LAB 5. Fermentation and Respiration

Protocols for Anaerobic growth, including use of Anaerobe Chamber, Catalase Assay, Oxidase Assay, Assay for Carbohydrate Utilization, Use of Oxidative-Fermentation tubes.

INTRODUCTION

Organisms that use preformed organic compounds as their source of carbon and energy are called **chemoheterotrophs**. Chemoheterotrophs exhibit two basic strategies of energy production: respiration and fermentation. To produce energy, both processes depend upon the flow of electrons from a substrate to a terminal electron acceptor. The electron acceptor (having a negative reduction potential) accepts electrons readily from the substrate (having a positive reduction potential). **Respiration** is the more efficient process whereby the substrate molecule is completely oxidized to CO_2, and a maximum amount of energy is released. This process most commonly uses an *inorganic* molecule to serve as the final electron acceptor (O_2 in aerobic respiration). **Fermentation** is less efficient. The substrate, commonly a sugar such as glucose, is not completely oxidized. Rather, the substrate is broken down to form pyruvate which still harbors potential energy. In fermentation the electrons from oxidation flow to an *organic* electron acceptor (pyruvate, or a byproduct of pyruvate) resulting in the formation of fermentation end products. The chemistry of endproducts formed during fermentation will vary depending upon the steps in this second portion of the fermentation pathway.

The diversity within heterotrophic organisms can be seen in the pathways used for energy metabolism (respiration and/or fermentation), in the substrates used, in the specific pathways available, and in the products produced. Particularly with fermentation, a variety of pathways have been observed among chemoheterotrophs. As these pathways produce characteristic products such as organic acids (lactic, acetic, butyric, propionic); neutral products (acetone, butyl alcohol, ethyl alcohol); and various gases (carbon dioxide, hydrogen, and methane), fermentation pathways are commonly named for the endproducts produced *e.g.* Mixed Acid Fermentation, and Butanediol Fermentation. Characteristics of energy producing pathways are important in classification and identification of bacteria.

Explanation of Protocols

Assay for oxidation/fermentation catabolism - OF media ~ Page 83

OF media is used to distinguish whether chemoheterotroph utilizes carbohydrates aerobically (Oxidation) or anaerobically (Fermentation). The direct oxidation pathway is an alternative to the Embden Meyerhoff pathway of glycolysis and occurs primarily in strict aerobes. To distinguish between an organism capable of fermentation, and one that will perform oxidation, the OF test is performed. OF media contains a small amount of peptone, a test carbohydrate (such as glucose) and the pH indicator bromothymol blue. The test organism is inoculated into two identical OF tubes. Tube 1 is sealed with sterile mineral oil to create an anaerobic environment. Tube 2 is exposed to the air. After incubation, the tubes are observed for acidity. Acidity in the open tube only, indicates oxidation. Acidity in both tubes, indicates either fermentation only, or both fermentation and oxidation. If neither tube is acidified, the organism is unable to catabolize the sugar.

Assay for Carbohydrate Utilization ~ Page 69

Data from the OF test will reveal if organisms are capable of fermentation. In the second period of this lab you will have the opportunity to examine more closely the characteristics of any fermentation positive organism. The range of sugars fermented by a particular organism is commonly used as an assay to identify and characterize bacteria. For example, *E. coli* can be discriminated from other Gram negative fermentative organisms such as *Serratia marcescens*, and *Proteus vulgaris* by observing the pattern of sugars each is capable of fermenting.

The ability of organisms to ferment carbohydrates is determined by observing the production of acid (+/- gas). This is accomplished by using a medium that contains a pH indicator and an inverted tube that can catch evolved gas. A commonly used pH indicator is phenol red. Phenol red is red at pH 7.4 (the original pH of the medium), and yellow below pH 6.8.

Assay for catalase enzyme ~ Page 71

The presence of the enzyme catalase allows organisms to break down toxic products of oxygen. Most organisms that tolerate atmospheric oxygen will have this enzyme. Another enzyme that destroys toxic products of oxygen is superoxide dismutase.

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

Catalase is detected through a slide test. Hydrogen peroxide is added to a small amount of culture on a glass slide. The evolution of bubbles (oxygen) indicates the presence of the enzyme catalase.

Assay for oxidase enzyme ~ Page 82

Oxidase enzyme, or cytochrome oxidase, is found in the electron transport chain of many bacteria. If the test for oxidase is positive, the test organism has an electron transport chain with cytochrome oxidase. If the test is negative, the organism may have an electron transport chain, but it will not contain cytochrome oxidase.

To test for oxidase, an artificial substrate (*p*-phenylenediamine) is added to the culture. If the organism has the oxidase enzyme, an electron will be passed to the artificial substrate, reducing it, and causing a change in color (from colorless to blue).

Assay for Growth in presence and absence of oxygen ~ Page 50

As mentioned in Lab 4 the an oxygen requirement for growth relates to the energy metabolism of an organism. An aerobe will use respiration with oxygen as the terminal electron acceptor. A heterotrophic anaerobe will perform fermentation, which does not require oxygen. A facultative anaerobe will have the option to ferment in the absence of oxygen or respire in its presence.

A variety of tactics are available to grow anaerobes in the laboratory:

(1) *Growth in media containing a strong reducing agent such as sodium thioglycolate.* Sodium thioglycolate has a low oxidation reduction potential which allows the sulfhydryl groups of this chemical to bind oxygen and allow anaerobes to grow.

(2) *Growth in an environment without oxygen.* Such an environment can be found in:

a. the bottom of agar deeps, where little air circulation occurs, or
b. in an anaerobic culture jar that can be used to exclude atmospheric oxygen.

The Gas Pak anaerobic culture system uses a jar with a sealed top. Disposable envelopes placed in the jar generate hydrogen. The hydrogen reacts with oxygen to produce water. The reaction requires a palladium catalyst. The catalyst is placed in the lid of the anaerobe jar. Carbon dioxide is also generated to replace the depleting oxygen. A methylene blue indicator placed in the jar turns blue if oxygen is present or white if oxygen is absent.

Facultative anaerobes can grow under conditions where oxygen is present *or* absent. Aerobes will grow only when oxygen is readily available. Conditions would include: on the top of a broth or nutrient deep, in a well aerated broth, or on an agar plate. (See figure 1 and 2)

OBJECTIVES

- Learn to effectively complete protocols and recognize positive and negative results.
- Learn the usefulness of biochemical tests in the characterization of bacteria.
- Learn the role of catalase and oxidase enzymes in bacterial metabolism
- Learn methods for effective culture of aerobes, anaerobes and facultative anaerobes.

Table 1: Control organisms and their characteristics.

	OF Test	**Fermentation**			**catalase**	**oxidase**
Control Organisms		**glucose**	**lactose**	**mannitol**		
Escherichia coli	**O/F**	**A/gas**	**A**	**A**	**positive**	**negative**
Enterobacter aerogenes	**O/F**	**A/gas**	**A**	**A**	**positive**	**negative**
Pseudomonas aeroginosa	**O**				**positive**	**positive**
Serratia marcescens	**O/F**	**A/gas**	**----**	**A**	**positive**	**negative**
Proteus vulgaris	**O/F**	**A/gas**	**-----**	**-----**	**positive**	**negative**
Streptococcus (Enterococcus) durans	**F**	**A**	**A**	**-----**	**negative**	**negative**

"A" refers to the production of Acid.

MATERIALS/ Group:

6 OF glucose tubes
Carbohydrate Utilization tubes:
2 glucose broth tubes, **2** lactose broth tubes**, 2** mannitol broth tubes
6 slides
1 vial oxidase substrate/lab section
dropper bottles with peroxide
Sterile cotton applicators
pH paper
Control cultures in test media as demonstration.

Protocol: Oxidation/Fermentation

1. For each organism to be tested, obtain 2 tubes of OF broth. Label tubes with your name and name of test organism.

2. Inoculate each tube: Use an inoculating needle to remove one isolated colony of culture and transfer to the OF tube. To inoculate, stab to approximately one centimeter from tube bottom. (The media is semisolid and will serve as a confirmation of a motility test as well as an indication of fermentation and oxidation.)

3. To one tube add sterile mineral oil to give a 1 cm layer.

4. Incubate. For most cultures in BSCI 223 incubation at 37^{o}C for 24 hours is sufficient to allow growth.

5. Observe tubes for growth and acidity. Compare growth of test cultures to control cultures. A positive result for acidity is a color change (green to yellow). Record results:

   Record "O" for cultures that display acidity in the aerobic tube only.
   Record "F" for cultures that display acidity in aerobic and anaerobic cultures.

Protocol: Catalase Assay

1. Label one clean slide for each organism to be assayed. For control results use control cultures: *Psuedomonas aeroginosa*, *E. coli* and *Streptococcus durans* .

2. Using a sterile inoculating loop transfer culture from an isolated colony to the clean slide.

3. To the culture, add a drop of hydrogen peroxide. Observe. Compare test cultures to control cultures. The appearance of bubbles indicates a positive reaction.

4. Record observations.

Protocol: Oxidase Assay

1. Use control cultures: *Psuedomonas aerogenes*, *E. coli* and *Streptococcus durans*.

2. Using a sterile cotton applicator, remove a small amount of culture from an isolated colony.

3. Hold the inoculated applicator over a clean, sterile, empty petri dish. Add one small drop of oxidase reagent. Observe. Compare test cultures to control cultures. A purple color appearing within 10-15 seconds will indicate a positive reaction.

4. Record Observations .

Protocol: Carbohydrate Utilization

1. Label Carbohydrate Utilization tubes with name of sugar, name of organism, date, and your name. For each sugar to be assayed, one tube/organism is needed.

2. Using a sterile inoculating loop transfer culture from an isolated colony into the appropriately labeled sugar tubes.

3. Incubate. . For most cultures in BSCI 223 incubation at 37^{o}C for 24 hours is sufficient to allow growth..

5. After incubation, observe fermentation tubes and compare each of the inoculated tubes with the control tubes provided.

   Positive control—growth of organism, acid production in the absence or presence of gas production;
   Negative control—growth of organism, no acid;
   False negative control—no growth, no acid.

   If acid was produced, the tube should be yellow;
   If gas was produced, a bubble should appear in the inverted durham tube;
   Note the color of the media. To determine the pH of the media after incubation. Use pH paper.

6. Record results.

Protocol: Aerobic and Anaerobic culture.

MATERIALS/group

Control cultures:
Clostridium sporogenes - anaerobe
Staphylococcus aureus - facultative anaerobe
Bacillus subtilis - aerobe
3 nutrient agar deep tubes (melted and stored in 50^{o} water bath)
3 tubes nutrient broth
3 tubes thioglycolate broth (steamed to release dissolved oxygen)
2 nutrient agar plates

Assay – growth in Nutrient Agar Deep:

1. For every culture to be tested, remove a molten deep from the water bath. Inoculate the deep with one loopful of culture. Mix culture well using Vortex. Allow medium to cool and solidify. Incubate. For most cultures in BSCI 223 incubation at 37^{o}C for 24 hours is sufficient to allow growth.
2. Observe. Compare to growth of Control cultures:
    - Aerobe would be expected to grow at the top only.
    - Anaerobe would be expected to grow at the bottom only.
    - Facultative anaerobe would be expected to grow throughout the tube.
3. Record Results.

Use of Thioglycolate broth to support the growth of anaerobles

1. For each culture tested, procure one tube of nutrient broth and one tube of thioglycolate broth. Inoculate one loopful of culture into each tube. Mix using vortex. Incubate..
2. Observe. Compare test cultures to control cultures.
    - Aerobic bacteria will grow **on the surface** of nutrient broth and on thioglycolate.
    - Anaerobic bacteria will grow in thioglycolate but not in nutrient broth
    - Facultative anaerobes will grow in thioglycolate and nutrient broth.
3. Record results.

Growth in Anaerobe Chamber

1. Use two agar plates. Label one plate with "+oxygen" and the other plate with "No Oxygen". Mark plates in sectors for each organism to be tested: 3 sectors for 3 organisms.
2. Inoculate one bacterium per sector.
3. Place the "no oxygen" plate in the anaerobe chamber
   After the anaerobic jar is filled with plates, set up the Gas Pak.
   - Î add three Gas Pak envelopes,
   - Ï cut off corner of each envelope and add 10 ml of water
   - Ð add methylene blue indicator.
   - Ñ seal jar and incubate at 37^{o} C for 48 hours.
4. Place anaerobe chamber and the second plate into an incubator for incubation. Incubate. For most cultures in BSCI 223 incubation at 37^{o}C for 24 hours is sufficient to allow growth. Cells may grow more slowly in an anaerobic environment. Incubate plates for 48 hours.

5. Observe plates. Compare test cultures to control cultures. . Record data.

/ Assignment 1 Check Point

What can you learn about your environmental isolates by using the assays in this exercise?

From your accumulated results, what do you know about your isolates?

Can you clearly recognize each organism?

Determine which tests have been most significant in the characterization of your isolates. Prepare for naming your organism and writing a Bergey's Manual description! .

Exercise 5_____Laboratory Report Questions

Respiration and Fermentation Name _____

Results: Record detailed results in tables below.

Table 1: Results from O/F catalase and oxidase tests.

Media:	O/F Results	Catalase	Oxidase
Culture 1			
Culture 2			
Culture 3			

Table 2: Results from growth in varying oxygen environments.

Media:	Growth in NA Deep	Growth in NA broth	Growth in thioglycolate	Growth on plate in aerobic environment	Growth on plate in anaerobic environment
Culture 1					
Culture 2					
Culture 3					

Table 3: Results from Carbohydrate Utilization experiments.

Media:	Mannitol	Glucose	Lactose
Culture 1			
Culture 2			
Culture 3			

Questions:

1. The evolution of bubbles in the catalase test indicates:

2. The pH indicator, bromothymol blue is added to OF tubes to:

3. In the gas pak, the evolution of hydrogen is required. Explain.

4. Define
   facultative anaerobe

   fermentation