Unit-2

Pharmaceutical Microbiology

Unit II **10 Hours**

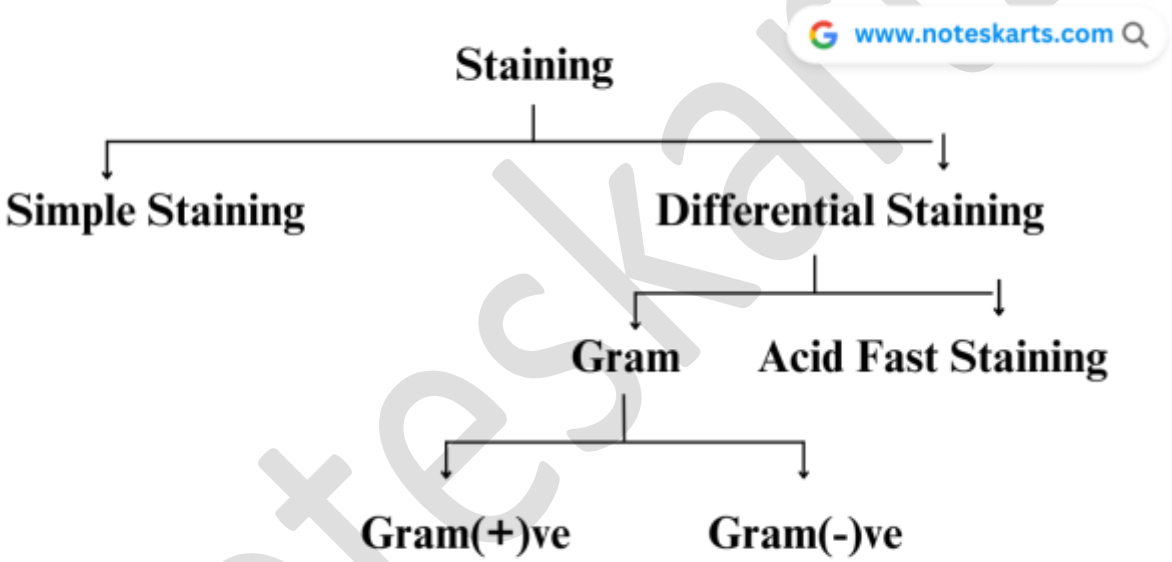
- Identification of bacteria using staining techniques (simple, Gram's &Acid fast staining) and biochemical tests (IMViC).
- Study of principle, procedure, merits, demerits and applications of physical, chemical gaseous, radiation and mechanical method of sterilization.
- Evaluation of the efficiency of sterilization methods.
- Equipment employed in large scale sterilization.
- Sterility indicators.

Staining Techniques:

- Staining techniques are used to enhance the contrast of microorganisms so that they can be visualized under a light microscope.
- Bacteria are typically colorless and transparent, making them difficult to see without staining.

Type of staining:

There are many different types of staining techniques,



Simple staining:

- In simple staining is perform to the morphological characteristic by comparing the shape and size of bacteria.
- In simple staining different basic dye solution are used such as crystal violet, safranin, methylene blue.

Principle

- Simple staining is a microscopic staining technique that uses a single dye to color all bacterial cells on a slide the same color.
- This technique is based on the principle of electrostatic attraction, whereby positively charged dye molecules are attracted to and bind to negatively charged components of bacterial cells, such as nucleic acids and cell walls.

Procedure

The following is a general procedure for simple staining:

- Prepare a smear of the bacteria to be stained on a clean glass microscope slide.
- Allow the smear to air dry completely.
- Fix the smear by passing it through a flame or by immersing it in a fixing solution.
- Flood the smear with the stain and allow it to sit for 1-2 minutes.
- Gently rinse the slide with water to remove excess stain.
- Blot the slide dry with filter paper.
- View the slide under a microscope.

Applications

Simple staining is a simple and rapid technique that is used in a variety of applications, including:

- **Microbiology:** Simple staining is used to visualize bacteria in clinical samples, such as stool, urine, and sputum. It can also be used to identify bacteria in food and water samples.
- **Mycology:** Simple staining can be used to visualize fungi in clinical samples, such as skin scrapings and nail clippings.
- **Parasitology:** Simple staining can be used to visualize parasites in clinical samples, such as blood and stool.
- **Histology:** Simple staining can be used to visualize tissues and cells in histological sections.

Gram's staining:

- On the basis of there structure bacteria is classified into two class gram +ve and –ve.
- The differential staining technique by which gran +ve and –ve bacteria is identify is called gram staining.
- This staining technique was developed by Han's Christian gram in 1884.

S.No	Gram +ve	Gram –Ve
1.	In this peptidoglycan is present multilayer	Single layer
2.	In this outer membrane is absent	Outer membrane is present
3.	Cell wall 20-30nm thick and single layer	Cell wall 5-12nm thick and two layer.
4.	After gram staining it gives purple/blue color.	It gives Red/Pink color.
5.	Eg. Streptococcus, bacillus etc.	Eg. Eschercheia coli

Procedure

The Gram staining procedure can be summarized in the following steps:

1. **Prepare a smear of the sample:** A small amount of the sample is spread on a clean glass slide and allowed to air dry.

2. **Heat-fix the smear:** The smear is passed through a flame to heat-fix the bacteria to the slide.
3. **Apply the crystal violet stain:** The crystal violet stain is applied to the smear for 1 minute.
4. **Rinse with water:** The smear is rinsed with water to remove excess crystal violet stain.
5. **Apply Gram's iodine:** Gram's iodine is applied to the smear for 1 minute.
6. **Rinse with water:** The smear is rinsed with water to remove excess Gram's iodine.
7. **Decolorize the smear:** The smear is decolorized with ethanol or acetone for 10-20 seconds.
8. **Rinse with water:** The smear is rinsed with water to remove excess decolorizer.
9. **Apply the safranin counterstain:** The safranin counterstain is applied to the smear for 1 minute.
10. **Rinse with water:** The smear is rinsed with water to remove excess safranin counterstain.
11. **Air dry the smear:** The smear is allowed to air dry.
12. **Observe the smear under the microscope:** The smear is examined under a microscope with oil immersion objective.

Applications

Gram staining is a versatile technique with a wide range of applications in microbiology. It is used to:

- Identify bacteria in clinical samples, such as sputum, urine, and blood cultures.
- Diagnose bacterial infections.
- Classify bacteria into different groups based on their cell wall structure.
- Monitor the effectiveness of antibiotic treatment.
- Perform quality control checks on food and water samples.

Acid Fast Staining:

- The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups.
- This method is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus Mycobacterium, are resistant and can only be visualized by acid-fast staining.
- It is the differential staining techniques which was first developed by Ziehl and later on modified by Neelsen. So this method is also called Ziehl-Neelsen staining techniques.
- Neelsen in 1883 used Ziehl's carbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue. Thus Ziehl-Neelsen staining techniques was developed.

Principle:

- Acid-fast staining is a microbiological technique used to differentiate acid-fast bacteria from non-acid-fast bacteria based on their cell wall characteristics.
- This staining method relies on the property of certain bacteria to resist decolorization by acid-alcohol (a mixture of hydrochloric acid and ethanol) due to the unique composition of their cell walls.

Summary of Acid-Fast Stain:

Application of	Reagent	Acid fast	Non-acid fast
Primary dye	Carbon fuchsine	Red	Red
Decolorizer	Acid alcohol	Red	Colorless
Counter stain	Methylene blue	Red	Blue

Procedure:

- Firstly take glass slide, cover slip, inculation loop, Culture media (Bacteria present), Microscope.
- Now wash all these with ethanol solution then allow for drying after drying put all these under flaming for complete sterilization.
- Now take inoculation loop, streak into culture media in which bacteria attached on loop, then streak inoculation loop on glass slide and make smear on it.
- Now add few Carbon fuchsine dry (primary dry) on surface of bacteria and allow for dry.
- Then wash it with alcohol.
- Then further and some methylene blue (Counter stain) (Non-acid fast attached methylene blue and colored as blue but acid fast not get it because it is already colorized (red))
- Again wash the slide with water then dry and observe it by seeing it on microscope.

Observation:

- If bacteria give **Red/Pink color** it acid fast.
- If bacteria give **blue color** it non-acid fast.

IMViC tests:

- IMViC is a series of four biochemical tests used in microbiology to identify and differentiate certain groups of bacteria, primarily Enterobacteriaceae, which are a family of Gram-negative bacteria commonly found in the intestinal tract of humans and animals.
- The IMViC tests are essential for the identification and classification of these bacteria, as they help distinguish between different species and strains within this family.

IMViC is an acronym that stands for:

1. Indole Test
2. Methyl Red Test
3. Voges-Proskauer Test
4. Citrate Utilization Test

Indole Test:

- Many bacterial species which possess enzyme tryptophanase.
- Tryptophanase degrade amino acid tryptophan to indole, Pyruvic acid and ammonia.

Tryptophane → Indole + Pyruvic acid + ammonia

- Inoculate the test microorganism into peptone water for check indole production, incubating at 37^{o}C for48-96 hours. (2-4 Days)
- Then and 0.5ml of Kovac's reagent and mix gently.
- A red colour layer formed which Indicates that positive reaction (Production of indole).
- E.coli give Indole test positive, so it identified that E.coli present, if bacteria give indole test positive.

Methyl Red Test:

- This test is performed to detect the production of acid during the fermentation of glucose.
- Due to production of acid, pH of the medium falls and it maintained below 4.5.
- Inoculate the test micro-organism in glucose phosphate both then incubate at 37^{o}C for 2-5 Days.
- Then add few drops of 0.04% solution of methyl red, mix and abserb the result in the form of change in color.
- Red colour signifies positive (MR test)
- Yellow Colour signifies negative.
- If test is Positive then E.Coli bacteria.
- If negative then E. Aerogens bacteria.

Voges-Proskauer test (VP test)

Objective:

- To check whether the microorganism follow 2,3 butanediol production pathway for glucose metabolism or not

Principle of VP test:

- The basic principle for Voges-Proskauer test is to determine the ability of some microorganisms to produce a neutral end product 2,3 butanediol from glucose fermentation.
- Those microorganisms which follows 2,3 butanediol formation pathway give positive VP test and negative MR test. 2,3 butanediol is a neutral end product of glucose fermentation pathway which does not change the pH of media so color of methyl red indicator does not changes and remain yellow.
- The VP test is given by the intermediate product ie Acetion formed just before butanediol in the pathway. Acetion reacts with VP reagent (α-napthol + KOH) to give pinkish red complex, which indicates positive test.
- The reagent α-naptholl is known as VP-I/ Barrit's reagent A and the reagent 40% KOH is known as VP-II/ Barrit's reagent B.

Requirements:

- 24 hours Culture of E.*coli* and *Klebsiella* spp
- α-napthol
- Potassium hydroxide (40% KOH)
- Absolute alcohol
- Deionized water

Procedure for VP test:

1. Prepare MRVP broth or Clark and Lub's media in test tubes
2. Inoculate the broth aseptically 2 loopful of respective bacterial culture
3. Label the test tubes with name of organism inoculated
4. Incubate the test tubes at 37°C for 48-72 hours.
5. Add Barrit's reagent A (α-napthol) and Barrit's reagent B (40% KOH) in the ratio 3: 1
6. Remove the cotton plug and shake the tubes for aeration.
7. Observe the results after 10-15 minutes

Results:

VP positive: Pinkish red color at the surface (*Klebsiella* spp)

- VP positive organisms are: *Klebsiella, Serratia marcescens, Hafnia alvei,*Viridans group streptococci (except *Streptococcus vestibularis*), *Listeria, Enterobacter, Vibrio eltor, Vibrio alginolyticus*, etc.

VP negative: whitish yellow (*E.coli*)

- VP negative organisms are: *E. coli, Streptococcus mitis, Citrobacter sp., Shigella, Yersinia, Edwardsiella, Salmonella, Vibrio furnissii, Vibrio fluvialis, Vibrio vulnificus, and Vibrio parahaemolyticus*

** Both MR and VP positive organisms: *Hafnia* spp, *Proteus* spp, *Serratia* spp

Precautions:

- The culture must be incubated nit less than 48 hours
- The reagents should be used in suitable amount ie. VP-I and VP-II in ratio 3:1
- Aeration must be maintained by opening cotton plug and shaking the tubes
- Observe the color change carefully.

Citrate Utilization Test:

- In this test check the use of citrate as a carbon source for growth.
- Koser's cetrate medium has citrate as the source of carbon.
- Ability of micro-organism to use citrate as the carbon source is indicated by the production of turbidity in the medium.
- So finally if organism have ability to use citrate as carbon source then it give positive test if not then negative.
  - E. aerogen gives positive
  - E. Coli gives negative

- Indole, MR, VP and citrate tests are done in routine for the classification of Gram Negative enteric bacteria.

Study of principle, procedure, merits, demerits and applications of physical, chemical gaseous, radiation and mechanical method of sterilization.

Sterilization:

- Sterilization is a process that kills or inactivates all forms of microbial life, including their spores.
- It is used in a variety of settings, including hospitals, laboratories, and food processing plants, to prevent the spread of infection.

Types of Sterilization:

- Physical Method
- Chemical Method
- Radiation Method
- Mechanical Method

Physical Method:

This method is used for phase subs which are thermostable and these products which are heat sensitive. They are not sterilized by heating method.

Heat sterilization: Heat is the most common method of sterilization.

- **Dry heat sterilization** is typically done in an oven at a temperature of 170°C (338°F) for at least two hours.
    - Eg: Hot air Oven
- **Wet heat sterilization** is typically done in an autoclave at a temperature of 121°C (250°F) with a pressure of 15 psi for at least 15 minutes.
    - Eg: Auto clave

Dry heat sterilization:

- In this method the material is kept under the flame or heat and after longer exposer micro-organism bacteria and viruses are kill.
- This method is used for glass wares, oils, powders, metal instruments and other items unrapped in paper.
- The temp and time is based on their thermal deathtime (TDT).

Hot Air Oven:

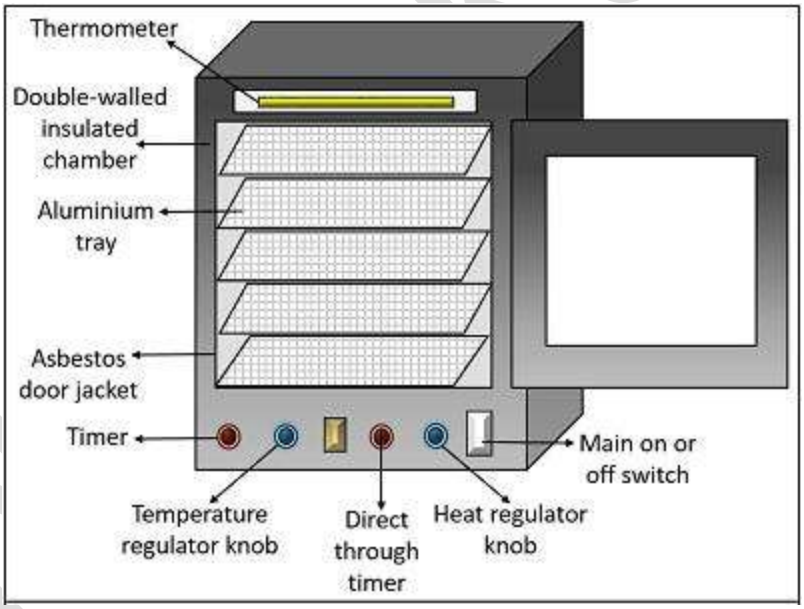
Principle:

- Hot Air Oven is work on the principle of bacteria are denatured and oxidised and the bacteria kills.

Construction:

Physically it appears similar to Incubator.

- **Cabinet:** The cabinet body is made is up of stainless steel. The cabinet is coupled with insulator that does not allow heat to escape and hence maintains the temperature.
- **Shelves:** The cabinet space is divided by the shelves, which provide space for placing the microbial culture.
- **Door:** The Incubator looks like small cupboard, which has insulated door with handle. The Door is air tight because of asbestos gasket that allows keeping heat trapped inside the incubator.
- **Thermostat:** It plays important role in maintaining the desired temperature. Thermostat contains the censor that senses the temperature and maintain as per the setting.
- **Control panel:** The control panel is placed on the front side of the cabinet, just above the door. The control panel contains switches for all the parameters. We can set the parameter as per our need.



Application of Hot Air Oven

It is used for the sterilization of heat resistant material like glasswares, forceps, scalpel, scissors, syringes etc.

Advantages:

1. It is Eco-friendly
2. It is most efficient method to degrade microbial endotoxins.

3. It is safer than autoclave.
4. It is the ideal instrument for sterilizing oil and powders.

Disadvantages:

1. It is time consuming because the dry heat penetrate slowly as compared to moist heat.
2. It may not be efficient to degrade prions.
3. The heat sensitive or heat labile material cannot be sterilized.
4. It cannot be operated without electricity.

Moist Heat Sterilization:

- Moist heat is more effective then dry heat sterilization. It kills the micro-organism of enzyme and protein.
- It is more and effective than dry heat sterilization.
- Moist heat sterilization is divided into three form.
- Temp below 100oC (Posterization, Serum bath, vaccine bath)
- Temp at 100oC (Boiling, Tyndallization)
- Temp above 100oC (Autoclave)

Autoclave:

Principle:

- It is based on the principle steam at high pressure.
- The high pressure is used to kill the micro-organism including spares.
- It is the best and most widely used method of sterilization.

Construction:

- It consist of a vertical cylinder of gun metal or stainless steel in a supporting frame or case.

1. Vessel/Pressure chamber –

- The vessel is made from stainless steel.
- The inner chamber is protected by outer jacket. The inner chamber is the place where we keep the autoclavable material for sterilization.
- The size of the chamber varies and selected based on the motive of use.

2. Lid/Door –

- The Vessel mouth is covered by lid or door. It is also made from stainless steel.

- The lid allows trapping and retaining the heat and pressure inside the chamber and producing favorable environment for sterilization.
- The lid is tightly closed with the help of airtight screw.

3. Pressure Gauge –

- It is present on the upper surface of lid. Its function is to indicate the level of pressure that is produced during autoclaving.
- It is vital part because it allows us to visually see the rise of pressure and alert for any forthcoming mishap hence it ensures the safety.

4. Pressure releasing unit/whistle –

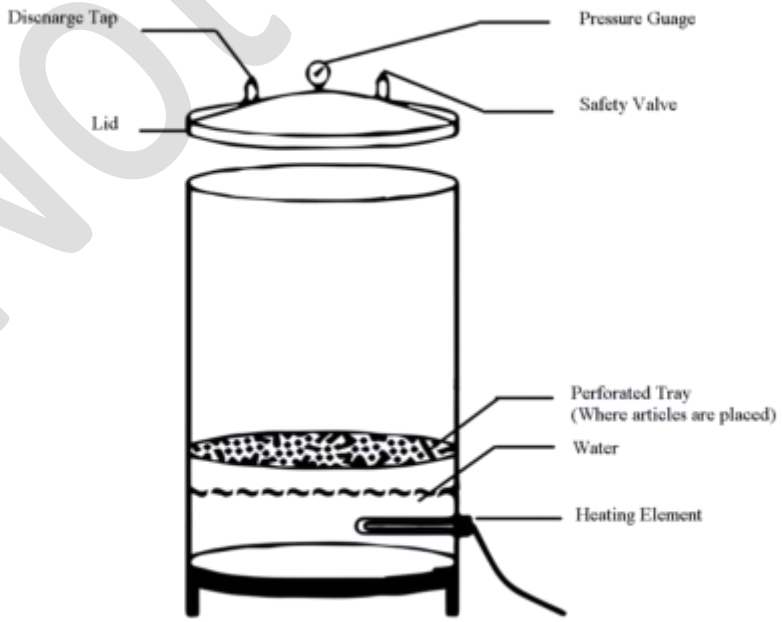
- The whistle is placed on top of the surface of the lid, just like pressure cooker.
- The whistle allows us to release the pressure whenever required.

5. Safety Valve:

- It is present on the surface of the lid.
- Their function is to avoid catastrophic accident especially when pressure inside the chamber is uncontrollable.

6. Heater –

- The electric heater is placed beneath the chamber.
- The electric heater working principle is similar to geezer. The electric heater start heating it causes boiling of water.
- The user need to maintained the water level as per the marking. Less water may cause burning and more water may lead to enter water in the experimental material.



Procedure

- Sufficient amount of water is placed inside the autoclave.
- Pack the material properly before putting inside the autoclave for sterilization.
- The steam outlet is kept open till air from inside autoclave has been evacuated and then close the steam outlet.
- The pressure is allowed to remain at 15 LBS per square inch for 15 to 30 minute is done by controlling the steam.
- Now, off the plug leave the autoclave for cooling down and thus the pressure is reached down to zero mark.
- Then open the lid and take out the materials.

Applications:

It is used for sterilization of media, glasswares, solutions and heat resistant numerous laboratory accessories. It is also used to decontaminate the media before discarding.

Chemical method of Sterilization:

When sterilization process is perform by using certain chemicals is called chemical method of sterilization.

In chemical method different gas and liquid are used to kill the micro-organism.

Chemicals can act as disinfectants to destroy pathogenic bacteria from the upper surface.

- **Liquid-** chemical method of sterilization involves the application of liquid to destroy the microbes permanently.
- **Alcohols**– usually, 70% of alcohols are used as a chemical to kill bacteria. Methyl alcohol, isopropyl alcohol, and ethyl alcohol are some important chemicals used in this method.
- **Aldehydes**– About 40% formaldehyde solution is used as surface disinfection. Formaldehyde and Glutaraldehyde are some of the best aldehydes used in this process. Similarly, 50% phenol can be used.
- **Halogens** – chlorination can impact the bacteria directly. The blend of iodine compounds and chlorine compounds can act as an antiseptic. Chlorine compounds are hydrochloride, chlorine bleach and iodine compounds are tincture, iodine, and iodophors.
- **Heavy Metals**– Not just chemicals, some heavy metals can be effectively used in the sterilization process. Heavy metals such as copper sulfate, mercuric salts, silver nitrate, mercuric chloride are used in the sterilization method. Similarly, dyes like aminacrine, acriflavine, acridine dyes are used to interact with bacterial nucleic acids.
- **Gaseous-** Gas such as formaldehyde and ethylene oxide are effective in killing bacterial spores.

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.

Advantage:

- It is very light weight and not more expensive.
- It sterilized all places even small places like holes etc.

Disadvantage:

- More Expensive
- Need trained person.

Application:

- Used for sterilization of medical and biological preparations.
- Used for sterilization of heat sensitive substance.

Radiation Method of Sterilization:

Radiation method involves the application of radiation on the substance.

- **Non-Ionizing Rays**– since non-ionizing rays are of low energy and have poor penetration power. The wavelength of ultraviolet rays lies between 260nm and 280nm. They are exposed on the substance to remove bacteria and microorganism.
- **Ionizing Rays**– Unlike Non-Ionizing rays, ionizing radiations come with good penetration power, thus it can be used to remove bacterial spores.

Sonic and Ultrasonic Vibrations

Ultrasonic waves are sound waves of a high frequency that is inaudible to the human ear. Hence, it can be used to kills virus and bacteria. The same way, sonic vibrations are used.

Electromagnetic Radiation

In this process, the cathode material is used for accelerating high-speed electrons. The electromagnetic rays are produced to kills all forms of the virus, bacteria, fungi, bacterial spores etc. This type of killing bacteria by exposing radiation is called cold sterilization.

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:

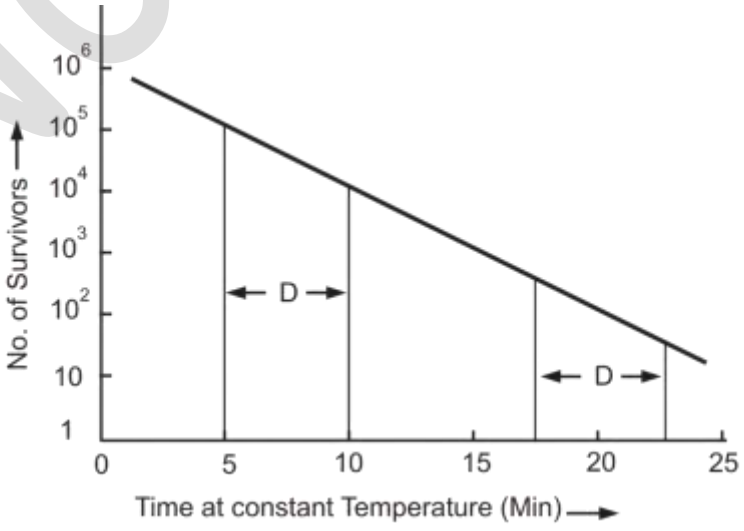
- UV light is the most lethal component in ordinary sun light used in sanitation of cloths and utensils.
- Gamma rays are used to sterilize antibiotics, hormones, sutures, plastics etc.

Evaluation of the Efficiency of Sterilization Methods:

- 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.
- Evaluation can be determine by following three value:
    - D-Value
    - Z-Value
    - F-Value

D-Value:

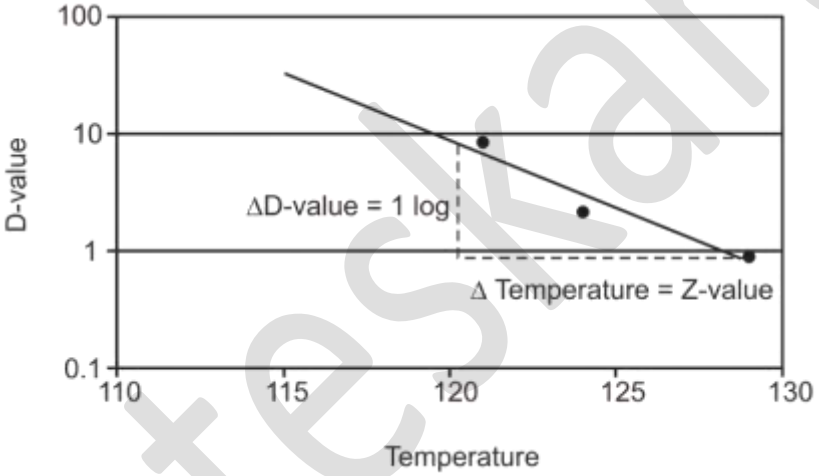
- It determines the time required to reduce the microbial population by one decimal point i.e. it is the time required for 90% reduction in the microbial population.
- Hence, the time or dose it takes to reduce thousand microbial cells to hundred cells is the D value.
- It is the rate of killing of microorganism.



- If the value of D less then efficiency of sterilization very effective, because D value is time required for destroy micro-organism, So if in less time (D Value) more bacteria kill then sterilization very efficient.

Z value:

- 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.
- The accepted standard (Z value) for steam sterilization of Bacillus stearothermophilus spores and dried heat sterilization for Bacillus subtilis are 10°C and 22°C respectively.
- These plots are important because one can determine D value of the indicator microorganism at any temperature of interest.



- The magnitude of slope indicates the relative degree of lethality as temperature is increased or decreased

F value:

- The time required in heating method of sterilization to kill the population of bacterial spores in minutes is called F value.
- The F value can be used to calculate the probable number of survivors remaining in a load as:-

F= D (log No - logN)

Where,

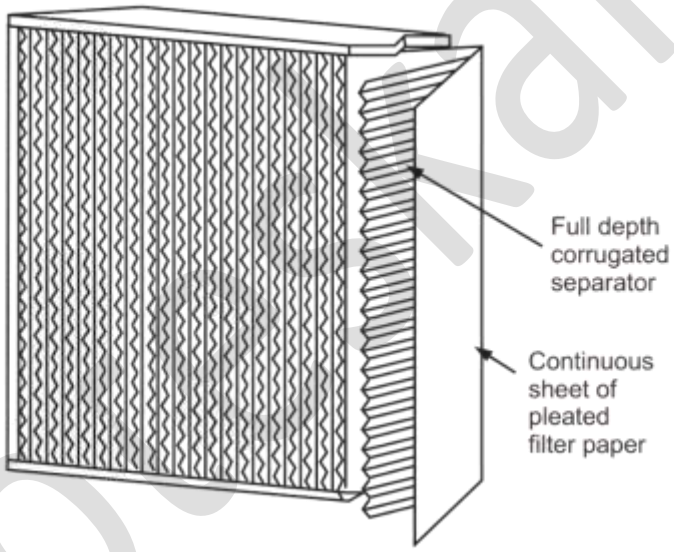
- D= D-Value at 121^{oC} of the organism.
- No= initial population number/unit volume
- N= Final Population number/unit volume

Equipments employed in large scale sterilization.

- Autoclave
- Hot Air Oven
- Microwave
- Air Filter

Air 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.



Sterility indicators:

- Sterility Testing is defined as a test that confirms that the products are free from the presence of viable microorganisms. It is very important for medical devices, pharmaceuticals, preparations, tissue materials and other materials that claim to be free from viable microorganisms.
- Sterility testing for products are mainly carried out by direct inoculation and by membrane filtration.
- In direct inoculation method, the test article is directly inoculated into two types of media to allow for the detection of both aerobic and anaerobic microorganisms.
- Then after inoculation the media is incubated for 14 days and finally observed for any microbial contamination.

- In membrane filtration method, the sterile enclosed units are allowed for the simultaneous filtration of equal volumes of test samples through two membrane filters.
- Then samples are incubated in two types of media for 14 days and finally observed for growth of any aerobic and anaerobic microorganisms. Hence, sterility indicators are required to check whether microbial growth occurs or not in terms of sterilization quality and process.
- So, as definition of Sterility indicators, are the indicators that are used for check the quality of sterilization and monitoring of the sterilization process.