

BIOPROSPECTING: INDIVIDUAL ISOLATE METHOD



Overview: Students investigate locations they believe harbor cellulose-digesting microbes, collect samples, isolate them on selective media, and screen them for cellulase activity. These novel microbes may be useful for the production of cellulosic ethanol. In the process they learn about plating techniques, serial dilutions, symbiotic relationships and enzyme specificity.

LEVELS

High school-Undergraduate

SUBJECTS

Science, Biotechnology

OBJECTIVES

- Identify the role of cellulose-degrading microbes in biofuels production.
- Explain the function of cellulase enzymes and the challenges in breaking down the cellulose within the plant.
- Identify the symbiotic relationships within an environmental sample that allows for the breakdown of plant cell wall components into food (glucose).
- Justify their environmental sample choice.
- Demonstrate how to isolate microbes from an environmental sample.
- Evaluate an environmental sample for cellulose-degrading microbes.

MATERIALS

Bioprospecting package

ACTIVITY TIME

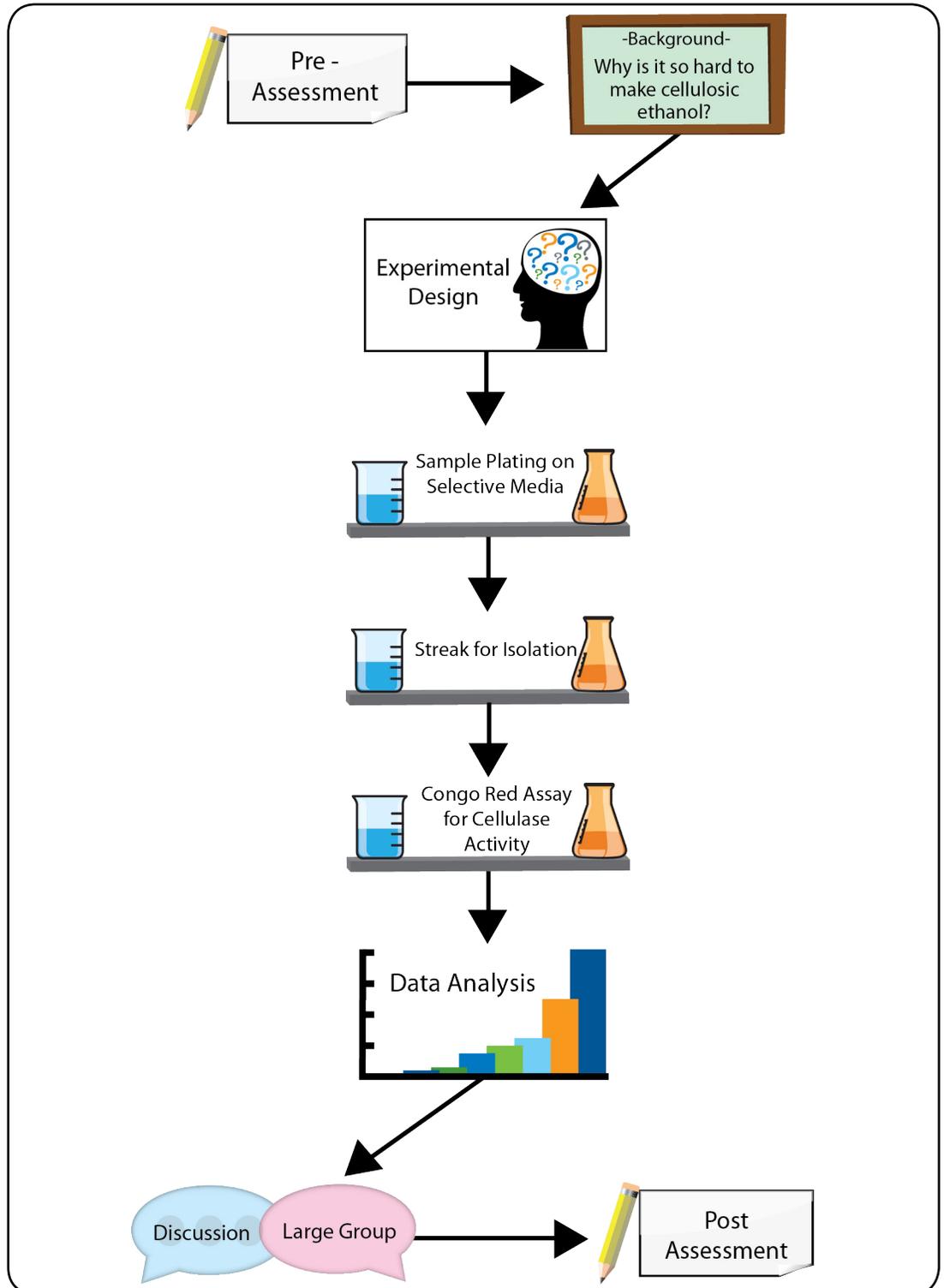
Seven 50-minute class periods over 12-18 days

STANDARDS

Next Generation Science Standards (2013)

- Scientific and Engineering Practices: planning and carrying out investigations; analyzing and interpreting data; constructing explanations and designing solutions
- Disciplinary Core Ideas: ecosystems; engineering design
- Crosscutting Concepts: patterns; cause and effect; energy and matter
- Performance Expectations: See page 3 for details

NGSS Lead States. 2013. Next Generation Science Standards: For States by States. Washington DC: The National Academies Press



For Teachers - Bioprospecting: Individual Isolate Method

Overview:

Students investigate locations they believe might harbor cellulose-digesting microbes, collect samples, isolate them on selective media, and screen them for cellulase activity. These novel microbes may be useful for the production of cellulosic ethanol. In the process they learn about plating techniques, serial dilutions, symbiotic relationships, and enzyme specificity.

This lesson is designed to run over the course of seven 50-minute class periods, which span 12 to 18 calendar days, while waiting for visible microbial growth. This activity provides flexibility based on time constraints, prior knowledge, equipment, and funds.

Learning Outcomes: Students will...

- Identify the role of cellulose-degrading microbes in biofuels production.
- Explain the function of cellulase enzymes and the challenges in breaking down the cellulose within the plant.
- Identify the symbiotic relationships within an environmental sample that allows for the breakdown of plant cell wall components into food (glucose).
- Justify their environmental sample choice.
- Demonstrate how to isolate microbes from an environmental sample.
- Evaluate an environmental sample for cellulose-degrading microbes.

This lesson assumes prior knowledge in symbiotic relationships, types of carbohydrates, and enzymes.

Standards

Next Generation Science Standards (2013)

Performance Expectations

Middle School:

- **MS-LS2-1.** Analyze and interpret data to provide evidence for the effects of resource availability on organisms and populations of organisms in an ecosystem.
- **MS-LS2-3.** Develop a model to describe the cycling of matter and flow of energy among living and nonliving parts of an ecosystem.

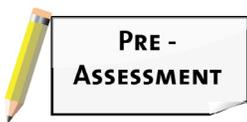
High School:

- **HS-LS2-7.** Design, evaluate, and refine a solution for reducing the impacts of human activities on the environment and biodiversity.
- **HS-ETS1-2.** Design a solution to a complex real-world problem by breaking it down into smaller, more manageable problems that can be solved through engineering.

Scientific and Engineering Practices	Disciplinary Core Ideas	Crosscutting Concepts
Planning and carrying out investigations Analyzing and interpreting data Constructing explanations and designing solutions	LS2: Ecosystems: Interactions, energy, and dynamics ETS1: Engineering design	Patterns Cause and effect: Mechanism and explanation Energy and matter: Flows, cycles and conservation

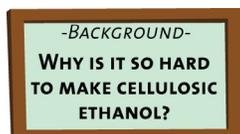
See Appendix for alignment with other standards.

Sequence:



Part 1. Pre-Test – (pages 1-2) (50 min)

Give the pre-test as a formative assessment tool. Provide background on symbiosis, cell wall structure, and the scientific challenge behind making cellulosic ethanol, using both the handout ‘Why is it so difficult to make cellulosic ethanol?’ and the PowerPoint included in this package. The PowerPoint also introduces the lab steps. Additional background material is suggested in the appendix.



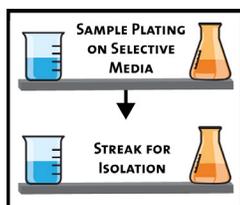
Part 2. Introductory Questions, Choosing a Sample & Experimental Design – (pages 3-6) (15 min day 1, then 1-2 additional class periods)

Hand out pages 3-4, *Introductory Questions* and *Choosing a Sample*.

Complete page 3 together and then assign each student to bring in an environmental sample that they believe to contain cellulose-degrading microbes. Brainstorming where to find cellulose-degraders may be helpful, but may hinder creativity. Caution students to bring samples in sealed containers as samples may contain mold spores and other organisms.



The next day, divide the class into lab groups of four students and allow them time to discuss the samples they brought and decide which one their group will test. Bring the class together and compare the choices from each group using the questions on page 4.



Lab Procedure

Each group should design how to plate their chosen sample. They need to determine 6 ways to test for microbial activity from each sample. The *Processing Techniques* page describes ways they might prepare their sample before plating it. For example, if they bring in rabbit droppings, different microbes might be found on the outside of the sample as opposed to the inside of the sample. They might do a rub of the outside and a serial dilution of 2 concentrations, then sterilize the outside and grind up the remaining sample from the inside and do a serial dilution of 3 different concentrations. The methods for processing the samples should have justification, but there is no right or wrong way to process the sample. Each group should do the serial dilution step on at least one plate to avoid lawns of bacteria, but they do not have to plate all 3 dilutions. (You may do fewer than 6 preparations to save time and resources).



Be sure students follow the example on page 6 for labeling their plates. These labels will carry through to more plates later in the experiment and a clear, concise coding system is helpful.

Before each of the following labs, be sure to discuss safe, sterile technique with the students as they will be working with fungus and bacteria. Students should wear protective eyewear, gloves and aprons as appropriate.

Part 3. Processing Sample, Preparing Media & Spreading Plates – (pages 7-11) (50 min + 3-5 days for growth)



Hand out pages 9-10 so students are familiar with the different types of media used in this experiment. It is your choice whether or not students actually make and/or pour the plates themselves, but they should be expected to know why different types of media are used in different steps. See ‘Tips for pouring and storing agar plates’ and ‘Streaking microbial cultures on agar plates’ in the appendix. After processing their samples as they designed in their experiments, follow the directions from page 11 to transfer the samples to the microcrystalline cellulose plates. Be sure students understand why this media is used here. Emphasize sterile technique to avoid contamination.

Part 4. Streaking for Isolation to Obtain a Monoculture – (page 12) (50 min + 3-5 days for growth)

Record results on Table 1. Students will choose 4 microbes from each treatment to isolate. You may choose only 1 or 2 to save time and number of plates required.



Microcrystalline cellulose media (“micro”) can be used here, which is a minimal media and takes longer to grow. Yeast malt extract media could be used, as microbes will grow more quickly and allow for better viewing of morphology, but it is a nutrient rich media that may become more easily contaminated. Save the original plates in case students need to return to them.

After the incubation period ends, use Table 2 to record results for each plate.

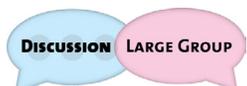
Part 5. Congo Red Assay or Supernatant Congo Red Assay – (page 13-14) (20 min + 3-5 days for growth, then 50 min)

Follow instructions on page 13 or 14, depending upon your preference.

Make sure that the students follow protocol for this step and only streak a small ‘x’ in the center of the plate. It may help to have them draw a circle on the bottom of plate and then streak within that circle. If students have good technique, you can use smaller plates for this step. Growth at this stage should also be watched carefully. Record growth results prior to staining on Table 3.



Students should wear goggles, gloves and aprons when using the congo red stain. It binds to cellulose, the main component of most t-shirts! You may also want to line the edge of your sinks with paper to avoid staining the table surfaces.



Positive results should show a clear or white halo around where the microbe was inoculated. If students transfer too much sample, it is possible none of the plate will stain because cellulase will degrade all of the cellulose on the plate. Record results on Table 3.

Part 6. Data Analysis, Discussion & Post-Test – (pages 15-23) (50+ minutes)

Have students complete pages 18-20 on their own or in groups. Then bring the class together to pool results and check for comprehension. Complete page 21. The post-test is a summative assessment that can be done following the lab.

Be sure to properly dispose of or sterilize all equipment used in this activity.

Extensions

1. If all students use the same serial dilution, they can count CFU's and compare abundance in a quantitative way.
2. Choose a colony from the 'pure' micro plate and perform a gram staining (protocol in supplemental materials). The gram-negative bacteria allows for better results in the PCR.
3. Perform Colony PCR from the gram-negative micro plates (protocol in supplemental materials).
4. PCR products can be sent for sequencing. Once the sequencing is complete, students can upload the sequence to Genbank (or other sequence database) to compare the most closely related known sequence. The students can then research their microbe for its general characteristics, and what it is known to do. Students may also use a bio-editing program to look at comparisons between different microbes.

Master Materials List

For a class of 24 students with 6 groups

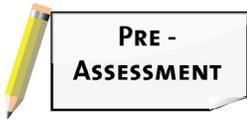
- 144 – 2mL centrifuge tubes
- 600 – Petri Dishes
- 20g -Carboxymethyl Cellulose (CMC) - purchased through VWR International, LLC
- 30g – Microcrystalline Cellulose (Micro)
- 120g – Agar- (Acros Organics - #AC40040-2500 ordered through Fisher Scientific)
- 6-24 Small pestles (to fit in centrifuge tubes--# depends on exp design)
- 6-12 Bacteria spreaders (or up to 72 disposables)
- 6-12 Inoculating loops (or box of 300 disposables)
- 300mL- PBS Buffer or TRIS buffer (or salt water solution could be used as well)
- 2g – Congo Red (powder form needs to be mixed before students can handle it- could also be ordered in liquid form – 1 L)
- P200 pipette tips
- 10uL dispensing micropipettes
- Parafilm
- Ethanol
- Bleach
- 180g – NaCl (table salt works fine for this)

For liquid media only

- 144- 15mL conical tubes

PCR Extensions

- 100- thin-walled PCR tubes
- 1.3mL – Master mix
- 50 uL-16s primers (forward and reverse) (536f, 907r)



Pre-Test

1. A 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. B Cellulose a. the raw material used to make biofuel
3. C Cellulase b. a carbohydrate found in plants
4. A Feedstock c. an enzyme found in some decomposers that help break down plant material

5. Most biofuel ethanol sold today is made from

corn grain or sugar cane

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

Cellulose, hemicellulose, lignin

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

Lignin and hemicellulose must first be separated from cellulose to allow enzymes to access carbohydrates. Then, an efficient set of enzymes must be used to break down cellulose and hemicellulose into fermentable sugars. We can use amylase for corn grain, and sugar cane doesn't require enzymes; yeast can digest the sugars already.

8. What is an enzyme?

An enzyme is a protein molecule that speeds up chemical reactions.

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

Answers will vary and can lead to class discussion. Compost piles, rotten vegetables in the refrigerator, herbivore droppings, herbivore stomachs, rotting logs, etc. Students should mention the role of microbes in returning nutrients to the soil or aiding in digestion.

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

A close, long-term interaction between two or more different species

- *Mutualism- both benefit*
- *Predation/Parasitism – one benefits, one is harmed*
- *Commensalism – one benefits, one is unchanged*
- *Competition – neither one benefits*
- *Neutralism – both are unchanged*



Introduction Questions (page 3)

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

Cellulose, hemicellulose, lignin

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

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

Glucose is a carbohydrate providing food/energy for organisms.

Glucose is readily fermentable by yeast and is converted to ethanol as a byproduct.

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

Digestion-aid, Decomposers – recycling nutrients.



Analysis Questions (pages 21-24)

Review the procedures from this lab. Why did you perform each step? What did each step teach you about your microbe?

1. Choosing a sample – *Environmental samples should come from a source that exhibits signs of natural cellulose degradation. If our sample gave successful results then the natural cellulose degradation was accurately identified.*
2. Processing the sample – *Performed to identify the location within the sample of the cellulose-degrading microbes. The processing methods identified the location within/on the sample where the microbes are present (i.e. if surface rub gave the most positive results, we can conclude that there are several cellulose degrading microbes on the surface of the sample).*
3. Serial Dilution – *Performed to reduce the number of microbes in the sample, to make them easier to count and/or identify. This step gives an idea of how many microbes are present within the sample.*
4. Spread on Micro Plate – *This selective media allows little growth, and only specific growth. This step reduces the number of non-cellulose-degrading microbes. If a microbe grows on microcrystalline cellulose media, we can conclude that the microbe contains cellulase.*
5. Isolate onto Micro Plate – *Performed to separate microbes and create a monoculture. This step is another screen that shows that the microbe we have chosen can break down cellulose.*
6. Streak onto CMC Plate – *This step is performed in order to do the Congo Red Assay. It is another screen to show that the microbe we have chosen can break down cellulose.*
7. Congo Red Assay – *Congo Red binds to the Carboxymethyl cellulose in the plate, and if the cellulose is degraded by the isolate that was inoculated, we can conclude that it contains cellulase.*

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

- *Microcrystalline Cellulose Agar (Micro)* – selective media used as the first screen for cellulose-degrading microbes. All microbes growing on this plate are assumed to contain cellulase enzymes.
- *Carboxymethyl Cellulose Agar (CMC)* – selective media used as a second screen for cellulose-degrading microbes. All microbes growing on this plate are assumed to contain cellulase enzymes. This agar is also used in the Congo Red Assay because the dye binds to Carboxymethyl cellulose.
- *Microcrystalline Cellulose Liquid Media (Micro Liquid)* – selective media used as a screen for extracellular cellulase enzymes. It is more difficult to grow in a liquid media, and when the tubes are spun down in the centrifuge, the cells will be pulled to the bottom of the tube and the supernatant will contain the extracellular enzymes.
- *Yeast Malt Extract Agar (YMEA)* – nutrient rich agar is used primarily to speed up the growth and to better see the morphology of the microbes.

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

Answers will vary. Acceptable student responses should contain a yes or no answer and then the data that supports the answer.

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

These microbes break down material to replenish the nutrients in the environment or are part of a symbiotic relationship with another organism. Student answers may vary for this question, but they should use what they have learned to justify their response.

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

Answers will vary.

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)

Answers will vary. Students should justify their response. The processing method should make a difference. Processing matters because the cellulose-degrading microbes may be located in different places on the environmental sample (inside or outside). Also, surface sterilizing will remove most of the microbes on the exterior of the sample, which may be a prime location for symbiotic microbes.

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)

Answers will vary. Students should justify their response.

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

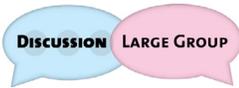
Answers will vary. Students should justify their response.

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.

Answers will vary. Students should justify their response.

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

After the pretreatment to get through the hemicellulose and the lignin, the cellulose needs to be broken down. Cellulose-degrading microbes are able to break down the cellulose more efficiently and are more environmentally friendly than chemical methods.



Class Discussion Questions (page 25)

Discuss the following questions as a class and answer them below.

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

Answers should be based on class discussion and contain data.

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

Answers should be based on class discussion and contain data.

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

Answers may vary, but they should refer to their hypotheses from page 4 (questions 3 and 4), identify whether they were correct or not, and compare them.

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

Answers will vary based on data.

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

Answers will vary based on data.

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

Answers will vary based on data. Students should include strategies to change the processing method(s).

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

Answers may vary, but they should refer to their hypotheses from page 6, identify whether they were correct or not, and compare them.



Post-Test (pages 26-7)

(answers only given for questions added to pre-test)

1. Is pure cellulase enough to break fresh plant material into simple sugars?

Why or why not?

No, cellulase cannot digest lignin or hemicellulose, the other two main components of cell wall. There are also many kinds of endo and exo-cellulases that digest different parts of cellulose.

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

Explain.

Answers will vary, but mutualisms are likely if there is an herbivore-microbe relationship or microbe/plant relationship.

Appendix

Lab Procedures

‘Tips for pouring and storing agar plates,’ ‘Sterilizing Laboratory Materials for the Classroom,’ & ‘Streaking Microbial Cultures on Agar Plates’ Microbes in Action Program by the University of Missouri St.Louis online resource and downloadable pdfs for use in the classroom.

<http://www.umsl.edu/~microbes/techniques.html>

Video Resources:

NREL video—Excellent 5-minute summary of difference between corn and cellulosic ethanol and process currently used to make cellulosic ethanol. Be aware the process discussed here is evolving and may vary from other information sources you read.

http://www.nrel.gov/learning/re_biofuels.html

Fields of Energy video: From the Minnesota Department of Agriculture, a free DVD with student hosts. Two short segments show how corn ethanol is made and the research into cellulosic ethanol. These two segments are currently available online as well.

<http://www.mda.state.mn.us/kids/>

Text/Online Resources:

Cellulosic Ethanol

US Department of Energy Office of Science (2007) “Biofuels for Transportation.”

<http://genomicsgtl.energy.gov/biofuels/index.shtml>

FAQ-style pages with overview material such as “What is biomass?”, “How is ethanol produced from cellulosic biomass”, “Can one gallon of ethanol displace one gallon of gasoline?” Links to many other quality resources available from the Department of Energy.

US Department of Energy. “Biomass Basics.” 2013.

http://www1.eere.energy.gov/bioenergy/biomass_basics_faqs.html

Information aimed towards high school students about the production steps involved in making ethanol and other biofuels. Also includes an appendix of additional teacher lesson plans on biofuels for middle and high school students.

Enzymes

“What is an Enzyme?” The department of Biology at Northland College has a great animation explaining the basics of enzymes.

<http://programs.northlandcollege.edu/biology/Biology1111/animations/enzyme.swf>

Enzymes in Biochemical Pathways – 2005. McGraw-Hill Higher Education, McGraw-Hill online learning center provides many animations with basic explanations on the workings of enzymes.

http://highered.mcgraw-hill.com/sites/0072437316/student_view0/chapter8/animations.html

Cellulase – Advanced Enzymes. 2008. - This website has information on the different types and actions of cellulase and many other enzymes. Appropriate for high school and higher education students.

<http://www.enzyme-india.com/cellulase-enzymes.html>

Symbiosis

Cameron Currie leads research into leaf cutting fungus farming ants

<http://www.youtube.com/watch?v=6qKySmzGgwo>

Leaf Cutting Ants Make Antibiotics

<http://www.youtube.com/watch?v=xblBLi-arY4>

Evolution coevolution of the ant and fungi

<http://www.youtube.com/watch?v=R5piJCyHwtw>

Standards:

WI MODEL ACADEMIC STANDARDS

SCIENCE:

C.12.2 Identify issues from an area of science study, write questions that could be investigated, review previous research on these questions, and design and conduct responsible and safe investigations to help answer the questions.

C.12.3 Evaluate the data collected during an investigation, critique the data-collection procedures and results, and suggest ways to make any needed improvements

F.12.7 Investigate how organisms both cooperate and compete in ecosystems

AGRICULTURE, FOOD AND NATURAL RESOURCE STANDARDS:

- D.8.7 Explain the emerging technologies within hydroponics, aquaculture, and biotechnology
- D.12.5 Describe how biotechnology can enhance food and fiber production
- D.9-12.1 Engage in applied learning opportunities emphasizing agriscience and production principles
- E.12.1 Understand the application of agricultural technologies that can sustain production while reducing environmental impact

ENVIRONMENTAL EDUCATION:

- A.12.2 Suggest possible investigations and describe the results that might emerge from the investigations
- A.12.3 Evaluate personal investigations and those of others, critiquing procedures, results, and sources of data and suggest improvements to the investigation
- A.12.4 State and interpret their results accurately and consider other explanations for their results
- B.12.9 Evaluate ways in which technology has expanded our ability to alter the environment and its capacity to support humans and other living organisms

AAAS PROJECT 2061 (1993):

- 1B - The Nature of Science: Scientific Inquiry
- 3C - The Nature of Technology: Issues in Technology
- 5D - The Living Environment: Interdependence of Life
- 12C - Habits of Mind: Manipulation and Observation
- 12D - Habits of Mind: Communication Skills

See page 3 for Next Generation Science Standards (2013) alignment.

Activity developed by Rhonda Knapp, Waunakee High School, Waunakee, WI while working in Dr. Cameron Currie's lab at the University of Wisconsin- Madison. Funding and additional support provided by the Great Lakes Bioenergy Research Center.



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