

Exercise 12

Selected Biochemical Reactions Used to Identify the Physiological Characteristics of Bacteria (with special reference to Gram Negative Enteric Rods)

INTRODUCTION:

Student Learning Objectives: After completing this exercise students will:

- a. Demonstrate the ability to recognize physiological differences between different bacterial species.
- b. Describe and define the principles of the biochemical tests used to identify bacteria based on their enzymatic properties and their byproducts.
- c. Set up a battery of tests to identify specific Gram Negative Enteric Rods.

Activities:

- Perform the Oxidase test.
- Inoculation of carbohydrate fermentation tubes (Glucose, Lactose, Sucrose).
- Inoculation of TSI agar.
- Inoculation of Urea broth.
- Inoculation of Simmons citrate agar.
- Inoculation of SIM agar.
- Inoculation of MR-VP medium for the Methyl Red and Voges-Proskauer tests.
- Inoculation of Phenylalanine Agar.
- Inoculation of Moeller Lysine Decarboxylase Broth.
- Inoculation of MIO (Motility, Indole, Ornithine) deeps.
- Inoculation of Starch agar plate.
- Inoculation of DNase agar plate.
- Inoculation of Gelatin agar.

Materials

- Work in groups of 4 per table. This is a group activity.

<p>The following agar/broth media:</p> <p>Fermentation tubes:</p> <p>Glucose broth w/ Durham tube</p> <p>Lactose broth w/ Durham tube</p> <p>Sucrose broth w/ Durham tube</p> <p>TSI agar slants</p> <p>Urea broth</p> <p>Simmons Citrate agar slants</p> <p>SIM agar tubes</p> <p>MR-VP broth tubes</p> <p>PAD agar slants</p> <p>Lysine Decarboxylase broth (and base)</p> <p>Optional</p> <p>(Arginine Decarboxylase broth)</p> <p>(Ornithine Decarboxylase broth)</p> <p>MIO agar tubes</p> <p>Starch agar plate</p> <p>DNase agar plate</p> <p>Gelatin agar tubes</p> <p>Oxidase strips</p>	<p>Slant cultures of the following bacteria:</p> <p><i>Alcaligenes faecalis</i></p> <p><i>E. coli</i></p> <p><i>Salmonella</i></p> <p><i>Proteus vulgaris</i></p> <p><i>Enterobacter cloacae</i></p> <p><i>Pseudomonas aeruginosa</i></p> <p><i>Klebsiella pneumoniae</i></p> <p><i>Citrobacter freundii</i></p> <p><i>Serratia marcescens</i></p> <p><i>Morganella morganii</i></p> <p><i>Bacillus subtilis</i>.</p>
	<p>Iodine solution</p> <p>10% Ferric Chloride</p> <p>Kovac's Reagent</p> <p>Barrit's reagent A and B</p> <p>Sterile toothpicks</p> <p>Inoculating loop and needle</p> <p>Tube rack</p> <p>Microbiology Atlas</p> <p>Sterile mineral oil</p> <p>Sterile cotton swabs</p>

Introduction:

Many bacterial species, unlike mammalian and plant species, can share the same morphology (rods, cocci, spirilli), so in other words can look the same under the microscope. How do you differentiate between the species, especially if they are morphologically similar? Bacteria are placed in different families based on Gram reaction, and other biochemical reactions that they carry out utilizing specific substrates in the media they grow on. Classification at the species level takes into consideration more of these biochemical reactions, based on enzymes and byproducts of these reactions. Examples of these enzymes are urease, decarboxylase, citrase, deaminase, reductase, gelatinase, DNase, tryptophanase, and desulfurase. Sugar fermentation into acids and alcohols are also considered among these reactions.

In this exercise, you will demonstrate a positive and a negative reaction to several biochemical tests used in the identification of bacteria. While no single test can identify a particular species, many tests are utilized to do so. This referred to as a Test Battery, which you will use to identify your unknown bacteria in this lab.

Make sure you READ and UNDERSTAND the methods of inoculation and incubation, and the principle of each test. Read the material in your Atlas and look for the photos of both positive and negative reactions, and always know what the uninoculated media looks like without any growth. Use coloring pencils to draw the reaction color changes.

1. Oxidase Test

The purpose of this test is to determine the presence of the oxidase enzymes. This test was originally devised to identify all *Neisseria* species, but later came into use to separate the *Pseudomonas*, a Gram negative rod, from the oxidase-negative Enterobacteriaceae.

The oxidase test is based on the bacterial production of an intracellular oxidase enzyme. This oxidase reaction is due to the presence of a cytochrome oxidase system, which activates the oxidation of reduced cytochrome by molecular oxygen, which in turn acts as an electron transfer system. In the oxidase test, cytochrome oxidase produced by the microorganism does not directly oxidize the p-phenylenediamine reagent, but rather oxidizes cytochrome c, which in turn oxidizes the reagent to form a purple-colored compound. In essence, the oxidase test determines the presence or absence of cytochrome c.

Lab Procedure:

1. You will use oxidase paper strips for this test unless indicated otherwise.
2. Using a sterile wooden tooth pick, pick a portion of the colony to be tested and rub directly onto a portion of a reaction area of the oxidase strip. Up to three tests can be accommodated. Use *Pseudomonas aeruginosa* and *E. coli* as test organisms for this exercise.
3. You may need to add a very small drop of water if the colony is too dry.
4. Examine the reaction area for the appearance of a dark purple color within 20 – 30 seconds. NOTE: Color development after 30 seconds should be disregarded.
5. Record your results below.

Oxidase-positive colonies => colony becomes dark purple.

Oxidase-negative colonies => no color change in colonies or a light pink color imparted due to the reagent.

Species	Oxidase +/-	Enteric or Non-enteric
<i>Pseudomonas aeruginosa</i>		
<i>E. coli</i>		

Precautions

The use of a platinum inoculating loop or needle or wooden toothpick to remove colonies for oxidase testing is recommended, because the presence of any trace of iron (nichrome) can catalyze the oxidation of the phenylenediamine reagent, resulting in a false positive reaction.

- Weak oxidase producers may appear negative within the time limits of the test.
- Mucoid *Pseudomonas aeruginosa* may require longer than 10-20 seconds to become positive.
- Oxidase reactions of gram-negative bacilli should be determined on colonies obtained from nonselective and non-differential media to ensure valid results.
- Media with bile (like MacConkeys) will inhibit oxidase. Use BAP or Chocolate Agar.
- Needs 24 to 48 hr. fresh oxidase enzyme. Oxidase negative will become oxidase positive with time.

2. Carbohydrate fermentation tests

In this exercise, you will be using a fermentation tube test which contains a carbohydrate (Glucose, Lactose, or Sucrose in this case), a phenol red broth (pH indicator and nutrient broth medium), and an inverted small Durham tube to collect the gas if it is produced as a result of fermentation.

Lab Procedure:

Label four sets of tubes (Each set has Glu, Lac, Suc) with the corresponding species and pertinent information; *E. coli*, *P. vulgaris*, *E. cloacae*, and *P. aeruginosa*. Inoculate them accordingly, and incubate 24-48 hours at 37 C. Read the results and record.

Positive fermentation reaction => Yellow (acid production)

Positive fermentation reaction + Gas => Yellow and bubble in Durham tube

Negative reaction => Red with Growth but no change (No acid production)

Purpose:

Principle:

Results:

Conclusion:

Draw your tubes below with the reactions

3. Triple Sugar Iron (TSI) test

In this exercise, you will be using this medium to determine the ability of an organism to break down a specific carbohydrate (glucose, lactose, or sucrose), with or without the production of gas, along with the determination of possible hydrogen sulfide.

Lab Procedure:

Label 5 TSI slants with the following species: *Alcaligenes faecalis*, *Morganella morganii*, *Salmonella typhimurium*, *E. coli*, and *Citrobacter freundii*.

Inoculate this slant agar medium with the inoculating needle; aseptically, pick up a colony with the tip of the needle and stab the medium all the way down to the bottom of the tube (butt). Then, withdraw the needle from the butt and streak the slant surface (fishtail). Incubate 24- 48 hours at 37 C. Observe for changes in color in the regions of the slant and the butt. Results are displayed as Slant/Butt, with the letter A for acid (Yellow color), and K for alkaline. Hydrogen sulfide production is indicated by a black color in the medium, and gas production shows as cracks and bubbles in the medium.

Interpretation of results:

Red slant/Red butt => No carbohydrate fermentation, no hydrogen sulfide production.

Red slant/Yellow butt => Glucose fermentation only, no H₂S production.

Red slant/Yellow butt with Black color => Glucose fermentation and H₂S production.

Yellow slant/Yellow butt => Glucose, Lactose and/or Sucrose fermentation only.

Yellow slant/Yellow butt with Black color => Glucose, Lactose and/or Sucrose fermentation, with H₂S production.

Purpose:

Principle:

Results: Draw slants below indicating color of medium and display results A,K,H₂S, gas.

4. Starch hydrolysis by amylase

In this exercise, you will be using a Starch agar plate to demonstrate the production of the enzyme amylase by specific bacteria. Amylase is the enzyme used to break down starch which is a polymer (polysaccharide) into disaccharides (and eventually monosaccharides by maltase) for absorption and use inside the cells for energy. The presence of starch is detected by adding iodine solution to the medium where the bacteria are growing; if the medium turns a purplish-blueish color, the starch has not been broken down. If a clear zone appears around the colonies, then that indicates the starch has been changed into simpler sugars, indicating the production of amylase.

Lab Procedure:

On the back of a Starch agar plate, draw a line with your marker bisecting the plate and label each half with *E. coli* and *B. subtilis*. Make a single streak of each in its respective half, as shown in the diagram below. Incubate 24- 48 hours at 37 C. Apply a few drops of iodine to cover the plate surface with a thin layer. Observe for color.

Positive reaction => Clear zone around the colonies = amylase production

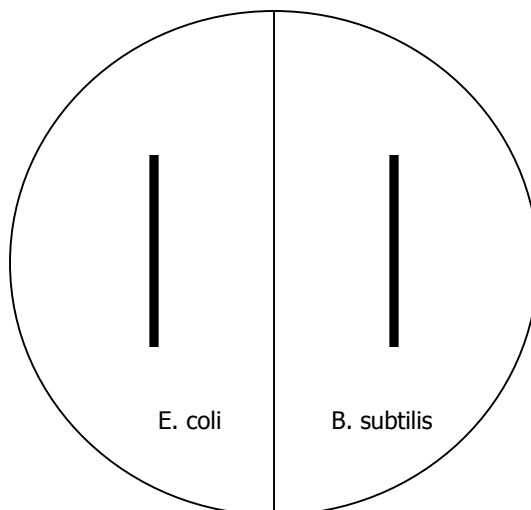
Negative reaction => Medium purplish-blueish uniformly = No amylase production

Purpose:

Principle:

Results:

Conclusion:



5. DNase production

In this exercise, you will be using a DNase agar plate to demonstrate the production of the enzyme deoxyribonuclease by specific bacteria. DNase is the enzyme used to break down DNA which is a polymer. Methyl green, which is incorporated into the medium, complexes with DNA producing a greenish color. Organisms producing DNase cause the breakdown of the DNA polymer, resulting in a clear zone around these colonies.

Lab Procedure:

On the back of a DNase agar plate, draw a line with your marker bisecting the plate and label each half with *S. marcescens* and *E. coli*. Make a single streak of each in its respective half, as shown in the diagram below. Incubate 24- 48 hours at 37 C. Observe for a clear zone around the colonies.

Positive reaction => Clear zone around the colonies = DNase production

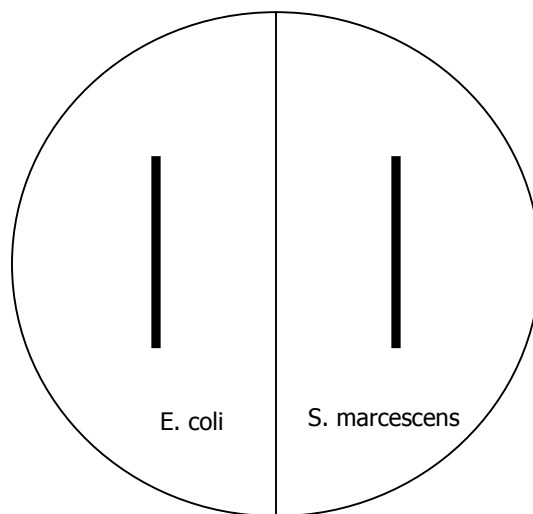
Negative reaction => Medium is uniformly greenish = No DNase production

Purpose:

Principle:

Results:

Conclusion:



6. Simmons Citrate Test

In this exercise, you will be using Simmons Citrate medium to determine if the bacterium utilizes citrate as a sole source of carbon, using the enzyme citrase. If the enzyme breaks down citrate, the resulting end-products cause the medium to change color from green to blue due to the pH indicator. If no citrase is produced, the medium remains green.

Lab Procedure:

Label two citrate slants and inoculate them with *E. coli* and *Enterobacter cloacae*. This is done using the loop to pick up a colony and streak it on the surface of the slant by fishtailing the loop on the surface several times. Incubate 24- 48 hours at 37 C. Observe for a change in the color of the medium. Draw your results below in color.

Positive reaction => Blue

Negative reaction => Green

Purpose:

Principle:

Results:

Conclusion:

7. Phenylalanine Deaminase Test

In this exercise, you will be using PAD medium to determine if the bacterium produces the enzyme phenylalanine deaminase. If the enzyme breaks down phenylalanine, the resulting end-products cause the medium to change color to a deep green after a 10% solution of ferric chloride is added. No change in color is observed if the enzyme is not produced.

Lab Procedure:

Label two PAD slants and inoculate them with *E. coli* and *P. vulgaris*. This is done using the loop to pick up a colony and streak it on the surface of the slant by fishtailing the loop on the surface several times. Incubate 24- 48 hours at 37 C. After incubation, add 4 – 5 drops of 10% ferric chloride solution and allow this to react and read within 5 minutes, since the positive green color can fade away if read later. Observe for a change in the color of the medium. Draw your results below in color.

Positive reaction => Green (Deep green)

Negative reaction => No color change (yellowish color)

Purpose:

Principle:

Results:

Conclusion:

8. Gelatinase (Proteinases) Test

In this exercise, you will be using gelatin medium to determine if the bacterium produces gelatinases. If the enzyme breaks down gelatin, the medium will be liquified. Otherwise, the medium will remain semi-solid. However, it is important to chill the medium in the refrigerator after incubation and before reading the results.

Lab Procedure:

Label two gelatin agar tubes and inoculate them with *E. coli* and *P. aeruginosa*. This is done using the needle to pick up a colony and carefully without wiggling the needle, stab straight down once or twice without disturbing or mixing the medium. Incubate 48 hours or more at 37 C. Remove from the incubator and chill in the refrigerator for 30 minutes. Observe for a change in the consistency of the medium. Draw your results below.

Positive reaction => Medium is liquified due to the presence of gelatinases.

Negative reaction => Medium is semi-solid.

Purpose:

Principle:

Results:

Conclusion:

9. Motility Indole Ornithine (MIO) Tests

In this exercise, you will be using MIO medium. This medium can be used to perform three tests at the same time: motility, the production of indole, and the decarboxylation of ornithine to aid in the identification of the Enterobacteriaceae.

Motility can be detected by looking at the stab line. Motile organisms move away from the stab line producing turbidity throughout the medium. Non-motile organisms grow only where they were inoculated in the stab line; the medium appears clear away from the stabline.

Indole is produced by degrading typtophan contained in the medium. Indole can be detected by the addition of Kovacs reagent. Indole combines with the Kovacs reagent(p-dimethylaminobenzaldehyde) and produces a red or pink color on top of the agar. If the organism does not produce indole the color will not be observed.

The ornithine decarboxylation test detects the enzymatic ability of an organism to decarboxylate the amino acid ornithine to form an amine with resulting alkalinity. Ornithine and glucose are added to the decarboxylase base. During the initial stages of incubation, the tube turns yellow, owing to the fermentation of the small amount of glucose in the medium. If the amino acid is decarboxylated, alkaline amines are formed and the medium reverts to the original purple color.

Lab Procedure:

Label 4 MIO agar tubes and inoculate them with *Morganella morganii* (or *Edwardsiella tarda*), *P. vulgaris*, *E. cloacae*, and *K. pneumoniae*. This is done using the **needle** to pick up a colony and carefully without wiggling the needle, stab straight down ONLY once without disturbing or mixing the medium. Incubate 24- 48 hours 37 C.

Read the results in the following order:

1. Motility
2. Ornithine
3. Add 5 drops Kovac's reagent and read within minutes.

Don't read motility and ornithine after the addition of Kovac's reagent.

Purpose:

Principle:

Results:

Conclusion:

10. Sulfide Indole Motility (SIM) Tests

In this exercise, you will be using SIM medium. This medium can be used to perform three tests at the same time: Sulfide production, motility, and the production of indole, to aid in the identification of the Enterobacteriaceae.

Hydrogen sulfide is produced as an end-product of metabolism of cysteine or thiosulfate. If hydrogen sulfide is produced, the medium turns black in color.

Indole is produced by degrading typtophan contained in the medium. Indole can be detected by the addition of Kovacs reagent. Indole combines with the Kovacs reagent(p-dimethylaminobenzaldehyde) and produces a red or pink color on top of the agar. If the organism does not produce indole the color will not be observed.

Motility can be detected by looking at the stab line. Motile organisms move away from the stab line producing turbidity throughout the medium. Non-motile organisms grow only where they were inoculated in the stab line; the medium appears clear away from the stabline.

Lab Procedure:

Label 3 SIM agar tubes and inoculate them with *P. vulgaris*, *E. coli*, and *Salmonella typhimurium*. This is done using the **needle** to pick up a colony and carefully without wiggling the needle, stab straight down ONLY once without disturbing or mixing the medium. Incubate 24- 48 hours 37 C. Draw the results below using color pencils.

Read the results in the following order:

1. Motility
2. Hydrogen sulfide
3. Add 5 drops Kovac's reagent and read within minutes.

Purpose:

Principle:

Results:

Conclusion:

11. Urea Hydrolysis (Urease) test

In this exercise, you will be using urea broth determine if the bacterium produces urease. If the enzyme breaks down urea, the medium will turn pink in color due to the alkaline end-products. Otherwise, the medium will remain alight orange color. The importance of this test is to differentiate *Proteus species*, non-lactose fermenters, from enteric pathogens (such as Salmonella or Shigella) which are also non-lactose fermenters.

Lab Procedure:

Label two urea broth tubes and inoculate them with *E. coli* and *P. vulgaris*. This is done using the loop with a heavy inoculum. Incubate 24 hours at 37 C. Observe for a change in the color of the medium. Draw your results below using color pencils.

Positive urease test (urea hydrolysis) => Medium color is pink.

Negative urease test (no urea hydrolysis) => no change in medium color.

Purpose:

Principle:

Results:

Conclusion:

12. Decarboxylase Tests (Lysine-Arginine-Ornithine)

The decarboxylase tests are used primarily to determine bacterial groups among the Enterobacteriaceae by measuring the enzymatic ability of an organism to decarboxylate an amino acid to form an amine with resulting alkalinity.

Decarboxylation is the process whereby bacteria that possess specific decarboxylase enzymes are capable of attacking amino acids at their carboxyl end (-COOH) yielding an amine and carbon dioxide. The decarboxylase enzymes are numerous and each is specific for a given substrate.

Moeller decarboxylase medium is the base used. The amino acid to be tested is added to the decarboxylase base. A control tube consisting of only the base without amino acid, must also be set up in parallel. Both tubes are anaerobically incubated by overlaying with sterile mineral oil. During the initial stages of incubation, both tubes turn yellow, owing to the fermentation of the small amount of glucose in the medium. If the amino acid is decarboxylated, alkaline amines are formed and the medium reverts to the original purple color. Non-fermenters do not produce the initial yellow color change and use of the amino acid containing tube becomes a deeper purple than the control.

Lab Procedure:

The test organisms to be used with the lysine decarboxylase test are *Klebsiella pneumoniae* and *Enterobacter cloacae*.

From a well-isolated colony of the test organism, inoculate two tubes of Moeller decarboxylase medium, one containing the amino acid to be tested, the other a control tube containing only base. Overlay both tubes with sterile mineral oil to cover about 1 cm of the surface and incubate at 37°C for 18 to 24 hours.

Positive test: turbid purple to a faded out yellow-purple color. Indicates the release of amines from the decarboxylase reaction.

Negative test: bright, clear, yellow color (only glucose fermented).

Control tube (no amino acid substrate): remains yellow (only glucose fermented). Indicates organism is viable and that the pH of the medium has been lowered sufficiently to activate the decarboxylase enzymes.

Precautions

Since decarboxylase media contain peptones, it is necessary to exclude air by sealing to prevent a false positive alkaline reaction due to oxidation and deamination of peptones which starts at the surface of the media. If tubes are not sealed, reactions are not valid after a 24 hour incubation and the control tube may also be alkaline making test results invalid and impossible to interpret.

Results

Organism	Lysine	Arginine	Ornithine	Base
<i>Klebsiella pneumoniae</i>				
<i>Enterobacter cloacae</i>				

13. Methyl Red – Voges Proskauer tests

In this exercise, you will be using the MR-VP medium to perform two tests at the same time, but in different tubes. The methyl red test is used to characterize enteric bacteria based on the pathways of glucose metabolism; some enterics use the mixed acid pathway, and are called MR positive (*E. coli*, *P. vulgaris*), while other enterics use the butylene glycol pathway, and are called MR negative (*Enterobacter cloacae*). The VP test is also used to characterize enteric bacteria based on the pathways of glucose metabolism, but addresses the production of neutral end-products, such as acetoin. This test distinguishes between acetoin-producers such as *Enterobacter cloacae*, and non-acetoin producers such as *E. coli*.

Lab Procedure:

Label two MR-VP broth tubes and inoculate them with *E. coli*. Label a second set of tubes with *E. cloacae*. This is done using the loop with a heavy inoculum. (You may use only one tube per species, but you will have to split the amounts for the tests using a sterile tube.) Incubate 48 hours at 37 C. Then perform the tests below.

Methyl Red test: Add 3-5 drops of methyl red to one tube.

Positive MR test: color of medium is red (acid)

Negative MR test: color of medium is yellow (neutral)

Voges-Proskauer test: Done on 2.5 ml amounts of the incubated medium. Add 6 drops of Barritt's Reagent A (alpha-naphthol) and 2 drops of Barritt's Reagent B (KOH). Mix gently and allow to sit for about 10 – 15 minutes.

Positive VP test: color of medium is red (acetoin produced)

Negative VP test: color of medium is copper (acetoin not produced)

Results		
Organism	Methyl Red	Voges-Proskauer
<i>Escherichia coli</i>		
<i>Enterobacter cloacae</i>		