Basic Techniques in Microbiology: A Laboratory Handbook (Second Edition)

Preface

Microbiology is a critical field that advances our understanding of health, disease and the environment through the study of microorganisms. This manual serves as a comprehensive guide to essential techniques used in microbiological laboratories, starting with foundational practices and safety protocols that ensure a safe and efficient working environment. Proper handling and maintenance of the microscope, a vital tool in microbiology, are also covered to ensure accurate and effective observation of microorganisms.

The manual explores the practical aspects of aseptic techniques to prevent contamination, culture media preparation to support microbial growth and methods for isolating pure cultures. Detailed instructions on various staining techniques enhance the visibility and contrast of microorganisms, while enumeration methods provide accurate quantification essential for many microbiological studies. Each section is designed to provide thorough guidance on specific laboratory practices, from sterilization methods to the interpretation of staining results.

Additionally, the manual explores the cultural characteristics of bacteria, crucial for their identification and classification based on observable traits such as shape, size, colour and texture. This resource aims to equip students, researchers and professionals with the necessary skills and knowledge to conduct microbiological research effectively, fostering the development of proficient microbiologists capable of advancing our understanding of the microbial world.

To facilitate a quick learning platform for students, immediate **Exercise**s following practical classes are included, reinforcing the concepts and techniques learned to foster their expertise in the microbial world.

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Introduction to General Practice in Microbiological Laboratories

Welcome to the world of microbiology laboratories. This guide provides an overview of the general practices you will encounter in a microbiology lab. Practice in microbiological laboratories encompasses a wide range of techniques and protocols aimed at studying microorganisms, identifying pathogens and promoting clinical and public health initiatives. From safety protocols to data management, these practices are essential for ensuring accurate and reliable laboratory results.

A. **Laboratory Setup and Safety Protocols**

Microbiological laboratories are designed with careful consideration for safety and efficiency. They are typically equipped with biosafety cabinets, autoclaves for sterilization, incubators for culturing microorganisms and microscopy facilities.

Safety protocols are paramount to prevent accidental exposure to hazardous microorganisms. Personnel must adhere to guidelines for wearing appropriate personal protective equipment (PPE) such as lab coats, gloves and safety goggles.

B. **Sterilization and Aseptic Technique**

Before any experimental work, all equipment and surfaces must be sterilized to eliminate any potential contaminants. Autoclaving, the use of chemical disinfectants and UV sterilization are common methods.

Aseptic technique involves handling microbial cultures in a way that minimizes the risk of contamination. This includes flame-sterilizing tools, working near a Bunsen burner flame and avoiding unnecessary exposure of cultures to the environment.

C. **Media Preparation**

Microorganisms require specific nutrients to grow and thrive. Microbiologists develop various types of growth media that meet the nutritional needs of different organisms. This can include agar plates, broth cultures and specialized media for selective or differential growth.

Media preparation involves careful measurement of ingredients, sterilization to eliminate any existing contaminants and pouring or plating the media into sterile containers under aseptic conditions.

D. **Culturing Techniques**

Culturing microorganisms involves inoculating a sample onto a suitable growth medium and providing optimal conditions for growth. Techniques such as streak plating, spread plating and pour plating are used to isolate and quantify microbial colonies.

Incubators provide controlled environments of temperature, humidity and sometimes atmospheric conditions (such as CO_2 or anaerobic conditions) to support the growth of specific microorganisms.

E. **Microscopic Examination**

Microscopes are essential tools for observing and studying microorganisms. Microbiologists stain samples using various staining reagents to enhance contrast and visualize cell structures.

Common staining methods include Gram staining, which differentiates bacteria into Gram-positive and Gram-negative groups based on cell wall composition and acid-fast staining, used to detect mycobacteria.

F. **Identification Methods**

Once cultured, microorganisms need to be identified. This can be achieved through a variety of methods including biochemical tests, serological assays and molecular techniques.

Biochemical tests involve observing reactions to specific substrates to determine metabolic characteristics, while serological assays detect antibodies or antigens specific to particular microorganisms.

G. **Antibiotic Sensitivity Testing**

Antibiotic susceptibility testing is crucial for determining the most effective treatment for bacterial infections. Microbiologists perform tests to assess the susceptibility of bacterial isolates to various antibiotics using methods such as disk diffusion or broth microdilution.

Results guide clinicians in prescribing appropriate antibiotic therapy and help combat antimicrobial resistance.

H. **Quality Control and Assurance**

Quality control measures ensure the reliability and accuracy of laboratory results. This includes regular calibration of equipment, monitoring of environmental conditions and participation in proficiency testing programs to validate testing procedures and maintain standards.

I. **Data Management and Reporting**

Microbiological laboratories generate vast amounts of data from experiments and tests. Efficient data management systems are essential for recording, organizing and analyzing this information.

Accurate and timely reporting of results is critical for patient care, disease surveillance and public health interventions. Microbiologists communicate their findings to healthcare providers, public health officials and other stakeholders.

H. Continual Learning and Improvement

Microbiology is a dynamic field with constant advancements in techniques and understanding. Laboratory personnel engage in ongoing education and training to stay updated on the latest developments and best practices.

Continuous quality improvement efforts ensure that laboratories are always striving to enhance the quality, efficiency and safety of their operations.

Exercise:

1. What are the essential components of laboratory setup and safety protocols in microbiological laboratories and why is safety protocols considered paramount?

2. Describe the importance of the aseptic technique in microbiology laboratories.

3. How do microbiologists typically identify microorganisms cultured in the laboratory and why is antibiotic sensitivity testing important for bacterial infections?

Safety and Safe Practice Methods in Laboratories

Microbiology labs deal with living organisms, some of which can cause harm. While working in a laboratory can be rewarding, it is important to prioritize safety to prevent accidents and minimize risks. Safety is paramount in microbiology laboratories to protect personnel from potential hazards. Persons who work in a microbiology lab may handle infectious agents in addition to other hazards such as chemicals and radioactive materials.

Precisely, in the microbiological laboratory there are many hazards, however, most problems and accidents can be prevented by following the rules, being prepared for the lab and thinking about What to do and what not to do in the Lab. Always behave thoughtfully and with consideration for others' safety.

• Make sure to read the laboratory **Exercise** before class and plan your work.

Safety in the laboratory is the primary concern. Procedures for microbiological safety involve the following three (3) aspects of laboratory practices:

(1) good laboratory practice,
(2) safety equipment and
(3) Clean up.

There are many rules we must follow through our work in the microbiology laboratory, these rules are as follows:

(1) Good Laboratory Practice

- **Personal Habits**

1. Do not eat, drink, smoke, or apply cosmetics in the laboratory. Never put anything in your mouth. Use pipette aids when appropriate. Never pipette by mouth.
2. Wash your hands thoroughly with soap and water before leaving the laboratory and any time they become contaminated with culture material.
3. Disinfect your lab bench before you start, if you spill culture and before you leave the lab, dispose used items properly.
4. Always Wear a lab coat within the microbiology lab.

- **Labelling**

Label all cultures and solutions properly with your name or initials, date, course / lab section and experiment information. The experiment information may include the composition of the solution, identity of the culture, or the experiment number.

- **Aseptic Techniques**

Because microorganisms are so small, large populations are generally needed in order to monitor microbial activity. Aseptic techniques help ensure that only one type of microorganism is present in a

container (pure culture). These methods also ensure that the microorganisms do not escape from the container, contaminating the laboratory and possibly causing disease.

Clean Up

For your safety and the safety of others, glassware and trash must be disposed of properly.

- Items contaminated with microorganisms or body fluids must be autoclaved before disposal or washing.
- Needles and syringes must be thrown into the red sharps container. Syringes without needles must be disposed of in the sharp's container.
- Plastic Petri plates and other disposable plastic items used to culture or contain microorganisms must be placed in the red biohazard bags for autoclaving prior to disposal.
- Chemicals must be disposed of properly. Some chemicals must be placed in special containers for disposal to prevent environmental contamination.
- Never pick up broken glass with your hands; use a broom. Disposable glass items that are not contaminated with culture must be placed in the broken glass container - even when they are not broken.
- Paper towels should be thrown in the ordinary trash unless they are contaminated with culture.

General Protective Procedures

- Read the laboratory **Exercise** before class and plan your work. This creates awareness of the special safety concerns for the laboratory class and permits efficient use of class time.
- Come properly dressed to the lab.
- Disinfect your lab bench before you start, if you spill culture and before you leave the lab.
- Use pipette aids when appropriate. Never pipette by mouth.
- Student needs to understand the safety signs in the lab (Figure 1).



Figure 1: Safety Signs

Exercise:

1. How does prioritizing safety in a microbiology laboratory help to prevent accidents and minimize risks associated with handling living organisms and hazardous materials?

2. What are the three aspects of laboratory practices involved in microbiological safety procedures and how do they contribute to maintaining a safe working environment?

3. Describe three specific rules or practices outlined for maintaining safety in a microbiology laboratory, emphasizing their importance in preventing accidents and ensuring the well-being of personnel.

Practical Procedures for Handling, Use and Maintenance Microscope

A microscope is a tool used by many biologists. Its development has made possible the exploration of the world of living things too small to be seen with the naked eye. Without the microscope, the human eye cannot distinguish objects smaller than 0.1 mm. The microscope acts as an eye extension, allowing it to see beyond this range. The compound light microscope is the type we will be using in this course. Since the objects observed with microscopes are often too small to be measured conveniently in millimetres, it is necessary to convert your measurement to micrometres. Remember that **1 μm = 0.001 mm**.

Before we delve into details on practical procedures for handling, use and maintenance of the microscope, let's quickly review the components of compound microscopes (Table 1 & Figure 2).

Table 1: The Function of each Microscope Part

S/N	Microscope Part	Function
1	Eyepiece or Ocular Lens	- They are used to look through the specimen. - It also magnifies the image formed by the objective lens, usually ten times (10x) or 15 times (15x).
2	Eyepiece Tube or Body Tube	The tube holds the eyepiece
3	Nosepiece	The nosepiece holds the objective lenses and allows it to revolve.
4	Objective Lenses	They are the primary optical lenses for specimen visualization on a microscope. Most compound microscopes come with three or four objective lenses (with a magnifying power of 4X, 10X, 40X and 100X) that revolve on the nosepiece.
5	Arm	The Arm connects the base to the nosepiece and eyepiece. It is the structural part that is also used to carry the microscope.
6.	Stage	The stage is a flat surface where the specimen is placed for observation.
7.	Stage Clips	Stage clips are the supports that hold the slides in place on the stage
8.	Diaphragm/iris	This apparatus can be adjusted to vary the intensity and size, of the cone of light projected through the slide.
9.	Illuminator	Light source (low voltage bulb or mirror); Built into the base.
10.	Coarse focus	The coarse focus moves the stage to provide a general focus on the specimen.
11.	Fine focus	The fine focus moves the stage in smaller increments to provide a clear view of the specimen.
12.	Base	The base is the main support of the microscope.

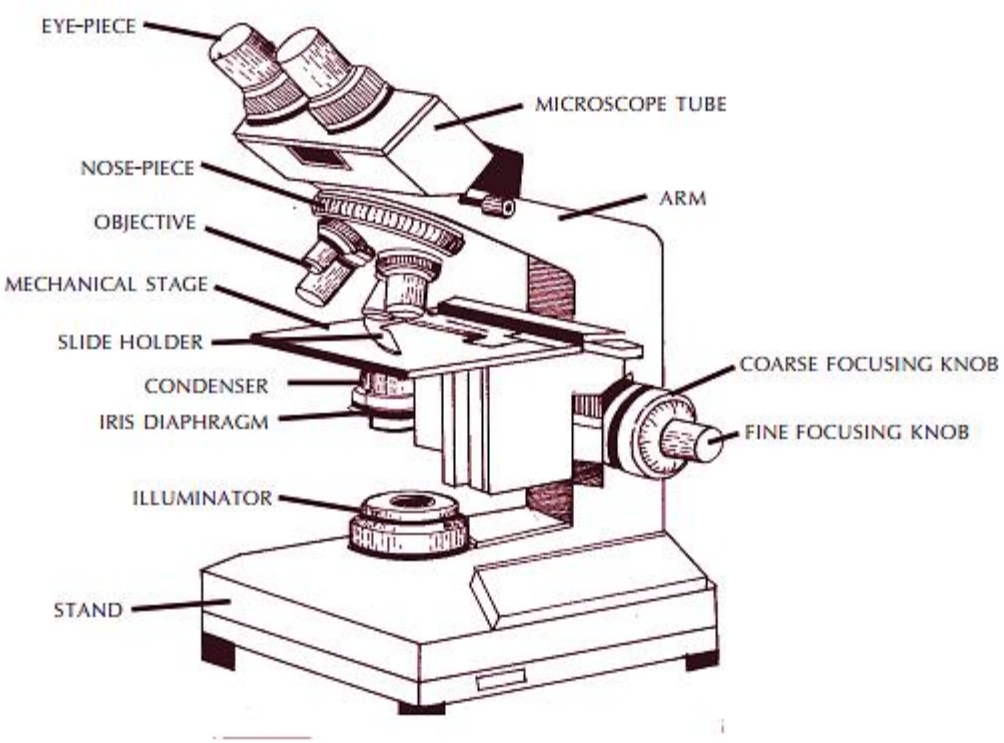


Figure 2: A Compound Microscope

Use of Microscope

Materials Required:

Microscope, slides, cover slide, specimen, normal saline and oil immersion

Laboratory Demonstration on the Use of a Compound Microscope

Procedure

The primary objective of this **Exercise** is to gain experience in using the bright field microscope. You will observe the microbial life present in pond water and hay infusions as you practice working with the microscope.

1. Place a slide on the lab bench frosted side up. The frosted section should feel rough.

2. Draw a line on the slide with a Sharpie marker. This line will be used to help you focus. Frosted glass (rough side up) Marker line

3. Choose to observe either the pond water or hay infusion. Collect some of the chosen samples using a transfer pipette. Be sure to stir it and then pick up some of the "gunk" from the bottom of the jar. Put a drop of the sample next to the line on the slide.

4. With your forceps, pick up a coverslip and place it on top of the sample. Avoid bubbles by putting the cover slip down at an angle.

5. View the sample using your microscope:

a. Lower the microscope stage a little to secure the prepared slide onto the stage using the spring-loaded slide holder.

b. Turn on the main power switch and adjust the light until the Voltmeter reads. Be certain that both the field iris diaphragm and the aperture iris diaphragm are open and that the condenser is set to 0.

c. Position the 10X objective lens directly above the focus line on the slide. Use the coarse adjustment knob to bring the stage as close to the 10X lens as possible. Now use the fine adjustment knob to back the stage away until the line comes into focus (*Note - If you see more than one circle of light when looking in the oculars, move these lenses until only one circle of light is present).

d. Move the slide to view the sample. Try to identify some of the organisms. You may move the high dry objective into place to get a better look at the tiny microbes, but please do not use the oil immersion lens (100X) at this point.

6. Draw some of the organisms you observed in the results section. When you have finished observing the slide, remove it from the mechanical stage and discard the coverslip in the glass disposal container. Wash and save your microscope slide.

7. Wash the slides under tap water and store them in a slide box provided in your drawer. Do not place the slides back into the original box.

Maintenance of the Microscope

1. Cover the microscope when not in use.

2. Remove immersion oil from the objective after use by wiping with lens tissue

3. Clean the optics with lens paper before use

4. Don't rack the objective downwards to focus the object while looking down the microscope

5. Don't attempt to dismantle the object

6. Don't use high-powered objectives when low power is sufficient

8. Don't lubricate the microscope with any oil other than that provided for the purpose

9. Don't place wet preparation on the stage without wiping under the surface of the slide

Exercise:

1. What is the primary purpose of using a compound light microscope in biological studies and why is it essential for observing microscopic organisms?

2. Describe the procedure for preparing a microscope for use, including steps for plugging it in, adjusting the specimen slide and optimizing light intensity.

3. Explain the significance of using immersion oil with the oil immersion objective lens when observing stained slides under high magnification.

4. What are the recommended maintenance practices for ensuring the longevity and optimal performance of a microscope and why are these practices important?

5. List three common mistakes or misuse practices to avoid when handling a microscope and explain the potential consequences of each.

6. How does the conversion of measurements from millimetres to micrometers relate to the precision of observations made using a microscope and why is this conversion necessary in microscopy?

Aseptic Techniques

Aseptic techniques are a fundamental and important laboratory skill in the field of microbiology. Microbiologists use aseptic techniques for a variety of procedures such as transferring cultures, inoculating media, isolation of pure cultures and for performing microbiological tests. Proper aseptic technique prevents contamination of cultures from foreign microbes inherent in the environment. For example, airborne microorganisms (including fungal spores), microbes picked up from the researcher's body, the lab bench-top or other surfaces, microbes found in dust, as well as microbes found on unsterilized glassware and equipment, etc. may potentially contaminate cultures, thus interfering with the lab results.

Using proper aseptic techniques can greatly minimize or even eliminate the risk of microbial contamination. In addition, the aseptic technique is of utmost importance to maintain pure stock cultures while transferring cultures to new media. The aseptic technique is also essential for the isolation of a single species of microorganism from a mixed culture to obtain a pure culture. Furthermore, proper aseptic technique prevents microbes used in the laboratory from accidentally being released into the environment and/or infecting people working in the laboratory. This is especially relevant when pathogens are being handled.

As microorganisms are ubiquitous, it is always safe to assume that any environment we are in is filled with them.

Before starting today's **Exercise** let us acquaint ourselves with certain important aspects of asepsis.

a) Work Area Disinfection: Before starting the experiment, the workbench surface must be sprayed with 70% ethanol to disinfect the place. It must be rubbed down into all regions. The mistake of wiping the workbench with towels or tissues to spread the ethanol will only introduce new microbes. Therefore, the best thing to do is to let the volatile ethanol evaporate on its own.

b) Transfer Instruments (Loops and Needles): Wire loops and needles are made from inert materials such as platinum. They are extremely durable and can be sterilized by flaming. To do this, place the loop in the blue part of the Bunsen burner flame as it is the hottest and allow the loop to get red hot and then remove (Figure 3). Cool the transfer instrument down before inserting it into the culture. This procedure has to be carried out before any inoculation and after any inoculation.

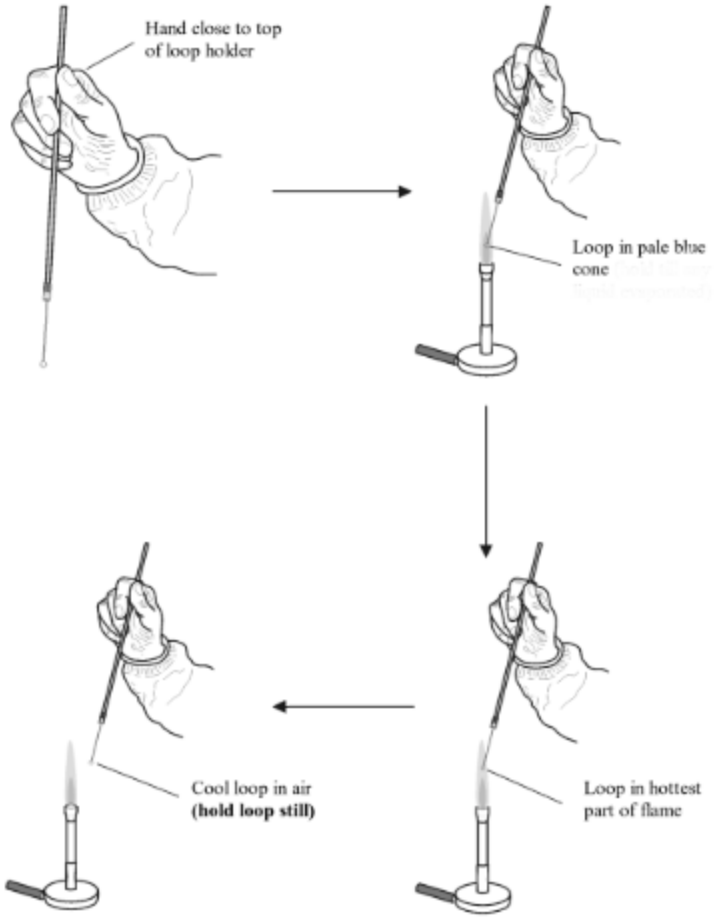


Figure 3: Flaming of Wire Loop

c) Culture Tube Flaming: Prior to inserting the loop into the culture tube, remove the lid of the tube and flame the tip of the tube to avoid entry of any air contaminants. This has to be carried out before and after the insertion of the loop into the tube culture.

d) General Sterilization: Sterilization is the process of destroying all forms of microbial life-vegetative and sporulation. It is important that all equipment used in a microbiological experiment is sterilized in order that a particular organism of interest is grown, without contamination from the surrounding environment. Disinfection is the process of removing most pathogenic microorganisms (excluding bacterial spores) from inanimate objects by physical or chemical methods. Chemicals used in disinfection are called disinfectants. Different disinfectants have different target ranges, not all disinfectants can kill all microorganisms. Some methods of disinfection such as filtration do not kill bacteria, they remove them. Sterilization is an absolute condition while disinfection is not. The two are not synonymous.

Several methods are employed in the process of sterilization (Figure 4) to sterilize the various equipment used in an experiment. These methods are broadly classified as PHYSICAL and CHEMICAL methods.

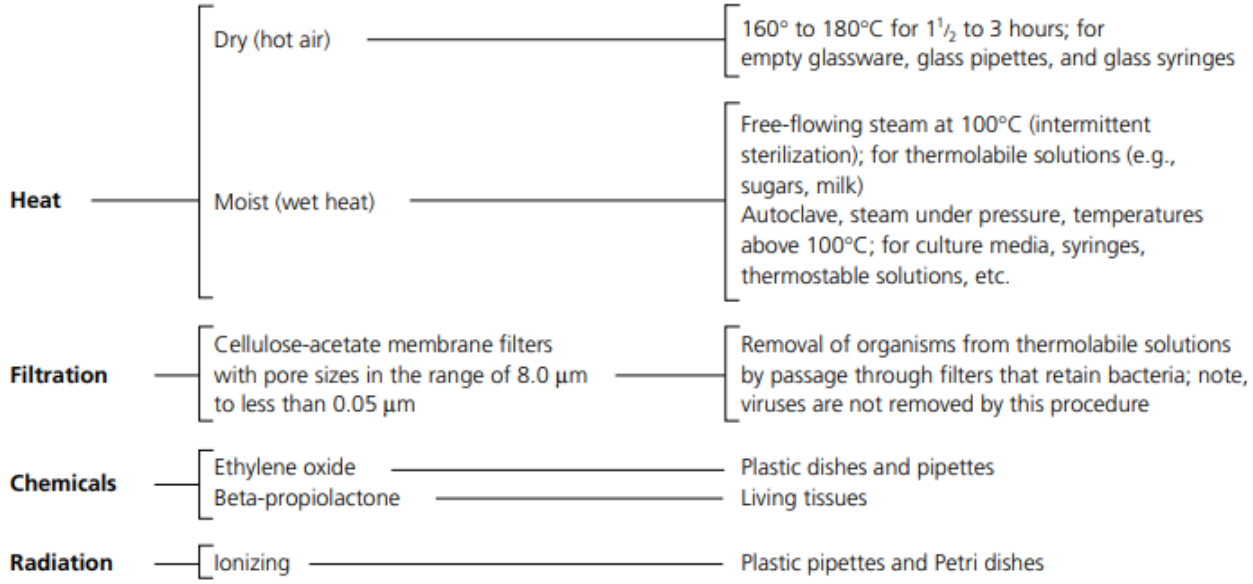


Figure 4: The Sterilization Techniques

I) Physical Agents:

The major physical agents used for the control of microorganisms are TEMPERATURE, RADIATION and FILTRATION.

1. TEMPERATURE: Microorganisms can grow over a range of temperatures, from very low temperatures characteristics of psychrophiles, to very high temperatures characteristics of thermophiles. Temperatures above a maximum generally kill microbes; sterilization can be achieved by high temperatures as DRY HEAT or MOIST HEAT.

- Dry Heat Sterilization: Dry heat sterilization is achieved in hot air oven. Laboratory items like petriplates, Erlyn Meyer flasks, pipettes, test tubes, dry powders, glycerol and other oils etc. are sterilized in hot air oven. Such material can be sterilized by placing in hot air, at a temperature of 160°C for 120 mins.
  Alternatively, equipment like forceps, inoculation needle etc. can be sterilized by direct heating on a flame till red hot. Thus it brings about destruction of unwanted organisms without changing the nature (flavor) of the material. This process involves heating at that temperature for 15 mins and then cooling it quickly to 0-5°C.
  Principle Involved in Dry Heat Sterilization: Dry heat oxidizes chemical components of organisms and thus destroying them.

- Moist Heat Sterilization: High temperatures combined with high moisture are one of the most effective ways of sterilization.

Principles Involved in Moist Heat Sterilization: Moist heat coagulates the microbial proteins and is hence more rapid in killing microbes. Moist heat can be applied in the following ways to bring about sterility:

- Steam Under Pressure: Provides temperatures higher than those obtainable by any coagulation of proteins. An autoclave is a device used in the laboratory to sterilize media solutions and to kill discarded cultures. It is operated at 151lbs/sq. inch pressure, which yields a temperature of 121°C effective in bringing about sterility in 15 min.
- Fractional Sterilization or Tyndallisation: Some microbial solutions cannot be heated over 100°C without being damaged. Such materials are sterilized by tyndallisation, which involves heating at 100°C on three successive days with incubation periods in between. Resistant spores germinate during the period of incubation that is killed on heating the subsequent day.
- Pasteurisation: Milk, cream and other alcoholic beverages are subjected to controlled heat treatment which kills microbes of a certain type alone.

2. Radiation: When ionizing radiation passes through cells, they create free hydrogen radicals, hydroxyl radicals and peroxides that cause intracellular damage, resulting in destruction of microbes. This method of sterilization is effective for sterilizing heat-liable materials. Thus, it is also called COLD STERILIZATION.

UV light is the most effective region of the electromagnetic spectrum and is employed in the disinfecting of inoculation chambers and hospital operating rooms. UV light alters nucleic acids and results in a pyrimidine dimer, thus inhibiting DNA replication.

3. Filtration: This technique is used when the material to be used is heat liable and cannot be sterilized by heating for e.g. Solutions of proteins, vitamins etc. Filters of pore size 0.03μ - 0.08μ are used to filter off microbes, thus rendering the filtrate sterile. Pore size, electric charge of the filter, the charge carried by the organisms and the nature of the fluid being filtered affect the efficiency of filtration. E.g. Acity filters, Berkefeld filters, membrane filters are all microbial filters.

II. Chemical Agents: Ethyl Alcohol (70%) finds indispensable use for disinfecting hands before and after a microbiological experiment and to disinfect the inoculation chamber or area where the experiment is conducted.

Table 2: Examples of Chemicals Used as Antimicrobial Agents

S.No.	Group	Action	Example
1.	Alcohol	Denature proteins and solubilize lipids	Ethanol, Glutaraldehyde
2.	Aldehyde	Alkylate, reacts with-NH_2,SH-	I_2
3.	Halogens	COOH	Cl_2
4	Heavy Metals	I_2 inactivates proteins oxidize cells.	$HgCl_2$
5	Gases	Precipitates and inactivates proteins (used for surface sterilization)	Ethylene Dioxide
6	Detergents	Alkylates organic compounds; Disrupt cell membrane	
7	Phenols	Denature protein & disrupt cell membrane	

Laboratory Demonstration of Autoclaving

Procedure for Autoclaving


Autoclave

Your instructor will demonstrate the use of the autoclave.

1. Load the autoclave with the freshly prepared culture media.
2. Close and lock the autoclave door.
3. Set the autoclave time for 15 minutes or longer and select a slow rate of exhaust.
4. Make certain that the autoclave temperature is set to 121°C.
5. Start the autoclave by pushing the start button or twisting the knob to the start position.
6. When the period of sterilization is completed and the pressure in the chamber reads, carefully open the door and remove the containers, using heat-proof gloves.

Exercise:

1. Explain the importance of aseptic techniques in microbiology laboratories and describe how they contribute to maintaining the integrity of laboratory experiments.

2. Detail the steps involved in disinfecting the workbench surface before starting a microbiological experiment and explain why the use of towels or tissues for spreading ethanol is discouraged.

3. How are wire loops and needles sterilized before and after each use in microbiological procedures and why is it important to allow them to cool down before insertion into a culture?

4. Describe the procedure for flaming the tip of a culture tube before and after inserting a wire loop into it and explain how this practice helps maintain sterile conditions.

5. Discuss the various physical agents used for sterilization in microbiology laboratories, including dry heat, moist heat, radiation and filtration, highlighting their principles and applications.

6. Explain the significance of using chemical agents such as ethyl alcohol (70%) for disinfecting hands and surfaces in microbiological experiments and provide examples of other chemical antimicrobial agents used in laboratory settings.

Microbiological Culture Media Preparation

To study microorganisms, we need to isolate them from their natural environments and grow them in the laboratory on synthetic media. Therefore, it is important to learn about the composition, types and preparation of culture media.

A culture medium (plural, media) is a solid or liquid mixture of chemicals that can support the growth of microorganisms. In terms of composition, culture media must contain a source of carbon, nitrogen, water, buffer system, other minerals, vitamins and growth factors.

- Water: used to dissolve materials to be transported across the cytoplasmic membrane

- Carbon source: required for the construction of all organic molecules, usually glucose

- Nitrogen source: obtained from an inorganic source or an organic source e.g. proteins, broken down into amino acids, or beef extract broken to nucleic acid.

- Buffer system: most bacteria can grow at 7.0 pH. This is achieved by using a buffer system e.g. carbonate or phosphate buffer.

Types of the Culture Media

The culture media may be in liquid (broth), semisolid or solid-state (figure 4). The major difference among these media is that solid (1.5-1.8% agar) and semisolid (< 1.0% agar) media contain a solidifying agent (usually agar), whereas a liquid medium does not.

Liquid media, such as nutrient broth, tryptic soy broth, or brain-heart infusion broth, can be used to propagate large numbers of microorganisms in fermentation studies and for various biochemical tests. Semisolid media can also be used in fermentation studies, in determining bacterial motility and in promoting anaerobic growth.

Solid media, such as nutrient agar or blood agar, are used for the surface growth of microorganisms to observe colony appearance, for pure culture isolations, for storage of cultures and to observe specific biochemical reactions. Solid media can be poured into either a test tube or Petri plate (dish). If the medium in the test tube is allowed to harden in a slanted position, the tube is designated an agar slant ; if the tube is allowed to



harden in an upright position, the tube is designated an agar deep tube and if the agar is poured into a Petri plate, the plate is designated an agar plate.

Media Preparation

Microbiological media in powder form can be obtained from a scientific supplier. Instructions for preparing the medium are provided on the container. Generally, this entails dissolving a specified quantity of media powder in one litre of distilled or deionized water, denoted as "grams per liter" (g/L). However, it's not obligatory to prepare one liter of medium each time. The quantity can be adjusted by determining the amount of microbiological media powder needed for the specified concentration (g/L) mentioned in the container.

Calculating the Amount of Medium

To determine the amount of powdered medium required for preparation of culture medium, use the following formula.

A= B x C

Where,

- A = Amount of media powder to weigh out (g)
- B = Amount of solution you want to make (mL)
- C = [concentration (g/1000 mL)]

Note that 1 L ("one liter") is the same thing as 1000 mL ("one thousand milliliters"), when the instructions on the media powder bottle tell to make a concentration in g/L ("grams per liter"), this is the same thing as g/1000 mL ("grams per thousand milliliters").

Calculation Example

In this example, you want to make 500 mL of medium. Instructions on the bottle state to make a concentration of 30 g/L.

Solution:

30 g/L = 30 g/1000 mL

From the formula above:

Amount of media powder to weigh out (g) = 500 mL x [30 g / 1000 mL]

Therefore, the amount to weigh out in g ("grams"): 15 g

Practical Demonstration: Preparation of Microbiological Media

In this practical demonstration, we will illustrate the step-by-step process of preparing both broth and agar media, adhering to the manufacturer's instructions for optimal results.

Preparing Broth Medium

1. Weigh the dehydrated medium as per the manufacturer's guidelines and dissolve it in a specified amount of distilled water.

2. Check the pH of the solution and adjust it if necessary.

3. Add additional distilled water to bring the total volume to 100 ml in a 250 ml flask.

4. Apply heat to the solution until it reaches a clear consistency, indicating proper dissolution.

5. Dispense the medium into test tubes (5 or 10 ml) and seal them with cotton wool for subsequent autoclaving, or sterilize directly in a 250 ml flask.

6. Autoclave the prepared medium at 121°C under 15 Ibs pressure for 15 minutes to ensure sterility.

Preparing Agar Medium

1. Weigh the dehydrated agar medium and dissolve it in the specified volume of water, adjusting the pH according to the manufacturer's instructions.

2. Measure the materials to prepare 500 ml of agar and transfer them into a 1000 ml Duran bottle.

3. Heat the mixture on a hot plate until all ingredients are fully dissolved, ensuring a gentle boil.

4. Autoclave the agar medium at 121°C under 15 Ibs pressure for 15 minutes to achieve sterilization.

By meticulously following these procedures, we ensure the preparation of microbiological media that is both sterile and conducive to the growth of microorganisms for laboratory experiments and analysis.

Exercise:

A. In order to make 250 mL of medium with a concentration of 30 g/L, how much medium powder would you weigh in grams?

Answer:

B. In order to make 400 mL of medium with a concentration of 45 g/L, how much medium powder would you weigh in grams?

Answer:

C. In order to make 500 mL of medium with a concentration of 35 g/L, how much medium powder would you weigh in grams?

Answer:

Isolation of Pure Culture (Streaking; Pour Plate Methods)

Microorganisms are found throughout the environment: in the air and water; on the surface of any object such as clothes, walls and furniture; in soil and dust; and on and in our bodies (skin and mucous membranes).

Demonstrating the Ubiquity of Microorganisms

Microorganisms are ubiquitous; that is, they are present nearly everywhere. In this lab, you will try to isolate bacteria and fungi.

PROCEDURE: (Per Group) Collect 2 Nutrient agar (NA) plates, 2 Potato dextrose agar (PDA) and 1 Nutrient broth (NB) tube from the side bench.

1. Moisten a sterile swab with sterile water (see bottle on your bench). Using this swab, collect a sample from any surface or object (e.g. doorknobs, shoes, drinking fountain, a strand of hair, various body parts, etc.).

2. After the sample has been collected, inoculate an NA plate by gently rolling the swab over the surface of the agar. Discard the used swab into a biohazard container.

3. Label the bottom of the plate with your name, lab section, date and the source of the sample. Write on the outer edge so that the markings won't interfere with observing the colonies growing on the plate.

4. Inoculate a second NA plate by the following procedure. Open the lid of the plate, place it close to your mouth and cough hard 3 times onto the plate. Place the lid back on. Correctly label your plate.

5. Inoculate a tube of Nutrient Broth by removing the cap, putting your thumb over the top of the tube and inverting the tube several times. Replace the cap. Label the tube with your name and lab section using a piece of tape. Do not write directly on the cap or tube.

6. Repeat the procedure 1 to 4 for the PDA plates.

7. After inoculating culture media with microorganisms, it is usually incubated at a temperature that most closely mimics the organisms' natural environments.

NB: Plates are always incubated in an inverted position (agar side up).

Serial Dilution

Serial dilution is a fundamental technique in microbiology that enables accurate quantification, isolation and characterization of microorganisms. This method involves sequential dilution of a bacterial suspension in sterile water blanks, which serve as a diluent of known volume (Figure 5). Once diluted, the suspensions are placed on suitable nutrient media.

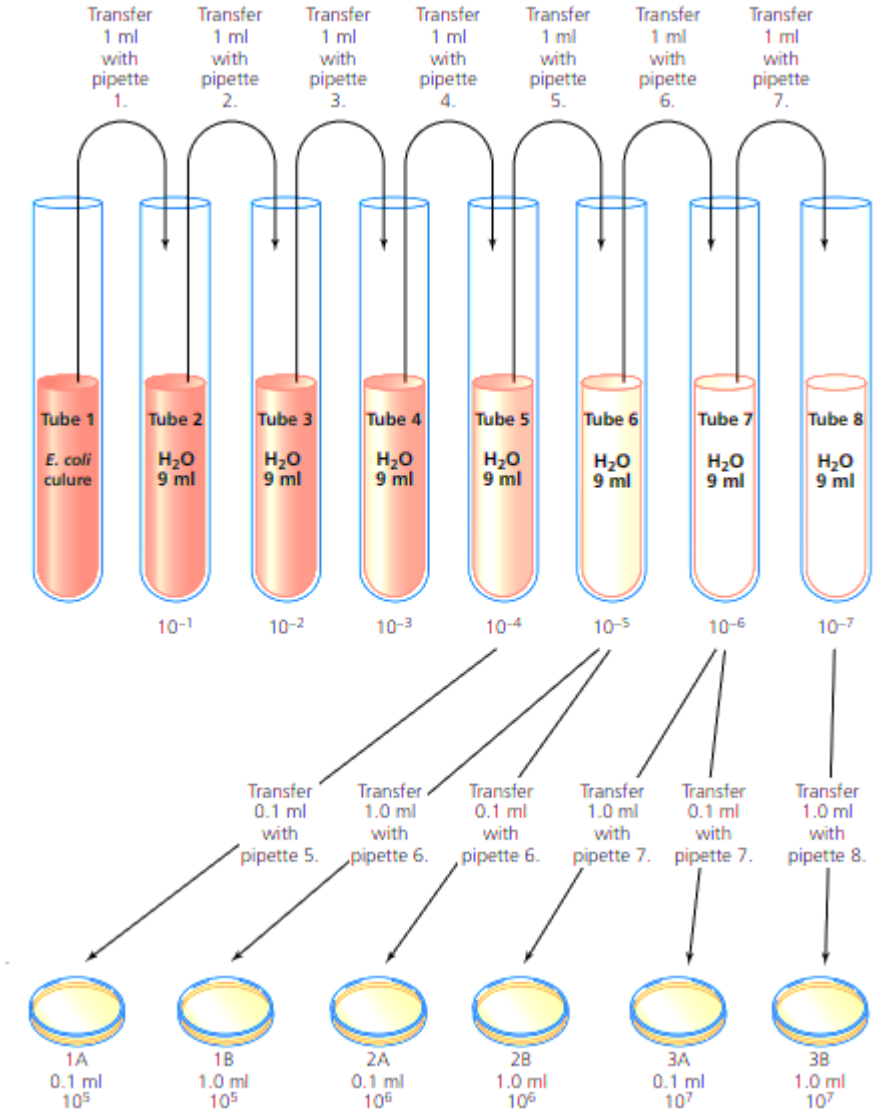


Figure 5: Serial Dilution Illustration

Techniques for Isolation of Pure Culture

The following are viable techniques that are used to isolate pure colonies.

The Streak–Plate Method

In this technique, the bacterial suspension is transferred to the edge of an agar plate with an inoculating loop and then streaked out over the surface in one of several patterns. At some point on the streaks, individual cells will be removed from the loop as it glides along the agar surface and will give rise to

separate colonies Again, one assumes that one colony comes from one cell. The key principle of this method is that by streaking, a dilution gradient is established on the surface of the plate as cells are deposited on the agar surface.

Lab Demonstration on Streak-Plate Technique

1. Flame the inoculating loop to red-hot and allow it to cool near the burner in the air.

2. Hold your isolated culture (previous **Exercise**) in your left hand near the flame. Open the lid of the plate just enough to insert a wire loop. Aseptically withdraw a loopful culture with a loop.

3. Place the inoculum on the agar plate at least 1 cm away from the sides. Spread it in two to three square cm areas.

4. With a sterile cool needle streak or spread the culture from one corner to another and rotate the plate by 90° after streaking 4-5 times in one direction without overlapping the previous streak as demonstrated by the instructor or as shown in the figure below.

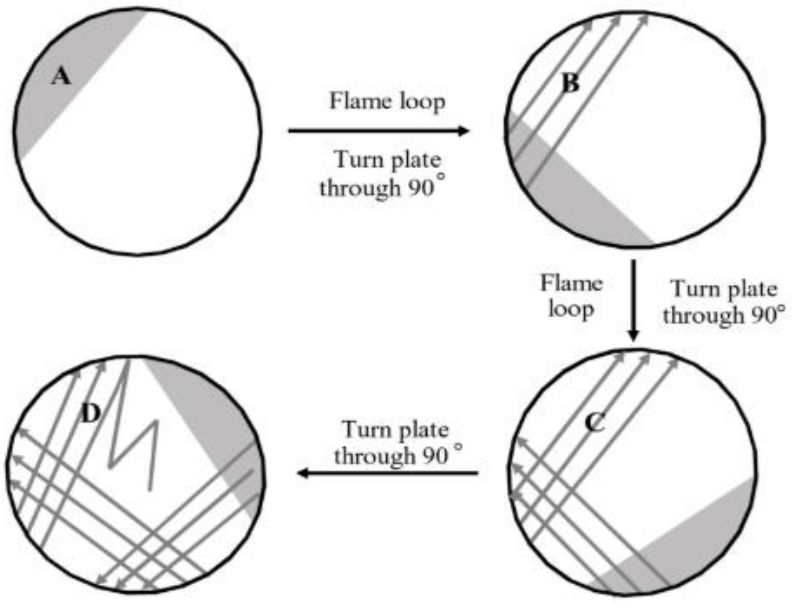


Figure 6: The Streak Plate Method

Pour Plate Method

In this method, we will be able to understand the pour plate method in which the bacteria are mixed evenly and separated evenly throughout the liquid. The molten agar is then poured into an empty plate and allowed to solidify. After inoculation, discrete bacterial colonies can then be formed growing both on the agar media and within the agar. Then, the formed colonies can be separately picked and transferred to a new sterile medium. One advantage of this method is that it requires somewhat less skill than that required for a good streak plate; a disadvantage, however, is that it requires more media, tubes and plates.



Figure 7: The Pour-Plate Technique

Spread Plate Method

Spread plates, also known as lawn plates, should result in a culture spread evenly across the surface of the growth medium. This means that they can be used to carry out microbial sensitivity tests to many antimicrobial substances, for example mouthwashes, garlic, disinfectants and antibiotics.

The spread plate can be used for quantitative work (colony counts) if the inoculum is a measured volume, usually 0.1 cm3, of each of a dilution series, delivered by pipette.

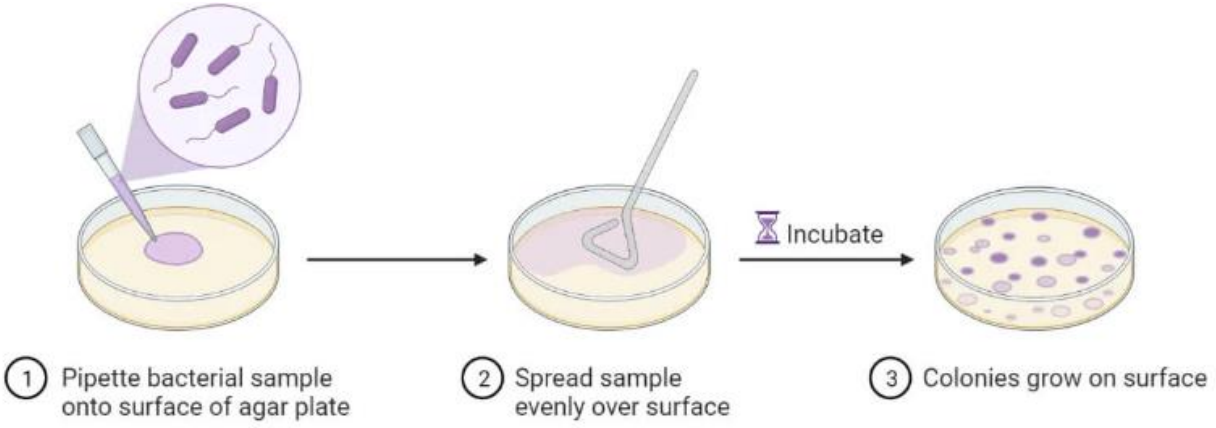


Figure 8: Spread Plate Method

Exercises:

1. - Explain the importance of serial dilution in microbiology. How does this technique aid in the quantification, isolation and characterization of microorganisms? Provide a step-by-step description of the serial dilution process and its application in microbiological experiments.

2. - Describe the streak-plate method for isolating pure colonies of microorganisms. What is the principle behind this technique and how does it work to achieve isolation? Outline the procedural steps involved in performing a streak-plate experiment, including aseptic techniques and precautions to prevent contamination.

3. - Compare and contrast the pour plate and spread plate methods for isolating bacterial colonies. Discuss the advantages and disadvantages of each method in terms of ease of execution, efficiency and resource requirements. Provide examples of scenarios where one method might be preferred over the other based on experimental goals and constraints.

Stains and Staining Techniques

Staining techniques are essential tools in microbiology used to enhance the visibility of microorganisms under a microscope. These techniques involve the application of specific dyes or stains to highlight different structures or components of microbial cells.

Numerous staining techniques are available for visualization, differentiation and separation of bacteria in terms of morphological characteristics and cellular structures. Staining methods are broadly divided into simple staining and differential staining techniques.

Simple staining is defined as the process of colouring bacteria or other cells by applying a single solution of a stain to a fixed smear. It is used for visualization of morphological shape (cocci, bacilli and spirilli) and arrangement (chains, clusters, pairs and tetrads) of bacteria. However, differential staining is the use of more than one staining reagent to bring out differences in microbial cell types or to differentiate particular cellular components from the rest of the cell body

The most common staining techniques include:

Gram Staining: Developed by Hans Christian Gram, this technique categorizes bacteria into Gram-positive (retains the violet stain) and Gram-negative (takes on a red or pink color) based on differences in their cell wall composition.

Acid-Fast Staining: Used to detect bacteria that have a waxy cell wall, such as Mycobacterium tuberculosis. Acid-fast bacteria retain the primary stain (carbolfuchsin) even after treatment with acid-alcohol.

Endospore Staining: Highlights the presence of endospores, which are dormant structures formed by certain bacteria as a survival mechanism. The most common method is the Schaeffer-Fulton stain, which uses malachite green as the primary stain.

Capsule Staining: Capsules are slimy outer layers produced by some bacteria for protection. Capsule staining techniques, like Maneval's method, use acidic and basic dyes to make the capsules more visible under the microscope.

Flagella Staining: Flagella are long, whip-like appendages that some bacteria use for movement. Flagella staining techniques involve staining the flagella with specific dyes, such as tannic acid and silver salts, to visualize them.

These staining techniques provide invaluable information about microbial morphology, cell structure and physiological characteristics, aiding in microbial identification, classification and diagnosis of infectious diseases.

Preparation of Bacteria Smear

The preparation of bacteria for the staining process involves smear preparation. In this step, the bacteria sample must firmly attach to a glass slide. However, there are two important things to consider when preparing a slide for staining.

- The bacteria must be evenly and lightly dispersed. If there are too many bacteria on the slide they will form a big blob and you will not be able to see the morphology of the individual cells. Large blobs of cells also do not stain properly and could yield erroneous results from improper staining.
- The bacteria need to be firmly attached to the slide so they are not washed off during the staining procedures. All procedures that attach the bacteria to the slide result in some morphological changes. The cells typically shrink in size and will exhibit some changes in shape and extra-cellular matrices.

Laboratory Demonstration of Bacteria Smear Preparation

1. Clean a plain microscope slide thoroughly using lens tissue.

2. Label a microscope slide with a marker pen to record the culture being used, date and initials; this is also a useful reminder of which side of the slide is being used.

3. Flame a wire loop to ensure that no culture accidentally remains from a previous operation.

4. Transfer one or two loopfuls of water onto the centre of the slide.

5. Flame the loop and allow it to cool.

6. Using an aseptic technique, transfer a very small part of a single colony from a plate or slope of agar medium into the water.

Note: If the amount of culture on the loop is easily visible you have taken too much!

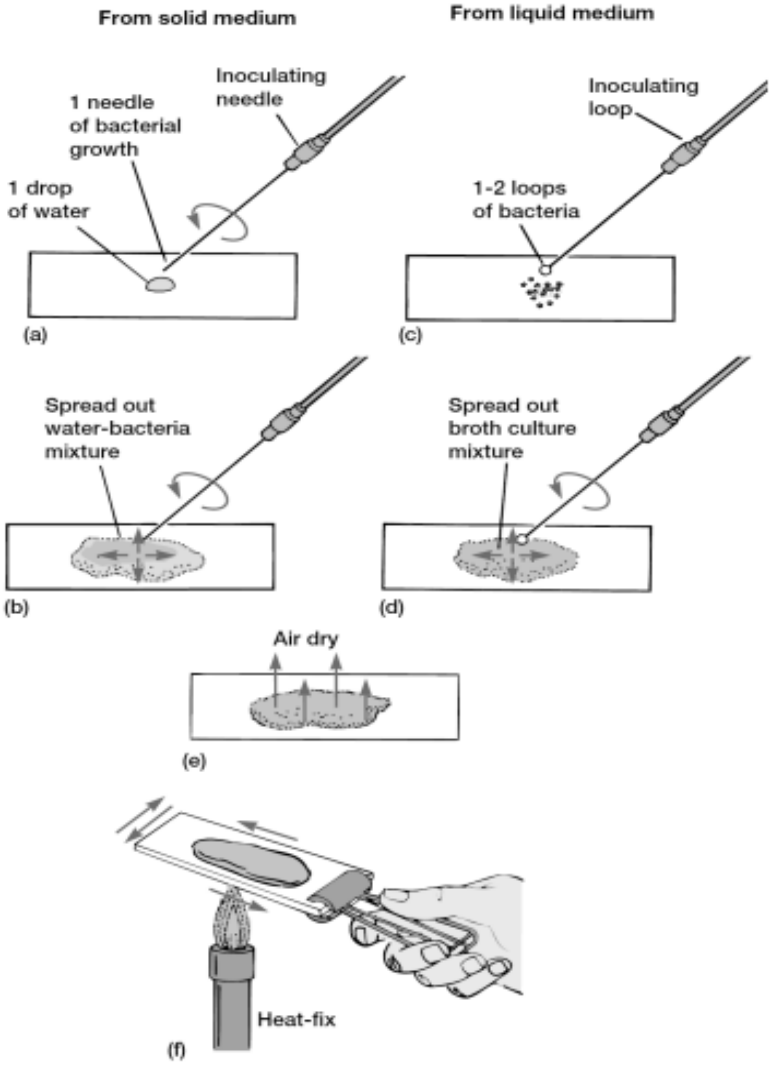


Figure 9: Smear Preparation from Bacterial Culture

7. Make a suspension of the culture in the water on the slide and thoroughly but gently spread it evenly over an oval area of up to 2 cm in length.

8. Flame the loop.

Note: If it is necessary to use a liquid culture or sample, the use of water to prepare the smear will probably be unnecessary and may result in a smear with too few cells.

9. Dry the suspension by warming it gently over a Bunsen burner flame and then "fix" it by quickly passing it through the flame a few times.

This is called a heat-fixed smear; it should be visible to the naked eye as a whitish area. Fixing is necessary to ensure that cells adhere to the slide and to minimise any changes before staining. The smear is now ready to be stained.

Laboratory Demonstration of Gram Staining Techniques

1. Put the slide with the fixed smear uppermost on a staining rack over a sink or staining tray.

2. Thoroughly cover the smear with crystal violet solution and leave for 1 minute.

3. Hold the slide with forceps (optional but avoids stained fingers), at a 45° angle over the sink.

4. Pour off the stain and wash off any that remains (and any on the back of the slide) with an iodine solution.

5. Put the slide back on the staining rack.

6. Cover the smear with iodine solution and leave for 1 minute. Iodine solution acts as a "mordant" (a component of a staining procedure that helps the stain to adhere to the specimen), a crystal violet-iodine complex is formed and the smear looks black.

7. Hold the slide with forceps at a 45° angle over the sink wash off the iodine solution with 95% (v/v) ethanol (not water); continue treating with alcohol until the washings are pale violet.

8. Rinse immediately with tap water.

9. Put the slide back on the staining rack. 10. Cover the smear with the counterstain, e.g. safranin solution, 0.5% w/v, for 30 seconds.

11. Rinse off the stain with tap water.

12. Blot dry the smear with filter/fibre-free blotting paper using firm pressure but not sideways movements that might remove the smear.

13. Examine under oil immersion.

14. When finished, dispose of the slides into a discarded jar.

Exercise:

1. Describe the difference between simple staining and differential staining techniques in microbiology. How do these staining methods aid in the visualization and differentiation of microbial cells? Provide examples of differential staining techniques and explain how they are used to highlight specific cellular components or characteristics.
2. Outline the steps involved in the Gram staining technique for bacterial identification. Explain the purpose of each step in the staining process and the role of specific reagents used, such as crystal violet, iodine, ethanol and safranin. Discuss the significance of Gram staining in bacterial classification and clinical diagnosis.
3. Discuss the importance of bacteria smear preparation in the staining process. Why is it necessary to ensure that bacteria are evenly dispersed and firmly attached to the slide? Describe the steps involved in preparing a bacterial smear on a microscope slide, including the use of aseptic techniques and heat fixation. What are the potential consequences of improper smear preparation?
4. Record all your observations in your lab workbook.

Enumeration of Microorganisms

The microbial enumeration technique is a fundamental laboratory procedure in microbiology that involves determining the number of microorganisms in a given sample. This process is essential for a variety of applications, including clinical diagnostics, food safety, environmental monitoring and scientific research. Accurate microbial counts are crucial for assessing contamination levels, evaluating the efficacy of antimicrobial treatments and ensuring compliance with health and safety standards.

Techniques employed in the enumeration of microorganisms in various samples (food, water, soil, air etc) can range from simple plate counts to advanced automated systems, each with specific methodologies and applications tailored to the type and context of the sample being analyzed. These include direct microscopic count, viable plate count, turbidimetric method, flow cytometry and molecular methods.

Direct Microscopic Count

The direct microscopic count is used for quantitative enumeration of bacteria in water, food, milk and air samples. It is done by spreading a measured volume of the sample over a predetermined area of a slide, counting representative microscopic fields and transforming the average values into appropriate volume-area factors.



Figure 10a: Counting chamber.

The counting chambers used for the procedures include Petroff-Hauser and Levy counting chambers). The Petroff-Hauser is a thick microscope slide with a chamber in the middle of 0.02 mm (1/50 mm) deep and it also has improved Neubauer rulings and an etched grid in the chamber (Figure 10b). The ruling is the centerline of a group of three squares in which cells are counted.

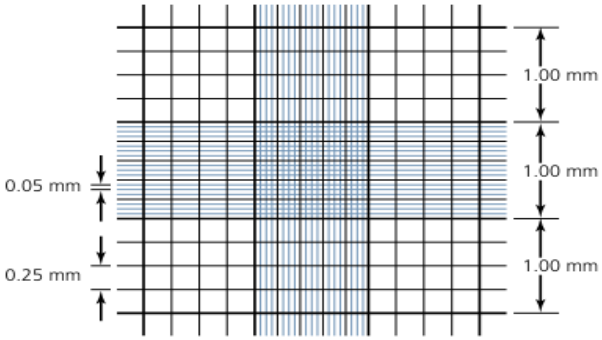


Figure 10b: Counting Chambers Grids.

Standard Plate Count (Viable Counts)

The standard or viable plate count is the most common method of measuring viable bacterial cell numbers in a sample. Contrary to a total cell count, the viable count reveals information related only to viable or live bacteria. Using this method, a small volume (0.1 - 1.0 mL) of liquid containing an unknown number of bacteria is spread over the surface of an agar plate, creating a "spread plate." The spread plates are incubated for 24-36 hours. During that time, each individual viable bacterial cell multiplies to form a readily visible colony. The number of colonies is then counted and this number should equal the number of viable bacterial cells in the original volume of sample, which was applied

to the plate. The total number of viable cells obtained from this procedure is usually reported as the number of colony-forming units (CFUs).

A bacterial culture and many other samples usually contain too many cells to be counted directly. Thus, to obtain plates, which are not hopelessly overgrown with colonies, it is often necessary to dilute the sample and spread measured amounts of the diluted sample on plates. Dilutions are performed by careful aseptic pipetting of a known volume of sample into a known volume of a sterile buffer or sterile water (Figure 5 & 11 respectively). This is mixed well and can be used for plating and/or further dilution. If the number of cells in the original sample is unknown, then a wide range of dilutions are usually prepared and plated.

The final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming units (CFUs).

The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of live bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10^{-3} to 10^{-10}) is normally plated because the exact number of live bacteria in the sample is usually unknown. Greater precision is achieved by plating duplicates or triplicates of each dilution.

The number of colonies is counted and used to estimate the number of viable bacteria in the original sample.

$$CFU(per\ mL) = \frac{(\#\ colonies\ present\ on\ the\ plate)(dilution\ factor)}{volume\ plated\ (mL)}$$

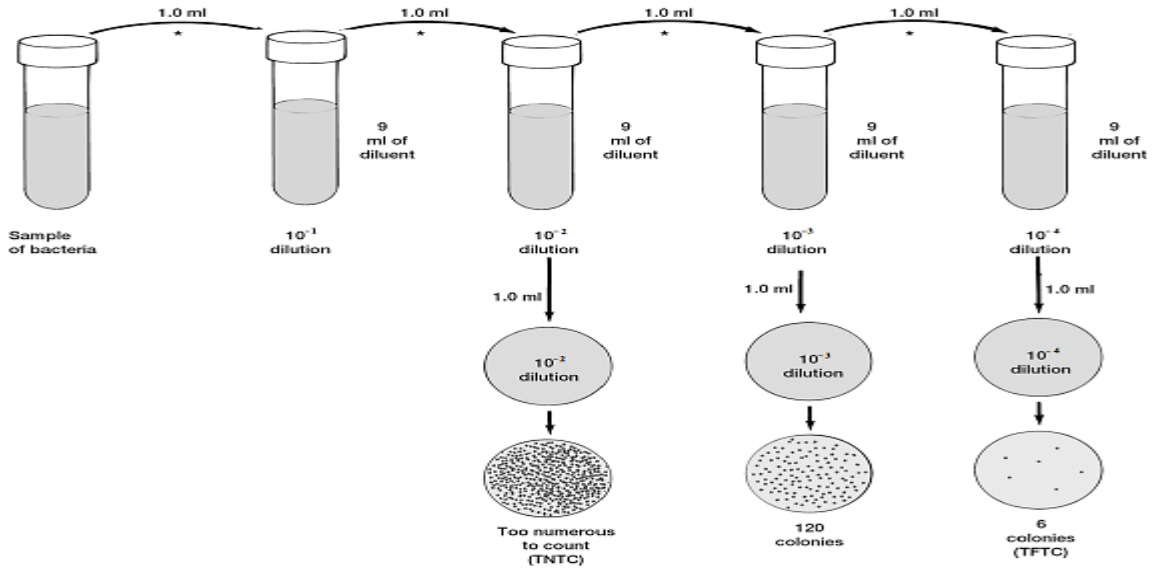


Figure 11: Procedure Standard Plate Count

Laboratory Demonstrations

Exercise 1: Direct Count Using a Counting Chamber

1. Clean a counting chamber with methanol and lens paper and then place it on the microscope stage.

2. Using the 4X objective find the ruled area on one side of the chamber and **note** the size and arrangements of larger squares and their small square subdivisions.

3. Shake the yeast suspension to distribute the cells evenly. Take out the counting chamber without changing the focus on the 4X objective. Place a coverslip over the calibrated surface of the counting chamber.

4. Using a transfer pipette, transfer some of the yeast suspension to the groove of the counting chamber to fill the chamber by capillary action.

5. Carefully place the counting chamber back onto the microscopic stage and observe the cells under 4X. You may need to reduce the amount of light by closing the diaphragm of the condenser to be able see the cells.

6. Switch to the high-dry objective (40 X) and count the number of yeast cells in at least 50 of the small squares.

NOTE:

If cells fall on a line, include in your count those on the top and left-hand lines and exclude those on the bottom and right-hand lines. (If the yeast cells are too dense to count, dilute your sample and start again.)

7. Calculate the average number of yeast cells per small square. Then calculate the number of yeasts per ml by dividing the average number of yeasts per small square by the volume of each small square which is 0.00025 μl.

NOTE:

If you diluted the sample, you must also multiply your results by the dilution factor to determine the concentration of yeast cells in the original sample.

Record your calculations and results.

Excersice 2:

Viable plate counts

1. Prepare serial dilutions of the sample as previously described in this manual Transfer 0.1 ml of the final three dilutions (10^{-4}, 10^{-5}, 10^{-6}) to duplicate nutrient agar plates and label the plates.

2. Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates until the medium no longer appears moist. Return the spreader to the alcohol.

3. Repeat the flaming and spreading for each of the remaining five plates.

4. Invert the six plates and incubate at room temperature until the next lab period (or ~ 48 hours, whichever is the shortest).

Remember that only plates with 30 to 300 colonies are statistically valid.

Note: Record all your observations

Cultural Characteristics of Bacteria

Colony morphology is the visual culture characteristics of a bacterial colony on an agar plate. Observing colony morphology is an important skill used in the microbiology laboratory to identify microorganisms. Colonies need to be well isolated from other colonies to be able to observe their characteristics such as shape, size, colour, surface appearance and texture.

By looking closely at the colonial growth on the surface of a solid medium, characteristics such as surface texture, transparency and the colour or hue of the growth can be described. The following three characteristics are readily apparent whether you're looking at a single bacterial colony or more dense growth, without the aid of any type of magnifying device.

Texture: This describes how the surface of the colony appears. Common terms used to describe texture may include smooth, glistening, mucoid, slimy, dry, powdery, flaky etc.

Transparency / Optical Characteristics: In this case, the colonies may be described as transparent (you can see through them), translucent (light passes through them), or opaque (solid appearing).

Colour or Pigmentation: many bacteria produce intracellular pigments which cause their colonies to appear a distinct colour, such as yellow, pink, purple or red. Many bacteria do not produce any pigment and appear white or grey.

Likewise, other features such as overall size, shape or form, edge or margin of the colony (what a close-up of the edges of the colony looks like) and elevation (how the colony appears when you observe it from the side) are used to describe the cultural characteristics of the colonies (Figure 12).

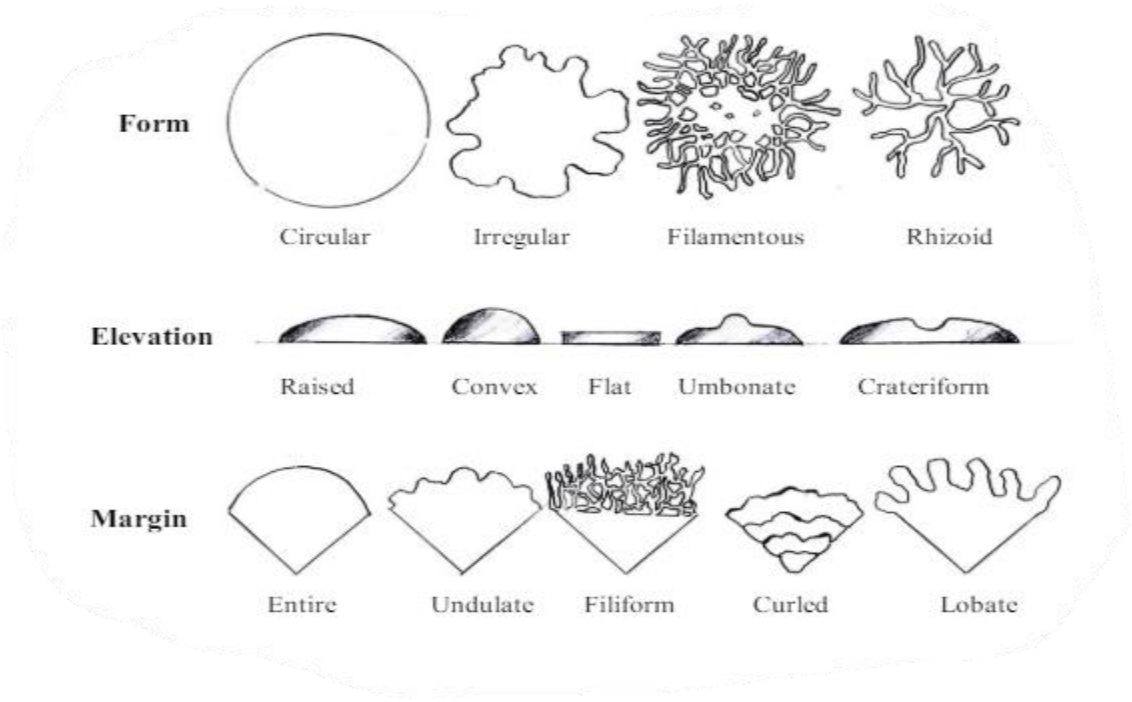


Figure 12: Cultural Characteristics of Bacterial Colonies on Plate.

Furthermore, on nutrient agar slants, in nutrient broth and in nutrient gelatin. The bacterial cultures have specific patterns of growth that can serve as guide towards their identification (Figure 13a & b).

While these growth patterns are an important piece of information when identifying a bacterial species, they are not sufficient for a positive identification. Staining procedures and metabolic tests must be used for a definitive identification.

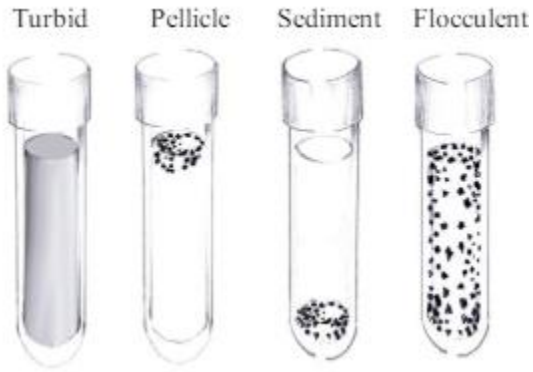


Figure 13a: Growth Characteristics in Broth Media.

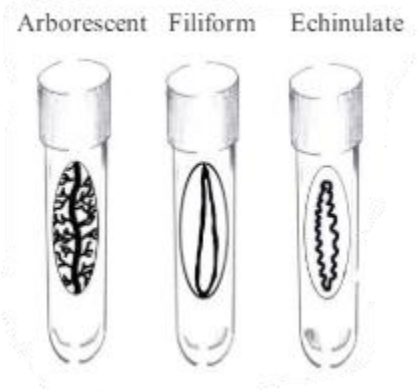


Figure 13b: Growth Characteristics on Agar Slant.

Exercise:

1. Observe the culture plate provided and describe fully the colonial morphology of at least three distinct colonies. A full description will include texture, transparency, colour and form (size, overall shape, margin and elevation).

2. Describe the colonial morphology of *Bacillus* spp, using the NSA plate culture of this bacterium provided to your group at the beginning of the lab: