
eScience Labs LLC
More Than An Experiment, An Experience
Microbiology
Student Manual

Fundamentals of Microbiology

Lab 8 Selective Media and Agar

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Concepts to Explore

- Fundamental Nutrients
- Chemically Defined Media
- Complex Media
- Inhibitors
- Differential Media
- Bioprospecting
- Selecting Gram-Positive Organisms
- Selecting Gram-Negative Organisms
- Industry Applications

Introduction

Selective media is used to grow microorganisms in many different types of experiments. It is unique in its class because it can encourage the growth of certain microorganisms, but inhibit the growth of others. In other words, these types of media contain a mix of nutrients that allows only specific types of species to survive. This gives it a lot of specific value when conducting experiments on bacterial tolerance, interactions, species-specificity, etc.

Fundamental Nutrients

All living things require certain elements for survival and growth; bacterial species are no exception. Therefore, growth media must also contain certain elements to grow bacterial species. These elements may vary by species, but some elements are required for all species. To begin, growth media must contain a **carbon** source. Carbon is essential for the organic compounds that comprise a living cell. It is estimated to make up half of the dry weight of a typical bacterium. The carbon in media and agar is typically supplied as a combination of glucose and proteins from either plant or meat extracts. In nature, some bacteria can obtain carbon from proteins, carbohydrates, and lipids. These bacteria are called chemoheterotrophs. Other types of bacteria can obtain their carbon from carbon dioxide. These types of bacteria are called chemoautotrophs and photoautotrophs.

*Figure 1: **Wort, used to make beer, is considered a growth medium. It contains required nutrients for yeast to both survive and produce alcohol under anaerobic conditions (called fermentation). When fermentation is complete, the media and dormant microbes can be consumed as beer.***

Nitrogen, sulfur, and phosphorus are three additional elements that are required for survival and growth of bacteria. A variety of salts and amino acids are added to media to provide these essential elements. Nitrogen and sulfur are re-

quired for protein synthesis. Nitrogen and phosphorous are required for DNA and RNA synthesis. It is estimated that nitrogen comprises approximately 14% of a bacterium cell's dry weight, with sulfur and phosphorus combined comprise another approximately 4% of the cell.

Table 1: Example of a Chemically Defined Minimal Media Agar for *Bacillus sphaericus*

Ingredient	Chemical Formula	Purpose
Sodium Acetate	CH_3COONa	Carbon source and energy
Ammonium Sulfate	$(NH_4)_2SO_4$	Nitrogen and sulfur source
Sodium Phosphate Dibasic	Na_2HPO_4	Phosphorus and sodium
Potassium Phosphate Monobasic	KH_2PO_4	Phosphorus and potassium
Agar	Varies	Polymerization
Water	H_2O	Hydrogen and oxygen

Chemically Defined vs. Complex Media

Media can be either chemically defined or chemically complex. Chemically defined media is a media in which the exact chemical composition is known and is made with purified ingredients. Different bacterial species can have different nutritional requirements. Therefore, using different chemically defined mediums can be helpful to grow different microorganisms. Chemically defined media is often used to grow autotrophic bacteria; meaning, bacteria which can produce their own complex organic molecules from simple, inorganic ones.

Table 2: Example of a Complex Media Agar for Growth of Heterotrophic Bacteria

Ingredient	Purpose
Beef Extract	Carbon, nitrogen, sulfur, vitamins, trace elements, energy
Peptone	Peptides and single amino acids
Sodium Chloride	Isotonic environment (prevents cell lysis)
Agar	Polymerization
Water	Diluent

Complex Media

In contrast, complex media is composed of yeast, plants, and/or animal extracts. Unlike the complete definition of ingredients found in defined media, the complete composition of this type of complex media is not fully known. Carbon, nitrogen, and sulfur are provided when proteins from the extracts are broken down. The extracts also provide vitamins and other essential organic growth factors for bacteria. Complex media is frequently used for the growth of heterotrophic bacteria; meaning, bacteria which require a source of organic carbon to produce their organic molecules.

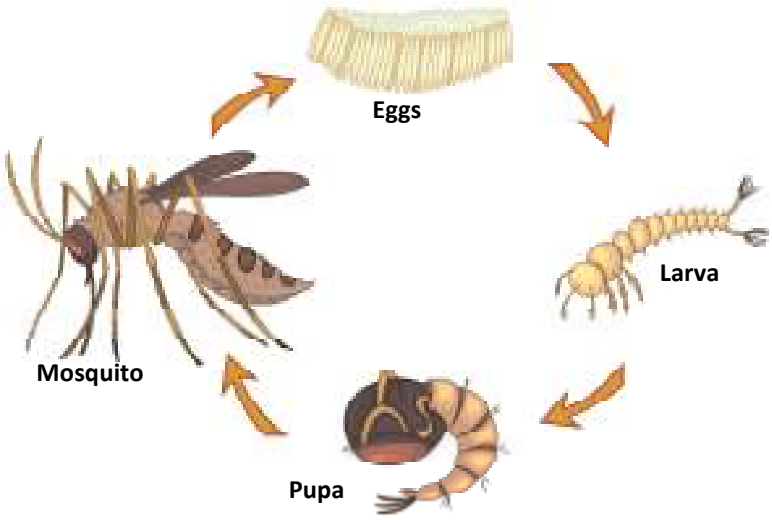

Figure 2: ***Bacillus sphaericus is used to control mosquito populations as a microbial larvicidal insecticide. B. sphaericus is a naturally occurring bacterium that mosquito larva ingest. The bacteria then produce a toxin that disrupts the gut and kills the larva, resulting in a reduced adult mosquito population.***

Media Inhibitors

Selective media contains nutrients required for optimal growth of a selected bacteria species and also inhibitor element(s) to make the media selective. Inhibitors are targeted at the specific types or groups of bacteria that a microbiologist *does not* want to grow. Inhibitors can function in a variety of ways, but they typically are used to attack the structure or function of the unwanted species. For example, an inhibitor might be used to block DNA synthesis and/or protein expression. They may also be used to decrease membrane stability and/or permeability

Differential Media

Differential media contains compounds that allow microbiologists to distinguish amongst various microorganisms growing on the same culture plate. Bacterial types can be distinguished (differentiated) by virtue of colony appearance (e.g., color) or by a functional effect on the media (e.g., hemolysis by gram-positive bacteria on blood agar plates). Media can be either selective, differential, or both selective and differential.

Clinical and Environmental Use

Selective media is used by microbiologists to help identify types of bacteria in a sample. Clinical microbiologists use selective media to eliminate bacteria that may normally reside in or on the body. They may also use selective media to help detect pathogenic bacteria that can cause disease.

Environmental microbiologists may use selective media to test for the presence of coliform bacteria in water samples. Coliform bacteria often indicates that a water sample has been exposed to fecal contamination. In general, media can be made to be selective by the addition of certain dyes (crystal violet in MacConkey's agar; methylene blue in EMB), by high salt (7% NaCl in MSA) in normal media, or by manipulating the pH of the media.

Although these different media make bacterial identification and investigation much easier, there are still many obstacles

Figure 3: ***A wooden house frame damaged by termites. Termites harbor cellulose degrading bacteria in their digestive systems that allows the termites to use wood cellulose as an energy source. Cellulose comprises approximately 50% of the total biomass of wood.***

to overcome. For example, one potential stumbling block when selecting for gram-positive cocci (spheres) from a mixed sample is the fact that Gram-negative microorganisms can block the gram-positive cell growth.

Experiment 1: Bio-Prospecting for Starch Degrading Bacteria

Ethanol derived from plant material is currently used as an alternative fuel source for petroleum-based products. Plant biomass is approximately 50% cellulose, which is composed of repeating linked subunits of glucose. Glucose is the primary carbohydrate used by most bacteria as an energy source. When joined together, glucose can also form starch, a great energy store. However, starch can be difficult to break down, and only a few types of identified bacteria are capable of doing so.

However, starch requires a specific enzyme to be broken down that not all bacteria have .The enzyme responsible for degrading starch is called amylase. In this experiment, you will select for bacteria that are capable of digesting starch by using starch agar petri plates and Gram Iodine. Starch agar is very similar to nutrient agar, but it has 0.4% soluble starch added to the media. This alone is not enough to indicate if a microorganism possesses amylase. However, when Gram Iodine is added to the plate the iodine in the solution reacts with the starch in the agar and creates a blue coloration on the plate. Blue coloring indicates that the starch is still present, and was not degraded by amylase. Clear coloring indicates that the starch has been degraded, which can be interpreted to mean that amylase is present.

Materials

- 1 tsp. Cow manure
- 4 Sterile, snap-cap tubes
- 40 mL PBS
- 8 Pipettes
- 4 Sterile bacterial spreaders
- Nutrient agar
- Starch agar
- Gram iodine
- (8) 5 cm. Petri dishes
- Parafilm™
- Permanent marker
- Hot pad
- 10 mL Graduated cylinder
- 4 Sterile inoculating loops
- *10% Bleach solution

*You must provide

Procedure:

Prepare Agar Plates:

1. Loosen or remove the cap on the nutrient agar bottle.
2. Place the bottle in the microwave (if you do not have a microwave, place the bottle in a heat-safe bowl and pour boiling water around the bottle) and heat until the entire agar bottle is liquefied. You will need to remove the bottle from the microwave and swirl it every 10 seconds to distribute the heat.

   Note: If you notice the agar boiling over, STOP the microwave and let the bottle cool down before handling. Hot agar can violently explode out of the bottle if heated too quickly and/or shaken. After boiling has stopped, use a hot pad to remove the bottle from the microwave. Remember, the bottle will be HOT!
3. Gently swirl the bottle to mix the solution.
4. Turn four of the petri dishes over and use the permanent marker to label them as “Nutrient”. Slowly pour the liquefied nutrient agar into the bottom half of four petri dishes so that it covers the entire bottom of the dish. It is important that the entire bottom is coated and that the agar is given time to spread out over the dish.
5. Place the lids onto the dishes and allow the agar to gel undisturbed. If you will not be using the dishes immediately, store them upside down in the refrigerator after they have fully gelled. Remove from the refrigerator and allow them to sit at room temperature for at least one hour prior to use.
6. Repeat Steps 1 - 5 using starch agar instead of nutrient agar, to prepare four more petri plates.

Prepare Stock Solution:

7. Place 10 mL PBS into one 15 mL conical tube; add the cow manure sample to it. Tightly screw on the cap and invert 10 times to mix thoroughly. Label this tube “Stock Solution”.
8. Place 9 mL of PBS into three 15 mL conical tubes (each tube should receive 9 mL).
9. Label one tube “10% Solution”, the second tube “1% Solution”, and the third tube “0.1% Solution”.
10. Perform serial dilutions of stock solution:
    a. Using a pipette, transfer 1 mL from the “Stock Solution” tube to the “10% Solution” tube. Tightly screw on the cap and invert 10 times to mix thoroughly.
    b. Using a pipette, transfer 1 mL from the “10% Solution” tube to the “1% Solution” tube. Tightly screw on the cap and invert 10 times to mix thoroughly.
    c. Using a pipette, transfer 1 mL from the “1% Solution” tube to the “0.1% Solution” tube. Tightly screw on the cap and invert 10 times to mix thoroughly.

Inoculating the Plates:

11. Label one nutrient plate “Stock”, one nutrient plate “10%”, one nutrient plate “1%”, and one nutrient plate “0.1%”.
12. Using a clean pipette for each tube to transfer approximately 4 drops from each serial dilution tube to the corresponding nutrient plate. You may need to re-invert the tubes the ensure that the manure remains in the solution.
13. Spread the diluted manure solution evenly over the plates with a sterile spreader. Remember to use a new spreader for each plate!
14. Allow to air dry then place tops on petri dishes.
15. Seal the plated with Parafilm™ and incubate them in a warm location (not to exceed 37.7 °C or 100 °F) for 1 - 2 days (until well defined bacterial colonies appear).
16. Label the starch plates as Stock, 10%, 1%, and 0.1%.
17. Choose one colony from the “Stock” nutrient agar plate and use an inoculating loop to individually streak the colony in an X shape onto the center of the “Stock” starch plate. It may be useful to draw a circle on the bottom of the plate and streak an X onto this area. See Figure 4.

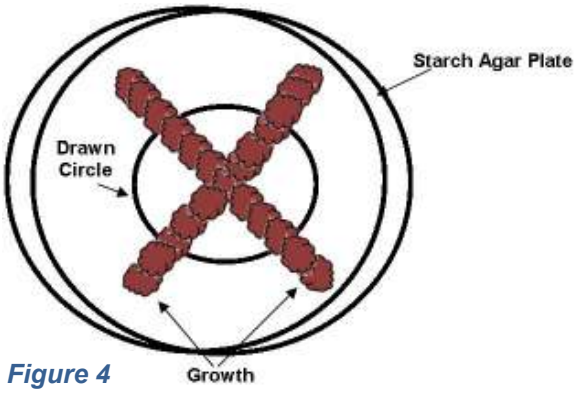

Figure 4

18. Repeat Step 17 for the 10%, 1%, and 0.1% plates.
19. Seal the plates with Parafilm™ and incubate them in a warm location (not to exceed 37.7 °C or 100 °F) for 2 - 3 days or until well-defined, isolated bacterial colonies appear (this may take up to 1 week if incubated at room temperature).
19. Flood the nutrient agar plates with a 10% bleach solution, incubate for 20 minutes, and pour the bleach down the drain with running water. Wrap Parafilm™ around the plates and dispose of them in the trash.

Perform the Gram Iodine Staining:

20. Record the growth on the starch agar plates in Table 3.
21. Cover each plate with Gram iodine (this should take approximately 1 mL of fluid).
22. Immediately examine the plate for any cleared areas surrounding the bacteria. The iodine will not stain the bacterial growth itself, so look for a halo-like effect of “no-staining” near the edges of the growth. Cleared areas indicate starch hydrolysis.
23. When you are finished with the experiment, flood the starch plates with a 10% bleach solution, incubate for 20 minutes, and pour the bleach down the drain with running water. Wrap Parafilm™ around the plates and dispose of them in the trash.

Table 3: Congo Red Analysis			
Sample	**Growth on Starch Agar (Yes or No)**	**Clear Area around Bacteria?**	**Is the Sample Capable of Degrading Starch?**
A			
B			
C			
D			

Post-Lab Questions:

1. Why was cow manure used as a potential source of starch degrading bacteria?

2. What are some other potential sources for starch degrading bacteria?

3. What component makes starch agar selective for starch degrading bacteria?

4. Why were each of the following steps performed in this experiment:

   Serial dilution?

   Growth on the nutrient agar plates?

   Streak on the starch agar plates?

Experiment 2: Selection and Differentiation of Body Inhabiting, Gram-Positive Bacteria

Mannitol Salt Agar (MSA) is both selective and differential for gram-positive bacteria, containing a high (7.5%) salt (sodium chloride) concentration, which makes it selective, and mannitol as the carbohydrate source for fermentation, which makes it differential. Most bacteria cannot grow in the high salt environment; however, *Staphylococcus* species have adapted to high salt environments, such as human skin. The differentiation agent mannitol can be effectively fermented by *Staphylococcus aureus*, but not by other *Staphylococcus* species. MSA also includes the pH indicator dye phenol red, which is red in basic conditions and yellow in acidic conditions. Fermentation of mannitol generates an organic acid which lowers the pH of the agar and changes the dye from red to yellow.

***Figure 5:** Different colonies can be distinguished by color and/or growth amount. Note, your colonies will vary in color and growth from the picture above.*

Materials

MSA agar	4 Sterile cotton swabs
Nutrient agar	Parafilm™
(4) 5 cm. Petri plates	Permanent marker

Procedure:

1. Turn four petri plates over, draw a line down the center of each plate, and label the bottom of two plates as “MSA” and the bottom of two plates as “Nutrient”. Return the lids to the plates and set them aside.
2. Loosen or remove the cap on the MSA agar bottle. Place the bottle in the microwave (if you do not have a microwave, place the bottle in a heat-safe cup and pour boiling water into the bowl around the bottle).
3. Heat the bottle in 10 second increments until the agar is liquefied. You may need to remove the bottle from the microwave and swirl it every 10 seconds to evenly distribute the heat.

   Note: If you notice the agar boiling over, STOP the microwave and let the bottle cool down before handling. Hot agar can violently explode out of the bottle if heated too quickly and/or shaken. After boiling has stopped, use a hot pad to remove the bottle from the microwave. Remember, the bottle will be HOT!
4. Gently swirl the bottle to ensure that the contents are fully liquefied.

5. Slowly pour the liquefied MSA agar into the bottom half of the two MSA plates so that it covers the entire bottom of the dish. Return the lids to the petri dishes and set aside.
6. Repeat Steps 2 - 5 for the nutrient agar. Be sure to loosen or remove the cap on the bottle to allow for heat expansion.
7. Pour the agar into the bottom of the two nutrient petri plates. It is important that the entire bottom is coated and that the agar is given time to spread out over the plate.
8. Place the lids onto the plates and allow the agar to gel undisturbed.

   Note: If you will not be using the dishes immediately, store them upside down in the refrigerator after they have fully gelled. Remove from the refrigerator and allow them to sit at room temperature for at least one hour prior to use.
9. Use a sterile cotton swab to gently rub a portion of your skin (such as your arm, cheek, etc.).
10. Lightly rub the exposed swab over half the surface of one MSA plate and half of the surface of one nutrient plate.
11. Use a permanent marker to label the respective half of each dish as “Skin”.
12. Use a new sterile cotton swab to gently swab the inside of your nose.
13. Lightly rub the exposed swab over the other half of the same MSA plate and half of the same nutrient plate.
14. Use a permanent marker to label the respective half of each dish as “Nose”.
15. Use a new sterile cotton swab to gently swab a non-porous surface (such as a countertop).
16. Lightly rub the exposed swab over half of the second MSA plate and half of the second nutrient plate.
17. Use a permanent marker to label the respective half of each dish with surface you swabbed.
18. Lightly rub a new sterile cotton swab over the remaining halves of the second MSA plate and second nutrient plate. Do not touch the cotton swab to anything!!
19. Use a permanent marker to label this half of each dish as “Control”.
20. Seal the plates with Parafilm™ and incubate them at room temperature (up to 37.7 °C or 100°F) for 1 - 2 days, or until colonies appear.
21. Examine the plates for amount of growth and colony/agar color. Record your results in Table 4.
22. When you are finished with the experiment, flood the plates with a 10% bleach solution, incubate for 20 minutes, then pour the bleach down the sink with running water.
23. Seal the plates with Parafilm™ and dispose of them in the trash.

Table 4: Experiment 2 Growth Observations				
Surface Tested	Growth (Good or Poor?)		Color (Red or Yellow?)	Other Observations
	LB/Nutrient Agar	MSA Agar		
Skin				
Nose				
Countertop/Other				
Control				

Post-Lab Questions:

1. What substance in MSA confers selectivity? Why?

2. What substance makes MSA differential? Why?

3. What purpose does phenol red serve in MSA?

4. Why are the Nutrient agar plates used in this experiment?

Experiment 3: Selection and Differentiation of Gram-Negative Bacteria from Liquid Samples

Water is often screened for the presence of coliform bacteria as an indicator of fecal contamination. Contaminated water can transmit a number of human diseases, including cholera, salmonella, dysentery, shigella, and many others. Coliform bacteria are Gram-negative rods, many of which can ferment lactose (the same sugar found in milk). MacConkey agar is a selective and differential media that contains a pH indicator dye (neutral red), lactose, bile salts, and crystal violet. The bile salts and crystal violet select for Gram-negative bacteria by inhibiting the growth of Gram-positive organisms. The lactose and neutral red demonstrate which bacteria can ferment lactose as lactose fermentation produces acidic compounds that lower the pH of the media and turn the lactose fermenting colonies red (neutral red is colorless at pH above 6.8 and red at pH less than 6.8). In this experiment, you will collect liquid samples and test them for the presence of coliform bacteria using MacConkey agar plates.

Lab 8 Selective Media and Agar

Materials

(3) 15 mL Sterile screw-top conical tubes
MacConkey Agar
3 Pipettes
3 Sterile disposable spreaders
(3) 5 cm. Petri dishes
Permanent marker
Hot pad
10 mL Graduated cylinder
Parafilm™

Procedure:

1. Prepare three MacConkey agar plates according to Steps 1 - 5 as listed in Lab 9 – Experiment 1.
2. Use your 15 mL conical tubes to collect 3 water-based liquid samples from different sources. These can come from a faucet in your home, a water fountain, a flowing stream (be sure to not collect any of the sediment at the bottom of the stream), iced tea from a restaurant, etc. Collect approximately 8-10 mL of each sample.
3. Use a permanent marker to label each tube with the sample type and location.
4. Label each MacConkey agar dish with the same information you put on the collection tubes (i.e., each petri dish should have the same information as each corresponding test tube).
5. Use a pipette to transfer approximately 4 drops of each sample onto the corresponding petri dish.
7. Use a disposable spreader to gently spread the liquid sample over the agar surface. Use a new spreader for each sample/plate combination.
8. Allow to air dry. This should take approximately 5 - 10 minutes.
9. Cover the plates with the petri dish lid and incubate them in a warm location (not to exceed 37.7 °C or 100 °F) for 1 - 2 days; or, until well defined, isolated bacterial colonies appear.
10. Examine the plates and record the bacterial growth and color of the colonies in Table 5.
11. When you are finished with the experiment, flood the plates with the 10% bleach solution, incubate them for 20 minutes, and then pour the bleach down the sink with running water.
12. Seal the plates with Parafilm™ and dispose of them in the trash.

Table 5: MacConkey Agar Results

Sample	Growth (Yes or No)	Colony Color	Analysis

Post-Lab Questions:

1. What ingredient(s) makes MacConkey agar selective for Gram-negative bacteria?

2. What types of bacteria are inhibited on MacConkey agar?

3. What ingredient(s) makes MacConkey agar differential?

4. Why is an indicator dye used in MacConkey agar?

5. What are some potentially pathogenic bacteria that are lactose fermenters that will grow on MacConkey agar?

6. What are some potentially pathogenic bacteria that do not ferment lactose that will grow on MacConkey agar?

7. How would you verify that the colonies that grew on a MacConkey agar plate were Gram-negative

8. Look up the formulation for MacConkey agar either in a microbiology text book or online (http://www.bd.com/ds/productCenter/221172.asp).

   Is this a chemically defined or complex media?

   Why is that important?

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