Name_____ Date_____ Hour_____

Bioprospecting: Individual Isolate Method

Part 1: Pre-Test

1. What is the most abundant organic compound on Earth?
   a. Cellulose
   b. Water
   c. Starch
   d. DNA

Write the letter of the correct definition in the blank in front of each term.

2. _____ Cellulose
3. _____ Cellulase
4. _____ Feedstock

a. the raw material used to make biofuel
b. a carbohydrate found in plants
c. an enzyme found in some decomposers that help break down plant material

5. Most biofuel ethanol sold today is made from _____ or _____.

6. What are the 3 main components of plant cell walls?

7. Why is it more difficult to make ethanol from fibrous plant material rather than corn grain or sugar cane?

8. What is an enzyme?

9. Where would you look in the environment to find microbes that break down plant material? Why?

10. Define symbiosis. List and explain at least 3 different types of symbiosis.

Name_____ Date_____ Hour_____

Part 2: Introductory Questions, Choosing a Sample & Experimental Design

Introductory Questions

Termites eat wood, yet they cannot access energy from the complex carbohydrates in their food on their own. Microbes, such as fungi and bacteria, co-evolved in the gut of the termite to break down the cell walls to allow the termites to access the resources they need from the wood. Many examples of similar symbiotic relationships exist where communities of microbes break apart the lignin, hemicellulose, and cellulose in the plant cell walls to expose the glucose for other organisms. The microbes that consume plant material are an important part of the nutrient cycle as the raw materials are returned to the environment for new plant and microbial growth. Fungi and bacteria are the only organisms that are capable of digesting lignin, hemicellulose and cellulose.

In this activity, we will be exploring these symbiotic relationships as we isolate microbes that have evolved to degrade the cellulose component of plant material. Cellulases, which break complex cellulose strings into simple glucose molecules, from these microbes are highly sought after for use in industrial biofuel production. Once the plant material has been broken down, the remaining glucose and other simple sugars can be fermented into ethanol or other fuels. *Bioprospecting* is the process used to search for these organisms, which are then studied for their potential to increase the efficiency with which we make biofuels from a variety of plant materials.

1. What are the major components of a plant cell wall?

2. Which of these components is most easily broken down into glucose monomers?

3. Why is glucose useful for organisms? Why is glucose useful for biofuel production?

4. What role do these microbes play in their ecosystem?

Name_____ Date_____ Hour_____

Choosing a Sample

Each student should bring in an environmental sample and be prepared to share their answer to Question #1.

In groups of 2-4 students, discuss the following questions.

1. Why would you expect to find cellulose-degrading microbes in the sample? (List the name of the sample and the answer for each.)
   -
   -
   -
   -

2. Of the samples within your group, which one do you believe will be the best candidate for locating cellulose-degrading microbes? List your group answer and be prepared to defend your group decision in class discussion.

Class Discussion – Discuss the following questions and answer them as a class.

3. Which environmental sample will have the greatest abundance of cellulose-degrading microbes? Why?

4. Which environmental sample will have the greatest variety (diversity) of cellulose-degrading microbes? Why?

Name_____ Date_____ Hour_____

Experimental Design

In your group**,** design your experiment setup by answering the following questions. This design must be approved by your instructor before you begin. Your group will be able to test 6 different sampling processes, and you will prepare two plates of each for a total of 12 plates.

1. Where in your environmental sample do you expect to find the highest concentration of microbes containing cellulase? (surface, interior, top, bottom, inside/outside cell, etc.)

2. How will you process your environmental sample to reach those microbes (rub it onto the plate, grind it, dilute it, heat it, etc.)?

3. Will you surface sterilize your sample or not? Why?

4. Your group will be performing a serial dilution of your processed sample. Which of the dilutions do you believe will be most useful (0, -1, -2, -3)? Explain. (see the serial dilution step in the *Processing Techniques* page to help make your decision)

5. Are there any safety concerns with your chosen sample that you will need to consider? Explain.

6. List any other questions that arose during your group discussion and list them and the reasoning here.

7. List the 6 different sampling processes that you will use and the labels you will use. (You will have 2 of each plate, label accordingly)

Sampling Process	**Label**
Example: surface sterilize and grind - 1 dilution	SSG - 1 #1 SSG - 1 #2

8. Hypothesis A: Which sampling process will reveal the greatest diversity of microbes? Why?

9. Hypothesis B: Which of your sampling processes will give you the highest overall number (abundance of) cellulase-producing microbes? Why?

_____ _____

Teacher Approval Date

Name_____ Date_____ Hour_____

Part 3: Processing Samples, Preparing Media & Spreading Plates

Processing Samples

After selecting your environmental sample, follow your experiment design plan to process your samples. Below is a list of several procedures that you may use depending on the processing method that you have chosen. Every group will do the serial dilution, but you may be using different concentrations.

Surface Sterilization

1. Place sample in a 0.8% bleach solution (enough to cover the sample) for 3 minutes.

2. Remove the sample and rinse with distilled water.

Rub

The sample may be rubbed directly onto the plate using a sterile spreader or forceps. This can be done before or after it has been surface sterilized. You can remove the sample from the plate or leave it on the plate after you rub it.

Serial Dilution (see figure below)

1. Prepare 2mL tubes, one tube for each of the processes you have chosen in your design (this DOES NOT include the serial dilutions themselves or the rubs). Label them according to your table.

2. Using a sterile pipette, aliquot 1mL of 1X PBS Buffer into each of the tubes.

3. Place a small amount of the processed sample into each of their corresponding tubes.

4. Using a small sterile pestle, crush the sample into the buffer. Add a '0' to the end of the label on these tubes.

5. Prepare three 2mL tubes for each original tube you just created. They should contain the same label as the original tube. Add one of the following labels to each of the three tubes to represent their dilution (-1, -2, -3).

6. Using a sterile pipette, aliquot 900uL of 1X PBS buffer into each of these tubes.

7. To create a -1 dilution (1:10), use a sterile pipette and remove 100uL from the '0' tube and place into a tube with -1 label. Pipette up and down several times to mix.

8. Using the same pipette tip, remove 100uL of solution from -1 and add it to the tube labeled -2. Pipette up and down several times to mix.

9. Using the same pipette tip, remove 100uL of solution from -2 and add it to the tube labeled -3. Pipette up and down several times to mix.

10. Repeat steps #3-#9 for each of the processing methods.

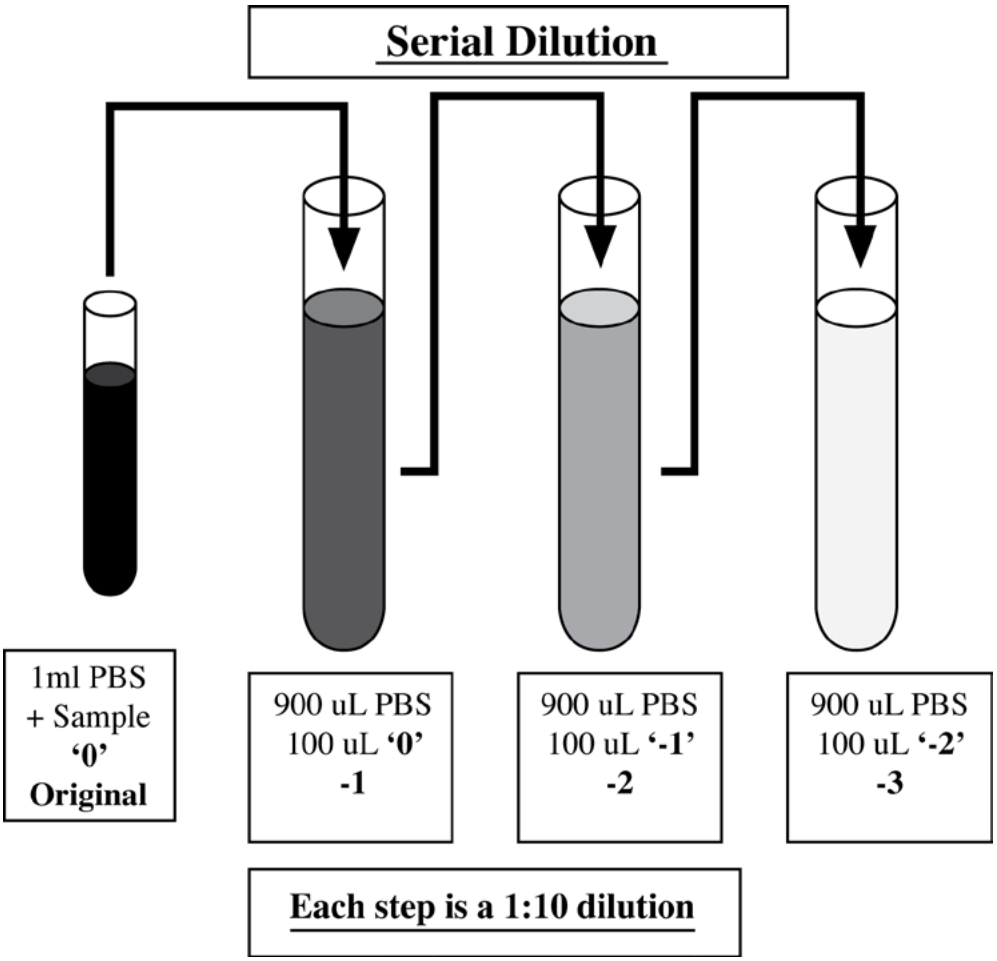


Image adapted from - Resource materials for the Biology core courses - Bates College 'How to Make Simple Solutions and Dilutions' *http://abacus.bates.edu/~ganderso/biology/resources/dilutions.html*

Name_____ Date_____ Hour_____

Preparing Media

Media is the material that the microbes will be growing on. Most media contain a supply of nutrients and moisture, and some contain agar (a gelatinous substance derived from seaweed). In this lab you will be using several types of media depending on the results that you want to achieve. Nutrient-rich media are easy to see growth on, but they are also very easily contaminated. A nutrient-poor or minimal media has limited nutrients so as to select a specific microbe that can break down the chosen nutrient. Minimal media is less likely to become contaminated, but you may need to wait longer to see growth. Discuss with your instructor which types of media your class will be using. Your instructor may have already prepared the media for you. Below are recipes for the different types of media that you may need. All media should be prepared in a glass flask twice the volume of media that you are making to prevent boiling over during heating.

Microcrystalline Cellulose Agar (Micro) – minimal media used to test for the presence of cellulose degrading microbes. The cellulose present is very difficult to break down. Microbes need both endocellulases and exocellulases to grow on this media. Therefore, it is a selective media for the cellulose degrading microbes.

5g Microcrystalline Cellulose
15g Agar
1000mL H_2O

Cover with aluminum foil and place a hotplate and stir to dissolve agar (or Autoclave).

Carboxymethyl Cellulose Agar (CMC) – minimal media used to test for the presence of cellulose degrading microbes. This cellulose media is used for the Congo Red Assay to screen for the presence of cellulases.

5g Carboxymethyl Cellulose
15g Agar
1000mL H_2O

Cover with aluminum foil and place a hotplate and stir to dissolve agar (or Autoclave).

Microcrystalline Cellulose Liquid Media (liquid micro) – minimal media, used to screen for extra cellular enzymes in the supernatant Congo Red Assay.

5g Microcrystalline Cellulose
1000mL H_2O

Stir vigorously to dissolve. The microcrystalline does not stay in solution, so you will have to shake/stir it before each use.

Yeast Malt Extract Agar (YMEA) – nutrient rich media, non-selective. This media is very easy to grow on. There are lots of different sugars, so it is easier to identify the microbes and you will get greater numbers of colonies. Microbes will look differently on the YMEA than on the cellulose media.

4g Yeast Extract
10g Malt Extract
4g Dextrose
15g Agar
1000mL H2O

Cover with aluminum foil and place a hotplate and stir to dissolve agar (or Autoclave).

Pouring Agar Plates

1. Set out the Petri dishes and make sure they are right-side up. One liter of agar should pour between 24 and 30 plates depending on the thickness.
2. 50°C is the optimum temperature to pour plates, any cooler and the agar may solidify before you are done pouring or can form bubbles.
3. Open the plates only far enough to pour. Pour plates to half the volume of the Petri dish and cover immediately to prevent contamination.
4. Leave plates at room temperature for 24 hours to cool.
5. After 24 hours, plates should be stored at room temperature. They can be stored directly in the sleeves that the plates came in. Be sure to label which media is in which plate. A colored line on the side of each plate works well; color-code the different types of media being used. Plates can be refrigerated if they are not going to be used within a month.

- For more tips on pouring and storing agar plates see 'Tips for Storing and Pouring Plates' *http://www.umsl.edu/~microbes/techniques.html*

Name_____ Date_____ Hour_____

Procedure #1 – Spreading the plates

After processing the environmental sample, you will spread each of your different processing methods on two plates. Make sure that your plates are labeled accordingly. Your duplicate plates should be identified (i.e. #1 and #2).

1. Each group should pre-label 12 microcrystalline cellulose (micro) plates. These labels should be on the outside edge of a pre-poured Petri dish. Don't forget the date and the group name or initials.
2. Put on safety goggles and gloves. To plate samples from the 2mL centrifuge tubes, use a sterile pipette tip to aliquot 10uL of processed sample into the center of the plate.
3. Using a sterile spreader, spread the sample all over the plate until it looks like it is beginning to be absorbed into the media.
4. Plates should be left right-side up for several minutes to allow the sample to have good contact with the agar.

5. Parafilm should be used to seal the edge of the plates, and they should be stored upside-down for 3-5 days at 30-33°C. (If there is not an incubator available, they should be stored at room temperature for one week).

- If you are performing a rub, the sample should be rubbed directly onto the microcrystalline plate using a sterile spreader. You can choose to leave the sample embedded into the center of the plate or remove it after you have rubbed it onto the plate.

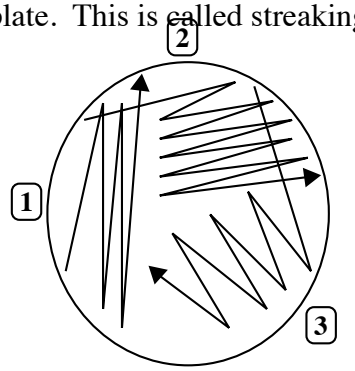
Name_____ Date_____ Hour_____

Part 4: Streaking for Isolation

Procedure #2 – Streak for Isolation

After 3-5 days of incubation, you should start to see some growth on the microcrystalline cellulose plates. This is an indication that these microbes can degrade cellulose. You will most likely have a variety of different microbes on this plate. In this procedure you are going to select the most interesting colonies and try to separate them from the others.

1. Put on safety goggles and gloves. Prepare 24 microcrystalline cellulose (micro) plates for each group. This time you will label as you isolate, not before.
2. Choose 4 interesting microbes from each of the 6 different processes. These should not be touching other microbes on the plate, this will make it easier to avoid getting more than one microbe when you isolate. Using a permanent marker, on the bottom of the Petri dish; circle the colonies you are going to choose and label them A, B, C, D. You can choose from either of your duplicate plates for each process.
3. Label the plates immediately before you isolate. The label on the second micro plate should match the plate the microbe was taken from, but add the letter of the microbe that was taken from that plate. Using a sterile inoculating loop, carefully open the cover of the original Petri dish and pick a small amount of the chosen microbe. Close the plate, and open the new microcrystalline cellulose plate just enough to streak the microbe onto one quarter or one third of the plate. This is called streaking for isolation or quadrant streaking. See figure below.



4. Allow the plate to set right-side up for the microbe to get a good contact with the plate.
5. Parafilm the edge of the Petri dish and store upside-down and incubate for 3-5 days at 30-33°C. (Incubate for one week at room temperature if an incubator is not available).

YMEA agar plates could be used at this step to speed up the process. It also allows for better morphological identification of microbes. However, it is a nutrient rich media that is more easily contaminated and may need to be re-isolated.

Name_____ Date_____ Hour_____

Part 5: Congo Red Assay

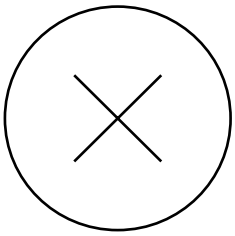
Procedure #3 – Congo Red Assay

After 3-5 days of incubation, you should start to see some growth on the microcrystalline cellulose plates. This is an indication that the microbes you chose can degrade cellulose. You should only see one type of microbe on your plate; this is called a monoculture. If the plate does not have a monoculture, you will need to isolate the microbe again until you get a monoculture. Remember that you are basing your decision of what a monoculture is by what is visually on the plate. There may be contamination that you cannot see, or you may be seeing a group of organisms that are working so closely together that you cannot differentiate visually between them.

Congo Red Assay is used as another screen to check that the selected microbe can degrade cellulose. Carboxymethyl Cellulose (CMC) plates are used for this screening because the Congo Red stain binds to the CMC. Then a NaCl rinse is used to wash away any stain that is not bound to the CMC. If there has been degradation of the cellulose, there should be a clear 'halo' around the sample where there is no longer cellulose for the Congo Red to bind. The microbe present on the plate has used or degraded the cellulose while it was growing on the plate. There are two versions of the Congo Red Assay. The first is the basic Congo Red assay, which looks at the microbe as a whole. The first assay is less sensitive. The second is a supernatant Congo Red assay, which looks for extracellular cellulase enzymes. The supernatant assay is more sensitive and will have fewer positive results.

Wear goggles, gloves and apron. Follow your teachers instructions to avoid staining the table, sinks or your clothes!

1. Basic Congo Red Assay
   a. Choose a colony to isolate and circle the colony using a marker on the bottom of the petri dish. This will make it easier to see. You also want to label the colony if you chose more than one a plate.
   b. Using sterile technique, streak one colony from the microcrystalline cellulose plate into an 'X' in the center of the CMC plate. See figure below. Label your plates accordingly.



c. Incubate for 3-5 days at 30-33°C. (Incubate for one week at room temperature if an incubator is not available).
d. After incubation, record growth on Table 3 before adding the Congo Red dye.
e. Flood the plates with Congo Red Solution (1ug/mL = 0.5g/500mL water), and let sit for 15 minutes.
f. Dump the Congo Red Solution out; make sure you have water running in the sink to prevent staining.
g. Flood the plates with a 3 molar NaCl Solution (87.8g NaCl/500mL water), wait 15 minutes. Dump the NaCl Solution.
h. View results under white light or on a light box. Look for a zone of clearing or no stain where the supernatant was inoculated; this is a positive result.
i. Record the results on Table 2 on the Analyzing Data Sheet.

2. Supernatant Congo Red Assay
   j. Perform a serial dilution adding one colony from the microcrystalline cellulose into 1mL PBS buffer. (Follow serial dilution procedure used earlier in the lab).
   k. Label the 2 mL tubes accordingly.
   l. Aliquot 100uL of solution from each of the -2 and -3 tubes into a 15mL conical tube containing 10mL microcrystalline liquid media. Label the 15mL tubes accordingly.
   m. Incubate for 3-5 days at 30-33°C. (Incubate for one week at room temperature if an incubator is not available).
   n. Record the growth on Table 3 on the Analyzing Data Sheet
   o. Centrifuge the tubes 2500rpm for 15 minutes.
   p. Extract 100uL of the supernatant using a sterile pipette tip and place it in the middle of a Carboxymethyl Cellulose (CMC) plate.
   q. Let the plate sit for 1 hour. They can sit over night, but results may vary.
   r. Flood the plates with Congo Red Solution (1ug/mL = 0.5g/500mL water), and let sit for 15 minutes.
   s. Dump the Congo Red Solution out. Make sure you have water running in the sink to prevent staining.
   t. Flood the plates with a 3 molar NaCl Solution (87.8g NaCl/500mL water) and wait 15 minutes. Dump the NaCl Solution.
   u. View results under white light. Look for a zone of clearing or no stain where the supernatant was inoculated; this is a positive result.
   v. Record the results on Table 3 on the Analyzing Data Sheet.

Name_____ Date_____ Hour_____

Part 6: Data Analysis, Discussion & Post-Test

Observational Data

Table 1. Microcrystalline Cellulose Growth

Label on Plate	**Growth** +/-	**# Different Microbes <u>B</u>acteria vs <u>F</u>ungi**	**Describe colonies chosen for isolation (B/F, color, texture, etc)**

Name_____ Date_____ Hour_____

Table 2. Streaking for Isolation

Label on Plate	**Growth** +/-	**Individual Colonies or Lawn?**	**Does the morphology of the CFU match the original plate?**

Name_____ Date_____ Hour_____

Table 3. Congo Red Assay

Label on Plate	**Growth** +/-	**Contamination** +/-	**Halo** +/-	**Comments**

Name_____ Date_____ Hour_____

Analysis Questions

Review the procedures from this lab. For each step answer the following questions:

- *Why did you perform each step?*
- *What did each step teach you about your microbe?*

1. Choosing a sample –
   -
   -

2. Processing the sample –
   -
   -

3. Serial Dilution –
   -
   -

4. Spread on Micro Plate –
   -
   -

5. Isolate onto Micro Plate –
   -
   -

6. Streak onto CMC Plate –
   -
   -

7. Congo Red Assay –
   -
   -

8. What is the purpose of using each of the different types of media for this experiment?

9. Did you find cellulose-degrading microbes in your environmental sample? Use data to justify your answer.

10. What role do these microbes play in their ecosystem?

11. Was there a difference in cellulase results due to different methods of processing your environmental sample? Explain your answer.

12. Which processing method yielded the most positive results for cellulase containing microbes? Why? (Positive results can refer to the Congo Red Assay or growth on the CMC or Micro media)

13. Which processing method yielded the least positive results? Why? (Positive results can refer to the Congo Red Assay or growth on the CMC or Micro media)

14. Were the results of the two methods comparable? Explain which method gave the most positive results? Use data to justify your answer.

15. If you were to repeat this experiment, what would you do differently? Why? Think both about mistakes that occurred and other questions to investigate.

16. What role do cellulose-degrading microbes serve in the production of biofuels?

Class Discussion Questions

1. Which environmental sample had the greatest abundance of cellulose-degrading microbes? Which had the least? Why do you think that happened? Use data to justify.

2. Which environmental sample had the greatest variety of cellulose-degrading microbes? Why? Which had the least? Why? Use data to justify.

3. How do these results compare to our original class predictions?

4. Which processing method(s) worked the best to isolate microbes?

5. Which processing method(s) worked best for overall growth of the microbes?

6. Which processing method(s) were less successful, and how could you change them to get better results?

7. How do your answers to #4-6 compare to your group hypothesis from page 6?

Name_____ Date_____ Hour_____

Post-Test

1. What is the most abundant organic compound on Earth?
   a. Cellulose
   b. Water
   c. Starch
   d. DNA

Write the letter of the correct definition in the blank in front of each term.

2. _____ Cellulose
3. _____ Cellulase
4. _____ Feedstock

a. the raw material used to make biofuel
b. a carbohydrate found in plants
c. an enzyme found in some decomposers that help break down plant material

5. Most biofuel ethanol sold today is made from _____ or _____.

6. What are the 3 main components of plant cell walls?

7. Why is it more difficult to make ethanol from fibrous plant material rather than corn grain or sugar cane?

8. What is an enzyme?

9. Is pure cellulase enough to break fresh plant material into simple sugars? Why or why not?

10. Where would you look in the environment to find microbes that break down plant material? Why?

11. Define symbiosis. List and explain at least 3 different categories of symbiosis.

12. What type of symbiosis did we investigate during our experiment? Explain.

13. Reflect on <u>at least</u> one thing that you learned from this lab experience that was not mentioned above.