CULTURE MEDIA AND CULTIVATION OF BACTERIA

The study of microorganisms requires techniques for isolating cells from natural sources and growing them in the laboratory on synthetic media. Thus, development of synthetic culture media and culture techniques have played important roles in the advance of this field. Microbiologists use bacterial culture media for many purposes and applications. Media are used to isolate and identify bacteria, reveal their metabolic properties, and allow long term storage of pure cultures. Taxonomic descriptions of bacteria commonly include information about their cultural requirements; species that are poorly characterized are frequently those most difficult to culture under laboratory conditions. Indeed, Koch's second postulate requires culturing of a suspected pathogen in pure form. In this laboratory exercise you will learn about composition and types of culture media and how different types of media can be used to study the properties of bacteria. You will be asked to drawn upon this knowledge frequently in future laboratory exercises.

Our ability to study different types of bacteria ultimately relies upon knowing their nutritional requirements. The bacteria with which we are most familiar are **generalists** (able to use a wide range of nutrients) and/or can use nutrients in forms that are easy to provide. However, when if swab a sample of saliva on a plate of culture medium, only a small percentage of the hundreds of different bacteria in you mouth will form colonies. This is because most bacteria are **fastidious**, meaning that they have very specific and/or complex nutritional requirements. These species do not grow because they cannot use one or more nutrients in the form provided in the medium (e.g, they might require H_2S rather than SO_4^{2-} as a sulfur source), have requirements for very specific types of nutrients (such as certain complex organic molecules), and/or require unusual growth conditions (such as growth in living cells or at high temperature or pressure). We presently know very little about many of these bacteria because we do not know how to grow them under artificial laboratory conditions.

One factor that greatly influences bacterial growth in their oxygen requirements. Clearly, the techniques used to culture and study an obligate anaerobe must be different from those used when culturing an aerobe. Thus, in the final section of this lab exercise you will study bacterial oxygen requirements and methods used to culture anaerobes.

Summary of exercise

1. You will prepare a complex-type medium from a preformulated mixture and use it to culture bacteria.

2. You will prepare 'defined' media that will be used to determine the carbon and nitrogen requirements of bacteria.

3. Kligler Iron Agar will be used to illustrate the properties of a differential medium.

4. You will use two techniques to culture anaerobic bacteria.

Types of culture media

Media are classified on the basis of composition or application. Usually, a particular medium may fall into two or more categories, which leads to some confusion among students. Thus, for example, a "defined" medium might be also classified as "selective" or "differential" depending upon its specific properties and application in a particular situation. The four principal types of culture media are described below.

COMPLEX MEDIA. Pasteur, Koch, and Tyndall typically prepared media by boiling animal or plant materials to extract nutritive molecules. Today, many modern complex media (such as **Tryptic Soy Agar)** contain extracts of beef (**peptone**), milk (**tryptone**), soybean meal (**soytone**), or yeast. The protein in these extracts are broken down into small peptides and amino acids. Although the specific amount of these molecules is not precisely determined, their wide assortment allows complex media to support a wide range of bacterial types.

DEFINED MEDIA. Defined media are formulated from pure substances at predetermined concentrations. Thus, unlike complex media, the exact chemical composition of defined media is known precisely. Because the composition is precisely established, defined media are often used to determine the nutritional requirements of bacterial species.

SELECTIVE MEDIA. Complex or defined media may also be classified as selective (or ENRICHMENT) media, which support the growth of only certain types of bacteria. Media can be made selective through the addition of substances that enhance or inhibit the growth of particular types of bacteria. Media have been developed that are selective for an astonishing diversity of bacteria, and we will be using many of these media throughout the semester.

DIFFERENTIAL MEDIA. Any of the above types of media might also be formulated as a differential medium. A differential medium reveals specific metabolic or metabolic characteristics of bacteria grown on it. Differential media are among the most powerful tools available to a microbiologist, revealing a wide range of information about an organism very quickly. Some media are both selective and differential. For example, the medium called **MacConkey Agar** is selective for gram-negative bacteria and will indicate whether bacteria can ferment lactose.

I. PREPARING 'COMPLEX TYPE' CULTURE MEDIA

Microbiologists traditionally mixed media by combining the individual components listed for a recipe. For a complicated medium, this could be a very time-consuming process. Today, most media are available commercially in premixed and dehydrated form. The media are prepared by simply dissolving the powder in water, sterilizing the solution, and then dispensing it into culture vessels. Two of the largest suppliers are **BBL** and **Difco** laboratories. Difco publishes a book (cleverly named the "Difco Manual") that lists the application, composition, history of development and other useful information for each medium it supplies. Copies of the 9th edition of the Difco Manual are in the laboratory, and you are encouraged to peruse one at your convenience during the laboratory period.

Your objective in this exercise is to gain experience in preparing bacterial culture media. The medium that you will prepare is called **Tryptic Soy Broth (TSB)** which is designed to support the growth of a wide range of bacterial types. The composition of this medium is given below:

TRYPTIC SOY BROTH (TSB)

Component	g/L medium
Tryptone	17.0
Soytone	3.0
NaCl	5.0
K_2HPO_4	2.5
Glucose	2.5
	30.0

TSB is a "complex" type medium. How can you tell? To prepare 1 liter of Tryptic Soy Broth (TSB), 30.0 g of the medium would be dissolved in 1 liter of H_2O and then autoclaved. This would yield a liquid (broth) culture medium.

Solidified media

Often, culture media is prepared in a solid form, such as that in the slants and petri plates which you used previously this semester. Generally, culture media is solidified with the addition of AGAR, a purified carbohydrate obtained from a marine seaweed. Agar is the most widely used solidifying agent for two reasons: 1) very few bacteria can metabolize it, and thus, it does not serve as a nutrient source, and 2) it has the unusual property of melting at 100°C but not resolidifying until the temperature decreases to 45°C. Thus, depending upon its previous temperature history, media containing agar can exist as either a liquid or solid at a temperature between 45° - 60°C . In future laboratory exercises we will take advantage of this property.

Supplies

1 empty 250 ml flask
1 empty 16mm test tubes (red cap)
4 sterile petri plates
agar (white cap)
paper tag (for labeling bottle)
TSB (clear cap)
1 5 ml pipet

Procedure

In this part of the lab exercise you will prepare and sterilize Tryptic Soy Broth (TSB) and solid **Tryptic Soy Agar (TSA)** culture media. We will begin by preparing 100 ml of TSB which is prepared at a ratio of 3 g of the dehydrated powder per 100 ml of medium.

A. Preparing TSB broth medium

1. Transfer the dehydrated TSB medium to a 250 ml flask. The proper use of the laboratory scales will be demonstrated during the lab period.
2. Add 100 ml of deionized water and swirl until all of the powder is completely dissolved.
3. To emphasize the importance of media sterilization, pipet 5 ml of TSB from the bottle into a 16mm test tube. Label and place it in your drawer.
4. Examine the appearance of the medium next time you come to lab, and then dispose of this medium.

B. Preparing solid medium (TSA)

You will now add agar to the remaining 95 ml of TSB to produce "Tryptic Soy Agar". Like most solid media TSA has an agar concentration of 1.5% (w/v), which is 1.4 g per 95 ml.

1. Transfer the agar to the TSB solution.
2. Cover the mouth of the flask with foil, and label it on a paper tag as "TSA" with your name and date.
3. Place the flask in the tray to be autoclaved.

Sterilizing the media will take about one hour. During this period you should continue with the other parts of the exercise. When the sterilization is completed, the TSA flasks should be transferred to a 50°C water bath to cool. Note that at this temperature they will not solidify. After 10 to 15 minutes the media will be cool enough to handle.

C. Pouring solid medium into petri plates

In this part of the exercise you will pour your sterilized TSA medium aseptically into petri plates.

1. Label the base of 4 petri plates with your name and date.
2. Remove your flask from the water bath, DRY THE OUTSIDE with a paper towel, and SWIRL THE MEDIA to mix the dissolved agar completely.
3. Return to your work station. You will need to work quickly but carefully now because the medium will begin to solidify when the temperature drops to 45°C.
4. Remove the foil from the flask and hold the flask at an angle at all times to minimize the chance for contamination.
5. Sequentially, lift the lid of each plate just enough to allow the medium to be poured in. Add enough to each plate to cover the base.
6. Let the media solidify for about 15 minutes before moving the plates.
7. Save the solidified plates in your drawer. **You should use two of them to restreak your semester unknown to check for contamination.**

II. USING DEFINED MEDIA TO STUDY NUTRITIONAL REQUIREMENTS

Many species of bacteria are identical in appearance and can only be distinguished by their biochemical of metabolic properties. One such property is their nutritional requirements. While all bacteria require similar essential mineral nutrients, such as carbon and nitrogen, species must obtain these nutrients in particular chemical forms. For example, some species can obtain nitrogen from nitrite (NO_2) but not ammonia (NH_3), vice versa, either, or neither. DEFINED media, which contain specific quantities of known substances are commonly used to determine the specific nutritional requirements and capabilities of bacteria. Since defined media typically provide the 'minimal required' source of nutrients, even generalist bacteria may grow more slowly than on a complex type of medium.

In this exercise, you will prepare a series of defined media that contain different nitrogen sources. The components of the media you will prepare are provided as sterile stock solutions. You will combine these stock solutions in various combinations to yield the media with the desired compositions. The carbon and nitrogen sources will be added to the following base salts medium (solutions A and b are sterilized separately and then combined together):

BASAL SALTS MEDIUM

Solution A	g/L	Solution B	g/L
K_2HPO_4	2.0	$FeCl_3$	0.002
NaCl	5.0	$MgSO_4 \cdot 7H_2O$	0.06
pH 7.1		$MnSO_4 \cdot H_2O$	0.0008

Supplies

12 sterile 13mm test tubes (light blue caps)
six 5 ml pipets
your bacterial unknown
Enterobacter aerogenes (light blue cap)
Pseudomonas aerogenes (white cap)

1 tube of basal salts medium A (black cap)
1 tube of basal salts medium B (red cap)
1 tube of NO_2 stock (white cap)
1 tube of NH_3 stock (turquoise cap)
1 tube of mannitol stock (blue cap)
1 tube of lactose stock (avacado cap)

Procedure

BEFORE STARTING THIS EXERCISE, READ THE HELPFUL HINT GIVEN BELOW.

1. Using 5 ml pipets, add 0.5 ml of the basal salts media (A and B) to each of 12 sterile 13mm tubes.
2. Using a single 5 ml pipet per component, add 0.5 ml of each to the appropriate tubes according to the table below.
3. Incubate these tubes at 37°C 24-48 hr

A positive result is indicated by a distinct turbidity (cloudiness) of the medium due to bacteria; a small, clumpy precipitate may form at the bottom of the tubes, and this should not be interpreted as growth. Record and interpret your results in Tables 1 and 2.

Protocol for preparing defined media for nutritional requirements exercise.

Tube #:	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
Stock solution	These tubes receive *Enterobacteria*				These tubes receive *Pseudomonas*				These tubes receive your semester unknown			
Mannitol (blue cap)	X	X			X	X			X	X		
Lactose (avocado cap)			X	X			X	X			X	X
NH_3 (turquoise cap)	X		X		X		X		X		X	
NO_2 (white cap)		X		X		X		X		X		X

Helpful hints

Even experienced scientists occasionally 'screw-up' experiments, particularly if distracted while multiple components are being added to a series of tubes. The following technique will help to avoid this problem while adding the carbon and nitrogen sources. Start with all of the tubes aligned in the front row of the rack, and move tubes 3, 4, 7, 8, 11, and 12 back one row. With a five ml pipet add 0.5 ml of the mannitol solution to all of those tubes for which mannitol is required, moving each back one row after the addition. All the tubes will again be in the same row. Repeat this process with each of the other ingredients; e.g. before adding the lactose, move tubes 1, 2, 5, 6, 9 and 10 back one row, etc.

III. DIFFERENTIAL AND SELECTIVE MEDIA

There are many media available that visually distinguish bacteria with particular metabolic characteristics, and many media that will selectively grow only certain types of bacteria. MacConkey Agar is one of the most widely used media in clinical settings and has both differential and selective properties.

MacConkey Agar

Component	g /L medium
Peptone	17.0
Protease peptone	3.0
Lactose	10.0
Bile salts No. 3	1.5
NaCl	13.5
Neutral red	0.003
Crystal Violet	0.0001
Agar	13.5

MacConkey agar medium is primarily used to differentiate between Gram negative enteric bacteria, such as from stool samples, while inhibiting the growth of most Gram positive bacteria. The medium differentiates bacteria that can ferment lactose, which include fecal coliform (see water analysis lab exercise) and a variety of potential pathogens.

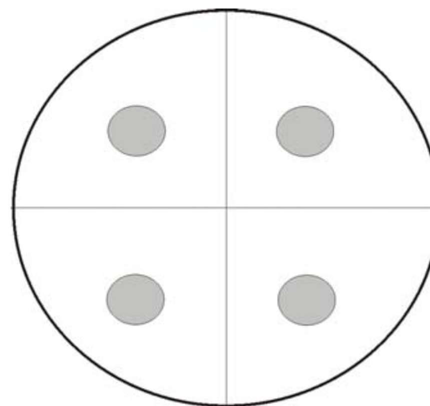
The selective property of the medium comes from the presence of bile salts and crystal violet that inhibit most Gram positive bacteria. Fermentation of lactose is differentiated by the neutral red, a pH indicator. When lactose is fermented, acid products lowers pH below 6.8, neutral red causes the medium to turn pinkish-red. Colonies of lactose-nonfermenting bacteria will be colorless.

Supplies

Escherichia coli broth culture (brown cap)
Proteus vulgaris (violet cap)
Bacillus subtilus (green cap)
Your semester unknown
1 MacConkey agar plate

Procedure

1. Quadrasect and label the bottom of the MacConkey agar plate as shown to the right.
2. Inoculate the bacteria as spots in the appropriate sectors.
3. Incubate the plate at 37°C for 24 -48 hours.
4. record your observation in Table 3



Innoculation of MacConkey Agar

IV. CULTURING ANAEROBES

Bacteria will only grow in an environment that contains an appropriate concentration of oxygen. For example, obligate aerobes grow only in the presence of oxygen, whereas obligate anaerobes grow only in an oxygen-free environment. Culturing of bacteria therefore requires cultivation under an atmosphere that supports their growth. While it initially may seem that achieving anaerobic conditions would be difficult, in fact a variety of special media and apparatuses can be used to support growth of anaerobes. Some of these techniques are discussed below.

1. Anaerobic container. Systems have been developed that allow bacteria to be cultured on petri plates under anaerobic conditions The Bio-Bag system consists of an air-tight plastic bag in which a petri plate can be sealed. Also placed in the bag is a disposable chemical H_2 gas generator and a catalyst which the H_2 reacts with O_2 to form water. Since the bag is sealed, eventually all of the O_2 is consumed, creating an anaerobic environment. Finally, an indicator (pink in the presence of O_2 and colorless in its absence) is also included to confirm that anaerobic conditions are achieved. This methodology has the advantage of allowing streak plating, colony identification and isolation, etc. – i.e., procedures that require that bacteria be cultured on solid medium,

2. Shake culture technique. Autoclaving a tube of medium drives off most of the dissolved oxygen. Reoxygenation of the medium occurs slowly, allowing the medium below the surface to remain anaerobic. In the "shake culture" technique, a tube of Fluid Thioglycollate Medium(FTG) held at $48^{O}C$ is inoculated before the medium solidifies. FTG contains a small amount of agar to increase viscosity and reduce diffusion of oxygen into the medium. The position in the tube where growth occurs indicates the oxygen requirements of the cells: strict anaerobes will only grow below the surface, facultative anaerobes will grow throughout the medium, microaerophiles will grow in a narrow zone just below the surface, and strict aerobes will only grow at the surface. This methodology has the advantage of allowing all of these oxygen requirements to be interpreted from a single culture.

3. Chemical reducing agents. The addition of a reducing compound helps to keep the medium poised at a low oxidation-reduction potential. The most commonly used compound is thioglycollic acid.

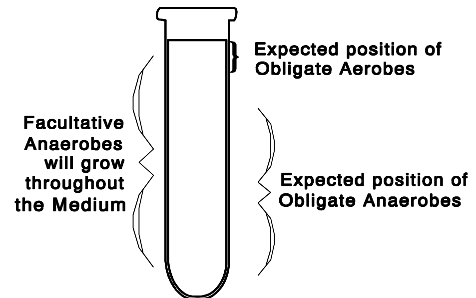
Supplies

4 tubes containing Fluid Thioglycollate (FTG) medium (blue caps) held at 48°C in water bath.
Broth cultures of
- *Clostridium sporogenes* (pink cap)
- *Escherichia coli* (brown cap) and
- *Bacillus subtilus* (green cap)

your bacterial unknown
2 plates of Thioglycollate medium

Procedure

A. Shake Culture technique



1. Obtain 4 tubes of FTG medium from the 48°C water bath. The following steps must be performed before the agar solidifies.
2. Inoculate one tube each with *E. coli*, *Bacillus*, and *C. sporogenes* and your unknown using your inoculating loop. Avoid introducing air bubbles.
3. Mix each culture by gently rotating the tubes between the palms of your hands.
4. Allow the medium to solidify, and then incubate the tubes at 37°C for 24 - 48 hours.
5. Record your results in Table 4.

B. Bio-bag procedure

For this part of the exercise, each group will inoculate 2 plates of solid agar. One will be cultured under aerobic conditions. The other plate will be cultured in a Bio-bag under anaerobic conditions.

1. Using a marking pen, divide the bottoms of the two thioglycollate medium plates into 4 sectors. Label each sector with either *Escherichia coli*, *Bacillus subtilus*, *Clostridium sporogenes* or 'unknown.'
2. Inoculate the centers of each sector with samples from the appropriate cultures.
3. Place one plate directly into the 37°C incubator (aerobic conditions).
4. Place the other plate in Bio-bag. The Bio-bag will contain an anaerobic generator tube, a catalyst and an indicator tube. Make sure the arrows on the two tubes face upward.
5. Seal the bag in the heat seal apparatus.
6. Crush the internal vials of the anaerobic generator and the indicator.
7. Incubate both plates at 37°C for 24 - 48 hours. The indicator tube should be white; if the indicator is blue, then anaerobic conditions were not achieved.
8. Record your observations in TABLE 4.

Names: ______________________________

✶✶Describe where your results did not agree with your expectations, and write a brief explanation of the discrepancy.✶✶

TABLE 1. Growth of bacteria in the presence of different carbon and nitrogen sources

Tube #	1	2	3	4	5	6	7	8	9	10	11	12
C source: N source:	Man NH_3	Man NO_2	Lac NH_3	Lac NO_2	Man NH_3	Man NO_2	Lac NH_3	Lac NO_2	Man NH_3	Man NO_2	Lac NH_3	Lac NO_2
Enterobacter												
Pseudomonas												
unknown												

"+" = heavy turbidity "±" = slight turbidity "—" = no growth

Interpreting results in Table 1.

We can conclude that a bacterium can use a particular carbon or nitrogen source if it can grow in the presence of the nutrient in any of the three culture media. If, for example, you find growth for your unknown in the presence of mannitol + NH_3 but no growth in the presence of lactose + NH_3, then you can conclude that the bacterium can utilize mannitol (but not lactose) as a carbon source and NH_3 as a nitrogen source.

TABLE 2. Summarize in this table interpretations of the data you tabulated above. Carefully interpret the results indicating if the bacteria could use each carbon and nitrogen source.

Organism	Sources of Carbon: Mannitol	<u>Lactose</u>	Sources of Nitrogen: <u>Ammonia</u>	<u>Nitrite</u>
Enterobacter				
Pseudomonas				
UNKNOWN				

Indicate “usable”, “slowly usable”(i.e., ± from Table 1), “not usable”

Explain any unexpected results; i.e, results that do not make sense in terms of the pattern of carbon and nitrogen utilization. If appropriate, explain why no growth occurred for your unknown.

Table 3. Results for MacConkey agar

	Did the bacteria grow on the medium (Yes / no)	If so, describe appearance of colonies & medium	Lactose fermentation? (+/-)
Escherichia coli			
Proteus vulgaris			
Bacillus subtilus			
Unknown			

Do these results agree with the Gram stain results for your unknown? Explain.

Results of Anaerobic cultures

TABLE 4. Oxygen requirements of selected bacterial species

Organism	Position of growth in shake culture tube			Growth characteristics on agar plates	
	Only grew near surface of the shake culture	Did not grow closer than approx. 5 mm to the surface	Growth occurred throughout shake culture	Significant growth in anaerobic bag*	Growth under aerobic conditions
Escherichia coli					
Bacillus subtilus					
C. sporogenes					
Unknown					

For each organisms, check (√) the <u>only</u> boxes that correctly describe the observed growth pattern

*Sometimes an obligate aerobe will growth slowly until O_2 is depleted from the medium, leaving small punctiform colonies; the growth under aerobic conditions will be much greater.

Based upon the results of Part III, what would you conclude about the oxygen requirements of each of these bacterial species; are they obligate aerobic, obligate anaerobic, or facultative anaerobic?

Are any of the results contradictory? Explain how you reached your conclusions.

E. coli:

Clostridium sporogenes:

Bacillus subtilus:

Unknown:

Also turn in answers to these two questions: (typed)

1. Suppose that you cultured a throat swab on Tryptic Soy Agar and on MacConkey Agar, and you see abundant colonies on the TSA but very few on the MacConkey Agar. If you performed a Gram stain for cells from one of the abundant colonies on the TSA plate, what Gram-reaction would you expect? Explain.

2. Suppose you obtained the following results in an analysis of the carbon and nitrogen requirements of a bacterium using defined media (NO_2 = nitrite; NH_3 = ammonia):

carbon source:	glucose	glucose	arabinose	arabinose
nitrogen source:	NH_3	NO_2	NH_3	NO_2
growth:	yes	no	no	yes

A. Do these results make sense? Explain.
B. How would you explain an inability of a species of bacteria to grown in any of these media; what are several explanations for "fastidious growth."