Exercise 15-A
PHYSIOLOGICAL CHARACTERISTICS OF BACTERIA: CARBOHYDRATE METABOLISM, MR-VP, ESCULIN & STARCH HYDROLYSIS, CATALASE & OXIDASE TESTS

Special Note: During this portion of the laboratory, students will learn how to perform and interpret the results of a variety of enzymatic tests developed for determining specific physiological characteristics of bacteria. The results of these tests will be used to complete the identification of unknown cultures as described in the supplement (The Scientific Method and Identification of Unknown Organisms). All students are expected to gain a basic understanding of how these tests work, and are expected to be able to recognize both positive and negative test results regardless of the individual results they obtain. Therefore, it is **your** responsibility to observe different examples of data and results.

Introduction

The metabolism of microorganisms, like that of all organisms, is a complex of chemical reactions that are mediated by catalysts, **enzymes** and **ribozymes**. The metabolic activities of microbial cultures often create changes in their environments (media) that can be measured as an increase in end products, or as a disappearance of certain substances from the media. By conducting a variety of tests using specifically designed media, it is possible to demonstrate specific aspects of a culture's metabolism, and thereby determine a part of its enzymatic composition. The functional capabilities of organisms are called **physiological characteristics** and along with **morphological features** determine the **phenotype** (observed characteristics) of the organisms. This in turn provides some information about the **genotype** (genetic potential) of the culture. **Enzymatic testing** has been used extensively as a method for identifying and categorizing microorganisms as well as for determining the functional potential of the genes they carry (some of which have important applications outside cells). In this section students will be introduced to a variety of enzymatic tests, how these tests are designed to work, and some of the enzymes involved.

Carbohydrate Metabolism – Oxidation and/or Fermentation

Many microorganisms, like humans, are **chemoheterotrophs** and tend to utilize various carbohydrates (monosaccharides, disaccharides, and polysaccharides) as their primary source of energy and carbon. Some microorganisms can catabolize a wide variety of these substances, while others utilize only a few. The metabolic pathways used to catabolize carbohydrates may be categorized as either **respiratory** (**oxidative**) or **fermentative** depending upon the final electron acceptors involved, and these will influence the end products formed. Respiratory organisms often use molecular oxygen as a final electron acceptor, and form carbon dioxide and water as the end products of their metabolism, but they can also release organic acids, that can be mistaken for fermentation products. Fermentative organisms use organic compounds (pyruvate or acetaldehyde) as final electron acceptors, and the end products formed can vary considerably. Some of these are acidic, e.g., lactic acid, acetic acid, butyric acid, etc.; while others are neutral, e.g., acetone, acetoin (acetylmethylcarbinol), alcohols, etc.; and some are gases, e.g., methane (CH_4), carbon dioxide (CO_2), and hydrogen (H_2). The carbohydrates organisms can utilize, and more importantly the specific metabolic pathways used and the products formed, can be important factors involved in microbial identification.

Glucose Catabolism – The Oxidation/Fermentation or O/F test

Organisms that can ferment glucose often produce metabolic end products (**acids**) that will react with **pH indicators** in culture media to bring about color changes. Respiratory organisms such as *Pseudomonas* cannot ferment, but can utilize carbohydrates via respiratory pathways. These organisms can also produce acids when they are actively catabolizing carbohydrates (review glycolysis and the Krebs Cycle), and some of these may escape into the surrounding medium. Because the acids produced in association with oxidative metabolism (cellular respiration) sometimes cause false positive results in fermentation tests, a more sensitive means of determining metabolic activity is necessary. One example of a more sensitive test is the **oxidation/fermentation** or **O/F test**.

In the O/F test, two agar deeps containing glucose as a carbon source and **Bromothymol blue** as a pH indicator are inoculated with the same organisms. One of these tubes contains an inverted glass tube (**Durham tube**) and both tubes are sometimes heated prior to inoculation to drive off excess oxygen. After inoculation, one tube (the one without the Durham tube) is sealed with **vaspar** (a 50:50 mixture of vasoline and parafin) and both tubes are incubated for 48 hours. Organisms that are fermentative will produce **acids** causing the pH indicator to turn **yellow**, and this bright yellow color will be present in both tubes throughout the medium (inside and outside the Durham tube). Organisms that are using a respiratory metabolism will grow and produce acids only where oxygen is available to them. Yellow coloration will occur only near the surface of the medium in the unsealed tube, and no growth will occur in the sealed tube. The medium in the sealed tube (as well as the medium inside the Durham tube) will remain green (neutral). Some organisms can grow only under anaerobic conditions. These organisms will ferment and cause the medium in the sealed tube to turn yellow while that in the unsealed tube remains green (there may be yellow color inside the Durham tube). **Gas production** can be indicated by **bubbles** inside the Durham tube, or by elevation of the vaspar seal (gas can readily lift the vaspar seal, moving it up the tube away from the medium).

Note – Some types of respiratory organisms will produce **alkaline end products** in the O/F medium by breaking down the peptone present. These organisms will cause the pH indicator in the medium to turn **blue** at the surface of the unsealed tube.

Procedure:

1. Obtain two tubes of O/F medium (one with a Durham tube and one without), for each different culture to be tested. Carefully label both tubes to indicate the date, the culture being used, and your name. Keep O/F tubes in a vertical position as this medium has a low agar content.

2. Aseptically inoculate the medium in both tubes with the organisms to be tested (stab a loopful of culture to each tube bottom). Note that the Durham tube is bottom-end-up within the culture medium, and that to reach the tube bottom, you must stab your loop down beside it.

3. Seal one tube (the one without the Durham tube) by adding molten vaspar to a depth of about 5mm (1/4 inch). This seal will prevent the diffusion of oxygen into the medium. Alternatively, your instructor may ask that all O/F tubes be placed in the same basket, so that vaspar can be added to the entire class set.

4. Incubate both tubes at 37^{o} C for 48 hours, and then observe and record your **data** and **results**. Remember, **data are what you see** (colors and gas bubbles within tubes), and must be recorded. The results obtained (positive or negative for fermentation or for respiration, and **you must specify which**), are dependent upon your interpretation of the data.

Acid and Gas Production from Specific Carbohydrates in Agar Deeps

The formation of organic acids as end products of fermentation can readily be demonstrated by adding a pH indicator (phenol red or bromocresol purple) to the culture medium. Gas formation may be detected through the use of a vaspar seal on, or an inverted vial (Durham tube) within a broth medium. Gas production in agar media is accompanied by the formation of gas pockets that appear as bubbles or cracks in the agar, lifted agar, or may appear as a shiny surface along the stab line. Throughout this and later laboratories we will be determining the fermentation abilities of various microbes using media containing a carbohydrate (various specific ones will be used) in agar or broth with phenol red as the pH indicator. **Note** - Phenol red is red in neutral media, pink in alkaline media, and turns yellow in the presence of acids, thus indicating fermentation with acidic end products.

Procedure:

1. Each student should obtain one tube of each type of carbohydrate medium made available. **Please note** - All of the carbohydrate agar deeps look alike, and must be labeled as soon as they are taken from the individual baskets in order to prevent mix-ups. **Unidentified agar deeps are worthless and a waste of time and media.**

2. Inoculate each type of carbohydrate with the organisms you have been provided. To inoculate an agar deep, touch a **single colony** of the culture to be tested (or obtain a loopful of broth culture), and stab the loop to the **bottom** of the tube. Be sure to practice aseptic technique whenever handling tubes and Petri plates.

3. Label all tubes as indicated and incubate them at 37^{o} C for 24 hours. Note that the data obtained from carbohydrate deeps are most accurate if read after only 24 hours of incubation.

4. Examine all tubes for acid and gas production, and record your data and results. Compare your data and results with those obtained by other members of the class.

Glucose Catabolism – The Methyl Red - Voges-Proskauer (MR-VP) Test

The Methyl Red-Voges-Proskauer (MR-VP) test is actually two tests that can be performed on a single culture in MR-VP broth medium. The MR-VP medium is composed of 0.7% peptone, 0.5% dextrose (glucose) and 0.5% K_2HPO_4 in distilled water. The phosphate serves as a buffer and inhibits pH change unless the bacteria present produce a **large quantity of acid**. When the glucose in the MR-VP medium is catabolized by **heterofermentative** bacteria, e.g., *Escherichia coli,* there is a rapid production of lactic and acetic acids, ethanol, hydrogen, and carbon dioxide. This **mixed-acid fermentation** causes the medium to become acidic despite the buffer. Bacteria such as *Enterobacter aerogenes* form fermentation products similar to those of *E. coli*, but can also form **acetylmethylcarbinol** or **acetoin**, which is **neutral**. Bacteria with this capability are sometimes called **butanediol fermenters**, because butanediol is formed along with acetoin (the two are in equilibrium). Since some of the material used in the production of acetylmethylcarbinol are the same ones used by *E. coli* to form acids, MR-VP medium supporting the growth of *E. aerogenes* does not become as acidic as that inoculated with *E. coli*. When enough acid is produced, methyl red indicator added to the medium will stay red (**red = MR-positive**), but if acid levels are low, the methyl red indicator will turn yellow (**yellow = MR negative**). Most enteric bacteria that test MR-positive, will be negative for the VP test and vice versa; however, this is not always true, since *Klebsiella oxytoca* and *Serratia marcescens* will routinely test positive for both. Respiratory organisms, i.e., those not capable of fermentation, will test negative in both the MR and VP tests, and facultative anaerobes grown aerobically (on a shaker) will typically do the same.

Procedure:

1. Inoculate one tube of MR-VP medium (add a visible blob of cells) with each unknown culture you are required to test. Label your tube/tubes accurately, and incubate it/them at optimum temperature for at least 48 hours. After turbidity developes you will complete the Methyl Red and Voges-Proskauer tests as indicated below.

2. Completion of the **Voges-Proskauer Test**:

   Use a digital pipette (blue top) to transfer 1mL (1000μL) of your culture into a clean glass (screw top) tube. Test for the presence of acetylmethylcarbinol (acetoin) by adding to this tube 18 drops (about 0.5 mL) of Barritt's reagent A (5% alpha-naphthol in 95% ethanol) and 18 drops of Barritt's reagent B (40% potassium hydroxide). Secure the cap, and then shake the tube **vigorously** (or hold it on a vortex mixer) for one minute to thoroughly aerate the medium. Allow the tube to rest for 10 to 15 minutes and then shake it again. As an alternative, you may shake the tube every 20 seconds or so for the next ten minutes. The development of a pink to wine-red color indicates the presence of acetoin (acetylmethylcarbinol), and should be recorded as a positive result (VP-positive). If the mixture remains yellowish or tan, acetoin is not present and the result is VP-negative. **Note** - You may have to allow some tubes to stand for an hour or more to observe a positive test result, and this test works best if the Barrit's reagent A is fresh. Compare your data and results with those obtained by other members of the class.

   CAUTION: When aerating the MR-VP medium as described above, keep in mind that our plastic capped tubes do not seal, and that screw top tubes must be used.

3. Completion of the **Methyl Red Test**:

   Add several drops (5-8) of methyl red indicator to the culture medium remaining in your original MR-VP culture tube. If the pH indicator turns a red color, the organisms present have produced a considerable amount of acid and the result should be recorded as methyl red positive. If the indicator remains yellow or slightly orange, the culture is not producing enough acid to overcome the buffers in the medium, and the result is recorded as methyl red negative. Compare your data and results with those obtained by other members of the class.

   Note – It is possible to obtain accurate results for the Voges-Proskauer test with a culture that has been previously tested with methyl red indicator. It is not possible to obtain accurate results for a methyl red test with a culture containing Barritt's reagent B.

Esculin Hydrolysis

Esculin (aesculin) is a fluorescent, water-soluble glycoside that can be cleaved (hydrolyzed) to yield a sugar (glucose) and a non-sugar component (esculetin). Esculin can be added to culture media containing ferric citrate, and when hydrolyzed will release esculetin that combines with ferric ions to produce a dark brown or black complex readily visible within the medium.

The esculin hydrolysis test may be used in the identification of various pathogenic bacteria including Enterococci, Actinobacilli and members of the family Enterobacteriaceae. When bile salts are added, the medium is selective. *Enterococcus faecalis*, formerly classified within the genus *Streptococcus*, (*S. faecalis*) can hydrolyze esculin in the presence of bile salts, while many other streptococci cannot. Since this test is easy to perform and the results are readily interpreted, it is commonly used in diagnostic labs.

Note – You may find the esculin hydrolysis test useful in the identification of semester project organisms. In most instances, esculin media without bile salts is required.

Procedure:

1. Inoculate one slant of esculin agar with the culture you have been provided. (Organisms used commonly for demonstration purposes are *Enterococcus faecalis*, and *Streptococcus pyogenes*). Inoculate the esculin medium by streaking the culture over the slant surface and stabbing it to the tube bottom.

2. Label your tube accurately, and incubate it at 37^{o} C until the next laboratory period (tubes should be incubated for 24-48 hours).

3. Observe and record your data and results. The presence of a dark brown to black color within the esculin agar is indicative of hydrolysis and should be recorded as a positive test result. No color change indicates that your organisms cannot hydrolyze esculin.

Starch hydrolysis

Starch (amylose combined with amylopectin) is a polysaccharide made up of many glucose molecules. The starch hydrolysis test is used to determine the ability of organisms to hydrolyze starch through the production of **amylase enzymes**. This test is useful in the identification of a variety of microorganisms including species within the genera *Bacillus* and *Pseudomonas*. The presence or absence of starch can be indicated by the application of an iodine solution. Iodine will reach with starch to form a dark purple-black color. If starch has been hydrolyzed on a starch plate, this dark color will not form in the region behind colonies, and the agar will remain light. We will use Gram's iodine reagent to test for the disappearance of starch.

Procedure:

1. Inoculate a starch agar plate with the organisms to be tested. A typical streak pattern may be used, or you may choose to inoculate a single plate with more than one type of organisms (testing multiple cultures at the same time). Caution, starch agar will tear easily with wire loops.

2. Allow the cultures to incubate for 24-48 hours or more, depending on the growth time requirements of your organisms. Various temperatures may be optimal depending on the temperature requirements of your organisms.

3. When growth is clearly evident (colonies have formed) apply a few drops of iodine reagent (Gram's iodine) to the surface of the plate in a region where colonies have formed. Watch for a color change to dark purple-black. If the agar remains clear to yellowish under and around the colonies, the organisms can hydrolyze starch (starch hydrolysis is positive). If the agar under the colonies turns dark purple-black, they cannot hydrolyze starch (starch hydrolysis is negative). Record your data and results.

Catalase Activity test

Catalase enzymes are found in most species of bacteria. Their function is to catalyze the breakdown of hydrogen peroxide (a powerful oxidizing agent) with the release of water and free oxygen gas.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

In many cases oxygen gas can be readily observed as a white froth (bubbles) if a few drops of 3% **hydrogen peroxide** (H_2O_2) are added to a microbial colony on a plate or to a small piece of a colony placed on a clean glass slide. In the event of a questionable or apparently negative reaction, you can put a bit of culture on a slide, bring it into focus under the low-power (10X) objective of your microscope, and add hydrogen peroxide to the culture as you observe it under magnification. Most microbial cultures taken from ordinary media give strong, unmistakable reactions; however, catalase activity seems to be inhibited to some extent in strongly acidic media. Five important catalase-negative genera are *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, and *Clostridium*. Catalase-negative organisms tend to be anaerobic, but many of them are facultative anaerobes.

Since certain terminal respiratory enzymes react with atmospheric oxygen to form hydrogen peroxide, which is toxic to living cells, perhaps catalase enzymes are essential for the aerobic growth of most microorganisms. Catalase enzymes contain a porphyrin ring structure that is similar to that found in cytochromes (respiratory enzymes), and chlorophylls (photosynthetic pigments found in cyanobacteria, algae and green plants).

Procedure:

1. Remove a bit of growth from each of the cultures to be tested and place these on the surface of a clean glass slide. Do not mix the cells with water.

2. Add a drop of 3% hydrogen peroxide (H_2O_2) to each mass of cells on the slide and observe the presence or absence of catalase activity (appearance of bubbles). **Note** - Cultures that are catalase-positive typically bubble so vigorously that their cells are dispersed into the drop of reagent, and no cell mass remains. Record your data and results.

Oxidase test

The oxidase test is used to determine if or not bacteria can produce **cytochrome c**, an enzyme involved in oxidative phosphorylation (part of the electron transport chain); however, the reagent used in this lab, *N,N,N′,N′*-tetramethyl-*p*-phenylene-diamine, actually indicates the presence of **cytochrome c oxidase** or **indophenol oxidase** enzymes. These are large, transmembrane protein complexes that normally transfer electrons from cytochrome c to molecular oxygen (O_2) resulting in the formation of water. They can also transfer electrons to the test reagent, turning the colorless reagent (oxidized state) to purple (reduced state). The oxidase test is useful in distinguishing the enterobacteriaceae from aerobic Gram-negative bacilli such as *Pseudomonas* and *Alcaligenes*. Portions of oxidase-positive colonies brought into contact with the oxidase test reagent will turn purple usually within a minute or two. Oxidase-negative colonies do not change color. For best results, the oxidase test should be performed on cultures that are actively growing (not several weeks old), and maintained at room temperature.

Procedure:

1. Using the large end of a **clean toothpick**, transfer a fair sized mass of the organisms being tested onto the test surface (filter paper treated with oxidase test reagent, or an oxidase test strip) and rub this into a **small** area to make contact with the test chemical.

2. Watch for a color change on the paper surface and on the toothpick. Organisms that are capable of forming cytochrome C will turn dark purple within a few minutes, while organisms that are not will remain their original color.

3. Record your data and results. **Note** – Culture samples taken from MAC or EMB plates will turn pink on the oxidase test paper, but this should not be recorded as a positive result as the color is associated with components of the media (neutral red and eosine).

   Note - It is not advisable to use a wire loop for the transfer of colonies being tested for the presence of oxidase enzymes, as these loops will sometimes yield false-positive results.

Students who have successfully isolated colonies of *Pseudomonas* and/or *Azotobacter* from soil may wish to test these cultures for the presence of oxidase enzymes. Both of these genera contain respiratory organisms that usually test oxidase-positive.

Note - The media and materials used in the identification of physiological characteristics are expensive, as is their preparation. Please be conservative and efficient in your use of these materials. Thank you!

Questions:

1. How would you interpret the results of an O/F test if the sealed tube showed no change in color and the unsealed tube was yellow only near the surface of the medium and not within the Durham tube?

2. How can you determine if or not organisms are able to ferment a specific carbohydrate with the production of acid and gas in a tube of solid media containing phenol red as the pH indicator?

3. How would you determine that gas production had taken place if you were using a broth medium?

4. What carbohydrate fermentation test could you use to test for the formation of acetylmethylcarbinol or acetoin?

5. What is esculin and what happens to a slant of esculin agar if the organisms growing there can hydrolyze esculin?

6. Describe the reaction you observed when you subjected your culture to 3% hydrogen peroxide. Is *Staphylococcus aureus* catalase-positive or catalase-negative?

7. What is being tested for in the oxidase test? Is *Escherichia coli* oxidase-positive or oxidase-negative? How do you know?

NOTES, OBSERVATIONS & ADDITIONAL INFORMATION

STUDY GUIDE
Exercise 15A
CARBOHYDRATE METABOLISM, (O/F, MR-VP, CARBOHYDRATE DEEPS)
ESCULIN HYDROLYSIS, CATALASE & OXIDASE TESTS

Materials, Methods & Data:

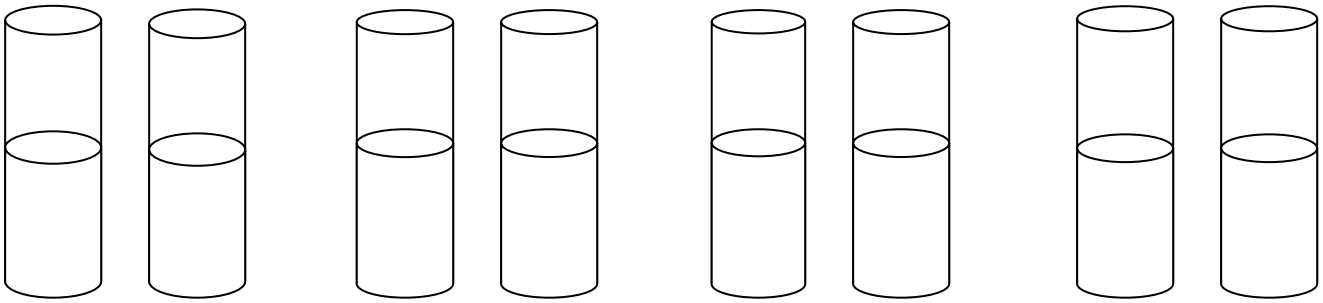
Oxidation/Fermentation test **Date** _____

Carbohydrate present _____ pH indicator present _____

Color variation expected - Neutral = ☐ Acid = ☐ Alkaline = ☐

Gas production can be indicated in two different ways within O/F tubes, what are they?

_____



Initial color Respiratory Ferments with gas Ferments w/out gas

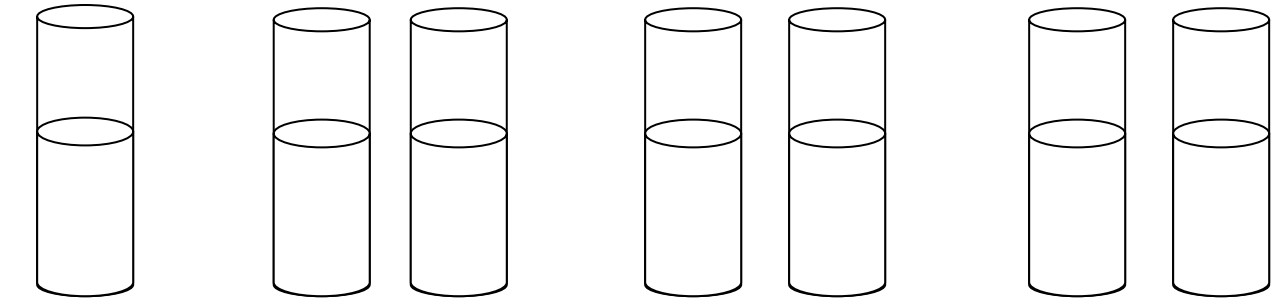
Methyl-Red, Voges-Proskauer Test **Date** _____

Carbohydrate present _____ pH indicator in MR _____

Color variation expected - Neutral = ☐ Acid = ☐ Alkaline = ☐

Reagents in VP _____ Action required? _____

End product tested for? _____ Positive = ☐ Negative = ☐



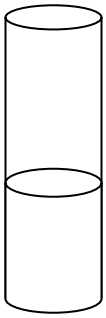
Initial color MR (+) VP (–) MR (–) VP (+) MR (–) VP (–)
No rxn Mixed acid fermenter Butanediol fermenter Non-fermenter

Remember – What is being fermented is glucose, not mixed acids or butanediol, and in the VP test we are testing for the presence of acetoin (acetylmethylcarbinol), and not butanediol.

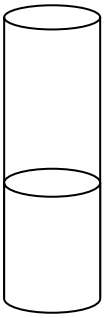
Carbohydrate deeps: **Date** _____

End products tested for? _____

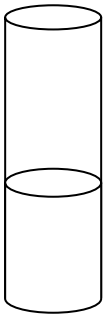
pH indicator present _____ Neutral = ☐ Acid = ☐ Alkaline = ☐



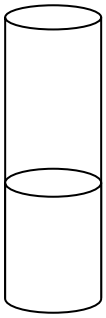
Initial color



Forms acid and gas



Forms acid no gas



Cannot ferment

Catalase test: **Date** _____

Reagent used _____ Indicate possible reactions below
_____ = Has catalase enzyme _____ = Does not have catalase

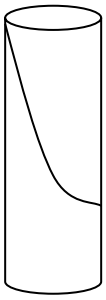
Oxidase test: **Date** _____

Reagent used _____ Testing for? _____

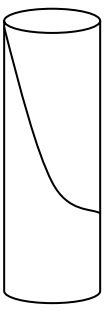
Possible reactions as observed on filter paper wetted previously with appropriate reagent.
_____ = Organisms have enzyme in their ETC (+)
_____ = Organisms do not have enzyme in their ETC (–)

Esculin Hydrolysis (using Bile Esculin Agar or Esculin Agar without Bile Salts):

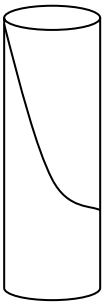
The medium used in this test is called _____ agar.
When bile salts are present, what type of organism is the medium selective for? _____
_____



Initial color



Esculin (+)



Esculin (–)