IDENTIFICATION OF BACTERIA

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

SIMPLE STAINING PROCEDURE AND ITS MECHANISMS

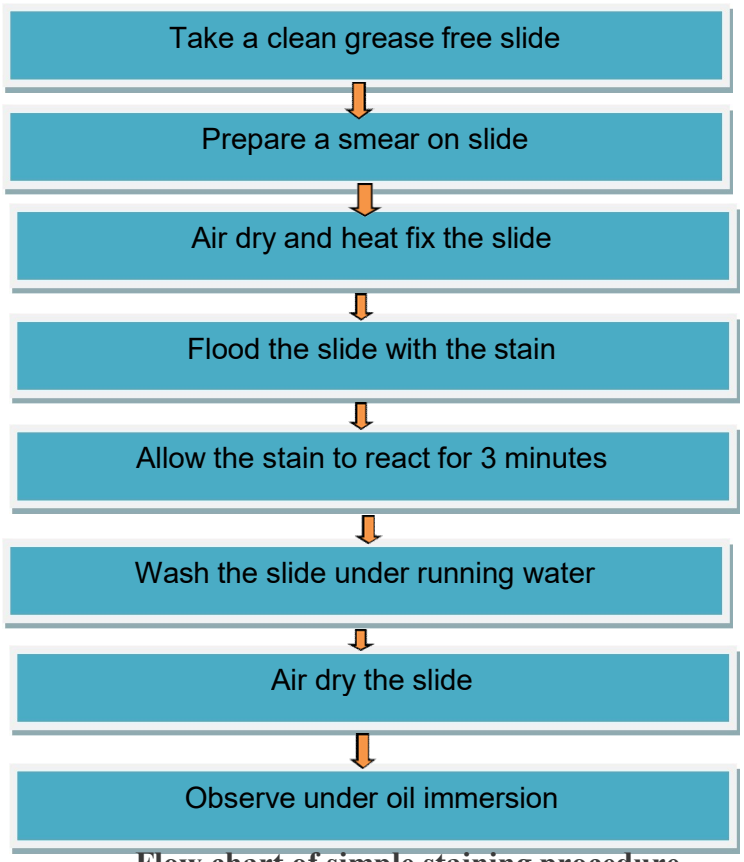
INTRODUCTION

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

PROCEDURE

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

SIMPLE STAINING PROCEDURE



Flow chart of simple staining procedure

MECHANISM

- A stain has ability to bind a cellular component. These abilities depend upon the charges present on cellular component and charges present on chromophore group of stain.
- Bacteria have large number of carboxyl group on its surface and these carboxyl group have negative charge.
- When these carboxyl group carry out ionization reaction it shows COO^- and H^+
- That is $COOH = COO^- + H^+$.
- In nature these H^+ ions are present on cell surface and further replaced by other positively charged ions like Na^+ or k^+.
- Now when these simple stains are used it has chloride group
- Further these stain carry out dissociation for example if the stain is Malachite green it will carry out dissociation and give free radicals
- That is $MgCl = Mg^+$ and Cl^-
- Now these free Mg^+ ions give positive charge on chromophore group.
- When these stain is applied to a cell these positively charged Mg^+ ions replace the K^+ or Na^+ present on cell surface.
- Thus a ionic bond is formed in between positively charged Mg + ions and cell surface.
- Thus it results in staining of cell.

APPLICATIONS

- Simple staining procedure stains bacteria easily and helps in observation under microscope.
- It is useful in preliminary studies of morphological characters of cell that is its size, shape and arrangement.[1]

GRAM'S STAINING PROCEDURE AND ITS MECHANISM

INTRODUCTION

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

REQUIREMENTS

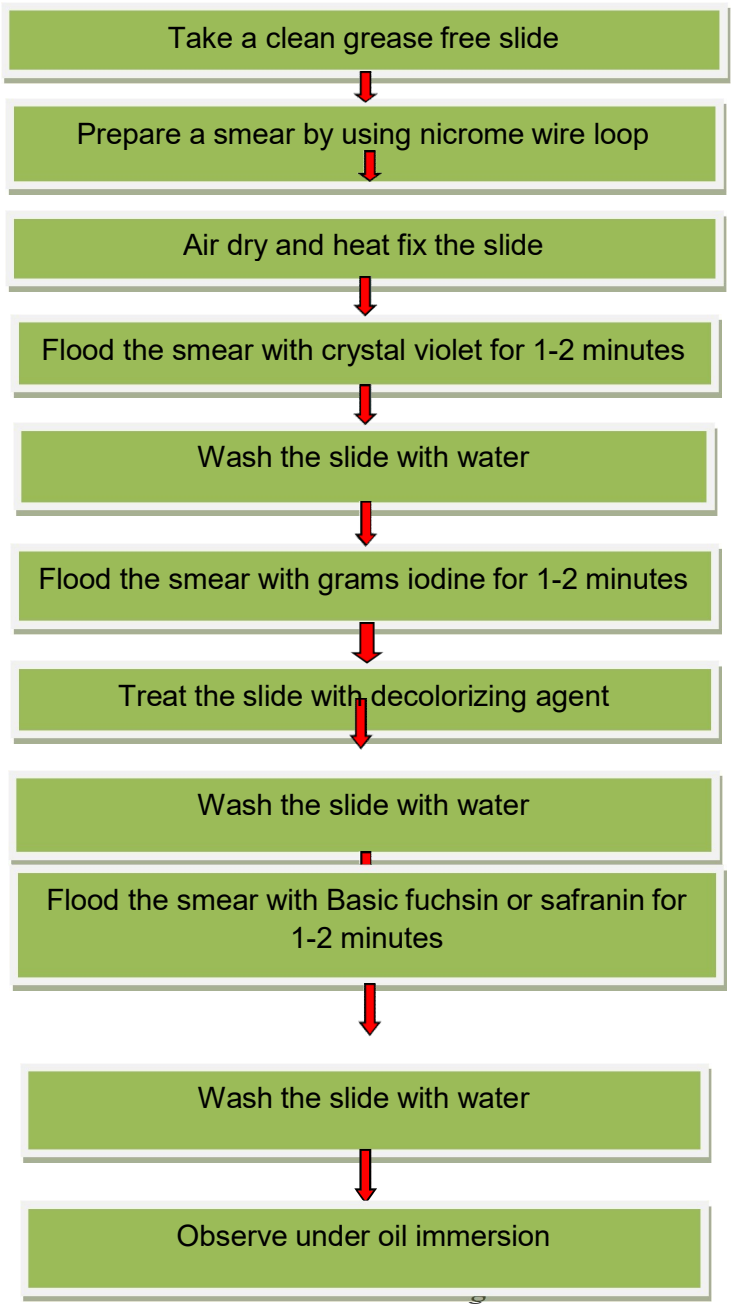
1. A clean grease free slide.
2. Bacterial cell suspension.
3. Nichrome Wire loop.
4. Primary stain - Crystal violet.
5. Mordant- Gram's Iodine.
6. Decolorizing agent - 95% alcohol (95% Ethanol).
7. Counterstain- Basic fuschin or Safranin.

PROCEDURE

1. Take a clean grease free slide.
2. Prepare a smear from a bacterial cell suspension on a slide by using nichrome wire loop.
3. Air dry and heat fix the smear.

4. Flood the smear with a primary stain that is Crystal violet and allow it to react for 1-2 minutes.
5. After Crystal violet treatment water wash treatment is given to the slide.
6. Further, the smear is treated with the mordant that is Gram's Iodine for 1-2 minutes.
7. Excess Gram's Iodine is removed and the slide is further treated with a decolorizing agent that is 95 % Ethanol.
8. After Ethanol treatment the smear is water washed and flooded with counter stain that is Basic fuchsin or Safranin for 1-2 minutes.
9. Finally, the slide is washed with water, air dried and observed under oil immersion.

GRAM STAINING PROCEDURE



GRAM STAINING PROCEDURE

1. Cells stained with crystal violet appear violet color are Gram-positive cells.
2. Cells stained with counter stain i.e Basic fuchsin or safranin appear pink in colour are Gram-negative cells.

FUNCTION OF AGENTS USED IN GRAM'S STAINING

1. Crystal violet – It is a primary stain and a basic dye it stains all micro-organisms.
2. Gram's Iodine – Gram's Iodine acts as a mordant and it forms a complex with crystal violet that is CV-I complex. This complex increases affinity between cell and stain.
3. 95% Alcohol (95% Ethanol) – It is a decolorizing agent as well as a lipid solvent. It tries to decolorize the cell by removing the CV-I complex from the cell.
4. Basic fuschsin or Safranin – It acts as a counterstain. It stains the cells that are decolorized by alcohol. Only Gram-negative bacteria get decolorize and this counter stain gives pink color to these cells.

MECHANISM

1. When a smear is stained with crystal violet it stains all cells to violet color.
2. After application of Gram's Iodine, its molecules acts as a mordant and forms a crystal violet – Gram's Iodine complex that is CV-I complex.
3. After CV – I complex formation this smear is subjected to decolorizing treatment by using 95% Ethanol for 30 seconds.
4. The gram-positive cell has some special features due to which CV – I complex is unable to come outside the cell they are-

- The gram-positive cell has 1 to 4 % of lipid content due to low lipid content the cell get dehydrated by alcohol treatment and its pore size decreases so CV – I complex is trapped inside the cell.
- Peptidoglycan layer account about 40 to 90% of the dry weight of Gram-positive cell so due to extremely dense cross-linkage CV – I complex is trapped inside the cell.
- The gram-positive cell contains Magnesium ribonucleate so this compound Magnesium ribonuclease molecule forms a covalent bond with CV – I complex and thus it doesn't allow CV – I complex to come outside the cell.
- The gram-negative cell contains 11 to 20 % of lipid content when Gram-negative cells are suspended in alcohol it dissolves the lipid and thus CV – I complex comes out.
- Peptidoglycan content in Gram-negative cell wall is 5 to 10 % so due to less amount of cross-linkage CV – I complex comes out easily.
- Gram-negative cell lacks Magnesium ribonucleate molecules so CV – I complex is extracted easily from the cell.

7. The cells which get decolourised by alcohol take the counterstain and appear pink in color these cells are Gram-negative cells.

6. After decolourisation treatment, the smear is treated with counterstain i.e Basic fuschin and Safranin.

APPLICATIONS

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

EXAMPLES

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

ACID FAST STAINING TECHNIQUES AND ITS DETAILS

INTRODUCTION

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

DEFINITION

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

REQUIREMENT

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

PROCEDURE

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

Flowchart of the Acid Fast staining procedure

FUNCTION OF REAGENTS USED IN ACID FAST STAINING

1. ZNCF (ZIEHL NEELSON OR CARBOL FUCHSIN)

- It is a primary stain.
- Many acid-fast bacteria are not stained with the common stain like carbol fuchsin because they are prepared in aqueous solution.
- These acid fast bacteria require a stain that is prepared in phenolic stain and ZNCF stain is prepared in phenolic solution.
- As these acid-fast bacteria have a waxy covering on their surface and phenolic stain solubilizes waxy covering and stains the cell.
- The cells stained with ZNCF appear pink in color.

2. ACID ALCOHOL

- It is the decolorizing agent.
- It is prepared from the combination of acid that is 3% hydrochloric acid and alcohol that is 95% ethanol.

3. 1% MALACHITE GREEN or 0.3 % MYTHELENE BLUE

- It acts as a counter stain.
- It stains the decolorized cell and these cells appear green or blue in colour.

MECHANISM

1. Acid-fast bacteria have a waxy covering on its surface or we can say it has high lipid content in the cell wall.
2. The cell wall of acid-fast bacteria is made up of lipids like Mycolic acid and Glycolipids.
3. Due to these high lipid content in the cell wall, these cell wall has less permeability.
4. So first it is necessary to increase the permeability of the cell wall so the stain can easily penetrate in the cell.
5. The permeability of the cell wall is increased by using phenolic solution and steaming.

6. After the permeability of the cell increases the cells get the stain.
7. Now once the acid-fast bacteria get stained it doesn't decolorize even by the strong decolorizing agent and appear pink in color.
8. But the non-acid fast bacteria get decolorize easily and get stained by counter stain and appear green or blue in color.
9. If we use Malachite green stain cells get the stain and appear green in color and if we use Methylene blue stain cells get the stain and appear blue in color.

OBDERVATION

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

SOME IMPORTANT POINTS

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

APPLICATIONS

1. Acid-fast staining is useful in the diagnosis of Tuberculosis and leprosy.[3]

BIOCHEMICAL TESTS

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

IMViC Tests

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

GENERAL PROCEDURE FOR PERFORMING IMViC Tests

INDOLE TEST:

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

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

METHYL RED (MR) TEST:

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

VOGES-PROSKAUR (VP) TEST:

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

CITRATE UTILIZATION TEST:

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

STERILIZATION

- Sterilization is the complete removal of microorganisms from an object or surfaces.
- Sterilization is obtained when microorganisms are subjected to antimicrobial agents for sufficient time and at optimum conditions.

Some physical methods associated with sterilization are explained below

PHYSICAL METHODS OF STERILIZATION

HEAT STERILIZATION

- Heat sterilization is the most effective and widely used method of sterilization, where the bactericidal activity results through the destruction of enzymes and other essential cell constituents.
- The effects of heat sterilization occur more rapidly in a fully hydrated state, as it requires a lower heat input, with low temperature and less time, under high humidity conditions where the denaturation and hydrolysis reactions are predominant, rather than in the dry state where oxidative changes take place.
- Under circumstances where thermal degradation of a product is possible, it can usually be minimized by adopting a higher temperature range, as the shorter exposure times generally result in a lower partial degradation.
- This method of sterilization is applicable to thermostable products. Still, it can be applied to both moisture-sensitive and moisture-resistant products, for which dry (160–180°C) and moist (121–134°C) heat sterilization procedures are respectively used.

A) DRY HEAT STERILIZATION

- Dry sterilization is the process of removing microorganisms by applying moisture-free heat which is appropriate for moisture-sensitive substances.
- The dry heat sterilization process is based on the principle of conduction; that is the heat is absorbed by the outer surface of an item and then passed onward to the next layer. Ultimately, the entire item reaches the proper temperature needed to achieve sterilization.
- Dry moisture-less heat destroys microorganisms by causing denaturation of proteins and also lyses the proteins in many organisms, causes oxidative free radical damage, causes drying of cells, and can even burn them to ashes, as in incineration
- Dry heat sterilization is used for the sterilization of materials which are difficult to sterilize by moist heat sterilization for several reasons.
- Substances like oil, powder, and related products cannot be sterilized by moist heat because moisture cannot penetrate into deeper parts of oily materials, and powders are destroyed by moisture.
- Similarly, laboratory equipment like Petridishes and pipettes are challenging to sterilize by moist heat due to the penetration problem.
- The lethal effects of dry heat on microorganisms are primarily due to oxidative processes which are less effective when compared to the hydrolytic damage that results from exposure to steam in moist heat sterilization.
- Thus, in dry heat sterilization usually higher temperatures in the range 160–180°C are employed and also require exposure times of up to 2 hours depending upon the temperature employed.
- This principle is used in instruments like hot air oven and incineration, which generates very hot moisture-free air.
- The primary industrial application of dry heat sterilization is in the sterilization of glass bottles which are to be filled aseptically.

- In addition to the fact that this method achieves an adequate sterility assurance level, this method also destroys bacterial endotoxins (which are the products of Gram-negative bacteria also called pyrogens, which cause fever when injected into the body) which are difficult to eliminate through other sterilization techniques.
- For the purposes of depyrogenation of glass, temperatures of approximately 250°C are used.
- There are different types of dry heat sterilization which are explained below:

Table 1:(Temperature time relationship in hot air oven)

Working
• The most common time –temperature relationships for sterilization with hot sterilizers are
• 170°C(340°F) for minutes,
• 160°C(320°F) for 60 minutes, and
• 150°C(300°F) for 150 minutes or longer depending up the volume

RED HEAT

- Red heat sterilization is the process of instant sterilization by holding the instruments in a Bunsen flame till they become red hot.
- This method is based on dry heat sterilization is commonly used for sterilization of instruments like incubation loops, wires, and points of forceps.
- This process ensures effective sterilization; however, it is only limited to substances that can endure heating until redness in flame.

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.
- This process doesn't ensure sterility and is not as effective as red heat sterilization.

INCENERATION

- Incineration is the process of sterilization along with a significant reduction in the volume of the wastes.
- It is usually conducted during the final disposal of the hospital or other residues.
- The scraps are heated till they become ash which is then disposed of later.
- This process is conducted in a device called incinerator.

HOT AIR OVEN

- Hot air oven is a method of dry heat sterilization which allows the sterilization of objects that cannot be sterilized by moist heat.
- It uses the principle of conduction in which the heat is first absorbed by the outer surface and is then passed into the inner layer.
- A hot air oven consists of an insulated chamber that contains a fan, thermocouples, temperature sensor, shelves and door locking controls.
- The commonly-used temperatures and time that hot air ovens need to sterilize materials are 170°C for 30 minutes, 160°C for 60 minutes, and 150°C for 150 minutes.

- These ovens have applications in the sterilization of glassware, Petri plates, and even powder samples.

Fig.1:(HOT AIR OVEN)

B) MOIST HEAT STERILIZATION

- Moist heat sterilization is one of the most effective method of sterilization where the steam under pressure acts as a bactericidal agent.
- Moist heat sterilization usually involves the use of steam at temperatures in the range 121–134°C.
- High pressure increases the boiling point of water and thus helps achieve a higher temperature for sterilization.
- High pressure also facilitates the rapid penetration of heat into deeper parts of material and moisture present in the steam causes the coagulation of proteins causing an irreversible loss of function and activity of microbes.
- The high temperature-short time cycles not only often result in lower fractional degradation, but they also provide the advantage of achieving higher levels of sterility assurance due to more significant inactivation factors.
- The most commonly used standard temperature-time cycles for clinical porous specimens (e.g. surgical dressings) and bottled fluids are 134°C for 3 minutes and 121°C for 15 minutes, respectively.
- An autoclave is a device that works on the principle of moist heat sterilization through the generation of steam under pressure.

- In this method, the microorganisms are killed by coagulating their proteins, and this method is much more effective than dry heat sterilization where microbes are killed through oxidation.
- In the pharmaceutical and medical sectors, it is used in the sterilization of dressings, sheets, surgical and diagnostic equipment, containers, and aqueous injections, ophthalmic preparations, and irrigation fluids, in addition to the processing of soiled and contaminated items.
- Moist heat can be used in sterilization at different temperatures:

AT TEMPERATURE BELOW 100°C

- The sterilization technique employed at a temperature below 100°C involves pasteurization.
- In this process, all non-spore forming microbes are killed in milk by subjecting the milk to a temperature of 63°C for 30 minutes (the holder method) or 73°C for 20 seconds (the flash method).
- In pasteurization, however, not all the pathogenic organisms are killed. The principle of pasteurization is the logarithmic reduction in the number of viable microbes so that they can no longer cause diseases.
- All mesophilic non-sporing bacteria can be killed by exposure to a moist heat at 60°C for half an hour with the exception of some organisms which require different temperature-time cycles.
- The milk is not heated above its boiling point as the milk might curdle, and its nutritional value might be destroyed.
- Besides milk, other fluids and equipment like vaccines of non-sporing bacteria are also pasteurized at 60°C for 1 hour in special water baths.
- Similarly, serum and body fluids with congealable proteins are also sterilized at 56°C for 1 hour in water baths.

AT A TEMPERATURE OF 100°C

- Boiling at 100°C is a moist heat sterilization technique that doesn't ensure complete sterility, but is enough for the removal of pathogenic vegetative microbes and some spores.
- In this case, the items to be sterilized are immersed in boiling distilled water for 30-40 minutes.
- Distilled water is preferred because hard water might result in the formation of a film of calcium salts on the instruments.
- Tyndallization 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.
- Moist heat at 100°C is applicable for contaminated dishes, beddings, pipettes, and other instruments that are not soiled or contaminated as well as for objects that are temperature sensitive.

AT TEMPERATURES ABOVE 100°C

- Moist heat sterilization above 100°C involves sterilization by steam under pressure.
- Water usually boils at 100°C under normal atmospheric pressure (760 mm of Hg); however, the boiling point of water increases if the pressure is to be increased.
- This principle is employed in an autoclave where the water boils at 121°C at the pressure of 15 psi or 775 mm of Hg.

- As a result, the steam under pressure has a higher penetrating power. When this steam comes in contact on the surface, it kills the microbes by giving off latent heat.
- The condensed liquid ensures the moist killing of the microbes.
- Autoclaves are used for the sterilization of contaminated instruments along with different culture media as it ensures complete sterility.

Fig.2:(AUTOCLAVE)

Table 2:(Pressure temperature relationship in autoclave)

Pressure temperature relations in Autoclave		
Pressure in psi	Temperature in °C	Temperature in °F
5	109	228
10	115	240
15	121	250
20	126	259
25	130	267
30	135	275

Table 3:(Heat sterilization method, its mechanism, merits, demerits & applications)

Sl no	Method	Mechanism	Merits	Demerits	Applications
1	Heat sterilization	Destroys bacterial endotoxins	Most widely used and reliable method of sterilization involving destruction of enzymes and other essential cell constituents	Can be applied only to the thermastable products	Dry heat is applicable for sterilizing glasswares and metal surgical instruments and moist heat is the most dependable method for decontamination of laboratory waste and the sterilization of laboratory glassware, media and reagents.

IRRADIATION

- Irradiation is the process of exposing surfaces and objects to different kinds of radiation for sterilization.
- Mainly electromagnetic radiation is used for sterilization.
- The major target for these radiations is considered to be microbial DNA, where damage occurs as a result of ionization and free radical production (gamma-rays and electrons) or excitation (UV light).

A) NON IONOZING

INFRARED RADIATION

- Infrared radiation (IR) is a method of thermal sterilization in which the radiation is absorbed and then converted into heat energy.
- For this purpose, a tunnel containing an IR source is used. The instruments and glassware to be sterilized are kept in a tray are then passed through the tunnel on a conveyer belt, moving at a controlled speed.
- During this movement, the instruments will be exposed to the radiation, which will result in a temperature of about 180°C for about 17 minutes.
- IR is applicable for mass sterilization of packaged items like syringes and catheters.

ULTRAVIOLET RADIATION

- Ultraviolet radiation includes light rays from 150-3900 Å, of which 2600 Å has the highest bactericidal effect.
- Non-ionizing waves have a very little penetration power, so microorganisms only on the surface are killed.
- Upon exposure, these waves are absorbed by many materials, particularly nucleic acids.
- The waves, as a result, cause the formation of pyrimidine dimers which bring error in DNA replication and cause the death of microbes by mutation.
- UV radiation owing to its poor penetrability of conventional packaging materials is unsuitable for sterilization of pharmaceutical dosage forms.

- It is, however, applied in the sterilization of air, for the surface sterilization of aseptic work areas, and the treatment of manufacturing-grade water.

B) IONIZING RADIATION

- X-ray and gamma rays are the commonly used ionizing radiation for sterilization.
- These are high energy radiation which causes ionization of various substances along with water.
- The ionization results in the formation of a large number of toxic O_2 metabolites like hydroxyl radical, superoxide ion, and H_2O_2 through ionization of water.
- These metabolites are highly oxidizing agents and kill microorganisms by oxidizing various cellular components.
- With ionizing radiation, microbial resistance decreases with the presence of moisture or dissolved oxygen (as a result of increased free radical production) and also with elevated temperatures.
- Radiation sterilization is generally exposed to items in the dried state which include surgical instruments, sutures, prostheses, unit-dose ointments, plastic syringes, and dry pharmaceutical products.

FILTRATION

- The process of filtration is unique among sterilization techniques in that it removes, rather than destroys, microorganisms.
- Further, it is capable of preventing the passage of both viable and nonviable particles and can thus be used for both the clarification and sterilization of liquids and gases.
- The primary mechanisms involved in filtration are sieving, adsorption, and trapping within the matrix of the filter material.
- Filtration uses membranous filters that have tiny pores that let the liquid pass through but prevent bigger particles such as bacteria from passing through the filter. Therefore, the smaller the pore, the more likely the filter is to stop more things from going through it.
- Certain types of filter (membrane filters) also have an essential role in sterility testing, where they can be employed to trap and concentrate contaminating organisms from solutions under test.
- These filters are then placed in a liquid nutrient medium and incubated to encourage growth and turbidity.
- The principal application of sterilizing-grade filters is the treatment of heat-sensitive injections and ophthalmic solutions, biological products, air, and other gases for supply to aseptic areas.
- They may also be required in industrial applications where they become part of venting systems on fermenters, centrifuges, autoclaves, and freeze dryers.

FILTRATION STERILIZATION OF LIQUIDS

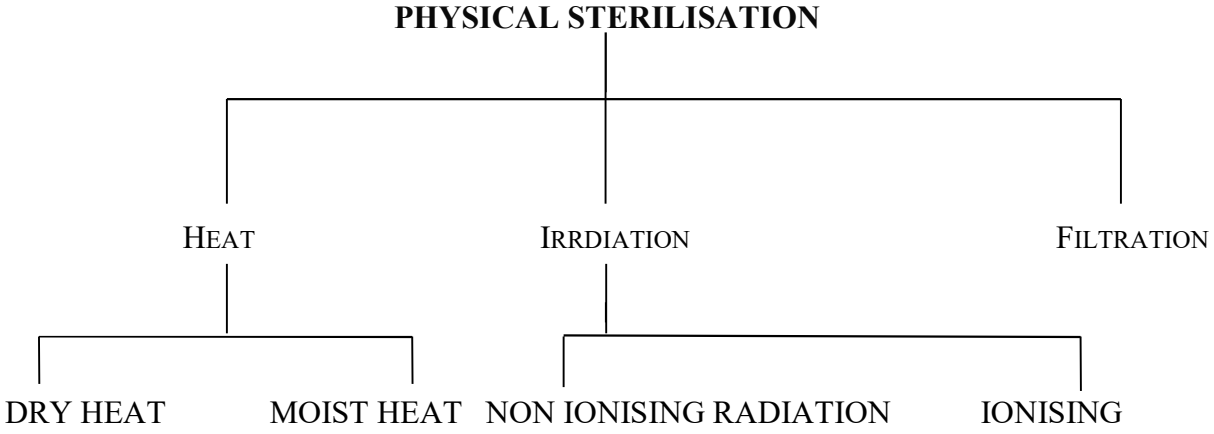
- Membrane filters, in the form of discs, can be assembled into pressure-operated filter holders for syringe mounting and in-line use or vacuum filtration tower devices for filtration of liquid.
- Filtration under pressure is generally considered most suitable, as filling at high flow rates directly into the final containers is possible without problems of foaming, solvent evaporation, or air leaks.
- Membrane filters are often used in combination with a coarse-grade fiberglass depth profiler to improve their dirt-handling capacity.

FILTRATION STERILIZATION OF GASES

- Filters employed for this generally consist of pleated sheets of glass microfibres separated and supported by corrugated sheets of Kraft paper or aluminum which are employed in ducts, wall or ceiling panels, or laminar air flow cabinets.
- These high-efficiency particulate air (HEPA) filters can remove up to 99.997% of particles >0.3mm in diameter and thus are acting as depth filters.
- In practice, their microorganism removal efficiency is rather better as the majority of bacteria are found associated with dust particles.
- Other applications of filters include sterilization of venting or displacement air in tissue and microbiological culture (carbon filters and hydrophobic membrane filters); decontamination of air in mechanical ventilators (glass fiber filters); treatment of exhausting air from microbiological safety cabinets (HEPA filters); and the clarification and sterilization of medical gases (glass wool depth filters and hydrophobic membrane filters).[6]

Table 4:(Filtration method, its mechanism, merits, demerits & applications)

Sl no.	Method	Mechanism	Merits	Demerits	Applications
1	Filtration	Does not destroy but remove the microorganisms	It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both vible and non viable particles	Does not differentiate between viable and non viable particles	In this method sterilizing grade filters are used in the treatment of heat sensitive injections and opthalmic solutions, biological products and air and other for supply to asceptic areas



Classification of physical sterilization

CHEMICAL STERILIZATION

- Chemical Sterilization is the process of removal of microorganisms by the use of chemical bactericidal agents.
- Even if physical methods of sterilization are more appropriate for effective sterilization, it is not always appropriate to use for heat-sensitive materials like plastics, fiber optics, and biological specimens.
- Under such conditions, chemical either in liquid or gaseous state can be used for sterilization. However, it is crucial to ensure that the materials undergoing sterilization are compatible with the chemical being used.
- Besides, it is important to adopt safety rules in the workplace safety during the use of chemical agents.
- The chemical method of sterilization can be categorized as liquid and gaseous sterilization.

GASEOUS STERILIZATION

- Gaseous sterilization involves the process of exposing equipment or devices to different gases in a closed heated or pressurized chamber.
- Gaseous sterilization is a more effective technique as gases can pass through a tiny orifice and provide more effective results.
- Besides, gases are commonly used along with heat treatment which also facilitates the functioning of the gases.
- However, there is an issue of release of some toxic gases during the process which needs to be removed regularly from the system.
- The mechanism of action is different for different types of gases.

Some of the common gases used for gaseous sterilization are explained below

ETHYLENE OXIDE

- Ethylene sterilize, pasteurize, or disinfect different types of equipment and surfaces because of its wide range of compatibility with different materials.
- EO treatment often replaces other sterilization techniques like heat, radiation, and even chemicals in cases where the objects are sensitive to these techniques.
- This method is a widespread method used for almost 70% of all sterilizations and around 50% for disposable medical devices.
- The mechanism of antimicrobial action of this gas is assumed to be through the alkylation of sulphydryl, amino, hydroxyl, and carboxyl groups on proteins and imino groups of nucleic acids.
- EO treatment is usually conducted at the temperature range of 30-60°C for several hours which aids in the activity of the gas.
- The efficacy of the gas depends on the concentration of gas available for each article which is greatly assisted by the good penetrating nature of the gas, which diffuses readily into many packaging materials including rubber, plastics, fabric, and paper.
- Ethylene oxide kills all known microorganisms, such as bacteria (including spores), viruses, and fungi (including yeasts and molds), and is compatible with almost all materials even when repeatedly applied.
- This process, however, is not without drawbacks as the level of gas in the sterilizer goes on decreasing due to absorption, and the treated articles need to undergo a process of desorption to remove the toxic residual wastes

- Organisms are more resistant to ethylene oxide treatment in a dried state, as are those protected from the gas by inclusion in crystalline or dried organic deposits.

Table 5: (Gaseous & Radiation sterilization methods, mechanism, merits, demerits & applications)

Sl no.	Method	Mechanism	Merits	Demerits	Applications
1	Gaseous sterilization	Alkylation	Penetrating ability of gases	Gases being alkylating agents and potentially mutagenic and carcinogenic	Ethylene oxide gas has been used widely to process heat sensitive devices
2	Radiation sterilization	Ionization of nucleic acids	It is a useful method for the industrial sterilization of heat sensitive products	Undesirable changes occur in irradiated products, an example is aqueous solution where radiolysis of water occurs.	Radiation Sterilization is generally applied to articles in the dry state; including surgical instruments, sutures, Prosthesis, unit doses.

FORMALDEHYDE

- Formaldehyde is another important highly reactive gas which is used for sterilization.
- This gas is obtained by heating formalin (37%w/v) to a temperature of 70-80°C.
- It possesses broad-spectrum biocidal activity and has found application in the sterilization of reusable surgical instruments, specific medical, diagnostic and electrical equipment, and the surface sterilization of powders.
- Formaldehyde doesn't have the same penetrating power of ethylene oxide but works on the same principle of modification of protein and nucleic acid.
- As a result of the low penetrating power, its use is often limited to paper and cotton fabrics.
- Formaldehyde can generally be detected by smell at concentrations lower than those permitted in the atmosphere and thus can be detected during leakage or other such accidents.

NITROGEN DIOXIDE

- Nitrogen dioxide is a rapid and effective sterilant that can be used for the removal of common bacteria, fungi, and even spores.
- NO_2 has a low boiling point (20°C) which allows a high vapor pressure at standard temperature.
- This property of NO_2 enables the use of the gas at standard temperature and pressure.
- The biocidal action of this gas involves the degradation of DNA by the nitration of phosphate backbone, which results in lethal effects on the exposed organism as it absorbs NO_2.

- An advantage of this gas is that no condensation of the gas occurs on the surface of the devices because of the low level of gas used and the high vapor pressure. This avoids the need for direct aeration after the process of sterilization.
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OZONE

- Ozone is a highly reactive industrial gas that is commonly used to sterilize air and water and as a disinfectant for surfaces.
- Ozone is a potent oxidizing property that is capable of destroying a wide range of organisms including prions, without the use of hazardous chemicals as ozone is usually generated from medical-grade oxygen.
- Similarly, the high reactivity of ozone allows the removal of waste ozone by converting the ozone into oxygen by passing it through a simple catalyst.
- However, because ozone is an unstable and reactive gas, it has to be produced on-site, which limits the use of ozone in different settings.
- It is also very hazardous and thus only be used at a concentration of 5ppm, which is 160 times less than that of ethylene oxide.

LIQUID STERILIZATION

- Liquid sterilization is the process of sterilization which involves the submerging of equipment in the liquid sterilant to kill all viable microorganisms and their spores.
- Although liquid sterilization is not as effective as gaseous sterilization, it is appropriate in conditions where a low level of contamination is present.

Different liquid chemicals used for liquid sterilization includes the following

HYDROGEN PEROXIDE

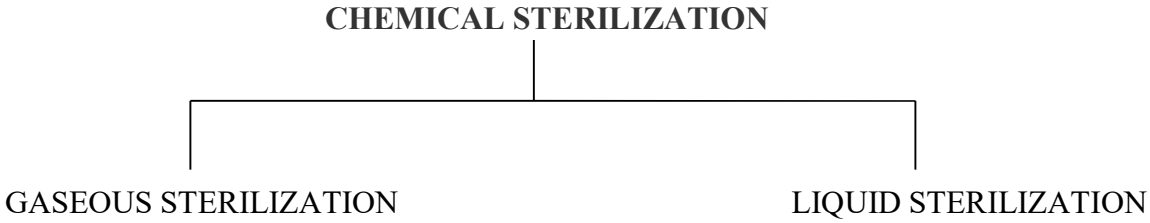
- Hydrogen peroxide is a liquid chemical sterilizing agent which is a strong oxidant and can destroy a wide range of microorganisms.
- It is useful in the sterilization of heat or temperature-sensitive equipment like endoscopes. In medical applications, a higher concentration (35-90%) is used.
- H_2O_2 has a short sterilization cycle time as these cycles are as short as 28 minutes where ethylene oxide has cycles that as long as 10-12 hours.
- However, hydrogen peroxide has drawbacks like low material compatibility, lower capacity of penetration, and associated health risks.
- Vaporized hydrogen peroxide (VHP) is used to sterilize largely enclosed and sealed areas, such as entire rooms and aircraft interiors.

GLUTERALDEHYDE

- Glutaraldehyde is an accepted liquid sterilizing agent which requires comparatively long immersion time. For the removal of all spores, it requires as long as 22 hours of immersion time.
- The presence of solid particles further increases the immersion time.
- The penetration power is also meager as it takes hours to penetrate a block of tissues.
- The use of glutaraldehyde is thus limited to certain surfaces with less contamination

HYPOCHLORITE SOLUTION

- Hypochlorite solution, which is also called liquid bleach, is another liquid chemical that can be used as a disinfectant, even though sterilization is difficult to obtain with this chemical.
- Submerging devices for a short period in liquid bleach might kill some pathogenic organisms but to reach sterilization submersion for 20-24 hours is required.
- It is an oxidizing agent and thus acts by oxidizing organic compounds which results in the modification of proteins in microbes which might ultimately lead to death.
- Appropriate concentrations of hypochlorite can be used for the disinfection of workstations and even surfaces to clean blood spills and other liquids.[7]



Classification of chemical sterilization

EVALUATION OF EFFICIENCY OF STERILIZATION METHODS

Evaluation of the efficiency of sterilization methods: The term 'sterile' in a microbiological context, means no surviving organisms, whatsoever. Thus, there are no degrees of sterility; an item is either sterile or it is not, and so there are no level of contamination which may be considered negligible or insignificant and therefore acceptable. True sterility, represented by zero survivors, can only be achieved after an infinite exposure period or radiation dose. Clearly then, it is illogical to claim, or expect, that a sterilization procedure will guarantee sterility. Thus, the likelihood of a product being produced free of microorganisms is best expressed in terms of the probability of an organism surviving the treatment process, a possibility not entertained in the absolute term 'sterile'. From this approach has arisen the concept of 'sterility assurance' or a microbial safety index which gives a numerical value to the probability of a single surviving organism remaining to contaminate a processed product. For pharmaceutical products, the most frequently applied standard is that the probability, post sterilization, of a non-sterile unit is 1 in 1 million units processed (i.e., <=10 -6). The sterilization protocol necessary to achieve this with any given organism of known D-value (decimal reduction time) can be established from the inactivation factor (IF) which may be defined as

$$IF = 10 \times {}^{t}/_{D}$$

where 't' is the contact time (for a heat or gaseous sterilization process) or dose (for ionizing radiation; and 'D' is D-value (time taken at a fixed temperature or the radiation dose required

to achieve a 90% reduction in viable cells. (D-value is one the functions to indicate the efficiency of sterilization process)

IF for selected sterilization protocols and their corresponding biological indicator organisms-

Moist heat (121℃ for 15 min) - ***B. stearothermophilus***, D-value - 1.5 min, Log IF - 10;
Dry heat (160℃ for 120 min) - ***B. subtilis var niger***, D-value - max. 3 min,
Log IF - Min. 40;
Irradiation (25 kGy) - ***B. pumilus***, D-value - 1.9 kGy,
Log IF - 13.2

This is the simplest method of calculating the probability of achieving sterility for any given initial survival level.

From the above-mentioned D-values and Log IF (or IF) values, it is clear that moist heat.[8]

STERILITY INDICATORS

PHYSICAL INDICATORS

- Monitoring physical indicators involves observing the gauges or displays on the sterilizer and recording the time, temperature, and pressure associated with each sterilization cycle for each load.
- Some sterilizers have recording devices that print out these parameters.
- Correct readings do not guaranty sterilization, but incorrect readings can be the first indication of a problem with the sterilization cycle and suggest the load may not be sterile. [9]

CHEMICAL INDICATORS

- Chemical indicators use sensitive chemicals to assess critical variables (e.g., time, temperature, or steam saturation) during a sterilization cycle.
- They are applied either to the outside or placed on the inside of each instrument unit (e.g., packs, peel pouches, containers, etc…).
- They do not prove that sterilization has been achieved, but they can provide an early indication of a problem and where in the sterilization process the problem might exist.[10]

BIOLOGICAL INDICATORS

- Biological indicators (BIs), or spore tests, assess directly the killing of known highly resistant, non pathogenic bacterial spores.
- ***Geobacillus stearothermophilus*** (***G. stearothermophilus***) spores test steam and unsaturated chemical vapor sterilizers.
- ***Bacillus atrophaeus*** (***B. atrophaeus***) spores test dry heat sterilizers.
- Bacterial spores in the test products are more resistant and are present in greater numbers than common microbial contaminants found on patient-care items.[11]

EQUIPMENTS EMPLOYED IN LARGE SCALE STERILIZATION ARE:

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

(A)

(B)

(C)

(D)

Fig. 3: (A) Steam sterilizer; (B) Dry heat sterilizer; (C) ETO Sterilizer; and (D) CIP System

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