# UNIT- II
# (CHAPTER-6)
# STAINING TECHNIQUES & BIOCHEMICAL TESTS

## Points to be covered in this topic

1. INTRODUCTION
2. TYPES OF STAINING TECHNIQUES
3. GRAMS STAINING
4. ACID FAST STAINING
5. BIOCHEMICAL TESTS



## INTRODUCTION

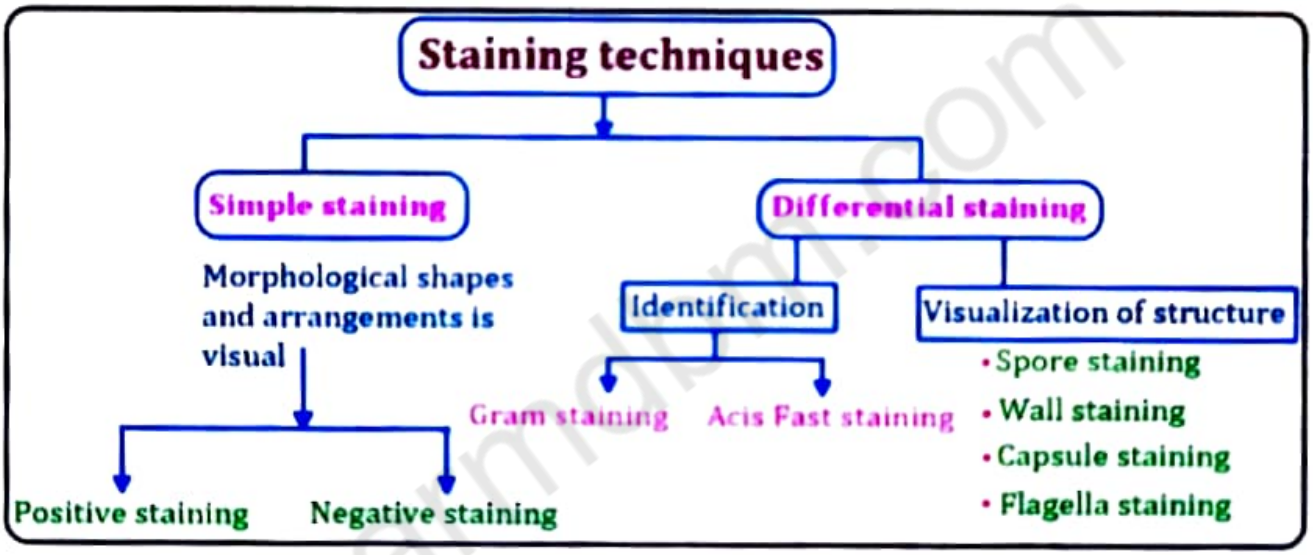
### STAINING TECHNIQUES

- Staining is a technique **used to enhance contrast in samples**, generally at the **microscopic level.**
- According to **microbiological definitions**, stains are **dyes or reagents** used for **differential colouring of clarity** under microscope.
- **Chemically** a stain / dye is defined as **an organic compound containing a benzene ring plus a chromophore group.**

## ❖ Types of stains:

1. **Acidic dyes:** **Anionic**, stain the cytoplasmic components of cells which are more alkaline in nature e.g., picric acid, acid fuchsin, eosin.
2. **Basic dyes:** **Cationic** and combine with those cellular elements which are acidic in nature (nucleic acids) e.g., methylene blue, crystal violet, safranin.
3. **Neutral stains:** Formed by mixing together aqueous solution of certain acidic and basic dyes.

## ❖ TYPES OF STAINING TECHNIQUES




## ❖ SIMPLE STAINING

### ✓ Types of simple staining

➢ **Positive staining :** In positive staining, the stain (methylene blue) is basic (cationic) having positive charge and attaches to the surface of the object that is negatively charged.

➢ **Negative staining :** In negative staining, the stain (India ink, nigrosine) is acidic (anionic) having negative charge and is repelled by the object (bacteria) that is negatively charged and therefore bacterial cell appears transparent. The stain stains only background but not bacteria.

### ✓ PRINCIPLE

➢ Simple staining involves single dye or staining reagent. The purpose of staining is to demonstrate cell size, shape and arrangement of bacterial cells.

- Since bacterial cells. usually have a negative charge on their surface, they are most readily coloured by basic stains.

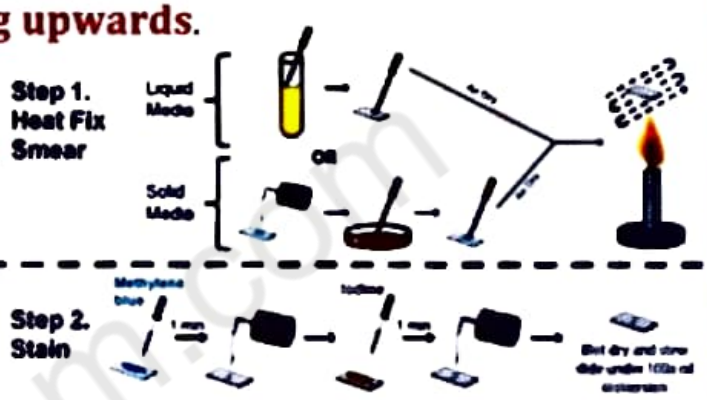
➢ **PROCEDURE -** **Reagent:** Loeffler's methylene blue solution, iodine.

- Prepare smear of a given culture (add few amount of bacteria in one to two drops of distilled water and mix it well) or material by spreading a thin film on a clean glass slide.

⬇

- Dry it and then heat fix by passing the slide 2 to 3 times through the flame with the smeared side facing upwards.

⬇




- Cover the smear with methylene blue and allow the dye to remain in the smear for approximately one minute.

⬇

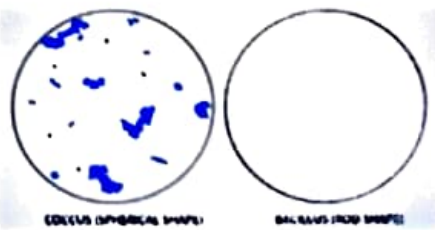
- Saturate the smear again but this time with Iodine. Iodine will set the stain.

⬇

- Wash any excess iodine with gently running tap water.
- Apply oil directly to the smear, and focus the smear first under low power objective and then under oil immersion objective

➢ **RESULT**

- The bacteria will appear blue or red depending upon the stain used with characteristic morphology and may be cocci, cacilli, coccobacilli, spiral, comma shaped etc.



## ❖ DIFFERENTIAL STAINING

- Differential staining is a staining process which uses more than one chemical stain. Using multiple stains can better differentiate between different microorganisms or structures/cellular components of a single organism

## Types of differential staining staining

- **Grams staining**
- **Acid fast staining**

## 1. GRAM STAINING

- **Gram staining is one of the most widely used technique for the differentiation and identification of bacteria.**
- **This differential staining technique was discovered by Dr. Christian Gram in 1884.**
- **It not only reveals the size and shape of bacteria but is also used to differentiate bacteria into Gram-positive and Gram-negative cells.** Hence, it is known as differential staining

### PRINCIPLE

- Gram staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in the cell wall of Gram-positive bacteria.
- Gram-negative cells contain a very small layer of peptidoglycan that is dissolved when the alcohol is added. This is why the cell loses its initial colour from the primary stain.
- Gram-positive bacteria retain the crystal violet dye, and thus are stained violet, while the Gram-negative bacteria do not and it gives pink colour after adding counter stain

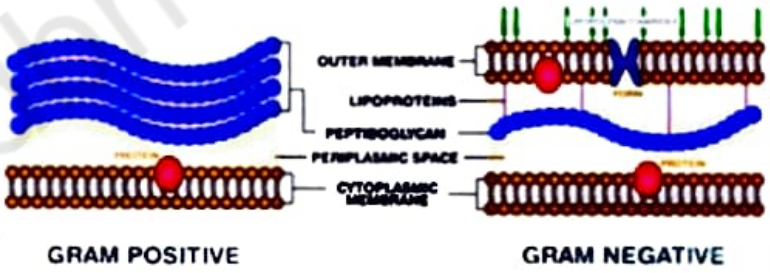
### Reagents used:

- **Primary stain- Crystal violet**
- **Mordant- Grams iodine, (A mordant may be defined as 'any substance that forms an insoluble compound with stain and serves to fix the colour to bacterial cell.**
- **It leads to form crystal violent iodine magnesium ribonuclease [CV-1-Mg ribonuclease] complex in Gram-positive bacteria.**
- **This complex is not formed in Gram-negative bacteria, as Mg-ribonucleate is absent in the cell wall. Hence, only CV-1 complex is formed in Gram-negative bacteria.)**

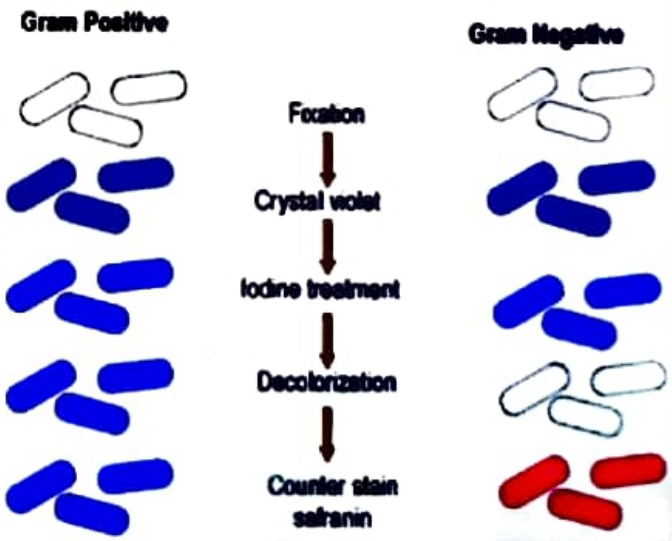
- **Decolourising agent- Ethyl alcohol, On application of decolourising agent like alcohol or acetone, shrinkage of cell wall takes place due to dehydration and decreases the permeability for CV-1-Mg ribonucleate complex.**
- **Thus, the complex is retained in the cell and hence cell is stained deep violet in colour.**
- **On the other hand, the treatment of decolourising agent extracts lipid from cell wall of Gram-negative cell and there is increase in permeability property of cell wall. Due to this, CV-1 complex is extracted and cells gets decolourised (lose violet colour).)**
- **Counter stain- Safranin, (This is the final reagent to stain red, those cells that have been previously decolourised. Since only Gram-negative cells are decolourised, they may now absorb the counter stain. Gram-positive cells retain the violet colour of the primary stain.)**

➢ **PROCEDURE**

- **Make smears of a given culture on a clear glass slide.**




- **Air dry the smear and heat fix it.**
- **Cover the smear completely with crystal violet stain and leave the stain on the slide for one minute.**
- **Wash the slide gently in distilled water or tap water.**
- **Flood the smear with Gram (or Lugol's) iodine solution and wait for one minute. Wash with tap water gently and drain carefully.**




- **Add ethyl alcohol (95%) or alcohol-acetone (1:1) solution drop by drop, until the smear becomes free from any colorization**

- Wash the slide gently under **running tap water and drain.**
- Now counter stain with **safranin** and wait for 30 seconds.
- Wash again and **blot dry with blotting paper** or simply air dry the slide and observe under oil immersion objective.

➢ **EXAMPLES**

- **Gram negative bacteria**: *Xanthomonas, acetobacteracea(vinegar), Escherichia coli, Klebsiella pneumonia, salmonella, pseudomonas aeruginosa.*
- **Gram positive bacteria**: *lactobacillus acidophilus, Bifidobacterium, staphylococcus aureus, anthrax, mycobacterium tuberculosis*

➢ **RESULT**

- Bacteria that **appear blue/violet /purple** are assigned as **Gram positive** while as those appearing **red/pink** are assigned as **gram negative.**

## 2. ACID FAST STAINING

- The technique was developed by **Paul Ehrlich(1882)** and was modified later by **Zichl Neelsen** and therefore **also known as Zichl-Neelsen staining.**
- This is a differential staining used to identify mainly the members **of Mycobacterium especially *Mycobacterium tuberculosis* and *Mycobacterium leprae.***
- These organisms are difficult to satin by ordinary staining methods due to **presence of high lipid content** (mycolic acid) in their cell wall.

**BACTERIA ARE CLASSIFIED AS:**

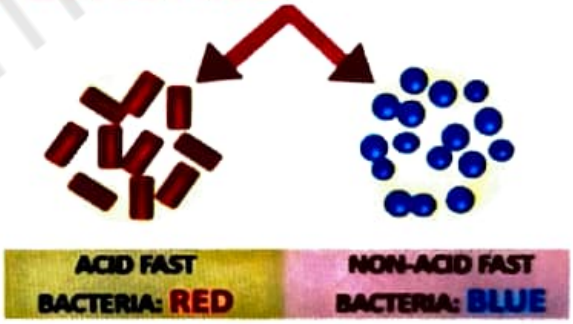
**(1) Acid fast :** If they retain the **primary stain (carbol fuchsin)** after the application of strong acid and appear red.

**(ii) Non-acid fast:** If they do not retain the primary stain and are counterstained by **methylene blue.**

## PRINCIPLE

- When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm. After that all cell appears red.
- Then the smear is decolorized with decolorizing agent (3% HCL in 95% alcohol) but the acid fast cells are resistant due to the presence of large amount of lipoidal material in their cell wall which prevents the penetration of decolorizing solution.
- The non-acid fast organism lack the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colourless. Then the smear is stained with counterstain, methylene blue.
- Only decolorized cells absorb the counter stain and take its colour and appears blue while acid-fast cells retain the red colour.

## PROCEDURE




- Prepare a smear of a purulent portion of the specimen on a clean glass slide.
- Air dry and heat fix the smear.

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- Flood the smear with freshly filtered carbol fuchsin till steam rises. Continue to heat for 5 minutes so that steam is seen but without boiling. Do not allow the side to dry and add more stain from time to time to prevent this drying.

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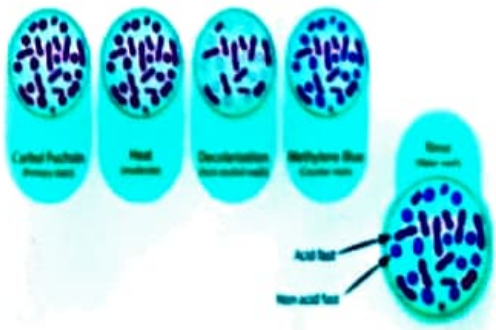
- Cool and wash the stain off the slide with water.
- Cover the side with acid alcohol solution for 3 minutes. Wash with tap water and drain. Repeat decolourization process until smear becomes faint pink. Finally wash with water.



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- Cover the slide with methylene blue stain and leave it for 2 minutes. Wash with tap water, blot dry or air dry the slide and observe under oil immersion objective.

✓ **RESULT**

- Acid fast organisms will appear **bright red on a blue background** while as non-acid fast organism will **appear dark blue in colour.**

## ❖ SPORE STAINING

- Spore staining technique is used for **detection of spore and type of spores.** It is not easy to stain spore by **primary stain malachite greens.** The smear is **heated** to accept the stain. Once the spore accepts the malachite green, it cannot be decolourised by tap water

- Vegetative cells easily get decolourized by tap water and take counter stain and appear red (safranin). This method is called **Schaeffer and Fulton method modified by Ashby.**
- **Donor method** is also used for spore staining in which **carbol fuchsin is used as primary stain.** After heating the side with stain for 5 to 10 minutes, wash it and **perform negative staining.**

## ❖ CAPSULE STAINING

- Capsule staining is used to detect capsules. It can be performed by using positive as well as negative staining techniques.
- Due to the **non ionic nature of** capsule, it has very less affinity for dye.
- In **positive stains, crystal violet** is applied which stains bacterial cell and also **copper sulphate is applied** and it allows the stain (light violet) to retain.

- Capsules are also easily demonstrated by **negative staining** techniques. When stain solution is applied, it stains the cell and on application of **nigrosine** background is stained leaving the **capsule colourless.**

## ❖ FLAGELLA STAINING

- Flagella staining is used to **detect presence and arrangement of flagella and cytoplasmic inclusion.**
- Staining is used to **identify intracellular deposits of starch, glycogen, polyphosphates, Hydroxyl butyrate and other substances.**

# ❑ BIOCHEMICAL TESTS (IMVIC)

- The IMVIC series is a group of **four individual tests** that are commonly used to identify bacterial species, especially **coliforms.**
- A coliform is a **gram negative, aerobic anaerobic rod** which produces **gas from lactose within 48 hours.** The presence of some coliforms indicate faecal contamination.
- Each of the letters in "IMViC" stands for one of these tests.
- "I" is for **indole**; (test for production of indole from **tryptophan**)
- "M" is for **methyl red**;"(methyl red test for acid production from **glucose**)
- "V" is for **Voges - Proskauer**, (test for production of **acetoin from glucose**)
- "C" is for **citrate**,(test for use of citrate for **sole carbon**)
- Lowercase" **(i)** is added for the ease of pronunciation.
- IMVIC is an acronym that stands for four different tests

## ➢ PURPOSE FOR TEST

- To find out the **procedure of treatment or prevention of a disease** we need to find out the causative bacteria responsible, this methods helps to detect the bacteria.
- All the bacteria present in intestine of a human or other mammals belong to the family of **Enterobacteriaceae**. These enterobacteria are short, **gram-negative, and non sporing bacilli.**
- The identification of these fermentative and non fermentative bacteria can be accomplished by **IMViC test.**
- In medical research, it will help to **identify the pathogen** responsible for the disease.

## 1. INDOLE TEST

### ➢ PRINCIPLE

- Some bacteria can produce **indole from amino acid tryptophan** using the enzyme **typtophanase.**

$$\text{Trptophan} \xrightarrow[\text{water}]{\text{Typtophanase}} \text{Indole} + \text{Pyruvic acid} + NH_3$$

- Production of indole is detected using **Ehrlich's reagent or Kovac's reagent.**
- Indole reacts with the aldehyde in the reagent to give a **red colour.** An alcoholic layer concentrates the **red colour as a ring at the top.**

## ➢ PROCEDURE

- Bacterium to be tested is inoculated in **peptone water**, which contains **amino acid tryptophan** and incubated overnight at 37oC. Prepare 1% tryptophan broth are one .
- Incubate one set of test tube with test organism and maintain one set as negative control without inoculation . inoculate one set of test tube with **E.coli use as positive control.**
- Following incubation few drops of **Kovac's reagent** are added. Shake gently.
- Kovac's reagent consists of **para-dimethyl amino benzaldehyde** 10 gm, isoamyl alcohol 150gm and con. HCl 50 ml .
- Allow the tubes to stand for 2 min. so that the reagent comes to the top and then compare test culture with the control tubes.
- Ehrlich's reagent is more sensitive in detecting **indole production in anaerobes and non-fermenters.**

## ➢ RESULT

- Formation of a **red or pink coloured ring** at the top is taken as positive.
- Escherichia coli: **Positive;**
- Klebsiella pneumoniae: **Negative**

## 2. METHYL RED TEST

- Bacteria which produces acid through fermentation of glucose can be identified using this test. Methyl red indicator produces red coloration in acidic pH while it produces orange coloration in non-acidic PH.

### ➢ PRINCIPLE

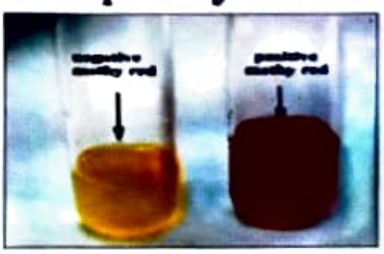
- The basic principle of MR test is to check the **ability of the organism to produce and maintain sufficient amount of stable acid as end product from glucose fermentation** and to overcome the buffering capacity of the system.
- Among different fermentation pathway followed by bacteria, MR test works upon the **mixed acid fermentation pathway**.

**Glucose——–> 2 pyruvate——–>** Succinic acid, lactic acid, acetic acid, formic acid + $CO_2$ + $H_2O$

- These acids **lowers the pH** of the medium which is nearly neutral so that the methyl red indicator **changes its colour to red.**
- The pH range of the methyl red **indicator is 4.4-6**
- but if there is no or low production of acids, the pH of the medium **remains unchanged and the indicator shows yellow colour.**
- Hence, the red colour of the broth medium after adding indicator is **positive MR test.**

### ➢ PROCEDURE

- Prepare MRVP broth in test tubes. Inoculate the broth aseptically with 2 **loop full** of respective bacterial culture.
- Label the test tubes with name of organism inoculated.
- Incubate the test tubes at **37°C for 48-72 hours.**
- Add few drops of **methyl red indicator** in the incubated tubes.



### ➢ RESULT

- ✓ **MR test positive:** bright red colour (E.coli)
- ✓ **MR test negative:** yellow colour ( Klebsiella )

# 3. VOGUES PRUSKAUER TEST

- The Voges-Proskauer (VP) test is used to determine if an organism produces **acetyl methyl carbinol from glucose fermentation**.

## PRINCIPLE

- The Voges-Proskauer (VP) test is used to determine if an organism produces **acetylmethyl carbinol** from glucose fermentation. If present, **acetylmethyl carbinol** is converted to **diacetyl** in the presence of **α- naphthol**, strong alkali (**40% KOH**), and atmospheric oxygen.

## PROCEDURE

- Prior to inoculation, allow medium to equilibrate to room temperature.
- Using organisms taken from an **18-24 hour pure culture**, lightly inoculate the medium.
- Incubate aerobically at **37 degrees C. for 24 hours**.
- Following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube.
- Re-incubate the remaining broth for an **additional 24 hours.**
- Add 6 drops of **5% alpha-naphthol**, and mix well to aerate.
- Add 2 drops of **40% potassium hydroxide**, and mix well to aerate.
- Observe for a **pink-red colour at the surface within 30 min**. Shake the tube vigorously during the 30-min period.

## RESULT

- **Positive Reaction:** A pink-red colour at the surface **Examples:** Viridans group streptococci (except *Streptococcus vestibularis*), Listeria, Enterobacter, Klebsiella, Serratia marcescens, Hafnia alvei, Vibrio eltor, Vibrio alginolyticus, etc.
- **Negative Reaction:** A **lack** of a pink-red colour **Examples:** Streptococcus mitis, Citrobacter sp., Shigella, Yersinia, Edwardsiella, Salmonella, Vibrio furnissii, Vibrio fluvialis, Vibrio vulnificus, and Vibrio parahaemolyticus etc.
- **A copper colour should be considered negative.** A rust colour is a weak positive reaction.

# 4. CITRATE UTILIZATION TEST

- **Citrate testing is used to determine the ability of the bacteria to use sodium citrate as the only source of carbon and inorganic ammonium hydrogen phosphate ($NH_4H_2PO_4$) as a source of nitrogen.**

## PRINCIPLE

- **This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy.**

**Citrate** $\longrightarrow$ **Oxaloacetate + acetate**

**Oxaloacetate** $\longrightarrow$ **Pyruvate +** $CO_2$

$CO_2 + Na + H_2O \longrightarrow Na_2CO_3$

- **Production of Na2CO3 as well as NH3 from utilization of sodium citrate and ammonium salt respectively results in alkaline ph.**
- **This results in change of medium's colour from green to blue.**

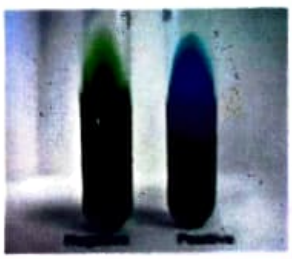
## PROCEDURE

- **Consider a slant, inject Simmons citrate agar gently on the slant by slightly touching the tip of the needle to the colony, which is 18 – 24 hours old.**
- **In the citrate medium, organisms require more time to grow. Therefore, the solution must be incubated at 35°C to 37°C for 18 – 24 hours.**
- **Some organisms may also require seven days of incubation.**
- **Observe the colour change in the solution, the development of blue colour indicates the alkylation process.**

## RESULT

**Positive result :**

- **The reagent used – Bromothymol blue, Growth on the medium indicates the change in the colour of the reagent from green to blue.**



**Negative result:**

- **Very less or no growth in the medium and colour of the solution remains the same.**

# UNIT- II
## (CHAPTER-7)
## STERILIZATION TECHNIQUES

## Points to be covered in this topic

1. INTRODUCTION
2. TYPES OF STERLIZATION METHODS
3. PHYSICAL STERLIZATION
4. RADIATION STERLIZATION
5. CHEMICAL STERLIZATION
6. MECHANICAL STERLIZATION



## ❑ INTRODUCTION

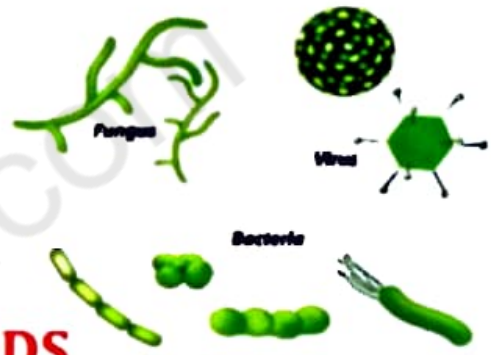
### ❖ STERLIZATION

- It is the process by which an **article, surface or medium** is made **free** of all **microorganisms either in vegetative or spore form.**
- It is the process of **absolute kill** of microorganisms.

## ❖ NEED FOR STERLIZATION

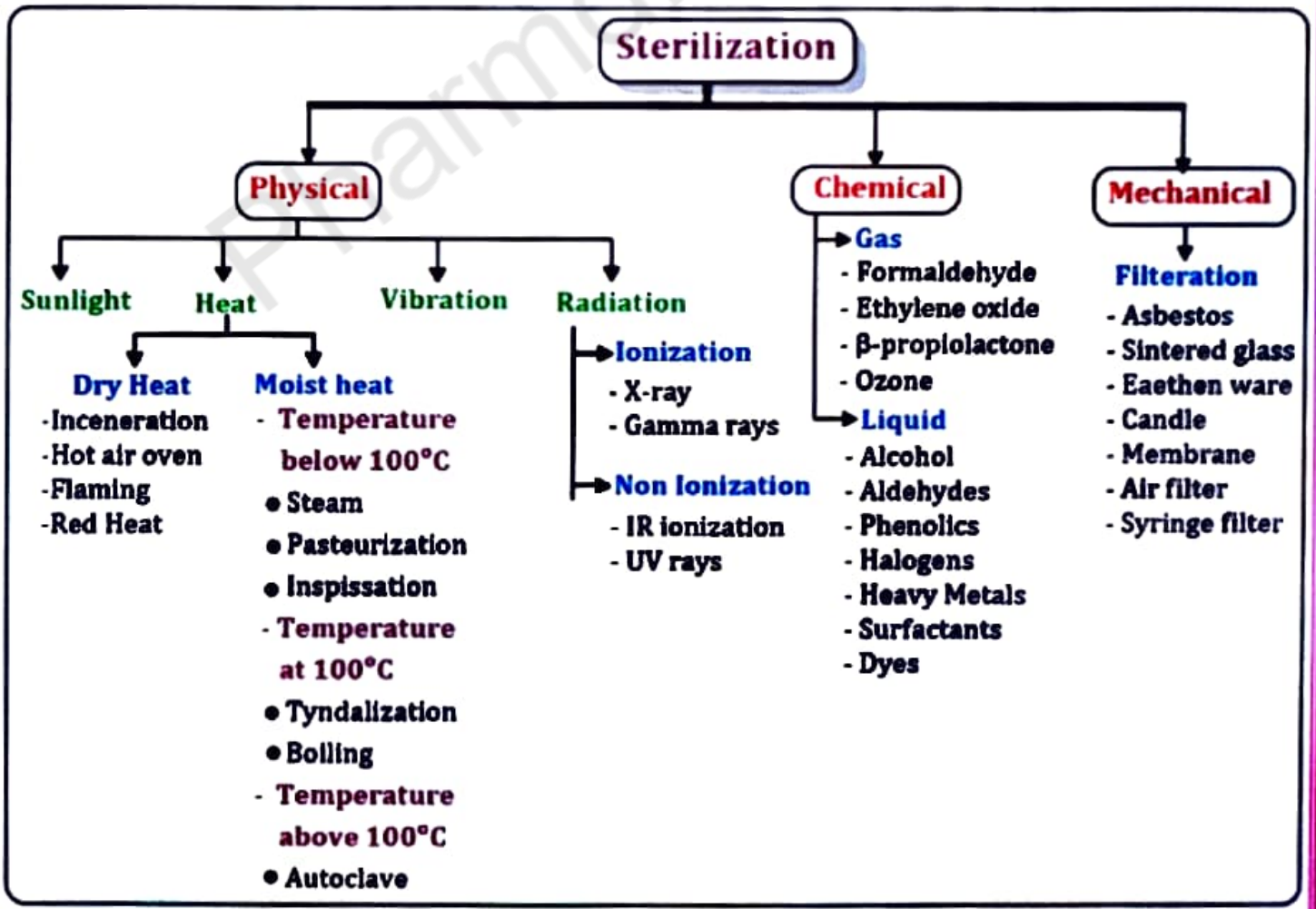
The growth of microorganisms can be controlled by two basic ways: -

- By either killing the microorganism,-
- Or by inhibiting the growth of microorganism.
- This is achieved by using physical or chemical agents which either kill or prevent the growth.
- The agents which kill the microorganisms are called 'cidal' agent
- & the agents which inhibit the growth (without killing them) are called static agents.




- ✓ **Bactericidal**-agents killing bacteria
- ✓ **Virucidal**-agents killing viruses
- ✓ **Fungicidal**-agents killing fungi

## ❖ TYPES OF STERLIZATION METHODS



## ❖ PHYSICAL STERLIZATION

- These methods involve processes by the use of **physical means**. They may involve the utilization of **heat in the presence or in the absence of moisture** or the applications of radiations or mechanical filtration.

### 1. DRY HEAT:

- Dry heat kills microorganisms by causing **protein denaturation & destructive oxidation of essential cell constituents.**
- Microorganisms are more resistance to dry heat thus this process requires **higher temperature** and **longer exposure time.**

### ➢ INCENERATION

Incineration is the process of sterilization along with a significant **reduction in the volume of the wastes.**

- The scraps are heated till they **become ash** which is then disposed of later.
- This process is conducted in a device called **incinerator**

➢ **PRINCIPLE :** It kills microorganisms by **destructive oxidation of essential cell constituents.**

➢ **ADVANTAGES :**

- Potentially destroy any material **containing organic carbon** including pathogens.
- Typically reduce the **volume and mass of material** that must be disposed off in landfills by 80 to 95 percent.
- Heat of combustion can be recovered and used to generate **steam or hot water.**

➢ **DISADVANTAGES:**

- Incineration may emit traces **amounts of unwanted pollutants** such as the **polychlorinated dibenzo dioxins** and **furans** and matter particulates if incinerators are not well designated and operated.
- The ash and waste water produced by the process also **contain toxin compounds**, which have t be **treated** to avoid adverse effects on health

and the environment.

- **EXAMPLES:** This is an excellent method for rapidly destroying materials e.g. pathological materiel, bedding, animals carcassec soiled dressing etc Polystyrene type of materials emit

clouds of dense black smoke and hence should be autoclaved in appropriate containers.

## ❖ FLAMING

Flaming is a type of dry sterilization that involves exposure of metallic objects to flame for some time where the flame burns microbes and other dust presents in the instrument.

In the case of flaming, the instrument is dipped in alcohol or spirit before burning it in a gas flame.

- **PRINCIPLE:** It kills microorganisms by destructive oxidation of essential cell constituents. ie: Glass slides, scalpels, and mouths of culture tubes or conical flasks are passed through Bunsen flame without allowing them to become red hot.

## ❖ RED HEAT

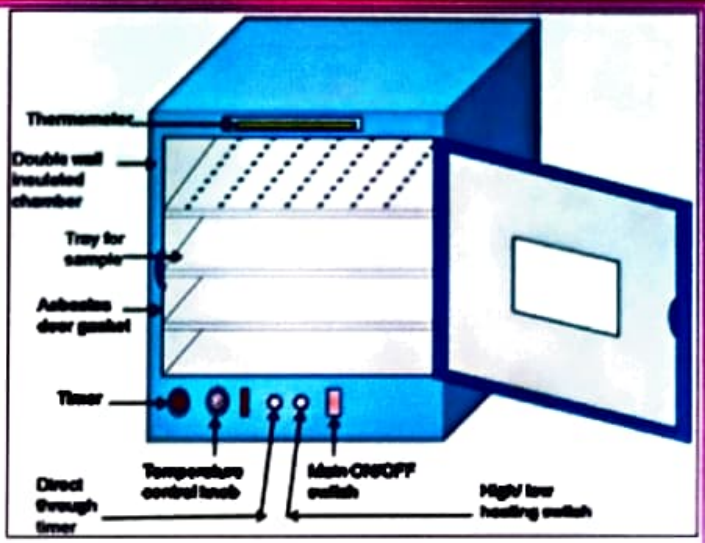
Red heat sterilization is the process of instant sterilization by holding the instruments in a Bunsen flame till they become red hot.

It is used to sterilize metallic objects by holding them on a flame till they are red hot. EX: inoculating wires, needles, forceps etc.

## ❖ HOT AIR OVEN

- ✓ **PRINCIPLE:** It kills microorganisms by destructive oxidation of essential cell constituents.
- ✓ **CONSTRUCTION:**
- The modern hot air ovens consist of a double walled chamber of aluminium or stainless steel separated from the outer case by a thick layer of insulation made of fiberglass.

- Insulation is also filled in the hollow flanged door, which carries an asbestos jacket that provides a tight seal.
- Heating is affected by electrical heating elements and thermostats automatically control temp.



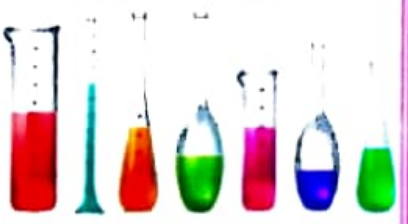

✓ **WORKING:**

The sterilization temperature & holding time for sterilization in hot air oven is 160°C for two hours is required for sterilization. 180°C for 30 minutes.

- 3 hours at 140°C.
- 1 hour at 160°C.
- 30 minutes at 1800C.

✓ **ADVANTAGES:**

- A dry heat cabinet is easy to install and has relatively low operating costs.
- It penetrates materials.
- It Is nontoxic and does not harm the environment.
- It is non-corossive for metal and sharp instruments.
- Does not require water.



✓ **DISADVANTAGES:**

- Time consuming method because of slow rate of heat penetration and microbial killing.
- High temperatures are not suitable for materials like rubber, plastic, etc.

✓ **APPLICATION:**

- Used to sterilize the equipment's like glassware, forceps, scissors, spatula, swabs, some pharmaceutical substances such as glycerin, fixed oil, paraffin propylene.
- Used in many industries for drying and baking and curing process.
- Used to sterilize powders and other non volatile compounds.

## 2. MOIST HEAT:

### ➢ PRINCIPLE

Sterilization by moist heat means killing of microorganisms with **hot water or steam.** The lethal effect of moist heat is due to the **denaturation and coagulation of proteins.**

### ➢ ADVANTAGES:

- High heat content plus **rapid heat transfer.**
- Destroys micro-organism **more efficiently** than dry heat.
- It can be used for a large number of **injections, Ophthalmic solutions, irritants, dialysis fluids etc.**
- It rapidly penetrates **porous materials** and is therefore very suitable for sterilizing surgical dressings and materials.
- The process is adaptable for **plastic containers** and some other special dosage forms.

### ➢ DISADVANTAGES:

- It is **not suitable** for anhydrous materials such **as powders and oils.**
- It cannot be used for **thermolabile substances.**
- It does not destroy pyrogen's.

### ❖ Temperature below 100 degree Celsius:

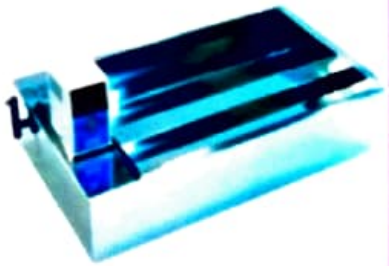
### ➢ PASTEURIZATION

- Pasteurization is done with **milk.**
- There are two different types of pasteurization methods that are used for sterilization of milk; **Holder method (63°C for 30 minutes) and flash method (72°C for 20 seconds).**
- This method is effective against all **non-sporing pathogens** such as **mycobacteria, Salmonella, etc.** except ***Coxiella burnetii*** which survives the **holder method** due to **heat resistant characteristics.**

- Similarly, **serum and body fluids** with congealable proteins are also sterilized at **56°C for 1 hour** in water baths.

## ➢ INSPISSATION

By this method **slow solidification of serum or egg** is carried out at **80°C to 85°C for one hour** in an inspissator e.g. **Lowestein-Jensen's medium, Serum slopes etc.**



This heating is used to **solidify & not sterilize** it. However, if the process is **repeated for days, some degree of sterilization is achieved** & is called **fractional sterilization.**

## ❖ Temperature at 100 degree Celsius:

## ➢ TYNDALLIZATION

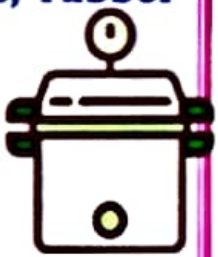
- Tyndallisation is also called as **intermittent sterilization or fractional sterilization.** Tyndallisation is a method that is used for sterilization of media with **sugar and gelatin** at **100°C for 30 minutes on three successive days** so as to preserve **sugar** which might be decomposed at a higher temperature.
- It is **not** recommended for **sterilization of instruments.** This method is **used to sterilize egg or serum, gelatin etc.**

## ➢ BOILING

Boiling for **10 to 30 minutes kills** mostly **the vegetative forms of bacteria, fungi and viruses** at 50-70 degree in short time, but many spores withstand boiling for considerable time.

Therefore boiling is **not adequate for sterilization** purpose.

This is not suitable for absolute sterility. **Glass syringe, tubes, rubber stopper or surgical instruments** are sterilized by this method.



## ❖ Temperature above 100 degree Celsius:

## ➢ AUTOCLAVE

The autoclave works on the principle of moist heat sterilization. The high **pressure inside the chamber increases** the boiling point of water for the

sterilization of equipment.

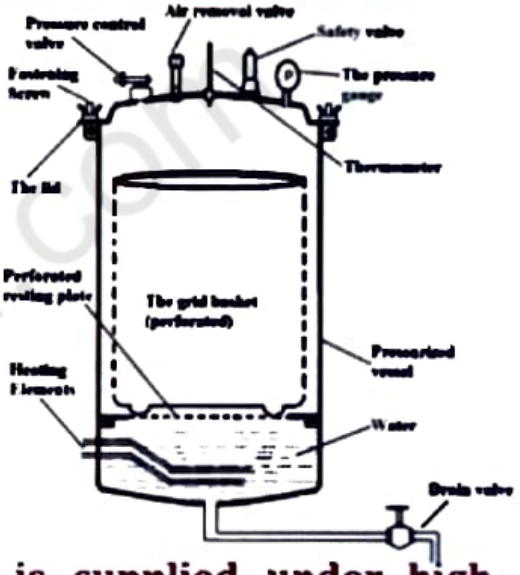
## ➢ PRINCIPLE

The higher pressure also ensures the rapid penetration of heat into the deeper parts of equipment. The moisture present in the steam causes coagulation of proteins of microbes causing irreversible loss of their activity and functions.

## ➢ CONSTRUCTION

The laboratory autoclave or pressure cooker type autoclave consists of a vertical or horizontal cylinder of gun metal or stainless steel in a supporting frame or case.

The lid is fastened by screw clamps and rendered airtight by a asbestos gasket. The autoclave has on its lid or upper side a discharge tap for air and steam a pressure gauge and a safety valve that can be set to blow off at any desired pressure.




## ➢ WORKING

- The steam circulates within the jacket & is supplied under high pressure to closed inner chamber where articles for sterilization are kept.

⬇

- One fifth of the cylinder is filled with water, materials to be sterilized are kept inside, lid closed & heater is put on. Safety valve is adjusted to required pressure.

⬇

- The boiling of water inside the chamber after sometime results in steam which is allowed to escape with air mixture fill the cylinder becomes air free.

⬇

- The discharge tap is closed and the desired pressure inside in chamber is allowed to rise to the one chosen for autoclaving for a fixed time Is thus complete sterilization achieved.

Standard operating procedures for autoclaving is: **115 °C, half an hour. 121 °C, 15 minutes.**

| S.NO | TIME | TEMPERATURE in degree Celsius | PRESSURE |
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| 1 | 80 min | 110 | 0 lbs |
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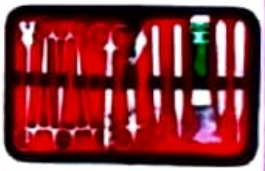
| 6 | 3 Min | 130 | 25- 30 lbs |

## ➢ ADVANTAGES

- **Rapid and effective**
- Effective for sterilizing cloth surgical packs and towel packs.
- Less time consuming.
- Applicable for both **thermoplastic and thermolabile components.**



## ➢ DISADVANTAGES

- Items sensitive to heat cannot be sterilized
- It tends to corrode carbon steel bum and instruments.
- It is **costly technique.**

## ✓ APPLICATIONS

- It is used to sterilize anything, which is **not injured by steam and high temperature of sterilization.** These includes, Aqueous parenteral solutions e.g. **distilled water, saline solutions**
- Aqueous liquid media e.g. **liquid media with or without carbohydrate and gelatin.**

- Surgical dressings and fabrics.
- Plastic and rubber closures.
- Metal instruments.
- Glass apparatus and containers.

## ❖ RADIATION STERLIZATION

- Sterilization by radiation is **also called as cold sterilization** because ionizing radiations produce relatively **little heat in the material being irradiated.**
- It is possible to sterilize **heat-sensitive substances.**
- Based on their wavelength and penetration power, radiation can be divided into two categories as **non-ionizing radiations& ionizing radiations.**
- **Non - ionizing radiations** - have **less energy** and do not disturb the atomic configuration of the target molecule.
- **Ionizing radiations - have energy and ionizing** the target molecules.

### ✓ PRINCIPLE

Breakage of **DNA and degradation of enzymes** lead to the death of the irradiated cells.

### ✓ ADVANTAGES

- **No degradation of media** during sterilization, thus it can be used for **thermally labile media.**
- **Leaves no chemical residue Administration** of precise dosage and **uniform dosage distribution.**
- Immediate availability of the media after sterilization.

### ✓ DISADVANTAGES

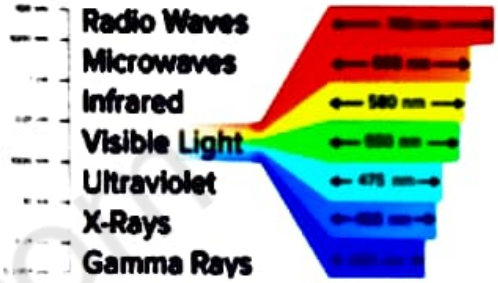
- This method is a more costly alternative to heat sterilization.
- **Requires highly specialized equipment.**

## 1. Ionizing radiations

They have **very high penetration power** and considerable energy. thus it can be used to **remove bacterial spores.**

### ➢ X RAYS

X-rays are lethal to microorganisms and higher forms of life but are rarely used in sterilization because their **production is expensive and efficient utilization is difficult** (since radiations are given off in all directions from the point of origin).




### ➢ GAMMA RAYS

Gamma radiations are **high-energy radiations** emitted from certain **radioisotopes such as Caesium-137 ($^{137}Cs$) and Cobalt-60 ($^{60}Co$),** both relatively inexpensive bioproducts of nuclear fission. Gamma rays are attractive for use in commercial sterilization of materials of considerable thickness or volume, **e.g. packaged food or medical devices.**

### ✓ ADVANTAGES OF IONIZING RADIATION

High penetrating power , Rapidity of action , **Temperature is not raised** ,Flexibility regarding **size, density**, matter of state.

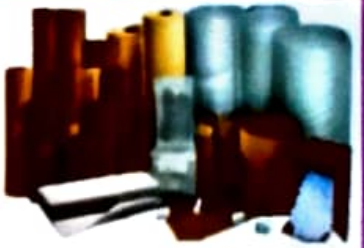
### ✓ DISADVANTAGES OF IONIZING RADIATION

- Capital costs are **high and specialized facilities are often needed e.g. for gamma irradiation.**
- Use of gamma radiation requires handling and disposal of **radioactive material**
- Not compatible with all materials and can **cause breakdown of the packaging material and/or product..**

### ✓ APPLICATIONS OF IONIZING RADIATION

- Sterilization of medical products (**e.g. insulin syringes**)
- Sterilization of **blood products , pharmaceutical products.**

- Preservation of foodstuffs (spices etc)
- Irradiation of cell cultures for research purposes.



## 2. Non - ionizing radiations

- X-ray; gamma rays and cathode rays are **highly lethal to DNA** and other vital cell constituents. since non-ionizing rays are of **low energy and have poor penetration power.**
- They are exposed on the substance to **remove bacteria** and microorganism

### ➢ ULTRAVIOLET RAYS

- Many cellular materials including **nucleic-acids** absorb ultraviolet light.

- It causes bonding of **two adjacent pyrimidines** i.e., the formation of pyrimidine dimer.
- Resulting in the **inhibition of DNA replication**. This leads to **mutation and death** of exposed organisms.
- Ultraviolet radiation is used for **disinfecting enclosed areas** such as bacterial **laboratory, nurseries, inoculation hood, laminar flow, and operation theatres.**
- Conventional UV light can **penetrate and damage skin and also cause cataracts.**
- Does not penetrate paper, glass, and cloth.

### ➢ INFRA- RED RAYS

Infra-red rays are **low energy type electromagnetic rays**, having wavelengths longer than those of visible light. They kill microorganisms by **oxidation of molecules as a result of heat generated.**

Infra-red rays are used for the rapid **mass sterilization of syringes and catheters.**

## ❖ CHEMICAL STERLIZATION

Chemical methods are **easy and economic-friendly**. They are of two types: gaseous and liquid.

### 1. LIQUID

Liquid sterilization destroy the microbes **permanently.**

#### ➢ ALCOHOL

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.

#### ➢ 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.

#### ➢ ALDEHYDE

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.

#### ➢ HEAVY METALS

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.**

### 2. GASEOUS

- Gaseous sterilization may be defined as the destruction of all living microorganisms with a **chemical in a gaseous or Vapour state.**
- Although **ethylene oxide is the most widely used gaseous sterilization agent.** other chemicals used are formaldehyde and B propiolactone.
- In addition to these, various glycols, methyl bromide and alcohol have

been used for room sterilization.

## ➢ FORMALDEHYDDE

- This gas is generated by heating a concentrated solution of formaldehyde.
- Formaldehyde in aqueous solution is known as formalin and contains 37 to 40% formaldehyde.
- Vaporization of formaldehyde, either from formalin or paraformaldehyde, is used to sterilize an enclosed area.
- It combines readily with vital organic nitrogen compounds such as proteins and nucleic acids. It is a bactericidal agent with poor penetrating power. It kills both vegetative cells and spores.

## ➢ B-PROPIOLACTONE

- It is capable of killing all microorganisms and is very active against viruses.
- It is highly bactericidal and used in concentrations of 2 to 5 mg/litre.

## ➢ ETHYLENE OXIDE

- Action of ethylene oxide is due to its power of alkylating the amino, carboxyl, hydroxyl and sulfhydryl groups in the enzymes and protein molecule. It reacts with DNA and RNA.
- Ethylene oxide is a powerful sterilizing agent for heat and moisture sensitive materials.
- It can be used for sterilization of medical and biological preparations, catgut, plastic equipment's, antibiotics, plaster bandages, culture media, hospital bedding, food stuff, heavy equipment, books, clothing and soil.

# ❖ MECHANICAL STERLIZATION

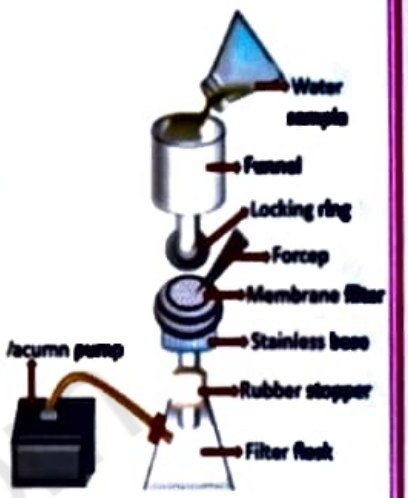
## ➢ FILTRATION

1. This method is used for sterilization of liquid substances or fluids such as sera & solutions of heat-labile substances such as sugars, urea, enzyme

& antibiotics which get damaged by heat process.

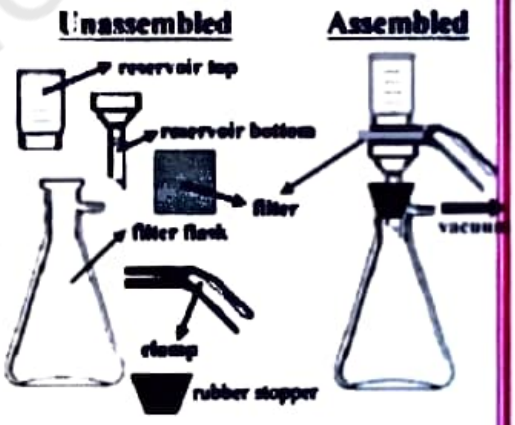
2. The method is also used for separation of bacteriophages & bacterial toxins from bacteria.
3. The following types of filters have been used for sterilization of different pharmaceuticals.

## ➢ MEMBRANE FILTERS



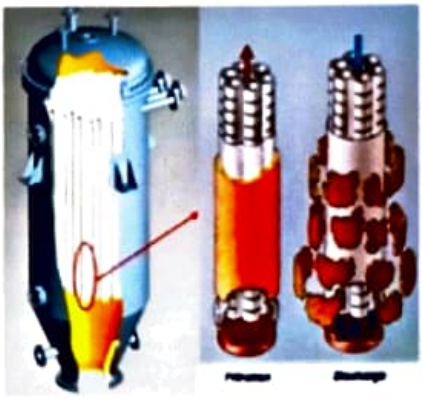

- The membrane filters are made of cellulose material.
- The membrane should be placed between needle and
- syringe while sterilizing the substance.
- This filter is effectively used in the sterilization of gas, solvent, and fluids.

## ➢ SEITZ FILTERS




The Seitz filters are made of asbestos material thus it has thick structure and strong enough to filter the solution. When solution passes through the Seitz filter, filter pad absorbs it and leaving the bacteria and residues on the top of the filter.

## ➢ SHINTERED GLASS FILTERS



Since the sintered glass filters are made of glass, it doesn't absorb liquids during filtration. The main drawback of practicing this method is that the filter is very soft and brittle and tend to break easily.

## ➢ CANDLE FILTERS

This modern mechanical filter is made of diatomous mud. It has minute pores that have the tendency to absorb microbes. When the fluid passes through the filters, microbes get stuck in the pores of candle filters.

| S.NO | CHEMICAL AGENT | MECHANISM OF ACTION | APPLICATION |
|---|---|---|---|
| 1 | Phenol and Phenolics | Plasma membrane; enzyme—denaturation and inactivation. | For instruments, mucous membranes, skin sur-faces, and environmental surfaces. |
| 2 | Chlorohexidine | Plasma membrane disrupted. | |
| 3 | Halogens | 1. Iodine inhibits protein function, a strong oxidising entity.<br>2. Chlorine forms the strong oxidizing agent hypochlorous acid that changes cellular constituents. | Used to disinfect glass wares, utensils, water. |
| 4 | Alcohol | Lipid dissolution and protein denaturation. | Disinfecting instruments. |
| 5 | Heavy metals and derivatives. | Denaturation of enzyme and other essential proteins. | Used as germicidal and antiseptics. |
| 6 | Surface active agents | 1. Acid anionic detergent-enzyme disruption and inactivation may take place.<br>2. Cationic detergents- protein denaturation, enzyme inhibition, disruption of plasma membrane. | Powerful antiseptics. |
| 7 | Organic acids | Metabolic inhibition- largely affecting moulds. | Effective at low PH, effective against microbes. |
| 8 | Aldehydes | Affords protein denaturation | Sterilizing medical equipment's |

# UNIT- II
## (CHAPTER- 8 )
## STERLITY EVALUATION

## Points to be covered in this topic

1. INTRODUCTION
2. MEMBRANE FILTRATION
3. DIRECT INOCULATION FILTRATION
4. EQUIPMENTS EMPLOYED IN LARGE SCALESTERLIZATION
5. STERLITY INDICATOR

## ❑ INTRODUCTION

- Sterility test is defined as **Microbiological test** applied to sterile product to show are products manufactured and processed under specification guided by cGMP, Or to **confirm the products either sterile or nonsterile.**
- Evaluation of efficiency of sterilization / Sterility testing Sterility tests can be carried out using following **methods**:

Method A: Membrane filtration.

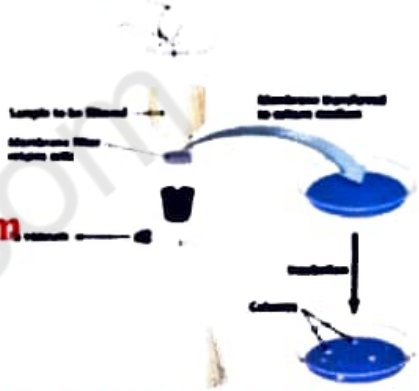
Method B: Direct Inoculation.

## ➢ Membrane filtration

- First clean the membrane filter unit and sterilize the unit and membrane filter separately by moist heat sterilization.

⬇

- Transfer the unit on laminar air flow bench or aseptic area and place the membrane filter in the unit.



⬇

- Pass all the solution through filter under vacuum

⬇

- After filtration, the membrane is removed aseptically and cut into two parts using sterile scissors.

⬇

- One half part of membrane filter is placed in 100ml of fluid thioglycollate medium (FTM) and incubate at 30-350C for not less than 7 days.

⬇

- Other half part of membrane filter is placed in soyabean casein digest medium (SCDM) and incubate at 20-250C for not less than 7 days.

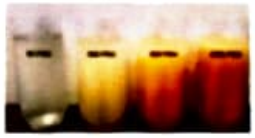
Observe the turbidity in the medium by comparing with the standard tube. If it has no turbidity in fluid thioglycollate medium and soyabean casein digest medium means it is free from microorganisms and suitable for use.

## ➢ Direct inoculation filtration

Cotton or prefilled syringe is transferred directly to culture media using sterile instruments such as sterile forceps.

- Incubate sample containing **fluid thioglycollate medium** (FTM) at **30-350C for not less than 7 days and soyabean casein digest medium (SCDM) at 20-250C for not less than 7 days.**

- Observe the **turbidity in the medium** by comparing with the standard tube.



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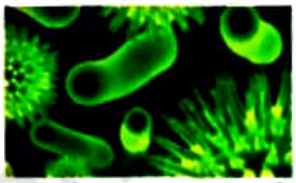


## Thermocouples:

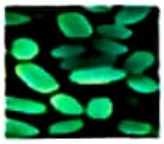
An autoclave's temperature can be measured using thermocouples. A particular autoclave temperature is deemed to be the appropriate temperature for sterilization,

## Brown Tubes:

Along with the articles, these tubes go into the autoclave. When the temperature inside the autoclave **reaches 121°**, these **tubes turn green**, which helps determine whether the **articles are properly sterilized.**

## Bacillus Stereo thermophilus Spores

These spores must be exposed to **121°C for 12 minutes** in order to be killed. Within the envelope are **placed paper strips containing 106 spores** before being placed in the autoclave. Inoculated with culture media, these strips are autoclaved and then inoculated. It is determined if these spores are not growing in the culture media it means **sterilization has been properly performed.**



## Autoclave Tape

In the autoclave, the product changes color when heated **to 121°C** due to lead carbonate. To check whether **the sterilization process has the ability to kill bacteria (microorganisms), and then to ensure that all microorganisms have been killed.**

• This can be measured by the following three factors:

➢ **D value (decimal reduction time):**

- The D value indicates the **time in minutes** it takes to destroy **90% of viable microorganisms at a constant(defined) temperature**. The less the value of D decreases (less).
- Then the more efficient sterilization will be. This is because D value is the time required to destroy microorganisms. **So less time (D value) results in killing more bacteria**, so sterilization becomes very efficient.

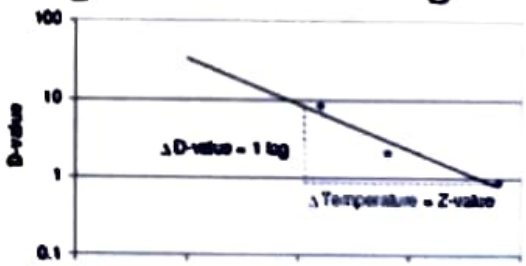
➢ **Z value:**

- This represents the **amount of temperature change** required to **decrease one point in D- value.**
- Change in temperature is the Z- value
- As the temperature **rises, more bacteria are killed** and the D- value decreases and **the Z- value increases.**
- As Z value is **proportional to the D- value and a lower D value** will result in a more efficient sterilization.

➢ **F- value:**

- Efficacy of a sterilization method is determined by the number **of minutes it takes to kill bacterial spores through heating**. It is possible to calculate the probability of survivors remaining from a load using the F value s follows;

$$[F = D(\log No - \log N)]$$




**Where,**

**No** = initial population volume original population number

**N** = number of final residents volume of units

**D**= D the organism's temperature at 121 degree Celsius.

# ❑ EQUIPMENTS EMPLOYED IN LARGE SCALE STERILIZATION

**List of equipment's employed in large scale sterilization:**

1. Autoclave
2. Hot air oven
3. Microwave
4. HEPA filter

## 1. AUTOCLAVE

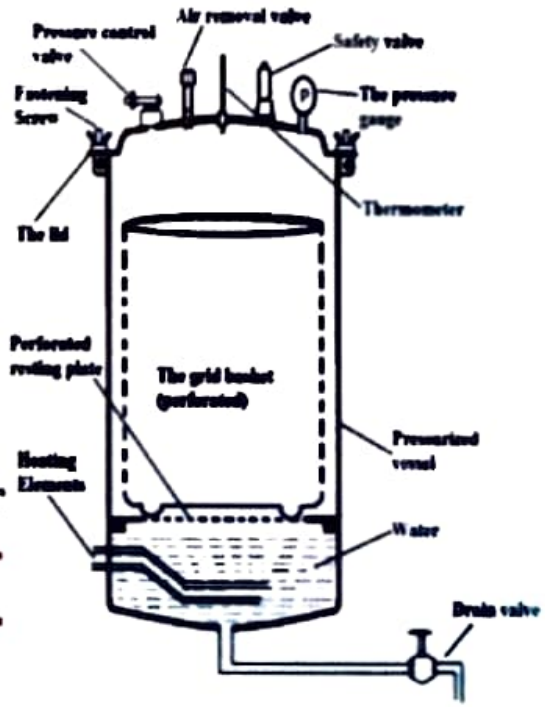
### ➢ Principle

The **higher pressure** also ensures the **rapid penetration of heat into the deeper parts of equipment**. The **moisture present in the steam causes coagulation of proteins** of microbes causing irreversible **loss of their activity** and functions

### ➢ Construction

**The autoclave is made of following components-**

1. Vessel or pressure chamber
2. Lid or door
3. Pressure gauge
4. Pressure releasing unit (whistle)
5. Safety valve
6. Electrical heater

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- The lid is fastened by screw clamps and rendered airtight by a asbestos gasket.
- The autoclave has on its lid or upper side a discharge tap for air and steam a pressure gauge and a safety valve that can be set to blow off at any desired pressure.

## ➢ Working

**Steps in Autoclave cycle-**

1. **Boiling phase:** The electric heat causes boiling of water and generate the steam. The produced steam replaces the trapped air by displacement.
2. **Rising temperature phase**: The temperature rises and reaches up to the set level i.e. 121°C.
3. **Sterilization time**: This is the time when microbes are killed.
4. **Release the pressure:** The entrapped pressure is released by opening the valve.

- The steam circulates within the jacket & is supplied under high pressure to closed inner chamber where articles for sterilization are kept.

⬇

- One fifth of the cylinder is filled with water, materials to be sterilized are kept inside, lid closed & heater is put on. Safety valve is adjusted to required pressure.

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- The boiling of water inside the chamber after sometime results in steam which is allowed to escape with air mixture fill the cylinder becomes air free.

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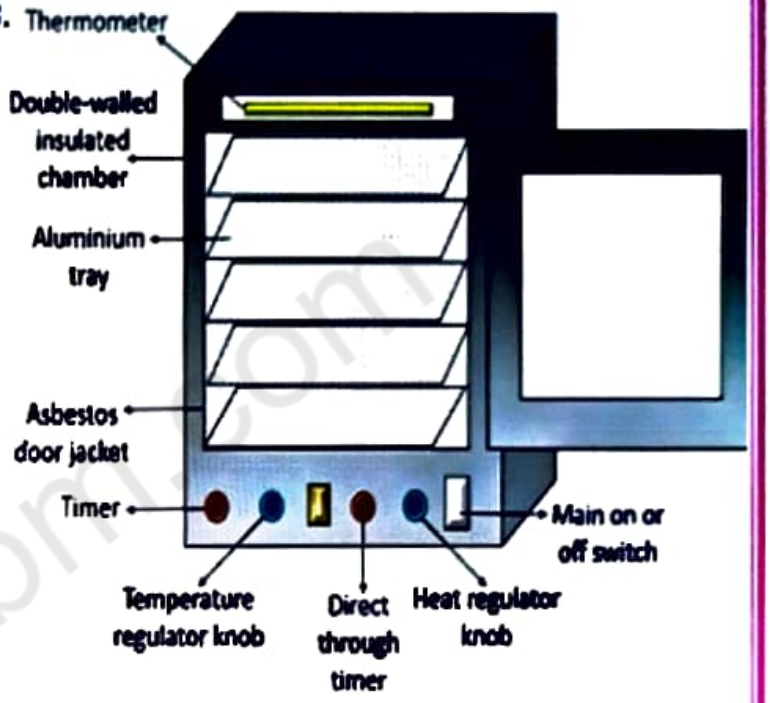
- It is used to sterilize anything, which is **not injured by steam and high temperature of sterilization.** These includes, Aqueous parenteral solutions e.g. distilled water, saline solutions
- Aqueous liquid media e.g. **liquid media with or without carbohydrate and gelatin.**
- Surgical dressings and fabrics.
- **Plastic and rubber closures.**
- Metal instruments.
- Glass apparatus and containers.

# 2. HOT AIR OVEN

➢ **Principle:** It kills microorganisms **by destructive oxidation of essential cell constituents.**

➢ **Construction:**

- The modern hot air ovens consist of a **double walled chamber of aluminium or stainless steel** separated from the outer case by a thick layer of insulation made **of fiberglass.**
- Insulation is also filled in the hollow flanged door, which carries an **asbestos jacket that provides a tight seal.**
- Heating is affected by electrical heating elements and **thermo-stats automatically control temperature.**




➢ **Working**

- Oven is an electrical devise that works on **Heat convection principle.** It consist of electrical heating coil that produces dry heat. In the chamber, the produced **dry heat air displaces the cooled air forming heat gradient.** The use of fan allows to the uniform distribution of heat.

| S. NO. | TEMPERATURE | TIME |
|---|---|---|
| 1 | 170°C | 30 Min |
| 2 | 160°C | 60 Min |
| 3 | 150°C | 150 Min |

## ➢ Application

- Used to sterilize the equipment's like glassware, forceps, scissors, spatula, swabs, some pharmaceutical substances such as glycerin, fixed oil, paraffin propylene.
- Used in many industries for drying and baking and curing process.
- Used to sterilize powders and other non volatile compounds.

## ➢ Advantages

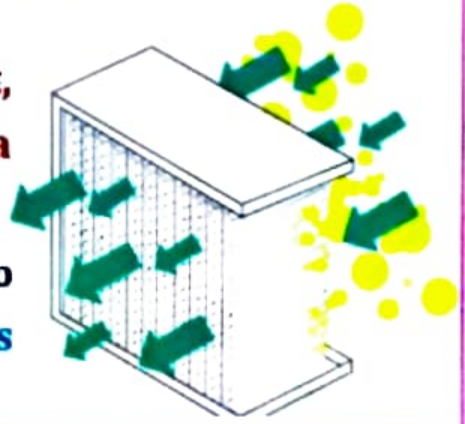
1. It is Eco-friendly
2. It is most efficient method to degrade microbial endotoxins.
3. It is safer than autoclave.
4. It is the ideal instrument for sterilizing oil and powders.

## ➢ 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.

## 3. HEPA FILTER

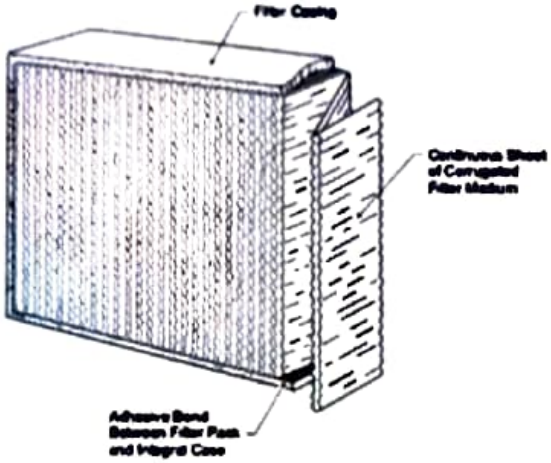
Air purifiers reduce air pollutants by removing particulate matter (HEPA), or High-Efficiency Particulate Absorbers. An example of a filter with 99.97 percent efficiency would be one that can trap particles smaller than 93 microns. During the pass of a HEPA filter, particles are emitted in four directions :

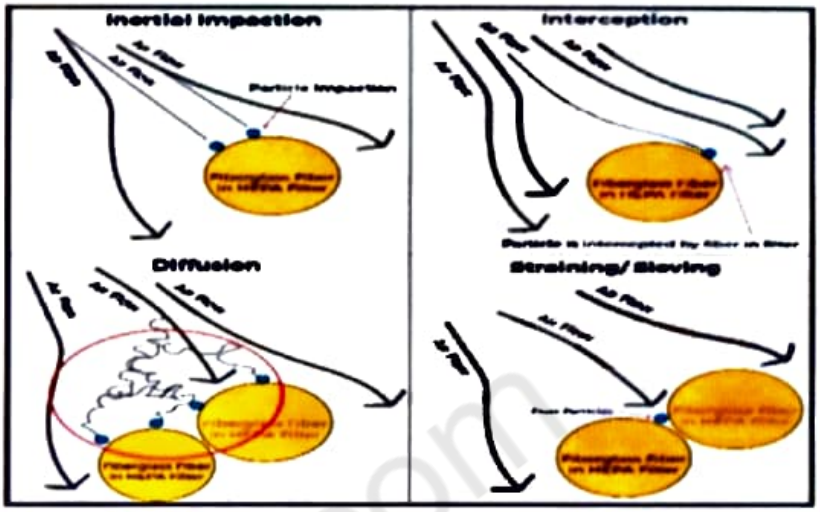


1. Direct impaction - large contaminants like dust, mold, pollen, and mold adhere to fiber In a straight line and travel directly through It.
2. Sieving - When an air particle moves between two fibers, ifs bigger than the gaps, and thus becomes ensued .

**3. Interception -** The air is capable of **rerouting around fibers**, but due to inertia, particles continue on **their path and remain attached to the fibers.**

**4. Diffusion -** It is more likely that **ultrafine particles will hit** and stick to fibers because they move **more erratically than larger ones.**




## ➢ ADVANTAGES

- In addition to sterilizing thermolabile medications, **such as blood products, insulin, and enzymes,** the method is also suitable for sterilizing blood plasma.
- During the preparation, **all types of bacteria are removed** including those that are alive and those that are dead.
- The sterilization process is **performed simultaneously** with the clarification process.
- A parenteral solution in a small quantity can be rapidly supplied in an emergency with this method.

## ➢ DISADVANTAGES

- Sterility testing is necessary because the **method is not reliable.**
- With this method, it is not possible to **sterilize suspensions and oily preparations.**
- Filters may allow medicaments to **be absorbed from a solution.**
- There are no immediate indications when the media is defective. **Aseptic techniques must be used .**
- Staff must be highly trained.

## ➢ APPLICATIONS

Parenteral solution containing **thermolabile medicines** can be sterilized using this method without decomposition, **such as insulin, blood stream, and other products containing protein matter**, such as heat sensitive injections and biological products.

# ❑ STERLITY INDICATORS

It is essential that strict controls are carried out on products to be labeled **'sterile.'** Such controls must then ensure, the absence of viable microorganisms from these products.

There are basically two types of controls:

1. Controls on the process of **sterilization i.e. sterilization monitors or sterilization indicators.**
2. Sterility testing of the products.
3. Monitoring of the sterilization process can be achieved by the use of **physical, chemical or biological indicators** of the sterilization performance.

## ➢ PHYSICAL INDICATORS

### 1. Moist heat:

A master process record (MPR) is prepared as **part of the validation procedure for a particular** autoclave and for each specified product and load configuration. The MPR should be checked at annual intervals and whenever **significant changes occur in the BPR** when compared with the MPR.

### 2. Dry heat:

In dry heat sterilization processes, a **temperature record chart made of each sterilization cycle** and is compared against a master temperature record.

### 3. Gaseous:

- For gaseous sterilization procedures, elevated **temperatures are monitored for each sterilization cycle by** temperature probes and routine leak. tests are performed to ensure **gas-tight seals.**
- Gas concentration is measured independently of pressure rise, often by reference to the weight of gas used. **Pressure and humidity measurements are recorded.**

### 4. Filtration:

- **Bubble point pressure test** is a technique employed for determining the pore size of filters and may also be used to **check the integrity of certain types of filter devices immediately after use.**

- The principle of the test is that the filter is soaked in an appropriate fluid and **pressure is applied to the filter.**
- The pressure difference when the **first bubble of air breaks away from the filter is equivalent to the maximum pore size.** When the air pressure is further increased slowly, there is general eruption of bubbles over the entire surface. **The pressure difference is equivalent to the mean pore size.**

## ➢ CHEMICAL INDICATORS

Chemical monitoring of a sterilization process is based on the **ability of heat, steam, sterilant gases and ionizing radiation** to alter the chemical or physical characteristics of a **variety of chemical substances.**

### 1. Browne tubes

Each tube consists of a **sealed glass tube** which contains a **red fluid (an ester and acid - base indicator) that changes to yellow, brown** and finally green on heating(the ester undergoes heat hydrolysis to form an acid + alcohol. **The acid will change the color of the indicator.**

## 2. Witness tubes:

- Witness tubes consist of single crystalline substances of known melting point contained in glass tubes e.g. Sulphur (115°C), succinic anhydride (120°C), benzoic acid (121°C) etc. A dye may be included to show more clearly that the crystals have melted.
- Such a device only indicates that a certain temperature has been reached. Exposure time can be calculated by putting the crystals in one end of an 'hour-glass' tube, the volume of the crystals and the diameter of the constriction of the tube being adjusted so that the time for transfer of the melt is the same as that required for the sterilization at the required temperature

## 3. Royce sachet:

- The Royce sachet is a chemical indicates for ethylene oxide sterilization. This consists of a polythene sachet containing magnesium chloride, HCl and a bromophenol blue indicator. A given concentration-time exposure to ethylene oxide results in the formation of ethylene chlorohydrin and a color change from yellow to purple.

## ➢ BIOLOGICAL INDICATORS

1. Biological indicators consist of a suitable organism deposited on a carrier and are distributed throughout the sterilizer load.
2. At the end of the sterilization process, the units are recovered and cultured to determine the presence or absence of survivors. The biological indicator measures sterilization processes directly and is able to integrate all sterilization parameters.
3. The selected organism should possess high and reproducible resistance to the sterilizing agent, should be genetically stable, readily characterizable and non-pathogenic.
4. The viability of the organisms, the storage conditions before use and the incubation and culture conditions after sterilization must be standardized for the results. The organisms used as biological indicators are usually resistant bacterial spores.