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BIOL 305L
Laboratory Six
An Investigation of Biofuels

Introduction:

To me, this is the ultimate use of the plant cell wall - the potential to obtain an alternative fuel from all of those structural carbohydrates. Most recently, the cellulose component, in particular, has been considered as a possible starting point for a biofuel. A biofuel is a renewable energy source unlike other natural resources such as petroleum, coal, and nuclear fuels. One legal definition of biofuel is any fuel with an 80% minimum content by volume of materials derived from living organisms harvested within the ten years preceding its manufacture. Like coal and petroleum, biomass is a form of stored solar energy. The energy of the sun is "captured" through the process of photosynthesis in growing plants. One of the major advantages of biofuel over most other fuel types is that it is biodegradable and so virtually harmless to the environment if spilled. The production of biofuels to replace oil and natural gas is in active development around the world focusing on the use of cheap organic matter in the efficient production of liquid and gas biofuels, which yield high net energy gain. The carbon in biofuels was recently extracted from atmospheric carbon dioxide by growing plants, so burning it does not result in a net increase of carbon dioxide in the Earth's atmosphere. Therefore, many people believe that a way to reduce the amount of carbon dioxide released into the atmosphere is to use biofuels to replace non-renewable sources of energy. The traditional use of plant biomass is combustion in stoves or boilers for heat. However, biomass can also be converted to biofuels - liquid and gaseous fuels such as ethanol, methanol, gasoline, diesel fuel, and methane. Making ethanol from corn is a major U.S. industry, producing nearly 1 billion gallons each year. The processes for converting biomass to fuels include a broad range of thermal, chemical, and biological processes.

Currently, in the Red River Valley, Biodiesel is being used in public transportation. However, this is very different from biofuel, as it is derived from oils (and not carbohydrates) extracted from plants such as soy, peanut, rapeseed, and some species of aquatic algae. So far, the biofuel market is untapped in this area of Minnesota. Various local prairie plant species, corn, and sugar-beet all have the potential to be a source of biofuel. The first step would be to extract the

carbohydrates from the plant cell wall and determine which of the three main carbohydrates is the best source to make a biofuel. Although corn is an obvious plant material to work with, sugar-beet pulp should not be ruled out. Its relatively low cost and availability have long made sugar-beet pulp a potential source of carbohydrates. However, because of the acetylation and feruloylation of the polymers, the applications for sugar-beet carbohydrates in the food industry have been limited. The pulp is almost exclusively used as animal feed or combustion in stoves or boilers for heat. When one considers the potential of biofuel research, it is clear that sugar-beet pulp would be a cheap and logical choice.

So, in nature, various enzymes are required to breakdown plant cell wall material to basic units which could be used as a source material for a potential biofuel. In this lab section we will look at just one of them, a class of enzymes known as *Cellobiases*.

These are a part of a group of enzymes collectively known as *cellulases* that are actively being studied and produced for use in the biofuel industry. These enzymes are capable of breaking down cellulose, a complex macromolecular construct of sugar chains.

Cellobiases are naturally produced by **fungi and bacteria** present in ruminants, termites, and some plants or plant products. They are being investigated for use in breaking down non-food/feed plant products, such as corn stover, switch grass, and waste wood products, along with fast-growing trees, such as poplar, for production of ethanol to replace fossil fuels.

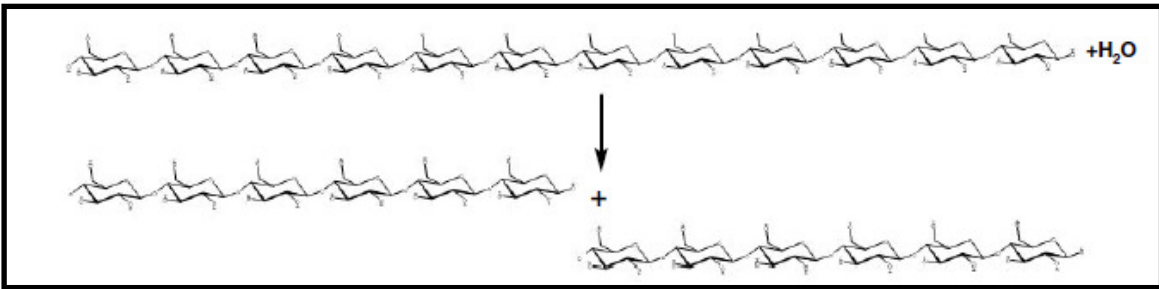
This lab uses the enzyme cellobiase to increase the degradation rate of a sugar compound p-nitrophenyl glucopyranoside to glucose and p-nitrophenol which is a yellow substance. The enzyme activity is indirectly measured by monitoring the change in color intensity of the solution.

Types of Cellulases Needed to Break Down Plant Cell Walls into Glucose

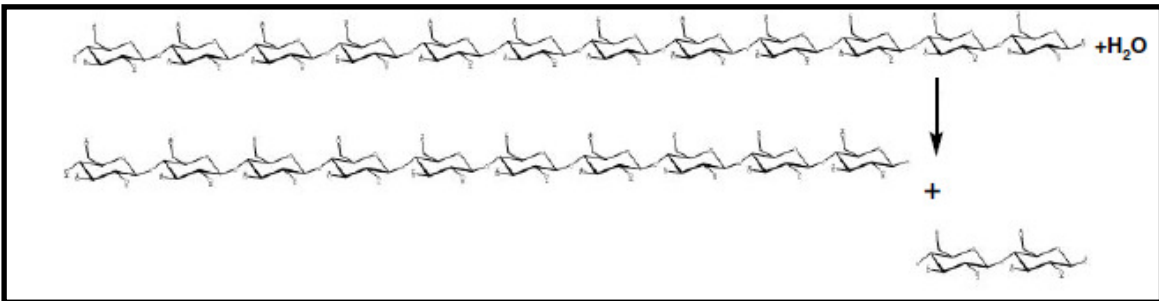
The production of ethanol from plant material is a very complex procedure requiring multiple steps. Plant material is first processed mechanically, as well as with acids or enzymes and heat to remove lignin.

Lignin is a highly complex, aromatic macromolecule found in high quantities in secondary cell walls of fibrous and woody plant tissue in close association with cellulose. Once the lignin is removed, the cellulose is more exposed and can be more readily broken down. Cellulose is broken down into glucose in three steps by three different types of enzymes.

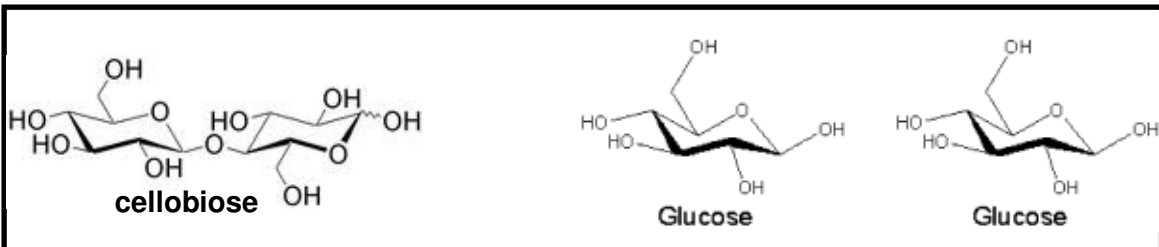
Endocellulases – these enzymes break down the internal bonds of the long chains of glucose molecules that form cellulose.



Exocellulases – these enzymes break the covalent linkages between the glucose units of cellulose that are on the end of the cellulose molecules, releasing cellobiose.



Cellobiases (β -glucosidases) – these enzymes break down the cellobiose left behind as a result of the work of the first two enzymes.



Cellobiase Enzyme - Cellobiase, the enzyme provided in this lab, breaks down cellobiose, a disaccharide made up of two glucose molecules connected together by a β - (1,4) glucoside linkage. The breakdown of cellobiose by cellobiase is the final step in producing glucose from cellulose. Glucose is the preferred source of sugar for microbial fermentation, an additional enzymatic reaction that produces ethanol.

Detecting the substrate used in this lab:

Although cellobiose is the natural substrate of cellobiase, there is no simple method to quantitatively detect the product (glucose) or the disappearance of cellobiose. A simple colorimetric assay using an artificial substrate, p-nitrophenyl glucopyranoside, can be used to detect enzymatic activity of cellobiase.

The substrate p-nitrophenyl glucopyranoside is composed of a beta glucose covalently linked to a molecule of nitrophenol. When the bond connecting these two molecules is cleaved with the help of cellobiase, the p-nitrophenol is released. To stop the activity of the enzyme and to create a colored product, the reaction mixture is added to a basic solution. When the p-nitrophenol is placed in a basic solution, the hydroxyl group on the nitrophenol loses an H^+ to the OH^- of the base, which changes the bonding within the phenolic ring, so that the molecule will absorb violet light (and reflect yellow light). This makes the solution yellow, which can be detected visually by comparing the deepness of the yellow color to a set of standards of known concentration of p-nitrophenol or by using a spectrophotometer to produce more accurate, quantitative results.

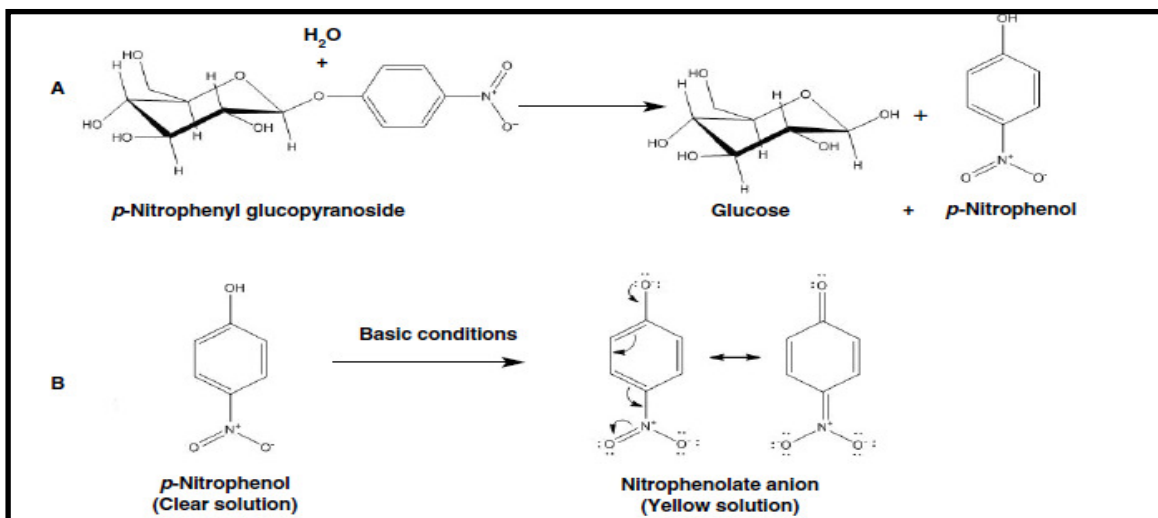


Figure 1: Detecting glucose from the breakdown of cellobiose by cellobiase enzyme. (A). An analog to cellobiose called p-nitrophenyl glucopyranoside is used to detect enzymatic activity and accumulation of glucose. Once cleaved by cellobiase, the p-nitrophenol is released, which can be detected by its yellow color in basic solutions. (B). p-nitrophenol is colorless to slightly yellow at pH 5. However, under basic conditions, the hydrogen ion of the hydroxyl group (OH^- group) is removed, resulting in a negative charge due to an extra pair of electrons on the remaining oxygen group. This pair of electrons travels along the nitrophenolate anion, creating a resonance structure that produces the yellow color.

Lab Activities:

So, for this lab we will be looking the key role cell wall associated enzymes play in the puzzle of Biofuels. We will use an artificial substrate which mimics cellobiose to look at plant enzyme reactions in terms of:

- Temperature
- PH
- Substrate and enzyme concentration
- Commercial and freshly isolated enzymes

All of these conditions will help you understand the complexities of enzyme actions and their role in the production of a biofuel.

**NOTE: ALL assays will be measured
at 410 nm**

Objectives for week one:

Activity 1: Determine the Reaction Rate in the Presence or Absence of an Enzyme

- a. Find your 15 ml conical tubes labeled "Stop Solution", "1.5 mM Substrate", "Enzyme" and "Buffer". Write your initials on each tube.
- b. Label five cuvettes E1-E5.
- c. Label the two remaining cuvettes "Start" and "End".
- d. Using a clean DPTP, pipette 500 μ l of stop solution into each labeled cuvette. Rinse the DPTP well with water.
- e. Label one empty 15 ml conical tube "Enzyme Reaction" and the other "Control".
- f. Using a clean DPTP, pipette 2 ml of 1.5 mM substrate into the 15 ml conical tube labeled "Enzyme Reaction". Use the same DPTP and pipette 1 ml

of 1.5 mM substrate into the conical tube labeled "Control". Rinse the DPTP well with water.

g. Label one DPTP "E" for enzyme and the other "C" for control. Only use the DPTP labeled "E" for the enzyme reaction tube and the DPTP labeled "C" for the control reaction tube.

h. Using the DPTP labeled "C", pipette 500 μ l of buffer into the 15 ml conical tube labeled "Control" and gently mix. Once you have mixed the buffer with the substrate, remove 500 μ l of this solution and add it to your cuvette labeled "Start".

i. Using the DPTP labeled "E", pipette 1 ml of enzyme into the 15 ml conical tube labeled "Enzyme Reaction". Gently mix, then **START YOUR TIMER**.

j. At the times indicated, use the DPTP labeled "E" to remove 500 μ l of the solution from the "Enzyme Reaction" tube and add it to the appropriately labeled cuvette containing the stop solution.

k. After all the enzyme samples have been collected, use the DPTP labeled "C" to remove 500 μ l of the solution from the "Control" reaction tube and add it to the cuvette labeled "End".

Activity 2: Determine the Effect of Temperature on the Reaction Rate

a. Label your cuvettes "0°C", "22°C", and "37°C".

b. Using a clean DPTP, pipette 500 μ l of stop solution into each cuvette. Wash the DPTP out thoroughly with water.

c. Label three 1.5 ml microcentrifuge tubes "0°C Enzyme", "22°C Enzyme", and "37°C Enzyme". Using a clean DPTP, pipet 250 μ l of enzyme into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.

d. Label three 1.5 ml microcentrifuge tubes "0°C Substrate", "22°C Substrate", and "37°C Substrate". Using a clean DPTP, pipette 500 μ l of 1.5 mM substrate into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.

e. Place the tubes labeled "0°C Enzyme" and "0°C Substrate" in the ice cup. Place the tubes labeled "22°C Enzyme" and "22°C Substrate" on your lab bench. Place the tubes labeled "37°C Enzyme" and "37°C Substrate" in the beaker of warm water at 37°C. Allow the tubes to equilibrate to their respective temperatures for at least 5 minutes

f. Have a stopwatch ready. Using a clean DPTP, pipette the 250 µl of enzyme from the tube labeled "0°C Enzyme" into the tube labeled "0°C Substrate", and place the tube now containing your enzyme and substrate mix back on ice. Add the 22°C enzyme to the 22°C substrate solution, and place that tube back on the lab bench. Add the 37°C substrate to the 37°C enzyme solutions, and put that tube back into the 37°C water bath. **START YOUR TIMER.**

g. After 2 minutes, use a clean DPTP for each temperature reaction to transfer 500 µl of your reaction to the appropriately labeled cuvette containing the stop solution.

Activity 3: Determine the effect of pH on the Reaction Rate

a. Label your cuvettes "pH 5.0", "pH 6.3", and "pH 8.6".

b. Using a clean DPTP, pipette 500 µl of stop solution into each cuvette. Wash the DPTP out thoroughly with water.

c. Using a clean DPTP, pipette 250 µl of 3.0 mM substrate into each microcentrifuge tube labeled "pH 5.0", "pH 6.3" and "pH 8.6" by your instructor. Wash the DPTP out thoroughly with water.

d. Have a stopwatch ready. Using a clean DPTP, add 250 µl of enzyme to each of the labeled microcentrifuge tubes. **START YOUR TIMER.**

e. After 2 minutes, using a clean DPTP for each pH reaction, transfer 500 µl of your reaction to the appropriately labeled cuvette containing the stop solution.

Objectives for week two:

Activity 4: Determine the Effect of Enzyme Concentration on the Reaction Rate

- a. Label one 15 ml conical tube "Low Concentration Enzyme". Using a clean DPTP, pipette 1 ml of buffer into the tube. Wash out the DPTP with water. Pipette 1 ml of high concentration enzyme to your tube labeled "Low Concentration Enzyme" and mix. Wash out the DPTP thoroughly with water.
- b. Label three cuvettes "H1-H3" (for high enzyme concentration time points) and the remaining three cuvettes "L1-L3" (for low enzyme concentration time points). Only label on the upper part of the cuvette face.
- c. Using a clean DPTP, pipette 500 μ l of stop solution into each cuvette. Wash out the DPTP thoroughly with water.
- d. Label one clean DPTP with an "H" for high enzyme concentration and a second clean DPTP with an "L" for low enzyme concentration.
- e. Using the DPTP labeled with an "H", pipette 250 μ l of 1.5 mM substrate into your 15 ml conical tube containing enzyme labeled "High Concentration Enzyme".
- f. Using the DPTP labeled with an "L", pipette 250 μ l of 1.5 mM substrate into your 15 ml conical tube containing enzyme labeled "Low Concentration Enzyme". **START YOUR TIMER.**
- g. At the times indicated (**1 min, 2 min 5 min, 10 min, 20 min**), use the correctly labeled DPTP to remove 500 μ l from the 15 ml conical tubes labeled "High Concentration Enzyme" and "Low Concentration Enzyme", and add it to the appropriately labeled cuvette that already contains the stop solution.

Activity 5: Determine the Effect of Substrate Concentration on the Reaction Rate

- a. Label one clean 15 ml conical tube "Low Concentration Substrate" and one clean 15 ml conical tube "High Concentration Substrate".
- b. Using a clean DPTP, pipette 1.5 ml of 1.5 mM substrate into the 15 ml conical tube labeled "High Concentration Substrate". Rinse the DPTP thoroughly with clean water.
- c. Using a clean DPTP, pipette 1.25 ml of buffer into the 15 ml conical tube labeled "Low Concentration Substrate". Rinse the DPTP thoroughly with water and then pipet 250 μ l of 1.5 mM substrate into the 15 ml conical tube labeled "Low Concentration Substrate" and mix. Rinse the DPTP thoroughly with water.
- d. Label your cuvettes "H1-H3" (for high substrate concentration time points) and "L1-L3" (for low substrate concentration time points). Only label on the upper part of the cuvette face.
- e. Using a clean DPTP, pipette 500 μ l of stop solution into each cuvette. Rinse the DPTP thoroughly with water.
- f. Label one DPTP as "H" for high substrate concentration and a second DPTP as "L" for low substrate concentration.
- g. Using a clean DPTP, pipette 750 μ l of enzyme into your 15 ml conical tube of substrate labeled "High Concentration Substrate".
- h. Using a clean DPTP, pipette 750 μ l of enzyme into your 15 ml conical tube of substrate labeled "Low Concentration Substrate". **START YOUR TIMER.**
- i. At the times indicated (1 min, 2 min 5 min, 10 min, 20 min), use the correctly labeled DPTP to remove 500 μ l from the 15 ml centrifuge reaction tubes labeled "High Concentration Substrate" and "Low Concentration Substrate" and add it to the appropriately labeled cuvette that contains the stop solution.

Activity 6: Test the Ability of Mushroom Extracts to Increase the Reaction Rate

- a. Weigh out approximately 1 gram of mushroom and put it into a mortar.
- b. Add 2 ml of extraction buffer for every gram of mushroom into the mortar. To calculate the amount of extraction buffer you need, multiply the weight (in grams) of the mushroom by 2 and add that many milliliters.

Weight of mushroom _____ g \times 2 = _____ ml

- c. Using a pestle, grind your mushroom to produce a slurry.
- d. Strain the solid particles out of your slurry using a piece of filter paper or cheese cloth into a 1.5 ml microcentrifuge tube. Alternatively, if you have a centrifuge, scoop the slurry into a 1.5 ml microcentrifuge tube and then pellet the solid particles by spinning at top speed for 2 minutes. **Note:** You will need at least 250 μ l of extract to perform the enzymatic reaction.
- e. Label your cuvettes "1-6". Only label on the upper part of the cuvette face.
- f. Using a clean DPTP, pipette 500 μ l of stop solution into each cuvette. Rinse out the DPTP thoroughly with water.
- g. Label a 15 ml conical tube with the type of mushroom you are using and then using a clean DPTP, pipette 3 ml of substrate into the tube.
- h. Using a clean DPTP, pipette 250 μ l of your enzyme extract into your 15 ml conical tube of substrate. **START YOUR TIMER.**
- i. At the times indicated, (1 min, 2 min 5 min, 10 min, 20 min), remove 500 μ l of mushroom extract/substrate mixture from the reaction tube, and add it to the appropriately labeled cuvette that already contains the stop solution.
- j. Using a clean DPTP, add 500 μ l of extraction buffer to cuvette #6. Clean the DPTP and then add one drop of mushroom extract. This will serve as the "blank" for this experiment.

LASTLY:

For the following week: Individual 9-10 page write up of the lab section (1 inch margin, 12 pt font, 1 ½ spacing). You know what to include! Just in case:

- Intro with background info and hypothesis: at least three references
- Methods in detail
- Written description of data, including all graphs (pasted into your work document, legends and titles!
- Detailed conclusion, explain results, include info from introduction. Did the experiment work? Possible errors and/or improvements.
- All references listed. No required format for this just be consistent!