PRINCIPLES OF MICROBIOLOGICAL METHODS - I

Microscopy

Microscope :

It is an instrument used for magnifying an object.

Types of microscopes :

In order to view microorganism and microbial structures of different sizes we require different kinds of microscopes.

i) Light microscopes resolve images with the help of light. The specimen is viewed as dark object against a light background in bright field microscope.

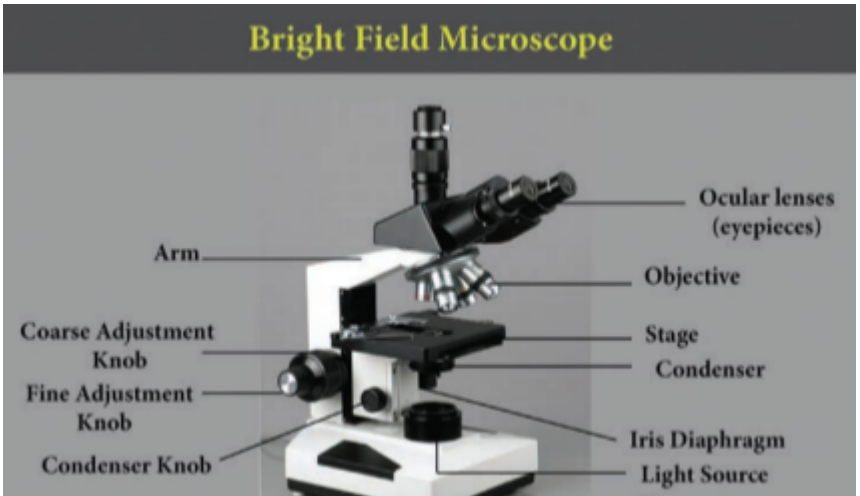
ii) Dark field microscope uses a special condenser and the specimen appears light against a black background.

iii) The other types of mircoscopes are Phase contrast and
Fluorescence microscope.

iv) Electron microscope uses a beam of electrons instead of light. Electrons pass through the specimen and form a two dimensional image.

v) Transmission Electron Microscope (TEM). Electrons are reflected from the specimen and produce a three dimensional image in Scanning Electron Microscope (SEM).

Bright Field Microscope :



The most commonly used microscope for general laboratory observations is the standard bright field microscope.

It contains the following components

i) A mirror or an electric illuminator is the light source which is located at the base of the microscope.

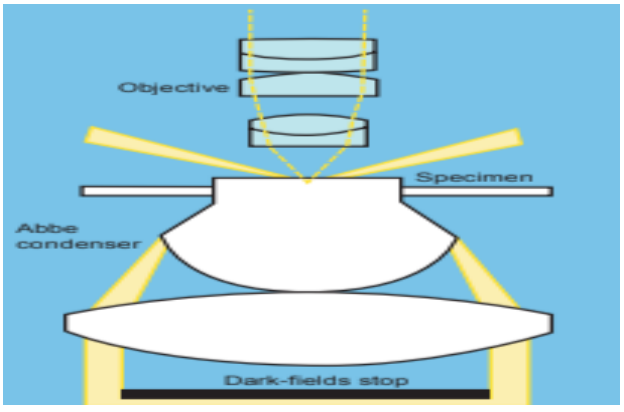
ii) There are two focusing knobs, the fine and the coarse adjustment knobs which are located on the arm. These are used to move either the stage or the nosepiece to focus the image.

iii) Mechanical stage is positioned about half way up the arm, which allows precise contact on moving the slide.

iv) The substage condenser is mounted within or beneath the stage and focuses a cone of light on the slide. In the simpler microscope, its position is fixed where as in advanced microscope it can be adjusted vertically.

Dark Field Microscope :

This is used for examining live unstained microorganisms. The distinct feature is the dark field condenser that contains an opaque disc. The disc blocks direct entry of light to the objective lens. The light rays reflected off the specimen enter the objective lens and in the absence of direct background light, the specimen appears light against a dark background. The microbes are visualized as halos of bright light against the darkness as stars are observed against the night sky.



Uses of Dark Field Microscope :

i) It is used to observe very thin bacteria. Eg: treponema pallidum (causes syphillis)
ii) It is used to observe unstained bacteria
iii) It is used to observe alage
iv) Used to observe structures of microorganisms like flagella and cilia
v) Used to observe aquatic organisms such as algae and plankton
vi) It is used in forensic science to examine pigments and tissues.
vii) It is used to analyse nanoparticles.

It uses 3 types of condenser lenses
i) Abbe condenser lens
ii) Paraboloid condenser lens
iii) Cardioid condenser lens

Phase Contrast Microscope :

Frits Zernike a Dutch Physicist invented the Phase Contrast Microscope and was awarded Nobel Prize in 1953. It is the microscope which allows the observation of living cell. This microscopy uses special optical components to exploit fine differences in the refractive indices of water and cytoplasmic components of living cells to produce contrast.

Principle :

The phase contrast microscopy is based on the principle that small phase changes in the light rays, induced by differences in the thickness and refractive index of the different parts of an object, can be transformed into differences in brightness or light intensity.The phase changes are not detectable to human eye whereas the brightness or light intensity can be easily detected.

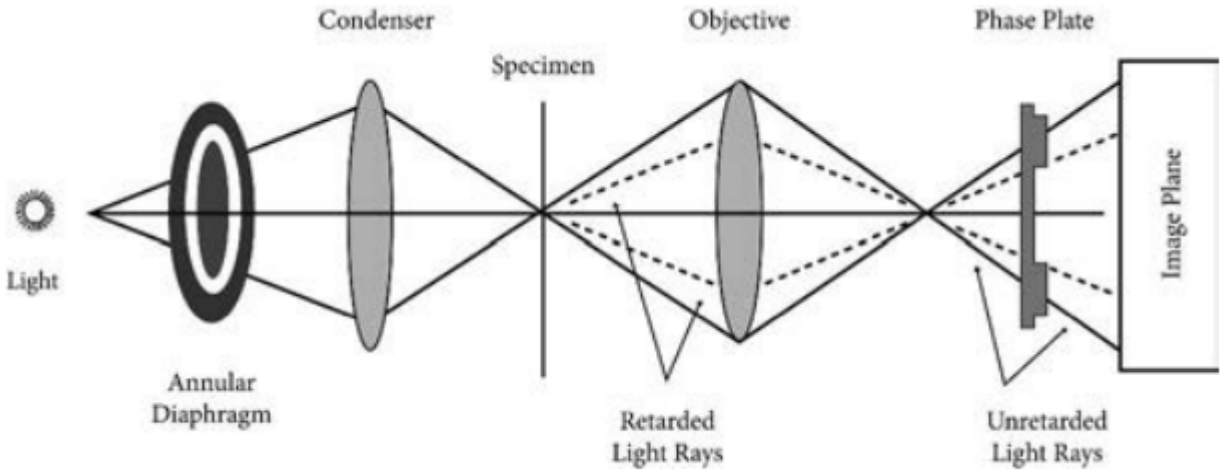


Figure 2.1: Components of phase contrast microscope

Optical Components of Phase Contrast Microscope (PCM) :

The phase contrast microscope is similar to an ordinary compound microscope in its optical components. It possesses a light source, condenser system, objective lens system and ocular lens system (Figure 2.1). A phase contrast microscope differs from bright field microscope in having,

i. Sub-stage annular diaphragm (phase condenser)
An annular aperture in the diaphragm is placed in the focal plane of the sub-stage which controls the illumination of the object. This is located below the condenser of the microscope. This annular diaphragm helps to create a narrow, hollow cone of light to illuminate the object.

ii. Phase – plate (diffraction plate or phase retardation plate)
This plate is located at the back focal plane of the objective lenses.The phase plate has two portions, in which one is coated with light retarding material (Magnesium fluoride) and the other portion devoid of light retarding material but can absorb light. This plate helps to reduce the phase of the incident light.

Working Mechanism of Phase Contrast Microscopy :

The unstained cells cannot create contrast under the normal microscope. However, when the light passes through an unstained cell, it encounters regions in the cell with different refractive indexes and thickness. When light rays pass through an area of

high refractive index, it deviates from its normal path and such light rays experience phase change or phase retardation (deviation). Light rays pass through the area of less refractive index remain non-deviated (no phase change).

The difference in the phases between the retarded (deviated) and un-retarded (non-deviated) light rays is about 1⁄4 of original wave length (i.e., λ/4). Human eyes cannot detect these minute changes in the phase of light. The phase contrast microscope has special devices such as annular diaphragm and phase plate, which convert these minute phase changes into brightness (amplitude) changes, so that a contrast difference can be created in the final image. This contrast difference can be easily detected by human eyes. In phase contrast microscope, to get contrast, the diffracted waves have to be separated from the direct waves.
This separation is achieved by the sub-stage annular diaphragm.The annular diaphragm illuminates the specimen with a hollow cone of light.
Some rays (direct rays) pass through the thinner region of the specimen and do not undergo any deviation and they directly enter into the objective lens. The light rays passing through the denser region of the specimen get regarded and they run with a delayed phase than the non- deviated rays. Both the deviated and non deviated light has to pass through the phase plate kept on the back focal plane of the objective to form the final image.
The difference in phase (Wavelength) gives the contrast for clear visibility of the object.

Application :

i) Phase contrast microscope enables the visualization of unstained living cells.
ii) It makes highly transparent objects more visible.
iii) It is used to examine various intracellular components of living cells at relatively high resolution.
iv) It helps in studying cellular events such as cell division.
v) It is used to visualize all types of cellular movements such as chromosomal and flagellar movements.

Fluorescence Microscope :

Fluorescence microscope is a very powerful analytical tool that combines the magnifying properties of light microscope with visualization of fluorescence.Fluorescence microscope is a type of light microscope which instead of

utilizing visible light to illuminate specimens, uses a higher intensity (lower wavelength) light source that excites a fluorescent molecule called a fluorophore (also known as fluorochrome). Fluorescence is a phenomenon that takes place when the substances (fluorophore) absorbs light at a given wavelength and emits light at a higher wavelength. Thus, fluorescence microscopy combines the magnifying properties of the light microscope with fluorescence technology.

The fluorophore absorbs photons leading to electrons moving to a higher energy state (excited state). When the electrons return to the ground state by losing energy, the fluorophore emits light of a longer wavelength
Three of the most common fluorophores used are Diamidino – phenylindole (DAPI) (emits blue), Fluorescein isothiocyanate (FITC) (emits green), and Texas Red (emits red).

Principle :

Light source such as Xenon or Mercury Arc Lamp which provides light in a wide range of wavelength, from ultraviolet to the infrared is directed through an exciter filter (selects the excitation wavelength). This light is reflected toward the sample by a special mirror called a dichroic mirror, which is designed to reflect light only at the excitation wavelength. The reflected light passes through the objective where it is focused onto the fluorescent specimen. The emissions from the specimen are in turn, passed back up through the objective where magnification of the image occurs and through the dichroic mirror. This light is filtered by the barrier filter, which selects for the emission wavelength and filters out contaminating light from the arc lamp or other sources that are reflected off from the microscope components. Finally, the filtered fluorescent emission is sent to a detector where the image can be digitized.

Components of Fluorescence Microscope :

The main components of the fluorescent microscope resemble the traditional light microscope. However, the two main difference are the type of light source used and the use of the specialized filter elements (Figure 2.6).
Light source Fluorescence microscopy requires a very powerful light source such as a Xenon or Mercury Arc Lamp. The light emitted from the Mercury Arc Lamp 10–100 times brighter than most incandescent lamps and provides light in a wide range of wavelengths from ultra-violet to the infrared. Lasers or high-power LEDs were mostly used for complex fluorescence microscopy techniques.

Filter elements :

A typical fluorescence microscope consists of three filters: excitation, emission and the dichroic beam splitter.

Excitation filters: It is placed within the illumination path of a fluorescence microscope. Its purpose is to filter out all

wavelength of the light source, except for the excitation range of the fluorophore in the sample or specimen of interest.

Emission filters: The emission filter is placed within the imaging path of a fluorescence microscope. Its purpose is to filter out the entire excitation range and to transmit the emission range of the fluorophore in the specimen.

Dichroic filter or beam splitter: The dichroic filter or beam splitter is placed in between the excitation filter and emission filter, at 45° angle. Its purpose is to reflect the excitation wavelength towards the fluorophore in the specimen, and to transmit the emission wavelength towards the detector.

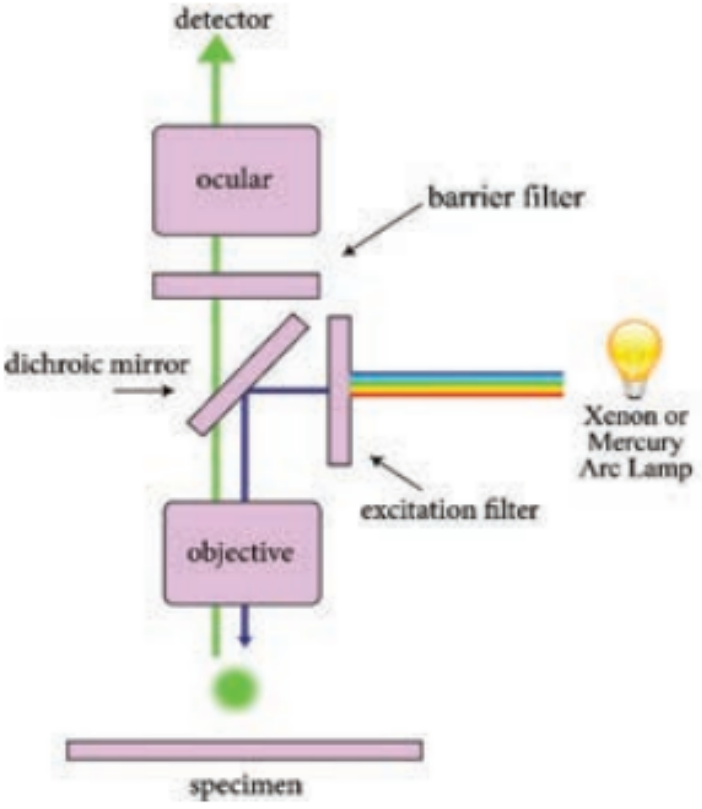


Figure 2.6: Components of fluorescence microscope

Working Mechanism :

The specimen to be observed are stained or labeled with a fluorescent dye and then illuminated with high intensity ultra violet light from mercury arc lamp. The light passes through the exciter filter that allows only blue light to pass through. Then the blue light reaches dichroic mirror and reflected downward to the specimen. The specimen labeled with fluorescent dye absorbs blue light (shorter wavelength) and emits green light. The emitted green light goes upward and passes through dichroic mirror, reflects back blue light and allows only green light to pass the objective lens, then it reaches barrier filter which allows only green light. The filtered fluorescent emission is sent to a detector where the image can be digitized

Application :

i) Fluorescence microscope has become one of the most powerful techniques in biomedical research and clinical pathology.
ii) Fluorescence microscope allows the use of multicolour staining, labeling of structures within cells, and the measurement of the physiological state of a cell.
iii) Fluorescence microscope helps in observing texture and structure of coal.
iv) To study porosity in ceramics, using a fluorescent dye.
v) To identify the Mycobacterium tuberculosis.

Electron Microscope :

Examining the ultra structure of cellular components such as nucleus, plasma membrane, mitochondria and others requires 10,000X plus magnification which was just not possible using Light Microscopes. This is achieved by Electron microscopes which have greater resolving power than light microscopes and can obtain higher magnifications. In an electron microscope, a focused electron beam is used instead of light to examine objects. Electrons are considered as radiation with wavelength in the range 0.001–0.01 nm compared to 400–700 nm wavelength of visible light used in an optical microscope. Optical microscopes have a maximum magnification power of 1000X, and resolution of 0.2 μm compared to resolving power of the electron microscope that can reach 1,000,000 times and resolution of 0.2 nm. Hence, electron microscopes deliver a more detailed and clear image compared to optical microscopes.

Types of Electron Microscopes :

i) Transmission electron microscopes (TEM)
ii) Scanning electron microscopes (SEM)
iii) Scanning transmission electron microscopes (STEM)

The electron microscope was invented in 1931 by two German scientists, Ernst Ruska and Max Knoll. Ernst Ruska later received Nobel Prize for his work in 1986.
The Transmission Electron Microscope (TEM) was the first type of Electron Microscope to be developed.

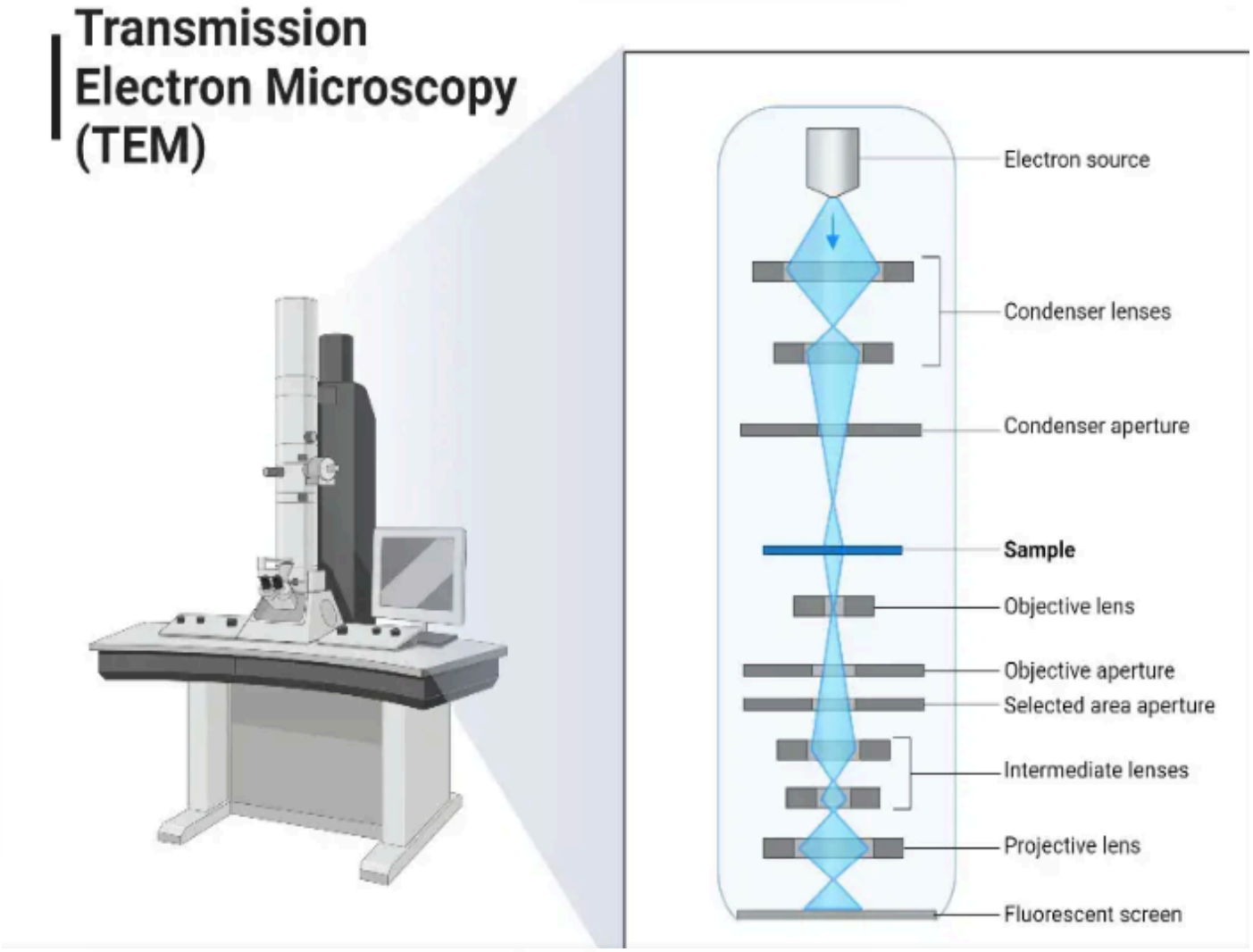
Principle :

The fundamental principle of electron microscope is similar to light microscope. In electron microscope, a high velocity beam of electrons is used instead of photons. In the electron gun, electrons are emitted from the surface of the cathode and accelerated towards the anode by high voltage to form a high energy electron beam. All lenses in the electron microscope are electromagnetic. Charged electrons interact with the magnetic fields and magnetic force focuses an electron beam. The condenser lens system controls the beam diameter and convergence angles of the beam incident on a specimen. The image is formed either by using the transmitted beam or by using the diffracted beam. The image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor.

Sample Preparation :

Preparation of specimens is the most complicated and skillful step in EM. The material to be studied under electron microscopy must be well preserved, fixed, completely dehydrated, ultrathin and impregnated with heavy metals that sharpen the difference among various organelles. The material is preserved by fixation with glutaraldehyde and then with osmium tetroxide. The fixed material is dehydrated and then embedded in plastic (epoxy resin) and sectioned with the help of diamond or glass razor of ultra-microtome. In TEM, sample sections are ultrathin about 50–100 nm thick. These sections are placed on a copper grid and exposed to electron dense materials like lead acetate, uranylacetate, phosphotungstate. In SEM, samples can be directly imaged by mounting them on an aluminum stub.

Diagrams :



Uses of Transmission Electron Microscope (TEM) in Microbiology

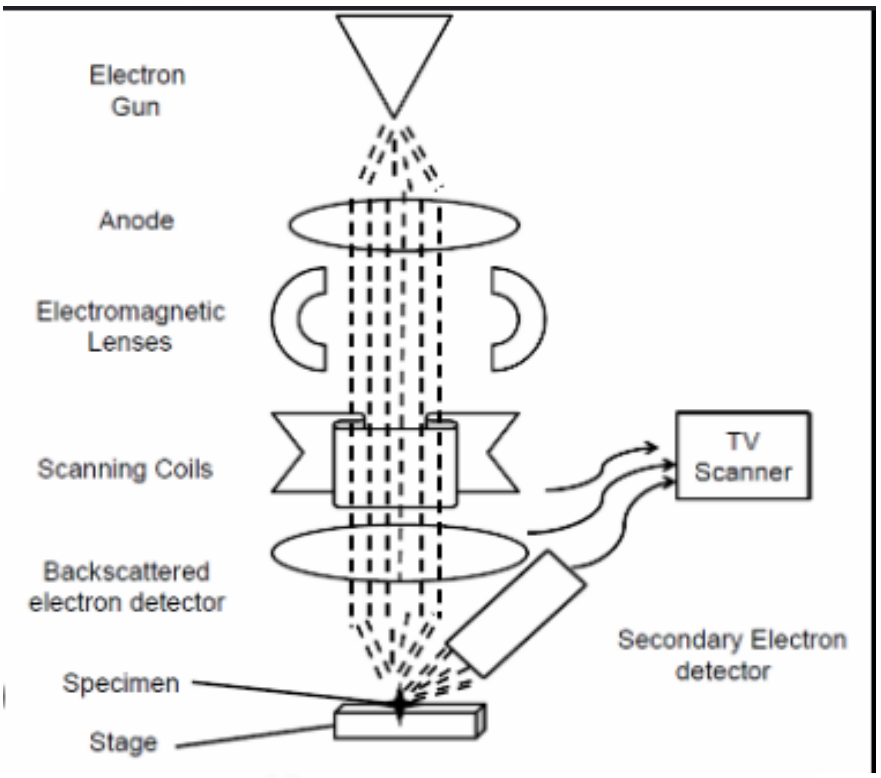
1. **Detailed study of bacterial ultrastructure** – Examines internal features like cell walls, membranes, and organelles.

2. **Virus visualization** – Allows observation of virus shape, size, and structure, which are too small for light microscopes.

3. **Observation of bacterial pili and flagella** – Shows fine surface structures important for motility and attachment.

4. **Study of microbial interactions** – Helps analyze interactions between microbes and host cells at the cellular level.

5. **Research on microbial organelles** – Investigates specialized structures such as endospores and inclusion bodies inside bacteria.



Magnification :

Magnification refers to the process of making a microscopic organism appear larger than it actually is, allowing for detailed observation. This is achieved through the use of lenses in a microscope, primarily the objective and ocular (eyepiece) lenses. Total magnification is calculated by multiplying the magnification power of the objective lens by that of the eyepiece.

Resolution :

It is the ability of a lens to separate or distinguish between small objects that are close together

Resolving power :

Ability of an lens to separate or distinguish between small objects that are close together.

$$D = \frac{\lambda}{2\,NA}$$

D = Resolution

λ = Wavelength of light

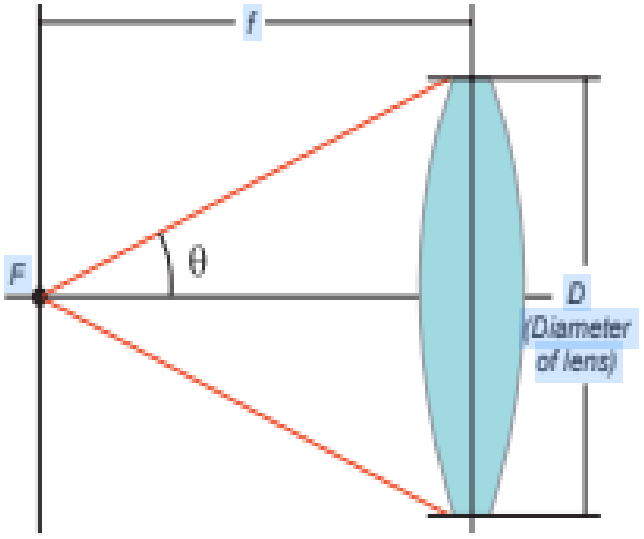
NA = Numerical aperture

Shortest wavelength of light + High numerical aperture = Gives the maximum resolution

Numerical aperture :

Numerical Aperture (NA) is the value representing the light gathering capacity of an objective lens. NA was first described by Ernst Abbe, and is defined by the following expression

Numerical Aperture (NA) =n × sin(θ) n = the refractive index of the medium between the specimen and objective; θ = half aperture angle or collection angle of the objective. (the maximum half angle of the cone of light that can enter or exit the lens).



The resolving power of a light microscope depends on the wavelength of light used and the NA of the objective lens.

Specimen Preparation for Light Microscope :

1. Smear Preparation (thin smear)
2. Fixation i) Heat fixation ii) Chemical Fixation
3. Simple Staining
4. Differential Staining (crystal violet or methylene blue)
5. Differential staining eg: Gram staining
6. Wet mount preparation or hanging drop technique

1. **Smear preparation** : involves spreading a thin layer of microbial sample on a slide, air drying, and heat fixing it. This process helps in observing microorganisms under a microscope after staining, preserving their shape and arrangement.

2. Fixation

   i) Heat fixation is a process in microbiology where a dried smear on a slide is briefly passed through a flame. It kills the microorganisms, adheres them to the slide, and preserves their shape for staining and microscopic examination.

   ii) **Chemical fixation** uses chemicals like formalin, ethanol, or methanol to preserve and fix microorganisms on a slide. It stabilizes cell structures, prevents decomposition, and maintains morphology for staining and microscopic analysis. It's often used for delicate specimens that may be damaged by heat.

3. **Simple staining** is a basic technique in microbiology where a single dye (like methylene blue or crystal violet) is used to color microbial cells. It helps to highlight the shape, size, and arrangement of bacteria, making them easier to see under a microscope.

4. **Differential staining** uses two or more dyes to distinguish between different types of microorganisms or cell structures. Common examples include **Gram staining** and **acid-fast staining**. It helps identify and classify bacteria based on their cell wall properties.

5. **Wet mount and hanging drop techniques** are used to observe live microorganisms in a liquid. A drop of the sample is placed on a slide (wet mount) or suspended from a coverslip over a cavity slide (hanging drop). These methods help study **motility**, **shape**, and **arrangement** of microbes in their natural state.

Uses of Simple Staining :

1. **To determine cell shape** – Identifies whether bacteria are cocci, bacilli, or spirilla.

2. **To observe cell arrangement** – Shows patterns like chains, clusters, or pairs.

3. **To measure bacterial size** – Helps estimate the size of individual cells.

4. **To make bacteria visible** – Enhances contrast between cells and the background.

5. **To quickly identify bacterial presence** – Confirms if bacteria are present in a sample.

Differential Staining Technique

1. **Differentiates Between Microorganisms**
   Differential staining helps distinguish different types of bacteria based on structural differences. For example, **Gram staining** separates bacteria into Gram-positive (purple) and Gram-negative (pink) based on their cell wall composition.

2. **Uses Multiple Dyes**
   Unlike simple staining, differential staining involves **two or more dyes** (e.g., crystal violet and safranin) to create contrast between different cells or cell parts, making it easier to compare and identify them.

3. **Reveals Structural Features**
4. It highlights **specific bacterial structures**, such as **endospores**, **capsules**, or **acid-fast cell walls**, which are not visible with simple stains. This is important for diagnosing certain bacterial infections like tuberculosis.

5. **Helps in Identification and Classification**
   The results of differential staining are used in **microbial classification and diagnosis**, helping microbiologists to **identify bacteria** and determine appropriate treatments.

6. **Examples of Techniques**
   Common differential staining methods include:

   - **Gram staining** (cell wall differences)
   - **Acid-fast staining** (mycolic acid in cell walls)
   - **Endospore staining** (detects spores within cells)
   - **Capsule staining** (visualizes the protective capsule around bacteria)

Gram Staining technique :

Gram staining is a differential staining technique developed by Hans Christian Gram in 1884. It is used to classify bacteria into two groups based on their cell wall structure: Gram-positive (which retain the crystal violet dye and appear purple) and Gram-negative (which do not retain crystal violet and appear pink/red after counterstaining with safranin). This method is essential for bacterial identification and guides treatment decisions in microbiology and medicine.

Principle :

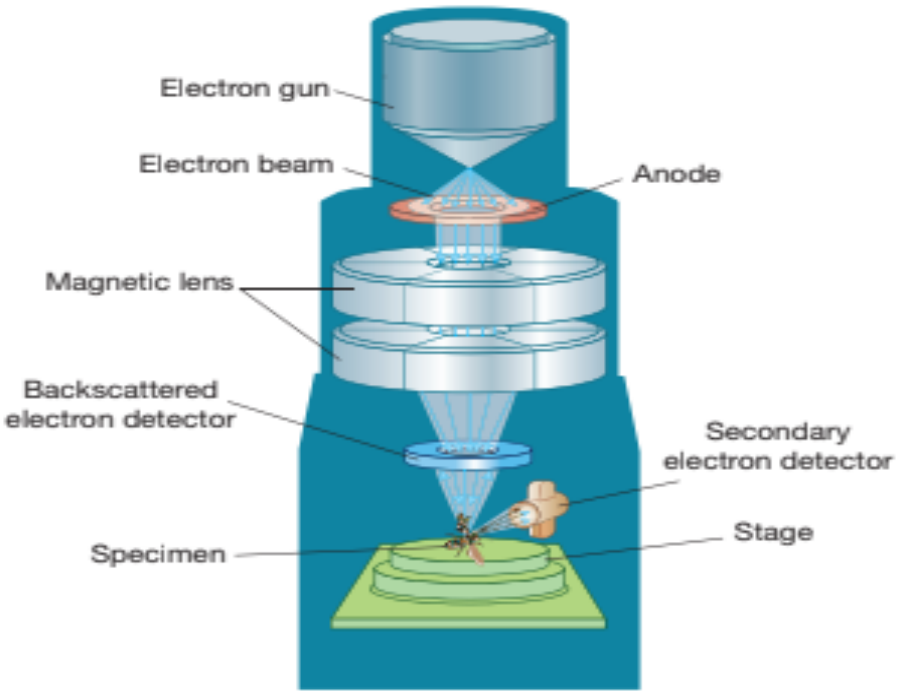
Based on gram staining reaction bacteria can be divided into two large groups called gram positive and gram negative. Gram positive bacteria have thicker peptidoglycan compared to the gram negative bacteria. The lipid content of the cell wall of gram negative bacteria is higher than that of gram positive bacteria. Both gram positive and gram negative bacteria take up the primary stain crystal violet and stain red. When decolorized the porosity and permeability of the gram negative bacteria increases due to which the crystal violet iodine complex is given out by the gram negative bacteria. Further the gram negative bacteria takes up the counterstain safranin and stains red. Hence when bacteria are observed under microscope after gram staining the gram positive cells appear violet and gram negative cells appear red.

28/07/2025

Working Principle and Instrumentation of SEM

It is first built by Knoll in 1935. It is used to study the three dimensional images of the surfaces of cells, tissues or particles. The SEM allows viewing the surfaces of specimens without sectioning. The specimen is first fixed in liquid propane at-180°C and then dehydrated in alcohol at-70°C. The dried specimen is then coated with a thin film of heavy metal, such as platinum or gold, by evaporation in a vacuum provides a reflecting surface of electrons. In SEMs, samples are positioned at the bottom of the electron column and the scattered electrons (back- scattered or secondary) are captured by electron detectors.

In SEM, there are several electromagnetic lenses, including condenser lenses and one objective lens. Electromagnetic lenses are for electron probe formation, not for image formation directly, as in TEM. Two condenser lenses reduce the crossover diameter of the electron beam. The objective lens further reduces the cross-section of the electron beam and focuses the electron beam as probe on the specimen surface. Objective lens thus functions like a condenser.



Uses of a Scanning Electron Microscope (SEM)

1. Biological Sciences:
SEM is useful in studying the surface of biological samples like cells, tissues, insects, and microorganisms. Unlike light microscopes, it gives a 3D-like view of the surface, which helps researchers see the texture and structure in much more detail.

2. Materials Science and Engineering:
This is one of the biggest areas where SEM is used. It helps scientists and engineers analyze the structure of materials—like metals, plastics, ceramics, etc.—to understand their properties. For example, it can be used to study why a material broke or how strong its surface is.

3. Electronics and Semiconductor Industry:
In electronics, SEM is often used to inspect microchips and circuit boards. Because these components are so tiny, SEM is ideal for checking for defects or failures at the microscopic level.

4. Forensics:
SEM plays an important role in forensic investigations. It can be used to analyze evidence like gunshot residue, hair, fibers, or tool marks. Since it's so sensitive, it can detect very small particles that might be missed with other tools.

5. Geology and Earth Sciences:
Geologists use SEM to study rocks, minerals, and soil. It helps identify the composition and surface features of geological samples, which is useful in understanding how they formed.

6. Nanotechnology:
When dealing with nanomaterials (like nanoparticles or nanotubes), SEM is often used to visualize them. Since SEM can zoom in at the nanoscale, it's a go-to tool for researchers working on advanced materials.

7. Education and Research Training:
In universities, SEM is also used to teach students about microscopic structures and techniques. It's a great tool for getting hands-on experience with imaging and analysis.

8. Chemical and Pharmaceutical Analysis:
SEM combined with other tools (like EDS for elemental analysis) can help in identifying the composition of substances. In pharma, it can be used to examine drug particles and detect any contamination or structural problems.

9. Art and Archaeology:
SEM is used to study historical artifacts. It can help examine the surface of paintings, metals, or pottery to understand how they were made or how they've deteriorated over time.

Sample Preparation

Preparation of specimens is the most complicated and skillful step in EM. The material to be studied under electron microscopy must be well preserved, fixed, completely dehydrated, ultrathin and impregnated with heavy metals that sharpen the difference among various organelles. The material is preserved by fixation with glutaraldehyde and then with osmium tetroxide. The fixed material is dehydrated and then embedded in plastic (epoxy resin) and sectioned with the help of diamond or glass razor of ultra-microtome.
In TEM, sample sections are ultrathin about 50–100 nm thick. These sections are placed on a copper grid and exposed to electron dense materials like lead acetate, uranylacetate, phosphotungstate. In SEM, samples can be directly imaged by mounting them on an aluminum stub.

Metal shadowing
The pores are coated from the metal, in this technique the greed specimen is exposed with heavy metals like platinum, palladium, gold as a result they produce the image reveals the 3 dimensional structure of the object.

Freeze etching
It is used to crystalize the internal structure of cells and other materials.The sample is quickly frozen by using liquid nitrogen.

Fracturing
The frozen sample is fractured like a knife to expose the internal surface and structures.

Etching
The fractured sample is placed in a vacuum chamber, and the temperature is increased to cause the ice to sublimate from the surface. This exposes the internal structures of the sample, revealing more detailed structural features.

Replication
A thin layer of heavy metal platinum or gold is placed on the etched surface, followed by a layer of carbon or stabilizer

Examination
The replication is then examined under an Electron Microscope.

An autoclave is a machine used to sterilize materials by using steam under pressure, killing bacteria, viruses, and spores. It works by heating the items inside to a certain temperature for a specific amount of time, making it a highly effective sterilization method, especially in healthcare and industries.

The main parts of an autoclave include:

- **Pressure Chamber**: The main part of the autoclave where materials are placed, made from stainless steel or gunmetal. The outer jacket helps speed up the process using steam.

- **Lid/Door**: Seals the autoclave, creating a sterile environment. It includes a pressure gauge, whistle, and safety valve.

- **Steam Generator**: Located below the chamber, this heats the water to create steam.

- **Vacuum Generator**: (in some models) removes air from the chamber to prevent microorganisms from growing.

- **Wastewater Cooler**: Cools the water before it leaves the autoclave to protect the drainage pipes.

The autoclave works on moist heat sterilization, where steam at high pressure increases the boiling point of water, allowing it to reach a higher temperature for deeper sterilization. This typically happens at 121°C and 15 psi, which kills microbes effectively.

To run an autoclave, you check for leftover items, add water, load the materials, close the lid, and adjust the pressure. After sterilization, the pressure is released, and the materials cool down before being removed.

There are different types of autoclaves:

- **Pressure Cooker Type**: Simple and often used in some places.
- **Gravity Displacement**: Common in labs, where steam is made inside the chamber.
- **Positive Pressure Displacement**: Faster, with a separate steam generator.
- **Negative Pressure Displacement**: Uses both steam and vacuum generators for high precision, but is the most expensive.

Uses of Autoclave

1.Autoclaves are essential for sterilizing materials that contain water, which cannot be sterilized by dry heat. They are also used for various other purposes:

2. Decontamination of Biological Waste: Autoclaves are used to safely sterilize biological waste, including those that may contain harmful bacteria, viruses, or other microorganisms, before disposal.

3.Sterilizing Medical Equipment: In medical labs, autoclaves sterilize equipment like surgical instruments, glassware, and other medical tools.

4. Sterilizing Culture Media: Autoclaves are commonly used to sterilize culture media, plastic tubes, autoclavable containers, and pipette tips in laboratories.

Advantages of Autoclave:

1. Highly Effective Sterilization: Autoclaves use steam under pressure, which is very effective at killing a wide range of microorganisms, including bacteria, viruses, and spores.

2.Fast Sterilization Process: Compared to dry heat methods, autoclaving is much faster, usually requiring only 15-30 minutes for complete sterilization.

3.Safe for Heat-Resistant Materials: Autoclaves can sterilize a wide variety of materials, including metals, glassware, and plastics that can withstand high temperatures.

4.Environmentally Friendly: Since autoclaves use steam and not harmful chemicals, they are a safer and more environmentally friendly option for sterilization.

5.Versatile: Autoclaves can sterilize a wide range of items like medical equipment, laboratory tools, biological waste, and even certain types of food.

Disadvantages of Autoclave:

1.Not Suitable for Heat-Sensitive Materials: Autoclaving is unsuitable for certain items like electronics or delicate fabrics that cannot withstand high heat or moisture.

2.High Initial Cost: The purchase and installation of autoclaves can be expensive, especially for larger or more advanced models.

3.Requires Regular Maintenance: To function effectively, autoclaves require regular maintenance and calibration to ensure proper pressure, temperature, and safety.

4.Energy Consumption: Autoclaves consume a significant amount of electricity to heat water and maintain high pressure, leading to higher operating costs.

5.Risk of Damage: Incorrect use, such as overloading the autoclave or improper sealing, can lead to inadequate sterilization or damage to the items being sterilized.

Hot air oven:

This is the most widely used method of sterilization using dry heat. The oven is usually heated by electricity and it has a thermostat that maintains the chamber air constantly at the chosen temperature. It has a fan or turbo-blower to assist the circulation of air and to ensure rapid, uniform heating of the load. In Hot Air Oven, the air is heated at a temperature
of 160oC for one hour. This is the best method of sterilizing dry glass ware such as test tubes, petri dishes, flasks, pipettes and instruments such as forceps, scalpels and

scissors. It is also used to sterilize some pharmaceutical products such as liquid paraffin, dusting powder, fats and grease.

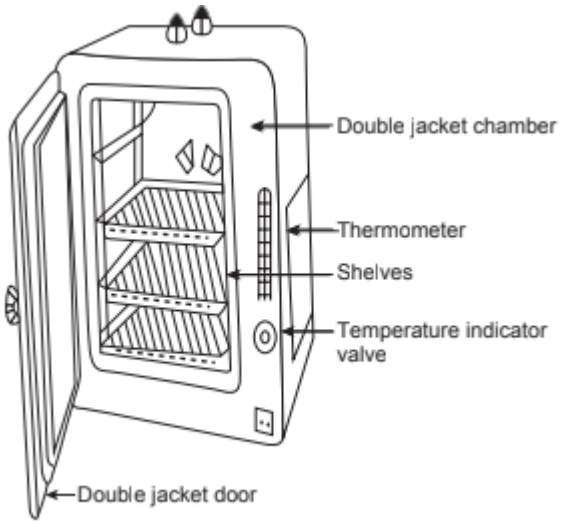


Figure 4.1: Hot Air Oven

Pasteurization :

The process of heating a liquid food or beverage either at 62.8°C for 30 seconds or 72°C for 15 seconds to enhance their self life and destroy harmful microorganisms. It should be noted that pasteurization process kills only vegetative cells but not spores.Pasteurization named in honour of its developer Louis Pasteur.

Pasteurization can be done in the following methods :

1. Low Temperature Holding Method (LTH)
   In this method milk,beer and fruit juices are maintained at 62°C for 30 minutes.
2. High Temperature Short Time Method (HTST)
   Products are held at 72°C for 15 seconds.
3. Ultra High Temperature (UHT)
   Milk can be treated at 141°C for 2 seconds (This method employs temperature above 100°C .

Electromagnetic radiation

It uses rays such as gamma rays and x rays (these are called as ionizing radiations) because they ionize the molecule.When such radiations pass through the cell hydrogen radicals, hydroxy radicals or some peroxides inside the cell will be formed.

Sterilization method

This sterilization method is called as cold sterilization, because ionizing radicals produce less amount of heat.

X-rays

- ➢ They are lethal microorganisms, and have penetration ability.
- ➢ They are not cost effective.
- ➢ They are used to produce microbial mutants.

Gamma rays

- ➢ Shorter wavelength, higher energy radiations emitted from radioactive isotopes.
- ➢ They have great penetration ability and are used for the sterilization for packaging materials (food materials) and medical equipments.

UV rays

- ➢ It ranges from 150 Å to 3900 Å
- ➢ Wavelength around 2650 Å
- ➢ Has the highest bactericidal effect
- ➢ Germicidal lamps are available.

Mode of action

- ➢ They cause mutation in nucleic acids (pyrimidine dimer) - (Thymine - Thymine dimer).
- ➢ Non-ionizing radiation (does not pass ionization)
- ➢ It has the highest bactericidal effect.

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Module - III

Sterilization using Chemical Agents

Chemical Substances (Physical and Chemical Agents)

1. For substances such as phenol, alcohol, Dettol, QACs, beta-propiolactone, and ethylene oxide, concentration is important.

2. Concentration of chemical agents.

3. Stability of chemical agents (structure).

4. Chemical structure.

5. Antimicrobial activity of chemical agents or chemical substances.

6. Solubility of chemical substances (soluble in water).

7. Should be cost-effective and cheap.

8. Availability and affordability.

9. To be used as antimicrobial agents and for sterilization.
10. Should be non-corrosive.

They kill microorganisms or inhibit the growth of microorganisms.

Terms:

- *Kill* means -cidal, and *inhibit* means -static.
- Bactericidal – kills bacteria
- Bacteriostatic – inhibits the growth of bacteria
- Fungicidal – killing of fungi
- Fungistatic – inhibition of fungi
- Sporicidal – killing of spores (e.g., endospores)
- Viricidal – killing of viruses

The killing or inhibition is mainly based on the concentration of the chemical agents.

Number of cells is based on the nature and concentration of the chemical substance.

Disinfectant and Antiseptic – Terms:

- Disinfectant – A chemical substance used for killing microorganisms, which is applied on non-living things or inanimate objects.
  Examples: phenol, soaps, detergents, etc.

- Antiseptic – A chemical substance used for killing microorganisms, which is applied on living tissues.
  Examples: used on wounds, cuts, infections, etc. Example: Dettol.

List of Chemical Agents

1) Phenol and phenolic compounds
2) Halogens
3) Heavy metals
4) Compounds of heavy metals
5) Gases
6) Alcohols
7) Dyes
8) Soaps and detergents
9) Aldehydes
10) Acid and alkali
11) Quaternery ammonium compounds

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1) **Phenol and Phenolic compounds**

Phenol and its derivatives, known as **phenolic compounds**, were first introduced by **Joseph Lister in the 1880s**. Lister is considered a pioneer in antiseptic surgery, and his discovery marked a major breakthrough in infection control. Phenol was initially used to reduce infections during surgical procedures.

Phenol is commonly used as a **disinfectant**, meaning it is applied to **non-living surfaces** to kill or inhibit microorganisms. However, it is **not suitable for use as an antiseptic** on living tissues due to its **toxic and irritating nature**.

At a **5% concentration**, phenol is effective in **killing vegetative bacterial cells** (the actively growing and dividing forms of bacteria). However, it is **not effective against bacterial spores**, which are highly resistant forms of bacteria. While phenol is **resistant to inactivation** by organic matter and has **strong bactericidal activity**, its **sporicidal action is limited**.

A derivative of phenol, called **hexyl resorcinol**, also has antimicrobial properties but, like phenol, **does not act as a sporicidal agent**.

Practical Applications:

Phenol and phenolic compounds have several uses in microbiology and clinical settings:

- ➢ They are often **bacteriostatic** at lower concentrations (inhibit the growth of bacteria) and **bactericidal** at higher concentrations (kill bacteria).

- ➢ Depending on the concentration, phenol can also exhibit **fungicidal activity**, meaning it can be used to **kill fungi**.

- ➢ These compounds are commonly used to **disinfect biological materials** and waste, including **urine, feces, and sputum**, especially in healthcare and laboratory environments.

Their ability to remain active in the presence of organic matter makes them useful in disinfecting dirty or contaminated surfaces.

Mode of Action:

The effectiveness of phenol comes from how it interacts with microbial cells:

- ➢ It **disrupts the cell membrane**, causing leakage of essential cellular contents.

- ➢ It causes **precipitation of cellular proteins**, which stops essential processes within the cell.

- ➢ Phenol **inactivates key enzymes**, which are vital for the metabolism and survival of the microorganism.

- ➢ It leads to **leakage of amino acids and other intracellular materials**, weakening the cell.

- ➢ This results in **denaturation of proteins** and **complete disruption of the cell structure**, leading to cell death.

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2) Alcohol

There are different types of alcohols used as antimicrobial agents, mainly:

- **Ethyl alcohol (ethanol)**

- **Methyl alcohol (methanol)**

Ethyl alcohol is the most commonly used. It is effective at concentrations between **50% to 90%**, with **70% being the most widely used concentration** in healthcare settings. At this level, it is highly effective against

vegetative cells (actively growing bacteria).

In addition to ethyl and methyl alcohol, **higher alcohols** such as **propyl alcohol, butanol**, and **amide alcohols** are also used for disinfection purposes due to their increased antimicrobial activity.

Practical Applications:

➢ Alcohol is widely used as an **antiseptic**, especially for application on **the skin**.

➢ It is commonly used to **disinfect the skin** of patients before procedures and the **hands of healthcare workers** before and after patient contact.

➢ It is also a key ingredient in **hand sanitizers**.

➢ A concentration of around **60% alcohol** has been found to be **very effective against viruses**, making it a strong **viricidal agent**.

Mode of Action:

Alcohols kill microorganisms through several mechanisms:

➢ **Denaturation of proteins**, which disrupts cellular function.

➢ **Damage to the cell wall and membrane**, leading to leakage of essential contents.

➢ **Inactivation of enzymes**, which are crucial for microbial metabolism.

➢ Acts as a **dehydrating agent**, drawing water out of cells and further disrupting cell processes.

➢ Also functions as a **solvent for lipids**, breaking down the lipid membranes of bacteria and enveloped viruses.

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3) Halogens

Examples:

➢ Chlorine

➢ Iodine

Halogens like chlorine and iodine are widely used as antimicrobial agents due to their strong germicidal properties.

Iodine

Iodine is a powerful germicidal agent, meaning it kills a wide range of microorganisms. It is commonly used in two forms:

➢ **Tincture of iodine**

➢ **Iodophors**

Iodine is primarily used as an antiseptic, applied to the skin to prevent infections before procedures such as surgeries or injections.

Preparation of Tincture of Iodine:

There are different formulations for preparing tincture of iodine:

1. 2% iodine + 2% sodium iodide
2. 7% iodine + 5% potassium iodide in 83% alcohol

These formulations help dissolve iodine and enhance its antimicrobial activity.

Mode of Action of Iodophors:

- ➢ Iodophors are a combination of iodine and surface-active agents (like detergents or surfactants).
- ➢ These surface-active agents act as carriers and solubilizers for iodine.
- ➢ Iodine acts as a strong oxidizing agent.
- ➢ It inactivates enzymes and denatures proteins, leading to cell death in microorganisms.

Practical Applications:

Iodine and iodophors are used for a wide range of antimicrobial purposes:

- ➢ As a bactericidal agent – kills bacteria
- ➢ As a fungicidal agent – kills fungi
- ➢ As a sporicidal agent – kills spores
- ➢ As a viricidal agent – kills viruses
- ➢ Used as a disinfectant for skin, especially before medical or surgical procedures

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Chlorine and Chlorine Compounds

Chlorine and its compounds are widely used for disinfection, especially in water treatment.

Common Chlorine Compounds:

1. Hypochlorides:
    - Sodium hypochlorite (NaOCl)

- Calcium hypochlorite ($Ca(OCl)_2$)

2. Chloramines:
    - Chloramine-T
    - Azochloramide

These are commonly used as disinfectants and sanitizing agents in various industries.

Mode of Action of Chlorine:

Chlorine reacts with water to form hypochlorous acid (HOCl), which is the main antimicrobial agent.

Chemical Reaction:
$Cl_2 + H_2O \rightarrow HOCl + HCl$
(*Hypochlorous acid acts as a strong oxidizing agent.*)

➢ Hypochlorous acid penetrates microbial cells and oxidizes essential proteins and enzymes, leading to enzyme inactivation.

➢ It binds to the proteins in the cell membrane, causing denaturation and disrupting cell function.

Applications of Chlorine Compounds:

➢ Water treatment (e.g., drinking water, swimming pools)

➢ Food industry sanitation

➢ Sanitizing dairy equipment and utensils in restaurants and food-processing facilities

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Heavy Metals and Their Compounds

Heavy metals like mercury, silver, and copper have antimicrobial properties and are used against various microorganisms.

1) Mercury

Mercury is used in both inorganic and organic compound forms.

Inorganic Mercury Compounds:

- ➢ Mercuric chloride ($HgCl_2$)
- ➢ Mercurous chloride (Hg_2Cl_2)
- ➢ Mercuric oxide (HgO)
  → These have bactericidal activity and are used in ointments and as antiseptics.

Organic Mercury Compounds:

- ➢ Mercurochrome (also known as merbromin)
  → Has bactericidal activity, used as a topical antiseptic.

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2) Silver

Silver compounds are widely used in the preparation of antiseptic ointments.

Common Silver Compounds:

- ➢ Silver nitrate ($AgNO_3$)
- ➢ Silver lactate
- ➢ Silver picrate

These have bacteriostatic and bactericidal properties.
Used as antiseptics for wounds, burns, and eye infections.

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3) Copper

➢ Copper sulfate ($CuSO_4$) is a well-known fungicidal and antifungal agent.

➢ Effective against algae and fungi, often used in swimming pools to control algal growth.

➢ Bordeaux mixture (a mixture of copper sulfate and lime) is used in agriculture to prevent fungal plant diseases.

Mode of Action of Heavy Metals:

➢ Heavy metals bind with cellular proteins and inactivate them.

➢ They interfere with enzyme function, particularly enzymes containing sulfhydryl (-SH) groups.

Key Reaction (Example with Mercury):

Active form of enzyme:

$$Enzyme–SH + SH$$

Reaction with mercuric chloride ($HgCl_2$):

$$(active\ form)\ Enzyme–SH + SH + HgCl_2 \rightarrow Enzyme–S–S–Hg + 2HCl\ (inactive\ form)$$

→ This forms an inactive enzyme, leading to enzyme inhibition and coagulation of cytoplasmic proteins, ultimately killing or inhibiting the microorganism.

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Aldehydes

There are two important aldehydes used in microbiology, and one of the most commonly used is **formaldehyde**. It acts as both a **bactericidal** and, though rarely, **sporicidal** agent.

Example: *Formaldehyde*

Formaldehyde is stable at high concentrations and can be effective at elevated

temperatures around **22°C**, with a **relative humidity of 60% to 80%**, which enhances its activity.

It is usually used in the form of **formalin**, which is a solution containing **37% to 40% formaldehyde** by weight.

Applications:

- ➢ Used for the **sterilization of certain medical and laboratory instruments**, especially those that can't withstand high heat.
- ➢ Commonly used for the **sterilization of enclosed spaces**, such as **laminar airflow cabinets**, **incubators**, and sometimes even rooms.
- ➢ Also used for the **preservation of biological specimens** in laboratories and museums.

Mode of Action:

- ➢ Formaldehyde works by reacting with **organic nitrogen-containing compounds** like **proteins** and **nucleic acids**.
- ➢ This reaction leads to the **denaturation of proteins and nucleic acids**, rendering **enzymes inactive** and thereby **killing the microorganisms**.
- ➢ This makes it effective for sterilization purposes, though proper safety precautions must be taken due to its toxic and irritating nature.

Glutaraldehyde

Glutaraldehyde is a widely used chemical disinfectant and sterilizing agent in both medical and laboratory settings. A typical working concentration is **2%**, which is prepared by dissolving **2 grams of glutaraldehyde in 100 mL of water**.

It is highly effective against a broad range of microorganisms, including:

- ➢ **Vegetative bacteria**
- ➢ **Fungi**
- ➢ **Bacterial spores**
- ➢ **Fungal spores**

➢ **Viruses**

Because of its strong action against spores, it can be classified as a **sporicidal agent**, similar to formaldehyde.

Applications:
Glutaraldehyde is commonly used for sterilizing heat-sensitive medical equipment, such as:

➢ **Optical instruments**, including **contact lenses and lens equipment**

➢ **Neurological surgical instruments**

➢ **Respiratory therapy equipment** (such as ventilator parts, tubing, etc.)

➢ Also used in hospitals for sterilizing instruments that cannot be autoclaved

Mode of Action:

Glutaraldehyde works by **alkylating the amino and sulfhydryl groups of microbial proteins and enzymes**, which disrupts their structure and function. This leads to microbial death, including in more resistant forms like spores.

01/09/2025

Gases

Two common gases used for sterilization are **ethylene oxide** and **beta-propiolactone**.

Ethylene Oxide

Properties:

➢ Highly flammable
➢ Usually mixed with carbon dioxide or Freon to reduce its flammability
➢ Suitable for sterilizing heat- and moisture-sensitive materials
➢ Commonly used in hospitals, laboratories, and various industrial settings
➢ Functions by inactivating enzymes and denaturing proteins

➢ Alters cell membrane permeability

Mode of Action:

Ethylene oxide acts primarily through **alkylation reactions**, where it interacts with organic molecules such as proteins and enzymes. It replaces hydrogen atoms in reactive groups (like carboxyl or sulfhydryl groups), disrupting enzyme activity.

Example reaction:

Active enzyme — SH + $H_2C–CH_2$ → Inactive enzyme —S–CH_2–CH_2–OH

Applications:

➢ Sterilization of large equipment and packaging

➢ Commonly used for sterilizing plastic materials, bundles of cloth, and medical devices that can't withstand high temperatures

Beta-Propiolactone

Applications:

➢ Used to sterilize surfaces rather than materials requiring deep penetration
➢ Effective against spores, fungi, and viruses
➢ A good alternative when ethylene oxide isn’t available
➢ Limited by its **poor penetrating ability**, so mostly used on exposed surfaces

Mode of Action:

Similar to ethylene oxide, beta-propiolactone also sterilizes by **alkylating nucleophilic groups** on proteins and nucleic acids, ultimately inactivating essential microbial enzymes.

Quaternary Ammonium Compounds

Quats are **cationic detergents** used for disinfection and sterilization, especially on surfaces.

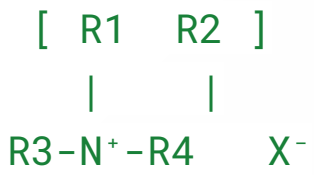
Types of Detergents:

1. **Anionic**
2. **Cationic**

3. **Non-ionic**

Among these, **cationic detergents**—especially quaternary ammonium compounds—are more effective as germicides.

General Structure:



- R1–R4: Organic (carbon-containing) groups
- X^-: Counter ion (commonly chloride or bromide)

Cetrimide (a Quaternary Ammonium Compound)

Properties:

➢ Bacteriostatic
➢ Particularly effective against **gram-positive bacteria**
➢ Fungicidal
➢ Active against certain pathogenic protozoa

Advantages:

➢ Low toxicity to humans
➢ Highly water-soluble
➢ Safe for general use

Mode of Action:

➢ Disrupts **plasma membrane permeability**
➢ Causes **protein denaturation**
➢ Inactivates essential microbial enzymes

Applications:

➢ Used as a **skin disinfectant**
➢ Incorporated into **ophthalmic solutions** and **cosmetic products** as a preservative
➢ Helps control microbial growth on hospital surfaces, nursing care facilities, etc.
➢ Used in **hand sanitizers**
➢ Applied in sterilization of **food utensils** (e.g., in restaurants)
➢ Used to disinfect **equipment in food processing plants**
➢ Applied in **dairy**, **egg**, and **fishing industries** for sanitation.

2/9/2025

Dyes and Their Antimicrobial Applications

Dyes can be classified into several chemical groups, some of which have **antimicrobial properties** and are used both in laboratory media and clinical applications.

Types of Antimicrobial Dyes:

❖ **Triphenylmethane Dyes**

➢ A broad group that includes several antimicrobial dyes.

❖ **Malachite Green**

➢ Effective against certain **Gram-positive bacteria**.

➢ **Example**: *Staphylococcus aureus* is inhibited by malachite green.

❖ **Brilliant Green**

❖ **Crystal Violet**

➢ More effective against **Gram-positive bacteria** than **Gram-negative bacteria**.

➢ **Mode of Action**:

- **Denatures proteins**.
- Disrupts the **cell's oxidative processes**, affecting metabolism and survival.

Applications:

- **Crystal Violet** is commonly used as a **fungicide**, especially effective against fungi such as *Monilia* and *Torula*.
- **Malachite Green** is also used in selective media for **suppressing Gram-positive organisms**.

Use in Laboratory Media:

In microbiological laboratories, dyes like **crystal violet**, **malachite green**, and **methylene blue** are often **incorporated into culture media** to **inhibit the growth of Gram-positive bacteria**. This allows for **selective isolation** of **Gram-negative organisms**, such as *Escherichia coli*.

- For example, **media containing crystal violet** can suppress unwanted Gram-positive contaminants, making it easier to detect and isolate **E. coli** from mixed cultures.

4/9/2025

Alkalis:

Alkalis such as **soaps** and other **alkaline cleaning agents** have antimicrobial properties. Their **high pH** helps to **disrupt microbial membranes** and denature proteins, which contributes to the destruction of microorganisms.

- **Applications**:
  Used in household and industrial cleaners, especially for cleaning **tiles, toilets, kitchens**, and other hard surfaces.

Acids:

Organic acids are commonly used as **food preservatives** because they **lower the pH** of the environment, creating conditions that inhibit the growth of bacteria and fungi.

Examples of Organic Acids:

- **Lactic acid** and **propionic acid**: Inhibit mold growth in foods and pharmaceutical preparations.

- **Benzoic acid**: Used to prevent fungal contamination in foods like **cheese**.

By lowering the pH, these acids **disrupt microbial metabolic activity** and help prevent spoilage and contamination.

- **Inorganic acids** like **hydrochloric acid (HCl)** and **sulfuric acid** are used for **surface cleaning and disinfection**, particularly in industrial settings.

Oxidizing Agents:

Oxidizing agents kill or inhibit microorganisms through **oxidation reactions**, which **damage cellular components**, especially **proteins and membranes**.

Common Oxidizing Agents:

- **Hydrogen peroxide (H_2O_2)**

- **Potassium permanganate ($KMnO_4$)**

Mode of Action:

- **Disrupt disulfide bonds** in proteins, causing **denaturation**.

- **Damage the cell membrane**, leading to cell lysis.

- **Inactivate bacterial enzymes**, halting essential metabolic processes.

Applications:

- Used for **sterilizing small rooms**, **instruments**, and even **skin disinfection** at **low concentrations**.
- Example: **Potassium permanganate** is used as a disinfectant for instruments and also for treating minor skin infections.

Alkylating Agents:

Alkylating agents destroy microorganisms by **adding alkyl groups** to proteins, DNA, and enzymes. This process, called **alkylation**, leads to the inactivation of essential cellular functions.

Examples of Alkylating Agents:

- **Formaldehyde**
- **Glutaraldehyde**
- **Ethylene oxide (ETO)**
- **Beta-propiolactone**

Applications:

- Used for **sterilizing medical equipment**, especially heat-sensitive instruments.
- **Ethylene oxide gas** is commonly used in hospital and pharmaceutical sterilization.
- **Glutaraldehyde** is used as a **liquid disinfectant** for surgical tools.

09/0/2025

Media Preparation

Types of Media:

1. Nutrient Agar Medium
2. Agar
3. Blood Agar
4. Smear Preparation

Purpose of Media Preparation:
Media is prepared to cultivate various microorganisms like bacteria, fungi, and algae. It is mainly used for growing bacteria in laboratory conditions.

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1. Nutrient Agar Medium Composition:

- Peptone – 5 g
- Beef extract (rich in vitamins) – 3 g
- Yeast extract – 2 g
- Sodium chloride (NaCl) – 5–9 g
- Agar – 1.5 g (used as a solidifying agent)
- Distilled water – 1000 ml

- pH – 7.2
- Temperature – Depends on the microorganism being cultured

Note:
Nutrient Broth is the same as nutrient agar but **without agar** (i.e., no solidifying agent).

2. Classification of Media (Based on Agar Concentration):

a) Solid Media

- Contains 1.5% to 2% agar (i.e., 1.5 to 2 grams per 100 ml of water)
- Used for isolating and growing colonies of bacteria

b) Semi-Solid Media

- Contains less than 1% agar (approx. 0.1%–0.9%)
- Mainly used to check bacterial motility

c) Liquid Media (Broth)

- Contains no agar
- Examples:
    1. Nutrient Broth
    2. Glucose Broth
    3. Peptone Broth
- Used for determining bacterial growth, production of enzymes, vitamins, and antibiotics
- Not suitable for industrial-scale production based solely on agar medium

3. Types of Media (Based on Function):

1) Transport Media

- ❖ Used to safely transport clinical samples from the collection site to the lab
- ❖ Maintains viability of pathogens without allowing them to multiply
- ❖ Examples:
  - ➢ Amies Transport Medium
  - ➢ Anaerobic Transport Medium
  - ➢ Peptone Broth (used in some cases)

2) Differential Media

- Helps differentiate between bacterial species based on their biochemical properties
- Examples:
  - **MacConkey Agar** – Differentiates between lactose fermenters (pink colonies, e.g., *E. coli*) and non-lactose fermenters (pale colonies)
  - **Blood Agar** – Differentiates hemolytic from non-hemolytic bacteria (e.g., *Streptococcus* is hemolytic)
  - **X-Gal Plate** – Another example of differential medium

3) Selective Media

- ❖ Promotes the growth of specific microorganisms while inhibiting others
- ❖ Examples:

➢ **EMB Agar (Eosin Methylene Blue)** – Selective for gram-negative bacteria like *E. coli*

➢ **Mannitol Salt Agar (MSA)** – Selective for *Staphylococcus aureus*

➢ **SS Agar (Salmonella-Shigella Agar)** – Used to isolate *Salmonella* and *Shigella*

4) Enriched Media

- Contains extra nutrients to support the growth of fastidious organisms
- Example: **Blood Agar** is also considered an enriched medium

5) Enrichment Media

- Enhances the growth of a particular microorganism while suppressing others
- Often used when the desired organism is in small numbers

6) Aerobic Media

- Media that supports the growth of organisms requiring oxygen

7) Assay Media *(possibly meant as assay media for testing antibiotics or vitamins)*

- Used for testing the effect of antibiotics, vitamins, etc., on microorganisms

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Indicator Media

Indicator (or differential) media are used to identify and distinguish microorganisms based on their ability to produce specific by-products or enzymes. These media contain pH-sensitive dyes or other indicators that change color in response to microbial activity.

Specialized Culture Media

Specialized media help differentiate microbes according to their metabolic reactions. Common examples include **MacConkey agar** and **Mannitol Salt agar**:

- **MacConkey agar** differentiates bacteria by their ability to ferment lactose. Lactose fermenters (such as *Escherichia coli*) produce acid, turning the colonies pink. Non-lactose fermenters (e.g., *Salmonella* and *Shigella*) remain colorless.

- **Mannitol Salt agar** selects for staphylococci and distinguishes *Staphylococcus aureus* (which ferments mannitol and turns the medium yellow) from other staphylococcal species.

- **Triple Sugar Iron (TSI) agar** detects hydrogen sulfide (H_2S) production. Bacteria like *Salmonella* form a black precipitate in the butt of the tube.

These media often include pH indicators such as **phenol red**, which changes color in response to acid or alkaline end products. Typical pH values for these media are around 7.5.

Biochemical Media

Biochemical test media identify microorganisms by detecting specific metabolic products or enzymatic activities. Examples include:

- **Indole test medium** (for tryptophanase activity)

- **Citrate agar** (for citrate utilization)

- **Urea agar** (for urease activity)

- **MR-VP broth** (Methyl Red and Voges–Proskauer tests)

Together, these indicators and biochemical media are essential tools for detecting and differentiating pathogenic bacteria such as *Staphylococcus aureus*, *E. coli*, *Salmonella*, and *Shigella*.

18/09/2025

Pure Culture Technique

A **pure culture** contains only a single species of microorganism, while a **mixed culture** contains more than one. Pure cultures are essential for accurately studying the characteristics of a specific bacterium. The three main techniques for obtaining a pure culture are:

1. **Streak plate method**
2. **Spread plate method**
3. **Pour plate method**

Streak Plate Method

In this method, an inoculating loop is used to spread the microbial sample across the surface of an agar plate in a way that gradually dilutes the cells, allowing individual colonies to develop.

Types of Streak Plate Methods and Short Procedures

- **Quadrant (or Quaternary) Streak**
  - ➢ Divide the plate into four quadrants.
  - ➢ Streak the first quadrant heavily.
  - ➢ Flame the loop, drag from quadrant 1 into 2, flame again, and repeat into 3 and 4, diluting each time.
- **T-Streak**
  - ➢ Draw a “T” on the plate’s underside to create three sections.
  - ➢ Streak the top section first, flame the loop, then streak each lower section by dragging from the previous area.
- **Radiant Streak**

➢ Start at the center of the plate.

➢ Streak outward in straight lines like spokes of a wheel.

❖ **Zigzag / Single-Line Streak**

➢ Move the loop back and forth across the agar surface in one continuous zigzag without crossing over.

➢ Often used for quick growth checks when isolation isn't critical.

❖ **Quaternary (four-way) Streak** *(if treated separately from quadrant)*

➢ Similar to the quadrant method but with more controlled, narrower streaks in four distinct sectors for finer isolation.

Spread Plate Method

- A **0.1 mL** portion of a previously **diluted** bacterial sample is pipetted onto the surface of a sterile agar plate.
- Using a **sterile L-shaped glass rod**, the inoculum is spread evenly across the surface while the plate is gently rotated on a turntable (or manually rotated).
- This produces well-separated colonies for **isolation** and allows accurate **enumeration of viable cells** (colony-forming units, CFU).

Before performing a spread plate (or a pour plate), a **serial dilution** of the original culture is usually prepared to reduce the cell concentration to a countable level.

Serial Dilution Technique – Short Procedure

1. **Prepare Diluent:** Use sterile saline or nutrient broth in a series of labeled test tubes (commonly 9 mL each).
2. **First Dilution (10^{-1}):** Pipette **1 mL** of the original sample into the first 9 mL tube. Mix thoroughly.

3. **Subsequent Dilutions:** Transfer **1 mL** from the 10^{-1} tube into the next 9 mL tube to make 10^{-2}, and continue the process for as many dilutions as needed (e.g., up to 10^{-6}).

4. **Plating:** From appropriate dilutions, take 0.1 mL for the **spread plate** or 1 mL for the **pour plate** to obtain isolated colonies and a countable range (typically 30–300 colonies per plate).

Pour Plate & Spread Plate

- **Purpose:** Both methods isolate and enumerate bacteria.

- **Difference:** In a **pour plate**, the diluted sample is mixed with molten agar before solidification, so colonies grow both on the surface and within the medium.

- **Spread plate** colonies grow only on the surface.