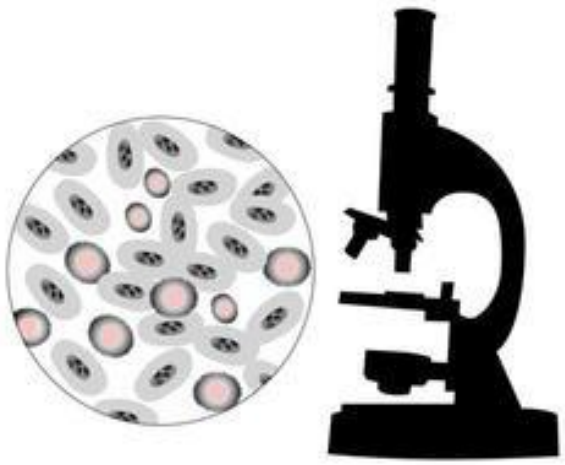
South Valley University
Faculty of Science
Department of Botany & Microbiology

PRACTICAL BACTERIOLOGY

For the 3rd year

Microbiology and Chemistry



Part (1) Laboratory basics

LABORATORY SKILLS

A student successfully completing basic microbiology will demonstrate the ability to:

1. Use a bright-field light microscope to view and interpret slides, including:

a. correctly setting up and focusing the microscope

b. proper handling, cleaning and storage of the microscope

c. correct use of all lenses

d. recording microscopic observations

2. Properly prepare slides for microbiological examination, including:

a. cleaning and disposal of slides

b. preparing smears from solid and liquid cultures

c. performing wet- mount and/ or hanging drop preparations

d. performing Gram stains

3. Properly use aseptic techniques for the transfer and handling of microorganisms and instruments, including:

a. sterilizing and maintaining sterility of transfer instruments

b. performing aseptic transfer

c. obtaining microbial samples

4. Use appropriate microbiological media and test systems, including:

a. isolating colonies and/ or plaques

b. maintaining pure cultures

c. using biochemical test media

d. accurately recording macroscopic observations

5. Estimate the number of microorganisms in a sample using serial dilution techniques, including:

a. correctly choosing and using pipettes and pipetting devices

b. correctly spreading diluted samples for counting

c. extrapolating plate counts to obtain correct CFU in the starting sample

6. Use standard microbiology laboratory equipment correctly, including:

a. using the standard metric system for weights, lengths, diameters, and volumes

b. lighting and adjusting a laboratory burner

c. using an incubator

Laboratory Thinking Skills

A student successfully completing basic microbiology will demonstrate an increased skill level in:

1. Cognitive processes, including:

a. formulating a clear, answerable question

b. developing a testable hypothesis

c. predicting expected results

d. following an experimental protocol

2. Analysis skills, including:

a. collecting and organizing data in a systematic fashion

b. presenting data in an appropriate form (graphs, tables, figures, or descriptive paragraphs)

c. drawing appropriate conclusions based on the results

3. Communications skills, including:

a. discussing and presenting laboratory results or findings in the laboratory

4. Interpersonal and citizenry skills, including:

a. working effectively in groups or teams so that the task, results, and analysis are shared

b. effectively managing time and tasks to be done simultaneously, by individuals and within a group

c. integrating knowledge and making informed judgments about microbiology in everyday life

LABORATORY RULES AND GENERAL SAFETY

A student successfully completing basic microbiology will demonstrate the ability to explain and practice safe:

1. <u>Microbiological procedures</u>, including:

a. reporting all spills and broken glassware to the instructor and receiving instructions for cleanup

b. methods for aseptic transfer

c. minimizing or containing the production of aerosols and describing the hazards associated with aerosols

d. washing hands prior to and following laboratories and at any time contamination is suspected

e. never eating or drinking in the laboratory

f. disinfecting lab benches prior to and at the end of each lab session

g. identification and proper disposal of different types of waste

h. never applying cosmetics, including contact lenses, or placing objects (fingers, pencils) in the mouth or touching the face

i. understands the safety rules of the laboratory

j. good lab practice, including returning materials to proper locations, proper care and handling of equipment, and keeping the bench top clear of extraneous materials

2. <u>Protective procedures</u>, including:

a. tying long hair back, wearing personal protective equipment (eye protection, coats, closed shoes; glasses may be preferred to contact lenses), and using such equipment in appropriate situations

b. always using appropriate pipetting devices and understanding that mouth pipetting is forbidden

3. <u>Emergency procedures</u>, including:

a. locating and properly using emergency equipment (first aid kits, fire extinguishers, chemical safety showers, and emergency numbers)

b. reporting all injuries immediately to the instructor

c. following proper steps in the event of an emergency

LABORATORY PROTOCOL

Scheduling

Each laboratory session will begin with a short discussion to brief you on the availability of materials and procedures. Since the preliminary instructions start promptly at the beginning of the period, it is **extremely important that you are not late to class.**

Personal Items

When you first enter the lab, place all personal items such as jackets, bags, and books in some out of the way place for storage. Don't stack them on your desktop. Desk space is minimal and must be reserved for essential equipment and your laboratory manual. The storage place may be a drawer, locker, coatrack, or perimeter counter. Your instructor will indicate where they should be placed.

Attire

A lab coat must be worn at all times in the laboratory. It will protect your clothing from accidental contamination and stains in the lab. When leaving the laboratory, remove the coat or apron. In addition, long hair must be secured in a ponytail to prevent injury from Bunsen burners and contamination of culture material.

Terminology

Various terms such as sterilization, disinfection, germicides, sepsis, and aseptic techniques will be used here. To be sure that you understand exactly what they mean, the following definitions are provided.

Sterilization is a process in which all living microorganisms, including viruses, are destroyed. The organisms may be killed with steam, dry heat,

or incineration. If we say an article is sterile, we understand that it is completely free of all living microorganisms. Generally speaking, when we refer to sterilization as it pertains here to laboratory safety, we think, primarily, in terms of steam sterilization with the autoclave. The ultimate method of sterilization is to burn up the infectious agents or **incinerate** them. All biological wastes must ultimately be incinerated for disposal.

Disinfection is a process in which vegetative, nonsporing microorganisms are destroyed. Agents that cause disinfection are called **disinfectants** or **germicides.** Such agents are used only on inanimate objects because they are toxic to human and animal tissues.

Sepsis is defined as the growth (multiplication) of microorganisms in tissues of the body. The term **asepsis** refers to any procedure that prevents the entrance of infectious agents into sterile tissues, thus preventing infection. **Aseptic techniques** refer to those practices that are used by microbiologists to exclude all organisms from contaminating media or contacting living tissues. **Antiseptics** are chemical agents (often dilute disinfectants) that can be safely applied externally to human tissues to destroy or inhibit vegetative bacteria.

Accidental spills

All accidental spills, whether chemical or biological, must be reported immediately to your instructor. Although the majority of microorganisms used in this laboratory are nonpathogens, some pathogens will be encountered. It is for this reason that we must treat all accidental biological spills as if pathogens were involved.

Chemical spills are just as important to report because some agents used in this laboratory may be carcinogenic; others are poisonous; and some can cause dermal damage such as blistering and depigmentation.

Decontamination Procedure Once your instructor is notified of an accidental spill, the following steps will take place:

1. Any clothing that is contaminated should be placed in an autoclavable plastic bag and autoclaved.
2. Paper towels, soaked in a suitable germicide, such as 5% bleach, are placed over the spill.
3. Additional germicide should be poured around the edges of the spill to prevent further aerosolization.
4. After approximately 20 minutes, the paper towels should be scraped up off the floor with an autoclavable squeegee into an autoclavable dust pan.
5. The contents of the dust pan are transferred to an autoclavable plastic bag, which may itself be placed in a stainless steel bucket or pan for transport to an autoclave.
6. All materials, including the squeegee and dustpan, are autoclaved.

Pipette Handling

Success in this experiment depends considerably on proper pipetting techniques. Pipettes may be available to you in metal cannisters or in individual envelopes; they may be disposable or reusable. In the distant past pipetting by mouth was routine practice. However, the hazards are obvious, and today it must be avoided. Your instructor will indicate the techniques that will prevail in this laboratory. If this is the first time that you have used sterile pipettes, keep the following points that are shown in the following figure.

Pipette-handling techniques

ASEPTIC PROCEDURES

The proper handling of materials in the Bacteriology lab requires special skills that you must master. It's all about aseptic technique. The procedures discussed above entitled **Laboratory Protocol** outline some of the specifics to be observed to ensure that you understand what is required in maintaining an aseptic environment when handling cultures of microorganisms. In this exercise you will have an opportunity to actually work with cultures and different kinds of media to develop those skills that are required to maintain asepsis. Aseptic transfer of a culture from one culture vessel to another is successful only if no contaminating microorganisms are introduced in the process. A transfer may involve the transport of organisms from an isolated colony on a plate of solid medium to a broth tube, or inoculating various media (solid or liquid) from a broth culture for various types of tests.

When you start handling bacterial cultures, you will learn the specifics of aseptic techniques. The general procedure is as follows:

Hand Washing Before you start working in the lab, wash your hands with a liquid detergent and dry them with paper toweling. At the end of the period, before leaving the laboratory, wash them again.

Tabletop Disinfection. The first chore of the day will be to sponge down your desktop with a disinfectant. This process removes any dust that may be present and minimizes the chances of bacterial contamination of cultures that you are about to handle. Your instructor will indicate where the bottles of disinfectant and sponges are located. At the end of the period before leaving the laboratory, perform the same procedure to protect students that may occupy your desk in the next class.

Work Area Disinfection. The work area is first treated with a disinfectant to kill any microorganisms that may be present. This step destroys

vegetative cells and viruses; endospores, however, are not destroyed in this brief application of disinfectant.

Bunsen Burner Usage. When using a Bunsen burner to flame loops, needles, and test tubes, follow the instructed procedures. Inoculating loops and needles should be heated until they are red- hot. Before they are introduced into cultures, they must be allowed to cool down sufficiently to prevent killing organisms that are to be transferred. If your burner has a pilot on it and you plan to use the burner only intermittently, use it. If your burner lacks a pilot, turn off the burner when it is not being used. Excessive unnecessary use of Bunsen burners in a small laboratory can actually raise the temperature of the room. More important is the fact that unattended burner flames are a constant hazard to hair, clothing, and skin.

The proper handling of test tubes, while transferring bacteria from one tube to another, requires a certain amount of skill. Test tube caps must never be placed down on the desktop while you are making inoculations. Techniques that enable you to make transfers properly must be mastered.

Pipetting. Transferring solutions or cultures by pipette must always be performed with a mechanical suction device. Under no circumstances is pipetting by mouth allowed in this laboratory.

Disposal of Cultures and Broken Glass. The following rules apply to culture and broken glass disposal:

1. Petri dishes must be placed in a plastic bag to be autoclaved.
2. Unneeded test tube cultures must be placed in a wire basket to be autoclaved.
3. Used pipettes must be placed in a plastic bag for autoclaving.
4. Broken glass should be swept up into a dustpan and placed in a container reserved for broken glass. Don't try to pick up the glass fragments with your fingers.
5. Contaminated material must never be placed in a wastebasket.

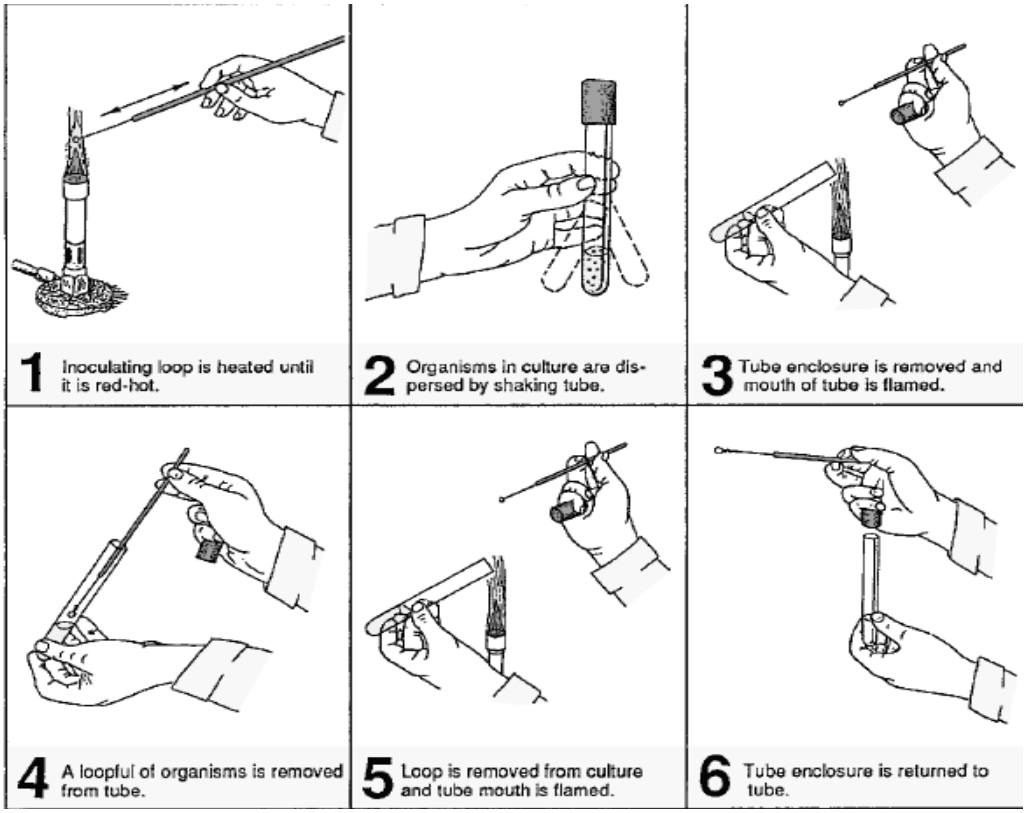
Loops and Needles. The transport of organisms will be performed with an inoculating loop or needle. To sterilize the loop or needle prior to picking up the organisms, heat must be applied with a Bunsen burner flame, rendering them glowing red- hot.

Culture Tube Flaming. Before inserting the cooled loop or needle into a tube of culture, the tube cap is removed and the mouth of the culture tube flamed. Once the organisms have been removed from the tube, the tube mouth must be flamed again before returning the cap to the tube.

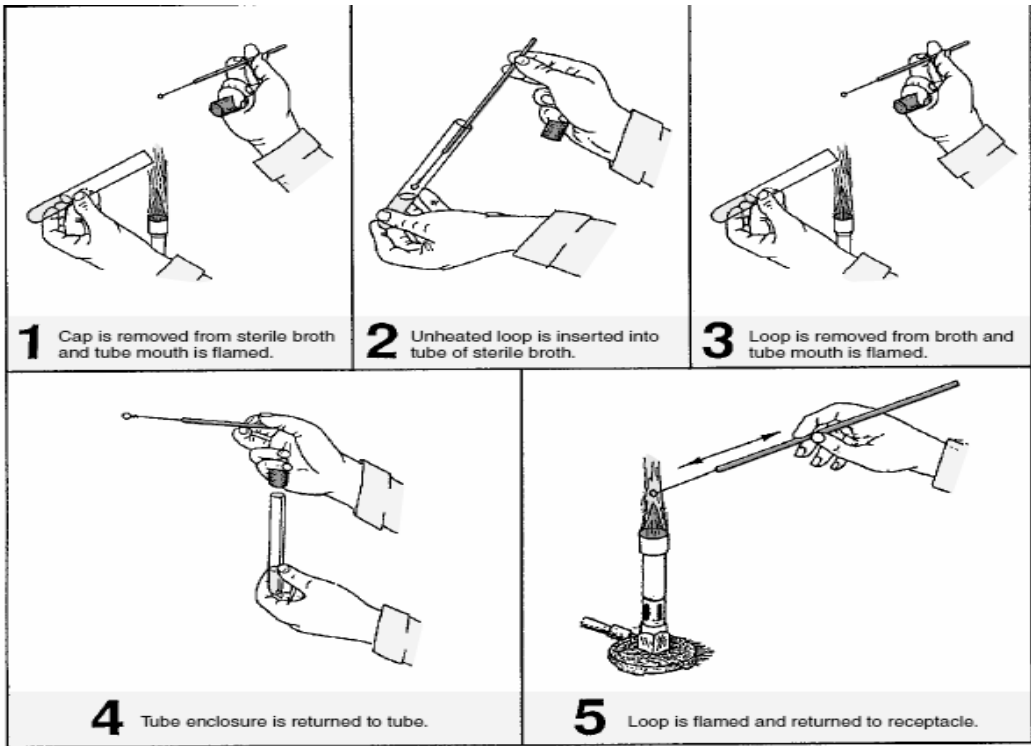
Liquid Medium Inoculation. If a tube of liquid medium is to be inoculated, the tube mouth must be flamed before inserting the loop into the tube. To disperse the organisms on the loop, the loop should be twisted back and forth in the medium. If an inoculating needle is used for stabbing a solid medium, the needle is inserted deep into the medium. Once the inoculation is completed, the loop or needle is removed from the tube, flamed as before, and returned to a receptacle. These tools should never be placed on the tabletop. The inoculated tube is also flamed before placing the cap on the tube.

Petri Plate Inoculation. To inoculate a Petri plate, no heat is applied to the plate and a loop is used for the transfer. When streaking the surface of the medium, the cover should be held diagonally over the plate bottom to prevent air contamination of the medium.

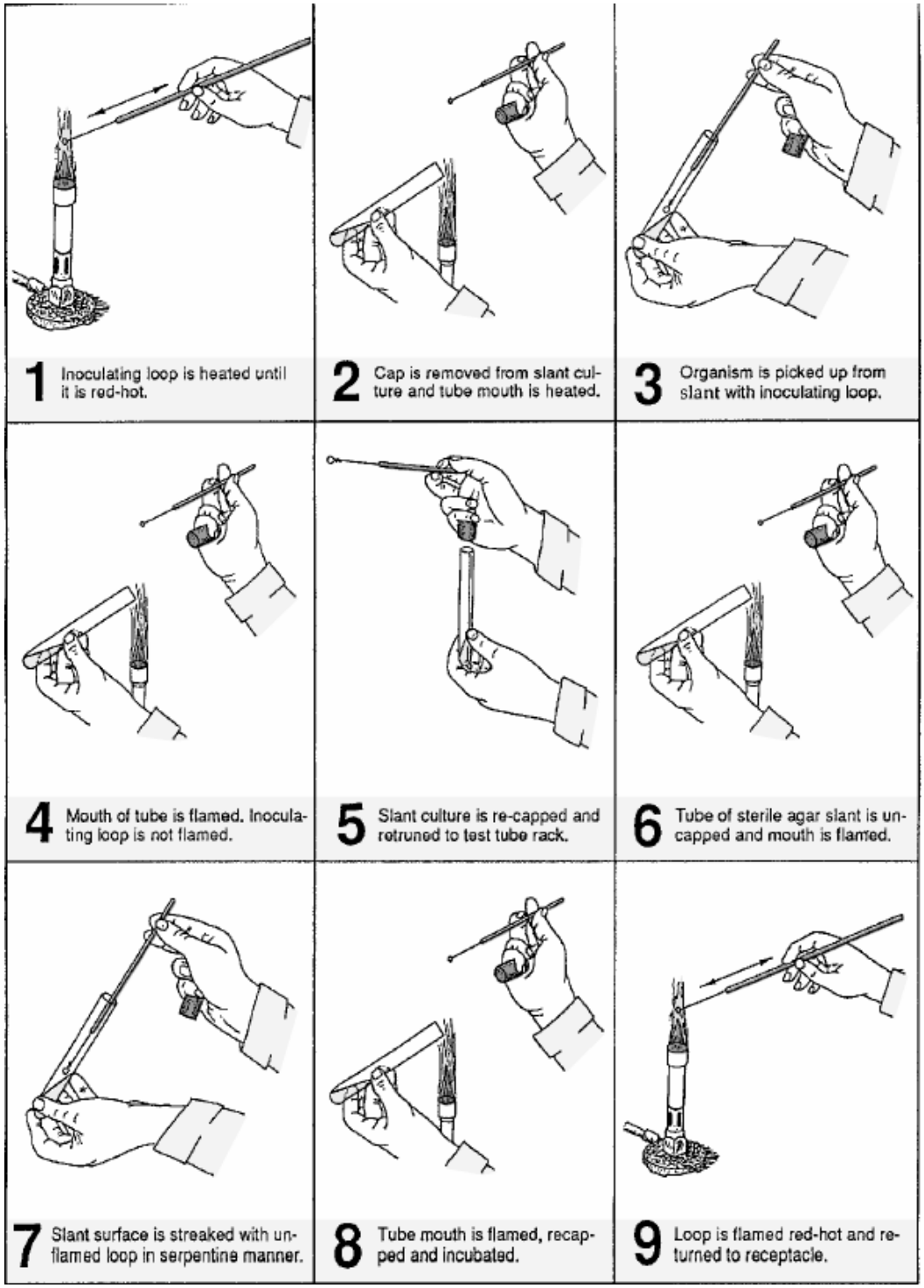
Final Disinfection. When all work is finished, the work area is treated with disinfectant to ensure that any microorganisms deposited during any of the procedures are eliminated. The following Figures illustrate some of these procedures.



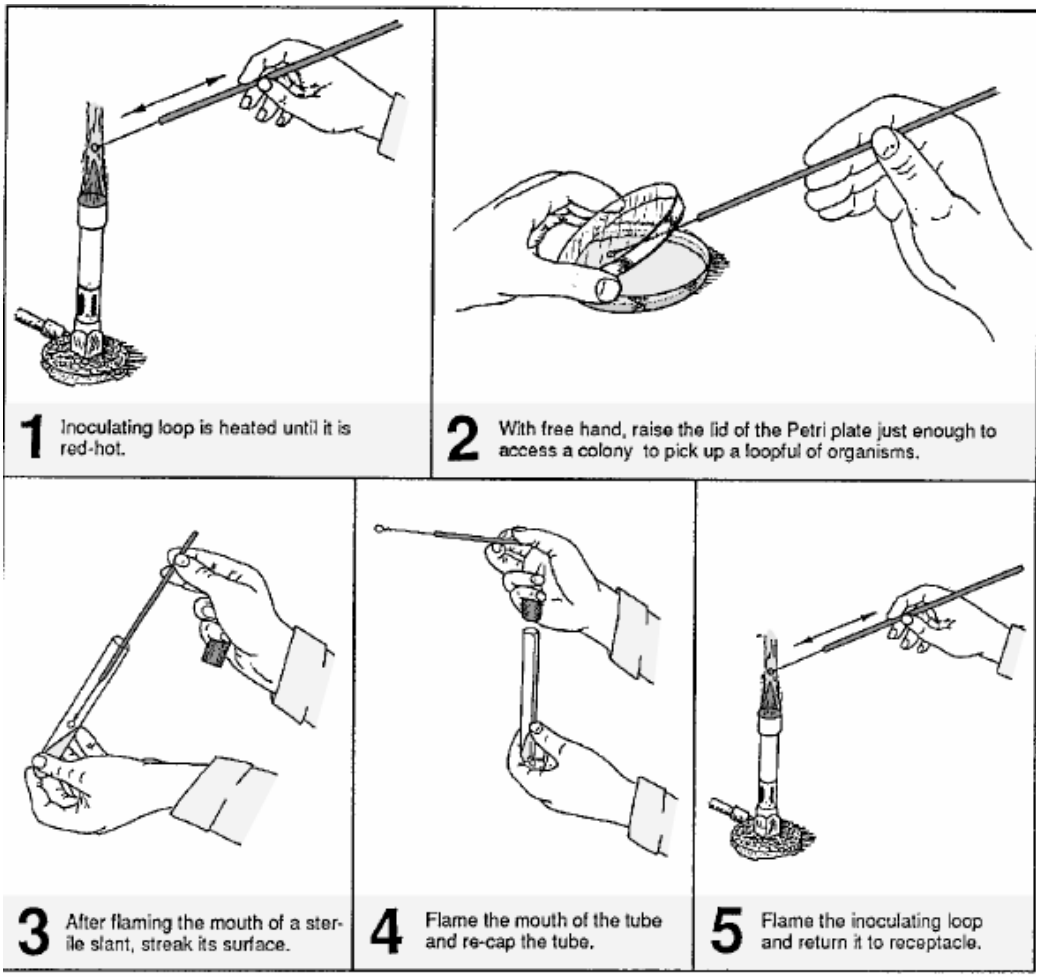
Procedure for removing organisms from a broth culture with inoculating loop



Procedure for inoculating a nutrient broth



Procedure for inoculating a nutrient agar slant from a slant culture



Procedure for inoculating a nutrient agar slant from an agar plate

ADDITIONAL IMPORTANT REGULATIONS

1. Don’t remove cultures, reagents, or other materials from the laboratory unless you have been granted specific permission.

2. Don’t smoke or eat food in the laboratory.

3. Make it a habit to keep your hands away from your mouth. Obviously, labels are never moistened with the tongue; use tap water or self-adhesive labels instead.

4. Always clean up after yourself. Gram stained slides that have no further use to you should be washed and dried and returned to a slide box. Coverslips should be cleaned, dried, and returned. Staining trays should be rinsed out and returned to their storage place.

5. Return all bulk reagent bottles to places of storage.

6. Return inoculating loops and needles to your storage container. Be sure that they are not upside down.

7. If you have borrowed something from someone, return it.

8. Do not leave any items on your desk at the end of the period.

9. Do not disturb another class at any time. Wait until the class is dismissed.

10. Treat all instruments, especially microscopes, with extreme care. If you don’t understand how a piece of equipment functions, ask your instructor.

11. Work cooperatively with other students in group assigned experiments, but do your own analyses of experimental results.

MICROSCOPY

Microscopes in a college laboratory represent a considerable investment and require special care to prevent damage to the lenses and mechanicals. The fact that a laboratory microscope may be used by several different individuals during the day and moved around from one place to another results in a much greater chance for damage and wear to occur than if the instrument were used by only one individual. The complexity of some of the more expensive microscopes also requires that certain adjustments be made periodically. Knowing how to make these adjustments to get the equipment to perform properly is very important. An attempt is made in the following exercises to provide the necessary assistance in getting the most out of the equipment.

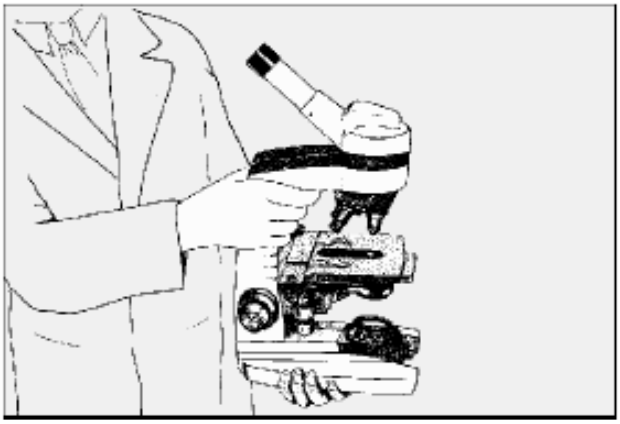
Brightfield Microscopy

A microscope that allows light rays to pass directly through to the eye without being deflected by an intervening opaque plate in the condenser is called a **brightfield microscope.** All brightfield microscopes have certain things in common, yet they differ somewhat in mechanical operation.

CARE OF THE INSTRUMENT

Microscopes represent considerable investment and can be damaged rather easily if certain precautions are not observed. The following suggestions cover most hazards.

Transport When carrying your microscope from one part of the room to another, use both hands when holding the instrument, as illustrated in the following figure. If it is carried with only one hand and allowed to dangle at your side, there is always the danger of collision with furniture or some other object. And, incidentally, under no circumstances should one attempt to carry two microscopes at one time.



The microscope should be held firmly with both hands while carrying it.

Clutter Keep your workstation uncluttered while doing microscopy. Keep unnecessary books, lunches, and other unneeded objects away from your work area. A clear work area promotes efficiency and results in fewer accidents.

Electric Cord Microscopes have been known to tumble off of tabletops when students have entangled a foot in a dangling electric cord. Don't let the light cord on your microscope dangle in such a way as to hazard foot entanglement.

Lens Care At the beginning of each laboratory period check the lenses to make sure they are clean. At the end of each lab session be sure to wipe any immersion oil off the immersion lens if it has been used.

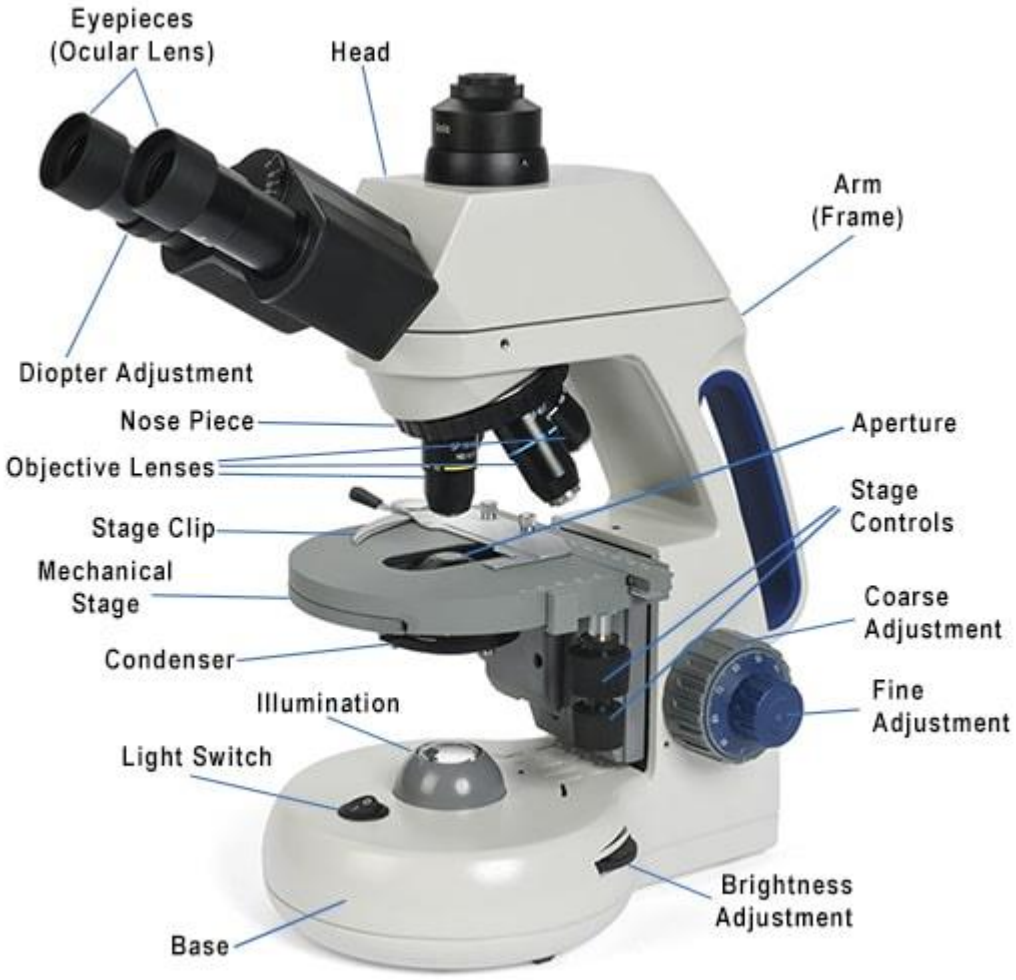
Dust Protection In most laboratories dustcovers are used to protect the instruments during storage. If one is available, place it over the microscope at the end of the period.

COMPONENTS

The principal parts of the instrument are illustrated in the following figure.

Framework All microscopes have a basic frame structure, which includes the **arm** and **base.** To this framework all other parts are attached. On many of the older microscopes the base is not rigidly attached to the arm; instead, a pivot point is present that enables one to tilt the arm backward to adjust the eyepoint height.

Stage The horizontal platform that supports the microscope slide is called the stage. Note that it has a clamping device, the **mechanical stage,** which is used for holding and moving the slide around on the stage. Note, also, the location of the **mechanical stage control** in figure

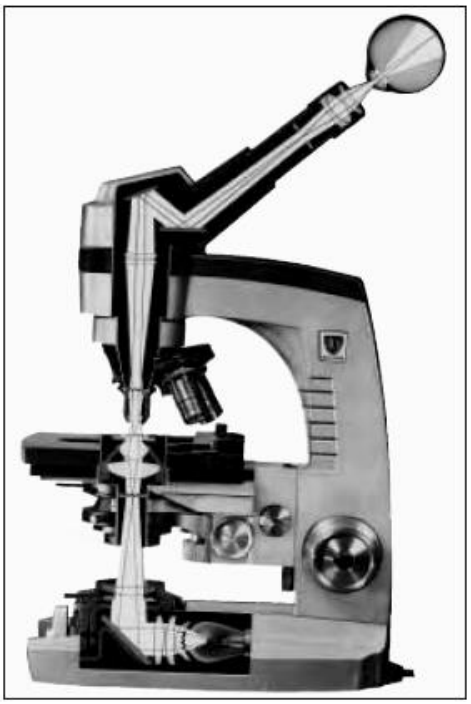


The compound microscope

Light Source In the base of most microscopes is positioned some kind of light source. Ideally, the lamp should have a **voltage control** to vary the intensity of light. The microscope in the above figure has a knurled wheel on its base to regulate the voltage supplied to the light bulb.

Most microscopes have some provision for reducing light intensity with a **neutral density filter.** Such a filter is often needed to reduce the intensity of light below the lower limit allowed by the voltage control.

Lens Systems All microscopes have three lens systems: the oculars, the objectives, and the condenser. The following Figure illustrates the light path through these three systems.



The light pathway of a microscope

The **ocular,** or eyepiece, is a complex piece, located at the top of the instrument, that consists of two or more internal lenses and usually has a magnification of 10X. Sometimes the microscope has two oculars (binocular) and others are monocular (as in the above figure).

Three or more **objectives** are usually present. Note that they are attached to a rotatable **nosepiece,** which makes it possible to move them into position over a slide. Objectives on most laboratory microscopes have magnifications of 10X, 45X, and 100X, designated as **low power, high-**

dry, and **oil immersion,** respectively. Some microscopes will have a fourth objective for rapid scanning of microscopic fields that is only 4X.

The third lens system is the **condenser,** which is located under the stage. It collects and directs the light from the lamp to the slide being studied. The condenser can be moved up and down by a knob under the stage. A **diaphragm** within the condenser regulates the amount of light that reaches the slide. Microscopes that lack a voltage control on the light source rely entirely on the diaphragm for controlling light intensity.

Focusing Knobs The concentrically arranged **coarse adjustment** and **fine adjustment knobs** on the side of the microscope are used for bringing objects into focus when studying an object on a slide. On some microscopes these knobs are not positioned concentrically. As the magnification of the lens increases, the distance between the objective lens and slide, called working distance, decreases, whereas the numerical aperture of the objective lens increases (see the following figure).

Ocular Adjustments On binocular microscopes one must be able to change the distance between the oculars and to make diopter changes for eye differences. On most microscopes the interocular distance is changed by simply pulling apart or pushing together the oculars.

RESOLUTION

The resolution limit, or **resolving power,** of a microscope lens system is a function of its numerical aperture, the wavelength of light, and the design of the condenser. The optimum resolution of the best microscopes with oil immersion lenses is around 0.2 µm. This means that two small objects that are 0.2 µm apart will be seen as separate entities; objects closer than that will be seen as a single object. To get the maximum amount of resolution from a lens system, the following factors must be taken into consideration:

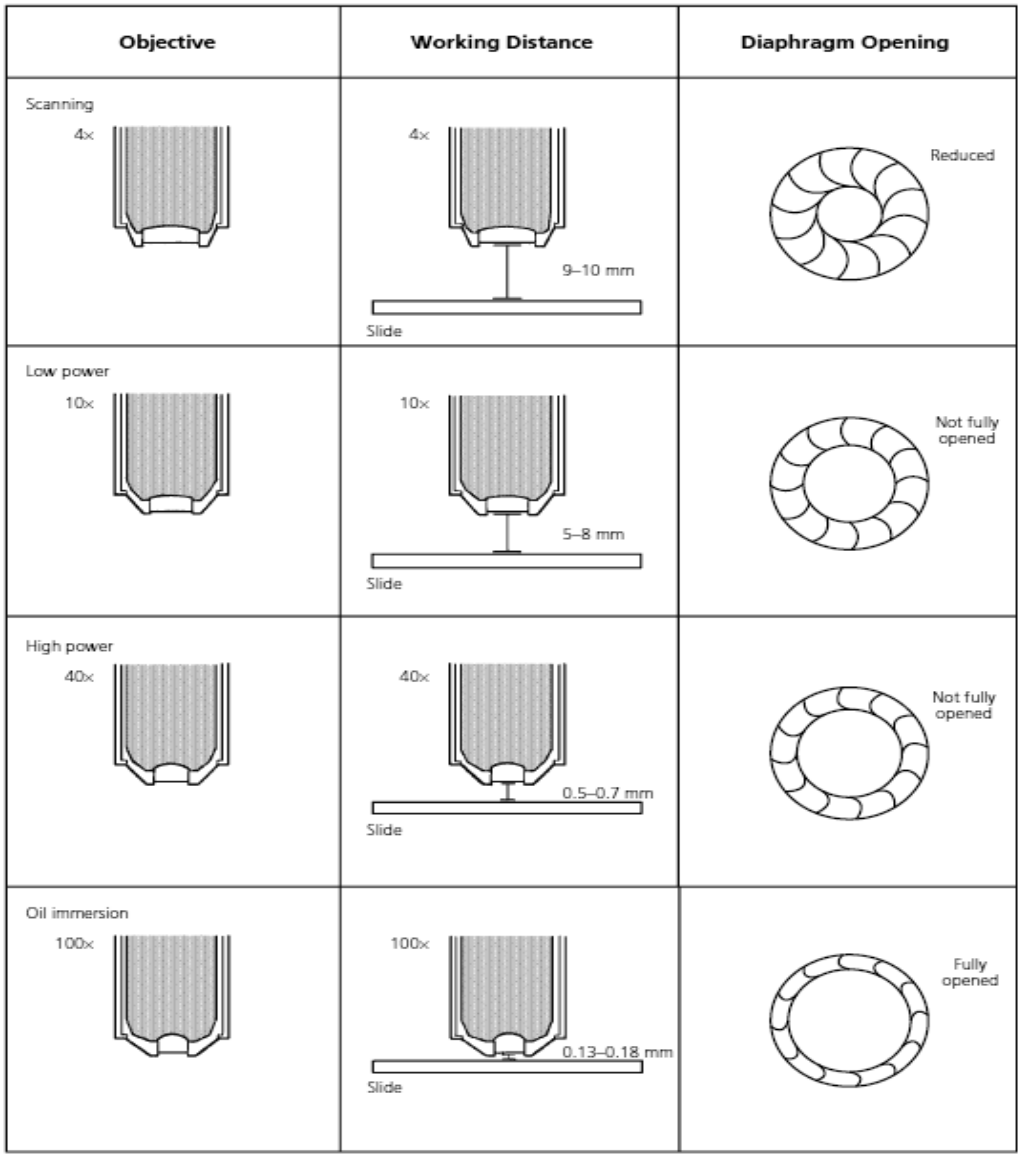
• A **blue filter** should be in place over the light source because the short wavelength of blue light provides maximum resolution.

• The **condenser** should be kept at its highest position where it allows a maximum amount of light to enter the objective.

• The **diaphragm** should not be stopped down too much. Although stopping down improves contrast, it reduces the numerical aperture.

• **Immersion oil** should be used between the slide and the 100X objective.

Of significance is the fact that increasing magnification won't increase the resolution.



Relationship between working distance, objective, and diaphragm opening

LENS CARE

Keeping the lenses of your microscope clean is a constant concern. Unless all lenses are kept free of dust, oil, and other contaminants, they are unable to achieve the degree of resolution that is intended.

Consider the following suggestions for cleaning the various lens components:

Cleaning Tissues Only lint- free, optically safe tissues should be used to clean lenses. Tissues free of abrasive grit fall in this category. Booklets of lens tissue are most widely used for this purpose. Although several types of boxed tissues are also safe, use only the type of tissue that is recommended by your instructor.

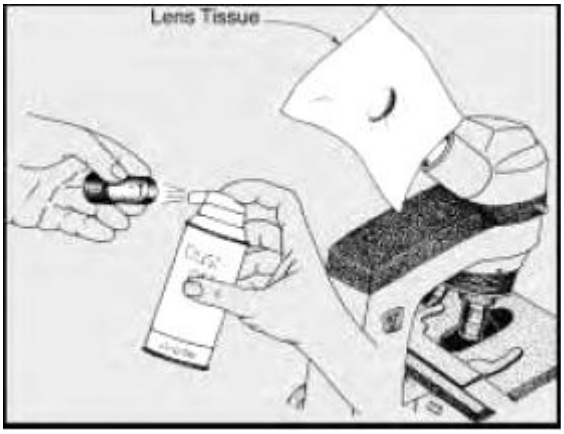
Solvents Various liquids can be used for cleaning microscope lenses. Green soap with warm water works very well. Xylene is universally acceptable. Alcohol and acetone are also recommended, but often with some reservations. Acetone is a powerful solvent that could possibly dissolve the lens mounting cement in some objective lenses if it were used too liberally. When it is used it should be used sparingly. Your instructor will inform you as to what solvents can be used on the lenses of your microscope.

Oculars The best way to determine if your eyepiece is clean is to rotate it between the thumb and forefinger as you look through the microscope. A rotating pattern will be evidence of dirt. If cleaning the top lens of the ocular with lens tissue fails to remove the debris, one should try cleaning the lower lens with lens tissue and blowing off any excess lint with an air syringe or gas cannister.

Whenever the ocular is removed from the microscope, it is imperative that a piece of lens tissue be placed over the open end of the microscope as illustrated in the following figure.

Objectives Objective lenses often become soiled by materials from slides or fingers. A piece of lens tissue moistened with green soap and water, or

one of the acceptable solvents mentioned above, will usually remove whatever is on the lens. Sometimes a cotton swab with a solvent will work better than lens tissue. At any time that the image on the slide is unclear or cloudy, assume at once that the objective you are using is soiled.



When oculars are removed for cleaning, cover the ocular opening with lens tissue. A blast from an air syringe or gas cannister removes dust and lint.

Condenser Dust often accumulates on the top surface of the condenser; thus, wiping it off occasionally with lens tissue is desirable.

PROCEDURES

If your microscope has three objectives you have three magnification options: (1) low- power, or 100X total (10x 10)

magnification, (2) high- dry magnification, which is 450X total with a 45X objective, and (3) 1000X total magnification with a 100X oil immersion objective. Note that the total magnification seen through an objective is calculated by simply multiplying the power of the ocular by the power of the objective.

Whether you use the low- power objective or the oil immersion objective will depend on how much magnification is necessary. Generally speaking, however, it is best to start with the low- power objective and progress to the higher magnifications as your study progresses. Consider the following

suggestions for setting up your microscope and making microscopic observations.

Low- Power Examination The main reason for starting with the low-power objective is to enable you to explore the slide to look for the object you are planning to study. Once you have found what you are looking for, you can proceed to higher magnifications. Use the following steps when exploring a slide with the low- power objective:

1. Position the slide on the stage with the material to be studied on the upper surface of the slide. The slide must be held in place by the mechanical stage retainer lever.

2. Turn on the light source, using a minimum amount of voltage. If necessary, reposition the slide so that the stained material on the slide is in the exact center of the light source.

3. Check the condenser to see that it has been raised to its highest point.

4. If the low- power objective is not directly over the center of the stage, rotate it into position. Be sure that as you rotate the objective into position it clicks into its locked position.

5. Turn the coarse adjustment knob to lower the objective until it stops. A built- in stop should prevent the objective from touching the slide. Be careful in this step because some microscopes has no built- in stop for lenses and this may cause permanent damage to the lens.

6. While looking down through the ocular (or oculars), bring the object into focus by turning the fine adjustment focusing knob. Don't readjust the coarse adjustment knob. If you are using a binocular microscope it will also be necessary to adjust the interocular distance and diopter adjustment to match your eyes.

7. Manipulate the diaphragm lever to reduce or increase the light intensity to produce the clearest, sharpest image. Note that as you close down the diaphragm to reduce the light intensity, the contrast improves and the

depth of field increases. Stopping down the diaphragm when using the low- power objective does not decrease resolution.

8. Once an image is visible, move the slide about to search out what you are looking for. The slide is moved by turning the knobs that move the mechanical stage.

9. Check the cleanliness of the ocular, using the procedure outlined earlier.

10. Once you have identified the structures to be studied and wish to increase the magnification, you may proceed to either high- dry or oil immersion magnification. However, before changing objectives, be sure to center the object you wish to observe.

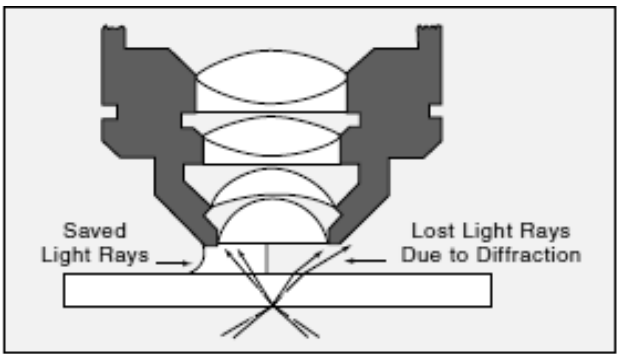
High- Dry Examination To proceed from low power to high- dry magnification, all that is necessary is to rotate the high- dry objective into position and open up the diaphragm somewhat. It may be necessary to make a minor adjustment with the fine adjustment knob to sharpen up the image, but the coarse adjustment knob should not be touched.

If a microscope is of good quality, only minor focusing adjustments are needed when changing from low power to high- dry because all the objectives will be **parfocalized.** Non parfocalized microscopes do require considerable refocusing when changing objectives. High- dry objectives should be used only on slides that have cover glasses; without them, images are usually unclear. When increasing the lighting, be sure to open up the diaphragm first instead of increasing the voltage on your lamp; reason: lamp life is greatly extended when used at low voltage. If the field is not bright enough after opening the diaphragm, feel free to increase the voltage. A final point: Keep the condenser at its highest point.

Oil Immersion Techniques The oil immersion lens derives its name from the fact that a special mineral oil is interposed between the lens and the microscope slide. The oil is used because it has the same refractive index as glass, which prevents the loss of light due to the bending of light rays as

they pass through air. The use of oil in this way enhances the resolving power of the microscope. The following Figure reveals this phenomenon.

With parfocalized objectives one can go to oil immersion from either low power or high- dry. On some microscopes, however, going from low power to high power and then to oil immersion is better.



Immersion oil, having the same refractive index as glass, prevents light loss due to diffraction.

Once the microscope has been brought into focus at one magnification, the oil immersion lens can be rotated into position without fear of striking the slide. Before rotating the oil immersion lens into position, however, a drop of immersion oil must be placed on the slide. An oil immersion lens should never be used without oil. Incidentally, if the oil appears cloudy it should be discarded.

When using the oil immersion lens it is best to open the diaphragm as much as possible. Stopping down the diaphragm tends to limit the resolving power of the optics. In addition, the condenser must be kept at its highest point. If different colored filters are available for the lamp housing, it is best to use blue or greenish filters to enhance the resolving power. Since the oil immersion lens will be used extensively in all bacteriological studies, it is of paramount importance that you learn how to use this lens properly. Using this lens takes a little practice due to the difficulties usually encountered in manipulating the lighting.

At the end of the laboratory period remove all immersion oil from the lens tip with lens tissue.

PUTTING IT AWAY

When you take a microscope from the cabinet at the beginning of the period, you expect it to be clean and in proper working condition. The next person to use the instrument after you have used it will expect the same consideration. A few moments of care at the end of the period will ensure these conditions. Check over this list of items at the end of each period before you return the microscope to the cabinet.

1. Remove the slide from the stage.

2. If immersion oil has been used, wipe it off the lens and stage with lens tissue. (Do not wipe oil off slides you wish to keep. Simply put them into a slide box and let the oil drain off.)

3. Rotate the low- power objective into position.

4. If the microscope has been inclined, return it to an erect position.

5. If the microscope has a built- in movable lamp, raise the lamp to its highest position.

6. If the microscope has a long attached electric cord, wrap it around the base or leave it as it is for the staff to do the job.

7. Adjust the mechanical stage so that it does not project too far on either side.

8. Replace the dustcover (if any).

9. If the microscope has a separate transformer, return it to its designated place.

10. Return the microscope to its correct place in the cabinet.

Part (2) Laboratory Exercises

MICROSCOPE SLIDE TECHNIQUES

(BACTERIAL MORPHOLOGY)

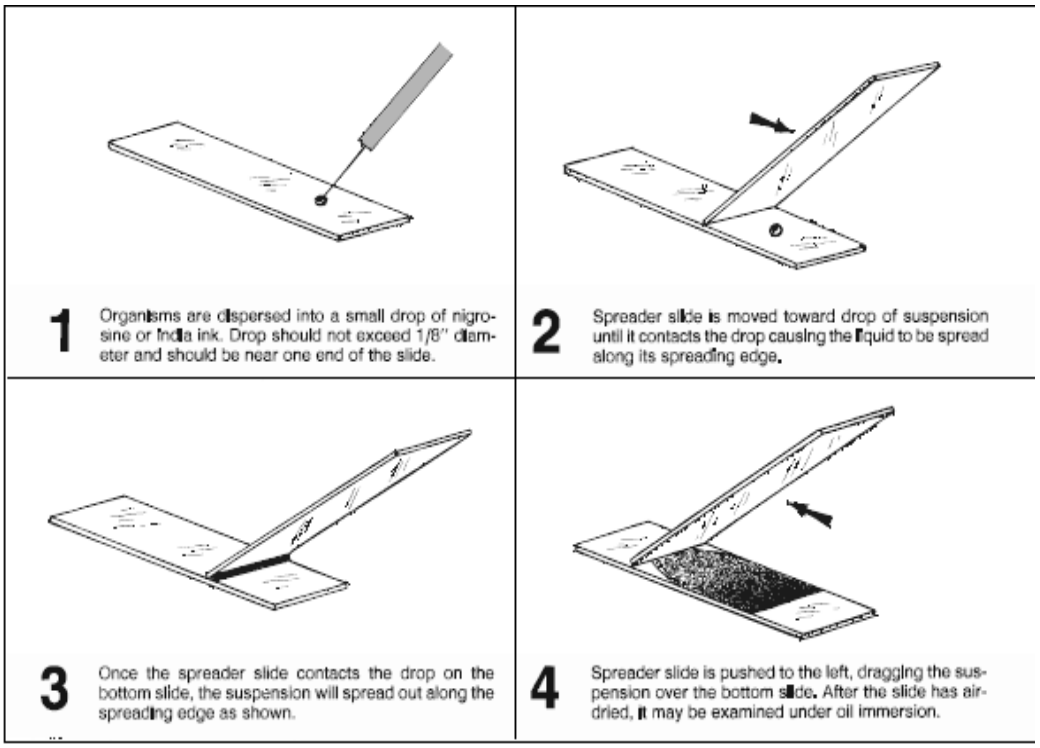
These exercises are intended to serve two important functions: (1) to help you to develop the necessary skills in making slides and (2) to introduce you to the morphology of bacteria.

Although the title of each exercise pertains to a specific technique, the organisms chosen for each method have been carefully selected so that you can learn to recognize certain morphological features. For example, in Gram staining you will observe the differences between cocci and bacilli, as well as learn how to execute the staining routine. Although one is seldom able to make species identification on the basis of morphological characteristics alone, it is a very significant starting point. This fact will become increasingly clear with subsequent experiments. Although the steps in the various staining procedures may seem relatively simple, student success is often quite unpredictable. Unless your instructor suggests a variation in the procedure, try to follow the procedures exactly as stated.

NEGATIVE STAINING

The simplest way to make a slide of bacteria is to prepare a wet mount. Although this method will quickly produce a slide, finding the bacteria on the slide may be difficult, especially for a beginner. The problem one encounters is that bacteria are quite colorless and transparent. Unless the diaphragm is carefully adjusted, the beginner usually has considerable difficulty bringing the organisms into focus. A better way to observe bacteria for the first time is to prepare a slide by a process called **negative,** or **background staining.** This method consists of mixing the microorganisms in a small amount of nigrosine or india ink and spreading the mixture over the surface of a slide. (Incidentally, nigrosine is far

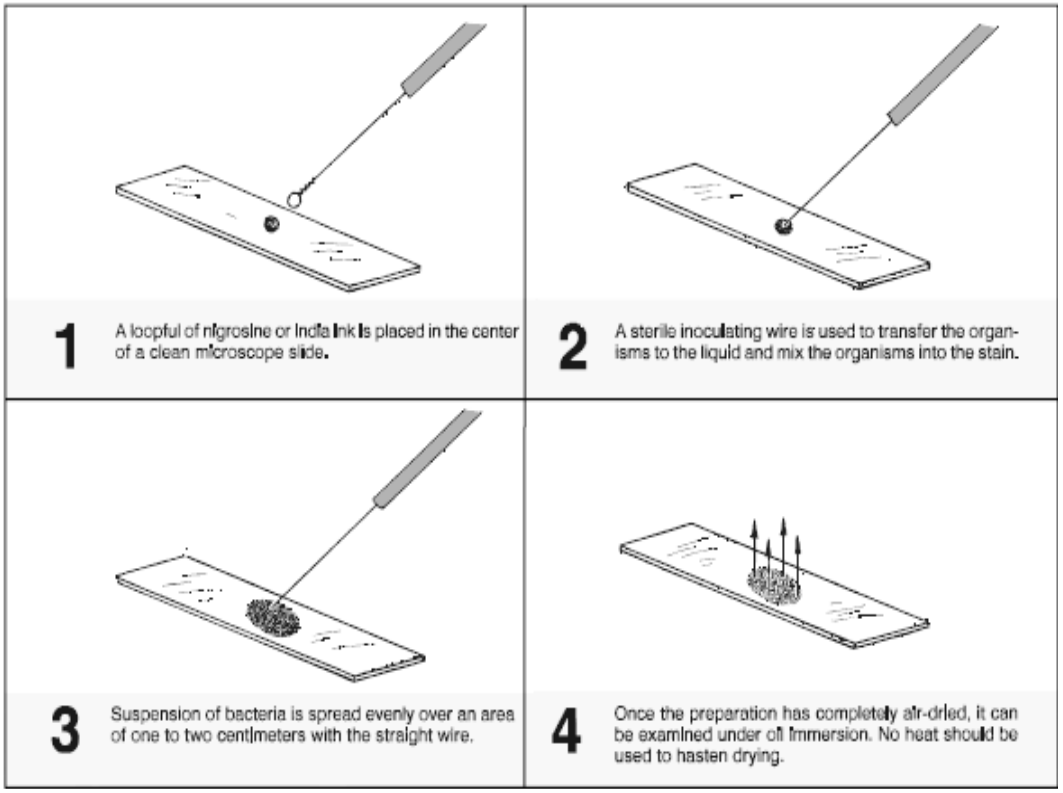
superior to india ink.) Since these two pigments are not really bacterial stains, they do not penetrate the microorganisms. Instead they stain the background, leaving the organisms transparent and visible in a darkened field. Although this technique has limitations, it can be useful for determining cell morphology and size. Since no heat is applied to the slide, there is no shrinkage of the cells, and, consequently, more accurate cell- size determinations result than with some other methods. This method is also useful for studying spirochaetes that don't stain readily with ordinary dyes.



Negative staining technique, using a spreader slide

Negative staining can be done by one of three different methods. The Figure above illustrates the more commonly used method in which the organisms are mixed in a drop of nigrosine and spread over the slide with another slide. The goal is to produce a smear that is thick at one end and feather- thin at the other end. Somewhere between the too thick and too thin areas will be an ideal spot to study the organisms.

The Figure below illustrates a second method, in which organisms are mixed in only a loopful of nigrosine instead of a full drop. In this method the organisms are spread over a smaller area in the center of the slide with an inoculating needle. No spreader slide is used in this method.



A second method for negative staining

The third procedure (Woeste- Demchick's method), which is not illustrated here, involves applying ink to a conventional smear with a black felt marking pen. If this method is used, it should be done on a smear prepared in the manner described in the next exercise. Simply put, the technique involves applying a single coat of felt- pen ink over a smear.

Note in the procedure below that slides may be made from organisms between your teeth or from specific bacterial cultures. Your instructor will indicate which method or methods you should use and demonstrate some basic aseptic techniques. Various options are provided here to ensure success.

Materials

- microscope slides (with polished edges)
- nigrosine solution or india ink
- slant cultures of *S. aureus* and *B. megaterium* (or as instructed)
- inoculating straight wire and loop
- sterile toothpicks
- Bunsen burner
- china marking pencil
- felt marking pen

Procedure

1. Swab down your tabletop with disinfectant in preparation for making slides.

2. Clean two or three microscope slides with alcohol to rid them of all dirt and grease.

3. Place the proper amount of stain on the slide (as in the figures above).

4. Oral Organisms: Remove a small amount of material from between your teeth with a sterile straight toothpick or inoculating needle and mix it into the stain on the slide. Be sure to break up any clumps of organisms with the wire or toothpick. When using a wire, be sure to flame it first to make it sterile.

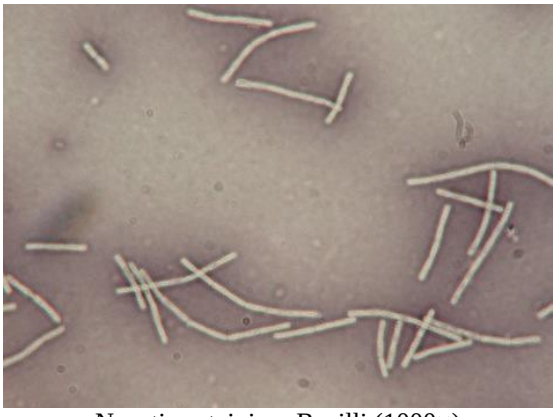
5. From Cultures: With a sterile straight wire, transfer a very small amount of bacteria from the slant to the center of the stain on the slide.

6. Spread the mixture over the slide according to the procedure used in figures above.

7. Allow the slide to air- dry and examine with an oil immersion objective

CAUTION

If you use a toothpick, discard it into a beaker of disinfectant.



Negative staining: Bacilli (1000×)

Notes and Results

(Draw and describe)

SMEAR PREPARATION

While negative staining is a simple enough process to make bacteria more visible with a brightfield microscope, it is of little help when one attempts to observe anatomical microstructures such as flagella, granules, and endospores. Only by applying specific bacteriological stains to organisms can such organelles be seen. However, success at bacterial staining depends first of all on the preparation of a suitable **smear** (thin film) of the organisms. A properly prepared bacterial smear is one that withstands one or more washings during staining without loss of organisms, is not too thick, and does not result in excessive distortion due to cell shrinkage.

The procedure for making such a smear is illustrated
in the following figure.

The first step in preparing a bacteriological smear differs according to the source of the organisms. If the bacteria are growing in a liquid medium (broths, milk, saliva, urine, etc.), one starts by placing one or two loopfuls of the liquid medium directly on the slide. From solid media such as nutrient agar, blood agar, or some part of the body, one starts by placing one or two loopfuls of water on the slide and then uses a straight inoculating wire to disperse the organisms in the water. Bacteria growing on solid media tend to cling to each other and must be dispersed sufficiently by dilution in water; unless this is done, the smear will be too thick. The most difficult concept for students to understand about making slides from solid media is that it takes only a very small amount of material to make a good smear. When your instructor demonstrates this step, pay very careful attention to the amount of material that is placed on the slide. Your instructor will indicate which cultures to use.

From liquid media (Broths, saliva, milk, etc.)

If you are preparing a bacterial smear from liquid media, follow this routine, which is depicted on the left side of the figure.

Materials

- microscope slides
- Bunsen burner
- wire loop
- marking pencil
- slide holder, optional

Procedure

1. Wash a slide with soap alcohol and hot water, removing all dirt and grease. Handle the clean slide by its edges.

2. Write the initials of the organism or organisms on the left- hand side of the slide with a china marking pencil.

3. To provide a target on which to place the organisms, make about 1 cm circle on the bottom side of the slide, centrally located, with a marking pencil. Later on, when you become more skilled, you may wish to omit the use of this "target circle."

4. Shake the culture vigorously and transfer two loopfuls of organisms to the center of the slide over the target circle. Follow the routine for inoculations. Be sure to flame the loop after it has touched the slide.

CAUTION

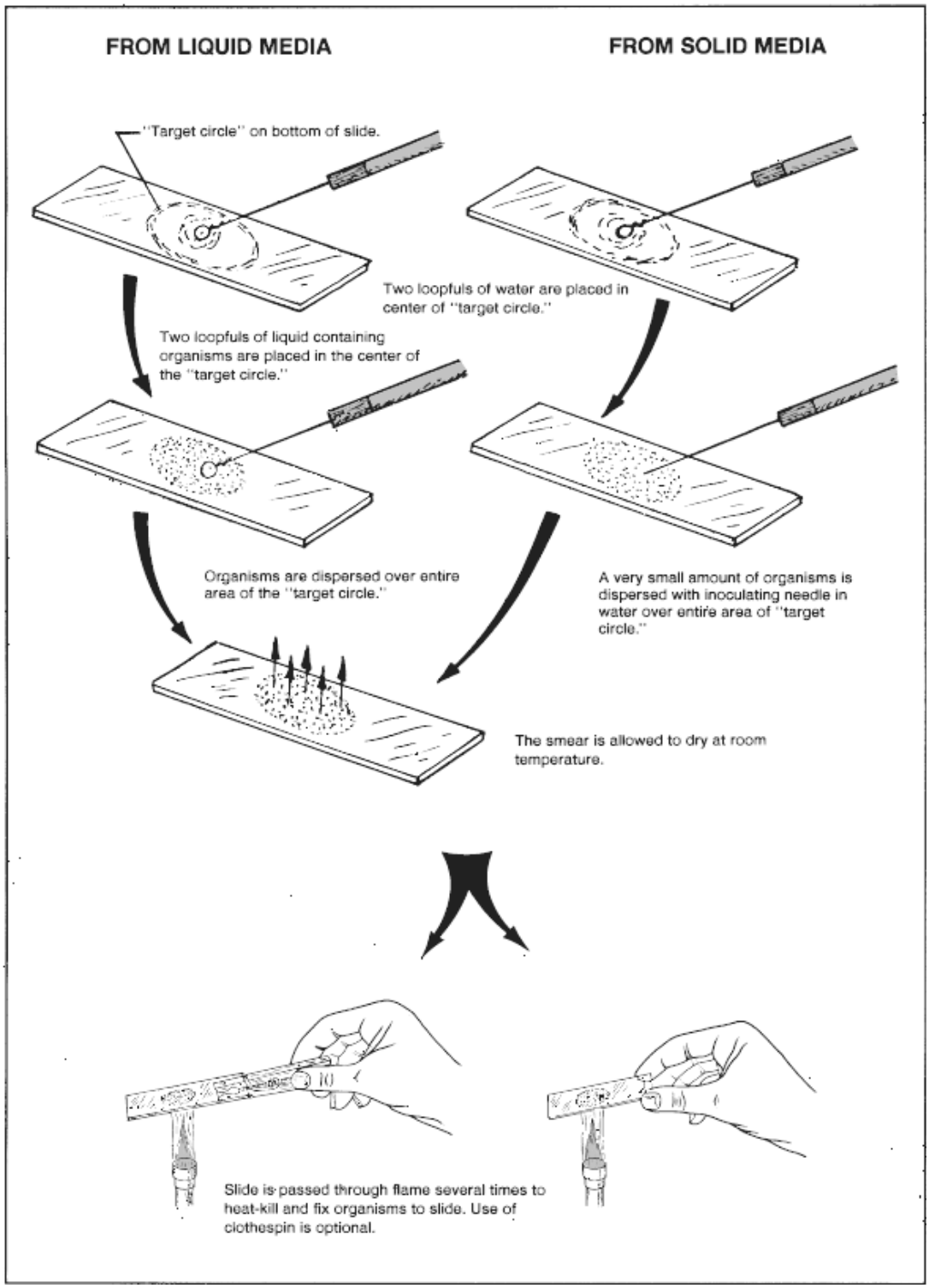
Be sure to cool the loop completely before inserting it into a medium. A loop that is too hot will spatter the medium and move bacteria into the air.

5. Spread the organisms over the area of the target circle.

6. Allow the slide to dry by normal evaporation of the water (at room temperature). Don't apply heat.

7. After the smear has become completely dry, pass the slide over a Bunsen burner flame to heat- kill the organisms and fix them to the slide.

Note that in this step one has the option of using or not using a slide holder.



Procedure for making a bacterial smear

FROM SOLID MEDIA

When preparing a bacterial smear from solid media, such as nutrient agar or a part of the body, follow this routine, which is depicted on the right side of the above figure.

Materials

- microscope slides
- inoculating needle and loop
- china marking pencil
- slide holder, optional
- Bunsen burner

Procedure

1. Wash a slide with soap or alcohol and hot water, removing all dirt and grease. Handle the clean slide by its edges.

2. Write the initials of the organism or organisms on the left- hand side of the slide with a china marking pencil.

3. Mark a "target circle" on the bottom side of the slide with a china marking pencil.

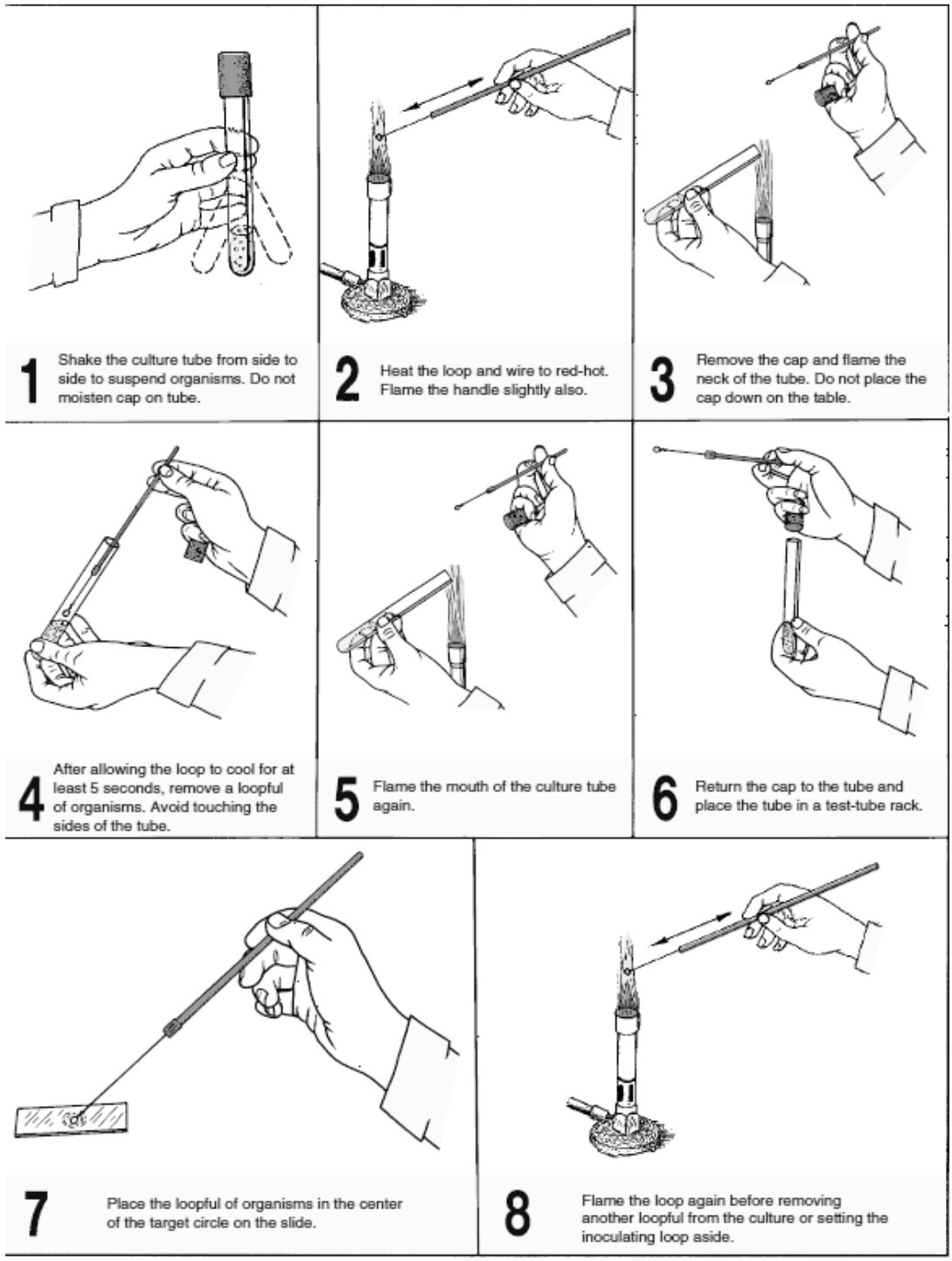
4. Flame an inoculating loop, let it cool, and transfer two loopfuls of water to the center of the target circle.

5. Flame an inoculating needle then let it cool. Pick up a very small amount of the organisms, and mix it into the water on the slide. Disperse the mixture over the area of the target circle. Be certain that the organisms have been well emulsified in the liquid. Be sure to flame the inoculating needle before placing it aside.

6. Allow the slide to dry by normal evaporation of the water. Don't apply heat.

7. Once the smear is completely dry, pass the slide over the flame of a Bunsen burner to heat- kill the organisms and fix them to the slide. Use a slide holder if it is preferred by your instructor. Some workers prefer to

hold the slide with their fingers so that they can monitor the temperature of the slide (to prevent overheating).



Aseptic procedure for organism removal

Notes and Results

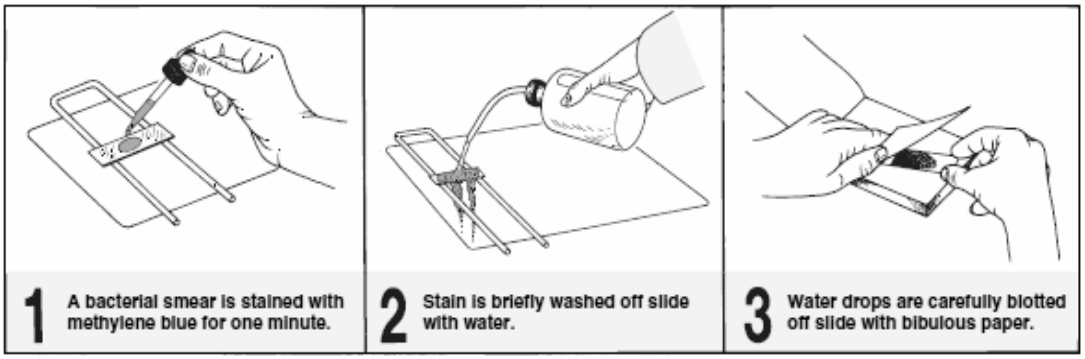
SIMPLE STAINING

The use of a single stain to color a bacterial organism is commonly referred to as **simple staining.** Some of the most commonly used dyes for simple staining are methylene blue, basic fuchsin, and crystal violet. All of these dyes work well on bacteria because they have color- bearing ions (chromophores) that are positively charged (cationic). The fact that bacteria are slightly negatively charged produces a pronounced attraction between these cationic chromophores and the organism. Such dyes are classified as **basic dyes.** Those dyes that have anionic chromophores are called **acidic dyes.** Eosin (sodium eosinate) is such a dye. The anionic chromophore, eosinate, will not stain bacteria because of the electrostatic repelling forces that are involved.

The staining times for most simple stains are relatively short, usually from 30 seconds to 2 minutes, depending on the affinity of the dye. After a smear has been stained for the required time, it is washed off gently, blotted dry, and examined directly under oil immersion. Such a slide is useful in determining basic morphology and the presence or absence of certain kinds of granules.

PROCEDURE

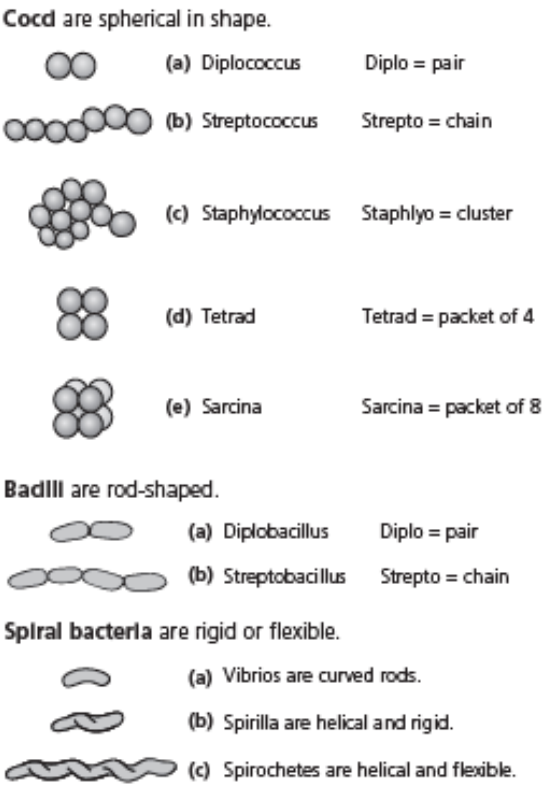
Prepare a slide of *Bacillus subtilis,* using the procedure outlined in the following figure.



Procedure for simple staining

Materials

- slant culture of *B. subtilis*
- methylene blue (Loeffler's)
- wash bottle
- bibulous paper



Bacterial shapes and arrangements

Notes and Results

CAPSULAR STAINING

Some bacterial cells are surrounded by a pronounced gelatinous or slimy layer called a **capsule.** There is considerable evidence to support the view that all bacteria have some amount of slime material surrounding their cells. In most instances, however, the layer is not of sufficient magnitude to be readily discernible. Although some capsules appear to be made of glycoprotein, others contain polypeptides. All appear to be water-soluble.

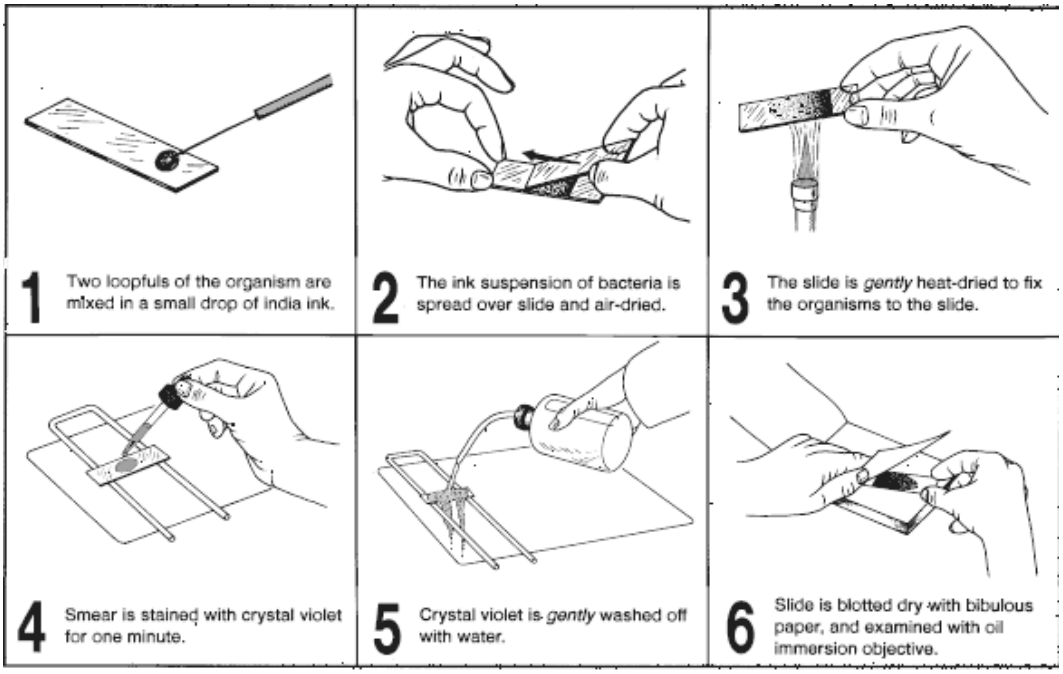
Staining the bacterial capsule cannot be accomplished by ordinary simple staining procedures. The problem with trying to stain capsules is that if you prepare a heat- fixed smear of the organism by ordinary methods, you will destroy the capsule; and, if you do not heat- fix the slide, the organism will slide off the slide during washing. In most of our bacteriological studies our principal concern is simply to demonstrate the presence or absence of a pronounced capsule. This can be easily achieved by combining negative and simple staining techniques, as in the following figure. To learn about this technique prepare a capsule "stained" slide of *Klebsiella pneumoniae* (or as instructed), using the procedure outlined in the figure.

Materials

- 36–48 hour milk culture of the used strain
- india ink
- crystal violet

Observation: Examine the slide under oil immersion and describe your results.

Note that you have always to draw the shape and arrangement of cells in each exercise.



Procedure for demonstration of capsule presence

Your Results

GRAM STAINING

In 1884 the Danish bacteriologist Christian Gram developed a staining technique that separates bacteria into two groups: those that are Gram-positive and those that are Gram- negative. The procedure is based on the ability of microorganisms to retain the purple color of crystal violet during decolorization with alcohol. Gram- negative bacteria are decolorized by the alcohol, losing the purple color of crystal violet. Gram- positive bacteria are not decolorized and remain purple. After decolorization, safranin, a red counterstain, is used to impart a pink color to the decolorized Gram- negative organisms. Note that crystal violet, the **primary stain,** causes both Gram- positive and Gram- negative organisms to become purple after 20 seconds of staining. When Gram's iodine, the **mordant,** is applied to the cells for one minute, the color of Gram-positive and Gram- negative bacteria remains the same: purple. The function of the mordant here is to combine with crystal violet to form a relatively insoluble compound in the Gram- positive bacteria. When the **decolorizing agent,** 95% ethanol, is added to the cells for 10– 20 seconds, the Gram- negative bacteria are leached colorless, but the Gram- positive bacteria remain purple. In the final step a **counterstain,** safranin, adds a pink color to the decolorized Gram- negative bacteria without affecting the color of the purple Gram-positive bacteria.

Of all the staining techniques you will use in the identification of unknown bacteria, Gram staining is, undoubtedly, the most important tool you will use. Although this technique seems quite simple, performing it with a high degree of reliability is a goal that requires some practice and experience. Here are two suggestions that can be helpful: first, don't make your smears too thick, and second, pay particular attention to the comments in step 4 on the next page that pertain to decolorization. When working with unknowns keep in mind that old cultures of Gram- positive bacteria tend

to decolorize more rapidly than young ones, causing them to appear Gram-negative instead of Gram- positive. For reliable results one should use cultures that are approximately 16 hours old. Another point to remember is that some species of *Bacillus* tend to be Gram variable i.e., sometimes positive and sometimes negative.

During this laboratory period you will be provided an opportunity to stain several different kinds of bacteria to see if you can achieve the degree of success that is required. Remember, if you don't master this technique now, you will have difficulty with your unknowns later.

Materials

- slides with heat- fixed smears
- Gram- staining kit and wash bottle
- bibulous paper

Procedure

1. Cover the smear with **crystal violet** and let stand for 20 seconds.

2. Briefly wash off the stain, using a wash bottle of distilled water. Drain off excess water (it is possible to use tap water).

3. Cover the smear with **Gram's iodine** solution and let it stand for one minute. (Your instructor may prefer only 30 seconds for this step.)

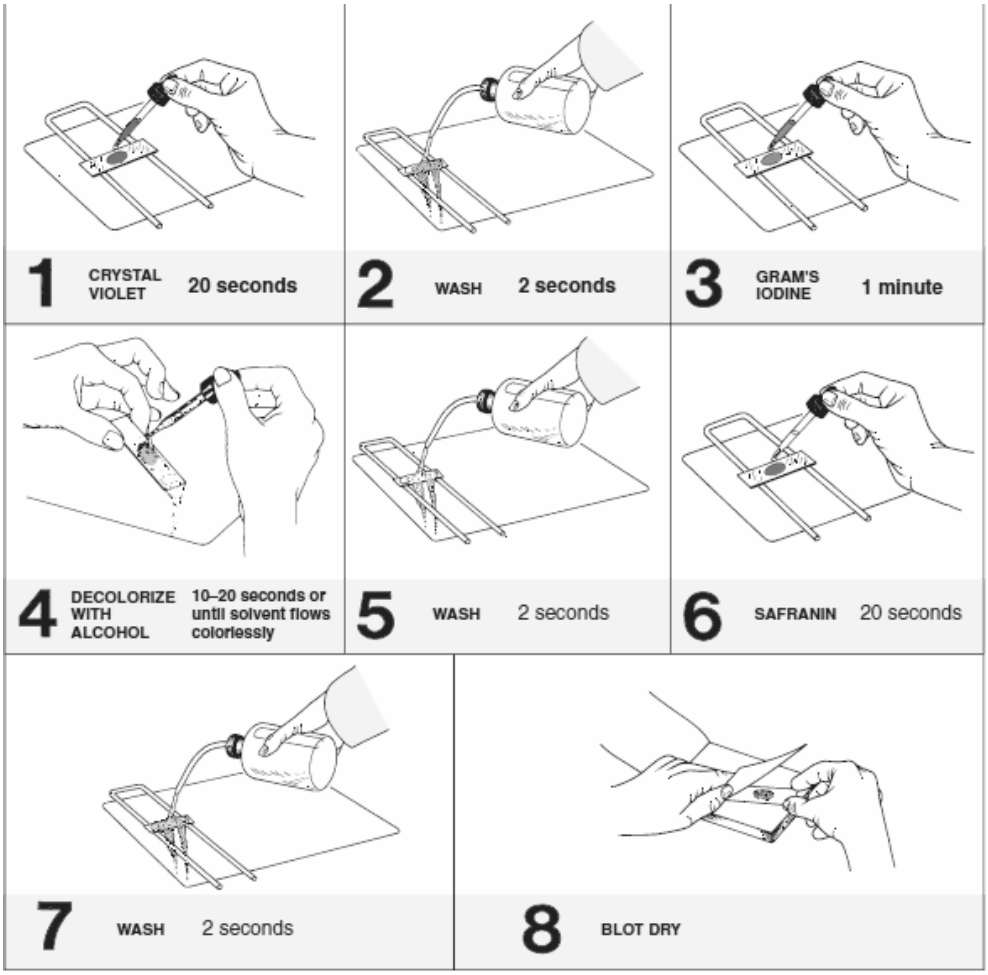
4. Pour off the Gram's iodine and flood the smear with **95% ethyl alcohol** for 10 to 20 seconds. This step is critical. Thick smears will require more time than thin ones. Decolorization has occurred when the solvent flows colorlessly from the slide.

5. Stop action of the alcohol by rinsing the slide with water for a few seconds.

6. Cover the smear with **safranin** for 20 seconds. (Some technicians prefer more time here.)

7. Wash gently (few seconds), blot dry with bibulous paper and air- dry.

8. Examine the slide under oil immersion.



The gram-staining procedure

Notes and Results

SPORE STAINING (TWO METHODS)

The genera *Bacillus* and *Clostridium*, produce extremely heat resistant structures called **endospores.** In addition to being heat- resistant, they are very resistant to many chemicals that destroy non spore forming bacteria.

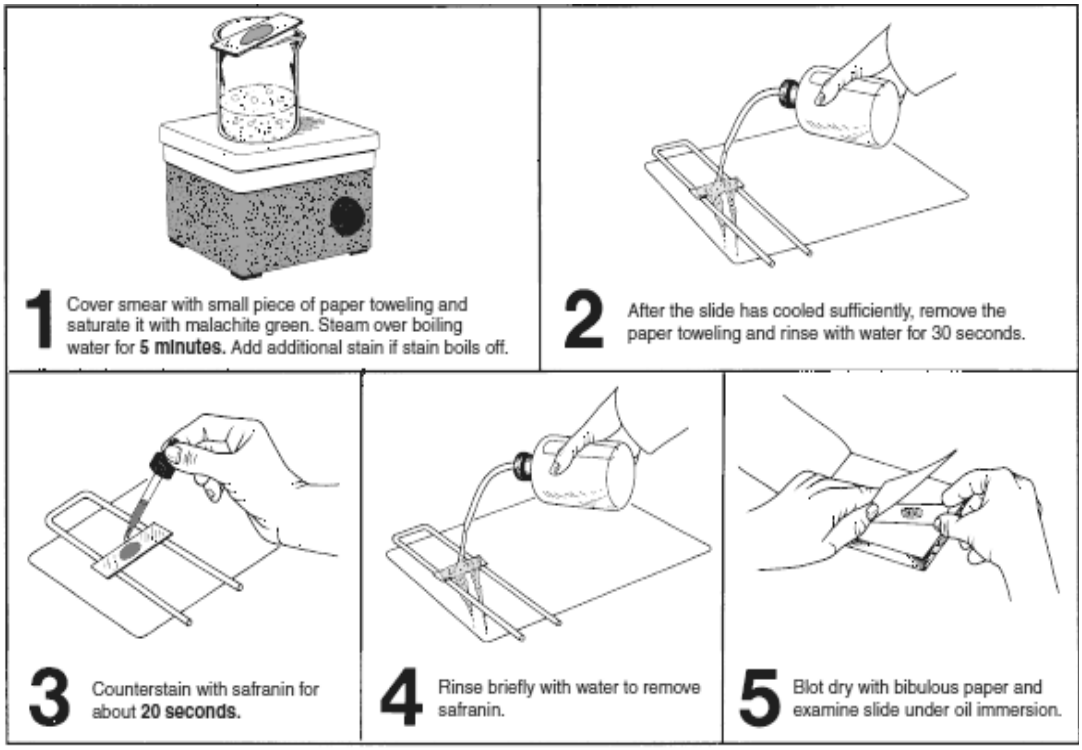
This resistance to heat and chemicals is due primarily to a thick, tough spore coat. It was observed in that Gram staining will not stain endospores. Only if considerable heat is applied to a suitable stain can the stain penetrate the spore coat. Once the stain has entered the spore, however, it is not easily removed with decolorizing agents or water. Several methods are available that employ heat to provide stain penetration. However, since the Schaeffer- Fulton and Dorner methods are the principal ones used by most bacteriologists, both have been included in this exercise. Your instructor will indicate which procedure is preferred in this laboratory.

SCHAEFFER- FULTON METHOD

This method (see the following figure) utilizes malachite green to stain the endospore and safranin to stain the vegetative portion of the cell. Utilizing this technique, a properly stained spore- former will have a green endospore contained in a pink sporangium. After preparing a smear of *Bacillus cereus,* follow the steps outlined in the figure to stain the spores.

Materials

- 24–36 hour nutrient agar slant culture of *Bacillus cereus*
- electric hot plate and small beaker (25 ml size)
- spore- staining kit consisting of a bottle each of 5% malachite green and safranin



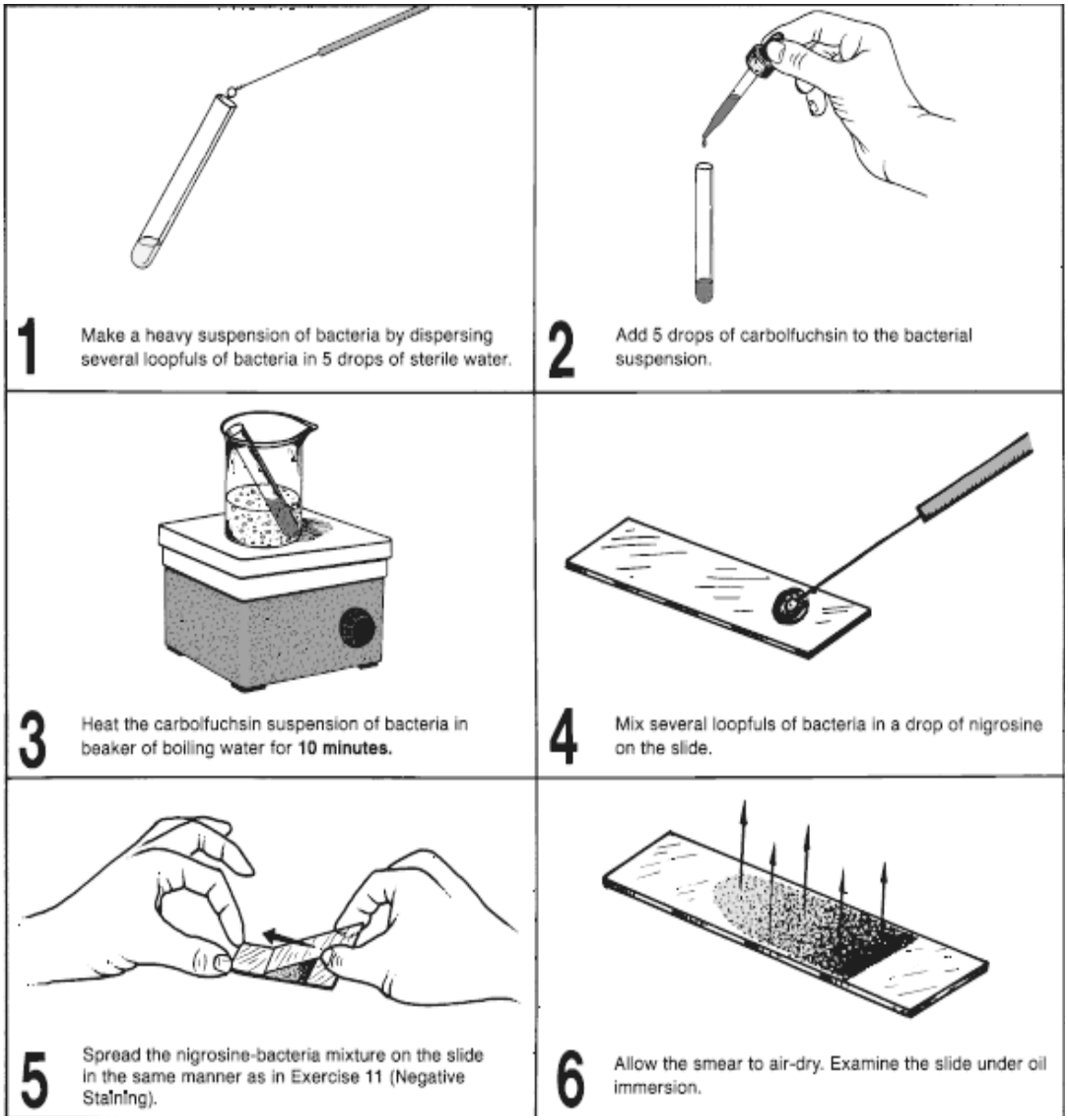
The Schaeffer-Fulton spore stain method

DORNER METHOD

The Dorner method for staining endospores produces a red spore within a colorless sporangium. Nigrosine is used to provide a dark background for contrast. The six steps involved in this technique are shown in the following figure. Although both the sporangium and endospore are stained during boiling in step 3, the sporangium is decolorized by the diffusion of safranin molecules into the nigrosine. Prepare a slide of *Bacillus cereus* that utilizes the Dorner method. Follow the steps in the figure.

Materials

- nigrosine
- electric hot plate and small beaker (25 ml size)
- small test tube
- test tube holder
- 24–36 hour nutrient agar slant culture of *Bacillus cereus*



The Dorner spore stain method

Notes and Results

ACID- FAST STAINING (Ziehl- Neelsen Method)

Most bacteria in the genus *Mycobacterium* contain considerable amounts of wax- like lipoidal material, which affects their staining properties. Unlike most other bacteria, once they are properly stained with carbol fuchsin, they resist decolorization with acid alcohol. Since they are not easily decolorized they are said to be **acid- fast.** This property sets them apart from many other bacteria. This stain is used primarily in the identification of the tuberculosis bacillus, *Mycobacterium tuberculosis,* and the leprosy organism, *Mycobacterium leprae.* After decolorization, methylene blue is added to the organisms to counterstain any material that is not acid- fast; thus, a properly stained slide of a mixture of acid- fast organisms, tissue cells, and non acid- fast bacteria will reveal red acid- fast rods with bluish tissue cells and bacteria. An example of acid- fast staining is shown in the following figure. The two organisms used in this staining exercise are *Mycobacterium smegmatis,* a nonpathogenic acid- fast rod found in soil, and *Staphylococcus aureus,* a non acid- fast coccus.

Materials

- nutrient agar slant culture of *Mycobacterium smegmatis* (48-hour culture) or as instructed.
- nutrient broth culture of *S. aureus*
- electric hot plate and small beaker
- acid- fast staining kit (carbol fuchsin, acid alcohol, and methylene blue)

Smear Preparation

Prepare a mixed culture smear by placing two loopfuls of *S. aureus* on a slide and transferring a small amount of *M. smegmatis* to the broth on the slide with an inoculating needle. Since the *M. smegmatis* bacilli are waxy and tend to cling to each other in clumps, break up the masses of

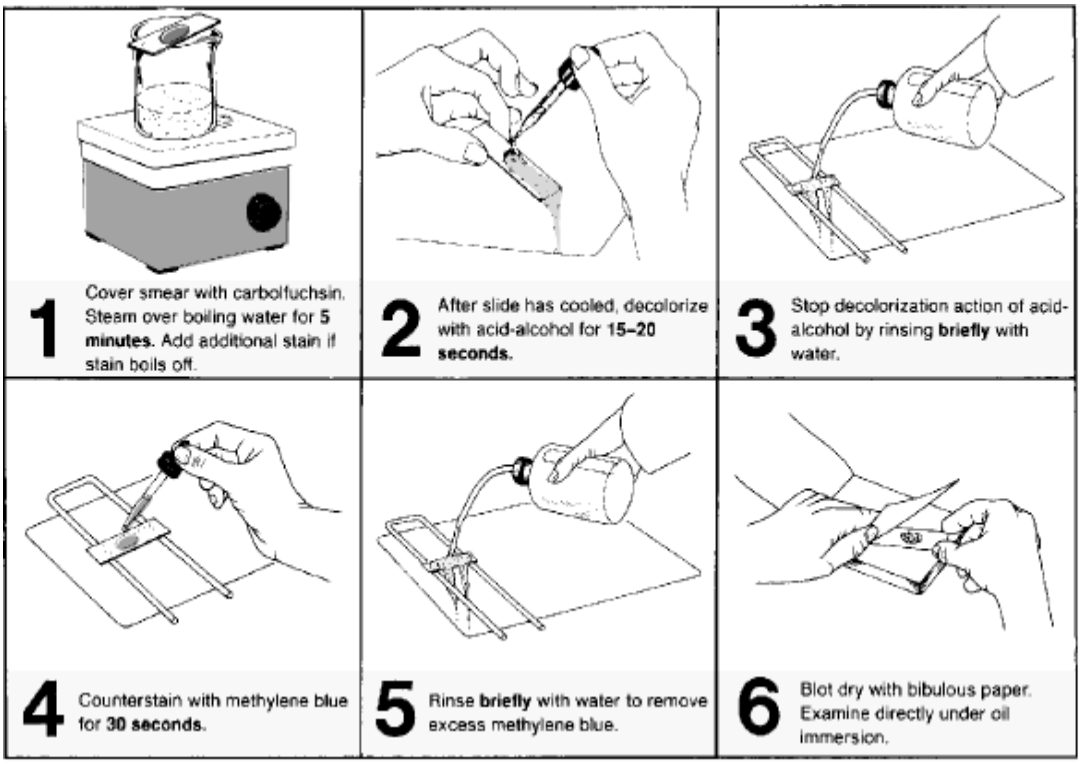
organisms with the inoculating needle. After air- drying the smear, heat-fix it.

Staining

Follow the staining procedure outlined in the figure.

Examination

Examine under oil immersion



Ziehl-Neelsen acid-fast staining procedure

Notes and Results

MOTILITY DETERMINATION

When attempting to identify an unknown bacterium it is usually necessary to determine whether the microorganism is motile. Although one might think that this determination would be easily arrived at, such is not always the case. For the beginner there are many opportunities to err.

FOUR METHODS

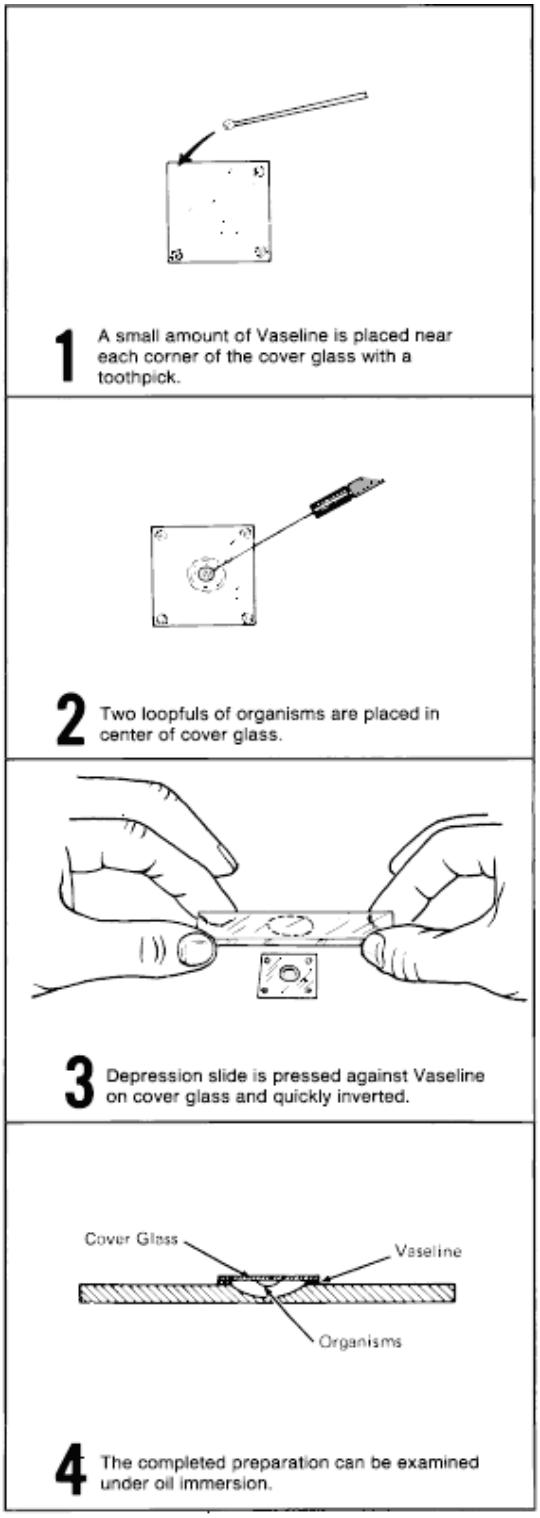
For non pathogens, there are two slide techniques that one might use. For pathogens, one tube and one Petri plate method can be used. Each method has its advantages and limitations. The method you use will depend on which one is most suitable for the situation at hand.

The Wet Mount Slide

When working with nonpathogens, the simplest way to determine motility is to place a few loopfuls of the organism on a clean slide and cover it with a cover glass. In addition to being able to determine the presence or absence of motility, this method is useful in determining cellular shape (rod, coccus, or spiral) and arrangement (irregular clusters, packets, pairs, or long chains). A wet mount is especially useful if **phase optics** are used. Unlike stained slides that are heat- fixed for staining, there is no distortion of cells on a wet mount. One problem for beginners is the difficulty of being able to see the organisms on the slide. Since bacteria are generally colorless and very transparent, the novice has to learn how to bring them into focus.

The Hanging Drop Slide

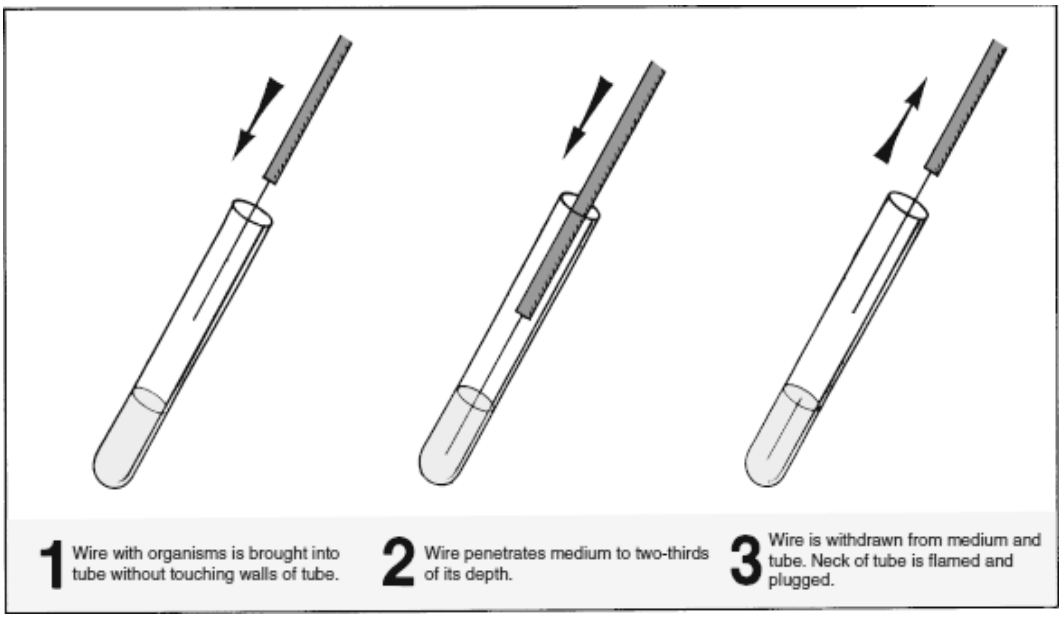
If it is necessary to study viable organisms on a microscope slide for a longer period of time than is possible with a wet mount, one can resort to a hanging drop slide. As shown in the following figure, organisms are observed in a drop that is suspended under a cover glass in a concave depression slide. Since the drop lies within an enclosed glass chamber, drying out occurs very slowly.



The hanging drop slide

Tube Method

When working with pathogenic microorganisms such as the typhoid bacillus, it is too dangerous to attempt to determine motility with slide techniques. A much safer method is to culture the organisms in a special medium that can demonstrate the presence of motility. The procedure is to inoculate a tube of semisolid medium that can demonstrate the presence of motility. The used medium has a very soft consistency that allows motile bacteria to migrate readily through it causing cloudiness. The following Figure illustrates the inoculation procedure.



Stab technique for motility test

Soft Agar Plate Method

Although the tube method is the generally accepted procedure for determining motility of pathogens, it is often very difficult for beginners to interpret. Richard Roller at the University of Iowa suggests that incubating a Petri plate of soft agar that has been stab inoculated with a motile organism will show up motility more clearly than an inoculated tube.

FIRST PERIOD

Tube Method

Inoculate tubes of semisolid medium with *M. luteus* and *E. coli* according to the following instructions:

1. Label the tubes of semisolid medium with the names of the organisms. Place your initials on the tubes, also.

2. Flame and cool the inoculating needle, and insert it into the culture after flaming the neck of the tube.

3. Remove the cap from the tube of medium, flame the neck, and stab it 2⁄3 of the way down to the bottom, as shown in the figure. Flame the neck of the tube again before returning the cap to the tube.

4. Repeat steps 2 and 3 for the other culture.

5. Incubate the tubes at room temperature for 24 to 48 hours.

Plate Method

Mark the bottom of a plate of soft agar with two one- half inch circles about one inch apart. Label one circle ML and the other EC. These circles will be targets for your culture stabs. Put your initials on the plate also. Using proper aseptic techniques, stab the medium in the center of the ML circle with *M. luteus* and the center of the other circle with *E. coli.* Incubate the plate for 24 to 48 hours at room temperature.

SECOND PERIOD

Assemble the following materials:

- culture tubes of motility medium that have been incubated
- inoculated Petri plate that has been incubated
- Compare the two tubes that were inoculated with *M. luteus* and *E. coli.* Look for cloudiness as evidence of motility.
- Does the plate method provide any better differentiation of results than the tube method?

Notes and Results

PURE CULTURE TECHNIQUES

When we try to study the bacterial flora of the body, soil, water, food, or any other part of our environment, we soon discover that bacteria exist in mixed populations. It is only in very rare situations that they occur as a single species. To be able to study the cultural, morphological, and physiological characteristics of an individual species, it is essential, first of all, that the organism be separated from the other species that are normally found in its habitat; in other words, we must have a **pure culture** of the microorganism.

Several different methods of getting a pure culture from a mixed culture are available to us. The two most frequently used methods involve making a streak plate or a pour plate. Both plate techniques involve thinning the organisms so that the individual species can be selected from the others. In this exercise you will have an opportunity to use both methods in an attempt to separate three distinct species from a tube that contains a mixture. The principal difference between the three organisms will be their colors: *Serratia marcescens* is red, *Micrococcus luteus* is yellow, and *Escherichia coli* is white.

STREAK PLATE METHOD

For economy of materials and time, this method is best. It requires a certain amount of skill, however, which is forthcoming with experience. A properly executed streak plate will give as good an isolation as is desired for most work. The following Figure illustrates how colonies of a mixed culture should be spread out on a properly made streak plate. The important thing is to produce good spacing between colonies.

Materials

- electric hot plate
- Bunsen burner and beaker of water
- wire loop, thermometer, and china marking pencil

- 1 nutrient agar pour and 1 sterile Petri plate
- 1 mixed culture of *Serratia marcescens, Escherichia coli,* and *Micrococcus luteus*

If your streak plate reveals well- isolated colonies you will have a plate suitable for subculturing.

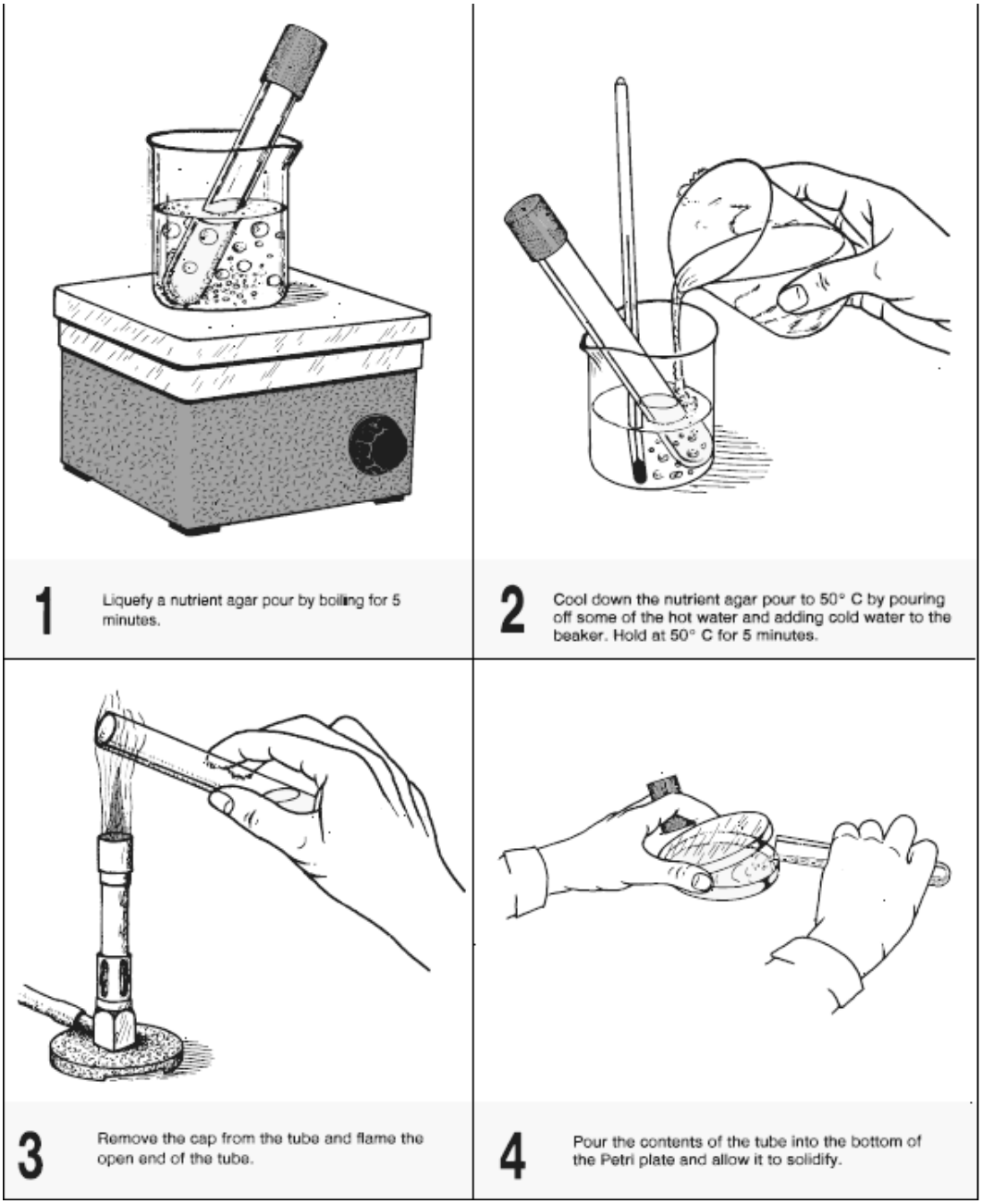
Procedure

1. Prepare your tabletop by disinfecting its surface with the disinfectant that is available in the laboratory. Use a sponge to scrub it clean.

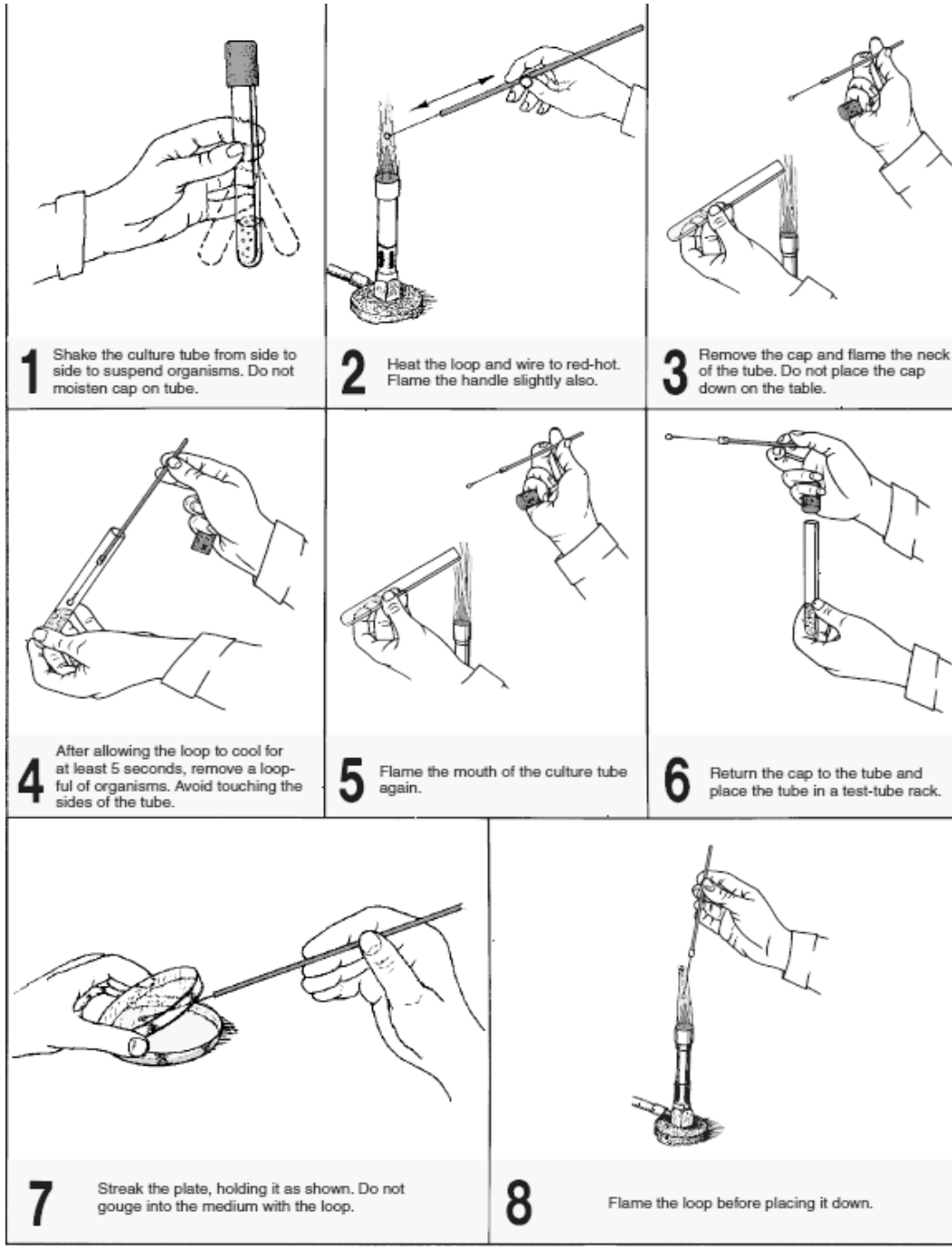
2. Label the bottom surface of a sterile Petri plate with your name and date. Use a china marking pencil.

3. Liquefy a tube of nutrient agar, cool to 50° C, and pour the medium into the bottom of the plate, following the procedure illustrated in the following figure. Be sure to flame the neck of the tube prior to pouring to destroy any bacteria around the end of the tube. After pouring the medium into the plate, gently rotate the plate so that it becomes evenly distributed, but do not splash any medium up over the sides. Agar- agar, the solidifying agent in this medium becomes liquid when boiled and resolidifies at around 42° C. Failure to cool it prior to pouring into the plate will result in condensation of moisture on the cover. Any moisture on the cover is undesirable because if it drops down on the colonies, the

organisms of one colony can spread to other colonies, defeating the entire isolation technique.



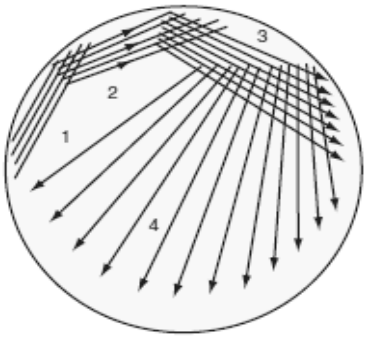
Procedure for pouring an agar plate for streaking



Routine for inoculating a Petri plate

4. Streak the plate by one of the methods shown in the following figure. Your instructor will indicate which technique you should use.

Caution: Be sure to follow the routine in the figure for getting the organism out of culture.



QUADRANT STREAK

(Method A)

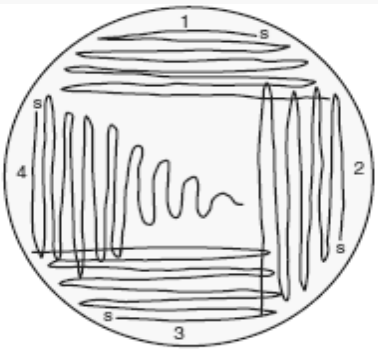
1. Streak one loopful of organisms over Area 1 near edge of the plate. Apply the loop lightly. Don't gouge into the medium.

2. Flame the loop, cool 5 seconds, and make 5 of 6 streaks from Area 1 through Area 2. Momentarily touching the loop to a sterile area of the medium before streaking insures a cool loop.

3. Flame the loop again, cool it, and make 6 or 7 streaks from Area 2 through Area 3.

4. Flame the loop again and make as many streaks as possible from Area 3 into Area 4, using up the remainder of the plate surface.

5. Flame the loop before putting it aside.



QUADRANT STREAK

(Method B)

1. Streak one loopful of organisms back and forth over Area 1, starting at point designated by "s". Apply loop lightly. Don't gouge into the medium.

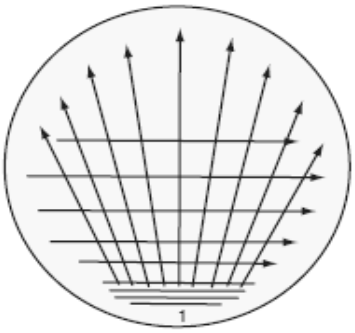
2. Flame the loop, cool 5 seconds and touch the medium in sterile area momentarily to insure coolness.

3. Rotate the dish 90 degrees while keeping the dish closed. Streak Area 2 with several back and forth strokes, hitting the original streak a few times.

4. Flame the loop again. Rotate the dish and streak Area 3 several times, hitting last area several times.

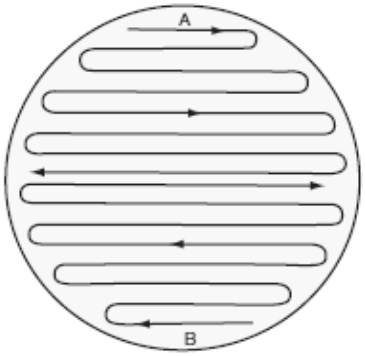
5. Flame the loop, cool it, and rotate the dish 90 degrees again. Streak Area 4, contacting Area 3 several times and drag out the culture as illustrated.

6. Flame the loop before putting it aside.



RADIANT STREAK

1. Spread a loopful of organisms in small area near the edge of the plate in Area 1. Don't gouge medium.
2. Flame the loop and allow it to cool for 5 seconds. Touching a sterile area of the medium will insure coolness.
3. **From the edge** of Area 1 make 7 or 8 straight streaks to the opposite side of the plate.
4. Flame the loop again, cool it sufficiently, and cross streak over the last streaks, **starting near Area 1.**
5. Flame the loop again before putting it aside.



CONTINUOUS STREAK

1. Starting at the edge of the plate (Area A) with a loopful of organisms, spread the organisms in a single continuous movement to the center of the plate. Use light pressure and avoid gouging the medium.
2. Rotate the plate 180 degrees so that the uninoculated portion of the plate is away from you.
3. Without flaming loop, and using the same face of the loop, continue streaking the other half of the plate by starting at Area B and working toward the center.
4. Flame your loop before putting it aside.

5. Incubate the plate in an inverted position at 25° C for 24– 48 hours. By incubating plates upside down, the problem of moisture on the cover is minimized.

POUR PLATE METHOD (Loop Dilution)

This method of separating one species of bacteria from another consists of diluting out one loopful of organisms with three tubes of liquefied nutrient

agar in such a manner that one of the plates poured will have an optimum number of organisms to provide good isolation. The following Figure illustrates the general procedure.

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. Proceed as follows to make three dilution pour plates, using the same mixed culture for streak plate.

Materials

- mixed culture of bacteria
- 3 nutrient agar pours
- 3 sterile Petri plates
- electric hot plate
- beaker of water
- thermometer
- inoculating loop and china marking pencil

Procedure

1. Label the three nutrient agar pours **I, II,** and **III** with a marking pencil and place them in a beaker of water on an electric hot plate to be liquefied. To save time, start with hot tap water if it is available.

2. While the tubes of media are being heated, label the bottoms of the three Petri plates **I, II,** and **III.**

3. Cool down the tubes of media to 50° C, using the same method that was used for the streak plate.

4. Following the routine in the figure, inoculate tube I with one loopful of organisms from the mixed culture. Note the sequence and manner of handling the tubes in the figure.

5. Inoculate tube II with one loopful from tube I after thoroughly mixing the organisms in tube I by shaking the tube from side to side or by rolling

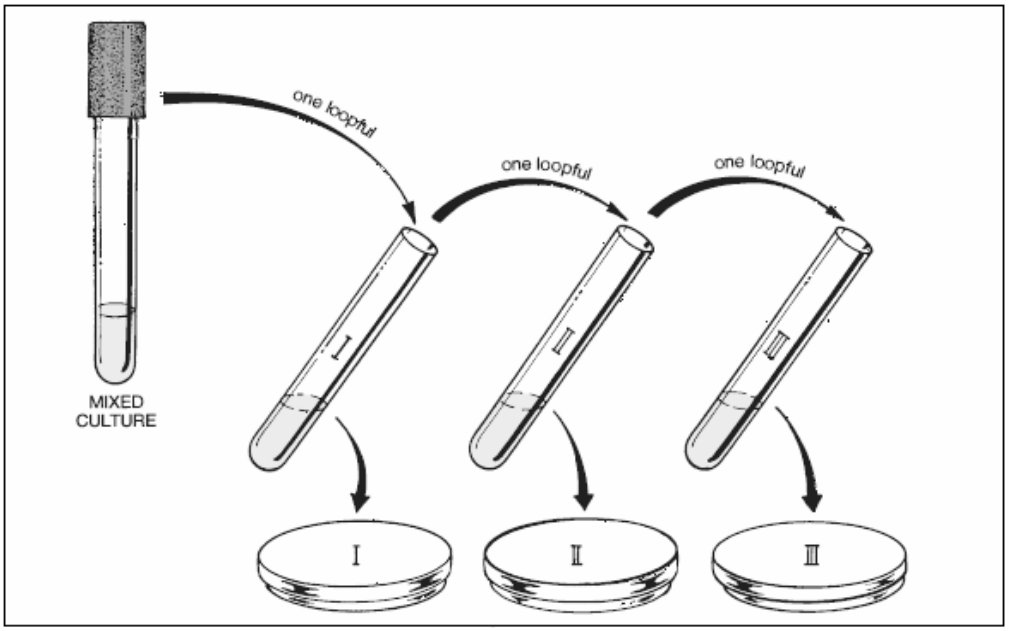
the tube vigorously between the palms of both hands. **Do not splash any of the medium up onto the tube closure.** Return tube I to the water bath.

6. Agitate tube II to completely disperse the organisms and inoculate tube III with one loopful from tube II. Return tube II to the water bath.

7. Agitate tube III, flame its neck, and pour its contents into plate III.

8. Flame the necks of tubes I and II and pour their contents into their respective plates.

9. After the medium has completely solidified, incubate the inverted plates at 25° C for 24–48 hours.



Three steps in the loop dilution technique for separating out organisms

EVALUATION OF THE TWO METHODS

Examine all four Petri plates after 24 to 48 hours of incubation. Look for colonies that are well isolated from the others. Note how crowded the colonies appear on plate I as compared with plates II and III. Plate I will be unusable. Either plate II or III will have the most favorable isolation of colonies. Can you pick out three well- isolated colonies that are white, yellow, and red? Draw the appearance of your streak plate and pour plates.

Notes and Results

BACTERIAL POPULATION COUNTS

Many bacteriological studies require that we be able to determine the number of organisms that are present in a given unit of volume. Several different methods are available to us for such population counts. The method one uses is determined by the purpose of the study. To get by with a minimum of equipment, it is possible to do a population count by diluting out the organisms and counting the organisms in a number of microscopic fields on a slide. Direct examination of milk samples with this technique can be performed very quickly, and the results obtained are quite reliable. A technique similar to this can be performed on a Petrof-Hauser counting chamber.

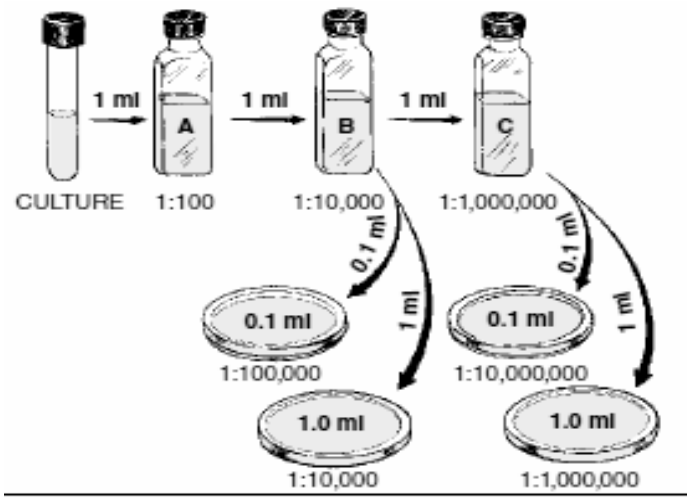
Bacterial counts of gas- forming bacteria can be made by inoculating a series of tubes of lactose broth and using statistical probability tables to estimate bacterial numbers. This method, used to estimate numbers of coliform bacteria in water samples, is easy to use, works well in water testing, but is limited to water, milk, and food testing.

In this exercise we will use **quantitative plating** (Standard Plate Count, or SPC) and **turbidity measurements** to determine the number of bacteria in a culture sample. Although the two methods are somewhat parallel in the results they yield, there are distinct differences. For one thing, the SPC reveals information only as related to viable organisms; that is, colonies that are seen on the plates after incubation represent only living organisms, not dead ones. Turbidimetry results, on the other hand, reflect the presence of all organisms in a culture, dead and living.

Quantitative plating method (Standard Plate Count)

In determining the number of organisms present in water, milk, and food, the **standard plate count** (SPC) is universally used. It is relatively easy to perform and gives excellent results. We can also use this basic technique to calculate the number of organisms in a bacterial culture. It is in this

respect that this assignment is set up. The procedure consists of diluting the organisms with a series of sterile water blanks as illustrated in the following figure. Generally, only three bottles are needed, but more could be used if necessary. By using the dilution procedure indicated here, a final dilution of 1:1,000,000 occurs in blank C. From blanks B and C, measured amounts of the diluted organisms are transferred into empty Petri plates. Nutrient agar, cooled to 50° C, is then poured into each plate. After the nutrient agar has solidified, the plates are incubated for 24 to 48 hours and examined. A plate that has between 30 and 300 colonies is selected for counting. From the count it is a simple matter to calculate the number of organisms per milliliter of the original culture. It should be pointed out that greater accuracy can be achieved by pouring two plates for each dilution and averaging the counts. Duplicate plating, however, has been avoided for obvious economic reasons.



Quantitative plating procedure

Notes and Results

Diluting and Plating Procedure

Proceed as follows to dilute out a culture of *E. coli* and pour four plates.

Materials

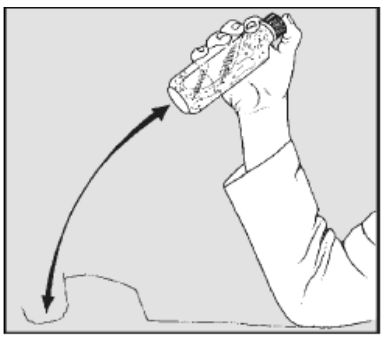
- 1 bottle (40 ml) broth culture of *E. coli*
- 1 bottle (80 ml) nutrient agar
- 4 Petri plates
- 1 pipettes 1 ml
- 3 sterile 99 ml water blanks
- cannister for discarded pipettes

procedure

1. Liquefy a bottle of nutrient agar. While it is being heated, label three 99 ml sterile water blanks **A, B,** and **C.** Also, label the four Petri plates **1:10,000, 1:100,000, 1:1,000,000,** and **1:10,000,000.** In addition, indicate with labels the amount to be pipetted into each plate (**0.1 ml** or **1.0 ml**).

2. Shake the culture of *E. coli* and transfer 1 ml of the organisms to blank A, using a sterile 1 ml pipette. After using the pipette, place it in the discard cannister.

3. Shake blank A 25 times in an arc of 1 foot for 7 seconds with your elbow on the table. Forceful shaking not only brings about good distribution, but it also breaks up clumps of bacteria.



Standard procedure for shaking water blanks requires elbow to remain fixed on table

4. With a different 1 ml pipette, transfer 1 ml from blank A to blank B.

5. Shake water blank B 25 times in same manner.

6. With another sterile pipette, transfer 0.1 ml from blank B to the 1:100,000 plate and 1.0 ml to the 1:10,000 plate. With the same pipette, transfer 1.0 ml to blank C.

7. Shake blank C 25 times.

8. With another sterile pipette, transfer from blank C 0.1 ml to the 1:10,000,000 plate and 1.0 ml to the 1:1,000,000 plate.

9. After the bottle of nutrient agar has boiled for 8 minutes, cool it down in a water bath at 50° C for **at least 10 minutes.**

10. Pour one- fourth of the nutrient agar (20 ml) into each of 4 plates. Rotate the plates **gently** to get adequate mixing of medium and organisms. **This step is critical!** Too little action will result in poor dispersion and too much action may slop inoculated medium over the edge.

11. After the medium has cooled completely, incubate at 35° C for 48 hours, inverted.

Counting and Calculations

Materials

- 4 culture plates
- colony counter
- mechanical hand counter
- felt pen (optional)

Procedure

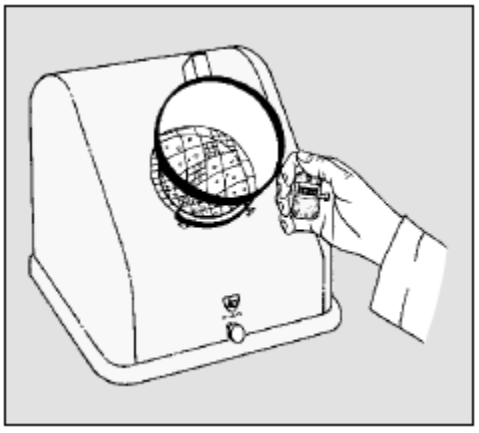
1. Lay out the plates on the table in order of dilution and compare them. Select the plates that have no fewer than 30 nor more than 300 colonies for your count. Plates with less than 30 or more than 300 colonies are statistically unreliable.

2. Place the plate on the colony counter with the lid removed. Start counting at the top of the plate, using the grid lines to prevent counting the same colony twice. Use a mechanical hand counter. Count every colony,

regardless of how small or insignificant and record your counts. **Alternative Counting Method:** Another way to do the count is to remove the lid and place the plate upside down on the colony counter. Instead of using the grid to keep track, use a felt pen to mark off each colony as you do the count.

3. Calculate the number of bacteria per ml of undiluted culture using the data recorded above. Multiply the number of colonies counted by the dilution factor (the reciprocal of the dilution).

Example: If you counted 220 colonies on the plate that received 1.0 ml of the 1:1,000,000 dilution: 220 x 1,000,000 (or 2.2 x 10^8) bacteria per ml. If 220 colonies were counted on the plate that received 0.1 ml of the 1:1,000,000 dilution, then the above results would be multiplied by 10 to convert from number of bacteria per 0.1 ml to number of bacteria per 1.0 ml (2,200,000,000, or 2.2 x 10^9). If the number of bacteria per ml was calculated to be 227,000,000, it should be recorded as 230,000,000, or 2.3 x 10^8.



Colony counts are made on a colony counter, using a mechanical hand tally

Notes and Results

ISOLATION OF ANAEROBIC PHOTOTROPHIC BACTERIA (the Winogradsky Column method)

The culture of photosynthetic bacteria requires special culture methods to promote their growth. These prokaryotes contain photopigments, such as chlorophyll and carotenoids, which convert solar energy into cellular constituents. There are two groups of phototrophic bacteria: (1) the aerobic phototrophic **cyanobacteria,** and (2) the anaerobic phototrophic bacteria, which include the **purple** and **green bacteria.** It is this latter group that will be studied in this exercise. The cyanobacteria contain chlorophyll *a,* carotenoids, and phycobilisomes. The nonchlorophyll pigments in this group are accessory pigments for capturing light. They resemble higher plants in that they split water for a source of reducing power and evolve oxygen in the process. The anaerobic phototrophic bacteria, on the other hand, differ in that they contain bacteriochlorophyll, which is chemically distinct from chlorophyll. Instead of utilizing water as a source of reducing power, the purple and green bacteria use sulfide or organic acids for the reduction of carbon dioxide. The purple bacteria that utilize organic acids instead of sulfide are essentially photoheterotrophic since they derive their carbon from organic acids rather than carbon dioxide. These bacteria are ubiquitous in the sediment of ditches, ponds, and lakes: i.e., mostly everywhere that freshwater lies relatively stagnant for long periods of time and subject to sunlight. In this environment, fermentation processes produce the sulfides and organic acids that are essential to their existence.

Characterization

According to *Bergey's Manual* (Section 18, Vol. 3), there are approximately 30 genera of anaerobic phototrophic bacteria. The purple bacteria belong to the family *Chromatiaceae.* The green ones are in the

family *Chlorobiaceae.* The morphological, cultural, and physiological differences between the purple and green sulfur bacteria are as follows:

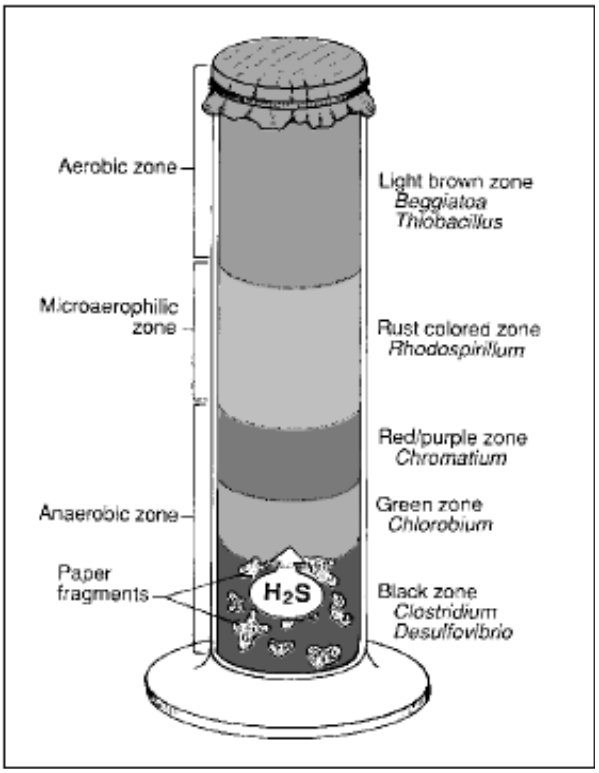
Purple Sulfur Bacteria Members of this group are all Gram negative, straight or slightly curved rods that are motile with polar flagellation. Colors of the various genera vary considerably—from orange- brown to brown, brownish- red to pink, and purple- red to purple- violet. Color variability is due to the blend of bacteriochlorophyll with the type of carotenoid present. All species contain elemental-sulfur internally in the form of globules. Some species are able to fix nitrogen. Sulfides are required as electron donors; bicarbonate, acetate, and pyrovate are also required. They cannot utilize thiosulfate, sugars, alcohols, amino acids, or benzoates.

Green Sulfur Bacteria All of these bacteria are Gram negative, spherical to straight, or curved rods. Arrangement of cells may be in chains like streptococci. Some are motile by gliding, others are nonmotile. Color may be grass- green or brown. Sulfur byproduct is excreted, not retained in cells. Some are able to utilize thiosulfate. Many are mixotrophic in that they can photoassimilate simple organic compounds in the presence of sulfide and bicarbonate.

WINOGRADSKY'S COLUMN

To create a small ecosystem that is suitable for the growth of these bacteria, one can set up a **Winogradsky column** as illustrated in the following figure. Sergii Winogradsky, a Russian microbiologist, developed this culture technique to study the bacteria that are involved in the sulfur cycle. From his studies he defined the chemoautotrophic bacteria. This setup consists of a large test tube or graduated cylinder that is packed with pond sediments, sulfate, carbonate, and some source of cellulose (shredded paper or cellulose powder). It is incubated for a period of time (up to 8 weeks) while being exposed to incandescent light. Note

that different layers of microorganisms develop, much in the same manner that is found in nature.



Winogradsky's Column

Observe that in the bottom of the column the cellulose is degraded to fermentation products by *Clostridium.* The fermentation products and sulfate are then acted upon by other bacteria (*Desulfovibrio*) to produce hydrogen sulfide, which diffuses upward toward the oxygenated zone, creating a stable hydrogen sulfide gradient. Note, also, that the *Chlorobium* species produce an olive- green zone deep in the column. A red to purple zone is produced by *Chromatium* a little farther up. Ascending the column farther where the oxygen gradient increases, other phototrophic bacteria such as *Rhodospirillum, Beggiatoa,* and *Thiobacillus* will flourish. Once the column has matured, one can make subcultures from the different layers, using an enrichment medium. The subcultures

can be used for making slides to study the morphological characteristics of the various types of organisms. Proceed as follows:

FIRST PERIOD

You will set up your Winogradsky column in a 100 ml glass graduate. It will be filled with mud, sulfate, water, phosphate, carbonate, and a source of fermentable cellulose. The cellulose, in this case, will be in the form of a shredded paper slurry. The column will be covered completely at first with aluminum foil to prevent the overgrowth of amoeba and then later uncovered and illuminated with incandescent light to promote the growth of phototrophic bacteria. The column will be examined at 2- week intervals to look for the development of different- colored layers. Once distinct colored layers develop, subcultures will be made to tubes of enrichment medium with a pipette. The subcultures will be incubated at room temperature with exposure to incandescent light and examined periodically for color changes.

Materials

- graduated cylinder (100 ml size)
- cellulose source (cellulose powder, newspaper, or filter paper)
- calcium sulfate, calcium carbonate, dipotassium phosphate
- mud from various sources (freshly collected)
- water from ponds (freshly collected)
- beaker (100 ml size)
- glass stirring rod
- aluminum foil
- rubber bands
- incandescent lamp (60–75 watt)

Procedure

1. Using cellulose powder or some form of paper, prepare a thick slurry with water in a beaker. If you are using paper, tear the paper up into small

pieces and macerate it in a small volume of water with a glass rod. If you are using cellulose powder, start with 1– 2 g of powder in a small amount of water. The slurry should be thick but not a paste.

2. Fill the cylinder with the slurry until it is one- third full.

3. To 200 g of mud, add 1.64 g of calcium sulfate and 1.3 g each of calcium carbonate and dipotassium phosphate. Keep a record of the source of the mud you are using.

4. Add some "self water" (pond water collected with the mud) to the mud and chemical mixture and mix the ingredients well.

5. Pour the mud mixture into the cylinder on top of the cellulose slurry.

6. With a glass rod, gently mix and pack the contents of the cylinder. As packing occurs, you may find that you need to add more "self water" to bring the level up to two- thirds or three- fourths of the graduate. Make sure all trapped air bubbles are released.

7. Top off the cylinder by adding pond water until 90% full.

8. Cap the cylinder with foil, using a rubber band to secure the cover.

9. Record the initial appearance of the cylinder.

10. Wrap the sides of the cylinder completely with aluminum foil to exclude light.

11. Incubate the cylinder at room temperature for one and a half to two weeks.

TWO WEEKS LATER

Remove the aluminum foil from the sides of the cylinder. Note the color of the mud, particularly in the bottom. Its black appearance will indicate sulfur respiration with the formation of sulfides by *Desulfovibrio* and other related bacteria. Record the color differences of different layers and the overall appearance of the entire cylinder. Place a lamp with a 75 watt bulb within a few inches of the cylinder and continue to incubate the cylinder at room temperature.

SUBSEQUENT EXAMINATIONS

Examine the cylinder periodically at each laboratory period, looking for the color changes that might occur. The presence of green, purple, red, or brown areas on the surface of the mud should indicate the presence of blooms of anaerobic phototrophic bacterial growth. Record your results.

SUBCULTURING

After 6 to 8 weeks, make several subcultures from your Winogradsky column.

Notes and Results

ENVIRONMENTAL FACTORS AND CONTROL OF MICROBIAL GROWTH

Microbial control by chemical and physical means involves the use of antiseptics, disinfectants, antibiotics, ultraviolet light, and many other agents. Some factors will be tested as examples of the environmental factors that include temperature, oxygen, pH, heavy metals and dyes.

OSMOTIC PRESSURE AND BACTERIAL GROWTH

Growth of bacteria can be profoundly affected by the amount of water entering or leaving the cell. When the medium surrounding an organism is **hypotonic** (low solute content), a resultant higher osmotic pressure occurs in the cell. Except for some marine forms, this situation is not harmful to most bacteria. The cell wall structure of most bacteria is so strong and rigid that even slight cellular swelling is generally inapparent.

In the reverse situation, however, when bacteria are placed in a **hypertonic** solution (high solute content), their growth may be considerably inhibited. The degree of inhibition will depend on the type of solute and the nature of the organism. In media of growth- inhibiting osmotic pressure, the cytoplasm becomes dehydrated and shrinks away from the cell wall. Such **plasmolyzed** cells are often simply inhibited in the absence of sufficient cellular water and return to normal when placed in an **isotonic** solution. In other instances, the organisms are irreversibly affected due to permanent inactivation of enzyme systems.

Organisms that thrive in hypertonic solutions are designated as halophiles or osmophiles. If they require minimum concentrations of salt (NaCl and other cations and anions) they are called **halophiles.** Obligate halophiles require a minimum of 13% sodium chloride. **Osmophiles,** on the other hand, require high concentrations of an organic solute, such as sugar.

In this exercise we will test the degree of inhibition of organisms that results with media containing different concentrations of sodium chloride.

To accomplish this, you will streak three different organisms on four plates of media. The specific organisms used differ in their tolerance of salt concentrations. The salt concentrations will be 0.5, 5, 10, and 15%. After incubation for 48 hours and several more days, comparisons will be made of growth differences to determine their degrees of salt tolerances.

Materials

- 1 Petri plate of nutrient agar (0.5% NaCl)
- 1 Petri plate of nutrient agar (5% NaCl)
- 1 Petri plate of nutrient agar (10% NaCl)
- 1 Petri plate of milk salt agar (15% NaCl)
- cultures of *Escherichia coli* (nutrient broth), *Staphylococcus aureus* (nutrient broth) and *Halobacterium salinarium* (slant culture) or as instructed.

Procedure

1. Mark the bottoms of the four Petri plates.

2. Streak each organism in a straight line on the agar, using a wire loop.

3. Incubate all the plates for 48 hours at room temperature with exposure to light (the pigmentation of *H. salinarium* requires light to develop). Record your results.

4. Continue the incubation of the milk salt agar plate for several more days in the same manner, and record your results again.

Notes and Results

EFFECT OF TEMPERATURE

Materials

- 24- to 48-hour tryptic soy broth cultures of *Escherichia coli* (ATCC 11229), *Bacillus stearothermophilus* (ATCC 7953), *Bacillus globisporus* (ATCC 23301), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 25923), and spore suspension of *Bacillus subtilis* (ATCC 6051). (To produce endospores, grow *B. subtilis* for 48 hours at 35°C on endospore agar, nutrient agar plus 0.002% $MnCl2 \cdot 4H2O$. Resuspend the paste in at least 7 ml of sterile diluent.)
- 12 tryptic soy agar slants
- Bunsen burner
- inoculating loop
- 15 tryptic soy broth tubes (9.9 ml per tube)
- test tube rack
- 18 sterile 1-ml pipettes with pipettor
- 3 sterile test tubes
- refrigerator set at 4°C
- incubators or water baths set at 4°, 23° to 25° (room temperature), 60°, 85°, and 100°C (The instructor or students are not limited to these temperatures. Modifications can be instituted based on incubators or water baths available.)
- wax pencil
- sterile water

Principles

Microbial growth is directly dependent on how temperature affects cellular enzymes. With increasing temperatures, enzyme activity increases until the three dimensional configuration of these molecules is lost because of denaturation of their protein structure. As the temperature is

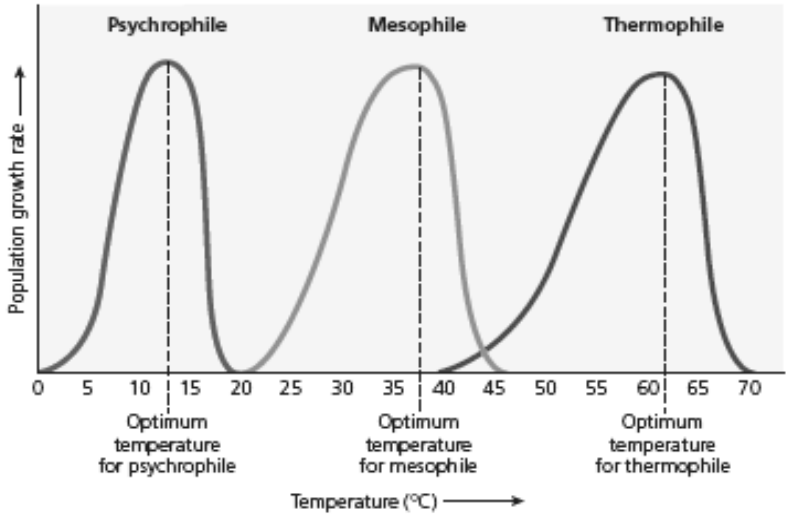
lowered toward the freezing point, enzyme inactivation occurs and cellular metabolism gradually diminishes. At 0°C, biochemical reactions cease in most cells.

Bacteria, as a group of living organisms, are capable of growth within an overall temperature range of minus 5°C to 80°C. Each species, however, requires a narrower range that is determined by the heat sensitivity of its enzyme systems. Specific temperature ranges consist of the following **cardinal (**significant**) temperature points** (see the Figure below):

1. Minimum growth temperature: The lowest temperature at which growth will occur. Below this temperature, enzyme activity is inhibited and the cells are metabolically inactive so that growth is negligible or absent.

2. Maximum growth temperature: The highest temperature at which growth will occur. Above this temperature, most cell enzymes are destroyed and the organism dies.

3. Optimum growth temperature: The temperature at which the rate of reproduction is most rapid; however, it is not necessarily optimum or ideal for all enzymatic activities of the cell.



The effect of temperature on the growth of microorganisms

All bacteria can be classified into one of three major groups, depending on their temperature requirements:

1. Psychrophiles: Bacterial species that will grow within a temperature range of −5°C to 20°C. The distinguishing characteristic of all psychrophiles is that they will grow between 0° and 5°C.

2. Mesophiles: Bacterial species that will grow within a temperature range of 20°C to 45°C. The distinguishing characteristics of all mesophiles are their ability to grow at human body temperature (37°C) and their inability to grow at temperatures above 45°C. Included among the mesophiles are two distinct groups:

a. Mesophiles with optimum growth temperature between 20°C and 30°C are plant saprophytes.

b. Mesophiles with optimum growth temperature between 35°C to 40°C are organisms that prefer to grow in the bodies of warmblooded hosts.

3. Thermophiles: Bacterial species that will grow at 35°C and above. Two groups of thermophiles exist:

a. Facultative thermophiles: Organisms that will grow at 37°C, with an optimum growth temperature of 45°C to 60°C.

b. Obligate thermophiles: Organisms that will grow only at temperatures above 50°C, with optimum growth temperatures above 60°C.

The ideal temperature for specific enzymatic activities may not coincide with the optimum growth temperature for a given organism. To understand this concept, you will investigate pigment production and carbohydrate fermentation by selected organisms at a variety of incubation temperatures.

1. The production of an endogenous red or magenta pigment by *Serratia marcescens* is determined by the presence of an orange to deep red coloration on the surface of the colonial growth.

2. Carbohydrate fermentation by *Saccharomyces cerevisiae* is indicated by the presence of gas, one of the end products of this fermentative process.

Detection of this accumulated gas may be noted as an air pocket, of varying size, in an inverted inner vial (Durham tube) within the culture tube.

Procedure

First Period

1. Work in groups of three to four students. Each group of students will be assigned one temperature to study: 4°, 23° to 25°, 60°, 85°, or 100°C.

2. Label each of the tryptic soy agar slants with the name of the test bacterium to be inoculated (*E. coli, B. stearothermophilus,* and *B. globisporus*), your name, and date.

3. Using aseptic technique, streak the surface of each slant with the appropriate bacterium. Incubate the slants for 24 to 48 hours at the temperature assigned to your group.

4. Take three sterile test tubes and label one *S. aureus,* the second *B. subtilis spores,* and the third *P. aeruginosa.* Add your name and date.

5. With a sterile pipette, aseptically add 1 ml of bacterial culture or spore suspension to the respective tubes.

6. Subject your tubes to the temperature you are studying for 15 minutes (i.e., either place them in the refrigerator, let them stand at room temperature, or put them in one of the water baths).

7. After 15 minutes, let the samples cool or warm up to room temperature. For each bacterial sample, make a dilution series as follows:

Pipette 0.1 ml of the incubated sample into the 9.9 ml tryptic soy broth (10^{-2} dilution). Mix the tube thoroughly. With a fresh pipette, transfer 0.1 ml of this 10^{-2} dilution into 9.9 ml of broth (10^{-4} dilution) and mix. In the same way, prepare a 10^{-6}, 10^{-8}, and 10^{-10} dilution.

8. Incubate all dilutions at 35°C for 24 to 48 hours.

Second Period

1. At the end of incubation, observe the slants for the presence of growth. Record your observations and those of your classmates; use a + for the

presence of growth and a – for the absence of growth in Part 1 of the exercise.

2. Observe your dilution series to see which tubes have bacterial growth as indicated by turbidity. The logic of this procedure is that reproduction will have occurred in each tube that received at least one living bacterium. The greater the number of bacteria present in the particular sample, the more such a sample can be diluted and still contain bacteria in the aliquot transferred. Thus, if bacteria **A** are less susceptible to heat than bacteria **B,** bacteria **A** will require more dilutions in order to obtain a sterile sample as indicated by no growth.

3. From your results and those of your classmates, indicate the last dilution in which growth occurred.

Notes and Results

EFFECT OF PH

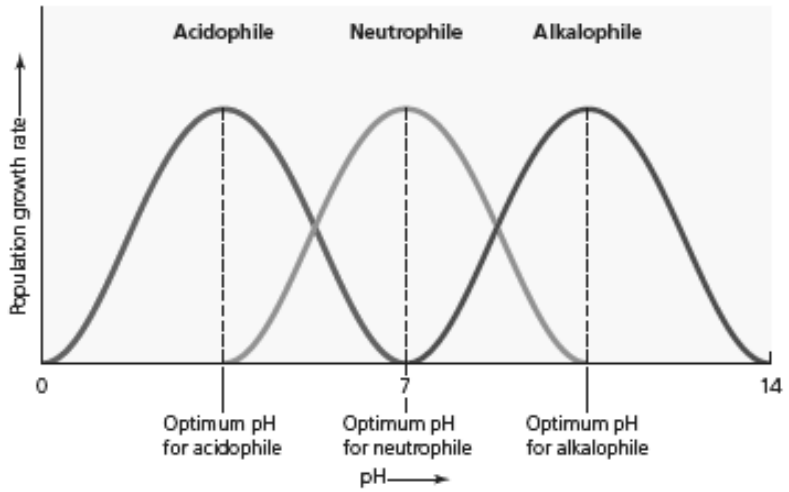
Materials

- saline suspensions of 24- hour tryptic soy broth cultures of *Alcaligenes faecalis* (ATCC 8750), *Escherichia coli* (ATCC 11229), and a Sabouraud dextrose agar slant (48 hours at 30°C) of *Saccharomyces cerevisiae* (ATCC 2366, a yeast). Add 3 ml of sterile saline to each *S. cerevisiae* slant and resuspend the yeast with a sterile inoculating loop. Transfer the suspension to a sterile culture tube. Adjust the suspensions to an absorbance of 0.05 at a wavelength of 550 to 600 nm by adding either more saline or culture.
- pH meter or pH paper
- 4 tryptic soy broth tubes, pH 3.0
- 4 tryptic soy broth tubes, pH 5.0
- 4 tryptic soy broth tubes, pH 7.0
- 4 tryptic soy broth tubes, pH 9.0
  (the pH of the above tubes is adjusted with either 1 N sodium hydroxide or 1 N hydrochloric acid)
- Bunsen burner
- sterile 1-ml pipettes with pipettor
- spectrophotometer
- cuvettes
- wax pencil
- test-tube rack

Principles

It is not surprising that **pH** (acidity; log 1/ $[H^+]$ dramatically affects bacterial growth. The pH affects the activity of enzymes—especially those that are involved in biosynthesis and growth. Each microbial species possesses a definite pH **growth range** and a distinct pH **growth**

optimum. Acidophiles have a growth optimum between pH 0.0 and 5.5; **neutrophiles** between 5.5 and 8.0; and **alkalophiles** 8.5 to 11.5. In general, different microbial groups have characteristic pH optima. The majority of bacteria and protozoa are neutrophiles. Most molds and yeasts occupy slightly acidic environments in the pH range of 4 to 6; algae also seem to favor acidity Many bacteria produce metabolic acids that may lower the pH and inhibit their growth. To prevent this, **buffers** that produce a pH equilibrium are added to culture media to neutralize these acids. For example, the peptones in complex media act as buffers. Phosphate salts are often added as buffers in chemically defined media.



The effect of ph on the growth of microorganisms

Procedure

First Period

1. Label each of the tryptic soy broth tubes with the pH of the medium, your name, date, and the microorganism to be inoculated.

2. Using a sterile pipette, add 0.1 ml of the *E. coli* saline culture to the tube that has a pH of 3.0. Do the same for the tubes that have pH values of 5.0, 7.0, and 9.0.

3. Repeat the above for *A. faecalis* and *S. cerevisiae.*

4. Incubate the *E. coli* and *A. faecalis* cultures for 24 to 48 hours at 35°C, and the *S. cerevisiae* culture for 48 to 72 hours at room temperature.

Second Period

1. By using the spectrophotometer, set the wavelength at 550 to 600 nm. Calibrate the spectrophotometer, using a tryptic soy broth blank of each pH for each respective set of cultures.

2. Fill each cuvette 2/ 3 full of the respective pH culture and read the absorbance. Blank the spectrophotometer with tryptic soy broth.

3. If no spectrophotometer is available, record your visual results as – (no growth), +, ++, +++, and ++++ (for increasing growth, respectively).

4. Record your.

Notes and Results

EFFECTS OF DISINFECTANTS

Materials

- 20- hour tryptic soy broth cultures of *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 10145)
- 2 sterile screw cap test tubes
- 1 sterile 5 ml pipette with pipettor
- 12 sterile 1 ml pipettes
- 48 tryptic soy broth tubes (10 ml per tube)
- sterile water in Erlenmeyer flask
- 12 sterile tubes for making dilutions
- commercial disinfectants such as 3% hydrogen peroxide, 70% isopropyl alcohol, bleach, or any cleaner or students can bring in their own to test. If commercial disinfectants are used, note the use dilution and active ingredients. Dilute with normal tap water. The tap water need not be sterilized for commercial disinfectants.
- phenol (carbolic acid)
- wax pencil
- 35°C incubator
- test-tube rack
- Bunsen burner
- inoculating loop

Principles

Many factors influence the effectiveness of chemical disinfectants and antiseptics. The **microbicidal** (to kill) or **microbiostatic** (to inhibit) **efficiency** of a chemical is often determined with respect to its ability to deter microbial growth. The first part of this exercise will examine this effect of several chemicals. More specifically, the microbicidal efficiency of a chemical is often determined with respect to phenol and is known as the **phenol coefficient (PC).**

The phenol coefficient is calculated by dividing the highest dilution of the antimicrobial of interest, which kills all organisms after incubation for 10 minutes but not after 5 minutes, by the highest dilution of phenol that has the same characteristics. Chemicals that have a phenol coefficient greater than 1 are more effective than phenol, and those that have a phenol coefficient less than 1 are less effective than phenol. However, this comparison should only be used for phenol- like compounds that do not exert bacteriostatic effects and are not neutralized by the subculture media used. The second part of this experiment will enable you to calculate a phenol coefficient for a select chemical.

Procedure

First Period

A- Growth Inhibition

1. Each group of students should select one of the disinfectants and, if necessary, dilute it according to the specifications on the label (the **use dilution**).

2. Place 5 ml of disinfectant into two sterile tubes. Add 0.05 ml of *P. aeruginosa* to one tube and 0.05 ml of *S. aureus* to the other.

3. Using the wax pencil, label the tubes with your name and those of the respective bacteria. Mix each of the tubes in order to obtain a homogeneous suspension.

4. At intervals of 1, 2, 5, 10, and 15 minutes, transfer 0.1 ml of the mixture containing the bacteria and disinfectant to separate tubes oftryptic soy broth. Do this for both bacteria. Also inoculate two tubes of broth with 0.1 ml of both bacteria and mark these "controls."

5. Incubate all tubes for 48 hours at 35°C.

B- Phenol Coefficient

1. Dilute phenol in sterile distilled water 1/ 80, 1/ 90,

and 1/ 100; dilute the used cleaner 1/ 400, 1/ 450, and 1/ 500 so that the final volume in each tube is 5 ml.

2. Label 18 tryptic soy broth tubes with the name and dilution of disinfectant, the time interval of the subculture (e.g., 5 minutes, phenol 1/ 80), and your name. Each dilution should be tested after 5, 10, and 15 minute incubations.

3. Place in order in a test tube rack, one test tube of each of the different cleaner and phenol dilutions for each time interval.

4. Add 0.5 ml of *S. aureus* to each tube of disinfectant and note the time. Mix each of the tubes in order to obtain a homogeneous suspension and allow the disinfectant to come into contact with the bacteria.

5. Using aseptic technique, at intervals of 5, 10, and 15 minutes, transfer one loopful from each disinfectant tube into the appropriately labeled tryptic soy broth tube.

6. Incubate all tubes for 48 hours at 35°C.

7. The experiment can be repeated with *P. aeruginosa.*

Second Period

A- Growth Inhibition

1. Shake and observe each of the tubes for growth. Record the presence of growth as + and the absence of growth as –. Tabulate your results as well as the results of the class.

B- Phenol Coefficient

1. Shake and observe all tryptic soy broth cultures for the presence (+) or absence (–) of growth.

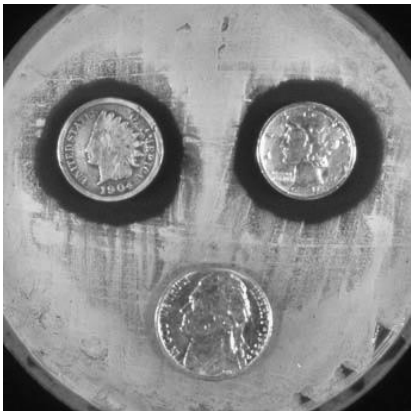
2. Record your observations.

Notes and Results

EFFECT OF HEAVY METALS

Principle

Metals such as mercury, silver, lead and copper are called **heavy metals** because of their large atomic weights and complex electron configurations. Salts of heavy metals catch onto sulfhydryl groups (–SH) on certain amino acids, thereby interfering with proteins and killing microbial cells (**see the following Figure**). Because many of the proteins involved are enzymes, cellular metabolism is disrupted, and the microorganism dies. However, heavy metals are not sporicidal.



The early-20th-century cent and dime were composed mainly of copper and silver, respectively. The clear zones around these two coins are "zones of inhibition" where the bacteria are unable to survive because these metals are present. The metal found in the nickel has little negative effect on the bacterial species in this culture.

In the following experiment, we will study the effect of both iron and copper on both Gram +ve and Gram –ve bacteria.

Materials

- Liquid cultures of *E. coli* and *B. cereus* or *subtilis*
- Two plates of sterilized nutrient agar
- Different metal coins (old and new preferably of different metals) and two nails (4- 6 cm long)
- Marker pen
- Triangular spreader
- Ethanol

- 2 sterile 1 ml pipettes
- Metal forceps
- Bunsen burner

Procedure

1- Sterilize the spreader by alcoholic flaming.

2- By using the sterile spreader, spread 0.1 ml of each cultures on one plates using a sterile pipette.

3- Hold the metal coin by forceps and sterilize by alcoholic flaming. Wait for seconds to cool and put in one plate and repeat for other coins. Repeat the procedure for metal nails in the second plate.

4- Incubate the plates inverted at 30°C until next day.

5- Observe and draw the growth and clear zones around the metal objects and record your results and notes.

Results

EFFECT OF DYES

Principle

The inhibitory effects of dyes on bacteria were first studied by Churchman and Kline in 1912, who tested gentian violet (crystal violet) and brilliant Green on bacterial cultures. Early works in this area were centered on the elucidation of the chemotherapeutic potential of dyes.

The incorporation of dyes into culture media for the purposes of isolation and differentiation of bacteria was described by Endo in 1904, who used basic fuchsin decolorized with sodium sulfite for the isolation of coliform organisms. Modifications of Endo's medium were developed in subsequent years, and a number of other dyes came into use. These dye-containing media found their greatest application in the field of sanitary microbiology where they were used for the detection of fecal coliforms from milk and water samples and for the isolation and detection of typhoid and other intestinal pathogens from fecal specimens. Dyes used extensively for their inhibitory and differential properties (especially selective for Gram negative organisms) are basic fuchsin, crystal violet, eosine Y, methylene blue, and Brilliant Green. Acridine orange, ethyl violet, aniline blue, and trypan blue were also used for selectivity for streptococci. Since these dyes are useful in differentiating between certain bacteria or groups of bacteria on the basis of inhibition, it is possible that many other dyes could be as effective or more effective in a similar manner.

Materials

- Solid or liquid cultures of *E. coli* and *B. cereus* or *subtilis*
- Inoculating loop
- Crystal violet solution (prepared for Gram staining)
- 4 test tubes containing 9 ml distilled water, sterilized
- 5 sterile Petri plates

- 5 sterile 1 ml pipettes
- Water bath adjusted to 45°C
- Sterile 10 ml measuring cylinder
- Marker pen

Procedure

1- Make a series of dilutions from the crystal violet solution using the tubes of distilled water by adding one ml in the first tube. Shake and transfer one ml to the second tube. Repeat this for the rest of the tubes until you have dilutions as follows: original stain, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}.

2- Pour 9 ml of nutrient agar in each plate.

3- Immediately add one ml of each dye dilutions to one of the plates and carefully shake in a "number 8 shape" until homogenous.

4- Now you have 5 agar plates with different dye dilutions as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}.

5- After solidification, divide the plates from the reverse side into two halves by the marker.

6- Inoculate *E. coli* in one half of each plate and the other with *Bacillus* in the middle of the used space (but not reaching the end). Try to make equal lines of inoculations in all plates.

7- Incubate all the plates at 30°C until next day.

8- Observe and draw the bacterial growth results for each plate and record your results.

Your Results

DETERMINATION OF BACTERIAL GROWTH CURVE (Two-Hour Method)

Materials

- 6-hour starter culture of *E. coli* (or as instructed)
- 500 ml flask containing approximately 300 ml of nutrient broth
- and 10-ml pipettes with pipettor
- water bath or incubator at 37°C

Principles

The four phases **(lag, logarithmic, stationary,** and **death or decline)** of growth of a bacterial population can be determined by measuring the **turbidity** of the population in a broth culture. Turbidity is not a direct measure of bacterial numbers but an indirect measure of biomass, which can be correlated with cell density during the log growth phase. Since about 10^7 bacterial cells per milliliter must be present to detect turbidity with the unaided eye, a spectrophotometer can be used to achieve increased sensitivity and obtain quantitative data. The construction of a complete **bacterial growth curve** (increase and decrease in cell numbers versus time) requires that aliquots of a shake- flask culture be measured for population size at intervals over an extended period. Because this may take many hours, such a procedure does not lend itself to a regular laboratory session. *E coli* is a rapid growth bacterium (a generation time of less than 20 minutes) makes this bacterium useful for determining a complete bacterial growth curve. The growth cycle, from lag through log and stationary phases, can be measured in approximately 2- 3 hours.

Procedure (Two-Hour Method)

1. Zero the spectrophotometer at 550 to 600 nm with the medium.

2. Place the flask containing the nutrient broth medium in the 37°C water bath or incubator for 15 minutes.

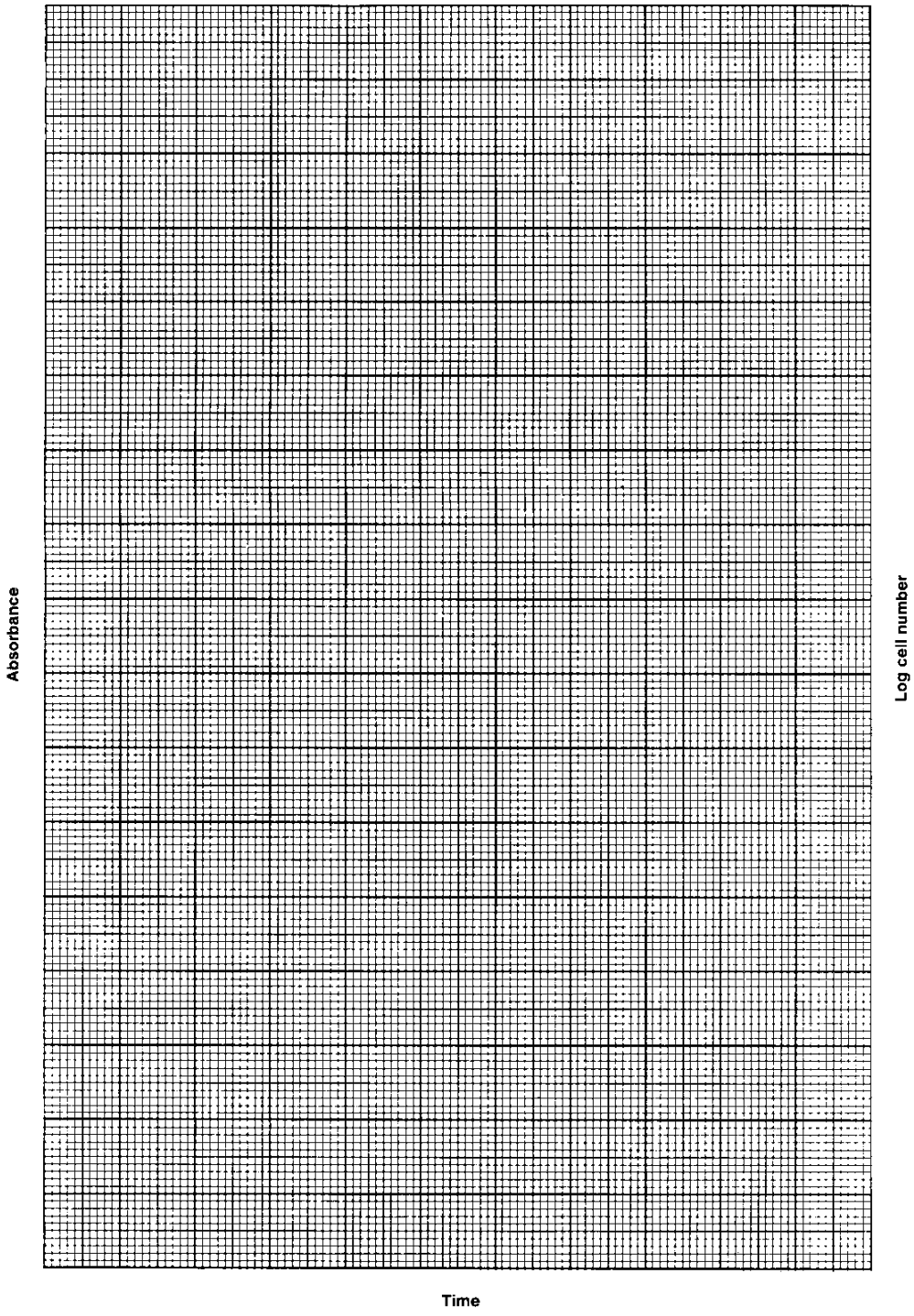
3. While slowly agitating the flask in the water bath, inoculate it with 10 ml of 6 hour *E. coli* culture.

4. Read and record the absorbance *(A)* of this initial culture (0 time) and every 20 minutes thereafter for about 2 hours. Be sure to suspend the bacteria thoroughly each time before taking a sample.

5. Construct a growth curve by plotting *A* against time on semilog graph paper.

Results

Graph paper



MICROBIOLOGY OF MILK AND FOOD PRODUCTS

Milk and food provide excellent growth media for bacteria when suitable temperatures exist. This is in direct contrast to natural waters, which lack the essential nutrients for pathogens. The introduction of a few pathogens into food or milk products becomes a much more serious problem because of the ability of these substances to support tremendous increases in bacterial numbers. Many milk- borne epidemics of human diseases have been spread by contamination of milk by soiled hands of dairy workers, unsanitary utensils, flies, and polluted water supplies. The same thing can be said for improper handling of foods in the home, restaurants, hospitals, and other institutions. Bacteriological testing of milk and food may also be performed in this same manner like water testing, using similar media and procedures to detect the presence of coliforms. However, most testing by public health authorities is quantitative. Although the presence of small numbers of bacteria in these substances does not necessarily mean that pathogens are lacking, low counts do reflect better care in handling of food and milk than is true when high counts are present.

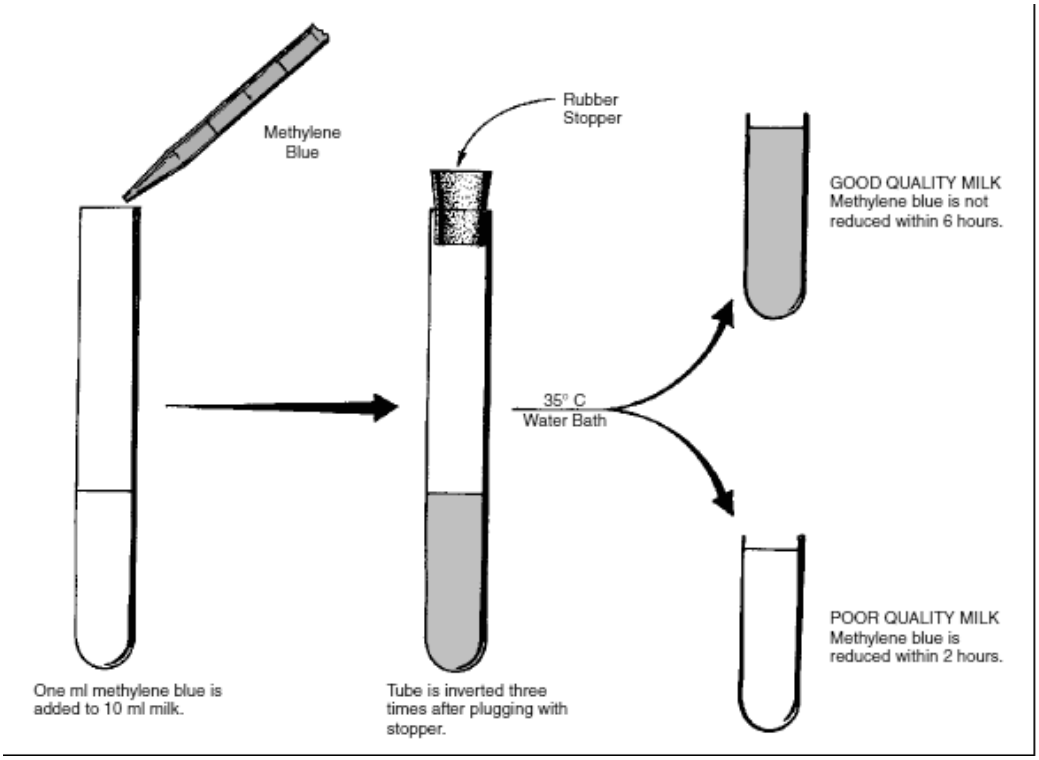
Standardized testing procedures for milk products are outlined by the American Public Health Association in Standard Methods for the Examination of Dairy Products. Since bacterial counts in foods are performed with some of the techniques you have learned in previous exercises, you will have an opportunity to apply some of those skills here.

Reductase Test

Milk that contains large numbers of actively growing bacteria will have a lowered oxidation- reduction potential due to the exhaustion of dissolved oxygen by microorganisms. The fact that methylene blue loses its color (becomes reduced) in such an environment is the basis for the **reductase test.** In this test, 1 ml of methylene blue (1:25,000) is added to 10 ml of milk. The tube is sealed with a rubber stopper and slowly inverted three

times to mix. It is placed in a water bath at 35° C and examined at intervals up to 6 hours (or up to the end of class). The time it takes for the methylene blue to become colorless is the **methylene blue reduction time** (MBRT). The shorter the MBRT, the lower the quality of milk. An MBRT of 6 hours is very good. Milk with an MBRT of 30 minutes is of very poor quality. The validity of this test is based on the assumption that all bacteria in milk lower the oxidation reduction potential at 35° C. Large numbers of psychrophiles, thermophiles, and thermodurics, which do not grow at this temperature, would not produce a positive test. Raw milk, however, will contain primarily *Streptococcus lactis* and *Escherichia coli*, which are strong reducers; thus, this test is suitable for screening raw milk at receiving stations. Its principal value is that less technical training of personnel is required for its performance.

In this exercise, samples of low- and high-quality raw milk will be tested.



Procedure for testing raw milk with reductase test

Materials:

- 2 sterile test tubes with rubber stoppers
- raw milk samples of low- and high-quality (samples A and B)
- water bath set at 35° C
- methylene blue (1:25,000)
- 10 ml pipettes
- 1 ml pipettes
- gummed labels

1. Attach gummed labels with your name and type of milk to two test tubes (a good- quality as well as a poor-quality milk).

2. Using separate 10 ml pipettes for each type of milk, transfer 10 ml to each test tube. To the milk in the tubes add 1 ml of methylene blue with a 1 ml pipette. Insert rubber stoppers and gently invert three times to mix. Record your name and the time on the labels and place the tubes in the water bath, which is set at 35° C.

3. After 5 minutes incubation, remove the tubes from the bath and invert once to mix. This is the last time they should be mixed.

4. Carefully remove the tubes from the water bath 30 minutes later and every half hour until the end of the laboratory period. When at least four-fifths of the tube has turned white, the end point of reduction has taken place. Record this time on the Laboratory Report. The classification of milk quality is as follows:

Class 1: Excellent, not decolorized in 8 hours.

Class 2: Good, decolorized in less than 8 hours, but not less than 6 hours.

Class 3: Fair, decolorized in less than 6 hours, but not less than 2 hours.

Class 4: Poor, decolorized in less than 2 hours.

Notes and Results

Microbiology of Yogurt Production

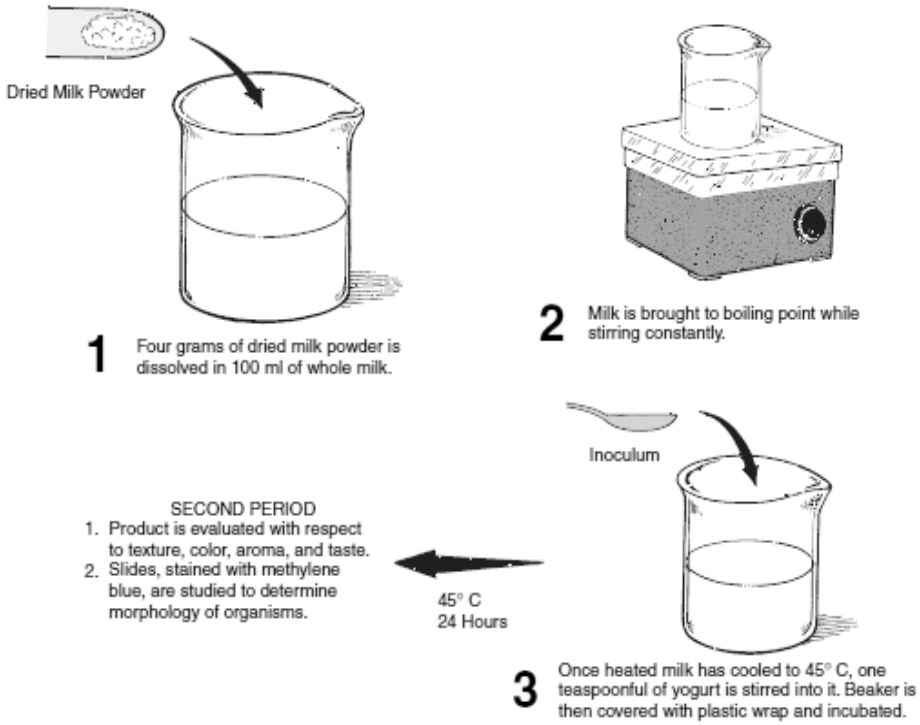
For centuries, people throughout the world have been producing fermented milk products using yeasts and lactic acid bacteria. The **yogurt** of eastern central Europe, the **kefir** of the Cossacks, the **koumiss** of central Asia, and the **leben** of Egypt are just a few examples. In all of these fermented milks, lactobacilli act together with some other microorganisms to curdle and thicken milk, producing a distinctive flavor desired by the producer.

Kefir of the Cossacks is made by charging milk with small cauliflower-like grains that contain *Streptococcus lactis, Saccharomyces delbrueckii,* and *Lactobacillus brevis.* As the grains swell in the milk they release the growing microorganisms to ferment the milk. The usual method for producing yogurt in large scale production is to add pure cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* to pasteurized milk.

In this exercise you will produce a batch of yogurt from milk by using an inoculum from commercial yogurt. Gram- stained slides will be made from the finished product to determine the types of organisms that control the reaction. If proper safety measures are followed, the sample can be tasted. Two slightly different ways of performing this experiment are provided here. Your instructor will indicate which method will be followed.

METHOD A (First Period)

The following Figure illustrates the procedure for this method. Note that 4 g of powdered milk are added to 100 ml of whole milk. This mixture is then heated to boiling and cooled to 45°C. After cooling, the milk is inoculated with yogurt and incubated at 45° C for 24 hours.



Yogurt production by Method A

Materials:

- dried powdered milk
- whole milk
- commercial yogurt (with viable organisms)
- small beaker, graduate, teaspoon, stirring rod
- plastic wrap
- filter paper (for weighing)

Procedure

1. On a piece of filter paper weigh 4 grams of dried powdered milk.

2. To a beaker of 100 ml of whole milk add the powdered milk and stir thoroughly with sterile glass rod to dissolve.

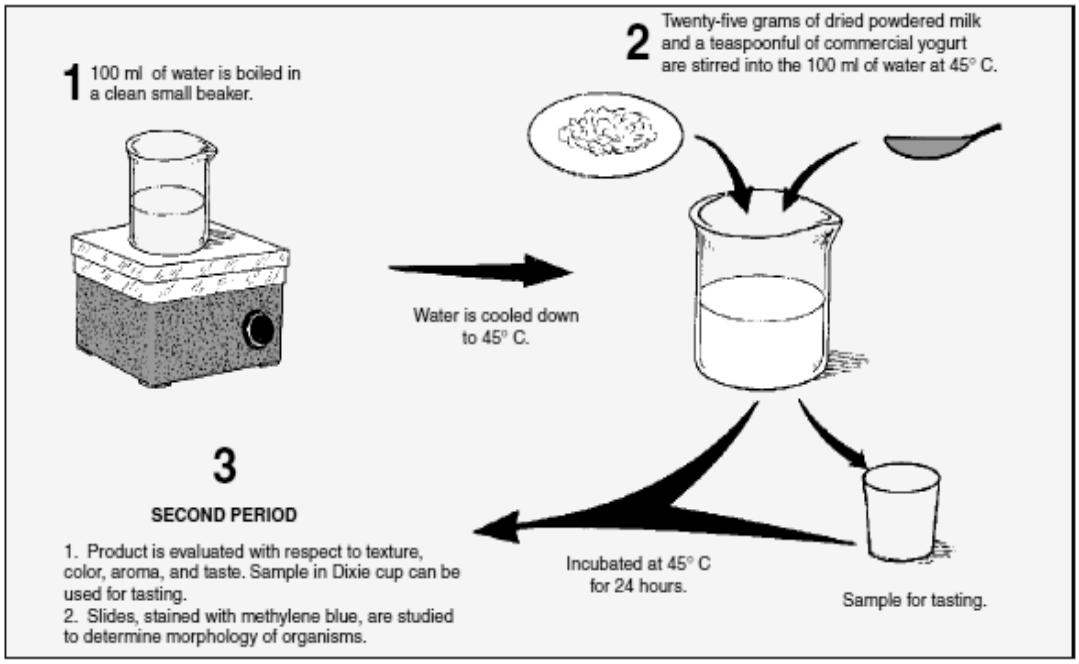
3. Heat to boiling, while stirring constantly.

4. Cool to 45° C and inoculate with 1 teaspoon of the commercial yogurt. Stir. Be sure to check the label to make certain that product contains a live culture. Cover with plastic wrap.

5. Incubate at 45° C for 24 hours.

METHOD B (First Period)

The following Figure illustrates a slightly different method of culturing yogurt, which, due to its simplicity, may be preferred. Note that no whole milk is used and provisions are made for producing a sample for tasting.



Yogurt production by Method B

Materials:

- small beaker, graduate, teaspoon, stirring rod
- dried powdered milk
- commercial yogurt (with viable organisms)
- plastic wrap
- filter paper for weighing
- paper cup (50 or 100 ml size) and cover
- electric hot plate or Bunsen burner and tripod

Procedure

1. On a piece of filter paper weigh 25 grams of dried powdered milk.

2. Heat 100 ml of water in a beaker to boiling and cool to 45° C.

3. Add the 25 grams of powdered milk and 1 teaspoon of yogurt to the beaker of water. Mix the ingredients with a sterile glass rod.

4. Pour some of the mixture into a sterile cup and cover loosely. Cover the remainder in the beaker with plastic wrap.

5. Incubate at 45° C for 24 hours.

SECOND PERIOD (Both Methods)

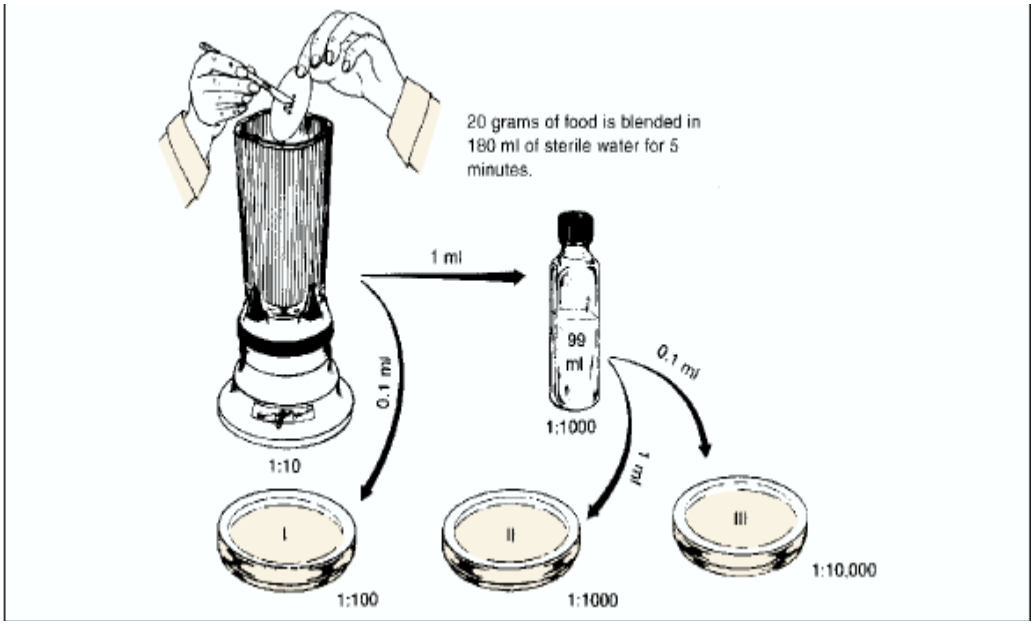
1. Examine the product and record the color, aroma, texture, and, if desired, the taste.

2. Make slide preparations of the yogurt culture. Fix and stain with methylene blue. Examine under oil immersion and record your results.

Notes and Results

Bacterial Count of a Food Product

The standard plate count, as well as the multiple tube test, can be used on foods much in the same manner that they are used on milk and water to determine total counts and the presence of coliforms. To get the organisms in suspension, however, a food blender is necessary. This will not be a coliform count. The instructor will indicate the specific kinds of foods to be tested and make individual assignments. The following Figure illustrates the general procedure.



Dilution procedure for bacterial counts of food

Materials

- 3 Petri plates
- 1 bottle (45 ml) of Plate Count agar or Standard Methods agar
- 1 99 ml sterile water blank
- 2 1 ml dilution pipettes
- food blender
- sterile blender jars (one for each type of food)
- sterile weighing paper

- 180 ml sterile water blanks (one for each type of food)
- samples of ground meat, dried fruit, and frozen vegetables, thawed for 2 hours

Procedure

1. Using aseptic techniques, weigh out on sterile weighing paper 20 grams of food to be tested.

2. Add the food and 180 ml of sterile water to a sterile mechanical blender jar. Blend the mixture for 5 minutes. This suspension will provide a 1:10 dilution. (Alternatively add 10g of food to 190 ml of water).

3. With a 1 ml dilution pipette dispense from the blender 0.1 ml to plate I and 1.0 ml to the water blank. See the figure.

4. Shake the water blank 25 times in an arc for 7 seconds with your elbow on the table as done in the Exercise of "Bacterial Population Counts.

5. Using a fresh pipette, dispense 0.1 ml to plate III and 1.0 ml to plate II.

6. Pour agar (50° C) into the three plates and incubate them at 35° C for 24 hours.

7. Count the colonies on the best plate and record the results.

Notes and Results

BIOCHEMICAL ACTIVITIES OF MICROORGANISMS

Microorganisms must be separated and identified for a wide variety of reasons, including:

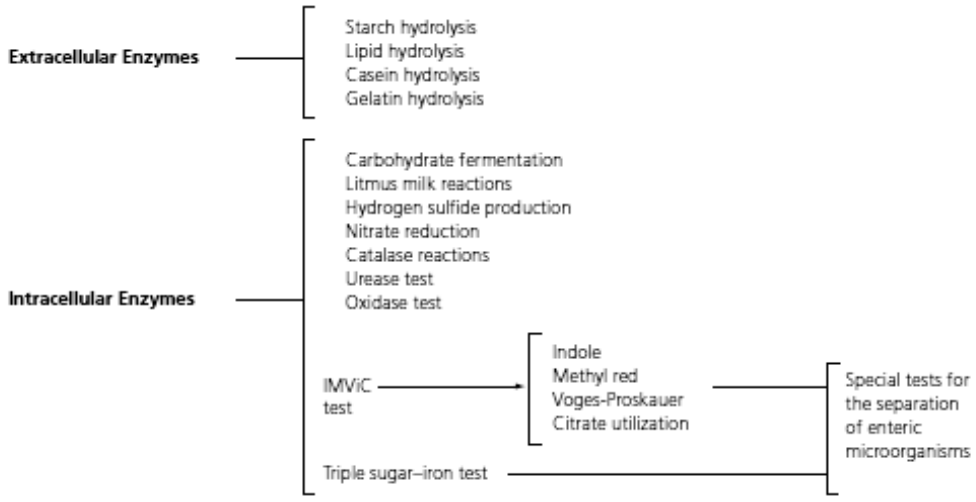
1. Determination of pathogens responsible for infectious diseases.

2. Selection and isolation of strains of fermentative microorganisms necessary for the industrial production of alcohols, solvents, vitamins, organic acids, antibiotics, and industrial enzymes.

3. Isolation and development of suitable microbial strains necessary for the manufacture and the enhancement of quality and flavor in certain food materials, including yogurt, cheeses, and other milk products.

4. Comparison of biochemical activities for taxonomic purposes.

To accomplish these tasks, the microbiologist utilized the fact that microorganisms all have their own identifying biochemical characteristics. These so- called biochemical fingerprints are the properties controlled by the cells' enzymatic activity, and they are responsible for bioenergetics, biosynthesis, and biodegradation. The sum of all these chemical reactions is defined as **cellular metabolism**, and the biochemical transformations that occur both outside and inside the cell are governed by biological catalysts called **enzymes**.



Biochemical activities of microorganisms

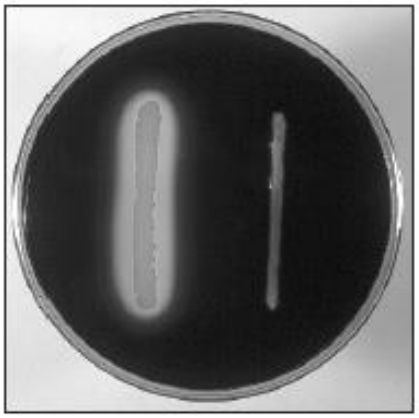
Extracellular enzymatic Activities of Microorganisms

Because of their large sizes, high molecular- weight nutrients such as polysaccharides, lipids, and proteins are not capable of permeating the cell membrane. These macromolecules must first be hydrolyzed by specific extracellular enzymes into their respective basic building blocks. These low molecular- weight substances can then be transported into the cells and used for the synthesis of protoplasmic requirements and energy production. The following procedures are designed to investigate the exoenzymatic activities of different microorganisms.

Starch Hydrolysis

Starch is a high molecular- weight, branching polymer composed of **glucose** molecules linked together by **glycosidic bonds**. The degradation of this macromolecule first requires the presence of the extracellular enzyme **amylase** for its hydrolysis into shorter polysaccharides, namely **dextrins**, and ultimately into **maltose** molecules. The final hydrolysis of this disaccharide, which is catalyzed by **maltase**, yields low molecular-weight, soluble **glucose** molecules that can be transported into the cell and used for energy production through the process of glycolysis.

In this experimental procedure, starch agar is used to demonstrate the hydrolytic activities of these exoenzymes. The medium is composed of nutrient agar supplemented with starch, which serves as the polysaccharide substrate. The detection of the hydrolytic activity following the growth period is made by performing the starch test to determine the presence or absence of starch in the medium. Starch in the presence of iodine will impart a blue- black color to the medium, indicating the absence of starch- splitting enzymes and representing a negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis will surround the growth of the organism. This is a positive result. Positive and negative results are shown in the following figure.



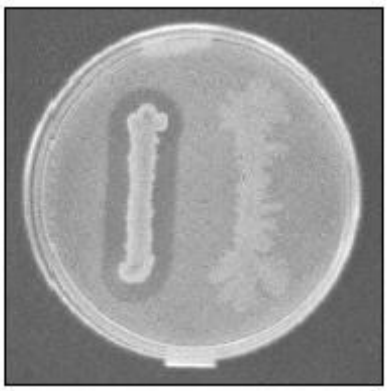
Starch agar plate. Starch hydrolysis on left; no starch hydrolysis on right.

Lipid Hydrolysis

Lipids are high molecular-weight compounds possessing large amounts of energy. The degradation of lipids such as **triglycerides** is accomplished by extracellular hydrolyzing enzymes, called **lipases** (esterases), that cleave the **ester bonds** in this molecule by the addition of water to form the building blocks **glycerol** (an alcohol) and **fatty acids**. The following figure shows this reaction. Once assimilated into the cell, these basic components can be further metabolized through aerobic respiration to produce cellular energy, adenosine triphosphate (ATP). The components may also enter other metabolic pathways for the synthesis of other cellular protoplasmic requirements.

In this experimental procedure, tributyrin agar is used to demonstrate the hydrolytic activities of the exoenzyme lipase. The medium is composed of nutrient agar supplemented with the triglyceride tributyrin as the lipid substrate. Tributyrin forms an emulsion when dispersed in the agar, producing an opaque medium that is necessary for observing exoenzymatic activity. Following inoculation and incubation of the agar plate cultures, organisms excreting lipase will show a zone of **lipolysis**, which is demonstrated by a clear area surrounding the bacterial growth. This loss of opacity is the result of the hydrolytic reaction yielding soluble

glycerol and fatty acids and represents a positive reaction for lipid hydrolysis. In the absence of lipolytic enzymes, the medium retains its opacity. This is a negative reaction. Positive and negative results are shown in the following figure.



Tributyrin agar plate. Lipid hydrolysis on left; no lipid hydrolysis on right.

Casein Hydrolysis

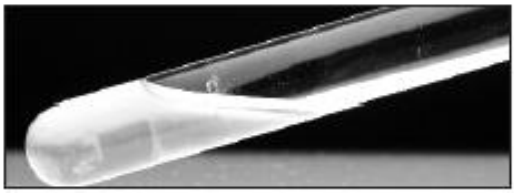
Casein, the major milk protein, is a macromolecule composed of **amino acid** subunits linked together by **peptide bonds** (CO—NH). Before their assimilation into the cell, proteins must undergo step-by-step degradation into **peptones**, **polypeptides**, **dipeptides**, and ultimately into their building blocks, **amino acids**. This process is called peptonization, or **proteolysis**, and it is mediated by extracellular enzymes called **proteases**. The function of these proteases is to cleave the peptide bond CO–NH by introducing water into the molecule. The reaction then liberates the amino acids. The low molecular-weight soluble amino acids can now be transported through the cell membrane into the intracellular amino acid pool for use in the synthesis of structural and functional cellular proteins.

In this experimental procedure, milk agar is used to demonstrate the hydrolytic activity of these exoenzymes. The medium is composed of nutrient agar supplemented with milk that contains the protein substrate casein. Similar to other proteins, milk protein is a colloidal suspension that gives the medium its color and opacity because it deflects light rays rather

than transmitting them. Following inoculation and incubation of the agar plate cultures, organisms secreting proteases will exhibit a zone of proteolysis, which is demonstrated by a clear area surrounding the bacterial growth. This loss of opacity is the result of a hydrolytic reaction yielding soluble, noncolloidal amino acids, and it represents a positive reaction. In the absence of protease activity, the medium surrounding the growth of the organism remains opaque, which is a negative reaction.

Gelatin Hydrolysis

Although the value of gelatin as a nutritional source is questionable (it is an incomplete protein, lacking the essential amino acid tryptophan), its value in identifying bacterial species is well established. Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Below temperatures of 25°C, gelatin will maintain its gel properties and exist as a solid; at temperatures above 25°C, gelatin is liquid. The following Figure shows gelatin hydrolysis.



(a) Positive for gelatin liquefaction

(b) Negative for gelatin liquefaction

Nutrient gelatin hydrolysis

Liquefaction is accomplished by some microorganisms capable of producing a proteolytic extracellular enzyme called **gelatinase**, which acts to hydrolyze this protein to **amino acids**. Once this degradation occurs, even very low temperatures of 4°C will not restore the gel characteristic.

In this experimental procedure, you will use nutrient gelatin deep tubes to demonstrate the hydrolytic activity of gelatinase. The medium consists of nutrient broth supplemented with 12% gelatin. This high gelatin concentration results in a stiff medium and also serves as the substrate for the activity of gelatinase. Following inoculation and incubation for 48 hours, the cultures are placed in a refrigerator at 4°C for 30 minutes. Cultures that remain liquefied produce gelatinase and demonstrate rapid gelatin hydrolysis. Re- incubate all solidified cultures for an additional 5 days. Refrigerate for 30 minutes and observe for liquefaction. Cultures that remain liquefied are indicative of *slow* gelatin hydrolysis.

Materials for the above experiments

Cultures

24- to 48-hour trypticase soy broth cultures of *Escherichia coli, Bacillus cereus, Pseudomonas aeruginos ,* and *Staphylococcus aureus.*

Media and Reagent

- Two plates each of starch agar, tributyrin agar, and milk agar, and three nutrient gelatin deep tubes per designated student group.
- Gram's iodine solution.

Equipment

Microincinerator or Bunsen burner, inoculating loop and needle, glassware marking pencil, test tube rack, and refrigerator.

Controls

Test	Positive Control	Negative Control
Starch Hydrolysis	*B. cereus*	*E. coli*
Lipis Hydrolysis	*S. aureus*	*E. coli*
Casein Hydrolysis	*B. cereus*	*E. coli*
Gelatin Hydrolysis	*B. cereus*	*E. coli*

Procedure for all experiments

Lab One (Inoculation and Incubation)

1. Prepare the starch agar, tributyrin agar, and milk agar plates for inoculation. Using two plates per medium, divide the bottom of each Petri dish into two sections. Label the sections as *E. coli*, *B. cereus*, *P. aeruginosa*, and *S. aureus*, respectively.

2. Using aseptic technique, make a single-line streak inoculation of each test organism on the agar surface of its appropriately labeled section on the agar plates.

3. Using aseptic technique, inoculate each experimental organism in its appropriately labeled gelatin deep tube by means of a stab inoculation.

4. Incubate all plates in an inverted position for 24 to 48 hours at 37°C. Incubate the gelatin deep tube cultures for 48 hours. Re- incubate all negative cultures for an additional 5 days.

Lab Two

Starch Hydrolysis

1. Flood the starch agar plate cultures with Gram's iodine solution, allow the iodine to remain in contact with the medium for 30 seconds, and pour off the excess.

2. Examine the cultures for the presence or absence of a blue- black color surrounding the growth of each test organism. Record your results.

3. Based on your observations, determine and record the organisms that were capable of hydrolyzing the starch.

Lipid Hydrolysis

1. Examine the tributyrin agar plate cultures for the presence or absence of a clear area, or zone of lipolysis, surrounding the growth of each of the organisms. Record your results.

2. Based on your observations, determine and record which organisms were capable of hydrolyzing the lipid.

Casein Hydrolysis

1. Examine the milk agar plate cultures for the presence or absence of a clear area, or zone of proteolysis, surrounding the growth of each of the bacterial test organisms. Record your results.

2. Based on your observations, determine and record which of the organisms were capable of hydrolyzing the milk protein casein.

Gelatin Hydrolysis

1. Place all gelatin deep tube cultures into a refrigerator at 4°C for 30 minutes.

2. Examine all the cultures to determine whether the medium is solid or liquid. Record your results.

3. Based on your observations following the 2-day and 7-day incubation periods, determine and record: (a) which organisms were capable of hydrolyzing gelatin and (b) the rate of hydrolysis.

Notes and Results

DIFFERENTIATION OF ENTERIC BACILLI

The IMViC Tests

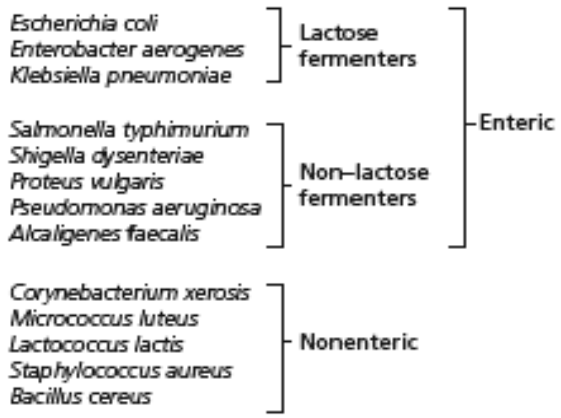
Identification of enteric bacilli is of prime importance in controlling intestinal infections by preventing contamination of food and water supplies. The groups of bacteria that can be found in the intestinal tract of humans and lower mammals are classified as members of the family **Enterobacteriaceae**. They are short, Gram negative, non spore- forming bacilli. Included in this family are:

1. Pathogens, such as members of the genera *Salmonella* and *Shigella.*

2. Occasional pathogens, such as members of the genera *Proteus* and *Klebsiella.*

3. Normal intestinal flora, such as members of the genera *Escherichia* and *Enterobacter*, which are saprophytic inhabitants of the intestinal tract.

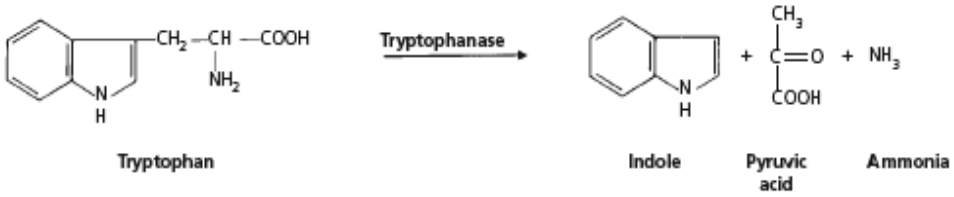
Differentiation of the principal groups of Enterobacteriaceae can be accomplished on the basis of their biochemical properties and enzymatic reactions in the presence of specific substrates. The **IMViC** series of tests (**indole**, **methyl red**, **Voges- Proskauer**, and **citrate utilization**) can be used. The biochemical reactions that occur during the IMViC tests are shown below. The enteric organisms are subdivided as lactose fermenters and non–lactose fermenters.



Principle

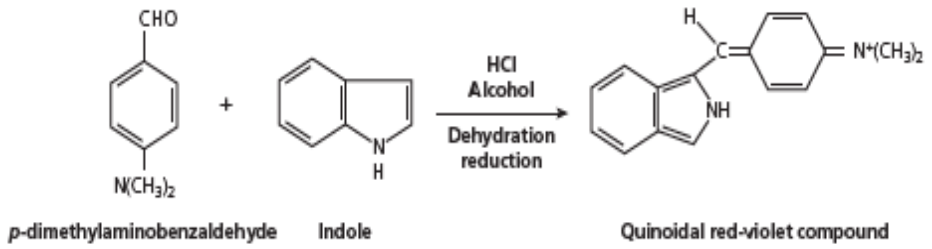
A) Tryptophanase test

Tryptophan is an essential amino acid that can undergo oxidation by way of the enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme **tryptophanase**. The chemistry of this reaction is illustrated in the following Figure. This ability to hydrolyze tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a biochemical marker.



Enzymatic degradation of tryptophan

In this experiment, SIM agar, which contains the substrate tryptophan, is used. The presence of indole is detectable by adding Kovac's reagent, which produces a cherry red reagent layer. This color is produced by the reagent, which is composed of *p*-dimethyl aminobenzaldehyde, butanol, and hydrochloric acid. Indole is extracted from the medium into the reagent layer by the acidified butyl alcohol component and forms a complex with the *p*-dimethyl aminobenzaldehyde, yielding the cherry red color. The chemistry of this reaction is illustrated in the following Figure. Cultures producing a red reagent layer following addition of Kovac's reagent are indole positive; an example of this is *E. coli*. The absence of red coloration demonstrates that the substrate tryptophan was not hydrolyzed and indicates an indole negative reaction.



Indole reaction with Kovac's reagent

Cultures

24- to 48-hour Trypticase soy broth cultures of *E. coli*, *P. vulgaris*, and *E. aerogenes* for the short version.

media

4 SIM agar deep tubes per designated student group:

reagent

Kovac's reagent.

Equipment

Microincinerator or Bunsen burner, inoculating needle, test tube rack, and glassware marking pencil.

Procedure Lab One

1. Using aseptic technique, inoculate each experimental organism into its appropriately labeled deep tube by means of a stab inoculation. The last tube will serve as a control.

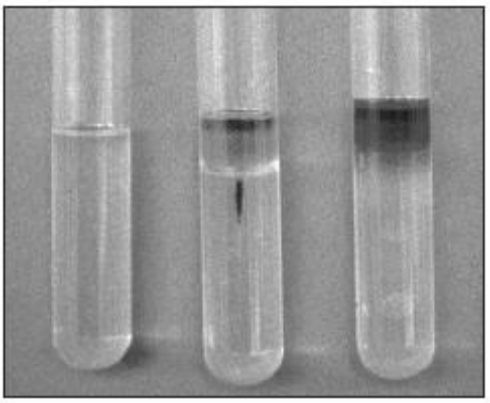
2. Incubate tubes for 24 to 48 hours at 37°C.

Procedure Lab Two

1. Add 10 drops of Kovac's reagent to all deep tube cultures and agitate the cultures gently.

2. Examine the color of the reagent layer in each culture (refer to the following Figure). Record your results in a chart.

3. Based on your observations, determine and record whether or not each organism was capable of hydrolyzing the tryptophan.



Indole production test.
(a) Uninoculated, (b) negative, and (c) positive.

B) Methyl Red Test

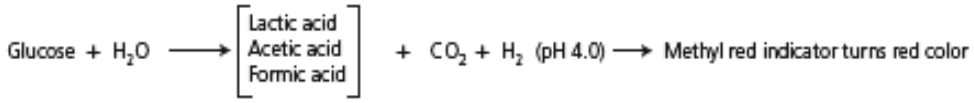
Principle

The hexose monosaccharide **glucose** is the major substrate utilized by all enteric organisms for energy production. The end products of this process will vary depending on the specific enzymatic pathways present in the bacteria. In this test, the pH indicator methyl red detects the presence of large concentrations of acid end products. Although most enteric microorganisms ferment glucose with the production of organic acids, this test is of value in the separation of *E. coli* and *E. aerogenes.*

Both of these organisms initially produce organic acid end products during the early incubation period. The low acidic pH (4) is stabilized and maintained by *E. coli* at the end of incubation. During the later incubation period, *E. aerogenes* enzymatically converts these acids to nonacidic end products, such as 2,3-butanediol and acetoin (acetylmethyl carbinol), resulting in an elevated pH of approximately 6. The glucose fermentation reaction generated by *E. coli* is illustrated

in the following Figure. As shown, at a pH of 4.4 or lower, the methyl red indicator in the pH range of 4 will turn red, which is indicative of a positive test. At a pH of 6.2 or higher, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicator turns yellow

and is a negative test. Production and detection of the nonacidic end products from glucose fermentation by *E. aerogenes* is amplified in Part C of this exercise, the Voges- Proskauer test, which is performed simultaneously with the methyl red test.



Glucose fermentation reaction with methyl red pH reagent

Cultures

24- to 48-hour Trypticase soy broth cultures of *E. coli*, *E. aerogenes*, and *K. pneumoniae.* In Lab Two, aliquots of these experimental cultures must be set aside for the Voges- Proskauer test.

media

4 MR- VP broth per designated student group.

reagent

Methyl red indicator.

Equipment

Microincinerator or Bunsen burner, inoculating loop, test tubes, and glassware marking pencil.

Procedure Lab One

1. Using aseptic technique, inoculate each experimental organism into its appropriately labeled tube of medium by means of a loop inoculation. The last tube will serve as a control.

2. Incubate all cultures for 24 to 48 hours at 37°C.

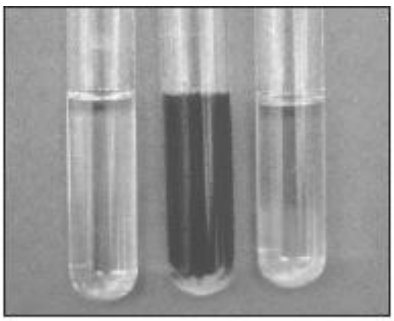
Procedure Lab Two

1. Transfer approximately one-third of each culture into an empty test tube and set these tubes aside for the Voges- Proskauer test.

2. Add five drops of the methyl red indicator to the remaining aliquot of each culture.

3. Examine the color of all cultures (refer to the following Figure). Record the results in a chart.

4. Based on your observations, determine and record whether or not each organism was capable of fermenting glucose with the production and maintenance of a high concentration of acid.



Methyl red test.
(a) Uninoculated, (b) positive, and (c) negative.

C) Voges- Proskauer Test

Principle

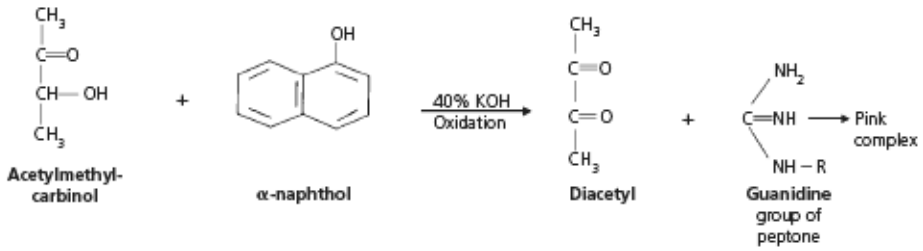
The Voges- Proskauer test determines the capability of some organisms to produce nonacidic or neutral end products, such as acetylmethylcarbinol, from the organic acids that result from glucose metabolism. This glucose fermentation, which is characteristic of *E. aerogenes*, is illustrated in the following Figure.

$$\text{Glucose} + O_2 \longrightarrow \underset{\text{acid}}{\text{Acetic}} \longrightarrow \left[\begin{array}{l}\text{2,3-butanediol} \\ \text{acetylmethylcarbinol}\end{array}\right] + CO_2 + H_2\ (\text{pH } 6.0)$$

Glucose fermentation by E. aerogenes

The reagent used in this test, Barritt's reagent, consists of a mixture of alcoholic anaphthol and 40% potassium hydroxide solution. Detection of acetylmethyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the α- naphthol catalyst and a guanidine group that is present in the peptone of the MR-

VP medium. As a result, a pink complex is formed, imparting a rose color to the medium. The chemistry of this reaction is illustrated
in the following Figure.



Acetylmethylcarbinol reaction with Barritt's reagent

Development of a deep rose color in the culture 15 minutes following the addition of Barritt's reagent is indicative of the presence of acetylmethyl carbinol and represents a positive result. The absence of rose coloration is a negative result.

Cultures

24- to 48-hour Trypticase soy broth cultures of *E. coli*, *E. aerogenes*, and *K. pneumoniae*. Note: Aliquots of these experimental cultures must be set aside from the methyl red test.

reagent

Barritt's reagents A and B.

Equipment

Microincinerator or Bunsen burner, inoculating loop, and glassware marking pencil.

Procedure Lab One

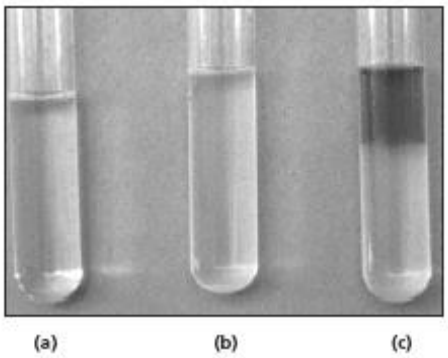
Refer to the methyl red test in Part B of this exercise.

Procedure Lab Two

1. To the aliquots of each broth culture separated during the methyl red test, add 10 drops of Barritt's reagent A and shake the cultures. Immediately add 10 drops of Barritt's reagent B and shake. Re shake the cultures every 3 to 4 minutes.

2. Examine the color of the cultures 15 minutes after the addition of Barritt's reagent. Record your results.

3. Based on your observations, determine and record whether or not each organism was capable of fermenting glucose with ultimate production of acetylmethyl carbinol.



Voges- Proskauer test
(a) Uninoculated, (b) negative, and (c) positive

Notes and Results

CITRATE UTILIZATION TEST

Principle

In the absence of fermentable glucose or lactose, some microorganisms are capable of using **citrate** as a carbon source for their energy. This ability depends on the presence of a **citrate permease** that facilitates the transport of citrate in the cell. Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme **citrase**, which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide. During this reaction, the medium becomes alkaline—the carbon dioxide that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of sodium carbonate changes the bromothymol blue indicator incorporated into the medium from green to deep Prussian blue. Following incubation, citrate- positive cultures are identified by the presence of growth on the surface of the slant, which is accompanied by blue coloration, as seen with *E. aerogenes*. Citrate-negative cultures will show no growth, and the medium will remain green.

Cultures

24- to 48- hour Trypticase soy broth cultures of *E. coli*, *E. aerogenes,* and *K. pneumoniae*.

media

4 Simmons citrate agar slants per designated student group.

Equipment

Microincinerator or Bunsen burner, inoculating needle, test tube rack, and glassware marking pencil.

Procedure Lab One

1. Using aseptic technique, inoculate each organism into its appropriately labeled tube by means of streak inoculation. The last tube will serve as a control.

2. Incubate all cultures for 24 to 48 hours at 37°C.

Procedure Lab Two

1. Examine all agar slant cultures for the presence or absence of growth and coloration of the medium. Record your results.

2. Based on your observations, determine and record whether or not each organism was capable of using citrate as its sole source of carbon.

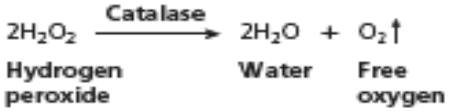
Note that negative tubes, showing no growth on slant surface, will have green colour and positive tubes, showing growth on slant surface will have blue colour.

Notes and Results

CATALASE TEST

Principle

During aerobic respiration, microorganisms produce hydrogen peroxide and, in some cases, an extremely toxic superoxide. Accumulation of these substances will result in death of the organism unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. Organisms capable of producing **catalase** rapidly degrade hydrogen peroxide as illustrated:



Aerobic organisms that lack catalase can degrade especially toxic superoxides using the enzyme **superoxide dismutase**; the end product of a superoxide dismutase is H_2O_2, but this is less toxic to the bacterial cells than are the superoxides. The inability of strict anaerobes to synthesize catalase, peroxidase, or superoxide dismutase may explain why oxygen is poisonous to these microorganisms. In the absence of these enzymes, the toxic concentration of H_2O_2 cannot be degraded when these organisms are cultivated in the presence of oxygen.

Catalase production can be determined by adding the substrate H_2O_2 to an appropriately incubated culture. If catalase is present, the chemical reaction mentioned is indicated by bubbles of free oxygen gas O_2. This is a positive catalase test; the absence of bubble formation is a negative catalase test. The following Figure shows the results of the catalase test using (a) the tube method, (b) the plate method, and (c) slide method.

Cultures

24- to 48-hour broth cultures of *Staphylococcus aureus* , *Micrococcus luteus*, and *Lactococcus lactis* for the short version.

media

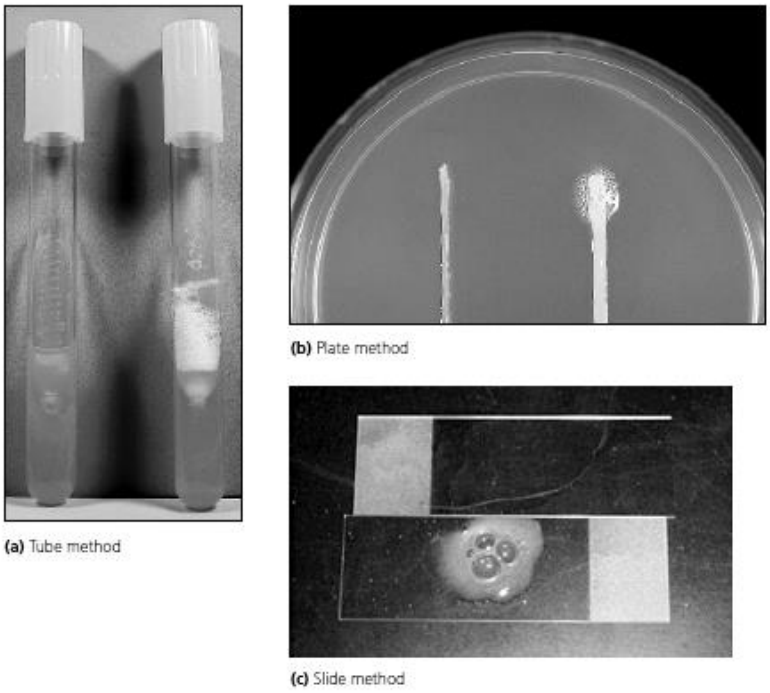
4 Trypticase soy agar slants per designated student group: for the short version.

reagent

3% hydrogen peroxide.

Equipment

Tube method: Microincinerator or Bunsen burner, inoculating loop, test tube rack, and glassware marking pencil.



Catalase test. Negative results are shown on the left and positive results on the right in the (a) tube method and (b) plate method. Negative results are shown on the top and positive results on the bottom in the (c) slide method.

Slide method: Microincinerator or Bunsen burner, inoculating loop, glassware marking pencil, 4 glass microscope slides, Petri dish and cover.

Procedure Lab One

(Tube method)

1. Using aseptic technique, inoculate each experimental organism into its appropriately labeled tube by means of a streak inoculation. The last tube will serve as a control.

2. Incubate all cultures for 24 to 48 hours at 37°C.

Procedure Lab Two

1. Allow three or four drops of the 3% hydrogen peroxide to flow over the entire surface of each slant culture.

2. Examine each culture for the presence or absence of bubbling or foaming. Record your results.

3. Based on your observations, determine and record whether or not each organism was capable of catalase activity.

Slide method

1. Label slides with the names of the organisms.

2. Using a sterile loop, collect a small sample of the first organism from the culture tube and transfer it to the appropriately labeled slide.

3. Place the slide in the Petri dish.

4. Place one drop of 3% hydrogen peroxide on the sample. Do not mix. Place the cover on the Petri dish to contain any aerosols.

5. Observe for immediate presence of bubble formation. Record your results.

6. Repeat Steps 2 through 5 for the remaining test organisms.

Notes and Results

MICROBIOLOGY OF SOIL

Soil is often thought of as an inert substance by the average person. However, contrary to this belief, it serves as a repository for many life forms, including a huge and diverse microbial population. The beneficial activities of these soil inhabitants far outweigh their detrimental effects. Life on this planet could not be sustained in the absence of microorganisms that inhabit the soil. This flora is essential for degradation of organic matter deposited in the soil, such as dead plant and animal tissues and animal wastes. Hydrolysis of these macromolecules by microbial enzymes supplies and replenishes the soil with basic elemental nutrients. By means of enzymatic transformations, plants assimilate these nutrients into organic compounds essential for their growth and reproduction. In turn, these plants serve as a source of nutrition for animals and man. Thus, many soil microorganisms play a vital role in a number of elemental cycles, such as the nitrogen cycle, the carbon cycle, and the sulfur cycle.

Nitrogen Cycle

The nitrogen cycle is concerned with the enzymatic conversion of complex nitrogenous compounds in the soil and atmosphere into nitrogen compounds that plants are able to use for the synthesis of essential macromolecules, including nucleic acids, amino acids, and proteins. The four distinct phases in this cycle are as follows:

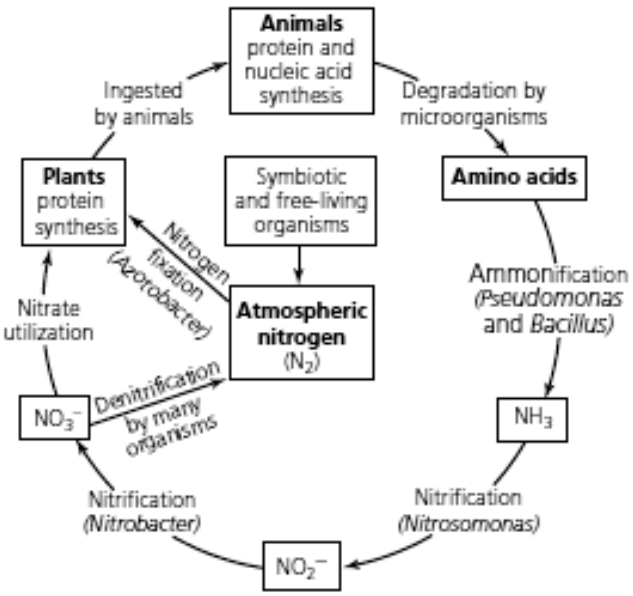
1. Ammonification: Soil microorganisms sequentially degrade nitrogenous organic compounds derived from dead plants and animals deposited in the soil. The degraded nitrogenous organic compounds are converted to inorganic nitrogen compounds and then to ammonia.

2. Nitrification: In this two- step process, (1) ammonia is oxidized to nitrite ions $NO2^{-2}$ by an aerobic species of *Nitrosomonas*, and then (2) nitrites are converted to nitrate ions $NO3^{-2}$ by another aerobic species,

Nitrobacter. Nitrates are released into the soil and are assimilated as a nutritional source by plants.

3. Denitrification: Nitrates $NO3^{-2}$ that are not used by plants are reduced to gaseous nitrogen (N_2) and are liberated back into the atmosphere by certain groups of microorganisms.

4. Nitrogen fixation: This vital process involves the chemical combination of gaseous nitrogen (N_2) with other elements to form fixed nitrogen (nitrogen- containing compounds), which are useful for plant growth.



The nitrogen cycle

The two types of microorganisms involved in this process are free- living and symbiotic. Free- living microorganisms include *Azotobacter, Pseudomonas, Clostridium,* and *Bacillus,* as well as some species of yeast. Symbiotic microorganisms, such as *Rhizobium*, grow in tumor- like nodules in the roots of leguminous plants, and use nutrients in the plant sap to fix gaseous nitrogen as ammonia for its subsequent assimilation into plant proteins. Animals then consume the leguminous plants and convert

plant protein to animal protein, completing the process. The nitrogen cycle is shown above.

Carbon Cycle

Carbon dioxide is the major carbon source for the synthesis of organic compounds. The carbon cycle is basically represented by two steps:

1. Oxidation of organic compounds to carbon dioxide with the production of energy and heat by heterotrophs.

2. Fixation of carbon dioxide into organic compounds by green plants and some bacteria, the autotrophic soil flora.

Sulfur Cycle

Elemental sulfur and proteins cannot be utilized by plants for growth. They must first undergo enzymatic conversions into inorganic sulfur-containing compounds. The basic steps in the sulfur cycle are:

1. Degradation of proteins into hydrogen sulfide (H_2S) by many heterotrophic microorganisms.

2. Oxidation of H_2S to sulfur (S) by a number of bacterial genera, such as *Beggiatoa.*

3. Oxidation of sulfur to utilizable sulfate SO_4 by several chemoautotrophic genera, such as *Thiobacillus.*

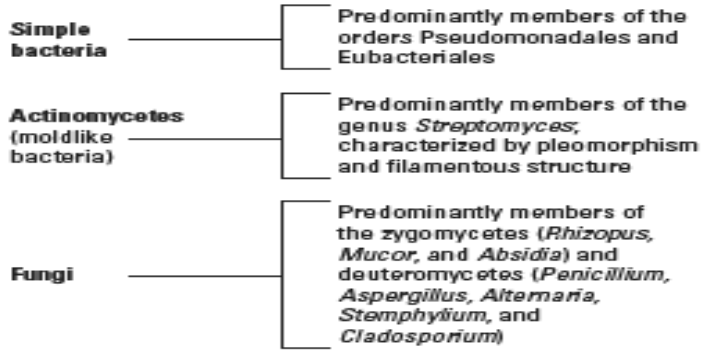
Some soil microorganisms also play a role in the enzymatic transformation of other elements, such as phosphorus, iron, potassium, zinc, manganese, and selenium. These biochemical changes make the minerals available to plants in a soluble form.

ENUMERATION OF SOIL MICROBES

Principle

Soil contains very diverse of microorganisms, including bacteria, fungi, protozoa, algae, and viruses. The most prevalent are bacteria, including the mold- like actinomycetes, and fungi. It is essential to bear in mind that the soil environment differs from one location to another and from one period

of time to another. Therefore, factors, including moisture, pH, temperature, gaseous oxygen content, and organic and inorganic composition of soil are crucial in determining the specific microbial flora of a particular sample.



Just as the soil differs, microbiological methods used to analyze soil also vary. A single technique cannot be used to count all the different types of microorganisms present in a given soil sample because no one laboratory cultivation procedure can provide all the physical and nutritional requirements necessary for the growth of a greatly diverse microbial population. In this experiment, only the relative numbers of bacteria, are determined. The method used is the serial dilution– agar plate procedure.

Materials

Soil

1 g sample of fine soil in a flask containing 99 ml of sterile water; flask labeled 1:100 dilution (10^{-2}).

Medium

four nutrient agar deep tubes, and two 99 ml flasks

of sterile water.

Equipment

Bunsen burner, 12 Petri dishes, colony counter, mechanical hand counter, sterile 1-ml pipettes, mechanical pipetting device, L- shaped bent glass

rod, turntable (optional), 95% alcohol in a 500 ml beaker, and glassware marking pencil.

Procedure Lab One

1. Liquefy the nutrient agar deep tubes in an autoclave or by boiling. Cool the molten agar tubes and maintain in a water bath at 45°C.

2. Using a glassware marking pencil, label the Petri dishes of nutrient agar as follows: 10−4, 10−5, 10−6, and 10−7 (to
be used for enumeration of bacteria).

3. With a glassware marking pencil, label the soil sample flask as Flask 1, and label the 99 ml sterile water Flasks 2 and 3.

4. Vigorously shake the provided soil sample dilution of 1:100 (10^{-2}) approximately 30 times, with your elbow resting on the table.

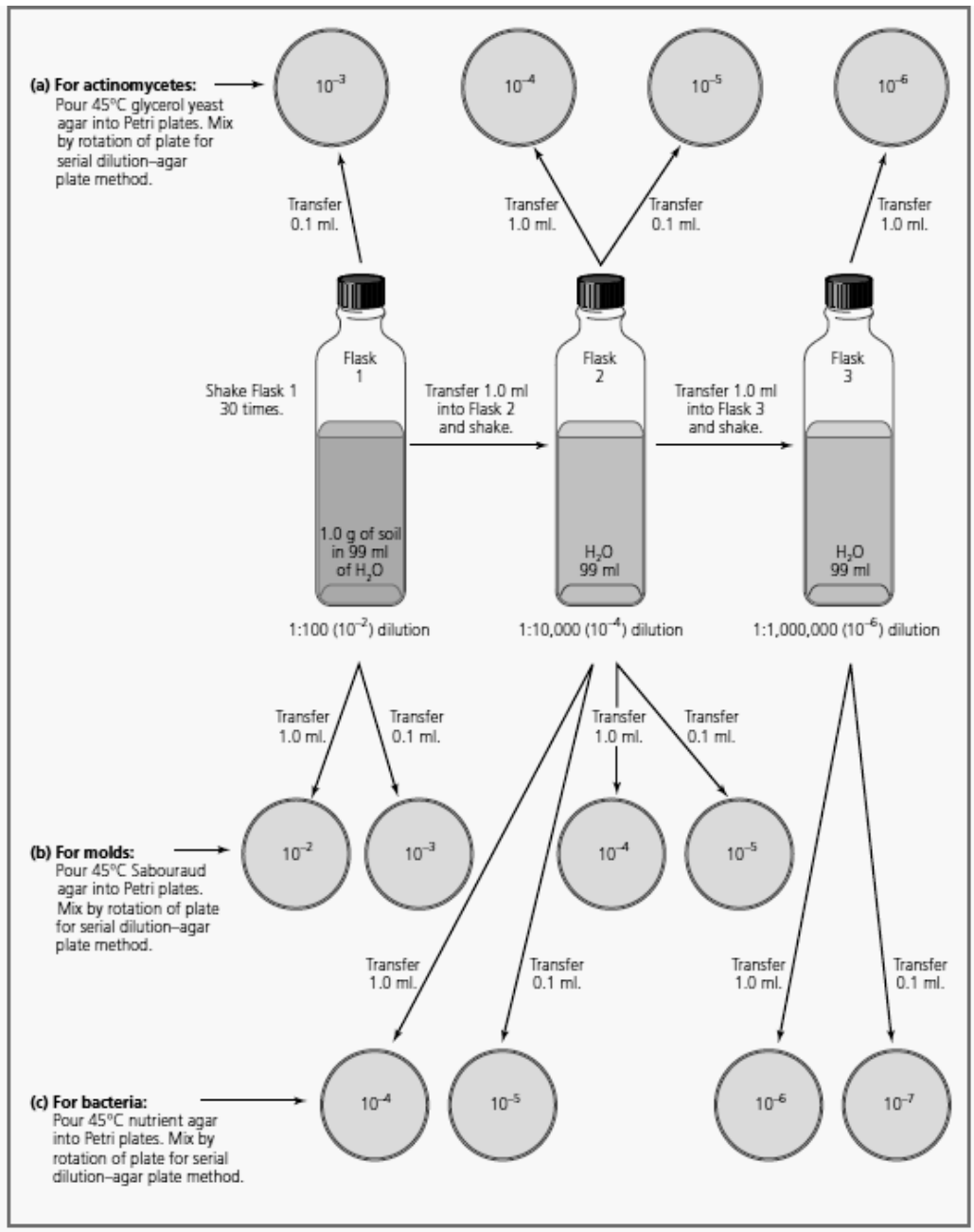
5. With a sterile 1 ml pipette, transfer 1 ml of the provided soil sample dilution to Flask 2 and shake vigorously as before. The final dilution is 1:10,000 (10^{-4}).

6. Using another sterile 1 ml pipette, transfer 1 ml of Dilution 2 to Flask 3 and shake vigorously as before. The final dilution is 1:1,000,000 (10^{-6}).

7. Using sterile 1 ml pipettes and aseptic technique, transfer 1 ml of dilution 2 into plate to effect a 10^{-4} dilution. Transfer 0.1 ml of Dilution 2 into plate to effect a 10^{-5} dilution. Transfer 1 ml of Dilution 3 into plate to effect a 10^{-6} dilution. Transfer 0.1 ml of Dilution 3 into plate to effect a 10^{-7} dilution.

8. Check the temperature of the molten agar medium to be sure that the temperature is 45°C. Remove the tubes from the water bath and wipe the outside surface dry with a paper towel. Using the pour plate technique, pour the liquefied agar into the plates as shown in the following Figure and rotate gently to ensure uniform distribution of the cells in the medium.

9. Incubate the plates in an inverted position at 25°C. Perform colony counts on nutrient agar plate cultures in 2 to 3 days.



Procedure for enumeration of soil microorganisms

Procedure Lab Two

1. Using an electronic colony counter or a colony counter and a mechanical hand counter, observe all the colonies on each nutrient agar plate 2 to 3 days after incubation begins. Plates with more than 300 colonies cannot be counted and should be designated as **too numerous to**

count (TNTC); plates with fewer than 30 colonies should be designated as **too few to count (TFTC)**. Count only plates with between 30 and 300 colonies.

2. Determine the number of organisms per milliliter of original culture on all plates other than those designated as TFTC or TNTC by multiplying the number of colonies counted by the dilution factor.

3. Record your observations and calculated cell counts per gram of sample in the Lab Report chart.

Organism	Dilution	Number of Colonies	Organisms per Gram of Soil
Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		
Actinomycetes	10^{-3}		
	10^{-4}		
	10^{-5}		
	10^{-6}		
Molds	10^{-2}		
	10^{-3}		
	10^{-4}		
	10^{-5}		

Results and observations

Nitrogen- Fixing Bacteria

Among the most beneficial microorganisms of the soil are those that are able to convert gaseous nitrogen of the air to "fixed forms" of nitrogen that can be utilized by other bacteria and plants. Without these nitrogen-fixers, life on this planet would probably disappear within a relatively short period of time. The utilization of free nitrogen gas by fixation can be accomplished by organisms that are able to produce the essential enzyme **nitrogenase**. This enzyme, in the presence of traces of molybdenum, enables the organisms to combine atmospheric nitrogen with other elements to form organic compounds in living cells. In organic combinations nitrogen is more reduced than when it is free. From these organic compounds, upon their decomposition, the nitrogen is liberated in a fixed form, available to plants either directly or through further microbial action.

The most important nitrogen-fixers belong to two families: **Azotobacteraceae** and **Rhizobiaceae.** Other organisms of less importance that have this ability are a few strains of *Klebsiella,* some species of *Clostridium,* the cyanobacteria, and photosynthetic bacteria.

In this exercise we will concern ourselves with two activities: the isolation of *Azotobacter* from garden soil and the demonstration of *Rhizobium* in root nodules of legumes.

AZOTOBACTERACEAE

Bergey's Manual of Systematic Bacteriology, volume 1, section 4, lists two genera of bacteria in family Azotobacteraceae that fix nitrogen as free-living organisms under aerobic conditions: *Azotobacter* and *Azomonas.* The basic difference between these two genera is that *Azotobacter* produces drought resistant cysts and *Azomonas* does not. Aside from the presence or absence of cysts, these two genera are very

similar. Both are large gram- negative motile rods that may be ovoid or coccoidal in shape (pleomorphic). Catalase is produced by both genera.

There are six species of *Azotobacter* and three species of *Azomonas.*

The following Figure illustrates the overall procedure for isolating Azotobacteraceae from garden soil. Note that a small amount of rich garden soil is added to a bottle of nitrogen- free medium that contains glucose as a carbon source. The bottle of medium is incubated in a horizontal position for 4 to 7 days at 30° C.

After incubation, a wet mount slide is made from surface growth to see if typical azotobacterlike organisms are present. If organisms are present, an agar plate of the same medium, less iron, is used to streak out for isolated colonies. After another 4 to 7 days incubation, colonies on the plate are studied and more slides are made in an attempt to identify the isolates.

The N_2- free medium used here contains glucose for a carbon source and is completely lacking in nitrogen. It is selective in that only organisms that can use nitrogen from the air and use the carbon in glucose will grow on it. All species of *Azotobacter* and *Azomonas* are able to grow on it. The metallic ion molybdenum is included to activate the enzyme nitrogenase, which is involved in this process.

FIRST PERIOD (ENRICHMENT)

Proceed as follows to inoculate a bottle of the nitrogen- free glucose medium with a sample of garden soil.

Materials:

- 1 bottle (50 ml) N_2-free glucose medium (Thompson-Skerman)
- rich garden soil (neutral or alkaline)
- spatula

1. With a small spatula, put about 1 gm of soil into the bottle of medium. Cap the bottle and shake it sufficiently to mix the soil and medium.

2. Loosen the cap slightly and incubate the bottle at 30° C for 4 to 7 days. Since the organisms are strict aerobes, it is best to incubate the bottle horizontally to provide maximum surface exposure to air.

SECOND PERIOD (PLATING OUT)

During this period a slide will be made to make certain that organisms have grown on the medium. If the culture has been successful, a streak plate will be made on nitrogen- free, iron- free agar. Proceed as follows:

Materials:

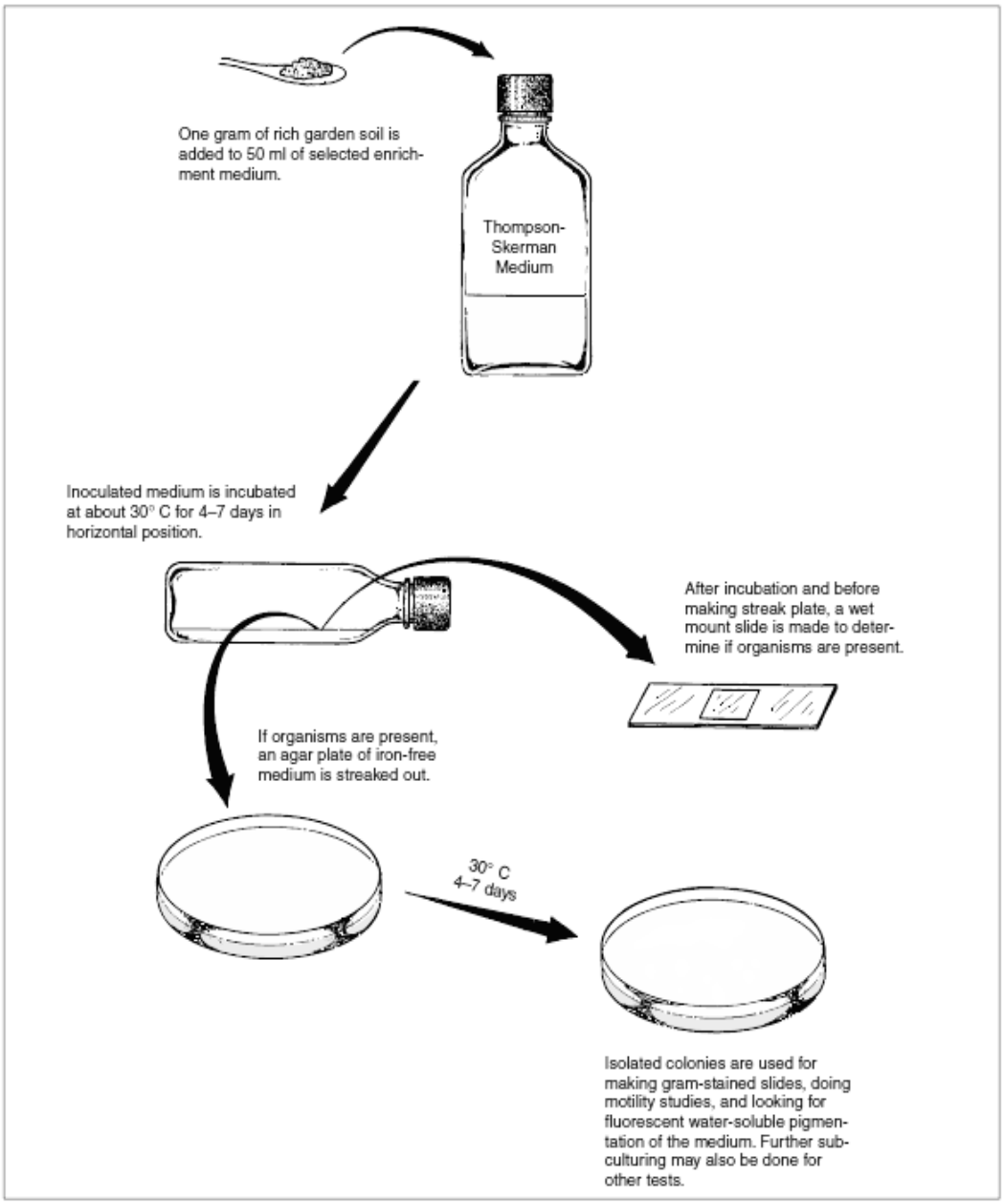
- microscope slides and cover glasses
- microscope with phase-contrast optics
- 1 agar plate of nitrogen- free, iron- free glucose medium

1. After 4 to 7 days incubation, carefully move the bottle of medium to your desktop without agitating the culture.
2. Make a wet mount slide with a few loopfuls from the surface of the medium and examine under oil immersion, preferably with phase- contrast optics. Look for large ovoid to rod- shaped organisms, singly and in pairs.
3. If azotobacter- like organisms are seen, note whether or not they are motile and if cysts are present. Cysts look much like endospores in that they are refractile. Since cysts often take 2 weeks to form, they may not be seen.
4. If the presence of azotobacter- like organisms is confirmed, streak an agar plate of nitrogen- free, iron- free medium, using a good isolation streak pattern. Ferrous sulfate has been left out of this medium to facilitate the detection of water- soluble pigments.
5. Incubate the plate at 30° C for 4 or 5 days. A longer period of incubation is desirable for cyst formation.

Alternatively, a more simple procedure can be as follows:

The N- free medium is inoculated in one or two plates by spreading some soil o the surface. After incubation for one to two days mucous colonies

on the surface of solid medium is indicative of N- fixing organisms. Take some of the mucous growth on a clean slide , stain with Gram staining and observe under light microscope by using oil immersion lens then proceed with step (3) above.



Enrichment and isolation procedure for *Azotobacter* and *Azomonas*

Microbiology of Water

The microorganisms of natural waters are extremely diverse. The numbers and types of bacteria present will depend on the amounts of organic matter present, the presence of toxic substances, the water's saline content, and environmental factors such as pH, temperature, and aeration. The largest numbers of heterotrophic forms will exist on the bottoms and banks of rivers and lakes where organic matter predominates. Open water in the center of large bodies of water, free of floating debris, will have small numbers of bacteria. Many species of autotrophic types are present, however, that require only the dissolved inorganic salts and minerals that are present.

The threat to human welfare by contamination of water supplies with sewage is a prime concern of everyone. The enteric diseases such as cholera, typhoid fever, and bacillary dysentery often result in epidemics when water supplies are not properly protected or treated. Thus, our prime concern in this unit is the sanitary phase of water microbiology.

Bacteriological Examination of Water:

Qualitative Tests

Principle

The three basic tests to detect coliform bacteria in water are presumptive, confirmed, and completed. The tests are performed sequentially on each sample under analysis. They detect the presence of coliform bacteria (indicators of fecal contamination), the Gram -ve, non–spore- forming bacilli that ferment lactose with the production of acid and gas that is detectable following a 24-hour incubation period at 37°C.

The Presumptive test

The **presumptive test** is specific for detection of coliform bacteria. Measured aliquots of the water to be tested are added to a lactose fermentation broth containing an inverted gas vial. Because these bacteria

are capable of using lactose as a carbon source (the other enteric organisms are not), their detection is facilitated by the use of this medium. In this experiment, you will use lactosefermentation broth containing an inverted Durhamtube for gas collection.

Tubes of this lactose medium are inoculated with 10-ml, 1-ml, and 0.1-ml aliquots of the water sample. The series consists of at least three groups, each composed of five tubes of the specified medium. The tubes in each group are then inoculated with the designated volume of the water sample, as described under "Procedure: Lab One."

The greater the number of tubes per group, the greater the sensitivity of the test. Development of gas in any of the tubes is **presumptive** evidence of the presence of coliform bacteria in the sample. The presumptive test also enables the microbiologist to obtain some idea of the number of coliform organisms present by means of the **most probable number (MPN) test**. The MPN is estimated by determining the number of tubes in each group that show gas following the incubation period (**MPN Tables are shown later**).

The Confirmed test

The presence of a positive or doubtful presumptive test immediately suggests that the water sample is nonpotable. Confirmation of these results is necessary because positive presumptive tests may be the result of organisms of noncoliform origin that are not recognized as indicators of fecal pollution. The **confirmed test** requires that selective and differential media (e.g., eosin–methylene blue (EMB) or Endo agar) be streaked from a positive lactose broth tube obtained from the presumptive test. The nature of the differential and selective media was discussed before. Eosin–methylene blue contains the dye methylene blue, which inhibits the growth of Gram +ve organisms. In the presence of an acid environment, EMB forms a complex that precipitates out onto the coliform colonies,

producing dark centers and a green metallic sheen. The reaction is characteristic for *Escherichia coli*, the major indicator of fecal pollution. Endo agar is a nutrient medium containing the dye fuchsin, which is present in the decolorized state. In the presence of acid produced by the coliform bacteria, fuchsin forms a dark pink complex that turns the *E. coli* colonies and the surrounding medium pink.

The Completed test

The **completed test** is the final analysis of the water sample. It is used to examine the coliform colonies that appeared on the EMB or Endo agar plates used in the confirmed test. An isolated colony is picked up from the confirmatory test plate and inoculated into a tube of lactose broth and streaked on a nutrient agar slant to perform a Gram stain. Following inoculation and incubation, tubes showing acid and gas in the lactose broth and presence of Gram -ve bacilli on microscopic examination are further confirmation of the presence of *E. coli,* and they are indicative of a positive completed test.

Media

Lab One (per student group): 15 double strength lactose fermentation broths (LB2X) and 30 single strength lactose fermentation broths (LB1X).
Lab Two (three each per student group): eosin–methylene blue agar plates or Endo agar plates.
Lab Three (three each per student group): nutrient agar slants and lactose fermentation broths.

Reagents

Lab Three: Crystal violet, Gram's iodine, 95% ethyl alcohol, and safranin.

Equipment

Lab One: Bunsen burner, 45 test tubes, test tube rack, sterile 10-ml pipettes, sterile 1-ml pipettes, sterile 0.1-ml pipettes, mechanical pipetting device, and glassware marking pencil.

Lab Two: Bunsen burner, glassware marking pencil, and inoculating loop.

Lab Three: Bunsen burner, staining tray, inoculating loop, lens paper, bibulous paper, microscope, and glassware marking pencil.

Procedure Lab One

Presumptive test

Exercise care in handling sewage waste water sample because enteric pathogens may be present**.**

1. Set up three separate series consisting of three groups, a total of 15 tubes per series, in a test tube rack; for each tube, label the water source and volume of sample inoculated as illustrated below.

Series 1: Sewage water	5 tubes of LB2X-10 ml 5 tubes of LB1X-1 ml 5 tubes of LB1X-0.1 ml
Series 2: Pond water	5 tubes of LB2X-10 ml 5 tubes of LB1X-1 ml 5 tubes of LB1X-0.1 ml
Series 3: Tap water	5 tubes of LB2X-10 ml 5 tubes of LB1X-1 ml 5 tubes of LB1X-0.1 ml

2. Mix sewage plant water sample by shaking thoroughly.

3. Flame bottle and then, using a 10-ml pipette, transfer 10-ml aliquots of water sample to the five tubes labeled LB2X-10 ml.

4. Flame bottle and then, using a 1-ml pipette, transfer 1-ml aliquots of water sample to the five tubes labeled LB1X-1 ml.

5. Flame bottle and then, using a 0.1-ml pipette, transfer 0.1-ml aliquots of water sample to the five tubes labeled LB1X-0.1 ml.

6. Repeat Steps 2 through 5 for the tap and pond water samples.

7. Incubate all tubes for 48 hours at 37°C.

Procedure Lab Two

Presumptive test

1. Examine the tubes from your presumptive test after 24 and 48 hours of incubation. Your results are positive if the Durham tube fills 10% or more

with gas in 24 hours, doubtful if gas develops in the tube after 48 hours, and negative if there is no gas in the tube after 48 hours. Record your results in the Lab Report.

2. Determine the MPN using the following Table, and record your results in the Lab Report.

Confirmed test

1. Label the covers of the three EMB plates or the three Endo agar plates with the source of the water sample (sewage, pond, and tap).

2. Using a positive 24-hour lactose broth culture from the sewage water series from the presumptive test, streak the surface of one EMB or one Endo agar plate, as described in Experiment 3, to obtain discrete colonies.

3. Repeat Step 2 using the positive lactose broth cultures from the pond and tap water series from the presumptive test to inoculate the remaining plates.

4. Incubate all plate cultures in an inverted position for 24 hours at 37°C.

Procedure Lab Three

Confirmed test

1. Examine all the plates from your confirmed test for the presence or absence of *E. coli* colonies (refer to the description of the confirmed test in the experiment introduction). Record your results in the Lab Report.

2. Based on your results, determine whether each of the samples is potable or nonpotable.

The presence of *E. coli* is a positive confirmed test, indicating that the water is nonpotable. The absence of *E. coli* is a negative test, indicating that the water is not contaminated with fecal wastes and is therefore potable. Record your results in the Lab Report.

Completed test

1. Label each tube of nutrient agar slants and lactose fermentation broths with the source of its water sample.

2. Inoculate one lactose broth and one nutrient agar slant with a positive isolated *E. coli* colony obtained from each of the experimental water samples during the confirmed test.

3. Incubate all tubes for 24 hours at 37°C.

Procedure Lab Four

Completed test

1. Examine all lactose fermentation broth cultures for the presence or absence of acid and gas. Record your results in the Lab Report.

2. Prepare a Gram stain, using the nutrient agar slant cultures of the organisms that showed a positive result in the lactose fermentation broth.

3. Examine the slides microscopically for the presence of Gram -ve short bacilli, that are indicative of *E. coli* and thus nonpotable water. In the Lab Report, record your results for Gram stain reaction and morphology of the cells.

The MPN Index per 100 ml for Combinations of Positive and Negative Presumptive Test Results When Five 10-ml, Five 1-ml, and Five 0.1-ml Portions of Sample Are Used

NUMBER OF TUBES WITH POSITIVE RESULTS						NUMBER OF TUBES WITH POSITIVE RESULTS					
FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	95% CONFIDENCE LIMITS		FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	95% CONFIDENCE LIMITS	
				LOWER	UPPER					LOWER	UPPER
0	0	0	<2	0	6	4	2	1	26	7	67
0	0	1	2	<0.5	7	4	3	0	27	9	78
0	1	0	2	<0.5	7	4	3	1	33	9	78
0	2	0	4	<0.5	11	4	4	0	34	11	93
1	0	0	2	0.1	10	5	0	0	23	7	70
1	0	1	4	0.7	10	5	0	1	31	11	89
1	1	0	4	0.7	12	5	0	2	43	14	100
1	1	1	6	1.8	15	5	1	0	33	10	100
1	2	0	6	1.8	15	5	1	1	46	14	120
2	0	0	5	<0.5	13	5	1	2	63	22	150
2	0	1	7	1	17	5	2	0	49	15	150
2	1	0	7	1	17	5	2	1	70	22	170
2	1	1	9	2	21	5	2	2	94	34	230
2	2	0	9	2	21	5	3	0	79	22	220
2	3	0	12	3	28	5	3	1	110	34	250
3	0	0	8	2	22	5	3	2	140	52	400
3	0	1	11	4	23	5	3	3	180	70	400
3	1	0	11	5	35	5	4	0	130	36	400
3	1	1	14	6	36	5	4	1	170	58	400
3	2	0	14	6	36	5	4	2	220	70	440
3	2	1	17	7	40	5	4	3	280	100	710
3	3	0	17	7	40	5	4	4	350	100	710
4	0	0	13	4	35	5	5	0	240	70	710
4	0	1	17	6	36	5	5	1	350	100	1100
4	1	0	17	6	40	5	5	2	540	150	1700
4	1	1	21	7	42	5	5	3	920	220	2600
4	1	2	26	10	70	5	5	4	1600	400	4600
4	2	0	22	7	50	5	5	5	≥2400	700	– – –

Observations and results

Presumptive test

Using he above Table, determine and record the MPN.

Example: If gas appeared in all five tubes labeled LB2X–10, in two of the tubes labeled LB1X–1, and in one labeled LB1X–0.1, the series would be read as 5-2-1. From the MPN table, such a reading would indicate approximately 70 microorganisms per 100 ml of water, with a 95% probability that between 22 and 170 microorganisms are present.

	GAS																	
	LB2X–10					LB1X–1					LB1X–0.1							
	Tube					Tube					Tube							
Water Sample	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**Reading**	**MPN**	**95% Probability Range**
Sewage																		
Pond																		
Tap																		

Confirmed test

	COLIFORMS			
Water Sample	**EMB Plate**	**Endo Agar Plate**	**Potable**	**Nonpotable**
Sewage				
Pond				
Tap				

Completed test

		GRAM STAIN	POTABILITY	
Water Source	**Lactose Broth A/G (+) or (–)**	**Reaction/ Morphology**	**Potable**	**Nonpotable**
Sewage				
Pond				
Tap				