Experiment # 20 - Title: **Biochemicals II: Carbohydrate Fermentation (Phenol Red broth), Indole Production, Methyl Red, Voges/Proskauer, Citrate, Hydrogen Sulfide, Urease Tests***

Learning Outcomes:

a) Students will demonstrate mastery of biochemical techniques.

b) Students will be able to apply their knowledge of and interpret biochemical test results in order to identify unknown bacterial species, given a dichotomous key.

Learning Objectives: After completing this experiment, you should understand:

i) The characteristics / features and activities of endoenzymes.

ii) Experimental procedures for differentiation of intestinal (enteric) bacteria.

iii) Biochemical test procedures for identification of bacteria.

Introduction / theory/overview: Finding the nutritional and metabolic capabilities of bacteria is the most widely used technique to study bacteria. Conventional methods are based on a combination of biochemical tests and selective and/or differential media to establish (i) presence or absence of enzymes (e.g., catalase, coagulase, nitrate reductase, urease, indole etc.), (ii) ability to use single substrate (citrate) and grow or survive in presence of certain inhibitors (selective media) and (iii) carbohydrate fermentation (phenol red broth, MR test, VP test). These tests are used to separate and identify microorganisms which can be used for many applications including:

a. Ascertain the pathogenic microbe responsible for an infectious disease.
b. Selection and isolation of strains of fermentative microbes needed for industrial production of enzymes, antibiotics, alcohols, solvents, organic acids, vitamins and certain foods such as yogurt, cheeses and other milk products.
c. Comparison of biochemical activities for classification / taxonomic purposes.

In order to accomplish the above applications, microbiologists exploit the fact that microbes have their own unique identifying biochemical characteristics (kind of like a specific set of fingerprints found in humans). The "biochemical fingerprints" of a microbe are determined by its unique genetic program that controls the cell's enzymatic activity for bioenergetics, biosynthesis, and biodegradation.

The *Enterobacteriaceae* are a large diverse family of gram-negative rods that inhabit the human intestinal tract. Most enteric species have the following characteristics: growth on MacConkey agar, gram-negative rods, facultative anaerobes, oxidase (-), acid production from glucose with or without gas, catalase (+), most reduce NO_3 to NO_2, and peritrichous flagella if motile. The *IMViC* series of tests (*I*ndole, *M*ethyl red, *V*oges-Proskauer, and *C*itrate) are used to distinguish between members of the family *Enterobacteriaceae* and differentiate them from other Gram (-) rods.

***References for all biochemical tests came from:**

1. Cappuccino, J.G. & Sherman, N. Microbiology: A Laboratory Manual, 8th ed., Pearson Ed. Inc., 2008.

2. Leboffe, M.J. & Pierce, B.E. Microbiology: Laboratory Theory & Application, 2nd ed., Morton Publishing Company, 2012.

20a. Phenol Red Broth Carbohydrate Fermentation (Glucose, Lactose, and Mannitol Broth)

Learning Objectives: After completing the experiment, you will be able to:

1. Determine if a bacterial species has the capability of degrading and fermenting a carbohydrate with the production of acid and gas.
2. Comprehend the difference between cellular respiration and fermentation.

Introduction / theory / overview:
Phenol Red broth (PR) is a differential test medium containing one carbohydrate (glucose, lactose or mannitol), peptone and the pH indicator phenol red. PR is yellow below pH 6.8, pink to magenta above pH 7.4, and red in between. If a species can ferment the sugar, then acids will be produced lowering the pH below the neutral range, giving you a yellow color. This is codified as a positive result by the symbol "A" standing for "acid production". Some species cannot ferment the carbohydrate but can use the peptone. As they do this, they will deaminate the amino acids in the peptone, which produces ammonia (NH_3), raising the pH above 7.4. The pH indicator will turn PR pink. This is codified as "K" standing for the letter k in the word alkaline and represents a negative result. Any red non-fermented broth is codified as a negative sign (-).

There is also a tiny inverted glass tube called a Durham tube inside of each PR broth tube. It is there to capture gas, evidenced by a gas bubble in the upper end of the tube. A positive gas result must be a bubble of at least 10% volume of the Durham tube and is codified as a "G" standing for "gas production". Negative gas production is codified as (-). Sample complete codes for a given species are: A/G, A/-, -/-, k/-. See figure below for examples.

Materials, equipment and organisms (per student/ per group): **Work in groups of 3 students.**
List of organisms: *E. coli*, *M. luteus*, *P. vulgaris*, *S. aureus*
Materials / Reagents Needed: Four each of glucose, lactose and mannitol carbohydrate fermentation tubes, test tube rack, four sterile cotton-tipped applicators.

Procedure:
i. Tap mix each bacterial broth.
ii. Using one cotton-tipped applicator per species, inoculate all three different PR broths.
iii. Change cotton-tipped applicator for different species only.
iv. Incubate at 37°C for 1-2 days.

Safety and disposal: Dispose used cotton-tipped applicators in the red bio-hazard bags and throw away the wrapper in the regular trash.

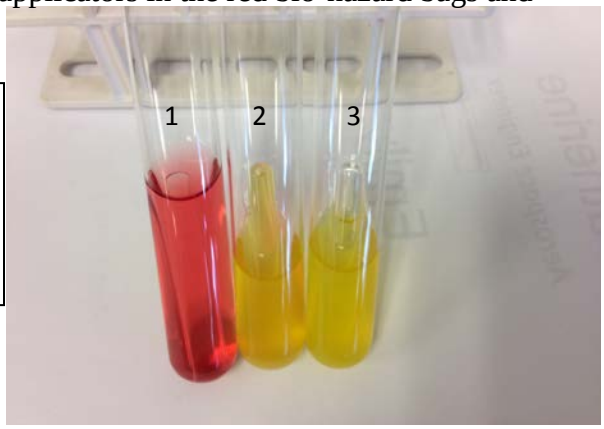
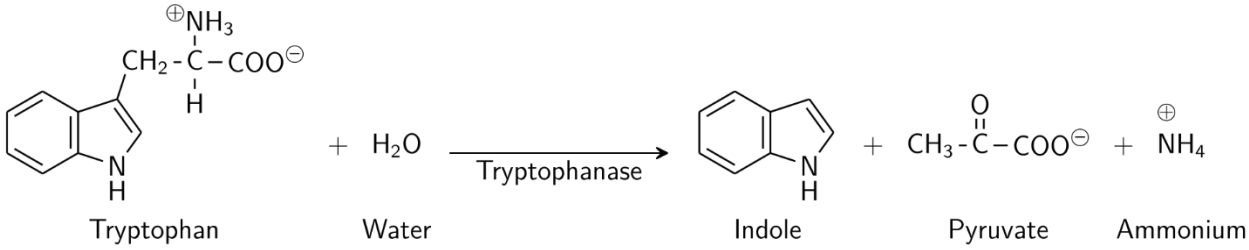


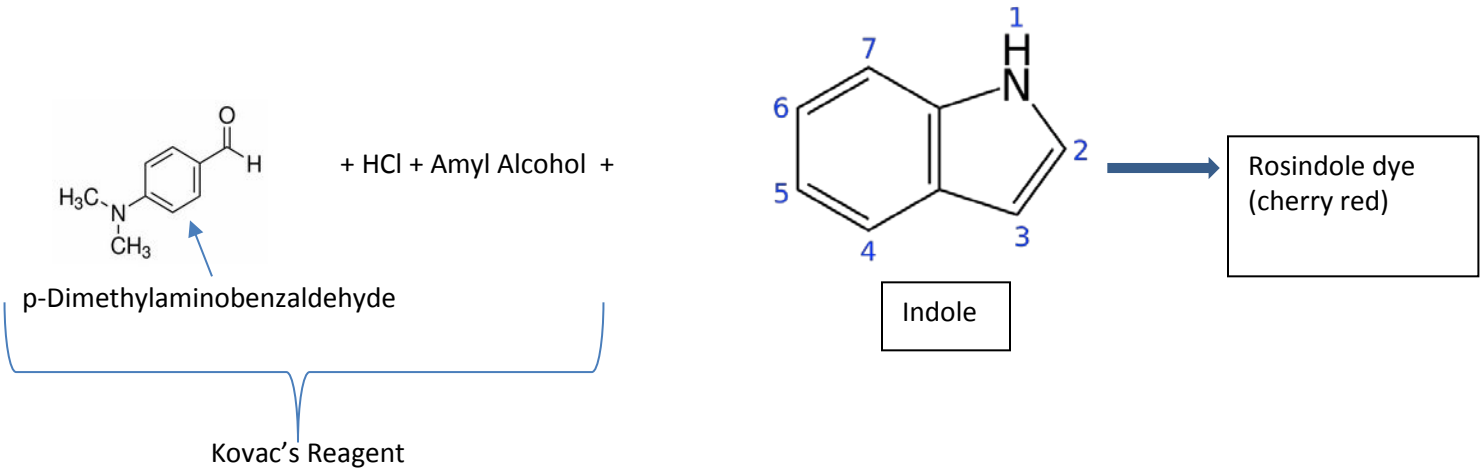
Figure. Carbohydrate fermentation tube samples after an incubation time of two days.
Tube 1 = -/-
Tube 2 = A/-
Tube 3 = A/G

20b. Indole (Tryptone broth)

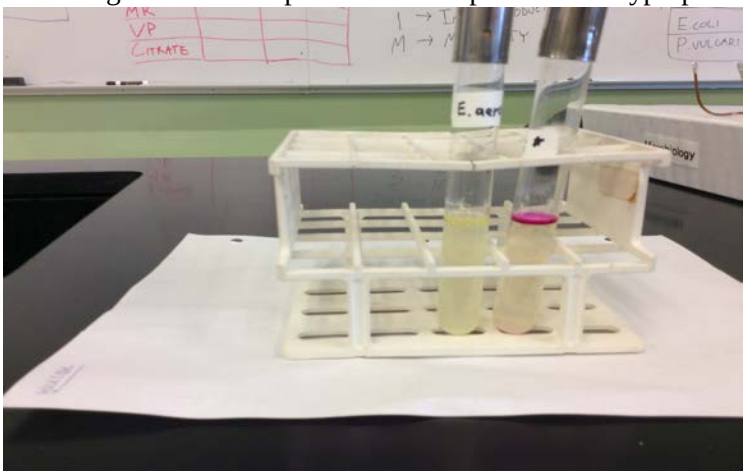
Learning Objective: Upon completion of the indole test, you should be able to determine if a bacterial species can catabolize the amino acid tryptophan.

Introduction / theory / overview: Tryptophan is an essential amino acid that can be oxidized by some bacteria if they have the enzyme tryptophanase. The metabolic products produced are shown below.





An indirect test for the presence of the tryptophanase is to test for one of its end-products (indole). When added, Kovac's Reagent will react with indole to produce a cherry red ring on the top of tryptone broth, indicating "Indole^{+}" or positive for the presence of tryptophanase. No red color is "Indole^{-}" or negative.



Tryptone broth with Kovac's Reagent added. Left tube is Indole $^{(-)}$ and tube on the right is Indole $^{(+)}$.

Materials, reagents, organisms:
2 tryptone broth tubes, *E. coli* and *E. aerogenes*, Kovac's reagent.

Procedure:
i. To inoculated tryptone broth incubated 1-2 days, add 10 drops of Kovac's reagent.
ii. The presence of a cherry red ring is a positive result (Indole $^{+}$). No red color = indole $^{-}$.

Safety and disposal: Remove tape label and dispose of used tubes in wire basket on the cart.

20c: SIM Medium

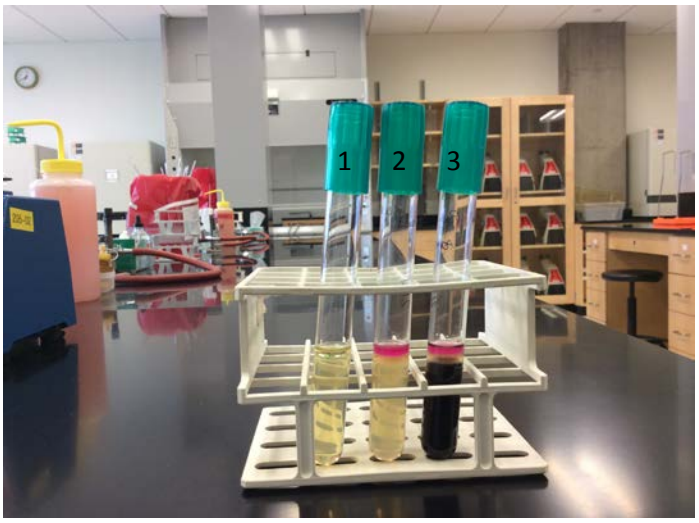
Learning Objectives: Upon completion of the SIM tests, you should be able to:

1. Determine the ability of a bacterial species to produce hydrogen sulfide gas (H2S) from inorganic sulfur compounds or sulfur-containing amino acids.
2. Determine if a bacterial species is motile.
3. Determine if a bacterial species has the ability to hydrolyze tryptophan to metabolic products such as indole.

Introduction / theory / overview: SIM media can be used for analysis of three different bacterial activities: sulfur reduction (S), indole production (I), and motility (M). SIM is a semisolid media containing casein and animal tissues as sources of amino acids, an iron-containing compound and sulfur in the form of sodium thiosulfate.
Sulfur reduction to hydrogen sulfide gas (H_2S) can be carried out by bacteria in two different ways depending on whether they have:

1. Cysteine desulfurase enzyme catalyzes the putrefication of cysteine amino acid to pyruvate.
2. Thiosulfate reductase enzyme catalyzes the reduction of sulfur (in the form of sulfate) at the end of the anaerobic electron transport chain (ETC).

Both of these will produce H_2S gas which will react with iron, in the form of ferrous ammonium sulfate, to form ferric sulfide (FeS), an insoluble black precipitate. Any black color in the SIM media indicates sulfur reduction and is a positive result (sulfur reduction positive). No black in the media indicates sulfur reduction negative. See tube 3 below for positive sulfur reduction.



The Indole test for tryptophanase can also be carried out in the SIM tube. See protocol for the indole test (20b). The second and third test tubes in the picture above are positive for indole (cherry red ring).

Determination of motility resides in the fact that SIM media has a reduced agar concentration and the method of inoculation (stab) with a needle. Motile organisms can move about the semisolid medium and can be detected by their radiating growth pattern projecting outward in all directions from the central stab line. Growth that radiates out in all directions (either black or turbid) is positive for motility. Test tubes 2 and 3 in the figure are positive for motility. Tube 1 is negative (clear). Motility should not be confused with the apparent spreading growth along the stab line only produced by lateral movement of the inoculating needle when stabbing.

Materials, reagents, organisms:
2 SIM tubes, Inoculating needle, *E. coli* and *P. vulgaris*, Kovac's reagent

Procedure:
i. To inoculated SIM tube incubated 1-2 days, add 10 drops of Kovac's reagent.
ii. The presence of a cherry red ring is a positive result. Indole $^{(+)}$. No red color = indole $^{(-)}$.

Safety and disposal: Remove tape label and dispose of used tubes in wire basket on the cart.

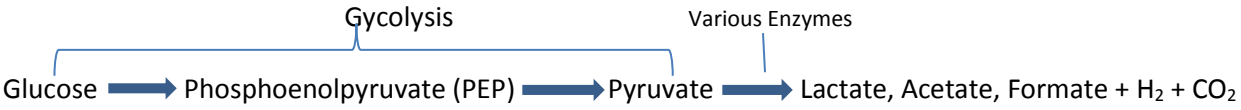
20d: Methyl Red – Voges-Proskauer (MR-VP) broth

Learning Objectives: Upon completion of the MR-VP tests, you should be able to:

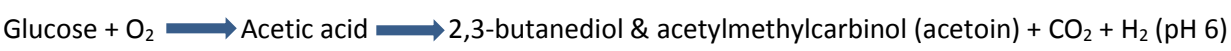
1. Determine the ability of microbes to ferment glucose with the production and stabilization of large quantities of acid end-products.
2. Differentiate between glucose-fermenting enteric organisms, using *E. coli* and *E. aerogenes* as examples.

Introduction / theory / overview: MR-VP medium is a dual-purpose medium that can test for a microbe's ability to follow either (or both) of two specific fermentation pathways. The methyl red test screens for what is called a mixed acid fermentation. The Voges-Proskauer (VP) test identifies bacteria that are able to produce acetoin as part of a 2,3-butanediol fermentation. MR-VP broth contains peptone, glucose and a phosphate buffer. The glucose and peptone provide respectively, a fermentable sugar and protein while the potassium phosphate resists changes in pH.

Glucose is a major nutrient used by all enterics for energy production. The end-products of this process can vary depending on the specific enzymatic pathways present in the bacteria. Most enterics ferment glucose with the production of organic acids. Some microbes produce the organic acid end-products during the early incubation period, dropping the pH to ~ pH 4, where they are stabilized and maintained. *E. coli* is an example. See mixed acid fermentation pathway below.



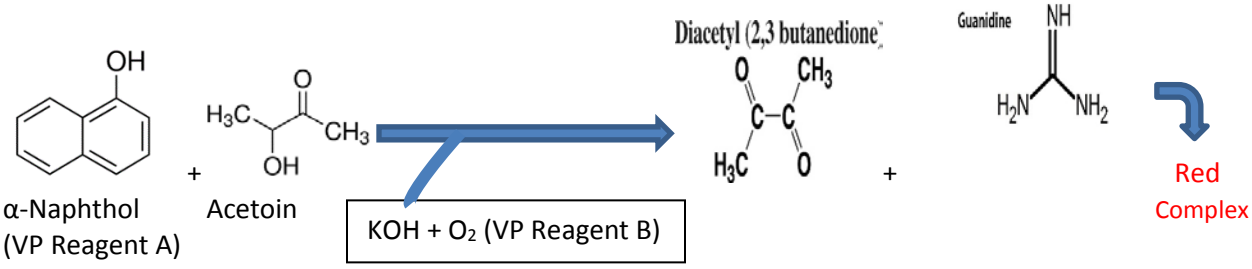
During the later incubation period, other microbes (*E. aerogenes*), enzymatically convert these acids to nonacidic end-products such as 2,3-butanediol and acetoin (acetylmethylcarbinol), resulting in a raised pH of ~6. See this biochemical path below.



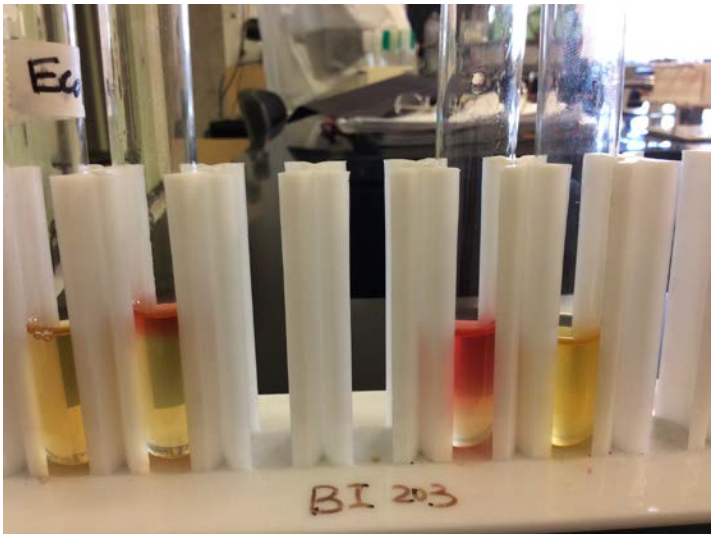
The MR test detects microbes capable of performing a mixed acid fermentation (acids are stabilized). The mixed acids overcome the phosphate buffer in the medium and lower the pH to at least pH 4.4. Addition of methyl red indicator dye will remain red and turn the media red at pH 4.4. Methyl red becomes yellow in color at pH 6.2 and various shades of orange between these two pH values. A red color is the only positive result (MR^+) indicating the microbe performed a mixed acid fermentation only. An orange color is negative or inconclusive. Yellow is negative (MR^-). See figure on next page for examples.

The VP test is used for microbes that ferment glucose but quickly convert their acid products to acetoin and 2,3-butanediol. Addition of VP reagents to the medium will oxidize the acetoin to

diacetyl, which in turn reacts with guanidine nuclei from the peptone, producing a red color ring on top of the tube. See Chemical pathway below. A true red color ring indicates a positive result and is VP^+. No color change or a copper color is VP^-. See examples of a VP test below in the figure.



MR/VP Tests



VP test is shown on the left. The first tube is VP - . The second tube is VP + (Red ring on top) after 30 min.

MR test is shown on the right. The two tubes on the right show the results of a methyl red test. The first tube is MR $^+$ (Red media) and the last tube is MR $^-$.

Materials, reagents, organisms:
2 MR/VP broth tubes, *E. coli* and *E.aerogenes*, VP reagent A, VP reagent B, two clean uncapped glass test tubes.

Procedure:

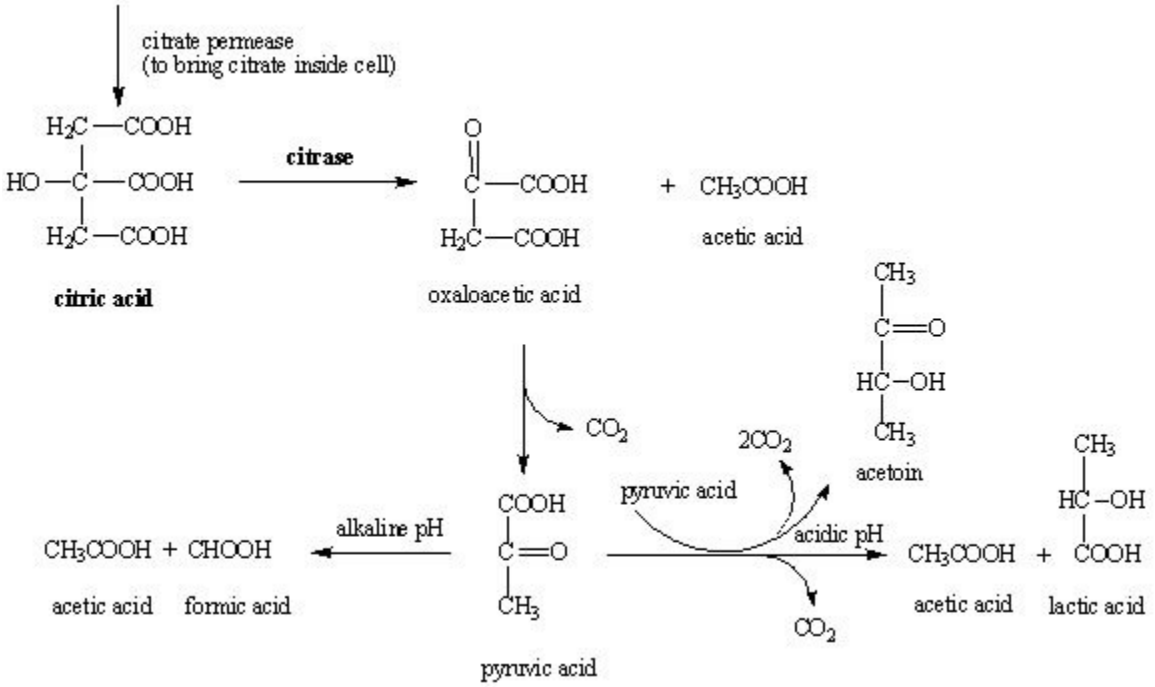
i. Split inoculated MR-VP broth tube incubated for 1-2 days, into approximately equal portions by pouring half of the growth into a clean test tube. To the original tube, you will perform a VP test. To the broth in the clean test tube, you will perform a MR test.
ii. To the MR tube, add 15 drops of methyl red (or whole dropperful) and mix tube between the palms of your hands ten times. If media remains red, it is MR^+. Orange or colors other than red is respectively an inconclusive and negative result.
iii. To the original broth tube, add 10 drops of VP reagent A (Barritt's A), and then add 10 drops of VP reagent (Barritt's B) to the tube and mix. Rack the tube.
iv. Let tube remain undisturbed in rack for at least 30 minutes.
v. If positive, a red ring will slowly develop at the top of the tube over the course of 30 minutes (VP^+). Any other color or no color is VP^-.

Safety and disposal: Remove tape label and dispose of used tubes in wire basket on the cart.

20e: Citrate Utilization

Learning Objective: Upon completion of the experiment you will know how to differentiate enteric microbes on the basis of their ability to ferment citrate as a sole source of carbon.

Introduction / theory / overview: Some microbes in the absence of glucose or lactose, have the capability of using citrate as their sole source of carbon for their energy. Simmons Citrate Medium is a defined medium, meaning the amount and source of all ingredients are carefully controlled. Sodium citrate being the only carbon source in Simmons Citrate media will not support a high energy-yielding respiratory reaction like the Citric Acid Cycle but it does provide a way for a few bacterial species that possess the enzyme citrate-permease to transport the citrate into the cell and metabolize it via the following fermentative pathway:



Source of citrate utilization pathway above: https://microbiologyinfo.com/citrate-utilization-test-principle-media-procedure-and-result/

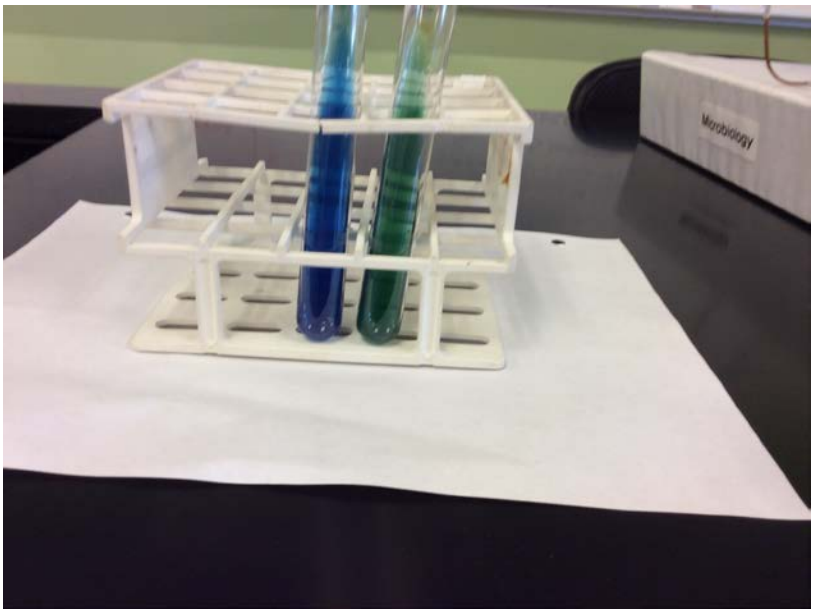
If the enzyme citrate-permease (citrase) is present, a bacterium can transport the citrate inside and then convert it to pyruvate. The pyruvate will then be converted to a variety of products, which depend on the pH of the environment. As Simmons Citrate Medium starts at or near neutral pH and the alkalizing effect of the ammonium salt breakdown, positive bacteria for this test drive the reaction to the left, producing acetate, formate and CO_2. Bacteria that do not have citrase in Simmons Citrate Medium will not survive.

Simmons Citrate Media contains sodium citrate, ammonium dihydrogen phosphate and bromthymol blue dye. Bromthymol blue is a pH indicator and is green at pH 6.9 and blue at pH 7.6. Ammonium dihydrogen phosphate is the sole source of nitrogen in this media. When taking

the nitrogen from the ammonium salt ($NH_4H_2PO_4$), citrate-positive bacteria also produce ammonia (NH_3) and ammonium hydroxide (NH_4OH). These alkalinize the medium and turn it blue, which is a positive result "Citrate $^+$." See examples below in the figure.

Note*: Sometimes a citrate positive bacteria grows on Simmons Citrate Media but does not produce a color change. One explanation of this is because of incomplete incubation or the starting pH of the media was not correct. Growth on the slant in the absence of a color change, still indicates that citrate is being utilized and would be considered a positive result. In order to avoid any confusion between actual growth and a heavy inoculum (which may fool you into thinking it is real growth), citrate slants should be inoculated lightly with an inoculating needle instead of a loop.

Citrate Utilization Test



Simmons Citrate Tubes. The tube on the right shows the normal green color of the media when inoculated by a Citrate ($^-$) bacteria. The tube on the left shows a bright blue color indicative of a bacteria that is Citrate ($^+$) and has the enzyme citrate permease.

Materials, reagents, organisms:
2 Simmons Citrate Slant tubes, *E. coli* and *E. aerogenes*.

Procedure:

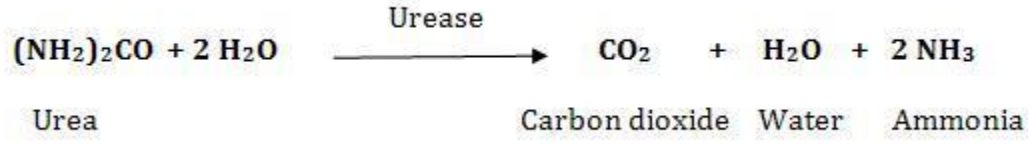
i. Lightly inoculate Simmons Citrate with a needle and incubate for 1-2 days.
The presence of a blue media is a positive result (Citrate $^+$). Green color = Citrate $^-$.

Safety and disposal: Remove tape label and dispose of used tubes in wire basket on the cart.

20f: Urea Hydrolysis Test

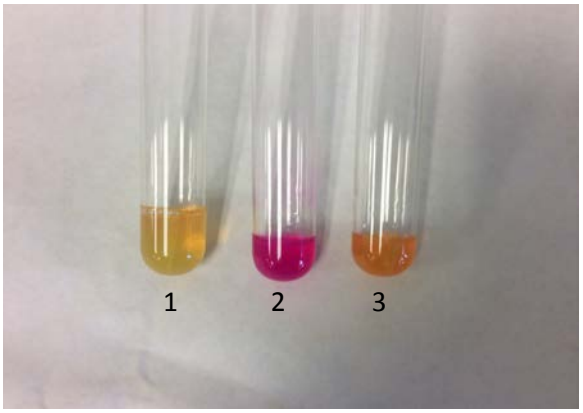
> **Learning Objective:** After completion of the experiment, a student will know how to determine the ability of a microbe to degrade urea by means of the urease enzyme.

Introduction / theory / overview: The Urea Hydrolysis test differentiates microbes by their ability to hydrolyze urea because they possess the enzyme urease. This test is used especially to identify and distinguish urinary tract pathogens from the genus *Proteus*, especially *Proteus vulgaris* from other enteric bacteria.
Urea is the product of decarboxylation of **amino acids**. Urease is a hydrolytic enzyme that acts upon the nitrogen and carbon bond in amide compounds such as urea, releasing the alkaline end product ammonia. See the urea degradative pathway below.



Urea agar* contains only urea, a trace amount of yeast extract (0.0001%), 0.1% dextrose (glucose), agar, and the pH indicator phenol red. It also contains buffers that inhibit alkalinization by all but the most rapid urease-positive organisms. Only organisms that can rapidly metabolize urea can overcome the buffer and change the pH to alkaline (ammonia is alkaline). The medium is yellow if it is acidic, orange if it is around a neutral pH, red if it is slightly above neutral, and pink if it is alkaline (pH > 8.3). The normal color of the agar (due to phenol red at neutral pH) is a light reddish-orange color at ~pH 7. When urease is present, urea releases ammonia, raising the pH above 8.4. The phenol red will then turn a fuchsia color and this is indicative of a positive Urease $^{(+)}$ test. A red or yellow color is a negative result. See examples below in the figure.

Urease Test



These tubes have been inoculated and incubated for two days. Tube #3 on the right shows the normal color of the urea agar and is urease negative. Tube #1 on the left has turned yellow and is indicative of a drop in pH below 6.8 due to acid production, and is urease negative. Dextrose is included in the formulation to stimulate urease activity in organisms that hydrolyze urea slowly, and to exclude false-negative reactions. Tube #2 (middle) has a fuchsia (hot pink) color because

the pH has been raised above 8.4 via urease enzyme releasing ammonia from urea and is a positive result.

Materials, reagents, organisms:
2 Urea agar stab tubes, *E. coli* and *P. vulgaris.*

Procedure:

ii. Stab the urea media to the bottom with a loopful of bacteria and incubate for 1-2 days.
The presence of a fuchsia color to the media is a positive result. Urease $^{(+)}$. Red / Orange / Yellow colors = Urease $^{(-)}$.

Safety and disposal: Remove tape label and dispose of used tubes in wire basket on the cart.

*Christensen's urea agar has a reduced buffer content and contains peptones and glucose. This medium supports the growth of many enterobacteria allowing for the observation of urease activity. Christensen's urea agar is used to detect urease activity in a variety of microorganisms (who deaminate urea at a much slower rate) compared to Proteus.