

ACP101: MICROBIOLOGY PRACTICALS

PRACTICAL 1: INTRODUCTION TO LABORATORY – RULES AND PROCEDURES, LABORATORY EQUIPMENT AND APPARATUS

The first session of this practical will be a demonstration and the class will be divided into four groups, each of which will visit the following stations:

- i. Parts and use of microscopes
- ii. Equipment used in microbiology
- iii. Apparatus used in microbiology
- iv. Reagents and culture media

Each group will spend about 15 minutes (total time 60 minutes in all the 4 stations) in each and a technician will demonstrate the above and how they are used in microbiology.

In second session on use of microscopes (about 45 to 60 minutes), the students will work within their designated groups of about 10 to 15 students.

1. Objectives

- i. To understand the rules and procedures to be observed in a laboratory
- ii. To know and familiarize with equipment and apparatus used in microbiology practical exercises
- iii. To familiarize and understand the parts and use of microscopes

2. Required for this practical exercise

- i. List of rules and procedures
- ii. Apparatus – petri dishes, microscope slides, cover slips, inoculating needles, wire loops, spirit lamps, paper towel, universal bottles, magnifying glass, scalpel with holder, beakers, conical flasks, media bottles, V-shaped glass rod, L-shaped glass rods, staining rack, microscope lens cleaning tissue, muslin cloth, filter paper, pipettes (various sizes), pipette filler,
- iii. Reagents – absolute ethanol, 70% ethanol, 2.5% sodium hypochlorite, sterile distilled water
- iv. Stains – methylene blue, water, cotton blue in lactophenol, safranin, iodine, crystal violet, nigrosin, carbol fuchsin
- v. Equipment – dissecting microscope, compound microscope, weighing balance, hot air oven, autoclave, pH meter,
- vi. Culture media – potato tubers, sugar (ordinary), dextrose, glucose, agar, fat free beef (a few pieces), mushroom, commercial potato dextrose agar (PDA), commercial nutrient agar (NA)

3. Laboratory rules and procedures

1. Always wear a laboratory coat or apron to protect clothes from contamination or accidental discolouration.
2. Before and after each laboratory session, clean the laboratory bench with disinfectant like Lysol, or 70% ethanol.

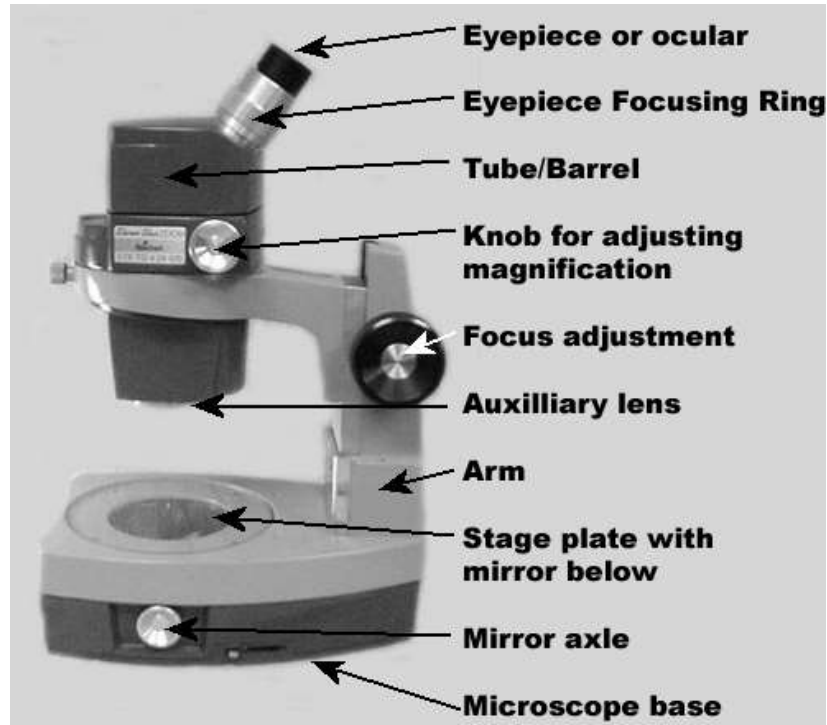
3. Keep the laboratory bench clean (no books, bags, papers, clothing etc) except laboratory apparatus and your note book.
4. Never smoke, eat or drink in the laboratory. Food and drink must not be taken in the laboratory, as it could be contaminated.
5. Never place pencils, pens, fingers etc in your mouth during laboratory sessions.
6. Long hair should be tied back to minimize contamination of cultures and fire hazards.
7. Wash hands with soap and water before and after laboratory sessions.
8. Liquid cultures and reagents must never be pipetted with mouth; use pipette fillers.
9. Aseptic techniques must be observed at all times.
10. Label all plates, tubes, cultures etc before starting an experiment. Indicate date of experiment.
11. Materials such as stains, reagent bottles, Petri plates, pipettes, microscopes etc must be returned to their original place after use.
12. All cultures should be kept covered and the Petri dish lids should be on whenever cultures are not in use.
13. All microbial cultures must be handled as being potentially pathogenic.
14. Never pour cultures down the water sink.
15. If a live culture is spilt, cover the area with a disinfectant for 15 minutes and then clean it.
16. Used and contaminated glassware should be kept in the containers provided. Old cultures must be disposed off promptly by sterilization in autoclave.
17. Be careful of laboratory burners: never spray ethanol near flame; turn the burner off when not in use. Equipment like ovens, microscopes, etc must be switched off whenever not in use.
18. You must familiarize yourself with the exercise to be performed.
19. Handle all equipment with care: never open or dismantle parts of equipment. Microscope stage, lenses and objectives should be cleaned with special lens tissue before and after use.
20. Any breakages, accidents or out of the ordinary occurrences must be reported immediately to the person in charge of the practical.
21. Keep the laboratory doors and windows closed when experiments are in progress.
22. As you perform the exercise, record your data in ink and make sketches and labels in pencil.
23. Laboratory exercise reports should be written immediately the observations are complete.

4. Handling and use of microscopes

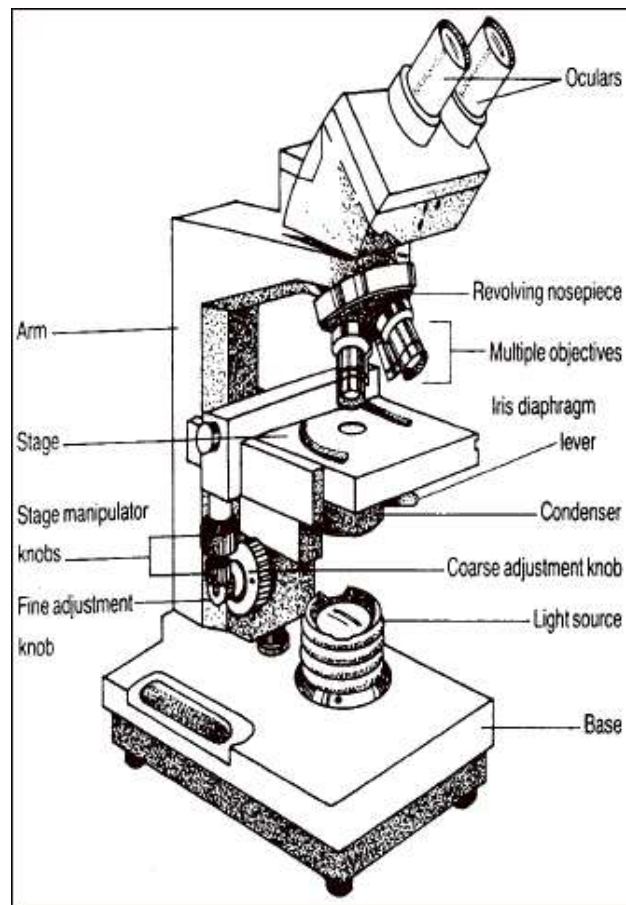
Required: Magnifying glass, dissecting microscope, compound microscope, glass slides, cover slips, microscope lens cleaning tissue.

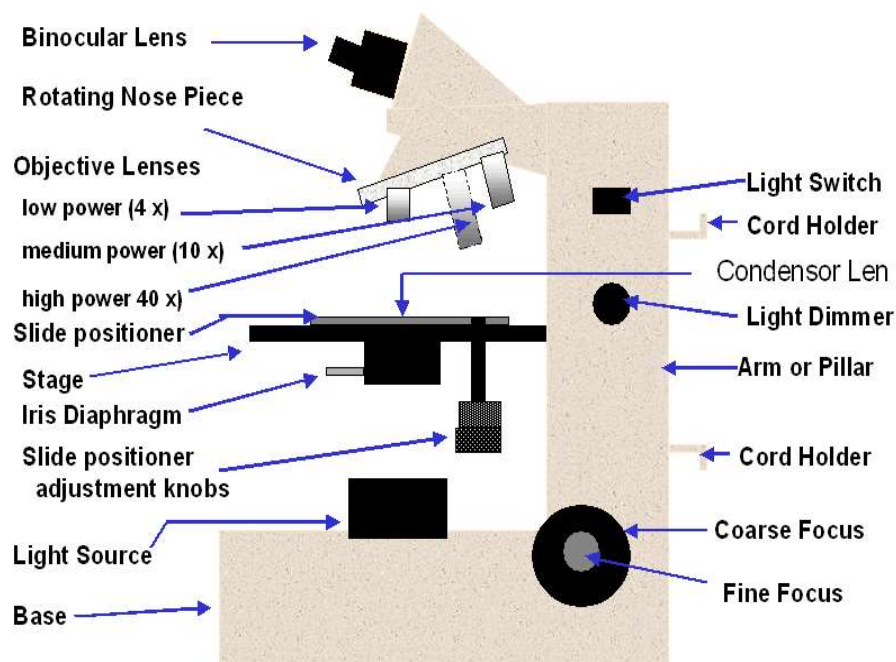
- The parts of the microscope
- Differences between magnifying glass, dissecting and compound microscopes
- Use of the different parts
- Viewing of objects using the microscope – when to use magnifying glass, dissecting or compound microscope
- When and how to use the different objective lenses and how focus objects for viewing
- Cleaning of the objectives
- Handling and care in the use of microscopes

Parts of a compound microscope



Dissecting microscope: <http://www.apsnet.org/edcenter/K-12/NewsViews/Pages/DissectingMicrosocpe.aspx#>





5. Equipments used in microbiology

- Show the different types of equipment
- Sterilization – autoclave, oven,
- Observation – microscopes
- Measurement equipment – weighing balances, pH meters, colony counter, haemocytometer, spectrophotometer
- Incubation – Incubator (to maintain specific incubation temperature); water bath (for cooling and maintaining media required temperature)

6. Apparatus used in microbiology

- petri dishes – used to hold culture media for growth of the microorganisms
- microscope slides and cover slips – mounting microbiological specimens for observation under microscope
- inoculating needles and wire loops – for transfer of microbiological specimens
- spirit lamps – for sterilization of needles, wire loops, spatula
- beakers, universal bottles, conical flasks and media bottles – for holding culture media and solutions
- paper towel and filter paper – for wiping surfaces, to hold moisture in closed micro-environments to create high relative humidity for microbial growth and filtration of solutions.
- magnifying glass – for enhancing the magnification of small macroscopic objects that are too large for viewing under microscope,
- scalpel blade with holder – for cutting objects
- V-shaped glass rods – used in assembly of slide culture (a special technique of growing fungi)
- L-shaped glass rods – used during inoculation to spreading bacterial suspensions on surface of media.

- staining rack – a glass rack where microscope slides are placed during staining procedures; the rack is usually placed over a water sink or open container to hold the dripping stains
- microscope lens cleaning tissue – special tissue paper for cleaning microscope lenses.
- muslin cloth – a porous cloth for filtering/ straining suspensions
- pipettes – for transferring required volumes of a solution.
- Reagents and culture media – when, where and how they are used.

Questions

1. In one sentence, explain the difference between dissecting and compound microscope?
2. Why is it not possible to observe bacteria under a dissecting microscope?
3. Why is it necessary to wipe lab bench surface with 70% ethanol before starting an experiment?
4. It is not allowed to come to the laboratory wearing open shoes – why?

PRACTICAL 2: ASEPTIC PROCEDURES, CULTURE MEDIA AND HABITAT OF MICROORGANISMS

Objectives

- i. To learn and familiarize with the sterilization methods and procedures
- ii. To learn the ingredients and preparation of general purpose culture media
- iii. To learn the skills used in isolating and culturing microorganisms
- iv. To learn the procedures in making slides for observation under microscope
- v. To appreciate the abundance and diversity of microorganisms in different habitats

Parts A to D of today's practical will be demonstrations – the class will be divided into four groups. Each group will spend about 15 to 20 minutes in each station (total maximum 80 minutes in all four demonstrations).

In part E, students will work in their normal groups (10 – 15 students per groups).

A. Demonstration of aseptic / sterilization procedures

Required: autoclave, hot air oven, spirit lamp or Bunsen burner, absolute ethanol, 70% ethanol, 2.5% sodium hypochlorite, Laminar flow hood (may use improvised chamber),

The following will be demonstrated:

- Importance of starting with clean surfaces and clean laboratory free of dust
- Sterilization of working surface with 70% ethanol
- Sterilization of needles, wire loops, scalpels, spatula by placing in absolute ethanol and flaming
- Use of sodium hypochlorite to sterilize tissues
- Working of autoclave, hot air oven etc
- Working of laminar flow (may use improvised chamber)

B. Demonstration of preparation of culture media

Required:

- i. Ingredients for making culture media: commercial media, agar, mineral salts, distilled water, fresh potato tubers, sugars (sucrose, glucose).
- ii. Apparatus used in media preparation – media bottles, analytical, weighing balance, hot plate, magnetic stirrer, beakers, graduated cylinders, pipettes, Petri dishes (glass, plastic), autoclave, water bath etc.

Preparation of potato dextrose agar (PDA) and nutrient agar (NA) from raw, locally available ingredients will be demonstrated.

Potato dextrose agar (for growth of fungi)

Potato	200 g
Dextrose (may use ordinary sugar)	20 g
Agar	20 g
Water	1,000 ml

- Peel potato and cut into small cubes.
- Weigh 200 g of the potato cubes
- Boil the potato in 1,000 ml of water until soft

- Allow to cool and strain through cheese cloth into a clean beaker
- Add 20 g of dextrose (or ordinary sugar) and 20 g agar to the potato extract and heat the mixture on a hot plate until agar dissolves.
- Place the mixture in a media bottle and make up volume to 1000 ml
- Autoclave at 121 °C for 15 minutes
- Allow to cool and pour into Petri dishes

Nutrient agar (for growth of bacteria)

Fat free beef	300 g
Potato	200 g
Mushroom	100 g
Sugar	2.5 g
Agar	20 g
Water	1,000 ml

- Cut the beef into small pieces and potato in four pieces; cut the mushroom in half
- Add the cut pieces into a beaker containing 1000 ml water and cook for 2 hours
- Allow to cool and filter through cheese cloth
- While stirring, add agar and sugar until all the agar is dissolved
- Make the solution to 1000 ml and autoclave at 121 °C for 15 minutes
- Allow to cool and pour into Petri dishes

C. Demonstration on isolating and culturing microorganisms

Materials required for isolation – inoculating needles, wire loops, spirit lamp, prepared plates (with set media), diseased plant materials (with fungal and bacterial infection), cultures (bacterial and fungal), forceps, scissors, scalpels, 70% ethanol, sodium hypochlorite, sterile distilled water, wire loops, inoculating needles, incubator.

The following will be demonstrated:

- Preparing for isolation – washing and cutting of materials into small piece
- Surface sterilization and rinsing in sterile distilled water
- Maceration of tissues in sterile water for bacteria;
- Plating for fungi
- Streaking for bacteria
- Sub-culturing from mixed culture for both bacteria and fungi

D. Demonstration of and making slides, mounting and focussing for microscopic examination

Materials required – cultures of bacteria and fungi; mounting needles; wire loops; methylene blue; cotton blue in lactophenol; microscope slides and cover slips; spirit lamp; microscope.

The following will be demonstrated:

- How much fungal mycelium specimen to collect with a needle from the culture for mounting on slide
- How to stain and place cover slip above fungal specimen
- How much of the bacterial specimen to collect with wire loop from culture for making a smear
- How to make a smear and stain bacterial specimen
- Placing slides on microscope stage, adding oil for bacteria and adjustments on stage
- Steps in focussing – locating the specimen; start with lowest (x5 or x10) to the highest (x40) for fungi; focussing by moving stage downwards

E. Habitats of microorganisms

The purpose of this experiment is to help students appreciate that microorganisms are found in all kinds of environments and how different habitats contain varying numbers and types of microorganisms. Each group will be provided with 12 plates containing potato dextrose agar (PDA) medium. Potato dextrose agar is a general purpose medium that can support growth of both fungi and bacteria.

Perform the following tests:

1. Open and expose 2 plates to the air inside the laboratory for about 5 minutes
2. Open and expose 2 plates to the air outside the laboratory for about 5 minutes
3. Collect soil from outside, pulverize a pinch of the soil between your index finger and thumb and sprinkle a little bit on the plate surface (use 2 plates).
4. Collect some fresh, green leaves, cut them into small fragments, and place five pieces on the plate surface (use 2 plates).
5. Collect some dry, rotting plant materials, cut them into small fragments, and place five pieces on the plate surface (use 2 plates).
6. Leave 2 plates intact – make sure they are not opened at all (these will act as control).

Label the plates (kind of treatment, group number, date) and Incubate at room temperature at room temperature for about one week. Examine the plates during the next practical session and record your observations. Record the number and types of fungi and bacteria, which develop.

NB: The microorganisms obtained from this exercise will be used for all the other tests in practicals 3 to 8. Each group must take care that their plates are not mishandled since any loss or bad contamination of the cultures will affect future practicals.

Questions

- i. After one week, observe the plates and complete the following table:

	BACTERIA		FUNGI	
	No of bacterial colonies	No of bacterial colony types based on colour	No of fungal colonies	No of fungal colony types based on colour and growth habit
Exposed to air inside				
Exposed to air outside				
Sprinkled with soil				
Dry, rotting vegetation				
Fresh, green leaves				
Control (un-opened plate)				

- ii. Which plate has the highest number of colonies of both bacteria and fungi combined – explain why?
- iii. Which plate has the least number of colonies of both bacteria and fungi combined – explain why?

- iv. Which habitat has the greatest diversity (the number of different colony types based on colour and growth habit) – explain why?
 - v. Which habitat has the least diversity (the number of different colony types based on colour and growth habit) – explain why?
 - vi. What is the use of the control (the un-opened plate)?
 - vii. In your own words, describe the appearance of the colonies of fungi that you isolated
 - viii. In your own words, describe the appearance of the colonies of bacteria that you isolated
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PRACTICAL 3: ISOLATION AND PREPARATION OF PURE CULTURES OF BACTERIA AND FUNGI

Introduction

A pure culture is a culture which contains only one kind of a microorganism. A culture that contains more than one kind of microorganism is called a mixed culture. Pure cultures are essential in the study of the following aspects of microorganisms: (i) colony characteristics, (ii) biochemical and DNA-based identification, (iii) morphology (iv) staining reactions. Pure cultures of microorganisms in the form of discrete colonies on solid media may be obtained by separation of individual organisms on or in a nutrient agar medium. Each viable cell gives rise, through growth to form a colony. The most commonly used methods for obtaining pure cultures of microorganisms are: (i) Streak-plate; (ii) Pour plate; (iii) Spread plate; (iv) use of differential and selective media; (v) use of enrichment media.

Objectives

- i. To appreciate the appearance of different microorganisms when growing together on culture medium
- ii. To gain skill in separating one microorganism from a mixture of different microorganisms growing on culture media.
- iii. To practically learn the methods of making pure cultures of bacteria and fungi

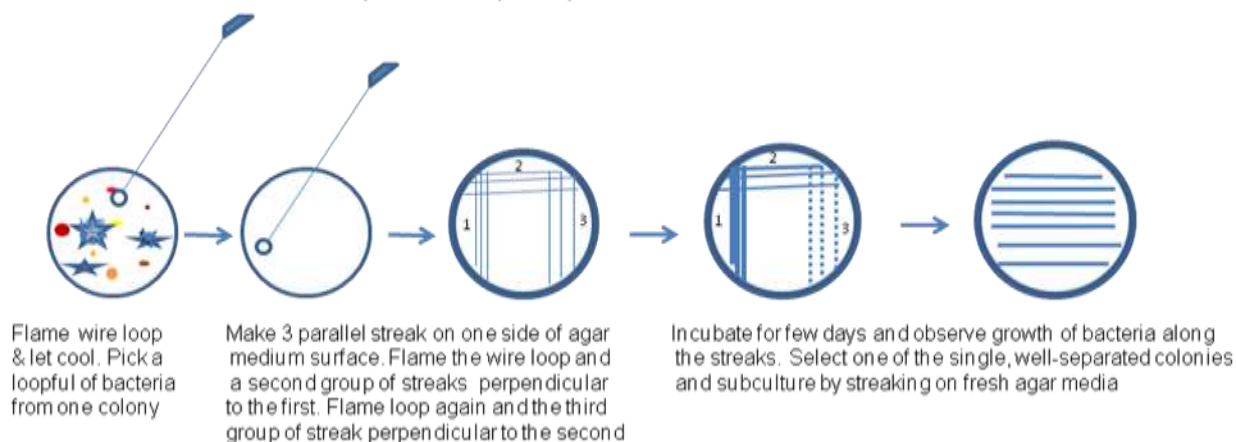
Requirements

Nutrient agar plates; mixed cultures of bacteria and fungi (from previous practical); Inoculating wire loop and needles; Spirit lamp or Bunsen burner

Streak-plate method of separating bacteria from a mixture of microorganisms

1. Label all the plates on the bottom.
2. Sterilize a wire loop on spirit lamp flame and allow it to cool
3. Holding the wire loop in your right hand, pick bacterial growth from a well-isolated colony (for culture on solid agar) or dip the loop into a culture broth (for broth cultures) and withdraw a loopful of the culture.
4. Lift the Petri dish cover with the left hand and open by holding at an angle of 60°
5. Place the loop containing the inoculum on the agar surface at the edge on the left hand side of the plate and streak the inoculum from side to side in parallel lines across the surface of the area.
6. Re-flame and cool the wire loop and turn the Petri dish at 90° . Make another group of parallel streaks perpendicular the first; re-flame the loop, allow to cool and make a third group of streak perpendicular to the second group.
7. Replace the lid of the Petri dish and incubate the plate in an inverted position at 25°C for 48-72hours.
8. Examine the plates for growth of bacterial colonies.

Streak-plate technique for purification of bacteria culture



NB: (i) Avoid pressing the loop too firmly against the agar surface as this will damage the agar surface; (ii) inoculating loop should be cooled by touching the agar surface at the middle of the plate away from the set of streaks; (iii) the Petri dish lid should never be lifted completely; (iv) the agar media should be prepared 24 hours before plating to complete drying of agar surface.

Results

A confluent growth will be seen where the initial streak was made but the growth is less dense away from the streaks. Discrete colonies are formed on the third group of streaks. Any colony not growing on the streak marks is regarded as a contaminant. If discrete pigmented and non-pigmented colonies are observed on the plates inoculated with mixed cultures, it shows that the components of the mixed **broth** have been successfully separated. Select a well isolated colony and transfer with a sterile wire loop onto fresh media.

Preparing pure cultures

Sub-culturing is the procedure of transferring microorganisms from their parent growth source to a fresh one or from one medium to another. After incubation and appearance of the discrete, well separated colonies, the next step is to subculture some of the cells from one of the colonies to a separate agar plate or nutrient agar slants. The culture obtained represents the growth of a single species and is called a pure culture.

Procedure

Bacteria

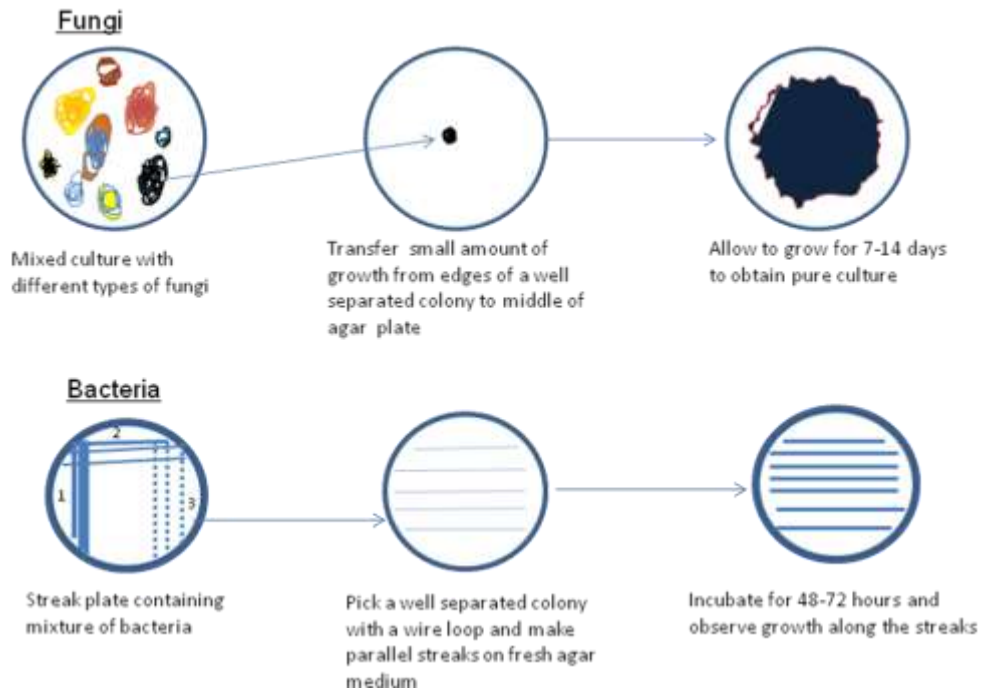
1. Flame a wire loop to red hot on a spirit lamp flame and cool it by dipping in a fresh agar plate.
2. Touch the tip of the loop to the surface of a selected discrete colony.
3. Lift the lid of the agar plate at 45° and inoculate by making parallel streaks on the agar surface.

Fungi

1. Flame an inoculating needle to red hot on a spirit lamp flame and cool it by dipping in a fresh agar plate.
2. Cut a very small agar block containing fungal growth at the growing edge of a well separated colony.
3. Lift the lid of the agar plate at 45° and place the agar block at the middle of fresh agar medium surface.

Incubate the cultures at 25°C for 48-72 hours (for bacteria) or for 7-14 days (for fungi).

Preparation of pure cultures



Questions

- Why is it necessary to flame the wire loop or the needle before transferring the microorganism from a mixed culture to new media?
- Give the reason for making three groups of streaks in purifying bacteria
- List the points at which you required to flame the wire loop in the streak plate method.
- In the streak plate method, after each group of streaks, did you collect another loopful of bacteria to make the next group of streaks [Yes / No]? Explain your answer.
- In purifying fungi, why was it recommended to take small fragments of mycelia only from the edges of a colony?
- What is likely to happen if you collected mycelia from the middle of a fungal colony and transferred to new media?

PRACTICAL 4: STAINING AND MORPHOLOGICAL STUDY OF BACTERIA

Introduction

Most microorganisms cannot be studied properly because they are transparent (colourless) and therefore difficult to see when viewed under the microscope. Specimens are, therefore, routinely stained to increase visibility and to reveal additional information to help identify the microorganisms. Stains are coloured dyes that impart colour to the colourless microorganisms. Staining may be done for the purpose of:

- (i) examination of shape and arrangement of bacterial cells – **simple staining**
- (ii) separation of bacteria into groups – **Gram stain** and **acid fast stain** or
- (iii) visualization of structures – **flagella stain, capsule stain, spore stain, nuclear stain**

Simple stains employ a single dye (e.g. methylene blue, crystal violet, safranin, nigrosin) and the cells and structures within the cell will attain the colour of the stain.

Differential stains require more than one dye and distinguish between structures within a cell or types of cells by staining them different colours

Objectives

- i. To familiarize with the staining procedures used in the study of bacteria
- ii. To gain skill making bacterial smear
- iii. To gain knowledge and skill in differentiating bacteria on the bases of cell size, shape and staining reaction

Requirements

24-hr old cultures of *Bacillus* Spp., *Xanthomonas campestris* p.v *campestris* and mycobacteria; Staining solutions (methylene blue, Nigrosin, crystal violet, Gram's iodine solution, 95% ethyl alcohol, safranin, carbol fuchsin); Clean glass slides Wire loops, Spirit lamp or Bunsen burner; Blotting paper, Staining racks, Microscopes with X100 objective; Immersion oil, Wash bottles of distilled water.

1. Preparation of a bacterial smear

A smear is a thin film of microorganism spread out on a microscope slide. The smear is air dried and then passed with smear side up, through a flame 2 or 3 times to heat fix the bacteria. Heat fixing denatures bacterial enzymes, preventing autolysis and also enhances the adherence of bacteria to the slide. The preparation is then ready for staining procedures.

Procedure

1. Take a clean glass slide and wipe with 70% ethanol and let ethanol evaporate.
2. Flame a wire loop to red hot in spirit lamp flame and allow to cool.
3. Put a loopful of sterile distilled water on the cleaned glass slide.
4. Using the wire loop, transfer a small amount of bacterial growth from one of the colonies grown on nutrient agar into the sterile distilled water droplet.
5. With the loop, emulsify the bacterial growth with the water droplet.
6. Allow the smear to air dry at room temperature.
7. Fix the smear by passing rapidly through the tip of the blue portion of the spirit lamp flame 4 to 5 times (do not burn the smear).
8. Allow the slide to cool.

Results

The smear appears as a thin, semi-transparent, whitish layer or film fixed to the glass surface that is ready for staining.

2. Negative staining

Negative staining provides the simplest and often the quickest means of gaining information about the cell shape. A simple stain that does not interact with the bacterial cell is used. Therefore, only the background is stained. Nigrosin or Indian ink (an acidic stain) is used. The negative charge of the stain is repelled by the bacteria which too carry a negative charge on their surface, and therefore, the bacterial cell appears transparent and unstained upon examination under microscope. Negative staining is advantageous because: (i) the cells appear less shriveled or distorted because no heat fixing is done; (ii) capsulated bacteria that are difficult to stain can be observed by this technique.

Procedure

1. Place one drop of nigrosin at one end of a clean glass slide.
2. With the help of a sterile wire loop, transfer a loopful of bacterial growth and mix with the drop of nigrosin. Then add a drop of water and mix.
3. Take another clean slide, place it against the drop at an angle of 30° and allow the droplet to spread across the edge of the top slide.
4. Spread the mixture of the stained bacterial suspension out into a thin wide smear by pushing the top slide to the left along the entire surface of the bottom slide.
5. Allow the smear to air dry.
6. Examine the preparation under oil immersion objective. Note the shape, arrangement and size of cells.

NB: Do not spread the drop during mixing it with bacteria; the thickness of the smear should be uniform; never heat fix the smear;

Results

Bacterial cells appear transparent (colourless) against a blue background.

3. Simple staining

- (a) Take clean glass slides, swab with 95% ethanol using absorbent paper and air dry
- (b) Negative staining. Place a loopful of any available bacterium e.g. *Bacillus* sp. on a slide. Add an equal amount of 30% dilution of India ink or Nigrosin. Mix and spread out. Allow to dry and observe with oil immersion objective.
- (c) Methylene blue staining: Make a smear of the available bacteria on a slide. Fix the smear by first allowing it to air dry and then passing it gently over a flame. Place on staining rack and apply a few drops of Methylene blue in smear and let the stain act for 1 min. Pour off the stain and wash the smear gently with slowly running tap water distilled water. Blot dry with absorbent paper (Do not wipe the slide). Examine under oil immersion.

4. Gram Stain

- (a) Make thin smears of the available bacteria.
- (b) Let the smear air dry
- (c) Heat fix the smear
- (d) Place on staining rack. Stain with crystal violet for 1 min. Wash off the stain with distilled water for a few seconds using a wash bottle..

- (e) Flood the smear with gram's iodine for 1 min. Wash off iodine solution with tap water.
- (f) Add ethyl alcohol drop by drop, until no more colour flows from the smear.
- (g) Wash with distilled water and drain.
- (h) Apply safranin to smear for 30 seconds (counter staining)
- (i) Wash with distilled water and blot dry with absorbent paper. Let the stained slides air dry and examine under oil immersion with x100 objective. Note that gram positive bacterium takes the colour of crystal violet (stained violet to purple) and the gram negative takes on the colour of the safranin counter stain (red).

Identify the gram reaction of each bacterium; make sketches for morphology of the bacterium; describe the morphology and arrangement of cells.

5. Acid-fast stain

- (a) Prepare a smear of the bacterium on a slide.
- (b) Air dry and heat fix over a flame.
- (c) Flood the smear with carbol fuchsin and gently heat (not boil) over a flame for 3 to 5 mins. From time to time, add more stain to prevent the smears from becoming dry.
- (d) Cool the slide and then wash off excess stain with distilled water.
- (e) Decolourize the smear with acid-alcohol until all red colour is removed.
- (f) Wash with distilled water.
- (g) Counter stain with methylene blue for 1-2min.
- (h) Wash with distilled water and blot dry with absorbent paper. Observe under oil immersion at objective x100.

Record the colour of the test bacterium and classify it as to reaction – acid-fast or non-acid-fast; describe the morphology and arrangement of cells. (Non acid fast bacteria stain a deep blue. Acid fast bacteria remain red, being saturated with the red carbolfuchsin).

Questions

- i. Why is it necessary to wipe the microscope slide with 70% ethanol before starting to make a bacterial smear?
- ii. In one sentence, explain why you have to place the slides on a rack above the sink while performing the staining procedures.
- iii. When observing bacteria under the microscope, why is it not necessary place a cover slip over the specimen?
- iv. What is the purpose of oil in observation of bacteria under a microscope?
- v. Complete the following table to show results of today's practical using the two bacteria provided:

Stain	Bacillus		Xanthomonas	
	Size & shape (draw)	Colour	Size & shape (draw)	Colour
Nigrosin				
Methylene blue				
Gram stain				
Carbol fushsin				
Gram stain reaction (+ve or -ve)				

vi. Complete the following table to show results of today's practical using two bacteria that you isolated and purified:

Stain	Bacteria 1		Bacteria 2	
	Size & shape (draw)	Colour	Size & shape (draw)	Colour
Nigrosin				
Methylene blue				
Gram stain				
Carbol fushsin				
Gram stain reaction (+ve or -ve)				

PRACTICAL 5: METHODS IN STUDY AND IDENTIFICATION OF BACTERIA

Objectives

- To recognize the differences among bacteria based on colonies formed on culture media
- To familiarize with the simple biochemical tests used in differentiating bacteria
- To perform the biochemical tests

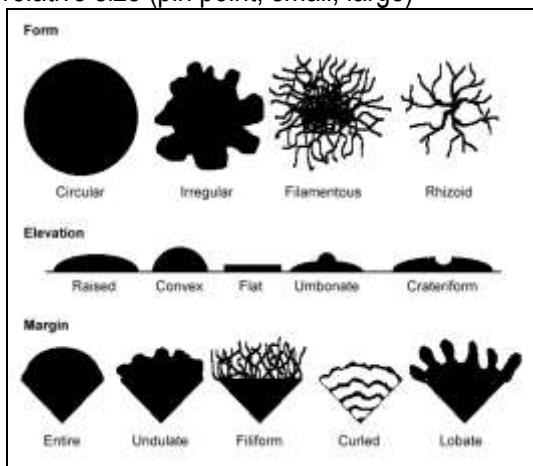
Requirements

Cultures of Bacteria (*Streptomyces sp*; *Xanthomonas campestris p.v campestris*; *Bacillus sp.*); Sterile fermentation tubes of glucose broth and sucrose broth; wire loops; Spirit lamps; Sterile liquid paraffin (or mineral oil); Nutrient broth (Beef extract 3.0g, peptone 10g, distilled water 1 lt; glucose and sucrose each at 1% concentration; Indicator = 0.7 ml of 1.5% alcoholic bromothymol blue or bromocresol purple); hydrogen peroxide

1. Colony Characteristics

Provided are bacteria cultures on solid medium. Observe the colonies of the different bacteria and note the following:

- Colony colour
- Colony shape (circular, irregular, filamentous)
- Colony elevation (flat, raised, convex)
- Colony margins (entire, undulate, filiform, curled, lobate)
- Pigment production
- Colony relative size (pin point, small, large)



2. Sugar oxidation and fermentation of glucose

Many bacteria are capable of utilizing simple sugars like glucose, sucrose etc as a carbon and energy source. Acid and gas are produced but sometimes only acid is produced. Some bacteria fail to metabolize any of these carbohydrates, hence neither acid nor gas is produced.

Method

1. Prepare nutrient broth with either glucose or sucrose plus pH indicator and dispense 10 ml into universal bottles.

2. Sterilize at 121°C for 15 minutes. Allow the media to cool.
3. Label each tube with name of bacteria to be inoculated (2 tubes per bacteria)
4. Take a loopful of bacteria and inoculate into each of the tubes. Leave 2 tubes un-inoculated as controls. I
5. Pour liquid paraffin over the medium to form a layer about 1cm in depth into one of the tubes of each pair
6. Incubate at room temperature for 2-3 days.
7. Observe the tube and record for the presence of growth of the bacterium, colour of the medium and type of metabolism. Tabulate the results.

NB: Due to lack of enough time, the practical has been shortened. The media has been sterilized for you and you will be required to do the inoculation and record results after 2-3 days.

3. Starch hydrolysis

Nutrient agar – 28 g

Distilled water – 1 litre

Soluble starch – 2.0g

Method

Dissolve the nutrient agar powder in the water by heating. Dissolve the starch in 10 ml distilled water and add to the molten agar. Sterilize by autoclaving at 115°C for 10 minutes. Pour into plates. After cooling and solidifying, inoculate by streaking with a single streak across the middle of the plate, then incubate.

Flood the plates with Lugol's iodine and record presence (or absence) of starch hydrolysis indicated by the presence of clear zones in the black – stained medium around or under the colonies. Reddish zones indicate that starch has been partially hydrolyzed to dextrin, and is usually considered a negative reaction.

4. Catalyze test

Bacteria cultures on nutrient agar

3% hydrogen peroxide

Method

Add a few drops of 3% hydrogen peroxide to the bacterial growth. Look for gas bubbles immediately and if absent look again after 5 minutes. Record as catalyze – positive if bubbles of gas form.

5. Gelatin Hydrolysis

Method

Dissolve nutrient agar in distilled water and add 0.4% gelatin (about 5g gelatin per 100 ml media). Sterilize by autoclaving at 121°C for 15 minutes and dispense into petridishes. Inoculate by making a single streak across the middle and incubate for 16-24 hours.

Test

Flood the surface of medium with 5-10 ml of acid mercuric chloride solution. A clear zone indicated positive reaction for gelatin hydrolysis. Use uninoculated agar plate for control.

Questions

1. Record the colony characteristics of each bacteria in a table as follows:

	Colony characteristic					
	Size	Colour	Shape	Elevation	Margins	Pigment
<i>Bacillus</i> sp.						
<i>Xanthomonas</i> sp.						
<i>Streptomyces</i> sp.						

2. Record the results of the other tests in a table as follows:

	Test			
	Oxidative	Starch	Catalyse	Gelatine
<i>Bacillus</i> sp.				
<i>Xanthomonas</i> sp.				
<i>Streptomyces</i> sp.				

3. Based on the results obtained in 1 and 2 above, explain the observed differences/ similarities among the three bacteria tested

PRACTICAL 6: METHODS OF DETERMINING CONCENTRATION OF MICROORGANISMS IN A SAMPLE

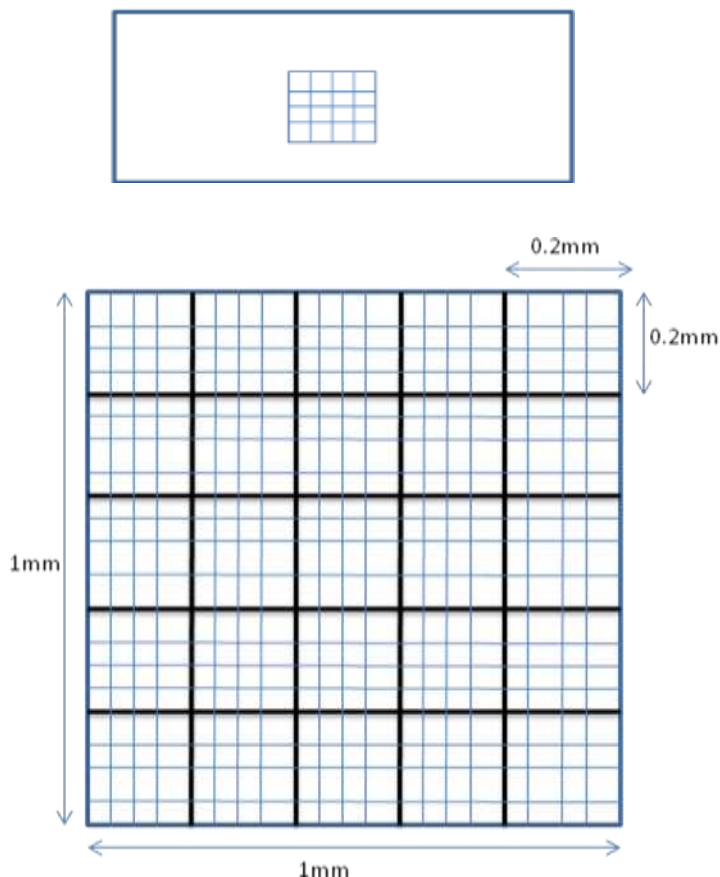
Objectives

- i. To learn how to use a haemocytometer to determine concentration of microorganisms
- ii. To perform serial dilution and determine concentration of bacteria in the dilutions by plating out on agar media.
- iii. To learn how calculate the concentration of microorganisms from readings made on haemocytometer and serial dilution plates.

Requirements

Counting chamber (haemocytometer); suspension of two bacteria (bacteria A and bacteria B); spore suspension of two fungi (fungus A and fungus B); Clean sterile Pipettes; 9ml dilution blanks (sterile distilled water in universal bottles); Sterile Petri dishes; Nutrient agar medium (molten in water baths); Colony counter; microscopes; cover slips

A. Total Count Using Chamber Method



The haemocytometer is used for counting fungal spores in liquid suspension. It is a special microscopic slide with a counting chamber 0.1mm deep so that the volume of liquid over a one mm² is 0.1mm³. The chamber has a total of nine large squares, each of 1mm x 1mm engraved over it but only one square per field is visible under 100microscope magnification (10x ocular and 10x objective). Each one mm² large square is divided into 25 medium sized squares (groups) (0.2 x 0.2mm each), each of which is further subdivided into 16 small squares (0.05 x 0.05mm each), thus a total of 400 squares in 1mm². The dimensions of 1 small square is 0.2÷4 = 0.05mm; the area of one small square is 0.05 x 0.05 = 0.0025mm² and the total area of the grid is 1mm x 1mm = 1mm². If

the depth of the grid is 0.02mm, the total volume above the grid is $1\text{mm}^2 \times 0.02\text{mm} = 0.02\text{mm}^3$. The volume of 1 large square is $0.2\text{mm} \times 0.2\text{mm} \times 0.02 = 0.0008\text{mm}^3$ (or $0.02\text{mm}^3 \div 25$ squares). The number of cells in a given volume of sample can therefore be counted. For example, if 30 cells are counted in one large square (i.e. 30 cells in 0.0008mm^3), how many cells are there in 1ml (or 1000mm^3)? = 3.75×10^7 cells/ml.

Procedure.

1. Place a drop of spore suspension at the centre of haemocytometer and let the preparation stand on level surface for 1-2 minutes to allow the spores to settle at the bottom.
2. Put the cover slip over the grid carefully so that no air bubbles enter between the slide and cover slip.
3. Mount on stage of microscope and focus to see the squares and spores.
4. Count the spores in 5 medium sized squares.
5. Calculate the number of spores per ml of the suspension as follows:

Spores/ml = average number of spores in one large square $\times (1.25 \times 10^6)$

i.e. $\frac{\text{Number of spores in sq 1 + sq 2 + sq 3 + sq 4 + sq 5}}{5} \times (1.25 \times 10^6)$ spores per cm^3

Work on haemocytometers will be in form of demonstrations. There are a number of microscopes provided and haemocytometers containing spores for two fungi are mounted showing different number of cells. Each group will be expected to make counts of the cells and calculate the number of cells per ml.

NB: Haemocytometers are expensive and must be handled with a lot of care – the specimens have been mounted and focussed. Do not try to adjust the focus - in case of any problem, a technician will be nearby to assist.

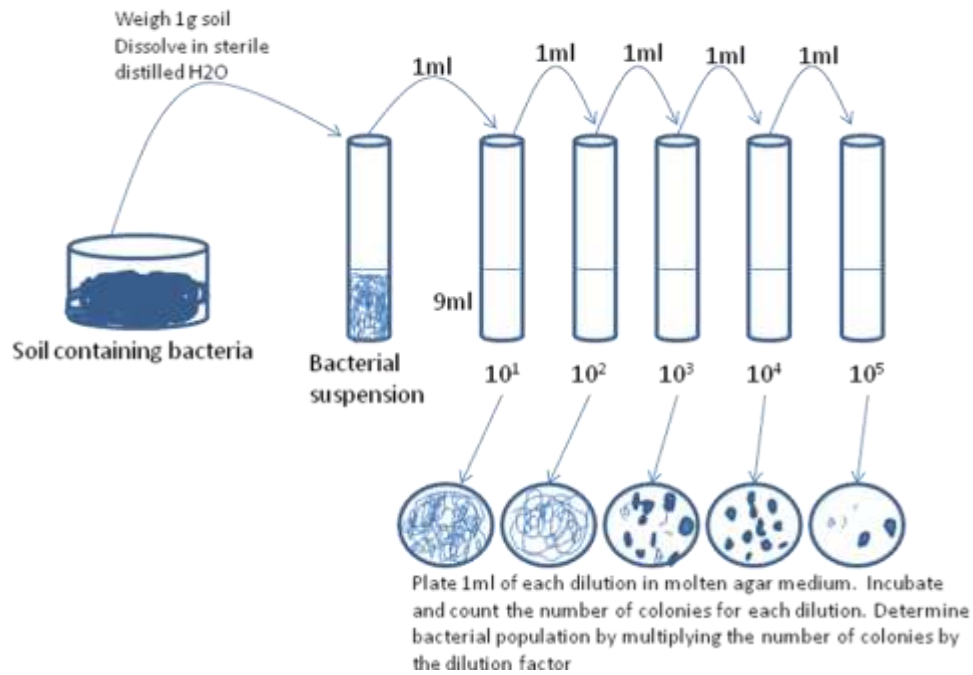
B. Viable Count Method

The plate count method is one of the most routinely used procedures to enumerate viable (living) cells. The method is based on the principle that when material containing bacteria are cultured, every viable bacterium develops into a visible colony on nutrient agar medium.

Procedure:

1. Label the dilution blanks (each 9ml sterile distilled water) as 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} .
2. Prepare the initial dilution by adding 1ml of bacterial suspension into a 9ml dilution blank labelled 10^{-1} , thus diluting the original suspension 10 times. Mix the contents by shaking.
3. From the first dilution, transfer 1ml of the suspension to dilution 10^{-2} with a sterile and fresh pipette. This dilutes the original suspension by 100 times (10^{-2}).
4. From the 10^{-2} suspension, transfer 1ml of the suspension to 10^{-3} dilution blank with fresh sterile pipette, thus diluting the original suspension by 10^{-3} .
5. Repeat this procedure till the original sample is diluted 1,000,000 (10^{-6}) times, using every time a fresh sterile pipette.
6. From each of the dilutions 10^{-1} to 10^{-6} , transfer 1ml of suspension to sterile Petri dishes. Two Petri dishes should be used for each dilution.
7. Add about 15ml of the nutrient agar medium, melted and cooled to 45°C , to each Petri plate and rotate gently to distribute the cells throughout the medium.
8. Allow the plates to solidify and incubate in an inverted position for 24 to 48hrs at room temperature

USE OF SERIAL DILUTION IN ISOLATION AND ENUMERATION OF BACTERIA IN SOIL SAMPLES



Observations

1. Observe all the plates for the appearance of bacterial colonies
2. Count the number of colonies in each plate and calculate the number of bacteria per ml of the original suspension as follows:

$$\frac{\text{Number of colonies (average of 2 plates)}}{\text{Amount plated} \times \text{dilution}}$$

For example, if 60 colonies were counted on 10⁻⁵ dilution, then

$$\text{Number of cells per ml} = \frac{60 \text{ colonies}}{1 \text{ ml} \times 10^{-5}} = 6 \times 10^6 \text{ bacteria /ml or gram of sample}$$

Questions

1. Calculate of concentration of fungal spores in a suspension using haemocytometer

	Number of spores counted in one large square						Spores/ml Average x (1.25 x 10 ⁶)
	Square 1	Square 2	Square 3	Square 4	Square 5	Average	
Fungus A							
Fungus B							

2. Calculate the number of bacteria colony-forming units per ml (cfu/ml)

For each of the dilutions 10³, 10⁴, 10⁵, and 10⁶, count the number of corresponding bacterial colonies in each plate, calculate the average number of colonies for two plates in each dilution and finally calculate the concentration (colony-forming units) per ml for each dilution.

	Average number of bacteria colonies per plate				Concentration (cfu/ml) Number of colonies x dilution factor			
	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ³	10 ⁴	10 ⁵	10 ⁶
Bacteria A								
Bacteria B								

3. Which dilution had the highest number of colonies? Explain the reason

4. Which dilution had the least number of colonies? Explain the reason

5. Did the different dilutions give approximately same number of colony-forming units per ml? Explain

PRACTICAL 7: MICROSCOPIC EXAMINATION AND GENERAL MORPHOLOGY OF FUNGI

Introduction

A considerable amount of information can be obtained by careful microscopic examination of microorganisms. Two techniques are employed to study microbial cells: (i) examination of living cells and, (ii) the study of stained cells. Living cells can be studied to determine the natural size and shape of cells and cellular arrangement. However, observation of cells in their natural or unstained state is sometimes difficult because microbial cells are semitransparent.

Objectives

- i. To gain skill in mounting specimens of fungi for microscopic examination
- ii. To observe the general external and internal morphology of fungi

Requirements

14-21 day-old cultures of two fungi you isolated and purified in practical three (3), clean glass slides, Cover slips, Mounting needles, Distilled water, Cotton blue in lactophenol in a dropper bottle, Spirit lamp or Bunsen burner, 70% alcohol, Nail polish, Microscopes, Mounting needles.

iii. Temporary wet mount technique for microscopic observation of fungi

Procedure

- i. Place a small drop of distilled water on the centre of a clean glass slide
- ii. Using an inoculating needle, transfer a small amount of fungal growth onto the drop of water on glass slide
- iii. Hold a cover slip by its edges and place it on the drop
- iv. Press the cover slip gently with the end of a pencil.
- v. Observe under objective x20 and 40 of microscope

Observations

Note the size, shape and characteristics of the fungus (spores, hyphae)

2. Cotton blue in lactophenol mounting of fungi

Introduction

Cotton blue in lactophenol stain is commonly used for making semi-permanent microscopic preparations of fungi. It stains the fungal cytoplasm and provides a light blue background, against which the walls of hyphae can readily be seen. The stain contains four constituents: (i) phenol, which serves as a fungicide; (ii) lactic acid, which acts as a clearing agent, and (iii) cotton blue, which stains the cytoplasm of the fungus; and (iv) glycerine, which gives a semi-permanent preparation. A permanent preparation may be made by incorporating polyvinyl alcohol in place of glycerine into the mounting medium. Lactophenol alone (without cotton blue) may be used in case of dark coloured fungi.

Procedure

1. Place a drop of cotton blue in lactophenol on a clean slide
2. Transfer a small tuft of a well-sporulated fungus into the drop, using a flamed, cooled mounting needle.
3. Gently tease the material using the two mounting needles.
4. Mix gently the stain with the fungal material
5. Place a cover slip over the preparation, taking care to avoid trapping air bubbles in the stain. A gentle pressure can be applied to the cover slip by tapping with back of mounting needle or pencil in order to spread the fungal structures and expel air bubbles.

6. Examine the preparation under low –power (X10 and X20) and high-power (x40) objectives.
7. Sealing lactophenol mounts to keep the slides for many years – the cover slip is sealed with nail clear varnish as follows:
 - a. Remove all air bubbles from the preparation by pressure, gentle heating or addition of more cotton blue in lactophenol.
 - b. Remove the excess stain from around the cover slip with 70% alcohol on cotton swab or with blotting paper.
 - c. Apply a thin layer of nail varnish around the edge of the cover slip.
 - d. Allow the preparation to dry overnight.
 - e. Apply a second coat of nail varnish over the first coat.

The fungal cytoplasm is seen as a lightly stained blue region forming a layer inside the unstained cell wall of hyphae, conidiophores, and conidia that is surrounded by light blue background on the slide.

Questions

- i. From the observations of the fungal cultures and under the microscope, list at least four (4) ways the fungus you isolated differs from the bacteria you observed in the earlier class.
- ii. How do the specimens mounted with water differ from those stained with cotton blue in lactophenol when observed under microscope?
- iii. List four (4) ways in which the staining of the fungi specimens differed from that you did for bacteria.
- iv. How many times was the fungal specimen magnified when you observed under microscope – explain how you arrived at the magnification you have indicated.
- v. Draw a large, clear diagram of what you have observed and label to indicate hypha, sporangiophores, sporangiospores, conidia, conidiophores and septa

PRACTICAL 8: CHARACTERISTICS OF DIFFERENT GROUPS OF FUNGI

Objectives

- i. To make make slides for microscopic examination from infected plant materials and fungal cultures
- ii. To learn and describe the morphological differences among different groups of fungi

Requirements for the practical

Microscopes, microscope slides and cover slips, cotton blue in lactophenol, mounting needles, spirit lamps, plant materials infected with fungi, cultures of various fungi

Oomycetes

Required materials – *Phytophthora infestans* infected plant materials incubated in moist chamber, cultures of *Pythium*, cultures of *Phytophthora infestans*

- Using a dissecting microscope, observe the surface of the infected plant material to see the fungal mycelium
- Using a sterile inoculating needle, pick small amount of mycelium from the diseased plant material or from cultures
- Place the mycelia on a small drop of water or cotton blue in lactophenol on microscope slide
- Gently apply the cover slip, taking care not to trap air bubbles
- Observe under microscope – start with low power objective (x10), then x20 and x40
- Make labelled diagrams of the structures you observe – look for hyphae (whether septate or coenocytic), sporangia, and sporangiospores

Zygomycetes

Bread mould (bread incubated in moist chamber), cultures of *Rhizopus*

- using a sterile inoculating needle, pick small amount of mycelium from the diseased plant material or from cultures
- place the mycelia on a small drop of water or cotton blue in lactophenol on microscope slide
- gently apply the cover slip, taking care not to trap air bubbles
- observe under microscope – start with low power objective (x10), then x20 and x40
- Make labelled diagrams of the structures you observe – look for hyphae (whether septate or coenocytic), sporangia, and sporangiospores

Ascomycetes

Powdery mildew infected leaves, cultures of *Sclerotinia sclerotiorum* of legumes (with sclerotia)

- Follow the steps as described above
- Make labelled diagrams to show hyphae, conidiophores, conidia
- Did you see any fruiting structures?

Basidiomycetes

Coffee leaf rust (infected leaves), maize smut, mushroom (unopened)

- Using a scalpel, make very thin slices through the mushroom tissue (make sure the section includes part of the gills).
- Mount the section in small amount of water on microscope

- Apply cover slip and observe under the microscope
- For the rust and smut, scrap off small amounts of the powder and apply in small amounts of water on microscope slide; apply cover slip and observe
- Make labelled diagrams to show the basidiospores, urediospores and spore bearing structure in mushroom

Mitosporic fungi

Coffee berry diseased berries incubated in moist chamber, cultures of bean anthracnose (*Colletotrichum lindemuthianum*), northern leaf blight infected maize leaves incubated in moist chamber

- Follow the steps as described for Oomycetes
- Make labelled diagrams to show hyphae, conidiophores, conidia

Questions

1. Using a table list the key features you observed in each group of fungi

	Oomycetes	Zygomycetes	Ascomycetes	Basidiomycetes	Mitosporic fungi
Under dissecting microscope					
Mycelia					
Type of spores					
Spore-bearing structures					

2. Make well labelled diagrams for at least of fungus in each group

PRACTICAL 9: SPECIAL TECHNIQUES FOR THE STUDY OF MORPHOLOGY OF FUNGI

Introduction

In today's practical session we are going to observe the undisturbed structures of fungi using three techniques: (i) slide culture technique, and (ii) Scotch tape preparation. The slide culture technique is used to observe structures of fungi without disturbing the arrangement of mycelia, spores and conidiophores. The slide culture technique is based on the ability of fungal aerial mycelia to adhere to a glass surface.

Objectives

- i. To gain knowledge on methods of microscopic study of fungal structures in situ (in their original, undisturbed state)
- ii. To gain skills in the preparation of slide culture and scotch tape preparations
- iii. To observe fungal mycelia septations, formation of spore-bearing hyphae, conidiophores, conidia, sporangiophores, and sporangiospores.

Requirements

Cultures of fungi on PDA and SNA; Czapek-Dox agar media plates; Sterile Petri dishes; sterile glass microscope slides; Sterile cover slips; Whatman filter paper; Forceps; Transfer needle; Sterile scalpel blades; 95% ethanol; Sterile distilled water; V-shaped glass rods; Masking tape; Cotton blue in lactophenol; spirit lamp or Bunsen burner; Glass marking pencil; clear cellotape. (10cm).

1. Slide culture technique (Riddel slide)

1. Place one or two filter paper in the bottom of Petri dish; place a V-shape glass rod, a glass slide and a cover slide in each of the Petri dishes.
2. Sterilize the assembly in oven and allow to cool.
3. Moisten the filter paper with sterile distilled water; using a sterile forceps, place the glass slide across the V-shaped glass rod.
4. Cut a 10mm square agar block with a flamed scalpel blade.
5. Gently place the agar block on the centre of the slide.
6. With a mounting needle, inoculate a very small portion of fungus onto each of the four corners of the agar block.
7. Gently place the cover slip on the surface of the inoculated agar block.
8. Replace lid of the Petri dish and incubate, right side up at 25°C.
9. Examine daily for the growth of the fungus and re-moisten the filter paper with sterile distilled water when it becomes dry.
10. Make mounts for microscopic examination as follows:
 - a. From cover slips
 - i. Gently lift the cover slip from the surface of the agar with forceps.
 - ii. Put a drop of 95% ethanol on fungus side of the cover slip; drain off the excess alcohol.
 - iii. Place a drop of cotton blue in lactophenol on a clean glass slide and put the cover slip over it with the fungus side down.
 - iv. Examine under the microscope.
 - b. From glass slide
 - i. Using a forceps, gently remove the agar block from the slide and discard it.
 - ii. Put a drop of 95% ethanol on the fungal growth on the slide; drain the ethanol.
 - iii. Place a drop of cotton blue in lactophenol on the fungal growth and place a clean cover slip.

- iv. Examine under microscope using low (X10, X20) and high power (X40) objectives. Observe the arrangement of conidia or spores, conidiophores and identify the fungus by consulting monographs and books on fungal taxonomy.

2. Scotch tape preparation for studying morphology of fungi

This is a rapid and simple method for preparing a temporary microscopic mount of a fungus without disturbing the arrangement of conidia and conidia-bearing hyphae.

1. Take a clean glass slide and place a drop of cotton blue in lactophenol or aniline blue stain in the centre of the slide.
2. Hold a strip of clear cello tape (10cm) with the sticky side down, between the thumb and forefinger of each hand.
3. Press firmly the centre of the sticky side of the cello tape onto the surface of the sporulated fungal growth on SNA medium.
4. Pull away the cello tape gently from the growth and place it on the drop of cotton blue in lactophenol stain.
5. Fold over the extended ends of the cello tape over the ends of the slide.
6. Examine the slide under low (x10 and 20) and high (x40) power objectives of microscope for fungus structures.

Questions

- i. What is the purpose of adding sterile distilled water to filter paper during incubation of the slide cultures?
- ii. Draw a large, clear diagram of what you have observed for two different fungi. Label to indicate hypha, sporangiophores, sporangiospores, conidia, conidiophores and septa.
- iii. From your observations, how does the appearance of the fungal structures differ from those observed on temporary squash mount preparations you made in the last practical?
- iv. Indicate the magnification for each of the illustrations