

# LAB 4. Cultivation of Bacteria

Protocols for use of cultivation of bacteria, use of general growth, enriched, selective and differential media, plate pouring, determination of temperature range for growth and determination of salt tolerance capacity of bacteria.

## INTRODUCTION

In nature, bacteria exist as mixed populations. In the laboratory these populations must be separated so that characteristics of individual species may be observed. A number of basic techniques are used in microbiology with this end in mind.

- First, microorganisms must be removed from natural environments and cultured in the laboratory. This requires artificial media and surfaces on which bacteria may grow. This also requires knowledge of nutritional requirements and environmental requirements (such as temperature of incubation and the requirement of oxygen).
- Second, bacteria of interest must be separated from all other bacteria in the environmental sample. This requires separation techniques that allow isolation of a pure culture of one type of bacteria.
- Third, once a pure culture is achieved, no contaminating bacteria can be introduced from the environment. This requires that all media and lab supplies be sterile (that is contain no bacteria that may contaminate the culture of interest).
- Fourth, techniques are needed that facilitate working with pure cultures. This requires aseptic technique and techniques of storage for pure cultures.

When microbial ecologists seek to isolate new bacteria from the environment, they must experiment with many nutrients and growth conditions to culture the newly isolated bacteria in the lab. It is often very difficult to replicate bacterial growth conditions in the lab. It is estimated that only 0.1% of all bacteria have been successfully cultured.

## A. Use of Media: Types of media.

The fundamental function of bacterial media is to provide nutrients for the growth of microbes in the lab. Media designed for growth are defined below.

**General Growth** media support the growth of a broad range of organisms. General growth media usually have **complex** constituents. Examples of General Growth media that are also complex include: *Trypticase-soy* and *Nutrient media*. Media may be designed to support the growth of one particular organism. Constituents of such media are generally defined and the media is referred to as **Chemically Defined media**. An example is the *Glucose Minimal Salts Broth* designed to support growth of *E.coli* (see Figure 1).

**Enriched** media is designed to support the growth of organisms with unusual growth requirements. Such organisms are referred to as **fastidious**. Special nutrients are added to a *general growth media* to support the needs of organisms with demanding nutritional requirements. The supplements added will vary dependent upon the particular requirements of the fastidious organism. An example of an enriched medium is *T-soy medium supplemented with sheep blood* (when agar is added to this medium it is commonly called “**blood agar**”).

Because the task of isolating a given bacterial species from a mixture can often be simplified by taking advantage of a particular nutritional requirement of a given species, constituents of media can be varied to select for, or differentiate among bacterial types. Media designed for selection and differentiation are described below.

**Selective media** will permit the growth of one type of bacteria while preventing the growth of other types. This will facilitate the isolation of a desired species. *MacConkey's medium* is selective. It contains bile salts and crystal violet, these will inhibit the growth of Gram-positive organisms. Other examples include *phenylethyl alcohol agar*, which inhibits or slows the growth of Gram-negative organisms, and *mannitol salt agar* which inhibits the growth of salt intolerant organisms.

**Differential media** will allow visual differentiation between two or more species of bacteria. Examples include *blood agar*, *MacConkey's medium* and *Mannitol Salt agar*.

**T** Colonies growing on blood agar are differentiated by hemolysis patterns (greening - alpha hemolysis, clearing - beta hemolysis and no hemolysis - gamma). ~ Figure 4.22

**T** Using MacConkey's medium, lactose fermenting and lactose non-fermenting bacteria can be distinguished. Organisms that are able to ferment lactose will produce an acid end-product that causes a change in the pH of the surrounding media. A pH indicator (neutral red), is present in the media and changes from yellow to red in an acidic environment. Lactose fermenting bacteria growing on

MacConkey's media will appear pink whereas non-lactose fermenters will be white. ~ Figure 4.39.

**T** Mannitol salt agar allows discrimination among salt tolerant organisms. Those that ferment mannitol will produce acid turning the pH indicator (phenol red) to a yellow color. Those that cannot ferment mannitol will leave the media the red color of phenol red at neutral pH. ~ Figure 4.28

### Solid vs liquid media

Organisms can be grown in liquid media (broth) or on solid media. For example, Nutrient media is referred to as *Nutrient Broth* when in the liquid form, and *Nutrient Agar* when in the solid form. Agar, a galactan obtained from marine algae is used as the hardening agent in solid media. Broth, with added agar powder, is heated to 121°C in an autoclave, dissolving the agar and sterilizing the medium. The molten medium may be poured into plates or tubes. The agar-media will remain liquid at temperatures above 45°C. Below 45°C the agar will harden, and supply a solid surface for the growth of bacteria. Agar plates and slants may be inoculated after they have solidified. To avoid any condensation-droplets from falling onto agar surfaces and smearing the inoculum, agar plates are inverted during incubation.

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#### Figure 1. Media constituents

Nutrient Agar: Example of a complex, general growth media

<i>Constituent</i>	<i>Amount</i>
Peptone	5.0g
Beef extract	3.0 g
Sodium Chloride	8.0 g
Agar	15.0 g
Water	1 liter

Glucose Minimal Salts Broth: Example of chemically defined medium designed for the growth of *E.coli*.

<i>Constituent</i>	<i>Amount</i>
Glucose	5.0 g
Ammonium phosphate monobasic	1.0 g
Sodium chloride	5.0 g
Magnesium sulfate	0.2 g
Potassium phosphate, dibasic	1.0g
Water	1 liter

Reference: Tortura, Funke, Case, 1998, **Microbiology, An Introduction**, 6<sup>th</sup> edition, Addison Wesley Longman Inc.

To study bacteria from an environmental sample or a mixed culture it is necessary to isolate a pure culture of each bacterium of interest. Using differing growth conditions and a variety of media, one can *begin* to separate bacteria, but, generally, methods to *physically* separate bacteria are also needed.

**B. Use of Physical Separation Procedures:**

**i Streak Plate technique**

As we have seen in previous labs, single colonies may be achieved by using the **streak plate technique**. Additional methods used to physically separate bacteria include; dilution in liquid, the **pour plate** (where diluted cultures are *poured* onto a solid medium spreading over its surface) and the **spread plate** (where diluted cultures are *spread* onto a solid medium and thus distributed over the medium surface). These additional methods will be described later in this manual.

### C. Growth Conditions

To grow bacteria in the lab, environmental conditions, as well as nutrients, must be considered. Bacteria may be isolated from a variety of environments. For cultivation of bacteria in the lab, the conditions of the environments must be mimicked. Prokaryotes that live in extreme environments are generally in the Domain: Archaea. Those that live in more moderate environments are generally in the Domain: Bacteria.

Consider **temperature**.

Microorganisms whose optimal temperature for growth is near 0°C are called **psychrophiles**. Those that grow optimally between 20°C and 45 °C are called **mesophiles**. Those growing best at high temperatures, 55°C to ~ 70 °C and higher, are called **thermophiles**. Prokaryotes that live at very high temperatures ( up to 113 °C ) are **hyperthermophiles**. Consider **salt tolerance**.

Some bacteria require relatively high concentrations of salt for growth (10-20%); these organisms are called **halophiles**. Halophiles must possess special membranes and enzyme mechanisms to survive in salty environments. Some organisms are **salt tolerant**. They do not require salt for growth but may grow in its presence. An example is *Staphylococcus aureus*. *S. aureus* is found on skin, which often has a high salt concentration (10% NaCl). Consider the requirement for **oxygen**.

An organism that *requires* oxygen for growth is called **aerobic**. An organism whose growth cannot occur in the presence of oxygen is **anaerobic**. An organism that can grow under either aerobic or anaerobic conditions is a **facultative anaerobe**. **Microaerophilic** organisms require free oxygen but at a very limited concentration.

The requirement for oxygen relates to the energy metabolism of an organism and the presence of enzymes that destroy toxic products of oxygen. Aerobes use respiration as a mechanism for energy metabolism, and oxygen serves as the terminal electron acceptor. Aerobes have enzymes such as catalase that help the organism deal with toxic forms of oxygen. Anaerobes do not use oxygen for energy metabolism and have no enzymes to cope with toxic forms of oxygen. Facultative anaerobes do not require oxygen for energy metabolism but many will use it if it is present. They have enzymes to cope with toxic forms of oxygen. Because the requirement for oxygen relates so closely to the energy metabolism of an organism, the requirement for oxygen will be investigated in Lab 5.

**Objectives:**

To investigate the requirement of media constituents and environmental conditions in the cultivation of bacteria.

- Learn to complete all protocols effectively.
- Learn to recognize positive and negative results for growth on general growth, enriched, selective and differential media
- Learn to Pour plates.
- Learn how to determine environmental requirements for bacterial growth
- Learn an appreciation for culture characteristics of bacteria.

**Protocol: PREPARATION OF SOLID MEDIA: Plate Pouring****MATERIALS/group.**

- 1 Sterile bottle containing 75ml Nutrient Agar  
(melted, autoclaved, in water bath at 60°C)
- 3 sterile petri plates
- Jar of powdered Nutrient Agar

**METHOD**

1. Your TA will demonstrate how molten media was prepared from powdered media, agar and water. The agar media was solubilized by boiling, and sterilized by autoclaving.
2. Your TA will demonstrate how plates are poured and then each student will pour one plate.
3. Allow plates to cool.

*Plates prepared in this section should be used for maintenance of Assignment 1 Organisms.*

## **Protocol: Use of Selective, Differential and Enriched Media**

### **MATERIALS/group**

- E. coli* - gram negative, lactose fermenting
- Salmonella typhimurium* - Gram negative, does not ferment lactose.
- Staphylococcus aureus* - Gram positive, mannitol fermenting
- Staphylococcus epidermidis* - Gram positive, does not ferment
- 1 Nutrient agar plate
- 1 Blood plate
- 1 MacConkey's agar plate
- Control examples

### **PROCEDURE**

1. Label the bottom of each plate with the name of the medium. Your name and lab section number.
2. To test for the growth of numerous bacterial species on one petri dish, the plate may be streaked in sectors. The streaking will not allow observation of isolated colonies, but will allow the determination of positive or negative growth. On the bottom of the petri dish, use a marker to divide each plate into sectors: e.g. three sectors for three bacterial test species.
3. Inoculate by streaking each sector with test culture. Streak each sector carefully, staying within the sector margins to minimize the possibility of cross contamination.
4. Incubate plate in an inverted position and wrapped in a plastic bag. For most cultures in BSCI 223 incubation at 37°C for 24 hours is sufficient to allow growth.
5. After incubation time is complete, observe plates for
  - Presence of absence of growth. For BSCI 223 organisms that you know will grow well on Nutrient Agar, use growth on Nutrient Agar as a control for testing growth of organisms on enriched, selective, or differential media.
  - color of growth
  - color of media
6. Compare observations of test organisms to control organisms.

### **/ Assignment 1 Check Point :**

Nutrient Agar, Trypticase Soy agar enriched with sheeps blood, MacConkey agar are provided. What will you learn by inoculating your environmental isolates on these media?

## Protocol: Determination of Range of Temperature for Bacterial Growth

### MATERIALS

Control examples  
4 Nutrient agar plates per group of 3 students  
Incubators set at 25 °C, 30 °C, 37 °C, and 55°C.

### PROCEDURE

1. Label the bottom of each plate with the name of the medium. Your name and lab section number. Label one plate for every temperature to be tested.
2. To test for the growth of numerous bacterial species on one petri dish, the plate may be streaked in sectors. The streaking will not allow observation of isolated colonies, but will allow the determination of positive or negative growth. On the bottom of the petri dish, use a marker to divide each plate into sectors: e.g. three sectors for three bacterial test species.
3. Inoculate by streaking each sector with test culture. Streak each sector carefully, staying within the sector margins to minimize the possibility of cross contamination.
4. Incubate plate in an inverted position and wrapped in a plastic bag. For most cultures in BSCI 223 a temperature of 37°C is considered optimal. To determine the range at which the culture may grow: incubate plates at a variety of temperatures. At lower temperatures, organisms may grow more slowly. Allow 48 – 36 hours for growth. Suggested temperatures for testing: 25 °C, 30 °C, 37 °C, and 55°C. For incubation at high temperatures, include a wet paper towel in the bag with the plates. This will keep the media moist during incubation.
5. After incubation, observe plates for growth. Compare growth at test temperatures to growth at a control temperature.

### / *Assignment 1 Check Point :*

All environmental isolates are **mesophiles**. Each has its *optimum temperature* for growth at 37°C, however, each may have a different *range of temperatures* where they can grow. Can you distinguish among your organisms by growth at different temperatures? What control temperature will you use?



## **Protocol: Determination of Growth at a variety of salt conditions**

### **MATERIALS/group**

Control examples

*Staphylococcus aureus* - salt tolerant

*Planococcus halophilus* - halophile

*E. coli* - salt intolerant

4 nutrient agar plates containing either 0%, 7.5%, 10%, or 15% NaCl

### **PROCEDURE:**

1. Label the bottom of each plate with the name of the medium. Your name and lab section number.
2. To test for the growth of numerous bacterial species on one petri dish, the plate may be streaked in sectors. The streaking will not allow observation of isolated colonies, but will allow the determination of positive or negative growth. On the bottom of the petri dish, use a marker to divide each plate into sectors: e.g. three sectors for three bacterial test species.
3. Inoculate by streaking each sector with test culture. Streak each sector carefully, staying within the sector margins to minimize the possibility of cross contamination. To determine the range of salt tolerance, streak bacteria on plates with a range of salt concentrations. Inoculate a 0% salt plate as a control for growth.
4. Incubate plate in an inverted position and wrapped in a plastic bag. For most cultures in BSCI 223 incubation at 37°C for 24 hours is sufficient to allow growth

### **/ Assignment 1 Check Point :**

Can you distinguish among your environmental isolates based upon a requirement for, or tolerance to, salt?

What new information about your organisms have you gained from this lab?

Can you write sentences like this:

“Isolate A is a salt tolerant, alpha hemolytic, Gram positive cocci. It grows best in the mesophilic range and is lactose fermenting.” ?

Did the MacConky plate data agree with Gram stain results? (If not, repeat your Gram stain!)

With new information gained in this lab, can you distinguish among your isolates?

**Exercise 4** \_\_\_\_\_ **Laboratory Report Questions**  
**Cultivation of Bacteria**

Name \_\_\_\_\_

Results:

Record observations:

Results Table 1: Growth characteristics of environmental isolates on culture media.

Media:	Nutrient Agar	Blood Agar	MacConkey's agar
Culture 1			
Culture 2			
Culture 3			

Results Table 2: Growth of environmental isolates at varying temperatures.

Temperature:				
Culture 1				
Culture 2				
Culture 3				

Results Table 3: Growth of environmental isolates at a variety of salt concentrations.

Salt Concentrations:	0%	7.5%	10%	15%
Culture 1				
Culture 2				
Culture 3				

Questions:

1. An organism grows on Nutrient Agar plates with 7.5%, 10%, and 15% Na Cl but does not grow on Nutrient Agar without added salt. This organism would be characterized as a \_\_\_\_\_.

2. Which type of organism would you most likely find to grow in food in your refrigerator?

Mesophile? Psychrophile? Thermophile?

Why?

3. Which type of organism would you most likely expect to cause disease in humans?

Mesophile? Psychrophile? Thermophile?

Why?

4. Define:           General Growth media           Alpha hemolysis.

