LAB: Turning Cellulose into Ethanol: A Study of Biofuels

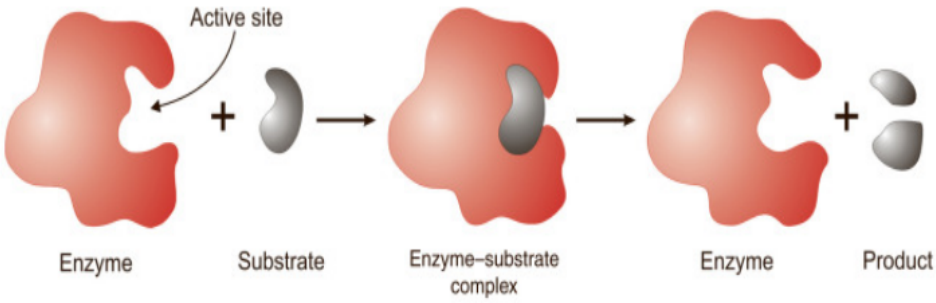
Background:

Enzymes

Enzymes are typically proteins that act as catalysts, meaning that they speed up chemical reactions that would take far too long to occur on their own.

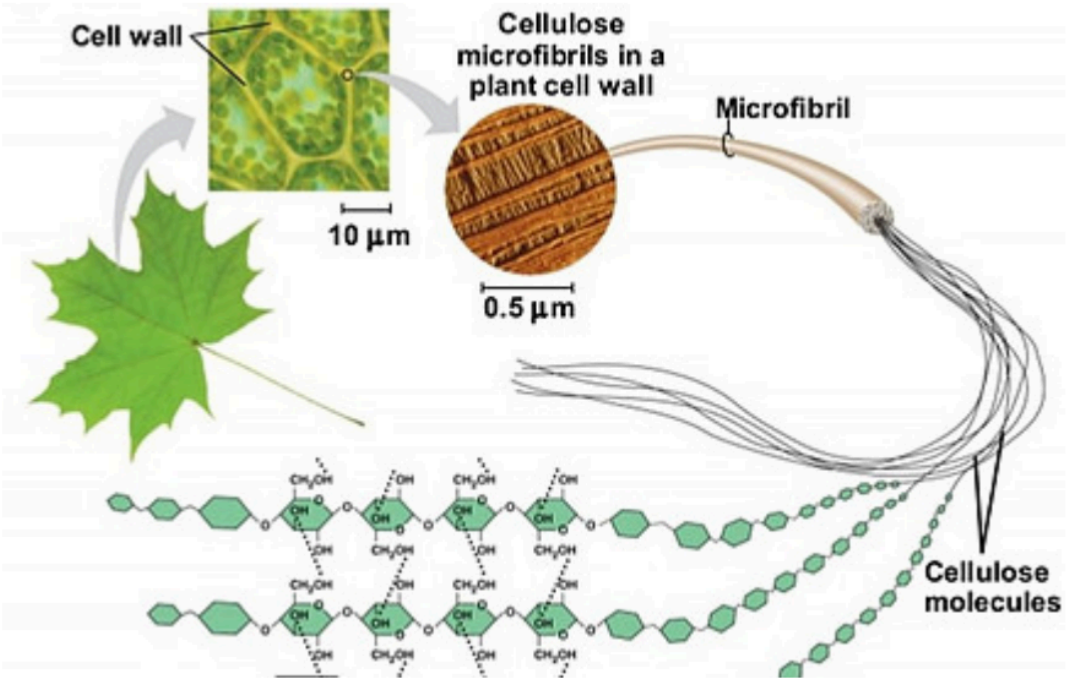
Each type of enzyme has a specific shape that compliments the structure of its substrate. An enzyme is like a baseball glove. The ball is like the substrate, it fits into the glove.

The substrate is the molecule that the enzyme converts into a product.



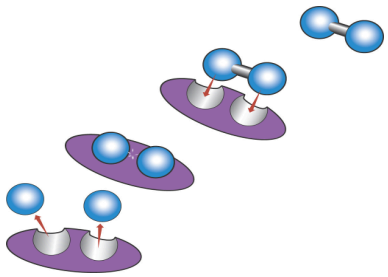
The substrate fits into an indentation (pocket) in the globular protein called the **active site.** The shape of the active site is critical to the enzyme's function.

Cellulose

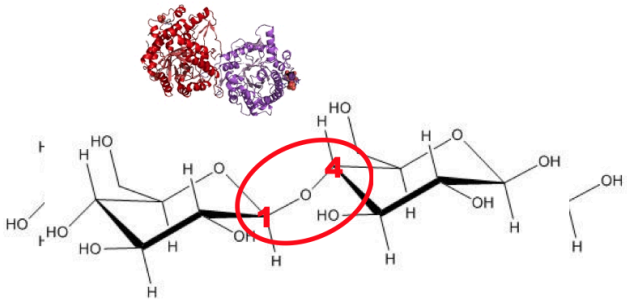


Breaking down the cellulose from plants into sugar is also an important step in the creation of ethanol for fuel.

Cellobiose is composed of two glucose molecules covalently connected.



Cellobiase has a pocket that fits the cellobiose molecule.



Cellobiose + H_2O ⟶ 2 Glucose

Once the **beta** 1–4 bond in cellobiose has been broken, the two glucose molecules are released from the cellobiase, and the enzyme is free to bind to another molecule of cellobiose and begin the cycle again.

Introduction

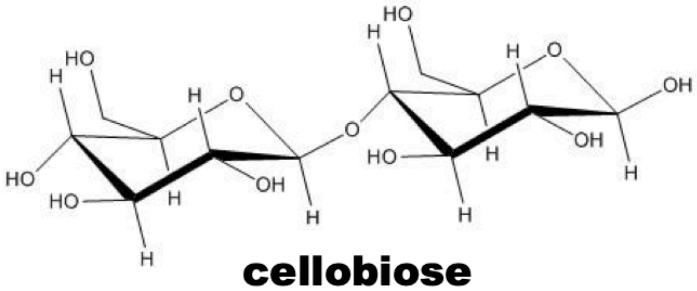
Cellobiase Enzyme

In this experiment, you will be studying **cellobiase**. Cellobiase is an enzyme that is involved in the last step of the process of breaking down cellulose, a molecule made up of bundled long chains of glucose that are found in plant cell walls, to glucose.

This is a natural process that is used by **many fungi** as well as bacteria (some present in termite guts, others in the stomachs of ruminants and also in compost

piles) to produce glucose as a food source. See video:

Cellobiase Substrates

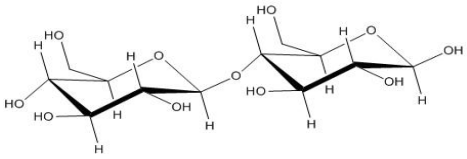


cellobiose

The natural substrate for the enzyme cellobiase is cellobiose. This is a disaccharide composed of two beta glucose molecules.

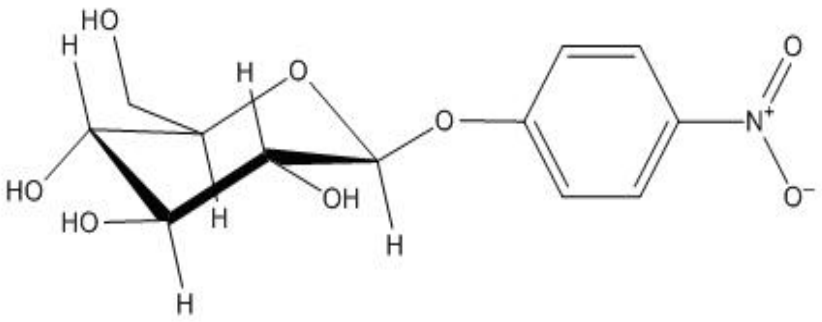
However, when scientists study enzyme function, it is best if there is an easy way to detect either the amount of substrate that is used up or the amount of product that is formed.

H_2O +



Solutions of cellobiose (substrate) and glucose (product) are clear, and there are not many simple, inexpensive, fast methods to detect these molecules quantitatively.

So, to make this reaction easier to follow,
an **artificial substrate, p-nitrophenyl glucopyranoside, will be used.**



This artificial substrate can also bind to the enzyme and be broken down in a manner similar to the natural substrate cellobiose. When the artificial substrate, p-nitrophenyl glucopyranoside, is broken down by cellobiase, it produces glucose and **p-nitrophenol**.

When p-nitrophenol is mixed with a solution that is basic in pH (such as the stop solution), it will turn yellow.

The amount of yellow color is proportional to the amount of p-nitrophenol present.

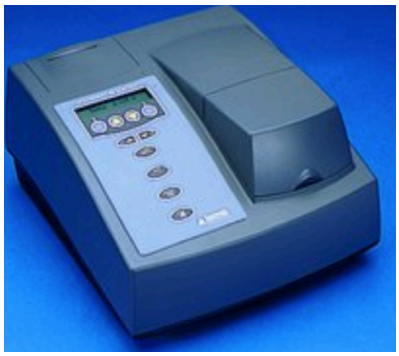
And for every molecule of p-nitrophenol present, one molecule of p-nitrophenyl glucopyranoside is broken apart. For the cellobiase reactions being run, another advantage of using a basic solution to develop the color of the p-nitrophenol is that the basic pH will also denature the enzyme and stop the reaction.

Measuring the Amount of Product Produced

Since the product (p-nitrophenol) of the artificial substrate reaction turns yellow once base is added, you can tell how much product is being produced. **The deeper the color, the higher the amount of product made**.

One simple method of estimating how much product is formed is to compare the yellowness of enzyme reaction samples to a set of known standards, which contain a known amount of colored product. You can estimate which tube in the set of standards most closely matches your samples in color. This will give you an estimated amount of product.

A spectrophotometer, which quantitatively measures the amount of yellow color by shining a beam of light (wavelength of 410 nm) through the sample.



The spectrophotometer measures the amount of light that is absorbed by the sample. **The darker the color of yellow the sample is, the more light that is absorbed, and thus the more concentrated the sample**. The absorbance values of a set of standards can first be measured to create a standard curve, a plot of the absorbance values of samples of known concentration of p-nitrophenol.

The absorbance values of the reaction samples can then be measured, and the standard curve can be used to convert the absorbance value to a concentration value.

Determining the Standard Curve

By plotting the absorbance values for the standards with known amounts of p-nitrophenol (called a standard curve), you can determine how much p-nitrophenol is present

Draw this table in your lab notebook.

***p*-Nitrophenol standards.**

Standard	Amount of *p*-Nitrophenol (nmol)	Absorbance at 410 nm
S1	0	0.00
S2	12.5	
S3	25	
S4	50	
S5	100	

Methods:

Methods for determining the Standard Curve

1. Each group
   Obtain the five cuvettes and label them S1–S5.

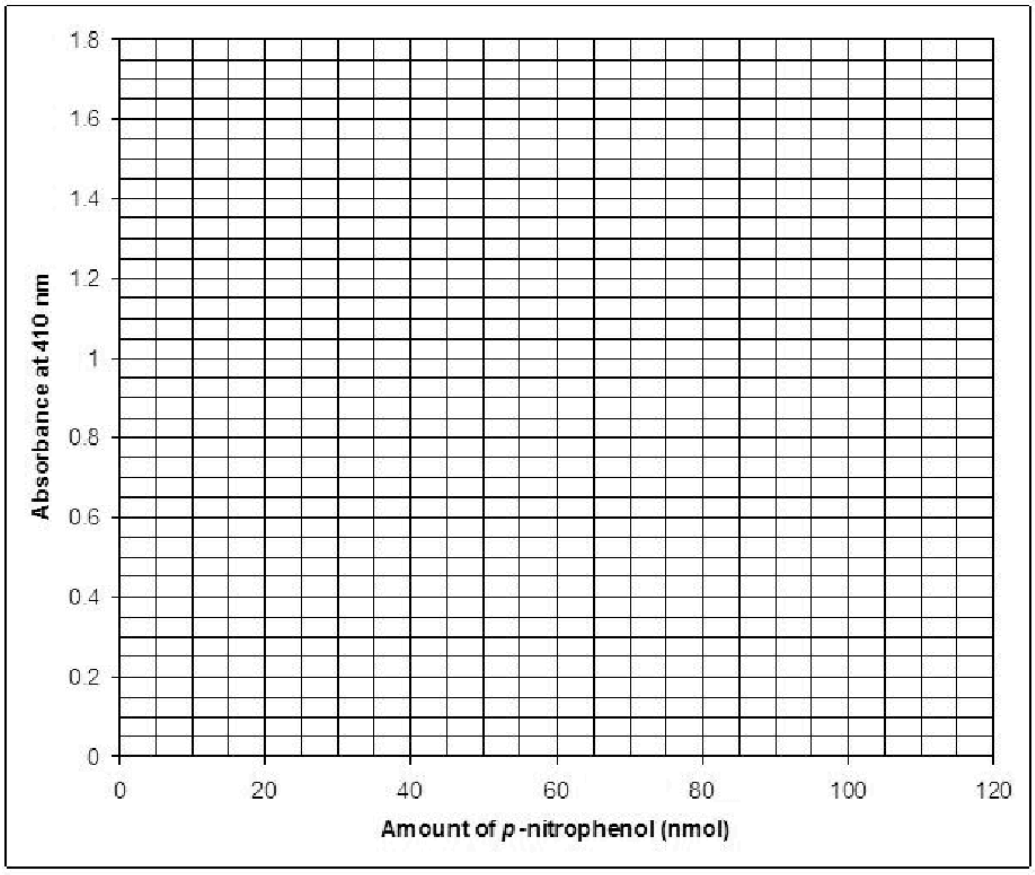
2. **Pipet 1 ml of the standard into your cuvettes**

3. **Blank the spectrophotometer at 410 nm with the cuvette labeled S1.**
4. Then measure and record the absorbance at 410 nm for the remaining standards in your data table. You will use this information to generate a standard curve that correlates the absorbance at 410 nm with the amount of p-nitrophenol present.

Table: Absorbance values for standards.

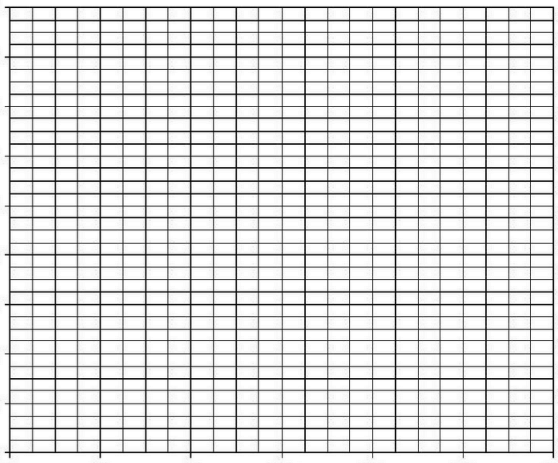
Use the data in your table to create a standard curve in your lab notebook.

Plot the absorbance values for each standard, and then draw the line that best goes through all the data points.



Practice Graphing and Rate Analysis

Time in (seconds)	Product formed in (mg/ml)	Product formed in (mg/ml)
0	0	0
10	5	8
20	10	17
30	15	26
40	20	35
50	25	44
60	30	53



Determining the Reaction Rate in the Presence or Absence of an Enzyme

BACKGROUND:

Enzymes are molecules that increase the rate of a reaction, but are not used up in the reaction. Because the enzyme can keep processing the substrate over and over again, very few molecules of enzyme are needed relative to the number of molecules of the substrate.

INTRODUCTION:

In this investigation, you will compare the rate of breakdown of the substrate into glucose and p-nitrophenol in the **presence** and **absence** of cellobiase, (the enzyme)

"Enzyme Reaction".

In a 15 ml tube, you will add an **enzyme** into a solution of substrate and determine the **rate of reaction** (product formation over time).

"Control Reaction"

The second tube is the **control** reaction. You will add a **buffer** to the same substrate, but **does not include enzymes**. This way, you will be able to compare the breakdown rate in the presence of enzymes and the presence of a control buffer.

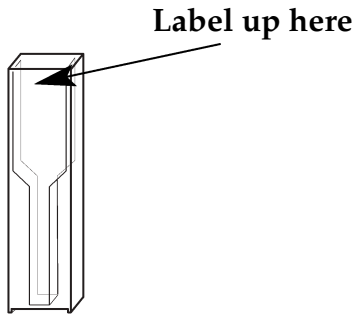
Methods

1. Locate the reagents at the rolling lab cart in the room.

    - Stop Solution
    - 1.5 mM Substrate
    - Enzyme
    - Buffer

2. **Label five cuvettes E1–E5 (for five time points).** These will be used for the enzyme reaction.

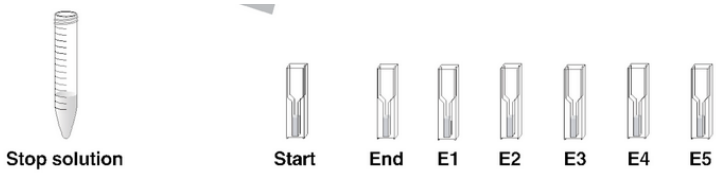
    Label only the upper part of the cuvette face.



3. **Label two more cuvettes "Start" and "End"** on the upper part of the cuvette. These two will be used for the control reaction.

These cuvettes will serve as control time points at the start and end of the reaction.

4. **Pipet 500 µl of stop solution into each of the seven labeled cuvettes**. The stop solution is a strong base. Goggles must be worn at all times.

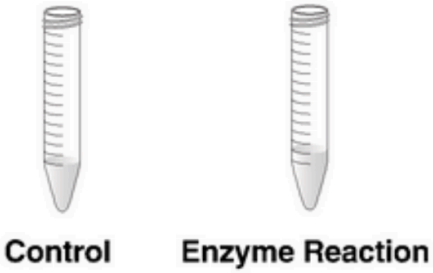


5. **Label two** 15 ml conical tubes.

Label one "**Enzyme Reaction**" and the other "**Control**".

6. **Pipet 2 ml of $_{1.5\ mM}$ substrate into the 15 ml conical tube labeled "Enzyme Reaction".**

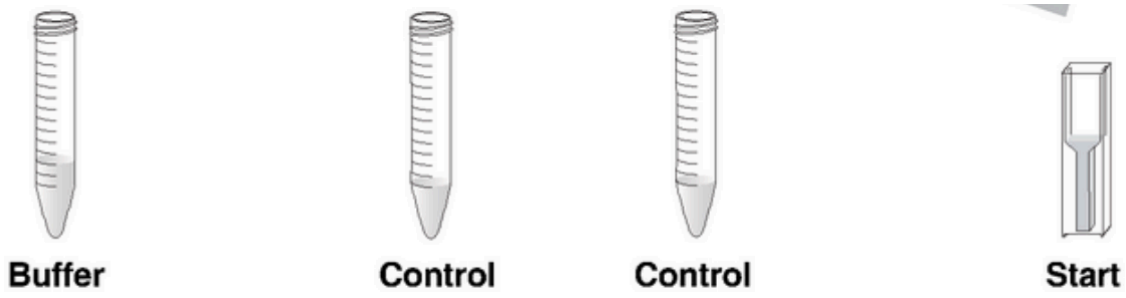
7. **Pipet 1 ml of $_{1.5\ mM}$ substrate into the 15 ml conical tube labeled "Control".**



Read and understand steps 8–11 fully before proceeding. These steps are time sensitive!

8. **Pipet 500 µl of buffer into the 15 ml conical tube labeled "Control"** and gently mix.

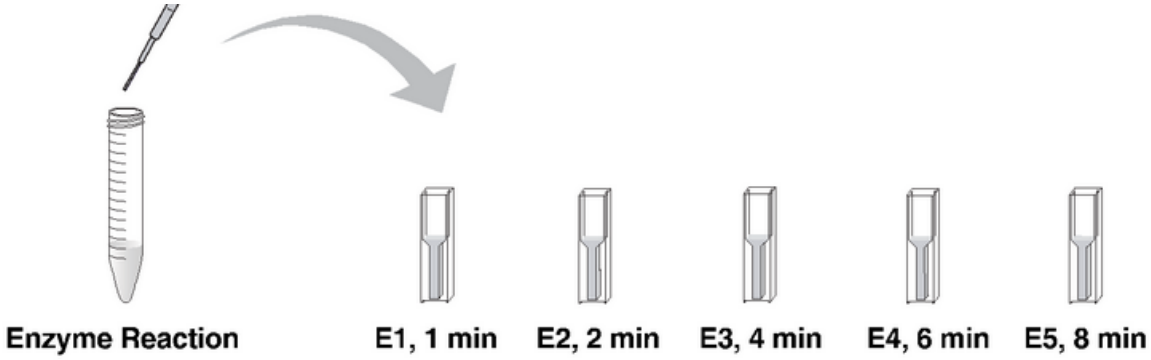
Once you have mixed the buffer with the substrate, **remove 500 µl of this solution and add it to your cuvette labeled "Start".**



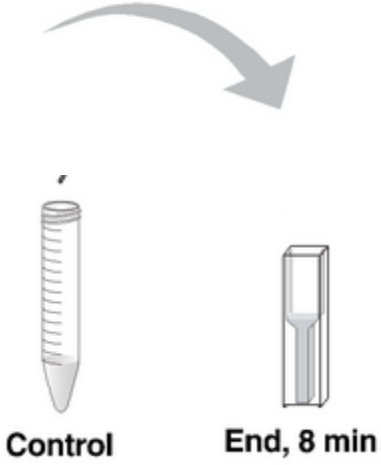
9. **Pipet 1 ml of enzyme into the 15 ml conical tube labeled "Enzyme Reaction"**. Gently mix, then **START YOUR TIMER**. This marks the beginning of the enzymatic reaction.

Enzyme Reaction

10. At the times indicated below, remove **500 µl** of the solution from the "Enzyme Reaction" tube and **add it to the appropriately labeled cuvette containing the stop solution.**



11. After all the enzyme samples have been collected, remove **500 µl of the solution from the "Control" reaction tube and add it to the cuvette labeled "End".**



Measure the absorbance of your enzyme-catalyzed reaction cuvettes (E1–E5) and your control cuvettes (Start, End) at 410 nm, and record your results in your table. You will use this information to determine the amount of product, p-nitrophenol, formed in the reaction cuvettes.

Determining *p*-nitrophenol produced using a standard curve.

Time (minutes)	Cuvette	Amount of *p*-Nitrophenol (nmol) from the Standard Curve	Absorbance at 410 nm
0	Start		
8	End		
1	E1		
2	E2		
4	E3		
6	E4		
8	E5		

To determine the rate of the reaction graph the Amount of p-nitrophenol over time in minutes.