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FUNDAMENTALS OF MICROBIOLOGY – A LABORATORY MANUAL

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Fundamentals of Microbiology - A Laboratory Manual

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THIS BOOK

IS DEDICATED

TO

MY FAMILY

MY FATHER (*Late*), MY MOTHER, MY SISTERS & MY BROTHERS

&

FRIENDS

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Practical 1

INTRODUCTION TO LABORATORY INSTRUMENTS AND EQUIPMENT

Equipment: These are basically automated devices which give results themselves.

- Incubator (adjust particular temperature)

Instruments: These are handling devices and their function is dependent on user.

- Inoculating loop

Media (Pleural; Medium; Singular)

Nutrition of microorganisms and can be provided in multiple forms;

- Liquid (Broth)
- Solid (Agar)
- Semi-solid (deep agar)

Culture

Growth of microorganisms in lab is called culture.

Microscope

It is used to study shapes and arrangements of microbes with the help of magnifying lenses and resolution power.

Falcon Tubes

These are basically ideal for cell centrifugation, pelleting and separation by density gradients. Their size is about 15 and 50 mL. These are already sterilized.

Micro Centrifuge Tube (Eppendorf)

It handles small volumes of liquids. It is used for sample storage, running reactions and spinning down or separating samples.

Petri Dish

It is widely used in microbiology for cultivation of colonies of microorganisms. To do this, it is filled with a layer of solid medium (agar), on which a culture of micro-organism is shown. It is used to grow bacteria on solid agar.

Pasteur Pipette

Pasteur pipette is used to transfer small quantities of liquids. They are usually glass tubes tapered to a narrow point and fitted with a rubber bulb at the top.

Inoculating Loop

Also called a smear loop, is a sample tool used mainly by microbiologists to retrieve an inoculum from a culture of micro-organisms. It is used in cultivation of microbes on plates by transferring inoculum for streaking. • Also used to transfer microscopic organisms. The loop is made up of Platinum which becomes red hot very quickly and is also cooled down immediately in few seconds. Other property of platinum loop is that it does not kill or affect the microbes being inoculated.

Inoculating Needle

For transfer of microbial growth from one medium to other.

Measuring Cylinder

It is a piece of laboratory glassware used to measure the volumes of liquids accurately.

Micropipette

These accurately dispense tiny volumes of liquid. They measure volumes in microliters, which represent millionths of a liter. Common micropipette sizes include 20, 100, 200 and 1000 microliters. It uses the tips which contain liquid being transferred.

Syringe Filters

These are generally used to remove particles from a liquid sample prior to some kind of analysis to avoid damage to equipment (e.g., ion chromatography). They are relatively affordable, can be used for small volumes, and avoid the difficulties because of filter set ups or similar. These may be of 0.25- or 0.45-micron meter. These are made up of nitrocellulose membrane.

Autoclave

An autoclave is a pressure chamber used to carry out steam heat sterilization. In autoclave, sterilization is carried out by 121°C for 15 minutes with 15 pounds per inch square pressure. It works on principle of pressure cooker.

Universal, McCartney and Bijoux Bottles

These are specially used to carry culture medium and bacteria can be growth in these bottles. These are set of thick clear glass bottles. Overall, these bottles can be sterilized with the medium present inside it.

- Universal bottle (30 mL)
- McCartney bottle (15 mL)
- Bijoux (7.5 mL)

Hot Air Oven

Hot air ovens are electrical devices use dry heat to sterilize. They use thermostat to control temperature. Their double walled insulation keeps the heat in and conserves energy. The inner layer is a poor conductor while outer layer is metallic in nature. Their temperature range is either;

- 160 Celsius for 1 hour
- 180 Celsius for 30 minutes

Screw Capped Test Tubes

Test tubes with screw caps are suitable for transporting and growing cultures; made of Borosilicate glass; very good chemical resistance. These have round bottoms. These are also used to grow bacteria on agar in them which is called Slant.

Whatman Filter Paper

It is barrier that is semi-permeable in nature. These are made up of cellulose paper. These are used for filtration.

Dropper

Dropper is a pipette consisting of a small tube with a vacuum bulb at one end for drawing liquid in and releasing it a drop at a time. Basically, used for random measuring out drops of medicine or other liquids.

Incubator

Laboratory incubators provide a controlled

- Temperature
- Humidity
- Gases (Oxygen and Carbon dioxide)

Microbiological incubators are used for the Growth of bacterial cultures.

Biosafety Cabinet

Biological safety cabinets (BSCs) are used in laboratories to work with infectious agents. These act as primary barriers to prevent the escape of biological aerosols into the laboratory environment.

Conical Flasks

Conical flasks are widely used for holding liquids and mixing these. These are used for preparation of medium for microbial growth.

Microscopic Glass Slides and Cover Slips

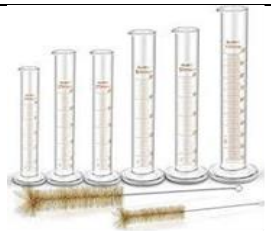
A glass slide is a thin, flat, rectangular piece of glass that is used as a platform for microscopic specimen observation. A typical glass slide and cover slip are designed to fit under the stage clips on a microscope stage.

Centrifuge Machine

It is used to separate particles from liquid on basis of density, size and weight. Particles settle down in bottom in form of pellet and liquid remain on top as supernatant. Centrifuge tubes are used for this purpose. These are microfuge (1.5mL) and Falcon tubes (15 and 50mL).

Glass Pipettes and Pipetting Aid (Suction Bulb)

These are used for transferring of liquids in measured amount. To take help pipetting aids are used that assist in pipetting process.

 Inoculating loop	 Inoculating needle	 Plastic dropper
 Pasteur pipette	 Membrane filters (Syringe filters)	 Whatmann filter paper
 Micropipette	 Tips for micropipette	 Measuring cylinders
 Petri plate	 Pipetting aid (roller & Suction bulb)	 Glass pipette
 Glass test tubes	 Glass bottles (Bijux, McCartney and universal-left to right)	 Conical flask

Glassware and Plastiwares used in Microbiology Laborator

 Microscopic glass slides (frosted)	 Cover slips	 Cavity slide
 Micro-centrifuge	 Falcon tube (50mL)	 Falcon tube (15mL)

Glassware and Plastiwares used in Microbiology Laborator

 Autoclave	 Biological safety cabinet	 Microscope
 Centrifuge machine	 Incubator	 Hot air oven

Laboratory Equipment used in Microbiology

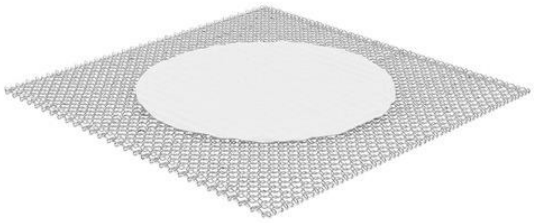
 Spirit lamp	 Tripod stand
 Wire gauze	 Aluminium foil
 Thraed	 Wash bottle
Miscellaneous Items Required in Microbiology Laborator	

Figure 1: Glasswares, Plastiwares, Equipment and Miscellaneous Items Used in Microbiology

Practical 2

MICROSCOPE: PARTS AND USE

Microorganisms: These organisms are small in size and cannot be seen with naked or unaided eye. These are either unicellular or multicellular

- Bacteria
- Viruses
- Fungi

These could be yeast (unicellular) or Mold (multicellular or filamentous form)

- Protozoans
- Microscopic algae

To observe microorganisms an instrument is used called Microscope; It is a combination of two words

Micro: small

Scope: To see

Microscope: So, microscope is optical an instrument used to observe objects/organisms cannot be seen with unaided eye.

Microscopes have magnifying lens or lenses by which these enlarges the size of microorganisms and due to enlarges size these can be seen under Microscope.

Microscope can be

- Simple
- Compound

Simple Microscope

It is a microscope with one magnifying glass**.** It is a magnifying glass that has a double convex lens.

Compound Microscope

It is a microscope with more than one magnifying glass.

Use of Microscope

Microscope helps to observe the

- Shape microorganisms
- Size microorganisms
- Arrangement of microorganisms
- Presence of special structures like flagella, endospores and capsule

There are two properties of a Microscope that helps to above mentioned observations. These are;

Magnification

It is the ability of a lens to enlarge an object. In compound microscope object is enlarges by two lenses

Objective lens: Objective lenses are of following magnifying powers

4X, 10X, 40X and 100 X

X means, how many times it enlarges

If we have a bacterium of 1 um size, it will appear of 4um under 4X objective lens.

Ocular lens: These are 5X, 10X or 15X. Mostly microscopes have 10X ocular lenses

As compound microscope has both objective and ocular lenses so term Total magnification is used.

Total Magnification

It is the product of magnification of objective lens and magnification of ocular lens.

Total magnification= Magnification of Objective lens X Magnification of Ocular lens

100 X 10

1000X

It means an object of 1 um will appear of 1000um in compound microscope if we use 100X objective lens and 10X ocular lens.

Resolution

It is defined as "The shortest distance between two points on a specimen that can still be distinguished by the observers or optical system as separate entities.

Resolving Power

It is the ability of a microscope to distinguish two close points as separate entities. Resolving power of compound microscope is **0.2 micron-meter.** It means if two objects are at a distance of 0.2um apart from each other, these will appear as separate entities in compound microscope.

Parts of Bright filed Binocular Compound Microscope

1- Arm / Frame

Supports the microscope and connects the base and body tube of the microscope. It is also used to hold microscope.

2- Base

The base is bottom of microscope that supports the whole microscope just like feet in human body. It also carries Illuminator (lamp) and Power (ON/OFF) switch.

3-Illuminator

It is light source for a microscope. It contains a bulb of the tungsten filament and provides a light of visible spectrum.

4- Power Button (On/Off Switch)

It is present on base to turn light on and off.

5-Adjustment Knob

These two knobs are present on lower part of frame/arm.

a. Course Knob

It is used for general focusing. It is used to move the stage up and down to focus the specimen.

b. Fine Adjustment Knob

It is used to bring the specimen into sharp focusing. So that fine details can be observed

6- Eye Piece (Ocular lens)

It is the lens present at the top of the microscope through which observer look. These are used to magnify a microscopic object. It also further magnifies specimen usually 10X, 5X or 15X in compound microscope. Mostly 10X are used. Old microscopes had one ocular lens (uniocular). But now two ocular lenses are present so now day microscopes are binocular.

7-Turret or Revolving Nose Piece

This is the part of the microscope that holds objective lenses and can be rotated easily to change objective lens of desired magnification power.

8- Objective Lens

Microscopes usually have 3 or 4 objective lenses on a microscope. These play role in magnification of microscopic objects. These are 4X, 10X, 40X and 100X powers. These are near objects that's why called objective lens. Each magnifying lens has a particular colour ring.

- White ring: 100X (It is oil immersion lens)
- Blue ring: 40X
- Yellow ring: 10X
- Red ring: 4X

9- Stage

It is a flat platform where microscopic objects/specimens are placed using a microscopic glass slide. The stage has clips (steel/plastic) hold the microscopic glass slide in place. The stage may be fixed or mechanical. If your microscope has a mechanical stage, you will move the slide around by turning two knobs. One moves it left and right, the other moves it forward and backward.

10-Aperture

The hole in the middle of the stage that allows light from the illuminator to reach the specimen

11-Condenser Lens and Iris Diaphragm

These are two combined parts; present beneath the stage. The purpose of the condenser lens is to focus the light in the specimen to be viewed. The iris diaphragm (shutter with a knob) is to regulates/controls the amount of light coming from light source.

12-Diopter Adjustment Knobs

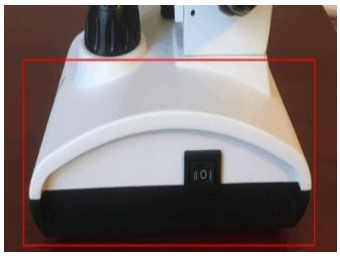
In this type of adjustment, eye piece lens rotates up and down to control the variations in eye sight. If there is variation or inconsistencies in observer's eye sight, it can be overcome using diopter adjustment.

13-Interpupillary Adjustment Knobs

Every individual has different distance between two eyes. Binocular microscopes have inter-pupillary adjustment to adjust the ocular lens to that distance. So that both ocular lenses are in front of eyes. If we see a single microscopic field (Circular area under observation in Microscope (Just like a full moon), it means distance between eyes matches to distance between ocular lenses. It adjusts the ocular lens according to eye distance.

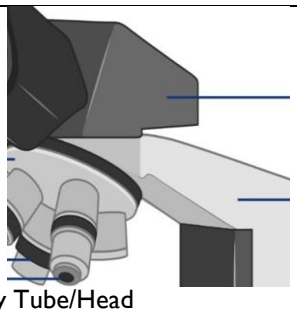
14-Working Distance

Distance between the objective lens and object/ specimen is called working distance. It is inversely proportional to magnification.



Base

Illuminator & Power cord



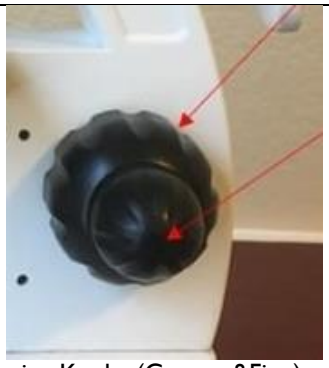
Body Tube/Head

Revolving nose piece (Taurret)

Condenser + Iris Diaphragm

Stage & Clips

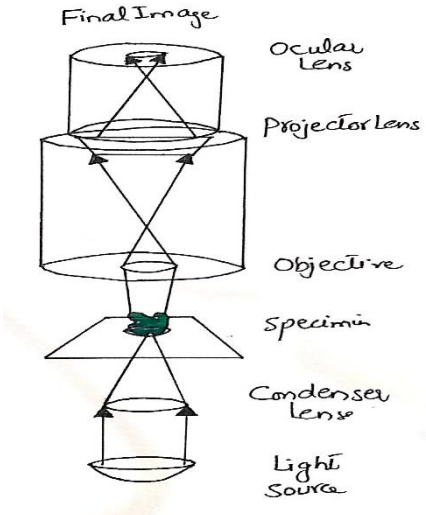
Arm



Focusing Knobs (Course &Fine)

Objective Lenses

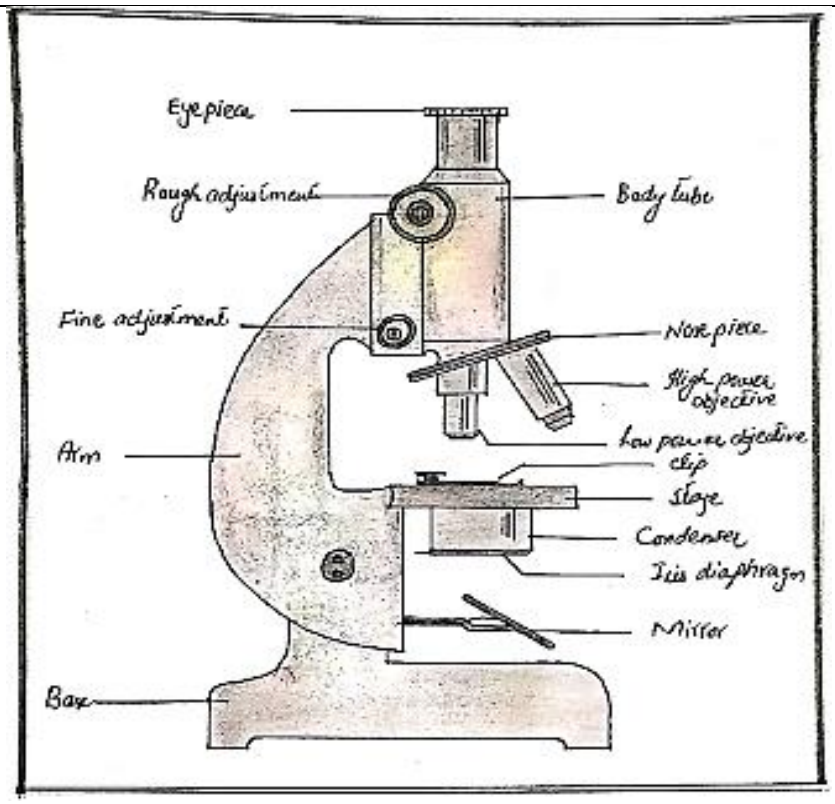
Ocular Lense



Light-path of Compound Microscope

Parts and Light Path of Compound Microscope

Figure 2: Parts and Ray Diagram of Binocular Bright-Field Compound Microscope



A

B

Compound Microscope

(A): Uniocular bright-field, (B): Binocular bright-field

Practical 3

STERILIZATION TECHNIQUES

Sterilization: Sterilization is the complete removal or killing of all life forms of microorganisms.

Microorganisms and Their Forms

Microorganisms exist in following forms in nature

Bacteria

Bacteria has two forms

1. **Vegetative;** it is metabolically **active forms**. In favorable conditions bacteria live in this form.
2. **Endospores;** It is resistant form. When conditions are unfavorable (deficiency of nutrition, inappropriate temperature) bacteria form endospores to survive in these harsh conditions. It is metabolically **inactive form**. Two genera form enodspores under harsh conditions. These are *Bacillus* and *Clostridium*. Their spores have resistance against heat, desiccation and pH. They also have osmotic resistance. Rests bacterial genera undergo the process of death.

Fungi

It is eukaryotic microorganism. It may be yeast (unicellular) or multicellular/filamentous form) mold.

Other forms of microbes include **protozoa, viruses, microscopic algae,** and **parasites**

In microbiology laboratory, our main objective is the isolation and identification of microorganisms. We use different sterilization techniques to make our instruments, glassware and plastiware free of microorganisms. Because, we isolate microorganisms from a desired sample or source. We are interested to grow the microbes present in sample only. So, material

used from processing of sample till the growth of microorganisms, must be sterile (free of microbes).

The materials that need to be sterile are;

- Inoculating loop/spreader/inoculating needle
- Petri plates
- Medium (agar and broth)
- Surface
- Area in which we work

Methods of Sterilizations

There are two basic methods of sterilization.

- Physical method
- Chemical method

Physical Methods

The physical methods of sterilizations used in microbiology laboratory are enlisted below.

1. **Heat**
2. **Filtrations**
3. **Radiations**

1. Heat

It is the most common method of sterilization. The heat kills the microbes in substance. In heat sterilization, the longer is the exposure to heat better in the sterilization at given temperature

Two types of heat used in this method. Both help to sterilize the material but by acting in different ways.

a. **Moist heat**
b. **Dry heat**

a. Moist heat:

In this method, heat applied in the form of **"steam".** It coagulates the proteins of microbes and distorts the structure of the microbial cell. Moist heat (steam) has **more penetration power.** It is more **effective method** than the dry heat.

Moist heat is used in three ways;

- Autoclaving
- Boiling
- Tyndalization

Autoclaving

The equipment that is used to provide moist heat for sterilization in laboratory is **autoclave**. The process of steam heat sterilization using autoclave is called **autoclaving**. It is used for sterilization of medium for cultivation of microorganisms. It is also used for sterilization of waste material of microbiology laboratory and hospitals before discard.

Autoclave:

- It is the application of the moist heat.
- It is double wall chamber made up of steel with a lid.
- The Conditions adjusted for autoclaving in autoclave are; temperature **121°C** for **15 minutes** at a pressure of **15 pound per square inch.**
- This equipment works on the principle of pressure cooker

Boiling

It is the method of moist heat. In it, temperature of **100°C** is applied for **10mins**. No pressure is used. It is a method of free-flowing steam heat sterilization.

Tyndalization

It is used to sterilize heat labile substances. The substances which are unable to tolerate autoclaving temperature. This is an old method. Now a day for heat labile substances filtration

is used. This method was developed by a scientist Tyndal. It is the lengthy process of **3 days.** It is also called "**intermittent heat treatment"**

Tyndalization essentially consist of heating the substance to boiling point (100°C) and holding for 45 minutes to kill metabolically active form of microbes three days in succession. During boiling vegetative forms kill but endospores are not killed at 100°C. After each heating, the substance/material/solution is incubated at 37°C for 24 hours. During this incubation endospores are germinated into vegetative form which is killed by heat. So, 2nd days this material is also kept on boiling for 45 minutes. This process is repeated on 3rd day as well.

b. Dry heat

It causes the killing of microbes by oxidation of cellular components of microbial cells

Following methods are used for dry heat sterilization.

Flaming

It is the process of exposing metallic devices to flame for few minutes. This is used for sterilization of inoculating needle and loop. Inoculating loop and needle are placed over flame until it becomes **red hot.** The allow it to cool before use for 15 seconds.

Hot air oven

In hot air oven, dry heat is used for killing all life forms. The temperature provided is **160°C and180°C** for **1hr** and **30** minutes respectively (160°C/1hr and 180°C/30min). This is used to sterile petri plates and glass pipettes etc.

Pasteurization

In pasteurization, Heat of temperature below 100°C is used. Pasteurization could be for **62°C for 30 mins which is called LTLT** (low temperature for long time) or **72°C for 15 seconds** it is called **HTST** (high temperature for short periods). While **UHT (**ultra-heat treatment) is process of sterilization in which **138°C** temperature is provided for just **2 seconds.** It is used on commercial level for sterilization of milk and juices or dairy products.

Filtration

This is a process of filtration used a membrane (**Nitrocellulose membrane)**. This is used to remove microbial forms form heat labile solution. Filters are available in different pore size. The filters used for sterilization has a pore size of **0.25** micron-meters (um). Membrane filters are also use with the help of syringe called syringe filter. The solutions to be sterilized taken in syringe, then filter is applied in place of needle of syringe. The filtrate is collected in sterile container. This method is commonly used to sterile sugar solutions and antibiotic solutions used in laboratory.

Radiation

This method involves exposing the packed material to radiation for sterilization i.e., **UV** and gamma radiation are used. Radiations are used to sterile plastic material. UV is mostly used in laboratory for surface sterilization.

Chemical Methods

The method of sterilization in which chemicals are used for sterilization are called chemical methods of sterilization

Following are the chemical methods of sterilization

Antisepsis

Sanitization

Disinfection

Degerming

Antiseptic

It is complete removal of microorganisms from living tissues. This method is called antisepsis (absence of microbes)

Disinfection

It is the complete removal of microorganisms from non-living bodies

Degerming

It is basically a method to remove microorganisms from a localized area of living tissue

Sanitization

It is actually a method which is used to reduce the number of microorganisms.

i.e., hand sanitizers are available in markets

Procedures

1. **Worker hands**

First use **70 percent alcohol** to sanitize your hand

2. **Surface for work**

Use cotton to make the surface clean. Soak cotton in 70 percent ethyl alcohol and rub the surface.

3. **Work area (air)**

Burn the flame and wait for **5min** to sterile the area for work free. Heat of flame removes microbes in **6 inches** area around flame.

4. **Inoculating loop and needle**

Inoculating loop and needle are placed over flame until it becomes **red hot.** The allow it to cool before use for 15 seconds.

5. **L-shape glass spreader**

Dip the glass spreader in 70 % ethyl alcohol and evaporates this just by passing over flame.

6. **Syringe filtration method**

This method is used to filter the sample present in the injections. In this method following steps are performed.

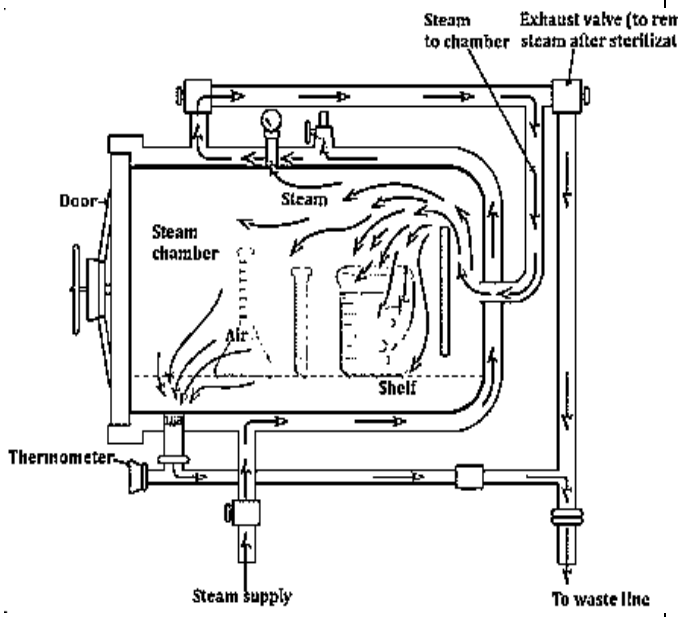
1. Load the sample into syringe.
2. Attach the **filter** securely with twisting motion.
3. Hold the assembled string and filter vertically to wet the membrane evenly.
4. Press the syringe plunger gently to push sample through the filter.
5. Then the sample goes to its destination after filtration.

7. Sterilization of Petri dish

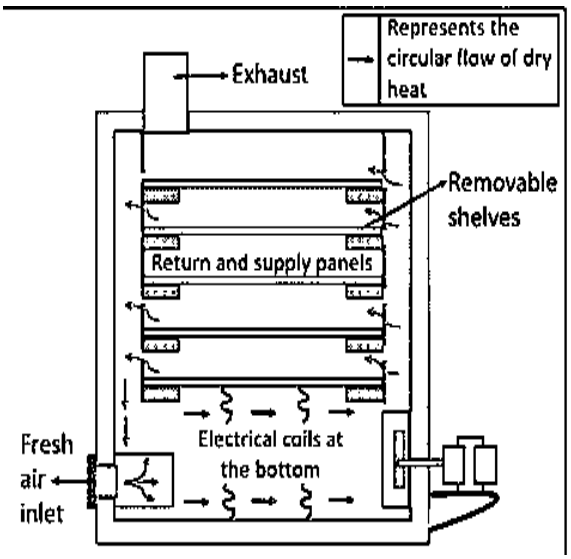
1. First of all, wash the Petri dish with detergent
2. Then, dry it with some soft and clean cloth
3. After drying it close the dish with lid and start wrapping it with paper
4. Place the Petri dish inverted on the paper in center
5. Now cover the dish with paper left on both (forward and backward) side
6. Then, from corners by making a ship like folds and then press them upside down

Note: Wrapping of petri-plates is just like the wrapping of gift in gift paper.

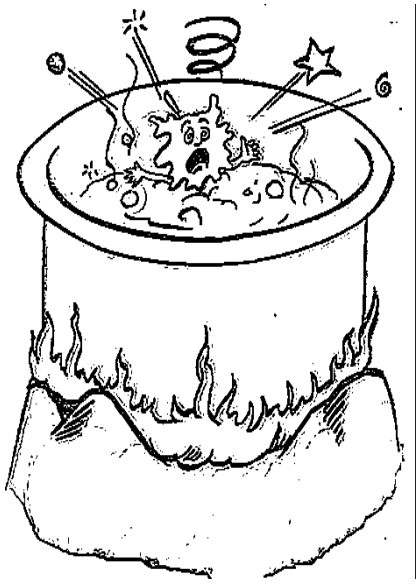
7. After packing, place the Petri dishes in hot air oven (temperature 180^{o}C/30 minutes).
8. Petri-plates must be places at some distance in hot air hoven.



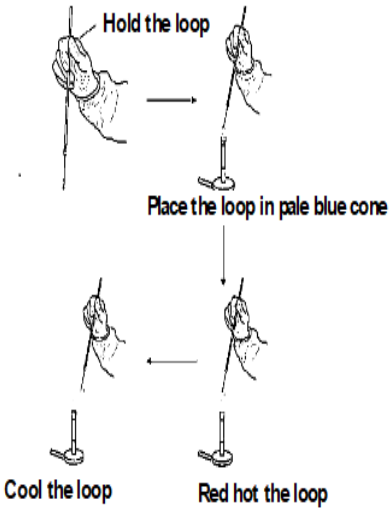
A: Autoclave



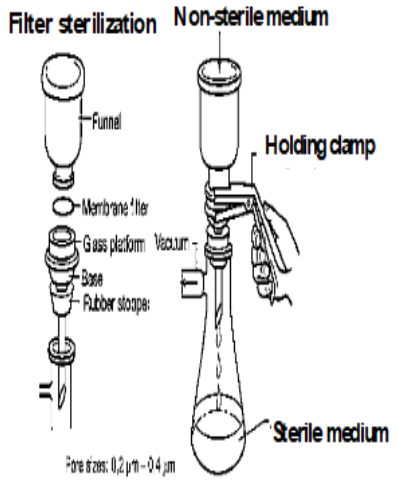
B: Hot air oven



C: Boiling



D: Dry heat Sterilization



E: Filtration

Figure 3: Sterilization Techniques

Practical 4

CULTURE MEDIA TYPES, USES & PREPARATION

Culture Media (Singular; Medium)

A growth media or culture media is a solid, liquid or semi-solid designed to support the growth of microorganism. There are different types of media suitable for growing different types of cells.

Microorganisms require C, H, N, S, P, vitamins and minerals for their growth. All this nutrition is provided either by nature (where microorganisms exist naturally) or in the form of artificial medium (for cultivation of microorganisms in laboratory).

Most of microorganisms grow using basic nutrient, however some microorganisms are fastidious.

Fastidious

Those microbes which require extra nutrients along with basic nutrients for their growth are called fastidious.

Example:

- ***Streptococcus*:** It feeds on blood and blood contains additional nutrients
- ***Haemophilus*:** It feeds on the materials present inside red blood cells

Classification of Media

Media are classified on the basis of:

1. Composition
2. Form
3. Nutrition

1. Composition

These are further classified into two types:

- Chemically defined media
- Complex media

Chemically Defined Media: Media having "known" chemical composition are called as chemically defined media. For example, we have one litre phosphate buffer saline having 20-gram glucose. It is chemically defined medium.

Complex Media: Media having "unknown" chemical composition is called complex media. Complex media have pancreatic digest, Yeast extracts and Peptones. We know that peptone is source of Nitrogen, Vitamins but how much amount will be provided by 20g of peptone, is unknown to us. So, this is example of complex medium.

2. Form

On the basis of form media are classified into three types:

- Liquid
- Solid
- Semi-solid

Liquid: The medium in liquid form is called broth. It is prepared and used in test tubes, flasks, bottles.

Solid: The medium in solid form is called agar. The medium in solid form contains a solidifying agent called agar. Agar medium is prepared in flask, bottles and test tubes but finally pour into petri-dishes before using for cultivation of microbes.

Agar (complex polysaccharide obtained from sea weed). It is present in concentration of **1.5 to 3 percent** in solid form of media.

Melting and solidifying temperature of agar are ≥92 and ≤ 42°C.

The same medium can be in broth form and in agar form. For example, Nutrient medium in broth form is called Nutrient broth and in solid form called Nutrient agar. Composition is same but form is different.

Semi-Solid: Semi solid media are prepared with agar at concentration of <1 percent. It is prepared in test-tubes.

3. Nutrition

On the basis of nutrition, media are classified into 4 types:

- Basal medium
- Enriched medium
- Differential medium
- Selective medium

Basal Medium: These provide "basis nutrients". It allows the growth of "non-fastidious"

Examples include;

- Nutrients agar
- Nutrient broth
- Peptone water

Enriched Medium: These provide basic nutrients and "additional nutrients". These are used for growth of fastidious microorganisms.

Examples include;

- Blood agar
- Chocolate agar (named because of the colour of RBC after lysis in case of *Haemophilus*)

Differential Medium: More than one type of micro-organism can grow in this differential medium but their growth (*colony colour) is different. Such media have some differential agent

in it (Mostly sugars, salts or antibiotics). Some microbes use this differential agent and some not, so behavior is different.

*Colony: It is visible mass of microbial growth on agar.

Example includes;

Manitol Salt Agar: Manitol is selective agent in it. Some staphylococcus uses manitol and produce yellow colour colonies on it (manitol fermenter) and Staphylococcus which do not use manitol produce red colour colonies on it (Non-fermenter of manitol). This medium has phenol red a pH indicator.

Macckonkeys Agar: It has lactose sugar as selective ingredient. E. coli ferment lactose and give red colour colonies, while Salmonella does not ferment lactose and give yellow (off-white) growth on this medium. This medium has neutral red as pH indicator.

Selective Medium: That allows only one type of bacterium to grow and inhibit the growth of all other microorganism. Such medium has selective agent in its composition.

Examples include;

Manitole Salt Agar: It Contain 7.5 percent NaCl in it with basic nutrients. Only *Staphylococcus* (bacterium) can tolerate this high salt concentration. So only *Staphylococcus* grows on it. Other bacteria are killed. So, this is selective medium for *Staphylococcus.*

Macconkey`s Agar (selective agent is bile salts) selective for enteric bacteria like *Escherichia coli* and *Salmonella.*

A medium can be selective and differential at the same time like manitol salt agar and Macckonkey's agar

Preparation of Culture Media

Now a day, media for microbiology work are commercially available in powder form. We have to prepare broth or agar using this commercially available medium.

Following are the steps to prepare the media:

1. Read the instructions written on the box. Medium box has medium name like, **Nutrient agar**. It has instructions regarding the quantity of medium for one litre (**for example 28g/ litre for nutrient agar**), about pH (**7.2 for nutrient agar**) of that medium and method of sterilization (**autoclaving for nutrient agar)** to be used for that medium

2. Weigh required amount of media
3. Dissolve it in desired volume of distilled H_2O in a conical flask. For this continuous stirring with heat is used
4. Adjust the pH with help of pH meter using 0.1 M HCl & 0.1 M NaOH. Required pH is mentioned on the box of that medium. Wrap the flask with cotton plug or brown paper using thread

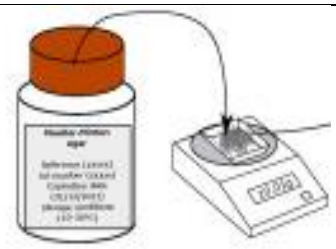
5. Sterilization the medium; Place medium flask in Autoclave (121°C, 15pond per inch square pressure for 15 minutes)

6. Powering into sterile petri dishes. This step is done for agar not for broth. Petri-dishes are sterilized by hot air oven. Pour 25-30 mL medium in petri-dish of 9cm diameter. Allow the medium to solidify. Once it is solidified, place the plates in inverted position. (Step 6 only for solid medium called Agar)

7. Sterility test. Incubate the prepared medium petri plates with agar or test tubes, bottles, flasks for broth at 37°C for 24 hours without any inoculation (without transferring any growth on it).

Select those plates/test tubes that are free of any growth. After incubation observe all plates (agar) or test tubes (broth) etc. If colonies (growth) on agar petri-plates or turbidity in broth. It indicates that something went wrong and undesired microbes enter into medium from environment. These unwanted microbes are contaminants and our medium has become contaminated. So, we use sterile (free of contamination) agar plates and broth tubes for growth of our desired bacteria.

1-Read instructions



2-Calculate & Weighing

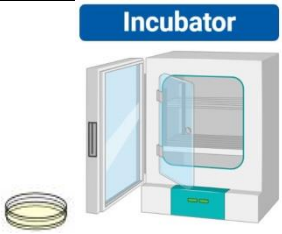
3-Dissolve in dH_2O

4-pH Adjustment

5- Sterilization

6-Pouring into Petri-plates

(Only for Agar)



7-Sterility testing

Packing & storage

OR

Use for inoculation

Figure 4: Preparation of Culture Media

Practical 5

INOCULATION TECHNIQUES FOR PRIMARY CULTURE

Inoculation means to transfer microorganisms (sample/growth on medium) into new sterile medium. The initial growth/initial no. of microorganisms that is transferred and starts growth in new medium is termed as **inoculum.**

Primary Culture

When a sample from natural environment is inoculated on culture media in laboratory, microorganisms in that sample grow on media. The growth or culture observed is called primary culture. Primary culture contains **mixed growth** having different types of organisms. However, if we use selective medium for primary culture, only selected microorganisms will grow on it.

Sub-culture

When microorganisms are transferred from primary culture or previous culture into new sterile media then this inoculation is called as subculture. The purpose of subculture is performed;

- To obtain pure growth (one type of microorganisms on one plate/in one test tube)
- To revive (fresh) microorganism

Primary Culture Techniques

In microbiology, following techniques are used for primary culture or primary inoculation;

1. Swabbing
2. Spreading
3. Sprinkling

1. Swabbing

Sterile cotton swab is used for this purpose. Swabs are commercially available. The whole procedure is carried out in **6 inches** radius of a Bunsen burner flame to avoid any contamination. Samples for primary culture using swab can be collected from surfaces, wound, juice, milk, water, soil suspension etc.

Procedure:

1. Remove the packaging of the swab and hold it in your right hand. Keeping it within 6 inches radius of the flame. Take the test tube in which the sample/ broth culture is present.
2. Open the cap of the test tube and hold it between your fingers facing it upwards.
3. Dip the cotton swab into sample/broth culture.
4. Remove the extra liquid by squeezing the swab on the walls of test tube.
5. Close the cap of the test tube securely and place it away in test tube holder.
6. Now take Nutrient agar plate for bacterial growth and transfer the inoculum to it by swabbing the cotton plug on the medium. Make sure to cover the whole plate uniformly.
7. Carefully close the lid of petri dish and discard the used cotton swab.
8. Inoculated plates are incubated at 37°C for 24 hours in inverted position.

2. Spreading

An **L-shaped glass spreader** is used in this technique. Samples for primary culture using spreading can be collected from surfaces, juice, milk, water, sample suspension (suspension can be prepared from solid food/feed material).

Procedure

1. First sterilize the spreader with **ethanol (70%)** and pass it over the flame (flame is not used for sterilization in this process but to fasten the evaporation of ethanol from spreader).
2. Using a micropipette transfer the desired amount of the sample/ broth culture to nutrient agar plate. A volume of 100uL is transferred on 100mm diameter petri-plate.

3. Now spread it with the sterilized spreader by moving it up and down in the petri dish make sure that broth is spread uniformly.
4. Spreading is continued till the inoculated sample is completely adsorbed over the surface of agar.
5. Inoculated plates are incubated at 37°C for 24 hours in inverted position.

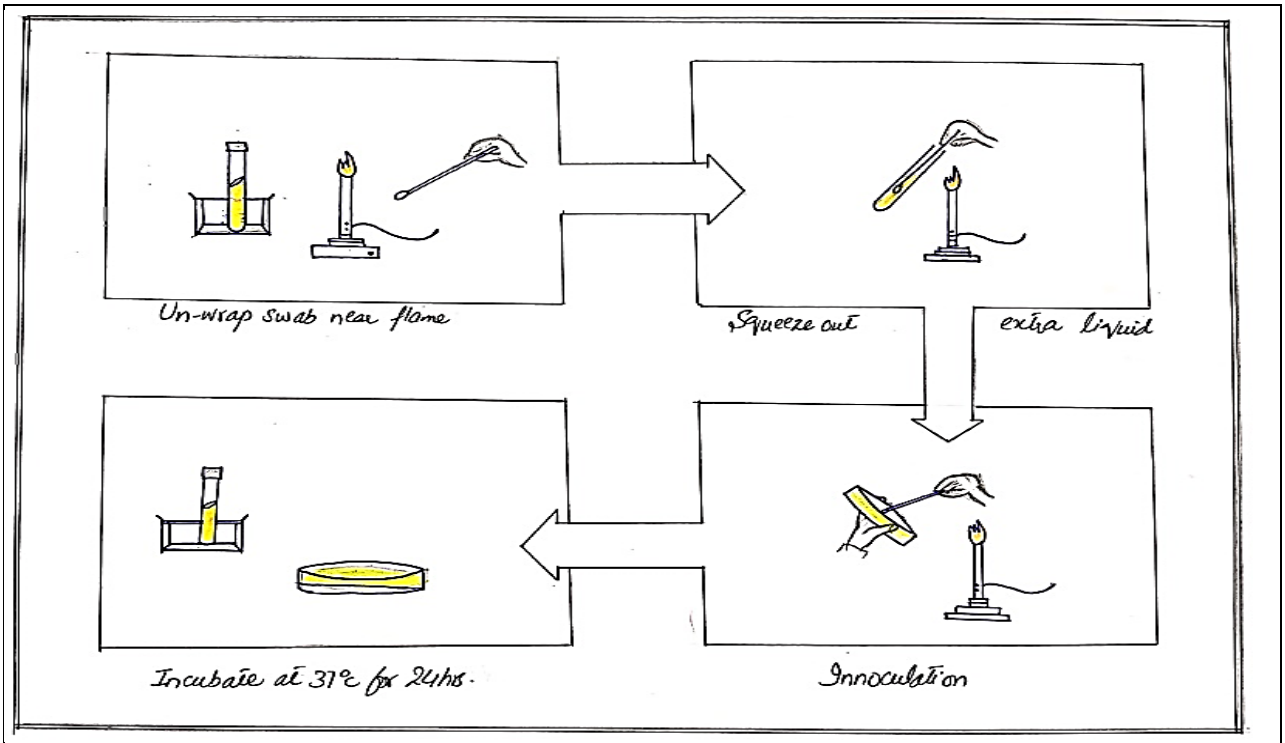
Note: Both swabbing and spreading are also used for uniformly distributed microbial growth on agar surface.

3. Sprinkling

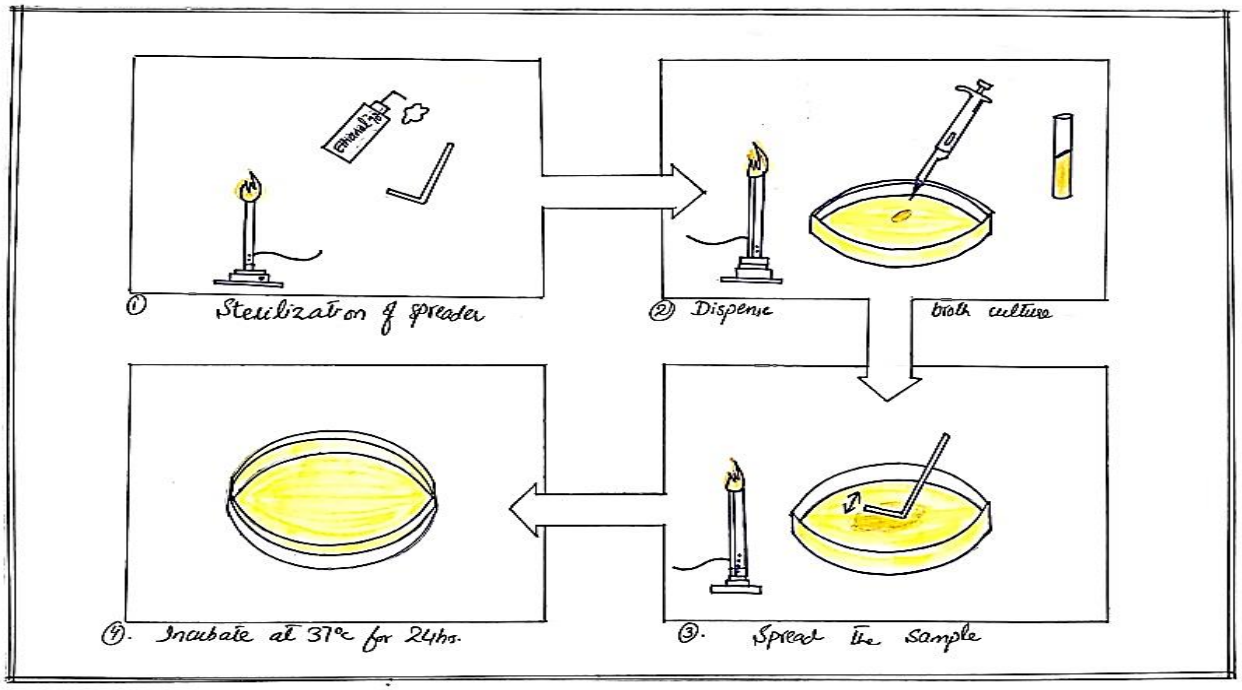
Sprinkling is mostly used for primary culture of fungi from soil.

Procedure

1. Collect a sample of **soil** in a petri dish.
2. Sprinkle soil (2-3mg) on surface of Sabouraud's dextrose agar plate.
3. Incubate the inoculated petri-plates at 22±3°C for 3 days in upright position.

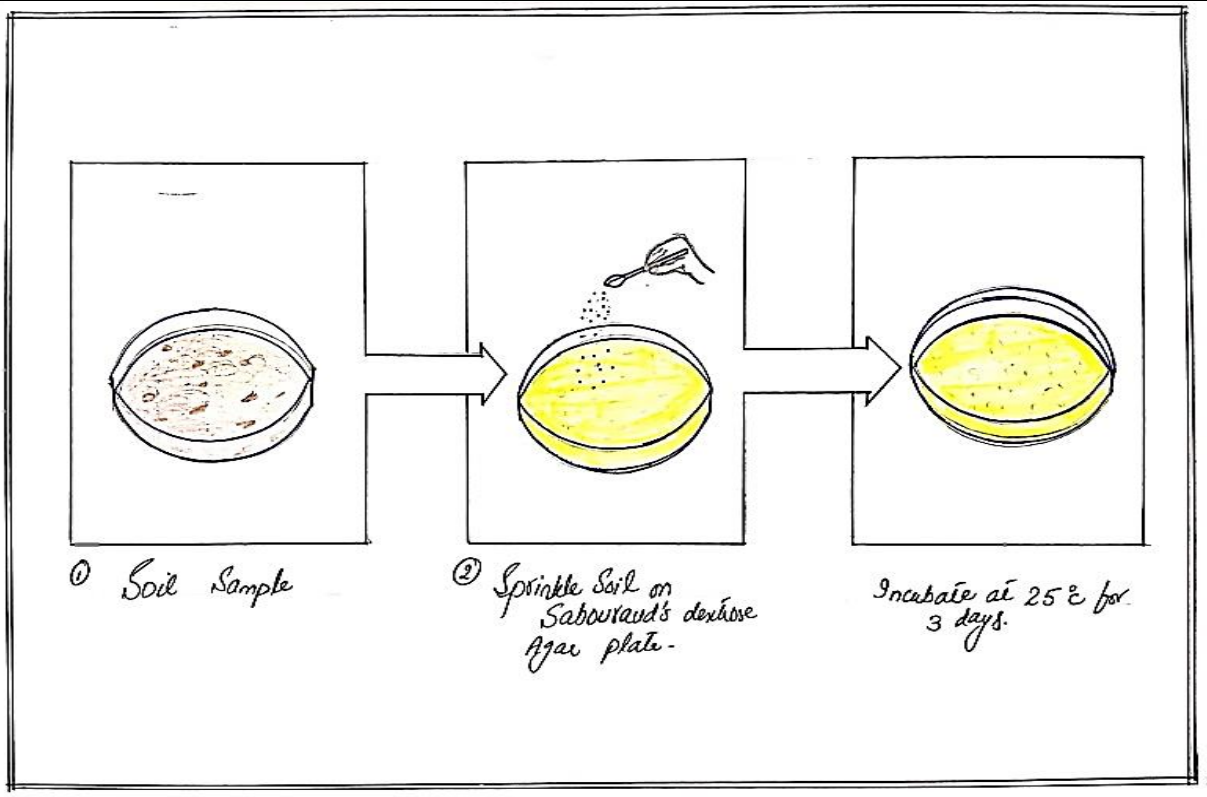


A: Swabbing on nutrient agar

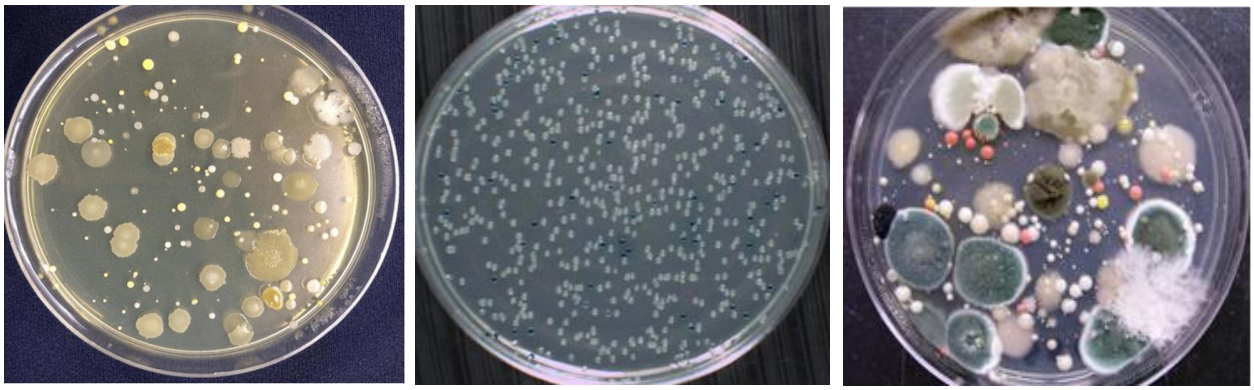


B: Spreading on nutrient agar

Figure 5: Primary cultivation techniques



C: Sprinkling on Sabouraud's dextrose agar



A **B** **C**

D: Primary culture

Figure 5: Primary cultivation techniques

Practical 6

SUBCULTURE TECHNIQUES

A subculture is a microbiological culture made by transferring cells from a previous culture to fresh culture medium. This action is called "***sub culturing***"

Sub-culturing is performed;

- To prolong the life and/or expand the number of micro-organisms in the culture.
- To purify the growth from primary culture. In sub-culture techniques, we transfer inoculum from primary or old culture to new plate.
- **Primary culture** contains the growth of different types of bacteria on a single plate. Primary culture is a mixed culture.

Basic Techniques of Sub-Culturing

There are following three techniques of sub-culturing

1. Streaking
2. Stabbing
3. Spotting
4. Broth inoculation

1. Streaking

It is a method of sub-culturing. It is used to produce **discrete** or **isolated colonies**. This method of sub-culturing is used for bacteria and yeast. Streaking is done with help of **inoculating loop.**

Types of Streaking;

Following are the two main types of streaking

- Continuous (Streak line on agar plate are connected to each other)
- Discontinuous (Streak line on agar plate are not connected to each other)

We will do continuous streaking.

Continuous Streaking

This type of streaking is carried out in four ways.

a) One-way streaking
b) Two-way streaking
c) Three-way streaking (T-streaking)
d) Four-way streaking (Quadrant streaking)

a. One-way streaking

In one way, streak line is applied in a single direction on agar plate

- Sterilize the inoculating loop
- Pick the colony from previous culture just by touching the colony
- Place inoculating loop at one point at top of agar
- Make a small circle on that point
- Distribute the growth in zigzag pattern from **top to bottom** of plate
- Incubate inoculated plates at 37◦C for 24 hours in inverted position

b. Two-way streaking

In two ways streaking, streak line is applied in two different directions on agar plate

- We hypothetically divide the plate in two halves
- Sterilize the inoculating loop
- Pick the colony from previous culture just by touching the colony
- Place inoculating loop at one point at top of agar
- Distribute the growth in zigzag pattern from **top to half** of plate
- Rotate the plate at a 90˚ angle and sterilized the loop
- Cool the loop

- Now distribute the cells in zigzag pattern on **second half by touching the previous streak** lines
- Incubate inoculated plates at 37°C for 24 hours in inverted position

c. Three-way streaking (T-streak)

In three ways streaking, streak line is applied in three different directions on agar plate

- We hypothetically divide the plate in three halves (one complete half and second half is divided into two)
- Sterilize the inoculating loop
- Pick the colony from previous culture just by touching the colony
- Place inoculating loop at one point at top of agar
- Distribute the growth in zigzag pattern from **top to half** of plate
- Rotate the plate at a 90˚angle and sterilized the loop
- Cool the loop
- Now distribute the cells in zigzag pattern on **half portion of second half by touching the previous streak** lines
- Again, rotate the plate at a 90˚angle and sterilized the loop
- Cool the loop
- Now distribute the cells in zigzag pattern on 3rd half on agar plate **by touching the previous streak** lines

Incubate inoculated plates at 37°C for 24 hours in inverted position

d. Four-way streaking (Quadrant streaking)

In four ways, we divide the in four halves and produce streaks in four different directions. After every part, we rotate plate at 90 degrees, sterilize the inoculating loop.

- We hypothetically divide the plate in four halves
- Sterilize the inoculating loop
- Pick the colony from previous culture just by touching the colony
- Place inoculating loop at one point at top of agar
- Distribute the growth in zigzag pattern in first half of plate

- Rotate the plate at a 90º angle and sterilized the loop
- Cool the loop
- Now distribute the cells in zigzag pattern on **second half by touching the previous streak** lines
- Again, rotate the plate at a 90˚angle and sterilized the loop
- Cool the loop
- Now distribute the cells in zigzag pattern on 3rd half on agar plate **by touching the previous streak** lines
- Again, rotate the plate at a 90˚angle and sterilized the loop
- Cool the loop
- Now distribute the cells in zigzag pattern on 4th half on agar plate **by touching the previous streak** lines

Incubate inoculated plates at 37°C for 24 hours in inverted position

2. Stabbing

Stabbing is another technique of sub-culturing. This technique is performed on solid medium in test tube (Solid medium in test tube is in *Slant* form with *Butt* or only in form of *Butt*). Stabbing is performed with **inoculating needle.** This technique is used for motility testing of bacteria (either bacteria is motile or not motile. Motile bacteria have flagella)

- Sterilized the inoculating needle
- Allow the needle to cool down
- Pick up a bacterial colony from agar plate
- Insert the needle having inoculum into the center of the agar in the tube and pushing it down to the bottom and then take it back in same direction.
- Incubate inoculated agar tube at 37ºC for 24 hours

If tube contains slant of agar, in that case we first do stabbing in butt and then streaking on slant.

3. Spotting

This technique is performed for sub-culturing of filamentous fungi with help of inoculating needle.

- Sterilize the inoculating needle
- Allow it to cool
- Touch the growth/colony of fungi from previous culture
- Now touch the surface of Sabouraud's dextrose agar plate with inoculating needle in center of plate
- Incubate inoculated plate at 22±3°C for 3 to 5 days in upright position

4. Broth Inoculation

Transfer of microbial growth into broth using inoculating loop is called broth inoculation.

- Sterilize the inoculating loop
- Allow it to cool down
- Pick the colony from previous culture
- Transfer it into sterile broth test tube in sterile area
- Incubate the inoculated tube at 37°C for 24 hours

The growth in broth after incubation is called broth culture

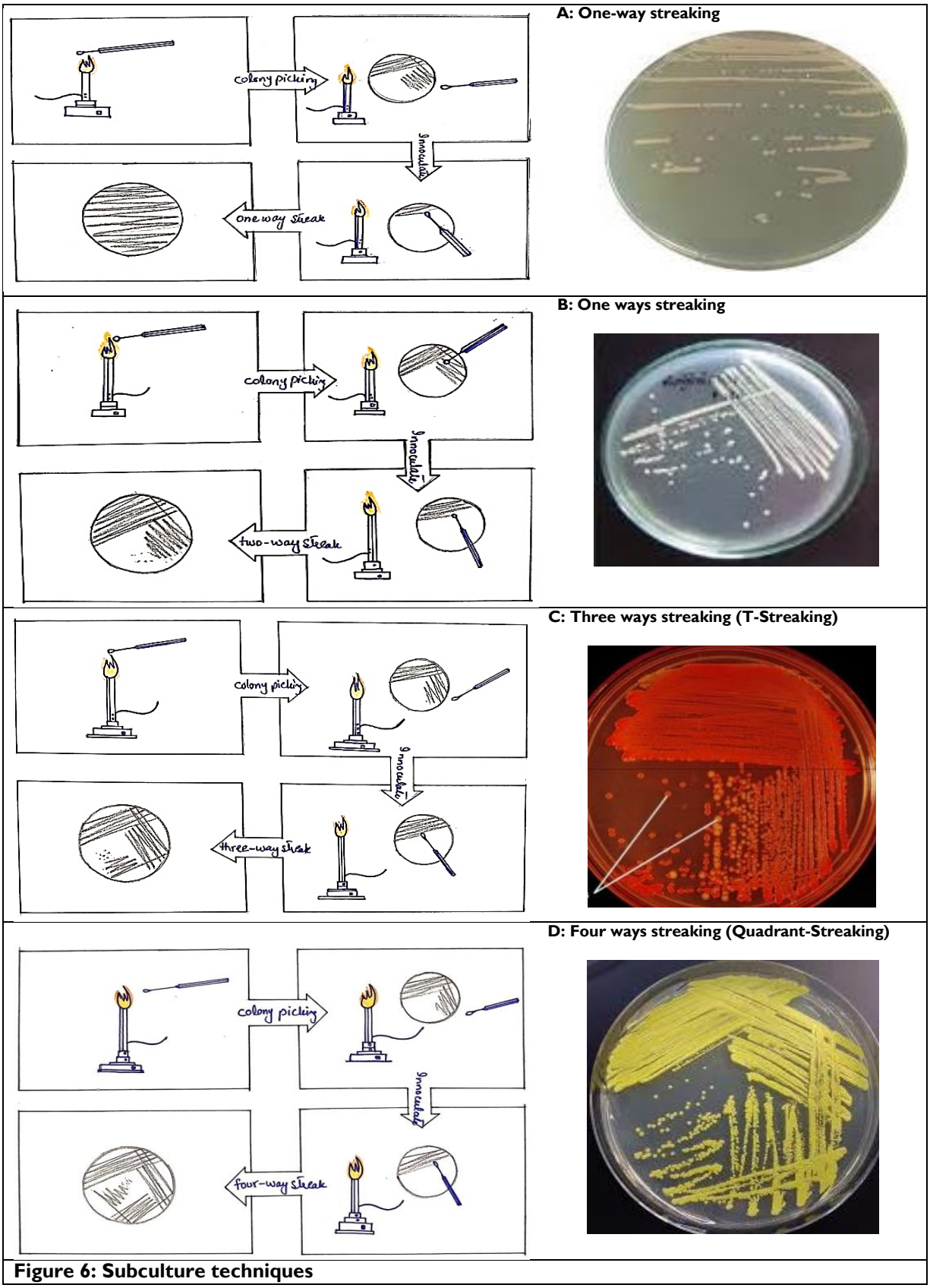
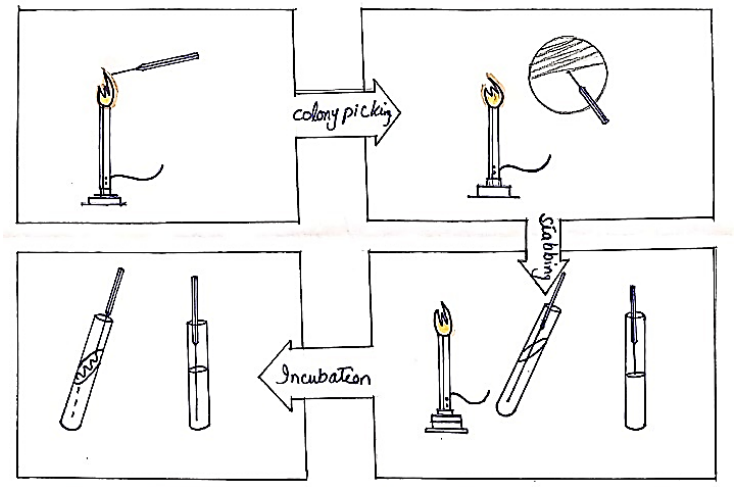
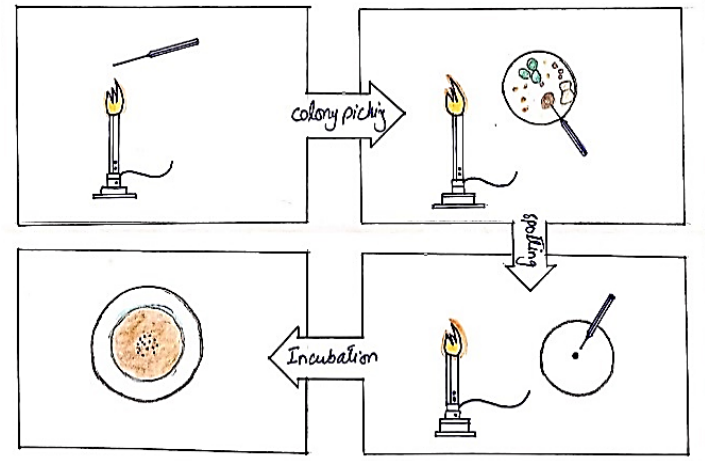


Figure 6: Subculture techniques



E: Inoculation on slant and deep agar



E: Single spot technique

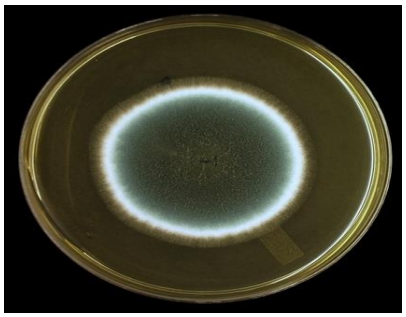


Figure 6: Subculture techniques

Practical 7

STUDY OF COLONY MORPHOLOGY

Colony

Visible mass of microorganisms on agar is called colony. Approximately 10^6 to 10^9 cells are present in a colony. A single microbial cell is observed under microscope only but a colony (collection of millions of cells) can be observed with unaided eye.

Morphology

Morphology is shape, size, form and margins of colony. Every microorganism has its characteristic colony morphology. So, it can be helpful to differentiate microorganisms to some extent.

Bacterial colonies may have differences are on the basis of:

1. Form (Shape)
2. Elevation (side view of colony)
3. Margins
4. Color (Pigmentation)
5. Size
6. Smell

1. Form (Shape)

This is an apparent character. Mostly colonies are in circular form. Some are in irregular form and some can be either lobulated or rhizoid.

Elevation

This is side view of the colony. When large number of cells is present, these are mounted on one another.

Convex elevation: peaks are sharper

- Raised elevation: peaks are broader
- Flat elevation

- Umbonate: fried egg shape colony
- Crateriform (Depression in center and periphery is raised)

2. Margins

It is the periphery of colony. Margins may be entire (smooth), curled, undulate or lobate.

3. Color (Pigmentation)

Some bacteria produce pigments. It could be

- Intracellular
- Extracellular

Intracellular: Cells produce colour which retain inside the cell. So, colony will be coloured in this case. For Example, *Serratia Marcescens* gives red color pigments at room temperature.

Extracellular: Some microbes produce extracellular pigments and excrete it outside. In case of extracellular pigment colour can be observed in agar around the colony. For Example, is *Pseudomonas aeruginosa* produces green color extracellular pigments.

4. Size

For size, the diameter of colony is observed. These can be in different sizes such as large, medium, small and pinpoint.

5. Smell

Some bacteria when grow in medium produce volatile substances which results in smell.

***Staphylococcus aureus*:** Its colonies smell like dirty socks

Pseudomonas aeuroginosa: Colonies give fruity smell mostly grapes like

Pasteurella moltocida: It produces sweat smell

Practical 8

STAINA AND STAINING; SIMPLE AND NEGATIVE STAINING

For observing morphology of single cell, microscopy is performed because single cell. Microscopy determined the following characters of a cell;

- Shape (Cocci, bacilli, spiral)
- Arrangement (diplo, tetrad, sarcinae, chains or clusters)
- Size
- Presence or absence of special structures (Capsule, endospores)

Microbial cells are transparent, so in order to observe microorganisms under microscope, cells have to be stain (impart of colour) in order to develop contrast with background light.

Stain: it is a coloring solution which imparts colour to microbial cell. Following are components of a stain solution

- **Organic solvent**: Colorless solvent (Benzene solution).
- **Chromophore** Basically, it's a salt (dye), a component that will impart colors.

Chromophore is mixed with organic solvent, this solution is "**chromogen** "Chromogen is unable to stain bacterial cell. As it is a non-ionic part. It cannot bind with bacterial cell as bacterial cell have negative charge on its cell wall

- **Auxochrome**: A chemical agent which causes ionization of chromogens. It given positive or negative ion to chromogen.

On basis of charge present on chromogen, stains can be divided into three types;

1. Acidic stain
2. Basic stain
3. Neutral Stain

BASIC STAIN: If positive charge is present on the chromogens (cationic) then the satin will be a basic stain.

EXAMPLES:

- Crystal Violet
- Safranin
- Methylene blue
- Basic Fuchsin
- Malachite green

ACIDIC STAIN: If negative charge is present on the chromogens (anionic) then the stain will be an acidic stain.

EXAMPLES:

- India Ink
- Nigrosine
- Picric Acid
- Acidic Fuchsin
- Eosin

Neutral Stain: Having both positively charged and negatively charged chromogen

EXAMPLES: Giemsa stain

Staining: The process of giving colour (stain) to cells is called staining. There are three categories of staining

- Simple Staining
- Differential Staining
- Special staining

A. SIMPLE STAINING

It is a type of staining in which we use single type of stain (basic stain). Its basic principle is that a bacterial cell being negative in charge due to its cell wall will acquire the color of basic stain and will appear colored while background will remain colorless.

Simple staining is basically done to examine the,

- Shape
- Arrangement
- Size

- Microscopic counts

Procedure

1. Form a smear on the glass slide.

- Dewax the glass
- Place a drop of water with the help of inoculating loop (Only in case of growth on agar)
- Pick the microbial culture after sterilizing the loop on flame
- Mix the colony in the drop of water on slide and spread on slide in circular fashion.
- Let the smear to air dry
- Fix the smear by heat
- Smear is prepared Now move for simple staining

2. Cover the smear with crystal violet and wait for one t minutes.

Time will be different for different stains like **1 minute** for CV, **1-3 minutes** for Safranin and **1-2 minutes** for Methylene Blue.

3. Rinse the glass slide with water
4. Now blot dry the slide.
5. Examine your slide under compound microscope at 100X objective lens using cedar wood oil on stained smear.

Observations: Cells will be colored and background will be white

B. NEGATIVE STAINING

A type of staining in which acidic stain is used and as a result of it, cells become colorless as they don't take up acidic stains (having negative charge on its cell wall) and background will be appeared colored.

It helps to observed

- Shape

- Arrangements
- Size
- Capsule of microbial cell

Procedure

1. Place a very small drop (more than a loop full, less than a free falling drop from the dropper) of **Nigrosin** near one end of a well-cleaned and flamed slide.
2. Remove a small amount of the culture from the pure growth with an inoculating loop and disperse it in the drop of stain without spreading the drop
3. Use another clean slide (called as spreader slide) to spread the drop of stain containing the organism using the following technique
4. Place one end of the clean slide on the center of the slide with the stain mixed culture. Tilt the clean slide toward the drop forming an angle (45º) and draw that slide toward the drop until it touches the drop and causes it to spread along the edge of the spreader slide. Maintaining an angle between the slides, push the spreader slide toward the clean end of the slide being stained dragging the drop behind the spreader slide and producing a broad, even, thin smear
5. Allow the smear to dry without heating
6. Observe slide at 1000x magnification using oil immersion lens

Observations

In it, cell will remain colorless while background will appear colored (purplish).

Practical 9

DIFFERENTIAL STAINING TECHNIQUES

GRAM'S STAINING AND ACID-FAST STAINING

Staining used to differentiate bacteria into different groups on the basis of differences present in structural components like cell walls. In differential staining two stains are used. Differential staining includes;

- Primary stain
- Mordant (It intensifies the effect of primary stain. Mordant may be chemical or physical agent)
- Decolourizer (It tries to remove the primary stain from cell)
- Secondary/Counter stain

1. GRAM'S STAINING

Grams's staining differentiated the bacteria into gram's positive and gram's negative group.

Principle

When the bacteria are stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of gram-positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink which closes the pores in the cell wall and prevents the stain from exiting the cell. So, the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram-positive bacteria and appears blue or purple in colour.

In case of gram-negative bacteria, cell wall also takes up the CV-Iodine complex but due to the thin layer of peptidoglycan and thick outer layer which is formed of lipids, CV-Iodine complex gets washed off. When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then when again stained with safranin, they take the stain and appear red in color.

Gram's Positive

These bacteria have multiple layers of peptidoglycan in cell wall. Lipids (Lipo Poly Saccharides; LPS) is present in lesser quantity

- *Staphylococcus aureus*
- *Streptococcus pneumonae*
- *Staphylococcus epidemidis*
- *Clostridium (C.tetni, C.perfringens)*
- *Bacillus* (*B.subtilus, B.antheracis, B.cereus)*

Gram's Negative

These bacteria have two to three layers of Peptidoglycan in cell wall and LPS is present in greater amount

- *Escherichia coli*
- *Shigella dysentrae*
- *Salmonella enteritidis*
- *Pseudomonas aeruginosa*
- *Pasteurella multocida*
- *Proteus vulgarus*

Procedure

1. Prepare a smear on a glass slide followed by fixation
2. Crystal violet (primary stain; violet colour stain) for 1 minute on smear
3. Rinse with water to remove excessive stain
4. Add Legules Iodine (mordant)
5. Rinse with water
6. Add 95% ethyl alcohol (decolorizing agent) for about 30 seconds on smear
7. Now apply Safranin (counter stain; red color stain) for 1-3 minutes
8. Rinse with water to remove excessive stain
9. Blot dry/air dry

10. Examine the slide under microscope at 100x objective lens

Observations

Bacteria having more peptidoglycan in cell wall will retain the color of primary stain and appear **violet** or **purple** in color (**Gram's positive**)

Bacterial cell with high lipids contents and less peptidoglycan will take secondary stain colour and appear as **red** (**Gram's negative**)

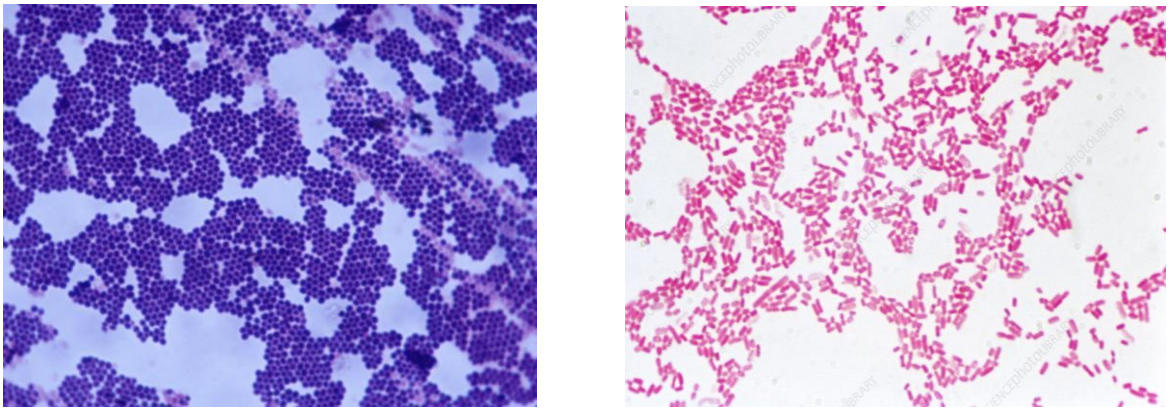


Figure 9: Gram's positive *Staphylococcus aureus* (Cocci arranged in cluster having violet color) Left side
Gram's negative *Escherichia coli* (Bacilli having round edges (coccobacilli), appear as red) Right side

2. ACID-FAST STAINING (Ziehl neelsen staining)

Some bacteria like *Mycobacterium* and *Nocardia* have Mycolic acid (60%) in their cell wall. In order to differentiate bacteria having Mycolic acid to those which don't have Mycolic acid in cell wall, acid fast staining is performed. Bacteria having Mycolic acid are called acid-fast and others are non-acid fast.

Principle

Smear is stained with carbol fuchsin, it solubilizes the mycolic acid present in the Mycobacterial cell wall by the application of steam heat, carbol fuchsin further penetrates through mycolic acid

wall and enters into cytoplasm. Then after all cell appears red. Then the smear is decolorized with decolorizing agent (3% HCL in 95% alcohol) but the acid fast cells are resistant due to the presence of large amount of mycolic acid in their cell wall which prevents the penetration of decolorizing solution. The non-acid fast organism lack the mycolic acid in their cell wall due to which these are easily decolorized, leaving the cells colorless. Then the smear is stained with counterstain, methylene blue. Only decolorized cells absorb the counter stain and take its color, appear blue while acid-fast cells retain the red color.

Procedure

1. Prepare a smear on a glass slide
2. Add Carbol Fuchsin (primary stain. It is of red color) on smear
3. Apply mordant (steam heat; physical mordant). For this place slide on steam heat for 5 minutes (It will increase the penetration of primary stain into cell)
4. Now rinse it with water
5. Add acid alcohol (95% alcohol + 3% HCL) on the glass slide as a decolorizing agent for 30 seconds. (It will wash primary stain out of the cells of non-acid fast bacterium and make it colorless but couldn't remove it out of the cells of acid-fast bacterium because of more affinity of carbol fuchsin to mycolic acid than to acid alcohol)
6. Rinse slide with water
7. Add methylene blue (counter stain) for about 2 minutes
8. Rinse it with water
9. Blot dry/air dry
10. Examine your slide under microscope at 100x objective lens

Observations

Acid-Fast bacteria will retain color of primary stain and will appear **dark red** while **non-acid fast** bacteria will take color of secondary stain and will appear **blue**.

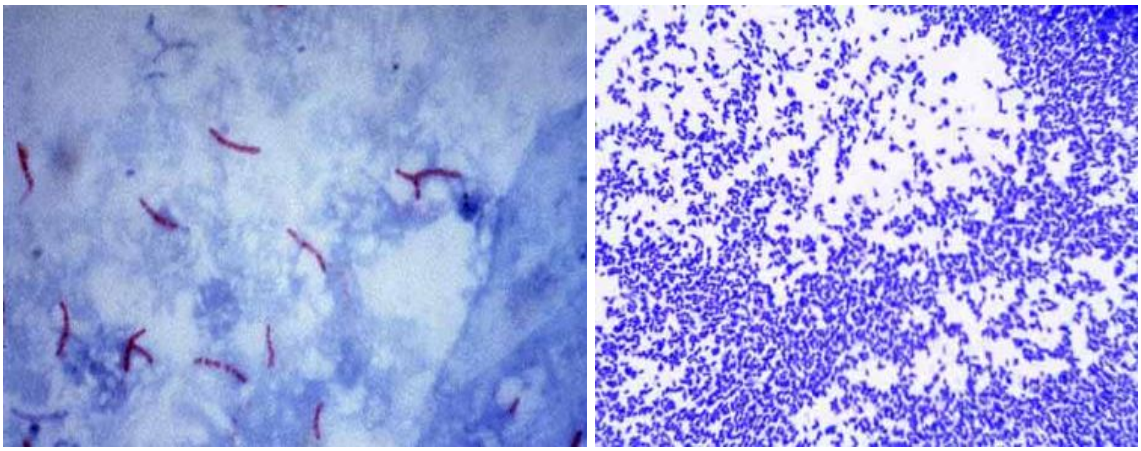
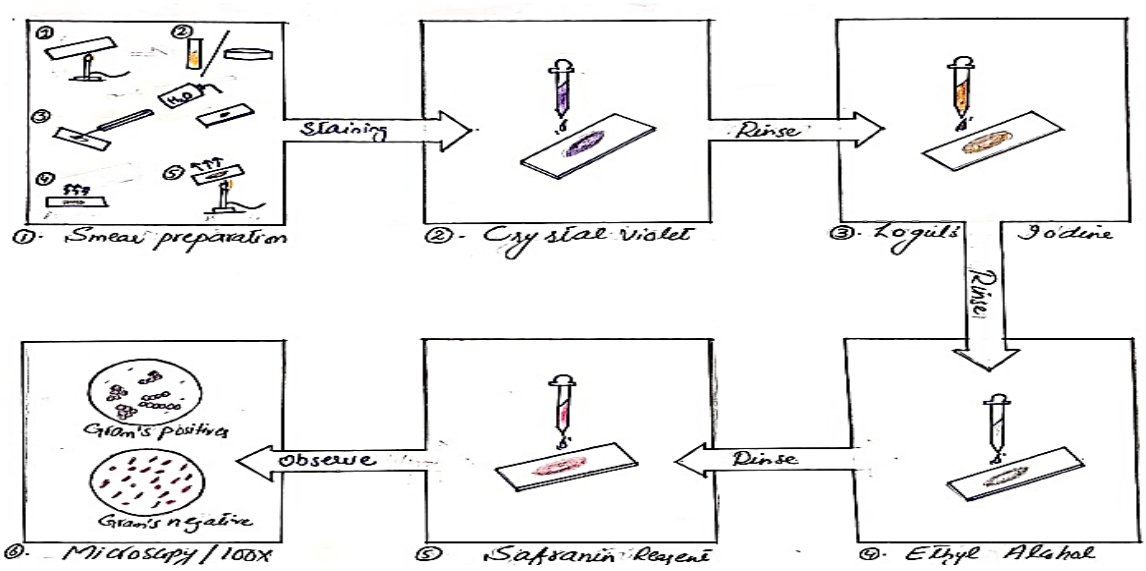
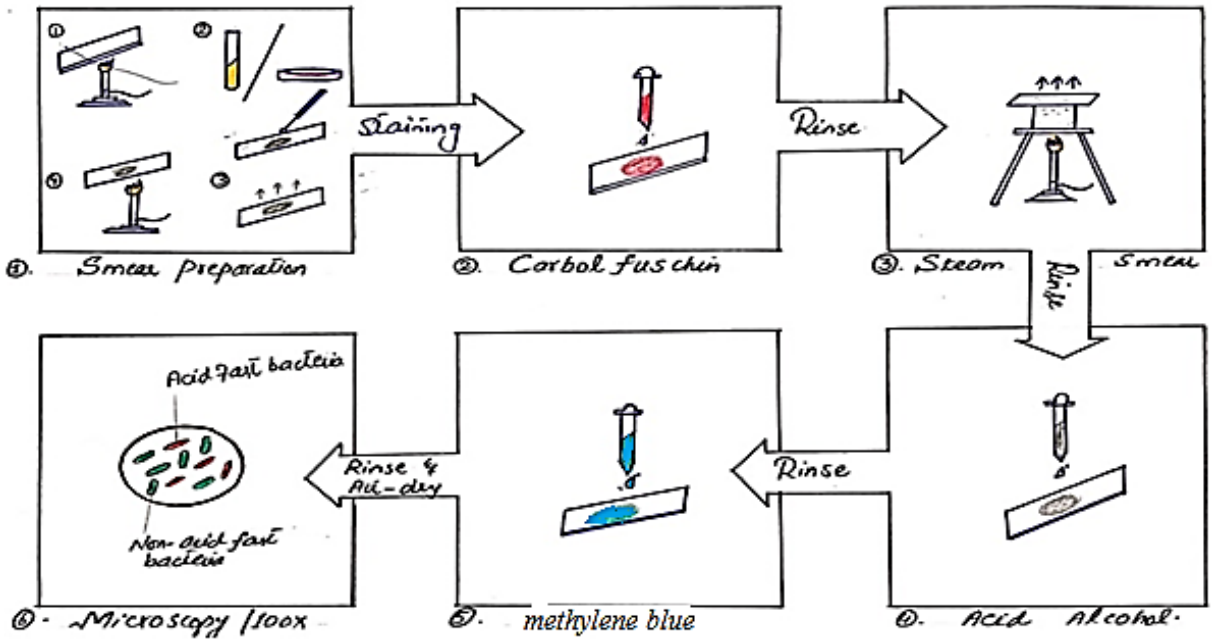


Figure 2: Acid-fast bacteria having red colour (left side); Blue color cells are non-acid fast (Right side)



A: Gram's staining



B: Acid-fast staining

Figure 9: Differential staining techniques

Practical 10

SPECIAL STAINING TECHNIQUES

SPORE STAINING AND CAPSULAR STAINING

Special staining is basically performed to observe the special structures of cell including flagella, pilli, fimbriae, capsule, slime and endospores under microscope. In this type of staining, both acidic and basic stains are used.

Here we are explaining the staining of following special structures.

- Spore Staining
- Capsular Staining
- Flagellar Staining

1. SPORE STAINING

Spores are formed under unfavorable conditions. Two species that form spores are Clostridium and Bacillus. Spore staining is done to check

- Presence or absence of spore.
- Spore position (Terminal, sub-terminal, Central)
- Bulging or non-bulging nature of spores
- Shapes of spores (*Clostridium perfringens* has lemon shaped spores)

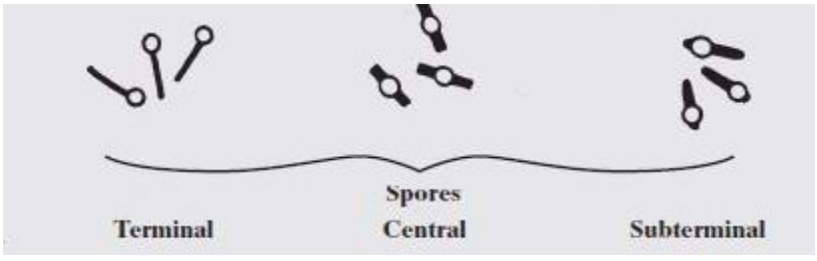


Figure: Spores of *Clostridium* species

Requirements:

- Malachite green is used as primary stain which has more affinity with spores as compared to vegetative cell.
- A physical mordant is used when we want to increase the penetration of stain into the cell. Mordant in spore staining is steam heat for 5 minutes.
- Decolorizer is used to remove the primary stain out of the cell. Water is used for this purpose in spore staining.
- Safranin is used as Counter or Secondary stain.

Principle

A primary stain (malachite green) is used to stain the endospores. Because endospores resist staining, the malachite green will be forced into *(i.e, malachite green permeate the spore wall)* the endospores by heating. In this technique, heating acts as a mordant. Water acts as decolorizer in this spore staining as the primary dye malachite green bind relatively weakly to the cell wall. In fact, if washed well with water the dye comes right out of cell wall however not from spore wall once the dye is locked in. Water is used to decolorize the vegetative cells. Colour-less cells are stained by safranin.

Procedure

1. Make a smear on glass slide.
2. Add few drops of primary stain on smear.
3. After it, steam heat is applied as a mordant for almost 5 minutes. Primary stain will adhere at some places to cell but form strong bonds with spores.
4. Rinse with water. Water will remove the primary stain out of the cell and make it colorless.
5. Apply Safranin for almost 3 minutes which form strong bonds with the cell because of its basic nature.
6. Rinse the glass slide with water
7. Blot dry /air dry it.
8. Examine your slide at 100X under microscope using cedar wood oil.

Observations:

Spores will appear **green** in color and **cells** will be seen **pink** in color. **Examples:** Species from genus *Clostridium* and *Bacillus*

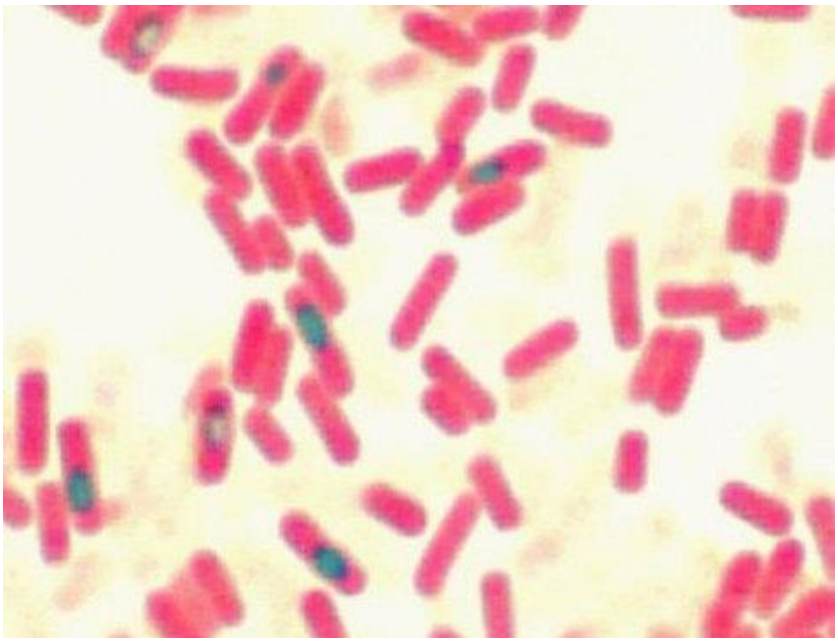


Figure 10: Green colour inside cells is endospores. This is *Bacillus* specie

2. CAPSULAR STAINING

Capsules are a special structure external to cell wall. It is made up of Polysaccharide or Polypeptide or both. It helps cell to survive in harsh conditions. It also acts as virulence factor (help in pathogenicity). Presence of capsule can be detected by *Negative staining* or *Capsular staining*

Requirements;

Primary stain; crystal violet (CV)

De-colorizer and counter stain; 20% CuSO4

Principle

Capsule is non-ionic structure, so when CV is added to smear, it will just adhere to capsule. Washing with $CuSO_4$ solution (acts as de-colourizer and counter stain) will remove the CV from capsule and will reside itself in capsule. So, cell will appear purple and capsule (if present) will be light blue colour around cell.

Precautions

- Don't use water while performing capsular staining because capsules are miscible in water and staining this way will give false negative results.
- Don't apply heat both directly and indirectly. Heat causes the shrinking of cell, so cells shrink leaving it actual place forming a false boundary. This boundary may be mistaken as capsule. It will give false positive results.

Procedure

1. Make smear in a drop of primary stain that's crystal violet.
2. Mix culture in primary stain drop gently and uniformly and a smear will form.
3. Keep spreader slide at the angle of 45 and slide it on the slide having a smear on it.
4. Allow the smear to air dry.
5. Gently wash the slide with CuSO4 solution (20%)
6. Blot dry/ air dry.
7. Examine your slide at 100X under microscope using cedar wood oil.

Observations;

Light **blue** periphery shows presence of **capsule** because of **copper sulphate** while cells will be appeared **dark purple** in color.

Examples; of capsulated bacteria are

- *Streptococcus mutants*
- *Streptococcus pneumoniae*
- *Klebsiella pneumoniae*
- *Pasteurella multocida*

- *Bacillus anthracis* (its capsule is polypeptide in nature and is made up of **poly-D-glutamic acid.**)

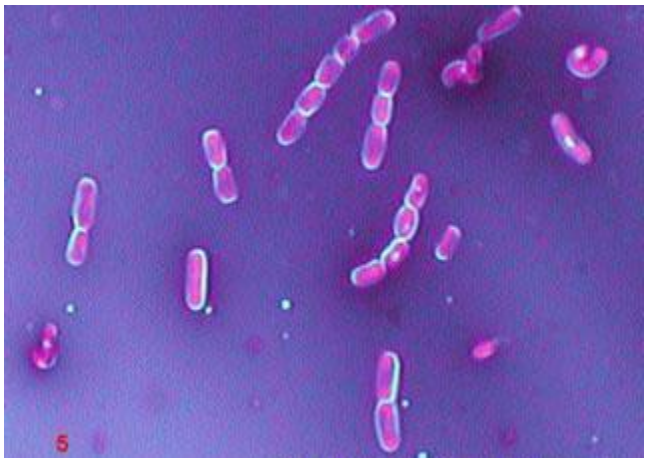
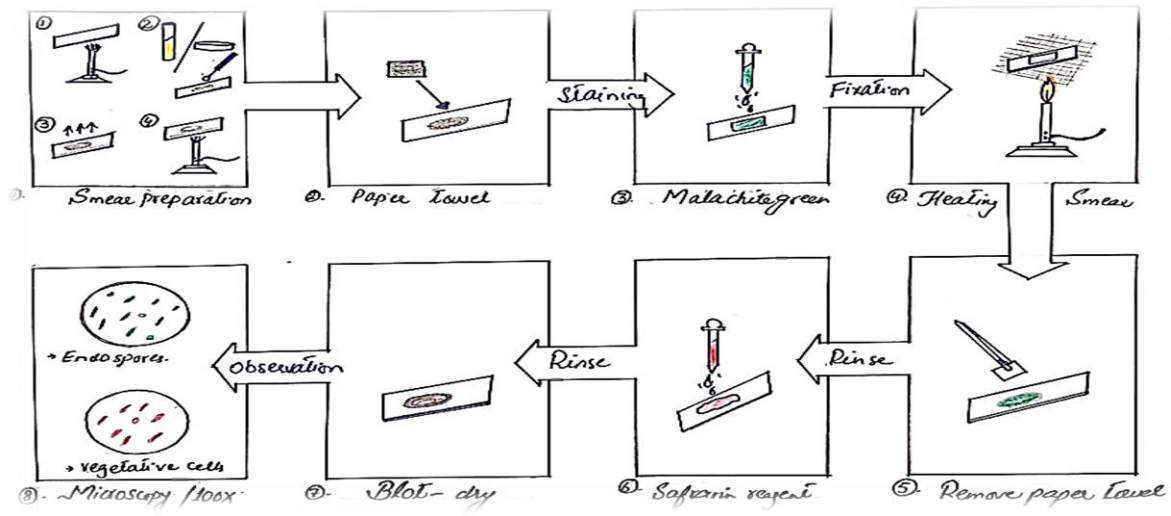
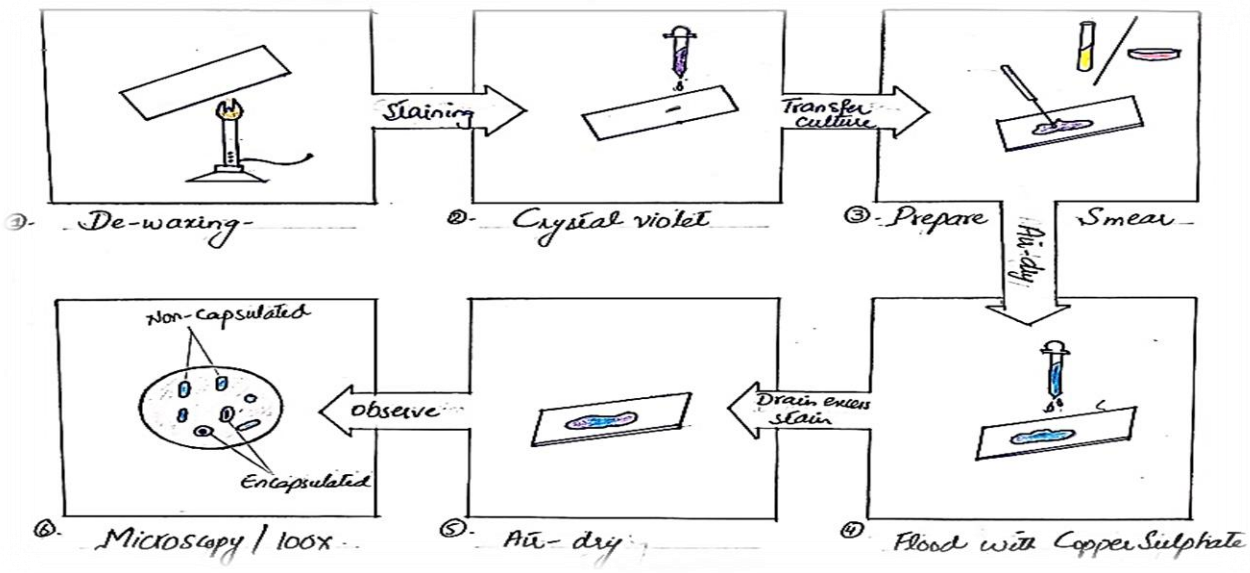


Figure 10: Bacterial cell dark purple having light blue capsule around cells



A: Spore staining



B: Capsular staining

Figure 10: Special staining techniques

Practical 11

FLAGELLAR STAINING AND MOTILITY TESTING

Motile bacteria possess flagella (A structure external to cell wall helps in motility). In order to find out that a bacterium is motile or non-motile, one method is to detect the flagella by special staining technique for flagella.

However, there are other methods by which we can determine either a bacterium is motile or non-motile. These methods are hanging drop method and inoculation into stab (stabbing). So, we will discuss;

1. Flagellar Staining
2. Hanging drop method
3. Stabbing for motility testing

1. FLAGELLAR STAINING BY LEIFSON'S METHOD

It is a type of special staining technique performed to determine the presence or absence of flagella. Flagellum is a thin hair-like external structure, which helps in locomotion of organisms. All bacteria don't have flagella. Difference is found in number and position of flagella in those has flagella. Various arrangements of flagella are seen on different cells.

Principle

Bacterial flagella are very thin and fragile, so a special stain is prepared which contains a mordant along with coloring component. This mordant allows piling of the stain on the flagellum, increasing the thickness until flagellum becomes visible.

Leifson'stain Composition

1. Sodium chloride in distilled water (1.5%)
2. Tannic acid in distilled water (3%)
3. Basic fuchsin in 95% ethanol (1.2%)

Mixed three components in equal proportions (1:1:1). This stain will remain stable for 1 month at 4°C and for at least 1 year at −20°C

Procedure

- Take a fresh culture of bacteria
- Prepared cell suspension by gently mixing the colony into sterile normal saline or adding liquid into growth on slant
  Avoid vigorous mixing; it may cause distortion of flagella, as flagella are fragile.
- Place cell suspension at room temperature (RT) undisturbed.
  This will facilitate to extend the flagella; extension of flagella is important to stain and then visualize it.
- Then take a drop of suspension and place the drop on a clean slide which is kept in slanting position.
- The drop should flow slowly from one end of slide to other end to avoid folding of flagella on cell.
- Allow smear to air dry here
  Don't heat for fixation.
- Flood smear with Leifson's stain for 10-15 minutes (a thin film of shinny surface will appear).
- Gently rinse with water
- Add methylene blue (1%) for 1 minute.
- Rinse with water
- Air dry and observe under oil immersion lens.

Observation

Flagella appear red in color and cell appears dark red or black-blue.

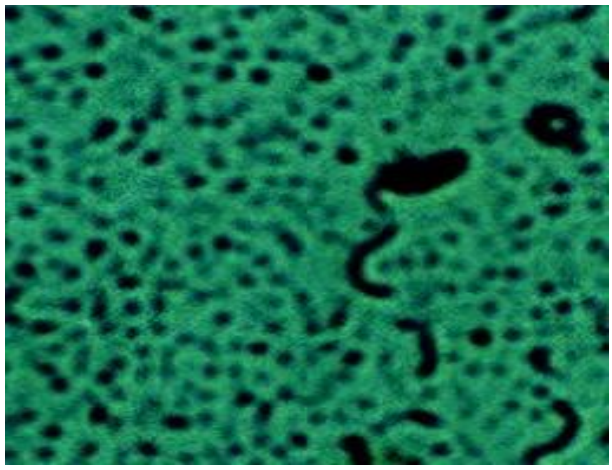


Figure 11: Bacterium having flagellum

2. HANGING DROP METHOD

- Take a fresh culture of bacteria
- Prepared cell suspension by gently mixing the colony into sterile normal saline
- Take a cavity slide. Apply Vaseline at the periphery of cavity carefully using a tooth pick or matchstick.
- Place a loop-full of the cell suspension/broth culture to be tested in the center of coverslip.
- Place slide on cover slip so that drop is under cavity. Drop must be in middle of cavity
- Turn the slide over so the coverslip is on top and the drop can be observed hanging from the coverslip over the concavity.
- Observe the slide under 100X objective lens

Observations

Motile bacteria will show directional movement. The movement must not be confused with Brownian movement.

3. Stabbing

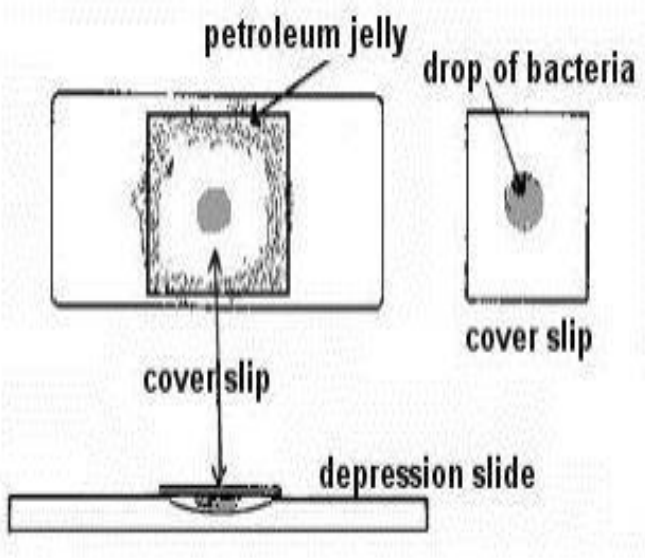
Stabbing is another technique of sub-culturing. For motility testing this technique is performed on semi-solid medium in test tube (in form of *Butt*). Stabbing is performed with **inoculating needle.** This technique is used for motility testing of bacteria (either bacteria is motile or not motile. Motile bacteria have flagella)

- Sterilized the inoculating needle
- Allow the needle to cool down

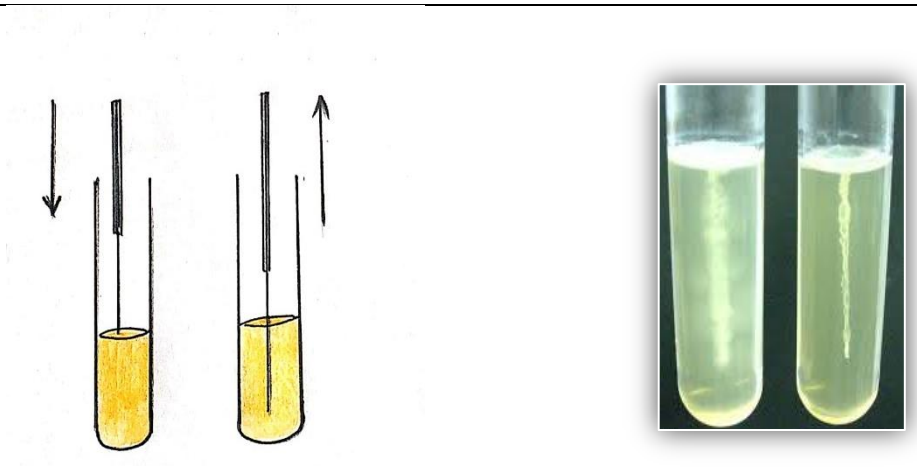
- Pick up a bacterial colony from agar plate
- Insert the needle having inoculum into the center of the agar in the tube and pushing it down to the bottom and then take it back in same direction.
- Incubate inoculated agar tube at 37°C for 24 hours
- Observe the growth

Observations

Motile bacteria have growth along with stab line and also in surroundings of stab line.



A: Hanging drop method



B: Stabbing

Figure 11: Flagellar staining and Motility Testing

Practical 12

MEASUREMENT OF BACTERIAL CELLS (MICROMETRY)

Micrometry is to measure the size of microscopic objects with the help of microscope and microscopic scales. The microscopic scales are called micrometers.

Two micrometers are used;

1. Stage micrometer (**Definite Scale**)
2. Ocular micrometer (**Arbitrary Scale**)

1. Stage micrometer

Stage micrometer is a glass slide having graduation of known distance. It is adjusted on stage under objective lens. Its measurements are known. When this scale is observed under microscope, it has 100 divisions. Complete scale is of 1mm and each stage division (distance between two lines) is of 0.01mm (10µm). However, on microscopic scale, graduations are not marked.

2. Ocular Micrometer

It is a disc inserted in under eyepiece (ocular lens), that's why it is called ocular micrometer. This has 100 divisions engraved on disc and divisions are marked after every 10^{th} division. But the distance of each division is unknown.

Procedure

We will measure the size of microscopic objects with help of ocular lens. As we have to remove stage micrometer, to place the slide having microorganism, which size we are going to determine. But the ocular micrometer division distance is unknown to us. So first we will determine the distance of one ocular division.

1. First step is to calibrate ocular micrometer with the help of stage micrometer.

- First insert ocular micrometer in tube below ocular lens and place stage micrometer on stage. Use objective lens which we are going to use for determination of measurement of bacteria.

- Adjust microscope using coarse and fine focus knobs under 1000x magnification
- Now, by moving stage coincide any division of stage with any division of ocular micrometer.
- Observe the next division of stage micrometer itself coinciding with the division of ocular micrometer.
- Measure number of stage and ocular divisions between coinciding area (Suppose we have 10 stage divisions and 1 ocular division in coinciding area)
- Determine the distance of ocular division using above information as below;

No. of ocular divisions= No. of stage divisions

10 = 1

As we know that 1 division of stage micrometer is equal to 10µm. So,

10 = 1X 10µm

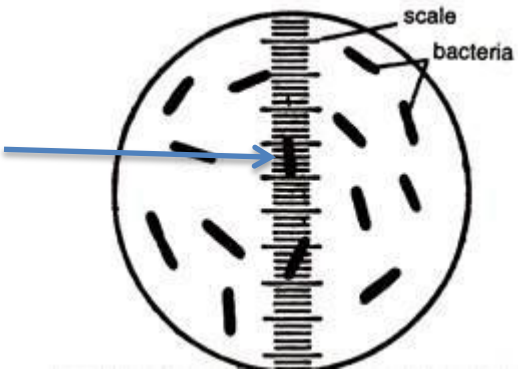
10 = 10µ

So, one ocular division = 10µm/10

= 1µm

The distance of one ocular division is 1µm

2. Prepare slide from a purified culture either by Negative staining or Simple staining
3. Remove the stage micrometer and place slide having bacterial stained smear
4. Fine focus slide under 1000x magnification using cedar wood oil
5. Bring cell length wise to ocular micrometer and count the no. of ocular divisions covering whole cell length. (**Suppose cell length covers 6 ocular divisions**).



6. Multiply the no. of divisions covering the cell length to the distance of one division of ocular lens as calculated above.

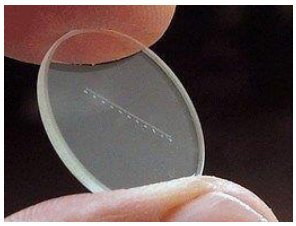
Cell length = No of division covered by cell length X Distance of one ocular division

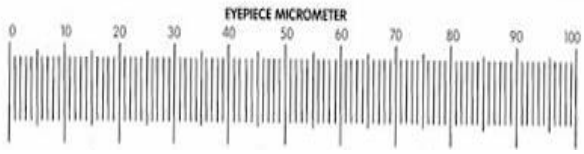
= **6X1** µm

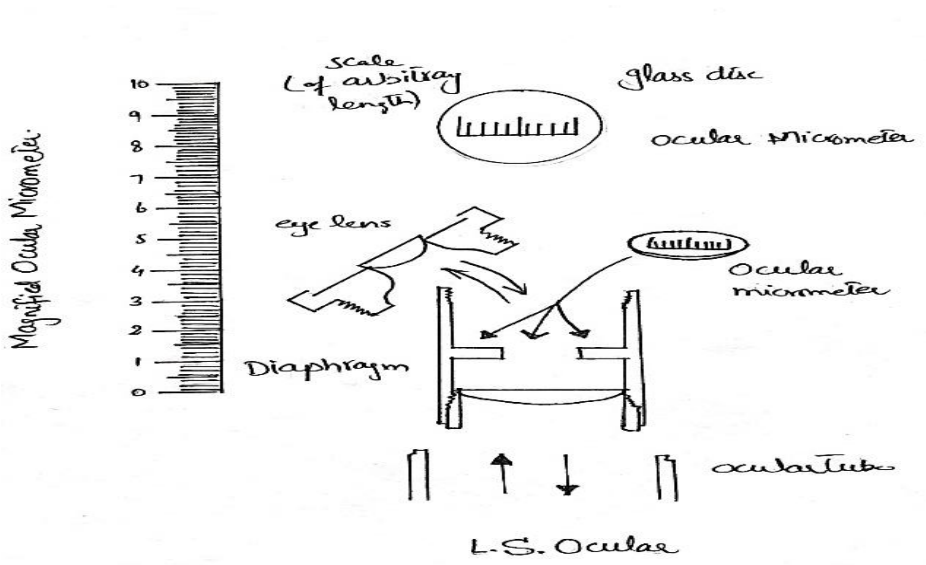
= **6** µm

So, cell length is 6µm.

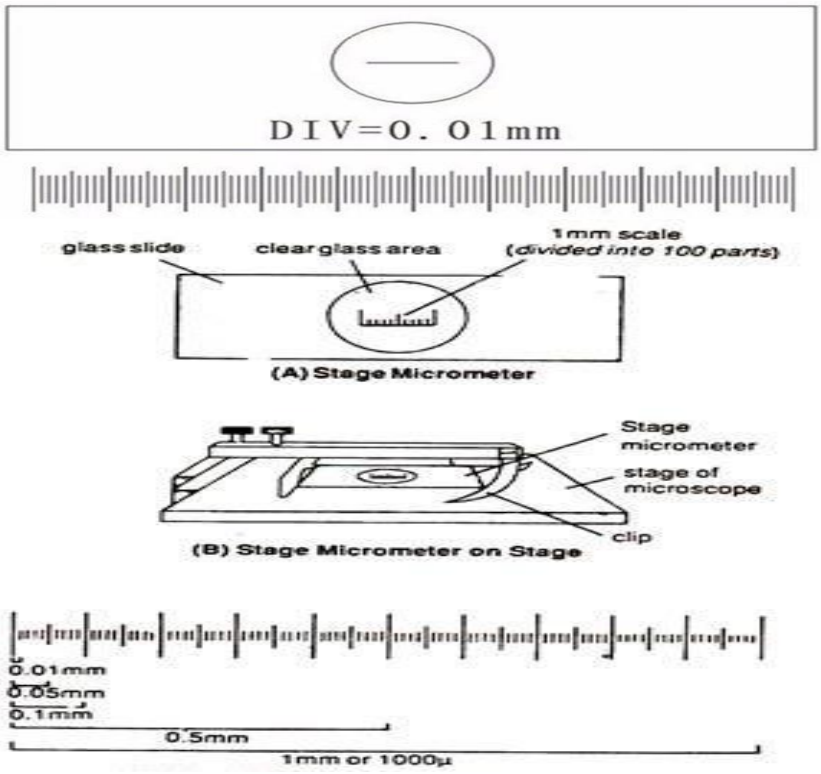
Similarly, cell width can be measured by bringing the cell in width wise under ocular micrometer.







A: Ocular Micrometer



B: Stage micrometer

Figure 12: Micrometery

Practical 13

BACTERIAL COUNT: DIRECT MICROSCOPIC COUNT

Sometimes, there is need to count the bacteria in sample. One objective is to determine the microbiological quality of water and food/feed. We have to give the results in microbial count/unit volume; like bacterial cells/mL.

There are several Direct and Indirect methods to enumerate bacteria. These are;

- Direct Microscopic count
- Viable Count
- Spectrophotometric methods

Direct Microscopic Count

To count the bacteria with the in a sample with the help of Microscope is direct microscopic count. It counts both live and dead bacteria in sample.

Procedure

- Mark an area of **1cm**^{2} on a slide.

  For direct microscopic count, we have to determine total no. of microscopic fields (MF)/cm^2 using following formula;

No. of MF in 1 cm^2 = $\frac{\text{Total area}}{\text{Area of 1 MF}} = \frac{1\text{cm}^2}{\Pi r^2}$

We can write it as;

$$= \frac{1\text{cm} \times 1\text{cm}}{\Pi r^2\ (\mu\text{m}^2)}$$

$$= \frac{10000 \times 10000(\mu\text{m}^2)}{3.14\ r^2\ (\mu\text{m}2)}$$

$$= \frac{10^8\ (\mu\text{m}^2)}{3.14\ r^2\ (\mu\text{m}2)}$$

Now the missing information in formula is r^2. It is the radius of MF. Radius of MF can be measured with the help of stage micrometer.

We will simply place the stage micrometer on stage under 100X and will count the no. of divisions in one MF. Suppose 20 divisions are visible when we observe in microscope at 1000X magnification. Each division on of stage micrometer is 10um. So, total distance is 200um (20X10um). This is the diameter of MF. To calculate radium, divide it by 2;

r^2 =diameter/2 = 200/2= 100um

No. of MF in 1 cm^2 $= \frac{10^8\ (\mu m^2)}{3.14\ (100\mu m)^2}$

=100000000/31400

=3184.71

So, the Total no. of MF_s /cm^2 area on glass slide are 3184.71.

- Take **10 µl** volume of sample and put it in the 1cm^2 area. Make smear within defined area with the help of inoculating loop. Air dry and heat fix the bacterial smear.
- Once smear is formed, do simple staining using any basic stain. We will use crystal violet.
- Adjust the slide under 1000X magnification.
- Now observe the slide at five different MF, and count stained bacterial cells in each MF.
- Calculate the average no. of cells per MF using following formula;

Avg. no. of cells/MF = $\frac{MF1+MF2+MF3+MF4+MF5}{5}$

Let's Suppose;

Average no. of cells per MF = **$\frac{10+5+10+5+5}{}$ = 7**

- Calculated the no. of cells/cm^2 using following formula;
  Cells/cm^2 = Average no. of cells/ MF X Total no. of Microscopic fields

= 7 X 3184.71

= 22292.99 cells/cm^2

We charged 10uL sample in 1cm^2 area. So, we can write as;

Cells/cm^2 = Cells/10uL

We have to give results cells/mL. We can convert this information using unit method;

To calculate Cells/mL;

10µl contains no. of cells = 22292.99

1µl contains no. of cells = (22292.99 /10)*1

1000µl (1 mL) contains no. of cells = (22292.99 /10)*1*1000

= 2229299 cells/mL

So, the no. of bacterial cells in each mL of sample is_2229299 cells (2.2 x 10^6 cells/mL)

Practical 14

BACTERIAL COUNT: PLATE COUNT

Plate count is the method of viable count of microorganisms. Only live microorganisms present in a sample are counted. Plate count is performed by;

- Spread plate technique
- Pour plate technique

Results are given in form of Colony forming units per mL of sample (CFU/mL) or (CFU/g)

Procedure

In both techniques of plate count (spread and pour plate), the first step is the preparation of ten-fold serial dilution.

A. Serial Dilutions (10-fold)

Now prepare ten-fold serial dilutions of milk sample

- For ten-fold serial dilution take ten test tubes. Each tube having 9 mL sterile normal saline in it, label these tubes as (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}).
- Now add one mL of milk sample in first tube (10^{-1}), mix it well by pipetting.
- Transfer one mL from 1st tube to second tube labeled as 10^{-2}.
- Similarly, from second tube after mixing transfer one mL to 3rd tube (10^{-3}), from 3rd to fourth tube (10^{-4}) and from fourth tube to 5th tube (10^{-5}). Similarly do this procedure up-to 10th test tube.
- From 10th tube discard one mL so that each tube has equal volume of diluted sample. These are ten-fold serial dilutions.

Table: Ten-fold Serial Dilutions

Test tube	1	2	3	4	5	6	7	8	9	10
ratio	1:10 1/10	1:100 1/100	1:1000 1/1000	1:10000 1:10000	$1:10^5$ $1/10^5$	$1:10^6$ $1/10^6$	$1:10^7$ $1/10^7$	$1:10^8$ $1/10^8$	$1:10^9$ $1/10^9$	$1:10^{10}$ $1/10^{10}$
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Dilution factor	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}

1. Spread Plate for Plate/Viable Count

- Prepare ten-fold serial dilutions of sample as mentioned above in section A
- Take ten sterile nutrient agar (NA) plates and label these as (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10})
- From each dilution spread 100uL on respectively labeled sterile Nutrient agar plates
- Incubate the plates at 37 ºC for 24 hours
- Count the colonies on plate having countable colonies

  Countable range;

  25-250 CFU

  30-300 CFU

- Calculate CFU/mL using following formula.

  CFU/mL= No. of colonies counted x dilution factor x corrective factor

Corrective factor is 10 if we inoculate 100uL from each dilution.

Dilution factor is the reciprocal of dilution. If you are choosing a plate on which you spread 10^{-2} dilution. You select that plate and count colonies in that plate. Suppose you count 20 colonies. The no. of colonies will be 20 to put in formula and dilution factor will be 10^{+2}.

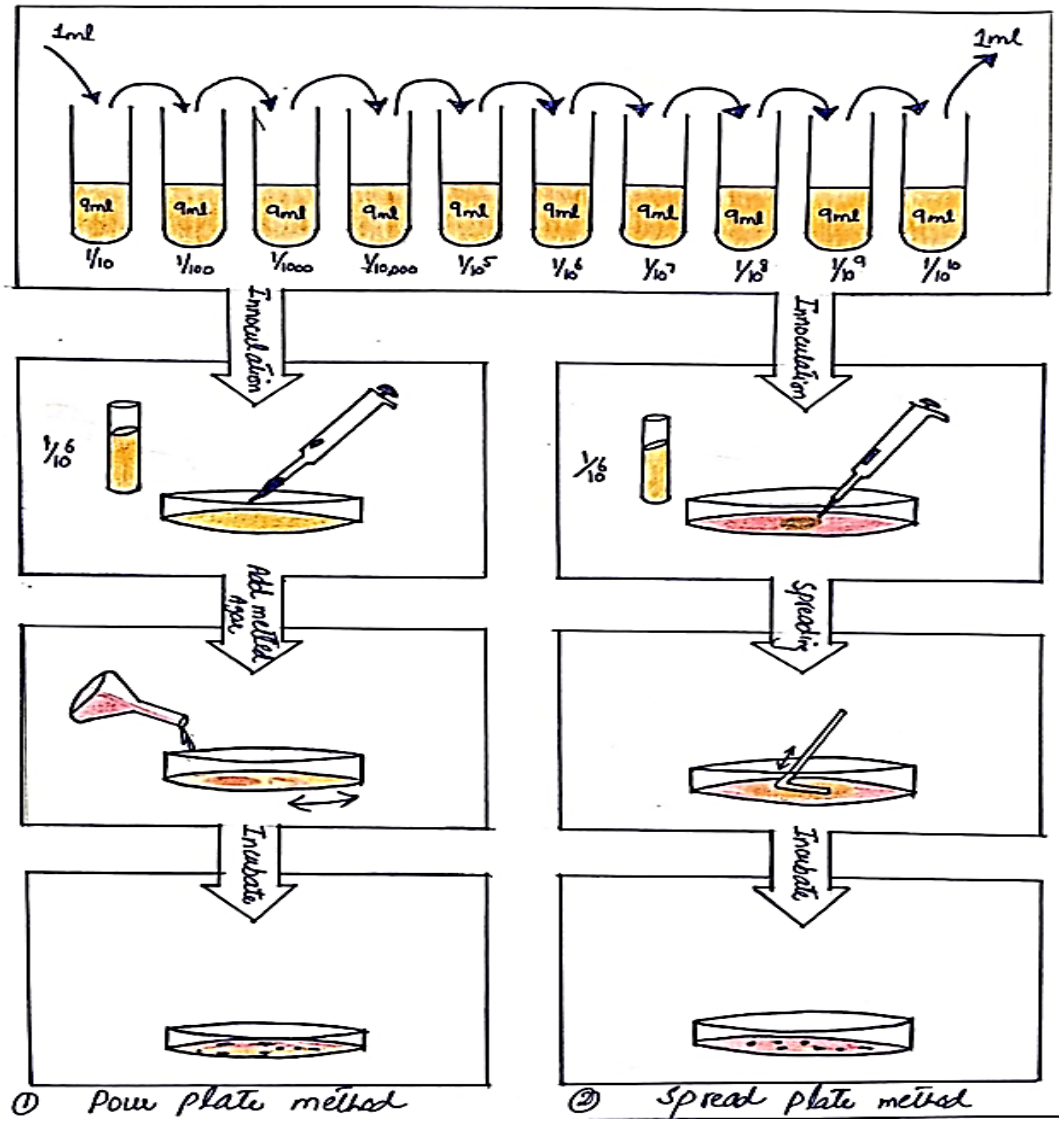
So;

$$CFU/mL = 20 \times 10^{+2} \times 10$$

$$CFU/mL = 20000 \text{ or in scientific notation } \quad 2x10^{4}$$

2. Pour Plate for Plate/Viable Count

- Prepare ten-fold serial dilutions of sample as mentioned above in section A
- Take ten flasks, each flask has 25mL sterile nutrient agar (NA) in it. Label these as (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}). Keep NA in molten form at 45 ºC.
- Take 10 sterile petri-plate (100mm diameter) and label these as (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10})
- Now from each dilution transfer one mL into receptively labeled NA flask and mix dilution into NA or directly add one mL of each dilution into respectively labeled empty sterile plate.
- Pour NA (mixed with a diluted sample) or into respectively labeled sterile petri-palate. If added dilution into plate pour 25 mL sterile NA into plate and swirl plate to mix molten agar and sample dilution.
- Allow the NA to solidify
- Incubate the plates at 37 °C for 24 hours
- Count the colonies on plate having countable colonies.
- Calculate CFU/mL as mentioned above



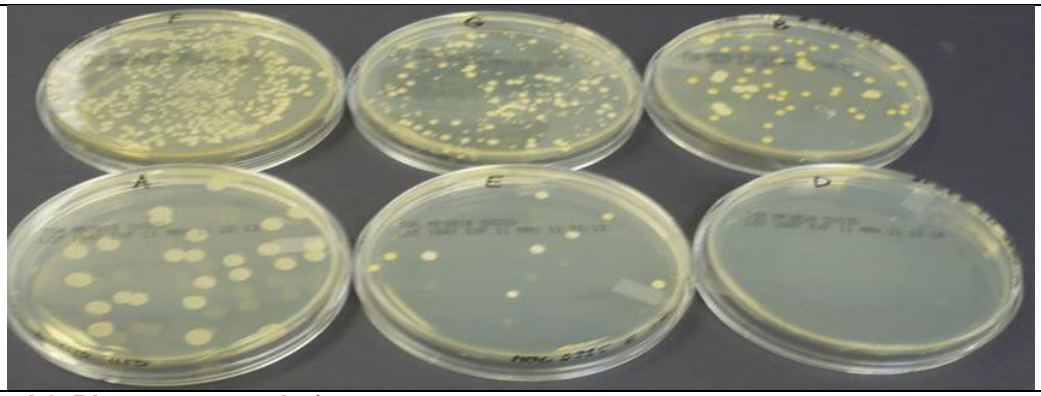


Figure 14: Plate count technique

Practical 15

ANTIBIOTIC SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing is performed to evaluate the effect of antimicrobial on pathogens. The antimicrobial are drugs which either kill (static effect) or inhibit the growth of microorganisms (Cidal effect).

- Antimicrobial effective against **bacteria** are **antibiotics**
- Antimicrobial effective against **fungi** are **antifungals**
- Antimicrobial effective against **viruses** are **antiviral**

In order to determine either a drug is effective against pathogen or not, antimicrobial susceptibility testing is performed. This will help to find out, pathogen is

- Resistant (Drug does not kill or inhibit growth of microorganisms)
- Sensitive (Drug kills or inhibits growth of microorganisms)
- Intermediate (Incomplete effect of drug on pathogens)

Antibiotic susceptibility testing (ABST) is included in our course

Methods for ABST

There are several methods for antibiotic testing. The methods included in our course are;

- Agar Disc diffusion method (Kirby Bauer Disc Diffusion Test)
- Agar Well diffusion method
- Micro-Broth dilution method

Which one we choose for performing ABST, first common step is sam in all methods. This is to adjust a particular bacterial number to be used as inoculum in this test. This is called inoculum standardization.

A. Standardization of Inoculum

This method is same for bacteria and yeast. In this method, cell suspension is prepared from purified fresh growth (24 hours) of bacteria in sterile normal saline. This suspension is compared with a standard solution (0.5 McFarland solution). Turbidity is matched, if bacterial suspension is less turbid then more bacterial growth is transferred to cell suspension and vice versa until turbidity of standard solution and cell suspension exactly becomes the same.

In second method we can take the optical density of bacterial cell suspension at 600nm wavelength using spectrophotometer or ELISA reader. The OD value must be between 0.085-0.1 at 600 nm.

We require $1x10^6$ bacterial cells /mL. The turbidity of 0.5 McFarland solution is equal to $1x\ 10^6$ bacterial cells /mL and 0.085-0.1 O.D value.

1. Agar Disc Diffusion Method

Antibiotic discs are commercially available. These discs have a particular concentration of Antibiotic absorbed on it. In dis diffusion these discs are used. This is called disc diffusion because Antibiotic starts diffusion from disc into the agar. If it is effective against Antibiotic, it inhibits the growth around itself to the area where effective concentration is diffused. This area appears as zone of inhibition (ZOI). In diagnostic laboratories this test is usually called **Culture Sensitivity**. To perform Disc diffusion followings are the steps;

1. Standardization of inoculum (10^6 bacteria / mL)
2. Make a uniform bacterial lawn on Muller Hinton agar by swabbing
3. Apply discs with forceps or disc dispenser
4. Incubate at 37°C for 24 hours.

Observation

Observe the plates for the presence or absence of zone of inhibition (Area around the disc where no growth is present). If present, diameter of zone of inhibition is measured in mm and compared with CLSI (clinical laboratory standards institute) available standards to find out whether our test bacterial pathogens is sensitive or resistant to that particular antifungal.

Sensitive: it means fungus is killed by that drug

Resistant: Fungus is not killed by that drug

Drugs to which bacteria is sensitive is recommended for treatment of disease caused by that particular bacterial species.

2. **Agar Well Diffusion Method**

Principle is the same as for disc diffusion. Drug diffuses into medium. But instead of disc, antibiotic solutions of required concentrations are poured into wells made in Muller Hinton agar plate. This test is carried out as;

1. Standardization of inoculum (10^6 bacterial cells / mL)
2. Make wells with the help of well borer. Three wells on 6cm diameter plate and 5 wells may be prepared on 9cm diameter Muller Hinton agar plate. Well base is sealed by molten agar so that when we pour antibiotic solution, it does not seep under the agar. It must be diffused into medium that's why we seal the base of agar with minute (10uL) of molten agar.
3. Make a uniform bacterial lawn on Muller Hinton agar by swabbing
4. Pour antibiotic solution into wells so that well is completely fills.
5. Incubate at 37°C for 24 hours. Plates are incubated in upright position.

Observation

Observe the plates for the presence or absence of zone of inhibition (Area around the well where no growth is present). If present, diameter of zone of inhibition (ZOI) is measured in mm and compared with CLSI (clinical laboratory standards institute) available standards to find out whether our test bacteria is sensitive or resistant to that particular antifungal.

If are testing a natural product/herbal product then presence of zone of inhibition indicates that the tested product has antibacterial activity.

3. **Broth Dilution Method**

This test is performed using broth (Muller hinton broth) instead of agar. If it is performed using small quantity (uL), then it is termed as **micro-broth dilution method**. In

this method, Minimum inhibitory concentration (MIC) of drug is determined. MIC value just like Zone of Inhibition (ZOI) helps to determine either fungus is sensitive or resistant to a particular antifungal. CLSI also provided standards based on MIC along with ZOI.

MIC: Minimum **Inhibitory** Concentration. It is the minimum concentration of drug which **inhibits** bacterial growth.

MBC: Minimum **bactericidal** Concentration. It is the minimum concentration of drug which **Kills** bacterial growth.

Micro-titration plate: 96 wells micro-titration plate flat bottom is used in this method. This plate has rows and columns. There are 12 columns and 8 rows. Rows are designated by letters (A-H), while columns are designated by number (1-12). This plate has 96 small pockets having capacity to accommodate 250uL volume. These pockets are called wells. So, this is 96 wells micro-titration plate.

This test is performed as mentioned below;

1. Standardization of inoculum (10^6 bacterial cells / mL)
2. Take 96 wells flat bottom micro-titration plate
3. Add 100uL sterilized Muller hinton broth in each well (A1-A12) as a diluent
4. Add100uL antibiotic agent in first well (A1) and mix properly
5. Now prepare two-fold serial dilutions till 10^{th} well (A10). Two-fold dilutions are prepared by transferring 100uL from 1^{st} well to second well. Then mix it in second well and transfer 100uL to 3^{rd} well and mix. Similarly transfer 100uL from previous well to next well (have 100uL diluent. already added in first step) till 10^{th} well. Now discard 100uL from 10^{th} well to keep the uniform volume in all wells. Now all wells have 100uL volume after this step. But first 10 wells have antibiotic in it. The highest concentration of antibiotic is in 1^{st} well and the lowest in the 10^{th} well of plate.

If we know the concentration of antibiotic, we are using then we can determine the concentration of antibiotic in each well. In above figure, we used antibiotic stock having concentration of 100mg/mL. It was two-fold diluted serially up-to 10th well. So, in first well its concentration remains half (50mg), in second well it again diluted two times, so concentration become 25 and so on.

6. Now add 100uL of standard inoculum in each well up-to 11th well.
7. Incubate this plate at 37ºC for 24 hours

Observations

According to performed procedure;

A12: Negative control; must be sterile after incubation, there must be no growth. It will remain transparent

A11: Growth control; add broth and inoculum. No antibiotic. So, after incubation there must be growth, which is represented by turbidity. It must be turbid

A1-A10: are test wells having broth + antibiotic (varying concentration in each well) + inoculum. These are test wells.

We will check test wells for presence or absence of growth.

MIC: Highest dilution (Lowest concentration) of drug that inhibits the fungus growth is MIC.

Example: Suppose the drug we tested have antibiotic activity proven by well diffusion. Now we want to determine its minimum concentration that inhibits bacteria (MIC). We will perform micro-broth dilution as mentioned.

Suppose that there is no growth from A1-A7, from A8 growth starts (Turbidity present in A8 to A10 test wells). This result indicates that this antibiotic is able to stop bacterial growth up-to concentrations present in A1-A7. Among all these concentrations the lowest or minimum concentration is in 7^{th} well. So, the concentration in 7^{th} well is MIC of that particular drug.

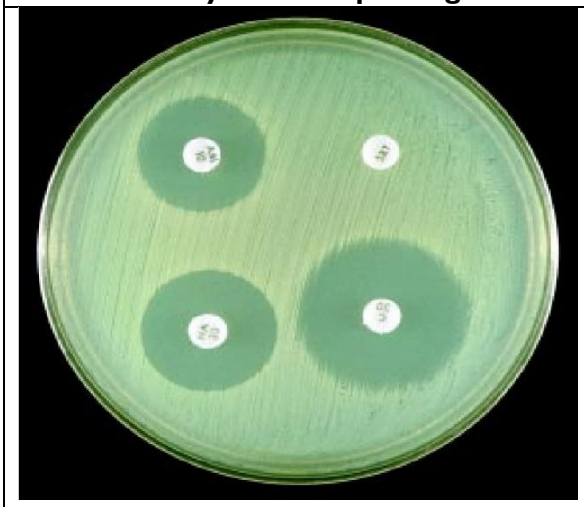
MIC is written as;

MIC ≥ Concentration in 7^{th} well (example only)

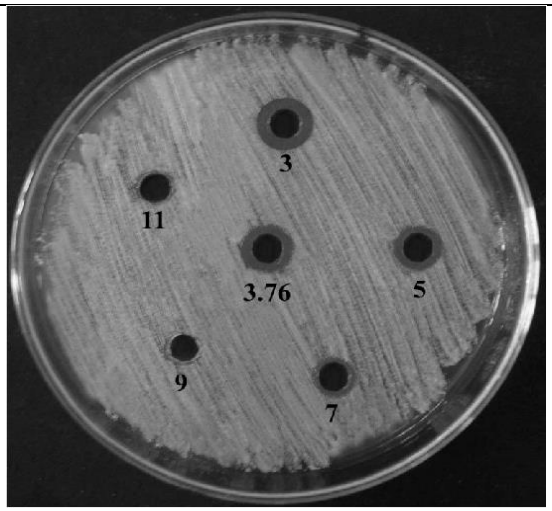
MIC ≥ 0.78mg

It means 0.78mg concentration of antibiotic and all concentrations higher than that are able to inhibit bacterial growth. But minimum concentration is 0.78mg.

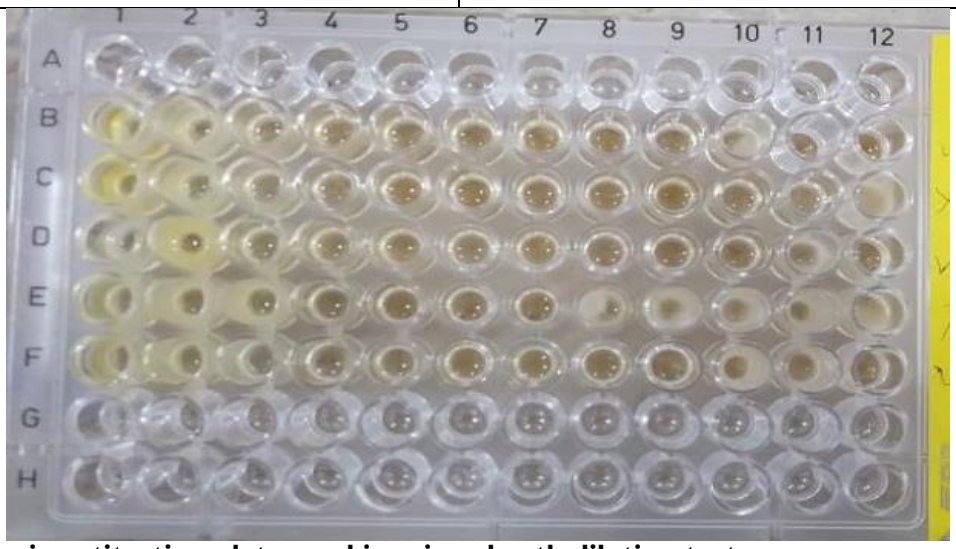
Commercially available packing of antibiotic discs



Agar DISC diffusion showing ZOI



Agar WELL diffusion showing ZOI



96 wells micro-titration plate used in micro-broth dilution test

Figure 15: Antimicrobial susceptibility testing

Practical 16

PHENOL CO-EFFICIENT TEST

Phenol is an aromatic organic compound consisting of a phenyl group attached to a hydroxyl group. It is also known as carbolic acid. It is the typical disinfectant used in determining the germicidal strength of another disinfectant. As phenol is a disinfectant having strong antimicrobial activity. One way to evaluate efficacy of disinfectant, their antimicrobial activity is compared with Phenol.

Phenol Co-efficient (PC-Value)

Ability of a disinfectant to kill microorganisms compared to the ability of the phenol to kill microorganisms is called **Phenol Co-efficient**. PC value can be calculated using following formula;

$$\text{Phenol coefficient} = \frac{\text{Highest dilution of test disinfectant that kill bacteria in10 min not in five min}}{\text{Highest dilution of phenol that kill bacteria in10 min not in five min}}$$

If;

$P_C = 1$

It means both phenol and disinfectant show same activity

$P_C < 1$

Test disinfectant is less effective as compared to phenol.

$P_C > 1$

Test disinfectant is more effective as compared to phenol.

Procedure

- Make 5 different dilution of phenol and test disinfectant using nutrient broth as diluent. Different dilutions are given in below mentioned table.

Disinfectant	Dilutions	Effect of Interaction Time *of S. typhymurium* with Disinfectants in minutes		
		5	10	15
Phenol	1:50			
	1:60			
	1:70			
	1:80			
	1:90			
Test Disinfectant	1:150			
	1:175			
	1:200			
	1:222			
	1:250			

- Add one mL from fresh growth *Salmonella typhymurium* broth culture into each dilution of phenol and test disinfectant.
- Incubate this mixture at Room Temperature (RT)
- After 5, 10 and 15 minutes, Transfer 200uL (0.02mL) from each tube into sterile nutrient broth tube. Each tube must be properly labeled to the type of disinfectant and interaction time.
- Incubate all inoculated tubes at 37°C/24 hours
- After incubation, observe the tubes for presence or absence of bacterial growth.
- If turbidity is present in tube, consider this tube positive (+) for growth. Tubes showing no turbidity are considered negative (-) for bacterial growth
- Record your observations and fill the following table to calculate PC value.
  We will fill the table from a previous experiment.

Disinfectant	Dilutions	Effect of Interaction Time of *S. typhymurium* with Disinfectants in minutes		
		5	10	15
Phenol	1:50	+	-	-
	1:60	**+**	**-**	**-**
	1:70	+	+	-
	1:80	+	+	+
	1:90	+	+	+
Test Disinfectant	1:150	+	-	-
	1:175	**+**	**-**	**-**
	1:200	+	+	-
	1:222	+	+	+
	1:250	+	+	+

(+) presence of turbidity, indicates growth

(-) Indicates absence of growth (killing of bacteria)

According to the table, the highest dilution of phenol that kills bacteria in 10 minutes of exposure but not in 5 minutes is 1:60.

While the highest dilution of test disinfectant that kills bacteria in 10 minutes but not in 5 minutes is 1:175.

We will put these values in formula to calculate PC value;

Phenol coefficient = Highest dilution of test disinfectant that kill bacteria in10 min not in 5 mins
Highest dilution of phenol that kill bacteria in10 min not in five min

$$= \frac{1:175}{1:60}$$

Re-arrange the values for calculations;

$$= \frac{1 \times 60}{1 \times 175}$$

Or;

$$= \frac{60}{175}$$

$$= 0.34$$

As the PC value is 0.34, less than 1. So, we can conclude that Test disinfectant is less effective as compared to phenol.

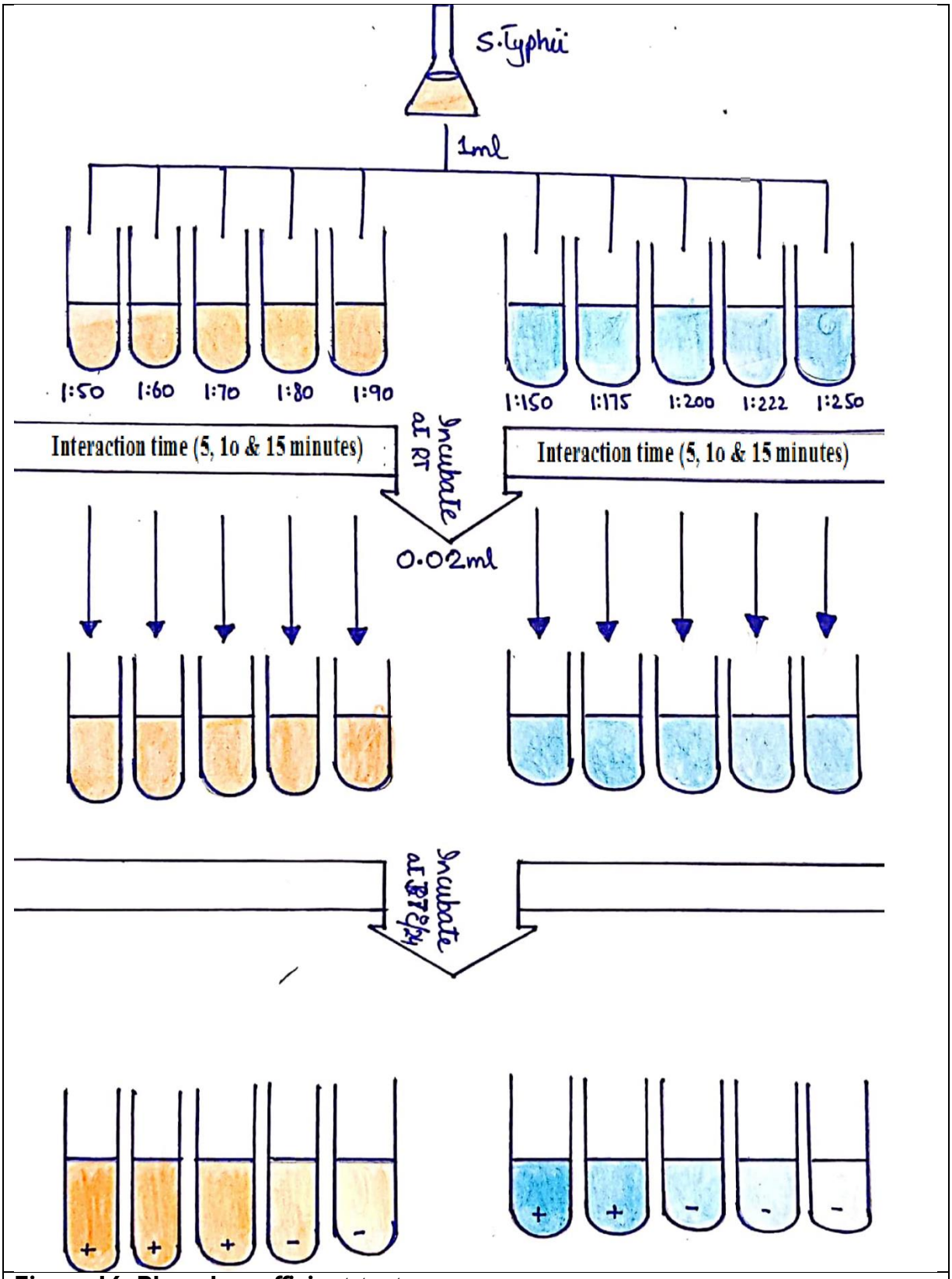


Figure 16: Phenol co-efficient test

Author Contributions

Conceptualization; S.S; Z.M validation; A.A.S writing—original draft preparation S.S; Z.M, I.L writing-review and editing S.S; A.A.S; I.L and visualization: N.M; T.Y; S.A; N.A.

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Conflicts of Interest

The authors declare no conflict of interest.