

## Experiment #4 - Requirements for Oxygen, Temperature, NaCl and pH

Do all living things require oxygen? Pasteur, studying clostridia, observed bacteria swimming in the center of a wet mount preparation. He saw that they slowed and then died at the edge of the coverslip where they contacted air. Correctly he surmised that oxygen killed the microbes. Pasteur coined the term “**anaerobe**” to describe an organism that is poisoned by oxygen. In this exercise you cultivate anaerobes and examine the oxygen and temperature requirements of microorganisms.

### Oxygen Requirements

You might anticipate that because humans and other familiar animals require oxygen, microbes would, too. But in reality—since liquid environments cover much of the earth, and oxygen is not highly soluble in water—organisms that require this gas are at a disadvantage in nature. Microorganisms need water; only a few actually require oxygen.

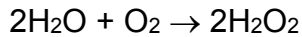
In the microbial world, there is great variation in the amount of oxygen that organisms require or can tolerate. Five oxygen requirement groupings are generally recognized: **aerobes, microaerophiles, facultative anaerobes, aerotolerants, and anaerobes.**

**Aerobes** must have oxygen to live. For these organisms, oxygen is the final acceptor of electrons that are released as foods are oxidized for energy during the process of **aerobic respiration**. Often the enzyme Cytochrome c oxidase reduces  $O_2$  to  $H_2O$ . This enzyme can be detected using the **Oxidase test**. In this test, a colorless substrate (N,N,N',N'-tetramethyl-p-phenylene diamine dihydrochloride) is used as an alternate electron acceptor and yields a purple pigment. **Micrococcus**, is an example of an obligate aerobe.

**Microaerophiles** are organisms that respire, requiring oxygen. But they demand a higher concentration of carbon dioxide ( $CO_2$ ), and less oxygen ( $O_2$ ), than is usually found in air.

**Facultative anaerobes** have the enzymes that are necessary for respiration, and they can utilize oxygen as the terminal electron acceptor. But facultative anaerobes do not require this gas. Utilizing **anaerobic respiration**, they can substitute  $NO_3^-$  (nitrate),  $SO_4^{2-}$  (sulfate), or other molecules when oxygen is depleted from an environment. Most facultative anaerobes are also able to **ferment**. Fermentation does not require oxygen. This process is an incomplete breakdown of **organic compounds** (most molecules that contain carbon) in which some but not all of the energy held in the compounds is released. In fermentation, the final acceptor of electrons is another organic molecule, often producing an acid or an alcohol. In this exercise you observe the oxygen tolerance and requirements of the facultative anaerobe **Staphylococcus epidermidis**, a common inhabitant of human skin.

**Obligate Anaerobes** are actually poisoned by various forms of oxygen. For instance, oxygen reacts with water to form the toxic molecule H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide):



Anaerobes do **not** produce the enzyme **catalase**, which reverses that reaction,  $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ , turning hydrogen peroxide back into water and oxygen. Most organisms that survive the presence of oxygen produce catalase. Anaerobes also lack the enzyme **superoxide dismutase**. Organisms growing in air produce the free radical **superoxide, O<sub>2</sub><sup>-</sup>**. This form of oxygen is so toxic that, to survive, organisms in air must immediately utilize their superoxide dismutase to neutralize it by forming H<sub>2</sub>O<sub>2</sub>. Catalase then rids the cell of hydrogen peroxide.



Lacking the ability to produce catalase and superoxide dismutase, anaerobes soon die in the presence of oxygen.

**Aerotolerants** do not utilize oxygen, but neither are they killed by it. They ferment and also produce superoxide dismutase or other enzymes that neutralize toxic forms of oxygen. The lactic acid-producing bacteria, including ***Streptococcus*** and ***Lactobacillus***, are aerotolerants.

Pathogenic aerotolerants include ***Streptococcus pneumoniae***, which causes pneumococcal pneumonia, and ***Streptococcus pyogenes***, the etiologic agent of septic sore throat as well as many other diseases.

## Oxidation Reduction Potential

In this exercise you cultivate anaerobes by reducing the **oxidation reduction potential** of their environment. The oxidation reduction potential is a means of expressing a compound's affinity for electrons. This affinity is compared with the attraction of H<sub>2</sub>, as in H<sub>2</sub>O, for electrons. Chemists often refer to an environment as either **oxidized** or **reduced**. A highly oxidized environment, air for example, has a great affinity for electrons; it has a high oxidation reduction potential. When, in an environment, there is a greater proportion of hydrogen than in H<sub>2</sub>O, that environment has a negative oxidation reduction potential. Thus an electron-rich environment is a reduced environment. Another name for the oxidation reduction potential is the **redox potential**.

## Cultivating Anaerobes

Microbiologists use a variety of methods to cultivate anaerobes. An environment with a reduced oxidation reduction potential may be created by boiling media to drive out dissolved oxygen. For the **agar shake culture**, one inoculates bacteria into the liquified deep. To prepare a **stab culture**, a freshly boiled, then solidified, deep is inoculated. What prevents anaerobes from growing near the top surface of the medium in a stab or agar shake culture?

One may also utilize a **GasPak (BBL) anaerobic system** to cultivate anaerobes. Cultures are placed in the jar along with an envelope containing chemicals that release hydrogen and carbon dioxide when activated by water. Water is added to the envelope; the jar is then sealed. In the presence of the palladium catalyst, hydrogen reacts with oxygen forming water. This reaction removes free oxygen. A haze of water droplets forms on the

inside walls of the jar, and the lid above the catalyst chamber warms slightly as the reaction progresses. But how does the microbiologist know that the jar is providing a sufficiently reduced environment? To evaluate the oxidation reduction potential in the jar, the scientist always includes a **redox potential indicator** (a device that shows whether free oxygen is present).

There are dyes that are colorless in a reduced environment. **Litmus, resazurin, and methylene blue**, for example, show color only when in an oxidized environment. These dyes are excellent redox potential indicators. The standard GasPak anaerobic indicator is a methylene blue-saturated pad in a sealed, peel-apart foil package.

One type of medium often used to grow anaerobes is **Fluid Thioglycollate Medium**. Reducing compounds such as **sodium thioglycollate** are incorporated into certain media so that the medium itself provides a reduced environment. Sodium thioglycollate binds free oxygen. Fluid Thioglycollate Medium also contains the redox potential indicator **resazurin**, which turns pink in an oxidized environment. For cultivation of anaerobes, special precautions are taken. In a large laboratory, the microbiologist may inoculate anaerobic cultures inside an **anaerobic glove box**. Some laboratories utilize **pre-reduced anaerobically sterilized (PRAS)** media. These are sealed, oxygen-free tubes of resazurin-laced, reduced media. The media are liquified, tempered, and then injected with anaerobes. By rolling the tubes as the agar solidifies, the **Hungate roll-tube method** produces a thin layer of medium, with separate, well-isolated colonies in PRAS media.

### **The Candle Jar**

A **candle jar** is a wide mouthed jar into which the microbiologist places a damp piece of paper, cultures, and a lighted candle. The apparatus is then tightly sealed; after a few minutes the flame goes out. The humid atmosphere that remains in the jar provides higher CO<sub>2</sub> and lower oxygen concentrations than are normally found in air. The usual levels of these gases in air are approximately 21% O<sub>2</sub> and 0.3% CO<sub>2</sub>. In a candle jar, the amounts are altered to about 16% O<sub>2</sub> and 4% CO<sub>2</sub>. Some **fastidious** (delicate, requiring special nutrients and/or growth conditions) organisms grow best in this environment. Pathogens that are generally cultured in a candle jar include ***Neisseria gonorrhoeae***, which causes gonorrhoea, and ***Haemophilus influenzae***, frequently the etiologic agent of meningitis and otitis media in infants.

## A. Procedure. – Oxygen, temperature, pH and NaCl

### Day 1

1. Draw lines on the bottom of 6 R2A plates to divide them into 2 sectors. Label each sector of the plate with the name of your known or unknown organisms.
2. Using your original streak plate as the source of the inocula, streak the organisms in the appropriate sector of each plate.  
 Label 1 plate “anaerobic” and place in the GasPak chamber on the front bench (the chambers will be “activated” after all of the plates are ready).  
 Label 1 plate “4°C” and place in the box to go into the refrigerator  
 Label 1 plate “22°C” and place in the RT incubator  
 Label 1 plate “30°C” and place in the 30°C incubator  
 Label 1 plate “37°C” and place in the 37°C incubator  
 Label 1 plate “44°C” and place in the 44°C incubator
3. Label the variable pH and NaCl R2A plates such that 4 students can “patch” their known and unknown organisms onto the plate. Use an inoculating needle to draw lines with each organism onto the plates.

### Day 2

4. Pipette 1 mL of hydrogen peroxide into two 13 x 100mm glass tubes. Label one for your known and one for unknown.
5. Draw lines on a piece of filter paper to divide it into 2 sectors. Label 1 sector for each known and unknown.
6. Obtain a large inoculum of each organism from the 30°C plate on a cotton swab, smear onto the appropriate sector of the filter paper, then place the swab into a tube of hydrogen peroxide and observe for the presence of bubbles (a positive catalase reaction). What are the bubbles that are produced?
7. Break an ampule of oxidase reagent and place one or two drops onto the bacteria that were smeared on the filter paper. A rapidly developing purple color is a positive result.

Organism	4°C	20°C	30°C	37°C	44°C	gaspak	oxidase	catalase

Organism	Growth at pH	No growth at pH	Growth at [NaCl]	No growth at [NaCl]