Biology 196 Laboratory
Enzyme Activity

Preparation and Background

Students should study or review the core concepts of chapter 8 in their textbook (Campbell Biology, 9th ed.). A basic comprehension of Gibb's **free energy** changes (**ΔG**) will greatly increase understanding of the laboratory background.

Enzymes

Enzymes are biological macromolecules (usually **proteins**) that facilitate biochemical reactions by dramatically increasing the *rate* of a reaction. Specifically, they function by lowering the **energy of activation** (E_a) of a reaction. Enzymes are **catalysts** - they are neither a reactant nor product, and are not used up in the reaction process. The reactants in an enzyme reaction are referred to as **substrates**. The shape and properties of each enzyme make it very specific to the type of substrate(s) it can convert into product. In addition to the specificity of substrates, most enzymes are extremely sensitive to environmental conditions. In this exercise we will test varying conditions on the rate of an enzymatic reaction.

The enzyme used for this exercise is **cellobiase**, which catalyzes the **hydrolysis** of glucose ß 1-4 glycosidic bonds (Fig. 1). This is the last step in the hydrolysis of **cellulose**, the most prominent structural carbohydrate of plants. This enzyme is crucial to the life cycles of any organism that obtains energy by hydrolyzing cellulose (termites, for example). This is also a potentially important commercial enzyme, since breaking down cellulose to glucose is the first step in turning plant material into ethanol for fuel.
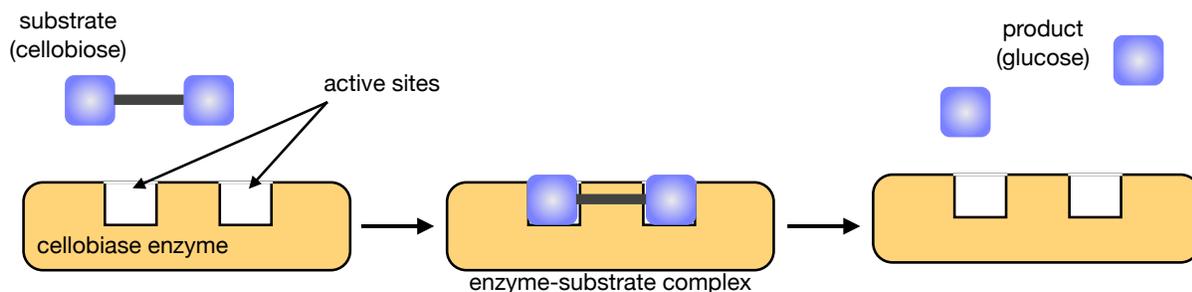

Fig. 1 The cellobiase reaction. Cellobiase has two **active sites** that bind to cellobiose (cellulose) and catalyzes the hydrolysis of the ß 1-4 glycosidic bond. Notice that the enzyme is unchanged by the reaction and is free to bind to another cellobiose molecule and repeat the process again.

Cellobiase Substrates and Detection

Cellulose and cellobiose (substrate) and glucose (product) are colorless in solution. Therefore, to to make it easier to detect the activity of the enzyme this exercise will use an artificial substrate, **p-Nitrophenyl glucopyranoside**, (pNG) that produces a colored product when exposed to a solution that is **basic** in pH (Fig. 2). It is important to realize that the enzyme is still catalyzing the same reaction, but by using this substrate we can measure its activity easier.

The yellow color of the **p-nitrophenol** can be quantified using a **spectrophotemeter**. Since *p*-nitrophenol absorbs light at the wavelength of 410 nm, the spectrophotometer will transmit a beam of light at 410 nm through the substance and measures how much of the light is absorbed. Since more product will produce a darker yellow color, this will allow us to quantitate how much product is formed and also the rate of our enzyme reaction. You will use a set of known quantities of product (**standards**) to create a **standard curve**, which is a graphical plot of the absorbance vs. concentration. The absorbance values of your experimental group can then be interpolated to concentrations by using this standard curve.
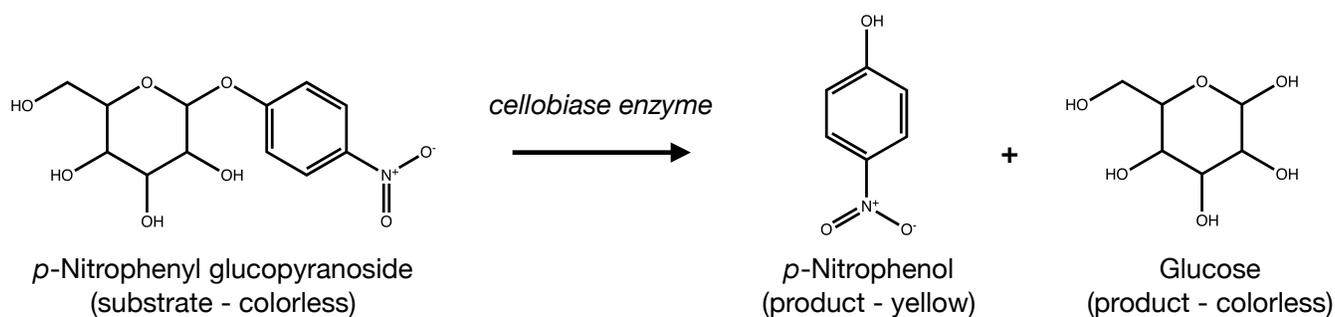
p-Nitrophenyl glucopyranoside
(substrate - colorless)

cellobiase enzyme

p-Nitrophenol
(product - yellow)

+

Glucose
(product - colorless)

Fig. 2 Hydrolysis of *p*-Nitrophenyl glucopyranoside by cellobiase produces a colored product. In order to assess the activity of cellobiase we will be using the artificial substrate *p*-Nitrophenyl glucopyranoside (pNG) that when hydrolyzed produces the yellow product, *p*-nitrophenol.

Experimental Activities

Preparation of a Standard Curve

All groups must complete this portion of the lab. The data from the standard curve is what will allow you to determine the activity of the enzyme under all conditions.

You should have the necessary supplies at your group's workstation before beginning the procedure. *Always read through a procedure before beginning it.*

1. Locate the five samples of standards labeled S1–S5 at your lab bench; their concentrations of *p*-Nitrophenol are noted in Table 1. Label 5 cuvettes S1-S5, and using a fresh pipet, add each of the solutions to the corresponding cuvette. Label only the upper part of the cuvette. Try not to get ink or fingerprints on the bottom portion of the cuvette. The bottom half is where the spectrophotometer will focus a beam of light.

Supplies needed

1.5 mM substrate
Low concentration enzyme
1x stop solution
Buffer
Transfer pipets (4)
Cuvettes (12)
Marker
Beaker with deionized water
15 mL conical tubes (2)
Standards labeled S1-S5
Stopwatch (a cell phone is fine)

Label up here

2. You must first "blank" the spectrophotometer. Load the S1 cuvette, close the door and hit the "0 ABS / 100% T" button. Now measure and record the absorbance for the remaining standards in Table 2 below.

Table 2. Absorbance values for standards

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

3. Create a standard curve by plotting the amount of *p*-Nitrophenol (X axis) vs. Absorbance at 410 nm (Y axis) on the supplied graph sheet below. It should produce a fairly straight line. Now move on to Activity 1. You will come back to this graphical plot later.
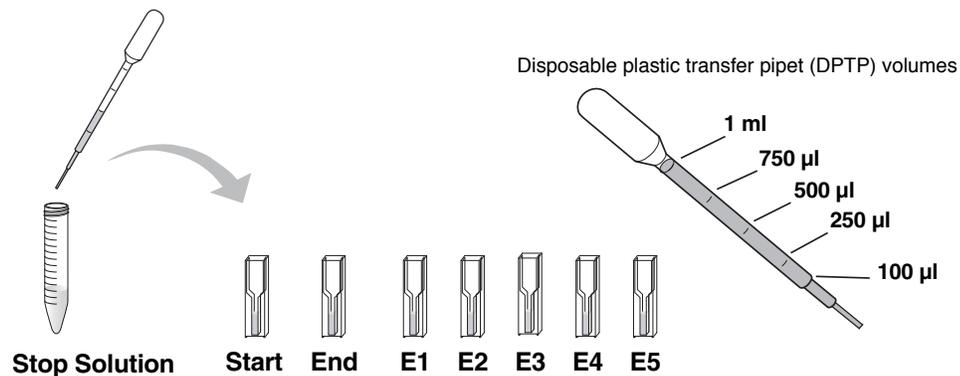

Amount of *p*-Nitrophenol (nmols)
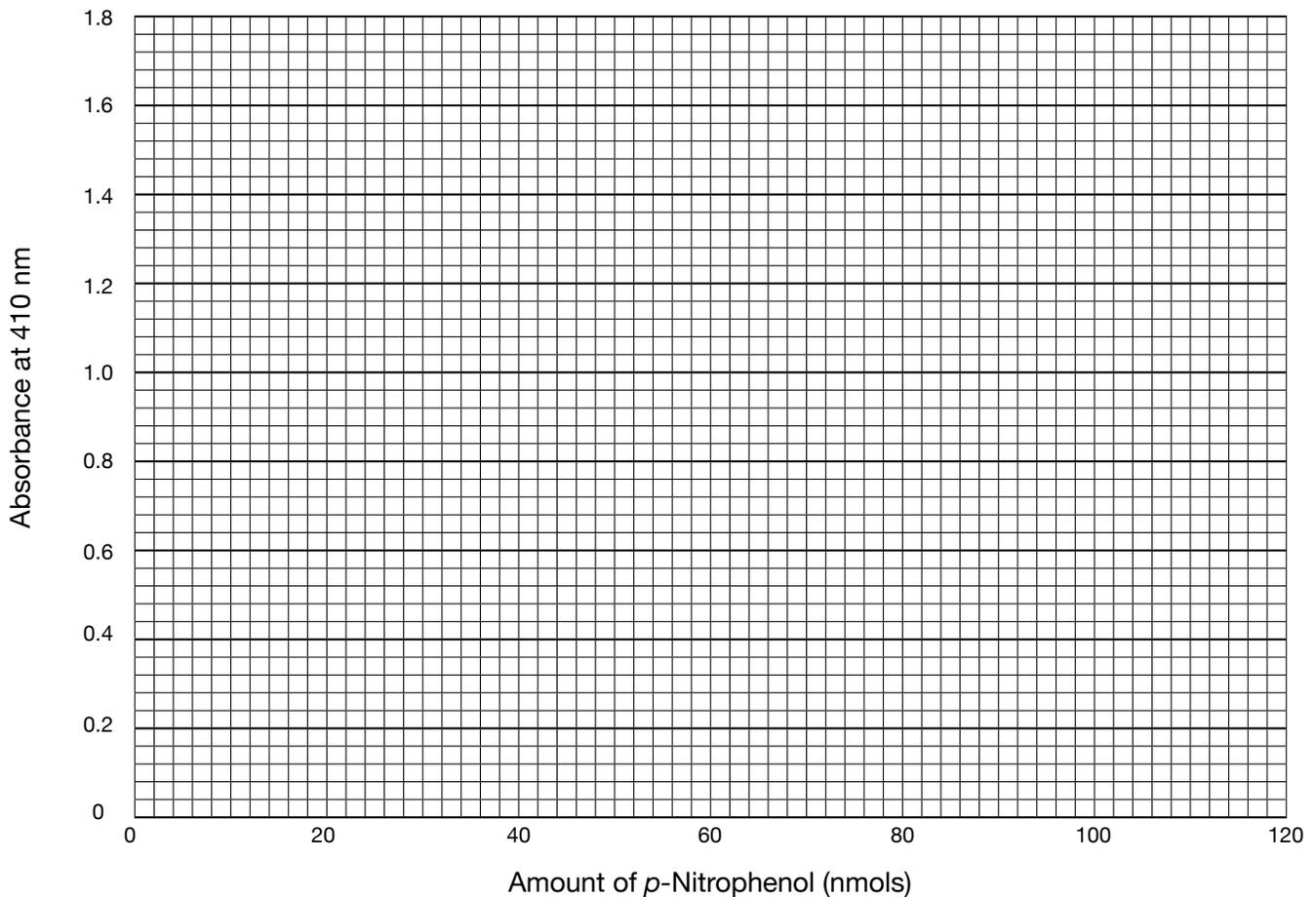
Activity 1 - Determine the Reaction Rate in the Presence or Absence of an Enzyme

In this activity you will determine two things:

✓ Does the enzyme you are supplied with cause hydrolysis of pNG?
✓ What is the rate of product formation with and without the enzyme present?

1. Locate the 15 ml conical tubes labeled "Stop Solution", "1.5 mM Substrate", "Low enzyme concentration" and "Buffer". Label each of the tubes with your initials.

2. Label five cuvettes E1–E5 (for five time points). Label only the upper part of the cuvette face.

3. Label the two remaining cuvettes "Start" and "End" on the upper part of the cuvette. These two cuvettes will serve as control time points at the start and end of the reaction and neither cuvette will contain any enzyme.

4. Label a new disposable plastic transfer pipet (DPTP) as "stop" for stop solution, and pipet 500 µl of stop solution into each of the seven labeled cuvettes. The stop solution is a strong base, so avoid getting it on your skin or clothes.

Disposable plastic transfer pipet (DPTP) volumes

1 ml
750 µl
500 µl
250 µl
100 µl

Stop Solution   **Start** **End**   **E1** **E2** **E3** **E4** **E5**

5. Locate two empty 15 ml conical tubes. Label one "Enzyme Reaction" and the other "Control".

6. Using a new DPTP, label it as "substrate", and pipet 2 ml of 1.5 mM substrate into the 15 ml conical tube labeled "Enzyme Reaction". Use the same DPTP and pipet 1 ml of 1.5 mM substrate into the conical tube labeled "Control".


1.5 mM Substrate   **Control**   **Enzyme Reaction**
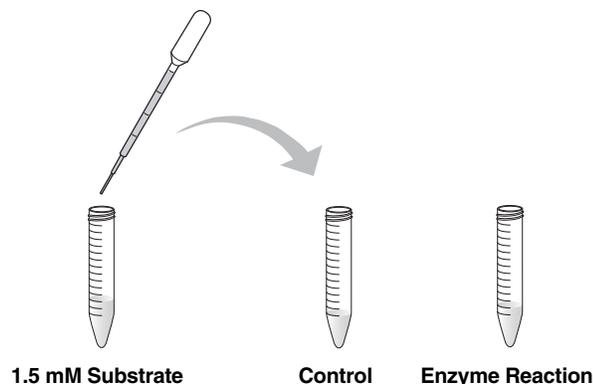
7. Label one new DPTP "E" for enzyme and another "C" for control. **Only use the DPTP labeled "E" for the enzyme reaction tube and the DPTP labeled "C" for the control reaction tube.**

    The next steps are time-sensitive. Be sure to read ahead before beginning!

8. Using the DPTP labeled "C", 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. Using the DPTP labeled "E", pipet 1 ml of "Low enzyme concentration" into the 15 ml conical tube labeled "Enzyme Reaction". Gently mix, then **START YOUR TIMER.** This marks the beginning of the enzymatic reaction.

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

Enzyme Reaction   E1, 1 min   E2, 2 min   E3, 4 min   E4, 6 min   E5, 8 min
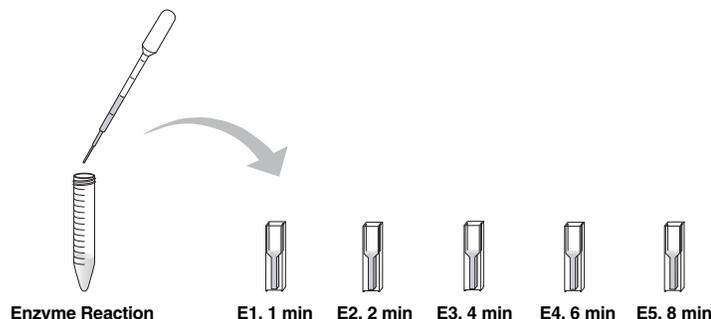
11. After all the enzyme samples have been collected, use the DPTP labeled "C" to remove 500 µl of the solution from the "C" reaction tube and add it to the cuvette labeled "End".

## Quantitative determination of product formed in Activity 1

12. 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 Table 3.

13. Refer back to your standard curve and interpolate the amount of product formed in cuvettes E1-E5. Record your data in Table 3.

Table 3. Determining *p*-nitrophenol produced using a standard curve.

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

Determine the *initial* rate of product formation in Activity 1

At the beginning of the reaction there is a large amount of substrate present, but as the reaction proceeds there is less substrate available for the enzyme. To determine the initial *rate* you must first graph the amount of product formed vs. time. The *slope* of the linear portion of the line will be the rate. Use the supplied graphing plot below.
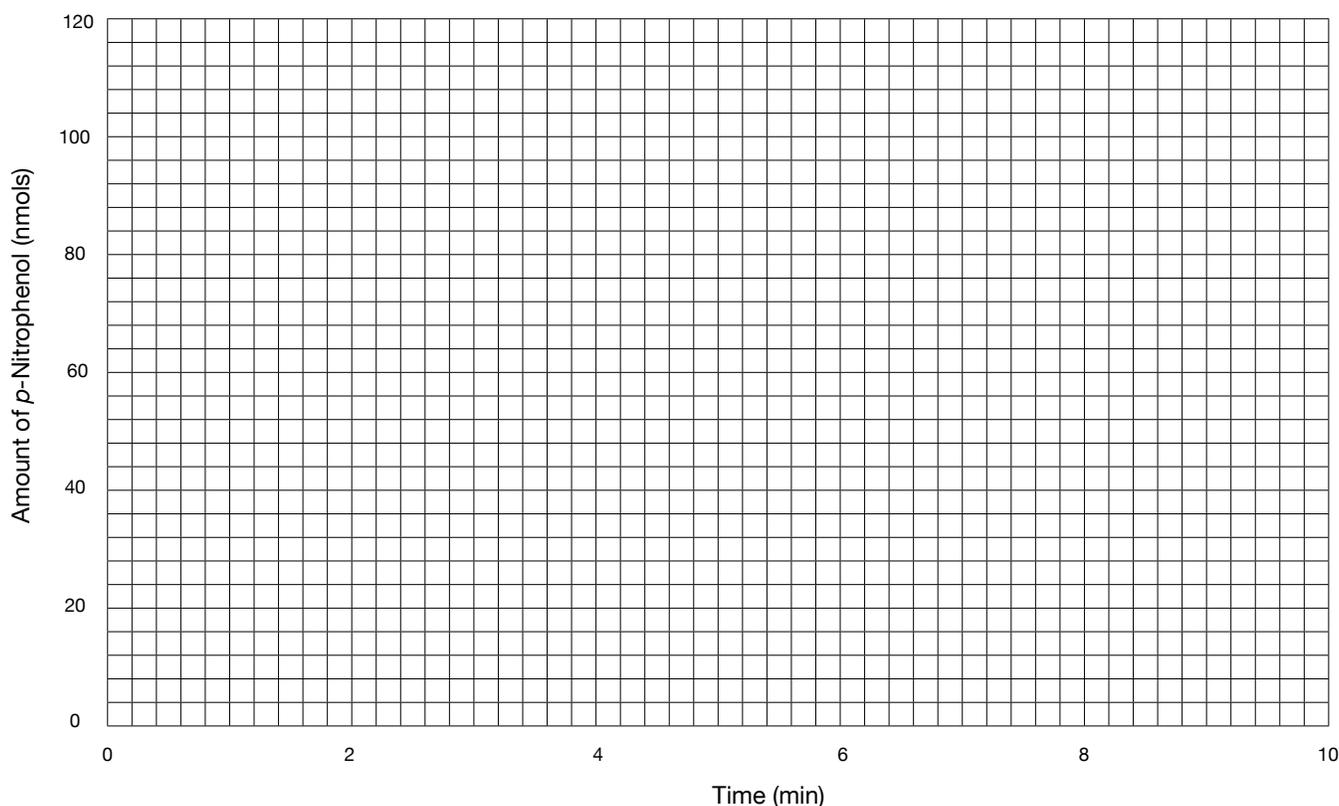
Finally, to figure out the initial *rate* of the enzymatic reaction using the above graph we use the simple formula for slope.

Saturday, January 26, 19 *Rate = Slope of the line = (change in Y) / (change in X) also known as "rise/run" in algebra.*

Look at the portion of your graph that is linear (straight line). It will probably consist of times 1min to 8min. We'll use only the linear portion of the line to figure out the rate.

Rate = (product at 8min – product at 1min) / (8 min – 1 min) = _____ nmol/min

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

In this activity you will determine the effect that temperatures have on the rate of cellobiase activity. Remember that every enzyme has different properties, so it isn't always obvious which temperatures are optimal for a particular enzyme.

> **Additional supplies needed**
>
> Boiling water bath (100ºC)
> Ice bath (4ºC)
> Water bath at 37ºC
> microcentrifuge tubes

1. Label your cuvettes "0°C", "22°C", "37°C" and "100ºC". Only label on the upper part of the cuvette face.

2. Using the DPTP labeled "stop" pipet 500 µl of stop solution into each cuvette.

3. Label four 1.5 ml microcentrifuge tubes with "0°C Enzyme", "22°C Enzyme", "37°C Enzyme" and "100ºC Enzyme". Label a new DPTP "enzyme" and pipet 250 µl of enzyme into each microcentrifuge tube.

4. Label four 1.5 ml microcentrifuge tubes with "0°C Substrate", "22°C Substrate", "37°C Substrate" and "100ºC Substrate". Using the DPTP labeled "substrate" pipet 500 µl of the 1.5 mM substrate into each microcentrifuge tube.

5. Place the microcentrifuge tubes labeled "0°C Enzyme" and "0°C Substrate" on ice. Place the microcentrifuge tubes labeled "22°C Enzyme" and "22°C Substrate" on your lab bench. Place the microcentrifuge tubes labeled "37°C Enzyme", the "37°C Substrate" in the water bath at 37°C and place the tubes labeled "100ºC Enzyme" and "100ºC Substrate" in the boiling water bath. Allow the tubes to equilibrate to their respective temperatures for at least 5 minutes.

    The next steps are time-sensitive. Be sure to read ahead before beginning!

6. Have a stopwatch ready. Using the DPTP labeled "E" pipet the 250 µl of enzyme from the tube labeled "0°C Enzyme" into the tube labeled "0°C Substrate", and then place the tube now containing your enzyme and substrate mix back on ice. Use the same DPTP to combine the room temperature enzyme and substrate solutions, and place that tube back on the lab bench. Using the same DPTP, combine your 37°C substrate and enzyme solutions, and put that tube back into the 37ºC water bath. Using the same DPTP, combine your 100°C substrate and enzyme solutions, and put that tube back into the boiling water bath. **Start your stopwatch.**

7. After 2 minutes use a *clean* DPTP for each reaction to transfer 500 µl of your reactions to the appropriately labeled cuvettes containing stop solution. Mix the solutions gently with the DPTP. Allow all solutions in the cuvettes to reach room temperature for approximately 5 minutes. During this time you can move all of your enzymatic reactions from their temperature locations (boiling water bath, ice bath, etc.) to your table top.

8. Analyze your samples using the the spectrophotometer as was performed in Activity 1. Use your standard curve to interpolate the product formed and the rate of each reaction. Graph the amount of product formed vs. temperature (X axis) on one of the supplied graphing plots in the Appendix.

9. Calculate the initial rate of reaction for each of the three different temperatures. Since you only had one time point (2 minutes), you can assume the amount of *p*-Nitrophenol at 0 minutes is 0 nmol.

Initial rate at 0ºC = (product at 2min – 0 nmol) / (2 min – 0 min) = _____ nmol/min
Initial rate at 22ºC = (product at 2min – 0 nmol) / (2 min – 0 min) = _____ nmol/min
Initial rate at 37ºC = (product at 2min – 0 nmol) / (2 min – 0 min) = _____ nmol/min
Initial rate at 100ºC = (product at 2min – 0 nmol) / (2 min – 0 min) = _____ nmol/min

10. Fill out Table 4 and then put the results on the classroom board to share your data with the other student groups

Table 4. Effects of temperature on cellobiase

Temperature	Absorbance at 410 nm	Amount of *p*-Nitrophenol (nmol)	Initial rate of product formation (nmol/min)
0ºC			
~22ºC (room temperature)			
37ºC			
100ºC			

Activity 3 - Determine the Effect of pH on Reaction Rate

In this activity you will determine the effect that pH has on the rate of cellobiase activity. Recall how enzymes interact with their substrates. Because charges are used in hydrogen bonds and salt bridges, different levels of H+ ion can dramatically alter those bonds.

> **Additional supplies needed**
>
> 3.0 mM substrate
> pH 5.0 adjustment buffer
> pH 6.3 adjustment buffer
> pH 8.6 adjustment buffer
> microcentrifuge tubes

1. Label your cuvettes "pH 5.0", "pH 6.3", and "pH 8.6". Only label on the upper part of the cuvette face.

2. Using the DPTP labeled "stop" pipet 500 µl of stop solution into each cuvette.

3. Using a *new* DPTP, pipet 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.

    The next steps are time-sensitive. Be sure to read ahead before beginning!

4. Have a stopwatch ready. Using the DPTP labeled "enzyme" add 250 µl of "low concentration enzyme" to each of the labeled microcentrifuge tubes and **start your stopwatch**.

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

6. Analyze your samples using the the spectrophotometer as was performed in Activity 1. Use your standard curve to interpolate the product formed and the rate of each reaction. Graph the amount of product formed vs. pH (X axis) on one of the supplied graphing plots in the Appendix.

7. Calculate the initial rate of reaction for each of the three different pH levels. Since you only had one time point (2 minutes), you can assume the amount of *p*-Nitrophenol at 0 minutes is 0 nmol.

Initial rate at pH 5.0 = (product at 2min – 0 nmol) / (2 min – 0 min) = _____ nmol/min
Initial rate at pH 6.3 = (product at 2min – 0 nmol) / (2 min – 0 min) = _____ nmol/min
Initial rate at pH 8.6 = (product at 2min – 0 nmol) / (2 min – 0 min) = _____ nmol/min

8. Fill out Table 5 and then put the results on the classroom board to share your data with the other student groups.

Table 5. Effects of pH on Cellobiase

pH	Absorbance at 410 nm	Amount of *p*-Nitrophenol (nmol)	Initial rate of product formation (nmol/min)
pH 5.0			
pH 6.3			
pH 8.6			

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

The association of an enzyme with its substrate is dependent on many things, including an empty active site on the enzyme. Enzymes come into contact with their substrate(s) by random, **Brownian Motion**. If all the enzymes in solution are actively catalyzing the formation of product then the reaction rate will have reached its maximum speed (i.e. V_{max}). The only way to increase the V_{max} under set conditions is to add more enzyme. In this activity you will examine the effect that differing levels of

> **Additional supplies needed**
>
> High concentration enzyme
> 15 mL conical tube

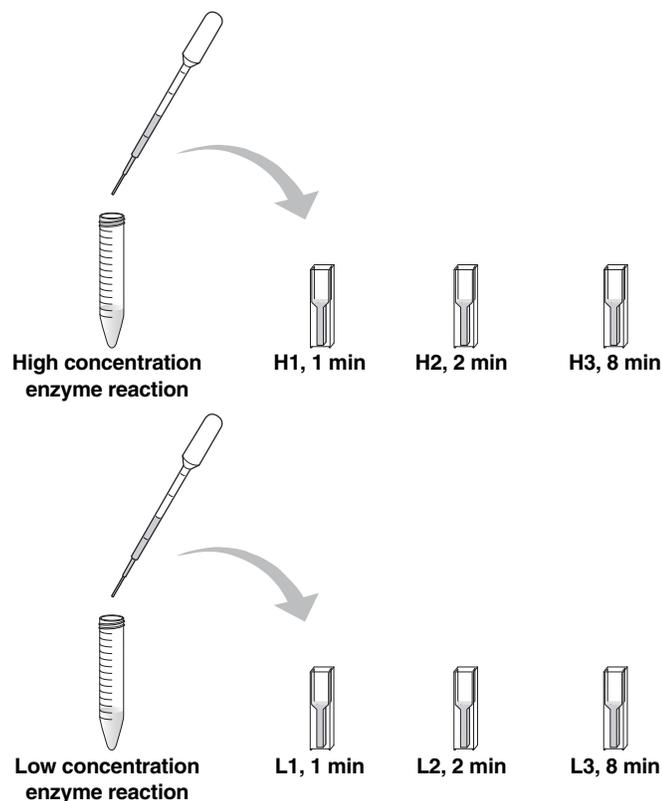enzyme have on a steady-state substrate concentration.

1. Obtain a tube of "High Concentration Enzyme".

    Label one, clean 15 ml conical tube "Low Concentration Enzyme". Using a clean pipet place 1 ml of buffer into this tube. Then pipet 1 ml of the high concentration enzyme to your tube labeled "Low Concentration Enzyme" and swirl to mix.

2. 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.

3. Using the DPTP labeled "stop" pipet 500 µl of stop solution into each cuvette.

4. Label one clean DPTP with an "H" for high enzyme concentration and a second clean DPTP with an "L" for low enzyme concentration.

    The next steps are time-sensitive. Be sure to read ahead before beginning!

5. Using the DPTP labeled with an "H", pipet 250 µl of 1.5 mM substrate into your 15 ml conical tube containing enzyme labeled "High Concentration Enzyme".

6. Using the DPTP labeled with an "L", pipet 250 µl of 1.5 mM substrate into your 15 ml conical tube containing enzyme labeled "Low Concentration Enzyme". **START YOUR TIMER**.

7. At the times indicated in the figure below, use the correctly labeled DPTP to remove 500 µl 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.

High concentration enzyme reaction   **H1, 1 min**   **H2, 2 min**   **H3, 8 min**

Low concentration enzyme reaction   **L1, 1 min**   **L2, 2 min**   **L3, 8 min**

8. Analyze your samples using the the spectrophotometer as was performed in Activity 1. Use your standard curve to interpolate the product formed.

9. Calculate the initial rate of reaction for the two different concentrations of enzyme. Assume the amount of *p*-Nitrophenol at 0 minutes is 0 nmol, and use the amount of product after 1 minute for your calculations.

Initial rate of low enzyme concentration = (product at 1min – 0 nmol) / (1 min – 0 min) = _____ nmol/min

Initial rate of high enzyme concentration = (product at 1min – 0 nmol) / (1 min – 0 min) = _____ nmol/min

10. Fill out Table 6 and then put the results on the classroom board to share your data with the other student groups.

Table 6. Effects of Enzyme Concentration on Cellobiase

Cuvette	Absorbance at 410 nm	Amount of *p*-Nitrophenol (nmol)
H1		
H2		
H3		
L1		
L2		
L3		

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

As discussed in the previous activity, enzymes come into contact with their substrate(s) by random, **Brownian Motion**. When concentrations of substrate are high the chances that the enzyme will collide with a substrate is much more likely than when substrate levels are low. In this activity you will examine the effect that differing substrate concentrations have on the reaction rate of the cellobiase enzyme.
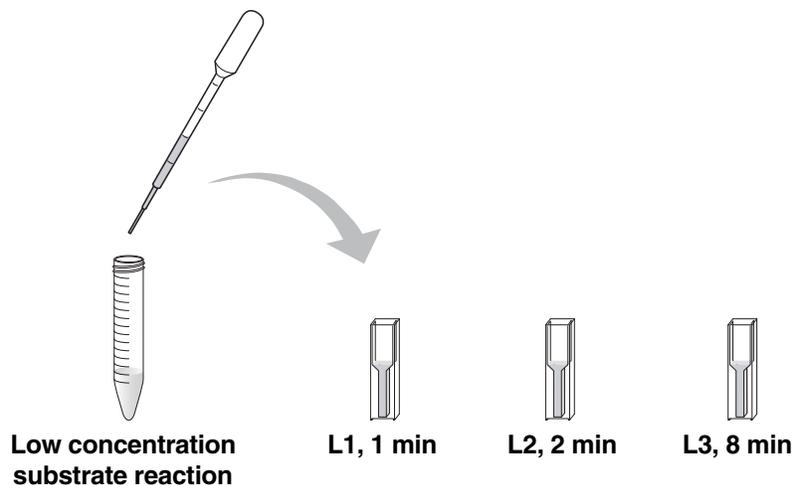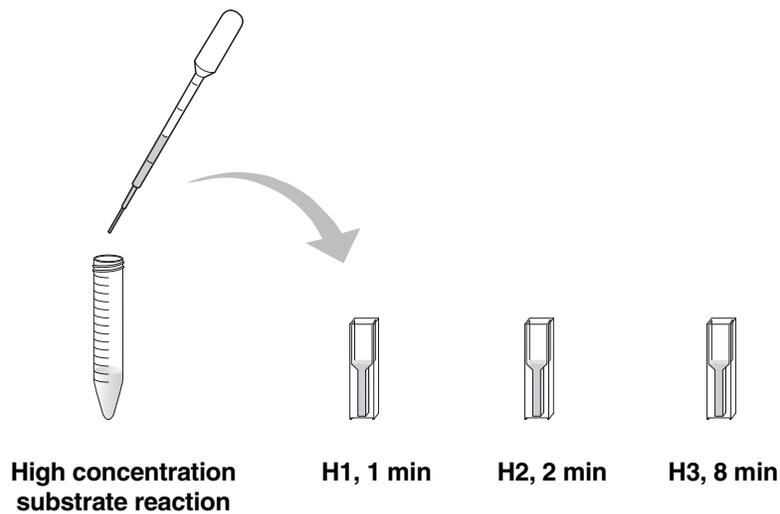
Additional supplies needed

2 x 15 mL conical tube

1. Label one clean 15 ml conical tube "Low Concentration Substrate" and one clean 15 ml conical tube "High Concentration Substrate".

2. Label a new DPTP "high substrate" and pipet 1.5 ml of 1.5 mM substrate into the 15 ml conical tube labeled "High Concentration Substrate".

3. Label a new DPTP "buffer" and pipet 1.25 ml of buffer into the 15 ml conical tube labeled "Low Concentration Substrate". Label a new DPTP "low substrate" and then pipet 250 µl of 1.5 mM substrate into the 15 ml conical tube labeled "Low Concentration Substrate" and mix.

4. Label your cuvettes "H1–H3" (for high concentration substrate time points) and "L1–L3" (for low concentration substrate time points). Only label on the upper part of the cuvette face.

5. Using the DPTP labeled "stop" pipet 500 µl of stop solution into each cuvette.

The next steps are time-sensitive. Be sure to read ahead before beginning!

6. Using a clean DPTP, pipet 750 µl of low concentration enzyme into your 15 ml conical tube of substrate labeled "High Concentration Substrate".

7. Using a clean DPTP, pipet 750 µl of enzyme into your 15 ml conical tube of substrate labeled "Low Concentration Substrate". **START YOUR TIMER**.

8. At the times indicated in the graphic below, use the correctly labeled DPTP to remove 500 µl from the 15 ml centrifuge reaction tubes labeled "High Concentration Substrate" and add it to the appropriately labeled cuvette that contains stop solution. Then use the correctly labeled DPTP to remove 500 µl from the 15 ml centrifuge reaction tubes labeled "Low Concentration Substrate" and add it to the appropriately labeled cuvette that contains stop solution.

High concentration   H1, 1 min   H2, 2 min   H3, 8 min
substrate reaction

Low concentration   L1, 1 min   L2, 2 min   L3, 8 min
substrate reaction

9. Analyze your samples using the standards and the spectrophotometer as was performed in Activity 1. Use your standard curve from Activity 1 to interpolate the product formed and the rate of each reaction.

10. Calculate the initial rate of reaction for the two different concentrations of substrate. Assume the amount of *p*-Nitrophenol at 0 minutes is 0 nmol, and use the amount of product after 1 minute for your calculations.

Initial rate of low substrate concentration = (product at 1min – 0 nmol) / (1 min – 0 min) = _____ nmol/min

Initial rate of high substrate concentration = (product at 1min – 0 nmol) / (1 min – 0 min) = _____ nmol/min

11. Fill out Table 7 and then put the results on the classroom board to share your data with the other student groups.

Table 7. Effects of Substrate Concentration on Cellobiase

Cuvette	Absorbance at 410 nm	Amount of *p*-Nitrophenol (nmol)
H1		
H2		
H3		
L1		
L2		
L3		

Extra graph plots

Amount of *p*-Nitrophenol (nmols)

Time (min)

Saturday, Ja

Amount of *p*-Nitrophenol (nmols)

Time (min)