MOLEBIO LAB #5: Enzyme Kinetics

Introduction:

Enzymes speed up the rate of chemical reactions. Since they do not chemically react with the substrate, they can work again and again to help convert reactants to products. Enzymes are generally proteins (some nucleic acid-based enzymes exist) with a specific 3-D structure (tertiary structure). The active site is a cleft in the protein where the chemical reaction takes place. The charges and positions of the R groups (variable side chains) of the amino acids are critical for the activity of the enzyme. The properties of the active site are important because it is where the reactant(s) binds. The reactant in an enzyme-catalyzed reaction is called the substrate. The substrate fits into the active site because the amino acids facing the active site are attracted to the chemical groups on the substrate.

An enzyme speeds up the chemical reaction by positioning the substrate in such a way that the transition state of the reaction is stabilized. The enzyme reduces the energy needed to make the reaction occur (activation energy). Once the activation energy is lowered, the chemical reaction occurs at a much faster rate.

Changes in salinity and pH can affect the charges of the side chains in the active site, which can decrease the enzyme's effectiveness dramatically by influencing its affinity for its substrate. Temperature can also affect the speed of the reaction. Heat speeds up the movement of substrate and enzyme molecules in solution, which increases the number of collisions and therefore speeds up the reaction. However, at some point, the non-covalent attractions between the amino acids of the enzyme will begin to break, changing the shape of the enzyme. The point at which an enzyme changes shape (becomes denatured) will depend on the particular properties of that enzyme. Some enzymes can still efficiently convert substrate to product at temperatures close to boiling, whereas others are denatured at room temperature. Most enzymes, however, function best at moderate temperatures (20–40° C).

Optimal laboratory conditions for enzyme activity can be predicted by determining the conditions under which it operates in nature. For instance, the enzymes produced by bacteria living in hot springs will function best at a high temperature and the enzymes produced in a person's stomach work best at a very low pH.

The relative concentration of all molecules involved in the reaction affects the reaction rate as well. The higher the concentration of an enzyme, the faster the reaction will take place until there is excess enzyme. Similarly, increasing the concentration of a substrate will speed up the reaction until the point at which all the enzyme present is saturated with the substrate. Sometimes it helps to think of an analogy of workers (enzymes) producing a product from raw materials (substrate). If you increase the number of workers, the amount of product produced will increase until there are excess workers and not enough raw materials to work on. In the same way, increasing the amount of raw materials, while keeping the number of workers constant, will increase the rate of product production until you have given the workers excess raw materials. There is a maximum rate at which the product can be produced given a particular "concentration" of workers (enzyme) and raw materials (substrate). In biochemical terms, this is called V_{max}.

In this activity, we will study the reaction rate of cellobiase, an enzyme involved in breaking down cellobiose to glucose. We will also analyze how the sheer presence of the enzyme, along with changes in pH, enzyme concentration, and substrate concentration affect the activity of cellobiase.

Organisms That Produce Cellulases

Cellulose, the structural polysaccharide found in the cell walls of plants, is a source of sugar to organisms that produce a family of enzymes known as cellulases. Cellulases catalyze the breakdown of cellulose to glucose. Humans and other animals do not produce cellulases. Many plant eating animals are hosts to other organisms that do possess these enzymes. For instance, termites have the protozoan *Trichonympha* living inside their gut. *Trichonympha* has a bacterium called Rs-D17 living inside it that produces cellulose enzymes that break down cellulose, the main component of wood (http://www.genomeweb.com/genome-termite-gut-bacteria-sequenced; http://www.sciencelinks.jp/content/view/826/258). Ruminants, such as cows, harbor a team of anaerobic microorganisms that digest the plants they eat. *Bacteroides succinogenes* is a common bacterium in the gut of cows that produces cellulases (http://sci.waikato.ac.nz/farm/content/microbiology.html). Many types of fungal decomposers derive much of their food from the cellulosic cell walls of plants. The filamentous fungus *Aspergillus niger* produces cellulases that it exudes from its hyphae to digest cellulose in its surroundings to use as a food source.

Cellulosic Ethanol: A Practical Application for Cellulases

The biofuel industry uses cellulases to convert the cellulose in plant cell walls to sugars, such as glucose. The sugar can then be converted to ethanol by microbial fermentation. This ethanol in turn can be used alone in certain engines or in combination with gasoline to power car, truck and airplane engines. To understand the process of cellulosic ethanol production in detail, a journey into the biochemical makeup of cell walls is helpful. A plant's biomass is mostly cell wall material. Plant cell walls are made up of a variety of polysaccharides and other compounds, but the primary component is cellulose. Cellulose is made up of a very long chain of glucose molecules. Each cellulose molecule is attracted to other cellulose molecules by the hydrogen bonds that form between their respective glucose molecules. These attractions form cellulose microfibrils made up of 60–80 individual strands of cellulose.

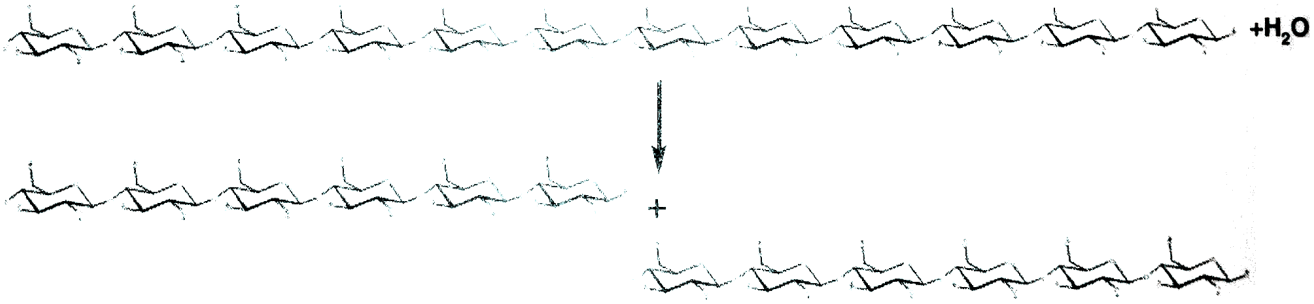
Plant cells can be alive or dead at maturity. Living plant cells, such as photosynthesizing mesophyll cells in leaves, have primary cell walls surrounding them. Primary cell walls are made up of cellulose microfibrils embedded in a matrix of other polysaccharides and protein. These cell walls are thick and relatively stretchy to allow for elongation. Cells with only primary cell walls are fairly soft like the majority of the cells found in a leaf.

Other plant cells are dead at maturity. They strengthen the plant and/or function to conduct water through the plant. These plant cells develop a second type of cell wall called the secondary cell wall before they die. Secondary cell walls are more rigid than primary cell walls. Plant tissues with secondary cell walls have water transport tissues such as xylem, the fibrous or hard tissues covering a coconut or walnut seed, and the stringy part of a celery stalk. These cell walls have additional molecules other than cellulose that contribute to their rigidity. Hemicellulose and lignin are found in high quantities in the secondary cell walls of woody or fibrous plant tissue. For cellulosic ethanol production, lignins must be removed because they inhibit enzymatic activity of cellulases. Hemicelluloses must be cleaved from the cellulose to allow enzymatic breakdown of the cellulose.

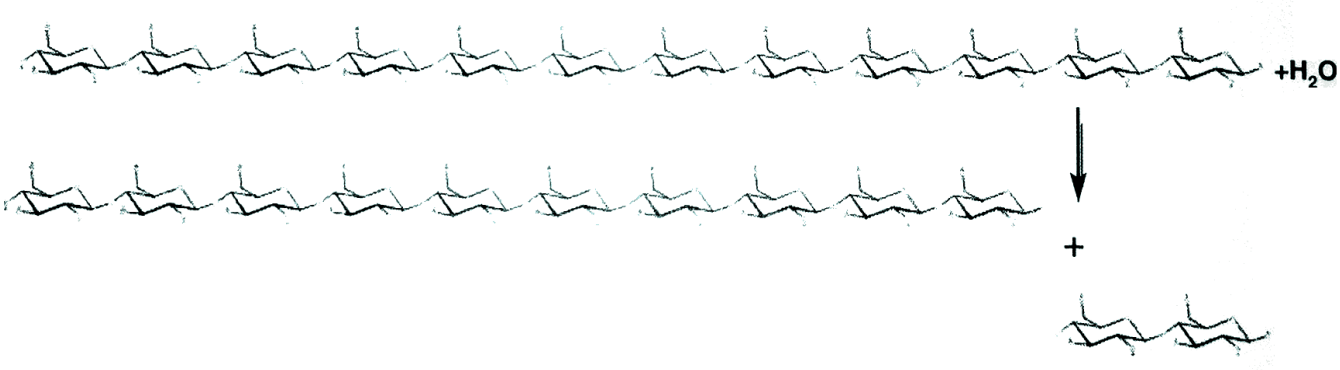
Types of Cellulases Needed to Break Down Plant Cell Walls into Glucose

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.

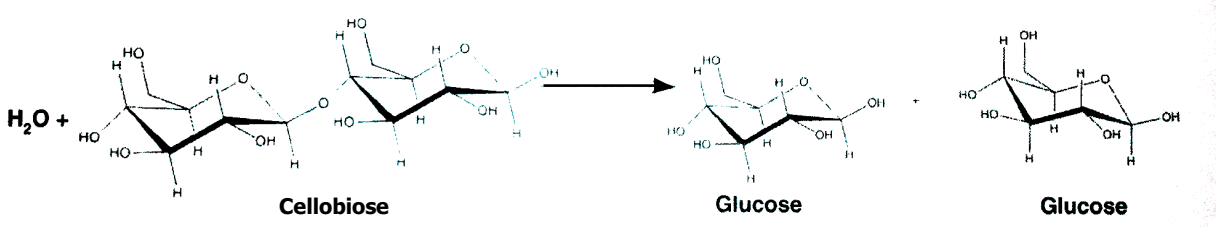
- Endocellulases — These enzymes break down the internal bonds of the long chains of glucose molecules that form cellulose.



- 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 kit, breaks down cellobiose, a disaccharide made up of two glucose molecules connected together by a 1,4 β–glucoside linkage (**Figure 1**). 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.

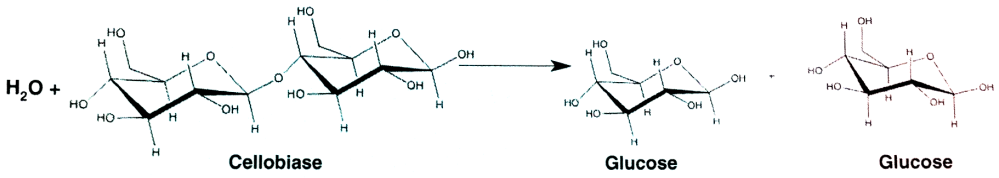


Figure 1: Breakdown of cellobiose into two glucose molecules. Cellobiose is linked at carbon 1 and carbon 4 of two separate glucose units. The enzyme cellobiase can break this linkage resulting in two glucose molecules.

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 (**Figure 2**). 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.

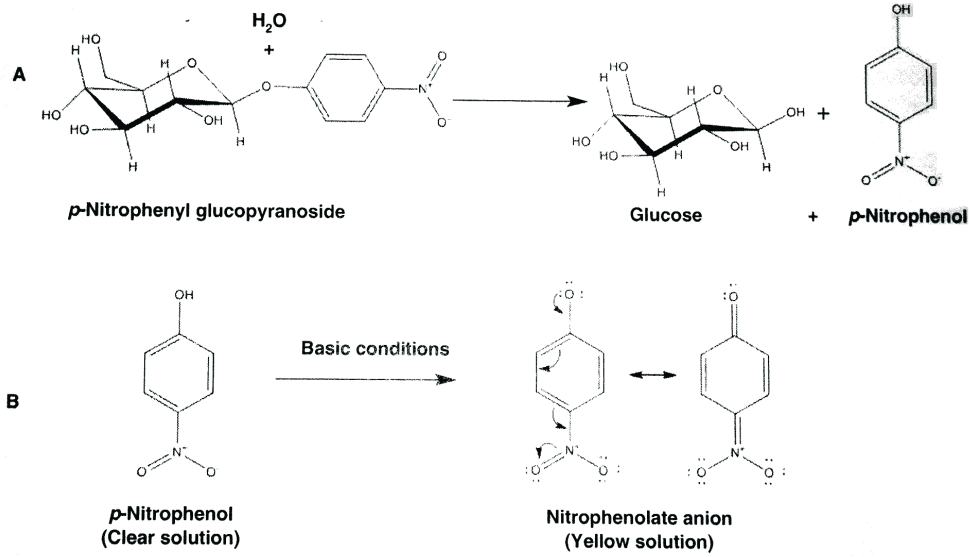


Figure 2: Detecting glucose from the breakdown of cellobiose by cellobiase enzyme. A. An analog to cellobiose called *p*-nitrophenyl glucopyranoside is used to detect enzymatic activity and accumulation of glucose. Once cleaved by cellobiase, the *p*-nitrophenol is released, which can be detected by 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.

PRE-LAB QUESTIONS

1. What type of molecule is an enzyme?

2. Why is an enzyme's shape important to its function?

3. How does an enzyme speed up chemical reactions?

4. What is the name of the enzyme involved in this experiment?

5. What is one practical, industrial application of this enzyme?

6. What is the natural product of this enzyme?

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7. What is the natural substrate of this enzyme?

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8. Explain how you will be able to determine the amount of product that is produced at each time period.

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9. How can you measure the rate of product formation?

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PROCEDURE:

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

In this activity, you will compare the rate of breakdown of *p*-nitrophenyl glucopyranoside into glucose and *p*-nitrophenol in the presence and absence of cellobiase. 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 substrate.

Because it is difficult to add really small volumes, your instructor has diluted the enzyme with a buffer solution — this will allow you to easily add the required volume that still contains a very small number of molecules of enzyme. However, to ensure that the buffer in which the enzyme was diluted does not affect the rate of formation of the product, a control reaction containing just the buffer will be run alongside the reaction containing the diluted enzyme.

To the first reaction tube, you will add enzyme into a solution of substrate and determine the initial rate of reaction (product formation). The second reaction, which is the control reaction, will have the same buffer added to the same substrate, but does not include enzyme. This way, you will be able to compare the breakdown rate of *p*-nitrophenyl glucopyranoside to glucose and *p*-nitrophenol in the presence of a control buffer.

1. Locate the conical tubes labeled "stop solution", "1.5 mM substrate", "enzyme", and "buffer". Label each of these tubes with your group number (G#).
2. Label five cuvettes E1-E5 (for the five time points: E1 = 1 minute after start of reaction, E2 = 2 minutes after start of reaction, E3 = 4 minutes after start of reaction, E4 = 6 minutes after start of reaction, E5 = 8 minutes after start of reaction). Label only the upper part of the cuvette.
3. Label the two additional cuvettes "Start" and "End" on the upper part of the cuvette. The cuvettes will serve as control time points at the start and end of the reaction and neither cuvette will contain enzyme.
4. Using a clean DPTP, pipet 1.0 mL (1000 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. Rinse the DPTP well with water and save it for future activities.
5. Locate two empty large test tubes. Label one "Enzyme Reaction" and the other "Control".
6. Using a clean DPTP, pipet 4.0 ml of 1.5 mM substrate into the test tube labeled "Enzyme Reaction". Use the same DPTP and pipet 2.0 ml of 1.5 mM substrate into the test tube labeled "Control". Rinse the DPTP well with water and save it for future activities.
7. 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**.

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

8. Using the DPTP labeled "C", pipet 1.0 mL of buffer into the 15 ml test tube labeled "Control" and gently mix. Once you have mixed the buffer with the substrate, remove 1.0 mL of this solution and add it to your cuvette labeled "Start".

9. Using the DPTP labeled "E", pipet 2.0 ml of enzyme into the 15 ml test 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 1.0 mL 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, use the DPTP labeled "C" to remove 1.0 mL of the solution from the "C" reaction tube and add it to the cuvette labeled "End".
12. Using a clean DPTP, add an additional 2.0 mL of buffer to each cuvette. This should bring the volume of each cuvette to 4.0 mL.
13. Rinse out all DPTPs with copious amounts of water and save them for later activities. After you have finished your analysis, rinse out your reaction (test) tubes and cuvettes with copious water and save them for later activities. The function of each cuvette is listed in **Table 1** below.

<u>Note:</u> Do not discard the unused stock solutions or cuvettes containing standards. They will be used for the next activity.

Table 1: Function of Cuvettes for Enzyme Reaction Rate

Time (min)	Enzyme Cuvette	Control Cuvette
0 (Start)	Start	Start
1	E1	
2	E2	
4	E3	
6	E4	
8	E5	End

ACTIVITY 1 RESULTS

Qualitative Determination of the Amount of Product Formed

A. Locate the five cuvettes of standards labeled S1–S5 at your lab bench; their concentrations are noted in **Table 2**. Compare all 7 cuvettes (control and reaction cuvettes) to the standard cuvettes by holding them against a white background. Record in **Table 3** the standard that is most similar to your control and enzyme reaction cuvettes.

Table 2: *p*-Nitrophenol standard concentration.

Standard	Amount of *p*-Nitrophenol (μmol)
S1	0.00
S2	12.50
S3	25.00
S4	50.00
S5	100.00

Table 3: The qualitative comparison of colorimetric standards with Activity 1 cuvettes.

Time (min)	Cuvette	Standard that is Most Similar	Estimated Amount of *p*-Nitrophenol (μmol)
0	Start		
8	End		
1	E1		
2	E2		
4	E3		
6	E4		
8	E5		

ACTIVITY 1 RESULTS

Quantitative Determination of the Amount of Product Formed

B. Locate the five cuvettes of standards labeled S1–S5 at your lab bench; their concentrations are noted in **Table 2**. Blank your spectrophotometer at 410 nm with the cuvette labeled S1. Then record the percent transmittance at 410 nm and then convert to absorbance for the remaining standards in **Table 4**. Using Excel, generate a standard curve called "**Graph 1**" that correlates the absorbance at 410 nm with the amount of *p*-nitrophenol (in μmol) present. Be sure to set the x-intercept to 0. Use the equation from the linear regression analysis to determine the amount of *p*-nitrophenol present in our reaction tubes in the next step.

Table 4: Absorbance values for standards at 410nm.

Standard	Amount of *p*-Nitrophenol (μmol)	% T at 410 nm	Absorbance at 410 nm
S1	0.00		
S2	12.50		
S3	25.00		
S4	50.00		
S5	100.00		

C. Record the percent transmittance and then convert to the absorbance of your enzyme-catalyzed reaction cuvettes (E1–E5) and your control cuvettes (Start, End) at 410 nm in **Table 5**.

Table 5: The qualitative comparison of colorimetric standards with Activity 1 cuvettes.

Standard Curve Equation from YOUR Linear Regression Analysis →				
Time (min)	Cuvette	% T at 410 nm	Absorbance at 410 nm	Determined Amount of *p*-Nitrophenol (μmol)
0	Start			
8	End			
0	Start			
1	E1			
2	E2			
4	E3			
6	E4			
8	E5			

D. The absorbance of the product, *p*-nitrophenol, is directly related to the amount of *p*-nitrophenol present in the cuvette. In other words, the more yellow a solution appears, the more *p*-nitrophenol in the solution and the higher its absorbance value at 410 nm. At the beginning of the reaction, there is plenty of substrate available for the enzyme to encounter. However, as the reaction progresses, there is less substrate readily available, because it is being converted to product. If you graph the amount of product formed at each time point, the data can be used to calculate the rate of product that is formed in the presence or absence of enzyme. The unit of rate is μmol/min. There is a region where the amount of product formed increases in a linear fashion. This is called the rate of reaction – initial rate is called the initial velocity; V_o during the first minutes of the reaction.

Rate of Product Formation = Slope of the Line = Change in Y / Change in X

E. Using Excel, plot the amount of *p*-nitrophenol (in μmol) produced over time for both your control and enzyme containing cuvettes (from **Table 5**). Call this "**Graph 2**".

F. Using the data from **Table 5** and **Graph 2** that you generated for concentration of product as a function of time, you will be able to determine the rate that the product is produced when there is plenty of substrate.

Perform these calculations for your data – <u>SHOW YOUR WORK OR NO CREDIT</u>!

Overall rate of product formation with enzyme present (<u>over the entire 8 minutes of reaction time</u>)

= _____ μmol/min

Overall rate of product formation with no enzyme present (<u>over the entire 8 minutes of reaction time</u>)

= _____ μmol/min

ACTIVITY 1 QUESTIONS

1. Did you observe any changes in the enzyme reaction and control reaction test tubes during the time that the reaction was occurring? If so, describe them.

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2. What happened to the solution in each cuvette after you added the enzyme/substrate mixture to the stop solution?

_____

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_____

3. Describe/diagram the chemical reaction that occurred in this experiment?

4. Describe the amount of product produced in the enzyme-catalyzed reaction compared to the control where no enzyme was added.

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5. If you took a time point at 15 minutes, do you think more product would be produced than at 8 minutes? Explain your answer… **based on your data!**

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6. How did we ***qualitatively*** estimate the amount of product (in μmol) produced by the reaction?

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7. Determine the initial rate (initial velocity V_o) from your quantitative absorbance measurements. HINT: Only use the time up to 1 minute. **SHOW WORK OR NO CREDIT!**

= _____ μmol/min

8. Is the rate of product production constant over time? What is your evidence for your answer? **HINT: can use graph and/or multiple rate calculations spanning different time intervals.**

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PROCEDURE:

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

Temperature can affect the speed of the reaction. Heat can speed up the movement of the substrate and enzyme molecules, which would increase the number of collisions and therefore speed up the reaction. However, at some point, the forces that allow the enzyme to maintain its proper shape will be broken, changing the shape of the enzyme. The point at which an enzyme changes shape (becomes denatured) will depend on the particular properties of that enzyme. Some enzymes are stable at temperatures close to boiling, whereas others are denatured at room temperature. Most enzymes, however, function best at moderate temperatures (20–40°C). Usually the environment in which the enzyme functions in nature can be a good predictor of the conditions at which it works best in the laboratory. For instance, enzymes produced by bacteria living in hot springs or compost piles can still function at a high temperature, while enzymes produced by bacteria living in arctic ice can function at low temperatures (Groudieva, 2004).

1. Label your cuvettes "XX°C" and "YY°C". (These temperatures will be assigned to your group as we will compile a class set of data.) Only label on the upper part of the cuvette face.
2. Using a clean DPTP, pipet 1.0 mL of stop solution into each cuvette. Wash the DPTP out thoroughly with water and save.
3. Label two 1.7 mL microcentrifuge tubes with "XX°C E" and "YY°C E". Using a clean DPTP, pipet 500 µL of enzyme into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.
4. Label two other 1.7 mL microcentrifuge tubes with "XX°C S" and "YY°C S". Using a clean DPTP, pipet 500 µL of the 1.5 mM substrate into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.
5. Place the microcentrifuge tubes labeled "XX°C and YY°C Enzyme" and "XX°C and YY°C Substrate" in their respective temperature controlled baths. Allow the tubes to equilibrate to their respective temperatures for at least 5 minutes.
6. Have a stopwatch ready. Using a clean DPTP, pipet the 500 µL of enzyme from the tube labeled "XX°C E" into the tube labeled "XX°C S", and then place the tube now containing your enzyme and substrate mix back in its regulated water bath. **Start your timer for that reaction.** Use the same DPTP to combine the other set of enzyme and substrate solutions, and place that tube back in its regulated water bath. **Start your timer for that reaction.**
7. After 2 minutes, use a clean DPTP for each temperature reaction to transfer 1.0 mL of your reactions to the appropriately labeled cuvettes containing stop solution. Allow all solutions in the cuvettes to reach room temperature for approximately 5 minutes.
8. Using a clean DPTP, add an additional 2.0 mL of buffer to each cuvette. This should bring the volume of each cuvette to 4.0 mL.
9. Quantitatively analyze the amount of product formed at your two assigned temperatures. Remember to use your S1 tube as a 'blank' to calibrate your spectrophotometer at 410 nm. Record the percent transmittance and then convert to the absorbance for your two cuvettes in **Table 6**. Gather the data from the other groups as well and also record this in the second column of **Table 6**.

<u>Note:</u> Do not discard the unused stock solutions or cuvettes containing standards. They will be used for the next activity.

ACTIVITY 2 RESULTS

Quantitative Determination of the Amount of Product Formed at Different Temps

A. Following the same protocol for utilizing your standard curve (**Graph 1**) used in Activity 1, convert the units of absorbance measured on the spectrophotometer to the total amount of product formed in μmol, and fill these values in the third column of **Table 6**.

B. Calculate the overall rate of reaction (covering the first two minutes of the reaction) at each of the different temperatures. Assume that the amount of *p*-nitrophenol at 0 minutes is 0 μmol. Record this data in the fourth column of **Table 6**.

Rate of Product Formation = Change in Y / Change in X

Table 6: Determination of total amount and rate of production of *p*-nitrophenol at different temperatures based on a standard curve.

Temperature (°C) of Cuvette	% T at 410 nm	Absorbance at 410 nm	Total Amount of *p*-Nitrophenol Produced (μmol) after 2 Minutes	Rate of *p*-nitrophenol Production (μmol/min)

C. Using Excel, plot the effect of temperature on the **rate** of the enzymatic reaction from your data in **Table 6**). Call this "**Graph 3**".

ACTIVITY 2 QUESTIONS

1. At what temperature do you think this enzyme works best? How did you come up with your answer?

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2. Why do chemical reactions occur faster at higher temperatures?

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3. Why do chemical reactions occur more slowly at low temperatures?

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4. Why do most enzymatic reactions slow down at extremely high temperatures?

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5. What types of bonds within the tertiary structure of an enzyme will break at high temperatures? Which ones will not break?

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PROCEDURE:

Activity 3: Determining the Effect of pH on the Reaction Rate

One of the ways that enzymes interact with their substrates is by charge groups on one molecule attracted to the oppositely charged groups on the other molecule. However, if the pH which the substrate and enzyme see is changed, it is possible that the positively and negatively charged groups can change or lose their charge. Not only is it possible for the pH to affect the enzyme, it can also affect the substrate. The net result for this is that the enzyme and substrate will no longer interact in an optimal fashion. Similar to enzymes optimized to work at high or low temperatures in organisms that live in these conditions, different enzymes are optimized to work at different pH values. Enzymes that are present in the stomach, for example, are optimized to work at low pH values around pH 3 (acidic conditions), while pancreatic enzymes that are secreted into the small intestine only work in neutral to basic conditions (pH 7.2–9.0).

<u>Note:</u> The concentration of substrate used in this activity is different from the one used in previous activities. The substrate you should be using for this activity is 3.0 mM to account for the dilution step that occurs when you add the pH adjustment buffers.

1. Obtain two cuvettes and label them "pH XX" and "pH YY". (These pH values will be assigned to your group as we will compile a class set of data.) Only label on the upper part of the cuvette face.
2. Using a clean DPTP, pipet 1.0 mL of stop solution into each cuvette. Wash the DPTP out thoroughly with water.
3. Obtain two 2.0 mL microcentrifuge tubes and label them "pH XX" and "pH YY". Add 1.0 mL of your assigned pH buffers into their respective tubes.
4. Using a clean DPTP, pipet 500 µL of enzyme into each microcentrifuge tube labeled "pH XX" and "pH YY". Wash the DPTP out thoroughly with water.
5. Have a stopwatch (timer) ready. Using a clean DPTP, add 500 µL of 3.0 mM substrate to each of the labeled microcentrifuge tubes and start your stopwatch.
6. After 2 minutes, using a clean DPTP for each pH reaction, transfer 1.0 mL of your reaction to the appropriately labeled cuvette containing stop solution.
7. Using a clean DPTP, add an additional 2.0 mL of buffer to each cuvette. This should bring the volume of each cuvette to 4.0 mL.
8. Rinse out the DPTPs and cuvettes used in this activity with copious amounts of water and save them for future activities.
9. Quantitatively analyze the amount of product formed at your two assigned temperatures. Remember to use your S1 tube as a 'blank' to calibrate your spectrophotometer at 410 nm. Record the percent transmittance and then convert to the absorbance values for your two cuvettes in **Table 7**. Gather the data from the other groups as well and also record this in the second column of **Table 7**.

<u>Note:</u> Do not discard the unused stock solutions or cuvettes containing standards. They will be used for the next activity.

ACTIVITY 3 RESULTS

Quantitative Determination of the Amount of Product Formed at Different pHs

A. Following the same protocol for utilizing your standard curve (**Graph 1**) used in Activity 1, convert the units of absorbance measured on the spectrophotometer to the total amount of product formed in μmol, and fill these values in the third column of **Table 7**.

B. Calculate the overall rate of reaction (covering the first two minutes of the reaction) at each of the different pHs. Assume that the amount of *p*-nitrophenol at 0 minutes is 0 μmol. Record this data in the fourth column of **Table 7**.

Rate of Product Formation = Change in Y / Change in X

Table 7: Determination of total amount and rate of production of *p*-nitrophenol at different pHs based on a standard curve.

pH of Cuvette	% T at 410 nm	Absorbance at 410 nm	Total Amount of *p*-Nitrophenol Produced (μmol) after 2 Minutes	Rate of *p*-nitrophenol Production (μmol/min)

C. Using Excel, plot the effect of pH on the **rate** of the enzymatic reaction from your data in **Table 7**). Call this "**Graph 4**".

ACTIVITY 3 QUESTIONS

1. At what pH do you think this enzyme works best? How did you come up with your answer?

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2. Why do chemical reactions slow down at extremely high or low pH values?

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PROCEDURE:

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

For this activity, you will determine the effect of changing the enzyme concentration on the initial rate of the reaction and on the amount of product formed. One simple way to think of the effect of enzyme concentration is to consider squirrels in a small forest with a known number of trees and a set number of nuts on those trees. The goal of the squirrels is to gather up all of the nuts. If there was just one squirrel, it would take it longer to move from tree to tree and gather up all the nuts, but given enough time, the squirrel would make it to all the trees and find and gather all the nuts. However, if there were many squirrels, they could gather up the nuts much more quickly, but in the end, they would have the same number of nuts as the single squirrel. Increasing enzyme concentration has an analogous effect. Initially, if there is plenty of substrate, the reaction will go faster in the presence of more enzyme. However, both a reaction with a lot of enzyme and a reaction with less enzyme have a set amount of substrate to work on (nuts to find), and if enough time is allowed, all the substrate will be converted to product.

1. Obtain two large test tubes. Label one test tube "Low Concentration Enzyme" and the other "High Concentration Enzyme".
2. Using a clean DPTP, pipet 2.5 mL of buffer into the tube labeled "Low Concentration Enzyme". Wash out the DPTP with water.
3. Pipet 2.5 mL of high concentration enzyme from the stock to the test tube "Low Concentration Enzyme" and mix. Wash out the DPTP thoroughly with water.
4. Pipet 5.0 mL of high concentration enzyme from the stock to the test tube "High Concentration Enzyme" and mix. Wash out the DPTP thoroughly with water.
5. Label five cuvettes "H1–H5" (for high enzyme concentration time points) and the remaining five cuvettes "L1–L5" (for low enzyme concentration time points). Only label on the upper part of the cuvette face.
6. Using a clean DPTP, pipet 1.0 mL of stop solution into each cuvette. Wash out the DPTP thoroughly with water.
7. Label one clean DPTP with an "H" for high enzyme concentration and a second clean DPTP with an "L" for low enzyme concentration.

Read and understand steps 6-8 fully before proceeding.
These steps are time sensitive!

8. Using the DPTP labeled with an "H", pipet 500 µL of 1.5 mM substrate into your large test tube containing enzyme labeled "High Concentration Enzyme". **START YOUR TIMER**.
9. Using the DPTP labeled with an "L", pipet 500 µL of 1.5 mM substrate into your large test tube containing enzyme labeled "Low Concentration Enzyme". **START YOUR TIMER**.
10. At the times indicated in **Table 8** on the next page, use the correctly labeled DPTP to remove 1.0 mL from the large test tubes labeled "High Concentration Enzyme" and "Low Concentration Enzyme", and add it to the appropriately labeled cuvette that already contains the stop solution.

Table 8: Function of Cuvettes for Enzyme Concentration Study

Time (min)	High [Enzyme] Cuvette	Low [Enzyme] Cuvette
1	**H1**	**L1**
2	**H2**	**L2**
4	**H3**	**L3**
6	**H4**	**L4**
8	**H5**	**L5**

11. Using a clean DPTP, add an additional 2.0 mL of buffer to each cuvette. This should bring the volume of each cuvette to 4.0 mL.
12. Rinse out all DPTPs, your 2 reaction (large test) tubes, and all cuvettes with copious amounts of water and save them for later activities.
13. Quantitatively analyze the amount of product formed at the designated time periods for both enzyme concentrations. Remember to use your S1 tube as a 'blank' to calibrate your spectrophotometer at 410 nm. Record the percent transmittance and then convert to the absorbance values for your cuvettes in the second column in **Table 9**.

<u>Note:</u> Do not discard the unused stock solutions or cuvettes containing standards. They will be used for the next activity.

ACTIVITY 4 RESULTS

Quantitative Determination of the Amount of Product Formed at Different Enzyme Concentrations

A. Following the same protocol for utilizing your standard curve (**Graph 1**) used in Activity 1, convert the units of absorbance measured on the spectrophotometer at that time interval to the total amount of product formed in μmol, and fill these values in the third column of **Table 9**.

B. Calculate the rate of reaction (covering the time interval of each tube) for each enzyme concentration. Assume that the amount of *p*-nitrophenol at 0 minutes is 0 μmol. Record this data in the fourth column of **Table 9**.

Rate of Product Formation = Change in Y / Change in X

Table 9: Determination of total amount and rate of production of *p*-nitrophenol at different enzyme concentrations based on a standard curve.

Cuvette	% T at 410 nm	Absorbance at 410 nm	Total Amount of *p*-Nitrophenol Produced (μmol) at Time Interval	Rate of *p*-nitrophenol Production (μmol/min) at Time Interval
H1				
H2				
H3				
H4				
H5				
L1				
L2				
L3				
L4				
L5				

C. Using Excel, plot the total amount of product formed versus time for both the high and low concentration of enzyme on the same graph. Call this "**Graph 5**".

ACTIVITY 4 QUESTIONS

1. Does the amount of enzyme change the initial rate of reaction? Explain why based on your data.

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2. Does the amount of enzyme change the final amount of product, assuming that you start with the same amount of substrate and that you let each reaction proceed for a really, really long time? Explain.

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PROCEDURE:

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

For this activity, you will determine the effect that changing the substrate concentration has on the initial rate of the reaction. We can again use the simple analogy of squirrels gathering nuts in the forest to understand how substrate concentration affects the initial rate of the reaction and the final amount of product. In this case, we are determining the effect of the number of nuts in the trees on the speed at which the squirrels gather them and how many nuts will be gathered after a long time. In this scenario, we have the same number of squirrels gathering nuts in a small forest with a constant number of trees. When there are a lot of nuts, the squirrels will initially be able to find them more quickly, and given enough time, they would gather all of them. When there are fewer nuts, it will take the same number of squirrels more time to go from tree to tree to find them, and given enough time, they would only be able to gather fewer nuts. Decreasing the substrate concentration has an analogous effect — the rate of initial reaction should be slower because the enzyme would be less likely to find the substrate, and even with a lot of time, there is less substrate to turn into product, so the final concentration of product would be lower. For a more complex analysis of the effects of varying substrate concentration, please see your notes regarding Michaelis – Menten kinetics.

1. Label one clean large test tube "XX mM" and one clean large test tube "YY mM". (These substrate concentrations will be assigned to your group as we will compile a class set of data.)
2. Using a clean DPTP, pipet 4.0 mL of XX mM substrate into the large test tube labeled "XX mM". Rinse the DPTP thoroughly with clean water.
3. Using a clean DPTP, pipet 4.0 ml of YY mM substrate into the large test tube labeled "YY mM".
4. Label ten cuvettes XX1 – XX5 (for the XX mM substrate) and YY1 –YY5 (for the YY mM substrate). Only label on the upper part of the cuvette face.
5. Using a clean DPTP, pipet 1.0 mL of stop solution into each cuvette. Rinse the DPTP thoroughly with water.
6. Label one DPTP as "H" for high concentration substrate and a second DPTP as "L" for low concentration substrate.
7. Using a clean DPTP, pipet 1.0 mL of enzyme into your large test tube of substrate labeled "XX mM". **START YOUR TIMER FOR THIS REACTION TUBE**.
8. Using a clean DPTP, pipet 1.0 mL of enzyme into your 15 large test tube of substrate labeled "YY mM". **START YOUR TIMER FOR THIS REACTION TUBE**.
9. At the times of each reaction indicated on **Table 10** below, use the correctly labeled DPTP to remove 1.0 mL from the large test tubes labeled "XX mM" and "YY mM" and add it to the appropriately labeled cuvette that contains stop solution.

Table 10: Function of Cuvettes for Substrate Concentration Study

Time (min)	XX mM [Substrate] Cuvette	YY mM [Substrate] Cuvette
1	XX1	YY1
2	XX2	YY2
4	XX3	YY3
6	XX4	YY4
8	XX5	YY5

14. Using a clean DPTP, add an additional 2.0 mL of buffer to each cuvette. This should bring the volume of each cuvette to 4.0 mL.
15. Rinse out all DPTPs, your 2 reaction (large test) tubes, and all cuvettes with copious amounts of water and save them for later activities.
16. Quantitatively analyze the amount of product formed at the designated time periods for both enzyme concentrations. Remember to use your S1 tube as a 'blank' to calibrate your spectrophotometer at 410 nm. Record the percent transmittance and then convert to the absorbance values for your cuvettes in the second column of **Table 11**.

ACTIVITY 5 RESULTS

Quantitative Determination of the Amount of Product Formed at Different Substrate Concentrations

A. Following the same protocol for utilizing your standard curve (**Graph 1**) used in Activity 1, convert the units of absorbance measured on the spectrophotometer at that time interval to the total amount of product formed in μmol, and fill these values in the third column of **Table 11**.

B. Calculate the rate of reaction (covering the time interval of each tube) for each substrate concentration. Assume that the amount of *p*-nitrophenol at 0 minutes is 0 μmol. Record this data in the fourth column of **Table 11**.

Rate of Product Formation = Change in Y / Change in X

Table 11: Determination of total amount and rate of production of *p*-nitrophenol at different substrate concentrations based on a standard curve.

Cuvette	% T at 410 nm	Absorbance at 410 nm	Total Amount of *p*-Nitrophenol Produced (μmol) at Time Interval	Rate of *p*-nitrophenol Production (μmol/min) at Time Interval
XX1				
XX2				
XX3				
XX4				
XX5				
YY1				
YY2				
YY3				
YY4				
YY5				

C. Using Excel, plot the total amount of product formed versus time for both of your substrate concentrations tested on the same graph. Call this "**Graph 6**".

D. Gather data of the **initial rates** (**Vo**) of reaction (covering just the first minute of the reactions) from the other groups and compile it with your two substrate concentration rates of reaction that you run. Record this data on **Table 12**.

Table 12: Initial velocity of the reaction that produces *p*-nitrophenol at different substrate concentrations.

[S] **Substrate Concentration**	**V_o** **Initial Rate of *p*-nitrophenol Production at 1 min (μmol/min)**	**1/[S]**	**$1/V_o$**

E. Using Excel, plot the Michaelis-Menten curve of initial velocity versus substrate concentration. Call this "**Graph 7**".

F. Calculate the reciprocal of the substrate concentration and initial velocity from first two columns in **Table 12** and place these values in the final two columns of the aforementioned table.

G. Using Excel, plot the Lineweaver-Burk curve from the above table. Call this "**Graph 8**".

ACTIVITY 5 QUESTIONS

1. How does the amount of substrate present change the **initial rate** of the reaction? Use data to support your answer.

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2. Does the initial amount of substrate change the final amount of product, assuming that you start with the same concentration of enzyme and that you let each reaction proceed for a really, really long time? Explain.

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3. From the Lineweaver-Burk plot, what are the V_{max} and K_m of the cellobiase enzyme that we studied? Show how you obtained that answer by sketching the graph below and include any calculations used.