MICROBIOLOGY

UNIT 2 NOTES

STAINING IN MICROBIOLOGY

- SIMPLE STAINING
- GRAM STAINING
- ACID FAST STAINING
- IMVIC TEST

STERILIZATION

- METHOD OF STERILIZATION
- EVALUATION OF STERILIZATION
- EQUIPMENT IN LARGE SCALE STERILIZATION
- EQUIPMENTS EMPLOYED IN LARGE SCALE STERILIZATION

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STAINING

- Bacterias are microscopic organism.
- Most of the bacterias are transparent & colourless in nature so it becomes very difficult to identify their shape, size and types even under the microscope.
- To identify & classify microorganism based on their physical & chemical characteristics it becomes very important to stain them.
- In Microbiology, Staining is a technique used to color bacteria or other microorganism with the help of various dyes to make them easier to see under the microscope

OBJECTIVE

- To make microorganism visible under a microscope.
- To determine shape, size & arrangement of microorganism.
- To study specific cellular structure & components (Flagella, Capsule, Endospores etc.)
- To differentiate between various types of Microorganisms.
- To detect specific molecules or substances in Microorganisms (specific proteins, antigens etc.)

TYPES OF STAINING

There are mainly two types of staining techniques used in Microbiology for identification of bacteria :

① Simple Staining
② Differential Staining

SIMPLE STAINING

- Simple staining technique is a basic staining technique used in Microbiology to enhance the contrast of cells or microorganism, making them more visible under a Microscope.
- This technique as the name suggest very simple staining technique involves the use of a single dye or stain to color all cells uniformly, allowing for easy observation of their shape, size and arrangement.
- Some most commonly used dyes for simple staining include Methylene Blue, Crystal Violet & Safranin.

PRINCIPLE

- The principle of simple staining is based on the use of a single basic dye that binds to the negatively charged surface of bacterial cells.
- Most bacteria have negatively charged cell surfaces so when a positively charged dye (such as Methylene Blue, Crystal Violet) is applied, it is attracted towards the cell, coloring them.
- This makes the bacteria stand out against the background, allowing for easy observation.

PROCEDURE

The procedure for simple staining includes 5 simple staining :

- Preparation
- Fixation
- Staining
- Rinsing
- Observation

① Preparation Of Smear

- For preparation of smear , use a sterilized inoculating loop & transfer a small amount of bacterial culture from Culture Media to a clean glass slide
- For Solid Culture : Add a drop of sterile water along with sample.
- For Liquid Culture : Place the sample directly on slide.
- Now gently spread the sample over the slide using inoculation loop.
- Leave the smear for Air Drying.

② Fixation

- After the smear has dried , pass the slide through the flame of the Bunsen Burner 2-3 times.
- It fixes the bacterial cells to the slide.

③ Staining

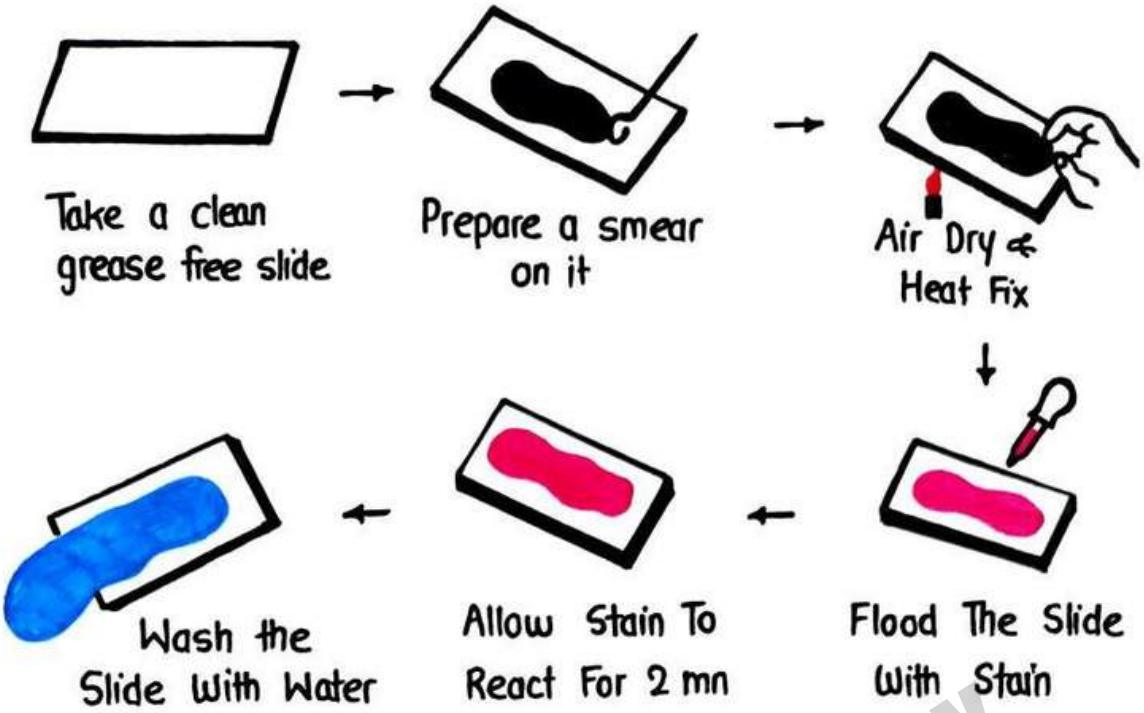
- This step involves applying a single dye to the prepared smear to make the microorganism visible under a microscope.
- Cover the smear with a few drops of basic dye such as Methylene Blue, Crystal Violet or Safranin.
- Allow the stain to sit on the smear for a specific amount of time (usually 30 seconds to 1 minute)
- This time allows the dye to penetrate the bacterial cells & color them.

④ Rinsing

- After the appropriate staining time, gently rinse the slide with distilled water to remove the excess dye.
- The slide should be kept parallel to stream of water, ensuring that it doesn't wash away the bacteria

⑤ Observation

- Once the slide is dry, place it under a microscope for observation.
- The bacteria should now be stained & easily visible, showing their shape, size and arrangement.



SUMMARY

Take a Clean Glass Slide

↓

Prepare a smear of given culture by spreading a thin film on Glass Slide

↓

Airdry & Heat Fix the smear

↓

Stain the Smear with appropriate Dye i.e., Methylene Blue, Crystal Violet For 30 sec- 1 mn.

↓

Wash the slide with distilled water to remove the excess stain

↓

Observe the slide under the microscope

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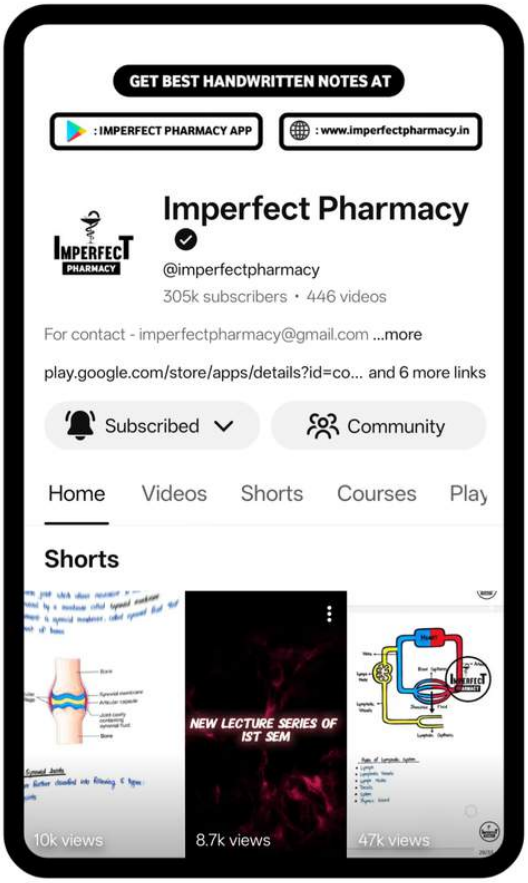

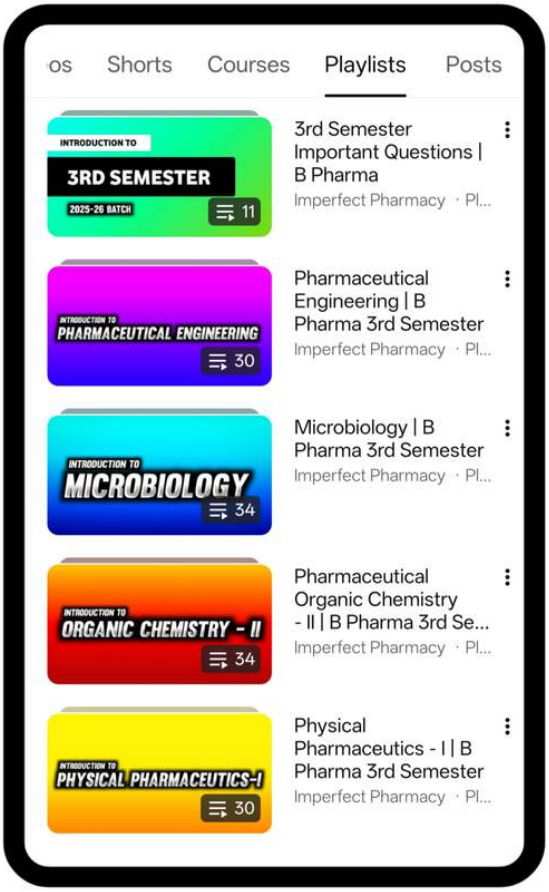

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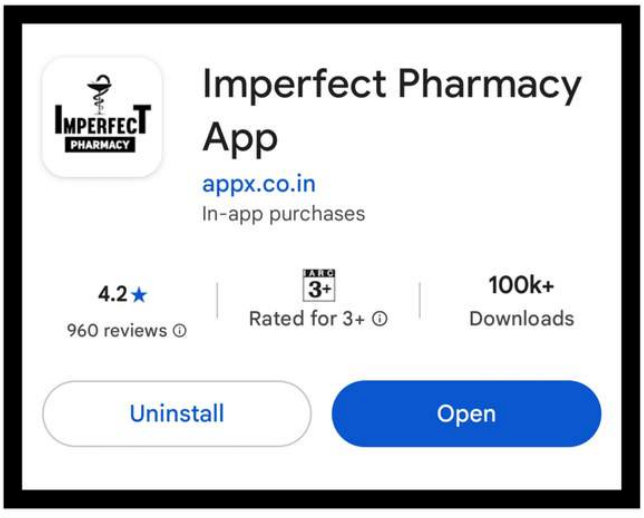


ADVANTAGES

- Easy to perform with minimal steps & equipments.
- Cost effective as requires only a single dye & basic laboratory equipments.
- Provide fast results for observing cell morphology.

LIMITATIONS

- Doesn't differentiate between different types of cells or cellular components.
- Does not provide information on cell wall structure, internal structure or metabolic activity.

APPLICATION

- Used for initial examination of bacterial morphology & arrangement.
- Commonly employed in academics to introduce students with Microscope & staining technique
- Provide a quick overview before more complex staining or biochemical test are performed.

DIFFERENTIAL STAINING

- Differential Staining is a technique used in Microbiology to distinguish between different types of microorganism or different structures within a single microorganism by using multiple stains.
- Unlike simple staining that uses only 1 dye differential staining involves 2 or more dyes that reacts differently with various types of Bacterias or cell structures allowing for differentiation based on colour.
- There are mainly two types of diffential staining used in Microbiology as follows:

① Gram's Staining
② Acid Fast Staining

GRAM'S STAINING

- Gram Staining is one of the most widely used differential staining technique in Microbiology
- Gram Staining is a universal staining technique used to differentiate between Gram Positive & Gram Negative bacteria.
- Gram Staining technique is developed by Han's Christian Gram in 1884.
- Gram differtiates the bacteria into two major groups:

① Gram Positive Bacteria
② Gram Negative Bacteria

GRAM +VE	GRAM -VE
• Have a thick peptidoglycan layer (20-80 nm) • They lack an outer membrane • The lipid content is very low • They are very susceptible to Antibiotics • The thick cell wall retains the crystal violet iodine complex during Gram staining, makes them appear purple. 	• Have a thin peptidoglycan layer (2-10 nm). • They have an additional outer membrane. • Lipid content is 20-30%. • They are very resistant to Antibiotics. • The thin Peptidoglycan layer does not retain the crystal violet iodine complex during Primary staining & cell are decolorized & then counterstained with safranin, appearing Pink/red. 

PRINCIPLE

- The principle of Gram Staining is based on ability of bacterial cell to retain the crystal violet dye during decolorization.
- Gram Positive bacteria retains the crystal violet dye due to their thick peptidoglycan layer & appear purple under the microscope.
- Gram Negative bacteria lose the crystal violet dye during decolorization due to their thin peptidoglycan layer and appear pink due to counterstain.

PROCEDURE

The procedure of Gram's Staining involves 6 steps:

① Preparation of Bacterial Smear
② Primary Stain
③ Mordant
④ Decolorization
⑤ Counterstain
⑥ Observation

① Preparation Of Bacterial Smear

It involves following steps:

- Clean a glass slide throughly
- Using a sterilized inoculating loop, pick a small amount of bacterial culture & place it on the slide
- Spread the sample.
- Air dry the sample.
- Heat fix the smear by passing it quickly through a flame for few minutes

② Primary Stain (Crystal Violet)

- Stain the smear with Crystal Violet & let it sit for 1 minute.
- Rinse the slide gently with distilled water to remove excess stain.

③ Mordant

- Add iodine solution to slide (it acts as a mordant) & let it sit for 1 minute.
- The iodine binds to crystal violet, forming a crystal violet - iodine complex inside the cell.
- Rinse the slide gently with distilled water.

④ Decolorization

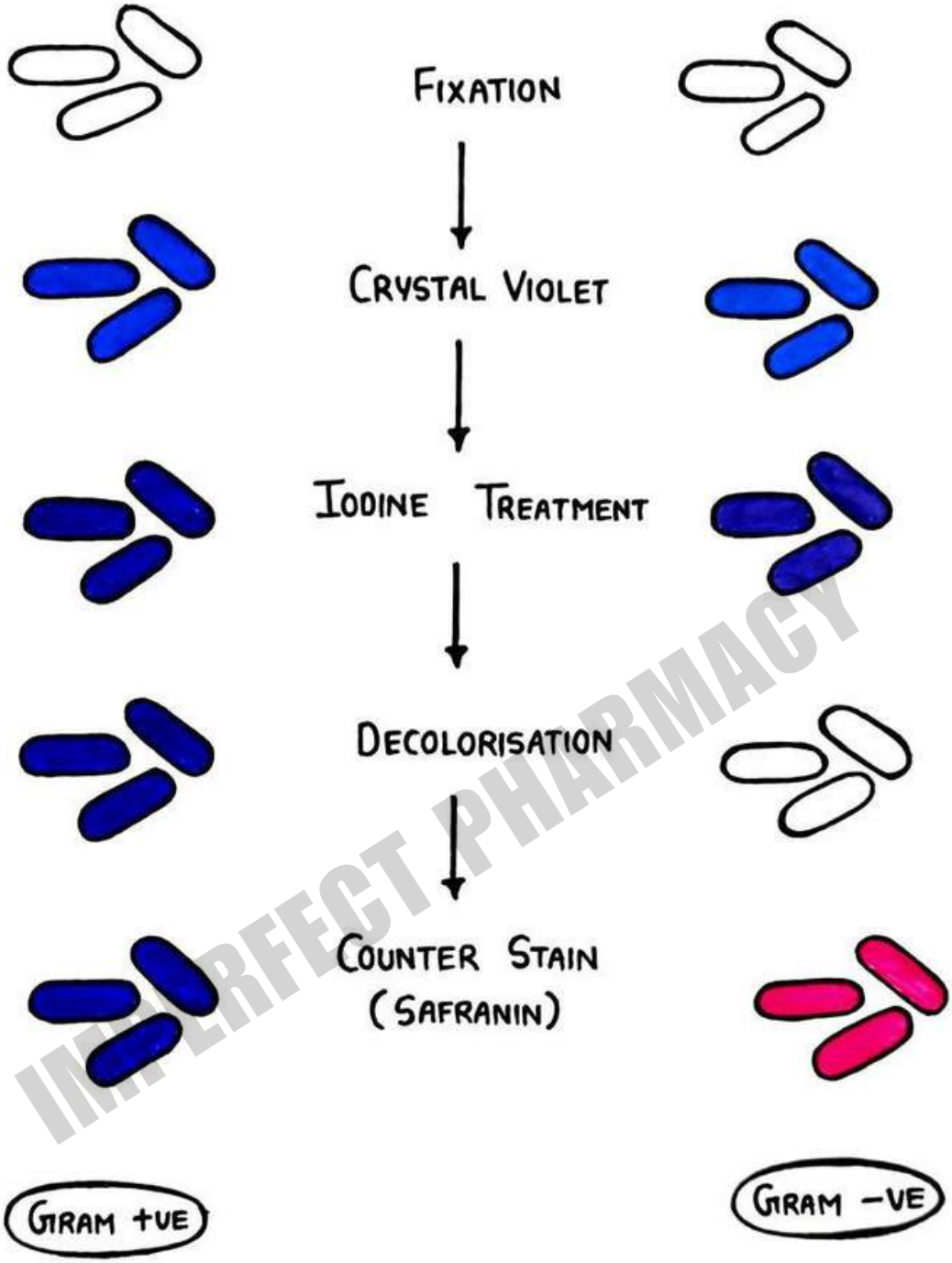
- Decolorize the smear by washing it with 95% ethanol or acetone for 10-20 seconds:
- Gram Positive Bacteria retains the crystal violet iodine complex.
- Gram negative bacteria loss the stain due to thin pephidoglycan layer.
- Rinse immediately with distilled water to stop the decolorization.

⑤ Counterstain (Safranin)

- Apply safranin (counterstain) to the smear for 30-60 seconds.
- Safranin stains Gram Negative Bacteria Pink.
- Rinse the slide with distilled water.

⑥ Observation

- Observe the slide under a microscope using oil immersion (100x objective lens).
- Gram +ve bacteria will appear purple or blue.
- Gram -ve bacteria will appear pink or red.



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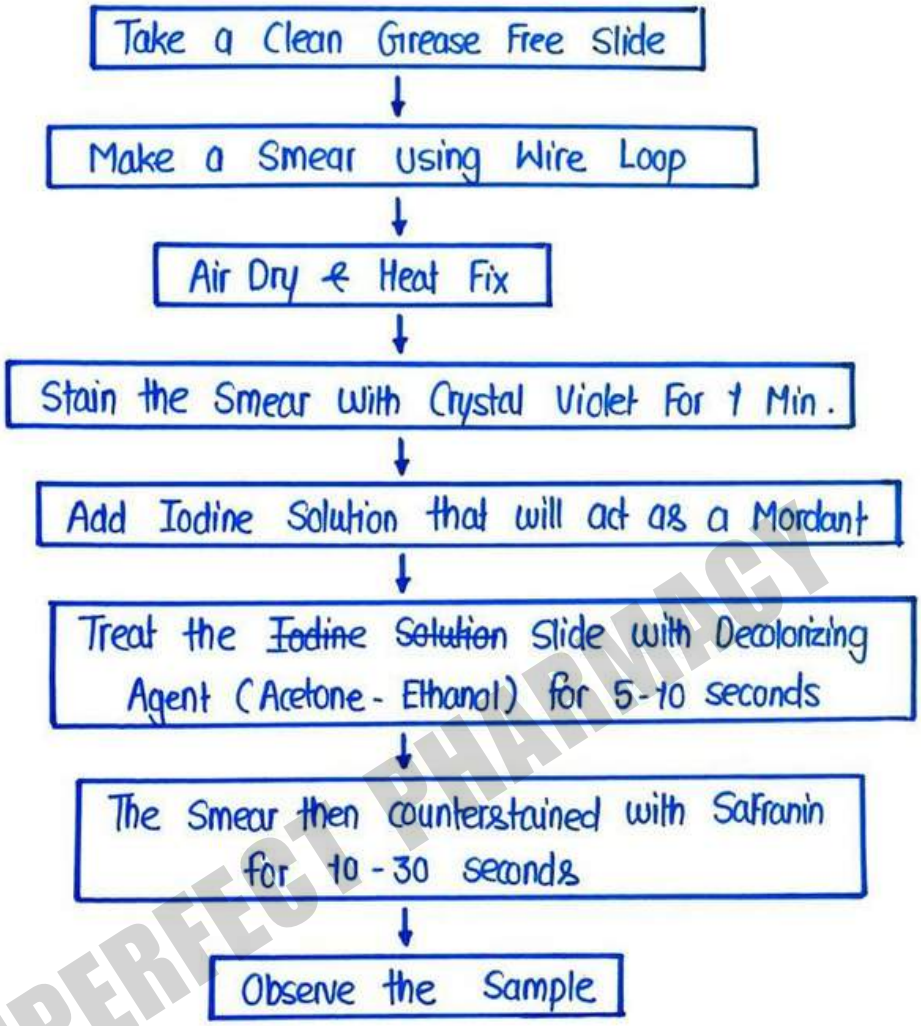


APPLICATION

- Help in identifying & classifying bacterial species based on cell wall.
- Used to determine the cause of infections to guide antibiotic treatment.
- Helps predict which Antibiotics are likely to be effective, as Gram-positive & Gram-negative bacteria often have different susceptibility profiles.
- Assist in studying bacterial physiology & pathogenesis

ADVANTAGES

- Differentiates bacteria into two major groups : Gram Positive & Gram Negative .
- Provides quick information about bacterial classification within minutes.
- The technique is relatively easy to perform & doesn't require advanced equipments .

DISADVANTAGES

- It doesn't work for all bacterias like Mycobacterium species .
- It doesn't differentiate between live & dead bacteria .
- The staining is specific to bacterial cell walls & does not apply to Fungi & Protozoa.

ACID - FAST STAINING

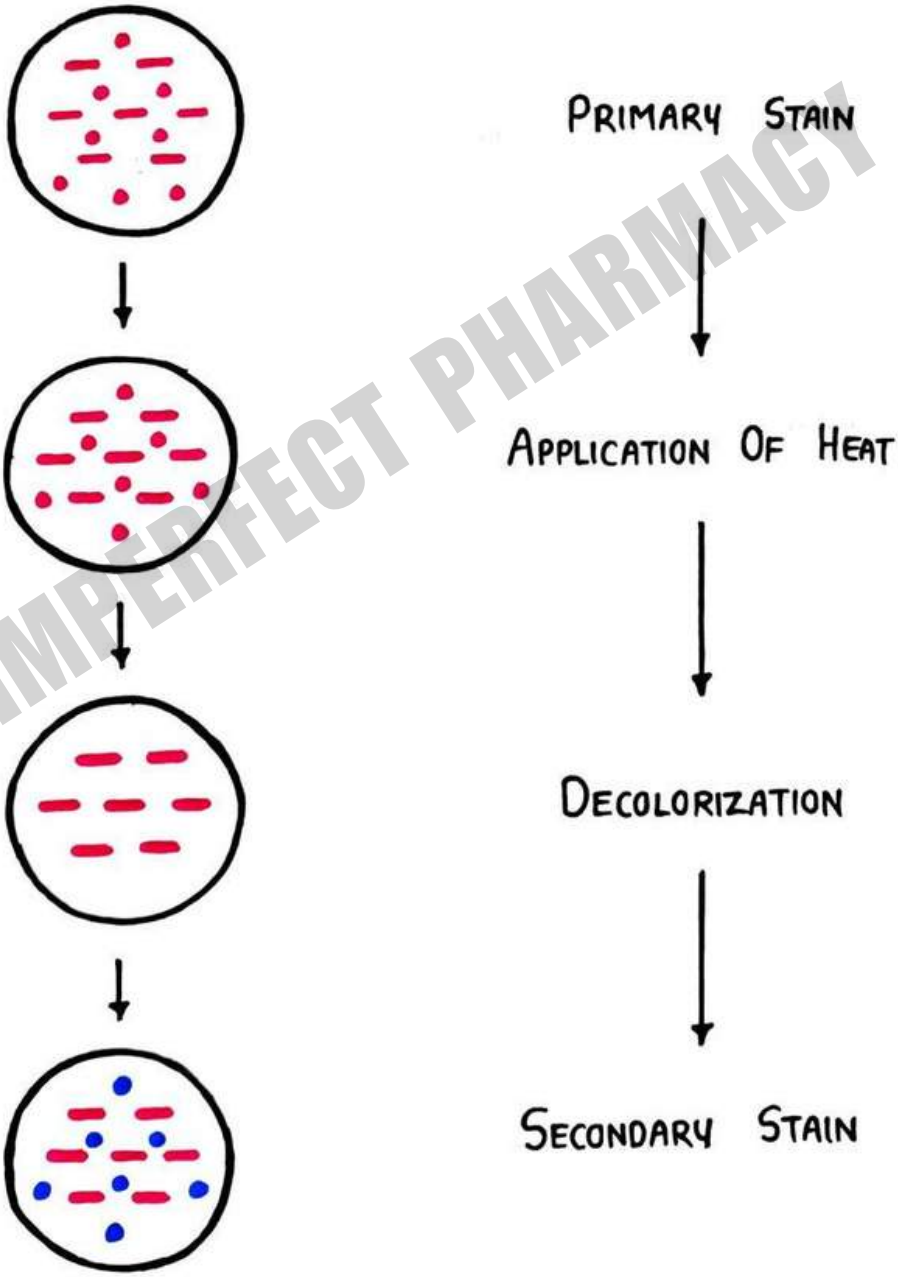
- This technique was developed by **Paul Ehrlich (1882)** and was later modified by Ziehl - Neelsen, hence it is also known as **Ziehl - Neelsen's Staining.**
- This is a special differential staining used to identify mainly the members of Mycobacterium species especially Mycobacterium Tuberculosis and Mycobacterium Leprae.
- These organism are difficult to stain by ordinary staining method due to presence of high lipid content in their cell wall.
- According to this staining bacteria are classified as:

① Acid Fast Bacteria
② Non- Acid Fast Bacteria

ACID FAST	NON ACID FAST
• They contain High Lipid Content (Myolic Acids) • They retain the primary stain after decolorization • Final color is Pink/ Red • Stained with Primary Stain • Example: Mycobacterium	• Low Lipid Content compare to Acid Fast Bacteria • They do not retain the primary stain after decolorization • Final color is Blue • Stained with Counter Stain. • Example: E. Coli

⑤ Observation

- Gently blot the slide with absorbent paper or allow it to air dry.
- Examine the slide under a light microscope using oil immersion.
- Acid Fast bacteria will appear red or pink due to retained carbol fuschin.
- Non- Acid Fast bacteria will appear blue or green due to counterstain.



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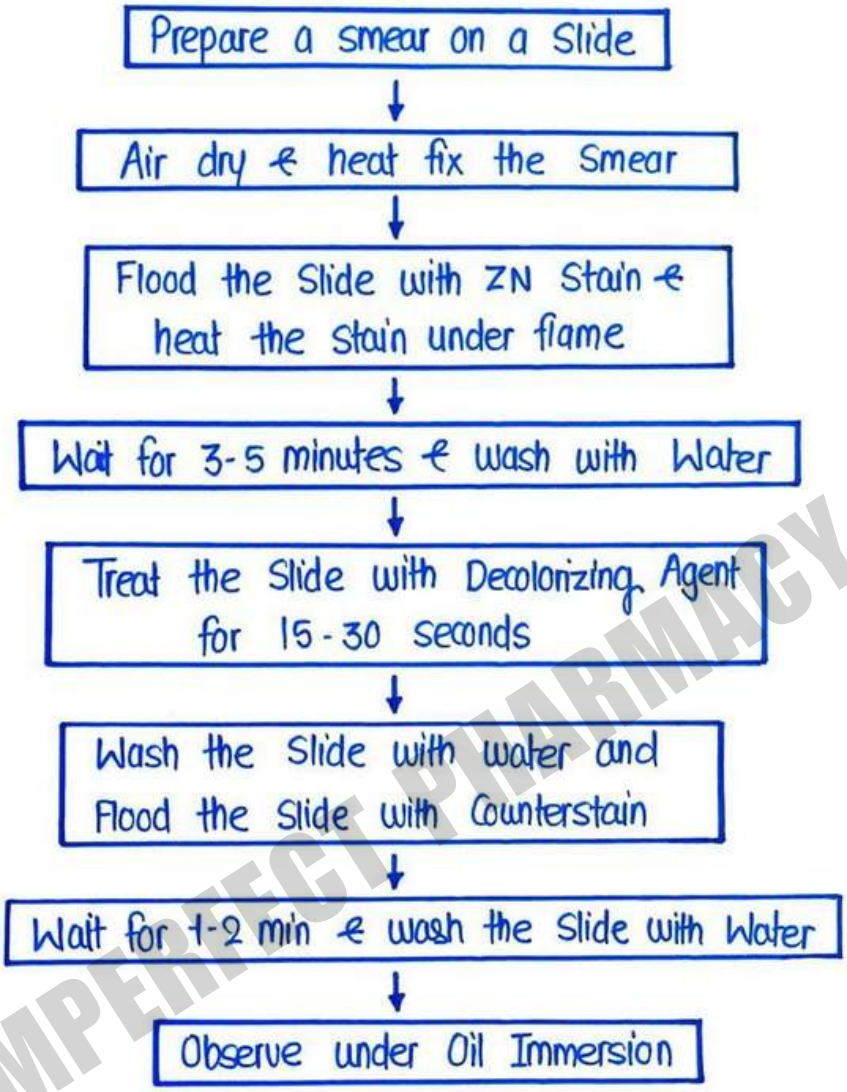


APPLICATION

- Diagnosis of Tuberculosis
- Diagnosis of Leprosy
- Identification Non - Tuberculous Mycobacterial Infections.

ADVANTAGES

- Early diagnosis of Tuberculosis
- Effective Management of Leprosy
- Research & Development

DISADVANTAGES

- Complex & Time consuming diagnosis.
- Limited to specific bacteria.

IMViC TEST

- The IMViC test is a series of 4 biochemical tests used in microbiology to identify and differentiate between members of the **Enterobacteriaceae** family, which are Gram Negative Bacteria.
- The term IMViC is an acronym stands for 4 different test

① I : Indole Test
② M : Methyl Red Test
③ V : Voges - Proskauer Test
④ C : Citrate Utilization Test

SIGNIFICANCE OF IMViC TEST

- The IMViC test is widely used to distinguish between closely related bacteria that inhibit the gastrointestinal tract, such as Escherichia coli, Enterobacter, klebsiella, Serratia and Citrobacter.
- In medical microbiology, the IMViC test is useful in identifying pathogens responsible for UTIs and other infections caused by enteric bacteria.
- The test is essential in food microbiology to detect spoilage organisms or pathogens in food & dairy products.
- The IMViC test is a quick, cost effective and relatively simple biochemical assay, making it a practical choice for many laboratories without the need of expensive equipments.

INDOLE TEST

- The Indole Test is a biochemical test used to determine the ability of an organism to produce Indole from the amino acid Tryptophan
- The test is primarily used in microbiology for the identification of bacteria, particularly to distinguish between members of the family Enterobacteriaceae.

PRINCIPLE

- Certain bacteria possess the enzyme Tryptophanase which catalyzes the breakdown of Tryptophan into Indole, Pyruvate & Ammonia.

$$\text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{Pyruvate} + NH_3$$

- The presence of indole in the culture can be detected by adding Kovac's Reagent, which react with indole to produce a red or pink coloured compound.

$$\text{Indole} + \text{Kovac's Reagent} \longrightarrow \text{Cherry Red Compound}$$

PROCEDURE

- A bacterial colony is inoculated into a tryptone - containing broth, such as tryptone broth.
- The culture is incubated at 37°C for 24-48 hours to allow for bacterial growth and Tryptophan metabolism.
- After incubation kovac's reagent is added into broth.

RESULT

- Positive : Formation of Pink/ Red coloured ring (E. Coli)
- Negative : No Colour change (klebsiella pneumoniae)

METHYL RED TEST

- The Methyl Red test is a biochemical test used to determine the ability of an organism to form stable mixed acids (such as lactic acid, acetic acid & formic acid) through Glucose fermentation.
- This test is particularly useful for distinguishing between different types of bacteria, especially Enterobacteriaceae family.
- It is part of IMViC series of test.

PRINCIPLE

- The principle of MR test is based on ability of certain bacteria to form stable mixed acids through Glucose fermentation.

Glucose ⟶ Mixed Acids (lactic, acetic, formic acids)

- The methyl red indicator, is used to detect this acidic environment, it turns red in acidic environment, indicating a positive result.

PROCEDURE

- A bacterial colony is inoculated into a **MR-VP Broth**, which contains glucose and other nutrients.
- The culture is incubated at 37°C for 24-48 hours (sometimes upto 5-7 days for certain bacteria)
- After incubation, add few drops of Methyl red indicator.

RESULT

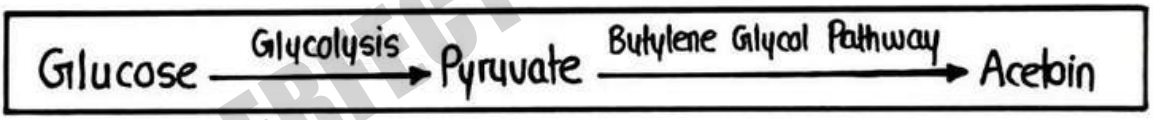
- Positive Result : A red colour develops, indicating a low pH due to production of stable acids (E. Coli).
- Negative Result : No Colour change (Klebsiella Pneumoniae)

VOGES - PROSKAUER TEST

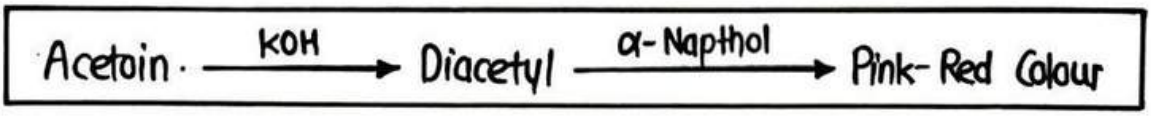
- The Voges - Proskauer test is a biochemical test used to detect the ability of an organism to produce Acetoin (Acetylmethylcarbinol) through glucose fermentation.
- The test is typically used in combination with Methyl Red Test to differentiate between bacteria in the Enterobacteriaceae family.
- It belongs to the series of IMViC Test.

PRINCIPLE

- The principle of Voges - Proskauer test is based on ability of certain bacteria to form Acetoin from Glucose fermentation through Butylene Glycol Pathway.



- When acytoin is formed, it is oxidized to diacetyl in the presence of oxygen and Potassium Hydroxide (KOH)
- Diacetyl then react with guanidine nuclei (present in peptone of the medium) to produce red colours in the poesence of α- Napthol, indicating a positive result.



PROCEDURE

- A bacterial colony is inoculated into a MR-VP broth containing glucose and peptone
- The culture is incubated at 37°C for 24-48 hours to allow for bacterial growth & fermentation.
- After incubation, VP reagent A (α-napthol) & VP reagent B (KOH) are added to broth.

RESULT

- Positive : A red colour develops at the surface of broth, indicating the presence of acetoin. (Klebsiella)
- Negative : No red colour appears, indicating that acetoin is not produced (E. Coli)

CITRATE UTILIZATION TEST

- The Citrate Utilization Test is a biochemical test used to determine if an organism can utilize Citrate as only source of carbon & ammonium ions as its only Nitrogen source.
- This test helps differentiate between members of Enterobacteriaceae family.
- It belongs to the series of IMViC test.

PRINCIPLE

- Some bacteria produce the enzyme Citrate Permease (or citrate lyase) which allows them to transport and metabolize citrate as a carbon source

$$\text{Citrate} \xrightarrow{\text{Citrate Lyase}} \text{Oxaloacetate} + \text{Acetate}$$

$$\text{Oxaloacetate} \longrightarrow \text{Pyruvate} + CO_2$$

- The CO_2 produced then reacts with sodium (present in medium) to form sodium carbonate, which turns medium alkaline.

$$CO_2 + H_2O + Na \longrightarrow Na_2CO_3$$

- When bacteria utilize citrate, they also use ammonium salts as nitrogen source, producing alkaline by-products.
- The medium used in the test contains bromomethyl blue, a pH indicator that turns from Green to Blue when pH rises above 7.6, indicating alkalinity.

PROCEDURE

- A light inoculum of the test organism is streaked onto the surface of simmons citrate agar slants.
- The inoculated medium is incubated at 37°C for 24-48 hours, and sometimes upto 7 days for certain organisms.
- observe the colour change.

RESULT

Positive : Colour change from Green to blue indicate citrate utilization & production of alkaline by products (Salmonella)

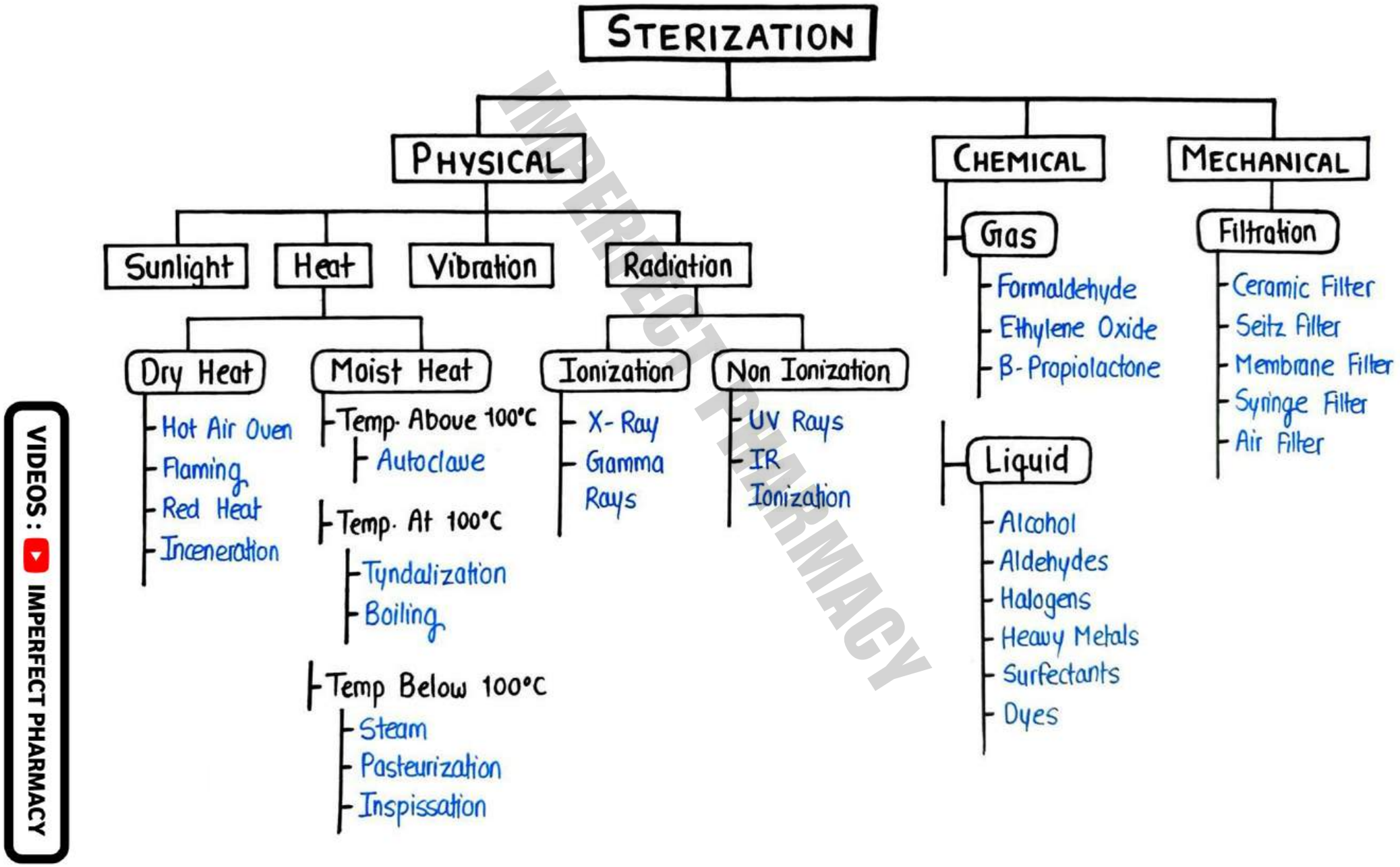
Negative : No Colour Change (E. Coli)

STERILISATION

- Sterilization is defined as a process of complete elimination or destruction of all forms of microorganism including bacteria, viruses, fungi and their spores from any surface, food, equipment, medications or culture medium.
- It is a critical process used to prevent contamination in laboratory, particulary while working with cultures or samples where presence of other microbes could interfere with results.
- It ensures complete elimination of microorganism, making it an essential step im Microbiology.

IMPORTANCE OF STERILIZATION

- Sterilization prevents infections by ensuring that surgical instruments, medical devices & surfaces are free of harmful microorganisms.
- Sterilization is essential to maintain pure cultures & avoid contamination that could interfere with results & lead to false data.
- It ensures that drugs, vaccines & medical devices are produced in sterile conditions to prevent introduction of harmful microbes in Human Body.
- It extends the shelf life of products by eliminating spoilage causing microorganisms.



PHYSICAL METHODS

- Physical Methods of sterilization involves the use of physical agents to eliminate or kill all forms of microorganisms including bacteria, viruses, fungi and spores.
- They may involve the use of heat in the presence or in the absence of moisture.

① HEAT

- Sterilization by Heat is the most widely used physical method of sterilization that uses high temperature to kill or inactivate all types microorganisms.
- There are two primary type of Heat Sterilization

ⓐ Moist Heat Sterilization
ⓑ Dry Heat Sterilization

ⓐ DRY HEAT STERILIZATION

- Dry Heat Sterilization is a method of sterilization that uses high temp in the absence of moisture to kill or inactivate microorganism
- It kills microorganisms by causing protein denaturation & destructive oxidation of bacterial cell components.
- It involves following methods :

① Hot Air Oven
② Flaming
③ Red Heat
④ Incineration

HOT AIR OVEN

- A Hot Air Oven, specifically designed for sterilization is an essential laboratory equipment used to eliminate microorganisms from heat resistant materials.
- It is a thermally insulated chamber that uses dry heat to sterilize laboratory instruments & materials.
- It is the most widely used equipment for performing Dry Heat Sterilization.

PRINCIPLE

- The Hot Air Oven works by heating the air inside the chamber, which then heats the material placed within it.
- The materials placed inside the oven are exposed to elevated temperatures (typically 160-180°C) for a predetermined time usually from 1 to 2 hours
- This process denatures proteins and destroys cellular structures of microorganisms.

CONSTRUCTION

- A Hot Air Oven is a thermally insulated chamber used for heating materials, often in libaratory or industrial setting to sterilize them.
- Its construction typically includes the following components:

① **Outer Casing** : It is made of metal or durable materials to ensure durability & heat resistance.

② **Insulation** : The space between outer casing & inner chamber is filled with high quality thermal insulation often fibreglass or mineral wool

③ **Inner Chamber** : The inner chamber is designed to hold racks or trays for samples & often made of stainless steel for easy cleaning.

④ **Heating Elements** : Electrical heating element typically made of nichrome generally located at base or sides of chamber, generate the required temperature.

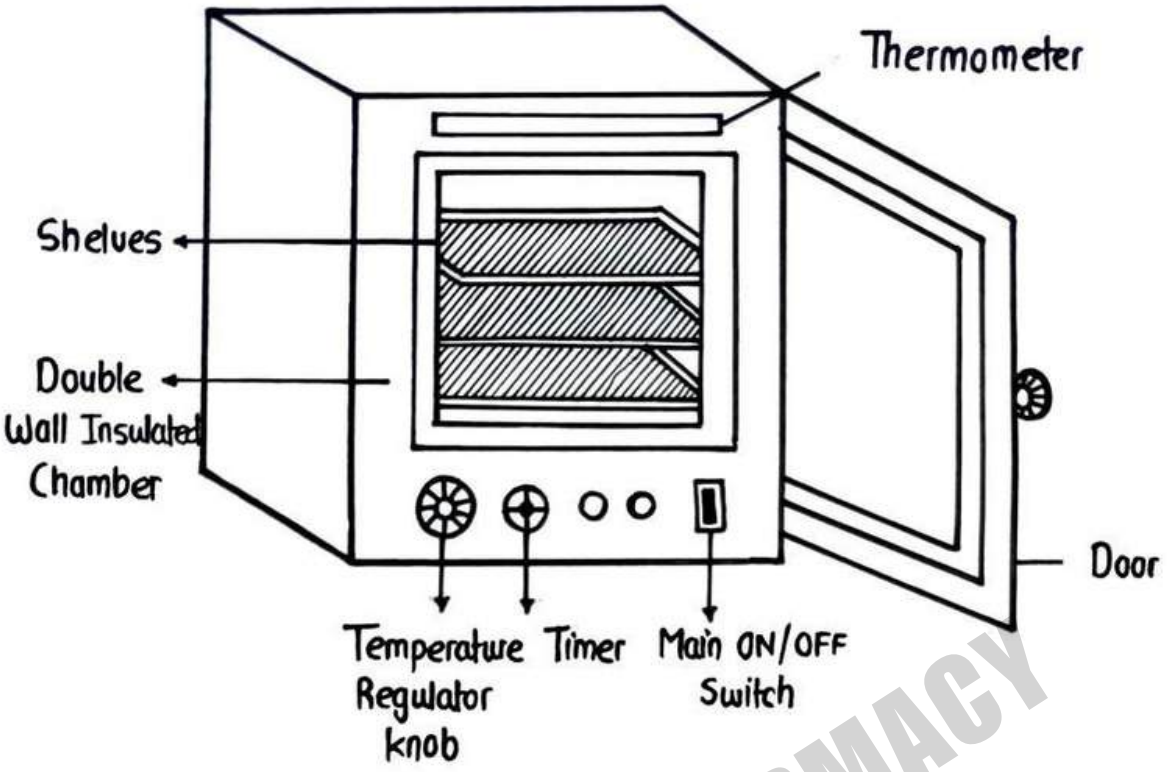
⑤ **Air Circulation** : A fan is usually included to circulate the hot air evenly within the chamber.
Uniform distribution of heat ensures that all parts of materials sterilized effectively.

⑥ **Temperature Control** : A thermostat or digital control system allows precise temperature setting & monitoring.

⑥ **Temperature Control** :

⑦ **Door** : The door is usually double-walled and it has a tight seal to prevent heat loss. It might have a glass window for visual monitoring.

⑧ **Safety Features** : These can include over temperature protection, alarms or an exhaust vent to release any pressure buildup & prevent overheating.



HOT AIR OVEN

WORKING

- The primary method of sterilization is dry heat, which usually requires a temperature of 160°C to 180°C for about 1-2 hours
- The heat effectively kills microorganisms by coagulating protein and causing oxidative damage.
- The fan ensures that heat is distributed evenly around the items, providing consistent sterilization.

TEMPERATURE	TIME
160°C	2 Hour
170°C	1 Hour
180°C	30 Minutes

APPLICATION

- Sterilization of Glassware like test tubes, petri dishes, beakers etc.
- Sterilization of Metal instruments i.e. scissors, dental tools etc.
- Sterilization of Powders & Oils
- Sterilization of Moisture Sensitive Items.

ADVANTAGES

- Effective for moisture sensitive items.
- Non - Corrosive
- Economical & Low Maintainance
- No need of Pressure

DISADVANTAGES

- Longer Sterilization Time
- Limited to Heat Resistant Items
- High Energy Consumption
- Cooling Period Required

INCENERATION

- Inceneration refers to the process of using high temperatures typically between 800°C - 1200°C to destroy microorganisms and pathogens by burning contaminated materials, such as biological waste, used medical supplies (e.g. syringes, bandages) and certain lab equipments
- Incineration completely eliminates microbial life, reducing the materials to ashes.

RED HEAT

- Red Heat refers to the process of exposing objects, typically metal instruments, to direct flame or high temperatures until they become visibly red-hot.
- This temperature is sufficient to kill microorganisms, including bacteria, viruses and spores.

FLAMING

- Flaming is a process where objects typically metal tools or glasswares are briefly passed through an open flame (such as from a Bunsen Burner) to kill microorganisms on their surface.
- This process is mainly used for quick and efficient surface sterilization.

ⓑ Moist Heat Sterilization

- Moist Heat Sterilization is a method of sterilization that uses steam or hot water to kill microorganisms including bacteria, viruses and spores through the process of denaturing proteins.
- It is one of the most effective & widely used methods for sterilizing a variety of items, particularly in medical and laboratory settings.

Types Of Moist Heat Sterilization

It is classified into following 3 categories :

① Temperature Below 100°C
- Steam
- Pasteurization
- Inspissation

② Temperature at 100°C
- Tyndalization
- Boiling

③ Temperature above 100°C
- Autoclave

AUTOCLAVE

- An Autoclave is a specialized, high pressure device used for sterilization and other industrial processes requiring elevated temperature & pressure.
- It works by using high pressure steam at a high temperature to kill bacteria, virus, fungi and spores, making it widely used device in medical, dental, microbiological & industrial settings.

PRINCIPLE

- The autoclave functions by heating water to create steam, which is pressurised in a sealed chamber.
- The combination of high temperature and high pressure allows the steam to penetrate objects & materials, effectively sterilizing them.
- According to Boyle's law when volume of steam is kept constant, Temperature $\propto$ Pressure.
- By increasing the pressure to around 15-30 pounds per square inch (psi) in the chamber, the boiling point of water rises, allowing the steam to reach temperature around 121°C or more.
- Most Common Setting
  - Pressure : 15 Psi
  - Temperature : 121°C

CONSTRUCTION

The construction of an autoclave typically includes the following key components :

① **Chamber** : The main body of Autoclave, typically made of stainless steel or other corrosion-resistant material, where apparatus needs to be sterilized placed.

② **Lid / Door** : It closes the chamber equipped with a locking mechanism to ensure safe operation.

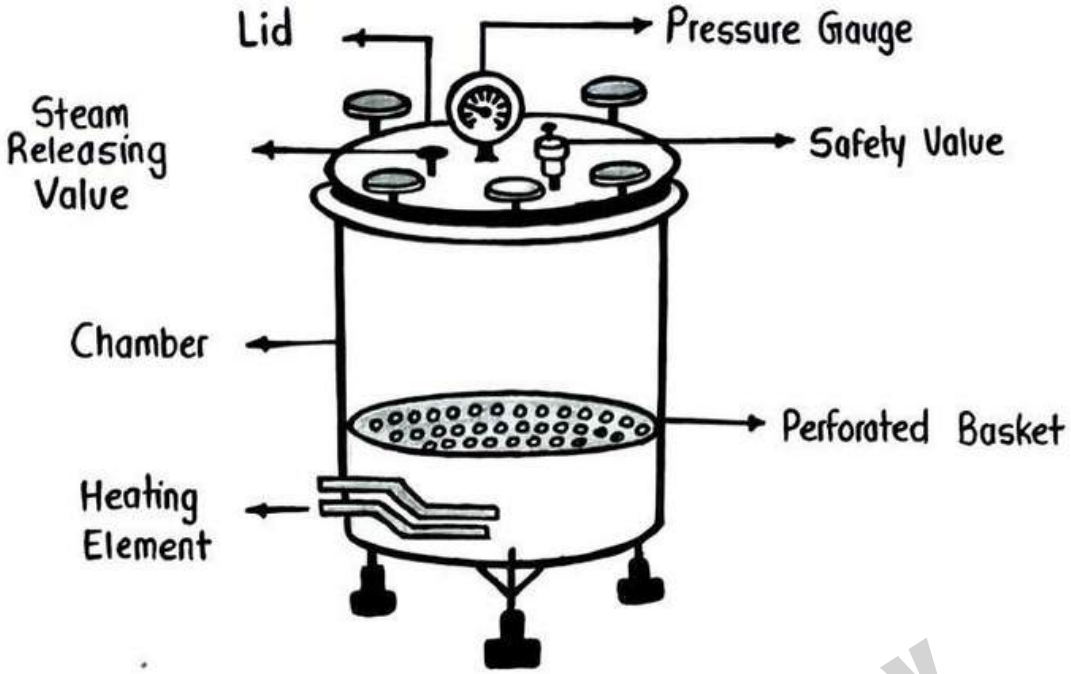
③ **Wire Basket** : It holds the materials to be sterilized.

④ **Pressure Gauge** : It displays the pressure inside the chamber.

⑤ **Safety Valve** : If the pressure becomes too high this valve let steam escape and prevents explosion.

⑥ **Heating Element** : It provide the heat necessary to generate steam. It can be either electric or gas powered.

⑦ **Steam Releasing Valve** : It allows the air & steam to go out when water is first heated & process is completed.



WORKING

The working of Autoclave typically involves following steps:

- Heating: First water is heated to generate steam, that fills the chamber containg object to be sterilized.
- Pressurization: The steam pressure increases, raising temperature above 100°C (usually around 121 - 134°C).
- Sterilization: The high temperature & pressure effectively kills bacteria, viruses, spores through protein denaturation and cell destruction.
- Cooling: The pressure is released & contents are allowed to cool before removal.

TEMPERATURE	PRESSURE
121°C	15 PSI
132°C	30 PSI

APPLICATION

- Sterilization of medical instruments & healthcare equipments.
- It is used for sterilization of majority of official injections
- It is used for sterilization of articles made of rubber, plastic & glass.
- In food production, autoclaves are used to sterilize containers and packing to prevent contamination.
- They are used to sterilize petri dishes, test tubes, pipettes and culture media before culturing microorganism.

ADVANTAGES

- Autoclaves use pressurized steam, which can kill bacteria, viruses, fungi and spores.
- Compared to other sterilization methods Autoclaves work faster, typically sterilizing items within 15-30 minutes.
- They can sterilize a wide range of materials, including medical instruments, laboratory equipments and glasswares.
- Autoclaves are relatively inexpensive to operate, particularly when handling large volume of items.

DISADVANTAGES

- Certain Heat sensitive materials like plastics can be damaged by high heat & moisture.
- High Energy consumption
- Require proper maintainance

② Temperature At 100°C

It involves :

- Tyndallization
- Boiling

BOILING

- Boiling for sterilization is a method is a method in which water is heated to its boiling point (100°C) to kill microorganisms
- The heat destroys most bacteria, viruses and fungi by denaturing their proteins and disrupting cellular processes.
- However, boiling is not considered a reliable method for sterilization in medical or laboratory settings because it may not destroy all bacterial spores or some heat-resistant pathogens.

TYNDALLIZATION

- Tyndallization is a sterilization process that involves intermittent heating, designed to destroy heat resistant bacterial spores.
- It was developed John Tyndall in 19th century & typically used for materials that cannot resist high temperature and autoclaving.
- In this material is first heated around 100°C for 10-30 mintues, which kills most vegetative cells.
- After heating, material is allowed to cool & incubated for 24 hours, during this any surviving bacterial spores, germinate into living cells.
- The process is repeated over 2-3 days, each time newly germinated cells killed by subsequent heat treatment.

③ Temperature Below 100°C

It includes :

- Steam
- Pasteurization
- Inspissation

Steam

- Sterilization by steam under temperature below 100°C in moist heat sterilization is generally less common and requires longer exposure time to achieve the same level of sterilization as higher temperature
- It is mainly used for sterilization of heat-sensitive instruments.

Inspissation

- Inspissation is a sterilization technique that involves heating media containing high amounts of protein to solidify & disinfect it.
- In this, medium are placed in the slopes of an inspissator and heated at 80-85°C for 30 minutes on three successive days.
- It is mainly useful for sterilization of egg and serum based media which generally get destroyed at higher temperature.

PASTEURIZATION

- Pasteurization is a process of heating food or liquid to a specific temperature generally below 100° C for a fix period of time to kill or inactivate harmful pathogens
- Pasteurization is named after the French scientist Louis Pasteur, who developed the process in mid 19th century.
- It is generally used for sterilization of milk.
- It kills almost 90% of bacteria in milk.
- There are generally two methods for pasteurization :

① Holder Method : 63°C for 30 minutes
② Flash Method : 72°C for 15-20 seconds.

② RADIATION

- Radiation sterilization can be defined as use of various forms of radiation to eliminate or inactivate microorganisms.
- It is mainly classified into two main categories :

ⓐ Ionization Radiation
ⓑ Non- Ionization Radiation

Ionization Radiation

- Ionization radiation has enough energy to remove tightly bound electrons from atoms.
- This process can damage DNA and cellular structures in microorganisms.
- It is of two types :

(1) Gamma Radiation
(2) X- Rays.

Non- Ionization Radiation

- Non- Ionization radiation does not carry enough energy to ionize atoms or molecules, having poor penetration power.
- They are used for substance sensitive for Ionization Radiation.
- It is of two types :

(1) IR Ionization
(2) UV Rays

③ SUNLIGHT

- Sunlight for sterilization refers to the use of natural sunlight specifically its Ultraviolet (UV) rays to inactivate or eliminate microorganisms, including bacteria, viruses & fungi.
- UV radiation, particularly UV-C (wavelength between 200-280 nm) is most effective for sterilization, which can damage the DNA or RNA of pathogens, leading to cell death.

④ VIBRATION

- The vibration method of sterilization is a technique that employs mechanical vibrations to disrupt and inactivate microorganisms.
- The process involves applying high-frequency vibrations to a surface or medium that can damage the cellular structures of bacteria, viruses and fungi.

CHEMICAL METHODS

- Chemical Methods of Sterilization utilize various chemical agents to destroy all forms of microbial life including Bacteria, Viruses, Fungi & Spores.
- These methods are mainly used in case of heat - sensitive medical devices, certain laboratory equipment and some pharmaceuticals.
- They can be further subdivided in two categories :

① Gaseous Methods
② Liquid Methods

PROPERTIES OF IDEAL CHEMICAL AGENT

- Must be effective against all microorganisms.
- High Penetration Power
- Non - Corrosive in Nature
- Should not cause irritation.

MOA OF CHEMICAL AGENTS

- Protein Coagulation
- Disruption of Cell Membrane
- Interference with Enzyme function
- Destructive Oxidation

① Liquid Methods

- In this, chemical solutions in liquid form are used to sterilize medical instruments, surfaces or equipments.
- This method is particularly useful for heat-sensitive items that cannot resist high temperature sterilization techniques like autoclaving.
- There are following liquid chemicals used in Chemical Sterilization.

Alcohols

- Ethyl Alcohol (Ethanol) & Isopropyl Alcohol (Isopropanol) are commonly used.
- They act by denaturing proteins.
- They are effective at killing a wide range of microorganisms, including Bacteria, Fungi & some Viruses, but they are not effective against bacterial spores.
- They are generally considered disinfectants rather than sterilants. Effective concentration: 70-80% in water.

Aldehyde

- Glutaraldehyde & Formaldehyde are most commonly used.
- They act by alkylation of amino group in proteins, disrupting cell wall synthesis and enzymatic functions, leading to cell death.
- They are capable of destroying bacteria, viruses, fungi and most importantly bacterial spores, which make them true sterilants.
- However, they require careful handling due to their toxicity and potential Health risks.

Phenols

- Phenols are class of chemical compounds that were historically among the first disinfectants used in medical settings.
- However, Phenols are not typically used for chemical sterilization today but rather for disinfection due to their limited abillity to kill all microorganisms, especially bacterial spores.
- Example : Cresols, Hexachlorophenol.

Halogens

- Halogens specifically Chlorine and Iodine, are widely used in disinfection and sterilization processes due to their strong antimicrobial properties.
- They are capable of destroying bacteria, viruses, fungi & some spores.
- However, like alcohols and phenols, halogens are generally more effective as disinfectants rather than true chemical sterilants, although some forms (like chlorine dioxide) can acheive sterilization under specific conditions.

Heavy Metals

- Heavy Metals such as Silver, Mercury and Copper, have long been known for their antimicrobial properties
- However they are not commonly used for sterilization in modern healthcare due to toxicity, environmental concerns and development of safer, more effective alternatives.

② GASEOUS METHOD

- It involves using sterilizing agents in gas form to kill all microorganisms, including bacteria, viruses, fungi and bacterial spores.
- There are following gases commonly used for sterilization.

Ethylene Oxide

- Ethylene Oxide is one of the most widely used methods for sterilizing medical equipment and instruments, particularly for items that are heat sensitive.
- It is effective at killing a wide range of microorganisms, including bacteria, viruses, fungi and even the highly resistant bacterial spores, making it a true sterilizing agent.
- It acts by alkylating the amino, carboxyl, hydroxyl and sulphydryl groups in protein molecule & also by damaging the DNA & RNA.

FORMALDEHYDE

- Formaldehyde is a colorless, strong-smelling gas used for sterilization and disinfection.
- As a gas, it is effective against a wide range of microorganisms, including Bacteria, Viruses, Fungi & Spores.
- It is commonly used for sterilization & disinfection of Medical equipments, instruments, pharmaceuticals & biological materials.

Ozone

- Ozone (O_3) is a highly reactive gas used for sterilization and disinfection.
- It is a strong oxidizing agent that effectively kills Bacteria, Viruses, Fungi, Spores & Protozoa.
- It is used for sterilization of Medical Devices, healthcare settings, Air purification, food processing etc.
- It acts by denaturing proteins, disrupting cell membranes and oxidising cellular components.

MECHANICAL STERILIZATION

- Mechanical Sterilization refers to method of sterilization that physically removes microorganisms without the use of chemical agent or high heat.
- It mainly involves Filtration method that involves physical removal of microorganisms from liquids or gases by passing them through a filter with pores small enough to trap bacteria, viruses, fungi or other contaminants.
- This method is especially useful for heat-sensitive substances like culture media, pharmaceuticals and certain biological solutions which cannot be sterilized by heat or chemicals.

① MEMBRANE FILTER

- Membrane Filtration is one of the most widely used methods for sterilizing liquids.
- It involves passing a solution through a membrane with uniform pore sizes that physically block microorganisms.
- It typically have pore sizes of 0.22 µm or 0.45 µm
- Membranes are made from materials such as cellulose acetate, polyethersulfone and polyvinylidene fluoride.
- It mainly uses for sterilizing heat sensitive liquids like Culture Media, Vaccines, Sera and Antibiotics.

② DEPTH FILTER

- Depth Filtration works by trapping particles and microorganisms throughout the thickness of filter material rather than just on the surface, as in membrane filtration.
- It is made from thick materials like diatomaceous earth, glass fibres or polymers.
- It is typically used for industrial water treatment or biopharmaceutical manufacturing.

③ HEPA FILTER

- HEPA (High Efficiency Particulate Air) filtration is primarily used to sterilize air, removing microorganisms and particles larger than 0.3 μm with an efficiency of 99.97%.
- HEPA Filters have very fine pores that can capture airborne microorganisms like bacteria, viruses & fungal spores.
- It is used for air filtration in clean rooms, operating theaters, biosafety cabinets & ventilation systems etc.

EVALUATION OF EFFICIENCY OF STERILIZATION

- Evaluation of efficiency of sterilization methods involves determining how well a sterilization process eliminates all forms of microbial life, including Bacteria, Viruses, Fungi & Spores.
- It ensures that sterilized materials are safe for use, especially in critical settings like healthcare pharmaceuticals, Food Processing & Laboratories.

PARAMETERS FOR EVALUATION

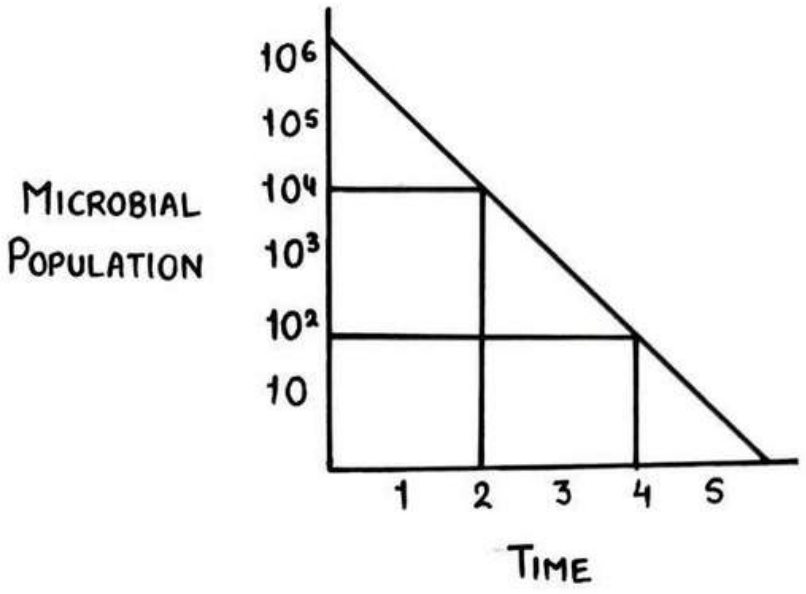
Following parameters are used for evaluating the efficiency of sterilization :

- Death Rate or Survivor Curve
- D Value
- Z Value
- F Value
- Q_{10} Value
- Inactivation Factor

① DEATH RATE OR SURVIVORS CURVE

A Death Rate or Survivor curve is a graphical representation of the number of microorganisms surviving over time when exposed to lethal conditions, such as heat, radiation or disinfection.

- X-Axis : Represents time of exposure
- Y-Axis : Represents number of surviving microorganism.



Factors Influencing Survivor Curves

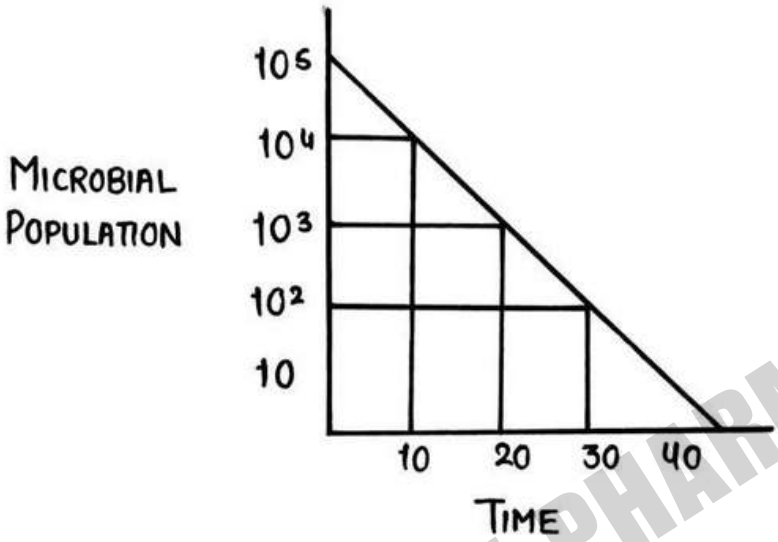
- Temperature
- pH
- Moisture
- Lethal Agent Concentration
- Microbial Species
- Growth Phase

Application

- Sterilization Validation
- Disinfection Efficacy Testing
- Pasteurization Optimization
- Radiation Sterilization
- Food Safety & Preservation

② D VALUE

- The D-value represents the time required to reduce the microbial population by 90% or by 1 logarithmic unit at a specific temperature.
- In other words, it's the time needed to kill 90% of microorganisms or spores at a certain temperature or condition



- A higher z value means that microorganism is more resistant to temperature changes, while lower z value indicates greater sensitivity.

Factors Affecting D value

- Microbial Type & Resistance
- Temperature
- pH
- Moisture
- Sterilization Method

③ Z VALUE

- The Z value measures resistance of microorganisms to changes in temperature.
- It is defined as temperature increase required to reduce the D value by 90%.

$$Z\ Value = \frac{(T_2 - T_1)}{\log\left(\frac{D_1}{D_2}\right)}$$

④ F VALUE

- F Value indicates the time (in minutes) required to reduce the number of viable microorganisms by one log (90% reduction) at a specified temperature i.e., 121°C.
- It helps in evaluating the effectiveness of a sterilization process allowing for comparison between different methods or conditions.

⑤ θ_{10} VALUE

- It is defined as increase in rate of reaction or killing rate of a sterilization process when temperature is increased by 10°C.

⑥ INACTIVATION FACTOR

- The inactivation factor is a measure of reduction in microbial population after applying a sterilization process.

STERILITY TESTING

- Sterility Indicators are tools or devices used to monitor and verify the effectiveness of sterilization processes.
- They provide essential feedback on whether a sterilization cycle has successfully eliminated all forms of microbial life including bacteria, viruses, fungi and spores.

TYPES OF STERILITY INDICATORS

There are 3 main types of sterility indicators :

① Biological Indicators
② Chemical Indicators
③ Physical Indicators

① BIOLOGICAL INDICATORS

- Biological Indicators contain live microorganisms that are highly resistant to sterilization process.
- After sterilization, the indicators are incubated to check for growth. a negative result confirms effective sterilization.
- They are most reliable method of validating sterilization process.
- Examples :

- Geobacillus Stearothermophilus : Used for Steam Sterilization
- Bacillus Atrophaeus : Used for Dry Heat & Ethylene Oxide Sterilization.

② Chemical Indicators

These indicators change colour or appearance when exposed to specific sterilization conditions (e.g., temperature, pressure, chemical) They are of following types :

- Class I Indicators : They are used for general monitoring of sterilization process (e.g. Autoclave Tape)
- Class II Indicators : They are specific for particular sterilization process (e.g. steam or ethylene oxide indicators).
- Class III Indicators : It is single parameter indicator, measures only one sterilization parameter, such as temperature or pressure.
- Class IV Indicators : It is multi-parameter indicator that respond to two or more variables such as time, temperature or pressure.
- Class V Indicators : They are Integrating Indicators that provide the most accurate assessment of sterilization effectiveness.

③ Physical Indicators

- Physical Indicators are mechanical or digital monitoring systems within sterilization equipment that track and record critical parameters like time, temperature, pressure & humidity.
- Examples : Thermometers, Pressure Gauges etc.

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