Student Manual

Background

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 far too long to occur on their own. Enzymes speed up the vast majority of the chemical reactions that occur in cells. Reactions that break down molecules (such as those involved in digestion and cellular respiration) and those that build up molecules (such as the ones involved in photosynthesis and DNA replication) all require enzymes. Each type of enzyme has a specific shape that compliments the structure of its substrate (Figure 5). 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.

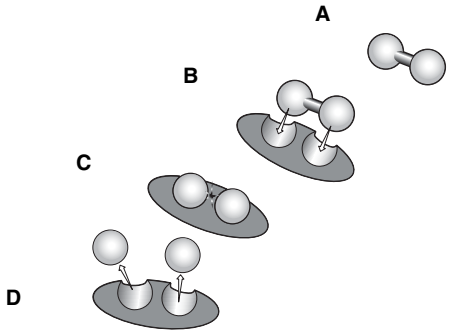


Fig. 5. A schematic of cellobiose and cellobiase in solution. A. Cellobiose in solution is composed of two glucose molecules covalently connected by a β 1–4 linkage. **B**. Cellobiase has a pocket that fits the cellobiose molecule. **C**. Cellobiase helps stabilize the cellobiose in a shape so that the bond between the two glucose molecules can be broken. **D**. 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 have also influenced the couple by pointing out the good points about each individual. Like enzymes, the matchmaker did not change and he/she was able to go on and make further matches. In chemical terms, the enzyme lowers the energy of activation 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 structure in the reaction with the highest energy. By lowering this energy, the reaction can take place much more easily.

Enzymes are "picky" about the conditions at 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. It increases the average kinetic energy (energy of movement) of the molecules so that more of them 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 non-covalent 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 this laboratory experiment, 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 for fuel.

Cellobiase Substrates

The natural substrate for the enzyme cellobiase is cellobiose (Figure 6). This is a disaccharide composed of two beta glucose molecules. However, when scientists study enzyme function, it is best if there is an easy way to detect either the amount of substrate that is used up or the amount of product that is formed. Solutions of cellobiose (substrate) and glucose (product) are clear, and there are not many simple, inexpensive, fast methods to detect these molecules quantitatively.

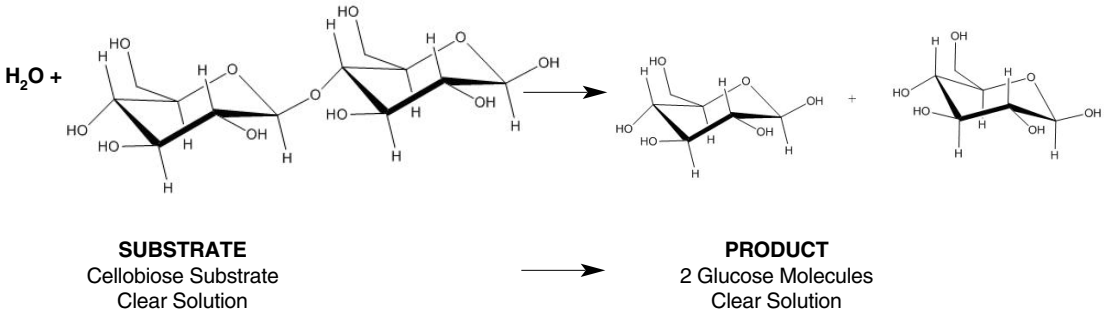


Fig. 6. Breakdown of cellobiose by cellobiase. The natural substrate of cellobiase is the dissacharide cellobiose. When cellobiose is bound by cellobiase, the cellobiase breaks apart the β 1–4 bond that connects the two glucose molecules and then releases two glucose molecules.

So, to make this reaction easier to follow, an artificial substrate, *p*-nitrophenyl glucopyranoside, will be used. This artificial substrate can also bind to the enzyme and be broken down in a manner similar to the natural substrate cellobiose. When the artificial substrate, *p*-nitrophenyl glucopyranoside, is broken down by cellobiase, it produces glucose and *p*-nitrophenol (Figure 7). 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 amount of yellow color is proportional to the amount of *p*-nitrophenol present. And for every molecule of *p*-nitrophenol present, one molecule of *p*-nitrophenyl glucopyranoside is broken apart. For the cellobiase reactions being run, another advantage of using a basic solution to develop the color of the *p*-nitrophenol is that the basic pH will also denature the enzyme and stop the reaction.

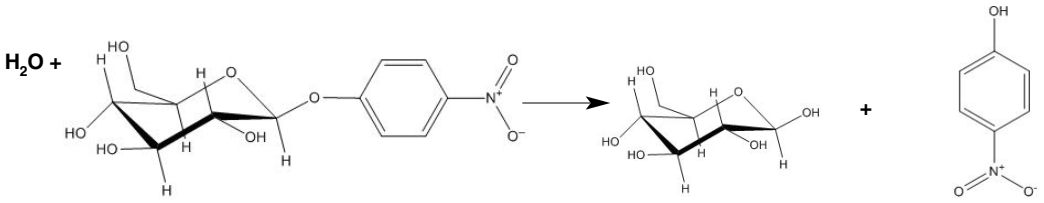


Fig. 7. Breakdown of *p*-nitrophenyl glucopyranoside into glucose and *p*-nitrophenol by cellobiase. When the *p*-nitrophenyl glucopyranoside is broken apart by cellobiase, one molecule of glucose and one molecule of *p*-nitrophenol are released. If the *p*-nitrophenol is put into a basic solution, it will produce a yellow color, which is detected 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 higher the amount of product made. One simple method of estimating how much product is formed is to compare the yellowness of enzyme reaction samples to a set of known standards, which contain a known amount of colored product. You can estimate which tube in the set of standards most closely matches your samples in color. This will give you an estimated amount of product. Alternatively, you can use an instrument called a spectrophotometer (or a colorimeter), which quantitatively measures the amount of yellow color by shining a beam of light (wavelength of 410 nm) through the sample. The spectrophotometer measures the amount of light that is absorbed by the sample. The darker the color of yellow the sample is, the more light that is absorbed, and thus the more concentrated the sample. The absorbance values of a set of standards can first be measured to create a standard curve, a plot of the absorbance values of samples of known concentration of *p*-nitrophenol. The absorbance values of the reaction samples can then be measured, and the standard curve can be used to convert the absorbance value to a concentration value.

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 is determined. For studying cellobiase activity, you will measure the rate of reaction by adding enzyme to the artificial substrate *p*-nitrophenyl glucopyranoside. The enzyme and substrate are dissolved in a buffer that is at an ideal pH (pH 5.0) for the reaction to occur. At set times, a sample of the enzyme reaction will be removed and added to a high pH stop solution which will help develop the color of the product *p*-nitrophenol, as well as stop the reaction by increasing the pH to above the range 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 is constant or whether it slows down toward the end as the amount of substrate decreases. You will also be able to detect any effects pH, temperature, substrate concentration or enzyme concentration have on the initial rate of reaction.

Pre-lab Questions

1. What type of molecule is an enzyme?

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

3. How does an enzyme speed up chemical reactions?

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

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

6. What is the natural product of this enzyme?

7. What is the natural substrate of this enzyme?

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

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

Activity 6: Test Ability of Mushroom Extracts to Increase Reaction Rate

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 to produce glucose for energy usage. 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. Cellobiase can also be found in the seeds of bitter almonds where it is known as emulsin. Emulsin is actually thought to be a combination of cellobiase and other enzymes. In this activity, you will choose a potential source of cellobiase, extract proteins from this source, and take this extract and combine it with the substrate, *p*-nitrophenyl glucopyranoside, to determine if your extract has any enzymatic activity that allows it to break down the substrate.

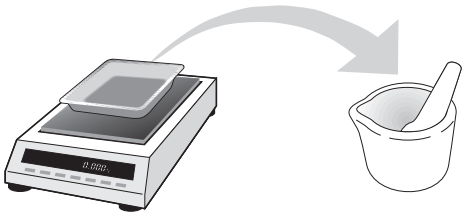
Student Workstation	**Quantity**	**(✔)**
Mushroom sample	1	❒
1.5 mM substrate	1	❒
Stop solution	1	❒
1x extraction buffer	1	❒
15 ml conical tube	1	❒
1.5 ml microcentrifuge tube	1	❒
DPTPs	4	❒
Cuvettes	6	❒
Marker	1	❒
Mortar and pestle	1	❒
Filter paper, cheese cloth, or strainer	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

Instructor's Workstation (Optional)	**Quantity**	**(✔)**
Spectrophotometer	1	❒

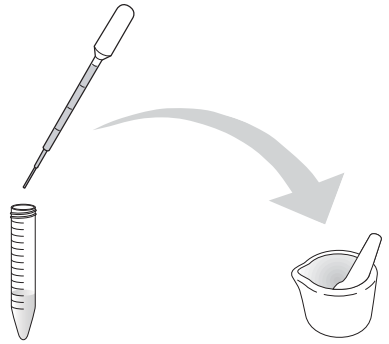
Protocol

1. Write down the name of your mushroom _____

2. Weigh out approximately 1 g of your mushroom and place it in a mortar. _____g



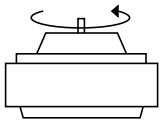
3. Add 2 ml of extraction buffer for every gram of mushroom into the mortar. _____ml



Extraction buffer

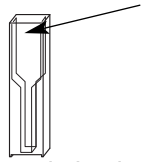
4. Using a pestle, grind your mushroom to produce a slurry.

5. Strain the solid particles out of your slurry using a piece of filter paper or cheese cloth into a 1.5 ml microcentrifuge tube. Alternatively, 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 minutes.

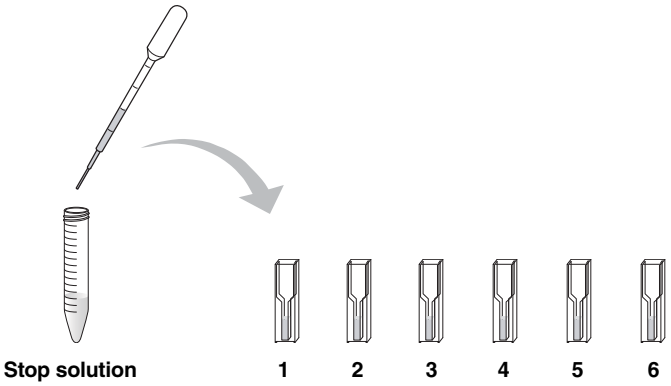


Note: You will need at least 250 µl of extract to perform the enzymatic reaction.

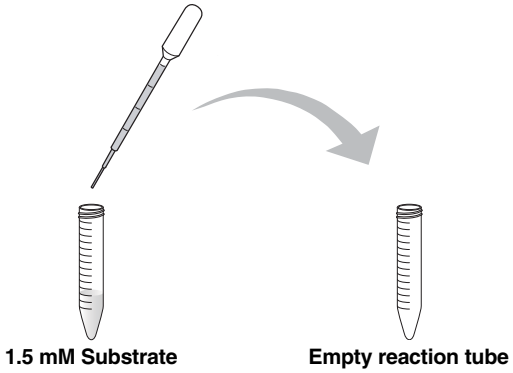
6. Label your cuvettes "1–6". Only label on the upper part of the cuvette face.



7. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Rinse out the DPTP thoroughly with water.

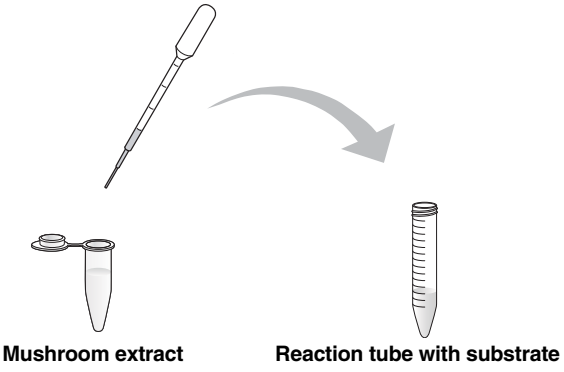


8. Label a 15 ml conical tube with the type of mushroom you are using. Using a clean DPTP, pipet 3 ml of substrate into the tube.

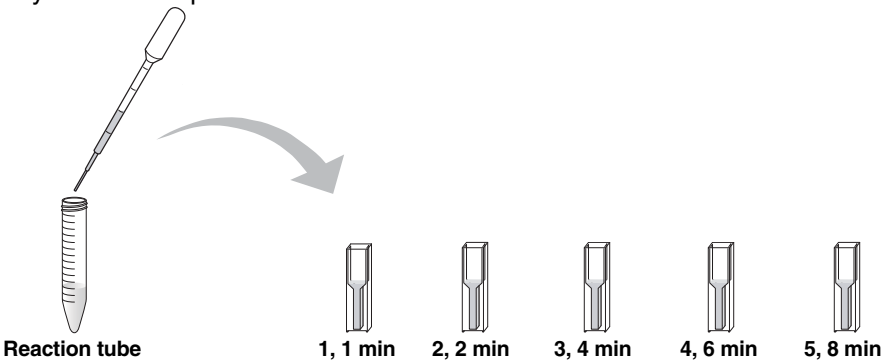


Please read and understand steps 10–11 fully before proceeding. These steps are time sensitive!

9. Using a clean DPTP, pipet 250 µl of your mushroom extract into the 15 ml conical tube containing 3 ml of substrate. **START YOUR TIMER**.

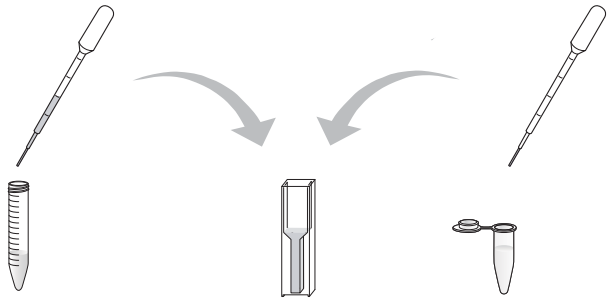


10. At the times indicated in the table below, remove 500 µl of mushroom extract/substrate mixture from the 15 ml conical tube, and add it to the appropriately labeled cuvette that already contains stop solution.



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

11. Using a clean DPTP, add 500 µl of extraction buffer to cuvette #6. Clean the DPTP and then add one drop of enzyme extract. This will serve as the "blank" for this experiment.



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

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

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

1. Blank your spectrophotometer with the blank (cuvette #6) at 410 nm. Measure the absorbance values for your five cuvettes and record the absorbance values in column 2 of Table 17.

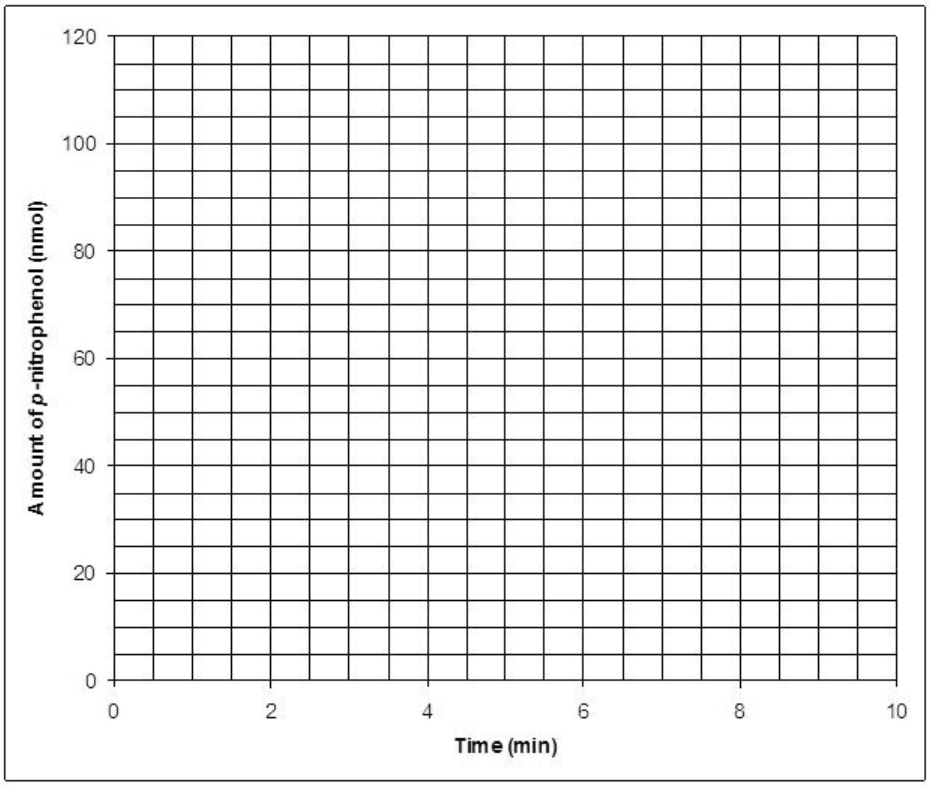
2. Using the protocols you learned in Activity 1, calculate the amount of *p*-nitrophenol formed in all of your samples and record it in column 3 of Table 17.

Table 17. Determination of *p*-nitrophenol produced by the mushroom extract breaking down the substrate based on a standard curve (similar to the one shown in Figure 8).

Cuvette	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
1		
2		
3		
4		
5		
6	0.00	

Analysis of Results

1. Plot the amount of product produced over time by your mushroom extract on the graph below.



2. Calculate the initial rate of reaction for mushroom extract

Initial rate of reaction = _____ nmol/min

Activity 6 Analysis Questions

1. Did your mushroom extract break down the substrate (that is, produce any yellow product)?

2. Why did we use a blank for this experiment that was different from the one used in earlier experiments? **Hint**: What would be the effect on your absorbance readings if a mushroom naturally had some yellow color to it?

3. Compare the initial rate of reaction of your mushroom extract to the enzyme included in this kit. From what you have learned about the effect of pH, temperature, and enzyme concentration, can you explain some factors that might influence your enzyme extract's initial rate of reaction?