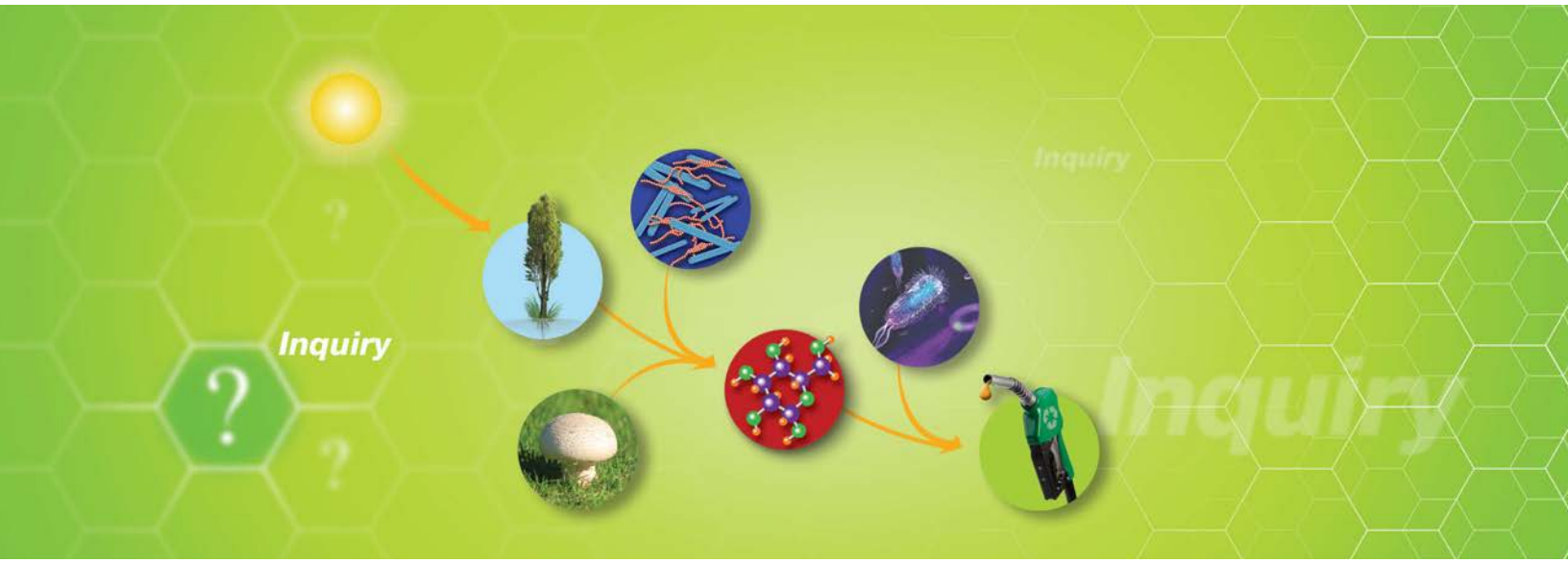
BIO-RAD Explorer™



Biofuel Enzyme Reactions Kit for AP Biology:

A ThINQ!™ Investigation

Student Manual

BIO-RAD

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Student Manual

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Background

Mushroom Power: Creating Fuels from Fungi

You've probably already heard that countries around the world are seeking renewable, clean sources of energy. You may even have heard of **biofuels** — fuels developed from plants such as corn, switchgrass, or sugarcane. The process of making biofuels from plant cellulose can be time-consuming and expensive. The biofuel industry uses special enzymes to convert the cellulose in plant cell walls to sugars, such as glucose. The sugar can then be converted to ethanol and other fuels by microbial fermentation. The ethanol or other fuels in turn can be used alone in certain engines or in combination with gasoline in other engines to power cars, trucks, and airplanes. The process of making biofuels typically requires three main steps (Figure 1):

1. **Pre-treatment**: Removes structural components of plant cells.
2. **Enzymatic hydrolysis**: Uses enzymes to break down cellulose and produce sugar molecules.
3. **Microbial fermentation**: Converts sugar products into biofuels.

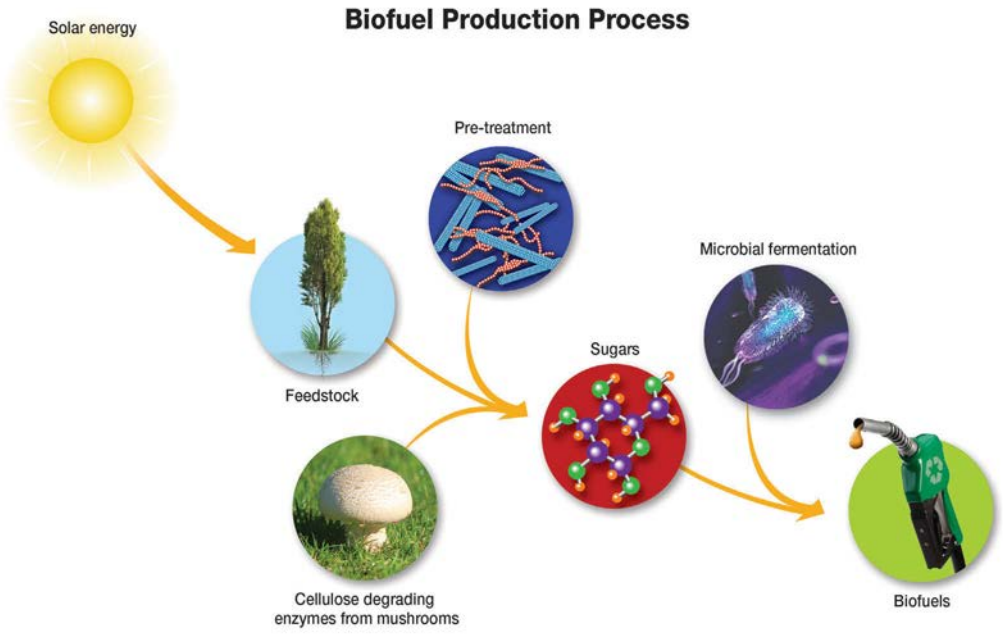


Fig. 1. Steps required for production of biofuels. Pre-treatment, enzymatic hydrolysis, and microbial fermentation.

Scientists and engineers are continually working to optimize this process so that biofuel production becomes time-, energy-, and cost-effective. The pre-treatment process often involves high temperatures and low pH, so though cooling the resulting product and adjusting pH for the next enzymatic step is expensive, it's also important because most enzymes currently used in this process work best at cooler temperatures and more neutral pH levels. Looking for new enzymes that work well under varying environmental conditions is one way researchers are attempting to make biofuel production more efficient and cost-effective.

Mushrooms are a promising source of a special group of enzymes called **cellulases** that break down cellulose into individual sugar molecules ready for biofuel production. Mushrooms are inexpensive to grow, require little energy, and reproduce quickly. For these reasons, mushrooms are potentially an excellent source of cellulases for biofuel production. However, not all mushrooms produce the same amount of cellulases. Mushrooms that grow on dense, woody surfaces must have large quantities of cellulase to break down the wood. Depending on a mushroom's environment, where it grows, and its primary nutrient source, the activity of its cellulases can vary widely. This variety in cellulase activity leads to questions about which mushroom cellulases to use for biofuel production and the optimal conditions for using those enzymes within the production process. In order to answer such questions, we first need to take a look at how enzymes work.

Enzymes

Enzymes are typically proteins (some nucleic acids have also been found to be enzymes) that act as **catalysts**, speeding up chemical reactions that would take a long time 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 complements the structure of its substrate (Figure 2). The **substrate** is the molecule or molecules that the enzyme converts into **product**. The substrate fits into an indentation in the globular protein called the **active site**. The shape and chemical properties of this active site are critical to the enzyme's function.

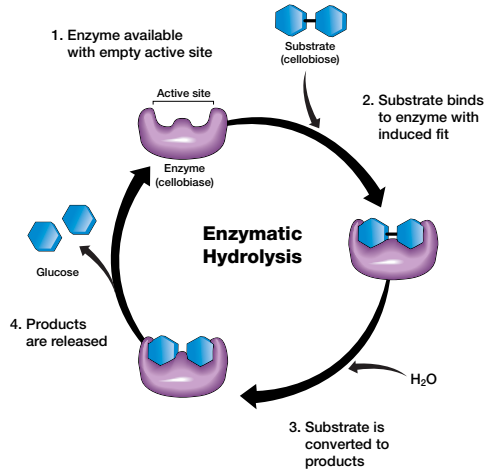


Fig. 2. Schematic illustration of cellobiose and cellobiase activity in solution. 1, cellobiase enzyme contains an active site that fits the cellobiose molecule, which is composed of two covalently linked glucose molecules. **2**, when the cellobiose substrate begins to bind to the enzyme active site, subtle structural changes in the active site occur for better binding and enhanced enzyme activity. This change is referred to as induced fit. **3**, with the addition of water, the bond between the two glucose molecules in cellobiose can be broken. **4**, once the β 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.

Many chemical reactions that enzymes speed up can occur at a much slower rate without the enzymes. Enzymes speed up reactions by positioning the substrates, adjusting their bonds so that they become unstable and reactive. Let's use the analogy of a friend setting up a blind date. The two people may have found each other on their own and made the connection, but the matchmaker sped up the process by putting the two people in the same room at the same time. The matchmaker may also have influenced the couple by pointing out to each the good points about the other. Like enzymes, the matchmaker did not change as a result of setting up the match, and he/she was able to go on and make further matches.

In chemical terms, the enzyme lowers the **activation energy** of a reaction. This is the amount of energy required to get the reaction going. Enzymes also stabilize the transition state of the reaction. The **transition state** is the most energetic structure. By lowering this energy, the reaction can take place much more easily.

Enzyme Effects on Activation Energy

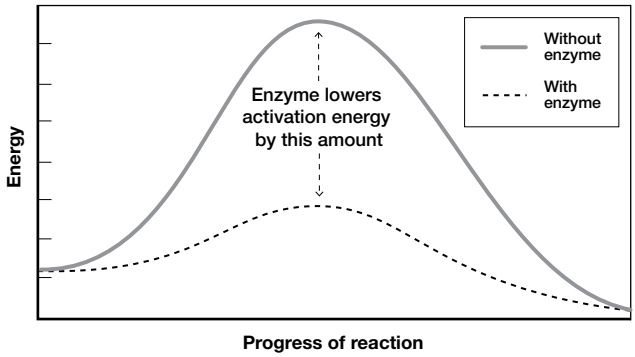


Fig 3. Enzymes catalyze reactions by lowering the activation energy required to make the reaction work. This speeds up the time it takes for the reactants to become products.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

An enzymologist — a scientist who studies enzymes — was interested in learning whether a new enzyme she discovered in kiwi fruit could break down proteins faster than digestive enzymes found in humans. She found that her new enzyme was very efficient and could break down a variety of proteins, whereas human digestive enzymes target specific substrates. What could explain why the kiwi fruit enzyme could break down a variety of substrates instead of just one? (Hint: Think about possible structural differences between the enzymes' active sites).

Enzymes are picky about the conditions in which they work best. The temperature and pH must be ideal for the enzyme to catalyze reactions efficiently. For any chemical reaction, raising the temperature will increase the movement of the molecules and cause more collisions to occur. This increases the average kinetic (movement) energy of the molecules so that more substrate and enzyme will be able to react. However, in an enzymatic reaction, too much heat is a bad thing. You may recall from studying about proteins that the noncovalent interactions within the protein, such as hydrogen and ionic bonds, can break at high temperatures. This will change the shape of the enzyme. If the enzyme changes shape, then the active site will not fit the substrate properly and the enzyme will not be able to function.

Cellobiase Enzyme

In these laboratory investigations, you will be studying **cellobiase**. Cellobiase 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. Breaking down the cellulose from plants into sugar is also an important step in the creation of ethanol and other biofuels.

Cellobiase Substrates

The natural substrate for the enzyme cellobi**ase** is cellobi**ose** (Figure 4). This is a disaccharide composed of two glucose molecules joined by a β-linkage. When scientists study enzyme function, they need 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, and fast methods to detect these molecules quantitatively.

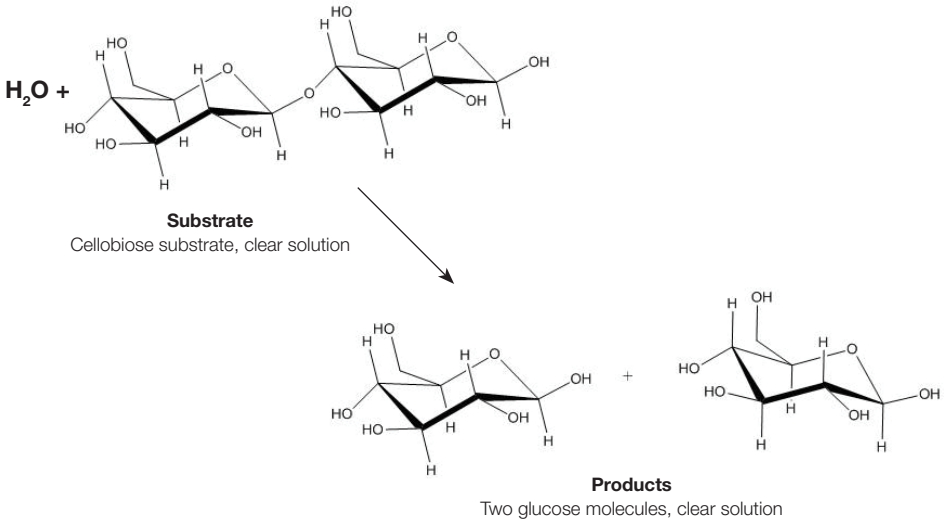


Fig. 4. Breakdown of cellobiose by cellobiase. The natural substrate of cellobiase is the disaccharide cellobiose. When cellobiose is bound by cellobiase, the cellobiase breaks apart the two glucose molecules and releases them as separate molecules. These glucose molecules can be used to make biofuel.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

Throughout this lab and later when you learn more about enzymes and substrates, you may notice that their names are very similar. How can you know which is the enzyme and which is substrate just based on its name?

What does the name of an enzyme typically tell you?

So to make this reaction easier to follow, an artificial substrate ***p*-Nitrophenyl glucopyranoside**, will be used. This artificial substrate can be catalyzed by the enzyme and broken down in a manner similar to the way the natural substrate cellobiose works. When the artificial substrate *p*-Nitrophenyl glucopyranoside is broken down by cellobiase, it produces glucose and *p*-Nitrophenol (Figure 5). When *p*-Nitrophenol is mixed with a solution that is basic in pH (such as the stop solution provided in the kit), it will turn yellow. The yellow product is an **indicator** that the reaction has taken place. The yellowness of the solution is proportional to the amount of *p*-Nitrophenol present. And for every molecule of *p*-Nitrophenol present, one molecule of *p*-Nitrophenyl glucopyranoside has been broken into two. Another advantage of using a basic solution to develop the color of the *p*-Nitrophenol is that the change in pH will also inhibit enzyme activity and stop the reaction.

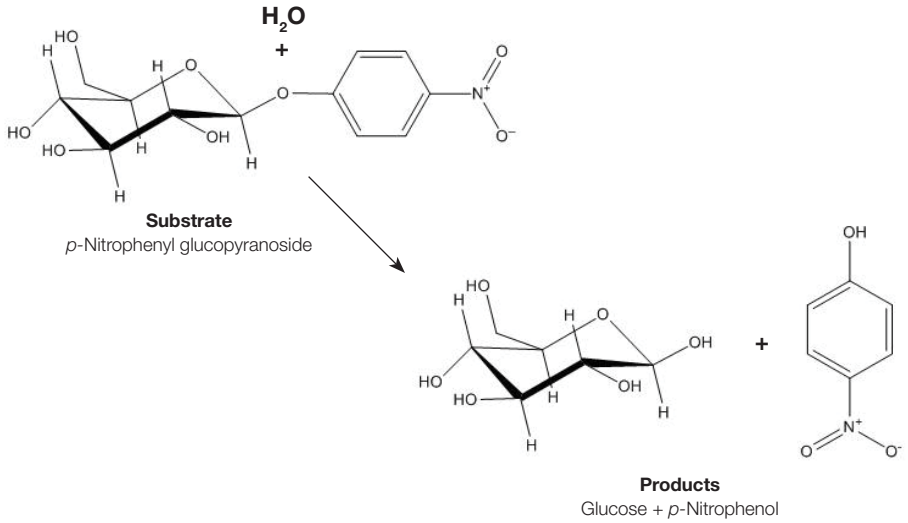


Fig. 5. Breakdown of *p*-Nitrophenyl glucopyranoside into glucose and *p*-Nitrophenol by cellobiase. When the *p*-Nitrophenyl glucopyranoside is broken apart by cellobiase, one molecule of glucose and one molecule of *p*-Nitrophenol are released. If the *p*-Nitrophenol is put into a basic solution, it will produce a yellow color, which is measured by a simple colorimetric quantitative method.

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 greater 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 standards that contain a known amount of colored product. Compare your samples to the set of standards to see which most closely matches the color of your samples. This will give you an estimate of the amount of product.

Alternatively, you can use a **spectrophotometer** (or a colorimeter), which quantitatively measures the amount of yellow color by directing a beam of light through the sample. The spectrophotometer measures the amount of light that is absorbed by the sample at a specific wavelength. The deeper yellow the color, the more concentrated the sample, the more light is absorbed. 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 of your samples can then be measured, and the standard curve can be used to convert the absorbance to a concentration.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

Using an indicator is a common practice in biology to study processes and phenomena that are difficult to observe directly. What other indicators can you think of?

Why was the indicator in your example chosen instead of observing the actual process directly?

Why is the use of p-Nitrophenol as an indicator of the enzymatic reaction in this lab useful?

Measuring the Rate of Cellobiase Activity

In order to determine what factors influence an enzyme's ability to break down its substrate, the rate of reaction, or how much product is formed in a set amount of time, must be determined. For studying cellobiase activity, you will measure the rate of reaction by adding enzyme to the artificial substrate *p*-Nitrophenyl glucopyranoside. At set times, a sample of the enzyme reaction will be removed and added to a high-pH (very basic) stop solution, which will help develop the color of the product *p*-Nitrophenol, as well as stop the reaction by increasing the pH above where the enzyme can work. By calculating how much *p*-Nitrophenol is produced over time, the rate of reaction can be calculated. By looking at small increments of time, you will be able to determine whether the rate of the enzyme-catalyzed reaction speeds up, is constant, or slows down over time. You will also be able to detect any effects of pH, temperature, substrate concentration, or enzyme concentration on the initial rate of reaction.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

What data would you need in order to calculate the rate of an enzymatic reaction?

How would you collect the data you need in order to calculate the rate of the reaction?

Pre-Lab Activity: Modeling Enzymatic Reactions

Enzymes function by lowering the amount of **activation energy** needed for a reaction to occur (Figure 3). With the help of enzymes to lower the activation energy of a reaction, reactions that would normally take years to happen on their own occur in just minutes or even seconds. However, enzymes can be very sensitive to their environment and require precise conditions to interact efficiently with a substrate. In your group, answer the following questions:

1. **Imagine you are working with the enzyme cellobiase that breaks down cellobiose into sugars in termite gut. Cellobiase from termite gut functions best at an acidic pH (4.0) and temperature (about 37°C). Describe what would happen to the enzyme under the following conditions:**

   - **Very basic pH (9.0)**:

   - **High temperature (100°C)**:

2. **If you were measuring the rate of reaction of cellobiase isolated from termite gut, what might happen if you:**

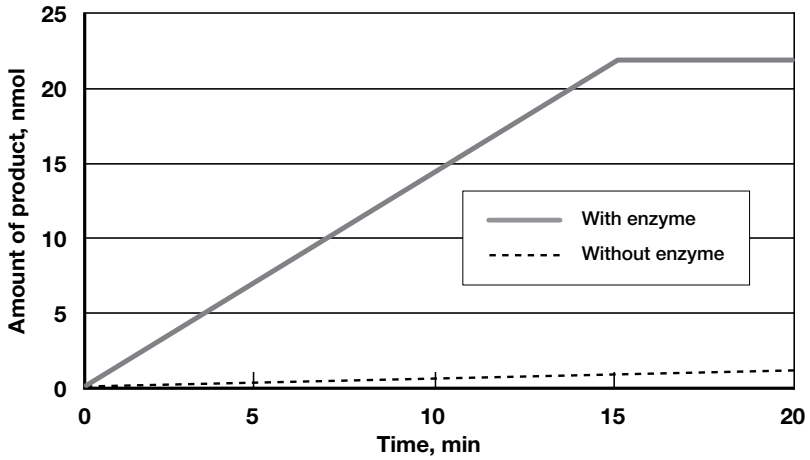
   - **Increase amounts of enzyme (cellobiase)**:

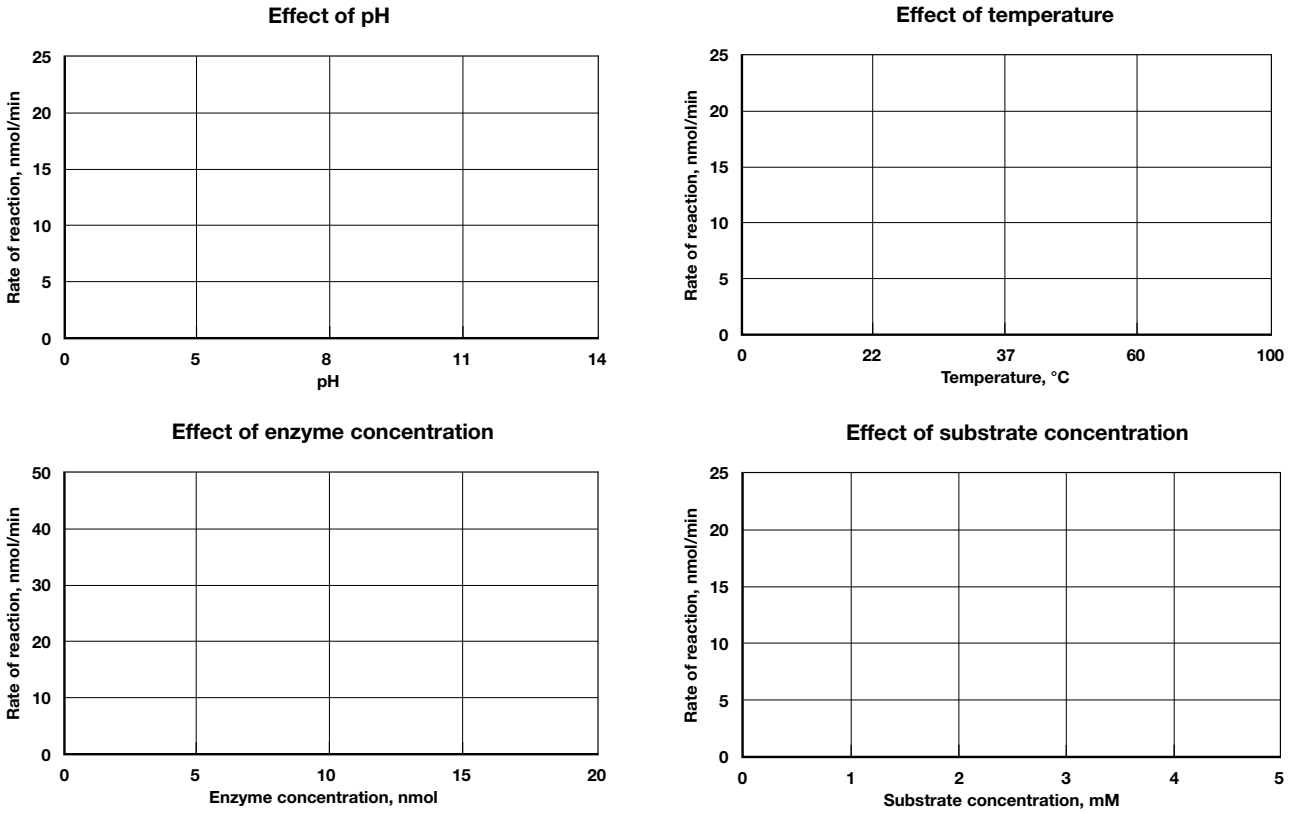
   - **Increase amounts of substrate (cellobiose)**:

Not all enzymes function under the same environmental conditions. Finding an enzyme that works under a broad variety of conditions will be useful for creating biofuels more efficiently. Imagine you are running a biofuels production facility and are considering using mushrooms for their cellobiase activity. However, mushrooms are not well studied for this process and it is unclear what the optimal conditions are for cellobiase activity from the fungi.

Your role is to answer the research question: Under what conditions does mushroom cellobiase generate glucose most efficiently?

3. **Examine the graph below. The amount of product is shown with and without cellobiase present. Using this graph as a starting point, complete the remaining graphs for mushroom cellobiase activity with changing pH, temperature, enzyme concentration, and substrate concentration. Substrate is in excess (high concentration).**





4. What evidence would you need to gather in order to determine whether your thinking is accurate? Describe your evidence below:

Investigation 1: Determine How Mushroom Extracts Compare in Terms of Cellobiase Activity

Cellobiase that breaks down the 1,4 β-glucoside linkages in cellobiose is produced by many organisms. Fungi such as molds, yeasts, and mushrooms produce this enzyme and can excrete it to digest cellobiose and produce glucose for energy use. Many bacteria also contain cellulytic enzymes and cellobiase to break down plant cell walls. These bacteria can be found in the second stomach (rumen) of many hoofed animals, such as cows, and also in the gut of termites. In this investigation you will choose a mushroom as a potential source of cellobiase, extract proteins from this source, and combine your extract with the substrate *p*-Nitrophenyl glucopyranoside to determine how your extract performs in terms of cellobiase activity. If more than one type of mushroom is available, compare your findings with those of your classmates.

Student Workstations	***Quantity***
☐ Mushroom sample	1
☐ 1.5 mM substrate	1
☐ Stop solution	1
☐ 1x extraction buffer	1
☐ 15 ml conical tube	1
☐ 1.5 ml microcentrifuge tube	1
☐ DPTPs	4
☐ Cuvettes	7
☐ Marker	1
☐ Mortar and pestle	1
☐ Filter paper, cheesecloth, or strainer	1
☐ Beaker with deionized or distilled water to rinse DPTPs	1
☐ Stopwatch or timer	1
☐ Colorimetric standards (S1–S5) in cuvettes	1 of each
☐ Spectrophotometer (optional)	1

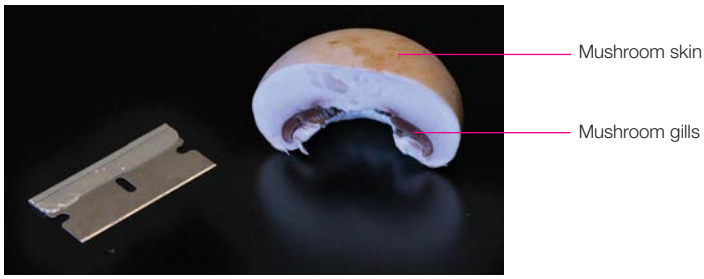
Protocol

1.1 What is the purpose of this protocol?

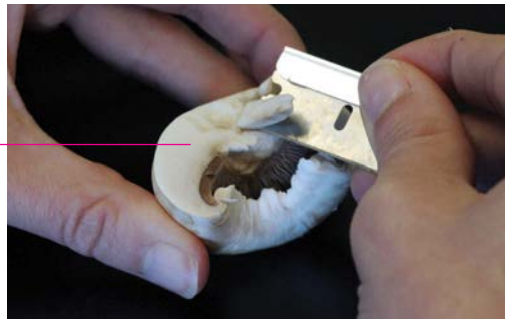
1. Write down your mushroom type _____.

2. Carefully remove the stem of your mushroom if you are using one with a woody stem like white button or shiitake. You will not need to remove the stem if you are using oyster or other soft-stemmed mushrooms.

3. Using a razor or knife, cut through the center of the cap of the mushroom. For mushrooms that do not have a distinguishable cap, cut through the meatiest section of the mushroom.

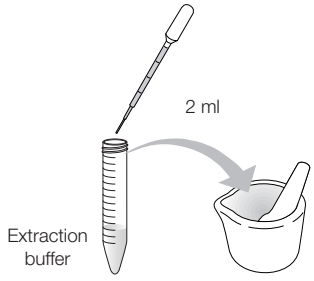


4. Using a cutting implement such as a razor cut out approximately 1 g (1,000 mg) of the internal flesh of the cap. Be careful to avoid including skin and gills in your sample.



5. Weigh out your sample and place it in a mortar: _____mg

6. Add 2 ml of extraction buffer to the mortar for every gram of mushroom.



7. Using a pestle, grind your mushroom to produce a slurry — a semiliquid mixture.

Tips & Tricks

It is recommended to use a mortar and pestle when preparing mushroom extract. If one is not available, you may also use a 1.5 ml tube and a pipet tip to crush mushroom (it may help to bend the tip of the pipet tip prior to crushing mushroom). In the latter case, weigh out 0.25 g (250 mg) of mushroom, cut the mushroom into small pieces to fit into the tube, add it to your tube, add 500 µl extraction buffer, and carefully crush the mushroom sample with a pipet tip. Take care not to spill the extraction buffer. Extraction buffer contains detergents and will cause foaming in the tube. Care should be taken that the buffer does not overflow from the tube and also that the tube does not slip from your hands.

You should remove and discard the stems of those mushrooms with a woody stem, like white button and shiitake, because the majority of active enzyme is found in the cap. You do not need to remove the stem of soft-stemmed mushrooms, like oyster mushrooms, since enzyme activity is uniform throughout the mushroom.

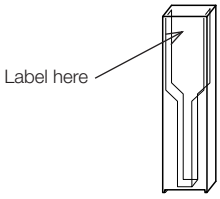
Tips & Tricks

Cutting implements other than razors can be used to cut mushroom samples. These include metal scoops, the large end of a 1,000 µl pipet tip, a plastic knife, and the cut bulb of a DPTP.

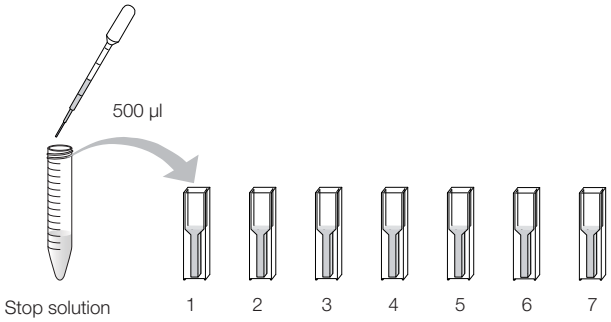
8. Strain the solid particles out of your slurry. If you have a centrifuge, scoop the slurry into a 1.5 ml microcentrifuge tube and then pellet the solid particles by spinning at top speed for 2 min. If you do not have a centrifuge, use a piece of cheesecloth to squeeze the extract into a 1.5 ml microcentrifuge tube. If you use the cheesecloth method, you will need to use 2 g mushroom and 4 ml of extraction buffer.

Note: You will need at least 250 µl of extract to perform the enzymatic reaction portion of this protocol.

9. Label your cuvettes 1–7. Write only on the upper part of the cuvette face so that your writing won't interfere with spectroscopy.

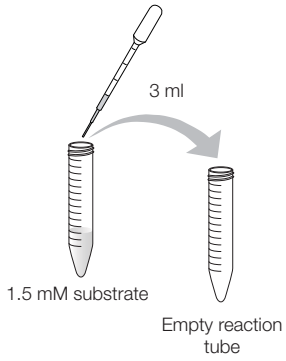


10. Using a clean DPTP, pipet 500 µl of stop solution into cuvettes 1–7. Rinse the DPTP thoroughly with water.

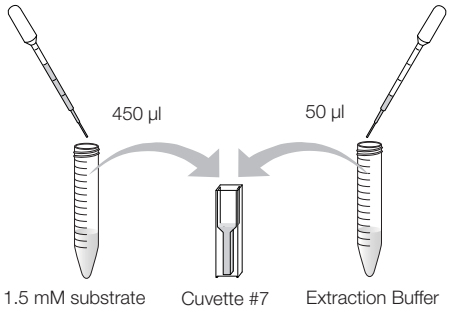


1.2 Why is it important that the stop solution is basic?

11. Label a 15 ml conical tube with the type of mushroom you are using. Using a clean DPTP, pipet 3 ml of substrate into the tube.



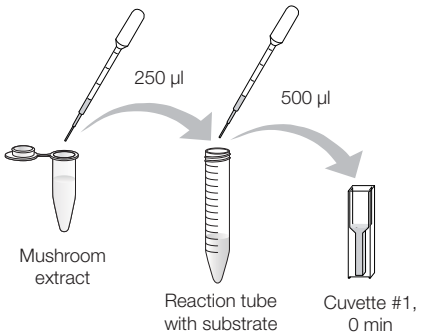
12. Using the same DPTP from the previous step, add 450 µl of substrate and 50 µl of extraction buffer to cuvette #7 and set it aside for analysis later on. This cuvette will act as a control during your investigation because it does not contain mushroom extract.



1.3 Why is including a control important during this investigation?

Please read and understand steps 13 and 14 fully before proceeding. These steps are time sensitive!

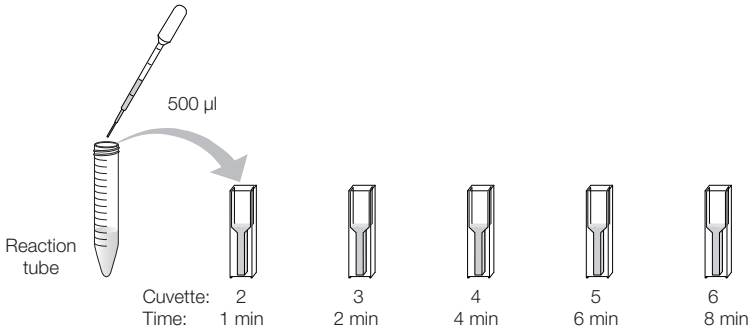
13. Using a clean DPTP, pipet 250 µl of your mushroom extract into the 15 ml conical tube containing 3 ml of substrate. This will now be referred to as the reaction tube. Pipet the liquid up and down to mix. Then pipet 500 µl from the 15 ml conical tube to cuvette #1. **START YOUR TIMER**.



1.4 Why is it important to have two DPTPs, one for mushroom extract and one for the substrate?

14. At the times indicated in the table remove 500 µl of mushroom extract/substrate mixture from the reaction tube and add it to the appropriately labeled cuvette that already contains stop solution.

Time	Cuvette
0 min	1
1 min	2
2 min	3
4 min	4
6 min	5
8 min	6



15. Rinse all DPTPs with copious amounts of water and save them for later investigations. After you have finished your analysis, rinse your reaction (conical) tubes and cuvettes with copious amounts of water and save them for later investigations.

Note: Do not discard the unused stock solutions or cuvettes containing standards. They will be used for the next investigation.

Results

Qualitative Determination of the Amount of Product Formed

1. Locate the five cuvettes of standards labeled S1–S5 at your lab bench; their concentrations are noted in Table 1. Hold the cuvettes against a white background to compare your one control and six reactions to each standard. Record in Table 2 the standard that is most similar to each of your experimental cuvettes.

Table 1. *p*-Nitrophenol standards.

Standard	Amount of p-Nitrophenol, nmol*
S1	0
S2	12.5
S3	25
S4	50
S5	100

* 1 nmol = 1 nanomole = 1 x 10^{-9} mol = 0.000000001

Table 2. Comparison of reaction cuvettes to standard cuvettes.

Time, min	Cuvette	Standard That Is Most Similar	Amount of p-Nitrophenol, nmol
0	1		
1	2		
2	3		
4	4		
6	5		
8	6		
8	7, Control		

1.5 In your own words, describe why having standards is useful for analysis of your reaction results:

1.6 How can knowing the amount of *p*-Nitrophenol in your enzyme reaction provide information about the amount of glucose produced? Hint: Look back at the reaction mechanism shown in Figure 5 of the background information.

2. If you are not using a spectrophotometer, please skip ahead to Data Analysis and Interpretation of Results.

Quantitative Determination of the Amount of Product Formed

1. Ask your teacher for assistance setting up the spectrophotometer. Also, see Appendices C or D for instructions on using Bio-Rad's SmartSpec™ Plus spectrophotometer or the Vernier SpectroVis spectrophotometer, respectively.

2. Locate the five cuvettes of standards labeled S1–S5 at your lab bench; their concentrations are noted in Table 1. Blank your spectrophotometer at 410 nm with the cuvette labeled S1. Then measure and record the absorbance at 410 nm for the remaining standards in Table 3. You will use this information to generate a standard curve that will allow you to calculate the amount of product formed.

Table 3. Absorbance values for standards.

Standard	*Absorbance at 410 nm*	*Amount of p-Nitrophenol, nmol*
S1	0.00	0
S2	0.21	12.5
S3	0.42	25
S4	0.82	50
S5	1.65	100

1.7 Why is 410 nm an appropriate wavelength to set on the spectrophotometer for analyzing your samples?

1.8 How might you determine the appropriate wavelength if it was not provided for you?

3. After recording the absorbance values for each standard, reblank your spectrophotometer at 410 nm with cuvette #1.

1.9 Why is it important to reblank the spectrophotometer before measuring the absorbance of your samples?

4. Measure the absorbance of your enzyme-catalyzed reaction cuvettes (#1–6) and your control cuvette (#7) at 410 nm and record your results in Table 4. You will use this information to determine the amount of product, *p*-Nitrophenol, formed in the reaction cuvettes.

Table 4. Determining *p*-Nitrophenol produced using a standard curve.

Time, min	*Cuvette*	*Absorbance at 410 nm*	*Amount of p-Nitrophenol, nmol, from the Standard Curve*
0	1		
1	2		
2	3		
4	4		
6	5		
8	6		
8	7, Control		

5. Determine nanomoles (nmol) of product formed from absorbance values. The absorbance value for a cuvette is directly related to the amount of *p*-Nitrophenol present in that cuvette. In other words, the more yellow a solution appears, the more *p*-Nitrophenol is in the solution and the higher its absorbance value at 410 nm. By plotting the absorbance values for the standards with known amounts of *p*-Nitrophenol to create a standard curve, you can determine how much *p*-Nitrophenol is present in your enzyme assay samples.

In the example shown in **Figure 4**, the solid filled diamonds represent the absorbance values for the five standards. The line drawn is the one that best connects all the data points; it is the standard curve. The circle symbol indicates a sample whose absorbance was measured to be 0.73. To use the standard curve to determine the amount of product corresponding to this value, absorbance value 0.73 was located on the y-axis and the value followed horizontally to where it intersects with the standard curve (the circle). Then a line was drawn down to the x-axis to find the amount of *p*-Nitrophenol. In this case, the amount of *p*-Nitrophenol in a sample with an absorbance value of 0.73 is approximately 44 nmol. Follow this process to create a standard curve with your standards and then use that standard curve to analyze your samples.

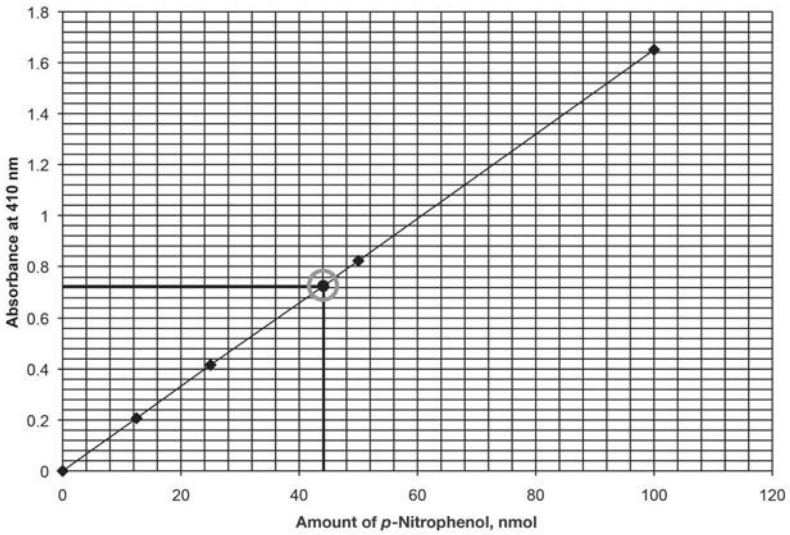


Fig. 4. Example of a standard curve. Absorbance of *p*-Nitrophenol is plotted against known the quantities of *p*-Nitrophenol in standards S1–S5.

Use the data in **Table 3** to create a standard curve. Plot the absorbance values for each standard in **Figure 5**, and then draw the line that best goes through all the data points.

As demonstrated in the previous example, plot the absorbance values for the six time points (#1–6) of your samples and the noncatalyzed reaction (control, #7), then determine the corresponding amount of product for each time point. Record this information in **Table 4**.

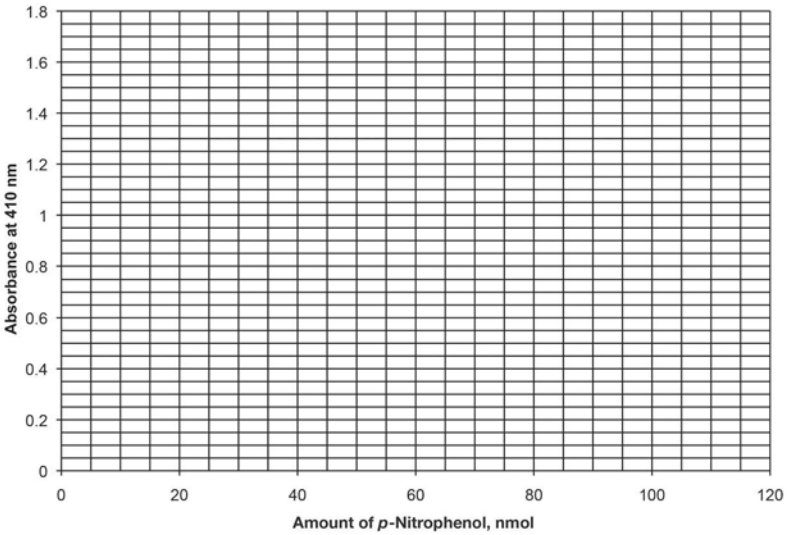


Fig. 5. Creating a standard curve. Plotting absorbance of *p*-Nitrophenol against the known quantities of *p*-Nitrophenol standards, S1–S5.

1.10 In your own words, what information does a standard curve provide?

Data Analysis and Interpretation of Results

1. Initial rate of product formation

At the beginning of the reaction there is plenty of substrate available for the enzyme to encounter. However, as substrate is converted to product and less of it is available for reaction, the rate of reaction slows. If you graph the amount of product formed at each time point, the data can be used to calculate the initial rate of product that is formed in the presence or absence of enzyme.

Calculating initial rates of reaction allows you to compare enzyme activity between different extracts. To calculate the initial rate of your reaction, see Figure 6 for an example.

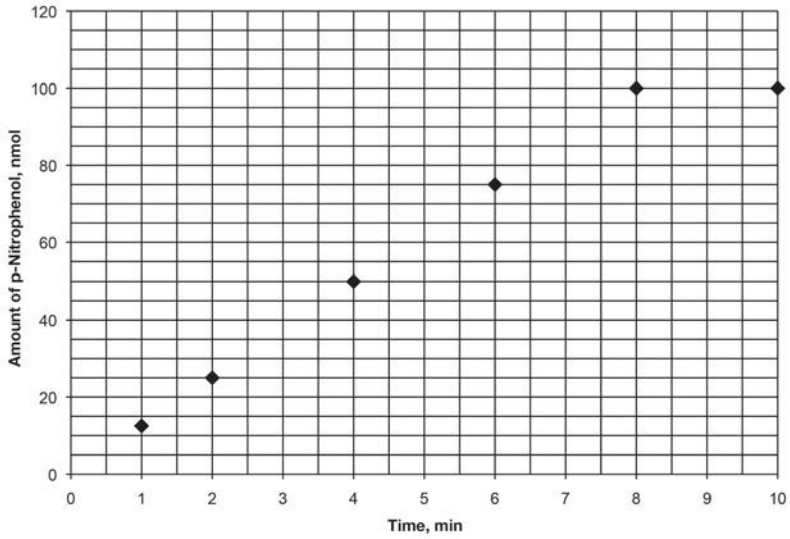


Fig. 6. Example of a rate curve for an enzyme reaction. The amount of product made is plotted against time to determine the initial rate of reaction.

In Figure 6, the amount of product, *p*-Nitrophenol, is plotted over time to determine the initial rate of product formed. The unit of measure is nmol/min.

There is a region where the amount of product formed increases in a linear fashion. This is called the initial rate of reaction. In the graph above, this linear region is between 1 and 8 minutes.

Initial rate of product formation = slope of the line = change in y/change in x

Initial rate of product formation = (100 nmol – 12.5 nmol) / (8 min – 1 min) = 12.5 nmol/min

2. Conversion of substrate to products

As demonstrated in **Figure 6**, plot the amount of *p*-Nitrophenol produced over time on the graph in **Figure 7** using data from either **Table 2** or **Table 4**. Draw a line that best fits through the data points.

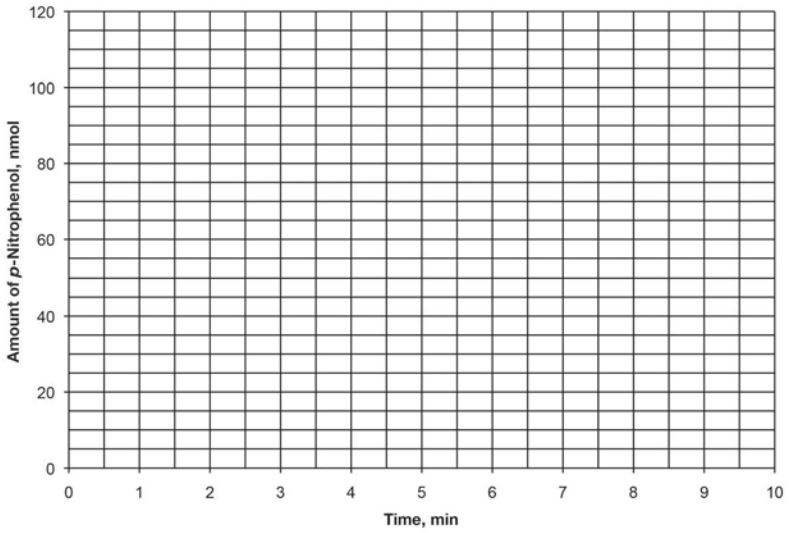


Fig. 7. Reaction rate curve for cellobiase. The amount of product made is plotted against time to determine the initial rate of reaction.

At the beginning of the reaction, there is plenty of substrate available for the enzyme to encounter and convert to product. Locate the region where the concentration of the product increases linearly.

Using the graph you generated for concentration of product as a function of time, you will be able to determine the rate at which the product is produced when there is plenty of substrate.

1.11 Perform this calculation for your data

Initial rate of product formation with enzyme present = _____ nmol/min

1.12 How does creating a standard curve (Figure 5) help determine the rate of *p*-Nitrophenol production in the presence of cellobiase (Figure 7)?

Post-Investigation Questions:

When scientists want to understand phenomena that are difficult to study (for instance, they may be very small, very large, dangerous, expensive, etc.), they often use models to represent the phenomena in an observable way. In this lab you cannot readily observe cellobiase turning the substrate cellobiose into glucose because both substrate and product are clear solutions. Instead you used a different substrate to stand in for cellobiose so that you could observe a color change when product was formed in the presence of cellobiase. This is a model system used to study the function of cellobiase.

1. For Investigation 1, describe the model system that you used to study glucose production with cellobiase enzyme extracted from mushrooms:

2. Name one drawback to and one benefit of using this model system and explain each.

During the pre-lab you were introduced to the research question guiding these investigations: **Under what conditions does mushroom cellobiase generate glucose most efficiently?** During Investigation 1, you examined the rate of product formation from various mushroom extracts.

3. How would you respond to the research question (Under what conditions does mushroom cellobiase generate glucose most efficiently?) given the data you generated from Investigation 1?

4. From your model in the pre-lab, suggest at least one follow-up question to research in order to better answer the guiding research question for the investigations:

5. Provide the steps you would take to answer your question:

One important component of creating an investigative protocol to answer a research question is including a **control**. A control is used as a comparison; typically all variables except the one under study are the same between the control sample and the experimental sample. This way, scientists can determine the effect that the variable under study has on a phenomenon.

1. If you included a control or controls in your protocol, what purpose do your controls serve?

2. If not, what control could you include in your procedure and why?

Assignment: Investigation 1 Wrap-Up

The data you generated during this investigation provide insight about how mushroom extracts compare in terms of cellobiase activity. However, further investigations are required to answer the research question: **Under what conditions does mushroom cellobiase generate glucose most efficiently?**

Looking back at your pre-lab models, decide with your group and your teacher's guidance which variable you would like to investigate in order to better answer the research question. During your investigation, you will be given purified cellobiase to work with. Having a purified form of the enzyme will allow you to compare your findings with those of your classmates who may be conducting investigations with a different variable.

Your assignment is to design a question to investigate about your chosen variable and develop a protocol to answer your question.

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

Temperature can affect the speed of an enzymatic reaction. Heat speeds up the movement of the substrate and enzyme molecules, which increases the number of collisions and therefore speeds up the reaction. However, at some point, the forces that preserve the enzyme's structure will be overcome, affecting the function of the enzyme. The point at which an enzyme 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 is 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). For this investigation, all of the reagents and equipment from Investigation 1 will be made available to you in addition to purified cellobiase and materials to create varying temperature conditions (for example, ice, water bath).

Student Workstations		*Quantity*
☐	1.5 mM substrate	1
☐	Low-concentration enzyme (0.18 U/ml)	1
☐	Stop solution	1
☐	DPTPs	4
☐	Colorimetric standards (S1–S5) from Investigation #1	1 of each
☐	1.5 ml microcentrifuge tubes	6
☐	Cuvettes	3
☐	Marker	1
☐	Beaker with ice water	1
☐	Beaker with warm water	1
☐	Thermometer	1
☐	Beaker with deionized or distilled water to rinse DPTPs	1
☐	Stopwatch or timer	1
☐	Experimental Design and Planning Worksheet (optional)	1
☐	Spectrophotometer (optional)	1

Pre-Investigation Questions

In this investigation you will design an experimental protocol to answer your questions and test your ideas about the effect of temperature on enzymatic reaction rate.

2.1 With your group, determine your **investigation question:**

2.2 Given what you learned about working with enzyme reactions in Investigation 1, what **procedural steps** will you take in order to answer your investigation question? Look back at the protocol and data analysis sections of Investigation 1 for ideas and draw and/or describe your steps below. Don't forget to include an experimental control.

2.3 **Predict your results.** In the space below draw and/or describe your expected results. A hypothesis is helpful in thinking about what you anticipate will occur during an investigation and should be revisited after you've collected your data. Before you begin, have your teacher review your protocol.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

How could increasing the temperature of an enzymatic reaction be useful for reducing the activation energy of that reaction?

When substrates are heated, sometimes they spontaneously form product without the presence of an enzyme. What might explain this phenomenon?

Data Collection

Record any results that are relevant to answering your investigation question. You may find it helpful to use a separate sheet of paper to create tables, similar to those in Investigation 1, in which to record data. As in Investigation 1, the standards provided to you contain a known amount of product (Table 1).

Table 1. *p*-Nitrophenol standards.

Standard	*Amount of p-Nitrophenol, nmol**
S1	0
S2	12.5
S3	25
S4	50
S5	100

* 1 nmol = 1 nanomole = 1 x 10^{-9} mol = 0.000000001

Data Analysis and Interpretation of Results

Before you began this investigation you generated an investigation question with your group, described procedural steps to answer that question, and predicted your results. Carefully analyzing the data you've collected will determine whether your ideas were accurate or require revision. You may choose to conduct your analysis in different ways. Remember that in Investigation 1 you generated a standard curve, graphed the amount of product formed given absorbance readings, graphed the amount of product formed at specific time points, and calculated the initial rate of the enzymatic reaction. These data analysis steps, and others you find useful, should be considered in this section.

Decide with your group which data analysis strategies will be most useful in answering your investigation question and carry out your analysis in the space below. Hint: this may include recording and graphing your data as in Investigation 1.

2.4 What do your results mean with regard to your investigation question?

2.5 Do your results support or refute your initial predictions? Explain your answer.

2.6 Another classmate reviews your data and states that you cannot know for sure from these data what the optimal temperature is for cellobiase to produce glucose. What reasoning might your classmate use to support her statement?

2.7 Remember that this investigation was designed to begin to answer the overarching research question: **Under what conditions does mushroom cellobiase generate glucose most efficiently?** How do the results of this investigation add to those of Investigation 1? Recall that this is a model system representing cellobiose conversion to glucose.

2.8 Look back at your model from the pre-lab describing the effect of temperature on reaction rate (page 7). How might you revise your model given the data you collected in this investigation? Be sure to include ideas relevant to structure and function in your revisions. Draw and describe your ideas below:

Investigation 3: Determine the Effect of pH on Reaction Rate

The presence of charge on enzymes and their substrates can strongly affect their interaction. Oppositely charged groups between the enzyme and substrate may increase the rate of the reaction. However, with changes in pH, it is possible that the positively and negatively charged groups can increase or decrease their net (total) charge. Changes in charge on the enzyme, substrate, or both can prevent the two from interacting and product from forming. Just as enzymes may work most rapidly within a specific temperature range, they may be most active within a particular pH range. 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 work only in neutral to basic conditions (pH 7.2–9.0). For this investigation, all the reagents and equipment from Investigation 1 will be made available to you as well as purified cellobiase and pH adjustment buffers at pH 5.0, 6.3, and 8.6.

Student Workstations		*Quantity*
☐	3.0 mM substrate	1
☐	Low-concentration enzyme (0.18 U/ml)	1
☐	Stop solution	1
☐	Colorimetric standards (S1–S5) in cuvettes from Investigation #1	1 of each
☐	pH adjustment buffer in 1.5 ml microcentrifuge tubes (labeled pH 5.0, pH 6.3, and pH 8.6)	1 of each
☐	DPTPs	4
☐	Cuvettes	3
☐	Marker	1
☐	Beaker with deionized or distilled water to rinse DPTPs	1
☐	Stopwatch or timer	1
☐	Experimental Design and Planning Worksheet (optional)	1
☐	Spectrophotometer (optional)	1

Pre-Investigation Questions

In this investigation you will design an experimental protocol to answer your questions and test your ideas about the effect of pH on enzymatic reaction rate.

3.1 With your group, determine your **investigation question:**

3.2 Given what you learned about working with enzyme reactions in Investigation 1, what **procedural steps** will you take in order to answer your investigation question? Look back at the protocol and data analysis sections of Investigation 1 for ideas and draw and/or describe your steps below. Don't forget to include an experimental control.

Note*: The concentration of substrate used in this investigation is different from the one used in previous investigations. The substrate you should be using for this investigation is 3.0 mM to account for the dilution step that occurs when you add the pH adjustment buffers.*

3.3 **Predict your results.** In the space below draw and/or describe your expected results. A hypothesis is helpful in thinking about what you anticipate will occur during an investigation and should be revisited after you've collected your data. Before you begin, have your teacher review your protocol.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

What causes molecules, including enzymes, to have an overall charge?

How can a change in pH affect the charge of a molecule?

What are some consequences of changing the overall charge of a molecule, such as an enzyme?

Data Collection

Record any results that are relevant to answering your investigation question. You may find it helpful to use a separate sheet of paper to create tables, similar to those in Investigation 1, in which to record data. As in Investigation 1, the standards provided to you contain a known amount of product (Table 1).

Table 1. *p*-Nitrophenol standards.

Standard	*Amount of p-Nitrophenol, nmol**
S1	0
S2	12.5
S3	25
S4	50
S5	100

* 1 nmol = 1 nanomole = 1 x 10^{-9} mol = 0.000000001

Data Analysis and Interpretation of Results

Before you began this investigation you generated an investigation question with your group, described procedural steps to answer that question, and predicted your results. Carefully analyzing the data you've collected will determine whether your ideas were accurate or require revision. You may choose to conduct your analysis in different ways. Remember that in Investigation 1 you generated a standard curve, graphed the amount of product formed at given absorbance readings, graphed the amount of product formed at specific time points, and calculated the initial rate of the enzymatic reaction. These data analysis steps, and others you find useful, should be considered in this section.

Decide with your group which data analysis strategies will be most useful in answering your investigation question and carry out your analysis in the space below. Hint: this may include recording and graphing your data as in Investigation 1.

3.4 What do your results mean with regard to your investigation question?

3.5 Do your results support or refute your initial predictions? Explain your answer.

3.6 Another classmate reviews your data and states that you cannot know for sure from these data what the optimal pH is for cellobiase to produce glucose. What reasoning might your classmate use to support her statement?

3.7 Remember that this investigation was designed to help answer the overarching research question: **Under what conditions does mushroom cellobiase generate glucose most efficiently?** How do the results of this investigation add to those of Investigation 1? Recall that this is a model system representing cellobiose conversion to glucose.

3.8 Look back at your model from the pre-lab describing the effect of changing pH on reaction rate (page 7). How might you revise your model given the data you collected in this investigation? Be sure to include ideas relevant to molecular charge in your revisions. Draw your ideas below:

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

For this investigation 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 were just one squirrel, it would take it a while to move from tree to tree and gather up all the nuts. However, given enough time, the squirrel would make it to all the trees and find and gather all the nuts. Now 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 a similar effect. Initially, when there is plenty of substrate, the reaction will go faster in the presence of more enzymes. However, both a reaction with a lot of enzyme and a reaction with less enzyme have the same amount of substrate to work on (nuts to find), and all the substrate will be converted to product given enough time.

For this investigation, all the reagents and equipment from Investigation 1 will be made available to you as well as purified cellobiase.

Student Workstations		*Quantity*
☐	1.5 mM substrate	1
☐	High-concentration enzyme (0.7 U/ml)	1
☐	Buffer	1
☐	Stop solution	1
☐	Colorimetric standards (S1–S5) in cuvettes from Investigation #1	1 of each
☐	DPTPs	4
☐	Cuvettes	6
☐	Marker	1
☐	Beaker with deionized or distilled water to rinse DPTPs	1
☐	Stopwatch or timer	1
☐	Experimental Design and Planning Worksheet (optional)	1
☐	Spectrophotometer (optional)	1

Pre-Investigation Questions

In this investigation you will design an experimental protocol to answer your questions and test your ideas about the effect of enzyme concentration on enzymatic reaction rate.

4.1 With your group, determine your **investigation question**:

4.2 Given what you learned about working with enzyme reactions in Investigation 1, what **procedural steps** will you take to answer your investigation question? Look back at the protocol and data analysis sections for Investigation 1 for ideas and draw and/or describe your steps below. Don't forget to include an experimental control.

4.3 **Predict your results.** In the space below draw and/or describe your expected results. A hypothesis is helpful in thinking about what you anticipate will occur during an investigation and should be revisited after you've collected your data. Before you begin, have your teacher review your protocol.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

How do enzymes reduce activation energy of a reaction?

Two students are debating what will happen when more enzyme is added to a reaction. Student A states that as more enzyme is added, the activation energy of the reaction will continue to decrease. Student B states that as more enzyme is added, the reaction will occur more quickly because there are many more enzyme molecules to interact with substrate. Which student do you agree with and why? Why do you disagree with the other student?

Name and describe one limiting factor that can prevent the continued production of glucose in this reaction.

Data Collection

Record any results that are relevant to answering your investigation question. You may find it helpful to use a separate sheet of paper to create tables, similar to those in Investigation 1, in which to record data. As in Investigation 1, the standards provided to you contain a known amount of product (Table 1).

Table 1. *p*-Nitrophenol standards.

Standard	*Amount of p-Nitrophenol, nmol**
S1	0
S2	12.5
S3	25
S4	50
S5	100

* 1 nmol = 1 nanomole = 1 x 10^{-9} mol = 0.000000001

Data Analysis and Interpretation of Results

Before you began this investigation you generated an investigation question with your group, described procedural steps to answer that question and predicted your results. Carefully analyzing the data you've collected will determine whether your ideas were accurate or require revision. You may choose to conduct your analysis in different ways. Remember that in Investigation 1 you generated a standard curve, graphed the amount of product formed given absorbance readings, graphed the amount of product formed at specific time points, and calculated the initial rate of the enzymatic reaction. These data analysis steps, and others you find useful, should be considered in this section.

Decide with your group which data analysis strategies will be most useful in answering your investigation question and carry out your analysis in the space below. Hint: this may include recording and graphing your data as in Investigation 1.

4.4 What do your results mean with regard to your investigation question?

4.5 Do your results support or refute your initial predictions? Explain your answer.

4.6 Another classmate reviews your data and notes that you cannot know for sure from these data what the optimal concentration of cellobiase is to produce glucose. What reasoning might your classmate use to support her statement?

4.7 Remember that this investigation was designed to help answer the overarching research question: **Under what conditions does mushroom cellobiase generate glucose most efficiently?** How do the results of this investigation add to those of Investigation 1? Recall that this is a model system representing cellobiose conversion to glucose.

4.8 Look back at your model from the pre-lab describing the effect of changing enzyme concentration on reaction rate (page 7). How might you revise your model given the data you collected in this investigation? Describe and/or draw your ideas below:

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

For this investigation, you will determine the effect that changing the substrate concentration has on the initial rate of the reaction. We can 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 in a specific amount of 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 quickly and, given enough time, 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 the same amount of time, they would still gather fewer nuts.

Decreasing the substrate concentration has an analogous effect. The rate of reaction should be slower because it will be harder for the enzyme 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 will be lower.

For a more complex analysis of the effects of varying substrate concentration, please see Appendix A. For this investigation, all the reagents and equipment from Investigation 1 will be made available to you as well as purified cellobiase.

Student Workstations		***Quantity***
☐	1.5 mM substrate	1
☐	Enzyme	1
☐	Buffer	1
☐	Stop solution	1
☐	Colorimetric standards (S1–S5) in cuvettes from Investigation #1	1 of each
☐	15 ml conical tubes	2
☐	DPTPs	4
☐	Cuvettes	6
☐	Marker	1
☐	Beaker with deionized or distilled water to rinse DPTPs	1
☐	Stopwatch or timer	1
☐	Experimental Design and Planning Worksheet (optional)	1
☐	Spectrophotometer (optional)	1

Pre-Investigation Questions

In this investigation you will design an experimental protocol to answer your questions and test your ideas about the effect of enzyme concentration on enzymatic reaction rate.

5.1 With your group, determine your **investigation question**:

5.2 Given what you learned about working with enzyme reactions in Investigation 1, what **procedural steps** will you take to answer your investigation question? Look back at the protocol and data analysis sections of Investigation 1 for ideas and draw and/or describe your steps below. Don't forget to include an experimental control:

5.3 **Predict your results**. In the space below draw and/or describe your expected results. A hypothesis is helpful in thinking about what you anticipate will occur during an investigation and should be revisited after you've collected your data. Before you begin, have your teacher review your protocol.

ThINQ! Exercises

Collaborate and use outside resources to answer the following question:

Given a fixed amount of enzyme, how might increasing the amount of substrate in a reaction tube affect the rate of an enzymatic reaction?

Data Collection

Record any results that are relevant to answering your investigation question. You may find it helpful to use a separate sheet of paper to create tables, similar to those used in Investigation 1, in which to record data. As in Investigation 1, the standards provided to you contain a known amount of product (Table 1).

Table 1. *p*-Nitrophenol standards.

Standard	*Amount of p-Nitrophenol, nmol**
S1	0
S2	12.5
S3	25
S4	50
S5	100

* 1 nmol = 1 nanomole = 1 x 10^{-9} mol = 0.000000001

Data Analysis and Interpretation of Results

Before you began this investigation you generated an investigation question with your group, described procedural steps to answer that question, and predicted your results. Carefully analyzing the data you've collected will determine whether your ideas were accurate or require revision. You may choose to conduct your analysis in different ways. Remember that in Investigation 1 you generated a standard curve, graphed the amount of product formed based on absorbance readings, graphed the amount of product formed at specific time points, and calculated the initial rate of the enzymatic reaction. These data analysis steps, and others you find useful, should be considered in this section.

Decide with your group which data analysis strategies will be most useful in answering your investigation question and carry out your analysis in the space below. Hint: this may include recording and graphing your data as in Investigation 1.

5.4 What do your results mean with regard to your investigation question?

5.5 Do your results support or refute your initial predictions? Explain your answer.

5.6 Another classmate reviews your data and notes that you cannot know for sure from these data what the optimal concentration is for substrate to produce glucose. What reasoning might your classmate use to support her statement?

5.7 Remember that this investigation was designed to help answer the overarching research question: **Under what conditions does mushroom cellobiase generate glucose most efficiently?** How do the results of this investigation add to those of Investigation 1? Recall that this is a model system representing cellobiose conversion to glucose.

5.8 Look back at your model from the pre-lab describing the effect of changing substrate concentration on reaction rate (page 7). How might you revise your model given the data you collected in this investigation? Draw or write your ideas below:

Assignment: Investigations 2–5 Wrap-Up

The data generated during Investigations 2–5 provide insight about the optimal conditions required for purified cellobiase to produce glucose. Using the results from each investigation (2–5), write your response to the research question: **Under what conditions is glucose generated most efficiently from mushroom cellobiase?**

Investigations 2–5 tested one variable each (temperature, pH, enzyme concentration, and substrate concentration) to determine the optimal conditions for purified cellobiase activity. However, during biofuel production all of the optimal conditions must be compatible with each other. Therefore, it is important to set up a final investigation that examines cellobiase activity using your best condition from each single-variable investigation (2–5).

Design an investigation question for this final experiment and develop a protocol to answer your question.

Investigation 6: Combine Results and Test an Optimized Protocol

Testing one variable at a time can be useful for scientists trying to understand how a system works. But when dealing with complex systems, it is often the case that variables interact with one another to generate unique outcomes. Therefore, it is important to understand not only how each variable works independently within a system (like in Investigations 2–5), but also how they interact with one another.

In this investigation you will pool results from your previous investigations and test the optimal conditions from each investigation together in one reaction. For this investigation, all the reagents and equipment from Investigations 2–5 will be made available to you.

Student Workstations		*Quantity*
☐	3 mM substrate	1
☐	High-concentration enzyme (0.7 U/ml)	1
☐	1x stop solution	1
☐	Buffer	1
☐	DPTPs	3
☐	15 ml conical tubes	1
☐	Colorimetric standards (S1–S5) in cuvettes	1 of each
☐	Cuvettes	6
☐	Marker	1
☐	Stopwatch or timer	1
☐	Beaker with water	1
☐	Experimental Design and Planning Worksheet (optional)	1
☐	Spectrophotometer (optional)	1
☐	Heat source for water (optional)	1

Pre-Investigation Questions

In this investigation you will design an experimental protocol to answer your questions and test your ideas about the effect of combining optimal conditions of several factors for cellobiase activity on enzymatic reaction rate.

6.1 With your group, determine your **investigation question**:

6.2 Given what you learned about working with purified cellobiase during Investigations 2–5, what **procedural steps** will you take in order to answer your investigation question? Look back at the protocol and data analysis sections for Investigation 1 for ideas and draw and/or describe your steps below.

6.3 **Predict your results**. In the space below draw and/or describe your expected results. A hypothesis is helpful in thinking about what you anticipate will occur during an investigation and should be revisited after you've collected your data. Before you begin, have your teacher review your protocol.

Data Collection

Record any results that are relevant to answering your investigation question. You may find it helpful to use a separate sheet of paper to create tables, similar to those in Investigation 1, in which to record data. As in Investigation 1, the standards provided to you contain a known amount of product (Table 1).

Table 1. *p*-Nitrophenol standards.

Standard	*Amount of p-Nitrophenol, nmol**
S1	0
S2	12.5
S3	25
S4	50
S5	100

* 1 nmol = 1 nanomole = 1 x 10^{-9} mol = 0.000000001

Data Analysis and Interpretation of Results

Before you began this investigation you generated an investigation question with your group, described procedural steps to answer that question, and predicted your results. Carefully analyzing the data you've collected will determine whether your ideas were accurate or require revision. You may choose to conduct your analysis in different ways. Remember that in previous investigations you generated a standard curve, graphed the amount of product formed given absorbance readings, graphed the amount of product formed at specific time points, and calculated the initial rate of the enzymatic reaction. These data analysis steps, and others you find useful, should be considered in this section.

Decide with your group which data analysis strategies will be most useful in answering your investigation question and carry out your analysis in the space below. Hint: this may include recording and graphing your data as in Investigation 1.

6.4 What do your results mean with regard to your investigation question?

6.5 Is it possible to compare the results of this investigation with those of Investigation 1 (mushroom extract)? Explain your answer.

6.6 Given your response to question 6.4 above, what is your best response to the research question of this lab: **Under what conditions does mushroom cellobiase generate glucose most efficiently?** Hint: remember that this investigation uses a model system that is similar but not identical to the cellobiase reaction taking place in mushrooms.

6.7 Look back at your model from the pre-lab describing the effect of varying conditions on enzyme function (page 7). How might you create a new model given the data you collected in this investigation? Describe and/or draw your ideas below:

Post-Lab Questions

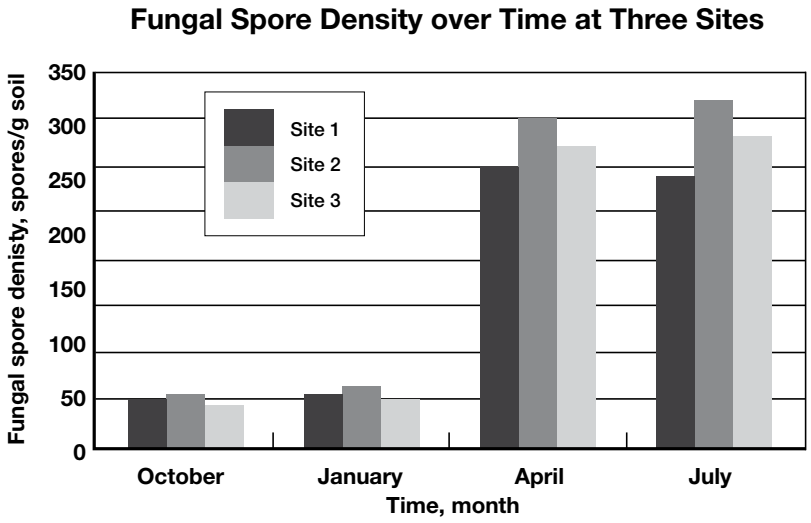
The Polynesian Islands, in the south central Pacific Ocean, are home to a variety of food crops from other continents. The sweet potato, a native plant of South America, was brought to the islands and became a food crop before Columbus traveled to the Americas. Sweet potatoes become sweet because of a special enzyme, called **beta-amylase** that breaks down starches the potatoes produce into sugars. The enzyme's action can be inhibited by things like heavy metals found in water and soil, certain types of bacteria and fungi, and plant viruses.

Last January, a typhoon battered the Polynesian Islands, dumping 12 inches of rain on the islands in less than 24 hours. Fortunately, much of the sweet potato crop was saved by local villagers who removed debris and standing water from the fields. However, at the next harvest, 4 months after the typhoon, the farmers noticed a change in their crops. The potatoes were no longer sweet! In fact their taste was dull and starchy. The farmers wondered what could have caused such a significant change in flavor.

CLAIM: Something in the environment was inhibiting the formation of sugars in the sweet potatoes.

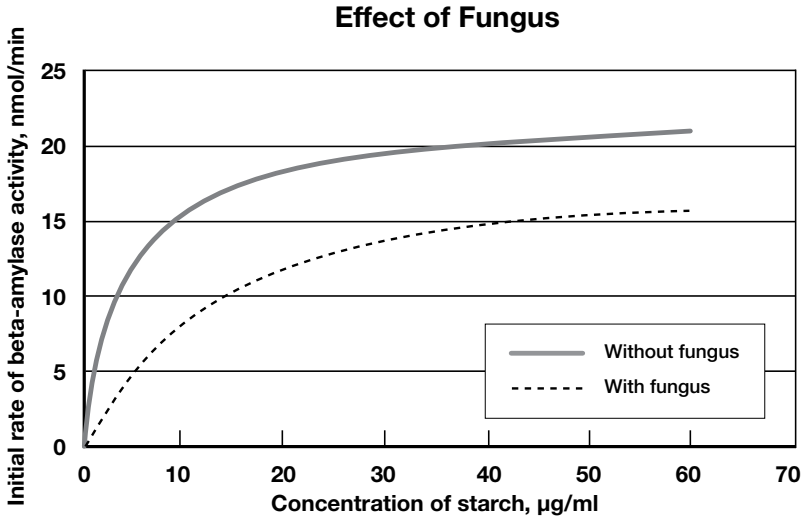
HYPOTHESIS 1: The farmers hypothesized that the something in the flood waters affected the growth, and thus the taste, of the potatoes.

EVIDENCE: The farmers called plant scientists at the local university to inspect the odd-tasting sweet potatoes. These botanists discovered a fungus growing on the skin of the tubers and the green parts of the potato plants. Researching the fungus, they found that it was common in the soil of the islands. The botanists analyzed data on fungal spore density that had been collected from three sites across the islands before and after the typhoon occurred. The data the scientists analyzed are shown in the graph below.



HYPOTHESIS 2: The botanists hypothesized that the fungus caused the change in flavor of the potatoes.

EVIDENCE: The scientists suspected that something in the fungus could be inhibiting the function of beta-amylase, ultimately preventing starches from turning into sugars in the sweet potatoes. So they conducted an experiment to determine the enzyme's rate of reaction in the presence and absence of the fungus. Their results are shown below:



EXPLAIN: Do you agree with the botanists' hypothesis regarding the potato fungus? Justify your answer using the evidence in the graph.

PREDICT: Given what you know about how enzymes work, how do you think the fungus may be inhibiting the function of the enzyme?

DESIGN: Describe what evidence you would need to collect in order to prove your prediction. What would your next research question be? What steps would you take in order to investigate how the fungus could be inhibiting the enzyme's function?

Appendix A

Enzyme Kinetics

How Enzymatic Rate Changes with Changing Substrate Concentration

If you were in charge of a cellulosic biofuels manufacturing plant, you would want to ensure that the enzyme you were using was the most efficient one available for the job. To compare how well one enzyme performs relative to another, you would want to compare the enzymatic investigation of the different enzymes under a given set of conditions. Enzymatic investigation, or how well an enzyme catalyzes a reaction, can be expressed in terms of the maximum rate of a reaction (V_{max}) and a second term that describes the efficiency of the conversion of substrate to product, which is called the Michaelis constant (K_m).

Theoretically, an enzyme can work at its maximum rate (V_{max}, also known as maximum velocity) when its active sites are completely saturated with substrate. This would occur only at extremely high substrate concentrations, when there is a negligible time delay in the enzyme binding to its substrate. However, the maximum velocity of the enzyme cannot be experimentally measured because it can occur only when substrate is constantly available for every active site of every enzyme molecule. In reality, there is always a little time delay required for the enzyme to find its substrate in solution.

A parameter that you can determine experimentally is the initial rate of the reaction when different starting substrate concentrations are used, as you did in Investigation 5. If you plot the initial velocity of the reaction vs. the concentration of substrate, you will generate a graph similar to the one shown in Figure 1.

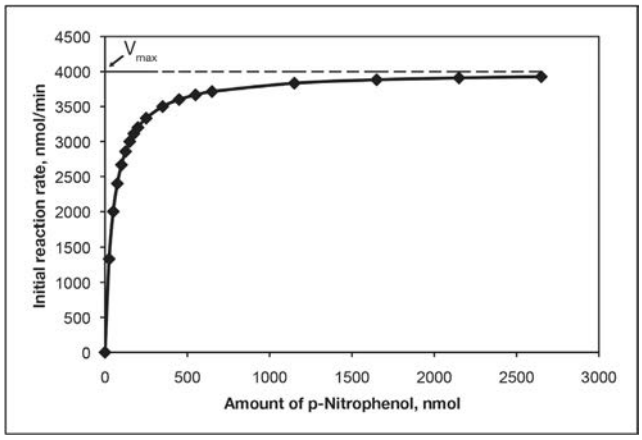


Fig. 1. The relationship between the initial velocity of an enzymatic reaction and the substrate concentration available at the beginning of the reaction. V_{max} is approached but never achieved at extremely high substrate concentrations.

Since V_{max} cannot be determined experimentally, it is useful instead to determine a mathematical relationship between V_{max} and factors that can be determined or controlled experimentally, namely the initial reaction rate (or initial velocity of the reaction), V_0, and the starting substrate concentration, [S]. The relationship between the maximum velocity (V_{max}), the initial velocity (V_0), the substrate concentration ([S]), and a constant term that is specific to each enzyme-substrate system (K_m) is the **Michaelis-Menten equation** that is represented below.

$$\text{Equation 1:} \quad V_0 = \frac{V_{max} * [S]}{(K_m + [S])}$$

Where

V_0 = the initial rate of reaction at a specific starting substrate concentration
V_{max} = the maximum velocity of the reaction
[S] = concentration of substrate
K_m = Michaelis constant (see derivation below) specific to each enzyme-substrate system

The Michaelis contant (K_m) is another parameter used to describe enzyme function. It is equivalent to the substrate concentration at which the reaction proceeds at half the maximum rate. When we plug an initial rate of reaction (V_0) equal to half the maximum rate of reaction into the Michaelis-Menten equation we get the following:

Equation 2: $$\frac{1}{2} V_0 = \frac{V_{max} * [S]}{(K_m + [S])}$$

Solving for K_m, we get $K_m = [S]$, when $V_0 = \frac{1}{2}V_{max}$. This means that the Michaelis constant is equal to the substrate concentration that results in an initial rate of reaction that is one half that of the maximum rate of reaction.

Deriving the Michaelis-Menten Equation

Where does the Michaelis-Menten equation come from and what does K_m mean in terms of the reactions actually occurring? K_m can also be understood in terms of the rate constants involved in an enzymatic reaction. When an enzyme (E) is added to a substrate (S), an enzyme-substrate complex (ES) is formed. The equilibrium constant for the formation of this complex is described as k_1. The enzyme-substrate complex can result in the formation of product (P) with a rate constant of k_2 and the liberation of E, or the ES complex can fall apart without the substrate being converted to product and a rate constant of k_{-1}.

$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_2} E + P$$

The rate at which product is formed is determined by the concentration of the enzyme substrate complex, [ES]. When there is excess substrate, then the concentration of [ES] is much higher than the concentration of free enzyme [E]. Under these conditions the rate of the reaction is at V_{max}.

In steady-state equilibrium, the rate at which the [ES] is formed is equal to the rate at which it is broken down. Since the rate of any reaction = (the equilibrium constant) * (concentration of reactants), then we can say that:

Equation 3: $$k_1 [E][S] = k_{-1} [ES] + k_2 [ES]$$

This equation can be rearranged algebraically by factoring out the [ES] on the right side of the equation and then dividing both sides of the equation by k–1 + k2, giving the following equation:

Equation 4: $$[ES] = \frac{k_1[E][S]}{k_{-1} + k_2}$$

At any point in time, the fraction of enzyme that is bound to the substrate (F) can be described by the following equation:

Equation 5: $$F = \frac{[ES]}{[ES] + [E]}$$

So if we algebraically combine Equations 4 and 5 (not an easy bit of algebra) we get:

Equation 6: $$F = \frac{[S]}{\{(k_{-1} + k_2)/k_1\} + [S]}$$

Since the fraction of the enzyme that is bound to substrate can be expressed in the following equation:

Equation 7: $V_0 = V_{max} * F$

We can do one more substitution to get:

Equation 8: $$V_0 = \frac{V_{max} * [S]}{\{(k_{-1} + k_2)/ k_1\} + [S]}$$

Compare Equation 8 to the Michaelis-Menten equation (Equation 1):

$$V_0 = \frac{V_{max} * [S]}{(K_m + [S])}$$

Thus, K_m is defined in terms of the rate constants of the reaction. So we have now defined K_m in terms of an actual reaction that is occurring:

Equation 9: $K_m = (k_{-1} + k_2)/k_1$

Making the Plot Linear

Once again, we would like to determine the V_{max} and K_m values for our enzyme so that it can be compared to other enzymes. Since the curve in Figure 1 is hyperbolic, it is difficult to determine exactly where V_{max} is. To solve that problem, taking the inverse of both the Y values (initial rates) and the X values (substrate concentrations) will produce a linear graph called the **Lineweaver-Burk** plot or double reciprocal plot (Figure 2).

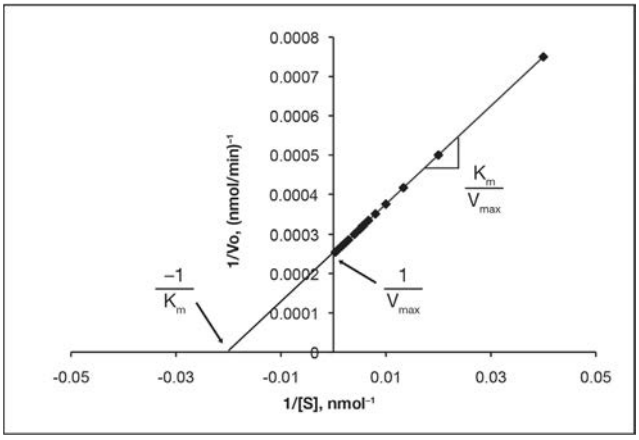


Fig. 2. A Lineweaver-Burk plot. The linear representation of the reaction rate data was used to calculate the Michaelis constant K_m and the maximum reaction rate V_{max}.

There are advantages to having a linear representation of the data. Not only can you see how good your data are (how close to linear they are with a linear regression calculation), but you can also more accurately determine V_{max} and K_m by taking the inverse values of the X and Y intercept. Using the components of this kit and a protocol similar to that in Investigation 5, it is possible to determine the V_{max} and K_m values for the cellobiase provided in the kit.

Appendix B

Biofuels

Types of Biofuels

When the term “biofuels” is used, it generally refers to a group of fuels that are produced from a biological source that was recently living, in contrast to fossil fuels, which are created from biological sources long dead. Current biofuel technologies tend to fall into four main categories: cellulosic biofuels, ethanol production from starch and sugar sources, biodiesels, and syngases. Cellulosic ethanol is primarily produced from the breakdown of cellulose to glucose followed by a fermentation step to ethanol. Another method of ethanol production involves breaking down starches to sugars followed by fermentation to ethanol. Biodiesels are fuels derived from oils, either recycled cooking oils or directly from plants that produce high levels of oils that can then be purified and burned in diesel engines. Work is also being done to genetically modify algae, yeast, and bacteria to produce fatty acids and oils that can be used as biodiesel sources. Syngas stands for synthetic gas and is a mixture of carbon monoxide and hydrogen gases that result from burning biomass. Syngas can be burned directly for power generation or chemically converted to be used in modified diesel engines. The remaining information will primarily deal with cellulosic ethanol but more references on starch-based ethanol production, syngas, and biodiesel can be found in Appendix G.

First-Generation Ethanol Production for Fuel

Currently, much of the ethanol used in the fuel industry is a result of the conversion of starch, such as that found in corn kernels, to sugar, which is then fermented into ethanol. Converting starches to sugar has two chief drawbacks. First, it takes away a food source from people and livestock. Second, a lot of the corn plant goes to waste, since the only polysaccharide that is converted to ethanol is the starch of the corn kernel. Converting the waste products of food plants or plants that are not used as a food or feed source into fuel is a potential method or producing fuel more sustainably. For these reasons, current research into the production of ethanol as a fuel source is concentrating on the use of cellulose rather than starches. However, the technology to produce ethanol from cellulose is much more complicated than that for converting starches. Appendix F has a debate structure to help investigate the future of biofuels.

Are Cellulosic Ethanol Biofuels a Carbon-Neutral Alternative to Petroleum?

Plants convert carbon dioxide from the air into the cellulose of their cell walls. If cellulose is broken down into sugar, converted into fuel, and later burned into carbon dioxide, then the process is neither adding nor removing any carbon dioxide from the biosphere. This is called a “carbon neutral” process. However, converting corn stalks, husks, grasses, or other nonfood plant products to ethanol does require some petroleum. Currently the fertilizers used to grow the crop, the tractors and other farm equipment involved in growing and harvesting, as well as the energy required to process the plants to create the ethanol all either directly or indirectly require petroleum. Experts calculate that it takes 1 gallon of gas to produce 5 gallons of cellulose-derived ethanol (Montenegro 2006).

Burning fossil fuels is not carbon neutral. This carbon was fixed from carbon dioxide millions of years ago. All the carbon in fossil fuels has been essentially locked out of the natural carbon cycle (Martin 2008). Burning fossil fuels adds carbon dioxide to the carbon cycle, which increases the amount of greenhouse gases contributing to the current global climate crisis (Figure 1). If the world could use less petroleum and more materials produced from plants in a sustainable manner, we could reduce greenhouse gas emissions.

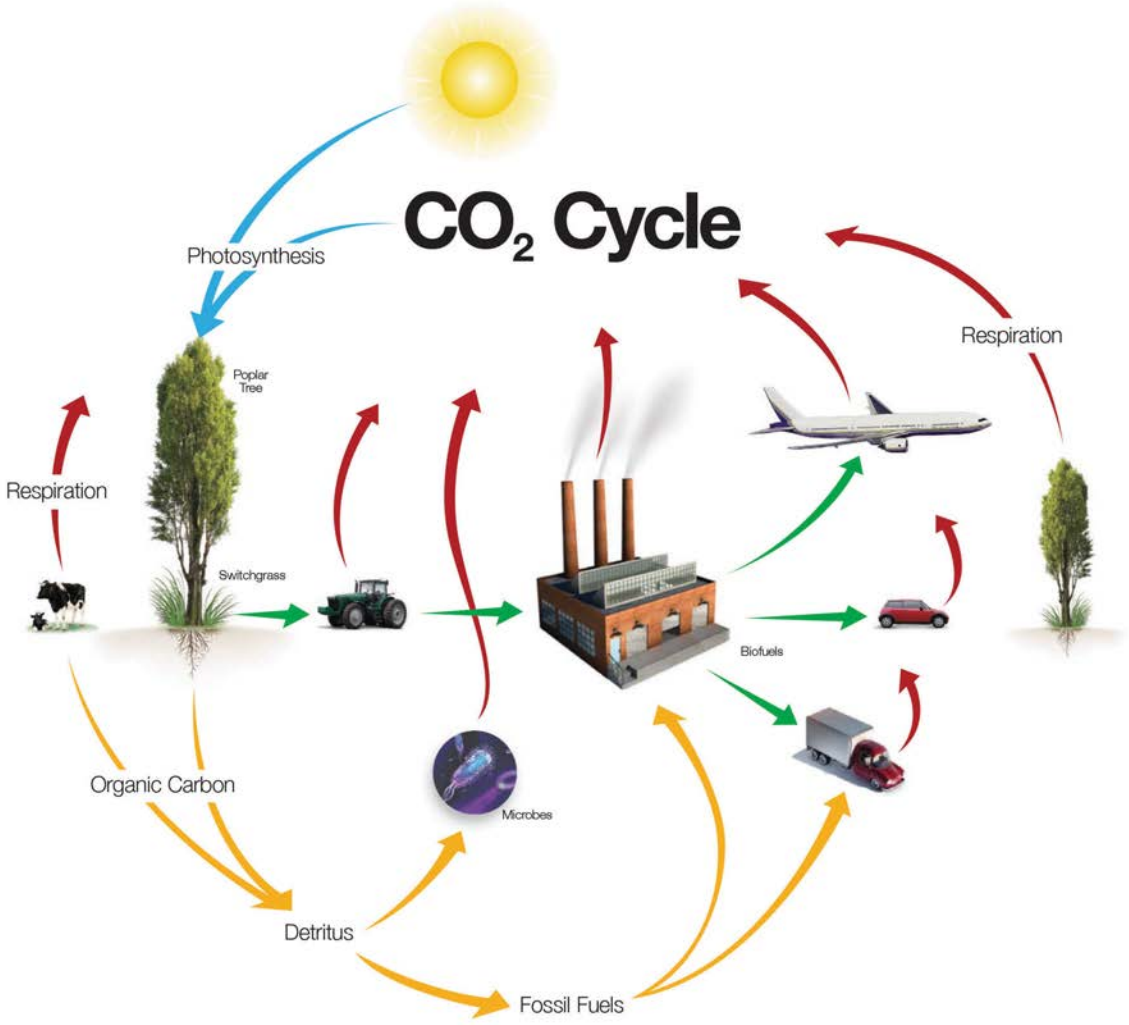


Fig. 1. A carbon cycle, including biofuel production and use. Plants remove carbon dioxide from the atmosphere and in the presence of sunlight they can convert it to complex polysaccharides including cellulose. Plants such as switchgrasses and poplar trees can then be harvested and processed in a cellulosic ethanol plant to produce ethanol to fuel planes, cars, and trucks. However, the tractors/vehicles used to process the switchgrasses, trees, and plants and the vehicles running on cellulosic ethanol still do produce carbon dioxide. Other sources of carbon dioxide are from respiration of animals, burning of fossil fuels, and breakdown of organic matter by microbes. Ideally, carbon dioxide created by the production and processing of biofuels will be reabsorbed by plants used to make the cellulosic ethanol. This is not the case with fossil fuel usage. Fossil fuels contribute only to carbon dioxide production.

Photosynthetically Efficient C4 Plants

Many of the plants that are considered biofuel crops are C4 plants. These plants, such as corn, sugarcane, and switchgrass (native prairie grass) photosynthesize with amazing efficiency, which means that they produce sugar at a much faster rate than other plants. This allows them to grow rapidly and incorporate the sugars into the production of cellulose more quickly than other plants.

What about Nitrogen?

It is also important to maintain the nitrogen cycle in a way that is sustainable and beneficial to the biosphere. If crops are harvested for the production of cellulosic ethanol, the nitrogen that was removed from the soil to become the biomolecules in the crops is lost from the soil. Replacing this loss with inorganic fertilizers has two drawbacks. First, it takes fuel to produce inorganic fertilizer. Second, nitrogen-based greenhouse gases (namely nitrous oxide) are up to 300 times more potent than carbon based greenhouse gases. These nitrogen-based gases are released from soils that have been fertilized with inorganic fertilizer (Smith 2009).

These problems can be solved by using either the wastewater and biomass from the cellulosic fuel plant to fertilize the fields (and thus return the same nitrogen to the soil) or a nitrogen-fixing crop such as alfalfa as a cover crop. Alfalfa, which is grown routinely as a feed crop for cattle, has the advantage over other grasses of being able to fix nitrogen from the air through a symbiotic relationship with bacteria living in its roots. Since alfalfa obtains nitrogen from the air, no nitrogen-based fertilizers are needed. In the future, both carbon and nitrogen could be recycled through the use of biofuels.

Engineering Enzymes for Hydrolysis

Ideally, the process required to produce ethanol from cellulose should be optimized to produce the largest amount of ethanol with the least amount of energy and at the lowest cost. Much work is being done to improve the efficiency of the enzymes used to break down cellulose. Since the process of producing sugars from plant material requires high temperatures or extreme pH, the enzymes involved in cellulosic ethanol production have to be able to function under these conditions. Scientists can use different methods to produce enzymes that work efficiently under extreme conditions.

One method is to find organisms, such as bacteria or fungi, that live in similar conditions and produce the desired enzymes. Hot springs, acidic bogs, or alkaline soda lakes (Tiago et al. 2004) are a wonderful place to find microbes that produce enzymes that function at high temperatures, low pH, or high pH, respectively. Another method is to mutate microbes with ultraviolet light or chemicals and select for those that produce enzymes with the desired characteristics. It is also possible to engineer a mutation only in the gene of interest by cloning the gene that codes for the cellulase enzyme of interest, and then copying it by a technique called "error prone PCR (polymerase chain reaction)." This will produce an assortment of mutations that can be ligated into plasmids and transformed into bacteria. The transformed bacteria can be screened for cellulases that work in the desired conditions (Arnold and Georgiou 2003).

Microbial Fermentation

Once glucose is produced from the enzymatically treated cellulose, it still needs to be processed into fuel. The most common fuel that is produced from glucose is ethanol. Yeast is an organism that is very efficient at converting sugar to ethanol in a process called alcohol fermentation. Bacteria such as *Zymomonas mobilis* and *Escherichia coli* are being engineered to efficiently produce ethanol from glucose. If hemicellulose is not separated from the cellulose after initial treatment of the plant sources, five-carbon sugars such as xylose and arabinose can be produced from the hemicellulose. Other organisms that have enzymatic pathways for these five-carbon sugars have been utilized to convert them to ethanol. Bio-engineered yeast strains that can metabolize all the sugars produced in the breakdown of plants are being developed as well.

Practicality of Ethanol as Fuel

Vehicles can run on 85–100% ethanol. In Brazil, most city buses run solely on ethanol. Many cars that use traditional gasoline can be converted to use fuel that is a mixture of 85% ethanol and 15% petroleum (E85).

Appendix C

Measuring the Amount of *p*-Nitrophenol Produced Using the Bio-Rad SmartSpec™ Plus Spectrophotometer

Operation of the SmartSpec Plus spectrophotometer is easy and intuitive. Brief instructions have been provided below. For more detailed information, please refer to the SmartSpec Plus spectrophotomer instruction manual. Text shown below in boxes is the text seen in the data window of the SmartSpec Plus spectrophotometer. The cuvettes must be inserted in the correct orientation to obtain a proper reading.

1. Turn the SmartSpec Plus spectrophotometer on and press λ.

   Enter number (1–3) of
   wavelengths to read: 1

   Type **1** and press **Enter**.

2. Choose the wavelength.

   Enter wavelength:
   _____nm

   Type **410** and press **Enter**.

3. Turn background subtraction off.

   Do you want to subtract
   background reading? **NO**

   Select **NO** and press **Enter**.

4. Place cuvette with the S1 (0 nmol *p*-Nitrophenol) standard into the chamber with the smooth side facing the light path.

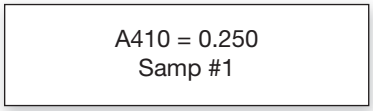
   Ready to read absorbance
   <= Exit Assay >= Options

   Press **Read Blank**.

   A410 = 0.00
   >=continue

   Press right arrow  to continue.

5. Remove the cuvette that contains the S1 standard and place the S2 standard cuvette in the chamber with the smooth side facing the light path.



Press **Read Sample**.

The A410 value will vary with the preparation of your standards, and if you have done more readings, then the Samp # will reflect this.

Record the A410 value. This is the absorbance that corresponds with 25 nmol of *p*-Nitrophenol.

6. Remove the S2 standard cuvette from the chamber and follow step 5 to read the remaining standards (S3–S5).

Standard	*Absorbance at 410 nm*	*Concentration, nmol/ml*
S1	0.000	0
S2		25
S3		50
S4		100
S5		200

Appendix D

Measuring the Amount of *p*-Nitrophenol in a Sample Using the Vernier SpectroVis Spectrophotometer

Using the Computer Procedure

1. Connect the spectrophotometer to your computer using a USB cable. Launch the Logger Pro software. Once the software is open, choose **New** from the File menu.

2. The S1 cuvette will serve as your blank. To correctly use cuvettes, remember to:

    a. Wipe the outside of each cuvette with a lint-free tissue.

    b. Handle cuvettes only by the top edge of the ribbed sides.

    c. Dislodge any bubbles by gently tapping the cuvette on a hard surface.

    d. Always position the cuvette so the light passes through the clear sides.

3. Calibrate the spectrophotometer

    a. Place the S1 cuvette into the cuvette slot of the spectrophotometer

    b. Choose **Calibrate Spectrometer** from the Experiment menu. The calibration dialog box will display the message **Waiting 60 seconds for lamp to warm up**. After 60 seconds, the message will change to **Warmup complete**.

    c. Select **Finish Calibration**. When the **Finish Calibration** button is grayed out, click **OK**.

4. Determine the optimum wavelength for examining the absorbance of *p*-Nitrophenol.

    a. Remove the cuvette from the spectrophotometer. Place the S5 cuvette into the spectrophotometer.

    b. Click **Collect**. A full-spectrum graph of the solution will be displayed. Note that one area of the graph contains an absorbance peak. Click **Stop** to complete the analysis.

    c. To save the graph of absorbance vs. wavelength, select **Store Latest Run** from the Experiment menu.

    d. To set up the data collection mode and select a wavelength for analysis, click the **Configure Spectrometer Data Collection icon**, (), on the toolbar.

    e. Click **Abs vs. Concentration** under Set Collection Mode. The wavelength of maximum absorbance (λ max) will be selected. Deselect any wavelengths below 410 nm that have been autoselected. Select the wavelength closest to 410 nm.

    f. Enter **Amount** for the column name. Enter **Amt**. for the short name. Enter nmol as the units.

    g. Click **OK**. Remove the cuvette from the spectrophotometer.

5. You are now ready to collect absorbance data for the standards. Click **Collect**. Find the S1 cuvette. Wipe the outside with a tissue and place it in the device. Wait for the absorbance value displayed on the monitor to stabilize. Then click **Keep**, type **0** in the edit box, and press **ENTER**. The data pair you just collected will now be plotted on the graph. Remove the cuvette from the device.

6. Wipe the outside of the cuvette labeled S2 and place it in the device. When the absorbance value stabilizes, click **Keep**, type **12.5** in the edit box, and press **ENTER**.

7. Wipe the outside of the cuvette labeled S3 and place it in the device. When the absorbance value stabilizes, click **Keep**, type **25** in the edit box, and press **ENTER**.

8. Wipe the outside of the cuvette labeled S4 and place it in the device. When the absorbance value stabilizes, click **Keep**, type **50** in the edit box, and press **ENTER**.

9. Wipe the outside of the cuvette labeled S5 and place it in the device. When the absorbance value stabilizes, click **Keep**, type **100** in the edit box, and press **ENTER**. When you have finished click **Stop**.

10. In the **Quantitative Analysis of Amount of Product Formed** section of the instruction manual, record the absorbance for each of your standards S1–S5 in Table 3.

11. Examine the graph of absorbance vs. concentration.

    a. To see whether the curve represents a direct relationship between these two variables, click  **Linear Fit**. A best-fit linear regression line will be shown for your data points. This line should pass through or near the data points.

    b. Print a copy of this graph to be used as your standard curve. You can also record the slope and y-intercept of the line and use the linear fit equation to calculate the concentrations of your reaction time points from the absorbance values.

12. You are now ready to measure the absorbance values of the reaction time points. Measure the absorbance values of the cuvettes and record them in Table 4. Use the standard curve to graphically calculate the amount of *p*-Nitrophenol in your reaction samples or carry out this calculation algebraically. See **Quantitative Determination of Amount of Product Formed** in Investigation 1 for more information.

Using the LabQuest Procedure

1. Connect the spectrophotometer to the LabQuest device using a USB cable. Choose **New** from the File menu.

2. The S1 cuvette will serve as your blank. To correctly use cuvettes, remember to:

    a. Wipe the outside of each cuvette with a lint-free tissue

    b. Handle cuvettes only by the top edge of the ribbed sides

    c. Dislodge any bubbles by gently tapping the cuvette on a hard surface

    d. Always position the cuvette so the light passes through the clear sides

3. Calibrate the spectrophotometer.

    a. Place the S1 cuvette in the spectrophotometer.

    b. Choose **Calibrate** from the Sensors menu. The following message is displayed: **Waiting 60 seconds for lamp to warm up**. After 60 seconds, the message will change to **Warmup complete**.

    c. Select **Finish Calibration**. When the message **Calibration completed** appears, select **OK**.

4. Determine the optimum wavelength for examining the absorbance of *p*-Nitrophenol.

    a. Remove the cuvette from the spectrophotometer. Place the cuvette labeled S5 in the spectrophotometer.

b. Start data collection by clicking **Start** on the lower left corner of the screen. A full-spectrum graph of the solution will be displayed. Stop data collection by clicking **Stop** on the lower left corner of the screen. The wavelength of maximum absorbance (λ max) is automatically identified and displayed in the lower right corner of the screen. Verify that the maximum absorbance is close to 410 nm. Select to read at around 410 nm by tapping on the displayed curve until the value displayed in the lower right corner is 410 nm ± 1 nm.

c. Tap the **Meter** tab in the upper left-hand corner of the screen. On the Meter screen, tap **Mode**. Change the mode to **Events with Entry**.

d. Enter the name as **Amount** and the units as **nmol**. Select **OK**. Remove the cuvette from the spectrophotometer.

5. You are now ready to collect absorbance data for the standards. Start data collection by tapping **Start** in the lower left corner. Wipe the outside of the cuvette labeled S1 with a tissue and place it in the device. Wait for the absorbance value to stabilize. Tap **Keep** and enter **0** as the concentration. Select **OK**. The absorbance and concentration values have now been saved for the first solution. Remove the cuvette from the device.

6. Wipe the outside of the cuvette labeled S2 and place it in the device. When the absorbance value stabilizes, tap **Keep**, enter **12.5** as the concentration, and select **OK**.

7. Wipe the outside of the cuvette labeled S3 and place it in the device. When the absorbance value stabilizes, tap **Keep**, enter **25** as the concentration, and select **OK**.

8. Wipe the outside of the cuvette labeled S4 and place it in the device. When the absorbance value stabilizes, tap **Keep**, enter **50** as the concentration, and select **OK**.

9. Wipe the outside of the cuvette labeled S5 and place it in the device. When the absorbance value stabilizes, tap **Keep**, enter **100** as the concentration, and select **OK**. When you have finished, stop data collection by tapping **Stop** in the lower left corner.

10. To examine the data pairs on the displayed graph, tap any data point. As you tap each data point, the absorbance and concentration values are displayed to the right of the graph. In the **Quantitative Determination of Amount of Product Formed** section of this instruction manual, record the absorbance for each of the standards S1–S5 in Table 3.

11. Display a graph of absorbance vs. concentration with a linear regression curve.

    a. Choose **Curve Fit** from the Analyze menu.

    b. Select **Linear** as the Fit Equation. The linear-regression statistics for these two data columns are displayed for the equation in the form

    $y = mx + b$

    where x is concentration, y is absorbance, m is the slope, and b is the y-intercept.

    c. Select **OK**. A best-fit linear regression line will be shown for your data points. This line should pass through or near the data points.

    d. Print a copy of this graph to use as your standard curve. You can also record the slope and y-intercept of the line and use the linear fit equation to calculate the concentrations of your reaction time points from the absorbance values.

12. You are now ready to measure the absorbance values of your reaction time points. Measure the absorbance values of your cuvettes and record the absorbance values in Table 4. Use your standard curve to graphically calculate the amount of *p*-Nitrophenol in your reaction samples or carry over this calculation algebraically. See **Quantitative Analysis of Amount of Product Formed** in Investigation 1 of your instruction manual for more information.

Appendix E

Glossary of Terms

β-**glucosidase**: An enzyme that breaks apart the β 1–4 bonds that link two glucose molecules of cellobiose, as well as other short polymeric chains of β 1–4 bonded glucose. These enzymes are stereospecific, meaning that they cannot also break apart α 1–4 bonds.

Cellobiase: A β-glucosidase enzyme that specifically breaks apart the β 1–4 bonds of the two glucose molecules of cellobiose.

Cellobiose: A molecule composed of two glucose molecules that are covalently linked with the β 1–4 bond.

Cellulose: A polymer that consists of multiple chains of D-glucose linked together at the β 1–4 groups. The long polymer chains of cellulose are not branched and the chains are stiff long rods. These glucose molecules in the long cellulose chains can hydrogen bond with each other to form long fibrils. Cellulose is one of the main components (along with hemicellulose and lignin) that make up plant cell walls.

Endocellulase: This is a family of enzymes that breaks the internal β 1–4 bonds of cellulose molecules (as opposed to the exocellulase family of enzymes that break the cellulose bonds at the ends of the polymer strands). The accepted name for this family of enzymes is cellulase. See expasy.org/enzyme/3.2.1.4 for alternative names.

Enzyme: A biological molecule (usually a protein, but can also be a nucleic acid) that increases the rate of reaction by reducing the activation energy of the reaction but which is not itself consumed in the reaction.

Exocellulase: This is a family of enzymes that cuts and releases cellobiose from the reducing (cellobiohydrolase I family) and nonreducing (cellobiohydrolase II family) ends of cellulose polymeric chains. The accepted name for this family of enzymes is cellulose 1,4-β-cellobiosidase. For alternative names see expasy.org/enzyme/3.2.1.91.

Hemicellulose: A polymer that consists of chains of five- and six-carbon sugar groups. Hemicellulose does not have a crystalline structure but instead consists of amorphous chains within plant cell walls. Hemicellulose is also a branched molecule in contrast to cellulose, which is linear.

Lignin: A complex aromatic polymer. Lignin adds stiffness and strength to plant cell walls. This polymer does not have a carbohydrate structure like cellulose and hemicellulose but does serve to bind those polymers together in the cell walls of plants.

Polysaccharide: A polymer chain made up of multiple sugar groups linked together.

Product: The compound or compounds that are produced when an enzyme acts upon a substrate.

Substrate: A compound that is acted upon by an enzyme, producing a product.

Biofuel Debate

Post-Lab Debate Investigation

Many people object to the use of crops for biofuel production. They argue that by using crops for fuel, resources that might produce food for people or animals are reduced. Many are concerned about the removal of essential nutrients from the fields if the nonfood portions of crops are used for fuel and are not left on fields to decay and act as natural fertilizer. Proponents of cellulosic ethanol argue that using nonfood portions of food crops such as corn stover and cover crops such as switchgrass to create fuel is better for the environment. These waste products of food and feed production can be harvested and used for ethanol production and would result in carbon-neutral transportation fuels. In addition, cellulosic portions of plants represent the largest source of biomass in the world, and hence this would be a renewable fuel source, unlike petroleum. Here we include a debate investigation to facilitate discussion of these issues.

Set the Stage

Your teacher will randomly divide the class into two groups and assign one group to support and the other to oppose the development and use of cellulosic ethanol technology for biofuel production. Your teacher will explain the format of the debate and have each team pick a captain.

Student Research

- In teams, conduct research on the development and use of cellulosic ethanol technology for biofuel production using the pro/con data sheet on the next page
- Compile research with your team members
- With your team, write a 4-minute opening statement and assign a spokesperson

The Debate

Opening Statement: Proponents of cellulosic ethanol technology for biofuel production present an opening statement outlining its benefits (4 min).

Break: Opponents assemble a list of questions they believe show holes in the proponents' argument (2 min).

Questions: Opponents present questions (2 min).

Opening Statement: Opponents of cellulosic ethanol technology for biofuel production present an opening statement outlining the reasons it should not be allowed (4 min).

Break: Proponents assemble a list of questions they believe show holes in the oppositions' argument (2 min).

Questions: Proponents present questions (2 min).

Rebuttal: The two sides present answers to each other's questions (2 min for each side).

Closing Arguments: Opposing view (3 min).

Closing Arguments: Supporting view (3 min).

Grading Rubric — Opening Statements

4 = Eloquent, very well organized, researched, and presented.
3 = Well organized, researched, and presented.
2 = Somewhat organized, researched, and presented.
1 = Lacking organization, partially correct research, not well presented.

Questions

4 = Questions were thoughtful, raised legitimate concerns, were research-based, and were well presented.
3 = Questions were somewhat thoughtful, raised some concerns, and were well presented.
2 = Questions were not research-based, did not raise legitimate concerns, and were not well presented.
1 = Questions were unrelated to the subject, did not raise legitimate concerns, and were not well presented.

Rebuttal

4 = Students used research to directly refute the questions.
3 = Students used research to partially refute the questions.
2 = Students used research improperly to attempt to refute the questions.
1 = Students did not refute the questions.

Closing Statements

4 = Closing statement was eloquent, very well organized, presented.
3 = Closing statement was well organized, researched, and presented.
2 = Closing statement was somewhat organized, researched, and presented.
1 = Closing statement lacked organization, used partially correct research, and was not well presented.

Working as a Team Member (as ranked by other team members)

4 = Fully participated and contributed to the team.
3 = Participated and contributed to the team.
2 = Partially participated, somewhat helpful.
1 = Little participation, little help.

Pro/Con Data Sheet

- Make a list of reasons we should use cellulosic ethanol technology for biofuel production (include references)
- Make a list of reasons we should not use cellulosic ethanol technology for biofuel production (include references)

If you are pro, find research to refute the con. If you are con, find research to refute the pro. Include these in your opening or closing statements.

Appendix G

References and Additional Resources

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