MCB 3020L **Lab Experiment 5a & 5b**

Winogradsky Column (5a) and Enrichment Culture (5b)

A long term (5a) and Three Session Experiment

5a Winogradsky Column. The Winogradsky column is made to provide enrichment culture for anaerobic photosynthetic bacteria and sulfur cycle bacteria from a natural assemblage of organisms and substrates (lake sediment). This column is made from the sediment mixed with certain nutrients (cellulose, phosphate, sulfate and carbonate) and placed under certain physiological conditions so that the growth of photosynthetic anaerobes is encouraged. When it is first made it is sealed and left for several days in the dark allowing the aerobic heterotrophs to "bloom" and utilize up all the available oxygen (where is it?) in the column thereby making it anaerobic. This is the first of a succession of organisms that will occur in the column. Each group of microbes will accumulate in particular regions of the column establishing different physiological areas and gradients: that is the column will contain many "niches".

After the first bloom of aerobic heterotrophs makes the column anaerobic, then the column is unwrapped and exposed to light. After removal of oxygen, there will occur a succession of anaerobes. Fermenters will begin to proliferate (producing what?), anaerobic respirers will also grow utilizing sulfate or thiosulfate as their terminal electron acceptor and reducing them ultimately to _____? After this, the growth of the photosynthetic anaerobes can occur: bacteria with either purple or green color that utilize either small molecular weight organics or reduced sulfur compounds for their electron donors in bacterial photosynthesis. These enrichments could also contain cyanobacteria that are anaerobic/sulfide tolerant, but use H_2O as their electron donor. Such cyanobacteria could create an aerobic zone (surrounded by the anaerobic conditions) in the column. The columns will be observed over several weeks and when photosynthetic bacteria (purple or green patches) appear, they will be examined and cultured further.

You will turn in separate reports for the Winogradsky Column and Enrichment Culture.

Materials per Pair

Lab Session One

1. One 16 x 150 mm culture tube.
2. Na_2SO_4, $NaHCO_3$, K_2HPO_4, Na_2SSO_3, NH_4Cl, Whatman filter paper, scissors.
3. 100 ml and 250 ml beakers, aluminum foil.
4. Incandescent lamp (30 to 60 watt).
5. Balances, weighing boats, and spatulas.
6. Three sediment samples (at least half of a 600 ml beaker with some overlying lake water): from OE Lake, Old Dorm Lake, Gracies Lake, New Dorm Lake, Trail Enterance Lake (East and West); Golden Panther Arena Lake. These samples should be in the lab BEFORE the lab session begins.
7. Rubber stoppers that fit a 16 mm culture tube tightly.

8. Other organic sources.

Some Later Lab Session

1. 1 13x100 mm screw cap tube completely filled with sterile PNSB broth.
2. 1 13x100 mm screw cap tube completely filled with sterile PSB broth.
3. Sterile disposable Pasteur pipettes.
4. Gram staining materials.

Last Lab Session

5. Spectrophotometer
6. 4 cuvettes
7. 1 tube of sterile PSB and PNSB broth for blanks
8. 1000 µl pipette tips

Procedure

Lab Session 1 **- Winogradsky Column**

1. Each pair will work as a group using one of the FIU lake sediment samples.

2. Place about 1 gram of *finely shredded* strips of filter paper in the 100 ml beaker. All other pairs will use a carbon-energy source of their own choosing...add 0.5 gram of this material to the 100 ml beaker. Be sure to record what you choose, and the exact amount.

2. Mix about 30 grams of lake sediment in the 100 ml beaker along with 0.1 gram of Na_2SO_4 or $Na_2S_2O_3$. Each group can decide on whether they will use sulfate or thiosulfate as the sulfur source for photosynthesis. What are the chemical structures and redox levels of these sulfur sources? Can they serve as electron donors or acceptors or both? Each group will then add 0.1 gram each of $NaHCO_3$, and NH_4Cl, and K_2HPO_4 to make a thick slurry with some of the overlying lake water. **Do not over aerate** (mix) the slurry. Make sure you record your chemical selections!

3. Carefully pour the amended slurry with the filter paper into a 16 x 150 mm tube almost to the very top. You will not use the entire mixture. Mix the slurry in so that no air bubbles remain. You might have to use some of the lake's overlying water. The tube should be more than 3/4 full of sediment after standing, the closer the sediment is to the top the better. Add more if needed. Fill to the top with the overlying slurry water and securely cap with a rubber stopper. This is a Winogradsky column.

4. Cover the entire column with aluminum foil so that no light can enter. Incubate at room temperature for two lab periods.

One Week Later**-Winogradky Column**

1. At the end of this time, remove the foil and position the column about 30 to 50 cm from an incandescent light. Do not get it so close as to heat the column. *BE SURE* to **mark the column** to indicate which side faces the LIGHT. It is extremely important that this side ALWAYS faces the light.

5. Observe the column at weekly intervals. When distinctly purple or green patches occur, begin the second "Winogradsky" lab. Be sure you have weekly documentation of changes in your Winogradsky column.

Some Later Lab Session - A month after making the Winogradsky Column

1. Remove a small portion of a red or green spot (or other chromogenic spot) to a slide and cover with a coverslip. Observe first with low, then with the high dry and oil immersion lens. What types of microbes are present? Is this a pure culture? Do any have sulfur granules? Are there spirilla or other morphological forms?

2. With a sterile Pasteur pipette, add a small drop of the red or green clump to each of the Purple Nonsulfur Bacteria medium (PNSB) and to the Purple Sulfur Bacterium medium (PSB). Note that these broth media completely fill the tubes; they are made anaerobic by autoclaving. Therefore try to minimize the time these tubes are open. Make sure they are closed immediately without introducing air. Incubate in front of the incandescent lamp as you did for the Winogradsky column.

Last Winogradsky Lab Session

1. Observe the tubes. Was growth obtained in both? Examine each medium that shows growth as wet mounts. Are they composed of the same cells? Is this a pure culture?

2. If available, place uninoculated medium in the reference and sample cuvettes of a double beam spectrophotometer. Blank the instrument. Remove the sample cuvette and replace it with the culture of the photosynthetic bacteria. You may have to adjust the concentration of cells if it is too thick...to do this set the spectrophotometer at 410 nm and adjust the concentration of cells by dilution with uninoculated medium so that there is an OD of less than 1.0, but more than 0.5. Then scan from 400 to 900 nm, every 50 nm. Draw a graph with nm on the X axis and absorbency units on the Y axis.

5b Enrichment Culture. This experiment will enrich for microbes that can utilize a particular (unusual?) carbon and energy source. You select your microbe source. The microbiological sample is material from the environment where you suspect that bacteria exist that can use your carbon and energy source. You now need to select your enrichment medium from the list at the back of this lab. Place your order for the unique carbon and energy source with the Lab Instructor. You will need to identify each of the following in your media:

A. *Carbon and energy sources* can be at high concentrations (1-3%) but for bacteria that live in low nutrient environments, this could be inhibitory.

B. *Nitrogen source*. Is your suspected microbe a prototroph or auxotroph? Nitrogen can come from many forms: the most reduced inorganic form is NH_4^+ to the most oxidized, NO_3^-. It can be neutral, N_2 or organic such as amine, imine and azole nitrogens in amino acids and nucleotides.

C. *Phosphorus and Sulfur sources*. These are the easiest: all microbes can use sulfate as a sulfur source, and phosphate as the phosphorus source. These can be added as simple sulfate and phosphate salts...what are those?

D. *Trace mineral sources*. Remember that life requires zinc, manganese, calcium, nickle, cobalt, etc....all at micro concentrations. How do these get into the medium?

You will turn in separate reports for the Winogradsky Column and Enrichment Culture.

Materials

Lab Session 1 - Enrichment Culture

1. Selected Enrichment Culture liquid broth (50 ml in a 125 ml screw cap flask).

Lab Session 2 - Enrichment Culture

1. 1 Enrichment Culture Agar plate
2. 1 Nutrient Agar plate
3. Gram staining reagents

Lab Session 3 - Enrichment Culture

1. 1 Enrichment Culture Agar plate
2. 1 Nutrient Agar plate
3. Gram staining reagents

Procedures

***Lab Session 1* - Enrichment Culture**

1. Students give oral presentations on a unique bacterium they will attempt to isolate. Their presentation will include the kind of ecological niche that bacterium could be found and how the enrichment medium is designed to allow pure culture of a totally unique bacterium.

2. Use a sample from nature, your home, FIU campus, where-ever to inoculate your enrichment broth with about a half gram. Decide what temperature you need to incubate this culture. Will the broth require shaking (aeration)? or standing (microaerophilic conditions). Is the broth clear or cloudy or colored? Incubate until next period.

***Lab Session 2* - Enrichment Culture**

1. Check the physical condition of the enrichment broth. Does it have turbidity? Observe it in the microscope using a live mount (see Lab 1 if you need to). Are your microbes motile? Then Gram stain the sample to get a rough count of the Gram positive and Gram negative cells in your original sample. This may require a dilution.

2. If there are bacteria growing in the medium, then isolate a pure culture. Streak both your medium agar plate and a Nutrient Agar plate. Do this by dipping a sterile loop into your culture and performing an isolation streak. Check it in the next period.

***Lab Session 3* - Enrichment Culture**

1. What is the difference between the colonies of your medium and Nutrient Agar. Gram stain these isolates. Are they the same? Do you have a pure culture? If so streak a slant to keep a stock culture of this isolate. If you do not, perform another isolation streak. Keep doing this until you obtain a pure culture. Although you will turn in your lab report, it is important to obtain a pure culture to use for Lab 9.

Questions

1. What is the function of each chemical (including the filter paper) added in the Winogradky Column? Your enrichment media?

2. What would have happened had the column not been wrapped in aluminum foil for the first incubation?

4. After a while, black zones often appear in the Winogradsky Column. What is that due to?

5. Why is it that the tubes can not be incubated very close to the light? (Two reasons!)

6. Identify the various pigments in the cells by the spectra obtained in the last Winogradsky Column period.

7. Did you isolate your target bacteria in your Enrichment Culture? How do you know?

Textbook Ref: Brock: Biology of Microorganisms, Sections 5.1-5.3, 18.1-18.2

Enrichment Culture Medias

1. Medium for Very Halophilic Bacteria: Tryptone, 1 g; Yeast Extract, 1 g; Solar Salt, 50 g; distilled water, 200 ml. pH, 7.5. Solid media 1.8% agar.
2. Medium for Proteolytic Bacteria: Skim milk, 2% in Nutrient Broth; distilled water, 200 ml. pH, 7.5. Solid media 1.5% agar.
3. Medium for Nitrifying Bacteria: $(NH_4)_2SO_4$, 0.01M; NaCl, 0.005M; KH_2PO_4, 0.001M; $MgSO_47H_2O$, 0.001 M; $CaCO_3$, 1 g; distilled water, 200 ml. Solid media 1.5% agar.
4. Medium for Actinomycetes: Glycerol, 2 mL; $NaNO_3$, 0.02; KH_2PO_4, 0.1 g; $MgSO_47H_2O$, 0.05 g; KCl, 0.05 g; $FeSO_47H_2O$ 1 mg; distilled water, 200 ml. pH 7.5. Solid media 1.5% agar.
5. Medium for Agar Digesting Bacteria: Solar Salts, 3%; Agar, 2 g; Yeast Extract, 0.02%; distilled water, 200 ml. pH, 7.5. Solid media only.
6. Medium for Lactic Acid Bacteria: Peptone, 1 g; beef extract, 1 g; glucose, 0.5 g; Tween 80, 0.1 ml; ammonium citrate, 0.2 g; Na-acetate, 0.05g; $MgSO_47H_2O$, 0.01 g; Na_2HPO4, 0.1 g; distilled water, 200 ml. pH 6.5. Solid media 1.5% agar.