BACTERIAL METABOLISM

As you are beginning to see so far this semester, bacteria are remarkably diverse in their biochemical properties. As we shall explore in this lab exercise, their metabolic capabilities even extend to the cell exterior. The sum of all metabolic processes is referred to as **cellular metabolism** which encompasses **catabolism**, the breaking down of molecules that serve as nutritional sources, and **anabolism**, the synthesis of new molecules to support cell growth and division. One of the most important catabolic reactions of bacteria is **fermentation**, which is coupled with the metabolic reactions through which they obtain energy. Many media and tests have been developed to identify specific types of bacterial fermentation and the types of byproducts that are produced, such as acids, gasses and other specific molecules. In this lab exercise you will be learning some of the techniques by which microbiologists study the metabolic properties of bacterial cells. The metabolic characteristics of bacteria afford an important means of identifying bacteria, and in this lab exercise you will see how these characteristics can form a biochemical "fingerprint" of a bacterial species.

Summary of laboratory exercise

1. You will examine the production of extracellular enzymes used by bacteria for the catabolism of starch.

2. You will study the fermentation of different types of sugars and the types of end-products released.

3. You will learn how metabolic capabilities can be used to identify bacteria by using the IMViC set of biochemical tests.

I. Extracellular enzymes

Bacteria must absorb molecules from the surrounding environment. Many macromolecules such as starch, proteins and lipids are too large to pass through the cell wall and membrane and, therefore, cannot be used directly as nutritional sources because the molecules. Many bacteria solve this problem by secreting enzymes called **extracellular** to the outside of the cell that degrade macromolecules into smaller compounds that can be taken up by the cells and further metabolized by **intracellular** enzymes. Examples of extracellular enzymes are amylase which degrades starch, protease for proteins, lipase for lipids, and DNase, which degrades DNA.

Starch is a large polysaccharide containing hundreds to thousands of glucose molecules ([glc]) linked end to end. The presence of starch can be determined using the iodine reagent (an aqueous solution of 2% potassium iodide and 0.2% I_2), which stains starch a bluish-black color. **Amylase** is an extracellular enzyme that hydrolyses (breaks down) starch molecules into molecular subunits called "maltose", each of which consists of two glucose sugars, as shown in Figure 1.

Figure 1. Manner in which amylase cleaves starch into maltose subunits. The "⇓" symbols indicate where the enzyme cleaves the starch molecule.

⇓ ⇓ ⇓ ⇓ ⇓

[glc]–[glc]–[glc]–[glc]–[glc]–[glc]–[glc]–[glc]–[glc]–[glc]–[glc]– ...

↓ products of amylase activity

[glc]–[glc] [glc]–[glc] [glc]–[glc] [glc]–[glc] [glc]–[glc] ...
(maltose)

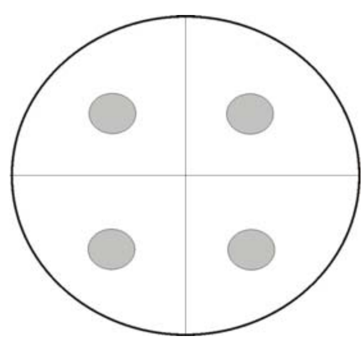
When bacteria that produce amylase are allowed to grow on Starch Agar, a medium containing starch, the starch in the medium surrounding the colony will be broken down. This can be shown by flooding the medium with the iodine reagent, which stains the medium a brownish-black color, accept in a zone around the bacterial growth will appear clear.

Starch Agar
Peptone . . . . 5 g
Beef extract. . . . . . 3 g
Soluble starch. . . . .5 g
Agar. . . . . 15 g

Supplies

1 plate of starch agar	*Bacillus subtilis* (white cap)
your semester unknown	*Escherichia coli* (brown cap)
	Proteus vulgaris (violet cap)

Procedure



1. Drawing on the bottom, partition the plate of starch agar into 4 quadrants.
2. Label each quadrant with the name of one of the following bacteria: *Proteus vulgaris*, *Escherichia coli*, *Bacillus subtilis*, and your unknown.
3. Innoculate bacteria to the centers of the appropriate quadrants.
4. Incubate the cultures at 37°C for 24 - 48 hours.
5. After incubation, carefully flood the surface of the agar with Iodine reagent.
6. Examine the medium around the bacterial growth for starch hydrolysis, which should will appear as a clear zone surrounded by a dark background.
7. Record your results in Table 1.

II. Fermentation of sugars

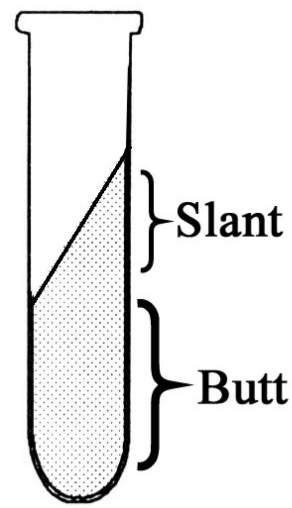
The types of sugars that bacteria can ferment and the fermentation byproducts that they produce are important in the identification of bacteria. In this section you will study two standard methods for studying carbohydrate fermentation.

A. Kligler Iron Agar

Kligler iron agar differentiates a number of metabolic traits. Lactose vs glucose fermentation can be distinguished based upon the coloration of the slant versus the 'butt' region of the medium. When fermentation occurs and acidifies the medium, the phenol red pH indicator turns yellow. If the test organism can ferment lactose or glucose, the acid produced will turn the entire medium yellow. However, if only glucose is fermented, the carbohydrate will be rapidly exhausted (there is only 1g/L), and within about 12 hours, the bacteria will begin using amino acids as a carbon source. This releases NH_3, which turns the medium basic and red again, first in the slant region. (Thus, the slant will be red and the rest of the medium will be yellow.) Bubbles forming in the medium indicate gas production as a product of fermentation Another process that is differentiated is sulfur reduction with the production of H_2S, which causes blackening of the medium.. Timing of the observations is critical, and should be made 24 - 36 hours after inoculation.

Kligler Iron Agar

Beef extract . . . . . .3 g/L	Fe Sulfate . . . 0.2
Yeast extract. 3	NaCl 5
Peptone 15	Na thiosulfate . 0.3
Proteose peptone . .5	Agar 12
Glucose 1	Phenol red . .0.024
Lactose 10	



Supplies

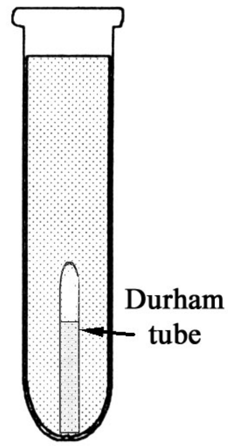
E. coli (brown cap)
Proteus vulgaris (violet cap)
your semester unknown
3 slants of Kligler Iron Agar (red cap)

Procedure

1. Inoculate each slant with one of the bacteria cultures list above. Using your transfer loop, inoculate thoroughly the surface of slant.
2. 'Reload' your loop, and then stab down into the center of the butt.
3. Incubate for 24 - 36 hrs at 37°C.
4. Record results in Table 2.

Purple Broth Media

Kligler iron agar is particularly useful for efficiently distinguishing different Gram-negative enteric bacteria. As you can see, it does not allow clear distinction between lactose and glucose fermentation, and does not allow testing for fermentation of other carbohydrates. For this, several other media are available to which different carbohydrates can be added. Purple Broth media is one example. The recipe for the base medium is shown below, and when the medium is prepared any type of carbohydrate can be added.



Purple Broth Base Medium

Beef extract	1 g/L
Proteose peptone #3	10
Sodium chloride	5
Brom cresol purple	0.015

We will use Purple Broth Media to study fermentation of three carbohydrates (the recipe given above is for the purple broth base medium, to which any type of sugar can be added). If fermentation of the sugar occurs and yields acids, acidification of the medium will turn the brom cresol purple indicator yellow. Gas production is detected by placing a small inverted vial called a **Durham tube** into the medium. The Durham tube is initially filled with medium; gas released by bacteria will be trapped in the vial and is readily observable. If the medium becomes turbid but remains purple, then the cells must be metabolizing amino acids.

Supplies

9 Durham fermentation tubes:
- 3 containing glucose, yellow caps
- 3 containing lactose, black caps
- 3 containing sucrose, avocado caps

Escherichia coli (brown cap)
Proteus vulgaris (violet cap)
your semester unknown

Procedure

1. Inoculate one set of tubes containing each sugar type with *E. coli*, one set with *P. vulgaris*, and one set with your unknown.
2. Incubate the tubes at 37°C for at least 48 hours.
3. Record the results of the exercise in Table 3.

III. The IMViC reactions

In this part of the exercise you will perform a set of biochemical tests referred to by the mnemonic "IMViC", which is an acronym for the names of the four tests:

I = Indole
M = Methyl red
Vi = Voges-Proskauer (the "i" facilitates euphony)
C = Citrate

The IMViC tests were developed to help distinguish morphologically and physiologically similar members of the **Enteric** group of bacteria, which includes may common pathogens of the intestine.

Supplies

<u>Cultures to be tested</u>
Pseudomonas aerogenes (pink cap)
Enterobacter aerogenes (light blue cap)
Escherichia coli (brown cap)
your bacterial unknown

<u>Indole test</u>
TSA plates (prepared last week)
DrySlide (in refrigerator)
filter paper pieces

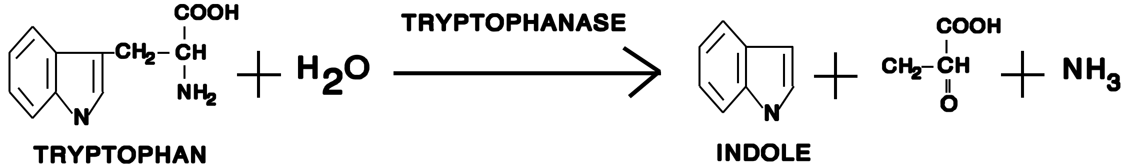
<u>Simmons citrate</u>
4 tubes of Simmons Citrate Agar (blue caps)

<u>Methyl red / Voges-Proskauer</u>
4 tubes of MR-VP broth (green cap)
Pasteur pipets and bulbs
4 clean, non-sterile 13mm tubes
α-naphthol and KOH reagents (on side bench)
methyl red reagent (on side bench)

Principles and Procedures

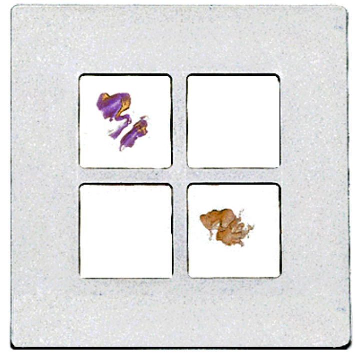
<u>A. Indole test</u>

This test reveals the presence of the enzyme **tryptophanase**, which cleaves the amino acid tryptophan into indole, pyruvic acid and ammonia, as shown in the reaction below. When amino acids are needed as a carbon source and tryptophan is present, some bacteria will begin to produce this enzyme and convert tryptophan to indole..



For the indole test, bacteria are cultured on tryptic soy agar (TSA), a medium that is rich in tryptophan. To test for tryptophanase activity, a small amount of the bacterial growth is transferred onto a BBL Indole DrySlide (Figure 4), which contains a reagent called DMABA. In a positive reaction, the reagent reacts with indole to form a pink to purplish color.

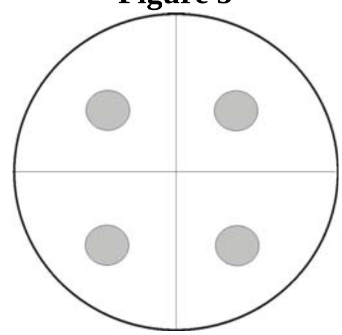
Figure 4. Indole Dryslide



Procedure for the Indole test

1. Quadrasect and label the bottom of a Tryptic Soy Agar plate as show in Figure 5.
2. Inoculate the bacteria as spots in the appropriate sectors.
3. Incubate the plate at 37°C for 24 - 48 hours.
4. When you are ready to perform the Indole test, obtain a single Indole DrySlide and allow it to equilibrate to room temperature (a few minutes).
5. To test each type of bacterium, using your inoculating loop smear a small amount of bacterial growth onto one of the reaction areas.
6. A positive result will be apparent WITHIN 30 SECONDS:
   Positive: pink to purplish color
   Negative: essentially colorless
7. Dispose of the DrySlide in the disposal jar so that it can be autoclaved.

Figure 5

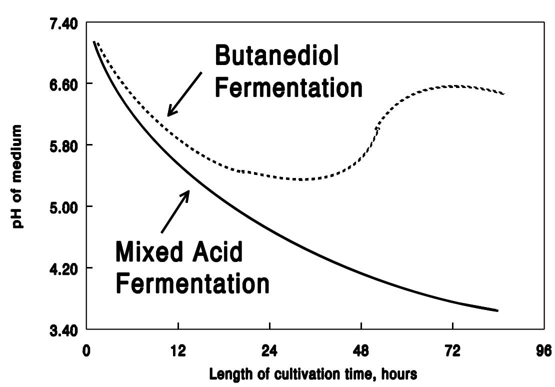


Note: The bacteria grown on this TSA plate will also be used for the Oxidase test described below.

B. Methyl Red and Voges-Proskauer

Most bacteria ferment glucose to acids. Some species further metabolize these acids to other types via **mixed acid fermentation**, a process that causes the pH of the medium to become very acidic. Certain species convert the acid by-products to more neutral substances such as **acetoin** (acetylmethylcarbinol) and butanediol (2,3-butylene glycol) via **butanediol fermentation**. The effects on the pH of the culture medium by these two types of metabolism are shown in the Figure 6.

Figure 6. pH change during bacterial fermentation.



The Methyl red and Voges-Proskauer tests are designed to detect these two modes of fermentation. The Methyl red indicator turns red only at relatively low pHs, as shown below. Thus, it is useful for detecting mixed acid fermentation.

pH of medium	Color of methyl red
4.5 and below	Red
around 5.1	Yellowish-Red
6.8 and above	Yellow

MR-VP Broth

Peptone	7.0 g/L
Glucose	5.0
K_2PO_4	5.0
pH 7.0	

The Voges-Proskauer tests for butanediol fermentation. The test is performed by adding a reagent containing alpha-naphthol that reacts with acetoin to form a red color. The two tests are performed from a single broth culture in MR-VP medium.

1. Inoculate one tube of MR-VP broth for each of the three bacteria.
2. Incubate the tubes at 37°C for AT LEAST 48 hours.
3. Using a Pasteur pipet, carefully transfer half of each culture to separate clean test tubes.
4. Perform the Methyl red test by adding to one half of each culture 3 drops of the methyl red reagent. Test results are immediately observable:
   Positive: distinct reddish color to the medium   Negative: no color change
5. Perform the Voges-Proskauer test by adding to the other tube 0.6 ml of α-naphthol reagent and 0.2 ml of 10 N KOH. Shake the mixture vigorously. The test results will develop within a period of 15 - 60 minutes.
   Positive: red color (beginning near the surface)   Negative: no color change

C. Citrate utilization

The Citrate test reveals whether the bacteria can utilize citrate as a sole carbon source. Citrate is produced intracellularly by most bacteria species during cellular respiration. However, bacteria must possess a specific transport enzyme in the cell membrane in order to utilize citrate present in the external medium as a nutritional source. The test for citrate utilization is performed on **Simmons Citrate Agar**, a defined, differential medium that contains citrate as the sole carbon source. As the Na Citrate is utilized by the bacteria, the pH of the medium increases, changing the bromethymol blue indicator from green to blue.

Simmons Citrate Agar

$NH_4HPO_4 \cdot 4H_2O$	1.0 g/L
K_2HPO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	5.0
Na Citrate	2.0
Bromthymol blue	0.08
Agar	15
pH 6.8	

1. Inoculate each tube of Simmons Citrate Agar with one of the bacteria to be tested.
2. Incubate the cultures for at 37°C for 48 hours, and then observe results:
   Positive: medium turns blue (starting along slant)
   Negative: medium remains green

IV. Oxidase activity

This test determines whether the organism possesses the enzyme cytochrome oxidase-C. This enzyme, part of the respiratory electron transport chain, is commonly found in aerobic organisms but not facultative or anaerobic bacteria. Cytochrome oxidase-C can be detected by testing cells for the ability to oxidize a reagent called tetramethyl-phenylenediamine using DrySlide technology. If the reagent is oxidized, a purple-colored product is formed.

Supplies

Your semester unknown
Bacteria from TSA plate prepared for Indole test
E. coli
Enterobacter aerogenes
Pseudomonas aerogenes

1 Oxidase DrySlide (in refrigerator)

Procedure

The procedure is very similar to that used to test for the Indole test described above

1. When you are ready to perform the oxidase test, obtain a 4-segment <u>oxidase DrySlide</u> panel from the refrigerator.
2. To test each type of bacterium, using your inoculating loop to smear some of the bacterial growth from plated cultures onto one of the reaction areas – the smear should be about 3 - 4 mm diameter. **Hint:** if bacterial growth is somewhat dry and hard to smear, wet it first with a loop of water.
3. A positive result will be apparent <u>WITHIN 20 SECONDS</u>:
   Positive: grey / purplish color
   Negative: essentially colorless; although many negatives will begin to turn purple after about 30 seconds.
4. Record the results in Table 4.
5. Dispose of the DrySlide in the disposal jar so that it can be autoclaved.

Names: ___________________________________

✶✶Indicate where your results do not agree with your expectations or are ambiguous, and write a brief explanation✶✶

Note: for a positive result, the clear zone should form a halo <u>around</u> colony and not just under the colony, which may result from iodine not spreading under the colony and staining the medium.

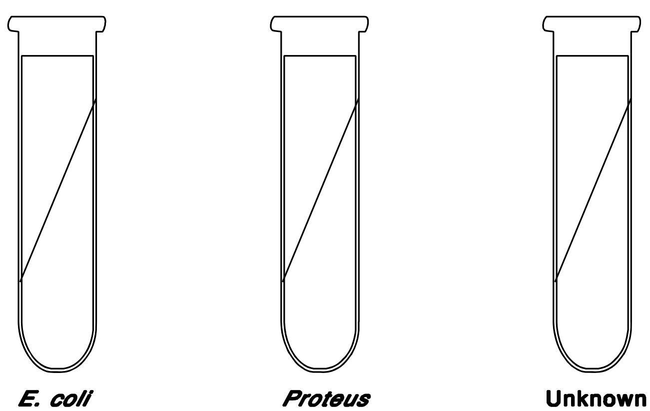
Table 1
Presence of Extracellular Amylase

Organism	Starch hydrolysis
P. vulgaris	
E. coli	
B. subtilis	
Unknown	

"+" = hydrolysis "–" = no hydrolysis

<u>Kligler Iron Agar results</u>

Diagram and label the coloration pattern and bubble formation in the Kligler Iron Agar cultures



Note: if only the very tip of the slant is red, do not interpret this as a red slant. If cultures are old, red slant may begin to fade to yellow.

Interpreting Kligler Iron Agar results (*** Look at image on Lab results web page***)

coloration pattern	interpretation
no color change:	probably no growth on medium
red slant + red butt:	no fermentation
red slant + yellow butt:	glucose fermentation only
yellow slant + yellow butt:	glucose and lactose fermentation
medium yellow but the very bottom of butt is orange	medium was not fully inoculated
Black coloration	H_2S production
Bubbles within medium	gas production

Table 2. Summary of results from Kligler Iron Agar

	lactose fermentation	glucose fermentation	H_2S production	gas formation
E. coli				
Proteus				
Unknown				

"+" = yes "–" = no "??" = Can't tell because black color masks fermentation results

Table 3
Fermentation of sugars in Purple Broth Media

Organism	TYPE OF SUGAR IN THE CULTURE MEDIA		
	Glucose	Lactose	Sucrose*
E. coli			
Proteus vulgaris			
Unknown			

Indicate results as: "+" = growth (turbid medium), "–" = no growth (clear medum), "A" = acid production, "AG" = acid + gas, "AA" = amino acid metabolism

*During autoclaving some sucrose may breakdown to release glucose and fructose, which, when fermented will yield a slight yellow coloration – this is negative for sucrose.

It does not make sense to have '–' plus "A","AA", or "AG": Why?

For your semester unknown: do the results for the Kligler Iron Agar, and Purple agar agree? ____
If not, explain.

Table 4. Results of Imvic and Oxidase Tests

Organism	Indole	Methyl red	Voges-Proskauer	Citrate	Oxidase
E. coli					
Enterbacter					
Pseudomonas					
Sem unknown					

"+" = positive result "–" = negative result

Note: Methyl red should be full red color, not just "orange-ish"

Are the IMVIC and Oxidase tests sufficient to distinguish your semester unknown from *E. coli* and *Enterobacter*?
Explain.

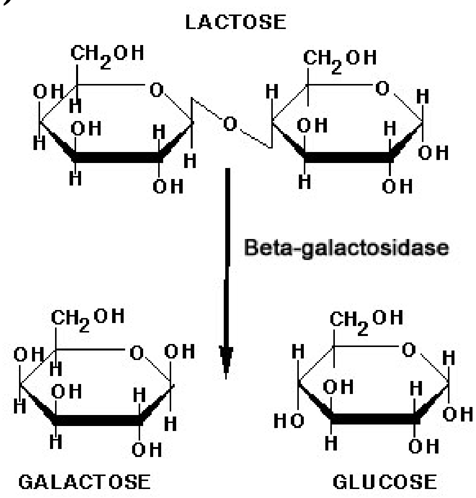
Table 5. Prepare a metabolic finger print for *E. coli, Proteus vulgaris, Enterbacter aerogenes* and your semester unknown. Use results from purple medium for fermentation tests.
Indicate '+' or '–' for each test; ("⊕" for acid and gas).

From Purple Broth

	lactose fermentation	glucose fermentation	sucrose fermentation	H2S production	starch hydrolysis	Indole production	Methy red	Voges Proskauer	Citrate metabolism	Oxidase activity
E. coli										
E. aerogenes										
Proteus vulgaris										
Semester unknown										

Suppose you wished to design a metabolic 'fingerprint' that could distinguish your semester unknown from *E. coli* and *Proteus* using the first 5 tests above. Can this be done? ______
If so, identify the smallest number of tests that would allow distinguishing these three bacteria:

Also turn in answers to these questions. (Typed)



1. As shown in the diagram to the right, in order for lactose to be metabolized through glycolysis and fermentation, it must first be broken down into it's two constituent monosaccharides, galactose and glucose. In order to do this, the cells must possess the enzyme beta-galactosidase.

A. Explain why some bacteria can ferment lactose but others cannot.

B. Suppose that you performed two purple broth tests for the same organism: one with glucose and one with lactose. Why it would be surprising to find that the organism could ferment lactose but not glucose? What is one possible explanation?

2. There is initially a high concentration of lactose and a low concentration of glucose in Kligler Iron Agar medium; why is this necessary to distinguish the ability to ferment glucose but not lactose?