SREE NARAYANA COLLEGE, KOLLAM

Affiliated to University of Kerala
NAAC Re-Accredited with 'A+' Grade

DEPARTMENT OF BIOTECHNOLOGY

LABORATORY MANUAL

B.Sc. Biotechnology (FYUGP)
Practical Record & Laboratory Manual
Semester I – V

BY

Ms. GREESHMA P (Asst Prof of Biotechnology)
Dr. SWETHA S (Asst Prof of Biotechnology)
Dr. POOJA N S (Asst Prof of Biotechnology)
Dr. NITHA ANAND (Asst Prof of Biotechnology)

SEM 1 ESSENTIALS OF BIOTECHNOLOGY

INDEX

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Experiment No-1

Biotechnology Laboratory: Basic Rules and Practices

A biotechnology laboratory is a place for working with a variety of micro-organisms, plants and animal tissue culture. Since several culture media and organic materials are present, chances exist for the presence of high spectrum of microbial activity & growth leading to contamination. Secondly, while working with these cultures, one should always follow good laboratory practices and rules, so that neither the experiment should be unsuccessful nor any hazard may occur. Therefore, the fresher such as students as well as teachers, laboratory assistant and helper must follow the guidelines given below:

1. Always wear an apron (a white coat or gown) before entering the microbiology laboratory to protect from the microbial contamination and laboratory hazards. At regular intervals, get the apron washed.
2. Cut nails regularly
3. Tie long hair back to avoid contamination and fire hazard.
4. Keep your working laboratory bench clean off everything. Nothing should be lying on the bench.
5. Never keep your books, purses, bag etc. on the laboratory working bench.
6. Always wash your hands with soap in running tap water before and after work.
7. Clean your working bench with ethanol (70 % v/v) or phenol (1:100) for cleaning.
8. Never spill the materials and never spit and smoke in the laboratory.
9. Do not put anything of the laboratory (pencil, threads, labels, inoculation loop or needle, pins etc.) in your mouth, ears, nose and eyes.
10. Do not put your finger in your eyes, ear and mouth during microbiological work. It may facilitate the chance of infection of pathogenic microorganisms.

11. Do not eat or drink or talk while working with microorganisms.
12. Do not mishandle any chemical solution, stain, spirit lamps, UV light, instrument, apparatus or electricity.
13. Always keep the burner at a distance from organic solvents. Your sincere care will avoid fire accident. The burner must be turned off soon after use.
14. Always maintain aseptic conditions while working with micro-organisms.
15. Always use flame sterilized inoculation needle or loop.

16. Do not open the culture tubes/ plates directly and never inhale them nor observe them with naked eye.
17. Always open the culture tube or plate near the vicinity of flame of the burner.
18. While working with the broth culture, do not suck the suspension with mouth. Always use pipette sucker.
19. After completion of work, always label the culture with name, code and date of work. This will help recording the data.
20. Always keep plates in tiers and culture tubes upright in racks or baskets. Finally transfer all the cultures in incubator at desirable temperature or wherever to keep.
21. Never leave your cultures on working table or bench.
22. Clean the working table or bench, when the work is completed.
23. Clean lenses of objectives of microscope with tissue paper.
24. Keep the stains, reagents, stock cultures to their respective places when the work is completed.
25. After the completion of work, keep your slides/ pipettes /culture tubes/ plates in containers and steam sterilize before washing.
26. Record your results at time.
27. For any difficulty, ask your laboratory assistant or concerned teachers.

Experiment No-2

Basic Requirements of a Biotechnology Laboratory

A biotechnology laboratory requires well-built room equipped with glassware, tools and equipment which are given as follows:

I. COMMON GLASSWARE

The most important glassware used in a biotechnology laboratory are test tubes, culture tubes, petridishes, Erlenmeyer flasks, measuring cylinder, pipette, glass spreaders, volumetric flasks, screw capped glass bottles, haemocytometer etc.

1. Test tube, Culture tube and Screw-capped tube

i. These are made up of glass, one end of which is closed and other end open.

ii. If the sidewall of the open end is slightly curved outside, it is called test tube. If the sidewall is smooth, it is called culture tube. When the side wall of the test tube has screws so that a plastic cap may be fitted, it is called screw capped tube.

iii. The test tubes are used for testing chemicals such as sugars, pH etc. Culture tubes are used for preparation of culture slants, and purification of microorganisms. The open end is plugged with non-absorbent cotton plug.

iv. Sometimes the microorganisms are purified and preserved in screw capped tube.

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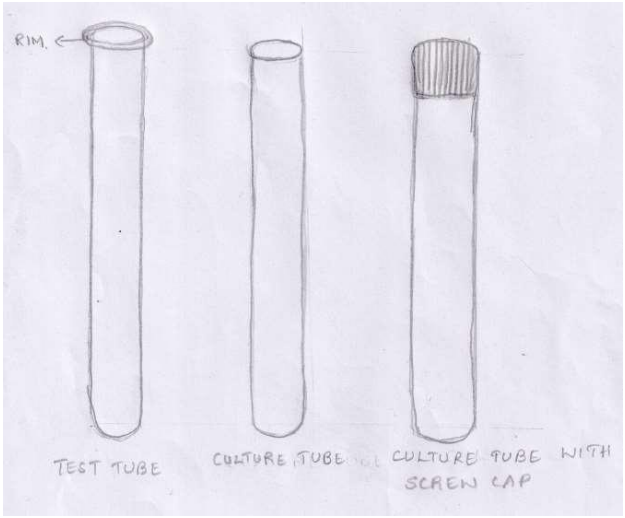

2. Petri-dish

i. As this glass dish was devised by R. J Petri, a student of renowned bacteriologist Robert Koch, it is known as Petri dish.

ii. It consists of two shallow glass dishes, the upper half or lid and the lower half or bottom half.

iii. For isolation and cultivation of different types of microorganisms these dishes are used in all microbiological laboratory

iv. According to the requirement its diameter can be varied

v. Molten agar medium is aseptically poured on the bottom half of the sterilized petridish and then covered with upper half.

vi. The petridishes are sterilized by putting them in a petridish container and placed in an oven or autoclave.

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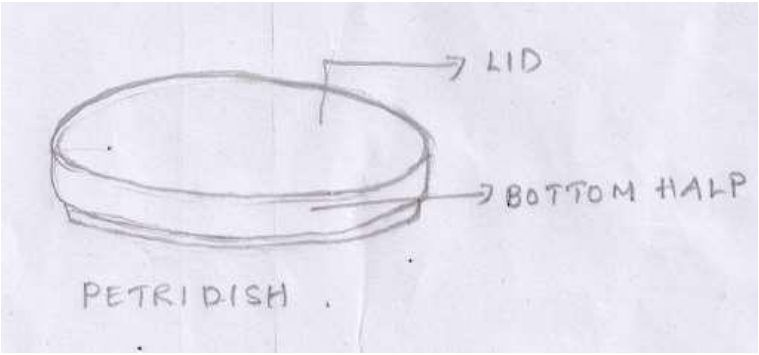

3. Pipette

i. It is a cylindrical and graduated glass apparatus.

ii. It's one end (lower side) tapers, while the other end is (mouth piece end) normal. The middle portion is wider or of the same size as mouth ending.

iii. It is graduated with numbers 1, 2, 3, 4….

iv. It has different measurements and has different measuring capacity such as 0.1, 0.5, 1, 5, 10 millilitres etc. and hence measures different quantity.

v. It is used for transferring appropriate amount of liquid in other containers.

vi. It should be sterilized in an oven or autoclave before use, by keeping in pipette container after being plugged with cotton.

vii. For safety point, liquid should be sucked by attaching pipette sucker at the normal end of pipette.

viii. Pipette should be sterilized by keeping them first in a steel can (steel container); the steel can then is sterilized at 121 °C for 30 minutes.

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4. Pasteur Pipette

i. Pasteur pipette is made by selecting the hollow glass tube of similar diameter as of standard pipette or graduated pipette, of which one end is heated so as to blow the glass to form a narrow end similar to 10 ml pipette. On other end, a similar rubber bulb is fitted.

ii. Before being sterilized all the pipettes are usually plugged with cotton in order to avoid contamination.

iii. Pasteur pipettes are generally used once and then transferred into disinfectant.

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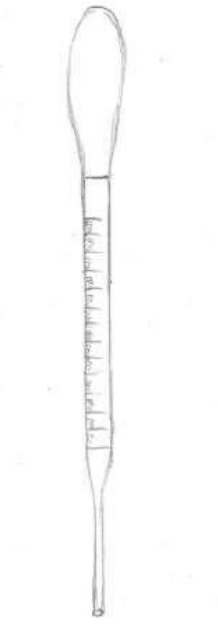

5. Erlenmeyer Conical flask

i. Erlenmeyer flask is named after the German chemist Emil Erlenmeyer who devised it.

ii. It has a narrow beak at the top with an opening and a broad bottom.

iii. The flasks are of different size, hence measure different volumes such as 100, 250, 500, 1000, 2000 ml of liquid.

iv. The flasks are of round bottom or flat bottom

v. Sometimes the flaks are also graduated to represent the volume of liquid.

vi. Certain modifications are made in Erlenmeyer flask according to requirement. For example, a beak is fabricated near the neck of the flask to connect to another equipment with rubber tubing. Such flasks are called side-arm flasks.

vii. During sterilization a cotton plug is inserted in the mouth of the flask.

viii. It must be sterilized before microbiological usage.

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6. Volumetric flask

i. It is used to prepare solutions of accurate strength.

ii. Its upper part is cylindrical and narrow, and marked at a point. This mark denotes the water level to be maintained at this point.

iii. The lower half is rounded and voluminous.

iv. Its base is flat, so that it may be properly placed on the surface.

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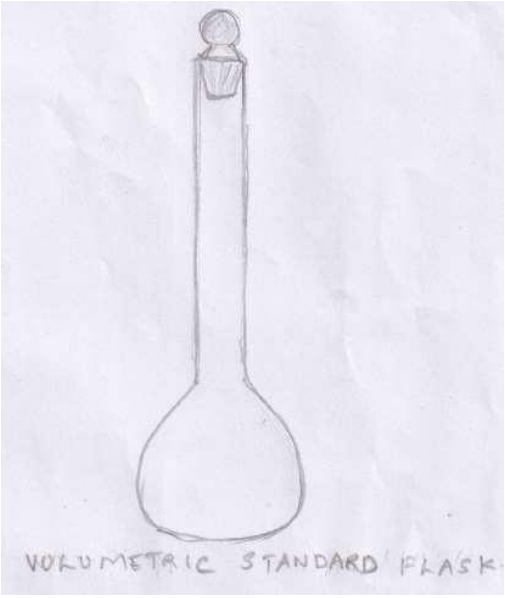

7. Glass Spreader or L-rod

i. A glass spreader or L-rod is made by bending a glass rod and making a L- shaped structure.
ii. It is used to spread evenly the microorganisms on agar surface, present in the liquid medium.
iii. The longer arm is held in hand and small arm is flame sterilized and put on agar surface.
iv. It is brought forth and back so that microorganisms present in the liquid may be dissociated and evenly spread on entire surface of agar.

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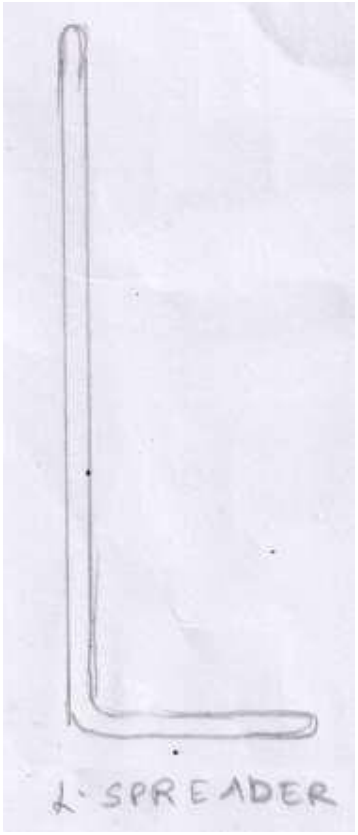

I. TOOLS & EQUIPMENTS IN MICROBIOLOGY LABORATORY

1. Inoculation needle and Inoculation loop

i. These are most commonly used tools.
ii. Inoculation needle or loop is made up of a long platinum or nichrome wire fixed in metallic rod.
iii. The wire loop has a handle with a steel screw shaft in which nichrome or platinum wire is to be fitted.
iv. The wire is to be wrapped around the small round objects such as pencil *etc*. to form a loop by twisting it mechanically. The loop should be such as, to retain a small circular

film in it by dipping in solution. For this the proper size 5-7 cm of the wire is recommended.

v. The straight wire or straight needle have a wire in place of the loop. The open or free end of it is blunt. Both loop and straight wire are to be sterilized either by using a Bunsen burner or hot heating coil wire till the needle or loop becomes red hot. After the loop or wire is cooled, these are generally used in transferring culture from liquid broth.

vi. The straight wire is used for transferring cultures from solid medium. Even smaller amount of liquid culture can be manipulated by using straight needles.

vii. The loop and wire are also used for picking smaller quantities of solid material from a microbial colony and can be used to inoculate either a liquid or a solid medium. Both the loop and straight wire must be flamed immediately after use so that contamination is avoided.

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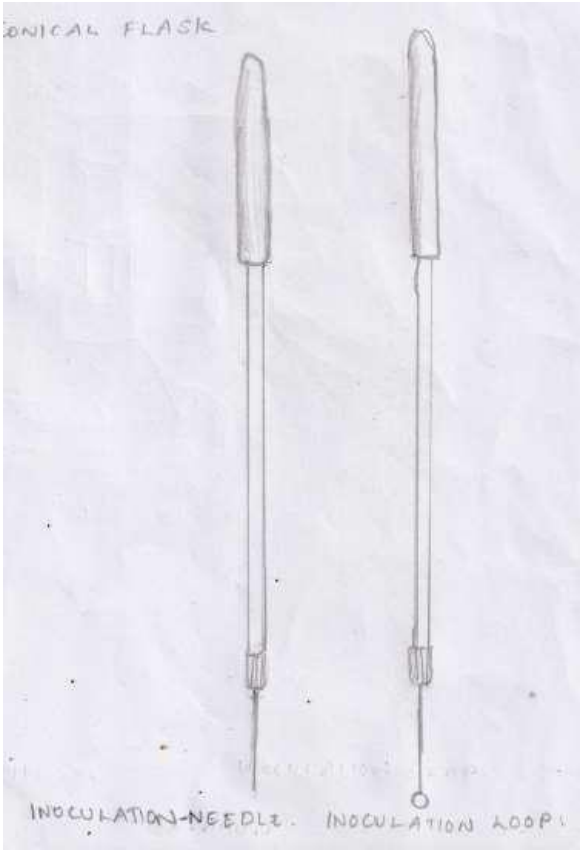

2. Bunsen Burner

i. A basic device for providing heat in the laboratory experiment is the Bunsen burner.

ii. The gas enters the burner at the base and its surprise is regulated externally by the gas cork. As the gas streams app what through a yet, inside the base, air in through the air intake holes just about the base.

iii. The amount of air can be controlled by rotating a sleeve that fits over the whole in the body of burner.

iv. To keep the flame from blowing out, special tips are frequently used to fit over the top end of the barrel.

v. The proper method of lighting the burner is to close of the air supply, turn on the gas and light the flame. The flame will be large and yellow. Gradually open the air intake until the flame takes a blue colour.

vi. For maximum heat, open further so that two distinct zones appear, the inner of which is light blue and cone shaped. The hottest point of the flame is just about the inner zone.

vii. In some instances, when the air supply to the burner is increased, the flame blows out or detaches itself from the burner tip. This means that the gas flow is top great and needs to be cut down. While relighting, remember to shut off the air supply.

viii. The inoculation needle or loop is sterilized before inserting into culture tubes or petri dishes or plates. It is also used during transfer and purification of microbial culture. After removing the cotton plug, mouth of the culture tubes or flask should be flamed so that the microorganisms if any present on mouth should be killed.

ix. Sterilization of tools by using spirit lamp or bunsen burner is called flame sterilization.

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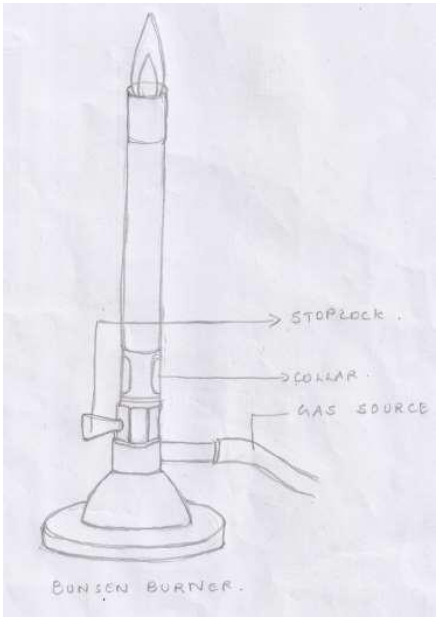

3. Water-bath

i. Water-bath is an instrument that is used to provide constant temperature to a sample. It consists of insulating box made up of steel fitted with the electric heating coil.

ii. Temperature is controlled through a thermostat.

iii. In some water-bath, the plate form rotates, then it is called water-bath shaker. It is more useful to microbiologist, because it provides uniform heat to the sample material meant for incubation.

iv. The main use of water bath is incubation of sample at a desired and constant temperature.

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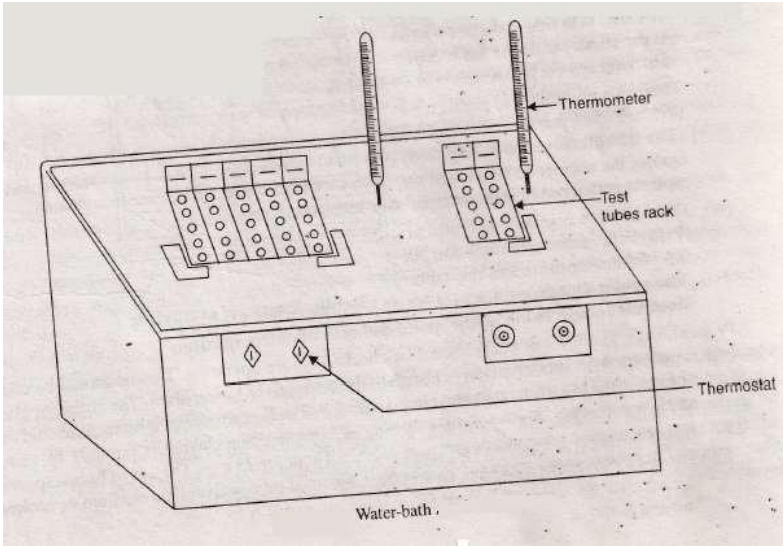

4. Laminar Air Flow Cabinet

i. Laminar air flow cabinet is an apparatus which consists of air blower in the rear side of the chamber which can air flow with uniform velocity along parallel flow lines. There is a special filter system of high efficiency particulate air filter (HEPA) which can remove particle as small as 0.3 micrometer

ii. In front of the blower, there lies a mechanism through which air blown from the blower produces air velocity along parallel flow lines.

iii. The laminar air flow cabinet is based on flow of air current of uniform velocity along parallel flow lines which help in transferring microbial cultures in aseptic condition. Air is passed through the filters into the enclosure and the filters does not allow any kind of microbes to enter into the system.

iv. Inside the chamber one fluorescent tube and another UV tube are fitted. Two switches for these tubes and a separate switch for regulation of air flow are fitted outside the apparatus. Due to the uniform velocity and parallel flow of air current, pouring of media, plating, slant preparation, streaking etc. without any kind contamination are performed.

v. Initially, best particles are removed from the surface of laminar air flow cabinet with the help of smooth cotton containing alcohol. Switch on the UV light for a period of 30 minutes so asked to kill the germs, any present in that area of working space.

vi. The front sheet of the apparatus is opened to keep the desired material inside. The air blower is set at the desired degree so that the air inside the chamber is expelled because the air inside the chamber may be contaminated or may bring contaminants.

vii. Sit properly in front of the chamber again, wipe the working table with alcohol to reduce the contaminants. All the work related to pouring, streaking etc are to be carried out in the plane zone of the burner or spirit lamp.

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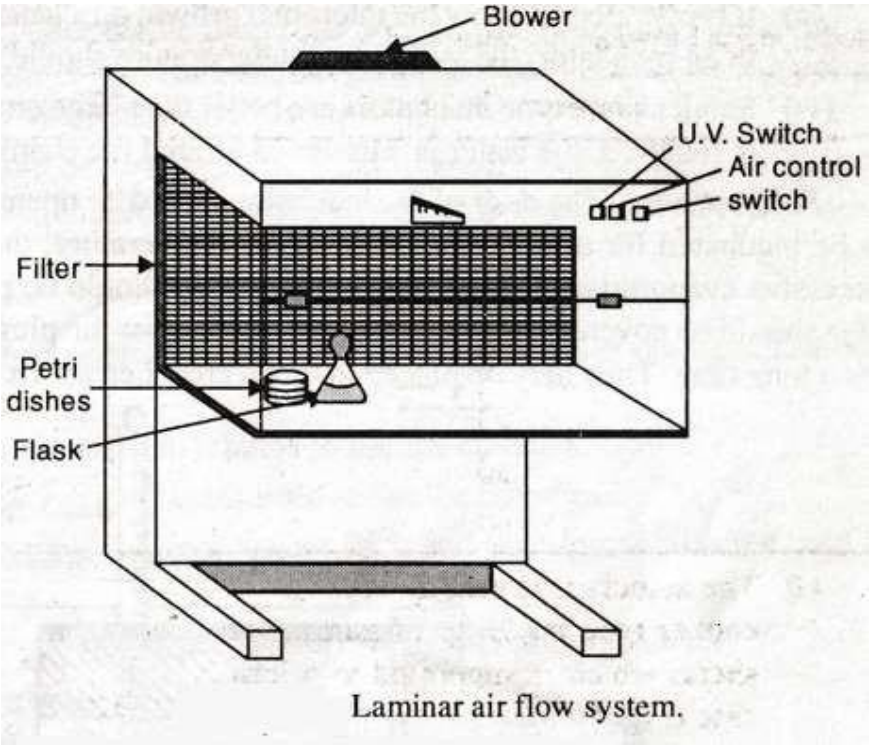

Laminar air flow system.

5. Incubator

i. An incubator is an instrument that consists of steel or copper chamber, around which warm water or air is circulated by electric current or by means of small gas flame. The temperature of the incubator is kept constant due to its control, by using thermostat.

ii. The incubator is made up of double walled chamber adjusted to a desired temperature. It is done by using and external knob controlling the thermostat system. The gap between the two walls is insulated to check the heat conduction. A thermometer is inserted from the top for recording the temperature. New sophisticated incubators are available with humidity and oxygen control system.

iii. Temperature greatly influences the microbial growth. Therefore, instrument is generally designed in a way that can allow the desired microorganisms to grow at particular temperature.

iv. It is operated to allow the microbial growth on a suitable medium under proper temperature the variation in temperature should not be more than 1 degree centigrade. Small square type into better or better than largest ones. If a low temperature incubation than room is required, then water is circulated around the chamber to pass through an ice chest.

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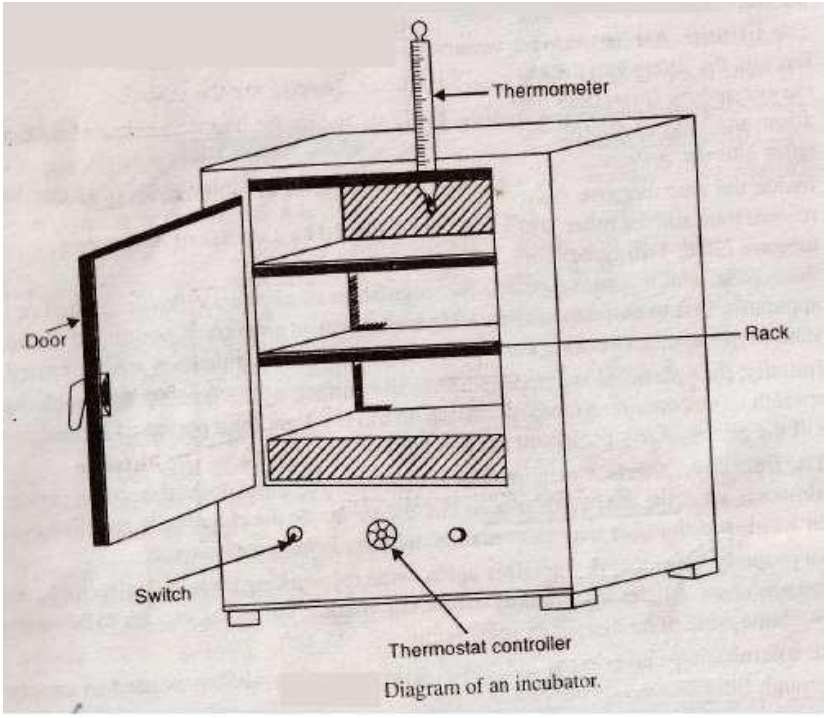

Diagram of an incubator.

Shaking incubator:

i. A thermostatically controlled shaker incubator is a type of incubator used to cultivate microorganisms.

ii. It consists of all parts of a common incubator along with a shaking platform or tray with circular clamps to fix conical flasks of various sizes. This platform permits mixing of contents in a flask with an orbital shaking motion.

iii. The rotation of the tray permits rotation from 30 to 250 rpm (revolutions per minute).

iv. Its advantage is that it provides a rapid and uniform transfer of heat to the culture vessel, and its agitation provides increased aeration, resulting in acceleration of growth.

v. This incubator, however, can only be used for broth or liquid culture media.

Precautions

The door of incubator should be opened only when necessary. If the tubes are to be incubated for a longer time or at high temperature, the medium may become too dry due to excessive evaporation. In such cases, cotton plug should be pushed the inside the neck of the tube. The tube should be covered by rubber cap so as to cover the plug. If the Petri dishes are to be incubated for a long time they may be placed in a moist chamber with a damp sterile cotton wool at the bottom.

6. Digital Colony Counter/ Quebec Colony Counter

i. It is a device used for counting the small or closely growing colonies on the surface of the media.

ii. For accuracy the lens fitted or mounted in it helps to see the colonies.

iii. The lens is movable on the box and can be adjusted to see colonies.

iv. The Petri dish is kept on a slanting platform meant for it and illuminated with the help of light source from beneath.

v. The number of undefined colonies are read out with the support of digital reading system.

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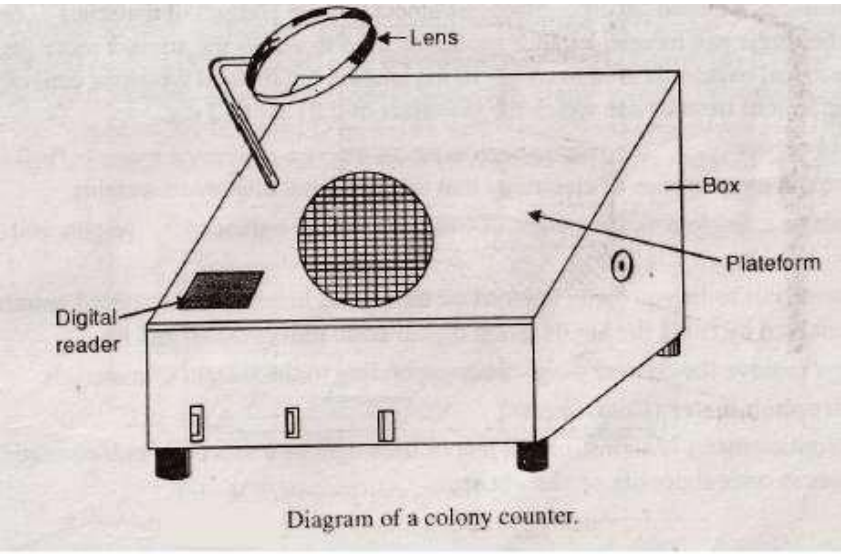

Diagram of a colony counter.

7. pH Meter

i. The pH can be defined as the negative logarithm of hydrogen ion concentration [H^+].

$$pH = -\log[H^+]$$

ii. pH is the degree of acidity and alkalinity of a solution on a scale 1 to 14.

iii. pH value of 1-7 shows the acid value, pH=7 indicates neutrality and pH value 7-14 shows alkalinity. pH of water if 7 at 25 °C.

iv. The acid is the proton donor and base is the proton acceptor [acid=base + H^+], i.e., acid dissociates to produce hydrogen ion concentration[H^+].

v. Maintenance of pH value is an important parameter for the growth and metabolic process of many organisms.

vi. pH is measured by using a pH meter which is an instrument calibrated against a series of buffers of known pH value.

vii. Standard pH meter has two electrodes, one glass electrode and the second mecury-mercurous chloride (Calomel) or silver- silver chloride reference electrode. The reference electrode is immersed in saturated potassium chloride (KCl) solution

viii. A normal pH meter consists of glass electrode made up of a thin glass membrane which is selectively permeable to H^+ ions.

ix. The function of electrodes is to form conductive bridge between the metallic element and the sample solution in which the electrodes are placed.

x. By using a pH meter, pH of unknown solution is measured.

xi. pH meters with combined electrodes are also available.

8. Balance

For chemical or biological experiments, accurate amount of chemicals should be weighed by using a balance. However, no experiment can be conducted without a balance. There are several types of balances used for weighing such as single pan, chemical or analytical and electronic balances. These balances are used according to the requirement and the amount of material to be weighed.

Electrical Balance

i. It works in the presence of electricity that shows digital display of weigh.

ii. It contains a single pan, the weight of which is counter balanced by weights and set as 0.

iii. The materials to be weighed is placed on the pan of the balance and required counter weights are removed by using the knobs. Soon digital scales move up and down.

iv. Always remove the counter weights corresponding to the weight of the material.

9. Spectrophotometer or Colorimeter

Spectrophotometer is an instrument that uses light as a source of radiation and measures changes in optical density or absorbance. It has three basic parts:-

i. Source of radiation

ii. A unit for dispersing radiation at different wavelength and
iii. A device that detect the amount of radiation at different wavelengths

Spectrophotometer uses monochromatic radiation, whereas colorimeter uses broad wavelength bands.

Principle of spectrophotometry

There are several atoms and their electron clouds in every chromophore (a functional group or molecule responsible for imparting colour to that substance) When light falls on to a substance, it absorbs some radiation of the electromagnetic spectrum and transmits the remaining radiation. These changes in light energy can be recorded by the detector of spectrophotometer. For quantitative estimation, the absorbance of different concentration of substrate can be measured following Beer's law. As per this law, absorbance is directly proportional to the concentration of a substance, i.e., when a ray of monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the concentration of absorbing medium increases.

It is expressed in an equation

$$\log[\frac{I_0}{I}] = KC$$

where I_0 = Original intensity of light beam

I = Intensity of beam passing through a solution

C = Concentration of the solution in moles/L

K = Constant

A plot of absorbance (Y-axis) versus concentration (X-axis) yields a calibration curve. This shows the range of beer's law. It may be used for quantitative estimation.

10. Centrifuge

Centrifuge is the instrument which operates through centrifugation technique. Centrifugation technique is based on molecular mass, shape and density of particle. The rate of velocity at which particle sediments is proportional to the particle size, difference in density between the particle and the surrounding medium and the applied centrifugal force. The larger the applied force than the earth's gravitational field, the greater the rate of sedimentation of particles. Different particles sediment separately due to variation in their density, shape and size. There are two techniques of centrifugation.

I. Preparative Centrifugation
II. Analytical Centrifugation

The separation, isolation and purification of whole cell, plasma membrane, mitochondria, nucleus and virus are carried out by preparative technique where as pure macromolecules or particles are separated by analytical technique.

Principle of sedimentation

The centrifugal force applied, includes gravitational force of the material present in a solution. The centrifugal force act in a direction away from the center of axis. If the spread of the rotation is larger, the force will be higher accordingly. However, the rate of sedimentation depends upon the applied centrifugal force. The relative centrifugal force, G is the function of the square of revolutions per minute (rpm) and angular velocity "ω" (radians per second) of the rotor and the radial distance of the particle ('r' in cm) present from the axis of rotation.

$$G = rpm^2 \times \omega^2 \times \square$$

Since one revolution is equal to 2π radians

$$\omega = \frac{2\square}{60}$$

$$\therefore G = \left(\frac{2\pi}{60}\right)^2 \times rpm^2 \times \square = \frac{4\pi^2}{3600} \times rpm^2 \times \square$$

The centrifugal force is expressed as relative centrifugal force (RCF) in g unit [g= 9.807 m/s^2 or 980 cm/s^2]

$$\therefore RCF = \square = \frac{4\pi^2}{3600} \times rpm^2 \times \square = 1.118 \times 10^{-5} \times rpm^2 \times \square$$

The sedimentation rate or velocity of particle varies accordingly to shape, size and density and hence sedimentation co-efficient, Svedberg unit (S)

Sedimentation velocity, $V = S\ \omega^2\ r$

Sedimentation co-efficient, $\square = \frac{V}{\omega^2 r}$

Sedimentation co-efficient 'S' of the most biological particles are very small and for convenience, its basic unit is taken as 10^{13} seconds. For example, types of RNA are 15S, 16S, 40S etc.

Date:…….

Experiment No-3

Microscopy

The cells of most animals, plants and bacteria are too small to be seen by naked human eye. The purpose of a microscope is to see what is invisible to the naked eye. By staining the cells, their different components could be made well contrasted. The aim of magnification of cells and their components was achieved by the lenses. The lenses could magnify the minute objects only up to a limited extend. So many lenses were combined together to form an instrument known as microscope.

Magnification and resolving power of the microscope are two important factors, that determines the quality of the microscope. Magnification is the increase in size of an optical image, over the size of an object being viewed. Resolving power is the ability to reveal closely adjacent structural details being actually separate and distinct.

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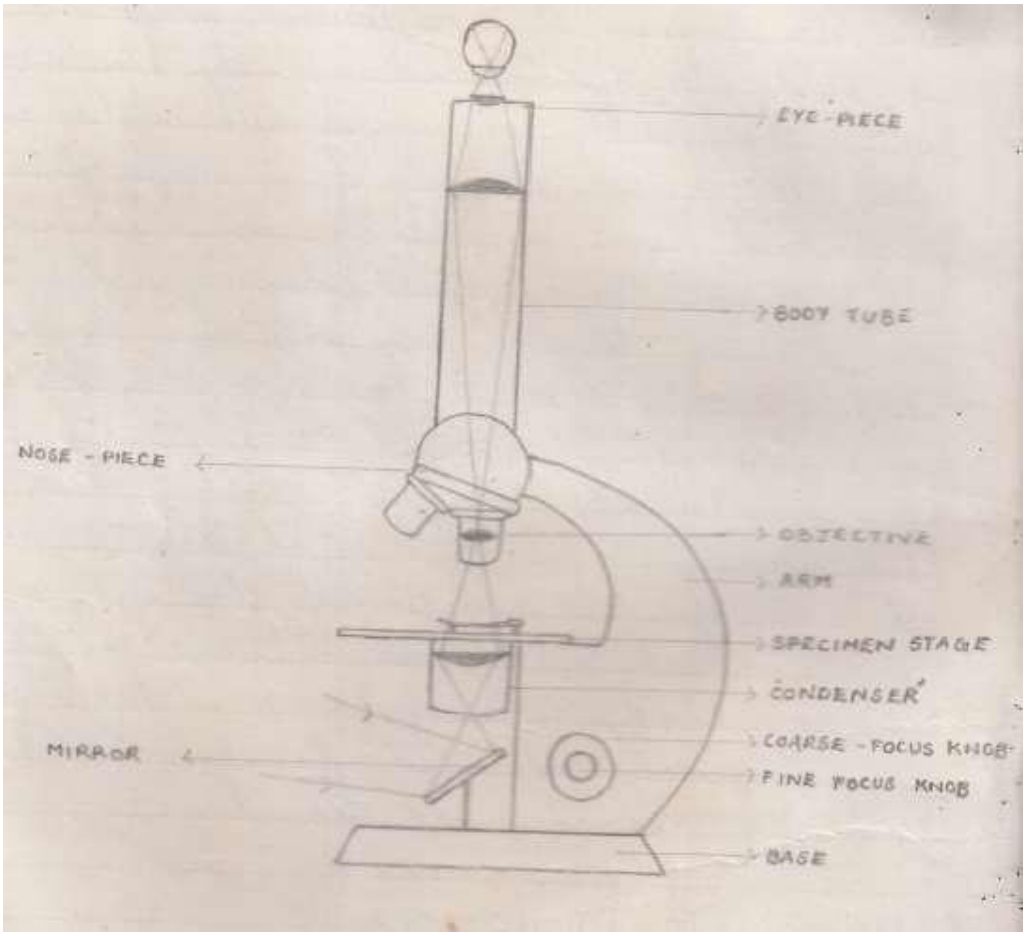


Light Microscope

PARTS OF A MICROSCOPE

A common bright field compound microscope has three parts of systems which are as follows:

I. Supporting system

II. Illuminating System

III. Magnifying System

I. SUPPORTING SYSTEM

This system comprises of the following components of the microscope:

i. **Arm**

It supports body tube. It is the part that one can grasp to carry the microscope. It gives correct height to the body tube.

ii. **Base**

A solid base that gives the microscope a firm steady state.

iii. **Body Tube**

A cylindrical tube with eyepiece at the upper end and nose piece at the lower end. The tube is moved up and down for focusing

iv. **Nose Piece**

This is the revolving mount to which objective lens of varying focal length can be screened.

v. **Stage**

It is a platform with a hole in the centre which admits lights from below. It has a pair of clips to hold the slide in position. In some microscopes, the stage can be removed up and down for focusing.

II. ILLUMINATING SYSTEM

The aim of using illuminating system in a microscope is to provide uniform and soft bright light or illumination of the entire field viewed under the microscope. The external light source can be in the form of electric lamp placed in a lamp-base with a window over it, which can be inbuilt as internal light source. If an external light source is used, the microscope will be provided with a mirror. The components of the illuminating system are as follows:

1. **Mirror**

It is situated below the stage and has a concave & flat surface. It reflects the light through the diaphragm to the condenser. Day light or electric light can be used to illuminate objects

2. **Iris diaphragm**

It regulates the light that goes through the specimen. It is controlled by a knob on the microscope that is moved back and forth.

3. **Condensor**

It is a lens that is located above the diaphragm and. beneath the specimen. Its function is the collection of light rays and focusing on to the object which is placed on the stage. Its position can be controlled by a lens knob on the microscope in which it is adjustable.

III. MAGNIFICATION SYSTEM

This system also known as optical system consists of parts of microscope that magnifies the objects. The components of magnification are as follows:

1. **Objective Lens**

These lenses are fitted to the nose-piece. Each lens has more than one plano-concave lens at iris. It is situated near and just above the specimen. It produces and magnifies the real image of the specimen. Three or more objective lens with varying power of magnification are mounted in nose-piece- 10X, 40X, 100X (oil immersion). Ideally a microscope should be parfocal—that is, the image should remain in focus when objectives are changed.

2. **Eyepiece Lens**

It is placed at the top of the body tube and remains near the eye of the observer. It is composed of two convex lenses which are arranged with their convex surfaces facing the objective. It magnifies and forms the virtual image of the real image previously formed by the objective lens. Each eyepiece lens are available in different magnification for brighter and sharper images.

3. **Coarse Adjustment**

Coarse adjustment knob moves the body tube or stage up and down depending on the design of the microscope to approximately the right position so that the specimen is perfectly in focus. This knob is used to achieve the focus and is used only with low power objective.

4. **Fine adjustment**

The fine adjustment knob moves the body tube or stage up and down respectively, the right position so that the specimen is perfectly in focus. It is used to achieve fine focus with the low power objective lens and focusing with high power (45 X) and oil immersion objective.

PRINCIPLE AND WORKING OF MICROSCOPE

The objective lens is a very high powered magnifying lens with a very short focal length. It is brought very close to the specimen being examined so that the light from the specimen comes to a focus about 160 mm inside the body tube. This creates an enlarged image of the object. This image is real & inverted which can be projected on a screen. It is this real image that is viewed by the eyepiece lens that provides further enlargement. It is this eye piece lens which enables human eye to focus on the erect and virtual image.

The basic limitation of microscope is not of magnification, but of resolution. Resolution is the ability of a lens to separate or distinguish between small objects that are close together. The resolution is inversely proportional to the limit of resolution (d) which is he minimum distance between the two objects that reveal them as separate entities. It is given by Abbe's equation,

$$d = \frac{0.5\,\lambda}{n \sin\theta}$$

where λ = wavelength of light used to illuminate the specimen

$n \sin \theta$ = numerical aperture (NA)

n= refractive index of the medium in which the lens works, as well as upon the objective

θ= half the angle of the cone of light entering an objective

The equation indicates resolution depends on the wavelength of light used and numerical aperture. The wavelength of light must be shorter than the distance between two objects or they will not be seen clearly. Thus the greatest resolution is obtained with light of the shortest wavelength, light at the blue end of the visible spectrum (in the range of 450 to 500 nm), the shortest wavelength of light.

The angle θ is defined as half the angle of the cone of light entering an objective. The light that strikes the microorganism after passing through a condenser is cone-shaped. When this cone has a narrow angle and tapers to a sharp point, it does not spread out much after leaving the slide and therefore does not adequately separate images of closely packed objects. Hence the resolution is low. If the cone of light has a very wide angle and spreads out rapidly after passing through a specimen, closely packed objects appear widely separated and are resolved.

The angle of the cone of light that can enter a lens depends on the refractive index (*n)* of the medium in which the lens works, as well as upon the objective itself. The refractive index for air is 1.00. Since $\sin\theta$ cannot be greater than 1 (the maximum is 90° and $\sin 90°$ is 1.00), no lens working in air can have a numerical aperture greater than 1.00. The only practical way to raise the numerical aperture above 1.00 is to increase the refractive index with immersion oil, a colorless liquid with the same refractive index as glass (1.5). Therefore, by using oil immersion objective lens, the resolution of microscope can be increased.

PROCEDURE FOR USING A MICROSCOPE

1. Place microscope on a stable laboratory bench. Always sit straight while working on a microscope, near the window, if external light source is used.
2. Adjust the illumination. Align the low power objective hook through the objective lens and turn the mirror in such a way that the field being viewed under microscope will be illuminated maximally.
3. Put the slide between the clips provided on the stage. Adjust the illumination to improve the contrast while moving the condenser up and down or controlling the diaphragm.
4. Put one hand on focusing knob that is coarse adjustment and other on the screen to move the stage. Use coarse adjustment for bringing the object close to the focus and get a sharp focus with a fine adjustment knob. The low power objective is commonly used to screen the field view and bring the object at the centre.
5. Switch to high power objective lens, if required, and increase the illumination, if needed. Repeat the process of focusing by fine adjustment.
6. To use oil immersion objective, turn away the high power objective, bring the condenser maximum up and place a drop of oil on the object. The objective lens must be in touch with the oil drop and hence bring down the objective lens, so that it touches the slide gently [avoid pressing hard on the slide]. Slowly bring the objective lens up while looking through the eyepiece and using the coarse adjustment knob. Stop when the object is in focus and sharpen the image using fine adjustment.

ROUTINE CARE AND MAINTENANCE OF MICROSCOPE

The points which should be carefully noted for the care and maintenance of microscope are as follows :-

1. Carry the microscope by holding the arm with one hand and with the other hand, hold the base.
2. Never swing the microscope while carrying it. Never allow direct sunlight to fall on the microscope. Put a plastic cover on it. At the end of the day, blow off dust particles from the surface and store it in a warm dry place.
3. While working with microscope, do not take out the glass slide without swinging back the oil immersion objective lens because, sliding against the glass slide many scratch the objective lens.
4. After working, clean the lens.

CLEANING OF LENS

1. Lens should be taken care to avoid dust. During cleaning, blow off the dust particles from the centre with the help of a point brush. The oil immersion objective lens requires

more frequent cleaning and make sure that there is no immersion oil left on the objective lens after use.

2. Use clean tissue paper for removing the oil by repeated gentle rubbing on the surface.
3. Do not use an organic solvent [Eg:- ether, ethanol, toluene etc…] to remove the oil on a regular basis.

Date:…….

Experiment No-4

Staining Techniques

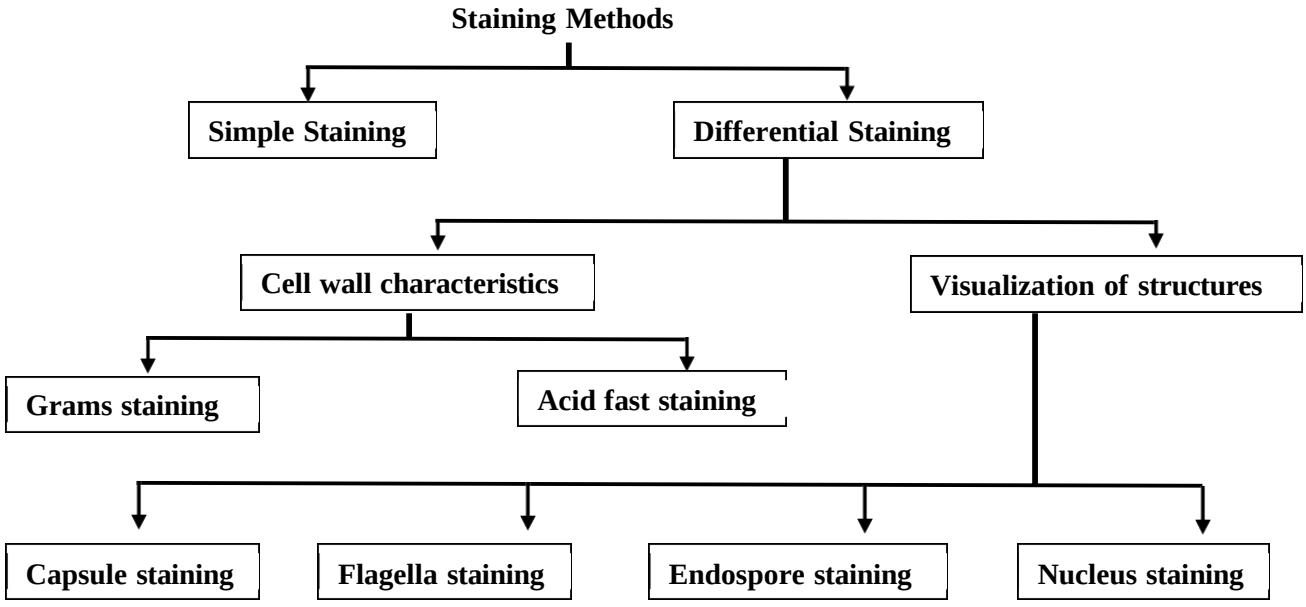
Live bacteria do not show much structural details, under the light microscope due to the lack of contrast. Hence it is customary to use staining techniques to produce colour contrast. Smear preparation is of great importance during staining. The slide used should be clearly wiped using cotton and any disinfectant, usually 70 % ethanol. Before the preparation of the smear, the end of the slide bearing the smear should be marked with number or letters to ensure the identity of smear.

Preparation of smear

In the case of liquid cultures, one loop-full of culture is taken up with the inoculating loop and spread thinly on the slide. The slide is now air dried first by leaving the slide on the working table for a few minutes. The slide is then held high over the Bunsen burner and dried further. Then film is fixed without charring, by passing it over the flame.

With a sterilized loop, single colony of the desired culture is placed over a drop of saline in the case of solid media. The smear is thoroughly mixed and the mixture is spread evenly on the slide. The resulting film is air-dried and then heat-fixed, without charring by passing it over the flame.

Work sheet	To be written/drawn on **the left side of the Practical Record**



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Experiment No-4 A

A. Simple Staining

This method of staining not only show the presence of organism but also the nature of cellular contents in exudates.

PROCEDURE OF STAINING WITH METHYLENE BLUE

A smear is prepared and it is flooded with Loeffler's methylene blue stain and is allowed to stand for 3 minutes. The stain is drained off and washed with water. Slide is air dried and observed under microscope, usually 40 or 45 X objective.

RESULT

The bacterial culture consists of blue coloured and rod shaped cells.

PROCEDURE OF STAINING WITH CRYSTAL VIOLET

A smear is prepared and the crystal violet stain is applied over the smear for 3 minutes, washed with water and observed under the microscope.

RESULT

Bacterial cells appear spherical shaped and violet in colour.

Left side of record

Composition of Loeffler's Methylene Blue Stain

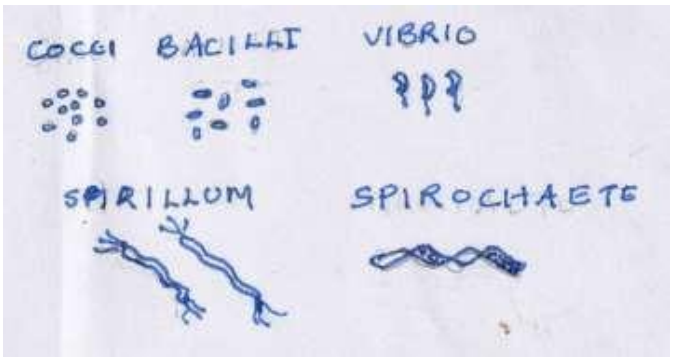
Saturated solution of Methylene Blue in Ethanol	:	300 ml
Potassium hydroxide (KOH, 0.01% in water)	:	make up to 1000 ml

Composition of Crystal Violet Stain

Crystal violet or methyl violet	:	10 g
Absolute alcohol	:	100 ml
Distilled water	:	make up to 1000 ml

Dissolve dye first in alcohol and then added to water.

Left side of record



Date:…….

Experiment No-4 B

E. NEGATIVE STAINING

AIM

To demonstrate the presence of cells in the given culture by negative staining.

PRINCIPLE

The cell wall of microorganisms such as yeast has low affinity for dyes such as India ink or nigrosin. Hence it remains unstained.

PROCEDURE

i. Place a large drop of undiluted India ink or nigrosine stain on the glass slide and emulsify a small amount of the yeast culture besides the ink.
ii. Mix the stain solution and culture on the slide surface.
iii. Place a clean coverslip on the ink drop and press it through the sheet of blotting paper.
iv. The film should be so thin that the yeast cells is gripped between the slide and coverslip, neither overlaid by ink nor cells moving about.
v. Examine under 45 X objective lens of microscope.

RESULT

The cells are seen as clear bright halos against black background.

Left side of record

Composition of Nigrosin Stain

Nigrosin	:	10 g
Distilled water	:	make up to 100 ml

Mix the nigrosine in water taken in a conical flask. The mouth of conical flask is tightly covered with an aluminum foil. Dissolve the stain by heating it in a boiling water bath about 30 min. Filter through a Whatman No.1 filter paper. Add 0.5 ml of formalin solution to the filtered solution.

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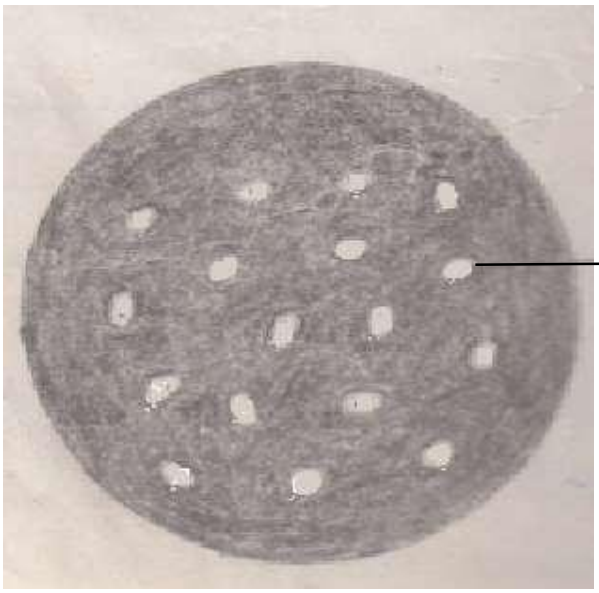

Circular clear bright cells

Date:…….

Experiment No-5

Demonstration of Microbial Production of Yogurt

In India, milk is being used for producing milk products using lactic acid producing bacteria. Yogurt is popular in Eastern European countries. The usual method of producing yogurt on large scale production is through addition of starter culture bacteria to homogenized and pasteurized milk. Many types of yogurts are available in the market for example, plain yogurt, fruit yogurt, flavoured and coloured yogurt, blended yogurt, sweetened yogurt, heated yogurt, frozen yogurt, dried yogurt, low-lactose yogurt, and carbonated yogurt.

AIM

To prepare yogurt from pasteurized milk.

PRINCIPLE

Yogurt is a fermented milk produced from homogenized and pasteurized milk through controlled fermentation with starter cultures such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus.* Plain yogurt has a semisolid mass due to coagulation of milk (skim, low~ or full fat) by starter-culture bacteria. It has a sharp acid taste with a flavour similar to walnuts and a smooth mouth feel. The flavour is due to the combined effects of acetaldehyde, lactate, diacetyl. and acetate, but 90% of the flavour is due to acetaldehyde. Most common method of yogurt production is tracing inoculum from previous batch to prepare the yogurt by fermenting the milk.

MATERIALS REQUIRED

1. Pasteurized milk
2. Inoculum- Yogurt from market
3. Sterile cotton plugged culture-tubes (150 × 16 mm)
4. Sterile cotton plugged 250 ml Erlenmeyer conical flask.
5. Sterile pipettes- 10 ml
6. Spatula

PROCEDURE

1. Pour pasteurized milk into sterile cotton plugged 250 ml conical flask.
2. Pipette out 10 ml of pasteurized using sterile pipette into a sterile cotton plugged culture-tube.

3. Inoculate it with a limited amount of yogurt purchased from market, using flame sterilized spatula.
4. Incubate the inoculated milk at 43 ℃ for about 8-12 hours.
5. After the fermentation process, examine the changes in the fermented milk such as colour aroma and texture

RESULT

Yogurt is successfully prepared which is identical to the yogurt from the market in colour, aroma, texture and taste.

Date:…….

Experiment No-6

Preparation of Agarose Gel for Electrophoresis

AIM

To prepare agarose gel for electrophoresis.

PRINCIPLE

Agarose gel electrophoresis is useful in identification of separation of DNA molecules between the size 100 bp – 25 kbp (25000 bp) and subsequent purification of DNA molecules of interest in heterozygous mixture.

Agarose is a polymer of β-galactose and 3,6-anhydro1-galactose. It forms a gel hydrogen bonding and the pore size of the gel depends on agarose concentration. The movement of DNA fragments within the gel matrix is influenced by gel concentration. The DNA molecules are separated by electrophoresis on the basis of their molecular size. DNA molecules have negative charge and migrate towards the anode. DNA is made visible using a dye called ethidium bromide. The ethidium bromide dye interacts between the bases of DNA molecules and produces a fluorescent bright orange colour when illuminated with ultra-violet light.

MATERIALS REQUIRED

1. Horizontal agarose gel electrophoresis apparatus- gel casting template or platform, gel comb, DC power supply
2. Microwave oven
3. Conical flask
4. UV transilluminator
5. Micropipette
6. Test sample for isolating DNA

REAGENTS REQUIRED

1. Tris-Acetate-EDTA (TAE) Buffer 50x Stock solution

Tris base	:	244 g
Glacial acetic acid	:	57.1 ml
0.5 M EDTA solution	:	100 ml
Distilled water	:	make up to 1000 ml

Dissolve 242 g of Tris base and 57.1 ml of glacial acetic acid into 400 ml of water. Then, add 100 ml of 500 mM EDTA solution and bring the final volume up to 1 liter with water. Dilute the 50x stock solution by 49:1 with water to achieve the 1x working concentration for running gels.

TAE buffer for DNA separation in agarose gel electrophoresis is of 1x composition of 40 mM Tris-acetate and 1 mM EDTA, with a pH around **8.0-8.3**

2. Ethidium bromide solution (10 mg/ml)

Ethidium bromide	:	0.25 g
Distilled water	:	20 ml

Dissolve 0.25 g of ethidium bromide in 20 ml of distilled water, mix well and store at 4 ℃

3. Loading buffer /Sample buffer solution

Sucrose	:	4 g
Bromophenol blue (0.025 %)	:	0.0025 g (2.5 mg)
TAE buffer	:	10 ml

PROCEDURE

1. Weigh out 1 g of agarose and transfer into a conical flask. Add 100 ml of 1x TAE buffer.
2. Melt agarose completely with a clear solution using a microwave oven or heating mantle. Cool the molten agarose. Avoid overheating as boiling agarose will bubble and may overflow its container.
3. Place the gel casting template horizontally on a table and put appropriate comb. The teeth of the comb should not touch the bottom of the plate leaving at least 1 mm distance between the comb and gel plate.
4. Agarose gel template must be previously cleaned with 70 % ethanol and sealed it with adhesive tapes.
5. Pour the molten agarose solution in the gel casting platform carefully without formation of air bubbles. It is important that there are no air bubbles in the gel as these will affect the migration of the DNA fragments during electrophoresis
6. Allow the gel to set by keeping it undisturbed for 30-45 min. Remove the comb and adhesive tapes from gel casting template carefully. Take care not to damage the gel, when the gel-comb is removed.
7. Mount the template carrying the gel in an appropriate electrophoretic apparatus and fill the tank with 1x TAE buffer just enough to immerse the gel.

Caution

Ethidium bromide is a powerful carcinogen. Wear gloves while handling gels. For disposal of materials containing ethidium bromide, adopt safe procedures. UV light can burn skin and eyes. Minimize exposure to UV light.

RESULT

Agarose gel is prepared in gel casting template/ tray for electrophoresis.

SEM 2 FUNDAMENTALS OF MICROBIOLOGY

INDEX

Exp No	Name of Experiment	Date	
1	Microscopy		
2	Cleaning of glassware and sterilization		
3	Culture media		
4	Culture methods		
5.	STAINING TECHNIQUES		
	i. Simple Staining		
	ii. Gram Staining		
	iii. Volutin granule Staining		
	iv. Endospore Staining		
	v. Negative Staining		
6	Examination of bacteria for motility		
7	Morphology of microbial colonies		
8	Isolation of pure culture by spread plate method		
9	Isolation of pure culture by pour plate method		
10	Determination of growth curve of bacteria		

Date:…….

Experiment No-1

Microscopy

The cells of most animals, plants and bacteria are too small to be seen by naked human eye. The purpose of a microscope is to see what is invisible to the naked eye. By staining the cells, their different components could be made well contrasted. The aim of magnification of cells and their components was achieved by the lenses. The lenses could magnify the minute objects only up to a limited extend. So many lenses were combined together to form an instrument known as microscope.

Magnification and resolving power of the microscope are two important factors that determine the quality of the microscope. Magnification is the increase in size of an optical image, over the size of an object being viewed. Resolving power is the ability to reveal closely adjacent structural details being actually separate and distinct.

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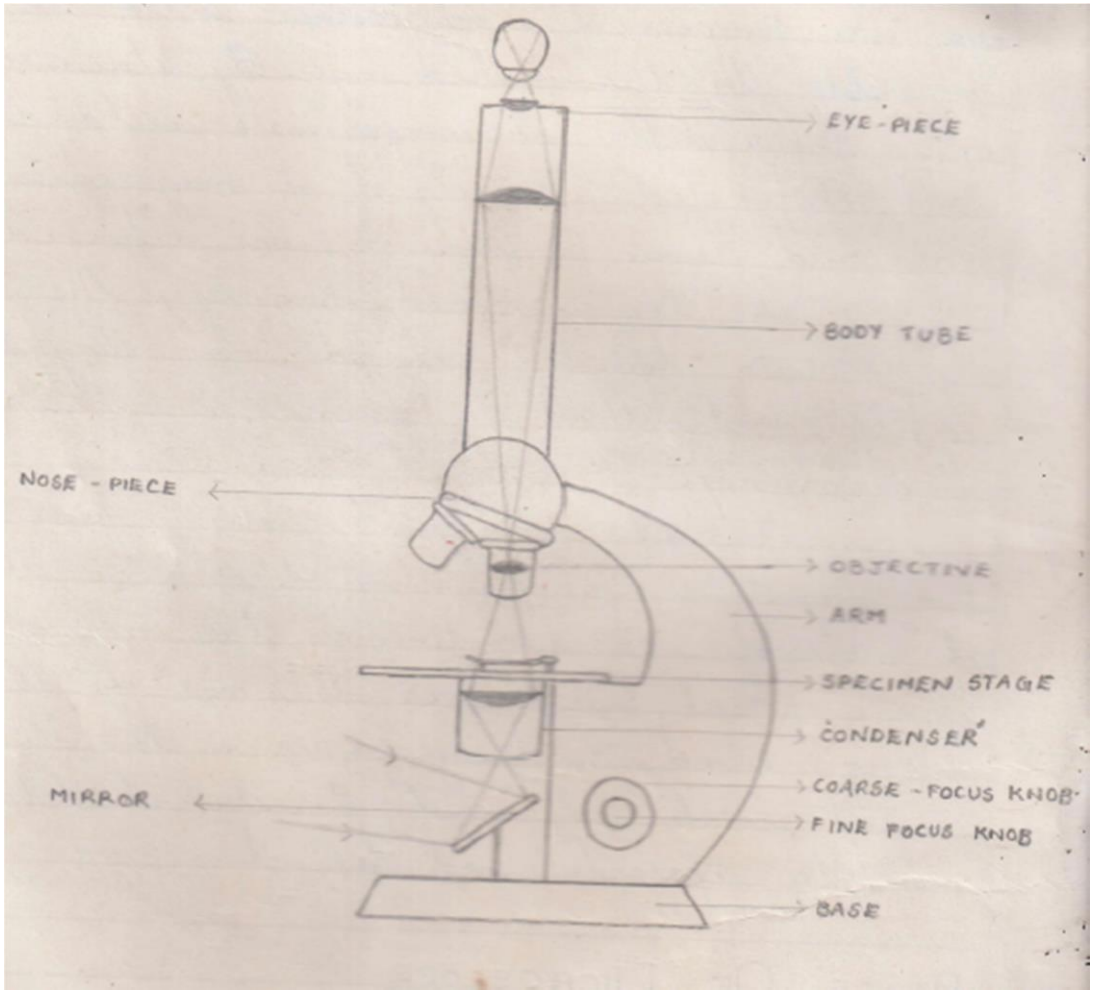

Light Microscope

PARTS OF A MICROSCOPE

A common bright field compound microscope has three parts of systems which are as follows:

I. Supporting system

II. Illuminating System

III. Magnifying System

I. *SUPPORTING SYSTEM*

This system comprises of the following components of the microscope:

i. Arm

It supports body tube. It is the part that one can grasp to carry the microscope. It gives correct height to the body tube.

ii. Base

A solid base that gives the microscope a firm steady state.

iii. Body Tube

A cylindrical tube with eyepiece at the upper end and nose piece at the lower end. The tube is moved up and down for focusing

iv. Nose Piece

This is the revolving mount to which objective lens of varying focal length can be screened.

v. Stage

It is a platform with a hole in the centre which admits lights from below. It has a pair of clips to hold the slide in position. In some microscopes, the stage can be removed up and down for focusing.

II. *ILLUMINATING SYSTEM*

The aim of using illuminating system in a microscope is to provide uniform and soft bright light or illumination of the entire field viewed under the microscope. The external light source can be in the form of electric lamp placed in a lamp-base with a window over it, which can be inbuilt as internal light source. If an external light source is used, the

microscope will be provided with a mirror. The components of the illuminating system are as follows:

1. Mirror

It is situated below the stage and has a concave & flat surface. It reflects the light through the diaphragm to the condenser. Day light or electric light can be used to illuminate objects

2. Iris diaphram

It regulates the light that goes through the specimen. It is controlled by a knob on the microscope that is moved back and forth.

3. Condensor

It is a lens that is located above the diaphragm and. beneath the specimen. Its function is the collection of light rays and focusing on to the object which is placed on the stage. Its position can be controlled by a lens knob on the microscope in which it is adjustable.

III. *MAGNIFICATION SYSTEM*

This system also known as optical system consists of parts of microscope that magnifies the objects. The components of magnification are as follows:

1. Objective Lens

These lenses are fitted to the nose-piece. Each lens has more than one plano-concave lens at iris. It is situated near and just above the specimen. It produces and magnifies the real image of the specimen. Three or more objective lens with varying power of magnification are mounted in nose-piece- 10X, 40X, 100X (oil immersion). Ideally a microscope should be parfocal—that is, the image should remain in focus when objectives are changed.

2. Eyepiece Lens

It is placed at the top of the body tube and remains near the eye of the observer. It is composed of two convex lenses which are arranged with their convex surfaces facing the objective. It magnifies and forms the virtual image of the real image previously formed by the objective lens. Each eyepiece lens are available in different magnification for brighter and sharper images.

3. Coarse Adjustment

Coarse adjustment knob moves the body tube or stage up and down depending on the design of the microscope to approximately the right position so that the specimen is perfectly in focus. This knob is used to achieve the focus and is used only with low power objective.

4. Fine adjustment

The fine adjustment knob moves the body tube or stage up and down respectively, the right position so that the specimen is perfectly in focus. It is used to achieve fine focus with the low power objective lens and focusing with high power (45 X) and oil immersion objective.

PRINCIPLE AND WORKING OF MICROSCOPE

The objective lens is a very high powered magnifying lens with a very short focal length. It is brought very close to the specimen being examined so that the light from the specimen comes to a focus about 160 mm inside the body tube. This creates an enlarged image of the object. This image is real & inverted which can be projected on a screen. It is this real image that is viewed by the eyepiece lens that provides further enlargement. It is this eye piece lens which enables human eye to focus on the erect and virtual image.

The basic limitation of microscope is not of magnification, but of resolution. **Resolution is the ability of a lens** to separate or distinguish between small objects that are close together. The resolution is inversely proportional to the limit of resolution (d) which is he minimum distance between the two objects that reveal them as separate entities. It is given by Abbe's equation,

$$d = \frac{0.5\,\lambda}{n\,sin\theta}$$

where λ = wavelength of light used to illuminate the specimen n sin

θ = numerical aperture (NA)

n= refractive index of the medium in which the lens works, as well as upon the objective θ= half

the angle of the cone of light entering an objective

The equation indicates resolution depends on the wavelength of light used and numerical aperture. The wavelength of light must be shorter than the distance between two objects or they will not be seen clearly. Thus the greatest resolution is obtained with light of the shortest wavelength, light at the blue end of the visible spectrum (in the range of 450 to 500 nm), the shortest wavelength of light.

The angle θ is defined as half the angle of the cone of light entering an objective. The light that strikes the microorganism after passing through a condenser is cone-shaped. When this cone has a narrow angle and tapers to a sharp point, it does not spread out much after leaving the slide and therefore does not adequately separate images of closely packed objects. Hence the resolution is low. If the cone of light has a very wide angle and spreads out rapidly after passing through a specimen, closely packed objects appear widely separated and are resolved.

The angle of the cone of light that can enter a lens depends on the refractive index (*n)* of the medium in which the lens works, as well as upon the objective itself. The refractive index for air is 1.00. Since $\sin\theta$ cannot be greater than 1 (the maximum is 90° and $\sin 90°$ is 1.00), no lens working in air can have a numerical aperture greater than 1.00. The only practical way to raise the numerical aperture above 1.00 is to increase the refractive index with immersion oil, a colorless liquid with the same refractive index as glass (1.5). Therefore, by using oil immersion objective lens, the resolution of microscope can be increased.

PROCEDURE FOR USING A MICROSCOPE

1. Place microscope on a stable laboratory bench. Always sit straight while working on a microscope, near the window, if external light source is used.
2. Adjust the illumination. Align the low power objective hook through the objective lens and turn the mirror in such a way that the field being viewed under microscope will be illuminated maximally.
3. Put the slide between the clips provided on the stage. Adjust the illumination to improve the contrast while moving the condenser up and down or controlling the diaphragm.
4. Put one hand on focusing knob that is coarse adjustment and other on the screen to move the stage. Use coarse adjustment for bringing the object close to the focus and get a sharp focus with a fine adjustment knob. The low power objective is commonly used to screen the field view and bring the object at the centre.
5. Switch to high power objective lens, if required, and increase the illumination, if needed. Repeat the process of focusing by fine adjustment.
6. To use oil immersion objective, turn away the high power objective, bring the condenser maximum up and place a drop of oil on the object. The objective lens must be in touch with the oil drop and hence bring down the objective lens, so that it touches the slide gently [avoid pressing hard on the slide]. Slowly bring the objective lens up while looking through the eyepiece and using the coarse adjustment knob. Stop when the object is in focus and sharpen the image using fine adjustment.

ROUTINE CARE AND MAINTENANCE OF MICROSCOPE

The points which should be carefully noted for the care and maintenance of microscope are as follows :-

1. Carry the microscope by holding the arm with one hand and with the other hand, hold the base.
2. Never swing the microscope while carrying it. Never allow direct sunlight to fall on the microscope. Put a plastic cover on it. At the end of the day, blow off dust particles from the surface and store it in a warm dry place.
3. While working with microscope, do not take out the glass slide without swinging back the oil immersion objective lens because, sliding against the glass slide many scratch the objective lens.
4. After working, clean the lens.

CLEANING OF LENS

1. Lens should be taken care to avoid dust. During cleaning, blow off the dust particles from the centre with the help of a point brush. The oil immersion objective lens requires more frequent cleaning and make sure that there is no immersion oil left on the objective lens after use.
2. Use clean tissue paper for removing the oil by repeated gentle rubbing on the surface.
3. Do not use an organic solvent [Eg:- ether, ethanol, toluene etc…] to remove the oil on a regular basis.

Date:…….

Experiment No-2

Cleaning of Glassware and Sterilization

A. Cleaning of Glassware

Cleaning, though a routine non-technical procedure, play an important preparing role before sterilization or disinfection by removing soil and other dust, thereby reducing the microbial burden and making sterilization more effective.

GENERAL CLEANING METHOD

Clean glassware is hydrophilic and will have a uniform wetted surface when distilled water is used a final rinse. Contaminants such as detergent residues, grease etc. will cause the water to bead and the cleaning process should be repeated. Wash glassware as quickly as possible after use. If cleaning is not immediately possible, place the glassware in water to soak. If the glassware is not cleaned immediately it may become impossible to remove the residue.

A non-alkaline detergent should be used for cleaning. The concentration of the detergent should be between 5 and 20 % depending on the residue. The water should be hot (80 °C). Do not use abrasive or steel wool in the cleaning process. They can scratch the surface of the glassware during washing. All parts of the glassware should be thoroughly scrubbed with a plastic or wooden handed brush which are recommended. Do not use brushes with metallic handle, as the metal arm will scratch the glassware.

Scratched glassware is more prone to breakage during experiments. If the glassware is clouded or contain coagulated organic material, it should be cleaned with chromic acid solution. Chromic acid solution is strongly acidic and will burn the skin. It is also a carcinogen. Extra care must be taken to make sure that the chromic acid solution is disposed of properly.

The glassware may be rinsed with chromic acid solution or it may be filled and allowed to stand. The length of the time for which the glassware is allowed to stand depends on the amount of contaminants. Relatively clean glassware may require just a few minutes to clean. If organic material is present, it may be necessary to let the glass-ware stand for whole night.

Removal of Grease

Grease is best removed by boiling glassware in a weak solution of sodium carbonate. Acetone or another fat solvent may also be used. Strong alkali should also be used. Silicone grease can be removed by soaking in the decahydronaphthalene for two hours, the king of all cleaning agents. Drain and rinse with acetone or use fuming sulfuric acid for 30 minutes. Be sure to rinse off all of the cleaning agents.

Rinsing and Drying

After cleaning, rinse the glassware with running tap water. Then the glassware is rinsed with distilled water after the removal of residues of cleaning agents, followed by the final rinse

with deionised water. Peg-board drying is not recommended as air-borne contaminants in the laboratory will be deposited in the clean glassware. Oven drying is recommended at temperature ranging from 110-140 °C. Open end glassware such as beakers, Erlenmeyer flask etc. should be covered with aluminium foil and stored in a dust free cabinet.

B. Sterilization

Sterilization is the process by which an article, surface or medium is freed of all living microorganisms either in vegetative or spore state. The method of sterilization employed depends upon the purpose of which sterilization is carried out, the material which has to be sterilized and the nature of micro-organisms that are to removed or destroyed. Microorganisms are ubiquitous in nature and hence cause contamination, infection and decay. It becomes necessary to remove or destroy them from materials or from areas. This is the objective of sterilization. Major agents used for controlling microorganisms are given below:

I. Physical Agents

1 Dry Heat : Flaming, Hot air oven

2 Moist Heat : Pasteurization, Boiling, Steam at atmospheric temperature, Steam under pressure

3 Filtration: : Candle filters, Sintered glass filters, Membrane filters

4 Radiations : Non-ionizing (UV), & Ionizing (Gamma rays)

II. Chemical Agents

1 Alcohols : Ethanol, Isopropanol

2 Aldehydes : Formaldehyde, Glutaraldehyde

3 Dyes

4 Halogens

5 Phenols

6 Surface active agents

7 Gases : Ethylene oxide, Formaldehyde, Betapropiolactone

I. Physical Agents

HEAT

Heat is the most reliable form of sterilization. The factors influencing sterilization by heat include:

a. Nature of heat
b. Temperature and time
c. Number of microorganism present
d. Characteristics of microorganism
e. Type of material from which the organisms are to be eradicated.

1. *DRY HEAT*

The killing effect of dry heat is due to the protein denaturation, oxidative damage and toxic effect of elevated levels of ions. In a completely moisture free atmosphere, bacteria are more resistant to heat. They are killed when oxidation of the cell constituents occurs and this requires much higher temperature than that needed for coagulation of proteins. The time required for sterilization is inversely proportional to the temperature of exposure. The sterilization time is related to the number of organisms in the suspension, presence or absence of spores and the strains and characteristics of microorganisms. The processes using dry heat for sterilization are as follows:

i. Flaming

Inoculation loop or wire, the tip of forceps, scalpels and spatulas are held in a flame of Bunsen burner till they become hot. Inoculation loop or wires are usually made red hot before they can be used for inoculation. Inoculation loops carrying infective material may be dipped in a disinfectant before flaming, to prevent spattering.

ii. Hot air oven

This is the most widely used method of sterilization by dry heat. The apparatus used for this type of sterilization may be a special electric or gas oven. The air in the oven is heated with electricity by heating elements in the wall of chamber. As hot air is a bad conductor of heat, it is usually fitted with a fan to ensure even distribution of air and elimination of air-pockets. The material should be arranged so as to allow free circulation of air in between objects. It should not be overloaded. Glass-wares should be perfectly dry before being placed in the oven. Test-tubes, Periplates, flasks, glass pipettes should be wrapped in a paper and kept for sterilization. A holding period of 160 °C for 2 hours or 171
°C for 1 hour is used to sterilize glassware, forceps etc. The oven must be allowed to cool slowly for 2 hours before the door is opened. This is done to prevent cracking of glass-wares due to sudden or uneven cooling.

Sterilization control: The spores of non-toxigenic strain of *Clostridium tetani* are used as microbiological test for dry heat efficiency. Paper strips impregnated with 10^6 spores are placed in envelopes and inserted into suitable packs. After sterilization, the strips are

removed and inoculated into thioglycollate media or cooked meat media and incubated for sterility test under strict anaerobic condition for 5 days at 37 °C.

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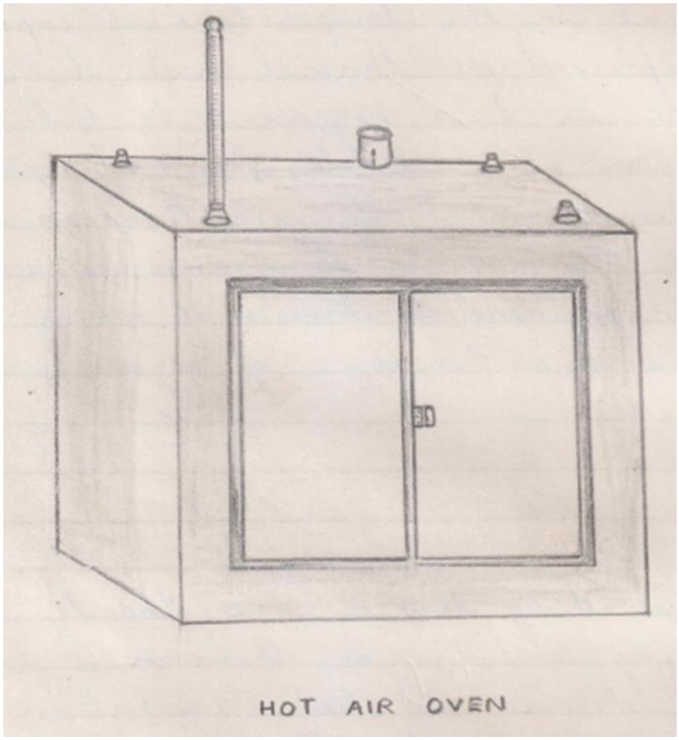

2. *MOIST HEAT*

The lethal effect of moist heat is due to the protein denaturation and coagulation. Moist heat sterilization requires attainment of temperature above boiling water. Such conditions are attained under controlled conditions by raising the pressure which can be done in an autoclave. The important qualities of steam for sterilization is that it is non-toxic, non-corrosive and highly penetrative sterilizing agent. The processes using moist heat for sterilization are as follows:

i. Temperature below 100 °C

Pasteurization is the process, in which the temperature employed for milk is either 63 °C for 30 minutes (Holder's method) or 72 °C for 15 to 20 seconds (Flash method) followed by immediate cooling to 4 °C. By these process all non-sporing pathogens in milk are destroyed.

ii. Temperature at 100 °C: Boiling

Vegetative cells of bacteria are killed by heating at 90-100°C. Sterilization may be promoted by the addition of 2 % sodium carbonate to the water. In cases where boiling is considered to be adequate, the material should be immersed in water and boiled for a period of 10-30 minutes. Glassware, forceps, scissors, scalpels, glass-syringes may be sterilized by this method.

iii. Steam at atmospheric pressure (100 °C)

An atmosphere of free steam is used to sterilize culture media which may be decomposed if subjected to high temperatures. Special devices such as Koch's steam sterilizer or Arnold steam sterilizer can be used for sterilization. The steamer consists of a tinned copper cabinet with walls suitably lagged. The lid is conical enabling drainage of condensed steam and a perforated tray fitted above water level ensures the material to be sterilized is surrounded by steam. One exposure of 90 minutes usually ensures complete sterilization.

For media containing sugars and gelatin, an exposure of 100°C for 20 minutes on three successive days is used. This is known as Fractional Sterilization or Tyndallization. The principle is that the first exposure kills all vegetative bacteria and spores present will germinate and be killed on subsequent exposures.

iv. Steam under pressure (100 °C)

The principle of autoclave is that water boils when its vapour pressure equals that of the surrounding atmosphere. Hence When pressure inside a closed vessel increases, the temperature at which water boils also increases. Saturated steam has penetrative power. When steam comes into contact with a cooler surface, it condenses to water and gives its latent heat of vapourisation to that surface (1600 ml steam at 100 °C & at 1 atmos pressure condenses into 1 ml of water). The large reduction in volume sucks in more steam to the area and the process continues till the temperature of that surface is raised to that of the steam. The condensed water ensures moist conditions for killing the microbes present. A holding period of 121 °C for 15 minutes at 15 lbs pressure is required for effective sterilization in an autoclave. Materials such as laboratory wares, culture media, dressings, etc. can be sterilized by this method. Several types of steam sterilizers in use are laboratory autoclaves, hospital dressing sterilizer, bowl and instrument sterilizer and rapid cooling sterilizers.

Sterilization control: For determining the efficiency of moist heat sterilization, endospores of *Bacillus stearothermophilus* are used as microbiological test organism. This a thermophilic organism with an optimum growth temperature of 55 - 60 °C and it' spores required an exposure of 12 minutes at 121 °C to be killed. Paper strips impregnated with 10^6 spores are

placed in envelopes and dried at room temperature. These paper strips are inserted in different parts of the load. After sterilization, the strips are removed and inoculated into a suitable recovering medium and
incubated for sterility test at 55 °C for 5 days.

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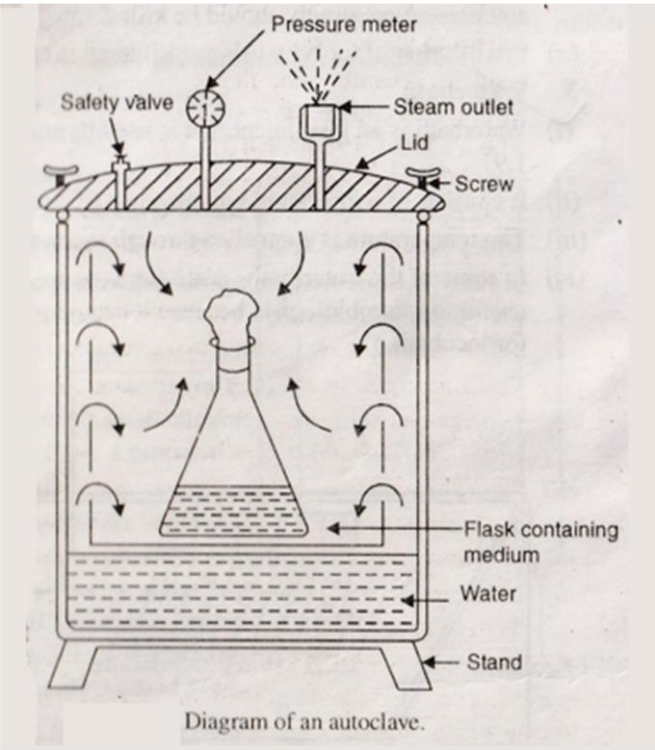

Diagram of an autoclave.

3. *FILTRATION*

Filtration helps to remove bacteria from heat labile liquids such as sera and solutions of antibiotics or sugar used for preparation of culture media. Filtration can be used to obtain bacteria-free filtrates of viruses and bacteria toxins. The filters help to retain the organism and the filter disc can be cultured. Various types of filters in use are as follows:

i. Candle filters

These are manufactured in different grades of porosity and have been widely used for purification of water, for drinking and industrial purpose. These are of two types:

a. Unglazed ceramic filters
b. Diatomaceous earth filters

a. Unglazed ceramic filters: They are made up of unglazed porcelain. These, after use can be cleaned with sodium hypochlorite solution. They can withstand scrubbing. Eg: Chamberland filters.

b. ***<u>Diatomaceous eath filters</u>****:* They are made up of diatomaceous earth. These can be cleaned with sodium hypochlorite solution, after use. But they cannot withstand scrubbing. Eg: Berkefeld and Mandler filters.

ii. <u>Sintered glass filters</u>

They are prepared by heat fusing finely powdered glass particles of graded size. They are available in the forms of discs and in different pore sizes. They have low absorptive property and can be cleaned easily but are brittle and expensive.

iii. <u>Membrane filters</u>

These circular filters are porous membranes, a little over 0.1 mm thick, made of cellulose acetate, nylon or other polymers. They have replaced other types of filters. These are routinely used in water purification and analysis, sterilization and sterility testing and for the preparation of solution for parenteral use.

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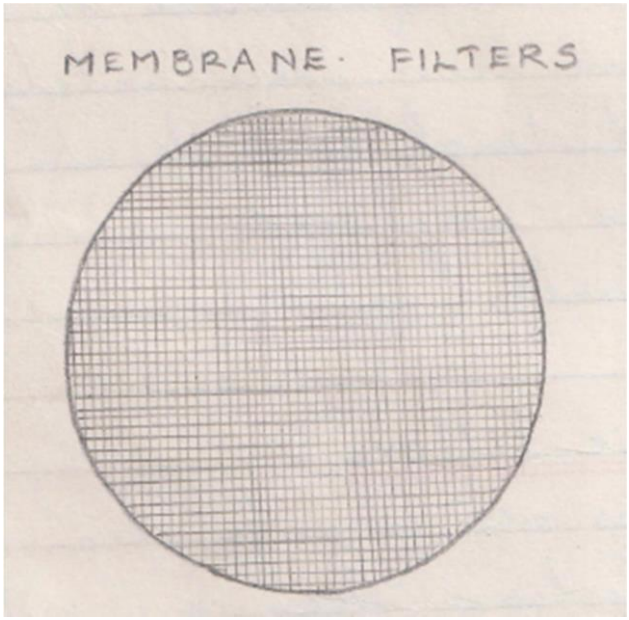

iv. **<u>High Efficiency Particulate Air (HEPA) filters</u>**

The development of high efficiency particulate air filters (HEPA) has made it possible to deliver clean air to an enclosure such as a room or chamber. These filters remove 99.97 % organisms and have pore size of 0.3 μm in diameter. This type of air filtration together with a system of laminar air flow is now used extensively to produce dust and bacteria free air in biosafety cabinets, burn units in hospitals etc.

4. *RADIATION*

Two types of radiation used for sterilization are as follows: Non-ionizing and Ionizing radiation.

i. Non-ionizing Radiation

In this type of radiation, electromagnetic rays with wavelength: (100-400 nm) known as ultraviolet (UV) rays and rays with wavelength longer than that of visible light are used for sterilization. UV rays and infrared rays are of non-ionizing low energy type. The most lethal UV radiation has a wavelength of 260 nm, the wavelength most effectively absorbed by DNA. The primary mechanism of UV damage is the formation of thymine dimers in DNA. Two adjacent thymines in a DNA strand are covalently joined to form a thymine dimer which inhibits DNA replication and function. Mercuric arc vapor lamp is used as the source for UV radiation. But UV radiation around 260 nm does not penetrate glass, dirt films, water, and other substances very effectively and hence its usage is limited. UV radiation is used for disinfection of enclosed areas such as entryways, operation theatres, biological safety cabinets and laboratories. Infra-red rays can be considered as a form of hot air sterilization as they are absorbed as heat to a large extent. It is used for rapid mass sterilization of prepacked items such as syringes and catheters.

ii. Ionizing Radiation

These are radiation of very short wavelength or high energy, which can cause atoms to lose electrons or ionize. Low levels of ionizing radiation will produce mutations and may indirectly result in death, whereas higher levels are directly lethal. It is an excellent sterilizing agent and penetrates deep into objects. Gamma rays and X-rays are of ionizing type. As there is no appreciable increase in temperature in this method, it is referred to as cold sterilization It will destroy bacterial endospores and vegetative cells, both procaryotic and eukaryotic However, ionizing radiation is not always as effective against viruses. These can be used to sterilize antibiotics, hormones, sutures, and plastic disposable supplies such as syringes etc.

II. Chemical Agents

A number of chemical agents are used as antiseptic and disinfectants. The major antimicrobial chemical agents used are as follows:

1. *ALCOHOL*

Ethyl alcohol (ethanol) and isopropyl alcohol are the most frequently used alcohols. They are used mainly as skin antiseptics and act by denaturing bacterial proteins. Isopropyl

alcohol is used for disinfection of clinical thermometers. Methanol is effective against fungal spores.

2. *ALDEHYDES*

In aqueous solutions, formaldehyde is bactericidal and sporicidal. It has lethal effect on viruses. It is used to preserve anatomical specimens and to sterilize clean metal instruments. Formaldehyde gas is used for sterilizing instruments and heat sensitive catheters. Glutaraldehyde can be used to treat corrugated rubber, anesthetic tubes, face masks, plastic endotracheal tubes, metal instruments and polythene tubing.

3. *DYES*

Two groups of dyes-aniline dyes and acridine dyes are bacteriostatic in high dilution but are of low bactericidal activity. The aniline dyes in use are malachite green and crystal violet. They are more active against gram positive organism than gram negative organisms. Their lethal effect in bacteria is due to their reaction with the acidic groups in the cell. These dyes in low concentrations are included in certain culture media to inhibit the growth of Gram-positive bacteria. The acridine dyes are also active against gram positive bacteria. The most important acridine dyes are proflavin, acriflavin etc. They impair the DNA of the organisms and thus kill or destroy the reproductive capacity of the cell.

4. *HALOGENS*

Iodine is an active bactericidal agent with moderate action against spores. Iodophores (complex of iodine with organic carrier) are used as antiseptics. Chlorine and its compounds have been widely used as disinfectants. Chlorine and salts of hypochlorites are markedly bactericidal. They yield hypochlorous acid (HClO) and atomic oxygen when dissolved in water. This leads to oxidation of cellular materials & destruction of vegetative bacterial cells, fungi. They have a wide spectrum of action against viruses too.

5. *PHENOLS*

A wide range of phenolic compounds have been developed as disinfectants. The lethal effect of phenols is due to their ability to disrupt cell membranes releasing cell contents and cell lysis. Low concentration of phenol precipitate proteins. Membrane bound oxidases and dehydrogenases are inactivated by concentration of phenol that are rapidly bactericidal. But its use is restricted due to corrosive and irritating nature.

6. *SURFACE ACTIVE AGENTS*

Substances that reduce surface tension or interfacial tension at interfaces are referred to as surface active agents. They are widely used as wetting agents, detergents and emulsifiers. They are classified into three main groups:- anionic, cationic, and ampholytic. The most important antibacterial agents are cationic surface active agents. These act on the phosphate group of cell membrane and also enter the cell. The membrane loses its selective permeability and the cell proteins are denatured. The cationic compounds are markedly active against gram positive bacteria and to a large extent on gram negative bacteria. The anionic compounds, for example common soap have moderate action. The ampholytic compounds known as "Tego" compounds are active against a wide range of gram positive and gram negative organisms.

7. *GASES*

Ethylene oxide is a highly penetrating non-corrosive and microbicidal gas, specially used for sterilizing heart-lung machine, respirators, dental equipment, books, clothing etc. Its action is due to the alkylation of amino, carbonyl, hydroxyl and sulfhydryl groups in proteins and other organic molecules. It also reacts with DNA and RNA. Formaldehyde gas is widely used for fumigation of operation theatres and laboratories. Betapropiolactone has a rapid biocidal action but it has carcinogenic activity. It is capable of killing all microorganism and is very active against viruses.

Disposal of Laboratory Wastes and Cultures

Any material which contains microorganisms should be decontaminated first and thereafter should be disposed of properly. The treatment is necessary due to the following reasons:

i. If the material contains pathogenic micro-organisms, this may result in transmission of diseases to healthy persons.

ii. It may contaminate the soil and cause soil, water and air pollution. Hence to check from such hazards, proper treatment is required to kill micro-organisms.

The infected material is usually a solid or liquid culture media used for cultivation of microorganism or it may be pure or mixed culture contained in reusable glassware or disposable plastic-ware. It may also contain cotton plugs, paper, cotton or cotton cloth, swabs, gloves, pins, needles, etc. These materials should be autoclaved first and then it is allowed to incinerate or burnt. But the material containing microbial contaminants should be treated with appropriate disinfectant and thereafter autoclaved by putting them in a suitable container. The cotton material should be discarded.

Sometimes hydrochloric acid is also used to hydrolyse agar, if present in the medium. This is added before autoclaving. The resistant forms such as bacteria endospores should be autoclaved for a prolonged period (30-40 minutes) to kill them before their surface disposal. All such laboratory materials should be disposed of after autoclaving.

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Experiment No-3

Culture Media

Culture media are of three types:

I. Solid media
II. Liquid media
III. Semisolid media

I. Solid Media

In order to obtain isolated colonies of bacteria, it is necessary to use solid media. Solidifying agents such as agar is added at a concentration of 2.0 to 2.5 % depending on the requirement. Organisms growing on solid media exhibit characteristic colony morphology and specific features like pigmentation, haemolysis etc. thereby enabling the identification of the organism more easily. For preparing stock cultures, solid media is widely used.

Preparation of Agar Plates

1. Prepare the required number of petri-dishes and the medium in a laminar air-flow, which had been wiped with a disinfectant and the area sterilized by U.V light for 20 min.
2. 10-15 ml of the melted sterile agar medium are poured into a sterile Petri-dish, care being taken to avoid contamination.
3. The mouth of the flask or tube containing the medium should be flamed after removal of the cotton plug and the lid of the Petri-dish raised only enough to allow easy access.
4. When the plates have been poured, the upper lid of Petri-dish is left slightly opened for hot vapor to escape and kept with air flow until the plates are completely dried. If condensed water droplets are present on the lid, this may prevent the clear visibility of colonies formed on the dish and may also interfere with the formation of discrete colonies.

The various solid media used in a microbiology laboratory are given as follows:

1. *NUTRIENT AGAR*

The most commonly used solid media in routine laboratory work is nutrient agar. Nutrient agar is used for isolation of common non-fastidious pathogens from clinical specimens. It is also used as a basal medium for the preparation of enriched media by supplementing with blood, serum or other nutrients. Routine antimicrobial sensitivity tests are performed on nutrient agar.

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Media Composition of Nutrient Agar

Peptone : 10 g
Beef extract : 5 g
Sodium chloride (NaCl) : 5 g Agar : 20 g
Distilled water : 1000 ml

Adjust pH to 7.2
Sterilize by autoclaving at 121 °C for 20 minutes at 15 lbs pressure

Preparation

i. Solid ingredients except agar is weighed and added to an Erlenmeyer conical flask.
ii. Add 500 ml of distilled water and stir well with glass-rod till all solids are dissolved completely (warmed if necessary).
iii. Adjust pH to 7.2 using 0.1 N sodium hydroxide (NaOH) or 0.1 N hydrochloric acid (HCl).
iv. Make upto 1000 ml with distilled water, weigh agar and add to the media.
v. Plug the mouth of the flask with a non-absorbent cotton and cover it with craft paper.
vi. Sterilize by autoclaving at 121 °C for 20 min at 15 lbs pressure.
vii. Cool the media to about 40 °C and transfer 15-20 ml of media to sterile Petri plates.
viii. Allow the media to solidify in the plate as mentioned earlier. It is used for culture after sterility check by incubation at 37 °C overnight.

2. *MAC CONKEY AGAR*

Mac Conkey agar is a selective and differential media. The presence of bile salt inhibits the growth of non-enteric bacteria. The presence of lactose along with the dye phenol red or neutral red helps to differentiate lactose fermenting group from that of non-lactose fermenting group. The omission of NaCl from the media prevents swarming of *Proteus* colonies. The medium prevents non-enteric bacteria and is mainly used for culturing enteric bacteria and coliform analysis of water. A modification of Mac Conkey agar containing crystal violet at a concentration of 0.001 g/100 ml medium inhibited all gram positive bacteria and therefore can be used for selective isolation of gram negative enteric organisms.

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Media Composition of Mac Conkey Agar

Peptone	:	20 g
NaCl	:	5 g
Bile salt (Sodium taurocholate)	:	5 g
Lactose	:	10 g
Agar	:	20 g
Distilled water	:	1000 ml

Adjust pH to 7.2

Sterilize by autoclaving at 121 °C for 15 minutes at 15 lbs pressure. Add 2.4 ml of 1 % aqueous solution of neutral red.

Preparation

i. Dissolve peptone, NaCl and bile salt in water and heat in boiling water bath till ingredients are completely dissolved.

ii. Add lactose to the warm mixture, adjust pH to 7.2 using 0.1 N NaOH or 0.1 N HCl.

iii. Make upto 1000 ml with distilled water, weigh agar and add to the media.

iv. Plug the mouth of the flask with a non-absorbent cotton and cover it with craft paper.

v. Sterilize by autoclaving at 121 °C for 15 min at 15 lbs pressure.

vi. Boil 1 % neutral red solution in a tube for 15 min.

vii. Under sterile condition, add 2.4 ml of neutral red solution into sterile media.

viii. Once the media is cooled to about 40 °C, transfer 15-20 ml of media to sterile Petri plates and allow to solidify.

ix. Plates can be used for culture after sterility check by incubation at 37 °C overnight.

When the medium is stored for longer time, the neutral red indicator tends to fade. To overcome this, medium can be made without neutral red and indicator has to be added only before pouring to the plate.

3. *PLATE COUNT AGAR (PCA)*

Plate Count agar is widely used in laboratories for enumeration of total viable micro-organisms particularly bacteria in the given sample. This is non-selective media with which when inoculated and incubated at different temperature helps in the enumeration of bacteria of different temperature specificities in the given sample. PCA is widely used in the

assessment of total microbial load in a given sample by Total Plate Count (TPC) method or Aerophilic Plate Count (APC) method.

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Media Composition of Plate Count Agar

Peptone or Tryptone	:	5 g
Yeast extract	:	2.5 g
Glucose	:	10 g
Agar	:	20 g
Distilled water	:	1000 ml

Adjust pH to 7.0

Sterilize by autoclaving at 121 °C for 15 minutes at 15 lbs pressure.

Preparation

i. Different ingredients such as tryptone, yeast extract and glucose have to be weighed accurately and dissolved in 500 ml distilled water.

ii. Adjust pH to 7.0 using 0.1 N NaOH or 0.1 N HCl and make up the volume to 1000 ml.

iii. Weigh 20 g agar and add to the media, heat in a boiling water-bath till agar is completely dissolved.

iv. Plug the mouth of the flask with a non-absorbent cotton and cover it with craft paper.

v. Sterilize by autoclaving at 121 °C for 15 min at 15 lbs pressure.

vi. Once the media is cooled to about 40 °C, transfer 15-20 ml of media to sterile Petri plates and allow to solidify.

vii. Plates can be used for culture after sterility check by incubation at 37 °C overnight.

4. *BLOOD AGAR*

Blood Agar is an enriched media. It is widely used in medical bacteriology. Media contains 5 - 10 % sterile blood as additional nutrient along with sterile nutrient agar medium. Either human or animal blood can be used. Commonly used blood for blood agar is sheep or horse blood. Blood agar supports the growth of nutritionally fastidious organisms like *Gonococcus*, *Haemophilus* etc. These media are also used for the demonstration of haemolysis which helps in the differentiation of bacterial strains in the genus *Streptococcus*.

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Media Composition of Blood Agar

Sterile nutrient agar (liquid), pH-7.2	:	95 ml

Freshly drawn blood : 5 ml

Preparation

i. Prepare nutrient agar and sterilize by autoclaving at 121 °C for 15 minutes at 15 lbs pressure.

ii. Once the media is cooled at 45 -50 °C, add 5ml blood aseptically to the media and mix well by gentle shaking

iii. Pour 15-20 ml media in sterile Petri plates.

iv. Plug the mouth of the flask with a non-absorbent cotton and cover it with craft paper.

v. Care should be taken to avoid trapping of air bubbles in the plate.

vi. Once solidified, media can be used for culturing.

vii. Prior checking of sterility of plates by incubation at 37 °C overnight is appreciable.

5. *CHOCOLATE AGAR*

Chocolate agar is enriched media, commonly you used for culturing nutritionally fastidious organisms such as *Haemophilus influenzae*, *Neisseria* and *Pneumococcus*. This media is prepared by heating blood agar until the blood becomes chocolate brown in colour. Application of heat ruptures the red blood cells and liberate the nutrients.

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Media Composition of Chocolate Agar

Sterile nutrient agar (liquid), pH-7.2 : 95 ml

Freshly drawn blood : 5 ml

Apply heat at 74 °C till the media turns chocolate brown in colour.

Preparation

i. Prepare nutrient agar and sterilize by autoclaving at 121 °C for 15 minutes at 15 lbs pressure.

ii. Cool the medium to 74 °C, while keeping in water-bath set at 74 °C

iii. Add 5 ml of freshly drawn blood to the media and kept at 74 °C with gentle shaking till the media turn chocolate brown in colour.

iv. Once the media is cooled at 40 °C, pour 15-20 ml media in sterile Petri plates and allow to solidify.

v. Media can be used for culturing after sterility check.

6. *POTATO DEXTROSE AGAR (PDA)*

Potato dextrose agar is used for primary isolation of fungi from source material. The low pH of media favors preferential growth of fungi when compared to bacteria. This media is used in the routine laboratory methods for the enumeration of fungal load in a given sample. It is also used for studying morphological features of fungi such as colony morphology and pigment production.

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Media Composition of Potato Dextrose Agar

Peeled potato	:	200 g
Dextrose	:	20 g
Agar	:	20 g
Distilled water	:	1000 ml

Adjust pH to 5.6

Sterilize by autoclaving at 115 °C for 10 minutes at 10 lbs pressure.

Preparation

i. Cut potatoes into small pieces.

ii. Add adequate amount of water and boil or steam in the autoclave for 30 minutes and strain the supernatant solution.

iii. To the potato extract, add 20 g of D-Glucose (Dextrose) and make up the volume into 1 litre.

iv. Add 20 g of agar and boil in water-bath until the agar is completely dissolved.

v. Plug the mouth of the conical flask with a non-absorbent cotton and cover it with craft paper.

vi. Sterilize by autoclaving at 115 °C for 10 min at 10 lbs pressure.

vii. Once the media is cooled to about 45- 40 °C, it can be poured into sterile Petri plates or tubes.

viii. The media can be used for culture after sterility check by incubation of plates/tubes at 37 °C overnight.

7. *EOSIN METHYLENE BLUE (EMB) AGAR MEDIUM*

Eosin Methylene Blue Agar medium is a selective medium used for isolating the bacteria *Escherichia coli*. The dyes Eosin Y and Methylene Blue act as selective agents, inhibiting the growth of Gram-positive bacteria while allowing Gram-negative bacteria to grow. The medium also contains fermentable carbohydrates like lactose and sucrose, allowing for differentiation based on lactose fermentation. *E. coli* form green colonies with metallic sheen on EMB agar medium.

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Media Composition of Eosin Methylene Blue Agar Medium

Peptone	:	10 g
Dipotassium hydrogen orthophosphate	:	2 g
Lactose	:	5g
Sucrose	:	5 g
Eosin Y	:	0.4 g
Methylene Blue	:	0.065 g
Agar	:	20 g
Distilled water	:	1000 ml

Adjust pH to 7.2

Sterilize by autoclaving at 115 °C for 10 minutes at 10 lbs pressure.

Preparation

i. Accurately weigh the required amount of dehydrated EMB agar powder, 1000 ml distilled water.

ii. Mix until the suspension is uniform. Heat to boiling to dissolve the medium completely.

iii. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

iv. Cool to 45 - 50°C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour) and to suspend the flocculent precipitate.

v. Pour into sterile Petri plates.

vi. Allow plates to cool to room temperature.

vii. The agar surface should be dry before inoculating.

II. Liquid Media

It is used for the method of bacterial culture called liquid culture or broth culture. In this case, the desired bacteria are suspended in the liquid media and incubated at appropriate temperature. Liquid cultures are preferable for inoculum containing antibiotics and other

inhibitory substances. Routinely used liquid media in common laboratory practice include nutrient broth and peptone water.

1. *NUTRIENT BROTH*

Nutrient broth is a liquid media used for study of common pathogenic and non-pathogenic organisms. It supports the growth of non-fastidious organisms. It is also used for obtaining bacterial growth from blood, wine, water etc. It is also used when large amount of inoculum has to be used for obtaining bulk culture for antigen or vaccine preparation.

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Media Composition of Nutrient Broth

Peptone	:	10 g
Beef extract	:	5 g
Sodium chloride (NaCl)	:	5 g
Distilled water	:	1000 ml

Adjust pH to 7.2
Sterilize by autoclaving at 121 °C for 15 minutes at 15 lbs pressure

Preparation

i. Weigh & add required amount of solid ingredients and add to a conical flask.
ii. Add 500 ml of distilled water and stir well with glass-rod till all solids are dissolved completely (warmed if necessary).
iii. Adjust pH to 7.2 using 0.1 N NaOH or 0.1 N HCl.
iv. Make up the volume to 1000 ml with distilled water.
v. Dispense the media into tubes or smaller flasks if required.
vi. Plug the mouth of the tubes or flasks with a non-absorbent cotton and cover it with craft paper.
vii. Sterilize by autoclaving at 121 °C for 15 min at 15 lbs pressure.
viii. Once the media is cooled, it can be used for culture.

2. *PEPTONE BROTH*

It is the minimal growth media. The formulation of peptone broth or peptone water permits cultivation of non-fastidious organisms. This non-selective medium has been used as

the basal medium for biochemical tests such as carbohydrate fermentation test (pattern with 1 % final sugar or carbohydrate concentration with phenol red indicator with Durham's tube) and production of indole. Peptone water contains peptone as sole source of carbon, nitrogen, vitamins and minerals. Sodium chloride maintain the osmotic balance.

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Media Composition of Peptone Broth

Peptone	:	10 g
Sodium chloride (NaCl)	:	5 g
Distilled water	:	1000 ml

Adjust pH to 7.2
Dispense into tubes or flasks and autoclave at 121 °C for 15 min.

Note: Alkaline peptone water (pH- 7.6 to 8.8) is used for the enrichment of *Vibrio* species from food, water and clinical samples.

Buffered peptone water is used for non-selective pre-enrichment of *Salmonella* species from food.

Preparation

i. Weigh required amount of solid ingredients and add to a conical flask.
ii. Add 500 ml of distilled water and stir well with glass-rod till all solids are dissolved completely (warmed if necessary).
iii. Adjust pH to 7.2 using 0.1 N NaOH or 0.1 N HCl.
iv. Make up the volume to 1000 ml with distilled water.
v. Dispense the media into tubes or smaller flasks, plug the mouth with a non-absorbent cotton and cover it with craft paper.
vi. Sterilize by autoclaving at 121 °C for 10 min at 15 lbs pressure and can be used for culture after cooling.

III. Semi-solid Media

Semi-solid media contains 0.2 - 0.5 % agar, which is sufficient to prevent convection currents and therefore helps to prevent the diffusion of oxygen into the medium. This enables the growth of anaerobes at the bottom of the tube. Stab-culture containing semi-solid media followed by addition of sterile mineral oil is greatly used as a method for preservation of culture for short term storage. Semi-solid media also enables motile organisms to spread.

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Media Composition of Semi-solid Media

Nutrient broth	:	100 ml
Agar	:	0.2 - 0.5 g

Preparation

i. Boil the media till agar is completely dissolved.

ii. Dispense into tubes and autoclave at 121 °C for 15 min at 15 lbs pressure.

Date:…….

Experiment No-4

Culture Methods

In clinical laboratory, the importance of culture method is to:-

1. Isolate bacteria in pure culture
2. Demonstrate their properties
3. Obtain sufficient growth for antigen preparation and for other tests,
4. Determine sensitivity to antibiotics
5. Estimate viable counts
6. Maintain stock culture

This method of culture ordinarily used in the laboratory are streak, lawn, stroke, stab, sweep, spread plate, pour plate and liquid culture. Special methods are used for culturing.

1. *STREAK CULTURE (SURFACE PLATING)*

This method is routinely employed for the isolation of bacteria in pure culture from clinical specimens or mixed cultures. A nichrome wire loop charged with one loopful of the specimen is transferred to the surface of the well dried plate on which it is spread over a small area at the periphery. The inoculum is then distributed evenly over the plate by streaking it with a loop in series of parallel lines in different segments of the plate. The loop should be flamed and cooled between the different sets of streaks. On incubating, growth may be confluent at the sides of the original inoculum, but becomes progressively thinner and well separated colonies are obtained over the final series of streaks.

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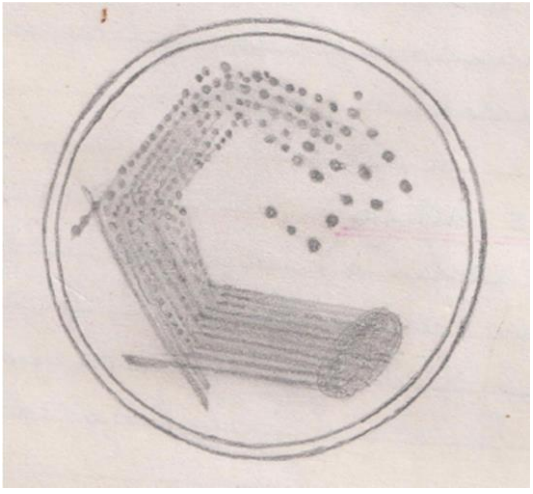

Streak culture

i. Continuous streak.

In this type, a continuous streak line was made in a zig-zag manner on the whole plate surface without any intermittent heating. This streak is usually done when the initial inoculum is diluted or to propagate pure cultures.

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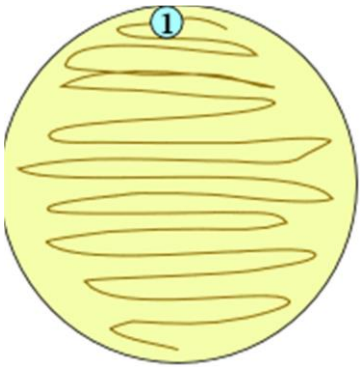
Continuous streak



ii. T streak.

In this type, zig zag streak lines made in three sections perpendicular to each other. First streak lines are made in a zig-zag manner drawn from the peripheral smear and cover half of the nutrient agar surface and then sterilize the loop. Then turn the plates to 90° and drag the loop through the area you have just streaked two to three times and continue to drag the loop in zig-zag formation in the remaining half of the section without touching that area again. The loop is sterilized again and the remaining quarter is streaked in the same manner but after turning the plates to 90°.

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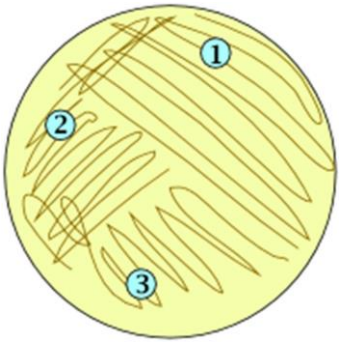
T-streak



iii. Radiant streak.

This method involves streaking the inoculum on one side of the plate, then spreading it in a vertical, ray-like pattern, and finally cross-streaking it diagonally to ensure even distribution. The loop is sterilized whenever you change the direction of the streak.

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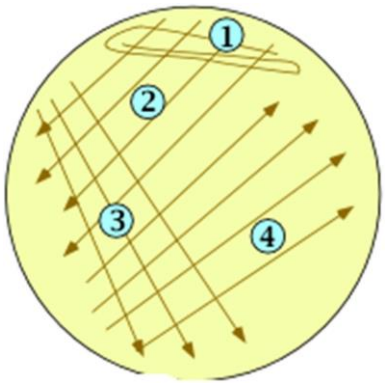
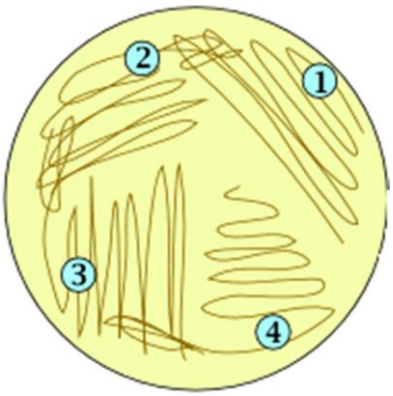
Radiant streak.



iv. Quadrant streak.

This method involves dividing a Petri-dish into four quarters and sequentially streaking the inoculum across each quadrant in zig-zag manner with intermittent heating. This gradually reduces the bacteria density to allow for isolated colonies to develop.

Left side of record Quadrant streak



2. LAWN or CARPET CULTURE

The lawn or carpet culture provides a uniform surface growth of bacteria. It is useful for bacteriophage typing and antibiotic sensitivity testing. This culture method can be done either by flooding the surface plate with liquid culture and pipetting out excess inoculum or by applying a swab suspended in liquid bacterial culture. Then the culture is spread evenly on the media surface and incubated at desired temperature.

3. *STROKE CULTURE*

It is made in a tube containing agar slant and is employed for providing a pure growth of bacteria, for slide agglutination and other diagnostic tests.

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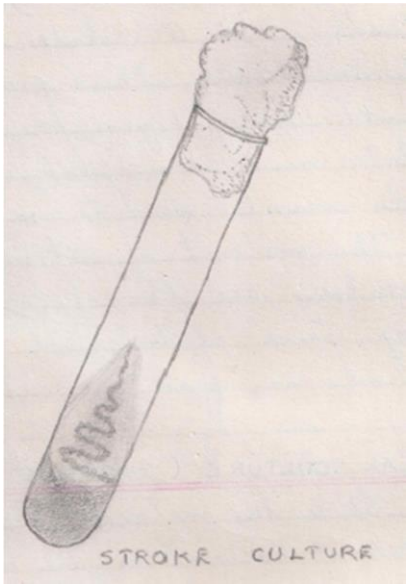

4. *STAB CULTURE*

It is prepared by puncturing the medium with a long straight wire charged with a bacterial suspension. The medium is set with the tube in a vertical position providing a flat surface at the top of the medium. They are used in the maintenance of stock culture.

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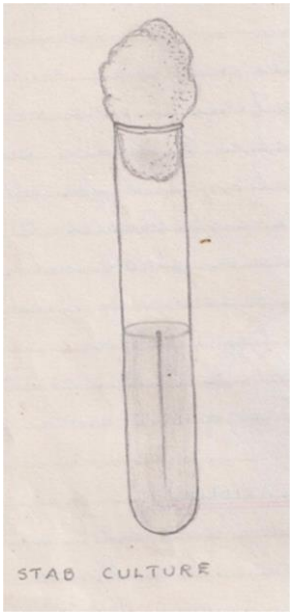

5. *SPREAD PLATE METHOD*

In this method, the mixed culture is serially diluted in a series of tubes containing sterile liquid, usually buffer or physiological saline. An aliquot of sample (1.0 or 0.1 ml) is removed from each tube, placed on the surface of the agar plate, by means of a sterile bent L-shaped rod. At least in one plate of the series, the bacteria will be in a number sufficiently low so as to allow development of well separated colonies. These may be counted, which gives an estimate of aerophilic viable counts in the suspension.

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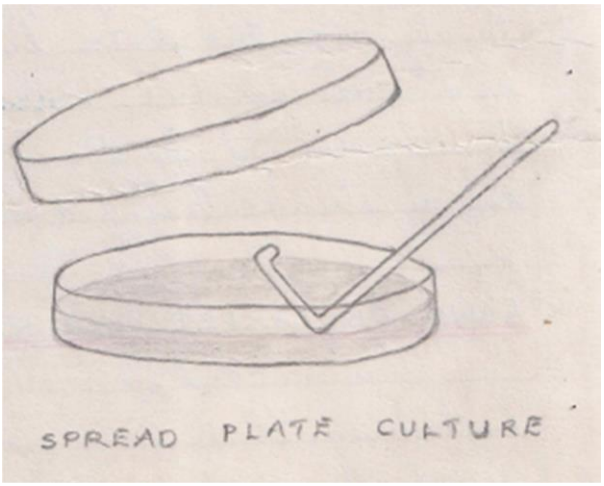

6. *POUR PLATE METHOD*

In this method, the mixed culture is serially diluted in a series of tubes containing sterile liquid, usually buffer or physiological saline. An aliquot of sample of volume 1 ml from each dilution is added to respective tubes containing 15 ml of melted agar medium, set at a temperature of 40 °C. The contents of the tubes are mixed well and are poured in a sterile Petri plate. Once the medium is set in the Petri plates, it is incubated. It also gives well separated colonies and hence an estimate of viable count of bacteria can be obtained.

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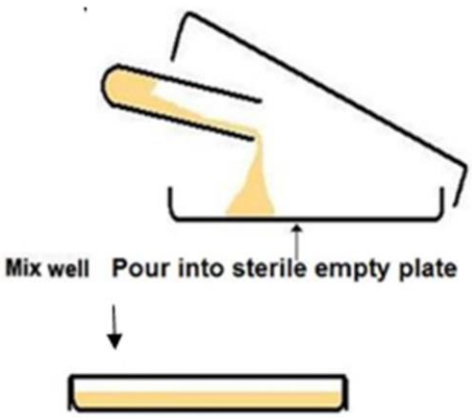

7. *SWEEP PLATE METHOD*

This method is used to prepare a replica plate of master plate obtained by spread plate technique or pour plate technique. A glass beaker or replica block whose diameter is same or slightly lesser than that of a Petri-dish is taken and its mouth is covered by a velvet cloth. This whole apparatus is sterilized in autoclave. A replica plate is prepared by pressing the replica block with velvet cloth on a culture plate with well separated colonies. This allows the cloth to pick up bacteria from each colony. Then the velvet is pressed to the surface of other fresh media plates and organisms are transferred to the same position as on the master plate. On incubation new colonies are developed with colonies positioned identical to that of master plate forming a replica. This method is known as sweep plate culture. This method uses a printing-like transfer to make an exact copy of the master plate.

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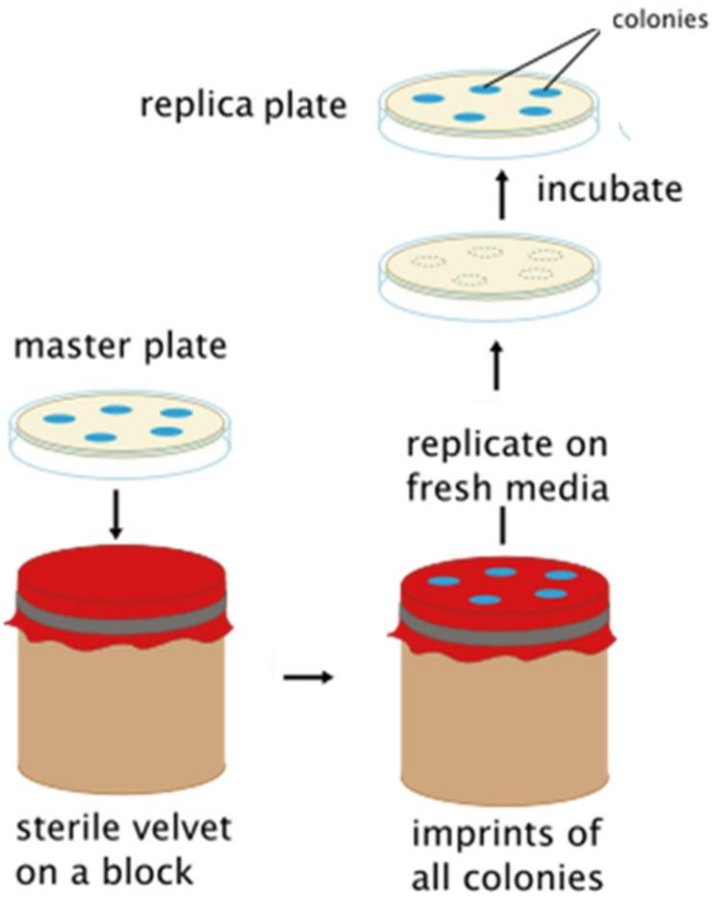
Sweep plate method



8. *LIQUID CULTURE*

Liquid culture in tubes, bottles or flasks may be inoculated by touching with a charged loop or by adding the inoculum with pipette and syringes. Liquid culture are preferred when large yields are desired. The yield can be enhanced by agitation, aeration etc. The major disadvantages of liquid culture are that it does not provide a pure culture from mixed inoculum.

9. *ANAEROBIC CULTURE*

Anaerobic bacteria differ in their requirement and sensitivity to oxygen. A number of methods have been described for achieving anaerobiosis, the principle being exclusion of oxygen, production of vacuum, displacement of oxygen with other gases, absorption of oxygen by chemical or biological methods and reduction of oxygen.

GASPAK ANAEROBIC JAR METHOD

A gaspak anaerobic system is used to create an oxygen free environment for culturing anaerobic microorganisms. It consists of a transparent jar made of polycarbonate with a air-tight lid. It utilizes a gas generator (GasPak sachet) containing the chemicals sodium bicarbonate ($NaHCO_3$) and sodium borohydrate ($NaBH_4$). These react with each other in the presence of water producing hydrogen and carbon dioxide. Thus oxygen is removed from chamber by combining with hydrogen to form water. This reaction is catalyzed by the palladium pellets attached to the lid. The carbon dioxide produced, replaces the removed oxygen thus creating a completely anaerobic environment. The inoculated plates or tubes are placed inside the jar along with GasPak envelope with water added. Along with these, a methylene blue indicator strip is also placed inside the jar, which acts as an anaerobic indicator. Then the lid is screwed air tight. The methylene blue of indicator strip becomes colorless (leucomethylene blue) when all oxygen is removed from jar, indicating complete anaerobisis inside the jar.

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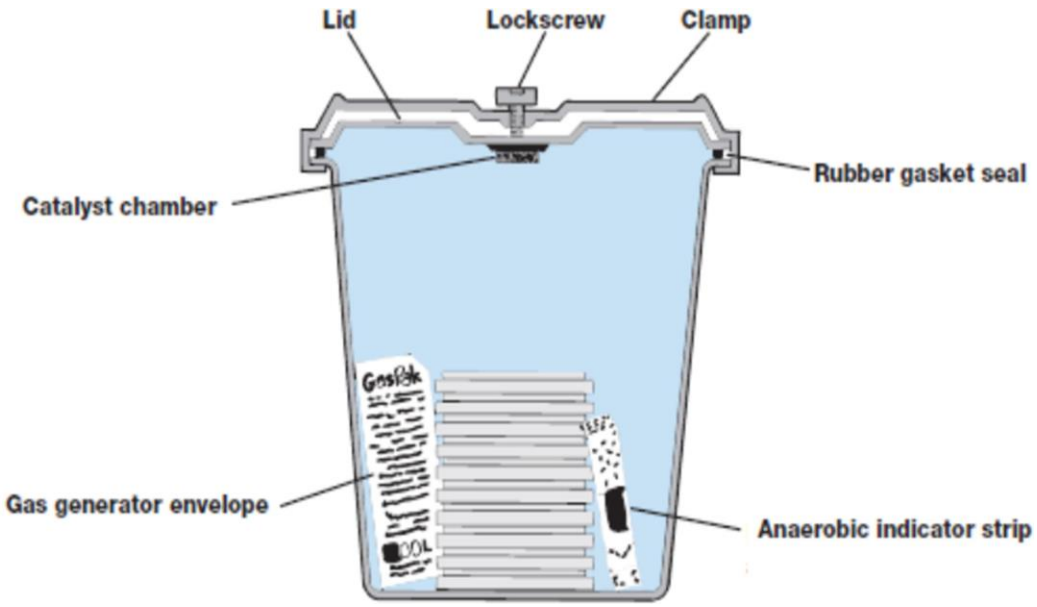
Gaspak Anaerobic Jar



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Experiment No-5

Staining Techniques

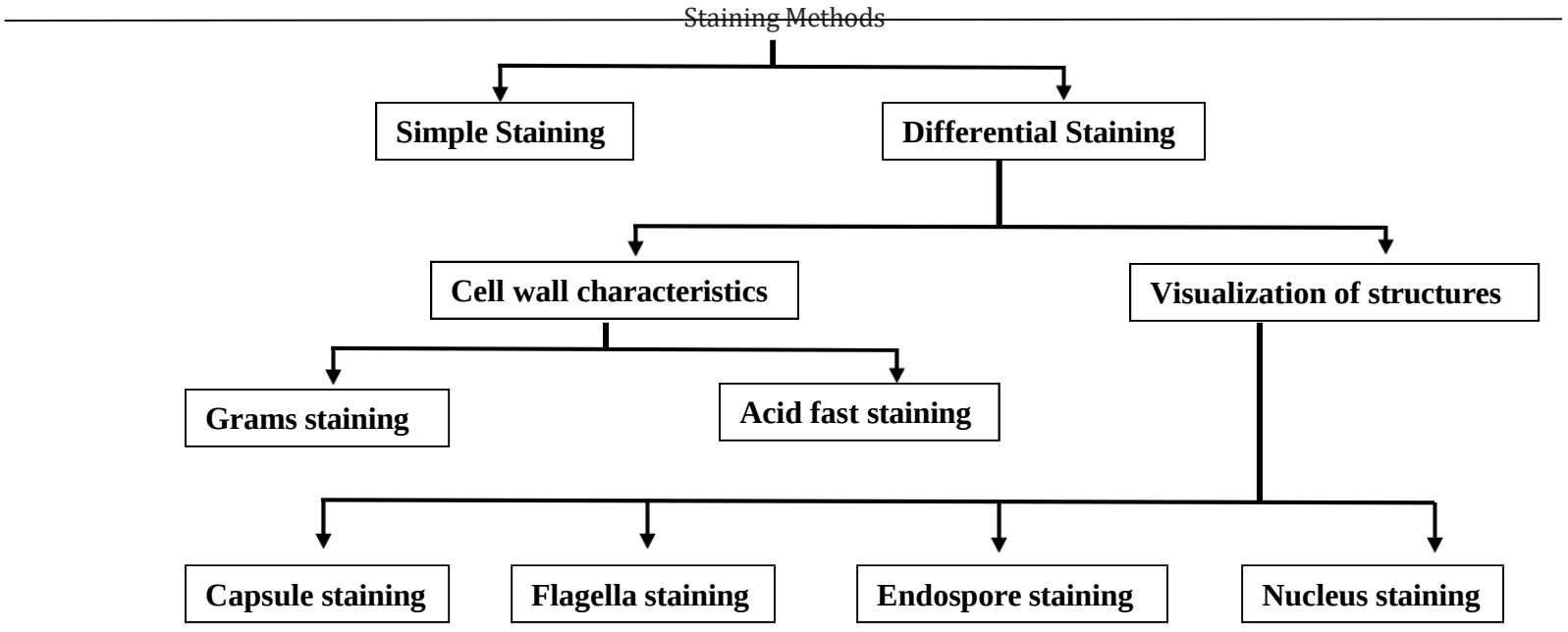
Live bacteria do not show much structural details, under the light microscope due to the lack of contrast. Hence it is customary to use staining techniques to produce colour contrast. Smear preparation is of great importance during staining. The slide used should be clearly wiped using cotton and any disinfectant, usually 70 % ethanol. Before the preparation of the smear, the end of the slide bearing the smear should be marked with number or letters to ensure the identity of smear.

Preparation of smear

In the case of liquid cultures, one loop-full of culture is taken up with the inoculating loop and spread thinly on the slide. The slide is now air dried first by leaving the slide on the working table for a few minutes. The slide is then held high over the Bunsen burner and dried further. Then film is fixed without charring, by passing it over the flame.

With a sterilized loop, single colony of the desired culture is placed over a drop of saline in the case of solid media. The smear is thoroughly mixed and the mixture is spread evenly on the slide. The resulting film is air-dried and then heat-fixed, without charring by passing it over the flame.

Work sheet	To be written/drawn on **the left side of the Practical Record**



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Experiment No-5 A

A. Simple Staining

This method of staining not only show the presence of organism but also the nature of cellular contents in exudates.

PROCEDURE OF STAINING WITH METHYLENE BLUE

A smear is prepared and it is flooded with Loeffler's methylene blue stain and is allowed to stand for 3 minutes. The stain is drained off and washed with water. Slide is air dried and observed under microscope, usually 40 or 45 X objective.

RESULT

The bacterial culture consists of blue coloured and rod shaped cells.

PROCEDURE OF STAINING WITH CRYSTAL VIOLET

A smear is prepared and the crystal violet stain is applied over the smear for 3 minutes, washed with water and observed under the microscope.

RESULT

Bacterial cells appear spherical shaped and violet in colour.

Left side of record

Composition of Loeffler's Methylene Blue Stain

Saturated solution of Methylene Blue in Ethanol : 300 ml

Potassium hydroxide (KOH, 0.01% in water) : make up to 1000 ml

Composition of Crystal Violet Stain

Crystal violet or methyl violet : 10 g
Absolute alcohol : 100 ml
Distilled water : make up to 1000 ml
Dissolve dye first in alcohol and then added to water.

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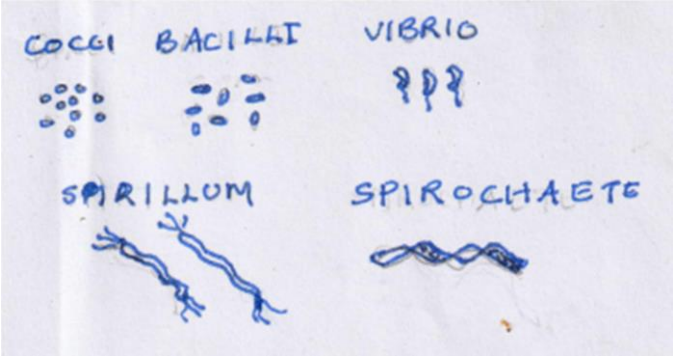

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Experiment No-5 B

Differential Staining

This staining method uses more than one stain for studying bacteria and its properties.

B. GRAM STAINING

AIM

To distinguish between gram positive and gram negative bacteria.

PRINCIPLE

There are two types of bacteria- gram positive and gram negative. Gram positive bacteria are those which resist decolourisation by retaining the primary stain and appear violet colour. Gram negative bacteria are those that are decolourised by alcohol and takes up the counter stain (safranin) and appear pink in colour. This is because the cell wall of gram negative bacteria contain high percentage of lipid than that of gram positive bacteria. When primary stain is applied, it causes the cell wall to appear violet or purple on the application of Gram's iodine reagent (mordant). The crystal violet- iodine (CV-I) complex is formed inside the cell. During decolourisation with alcohol, cell wall lipid gets extracted which results in increased porosity and permeability of gram negative cell wall. Thus CV-I complex comes out of the cell and cell is decolourised. On application with counter stain, the cell wall gets stained pink. In the case of gram positive bacteria, cell wall is made up of peptidoglycan and have very low amount of lipid. On decolourisation, the cell wall undergoes dehydration. This results in shrinkage of cell wall and its permeability to stain is decreased. So CV-I complex gets trapped inside the cell and do not get washed off. So Gram positive bacteria appear as violet in colour.

PROCEDURE

i. The smear is prepared on a glass slide and heat fixed.
ii. The slide is flooded with crystal violet stain for 1 minute and is washed off with running tap water.
iii. The slide is then flooded with Gram's iodine solution for 1 minute and is rinsed off with water.
iv. Decolourising agent (95 % acetone or ethanol) was added drop by drop at the upper end of the inclined slide until last drop do not contain violet colour and is immediately washed off with running tap water.
v. Then the slide is flooded with safranin for 1 minute and washed off with tap water.

vi. The slide is air-dried, a drop pf immersion oil is applied and observed under oil immersion objective lens.

RESULT

The cells which appeared violet in colour are gram positive bacteria and the cells which appear pink in colour are gram negative bacteria.

Left side of record

Composition of Crystal Violet Stain

Crystal violet or methyl violet : 10 g
Absolute alcohol : 100 ml
Distilled water : make up to 1000 ml
Dissolve dye first in alcohol and then added to water.

Composition of Gram's Iodine Reagent

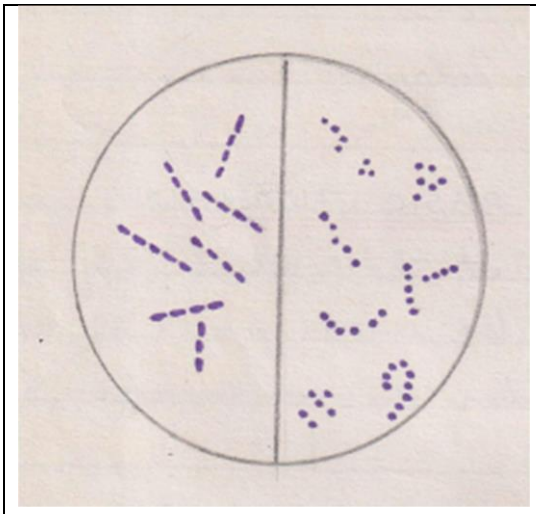
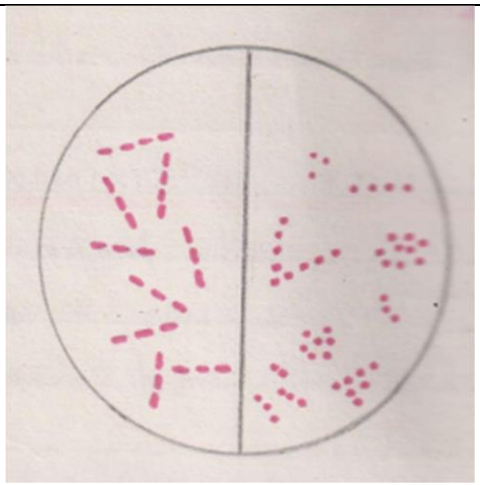
Iodine, resublimed : 10 g
Potassium iodide (KI) : 20 g
Distilled water : 1000 ml
Make a paste of 20 g of KI with a known volume of water (50 ml water) and the add 10 g of iodine. When the iodine is completely dissolved, make it up to 1000 ml.

Composition of Safranin Stain

Safranin solution (2.5 % in ethanol) : 10 ml
Distilled water : 1000 ml
2.5 % w/v solution of safranin in 95 % ethanol

Diagram

Gram positive bacilli	Gram positive cocci	Gram negative bacilli	Gram negative cocci
Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Neisseria gonorrhoeae*
Clostridium tetani	*Streptococcus pyogenes*	*Salmonella enterica*	*Neisseria meningitidis*
Corynebacterium diphtheriae	*Streptococcus pneumoniae*	*Klebsiella pneumoniae*	

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Experiment No-5 C

C. VOLUTIN GRANULE STAINING

AIM

To stain volutin or polyphosphate or metachromatic granules by Albert's technique.

PRINCIPLE

Volutin granules are also known as polyphosphate granules. They are formed of linear polymer of orthophosphate joined by ester bonds. These granules are also known as metachromatic granules because of its ability to change the colour of the dye, i.e., when stained with methylene blue, they appear as red in colour. Volutin granules are the most acidic component of the cell.

Albert's stain is made up of two dyes- toluidine blue and malachite green. Both of these dyes are basic dyes with high affinity for acidic components of the cell like cytoplasm. The pH of Albert's stain is adjusted to 2.8 by using acetic acid, which is still basic for volutin granules, which are highly acidic. When Albert's stain is applied to the smear, toluidine blue stains volutin granules and malachite green stains the cytoplasm green. The iodine in Albert's iodine reagent inhibits the metachromatic property and hence the volutin granules appear as blue in colour.

PROCEDURE

i. A thin smear is made on a glass slide and heat fixed.
ii. Cover the smear with Albert's stain and allowed to act on 5 minutes is rinsed off with running water.
iii. The smear is then covered with Albert's iodine for 1 minute.
iv. Slide is washed off with tap water, air-dried and observed under oil immersion objective lens.

RESULT

The cells appear as green in colour and volutin granules appear as bluish black granules within green cell.

Left side of record Composition of

Albert's Stain

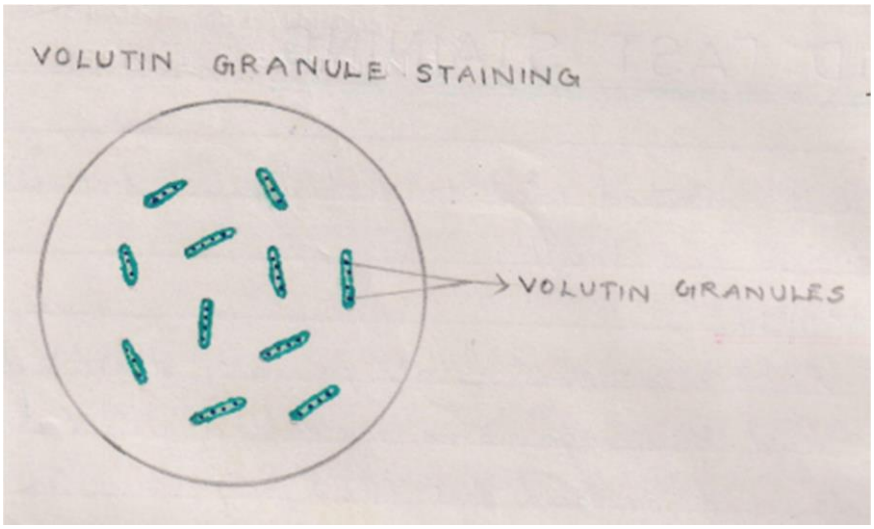
Toluidine blue	:	1.5 g
Malachite green	:	2 g

Glacial acetic acid	:	10 ml
Alcohol (ethanol)	:	20 ml
Distilled water	:	make up to 1000 ml

Composition of Albert's Iodine Reagent

Iodine, resublimed	:	6 g
Potassium iodide (KI)	:	9 g
Distilled water	:	900 ml

Make a paste of KI with a known volume of water and then add iodine. When the iodine is completely dissolved, make it up to desired volume.



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Experiment No-5 D

D. ENDOSPORE STAINING

AIM

To stain the endospore of bacteria by Schaeffer-Fulton method.

PRINCIPLE

Bacteria like *Bacillus* form endospores during unfavorable condition. Endospores resist staining, but once they are stained, they resist counter staining. The resistance of spores to physical and chemical agents is attributed to the extremely dehydrated state of spores and impermeability of spore coat. Due to the moist heat treatment, pores of endospores widens and it facilitates staining with strong basic stain solutions. Employment of decolourisers selectively decolorizes cytoplasm and not the spore. Contrast can be created for better observation by counter staining the cytoplasm with safranin or the background or leaving it colourless.

PROCEDURE

i. A thin smear is made on a glass slide and heat fixed.
ii. The slide is placed over a beaker of water, resting it on the rim of beaker with bacterial film uppermost.
iii. When large droplets get condensed on the underside of slide, flood it with 5 % aqueous solution of malachite green and leave it to act for 3 minutes while water continues to boil.
iv. Slide is washed off with tap water and the smear is treated with safranin for 1 min.
v. The stain is washed off, air-dried and observed under oil immersion objective lens.

RESULT

The endospores are coloured green in colour and the vegetative cell (spore mother cell) pinkish red in colour.

Left side of record Composition of

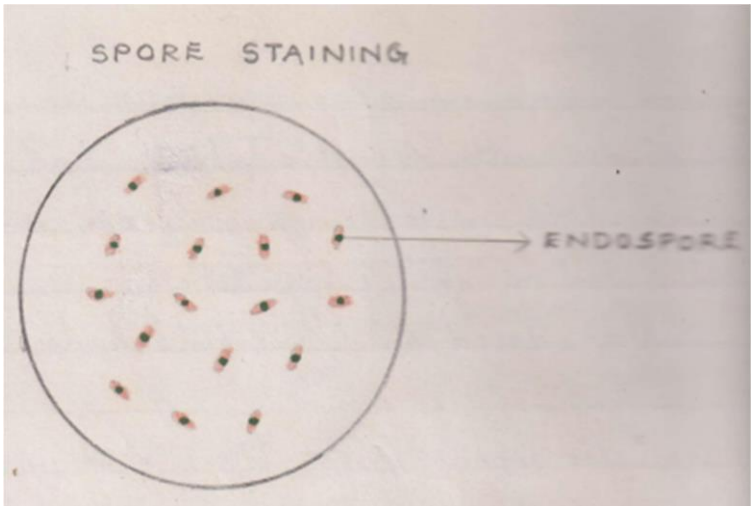
Albert's Stain

Malachite green	:	5 g
Distilled water	:	make up to 100 ml

Composition of Safranin Stain

Safranin solution (2.5 % in ethanol)	:	10 ml

Distilled water : 1000 ml
2.5 % w/v solution of safranin in 95 % ethanol



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Experiment No-5 E

E. NEGATIVE STAINING

AIM

To demonstrate the presence of cells in the given culture by negative staining.

PRINCIPLE

The cell wall of microorganisms such as yeast has low affinity for dyes such as India ink or nigrosin. Hence it remains unstained.

PROCEDURE

i. Place a large drop of undiluted India ink or nigrosine stain on the glass slide and emulsify a small amount of the yeast culture besides the ink.
ii. Mix the stain solution and culture on the slide surface.
iii. Place a clean coverslip on the ink drop and press it through the sheet of blotting paper.
iv. The film should be so thin that the yeast cells is gripped between the slide and coverslip, neither overlaid by ink nor cells moving about.
v. Examine under 45 X objective lens of microscope.

RESULT

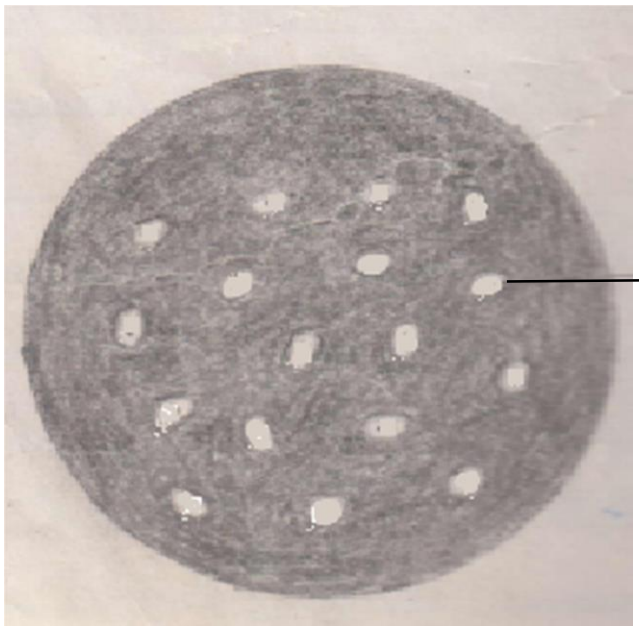
The cells are seen as clear bright halos against black background.

Left side of record Composition of

Nigrosin Stain

Nigrosin	:	10 g
Distilled water	:	make up to 100 ml

Mix the nigrosine in water taken in a conical flask. The mouth of conical flask is tightly covered with an aluminum foil. Dissolve the stain by heating it in a boiling water bath about 30 min. Filter through a Whatman No.1 filter paper. Add 0.5 ml of formalin solution to the filtered solution.


Circular clear bright cells

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Experiment No-6

EXAMINATION OF BACTERIA FOR MOTILITY

AIM

To observe the vibrating motion of bacteria in a liquid media by hanging drop technique.

PRINCIPLE

Knowing whether organisms are motile or non-motile helps in its identification. The vibrating movement of bacteria is due to particular stimulus. In hanging drop method, organisms are observed in a drop that is hanging under a coverslip, kept over the cavity slide so that microorganisms can move freely on the hanging drop. A majority of microorganisms are observed to be motile along the edge of the water drop, as the concentration of oxygen is higher at the edge.

PROCEDURE

i. Prepare a clear cavity slide and coveslip. Any grease on the slide as well as coverslip must be removed.
ii. Place a very small amount of grease near each corner of the coverslip.
iii. Transfer a loop full of young fluid culture on the coverslip.
iv. Place the slide in contact with coverslip with depression or cavity over the drop of suspended bacteria.
v. Invert the slide quickly so that the drop cannot runoff to one side and is examined without delay.
vi. Observe under low power objective lens (10 X) and focus on the edge of the drop. Then place the high power objective lens in position and focus the edge clearly.

RESULT

Bacteria are seen clearly moving, changing their position along the edge of the drop.

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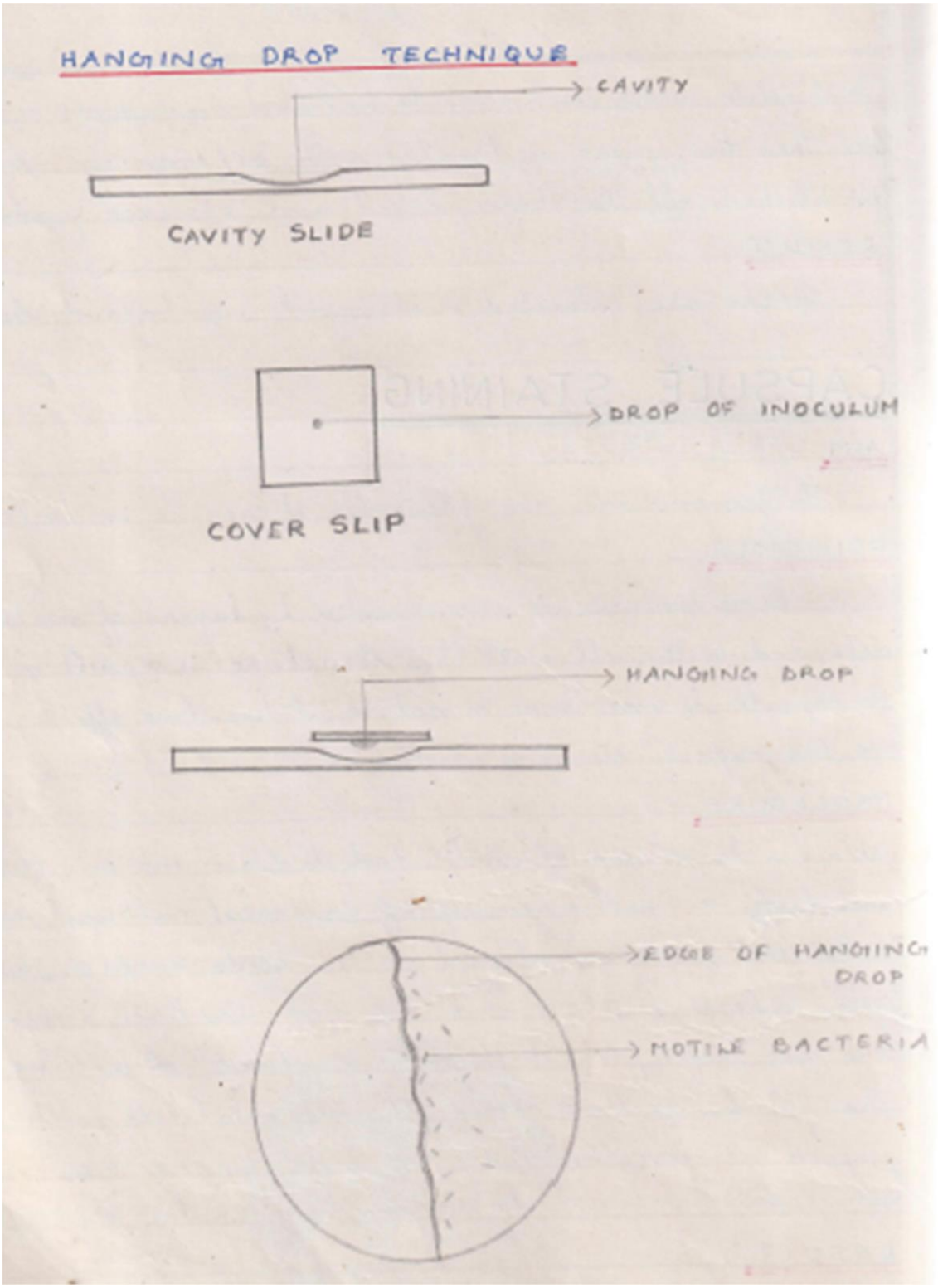

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Experiment No-7

MORPHOLOGY OF MICROBIAL COLONIES

A. *SURFACE COLONIES ON SOLID MEDIA*

Shape/Form	:	Irregular, circular , rhizoid, spindle, filamentous, punctiform Size
	:	Record diameter of colony in millimeter
Colour or chromogenesis	:	Colour of pigment, soluble or insoluble (impossible to detect on media containing indicator dye for pH, redox potential or inhibitor
Opacity	:	Transparent, translucent, opaque Elevation:
		Flat raised, convex, pulvinate, umbonate
Surface	:	Smooth, rough, dull, glistening, granular, waxy
Edge	:	Entire, undulate, lobate, erose, filamentous
Consistency	:	Tested by touching with a sterile loop- membraneous, friable, butyrous, viscous
Emulsifiability	:	Easily emulsifiable or diffusible in water forming uniform turbid suspension, granular suspension or do not emulsify.

B. *GROWTH ON LIQUID MEDIA*

Amount of growth	:	None, scanty, moderate, profuse
Surface growth	:	Present or absent, formation of pellicle that disintegrate on shaking or not on shaking.
Turbidity	:	Uniform, flocculant, turbid, viscoid
Deposits	:	Granular, flocculant, viscoid, disintegrate on shaking or not on shaking.

C. *GROWTH ON STAB CULTURE*

Observe growth on the surface and in depth of tubes (record depth to which growth occurs and mark position of optimal growth)

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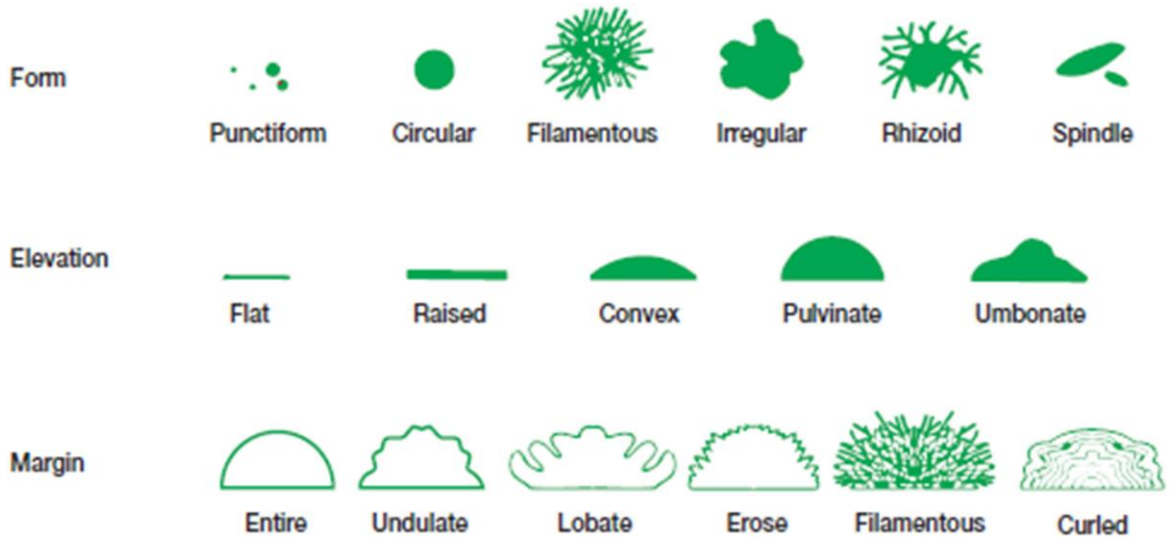

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Experiment No-8

Microbial Count Estimation by Spread Plate Culture

AIM

To enumerate the viable colonies of aerobic bacteria in the given culture or suspension.

PRINCIPLE

Generally, microorganisms present in food or water are higher in number. The examination of micro-organisms for quality evaluation of these sources involves the study of various kinds of microorganisms present and also to estimate the number of viable microorganisms. In a normal routine laboratory, the most sensitive method of detecting the presence of a viable bacterium is to allow it to reproduce itself to form a visible colony. Moreover, microorganisms such as bacteria exist in mixed population. It is rarely, pure form of a single species. It is difficult to study the culture, morphological and physiological characters of an individual species of bacteria from a mixed population. Therefore, it is essential to separate them from others, to get in the form called pure culture or axenic culture. Both isolation and enumeration studies of bacteria can be done easily by spreading them over a wide area such as a nutrient agar plate resulting in well separated microbial colonies.

There are two techniques mainly for isolation and enumeration of viable count (plate count) of bacteria.

i. Spread Plate Technique
ii. Pour Plate Technique

Microbial cell numbers are observed to be very high in sources such as soil, food etc., and hence plating out this original sample in an undiluted manner would result in confluent or evenly spreaded growth like that of a lawn. Based on statistical studies, it is widely accepted that reasonably accurate results are obtained when plates contain between 30 and 300 colonies. To obtain plates with this number of colonies it is often necessary to dilute an original sample before enumeration. The most widely used dilution technique is the ten-fold dilution series. With a completely unfamiliar sample it is necessary to plate-out a number of dilutions to ensure that some plates are obtained with counts in the desired range, but with experience of a particular material, plating only one dilution may be sufficient. The diluent used must not cause any damage, such as osmotic shock, to the micro-organisms. Sterile distilled water is therefore unsuitable. A commonly used diluent, known as maximum recovery diluent, contains 0.1% peptone and 0.85% sodium chloride.

Spread Plate Technique

In this method, a known amount of food or water sample (25 g /225 ml or 10 g /100 ml or 1 ml / 9 ml) is suspended into known volume of sterile physiological saline or peptone water so that the total volume of microbial suspension may become either 250 ml/ 100 ml / 10 ml. Known volume of suspension (1 ml) from the initial food or water suspension is transferred to additional test-tubes containing 9 ml of physiological saline to get serially diluted suspension of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-5}, 10^{-8} etc. Then a small amount (aliquot), usually 0.1 ml of respective diluted suspension is poured on the surface of a dry solid nutrient medium and is spread with the help of a sterile L-shaped glass rod. This results in the separation and isolation of propaganules of microorganisms, which can be counted from each plate. Each colony formed from a propaganule represents a "colony forming unit" (CFU). The counting of CFUs assumes that every colony is separate and founded by a single viable microbial cell. Each colony is carefully counted either manually or by using magnifying colony counter. The total colony counts obtained in CFUs from the incubated agar plates and the respective dilution factor used can then be combined to calculate the original number (extrapolated) of micro-organisms in the original sample in CFUs/ml.

The spread-plate technique provides an aerobic environment and is widely used for various plate count methods such as standard plate count, psychrophile or psychrotroph count. Surface plating offers advantages in determining the numbers of heat-sensitive psychrotrophs in a food product because the organisms do not come in contact with melted agar. It is the method of choice when the colonial features of a colony are important to its presumptive identification and for most selective media. Strict aerobes are obviously favored by surface plating, but microaerophilic organisms tend to grow slower. Among the disadvantages of surface plating are the problem of spreaders (especially when the agar surface is not adequately dry prior to plating) and the crowding of colonies, which makes enumeration more difficult.

STANDARD PLATE COUNT

The most widely used viable enumeration technique is standard plate count (SPC) or aerobic mesophilic count (APC) which indicates the sanitary and hygienic condition of a food product. A non-selective nutrient medium such as plate count agar medium (glucose tryptone yeast extract agar) is used for plating and is incubated at 30 °C aerobically to yield an aerobic mesophilic count earlier known as total plate count (TPC) or total viable count.

MATERIALS REQUIRED

1. Test sample
2. Sterile saline (0.85% NaCl)/ 0.1 % peptone water
3. Sterile Plate Count Agar or Nutrient Agar Plates- 10
4. Sterile cotton plugged culture-tubes (150 × 16 mm)

5. Sterile pipettes- 10 ml, 1.0 ml and 0.1 ml
6. Sterile L-shaped spreader
7. Alcohol in a beaker
8. Incubator

PROCEDURE

1. Preparation of sample: Solid samples are macerated using mortar and pestle or blended using a blender after suspending in required amount of saline or peptone water to make the dilution 10^{-1}. Liquid samples are mixed thoroughly and prepared 10^{-1} dilution.
2. Prepare ten plate count agar (PCA) plates, allow them to solidify and label the dilution.
3. Prepare nine sterile test-tubes containing 9 ml sterile saline or peptone water (0.1 %) and mark as 1-9
4. Holding a sterile blow-out pipette vertically, introduce the pipette tip not more than 2 cm below the surface of the sample and suck up and down 10 times to the 1 ml mark. Withdraw 1 ml of the sample, touching the tip of the pipette against the neck of the bottle to remove the excess of liquid adhering to the outside of the pipette. Transfer it to the first tube of the dilution series with the tip touching the side of the tube 2 cm above the level of the diluent. The pipette must not contact the diluting liquid. Blow out the contents of the liquid, wait three seconds and then blow out the remaining drops. Discard this pipette and label the first dilution tube as 10^{-1}.
5. Using a fresh sterile pipette, mix the contents of the first dilution tube by sucking up and down to the 1 ml mark 10 times (or by rotating the tube between the hands). The tip of the pipette should not be more than 2 cm below the surface of the diluents. Then withdraw 1 ml of this first dilution and transfer to a second tube of sterile diluent expelling the contents of the pipette as described in the step 5. Discard this pipette and label the first dilution tube as 10^{-2}.
6. Following the same procedure, dilute the suspension serially through all tubes, using fresh sterile 1ml pipette every time.
7. Dilution obtained are as follows:

Test-tube	1	2	3	4	5	6	7	8	9
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}

9. Using a fresh sterile pipette, mix the contents of the final dilution tube (i.e 10^{-9}) by sucking up and down ten times. Withdraw 1 ml of the dilution, touching the tip of the pipette against the side of the tube to remove the excess of liquid adhering to the outside of the pipette, and transfer the contents to respectively labeled PCA plates. The same

pipette can be used to transfer 1 ml from the 10^{-8} dilution and procedure may repeated for all the dilutions in the order 10^{-7} to 10^{-1}.

10. Sterilize the L-spreader by putting it first to a beaker containing 70 % ethanol and then on the flame of Bunsen burner and L-rod is cooled for 30 seconds. Remove the lid of Petri-plate in a laminar air flow and spread bacterial suspension using the L-rod while turning Petri-plates.
11. Sterilize L-rod and prepare the dilution plate (10^{-8}) as mentioned above. Cover the Petri-dish and continue the steps for all the dilutions following the steps 10-11.
12. Similarly make triplicate plates from 10^{-8} to 10^{-6} using fresh sterile pipettes.
13. Incubate the culture plates at 37 ℃ overnight and observe for growth.
14. Count the number of colonies present in all the plates and the viable count can be calculated using following equation

Viable counts = Number of colonies × Reciprocal of dilution =…… CFUs / ml

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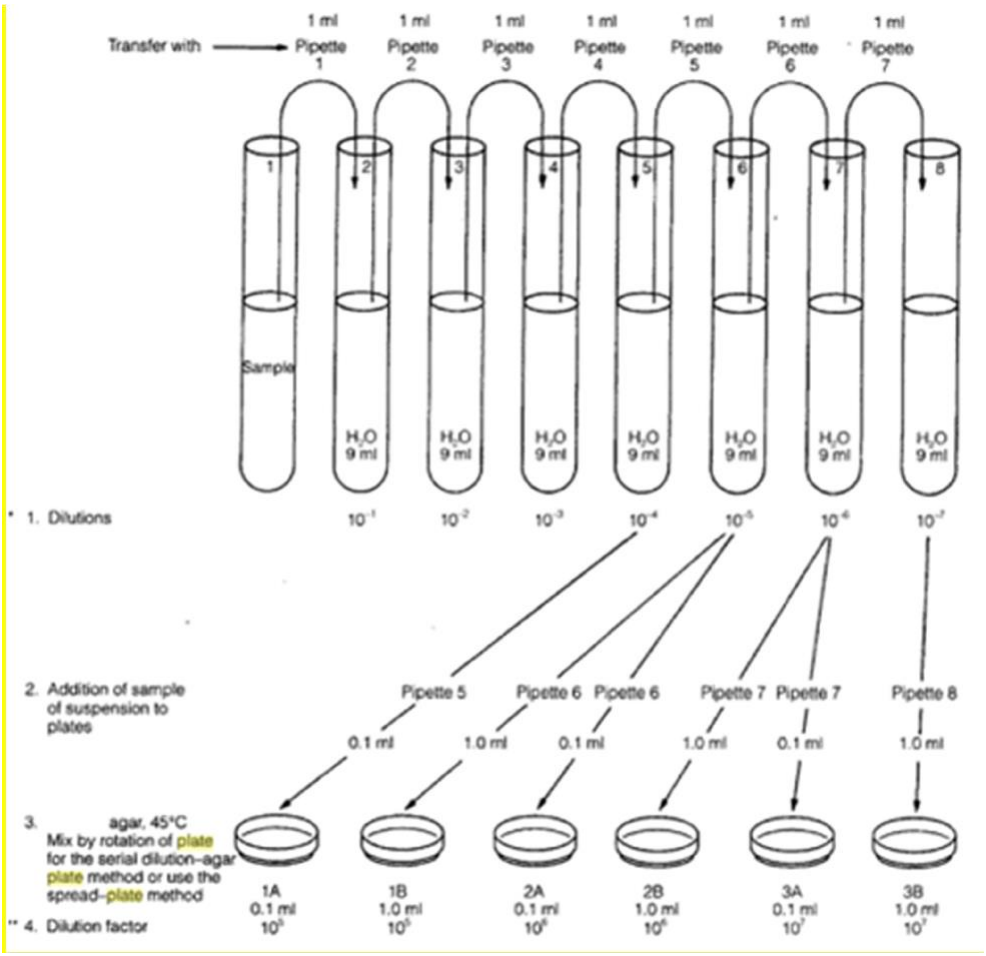
Work sheet	To be written/drawn on **the left side of the Practical Record**



OBSERVATION

Sl No	Dilution	Number of CFUs on Plate Count Agar
	10^{-1}	TNTC*
	10^{-2}	TNTC*

	10^{-3}	TNTC*
	10^{-4}	TNTC*
	10^{-5}	275
	10^{-6}	51

TNTC*- Too numerous to count

RESULT

Number of colony forming units of bacteria per 1 ml of milk = 5×10^{6}..... CFUs / ml.

Hence the aerophilic plate count per 1 ml of milk = 5×10^{6} ..... CFUs / ml

Date:…….

Experiment No-9

Microbial Count Estimation by Pour Plate Culture

AIM

To enumerate the viable colonies of anaerobic and microaerophilic bacteria in the given culture or suspension.

PRINCIPLE

Pour plate method is used to estimate the number of viable bacteria or groups of bacteria in liquid sample. Solid materials which are water soluble or give fine suspensions in water (soil, flour, milk powder, sugar) can be examined by shaking a known weight of the sample in sterile diluents. Some solid materials, including food materials (e.g. cheese, meat) need to be macerated in sterile diluent in order to prepare the suspension. In this viable count technique, the original sample is diluted in serial dilution tubes, followed by transferring a fixed amount of inoculum (generally 1 ml) from diluted tubes in the center of sterile Petri dish using a sterile pipette. Molten cooled agar (approx. 15 ml) is then poured into the Petri dish containing the inoculum and mixed well. After the solidification of the agar, the plate is inverted and incubated at 37°C for 24-48 hours. During incubation, microorganisms will grow both on the surface and within the medium. Each colony is carefully counted (using magnifying colony counter if needed). Each colony represents a "colony forming unit" (CFU). The counting of CFUs assumes that every colony is separate and founded by a single viable microbial cell. The total colony counts obtained in CFUs from the incubated agar plates and the respective dilution factor used can then be combined to calculate the original number (extrapolated) of micro-organisms in the original sample in CFUs/ml. The typical counting ranges are 30-300 colonies/ standard PCA medium plate for accuracy. The disadvantage is that there is loss of viability of heat-sensitive organisms coming into contact with hot molten agar and hence a lower count is observed when compared to spread plate technique.

MATERIALS REQUIRED

1. Test sample
2. Sterile test-tubes containing 9 ml saline (0.85% NaCl)/ sterile buffer
3. Sterile Plate Count Agar or Nutrient Agar
4. Sterile cotton plugged culture-tubes (150 × 16 mm)
5. Sterile Petri-plates

6. Sterile pipettes- 10 ml, 1.0 ml and 0.1 ml
7. Hot water bath
8. Incubator

PROCEDURE

8. Set the water-bath at 55 °C.
9. Melt the agar medium till it becomes completely fluid.
10. Using sterile 10 ml pipette, add 15 ml of sterile agar medium into each of the test-tube. Keep the tubes in water-bath at 45 °C.
11. Mark the sterile test-tubes containing 9 ml sterile saline as 1-8.
12. Holding a sterile blow-out pipette vertically, introduce the pipette tip not more than 2 cm below the surface of the sample and suck up and down 10 times to the 1 ml mark. Withdraw 1 ml of the sample, touching the tip of the pipette against the neck of the bottle to remove the excess of liquid adhering to the outside of the pipette. Transfer it to the first tube of the dilution series with the tip touching the side of the tube 2 cm above the level of the diluent. The pipette must not contact the diluting liquid. Blow out the contents of the liquid, wait three seconds and then blow out the remaining drops. Discard this pipette and label the first dilution tube as 10^{-1}.
13. Using a fresh sterile pipette, mix the contents of the first dilution tube by sucking up and down to the 1 ml mark 10 times (or by rotating the tube between the hands). The tip of the pipette should not be more than 2 cm below the surface of the diluents. Then withdraw 1 ml of this first dilution and transfer to a second tube of sterile diluent expelling the contents of the pipette as described in the step 5. Discard this pipette and label the first dilution tube as 10^{-2}.
14. Following the same procedure, dilute the suspension serially through all tubes, using fresh sterile 1ml pipette every time.
15. Dilution obtained are as follows:

Test-tube	1	2	3	4	5	6	7	8
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}

15. Using a fresh sterile pipette, mix the contents of the final dilution tube (i.e 10^{-8}) by sucking up and down ten times. Withdraw 1 ml of the dilution, touching the tip of the pipette against the side of the tube to remove the excess of liquid adhering to the outside

of the pipette, and transfer the contents to respectively labeled molten agar tubes. The same pipette can be used to transfer 1 ml from the 10^{-7} dilution and procedure may repeated for all the dilutions in the order 10^{-6} to 10^{-1}. Mix the agar tubes well by rotating the tube between the hands.

16. Pour the contents into respectively labeled sterile Petri-dishes.
17. Allow the agar to set and incubate at 37 °C for 24 hours. Make duplicate plates from the same dilutions.
18. Similarly make triplicate plates from 10^{-8} to 10^{-6} using fresh sterile pipettes.
19. Count the number of colonies present in all the plates and the viable count can be calculated using following equation

Viable counts = Number of colonies × Reciprocal of dilution =…… CFUs / ml

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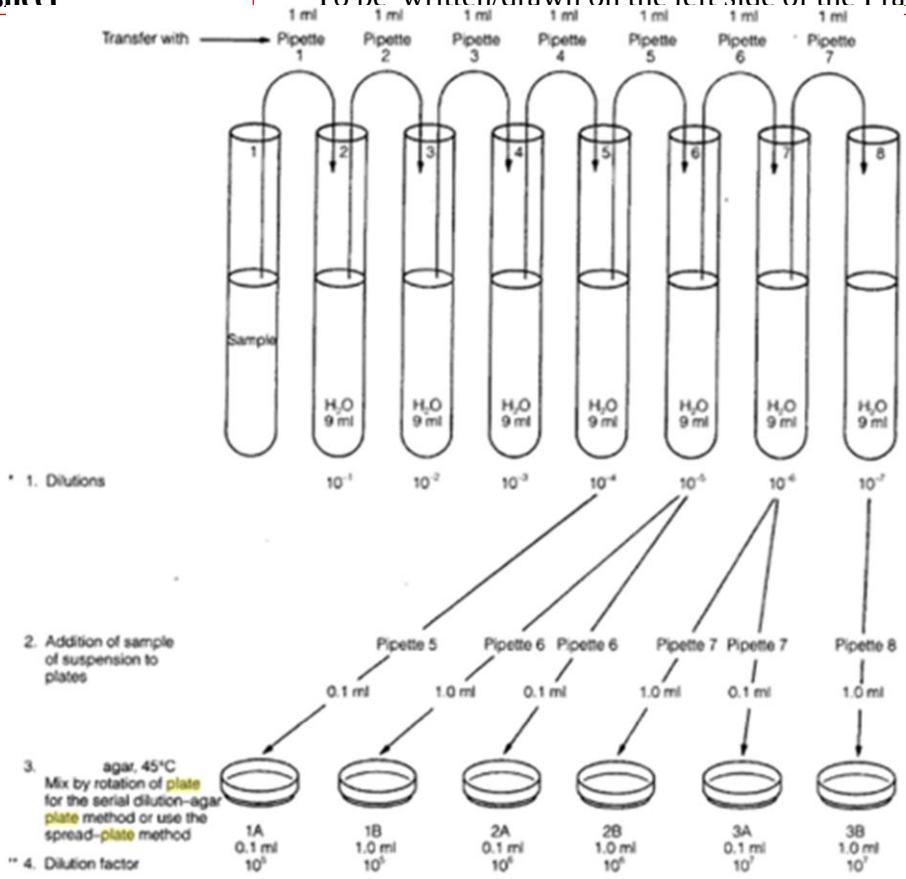
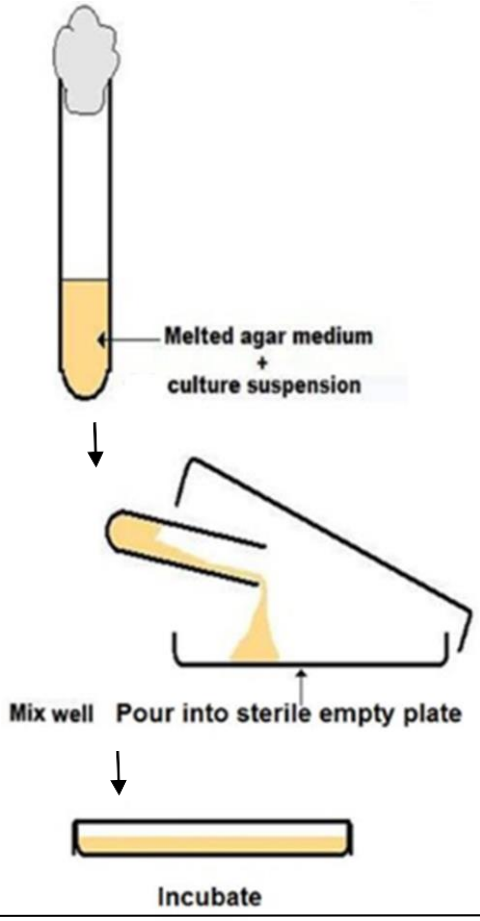
Work sheet To be written/drawn on the left side of the Practical Record





OBSERVATION

Sl No	Dilution	Number of CFUs on Plate Count Agar
	10^{-1}	TNTC*
	10^{-2}	TNTC*
	10^{-3}	288
	10^{-4}	41
	10^{-5}	2

TNTC*- Too numerous to count

RESULT

Number of colony forming units of bacteria per 1 ml of milk = 2×10^5..... CFUs / ml

Hence the pour plate count per 1 ml of milk = 2×10^5 ..... CFUs / ml

Date:…….

Experiment No-10

Determination of Growth Curve of Bacteria

AIM

To determine bacterial growth curve.

PRINCIPLE

Bacterial growth is usually referred to the reproductive increase in the population size rather than the enlargement of cell. Microbial growth can be measured by various techniques, i.e. dry weight determination of biomass, determination of total nitrogen by Kjeldahl's method, direct counting of cells under microscope or indirectly by viable count or plate count, turbidity measurements etc. Plate count method detects the number of microbes by their ability to form colonies on other surface of solidified growth medium while turbidity measurements relate to the cell number or turbidity of culture. Turbidity is not a direct measure of bacterial number, but increase in turbidity indicate bacterial growth. In this method, turbidity is measured with photoelectric colorimeter. With increase of bacterial number, the culture becomes turbid causing light to scatter off, thus less light to reach photoelectric cell. Bacterial growth curve construction requires a sample of 24 hours. Shake the culture before measuring the turbidity at certain time intervals during the incubation period. The bacterial growth curve shows 4 distinct stages: - lag phase, log phase, stationary phase and death phase. Actual length of each phase varies with organisms and environmental condition.

MATERIALS REQUIRED

1. Microbial culture- 10-20 hour old (100 ml)
2. Test tubes containing 9 ml Nutrient broth
3. Sterile pipette
4. Nutrient broth
5. Water-bath
6. Colorimeter

PROCEDURE

1. Take the lyophilized culture of microbial culture and inoculate in 100 ml nutrient broth and incubate at 37 ℃ for overnight (12hours).
2. Take 10 test tubes, each containing 9 ml nutrient broth.
3. From the stock culture, transfer 1 ml to each test-tube
4. The first reading of optical density was taken with a time interval of 1 hour, at 540 nm wavelength.
5. OD was then determined for different time periods having a time interval of 1 hour.
6. Graph was plotted between OD on Y-axis and incubation time of X-axis.

Work sheet	To be written/drawn on the left side of the Practical Record

OBSERVATION

Examination of sample

Sl No	**Time of Incubation**	**OD at 540 nm**

Paste the Graph (Write this exp in two pages)

RESULT

The organism initially had no growth, in lag phase and later it multiplied exponentially in log phase. After this, it reaches stationary phase and then decreased in growth when it reached death phase.

SEM 3 Cell and Molecular Biology

INDEX

Experiment No 1 Date…….

Study of Different Types of Cells: Prokaryotes vs Eukaryotes

Aim

To study different type of cells

Objectives

1. Identify and distinguish between prokaryotic and eukaryotic cells.
2. Observe key structural features of prokaryotic and eukaryotic cells via slides/models/charts.
3. Compare similarities and differences between prokaryotic and eukaryotic cells.
4. Sketch or model representative cells and label major parts

Background

Cells are the basic functional and structural units of life. Broadly, cells fall into two major classes: **prokaryotic** and **eukaryotic**.

Prokaryotic cells:

- Do not have a membrane-bound nucleus; their genetic material is found in a region called the nucleoid.
- Lack membrane-bound organelles (e.g., mitochondria, chloroplasts, endoplasmic reticulum) that are typical of eukaryotes.
- Are generally smaller (e.g., 0.1–5.0 μm) compared to eukaryotic cells.
- Examples include bacteria and archaea.

Eukaryotic cells:

- Have a true nucleus containing the genetic material, bound by a nuclear membrane.
- Contain a variety of membrane-bound organelles (mitochondria, Golgi apparatus, ER, chloroplasts in plants) and a cytoskeleton.
- Are typically larger (10–100 μm) and more complex.
- Include the cells of plants, animals, fungi and many protists.

Similarities: Despite differences, both types share key features: plasma membrane, cytoplasm, ribosomes, and genetic material (DNA).

Materials / Equipments

- Microscope(s) (compound light microscope)
- Prepared microscope slides: one slide of a prokaryotic cell (e.g., bacterial smear) and one or more slides of eukaryotic cells (e.g., onion epidermis, cheek epithelium)

- Models or charts showing detailed cell structure for prokaryotes and eukaryotes (either printed charts or 3D models)
- Drawing sheets or lab notebooks for sketches
- Labels/stickers or markers for parts of models/charts

Procedure

Part A: Observation of Slides

1. Label your microscope appropriately and make sure it is clean.
2. Place the prokaryotic cell slide on the stage, focus using low power, then higher magnification.
    - Observe the overall shape, arrangement (if bacteria), presence/absence of nucleus, size estimate.
    - Sketch what you see in your notebook. Label visible parts (e.g., cell wall, cell membrane, ribosomes (if visible), nucleoid region).
3. Switch to the eukaryotic cell slide (e.g., onion epidermis or cheek cells).
    - Focus and observe the nucleus, cytoplasm, cell membrane, possibly other organelles depending on stain.
    - Sketch the cell(s) and label the nucleus and other visible structures.
4. Use the models or charts of prokaryotic and eukaryotic cells to compare with your observations. Identify features that match or differ.

Part B: Model/Chart Study & Comparison

5. With the provided chart/model of a prokaryotic cell, identify key features: nucleoid, cell wall (if present), plasma membrane, possible flagella/pili.
6. With the chart/model of a eukaryotic cell (perhaps a plant cell and an animal cell), identify nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, chloroplast (in plant cell), etc.
7. On a comparison table in your notebook or worksheet, fill in similarities and differences between prokaryotic vs eukaryotic cells (size, presence of nucleus, organelles, cell wall, reproduction method, etc.). Use sources and your observation.

Results & Observations

- Sketches (attached or drawn in lab notebook)
- Table of comparisons
    - In prokaryotic slide, no distinct nucleus visible; cells were small and uniform
    - In eukaryotic slide, distinct nucleus stained dark, cell ~20 µm in diameter

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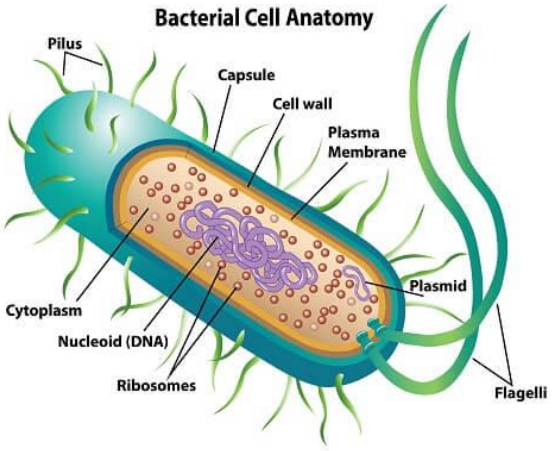
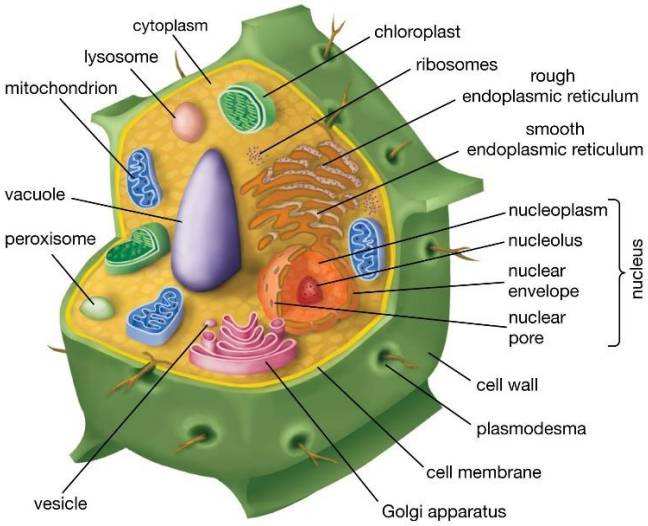
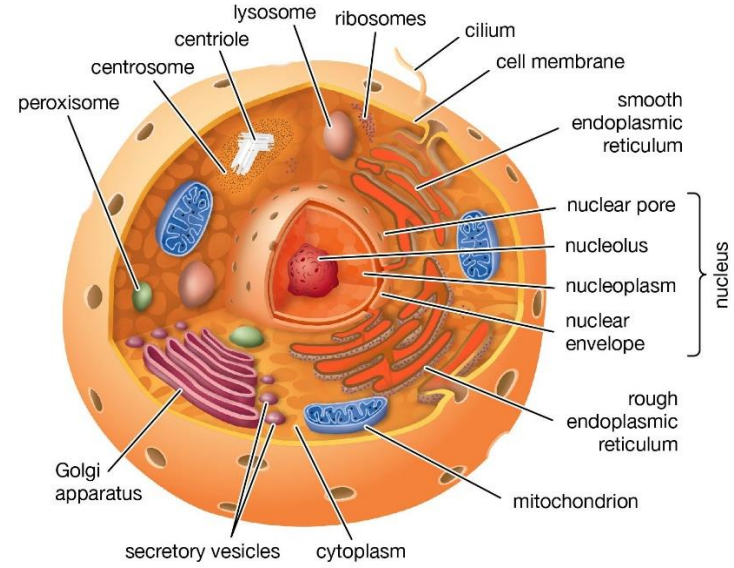
Left side



Plant cell



Animal cell



Comparison between Prokaryotic cell and Eukaryotic cell

Characteristics	Prokaryotic Cell	Eukaryotic Cell
Cell Size	Small, 1-10 µm	Large, 10-100 µm
Cell Wall	Usually present; chemically complex	Present in plant cells and fungi, chemically simple in nature
Nucleus	Absent. Instead, they have a nucleoid	Present
Nuclear membrane	Absent	Present
Cytoplasm	No streaming movement	Exhibits streaming movement
Chromosomes	only one, contains DNA & no proteins	More than one, contains DNA & proteins
DNA arrangement	Circular	Linear
Respiratory System	In plasma membrane (Mesosomes)	In mitochondria
Mitochondria	Absent	Present
E.R. & Golgi Complex	Absent	Present
Plasmids	Present	Very rarely found in eukaryotes
Ribosome	Small, 70S type	Large. 80S type
Lysosome	Lysosomes and centrosomes are absent	Lysosomes and centrosomes are present
Vacuoles	Absent	Present
Mitosis	does not occur	occurs
Exocytosis & Endocytosis	Absent	Present

Experiment No 2 **Date……..**

Study of Cytoplasmic Organelles & Cell Inclusions

Aim

To study cytoplasmic organelles and cell inclusions

Objectives

1. Identify major cytoplasmic organelles (membrane-bound and non-membrane-bound) via permanent slides, models or charts.
2. Identify cell inclusions (cytoplasmic inclusions) such as storage granules, pigments, lipid droplets etc., and differentiate them from organelles.
3. Sketch or model selected organelles and inclusions, label the parts, and explain their functions.

Background

- The cytoplasm of a cell comprises the **cytosol** (the fluid matrix), **organelles**, and **inclusions**.
- **Organelles** ("little organs") are sub-cellular structures that are often membrane-bound (in eukaryotes) or non-membrane-bound, which perform specific vital functions (e.g., mitochondria for energy, endoplasmic reticulum for protein/lipid synthesis, ribosomes for protein synthesis).
- **Cytoplasmic inclusions** are non-living components of the cytoplasm (not bound by membranes) that typically serve as storage (lipid droplets, glycogen granules), pigment granules, crystals, secretory products, etc.
- Distinguishing points:
    - Organelles carry out active metabolic functions; inclusions are inert storage or by-products.
    - Organelles often have membranes; inclusions do not (generally).
- Understanding these structures is essential for interpreting cell ultrastructure, physiology and pathology.

Materials / Equipments

- Compound light microscope (and if available, higher magnification or electron micrographs/charts)
- Permanent slides demonstrating cytoplasmic organelles (e.g., mitochondria, Golgi apparatus, endoplasmic reticulum) and slides showing cytoplasmic inclusions (e.g., liver tissue with glycogen granules, adipose tissue with lipid droplets, pigment granules)
- Models or charts of cell showing organelles (animal and plant cell)
- Chart/Poster showing cytoplasmic inclusions (glycogen, lipid, pigment crystals)
- Drawing sheets or lab notebook

Procedure

Part A: Observation of Slides — Organelles

1. Set up your microscope; clean lenses, ensure slide is clean.
2. Select a slide showing one or more organelles (for example: a slide of pancreatic cell with abundant rough ER, or liver cell with many mitochondria).
3. Place the slide, start with low magnification to locate region of interest; then increase magnification for detail.
4. Observe features such as: shape and size of organelle, location within cytoplasm, relation to nucleus or other organelles.
5. Sketch what you observe in your notebook: show outline of cell (if visible), location of organelle(s), label structure (e.g., mitochondrion, Golgi bodies, lysosome).
6. Using model/chart, identify the same organelle in the diagram/model; note features that you observed in slide vs model.
7. Record your observation (slide ID, magnification used, description of organelle, any special features).

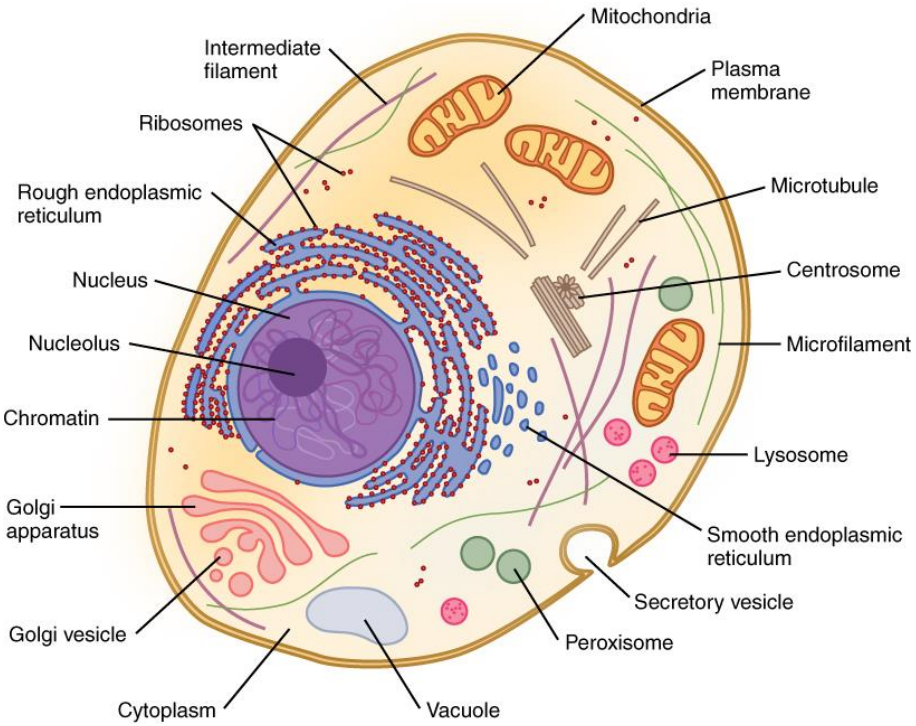
Part B: Observation of Slides — Cytoplasmic Inclusions

8. Replace with a slide showing cytoplasmic inclusions (for example: adipose tissue showing lipid droplets, liver showing glycogen granules, cells with pigment granules).
9. Focus similarly; locate inclusions, note their appearance: colour, refractility, size, distribution in cytoplasm, presence/absence of membrane boundary.
10. Sketch the inclusion(s) and cytoplasmic context; label them.
11. From the charts/models, identify the type of inclusion, note its biological significance (storage, pigment, secretory product).
12. Record your observations

Results & Observations

- Slide Observations: for each slide — magnification, description of structure observed, sketch.
- Model/Chart Observations: list of organelles and inclusions identified in models/charts.
- Comparative table: organelles vs inclusions with properties and examples.

Left side



Cell Organelles

Type	Name of Organelle	Structure / Representation in Model or Chart	Main Function
Membrane-bound organelles	Nucleus	Large spherical structure enclosed by nuclear membrane	Control center of the cell; contains genetic material (DNA)
	Mitochondria	Rod or oval-shaped with inner folds (cristae)	Site of cellular respiration and energy (ATP) production
	Endoplasmic Reticulum (ER)	Network of membranous tubules; smooth or rough	Protein (RER) and lipid (SER) synthesis, transport
	Golgi Apparatus	Stack of flattened sacs (cisternae)	Modifies, packages, and secretes cell products
	Lysosomes	Small spherical vesicles	Contain hydrolytic enzymes; involved in intracellular digestion
	Peroxisomes	Small single-membrane vesicles	Detoxification and breakdown of hydrogen peroxide
	Plastids (in plants)	Chloroplasts, chromoplasts, leucoplasts	Photosynthesis, storage, and pigment synthesis
	Vacuole	Large fluid-filled sac (prominent in plant cells)	Storage of water, food, and waste; maintains turgor pressure
Non-Membrane-bound organelles	Ribosomes	Small granules on RER or free in cytoplasm	Site of protein synthesis
	Centrioles	Cylindrical structures near nucleus (animal cells)	Help in spindle formation during cell division |
	Cytoskeleton	Network of microtubules and microfilaments	Provides shape, support, and movement to the cell

Cell Inclusions (Ergastic Substances)

Type	Example	Nature / Representation in Chart or Model	Function / Role
Reserve food materials	Starch grains (plants)	Irregular or oval white bodies	Storage form of carbohydrate
	Glycogen granules (animals)	Small granules in cytoplasm	Energy reserve
	Lipid droplets	Shiny spherical droplets	Fat storage
Pigments	Melanin, carotenoids	Colored granules	Provide color and protection
Secretory products	Tannins, resins, crystals	Irregularly shaped deposits	Protective or waste materials
Crystalline inclusions	Calcium oxalate, calcium carbonate crystals	Prismatic or raphide crystals	Structural or waste disposal role

Comparative Table: Cell Organelles vs Cell Inclusions

Feature	Cell Organelles	Cell Inclusions
Definition	Permanent, living subcellular structures performing specific metabolic functions	Non-living, temporary deposits or by-products in cytoplasm
Membrane presence	Many are membrane-bound (e.g., mitochondria, ER, Golgi)	Non-membranous (e.g., starch grains, lipid droplets)
Nature	Living components	Non-living components
Function	Perform vital metabolic activities like synthesis, respiration, secretion	Storage of reserve materials or waste products
Examples	Nucleus, mitochondria, ribosomes, Golgi apparatus	Starch grains, lipid droplets, crystals, pigments
Permanence	Usually permanent and essential for cell survival	Temporary and may appear/disappear based on metabolic state
Occurrence	Present in all living cells	Occur variably depending on cell type and function

Experiment No. 3 Date ……

Study of Stages of Mitosis – Squash Preparation of Onion Root Tip

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Aim

To study and identify different stages of mitotic cell division in onion (Allium cepa) root tip cells using the squash preparation technique.

Principle

Mitosis is the process of equational cell division in which a parent cell divides to form two daughter cells with the same number and type of chromosomes. The onion root tip is an ideal material because it contains actively dividing **meristematic cells**.
The root tips are softened using dilute HCl, stained with aceto-orcein or acetocarmine, and then gently squashed to spread the cells for microscopic observation. The chromosomes thus become clearly visible under the microscope.

Materials Required

- Fresh onion bulbs
- Beakers and watch glass
- Compound microscope
- Slides and cover slips
- Forceps and needles
- Scalpel or blade
- Filter paper or tissue paper
- **Reagents:**
    - 1 N Hydrochloric acid (HCl)
    - Aceto-orcein stain (or acetocarmine stain)
    - Distilled water
    - Ethanol (optional for cleaning slides)

Procedure

1. **Root tip preparation:**
    - Place a few onion bulbs in a beaker containing water to allow new roots (2–3 cm long) to grow.
    - Cut 1–2 cm of actively growing root tips for the experiment.
2. **Fixation:**
    - Transfer the root tips into a fixative (e.g., Carnoy's fluid or FAA) for about 24 hours to preserve cell structure.
3. **Hydrolysis (softening):**
    - Take a fixed root tip and place it in a test tube or watch glass containing **1 N HCl**.
    - Warm it gently in a water bath at **60 °C for 5 minutes** to soften the tissues.
    - Wash thoroughly with distilled water.
4. **Staining:**

- Place the softened root tip on a clean slide and add **1–2 drops of aceto-orcein** (or acetocarmine).
- Leave for **10–15 minutes** for proper staining of the chromosomes.

5. **Squash preparation:**
   - Cut off the very tip (1–2 mm) of the stained root.
   - Place a **cover slip** over the root tip.
   - Gently press (squash) with the blunt end of a needle or handle of a pencil using blotting paper to spread the cells evenly without breaking the cover slip.
6. **Microscopic observation:**
   - Observe the slide under a low-power microscope first, then under high power.
   - Identify the different **stages of mitosis** – *prophase, metaphase, anaphase,* and *telophase*.

Observations

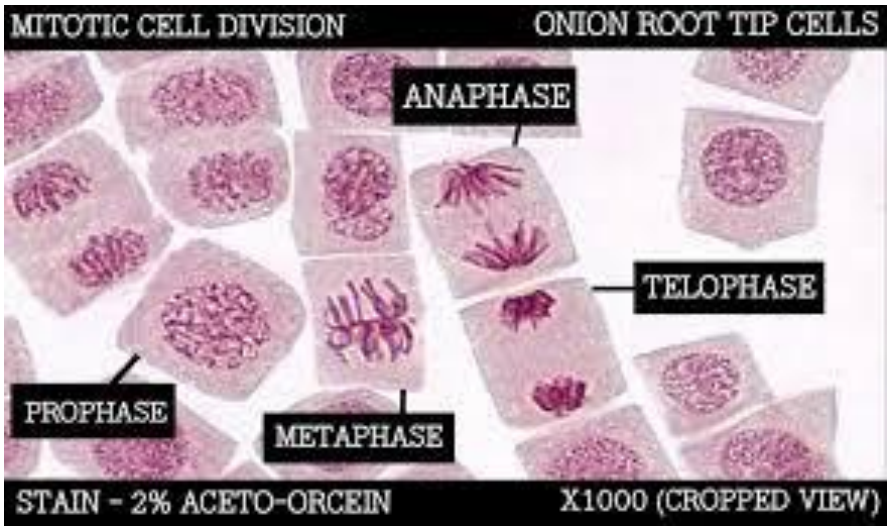
- Cells in different stages of mitosis can be seen in the prepared slide.
- Chromosomes appear as:

Stage	Observation
Prophase	Chromosomes condense and become visible; nuclear membrane starts disintegrating.
Metaphase	Chromosomes align at the equatorial plate; spindle fibers attached to centromeres.
Anaphase	Sister chromatids move to opposite poles.
Telophase	Chromosomes uncoil; nuclear membrane reappears; two daughter nuclei form.

Result

Different stages of mitosis in onion root tip cells were successfully observed under the microscope through the squash preparation technique.

Left side



Experiment No.: 4 Date ……

Preparation of Solutions and Buffers for DNA Isolation

Aim:

To prepare the required **solutions and buffers** used in the process of DNA isolation, ensuring correct concentration, pH, and sterility for effective extraction and purification of DNA from biological samples.

Principle:

DNA isolation requires specific **chemical environments** that protect DNA from degradation and help in its separation from proteins and other cellular components.
Buffers such as **Tris-HCl, EDTA, NaCl, and SDS** are commonly used:

- **Tris-HCl** maintains stable pH.
- **EDTA** chelates divalent cations (Mg^{2+}, Ca^{2+}), inhibiting DNases.
- **NaCl** stabilizes DNA and helps in protein removal.
- **SDS** (detergent) lyses cell membranes and denatures proteins.
  Correct buffer preparation ensures high-quality, intact DNA for downstream applications like PCR, electrophoresis, or sequencing.

Materials Required:

- Distilled or deionized water
- Beakers, measuring cylinders, volumetric flasks
- Magnetic stirrer and pH meter
- Pipettes and tips
- Glass bottles with labels
- **Chemicals:**
  - Tris base
  - EDTA (disodium salt)
  - Sodium chloride (NaCl)
  - Sodium dodecyl sulfate (SDS)
  - Glacial acetic acid or HCl (for pH adjustment)
  - Ethanol (for cleaning)

Procedure:

1. Preparation of 1 M Tris-HCl Buffer (pH 8.0)

1. Weigh **121.1 g Tris base** and dissolve in about 800 mL distilled water.
2. Adjust the pH to **8.0** using concentrated HCl.
3. Make up the final volume to **1 L** with distilled water.
4. Store at room temperature or 4 °C in a tightly closed bottle.

2. Preparation of 0.5 M EDTA Solution (pH 8.0)

1. Weigh **186.1 g disodium EDTA·$2H_2O$** and dissolve in 800 mL distilled water.

2. The solution will be cloudy; add NaOH pellets slowly while stirring until the solution clears and pH reaches **8.0**.
3. Make up the volume to **1 L** with distilled water.
4. Store at room temperature.

3. Preparation of 5 M NaCl Solution

1. Dissolve **292.2 g NaCl** in about 800 mL distilled water.
2. Make up to **1 L** with distilled water.
3. Sterilize by autoclaving and store at room temperature.

4. Preparation of 10% SDS Solution

1. Weigh **10 g SDS powder** and dissolve in 90 mL distilled water by gentle heating (do not boil).
2. Adjust pH to **7.2** if required.
3. Make up to **100 mL** and store at room temperature.

5. Preparation of TE Buffer (Tris-EDTA, pH 8.0)

1. Mix **10 mM Tris-HCl** and **1 mM EDTA** (using stock solutions prepared above).
2. Adjust pH to **8.0**.
3. Store in refrigerator at 4 °C.

Result:

Various buffers and solutions required for DNA isolation — **Tris-HCl, EDTA, NaCl, SDS, and TE buffer** — were successfully prepared with correct molarity and pH for use in the DNA extraction process.

Experiment No.: 5 Date……..

Isolation of Genomic DNA from Small Onion (*Allium cepa*)

Aim:

To isolate and visualize **genomic DNA** from small onion cells using a simple extraction procedure involving cell lysis, removal of proteins and polysaccharides, and DNA precipitation.

Principle:

Genomic DNA can be isolated from plant tissues by **breaking open the cells** and **releasing DNA** into solution. The onion is an ideal source because it has **large cells with a low starch content**, making DNA easy to extract.

The process involves:

1. **Cell lysis** using detergent (SDS or liquid soap) to disrupt cell membranes.
2. **Salt (NaCl)** to neutralize DNA's negative charges and aid aggregation.
3. **Chloroform isoamyl alcohol** is used for denaturation and precipitation of proteins and lipids.
4. **Chilled isopropanol** is used for the precipitation of DNA by engaging with the water molecule and not allowing DNA to get dissolved in water
5. **Filtration and protein removal** to obtain a clear extract.
6. **Cold isopropanol** addition, which precipitates DNA as visible white threads.

Materials Required:

Apparatus:

- Mortar and pestle
- Beakers, test tubes, and glass rods
- Measuring cylinder
- Funnel and filter paper
- Water bath and ice bath
- Micropipette or dropper

Reagents:

- Small onions (fresh)
- **Extraction buffer** containing:
    - 100 mM Tris-HCl (pH 8.0)
    - 50 mM EDTA
    - 500 mM NaCl
    - 1% SDS (or liquid detergent)

  Take 5.96g EDTA and dissolved in 100ml distilled water. To this add 9.68g of Tris salt and mix well. Add 23.36g sodium chloride to the above solution and dissolve. Weigh out 13.6g SDS and stir well. Adjust pH to 8.3 and make upto 800ml.

- **Pottassium acetate (250ml)**

122.68g potassium acetate in 100ml distilled water and adjust the pH to 5.8 using 0.1M HCl and make up the final volume to 250ml

- **Chloroform- isoamyl alcohol (24:1) – 800ml**
  Mix 768ml chloroform and 82ml isoamyl alcohol
- **Chilled Isopropanol**

Procedure:

1. **Sample preparation:**
   - Peel a small onion and cut into small pieces.
   - Grind 5g the onion pieces in a mortar with 10 ml of extraction buffer until a fine paste is obtained.
2. **Cell lysis:**
   - Transfer the paste into a beaker and add more extraction buffer (about 10 mL).
   - Stir gently for 5–10 minutes to lyse cells and release DNA into solution.
3. **Filtration:**
   - Filter the homogenate through a funnel lined with filter paper or cheese cloth into a clean test tube or beaker to remove debris.
   - The filtrate is incubated at 65^0C for 10 min. in a water bath.
4. **Protein removal:**
   - Add 5ml potassium acetate buffer and mix gently for 5 min.
   - Add equal volume of chloroform- isoamyl alcohol (24:1) and shake well for 5 minutes.
   - Centrifuge at 10000 rpm for 5 minutes at 4^0C
5. **DNA precipitation:**
   - Carefully collect aqueous phase and add an equal volume of **chilled isopropanol** along the side of the tube
   - The precipitated DNA can be seen as fibrous strands.
6. **DNA collection:**
   - Use a glass rod or pipette tip to spool and collect the DNA strands carefully.
   - Transfer to a small tube containing TE buffer or distilled water for storage.

Result:

White, fibrous strands of **genomic DNA** were successfully isolated from small onion tissue. The DNA precipitated as visible filaments in the isopropanol, confirming successful extraction.

Experiment No.: 6 Date…….

Examination of the Purity of DNA by Agarose Gel Electrophoresis

---

Aim:
To examine the **purity and integrity** of isolated DNA using **agarose gel electrophoresis**.

Principle:
Agarose gel electrophoresis is a technique used to **separate and analyze nucleic acids** (DNA or RNA) based on their size and charge.
DNA molecules are **negatively charged** due to their phosphate backbone and therefore migrate towards the **positive electrode (anode)** when an electric field is applied.
The agarose gel acts as a **molecular sieve**—smaller DNA fragments migrate faster, while larger fragments move slower. The separated DNA bands can be visualized by staining with **ethidium bromide** or **safe DNA stains** under UV or blue light.
The **purity of DNA** can be determined by observing:

- The sharpness and clarity of DNA bands
- Absence of smearing (which indicates degradation)
- Comparison of intensity with standard DNA ladder

Materials Required:
Apparatus:

- Agarose gel electrophoresis unit
- Power supply
- Micropipettes and tips
- Gel casting tray and combs
- UV transilluminator or blue light viewer
- Beakers and conical flasks
- Water bath or microwave oven

Reagents:

- Agarose powder
- 1× TAE or TBE buffer
- Ethidium bromide (or non-toxic DNA stain like SYBR Safe)
- DNA loading dye (bromophenol blue)
- DNA ladder (molecular weight marker)
- DNA sample (previously isolated)
- Distilled water

Procedure:

1. **Preparation of Agarose Gel:**
    - Weigh **0.8–1.0 g of agarose** and dissolve it in **100 mL of 1× TAE buffer** by gentle heating (microwave or water bath).

- Cool the solution to about **50–55 °C**, then add a few drops of **ethidium bromide (final concentration 0.5 µg/mL)** or use a safe DNA stain.
- Pour the gel into a casting tray with combs in place and allow it to solidify (15–20 minutes).

2. **Preparation of Samples:**
   - Mix **5 µL of DNA sample** with **1 µL of loading dye** in a microtube.
   - Prepare standard DNA ladder similarly for comparison.
3. **Setting up the Electrophoresis:**
   - Place the solidified gel in the electrophoresis tank and add **1× TAE buffer** until the gel is just submerged.
   - Carefully load DNA samples and the DNA ladder into the wells using a micropipette.
4. **Running the Gel:**
   - Connect the electrodes correctly (DNA migrates toward the positive/red terminal).
   - Run the gel at **80–100 V for 30–45 minutes** until the tracking dye migrates about 3/4th of the gel length.
5. **Visualization:**
   - After electrophoresis, view the gel under **UV transilluminator** or **blue light viewer**.
   - Photograph the gel if required for record.

Observation:

- Intact, pure DNA appears as a **sharp, high-molecular-weight band** near the loading well.
- Degraded DNA shows **smearing**.
- Contaminated DNA may appear with **multiple faint bands** or irregular migration patterns.

Result:

The agarose gel electrophoresis successfully demonstrated the **presence and purity of DNA**. A clear, distinct DNA band without smearing indicates **pure and intact genomic DNA**, suitable for further molecular biology applications.

Experiment No.: 7 **Date…….**

Quantification of DNA by UV–Spectrophotometer

Aim:

To determine the **concentration and purity of DNA** using a **UV–spectrophotometer** by measuring its absorbance at 260 nm and 280 nm.

Principle:

Nucleic acids (DNA and RNA) absorb ultraviolet (UV) light strongly at **260 nm** due to the presence of **aromatic bases** (adenine, guanine, cytosine, thymine). Proteins absorb maximally at **280 nm** due to aromatic amino acids like tryptophan and tyrosine.

By measuring the absorbance (optical density, OD) of a DNA solution at these two wavelengths:

- **A_{260}** gives an estimate of nucleic acid concentration.
- **A_{260}/A_{280} ratio** gives an indication of DNA purity.

Interpretation of Purity Ratio:

A_{260}/A_{280} Ratio	Interpretation
~1.8	Pure DNA
~2.0	Pure RNA
<1.7	Protein or phenol contamination

The DNA concentration is calculated using the formula:

$$\text{DNA concentration (µg/mL)} = A_{260} \times 50 \times \text{Dilution factor}$$

(Where **50 µg/mL** is the absorbance constant for double-stranded DNA at $A_{260} = 1.0$)

Materials Required:

Apparatus:

- UV–Visible spectrophotometer
- Quartz cuvettes
- Micropipettes and tips
- Vortex mixer or microfuge tubes

Reagents:

- Isolated DNA sample
- TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) or distilled water (as blank)

Procedure:

1. **Instrument setup:**
    - Switch on the UV–spectrophotometer and allow it to stabilize for 10–15 minutes.
    - Select **wavelengths 260 nm and 280 nm** for readings.
2. **Blank calibration:**
    - Fill a clean quartz cuvette with **TE buffer** or distilled water (used to dissolve DNA).
    - Place it in the spectrophotometer and set the instrument to **zero absorbance** (blanking).
3. **Sample loading:**

- Pipette **2–3 µL of DNA sample** into another quartz cuvette.
- Measure the **absorbance at 260 nm (A_{260})** and **280 nm (A_{280})**.
- Record the readings.

4. **Calculation:**
   - Calculate the **DNA concentration** using the formula:

     $$\text{DNA concentration (µg/mL)} = A_{260} \times 50 \times \text{Dilution factor}$$

   - Calculate the **A_{260}/A_{280} ratio** to assess DNA purity.

Result:

The absorbance readings at 260 nm and 280 nm indicated a **DNA concentration of approximately 45 µg/mL** with an **A_{260}/A_{280} ratio of 1.8**, confirming that the DNA sample is **pure and free from major protein contamination**.

Left side

Observation Table

Sample	**A_{260}**	**A_{280}**	**A_{260}/A_{280} Ratio**	**DNA Concentration (µg/mL)**	**Remark**
DNA Sample	0.90	0.50	1.8	45	Pure DNA

Experiment No.: 8 **Date…….**

Isolation and Purification of Plasmid DNA

Aim:

To isolate and purify **plasmid DNA** from bacterial cells using the **alkaline lysis method**.

Principle:

Plasmids are small, circular, double-stranded extrachromosomal DNA molecules found in many bacteria. They can replicate independently of chromosomal DNA and often carry genes for antibiotic resistance or other traits.

The **alkaline lysis method** is commonly used for plasmid DNA extraction. It involves the following steps:

1. **Cell lysis:** Bacterial cells are lysed by treatment with an alkaline SDS solution (containing NaOH and SDS).
2. **Denaturation:** The alkaline condition denatures both chromosomal and plasmid DNA.
3. **Neutralization:** When a neutralization buffer (containing potassium acetate) is added, small circular plasmid DNA renatures while the large chromosomal DNA and proteins precipitate.
4. **Separation:** The plasmid DNA remains in the supernatant and can be further purified by alcohol precipitation or column purification.

This method yields plasmid DNA free from major contaminants and suitable for restriction digestion, ligation, and transformation.

Materials Required:

Apparatus:

- Microcentrifuge tubes (1.5 mL)
- Micropipettes and tips
- Centrifuge
- Water bath or heating block
- Vortex mixer
- Ice bath

Reagents:

- Bacterial culture (E. coli with plasmid)
- **Solution I:** 50 mM Glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA
- **Solution II (Lysis buffer):** 0.2 N NaOH, 1% SDS
- **Solution III (Neutralization buffer):** 3 M Potassium acetate (pH 5.2)
- **Phenol:Chloroform:Isoamyl alcohol (25:24:1)**
- **Absolute ethanol** and **70% ethanol**
- **TE buffer** (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Procedure:

1. **Culture Preparation:**
    - Inoculate a single bacterial colony containing the desired plasmid into 5 mL LB broth with appropriate antibiotic.
    - Incubate overnight at 37°C with shaking (150–200 rpm).
2. **Cell Harvesting:**

- Transfer 1.5 mL of the culture into a microcentrifuge tube.
- Centrifuge at 8,000 rpm for 5 minutes to pellet the cells.
- Discard the supernatant.

3. **Cell Resuspension:**
   - Resuspend the pellet in **100 µL of Solution I** by gentle pipetting or vortexing.
4. **Cell Lysis:**
   - Add **200 µL of Solution II**, mix gently by inverting the tube (do not vortex).
   - Incubate on ice for 2–5 minutes until the solution becomes clear.
5. **Neutralization:**
   - Add **150 µL of Solution III**, mix gently by inversion.
   - A white precipitate will form.
   - Incubate on ice for 10 minutes.
6. **Centrifugation:**
   - Centrifuge at 10,000 rpm for 10 minutes.
   - Transfer the clear supernatant (containing plasmid DNA) to a new microtube.
7. **Purification:**
   - Add an equal volume of **phenol:chloroform:isoamyl alcohol (25:24:1)** to the supernatant.
   - Mix gently and centrifuge for 10 minutes at 10,000 rpm.
   - Transfer the upper aqueous layer to a new tube.
8. **DNA Precipitation:**
   - Add **2 volumes of cold absolute ethanol**, mix gently, and incubate at –20°C for 10–15 minutes.
   - Centrifuge at 10,000 rpm for 10 minutes.
   - Discard the supernatant carefully.
9. **DNA Washing:**
   - Wash the pellet with **70% ethanol**, centrifuge again for 5 minutes, and air-dry the pellet.
10. **DNA Re-suspension:**
    - Dissolve the purified plasmid DNA pellet in **30–50 µL of TE buffer**.
    - Store at 4°C for short-term or –20°C for long-term use.

Result:

Plasmid DNA was successfully isolated and purified from bacterial cells. The purified DNA appeared as a **white pellet** after ethanol precipitation and can be confirmed by **agarose gel electrophoresis** showing distinct plasmid bands.

Left side

Observation Table

Step	Observation
Cell lysis	Solution turned clear
Neutralization	White precipitate formed

Step	Observation
After centrifugation	Clear supernatant obtained
After ethanol precipitation	Visible white DNA pellet formed

Experiment No.: 9 **Date…….**

Agarose Gel Analysis of Plasmid DNA

Aim:
To analyze the integrity, purity, and conformation of plasmid DNA using agarose gel electrophoresis.

Principle:
Agarose gel electrophoresis is a technique used to separate and visualize nucleic acids based on their size, charge, and conformation.
Plasmid DNA exists in several forms —

- Supercoiled (covalently closed circular)
- Relaxed/nicked circular
- Linear

These forms migrate differently through the agarose matrix when an electric field is applied:

- Supercoiled DNA moves fastest (most compact form)
- Linear DNA migrates moderately
- Nicked circular DNA moves slowest

The agarose gel acts as a molecular sieve, and the negatively charged DNA migrates toward the positive electrode. After electrophoresis, DNA bands are visualized using a fluorescent dye (e.g., ethidium bromide or SYBR Safe) under UV or blue light.

Materials Required:
Apparatus:

- Agarose gel electrophoresis unit
- Power supply
- Gel casting tray and combs
- Micropipettes and tips
- UV transilluminator or blue light viewer
- Beakers, flasks, and measuring cylinders
- Water bath or microwave oven

Reagents:

- Agarose powder
- 1× TAE or TBE buffer
- Ethidium bromide (0.5 µg/mL) or non-toxic stain (e.g., SYBR Safe)
- DNA loading dye (bromophenol blue/xylene cyanol)
- DNA ladder (molecular weight marker)
- Plasmid DNA sample (isolated)
- Distilled water

Procedure:

1. **Preparation of Gel:**
    - Weigh **0.8–1.0 g agarose** and dissolve it in **100 mL of 1× TAE buffer** by gentle heating in a microwave or water bath.

- Cool to ~55 °C, then add a few drops of **ethidium bromide** (or use post-staining with a safe dye).
- Pour the gel into a **casting tray** with a comb in place.
- Allow it to **solidify (15–20 minutes)** at room temperature.

2. **Loading of Samples:**
   - Remove the comb carefully to create wells.
   - Place the gel in the electrophoresis tank and **add 1× TAE buffer** until the gel is submerged.
   - Mix **2–3 µL plasmid DNA** with **1 µL loading dye** and load into wells.
   - Load **DNA ladder** in one well as reference.
3. **Running the Gel:**
   - Connect the electrodes correctly (DNA migrates from **cathode to anode**).
   - Run the gel at **80–100 V for 45–60 minutes** until the tracking dye migrates 3/4th of the gel length.
4. **Visualization:**
   - After electrophoresis, visualize the gel under **UV transilluminator** or **blue light**.
   - Observe and record the **number, position, and brightness** of bands.
   - Photograph the gel for documentation.

Result:

Plasmid DNA was successfully analyzed by agarose gel electrophoresis. The gel showed distinct bands corresponding to supercoiled, relaxed, and linear forms of plasmid DNA. The clarity and integrity of these bands indicate that the plasmid DNA was intact and of good purity.

Left side

Observation Table:

Sample	Observed Bands	Type of Band	Interpretation
Plasmid DNA	2–3 bands	Supercoiled, Linear, Nicked	Different conformations of plasmid DNA
DNA Ladder	Multiple bands	Size standards	Used for molecular weight comparison

SEM 4 IMMUNOLOGY AND IMMUNOTECHNOLOGY

INDEX

LAB MANUAL

Experiment No 1

Hematological Reactions: Blood Grouping and Rh Typing

Aim

To determine the ABO blood group and Rh factor of a given blood sample using antigen–antibody agglutination reactions.

Principle

Blood grouping is based on the presence or absence of specific antigens on the surface of red blood cells (RBCs).

- **ABO System**:
  - Antigen A → Group A
  - Antigen B → Group B
  - Both antigens → Group AB
  - No antigen → Group O
- **Rh System**:
  - Presence of Rh (D) antigen → Rh positive (+)
  - Absence of Rh antigen → Rh negative (−)

When blood is mixed with specific antisera (Anti-A, Anti-B, Anti-D), **agglutination (clumping)** occurs if the corresponding antigen is present.

Materials Required

- Clean glass slide or tile
- Sterile lancet/needle
- Antisera: Anti-A, Anti-B, Anti-D
- Dropper or micropipette
- Toothpicks or applicator sticks
- Cotton and спирit/alcohol swab
- Marker pen
- Biohazard disposal container

Procedure

A. ABO Blood Grouping

1. Clean the fingertip with sprit and allow it to dry.
2. Prick the finger using a sterile lancet.
3. Place **three separate drops of blood** on a clean slide.
4. Label them as **A, B, and D**.
5. Add:
   - Anti-A serum to spot A
   - Anti-B serum to spot B

6. Mix each spot using separate applicator sticks.
7. Gently rock the slide for 1–2 minutes.
8. Observe for agglutination.

B. Rh Typing

1. Add Anti-D serum to the third blood drop (spot D).
2. Mix gently with a clean applicator stick.
3. Observe for agglutination.

Observation Table (Left side)

Sample	Anti-A	Anti-B	Anti-D	Blood Group
	+ / −	+ / −	+ / −	

(+ = Agglutination, − = No agglutination)

Interpretation

Agglutination Pattern Blood Group

Anti-A (+), Anti-B (−) A

Anti-A (−), Anti-B (+) B

Anti-A (+), Anti-B (+) AB

Anti-A (−), Anti-B (−) O

- **Anti-D (+)** → Rh positive
- **Anti-D (−)** → Rh negative

Result

The given blood sample belongs to…… **(ABO group) and Rh ……....._____ (positive/negative)**.

Experiment No. 2

Separation of Immune Cells from Lymphoid Organs / Blood

Aim

To isolate immune cells (lymphocytes) from lymphoid organs or peripheral blood using standard laboratory techniques.

Principle

Immune cells such as lymphocytes can be separated based on their **density and size**.

- From **blood**: Separation is commonly done using **density gradient centrifugation** (e.g., Ficoll method), where mononuclear cells form a distinct layer.
- From **lymphoid organs (spleen/lymph nodes)**: Cells are obtained by **mechanical disruption** followed by filtration to remove debris.

Materials Required

- Fresh blood sample / lymphoid tissue (spleen/lymph node)
- Anticoagulant (EDTA or heparin)
- Phosphate Buffered Saline (PBS)
- Ficoll or similar density gradient medium
- Centrifuge tubes
- Centrifuge
- Sterile scissors and forceps
- Nylon mesh or cell strainer
- Pasteur pipette / micropipette
- Glass slides
- Trypan blue (optional, for viability check)
- Hemocytometer (optional)

Procedure

A. Separation from Blood (Density Gradient Method)

1. Collect blood in a tube containing anticoagulant.
2. Dilute blood with equal volume of PBS.
3. Carefully layer diluted blood over Ficoll solution in a centrifuge tube.
4. Centrifuge at ~2000 rpm for 20–30 minutes.
5. After centrifugation, observe layers:
    - Plasma (top)
    - **Buffy coat (mononuclear cells)**
    - Ficoll layer
    - RBCs (bottom)
6. Carefully collect the buffy coat using a pipette.
7. Wash cells with PBS and centrifuge again to remove Ficoll.
8. Resuspend cells in PBS or culture medium.

B. Separation from Lymphoid Organs

1. Dissect the lymphoid organ (e.g., spleen) under sterile conditions.
2. Place tissue in cold PBS.

3. Gently mince the tissue using forceps/scissors.
4. Pass the homogenate through a nylon mesh or cell strainer.
5. Collect the cell suspension in a centrifuge tube.
6. Centrifuge at ~1500 rpm for 5–10 minutes.
7. Discard supernatant and resuspend the pellet in PBS.
8. (Optional) Remove RBCs using RBC lysis buffer.
9. Obtain a clean suspension of immune cells.

Observation

- Formation of a **distinct buffy coat layer** in blood samples.
- Turbid cell suspension from lymphoid organs indicating presence of cells.

Result

Immune cells (lymphocytes) were successfully isolated from the given **blood / lymphoid tissue sample**.

Experiment No. 3

Identification of Various Immune Cells by Morphology using Leishman and Giemsa Staining

Aim

To identify different types of immune cells in a blood smear based on morphological characteristics using Leishman and Giemsa staining techniques.

Principle

Leishman and Giemsa stains are **Romanowsky-type stains** used for differential staining of blood cells.

- These stains contain **eosin (acidic dye)** and **methylene blue/azure (basic dyes)**.
- Acidic components (e.g., cytoplasm, hemoglobin) stain **pink/red**.
- Basic components (e.g., nucleus) stain **blue/purple**.

Different immune cells (leukocytes) can be identified based on **size, nucleus shape, and cytoplasmic granules**.

Materials Required

- Fresh blood sample
- Clean glass slides
- Sterile lancet
- Leishman stain
- Giemsa stain
- Buffer solution (pH 6.8)
- Dropper / micropipette
- Distilled water
- Microscope
- спирit/alcohol swab
- Cotton

Procedure

A. Preparation of Blood Smear

1. Clean fingertip with sprit and allow to dry.
2. Prick the finger using a sterile lancet.
3. Place a small drop of blood on a clean slide.
4. Prepare a **thin smear** using another slide at a 45° angle.
5. Air dry the smear.

B. Leishman Staining

1. Flood the smear with Leishman stain.
2. Allow it to fix for 1–2 minutes.
3. Add equal volume of buffer solution.
4. Mix gently and leave for 8–10 minutes.
5. Wash with distilled water.
6. Air dry the slide.

C. Giemsa Staining

1. Fix the smear with methanol for 2–3 minutes.
2. Dilute Giemsa stain with buffer (1:10).
3. Flood the smear with diluted stain.
4. Leave for 15–20 minutes.
5. Wash gently with distilled water.
6. Air dry the slide.

Observation

Examine the stained smear under a microscope (oil immersion). Identify cells based on morphology.

Identification of Immune Cells (Left side)

Cell Type	Morphological Features
Neutrophils	Multi-lobed nucleus, fine granules
Lymphocytes	Large round nucleus, thin cytoplasm
Monocytes	Kidney-shaped nucleus, abundant cytoplasm
Eosinophils	Bilobed nucleus, large red/orange granules
Basophils	Irregular nucleus, dark blue granules

Add digram

Result

Different immune cells were identified based on their morphological characteristics using Leishman and Giemsa staining.

Experiment No 4

Enumeration of RBC and WBC using Haemocytometer

Aim

To determine the number of red blood cells (RBCs) and white blood cells (WBCs) per cubic millimeter of blood using a haemocytometer.

Principle

A haemocytometer is a specialized counting chamber used to count cells in a known volume of diluted blood.

- Blood is diluted using specific diluting fluids:
  - **RBC diluting fluid**: Preserves RBCs and prevents clumping
  - **WBC diluting fluid (Turk's fluid)**: Lyses RBCs and stains WBC nuclei
- Cells are counted in a defined grid area, and total cell count is calculated using a standard formula.

Materials Required

- Haemocytometer (Neubauer chamber)
- Cover slip
- Microscope
- Sterile lancet
- RBC pipette and WBC pipette
- RBC diluting fluid

- WBC diluting fluid (Turk's fluid)
- спирit/alcohol swab
- Cotton

Procedure

A. RBC Count

1. Clean fingertip with sprit and prick using a sterile lancet.
2. Draw blood up to 0.5 mark in RBC pipette.
3. Add RBC diluting fluid up to 101 mark (dilution 1:200).
4. Mix well by shaking the pipette.
5. Discard the first few drops.
6. Charge the haemocytometer by placing a drop under the cover slip.
7. Allow cells to settle for 2 minutes.
8. Observe under microscope.
9. Count RBCs in **5 small squares** of the central large square.

B. WBC Count

1. Draw blood up to 0.5 mark in WBC pipette.
2. Add WBC diluting fluid up to 11 mark (dilution 1:20).
3. Mix thoroughly.
4. Discard first few drops.
5. Charge the haemocytometer.
6. Allow cells to settle.
7. Count WBCs in **4 large corner squares**.

Counting Formula

RBC Count

$$\text{RBC count} = \frac{\text{Number of cells counted} \times \text{Dilution factor}}{\text{Area counted} \times \text{Depth}}$$

Final simplified form:
RBC count = Cells counted × 10,000 (cells/mm³)

WBC Count

Final simplified form:
WBC count = Cells counted × 50 (cells/mm³)

Observation Table (Left side)

Parameter	Cells Counted	Calculated Count (cells/mm³)
RBC		

Parameter	Cells Counted	Calculated Count (cells/mm³)
WBC		

Result

- RBC count of the given sample = _____ cells/mm³
- WBC count of the given sample = _____ cells/mm³

Normal Values (Left side)

Cell Type	Normal Range
RBC	4.5 – 6.0 million cells/mm³
WBC	4,000 – 11,000 cells/mm³

Experiment No.5

Agglutination Reactions: Latex Agglutination Test

Aim

To detect the presence of specific antigens or antibodies in a sample using the latex agglutination reaction.

Principle

Latex agglutination is based on an **antigen–antibody reaction** in which latex particles are coated with either antibodies or antigens.

- When mixed with a test sample containing the corresponding antigen or antibody, **visible clumping (agglutination)** occurs.
- If the specific antigen/antibody is absent, no agglutination is observed.

This reaction is rapid, sensitive, and widely used for diagnostic purposes.

Materials Required

- Latex reagent (coated latex particles)
- Test sample (serum/plasma)
- Positive and negative controls
- Glass slide or reaction card
- Mixing sticks/applicator sticks
- Dropper/micropipette
- Timer

Procedure

1. Bring all reagents and samples to room temperature.
2. Place a drop of test sample on a marked area of the slide/card.
3. Add one drop of latex reagent to the sample.
4. Mix gently using a clean applicator stick.
5. Spread the mixture over the test circle.
6. Gently rotate the slide for **2–3 minutes**.
7. Observe for visible agglutination.

Controls

- **Positive control**: Should show agglutination.
- **Negative control**: Should show no agglutination.

Controls must be tested alongside the sample to validate results.

Observation (Left side)

Sample	Agglutination	Interpretation
Test sample	+ / −	Positive / Negative
Positive control	+	Valid
Negative control	−	Valid

Result

- Presence of agglutination indicates a **positive reaction** (antigen/antibody present).
- Absence of agglutination indicates a **negative reaction** (antigen/antibody absent).

Experiment No.6

Precipitation Reactions in Gels: Ouchterlony Double Immunodiffusion (ODD) and Mancini's Single Radial Immunodiffusion (SRID)

Aim

To study antigen–antibody precipitation reactions in agar gel using:

1. Ouchterlony Double Immunodiffusion (qualitative analysis)
2. Mancini's Single Radial Immunodiffusion (quantitative analysis)

Principle

1. Ouchterlony Double Immunodiffusion (ODD):
Both antigen and antibody diffuse through agar gel from separate wells. Where they meet at optimal proportions, a **visible precipitin line** is formed.

- Identity → continuous line
- Non-identity → crossed lines
- Partial identity → spur formation

2. Single Radial Immunodiffusion (SRID):
Antibody is uniformly incorporated in agar gel, and antigen diffuses radially from a well.

- A **precipitin ring** forms around the well.
- The **diameter of the ring is proportional to antigen concentration**.

Materials Required

- Agar or agarose powder
- Glass slides or Petri dishes
- Template/well cutter
- Antigen samples
- Specific antiserum

- Micropipette/dropper
- Incubator (optional)
- Ruler or scale

Procedure

A. Ouchterlony Double Immunodiffusion (ODD)

1. Prepare 1% agar gel and pour onto a clean slide or plate.
2. Allow gel to solidify.
3. Punch wells in a pattern (central well surrounded by peripheral wells).
4. Add:
    - Antibody in central well
    - Different antigen samples in surrounding wells
5. Incubate at room temperature (24–48 hours).
6. Observe formation of precipitin lines between wells.

B. Mancini's Single Radial Immunodiffusion (SRID)

1. Prepare agar gel containing uniform antibody.
2. Pour onto a plate and allow to solidify.
3. Punch wells in the gel.
4. Add antigen samples of known and unknown concentrations into wells.
5. Incubate at room temperature (24–48 hours).
6. Measure the diameter of precipitin rings.

Observation

ODD

- Formation of precipitin lines between antigen and antibody wells.

SRID

Sample	Ring Diameter (mm)	Antigen Concentration
Standard		
Unknown		

Graph (SRID)

Plot **square of ring diameter (D^2)** against antigen concentration to determine unknown concentration.

Result

- In ODD, antigen–antibody relationships (identity/non-identity) were determined.
- In SRID, the concentration of antigen in the given sample was estimated.

Experiment No. 7

Immuno-electrophoresis and Staining of Precipitin Lines

Aim

To separate serum proteins by electrophoresis and identify them using antigen–antibody precipitation, followed by staining of precipitin lines.

Principle

Immuno-electrophoresis combines **electrophoresis** and **immunodiffusion**:

1. **Electrophoresis** separates antigens (proteins) based on their charge and size in an agar/agarose gel under an electric field.
2. **Immunodiffusion** occurs when antiserum diffuses into the gel and reacts with separated antigens.
3. At optimal antigen–antibody concentration, **precipitin arcs (lines)** are formed.
4. These lines are stained to improve visibility and identification.

Materials Required

- Agarose or agar gel
- Glass slides or electrophoresis plates
- Electrophoresis apparatus and power supply
- Buffer solution (e.g., barbitone buffer, pH ~8.6)
- Serum sample (antigen)
- Specific antiserum
- Micropipette
- Gel puncher
- Staining solution (e.g., Coomassie Brilliant Blue/Amido Black)
- Destaining solution (methanol + acetic acid)
- Wash bottles

Procedure

A. Electrophoresis

1. Prepare agarose gel on a clean slide or plate.
2. Allow the gel to solidify.
3. Cut a small well near one end of the gel.
4. Load the serum sample into the well.
5. Place the gel in the electrophoresis chamber with buffer.
6. Apply electric current (e.g., 100–150 V) for 30–60 minutes.
7. Proteins separate into distinct bands.

B. Immunodiffusion

1. Cut a longitudinal trough parallel to the line of protein separation.
2. Fill the trough with specific antiserum.
3. Incubate the plate in a moist chamber for 24–48 hours.
4. Allow antigen and antibody to diffuse and form precipitin arcs.

C. Staining of Precipitin Lines

1. Wash the gel with saline or distilled water to remove unreacted proteins.
2. Flood the gel with staining solution (e.g., Coomassie Brilliant Blue).
3. Leave for 10–15 minutes.
4. Destain using destaining solution until clear background is obtained.
5. Observe distinct stained precipitin lines.

Observation

- Formation of **curved precipitin arcs** between antigen and antibody.
- After staining, lines appear as **dark bands** against a clear background.

Result

Serum proteins were successfully separated and identified by immuno-electrophoresis, and precipitin lines were visualized after staining.

Experiment No. 8

Rocket Immuno-electrophoresis and Counter Current Immuno-electrophoresis

Aim

To study antigen–antibody reactions using:

1. Rocket Immuno-electrophoresis (quantitative method)
2. Counter Current Immuno-electrophoresis (rapid qualitative method)

Principle

1. Rocket Immuno-electrophoresis:

- Antibody is uniformly incorporated in agarose gel.
- Antigen migrates through the gel under an electric field.
- At equivalence, a **rocket-shaped precipitin peak** is formed.
- The **height of the rocket is directly proportional to antigen concentration**.

2. Counter Current Immuno-electrophoresis:

- Antigen and antibody are placed in opposite wells.
- Under an electric field, they migrate towards each other.
- When they meet at optimal concentration, a **precipitin line** forms rapidly.

Materials Required

- Agarose powder
- Glass slides or electrophoresis plates
- Electrophoresis apparatus and power supply
- Buffer solution (pH ~8.6)
- Antigen samples
- Specific antiserum
- Micropipettes
- Gel puncher
- Ruler or scale

Procedure

A. Rocket Immuno-electrophoresis

1. Prepare agarose gel containing uniform antibody.
2. Pour onto a slide/plate and allow to solidify.
3. Punch wells near one end of the gel.
4. Add antigen samples (standards and unknown) into wells.
5. Place gel in electrophoresis chamber with buffer.
6. Apply electric current (100–150 V) for 1–2 hours.
7. Observe formation of **rocket-shaped precipitin peaks**.
8. Measure the height of rockets.

B. Counter Current Immuno-electrophoresis

1. Prepare plain agarose gel on a slide.
2. Punch two opposite wells for antigen and antibody.
3. Add antigen in one well and antibody in the opposite well.
4. Place gel in electrophoresis chamber.
5. Apply electric current for 30–60 minutes.
6. Observe formation of a **precipitin line** between wells.

Observation

Rocket Immuno-electrophoresis

Sample	Rocket Height (mm)	Antigen Concentration
Standard		
Unknown		

Counter Current Immuno-electrophoresis

Sample	Precipitin Line	Interpretation
Test sample	+ / −	Positive / Negative

Result

- Rocket immuno-electrophoresis was used to estimate antigen concentration.
- Counter current immuno-electrophoresis confirmed the presence of antigen–antibody reaction.

Experiment No. 9

ELISA Technique – Dot Method (Dot ELISA)

Aim

To detect the presence of a specific antigen or antibody in a sample using the Dot ELISA technique.

Principle

Dot ELISA is a qualitative immunoassay based on **antigen–antibody interaction**.

- Antigen (or antibody) is immobilized as a **dot on a membrane** (e.g., nitrocellulose).
- The membrane is incubated with a specific antibody (or antigen).
- An **enzyme-linked secondary antibody** binds to the complex.
- Upon addition of substrate, the enzyme produces a **colored spot**, indicating a positive reaction.

Materials Required

- Nitrocellulose membrane / filter paper
- Antigen or antibody sample
- Primary antibody
- Enzyme-linked secondary antibody
- Blocking agent (e.g., BSA or skim milk)
- Washing buffer (PBS/Tween)
- Substrate solution (e.g., DAB, TMB)
- Micropipettes
- Incubation tray
- Forceps
- **Procedure**

1. Cut nitrocellulose membrane into small strips.
2. Spot a small volume of antigen (or antibody) onto the membrane.
3. Allow the spots to air dry.
4. Block the membrane using blocking solution for 30–60 minutes.
5. Wash with buffer to remove excess blocker.
6. Incubate with primary antibody for 1 hour.
7. Wash thoroughly with washing buffer.
8. Add enzyme-linked secondary antibody and incubate for 30–60 minutes.
9. Wash again to remove unbound antibodies.
10. Add substrate solution.
11. Observe for development of **colored dots**.

Controls

- **Positive control**: Should give a colored dot
- **Negative control**: Should show no color

Observation

Sample	Color Development	Interpretation
Test sample	+ / −	Positive / Negative
Positive control	+	Valid

Sample	Color Development	Interpretation
Negative control	–	Valid

Result

- Appearance of a **colored dot** indicates a positive result (presence of antigen/antibody).
- Absence of color indicates a negative result.

SEM 5 Food and Industrial Biotechnology

INDEX

Exp.No	Name of Experiment	Date	Page. No
1	Demonstration of setting laboratory fermentor-basic features, purpose, procedure		
2	Isolation and screening of amylase producing microbes from soil.		
3	Production of citric acid by Aspergillus		
4	Preparation of ethanol by Yeast fermentation		
5.	Separation and quantification of ethanol by distillation (demonstration)		
6	Production of wine (Demonstration)		
7	Isolation of microorganisms from spoiled food and identification		
8	Isolation of organisms from curd/ milk and fermentation of lactose		
9	Immobilization of yeast.		

Experiment: 1 **Date:**

Demonstration of Setting Up a Laboratory Fermentor

Aim

To study the basic features of a laboratory fermentor and demonstrate its setup for microbial cultivation.

Principle

A fermentor (bioreactor) is a vessel used for the controlled growth of microorganisms and production of valuable products such as enzymes, antibiotics, and organic acids. It provides optimal conditions such as temperature, pH, aeration, agitation, and sterility for microbial growth.

Basic Features of a Laboratory Fermentor

- Culture vessel
- Agitator (impeller)
- Sparger (air supply)
- pH probe
- Dissolved oxygen (DO) probe
- Temperature sensor
- Foam control system
- Sampling port
- Inlet and outlet ports
- Control panel

Materials Required

- Laboratory fermentor
- Sterile growth medium
- Microbial inoculum
- Distilled water
- 70% ethanol
- Air compressor
- pH and DO probes

Procedure

A. Preparation of the Fermentor

1. Clean the fermentor vessel and accessories thoroughly.
2. Assemble the fermentor with impeller, sparger, probes, and sampling ports.
3. Add the required volume of culture medium to the vessel.
4. Close all ports securely.

B. Sterilization

5. Sterilize the fermentor and medium by autoclaving or in situ sterilization.
6. Allow the fermentor to cool to room temperature.

C. Installation of Sensors

7. Connect the pH, temperature, and dissolved oxygen probes.
8. Calibrate the probes according to the manufacturer's instructions.

D. Setting Operating Conditions

9. Set the required temperature (e.g., 30–37°C).
10. Set the agitation speed (e.g., 100–300 rpm).
11. Adjust the aeration rate using the air supply system.
12. Set the desired pH control range.

E. Inoculation

13. Transfer the microbial inoculum aseptically into the fermentor through the inoculation port.
14. Start agitation and aeration.

F. Monitoring

15. Monitor temperature, pH, dissolved oxygen, and foam formation periodically.
16. Collect samples at regular intervals through the sampling port for analysis.

Observations

Parameter	Set Value	Observed Value
Temperature		
pH		
Agitation Speed (rpm)		
Aeration Rate (vvm)		
Dissolved Oxygen (%)		

Result

The laboratory fermentor was successfully assembled and operated under controlled conditions for microbial cultivation.

Precautions

1. Maintain aseptic conditions throughout the experiment.
2. Ensure proper sterilization before inoculation.
3. Calibrate probes accurately before use.
4. Avoid contamination during sampling.
5. Monitor operating parameters regularly.

Application

Laboratory fermentors are used for the production of antibiotics, enzymes, vaccines, organic acids, biofuels, and recombinant proteins in biotechnology industries.

Left Side Record Format

Experiment No.: _____

Date: _____

Title

Demonstration of Setting Up a Laboratory Fermentor

Diagram

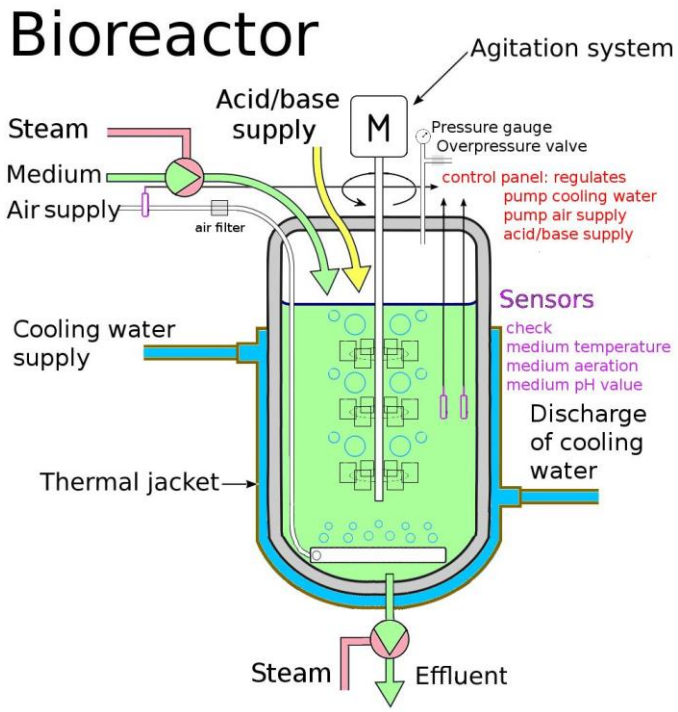


Fig. 1. Laboratory Fermentor (Stirred-Tank Bioreactor) Showing Major Components

Labels to Mark in the Diagram

1. Motor
2. Agitator shaft
3. Impeller
4. Sparger
5. Culture vessel
6. pH probe
7. Dissolved oxygen probe
8. Temperature probe
9. Sampling port
10. Air inlet
11. Exhaust outlet
12. Control panel
13. Foam sensor
14. Cooling/heating jacket

Principle (Short Note for Left Side)

A fermentor is a specialized vessel used for the cultivation of microorganisms under controlled conditions of temperature, pH, aeration, and agitation. It provides an optimal environment for microbial growth and product formation.

Flow Chart

Medium Preparation → Sterilization → Assembly of Fermentor → Calibration of Sensors → Inoculation → Aeration & Agitation → Monitoring → Sampling → Product Formation

Experiment: 2 **Date:**

Isolation and Screening of Amylase-Producing Microorganisms from Soil

Aim

To isolate amylase-producing microorganisms from soil and screen them for extracellular amylase production.

Principle

Amylase is an enzyme that hydrolyzes starch into simpler sugars. Microorganisms capable of producing amylase can be isolated from soil using nutrient agar. The isolated colonies are screened on starch agar plates. After incubation, the plates are flooded with iodine solution. Clear zones around colonies indicate starch hydrolysis and hence amylase production.

Materials Required

- Soil sample
- Sterile distilled water
- Nutrient agar plates
- Starch agar plates
- Sterile test tubes
- Micropipettes
- Sterile spreader
- Iodine solution (Gram's iodine)
- Incubator
- Conical flasks

Procedure

A. Isolation of Microorganisms

1. Collect soil sample from a garden or agricultural field.
2. Add 1 g of soil to 9 mL sterile distilled water and mix thoroughly.
3. Prepare serial dilutions up to 10^{-5} or 10^{-6}.
4. Transfer 0.1 mL of diluted sample onto nutrient agar plates.
5. Spread evenly using a sterile spreader.
6. Incubate at 30–37°C for 24–48 hours.
7. Observe colonies and isolate distinct colonies by streak plate method to obtain pure cultures.

B. Screening for Amylase Production

1. Spot inoculate the purified isolates onto starch agar plates.
2. Incubate at 30–37°C for 24–48 hours.
3. Flood the plates with iodine solution.
4. Allow to stand for 2–3 minutes.
5. Observe for clear zones around microbial growth.

Observation

Isolate No.	Zone of Clearance (mm)	Amylase Production
1		
2		
3		

Result

Microbial isolates showing a clear zone around the colony after iodine treatment are identified as **amylase-producing microorganisms**. The isolate with the largest zone of clearance is considered the best amylase producer.

Precautions

1. Maintain aseptic conditions throughout the experiment.
2. Use sterile glassware and media.
3. Perform serial dilution carefully.
4. Avoid contamination during inoculation.
5. Flood iodine solution uniformly over the plate.

Left-Side Record Work

Diagram

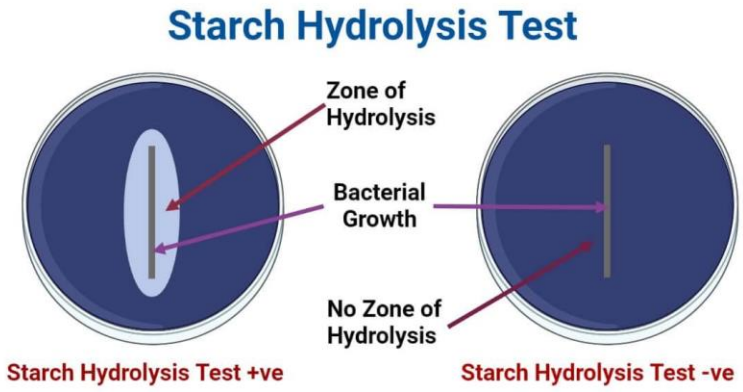


Fig. 1. Screening of Amylase-Producing Microorganisms on Starch Agar Using Iodine Test

Flow Chart

Soil Sample → Serial Dilution → Plating on Nutrient Agar → Isolation of Colonies → Streaking for Pure Culture → Inoculation on Starch Agar → Incubation → Iodine Treatment → Observation of Clear Zone → Identification of Amylase Producer

Application

Amylase-producing microorganisms are used in the food, brewing, textile, paper, detergent, and pharmaceutical industries for starch degradation and processing.

Experiment: 3 **Date:**

Production of Citric Acid by *Aspergillus niger*

Aim

To produce citric acid by fermentation using *Aspergillus niger*.

Principle

Citric acid is an important organic acid produced commercially by the fungus *Aspergillus niger* through submerged fermentation. Under conditions of high sugar concentration, acidic pH, and adequate aeration, the fungus converts sugars into citric acid, which accumulates in the fermentation medium.

Requirements:

Microorganism

- *Aspergillus niger* culture

Materials

- Fermentation medium
- Conical flask (250 mL)
- Cotton plugs
- pH meter
- Incubator
- Sterile pipettes
- Distilled water

Composition of Production Medium (100 mL)

- Sucrose – 15 g
- NH_4NO_3 – 0.2 g
- KH_2PO_4 – 0.1 g
- $MgSO_4 \cdot 7H_2O$ – 0.025 g
- Distilled water – 100 mL
- pH adjusted to 5.0–5.5

Procedure

1. Prepare the production medium and dispense 100 mL into a 250 mL conical flask.
2. Sterilize the medium by autoclaving at 121°C for 15 minutes.
3. Allow the medium to cool.
4. Inoculate aseptically with a spore suspension of *Aspergillus niger*.
5. Incubate at **28–30°C** for **5–7 days** under stationary conditions.
6. Observe fungal growth and acid production.
7. After incubation, filter the culture to remove fungal biomass.
8. Collect the filtrate containing citric acid.
9. Estimate citric acid production by titration against standard NaOH using phenolphthalein indicator.

Observation Table

Parameter	Observation
Initial pH	
Final pH	
Incubation period	
Colour of filtrate	
Citric acid produced	

Result

Citric acid was successfully produced by fermentation using *Aspergillus niger* and accumulated in the fermentation broth.

Precautions

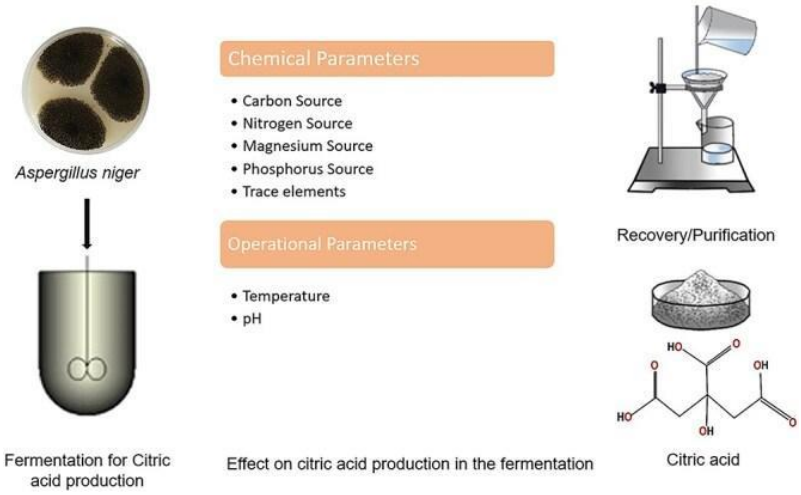
1. Maintain aseptic conditions throughout the experiment.
2. Use a pure culture of *Aspergillus niger*.
3. Ensure proper sterilization of medium and glassware.
4. Maintain optimum temperature and pH.
5. Avoid contamination during inoculation and incubation.

Left-Side Record Work

Flow Chart

Preparation of Medium → Sterilization → Inoculation with *A. niger* → Incubation (5–7 days) → Filtration → Citric Acid Recovery → Estimation

Diagram



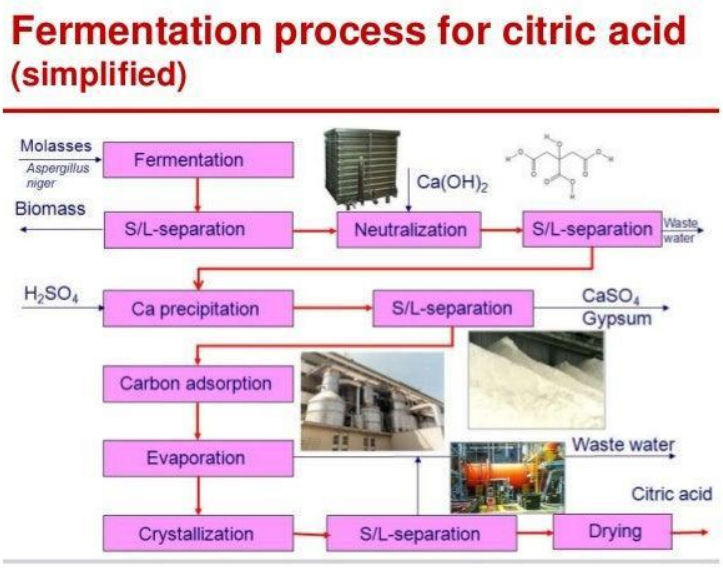


Fig. 1. Production of Citric Acid by *Aspergillus niger* through Submerged Fermentation

Applications of Citric Acid

- Food and beverage industry as an acidulant and preservative.
- Pharmaceutical formulations.
- Cosmetics and personal care products.
- Cleaning and chelating agent in industries.

Experiment:4 Date:

Preparation of Ethanol by Yeast Fermentation

Aim

To prepare ethanol from glucose using *Saccharomyces cerevisiae* (yeast) through fermentation.

Principle

Yeast converts glucose into ethanol and carbon dioxide under anaerobic conditions. This process is known as alcoholic fermentation. The enzyme complex zymase present in yeast catalyzes the conversion of sugar into ethanol.

Reaction

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + Energy$

(Glucose → Ethanol + Carbon dioxide)

Requirements

Materials

- Baker's yeast (*Saccharomyces cerevisiae*)
- Glucose – 10 g
- Distilled water – 100 mL
- Conical flask (250 mL)
- Cotton plug
- pH paper
- Incubator

Procedure

1. Prepare 100 mL of 10% glucose solution in a 250 mL conical flask.
2. Adjust the pH to 4.5–5.0.
3. Sterilize the medium by autoclaving at 121°C for 15 minutes.
4. Allow the medium to cool to room temperature.
5. Inoculate the sterile medium with 1–2 g of active yeast culture.
6. Plug the flask with cotton and maintain anaerobic conditions.
7. Incubate at 30°C for 48–72 hours.
8. Observe the evolution of carbon dioxide bubbles indicating fermentation.
9. After incubation, filter the culture to remove yeast cells.
10. Collect the fermented broth containing ethanol.
11. Ethanol production can be confirmed by characteristic alcoholic odour or by distillation.

Observations

Parameter	Observation
Initial pH	
Final pH	
Incubation Time	

Parameter	Observation
CO_2 Production	
Ethanol Odour	

Result

Ethanol was successfully produced from glucose by fermentation using *Saccharomyces cerevisiae*.

Precautions

1. Maintain aseptic conditions throughout the experiment.
2. Use fresh and active yeast culture.
3. Ensure anaerobic conditions during fermentation.
4. Maintain optimum tempera ture (30°C).
5. Avoid contamination of the fermentation medium.

Left-Side Record Work

Flow Chart

Glucose Solution Preparation → Sterilization → Inoculation with Yeast → Anaerobic Incubation → Fermentation → CO_2 Production → Ethanol Formation

Calculation Table

Component	Quantity Required
Glucose	10 g
Distilled Water	100 mL
Yeast Culture	1–2 g
pH	4.5–5.0
Temperature	30°C
Incubation Period	48–72 h

Diagram

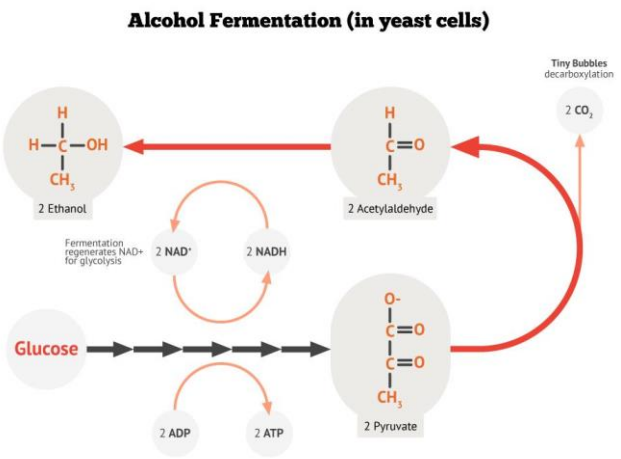


Fig. 1. Production of Ethanol by Yeast Fermentation

Applications of Ethanol

- Alcoholic beverage industry.
- Biofuel production.
- Pharmaceutical and chemical industries.
- Solvent in laboratories and industries.

Experiment: 5 Date:

Separation and Quantification of Ethanol by Distillation (Demonstration)

Aim

To separate ethanol from fermented broth by distillation and estimate the quantity of ethanol obtained.

Principle

Distillation is a separation technique based on differences in boiling points of liquids. Ethanol has a boiling point of **78.3°C**, which is lower than that of water (**100°C**). When the fermented broth is heated, ethanol vaporizes first and is condensed in a condenser to obtain purified ethanol. The quantity of ethanol can be estimated by measuring the volume of distillate collected.

Requirements

Apparatus

- Distillation flask
- Liebig condenser
- Thermometer
- Heating mantle/water bath
- Receiving flask
- Measuring cylinder

Materials

- Fermented broth containing ethanol
- Distilled water

Procedure

1. Transfer the fermented broth into a clean distillation flask.
2. Assemble the distillation apparatus with thermometer and condenser.
3. Start the flow of cold water through the condenser.
4. Heat the flask gently using a heating mantle.
5. Observe the temperature and maintain it around **78–80°C**.
6. Collect the distillate in a receiving flask.
7. Continue distillation until no more ethanol is collected.
8. Measure the volume of distillate obtained using a measuring cylinder.
9. Record the observations.

Observations

Parameter	Observation
Volume of fermented broth taken (mL)	
Temperature during distillation (°C)	
Volume of distillate collected (mL)	

Parameter	Observation
Appearance of distillate	

Calculation

Percentage of Ethanol

$$\text{Percentage of Ethanol} = \frac{\text{Volume of Ethanol Collected}}{\text{Volume of Fermented Broth Taken}} \times 100$$

Example:

If 10 mL ethanol is collected from 100 mL fermented broth:

$$\frac{10}{100} \times 100 = 10\%$$

Therefore, the ethanol content is **10% (v/v)**.

Result

Ethanol was successfully separated from the fermented broth by distillation and its quantity was determined by measuring the volume of distillate collected.

Precautions

1. Ensure proper assembly of the distillation apparatus.
2. Maintain continuous water flow through the condenser.
3. Do not overheat the fermentation broth.
4. Record the temperature accurately.
5. Handle hot glassware carefully.

Left-Side Record Work

Flow Chart

Fermented Broth → Distillation Setup → Heating (78–80°C) → Ethanol Vapour Formation → Condensation → Collection of Distillate → Measurement of Ethanol

Diagram

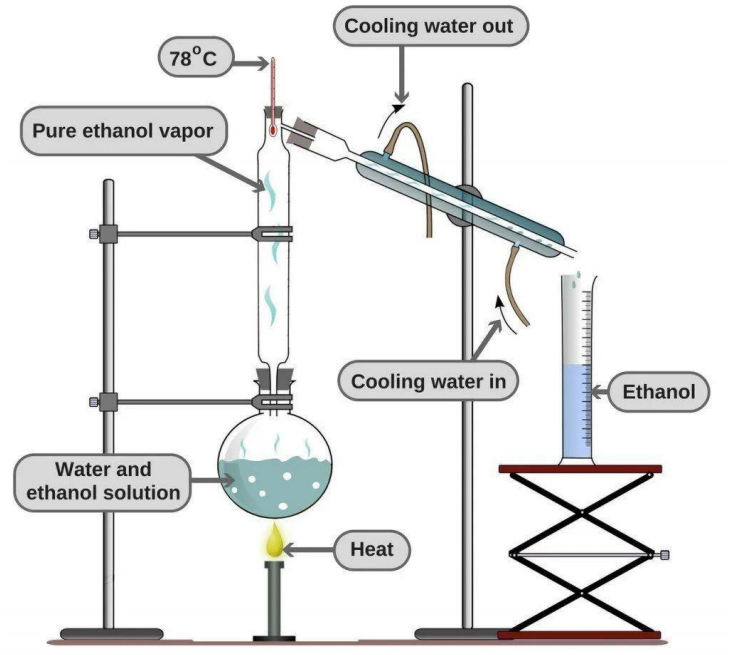


Fig. 1. Distillation Apparatus for Separation of Ethanol from Fermented Broth

Experiment: 6

Production of Wine (Demonstration)

Aim

To demonstrate the production of wine from fruit juice by yeast fermentation.

Principle

Wine is an alcoholic beverage produced by the fermentation of sugars present in fruit juices by **Saccharomyces cerevisiae**. Under anaerobic conditions, yeast converts sugars into ethanol and carbon dioxide. The fermented product is then clarified and aged to improve its flavor and quality.

Reaction

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + Energy$$

(Glucose → Ethanol + Carbon dioxide)

Requirements

Materials

- Fresh grape juice (or any fruit juice) – 500 mL
- Sugar – 50–100 g
- Yeast (*Saccharomyces cerevisiae*) – 5 g
- Fermentation bottle/flask
- Cotton plug or airlock
- Measuring cylinder
- pH paper

Procedure

1. Wash and crush fresh grapes to obtain juice.
2. Filter the juice through muslin cloth to remove solid particles.
3. Transfer 500 mL of juice into a sterile fermentation flask.
4. Add sugar and mix thoroughly.
5. Adjust the pH to about **4.0–4.5** if necessary.
6. Add activated yeast culture and mix well.
7. Close the flask with a cotton plug fitted with an airlock.
8. Incubate at room temperature (25–30°C) for **7–14 days** under anaerobic conditions.
9. Observe gas bubble formation indicating active fermentation.
10. After fermentation, filter the wine to remove yeast cells and sediments.
11. Store the clarified wine for maturation if required.

Observations

Parameter	Observation
Fruit used	

Parameter	Observation
Initial pH	
Incubation period	
Gas production	
Final appearance	
Alcoholic odor	

Result

Wine was successfully produced from fruit juice by fermentation using **Saccharomyces cerevisiae**.

Precautions

1. Use clean and sterile containers.
2. Maintain aseptic conditions throughout the experiment.
3. Use fresh fruit juice and active yeast culture.
4. Avoid contamination during fermentation.
5. Do not expose the fermenting mixture to excessive air.

Left-Side Record Work

Flow Chart

Fruit Selection → Juice Extraction → Filtration → Addition of Sugar → Inoculation with Yeast → Fermentation (7–14 Days) → Filtration → Wine Production

Calculation Table

Component	Quantity
Fruit Juice	500 mL
Sugar	50–100 g
Yeast	5 g
pH	4.0–4.5
Temperature	25–30°C
Fermentation Period	7–14 days

Diagram

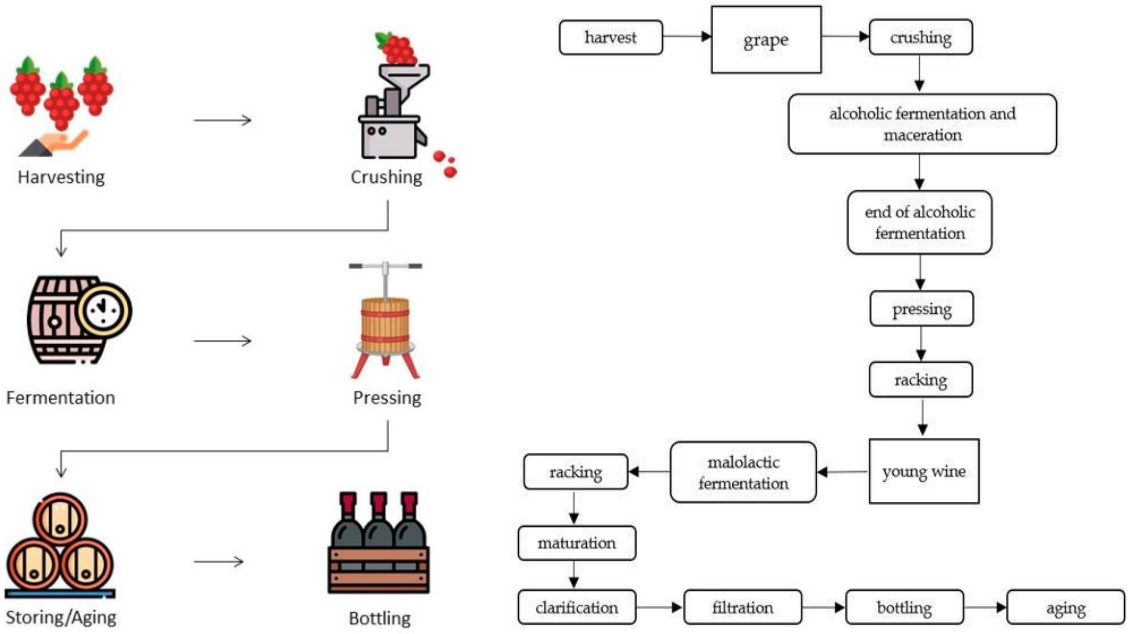


Fig. 1. Production of Wine by Yeast Fermentation

Applications

- Production of alcoholic beverages.
- Fermentation technology studies.
- Industrial biotechnology and food processing industries.

Experiment: 7 Date:

Isolation of Microorganisms from Spoiled Food and Identification

Aim

To isolate microorganisms from spoiled food samples and identify them based on their colony morphology and microscopic characteristics.

Principle

Spoiled food serves as a rich source of microorganisms such as bacteria, yeasts, and molds. These microorganisms can be isolated by culturing the food sample on suitable media. Identification is carried out by observing colony characteristics and microscopic features after staining.

Requirements

Materials

- Spoiled food sample (bread, fruit, vegetable, milk, etc.)
- Sterile distilled water
- Nutrient Agar (NA) plates
- Potato Dextrose Agar (PDA) plates
- Sterile test tubes
- Inoculating loop
- Micropipettes
- Glass slides
- Gram staining reagents
- Microscope

Procedure

A. Isolation of Microorganisms

1. Collect a small quantity (1 g) of spoiled food sample.
2. Transfer it into 9 mL sterile distilled water and mix thoroughly.
3. Prepare serial dilutions up to 10^{-5}.
4. Pipette 0.1 mL of appropriate dilution onto Nutrient Agar and PDA plates.
5. Spread evenly using a sterile spreader.
6. Incubate:
    - Nutrient Agar plates at **37°C for 24–48 h**
    - PDA plates at **28°C for 3–5 days**
7. Observe the growth of distinct colonies.

B. Purification of Isolates

8. Select well-isolated colonies.
9. Streak onto fresh agar plates to obtain pure cultures.
10. Incubate and maintain pure cultures for identification.

C. Identification

Colony Morphology

11. Observe colony characteristics such as:

- Size
- Shape
- Colour
- Margin
- Elevation
- Texture

Microscopic Observation

12. Prepare a smear from bacterial colonies.
13. Perform Gram staining.
14. Observe under a microscope and record cell shape and Gram reaction.
15. For fungal isolates, prepare a lactophenol cotton blue mount and observe fungal structures.

Observation Table

Colony Characteristics

Isolate	Colour	Shape	Margin	Elevation
1				
2				

Microscopic Characteristics

Isolate	Gram Reaction	Cell Shape
1		
2		

Result

Microorganisms were successfully isolated from the spoiled food sample and identified based on their colony morphology and microscopic characteristics.

Precautions

1. Maintain aseptic conditions throughout the experiment.
2. Sterilize all glassware and media before use.
3. Avoid contamination during inoculation.
4. Use proper staining techniques.
5. Handle microbial cultures carefully.

Left-Side Record Work

Flow Chart

Spoiled Food Sample → Serial Dilution → Plating on NA/PDA → Incubation → Colony Isolation → Pure Culture → Staining → Microscopic Identification

Diagram

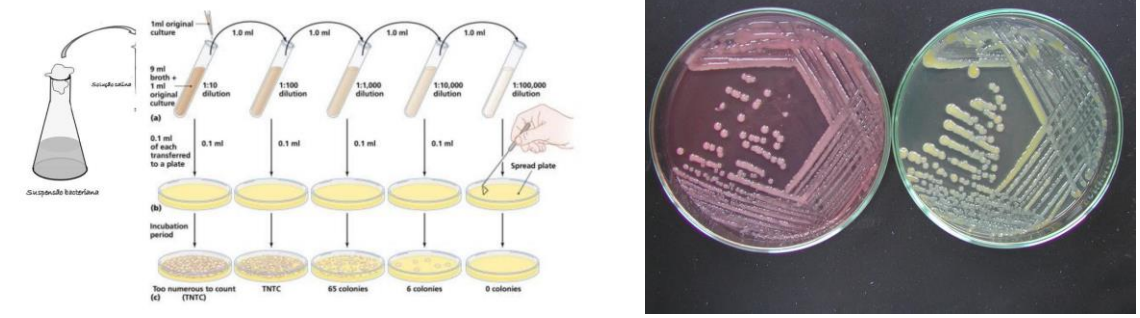


Fig. 1. Isolation and Identification of Microorganisms from Spoiled Food Samples

Applications

- Food microbiology studies.
- Identification of food spoilage organisms.
- Quality control in food industries.
- Detection of microbial contamination in food products.

Experiment: 8 **Date:**

Isolation of Organisms from Curd/Milk and Fermentation of Lactose

Aim

To isolate microorganisms from curd/milk and demonstrate lactose fermentation by the isolated organisms.

Principle

Curd contains beneficial microorganisms, mainly lactic acid bacteria such as *Lactobacillus* spp., which ferment lactose (milk sugar) into lactic acid. The production of acid lowers the pH of milk, resulting in curd formation. Isolation and cultivation of these microorganisms help in understanding the process of lactose fermentation.

Requirements

Materials

- Fresh curd or milk sample
- Sterile distilled water
- Nutrient Agar (NA) or MRS Agar plates
- Lactose broth containing Durham tube
- Sterile test tubes
- Inoculating loop
- Micropipettes
- Incubator

Procedure

A. Isolation of Microorganisms

1. Take 1 mL of curd/milk sample and add it to 9 mL sterile distilled water.
2. Prepare serial dilutions up to 10^{-5}.
3. Transfer 0.1 mL of diluted sample onto MRS agar or Nutrient Agar plates.
4. Spread evenly using a sterile spreader.
5. Incubate the plates at **37°C for 24–48 hours**.
6. Observe colony growth and select isolated colonies.
7. Purify the colonies by streak plate method.

B. Lactose Fermentation Test

8. Inoculate the purified culture into lactose broth containing a Durham tube.
9. Incubate at **37°C for 24–48 hours**.
10. Observe for:

- Change in colour of the medium (acid production).
- Gas accumulation in the Durham tube (gas production).

Observations

Isolation

Colony No.	Colour	Shape	Size
1			
2			

Lactose Fermentation

Isolate	Acid Production	Gas Production	Result
1	+ / –	+ / –	
2	+ / –	+ / –	

Result

Microorganisms were successfully isolated from curd/milk. The isolates fermented lactose, producing acid and/or gas, indicating their ability to metabolize milk sugar.

Precautions

1. Maintain aseptic conditions throughout the experiment.
2. Use sterile media and glassware.
3. Incubate cultures at the recommended temperature.
4. Avoid contamination during inoculation.
5. Record observations carefully.

Left-Side Record Work

Flow Chart

Curd/Milk Sample → Serial Dilution → Plating on MRS Agar → Incubation → Isolation of Colonies → Pure Culture → Inoculation into Lactose Broth → Incubation → Observation of Acid/Gas Production

Diagram

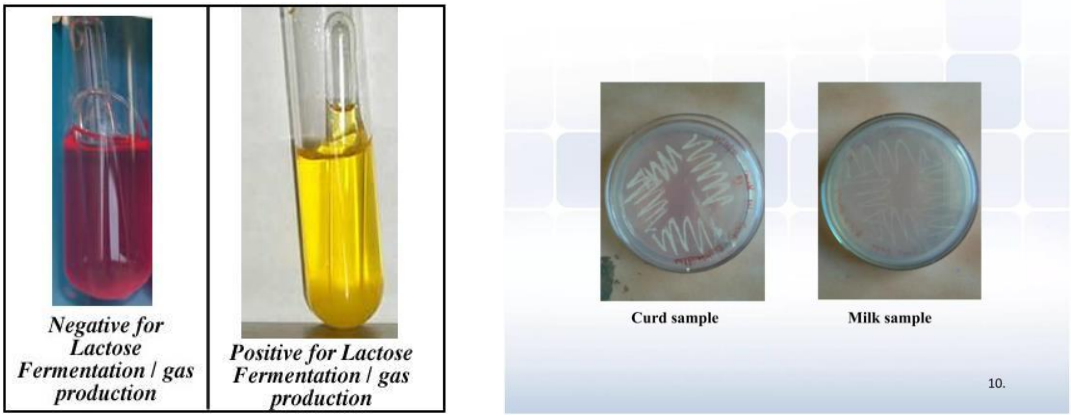


Fig. 1. Isolation of Lactic Acid Bacteria from Curd and Lactose Fermentation Test

Applications

- Production of curd, yogurt, and fermented dairy products.
- Probiotic food production.
- Industrial production of lactic acid.
- Food biotechnology and dairy microbiology studies.

Experiment: 9 Date:

Immobilization of Yeast

Aim

To immobilize yeast cells using the sodium alginate–calcium chloride method and study their application in fermentation.

Principle

Immobilization is the technique of entrapping living cells within a suitable matrix while retaining their metabolic activity. In this experiment, yeast cells are entrapped in calcium alginate beads formed by the reaction between sodium alginate and calcium chloride. Immobilized yeast cells can be reused for fermentation and are easier to separate from the fermentation medium.

Requirements

Materials

- Yeast suspension (*Saccharomyces cerevisiae*)
- Sodium alginate (2–3%)
- Calcium chloride solution (0.1 M)
- Distilled water
- Beakers
- Syringe/dropper
- Glass rod

Procedure

1. Prepare a **2% sodium alginate solution** by dissolving 2 g sodium alginate in 100 mL distilled water.
2. Prepare a fresh yeast suspension in sterile water.
3. Mix equal volumes of yeast suspension and sodium alginate solution thoroughly.
4. Prepare 100 mL of **0.1 M calcium chloride solution** in a beaker.
5. Using a sterile syringe or dropper, add the yeast-alginate mixture dropwise into the calcium chloride solution.
6. Allow the beads to harden for **20–30 minutes**.
7. Collect the formed calcium alginate beads containing immobilized yeast cells.
8. Wash the beads gently with sterile distilled water to remove excess calcium chloride.
9. Store the beads in sterile water until use.

Observation

Observation	Result
Appearance of beads	
Colour of beads	
Shape of beads	
Cell entrapment	

Result

Yeast cells were successfully immobilized in calcium alginate beads and can be used for fermentation studies.

Precautions

1. Prepare sodium alginate solution without lumps.
2. Add the alginate mixture slowly to obtain uniform beads.
3. Avoid breaking the beads during washing.
4. Use fresh yeast culture.
5. Maintain aseptic conditions throughout the experiment.

Left-Side Record Work

Flow Chart

Yeast Culture + Sodium Alginate → Mixing → Dropwise Addition into $CaCl_2$ Solution → Formation of Calcium Alginate Beads → Washing → Immobilized Yeast Beads

Diagram

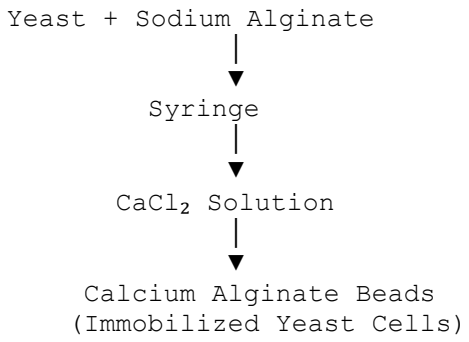


Fig. 1. Immobilization of Yeast Cells by Entrapment in Calcium Alginate Beads

Applications

- Alcohol production
- Brewing industry
- Continuous fermentation processes
- Biotechnology and bioprocess industries
- Production of bioethanol and fermented products

SEM 5 General Virology

INDEX

Exp.No	Name of Experiment	Date	Page. No
1	Introduction to biosafety protocols and laboratory safety.		
2	2. Introduction to microscopy techniques for viral visualization.		
3	3. Practice in handling viral cultures under biosafety cabinets.		
4	4. Introduction to cell culture techniques.		
5.	5. Isolation of bacteriophages from environmental samples.		
6	6. Plaque assay of bacteriophages.		
7	7. Study of plant viral symptoms.		
8	8. Mechanical Transmission of Plant Viruses (Demonstration)		
9	9. Study of Embryonated Egg Inoculation (Demonstration)		

Experiment: 1 Date:

Introduction to Biosafety Protocols and Laboratory Safety

Aim

To familiarize students with basic biosafety practices, laboratory safety guidelines, and safe handling of biological materials in a biotechnology laboratory.

Principle

Biosafety refers to the safe handling, storage, and disposal of biological materials to prevent accidental exposure to microorganisms, hazardous chemicals, and laboratory-related injuries. Following biosafety protocols ensures the protection of laboratory personnel, the environment, and experimental materials.

Objectives

- To understand basic laboratory safety rules.
- To learn the proper use of Personal Protective Equipment (PPE).
- To identify biological, chemical, and physical hazards in the laboratory.
- To understand waste disposal and emergency procedures.

Materials Required

- Laboratory coat
- Gloves
- Face mask
- Safety goggles
- Biosafety cabinet (demonstration)
- First-aid kit
- Disinfectants (70% ethanol)
- Biohazard waste bins

Procedure

A. Personal Safety Measures

1. Wear a laboratory coat before entering the laboratory.
2. Use gloves and safety goggles while handling cultures and chemicals.
3. Tie back long hair and avoid loose clothing.
4. Wash hands thoroughly before and after practical sessions.

B. Safe Laboratory Practices

5. Read the experimental procedure carefully before starting work.
6. Label all cultures, reagents, and samples properly.
7. Do not eat, drink, or use mobile phones in the laboratory.
8. Avoid mouth pipetting; always use pipette aids.
9. Maintain aseptic conditions while handling microorganisms.

C. Biological Safety

10. Handle microbial cultures carefully to avoid contamination.
11. Disinfect work surfaces before and after experiments using 70% ethanol.
12. Dispose of microbial cultures and contaminated materials in biohazard waste containers.
13. Sterilize waste materials by autoclaving before disposal.

D. Chemical Safety

14. Read chemical labels carefully before use.
15. Handle acids, alkalis, and solvents with caution.
16. Report chemical spills immediately to the instructor.

E. Emergency Procedures

17. Locate emergency exits, fire extinguishers, eye wash stations, and first-aid kits.
18. Report accidents, spills, or injuries immediately.
19. Follow emergency evacuation procedures when instructed.

Observation

Safety Item	Status (Observed/Not Observed)
Lab coat worn	
Gloves used	
Workbench disinfected	
Proper waste disposal	
Hand washing performed	

Result

Students became familiar with biosafety protocols, laboratory safety measures, proper use of PPE, and safe handling of biological materials.

Precautions

1. Always wear appropriate PPE.
2. Never handle cultures without supervision.
3. Dispose of laboratory waste in designated containers.
4. Follow aseptic techniques throughout the experiment.
5. Report accidents immediately.

Left-Side Record Work

Flow Chart

Wear PPE → Follow Laboratory Rules → Handle Samples Safely → Disinfect Work Area → Proper Waste Disposal → Emergency Response

Diagram

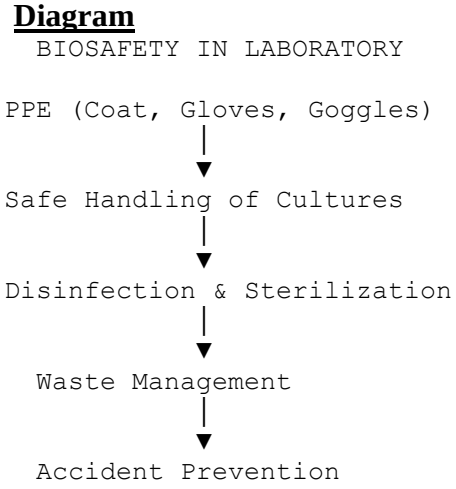


Fig. 1. Basic Biosafety Practices in a Biotechnology Laboratory

Application

Biosafety protocols are essential in microbiology, biotechnology, molecular biology, clinical laboratories, pharmaceutical industries, and research institutions to ensure safe and responsible laboratory practices.

Experiment: 2 Date:

Introduction to Microscopy Techniques for Viral Visualization (Demonstration)

Aim

To study the different microscopy techniques used for the visualization and analysis of viruses.

Principle

Viruses are extremely small particles, typically ranging from **20–300 nm**, which are below the resolution limit of a light microscope (≈200 nm). Therefore, specialized microscopy techniques, particularly electron microscopy, are used for their visualization. These techniques help in studying viral morphology, structure, size, and interaction with host cells.

Objectives

- To understand the limitations of light microscopy in virus observation.
- To learn the principles of electron microscopy and fluorescence microscopy.
- To study the applications of different microscopy techniques in virology.

Materials Required

- Charts/images of viruses
- Light microscope (demonstration)
- Electron microscopy images
- Fluorescence microscopy images
- Multimedia presentation/video (optional)

Microscopy Techniques for Viral Visualization

1. Light Microscopy

- Uses visible light and glass lenses.
- Resolution is approximately **0.2 µm (200 nm)**.
- Cannot directly visualize most viruses.
- Useful for observing virus-induced cytopathic effects in infected cells.

2. Fluorescence Microscopy

- Uses fluorescent dyes or antibodies tagged with fluorochromes.
- Allows detection of viruses within host cells.
- Commonly used in diagnostic virology.

3. Transmission Electron Microscopy (TEM)

- Electrons pass through a thin specimen.
- Provides detailed internal structure of viruses.
- Resolution up to **0.1–1 nm**.
- Widely used for viral identification and morphology studies.

4. Scanning Electron Microscopy (SEM)

- Electrons scan the specimen surface.
- Produces three-dimensional images.
- Useful for studying viral surface features and host-virus interactions.

5. Cryo-Electron Microscopy (Cryo-EM)

- Specimens are rapidly frozen and observed in a near-natural state.
- Provides high-resolution structural information.
- Widely used in modern virology and vaccine research.

Procedure (Demonstration)

1. Observe charts/images showing different virus structures.
2. Study prepared images obtained through TEM, SEM, and fluorescence microscopy.
3. Compare the resolution and applications of each microscopy technique.
4. Record observations regarding viral morphology and structural details.

Observation Table

Microscopy Technique	Resolution	Major Application
Light Microscope	~200 nm	Cytopathic effects
Fluorescence Microscope	High	Detection of viral antigens
TEM	Very High	Internal viral structure
SEM	High	Surface morphology
Cryo-EM	Extremely High	3D viral structure

Result

Different microscopy techniques used for viral visualization were studied. Electron microscopy, especially TEM and Cryo-EM, provides detailed visualization of viruses, while fluorescence microscopy is useful for virus detection in infected cells.

Precautions

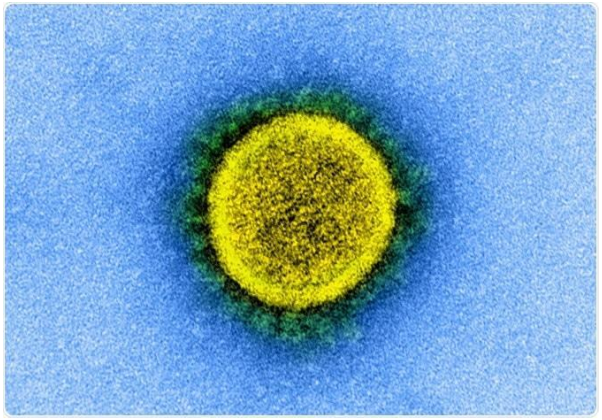
1. Handle microscopy equipment carefully.
2. Follow laboratory safety guidelines.
3. Avoid touching optical surfaces.
4. Record observations accurately.
5. Use proper protective measures when handling viral samples.

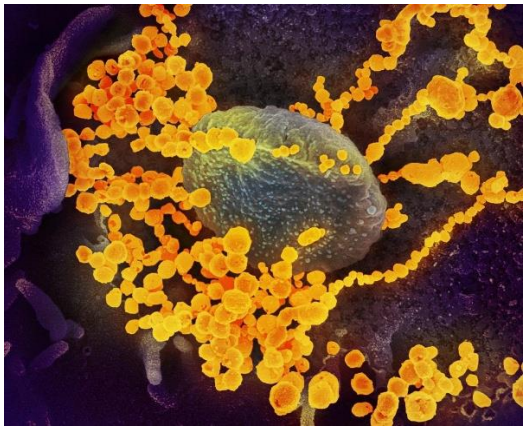
Left-Side Record Work

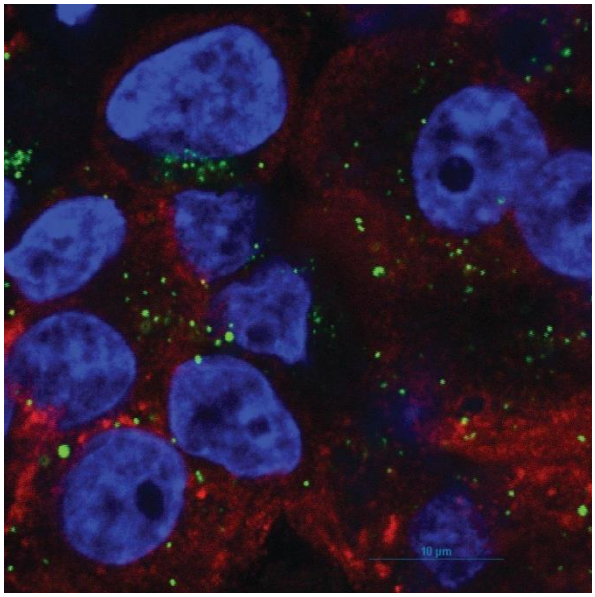
Flow Chart

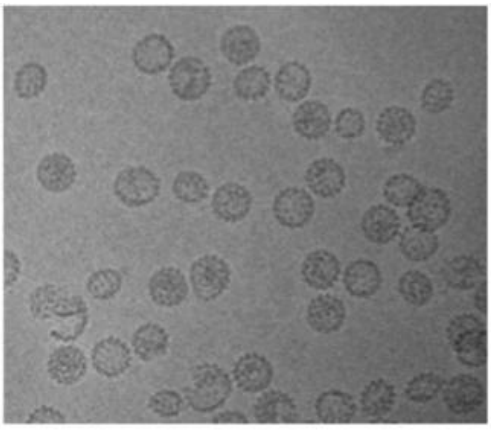
Virus Sample → Sample Preparation → Microscopy Technique Selection → Observation → Image Analysis → Viral Identification

Visual Examples of Viral Imaging

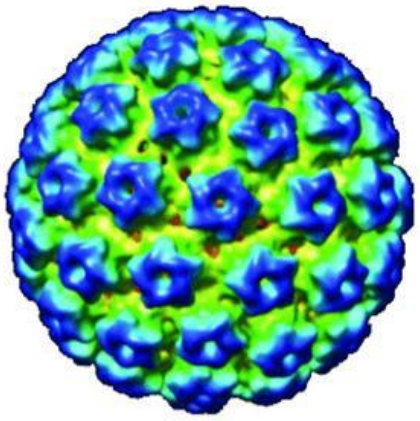








TEM images of VLPs



3D reconstruction of VLP

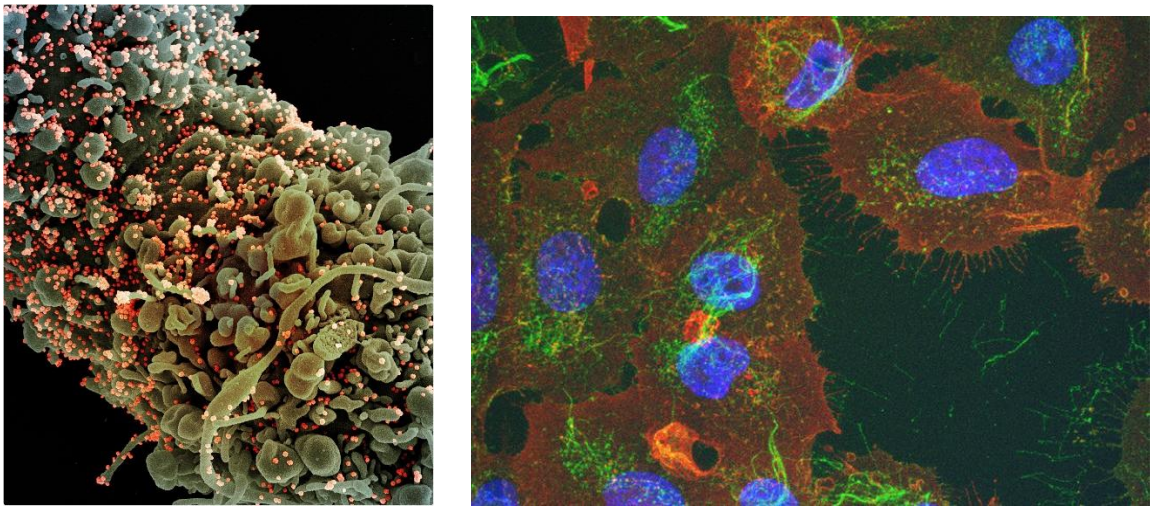


Fig. 1. Viral Visualization Using Different Microscopy Techniques

Application

Microscopy techniques are extensively used in **viral diagnosis, viral taxonomy, vaccine development, structural biology, and virology research** to study virus morphology, replication, and host-virus interactions.

Experiment: 3 Date:

Practice in Handling Viral Cultures Under Biosafety Cabinets (Demonstration)

Aim

To demonstrate the proper use of a Biosafety Cabinet (BSC) for the safe handling of viral cultures and infectious materials.

Principle

Viral cultures may contain infectious agents that pose risks to laboratory personnel and the environment. A **Biosafety Cabinet (BSC)** provides a sterile work environment and protects the operator, sample, and surroundings through **HEPA-filtered airflow**. Most virology laboratories use **Class II Biosafety Cabinets** for routine handling of viral cultures.

Objectives

- To understand the components and functions of a Biosafety Cabinet.
- To learn proper aseptic techniques while handling viral cultures.
- To understand biosafety practices for preventing contamination and exposure.

Materials Required

- Class II Biosafety Cabinet (Demonstration)
- Personal Protective Equipment (Lab coat, gloves, mask)
- 70% ethanol disinfectant
- Sterile culture tubes and pipettes
- Biohazard waste container
- Autoclave bags

Procedure (Demonstration)

A. Preparation

1. Wear appropriate PPE before entering the laboratory.
2. Switch on the Biosafety Cabinet and allow airflow to stabilize for 10–15 minutes.
3. Disinfect the work surface with 70% ethanol.
4. Arrange all required materials inside the cabinet without overcrowding.

B. Safe Handling of Viral Cultures

5. Perform all manipulations within the working area of the cabinet.
6. Minimize rapid hand movements that may disrupt airflow.
7. Keep culture containers closed when not in use.
8. Avoid blocking front and rear air grills.
9. Dispose of contaminated materials in designated biohazard containers.

C. Completion of Work

10. Seal and remove cultures as instructed by the supervisor.
11. Disinfect all work surfaces after completion.
12. Remove waste materials for autoclaving and disposal.

13. Allow the cabinet to run for a few minutes before switching it off.

Observation

Parameter	Observation
Type of BSC used	
PPE worn	
Surface disinfected	
Proper waste disposal observed	
Airflow maintained	

Result

The procedure for safe handling of viral cultures under a Biosafety Cabinet was demonstrated, and the importance of aseptic techniques and biosafety measures was understood.

Precautions

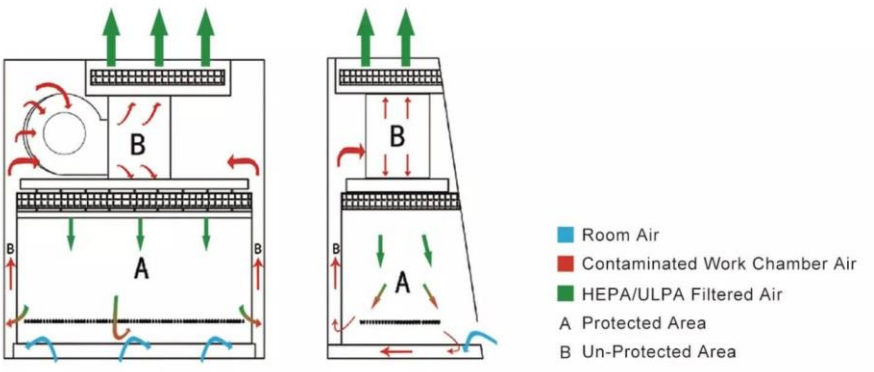
1. Always wear appropriate PPE.
2. Never obstruct the airflow grills of the cabinet.
3. Disinfect surfaces before and after use.
4. Dispose of biohazardous waste properly.
5. Follow institutional biosafety guidelines at all times.
6. Handle viral cultures only under trained supervision.

Left-Side Record Work

Flow Chart

Wear PPE → Switch on BSC → Disinfect Surface → Arrange Materials → Handle Viral Cultures Aseptically → Dispose Waste Safely → Disinfect Cabinet → Switch Off

Diagram



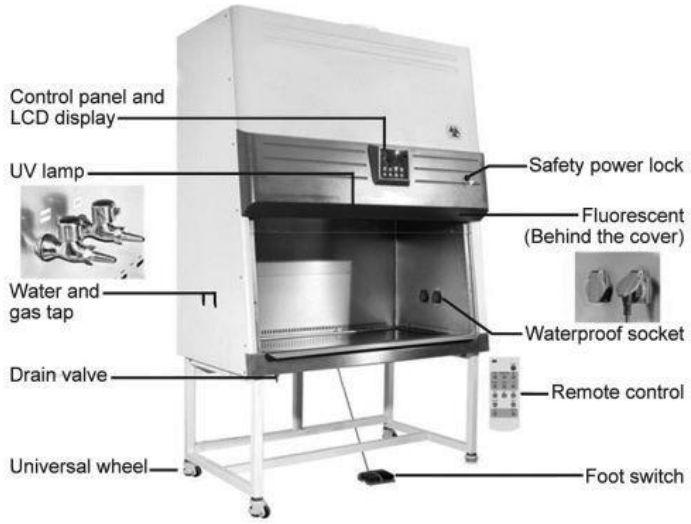


Fig. 1. Class II Biosafety Cabinet Showing HEPA-Filtered Airflow and Work Area

Application

Biosafety cabinets are extensively used in **virology, microbiology, molecular biology, vaccine production, diagnostic laboratories, and biomedical research** for the safe handling of infectious materials.

Experiment: 4 **Date:**

Introduction to Cell Culture Techniques (Demonstration)

Aim

To familiarize students with the basic principles, equipment, and procedures involved in animal cell culture techniques.

Principle

Cell culture is the process of growing animal or plant cells under controlled laboratory conditions outside their natural environment. Animal cells require sterile conditions, appropriate culture media, temperature, pH, and carbon dioxide levels for growth and maintenance. Cell culture techniques are widely used in biotechnology, vaccine production, drug testing, cancer research, and tissue engineering.

Objectives

- To understand the principles of cell culture.
- To learn the basic requirements for maintaining cell cultures.
- To identify the equipment used in a cell culture laboratory.
- To understand aseptic techniques used in cell culture.

Materials Required

- Cell culture flask or Petri dish
- Cell culture medium (DMEM/RPMI-1640)
- Fetal Bovine Serum (FBS)
- Phosphate Buffered Saline (PBS)
- CO_2 incubator
- Inverted microscope
- Laminar airflow cabinet
- Micropipettes and sterile tips
- Trypsin-EDTA solution (demonstration)
- Personal Protective Equipment (PPE)

Procedure (Demonstration)

A. Preparation of Work Area

1. Wear laboratory coat, gloves, and mask.
2. Switch on the laminar airflow cabinet and UV light (if available) before use.
3. Disinfect the work surface with 70% ethanol.

B. Observation of Cell Culture Setup

4. Observe the cell culture flask containing growing cells.
5. Examine the culture medium and note its colour and clarity.
6. Observe cell morphology under an inverted microscope.

C. Maintenance of Cell Culture

7. Remove old culture medium aseptically.
8. Wash cells gently with sterile PBS.
9. Add fresh culture medium containing serum.
10. Incubate the culture flask in a CO_2 incubator at **37°C and 5% CO_2**.

D. Subculturing (Demonstration)

11. Remove the medium and wash cells with PBS.
12. Add trypsin-EDTA solution to detach adherent cells.
13. Observe cell detachment under the microscope.
14. Transfer cells to a fresh culture flask containing new medium.

Observation

Parameter	Observation
Cell type observed	
Cell morphology	
Medium colour	
Incubation temperature	
CO_2 concentration	

Result

The basic principles and procedures involved in cell culture techniques were studied, including aseptic handling, cell maintenance, and subculturing.

Precautions

1. Maintain strict aseptic conditions.
2. Always work inside a laminar airflow cabinet.
3. Use sterile media and equipment.
4. Avoid contamination by minimizing exposure of cultures to the environment.
5. Handle cell cultures gently to prevent cell damage.

Left-Side Record Work

Flow Chart

Preparation of Medium → Sterilization → Cell Inoculation → Incubation → Observation of Cell Growth → Subculturing → Maintenance of Cell Line

Diagram

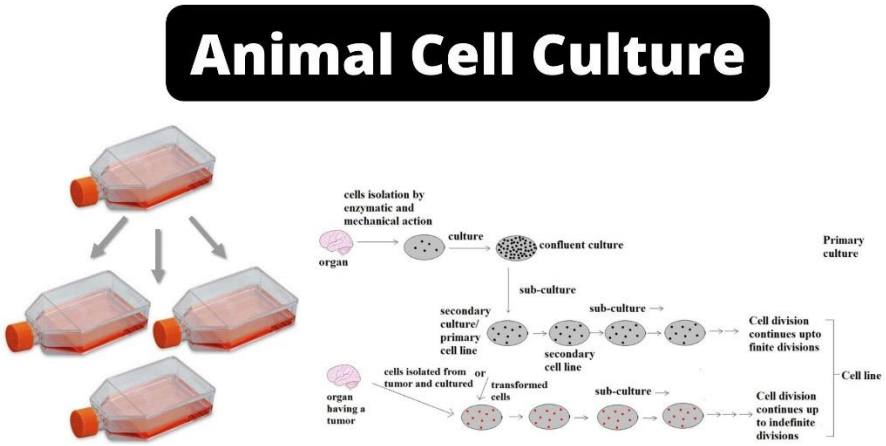


Fig. 1. Basic Cell Culture Technique and Laboratory Setup

Applications

- Vaccine production
- Monoclonal antibody production
- Cancer research
- Drug screening and toxicity testing
- Tissue engineering and regenerative medicine
- Recombinant protein production
- Stem cell research

Experiment: 5 **Date:**

Isolation of Bacteriophages from Environmental Samples (Demonstration)

Aim

To isolate bacteriophages from environmental samples such as sewage water or soil using a susceptible bacterial host.

Principle

Bacteriophages (phages) are viruses that infect bacteria. Environmental sources such as sewage, pond water, and soil are rich in bacteriophages. When a phage infects a susceptible bacterial host, it multiplies and lyses the bacterial cells, producing clear zones called **plaques** on a bacterial lawn. Isolation of bacteriophages is commonly performed using the plaque assay technique.

Objectives

- To understand the occurrence of bacteriophages in the environment.
- To learn the principles of phage isolation.
- To observe plaque formation caused by bacteriophages.

Materials Required

- Environmental sample (sewage water/soil extract)
- Host bacterial culture (*Escherichia coli*)
- Nutrient broth
- Nutrient agar plates
- Soft agar (0.7%)
- Sterile centrifuge tubes
- Micropipettes and tips
- Water bath
- Incubator

Procedure (Demonstration)

A. Preparation of Sample

1. Collect sewage water or soil extract in a sterile container.
2. Centrifuge the sample to remove debris.
3. Filter the supernatant through a membrane filter (0.22–0.45 µm) to remove bacterial cells.

B. Enrichment of Bacteriophages

4. Add 1 mL of filtered sample to 10 mL nutrient broth containing an actively growing *E. coli* culture.
5. Incubate at **37°C for 18–24 hours**.

C. Plaque Assay

6. Mix 0.1 mL of enriched culture with 3 mL molten soft agar maintained at 45°C.
7. Pour the mixture onto a nutrient agar plate.

8. Allow the agar to solidify.
9. Incubate at **37°C for 24 hours**.

D. Observation

10. Examine the plate for the presence of clear circular zones (plaques) on the bacterial lawn.
11. Record the number and appearance of plaques.

Observation Table

Plate No.	Presence of Plaques	Number of Plaques	Observation
1			
2			

Result

Bacteriophages were successfully isolated from the environmental sample as indicated by the formation of clear plaques on the bacterial lawn.

Precautions

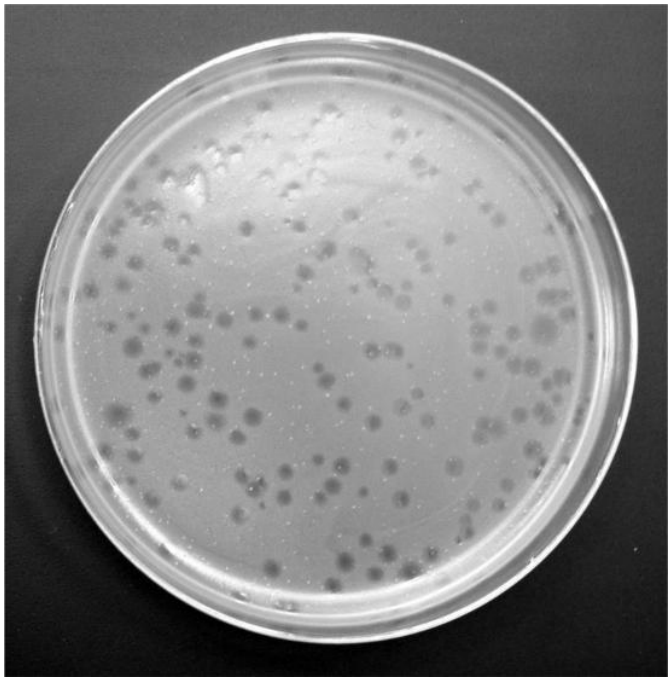
1. Maintain aseptic conditions throughout the experiment.
2. Use fresh bacterial cultures.
3. Avoid contamination during sample handling.
4. Handle environmental samples carefully.
5. Incubate at the recommended temperature.

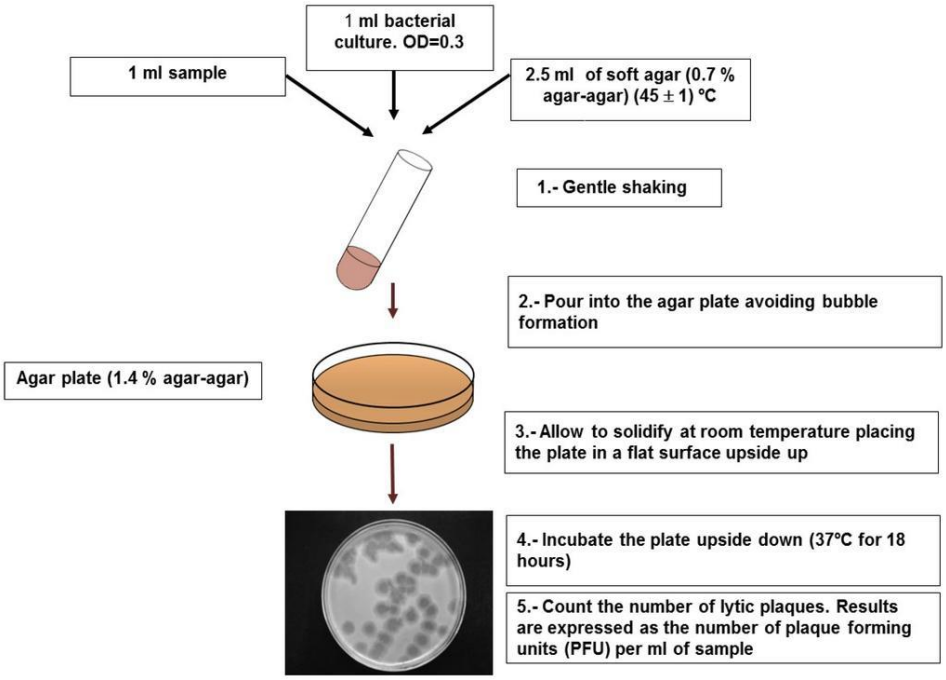
Left-Side Record Work

Flow Chart

Environmental Sample → Filtration → Enrichment with Host Bacteria → Mixing with Soft Agar → Pour Plate Method → Incubation → Plaque Formation → Phage Isolation

Diagram




1 ml bacterial culture. OD=0.3
1 ml sample
2.5 ml of soft agar (0.7 % agar-agar) (45 ± 1) ºC
1.- Gentle shaking
2.- Pour into the agar plate avoiding bubble formation
Agar plate (1.4 % agar-agar)
3.- Allow to solidify at room temperature placing the plate in a flat surface upside up
4.- Incubate the plate upside down (37ºC for 18 hours)
5.- Count the number of lytic plaques. Results are expressed as the number of plaque forming units (PFU) per ml of sample

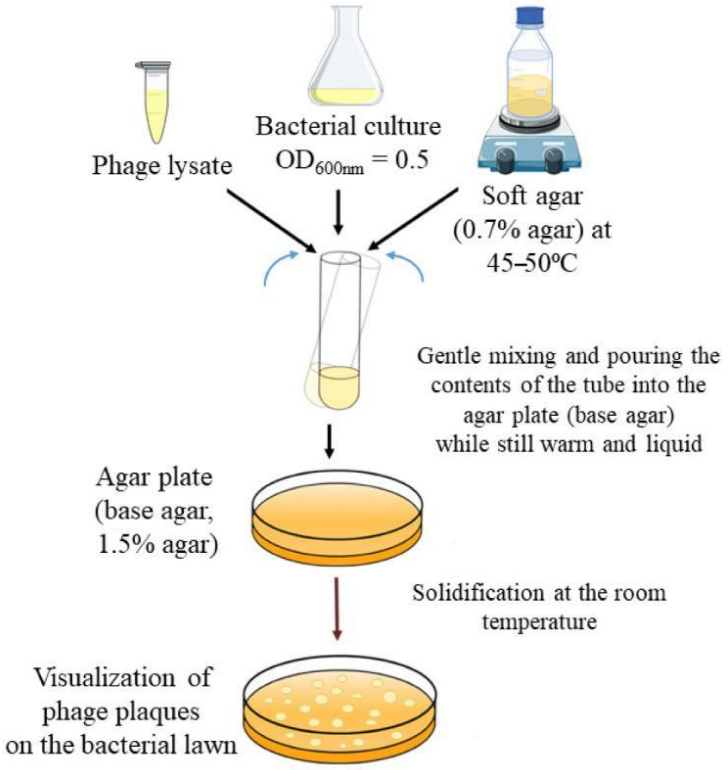


Fig. 1. Isolation of Bacteriophages by Plaque Assay Technique

Applications

- Phage therapy against antibiotic-resistant bacteria
- Molecular biology and genetic engineering research
- Bacterial detection and typing
- Biocontrol in food and agriculture
- Environmental microbiology studies

Experiment: 6 **Date:**

Plaque Assay of Bacteriophages

Aim

To determine the presence and quantify bacteriophages using the plaque assay technique.

Principle

A plaque assay is a method used to detect and quantify bacteriophages. When a phage infects susceptible bacterial cells, it replicates and causes lysis of the host cells, forming clear zones called **plaques** on a bacterial lawn. Each plaque is assumed to arise from a single infective phage particle and is expressed as **Plaque Forming Units (PFU)**.

Requirements

Materials

- Bacteriophage suspension
- Host bacterial culture (*Escherichia coli*)
- Nutrient agar plates
- Soft agar (0.7%)
- Sterile saline
- Sterile test tubes
- Micropipettes and tips
- Water bath (45°C)
- Incubator

Procedure

A. Serial Dilution of Phage Sample

1. Label sterile test tubes containing 9 mL sterile saline as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}.
2. Add 1 mL of phage suspension to the first tube and mix thoroughly.
3. Transfer 1 mL from the first tube to the second tube and continue serial dilution up to 10^{-5}.

B. Plaque Assay

4. Transfer 0.1 mL of diluted phage sample into a sterile tube.
5. Add 0.1 mL of actively growing *E. coli* culture.
6. Allow adsorption for 10 minutes.
7. Add 3 mL molten soft agar maintained at 45°C.
8. Mix gently and pour onto a nutrient agar plate.
9. Allow the agar to solidify.
10. Incubate at **37°C for 18–24 hours**.

C. Observation

11. Observe the plates for clear plaques.
12. Select plates containing **30–300 plaques** for counting.
13. Count the plaques and calculate phage concentration.

Observation Table

Dilution	Number of Plaques
10^{-1}	
10^{-2}	
10^{-3}	
10^{-4}	
10^{-5}	

Calculation

Plaque Forming Units (PFU/mL)

$$\text{PFU/mL} = \frac{\text{Number of Plaques} \times \text{Dilution Factor}}{\text{Voume of phage sample Plated(mL)}}$$

Example

If 50 plaques are observed at 10^{-4} dilution and 0.1 mL was plated:

$$\text{PFU/mL} = \frac{50 \times 10\ 4}{0.1}$$

PFU/mL=5×10 6

Result

Plaques were observed on the bacterial lawn, indicating the presence of bacteriophages. The phage concentration was determined in terms of PFU/mL.

Precautions

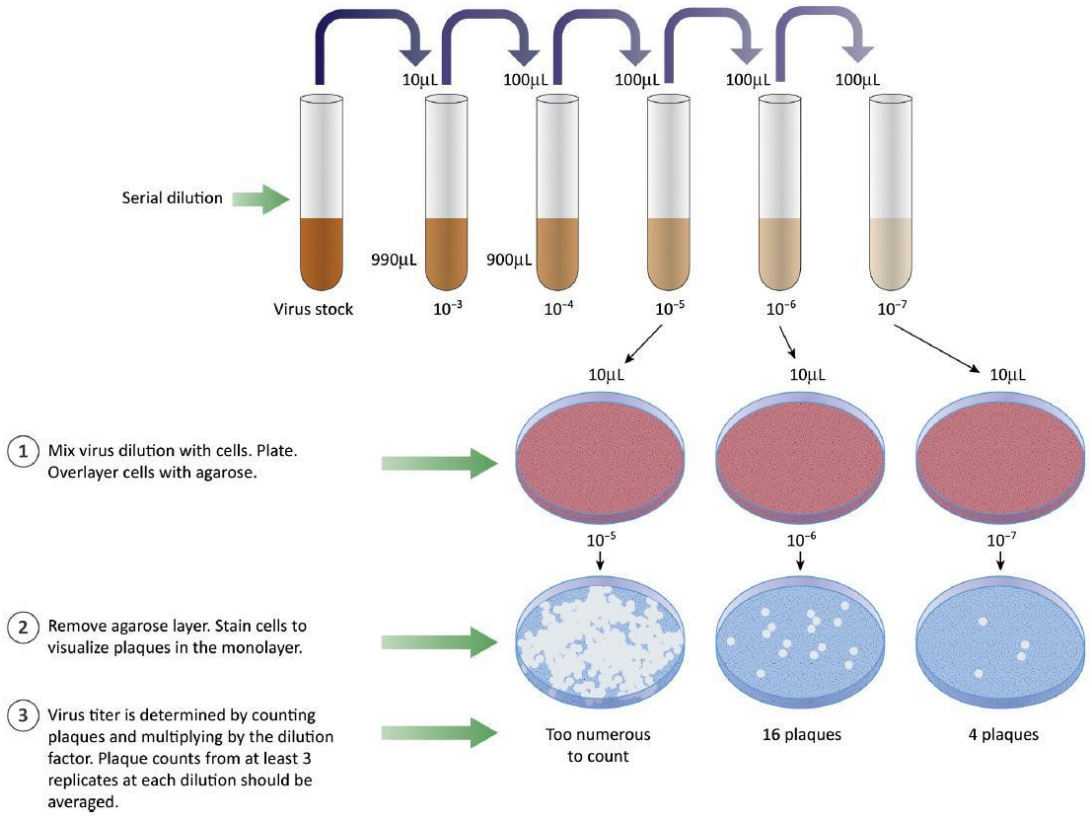
1. Maintain aseptic conditions throughout the experiment.
2. Use fresh host bacterial cultures.
3. Ensure soft agar is maintained at 45°C before use.
4. Mix cultures gently to avoid damaging phage particles.
5. Count plaques only on plates containing 30–300 plaques.

Left-Side Record Work

Flow Chart

Phage Sample → Serial Dilution → Mixing with Host Bacteria → Addition of Soft Agar → Pour Plate → Incubation → Plaque Formation → Plaque Counting → PFU Calculation

Diagram



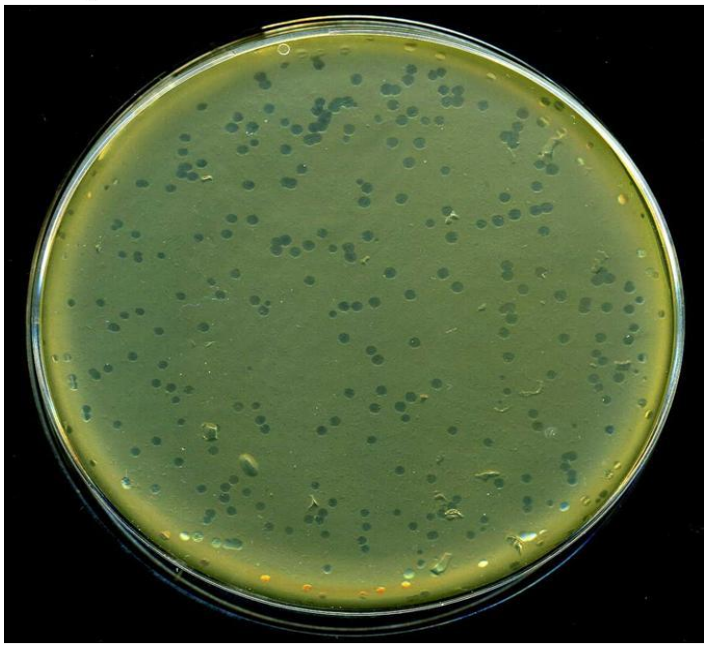


Fig. 1. Plaque Assay Showing Clear Plaques on a Bacterial Lawn

Applications

- Quantification of bacteriophages
- Phage therapy research
- Molecular biology studies
- Viral genetics and biotechnology
- Detection and characterization of bacteriophages in environmental samples

Experiment: 7 **Date:**

Study of Plant Viral Symptoms

Aim

To study and identify common symptoms caused by viral infections in plants.

Principle

Plant viruses infect host plants and interfere with their normal growth, metabolism, and development. Viral infections produce characteristic symptoms on leaves, stems, flowers, and fruits. Observation of these symptoms helps in the preliminary diagnosis of plant viral diseases.

Objectives

- To identify common symptoms of viral infections in plants.
- To differentiate viral symptoms from those caused by other pathogens.
- To understand the impact of viral diseases on crop productivity.

Materials Required

- Virus-infected plant specimens (fresh or preserved)
- Healthy plant specimens for comparison
- Hand lens
- Charts/photographs of viral diseases
- Observation notebook

Procedure

1. Collect healthy and virus-infected plant samples.
2. Observe the leaves, stems, flowers, and fruits carefully.
3. Compare infected plants with healthy plants.
4. Identify and record visible symptoms.
5. Examine specimens using a hand lens if necessary.
6. Note the type and severity of symptoms.
7. Record observations and identify the probable viral disease with the help of charts or manuals.

Common Viral Symptoms in Plants

1. Mosaic

- Alternating light and dark green patches on leaves.
- Example: Tobacco Mosaic Virus (TMV).

2. Chlorosis

- Yellowing of leaves due to loss of chlorophyll.

3. Leaf Curl

- Upward or downward curling of leaves.
- Example: Tomato Leaf Curl Virus.

4. Vein Clearing

- Veins appear transparent or lighter than surrounding tissues.

5. Ring Spots

- Circular chlorotic or necrotic rings on leaves or fruits.

6. Stunting

- Reduced growth and dwarfing of plants.

7. Necrosis

- Death of plant tissues resulting in brown or black lesions.

Observation Table

Plant Sample Symptom Observed Probable Viral Disease

Result

Various characteristic symptoms of plant viral infections such as mosaic, chlorosis, leaf curl, ring spots, and stunting were observed and studied.

Precautions

1. Handle plant samples carefully.
2. Compare infected plants with healthy controls.
3. Record observations accurately.
4. Avoid cross-contamination between samples.
5. Use fresh specimens whenever possible.

Left-Side Record Work

Flow Chart

Collection of Plant Samples → Observation of Symptoms → Comparison with Healthy Plants → Identification of Viral Symptoms → Recording of Obserations

Diagram

Fig. 1. Common Symptoms of Viral Diseases in Plants

Applications

- Diagnosis of plant viral diseases
- Crop disease management
- Agricultural biotechnology research
- Plant pathology studies
- Improvement of crop health and productivity

Experiment: 8 **Date:**

Mechanical Transmission of Plant Viruses (Demonstration)

Aim

To demonstrate the mechanical transmission of a plant virus from an infected plant to a healthy host plant.

Principle

Many plant viruses can be transmitted mechanically through sap inoculation. The virus present in the infected plant sap enters healthy plant tissues through small wounds created on the leaf surface. Once inside the host cells, the virus multiplies and spreads, producing characteristic disease symptoms. Mechanical transmission is commonly used in plant virology for studying virus-host interactions.

Objectives

- To understand the process of mechanical transmission of plant viruses.
- To observe the development of viral symptoms in inoculated plants.
- To learn basic techniques used in plant virology.

Materials Required

- Virus-infected plant leaves (e.g., Tobacco Mosaic Virus-infected leaves)
- Healthy susceptible plants (tobacco, tomato, or other suitable hosts)
- Mortar and pestle
- Phosphate buffer (pH 7.0)
- Carborundum powder (abrasive)
- Cotton swabs or soft brush
- Distilled water
- Gloves

Procedure (Demonstration)

A. Preparation of Inoculum

1. Collect fresh virus-infected leaves.
2. Grind the leaves in a mortar using a small quantity of phosphate buffer.
3. Filter the sap through muslin cloth to obtain a clear extract.

B. Inoculation of Healthy Plants

4. Select healthy young plants for inoculation.
5. Dust the upper surface of leaves lightly with carborundum powder.
6. Apply a small quantity of infected sap onto the leaf surface.
7. Gently rub the leaf using a cotton swab or gloved finger.
8. Wash the leaves with distilled water after 1–2 minutes to remove excess inoculum and abrasive.

C. Observation

9. Maintain the inoculated plants under suitable growth conditions.
10. Observe plants regularly for symptom development over 7–14 days.
11. Record the appearance of symptoms such as mosaic, chlorosis, mottling, or leaf distortion.

Observation Table

Plant No.	Date of Inoculation	Symptoms Observed	Days After Inoculation
1			
2			

Result

The plant virus was successfully transmitted mechanically through sap inoculation, as indicated by the appearance of characteristic viral symptoms in the inoculated plants.

Precautions

1. Use fresh infected plant material.
2. Avoid excessive rubbing that may damage leaves.
3. Use separate tools for infected and healthy plants.
4. Wash hands and equipment after inoculation.
5. Handle infected plants carefully to prevent accidental spread.

Left-Side Record Work

Flow Chart

Virus-Infected Leaf → Preparation of Sap Extract → Addition of Buffer → Dusting with Carborundum → Sap Inoculation → Incubation → Symptom Development

Diagram

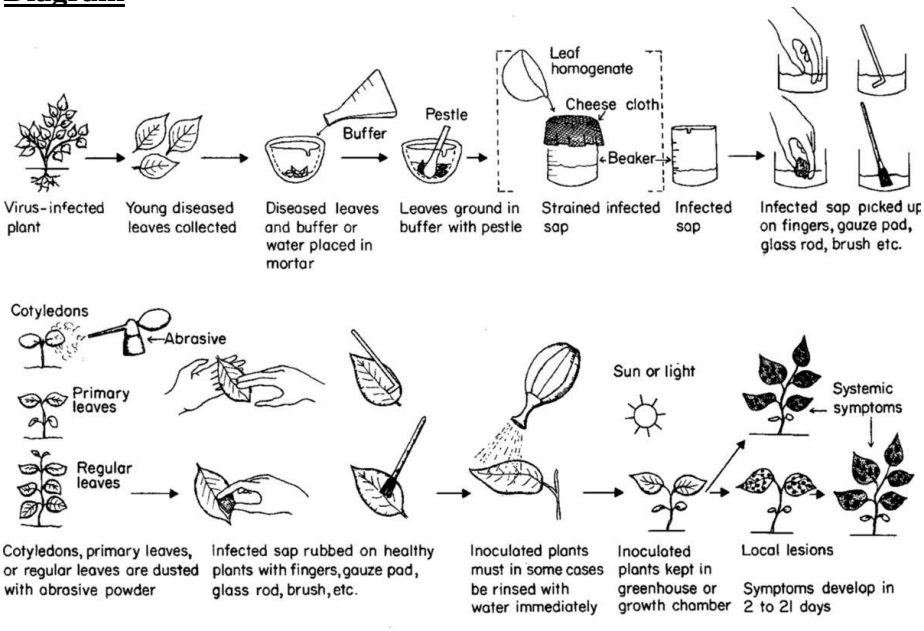


Fig. 1. Mechanical Transmission of Plant Viruses by Sap Inoculation

Applications

- Study of plant-virus interactions
- Identification and characterization of plant viruses

- Plant pathology research
- Screening of resistant plant varieties
- Teaching and demonstration in plant virology laboratories

Experiment: 9 **Date:**

Study of Embryonated Egg Inoculation (Demonstration)

Aim

To study the technique of inoculation of viruses into embryonated chicken eggs and understand its applications in virus cultivation.

Principle

Before the development of cell culture techniques, embryonated chicken eggs were widely used for the cultivation and propagation of viruses. Different viruses grow in specific regions of the egg such as the **chorioallantoic membrane (CAM), allantoic cavity, amniotic cavity, and yolk sac**. Embryonated eggs provide a sterile, nutrient-rich environment suitable for viral multiplication and vaccine production.

Objectives

- To understand the structure of an embryonated egg.
- To learn different routes of virus inoculation.
- To study the applications of embryonated eggs in virology and vaccine production.

Requirements

Materials

- Fertile embryonated chicken eggs (9–12 days old)
- Candling lamp
- Alcohol (70% ethanol)
- Sterile syringe and needle
- Adhesive tape or melted wax
- Marker pen
- Demonstration charts/models

Important Inoculation Sites

1. **Chorioallantoic Membrane (CAM)**
    - Used for poxviruses and herpesviruses.
    - Produces visible pocks on the membrane.
2. **Allantoic Cavity**
    - Used for influenza and Newcastle disease viruses.
    - Virus accumulates in allantoic fluid.
3. **Amniotic Cavity**
    - Used for primary isolation of influenza viruses.
4. **Yolk Sac**
    - Used for certain viruses and intracellular microorganisms.

Procedure (Demonstration)

A. Selection and Preparation of Eggs

1. Select healthy fertilized eggs of appropriate age (9–12 days).
2. Candle the eggs to locate the embryo and air sac.
3. Mark the inoculation site with a pencil.

4. Disinfect the shell surface with 70% ethanol.

B. Inoculation

5. Drill a small hole over the selected inoculation site.
6. Using a sterile syringe, inject the viral inoculum into the desired cavity (CAM, allantoic cavity, amniotic cavity, or yolk sac).
7. Seal the hole with wax or adhesive tape.

C. Incubation

8. Incubate the inoculated eggs at 37°C.
9. Observe the eggs daily by candling.

D. Harvesting

10. After the required incubation period, chill the eggs.
11. Open the eggs aseptically.
12. Collect the infected membrane or fluid for examination.

Observation Table

Inoculation Site	Virus Cultivated	Observation
CAM		
Allantoic cavity		
Amniotic cavity		
Yolk sac		

Result

The technique of inoculating viruses into embryonated chicken eggs and the various inoculation routes used for virus cultivation were studied.

Precautions

1. Use only healthy embryonated eggs.
2. Maintain sterile conditions during inoculation.
3. Handle eggs carefully to avoid breakage.
4. Properly disinfect the shell before inoculation.
5. Follow biosafety guidelines while handling viral materials.

Left-Side Record Work

Flow Chart

Selection of Embryonated Egg → Candling → Surface Disinfection → Inoculation into Desired Site → Sealing → Incubation → Harvesting of Virus

Diagram

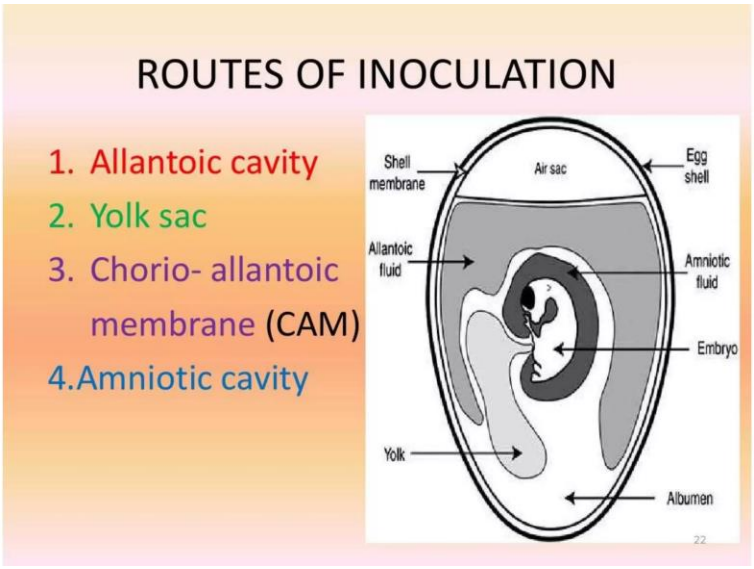


Fig. 1. Embryonated Chicken Egg Showing Different Sites of Viral Inoculation

Applications

- Isolation and cultivation of viruses.
- Production of viral vaccines (e.g., influenza vaccine).
- Viral identification and research.
- Study of viral pathogenicity and host-virus interactions.
- Teaching and training in virology laboratories.