Fermentation Challenge

Overview: Students will design their own experiment and collect data to investigate the ability of yeast to metabolize a variety of feedstocks originating from different carbohydrate sources. Teachers demonstrate yeasts' inability to metabolize certain food sources. Students are encouraged to think about potential feedstocks and the biochemical processes necessary to convert each type of carbohydrate into fuel.

Levels
High school - Undergraduate

Subjects
Science, Biotechnology

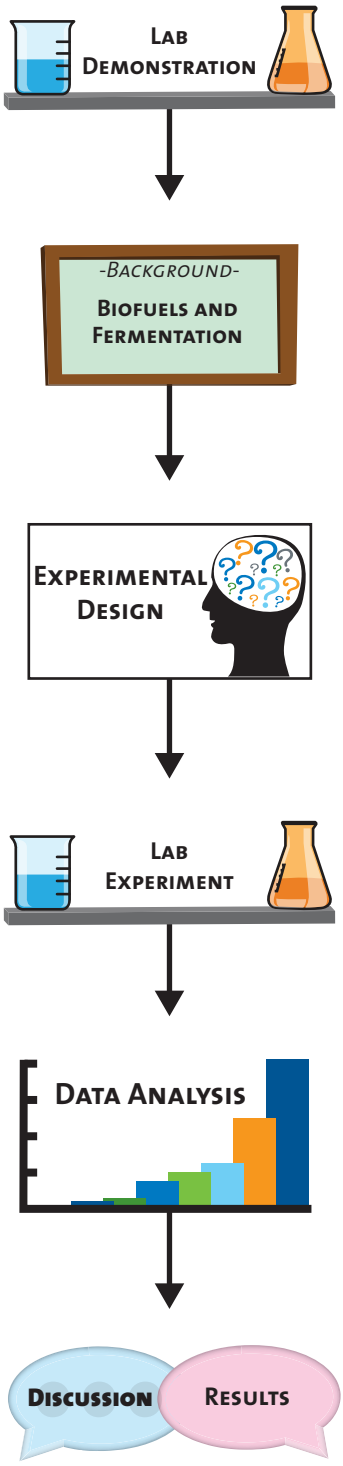
Objectives
- Identify carbohydrates found in different plant parts.
- List the products of fermentation.
- Explain that due to enzyme specificity yeast can only metabolize sucrose, not starch or cellulose.
- Infer metabolic rates through measurement of carbon dioxide production.
- Propose methods, such as heat and enzymes, to digest complex carbohydrates into simple sugars.

Materials
Fermentation Challenge package

Activity Time
Variable: Three to Five 50-minute class periods

Standards
Next Generation Science Standards (2013)
- Scientific and Engineering Practices: asking questions and defining problems; 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 - Fermentation Challenge

Overview:

Students will design their own experiment and collect data to investigate the ability of yeast to metabolize a variety of feedstocks originating from different carbohydrate sources. Teachers demonstrate yeasts' inability to metabolize certain food sources. Students are encouraged to think about potential feedstocks and the biochemical processes necessary to convert each type of carbohydrate into fuel.

Learning Outcomes: Students will...

- Identify different types of carbohydrates found in plants.
- List the products of fermentation.
- Explain that due to enzyme specificity yeast can only metabolize sucrose, not starch or cellulose.
- Infer metabolic rates through measurement of carbon dioxide production.
- Propose methods, such as heat and enzymes, to digest complex carbohydrates into simple sugars.

This lesson assumes prior knowledge in basic carbohydrate structures, and a brief introduction to photosynthesis, cellular respiration, and enzyme structure and function.

Standards

Next Generation Science Standards (2013)

Performance Expectations

High School:

- **HS-LS2-3.** Construct and revise an explanation based on evidence for the cycling of matter and flow of energy in aerobic and anaerobic conditions.
- **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
Asking questions and defining problems 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

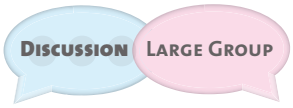
See Appendix for alignment with other standards.

Sequence:

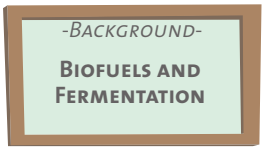
Part 1. Demonstration of fermentation rates of different feedstocks (pages 1-9) (50 minutes).

The demonstration is designed as a common exploration to generate discussion and questions amongst the large group by displaying the differing rates of fermentation between sugar and other feedstocks. Students should answer the *Introduction Questions* and create a hypothesis before viewing the demonstration. Instructions are provided in the student section using a Vernier Gas Pressure Sensor, or alternatively, using a balloon setup depending on classroom equipment availability. See *Sample Data* included in the supporting materials for a graph made with the Vernier Gas Pressure Sensor. Materials lists and teacher tips for running demonstrations can be found on pages 6T-9T. If students have done the GLBRC *Fermentation In A Bag* activity, the demonstration may not be necessary, except to show how quantitative methods work. Inform students that fermentation is one type of metabolism, and the words fermentation and metabolism will be used interchangeably throughout this document. Students should analyze the results of the demonstration using the *Analysis Questions* either individually or in small groups.

"Sample Data"



Discuss answers with students. Please see Teacher Answers (pages 10T-13T) for guidance.



Part 2. Provide background information on biofuels and fermentation (time varies by teacher choice).

In order to complete the exercise and design individual experiments, students will need a basic understanding of the fermentation process used to create cellulosic ethanol, including an introduction to the enzymes and chemical changes necessary in this process. The reading *Why is it so difficult to create cellulosic ethanol?* as well as information on biofuels and enzymes are included in the supplementary materials folder in the package. See the Appendix for other suggested background materials. Students may want to revisit their answers to the *Analysis Questions* in light of this new information.

"Why is it so difficult to create cellulosic ethanol?"

"What are biofuels?"

"Enzymes"

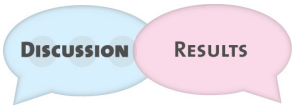
Part 3. Designing individual experiments (pages 10-12) (25-50 minutes). Students will work to design individual experiments to test methods for improving fermentation of alternative feedstocks. In order to brainstorm experiment ideas, students should work to complete *Experimental Pre-Design Questions* (page 10) individually, perhaps as homework, or in pairs. The *Experimental Design Questions* (pages 11-12) should be completed by students in small groups or pairs. Below is a list of possible changes students could make:

- pH
- Length of time the sample runs, measure rates over 24 hrs
- Feedstock choice (fruit juice, flowers, grasses, etc)
- Feedstock concentrations
- Try using a fungus, such as compost fungus, as a biodegrader
- Boil or freeze the sample feedstock before fermenting
- Temperature or concentration of the yeast solution
- Use enzymes (amylase or cellulase for example). See *Teacher Tips* (pages 7T-8T) for more information. Below are some options:
  - Cellulase from Flinn Scientific (www.flinnsci.com). Order #: C0172, $33.95 for 25g at the time of publication in 2010.
  - Alpha Amylase from Carolina Biological (www.carolina.com). Order #: 202350, $28.95 for 100g at the time of publication in 2010.

Depending upon your students' comfort level designing experiments you may need to help them through the steps of writing a formal procedure, designing a data table or data collection system, recording observations, analyzing and displaying their results, and drawing conclusions.

Part 4. Run student-designed experiments (minimum of one class period). Experiments should be run in small groups. Students should use data to draw and record conclusions, analyze the validity of their preliminary hypotheses, and refine these hypotheses if necessary. Timing in this experiment is extremely important so it may be helpful to designate one student as the timer.

Part 5. Share results (page 13) (minimum 20 minutes).
Ask students to present their findings to you in written form and/or directly to the class. If students do not make formal presentations, at a minimum they should describe their independent and dependent variables and conclusions to the class. Creating a master list of experiments for all students to read will aid in the overall discussion of results and further questions for investigation.



Students should participate in a large group discussion of results including *Post-Experiment Questions*. Teachers may also want students to include a formal report of results, including discussion of post-experiment questions.

Extensions

1. Run a revised experiment based on results.
2. Use the more advanced *Aqueous Ammonia Pretreatment* method included in the supporting materials of this package.
3. Connect this activity to a deeper discussion of carbohydrate structures and enzyme function.
4. Ethanol sensors from Vernier are available to approximate ethanol production levels.

"Aqueous Ammonia Pretreatment"

Materials Lists

Preview Demonstration with Vernier Setup

- Computer or Graphing Calculator or Vernier Lab Quest
- Vernier computer interface, Logger Pro software
- Vernier gas pressure sensor, rubber stopper, tubing for each set-up
- 2mL water dropper or small pipets
- 7% yeast solution – 12mL per set-up
- 5% feedstock solution of each carbohydrate source – 3mL per solution per set-up
- Hot Plate (optional, see below)
- Water bath (600-1000mL beaker or plastic container)
- Thermometer
- Four 15mL conical tubes or test tubes
- Test tube/ conical rack or ring stand set-up seen in Fig. 1
- Vegetable oil – 3-5mL per set-up

If you don't have a hot plate, you will need:

- Hot and cold water
- Beral pipet or basters to remove water

Preview Demonstration with Balloons

- 3 Balloons
- 3 cups very warm tap water
- 3 packets dry yeast
- Sugar
- Cornmeal or ground field corn
- Corn stover or other plant materials (grass clippings, composting materials, sawdust, etc)

Teacher Set Up for Demonstration/Student Activity (15-20 minutes)

Prepare two types of solutions before class begins.

1. Prepare a 7% yeast solution (7g or one packet of dry yeast for every 100mL of warm tap water). The yeast solution should be incubated in a 37-40°C water bath before students begin the activity. If students complete the demonstration in groups, each student group will require approximately 12mL yeast solution.
2. Prepare a 5% (5g substrate for every 100mL water) solution of each of the soluble feedstock sources (i.e. sucrose or glucose). Each student group will need to prepare 2mL of a 5% misture (.1g of substrate in 2mL of water) for each of the insoluble feedstock sources(i.e. cornmeal, ground field corn, ground sweet corn, and/or corn stover powder). Ideally, the class should at least observe the differences between sucrose, cornmeal, and a stover-like material. Note: Sucrose is the positive control. Potential feedstock sources include:
    - Sucrose
    - Glucose
    - Cornmeal (make sure to look at ingredient list of the cornmeal, note if sugar is an ingredient as it will affect your results).
    - Ground field corn
    - Ground sweet corn
    - Corn stover powder (You may be able to contact a local farmer for this material. If corn stover is not available, try using other plant materials like dead leaves, dried grass clippings, brush pile clippings, sawdust, etc. Pulverize samples in a food processor or spice grinder to achieve a powder-like consistency.)

Teacher Tips…

General

1. It is important to stagger the starting incubation times in both setups. The yeast and feedstock sources should be mixed and incubate together for the same amount of time for all samples before measurements are conducted. For example, combine yeast and feedstock #1 and allow to incubate for 10 minutes, take measurements for 4 minutes. Do not start incubating yeast and feedstock #2 until the first sample is at least half way complete. The goal is to allow fermentation to occur for the same amount of time in each test tube before taking measurements of the rates.
2. An airtight fit is important with the gas pressure sensor and can be achieved by gently twisting the rubber stopper into the test tube or conical tube. Make sure all plastic tubing is securely fit as well.
3. Make sure to keep the graph scales and labels consistent for easy comparison. Also, creating and using a linear average line (best-fit) from the data may be easier than using raw-data (jagged) lines.

Feedstocks

4. Read the ingredients when purchasing cornmeal. If the cornmeal contains sugar, results will be much more similar to the table sugar tested. If it contains lime or other ingredients, make a note, as these ingredients may act as catalysts to change metabolic rates.
5. Ground field corn produces similar results to cornmeal. Sweet corn is another option. It will produce different results due to the genetic differences between field corn and sweet corn (which contains more sugar).
6. The cornmeal and stover feedstocks are insoluble. Be sure to have students mix the samples before beginning the experiment.

Enzyme & Heat Pretreatments

7. If student choose to use enzymes to break down starch or cellulose, have them run two negative controls. One should be water and enzyme, with no feedstock. The other should just be water. Yeast may metabolize simple sugars that remain in the enzyme from the manufacturing process, creating false positive results.
8. To break down starch with amylase, boil a 5% cornmeal mixture on a hot plate for 10 minutes. Cool to 37°C. Combine 5mL boiled cornmeal mixture with 2mL of 1% amylase solution. Incubate for 30 minutes at 37°-40°C and run the fermentation.
9. Optimum temperatures for enzymes may differ based on manufacturer. (For example, alpha amylase from Carolina lists optimal temperature as 45°-55°C.) However, you can follow the directions in #7 and still see sufficient enzymatic breakdown of the starch.
10. To break down corn stover with cellulase, combine 5mL of 5% corn stover mixture and 2mL of 1% cellulase solution. Incubate at 50°C for 3 days before running fermentation at 37°-40°C.

Introduction Questions (pages 1-2)

1. Which parts of a plant contain carbohydrates? Do all these parts contain the same type of carbohydrate? Elaborate.
   Fruits contain simple sugars, such as fructose – this is the first one we usually think of because we can think of tasting a plant, and what will taste sweet. Plant cell walls also contain carbohydrates – almost 50% in the form of cellulose, which looks like starch but has inverted bonds between the glucose molecules. Starch can be found in plant parts such as endosperm (grains) and tubers.
2. Look at the materials list provided by your teacher for this activity. How will we measure the metabolic activity of the yeast? Why is this measurement an indication of metabolic rate?
   We will measure metabolic activity of the yeast using a gas pressure sensor. This works as an indicator of metabolic rate because carbon dioxide gas is a product of yeast metabolism. The greater the amount of carbon dioxide produced per minute, the higher the metabolic rate.
3. What are the plant materials (also known as feedstocks) to be tested in this experiment? Compare the carbohydrate composition of table sugar (sucrose) with the other feedstocks and hypothesize what some of the differences may be.
   Cornmeal contains starch, and corn stover contains mainly cellulose, but also starch and other sugars. Sucrose is a disaccharide sugar that can be broken into two monosaccharides by an enzyme in yeast called invertase (sucrase). Starch and cellulose are both chains of six-sided glucose molecules, but the bonds between molecules in the cellulose chain are inverted compared to starch.
4. Which of the feedstocks to be tested in this experiment do you think will be metabolized fastest by the yeast? Why? Make your hypothesis below.
   Student hypotheses should be supported with rational information about carbohydrates, enzymes, or cellular respiration.

Analysis Questions (pages 8-9)

1. What is the chemical formula for the metabolism observed in this experiment?

   $C_6H_{12}O_6$ ---> 2 CH_3CH_2OH + 2 CO_2 + ATP

   glucose ---> ethanol + carbon dioxide + energy

2. Which feedstock fermented the most? How do you know?

   Sucrose fermented the most because that mixture produced the most CO_2. CO_2 is a product of fermentation, so when large quantities are produced in the chamber, the probe detects the increase in pressure, indicating high rates of fermentation.

3. Was your hypothesis supported by the experimental results? Use data to support your answer.

   Answers will vary.

4. Think about the differences in metabolic rates for the feedstocks you tested. What can you infer about the enzymes in yeast from the different rates you measured?

   Enzymes in yeast are substrate specific and convert only specific forms of sugars. Yeast must only have enzymes for sucrose, not starch or cellulose (or whatever students think is in the other feedstocks), because that fermented best when compared to the other feedstocks. In this experiment, sucrose is a disaccharide, which can be broken up into monosaccharides of glucose and fructose by the yeast enzyme invertase (sucrase). See supporting material on Enzymes for more information.

5. Table sugar is pure sucrose, which is fermentable by yeast. What do you think the carbohydrate content is for the other feedstocks tested? Are they homogeneous or heterogeneous? What evidence do you have?

   Acceptable student answers should explain that other feedstocks (plant materials) contain sugar, but it might not be pure sucrose, so they may not ferment as well. Teachers may want to explain that corn stover is heterogeneous in carbohydrate makeup. It contains cellulose (44%),

hemicellulose (30%), and lignin (26%). Cellulose is made of chains of linked glucose molecules. This glucose is fermentable by yeast, but only when cellulose is broken down into glucose. Sucrose ferments best of all the feedstocks (because yeast have enzymes to break down sucrose into glucose and fructose), while corn, stover, and other feedstocks may show some (but not much) fermentation in comparison.

6. The demonstration is meant to model the fermentation of carbohydrates into ethanol using yeast. What are some of the limitations of this demonstration as a model?
   The model we use does not actually measure ethanol (fuel) production. We are measuring CO_2 production, which implies ethanol production, as they are both products of fermentation. Experiment is too short, fermentation may occur over much longer periods of time. The rate in the first few minutes may not be representative of a longer batch fermentation because there could be a small amount of simple sugar in the mix which is fermented first.

Experimental Pre-Design Questions (page 10)

1. What feedstocks are used to create biofuels?
   Biofuels can be created from sugar cane, corn, or cellulosic biomass. Cellulosic ethanol is made from sugars in plant cell walls. Cellulosic ethanol can be made from anything that is or ever was plant material, including wood chips, corn stover, switchgrass, straw, hay, yard trimmings, and urban waste.
2. What are the challenges associated with making biofuels from corn grain (corn ethanol) or cellulosic material (cellulosic ethanol)?
   The challenges when making corn ethanol include efficiency of production, land use changes to plant corn grain, and massive inputs into the system (fertilizers, fuel for product transport, etc.) Challenges in making cellulosic ethanol include the difficulties of breaking down cellulose plant

material to release plant sugars, conversion of these sugars into ethanol, and efficiency of production process. In both cases, environmental and economic impacts must be taken into consideration for large-scale production and use.

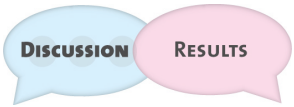
3. What could be done to improve fermentation rates seen in the demonstration? Make 2 hypotheses below, and explain why you think each idea would work.

Pretreatment of feedstocks (using heat, chemicals, or enzymes) are important steps researchers must take in converting biomass to fuels. These steps break down cellulose, hemicellulose, and lignin complexes in the plant material in order to release sugars for fermentation. Changing concentrations of yeast or feedstock sources may change results, as well as changing water temperature or pH of the system.

Hypotheses will vary. A list of possible experiments to consider if students struggle with creating ideas can be found on page 2T.

Experimental Design Questions (pages 11-12)

Answers will vary.



Post-Experiment Questions (page 13)

Answers will vary.

Extensions and Variations

1. Read and discuss a research story about Dr. Donna Bates, GLBRC scientist currently investigating fermentation in A Modern Scientist-Engineer in the World of Fermentation.
2. Extend the learning with the Data Dive: Boosting Yeast's Appetite for Sugars by having students learn about how scientists are using directed evolution techniques to create mutant yeast strains than can ferment all of the sugars in plant biomass, not just the glucose.
3. Conduct the Fermentation in a Bag investigation comparing simple sugars

like sucrose, glucose, and xylose as the food source for yeast.

4. Have students conduct their own fermentation experiment through CB2E: Converting Cellulosic Biomass to Ethanol by investigating the process of converting cellulosic biomass into sugars (glucose) and then into ethanol.

Appendix:

Video Resources:

Measuring Ethanol in the Classroom: Tips & Tricks - A 2-minute video on how to calibrate, operate, and troubleshoot Vernier and Pasco ethanol sensors to allow for qualitative comparisons between ethanol samples.
https://youtu.be/8iNAWPy7xS8

Biofuels: Beyond Ethanol - A 10-minute piece by KQED which provides background on the lab science behind the biofuel challenge and focuses on how the areas of synthetic biology and directed evolution can create microbes that produce fuels similar to those we currently use in our vehicles. Features JBEI, another Bioenergy Research Center.
http://www.kqed.org/quest/television/view/819

Converting Biomass to Liquid Fuels - Excellent 5-minute summary of difference between corn and cellulosic ethanol and process currently used to make cellulosic ethanol.
http://www.nrel.gov/learning/re_biofuels.html

Fields of Energy - 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 Resources:

US Department of Energy Office of Science. Biofuels for Transportation. 2007. - 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.
http://genomicsgtl.energy.gov/biofuels/index.shtml

US Department of Energy: ABC's of Biofuels. 2009. - Information aimed towards high school students about the production steps involved in making bioethanol and other biofuels. Also includes an appendix of additional teacher lesson plans on biofuels for middle and high school students. *http://www1.eere.energy.gov/biomass/abcs_biofuels.html#prod*

Redding, K., D. Masterman. Biology with Vernier. Beaverton, OR: Vernier Software & Technology. 2007. - Biology with Vernier Lab 12B is the basis for the preview activity procedures used in this activity guide. *http://www2.vernier.com/sample_labs/BWV-12B-COMP-sugar_fermentation.pdf*

Enzymes

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*

"What is an Enzyme?" - The department of Biology at Northland College has a great animation explaining the basics of enzymes. *http://www.northland.cc.mn.us/biology/Biology1111/animations/enzyme.swf*

Follow-Up Resources:

These are primary source articles by authors who study various parts of the fermentation process in creating cellulosic ethanol, especially the pretreatment process. University Library permissions may be necessary to access full text articles.

Yang, B. and E. Wyman. "Pretreatment: the key to unlocking low-cost cellulosic ethanol." Wiley Interscience: Biofuels, Bioproducts, and Biorefining 2:26-40. 2007. - Gives a nice background of the use of petroleum and biofuels in the introduction. Also provides a comparison each pretreatment method, including pros and cons, on page 31. Figure 1 (page 29) provides an outline of the biological conversion of cellulosic biomass into ethanol, including effects of pretreatment processes on other operations. Best suited for high school students or teachers who want to know more about pretreatment methods.

Mosier, N., Wyman, C., Dale, B., et al. "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource Technology 96.6

673-686. 2005. - Overview of plant structure and challenges of accessing cellulose provided on pages 673-676. Also provides detailed descriptions of pretreatment options. Useful for high school students and teachers, especially chemistry teachers who may be interested in mimicking procedures outlined for pretreatment.

Standards:

AAAS PROJECT 2061 (1993):

1B - The Nature of Science: Scientific Inquiry
3A - The Nature of Technology: Technology and Science
4D - The Physical Setting: Structure of Matter
4E - The Physical Setting: Energy Transformations
5C - The Living Environment: Cells
5E - The Living Environment: Flow of Matter and Energy
8B - The Designed World: Materials and Manufacturing
12B - Habits of Mind: Computation and Estimation

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

DOE Bioenergy Research Centers

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U.S. DEPARTMENT OF ENERGY

Name_____ Date_____ Hour_____

Introduction Questions

Yeast metabolize carbohydrates for energy for their own growth and reproduction. In anaerobic conditions they produce ethanol as a by-product, which can be used as a transportation fuel. Due to concerns of dwindling supplies of fossil fuels and global climate change, scientists are investigating using yeast to produce large quantities of ethanol for transportation fuels. However, yeast can only metabolize certain food sources due to biological constraints. In this activity, you will investigate the ability of yeast to metabolize a variety of carbohydrates originating from different feedstocks (plant materials).

In order to create ethanol, yeast must have a good source of digestible sugars. Plants contain a tremendous amount of carbohydrates (about 50% of a plant is carbohydrates) but not all of this is digestible for yeast. The chemical formula for fermentation is:

$$\mathbf{C_6H_{12}O_6 \text{ ---> } 2\ CH_3CH_2OH + 2\ CO_2 + ATP}$$

Glucose ---> ethanol + carbon dioxide + energy

1. Which parts of a plant contain carbohydrates? Do all these parts contain the same type of carbohydrate?

2. Look at the materials list provided by your teacher for this activity. How will we measure the metabolic activity of the yeast? Why is this measurement an indication of metabolic rate?

3. What are the plant materials (also known as feedstocks) to be tested in this experiment? Compare the carbohydrate composition of table sugar (sucrose) with the other feedstocks and hypothesize what some of the differences may be.

4. Which of the feedstocks to be tested in this experiment do you think will be metabolized the fastest by the yeast? Why? Make your hypothesis below.

Name_____ Date_____ Hour_____

Simple Demonstration to Compare Metabolic Rates of Different Feedstocks: Method A (Vernier Gas Pressure Sensor)

This introductory activity demonstrates that yeast can digest some sugars but not others. Your job is to determine why this happens. After the demonstration, discuss why the results varied for different feedstocks (plant materials) and join other scientists in the field of biofuel production to develop your own experimental methods to increase CO_2, and therefore ethanol, production rates from cellulosic biomass.

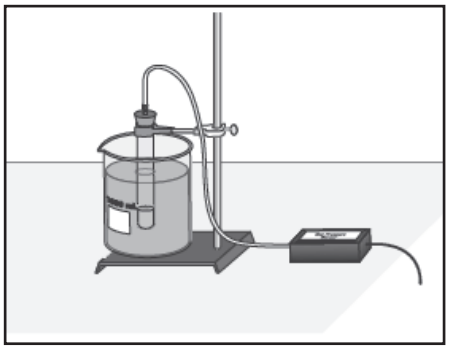


Figure 1. Vernier Setup

Procedure - Anaerobic Respiration with a Vernier Gas Pressure Sensor

Wear goggles or safety glasses when working with glassware or rubber stoppers

1. Set up a water bath for the yeast solution in a large glass beaker that will fit the test tubes. To maintain the water-bath at 37°-40°C, place the beaker on a hot plate and set it to a low temperature. The temperature that maintains the water bath will vary depending on the air temperature and rate of heat loss from the beaker. Monitor the water bath with a thermometer during the experiment. If you don't have access to a hot plate, combine warm and cool tap water until a temperature in this range is achieved. Use a beral pipet or baster to maintain the water bath by removing the cooling water and adding warm water.
2. Label 4 test tubes – sucrose (S), cornmeal (CM), stover (CS), and a negative control (water).
3. Obtain 2mL of 5% sucrose solution. In addition, obtain .5g of CM and place it in the corresponding test tube and add 2mL of water. Repeat this process for the CS test tube. Be sure to also add 2mL of warm tap water to the negative control test tube.
4. Test only one feedstock source at a time. Add 2mL of the yeast solution to the first of the four test tubes feedstock solution for a total volume of 4mL per test tube (2mL feedstock + 2mL yeast). Use the same procedure with the control test tube as with the ones containing feedstock solutions.

5. Gently swirl the test tube to mix the contents.
6. In the test tube, add enough vegetable oil to cover the surface of the mixture. Be careful not to get oil on the inside walls of the test tube. The addition of vegetable oil is to create an anerobic environment. Place the test tubes in the water bath.

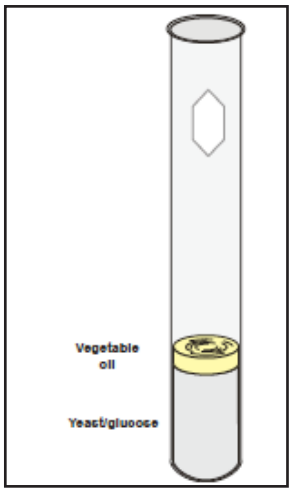


Figure 2. Cover yeast solution with oil (step 6).

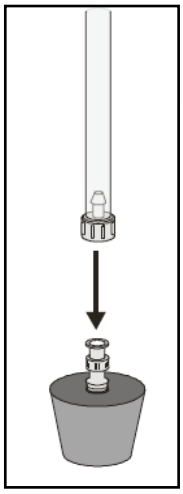


Figure 3. Connect tubing to stopper (step 11).

7. Insert the single holed rubber stopper into the test tube, firmly twisting it in for an airtight fit.
8. Incubate the test tube in the water bath for 10 minutes. Carefully monitor the temperature of the water bath. The water should surround the solutions inside the test tubes. While the test tube is incubating, begin computer setup for data recording.
9. Open the Vernier Logger Pro or Logger Lite software on the computer. Connect the gas pressure sensor to the computer interface. Connect the plastic tubing to the valve on the gas pressure sensor, leaving the other end to be connected to the rubber stopper once incubation is complete.
10. Select 'Data Collection' from the *Experiment* menu. Set the experiment length to be 15 minutes. Set the sampling rate at 12 samples/minute (a sample every 5 seconds).
11. When incubation is complete, connect the free end of the plastic tubing to the rubber stopper.
12. Click the green collect data button to begin recording data. Make sure to keep the water bath temperature constant during the course of data collection.
13. Monitor the pressure readings displayed on the computer. If the pressure exceeds 130kPa, the pressure inside the tube may cause the rubber stopper to pop off. Disconnect the plastic tubing from the gas pressure sensor if it exceeds 130kPa.

14. When data collection has finished, disconnect the plastic tubing from the rubber stopper. Remove the rubber stopper from the test tube and discard the contents in a waste beaker or sink.
15. Determine the rate of fermentation.
    a. Move the mouse to the point on the graph where values begin to increase. Drag the mouse to the point on the graph where data values end and release.
    b. Click the Linear Fit button to perform a linear regression of the data. A box will appear with the formula for the line of best fit.
    c. Record the slope of the line, m, as the fermentation rate in the table below.
16. Store this data run by choosing Store Latest Run from the Experiment drop down menu. Your teacher may ask you to print your final results or save your data for further analysis, but be sure to also record results in the table below.
17. Repeat steps 4-15 for the each of the other test tubes, remembering to always monitor and adjust the temperature of the water bath. If each group only does a few samples, average class data to obtain the missing values.

Record data in the table below.

Table 1.

Feedstock Tested	Fermentation Rate (kPa/min)
Water (control)	
Sucrose	
Cornmeal	
Stover	

Calculate class average respiration rates and record results in the table below.

Table 2.

Feedstock Tested	Class Average Fermentation Rate (kPa/min)
Water (control)	
Sucrose	
Cornmeal	
Stover	

Name_____ Date_____ Hour_____

Simple Demonstration to Compare Metabolic Rates of Different Feedstocks: Method B (Balloons)

This introductory activity demonstrates that yeast can digest some sugars but not others. Your job is to determine why this happens. After the demonstration, discuss why the results varied for different feedstocks (plant materials) and join other scientists in the field of biofuel production to develop your own experimental methods to increase CO_2, and therefore ethanol, production rates from cellulosic biomass.

Procedure – Anaerobic Respiration with Balloons

1. Stretch out 3 balloons by blowing them up a few times and then lay them aside.
2. Add one packet of dry yeast to one cup of very warm tap water and stir. Repeat this twice so that 3 cups of warm water are activating yeast. Allow yeast to activate for about 5 minutes.
3. Add 2 tablespoons of sugar to the 1st bottle, 2 tablespoons of ground corn or cornmeal to the 2nd bottle, and add 2 tablespoons of corn stover or other plant material to the 3rd bottle.
4. Add the one cup of the yeast water mixture to each bottle and gently swirl until the sugar/corn/plant is as dissolved as possible.
5. Attach a stretched out balloon to the mouth of each bottle, securing with a rubber band if necessary.
6. After 10-20 minutes, the balloons may stand upright. Eventually the balloons may begin to inflate. Allow experiment to run for a minimum of 1 hour, and for as long as desired afterwards.
7. Record visual results at 20-minute intervals. Measurements of balloon circumference maybe taking for quantitative analysis.

Name_____ Date_____ Hour_____

Record data in the table below.

Feedstock Tested	Balloon Circumference
Water (control)	
Sucrose	
Cornmeal	
Stover	

Calculate class average respiration rates and record results in the table below.

Feedstock Tested	Class Average Balloon Circumference
Water (control)	
Sucrose	
Cornmeal	
Stover	

Name_____ Date_____ Hour_____

Analysis Questions

1. What is the chemical formula for the metabolism observed in this experiment?

2. Which feedstock fermented the most? How do you know?

3. Was your hypothesis supported by the experimental results? Use data to support your answer.

4. Think about the differences in metabolic rates for the feedstocks you observed. What can you infer about the enzymes in yeast from the different results you observed?

5. Table sugar is pure sucrose, which is fermentable by yeast. What do you think the carbohydrate content is for the other feedstocks you tested? Are they homogeneous or heterogeneous? What evidence do you have?

6. What are some of the limitations of this demonstration as a model of fermentation?

Name_____ Date_____ Hour_____

Experimental Pre-Design Questions

Use data from this experiment and the information your teacher provided to answer the following questions and begin thinking about experiments to test your ideas.

1. What feedstock sources are used to create biofuels?

2. What are the challenges associated with making biofuels from corn grain (corn ethanol) or cellulosic material (cellulosic ethanol)?

3. What could be done to improve fermentation rates seen in the demonstration? Make 2 hypotheses below, and explain why you think each idea would work.

Name_____ Date_____ Hour_____

Experimental Design Questions

Design a new experiment that could be done to test one of your hypotheses from the questions in the Pre-Design section.

1. Which hypothesis will you test?

2. Write a paragraph or draw a picture of your experimental setup including your control.

What measurements will you need to make when you record your data? Will you measure metabolic rate or another variable? What equipment will you need to make quantitative measurements (Vernier probes or other)?

3. How will you measure the success of your experiment?

Name_____ Date_____ Hour_____

Post-Experiment Questions

1. Rate the success of the techniques you attempted in your experiment. Use data to support your claims.

2. Using evidence from the experiments to support your answer, discuss which variables or techniques should be investigated further.

3. Using your experiment results and what you have read or learned about biofuels, speculate on why certain techniques worked better than others.

4. If you could speak with an expert in the field of biofuels, what would you want to ask them about this experiment or about biofuels in general? Why?

Aqueous Ammonia Pretreatment

This technique is a lower-tech version of the AFEX (Ammonia Fiber Expansion) pretreatment being researched at GLBRC, which requires high pressure and temperatures not suitable for classroom use. The process helps open up the cell wall and exposes the cellulose (and hemicellulose) for the enzymes to attach more effectively. See the *Fermentation Challenge* Appendix for more information on pretreatment techniques.

Materials:

Pretreatment

- Hot plate set to 60°C, or same 50°C water bath for hydrolysis
- 250mL Erlenmeyer flask with stopper
- Duct tape
- 5 g of each feedstock sample (dried, cut or chopped)
- 15 wt% aqueous ammonia solution (NH_4OH) (15g NH_3 in 100g total solution)
- Maintain a 1:9 Solid : Liquid Ratio (1g solid : 9g liquid = 10g total weight)

Enzymatic Hydrolysis

- 50°C water bath
- Optional – Large beaker, stir bar, clamp and stand
- pH probe
- Burette
- Concentrated hydrochloric acid (HCl) – make sure you wear gloves and splash goggles!
- Cellulase enzyme (see information below)

Fermentation

- 37°C water bath
- Cheesecloth
- pH probe
- 125mL flasks with stoppers
- Duct tape
- Brewer's Yeast (1.75g per flask)

Cellulase enzyme:

Buy enzymes from a chemical company, for example: Sigma: 50mL = $67.00 - C2730 Sigma Cellulase from *Trichoderma reesei* ATCC 26921

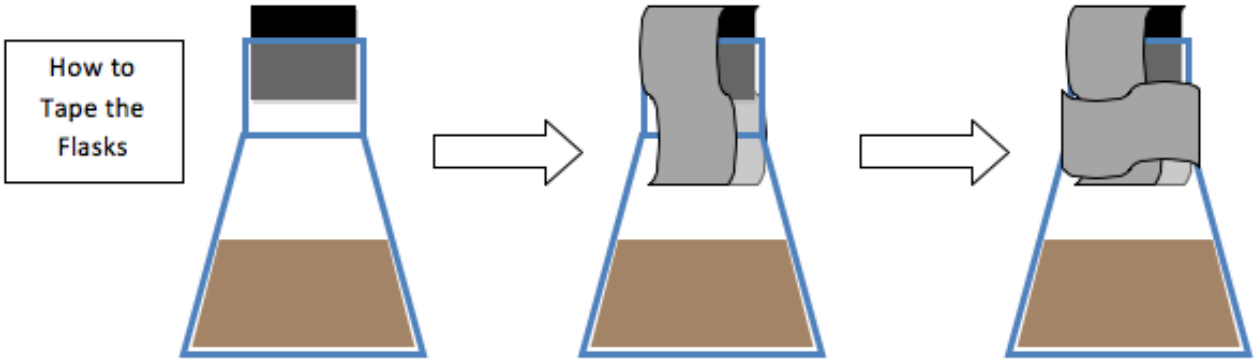
or ask Genencor donate some "Accelerase" enzyme. These are in solution.
Genencor, A Danisco Division
925 Page Mill Rd., Palo Alto, CA 94304
phone: 650-846-7645 fax: 650-845-6524

Aqueous Ammonia Pretreatment

Use proper safety equipment--goggles, gloves, apron.

Pretreatment (50mL):

1. Set a hot plate at 60°C or preheat the water bath to 50°C.
2. Weigh out 5g of feedstock sample into a 250mL flask.
3. Add 45g of 15wt% aqueous ammonia (NH4OH) **(all steps involving ammonia must be done in the fume hood).**
4. You can try to mix using a stir bar on a heated stir plate, but the slurry may be too thick for this.
5. Place the stopper in the flask and duct tape the stopper down.
6. Place on hot plate or in water bath. Allow the reaction to proceed for 24hours.



Enzymatic Hydrolysis (100mL):

1. Preheat the water bath to 50°C.
    a. Note: Mixing is important for both hydrolysis & fermentation. Ideally, add water to a beaker that is large enough to hold the flask. Heat this water on a stir plate to 50°C and add a stir bar to the hydrolysate. Use a clamp and stand to support the flask in the beaker, close enough to the stir plate to allow for mixing. Do not place the flask directly on the hot plate. If you cannot do this, it's more important to maintain the correct temperature than it is to stir.
2. Remove the tape and the stopper – perform all of the following steps in the fume hood.
3. Place a pH probe into the slurry (record the pH) and continuously monitor the pH for the next few steps.
4. Add 30mL of water to the flask and swirl gently to mix.

5. To neutralize the solution, fill a buret with HCl. Either while stirring with a hot plate, or swirling the mixture every so often, slowly add HCl to the slurry until the pH is between 4.8 – 5.0.
6. Subtract the volume of HCl added from 20mL and, using this amount of water, rinse off the pH probe into the flask (HCl + water=20 mL).
    a. The final solids concentration of your hydrolysate should be ~5g solids /100g total weight of solution for each flask.
7. Stopper the flasks, seal with duct tape, and place in a 50°C water bath. After 10 minutes, add 3mL of cellulase enzyme to each sample. Restopper and tape the flasks.
8. Allow the hydrolysis to run for 72 hours.

Fermentation (50mL):

1. Heat the water bath to 37°C.
    a. See the previous note in the hydrolysis section.
2. Filter the hydrolysate through 2 – 10 layers of cheesecloth. Try to remove as much of the solids as possible.
3. Measure the pH of the liquid. (It should be between pH4.5 – 5.5 for adequate yeast fermentation).
4. Transfer 50mL of hydrolysate into a clean 125mL flask (sterilize the flask if possible).
    a. If adding a stock solution of yeast, you can either decrease the amount of hydrolysate or add it to the 50mL – but remember the sugar concentration will change. The final amount of yeast in the flask should be at the concentration of 3g/100mL of liquid.
5. Dissolve 1.75g yeast in hydrolysate and control solution (make sure the temperature is between 35-38°C).
6. Conduct the fermentation at 37°C under anaerobic conditions. (Stopper & tape the flasks.)
7. Observe gas formation, recording observations.
    a. Normally our fermentation experiments run for 72hours. This isn't necessary for these experiments, but you could observe them for a few hours, making observations every 10-20 minutes or so and then leave them for 12 or 24 hours and observe them at the final time point.
    b. To provide actual CO2 measurements, connect the flasks with tubing to a eudiometer and measure water displacement, or build a mini-fermenter and use a CO2 probe to quantify fermentation rate (see GLBRC educational materials page for directions).

Frequently Asked Questions

Can you explain what you mean by 15wt% aqueous ammonia ? *Ammonium hydroxide solutions are generally sold as wt%, not molarity. The standard concentration if you purchase from a chemical company is 28-30wt%. It would be possible to just use this concentration as is, without diluting to 15wt%. But if wanted to use the dilution: in the original sample, at ~30wt%, you would have 30g NH3 in 100g of solution. So to get ~15wt%, you would just mix an equal weight (volume) of water and ammonium hydroxide solution (30g in 200g solution 15g in 100g solution).*

Does the feedstock sample need to be prepared before using? *Use dry feedstocks, chopped or cut. You can use sawdust, woodchips (like rodent bedding), grass clippings, and paper punch holes. Or you can use scissors or a paper cutter to cut up grass or paper to specific sizes. One possible experiment could be to look at the effect of different particle sizes on the results. (2 inch lengths, 1 inch lengths, 0.5 inch lengths, etc...) You can use powdered biomass, but if you do, I would recommend cutting the time for pretreatment in half (unless you're comparing pretreatment sizes in which case the length of time should be held constant). The pretreatment and hydrolysis will be extremely effective on powdered materials.*

What are biofuels?

Biofuels are produced from biological carbon sources such as sugar cane, corn, switchgrass, and cellulosic plant material. They differ from fossil fuels because they are produced from recently dead organic material, whereas fossil fuels come from long dead (millions of years!) sources. The two most common types of biofuels are biodiesel and ethanol. Biodiesel is made from recycled cooking grease or a variety of plant oils. Ethanol is an alcohol made from sugar using a process similar to brewing beer or fermenting wine. Ethanol can be made from many feedstocks – corn grain in the US and sugarcane in Brazil are currently the most common, but other substances like cellulosic materials have potential to be used in the future. The biggest challenge in creating useful feedstocks is efficiently converting biomass into sugars to be fermented into ethanol.

What are sugar, ground corn, corn meal, and stover, and what are their uses in biofuels?

Table sugar, or sucrose, is one of the easiest substrates for yeast to convert into ethanol. Through fermentation, yeast breaks down sugar into carbon dioxide and ethanol. Sucrose ($C_{12}H_{22}O_{11}$) is a disaccharide made of fructose and glucose.

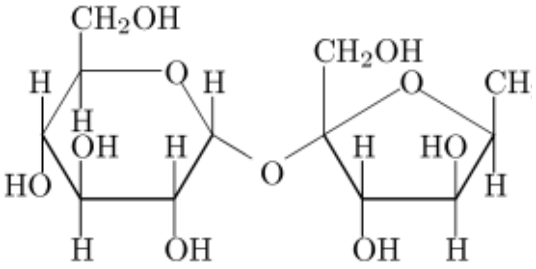


Figure 1. Sucrose is a disacchride made from the monosaccharides fructose and glucose.

Both ground corn and corn meal contain high quantities of starch, which can be broken down into simple sugars with use of enzymes or heat treatment. Ground corn and corn meal are similar products, but depending on processing and added ingredients, corn meal may be chemically different than ground corn, and may produce different results. Grain corn is genetically different from sweet corn, which contains more sugar than starch in the ear and is consumed as a vegetable.

Stover is the stalks, leaves, cobs, and husks of the corn plant, which is often discarded as waste. Stover contains a large amount of cellulose, which is the most abundant organic compound on earth. Breaking down this cellulose into usable sugars requires chemical treatments, enzymes, or heat. Figuring out how to efficiently convert cellulosic material into ethanol would unlock an enormous source of renewable energy. The main difference between stover (cellulose) and corn (starch) are the types of bonds that hold the chains together.

Enzymes

Enzymes are molecules that catalyze (or speed up the rate of) chemical reactions. Most enzymes are proteins that act on specific substrates, converting them into different molecules. Enzymes are not consumed, but are reused in the reactions they catalyze, and do not change equilibrium rates. Enzymes are simply responsible for lowering the activation energy for the specific reactions they catalyze. They can be affected by other molecules – inhibitors decrease enzymatic activity while activators increase this activity. Enzymes contain an active site, where substrates may temporarily bind to form an enzyme/substrate complex. Then the enzyme works to change this molecule into an enzyme/product complex, and the products eventually leave the enzyme, which may be reused. In the case of yeast, they use their natural enzyme, invertase (sucrase) to catalyze the hydrolysis of sucrose (a disaccharide) into glucose and fructose (both monosaccharides). Yeast cannot metabolize sucrose for energy until the enzymes have broken the disaccharides into glucose and fructose.

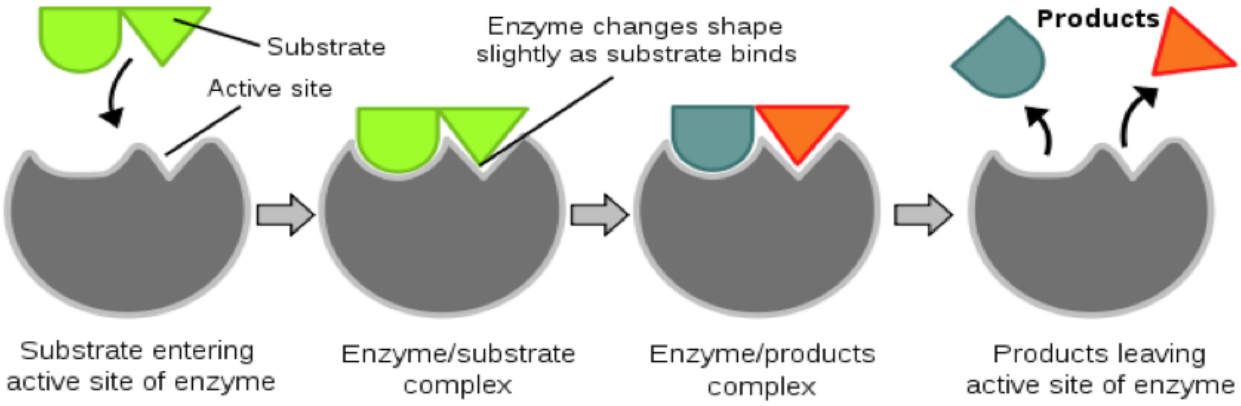


Figure 3: Enzyme function. The enzyme in this diagram represents invertase; the substrate is sucrose. Sucrose is a disaccharide, which is broken into two monosaccharide products, glucose and fructose, by the enzyme.

Yeast metabolize sugars as a food source, producing ethanol and CO2.

Yeast are small organisms that are classified as fungi. Most reproduce asexually by budding, and a few reproduce through binary fission. Their main sources of nutrition are hexose sugars, such as fructose and glucose, or disaccharides like maltose and sucrose (table sugar). They prefer glucose, but have enzymes to change some other forms of carbohydrates into glucose for use. Yeast can grow over a wide temperature range, but prefer warm conditions, typically between 30-37 °C. Yeasts perform both aerobic cellular respiration (requires oxygen) or anaerobic respiration, depending on the conditions; however, ethanol is produced in the anaerobic state.

Fermentation Challenge Data