Antelope Valley College

Microbiology Laboratory Manual

Summer 2020

Dr. Jedidiah Lobos

Media Preparation

Introduction

A culture medium is a nutrient preparation used to grow microorganisms. Chemically defined media, also called synthetic, are made up of known amounts of pure chemicals. Complex, also called non-synthetic, are composed of complex materials rich in vitamins and nutrients. There are media types for different microbiological applications. There are media types for different microbiological applications. Media can be liquid (also called broth), semi-solid, or solid. Liquid media do not contain agar and can be used to grow a large number of microorganisms. Semi-solid media contains agar and can be used to determine motility and/or anaerobic growth. Solid media contains agar, but more than semisolid. Solid media can be used for surface growth to observe colony appearance, for isolation of pure cultures, and to observe specific biochemical reactions. There are different forms of solid media:

- Slant: media with agar in a test tube left in slanted position until it becomes solidified.
- Deep: media with agar in a test tube kept upright until solidification.
- Plate: media with agar poured into dish and left to solidify.

When preparing media, the following needs to be considered:

- Always use a flask that holds twice the volume of media you are preparing. This will prevent boil-over of the media.
- Mix ingredients and water together by swirling the flask or using a Teflon-coated magnetic stir bar.
- If appropriate, add agar if needed.

In order to use culture media, it needs to be sterilized. Sterilization is the process of rendering a medium or object free from all forms of life. Sterilization techniques include:

- Autoclaving: exposure to 121°C and 15 pounds of pressure for 15 minutes
- Dry-heat: electric oven set to 160°-170°C for two or more hours
- Bacteriological filter: passing of media through filter, which traps microorganisms. Sterilizes without heat.
- Ultraviolet radiation: exposure to 260 nm light. Unfortunately, cannot penetrate glass, dirt films, water, or other substances very effectively.
- Ethylene oxide gas: exposure to gas kills both microorganisms and spores by attaching to cell proteins. Can penetrate packing materials, including plastic wrap.
- Flaming: The flame from a gas burner will effectively sterilize items such as metal inoculating loops. For glass spreaders, dip the items in alcohol and then ignite by flaming.

Materials

- Culture medium
- Water
- Electronic scales
- Graduated cylinders
- Stirring hot plate
- Magnetic stir bar
- Beaker
- Weigh boat/paper
- Pipettes
- Pipettors

Procedure

A. Tryptic Soy Agar

1. Read the directions on the label of the culture medium. In many cases, the recipes on the label call for a total volume of one (1) liter. Often, a liter of medium is not desired, so you may need to calculate the appropriate amount of medium to solvent (usually deionized (DI) water). Today you will prepare **500 ml of Tryptic Soy Agar (TSA).**
2. Confirm with the instructor the proper amount of TSA powder needed to make the required 500 ml.
3. Measure the TSA and the 500 ml of DI water and add both to a beaker **larger than 500 ml, (at least 800 ml)**.
4. Add a magnetic stir bar and place the beaker on the stirring hot plate. Set the temperature high and gradually turn the "stir" knob until the stir bar is adequately mixing the medium.
5. Once the medium begins to boil, look for the medium to clarify. Once it does, turn the heat and stir controls off.
6. Using hot gloves, remove the beaker from the hot plate and place it on a stack of paper towels or on a pad/trivet.
7. Allow the medium to cool slightly before pipetting.
8. Dispense TSA in the following manner:
    a. **10 ml (each) into a total of 15 test tubes.** These will be placed into a Kimrack to make slants.
    b. Equally dispense the remaining medium into two (2) screw capped bottles and keep the caps loose.
9. Label all the tubes and bottles using a permanent marker. **Labels should have your initials, name of medium, and date.**
10. The slants go into a Kimrack and the bottles go into a stainless steel tray.

11. Once the beaker is cool, wash the beaker and the magnetic stir bar. Dry the materials and return them to where they were taken from.

B. Tryptic Soy Broth

1. Now, you will prepare **100 ml of Tryptic Soy Broth (TSB).**
2. Confirm with the instructor the proper amount of TSB powder needed to make the required 100 ml.
3. Measure the TSB and the 100 ml of DI water and add both to a **250 ml beaker**.
4. Add a magnetic stir bar and place the beaker on the stirring hot plate. Gradually turn the "stir" knob until the stir bar is adequately mixing the medium. The broth will readily dissolve in the water **without** heat.
5. Once the medium is completely dissolved, turn the stir control off.
6. Dispense 10 ml (each) into 10 test tubes and cap each one. Label the tubes appropriately and place them into a plastic basket.
7. Wash the beaker and the magnetic stir bar. Dry the materials and return them to where they were taken from.
8. All of the media will be autoclaved and used for later lab exercises.

Microscope

Introduction

The microscope is an instrument for viewing objects that are too small to be seen easily by the naked eye. Anton van Leeuwenhoek is generally credited with bringing the microscope to the attention of biologists, even though simple magnifying lenses were already being produced in the 1500's, and the magnifying principle of water-filled glass bowls had been described by the Romans. In its simplest form - as used by Robert Hooke, for example - the compound microscope would have a single glass lens of short focal length for the objective, and another single glass lens for the eyepiece or ocular. Modern microscopes of this kind are usually more complex, with multiple lens components in both objective and eyepiece assemblies. These multi-component lenses are designed to reduce aberrations, particularly chromatic aberrations and spherical aberrations. In modern microscopes, the mirror is replaced by a lamp unit providing stable, controllable illumination. Compound optical microscopes can magnify an image up to 1000x and are used to study thin specimens as they have a very limited depth of field. Typically, microscopes are used to examine a smear or a thinly sectioned slice of some material. With a few exceptions, light passes through the sample from below and special techniques are usually necessary to increase the contrast in the image to useful levels.

Typically, on a standard compound optical microscope, there are three objective lenses: a scanning lens (4x), low power lens (10x), and high-power (or high-dry) lens (40x). Advanced microscopes often have a fourth objective lens, called an oil immersion lens (100x). Generally, from top to bottom, the parts are: Ocular Lenses (housed in the head of the microscope), arm of the microscope, rotating nosepiece (containing objective lenses), mechanical stage with stage clips, stage knobs (that control the stage), condenser with iris diaphragm, condenser focusing knob, course adjustment knob, fine focusing knob, rheostat, switch, lamp, and base.

A few things to remember:

- To obtain the degree of magnification, multiply the ocular magnification by the objective lens magnification.
- Bacteria and other microbes are not colored! Most times, in order to actually see them using light microscopy, you would need to stain the organisms.

More importantly:

- **Immersion oil is only to be used with the oil immersion (100x) lens. Never use oil on the 40x lens.**
- **ONLY clean microscope lenses with lens paper**

Procedure

A. We will go over how to use the microscope in class

B. Putting the microscope away

a. The following needs to be done to the microscope before putting it away:

i. Switch off

ii. Rheostat at lowest setting
iii. No slide in place
iv. Oil immersion lens free of oil
v. Stage all the way down
vi. Condenser at its highest position
vii. Microscope cover on
viii. Cord put away

Wet Mount

Introduction

Microbes are, well, microscopic. We can view microbial specimens under light microscopy using two techniques: wet mounts and stained smears. Problems can occur when using these techniques. Dirty slides may interfere with the object being viewed. In this lab, a wet mount will be prepared. Wet mounts allow you to see live bacteria. In order for them to be seen alive they either need to be in liquid culture or need to be resuspended in a drop of water from a fresh solid culture medium.

Materials

- Hay infusion
- Broth cultures of :
    - *Bacillus subtilis*
    - *Staphylococcus epidermidis*
    - *Rhodospirillum rubrum* (depending on availability)
    - Other unknown organisms

Procedure

A. Wet mount from a liquid culture medium

a. Take a clean glass slide.
b. Using a pipet or medicine dropper, take a drop of culture and add it to the slide.
c. Take a clean cover slip and place it on top of the water droplet.
d. View the wet mount using light microscopy. Do not go past the high-dry objective lens (40x).

Ubiquity of Microorganisms

Introduction

Bacteria are ubiquitous in nature. This means that they are EVERYWHERE. Bacteria, fungi, and microscopic unicellular and multicellular organisms inhabit the soil, air, water, and even the human body. There are microorganisms that live on your body's outer surface and even in your gut! We can use a variety of techniques to determine what kinds of organisms inhabit certain environments. Remember that the environment plays a large role in how the organism grows. For this laboratory period, you will survey the environment around you for different bacteria.

Materials

- (7) Nutrient agar (NA) plates
- (1) TSA plate
- (1) tube of Nutrient Broth (NB) or TSB
- Sterile saline
- Sterile swabs

Note: For all inoculated media, label them in the following manner: **initials, name of medium (abbreviated), source of culture (soil, etc.) or organism name (underlined), temperature incubated, and date.**

Procedure

A. Microorganisms from soil

1. Label an NB or TSB tube with a piece of tape.
2. Go outside and a pinch of soil into the tube.
3. Go back to the lab and incubate the tube at 30 °C. All tubes will be incubated for 24-48 hours.

B. Microorganisms from air

1. Label an NA plate on the "bottom" of the plate (the part opposite the lid). Label directly on the plate; do not use tape.
2. As instructed place the plate in either a dynamic or static air environment, **uncovered**.
3. Leave the plate exposed for 15 minutes, then put the lid back on.
4. Go back to the lab and incubate the plate **LID SIDE DOWN** at 30 °C. All plates will be incubated for 24-48 hours.

C. Microorganisms from benchtop

1. Rub the benchtop with a sterile swab dipped in sterile saline.
2. Obtain and label an NA plate and perform a "lawn" streak with the swab.
3. **Disinfect the benchtop.** Repeat steps 1 and 2.

4. Incubate both plates **LID SIDE DOWN** at 30 °C. All plates will be incubated for 24-48 hours.

D. Microorganisms from yourself

1. **Unwashed Fingers**
    a. Obtain and label an NA plate and press your fingers onto the plate.
2. **Washed Fingers**
    a. Wash your hands well with soap and water.
    b. Obtain and label an NA plate and press your fingers onto the plate.
3. **Lips**
    a. Obtain and label an NA plate and press your lips or kiss the plate.
4. **Fingernails**
    a. Obtain and label an NA plate and using a saline-dipped swab, "clean" underneath your fingernails.
    b. Perform a "lawn" streak with the swab.
5. Incubate all plates **LID SIDE DOWN** at 30 °C. All plates will be incubated for 24-48 hours.

E. Microorganisms from mouth

1. Obtain and label a TSA plate and place a sterile swab inside your mouth.
2. Swab your tongue and the inside your cheeks. Perform a "lawn" streak with the swab.
3. Incubate the plate **LID SIDE DOWN** at 37 °C. All plates will be incubated for 24-48 hours.

Aseptic Technique

Introduction

Aseptic technique is a procedure used to prevent contamination of cultures when the media is handled in any way. It also prevents the handler and those around the handler of the cultures from being contaminated or otherwise exposed. Aseptic technique ensures that no contamination remains after cultures have been handled. Practicing the following procedures will, in time, lead to this technique being second nature. Aseptic technique does not just involve the transferring of cultures, however. Keep in mind that preparing your work environment as well as the tools you are using are also involved in aseptic technique. In other words, make sure that you continue to use disinfectant when preparing the benchtop for lab work.

Materials

- (3) TSA slants (prepared on the first day)
- (1) TSB tube (prepared on the first day)
- (1) slant culture of *Serratia marcescens*

Reminder: For all inoculated media, label them in the following manner: **initials, name of medium (abbreviated), organism name (underlined), temperature incubated, and date.**

Note: The procedures listed below are written for those who are right-hand dominant. For those who are left-hand dominant, just switch appropriately.

Procedure

A. Culture transfer from slant to slant

1. Label a sterile TSA slant appropriately.
2. Carefully ignite a Bunsen burner.
3. Using your right hand, grab the inoculating loop and hold it as you would a pencil.
4. Put the loop into the flame of the burner until the loop is glowing (a red-orange color), and pull it out. It is not necessary to put the **whole** loop into the flame. Just insert the loop and about 3-4 cm of wire leading to it. Once the loop is glowing, pull it out. DO NOT leave the loop in the flame for an extended period of time- this may shorten the integrity of the loop over time.
5. Take the tube that has bacteria in it with your left hand.
6. While holding the loop in your right hand, using your pinky, remove the cap of the tube with bacteria in it.
7. Pass the neck of the tube across the open flame.
8. Take the cooled, sterile loop and insert it into the tube with bacteria in it. Touch the loop to the bacteria. It is not necessary to "scoop" a large amount of bacteria. Touching the culture or grabbing a small amount is all you need to do.

9. Re-flame the neck of the culture tube and then place the cap (that you are still holding) back on.
10. Take a sterile TSA slant with your left hand.
11. While holding the loop (with bacteria on it) in your right hand, using your pinky, remove the cap of the TSA slant.
12. Pass the neck of the tube across the open flame.
13. Take the loop, insert it into the TSA slant and place it at the bottom of the slant.
14. Using a serpentine motion (a zig-zag or snake-like motion), streak upward on the slant.
15. Remove the loop, re-flame the neck of the TSA slant, and then place the cap (that you are still holding) back on.
16. Sterilize the loop as you did in step 4.
17. Allow the loop to cool before returning it to your drawer or before handling it again.
18. Incubate media at 30 °C for 24-48 hours.

B. Culture transfer from slant to broth

1. Label a sterile TSB tube appropriately.
2. Carefully ignite a Bunsen burner.
3. Using your right hand, grab the inoculating loop and hold it as you would a pencil.
4. Put the loop into the flame of the burner until the loop is glowing (a red-orange color), and pull it out. It is not necessary to put the **whole** loop into the flame. Just insert the loop and about 3-4 cm of wire leading to it. Once the loop is glowing, pull it out. DO NOT leave the loop in the flame for an extended period of time- this may shorten the integrity of the loop over time.
5. Take the tube that has bacteria in it with your left hand.
6. While holding the loop in your right hand, using your pinky, remove the cap of the tube with bacteria in it.
7. Pass the neck of the tube across the open flame.
8. Take the cooled, sterile loop and insert it into the tube with bacteria in it. Touch the loop to the bacteria. It is not necessary to "scoop" a large amount of bacteria. Touching the culture or grabbing a small amount is all you need to do.
9. Re-flame the neck of the culture tube and then place the cap (that you are still holding) back on.
10. Take a sterile TSB tube with your left hand.
11. While holding the loop (with bacteria on it) in your right hand, using your pinky, remove the cap of the TSB tube.
12. Pass the neck of the TSB tube across the open flame.
13. Take the loop, insert the loop end into the TSB tube.
14. It will be sufficient to simply dip the loop in, but you may shake the loop a few times.

15. Remove the loop, re-flame the neck of the TSB tube, and then place the cap (that you are still holding) back on.
16. Sterilize the loop as you did in step 4.
17. Allow the loop to cool before returning it to your drawer or before handling it again.
18. Incubate media at 30 °C for 24-48 hours.

Bacterial Enumeration (Standard Plate Count)

Introduction

The standard plate count is a technique used to estimate the number of microorganisms in a liquid sample by performing a series of dilutions and then culturing an aliquot of each dilution (or certain tubes in the series) on solid media. The inoculum taken from the dilution is a known proportion, in itself representing another dilution of the sample. After incubating the plate(s) at a proper temperature for a given length of time, colonies are counted. If colonies fall within a countable range, usually between **25-250** colonies, the plate is used to estimate the quantity of cells found in the original culture. The **colony forming unit (CFU)** is a term used to describe the number of viable cells that would lead to the formation of a colony on growth medium. Since one cell or a cluster of cells (depending on cellular arrangement) can lead to the formation of a colony, the CFU is treated as if it can represent an individual cell.

Materials

- Broth culture of *Escherichia coli*
- 3 bottles containing 99 ml of sterile water
- 5 TSA plates
- Micropipettes and micropipette tips

Procedure

A. Performing the serial dilution

1. Aseptically transfer 1 ml to the first 99 ml bottle and label the bottle "**A**".
2. Carefully swirl the bottle **A** to mix it. This is a 10^{-2} dilution.
3. Aseptically transfer 1 ml of bottle "**A**" to a second 99 ml bottle and label the bottle "**B**".
4. Carefully swirl the bottle **B** to mix it. This is a 10^{-4} dilution.
5. Aseptically transfer 1 ml of bottle "**B**" to the last 99 ml bottle and label the bottle "**C**".
6. Carefully swirl the bottle **C** to mix it. This is a 10^{-6} dilution.
7. Take 1 ml of bottle **B** and plate it on TSA plate. Achieve a "lawn culture" using a hockey stick spreader or a loop. The final dilution for plate **1** will be 10^{-4}.
8. Take 0.1 ml of bottle **B** and plate it on TSA plate. Achieve a "lawn culture" using a hockey stick spreader or a loop. The final dilution for plate **2** will be 10^{-5}.
9. Take 1 ml of bottle **C** and plate it on TSA. Achieve a "lawn culture" using a hockey stick spreader or a loop. The final dilution for plate **3** will be 10^{-6}.
10. Take 0.1 ml of bottle **C** and plate it on TSA. Achieve a "lawn culture" using a hockey stick spreader or a loop. The final dilution for plate **4** will be 10^{-7}.
11. Take 0.01 ml of bottle **C** and plate it on TSA. Achieve a "lawn culture" using a hockey stick spreader or a loop. The final dilution for plate **5** will be 10^{-8}.

Aseptic Plate Pouring

Introduction

In modern microbiology labs, media-making, including the pouring of solid media, has become an automated process. In many academic labs, however, solid media is still poured by the students. In this lab, you will be tasked to pour agar plates. In order to pour agar into empty Petri plates, the agar is taken straight from the autoclave (or molten from a previous prep) and placed into a 50 °C water bath. This temperature is ideal for pouring agar and it should be noted that agar solidifies around 40 °C. Therefore, the medium has to be poured before the agar solidifies.

Materials

- (1) TSA deep (prepared on the first day)
- (1) TSA bottle (prepared on the first day)

Note: The procedures listed below are written for those who are right-hand dominant. For those who are left-hand dominant, just switch appropriately.

Procedure

A. Pouring from a tube

1. Obtain 2 sterile Petri dishes.
2. Ignite a Bunsen burner.
3. Grab a paper towel and go to the water bath and obtain a TSA deep.
4. Wipe the deep, put it in a test tube rack, and back to your benchtop.
5. While holding the tube with your right hand, uncap the tube with your left pinky and flame the tube.
6. Holding the plate at a 45° angle, pour the deep into the sterile plate. The deep should fill most, if not all, of the dish.
7. Completely cover the dish and then lightly press down on the plate. Make a "figure-eight" motion to ensure that the media is evenly dispersed throughout the dish.
8. Leave the plates on the benchtop to solidify for at least 20 minutes.
9. Label all the media with "TSA" on the **bottom of the plate** (not the lid).

B. Pouring from a bottle

1. Obtain 8-10 sterile Petri dishes.
2. Ignite a Bunsen burner.
3. Grab a paper towel and go to the water bath and obtain a TSA bottle.
4. Wipe the bottle and bring it back to your benchtop.
5. While holding the bottle with your right hand, uncap the bottle with your left pinky and flame the neck of the bottle.

6. Holding the plate at a 45° angle, pour enough of the medium to fill about ¾ of the bottom of the sterile plate.
7. Completely cover the dish and then lightly press down on the plate. Make a "figure-eight" motion to ensure that the media is evenly dispersed throughout the dish.
8. Leave the plates on the benchtop to solidify for at least 20 minutes.
9. Label all the media with "TSA" on the **bottom of the plate** (not the lid).

Streaking for Isolation (Quadrant Streak)

Introduction

In nature, bacteria exist as mixtures of different populations. Rarely would you find bacteria existing as a single species. With a pure culture, however, we would be able to study the organism's cultural, physical, and morphological characteristics. Several methods can be used to obtain pure cultures. Two of the most commonly used techniques for obtaining a pure culture are the pour plate technique and the streak plate technique. In both cases, a pure colony can be isolated from a mixed culture. In both cases the bacterial culture is being diluted, giving rise to a single colony. The single colony, then, is assumed to be a pure representation of one particular genus and species. Although one colony may contain millions of bacterial cells, it is usually counted as one colony or one **colony forming unit (CFU).**

Materials

- Broth culture containing two (2) different bacteria
- Slant cultures of:
    - *Serratia marcescens*
    - *Escherichia coli*
    - *Bacillus subtilis*
- TSA plates

Note: The procedures listed below are written for those who are right-hand dominant. For those who are left-hand dominant, just switch appropriately.

Procedure

A. Streak plate technique from broth

1. Ensure that the broth culture is mixed well
2. Ignite a Bunsen burner.
3. Using your right hand, grab the inoculating loop and sterilize it.
4. With your left hand, grab the broth culture.
5. While holding the loop in your right hand, using your pinky, remove the cap of the tube with bacteria in it.
6. Pass the neck of the tube across the open flame.
7. Take the cooled, sterile loop and insert it into the tube with bacteria in it.
8. Remove the loop and re-flame the neck of the culture tube and then place the cap (that you are still holding) back on.
9. Using the loop with bacteria on it, perform the streak plate as demonstrated by the instructor.
10. When done, sterilize the loop.

B. Streak plate technique from slant

1. Ignite a Bunsen burner.
2. Using your right hand, grab the inoculating loop and sterilize it.
3. With your left hand, grab the slant culture.
4. While holding the loop in your right hand, using your pinky, remove the cap of the tube with bacteria in it.
5. Pass the neck of the tube across the open flame.
6. Take the cooled, sterile loop and insert it into the tube with bacteria in it. Touch the loop to the bacteria. It is not necessary to "scoop" a large amount of bacteria. Touching the culture or grabbing a small amount is all you need to do.
7. Remove the loop and re-flame the neck of the culture tube and then place the cap (that you are still holding) back on.
8. Using the loop with bacteria on it, perform the streak plate as demonstrated by the instructor.
9. When done, sterilize the loop.

C. Pour plate technique (demo)

1. Ensure that the broth culture is mixed well
2. Aseptically take 1 ml of the culture and add it to empty Petri dish.
3. Obtain the molten agar from the water bath, dry the outside of the tube, and bring it to the benchtop.
4. While the agar is still liquified, aseptically pour the entire contents of the tube into the plate. Cover the plate and hold it down onto the benchtop while performing a figure 8 motion.
5. Once the plates are solidified (about 20 minutes), label the bottom of the plates appropriately.

D. Pour plate technique- alternative (demo)

1. Ensure that the broth culture is mixed well
2. Obtain the molten agar from the water bath, dry the outside of the tube, and bring it to the benchtop.
3. Aseptically take 1 ml of the culture and add it to the agar tube.
4. Quickly "roll" the tube in your hands 5-10 times.
5. While the agar is still liquified, aseptically pour the entire contents of the tube into the plate. Cover the plate and hold it down onto the benchtop while performing a figure 8 motion.
6. Once the plates are solidified (about 20 minutes), label the bottom of the plates appropriately.

Incubate the *E. coli* and *B. subtilis* agar plates at 30 °C and *S. marcescens* at 25 °C for 48 hours. Incubate any media containing the mixed culture at 30 °C for 48 hours.

Bacterial Cultural Characteristics

Introduction

The cultural characteristics of a microorganism refer to the macroscopic appearance of the organism on solid media. In Bergey's manual there are terms that bacteriologists have used to describe the cultural characteristics of different organisms. Typically, these determinations are made on some type of rich medium such as NA or TSA and growth in nutrient broth can also be used to some extent. Usually, the characteristics are used to describe an organism: size, shape, margin, elevation, and pigmentation.

Materials

- Broth culture of *Pseudomonas aeruginosa*
- Slant cultures of:
  - *Bacillus subtilis*
  - *Streptococcus pyogenes*
  - *Staphylococcus aureus*
  - *Escherichia coli*
  - *Proteus vulgaris*
- (4) TSA plates
- (4) TSB tubes
- (2) NA 1.5% plates

Procedure

A. Transfer to TSB

1. Aseptically transfer each of the following to a TSB tube:
   a. *Bacillus subtilis*
   b. *Escherichia coli*
   c. *Streptococcus pyogenes*
   d. *Staphylococcus aureus*
2. Make sure to label each test tube appropriately
3. Incubate *B. subtilis* at **30 °C** for 48 hours.
4. Incubate the other organisms at **37 °C** for 48 hours.

B. Transfer to TSA

5. Aseptically streak each of the following to a TSA plate:
   a. *Pseudomonas aeruginosa*
   b. *Bacillus subtilis*
   c. *Streptococcus pyogenes*
   d. *Staphylococcus aureus*
6. Make sure to label each plate appropriately

7. Incubate *B. subtilis* at **30 °C** for 48 hours.
8. Incubate the other organisms at **37 °C** for 48 hours.

C. Transfer to NA 1.5%

9. Aseptically streak each of the following to a NA 1.5% plate:
    a. *Bacillus subtilis*
    b. *Proteus vulgaris*
10. Make sure to label each plate appropriately
11. Incubate *B. subtilis* at **30 °C** for 48 hours.
12. Incubate *P. vulgaris* at **37 °C** for 48 hours.

Fungi: Yeasts and Molds

Introduction

Kingdom Fungi is a large group of organisms that consists of over 70,000 species. Fungi are ubiquitous, growing almost in every habitat where organic compounds are found. Fungi are heterotrophic with most being saprotrophs. In this lab, we will specifically examine yeasts and molds.

Yeasts do not have true hyphae. Instead, they form structures called pseudohyphae. Yeasts reproduce asexually by a process called budding. Because a bud scar is formed after each bud, there is a limit as to how many offspring a single mother yeast cell can produce. In the event a bud does not pinch off, the result is the formation of a pseudohypha.

Molds *do* have true hyphae. Therefore, the formation of a mycelium (a mass of hyphae) will be present. The macroscopic presentation of these organisms tends to be filamentous. The hyphae of molds may be septate or coenocytic, meaning that there may be cell wall partitions found in some hyphae (septate) or there may be one continuous stretch of hyphae (coenocytic). Molds have a more characteristic type of fungal reproduction, displaying both asexual and sexual reproduction, depending on environmental factors.

Materials

- Broth culture of:
  - *Candida albicans*
  - *Saccharomyces cerevisiae*
- Slant cultures of:
  - *Rhizopus stolonifer*
  - *Penicillium notatum*
- (5) Sabouraud Dextrose Agar (SDA) plates
- 100 ml bottle of sterile water

Procedure

A. Growing yeast on SDA

1. Aseptically streak for isolation each of the following to an SDA plate:
   a. *Candida albicans*
   b. *Saccharomyces cerevisiae*
2. Make sure to label each plate appropriately
3. Incubate both plates at **30 °C** for 48 hours.

B. Growing mold on SDA

1. Using an inoculating needle, aseptically place a **dot** of each of the following to an SDA plate:
    a. *Rhizopus stolonifer*
    b. *Penicillium notatum*
2. Make sure to label each plate appropriately
3. Incubate both plates at **25 °C (room temp)** for 48 hours.

C. Microscopic visualization of molds

a. Use two sterile microculture plates, one for each mold culture. Each plate contains a glass slide placed on a bent glass rod.
b. Prepare two 1 cm blocks of Sabouraud dextrose agar using a thick plate of SDA.
c. Using a sterilized spatula, lift the agar block from the plate and place it on the center of the sterile glass slide.
d. Inoculate all four sides of the block with *Rhizopus stolonifer*.
e. Sterilize a cover slip by holding it with forceps and dipping it into a beaker containing alcohol. Pass the cover slip once over the flame of a Bunsen burner to burn off the alcohol.
f. Place the sterile cover slip on top of the inoculated block.
g. Pour 10 ml of sterile water into the bottom of the plate. Cover the plate.
h. Place the plate right side up in the tray available on the counter. Leave the tray at room temperature for one week.
i. Repeat same procedure using *Penicillium notatum*.
j. After one week of incubation use two clean glass slides and place one drop of lactophenol cotton blue on the center of each.
k. Remove the cover slip from each block and place it on each drop of lactophenol cotton blue.
l. Observe the structures of each of the molds under the 40x objective lens.

D. Fermentation

a. Aseptically pipet 10 ml of grape juice into each of two clean screw-cap culture tubes.
b. One tube of culture will contain *Saccharomyces cerevisiae*
c. One tube of culture will contain *Candida albicans*
d. Incubate the uninoculated bottle of grape juice with the two cultures at **30 °C** for 48 hours.

Smear Preparation

Introduction

It is difficult to see bacterial cells because of their size and because of their lack of color. In order to view these cells, a bacterial smear must be prepared on a clean glass slide and then stained. Considerations should be made in order to prepare a good bacterial smear. First, the cells need to stick to the glass slide and must resist being washed off during staining. Also, thick smears do not allow for a good visualization of the individual cells. Therefore, do not use a heavy inoculum when preparing the smear. The smear needs to be air-dried before being heat-fixed. Heat-fixing is necessary in order to kill the bacteria and in order to allow for the cells to completely adhere to the slide.

Materials

- Broth culture of:
  - *Bacillus subtilis*
- Slant culture of:
  - *Escherichia coli*

Procedure

A. Preparing a Bacterial Smear from a Broth Culture

a. Obtain a glass slide.
b. Aseptically transfer a "loopful" of *Bacillus subtilis* to the slide.
c. Spread the inoculum throughout the slide.
d. Allow the smear to air-dry at room temperature.
e. Once dry, heat-fix the slide (as demonstrated by the instructor).
f. The slide is now ready to be stained.

B. Preparing a Bacterial Smear from a Slant Culture

a. Obtain a glass slide.
b. Aseptically transfer a "loopful" of water to the slide.
c. Aseptically transfer a small amount of *Escherichia coli* to the drop of water.
d. Spread the inoculum throughout the slide.
e. Allow the smear to air-dry at room temperature.
f. Once dry, heat-fix the slide (as demonstrated by the instructor).
g. The slide is now ready to be stained.

C. Preparing a Mixed Smear from a both sources

a. Obtain a glass slide.
b. Aseptically transfer a "loopful" of *Bacillus subtilis* to the slide.
c. Aseptically transfer a small amount of *Escherichia coli* to the *Bacillus subtilis.*

d. Spread both inocula throughout the slide.
e. Allow the smear to air-dry at room temperature.
f. Once dry, heat-fix the slide (as demonstrated by the instructor).
g. The slide is now ready to be stained.

Simple Stain

Introduction

Since bacteria can be hard to see, stains can be used to increase contrast between bacteria and their background. A simple stain is a procedure using a single dye or stain. In many cases, the dye reacts with the surface of the bacterium giving the cell color. Chromophores are the colored portion of the dye molecule. The chromophores can have a positive charge and therefore reacts with the negatively charged molecules found on the surface of bacteria. These dyes are known as basic dyes. Examples of basic dyes include methylene blue, crystal violet, safranin, and malachite green. Dyes can also be acidic. Acidic stains have negatively charged chromophores and are repelled by the surface of bacteria. They are also known as negative stains or background stains because they stain the background and leave the bacteria transparent. Examples of such dyes include Congo red and India ink. A combination of both dyes has been and can be used in some instances.

Materials

- Broth culture of:
    - *Bacillus subtilis*
- Slant culture of:
    - *Escherichia coli*

Procedure

A. Simple staining

a. Stain one slide at a time, using the slides that were prepared in the previous exercise.
b. Place the heat-fixed smear on a staining rack over the metal staining bowl.
c. Liberally flood the smear with methylene blue.
d. Allow the dye to react for one (1) minute.
e. Tilt the slide to allow the dye to drain into the bowl.
f. Rinse the slide with deionized (DI) water.
g. Blot the slide dry using bibulous paper.
h. Repeat this procedure for all prepared slides.
i. View your slides under the microscope.

Gram Stain

Introduction

Gram staining is a procedure invented in the late 1800s by Hans Christian Gram that is the arguably the most useful and widely used staining procedure in microbiology. It is a differential staining technique in that it distinguishes between types of bacteria based on their physical and chemical differences. This technique divides bacteria into two groups: Gram-positive bacteria and Gram-negative bacteria. There are four solutions involved in the Gram stain: the primary stain, the mordant, the decolorizer, and the counterstain. The primary stain is crystal violet. Recall that crystal violet is a positive stain and will interact with the cell wall of bacterial cells. The mordant increases the interaction between the bacterial cell and the primary stain and is arguably the most important step in this process. The decolorizer is either 95% ethanol *or* 70% ethanol + 30% acetone. This is also an extremely important part of this process. In Gram-positive cells, the decolorizer shrinks the pores in the peptidoglycan layer which increases the retention of the crystal violet. In Gram-negative cells, the decolorizer extracts lipids from the cell wall which increases the porosity of the cell wall. This then leads to the decolorization of Gram-negative cells. The counterstain, safranin, then stains all colorless cells pink.

Materials

- Slant culture of:
    - *Bacillus subtilis*
    - *Escherichia coli*
    - *Moraxella catarrhalis*
    - *Staphylococcus aureus*
    - Unknown bacteria

Procedure

A. Gram staining

a. Prepare smear. Make sure the smear is well prepared (air-dried and heat-fixed)
b. Flood smear with crystal violet and let sit for **30-60 seconds**.
c. Rinse with DI water thoroughly.
d. Flood smear with iodine and let sit for **60 seconds**.
e. Rinse with DI water thoroughly.
f. Decolorize the slide by **splashing it or adding drop by drop** with the decolorizer until it runs clear. Rinse with DI water **immediately**.
g. Flood smear with Gram Safranin and let sit for about **60 seconds**.
h. Rinse with DI water thoroughly.
i. Blot dry with bibulous paper.
j. View.

Capsule Stain

Introduction

The cell walls of some bacteria such as *Streptococcus pneumoniae* and *Klebsiella pneumoniae* may be surrounded by an extrapolymeric substance. Some bacteria are covered with either a loose layer of polysaccharides called a slime layer or a tighter, better organized layer called a capsule. Both may protect bacterial cells from desiccation or may aid in the pathogenicity of the organism. In many cases, the capsule can protect bacteria against phagocytic white blood cells. As mentioned before, the capsule stain is designed to stain everything but the organism (the background). The polysaccharide or polypeptide composition of the capsules makes staining of the cell difficult. Therefore, the background is stained black, leaving the cell transparent. The smear is prepared in the same manner as a negative stain, with the slide not being heat-fixed, since application of heat may destroy or distort the capsule. In many cases, a basic stain may be applied in order to attempt to stain the cells to add contrast. The capsule stain is air-dried before a basic stain is applied, which in many cases is crystal violet.

Materials

- Slant culture of:
    - *Klebsiella pneumoniae*

Procedure

A. Capsule staining

a. Place a drop of India Ink on a slide.
b. Aseptically, take a small inoculum of the organism and mix it into the drop of India Ink.
c. Spread the drop around the slide using a loop and allow it to air-dry.
d. **DO NOT HEAT-FIX.**
e. Because the slide is not heat-fixed, remember that the bacteria are still alive!
f. Flood smear with crystal violet for **60 seconds**.
g. Rinse with DI water thoroughly.
h. **Allow the slide to air-dry, do not blot.**
i. View.

B. Capsule staining (alternative method)

a. Place a drop of India Ink on a slide.
b. Aseptically, take a small inoculum of the organism and mix it into the drop of India Ink.
c. Take another slide to spread a thin film as demonstrated by the instructor.
d. Follow **steps d-h** as written in the previous method.
e. View.

Endospore Stain

Introduction

The endospore stain is a differential stain used to detect the presence and location of spores. Few genera produce spores, including Bacillus and Clostridium. Pathogenic strains include Clostridium tetani, Clostridium botulinum, Clostridium perfringes, and Clostridium difficile. An endospore is a structure produced by bacteria that can withstand harsh environmental conditions. Examples of harsh conditions include heat, UV radiation, disinfectants, toxins, waste lack of nutrients, and desiccation. Bacteria can form endospores in approximately 6 to 8 hours after being exposed to adverse conditions. The normally growing cell that forms the endospore is called a vegetative cell. Spores are metabolically inactive and dehydrated and can remain viable for thousands of years. When spores are exposed to favorable conditions, they can germinate into a vegetative cell within 90 minutes. Endospores can form within different areas of the vegetative cell. They can be central, subterminal, or terminal. Central endospores are located within the middle of the vegetative cell. Terminal endospores are located at the end of the vegetative cell. Subterminal endospores are located between the middle and the end of the cell. Endospores can also be larger or smaller in diameter than the vegetative cell. Those that are larger in diameter will produce an area of "swelling" in the vegetative cell. These endospore characteristics are consistent within the spore-forming species and can be used to identify the organism. Because of their tough protein coats made of keratin, spores are highly resistant to normal staining procedures. There are three components in the endospore stain. The primary stain is malachite green. Malachite green is a stain that has to be driven into the spores and is done so using heat. It is also a stain that has low cellular affinity and therefore the decolorizer for this procedure is DI water. Since the cells wash out easily with water, they need to be counterstained. The counterstain in this procedure is Gram safranin.

Materials

- 72-hour slant culture of:
    - *Bacillus subtilis*

Procedure

A. Endospore staining

a. Smear prep two (2) bacterial smears from a 72-hour culture of *Bacillus subtilis*
b. Place a small strip of paper towel on the slide and flood both smears with malachite green.
c. Heat the dye for **5-7 minutes** and keep the slide wet during heating by replacing the evaporated dye.
d. Carefully remove the slide from the rack using forceps.
e. Allow the slide to cool for **3 minutes**.
f. Rinse the slide with DI water to wash the malachite green.

g. Flood the slide with safranin for **60 seconds**.
h. Rinse the slide with DI water.
i. Blot the slide with bibulous paper.
j. View.

Acid Fast Stain

Introduction

The acid-fast stain is a differential stain used to identify cells capable of retaining a primary stain when treated with acid alcohol. Members of the genus Mycobacterium including M. tuberculosis, the causative agent of tuberculosis, and M. leprae, the causative agent of Hansen's disease, are acid -fast bacteria. Recall that this means that the cell wall of these bacteria has a substance called mycolic acid. Mycolic acid is a waxy substance which does not allow the cells to be stained by simple stains, but when stained by carbolfuchsin can retain this stain even acid alcohol decolorizer is used. The acid-fast stain is also useful in identifying other organisms which could be pathogenic such as members of the *Nocardia* genus and parasites in the genus *Cryptosporidium* and the genus *Isospora*. Few organisms are acid-fast, so this stain is run only when there is suspicion of an infection by an acid-fast organism.

There are two (2) methods used for acid-fast staining: the Ziehl-Neelsen and the Kinyoun method. Both methods use carbolfuchsin containing 5% phenol as the primary stain and methylene blue as the counterstain. The Ziehl-Neelsen method uses heat to drive carbolfuchsin into the mycolic acid containing cell walls while the Kinyoun method uses a more concentrated, more lipid soluble form of carbolfuchsin. There are three components in the acid-fast procedure. Again, carbolfuchsin is the primary stain that contains a phenolic compound that is lipid soluble. This will stain cells a reddish-purple color. The decolorizer for this technique is acid-alcohol which will decolorize acid-fast negative cells. Finally, methylene blue is the counterstain that stains acid-fast negative cells blue. In our class, the Kinyoun method is utilized.

Materials

- Slant culture of:
    - *Mycobacterium smegmatis*
    - *Staphylococcus epidermidis*

Procedure

A. Acid-fast staining

a. Smear prep one (1) slide with both organisms.
b. Flood smear with carbolfuchsin and let sit for **5 minutes**.
c. Rinse with DI water thoroughly.
d. Decolorize the slide with acid-alcohol by adding decolorizer **until it runs clear**.
e. Rinse with DI water.
f. Flood smear with methylene blue and let sit for about **2 minutes**.
g. Rinse with DI water thoroughly.
h. Blot dry with bibulous paper.
i. View.

Protozoa

Introduction

Protozoa are thought of as the "animal-like" members of Kingdom Protista. Protozoa include approximately 65,000 species with most species being free-living inhabitants of soil and water. Some protozoa are parasites of the human intestine, liver, brain, blood and other organs. Recall that the protozoa may use several different structures in order to move. The means of locomotion for these organisms can be used to classify protozoa and, in this lab, we will study several members from different phyla based on their locomotive apparatuses.

Materials

- Prepared slides of:
  - *Amoeba proteus*
  - *Entamoeba histolytica* (cyst)
  - *Trypanosoma brucei gambiense*
  - *Trypanosoma cruzi*
  - *Trichomonas vaginalis* (trophozoite)
  - *Giardia lamblia* (trophozoite)
  - *Giardia lamblia* (cyst)
  - *Plasmodium falciparum* (ring stage)
  - *Plasmodium falciparum* (male and female gametocytes)
  - *Balantidium coli* (cyst)

Procedure

A. View all slides at magnifications appropriate for each organism (in order to view the organism in great detail).
B. Record your observations.

Platyhelminthes & Nematodes

Introduction

The general life cycle of helminths involves the transmission of an egg or larva to the body of a host. The host species may vary and transmission can occur between organisms of the same and of different species. Usually an intermediate host, a host in which larval development occurs, is found during transmission while a definitive host is the host in which adulthood and mating occur. Recall that Phylum Platyhelminthes is characterized by helminths that are acoelomates and Phylum Nematoda are pseudocoelomates.

About 50 species of helminths parasitize humans. These are distributed in all areas of the world that support human life and may be geographically restricted, with a higher incidence of them occurring in tropical areas. There are numerous cases of worldwide helminth infections, with numbers found usually in the billions. Humans have evolved in the constant presence of helminths and only recently have humans evolved into a "helminth-free" existence. There is evidence showing that an absence of helminth infections may contribute to autoimmunity.

Materials

- Prepared slides of:
    - Phylum Platyhelminthes
        - Trematodes
            - *Clonorchis sinensis* (adult and ova)
            - *Schistosoma* (male and female)
        - Cestoda
            - *Echinococcus granulosis* (adult, scolex, ova, and hydatid cyst)
            - *Taenia saginata* (proglottid and scolex)
            - *Taenia solium* (proglottid and scolex)
- Prepared slides of:
    - Phylum Nematoda
        - *Ascaris lumbricoides* (ova)
        - *Necator americanus* (ova)
        - *Trichuris trichiura* (ova)
        - *Trichinella spiralis* (ova)
        - *Enterobius vermicularis* (adult and ova)

Procedure

A. View all slides at magnifications appropriate for each organism (in order to view the organism in great detail).
B. Record your observations.

The Effect of Temperature on Microbial Growth

Introduction

There are many environmental factors that can influence microbial growth. These factors may include temperature, pH, oxygen concentration, osmotic pressure, and ionizing or non-ionizing radiation. Bacteria do not have a way to internally control temperature and therefore survival depends on adapting to the temperature in the environment. Temperature ranges for microbial growth can be expressed as three (3) cardinal temperatures: minimum, maximum, and optimum. The minimum temperature is the lowest temperature at which a microbe will grow. The maximum temperature is the highest temperature at which a microbe can grow. The optimum temperature is the temperature at which a microbe grows best. Recall that there are several temperature classes of microorganisms, with each one corresponding to a specific temperature range.

Heat resistance varies among microbes. Two measurements can be used to compare the susceptibility of different microorganisms to the effect of temperature:

- Thermal Death Point (TDP): the temperature at which a microbe will be killed in 10 minutes
- Thermal Death Time (TDT): the time for a microbe to be killed at a given temperature.

Materials

- Slant culture of *Serratia marcescens*
- Nutrient broth cultures
    - *Bacillus subtilis*
    - *Escherichia coli*
- TSA slants (2 per pair)
- TSA plates (2 per pair)

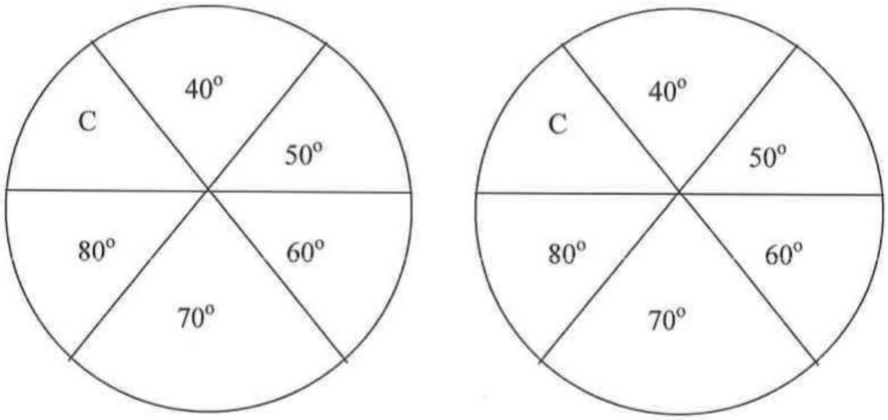
Procedure

A. The Effect of Temperature on Pigment Production

1. Streak two (2) TSA slants with *Serratia marcescens*.
2. Incubate one slant at **40 °C** and incubate the other slant at room temperature (**25 °C)** for 48 hours.

B. The Effect of Temperature on Bacterial Growth

1. Divide the bottom of two (2) TSA plates into six sectors and label them as shown below:



B. subtilis *E.coli*

2. Streak the sector marked "C" (control) on each TSA plate using the appropriate corresponding organism. Use a simple line streak to inoculate the plate.
3. Using a 10 ml pipette, aseptically transfer 2 ml of *B. subtilis* broth from the flask into each of five (5) sterile test tubes. Label them 40°, 50°, 60°, 70°, and 80°, accordingly.
4. Using a 10 ml pipette, aseptically transfer 2 ml of *E. coli* broth from the flask into each of five (5) sterile test tubes. Label them 40°, 50°, 60°, 70°, and 80°, accordingly.
5. On the countertop you will see five water baths. The temperature in the first one is set at 40 °C, in the second is set at 50 °C, in the third is set at 60 °C, in the fourth is set at 70 °C, in the last is set at 80 °C.
6. Place both tubes of *E. coli* and *B. subtilis* labeled 40 °C in the first water bath for 10 minutes.
7. After 10 minutes, remove both tubes from the water bath and streak sectors labeled 40 °C. Discard both tubes.
8. Repeat the same procedure for 50 °C, 60 °C, 70 °C, and 80 °C temperatures.
9. Incubate the inoculated plates at **30° C for 48 hours**.
10. After incubation, examine the growth in each sector and determine thermal death point (TDP) for each bacterium.

The Effect of Ultraviolet (UV) Radiation on Bacterial Growth

Introduction

As mentioned in the last lab experiment, ultraviolet (UV) light can affect growth. Traditionally, the wavelengths of UV range between 100 to 400 nm, however, ultraviolet wavelength has been characterized as low as 10 nm (extreme UV). The UV range that we typically focus on is between the former as opposed to the latter. UV light is divided into three bands: UVA 315-400 nm, UVB 280-315 nm, and UVC 100-280 nm. The most lethal of these bands is UV-C which is, fortunately, completely absorbed by the ozone found in the atmosphere. Being the most lethal, UVC is also the most germicidal.

UV light damages DNA molecules by breaking the hydrogen bonds between the two strands of DNA and forming new harmful covalent bonds between two adjacent pyrimidines (thymine and cytosine bases) and most commonly between two adjacent thymine bases forming so called thymine dimers. These dimers produce what are called DNA lesions and, if left unrepaired, they can block DNA replication and transcription resulting in the death of the cell.

Other radiations, such as gamma rays and X-rays, can damage DNA molecules by breaking the covalent bonds between the phosphate and sugar in the nucleotide (the basic unit of DNA). This type of radiation is known as ionizing radiation while UV is commonly known as non-ionizing (with the exception of extreme UV). While gamma rays and X-rays can penetrate solid objects, UV radiation can damage cells only by direct contact. Therefore, UV radiation can be used as a germicidal agent, for disinfection of surfaces.

Materials

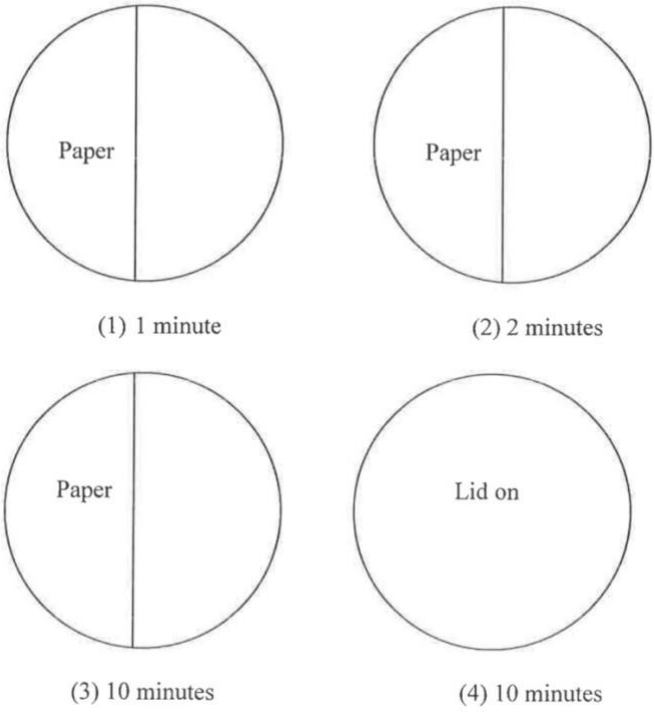
- Broth cultures
  - *Bacillus subtilis*
  - *Serratia marcescens*
- TSA plates (4 per pair)

Procedure

A. The Effect of Temperature on Pigment Production

1. Label 4 TSA plates 1, 2, 3, and 4.
2. Dip a sterile swab in NB culture of the bacterium.
3. Perform a “lawn” culture as described early in the semester. Repeat same procedure for each plate.
4. Cover the plates with the lid and take them to the workroom where the source of UV light is located.
5. Remove the lids of the first three plates, then cover half of each plate with paper and expose them to UV radiation for 1 minute, 2 minutes, and 10 minutes, respectively.

6. Expose the fourth plate with cover on to UV radiation for 10 minutes, as shown below:



7. Replace the covers of the plates; 1, 2, and 3.
8. Incubate plates of *S. marcescens* at 25 °C (room temperature) for 48 hours.
9. Incubate plates of *B. subtilis* at 30 °C for 48 hours.
10. Each table will use an uninoculated plate as a negative control.

Cultivation of Aerobes, Anaerobes, and Microaerophiles

Introduction

Oxygen and carbon dioxide are gases that have a strong influence on bacterial growth. With respect to oxygen requirements several general categories are recognized:

- Obligate (strict) aerobe: Unable to grow without high oxygen concentrations.
- Aerobe: Grows well in the presence of oxygen and possesses the enzymes needed to process toxic oxygen products.
- Microaerophile: Requires oxygen at a concentration less than that in the atmosphere.
- Aerotolerant anaerobe: Does not utilize oxygen but can survive in its presence.
- Facultative anaerobe: An aerobe that does not require oxygen for its metabolism and is capable of growth in the absence of oxygen.
- Anaerobe: Does not grow in normal atmospheric oxygen.

One of the most important media for the study of oxygen tolerance of bacteria is thioglycolate broth. The medium contains sodium thioglycolate that removes oxygen from the environment of bacteria, a small amount of agar 0.05% and either methylene blue or, usually, resazurin. These dyes are colorless in an anaerobic environment and greenish-blue (methylene blue) or pink (resazurin) in the presence of oxygen.

For this exercise, we use an OXOID AnaeroGen Anaerobic Gas generator. The active component within each AnaeroGen sachet is ascorbic acid. When an AnaeroGen is placed in a sealed jar and exposed to air, the reaction will start and oxygen in the jar is rapidly absorbed with the simultaneous generation of carbon dioxide. The concentration of oxygen will be reduced to less than 1% within 30 minutes. The resulting carbon dioxide will be between 9% and 13%.

A candle jar is usually a container large enough to hold several agar plates. After inoculation, Petri dishes are placed upside down in the jar. A candle is lit and placed in the jar. The jar is then sealed tightly. The candle's flame will consume most of the oxygen in the jar and will produce a higher level of carbon dioxide. These conditions allow the growth of capnophiles.

Materials

- Broth cultures
    - *Clostridium sporogenes*
    - *Escherichia coli*
    - *Micrococcus luteus*
    - *Streptococcus pyogenes*
- Thioglycolate Broth (10 ml screw-capped tubes) (3 per pair)
- Brewer anaerobic agar plates (3 per pair)
- TSA plate (2 per pair)

Procedure

A. Growth in Thioglycolate Broth

1. Inoculate the first thioglycolate broth with *Micrococcus luteus*.
2. Inoculate the second thioglycolate broth with *Escherichia coli*.
3. Inoculate the third thioglycolate broth with *Clostridium sporogenes*.
4. Incubate *M. luteus* at 30 °C. Incubate *E. coli* and *C. sporogenes* at 37 °C for 48 hours.

B. Growth in Anaerobic Jar/Chamber

1. Streak three (3) Brewer's anaerobic agar plates with *Micrococcus luteus*, *Clostridium sporogenes*, and *Escherichia coli*.
2. Place the inoculated plates in the anaerobic jar.
3. Tear open an anaerobic foil sachet at the indicated area, and remove the **AnaeroGen** paper sachet from within.
4. Immediately place the **AnaeroGen** paper sachet in the jar.
5. It is recommended that an **OXOID** anaerobic indicator is also used in the jar as a visual check that anaerobic conditions have been achieved and maintained.
6. Seal and close the jar lid immediately.
7. Incubate the jar at 37 °C for 48 hours.

C. Growth in Candle Jar

1. Streak two (2) TSA plates with *Streptococcus pyogenes*.
2. Place one in the candle jar (as directed above), and incubate at 37 °C for 48 hours.
3. Incubate the other TSA plate in the incubator at 37 °C for 48 hours.
4. Compare the amount of growth after incubation.

Disinfectants and Antiseptics

Introduction

In this lab exercise we will study how some disinfectants and antiseptics affect the growth of bacteria. Disinfectants are substances that are applied to inanimate (non-living) objects such as instruments, glass, and/or a benchtop to destroy microorganisms. Antiseptics are antimicrobial agents that are applied to animate (living) tissues such as skin to inhibit (sometimes kill) the growth of microorganisms.

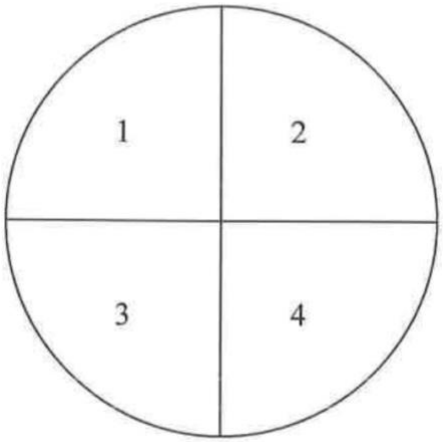
Materials

- Broth cultures
  - *Escherichia coli*
  - *Staphylococcus aureus*
- TSA plate (2 per pair)

Procedure

A. Disinfectants and Antiseptics

1. On the bottom of each TSA plate, mark four sectors as shown below:



2. Label one plate with *S. aureus* and the second plate with *E. coli*.
3. Dip a sterile swab into the broth of *S. aureus*, inoculate the surface of the first agar plate using the "lawn" culture technique.
4. Dip another sterile swab into the broth of *E. coli*, inoculate the surface of the second agar plate using the "lawn" culture technique.
5. Flame the tip of forceps.
6. Using the flamed forceps, pick up a sterile filter paper disk and dip it into disinfectant # 1, then place the disk in the center of sector marked "1" of the first plate.
7. Using the same disinfectant, place another disc in sector "1" of the second plate.

8. Repeat the previous step; place the other disinfectants 2, 3, and 4 in the corresponding sectors of the two plates.
9. Incubate both plates at 30 °C for 48 hours.
10. After incubation, measure the diameter of each no growth zone around each disinfectant or antiseptic and record it in millimeters.

The Effect of Antibiotics on Bacterial Growth

Introduction

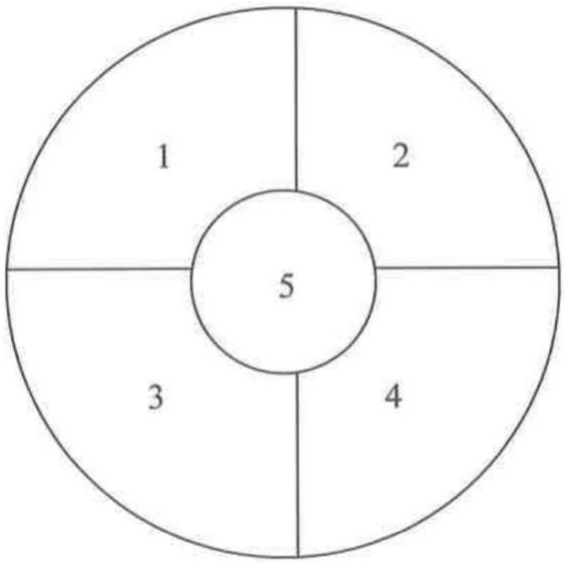
In this lab exercise we will study the effectiveness of some antibiotics on the growth of bacteria. In order to do this, the Kirby-Bauer method will be employed using Mueller Hinton Agar. This medium is inoculated with an organism and disks containing relevant antibiotics are placed on top of the organism. Antibiotics in the disks diffuse into the agar during incubation. If the organism is susceptible to one of the antibiotics, an area of clearing around the disk will result. This clearing is called a zone of inhibition. Larger zones indicate that an organism is more sensitive to an antibiotic than smaller zones. Zone sizes have been standardized in order to interpret the most effective antibiotic against a particular organism. Established zone diameters can then be used to interpret resistance for pathogenic microorganisms and this data can be used in patient care.

Materials

- Broth cultures
    - *Escherichia coli*
    - *Staphylococcus aureus*
- Mueller Hinton with Blood Agar Plates (2 per pair)

Procedure

1. **Disinfectants and Antiseptics**
    1. On the bottom of each plate, mark five sectors as shown below:



2. Label one plate with S. aureus and the second plate with *E. coli.*
3. Dip a sterile swab into the broth of *S. aureus* and inoculate the surface of the first agar plate using the lawn streak technique.
4. Dip another sterile swab into the broth of *E. coli* and inoculate the surface of the first agar plate using the lawn streak technique.
5. Flame the tip of forceps and place the five different antibiotic disks on the surface of each plate.
6. Incubate all plates at 37 °C for 48 hours.
7. After incubation, measure the diameter of no growth zone around each antibiotic and record it in millimeters.

Exoenzymes in Bacteria

Introduction

In order for some bacteria to utilize macromolecules for nutrition, they need to produce exoenzymes that are secreted outside the cell to degrade the large molecules into smaller ones. This allows the smaller molecules to then enter the bacterial cell through the plasma membrane and become available for different functions. This lab will illustrate how bacteria use certain exoenzymes to use certain macromolecules. This lab will also be one of the first labs using differential media plates.

TSA, Nutrient agar, and Luria-Bertani (LB) are all examples of enrichment media. They are designed to provide the nutrients needed to support the growth of microbes. Media can contain selective agents that will result in media that is specific for certain microorganisms. Selective media "selects" for a specific bacteria or type of bacteria and the media usually inhibits the growth of other bacteria. Usually, a selective agent works by inhibiting growth of the unwanted organisms. Examples of selective media include azide agar and sodium chloride agar or broth. Differential media allows you to distinguish between different groups of bacteria. It is commonly seen as a color change in the media or "zones" of clearing. Color change and zones of clearing or inhibition are not the only ways that media can be differential. Hemolytic reactions like that exhibited by Staphylococcus aureus on blood agar is a differential reaction. Other examples of differential media include starch agar and skim milk agar. There are media which are both selective and differential. Examples of these media types are Eosin Methylene Blue Agar, MacConkey Agar, and Bile Esculin Agar.

The purpose of the **starch hydrolysis** test is to detect production of amylases. Amylase is an enzyme that breaks down 1.5% starch that is incorporated in the agar. Starch is a polysaccharide molecule which is too large to pass through the bacterial cell membrane. These large molecules can be hydrolyzed into smaller fragments or individual glucose molecules by extracellular enzymes amylase and oligo-1,6-glucosidase. The smaller molecules can then enter the cells. Starch is soluble, and therefore a reagent, iodine, must be added to visualize the reaction. Since iodine reacts with starch to produce a blue/black color, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth. The following is the reaction that is occurring:

$$\text{Starch} + H_2O \xrightarrow{Amylase} \text{Maltose}$$

The **casein hydrolysis** test is to detect organisms that can break down casein. Casein is a large milk protein incapable of permeating the plasma membrane of bacteria. Before casein

can be used by some bacteria as their carbon and energy source, it must be degraded into amino acids. Proteolytic enzymes (e.g. caseinase) catalyze the hydrolysis of casein to yield amino acids, which are then transported into the cell and catabolized. The medium used is usually skim milk mixed in with plate count agar (PCA) or nutrient agar. Organisms that break down the casein have a zone of clearing around the growth. The following is the reaction that is occurring:

$$\text{Casein} + H_2O \xrightarrow{Caseinase} \text{Amino Acids}$$

The purpose of the **lipid hydrolysis** test is to identify bacteria capable of producing the enzyme lipase. Lipase is responsible for hydrolysis of triglycerides into glycerol and fatty acids. Tributyrin Agar, which contains an oil which can be hydrolyzed by lipase-producing bacteria, is the media used in this experiment. If the triglycerides are hydrolyzed, there will be a zone of clearing. The following is the reaction that is occurring:

$$\text{Lipid} + H_2O \xrightarrow{Lipase} \text{Glycerol} + \text{Fatty Acids}$$

The **gelatin hydrolysis** test is used to determine whether an organism produces gelatinase. Gelatinases are extracellular enzymes produced and secreted to hydrolyze gelatin. Once the gelatin is hydrolyzed, the individual amino acids can be taken up by the cell and used for metabolic purposes. The medium used is a gelatin plate or gelatin deep. Organisms that hydrolyze gelatin have a zone of clearing around the growth or will liquefy the deep. The following is the reaction that is occurring:

$$\text{Gelatin} + H_2O \xrightarrow{Gelatinase} \text{Amino Acids}$$

Materials

- Slant cultures
    - *Bacillus subtilis*
    - *Corynebacterium xerosis*
    - *Escherichia coli*
    - *Pseudomonas aeruginosa*
    - *Staphylococcus aureus*
- Per pair:
    - Starch Agar
    - Skim Milk Agar
    - Tributyrin Agar
    - Nutrient Gelatin Stabs

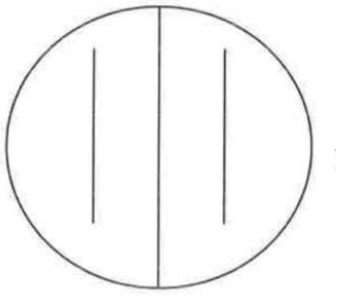
Media Preparation

1. **Starch agar:** Add 6.9 g of nutrient agar to 300 ml of water, and add 0.9 g of starch. Turn on the heat and boil the medium. Dispense into each of two bottles equally. This amount is enough to pour 12 plates **for the whole class**.
2. **Skim milk agar:** Add 6.9 g of nutrient agar to 300 ml of water. Boil and dispense into each of two bottles equally. Dissolve 3 g of skim milk in 30 ml of water. Dispense into each of two screw-cap tubes equally. Once autoclaved, using aseptic technique, pour the sterile skim milk of each tube into the bottle of the sterile nutrient agar when you are ready to pour the plates. The two bottles are enough to pour 12 plates **for the whole class**.
3. **Nutrient gelatin:** Add 25.6 g of nutrient gelatin to 200 ml of water. Warm slightly until the powder dissolves completely. Dispense 7 ml into each test tube for stabs (dispense the whole amount to make approximately 27 tubes).

Procedure

A. Starch Hydrolysis

1. Divide the bottom of a starch agar plate in half. Label one half *E. coli* and the other *B. subtilis*
2. Make a single streak inoculation of *B. subtilis* and *E. coli* on each side of the starch agar plate:



3. Incubate the plate at 37 °C for 48 hours.
4. **After incubation**, flood the plate with iodine.

B. Casein Hydrolysis

1. Divide the bottom of a skim milk agar plate in half. Label one half *C. xerosis* and the other half *B. subtilis* as shown above.
2. Make a single streak inoculation of *Corynebacterium xerosis* and *Bacillus subtilis* on each side of the plate.
3. Incubate the plate at 37 °C for 48 hours.

C. Lipid Hydrolysis

1. Divide the bottom of a tributyrin agar plate in half. Label one half *E. coli* and the other *P.aeruginosa* as shown above.
2. Make a single streak inoculation of *E. coli* and *P.aeruginosa* on each side of the plate.
3. Incubate the plate at 37 °C for 48 hours.

D. Gelatin Hydrolysis

1. Stab three nutrient gelatin tubes using *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Escherichia coli*.
2. Incubate the tube of S. marcescens at 25° C for 48 hours and the other two tubes at 37 °C for 48 hours
3. **After incubation**, place the tubes in the refrigerator for 30 minutes.

Carbohydrate Fermentation

Introduction

Fermentations are energy-producing reactions in which organic molecules serve as both electron acceptors and donors. It is important to understand that a microorganism's ability to ferment carbohydrates may be important in their identification. A carbohydrate may be fermented into different end products depending on what microorganism is involved in the process. The media used in the fermentation exercises in the lab will consist of a carbohydrate and a pH indicator. If the organism ferments the sugar, organic acids may result and cause the pH to drop and change the color of the medium. Gases can also be produced and they can be detected by way of a Durham tube. The Durham tube is in the medium and if gas is produced, the medium inside the tube is displaced entrapping the gas in a bubble form.

Materials

- Broth cultures
  - *Enterobacter cloacae*
  - *Escherichia coli*
  - *Proteus vulgaris*
  - *Pseudomonas aeruginosa*
  - *Staphylococcus aureus*
- Six (6) tubes per pair of:
  - PR-Glucose
  - PR-Lactose
  - PR-Sucrose

Media Preparation (for each carbohydrate)

1. Add 4.8 g of phenol red base to 300 ml of water.
2. Allow the powder to dissolve completely (warm slightly).
3. Then add 2.1 g of glucose to the rehydrated 300 ml of phenol red base (0.7g of sugar per 100 ml of broth).
4. Mix to dissolve the glucose.
5. Prepare two sets for the whole section.
6. Place an inverted Durham tube in each test tube.
7. Dispense 7 ml of glucose broth into each test tube (dispense the whole amount to make approximately 42 broth tubes).
8. Repeat the same preparation for lactose.
9. Repeat the same preparation for sucrose.

Procedure

1. Obtain six (6) test tubes of each carbohydrate broth.

2. **Ensure that you transfer the correct bacterium into the labeled tube. Always double check the name of the bacterium on the tube containing the bacterial culture.**
3. Inoculate the first 3 broth tubes (glucose, lactose and sucrose broth) with *Escherichia coli*. Label and place them in the tube rack.
4. Inoculate the second set of broth tubes with *Enterobacter cloacae*. Label and place in the tube rack.
5. Inoculate the third set with *Staphylococcus aureus*. Label and place in the tube rack.
6. Inoculate the fourth set with *Proteus vulgaris*. Label and place in the tube rack.
7. Inoculate the fifth set with *Pseudomonas aeruginosa*. Label and place in the tube rack.
8. Leave the last set of broth tubes uninoculated as a comparative control and place in the tube rack.
9. Incubate all at 37 °C for 48 hours.

Hydrogen Sulfide Production

Introduction

Hydrogen sulfide production can be used to differentiate between enteric bacteria. *Salmonella*, *Francisella*, and *Proteus* are sulfur reducers. Proteins containing amino acids with sulfur are hydrolyzed by some bacteria. The amino acids that are released are taken up by the bacteria as nutrients. The medium used to help determine this is called peptone iron agar. This medium contains proteose peptone, sodium thiosulfate, and ferric ammonium citrate. If hydrogen sulfide (H_2S) is produced, it combines with the ferric ammonium citrate and the sodium thiosulfate to form an insoluble black precipitate.

Materials

- Slant cultures
    - *Escherichia coli*
    - *Proteus vulgaris*
    - *Salmonella typhimurium*
- Peptone Iron Agar (PIA) Deeps (4 per pair)

Note: For the following procedure, stab the needle to within 1-2 inch of the bottom of the tube and withdraw the needle following the initial line of inoculation.

Procedure

1. Stab the first tube of PIA with *Salmonella typhimurium*.
2. Stab the second tube of PIA with *Proteus vulgaris*.
3. Stab the third tube of PIA with *Escherichia coli*.
4. Keep the fourth tube of PIA as a control medium (uninoculated).
5. Incubate all tubes at 37 °C for 48 hours.

Urea Hydrolysis

Introduction

Urea hydrolysis is a test that identifies bacteria that can degrade urea using the enzyme urease. Urea agar contains phenol red as a pH indicator. Under acidic conditions, phenol red turns yellow. Under neutral conditions, phenol red remains peach-like (or slightly peach-pink. Under alkaline conditions, phenol red turns hot pink/fuchsia. The following is the reaction that takes place:

$$\text{Urea} + H_2O \xrightarrow{Urease} NH_3 + CO_2 + H_2O$$

Materials

- Slant cultures
  - *Escherichia coli*
  - *Proteus vulgaris*
  - *Salmonella typhimurium*
- Urea broth (4 per pair)

Media Preparation

1. Sterilize 90 ml of water.
2. Add 10 ml of urea concentrate to the 90 ml of sterile water and mix well.
3. Use 10 ml sterile pipette.
4. Dispense 3 ml into each sterile test tube. Dispense, approximately, 33 tubes for the whole class.

Procedure

1. Stab the first tube of urea broth with *Salmonella typhimurium*.
2. Stab the second tube of urea broth with *Proteus vulgaris*.
3. Stab the third tube of urea broth with *Escherichia coli*.
4. Keep the fourth tube of urea broth as a control medium (uninoculated).
5. Incubate all tubes at 37 °C for 48 hours.

Nitrate Reduction

Introduction

Many enterics are nitrate reducers. Nitrate used as terminal electron acceptor in anaerobic respiration. Nitrate reducers have the enzyme nitrate reductase. Nitrate reacts with the reagents to give a pink/red color. If no red color forms after the addition of the reagents, it is possible that nitrate has reduced further past nitrite. If this is the case, zinc powder is added. If, after the addition of zinc, there is a red color, then there is nitrate present (negative result). If there is no color after the addition of zinc, then the nitrate has been reduced past nitrite. In many cases, a Durham tube is placed to indicate if nitrogen gas is present. The reaction below is what occurs:

Materials

- Slant cultures
  - *Corynebacterium xerosis*
  - *Escherichia coli*
  - *Pseudomonas aeruginosa*
- Nitrate broth (4 per pair)

Procedure

1. Stab the first tube of nitrate broth with *Corynebacterium xerosis.*
2. Stab the second tube of nitrate broth with *Pseudomonas aeruginosa*.
3. Stab the third tube of nitrate broth with *Escherichia coli*.
4. Keep the fourth tube of nitrate broth as a control medium (uninoculated).
5. Incubate all tubes at 37 °C for 48 hours.

IMViC

Introduction

The IMViC tests are a series of 4 tests that is considered as 1 test (but we use four different sets of media for each). The IMViC is used to differentiate between enteric organisms. IMViC stands for:

- I= Indole
- M=Methyl red
- V= Voges-Proskauer
- C= Citrate

The following are the media, developers, and reactions for each component of the IMViC:

1. Indole: This test determines bacteria that can utilize tryptophan.

   Medium: Tryptone broth
   Developer: Kovac's Reagent (5 to 10 drops)

   $$\text{Tryptophan} \xrightarrow{\textit{Tryptophanase}} \text{indole + pyruvic acid + ammonia}$$

   What a positive result looks like: A red (cerise) color appears when the Kovac's Reagent reacts with the indole

2. Methyl red (MR): Tests for mixed acid fermenters of glucose.

   Medium: MR/VP broth (w/ peptone, glucose, and phosphate buffer)
   Developer: MR pH indicator (3 drops)

   $$\text{Glucose} \xrightarrow{\textit{Fermentation}} \text{Mixed acids}$$

   What a positive result looks like: Methyl red color remains red at a pH of 4.4 or less (if the pH is any higher, a yellow color is formed, which is negative).

3. Voges-Proskauer: Identifies bacteria that produce acetoin from fermentation of glucose into 2,3-butanediol (butylene glycol).

   Medium: MR/VP broth (w/ peptone, glucose, and phosphate buffer)
   Developer: Barritt's reagent A (15 drops) + Barritt's reagent B (5 drops). Shake the tube gently and let sit for 10-15 minutes

Barritt's Reagent A= α-naphthol
Barritt's Reagent B= Potassium hydroxide

Acetoin + A + B $\longrightarrow$ Red color

What a positive looks like: A red color appears when Barritt's A and B react with the acetoin formed.

4. Simmons citrate: To identify bacteria that can use citrate as its sole carbon source.

Medium: Sodium Citrate slant w/pH indicator bromothymol blue.

Citrate $\xrightarrow{Citritase}$ alkaline end products

What a positive result looks like: The slant changes from green to blue when the pH rises.

Materials

- Slant cultures
  - *Enterobacter cloacae*
  - *Escherichia coli*
  - *Klebsiella pneumoniae*
- Four (4) each, per pair:
  - Tryptone broth (5 ml/ tube)
  - MR/VP broth (3 ml/ tube)
  - MR/VP broth (2 ml/ tube)
  - Simmons citrate agar slant

Procedure

1. Inoculate a tryptone broth, MR/VP (3 ml), MR/VP (2 ml), and Simmons citrate slant with *E.coli.*
2. Repeat this procedure using *Enterobacter cloacae.*
3. Repeat this procedure using *Klebsiella pneumoniae*
4. Incubate all tubes at 37 °C for 48 hours.
5. **After incubation,** develop each tube with the appropriate developer.

Other Selective and Differential Media I

Introduction

In the exoenzymes lab, you were first introduced to selective and differential media and their importance. This lab will continue to introduce media that are selective, differential, or both.

Sodium chloride agar is a selective medium that is composed of nutrient agar with a 7% concentration of NaCl. This medium, selects for halophiles or organisms that are salt-tolerant.

Eosin methylene blue (EMB) agar is a selective and differential medium that contains lactose, sucrose, Eosin Y, and methylene blue. Methylene blue and eosin Y are dyes that both inhibit the growth of Gram-positive bacteria and therefore is selective for Gram-negative bacteria (usually used to help isolate enterics). It is differential for lactose and sucrose fermenters. The methylene blue-eosin Y complex will go from colorless to blue-black under acidic conditions. When lactose or sucrose are fermented, a dark purple complex is formed. If the organism is a **strong** lactose/sucrose fermenter, a metallic green sheen is formed. If non-lactose fermenters are present, the colonies will be translucent.

MacConkey agar contains lactose, bile salts, crystal violet, and neutral red (as a pH indicator). The bile salts and the crystal violet inhibit the growth of Gram-positive bacteria and therefore is selective for Gram-negative bacteria (usually used to help isolate enterics). It is differential for lactose fermenters. When lactose is fermented, the drop in pH will result in pink/red colonies (darker depending on the amount of fermentation). If non-lactose fermenters are present, the colonies will be translucent.

Blood agar is a differential medium that is composed of TSA with 5% defibrinated sheep blood. This medium determines whether or not an organism produces hemolysins (substances that destroy red blood cells). This medium will determine if the bacterium will exhibit:

- No hemolysis (gamma-hemolytic)
- Slight hemolysis (alpha-hemolytic)
- Complete hemolysis (beta-hemolytic)

In the **motility test**, a semi-solid medium is used to allow for the macroscopic examination of bacterial motility. Organisms are stabbed into the medium using a needle. Motility Test Medium contains Triphenyltetrazolium Chloride (TTC) which is used to add visual enhancement of bacterial growth. TTC is a colorless dye that gets reduced by bacterial cells to produce formazan, an insoluble red pigment. The red color forms only in the area of bacterial growth. An organism that is positive for motility will extend from the stab line and produce an area of red turbidity or cloudiness throughout the medium. Non-motile organisms grow only along the stab line (it will look like a red stab line) and leave the rest of the medium clear.

Materials

- Slant cultures

- *Enterobacter cloacae*
- *Escherichia coli*
- *Klebsiella pneumoniae*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Staphylococcus epidermidis*

- Per pair:
  - Sodium chloride agar plates (2)
  - Eosin methylene blue plates (5)
  - MacConkey Agar plates (5)
  - Blood agar plate (2)
  - Motility deep (2)

Media Preparation (per table)

1. **Sodium chloride agar:** Combine 2.3g of nutrient agar with 100 ml of water and add 7g of sodium chloride. Boil for 1 minute and dispense into a bottle.
2. **EMB agar:** Combine 5.4g of EMB with 150ml of water. Boil for 1 minute and dispense into a bottle.

Procedure

A. Sodium chloride agar

1. Streak one (1) NaCl agar plate for isolation with *Staphylococcus aureus*.
2. Streak the other NaCl agar plate for isolation with *Enterobacter cloacae*.
3. Incubate the plates at 37 °C for 48 hours.

B. Blood agar

1. Streak one (1) blood agar plate for isolation with *Staphylococcus aureus*.
2. Streak the other blood agar plate for isolation with *Staphylococcus epidermidis*.
3. Incubate the plates at 37 °C for 48 hours.

C. EMB agar

1. Streak one (1) EMB agar plate for isolation with *Staphylococcus aureus*.
2. Streak the second EMB agar plate for isolation with *Klebsiella pneumoniae*.
3. Streak the third EMB agar plate for isolation with *Enterobacter cloacae*.
4. Streak the fourth EMB agar plate for isolation with *Escherichia coli*.
5. Streak the fifth EMB agar plate for isolation with *Pseudomonas aeruginosa*.
6. Incubate the plates at 37 °C for 48 hours.

D. MacConkey agar (Mac)

1. Streak one (1) Mac agar plate for isolation with *Staphylococcus aureus*.
2. Streak the second Mac agar plate for isolation with *Klebsiella pneumoniae*
3. Streak the third Mac agar plate for isolation with *Enterobacter cloacae*.
4. Streak the fourth Mac agar plate for isolation with *Escherichia coli*.
5. Streak the fifth Mac agar plate for isolation with *Pseudomonas aeruginosa*.
6. Incubate the plates at 37 °C for 48 hours.

E. Motility test

1. Streak one (1) motility deep with *Staphylococcus aureus*.
2. Streak another motility deep with *Escherichia coli*.
3. Incubate the tubes at 37 °C for 24 hours.

Other Selective and Differential Media II (Staphylococci)

Introduction

Genus *Staphylococcus* is characterized by Gram positive, facultative anaerobic, catalase positive bacteria. Staphylococci will occur in grape-like clusters and, as mentioned, possess the ability to degrade hydrogen peroxide. Several members of genus *Staphylococcus* cause different types of infections. These include many different skin infections but can also include many severe internal infections. Normally, staphylococci are harmless skin microorganisms but can cause these diseases once given the chance to enter the body.

The **catalase test** determines if a bacterium possess the enzyme catalase, which is responsible for breaking down hydrogen peroxide into oxygen and water.

$$H_2O_2 \xrightarrow{Catalase} H_2O + O_2$$

Mannitol salt agar is a medium that is both selective and differential. What makes this medium selective is the presence of a high salt concentration (7.5%). This is a high enough concentration of salt to inhibit the growth of most bacteria, but members of the genus *Staphylococcus* can tolerate this concentration. What makes this medium differential is the presence of mannitol. *Staphylococcus aureus* is an organism that can ferment the mannitol, releasing acid by-products. This will lower the pH and will be indicated on this medium by phenol red.

The **coagulase test** is a method used to differentiate between pathogenic and non-pathogenic strains of *Staphylococcus*. It identifies whether an organism produces the exoenzyme coagulase, which causes the fibrin of blood plasma to clot. Organisms that produce coagulase can form protective barriers of fibrin around themselves, making them highly resistant to phagocytosis, other immune responses, and some antimicrobial agents. There are two ways this test can be performed: The slide test or the tube test. In both cases, the coagulase test is used to identify the presence of bound coagulase, which is attached to the cell walls of bacteria. Bound coagulase reacts with the fibrinogen in plasma, causing the fibrinogen to precipitate. This causes the cells to agglutinate, creating a “lumpy” look of a positive coagulase test.

The **oxidase test** identifies whether the organism has the enzyme cytochrome C oxidase in its electron transport chain. If the organism has the enzyme, it will oxidize the reagent that is impregnated on the oxidase strip. The reagent is tetramethyl-p-phenylenediamine (TMPD) and will turn blue/purple when oxidized. If the organism DOES NOT have the enzyme, the reagent will remain reduced and will not turn color. A result will occur and should be read within one minute. It is important to note that when performing this test, you MUST use a toothpick to ”grab” the organism. Using a loop can result in a false-positive.

Materials

- Slant cultures
    - *Micrococcus luteus*
    - *Staphylococcus aureus*
    - *Staphylococcus epidermidis*

- Three (3) plates per pair:
    - Mannitol Salt Agar
    - Blood Agar
- Rabbit plasma
- Oxidase test strips

Procedure

A. Catalase test

1. Perform the catalase test on each of the organisms.
2. Aseptically smear the organism on a glass slide.
3. Place a drop of 3% hydrogen peroxide on top of the smear.
4. Observe for bubbling immediately.

B. Blood agar

1. Streak a blood agar plate for isolation using *S. aureus*.
2. Repeat the procedure using *S. epidermidis*.
3. Repeat the procedure using *M. luteus*.
4. Incubate the first two plates at 37 °C for 48 hours.
5. Incubate the *Micrococcus* at 30 °C for 48 hours.

C. Mannitol salt agar

1. Streak a mannitol salt agar plate for isolation using *S. aureus*.
2. Repeat the procedure using *S. epidermidis*.
3. Repeat the procedure using *M. luteus*.
4. Incubate the first two plates at 37 °C for 48 hours.
5. Incubate the *Micrococcus* at 30 °C for 48 hours.

D. Coagulase test

1. Take a test tube and add 2 ml of rabbit plasma into the tube.
2. Label the test tube as *S. aureus.* Inoculate the test tube with *S. aureus*.
3. Repeat the procedure using *S. epidermidis*.
4. Incubate the tubes at 37 °C for 1 hour.

E. Oxidase Test

1. Inoculate an oxidase test strip with *S. aureus*.
2. Inoculate the other end of the strip with *M. luteus*.
3. Wait 60 seconds and record the results.

Other Selective and Differential Media III (Streptococci)

Introduction

Genus *Streptococcus* is characterized by Gram positive, facultative anaerobic, catalase **negative** bacteria. Staphylococci will occur in chains of three or more cocci. While there are members of genus *Streptococcus* that are part of the normal human microbiome, there are others that are pathogenic and can cause diseases such as pneumonia, pharyngitis, and endocarditis. There are a few ways to classify streptococci. Streptococci can be characterized based on their hemolytic reactions or using the Lancefield classification. The Lancefield classification is based on a specific carbohydrate found on the bacterial cell surface. There are currently 15 groups under this classification with any other streptococci falling under the category "non-Lancefield streptococcus".

The **bacitracin test** uses the antibiotic bacitracin to differentiate *Streptococcus pyogenes* (Group A streptococcus) from other beta-hemolytic streptococci. Since *Streptococcus pyogenes* is sensitive to bacitracin, there will be a zone of inhibition around the bacitracin disk that is placed on the agar plate.

The **CAMP test** is used to identify *Streptococcus agalactiae* (Group B streptococcus). *Streptococcus agalactiae* is known to cause neonatal meningitis, sepsis, and pneumonia in newborns. This organism can also be found to colonize the skin, intestine, and female reproductive tract of pregnant women and can be transmitted from mother to infant. Women who test positive for *S. agalactiae* are normally given prophylactic antibiotics either during labor or a few days before to prevent transmission of the organism. *Streptococcus agalactiae* produces a compound known as CAMP factor. While *S. agalactiae* is beta-hemolytic, it is not strongly beta-hemolytic. When inoculated close to *S. aureus*, an increased zone of hemolysis is produced between the two organisms, presenting itself as an arrowhead.

Group D streptococci originally included one genus- *Streptococcus*. They have since been reclassified into two groups- *Streptococcus* and *Enterococcus.* The remaining group D streptococcus is *Streptococcus bovis*. Both *Streptococcus bovis* and *Enterococcus faecalis* inhabit the gastrointestinal tract of humans and other animals. In order to differentiate between the different Group D streptococci, salt tolerance can be used. *Enterococcus* can grow in **6.5% NaCl** while *Streptococcus bovis* cannot. *Enterococcus* are also generally more resistant to penicillin compared to *Streptococcus bovis*.

Bile esculin agar is a selective and differential medium. It is used to differentiate between Group D streptococci and non-group D streptococci. The Group D strep have the ability to grow in the presence of bile, an emulsifying agent produced in the liver. They also have the ability to hydrolyze esculin. This hydrolysis of esculin turns the medium black and denotes a positive test.

Bile esculin agar comes in a slant form. It is 2 tests in one---resistance to 40% bile AND the use of the sugar esculin.

$$\text{Esculin} + H_2O \xrightarrow{Esculinase} \text{Esculetin} + \text{Dextrose}$$

$$\text{Esculetin} + \text{Ferric Citrate} \longrightarrow \text{Black precipitate}$$

Materials

- Slant cultures
  - *Enterococcus faecalis*
  - *Streptococcus agalactiae*
  - *Streptococcus bovis*
  - *Streptococcus mitis*
  - *Streptococcus pyogenes*

- Three (3) per pair:
  - Bile Esculin Agar slants
  - 6.5% sodium chloride broth

- Six (6) per pair:
  - Blood Agar plates

Media Preparation of 6.5% NaCl broth

1. Combine 3g of tryptic soy broth to 100 ml of water and add 6g of sodium chloride. Dispense 10 ml of the broth into test tubes.

Procedure

A. Hemolysis

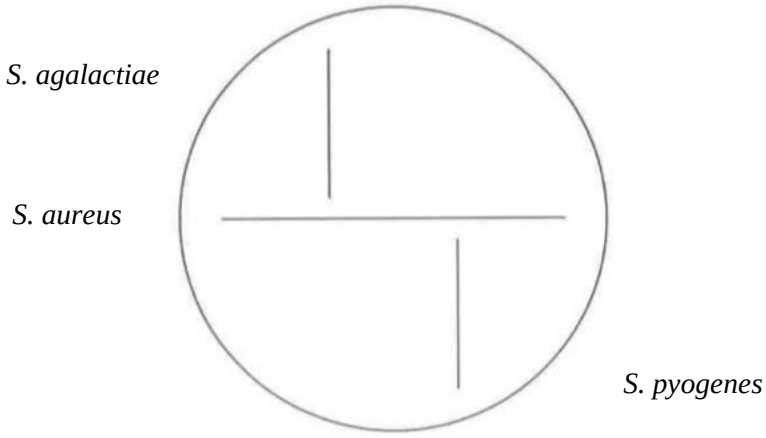
1. Streak for isolation on a blood agar plate with *E. faecalis.*
2. Make 3-4 stabs into the agar in quadrant 1 with a sterile loop. Then make 3-4 stabs into the agar in quadrant 4.
3. Streak for isolation on a blood agar plate with *S. bovis.*
4. Make 3-4 stabs into the agar in quadrant 1 with a sterile loop. Then make 3-4 stabs into the agar in quadrant 4.
5. Streak for isolation on a blood agar plate with *S. mitis.*
6. Make 3-4 stabs into the agar in quadrant 1 with a sterile loop. Then make 3-4 stabs into the agar in quadrant 4.
7. Incubate these plates at 37 °C for 48 hours.

B. Bacitracin Test

1. Streak for isolation on a blood agar plate with *S. pyogenes.*
2. Make 3-4 stabs into the agar in quadrant 1 with a sterile loop. Then make 3-4 stabs into the agar in quadrant 4.
3. Place a disk of bacitracin in the center of quadrant 1 of the plate.
4. Streak for isolation on a blood agar plate with *S. agalactiae.*
5. Make 3-4 stabs into the agar in quadrant 1 with a sterile loop. Then make 3-4 stabs into the agar in quadrant 4.
6. Place a disk of bacitracin in the center of quadrant 1 of the plate.
7. Incubate these plates at 37 °C for 48 hours.

C. CAMP Test

1. Streak *S. aureus* in a straight line in the center of a blood agar plate as shown below:



2. Streak *S. agalactiae* at a right angle to *S. aureus.* **BE CAREFUL NOT TO TOUCH *S. aureus.***
3. Streak *S. pyogenes* at a right angle to *S. aureus.* **BE CAREFUL NOT TO TOUCH *S. aureus.***
4. Incubate these plates at 37 °C for 48 hours.

D. Bile Esculin Hydrolysis

4. Inoculate a bile esculin agar slant with *E. faecalis*.
5. Inoculate a second bile esculin agar slant with *S. bovis*.
6. Inoculate a third bile esculin agar slant with *S. mitis*.
7. Incubate these plates at 37 °C for 48 hours.

E. 6.5% NaCl Tolerance

1. Inoculate a tube of 6.5% NaCl broth with *E. faecalis*.
2. Inoculate a second tube of 6.5% NaCl broth with *S. bovis*.

3. Inoculate a third tube of 6.5% NaCl broth with *S. mitis*
4. Incubate these plates at 37 °C for 48 hours.