MICROBIOLOGY

A Laboratory Manual

Fifth Edition

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Part I

Basic Laboratory Techniques for Isolation, Cultivation, and Cultural Characterization of Microorganisms

PURPOSES

To become familiar with

1. The types of laboratory equipment and culture media needed to develop and maintain pure cultures.
2. The concept of sterility and the procedures necessary for successful subculturing of microorganisms.
3. Streak-plate and spread-plate inoculations for separation of microorganisms in a mixed microbial population for subsequent pure culture isolation.
4. Cultural and morphological characteristics of microorganisms grown in pure culture.

INTRODUCTION

Microorganisms are ubiquitous. They are found in soil, air, water, food, sewage, and on body surfaces. In short, every area of our environment is replete with them. The microbiologist separates these mixed populations into individual species for study. A culture containing a single unadulterated species of cells is called a **pure culture.** To isolate and study microorganisms in pure culture, the microbiologist requires basic laboratory apparatus and the application of specific techniques, as illustrated in Figure I.1.

Media

The survival and continued growth of microorganisms depend on an adequate supply of nutrients and a favorable growth environment. For the former, most microbes must use soluble low-molecular-weight substances that are frequently derived from the enzymatic degradation of complex nutrients. A solution containing these nutrients is a **culture medium.** Basically, all culture media are liquid, semisolid, or solid. A liquid medium lacks a solidifying agent and is called a **broth medium.** A broth medium supplemented with a solidifying agent called **agar** results in a solid or semisolid medium. Agar is an extract of seaweed, a complex carbohydrate composed mainly of galactose, and is without nutritional value. Agar serves as an excellent solidifying agent because it liquefies at 100°C and solidifies at 40°C. Because of these properties, organisms, especially pathogens, can be cultivated at temperatures of 37.5°C or slightly higher without fear of the medium liquefying. A completely solid medium requires an agar concentration of about 1.5 to 1.8%. A concentration of less than 1% agar results in a

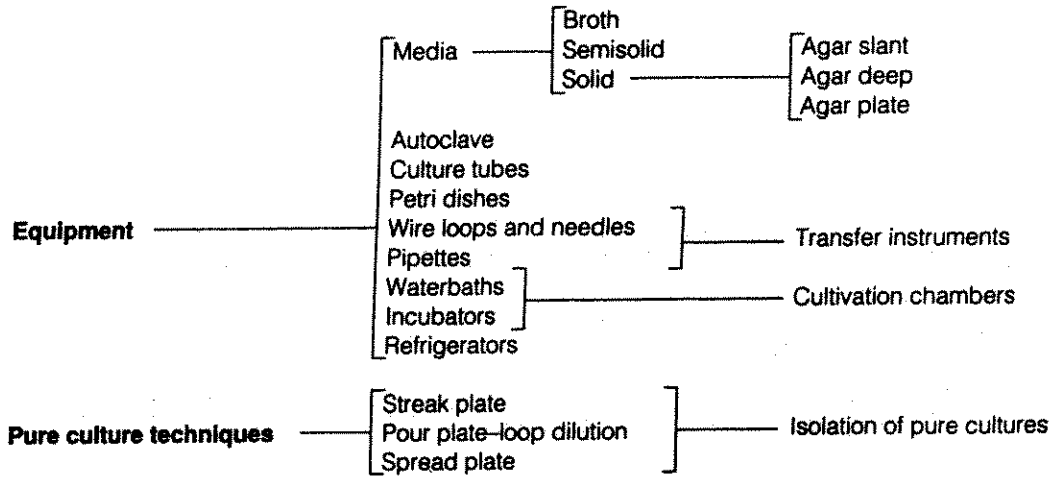


Figure I.1 Laboratory apparatus and culture techniques

semisolid medium. A solid medium has the advantage that it presents a hardened surface on which microorganisms can be grown using specialized techniques for the isolation of discrete colonies. Each colony is a cluster of cells that originates from the multiplication of a single cell and represents the growth of a single species of microorganism. Such a defined and well-isolated colony is a **pure culture.** Also, while in the liquefied state, solid media can be placed in test tubes, which are then allowed to cool and harden in a slanted position, producing **agar slants.** These are useful for maintaining pure cultures. Similar tubes that, following preparation, are allowed to harden in the upright position are designated as **agar deep tubes.** Agar deep tubes are used

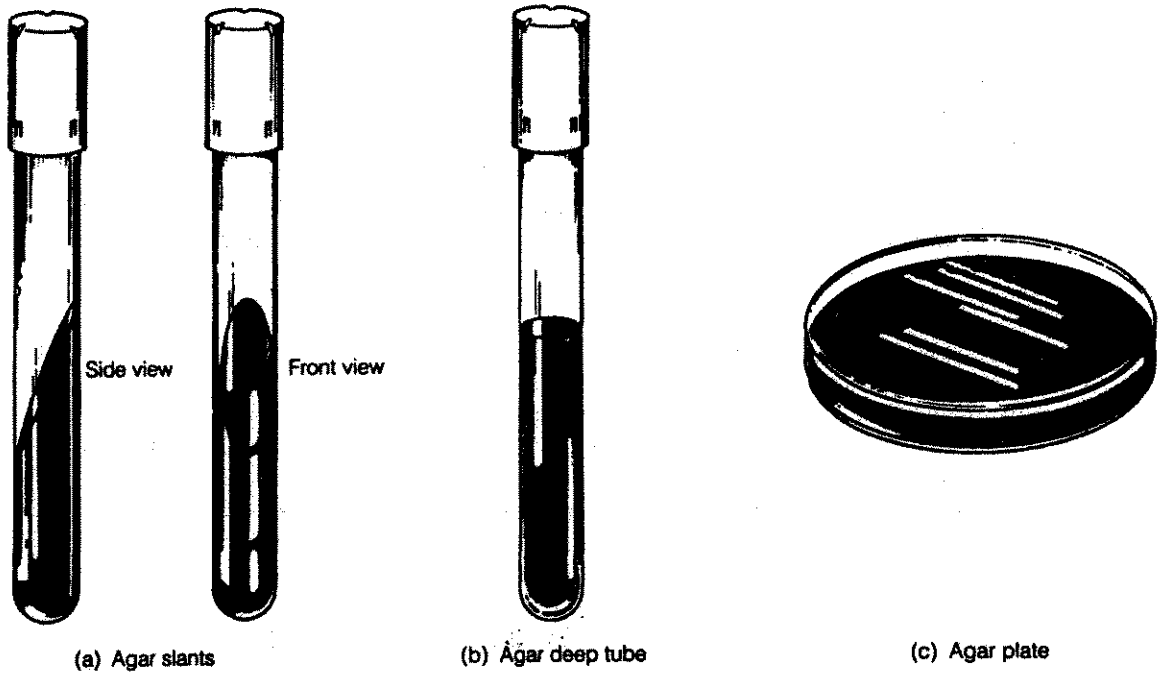


Figure I.2 Forms of solid (agar) media

primarily for the study of the gaseous requirements of microorganisms. However, they may be liquefied in a boiling water bath and poured into Petri dishes, producing **agar plates,** which provide large surface areas for the isolation and study of microorganisms. The various forms of solid media are illustrated in Figure I.2.

In addition to nutritional needs, the environmental factors must also be regulated, including proper pH, temperature, gaseous requirements, and osmotic pressure. A more detailed explanation is presented in Part IV, the section dealing with cultivation of microorganisms; for now, you should simply bear in mind that numerous types of media are available.

Sterilization

Sterility is the hallmark of successful work in the microbiology laboratory. To achieve sterility, it is mandatory that you use sterile equipment and sterile techniques. **Sterilization** is the process of rendering a medium or material free of all forms of life. Although a more detailed discussion is presented in Part IX, the section dealing with control of microorganisms, Figure I.3 is a brief outline of the routine techniques used in the microbiology laboratory.

Culture Tubes and Petri Dishes

Glass **test tubes** and glass or plastic **Petri dishes** are used to cultivate microorganisms. A suitable nutrient medium in the form of broth or agar may be added to the tubes, while only a solid medium is used in Petri dishes. A sterile environment is maintained in culture tubes by various types of closures. Historically, the first type, a cotton plug, was developed by Schroeder and von Dusch in the nineteenth century. Today most laboratories use sleevelike caps made of metal, such as stainless steel, or heat-resistant plastics. The advantage of these closures over the cotton plug is that they are labor-saving and, most of all, slip on and off the test tubes easily.

Petri dishes provide a larger surface area for growth and cultivation. They consist of a bottom dish portion that contains the medium and a larger top portion that serves as a loose cover. Petri dishes are manufactured in various sizes to meet different experimental requirements. For routine purposes, dishes approximately 15 cm in diameter are used. The sterile agar medium is dispensed to previously sterilized dishes from molten agar deep tubes containing 15 to 20 ml of medium, or from a molten sterile medium prepared in bulk and contained in 250- to 500-ml flasks. When cooled to 40°C, the medium will solidify. Remember that **after inoculation, Petri dishes are incubated**

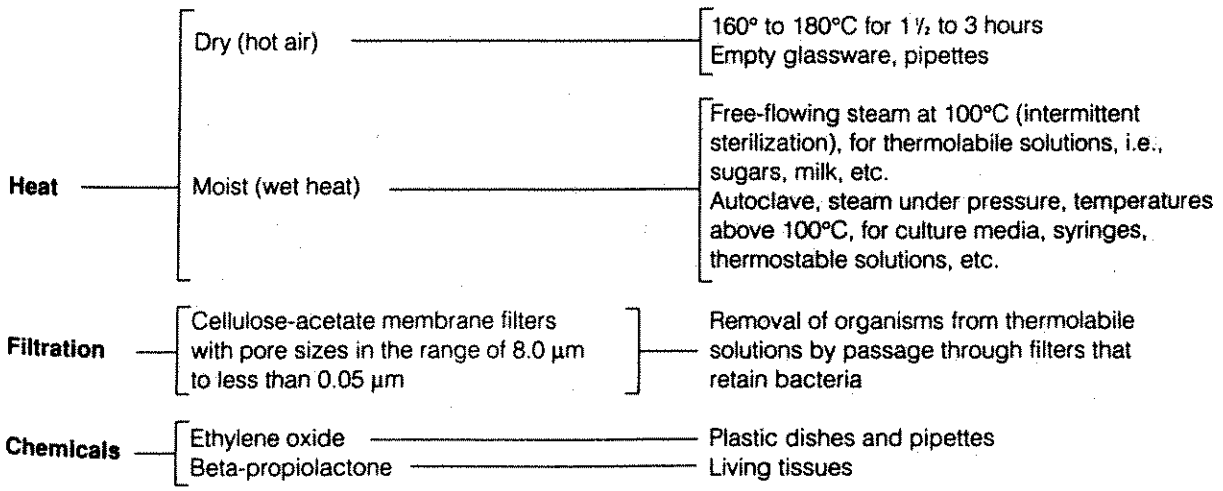


Figure I.3 Sterilization techniques

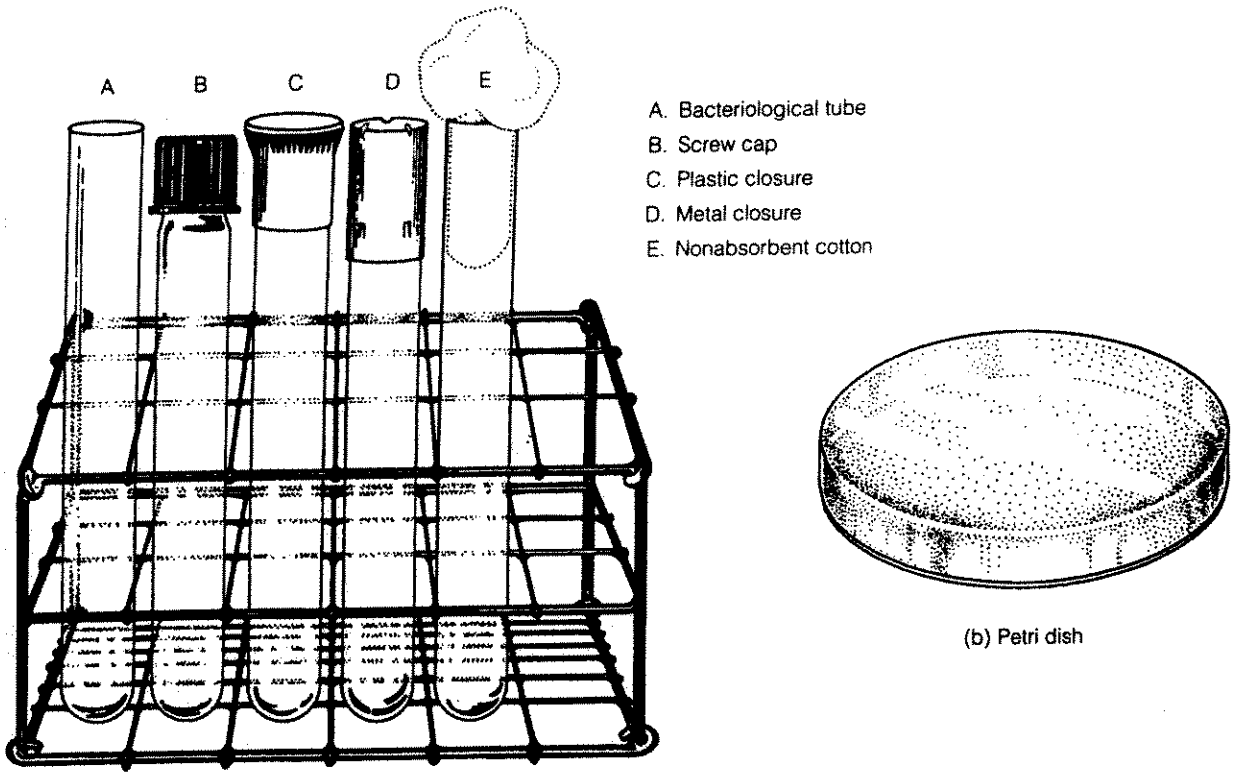


Figure I.4 Culture vessels

in an inverted position (top down) to prevent condensation that forms on the cover during solidification from dropping down onto the surface of the hardened agar. Figure I.4 illustrates some of the culture vessels used in the laboratory.

Transfer Instruments

Microorganisms must be transferred from one vessel to another or from stock cultures to various media for maintenance and study. Such a transfer is called **subculturing** and must be carried out under sterile conditions to prevent possible contamination.

Wire loops and needles are made from inert metals such as nichrome or platinum and are inserted into metal shafts that serve as handles. They are extremely durable instruments and are easily sterilized by incineration in the blue (hottest) portion of the Bunsen burner flame.

A **pipette** is another instrument used for sterile transfers. Pipettes are similar in function to straws; that is, they draw up liquids. They are made of glass or plastic drawn out to a tip at one end and with a mouthpiece forming the other end. They are calibrated to deliver different volumes depending on requirements. Pipettes may be sterilized in bulk inside canisters, or they may be wrapped individually in brown paper and sterilized in an autoclave or dry-heat oven.

Figure I.5 illustrates these transfer instruments. **Note: Pipetting by mouth is not permissible! Pipetting is to be performed with the aid of mechanical devices.** The proper procedure for the use of pipettes will be demonstrated by your instructor.

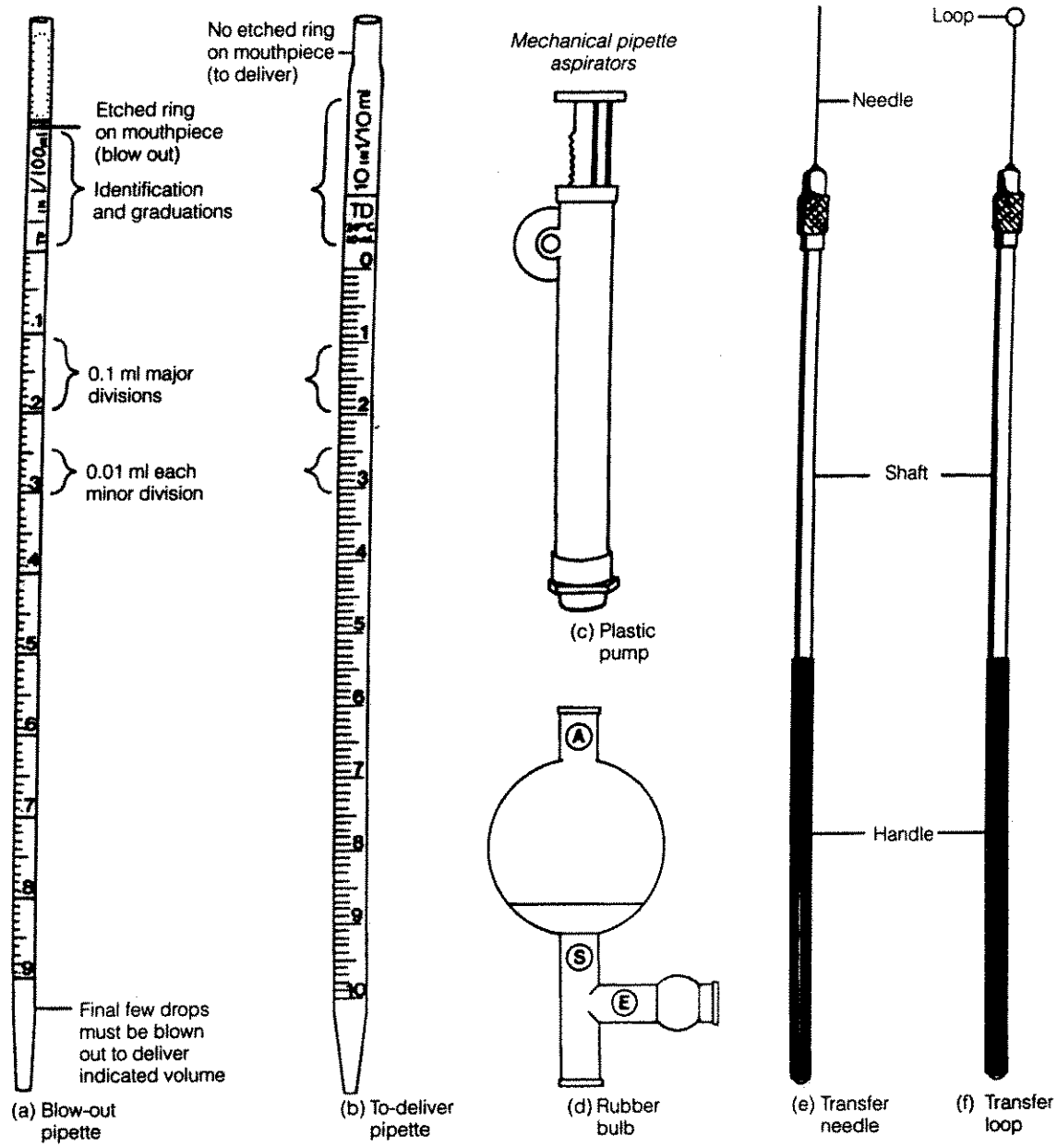


Figure I.5 Transfer instruments

Cultivation Chambers

The specific temperature requirements for growth are discussed in detail in Part IV. However, a prime requirement for the cultivation of microorganisms is that they be grown at their optimum temperature. An **incubator** is used to maintain optimum temperature during the necessary growth period. It resembles an oven and is thermostatically controlled so that temperature can be varied depending on the requirements of specific microorganisms. Most incubators use dry heat. Moisture is supplied by placing a beaker of water in the incubator during the growth period. A moist environment retards dehydration of the medium and thereby avoids spurious experimental results.

A thermostatically controlled **shaking waterbath** is another piece of apparatus used to cultivate microorganisms. Its advantage is that it provides a rapid and uniform

transfer of heat to the culture vessel, and its agitation provides increased aeration, resulting in acceleration of growth. The single disadvantage of this instrument is that it can be used only for cultivation of organisms in a broth medium.

Refrigerator

A refrigerator is used for a wide variety of purposes such as maintenance and storage of stock cultures between subculturing periods and storage of sterile media to prevent dehydration. It is also used as a repository for thermolabile solutions, antibiotics, serums, and biochemical reagents.

Culture Transfer Techniques

PURPOSE

To become familiar with the technique of aseptic removal and transfer of microorganisms for subculturing.

PRINCIPLE

Microorganisms are transferred from one medium to another by **subculturing.** This technique is of basic importance and is used routinely in preparing and maintaining stock cultures, as well as in microbiological test procedures.

Microorganisms are always present in the air and on laboratory surfaces, benches, and equipment. They can serve as a source of external contamination and thus interfere with experimental results unless proper techniques are used during subculturing. Described below are essential steps that you must follow for aseptic transfer of microorganisms. The complete procedure is illustrated in Figure 1.1.

1. An inoculating needle or loop must always be sterilized by holding it in the hottest portion of the Bunsen burner flame, the inner blue cone, until the entire wire becomes red hot. Then rapidly pass the upper portion of the handle through the flame. Once flamed, the loop is never put down but is held in the hand and allowed to cool for 10 to 20 seconds. The stock culture tube and the tube to be inoculated are held in the palm of the other hand and secured with the thumb. The two tubes are then separated to form a V in the hand.
2. The tubes are uncapped by grasping the first cap with the little finger and the second cap with the next finger and lifting the closures upward. Once removed, these caps must be kept in the hand that holds the sterile inoculating loop or needle, thus the inner aspects of the caps point away from the palm of the hand. They must never be placed on the laboratory bench, because doing so would compromise the sterile procedure. Following removal of the closures, the necks of the tubes are briefly passed through the flame and the sterile transfer instrument is further cooled by touching it to the sterile inside wall of the culture tube before removing a small sample of inoculum.
3. Depending on the culture medium, a loop or needle is used for removal of the inoculum. Loops are commonly used to obtain a sample from a broth culture. Either instrument can be used to obtain the inoculum from an agar slant culture by carefully touching the surface of the solid medium in an area exhibiting growth so as not to gouge into the agar. A straight needle is always used when transferring microorganisms to an agar deep tube from both solid and liquid cultures.
4. The cell-laden loop or needle is inserted into the subculture tube. In the case of a broth medium, the loop or needle is shaken slightly to dislodge the organisms; with an agar slant medium, it is drawn lightly over the hardened surface in a straight or zigzag line. For inoculation of an agar deep tube, a straight needle is inserted to the bottom of the tube in a straight line and rapidly withdrawn along the line of insertion.
5. Following inoculation, the instrument is removed, the necks of the tubes are reflamed, and the caps are replaced in the order in which they were removed.
6. The needle or loop is again flamed to destroy remaining organisms.

In this experiment you will master the manipulations required for aseptic transfer of microorganisms in broth-to-slant, slant-to-broth, and slant-to-agar deep transfers. The technique for transfer to and from agar plates is discussed in Experiment 2.

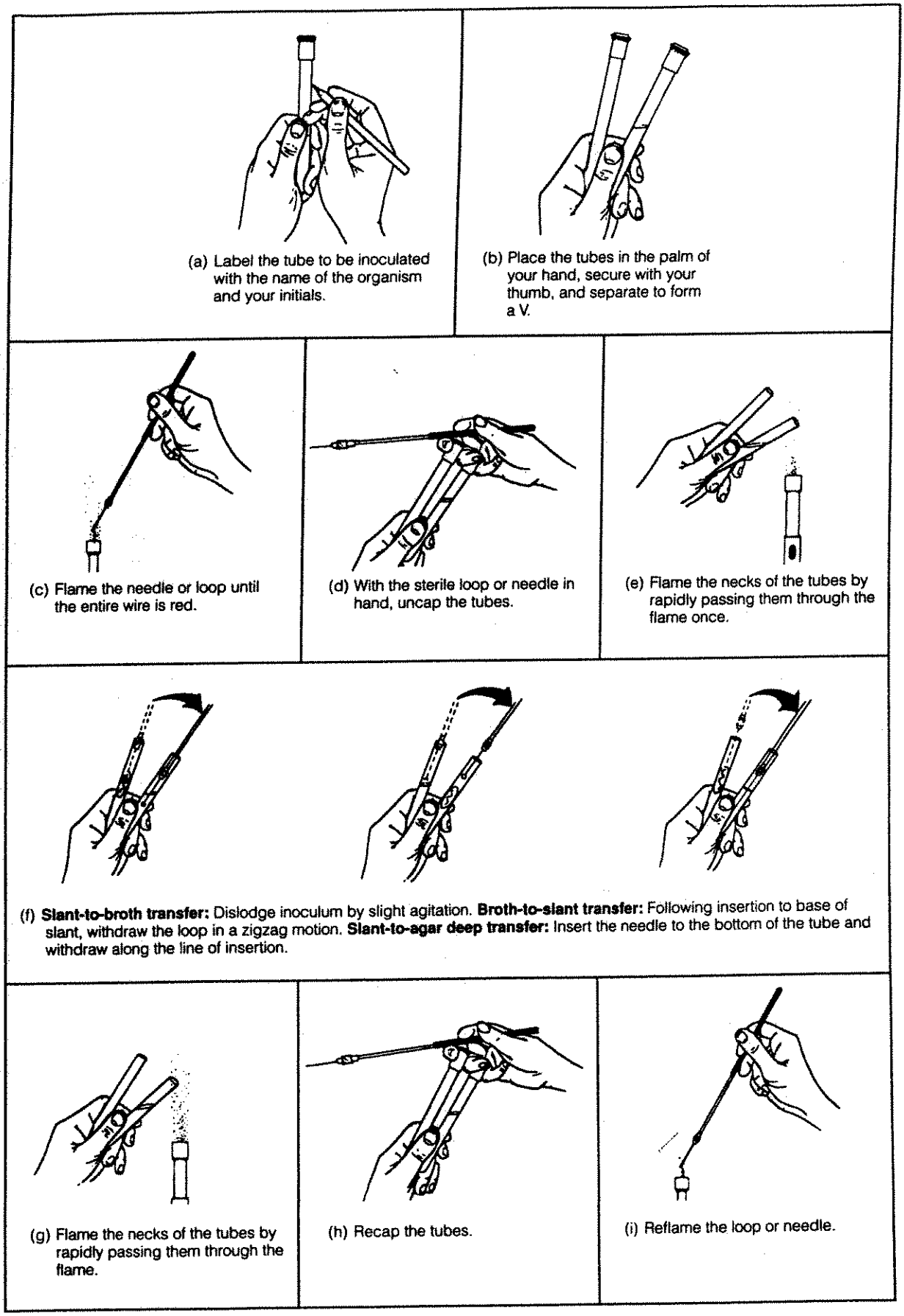
MATERIALS

Cultures

24-hour nutrient broth and nutrient agar slant cultures of *Serratia marcescens.*

Media

Per designated student group: one nutrient broth, one nutrient agar slant, and one nutrient agar deep tube.



(a) Label the tube to be inoculated with the name of the organism and your initials.

(b) Place the tubes in the palm of your hand, secure with your thumb, and separate to form a V.

(c) Flame the needle or loop until the entire wire is red.

(d) With the sterile loop or needle in hand, uncap the tubes.

(e) Flame the necks of the tubes by rapidly passing them through the flame once.

(f) **Slant-to-broth transfer:** Dislodge inoculum by slight agitation. **Broth-to-slant transfer:** Following insertion to base of slant, withdraw the loop in a zigzag motion. **Slant-to-agar deep transfer:** Insert the needle to the bottom of the tube and withdraw along the line of insertion.

(g) Flame the necks of the tubes by rapidly passing them through the flame.

(h) Recap the tubes.

(i) Reflame the loop or needle.

Figure 1.1 Subculturing procedure

Equipment

Bunsen burner, inoculating loop and needle, and glassware marking pencil.

PROCEDURE

1. Label all tubes of sterile media as described in the Laboratory Protocol section on page xi.
2. Following the procedure outlined and illustrated above, perform the following transfers:
   a. *S. marcescens* broth culture to a nutrient agar slant, nutrient agar deep tube, and nutrient broth.
   b. *S. marcescens* agar slant culture to a nutrient broth, nutrient agar slant, and nutrient agar deep tube.
3. Incubate all cultures at 25°C for 24 to 48 hours.

E. coli
Strep. sp.
B. licheniformis

Techniques for Isolation of Pure Cultures

In nature, microbial populations do not segregate themselves by species but exist with a mixture of many other cell types. In the laboratory, these populations can be separated into **pure cultures.** These cultures contain only one type of organism and are suitable for the study of their cultural, morphological, and biochemical properties.

In this experiment, you will first use one of the techniques designed to produce discrete colonies. Colonies are individual, macroscopically visible masses of microbial growth on a solid medium surface, each representing the multiplication of a single organism. Once you have obtained these discrete colonies, you will make an aseptic transfer onto nutrient agar slants for the isolation of pure cultures.

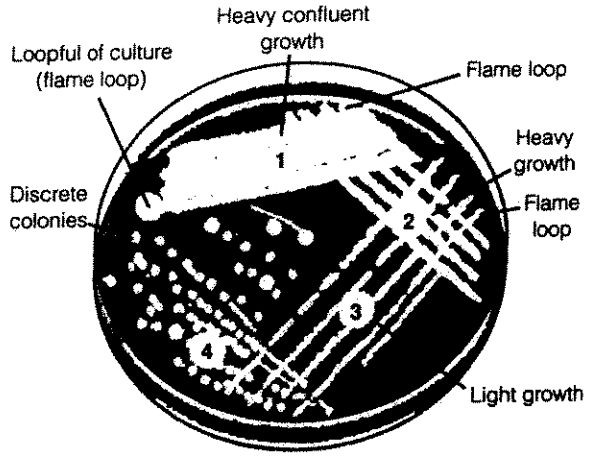


Figure 2.1 Four-way streak-plate inoculation

PART A: ISOLATION OF DISCRETE COLONIES FROM A MIXED CULTURE

PURPOSE

To perform the spread-plate and/or the streak-plate inoculation procedure for the separation of the cells of a mixed culture so that discrete colonies can be isolated.

PRINCIPLE

The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculum be reduced. The resulting diminution of the population size ensures that, following inoculation, individual cells will be sufficiently far apart on the surface of the agar medium to effect a separation of the different species present. The following are techniques that can be used to accomplish this necessary dilution:

1. The **streak-plate** method is a rapid qualitative isolation method. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate. Although many types of procedures are performed, the four-way, or quadrant, streak is described. Refer to Figure 2.1, which schematically illustrates this procedure.

   a. Place a loopful of culture on the agar surface in Area 1. Flame and cool the loop and drag it rapidly several times across the surface of Area 1.

   b. Reflame and cool the loop and turn the Petri dish 90°. Then touch the loop to a corner of the culture in Area 1 and drag it several times across the agar in Area 2. The loop should never enter Area 1 again.

   c. Reflame and cool the loop and again turn the dish 90°. Streak Area 3 in the same manner as Area 2.

   d. Without reflaming the loop, again turn the dish 90° and then drag the culture from a corner of Area 3 across Area 4, using a wider streak. Don't let the loop touch any of the previously streaked areas. The flaming of the loop at the points indicated is to effect the dilution of the culture so that fewer organisms are streaked in each area, resulting in the final desired separation.

2. The **spread-plate** technique requires that a previously diluted mixture of microorganisms be used. During inoculation, the cells are spread over the surface of a solid agar medium with a sterile, L-shaped bent rod while the Petri dish is spun on a "lazy-Susan" turntable (Figure 2.2). The step-by-step procedure for this technique is as follows:
   a. Place the bent glass rod into the beaker and add a sufficient amount of 95% ethyl alcohol to cover the lower, bent portion.
   b. With a sterile loop, place a loopful of *Micrococcus luteus* culture in the center of the appropriately labeled nutrient agar plate that has been placed on the turntable. Replace the cover.
   c. Remove the glass rod from the beaker and pass it through the Bunsen burner flame, with the bent portion of the rod pointing downward to prevent the burning alcohol from running down your arm. Allow the alcohol to burn off the rod completely. Cool the rod for 10 to 15 seconds.
   d. Remove the Petri dish cover and spin the turntable.
   e. While the turntable is spinning, lightly touch the sterile bent rod to the surface of the agar and move it back and forth. This will spread the culture over the agar surface.
   f. When the turntable comes to a stop, replace the cover. Immerse the rod in alcohol and reflame.
   g. In the absence of a turntable, turn the Petri dish manually and spread the culture with the sterile bent glass rod.
3. The **pour-plate** technique requires a serial dilution of the mixed culture by means of a loop or pipette. The diluted inoculum is then added to a molten agar medium in a Petri dish, mixed, and allowed to solidify. The serial dilution and pour-plate procedures are outlined in Experiment 20.

MATERIALS

Cultures

24- to 48-hour nutrient broth cultures of a mixture of one part *Serratia marcescens* and three parts *M. luteus* and a mixture of one part *Escherichia coli* and ten parts *M. luteus*. For the spread-plate procedure, adjust the cultures to an optical density (O.D.) of 0.1 at 600 mμ.

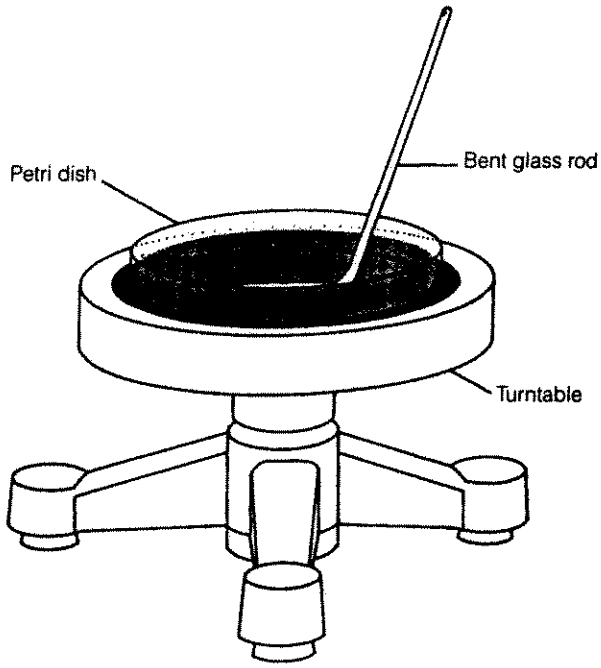


Figure 2.2 Petri dish turntable

Media

Two trypticase soy agar plates per designated student group for each inoculation technique to be performed.

Equipment

Bunsen burner, inoculating loop, turntable, 95% ethyl alcohol, 500-ml beaker, L-shaped bent glass rod, and glassware marking pencil.

PROCEDURE

1. Following the procedures previously described, prepare a spread-plate and/or streak-plate inoculation of each test culture on an appropriately labeled plate.
2. Incubate all plates in an inverted position for 48 to 72 hours at 25°C.

PART B: ISOLATION OF PURE CULTURES FROM A SPREAD-PLATE OR STREAK-PLATE PREPARATION

PURPOSE

To prepare a stock culture of an organism using isolates from the mixed cultures prepared on the

agar streak-plate and/or the spread plate in Part A of this experiment.

PRINCIPLE

Once discrete, well-separated colonies develop on the surface of a nutrient agar plate culture, each may be picked up with a sterile needle and transferred to separate nutrient agar slants. Each of these new slant cultures represents the growth of a single bacterial species and is designated as a **pure or stock culture.**

MATERIALS

Cultures

Mixed-culture, nutrient agar streak-plate, and/or spread-plate preparations of *S. marcescens* and *M. luteus* and of *M. luteus* and *E. coli* from Part A.

Media

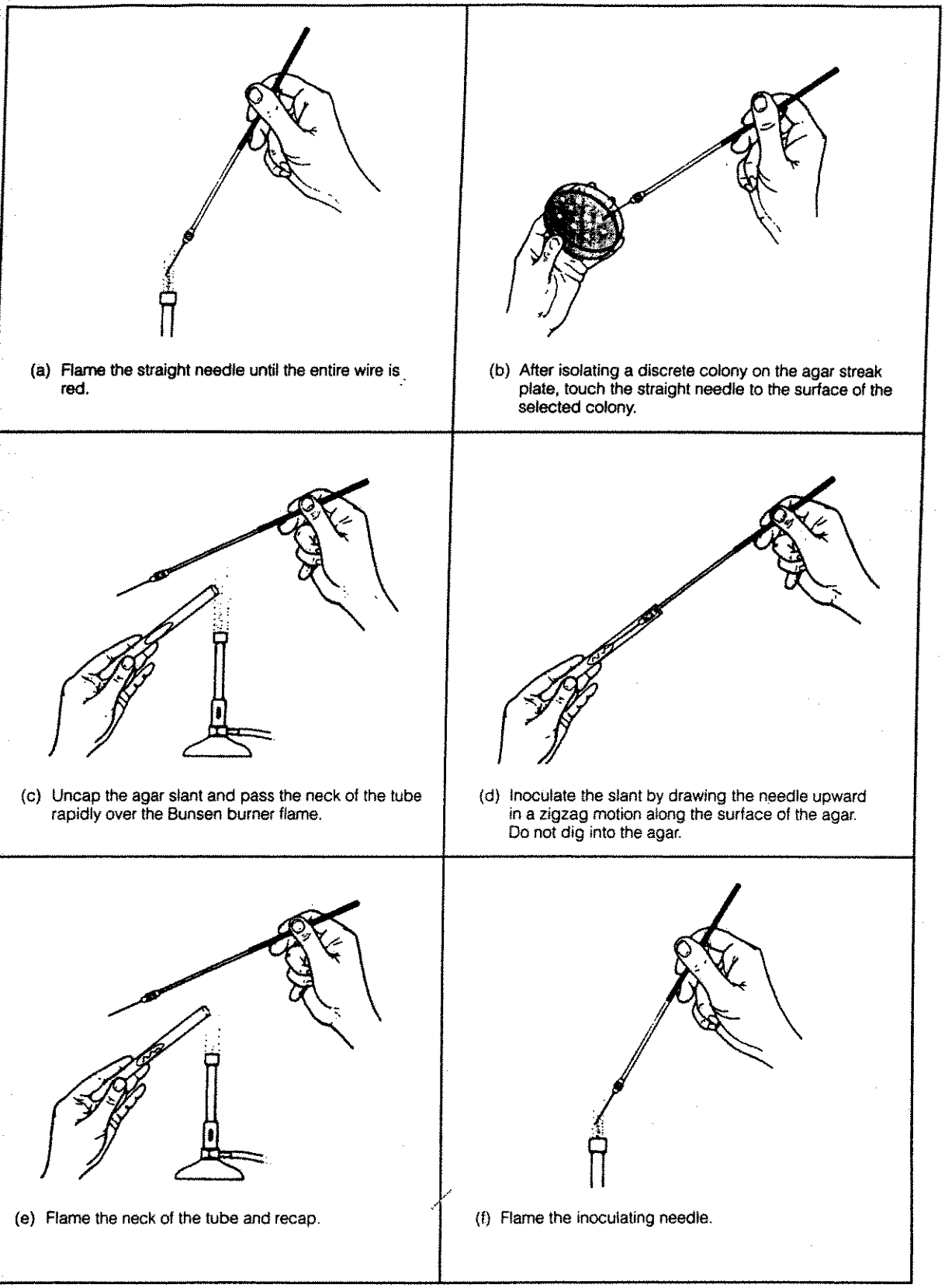
Three trypticase soy agar slants per designated student group.

Equipment

Bunsen burner, inoculating needle, and glassware marking pencil.

PROCEDURE

1. Aseptically transfer, from visibly discrete colonies, the yellow *M. luteus,* the white *E. coli,* and the red *S. marcescens* to the appropriately labeled agar slants as shown in Figure 2.3.
2. Incubate the cultures for 48 to 72 hours at 25°C.



(a) Flame the straight needle until the entire wire is red.

(b) After isolating a discrete colony on the agar streak plate, touch the straight needle to the surface of the selected colony.

(c) Uncap the agar slant and pass the neck of the tube rapidly over the Bunsen burner flame.

(d) Inoculate the slant by drawing the needle upward in a zigzag motion along the surface of the agar. Do not dig into the agar.

(e) Flame the neck of the tube and recap.

(f) Flame the inoculating needle.

Figure 2.3 Procedure for the preparation of a pure culture

Cultural Characteristics of Microorganisms

PURPOSE

To determine the cultural characteristics of microorganisms as an aid in identifying and classifying organisms into taxonomic groups.

PRINCIPLE

When grown on a variety of media, microorganisms will exhibit differences in the macroscopic appearance of their growth. These differences, called **cultural characteristics,** are used as the basis for separating microorganisms into taxonomic groups. The cultural characteristics for all known microorganisms are contained in *Bergey's Manual of Systematic Bacteriology.* They are determined by culturing the organisms on nutrient agar slants and plates, in nutrient broth, and in nutrient gelatin. The patterns of growth to be considered in each of these media are described below, and some are illustrated in Figure 3.1.

Nutrient Agar Slants

These have a single straight line of inoculation on the surface and are evaluated in the following manner:

1. **Abundance of growth:** The amount of growth is designated as none, slight, moderate, or large.
2. **Pigmentation:** Chromogenic microorganisms may produce intracellular pigments that are responsible for the coloration of the organisms as seen in surface colonies. Other organisms produce extracellular soluble pigments that are excreted into the medium and that also produce a color. Most organisms, however, are nonchromogenic and will appear white to gray.
3. **Optical characteristics:** Optical characteristics may be evaluated on the basis of the amount of light transmitted through the growth. These characteristics are described as **opaque** (no light transmission), **translucent** (partial transmission), or **transparent** (full transmission).
4. **Form:** The appearance of the single-line streak of growth on the agar surface is designated as:
   a. **Filiform:** Continuous, threadlike growth with smooth edges.
   b. **Echinulate:** Continuous, threadlike growth with irregular edges.
   c. **Beaded:** Nonconfluent to semiconfluent colonies.
   d. **Effuse:** Thin, spreading growth.
   e. **Arborescent:** Treelike growth.
   f. **Rhizoid:** Rootlike growth.

Nutrient Agar Plates

These demonstrate well-isolated colonies and are evaluated in the following manner:

1. **Size:** Pinpoint, small, moderate, or large.
2. **Pigmentation:** Color of colony.
3. **Form:** The shape of the colony is described as follows:
   a. **Circular:** Unbroken, peripheral edge.
   b. **Irregular:** Indented, peripheral edge.
   c. **Rhizoid:** Rootlike, spreading growth.
4. **Margin:** The appearance of the outer edge of the colony is described as follows:
   a. **Entire:** Sharply defined, even.
   b. **Lobate:** Marked indentations.
   c. **Undulate:** Wavy indentations.
   d. **Serrate:** Toothlike appearance.
   e. **Filamentous:** Threadlike, spreading edge.
5. **Elevation:** The degree to which colony growth is raised on the agar surface is described as follows:
   a. **Flat:** Elevation not discernible.
   b. **Raised:** Slightly elevated.
   c. **Convex:** Dome-shaped elevation.
   d. **Umbonate:** Raised, with elevated convex central region.

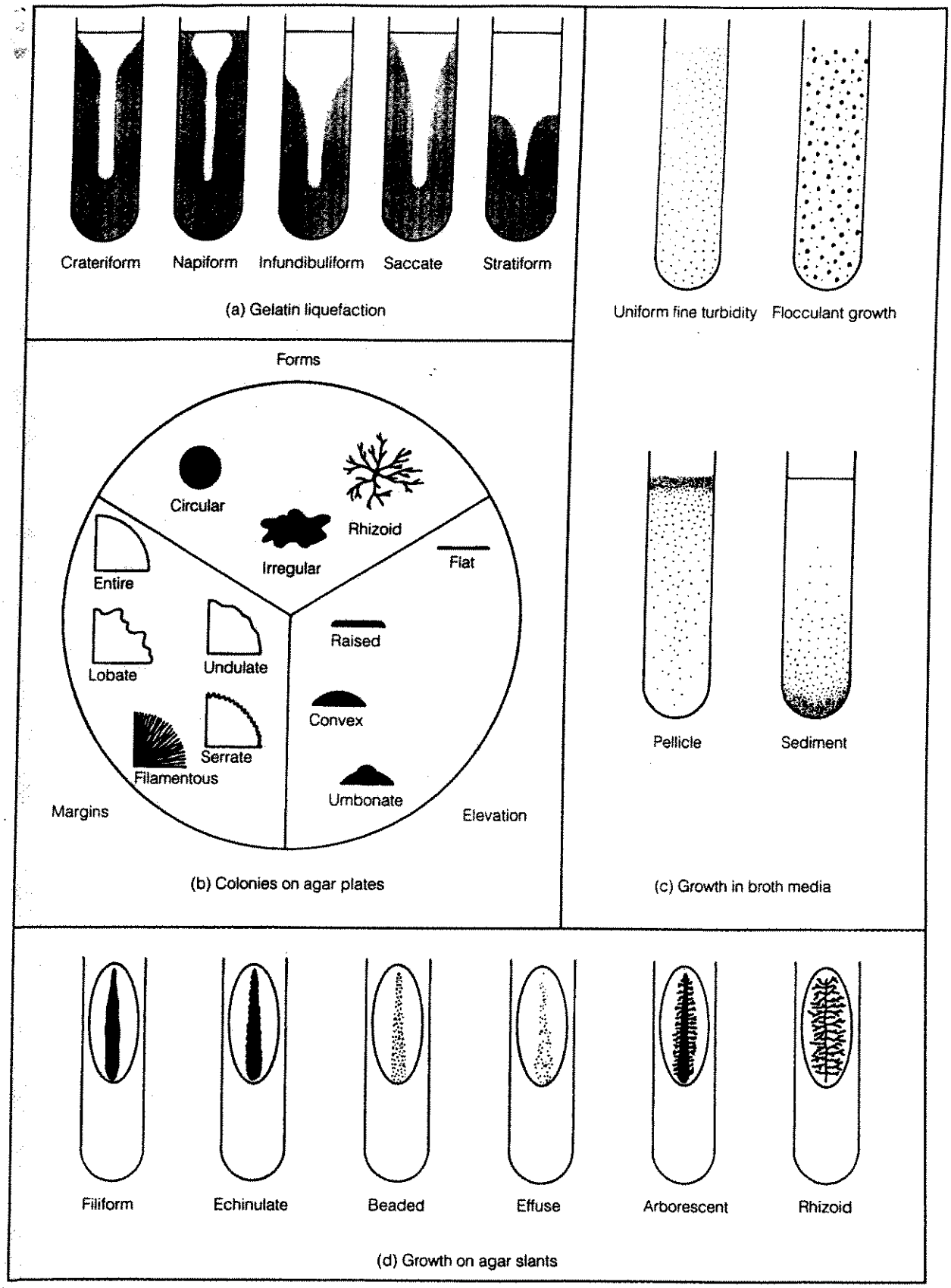


Figure 3.1 Cultural characteristics of bacteria

Nutrient Broth Cultures

These are evaluated as to the distribution and appearance of the growth as follows:

1. **Uniform fine turbidity:** Finely dispersed growth throughout.
2. **Flocculant:** Flaky aggregates dispersed throughout.
3. **Pellicle:** Thick, padlike growth on surface.
4. **Sediment:** Concentration of growth at the bottom of broth culture may be granular, flaky, or flocculant.

Nutrient Gelatin

This solid medium may be liquefied by the enzymatic action of gelatinase. Liquefaction occurs in a variety of patterns:

1. **Crateriform:** Liquefied surface area is saucer-shaped.
2. **Napiform:** Bulbous-shaped liquefaction at surface.
3. **Infundibuliform:** Funnel-shaped.
4. **Saccate:** Elongated, tubular.
5. **Stratiform:** Complete liquefaction of the upper half of the medium.

MATERIALS

Cultures

24-hour nutrient broth cultures of *Pseudomonas aeruginosa, Bacillus cereus, Micrococcus luteus, Escherichia coli,* and *Staphylococcus aureus.*

Media

Per designated student group: five each of nutrient agar plates, nutrient agar slants, nutrient broth tubes, and nutrient gelatin tubes.

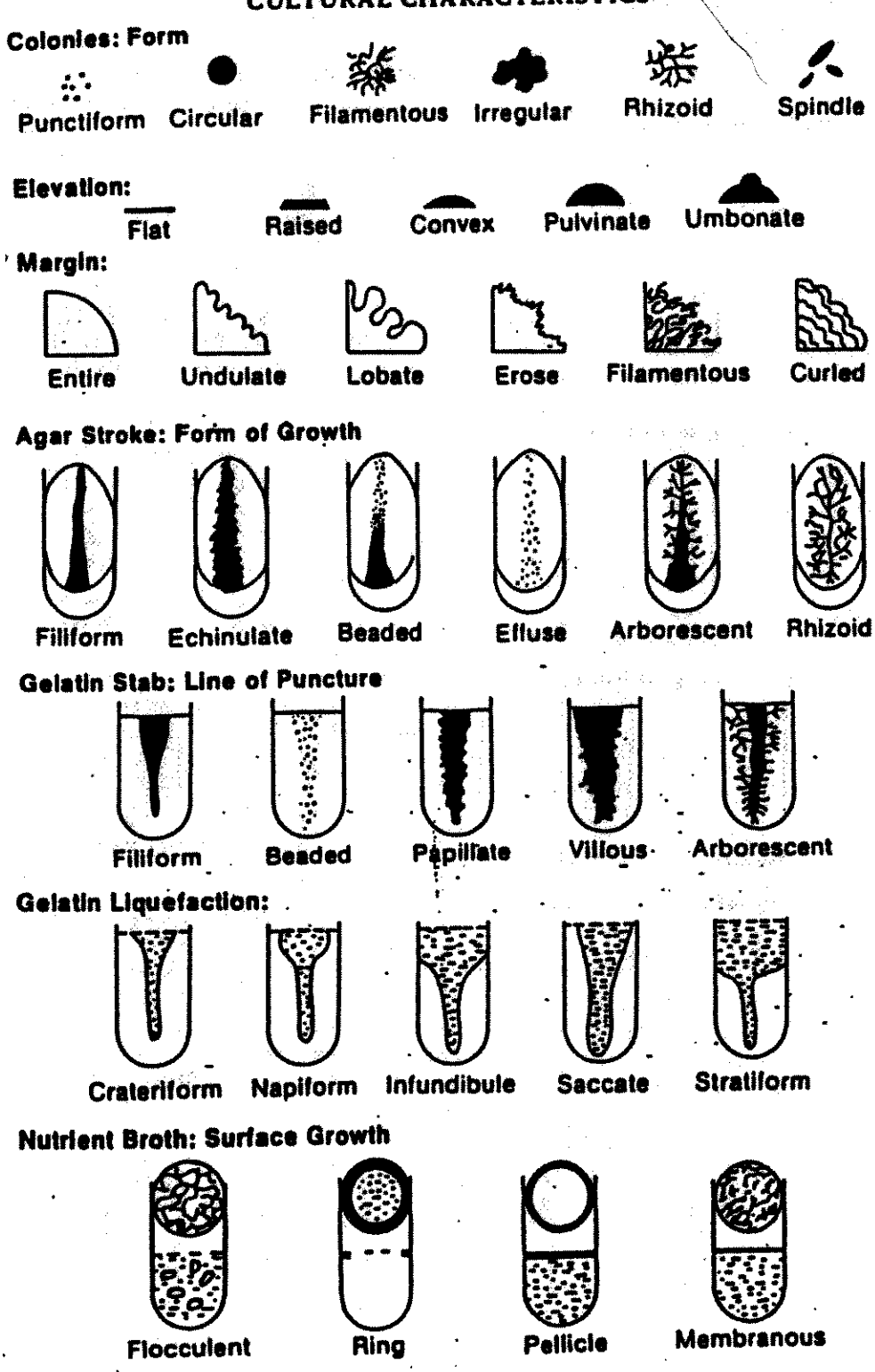
Equipment

Bunsen burner, inoculating loop and needle, and glassware marking pencil.

PROCEDURE

1. Using sterile technique, inoculate each of the appropriately labeled media listed below in the following manner:
   a. Nutrient agar plates: With a sterile loop, prepare a streak-plate inoculation of each of the cultures for the isolation of discrete colonies.
   b. Nutrient agar slants: With a sterile needle, make a single-line streak of each of the cultures provided, starting at the butt and drawing the needle up the center of the slanted agar surface.
   c. Nutrient broth: Using a sterile loop, inoculate each organism into a tube of nutrient broth. Shake the loop a few times to dislodge the inoculum.
   d. Nutrient gelatin: Using a sterile needle, prepare a stab inoculation of each of the cultures provided.
2. Incubate all cultures at 37°C for 24 to 48 hours.

Fig. 1-1. Characteristics of broth and slant cultures of bacteria and of isolated bacterial colonies on agar plates.



Preparation of Bacterial Smears

PURPOSE

To become familiar with the preparation of bacterial smears for the microscopic visualization of bacteria.

PRINCIPLE

Bacterial smears must be prepared prior to the execution of any of the staining techniques listed in Figure III.3 on page 47. Although not difficult, the technique requires adequate care. Meticulously follow the rules listed below.

1. **Preparation of the glass microscope slide:** Clean slides are essential for the preparation of microbial smears. Grease or oil from the fingers on slides must be removed by washing the slides with soap and water or scouring powders such as Bon Ami, followed by a water rinse and a rinse of 95% alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use. **Remember to hold the clean slides by their edges.**
2. **Labeling of slides:** Proper labeling of the slide is essential. The initials of the organism can be written on either end of the slide with a glassware marking pencil, on the surface on which the smear is to be made. Care should be taken that the label does not come into contact with staining reagents.
3. **Preparation of smear:** It is crucial to avoid thick, dense smears. A thick or dense smear occurs when too much of the culture is used in its preparation, which concentrates a large number of cells on the slide. This type of preparation diminishes the amount of light that can pass through and makes it difficult to visualize the morphology of single cells. **Smears require only a small amount of the bacterial culture.** A good smear is one that, when dried, appears as a thin whitish layer or film. The print of your textbook should be legible through the smear. Those made from broth cultures or cultures from a solid medium require variations in technique.
   a. **Broth cultures:** Resuspend the culture by tapping the tube with your finger. Depending on the size of the loop, one or two loopfuls should be applied to the center of the slide with a sterile inoculating loop and spread evenly over an area about the size of a dime. Set the smears on the laboratory table and allow to air dry.
   b. **Cultures from solid medium:** Organisms cultured in a solid medium produce thick, dense surface growth and are not amenable to direct transfer to the glass slide. These cultures must be diluted by placing one or two loopfuls of water on the center of the slide in which the cells will be emulsified. Transfer of the cells requires the use of a sterile inoculating loop or a needle if preferred. Only the tip of the loop or needle should touch the culture to prevent the transfer of too many cells. Suspension is accomplished by spreading the cells in a circular motion in the drop of water with the loop or needle. The finished smear should occupy an area about the size of a nickel, and should appear as a translucent, or semitransparent, confluent whitish film. At this point the smear should be allowed to dry completely. **Do not blow on slide or wave it in the air.**
4. **Heat fixation:** Unless fixed on the glass slide, the bacterial smear will wash away during the staining procedure. This is avoided by heat fixation, during which the bacterial proteins are coagulated and fixed to the glass surface. Heat fixation is performed by the rapid passage of the air-dried smear two or three times over the flame of the Bunsen burner.

The preparation of a bacterial smear is illustrated in Figure 7.1 on page 51.

MATERIALS

Cultures

24-hour nutrient agar slant culture of *Bacillus cereus*, and a 24-hour nutrient broth culture of *Staphylococcus aureus*.

Equipment

Glass microscope slides, Bunsen burner, inoculating loop and needle, and glass marking pencil.

PROCEDURE

Broth cultures:

1. Label three clean slides with the initials of the organism, and number them 1, 2, and 3.
2. Resuspend the sedimented cells in the broth culture by tapping the culture tube with your fingers.
3. With a sterile loop, place one loopful of culture on slide number 1, two loopfuls on slide 2, and three loopfuls on slide 3, respectively. Allow all slides to air dry completely; then heat fix.

Solid medium cultures:

1. Label four clean glass slides with the initials of the organism. Label slides 1 and 2 with an "L" for loop and slides 3 and 4 with an "N" for needle.
2. Using the loop, add two loopfuls of water to each slide.
3. With a sterile loop, touch the entire loop to the culture and emulsify the cells in the drop of water on slide 1.
4. With a sterile loop, just touch the tip of the loop to the culture and emulsify the cells in the drop of water on slide number 2.
5. Repeat steps 3 and 4 using a sterile inoculating needle on slides number 3 and number 4.
6. Air dry completely and heat fix.

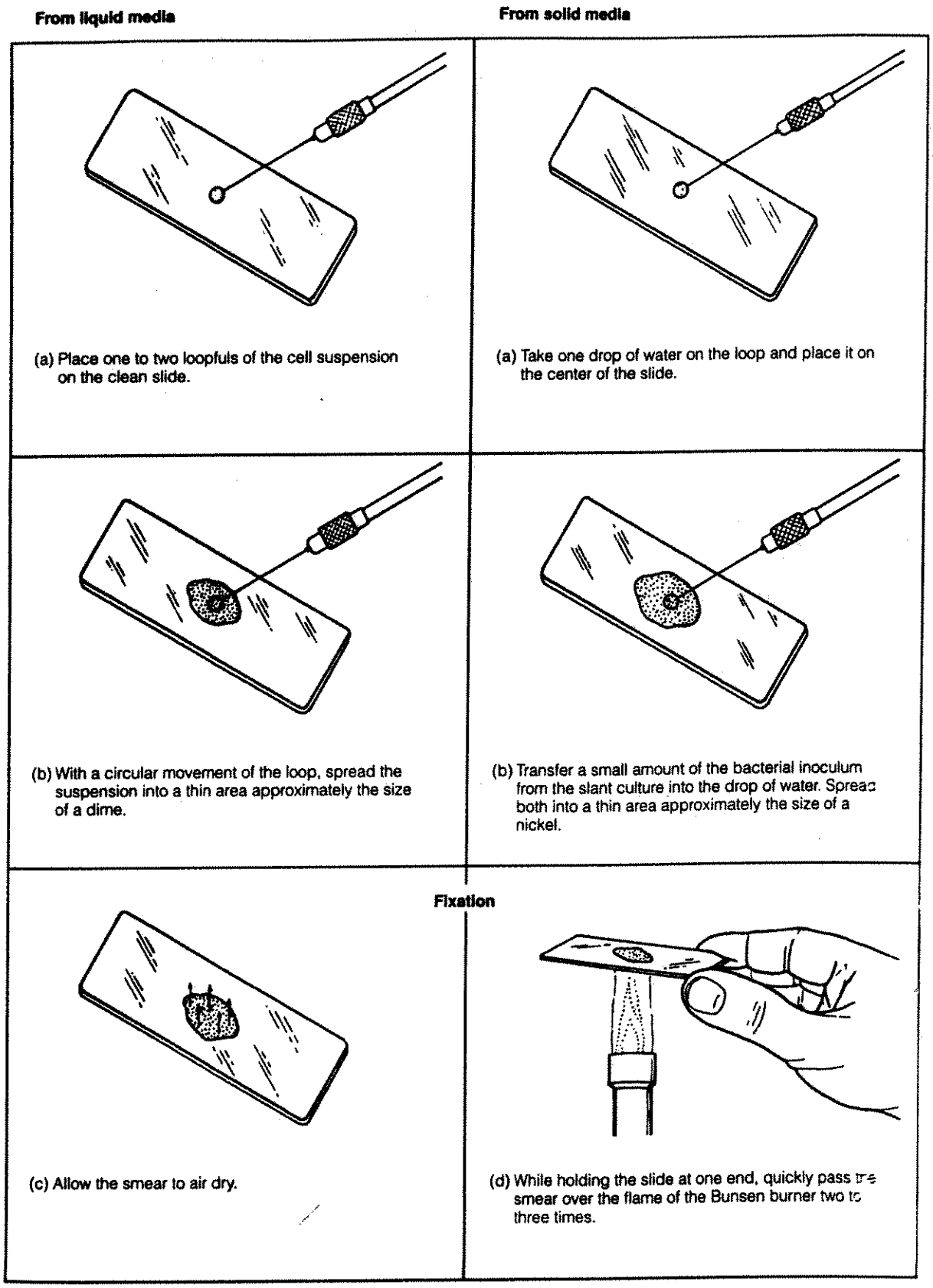


Figure 7.1 Bacterial smear preparation

Gram Stain

PURPOSES

To become familiar with

1. The chemical and theoretical bases for differential staining procedures.
2. The chemical basis of the Gram stain.
3. Performance of the procedure for differentiating between the two principal groups of bacteria: gram-positive and gram-negative.

PRINCIPLE

Differential staining requires the use of at least three chemical reagents that are applied sequentially to a heat-fixed smear. The first reagent is called the **primary stain.** Its function is to impart its color to all cells. In order to establish a color contrast, the second reagent used is the **decolorizing agent.** Based on the chemical composition of cellular components, the decolorizing agent may or may not remove the primary stain from the entire cell or only from certain cell structures. The final reagent, the **counterstain,** has a contrasting color to that of the primary stain. Following decolorization, if the primary stain is not washed out, the counterstain cannot be absorbed and the cell or its components will retain the color of the primary stain. If the primary stain is removed, the decolorized cellular components will accept and assume the contrasting color of the counterstain. In this way, cell types or their structures can be distinguished from each other on the basis of the stain that is retained.

The most important differential stain used in bacteriology is the **Gram stain,** named after Dr. Christian Gram. It divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an essential tool for classification and differentiation of microorganisms. The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer, whereas the peptidoglycan layer in gram-negative cells is much thinner and surrounded by an outer lipid containing layers. Early experiments have shown that if the gram-positive cell is denuded of its cell wall by the action of lysozyme or penicillin, the gram-positive cell will stain gram-negative. The Gram stain uses four different reagents. Descriptions of these reagents and their mechanisms of action follow. Figure 10.1 shows the microscopic observation of the cell at each step of the Gram staining procedure.

Primary Stain

Crystal Violet (Hucker's) This violet stain is used first and stains all cells purple.

Mordant

Gram's Iodine This reagent serves as a mordant, a substance that increases the cells' affinity for a stain. It does this by binding to the primary stain, thus forming an insoluble complex. The resultant crystal-violet-iodine complex (CV-I) serves to intensify the color of the stain. At this point, all cells will appear purple-black.

Decolorizing Agent

Ethyl Alcohol, 95% This reagent serves a dual function as a protein-dehydrating agent and as a lipid solvent. Its action is determined by two factors, the concentration of lipids and the thickness of the peptidoglycan layer in bacterial cell walls. In gram-negative cells, the alcohol increases the porosity of the cell wall by dissolving the lipids in the outer layers. Thus, the (CV-I) complex can be more easily removed from the thinner and less highly cross-linked peptidoglycan layer. Therefore, the washing-out effect of the alcohol facilitates the release of the unbound (CV-I) complex, leaving the cells colorless or unstained. The much thicker peptidoglycan layer in gram-positive cells is responsible for the more stringent retention of the (CV-I) complex, as the pores are made smaller due to the dehydrating effect of the alcohol. Thus the tightly bound primary stain complex is difficult to remove and the cells remain purple.

Counterstain

Safranin This is the final reagent, used to stain red those cells that have been previously decolorized. Since only gram-negative cells undergo decolorization, they may now absorb the counterstain. Gram-positive cells retain the purple color of the primary stain.

The preparation of adequately stained smears requires that you bear in mind the following precautions:

1. The most critical phase of the procedure is the decolorization step, which is based on the ease with which the CV-I complex is released from the cell. Remember that over-decolorization will result in loss of the primary stain, causing gram-positive organisms to appear gram-negative. Under-decolorization, however, will not completely remove the CV-I complex, causing gram-negative organisms to appear gram-positive. Strict adherence to all instructions will help remedy part of the difficulty, but individual experience and practice are the keys to correct decolorization.
2. It is imperative that, between applications of the reagents, slides be thoroughly washed under running water or water applied with an eye dropper. This removes excess reagent and prepares the slide for application of the subsequent reagent.
3. The best Gram stained preparations are made with fresh cultures, that is, not older than 24 hours. As cultures age, especially in the case of gram-positive cells, the organisms tend to lose their ability to retain the primary stain and may appear to be **gram-variable;** that is, some cells will appear purple, while others will appear red.

MATERIALS

Cultures

24-hour nutrient agar slant cultures of *Escherichia coli, Staphylococcus aureus,* and *Bacillus cereus.*

Reagents

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

Equipment

Bunsen burner, inoculating loop or needle, staining tray, glass slides, bibulous paper, lens paper, and microscope.

PROCEDURE

The steps are pictured in Figure 10.2.

1. Obtain four clean glass slides.
2. Using sterile technique, prepare a smear of each of the three organisms and on the remaining slide prepare a smear consisting of a mixture of *S. aureus* and *E. coli.* Do this by placing a drop of water on the slide and then transferring each organism separately to the drop of water with a sterile, cooled loop. Mix and spread both organisms by means of a circular motion of the inoculating loop.
3. Allow smears to air dry and then heat fix in the usual manner.
4. Flood smears with crystal violet and let stand for 1 minute.
5. Wash with tap water.
6. Flood smears with the Gram's iodine mordant and let stand for 1 minute.
7. Wash with tap water.
8. Decolorize with 95% ethyl alcohol. **Caution: Do not over-decolorize.** Add reagent drop by drop until alcohol runs almost clear, showing only a blue tinge.
9. Wash with tap water.
10. Counterstain with safranin for 45 seconds.
11. Wash with tap water.
12. Blot dry with bibulous paper and examine under oil immersion.

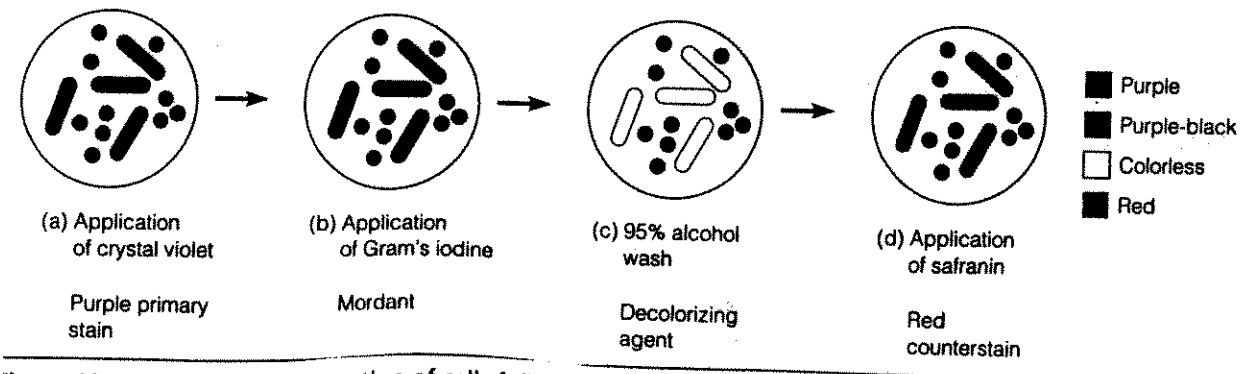


Figure 10.1 Microscopic observation of cells following the Gram's staining procedure

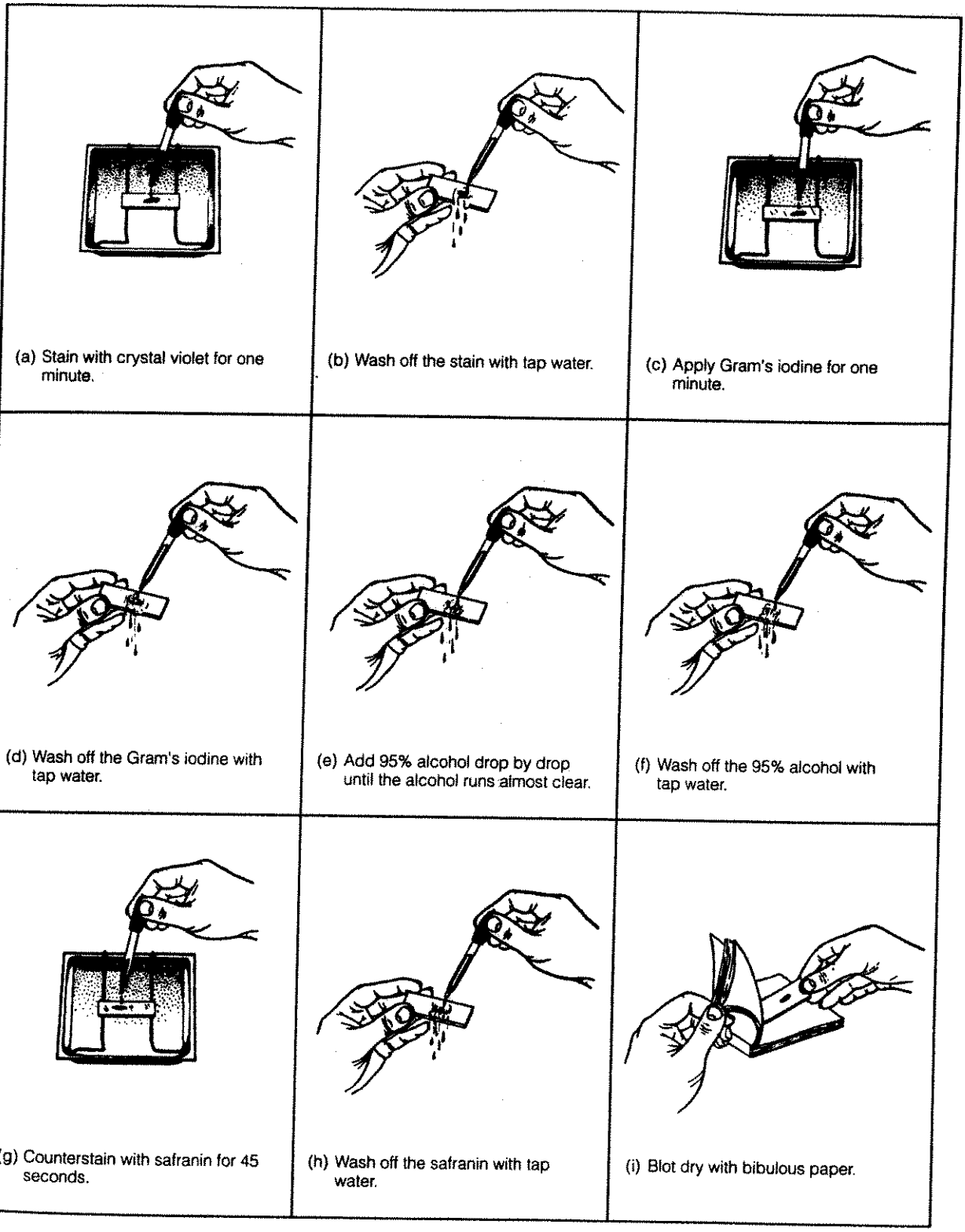


Figure 10.2 Gram staining procedure

Name _____

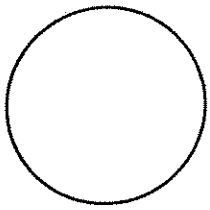
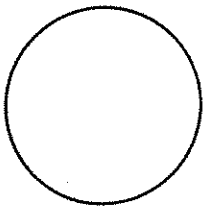
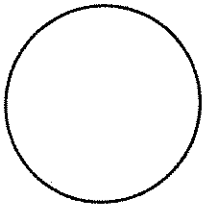
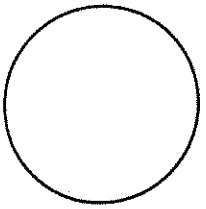
Experiment 10

OBSERVATIONS AND RESULTS

Following your observation of all slides under oil immersion, record your results in the chart.

1. Make a drawing of a representative microscopic field.
2. Describe the cells according to their morphology and arrangement.
3. Describe the color of the stained cells.
4. Classify the organism as to the Gram reaction: Gram-positive or gram-negative.

Refer to photo numbers 2–4 in the color-plate insert for illustration of this staining procedure.

	E. coli	*B. cereus*	*S. aureus*	Mixture
Drawing of a representative field				
Cell morphology: Shape Arrangement Cell color Gram reaction	_____ _____ _____ _____	_____ _____ _____ _____	_____ _____ _____ _____	_____ _____ _____ _____

REVIEW QUESTIONS

1. What are the advantages of differential staining procedures over the simple staining technique?

2. Cite the purpose of each of the following reagents in a differential staining procedure.
   a. Primary stain:

   b. Counterstain:

c. Decolorizing agent:

d. Mordant:

3. Why is it essential that the primary stain and the counterstain be of contrasting colors?

4. Which is the most crucial step in the performance of the Gram staining procedures? Explain.

5. Because of a snowstorm, your regular laboratory session was cancelled and the Gram staining procedure was performed on cultures incubated for a longer period of time. Examination of the stained *Bacillus cereus* slides revealed a great deal of color variability, ranging from an intense blue to shades of pink. Account for this result.

Staining Reagents

Acid-Fast Stain

Carbol fuchsin (Ziehl's)

Solution A

Basic fuchsin (90% dye content)	0.3 gm
Ethyl alcohol (95%)	10.0 ml

Solution B

Phenol	5.0 gm
Distilled water	95.0 ml

Note: Mix solutions A and B. Add 2 drops of Triton X per 100 ml of stain for use in heatless method.

Acid Alcohol

Ethyl alcohol (95%)	97.0 ml
Hydrochloric acid	3.0 ml

Methylene blue

Methylene blue	0.3 gm
Distilled water	100.0 ml

Capsule Stain

Crystal violet (1%)

Crystal violet (85% dye content)	1.0 gm
Distilled water	100.0 ml

Copper sulfate solution (20%)

Copper sulfate ($CuSO_4 \cdot 5H_2O$)	20.0 gm
Distilled water	80.0 ml

Fungal Stains

Lactophenol–cotton-blue solution

Lactic acid	20.0 ml
Phenol	20.0 gm
Glycerol	40.0 ml
Distilled water	20.0 ml
Aniline blue	0.05 gm

Note: Heat gently to dissolve in hot water (double boiler). Then add aniline blue dye.

Water-iodine solution

Gram's iodine (as in Gram's stain)	10.0 ml
Distilled water	30.0 ml

Gram Stain

Crystal violet (Hucker's)

Solution A

Crystal violet (90% dye content)	2.0 gm
Ethyl alcohol (95%)	20.0 ml

Solution B

Ammonium oxalate	0.8 gm
Distilled water	80.0 ml

Note: Mix solutions A and B.

Gram's iodine

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 ml

Ethyl alcohol (95%)

Ethyl alcohol (100%)	95.0 ml
Distilled water	5.0 ml

Safranin

Safranin O	0.25 ml
Ethyl alcohol (95%)	10.0 ml
Distilled water	100.0 ml

Negative Stain

Nigrosin

Nigrosin, water-soluble	10.0 gm
Distilled water	100.0 ml

Note: Immerse in boiling water bath for 30 minutes.

Formalin	0.5 ml

Note: Filter twice through double filter paper.

Spore Stain

Malachite green

Malachite green	5.0 gm
Distilled water	100.0 ml

Safranin

Same as in Gram stain