Isolation of Bacteria by Dilution Techniques

Experience is the father of wisdom, and memory the mother.

THOMAS FULLER

Objectives

After completing this exercise, you should be able to:

1. Isolate bacteria by using the streak plate and pour plate techniques.
2. Prepare and maintain a pure culture.

Background

In nature most microbes are found growing in environments that contain many different organisms. Unfortunately, mixed cultures are of little use in studying microorganisms because of the difficulty they present in determining which organism is responsible for any observed activity. A **pure culture,** one containing a single kind of microbe, is required in order to study concepts such as growth characteristics, pathogenicity, metabolism, and antibiotic susceptibility. Because bacteria are too small to separate directly without sophisticated micromanipulation equipment, indirect methods of separation must be used.

In the 1870s, Joseph Lister attempted to obtain pure cultures by performing serial dilutions until each of his containers theoretically contained one bacterium. However, success was very limited, and **contamination,** the presence of unwanted microorganisms, was common. In 1880, Robert Koch prepared solid media, after which microbiologists could separate bacteria by dilution and trap them on the solid media. An isolated bacterium grows into a visible colony that consists of one kind of bacterium.

Currently there are three dilution methods commonly used for the isolation of bacteria: the streak plate, the spread plate, and the pour plate. In the **streak plate technique,** a loop is used to streak the mixed sample many times over the surface of a solid culture medium in a Petri plate. Theoretically, the process of streaking the loop repeatedly over the agar surface causes the bacteria to fall off the loop one by one and ultimately to be distributed over the agar surface, where each cell develops into a colony. The streak plate is the most common isolation technique in use today.

The spread plate and pour plate are quantitative techniques that allow determination of the number of bacteria in a sample. In the **spread plate technique,** a small amount of a previously diluted specimen is spread over the surface of a solid medium using a spreading rod.

In the **pour plate technique,** a small amount of diluted sample is mixed with melted agar and poured into empty, sterile Petri dishes. After incubation, bacterial growth is visible as colonies in and on the agar of a pour plate. To determine the number of bacteria in the original sample, a plate with between 25 and 250 colonies is selected. Fewer than 25 colonies is inaccurate because a single contaminant causes at least a 4% error. A plate with greater than 250 colonies is difficult to count. The number of bacteria in the original sample is calculated using the following equation:

$$\text{Colony-forming units per ml} = \frac{\text{Number of colonies}}{\text{Dilution}^* \times \text{Amount plated}}$$

Materials

Petri plates containing nutrient agar (2)

Tubes containing melted nutrient agar (3)

Sterile Petri dishes (3)

250-ml beaker

Sterile 1-ml pipettes (3)

Propipette or pipette bulb

Nutrient agar slant (second period)

*In this exercise, 1 ml of sample is put into each plate. Dilution refers to the dilution of the sample (Appendix B). For example, if 37 colonies were present on the 1:8000 plate, the calculation would be as follows:

$$\text{Colony-forming units per ml} = \frac{37}{1{:}8000 \times 1} = 37 \times 8000 = 296{,}000 = 2.96 \times 10^5$$

Cultures

Mixed broth culture of bacteria

Turbid nutrient broth from Exercise 9

Techniques Required

Compound light microscopy, Exercise 1

Aseptic technique, Exercise 10

Pipetting, Appendix A

Serial dilution techniques, Appendix B

Procedure

Streak Plate

1. Label the bottoms of two nutrient agar plates to correspond to the two broth cultures: mixed culture and turbid broth.
2. Flame the inoculating loop to redness, allow it to cool, and aseptically obtain a loopful of one broth culture.
3. The streaking procedure may be done with the Petri plate on the table (Figure 11.1a) or held in your hand (Figure 11.1b).
   a. To streak a plate (Figure 11.2), lift one edge of the Petri plate cover, and streak the first sector by making as many streaks as possible without overlapping previous streaks. Do not gouge the agar while streaking the plate. Hold the loop as you would hold a pencil or paintbrush, and gently touch the surface of the agar.
   b. Flame your loop and let it cool. Turn the plate so the next sector is on top. Streak through one area of the first sector, and then streak a few times away from the first sector.
   c. Flame your loop, turn the plate again, and streak through one area of the second sector. Then streak the third sector.
   d. Flame your loop, streak through one area of the third sector, and then streak the remaining area of the agar surface, being careful not to make additional contact with any streaks in the previous sections. Flame your loop before setting it down. Why? _____
4. Streak two plates: one of the mixed culture provided and one of the turbid broth from an environmental sample. Label each plate on the bottom with your name and lab section, the date, and the source of the inoculum.
5. Incubate the plates in an inverted position in the 35°C incubator (or at room temperature, depending on the inoculum) until discrete, isolated colonies develop (usually 24 to 48 hours). Why inverted? _____
6. After incubation, record your results. Use proper terms to describe the colonies. (Refer to Color Plates VI.2 and VI.3, and Figure 9.2.)
7. Prepare a subculture of one colony. Sterilize your needle by flaming it. Let it cool. Why use a needle instead of a loop? _____
   To subculture, touch the center of a small isolated colony located on a streak line, and then aseptically streak a sterile nutrient agar slant. How can you tell whether you touched only one colony and whether you have a pure culture? _____
8. Incubate the slant at 35°C until good growth is observed. Describe the growth pattern (Figure 10.7).

Pour Plate (Figure 11.3)

1. Label the bottoms of three empty, sterile Petri dishes with your name and lab section and the date. Label one dish "1:20," another "1:400," and the third one "1:8000." Place the labeled dishes on your workbench right-side up.
2. Fill a beaker with hot (45–50°C) water (about 3–6 cm deep), and place three tubes of melted nutrient agar in the beaker. Each tube contains 19 ml of nutrient agar.
3. Select a mixed broth culture.
4. Remove a pipette, attach a bulb, and aseptically transfer 1 ml of the broth to a tube of melted agar. Mix well, as shown in Figure 11.4. Using a different pipette, transfer 1 ml from this tube to a second tube. Work quickly so the agar does not solidify in the pipette. Aseptically pour the contents of the first tube into Petri dish 1:20. Discard the pipettes in the container of disinfectant.
5. Mix the second tube. With the third pipette, aseptically transfer 1 ml to the third tube. Pour the contents of the second tube into dish 1:400. Mix the third tube and pour its contents into the remaining dish, 1:8000.
6. Discard the tubes properly. Let the agar harden in the plates, and then incubate them at 35°C in an inverted position until growth is seen. *Suggestion:* When incubating multiple plates, use a rubber band to hold them together.
7. After incubation, count the number of colonies on the plates. Remember that more than 250 is too numerous to count, and less than 25 is too few to count.

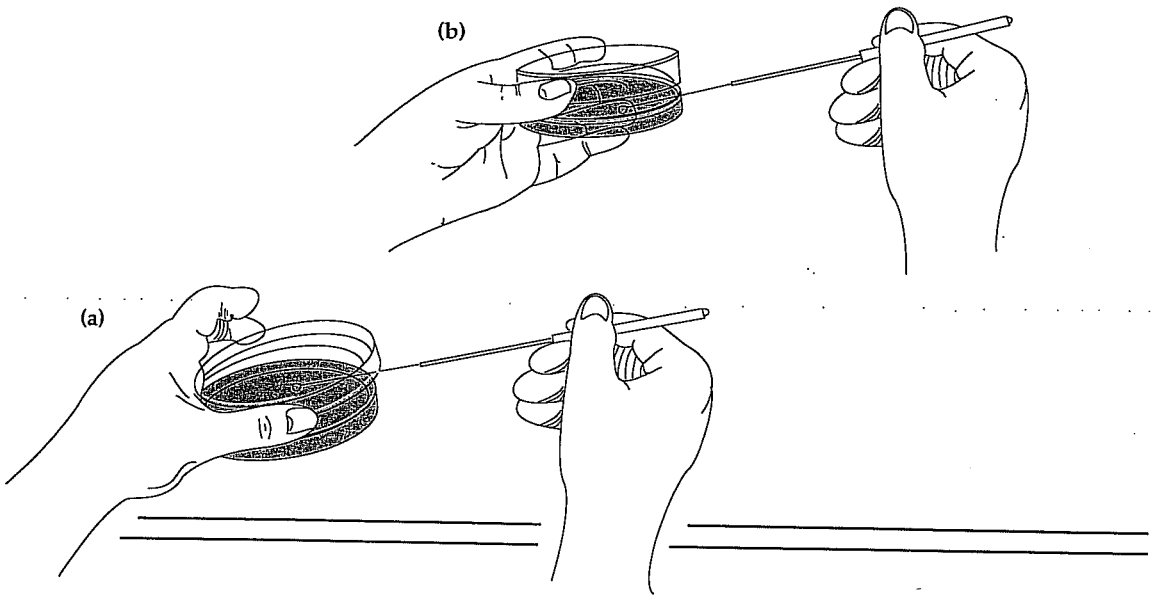


Figure 11.1

Inoculation of a solid medium in a Petri plate. Lift one edge of the cover while the plate **(a)** rests on the table or **(b)** is held.

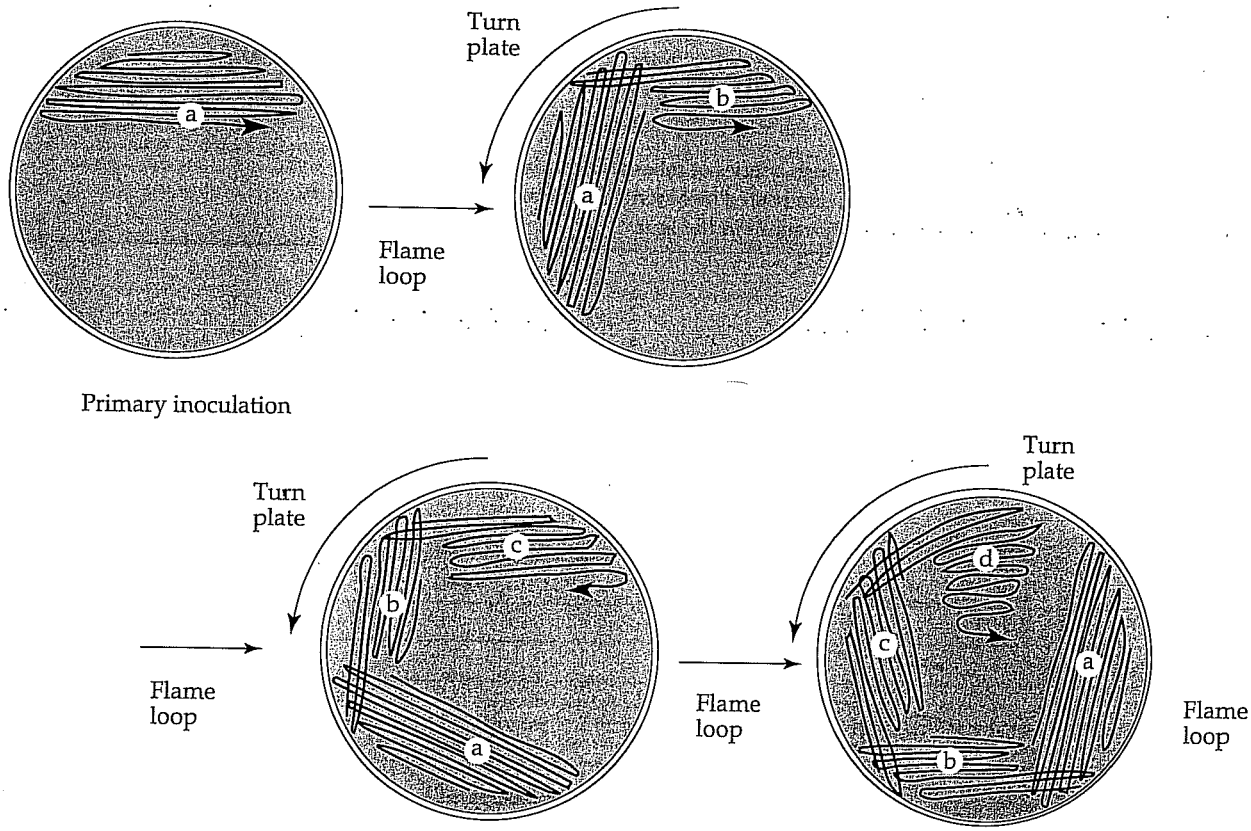


Figure 11.2

Streak plate technique for pure culture isolation of bacteria. The direction of streaking is indicated by the arrows. Between each section, sterilize the loop and reinoculate with a fraction of the bacteria by going back across the previous section.

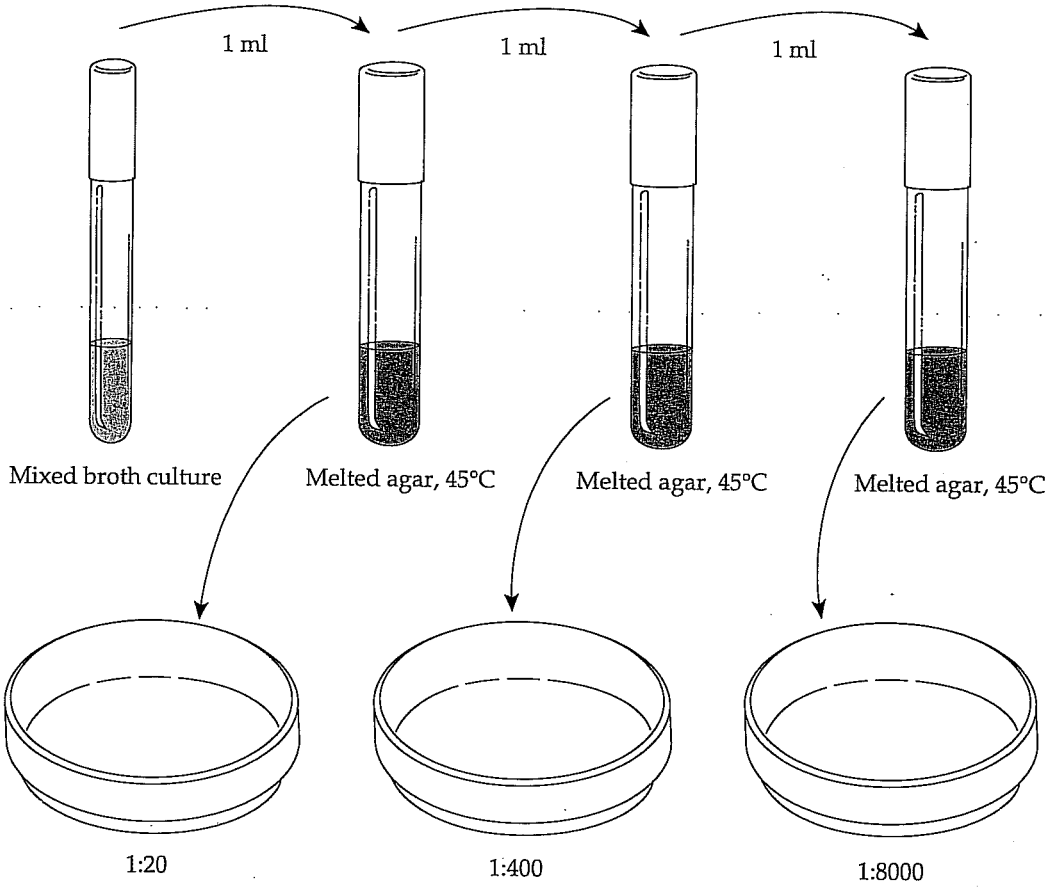


Figure 11.3

Pour plate technique. Bacteria are diluted through a series of tubes containing 19 ml of melted nutrient agar. The agar and bacteria are poured into sterile Petri dishes. The bacteria will form colonies where they are trapped in the agar.

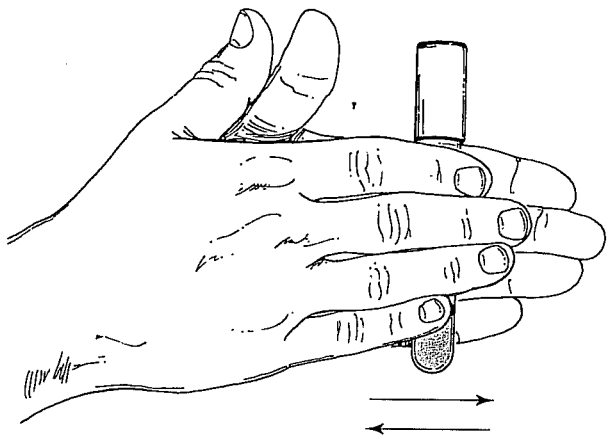


Figure 11.4

Mix the inoculum in a tube of melted agar by rolling the tube between your hands.

Exercise 11

Isolation of Bacteria by Dilution Techniques

LABORATORY REPORT

NAME _____

DATE _____

LAB SECTION _____

Purpose

_____

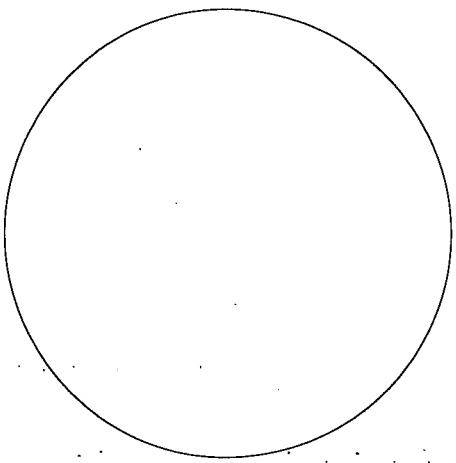
_____

_____

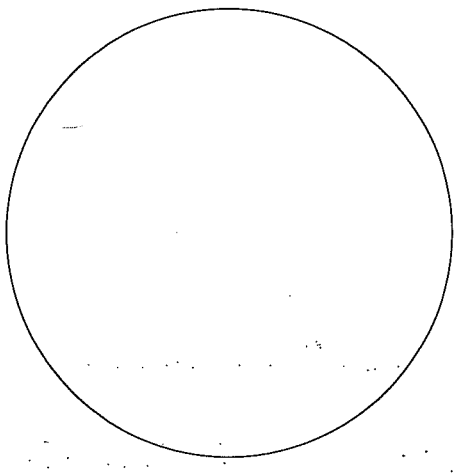
Data

Streak Plate

Sketch the appearance of the streak plates.



Mixed culture



Turbid broth

Fill in the following table using colonies from the most isolated streak areas.

Culture	Colony Description (Each different-appearing colony should be described.)				
	Diameter	Appearance	Margin	Elevation	Color
Mixed culture					
Turbid broth					

Pour Plate

Dilution	Number of Colonies
1:20	
1:400	
1:8000	

Calculate the number of colony-forming units per milliliter in the mixed culture. Which plate will you use for your calculations? _____
Show your calculations.

_____ Colony-forming units per ml

Subculture

Describe the growth on your slant. _____

Do you appear to have a pure culture? _____

Questions

1. How many different bacteria were in the mixed culture? _____ How many in the turbid broth? _____ How can you tell? _____

2. How do the colonies on the surface of the pour plate differ from those suspended in the agar?

3. What is a contaminant? _____

4. How would you determine whether a colony was a contaminant on a streak plate? _____

   On a pour plate? _____

5. What would happen if the plates were incubated a week longer? _____

   A month? _____

6. How could your streak plate technique be improved? _____

Critical Thinking

1. Could some bacteria grow on the streak plate and not be seen using the pour plate technique? Explain.

2. What is a disadvantage of the streak plate technique? Of the pour plate technique?

3. Will the isolated colonies always be in the fourth sector on the streak plate?