PHARMACEUTICAL MICROBIOLOGY (BP303T)
UNIT-1

INTRODUCTION

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

HISTORY OF MICROBIOLOGY

- 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.
- **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.**

SCOPE OF MICROBIOLOGY

- 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..
- The study of microbiology contributes greatly to the understanding of life through enhancements and intervention of microorganisms.

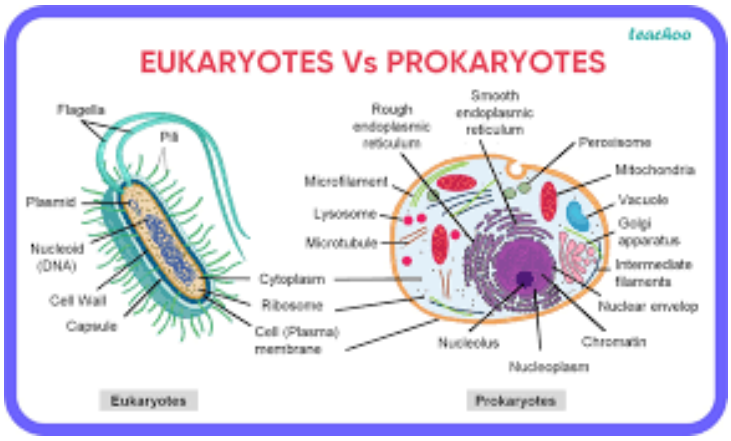
ROLE AND APPLICATION OF MICROBIOLOGY IN DIFFERENT FIELDS:

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: 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.



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):

- *"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

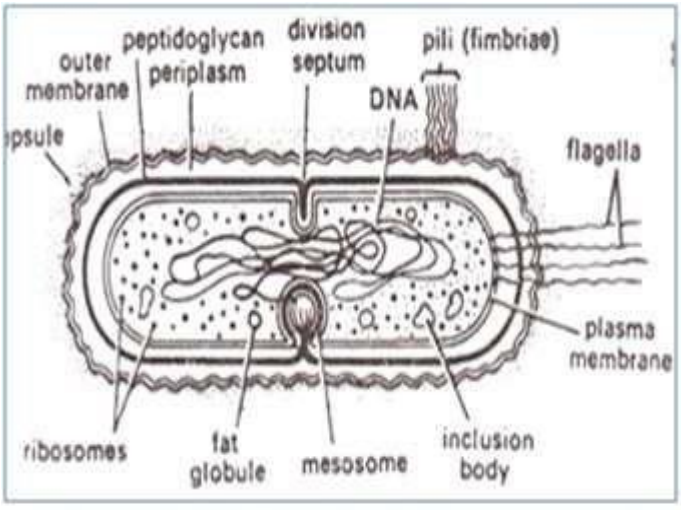


Fig. 1: Bacterial cell

CLASSIFICATION OF BACTERIA :

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.

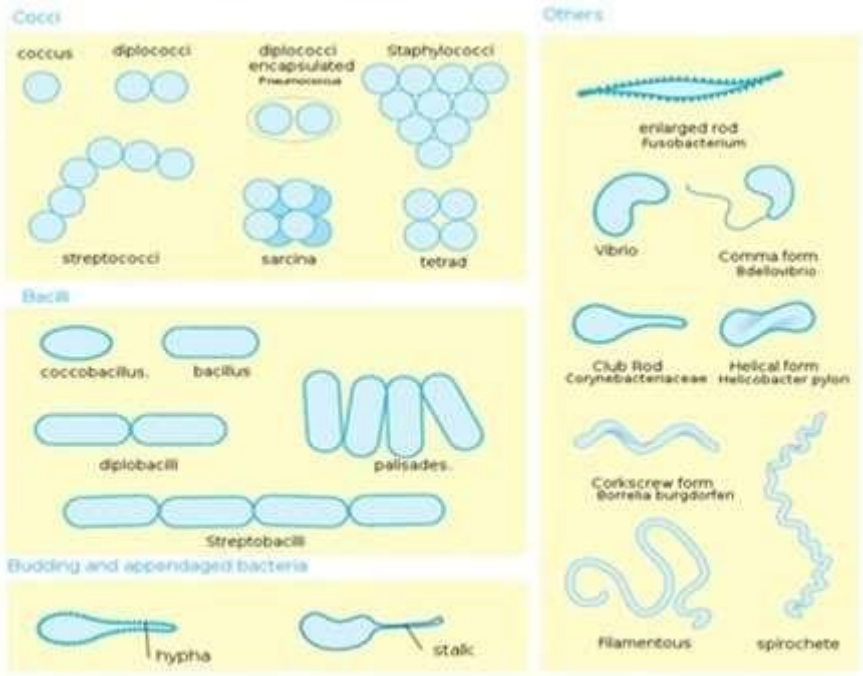


Fig. 8: Bacteria in different shapes and arrangements

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

Chemical Requirements:

- **Carbon**
- **Nitrogen**
- **Sulfur**
- **oxygen**
- **Phosphorus**
- **Other elements**
- **Traceelements**

Microbial Growth Media/ Microbial Culture Media): 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

PHYSICAL PARAMETERS FOR GROWTH

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

Bacterial Growth Curve

- **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.
- 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.

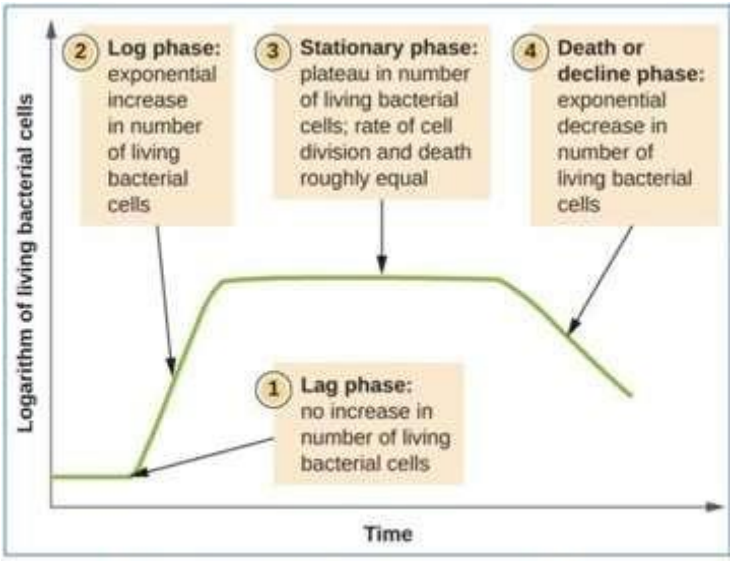


Fig. 9: Four Phases in Bacterial Growth Curve

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
     - Acidic pH of media
     - Limited nutrients
     - 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

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.
  - Pure culture spontaneous mutation rate is low
  - 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.

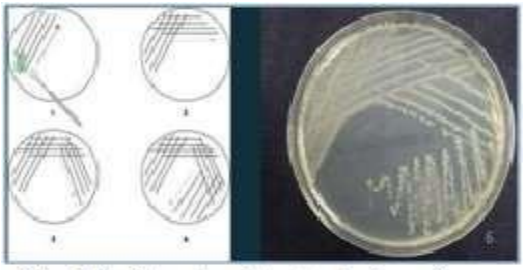


Fig. 10: Streak plate technique for pure culture

- 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℃.
- Unsuitable for *Psychrophile*

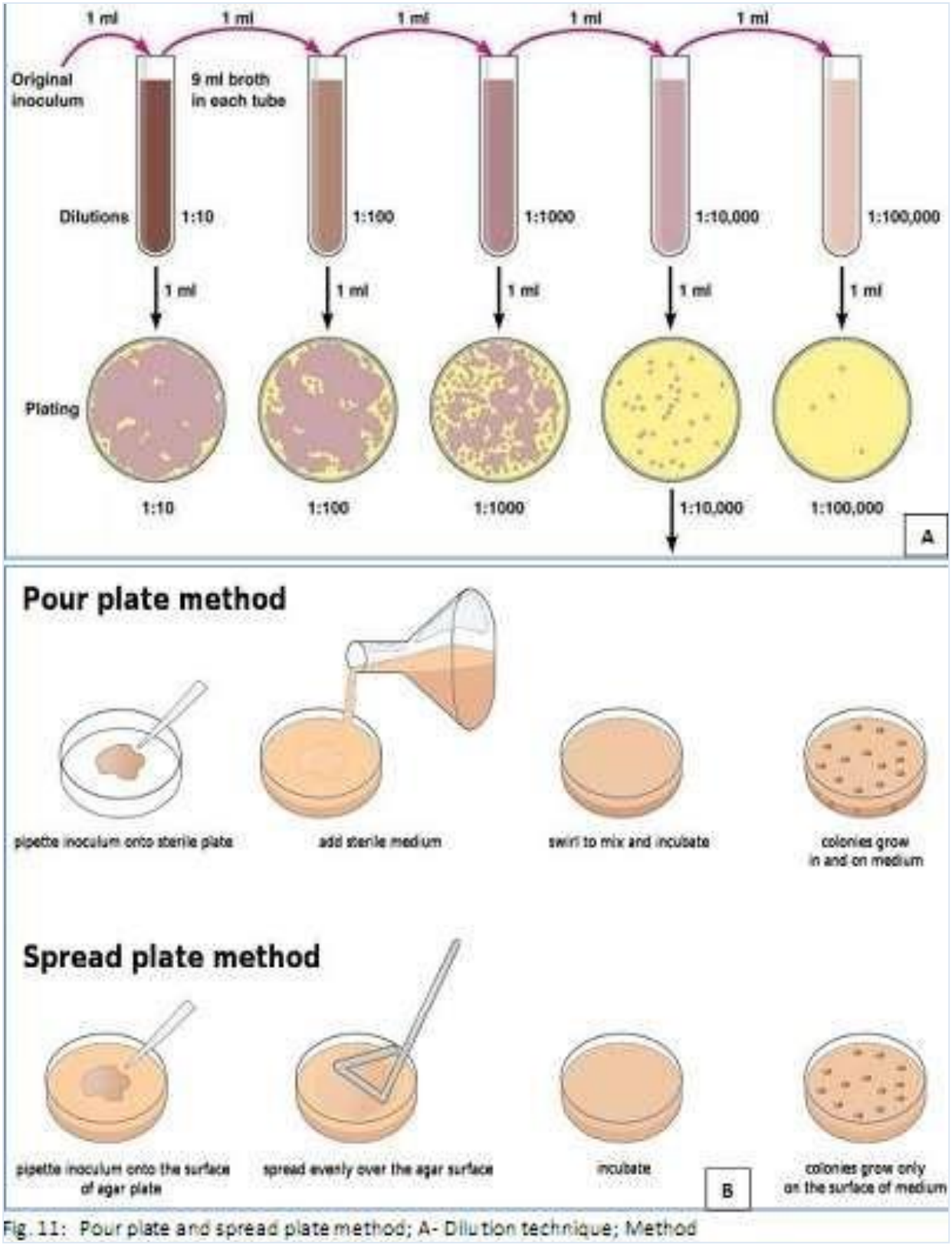


Fig. 11: Pour plate and spread plate method; A- Dilution technique; Method

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.

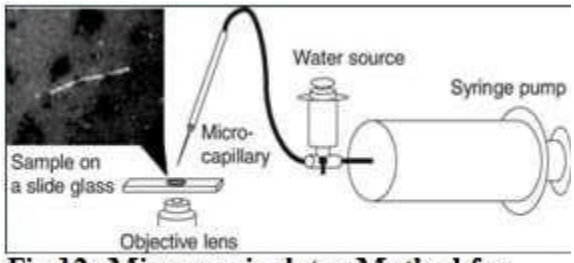


Fig.12: Micromanipulator Method for pure culture

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.

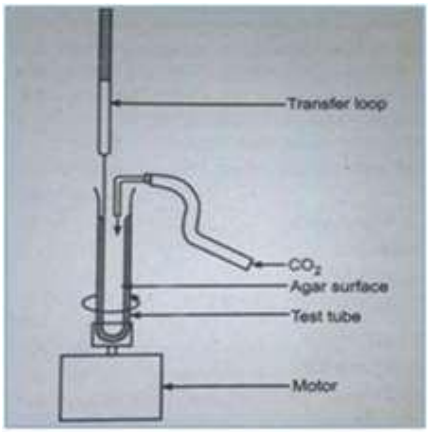


Fig. 13: Roll tube technique for pure culture

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:

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:

- Simple and cost effective
- can preserve for 10-15 years
- 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:

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

Disadvantage: Costly equipment

CULTIVATION OF ANAEROBIC BACTERIA

- 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).

Anaerobic culture methods:

1. **Use of media containing reducing substances**
   A. Robertson Cooked Meat broth B. Thioglycolate broth
2. **Culture away from O2**
   →Deep agar tubes
3. **Chemical exclusion of O2**
   A. Anaerobic gas pak system B. Candle jar bottle
4. **Mechanical exclusion of O2**
   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.

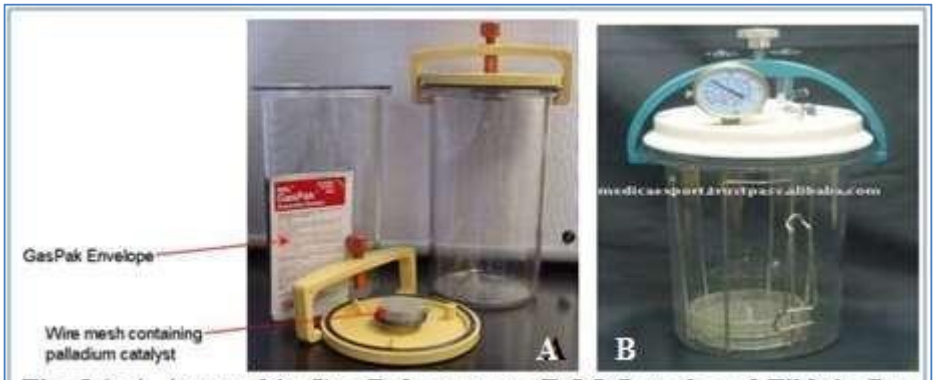


Fig. 14: A-Anaerobic Gas Pak system; B-McIntosh and Filde's Jar

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.

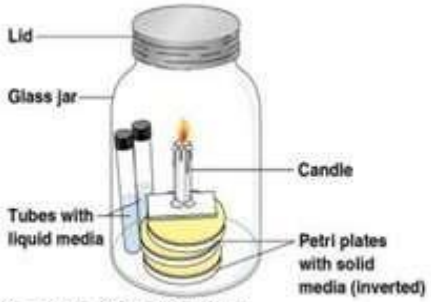


Fig. 15: Candle jar

- 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.

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 37^0C 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 H2O when H2 combine free O2
- H2 gas (5 to 10%)
- CO2 gas (10%)
- N2 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 O2-free N2 or N2 with CO2 to exclude O2.
- 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)

- **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 O2 Uptake**
   - **C. Determination of Lactic acid production**
   - **D. Determination of CO2 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.

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:

- **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

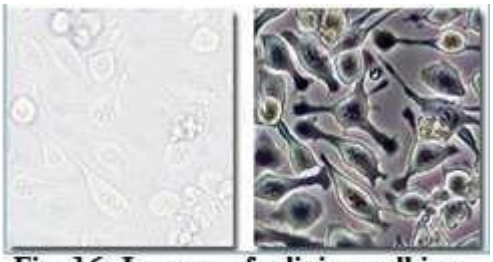


Fig. 16: Images of a living cell in a bright field and phase contrast

- 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.
- 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 ground microscope:

- 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.

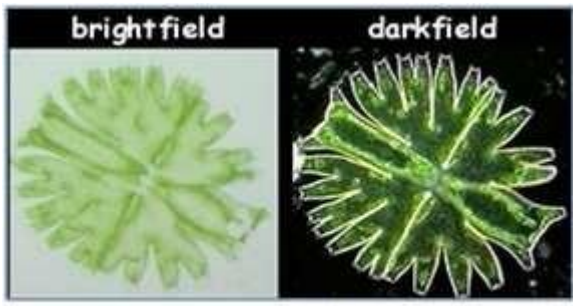


Fig. 17: Images in a dark field microscope

- 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 anelectromagnetic 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 theelectron beam
v. An objective lens
vi. A projector lens
vii. Fluorescent screen on which finalimage is observed.

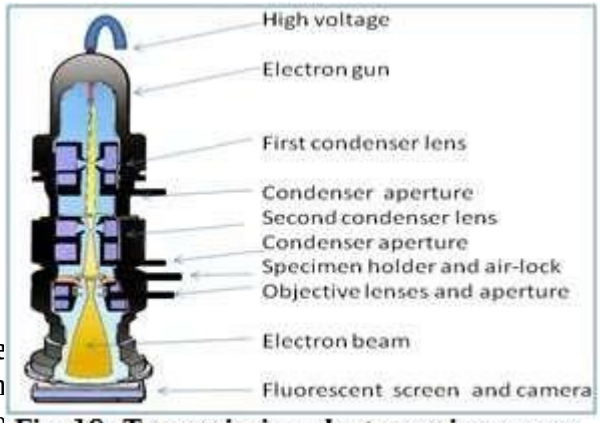


Fig. 18: Transmission electron microscope

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 witha 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 ofthe 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 themagnified electron image onto a fluorescent viewing screen.
- Alternatively, the image can be photographically recorded by exposing a photographicfilm or plate directly to the electron beam.

PHARMACEUTICAL MICROBIOLOGY (BP303T)
UNIT-2

IDENTIFICATION OF BACTERIA

Once a bacterium has been obtained in a pure culture, it has to be identified. There are different techniques for Identification of bacteria, out of those techniques staining technique is one of them.

SIMPLE STAINING PROCEDURE:

- Simple staining is a method of staining in which bacteria are stained by using a single stain.
- Simple staining is also called as monochrome staining or positive staining.
- Examples of simple stain are Methylene blue, Safranin, Malachite green, Basic fuchsin and crystal violet etc. In simple staining procedure cell are uniformly stained.

PROCEDURE

1. A clean grease free slide is taken .A grease free slide is made by first washing the slide with detergent wiping the excess water and the slide is passed through flame.
2. On these grease free slide smear is made by using a sterile wireloop and cell suspension.
3. These slide is allowed to air dry.
4. After air drying these slide is rapidly passed through a flame for three to four times for heat fixation.
5. After heat fixation the slide is placed on the staining rack and flooded with a particular stain and this stain is allowed to react for three minutes.
6. Further the slide is washed under running water.
7. The slide is air dried and washed under oil immersion.

APPLICATIONS

- Simple staining procedure stains bacteria easily and helps in observation under microscope.
- It is useful in preliminary studies of morphological characters of cell that is its size, shape and arrangement.

GRAM'S STAINING PROCEDURE AND ITS MECHANISMINTRODUCTION

- Gram staining procedure was discovered by Han's Christian Gram in 1884.
- Gram staining is a universal staining technique used for identification and classification of organisms.
- In this staining, method bacteria are classified into two groups that are-
    1. Gram-positive bacteria
    2. Gram-negative bacteria
- This classification of bacteria depends upon the property of a cell to retain or lose the primary stain after the treatment of decolorizing agent.
- Gram staining is a basic and widely used technique.
- This technique was modified by many scientists but the best result was obtained by Hucker and Conn's modification.

REQUIREMENTS

1. A clean grease free slide.
2. Bacterial cell suspension.
3. Nichrome Wire loop.
4. Primary stain - Crystal violet.
5. Mordant- Gram's Iodine.
6. Decolorizing agent - 95% alcohol (95% Ethanol).
7. Counterstain- Basic fuschin or Safranin.

PROCEDURE

1. Take a clean grease free slide.
2. Prepare a smear from a bacterial cell suspension on a slide by using nichrome wire loop.
3. Air dry and heat fix the smear.
4. Flood the smear with a primary stain that is Crystal violet and allow it to react for 1-2 minutes.
5. After Crystal violet treatment water wash treatment is given to the slide.
6. Further, the smear is treated with the mordant that is Gram's Iodine for 1-2 minutes.
7. Excess Gram's Iodine is removed and the slide is further treated with a decolorizing agent that is 95 % Ethanol.
8. After Ethanol treatment the smear is water washed and flooded with counter stain that is Basic fuchsin or Safranin for 1-2 minutes.
9. Finally, the slide is washed with water, air dried and observed under oil immersion.

APPLICATIONS

- Gram staining is a basic technique used for identification and classification of the cell
- It is a useful technique in the diagnosis of the causative agent of a clinical infection.
- It is also helpful in studying morphological characters of cells.

EXAMPLES

1. Gram positive bacteria – ***Bacillus, Staphylococcus, Streptococcus, Micrococcus*** etc.
2. Gram negative bacteria – ***Pseudomonas, E.coli, Salmonella, Shigella, Proteus, Xanthomonas***

ACID FAST STAINING TECHNIQUES AND ITS DETAILS

INTRODUCTION

- In nature, there is a variety of micro-organism each micro-organism have some special characters.
- Most of the microorganisms are easily stained by simple staining procedures.
- But there is some micro-organism that is not easily stained by this technique because they have a waxy covering on its surface. If anyhow they get stained they don't get decolorize even by strong acid.
- Such organism requires a special staining technique.
- Acid-fast staining technique is a differential staining technique in bacteriology.
- This staining technique was discovered by scientist Paul Ehrlich in 1883.
- Acid-fast staining technique helps us to differentiate the organism as acid-fast and non-acid fast organisms.
- For staining such organism Ziehl- Neelsen staining method is used. It is also called as Acid-fast staining method

DEFINITION

1. Acid-fast organism- The organism that get stained by acid-fast staining technique but don't get decolorized even by strong acid are called as an acid-fast organism.
2. Non-acid-fast organism- The organism that easily gets stained by a staining procedure as well as decolorizes easily by a strong acid are a non-acid fast organism.

REQUIREMENT

1. A clean grease free slide.
2. A bacterial cell suspension.
3. Staining agent- Ziehl Neelsen, carbol fuchsin.
4. Boiling water bath.
5. Decolorizing agent – Acid alcohol.
6. Counterstain – 1% Malachite green or 0.3 % Methylene blue.

PROCEDURE

1. Take a clean grease free slide and prepare a smear using nichrome wire loop.
2. Air dry and heat fix the slide.
3. The slide is flooded with ZNCF stain and placed on a boiling water bath for steaming for about 3-5 minutes.
4. During steaming the stain is repeatedly added on the slide to avoid drying of smear.
5. Further, the slide is treated to the decolorizing agent that is acid alcohol until the stain disappears in washing.
6. After decolourisation, the slide is given a water wash treatment.
7. Further, the smear is flooded with the counterstain that is 1% Malachite green or 0.3 % Methylene blue for about 2 minutes.
8. After 2 minutes the slide is washed with water, air dried and observed under oil immersion objective.

OBDERVATION

1. Acid-fast bacteria appear pink in color.
2. Non-acid fast bacteria appear green or blue in color.

SOME IMPORTANT POINTS

1. The permeability of acid-fast cell is increased by phenolic stain because phenolic stain have high affinity towards the waxy covering and it is more soluble in waxy covering.
2. For increasing the permeability we use heat steaming because steaming softens the waxy material and allow easy penetration of stain.

APPLICATIONS

1. Acid-fast staining is useful in the diagnosis of Tuberculosis and leprosy.

BIOCHEMICAL TESTS

Many biochemical tests are performed for identification of bacteria, Out of which IMViC tests is very important.

IMViC Tests

Each of the letters in "IMViC" stands for one of these tests. "I" is for indole; "M"is for methyl red;"V" is for Voges-Proskauer, and "C" is for citrate, lowercase "I" is added for the ease of pronunciation. "IMViC" is an acronym that stands for four different tests.[4]

GENERAL PROCEDURE FOR PERFORMING IMViC Tests

INDOLE TEST:

This is tested in a peptone water culture after 48 or 96 hours incubation at 37°C.This test demonstrates the production of indole from tryptophan. Add 0.5 ml Kovac's reagent andshake gently.

Red colour in the top of the tube indicates a positive reaction.Kovac's reagent consists of
Paradimethylaminobenzaldehyde ……10gm Amyl or isoamyl alcohol 150ml
Concentrated HCL 50ml
This is prepared in small quantities and stored in the refrigerator.

METHYL RED (MR) TEST:

This test is employed to detect the production of acid during the fermentation of glucose and the maintenance of pH below 4.5 in an old culture. Five drops of 0.04% solution of methyl red are added to the culture in glucose phosphate medium which had been incubated at 30°C for five days, mixed well and read at once. Red color is positive while yellow signifies a negative test.

VOGES-PROSKAUR (VP) TEST:

This test depends on the production of acetyl methyl carbinol from pyruvic acid, as an intermediate stage in its conversion to 2:3 butylene glycol. In the presence of alkali and atmospheric oxygen, the small amount of alkylmethyl carbinol present in the medium is oxidized to diacetyl which reacts with the peptone of the broth to give a red colour.
The test is performed by adding 0.6 ml of a 5% solution of α-napthol in ethanol and 0.2 ml of 40% KOH to one ml of a glucose phosphate medium culture of the organism incubated at 30°C for five days or 37°C for 48 hours. In a positive, a pink colour appears in 2-5 minutes, depening to magenta or crimson in half an hour. Traces of pink colouration should be ignored.

CITRATE UTILIZATION TEST:

Kosers citrate medium has citrate as the sole source of carbon. Ability to use this substance is indicated by the production of turbidity of the medium.
Indole, MR, VP and citrate tests are very useful in the identification and classification of enteric Gram negative bacteria. [5]

Sterilization: Sterilization is the process that eliminates, removes, kills, or deactivates all forms of life and other biological agents such as fungi, bacteria, viruses, spore forms, unicellular eukaryotic organisms such etc.

Need for sterilization:
Microorganisms are capable of causing infection & responsible for contamination.

Autoclaves: They are highly effective and inexpensive. Unsuitable for heat sensitive objects.
Hot air ovens: They are inefficient compared to autoclaves.
Ethylene oxide: It is suitable for heat sensitive items but leaves toxic residue on sterilized items.
Low-temperature steam and formaldehyde: They are effective for instruments with cavities or tubular openings.
Sporicidal chemicals: They are often used as disinfectants but can also sterilize instruments if used for prolonged periods.
Irradiation: Gamma rays and accelerated electrons are excellent at sterilization.
Gaseous Agent: Many types of gases are used for inactivation of microorganisms.

Mechanism:
- Denaturation of protein
- Oxidation
- Filtration
- Interference with protein synthesis
- Disruption of cell membranes
- Interruption of DNA synthesis

Some Important Terminologies:
Disinfectant: The process of destruction or removal of all pathogenic organisms.
Examples: Alcohol, formaldehyde, chloramines etc.
Asepsis: It is two types viz. medical and surgical. Medical asepsis is the process that helps to reduce the number and spread of microorganisms. Surgical asepsis is the process by which complete removal of microorganism and their spores from the surface of an object.
Antisepsis: The procedure of antiseptic solution which inhibits growth of microorganism with their contact.
Example: Betadiene.

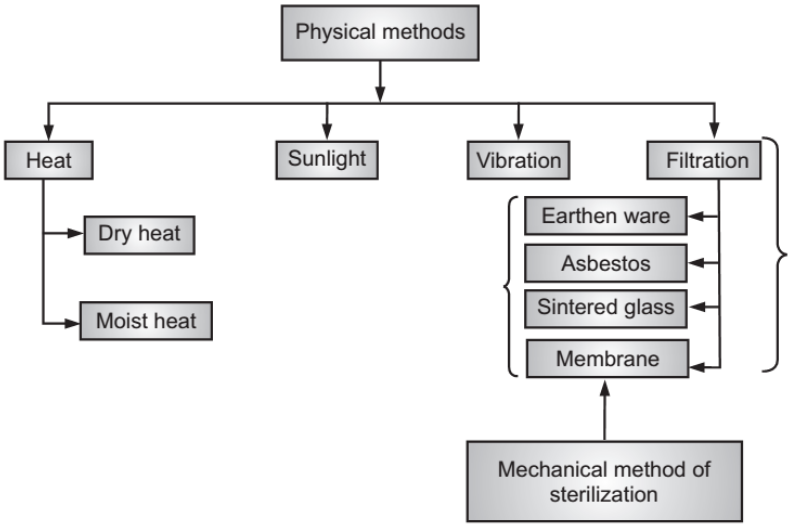
Pharmaceutical Importance of Sterilization:
- Moist heat sterilization is the most efficient biocidal agent. In the pharmaceutical industry, it is used for Surgical dressings, Sheets, Surgical and diagnostic equipment, Containers, Closures, Aqueous injections, Ophthalmic preparations and Irrigation fluids etc.
- Dry heat sterilization is used for thermo-stable, moisture-sensitive or moisture impermeable pharmaceutical and medicinal. These include products like; Dry powdered drugs, Suspensions of drug in non-aqueous solvents, Oils, fats waxes, soft hard paraffin silicone, Oily injections, implants, ophthalmic ointments and ointment bases etc.
- Gaseous sterilization is used for sterilizing thermolabile substances like; hormones, proteins, various heat sensitive drugs etc.
- U.V. light is the most lethal component in ordinary sunlight used in sanitation of garments or utensils.
- Gamma-rays from Cobalt 60 are used to sterilize antibiotic, hormones, sutures, plastics and catheters etc.
- Filtration sterilizations are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products, air and other gases for supply to aseptic areas.

They are also used in industry as part of the venting systems on fermenter, centrifuges, autoclaves and freeze driers. Membrane filters are used for sterility testing.

Classification of Sterilization: Broadly sterilization is classified as:

- Physical Methods
- Chemical Methods
- Gaseous Methods
- Mechanical Methods
- Radiation Methods

Physical Methods:



Filtration methods for sterilization also comes under Mechanical methods.

Heat: Heat is considered as most reliable method of sterilization of objects that can withstand heat.
It is carried out by two ways via.

- ✓ **Dry heat Sterilization**
- ✓ **Moist heat Sterilization**

They are the most common sterilizing methods used in hospitals and are indicated for most materials.
This method of sterilization is applied only to the thermostable products.
It can be used for moisture sensitive materials for which dry heat (160-180°C) sterilization, and for moisture resistant materials for which moist heat (121-134°C) sterilization is used.

Advantages:

- Most common method for controlling microbial growth.
- Very effective in destroying unwanted microbes.
- Low cost.

Disadvantages:

- It is probabilistic i.e. not absolute sterilization.

Dry Heat Sterilization:

Dry heat sterilization requires higher temperatures and longer exposure times than moist heat sterilization.
It uses high temperatures to kill microorganisms and bacterial spores and is used on items that cannot get wet and for glassware, oils, powders, metal instruments, and items wrapped in paper.
There are three types of temperatures are recommended via.

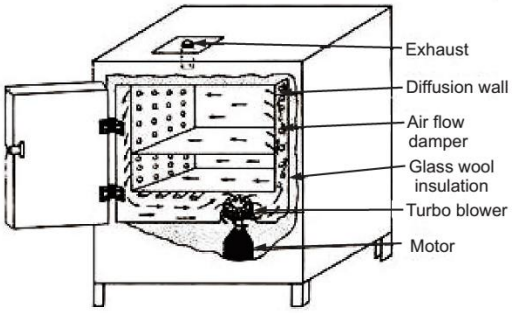
Temperature	Exposure time
50°C	150 minutes
160°C	60 minutes
170°C	30 minutes

Thermal death time (TDT): It is the minimum time required to kill a suspension of organism at a predetermined temperature at a specified environment.
Thermal death time is inversely proportional to temperature.
It is increased in presence of organic substance, proteins, nucleic acid, starch gelatin etc.

Principle: It causes denaturation of proteins and oxidative damage. There are several different types of dry heat sterilization such as:

- **Red Heat:** It is used for straight wires, bacterial loops and spatulas.
- **Flaming:** It is the process of heating over fire till they become red hot. It is used for bacterial loops, wires and spatulas.
- **Hot Air oven:** It is an electrical device. The oven uses dry heat to sterilize articles at 50 to 300°C. Thermostat is used to control the temperature. It is commonly used in dairy industry.

Glasswares, forceps, scissors, scalpels etc. are sterilized by this method but surgical dressings, rubber items, or plastic materials are not sterilized by this method.
Temperature used in hot air oven with holding time of articles are described as 160°C for 45 minutes, 170°C for 18 minutes, 180°C for 7.5 minutes etc.



Operation:
(i) Articles to be sterilized are first wrapped or enclosed in containers of cardboard, paper or aluminium.
(ii) Then, the materials are arranged to ensure uninterrupted air flow.
(iii) Oven may be pre-heated for materials with poor heat conductivity.
(iv) The temperature is allowed to fall to 40°C, prior to removal of sterilized material.

Types of Hot Air Oven:
There are two types of hot air ovens. **(i) Forced air hot air oven and (ii) Static air hot air oven.**
The forced air hot air oven is more effective than the static air hot air oven. Forced hot air oven works by heating the oven and using a fan to move the hot air around whereas the static air hot air oven works by using a heating coil at the bottom of the oven. The heat rises throughout the oven and takes a longer time to reach the desired temperature.

Advantages:

- It does not cause metals to corrode or rust.
- It is relatively inexpensive.
- It does not release any harmful or hazardous fumes or pollutants.

Disadvantages:

- It is relatively slow (can take a couple hours).
- Many objects can not withstand the very high temperatures required for dry heat sterilization (example: some plastics would melt).

Applications of Dry Heat Sterilization: The method is applicable for thermo stable, moisture-sensitive pharmaceutical and medical devices and materials such as dry powdered drugs, suspension of drugs in non-aqueous solvents, oils, fats, waxes, oily injections, implants, ophthalmic ointments etc.

Moist Heat:

Principle: Moist heat is more efficient for sterilization in contrast to dry heat; it destroys microorganisms by the irreversible denaturation of enzymes and structural proteins

At temperature below 100°C:

- Pasteurization: Food industry. (It is the process for heating liquids)

At temperature 100°C:

- Boiling: (100°C). It is the process of heating the sample to be sterilized material in a liquid at its boiling point
- Tyndallization (100°C): Discovered by John tyndall, effective for spores killing, Continue heats time to time for 20 min till three days at boiling point.

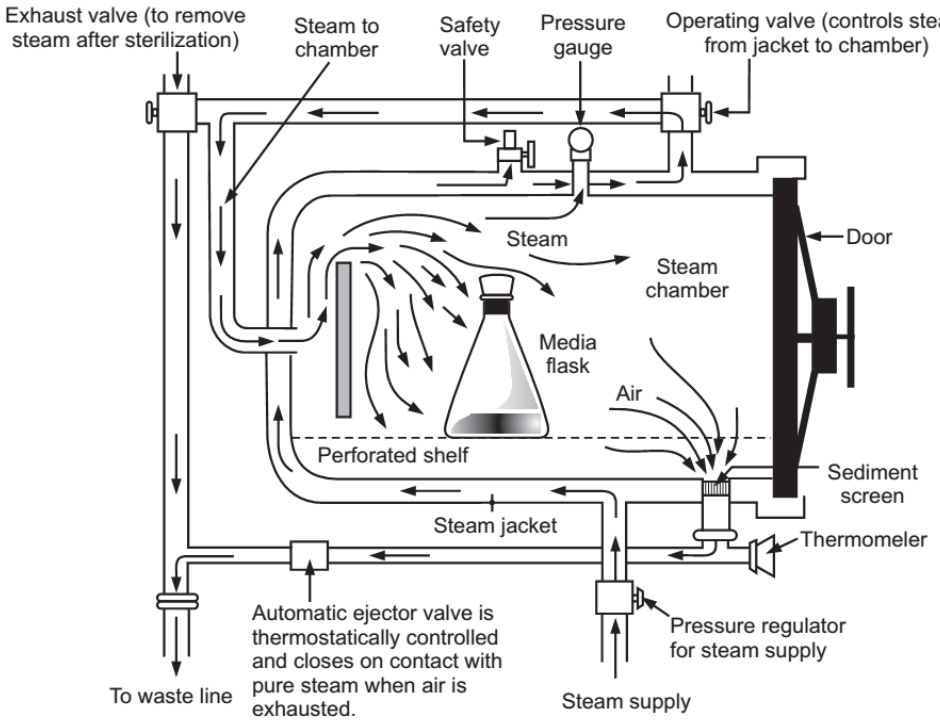
At temperature above 100°C:

- **Autoclave:** It is also known as steam under pressure.

Principle (Autoclave):

High-pressure steam sterilization is used to kill all microorganisms, including spores. It is the best and most widely used methods of sterilization. Example: Pressure cooker.

This method is used for physiological saline, surgical instruments, containers, glass syringes, dressings.



Advantages of Autoclave:

- It is rapid and effective, destroys microorganisms more efficiently than dry heat and therefore a shorter exposure at a lower temperature is possible.
- It can be used for a large proportion of the official injections.
- It is supplied with dry saturated steam porous materials and can be sterilized without damage.
- Equipment or components of rubber and certain plastics such as nylon and P.V.C will withstand the conditions.

Disadvantages of Autoclave:

- Items sensitive to heat cannot be sterilized.
- It is unsuitable for anhydrous materials such as powders and oils.

• It cannot be used for injections and articles such as plastics that deteriorate at 115°C.

Advantages of Moist Heat: It maintains integrity of liquids due to the 100% humidity within the chamber.

Disadvantages of Moist Heat:

• Non-stainless steel metal items corrode in moist heat.
• It may damage rubber and plastic items.
• This method is not suitable for oils, fats, ointments, oily injections etc.

Applications of Moist Heat Sterilization:

• This method is most essential biocidal agent. It is used for surgical dressings, sheets, surgical and diagnostic equipments, containers, closures, aqueous injections, ophthalmic preparations etc.

Differences between dry heat and moist heat sterilization:

Moist heat	Dry heat
1. It has water and steam.	1. It do not require water and steam.
2. It is based on protein denaturation and coagulation.	2. It is based on protein denaturation.
3. Latent heat liberated when steam condenses on cooler surface.	3. It is based on oxidative damage.
4. Spores are killed by exposure to heat at 121°C for 10-30 minutes.	4. Spores are killed by exposure of temperature at 160°C for 1 hour.
5. This process is under pressure.	5. This process is on direct flame.
6. This process takes less time.	6. This process takes more time.
7. It is mainly dependent on boiling and autoclaving.	7. It is mainly dependent on flame and incineration.

Sunlight:

It possesses bacterial activity. The action is due to its content of UV rays.

Direct sunlight has a powerful germicidal action. Exposure to direct sunlight for a sufficient time will kill spores as well as vegetative cells. Tuberculosis germs are killed in few hours.

UV rays destroy bacteria in a few seconds or minutes; molds and yeasts are some what more resistant.

Ultraviolet light is also used for purification of drinking water and swimming pool water.

Ultrasonic Vibration:

It is the sound waves above the frequencies.

Vibrations have the property to disrupt the cells.

Gram negative rods are more sensitive to ultra sonic vibration whereas Gram positive cocci, spores of fungi and resistant to the vibration.

The current trend is to use ultrasonic as a cleaning agent to follow the process by sterilization in an autoclave.

Mechanical methods of sterilization:

Filtration:

Filtration allows for the exclusion of organisms based upon size. There are many types of filtration techniques, but when sterilizing a system membrane filtration is used.

To obtain a sterile filtrate it is necessary that the filter and all connecting parts likely to come into contact with the filtrate must be sterile.

The major mechanisms of filtration are sieving, adsorption and trapping within the matrix of the filter material.

Four steps for sterilization by filtration:

1. Filtration of the solution through one of the bacteria-proof filters.
2. Aseptic distribution of the filtered solution into the previously sterilized final containers.
3. Aseptic closure of the containers.
4. Performing the sterility test.

There are various types of filtrations used for various, are as follows:

1. Ceramic filters: These are also known as filter candles, The candle is placed in the solution to be sterilized and its opening is attached to the vacuum system. When vacuum is applied the pressure inside the candle is decrease. Due to difference in pressure between the outside and inside of the candle, the solution moves into the candle. The filtrate is collected in sterile container.

The main disadvantage of ceramic filter is its tendency to absorb materials from aqueous solutions.

2. Seitz filter: It consists of two parts. The lower part holds a perforated disc and the upper part is compressed asbestos sheet. Two parts are joined together with the help of nuts. There is a valve on the upper part through which vacuum is applied. Due to the fibrous nature of asbestos pads, it may shed fibres into the filtrate and also absorb drugs from solution. Hence, few ml of filtrate should always be rejected and sintered glass disc may also be fixed in the filtration unit immediately after seitz filter.

3. Sintered glass filters: They are made from borosilicate glass. The glass is finely powdered and particles of the required sizes are separated and are then packed into disc moulds. These discs are fused to funnels of suitable shape and size. Sintered glass filters are available in different pore sizesand are numbered accordingly. For bacteria proof filtration number 5 or 3 is used. The filtration is carried out under a reduction pressure. Sintered glass filter do not absorb the medicaments from the solution. These filters are made from borosilicate glass, so change of pH of the solution not occurred.

4. Sintered metal filters: They are the metallic counter part of sintered glass filters. These are usually made from stainless steel. They have the advantage of having greater mechanical strength.

5. Membrane filters: These are made of cellulose acetate or cellulose nitrate. These are fixed in metallic holders similar to those used with asbestos pads. The pore size in the membranes lies in the range of 100-150 µ. They are also called millipores filters. They are suitable for sterilizing aqueous and oily solutions but are not suitable for organic solvents like alcohol, ketones, esters or chloroform.

6. Air filter: (HEPA filter): It is high efficiency particulate air or originally called **High-Efficiency Particulate Absorber (HEPA)**. It is used to describe filters that are able to trap 99.97 per cent of particles that are 0.3 microns. Air particles are circulated through HEPA filter by four directions viz.

(a) Direct Impaction: Large contaminants, such as certain types of dust,mold, and pollen, travel in a straight path, collide with a fibre, and stick to it.

(b) Sieving: The air stream carries a particle between two fibres, but the particle is larger than the gap, so it becomes ensured.

(c) Interception: Airflow is nimble enough to reroute around fibres,but, thanks to inertia, particles continue on their path and stickto the sides of fibres.

(d) Diffusion: Small, ultrafine particles move more erratically than larger ones, so they are more likely to hit and stick to fibres.

Advantages:

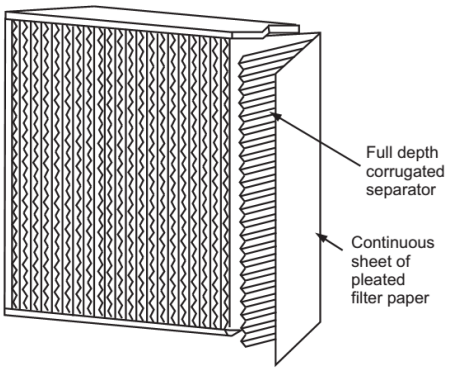
- The method is suitable for sterilization of thermolabile medicaments, such as, blood products, insulin & enzymes.
- All types of bacteria i.e., living as well as dead, are removed from the preparation.
- Both clarification and sterilization are done side by side.
- It is an excellent method for the rapid supply of a small volume of a parenteral solution in an emergency.

Disadvantages:

- The method is not a reliable one and therefore a sterility test is necessary.
- The suspension and oily preparations cannot be sterilized by this method.

- There are chances of absorption of medicaments from a solution by the filter.
- Highly trained staff is required.
- The process is only suitable for medicaments which are in solution form.

HEPA FILTER

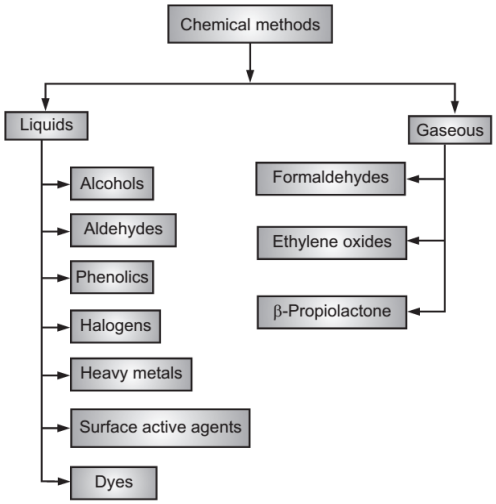


Applications:

This filtration method is useful for sterilization of parenteral solutions containing thermolabile medicaments without any decomposition e.g. insulin, blood serum and other products containing protein matters, heat sensitive injections, biological products etc.

CHEMICAL METHODS OF STERILIZATION:

Disinfection is known as partial removal of pathogens whereas complete removal of microorganisms is called sterilization. Chemical sterilization is the process of elimination of all viable microorganisms and their spores. It is of two types viz. using liquids and gaseous compounds.



Ideal Properties of Chemical Sterilization:

- Wide spectrum of activity.
- Active in presence of organic matter.
- Effective in acid as well as alkaline media.
- High penetration power.
- Stable.
- Speedy action.
- Compatible with other antiseptics and disinfectants.
- Safe and easy to use.
- Cheap and easily available.
- Not corrode metals.

Mode of Action of Chemical Disinfectants:

- It acts by protein coagulation, Disruption of cell membrane resulting in exposure, damage or loss of the contents.
- Removal of free sulfhydryl group essential for the functioning of enzymes.
- **Substrate competition:** A compound resembling the essential substrate of the enzyme diverts the enzymes necessary for the metabolism of the cell and causes cell death.

Alcohol:

Mainly ethanol (80% v/v ethanol) or isopropyl alcohol (60-70% v/v) solutions are used to disinfect skin and decontaminate clean surfaces. Methanol is also used as disinfectant but less bactericidal than ethanol and highly poisonous.

Other alcohols such as propyl, butyl, amyl alcohols are more powerful germicidal than ethanol.

Alcohols are effective against fungi, vegetative bacteria, mycobacterium species and some lipid containing viruses. They are more effective at concentration 70% in water. In tinctures, alcohols enhance the effectiveness of other antimicrobial chemicals.

Principle: It is based on denaturing coagulating proteins and dissolving membrane lipid of microorganisms.

Advantages:

- It is inexpensive and non-toxic.
- Widely available & Rapidly effective.
- Active against bacteria, viruses, mycobacterium.

Disadvantages:

- Not effective against bacterial spores.
- Not effective with organic materials.

Aldehydes:

They are low molecular weight compounds and act as antimicrobial. The most important two aldehydes are formaldehyde and glutaraldehyde. 2% solution of glutaraldehyde is known as Cidex which is used for bactericidal and viricidal in 10 minutes and sporicidal in 3-10 hours.

Both these compounds are highly microbicidal and also kill the spores.

Formaldehyde is also a common aldehyde which is a gas in high concentration but at room temperature it polymerizes and form solid substance. Formaldehyde solution as well as in gaseous form is used for sterilization and disinfection of enclosed area respectively where vegetative cells are killed more quickly than spores.

Formalin and paraformaldehyde are two important sources of formaldehyde when it is used for gaseous disinfection. Formalin is the aqueous solution of 40% formaldehyde. Formalin is extensively used for preservation of specimens, inactive viruses and bacteria in vaccines.

Principle:

Aldehyde combines with important proteins and nucleic acids of the bacterial cells. These interactions of aldehyde with these cellular substances produce antimicrobial action. Formaldehyde inactivates microorganisms by alkylating the amino acids and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases.

Advantage:

- They have good activity against spores, viruses andfungi.

Disadvantages:

- They are toxic.
- They need long exposure time for the action, minimum 3 hours.
- They are irritant and carcinogenic.

Phenolics and Phenolic Agents:

Phenol or carbolic acid was first used by Lister as a disinfectant. Phenolics are the chemical derivatives of phenols. They are very effective disinfectants. Examples: cresols, biphenols etc.

Cresols are derived from coal tar. A 5% aqueous solution of phenol rapidly kills the vegetative cells of microorganisms but spores are resistant. Phenols are used as standard chemical to determine antimicrobial activity of other similar chemical compounds by Phenol coefficient method.

Pure crystalline phenol is colourless, its 5% aqueous solution is used as disinfectant of sputum, urine, feces etc. Some other examples of phenolic compounds are amyl phenol, benzyl-4-chlorophenol etc.

Principle:

They are having various modes of action. They act by destroying plasma membranes and denature proteins. They also affect plasma membrane, inactivate enzymes, and denature proteins. Some phenolics are mild enough for use asantiseptics.

Advantages:

- They are stable, persist for long times after application, and remain active in the presence of organic compounds.
- They are active against wide range of organisms.
- They have good antimicrobial activity and are rapidbactericidal.
- They are more active in acid pH.
- They remain active on surfaces long after application.

Disadvantages:

- They are having caustic effects on skin.
- They are having systemic toxicity.
- They are not effective against spores.
- They are not effective at low temperature.
- They are incompatible with non-ionic and cation surfactants.
- They have a disagreeable odor.

Halogens:

Halogens, mainly, iodine and chlorine are used as antimicrobial agents. Iodine is used as antiseptic against all microbes, fungi and viruses. It inhibits protein synthesis and oxidizes sulphydryl group of amino acids.

There are various preparations of iodine such as 2% iodine plus 2% sodium iodide diluted in alcohol, 7% iodine plus 5% potassium iodide in 83% alcohol, 5% iodine plus 10% potassium iodide in aqueous solution.

Iodine is also used in the form of iodophors. Iodophors are mixtures of iodine with surface active agents, used forgermicidal activity. Chlorine is used as disinfectant. Hypochlorous acid (HOCl) is an aqueous solution of chlorine which is used as disinfectant.

Some other compounds like calcium hypochlorite [Ca(OCl)2], sodium hypochlorite (NaOCl) etc. are the preparation of chlorine. Chloramines are also chlorinated compound (chlorine and ammonia) which acts as antiseptics.

Principle:

Iodine is an oxidizing agent. These agents can irreversibly oxidize and inactivate essential metabolic compounds like proteins with sulphhydryl groups. It also has halogenations action of tyrosine unit of enzymes and other cellular proteins require tyrosine for activity.

Chlorinated compounds show antimicrobial activity due to formation of hypochlorous acid in water. This hypochlorous acid is further decomposed in to nascent oxygen which shows strong oxidation reaction.

Advantages of Iodine compounds:

- Iodine compounds are effective against gram positive bacteria.
- They produce residual activity.
- They retain microbial action in the presence of organic debris.
- They are available in solutions, sprays and in gel preparations.

Disadvantages of iodine compounds:

- Iodine compounds when used alone are major irritants.
- They are weak against myco bacteria, fungi, viruses.
- They are absorbed into skin and can be toxic.

Advantages of Chlorine Compounds:

- They are cheap and readily available.
- Chlorine is highly soluble in water.
- They leave a residue in solution.
- They are toxic to most microorganisms.
- They remove iron and manganese and ammonia nitrogenduring oxidation.
- They destroy taste and other odour compounds.

Disadvantages of Chlorine Compounds:

- Chlorine is a poisonous and toxic gas.
- They are corrosive, requires special non-metal conduits.
- They require careful handling, operation and storage.
- The vapours of chlorine are irritant.
- They are strong oxidizing agents, react with most elements and compounds.

Heavy Metals:

Most of the heavy metals have detrimental effect on microorganisms. The compounds such as mercury, silver, copper are more effective against microorganisms. Mercury compounds such as mercuric chloride, mercurous chloride, mercuric oxide are in dilution form act as bactericidal. They are highly toxic to animals but used in ointments as antiseptics. Some of the organic compounds of mercury are Mercurochrome, metaphen, mercresin are used as antiseptics. They are less irritants and less toxic.

Silver compounds such as silver nitrate, silver lactate, silver picrate act as bacteriostatic as well as bactericidal when these metals are in contact with proteins of microorganisms. Silver nitrate is mainly used as antiseptics and other compounds are act as germicidal.

Copper compound such as copper sulfate is more effective against algae and molds than bacteria. 2 ppm in water prevents algal growth. It is used in the form of Bordeaux mixture as a fungicide.

Zinc salt controls infection caused byanaerobic bacteria.

Principle:

Heavy metals and their compounds have antimicrobial activity due to combination with cellular proteins and inactivating their function. Example: mercuric chloride inhibits the action of enzyme by acting on sulfhydryl group of enzyme and form inactive enzyme. High concentration of heavy metal salt coagulates cytoplasmic proteins of microorganisms that cause death of the cells.

Advantages:

- They are powerful biocides.
- They form complex with proteins of microorganisms and converts inactive form of microorganisms.

Disadvantage:

- They are highly toxic to the animals.

Surface Active Agents:

Soaps and detergents are mainly used as surfactant. It depends on the alkali content in the soap how they show their action. They act as germicidal for pneumococci, streptococci, gonococci etc. Detergents are ionized in water and are made up of fats. They quickly dissolve in cold and hard water.

Quaternary ammonium compounds are important cationic
detergents which act as germicidal as well as bactericidal, mainly against Gram positive bacteria. They also show fungicidal activity and destroy the pathogenic protozoa. Benzalkonium chloride is used as disinfectant. Diaparene chloride acts as bacteriostatic against Brevibacterium ammoniagenes.

Principle:

They denature proteins, interference with glycolysis and damage membrane of the microorganisms. They also damage the cell cytoplasmic membrane and alter the cell structure.

Advantages:
- They are effective against vegetative bacteria.
- They are widely available.
- They are less expensive.
- They are non-irritant.

Disadvantages:
- They are ineffective against spores.
- They are not effective against non-enveloped viruses.
- They may become contaminated.
- They are easily inactivated by presence of anionic detergents, soaps and hard water.

Dyes:

There are two types of dyes exhibiting antimicrobial activity viz. triphenylmethane and acridine.

Triphenylmethane dyes such as brilliant green, crystal violet, malachite green inhibit gram positive bacteria and fungi. Malachite green is used to inhibit Staphylococcus aureus.

Acridine dyes are the compounds that are derived from acridine such as acriflavine, tryptoflavine, proflavine etc. They inhibit bacteria especially Staphylococci and gonococci.

Principle: They have their inhibitory effect by interfering with cellular oxidation processes.

Advantages:
- They are bacteriostatic in high dilution.
- They are more active against Gram positive bacteria.

Disadvantages:
- They are less active against Gram negative bacteria.
- They have low bactericidal activity.

GASEOUS AGENTS FOR STERILIZATION:

There are some important medical devices which are required for sterilization but not possible through heat sterilization due to damage of the materials. In such cases, gaseous sterilization is required. Like Plastic syringe, blood transfusion apparatus, plastic pipettes, petri dishes etc.

The main gaseous agents are used for sterilization are ethylene oxide, formaldehyde, beta-propiolactone etc.

Principle: They act on principle of denaturing proteins and DNA by cross linking functional groups.

Disadvantages:
- Gas can be hazardous to people.
- They often highly explosive.
- Some time gases are extremely poisonous.
- They are potentially carcinogenic.

Factors affecting gaseous sterilization:
- Efficiency of sterilization method is influenced by the concentration of ethylene oxide.
- The humidity of the sterilizing atmosphere.
- The temperature of sterilization.
- Time of exposure.
- Physical nature and permeability of the load.
- Atmospheric preconditioning of the load before sterilization.

Formaldehydes:

It is also a group of alkylating agent. It inactivates microorganisms by alkylating the amino acid and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases. It is a gaseous disinfectant and biocide. It is a strong, broad-spectrum disinfectant and biocide that has the ability to kill bacteria, viruses, fungi and endospores. It is very irritating to living tissues and also carcinogenic, therefore it is not used as an antiseptic.

Ethylene Oxides:

It is a type of alkylating agent that is used for gaseous sterilization. It is highly penetrating and can sterilize items within plastic bags such as catheters, disposable items in laboratories and clinical settings.

It is gaseous above 10.8°C.

Ethylene oxide have high antimicrobial activity, it kills even endospores.

It is used for sterilization of heat sensitive materials such as spices, oils, plastics etc.

Ethylene oxide is used in formulation with CO_2 as Freon (CClFe).

β-Propiolactone:

It binds to DNA, thereby inactivating it. It is a clear liquid with a strong odor and has the ability to kill endospores. It has been used in either liquid form or as a vapor for the sterilization of medical instruments and tissue grafts, and it is a common component of vaccines, used to maintain their sterility. It is also used for the sterilization of nutrient broth, as well as blood plasma, milk and water.

It is gas above 15.5°C.

Penetration power of β-propiolactone is less than ethylene oxide but it is more active in killing microorganisms.

Due to its carcinogenic effects, it is not commonly used.

Applications of Gaseous Sterilization:

• This method is used for sterilizing thermolabile substances like hormones, proteins, various heat sensitive drugs etc.

REDIATION METHOD FOR STERILIZATION:

This type of method is carried out for surface sterilizations. Many types of radiations are used for sterilization like electromagnetic radiation (examples: UV rays and Gamma rays).

There are two types of radiations used for sterilization, ionizing radiation and non-ionizing radiation.

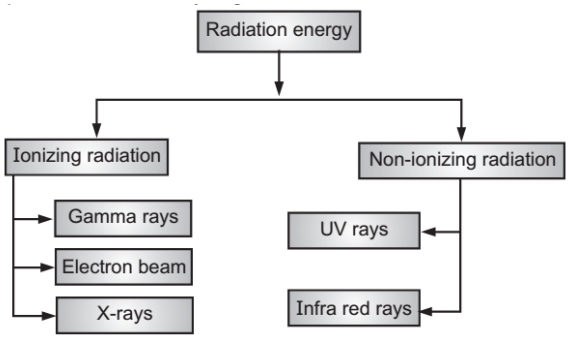
Ionizing radiation is the use of short wavelength, high-intensity radiation to destroy microorganisms.

This radiation is comes in the form of gamma or X-rays that react with DNA resulting in a damaged cell.

Non-ionizing radiation uses longer wavelength and lower energy, and is only used for sterilizing surfaces.

The most common form of non-ionizing radiation is ultraviolet light. Gamma radiation is very penetrating and are commonly used for sterilization of medical equipments like syringes, needles etc.

Infrared radiation is considered as a form of hot air sterilization. It is used for rapid mass sterilization of pre packed items like syringes, catheters etc.



Principle of Ionized Radiation:

It causes death or mutations in microorganisms as they damage the DNA and protein.

Principle of Non-ionized Radiation:

When microorganisms are comes in contact with UV light, than UV rays disturbs protein synthesis and finally no longer produce protein. UV light also inactivates viruses. UV wavelength in the 200 nm to 265 nm range is most effective in killing microorganisms.

Advantages:

• It effectively reduces the number of airborne microorganisms and kills them.

- No degradation of media during sterilization, thus it can be used for thermally labile media.
- It leaves no chemical residue.
- Its administration of precise dosage and uniform dosage distribution.
- Its immediate availability of the media after sterilization.

Disadvantages:

- This method is a more costly alternative to heat sterilization.
- This method requires highly specialized equipment.

Applications of Radiation Sterilization:

- UV light is the most lethal component in ordinary sun light used in sanitation of cloths and utensils.
- Gamma rays are used to sterilize antibiotics, hormones, sutures, plastics etc.

Methods	Mechanism	Advantages	Disadvantages	Applications
Physical: Heat sterilization	Destroys bacterial endotoxins by denaturation of proteins and oxidative damage	Most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents.	Can be applied only to the thermostable products	Dry heat is applicable for sterilizing glasswares and metal surgical instruments and moist heat is the most dependable method for decontamination of laboratory waste and the sterilization of laboratory glassware, media and reagents.
Chemical: Gaseous sterilization	Alkylation	Penetrating ability of gases	Gases being alkylating agents are potentially mutagenic and carcinogenic	Ethylene oxide gas has been used widely to process heat-sensitive devices.
Radiation: Radiation sterilization	Ionization of nucleic acids	It is a useful method for the industrial sterilization of heat sensitive products.	Undesirable changes occur in irradiated products, an example is aqueous solution where radiolysis of water occurs.	Radiation sterilization is generally applied to articles in the dry state; including surgical instruments, sutures, prostheses, unit dose ointments, plastics
Mechanical: Filtration sterilization	Does not destroy but removes the microorganisms	It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non-viable particles.	Does not differentiate between viable and non-viable particles	This method is Sterilizing grade filters are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products, air and other gases for supply to aseptic areas.

EQUIPMENTS EMPLOYED IN LARGE SCALE STERILIZATION ARE:

- Steam sterilizer
- Dry heat sterilizer
- ETO Sterilizer
- Sterilizing tunnel
- CIP System
- SIP System[12]

(A)

(B)

(D)

(A) Steam sterilizer; (B) Dry heat sterilizer; (C) ETO Sterilizer; and (D) CIPSystem

Sterility Testing: Sterility Testing is defined as a test that confirms that the products are free from the presence of microorganisms.

It is very important for medical devices, pharmaceuticals preparations, and other materials etc.

Sterility testing for products are mainly carried out by Direct inoculation and by Membrane filtration method.

Hence, Sterility indicators are required to check whether microbial growth occurs or not in terms of sterilization quality and process.

STERILITY INDICATORS / MONITORS:

Sterility indicators, are the indicators that are used for check the quality of sterilization and monitoring of the sterilization process.

The Sterility indicators are following types:

1. Physical Indicators
2. Chemical Indicators
3. Biological Indicators

1. Physical Indicators:

The guages or display or recording device on sterilizer that can print the parameters like temperature, time & pressure associated with each sterilization cycle for each load, such indicators are consider as physical indicators.

The correct reading of the physical indicators do not guaranty sterilization but incorrect reading can be the first indication of a problem with the sterilization cycle and suggest the load may not be sterile.

S.NO.	METHOD	DEVICE
1	Dry heat sterilization	Temp recording chart
2	Moist heat sterilization	Temp recording chart
3	Filteration sterilization	Bubble point chart
4	Gaseous sterilization	Temp recording chart
5	Radiation sterilization	Recording charts

2. Chemical Indicators:

Chemical indicators are chemical substances that are used in the process of sterilization in order to indicate that the process of sterilization is going as per requirements or it may indicate that the products are sterile.

The chemical indicators that are used in dry heat or moist heat sterilization, may melt or change it's colour only when satisfactory condition that the product is sterile.

In case of gaseous sterilization, Royach sacket is used, it is an indicator paper which is soaked with reactive chemical, its job is to show a colour change and indicate the prevailing condition.

3. **Biological Indicators:**

The biological indicators are standardised bacterial spores used in the form of suspension in water or culture media or dried spores on the paper or plastics carries that are place in sterilizer.

According to the sterilization process, we use that kind of bacterial spores like spores of bacillus subtilis is used as biological indicators.

In dry heat sterilization process to determine D-value (Decimal Reduction Time), while bacillus stereo-thermophilus spores are used in case of moist heat sterilization.

S/N	METHOD	PRINCIPAL	MICROORGANISM	PARAMETER
1	Dry heat sterilization	Temp sensitive microbes	Bacillus subtilis	D-value
2	Moist heat sterilization	Temp sensitive microbes	Bacillus stereo-thermophilus	D-value
3	Radiation sterilization	Radiation sensitive microbes	Bacillus pumilus	D-value
4	Gaseous sterilization	Temp sensitive microbes	Bacillus subtilis	D-value
5	Filtration sterilization	Retention of bacteria	Pycnoclavella diminuta	Size of microorganism

EVALUATION OF THE EFFICIENCY OF STERILIZATION METHODS:

Evaluation of sterilization methods is carried out to ensure that the product produced by design process should be of best quality.

Evaluation of sterilization methods provide a high degree of assurance that indicates whether a specific process will consistently produce a product that will meet it predetermined specifications and quality assurance.

So this action proves that any procedure, process, equipments, material activity or system actually leads to the expected result and produce quality product.

Process of Microbial Destruction:

Destruction of microbes are carried out by several methods such as heat, chemical, and radiation sterilization are used. Upon exposure of such treatment, microorganisms die according to logarithmic relationship between population of the living cells and the time exposure.

The relationship between microbial population and time may be linear or non-linear.

D value:

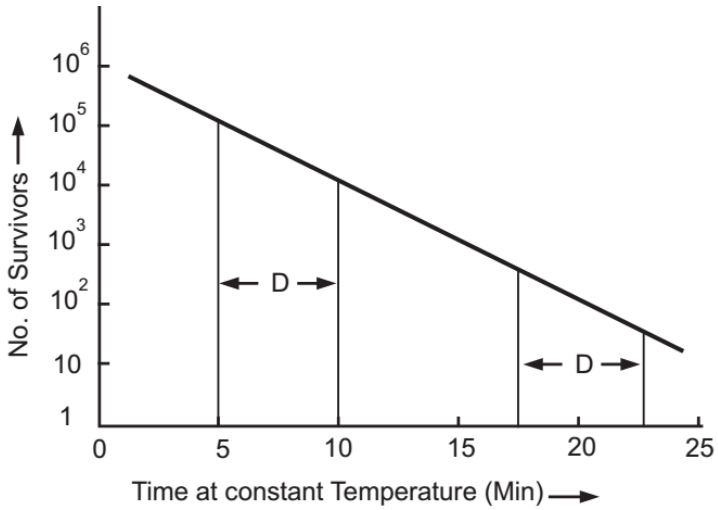
It is the rate of killing of microorganism.

It is the time required for 90% reduction (one log value) in the microbial population. Hence, the time or dose it takes to reduce thousand microbial cells to hundred cells is the D value.

D value is inversely proportional to time, mean if D value is short than efficiency of method/chemical is high.

D value is determined by:

Survival curve method: The survival curve method is based on plotting the log number of the surviving micro-organism versus time.



(D-VALUE GRAPH)

Importance of D value validation:

- D value is specific for each microorganism.
- D value is necessary for the calculation of Z value & F value.

Factors affecting D value:

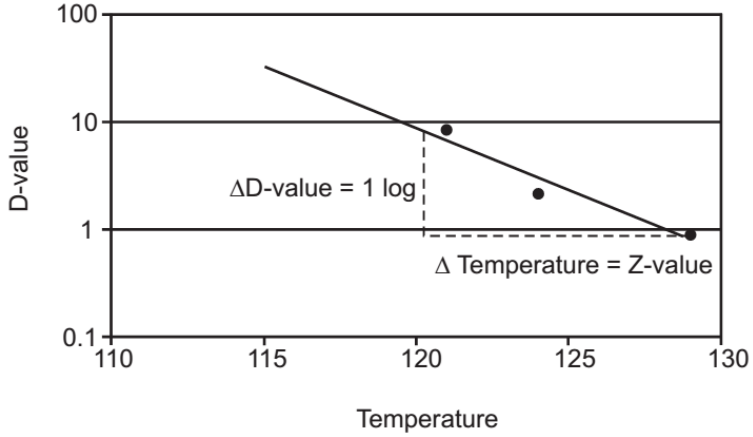
- Types of micro organism.
- Composition of micro organism.
- Surface using for sterilization.
- Temperature using for sterilization.

Z value:

This value is used in the validation of heat sterilization process.

The Z value is the reciprocal of slope resulting from the plot of the logarithm of D value versus the temperature at which the D value was obtained.

The Z value may be defined as the temperature required for one log reduction in the D value.



(Z-VALUE GRAPH)

F value:

It is the time in minutes at a specific temperature is needed to kill a population of micro organism or spores. The F value measures equivalent time, that a monitored article is exposed to the desired temperature e.g. 121.1^{o}C.

Mathematically, the F value is expressed by the rate of motility per minute in function of temperature for a given pressure.

F value is calculated from the following equation:

$$\mathbf{F = \Delta t \sum 10^{(T-To)/Z}}$$

Where,

Δt is the time interval for the measurement of product temperature t,

T is reference temperature

To is 121.1^{o}C for steam sterilization.

PHARMACEUTICAL MICROBIOLOGY (BP303T)
UNIT-3

INTRODUCTION OF FUNGI:

Mycology is the branch of biology concerned with the systematic study of fungi,

Fungi (Singular: Fungus & Plural: Fungi) are a kingdom of usually multicellular eukaryotic organisms.

Fungi are heterotrophs (cannot make their own food) and have important roles in nutrient cycling in an ecosystem.

Fungi reproduce both sexually and asexually, and they also have symbiotic associations with plants and bacteria.

Fungi have thick wall usually made of polysaccharides.

Fungi obtain their food in a soluble form by uptake through plasma membrane.

Fungus are more than 1,44,000 in species of organisms of the kingdom Fungi. (Exp: Yeast, Rusts, Molds & Mushrooms)

MORPHOLOGY OF FUNGI:

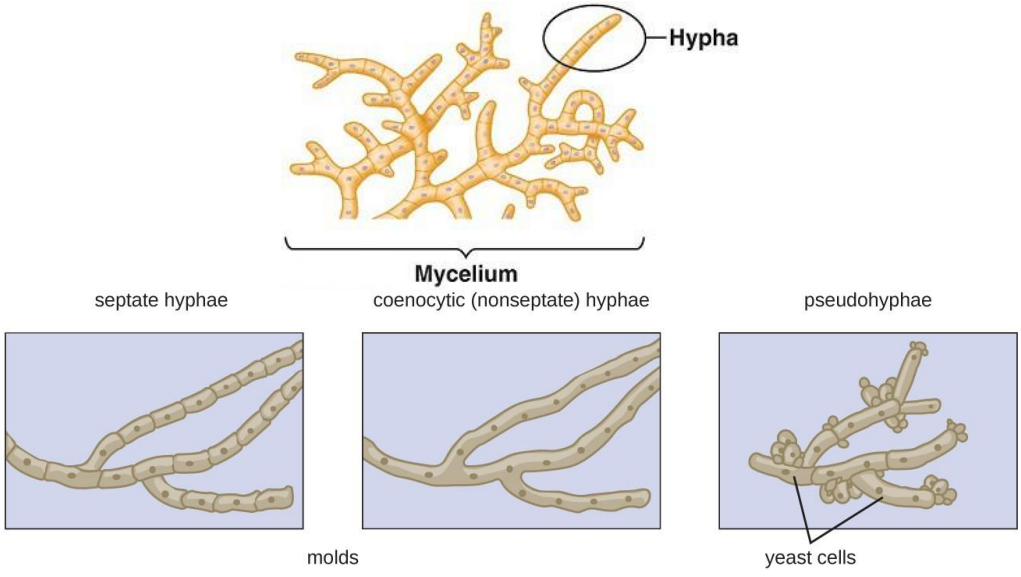
General: Fungi vary widely in size and shape, from unicellular, microscopic organisms to multicellular forms easily seen with the naked eye. Individual cells range from 1 µ to 30 µ. Microscopic fungi exist as either molds or yeasts or both. Internally, fungal cells are fairly typical eucaryotic cells.

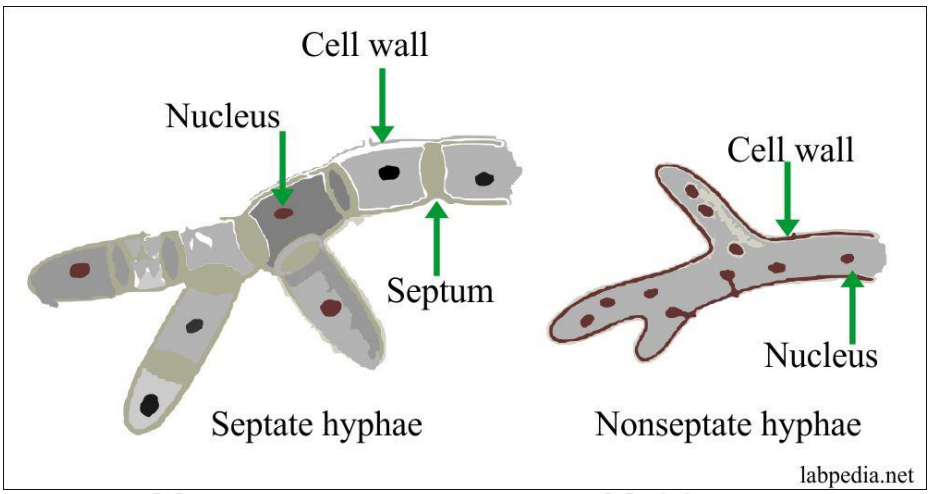
Molds: The molds form large multicellular aggregates of long branching filaments, called hyphae. There are vegetative hyphae and reproductive hyphae. Spores are borne on the reproductive hyphae.

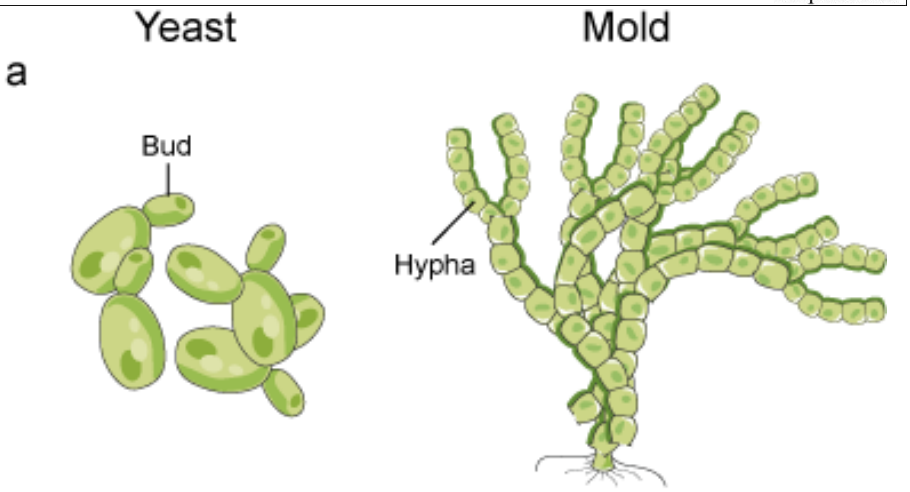
Spore size, shape and structure are used in the classification and identification of fungi.

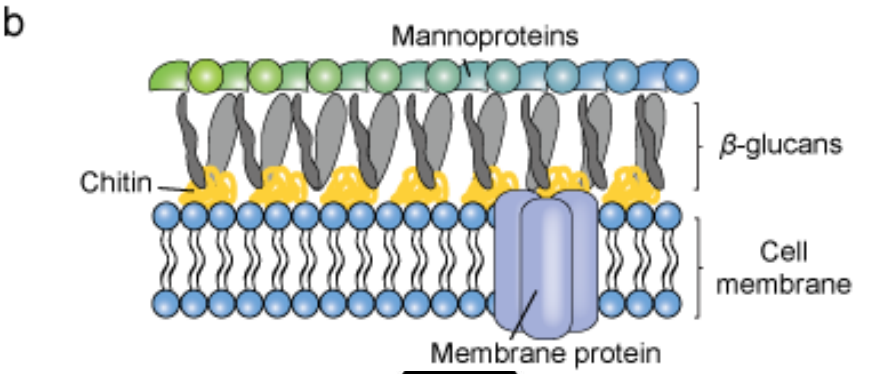
The tube-like hyphae are responsible for the fluffy appearance of the macroscopic mold colony.

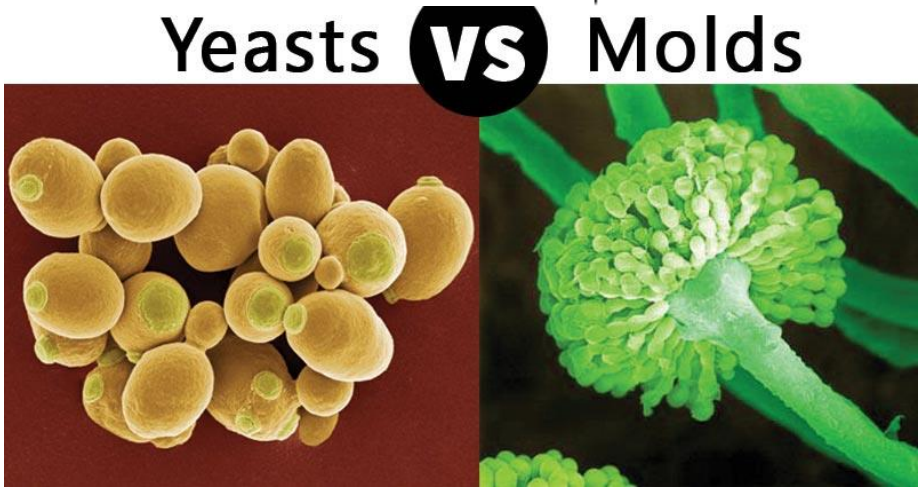
The hyphae and other structures combine to form an elaborate network called a mycelium.



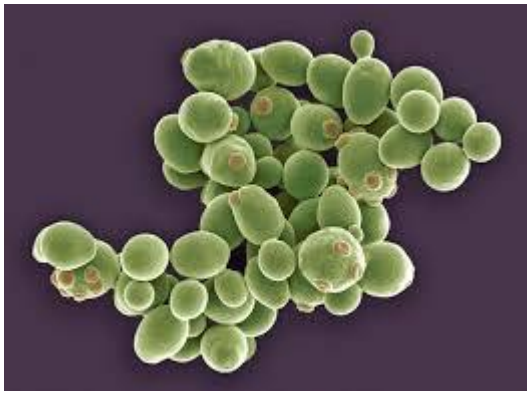

Cell wall
Nucleus
Septum
Septate hyphae
Cell wall
Nucleus
Nonseptate hyphae
labpedia.net


Yeast
Mold
a
Bud
Hypha


b
Mannoproteins
β-glucans
Chitin
Cell membrane
Membrane protein


Yeasts
VS
Molds

Yeasts: These are large (5 to 8 µ), single-celled organisms, nonfilamentous or rarely form filaments.
Yeast are frequently found as a white powdery coating on fruits and leaves.
The yeast cell wall consists of two or three layers, and contains chitin.
Yeast cells sometimes contain large quantities of fat.
Most yeasts reproduce by the asexual process of budding. Yeast colonies are usually characterized by a smooth surface similar to that of many bacteria.



CLASSIFICATION OF FUNGI:
Fungi are usually classified in four divisions: the Chytridiomycota (chytrids), Zygomycota (bread molds), Ascomycota (yeasts and sac fungi), and the Basidiomycota (club fungi). Placement into a division is based on the way in which the fungus reproduces sexually. The shape and internal structure of the sporangia, which produce the spores, are the most useful character for identifying these various major groups.

Chytridiomycota:

Chytrids, the organisms found in Chytridiomycota.

They are usually aquatic and microscopic.

They are usually asexual, and produce spores that move around using flagella, small tail-like appendages.

Zygomycota:

Zygomycetes are mainly connected with earth and feed off of waste plants or decaying animal material.

They also cause problems by growing on human food sources.

One example of a zygomycte is *Rhizopus stolonifer*, a bread mold

Glomeromycota:

Glomeromycetes make up half of all fungi found in soil, and they often form symbiotic association with plants.

The fungi obtain sugars from the plant, and returns dissolve minerals, nutrients to plants.

These fungi also reproduce asexually.

Ascomycota:

Ascomycetes are often pathogens of plants and animals, including humans.

They are responsible for infections like athlete's foot, ringworm, which causes vomiting, Muscles spasm, Confusion, Itching and sometimes even death.

However, some ascomycetes normally are found inside humans, such as *Candida albicans*, a yeast which lives in the respiratory, gastrointestinal, and female reproductive tracts. Ascomycetes reproduce asexually.

Basidiomycota: Like ascomycetes, basidiomycetes also produce sexual spores called basidiospores in cells called basidia. Basidia are usually club-shaped, and basidiomycetes are also known as club fungi.

Most basidiocytes reproduce sexually.

Mushrooms are a common example of basidiomycetes.

REPRODUCTION / REPLICATION OF FUNGI:

Mostly in fungi, two type of reproduction way are possible:

1) Asexual Reproduction
2) Sexual Reproduction

Asexual Reproduction: Asexual reproduction of fungi may take place by a variety of ways.

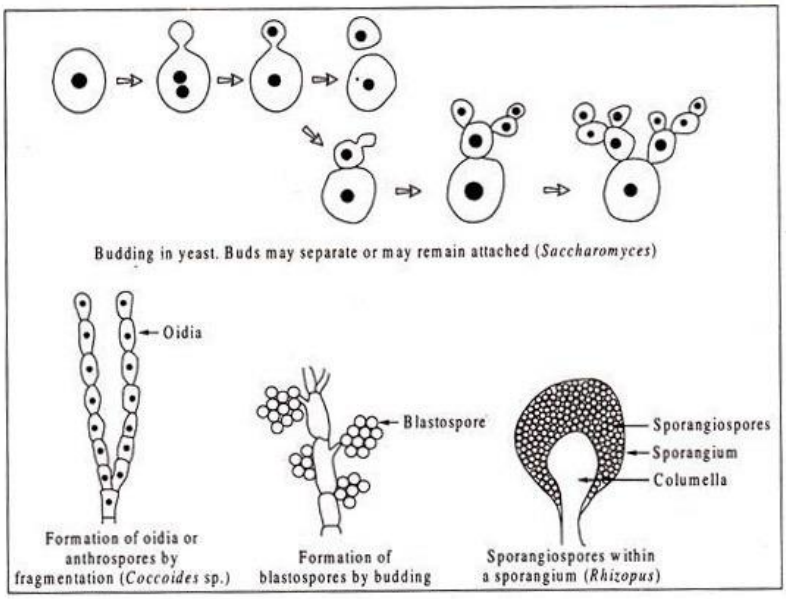
The unicellular forms may multiply by cell division, fission or budding.

Yeasts multiply either by fission as observed in the species of Schizosaccharomyces, or by budding in members of Saccharomyces. Some ascospores and basidiospores may also multiply by budding.

In many fungi, asexual reproduction occurs through production of more specialized spores.

Such spores may be produced in special sacs, known as sporangia and the spores are known as sporangiospores.

Spores may also be motile and, in that case they are called zoospores.



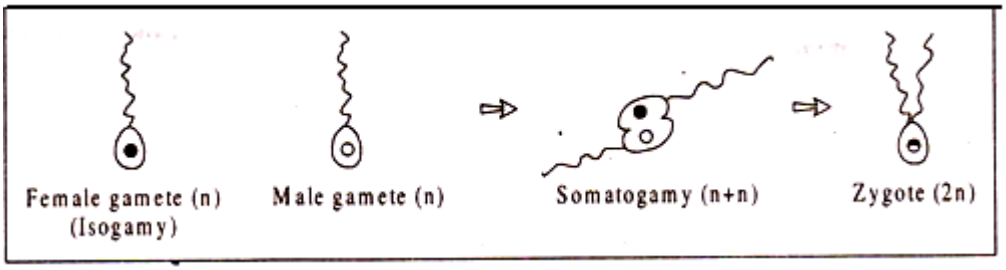
Sexual Reproduction:

Sexual reproduction takes place by fusion of two nuclei originating from two individuals of opposite mating types, generally designated as male and female.

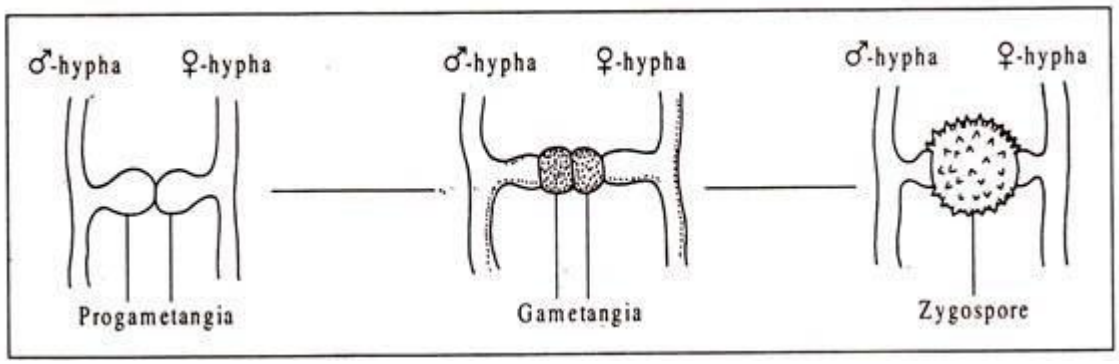
Fusion of the nuclei is preceded by fusion of two protoplasts, the process is called plasmogamy.

Fusion of the nuclei is known as karyogamy.

It leads to production of a diploid cell, called zygote.



Gasmetangial copulation in mucor sp. The male and female gametangia fuse with each other to produce a zygospore. In the zygospore, numerous male and female nuclei pair with other (n+n).



CULTIVATION OF FUNGI:

Fungi are not plants. While plants make their own food in their leaves using sunlight and carbon dioxide (CO_2), fungi can't do this. Instead, fungi have to get their food from other sources, living or dead. Animals, like fungi, cannot make their own food but they can at least move to find the food they need.

All fungi need the physical presence of water for uptake of nutrients through the wall and cell membrane, and often for the release of extracellular enzymes. Fungi also need intracellular water for metabolic reactions.

General purpose media that are commonly used for fungal culture are Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) & Malt Extract (Corn uses as starch source).

To prevent contamination of the medium by bacteria, Chloramphenicol / Tartaric Acid is uses.

Cultures are routinely incubated at 25° to 30° C for up to 4 weeks. (pH 5-6). Humid atmosphere is best atmosphere for fungi cultivation.

We can follow following steps for cultivation of fungi:

Identification & Selection of species of fungi which want to cultivate

⇩

Culture Media Preparation as per species requirement

⇩

Transfering of selected culture with the help of transferring tube

⇩

Providing require temperature, pH Medium

⇩

Keep for require time period for Fertilization / Growing (Observation should be occure time to time at every step)

⇩

Collection / harvesting as per need

CULTURE MEDIA PREPARATION:

The media most commonly used are nutrient agar (bacteria), Potato Dextrose Agar (PDA), & Sabouraud Dextrose Agar (SDA).

1) POTATO DEXTROSE AGAR (PDA) CULTURE MEDIA:

REQUIREMENTS:

Dextrose: 20gm
Potato extract: 040gm
Agar: 15 gm
Distilled Water: 1 ltr
Chloramphenicol: 25 mg or Tartaric Acid : 1.4 gm(if require)
Note: 04 GM of potato extract is equivalent to 200 ml of potato infusion.

Appearance: Light amber colored clear to slightly opaque gel
pH: 5.6 ± 0.2

PROCESS:

1. Boil 200 g of sliced unpeeled potatoes in 1 liter of water for 30 minutes.
2. Filter through cheesecloth, saving effluent, which is potato infusion.
3. Add dextrose, agar, and water to effluent. Boil to dissolve completely.
4. Sterilize media by autoclaving at 121ºC for 15 minutes.
5. Aseptically dispense into sterile Petri dishes.

STORAGE:

Media is both light and temperature sensitive. Store plates away from direct light at 2-8ºC. Plates may be used for one week when stored in a clean sterile area. Media should not be used if any signs of deterioration, contamination, and/or expiration date has passed.

2) SABOURAUD DEXTROSE AGAR (SDA) CULTURE MEDIA:

Sabouraud Dextrose Agar contains digests of animal tissues (**peptones**) which provide a nutritious source of amino acids and **nitrogenous** compounds for the growth of fungi and yeasts. **Dextrose** is added as the energy and carbon source. **Agar** is the solidifying agent. **Chloramphenicol** and/or **tetracycline** may be added as broad spectrum antimicrobials to inhibit the growth of a wide range of gram-positive and gram-negative bacteria. **Gentamicin** is added to further inhibit the growth of gram-negative bacteria. The pH is adjusted to approximately 5.6 in order to enhance the growth of fungi.

REQUIREMETS:

DEXTROSE: 40 GM
PEPTONE: 10 GM
AGAR: 15 GM
pH: 5.6±0.2
Temp: 25°C

PREPARATION:

1. Suspend 65 g of the medium in one liter of distilled water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121° C for 15 minutes.
4. Cool to 45 to 50°C and pour into petri dishes or tubes for slants.

IDENTIFICATION & FUNGI COLONY MORPHOLOGY:

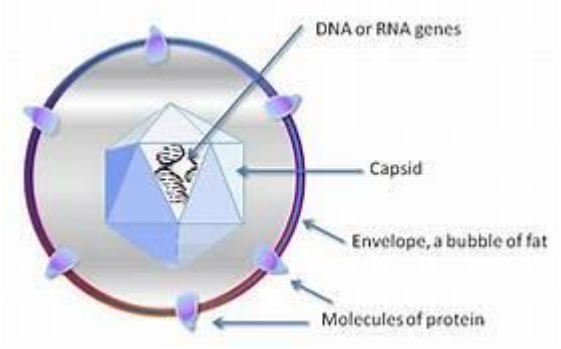
Candida albicans **in Sabouraud Dextrose Agar** ***Sporothrix schenckii*** **in Sabouraud Dextrose Agar**

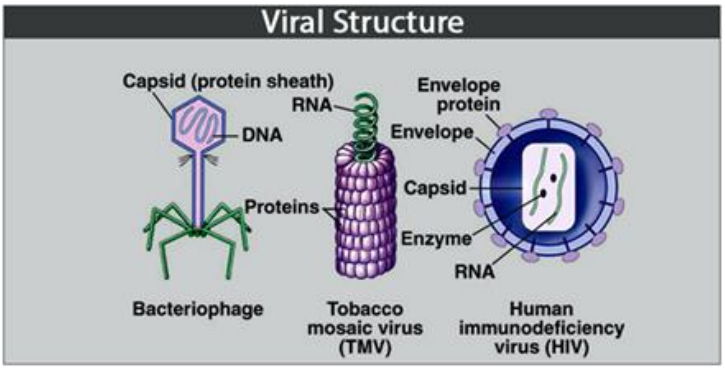
Fungi	Colony morphology
Candida albicans	Pasty opaque slightly domed, smooth, and cream or white colonies
Aspergillus flavus	Yellow-green powdery on front and pale yellowish on reverse
Aspergillus niger	The initial growth is white, becoming black later on giving "salt and pepper appearance" which results from darkly pigmented conidia borne in large numbers on conidiophores and reverse turning pale yellow
Aspergillus fumigatus	Bluish green powdery colonies on front and pale yellow on reverse .
Trichosporon mucoides	White to cream, yellowish, wrinkled
Geotrichum candidum	White to cream colored, flat with aerial mycelium

VIROLOGY: STUDY OF VIRUSES

- Virus is a small nucleoprotein complex and infectious agent.
- Virus replicates only inside the living cells of other organisms such as animals and plants.
- They are such smaller than bacteria (20-300 nm).
- They are obligate intracellular parasites of bacteria, protozoa, fungi, algae, plants and animals.

- They cannot be grown in sterile medium but grow in specific host cells.
- They have only a single type of nucleic acid that serves as genetic material but lack cellular structure and enzymes for metabolite processes.
- In 1890, D. Ivanovski and M. Beijerinck discovered Tobacco mosaic virus that caused disease in tobacco plant.
- Friedrich Leoffler and Paul Frosch have discovered animal viruses that cause foot and mouth disease incattle.



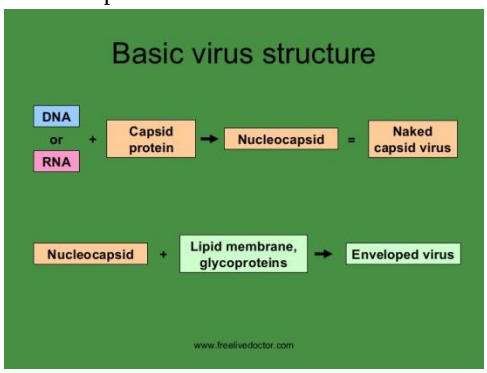


Properties of Viruses:

- They do not have cellular organization.
- They contain only one type of nucleic acid either DNA or RNA.
- They are obligate intracellular parasites.
- They lack enzymes for protein synthesis.
- They multiply by complex process but not by binary fission.
- They are unaffected by antibacterial antibiotics.

MORPHOLOGY:

- Viruses are acellular that means they do not have cells as it has no nucleus, cytoplasm or organelles.
- They consist of two main parts viz. nucleic acid and capsid. In nucleic acid, DNA or RNA is found at the core of virus.
- They need living host to survive. Smallest infectious virus is 20 nm to 300 nm.
- The smallest RNA virus is picornavirus, the smallest DNA virus is parvovirus (20 nm) and the largest virus is poxvirus (300 nm). They may be single or double stranded.
- The nucleic acid is linear or circular and either segmented or non-segmented.
- Capsid is a protein coat which is surrounded by the genetic material. Protein coat is made up of repeating sub-units known as capsomeres.
- Some viruses have an outer envelope which is made of lipids and proteins covering the capsid.
- They are parasites. Most of viruses are spherical shaped but some are different types like pox virus is brick shaped, bacteriophage is tadpole shaped, tobacco mosaic virus is rod shaped.



Functions of Viral Capsid:

- It protects the genetic materials.
- It serves as antigenic determinants.
- It induces antibody production.
- It provides the structural symmetry of the virus.
- It facilitates the assembly and packaging of viral genetic information.
- It serves as a vehicle of transmission from host toanother.

Viral Envelope: It is a lipoprotein derived from the host cell membrane and virus specific protein. It is composed of glycoproteins which look like spikes. It confers instability to the virus because of the loss of infectivity due to disruption of lipid. They are more sensitive to heat, lipid solvents and detergents.

Functions:

- They help to attach to host cell receptors.
- They are antigenic determinants.
- They stimulate antibody production.

Examples:Herpes virus, HIV virus, Hepatitis virus etc.

Virion: It is an entire virus particle, consisting of an outer protein shell called a capsid and an inner core of nucleic acid. They are present in naked viruses, identical to nucleo capsid whereas in enveloped viruses develops envelop.

Peplomers: In mature virus particle, the glycoprotein often appears as projecting spikes on the outer surface of the envelope, that are known as peplomers. Example: Influenza virus carries two types of peplomers viz. hemagglutinin and neuraminidase.

Functions:

- It helps for attachment of virus to the host cell receptors to initiate the entrance of the virion into the cell.
- It has antigentic properties.
- It has enzymatic activity like neuraminidase which cleaves neuraminic acid from host cell glycoproteins.
- It attaches to the receptors on the RBC, causes these cells to agglutinate.

CLASSIFICATION OF VIRUSES:

Viruses are classified by phenotypic characteristics, such as morphology, nucleic acid type, mode of replication, host organisms, and the type of disease they cause. They are as follows:

1. Classification on the basis of nucleic acid
2. Classification on the basis of structure or symmetry
3. Classification on the basis of replication properties and site of replication
4. Classification on the basis of host range
5. Classification on the basis of mode of transmission

1. **Classification on the Basis of Nucleic Acid**:

(a) DNA virus:

- Viral genome is DNA.
- Double stranded DNA virus: Examples: Adenovirus, Herpesvirus.
- Single stranded DNA virus: Example: Parvovirus.

(b) RNA virus:

- Genome is RNA.
- Double stranded RNA virus: Example: Reo virus.
- Single stranded RNA virus: These are further classified into two groups.

❖ Positive sense RNA (+RNA): Polio virus, Influenza virus.
❖ Negative sense RNA (−RNA): Rabies virus.

2. **Classification of Virus on the Basis of Structure :**

(a) Cubical virus: They are also known as icosahedral symmetry virus.

Examples: Reo virus, Picorna virus.

(b) Spiral virus: They are also known as helical symmetry virus.

Examples: Paramyxovirus, orthomyxovirus.

(c) Radial symmetry virus: They are look like spoke or wheel type.

Example: **Bacteriophage. (Bacteriophase is a type of virus that infects bacteria and destroy their host cell, Bacteriophase consists from nucleic acid molecule that is surrounded by a protein structure)**

(d) Complex virus: They possess a capsid that is neither purely helical nor purely icosahedral.

Example: Pox virus.

3. **Classification of virus on the basis of replication properties and site of replication :**

(a) Replication and assembly in cytoplasm of host:

Example: All RNA virus replicate and assemble in cytoplasm of host cell except Influenza virus.

(b) Replication in nucleus and assembly in cytoplasm of host:

Examples: Influenza virus, Pox virus.

(c) Replication and assembly in nucleus of host: All DNA viruses replicate and assemble in nucleus of host cell except Pox virus.

(d) Virus replication through ds DNA intermediate:

Examples: All DNA virus, Retro virus and some tumorcausing RNA virus replicates through ds DNA as intermediates.

(e) Virus replication through ss RNA intermediate:

Example: All RNA virus except Reo virus and tumor causing RNA viruses.

4. **Classification of Virus on the basis of Host Range:**

(a) Bacteriophage: Phages are virus infecting bacteria. Examples: λphage, T2, T4, φ174, MV-11.

(b) Plant virus: Those viruses that infects plants. Examples: TMV, cauliflower mosaic virus.

(c) Animal virus: Those viruses that infects animals. Examples: Poliovirus, Retro virus, Herpes virus, Adeno virus.

(d) Insect virus: Virus that infects insects. Examples: Baculovirus, Sacbrood virus, Entomopox virus, Granulosis virus.

5. **Classification of virus on the basis of mode of transmission:**

(a) Virus transmitted through respiratory route:

Examples: Swine flu, Rhino virus.

(b) Virus transmitted through faeco-oral route:

Examples: Hepatitis A virus, Polio virus, Rota virus.

(c) Virus transmitted through sexual contacts:

Example: Retro virus.

(d) Virus transmitted through blood transfusion:

Examples: Hepatitis B virus, HIV

(e) Zoonotic virus: Virus transmitted through biting of infected animals.

Examples: Rabies virus, Alpha virus, Flavi virus.

dsDNA viruses	ssDNA viruses	dsRNA viruses	ssRNA (+) viruses	ssRNA (-) viruses	RNA and DNA (RT) viruses
Poxviridae	*Circoviridae*	*Reoviridae*	*Picornaviridae*	*Bornaviridae*	*Retroviridae* (RNA)
Asfaviridae	*Anellovirus*	*Birnaviridae*	*Caliciviridae*	*Rhabdoviridae*	*Hepadnaviridae*
Iridoviridae	*Parvoviridae*		*Hepevirus*	*Filoviridae*	(DNA)
Herpesviridae			*Astroviridae*	*Paramyxoviridae*	
Adenoviridae			*Nodaviridae*	*Orthomyxoviridae*	
Polyomaviridae			*Coronaviridae*	*Bunyaviridae*	
Papillomaviridae			*Arteriviridae*	*Arenaviridae*	
			Flaviviridae	*Deltavirus*	
			Togaviridae		

CULTIVATION OF VIRUSES:

Viruses are cultured in embryonated egg, cell line culture and animal inoculation because they can only replicate inside host cell, so viruses cannot be cultured in non-living medium as bacteria and fungi (Bacteria and fungi are living creatures).

The main purposes of virus cultivation are identification and isolation of viruses in clinical samples, research on viral structure, replication, genetics and their effects on host cell and production of viral vaccines.

Hence, cultivation of viruses is essential which iscarried out by three methods via. Animal inoculation, Embryonated egg culture and Cell culture.

1. **Animal inoculation:** Animal inoculation is one of the primary methods for isolation of certain viruses and for study of pathogenesis of certain viral diseases.

This method is carried out for those viruses which are not cultivated in embryonated egg and tissue culture methods. In this method, healthy and disease free laboratory mice (white mice) are particularly used for virus cultivation. Suckling mice of age less than 48 hours are used for culture of Toga virus and Coxsackie virus.

The inoculation of viruses is carried out by intracerebral and intranasal route. The inoculation is also carried out by intraperitoneal and subcutaneous routes. Other healthy and disease free animals such as hamsters, Guinae pig, rabbits, Chimpanzee and primates are also used as alternative for virus culture. After inoculation of virus sample in to the host cell, the animals are observed for symptoms of disease till death and then virus is isolated from tissue of animal. Live inoculation was first used to study of yellow fever virus on human volunteers.

Advantages:

- Diagnosis, Pathogenesis and clinical symptoms are determined.
- Antibodies productions are identified.
- Mice are the reliable model for virus replication.
- This method helps to study of immune responses, epidemiology and oncogenesis.

Disadvantages:

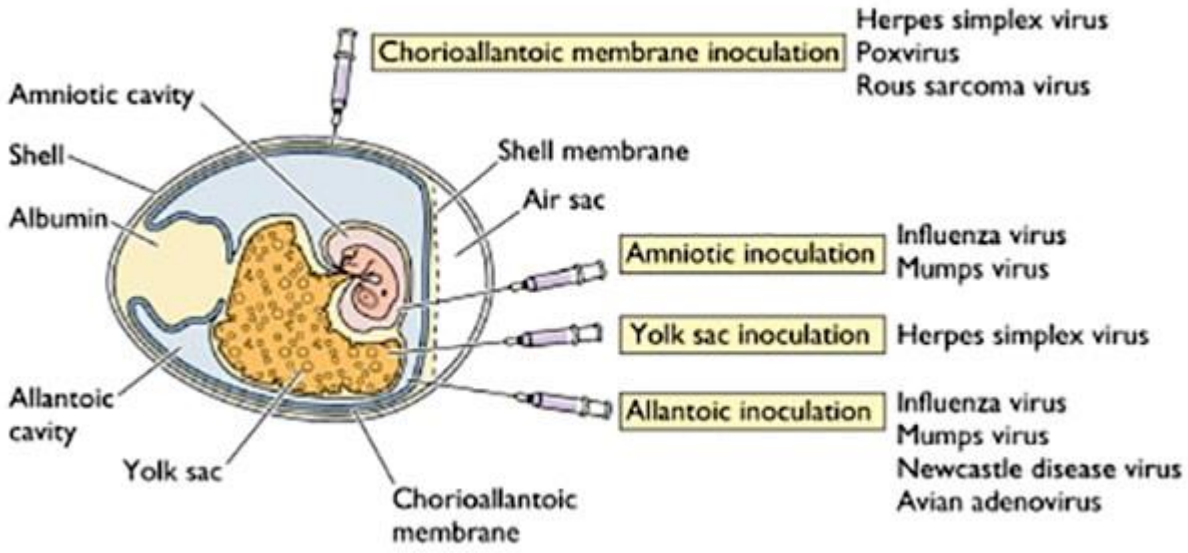
- Animal experiment is costly and difficult to maintain.
- Animal selection for specific viruses is difficult.
- Some human viruses are not grown in animals.
- Vaccine production is not possible with mice model.

2. Embryonated egg culture:

For virus cultivation, an egg embryo of 7-12 days is used. At first egg is kept in incubator for embryo development up to 7-12 days and then virus sample is inoculated into the egg. For inoculation, eggs are first prepared for cultivation and the shell surface

is first disinfected with iodine and penetrated with a small sterile drill. After inoculation, the opening is sealed with paraffin and incubated at 36°C for 2-3 days. The egg is broken after the incubation and virus is isolated from tissue of egg.

Virus can be cultured in different parts of embryonated egg, such as choriallontoic membrane (Pox virus arecultured), amniotic sac (Influenza virus, Mumps virus, Yellow fever virus and Rabies virus are cultivated), amniotic cavity (Influenza virus is cultured) or yolk sac (Herpes virus is cultured) depending upon types of virus. In 1931, Scientist Pasture was first used the embryonated hen's egg for the cultivation of virus.



Advantages:

- This method is widely used method for the isolationof virus.
- It is an ideal substrate for the viral growth and replication.
- This method is cost effective and maintenance is much easier.
- Less labor is needed.
- The embryonated eggs are readily available.
- They are free from contaminating bacteria and many latent viruses.

Disadvantages:

- Each virus has different sites for their growth andreplication hence the site of inoculation varies with the viruses.

3. Cell culture (tissue culture) technique:

This technique is most commonly used technique for cultivation of virus. There are three types of cell culture technique viz. organ culture,explant culture and cell culture.

(i) Organ culture: This culture method is mainly done for highly specialized parasites of certain organs. Like, tracheal ring culture is done for isolation of corona virus.

(ii) Explant culture: In this method, small fragment of tissue is extracted from human or animal and used for virus culture but this technique is very rarely used.

(iii) Cell line culture: This is the most commonly used technique. Cell line culture is routinely used in lab for virus culture, isolation and identification. In this method, the growth media is prepared by maintaining balanced salt concentration, all essential aminoacids, glucose, buffering agents, some antibiotic, serum etc. Thereafter some tissue fragment is obtained which is trypsionized to dissociate cells. These cells are washed and suspended in culture media in petriplates and incubated for sufficient time. On incubation, cell divides and spread out on the glass surface to form a confluent mono-layer of cells that is used for virus culture.

On the basis of origin, chromosomal characteristics and number of generation through which cell culture can be maintained, cell line culture is classified in three types viz. Primary cell line, Semi-continuous cell line and Continuous cell line.

I. Primary cell line:

These are normal cells, obtained from fresh organ of animals or human and cultured.
Examples: Monkey kidney cell line, Human amnion cell line, etc.

II. Semi-continuous cell line:

These cells are fibroblastic cells or also known as diploid cell because they contain same number of chromosome as the parent cell. These diploid cells are sub-cultured for limited generation. They are susceptible for wide range of Human virus culture and also used for vaccine production.
Examples: Rhesus embryo cell, human embryonic lung strain, etc.

III. Continuous cell line:

These are cells of single type capable of infinite growth in-vitro. These are usually cancer cells derived from cancerous tissue. These cells grow faster and they are haploid cells. These cells are maintained by serial sub-culture or by deep freezing at −7°C, so that these cells are reused when necessary. This cell line is used for virus culture but not used for vaccine preparation.
Examples: HeLa cell is obtained from cervical cancer, HEP-2 (Human Epithelioma of larynx cell line), Vero (Vervet monkey) kidney cell lines, BHK-21 (Baby Hamster Kidney cell line).

Advantage of Cell Culture:

- The method is easy, broad spectrum, cheaper and good sensitivity.

Disadvantages of Cell Culture:

- The process requires skilled person.
- Tissue or serum is sent central laboratories for analysis to identify virus.

Cultivation of Bacteriophages :

Bacteriophages are cultivated in either broth or agar cultures of young, actively growing bacterial cells.

Cultivation of Plant Viruses:

Plant viruses are cultivated by plant tissue cultures, cultures of separated cells, or cultures of protoplasts, etc. and viruses are grown in wholeplants. Leaves are mechanically inoculated by rubbing with a mixture of viruses and an abrasive and that time cell wall is broken and the viruses directly contact the plasma membrane and infect the exposed host cells. A localized necrotic lesion often develops due to the rapid death of cells in the infected area.

REPRODUCTION OF VIRUSES:

Viruses do not reproduce. The living cell in which the virus reproduces is called a host cell. They use these host cells to replicate themselves by creating an exact copy of the virus.
Two main types of reproductive cycle are observed in virus namely **Lytic Cycle** and **Lysogenic Cycle.**

Lytic Cycle: This cycle is completed with several steps which are as follows:

1. **Attachment:** In this first step, Virus is attached to the host cell.
2. **Entry:**Then genetic material is injected into the host cell.
3. **Replication:** The virus takes over the cell's metabolism, causingthe creation of new proteins and nucleic acids by the host cell's organelles.
4. **Assembly:** Then proteins and nucleic acids are assembled into new viruses.
5. **Release:** Finally virus enzymes cause the cell to burst and viruses are released from the host cell. These new viruses further infect to other cells and this process is continuing.

Lysogenic Cycle: This cycle is completed with several steps which are as follows:

1. **Attachment:** In the first step, virus is attached to the host cell.
2. **Entry:**Then genetic material is injected into the host cell.
3. **Integration:**Viral DNA integrates into the host cell's genome.
4. **Replication**: When the host cell replicates, viral DNA is copied along with host cell DNA. Each new daughter cell is infected with the virus.
5. **Induction:**When the infected cells are exposed to certain environmental conditions, viral DNA is activated and enters the lytic cycle.

6. Replication: The virus takes over the cell's metabolism, causing the creation of new proteins and nucleic acids by the host cell's organelles.

7. Assembly: Proteins and nucleic acids are assembled into new viruses.

8. Release: Then finally virus enzymes cause the cell to burstand viruses are released from the host cell. These new viruses can infect other cells.

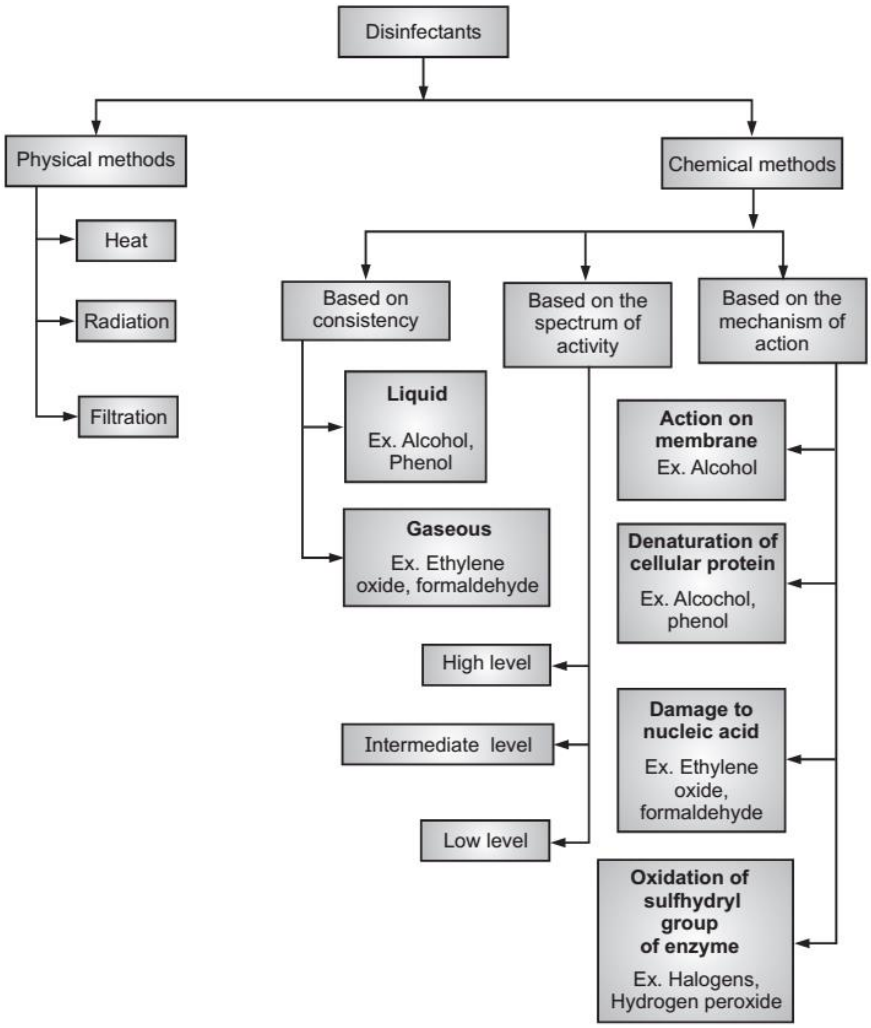
Disinfectants: They are chemical agents that are used to kill microorganisms (except spores & viruses).
But they are not to be used on living tissues such as skin or mucous membranes.
The process by which destruction or removal and killing of all pathogenic organisms are carried out is known as disinfection.
The main purpose is to prevent transmission , spreading of infection of certain microorganisms.

Ideal Properties:

- They should have wide spectrum of activity, should be active against all pathogens.
- They should be cidal (capable of killing bacteria) and chemically stable.
- They should be cheap and should not produce any stain.
- They should not damage any non-living materials.
- They should be non-toxic and non-corrosive.
- They should be relatively safe to human and other animals.
- They should be easily soluble in hard water and should be active at any pH.
- They should have good cleaning properties and should be non-inflammable.

CLASSIFICATION OF DISINFECTANTS:

Disinfectants are broadly classified into two categories viz. physical methods and chemical methods.



PHYSICAL METHOD:

Heat:

Mainly dry and moist heats are used for the disinfection action. Flaming is used for dry heat process whereas temperatures at various levelsare used in moist heat sterilization.

Filtration:

This method is carried out for fluids that are heat labile like antibiotics, vitamins and other growth factors with very fine pore membrane filters below 0.45 microns in diameter.

Radiation:

Ultra-violet lights are used in laboratories that reduce the number of organisms to low level in the air and on surfaces.
Chemical Methods.

CHEMICAL METHOD:

Based on Consistency:

Mainly liquids and gaseous substances are used for the disinfection activity. Liquids like alcohol and phenol are widely used as disinfectant. Alcohols like ethanol or isopropanol at a concentration of 50-70% are used for disinfectants.They are used for the disinfection of the surfaces and other laboratory equipment. Alcohols are low in sporocidal activity.

Gaseous agents like Ethylene oxide and aldehydes are used as disinfectant agents. Ethylene oxide is used as a sterilant, but in liquids, it shows antimicrobial action. Formaldehyde and glutaraldehyde are used as powerful disinfectants.

Based on the Spectrum of Activity:

Based on the activity, disinfectants are classified as high, intermediate and low levels.
High level disinfectants such as aldehydes and gases, are used for endoscope and disinfectants for surgical instruments. Some examples of high level disinfectants are like 2% glutaraldehyde for 20 minutes, 6% hydrogen peroxidefor 30 minutes, 0.2% per acetic acid for 30-45 minutes.
Intermediate level disinfectants are like alcohols and iodophore, used for disinfectant of laryngoscope.
Low level disinfectants are like quaternary ammonium compounds. They are also known as quats.

Based on Mechanism of Action:

(i) Action on membrane: Mainly alcohols such as ethanol, isopropanol and methanol are used for this purpose. They act on membrane of microorganisms and destroy the cell membrane. They are highly active in combination with water. 70% solution of Ethanol and isopropanol are used as hand disinfectants in pharmaceuticals.
Example: Triclogel contains 75% ethanol.

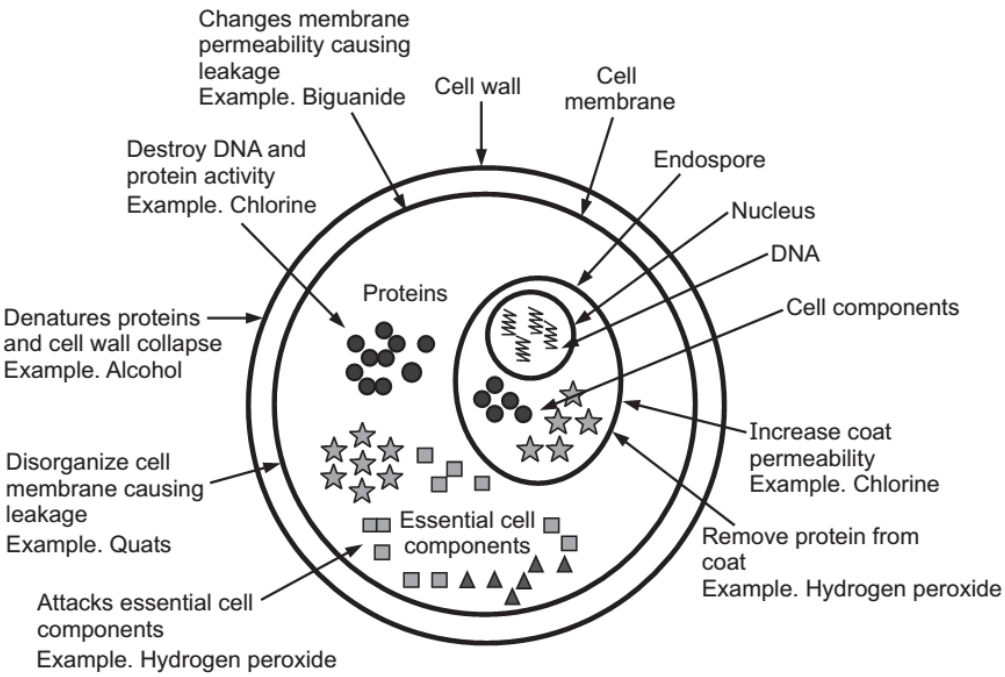
(ii) Denaturation of cellular proteins: Alcohols also denatures the cell wall proteins. Phenols such as chlorocresol and chloroxylenol are used as disinfectants. They also denature the proteins and enzymes of the cell.
Examples: Dettole contains chloroxylenol and Lysol contains para chloroorthobenzylphenol.

(iii) Damage to nucleic acid: Ethylene oxide is a colourless gas which is soluble in water. It has alkylating action on proteins and damages the nucleic acid. Inhibition produced by it is irreversible, resulting inhibition of enzyme activity.

(iv) Oxidation of sulfhydryl group of enzyme: Hydrogen peroxide acts by producing destructive hydroxyl free radicals that attack membrane lipid, DNA and other cell components. Halogen compounds such as chlorine and chlorinated compounds and iodine compounds act as disinfectants.

Mode of Action of Disinfectants:

The main function of disinfectants is to act on microorganisms and the action is two ways either growth inhibition (bacteriostasis, fungistasis) or lethal action (bactericidal, fungicidal or virucidal effects). But the main objective of the disinfectants is the lethal action or lethality.



DIFFERENCE BETWEEN STERILIZATION & DISINFECTION:

Sterilization	Disinfection
1. It is the method of complete removal of microorganisms.	1. It only reduces the number of microorganisms without killing.
2. It requires strict protocol to remove microbes.	2. It do not require any strict protocol.
3. It kills bacteria along with their spores.	3. It does not kills bacterial spores.

Sterilization	Disinfection
4. It is require in surgical operations or in sterile labs.	4. It is commonly use in daily life.
5. Usually physical methods are used for destruction of microorganisms (heat, chemical sterilants and gases).	5. Usually chemical methods are used.
6. Sterilization is an absolute condition.	6. Disinfection is an adequate method.
7. Some sterilants are like dry heat, steam, radiation, filtration.	7. Disinfectants are like phenol, chlorine, iodine, alcohol, hydrogen peroxide.

ANTISEPTICS:

They are the agents that cause destruction or inhibition of growth of microorganisms such as bacteria, viruses and fungi on living surfaces such as skin and mucous membranes.

Mechanisms of Action:
All antiseptics interfere with life processes of microorganisms. Example: Benzylalkonium interferes with intracellular signaling and chemistry to the point that the cell membrane begins to disintegrate.
Iodine compounds irreversibly deform, or denature, critical proteins in bacteria, starting at the cell membrane and moving inwards.

Alcohol also denatures and destroys cell membranes.

Some soaps and detergents cause bacterial cell membranes to lose integrity or denature critical proteins. Other antiseptics directly interfere with critical enzymes inside a microbe.

Types of Antiseptics:
Many antiseptics are used today and they are classified based on their active chemical ingredients.
1. Alcohol Antiseptics: They are generally composed of isopropyl or methylalcohols and are commonly used to sterilize hard surfaces such as medical tools at hospitals.
Medical personnel also use alcohol gels as hand sanitizers in order to prevent the spread of disease.
2. Chlorhexidine Antiseptics: It is used as both oral and skin cleansing products. It is the active ingredient in prescription mouthwashes.
3. Idochlor Antiseptics: They are applied as a topical treatment to unbroken skin or mucus membranes to prevent infection before surgery. Example: Betadine.
4. Peroxygen Antiseptics: They are used in bubbling reaction to blood and damaged cells. Example: hydrogen peroxide. Hydrogen peroxide is used to cleanse wounds and disinfect skin. It is also used as a gargle or mouthwash.
5. Phenol Antiseptics: They are used primarily as skin disinfectants and in medicated soaps and scrubs. In addition, phenol can also be used in mouthwashes and throat lozenges.

Ideal Properties of Antiseptics:
- They should have broad spectrum activity, should destroy microorganisms.
- They should have rapid bactericidal activity.
- They should be active at any pH.
- They should be non-irritant, non-corrosive.
- They should not stain the instruments.
- They should be soluble in water.
- They should penetrate deeply to the bacterial cells.
- They should not damage materials or living tissues when they are come in contact.

DIFFERENCES BETWEEN ANTISEPTICS & DISINFECTANTS:

Disinfectants	Antiseptics
1. They are manly used to kill the microbes on the non-living objects.	1. They are used to kill the microbes on the skin.
2. They have power to kill all the microorganisms and bacteria on the surfaces.	2. They do not have power to kill all the microorganisms and bacteria on the surfaces.
3. They can cause skin corrosion and inflammation.	3. They do not cause any skin damage and inflammation.
4. They are used in laboratory and house hold items cleaning.	4. They are used in surgery and hand washing.
5. They destroy the cell wall of microorganisms or interferes with the metabolism of microbes thrives on the surface of an object.	5. They transport through the lymphatic system and destroy bacteria within the living body.

FACTORS INFLUENCING DISINFECTION & ANTISEPTICS:

Several physical and chemical factors also influence disinfectant procedures namely temperature, pH, relative humidity and water hardness.

Temperature: It is one of the major factors that influence disinfection activity. Generally, disinfectant activity increases with the increased temperature but there are some exceptions.

Generally, higher temperature decreases the surface tension and also the viscosity and enhances the germicidal action. Furthermore very high temperature causes the degradation of disinfectant and weakens its germicidal activity and may produce health problems.

Example: Chlorine compounds are more corrosive in higher temperature and iodine tends to sublime at temperature 49°C or above.

pH: An increase in pH improves the antimicrobial activity of some disinfectants (e.g., glutaraldehyde, quaternary ammonium compounds) but decreases the antimicrobial activity of others (e.g., phenols, hypochlorites, and iodine).

Relative humidity: It is the most important factor influencing the activity of gaseous disinfectants, such as ethylene oxide, chlorine dioxide, and formaldehyde.

Water hardness: Hardness of water means high concentration of divalent cations present in water. Hardness reduces the rate of kill of certain disinfectants because divalent cations (e.g., magnesium, calcium) in the hard water interact with the disinfectant to form insoluble precipitates.

Other factors like Duration, Age of disinfectants, Concentration of disinfectants, Biofilms and Number of pathogens are also play an important role.

ANTIMICROBIAL AGENTS:

Based on mode of action, antimicrobials are mainly of two types either bactericidal or bacteriostatic.

Bactericidal:

They are the agents that disrupt the cell wall synthesis and kill the bacteria. They are less reliant on host resistance. MBC is mainly done for bactericidal activity. It is defined as the minimum concentration of the drug or antibiotic that kills the given test organism. Examples: Penicillins, cephalosporins.

Bacteriostatic:

They are the agents that inhibit the RNA synthesis or reproduction and hence inhibit the growth and reproduction of bacteria. They help the host defenses to take over. MIC is mainly done for bacteriostatic activity. MIC (Minimum Inhibiting Con.) is defined as the lowest concentration of drug or antibiotics that inhibits the growth of the test organism.

Examples: Tetracyclins, Chloramphenicol, Trimethoprim.

Some of other examples of bacteriostatic and bactericidal drugs are given below

Bacteriostatic	Bactericidal
▪ Clindamycin	▪ Isoniazid
▪ Macrolides	▪ Metronidazole
▪ Sulfonamides	▪ Polymyxins
▪ Tetracyclines	▪ Rifampin
	▪ Vancomycin
	▪ Aminoglycosides (at high doses)
	▪ Bacitracin
	▪ Quinolones

Factors Affecting Antimicrobial Activity:

Some antimicrobial agents are having cidal action under one set of conditions and static action under others. There are several factors that influence the activity of antimicrobial agents are:

(1) the susceptibility of the microorganism, (2) the concentration or dose of the agent, (3) the length of exposure, (4) the number of microorganisms, and (5) environmental conditions.

EVALUATION OF BACTERICIDAL AND BACTERIOSTATIC:

For bacteriostatic: Serial dilution test, Cup plate method, Ditch plate method

Serial Dilution Test: This test is carried out in liquid media as well as in solid media. In liquid media, serial dilution is any dilution where the concentration decreases by the same quantity in each successive step. Hence, serial dilutions are multiplicative. In this method, graded concentration of the test substance in a nutrient medium are inoculated with the test

organism and incubated. The medium concentration preventing detectable growth (MIC) is measured as bacteriostatic activity. MIC is defined as the lowest concentration of drug or antibiotics that inhibits the growth of the test organism. MIC mainly done for bacteriostatic activity.

Cup Plate Method: This method depends on the diffusion of an antibiotic from a vertical cavity through the solidified agar layer in a petriplate. In this method, agar is melted, cooled and then inoculated with the test organism and poured into a sterile petridish. In
this method when the inoculated agar is solidified, holes about 8 mm in diameter are cut in the medium with a sterile cork borer. Using the proper buffer solutions, prepared solutions of known concentrations of the antibiotic are examined and poured into cup of agar plate and then incubated at 37°C for 24 hours. In this method, zones of inhibition may be observed, the diameter of the zones giving a rough indication of the relative activities of different antimicrobial substance. If the antibiotic has any anti-bacterial effect it will show zone of inhibition.

Ditch Plate Method: In this method the agar is poured in a petri plate and then allowed to solidify. After that ditch cut out is made of the agar. A solution of the antimicrobial substance or a mixture of this with agar is carefully run into the ditch at about three
quarters fill it. A loopful of each test organism is then streaked outwards from the ditch on the agar surface. The drug is poured very carefully into the ditch. Various microorganisms are streaked on the sides of the ditch. The width of the zone of inhibition
indicates antimicrobial activity. The method is used to find out the potency of the drug against various microorganisms by the means of inhibition of growth on streaked area.

For Bacteriocidal: Phenol coefficient test, Chick martin test

Phenol Coefficient Test: The test is for the measurement of bactericidal activity of a chemical compound in relation to phenol. This test is carried out by measured the concentration at which a chemical is equal in effectiveness to phenol. Staphylococcus aureus or Salmonella typhi are used for this test which are inoculated at 20-37°C for 2-3 days. Phenol coefficient is calculated by:

$$\text{Phenol coefficient} = \frac{\text{Concentration of chemical}}{\text{Concentration of phenol}}$$

If a chemical is equal in effectiveness to phenol at the same concentration then its phenol coefficient is = 1. If the concentration of chemical is less than phenol then phenol coefficient is greater than 1.

Chick Martin Test: This test is carried out in the presence of organic matter like 3% human faecas or dried yeast. This test is modified method of RW test or qualitative suspension test.

Sterility Testing:

In pharmaceuticals products, All the products labeled sterile indicate that the product must pass through the sterility testing methods as per IP, BP and USP.

There are two methods are used namely Membrane filtration and Direct inoculation.

Minimum number of items are recommended as per IP to be tested in a batch are depicted in Table:

Number of test item in a batch as per IP, BP and USP

Preparations	Number of Items in a batch	Recommended number of items
Injectable preparations	Not more than 100 containers	4 containers (10%)
	More than 100 but not more than 500 containers	10 containers
	More than 500 containers	20 containers (2%)

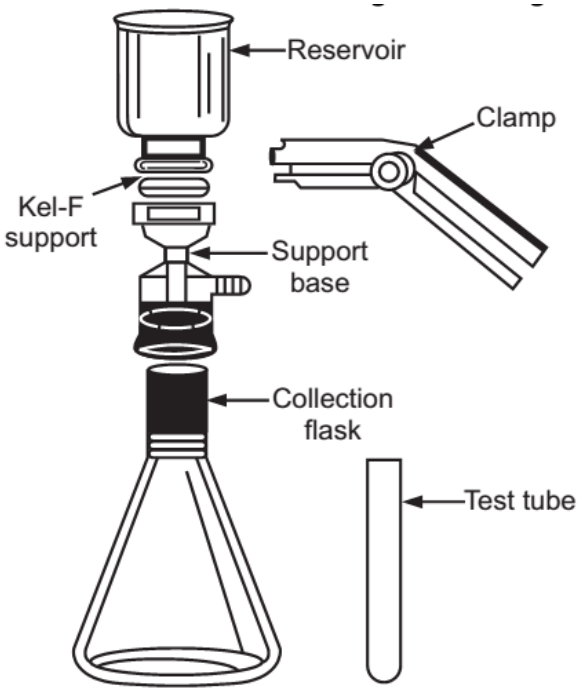
Preparations	Number of Items in a batch	Recommended number of items
Ophthalmic and other non-injectable preparations	Not more than 200 containers	2 containers (5%)
	More than 200 containers	10 containers
Devices	Catgut and other surgical sutures	5 packets (2%)
	Not more than 100 articles	4 articles (10%)
	More than 100 but not more than 500 articles	10 articles
	More than 500 articles	20 articles (2%)
Bulk solid products	Up to 4 containers	Each containers
	More than 4 containers but not more than 50 containers	4 containers (20%)
	More than 50 containers	10 containers (2%)
Liquids (other than antibiotics)	Less than 1 ml	Whole contents of container
	1-40 ml	Half the content in each container
	More than 40 ml but not more than 100 ml	20 ml
	More than 100 ml	10% of the content of the container but not less than 20 ml
	Antibiotic liquids	1 ml
	Insoluble preparations, creams and ointments	Not less than 200 mg
Solids Added as per BP	Less than 50 mg	Whole content of the container
	More than 50 mg but less than 300 mg	Not less than 50 mg
	300 mg to 5 g	150 mg
	More than 5 g	500 mg

(A) Membrane Filtration:

This method is used for those substances that are put in to the solution like oil, ointment, non-bacteriostatic solids and a soluble powder or liquids that have themselves bacteriostatic properties etc. This method is used for the liquid products which have the volume of the container 100 ml or more.

Apparatus:

The apparatus contains a reservoir and a container to collect the filtrate. In the junction of these two, a membrane is placed which has an proper porosity (0.45 µM) and filter diameter is 47 mm. The flow rate of fluid is 55-75 ml/minute at a pressure of 70 mm of mercury. Complete unit should be free from microorganisms.



(MEMBRANE FILTER)

Dilution Fluids:

Mainly two types of dilution fluids are used likely Fluid-A, which is prepared by dissolved 1 g of peptic digest animal tissue in 1 litre of water. Filtered and pH adjusted 7.1. Poured the fluid into 100 ml flask and then sterilized at 121°C for 20 minutes. Fluid-B, which is prepared by using fluid A, to each litre, is added 1 ml of polysorbate 80. This is prepared if the sample contains lecithin or oil. Then poured the fluid into 100 ml flask and then sterilized at 121°C for 20 minutes in autoclave.

For injectable preparations:

The uses of the contents of the containers are listed in below.

Quantities of injectable preparations for sterility testing

Preparations	Quantities of each containers	Minimum quantities to be used
Liquids	Less than 1 ml	Total content of the container
	1 ml to 4 ml	Half of the content
	4 ml to 20 ml	2 ml
	20 ml to 100 ml	10% of the content of the container
	100 ml or more	Not less than half of the content
Solids	Less than 50 mg	Whole content
	50 mg to 200 mg	Half the content
	200 mg or more	100 mg

For ophthalmic and other non-injectable preparations:
The uses of the contents of the containers are listed in below:

Quantities of ophthalmic and other non-injectable preparations for sterility testing

Preparations	Quantities to be mixed	Minimum quantities to be used
Ophthalmic and other non-injectable preparations	10 to 100 ml	5 to 10 ml
Insoluble preparations for emulsification or suspension (cream, ointments), preparation soluble in water	1 to 10 gm	0.5 to 1 gm
Absorbent cotton	–	Not less than 1 gm

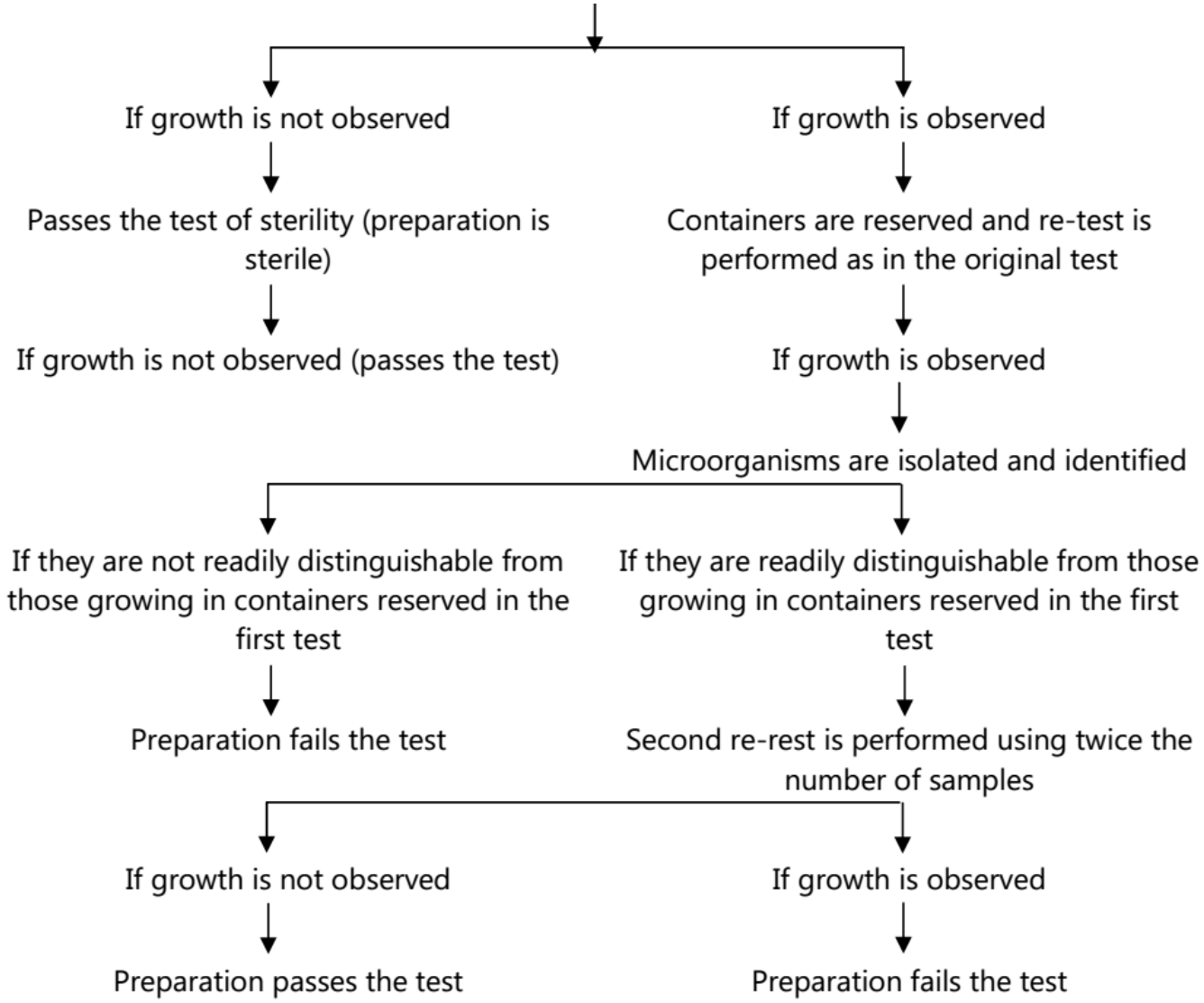
• **For aqueous solution:** Aseptically transferred the quantities of test preparations in the two media on to one membrane. Collected the filtrate through the vacuum filtration. The membrane is removed aseptically and cutted in to two pieces. One piece is then soaked in 100 ml soyabean casein digest medium and incubated at 25°C for 7 days. Other part of the paper is immersed in 100 ml of liquid thioglycollate medium and incubated at 35°C for 7 days.
• **For liquid immiscible with water:** This test is carried out with sufficient quantity of fluid A to the sample to get fast filtration.
• **For oils and oil solutions:** Low viscosity of oils and their solutions are filtered through drug membrane without dilution whereas viscus oils are filtered through filter membrane with suitable diluents.
• **For ointments and creams:** Ointments are dilute in a fatty base and water inoil type emulsions formed by heating at 40°C by added sterile isopropyl myristate as sterile diluents.
• **For soluble solids:** Solids are dissolved in a suitable sterile solventlike fluid A and then filtered.
• **For sterile devices:** Aseptically passed a sufficient volume of fluid B and then collected the liquid in sterile container and filtered through membrane filter.

(B) Direct Inoculation:
This method is used for small volume samples. The volume of the product is not more than 10% of the volume of the medium. It is suitable method for aqueous solutions, oily liquids, ointments and creams. The method involves aseptically opened each sample container from a sterilized batch of product. Then using sterile syringe and needle the required volume of sample is withdrawn for both media from the container. Half of the volume of the sample is injected in to a test tube containing the required volume of fluid thioglycollate medium and the other half volume of sample into a second test tube containing the required volume of Soybean Casein Digest Medium.

• **For aqueous solutions and suspensions:** The liquid from the test container is removed and specified volume is aseptically transferred to the vessel of culture medium. The inoculated medium is then incubated for 35°C for 14days in fluid thioglycolate medium and at 25°C in case of soyabean casein digest medium.
• **For oils and oily solutions:** For the oils and oily solutions the media is added with 0.1% w/v of polyethoxyethanol or 1% w/v of polysorbate 80 or other emulsifying agents in suitable concentration and the sterility test is performed.
• **For ointments:** Ointments are 10 fold diluted with sterile diluentslike fluid-B.
• **For solids:** Required quantity of material is transferred to thesterile medium.
• **For sterile devices:** The device is immersed in 1000 ml of culture medium. If not immersed properly then 20 units of lumen flushed with thioglycolate medium and soyabean casein digest medium separately and recovered 15 ml of each medium and incubated 100 ml of the each medium.

RESULT OBSERVATION:

Observation and interpretation of sterility testing result After the incubation and during the incubation period



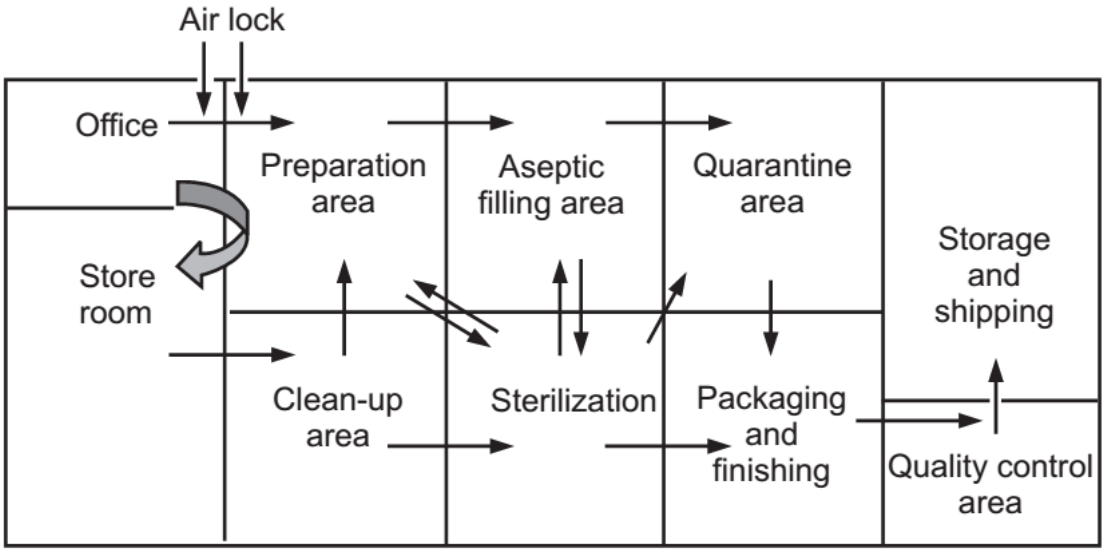
PHARMACEUTICAL MICROBIOLOGY (BP303T)
UNIT-4

ASEPTIC AREA:

Aseptic area is the area where strict control measures are adopted to avoid contamination of the preparations or any transfer of microorganisms. There are some differences between clean area, aseptic area and sterile area which are listed in Table:

	Clean	Aseptic	Sterile
Procedure space	On ward	Dedicated area	Dedicated room
Gloves	Clean	Sterile	Sterile surgical
Hand hygiene before the procedures	Routine	Antiseptic (Alcohol)	Surgical scrub, Iodophores
Skin antisepsis	No	Alcohol	Long acting agent
Sterile field	No	No	Yes
Sterile gown, mask, hand covers	No	No	Yes

Building Design, Construction and Production Facilities: Production for sterile products is carried out in a clean environment. The environment should be freed from quality microbes and dust particle contamination. The limit of contamination is essential for reduce the product contamination. The production area is having many other small areas like clean up area, compounding area, aseptic area, quarantine area and packaging area.



Floors, Walls and Ceilings: All the floors, walls and ceilings must be smooth, easy to clean, disinfected and be constructed to minimize microbial and particulate contamination. Flexing and non-flexing types of materials are used for construction of floor. Flexing materials are made up of synthetic elastromers like polyvinylchloride (PVC) are used for flooring which is easily repaired, cleaned, simple and cheap. Non-flexing flooring materials are made up of hard inorganic filler substances like concrete floor is properly sealed with chemical resistant materials, solvents and cleaning fluids.

Wall should be made up of non-inflammable materials like glass, stainless steel etc. For minimization of fungal growth, 1% 8-hydroxyquinolone or pentachlorophenol is added with the paint for painting the wall. Epoxy resin paint, polyurethane paints are applied on the wall that helps to reduce peeling of plaster and cracking of wall. The ceilings are sealed to prevent the entry of contaminants.

Doors, Windows and Other Services: Doors and windows are fitted much closed together with the wall. Windows are kept at minimum only for providing illumination; not for ventilation to minimize openings. Doors must be minimal, and well fitted by maintaining positive pressure air flow and self-closing. All pipes passing through the wall should be sealed properly and should be closely fitted. They should be cleaned easily. Gas cylinder should be avoided in the aseptic room. The gas connection should be through pipeline where cylinder be kept outside area. Sinks and drains must be avoided inside the aseptic room.

All lights are fitted with the ceiling or inside the ceiling and covered with glass so that to avoid deposition of dust particles, other contaminants and also to avoid the disturbance of the air flow pattern inside the room. All the switches of the lights should be outside the aseptic room.

Personnel and Protective Clothing: Contamination mainly comes from the skin surface of the operator of the room. Persons should be neat and clean and reliable to the selected work. They should be in good health, skilled for good manufacturing practices and free from any dermatological diseases otherwise it increases microbial load inside the aseptic area. Borne contamination can be controlled by limiting the more number of persons in clean area. They should also be skilled for aseptic techniques. They should wear sterile protective clothing like head wear, rubber or plastic gloves, non-fibre shedding face mask and foot wear. Cloths should be sterilized by moist heat sterilization or ethylene oxide sterilization. Eachtime when the persons are entering inside the aseptic room they should wear freshly sterile dresses.

Cleaning and Disinfection: Disinfection and cleaning procedures are used for the removal of microbes and other contaminations. Alkaline detergents, ionic and non-ionic surfactants are used as cleaning agents. Various types of disinfectants are used to prevent the development of the resistant strains of microorganisms. Different concentration of quats, sodium hypochloride, ethanol and formaldehyde solutions are used as disinfectants inside the aseptic room. Chlorhexidine in 70% alcohol is mainly used as skin disinfectant. Training is required for staff who works in clean room:

- ✓ They should know about microorganisms and controlling of microbial contamination.
- ✓ They should know the entry and exit rules inside the aseptic room.
- ✓ They should get personal hygiene training.
- ✓ They should know specific microbial risk associated with specific production tasks.
- ✓ Training must be effective and documented and regular wise review is required.

Air supply: In the aseptic room, the air supply should be filtered through high efficiency particulate air (HEPA) filter. The HEPA filter must be fitted at the inlet of the clean room and pre filter also is fitted upstream of the HEPA filters to increase the life span of the original filters. HEPA filters are used in the construction of vertical and horizontal laminar air flow bench which filters 99.97% of the dust particles against 0.3 μm particles. HEPA filters are tested for efficiency by hot DOP (di octyl phalate) (efficiency test), cold DOP and air flow resistance test. It is oil, used by air filter manufacturers and various testing agencies to make an aerosol to test the effectiveness of air filters. The DOP aerosol used to challenge HEPA filters to test for efficiency. The hot DOP aerosol has a very narrow particle size distribution and is used to produce a high concentration of 0.3 micron particles that considered the most penetrating of filter media. Cold DOP is having a broad particle size distribution aerosol which is useful for field testing for leaks and ensuring the integrity of an installation but does not provide the ultimate test of filter efficiency. The penetration or efficiency of a filter is strongly affected by the particle size of the challenge aerosol. A small change in particle size has significant effect on penetration. The smaller the particle has the lower efficiency until the maximum penetrating particle size is reached.

LAMINAR FLOW EQUIPMENTS: Laminar airflow is an enclosed bench cabinet. It isdesigned to prevent contamination of biological samples or any particle sensitive materials.

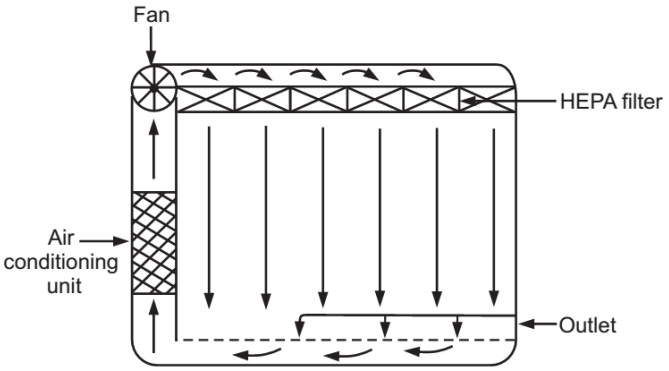
Principle: The air is passed through a HEPA filter which removes all airborne contamination to maintain sterile conditions.

Construction: It is low cost and does not require more space. The cabinet is made up of stainless steel without gaps or joints thereby preventing the build up of bacteria from collecting anywhere in the working zone. They are also known as clean benches because the air for the working environment is thoroughly cleaned by the precision filtration process. They consist of a filter pad, a fan, light with 100 lux and UV tube to disinfect the bacterial growth and a HEPA filter. The fan sucks the air through the filter pad to trap the dust particles. After that the pre-filtered air has to pass the HEPA filter where all the

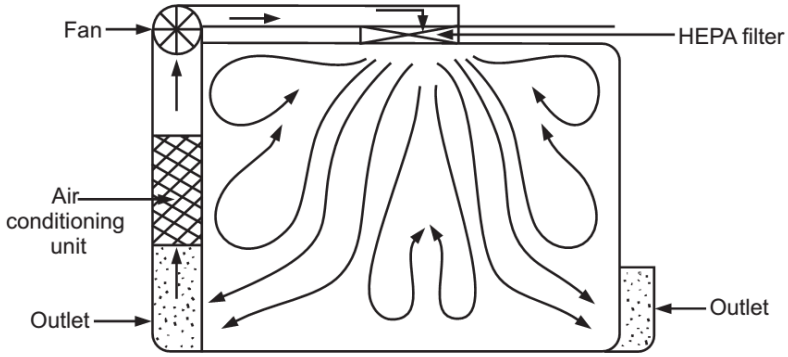
microorganisms are removed 99.97% of airborne particles 0.3 μm in diameter. Finally, sterile air flows into the working area without risk of contamination. The air flows at a speed of 100 ft/min and sweep dust particles making entire room free of particulate matter. This helps to perform work under aseptic condition.

Air Flow Pattern: Generally air flow pattern in the clean rooms are of three types viz. unidirectional air flow, non-unidirectional air flow and combined air flow. Unidirectional air flow is an air flow pattern in which essentially the whole body of the air within a confined area moves with uniform velocity and in single direction with parallel air streams that means it is an airflow where a fluid flows in one direction with no interruptions from turbulence. It should have uniformed airflow at a speed of ±15% and the speed of the air is measured as it exits the HEPA / ULPA filter.

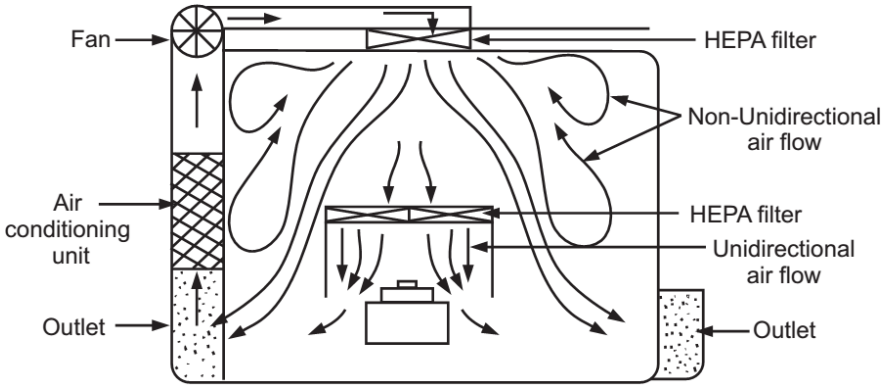
UNIDIRECTIONAL AIR FLOW



NON-UNIDIRECTIONAL AIR FLOW:



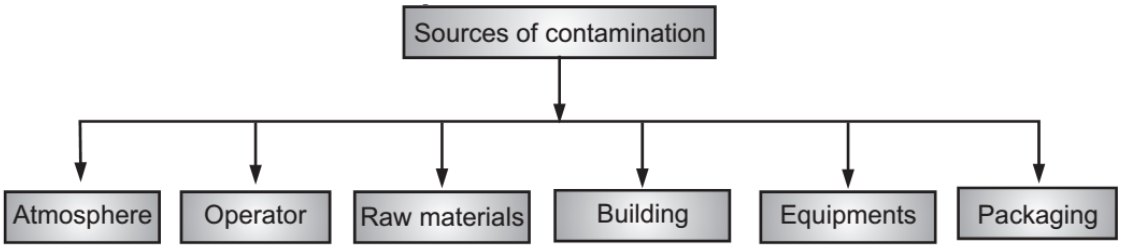
COMBINED AIR FLOW:



Advantages:

- ✓ The persons working in the area do not feel the movement of the air.
- ✓ The body of filters is made up of stainless steel which is not favorable for bacterial growth.
- ✓ The HEPA filter can reduce the contamination.

SOURCES OF CONTAMINATIONS:



CLEAN AREA CLASSIFICATION:

A clean room is a controlled environment that has a low level of pollutants such as dust, airborne microbes, aerosol particles, and chemical vapors. That means it has a controlled level of contamination, specified by the number of particles per cubic meter at a specified particle size. They are used in every industry for the safe manufacturing processes. They are constructed to minimize the introduction, generation and retention of air borne particles in the area.

CLASSIFICATION OF CLEAN AREAAS PER WHO:

Grade	Maximum permitted number of particles/m^3			
	At rest		In Operation	
	0.5 µm	5 µm	0.5 µm	5 µm
A	3520	20	3520	20
B	3520	29	352000	2900
C	352000	2900	3520000	29000
D	3520000	29000	---	----

AIR CLASSIFICATION: (AIR QUALITY CONTROL)

Clean Area Classification (0.5 µm particles/ft^3)	ISO Designation[b]	≥ 0.5 µm particles/m^3	Microbiological Active Air Action Levels[c] (cfu/m^3)	Microbiological Settling Plates Action Levels[c,d] (diam. 90 mm; cfu/4 hours)
100	5	3,520	1[e]	1[e]
1,000	6	35,200	7	3
10,000	7	352,000	10	5
100,000	8	3520,000	100	50

PRINCIPLES & METHODS OF DIFFERENT MICROBIOGICAL ASSAY:

Microbiological assay is the technique in which the potency or concentration of a compound is assessed by determines its effect on microorganism. It is a legal quality control requirement for the assay of a number of antibiotics in both the British Pharmacopoeia and United States Pharmacopoeia.

The microbiological assay of an antibiotic is based upon a comparison of the inhibition of growth of microorganisms by measured concentrations of the antibiotics under examination with that produced by known concentration of a standard preparation of the antibiotic having a known activity.

Microbiological assay of vitamins is a type of biological assay performed with the help of microorganisms.

Applications:

1. This method determines the potency.
2. The method controls antimicrobial chemotherapy.
3. Good in-vitro and in-vivo correlations are provided by this method.
4. This method is accurate, in expensive and convenient.
5. This method is easy to interpret results.

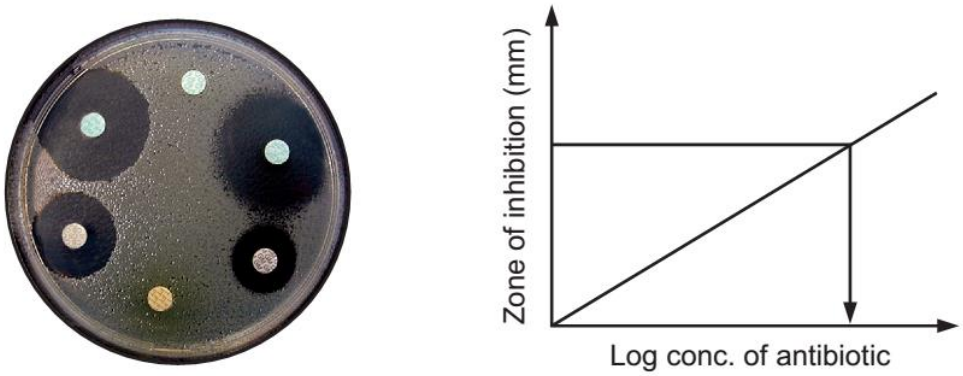
Two methods are used for the microbiological assay namely cylinder plate or cup plate method and tube assay method or turbidity method.

CYLINDER PLATE METHOD (CUP PLATE METHOD):

PRINCIPLE: This method depends on the diffusion of an antibiotic from a vertical cavity through the solidified agar layer in the petri plate. The growth of test microorganism is inhibited entirely in a zone around the cylinder containing antibiotic solution.

PROCEDURE:

- The nutrient agar is melted, cooled and poured into petri dish.
- 0.2 ml of known concentration of inoculum is spread on the surface of solidified agar by spread plate technique.
- Holes or cavities are made by sterile borer.
- 0.2, 0.4, 0.6, 0.8 and 1 ml of antibiotic is poured into the cup of agar plate and then incubated in 37°C for 24 hours.
- Zone of inhibition is observed for antibiotic that has antimicrobial activity.
- Zone of inhibition is measured by scale from the back side of the plate from the center of the cavity or hole.



CUP PLATE METHOD AND ZONE OF INHIBITION

TURBIDITY METHOD OR TUBE ASSAY METHOD:

The method is based on the correlation between turbidity and changes in the microbial cell number. The turbidity is measured in suitable liquid medium inoculated with microorganisms and finally standard optical density or turbidimetric curves are used to estimate the number of microbial cell.

Principle: This method depends on the inhibition of growth of microbial culture in a uniform solution of the antibiotic in the fluid medium that is favorable to its rapid growth in the absence of antibiotic.

Procedure:

- Five different concentrations of the standard solutions are prepared from the stock solution of standard as per step wise increased ratio 4 : 5.
- The concentration of media is selected and unknown substance solution is diluted to that concentration.
- 1 ml of each concentration of the standard solution as well as test sample solution in each of the tube is placed in duplicate.
- 9 ml of nutrient medium is added to each of the tube.
- Side by side three control samples are prepared. One contained the inoculated culture medium, another is blank (0.5 ml of dilute formaldehyde solution) and the third one contains uninoculated culture medium.
- All the tubes are placed in the incubator for 3-4 hours in 37°C and after incubation 0.5 ml of dilute formaldehyde is added in each tube.

- The growth of test microorganism is measured by determined the absorbance at about 530 nm of each of the solutions in the tubes against the blank.

Advantage: Shorter incubation period (3-4 hours) for the growth of the test organism.

Disadvantage: The presence of solvent residues inhibitory substances affects more in this assay. Further this method is not applicable for the turbid or cloudy preparation.

METHODS FOR STANDARDIZATION OF ANTIBIOTICS:

Antibiotic is a medical preparation, containing sufficient amount of chemical entity which is caused to produce naturally (by a microorganism) or by artificially (semi-synthetic way) and that possesses the inherent ability to either destroy (bactericidal) or inhibit (bacteriostatic) microorganisms in relatively dilute solutions.

There are mainly three important points are for standardization of antibiotics:

1) FDA (Food Drug Administration) regulations governing all aspects of antibiotics testing are completely detailed and are subject to periodic amendment.
2) FDA regulations need to be referred with regard to prescribed method for the assay of individual antibiotics and their preparations.
3) During the evaluation of potency of antibiotic substances, the actual and apparent measured effect is the degree of inhibition of the growth of a suitable strain of microorganism i.e. the prevention of the multiplication of the test organisms.

Two methods are usually applied, the cylinder-plate (or cup-plate) method and the turbidimetric method. The cylinder-plate method depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish. The growth of the added micro-organism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic. The turbidimetric method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.

TEST ORGANISMS FOR ASSAY OF ANTIBIOTICS:

Test organism	Antibiotics	*ATCC no.
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Gentamicin	*Styaphylococcus epidermidis*	12228
Kanamycin	*Bacillus pumilus*	14884
Neomycin	*Styaphylococcus epidermidis*	12228
Streptomycin	*Bacillus subtilis*	6633
Tetracycline	*Bacillus cereus*	11778

**American Type Culture Collection, 21301 Park Lawn Drive, Rockville, MD20852, USA*

METHODS FOR STANDARDIZATION OF VITAMINS:

Microbiological assay of vitamins is a biological assay method performed with the help of microorganisms. Vitamins and amino acids are the essential components for the growth of microorganisms. The basis of this assay is to measure the ability of test organism to utilize the substance being assayed under a proper nutritional condition. Some examples of microorganisms used for bioassay of vitamins are listed in Table:

Microorganism	Vitamin
Lactobacillus casei	Biotin, Folic acid, riboflavin
L. leichmannii	Cyanocobalamin
L. arabinosus	Nicotinic acid
L. viridans	Thiamine

MICROORGANISMS FOR VITAMINS ASSAY

Materials required: A stock solution, inoculum media and assay medium are required. Finally a standard curve is obtained.

Assay of Vitamin B12: Vitamin B12 is also known as cyanocobalamin. It is a water soluble vitamin. Its main sources are liver, eggs, milk, meat and fish. Vitamin B12deficiency causes Macrolytic anemia, Pernicious anemia.

Principle: The test organism selected is Lactobacillus liechmannii that is capable of utilizing free cyanocobalamin. Assay is performed by using either titrimetric or turbidimetric method.

Preparation of Standard Stock Solution: An accurately weighed amount of Cyanocobalamin reference standard is added to
sufficient 25% ethanol (resulting in a solution containing 1.0 µ g of cyanocobalamin per ml) and stored in refrigerator. Further dilutions of this stock solution (1 µg/ ml) are made by added 1 ml stock solution to 99 ml purified water (1 ml = 10 ng) and further added 1 ml of the above solution to 199 ml purified water (1 ml =0.05 ng).

Test Solution: Accurate amount of vitamin to be assayed is taken and dissolved in water, dilute HCl or NaOH is added to adjust pH at 6.0 and make up the volume with water up to the mark.

Inoculum Preparation: Transfer a loop full of Lactobacillus liechmannii from a recent sub-culture into two tubes each containing 10 ml of sterile culture medium.

Composition of culture media:

- Yeast extract - 0.75 gm
- Peptone - 0.75 gm
- Dextrose - 1 gm
- Potassium dihydrogen phosphate - 0.2 gm
- Tomato juice filtrate - 10 ml
- Sorbitan mono oleate solution - 1 ml
- Water up to - 100 ml

Media is incubated for 18 to 24 hours at 37oC. The culture is centrifuged and decanted the supernatant fluid, under aseptic condition. These cultured cells are suspended into 10 ml of sterile suspension of Basal medium stock solution and again centrifuged and decanted off supernatant fluid. The cells are uniformly suspended in 10 ml of sterile medium and then aseptically transferred 1 ml of the cell suspension to 10 ml of sterile medium and mixed.Finally, this resulting cell suspension is taken asinoculum.

Assay Procedure:

- In clean ten test tubes, added 0, 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5 ml of standard cyanocobalamin solution.
- To each tube added 5 ml of Basal medium solution and the volume of each is adjusted to 10 ml by water.
- In another four test tubes added 1, 2, 3, 4 ml of test solution which is to be assayed.
- To each of this also added 5 ml of Basal medium stock solution and adjusted volume to 10 ml with water.
- All test tubes are sterilized in autoclave at 121oC for 15 minutes after that cooled the test tubes at room temperature.
- Inoculate a drop of inoculum prepared of Lactobacillus liechmannii and incubated the test tubes for 64 to 72 hours at temperature range of 30 to 37oC.
- After incubation period titrated contents of each test tube with 0.05 N NaOH using bromothymol blue as indicator until green colour end point. Calculate the readings.
- The average of titration values of each level of both standard and test solutions are determined and plotted a graph considered average titration values (in ml) of 0.05 N NaOH against concentration of standard cyanocobalamin solution. A linear graph is obtained by interpolating the standard curve determine the concentration as activity per ml of vitamin B12

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METHODS FOR STANDARDIZATION OF AMINO ACIDS:

Amino acids are the basic building blocks of proteins. They constitute all proteinaceous material of the cell including the cytoskeleton and the protein component of enzymes, receptors, and signaling molecules and also used for the growth and maintenance of cells.

The amino acids are standardized by HPLC method. HPLC with pre-column derivatization is commonly used for the analysis of amino acids. Amino acid analysis is an important approach for the characterization of protein and peptide-based products.

ASSESSMENT OF A NEW ANTIBIOTICS:

Antibiotic Production: For production of new antibiotics there are few steps to be followed.

(i) Isolation or collection of culture.

(ii) Screening of cultures to detect antimicrobial activity.

(iii) Development of methods for submerged culture production.

(iv) Development of methods for isolation and purification of antibiotic.

(v) Determination of antibiotic properties like physical (adsorption, absorption) and chemical (reactions, solubility stability etc.)

(vi) Evaluation of antibiotics:

- Pharmacological test: To check the toxicity, activity at lower concentration, bacterial sensitivity etc.
- Antimicrobial activity: To check whether bacteriostatic or bactericidal.
- Comparison with existing antibiotic: To check the potency.

(vii) Development of pilot plant production method.

(viii) Submission of license for clinical trials.

(ix) Testing of purified antibiotics.

(x) Development of plant scale production method.

(xi) Obtaining a product license for clinical use.

(xii) Other considerations.

- Development of methods to control production of antibiotics.
- Development of new applications.
- Development of marketing and distribution system.
- Financing of business.

PHARMACEUTICAL MICROBIOLOGY (BP303T)
UNIT-5

INTRODUCTION: Spoilage is a complex reaction in which a combination of microbial and biochemical activities are interact. Legally they are known as spoilage.

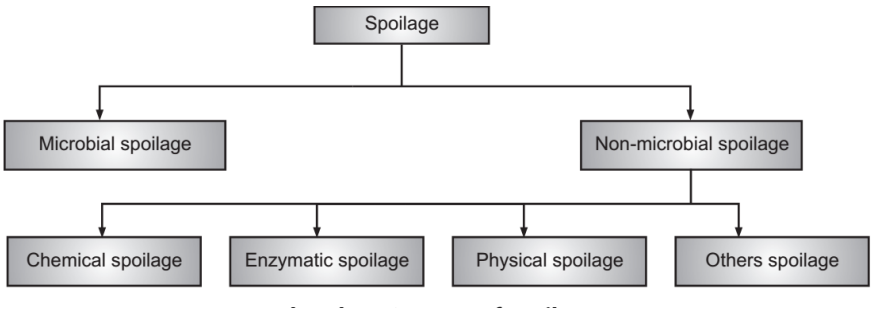
Spoilage is the term that indicates the goods that do not meet production standard. It is the method by which material or the amount of material is wasted or scrapped during the manufacturing process.

In another word, it is a form of substandard drug or food in which the quality or value or usefulness of the article is destroyed or impaired by the action of microorganisms like fungi or bacteria as to render the article unfit for human consumption.

TYPES OF SPOILAGE: Spoilages are mainly two types.

(A) Microbial spoilage

(B) Non-microbial spoilage



(A) Microbial spoilage:

It includes the contamination of Pharmaceutical products with the microbes that lead to spoilage of the product. It affects drug safety and quality, and is not intended for use. Hence, microbial spoilage is the deterioration of pharmaceutical products by the contaminant microbes such as bacteria, moulds, fungi and yeasts.

(B) Non-microbial spoilage:

These are the chemical reactions caused by foreign material in the foodstuff or by enzymes that occur in the foodstuff naturally resulting in rancidity, staling, discolouration, drying out or damage by insect.

Chemical Spoilage:

It is based on non-enzymatic chemical reaction occurred within the foods and resulted change in flavor.

Examples:

1. When trichloroanisol adds in bread than musty forms. (Mouldy, damp flavour)
2. When acetic acid or lactic acid adds in milk or vine than souring nature occurs.
3. When ethanol adds in fruits juice than alcoholic flavour occurs.
4. When 2- methoxy phenol chemical adds in juice or vine than medicinal flavour occurs.

Enzymatic Spoilage:

It is based on enzymatic reactions occurred in foods and changes in chemical nature and resulted rancidity. This rancidity occurred by two types of chemical reactions viz. hydrolytic and oxidative rancidity.

Examples:

1. When lipase adds in milk or oils than hydrolytic rancidity occurs.
2. When thiaminase adds in meat and fishes than thiamine destruction occurs.
3. Protease adds in egg than degradation of protein or shelf life reduction occurs.
4. Lipoxigenase adds in vegetables than destruction of vit A occurs.

Physical Spoilage:

It occurs due to temperature, light, relative humidity and results in mechanical damage of the food components.

Example: Oxidation of food occurs due to light and changes colour, flavour and chemical nature, like greening of potatoes, sunlight flavour in milk, loss of vitamin D, E etc.

Other Spoilage:

It occurs due to insects, rodents, birds, and otheranimals and resulting in changes in colour, odour, and chemical nature. Based on rate of spoilage, they are classified into three types like high perishable, semi-perishable and stable or non-perishable spoilage.

(i) High perishable: Meat, fish, poultry, eggs, milk, fruits and vegetables etc.

(ii) Semi-perishable: Potatoes, some apple varieties, nutmeats etc.

(iii) Stable or non-perishable: Sugar, flour, dry beans etc.

Spoilage of fruits and vegetables: Acid tolerant bacteria are responsible for the spoilage of fruits and vegetables such as Lactobacillus species.

Spoilage of cereals: Moisture content above 12 to 13 per cent causes spoilage of cereals due to the growth of molds, bacteria and yeasts. Molds grown in little moisture whereas in high moisture condition yeasts and bacteria grows. Aspergillus, Penicillium, Mucor, Rhizopus, Fusarium are some common molds that produces mycotoxins.

Spoilage of milk: Souring of milk is caused by Streptococcus lactis, Enterococci, Lactobacillietc. Proteolysis causes bitter taste of milk which is caused by Bacillus, Serratia, Pseudomonas etc.

Spoilage of meat: Raw meat is spoiled by microbial as well as enzymatic action. Excessive autolysis causes souring of meat.

Spoilage of fish: Fish is spoiled by autolysis, oxidation or by bacterial activity. Under anaerobic conditions, surface slime of fishes is caused by Pseudomonas, Streptococcus, Micrococci etc.

Spoilage of eggs: Spoilage of eggs is promoted by cracking, improper washing and storage. Egg yolk is good growth medium for microorganism due to the high nutrient. Molds that cause the spoilage of eggs are Penicillium, Mucor, Sporotrichum etc. Spoilage of eggs is also occurred by fungi.

Spoilage of canned foods: Canned foods are chemically spoiled due to hydrogen swells as a result discolouration of inside the can, cloudiness of liquors and that affects the nutritive value of the canned foods. Due to bacterial spoilage loss of nutritive value occurs by thermophilic and mesophilic bacteria.

FACTORS AFFECTING THE MICROBIAL SPOILAGE PHARMACEUTICAL PREPARATIONS:

Pharmaceutical products contain wide range of excipients in addition to active pharmaceutical ingredients for making the formulation.

In formulation process, microbial spoilage is required to be minimized which depends on many factors that are enlisted as:

1. **Types and size of contaminant inoculum.**
2. **Nutritional factors.**
3. **Water.**
4. **Storage temperature.**
5. **pH.**
6. **Oxidation-reduction balance (Redox potential).**
7. **Package design.**

1. **Types and size of contaminant inoculums:** Very low levels of contaminants which are unable to replicate in a product might not cause appreciable spoilage but, if a higher contaminant bio-burden occurs, the built in protection could be insufficient and spoilage occurs.

   This higher contamination arises if:

- Raw materials were unusually contaminated;
- A problem of the plant-cleaning protocol occurs;
- Biofilm detached itself from within supplying pipework;
- There was demolition or maintenance work in the manufacturing site
- Misuse of the product occuring during administration.

2. **Nutritional factors:** Many spoilage microorganisms have simple nutritional requirements and metabolic adaptability which enables them to utilize many formulation components as substrates for biosynthesis, growth and also trace materials contained in them. The use of animal products and crude vegetable materials in a formulation provides an additionally nutritious environment.
3. **Water:** It is the most important cause of the survival and growth of micro-organisms. Demineralized water prepared by ion-exchange methods, contains sufficient nutrients to allow significant growth of many water-borne Gram-negative bacteria like Pseudomonas spp.
4. **Storage temperature:** The actual storage temperature determines the spoilage by particular types of microorganisms. Spoilage of pharmaceuticals occurs potentially over the range of about 20°C to 60°C.
5. **pH:** Extremes of pH prevent microbial attack. They grow at neutral pH, therefore acidic or alkaline formulations are less susceptible to spoilage. pseudomonas and related Gram-negative bacteria growing in antacid mixtures, flavoured mouth washes and in distilled or demineralized water. Above pH 8, the spoilage is rare for soap-based emulsions.
6. **Oxidation-reduction balance (Redox potential):** The ability of microbes to grow in an environment is influenced by its oxidation reduction balance. The redox potential in viscous emulsion is high due to the high solubility of oxygen in most fats and oils.
7. **Package design:** For prevent spoilage, packaging such as strip foils are must be of water vapor-proof materials with fully efficient seals. Sacking, cardboard, card liners, corks and paper are unsuitable for packaging pharmaceuticals because they are heavily contaminated with bacterial or fungal spore. These are replaced by non-biodegradable plastic materials. In the past, packaging in hospitals is frequently re-used for economic reasons.

SOURCES & TYPES OF MICROBIAL CONTAMINATS:

In pharmaceutical formulations , many sources are available for microbial spoilage:

Atmosphere, Operators, Raw materials from which products are manufactured and Equipments.

Atmosphere: A poor HVAC (Heating ventilation and air-conditioning) system is a potential source of growth of microbes and a transportation mode for dispersing contaminants throughout the manufacturing facility.

Operators: The main reasons for the contamination from the operators includes mainly lack of training, Direct contact between the operator's hands and starting materials, primary packaging materials and intermediate or bulk product, Inadequate cleanliness, access of unauthorized operators into production, storage, and product control areas, malpractices like eating food, drinking beverages, or using tobacco in the storage and processing areas.

Raw materials: The raw materials used for production is a potential source of contamination. The main reasons for contamination from the raw materials includes storage and handling mistakes causing mix-ups or selection errors, Contamination with microorganisms or other chemicals, degradation fromexposure to excessive environmental conditions such as heat, cold, sunlight, moisture, etc., improper labeling, sampling and testing.

Equipments: The equipment used in processing, holding, transferring and packaging are the common source of pharmaceutical contamination. The main reasons for contamination includes, inappropriate design, size, material leading to corrosion and accumulation of static material, coolants, dirt, and sanitizing agents, improper cleaning and sanitization, design preventing proper cleaning and maintenance, improper calibration and irregular service, and, Deliberate use of defective equipment etc.

SOURCES OF CONTAMINANTS:

Air carrying dust: Dust is everywhere. Air is the carrier of the dust particles. Very small dust particles that present inside the aseptic room are the source of contamination. Hence, HEPA filter is used for reduction of dust particle inside the room.
Skin of the operators: Outer skin of the operators should smooth and covered properly. Any skin infections or rashes are the sources of microorganism growth inside the room.

Movement of persons inside the aseptic room: It is advisable that movement of persons inside the aseptic room should be restricted. It is evident that person walking inside the room liberate 5000 bacteria/minute.

Operators health: Persons those who are inside the aseptic room, should be healthy and free from any infections. A single sneeze will produce up to 1 million bacteria.

Manufacturing process: Manufacturing process generates contaminants. The main reasons for contamination during manufacturing process are lack of dedicated facilities to manufacture a single product, inappropriate cleaning in-between batches

to minimize the amount of product change overs, use of an open manufacturing system exposing the product to the immediate room environment, inappropriate zoning, lack of cleaning status labeling on all equipment and materials used within the manufacturing facility etc.

TYPES OF MICROBIAL CONTAMINANTS:

Microbial contamination is broadly classified in to direct contamination and cross contamination.

(a) Direct contamination: Contamination occurred by microbial components and poorly maintained heating, ventilation and air conditioning system.

(b) Cross contamination: It is the process by which microbes are spread indirectly from one to another through improper and unsterilized equipment.

Direct contamination is classified as follows:

(a) Direct Physical contamination: Examples: Particles, fibres, metal parts etc.

(b) Direct chemical contamination: Examples: Moisture, gases, vapors etc.

(c) Direct biological contamination: Example: Microorganisms like bacteria, viruses, molds, fungi etc.

Cross contamination is classified as follows:

(a) Physical cross contamination: Example: Leakage of oil seal from the reactor.

(b) Chemical cross contamination: Examples: Moisture content is increased when a product exposed to high relative humidity.

(c) Biological cross contamination: Example: Improper cleaning of equipment, unclean equipments.

ASSESSMENT OF MICROBIAL CONTAMINATION & SPOILAGE:

Early indications for microbial spoilage are gas production, pH changes, organoleptic changes like smell, sour, fishy amines, bad eggs, earthy taste, bitter etc.

Microbial polymerization of sugars and surfactant molecules can produce slimy, viscous, masses in syrups, shampoos and creams, and fungal growth in creams produce 'gritty' textures.

This contamination and spoilage is assessed by quality control tests.

Assessment of microbial contamination and spoilageis also known as microbial quality control. This includes laboratory applications like sterility testing, bioburden determination, air monitoring and product testing.

These methods are conducted to:

- Monitor microbial contamination of raw materials.
- Monitor and confirm the efficacy of sterilization.
- Control the risk from pathogenic microorganisms by confirming their absence.

It is possible that presence of the microorganisms can be detectable by:

- Their physical presence (cloudiness in liquid medicines, moulds on, or in, creams and syrups, or as discolouration of tablets stored in a damp environment).
- Changes in colour (pigment production).
- Smell (due, for example, to amines, acetic or other organic acids, or sulfides from protein breakdown).
- Gas accumulation without any obvious odour (bubbles of carbon dioxide following sugar fermentation).

Quality Control: The methods for counting and detecting some microbes in non-sterile products have poor accuracy and precision. For example, low number microorganisms sometimes damage the product and cannot be isolated.

Sterility Testing: Sterility testing of pharmaceutical articles is required during the sterilization validation process as well as for routine release testing. It is a very tedious and artful process that must be performed by trained and qualified laboratory personnel. Sterility testing is an essential part of sterilization validation.

Good Pharmaceutical Manufacturing Practice (GPMP): Quality Control (QC) is that part of GPMP dealing with specification, documentation and assessing conformance to specification. A high assurance of overall product quality is raised only from a detailed specification, control and monitoring of all the stages that contribute to the manufacturing process.

Quality Assurance (QA): It is a combined scheme of management which embraces all the procedures necessary to provide a high probability that a medicine will conform consistently to a specified description of quality.

The Preservative Challenge Test (Antimicrobial Effectiveness Test): It is required for assessment of the microbial preservation of multiple used cosmetics and pharmaceutical products. They are added to products to prevent or limit microbial contamination, which occurs during normal conditions of storage and use.

Post-market Surveillance: It is most important stage to follow up a medicine that is smooth floating in the market without any complain by the customers.

PRESERVATIVES:

- Preservatives are substances (natural or chemical) that are added to pharmaceutical products to prevent any kind of physical, chemical or biological changes.
- The term "preservative" means an antimicrobial component, the action of which is designed to extend the shelf life of medicines by destroying bacteria, yeast, and molds or inhibit considerably their growth and development.
- All chemical preservatives must be nontoxic and readily soluble, not impart off-flavors, exhibit antimicrobial properties over the pH range of the food, and be economical and practical.

Classification of preservatives:

Based on Mechanism of Action:

- Antioxidants
- Antimicrobial Agents
- Chelating Agents

Based on Source:

- Natural: Obtained through natural source. Example: Salt, Lemon etc
- Synthetic: Man made or chemically synthesized. Example: Benzoates etc

Antioxidants:

- They are self-reducing agents that oxidize themselves and prevent oxidation of the components that are sensitive to oxygen.
- They make an excellent guard against deterioration which considers two main factors oxygen and sunlight.
- Example: Vit C, Vit E, BHA (butylatedhydroxyanisole), BHT (Butylatedhydroxytoulene), Propyl gallate etc.

Antimicrobial agents:

- These agents act against microorganisms by affecting the various cellular portions of microbial cell and thereby inhibiting their growth. Cell wall, cytoplasmic membrane and cytoplasm are such portions of microbial cell wall that are mostly targeted by the preservatives.
- The factors that effect in choosing the antimicrobial preservative include: preservative dose, its effect on active ingredient and range of antimicrobial functionality.
- Examples are phenol, parabens, aryl and alkyl acids etc.

Chelating Agents:

- They term chelate derived from greek word "chele" which indicates "Crab's claw".

- In this the ligand molecule binds through donor groups and make a type of ring thus forming a complex with the pharmaceutical ingredient thus protecting it from any deterioration. Example EDTA, Citric acid etc.

Preservative		**Concentration in preparations (in %)**			
	Class	**Oral Liquid**	**Parenterals**	**Opthalmic/ Nasal**	**Ointments and creams**
Methyl Paraben	Amino aryl acid esters	0.25	0.01-0.5	0.1	0.001-0.2
Ethyl Paraben		0.1-0.25	0.01-0.5	0.1	0.001-0.2
Propyl Paraben		0.5-0.25	0.005-0.02	0.1	0.001-0.2
Butyl Paraben		0.1-0.4	0.015	0.1	0.001-0.2
Benzyl Alcohol	Alkyl/ aryl alcohols	3.0	0.5-10		
Chlorobutanol		0.5	0.25-0.5	0.5	0.5
Phenol	Phenols	0.1-0.5	0.065-0.02		0.25-0.5
Meta cresol		0.15-0.3	0.1-0.25		0.1-0.3
Chloro cresol		0.2	0.1-0.18		0.1-0.3
Benzoic acid	Alkyl/aryl	0.1-0.2			
Sorbic acid		0.1-0.2			
Thiomersal	Organic mercurials	0.1	0.01	0.01	0.01
Phenylmercuric nitrate		0.002-0.1	0.002	0.004	0.002
Bronopol		0.01-0.1			
Propylene Glycol	Diols	15-30			
Benzylkonium Chloride	Quatenary Ammonium Compounds	0.002-0.02	0.01	0.004-0.02	0.01
Benzethonium Chloride		0.01-0.02	0.01	0.004-0.01	0.01

Criteria for choosing antimicrobial preservatives include:

- Preservative dose
- Effects on the active ingredient
- Antimicrobial functionality

Common microbial preservatives:

- Phenol and Benzyl alcohol – Effective for peptide and protein products
- Phenoxyethanol – Vaccine preservation

A combination of propylparaben and methylparaben or benzyl alcohol is usually present in small volume parenteral formulations. It is required especially for multi-dose or semi-solid parenteral formulations.

Evaluation of preservatives:

- The evaluation of preservatives has traditionally involved time-consuming tests –Pharmacopoeial antimicrobial effectiveness tests (AET) or preservative efficacy tests (PET).
- Such tests involve challenging a product with a defined number of colony forming units (cfu) of a variety of test microorganisms (bacteria, yeasts and fungi), enumeration at time zero and then monitoring the kill / survival rate at defined time intervals up to 28-days.

Test organisms that are recommended by all of the pharmacopoeias include:

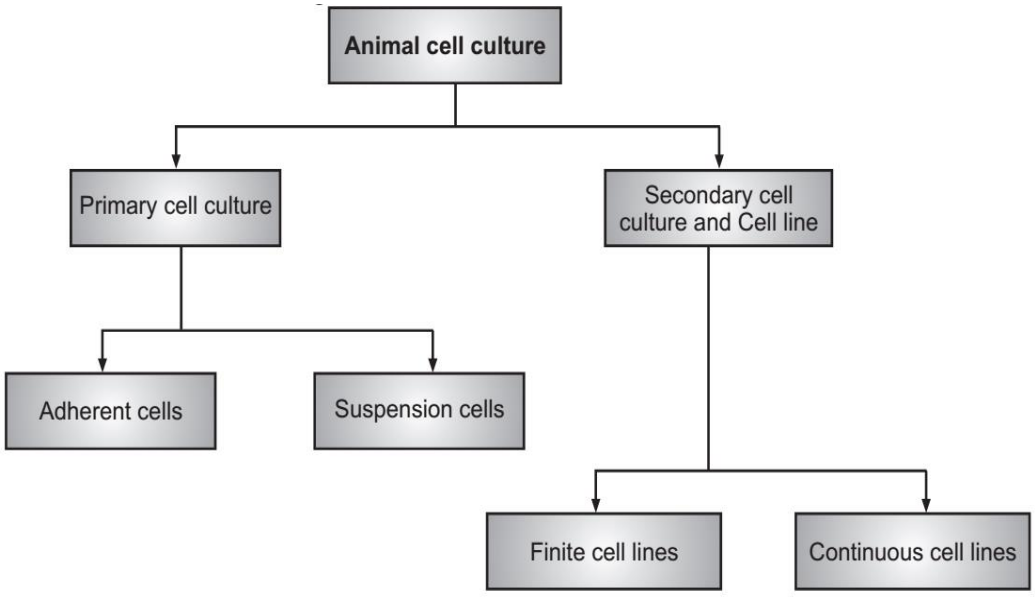
- ✓ Gram positive coccus, Staphylococcus aureus.
- ✓ Gram negative rod, Pseudomonas aeruginosa.
- ✓ Fungi / mould, Aspergillus niger.
- ✓ Yeast, Candida albicans.

GROWTH OF ANIMAL CELLS IN CULTURE:

Removal of cells from animals and their subsequent growth in a favorable artificial environment is animal cell culture. Animal cells are more difficult to culture than microorganisms because animal cells require many more nutrients and typically grow only when attached to specially coated surfaces.

The environment consists of a suitable glass or plastic culture vessel containing liquid or semisolid medium that supplies the nutrients essential for survival and extra proliferation.

TYPES OF ANIMAL CELL CULTURE:



Primary Cell Culture:
This cell culture is obtained from the cells of a host tissue. The cells dissociated from the parental tissue are grown on a suitable container and the culture thus obtained is called primary cell culture.

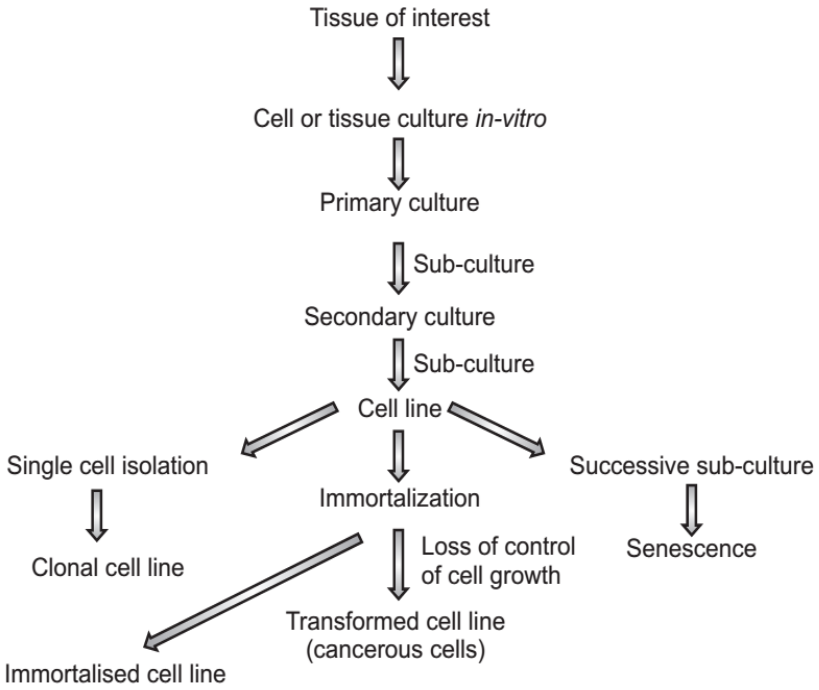
Adherent Cells:
These cells are propagated as a monolayer and are anchorage dependent. Monolayer cultures are defined as when the bottom of the culture vessel is covered with a continuous layer of cells of once need to be attached to a solid or semi-solid substrate for proliferation. They adhere to the culture vessel with the use of an extracellular matrix which is derived from tissues of organs that are immobile and embedded as connective tissue. For example: Fibroblasts and Epithelial cells.

Suspension Cells:
These types of cells do not attach to the surface of the culture vessels. Hence, they are also known as anchorage independent or non-adherent cells which are grown in liquid culture medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells are grown in suspension much faster which do not require frequent replacement of the medium. These cultures have short lag period.

Secondary Cell Culture and Cell Line:
Secondary culture is the culture when a primary culture is sub-cultured. It is also known as cell line or sub-clone. The process involves removing the growth media and disassociating the adhered cells by enzymatic treatment. Sub-culturing of primary cells to different divisions leads to the generation of cell lines.

Finite cell lines:
It is defined as the cell line that undergoes limited number of cell division with a limited life span. The cells passage several times and then lose their ability to proliferate, which is a genetically determined event known as senescence. Cell lines derived from primary cultures of normal cells are finite cell lines.

Continuous cell lines:
It is defined as a finite cell line which undergoes transformation and acquires the ability to divide indefinitely. This transformation occurs spontaneously or chemically or virally induced or from the establishment of cell cultures from malignant tissue. Prepared cell cultures are then sub-cultured and grown indefinitely as permanent cell lines. These cells are less adherent and fast growing as a result the cell density becomes higher and different in phenotypes from the original tissue. They grow more in suspension medium.



GENERAL PROCEDURE OF CELL CULTURE:

(a) Requirements: Vertical Laminar air flow, Incubator, Refrigerator,Microscope, Tissue culture ware.

(b) Temperature: The temperature sets at as the same as body temperature of the host from which cells are procured. Most animal cells required 36-37ºC.

(c) Substrate: Good compatible substrate is required for attachment and optimum growth. Glass and specially treated plastics are commonly used as substrate. Thereafter attachment factor such as collagen, gelatin, laminin etc. are used as substrate coating to improve growth and function of normal cells derived from brain, blood vessels, kidney, liver, skin etc.

(d) Culture medium: It is an important and complex factor for the cell growth. The culture medium is supplemented with various growth factors, pH and osmolality regulator and provides essential gases like oxygen and carbon dioxide. The medium is also supplemented with various nutrients like amino acids, vitamins, minerals and carbohydrates which are essential for growth of cells and provided energy for metabolism. Choice of media is used based on the cells being cultured. Generally, media like Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM), Glasgow Minimum Essential Medium (GMEM) are used for cell culture. Prepared media is filtered and incubated at 4ºC.

(e) Media and growth requirement: Temperature should maintain at 37°C and optimum pH is 7.2 to 7.5. The humidity is required to be maintained properly in the media with proper gas phase ratio (Bicarbonate concentration and carbon dioxide in equilibrium). For growth of cultured cells light intensity also plays a vital role. Inside environment cells are cultured in dark because light induced production of toxic compound. Commonly used antibiotics are penicillin,streptomycin, Kanamycin etc. Commonly used fungicides are Nystatin, Amphotericin B. Bulk ions like sodium, potassium, calcium, magnesium, chlorine, phosphorus, carbon dioxide, oxygen etc. Trace elements like iron, zinc, selenium, sugar, amino acids, vitamins, choline, inositol etc.

(f) Selection of organ: Different type of cells are grown in cultures including connective tissue elements such as fibroblasts, skeletal tissue, cardiac, epithelial tissue (liver, breast, skin, and kidney) and many different types of tumor cells. On the basis of morphology (shape and appearance) or on the functional characteristics of cells, they are divided into three types.

- Epithelial like - attached to a substrate and appears flattened and polygonal in shape.
- Lymphoblast like - cells do not attach remain in suspension with a spherical shape.
- Fibroblast like - cells attached to an substrate appears elongated and bipolar.

(g) Culturing of cell: Cells are cultured as anchorage dependent or independent. Cell lines derived from normal tissues are considered as anchorage-dependent which grows only on a suitable substrate e.g. tissue cells. Suspension cells are anchorage independent e.g. blood cells whereas Transformed cell lines either grows as monolayer or as suspension.

APPLICATION OF CELL CULTURE IN PHARMACEUTICAL AND RESEACH:

Cell culture is used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistryof cells (for example: metabolic studies, aging), the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis. It is also used in drug screening and large scale manufacturing of biological compounds (for example: vaccines, therapeutic proteins).

1. Model System: Cell culture is used as model system to study basiccell biology and biochemistry. It is also used to study the interaction between cell and disease causing agents like bacteria, virus. It helps to study the effect of drugs and to study triggers for ageing.

2. Cancer Research: Cell culture is one of the most important tools incancer research. The basic difference between normal cell and cancercell can be studied using animal cell culture technique. Normal cells are induced cancer cells by using radiation, chemicals, viruses and then cause of cancer is studied. Cell culture is also used to determine the effective drugs for selectively destroy only cancer cells.

3. Virology: Animal cell cultures are used to replicate the viruses instead of animals for the production of vaccine. Cell culture can also beused to detect and isolate viruses, and also to study growth and development cycle of viruses. It is also used to study the mode of infection. This technique is also used for production of human and veterinary viral vaccine against a variety of diseases.

4. Toxicity Testing: Animal cell culture is used to study the effects of new drugs, cosmetics and chemicals and growth of multiple cells, especially liver and kidney cells. This technique is also used to determine themaximum permissible dosage of new drugs.

5. Vaccine Production: Cultured animal cells are used for virus production and these viruses are used to produce vaccines. For example: Vaccines like polio, rabies, chicken pox, measles and hepatitis B are produced using animal cell culture. The polio vaccine produced in 1954 was first human vaccine is produced using large scale cell culture techniques.

6. Genetically Engineered Protein: Animal cell cultures are used to produce commercially important genetically engineered proteins such as monoclonal antibodies, insulin, hormones etc. Proteins extracted from biological sources are important for substitution therapy. Interferone is discovered in 1957 by cell cultured method by viral infection. Tissue plasminogen activator is produced in large scale from transferred CHO-K1 cells. It is used to prevent fibrin clots in the blood stream. Blood clotting factor VIII is produced in large scale by Bayer through transfection of the kidney cell line with an appropriate gene.

7. Replacement Tissue or Organ: Animal cell culture is used as replacement tissue or organs. For example: artificial skin is produced totreat patients with burns and ulcers. Recently artificial organ culture such as liver, kidney and pancreas are successfully carried out for transplantation. Organ culture techniques and research are also conducted on both embryonic and adult stem cell culture. These cells have the capacity to differentiate into many different typesof cells and organs.

8. Genetic Counseling: Fetal cell culture extracted from pregnant women is used to study or examine the abnormalities of chromosomes, genes using karyotyping and these findings are used in early detection of fetaldisorders.

9. Genetic Engineering: Cultured animal cells are used to introduce new genetic material like DNA or RNA into the cell. This methodis used to study the expression of new genes and its effect on the health of the cell.Insect cells are used to produce commercially important proteins by infecting them with genetically altered baculoviruses. With this method mass produced insulin, human growth hormone, human albumin, vaccines etc.

10. Gene Therapy: Cultured animal cells are genetically altered and are used in gene therapy technique. First cells are removed from the patient lacking a functional gene or missing a functional gene. These genes are replaced by functional genes and altered cells are culture and grown in laboratory condition and are introduced into the patient. Functional gene is inserted into the genome of viral vector and then they are allowed to infect the patient and the missing gene will be expressed with the help of the viral vector.

11. Drug Screening and Development: Animal cell cultures are used to study the cytotoxicity of new drug. This is also used to findout the effective and safe dosage of new drugs. Cell-based assay plays an important role in pharmaceutical industry. Originally these cell culture tests were done in 96well plates but recently these tests are conducted in 384 and 1536 well plates.