony-forming unit (CFU) at a gap of a few millimeters. When these inoculated plates are incubated, the isolated bacterium or a CFU will give rise to a well-isolated colony. This will allow us to get a pure culture as well as describe the colony morphology of the organism.

Based on the pattern of streaking, the streak plate method can be classified into 4 types, viz.: Quadrant Streaking, T-Streaking, Continuous Streaking, and Radiant Streaking.

Requirements:

1. **Streaking tool**

This is a sterile tool used to streak the specimen over the surface of culture media. The tools used for streaking are cotton swabs, inoculating loop (both metal and plastic), toothpicks, and wooden or metal or plastic sticks/wires. The most commonly used one is inoculating loop (nichrome wire loop).

(In this whole article, we will talk about inoculating loop.)

2. **Sample culture**
   Sample bacteria may be in the form of suspension, liquid broth, or colonies over solid media. The sample is picked by using an inoculating loop and transferred over the surface of fresh culture media to perform streaking.
3. **Solid culture media**
4. **Bunsen burner and other laboratory facilities**

Procedure:

The general procedure of the streak plate method can be summarized as:

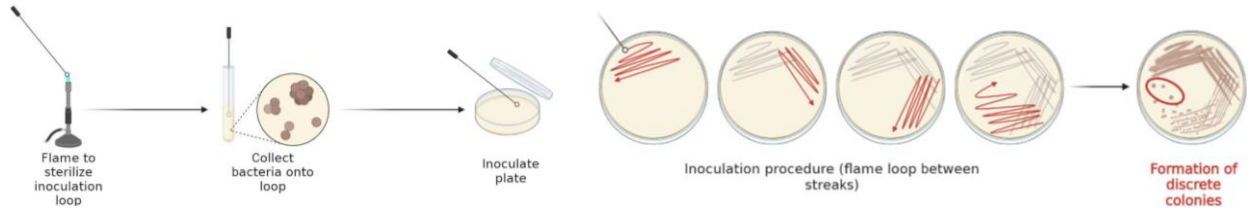
1. Arrange all the requirements, put on the PPE, sterilize the work surface, and allow all the samples and media to come to room temperature if were refrigerated.
2. If the sample is very concentrated then dilution can be helpful to get the isolated colonies. (But it is not compulsory as the sample will be diluted during the streaking process.)
3. Sterilize the inoculating loop by flaming and allow it to cool. Pick a small portion of the isolated colony. (if the sample is in the suspension then take a loopful of the sample)
   The inoculating procedure is different according to the method of streaking, let us deal with each type:

Continuous Streaking Procedure

1. Lift the Petri plate in your left hand and hold it at an angle of 60^0. (if you are left-handed, hold the plate in your right hand)
2. Place the loop at one end of the plate and start streaking the inoculum from that point in a continuous movement to the center of the plate.
3. Rotate the plate by 180^0 and without sterilizing the loop, follow the step (ii) to streak the remaining half of the plate.

Applications:

1. Used to obtain a pure culture from the mixed culture in order to perform morphological, biochemical, and molecular tests to identify and for other applications.
2. Used to define the specimen as pure or mixed species.
3. Used to study colony characters of bacteria.
4. Used to produce a colony of genetically identical individuals
5. Used in inoculation of clinical specimens in diagnostic laboratories to grow isolated colonies of pathogen
6. Used in urine culture to isolate pathogens and semi-quantify the uropathogens to determine the significance of the infection. A calibrated loop is used for this purpose.



Cultivation, maintenance and preservation/stocking 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.

Agar slant culture:

- 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.

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.

Paraffin method:

- This is a simple and most economical method of maintaining pure cultures of bacteria and fungi.
- In this method, sterile liquid paraffin in poured over the slant (slope) of culture and stored upright at room temperature.
- The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium.
- This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.

Saline suspension:

- Sodium chloride in high concentration is frequently an inhibitor of bacterial growth.
- Bacteria are suspended in 1% salt solution (sublethal concentration in screw cap tubes to prevent evaporation).
- The tubes are stored at room temperature. Whenever needed the transfer is made on agar slant.

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 $-78^{o}C$.
- 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.

Cultivation of anaerobic bacteria:

Aerobic Bacteria

Main Principle: Provide Oxygen

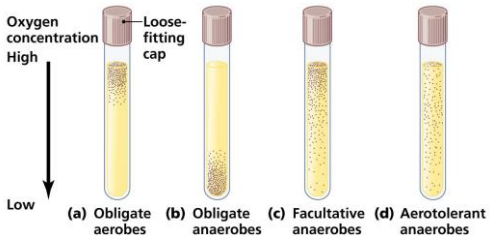
Atmospheric condition is generally satisfactory for the culture of aerobes or facultative anaerobes but for the growth of many aerobes, it is necessary to provide extensive aeration. Forced aeration of cultures is therefore frequently desirable and can be achieved either by vigorously shaking the flask or tube on a shaker or by bubbling sterilized air into the medium. When aerobic organisms are to be grown in large quantities, it is advantageous to increase the exposure of the medium to the atmosphere. This can be accomplished by dispensing the medium in shallow layers or by providing aeration by constantly shaking the inoculated liquid cultures.

B. Cultivation of Anaerobic Bacteria

Main Principle: reduce the O_2 content of the culture medium and remove any oxygen already present inside the system or in the medium.

Oxygen is ubiquitous in the air so special methods are needed to culture anaerobic microorganisms. A number of procedures are available for **reducing the O2 content** of cultures; some simple but suitable mainly for less sensitive organisms, others more complex but necessary for the growth of strict anaerobes.

- Bottles or tubes filled completely to the top with culture medium and provided with a tightly fitting stopper. Suitable for organisms not too sensitive to small amounts of oxygen.
- Addition of a reducing agent that reacts with oxygen and reduces it to water e.g., Thioglycolate in thioglycolate broth. After thioglycolate reacts with oxygen throughout the tube, oxygen can penetrate only near the top of the tube where the medium contacts air.
- Obligate aerobes grow only at the top of such tubes.
- Facultative organisms grow throughout the tube but best near the top.
- Microaerophiles grow near the top but not right at the top.



- Anaerobes grow only near the bottom of the tube, where oxygen cannot penetrate.

A redox indicator dye called resazurin is added to the medium because the dye changes color in the presence of oxygen and thereby indicates the degree of penetration of oxygen into the medium.

Strict anaerobes, such as methanogenic bacteria can be killed by even brief exposure to O_2. In these cases, a culture medium is first boiled to render it oxygen- free, and then a reducing agent such as H_2S is added and the mixture is sealed under an oxygen-free gas. All manipulations are carried out under a tiny jet of oxygen-free hydrogen or nitrogen gas that is directed into the culture vessel when it is open, thus driving out any O_2 that might enter. For extensive research on anaerobes, special boxes fitted with gloves, called anaerobic glove boxes, permit work with open cultures in completely anoxic atmospheres.

Stringent anaerobes can be grown only by taking special precautions to exclude all atmospheric oxygen from the medium. Such an environment can be established by using one of the following methods:

1. **Pre-reduced media**
   During preparation, the culture medium is **boiled** for several minutes to drive off most of the dissolved oxygen. A reducing agent e.g., cysteine, is added to further lower the oxygen content. Oxygen-free N_2 is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes which are flushed with oxygen-free nitrogen, stoppered tightly, and **sterilized by autoclaving**. Such tubes are continuously flushed with oxygen-free CO_2 by means of a cannula, restoppered, and incubated.

Of all the methods available for the cultivation of anaerobic bacteria, exclusion of oxygen from the medium is the simplest method. During preparation, the liquid culture medium is boiled by holding in a boiling water both for 10 minutes to drive off most of the dissolved oxygen.

Liquid media soon become aerobic thus a reducing agent (e.g., cysteine 0.1%, ascorbic acid 0.1%, sodium thioglycollate 0.1%), is added to further lower the oxygen content.

Oxygen-free N_2 is bubbled through the medium to maintain anaerobic condition. The medium is then dispensed into tubes, which are stoppered tightly and sterilized by autoclaving. Such tubes can be stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen free CO_2 by means of gas cannula, re-stoppered, and incubated.

2. **Anaerobic Chambers**
   This refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Culture media are placed within the chamber by means of an airlock which can be evacuated and refilled with N_2. Any oxygen in the media is slowly removed by reaction with hydrogen, forming water; this reaction is aided by a palladium catalyst. After being rendered oxygen-free, the media are inoculated within the chamber (by means of the glove ports) and incubated (also within the chamber).

   Anaerobic chamber is an ideal anaerobic incubation system, which provides oxygen- free environment for inoculating media and incubating cultures. It refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Glove ports and rubber gloves are used by the operator to perform manipulations within the chamber. There is an air-lock with inner and outer doors.

Culture media are placed within the air-lock with the inner door. Air of the chamber is removed by a vacuum pump connection and replaced with N_2 through outer doors.

The culture media are now transferred from air-lock to the main chamber, which contains an atmosphere of H_2, CO_2, and N_2. A circulator fitted in the main chamber circulates the gas atmosphere through pellets of palladium catalyst causing any residual O_2 present in the culture media to be used up by reaction with H_2.

When the culture media become completely anaerobic they are inoculated with bacterial culture and placed in an incubator fitted within the chamber. The function of CO_2 present in the chamber is that it is required by many anaerobic bacteria for their best growth.

Growth curve

Definition

When a few **bacteria** are inoculated into a liquid growth medium or any solid culture media and the population is counted at intervals, it is possible to plot a **bacterial growth curve** that shows the growth of cells over time.

Growth

- It is orderly increase in a cellular constituent.
- When microorganisms reproduce by binary fission or budding then it also leads to increase in the number of cells.

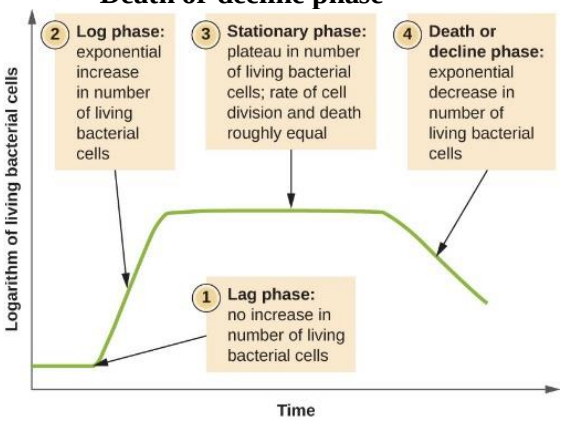
Growth curve

- Growth of microbial population is measured periodically by plotting log number of viable bacteria against time on a graph then it gives a characteristics curve which is also called as **growth curve** or **growth cycle.**
- Due to exponential bacterial growth pattern, the growth curve of microbial population is hyperbolic.
- In batch culture, there is an initial phase of no growth (**lag phase**), which is followed by rapid growth (**exponential growth**), then there is leveling off (**stationary phase**) and finally a decline in the viable cell count (**death or decline phase**).
- Between each of these phases, there is a transition period (curve portion).

Different phases of bacterial growth curve

- **Lag phase**
- **Log phase or exponential phase**

- **Stationary phase**
- **Death or decline phase**



Lag phase

- On adding microorganisms into a fresh culture medium, usually no immediate doubling of the population, so this period is called the **Lag phase.**
- During this phase microorganisms does not grow or divide immediately, but take sometimes to adjust to the environment, so this phase is also called **adaptation phase.**
- But this does not mean that the cells are dormant; on the contrary, during this stage the individual cells increases in size beyond their normal dimensions.
- At the end of the phase, microorganisms are well prepared for the for the cell division.

Exponential phase

- During **exponential phase**, microbial cells are divides at the maximal rate possible given their genetic potential, the nature of the medium and the conditions under which they are growing.
- Growth of microorganisms during this phase is constant and there is doubling in number at regular interval.
- Microbial Growth during this phase is balanced, means all the cellular constituents are produced at a constant rate relative to each other.
- During this period on plotting log of the number against time results produces straight line.
- In this of growth, population is most nearly uniform in terms of metabolic activity, chemical composition of cells and other physiological characteristics.

Stationary phase

- The phase during which growth reaches a state where there is no net increase in the population, is called **stationary phase**.
- Due to a closed system, eventually population growth of microorganisms ceases during this phase and the growth curve becomes horizontal.
- The population remains constant because of balance between reproduction rate and equivalent death rate or the growth of the population ceases but remain metabolically active.
- Microbial population enters into the stationary due to several reasons, increased bacterial cell density, depletion of nutrition, oxygen limitation, growth of microorganisms is slow and accumulation of the toxic secondary metabolites.

Death or decline phase

- During death phase, number of viable cells is decreases exponentially at constant rate.
- Bacteria die at different rate as they grow at different rates.
- It was assumed that the variety of **environmental** conditions causes death of the microorganisms like depletion of essential nutrients and the buildup of toxic wastes.

Measurements of microbial growth

Introduction

- When a few **bacteria** are inoculated into a liquid growth medium or any solid culture media and the population is counted at intervals.
- The growth of microbial populations can be measured in a number of ways. Some methods measure cell numbers; other methods measure the population's total mass, which is often directly proportional to cell numbers.
- It is orderly increase in a cellular constituent.
- When microorganisms reproduce by binary fission or budding then it also leads to increase in the number of cells.

Measurements of microbial growth

- There are various ways to measures microbial growth for the determination of growth rates and generation times.
- For the measurement of growth either mass or population number is followed because growth leads to increase in both.
- Growth can be measured by one of the following types of measurements:

1.
    1. **Cell count** this method involves the measurement of growth either by microscopy or by using an electronic particle counter or indirectly by a colony count.
    2. **Cell mass** in this growth can be measured directly by weighing or by a measurement of nitrogen concentration in cells or indirectly by the determination of turbidity using spectrophotometer.
    3. **Cell activity** in this growth can be measured indirectly by analysis of the degree of biochemical activity to the size of population.

Bacterial growth can be measured either by:
Colony counting or cell counting,

(ii) By weighing the cell i.e. cell mass measurement or

(iii) By cell activity (turbidity method) measurement.

(i) Cell Counting by Direct Microscopic Count Method

Direct microscopic counts are rapid but limited for their inability to distinguish between the living and dead cells unless differentiated by use of a vital staining technique. Bacterial cells can be accurately counted by using Petroff- Hausser counting chamber (the chamber includes a glass slide, a cover slip which is framed and kept 1/50 mm above the slide so that bacterial suspension is present in each ruled square of the slide.

The area of square is 1/400 mm^2; glass cover slip rests 1/50 mm above the slide hence volume over a square is 1/20,000 mm^3 or 1/20,000,000 cm^3, for example – if in one square, an average of five bacteria is present, then these are 5 × 20,000,000, or 10^8 bacteria per ml.

(ii) Colony Counting (Plate-Counting Technique):
This method is based on the fact that one viable cell gives rise to one colony. Therefore, a colony count on an agar plate reveals the viable microbial population. For carrying out this, a measured amount of the sample of bacterial suspension is mixed in the agar medium (when it is in liquid form at 40-45±°C).

It is plated after mixing thoroughly. Each organism grows, reproduces and forms a visible mass in the form of colony. These are counted either by using colony counter or with the aid of large magnifying lens. If too many colonies are appearing and overlapping each other, the sample is diluted so that the colonies are accurately counted.

This method is called pour plate method. The plate count is also performed by spread plate method. In this method 0.1 ml sample containing bacteria is spread over the surface of an agar plate using a sterile glass spreader.

In both pour plate and spread plate methods the plates containing bacterial suspension are incubated until the colonies appear and the colonies are counted. To obtain the appropriate colony number, the sample must be diluted.

Serial dilutions of the sample are usually adopted. To make a 10-fold (10^{-1}) dilution, 10 ml sample is mixed with 90 ml diluent. Serial dilution of soil sample is shown in Fig. 3.5. In most cases, serial dilutions are needed to obtain final dilution. Demerits of the above methods are not only the suitability of the culture medium and incubation conditions but sometimes bacterial cells are deposited on the plate, does not show their visibility in the form of colony if incubation period is short.

Further, viable counts, preparations of dilutions of the sample also give wrong information's. To get correct information's, viable counts are often expressed as the number of colony forming units (CFU) per millilitre rather than number of viable cells. This method is adopted in counting microorganisms in soil.

(iii) Measurement of Cell Mass and Turbidity:
Cell mass is directly proportional to cell number. This can be obtained after centrifugation of a known volume of culture and weighing the pellet obtained. This is called fresh weight but dry weight of cells is obtained by drying the pelleted cells at 90-110°C overnight.

(iv) Turbidity Measurement by Optical Density Method:
The cell mass and number is also obtained by using optical density method. Turbidity is developed in the liquid medium due to the presence of cells which make cloudy appearance to the eyes.

When sample is more turbid it means that more cells are present. Hence more light is scattered. Turbidity can be measured with a photometer or a spectrophotometer device that detects the amount of un-scattered light recorded in photometer unit. The method is indirect; hence some direct measurements of cell number should also be determined. Since OD is proportional to cell mass and thus also to cell number, therefore, turbidity reading acts as an estimate of cell number or cell mass.

The plotting between semi-logarithmic versus time, growth rate of microbial cultures is obtained and used to calculate the generation time of the growing culture. Such a curve can contain data for both cell number and cell mass, allowing for an estimate of both parameters from a single turbidity reading.

Stains and staining techniques: Principles of staining, Types of stains-simple stains, structural stains, negative stain and differential stains.

Staining technique
Introduction

It is very difficult to observe microorganisms by our naked eyes because they are very minute and transparent as well as colorless when they are suspended in aqueous medium. The refractive index of microorganism is not very different from the medium in which they grow, due to which they cannot be observed in unstained preparation. Staining helps to observe the organism clearly.

Chemically a stain (dye) can be defined as an organic compound containing an aromatic compound ie, benzene ring, chromphore and auxochrome group. According to the nature of stain, they are classified into three groups.

1. Acidic dye: those dyes which ionizes to give anionic chromogen portion and has a strong affinity towards positively charged constituents of the cell wall are called acidic dyes. Eg. eosin, picric acid, india ink etc
2. Basic dye: those dyes which ionizes to give cationic chromogen portion and therefore has strong affinity for negatively charged constituents of the cell wall are called basic dyes. Eg. Crystal violet, methylene blue
3. Neutral dye: they are formed by the suitable mixing of two types of dyes, so they contain both cationic and anionic chromogens. They produce precipitate with the cellular components. They are used to stain nucleic acids and cytoplasm. Eg. Sudan IV, eosinate of methylene blue.

Principle

Simple staining technique uses a single stain to visualize the bacteria, which produces a distinctive contrast between the organism and its background. Basic stains (such as methylene blue, crystal violet, and carbol fuchsin) with a positively charged chromogen are preferred in simple staining because bacterial nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen. They stains the microbial cell, not the background.

The purpose of simple staining is for visualization of morphological shape, size, and arrangement of microbial cells.

Mechanism of staining:
- The process of staining involves ion exchange reaction between the stain and component to be stained
- For example, bacterial cell is a negatively charged due to large number of protein having COO- group. This negative charged is balanced by positive charged ion presentoutside the cell wall.
- Therefore a bacterial cell is represented as (BACTERIAL CELL -) Na+.

- To stain the bacterial cell, cationic dye are used having positively charged chromogen. eg. Methylene blue, which is represented as (MB+)Cl-.
- During staining, bacteria cell is flooded with methylene blue and due to ion exchange mechanism acidic component of bacterial ie bacterial cell wall become stained.
- The reaction occurs as follows;
  (BACTERIAL CELL-)Na+ + (MB+)Cl- ======NaCl + (BACTERAIL CELL -)MB+

Simple Staining

Simple Staining- Principle, Procedure and Result Interpretation

Objectives of Simple Staining
- To perform a simple staining procedure.
- 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.

Reagents and Equipment's for Simple Staining
Methylene blue, crystal violet, and carbol fuchsin, Microincinerator or Bunsen burner, inoculating loop, staining tray, microscope, lens paper, bibulous (highly absorbent) paper, and glass slides.

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

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.

Reagents 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
The bacteria will show up as clear spots against a dark background.

Gram Staining

Gram Stain- Principle, Reagents, Procedure, Steps, Results
The Gram stain was developed by Christian Gram in 1884 and modified by Hucker in 1921.

Objectives 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).

Principle of Gram Stain
The two major groups of bacteria can be divided into gram-positive and gram-negative. The Gram stain technique is based on the differential structure of the cellular membranes and cell walls of the two groups.
Gram-positive organisms contain a highly cross-linked layer of peptidoglycan that retains the primary dye, crystal violet (CV), following the application of the mordant, iodine (I). The iodine and crystal violet form a complex within the peptidoglycan. When decolorizer is applied to the cells, the CV-I complex remains within the cell, making it appear dark purple to blue.
The gram-negative organisms do not contain a thick cross-linked layer of peptidoglycan. The peptidoglycan is loosely distributed between the inner cell and the outer cell membranes. Following the application of the crystal violet and iodine, the CV-I complexes are not trapped within the peptidoglycan. Application of the acid-alcohol decolorizer dehydrates the outer cellular membrane, leaving holes in the membrane and effectively washing or removing the CV-I complex from the cells. The cells appear colorless. To make the colorless cells visible, a secondary stain, safranin, is applied, leaving the gram-negative cells pink.

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

Procedure of Gram Stain
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

Limitations:

1. Over-decolorization may result in the identification of false gram-negative results, whereas under-decolorization may result in the identification of false gram-positive results.
2. Smears that are too thick or viscous may retain too much primary stain, making the identification of proper Gram stain reactions difficult. Gram-negative organisms may not decolorize properly.
3. Cultures older than 16 to 18 hours will contain living and dead cells. Cells that are dead will be deteriorating and will not retain the stain properly.
4. The stain may form a precipitate with aging. Filtering through gauze will remove excess crystals.
5. Gram stains from patients on antibiotics or antimicrobial therapy may have altered Gram stain reactivity due to the successful treatment.
6. Occasionally, pneumococci identified in the lower respiratory tract on a direct smear will not grow in culture. Some strains are obligate anaerobes.
7. Toxin-producing organisms such as Clostridia, staphylococci, and streptococci may destroy white blood cells within a purulent specimen.
8. Faintly staining Gram-negative organisms, such as Campylobacter andBrucella, may be visualized using an alternative counterstain (e.g., basic fuchsin).
9. Differences Between Gram Positive and Gram Negative Bacteria