H. R. Patel Institute of Pharmaceutical Education and Research, Shirpur

LAB MANNUAL

(S. Y. B. Pharmacy, Sem- III)

Prepared By

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COURSE OBJECTIVES

1. Introduction and study of different equipment's and processing, e.g., B.O.D. incubator, laminar flow, aseptic hood, autoclave, hot air sterilizer, deep freezer, refrigerator, microscopes used in experimental microbiology.

2. Sterilization of glassware, preparation, and sterilization of media.

3. Sub culturing of bacteria and fungus. Nutrient stabs and slants preparations.

4. Staining methods- Simple, Gram's staining and acid-fast staining (Demonstration with practical).

5. Isolation of pure culture of micro-organisms by multiple streak plate technique and other techniques.

6. Microbiological assay of antibiotics by cup plate method and other methods

7. Motility determination by Hanging drop method.

8. Sterility testing of pharmaceuticals.

9. Bacteriological analysis of water

10. Biochemical test.

Index

Experiment 1

Introduction to laboratory rules and procedures, Laboratory equipment and apparatus

The class will be split into four groups for the initial presentation of this practical, and each group will visit one of the following stations.

1.1 Objectives

1. To understand the regulations and practices that must be followed in a lab.
2. To be familiar with the tools and equipment used in practical microbiology activities.
3. To get familiar with, comprehend, and know how to utilize microscopes.

1.2 Parts and use of microscopes

• Reagents and culture medium; apparatus used in microbiology

A technician will show each group how the are used in microbiology for roughly 15 minutes (for a total of 60 minutes across all 4 stations).

The students will be work in their allotted groups of roughly 10 to 15 students during the second session on using microscopes (which should last 45 to 60 minutes).

1.3 Required for this practical exercise.

1.3.1 Equipment used in microbiology.

1.3.1.1 Apparatus: V-shaped glass rod, universal bottles, petri dishes, muslin cloth, filter paper, magnifying glass, microscope slides, cover slips, inoculating needles, wire loops, spirit lamps, paper towel, scalpel with holder, beakers, conical flasks, L-shaped glass rods, staining rack, microscope lens cleaning tissue, pipettes (various sizes), pipette filler, media bottles

1.3.1.2 Reagents: 70% ethanol, absolute ethanol, 2.5% sodium hypochlorite, sterile distilled water

1.3.1.3 Stains: carbol fuschsin, methylene blue, water, safranin, iodine, crystal violet, nigrosine, cotton blue in lactophenol

1.3.1.4 Equipment: pH meter, hot air oven, compound microscope, weighing balance, autoclave, dissecting microscope

1.3.1.5 Culture media: commercial nutrient agar, Blood agar, sugar, dextrose, glucose, agar, mushroom, commercial potato dextrose agar (PDA), Potato tubers

1.4 Apparatus used in microbiology.

- Petri dishes were used to hold culture medium for microorganism development.
- Cover slips and microscope slides for mounting microbiological specimens for microscopic inspection.
- Transferring microbiological specimens using wire loops and needles that have been inoculated.
- Spatula, wire loops, Spirit lamps for sterilization of needles.
- conical flasks, Beakers, universal bottles and media bottles for holding culture media and solutions.
- Magnifying glass for making small macroscopic objects that are too big to see under a microscope appear larger.
- L-shaped glass rods are used during inoculation to distribute bacterial suspensions across the surface of medium, whereas V-shaped glass rods are employed in the construction of slide cultures.
- A staining rack is a glass rack used to hold microscope slides during staining processes; it is typically positioned over a water basin or other open container to catch spilled stains.
- Special tissue paper for cleaning microscope lenses is called a microscope lens cleaning tissue.
- Muslin cloth a porous cloth for filtering/ straining suspensions
- Pipettes for moving the necessary amounts of a solution.

1.5 Norms and procedures in the lab.

- Keep workspace clear of unnecessary materials.
- Well Label laboratory chemicals with suitable warning signs for hazards.
- Wash hands with disinfectant soap upon arrival at the lab and before you leave.
- In the microbiology labs, no eating, drinking, chewing gum, or smoking or vaping of any type is permitted.
- To avoid contamination or unintentional coloration of clothing, always wear a lab coat or apron.
- Use a disinfectant like Lysol or 70% ethanol to wipe the lab bench before and after each lab session.
- Keep the laboratory bench clean except laboratory apparatus.
- Never put anything in your mouth during lab sessions, including fingers, pens, or pencils.
- To reduce the risk of fire and cultural contamination, long hair should be pulled back.
- Never pipette liquid reagents or cultures by mouth; instead, use pipette fillers.
- Aseptic procedures must always be followed.
- Before beginning an experiment, label all plates, tubes, cultures, etc. Indicate the experiment's date.
- After usage, tools including stains, reagent bottles, Petri plates, pipettes, microscopes, etc. must be put back where they came from.
- When not in use, all cultures should be kept covered, and the Petri dish lids should be closed.
- All microbial cultures must be treated with caution since they might be harmful.
- Never pour any cultures into the sink.
- If a live culture is spilled, clean the area after 15 minutes of disinfectant application.

- The containers provided should be used to store used and contaminated glassware. Old cultures must be disposed of right away by autoclave.
- Be cautious while using lab burners: avoid spraying ethanol near open flames and switch off the burner when not in use. When not in use, equipment like ovens and microscopes must be turned off.
- Get used to the workout you will be performing.
- Carefully handled all equipment; never opened or disassembled pieces of it. Before and after usage, the microscope stage, lenses, and objectives should be cleaned with a specific lens tissue.
- Any mishaps, breakages, or unusual events must be reported right away to the person overseeing the practical.
- Any breakages, accidents or out of the ordinary occurrences must be reported immediately to the person in charge of the practical.
- When conducting experiments, keep the lab doors and windows closed. As you complete the assignment, enter your findings in ink and create any drawings or labels in pencil. Reports on laboratory exercises should be produced as soon as the observations are finished.

Experiment 2

Overview on Microscope.

2.1 Introduction

- A microscope is a tool used to see things that are invisible to the naked eye. The science which investigates small objects by an optical instrument is called microscopy. Microscope is one of the essential devices for studying organisms that are not perceived by naked eye.
- The Dutch spectacles maker Hans Janssen and his son Zacharias credited for development of compound microscope. It was Antony Van Leeuwenhoek a Dutch, cloth merchant, very interested in lenses. He was used the magnifying glass to count the threads in woven cloth. By grinding and polishing, he was able to make small lenses with great curvature. Antony van Leeuwenhoek more involved in science and formation of microscope device. He developed simple microscope which consist of biconcave lens enclosed in two metal plates and explain bacteria and protozoa.
- The compound microscope uses lenses and light to enlarge the image and so it is called an optical microscope. The simple microscope utilises the magnifying glass to enlarge the image ten times (10X). While compound microscope involved two systems of lenses for higher magnification. Eyepiece and objective lens is equipped in compound microscope.

2.2 Important concepts in microscope:

2.2.1 Magnification:

Magnification is ability of microscope to enlarge the image of an object. It is denoted by symbol X. The magnification of object is influenced by type of objective and eyepiece. The total magnification of microscope is calculated by multiplying the magnification of objective by the

magnification of the ocular lens. The greatest useful magnification of a microscope is that which makes the smallest visible objects clearly resolvable.

2.2.2 Resolving power:

Resolving power means the ability of microscope to reveal closely adjacent elements as separate and distinct. It depends on the wavelength of the beam used for illumination and on the optical quality of the lenses. Shorter wavelength gives better resolution. Ultraviolet light or high voltage electron beam are two sources of illumination with wavelengths shorter than those of visible light and are used in the construction of electron microscope. Resolving power is determined by using formula as given below.

Resolving power (RP) =λ/ 2 x N.A.

Were,

λ= wavelength of light

N.A. = numerical aperture

The resolving power is like magnification which can be increased by numerical aperture.

2.2.3 Numerical Aperture:

Numerical aperture can be defined as function of the diameter of objective lens in relation to its focal length. Numerical aperture can be expressed by the formula.

Numerical aperture (N.A.) = n sin ɵ

Were,

n= Refractive index of the medium through which light passes before entering the objective lens

sin ɵ= Trigonometric sine of one-half the angle formed by light rays coming from the condenser and passing through the specimen.

N.A. is engraved on the barrel of objectives and specify the maximum obtainable resolving power. Numerical aperture is doubled using sub-stage condenser that illuminates the specimen with rays of light which pass through the specimen obliquely as well as directly.

2.2.4 Oil immersion lens:

Oil immersion lens is used to increase the resolving power of a microscope. Refractive index of air is 1 which is lower than glass (n= 1.55). As when light rays passes from the glass slide into air, light rays get refracted. It results into loss in resolution power of microscope. This refraction of light can be compensated by using immersion oil having refractive index same as that of glass slide. Oil replaces the air present between glass slide and objective lens. Cedar wood oil (n=1.5) is use most commonly for oil immersion lens.

2.3 Types of microscopes:

The microscopes are of two kinds depending upon the lens system.

(1) Simple microscope (2) Compound microscope

A simple microscope is consisting of a single lens system while a compound microscope consists of two or more lens system. Depending upon the source of illumination the microscope can be classified as

2.3.1 Light microscope: With the assistance of a light source or UV radiation, the item is visible. It comprises ultraviolet, fluorescence, and bright field microscopes as well as dark field, phase contrast, and fluorescent microscopes.

2.3.2 Electron Microscope: Instead of employing light rays and a magnetic field, an electron beam is used to create the picture. Since the electron beam's wavelength is substantially shorter than that of light, its resolution is much improved. Scanning electron microscopes (SEM) and transmission electron microscopes (TEM) are the two different types of electron microscopes.

2.4 Principle of Light microscope -The light source illuminates the object. The first image of the object is formed by objective lens which is a real, inverted, and magnified image. The image formed by the objective functions as the object for eyepiece, which produces the final, virtual, and magnified image. In this way, the final image produced is inverted with respect to the object.

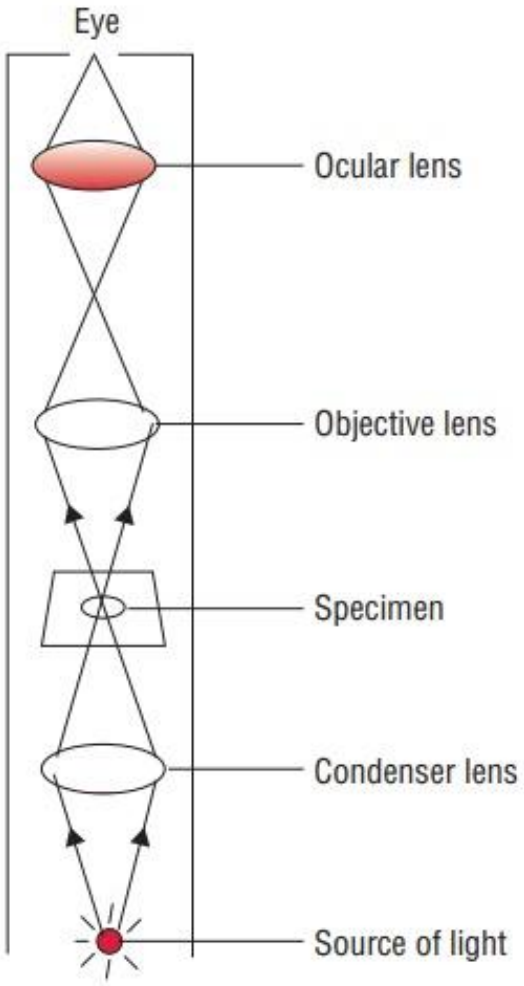


Fig 2.1: Ray Diagram of Light Microscope

2.5 Construction of Light Microscope:

Microscope is consisting of following parts.

2.5.1 Eyepiece /Ocular lens: It is optical lens which is present at the top of the body tube. They are usually 10X or 15X magnification power. Eye piece is used to see the objects.

2.5.2 Body tube: A body tube is an integral part of the microscope which holds eyepiece at the top while objective at the base. It connects eyepiece to the objective lens.

2.5.3 Arm: Arm is the part of microscope which supports the body tube of the microscope and connects to the base of the microscope. It also helps to carry the microscope easily.

2.5.4 Base: It is the bottom part of the microscope which supports the microscope to stand and provide stability to all parts of microscope.

2.5.5 Illuminator: It is consisting of light source or mirror, iris diaphragm and condenser. For illumination, light source is obtained by either plain or concave mirror or electrical lamp. Electrical illumination involves use of tungsten filament lamp or halogen lamp.

2.5.6 Mechanical Stage: It is a platform is flat surface used for placing the slide under observation. It is also fitted with knobs for movement of slides left and right as well as up and down.

2.5.7 Nosepiece /Turret: Nosepiece holds the two or more objective lenses and can be rotate and change the power according to need of the specimen to view the specimen in various dimensions.

2.5.8 Objective lenses: Microscope consist of two or three objective lenses at the base of the nose piece, which ranges from 10X, 45X and 100X. The shortest lens is the lowest magnification while the longest lens is greatest magnification power. Oil immersion lens (100X) magnifies the image 100 times using oil on the specimen. Main function of objective lens is to provide magnification of specimen.

2.5.8.1 Condenser lens: The light from the illuminator is collected by the condenser lens and focused on the specimen. It helps in- formation of clean and sharp image. Condenser lenses are most useful at the highest powers i.e., 400X and above. The main function of condenser is to collect and focus it on the specimen.

2.5.9 Diaphragm or Iris: The diaphragm is used to control the amount of light that is passing through the object. It is rotating disk present under the stage and above the condenser. The diaphragm has different sized holes and is used to vary the intensity and size of the cone of light that is projected upward into the slide.

2.5.10 Coarse adjustment Knob: It is present on the arm of a microscope. The main role of knob is to move the specimen and create proper focus on the object for best image formation.

2.5.10.1 Fine adjustment Knob: It is subbing part of coarse adjustment knob used for slight movement body tube.

2.5.11 Stage clips: Stage clips are used to holds the slides in proper place and also to gives stability.

2.5.12 Rack Stop: It is the part of microscope which determines the distance between the objective lens and the specimen.

2.5.13 Aperture: Aperture is a small hole in the stage through which the light is transmitted and passed on to the slide.

2.5.14 Power Switch: Power switch is an electrical switch present at the bottom of the microscope, which provide light source.

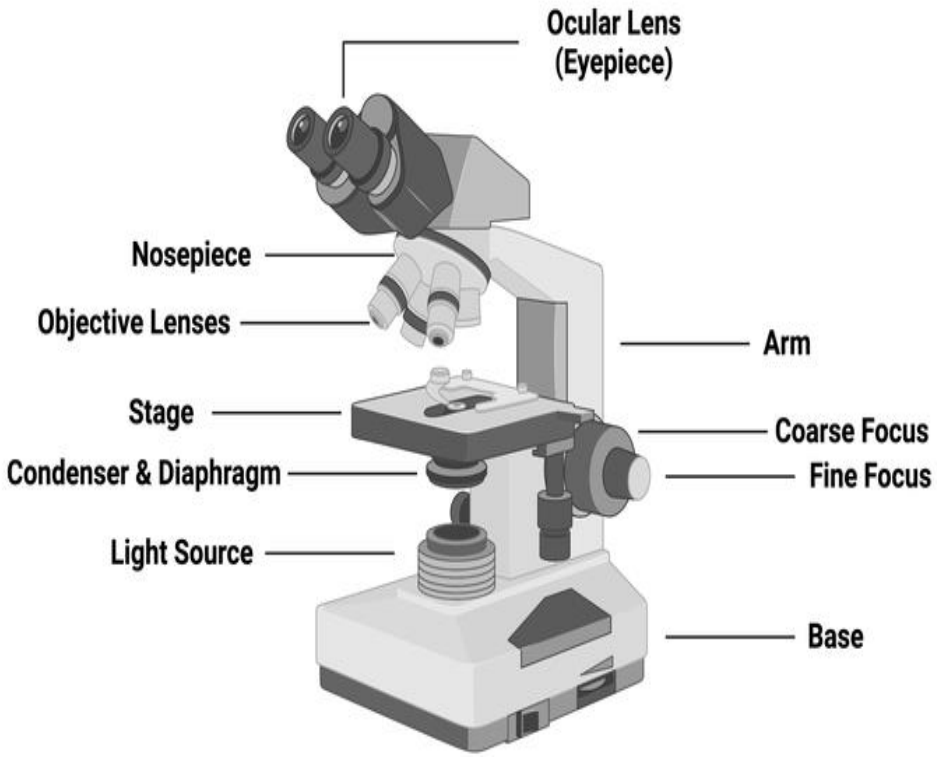


Fig 2.2: Parts of Microscope

2.6 Steps in Microscope operation:

- ✓ Remove the microscope from the cabinet and pick up the microscope firmly.
- ✓ Clean the lenses of the microscope with lens paper and remove dust and oily substances from the lens with the help of xylol.
- ✓ Place the microscope in front of light source.
- ✓ Prepare the slide of specimen and keep on stage and secure with stage clips.
- ✓ Adjust the light source with the help of mirror, their flat surface reflects the sufficient light into the barrel of the microscope.
- ✓ First adjust the low power objective 10X on the specimen by rotating nose piece of body tube.

- ✓ Observe with eye piece and raise the condenser until there is a minimum illumination i.e. light is seen as a circle in the centre of the field and called bright full moon.
- ✓ Observe through the ocular lens and bring the sharp focus on specimen with the help of adjustment knob.
- ✓ Adjust the diaphragm and to produce sufficient illumination. Once the image comes into focus, rotate the 10x objective and adjust the 100x lens on the specimen.
- ✓ Look the specimen and focus the object with the help of fine adjustment knob.
- ✓ After formation of specific image of specimen note down the observation details.
- ✓ After completion of microscopic work move the 10X on their original position and clean the oil immersion lens using lens paper.

2.7 Observation:

Observe the slide for the structural details and note the relative details of the microbes under objective lens.

2.8 Handling precautions and use microscope:

- To carry the microscope, grasp the arm with one hand and use other hand under the base.
- Place the microscope on a table with the arm towards you.
- Handle all the parts of microscope carefully and also remove the dust from eyepiece.
- Do not touch the glass part of the lenses with your finger.
- Place the slide on the stage and fix with the help of stage clips.
- Focus the slide carefully first with low power objective.
- Use proper side of mirror for proper illumination of specimen.
- Use the coarse adjustment knob properly to raise the body tube.
- Observe the slide with both eyes and to have less strain on observer eyes.
- When finished the work, raise the body tube and remove the slide from the microscope.
- Clean the microscope and keep in plastic bags.

2.9 Care of Microscope:

2.9.1 Transportation: Carry the microscope with both hands in laboratory. Grasp the metal arm with one hand and place the other hand under the base of support. Do not only pick it by the stage to avoid misalignment.

2.9.2 Cleaning of lenses: Low power objective lens is kept in their proper position and never allow the lens to touch the slide more deeply.

2.9.3 Cleaning of lens after using oil immersion oil: After use of oil immersion lens, clean the oil properly with the help of lens paper and lens cleaner.

2.9.4 Cover Microscope: Always covered the microscope with plastic bags and store in the cabinet.

2.9.5 Special lens care: Microscope lenses can easily be scratched and should be treated with great care. Sticky residues are removed with lens paper which is moistened with distilled water or lens solution.

2.10 Principle of Electron Microscopy

The first electron microscope was made by a German engineer, Ernst Ruska in 1931. It uses the beam of accelerated electrons to visualize the specimens and has a high resolution.

2.11 Parts of Electron Microscope

- Electron gun is heated tungsten filament which produces the beam of electrons.
- Electromagnetic lenses- Condenser lens directs the electron beam towards the specimen. The electron beam coming out from specimen goes through objective lens. The projector or ocular lens produces the final magnified image.
- The specimen holder is made up of thin layer of carbon held by metal grid.
- The final image is obtained on fluorescent screen, below which a camera is present to capture the image.

2.12 The working principle of electron microscope

Through two sets of condenser lenses, the electron beam that is generated by the electron cannon is focused on the specimen. The electrons are accelerated downward by applying a voltage between the filament and the anode. The specimen under observation is stored on a specimen holder as a thin segment of 20–100 nm. The denser parts scatter more electrons on the screen than the lighter portions when an electron beam passes through the material. The picture is further amplified by the ocular lens after the electron beam from the specimen scatters via the objective lens.

Fig 2.3: Electron Microscope

2.13 Applications of Microscope:

Microscope is essential tool in various fields including microbiology, medicine, material science and mineralogy.

- Microscope is useful to the study of structure, shape of microorganisms, which are not visible by naked eye.
- To study thin slice of rocks.
- To check shape of rocks.
- Contaminant analysis is also performed by microscopy.
- To identify dye in textiles.
- Petroleum analysis is carried out by microscopy.
- To carry out surface study microscope plays important role.

Table 2.1: Difference between Light and Electron Microscope

Light Microscope	Electron Microscope
Illumination of specimen by light	Illumination of specimen by electron beam
Microscope is simple and handy	Microscope is complex
Techniques are simple	High levels of technical skills are required.
Live and dead specimen may be seen	Only dead and dried specimen is required
Condenser, objective and eye piece lenses are made of glass	All lenses are electromagnetic lenses.
Light microscope has low resolution power	Electron microscope has high resolution power.
It has magnification from 500X to 1500X.	It has magnification as high as 160,000X.
In light microscope the image formation depends upon light absorption by objects.	In electron microscope, the image formation is depend upon electron scattering.
No magnetic field.	Development of magnetic field.
Image is seen by ocular lens.	The image is observed on fluorescent screen or photographic plate.

No vacuum pump is needed.	It operates at high vacuum.
Dehydrated specimen is not required	Dehydrated specimen is required.
No cooling system.	Cooling system is present.
Radiation risk is absent	Radiation risk in electron microscope.
Magnification is up to 4000	Magnification is up to 300,000.
It is used for the study of internal structure.	It is used in the external, ultra-structure.
There is no need of electron current.	High voltage electric current is required.
Cheap and low cost.	Costly and heavy cost.
Low resolving power.	High resolving power.

Experiments 3

Introduction to laboratory equipment's

3.1 Autoclave

An autoclave is a device that offers a physical way of sterilisation by utilising steam under pressure to destroy bacteria, viruses, and even spores contained in the material placed within the vessel. By heating the materials to a given temperature for a set amount of time, an autoclave sterilises the materials. The autoclave, which is also known as a steam steriliser, is frequently used in healthcare institutions and other commercial settings for a variety of functions. Because it relies on moist heat sterilisation, the autoclave is regarded as a more effective sterilisation technique. These days, modified autoclaves such as cold sterilisers, gas steriliser autoclaves, and UV autoclaves are also available.

Fig 3.1: Autoclave

3.1.1 Principle:

The autoclave operates according to the moist heat sterilisation concept. For equipment sterilisation, the high pressure inside the chamber raises the boiling point of water. Additionally, the increased pressure assures that heat will quickly reach the deeper components of the equipment. The moisture in the steam induces the coagulation of microbial protein, which results in an irreparable loss of activity and function. The length of time needed for sterilisation varies based on the temperature and pressure levels. Holding time and condition of temperature and pressure is given in Table 3.1.

Table 3.1: Autoclave Conditions

Temperature (°C)	Steam pressure (lb/sq. inch)	Holding time (minutes)
115-118	10	30
121-124	15	15
126-129	20	10
135-138	30	3

3.1.2 Parts of autoclave

The simplest form of the autoclave is the pressure cooker type or laboratory bench autoclaves. The following is the detailed description of different components/ parts of an autoclave:

3.1.2.1 Pressure Chamber

A steam autoclave's pressure chamber, which consists of an inner chamber and an outside jacket, is its primary part. The outer chamber is composed of an iron casing, while the inner chamber is made of stainless steel or gunmetal.

3.1.2.2 Lid/ Door

The autoclave lid or door is the next crucial part of an autoclave. The lid's main function is to produce a sterilised environment by sealing off the outside atmosphere. The asbestos washer and screw clamps seal the lid against airflow. Other parts of the lid include things like:

3.1.2.3 Pressure gauge

A pressure gauge is present on the lid of the autoclave to indicate the pressure created in the autoclave during sterilization. The pressure gauge is essential as it assures the safety of the autoclave and the working condition of the operation.

3.1.2.4 Pressure releasing unit/ Whistle.

A whistle is present on the lid of the autoclave is the same as that of the pressure cooker. The whistle controls the pressure inside the chamber by releasing a certain amount of vapor by lifting itself.

3.1.2.5 Safety valve

A safety valve is present on the lid of the autoclave, which is crucial in cases where the autoclave fails to perform its action or the pressure inside increases uncontrollably. The valve has a thin layer of rubber that bursts itself to release the pressure and to avoid the danger of explosion.

3.1.2.6 Steam generator/ Electrical heater

An electrical steam generator or boiler is present underneath the chamber that uses an electric heating system to heat the water and generate steam in the inner and the outer chamber. The level of water present in the inner chamber is vital as if the water is not sufficient; there are chances of the burning of the heating system. Similarly, if the water is more than necessary, it might interfere with the trays and other components present inside the chamber.

3.1.2.7 Vacuum generator

Some kinds of autoclaves have a separate vacuum generator that removes air from the interior of the chamber to generate a vacuum there.

3.1.2.8 Wastewater cooler

Many autoclaves are provided with a system to cool the effluent before it enters the draining pipes. This system prevents any damage to the drainage pipe due to the boiling water being sent out of the autoclave.

3.1.3 Working:

In general, an autoclave is run at a temperature of 121° C for at least 30 minutes by using saturated steam under at least 15 psi of pressure. The following are the steps to be followed while running an autoclave: Enough water is then put inside the chamber. Now, the materials to be sterilized are placed inside the chamber. The lid is then closed, and the screws are tightened to ensure an airtight condition, and the electric heater is switched on. The safety valves are adjusted to maintain the required pressure in the chamber. Once the water inside the chamber boils, the air-water mixture is allowed to escape through the discharge tube to let all the air inside to be displaced. The complete displacement can be ensured once the water bubbles cease to come out from the pipe.

The drainage pipe is then closed, and the steam inside is allowed to reach the desired levels i.e.,15 lbs Once the pressure is reached, the whistle blows to remove excess pressure from the chamber. After the whistle, the autoclave is run for a holding period, which is 15 minutes in most cases. Now, the electric heater is switched off, and the autoclave is allowed to cool until the pressure gauge indicates the pressure inside has lowered down to that of the atmospheric pressure. The discharge pipe is then opened to allow the entry of air from the outside into the autoclave. Finally, the lid is opened, and the sterilized materials are taken out of the chamber.

3.1.3.1 Applications of Autoclave:

- They are used to decontaminate specific biological waste and sterilize media, instruments, and labware.
- Regulated medical waste that might contain bacteria, viruses, and other biological materials is recommended to be inactivated by autoclaving before disposal.
- In medical labs, autoclaves are used to sterilize medical equipment, glassware, surgical equipment, and medical wastes.
- Similarly, autoclaves are used for the sterilization of culture media, autoclavable containers, plastic tubes, and pipette tips.
- Autoclaves are widely used in microbiology, veterinary science, mycology etc.
- It is used to sterilise wide range of material including but not limited to laboratory glass wares, laboratory equipment's and instruments, surgical material including needles, seizers, heat stable hand gloves, containers, and closures etc.
- Various pharmacopoeias have recommended autoclave for sterilization of number of Injectable.

3.1.3.2 Advantages:

1. Economical or cheap
2. Short procedure time
3. Provides good penetration on all surfaces.
4. No additional chemicals or disposables required.

3.1.3.3 Disadvantages:

1. Moisture retention
2. Carbon steel can get damaged due to moisture exposure.
3. Only stainless-steel instruments and plastics which can bear the heat be sterilized.

3.2 Hot Air Oven

A hot air oven is an essential laboratory equipment that uses to dry heat to sterilize laboratory objects and samples. This type of sterilization is also known as dry heat sterilization.

This mechanism of heat treatment was introduced by French scientist Louis Pasteur in the late 1800s where he used dry heat for a brief period to kill off harmful microorganisms from the wine without altering its taste.

Hot air ovens typically have a temperature range of ambient temperature to 250°C or higher and are equipped with a thermostat to maintain a consistent temperature. Some hot air ovens also have a fan to circulate the hot air inside the oven chamber, which can help to ensure that the materials being processed are heated or dried evenly.

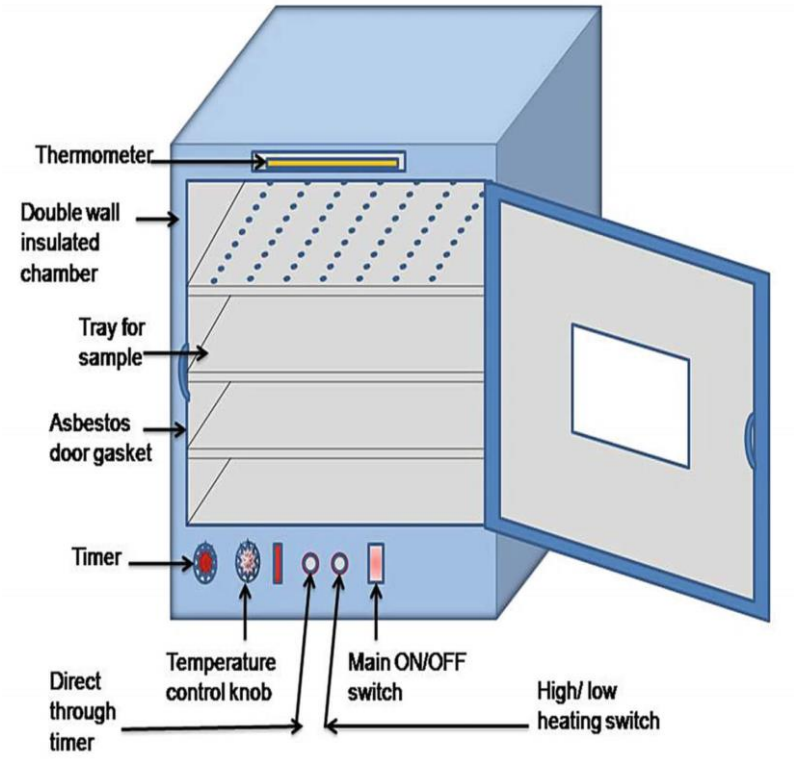


Fig 3.2: Hot air oven

3.2.1 Principle

Microorganisms and bacterial spores are destroyed in hot air ovens using extremely high temperatures maintained for several hours. By heating the item's outside surfaces, which the ovens then absorb and transport into the item's centre, conduction sterilises the object. the amount of time needed for sterilising depends on the temperature. Relation between holding time and temperature in Table 3.2

Table 3.2: Autoclave Conditions

Temperature (°C)	Time (minutes)
170	60
160	120
150	150
140	140

3.2.2 Construction

an exterior casing enclosing an insulated chamber with electric heaters Shelves, a thermostat, a fan, and door lock controls. Wall-mounted metallic cabinet with heating filament and fan. Thermostat, temperature control, double-walled construction, and insulation maintain heat and reduce energy use. electrically heated, with a fan or blower included to provide quick and even heating. the mechanism: The damaging oxidation of vital cell components, protein denaturation, and the poisonous effects of high electrolyte levels are what cause dry heat to kill microorganisms.

3.2.3 Working

- The oven is plugged into the socket and switched on.
- The oven is preheated for 30 minutes before placing the items on the trays or shelves.
- The temperature gauge is set at the desired time, depending on the volume of the contents to be sterilized.

- The items are loaded on the shelves (appropriate spacing should be maintained between the articles on the trays for efficient heat circulation).
- The door is closed by fastening the screws provided, after which the temperature begins to rise.
- The thermometer is checked in to determine whether the desired temperature is achieved after a certain holding time.
- The device is switched off once the temperature holding period is achieved, allowed to cool down before opening the door, and then the samples are removed using oven mitts or tongs.
- The door is closed after removing the samples.

3.2.4 Applications

- It is used for sterilization of laboratory equipment such as glassware flasks, pipettes, Petri-plates, and test tubes, metal items forceps, spatula, scalpel, scissors, non-volatile compounds zinc and starch powder, sulphonamide, and other materials that contain oils.
- It can be employed for testing food items, pharmaceutical products and other consumable materials in order to ensure their temperature stability during the shelf life.
- It can be used in research settings in the field of biology, chemistry and material science.
- It can be used in heat treatment and drying of samples, such as metals, alloys, soil, and other materials.

3.2.5 Advantages

1. It does not require water for sterilization purposes like an autoclave.
2. It is economical and easy to operate.
3. It can function at higher temperature and faster than autoclave.
4. The small size of the oven requires less space and has an easier installation process.
5. The dry heat does not corrode or rust metals or other sharp articles.

6. It is smaller in size and is convenient to work on.
7. Less pressure built in it ensures safety during operation.
8. It is non-toxic. No harmful chemical residues will be discarded.
9. The dry heat can penetrate deeply into thick objects such that it helps to achieve an in-depth sterilization effect.

3.2.6 Disadvantages

1. It is not suitable to sterilize items such as rubber, plastics, surgical dressing, etc., due to their low melting point.
2. It may not destroy the heat-resistant endospores and prions because they utilize dry heat instead of moist heat.
3. It is time-consuming relative to steam, flaming, chemical sterilization, or radiation.

3.2.7 Precautions

1. Glass apparatus must be wrapped with the Kraft paper or filter paper.
2. Do not keep the material at the bottom where it receives relatively more heat which may cause cracking of material.
3. Keep space in between material for proper circulation of hot air. Avoid over loading.

3.3 Incubator

A sealed, insulated apparatus called an incubator is employed in biological labs. By offering the ideal temperature, humidity, and other environmental factors, like the CO2 and oxygen content inside the atmosphere, it generates the ideal habitat needed for the development of microorganisms.

It is used in labs to develop and preserve cell or microbiological cultures. An incubator is used to develop organisms with bacterial and eukaryotic cells. A device called an incubator is used to develop and preserve microbial cultures. It is a warm cabinet that maintains ideal temperatures, humidity levels, and other factors including oxygen and carbon dioxide levels.

inside the incubator. Its main function is to maintain the favourable condition to promote growth of microorganisms.

3.3.1 Principle:

The thermostat in the incubator produces a thermal gradient to keep the temperature constant. Any conductor will produce voltage when it is exposed to a heat gradient, which is known as the thermoelectric effect. The incubator is heated to a predefined temperature (37°C) as power is applied to the circuit. The compatibility and efficient performance of the temperature sensor, temperature controller, and temperature contactor are key elements in maintaining this temperature.

3.3.2 Construction:

Incubator is made of material like stainless steel which is resistant to corrosion. It is insulated box equipped with heating element, fan, microprocessor or thermostat required to maintain desired condition inside the chamber. For most of bacterial cells 37ºC is favourable temperature while yeast like organism require 30ºC.

3.3.3 Operating Procedure

After preparation of culture, it needs to be stored in an incubator at the desired temperature, co2 concentration for a period.

1. Before running an incubator make sure there are no remaining items present from the previous cycles.
2. If different organisms require the same parameters, then keep them in the same incubator.
3. Then close the door and switch on the incubator. Make sure the door is properly closed.
4. Now set the desired temperature in the controller and keep monitoring it through the thermometer.
5. If any organism requires a specific concentration of CO2 or a specific humidity, then we also can set them in the controller.

6. After setting the parameters, place the culture plates on the perforated shelves upside down.
7. It is necessary to seal the plates with adhesive tapes or are placed in plastic bags to prevent contamination.

3.3.4 Application

In laboratories incubator is used for different purpose such as;

1. Used to maintain the growth of different microbial cultures or cell cultures, which we can use in later.
2. Sometimes they are used to enhance the growth rate of organisms.
3. Some advanced incubators are used for the reproduction of microbial colonies and subsequent determination of biochemical oxygen demand.
4. In zoology, they are also used for the breeding of insects and hatching of eggs.
5. It also used to store different medical samples. Incubator is most used equipment in cell culture, tissue culture, biochemical studies, food processing, haematological studies, fermentation studies etc.
6. Recently advanced technology made it possible to use incubator in genetic engineering.

3.3.5 Advantages

1. Incubators require less energy, which lowers costs and increases savings.
2. Depending on the type of cell culture being stored, the parameters can then be modified.
3. Incubators offer a stable environment that is conducive to the growth and survival of cultures and microorganisms by mixing natural and forced convection.

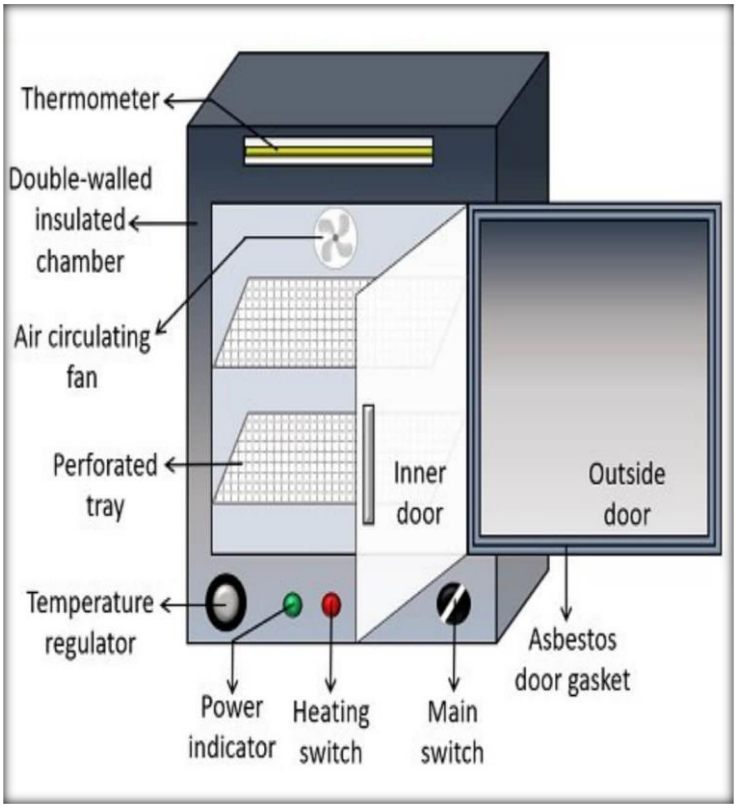


Fig 3.3: Incubator

3.4 Anaerobic Jar

McIntosh and Fildes' anaerobic jar is an instrument used in the production of an anaerobic environment. This method of *anaerobiosis* as others is used to culture bacteria which die or fail to grow in presence of oxygen.

3.4.1 Principle:

The McIntosh and Fildes anaerobic jar operates on the replacement and evacuation principle, in which the chamber's air is expelled and replaced with a mixture of gas composed of 5% CO2, 10% H2, and 85% N2. Two chemical tablets, sodium borohydride and sodium bicarbonate, are contained in anaerobic jars. When water combines with these chemicals, hydrogen gas and carbon dioxide are produced. In the chamber, liberated hydrogen gas reacts with unrestrained oxygen to form water. Anaerobic conditions are produced when carbon dioxide replaces the oxygen that has been withdrawn. Palladium is a catalyst for this reaction.

3.4.2 Construction:

It utilises ‘gas pack’ method to cultivate anaerobic microorganisms. Anaerobic condition is created by addition of water to a gas generator envelop that is placed in the jar before sealing. Anaerobic jar is airtight metallic body which contain lid at the top to close the jar properly. It is very important to maintain position of lid and hence lid is tightened with the help of screw. It is equipped with thermometer to measure temperature. It also contains vacuum pump as well as side tube for provision of gases. Catalyst device is present which makes the hydrogen react with some oxygen.

3.4.3 Procedure

- Keep the inoculated culture plates inside the jar along with an indicator.
- Screw tight the lid
- Close the inlet tube and connect outlet tube to a vacuum pump (at least three quarters of the air of the jar can be removed).
- Note the pressure on a vacuum gauze and when the pressure is reduced to 100 mm Hg (i.e., 600 mm below atmospheric), tightly close the outlet tap.
- Connect the inlet tap is to a hydrogen supply and then open it. Hydrogen is passed through a small wash bottle.
- Bring the reduced pressure up to 760 mm Hg (i.e., atmospheric) by monitoring the vacuum gauze as 0.
- Switch on the electric terminals for heating the platinised crystal *(When room temperature catalyst is used heating is not required).*
- The catalyst helps the combination of hydrogen and residual oxygen to form water. This process is allowed to continue for 20 minutes.
- Incubate the McIntosh and Fildes’ jar in an incubator at 37°C for 48 hours.

3.4.3 Application:

Anaerobic jar mainly found application in cultivation of anaerobic bacteria which are difficult to cultivate in normal incubator.

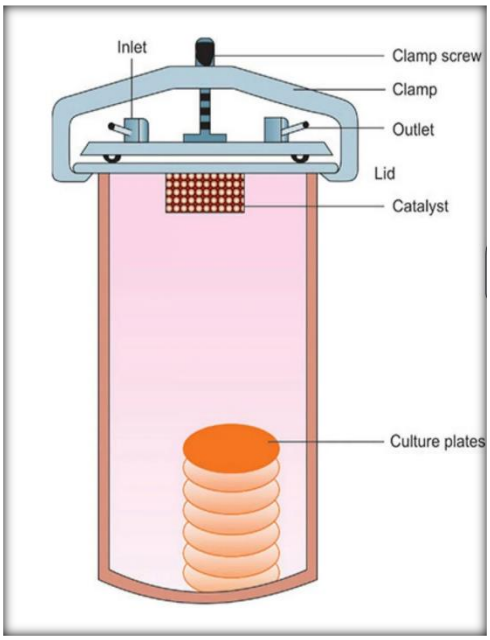


Fig 3.4: Anaerobic Jar

3.5 Refrigerator

In both commercial and residential settings, refrigeration systems are crucial because they provide cooling or maintain a specific room temperature. With a refrigerant flowing through the units, a refrigeration cycle entails heat exchange, compression, and expansion. To retain different strains of microbe, the refrigerator is most frequently employed in microbiology labs. It maintains a temperature of around 4 oC, below which water crystallises, and bacterial cells lose their viability but remain inactive.

3.5.1 Principle

The principle of refrigeration and cooling is very simple: it involves removing heat from one region and depositing it in another. When you pass a low-temperature liquid close to objects that you want to cool, heat from those objects is transferred to the liquid, which evaporates and takes away the heat in the process.

3.5.2 Working

A fluid refrigerant, a compressor, a condenser coil, an evaporator coil, and an expansion device are the five fundamental parts of the refrigeration cycle. The compressor raises the pressure of the refrigerant vapour by constricting it, forcing it into the coils on the refrigerator's outside. The heated gas in the coils turns into a liquid when it comes into contact with the kitchen's colder air temperature. The refrigerant now cools as it travels through the coils within the freezer and the refrigerator in liquid form under high pressure. The air within the refrigerator is cooled as a result of the refrigerant absorbing heat. Last but not least, the refrigerant dries up and goes back to the compressor, where the cycle is repeated.

3.5.3 Uses:

1. It is used to store bacterial culture.

2. It also used to store sterile media, biochemical reagents and thermolabile substances.

3.Refrigerator most used to store pharmaceuticals mainly antibiotics and injectables.

3.5.4 Limitation: This method is used for temporary storage of bacterial cultures and sub-culturing is necessary if the period exceeds to four or five weeks.

Fig 3.5: Refrigerator

3.6 Laminar Air flow

Equipment with a laminar air flow is frequently utilised in microbiology labs. It comprises of a chamber with an air blower attached to its back side that allows air to flow in straight, parallel lines at a constant pace. A laminar flow cabinet/hood's primary function is to create a clean working environment. It filters and collects all forms of impurity particles entering the cabinet for this purpose. It employs a filter pad and a specialised filter system called a high-efficiency particulate air filter, or HEPA filter, which can filter out airborne impurity particles as small as 0.3 micrometres. A laminar air flow chamber is also known as a laminar flow closet or a tissue culture hood.

3.6.1 Types of Laminar Air Flow:

There are different types of laminar cabinets are available with a variety of air flow patterns for different purpose.

1. Flow of vertical laminar air

2. Air Flow 2 Horizontal Laminar

3. Cabinets and Hoods with Laminar Flow

4. Benches and Booths for Laminar Flow

3.6.2 Advantages of a Laminar Air Flow Chamber

1. The laminar air flow devices have the benefit of being environmentally benign since they do not discharge any poisonous gas into the atmosphere.

2. Laminar air flow chambers don't need a lot of upkeep or repairs.

3. They are easily transportable and may be brought to new places.

4. Laminar air flow cabinets lessen the likelihood of environmental turbulence during an experiment.

5. A few laminar air flow devices include sophisticated security features that are designed to alert users and sound alarms in the event of any security breaches.

3.6.3 Disadvantages of a Laminar Air Flow Chamber

1. Placing hands or items on the machine interferes with air movement, creates turbulence, and lessens the machine's capacity to effectively sterilise the interior environment.

2. Some laminar air flow devices have a propensity to blast fumes in the user's face.

3. For optimal operation, the laminar air flow devices must be handled and cared for properly.

3.6.4 Applications of a Laminar Air Flow Chamber

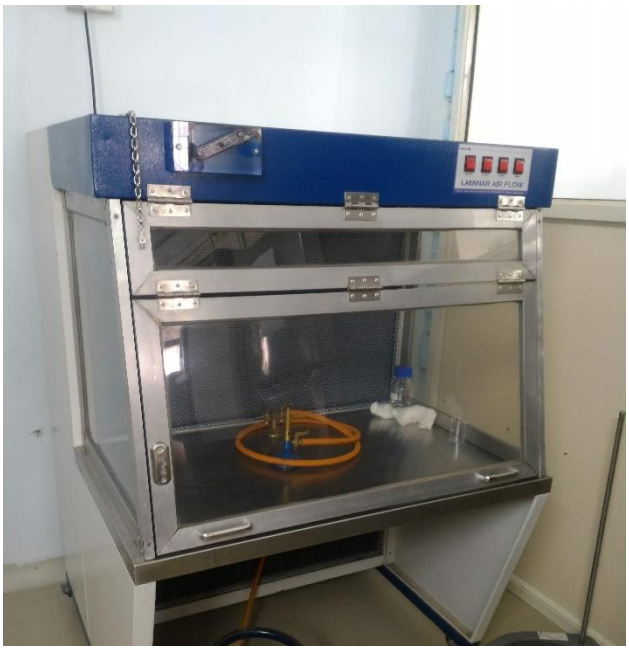
The following is a list of some common applications for a laminar air flow cabinet:

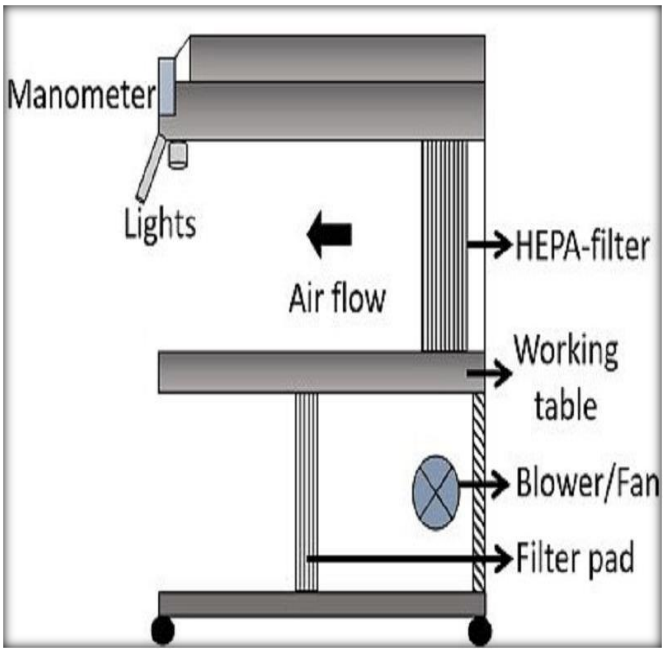
1. In laboratories, a laminar air flow cabinet is typically used to provide a sterile atmosphere for procedures like plant tissue culture.

2. Inside the air flow chambers, certain particle-sensitive electron devices are manufactured and operated.

3. One of the most important uses for a laminar air flow chamber is in the pharmaceutical business, where it is used for a variety of laboratory tasks including preparing media plates and cultivating microorganisms.

4. The most often utilised laminar air flow unit is in established clean room areas. Laminar flow benches are used for sterile product filling, microbiological research, and other purposes.





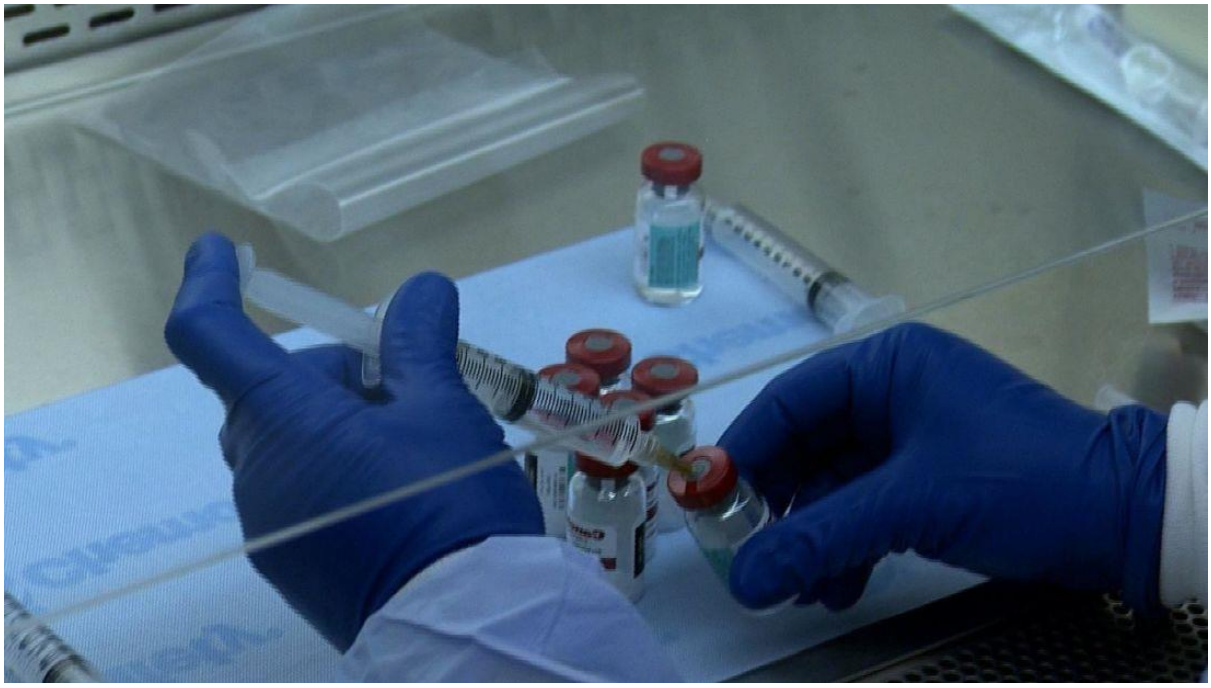


Fig 3.6: Horizontal Laminar Flow

3.7 Inoculating loops and Inoculating needles

Inoculation loops or an inoculation rod are used by microbiologists to mainly transfer inoculum. The apparatus comprises a fine handle with a tiny loop towards the terminal. The twisted wire loops have now been substituted by moulded plastic ones, commonly in use.

Inoculation needles are lab tools used in transferring inoculate living microbes. These can be re used or are disposable. A standard one is made from platinum or nichrome which is fixed with a metallic handle. Inoculation needles that can be disposed are usually made from plastic resins.

While inoculation needles are used to retrieve dense or solid media, an inoculation loop can retrieve liquid media.

3.7.1 Uses of Inoculating loops and Inoculating needles

Inoculation needles and loops are hand-held apparatus. They are of the following uses -

1. Inoculation needles are functional to sample and transfer small quantities of inoculum, they are used to study fungi and bacteria on semi-solid media.
2. Inoculation handles are used in inoculation, sterile sampling, serial dilution, spreading and transferring microbial samples.
3. Inoculation needles works on isolation of highly defined areas of the cultures and requirements of minimum disturbance between two densely crowded colonies.
4. Inoculation needles can be used in streaking on streak plates, inoculation of stab cultures, fish tail inoculation of slant cultures.
5. Loop threads can be used to take large samples.
6. Straight needles are functional in taking single colony samples of microbes.

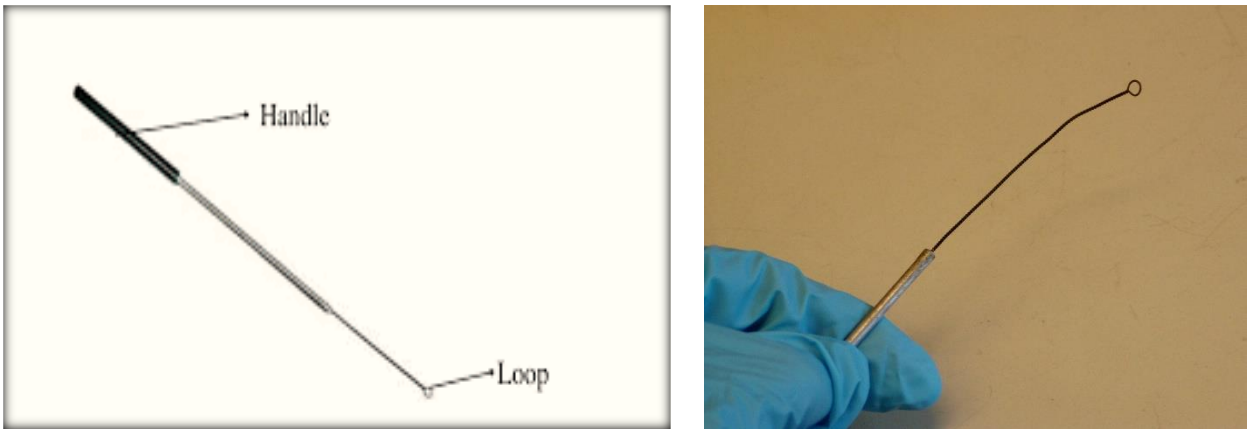


Fig 3.7: Inoculating loop

Experiment 04

To Preparation and sterilization of Laboratory Nutrient Agar media and Nutrient Broth

Requirements

Chemicals: Beef Extract, Peptone, Glucose, Sodium Chloride, Agar Agar Powder

Apparatus: Conical Flask, Glass rod, Burner, Petri plate, Test tubes, Measuring cylinder, Cotton, Autoclave, Balance.

Introduction:

- Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth.
- Nutrient media are used of cultivation of microorganisms. Media can be prepared in three different forms viz. Solid, liquid and semisolid. Consistency of media is determined by concentration of agar which act as solidifying agent.

Procedure

28gm Nutrient Powder + 1 L distilled Water

↓

Heat this mixture until fully dissolve all components.

↓

Kept in Autoclave at 121°C for 15 Min.

↓

Allow it to cool but not solidify.

↓

Pour nutrient agar into each plate and leave plates on the sterile surface until the agar has solidified.

↓

Replace the lid of each Petri dish and store the plates in a refrigerator.

Observation:

Clean and clear transparent yellow coloured nutrient agar form by sterilization.

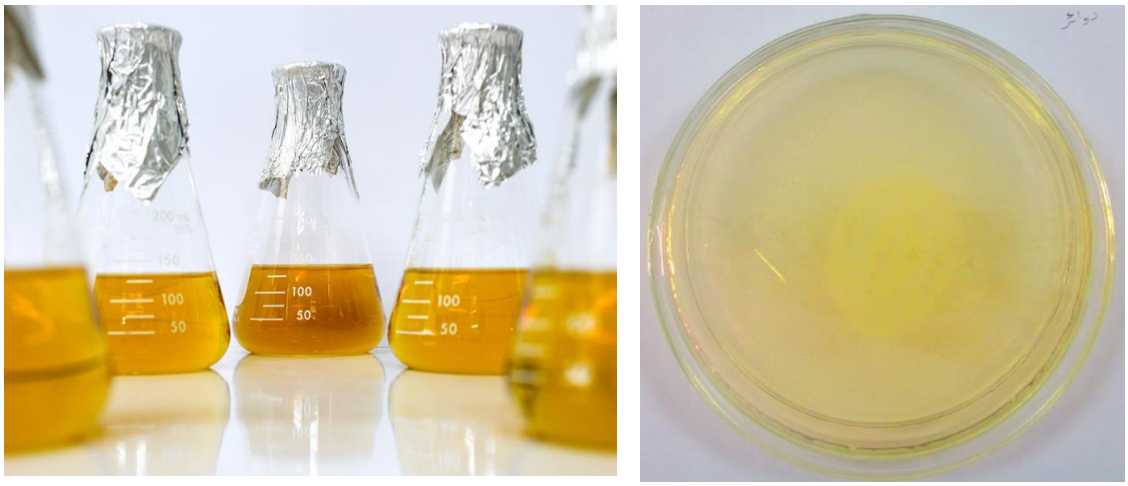


Fig 4.1: Nutrients Agar

Uses of Nutrients Agar

1. It is frequently used for isolation and purification of cultures.
2. It can also be used to create the bacterial lawns required for testing on antibiotic sensitivity.
3. Nutrient Agar/broth is the simplest and most common medium in routine diagnostic laboratories.
4. It is ideally used for the isolation, cultivation and maintenance of non-fastidious organisms.
5. It is used for checking the purity of the culture prior to biochemical or serological testing.
6. It aids in the enumeration of organisms in water, sewage, dairy products, faeces, and other materials.
7. It is used for producing bacterial lawns required for antibiotic sensitivity tests.
8. It provides a high-grade base for preparing special media.

PREPARATION OF NUTRIENT BROTH

Introduction

Nutrient Broth is a medium widely used for the culture of undemanding microorganisms. It is recommended in many standardized methods of analysis of foods, dairy products, water and other products. Nutrient broth has the same formulation as Nutrient Agar, only agar has been omitted which causes the medium to solidify at room temperature. Bacterial growth in the nutrient broth is evidenced by obtaining turbidity resulting from microbial multiplication.

Table 4.1: Composition of Nutrient broth

Ingredients	g/L
Peptones	10 g
Beef extract	1 g
Yeast extract	2g
Sodium chloride	5g
pH final	6.8 ± 0.2 at 25°C

Procedure

Follow the manufacturer's recommendations:

1. Add 13g to 15g of nutritious broth powder in 1L of distilled water.
2. Mix and dissolve completely.
3. Sterilize by autoclaving at 121°C for 15 minutes.

Fig 4.2: Nutrient Broth

Precautions:

1 Cotton plugs are to be kept loose when sterilize the media.

2 Prevent the media from contaminated area.

3 Store the media at low temperature.

Experiment 5
Stain and Staining Techniques

5.1 Simple Staining

5.1.1 Introduction

• A common approach for identifying the size, shape, and arrangement of bacterial cells is staining. The majority of bacteria have a distinctive form that belongs to one of three key morphological groups, including the spherical shape coccus, the rod shape bacillus, and the spiral or corkscrew shape.

Depending on the strains, we utilise various colours while simply staining. Examples of dyes include Malachite green for endospore, Crystal violet for gram-positive bacteria, Safranin for gram-negative bacteria, Carbol fuchsin for acid-fast bacteria, and Methylene blue for non-acid-fast bacteria.

5.1.2 Principle

- In simple staining, a single reagent is used to stain the bacterial smear, resulting in a pronounced contrast between the organism and its backdrop. Because bacterial nucleic acids and certain cell wall components have a negative charge that attracts and binds to the cationic chromogen strongly, basic stains with a positively charged chromogen are chosen.
- Simple staining is used to clarify the shape and organisation of bacterial cells. Methylene blue, crystal violet, and carboline fuchsin are the three most popular basic stains.

5.1.3 Requirements

- Bacterial culture *(24 hours old culture of bacteria like E. coli or Staphylococcus aureus)*
- Staining reagent *(Methylene blue stain)*
- Apparatus (*Staining tray, Glass slide, inoculating loop, Busen burner, Filter paper / Blotting paper, Glass marking pencil)*

5.1.4 Procedure

1. Clean the slide with distilled water first. Sterilise the slide by applying the flame.
2. Next, draw a circle in the centre of the slide and smear it.
3. Apply methylene blue to the stain, then wait about a minute for the dye to set.
4. Applying a mild spray of water to the slide's surface with a wash bottle filled with distilled water may help remove any remaining methylene blue.
5. Clean out any stains that ended up on the slide's bottom as well.
6. This time, saturate the smear with iodine. The stain will set with iodine.
7. Rinse off any extra iodine under softly running water. Thoroughly rinse.
8. Blot the discoloured area and wipe the slide's back with either bibulous paper or a paper towel.
9. Focus the stained smear using the 45X objective after placing it stained side up on the microscope stage.
10. Pick a section of the smear where the cells are evenly distributed in a monolayer.
11. Centre the region to be investigated, drizzle immersion oil over the smear, and then use a 100X objective to focus on the smear under oil.

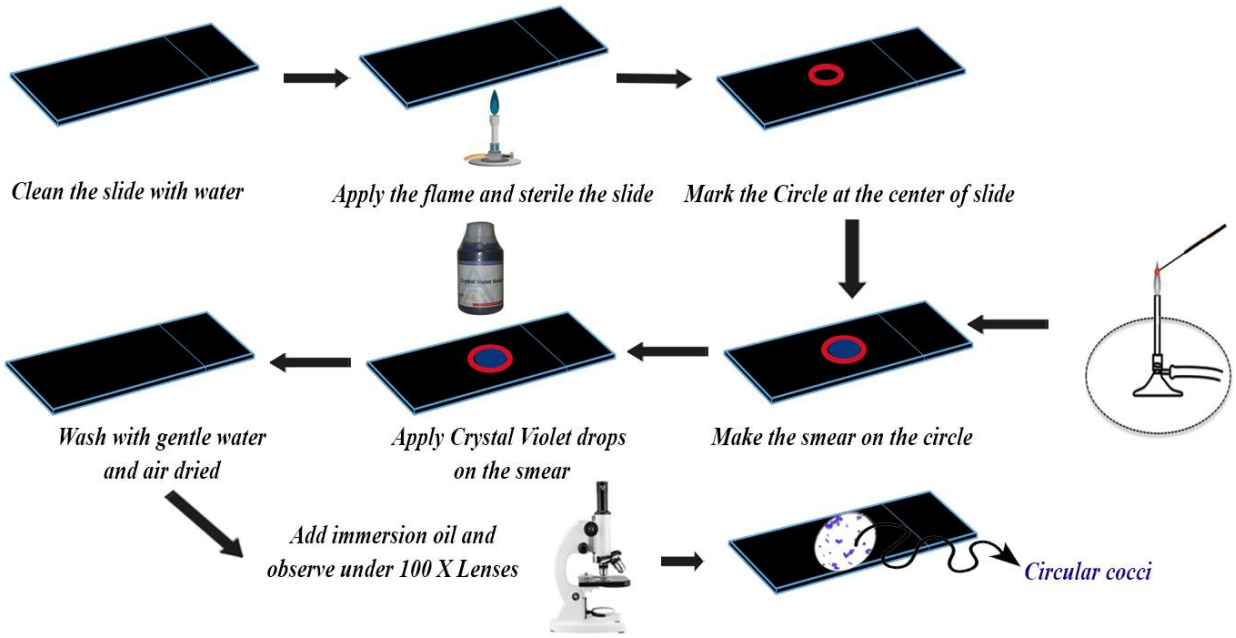
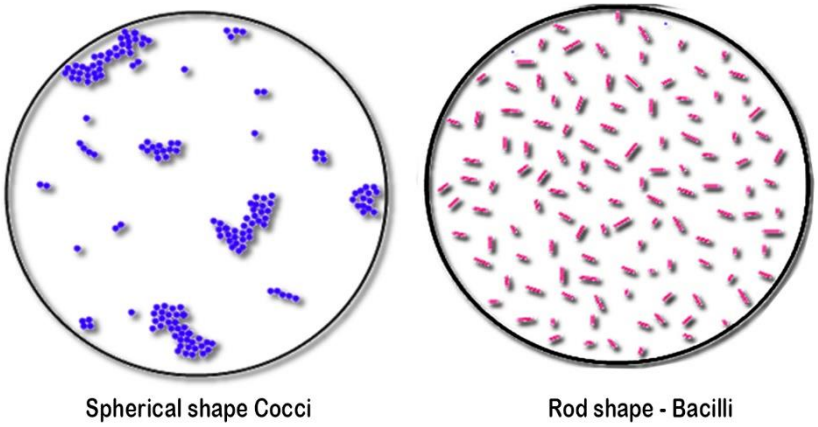


Fig 5.1: Sequential Diagrammatic steps of Simple Staining

5.1.5 Observation



5.1.6 Interpretation

Bacilli and diplobacilli: Rod-shaped bacteria, purple

Spirilla: spiral-shaped bacteria, purple

Cocci: spherical-shaped, bacteria, purple

5.2 Gram Staining

5.2.1 Introduction

- Gram stain is the most important and widely used microbiological differential stain, published by Hans Christian Gram in 1884, it allows bacteria to be differentiated according to 2 main criteria: their shape and their affinity for dyes.

5.2.2 Principle

- Gram-positive bacteria are those that trap the main stain-mordant complex and stain purple. These bacteria have a thick, strongly cross-linked coating of peptidoglycan that is between 20 and 80 nm in thickness.
- In Gram-negative bacteria, the outer membrane, which is a thin second layer that is 1 to 3 nm thick and has a lower proportion of cross-linkage than the peptidoglycan layer above it, does not preserve the primary stain-mordant complex after alcohol treatment.
- Both Gram-positive and Gram-negative staining of bacteria disclose the overall cell shape so that the cells may also be further labelled as rods or cocci. These bacteria with a thin cell wall are counterstained with safranin and are labelled as the Gram-negative bacteria.

5.2.3 Reagents Used in Gram Staining

a. primary stain and Crystal Violet
b. Iodine, the mordant
c. A decolourizer made of acetone and alcohol (95%)
d. Safranin, the counterstain

5.2.4 Applications of reagents

- Heating or applying methanol to fix clinical materials to the surface of the microscope slide.

- Applying the principal stain with crystal violet's assistance. Dark blue to purple dye known as crystal violet. All cells are stained blue.
- Applying the mordant: The iodine solution is applied to create a compound called crystal violet-iodine (CV-I), which keeps all of the cells' blue hues.
- The decolorization step: This stage separates gram-positive cells from gram-negative cells. The blue dye complex is more readily extracted from the lipid-rich, thin-walled gram-negative bacteria than from the lipid-poor, thick-walled gram-positive bacteria by an organic solvent like acetone or ethanol. Gram-positive bacteria stay blue whereas gram-negative bacteria look colourless.
- Using safranin as a counterstain: Gram-positive bacteria remain blue while the decolorized gram-negative cells are stained red/pink.

5.2.5 Procedure of Gram Staining

a) Use a grease-free, spotless slide.

b) With a loopful of the sample, prepare the smear of suspension on the clean slide.

c) Heat fix and air dry

d) Crystal Violet was poured, allowed to sit for 30 to 60 seconds, and then rinsed with water.

e) Soak the Gram's iodine in water for one minute.

f) Next, rinse with water after washing with 95% alcohol or acetone for 10 to 20 seconds.

g) After approximately a minute, add safranin and rinse with water.

h) Let dry naturally, blot, and see under a microscope.

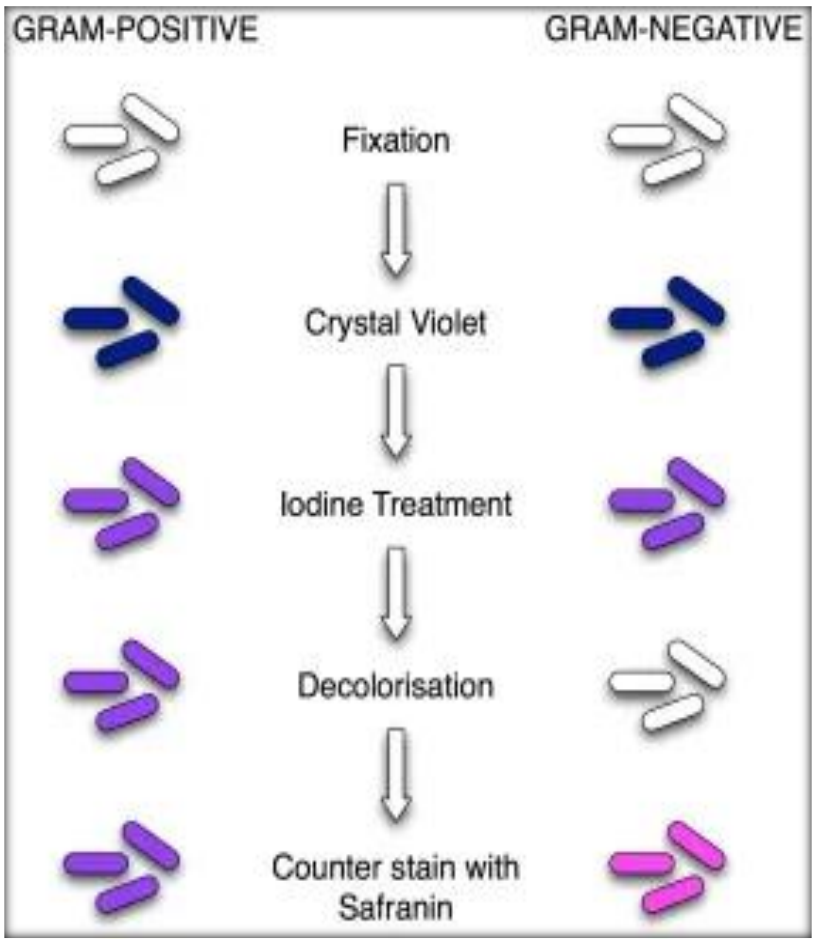
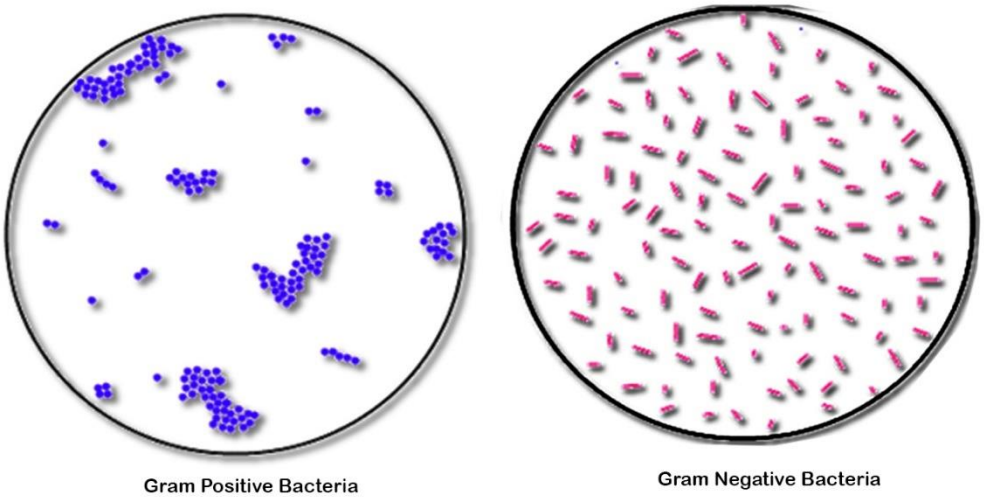


Fig 5.2: Procedure of Gram Staining

5.2.6 Observation



Gram Positive Bacteria

Gram Negative Bacteria

5.2.7 Interpretation

Gram Positive: **Blue/Purple Color**

Gram Negative: **Red Color**

5.2.8 Examples

Gram Positive Bacteria: *Actinomyces, Bacillus, Clostridium, Mycoplasma, Nocardia, Staphylococcus, Streptococcus, Streptomyces*,etc.

Gram Negative Bacteria: *Escherichia coli* (*E. coli*), *Salmonella, Shigella*, and Enterobacteriaceae, *Pseudomonas,Moraxella*, etc

Experiments 6

Isolation of fungi from the given soil sample

6.1 Introduction

There is no site free from the presence of one or more types of fungus or spores due to the widespread proliferation of fungus in numerous situations. Fungi, which may be separated from soil, air, or water, parasitize people and plants more than animals, which results in illnesses and financial losses. Therefore, it is vital to isolate and diagnose these fungi to lessen the risk.

6.2 Principle

The isolation and identification of soil fungi is an example in which a mycological ecosystem can be studied. It is usually too difficult to separate fungi from soil by directly picking them to place under a microscope for observation. Therefore, various techniques have been devised for stimulating growth and consequently easing fungal isolation and subsequently examination.

6.3 Requirements

1. Soil sample
2. Potato dextrose agar media / Czapek
3. Sterile Petri-plates
4. Sterile test-tubes
5. Lactophenol cotton blue
6. Sterile slides, coverslips
7. Sterile Spreader, needle
8. Bunsen burner
9. Micropipettes

6.4 Method -I

- Weigh 0.006-0.016 gm of soil sample and place it in a sterile Petri dish using a spatula.
- Crush the soil with spatula adding a drop of sterile water.
- Pour 8-10 ml of melted (45°C) fungal medium i.e., PDA/Czapek' into the plate and rotate the plate gently in order that the soil particles get evenly distributed in the agar medium.
- Incubate the plates at 37°C for 4—5 days. Pick up the colonies that come up, make pure cultures, and identify them.
- Preparation of the lactophenol cotton microscopic blue mount
- Place a drop of lactophenol cotton blue on a clean slide.
- Transfer a small tuft of the fungus, preferably with spore and spore bearing structures, into the drop, using a flamed, cooled needle.
- Mix gently the stain with the mould structures.
- Place a cover-glass over the preparation taking care to avoid trapping air bubbles in the strain.
- Examine the preparation under the low and high-power objectives of the microscope.

6.5 Method II- Burried Slide Technique

6.6 Requirements

1. Slides coated with malt agar
2. Electric plate (40°-50°C).
3. Knife
4. Stain-Rose Bengal.
5. Microscope.
6. Petri dishes.

6.7 Procedure:

- Place sterile slides in sterile Petri dishes.
- Make a thin coating with molten agar on ¾ th of the slide.
- Remove the slides, keep them on an electric plate at 40°-50°C until the agar is dried to a thin paper like layer.
- Make slits in soil with a sterile knife and introduce the slides vertically down.
- Press the soil around the slides gently so that the soil comes in contact with the slides. Leave the slides for 3-5 days. During this time, dried malt agar absorbs moisture and becomes softened.
- Remove the slides after 6—8 days, wash them gently, dry and stain with Rose Bengal and observe under the microscope.

6.8 Observations and results

Observe fungal growth on the plate culture and stain preparation for structure of hyphae and details of sporulating structures microscopically under low and high power.

Fig 6.1: Aspergillus flavus and Penicillium chrysogenum

6.9 Example of fungi- *Mucor, Rhizopus, Aspergillus, Penicillium, Fusarium, Cladosporium, Alternaria, Curvularia.*

6.10 Precautions

1. Soil should be in powered form.

2. Use a fresh sterile pipette in each dilution.

3. Each dilution must be thoroughly shaken before removing an aliquot for subsequent dilution.

4. The air bubble may also be removed by placing the slide in a vacuum chamber.

5. Plates are to be incubated in an inverted position.

Experiment 7

Isolation Techniques

7.1 Introduction

- All inanimate surfaces contain microorganisms, which makes them common sources of potential contamination in scientific settings. The outcome of an experiment depends on the scientist's capacity to sterilise tools and work surfaces as well as to keep sterile instruments and solutions away from non-sterile surfaces.
- Several plating techniques are often employed in laboratories to isolate, multiply, or count microorganisms including bacteria and phage. All five techniques use aseptic technique, or steps used to keep experimental materials sterile. Procedures described include.

  (1) streak-plating bacterial cultures to isolate single colonies

  (2) pour-plating

  (3) spread plating to enumerate viable bacterial colonies.

  (4) Isolation of fungi from the given soil sample

7.1.2 Principle

- It is a typical technique to identify and isolate a specific bacterial colony from a bacterial swarm. In the streak plate approach, the number of bacterial colonies is greater at the beginning of the streak and gradually decreases towards the end. Each colony is regarded as a pure colony, which aids in separating it from other colonies.

7.1.3 Requirements

1. Bacterial culture: Bacillus subtilis and Staphylococcus aureus 24-hour bacterial culture.

2. Instruments: sterile Petri plates, a nichrome wire loop for inoculation, a burner, and a marking pen.

3. The medium is nutrient agar.

4. Equipment: an incubator, a hot air oven, an autoclave, and a colony counter.

7.2 Streak Plate Method

The Culture techniques are commonly used in the laboratory for various purposes for which they are intended.

1. To show how the bacteria's cultural traits are expressed.
2. To separate the distinct colonies of bacteria from the specimen that contains several microorganisms.
3. To assess a bacterium's sensitivity to drugs, antibiotics, or test chemicals, as well as its resistance to them.
4. To ensure that the bacteria grows sufficiently for certain biochemical and other tests.
5. To calculate the number of bacteria in the specimen that are still alive.
6. To keep the stock cultures alive.
7. To convey biological materials or store specimens temporarily

7.2.1 Standard Procedure

- The bottom of the petri dish has the label. The name of the organism and the date are often on labels.
- Use the heat of the Bunsen burner to sterilise the nichrome wire loop.
- Use a sterile nichrome wire loop to collect a sample of the bacterial culture after opening the bacterial culture tube.
- Spread bacterial culture on the nutrient agar plate. To remove the designated quadrants, the agar plate cover must be opened between the Bunsen burners.
- The entire procedure is carried out in a laminar airflow cabinet under highly aseptic conditions.

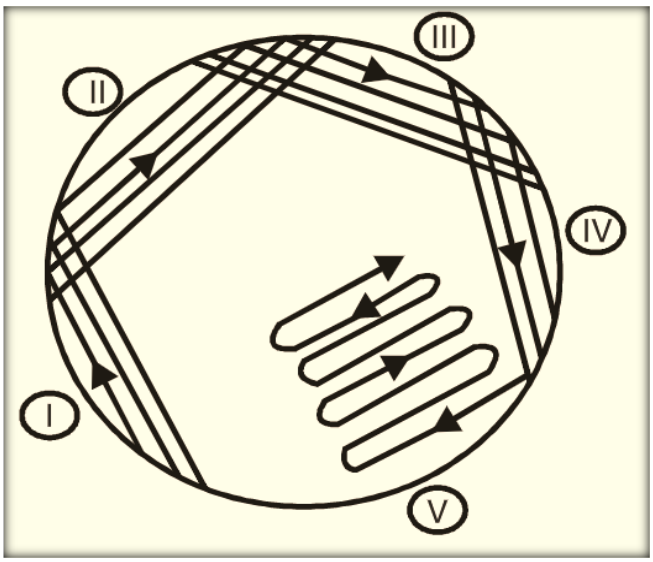


Fig 7.1: Streak Plate

7.2.3 Three Sector streak (t streak)

- Put the nichrome wire loop over Bunsen burners flame to sterilise it.
- Cool the loop of wire in-between the Bunsen burners.
- Submerge the wire loop in the broth culture that contains the assortment of bacteria.
- Streak the bacterial suspension in a zigzag pattern to create T-shaped streaks on the nutrient agar plate using the nichrome wire loop.
- After incubating the plate for 24 hours, the third sector will contain solitary colonies. The first sector will have the strongest growth, with the second sector experiencing less of it.

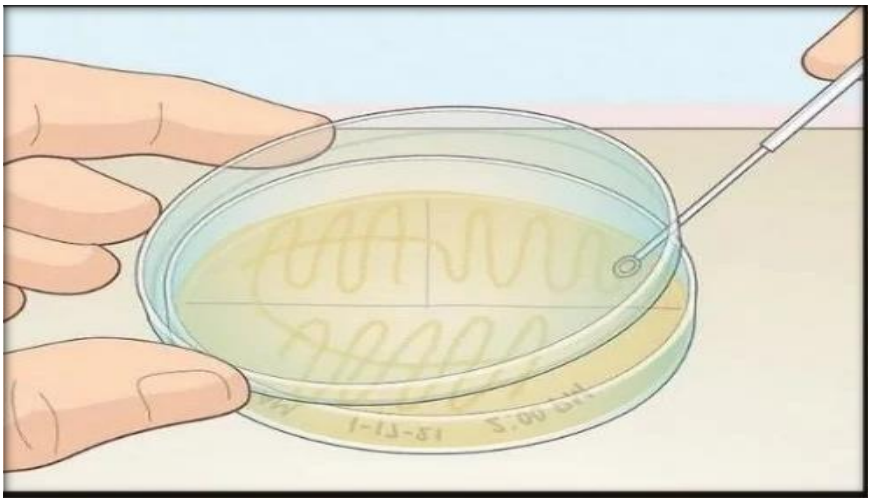


Fig 7.2: Three Sector streak (t streak)

7.2.4 Four – Quadrant streak

- Put the Nichrome wire loop over a Bunsen burner flame to sterilise it.
- Cool the loop of wire that runs between the burners.
- Use a marker to write the petri dish's name and draw a four-quadrant grid on the bottom of the dish.
- Set a test tube containing a bacterial culture on fire.
- Insert the culture tube with the nichrome wire loop.
- Spread the bacterial suspension across the plate's four quadrants, between the two burners.
- Incubate the plate for 24 hours at 37 °C.

7.2.5 Observation: Examine the growth of isolated colonies on the surface of the nutrient agar plate.

7.2.6 Result: Few numbers of isolated colonies appear along with the points of the streak.

7.2.7 Advantages

1. Distinct separate colonies are obtained by the streak plate method.
2. It is a simple method for the isolation of microorganisms.
3. Commonly used for isolation of colonies from pharmaceutical products.

7.2.8 Disadvantage

1. Before isolation, there was a higher possibility of contamination.
2. This approach only works in terms of quality.
3. The colony count is not relevant in other quadrants since only isolation is acquired in the fourth quadrant.

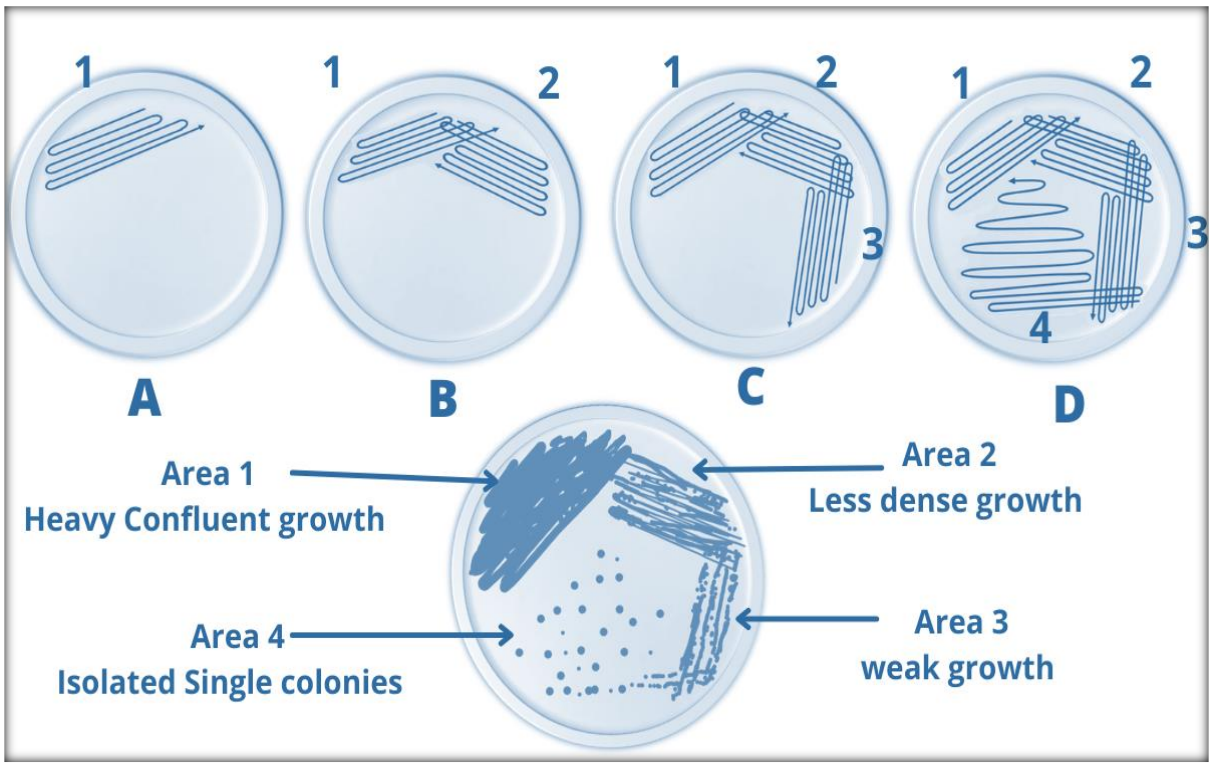


Fig 7.3: techniques of Streak plate method

7.3 Pour plate method.

7.3.1 Introduction

- For obligate and anaerobic microorganisms, the pour plate method is a typical plating technique. By serially diluting microbial colonies and then counting the colony forming units (CFUs), this approach is used to isolate the colonies. In this procedure, the liquid sample is added to the petri dish before the agar medium has a chance to set.
- Colonies develop within and outside of the medium after solidification. Confluent colonies, however, are developing within the medium; the colonies on the surface are counted as viable.

7.3.2 Principle

- The pour plate method involves using a sterile pipette to transfer a predetermined volume of inoculum, typically 1 ml, from a broth or sample into the centre of a sterile Petri dish. After that, 15mL of the molten, cooled agar is added and well mixed in the

Petri plate holding the inoculum. The plate is turned over and incubated at 37°C for 24-48 hours following the agar's solidification.

- Both on the surface and within the medium, microorganisms will proliferate. Most colonies that develop beneath the medium are typically tiny and may even be confluent; the few that develop on the agar surface are similar in size and resemble those on a streak plate. Each colony, big and little, is meticulously tallied (using a magnifying colony counter if necessary). The number of microorganisms present in the test sample is determined using the formula:

$$CFU\backslash ml = \frac{Total\ number\ of\ colonies\ obtained\ x\ dilution\ factor}{Volume\ of\ specimen\ used\ (aliquot)} \ldots$$

7.3.3 Requirements

1. Test sample
2. Nutrient agar or plate count agar (PCA)
3. A 45 °C hot water bath
4. Sanitised Petri plates
5. Flame
6. A colony counter with a microscope
7. Test tubes 16*150 mm with sterile caps
8. Pipettes in a range of sizes, such as 01, 1.0, and 2.0 mL

7.3.4 Procedure

- Sterilise every piece of equipment, flask, and medium needed for the streaking process.
- To reduce infection, sanitise the space where you operate.
- Carefully assemble the Bunsen burner at your workspace.
- Prior to handling any microbiological solution, wash your hands with an antiseptic solution.

- Write down all pertinent details on the petri dish, including your name, the date, the medium you used, and the culture that is being injected.
- Since the sample is already liquid, produce successive dilutions to reduce the concentration of microbial colonies, which vary from 20 to 300 CFU per millilitre. Dilutions up to 10-10 can be made.
- To inoculate, remove the Petri dish covers and add 1 millilitre of the diluted sample within. 15 to 18 ml of the molten agar should be poured onto the sample once it has been slightly heated. Agar shouldn't be too hot or too cold, so keep that in mind. Put the dish's cover on and give it a steady stir.
- Gently combining the diluted material with the agar medium and then pouring it onto the petri dish is another way for inoculation.
- Permit the plate to set.
- Flip the dish over and let it sit for 24 to 48 hours at the ideal temperature, which is often 37°C.

7.3.5 Result Interpretation

- After 24 to 48 hours of incubation, count all colonies. A magnifying colony counter may assist in the counting of tiny, embedded colonies.

7.3.6 Applications of the Pour Plate Method

- It is used by scientists to obtain microbial growth curves and in the calculation of the concentration of cells in a particular sample.
- It is also used to check the effect of various growth factors and environmental factors on the growth rate of the bacteria.
- It is used to separate pure cultures from mixed cultures.
- It is used to identify and count viable fungi and bacteria (calculate CFU per ml) from liquid samples.

- Utilized to create growth curves to study biochemical and microbial metabolic processes and the effect of environmental conditions on the growth of microbial species.

7.3.7 Advantages of the Pour Plate Method

1. It is useful for counting viable colonies.
2. It can detect very low loads of bacterial counts as well.
3. It does not require previously solidified agar plates.
4. It can also be used for clinical and environmental samples.

7.3.8 Precautions

1. The protocol must be followed in all aseptic conditions, particularly in Laminar air Flow (Safety Cabinet) to prevent contamination.
2. Make sure you accurately measure the amount when preparing the serial dilutions from the sample.
3. Use sterile pipettes each time to prevent any mistakes or contamination that may occur.
4. Determine the exact amount of dilute specimen prior to injecting it onto Plates of Solidified Media Plates.
5. Spread the specimen uniformly on the Media plate until you get clear and healthy colonies.

7.3.9 Limitations

1. The organisms that are heat sensitive are susceptible to being affected by molten media between 40-45degC.
2. Colonies could be smaller than those in spreading or streaking, which can increase the probability of ignoring them.

3. Obligate aerobes could have trouble growing towards the lower portion of the plate. Some don't even grow.
4. It is time-consuming since it requires dissolving these solids, a series of diluting, and then melting the media at a particular temperature 42 to 45degc.
5. Semisolid or solid samples should be suspended prior to the inoculation. It can be very difficult to do this to inoculate if the sample isn't easily dissolved.
6. It is necessary to perform regular dilutions of the sample or else many colonies will form that aren't able to be counted and recognized as distinct.
7. It takes time for the growth of the organisms and the creation of colonies.

7.4 Spread plate method.

7.4.1 Introduction

- A mixed sample is spread out across the surface of an agar plate, and a method called the spread plate is used to separate the bacteria from the material. To separate and isolate each bacterial colony, samples are diluted. In enrichment, selection, and screening experiments, the spread plate is frequently employed.

7.4.2 Principle

- One of the popular culture techniques for isolating microorganisms, particularly bacteria, in the lab is the spread plate culture method. Using a sterile L-shaped glass rod (Spreader), a serially diluted specimen containing two or more bacteria is applied in a thin layer to the solidified agar media plates while the media plate is being spun on a turntable.

7.4.3 Requirements

Nutritional agar, beaker, a busen burner, an L-shaped bent glass rod and a wax marking pencil, alcohol with a 95% purity Bacterial Culture: Bacillus subtilis, Chromobacterium indica, and

Staphylococcus album, magnifying colony counter, an autoclave, a hot air oven, and an incubator.

7.4.4 Procedure

- Set up the nutritional agar plate and label it with the appropriate bacteria, such as Staphylococcus aureus, Staphylococcus album, and Bacillus subtilis.
- Transfer a loopful of the bacterial culture aseptically from the medium in the appropriate Petri plate.
- Place the bent section of the glass rod in a beaker filled with 95% alcohol to sterilise it. Next, slide the beaker over the flame of a Bunsen burner.
- Let the rod cool for 15 to 30 seconds.
- Tilt the plate's cover, gently press a sterile rod on the agar surface, and disperse the bacterial suspension evenly.
- Use an alcohol bath to sterilise the bent rod, then reflame it over a Bunsen burner.
- Inoculate the remaining two plates with the bacterial sample by using the same procedure.
- For 24 to 48 hours, incubate all plates at 25°C while they are inverted.

7.4.5 Observation: Few colonies may be separate while the rest of the colonies are maybe in a bunch. Record the result of colonies their form, elevation, pigment formation by colonies, and their size.

7.4.6 Results: Isolated colonies have appeared on the surface of the nutrient agar plate.

7.4.7 Advantages

1. It is a simple, easy, and quick method of culturing microorganisms.
2. A very low microbial load can be detected.
3. The colony morphology of a microorganism can be studied by this method.
4. It is a qualitative and quantitative isolation approach that makes it easy to isolate and count.

5. It is used in preparing and maintaining stock culture.
6. It is the most appropriate method for culturing the aerobic microorganism.

7.4.8 Disadvantages

1. The spread plate method allows the growth of other microbes along with desired microbes.
2. The spread plate method allows the growth of obligate anaerobic microorganisms.
3. Accidental contamination and hence the growth of undesired microbes may be possible.

7.4.9 Limitations

- Additional tools, such as a spreader, are needed.
- The technique is a little complicated since the material must be in liquid or suspension form and must be serially diluted.
- Before inoculation, solid or semisolid materials must be suspended. If the sample is not readily soluble, it is quite challenging.
- Anaerobes and microaerophiles can't grow there in sufficient numbers.
- If the sample's microbial burden is too high, it is inappropriate. In order to lower the microbial burden at 20–300 CFU/mL, the sample must be serially diluted. We might even need to do pilot tests to obtain this dilution range.

7.4.10 Applications

1. Used to isolate bacteria and fungi from a given sample.
2. Used in antimicrobial sensitivity testing, and enrichment and screening experiments.
3. Used to calculate the number of viable microorganisms i.e., calculate CFU/mL in a sample.
4. Used in food industries, pharmaceutical industries, soil studies, etc.
5. Used to mass culture the stock culture or fresh specimen
6. Used in clinical laboratories to inoculate the clinical specimens.

7. Used to study growth curves, metabolic activities, and biochemical features of microorganisms,
8. Used in separating pure culture from a mixed culture.

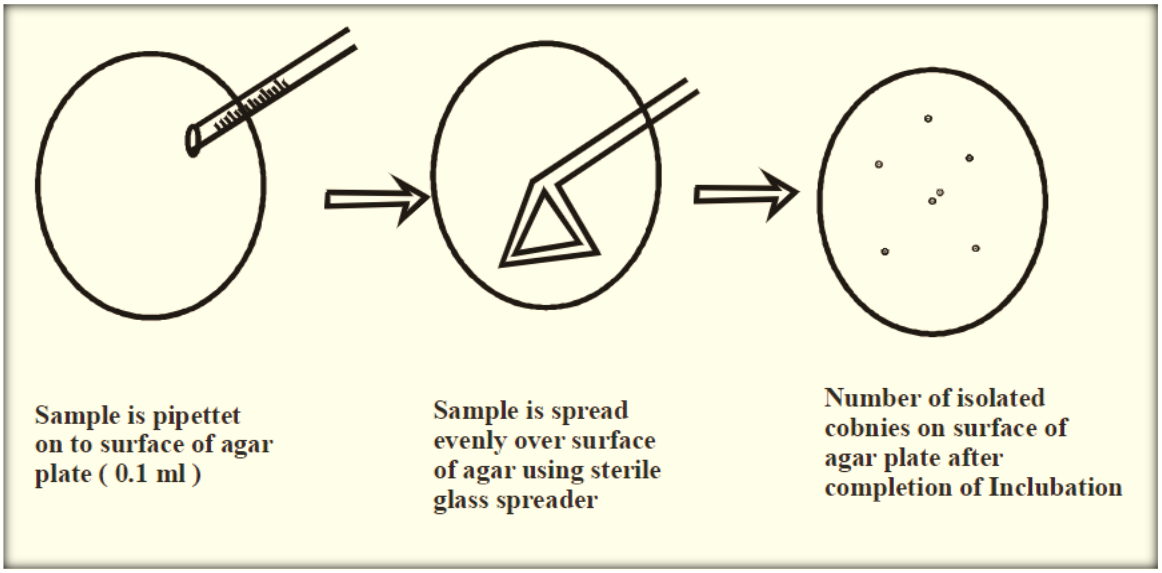


Fig 7.4 Spread Plate Method

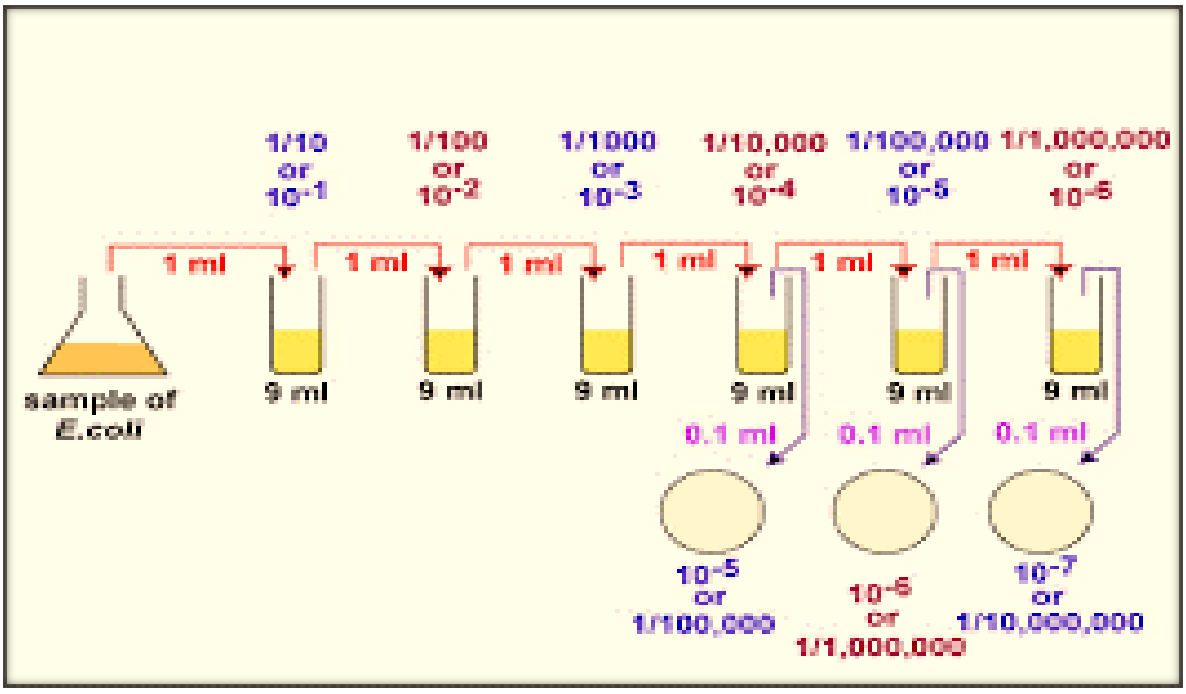


Fig 7.5 Serial dilution of sample

Experiment No 13

Biochemical Test

13.1 Introduction

IMViC is the most widely used primary biochemical test series. Though considered key to selectively differentiating Enterobacterales, it is also used for characterization and identification of several Gram-positive bacteria. It is routinely used in clinical laboratories for teaching and research purposes.

All the tests in the series are easy to perform and give results within 24 – 48 hours. Hence, used for the primary screening purpose. Though it can be used for the identification of any type of bacteria, it is mainly used for identifying Gram-negative bacteria. It is the key to identifying and differentiating members of the *Enterobacteriaceae* family.

IMViC is an acronym for four different biochemical tests; each letter except "I" represents an individual test making this series of biochemical tests. IMViC series contains the following biochemical tests:

I - Indole Test

M - Methyl Red (MR) Test

V - Voges – Proskauer (VP) Test

C - Citrate Utilization Test

The letter "i" is only for rhyming purpose. The four reactions are: Indole test, Methyl Red test, Voges Proskauer test and Citrate utilization test. The letter "i" is only for rhyming purpose. The letter **"i"** after 'V' is only for the rhyming purpose, it does not indicate any test.

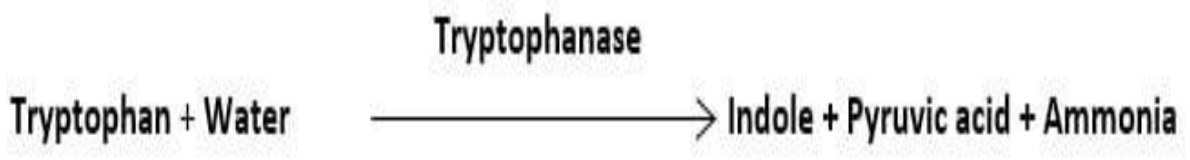
13.2 Principle

The IMViC test is based on the variations in the metabolic requirements and properties of different genera and species of bacteria. The 'indole test' and 'citrate utilization test' in the series detect the ability of bacteria to produce specific enzymes and utilize specific nutrients. On the other hand, the 'MR test' and 'VP test' in the series detect the final metabolic products produced by the bacteria utilizing specific nutrients. For this purpose, the test bacteria are cultured in specific culture media, different media for the different tests.

Indole Test

13.3 Principle of Indole Test

Some bacteria can produce an enzyme called 'tryptophanase' which helps them to metabolize the amino acid 'tryptophan' into 'indole, pyruvic acid, and ammonia'.



When the indole reagent is added to a medium with a bacterial culture that has produced indole, the indole combines with the aldehyde present in the reagent to give a distinctive colour.

If benzaldehyde is present in the reagent, a pink to a violet-red quinoidal compound is formed, hence; a pink to red colour ring is formed.

If cinnamaldehyde is present in the reagent, a blue to the green colour compound is formed, hence; a green to blue colour ring is formed. Kovac's indole reagent uses amyl alcohol and benzaldehyde. The amyl alcohol is water-insoluble and forms an oily layer, thus giving a cherry-red or pink – red ring on the top."

13.4 Requirements for Indole Test

Culture media:

- Tryptophan broth

Reagents:

- Kovac's Indole Reagent
- Ehrlich's Reagent
- 5% *p*-dimethylaminobenzaldehyde or 1% *p*dimethylaminocinnamaldehyde in 10% (v/v) concentrated HCl for the spot indole test.

Procedure

1. Test bacteria are cultured in the medium containing tryptophan for 24 – 48 hours
2. and an indole reagent is added following the incubation to read the result.
3. A positive result is indicated by the formation of a pink to violet-red or green to blue colour ring according to the type of reagent used.
4. A negative colour is indicated by the formation of a lack of colour change or a slight yellowish colour ring at the top.

Example

Indole Positive Bacteria: *Escherichia coli, Klebsiella oxytoca, V. cholerae*

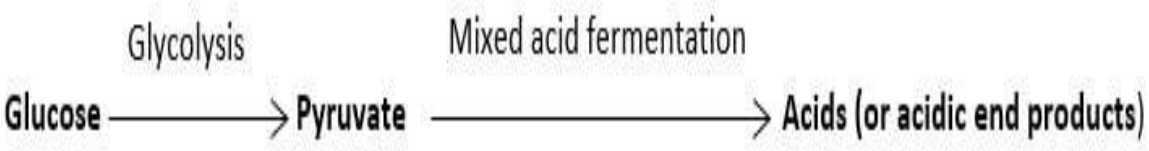
Indole Negative Bacteria: *Klebsiella pneumoniae, Proteus mirabilis, Salmonella spp., Shigella spp., Citrobacter freundii, Pseudomonas aeruginosa,*

Methyl Red (MR) Test

Methyl Red (MR) Test is a biochemical test that detects the ability of organisms to produce stable mixed acids as metabolic end products of glucose metabolism. It is indicated by the letter "M" of the IMViC.

13.5 Principle of MR Test

Some species of bacteria use the mixed acid fermentation pathway as their glucose metabolism process. Following this metabolic pathway, they convert pyruvate into stable mixed acids.



When such acid fermenters bacteria are grown in a medium containing glucose, they will release the acids, hence, decreasing the pH of the medium to 4.4 or lower. When methyl red indicator is added in a medium containing such acid fermenters, it will turn the medium red." Following the 24-hour incubation on the MR-VP broth, a methyl red indicator is added to the broth. A positive result is indicated by the development of red colour while a negative result is indicated by the development of yellowish colour.

13.6 Requirements for MR Test:

Culture media:

- MR-VP broth

Reagents:

- Methyl red indicator

13.7 Procedure

1. The bacterium to be tested in inoculated into glucose phosphate broth, which contains glucose and a phosphate buffer and incubated at 37oC for 48 hours.

2. Over the 48 hours the mixed acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acid.

3. The pH of the medium is tested by the addition of 5 drops of MR reagent.

4. Development of red colour is taken as positive.

5. MR negative organism produce yellow colour.

13.8 MR Test

Example

MR Positive Bacteria: *Escherichia coli, Salmonella spp., Shigella spp., Citrobacter spp., Proteus spp., Yersinia spp., Edwardsiella spp., Staphylococcus aureus,*

MR Negative Bacteria: *Klebsiella pneumoniae, Enterobacter spp., Hafnia spp., Serratia marcescens.*

13.9 Voges-Proskauer (VP) Test

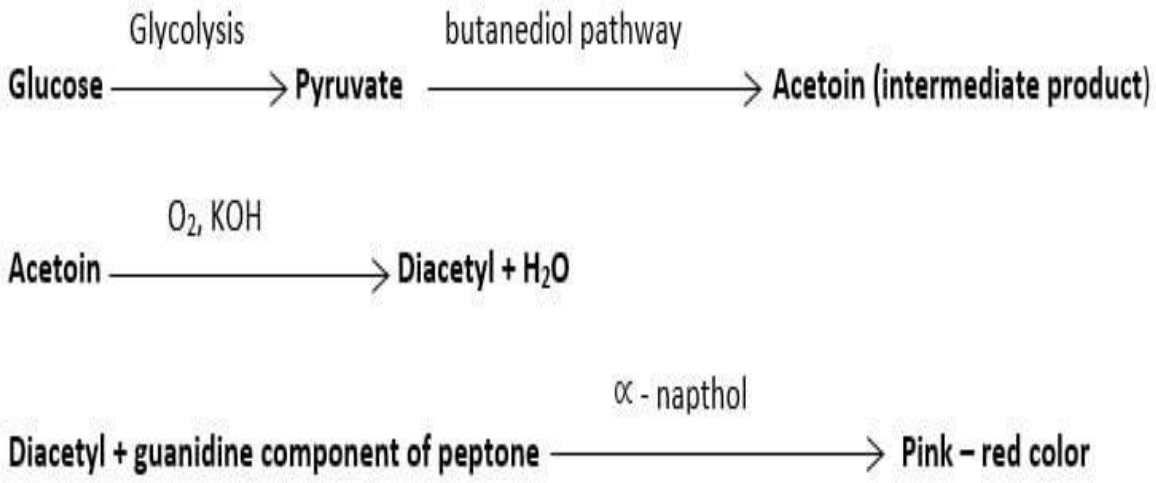
Voges-Proskauer (VP) Test is a biochemical test in the IMViC test series which detects the ability of organisms (bacteria) to metabolize the pyruvate into a neutral intermediate product called 'acetylmethylcarbinol' or 'acetoin'. It is indicated by the letter "V" of the IMViC.

13.10 Principle of VP Test

Pyruvate can be metabolized into a neutral intermediate product called 'acetyl methyl carbinol', commonly called the 'acetoin' during the butanediol pathway of 2,3-butanediol production.

If acetoin is present in the media, it is oxidized readily to diacetyl in presence of air and KOH. Thus, produced diacetyl, in the presence of $\propto$ – naphthol, will react with the guanidine component of peptone forming a pink to a red colored product.

$$Glucose \rightarrow pyruvate \rightarrow Acetoin\ (intermidate\ product)$$



13.11 Requirements for VP Test:

Culture media:

- MR-VP broth

Reagents:

- Barritt's A solution or VP reagent I (5% – α-naphthol solution)
- Barritt's B solution or VP reagent II (40% KOH solution)

13.12 Procedure

1. Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours.

2. 0.6 ml of alpha-naphthol is added to the test broth and shaken. 0.2 ml of 40% KOH is added to the broth and shaken. The tube is allowed to stand for 15 minutes. Appearance of red colour is taken as a positive test.

3. The negative tubes must be held for one hour since maximum colour development occurs within one hour after addition of reagents.

Example

VP Positive Bacteria: *Klebsiella spp., Enterobacter spp., Viridans Streptococci*

VP Negative Bacteria: *Escherichia spp., Proteus vulgaris, Citrobacter freundii,*

Citrate Utilization Test

13.13 Principle

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO2. 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.

13.14 Requirements for Citrate Utilization Test

Culture media

- Simmon's Citrate Agar

Reagent

- Bromothymol blue indicator (it is already incorporated in the Simmon's citrate medium)

- The simplified IMViC agar plate containing modified media containing all four IMVic test media is also prepared, but it is not applied widely.

13.15 Procedure

1. Bacterial colonies are picked up from a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37oC.

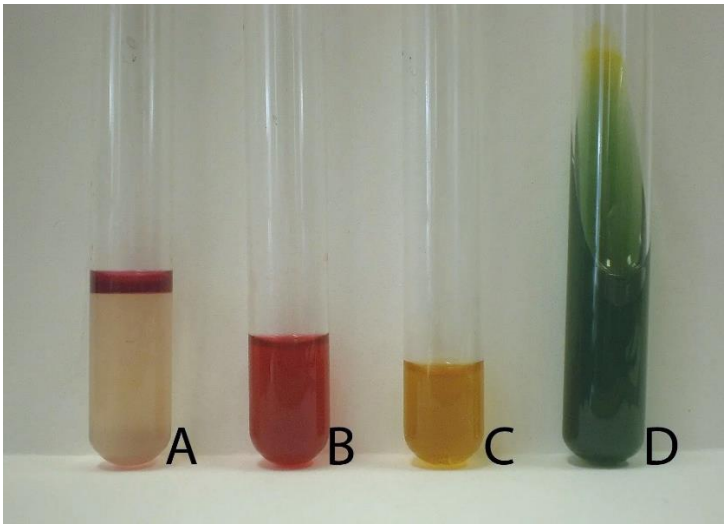
2. If the organism can utilize citrate, the medium changes its colour from green to blue.

13.16 Observation

If colour of the medium change to blue, it is citrate

Positive -*E. coli* is citrate

Negative- *Klebsiella pneumoniae*



IMViC Test

INDOLE TEST: Principle: Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase. 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 color. An alcoholic layer concentrates the red color as a ring at the top. Procedure: 1. Bacterium to be tested is inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at

37oC.Prepare 1% tryptophan broset thbate one . 2. Incubate one set of test tube withtest organism and maintain one set as negative control without inoculation . inoculate one set of test tube with E.coli use as positive control. 3. Following incubation few drops of Kovac's reagent are added. Shake gently. 4. Kovac's reagent consists of para-dimethyl aminobenzaldehyde 10 gm, isoamyl alcohol 150gm and con. HCl 50 ml 5. 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. 6. Ehrlich's reagent is more sensitive in detecting indole production in anerobes and non-fermenters. Observation:- Formation of a red or pink coloured ring at the top is taken as positive. Example: Escherichia coli: Positive; Klebsiella pneumoniae: Negative METHYL RED (MR) TEST: Principle: This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less. Procedure: 1. the bacterium to be tested in inoculated into glucose phosphate broth, which contains glucose and a phosphate buffer and incubated at 37oC for 48 hours. 2. Over the 48 hours the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acid. 3. The pH of the medium is tested by the addition of 5 drops of MR reagent. 4. Development of red color is taken as positive. MR negative organism produce yellow color. Example: Eschericihia coli: Positive; Klebsiella pneumoniae: Negative VOGES PROSKAUER (VP) TEST: Principle: While MR test is useful in detecting mixed acid producers, VP test detects butylene glycol producers. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In this test two reagents, 40% KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha- naphthol to produce red color.

Role of alpha-naphthol is that of a catalyst and a color intensifier Procedure: 1. Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours. 2. 0.6 ml of alpha-naphthol is added to the test broth and shaken. 0.2 ml of 40% KOH is added to the broth and shaken. The tube is allowed to stand for 15 minutes. Appearance of red color is taken as a positive test. 3. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents. Examples: Escherichia coli: Negative; Klebsiella pneumoniae: Positive CITRATE UTILIZATION TEST:

Experiment No 11

Bacterial culture preservation

11.1 Introduction:

As name indicates this method is used to preserve pure culture of microorganism in there viable and active form. Main target behind preservation is to avoid multiplication of microbes which intern control the change in generation. Control on generation changes helps to avoid any mutation in microorganism. Beside this main motto, control on growth of bacteria prevents formation and accumulation of toxic material in media.

11.2 Objectives of preservation:

a) To maintain pure culture in viable and active form

b) To avoid contamination

c) To prevent genetic changes.

Uses of preserved culture

a) Academic use.

b) Fermentation industry.

c) Biotechnology field.

d) Research purpose.

Paraffin method

11.3 Introduction

The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and,

therefore, the culture can be preserved form months to years. medium, and still preserve the original culture.

11.4 Procedure

1. This is a simple and most economical method of maintaining pure cultures of bacteria and fungi.
2. In this method, sterile liquid paraffin in poured over the slant (slope) of culture and stored upright at room temperature.
3. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium.
4. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.

Glycerol method

11.5 Introduction

Glycerol can be used as carbon source in media. maybe your isolates are slow growing bacteria. Generally, for actinobacterial isolation glycerol is carbon source and most of the actinobacteria are slow growing organism so it dints mean because of the glycerol.

11.6 Procedure:

1. Prepare fresh bacterial culture on specific media from max 48-72 h older culture.
2. Allow bacteria to grow enough and scrap bacterial colonies with sterile loop under aseptic condition.

3. Transfer the clumps of culture into the vial of broth containing 15% glycerol which results in formation of dense suspension.
4. Keep the broth at room temperature for approximately 30 minutes before placing in the freezing condition. Store the culture at -50ºC or -70ºC.

11.7 Precautions:

1. Perform all steps under aseptic condition only in order to avoid any cross contamination.
2. At -50ºC, the bacterial culture remains stable for NMT one year while at -80ºC it may remain stable for more than one year.
3. Avoid frequent refreezing and variation in storage temperature which may reduce shelf life.

11.8 Limitations:

1. It possesses limited shelf life.
2. High cost requires for preservation.
3. Require sophisticated deep freezer.

Culture preservation by Mineral oil

11.9 Introduction

The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture can be preserved form months to years. In most of microbiology laboratories, microbial cultures are preserved for longer time by oil method.

11.10 Procedure:

1. Isolate bacterial culture and streak on agar slant under aseptic condition.

2. Cover slant to a depth of 1cm above with sterile oil. (Note: Mineral oil is sterilized in hot air oven at 170ºC for 1 hour.)

3. Keep the culture at room temperature where mineral oil helps to prevent dehydration of medium.

4. Sub-culturing is performed by scraping of the bacteria from slant.

11.11 Advantages:

1. It is simple and economical method for preservation of bacteria as well as for fungi.

2. Viability of bacteria can be maintained for several years.

3. Culture is store at room temperature and hence no need of deep freezer.

Agar Slant Method

11.12 Introduction

Solid medium made with agar and various nutrients and indicators. Slanting gives the bacteria a greater surface area on which to grow in a tube. Agar slants are also useful in maintaining bacterial cultures, more so than stacks of Petri dishes. It is most commonly used method for preservation of bacterial cultures.

11.13 Procedure:

1. Pour the agar media into test tube and subject it for sterilization.
2. After sterilization, keep test tube in inclined position till agar get solidify to prepare slant.
3. Inoculate slant with culture of bacteria and incubate it under specific condition.
4. After incubation, keep slant in refrigerator.
5. Transfer this culture periodically in fresh media for supplements of nutrients to the organisms.

Precautions: Perform all steps under aseptic condition.

11.14 Advantages:

1. It is simple and economical method.

2. More suitable for small scale laboratory work.

11.15 Limitations:

1. Limited shelf life.

2. Require frequent transfer and preparation of sub-culture.

Saline Suspension

11.16 Introduction

This method involves preservation of bacterial culture by using normal saline solution for 2-3 days. Along with preparation of microbial culture, saline solution can also use for dilution of bacterial culture.

11.17 Procedure:

1. Prepare 0.85% normal saline solution.

2. Aseptically transfer bacterial culture in saline solution.

3. Incubate under specific condition.

4. Repeat the same procedure after 2-3 days.

5. Sodium chloride in high concentration is frequently an inhibitor of bacterial growth.

6. Bacteria are suspended in 1% salt solution (sublethal concentration in screw cap tubes to prevent evaporation).

7. The tubes are stored at room temperature. Whenever needed the transfer is made on agar slant.

11.18 Precaution: It is very important to prepare normal saline solution otherwise concentrated or diluted saline solution may causes shrinkage or swelling of cells.

11.19 Advantage: Simple and economical method.

11.20 Limitation: Preserve culture for only for 2-3 days.

Cooked-meat medium

11.21 Introduction

Cooked Meat Medium (R.C. Medium) is used for cultivation of aerobes and anaerobes, especially pathogenic Clostridia. This can also be used as a maintenance medium for stock cultures. Cooked medium is preferred for preservation of anaerobic bacteria.

11.22 Procedure:

1. Inoculate the bacterial culture in cooked neat medium and incubate it at 37ºC for 24 hours.
2. After sufficient growth, tubes are preserved at room temperature.
3. Perform sub-culturing process in time interval of one month.

11.23 Advantages:

1. Cooked neat media maintain the viability of cultures over long period of time.
2. It is excellent method for preservation of anaerobic as well as aerobic organisms.

Lyophilization

11.24 Introduction

Lyophilization, or freeze-drying, is a term applied to the procedure of freezing and subliming water from frozen preparations. “Lyophil method” is specifically applied to the condensation of water, sublimed from frozen preparations, on a cold surface as distinct from the use of desiccants. As most biological materials dried from the frozen state are lyophilic, regardless of how water is

removed, the term “lyophilization” is adopted by a number of workers to describe any process involving the sublimation of water from frozen preparations.

11.25 Procedure

1. Bacterial culture is rapidly frozen at very low temperature -70^{o}C and then dehydrated by vacuum.

2. Microbial cells are dehydrated and then transferred into dormant phase, so retain the viability of cells for longer periods.

3. Bacterial cultures which are freeze dried, preserved in the dark at 4^{o}C in refrigerator.

11.26 Advantages

1. Minimum storage space is required for preservation of lyophilized culture.
2. Small vials are sent conveniently to the other laboratories and culture centers.
3. Sterility can be maintained.
4. Reconstitution is easy.
5. Water is removed at low temperature and pressure.
6. Thermo-labile materials are operated and get dried.
7. Long term preservation method

11.27 Disadvantage

1. Due to freeze drying and stress, biological molecules may damage.
2. Cost is the big issue in lyophilization method.
3. It is time consuming method.

11.28 Application

Freeze drying method is often used for preservation of cultures in the national and international culture collection center and maintains the viability for several years.

Soil method

11.29 Introduction

This method is applied for spore forming microbes like Bacillus, Streptomyces, Penicillium etc. Pure cultures are kept in sterile soil medium and preserved for many months under refrigeration. Sterile garden soil is used for preservation of bacterial as well as fungal culture. Soil provides natural habitat for preservation of bacteria and fungi.

11.30 Procedure:

1. Soil is autoclaved at 15 lbs pressure for 30 minutes.

2. After sterilization soil is inoculated with aqueous suspension of cells or spores. 3. Organisms are allowed to grow for 7-10 days.

4. Soil culture is stored in refrigerator.

11.31 Advantages:

1. Pure culture of soil microbes are preserved for a number of months under refrigerator.

2. Suitable for bacterial as well as fungal preservation.

Experiment 10

Sterility Techniques

10.1 Introduction

- Sterility can be defined as the freedom from the presence of viable microorganisms. However, the conditions that guarantee absolute sterility are usually too harsh for active ingredients, and the definition of sterility for a medicinal product must be defined in functional terms.
- Sterility testing is a GMP microbiology testing requirement used to confirm sterile products do not contain viable microorganisms before release and patient administration. Sterility testing methods must be as accurate as possible, due to their importance for medical devices, pharmaceutical products, and formulations, tissue materials, and other products that claim to be sterile or free from viable microorganisms.
- Sterility testing procedures are applied to products in many industries, including food and beverage manufacturers, but the main industries are the pharmaceutical and medical sectors where the sterility testing of the products remains a vital and routine task for microbiologists.

10.2 Direct Inoculation

- Inoculation in two types of media for detection of aerobic and anaerobic microorganisms
- Inoculation period of 14 days with intermittent observations.
- Performed in a Grade A Isolator with optional VHP to reduce risk of naturally occurring bacteria and fungi contamination delivering false positives and out-of-specification results.

- If the sample appears cloudy or turbid after inoculation, it can be challenging to detect turbidity from microbial growth at the end of the incubation period.
- Additionally, if the product has antimicrobial properties, the sample must be neutralized so that microbial growth is not inhibited.

10.3 Membrane filtration sterility testing

- Enclosed sterile units allowing two samples of equal volume to filtered simultaneously through two membrane filters.
- Incubated in two types of media to facilitate detection of aerobic and anaerobic microorganisms.
- We test bulk and final drug products as well as raw materials. We also perform bacteriostasis/fungi stasis tests to assess if microorganism growth is inhibited.

10.4 Methods for testing the sterility of medical devices.

1. Direct transfer sterility testing is recommended for the sterility testing of medical devices.
2. The device to be tested is in direct contact with the test media throughout the incubation period, during which any microorganism in or on the device will grow and proliferate.
3. Furthermore, product flush sterility testing is preferred for products with hollow tubes, such as transfusion and infusion assemblies, where the fluid pathway is labelled as sterile.
4. The product lumen is flushed with a rinsing fluid, the elute is membrane filtered and is placed in a suitable media for incubation.

10.5 Observation and Interpretation of results

- Visually examine the media tubes daily to its conclusion for macroscopic evidence of microbial growth. If no evidence of growth observed in any of the tube the product to be examined for the test complies with the test for sterility.

Experiment 11

Hanging drop preparation

11.1 Introduction

- Some bacteria are motile and some other are non-motile. Motile bacteria usually use flagella as their locomotory organ. Bacteria tend to move towards or away from various chemotactic, phototactic, aerotactic or magneto tactic stimuli. Description of bacterial flagella is available here. There are various ways to demonstrate bacterial motility. These include, a simple wet mount, hanging drop preparation, or employment of soft gels (semi-solid agar).

11.2 Requirements for hanging drop preparation.

- Fresh broth culture of bacteria, Bunsen burner/spirit lamp,
- Bacteriological loop
- Glass slide with central concavity/paraffin ring/adhesive ring
- Cover slips
- Microscope

11.3 Procedure

1. A loopful of bacterial suspension is placed in the center of a cover slip.
2. The glass side with stick ring is placed over the coverslip such a way that coverslip sticks to the ring on the slide.
3. Immediately, the glass slide is lifted and turned around.
4. The drop of bacterial suspension now "hangs" on the lower surface of the coverslip.
5. The drop is then observed under the low power (10x) dry objective of the compound microscope.
6. The edge of the drop must be focused. Bacteria tend to accumulate at the edge of the drop.
7. Once the edge is located, it is then observed under 40x high power objective.

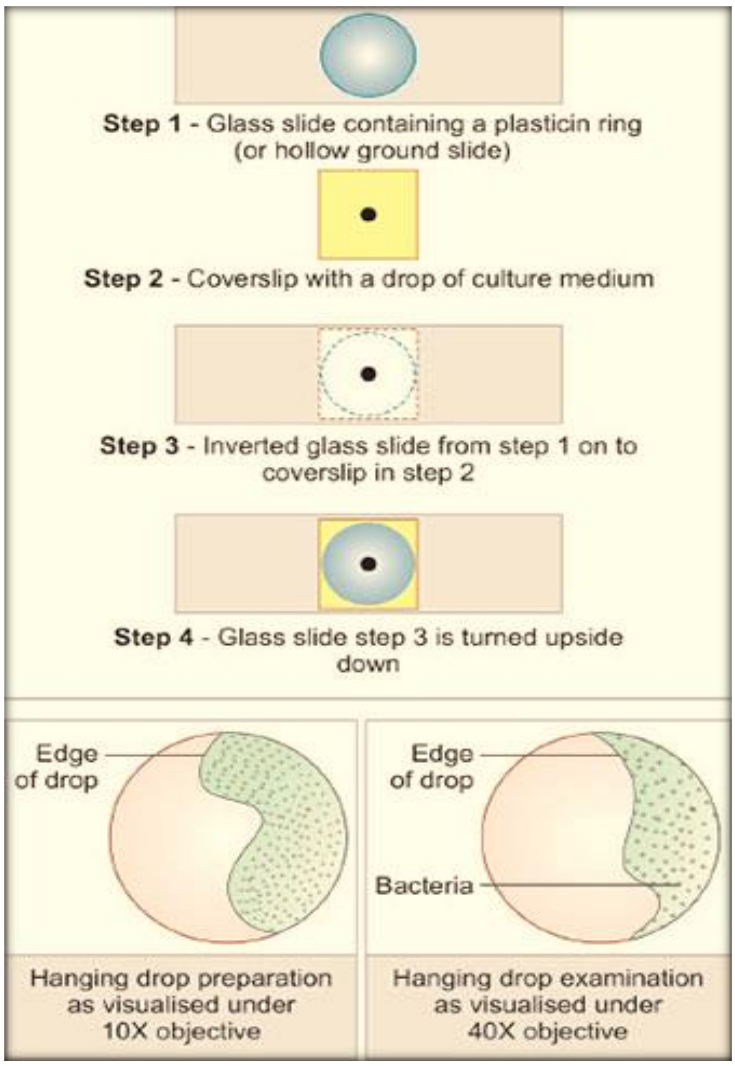
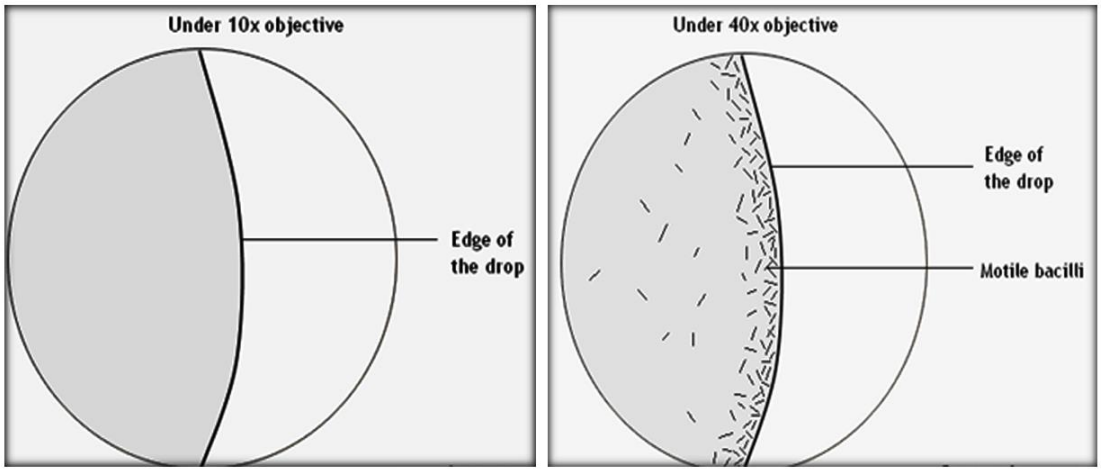


Fig 11.1: Hanging drop technique.

Observation



Observation under 10x objective

Observation under 40x objective

Motile bacilli are seen at the edge of the drop

Experiment 12

bacteriological testing of water by MPN method

12.1 Principle:

- Three basic test to detect colroform bacteria in water are presumitive, confirmed and completed. This test detects the pressure of coliform bacteria the gram-negative non-spore forming bacilli that fermit lactose (carbon source) with the production of acid and gas.
- In addition to lactose, the medium also contain a surface tension depressant, bile salt which is used to supress the growth of the microorganisms other than caliform bacteria. Development of gas in tube is presumptive evidence of the present of coliform bacteria in the sample.
- The presumptive test is also used to obtain some idea of number of microorganisms present by mean of the most probable number of tubes in each group that shows gas formation in the incubation period. This medium is selective because of presence of dye methylene blue which inhibits the growth of gram-positive bacteria.
- Eoisin- methylene blue (EMB) is differential in nature in that lactose fermenting bacterial gives coloured colonies (a positive confirmed test) due to formation of a complex. In presence of an acid environment BMB forms a complex that precipitate out into coliform colonies introducing dark corner produce colonies on EMB agar.
- Completed test is used in a confirmatory test for process of e-coli in a water sample. In this test, lactose positive colonies form EMB agar are isolated and incubated into a lactose broth tube and streaked on the nutrient to perform gram staining.
- If there is production of acid and gas is incubated lactose broth, there are rod shaped bacteria showing gram negative reaction, there reaction of E. coli in water sample and indicate of positive completed test.

12.2 Requirements:

12.2.1 Chemicals: single strength lactose broth, double strength lactose broth, water.

12.2.2 Apparatus: 3 bumper tubes, 6 test tubes, 10ml measuring cylinder, capillary tubes, forceps, test tubes sand, conical flask, beaker, absorbent cotton.

12.2.3 Equipment: incubator, autoclave, weighing balance.

12.3 Procedure.

- Collect a water form pond or sewage paint and use for detection of coliforms.
- See up three separate grows in test tube rack containing each group.
- First group (3) test tube and 10ml double strength lactose broth.
- 10ml water sample in first group of double strength lactose broth.
- 1ml in first group of single strength broth and 0.1ml in second group of single strength broth.
- Incubate all 9 tubes for 37-degree c for 48 hours.

12.4 Observation Chart

Number of 3 tubes giving positive reaction			MPN Index per 100ml
10ml	1ml	0.1ml	
0	0	1	3
0	1	0	3
1	0	0	4
1	0	1	7
1	1	0	7
1	1	1	11
1	2	0	11
2	0	0	9
2	0	1	14
2	1	0	15
2	1	1	20
2	2	0	21
2	2	1	28
3	0	0	23
3	0	1	39

3	0	2	64
3	1	0	45
3	1	1	75
3	1	2	120
3	2	0	93
3	2	1	150
3	2	2	210
3	3	0	240
3	3	1	460
3	3	2	1100
3	3	3	2400

*Source: standard methods for the examination of water and wastewater APHA New York 1999.

Experiments 13

Validation of Sterilization

13.1 Introduction

A sterilization validation test confirms the appropriate Sterility Assurance Level of the medical device. The testing establishes the pertinent sterilization parameters for health care reprocessing instructions.

Since the sterility of a medical device or product is based on acceptance criteria, the process that a product or device undergoes to become sterile must be validated to prove that sterility acceptance criteria are consistently met. Sterility can be assured only by using a validated sterilization process under current good manufacturing practices (cGMP). Chemical and biological indicators are used to validate whether an autoclave is working properly. Chemical indicators are quick to read, but generally, only demonstrate that the autoclave has reached a certain minimum temperature.

13.2 Validation of Autoclave

13.2.1 Chemical Indicators- Tape Indicators

Tape indicators are adhesive-backed paper tape with heat sensitive, chemical indicator markings. Tape indicators change color or display diagonal stripes, the words “sterile” or “autoclaved” when exposed to temperatures of 121°C. Tape indicators are typically placed on the exterior of the waste load. If the temperature sensitive tape does not indicate that a temperature of at least 121°C was reached during the sterilization process, the load is not considered decontaminated. If tape indicators fail on two consecutive loads, notify your Department Safety Manager.

Tape indicators are not designed nor intended to prove that organisms have actually been killed. They indicate that a temperature of 121°C has been achieved within the autoclave.

13.2.2Biological Indicators

13.2.3 Procedure

Biological indicator vials contain spores from B. ***stearothermophilus***, a microorganism that is inactivated when exposed to 121.1^{o}C saturated steam for a minimum of 20 minutes. Autoclaves used to treat biological waste will be evaluated with a biological indicator by EHS on a quarterly basis.

13.2.4 Results

If the autoclaved indicator exhibits growth, the validation has failed and will be repeated.

If the second validation indicator fails, EHS will notify the Department Safety Manager and request service on the autoclave. Autoclave should not be used until service has been conducted and the validation test passes.

13.3 Sterilization control for hot air oven

1. Biological controls: 10^{6} spores of ***Bacillus subtilis*** subsp. ***niger*** or spores of nontoxigenic strains of ***Clostridium tetani*** on paper strips are placed inside envelopes and then placed inside the hot air oven after complete sterilization inoculated in thioglycolate or cooked meat medium and incubated for sterility test under strictly anaerobic conditions for 3 to 5 days at 37°C. Growth in medium indicates the failure of sterilization.

2. Chemical control: Browne's tube No. 3 shows a green color after sterilization at 160°C for 60 minutes, color changes from red to green.