- **Introduction, history of microbiology, its branches, scope and its importance.**
- **Introduction to Prokaryotes and Eukaryotes.**
- **Study of:**
  - Ultra-structure and morphological classification of bacteria
  - Nutritional requirements, raw materials used for culture media
  - Physical parameters for growth
  - Growth curve
  - Isolation and preservation methods for pure cultures
  - Cultivation of anaerobes
  - Quantitative measurement of bacterial growth (total and viable count)
- **Study of different types of:**
  - Phase contrast microscopy
  - Dark field microscopy
  - Electron microscopy

INTRODUCTION [1, 2]

A **microbe**, or microorganism, is a microscopic organism that comprises either a single cell (unicellular); cell clusters; or multi cellular, relatively complex organisms.

Microbiology: The detailed study of microorganisms.

Microorganisms are very diverse; they include:

- Bacteria
- Fungi
- Algae
- Protozoa
- Microscopic plants (green algae)
- Animals such as rotifers and planarians.
- Some microbiologists also include **viruses**.

➢ Most microorganisms are unicellular, but this is not universal, since some multicellular organisms are microscopic.

➢ Some unicellular protists and bacteria, like *Thiomargarita namibiensis*, are macroscopic and visible to the naked eye.

➢ Most importantly, these organisms are vital to humans and the environment, as they participate in the Earth's element cycles, such as the carbon cycle and the nitrogen cycle.

➢ Microorganisms live in all parts of the biosphere:

- water
- soil
- hot springs
- on the ocean floor
- in the atmosphere
- deep inside the rocks, within the Earth's crust

<u>HISTORY OF MICROBIOLOGY</u> [1]

- Scientific evidence suggests that life began on Earth some 3.5 billion years ago.
- Since then, life has evolved into a wide variety of forms, which biologists have classified into a hierarchy of taxa.
- Some of the **oldest cells on Earth** are **single-cell**

Early history of microbiology

Historians are unsure who made the first observations of microorganisms.

Antonie van Leeuwenhoek (1632–1723):

- Father of microbiology" Father of bacteriology and protozoology (protistology), from Holland
- Developed microscope in 1673 and observed microorganisms, which he called **animalcules** and made one of the most important contributions to biology.
- Revealed accurate descriptions of protozoa, fungi, and bacteria.

Robert Hook: Developed compound microscope and observed first cork cell.

DIFFERENT ERA IN HISTORY OF MICROBIOLOGY

1. Discovery Era
2. Transition Era
3. Golden Era (1850 to 1915)
4. Modern Era

Abiogenesis vs. Biogenesis

- Theory of **spontaneous generation**, which stated that microorganisms arise from lifeless matter such as beef broth.
- An English cleric named **John Needham** advanced spontaneous generation.
- This theory was disputed by **Francesco Redi**, who showed that fly maggots do not arise from decaying meat (as others believed) if the meat is covered to prevent the entry of flies.
- **Lazzaro Spallanzani** disputed the theory by showing that boiled broth would not give rise to microscopic forms of life.
- Pasteur had to disprove spontaneous generation to sustain his theory, and he therefore devised a series of **swan-necked flasks** filled with broth.
- He left the flasks of broth open to the air, but the flasks had a curve in the neck so that microorganisms would fall into the neck, not the broth.
- The flasks did not become contaminated.
- Pasteur's experiments put to rest the notion of spontaneous generation.
- Pasteur, thus in 1858 resolved the controversy of spontaneous generation versus biogenesis and proved that microorganisms are not spontaneously generated from inanimate matter but arise from other microorganisms.
- **John Tyndall (1820–1893): An English physicist, Gave a final blow to spontaneous generation in 1877.**
- He conducted experiments in an aseptically designed box to prove that dust indeed carried the germs.
- He demonstrated that if no dust was present, sterile broth remained free of microbial growth for indefinite period even if it was directly exposed to air.
- He discovered highly resistant bacteria structure, later known as endospore.
- Prolonged boiling or intermittent heating was necessary to kill these spores, to make the infusion completely sterilized, a process known as **Tyndallisation.**

Chicken cholera experiment

- In 1880, Pasteur found that Chicken cholera germs from an old culture that had been around for some time lost their ability to transmit the disease → The inoculated chickens did not die.
- He repeated what he had done but with a fresh culture of chicken cholera germs.
- Pasteur reasoned that a new culture would provide more potent germs.
- He found a way of producing the resistance without the risk of the disease.
- Two groups of chickens were inoculated; one that had been given the old culture and one group that had not.
- Those chickens that had been given the old culture survived, those that had not died.
- The chickens that had been inoculated with the old culture had become immune to chicken cholera.
- Pasteur believed that their bodies had used the weaker strain of germ to form a defense against the more powerful germs in the fresher culture.

Koch's four postulates (1890):

- The organism causing the disease can be found in sick individuals but not in healthy ones.
- The organism can be isolated and grown in pure culture.
- The organism must cause the disease when it is introduced into a healthy animal.
- The organism must be recovered from the infected animal and shown to be the same as the organism that was introduced.
- The combined efforts of many scientists and most importantly Pasteur and Robert Koch established the **Germ theory of disease**.
- **The idea that invisible microorganisms are the cause of disease is called germ theory.**

The steps of Koch's postulates

a) Microorganisms are observed in a sick animal
b) Cultivated in the lab.
c) The organisms are injected into a healthy animal
d) The animal develops the disease.
e) The organisms are observed in the sick animal
f) Re-isolated in the lab.

Edward Jenner (1749-1823): An English physician was the first to prevent small pox.

- Impressed by the observation that countryside milk maid who contacted cowpox *(Cowpox is a milder disease caused by a virus closely related to small pox)* while milking were subsequently immune to small pox.
- In1796 he proved that inoculating people with pus from cowpox lesions provided protection against small pox.
- Jenner in 1798 published his results on 23 successful vaccinators.
- Eventually this process was known as vaccination, based on the latin word 'Vacca' meaning cow.
- He called the attenuated cultures as vaccines and the process as vaccination.
- Thus the use of cow pox virus to protect small pox disease in humans became popular replacing the risky technique of immunizing with actual small pox material.
- Jenner's experimental significance was realized by Pasteur who next applied this principle to the prevention of anthrax and it worked.
- Encouraged by the successful prevention of anthrax by vaccination, Pasteur marched ahead towards the service of humanity by making a vaccine for hydrophobia or rabies (a disease transmitted to people by bites of dogs and other animals).
- As with Jenner's vaccination for small pox, principle of the preventive treatment of rabies also worked fully which laid the foundation of modern immunization programme against many dreaded diseases like diphtheria, tetanus, pertussis, polio and measles etc.

Lord Joseph Lister (1827-1912):

- English surgeon is known for his notable contribution to the antiseptic treatment for the prevention and cure of wound infections.
- Lister concluded that wound infections too were due to microorganisms.
- In 1867, he developed a system of antiseptic surgery designed to prevent microorganisms by the application of phenol.
- He also devised a method to destroy microorganisms in the operation theatre by spraying a fine mist of carbolic acid into the air, thus producing an antiseptic environment.
- He first to introduce aseptic techniques for control of microbes by the use of physical and chemical agents which are still in use today.
- Because of this notable contribution, Joseph Lister is known as the **Father of Antiseptic surgery.**

Sir Alexander Fleming (Scottish physician and bacteriologist**):** The credit for the discovery of the first 'wonder drug' penicillin in 1929 goes to Fleming.

Dmitri Ivanovsky and **Virus:**

- Louis Pasteur was unable to find a causative agent for rabies and speculated about a pathogen too small to be detected by microscopes.
- In 1884, the French microbiologist Charles Chamberland invented the Pasteur-Chamberland filter with pores small enough to remove all bacteria from a solution passed through it.
- In 1892, the Russian biologist **Dmitri Ivanovsky** used this filter to study what is now known as the tobacco mosaic virus: crushed leaf extracts from infected tobacco plants remained infectious even after filtration to remove bacteria.
- Ivanovsky suggested the infection might be caused by a toxin produced by bacteria, but did not pursue the idea.
- At the time it was thought that all infectious agents could be retained by filters and grown on a nutrient medium—this was part of the germ theory of disease.
- In 1898, the Dutch microbiologist Martinus Beijerinck repeated the experiments and became convinced that the filtered solution contained a new form of infectious agent.
- He observed that the agent multiplied only in cells that were dividing, but as his experiments did not show that it was made of particles, he called it a *contagium vivum fluidum* (soluble living germ) and re-introduced the word *virus*.
- In the early 20th century, the English bacteriologist Frederick Twort discovered a group of viruses that infect bacteria, now called bacteriophages (or commonly 'phages').
- The development of bacterial resistance to antibiotics has renewed interest in the therapeutic use of bacteriophages.

- In 1931, when the American pathologist Ernest William Goodpasture and Alice Miles Woodruff grew influenza and several other viruses in fertilised chicken eggs.
- The first images of viruses were obtained upon the invention of electron microscopy in **1931 by the German engineers Ernst Ruska and Max Knoll**.
- In 1935, American biochemist and virologist Wendell Meredith Stanley examined the tobacco mosaic virus and found it was mostly made of protein.
- A short time later, this virus was separated into protein and RNA parts.
- The second half of the 20th century was the golden age of virus discovery and most of the documented species of animal, plant, and bacterial viruses were discovered during these years.

SCOPE OF MICROBIOLOGY [1]

- Microorganisms are present everywhere on earth which includes humans, animals, plants and other living creatures, soil, water and atmosphere.
- Microbes can multiply in all three habitats except in the atmosphere.
- Together their numbers far exceed all other living cells on this planet.
- Microorganisms are relevant to all of us in a multitude of ways.
- The influence of microorganism in human life is both beneficial as well as detrimental also.
- For example microorganisms are required for the production of bread, cheese, yogurt, alcohol, wine, beer, antibiotics (e.g. penicillin, streptomycin, chloromycetin), vaccines, vitamins, enzymes and many more important products.
- Microorganisms are indispensable components of our ecosystem.
- Microorganisms play an important role in the recycling of organic and inorganic material through their roles in the C, N and S cycles, thus playing an important part in the maintenance of the stability of the biosphere.
- There is vast scope in the field of microbiology due to the advancement in the field of science and technology.
- **The scope in this field is immense due to the involvement of microbiology in many fields like medicine, pharmacy, diary, industry, clinical research, water industry, agriculture, chemical technology and nanotechnology.**
- Microorganisms also have harmed humans and disrupted societies over the millennia.
- Many microbes spoil food and deteriorate materials like iron pipes, glass lenses, computer chips, jet fuel, paints, concrete, metal, plastic, paper and wood pilings.
- **The study of microbiology contributes greatly to the understanding of life through enhancements and intervention of microorganisms.**

ROLE AND APPLICATION OF MICROBIOLOGY IN DIFFERENT FIELDS [2]:

Table 1: Role and application of microbiology in different fields

Microbial physiology and Biochemistry	• Study the synthesis of antibiotics and toxins, microbial energy production, microbial nitrogen fixation, effects of chemical and physical agents on microbial growth and survival etc.
Immunology and Medicine	• *Immunology:* The study of immune system which protect the body from pathogens) • Deals with the identification and measures to cure diseases of human and animals which are infectious to them. • They have also provided us with the means of their control in the form of vaccine, antibiotics and other medically important drugs.
Molecular biology Microbial genetics and Genetic engineering	• Study of genetic information and how it regulated the development and function of cells and organisms. • New genes can be inserted into plants and animals. • Genetic engineering: microbes used to make hormones (insulin, human growth hormone), vaccine, antibiotics, and interferon and many other useful products for human being. • Development of new efficient microbial strains to synthesize useful products.

Agriculture	• The influence of microbes on agriculture; the prevention of the diseases that mainly damage the useful crops.
Food science	• Microorganisms have been used to produce food, from brewing and wine making, • Use of microbes to produce cheese, yoghurt, pickles and beer. • Microbes are also responsible for food spoilage so their studt helps in the prevention of spoilage of food and food borne diseases.
Industrial microbiology	• Involves use of microbes to produce antibiotics, steroids, alcohol, vitamins and amino acids etc.
Microbial ecology	• Bio-geochemical cycles: bioremediation (clean up the environment of toxic compounds) to reduce pollution effects • Microbes are responsible for the cycling of carbon, nitrogen phosphorus (geochemical cycles) • Maintain ecological balance on earth • Maintain soil fertility and may also be

Prokaryotes and Eukaryotes [1]: On the basis of genetic materials enclosed by a nuclear envelope, cells are divided into prokaryotes and eukaryotes. Prokaryotes don't have membrane bound organelles where as eukaryotes have.

Table 2: Difference between eukaryotes and prokaryotes

Prokaryotic Cell	Eukaryotic cell
Size is 0.1- 5.0 um	Size is 5-100 um
Nucleus is absent	Nucleus is present
Membrane bound nucleus absent.	Membrane bound Nucleus is present.
One chromosome is present	More than one number of chromosomes is present.
Unicellular	Multicellular
Lysosomes and Peroxisomes absent	Lysosomes and Peroxisomes present
Microtubules absent	Microtubules present
Endoplasmic reticulum absent	Endoplasmic reticulum present
Mitochondria absent	Mitochondria present
Cytoskeleton absent	Cytoskeleton present
Ribosomes smaller	Ribosomes larger
Vesicles present	Vesicles present
Golgi apparatus absent	Golgi apparatus present
Chloroplasts absent; chlorophyll scattered in the cytoplasm	Chloroplasts present in plants
Vacuoles absent	Vacuoles absent
Permeability of Nuclear membrane is not present	Permeability of Nuclear membrane is selective
Sexual reproduction is absent	Sexual reproduction is present
Endocytosis and exocytosis are absent.	Endocytosis and exocytosis occurred
It may have pili and fimbriae.	Pili and fimbriae are absent
Transcription occurs in the cytoplasm	Transcription occurs inside the nucleus.

- **ULTRA-STRUCTURE OF BACTERIA (bacteria-singular; bacterium: plural) [1, 2]:**
- *"Bacteria are unicellular prokaryotic organism where the organisms lack a few organelles and a true nucleus".*
- Bacterial cell have simpler internal structure, which lacks all membrane bound cell organelles such as mitochondria, lysosome, golgi, endoplasmic reticulum, chloroplast and true vacuole etc.
- All the action takes place in the cytosol or cytoplasmic membrane
- Bacteria also lacks true membrane bound nucleus and nucleolus. The bacterial nucleus is known as nucleoid.
- Most bacteria possess peptidoglycan, a unique polymer that makes its synthesis
- peptidoglycan is a good target for antibiotics.
- Protein synthesis takes place in the cytosol with structurally different ribosome's

Size
- 0.2 µm – 0.1 mm
- Most 0.5 – 5.0 µm

Shape
- Coccus (cocci)
- rod (bacillus, bacilli)
- spiral shapes (spirochetes; spirillum, spirilla)
- filamentous and various odd shapes.

Arrangement
- Clusters
- Tetrads
- Pairs
- chains

<u>Structures Outside the Cell Wall</u>
- Capsule
- Flagella
- Pili
- Sheath
- Prostheca
- Stalks

<u>Structures Inside the Cell Wall</u>
- Cell wall
- Cytoplasmic membrane
- Nucleoid
- Mesosome
- Ribosome
- cytoplasm

<u>Structure Outside the Cell Wall</u>

Capsule:
- Capsule is 0.2µm thick viscus layer outer layer to the cell wall.
- Capsule is 98% water and 2% polysaccharide or glycoprotein/ polypeptide or both.
- There are two types of capsule.
  - i. **Macro-capsule:** thickness of 0.2µm or more, visible under light microscope
  - ii. **Microcapsule:** thickness less than 0.2µm, visible under Electron microscope
- Capsule is very delicate structure.
- It can be removed by vigorous washing.
- Capsule is most important virulence factor of bacteria.

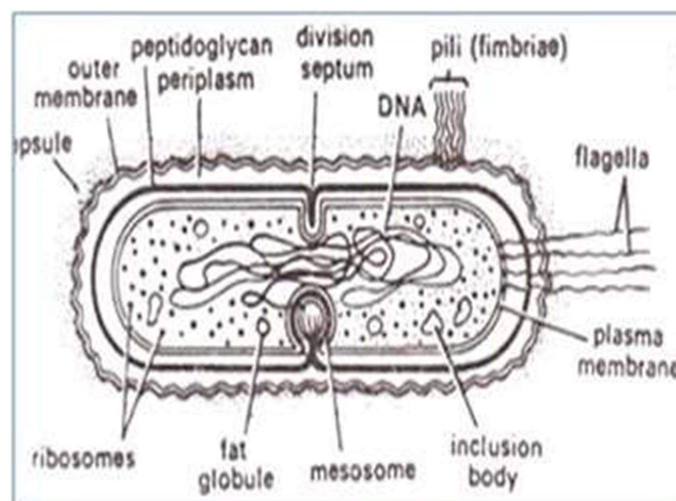


Fig. 1: Bacterial cell

Function of capsule:
- It helps in attachments as well as it prevent the cell from desiccation and drying.
- Capsule resist phagocytosis by WBCs

Flagella
- It is 15-20 nm filaments (hair like helical structure) extending from cytoplasmic membrane to exterior of the cell.
- The location of the flagella depends on bacterial species as polar situated at one or both ends which swims in back and forth fashion and lateral at along the sides.
- The parts of flagella are the filament, hook and the basal body.
- Filament is external to cell wall and is connected to the hook at cell surface, the hook and basal body are embedded in the cell envelope.
- Hook and filament is composed of protein subunits called as flagellin.

- It is composed of flagellin protein (globular protein) and known as H antigen.
- Flagellin is highly antigenic and functions in cell motility.
- Flagellin is synthesized within the cell and passes through the hollow centre of flagella.
- The arrangement of flagella may be described as
  i. *Monotrichous– single flagella on one side*
  ii. *Lophotrichous– tuft of flagella on one side*
  iii. *Amphitrichous–single or tuft on both sides*
  iv. *Peritrichous–surrounded by lateral flagell*

Function of Flagella

- Flagella are fully responsible for the bacterial motility.
- Deflagellation by mechanical means renders the motile cells immotile.
- The apparent movement of the bacterial cell usually takes place by the rotation of the flagella either in the clockwise or anticlockwise direction along its long axis.
- Bacterial cell possesses the inherent capacity to alter both the direction of rotation and the speed; besides, the meticulous adjustment of frequency of 'stops' and 'starts' by the appropriate movement of the flagella.

Pili / Fimbriae

- Hair-like proteinaceous structures that extend from the cell membrane to external environment are pili which are otherwise known as fimbriae.
- They are thinner, shorter and more numerous than flagella and they do not function in motility.
- There are two types of pili namely:
  i. Non-sex pili (Common pili) eg. fimbriae. **The fimbriae** are antigenic and mediate their adhesion which inhibits phagocytosis
  ii. The sex pili help in conjugation.

Cell Walls

- Beneath the external structures is the cell wall.
- Animal cells do not have a cell wall outside the cell membrane.
- Plant cells and fungal cells do have a cell wall.
- Most prokaryotic cells like bacteria (almost all) have a cell wall.
- They are essential structures in bacteria.
- A bacterium is referred as a protoplast when it is without cell wall.
- Cell wall may be lost due to the action of lysozyme enzyme, which destroys peptidoglycan. This cell is easily lysed and it is metabolically active but unable to reproduce.
- A bacterium with a damaged cell wall is referred as spheroplasts.
- It is caused by the action of toxic chemical or an antibiotic, they show a variety of forms and they are able to change into their normal form when the toxic agent is removed, i.e. when grown on a culture media.
- They are made of chemical components, which are found nowhere else in nature.
- Gram stain invented by Hans Christian Gram
- Divides Eubacteria into two main groups based on stain.
- Gram-positive cell wall is thick homogeneous monolayer
- Gram-negative cell wall is thin heterogeneous multilayer
- Correlates with two types of cell wall architecture.

Gram Positive Cell wall

- Composed of peptidoglycan.
- Mucopeptide (peptidoglycan or murien) formed by N acetyl glucosamine and N acetyl muramic acid alternating in chains, cross linked by peptide chains.
- Embedded in it are polyalcohol called Teichoic acids.
- Some are linked to Lipids and called Lipoteichoic acid.
- Lipotechoic acid link peptidoglycan to cytoplasmic membrane and the peptidoglycan gives rigidity.

Structure and Function of Peptidoglycan

- Single bag-like, seamless molecule
- Composed of polysaccharide chains cross linked with short chains of amino acids: "peptido" and "glycan".
- Peptidoglycan provides support and limits expansion of cell membrane
- Provides shape and structural support to cell

Fig. 2: Glycan chains cross-linked

Fig. 3: Arrangement of peptidoglycan layers

Fig. 4: Gram (+) cell wall

- Resists damage due to osmotic pressure
- Provides some degree of resistance to diffusion of molecules
- Lysozyme found in secretions cuts peptidoglycan
- Antibiotic penicillin prevents cross linking
- Cells in isotonic medium are not harmed
- Gram-positive cell walls contain teichoic acids lipoteichoic acid.
- Polymer of phosphate and ribitol or glycerol
- Lipoteichoic acid covalently attached to membrane lipids.
- Major contributor to negative charge of cell exterior.
- Appears to function in Ca++ binding
- Teichoic acids are thought to stabilize the Gram positive cell wall and may be used in adherence.

GRAM-NEGATIVE CELL WALL

- The cell walls of Gram-negative bacteria are more chemically complex, thinner and less compact.
- Gram-negative bacteria have relatively thin cell wall consisting of few layers of peptidoglycan and a outermembrane. Peptidoglycan comprises 5-10% of the wall material (thickness 2-6 nm).
- Outer membrane composed of: lipoprotein, phospholipid and lipopolysaccharide.

1. Outer Membrane (Outer Lipopolysaccharide and Phospholipids layer)

- Outer membrane is found only in Gram-negative bacteria.
- It functions as an initial barrier to the environment.
- It protects the cells from permeability by many substances including penicillin and lysozyme.
- It is the location of lipopolysaccharide (endotoxin) which is toxic for animals.

Fig. 5: Gram (-) Cell wall

- **This is composed of:**

  A. Lipopolysaccharide (LPS)

  B. Phospholipids

A. **Lipopolysaccharide (LPS):** Lipopolysaccharides are outer most part of Gram (-) bacterial cell wall, which acts as an endotoxin.

Lipopolysaccharides,are composed of:
- polysaccharides and lipid A (responsible for much of the toxicity of Gram -ve bacteria)
- core polysaccharide
- a terminal series of repeat units (O antigen).

B. **Phospho-lipid of outer membrane** (distinct from all other biological membranes):
- Its outer leaflet contains a lipopolysaccharides.
- This membrane has special channels (tiny holes or openings) called porins (consisting of protein molecules).
- Porins block the entrance of harmful chemicals and antibiotics, making Gram-ve bacteria much more difficult to treat than Gram+ve cells.

C. **Lipoprotein:** It cross-link the outer membrane and peptidoglycan layers.

2. PEPTIDOGLYCAN LAYER (Thin inner layer): Peptidoglycan makes up only 5 – 20% of the cell wall and is not the outermost layer, but lies between the plasma membrane and an outer membrane.

Primary function of the bacterial cell wall:
- To prevent the cell from expanding and eventually rupture or osmotic lysis of the cell protoplast.
- It is very rigid and gives shape to the cell.
- They may cause symptoms of disease in animals.
- They are the site of action of some of our most important antibiotics.

CELL MEMBRANE/ CYTOPLASMIC MEMBRANE
- Lies beneath the cell wall and separating it from the cell cytoplasm.
- Completely encloses the bacterial cell protoplast
- Composed of 60% protein and 40% phospholipid
- 5-10 nm thick, elastic and semipermeable layer
- Composed of Phospholipid, proteins and enzymes

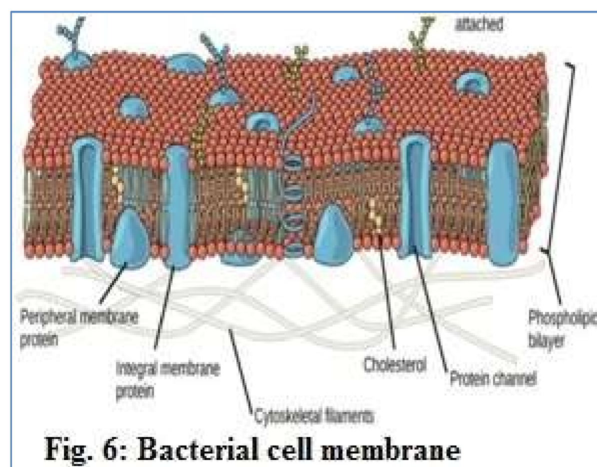


Fig. 6: Bacterial cell membrane

.Phospholipid (20-30%): Form bi-layered structure in which proteins are embedded and has two parts:
1. Hydrophilic head part 2. Hydrophobic tail part.

Proteins (70-80%), which are of two types: 1. peripheral proteins and 2, integral proteins
- **Plasma membranes in bacteria are composed of phospholipids contains a polar group attached to a 3 carbon glycerol back bone.**
- Arranged as a bi-layer
- Two fatty acid chains (hydrophobic) dangling from the other carbons of glycerol.
- The phosphate end of the molecule is hydrophilic and is attracted to water.
- The fatty acids are hydrophobic.
- Phospholipids arrange themselves spontaneously in water: lipid "tails" inward; glycerol "heads" outward.
- **The membrane proteins** associate with both sides of the membrane, or may imbed in the membrane, or pass through the membrane.

Functions of the Cytoplasmic Membrane
- Proteins in the cytoplasmic membrane have a variety of functions including transport and energy transformations.
- The plasma membrane is **selectively permeable** (control what moves into and out of cell).
- **Osmotic or permeability barrier:** the membrane is impermeable to molecules that are charged or greater than molecular weight of 100

- **Energy generation:** Location of the electron transport system (ETS) and the ATP synthsizing enzyme ATPase
- Specialized functions involving: cell wall synthesis, cell division and DNA replication.

Mesosome
- The outer membrane of cytoplasm forms much coiled invagination called mesosome.
- The surface of mesosome has many respiratory enzymes, which takes part in respiration.
- It is absent in eukaryotic cells.

Structure Inside the Cell wall: **Cytoplasm** and **Cytoplasmic Constituents of Bacterial Cells**

Cytoplasm:
- This is a Colloidal system containing a variety of organic and inorganic solutes containing 80% Water and 20% Salts, Proteins.
- They are rich in ribosomes, DNA and fluid.

Cytoplasmic Constituents of Bacterial Cells

1. **Genetic materials:** **2.Ribosomes** **3.Inclusions**

→Chromosome (DNA)
→Plasmids

Genetic Materials: In prokaryotes nucleus is not distinct.
- Nuclear membrane and nucleolus are absent.
- The genetic materials consists of DNA.
- **DNA (deoxyribonucleic acid):** This is the genetic material of the cell.
    - It contains a single chromosome consisting of a circular DNA filament and haploid.
    - They are highly coiled with intermixed polyamines and support proteins.
    - The genetic material DNA is present in the cytoplasm without histon proteins.
    - It can be replicated in a semi-conservative fashion and passed on to progeny cells.
- **Plasmids** are extra circular DNA.
- The cytoplasmic carriers of genetic information are termed plasmids or episomes.

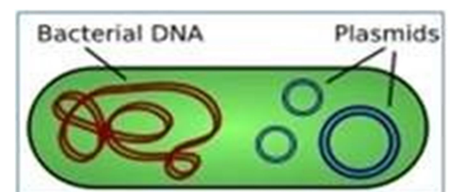


Fig. 7: Genetic materials in bacterial cell

Ribosome
- The procaryotic ribosome (L) is 70S in size, being composed of a 50S (large) subunit and a and 30S (small) subunit. The eucaryotic ribosome (R) is 80S in size and is composed of a 60S and a 40S subunit.
- Ribosomes are made of two subunits, a large subunit and a small subunit. Each subunit is made up of RNA and various proteins.

Function of Ribosome:
- **Ribosomes function in protein synthesis.**
- Amino acids are assembled into proteins according to the genetic code on the surfaces of ribosomes during the process of translation.

CLASSIFICATION OF BACTERIA [2]:

Bacteria can be classified into various categories based on their features and characteristics.
The classification of bacteria is mainly based on the following characters:
1. **Morphological Classification (Based on Shape)**
2. **Composition of the cell wall**
3. **Mode of respiration**
4. **Mode of nutrition**

Morphological Classification (Based on Shape)

- Bacteria and Archaea are classified by direct examination with the light microscope according to their morphology and arrangement.
- The basic morphologies are:
  1. Spheres (coccus)
  2. Round-ended cylinders (bacillus).
  3. Helically twisted cylinders (spirochetes)
  4. Cylinders curved in one plane (selenomonads)
  5. Unusual morphologies (such as the square, flat box-shaped cells of the archaean genus).
  - Arrangements include pairs, tetrads, clusters, chains and palisades.

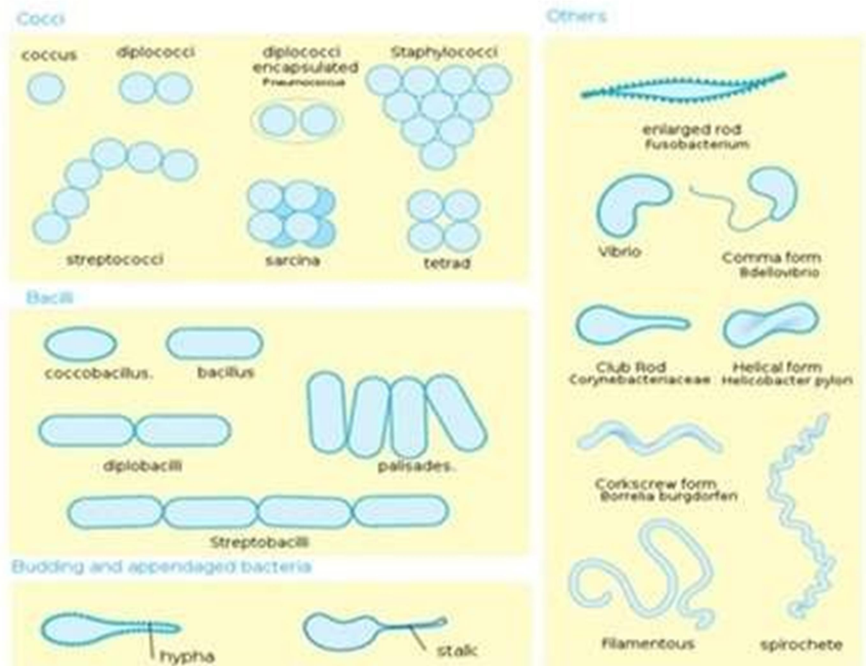


Fig. 8: Bacteria in different shapes and arrangements

1. **Coccus (Pleural – Cocci): Spherical bacteria**
   - may occur in pairs (**diplococci**)
   - in groups of four (**tetracocci**)
   - in grape-like clusters (**Staphylococci**)
   - in chains (**Streptococci**)
   - in cubical arrangements of eight or more (**sarcinae**).

   Example: *Staphylococcus aureus, S. pyogenes.*

2. **Bacillus (Pleural–Bacilli):** Rod-shaped bacteria; for example – *Bacillus cereus, Clostridium tetani.*
   - generally occur singly
   - but may occasionally be found in pairs (**diplo-bacilli**)
   - chains (**streptobacilli**).

3. **Spirillum (Pleural–Spirilla): Spiral-shaped bacteria.**
   - Spiral bacteria can be sub-classified on the basis of number of twists per cell, cell thickness, cell flexibility, and motility.
     - Spirilla
     - Spirochetes
     - vibrios

   For example – Spirillum, Vibrio, Spirochete species.

4. **Bacteria have Other Shapes Such as:**
   - Coccobacilli – Elongated spherical or ovoid form.
   - Filamentous – Bacilli that occur in long chains or threads.

NUTRITIONAL REQUIREMENTS, RAW MATERIALS USED FOR CULTURE MEDIA [3]

Chemical Requirements: Carbon, Nitrogen, Sulfur, and Phosphorus, other elements, trace elements and oxygen.

Carbon: Structural backbone of all organic compounds and Makes up 50% of dry weight of cell.

- **Chemoheterotrophs:** Obtain carbon from their energy source: lipids, proteins, and carbohydrates.
- **Chemoautotrophs and Photoautotrophs:** Obtain carbon from carbon dioxide.

Nitrogen: It makes up 14% of dry cell weight and used to form amino acids, DNA, and RNA.

Sources:

- Protein: Most bacteria
- Ammonium: Found in organic matter
- Nitrogen gas (N_2): Directly from atmosphere.
- Nitrates: Salts that dissociate to give NO_3^-

Sulfur: Used to form proteins and some vitamins (thiamin and biotin).
Sources:
- **Protein: Mostly**
- **Hydrogen sulfide**
- **Sulfates**

Phosphorus: Used to form DNA, RNA, ATP, and phospholipids.
Sources: Mainly inorganic phosphate salts and buffers.

Other Elements: Potassium, magnesium, and calcium are often required as enzyme cofactors. Calcium is required for cell wall synthesis in Gram positive bacteria.

Trace Elements:
- Many are used as enzyme cofactors.
- Commonly found in tap water.
- Iron, Copper, Molybdenum and Zinc

Oxygen: Organisms that use molecular oxygen (O2), produce more energy from nutrients than anaerobes.
Microorganism can be classified on the basis of their oxygen requirements:
A. **Obligate Aerobes:** Require oxygen to live. Example: Pseudomonas, common nosocomial pathogen.
B. **Facultative Anaerobes:** Can use oxygen, but can grow in its absence. Have complex set of enzymes. Examples: E. coli, Staphylococcus, yeasts, and many intestinal bacteria.
C. **Obligate Anaerobes:** Cannot use oxygen and are harmed by the presence of toxic forms of oxygen. Examples: Clostridium bacteria that cause tetanus and botulism.
D. **Aerotolerant Anaerobes:** Can't use oxygen, but tolerate its presence. Can break down toxic forms of oxygen. Example: Lactobacillus carries out fermentation regardless of oxygen presence.
E. **Microaerophiles:** Require oxygen, but at low concentrations. Sensitive to toxic forms of oxygen. Example: Campylobacter.

Microbial Growth Media/ Microbial Culture Media) [4]:
Culture: Microbes that grow and multiply in or on a culture medium.
Culture Media: Nutrient material prepared for microbial growth in the laboratory.
Requirements of a Microbial Culture Media:
- Must be sterile
- Contain appropriate nutrients
- Must be incubated at appropriate temperature

Types of Microbial Culture Media:
- **Culture Media Based on Consistency:**
  1. Solid Media
  2. Semisolid media
  3. Liquid Media
- **Culture Media Based on Composition:**
  1. Synthetic (chemically defined) media: Known chemical composition
  2. Non-synthetic/ Complex (chemically not defined) media: Unknown chemical composition
- **Culture Media Based on Application:**
  1. Basic Media: nutrient broth, nutrient agar
  2. Anaerobic media
  3. Enriched Media
  4. Enrichment Media
  5. Differential Media
  6. Transport Media
  7. Assay Media
  8. Selective Media:
     i. Thayer-Martin Media
     ii. Manittol- Salt Agar Media
     iii. Mac-Conkey's Agar Media
     iv. Wilson and blair Agar Media
     v. Crystal violet Blood Agar Media
     vi. Pseudosel Agar Media

Culture Media Based on Consistency:

1. **Liquid Media or nutrient broth:** Liquid consistency, Fast growth
2. **Solid Media or nutrient agar:**
   - Nutrient material that contains a solidifying agent (1.5-2% agar): plates, slants, deeps
   - The most common solidifier is agar, first used by Robert Koch.
3. **Semisolid Media: To study the motility; Agar: 0.5-0.7%**

Unique Properties of Agar:

- Melts above 95oC.
- Once melted, does not solidify until it reaches 40oC.
- Cannot be degraded by most bacteria.
- Polysaccharide made by red algae.
- Originally used as food thickener

Culture Media Based on Composition:

Nutrient material whose exact chemical composition is known (Defined Media):

- For chemoheterotrophs, must contain organic source of carbon and energy (e.g.: glucose, starch, etc.).
- May also contain amino acids, vitamins, and other important building blocks required by microbe.
- Expensive and not widely used.

Nutrient material whose exact chemical composition is known (Complex Media):

- Widely used for heterotrophic bacteria and fungi.
- Made of extracts from yeast, meat, plants, protein digests etc.
- Composition may vary slightly from batch to batch.
- Vitamins and organic growth factors provided by meat and yeast extracts.
  Example: Solid media (Nutrient agar), Liquid media (Nutrient broth)

Culture Media Based on Application:

Anaerobic Growth Media:

- Used to grow anaerobes that might be killed by oxygen.
- Reducing media
- Contain ingredients that chemically combine with oxygen and remove it from the medium.
- Example: Sodium thioglycolate
- Tubes are heated shortly before use to drive off oxygen.
- Plates must be grown in oxygen free containers (anaerobic chambers).

Selective Media: Used to suppress the growth of unwanted bacteria and encourage the growth of desired microbes.

- **Saboraud's Dextrose Agar:** pH of 5.6 discourages bacterial growth. Used to isolate fungi.
- **Brilliant Green Agar:** Green dye selectively inhibits gram-positive bacteria. Used to isolate gram-negative Salmonella.
- **Bismuth Sulfite Agar:** Used to isolate Salmonella typhi. Inhibits growth of most other bacteria.

Differential/ Indicator Media: Used to distinguish colonies of a desired organism.

- **Blood Agar:** Used to distinguish bacteria which destroy red blood cells (hemolysis). Hemolysis appears as an area of clearing around colony. Example: Streptococcus pyogenes.
- **Mannitol Salt Agar:**
  - Used to distinguish and select for Staphylococcus aureus.
  - High salt (7.5% NaCl) discourages growth of other organisms.
  - pH indicator changes color when mannitol is fermented to acid.
- **MacConkey Agar:**
  - Used to distinguish and select for Salmonella
  - Bile salts and crystal violet discourage growth of grampositive bacteria.
  - Lactose plus pH indicator: Lactose fermenters produce pink or red colonies, nonfermenters are colorless.
- **Enriched Media**
  - Enriched media contain the nutrients required to support the growth of a wide variety of organisms, including some fastidious ones.

- They are commonly used to grow as many different types of microbes as are present in the specimen.
- *Examples* are:
  - Blood agar is an enriched medium in which nutritionally-rich whole blood supplements the basic nutrients.
  - Chocolate agar is enriched with heat-treated blood (40-45°C), which turns brown and gives the medium that chocolate color.

- **Enrichment media**:
  - These media promotes the growth of a particular organism by providing it with the essential nutrients and rarely contains certain inhibitory substance to prevent the growth of normal competitors.
  - Used to favor the growth of a microbe that may be found in very small numbers.
  - Unlike selective medium, does not necessarily suppress the growth of other microbes.
  - Used mainly for fecal and soil samples.
- After incubation in enrichment medium, greater numbers of the organisms, increase the likelihood of positive identification.
  - ✓ An example is: Selenite F broth favors the growth of Salmonella also prevents the growth of normal competitors like *E. coli*. *E. coli* does not die in the medium but they do not flourish like Salmonella does.

PHYSICAL PARAMETERS FOR GROWTH [5]

Growth and Multiplication of Bacteria

- Refers to an increase in **cell number, not in cell size.**
- Bacteria divide by binary fission and cell divides to form two daughter cells.
- Nuclear division precedes cell division and therefore, in a growing population, many cells having two nuclear bodies can be seen. Bacterial growth may be considered as two levels, increase in the size of individual cells and increase in number of cells.
- Growth in numbers can be studied by bacterial counts that of total and viable counts.
- The total count gives the number of cells either living or not and the viable count measures the number of living cells that are capable of multiplication.

Many factors affect the generation time and growth of the organism, which are:

1. Nutrition
2. Temperature
3. Oxygen
4. Carbon dioxide
5. Light
6. pH
7. Moisture
8. Salt concentration

Nutrition: The principal constituents of the cells are water, proteins, polysaccharides, lipids, nucleic acid and mucopeptides.

- For growth and multiplication of bacteria, the **minimum nutritional requirement is water, a source of carbon, nitrogen and some inorganic salts.**
- Bacteria can be classified nutritionally, based on their energy requirement and on their ability to synthesise essential metabolites.
- Bacteria which derive their energy from sunlight-**phototrophs.**
- Bacteria which obtain energy from chemical reactions-**chemotrophs**.
- Bacteria which can synthesise all their organic compounds-**autotrophs**
- Bacteria which are unable to synthesise their own metabolites-**heterotrophs**.
- Bacteria require certain organic compounds in minute quantities. These compounds are known as growth factors or bacterial vitamins. Growth factors are called essential when growth does not occur in their absence, or they are necessary for it.

Oxygen: Depending on the influence of oxygen on growth and viability, bacteria are divided into aerobes and anaerobes.

- **Aerobic bacteria** require oxygen for growth.
- **Obligate aerobes** like cholera, vibrio, which will grow only in the presence of oxygen. These may even die on exposure to oxygen.
- **Facultative anaerobes** are ordinarily aerobic but can grow in the absence of oxygen. Most bacterial of medical importance are facultative anaerobes.

- **Microaerophilic bacteria** are those that grow best in the presence of low oxygen tension.

Carbon Dioxide

- All bacteria require small amounts of carbon dioxide for growth.
- This requirement is usually met by the carbon dioxide present in the atmosphere.
- Some bacteria like *Brucella abortus* require much higher levels of CO2.

Temperature

- Bacteria vary in their requirement of temperature for growth.
- The temperature at which growth occurs best is known as the optimum temperature.
- **Mesophilic bacteria -** grow best at temperatures of 25-40°C.
- **Psychrophilic bacteria -** grow best at temperatures below 20°C.
- **Thermophiles -** grow best at high temperatures, 55-80°C.
- The lowest temperature that kills a bacterium under standard conditions in a given time is known as thermal death point.

Moisture and Drying

- Water is an essential ingredient of bacterial protoplasm and hence drying is lethal to cells.
- Water stress, either from the concentration of solutes in the microbe's surroundings or from drying. As more solutes such as salts or sugar are dissolved in water, the concentration of water to solutes goes down.

Light

- Bacteria except phototrophic species grow well in the dark.
- They are sensitive to ultraviolet light and other radiations.
- Cultures die if exposed to light.

pH (H-ion concentration):

- Bacteria are sensitive to variations in pH.
- Each species has a pH range, above or below which it cannot survive and an optimum pH at which it grows best.
- Majority of pathogenic bacteria grow best at neutral or slightly alkaline pH.
- **Acidophiles:** "Acid loving" - Grow at very low pH (0.1 to 5.4).
- **Neutrophiles:** u Grow at pH 5.4 to 8.5.
- **Alkaliphiles:** "Alkali loving"- Grow at alkaline or high pH (7 to 12 or higher), for *Vibrio cholerae* optimal pH 9.

Salt concentration/ Osmotic Effect

- Bacteria are more tolerant to osmotic variation than most other cells due to the mechanical strength of their cell wall. Sudden exposure to hypertonic solutions may cause osmotic withdrawal of water and shrinkage of protoplasm called plasmolysis.
- **Halophiles:** Require moderate to large salt concentrations. Ocean water contains 3.5% salt. u Most bacteria in oceans.
- **Extreme or Obligate Halophiles:** Require very high salt concentrations (20 to 30%).
- **Facultative Halophiles:** Do not require high salt concentrations for growth, but tolerate 2% salt or more.

  <u>Hypertonic Solutions:</u> High osmotic pressure removes water from cell, causing shrinkage of cell membrane (plasmolysis). Used to control spoilage and microbial growth (Sugar in jelly and Salt on meat)

  <u>Hypotonic Solutions:</u> Low osmotic pressure causes water to enter the cell. In most cases cell wall prevents excessive entry of water. Microbe may lyse or burst if cell wall is weak.

Bacterial Growth Curve [5]

- **Bacterial Growth Curve :** When bacteria are inoculated into a liquid growth medium, and incubated, its growth follows a definite process, if bacterial counts are carried out at intervals after innoculation and plotted in relation to time, a growth curve is obtained.
- **Generation Time:** Time required for a cell to divide, and its population to double. Generation time varies considerably for example *E. coli* divides every 20 minutes whereas some bacteria require over 24 hours to divide.
- We can express the number of cells in a bacterial generation as 2^n , where n is the number of doublings that have occurred.

- The various stages of bacterial growth curve are associated with morphological and physiological alterations of the cells.

The Curve Shows the Following Four Phases in Bacterial Growth Curve

1. **Lag phase:**
    - Immediately following inoculation there is no appreciable increase in number, though there may be an increase in the size of the cells. This initial period is the time required for adaptation to the new environment and this lag phase varies with species, nature of culture medium and temperature.
    - Period of adjustment to new conditions.
    - Little or no cell division occurs, population size doesn't increase.
    - Phase of intense metabolic activity.
    - The maximum cell size is obtained towards the end of the lag phase.
    - May last from one hour to several days.

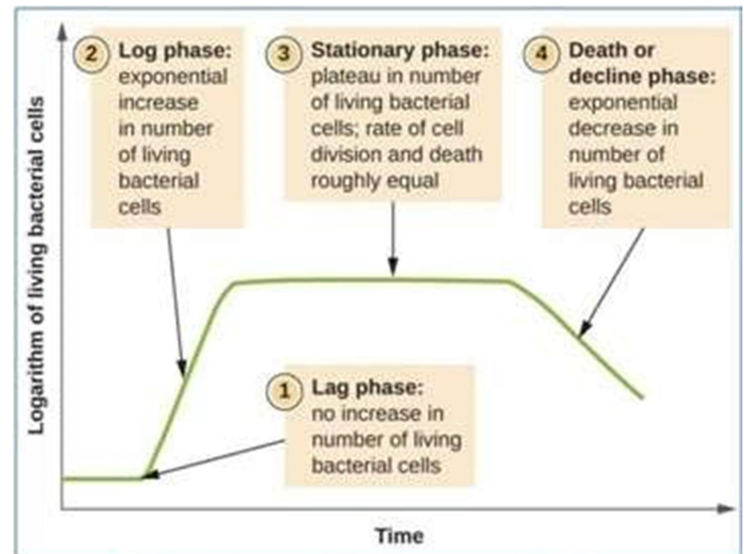


Fig. 9: Four Phases in Bacterial Growth Curve

2. **Log or exponential phase:**
    - Following the lag phase, the cell starts dividing and their numbers increase exponentially with time.
    - Cells begin to divide and generation time reaches a constant minimum.
    - Period of most rapid growth.
    - **Number of cells produced > Number of cells dying**
    - Cells are at highest metabolic activity.
    - In the log phase, cells are smaller and stained uniformly.
    - Cells are **most susceptible to adverse environmental factors at this stage.**
3. **Stationary Phase:**
    - After a period of exponential growth, cell division stops.
    - Overall cell number does not increase.
    - Cell division begins to slow down and population size begins to stabilize.
    - The viable count remains stationary as an equilibrium exists between the dying cells and the newly formed cells.
    - In the stationary phase, cells are frequently gram variable and show irregular staining due to the presence of intracellular storage granules.
    - Sporulation occurs at this stage.
    - Many bacteria produce secondary metabolic products such as exotoxins and antibiotics.
    - **Number of cells produced = Number of cells dying**
    - **Factors that slow down microbial growth:**
        - Accumulation of toxic waste materials
        - Limited nutrients
        - Acidic pH of media
        - Insufficient oxygen supply
4. **Death or Decline Phase:**
    - This is the phase when the population decreased due to cell death.
    - **Number of cells dying > Number of cells produced**
    - Cell number decreases at a logarithmic rate.
    - Cells lose their ability to divide.
    - A few cells may remain alive for a long period of time.
    - Involution forms are common in the phase of decline.

ISOLATION METHODS FOR PURE CULTURES [6]

Microbial Culture: Act of cultivating microorganisms or the microorganisms that are cultivated.

1. **Mixed culture:** Contains more than one type of microorganism in a culture.
2. **Pure culture:** Contains a single species of organism in a culture.
    - A pure culture is usually derived from a mixed culture (one containing many species) by transferring a small sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample many times before inoculating the new medium.

Pure cultures are important in microbiology for the following reasons:

- Once purified, the isolated species can then be cultivated with the knowledge that only the desired microorganism is being grown.
- A pure culture can be correctly identified for accurate studying and testing, and diagnosis in a clinical environment.
- Testing/experimenting with a pure culture ensures that the same results can be achieved regardless of how many times the test is repeated.
    - o Pure culture spontaneous mutation rate is low
    - o Pure culture clone is 99.999%identical

ISOLATION TECHNIQUE OF PURE CULTURE

- Cultures composed of cells arising from a single progenitor
- Progenitor is termed a CFU
- Aseptic technique prevents contamination of sterile substances or objects

Common isolation techniques:

1. **Streak plate method**
2. **Pour plate method**
    A. Loop dilution method
    B. Serial dilution method
3. **Spread plate method**
4. **Roll tube method**
5. **Special methods:**
    A. Single cell isolation method
        i. Cappilary pipette method
        ii. Micromanipulator method
    B. Enrichment culture method

Streak plate method

- Streaking is the process of spreading the microbial culture with an inoculating needle on the surface of the media.
- Sterilize the inoculating needle/ loop by flame to make red hot and allow it to cool for 30 seconds.
- The sample is streaked in such a way to provide series of dilution.
- Purpose - to thin out innoculum to get separate colonies.
- Sub-culturing can be done by streaking well isolated colonies from streak plate to new plate.
- Hold the broth culture containing tube in left hand and shake it.
- Sterilize the wire loop of the inoculation needle on burner flame.
- Remove the cotton plug of the broth culture tube by little finger of right hand.
- Flame the mouth of the test tube immediately.
- Insert the wire loop to form a thin film and replace the cotton plug.

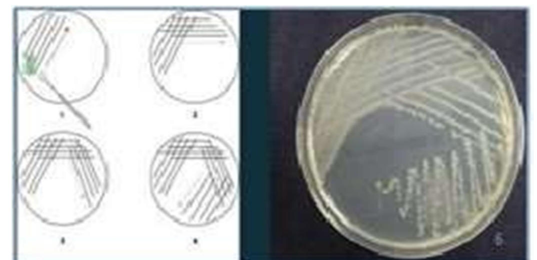


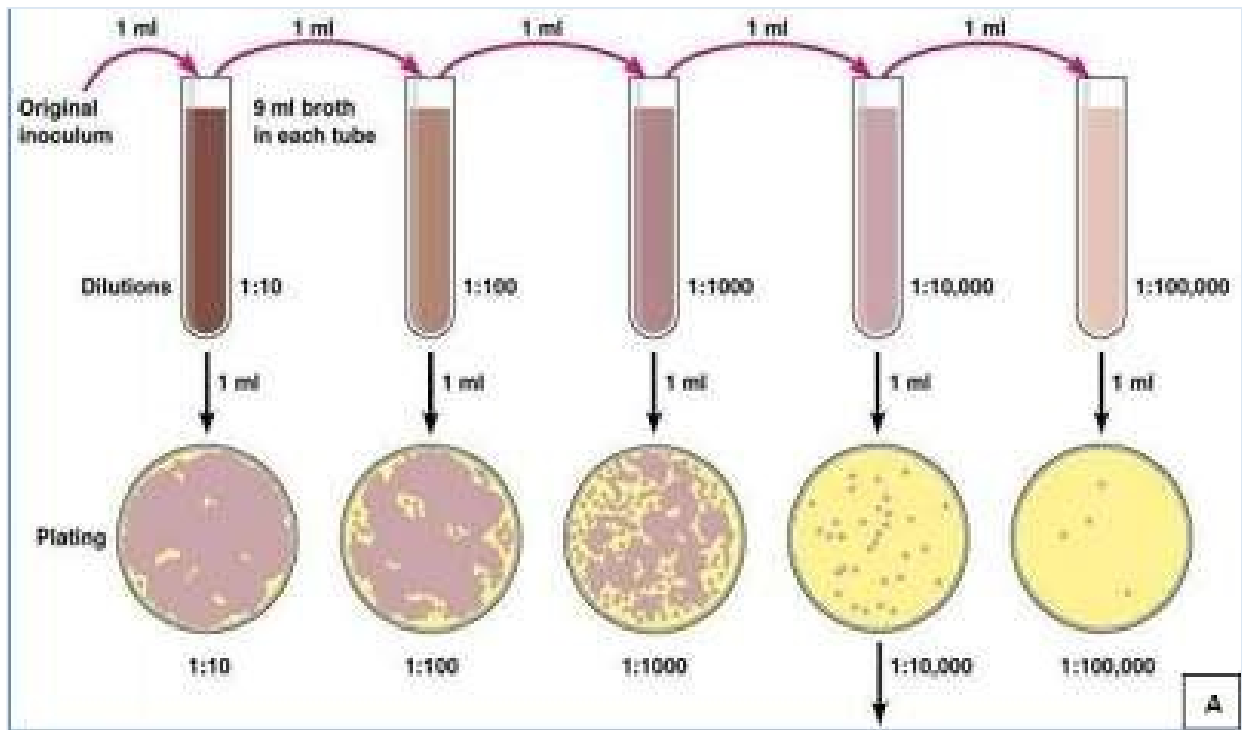
Fig. 10: Streak plate technique for pure culture

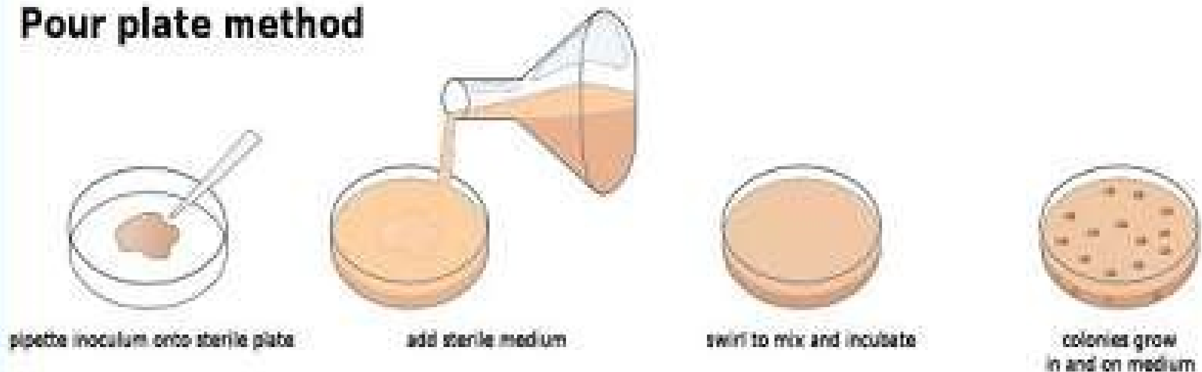
- The thin film in the loop is streaked in both a zig-zag manner by removing the loop backwards and forwards firmly.
- Care should be taken that loop should not be firmly pressed against the agar surface.
- Incubate the petri dish in incubator at a required temperature.
- Growth of the bacteria will be visible (after an overnight incubation)on the streaked marks.

Pour Plate Method:

- The bacterial culture and liquid agar medium are mixed together.
- The medium containing the culture poured into sterilized petri dishes, allowed solidifying and then incubated. After incubation colonies appear on the surface.

  Disadvantages:
- Microorganism trapped beneath the surface of medium hence surface as well as subsurface colonies are developed which makes the difficulties in counting the bacterial colony.
- Tedious and time consuming method
- Microbes are subjected to heat shock because liquid medium maintained at 45°C.
- Unsuitable for *Psychrophile*





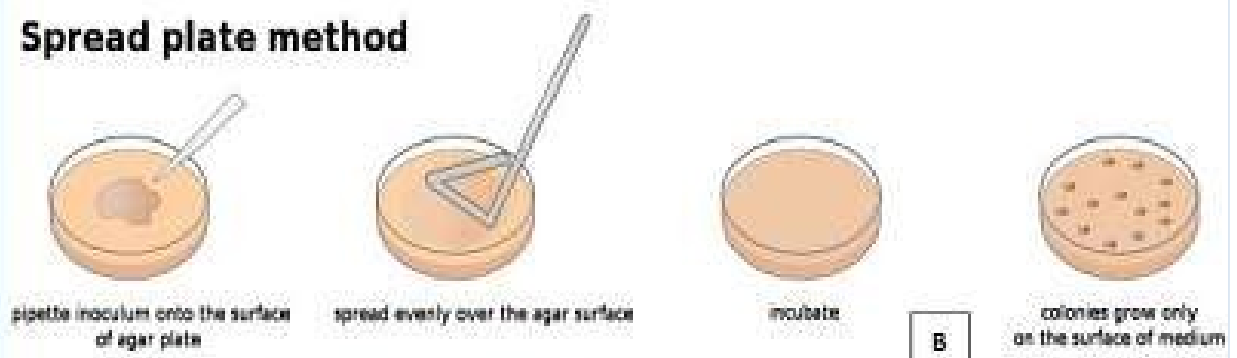


Fig. 11: Pour plate and spread plate method; A- Dilution technique; Method

Spread plate method

- This is the best method to isolate the pure colonies.
- In this technique, the culture is not mixed with the agar medium. Instead it is mixed with normal saline and serially diluted.

Advantages

- It is a simple method.
- In this method only surface colonies are formed.
- Micro-organisms are not exposed to higher temperature.

Micromanipulator Method:

- Micromanipulators have been built, which permit one to pick out a single cell from a mixed culture.
- This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation.
- The single cell of microbe sucked into micropipette and transferred to large amount of sterile medium.

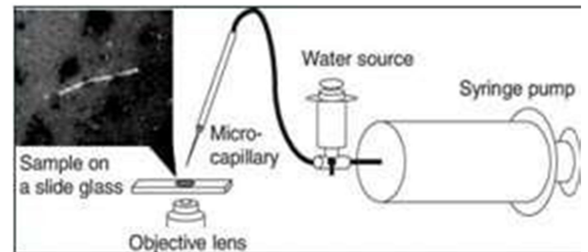


Fig.12: Micromanipulator Method for pure culture

Advantages

- The advantages of this method are that one can be reasonably sure that the cultures come from a single cell and one can obtain strains within the species.

Disadvantages

- The disadvantages are that the equipment is expensive, its manipulation is very tedious, and it requires a skilled person.

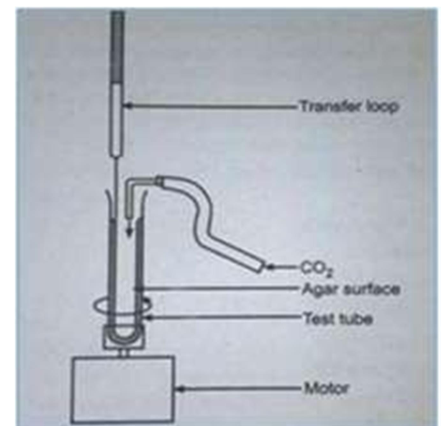


Fig. 13: Roll tube technique for pure culture

Roll Tube Method

- Agar medium is distributed as a thin layer over the internal surface of test tubes charged with an anaerobic atmosphere for the isolation of obligately anaerobic bacteria of the rumen.
- In this method, exposure of bacteria and culture medium to air is avoided by displacing the air in the culture vessel with an oxygen-free gas, such as carbon dioxide, hydrogen, nitrogen, or mixtures of these gases.
- Carbon dioxide is the gas of choice because it is heavier than air, relatively cheap, and valuable in buffering.
- Vessels are stoppered under conditions preventing access of air.
- The cultures require no special incubators and can be removed and examined with no anaerobic precautions if kept stoppered.
- If opened, anaerobiosis can be continuously maintained during necessary manipulations, and the culture again closed without exposure to oxygen.
- Media have an oxidation-reduction potential of –150 mv and are prepared, stored, and inoculated under oxygen-free gas.
- Specimens may be streaked on the surface of an agar layer on the wall of the tube.
- For quantitative work, molten agar is inoculated with dilutions of the specimen before the agar is spun and hardened on the tube wall (pour tube).
- The method is simple and is recommended for isolation of anaerobic bacteria in clinical or research laboratories.

PRESERVATION OF PURE CULTURE [6]:
Once a microorganism has been isolated and grown in pure culture, it becomes necessary to maintain the viability and purity of the microorganism by keeping the pure cultures free from contamination.

Objectives of Preservation
- To maintain isolated pure cultures for extended periods (future use) in a viable conditions.
- To avoid the contamination
- To restrict genetic change(Mutation)

Application of Preservation
1. *Academic Use*
2. *Research Purpose*
3. *Fermentation Industry*
4. *Biotechnological Field*

Methods of Preservation:
1. **Continuous Metabolism (slow rate, simple and less costly)**
   - Periodic transfer to fresh media (Sub- culturing)
   - Preservation by overlaying cultures with mineral oil
   - Storage in sterile soil
2. **Suspended (Stoppage) Metabolism (Drying and preservation at low temperature) Costly and modern:**
   - Storage at low temperature
   - Freeze dying/Lyophilization
   - Storage in silica gel
   - Vacume drying

Periodic Transfer to Fresh Media
- Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms.
- Strains can be maintained by periodically preparing a fresh culture from the previous stock culture.
- The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand.
- The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible.
- Many of the more common heterotrophs remain viable for several weeks or months on a medium like Nutrient Agar.
- The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

Advantages:
- It is a simple method; any special apparatus are not required.
- Easy to recover the culture

Disadvantages:
- The transfer is always subject to aseptic conditions to avoid contamination.
- Time consuming
- It becomes difficult to maintain a large number of pure cultures successfully for a long time
- In addition, there is a risk of genetic changes; therefore, it is now being replaced by some modern methods that do not need frequent sub-culturing.

Preservation by Oil Overlaying:
- Culture with mineral oil or liquid paraffin storage
- In this method sterile liquid paraffin is poured over the slant culture of microbes and stored upright at room temperature.
- cultures can also be maintained by covering agar slants by sterile mineral oil which is stored at room temperature or preferably at 0-5°C.
- It limits the oxygen access that reduces the microorganism’s metabolism and growth, as well as to cell drying during preservation.
- The preservation period for bacteria from the genera Azotobacter and Mycobacterium is from 7-10 years, for Bacillus it is 8-12 years.

Advantages:

- o Simple and cost effective
- o can preserve for 10-15 years
- o mainly used for anaerobic microorganisms

Storage in Sterile Soil

- It is mainly applied for the preservation of sporulating microorganisms (a single spore (endospore) within the cell).
- Fusarium, Penicillium, Alternaria, Rhizopus, Bacillus, Aspergillus, Penicillium, etc. proved successful for store in sterile soil.
- Viability of organisms found around 70- 80 years.
- Soil storage involves inoculation of 1ml of spore suspension into soil (autoclaved twice) and incubating at room temperature for 5-10 days.
- The initial growth period allows the fungus to use the available moisture and gradually to become dormant.
- The bottles are then stored at refrigerator.

Storage at Low Temperature

- Culture medium can be successfully stored in refrigerators or cold rooms, when the temperature is maintained at 4˚C.
- **Liquid nitrogen** can provide long term preservation of culture. In this method, dense suspension of microbes is prepared in a medium containing a protective agent(Glycerol or dimethyl sulfoxide) which prevent cell damage due to ice crystal formation. Suspension is sealed into small ampoules or vials and then frozen at - 150°c.
- At this temperature range the metabolic activities of microbes slows down greatly and only small quantity of nutrients will be utilized.
- This method cannot be used for a very long time because toxic products get accumulated which can kill the microbes.
- 10-30 Years without changing the characteristics.

Lyophilization (Freeze-Drying):

- Freeze-drying is a process where water and other solvents are removed from a frozen product via sublimation.
- Sublimation occurs when a frozen liquid goes directly to a gaseous state without entering a liquid phase.
- It is recommended using slow rates of cooling, as this will result in the formation of vertical ice crystal structures, thus allowing for more efficient water sublimation from the frozen product.

Procedure:

- In this process, a dense cell suspension is placed in small vials and frozen at -60 to -70°C.
- The vials are immediately connected to a high vacuum line.
- The ice present in the frozen suspension evaporates (sublime) under the vacuum.
- This result in dehydration of bacterial cell and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years.
- The vials are then sealed off under a vacuum and stored in the dark at 4°C in refrigerators.

Advantage:

- o Minimal storage space is required for process
- o Remained viable for more than 30 years.
- o Frequent sub-culturing is not required.
- o Maintained without contamination
- o Lyophilized strains remain genetically stable.
- o Small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in special sealed mailing containers.
- o Employed for the preservation of sera, toxin, enzymes and other biologicals

Disadvantage: Costly equipment

CULTIVATION OF ANAEROBIC BACTERIA [7]

- Anaerobic microorganisms are widespread and very important.
- Do not require oxygen for growth
- Oxygen
- is often extremely toxic for them.

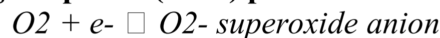
Different Types of Anaerobes:

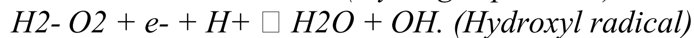
1. **Facultative anaerobes:** can grow in the presence or absence of oxygen
2. **Obligate or Strict Anaerobic Bacteria:** oxygen is toxic to these organisms, e.g. Clostridia.
   - Anaerobic environments exist in nature too.
   - **Anaerobic environments (low reduction potential) include:** Sediments of lakes, rivers and oceans; bogs, marshes, flooded soils, intestinal tract of animals; oral cavity of animals, deep underground areas, e.g. oil packets and some aquifers.

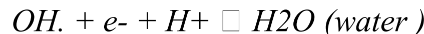
Oxygen Toxicity:

- Oxygen is used by aerobic and facultatively anaerobic organisms as its strong oxidising ability makes it an excellent electron acceptor.
- During the stepwise reduction of oxygen, which takes place in respiration, toxic and highly reactive intermediates are produced reactive oxygen species (ROS).

Reactive Oxygen Species (ROS) production during respiration:

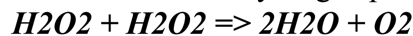






Chemical Dynamics in Anaerobic Bacteria: Organisms that use O_2 have developed defense mechanisms to protect themselves from these toxic forms of oxygen by different enzymes.

- ✓ **Catalase:** breaks down hydrogen peroxide (H_2O_2) to water and oxygen



- ✓ **Peroxidase:** peroxidase [by which 1NADH + H_2O_2 are converted to 2NAD and O_2] and superoxide dismutase [by which superoxide, O_2., is converted to H_2O_2

- ✓ **Superoxide dismutase:**

These enzymes detoxify peroxide and oxygen free radicals produced during metabolism in the presence of oxygen. Anaerobes are killed by oxygen as they lack these enzymes

Caution during anaerobic culture:

- ❖ Avoid shock to the anaerobic bacteria by exposing them to Oxygen or dryness of sample.
- ❖ Avoid exposure to cold as anaerobic microorganisms are sensitive to cold.
- ❖ Avoid swabs in sampling as Swab fibers contain ambient air and introduce oxygen to the sample.
- ❖ Avoid delay in transport of sample to the culture laboratory.

Anaerobic culture methods:

1. **Use of media containing reducing substances**
   A. Robertson Cooked Meat broth
   B. Thioglycolate broth
2. **Culture away from O_2**
   →Deep agar tubes
3. **Chemical exclusion of O_2**
   A. Anaerobic gas pak system
   B. Candle jar bottle
4. **Mechanical exclusion of O_2**
   A. Anaerobic incubator
5. **Exclusion of oxygen by flushing the tube with the desired gas**
   A. Roll tube method

1. **Use of media containing reducing substances (Thioglycolate broth):**
    - Media for anaerobes supplemented with nutrients like hemin and vitamin K, 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings.
    - Sterilize by autoclaving at 121°C for 15 minutes.
    - Cool to 25°C and store in a cool dark place preferably below 25°C.
    - Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin.
2. **Culture away from O2 (Deep agar tubes):** Simple way to produce anaerobic condition
    - The agar surface can be overlaid with oil to maintain the anaerobic condition.
    - Sterilization of the media can be carried out in the autoclave at 121^{oC} for 30 minutes.
    - Inoculation is by deep stabbing.

3. **Chemical exclusion of O2**

Anaerobic Gas Pak system:
- Uses H2 to convert air O2 to H2O in the presence of a catalyst.
- The reaction formula is (2H2 + 02 →2H20).
- The source of H2 is the gas Packet commercially supplied.
- The catalyst is palladium contained in the lid of the jar.
- Anaerobic indicator strips included to monitor the anaerobic condition.

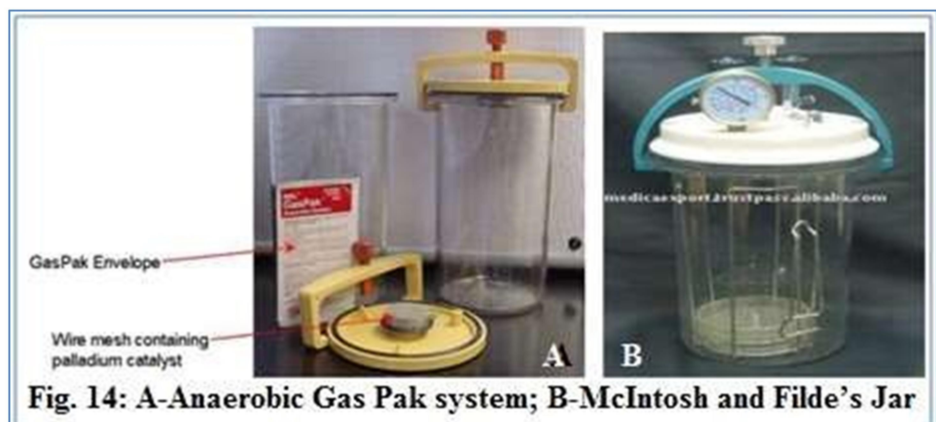


Fig. 14: A-Anaerobic Gas Pak system; B-McIntosh and Filde's Jar

McIntosh and Filde's Jar:
- Hydrogen gas is passed in through the jar.
- Catalyst helps to combine Hydrogen and O2
- Reduced Methylene blue remains colorless if anaerobiosis is achieved.

Candle jar:
- A microaerophile is a microorganism that requires oxygen to survive, but requires environments containing lower levels of oxygen than are present in the atmosphere (20% concentration).
- Many microphiles are also capnophiles, as they require an elevated concentration of carbon dioxide.
- In the laboratory they can be easily cultivated in a candle jar.
- A candle jar is a container into which a lit candle is introduced before sealing the container's airtight lid.
- The candle's flame burns until extinguished by oxygen deprivation, which creates a carbon dioxide-rich, oxygen-poor atmosphere in the jar.
- Many labs also have access directly to carbon dioxide and can add the desired carbon dioxide levels directly to incubators where they want to grow microaerophiles.
- Candle jars are used to grow bacteria requiring an increased CO2 concentration (capnophiles).
- Candle jars increase CO2 concentrations and still leave some O2 for aerobic capnophiles.

Fig. 15: Candle jar

4. **Mechanical exclusion of O2 (anaerobic incubator/** Anaerobic chamber**):**
- The ideal anaerobic incubation system provide O2 free environment for inoculation, incubation, identification and susceptibility tests.
- Use of gloves or sleeves forming airtight seals around the arms to handle items inside the chamber.
- Inside 37ºC incubator, enclosed heated block for loop sterilization.
- Anaerobic air is circulated by fan to maintain homogeneity.

All anaerobic chambers contain the followings:

- Catalyst usually palladium coated alumina pelletes
- Desiccant silica gel - absorb H_2O when H_2 combine free O_2
- H_2 gas (5 to 10%)
- CO_2 gas (10%)
- N_2 gas (80 to 90%)
- Indicator usually methylene blue

5. **Exclusion of oxygen by flushing the tube with the desired gas (Roll tube method):**

- Manipulations usually carried out under a jet of O_2-free N_2 or N_2 with CO_2 to exclude O_2.
- Exclude oxygen by flushing the tube with the desired gas
- Roll-tube (Hungate) method often used instead of conventional plates for isolation and culture of strict anaerobes
- Place 4.5ml of pre-reduced anaerobic agar medium into tube
- Seal the tube with the butyl rubber stopper and screw cap and autoclave.
- Inoculate with a syringe
- Prepare on roll tube spinner and Incubate in water bath
- Use of anaerobic cabinet/glove box allows conventional bacteriological techniques e.g. replica plating, antibiotic sensitivity testing etc. to be carried out anaerobically.

QUANTITATIVE MEASUREMENT OF BACTERIAL GROWTH (TOTAL AND VIABLE COUNT) [7]

- **Determination of growth (number of bacteria)**
- **Total counts:** Which include counting of both living cells and dead cells
- **Viable counts:** Which count living cells only

METHODS FOR TOTAL AND VIABLE COUNT

1. **DETERMINATION OF CELL MASS**
   A. **Direct Methods**
      i. Dry weight of cell
      ii. Wet weight of cell
      iii. Measurement of cell nitrogen
      iv. Volume of cells after centrifugation
   B. **Indirect Methods**
      i. Turbidometric or optical density Method
2. **DETERMINATION OF CELL ACTIVITY/ METABOLISM (INDIRECT METHODS):** ***Amount of metabolites formed are proportional to the population:***
   A. **Determination of Glucose metabolism**
   B. **Determination of O_2 Uptake**
   C. **Determination of Lactic acid production**
   D. **Determination of CO_2 production**
   E. **Determination of Total protein and Total DNA content**
3. **DETERMINATION OF CELL NUMBER:**
   A. **Direct Methods**
      i. Breed method or direct microscopic count
      ii. Counting chamber method
      iii. Coulter-Counter method
   B. **Indirect Methods**
      i. Plate count technique
      ii. Membrane filter technique.

Wet Weight Measurement:

- Measurement of cell mass is an easy step of cell growth measurement.
- A known volume of culture sample from the ferment or is withdrawn and centrifuged.
- Wet weight of pellets is measured by using pre-weighed filter paper.
- A pre-weighed filter paper of similar size is used to subtract the weight of wet filter paper.
- Thus wet-weight of cells is calculated.

Dry Weight Measurement:

- This is one of the simplest indirect methods in situations where determining the number of microorganisms is difficult or undesirable for other reasons.
- These methods measure some quantifiable cell property that increases as a direct result of microbial growth.
- Dry weight measurement of cell material is similar to that of wet weight.
- Portions of a culture can be taken at particular intervals and centrifuged at high speed to sediment bacterial cells to the bottom of a vessel.
- The sediment cells (called a cell pellet) are then washed to remove contaminating salt, and dried in an oven at 100-105°C to remove all water, leaving only the mass of components that make up the population of cells.
- An increase in the dry weight of the cells correlates closely with cell growth.
- However, this method will count dead as well as living cells.
- There might also be conditions where the dry weight per cell changes over time or under different conditions. For example, some bacteria that excrete polysaccharides will have a much higher dry weight per cell when growing on high sugar levels (when polysaccharides are produced) than on low.
- If the species under study forms large clumps of cells such as those that grow filamentous, dry weight is a better measurement of the cell population than is a viable plate count.
- Dry weight of about one million cells of E. coli is equal to 150 mg.
- Dry weight of bacterial cells is usually 10-20% of then- wet weight.

Determination of nitrogen content

- The major constituent of cell material is protein, and since nitrogen is a characteristic part of proteins, one can measure a bacterial population or cell crop in terms of bacterial nitrogen.
- Bacteria average approximately 14% nitrogen on a dry weight basis, although this figure is subject to some variation introduced by changes in culture conditions or differences between species.
- To measure growth by this technique, first harvest the cells and wash them free of medium and then perform a quantitative chemical analysis for nitrogen.
- Furthermore, the method is applicable only for concentrated populations.
  For these and other reasons, this procedure is used primarily in research.

Spectrophotometry - Turbidometric Analysis:

- The turbidity or optical density of a suspension of cells is directly related to cell mass or cell number.
- The method is simple but the sensitivity is limited to about 107 cells per ml for most bacteria.

Principle:

- **Incident light (passed through bacterial cell suspension) = Light absorbed by microorganisms + Light scattered by microorganisms + Light transmitted through microorganism suspension.**
- Absorbance is the intensity of light absorbed by the particles/ microorganisms, which is measured by using a spectrophotometer.
- Microbial cells scatter light that strikes them so when light is passed through bacterial cell suspension, light is scattered by the cells.
- Scattering of light increases with increase in cell number.
- As culture density increases (due to increase in cell number) the scattering (transmission) of light becomes less and can be measured by spectrophotometer.
- Thus cell growth of any bacterial suspension at a particular wavelength at different intervals can be measured in terms of absorbance and a standard graph (between absorbance and cell concentration) can be prepared.
- Determination of number of microorganism can be determined by extrapolating the test absorbance in the graph.

Detecting Acid and Gas Production

- The bacterial growth can be indirectly estimated by detecting specific changes caused in growth medium as a result of activity and multiplication of bacterial cells.
- It includes detecting activity cell products such as acid and gas production.

- Differential culture media can be used to differentiate between different kinds of bacteria by detecting acid or gas production.
- Differential media uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators.
- Amount of acid/ gas produced can be measured to detect the number of bacteria in the media.
- To measure acid production one can use a pH indicator in the media.
- The dye reduction tests such as methylene blue and resazurin reduction tests is based on the fact that the color imparted to milk by the addition of a dye such as methylene blue will disappear more or less quickly.
- Removal of the oxygen from milk and the formation of reducing substances during bacterial metabolism cause the color to disappear.
- The agencies responsible for the oxygen consumption are the bacteria.
- Though certain species of bacteria have considerably more influence than others, it is generally assumed that the greater the number of bacteria in milk, the quicker will the oxygen be consumed, and in turn the sooner will the color disappear.
- Thus, the time of reduction is taken as a measure of the number of organisms in milk although actually it is likely that it is more truly a measure of the total metabolic reactions proceeding at the cell surface of the bacteria.
- Gas production by bacteria is another major activity which can be taken up as an index of bacterial growth.
- Detection of gas production using Durham tube and change in color of the growth medium due to reduction of pH sensitive ingredients present in medium are commonly used for detection of acid and gas producing coliforms and yeasts.

Methods for Measurement of Cell Number

Direct microscopic count

- In the direct microscopic count, a counting chamber consisting of a ruled slide and a cover slip is employed.
- It is constructed in such a manner that the cover slip, slide, and ruled lines delimit a known volume.
- The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.
- The Petroff-Hausser counting chamber has small etched squares 1/20 of a millimeter (mm) by 1/20 of a mm and is 1/50 of a mm deep.
- The volume of one small square therefore is 1/20,000 of a cubic mm or 1/20,000,000 of a cubic centimeter (cc).
- There are 16 small squares in the large double-lined squares that are actually counted, making the volume of a large double-lined square 1/1,250,000 cc.
- The normal procedure is to count the number of bacteria in five large double-lined squares and divide by five to get the average number of bacteria per large square.
- This number is then multiplied by 1,250,000 since the square holds a volume of 1/1,250,000 cc, to find the total number of organisms per cc in the original sample.
- If the bacteria are diluted, such as by mixing the bacteria with dye before being placed in the counting chamber, then this dilution must also be considered in the final calculations.
- **The formula used for the direct microscopic counting:**
  The number of bacteria per cc = *The average number of bacteria per large double-lined square* **X** *The dilution factor of the large square (1,250,000)* **X** *The dilution factor of any dilutions made prior to placing the sample in the counting chamber.*
- The cell culture of high density can be diluted; otherwise clumps of cells would be formed which would create problem in exact counting of bacterial cells.
- A viable cell is defined as a cell which is able to divide and increase cell numbers.
- The normal way to perform a viable count is to determine the number of cells in the sample which is capable of forming colonies on a suitable medium.
- Here it is assumed that each viable cell will form one colony.
- Therefore, viable count is often called plate count or colony count.

Plate count method:

- Standard Plate Count (SPC) is a technique under this category which is commonly employed in microbiological laboratories for enumeration of bacteria.
- The SPC is the number of bacterial colonies that develop on a medium in a petri dish seeded with a known amount of inoculum.
- The number of bacteria in a given sample is usually too great to be counted directly.
- However, if the sample is serially diluted and then plated out on an agar surface in such a manner that single isolated bacteria form visible isolated colonies, the number of colonies can be used as a measure of the number of viable (living) cells in that known dilution.
- However, keep in mind that if the organism normally forms multiple cell arrangements, such as chains, the colony-forming unit may consist of a chain of bacteria rather than a single bacterium.
- In addition, some of the bacteria may be clumped together.
- Therefore, when doing the plate count technique, we generally say we are determining the number of Colony-Forming Units (CFUs) in that known dilution.
- By extrapolation, this number can in turn be used to calculate the number of CFUs in the original sample.
- Normally, the bacterial sample is diluted by factors of 10 and plated on agar either by pour plate or spread plate technique.
- After incubation, the number of colonies on a dilution plate showing between 30 and 300 colonies is determined. A plate having 30-300 colonies is chosen because this range is considered statistically significant.
- If there are less than 30 colonies on the plate, small errors in dilution technique or the presence of a few contaminants will have a drastic effect on the final count.
- Likewise, if there are more than 300 colonies on the plate, there will be poor isolation and colonies will have grown together.
- Generally, one wants to determine the number of CFUs per milliliter (ml) of sample.
- To find this, the number of colonies (on a plate having 30-300 colonies) is multiplied by the number of times the original ml of bacteria was diluted (the dilution factor of the plate counted).
- For example, if a plate containing a 1/1,000 dilution of the original ml of sample shows 159 colonies, then 159 represents 1/1,000 the number of CFUs present in the original ml.
- Therefore the number of CFUs per ml in the original sample is found by multiplying 159 x 103 (or preferably represented as 1.59 x 105).

Advantage: Its sensitivity (theoretically, a single cell can be detected), and it allows for inspection and positive identification of the organism counted.

Disadvantages:

- Only living cells develop colonies that are counted.
- Clumps or chains of cells develop into a single colony.
- Colonies develop only from those organisms for which the cultural conditions are suitable for growth.

Microscopic counting

- **Advantages:** Easy to perform, inexpensive and give information about size and morphology
- **Disadvantages:** Non viable cells are also counted

Electronic enumeration of cells (Coulter Counter):

- A Coulter counter is an apparatus for counting and sizing particles and cells.
- The counter detects change in electrical conductance of a small aperture as fluid containing cells is drawn through.
- Cells, being non-conducting particles, alter the effective cross-section of the conductive channel.
- It was an American inventor named Wallace H. Coulter who was responsible for the theory and design of the Coulter Counter.
- He first devised the theory behind its operation in 1947 while experimenting with electronics.
- Coulter determined that electrical charge could be used to determine the size and number of microscopic particles in a solution.

- This phenomenon is now known as the Coulter Principle.
- A typical Coulter counter has one or more micro-channels that separate two chambers containing electrolyte solutions.
- When a particle flows through one of the micro-channels, it results in the electrical resistance change of the liquid filled micro-channel.
  - This resistance change can be recorded as electric current or voltage pulses, which can be correlated to size, mobility, surface charge and concentration of the particles.

Membrane filter counting method

- This method is suitable for liquid or semi-liquid samples (e.g. water) and commonly used for enumeration of Coliform and Staphylococcus spp.
- Membrane filtration method is used with relatively low numbers.
- A known volume of liquid passed through membrane filter. Filter pore size retains organism.
- It filters microorganism of size more than 0.45 micrometer.
- Filter is placed on appropriate growth medium and incubated and cells are counted.

STUDY OF DIFFERENT TYPES OF MICROSCOPE [8]:

- **Phase contrast microscopy** • **Dark field microscopy** • **Electron microscopy**

Microscope is an instrument which provides a magnified image of an object, which is not visible with the naked eye.

TYPES OF MICROSCOPE

1. Optical microscope

A. Compound Microscope: Compound microscope achieves higher levels of magnification and is used to view smaller specimens such as cell structures which cannot be seen at lower levels of magnification.

B. Phase contrast microscope: A microscope that visualizes minute surface irregularities by using light interference. It is commonly used to observe living cells without staining them.

C. Polarizing microscope: A microscope that uses different light transmission characteristics of materials (crystalline structures), to produce an image.

D. Fluorescence microscope: A microscope that observes fluorescence emitted by samples by using special light sources such as mercury lamps and specimen stained with fluorescence materials.

2. **Electron microscope:** These microscopes emit electron beams, not light beams, toward targets to magnify them.

Phase-Contrast Microscope

- Phase contrast can be employed to distinguish between structures of similar transparency.
- Contrast is defined as the difference in light intensity between the specimen and the adjacent background relative to the overall background intensity.
- This is used to study the behavior of living cells, observe the nuclear and cytoplasmic changes taking place during mitosis and the effect of different chemicals inside the living cells.

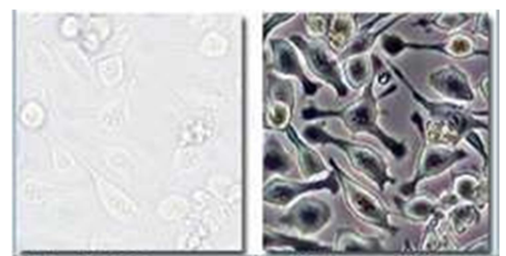


Fig. 16: Images of a living cell in a bright field and phase contrast

- The phase contrast technique employs an optical mechanism to translate minute variations in phase into corresponding changes, which can be visualized as differences in image contrast.
- One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without previously being killed, fixed, and stained. As a result, the dynamics of ongoing biological processes can be observed and recorded in high contrast with sharp clarity of minute specimen details.

The Dark Field Microscope

- **Dark-field microscopy** (also called **dark-ground microscopy**) describes microscopy methods, in both light and electron microscopy, which exclude the

unscattered beam from the image. As a result, the field around the specimen (i.e., where there is no specimen to scatter the beam) is generally dark.

- In optical microscopy, dark-field describes an illumination technique used to enhance the contrast in unstained samples. It works by illuminating the sample with light that will not be collected by the objective lens and thus will not form part of the image. This produces the classic appearance of a dark, almost black, background with bright objects on it.

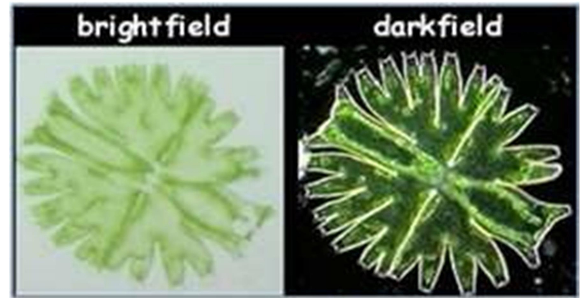


Fig. 17: Images in a dark field microscope

- Dark-field studies in transmission electron microscopy play a powerful role in the study of crystals and crystal defects, as well as in the imaging of individual atoms.
- Dark-field microscopy is a very simple yet effective technique and well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual, water-borne, single-celled organisms. Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.
- The main limitation of dark-field microscopy is the low light levels seen in the final image. This means that the sample must be very strongly illuminated, which can cause damage to the sample.

The Electron Microscope

- The organelles of the cell became known after the electron microscope was invented.
- The electron microscope was developed in **1932 by M. Knoll and Ruska in Germany.**
- This microscope utilizes a stream of high speed electrons which are deflected by an electromagnetic field.

It consists of:

i. A source of supplying, a beam of electron of uniform velocity
ii. A condenser lens for concentrating the electron on the specimen
iii. A specimen stage for displacing
iv. The specimen which transmits the electron beam
v. An objective lens
vi. A projector lens
vii. Fluorescent screen on which final image is observed.

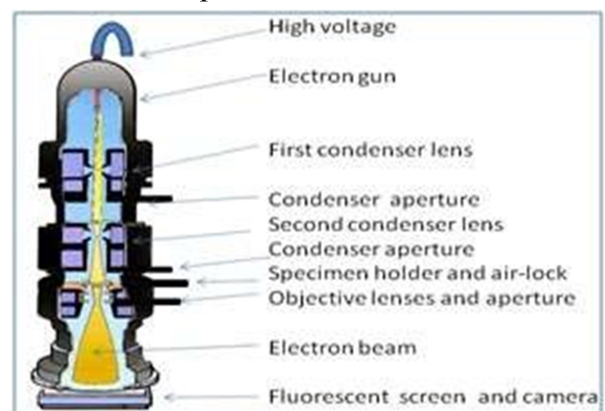


Fig. 18: Transmission electron microscope

There are two types of electron microscopes:

1. **Transmission electron microscope (TEM)**
2. **Scanning electron microscope (SEM)**

- The original form of electron microscope, **the transmission electron** microscope (TEM) uses a high voltage electron beam to illuminate the specimen and create an image.
- The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source.
- The electron beam is accelerated and focused by electrostatic and electromagnetic lenses.
- Electrons transmitted through the transparent part of the specimen scatters them out of the beam.
- When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope.
- The spatial variation in this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen.
- Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam.

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