PRELIMINARY ACTIVITY FOR

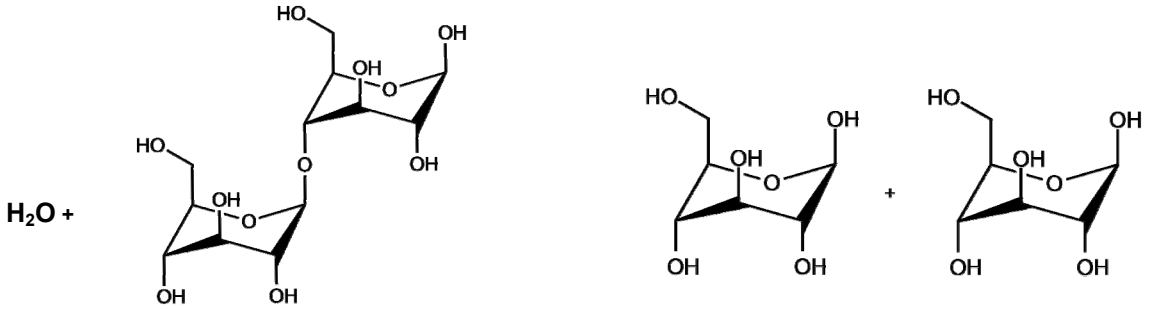
Introduction to Biofuels: Enzyme Action

Open Inquiry Version

Enzymes are typically proteins that act as catalysts, speeding up chemical reactions that would take far too long to occur on their own. Enzymes speed up the vast majority of the chemical reactions that occur in cells. Reactions that break down molecules (such as those involved in digestion and cellular respiration) and those that build up molecules (such as the ones involved in photosynthesis and DNA replication) all require enzymes. Each type of enzyme has a specific shape that compliments the structure of its substrate. The *substrate* is the molecule or molecules that the enzyme converts into product.

In this investigation, you will be studying cellobiase. Cellobiase is involved in the last step of the process of breaking down cellulose, a molecule made up of long chains of glucose that are found in plant cell walls, into glucose. This is a natural process that is used by many fungi as well as bacteria (such as those found in termite guts, the stomachs of ruminants, and compost) to produce glucose as a food source. Breaking down the cellulose from plants into sugar is also an important step in the creation of ethanol for fuel.

The natural substrate for the enzyme cellobiase is cellobiose (Figure 1). 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. Solutions of cellobiose (substrate) and glucose (product) are clear, and there are not many simple, inexpensive, fast methods to detect these molecules quantitatively.



Substrate
Cellobiose Substrate
Colorless Solution

Products
2 Glucose Molecules
Colorless Solution

Figure 1

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 (Figure 2). When *p*-nitrophenol is mixed with a basic solution referred to as the *stop solution*, it will stop the reaction and turn the solution yellow. The amount of yellow color is proportional to the amount of *p*-nitrophenol present. 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.

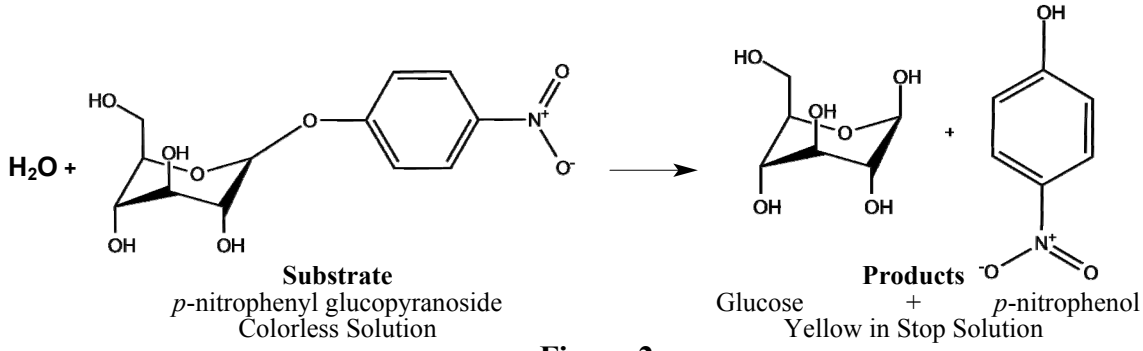


Figure 2

In this Preliminary Activity, you will use a spectrophotometer to determine *p*-nitrophenol concentration. You will first produce a *p*-nitrophenol containing sample with the reaction described in Figure 2. Next, you will determine the absorbance values for a set of *p*-nitrophenol solutions with known concentrations in order to create a standard curve. When a graph of absorbance *vs.* concentration is plotted for the standard solutions, a direct relationship should result, as shown in Figure 3. The direct relationship between absorbance and concentration for a solution is known as Beer's law. You will then determine the *p*-nitrophenol concentration of your prepared sample by measuring its absorbance and then finding the corresponding concentration on the standard curve. Because the relationship is linear, you could also calculate the
p-nitrophenol concentration using the formula for the standard curve. You will also determine the initial rate of your *p*-nitrophenol producing reaction.

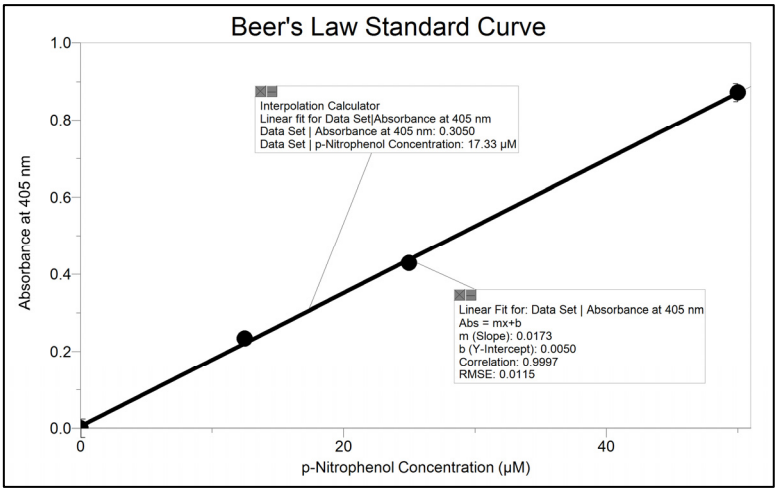


Figure 3

After completing the Preliminary Activity, you will first use reference sources to find out more about biofuels and the cellobiase catalyzed reaction that produces *p*-nitrophenol before you choose and investigate a researchable question. Some topics to consider in your reference search are:

- catalyst
- enzyme
- biofuel
- ethanol
- cellulose
- cellobiose
- cellobiase
- substrate
- *p*-nitrophenol
- Beer's law

PROCEDURE

The Cellobiase Catalyzed Production of *p*-Nitrophenol

1. Obtain and wear goggles.

2. Pipet 500 µL of stop solution into a cuvette.

3. Pipet 500 µL of 1.5 mM substrate into a 1.5 mL microcentrifuge tube.

4. Have a stopwatch ready. Pipet 250 µL of enzyme into the microcentrifuge tube containing the substrate and start your stopwatch.

5. When two minutes have elapsed, transfer 500 µL of the reaction mixture into the cuvette containing stop solution.

6. Place the cuvette containing the yellow *p*-nitrophenol solution aside for later use.

Producing a Beer's Law Standard Curve

7. Use a USB cable to connect the SpectroVis Plus spectrophotometer to your computer or LabQuest. Start the data-collection program, and then choose New from the File menu.

8. Obtain the *p*-nitrophenol standards set, S1–S4. cuvette S1, which contains stop solution, will serve as the blank. To correctly use cuvettes, remember:
   - Wipe the outside of each cuvette with a lint-free tissue.
   - Handle cuvettes only by the top edge of the ribbed sides.
   - Dislodge any bubbles by gently tapping the cuvette on a hard surface.
   - Always position the cuvette so the light passes through the clear sides.

9. Calibrate the spectrophotometer.
   a. Place the blank, cuvette S1, into the cuvette slot of the spectrophotometer.
   b. Choose Calibrate from the Sensors menu of LabQuest or the Experiment menu of Logger *Pro*.
   c. When the warmup period is done, select Finish Calibration. When the calibration procedure is complete, select OK.

10. Determine the optimum wavelength for examining the absorbance of *p*-nitrophenol.
    a. Remove the blank cuvette from the spectrophotometer. Place cuvette S4 into the spectrophotometer.
    b. Start data collection. A full spectrum graph of the solution will be displayed. Note that one area of the graph contains a peak absorbance. Stop data collection.

11. Set up the data-collection mode.

    Logger *Pro*

    a. Click Configure Spectrometer Data Collection, . Select Abs *vs.* Concentration as the Collection Mode. The wavelength of maximum absorbance (λ max) will be selected. Verify that the maximum absorbance is close to 405 nm.
    b. Enter **Concentration** as the Name, **Conc**. as the Short Name, and **µM** as the Units.
    c. Select OK. Remove the cuvette from the spectrophotometer and proceed to Step 12.

    LabQuest App

    a. The peak absorbance is automatically selected. On the Meter screen, tap Mode and change the data-collection mode to Events with Entry.
    b. Enter **Concentration** as the Column Name and **µM** as the Units.
    c. Select OK. Remove the cuvette from the spectrophotometer and proceed to Step 12.

12. You are now ready to collect absorbance data for the four *p*-nitrophenol standards. Start data collection. Obtain the cuvette labeled S1. Wipe the outside with a tissue and place it in the spectrophotometer. Wait for the absorbance value displayed on the screen to stabilize, and then select Keep to store the data point. Enter **0** as the concentration and select OK. The data pair you just collected will now be plotted on the graph. Remove the cuvette from the spectrophotometer.

13. Obtain the cuvette labeled S2. Wipe the outside and place it in the spectrophotometer. When the absorbance value stabilizes, keep the data point, enter **12.5**, and then select OK.

14. Obtain the cuvette labeled S3. Wipe the outside and place it in the spectrophotometer. When the absorbance value stabilizes, keep the data point, enter **25**, and then select OK.

15. Obtain the cuvette labeled S4. Wipe the outside and place it in the spectrophotometer. When the absorbance value stabilizes, keep the data point, enter **50**, and then select OK.

16. Stop data collection and record the absorbance values in your data table.

17. Examine the graph of absorbance *vs.* concentration. To see if the curve represents a direct relationship between these two variables, apply a Linear Fit to the data. A best-fit linear regression line will be shown for your data points. This line should pass near or through the data points.

18. Record the equation of this line in your data table.

Determining *p*-Nitrophenol Concentration

19. You are now ready to collect absorbance data for your *p*-nitrophenol sample. Wipe the outside of the cuvette and place it in the spectrophotometer. When the displayed absorbance value stabilizes, record the value in your data table. **Important:** The reading on the screen is live, so it is **not** necessary to start data collection to read the absorbance value.

20. To determine the concentration of the unknown, interpolate along the regression line to convert the absorbance value of the unknown to the concentration value.

    Logger *Pro*

    a. Choose Interpolation Calculator from the Analyze menu.
    b. Check that the absorbance value that you recorded is displayed. If it is not, enter the absorbance value in the correct space. The *p*-nitrophenol concentration expressed in units of µM will be displayed. Record the concentration value in your data table.
    c. Select OK. A point on the graph will be displayed. Proceed to Step 21.

    LabQuest App

    a. Tap the Graph tab and choose Interpolate from the Analyze menu.
    b. Find the absorbance value that is closest to the absorbance value that is closest to the absorbance reading you obtained for the cuvette containing your *p*-nitrophenol sample. The *p*-nitrophenol concentration in µM will be displayed.
    c. Record the concentration value in your data table and proceed to Step 21.

21. Print the graph as directed by your instructor.

22. You will use the standard curve in order to determine *p*-nitrophenol concentration throughout this investigation. Save it as directed by your instructor.

QUESTIONS

1. Calculate the initial rate of *p*-nitrophenol production. Since you only measured the amount of *p*-nitrophenol at one time point, 2 minutes, assume the amount of *p*-nitrophenol at 0 minutes is 0 nmol. **Note**: The amount of *p*-nitrophenol present in the cuvette, measured in nmol, has the same numerical value as the concentration expressed in µM. For example, 42.8 nmol corresponds to 42.8 µM.

   Example: Suppose that after 2 minutes your sample gave an absorbance value of 0.694. You would use the standard curve you generated to find the corresponding amount of *p*-nitrophenol, 39.8 nmol.

   The initial rate would be (39.8 nmol – 0 nmol)/(2 min – 0 min) = 19.9 nmol/min

2. What is the name of the enzyme studied in this investigation?

3. What is one practical industrial application of this enzyme?

4. Identify the natural product of this enzyme.

5. Identify the natural substrate of this enzyme.

6. List at least three factors that might influence the rate of p-nitrophenol production for the reaction being studied in this investigation.

7. List at least one researchable question concerning the rate of p-nitrophenol production.