Laboratory manual in microbiology with standard procedures, techniques, safety measures and technical information as a reference framework to enable efficient student practical laboratory work

Laboratory Manual in Microbiology

F.Y.B.Sc. CBCS Semester I
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St. Xavier's College, Mapusa- Goa

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Experiment No. 1

MICROBIOLOGY GOOD LABORATORY PRACTICES (GLP) AND BIOSAFETY

A rewarding laboratory experience demands strict adherence to prescribed rules for personal and environmental safety. Avoiding laboratory accidents, maintaining scrupulously clean laboratory setting, and mastering aseptic techniques form an integral part of most microbiological procedures. Although the virulence of microorganisms used in the academic laboratory environment has been greatly diminished because of their long-term maintenance on artificial media, all microorganisms should be treated as potential pathogens.

The following basic steps should be observed at all times in the laboratory:

1. Upon entering the laboratory, place coats, books in specified locations, never on bench tops.
2. Keep doors and windows closed during the laboratory session to prevent contamination from air currents.
3. At the beginning and termination of each laboratory session, wipe bench tops with a disinfectant solution.
4. Do not place contaminated instruments, such as inoculating loops, needles, and pipettes, on bench tops. Loops and needles should be sterilized and pipettes should be disposed of in designated receptacles.
5. On completion of the laboratory session, place all cultures and materials in the disposal area.
6. Wash your hands with liquid detergent and disinfectant upon entering and prior to leaving the laboratory.
7. A laboratory coat is necessary while working in the laboratory, to protect clothing from contamination or accidental discoloration by staining solutions.
8. Wear disposable gloves during the manipulation of test materials such as blood, serum, and other body fluids.
9. Tie back long hair to minimize its exposure to open flames, wear closed shoes at all times in the laboratory setting, never apply cosmetics or insert contact lenses in the laboratory.
10. Do not eat, or drink in the laboratory.
11. Carry cultures in a test-tube rack when moving around the laboratory and keep cultures in a test-tube rack on the bench tops when not in use.

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12. Report accidental cuts or burns to the instructor immediately.
13. Speak quietly and avoid unnecessary movement around the laboratory to prevent distractions that may cause accidents.

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Experiment No. 2

PRINCIPLE AND APPLICATIONS OF IMPORTANT LABORATORY INSTRUMENTS

Aim: To study the principle and applications of important instruments used in the Microbiology Laboratory

1. BIOLOGICAL SAFETY CABINET / LAMINAR AIR FLOW (LAF)

Biological safety cabinets work by the use of in-flow laminar air drawn through one or more High-Efficiency Particulate Air (HEPA) filters, which remove 99.97% of 0.3µ particles and are one of the most important air filtration systems. Laminar flow workstations force air through HEPA filters and then project a laminar curtain of sterile air across the cabinet. The cabinet is usually made of stainless steel, with no gaps or joints where spores might collect. Such hoods exist in both horizontal and vertical configurations, and there are many different types of cabinets with a variety of airflow patterns and acceptable uses. Laminar flow cabinets generally have a UV-C germicidal lamp to sterilize the interior and contents before usage to prevent contamination of the experiment. Germicidal lamps are usually kept on for 15 minutes to sterilize the interior before the cabinet is used. The light must be switched off when the cabinet is being used, to limit exposure to skin and eyes as stray ultraviolet light emissions can cause cancer and cataracts.

Laminar airflow biological safety cabinets are designed to create a particle-free working environment, prevent contamination of biological samples, and provide protection to any particle-sensitive materials. Biological safety cabinets render three levels of protection: 1) Personal Protection from harmful agents within the cabinet; 2) Product Protection to avoid contamination of the samples; and 3) Environmental Protection from contaminants contained within the cabinet.

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BIOLOGICAL SAFETY CABINET / LAMINAR AIR FLOW (LAF)

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2. AUTOCLAVE

An autoclave is a self-sealing vessel used to perform sterilization in high-pressure and high-temperature environments. It works on the principle of moist heat sterilization where steam under pressure is used to sterilize the material present inside the chamber. Boiling point of water is directly proportional to the pressure when volume is constant. When pressure is increased in a closed vessel, the temperature increases proportionally e.g. at 15 lbs/sq.in., the temperature kept constant at 121.6 ˚C, for 20 minutes, is sufficient to kill all the vegetative forms and spores of the organisms.

It is a cylindrical vessel made of gun metal or copper. The cylindrical shape withstands high pressure more easily than a box or a cube. It has a lid, which can be fastened to the body by means of screw-clamps. The lid is provided with the steam valve, a thermometer, a safety valve and a pressure gauge. On the rim of the cylinder is a rubber gasket to ensure an air tight closure. The cylinder contains water up to a certain level which is heated by means of an electrical or gas device. Above this level, there is a perforated diaphragm which is used for keeping the material to be sterilized. Materials to be sterilized are placed on the perforated diaphragm. Test tubes and flasks are plugged with non-absorbent cotton and covered with paper to avoid drenching of the plugs during the release of steam, when condensation occurs.

After adjusting the water level, the autoclave is switched on. The lid is kept in position and is closed tightly by means of the screw clamps. The steam valve is kept open until all the air is expelled and only steam comes out. The steam valve is then closed, so that pressure begins to rise. As the pressure rises temperature also rises accordingly. When the pressure is 15 lbs/sq.in (temperature 121.6 ˚C) the time is noted, one of the electric coils is switched off (when two coils are used) or the steam valve is opened partially so as to maintain the pressure constant at15 psi. After 20 minutes the autoclave is switched off and is allowed to cool down after all the steam has escaped from the open steam valve. Apparatus is then opened and all sterilized materials are removed.

Autoclaves are widely used for sterilization in microbiology, medicine, veterinary medicine, mycology, funerary practice, dentistry, and prosthetics fabrication. They vary in size and

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function depending on the media to be sterilized and are sometimes called retort in the chemical and food industries. Autoclaves are used in medical applications to perform sterilization and in the chemical industry to cure coatings and vulcanize rubber and for hydrothermal synthesis. Industrial autoclaves are used in industrial applications, especially in the manufacturing of composites. In the laboratory it is used for the sterilization of laboratory culture media, aprons, rubber tubing, etc.

AUTOCLAVE

3. INCUBATOR

An incubator is a device used to cultivate and maintain microbiological cultures or cell cultures at constant temperature and humidity. All incubators are based on the concept that when organisms are provided with the optimal condition of temperature, humidity, oxygen, and carbon dioxide levels, they grow and divide to form more organisms. The incubator maintains optimal temperature, humidity and other conditions such as the CO_2 and oxygen content of the atmosphere inside. It consists of a box with a double wall, insulated with asbestos. It is heated by an electric current and is maintained at constant temperature by means of a thermostat. The temperature is noted by means of a thermometer. The incubator is provided with a double door, the inner one is glass and outer one is of the same material as the rest of the chamber. It is generally maintained at 37 °C which is the optimum temperature of most human pathogens.

INCUBATOR

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4. BOD (BIOLOGICAL OXYGEN DEMAND) INCUBATOR

The BOD incubator is the most versatile and reliable low temperature incubator which is designed to maintain at 20°C, necessary for the determination of Biological Oxygen Demand (BOD). BOD incubators provide accurate, uniform and controlled temperature conditions for accelerated tests and exposures. The BOD incubator is widely used in microbiology laboratories for the applications that include cell culture and fungal growth, BOD test, fermentation, crop and physiology and various pharmaceutical tests etc. It is used to maintain temperature for test tissue culture growth, storage of bacterial cultures and incubation where high degree of constant temperature accuracy is required.

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5. HOT AIR OVEN

Hot air ovens are electrical devices which use dry heat to sterilize. They were originally developed by Pasteur. Generally, they use a thermostat to control the temperature. Their double walled insulation keeps the heat in and conserves energy, the inner layer being a poor conductor and outer layer being metallic. Hot air ovens use extremely high temperatures over several hours to destroy microorganisms and bacterial spores. The ovens use conduction to sterilize items by heating the outside surfaces of the item, which then absorbs the heat and moves it towards the centre of the item. It consists of a chamber with double walls insulated with asbestos or glass wool to prevent radiation of heat. The chambers are heated electrically and controlled thermostatically. The temperature is noted on the thermometer. The temperatures are generally used 180 °C for 30 min or 160 °C for 1 hour or148° C for 1-2 hours. This temperature is sufficient for destruction of vegetative cells and spores. Hot air ovens are generally used for dry sterilization of glassware e.g. test tubes, pipettes and petri plates. Culture media cannot be sterilized by this method.

Precautions:

- Glassware should be dry before sending for sterilization
- To prevent contamination after sterilization, the test tubes, flasks and pipettes are plugged and wrapped in paper; petri plates are also wrapped in paper
- The temperatures should not be allowed to increase above 180° C, otherwise cotton plugs and papers may get charred
- All sterilized articles must be cooled to room temperature before removal

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HOT AIR OVEN

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6. LIGHT MICROSCOPE

The microscope is an instrument which is absolutely essential for microbiologists. It is a combination of lenses so adjusted that minute objects invisible to the naked eye are magnified and made visible. The term microscope is compounded from two Greek words *micro*-small and *scope* –to view.

It is a complicated instrument with the different parts fitted in each other very accurately. The eyepiece fits into a graduated draw tube which in turn slides in the body tube. A revolving nose-piece is attached to the lower end of the body tube and to it, various objectives are fitted. The body tube and the objectives move up and down by means of a rack and pinion arrangement known as the course adjustment. The tube and its attachments are connected to the foot of the microscope by means of hinged joint. Below the objective, in the centre of the microscope, is a platform called the stage on which microscopic preparations are mounted. A mechanical stage may be fitted for the steady and controlled movements of the slide. Below the stage is a sub-stage, which is fitted with an Abbe's condenser, in order to increase the intensity of illumination at high magnification. The condenser has an iris diaphragm, which controls the intensity of the beam of the light, entering the condenser. The condenser can be raised or lowered. Below the condenser there is a horseshoe shaped base over which a plano-concave mirror is mounted. The plane side of the mirror is generally used with the condenser while the concave side is used in its absence.

The objective is of 3 types:-

Lower power lens	Focal length 16mm or 2/3''
High power lens	Focal length 4 mm or 1/6''
Oil immersion lens	Focal length 2 mm or 1/12''

The oil immersion lens must be used with a drop of cedar wood oil (preferable) for microscopic examination of cells up to 0.22/μm. A micrometer (or micron) is the unit of linear measurement on the microscopic scale symbolized by um and is equal to 0.001 mm. The total magnification of the objective magnification x eye-piece magnification. The total magnification of objective can be calculated as the ratio of mechanical tube length of the objective.

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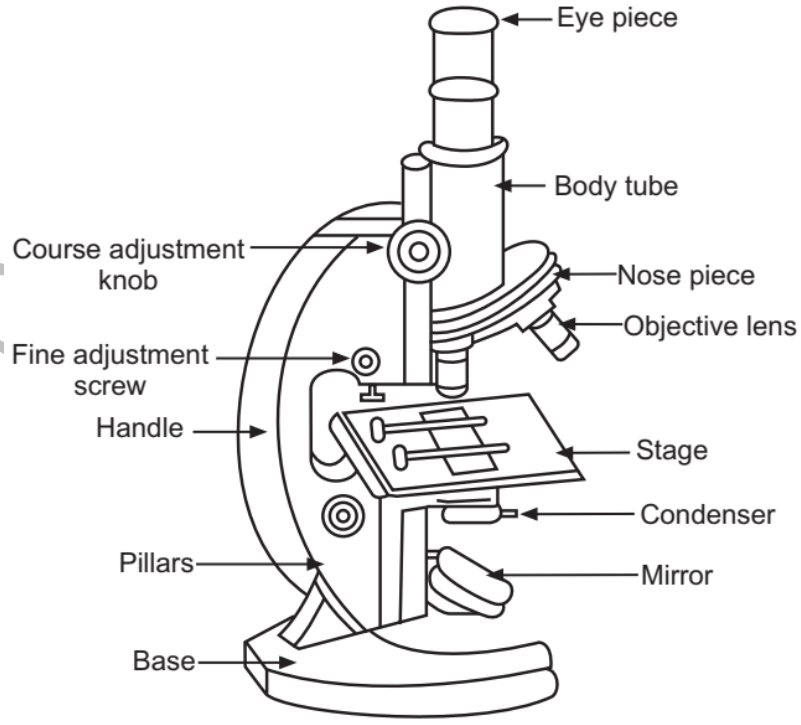
Objective	Magnification of the lens	Total magnification with 10 x ocular	Diameter of the field seen
Lower power lens	160/16=10	10 x 10=100	1.55mm
High power lens	160/4=40	40 x 10=400	0.31mm
Oil immersion lens	160/2=80	40 x 10=400	0.31mm

Nowadays oil immersion lenses with 100 x magnification are used. When oil immersion lens is used, a drop of cedar wood oil is placed on the object and the lens is immersed in it. This is because when an oblique ray emerges out from a denser to rarer medium it is refracted away from the normal and hence the light rays are scattered. If the space between the object and objective is filled with immersion oil which has a refractive index similar to that of glass, the rays do not undergo refraction and pass into the oil immersion lens giving clear, bright magnified image of the object.

Parts of the Microscope



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10X WIDE
4/0.10

7. pH METER

pH is defined as the concentration of hydrogen ions in the solution. When the solution contains more H^+ ions, it is said to be acidic; while a solution that contains more OH^- ions is deemed alkaline. The pH value ranges from 1 (highly acidic) to 14 (highly basic), pH 7 being neutral.

A pH meter is used to determine the acidity or alkalinity of a solution. It contains a pH probe, which when dipped into a solution, passes the electrical signals to the pH meter which displays the pH value of the solution. The glass pH probe contains two electrodes: a sensor and a reference electrode. These electrodes are in the form of glass tubes, one contains buffer 7 and the other contains saturated potassium chloride solution. The sensor electrode bulb is made up of porous glass or permeable glass membrane coated with silica and metal salts. A silver wire coated with silver chloride is immersed in pH 7 buffer in the bulb. Another silver wire coated with silver chloride is immersed in the saturated potassium chloride solution in the reference electrode. When the probe is placed in a solution to measure the pH, hydrogen ions accumulate around the bulb and replace the metal ions from the bulb. This exchange of ions generates some electric flow that is captured by the silver wire. The voltage of this electric flow is measured by the pH meter by converting it into pH value by comparing the generated voltage with the reference electrode. Increase in acidity of the solution has a greater concentration of hydrogen ions that increases the voltage. This increased voltage decreases the pH reading in pH meter. In the same manner, an increase in alkalinity decreases the hydrogen ions and increases the pH value in the pH meter.

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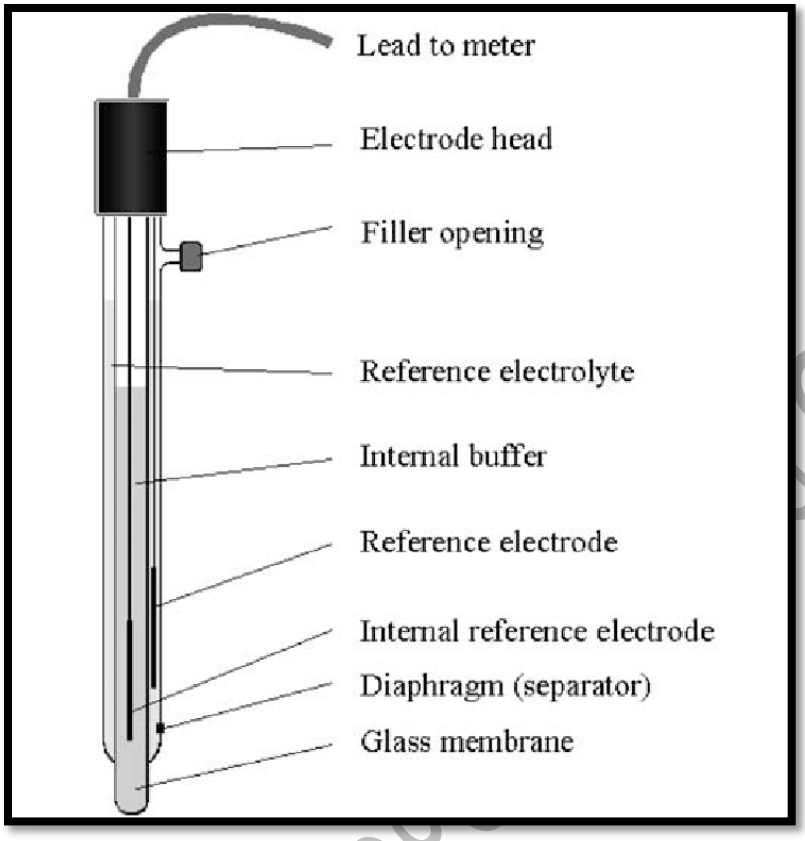

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Experiment No. 03

PREPARATION OF CULTURE MEDIA

Aim: To prepare routine laboratory media

Principle: A growth or culture medium is a solid, liquid or semi-solid designed to support the growth of microorganisms in the laboratory. A suitable medium provides essential nutrients, an energy source and appropriate growth conditions required by the microorganism to survive and proliferate. Laboratory media may be simple, complex, differential, defined or selective for specific microorganisms.

Requirements: Peptone, Meat extract, Agar-agar, glucose, sodium taurocholate, lactose, sodium chloride, 1% neutral red indicator, 0.1 N HCl, 0.1 N NaOH, Distilled water, pH paper

Procedure:

A) Peptone Water

Composition:

Peptone - 1 g
NaCl - 0.5 g
Distilled water - 100 mL
pH - 7.4

Weighed amounts of peptone and NaCl are dissolved in 100 mL of distilled water. The pH is adjusted to 7.4 by adding 0.1 N NaOH dropwise if required. The final pH of the medium is checked using pH paper. 5 mL of the prepared medium is then distributed into each test tube. The test tubes are plugged with cotton wool, wrapped in paper and sterilized in an autoclave at 15 psi for 20 minutes.

B) Nutrient Broth

Composition:

Peptone - 1 g
NaCl - 0.5 g

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Meat Extract - 0.3 g

Distilled water - 100 mL

pH - 7.4

Weighed amounts of the ingredients are dissolved in 100 mL of distilled water. The pH is adjusted to 7.4 by adding 0.1 N NaOH dropwise if required. The final pH of the medium is checked using pH paper. 5 mL of the prepared medium is then distributed into each test tube. The test tubes are plugged with cotton wool, wrapped in paper and sterilized in an autoclave at 15 psi for 20 minutes.

C) Nutrient agar

Composition:

Peptone - 1 g

NaCl - 0.5 g

Meat Extract - 0.3 g

Agar - 2 g

Distilled water - 100 mL

pH - 7.4

To prepare nutrient agar, add 2 g of agar powder to 100 mL of nutrient broth and sterilize the flask in an autoclave at 15 psi for 20 minutes.

D) MacConkey's broth and agar

Composition:

Peptone - 1.7 g

Proteose peptone - 0.3 g

Lactose - 1 g

Bile salts - 0.15 g

Sodium chloride - 0.5 g

Neutral red - 0.003 g

Crystal violet - 0.0001 g

Agar - 1.5 g

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Distilled water - 100 mL

pH - 7.4

Weigh the ingredients as given in the composition except sugar, agar and indicator and dissolve them in 90 mL of distilled water. Dissolve 1 g of lactose in 10 mL of distilled water.

Adjust the pH of the medium to 7.4. Now add the required quantity of neutral red indicator. If MacConkey's agar is being prepared, add 1.5 g agar powder to the medium before autoclaving. Sterilize the medium and sugar separately by autoclaving at 15 psi for 20 minutes. After autoclaving add the lactose solution to the molten medium.

E) Sabouraud's broth

Composition:

Glucose - 4 g

Peptone - 1 g

Distilled water - 100 mL

pH - 5.4

Weigh the ingredients as given in the composition and dissolve them in 100 mL of distilled water. The pH is adjusted to 5.4 by adding 0.1 N HCl dropwise if required. The final pH of the medium is checked using narrow range pH paper. The medium is sterilized by placing in a boiling water bath for 10-15 minutes and cooled.

F) Sabouraud's Agar

Composition:

Glucose - 4 g

Peptone - 1 g

Agar - 1.5 g

Distilled water - 100 mL

pH - 5.4

Weigh the ingredients as given in the composition (except agar) and dissolve them in 100 mL of distilled water. Add the agar powder. The pH is adjusted to 5.4 by adding 0.1 N HCl dropwise if required. The final pH of the medium is checked using narrow range pH paper. The medium is sterilized for three consecutive days by autoclaving at 10 psi for 10 minutes.

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To check the sterility of the media, observe after 24 hours and up to 4 days to check for any growth or contamination.

Results: No growth was observed in the sterilized media as preparation was done aseptically.

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Experiment No. 04

MONOCHROME STAINING

Aim: To study the morphology of bacteria using simple stains.

Principle: Any basic dye such as methylene blue, safranin, or crystal violet can be used to color the bacterial cells. These stains readily give up a hydroxide ion or accept a hydrogen ion, thus making the stain positively charged. Since the surface of most bacterial cells and cytoplasm is negatively charged, these positively charged stains adhere to the cell surface. The bacteria will show up as colored spots against a white background.

Requirements:

Culture: Bacterial suspension

Labware: Glass slides

Nichrome loop

Glass marking pencil / marker

Immersion Oil

Equipment: Microscopes

Stains: Crystal violet, Basic Fuchsin, Loeffler's Methylene blue, Safranin.

Protocol:

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Take a clean grease - free slide

Place a loopful of the suspension on the glass slide inside the marked area and spread into a thin, even film.

Air dry the film

Heat fix

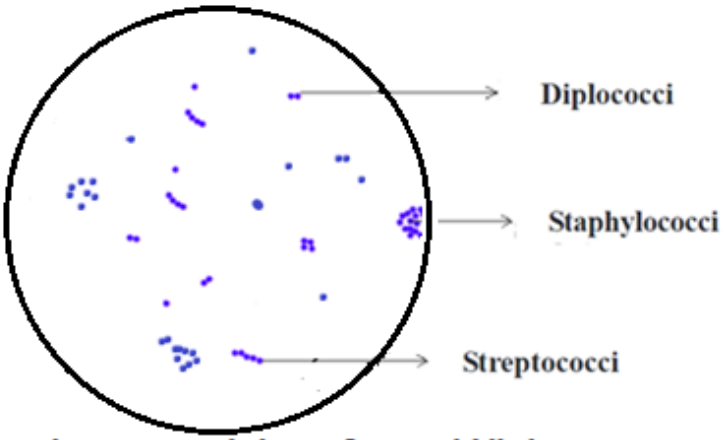
Keep the slide on a support and flood the smear with any one of the following stains and allow to stand for the following time intervals.

Crystal violet-30 seconds to 1 minute	Basic fuchsin-2-3 minutes	Loeffler's methylene blue-2-3 minutes	Safranin - 1 minute.

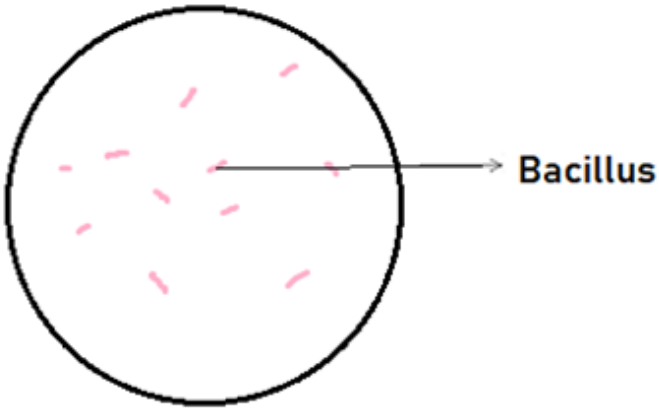
Gently wash the slide using an indirect stream of tap water.

Using a filter paper, blot dry (without rubbing) the smear.

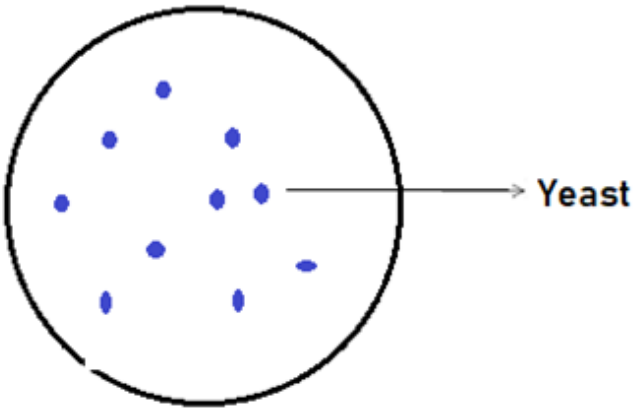
Examine the smear under oil immersion lens.



Monochrome staining- Crystal Violet



Monochrome Staining- Safranin



Monochrome Staining - Crystal Violet

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Results: Cocci, Bacilli and yeast cells were observed

Suspension	Morphology	Arrangement

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Experiment No. 05

NEGATIVE STAINING

Aim: To demonstrate the morphology of bacteria by negative staining technique.

Principle: India ink or nigrosin is an acidic stain. The stain readily gives up a hydrogen ion (proton) and the chromophore of the dye becomes negatively charged. Since the surface of most bacterial cells is negatively charged, the cell surface repels the stain. The glass of the slide will stain, but the bacterial cells will not. The bacteria will show up as clear spots against a dark background.

Requirements:

Culture: Bacterial suspension

Labware: Glass slides

Nichrome loop

Glass marking pencil / marker

Immersion Oil

Equipment: Microscopes

Stains: Nigrosine stain

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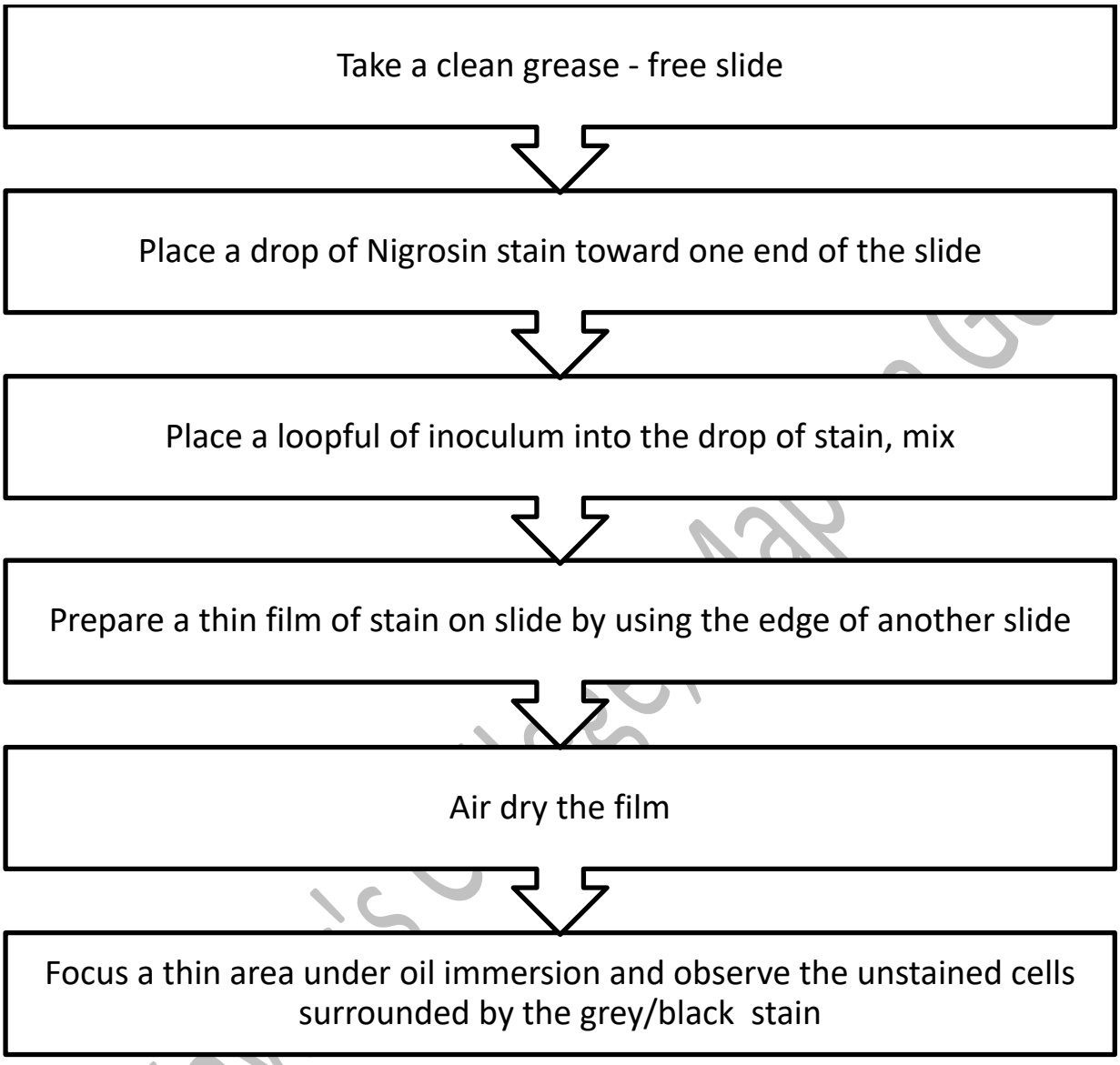
Protocol:


Take a clean grease - free slide
Place a drop of Nigrosin stain toward one end of the slide
Place a loopful of inoculum into the drop of stain, mix
Prepare a thin film of stain on slide by using the edge of another slide
Air dry the film
Focus a thin area under oil immersion and observe the unstained cells surrounded by the grey/black stain

Observation And Expected Results:

Colorless cocci, bacilli and yeast cells against a dark background

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Experiment No. 06

GRAM STAINING

Aim: To determine the Gram character of the given microorganism.

Principle: The structure of the organism's cell wall determines whether the organism is Gram positive or negative. Cell are stained with a primary stain Crystal violet (CV) which dissociates into CV^+ and Cl^- ions in aqueous solutions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells and the CV+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine (I), used as a mordant, interacts with CV+ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. Gram negative organism have additional lipopolysaccharide layer which gets dissolved due to the addition of alcohol, so Gram negative organisms fail to retain the complex and get decolorized as the complex is washed away. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. This closes the pores in the cell wall and thus traps the CV–I complex and prevents it from exiting the cell. After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Counterstain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color.

Requirements:

Culture: Bacterial suspension

Labware: Glass slides
Nichrome loop
Glass marking pencil / marker
Immersion Oil

Equipment: Microscopes

Stains: Crystal violet (Primary stain), Gram's iodine (Mordant), 95% alcohol (Decolourizer), Safranine or Basic fuchsin (Counter stain)

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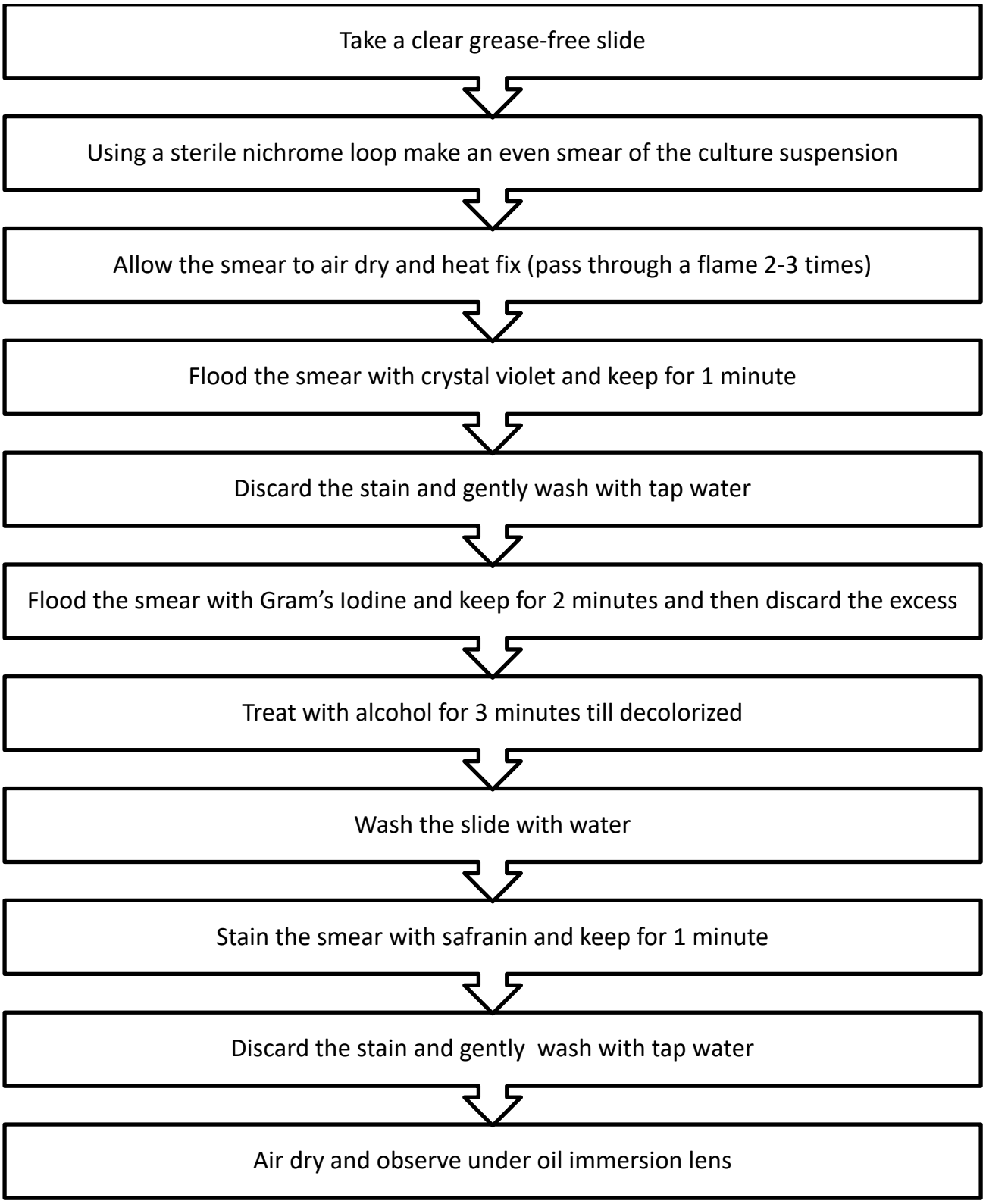
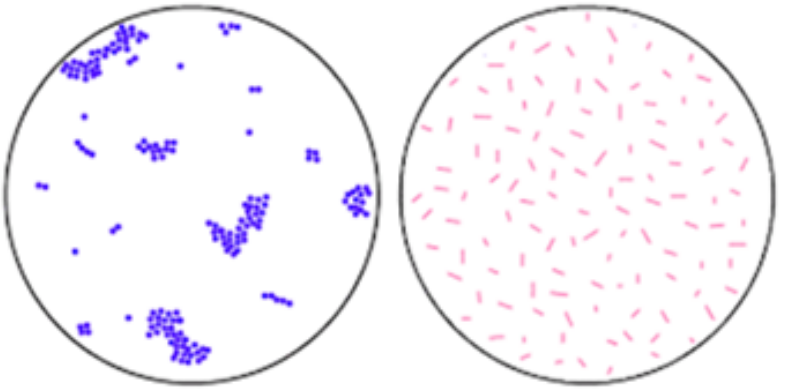
Procedure:


Take a clear grease-free slide
Using a sterile nichrome loop make an even smear of the culture suspension
Allow the smear to air dry and heat fix (pass through a flame 2-3 times)
Flood the smear with crystal violet and keep for 1 minute
Discard the stain and gently wash with tap water
Flood the smear with Gram's Iodine and keep for 2 minutes and then discard the excess
Treat with alcohol for 3 minutes till decolorized
Wash the slide with water
Stain the smear with safranin and keep for 1 minute
Discard the stain and gently wash with tap water
Air dry and observe under oil immersion lens

Observation And Expected Results:



Gram positive cocci Gram negative coccobacilli

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Experiment No. 07

ISOLATION OF PURE CULTURE BY STREAK PLATE METHOD

Aim: To obtain isolated colonies of bacteria/ fungi on an agar plate from a consortium of cells

Principle: The application of microbial cultures to the surface of the agar and spreading them by a loop is called streaking and the plates so prepared are called streak plates. During inoculation, the closely packed cells at the start of the streak form colonies that run together but as streaking continues, fewer and fewer cells remain in the droplet being carried on the loop. As this falls off and grows on the surface, well separated colonies develop. A good plate results from progressive movement of the loop, repeated several times.

Requirements:

Culture: Bacterial suspension

Labware: Nichrome loop

Glass marking pencil / marker

Media: Sterile nutrient Agar plates

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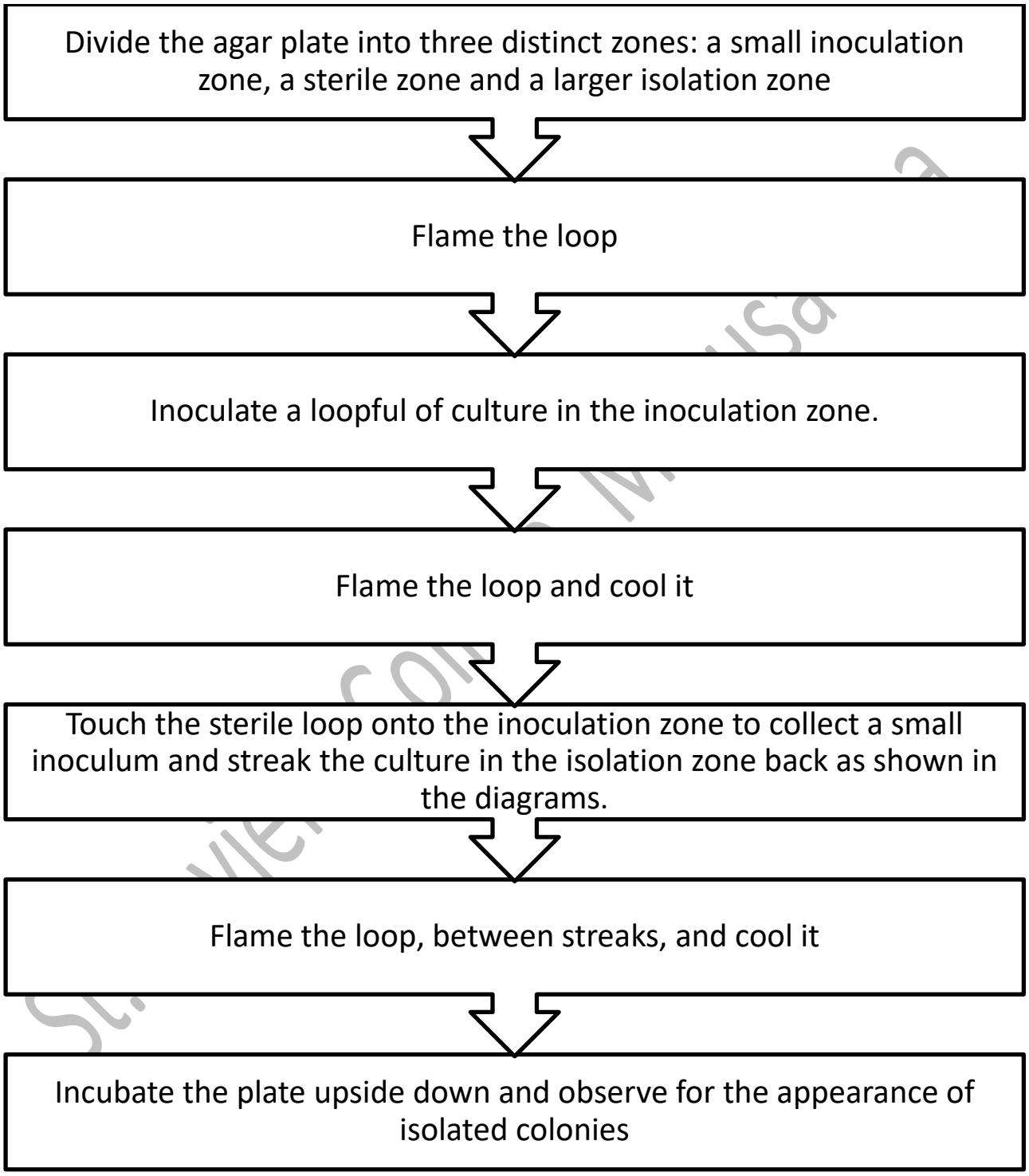
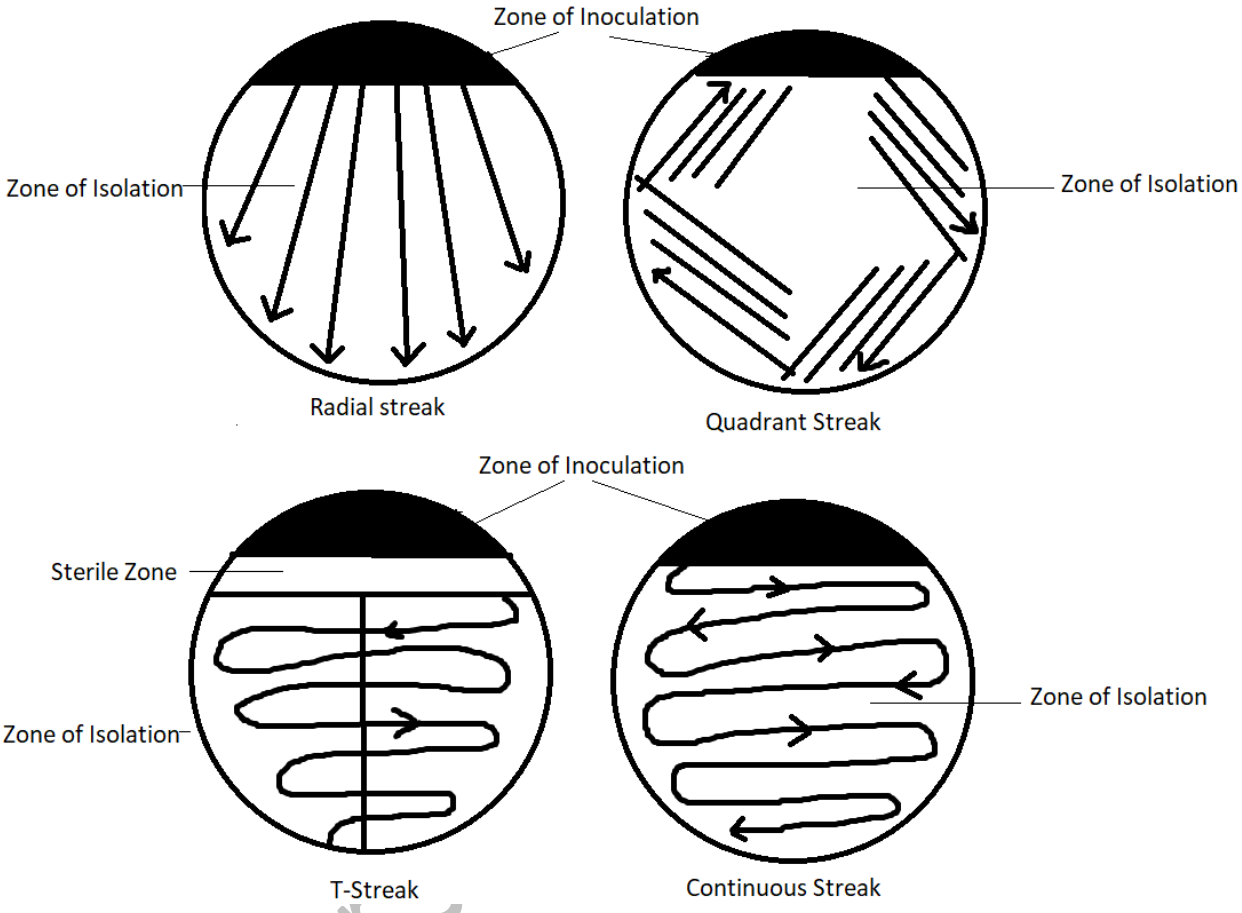
Protocol:


Divide the agar plate into three distinct zones: a small inoculation zone, a sterile zone and a larger isolation zone
Flame the loop
Inoculate a loopful of culture in the inoculation zone.
Flame the loop and cool it
Touch the sterile loop onto the inoculation zone to collect a small inoculum and streak the culture in the isolation zone back as shown in the diagrams.
Flame the loop, between streaks, and cool it
Incubate the plate upside down and observe for the appearance of isolated colonies

Observation And Expected Results:



Isolated Colonies along the line of streaking

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Experiment No. 08

VIABLE COUNT METHOD

Aim: To enumerate the bacteria in the given suspension using 1. Pour plate method and 2. Surface spreading method

Requirements:

Culture: Bacterial suspension18-24 hour old culture of E. coli

Labware: Glass marking pencil / marker

Sterile tubes

Sterile pipettes (1ml and 10 ml)

Media: Sterile nutrient agar plates.

Sterile nutrient agar butts (10 ml)

Chemicals: sterile saline

Procedure:

SPTREAD PLATE METHOD

a) Prepare the dilutions of the given culture as mentioned.

b) Transfer 0.1 ml of each of 10^{-5}, 10^{-4} and 10^{-3} dilutions to the surface of a sterile agar plate with a sterile pipette.

c) Using an alcohol sterilized glass spreader spread the dilutions evenly on the agar surface.

d) Incubate the plates inverted at 37ºC for 24-48 hours.

Result: The viable count of the given sample (*E. coli*) by spread plate method was found to be _____ cfu/ml.

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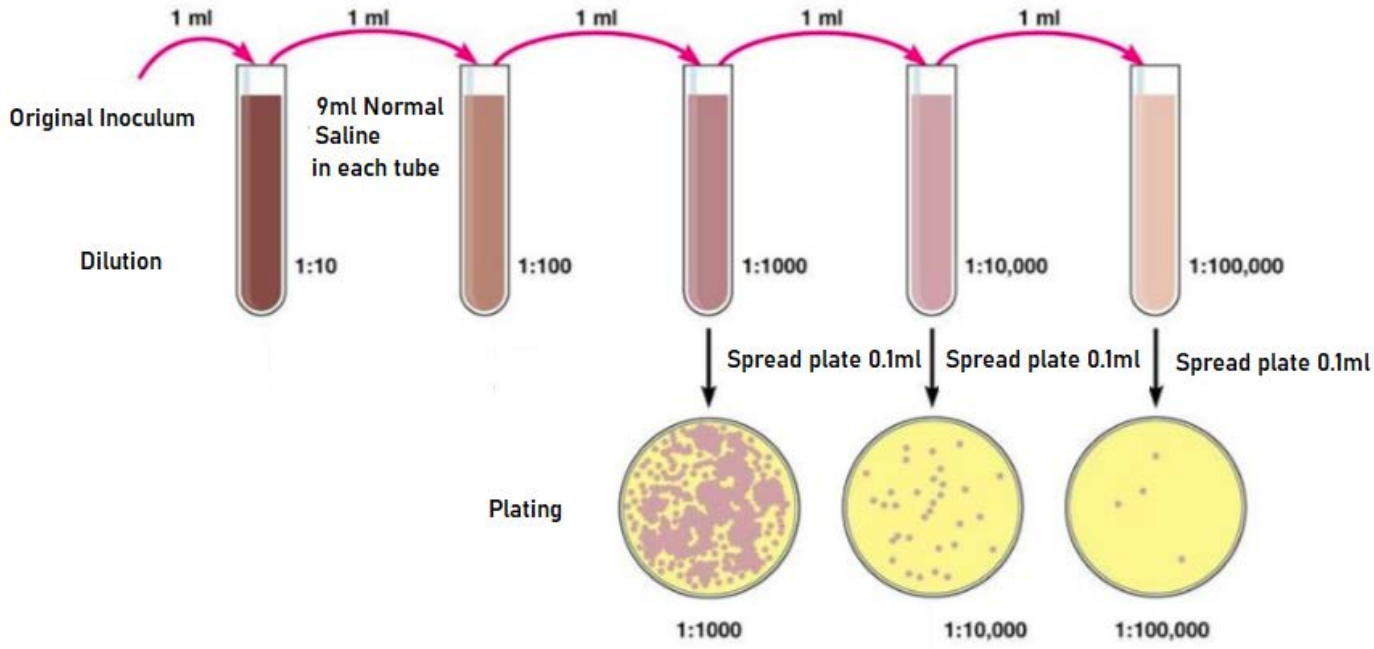
Viable Count



Calculations:

Viable count= <u>Average number of Colonies x Dilution factor</u>

Volume of Inoculum

Units- cfu/ml

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Experiment No. 09

HANGING DROP (MOTILITY TEST)

Aim: To demonstrate the motility of the given organism by means of the hanging drop technique.

Requirements:

Culture: 24 hour old culture of organisms

Glassware: Cavity slide, cover slips, nichrome loop, vaseline.

Principle: A very small drop of bacterial suspension is hung from the centre of a cover slip into the cavity of a cavity slide. The hanging drop is observed under a microscope using oil-immersion objective. If the bacteria are motile, its cells can be seen to have erratic movements in the surrounding medium. In contrast, if it is non-motile, its cells remain static in the medium without any movement or may show Brownian movement (resulting from the bombardment of cells with other cells or with surrounding water molecules in the medium).

Procedure:

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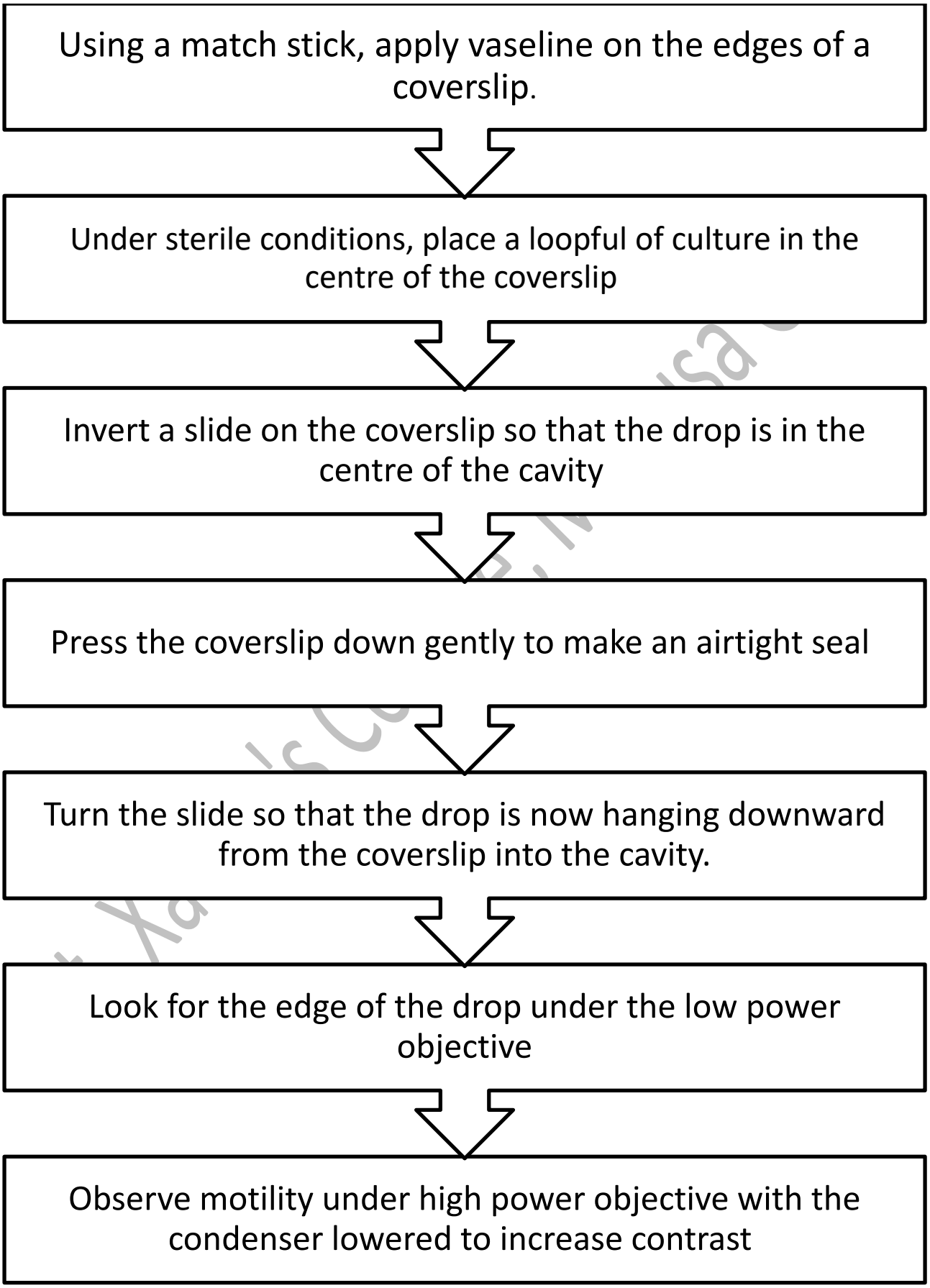
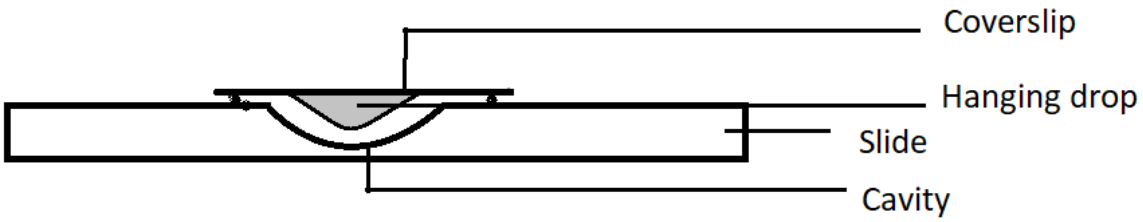
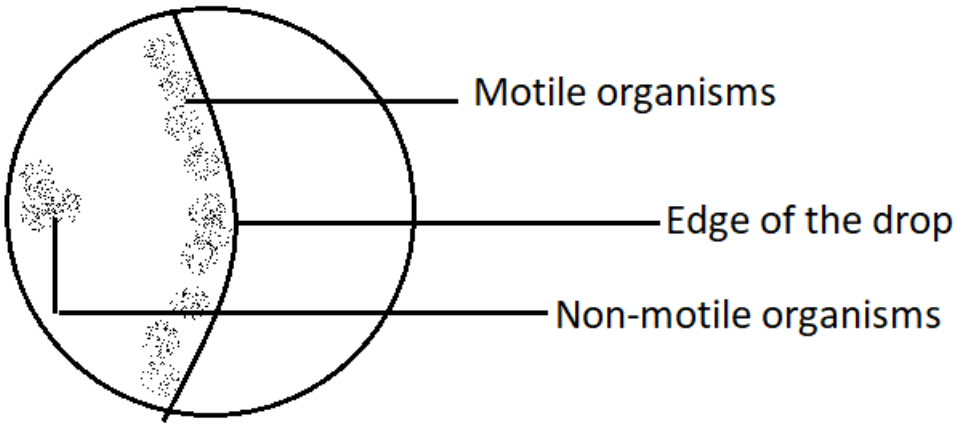

Using a match stick, apply vaseline on the edges of a coverslip.
Under sterile conditions, place a loopful of culture in the centre of the coverslip
Invert a slide on the coverslip so that the drop is in the centre of the cavity
Press the coverslip down gently to make an airtight seal
Turn the slide so that the drop is now hanging downward from the coverslip into the cavity.
Look for the edge of the drop under the low power objective
Observe motility under high power objective with the condenser lowered to increase contrast

Result:





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Experiment No. 10

QUALITATIVE TESTS FOR CARBOHYDRATES

1. MOLISCH TEST -

Aim: To detect the presence of carbohydrates in the sample.

Requirements:

*Chemicals***:** conc, H_2SO_4, α-naphthol, distilled water.

Glassware: test tubes

Sample: Test sample

Principle: Conc. H_2SO_4 hydrolysis the glycosidic bonds to give monosaccharides, which are then hydrolysed to furfural and its derivatives. These products combine with sulphonated α-naphthol to give a purple coloured complex. This reaction is seen in general with all carobydrates which can give furfural with conc. H_2SO_4.

Procedure:

a) Add 2 drops of α-naphthol solution (Molisch's reagent) to 2ml of test solution.

b) Carefully pour about 1ml of conc. H_2SO_4 along the sides of the tube so as to form 2 layers.

c) Carefully observe any colour change at junction of two layers.

d) Use D/W for the control.

e) Positive test is indicated by the formation of a violet ring at the junction of two layers.

Result: Violet ring is observed at the junction of 2 layers. Therefore carbohydrates are present.

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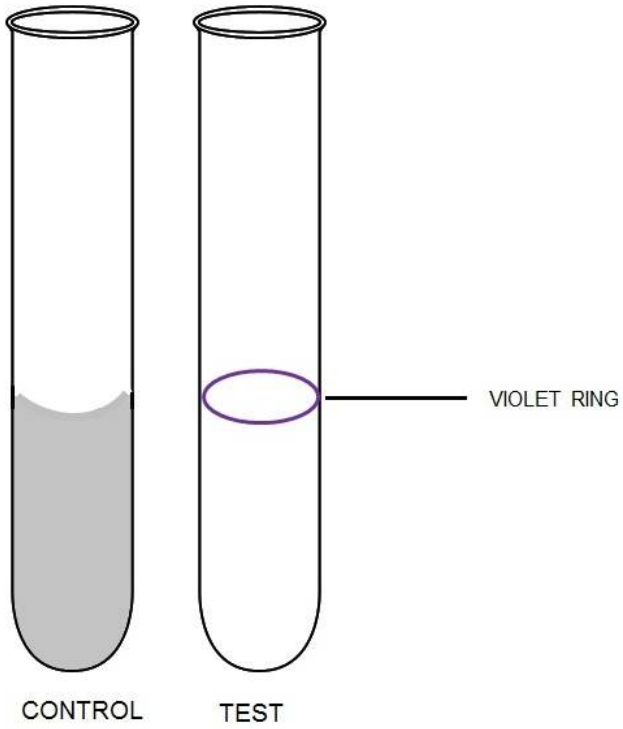
MOLISCH TEST (BLANK PAGE)



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2. BENEDICT'S TEST

Aim: To detect the presence of carbohydrates (Reducing sugars).

Requirements:

*Chemicals***:** Benedict's solution, distilled water

Glassware: test tubes

Sample: Test sample (Glucose)

Principle: Benedict's solution contains cupric ions (Cu^{+2}) in a stable complex form. If the carbohydrates have a reducing group, the cupric ion is reduced to cuprous ions (Cu^{+}) or even copper and this show up a rusty brown color or red precipitate.

Procedure:

a) Add 5 drops of test solution to 2 ml of Benedict's solution and place in a boiling water bath for 5 mins.

b) Use D/W for the control tube.

c) Positive test is indicated by the formation of a brick red color precipitate.

Result: Brick red / orange color precipitate is observed upon boiling indicating the presence of reducing sugars.

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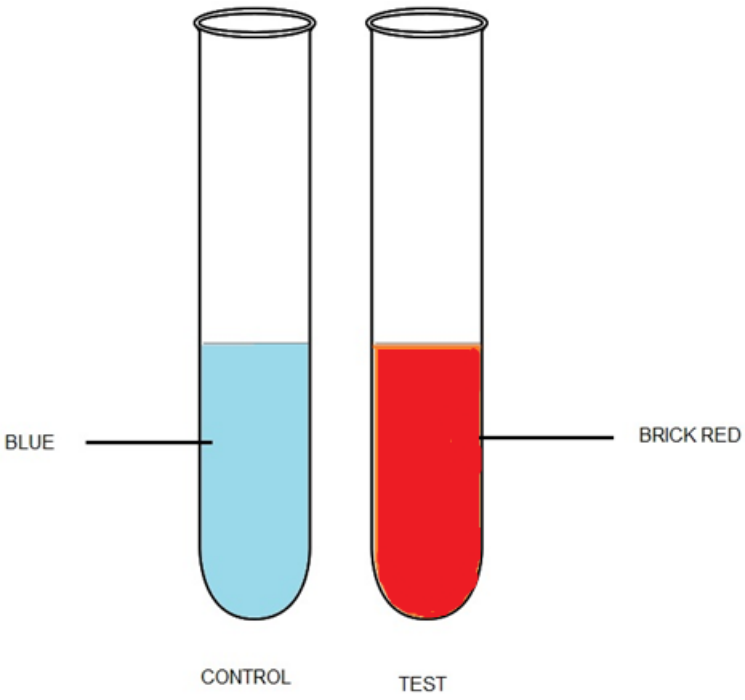
BENEDICT'S TEST



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3. IODINE TEST

Aim: To detect the presence of Starch.

Requirements:

*Chemicals***:** Iodine solution, dil. HCl, distilled water.

Glassware: test tubes

Sample: Test sample (starch)

Principle: Iodine forms colored complexes with polysaccharides. Starch gives a blue color with iodine. Glycogen forms wine red color upon reacting with Iodine.

Procedure:

1. Add 2 drops of Iodine solution to 1 ml of test solution and compare the color with the control comprising of Distilled water.
2. Positive test is indicated by the formation of a bluish black color.

Result: Bluish black color is obtained on addition of Iodine indicating the presence of Starch.

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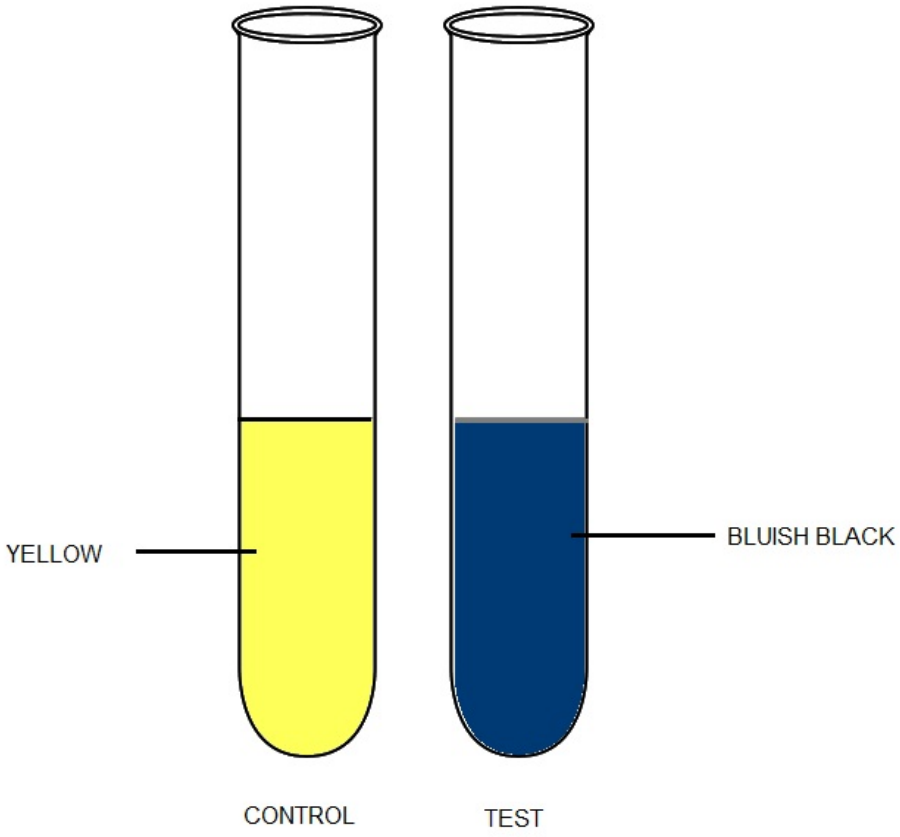
IODINE TEST



Experiment No. 11

QUALITATIVE TESTS FOR PROTEINS

1. BIURET TEST -

Aim: To detect the presence of proteins in the sample by detecting the presence of peptide bonds

Requirements:

*Chemicals***:** $CuSO_4$, NaOH, distilled water.

Glassware: test tubes

Sample: Test sample

Principle: Proteins are made up of many amino acids linked to each other by peptide bonds. Alkaline $CuSO_4$ reacts with compounds containing two or more peptide bonds to give a violet colored complex. In this reaction, the Cu^{+2} ions form a colored coordinate complex with the nitrogen atoms of the peptide bond.

Procedure:

a) Add 2 drops of $CuSO_4$ solution to 2ml of NAOH and 1 ml test solution.

b) Mix thoroughly and observe the color produced.

c) Use D/W for the control.

d) Positive test is indicated by the formation of violet color in the solution.

Result: Violet color is observed. Therefore proteins are present

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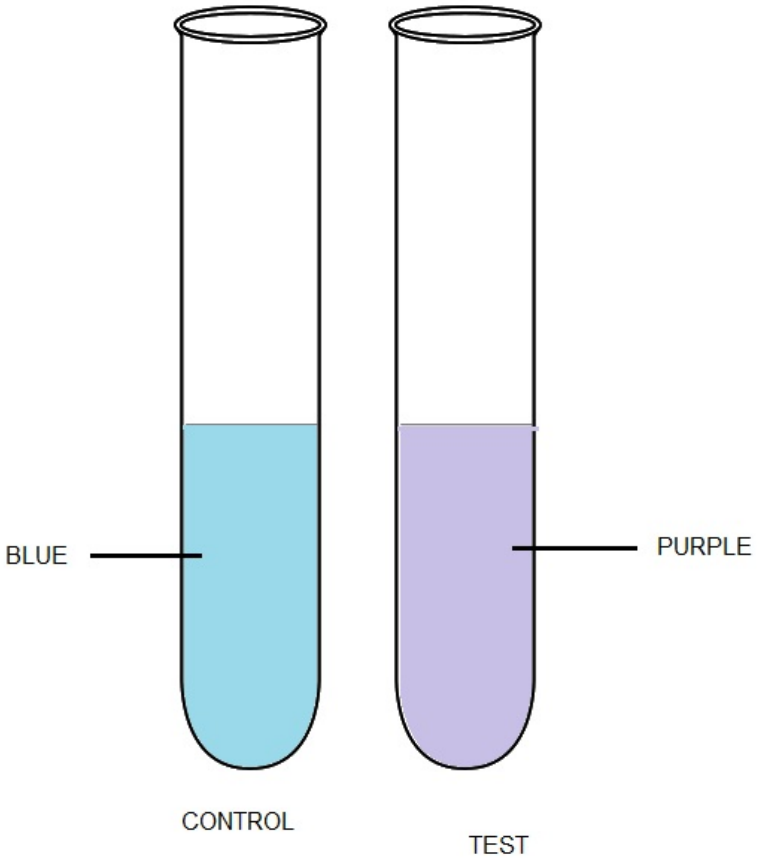
BIURET TEST



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2. NINHYDRIN TEST

Aim: To detect the presence of amino acids

Requirements:

*Chemicals***:** Ninhydrin reagent, distilled water

Glassware: test tubes

Sample: Test sample

Principle: Ninhydrin is a powerful oxidizing agent. It reacts with the α-amino group of free amino acids between pH 4 to 8 to give a purple colored complex. The amino group undergoes oxidative deamination to liberate ammonia, CO_2 and the reduced form of ninhydrin called hydrindantin. The formed products further react with ninhydrin to produce a purple colored complex. Amino acids like proline give a yellow colored reaction.

Procedure:

a) Take 1 ml of test solution in a test tube.

b) Add 5 drops of Ninhydrin solution and carefully boil for 2 minutes.

b) Use D/W for the control tube.

c) Positive test is indicated by the formation of a violet colored solution.

Result: Violet color is observed upon boiling indicating the presence of amino acids.

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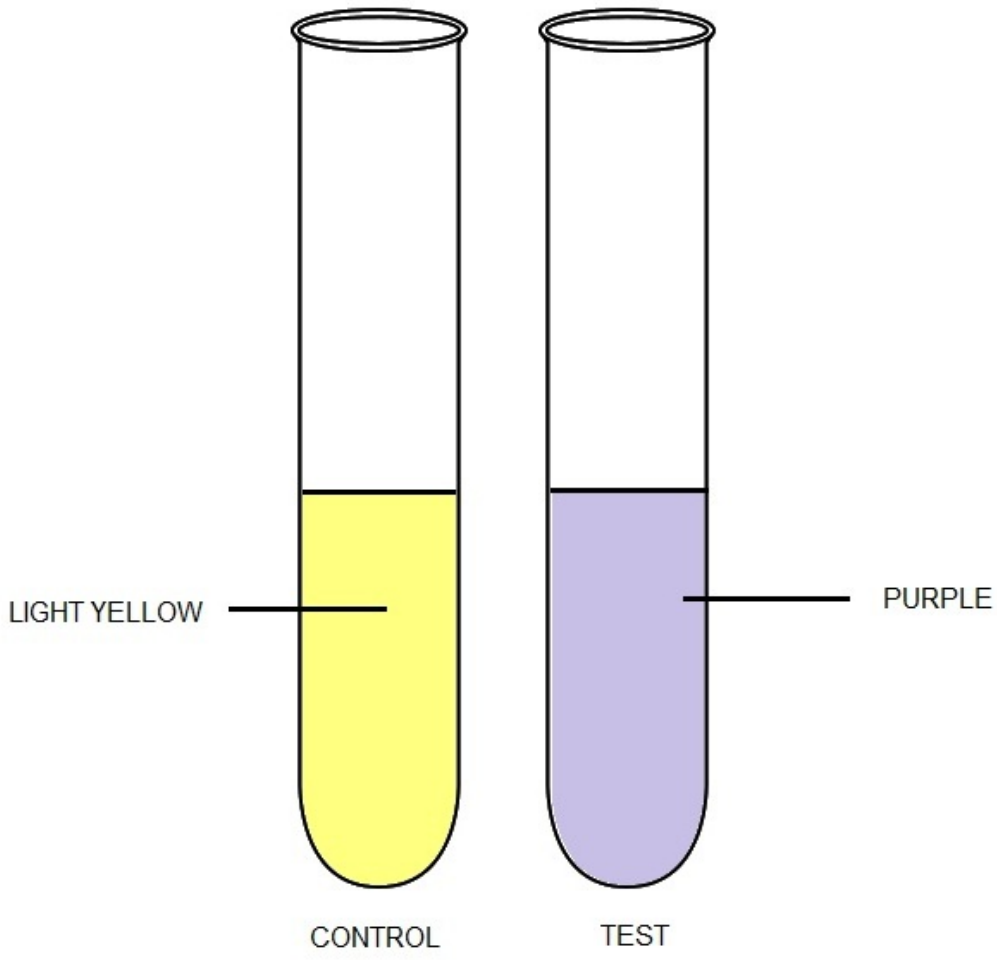
NINHYDRIN TEST



Experiment No. 12

QUALITATIVE TESTS FOR LIPIDS

1. SOLUBILITY TEST

Aim: To determine the solubility of lipids in different solvents.

Principle:

Lipids are a heterogeneous class of naturally occurring organic substances grouped together on the basis of a physical property namely solubility and not the functional group. They are widely distributed in both animal and plant systems and perform a wide variety of functions. These include energy storage, structural components (*e.g*. cell membranes), vitamins, metabolism regulators (*e.g*. steroid hormones), and emulsifying agents.

Lipids are all insoluble in polar solvents like water, but highly soluble in non-polar or weakly polar organic solvents, including ether, chloroform, benzene, and acetone. Upon addition of water, lipids form a milky white emulsion of fine droplets.

Lipids includes not only fats and oils, which are esters of the trihydroxy alcohol glycerol and fatty acids, but also compounds derived from phosphoric acid, carbohydrates, amino alcohols and steroids

Requirements:

*Chemicals***:** alcohol, ether, distilled water

Glassware: test tubes

Sample: Test sample or oil

Procedure:

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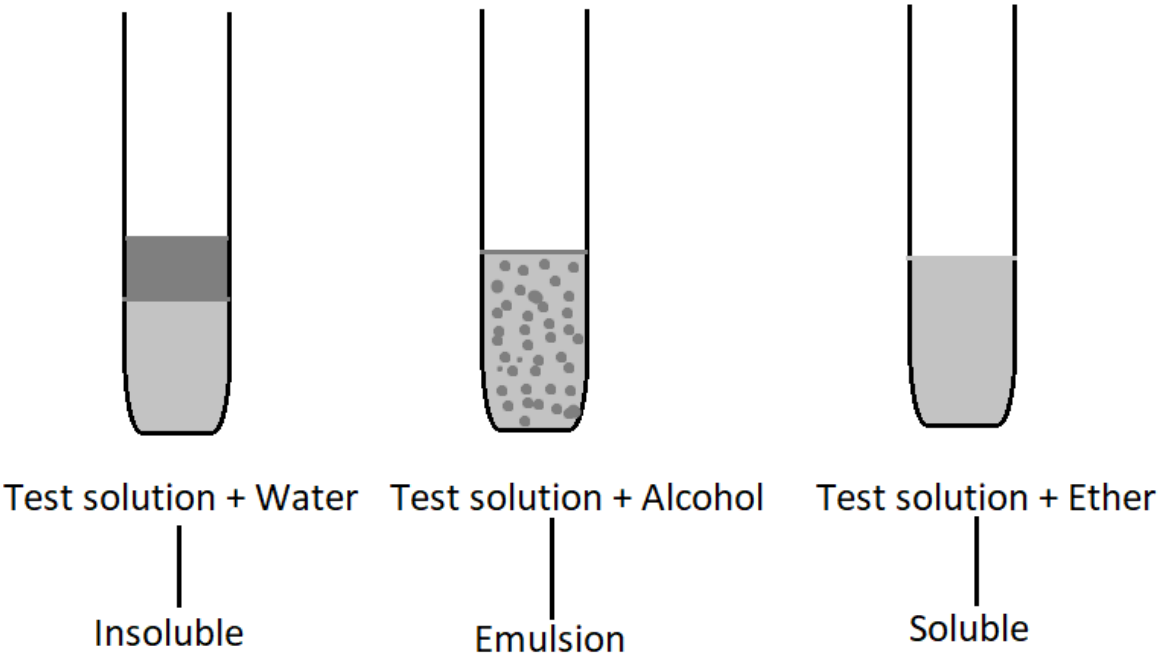
1. Place 1 ml of the test solution in 3 clean test tubes containing 2 ml of the solvents namely water, alcohol, and ether.
2. Shake the tubes thoroughly, then leave the solution for about one minute

Result: Lipids are insoluble in water, forms an emulsion with alcohol and is soluble in ether



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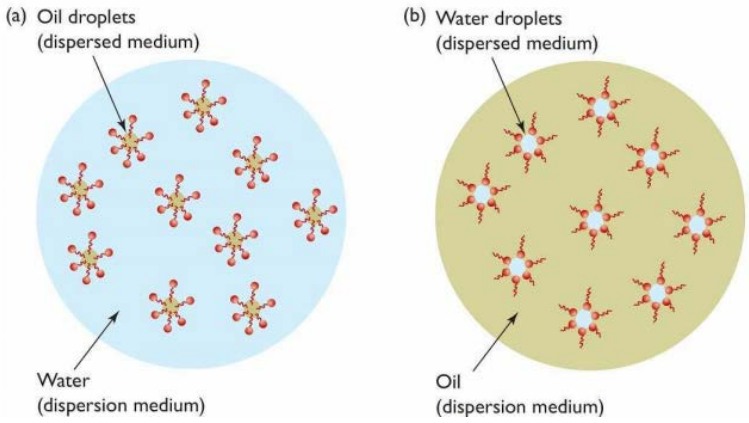


2. SAPONIFICATION AND SOAP FORMATION

Aim: To detect the presence of fatty acids.

Principle: Triglycerides (vegetable oils and animal fats) are greasy materials that are saponified. These triglycerides, are mixtures derived from diverse fatty acids. Triglycerides can be converted to soap by treating it with a strong base (e.g. lye), which cleaves the ester bond; releasing fatty acid salts (soaps) and glycerol.

Requirements:

*Chemicals***:** NaOH solution in alcohol (20% NaOH) Glassware: test tubes

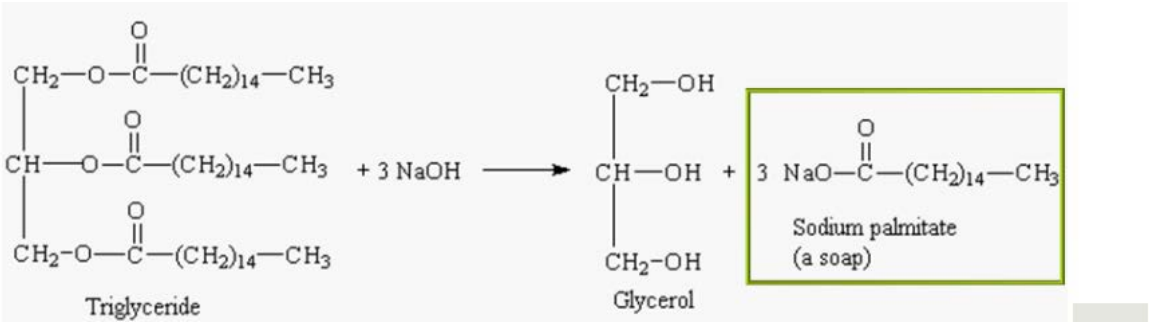
Sample: Test sample or oil

Equipment: Boiling water bath

Procedure:

1. Place 2 ml of test solution in a large test tube
2. Add 4 ml of alcoholic sodium hydroxide.
3. Boil the solution for 3 minutes.
4. After this period, make sure the process of saponification has occurred by taking a drop of the solution and mix it with water, (if the oil separates it indicates the non-completion of the saponification process).
5. In this case, continue to boil until all the alcohol evaporates.
6. Take the remaining solid material (soap) and add about 30 ml of water.
7. Shake the solution after it cools and note the formation of thick foam.

Result: Precipitation of insoluble salts (soap) was observed and therefore fatty acids are present.



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References:

1. Cappuccino, J.G. and Sherman, N. (2002) Microbiology: A Laboratory Manual. 6th Edition, Pearson Education Inc.
2. Negative Staining- Principle, Reagents, Procedure and Result (microbiologyinfo.com)

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