Exercise 4
ASEPTIC TECHNIQUE & STREAK PLATE PREPARATION

Introduction

In order to make laboratory studies with pure microbial cultures, microbiologists must start with sterile culture media and must be able to prevent contamination of this media. At the same time, they must be able to inoculate media with the desired pure cultures without introducing any other organisms. The procedure utilized to accomplish this is referred to as **aseptic technique** and is essential to any microbiology laboratory. **Remember** that all culture media must be sterile prior to inoculation. Media samples that appear to be contaminated with unknown cultures (are already supporting growth) must be discarded and not used for laboratory exercises.

Transfer Instruments

In order to inoculate a tube or plate with a desired microbial culture, students will use some type of transfer instrument, usually a wire loop or a pipette. If a wire loop or needle is being used, it must be sterilized (**heated to redness** by flaming) **before** and **after** making the microbial transfer. This heating destroys any living forms on the surface of the needle or loop, thus preventing unwanted contamination. To sterilize the loop or needle, hold it in the hottest part of the flame at an angle that will distribute heat to the **entire length** of the wire. After the loop or needle is sterilized in this manner, it must be allowed to cool briefly before being used to pick up the desired culture. **Remember that contact with hot metal will kill bacteria.** When using a wire loop to transfer bacteria from cultures grown on solid media it is recommended that students avoid filling the loop. Use a small volume of cells (just visible as a patch of color on the wire surface) for routine inoculations. This practice will allow most bacteria to be removed from the loop during inoculation and will prevent splattering when the loop is reheated. Avoid filling your loop, i.e., practicing "scoop-shovel" microbiology, as this will hinder your ability to obtain well-isolated colonies on streak plates.

In this laboratory students will occasionally use pipettes when making microbial transfers. Pipettes provided in air-tight plastic containers (sleeves) have been pre-sterilized and if handled appropriately will not introduce contaminants. Always open the plastic sleeve so that the pipette tip remains covered, and if a pipette bulb will be used, attach the bulb before removing the pipette from the sleeve. Do not allow the pipette tip to contact any foreign surface prior to making the transfer. When transfers are made with digital pipettes, the tips that will contact microbial cultures have been sterilized prior to use. Be careful to keep the pipette in a vertical position during use so the culture medium cannot contact the pipette body. **Keep the tip box closed to avoid the introduction of air contaminants**.

Microbial Transfers Using Tubes

When transferring microbial cultures to or from glass culture tubes, students are encouraged to use the following procedure. Hold the tube (or tubes) in your left hand and hold your transfer instrument with your right. Remove the plastic cap (or cotton plug) with the fingers of your right hand not engaged in holding the sterile loop or pipette. **Never lay a cap or plug down** on the surface of the table or bench. Keep the tube horizontal as much as possible during the transfer, and do not leave it uncapped longer than is necessary.

The mouths of tubes into which cultures are being transferred, as well as those from which cultures are being taken should be heated (rolled through a hot flame) immediately **before** and **after** the transfer instrument is introduced and withdrawn. In addition to destroying any organisms on the mouth of the

tube, this procedure tends to create convection currents that help to decrease the chances of air-borne contaminants entering the tube.

Microbial Transfer Using Plates

When transferring microbial cultures to or from plates, it is essential that exposure of the agar surface be kept to a minimum thus limiting contamination. Before transferring organisms from a plate, study the plate surface with the lid in place to determine which colony you wish to sample. To make the transfer, place the plate, top downward, on the counter or table surface, sterilize your loop or needle, lift the plate bottom and pick up the desired culture, then replace the plate bottom into the lid. When transferring organisms onto a plate, handle the plate in the same manner as described above.

Streak Plate Inoculation

The application of microorganisms to the medium surface within a plate, and the spreading of those organisms with a loop, needle or glass rod is called **streaking**, and the resulting preparation is called a **streak plate**. The objective of this technique is to dilute the culture, and to produce well-isolated colonies from a concentrated mass or suspension of cells. Streaking over a broad agar surface provides a technique by which mixed cultures can be separated since colonies of different types can usually be isolated by this means.

There are several patterns that may be used when streaking agar surfaces, all of which can yield well-isolated colonies. The diagram presented (Fig. 4.1) provides two examples.

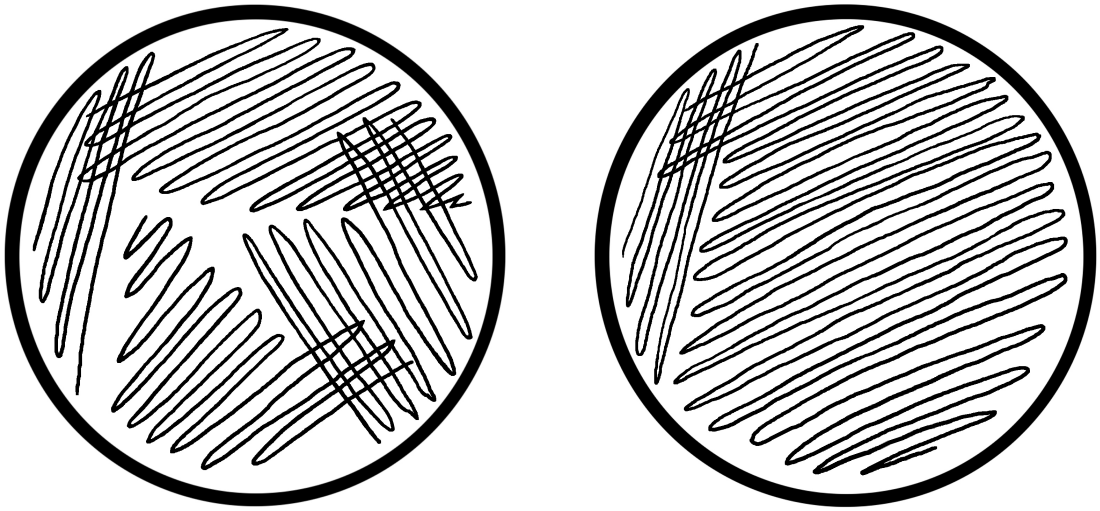

Fig. 4.1 - Successful Patterns - Streaking for Isolated Colonies

When applying these techniques, begin with a sample of culture on a wire loop. For pattern (A), streak the first section, flame the loop, streak a second section, flame the loop again, streak a third section, etc. After each flaming it is necessary to let the loop cool and then make two or three (NOT MORE) sweeps through the area already inoculated in order to pick up cells. For pattern (B), streak the first section, flame the loop, pick up cells from the first section, and then streak the loop back and forth across the remaining agar surface, at least ten times without interruption or overlap. The objective of these techniques is to **dilute the culture** and spread out the cells present using as much of the plate surface as possible. Since every viable cell in contact with the medium has the potential of producing a colony, only cells that are spaced well apart on the agar surface will yield well-isolated colonies.

Incubation of Tubes and Plates

Following inoculations, tubes or plates containing microbial cultures are stored or **incubated** in an environment suitable for growth, i.e. the development of populations. For many of the organisms used in this laboratory a temperature of 37^{o} C is optimal, for others, room temperature (25-27^{o} C) is best. In most cases, tubes are incubated in a vertical position in the racks provided, while plates are allowed to lay flat, agar-side up.

Because agar media contain considerable quantities of water, there is usually some condensation of moisture during incubation. If this moisture is allowed to drip onto the surface of the agar, it will spread out and cause the colonies forming there to grow together into a confluent mass. In order to avoid this, Petri plates are incubated bottom-side up.

NOTE: **ALWAYS INCUBATE PETRI PLATES BOTTOM-SIDE UP**
(unless otherwise instructed!)

Procedure:

Always use aseptic technique as demonstrated.

A. Inoculation of broth media (Please work in pairs or groups of three)

1. Obtain one tube of *Pseudomonas* enrichment medium and one tube of *Azotobacter* enrichment medium and label these tubes with your name, the medium types contained and the soil collection location.

2. Inoculate both tubes by placing a one-gram sample of soil into each. Gently swirl the liquid in your tubes to wet any soil that may be clinging to the inside surface of the glass. (Do not shake or invert these tubes because the plastic snap-caps will leak.)

3. Place both tubes in the labeled racks provided. They will be incubated at room temperature on the orbital shaker for the next few days.

B. Inoculation of Streak Plates

1. Use pattern A as indicated above (Fig 4.1) to inoculate three streak plates with the bacterial cultures provided as indicated below. **DO NOT WASTE MEDIA!**

   a. Streak a sample from Mix A onto **one** plate of tryptic soy agar (TSA). Note that TSA plates are marked with two blue-colored lines.

   b. Streak a sample from Mix B onto **one** plate of nutrient agar (NA). Note that NA plates are marked with one black-colored line.

   c. Streak a sample from your morphological unknown culture onto **one** plate of the medium type specified by your instructor.

   Remember that the object of streaking a broad plate surface is to dilute the culture so as to obtain well-isolated colonies. Use the surface as efficiently as possible and be careful not to gouge the agar. **DO NOT THROW PLATES AWAY** and take new ones because you think your streak technique was poor. **That is wasting media!**

2. Label your plates with waterproof markers or white tape on the bottom (agar containing side), not on the lids. Make sure your label includes:
   a. Your name (first initial and last name in full - do not abbreviate).
   b. The identity of the culture used (mix A, mix B or unknown by number).
   c. The current date.
   d. The type of medium used.

   Note that labels are applied to plate bottoms to insure that cultures cannot become separated from their labels. This practice will also allow labels to be visible during incubation and will keep the plate lids clear for easy observation of the colonies present.

3. Incubate your plates as directed until the next laboratory period.

Exercise 5
PURE CULTURE TECHNIQUE
(ISOLATION OF A PURE BACTERIAL CULTURE)

Introduction

In order to study the cultural and physiological characteristics of a microbial species, it is often necessary to separate that species from others living in the same habitat. In other words, a **pure culture** of the desired organisms must be obtained.

There are several methods by which a pure culture can be obtained, but one of the easiest and most commonly used is the streak plate method. A properly executed streak plate will yield well-spaced, isolated colonies from which several different species of organisms may be obtained. A **colony** is a mass of cells visible to the naked eye, and represents a **clone** of cells arising from a single vegetative cell, a spore, or a number of cells connected together in a chain or cluster (a colony-forming unit or cfu). Colonies arising from different microbial species often have distinct cultural characteristics and can be readily distinguished from one another. When cells taken from a single colony are inoculated into broth media, or onto the surface of a new plate, a pure culture of a single species will usually result. The purity of a broth culture can be checked by streaking a sample of it on the agar surface of a new plate. If all the colonies developing on the agar appear the same, the culture is probably pure.

Morphology may be defined as the science or study of form or external appearance (size, shape, color, etc.) without regard to function. In order to use morphological differences as a means of determining the purity of a microbial culture, one must be at least somewhat familiar with morphological features or **cultural characteristics**. Some important aspects of colony morphology are indicated below.

Note - Colonies growing within a streak plate will vary considerably in size depending on their density on the agar surface. In most instances, the largest, most isolated colonies will provide the best examples of cultural characteristics typical of the organisms present.

The **form** of whole colonies growing on a solid agar surface may be described as **punctiform** (pin-point), **circular**, **irregular**, **filamentous** or **rhizoid**. If the colonies are embedded in the agar they may be spindle-shaped or **biconvex**.

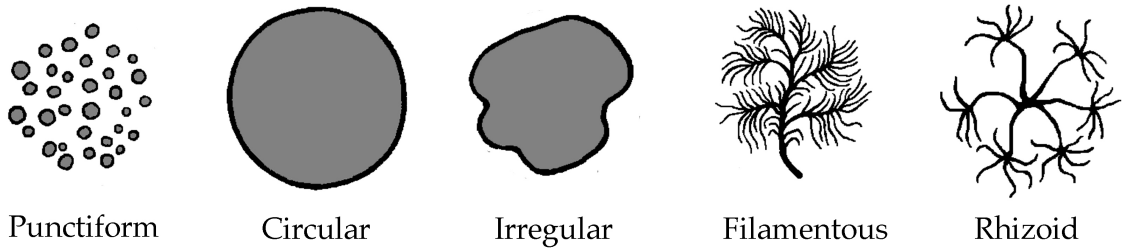

Fig. 5.1 - A variety of bacterial colony forms. Note that punctiform colonies are pin-point in size and that these illustrations are magnified 10X.

The edge or margin of an individual colony may be described as entire, undulate, lobate, serrate, filamentous or curled.

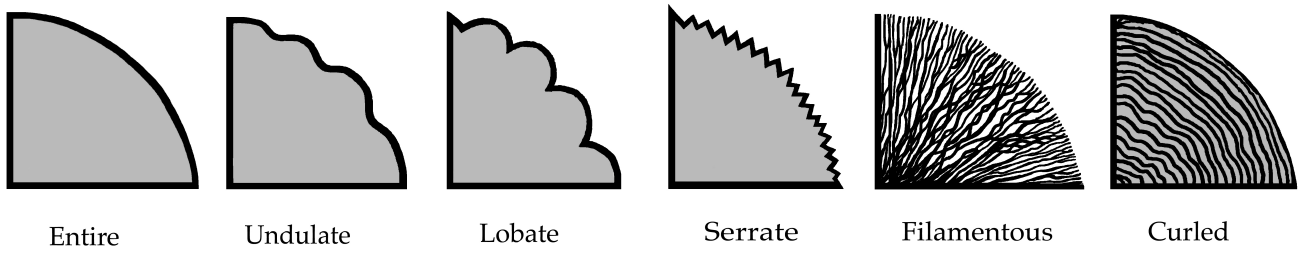

Fig. 5.2 - A variety of bacterial colony margins.

The **elevation** of a colony may be described as **flat**, **raised**, **convex**, **pulvinate** (dome-shaped) or **umbonate** (with a lump or indentation at the colony center).

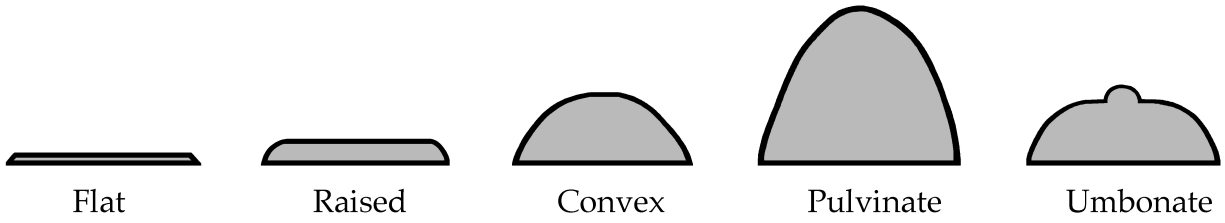

Fig. 5.3 - A variety of bacterial colony elevations.

The **surface texture** of colonies varies considerably and may be described as **smooth**, **shiny**, **glistening** (wet-looking), **dull** or **rough**, **wrinkled**, **dry** or **powdery**. Colonies also vary with respect to their **optical character** and may be described as **opaque**, **translucent**, **opalescent**, or **iridescent**. Some colonies are **fluorescent** (will glow when exposed to ultra-violet light) and some produce their own light (are **bioluminescent**). The color of colonies (which may or may not be uniform, and may change over time) is another morphological feature. Many colonies are **pigmented** (colored red, yellow, purple or some other color), and some produce water-soluble pigments that diffuse into the medium.

The **size** of microbial colonies (measured in millimeters) may be useful in their identification; however, the number of colonies present on an agar surface will influence their size. If colonies are crowded they tend to be small, and if they are distributed such that there is considerable space between them, they will be larger. Some cultures tend to spread or **swarm** over the agar surface and so form a thin or **effuse** layer rather than isolated colonies. Some colonies (especially filamentous and rhizoid forms), that are initially isolated, will continue to grow (enlarge) until they cover the entire agar surface. It is important to **remember** that morphological features often vary (sometimes considerably) depending upon the type of medium used for microbial growth.

Procedure:

1. Observe the streak plates of mix A and mix B prepared during the last laboratory session and determine the morphological features of the colonies present by comparing them to the examples described and illustrated above.

2. Record the morphological features that distinguish the organisms present on the mixed culture plates, and indicate which species are represented by each description. Although illustrations are not required, **remember** that making drawings will often enhance the perception of structural details.

3. Select one obviously distinct and well isolated colony (on either plate) and mark it by placing a circle around its location on the underside of the plate (use a wax pencil or lab marker).

4. Using aseptic technique, transfer some cells from this colony onto the agar surface of a new plate, and dilute the sample using a streak technique designed to yield well isolated colonies. **Note** that in order to pick up a large number of cells, it is only necessary to touch the colony with a cooled loop. **Do not try to scrape up the entire colony**, as it is not beneficial to transfer too many cells.

5. Observe the streak plate inoculated with your morphological unknown culture. If colonies are present, record their cultural characteristics and determine if or not the culture is pure. If the culture is pure, place the plate in your lab drawer for future investigation. If the culture is not pure, i.e., if there are contaminants present, select a well-isolated colony representative of the original culture and streak a new plate. If you are uncertain about which colony to restreak, ask your instructor.

6. Label your new streak plates with your name, the name of the microbial species present (or number of the tube from which the sample was taken), the date, and the type of medium used. Incubate the cultures at the appropriate temperature until the next laboratory period. Keep your original streak plates in your lab drawer.

6. Observe your streak plates during the next laboratory period. If all of the colonies that have developed on any plate appear to be the same, then you have probably established a pure culture. It will be necessary to make Gram stains of the morphological unknown cultures to determine microscopically if or not they are pure.

7. When you have obtained a properly streaked plate containing a pure culture with well-isolated colonies from Mix-A or Mix-B, check to be certain your label includes the correct name of the species present, and then turn the plate in. A properly prepared pure culture plate is worth 5 points.

Questions:

1. What is Aseptic technique? When do we use it in this laboratory?
2. What is the function of flaming the mouths of tubes prior to and following inoculation?
3. What is the objective of streaking microbial cultures over a broad agar surface?
4. Why are Petri plates normally incubated bottom-side up?
5. Where are labels applied to plates containing microbial cultures and why?
6. What is a pure culture and what method is most commonly used to obtain a pure culture?
7. Which method of streak plate preparation (streak from a broth mixture or directly from a colony on agar) seemed to yield the best results? What explanation can you give for the variation observed?

Name_____ **Lab Section**_____

WORKSHEET
Exercises 4 and 5
Streak Plate Preparation & Pure Culture Technique
"Mix A/Mix B Experiment"

Goals: _____
_____
_____

Part 1:Exercise 4 – Inoculation of Streak Plates
"Mix A/Mix B Experiment"

Hypothesis: _____
_____

Materials & Methods:

Medium type inoculated with "Mix A": _____
Incubation temperature: _____ Duration of incubation: _____

Medium type inoculated with "Mix B": _____
Incubation temperature: _____ Duration of incubation: _____

Data & Results:

How many different types of colonies do you see on your "Mix A" plate? _____

Cultural characteristics of colonies observed with Mix A:

	Colony 1	Colony 2
Form:		
Margin:		
Elevation:		
Surface Texture:		
Optical Character:		
Pigmentation:		
Size (mm):		
Other:		
Organism type:		

Part 1: Exercise 4 – Inoculation of Streak Plates (continued)

How many different types of colonies do you see on your "Mix B" plate? _____

Cultural characteristics of colonies observed with Mix B:

	Colony 1	Colony 2
Form:		
Margin:		
Elevation:		
Surface Texture:		
Optical Character:		
Pigmentation:		
Size (mm):		
Other:		
Organism type:		

Conclusions:

Was your hypothesis correct? _____ Explain. _____

_____

_____

_____

Part 2: Exercise 5 – Pure Culture Technique - Isolation of a Pure Bacterial Culture

"Pure Culture for 5 points"

Materials & Methods:

Organism chosen to isolate: _____

Medium type used: _____

Incubation temperature: _____ Duration of incubation: _____

Data & Results:

	At least 5 isolated colonies? (yes or no?)	Colonies are all the same morphology? (yes or no?)
Attempt #1:		
Attempt #2:		
Attempt #3:		
Attempt #4:		
Attempt #5:		

(If more attempts are required, please record your data on a separate sheet of paper.)

Conclusions:

Were you successful in acquiring a pure culture? _____ Explain. _____

_____

_____

Was your streaking technique successful? _____ Explain. _____

_____