Exercise 1 Anaerobic Microbiology Lab

Organisms can be divided into three groups based on their metabolism in the presence or absence of oxygen. Obligate aerobes are organisms which have only respiratory metabolism and require (usually) the presence of oxygen. Obligate anaerobes are unable to use oxygen as an electron acceptor for respiratory processes. Obligate anaerobes can be further divided into two groups: strict anaerobes are obligate anaerobes which cannot tolerate the presence of detectable concentrations of oxygen; aerotolerant anaerobes are also obligate anaerobes but these can grow in the presence of oxygen, often up to atmospheric concentrations. Facultative organisms can use oxygen as an electron acceptor for respiratory processes if it is present, however, in the absence of oxygen, they are able to gain energy through other processes.

For this exercise, you have four different organisms. Each organism will be used to inoculate two tube of aerobic medium (different sized tubes), one tube of anaerobic medium and two aerobic flasks or bottles containing media. The medium used for this exercise is Brain Heart Infusion (BHI). All tubes contain 15 ml of medium, the flasks contain 45 ml of medium. Label each tube with the organism code, your name, and the date. Each student (or pair) will need eight tubes of sterile aerobic medium (four of each size) and four tubes of sterile anaerobic medium, and eight flasks or bottles. Reversed-osmosis (R/O) water will be used for blanks. Sterile medium will be used to determine the absorbance of the uninoculated medium.

Inoculate each tube with 0.1 ml of the appropriate culture. Inoculate each flask with 0.3 ml of the appropriate culture. Zero the spectronic 20 at 600 nm using R/O water. Identify a second cuvette that will also read zero when filled with R/O water and use this for the sample cuvette. Read and record the optical density at 600 nm for the sterile medium in the aerobic tube. Read and record the optical density at 600 nm for each inoculated aerobic flask and tube (you will need to aseptically remove ~ 3 ml from the aerobic tubes and flasks and measure them using a cuvette). **Vortex or otherwise mix each tube/flask before sampling and each cuvette before reading the optical density to be sure all particles are well suspended.** Discard the liquid in the cuvette into a metal bowl and rinse with R/O water (also discarded into the metal bowl). **Remember, live cultures cannot go down the sink.** Repeat the procedure for the anaerobic tubes (zero spec using water, read and record the OD of the sterile anaerobic medium and each inoculated anaerobic tube). You will need to aseptically withdraw about 3 ml of liquid from each tube using a sterile syringe and needle. Be sure to always line up the cuvettes the same way each time (place the trademark in the center front of the cuvette holder). This will reduce problems with differing optical density due to differences in the glass, scratches, etc. which will influence the amount of light passing through the sample to the detector.

After inoculation, the half the flasks will be shaken to maximize surface area for diffusion of oxygen into the aerobic flasks, the other set will be incubated statically. Incubation will be at 30° C in the dark.

In your notebook record all data (include medium used, tubes types and sizes used (125 ml flasks for aerobic incubations; Bellco aluminum seal tubes (commonly known as Balch tubes) with 1 cm butyl rubber stoppers secured with aluminum crimp seals for anaerobic tubes), measure other tubes used, time and date

of inoculation, amount of inoculum added, instrument used for reading optical density (Milton Roy Spectronic 20), wavelength of light used (600 nm), type of blank used for zeroing spec, how samples are mixed before sampling). Record all readings. Be sure all information is clearly labeled. Record how the tubes are incubated (30° C, static conditions, dark, record the speed if shaken).

During the next laboratory period, repeat readings of the optical density on all sets of tubes using the appropriate blanks. The optical density of a cell culture is roughly proportional to bacterial concentration (dry wt./vol). In general, if the optical density (OD) of culture 1 is twice as high as the OD of culture 2 (1 and 2 are cultures of the same organism) the cell mass/vol in culture 1 is twice than in culture 2. At higher absorbance values, the linearity is lost and the line curves and flattens. For this reason, in order to get a better value, any culture reading above 0.4 absorbance units should be diluted, a reading obtained on this dilution using the dilution medium as a blank, and the resulting absorbance multiplied by the reciprocal of the dilution factor in order to correct for the dilution performed. For instance, if you add 1 ml of your culture to a dilution blank containing 5 ml of a diluent, the dilution factor is 1 ml/(1 ml + 5 ml) = 1/6. The reciprocal of the dilution factor is 1/(1/6) = 6. So if your optical density reading was 0.3 on the dilution, the corrected O.D. of your culture would be 0.3 x 6 or 1.8.

If your initial O.D. reading is too high and you dilute the sample and the O.D. is still above 0.4, you would dilute the dilution again. If your reading of the first 1/6 dilution was 0.6, you should make an additional dilution (in this case 1 ml of the dilution added to 5 ml of a fresh dilution blank). If the reading on the second dilution is 0.15, the OD of the first dilution is 0.15 x 6 = 0.9. However, the first dilution has to be corrected for that back to the original culture so the OD of the culture is 0.9 x 6 = 5.4.

You will need one dilution blank for each tube with an $OD_{600} > 0.4$. **Note: the cells may tend to settle out or clump. Be sure to vortex or otherwise mix the tubes before making dilutions.** Determine the OD_{600} for each of your four cultures under anaerobic and aerobic conditions.

Subtract the initial reading for each tube (OD_{600} for each tube - the OD_{600} of the media blank for that tube) to get an estimate of the starting OD. After taking the readings on Thurs. Sept. 19, determine the final OD_{600} for each tube by taking the final OD (corrected for dilution if necessary) - the OD of the media blank. Subtract the initial corrected OD for each tube from the final corrected OD to get an estimate of how much the culture has grown in the week of incubation.

Pool the class final data for all four organisms under both sets of conditions. Get a copy of the results for the entire class before you leave. Either individually or as a pair (or larger group), come to a conclusion as to which category each organism should be place (obligate aerobe, obligate anaerobe (strict), obligate anaerobe (aerotolerant), or facultative). **Hint: think about relative energy yields. Generally, the higher the energy yield obtained during metabolism of a substrate, the greater the cell mass produced. Use your text (or other resources available) to predict relative energy yields of aerobic respiration vs fermentative pathways.**

Individually, write a short paragraph assigning each organism to one of the four categories (strict anaerobe and aerotolerant anaerobe will be two separate categories) and explain your reasoning. This exercise will be worth five points. Clarity of expression is critical. Please write legibly and in complete sentences. Cite any information taken from

a book or journal using the format in the journal Applied and Environmental Microbiology. (You can access articles from this and other ASM journal that are over 6 months old at http://www.asmusa.org, go to the Search ASM journals link).

Use of Spectronic 20

The knob on the left front turns the instrument on. It should be allowed to warm up for ~ 15 minutes before use. To zero the instrument, be sure desired wavelength is selected. With the spectrophotometer empty and the cover in place, set the absorbance to ∞ (0% transmittance) using the left knob. Place the appropriate blank in the spec 20 and cover the top of the tube to prevent entrance of extraneous light from the room. Set the absorbance to 0 (100% transmittance) using the right knob. Remove blank, cover adaptor, and if the absorbance doesn't read ∞, then reset using the left knob. Check with the blank in place to see if the absorbance reads zero. If it does not, rezero with the right knob. Continue until the correct readings are obtained for the empty spec and for the blank. **(Note: there is a mirror behind the dial on the spec. To increase reproducibility, look at the dial when zeroing or making readings such that you see no reflection of the needle in the mirror.)**

Use some reference point on each cuvette or place a mark near the top. Each time the cuvette is placed in the spec, orient it in the same way with respect to the mark. Also, before placing tube in the spec, wipe the tube with a KimWipe to remove any extraneous dirt, grease, water, etc. on the outside of the tube which will negatively affect the reading obtaining. Always use the same type of tube for the blank as that containing your culture.