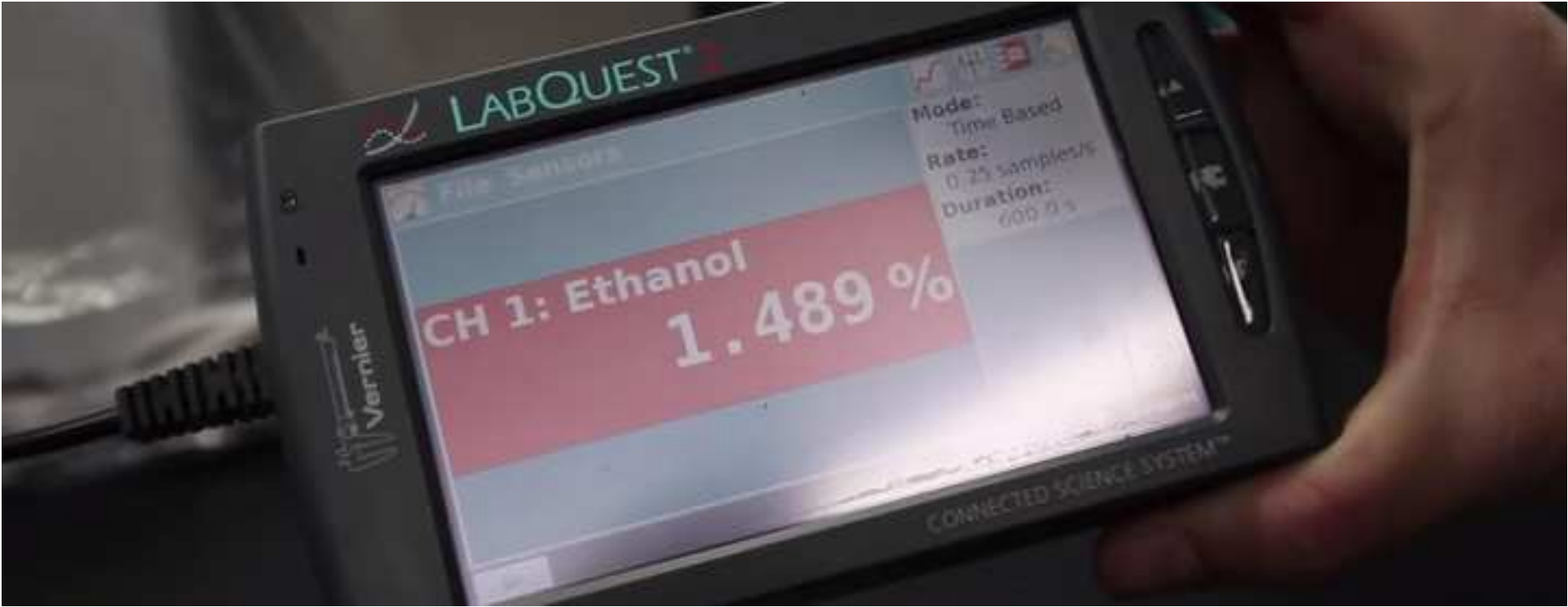


**JoVE: Science Education**  
**Biofuels: Producing Ethanol from Cellulosic Material**  
--Manuscript Draft--

Manuscript Number:	10014
Full Title:	Biofuels: Producing Ethanol from Cellulosic Material
Article Type:	Manuscript
Section/Category:	Manuscript Submission
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Feedstock	Ethanol produced
Sawdust	0.70%
Corn Stalks	0.60%
Cardboard	1.67%
Switchgrass	0.37%
Control	0.11%

**PI Name:** Margaret Workman and Kimberly Frye

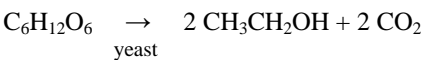
**Science Education Title** Biofuels: Producing Ethanol from Cellulosic Material

**Overview** In this experiment, we will use a cellulosic material (such as corn stalks, leaves, grasses, etc.) as a feedstock for the production of ethanol. The cellulosic material is first pretreated (ground and heated), digested with enzymes, and then fermented with yeast. Ethanol production is monitored using an ethanol probe. The experiment can be extended to optimize ethanol production by varying the feedstock used, pretreatment conditions, enzyme variation, yeast variation, etc. An alternative method of monitoring the reaction is to measure the carbon dioxide produced (using a gas sensor) instead of the ethanol. As a low tech alternative, glucose meters (found in any drug store) can be used to monitor the glucose during the process, if an ethanol probe or carbon dioxide gas sensor are not available.

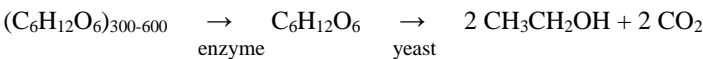
With an increased emphasis on ‘inquiry-based learning’, scientific probes are becoming more popular. Handheld devices like the Vernier Lab Quest used in conjunction with a variety of probes (such as those for conductivity, dissolved oxygen, voltage, and more) allow for less focus on collecting data and/or making graphs and more on analyzing the data and making predictions. Another advantage is that these are small and lightweight and can be taken into the field for measurements.

**Principles** The United States is looking to wean itself off of fossil fuels, especially petroleum used in gasoline. Global climate change, dependence on foreign countries, and increased political instability around the world are only a few reasons why. One possible way to decrease dependence on petroleum as a transportation fuel is by using more ethanol. Currently, regular gasoline contains approximately 10% ethanol as an additive. Special flex-fuel vehicles can use E85 gasoline, which is 85% ethanol.

In Brazil, ethanol is made by using sugarcane as the feedstock. The main product of sugarcane is sucrose, which is a disaccharide of glucose and fructose. The glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) is separated, then fermented by yeast to produce ethanol and carbon dioxide. The chemical reaction is as follows:

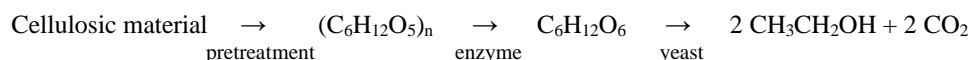


However, in the US, ethanol is made from corn. This requires more effort than making ethanol from sugarcane. To make ethanol from corn, first corn kernels need to be ground up, then the ground corn treated with enzymes to convert the starch in the corn to glucose. After this step, the process continues as in the sugarcane method above by using yeast to ferment the glucose into ethanol and carbon dioxide. The chemical reaction is as follows:



The production of ethanol from corn is problematic, however. For one thing, it takes some corn out of the food supply, thus driving up prices. It is also energy and fertilizer intensive to produce corn, decreasing its desirability as a transportation fuel alternative to petroleum. Therefore,

scientists are increasingly turning to cellulosic material to make ethanol. These materials include wood, grasses, and non-edible parts of plants. These are more desirable as they do not impact food supply. However, in order to release the glucose from the cellulosic material, much more effort is needed, as the glucose from cellulosic material is bound up in cellulose, which is then wrapped with hemicelluloses and lignin. First the cellulose needs to be extracted from the hemicelluloses and lignin bindings. This is done through a pretreatment of grinding and acid hydrolysis. Then, the cellulose is treated with enzymes to break it up into its component glucose. Finally, the glucose can be fermented with yeast to produce ethanol and water. The process can be summarized as follows:



This is currently too energy intensive to make it a practical method for ethanol production. However, research is underway to make the process better.

## **Procedure**

### Sample Preparation:

1. Select cellulosic material to be used as feedstock. This can be corn stalks, grasses, leaves, pet bedding, or paper.
2. Using a coffee grinder, grind feedstock into a fine powder with no large pieces remaining.
3. Measure 1.0 g of feedstock and place in a 50 mL centrifuge tube. Label the tube with the feedstock chosen.
4. Label a second 50 mL centrifuge tube as "Control". Do not put any feedstock in this tube.

### Pretreatment:

1. Set up a 500 mL beaker with approximately 400 mL of water on a hotplate and heat to a gentle boil.
2. Add 25 mL of distilled water to the 2 centrifuge tubes. Swirl to mix. Put the cap on the centrifuge tubes loosely.
3. Put the centrifuge tubes in the beaker full of gently boiling water. Be sure that water from the water bath does not leak into the tubes. Let boil for 30 minutes.
4. Let tubes cool to room temperature.

### Enzymatic Digestion:

1. Add 1 mL of cellulase enzyme to both tubes.
2. Put tubes in an incubator at 50°C for 24 hours.

### Fermentation:

1. Add 1 .0 g of active yeast to each of the centrifuge tubes. Swirl to mix.
2. Loosely cap the centrifuge tubes. Do NOT cap tightly because CO<sub>2</sub> will be generated, creating pressure in the tube. This could cause the cap to be ejected off the tube if some of the CO<sub>2</sub> is not allowed to escape, reducing the pressure.
3. Place the centrifuge tubes in a rack and put in an incubator at 37°C for 24 hours.
4. Using an ethanol probe, measure the ethanol concentration in the control tube. (Figure 1)
5. Using an ethanol probe, measure the ethanol concentration in the sample tube.

#### Extension:

1. Students can vary the feedstock used to determine which biomass material produces the most ethanol.
2. Students can vary the enzyme used to determine which enzyme produces the most ethanol.
3. Students can vary other conditions (temperatures, time, etc.) to try to optimize the ethanol produced.

**Comment [DM1]:** Not sure how you want to handle this Aaron. I suspect we'll cut it. If you wanted to include it but altered, let me know and I can work on it.

## Representative Results

The % ethanol in the solution will be displayed on the handheld tablet screen using the software related to the brand of the ethanol probe used. (Figure 2)

Representative results of the percent ethanol produced by various feedstocks can be seen in table 1.

**Applications** The Energy Independence and Security Act of 2007 set into law a renewable fuel standard. It created a phase-in for renewable fuel volumes starting at 9 billion gallons in 2008 and ending at 36 billion gallons in 2022. Of that 36 billion, it was expected that 16 billion of that would come from cellulosic materials. For 2014, the original proposal was for 18.15 billion gallons of renewable fuel, 1.75 billion of that coming from cellulosic material. Unfortunately, based on the volume of cellulosic ethanol that is feasible to be produced currently, this number has had to be reduced to 17 million gallons according to a recent EPA proposal. Improving the process of creating ethanol from cellulosic material is currently a very hot area of research. In this experiment, students will be emulating the scientific practices that scientists in the top research labs are following. This experiment is also a great example of incorporating engineering practices into scientific research, as recommended by the Next Generation Science Standards.

**Comment [DM2]:** I suspect this will be problematic but wanted to get your input on it Aaron. It isn't "evergreen" enough with all the dates, but at the same time, I'm not sure how to tackle the Application of this sort of technique without getting into some issues that are timely/require date benchmarks, if that makes sense.

**Legend:**

**Figure 1:** Ethanol probe measuring the ethanol concentration in the control tube.

**Figure 2:** Handheld tablet displaying % of ethanol.

**Table 1:** Representative results of the percent ethanol produced by various feedstocks



**PI Name:** Margaret Workman and Kimberly Frye

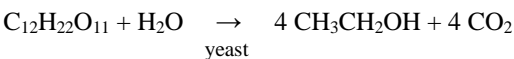
**Science Education Title:** Biofuels: Producing Ethanol from Cellulosic Material

**Overview:** In this experiment, cellulosic material (such as corn stalks, leaves, grasses, etc.) will be used as a feedstock for the production of ethanol. The cellulosic material is first pretreated (ground and heated), digested with enzymes, and then fermented with yeast. Ethanol production is monitored using an ethanol probe. The experiment can be extended to optimize ethanol production by varying feedstock, pretreatment conditions, enzyme variation, yeast variation, etc. An alternative method of monitoring the reaction is to measure the carbon dioxide produced (using a gas sensor) instead of the ethanol. As a low-tech alternative, glucose meters (found in any drugstore) can be used to monitor the glucose during the process, if an ethanol probe or a carbon dioxide gas sensor are not available.

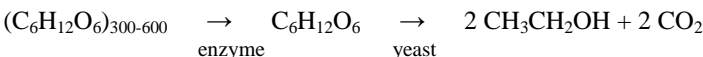
With an increased emphasis on “inquiry-based learning”, scientific probes are becoming more popular. Handheld devices, like the Vernier Lab Quest, used in conjunction with a variety of probes (such as those for conductivity, dissolved oxygen, voltage, and more) allow for less emphasis on collecting data and/or making graphs and more focus on analyzing the data and making predictions. Additionally, these devices are small, lightweight, and portable, which allows them to be taken into the field for measurements.

**Principles:** The United States is looking to wean itself off of fossil fuels, especially petroleum used in gasoline, for many reasons, including global climate change, dependence on foreign countries, and increased political instability around the world. One possible way to decrease dependence on petroleum as a transportation fuel is by using more ethanol. Currently, regular gasoline contains approximately 10% ethanol as an additive. Special flex-fuel vehicles can use E85 gasoline, which is 85% ethanol.

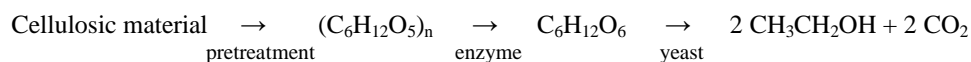
In Brazil, ethanol is made by using sugarcane as the feedstock. The main product of sugarcane is sucrose, which is a disaccharide of glucose and fructose. Most species of yeast have the enzyme sucrose and are able to cleave the glucose – fructose bond. The sucrose (C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>) is fermented by yeast to produce ethanol and carbon dioxide. The overall chemical reaction is as follows:



However, in the US, ethanol is made from corn. Corn stores carbohydrates as a polysaccharide called starch. This requires more effort than making ethanol from sugarcane, because yeast cannot cleave the bonds in starch. To make ethanol from corn, corn kernels need to be ground up, and then the ground-up corn is treated with enzymes to convert the starch in the corn to glucose. After this step, the process continues (as in the sugarcane method) by using yeast to ferment the glucose into ethanol and carbon dioxide. The chemical reaction is as follows:



The production of ethanol from corn is problematic. It removes some corn out of the food supply, thus driving up prices. It is also energy and fertilizer intensive to produce corn, decreasing its desirability as a transportation fuel alternative to petroleum. Therefore, scientists are increasingly turning to cellulosic material to make ethanol. These materials include wood, grasses, and non-edible parts of plants. These are more desirable as they do not impact food supply. However, in order to release the glucose from the cellulosic material, much more effort is needed, as the glucose from cellulosic material is bound up in cellulose, which is then wrapped with hemicelluloses and lignin. First, the cellulose needs to be extracted from the hemicelluloses and lignin bindings. This is done through a pretreatment of grinding and acid hydrolysis. Then, the cellulose is treated with enzymes to break it up into its component glucose. Finally, the glucose can be fermented with yeast to produce ethanol and water. The process can be summarized as follows:



This is currently too energy intensive to be a practical method for large-scale ethanol production. Research is underway to make the process better.

## **Procedure:**

### Sample Preparation:

1. Select cellulosic material to be used as feedstock. This can be corn stalks, grasses, leaves, pet bedding, or paper.
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3. Put the centrifuge tubes in the beaker full of gently boiling water. Be sure that water from the water bath does not leak into the tubes. Let boil for 30 minutes.
4. Let tubes cool to room temperature.

### Enzymatic Digestion:

1. Add 1 mL of cellulase enzyme to both tubes.
2. Put tubes in an incubator at 50°C for 24 hours.
3. Allow the tubes to cool to room temperature.

#### Fermentation:

1. Add 1.0 g of active yeast (regular grocery store yeast is fine) to each of the centrifuge tubes. Swirl to mix.
2. Loosely cap the centrifuge tubes. Do NOT cap tightly, because CO<sub>2</sub> will be generated, creating pressure in the tube. This could cause the cap to be ejected off the tube if some of the CO<sub>2</sub> is not allowed to escape, reducing the pressure.
3. Place the centrifuge tubes in a rack and put in an incubator at 37°C for 24 hours.
4. Using an ethanol sensor, measure the ethanol concentration in the control tube (Figure 1). Ethanol sensors can be purchased through Vernier or PASCO for approximately \$100 each.
5. Using an ethanol sensor, measure the ethanol concentration in the sample tube.

**Comment [A1]:** Do the tubes need to cool down to RT before adding the yeast?

**Comment [A2]:** Does the strain of yeast matter? Has this been studied?

**Comment [JR3]:** Mworkman: There will be slight differences depending on the yeast used. It is recommended to be consistent with the type of yeast used. Students could extend the lab and try various types of yeast. These can be purchased at the local brewing supply store.

**Comment [A4]:** Provide information about the sensor used in this experiment.

**Representative Results:** The % ethanol in the solution will be displayed on the handheld tablet screen using the software related to the brand of the ethanol sensor used (Figure 2).

Representative results of the percent ethanol produced by various feedstock can be seen in Table 1.

**Comment [A5]:** Is this by weight, by volume?

**Comment [maw6]:** This is the percent ethanol in the solution, so by volume.

**Applications:** The Energy Independence and Security Act of 2007 set into law a renewable fuel standard. It created a phase-in for renewable fuel volumes, starting at 9 billion gallons in 2008 and ending at 36 billion gallons in 2022. Of that 36 billion, it was expected that 16 billion would come from cellulosic materials. For 2014, the original proposal was for 18.15 billion gallons of renewable fuel, 1.75 billion of that coming from cellulosic material. Unfortunately, based on the volume of cellulosic ethanol that is feasible to be produced currently, this number has been reduced to 17 million gallons, according to a recent EPA proposal. Improving the process of creating ethanol from cellulosic material is currently a very hot area of research. In this experiment, students will be emulating the scientific practices that scientists in the top research labs are following.

A variety of biomass feedstock materials can be used to produce cellulosic ethanol for transportation. The U.S. Department of Energy's Bioenergy Technologies Office is focused on developing cellulosic feedstock from non-food based plant material and the related technologies that will allow an economically feasible conversion of this biomass to transportation fuel. They are investigating biomass sources ranging from agricultural residue, herbaceous energy crops, and forest materials to waste materials and algae. In this laboratory activity, students can vary the feedstock they use and compare the amount of ethanol that results. Possibilities include corn stover, grasses, leaves, cardboard, newspaper, paper, flowers, etc.

One roadblock to large scale production of cellulosic ethanol is the costly nature (both in terms of money and energy) of the pretreatment process. Much research is being done on reducing these costs and making the breakdown of the cellulosic material easier. Enzyme companies are spending a lot of time and money developing new enzymes to increase the yield of ethanol. In this laboratory activity, students can vary the enzymes they use and compare the amount of ethanol that results. A variety of Cellulase enzymes can be purchased from chemical companies that sell to schools, such as cellulase from *Aspergillus niger* or cellulase from *Trichoderma viride*. Proprietary enzymes can be purchased from specialty enzyme companies, but these can be costly. Other enzymes can be used, like amylase, to compare their ability of producing ethanol from cellulosic material to that of the cellulases.

Another emerging area of research is improving the ability of yeast to convert cellulosic biomass into ethanol. Lignocellulose has evolved in plants to provide stability. This is due to the crosslinking between cellulose and hemicellulose and the ester linkages in lignin. It is necessary to separate the cellulose from the lignin and then treat the cellulose to break it down into a monosaccharide. In addition, the hemicellulose has a high percentage of pentoses like xylose, which is more difficult to ferment than a hexose like glucose. In this laboratory activity, students can vary the yeast they use and compare the amount of ethanol that results. Many yeast types can be purchased at a local brewing supply store.

### Extension:

### Legend:

**Figure 1:** Ethanol probe measuring the ethanol concentration in the control tube.

**Figure 2:** Handheld tablet displaying % of ethanol.

**Table 1:** Representative results of the percent ethanol produced by various feedstocks.

**Comment [A7]:** All of these would be interesting to show (assuming there was a change in ethanol production). How many can you demonstrate? Replace the current Application section with a more flushed out version of this.

**Comment [maw8]:** I can test a variety of feedstocks, including corn stover, grasses, prairie plants, cardboard, waste paper, etc. We can vary the enzyme used. I could probably find 2 or 3 different enzymes to test. (Some would work, some might produce little ethanol.) In addition, I could vary the yeast. I could try brewers yeast, champagne yeast, grocery store yeast, etc. Our local brew store has several varieties of yeast. We could also do a corn starch feedstock and amylase enzyme to compare the cellulosic ethanol to corn ethanol production.