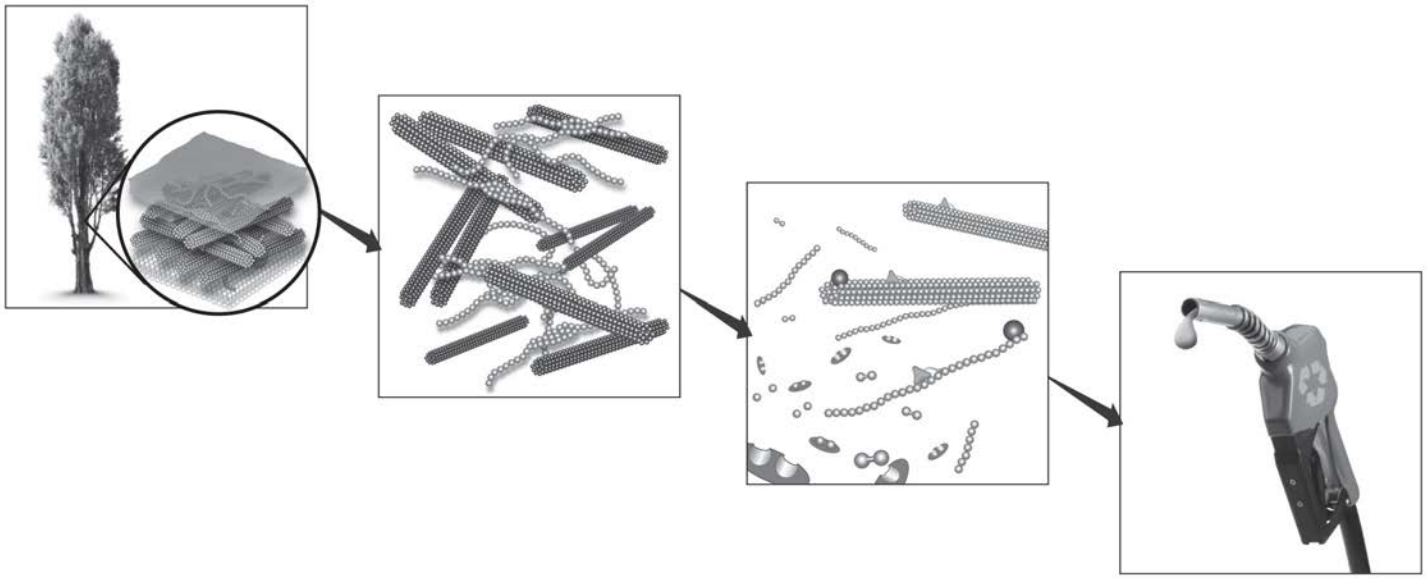

Bio-Rad Explorer™

Biofuel Enzyme Kit

Catalog Number
166-5035EDU

explorer.bio-rad.com

Note: Kit contains temperature-sensitive reagents. Open immediately upon arrival and store components at 4°C as indicated.

Please visit explorer.bio-rad.com to access our selection of language translations for Bio-Rad Explorer kit curricula.

BIO-RAD

Dear Educator:

Fossil fuels such as petroleum have been used extensively as sources of energy for transportation since the drilling of Drake Well in Pennsylvania in 1859. Early combustion engines were designed to run on ethanol, but the discovery of new oil fields in Texas and the resulting inexpensive petroleum, along with later politics, pushed petroleum in as the main fuel source. But supplies of fossil fuels are finite and as they decrease and the impact of their combustion on the environment is felt, sustainable, inexpensive petroleum replacements are once again being sought.

How to begin to solve some of our fuel issues, along with the environmental impact of different fuels, captures students' imaginations. This kit provides an in-depth instruction about cellobiase, one of the enzymes used for the production of ethanol from cellulose for use as a transportation fuel. Using cellobiase and a colorimetric substrate provided in the kit, students investigate the enzyme's ability to increase the reaction rate and determine the effect of factors such as pH, temperature, substrate concentration, and enzyme concentration on enzymatic activity and reaction rates.

Students can also conduct their own independent study by analyzing the ability of extracts from mushrooms to break down the colorimetric substrate. This can open the door to discussions about research and discovery of new enzymes for biofuel production and optimization of enzymatic reactions. Since students will be learning about enzyme function within the context of biofuels, this independent study also provides an opportunity for discussions about carbon cycles, fuel vs. food vs. feed usage of plants, as well as what sustainable fuel production means.

With these lab activities, students gain practical knowledge about conducting experiments to determine factors that affect enzymatic reaction rates. These activities are designed for any classroom environment and do not require any specialized equipment. The ability to colorimetrically track the generation of product allows students to directly see the progression of their reaction. Additionally, if a spectrophotometer is available, students can also gain an understanding of quantitative measurements.

This curriculum was developed in collaboration with Dr. Diane Sweeney at the Punahou School in Hawaii. We would like to thank Dr. Sweeney for her invaluable guidance and contribution to this curriculum.

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Create context. Reinforce learning. Stay current.
New scientific discoveries and technologies create more content for you to teach, but not more time. Bio-Rad Explorer kits help you teach more effectively by integrating multiple core content subjects into a single lab. Connect concepts with techniques and put them into context with real-world scenarios.

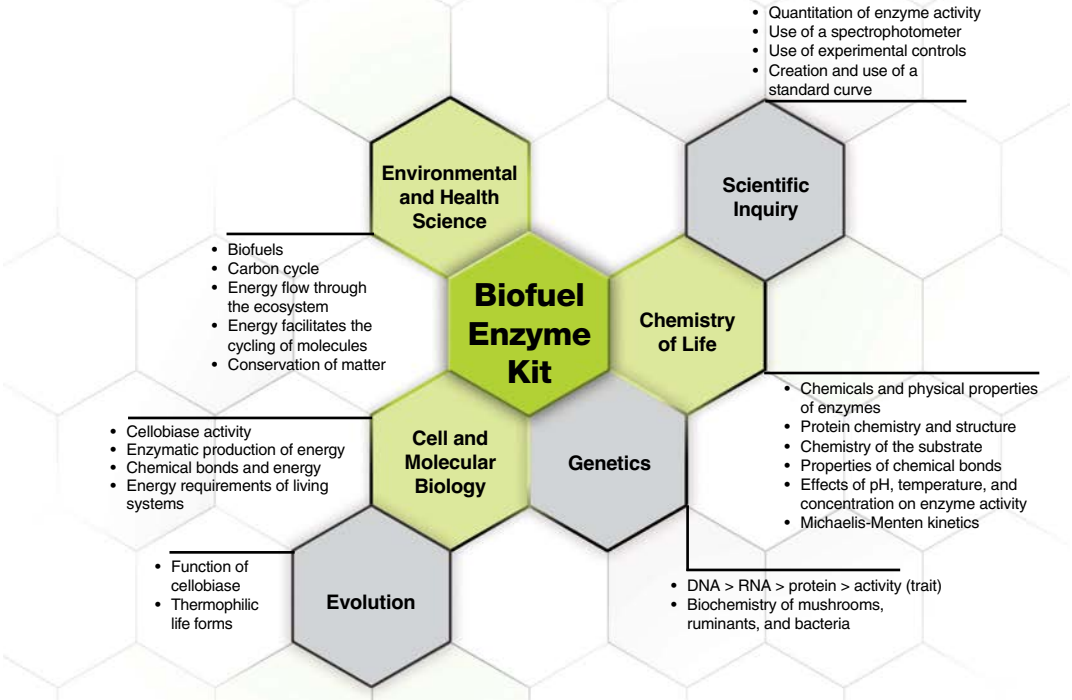


Table of Contents

Kit Summary

The Biofuel Enzyme kit tests the ability of an enzyme to increase the conversion rate of a clear substrate to a colored product. The kit contains sufficient materials for 8 student workstations to test and calculate the rate a sugar substrate (*p*-nitrophenyl glucopyranoside) is converted to *p*-nitrophenol and glucose in the presence or absence of the enzyme, cellobiase. The kit also provides sufficient materials to test the effects of various conditions, such as pH, enzyme concentration, substrate concentration, and temperature, on the rate of enzyme-catalyzed reactions. In addition, the kit guides students through an independent inquiry that tests fungal sources for cellobiase activity.

- Activity 1: Determine the reaction rate in the presence or absence of an enzyme
- Activity 2: Determine the effect of temperature on the reaction rate
- Activity 3: Determine the effect of pH on the reaction rate
- Activity 4: Determine the effect of enzyme concentration on the reaction rate
- Activity 5: Determine the effect of substrate concentration on the reaction rate
- Activity 6: Test the ability of mushroom extracts to increase the reaction rate

Cellobiases are part of a group of enzymes collectively known as cellulases that are actively being studied and produced for use in the biofuel industry. These enzymes are capable of breaking down cellulose, a complex macromolecular construct of sugar chains. Cellobiases are naturally produced by fungi and bacteria present in ruminants, termites, and some plants or plant products. They are being investigated for use in breaking down non-food/feed plant products, such as corn stover, switch grass, and waste wood products, along with fast-growing trees, such as poplar, for production of ethanol to replace fossil fuels. The Biofuel Enzyme kit uses the enzyme cellobiase to increase the degradation rate of a sugar compound *p*-nitrophenyl glucopyranoside to glucose and *p*-nitrophenol which is a yellow substance (Figure 1). The enzyme activity is indirectly measured by monitoring the change in color intensity of the solution.

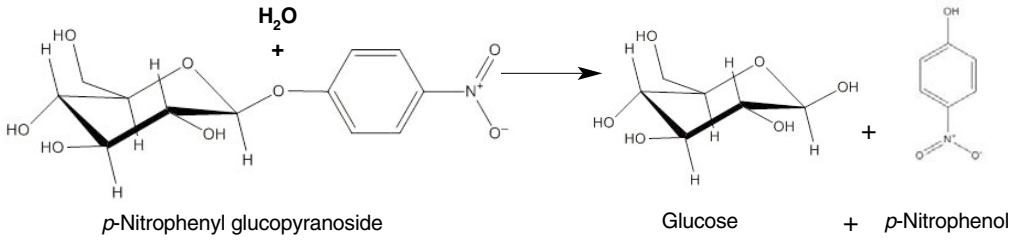


Fig. 1. The cleavage of *p*-nitrophenyl glucopyranoside results in glucose and *p*-nitrophenol as products. This reaction occurs at an extremely slow rate without an enzyme present, but occurs at a detectable rate with an enzyme with β-glucosidic activity present.

Storage Instructions

Place the reagent bag in the refrigerator (4°C) within 1 week of arrival. Once stock reagents are prepared, store the diluted/reconstituted solutions at 4°C to ensure stability (if not running all activities at the same time). The diluted enzyme is stable at 4°C for one week. The reconstituted substrate and standards are stable at 4°C for one month. Once diluted to 1x, the stop solution, resuspension buffer, and extraction buffer are stable for 3 years at 4°C.

Kit Inventory Checklist

This section lists the components provided in the Biofuel Enzyme kit. It also lists the required accessories. Each kit contains sufficient materials for 8 student workstations, 4 students per workstation. As soon as your kit arrives, open it and check off the listed components to familiarize yourself with the kit. **Immediately place the bag containing the enzyme, substrate, standard and buffers in the refrigerator (4°C)**.

Kit Components	Quantity	(✔)
Store at 4°C		
Enzyme, cellobiase, 1 ml	1 vial	❒
Substrate, *p*-nitrophenyl glucopyranoside, 90 mg	1 vial	❒
Standard, *p*-nitrophenol (1 mM, 4 ml)	1 bottle	❒
2x stop solution, 100 ml	1 bottle	❒
10x resuspension buffer, 50 ml	1 bottle	❒
Extraction buffer, 50 ml	1 bottle	❒
Store at room temperature		
Disposable plastic transfer pipets (DPTPs)	40	❒
1.5 ml microcentrifuge tubes	90	❒
15 ml conical tubes	50	❒
1.5 ml standard disposable polystyrene cuvettes, 100	1 box	❒
Instruction Manual available for download online		

Required Accessories (Not Included)	Quantity per Kit	(✔)
Instructor setup or lab equipment		
500 ml bottle for preparing 1x resuspension buffer	1	❒
200 ml bottle for preparing 1x stop solution	1	❒
100 ml bottle for preparing high concentration enzyme	1	❒
100 ml bottle for preparing low concentration enzyme	1	❒
100 ml bottle for preparing 3 mM substrate	1	❒
150 ml bottle for preparing 1.5 mM substrate	1	❒
50 ml tubes or bottles for preparing standards	5	❒
Serological pipettor and pipets or graduated cylinders to measure volumes ranging 4–450 ml		
Deionized or distilled water	1,000 ml	❒
Lab tape	1	❒
Parafilm	1 roll	❒
Balance (for Activity 6)	1	❒

Student Workstation (4 Students)	Quantity per Station	(✔)
All Activities		
Marking pens	1	❒
Timers or stopwatches	1	❒
Activity 2: Determine the effect of temperature on the reaction rate		
Beakers with 37°C water	1	❒
Beaker or ice bucket with chipped ice	1	❒
Thermometers	1	❒

Student Workstation (4 Students)	Quantity per Station (✔)	
Activity 6: Test the ability of mushroom extracts to increase the reaction rate		
Mortar and pestles	1	❒
Weight boats or weigh paper	1	❒
Filter paper, cheese cloth, or strainer	1	❒

Optional Accessories	Quantity
100–1,000 µl adjustable micropipet (catalog #166-0508EDU, 166-0553EDU)	8
100–1,000 µl pipet tips (catalog #223-9350EDU)	8
Spectrophotometer such as SmartSpec™ Plus spectrophotometer (catalog #170-2525EDU), Vernier SpectroVis spectrophotometer, or similar	1
Waterbath, 120 V (catalog #166-0504EDU)	1
Digital dry bath, 120 V (catalog #166-0562EDU)	1
Mini incubation oven, 120 V (catalog #166-0501EDU)	1
Mini centrifuge, 120 V (catalog #166-0603EDU)	1

Refills Available Separately

Biofuel Enzyme kit temperature sensitive reagents bag (catalog #166-5036EDU) containing enzyme (cellobiase), substrate (*p*-nitrophenyl glucopyranoside), standard (*p*-nitrophenol), 2x stop solution, 10x resuspension buffer, extraction buffer

1.5 ml standard disposable polystyrene cuvettes (catalog #223-9955EDU)

Conical centrifuge tubes, package of 50 (catalog #166-0475EDU)

Disposable plastic transfer pipets, non-sterile, 500 (catalog #166-0480EDU)

1.5 ml EZ Micro™ test tubes, pkg of 500 (catalog #223-9480EDU)

Timeline

Running all six activities requires approximately three, 50-minute laboratory periods, depending on whether or not results are analyzed during the allocated laboratory time. We also recommend 1– 2 days for background review and lectures to prepare your students for these exercises.

Prior to Laboratory

- Read manual (2 hours)
- Inventory required accessories (1 hour)
- Perform instructor's advance preparation – Preparation of stock reagents and initial setup for all activities (2 hours)
- Perform instructor's advance preparation – For any single activity (15–45 minutes)
- Set up student workstations (15 minutes)

50-Minute Lessons

- Activity 1: Determine the reaction rate in the presence or absence of an enzyme (45 minutes)
- Activities 2–5: Determine the effect of temperature, pH, and enzyme or substrate concentration on the rate of reaction (25 minutes for each activity)
- Activity 6: Test ability of mushroom extracts to increase the reaction rate (30 minutes)

90-Minute Block Lessons

- Activities 1–3: Determine the rate of reaction in the presence or absence of the enzyme, and determine the effect of temperature and pH on the rate of reaction (90 minutes)
- Activities 4–6: Determine the effect of enzyme and substrate concentration on the rate of reaction, and test the ability of mushroom extracts to increase the rate of reaction (90 minutes)

Safety Issues

Eating, drinking, smoking and applying cosmetics are not permitted in the work area. Wearing protective eyewear and gloves is strongly recommended. Students should wash their hands with soap before and after this exercise. There is a strong base solution (stop solution, pH 9.5) used in this lab, so safety protocols should be observed. If any solution gets into a student's eyes, flush with water for 15 minutes. Lab coats or other protective clothing should be worn to avoid any injury caused by spilled base. Also, if mushrooms are to be tested as independent inquiry sources of enzyme, **students should only use samples from the grocery store**. Field collection of mushrooms is not advised.

Background for Instructors

The Biofuel Enzyme kit measures the cellobiase enzyme activity of a beta glucosidase* and identifies the optimal conditions for the enzyme. The reaction of cellobiase breaking down cellobiose is important in the process of making cellulosic ethanol, which is an efficient, more sustainable fuel to replace petroleum. The reaction is simple to set up and produces reliable, quantifiable data. The practical applications of this enzyme give students a real world experience that will make the abstract concepts involved in enzymatic reactions relevant.

General Information about Enzymes

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

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

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

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

The relative concentration of all molecules involved in the reaction affects the reaction rate as well. The higher the concentration of an enzyme, the faster the reaction will take place until there is excess enzyme. Similarly, increasing the concentration of a substrate will speed up the reaction until the point at which all the enzyme present is saturated with the substrate. Sometimes it helps to think of an analogy of workers (enzymes) producing a product from raw materials (substrate). If you increase the number of workers, the amount of product produced will increase until there are excess workers and not enough raw

* The beta glucosidase enzyme used in this kit has specificity broader than just cellobiase activity; however, to clearly link cellobiase enzyme activity on cellobiose substrate for students, the enzyme in this kit will be referred to as cellobiase.

materials to work on. In the same way, increasing the amount of raw materials, while keeping the number of workers constant, will increase the rate of product production until you have given the workers excess raw materials. There is a maximum rate at which the product can be produced given a particular "concentration" of workers (enzyme) and raw materials (substrate). In biochemical terms, this is called V_{max}. More details about enzyme kinetics can be found in Appendix A.

Students will use this kit to study the reaction rate of cellobiase, an enzyme involved in breaking down cellobiose to glucose. They will also analyze how temperature, pH, enzyme concentration, and substrate concentration affect the activity of cellobiase.

Organisms That Produce Cellulases

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

Cellulosic Ethanol: A Practical Application for Cellulases

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

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

Other plant cells are dead at maturity. They strengthen the plant and/or function to conduct water through the plant. These plant cells develop a second type of cell wall called the secondary cell wall before they die. Secondary cell walls are more rigid than primary cell walls. Plant tissues with secondary cell walls have water transport tissues such as xylem, the fibrous or hard tissues covering a coconut or walnut seed, and the stringy part of a celery stalk. These cell walls have additional molecules other than cellulose that contribute

to their rigidity. Hemicellulose and lignin are found in high quantities in the secondary cell walls of woody or fibrous plant tissue. For cellulosic ethanol production, lignins must be removed because they inhibit enzymatic activity of cellulases. Hemicelluloses must be cleaved from the cellulose to allow enzymatic breakdown of the cellulose.

One way that ethanol can be produced from plant matter is by completing the following three tasks (Figure 2):

1. **Pretreatment**: Removal of non-cellulose biomolecules such as lignin
2. **Enzymatic hydrolysis**: Cellulases hydrolyze cellulose to produce six carbon sugars and enzymes can be added to hydrolyze hemicellulose to five carbon sugars
3. **Microbial fermentation**: Converts sugar products into ethanol

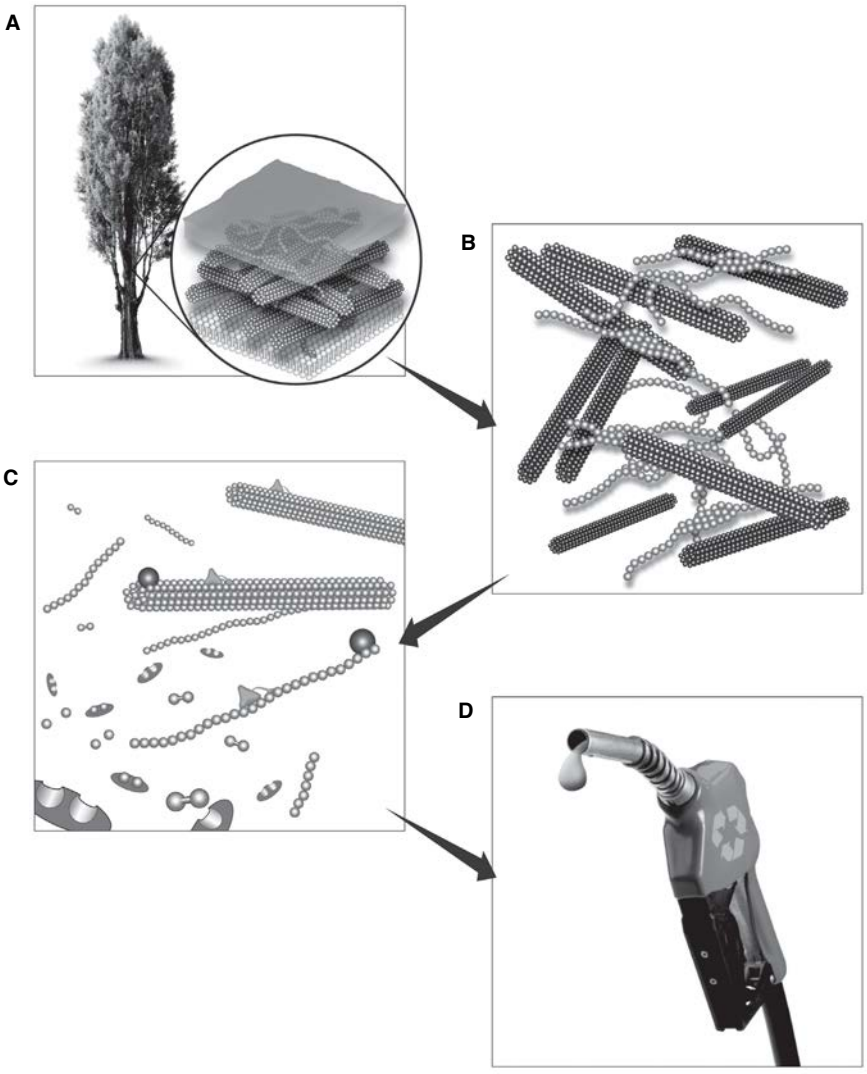
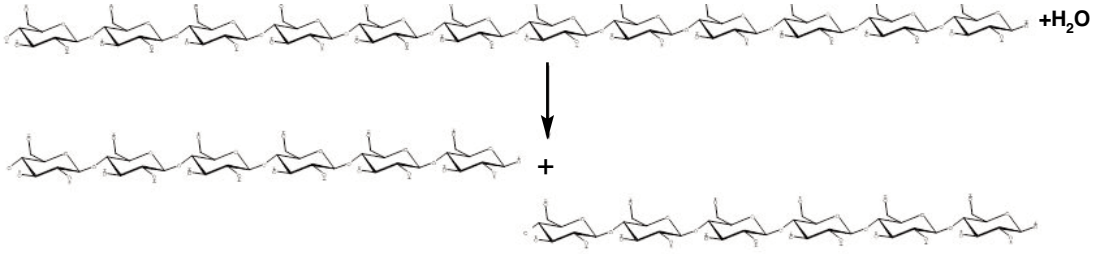


Fig. 2. The process of cellulosic ethanol production. A. Cellulose from sources such as poplar trees, switchgrasses, and corn stover is collected. **B**. The cellulose is isolated from these plant materials by heat treatment, physical crushing, acid or base treatment. Ideally, the cellulose is isolated from other compounds such as lignins that interfere with the breakdown of cellulose. **C**. The cellulose is enzymatically broken down to glucose. **D**. The glucose is fermented to ethanol and processed as fuel.

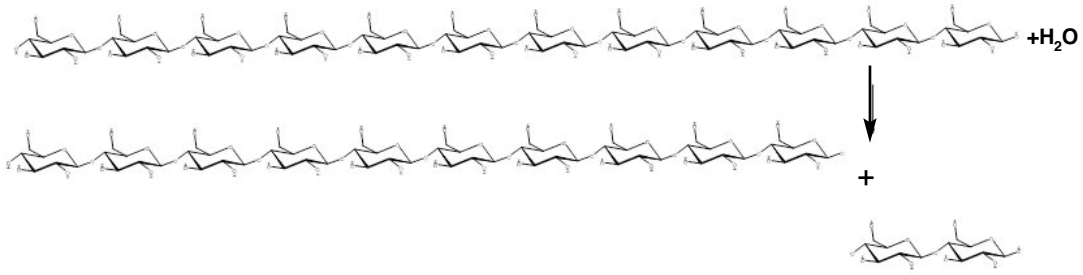
Types of Cellulases Needed to Break Down Plant Cell Walls into Glucose

The production of ethanol from plant material is a very complex procedure requiring multiple steps. Plant material is first processed mechanically, as well as with acids or enzymes and heat to remove lignin. Lignin is a highly complex, aromatic macromolecule found in high quantities in secondary cell walls of fibrous and woody plant tissue in close association with cellulose. Once the lignin is removed, the cellulose is more exposed and can be more readily broken down. Cellulose is broken down into glucose in three steps by three different types of enzymes.

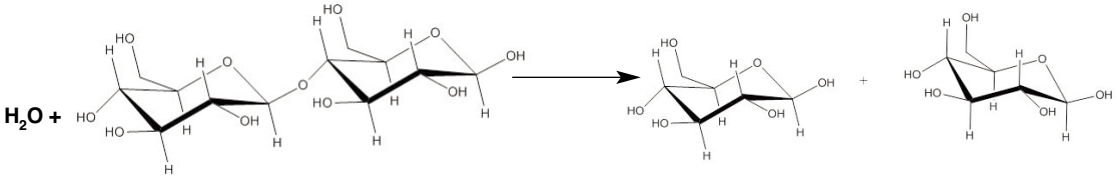
- Endocellulases — These enzymes break down the internal bonds of the long chains of glucose molecules that form cellulose.



- Exocellulases — These enzymes break the covalent linkages between the glucose units of cellulose that are on the end of the cellulose molecules, releasing cellobiose.



- β-glucosidases (Cellobiases) — These enzymes break down the cellobiose left behind as a result of the work of the first two enzymes.



Cellobiase Enzyme

Cellobiase, the enzyme provided in this kit, breaks down cellobiose, a disaccharide made up of two glucose molecules connected together by a 1,4 β–glucoside linkage (Figure 3). The breakdown of cellobiose by cellobiase is the final step in producing glucose from cellulose.

Glucose is the preferred source of sugar for microbial fermentation, an additional enzymatic reaction that produces ethanol.

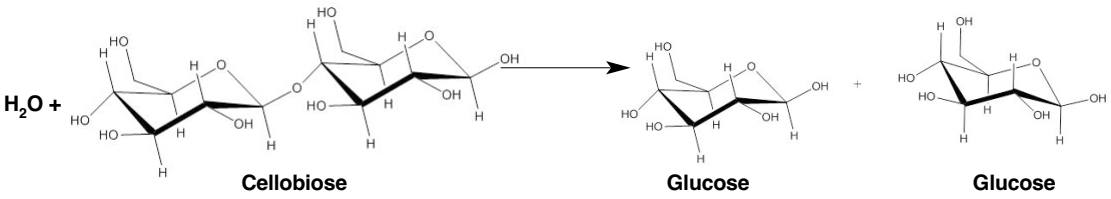


Fig. 3. Breakdown of cellobiose into two glucose molecules. Cellobiose is linked at carbon 1 and carbon 4 of two separate glucose units. The enzyme cellobiase can break this linkage resulting in two glucose molecules.

Detecting the Substrate Used in This Lab

Although cellobiose is the natural substrate of cellobiase, there is no simple method to quantitatively detect the product (glucose) or the disappearance of cellobiose. A simple colorimetric assay using an artificial substrate, *p*-nitrophenyl glucopyranoside, can be used to detect enzymatic activity of cellobiase. The substrate *p*-nitrophenyl glucopyranoside is composed of a beta glucose covalently linked to a molecule of nitrophenol (Figure 4). When the bond connecting these two molecules is cleaved with the help of cellobiase, the *p*-nitrophenol is released. To stop the activity of the enzyme and to create a colored product, the reaction mixture is added to a basic solution. When the *p*-nitrophenol is placed in a basic solution, the hydroxyl group on the nitrophenol loses an H^+ to the OH^- of the base, which changes the bonding within the phenolic ring, so that the molecule will absorb violet light (and reflect yellow light). This makes the solution yellow, which can be detected visually by comparing the deepness of the yellow color to a set of standards of known concentration of *p*-nitrophenol or by using a spectrophotometer to produce more accurate, quantitative results.

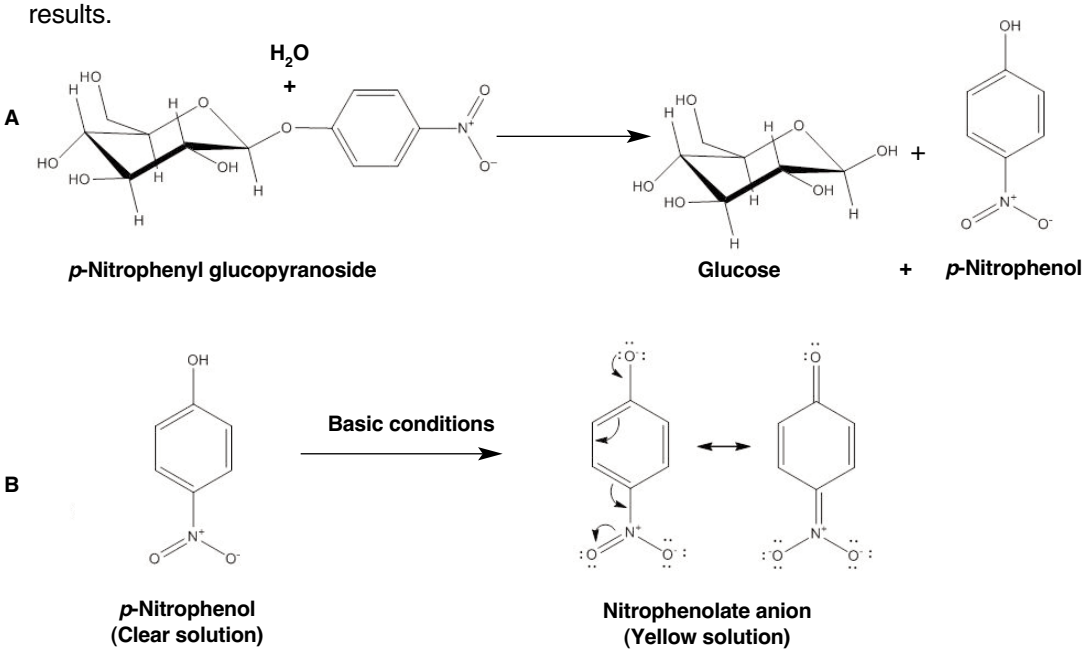


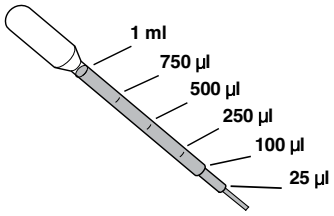
Fig. 4. Detecting glucose from the breakdown of cellobiose by cellobiase enzyme. A. An analog to cellobiose called *p*-nitrophenyl glucopyranoside is used to detect enzymatic activity and accumulation of glucose. Once cleaved by cellobiase, the *p*-nitrophenol is released, which can be detected by its yellow color in basic solutions. **B**. *p*-nitrophenol is colorless to slightly yellow at pH 5. However, under basic conditions, the hydrogen ion of the hydroxyl group (OH^- group) is removed, resulting in a negative charge due to an extra pair of electrons on the remaining oxygen group. This pair of electrons travels along the nitrophenolate anion, creating a resonance structure that produces the yellow color.

An Independent Inquiry Experiment

This kit can also be used for a guided independent inquiry experiment to determine the ability of naturally occurring (or manufactured) sources of cellobiase, such as an extract from a mushroom sample, to break down the model substrate included in the kit. Extensive research is currently being performed by the biofuel industry to find and study natural sources of cellulase enzymes that might be more stable at extreme conditions for degrading plant sources of cellulose. Bacteria from hot springs or fungi that can live at high or low pH conditions are being found and their enzyme activity tested. Your students can test the enzymatic ability of a common fungus to break down the provided substrate and determine how efficient they are when compared to the enzyme in the kit.

Volume Measurements

Sterile graduated disposable plastic transfer pipets (DPTPs) are supplied with this kit and can be used for volumes of 100, 250, 500, 750, and 1,000 µl. The illustration shows the marks on the DPTP corresponding to the volumes to be measured. Volumes over 1 ml will require multiple additions. It is recommended that students practice using the DPTPs to pipet water before actually beginning the laboratory activities.



Mortars and Pestles

If running Activity 6 to test the ability of mushroom extracts to increase the reaction rate, mushrooms will be ground using a mortar and pestle. Please ensure that the mortar and pestles have been thoroughly washed and rinsed to remove any residual chemicals that may interfere with the ability of extracted cellobiase to function. Soap followed by a thorough rinsing with water to remove any remaining soap should be sufficient.

Instructor's Advance Preparation

This section describes the preparation that needs to be performed by the instructor for each activity. Basic stock solutions used in all activities should be prepared in advance of the laboratory activity using the instructions provided below. Depending on which activities are being performed, volumes and preparation necessary for each individual activity are listed separately. An estimation of preparation time is also included.

Materials Needed for Advanced Preparation	Quantity
10x resuspension buffer	1 bottle
Enzyme	1 vial
Substrate	1 vial
2x stop solution	1 bottle
Standard	1 bottle
500 ml bottle for preparing 1x resuspension buffer	1
200 ml bottle for preparing 1x stop solution	1
100 ml bottle for preparing high concentration enzyme	1
100 ml bottle for preparing low concentration enzyme	1
100 ml bottle for preparing 3 mM substrate	1
150 ml bottle for preparing 1.5 mM substrate	1
50 ml tubes or bottles for preparing standards	5
Serological Pipettor and pipets or graduated cylinders to measure volumes ranging from 4–450 ml	
Disposable plastic transfer pipets (DPTPs)	2
Deionized or distilled water	1,000 ml
Lab tape	1

Procedure (Estimated time — 2 hours)

1. **Preparation of 1x resuspension buffer**: Label a bottle "1x Resuspension Buffer". Combine 50 ml of 10x resuspension buffer with 450 ml of deionized or distilled water in the bottle and mix by shaking.
2. **Preparation of high concentration enzyme**: Label a bottle "High Concentration Enzyme" and add 63 ml of 1x resuspension buffer to it. Add 1 ml of enzyme to the resuspension buffer in the bottle and mix by shaking.
3. **Preparation of low concentration enzyme**: Label a bottle "Low Concentration Enzyme". Combine 20 ml of high concentration enzyme with 60 ml of 1x resuspension buffer in the bottle and mix by shaking.
4. **Preparation of 3 mM substrate**: Label a bottle "3 mM Substrate" and add 98 ml of 1x resuspension buffer to it. Add 1 ml of 1x resuspension buffer to the vial of substrate and mix. Combine this 1 ml with the 98 ml of buffer in the bottle. Add another 1 ml of 1x resuspension buffer to the vial and mix. Add this 1 ml to the same bottle containing the substrate solution. Mix until particles are thoroughly dissolved. **Note**: The powder will take approximately 10–20 minutes to fully dissolve once it has been added to 98 ml of 1x resuspension buffer.
5. **Preparation of 1.5 mM substrate**: Label a bottle "1.5 mM Substrate". Combine 75 ml of 3 mM substrate with 75 ml of 1x resuspension buffer in the bottle and mix thoroughly by shaking.

6. **Preparation of 1x stop solution**: Label a bottle “1x Stop Solution”. Combine 100 ml of 2x stop solution with 100 ml of deionized or distilled water in the bottle and mix by shaking.
7. **Preparation of standard dilutions**:
    a. Label five 50 ml tubes or bottles S1–S5.
    b. Put 10 ml of deionized water into each tube.
    c. Add 4 ml of standard and 6 ml of deionized or distilled water to tube labeled S5 and mix.
    d. Transfer 10 ml of standard from tube S5 to tube S4 and mix.
    e. Transfer 10 ml of standard from tube S4 to tube S3 and mix.
    f. Transfer 10 ml of standard from tube S3 to tube S2 and mix.
    g. Remove 10 ml of standard from tube S2 and discard.
    h. Add 10 ml of 1x stop solution to all five tubes and mix.

Initial Setup for All Activities

Note: Solutions such as 1.5 mM substrate, low concentration enzyme, stop solution, and resuspension buffer will be used for multiple activities. The volume of each solution listed in the initial setup for all activities is the amount required for all six activities. In the setup information for each individual activity, the minimum amount of reagent needed for that one activity is listed.

Also, the set of 5 standards will be reused for each activity for quantitative or qualitative determination of the amount of product formed. Do not discard these solutions. The cuvettes can be covered with Parafilm and stored at 4°C between laboratory periods if necessary.

Finally, the cuvettes used to measure reaction time points, DPTPs, and the 15 ml conical tubes used for reactions during each activity will be reused by the students for subsequent activities. These should be washed out thoroughly with deionized or distilled water between each activity and saved for later use. For ease of reuse of these items, we recommend that students label them using laboratory tape rather than writing directly on the surfaces using a marker.

Material Needed for Advanced Preparation	Quantity
1.5 mM substrate	120 ml
Low concentration enzyme	40 ml
1x stop solution	120 ml
1x resuspension buffer	64 ml
Colorimetric standards (S1–S5)	8 ml each
15 ml conical tubes	32
Cuvettes	40
Serological pipettor and pipets	
Marking pen	1

Procedure (Estimated time — 1 hour)

1. Label eight 15 ml conical tubes “1.5 mM Substrate”. Add 15 ml of 1.5 mM substrate to each tube.
2. Label eight 15 ml conical tubes “Enzyme”. Add 5 ml of low concentration enzyme to each tube.
3. Label eight 15 ml conical tubes “Stop Solution”. Add 15 ml of 1x stop solution to each tube.
4. Label eight 15 ml conical tubes “Buffer”. Add 8 ml of 1x resuspension buffer to each tube.
5. Label (near the top) eight cuvettes S1. Add 1 ml of S1 standard to each cuvette and cover the top with parafilm.
6. Label (near the top) eight cuvettes S2. Add 1 ml of S2 standard to each cuvette and cover the top with parafilm.
7. Label (near the top) eight cuvettes S3. Add 1 ml of S3 standard to each cuvette and cover the top with parafilm.
8. Label (near the top) eight cuvettes S4. Add 1 ml of S4 standard to each cuvette and cover the top with parafilm.
9. Label (near the top) eight cuvettes S5. Add 1 ml of S5 standard to each cuvette and cover the top with parafilm.

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

In this activity, students will measure the rate of breakdown of a substrate in the presence of an enzyme or a buffer control. The product can be detected by the formation of yellow color.

Procedure (Estimated time – 15 minutes)

1. Set up student workstations.
2. If you will be using a spectrophotometer, turn it on and allow it to warm up for at least 15 minutes. Set the absorbance wavelength to 410 nm.

Student Workstation	Minimum Quantity Required	(✔)
1.5 mM substrate	3.25 ml	❒
Enzyme (low concentration enzyme)	1.25 ml	❒
1x stop solution	3.75 ml	❒
Buffer (1x resuspension buffer)	0.75 ml	❒
DPTPs	4	❒
15 ml conical tubes	2	❒
Colorimetric standards (S1–S5) in cuvettes	1 of each	❒
Cuvettes	7	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

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

In this activity, students will determine the effect of temperature on the ability of an enzyme to break down its substrate.

Procedure (Estimated time – 30 minutes)

1. Fill up 8 beakers with ice and label them "0°C".
2. Warm up water to 37°C. Right before the lab, pour the water into eight beakers and label them "37°C".
3. If you wil be using a spectrophotometer, turn it on and allow it to warm up for at least 15 minutes. Set the absorbance wavelength to 410 nm.
4. Set up student workstations.

Student Workstation	Minimum Quantity Required	(✔)
1.5 mM substrate	1.75 ml	❒
Enzyme (low concentration enzyme)	1 ml	❒
1x stop solution	1.75 ml	❒
DPTPs	4	❒
Colorimetric standards (S1–S5) in cuvettes	1 of each	❒
1.5 ml microcentrifuge tubes	6	❒
Cuvettes	3	❒
Marker	1	❒
Beaker with ice	1	❒
Beaker with 37°C water	1	❒
Thermometer	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

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

In this activity, students will determine the effect of pH on the ability of an enzyme to break down its substrate.

Procedure (Estimated time – 45 minutes)

1. Label eight 1.5 ml microcentrifuge tubes "3.0 mM Substrate". Add 1 ml of 3.0 mM substrate to each tube.
2. Label eight 1.5 ml microcentrifuge tubes "pH 5.0 Adjustment Buffer". Add 250 µl of 1x resuspension buffer to each tube.
3. Label eight 1.5 ml microcentrifuge tubes "pH 8.6 Adjustment Buffer". Add 250 µl of stop solution to each tube.
4. Prepare pH 6.3 adjustment buffer: Combine 1.25 ml of 1x stop solution with 3.75 ml of deionized or distilled water in a 15 ml conical tube tube and mix. Label eight microcentrifuge tubes "pH 6.3 Adjustment Buffer". Add 250 µl of the pH 6.3 adjustment buffer to the tubes.
5. If you will be using a spectrophotometer, turn it on and allow it to warm up for at least 15 minutes. Set the absorbance wavelength to 410 nm.
6. Set up student workstations.

Note: This activity requires a higher concentration of substrate (3.0 mM) prepared using the instructions provided in the Preparation of Stock Reagents section. If your students have run one of the other five activities, you should remove the tubes containing 1.5 mM substrate from their lab benches to avoid confusion.

Student Workstation	Minimum Quantity Required	(✔)
3.0 mM substrate	1 ml	❒
Enzyme (low concentration enzyme)	1 ml	❒
1x stop solution	1.75 ml	❒
Colorimetric standards (S1–S5) in cuvettes	1 of each	❒
1.5 ml microcentrifuge tube containing		
adjustment buffer, pH 5.0	250 µl	❒
adjustment buffer, pH 6.3	250 µl	❒
adjustment buffer, pH 8.6	250 µl	❒
DPTPs	4	❒
Cuvettes	3	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

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

In this activity, students will determine the effect of enzyme concentration on the initial rate of reaction and the final amount of product formed.

Procedure (Estimated time – 45 minutes)

1. Label eight clean 15 ml conical tubes "High Concentration Enzyme". Add 3 ml of high concentration enzyme to each tube.
2. If you have a spectrophotometer, turn it on and allow it to warm up for at least 15 minutes. Set the absorbance wavelength to 410 nm.
3. Set up student workstations.

Note: This activity requires the high concentration enzyme prepared using the instructions provided in the Preparation of Stock Reagents section. If your students have run one of the other five activities, you should remove their low concentration enzyme from their lab benches to avoid confusion for this activity.

Student Workstation	Minimum Quantity Required	(✔)
1.5 mM substrate	750 µl	❒
High concentration enzyme	3 ml	❒
Buffer (1x resuspension buffer)	1.25 ml	❒
1x stop solution	3.25 ml	❒
Colorimetric standards (S1–S5) in cuvettes	1 of each	❒
DPTPs	4	❒
15 ml conical tube	1	❒
Cuvettes	6	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

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

In this activity, students will determine the effect of substrate concentration on the initial rate of reaction and the final amount of product formed. It is also possible to use this general protocol to perform a Michaelis-Menten analysis, determining the maximum reaction rate (V_{max}) and the Michaelis parameter K_m. Please see Appendix A for more information.

Procedure (Estimated time – 15 minutes)

1. Set up student workstations.
2. If you will be using a spectrophotometer, turn it on and allow it to warm up for at least 15 minutes. Set the absorbance wavelength to 410 nm.

Student Workstation	Minimum Quantity Required	(✔)
1.5 mM substrate	2 ml	❒
Low concentration enzyme	1.75 ml	❒
Buffer (1x resuspension buffer)	1.5 ml	❒
1x stop solution	3.25 ml	❒
Colorimetric standards (S1–S5) in cuvettes	1 of each	❒
15 ml conical tubes	2	❒
DPTPs	4	❒
Cuvettes	2	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

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

In this activity, students will prepare an extract from a mushroom sample and determine if the extract has any cellobiase activity.

Procedure (Estimated time – 45 minutes)

1. Label eight clean 15 ml conical tubes “Extraction Buffer”. Add 5 ml of extraction buffer to each tube.
2. If you will be using a spectrophotometer, turn it on and allow it to warm up for at least 15 minutes. Set the absorbance wavelength to 410 nm.
3. Set up student workstations.

Student Workstation	Minimum Quantity Required	(✔)
Mushroom sample	1 g	❒
1.5 mM substrate	3.25 ml	❒
Stop solution	3.25 ml	❒
Extraction buffer	5 ml	❒
15 ml conical tube	1	❒
1.5 ml microcentrifuge tube	1	❒
DPTPs	4	❒
Cuvettes	6	❒
Mortar and pestle	1	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒
Filter paper, cheese cloth or strainer	1	❒

Typical Classroom Results

The data below represent typical classroom results for experiments run at 22°C and using a SmartSpec Plus spectrophotometer for quantitative analysis. However, please note that the enzyme used in this kit is highly temperature dependent. Because of this, changes in room temperature can have an effect on the rate of reaction relative to the results depicted. An increase in ambient room temperature will increase the reaction rate and can lead to saturation of absorbance at an earlier reaction time point. Conversely, a colder room temperature will lead to slower reaction rates. The trends depicted in the typical classroom data should be consistent irrespective of the exact temperature at which they were run.

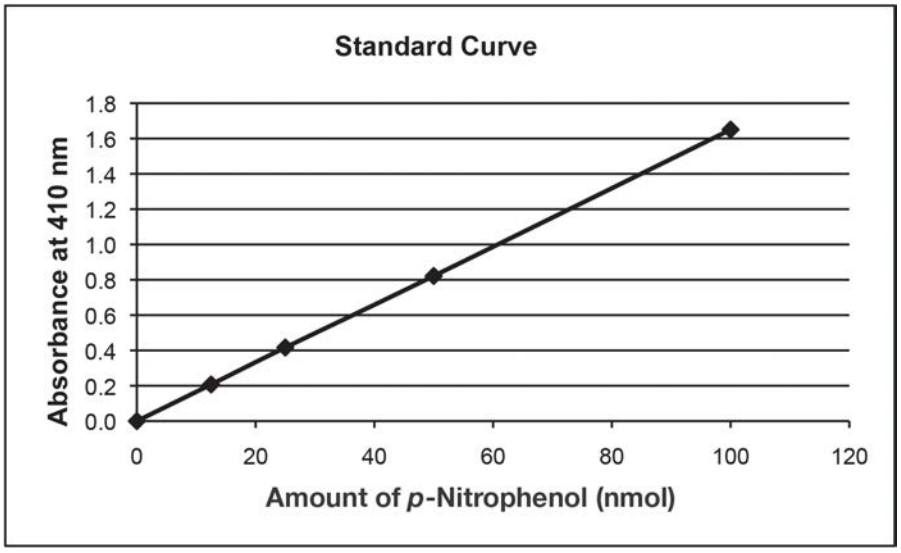
Activity 1: Determine the Reaction Rate in the Presence or Absence of an Enzyme

Qualitative data

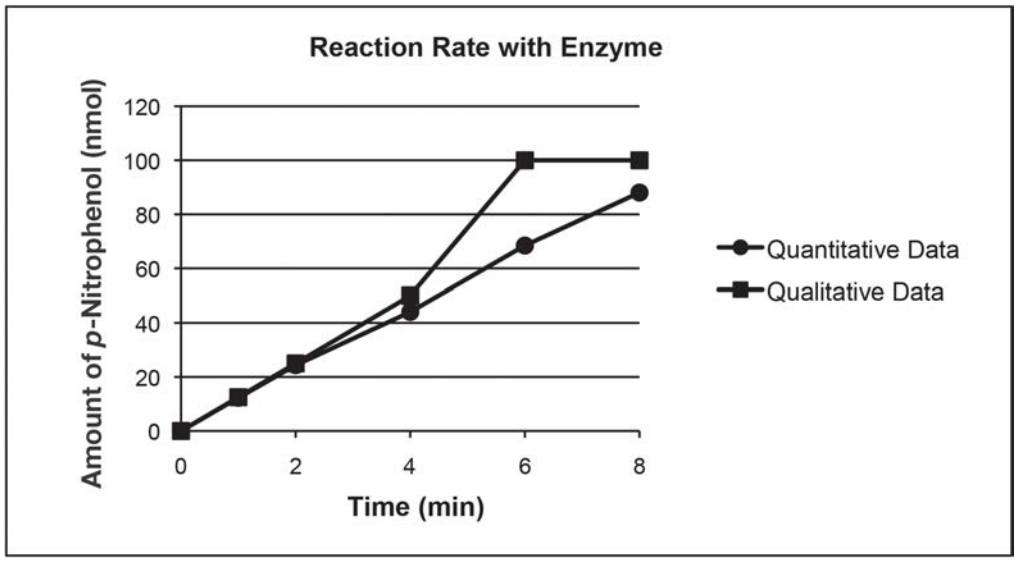
Time (minutes)	Cuvette	Standard That Is Most Similar	Amount of *p*-Nitrophenol (nmol)
0	Start	S1	0
8	End	S1	0
1	E1	S2	12.5
2	E2	S3	25
4	E3	S4	50
6	E4	S5	100
8	E5	S5	100

Quantitative data

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



Time (minutes)	Cuvette	Amount of *p*-Nitrophenol (nmol) from the Standard Curve	Absorbance at 410 nm
0	Start	0.0	0.00
8	End	0.0	0.00
1	E1	12.3	0.20
2	E2	24.3	0.40
4	E3	44.1	0.72
6	E4	68.4	1.12
8	E5	88.0	1.45



	Qualitative	Quantitative
Initial rate of product formation with enzyme present	12.5 nmol/min	11.2 nmol/min
Rate of product formation with no enzyme present	0 nmol/min	0 nmol/min

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

The enzyme provided in this kit increases reaction rate with increasing temperature. The reaction rate should be close to 0 nmol/min for experiments run at 0°C and increase as the temperature increases. The enzyme can be inactivated if it is heated to 95°C for at least five minutes.

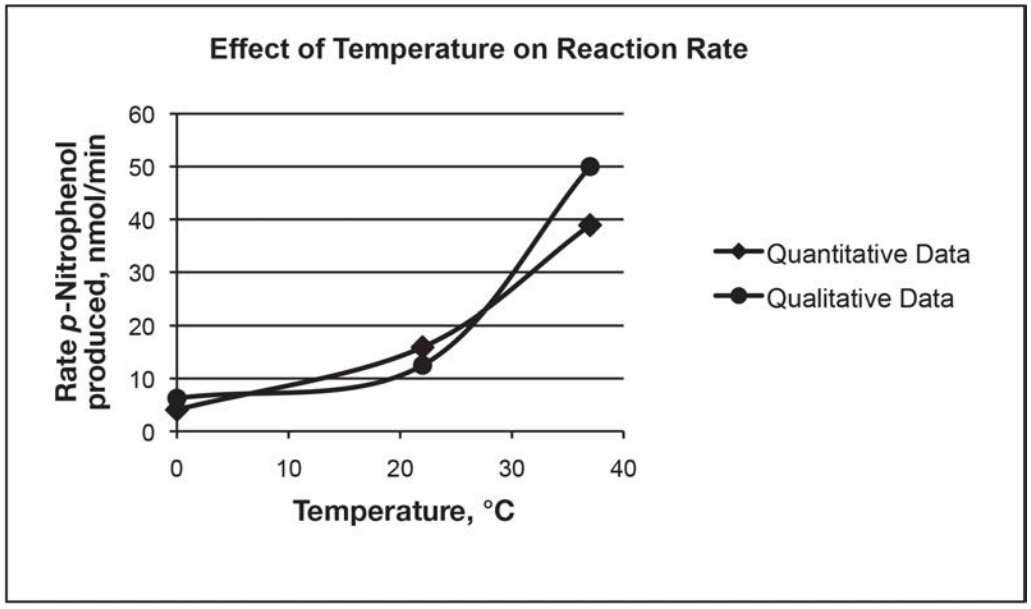
Qualitative Results

Temperature	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
0°C	S2	12.5
~22°C (room temperature)	S3	25
37°C	S5	100

Quantitative Results

Temperature	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
0°C	0.13	8.1
~22°C (room temperature)	0.53	31.2
37°C	1.28	77.8

	Qualitative	Quantitative
Initial rate of product formation at 0°C	6.2 nmol/min	4.1 nmol/min
Initial rate of product formation at room temperature	12.5 nmol/min	15.9 nmol/min
Initial rate of product formation at 37°C	50 nmol/min	38.9 nmol/min



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

The enzyme provided in this kit has optimal activity at pH 5 and is inactivated at higher pH values. Therefore, the highest reaction rate should be measured at pH 5 and should decrease with increasing pH values.

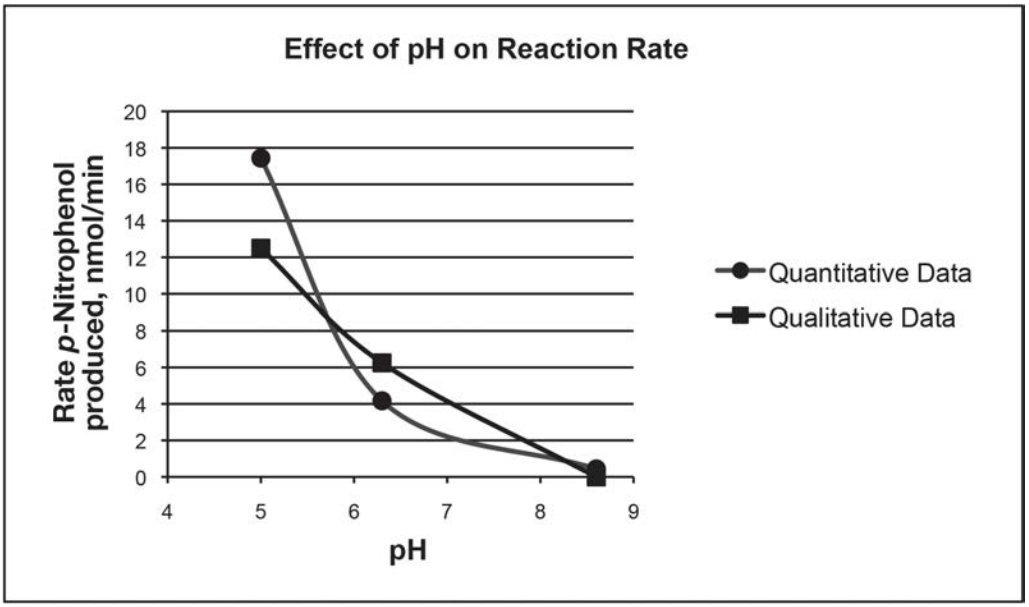
Qualitative Results

pH	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
pH 5.0	S3	25
pH 6.3	S2	12.5
pH 8.6	S1	0

Quantitive Results

pH	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
pH 5.0	0.58	34.9
pH 6.3	0.14	8.3
pH 8.6	0.01	0.9

	Qualitative	Quantitative
Initial rate of product formation at pH 5.0	12.5 nmol/min	17.4 nmol/min
Initial rate of product formation at pH 6.3	6.2 nmol/min	4.1 nmol/min
Initial rate of product formation at pH 8.6	0 nmol/min	0.4 nmol/min



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

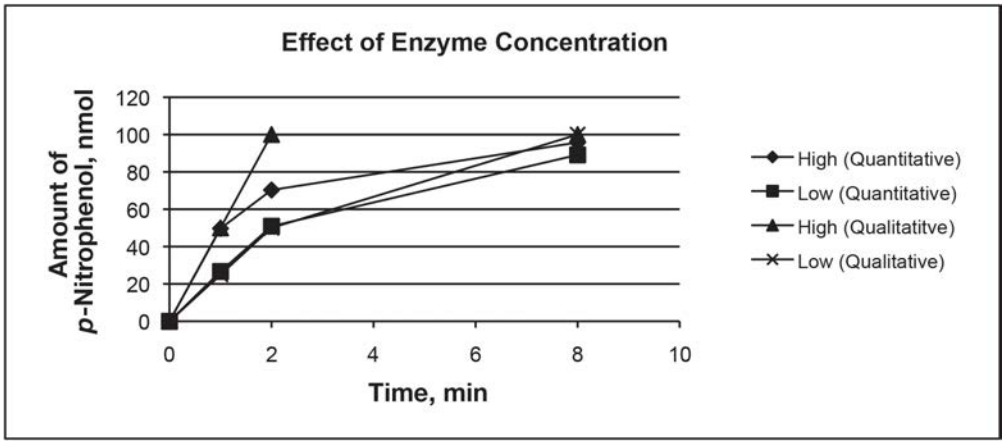
An increase in enzyme concentration will increase the initial reaction rate. If the reaction is allowed to proceed for long enough times, since the same amount of substrate was used for the high concentration enzyme and low concentration enzyme reactions, the final amount of product for both reactions will be comparable. The amount of substrate used for this activity was limited so that it will all be converted to product within the time frame of the experiment.

Qualitative Results

Cuvette	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
H1	S4	50
H2	S5	100
H3	S5	100
L1	S3	25
L2	S4	50
L3	S5	100

Quantitative Results

Cuvette	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
H1	0.82	49.7
H2	1.16	70.3
H3	1.58	95.8
L1	0.44	26.7
L2	0.84	50.9
L3	1.47	89.1



	Qualitative	Quantitative
Initial rate of reaction for high enzyme concentration	50 nmol/min	49.7 nmol/min
Initial rate of reaction for low enzyme concentration	25 nmol/min	26.7 nmol/min

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

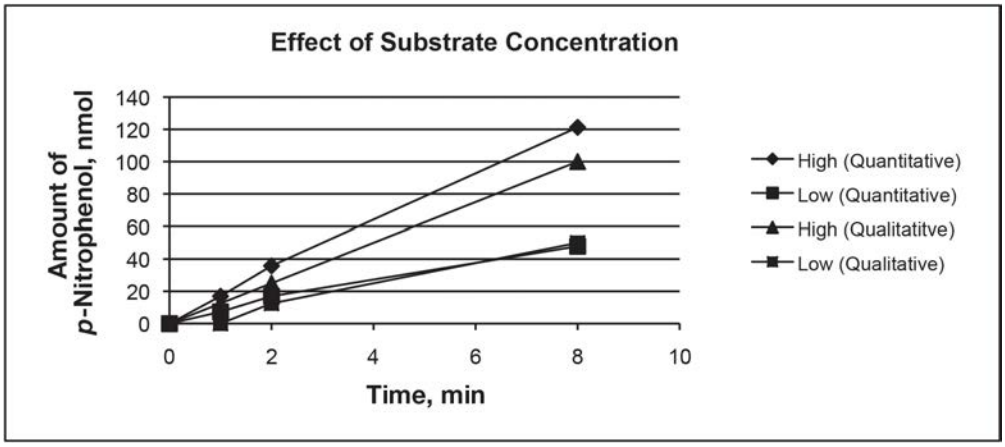
An increase in substrate concentration will increase the initial reaction rate assuming that the enzyme present is not completely saturated with substrate. The high and low substrate concentration reactions were designed so that the high concentration substrate reaction would be close to enzyme saturation while the low concentration substrate reaction will not completely saturate the enzyme. The final amount of product will be higher for the high substrate concentration reaction than the low substrate concentration reaction, even if the reaction is allowed to proceed for an extremely long time.

Qualitative Results

Cuvette	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
H1	S2	12.5
H2	S3	25
H3	S5	100
L1	S1	0
L2	S2	12.5
L3	S4	50

Quantitative Results

Cuvette	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
H1	0.28	16.9
H2	0.59	35.8
H3	2.00	121
L1	0.12	7.3
L2	0.28	16.9
L3	0.79	47.9



	Qualitative	Quantitative
Initial rate of reaction for high substrate concentration	12.5 nmol/min	16.9 nmol/min
Initial rate of reaction for low substrate concentration	0 nmol/min	7.3 nmol/min

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

Results will vary.

Tips and Frequently Asked Questions

1. **I have a spectrophotometer that requires 4 ml samples. Can I still use this kit?**

   The absorbance range for the reactions and standards in this kit is approximately 0.2–1.7. Therefore, if you want to run just a quantitative assay, it is possible to run the experiments with twice the volume of substrate and enzyme and also dilute the final solutions 1:1 with deionized or distilled water. This means that all samples will be 4 ml in volume and have an absorbance in the range of 0.1–0.85. It should be noted that if double the volume of solutions is used, there will not be enough solutions to run all six activities for eight workstations. Also, 4 ml sample tubes for the spectrophotometer should be used instead of the cuvettes included in this kit. The cuvettes will only hold ~2 ml.

2. **My spectrophotometer is no longer linear above an absorbance of 1. What can I do?**

   The following solution assumes that the activities will be run using only quantitative data. After reactions are completed and added to the stop solution, you can add 1 ml of deionized or distilled water to each cuvette. Mix the solution and read the absorbance at 410 nm. This should bring the absorbance down below 1.0 at 410 nm.

3. **Is it possible to use a wavelength other than 410 nm to read the samples?**

   p-Nitrophenol has a peak absorbance from 400–410 nm. At higher wavelengths, there is a decrease in the signal intensity. It is possible to read samples at a higher wavelength, but sensitivity at the lower concentration range of *p*-nitrophenol will be compromised.

4. **Can I store my diluted enzyme, standards, substrate, and buffers over multiple weeks?**

   If stored at 4°C, the diluted enzyme is stable for one week, the standards and reconstituted substrate for one month and the buffers for 3 years.

5. **I spilled my stop solution and need to run the lab today. Is there anything I can do?**

   Sodium hydroxide solution, with a pH of 9.5 or above can be used as a stop solution.

6. **Is it possible to run experiments at temperatures higher than 37°C or at other pH values?**

   If extra temperature or pH value data points are desired, these experiments can be run, but there will not be enough reagents to run all six activities for eight workstations. Also, the enzyme included in this kit has increasing activity at higher temperatures. It is possible that the absorbance will be beyond the linear range of the spectrophotometer at higher temperatures. Samples can be diluted in deionized or distilled water before being read on the spectrophotometer if this is occurring. Reactions run at higher temperatures should also use screwcap tubes (not included in this kit) for safety.

7. **What temperature will deactivate the enzyme?**

   Heating to 95°C for at least 5 minutes will deactivate the enzyme.

8. **We are interested in doing a full Michaelis-Menten study. Is there a way to do that with this kit?**

   The protocol used in Activity 5 can be used. Please see Appendix A for details. The 3 mM substrate and 1.5 mM substrate that were prepared during the Instructor's Advance Preparation can be diluted to various substrate concentrations. The initial reaction rate can be determined for these different starting substrate concentrations and used to generate a Lineweaver-Burk plot. There will not be enough reagents to run all six activities for eight workstations if full Michaelis-Menten analyses are run.

9. Why is picking wild mushrooms not recommended for this lab?

Since wild gathered mushrooms have the potential to be poisonous, it is recommended to use those from the supermarket. The following mushrooms have been found to work with this kit: white button, crimini, shiitake, dried shiitake, enoki, and oyster mushrooms.

10. Will other sources of cellobiase, such as health supplements, work with this lab?

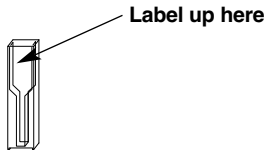
Health supplement sources have not been tested, but if they have β-glucosidic activity, they should work.

Quick Guide

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

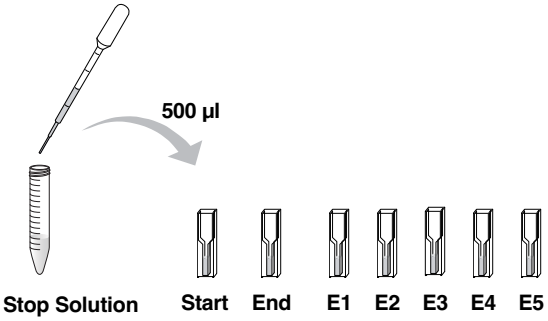
1. Find your 15 ml conical tubes labeled "Stop Solution", "1.5 mM Substrate", "Enzyme" and "Buffer". Write your initials on each tube.

2. Label five cuvettes E1–E5.



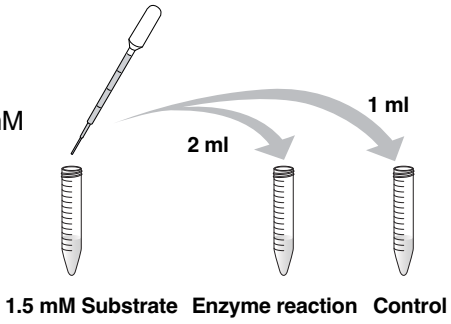
3. Label the two remaining cuvettes "Start" and "End".

4. Using a clean DPTP, pipet 500 µl of stop solution into each labeled cuvette. Rinse the DPTP well with water.



5. Label one empty 15 ml conical tube "Enzyme Reaction" and the other "Control".

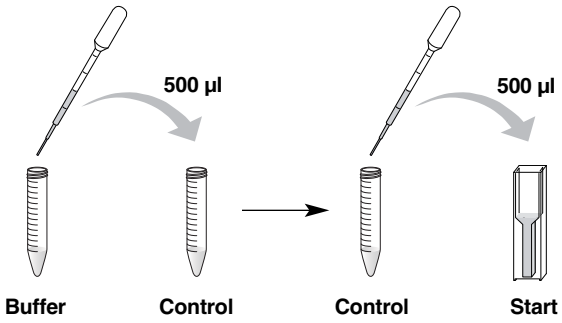
6. Using a clean DPTP, pipet 2 ml of 1.5 mM substrate into the 15 ml conical tube labeled "Enzyme Reaction". Use the same DPTP and pipet 1 ml of 1.5 mM substrate into the conical tube labeled "Control". Rinse the DPTP well with water.



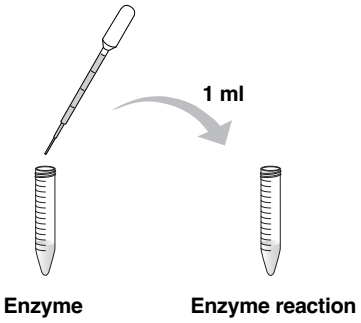
7. Label one DPTP "E" for enzyme and the other "C" for control. Only use the DPTP labeled "E" for the enzyme reaction tube and the DPTP labeled "C" for the control reaction tube.

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

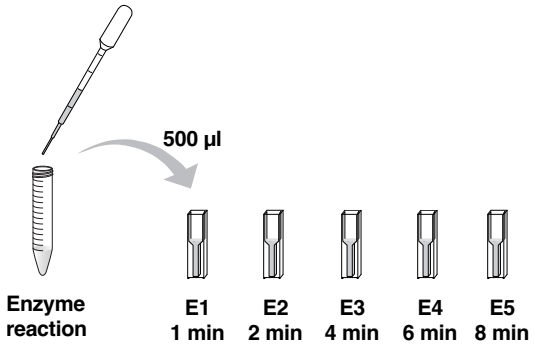
8. Using the DPTP labeled “C”, pipet 500 µl of buffer into the 15 ml conical tube labeled “Control” and gently mix. Once you have mixed the buffer with the substrate, remove 500 µl of this solution and add it to your cuvette labeled “Start”.



9. Using the DPTP labeled “E”, pipet 1 ml of enzyme into the 15 ml conical tube labeled “Enzyme Reaction”. Gently mix, then **START YOUR TIMER**.

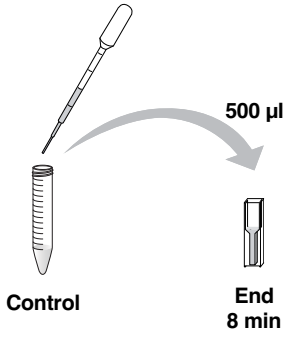


10. At the times indicated, use the DPTP labeled “E” to remove 500 µl of the solution from the “Enzyme Reaction” tube and add it to the appropriately labeled cuvette containing the stop solution.



11. After all the enzyme samples have been collected, use the DPTP labeled “C” to remove 500 µl of the solution from the “Control” reaction tube and add it to the cuvette labeled “End”.

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



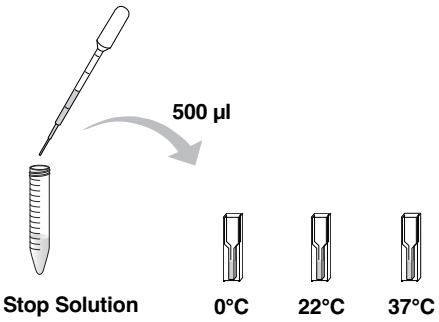
Note: Do not discard unused stock solutions or cuvettes containing standards. They will be used for the next activity.

Quick Guide

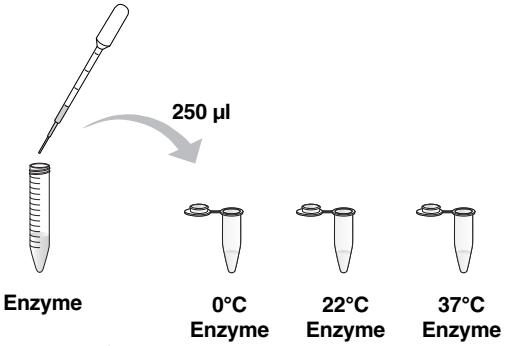
Activity 2: Determine the Effect of Temperature on the Reaction Rate

1. Label your cuvettes “0°C”, “22°C”, and “37°C”.

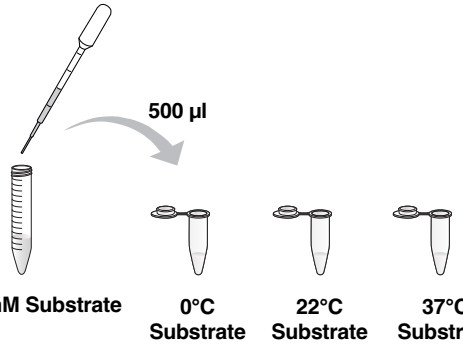
2. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Wash the DPTP out thoroughly with water.



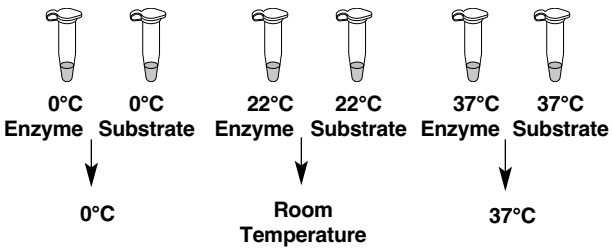
3. Label three 1.5 ml microcentrifuge tubes “0°C Enzyme”, “22°C Enzyme”, and “37°C Enzyme”. Using a clean DPTP, pipet 250 µl of enzyme into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.



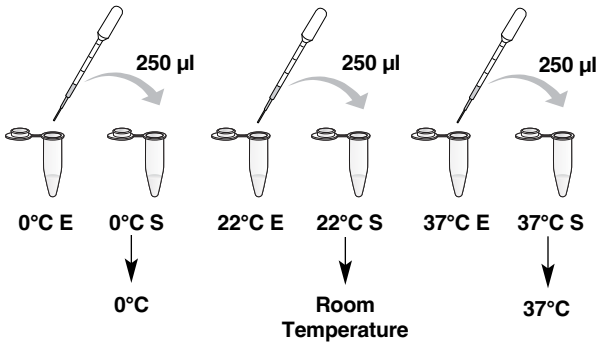
4. Label three 1.5 ml microcentrifuge tubes “0°C Substrate”, “22°C Substrate”, and “37°C Substrate”. Using a clean DPTP, pipet 500 µl of 1.5 mM substrate into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.



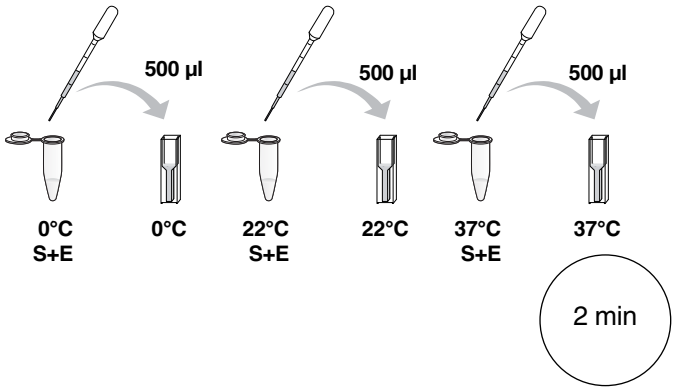
5. Place the tubes labeled “0°C Enzyme” and “0°C Substrate” in the ice cup. Place the tubes labeled “22°C Enzyme” and “22°C Substrate” on your lab bench. Place the tubes labeled “37°C Enzyme” and “37°C Substrate” in the beaker of warm water at 37°C. Allow the tubes to equilibrate to their respective temperatures for at least 5 minutes.



6. Have a stopwatch ready. Using a clean DPTP, pipet the 250 µl of enzyme from the tube labeled “0°C Enzyme” into the tube labeled “0°C Substrate”, and place the tube now containing your enzyme and substrate mix back on ice. Add the 22°C enzyme to the 22°C substrate solution, and place that tube back on the lab bench. Add the 37°C substrate to the 37°C enzyme solutions, and put that tube back into the 37°C water bath. **START YOUR TIMER**.



7. After 2 minutes, use a clean DPTP for each temperature reaction to transfer 500 µl of your reaction to the appropriately labeled cuvette containing the stop solution.



8. Proceed with the analysis of your samples. After you have finished your analysis, rinse out the cuvettes and DPTPs with copious water and save them for later activities.

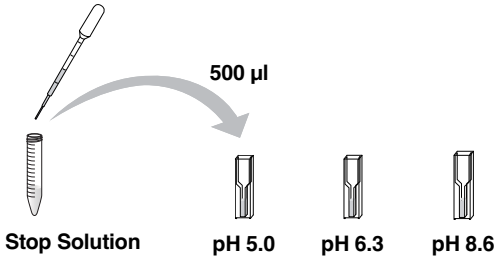
Note: Do not discard unused stock solutions or cuvettes containing standards. They will be used for the next activity.

Quick Guide

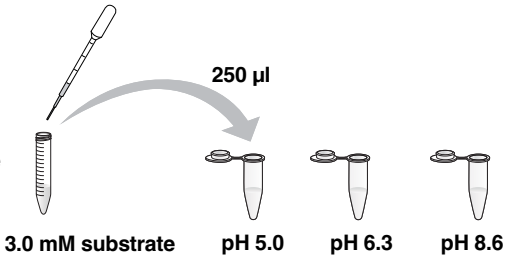
Activity 3: Determine the effect of pH on the Reaction Rate

1. Label your cuvettes “pH 5.0”, “pH 6.3”, and “pH 8.6”.

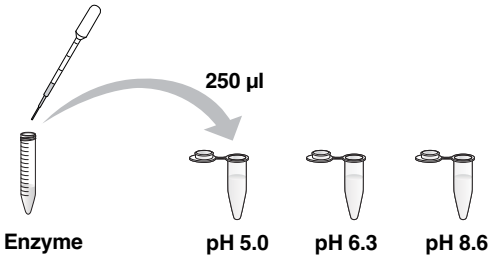
2. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Wash the DPTP out thoroughly with water.



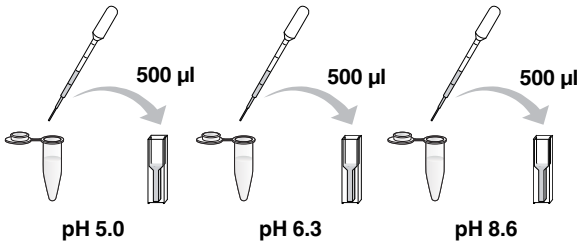
3. Using a clean DPTP, pipet 250 µl of 3.0 mM substrate into each microcentrifuge tube labeled “pH 5.0”, “pH 6.3” and “pH 8.6” by your instructor. Wash the DPTP out thoroughly with water.



4. Have a stopwatch ready. Using a clean DPTP, add 250 µl of enzyme to each of the labeled microcentrifuge tubes. **START YOUR TIMER**.



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



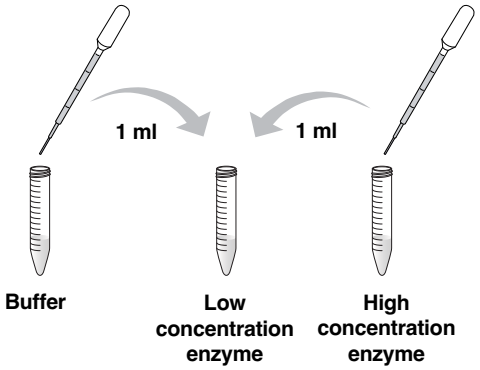
6. Proceed with the analysis of your samples. After you have finished your analysis, rinse out the cuvettes and DPTPs with copious water and save them for later activities.

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

Quick Guide

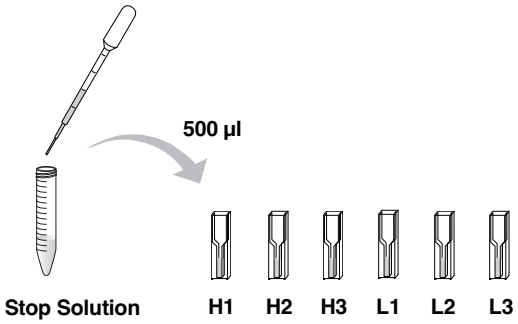
Activity 4: Determine the Effect of Enzyme Concentration on the Reaction Rate

1. Label one 15 ml conical tube “Low Concentration Enzyme”. Using a clean DPTP, pipet 1 ml of buffer into the tube. Wash out the DPTP with water. Pipet 1 ml of high concentration enzyme to your tube labeled “Low Concentration Enzyme” and mix. Wash out the DPTP thoroughly with water.



2. Label three cuvettes “H1–H3” (for high enzyme concentration time points) and the remaining three cuvettes “L1–L3” (for low enzyme concentration time points). Only label on the upper part of the cuvette face.

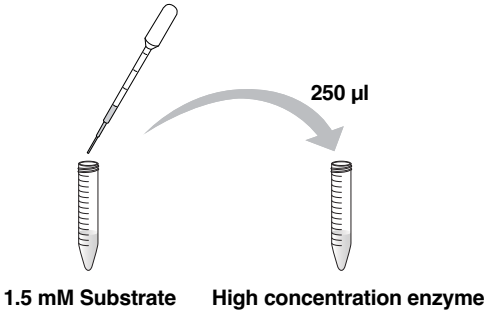
3. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Wash out the DPTP thoroughly with water.



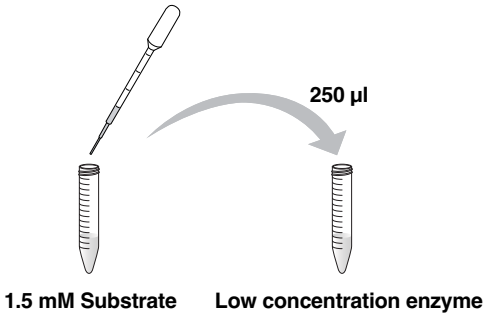
4. Label one clean DPTP with an “H” for high enzyme concentration and a second clean DPTP with an “L” for low enzyme concentration.

Please read steps 5–7 fully before proceeding. These steps are time sensitive!

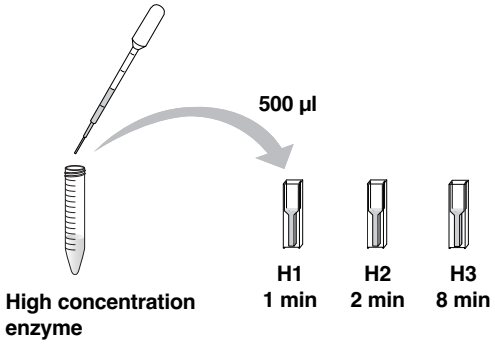
5. Using the DPTP labeled with an “H”, pipet 250 µl of 1.5 mM substrate into your 15 ml conical tube containing enzyme labeled “High Concentration Enzyme”.

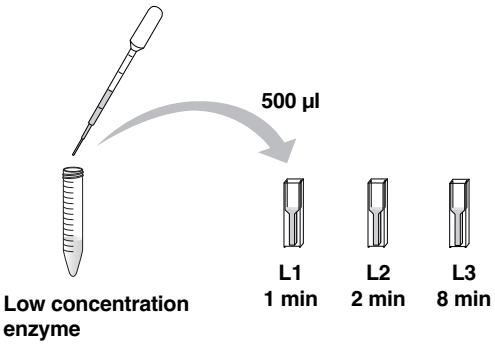


6. Using the DPTP labeled with an “L”, pipet 250 µl of 1.5 mM substrate into your 15 ml conical tube containing enzyme labeled “Low Concentration Enzyme”. **START YOUR TIMER**.



7. At the times indicated, use the correctly labeled DPTP to remove 500 µl from the 15 ml conical tubes labeled “High Concentration Enzyme” and “Low Concentration Enzyme”, and add it to the appropriately labeled cuvette that already contains the stop solution.





8. Proceed with the analysis of your samples. After you have finished your analysis, rinse out reaction tubes, cuvettes, and DPTPs with copious water and save them for later activities.

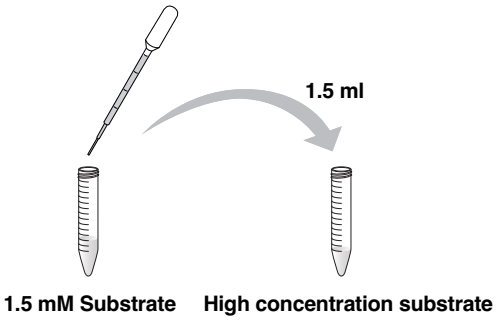
Note: Do not discard unused stock solutions or cuvettes containing standards. They will be used for the next activity.

Quick Guide

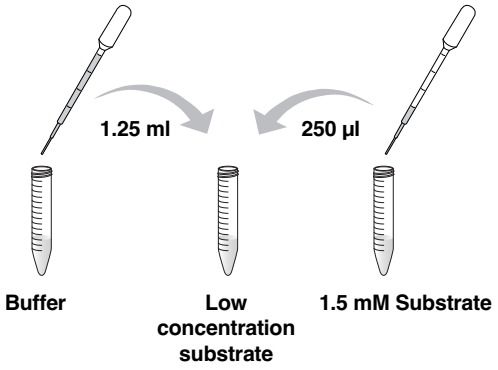
Activity 5: Determine the Effect of Substrate Concentration on the Reaction Rate

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

2. Using a clean DPTP, pipet 1.5 ml of 1.5 mM substrate into the 15 ml conical tube labeled “High Concentration Substrate”. Rinse the DPTP thoroughly with clean water.



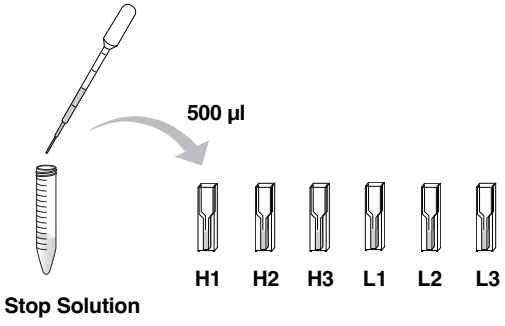
3. Using a clean DPTP, pipet 1.25 ml of buffer into the 15 ml conical tube labeled “Low Concentration Substrate”. Rinse the DPTP thoroughly with water and then pipet 250 µl of 1.5 mM substrate into the 15 ml conical tube labeled “Low Concentration Substrate” and mix. Rinse the DPTP thoroughly with water.



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

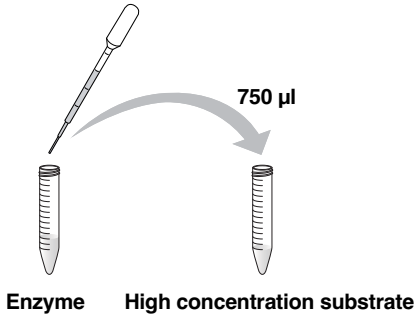
5. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Rinse the DPTP thoroughly with water.

6. Label one DPTP as “H” for high substrate concentration and a second DPTP as “L” for low substrate concentration.

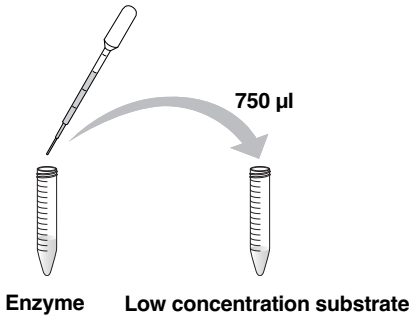


Please read and understand steps 7–9 fully before proceeding. These steps are time sensitive!

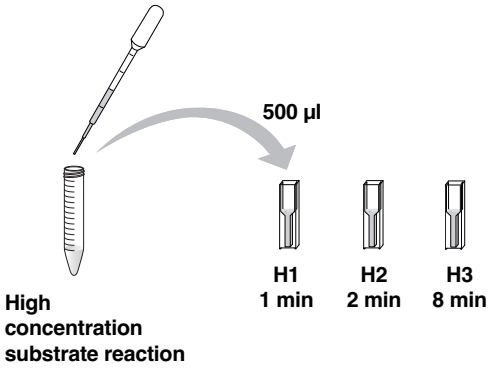
7. Using a clean DPTP, pipet 750 µl of enzyme into your 15 ml conical tube of substrate labeled "High Concentration Substrate".



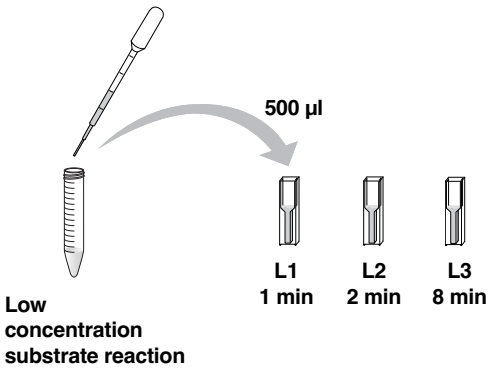
8. Using a clean DPTP, pipet 750 µl of enzyme into your 15 ml conical tube of substrate labeled "Low Concentration Substrate". **START YOUR TIMER**.



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



10. Proceed with the analysis of your samples. After you have finished your analysis, rinse out the reaction tubes, cuvettes, and DPTPs with copious water and save them for later activities.



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

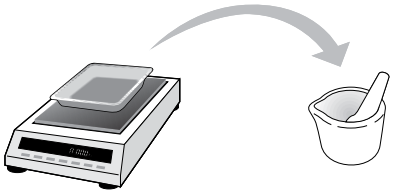
Quick Guide

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

1. Write down the name of your mushroom

   _____

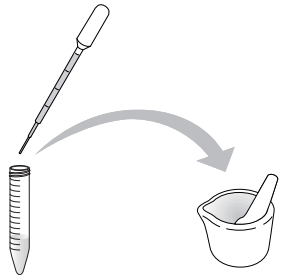
2. Weigh out approximately 1 gram of mushroom and put it into a mortar.



3. Add 2 ml of extraction buffer for every gram of mushroom into the mortar. To calculate the amount of extraction buffer you need, multiply the weight (in grams) of the mushroom by 2 and add that many milliliters.

Weight of mushroom ____ g x 2 = _____ ml

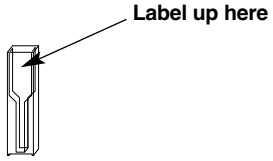
4. Using a pestle, grind your mushroom to produce a slurry.



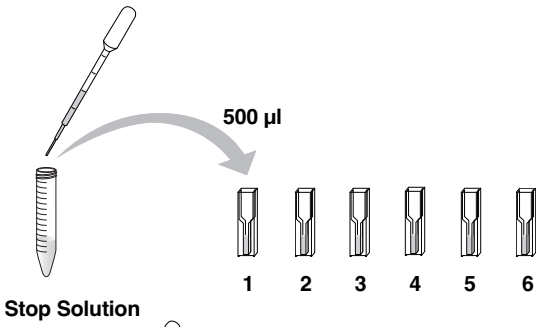
Extraction buffer

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.

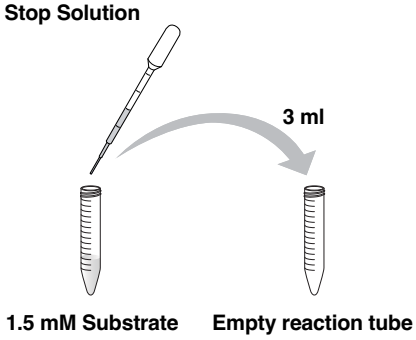
6. Label your cuvettes “1–6”. Only label on the upper part of the cuvette face.



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

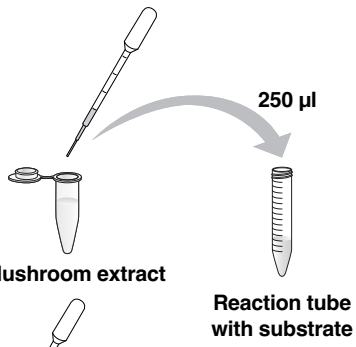


8. Label a 15 ml conical tube with the type of mushroom you are using and then using a clean DPTP, pipet 3 ml of substrate into the tube.

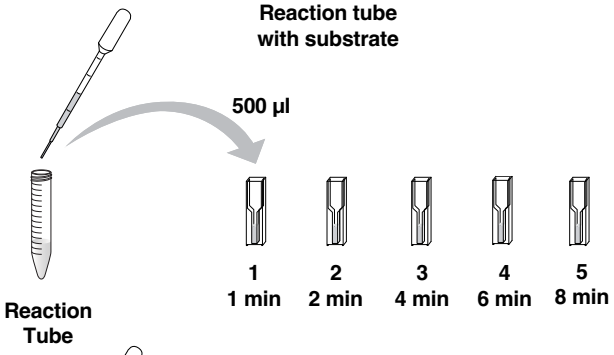


Please read and understand steps 9–10 fully before proceeding. These steps are time sensitive!

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

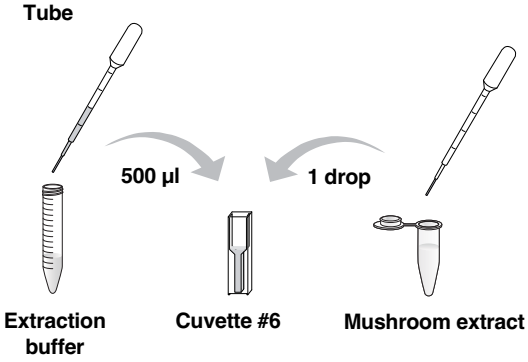


10. At the times indicated, remove 500 µl of mushroom extract/substrate mixture from the reaction tube, and add it to the appropriately labeled cuvette that already contains the stop solution.



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

12. Proceed with the analysis of your samples. After you have finished your analysis, rinse out the reaction tubes, cuvettes, and DPTPs with copious water and save them for later activities.



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

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.

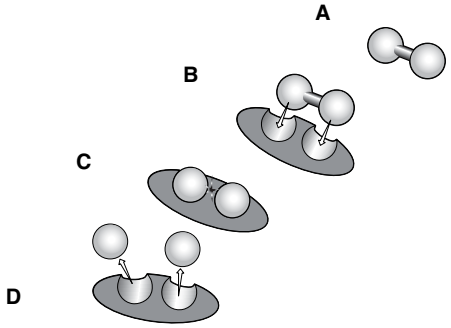


Fig. 5. A schematic of cellobiose and cellobiase in solution. A. Cellobiose in solution is composed of two glucose molecules covalently connected by a β 1–4 linkage. **B**. Cellobiase has a pocket that fits the cellobiose molecule. **C**. Cellobiase helps stabilize the cellobiose in a shape so that the 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 dissacharide 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.

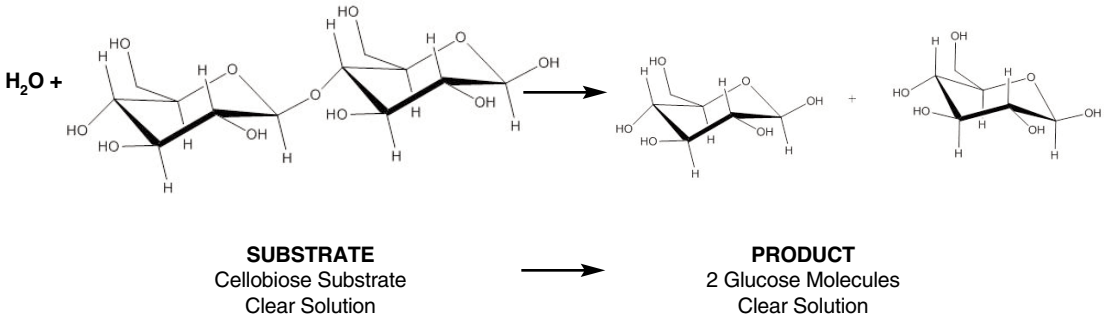


Fig. 6. Breakdown of cellobiose by cellobiase. The natural substrate of cellobiase is the dissacharide cellobiose. When cellobiose is bound by cellobiase, the cellobiase breaks apart the β 1–4 bond that connects the two glucose molecules and then releases two glucose molecules.

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.

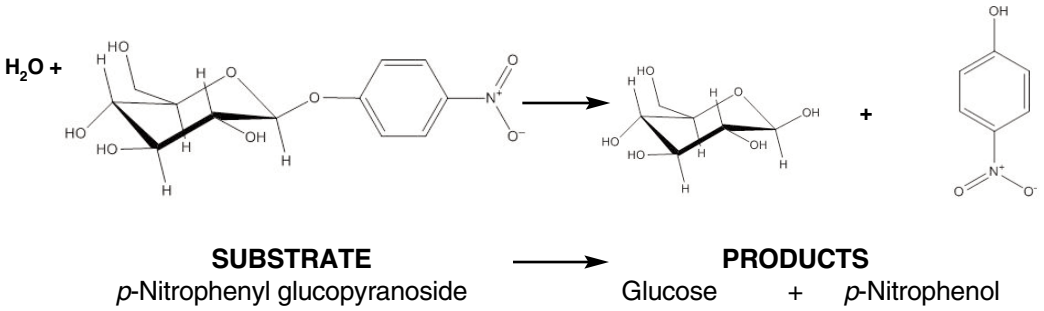


Fig. 7. Breakdown of *p*-nitrophenyl glucopyranoside into glucose and *p*-nitrophenol by cellobiase. When the *p*-nitrophenyl glucopyranoside is broken apart by cellobiase, one molecule of glucose and one molecule of *p*-nitrophenol are released. If the *p*-nitrophenol is put into a basic solution, it 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 1: Determine the Reaction Rate in the Presence or Absence of an Enzyme

In this activity, you will compare the rate of breakdown of *p*-nitrophenyl glucopyranoside into glucose and *p*-nitrophenol in the presence and absence of cellobiase. Enzymes are molecules that increase the rate of a reaction, but are not used up in the reaction. Because the enzyme can keep processing the substrate over and over again, very few molecules of enzyme are needed relative to the number of molecules of substrate.

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

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

Student Workstation	**Quantity**	**(✔)**
1.5 mM substrate	1	❒
Enzyme	1	❒
1x stop solution	1	❒
Buffer	1	❒
DPTPs	4	❒
15 ml conical tubes	2	❒
Colorimetric standards (S1–S5) in cuvettes	1 of each	❒
Cuvettes	7	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

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

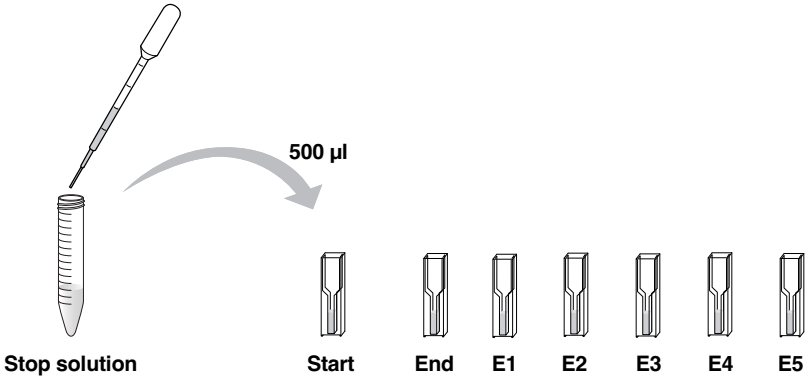
Protocol

1. Locate the 15 ml conical tubes labeled “Stop Solution”, “1.5 mM Substrate”, “Enzyme” and “Buffer”. Label each of the tubes with your initials.

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

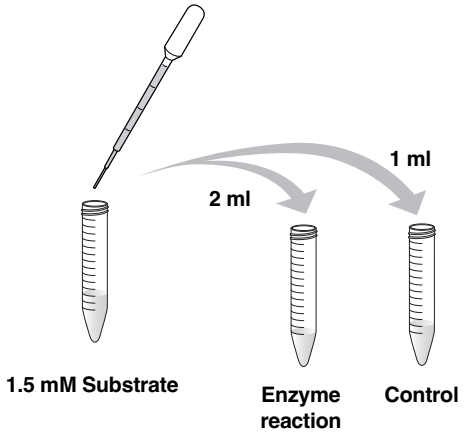
3. Label the two remaining cuvettes “Start” and “End” on the upper part of the cuvette. The cuvettes will serve as control time points at the start and end of the reaction and neither cuvette will contain enzyme.

4. Using a clean DPTP, pipet 500 µl of stop solution into each of the seven labeled cuvettes. The stop solution is a strong base, so avoid getting it on your skin or clothes. Rinse the DPTP well with water and save it for future activities.



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

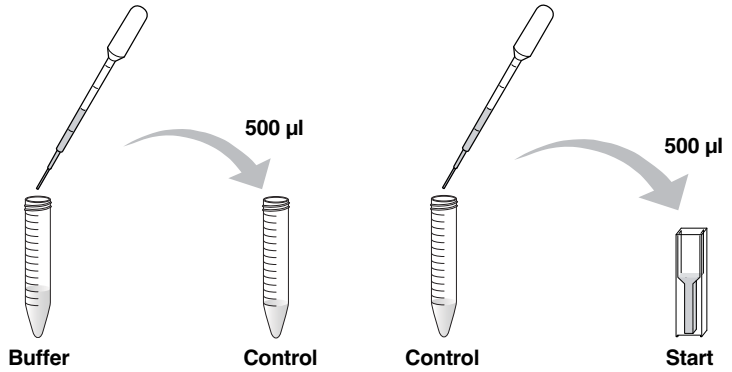
6. Using a clean DPTP, pipet 2 ml of 1.5 mM substrate into the 15 ml conical tube labeled “Enzyme Reaction”. Use the same DPTP and pipet 1 ml of 1.5 mM substrate into the conical tube labeled “Control”. Rinse the DPTP well with water and save it for future activities.



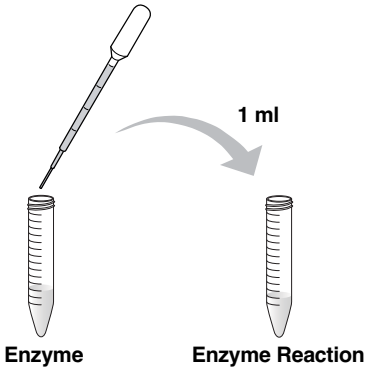
7. Label one DPTP "E" for enzyme and the other "C" for control. **Only use the DPTP labeled "E" for the enzyme reaction tube and the DPTP labeled "C" for the control reaction tube**.

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

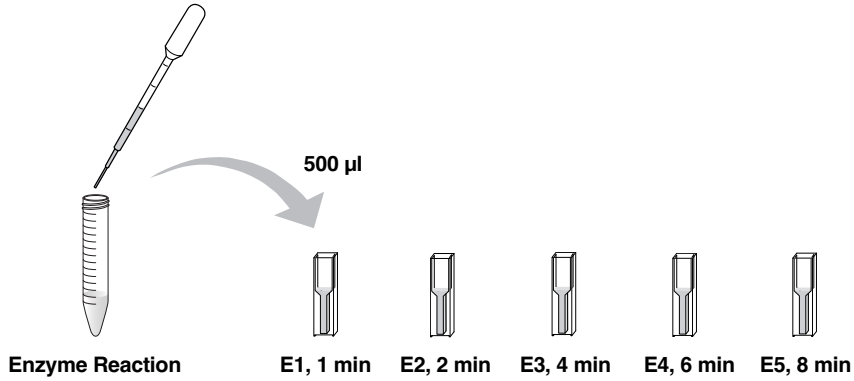
8. Using the DPTP labeled "C", pipet 500 µl of buffer into the 15 ml conical tube labeled "Control" and gently mix. Once you have mixed the buffer with the substrate, remove 500 µl of this solution and add it to your cuvette labeled "Start".



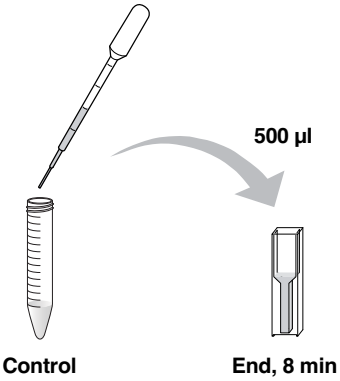
9. Using the DPTP labeled "E", pipet 1 ml of enzyme into the 15 ml conical tube labeled "Enzyme Reaction". Gently mix, then **START YOUR TIMER**. This marks the beginning of the enzymatic reaction.



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



11. After all the enzyme samples have been collected, use the DPTP labeled "C" to remove 500 µl of the solution from the "C" reaction tube and add it to the cuvette labeled "End".



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

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.

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. Compare all 7 cuvettes (control and reaction cuvettes) to the standard cuvettes by holding them against a white background. Record in Table 2 the standard that is most similar to your control and enzyme reaction 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 mol

Table 2. Comparison of reaction cuvettes to standard cuvettes.

Time (minutes)	Cuvette	Standard That Is Most Similar	Amount of *p*-Nitrophenol (nmol)
0	Start		
8	End		
1	E1		
2	E2		
4	E3		
6	E4		
8	E5		

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

Quantitative 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. 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 correlates the absorbance at 410 nm with the amount of *p*-nitrophenol present.

Table 3. Absorbance values for standards.

Standard	Amount of *p*-Nitrophenol (nmol)	Absorbance at 410 nm
S1	0	0.00
S2	12.5	
S3	25	
S4	50	
S5	100	

2. Measure the absorbance of your enzyme-catalyzed reaction cuvettes (E1–E5) and your control cuvettes (Start, End) at 410 nm, and record your results in Table 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 (minutes)	Cuvette	Amount of *p*-Nitrophenol (nmol) from the Standard Curve	Absorbance at 410 nm
0	Start		
8	End		
1	E1		
2	E2		
4	E3		
6	E4		
8	E5		

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

In the example shown in Figure 8, the solid filled diamonds represent the absorbance values for the five standards. The best line connecting all the data points is then drawn. The circle symbol represents the E3 data point; its absorbance was measured to be 0.73. To determine the amount of product corresponding to this value, locate the absorbance value of 0.73 (approximately) on the y-axis and then follow the value horizontally until it intersects with the standard curve. From this point, draw a line down to the x-axis (amount of *p*-nitrophenol) and read the value directly from the graph. In this case, it is approximately 44 nmol. This same process is done with the remaining data points.

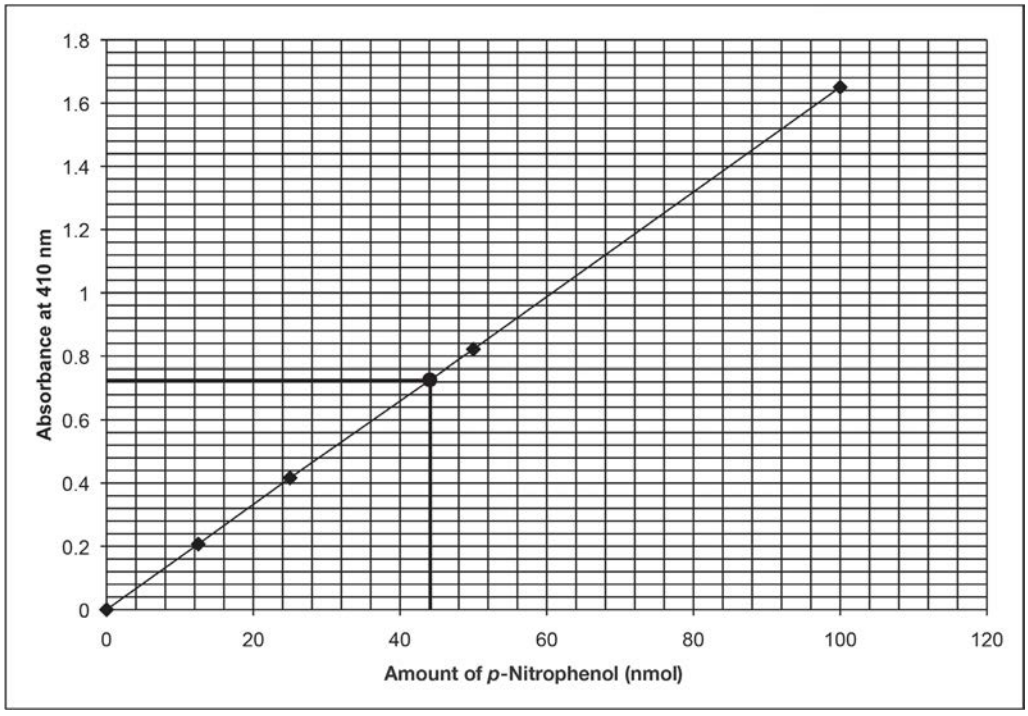


Fig. 8. Example of a standard curve. Absorbance of *p*-nitrophenol is plotted against known quantities of *p*-nitrophenol standards, S1–S5.

Use the data in Table 3 to create a standard curve. Plot the absorbance values for each standard in Figure 9, and then draw the line that best goes through all the data points. As described in the example on page 47, plot the absorbance values for the five time points (E1–E5) and non-catalyzed reaction (Start and End), then determine the corresponding amount of product for each time point. Record this information in Table 4.

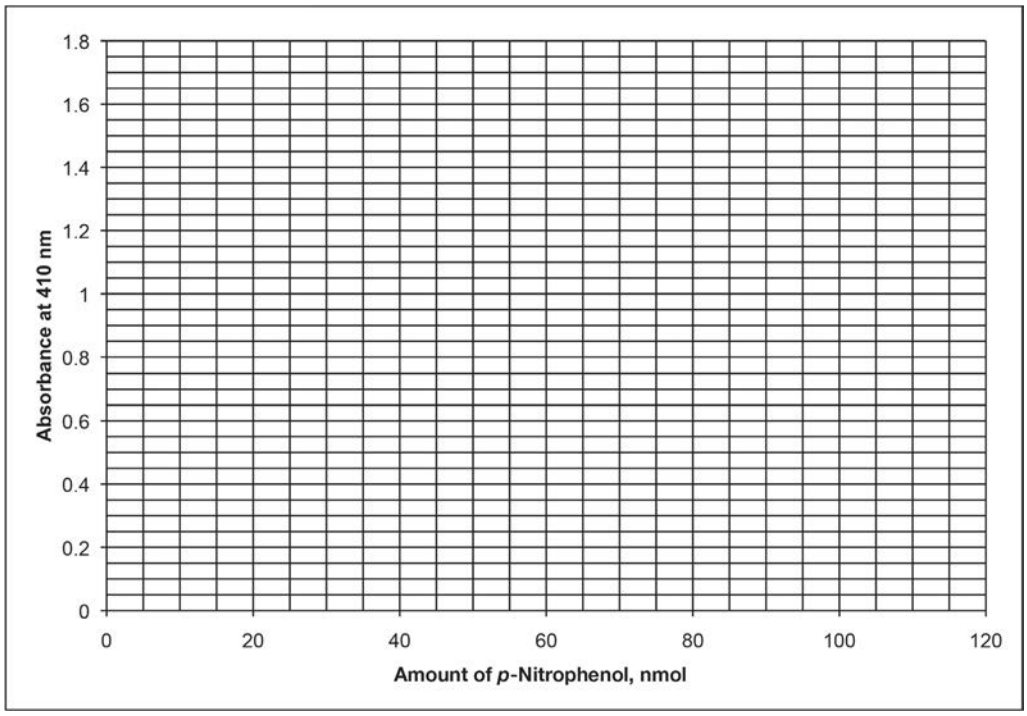


Fig. 9. Creating a standard curve by plotting absorbance of *p*-nitrophenol against known quantities of *p*-nitrophenol standards, S1–S5.

Analysis 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 the reaction progresses, there is less substrate readily available, because it is being converted to product. If you graph the amount of product formed at each time point, the data can be used to calculate the initial rate of product that is formed in the presence or absence of enzyme.

In Figure 10, the amount of product, *p*-nitrophenol, is plotted over time to determine the initial rate of product formed. The unit of rate is nmol/min.

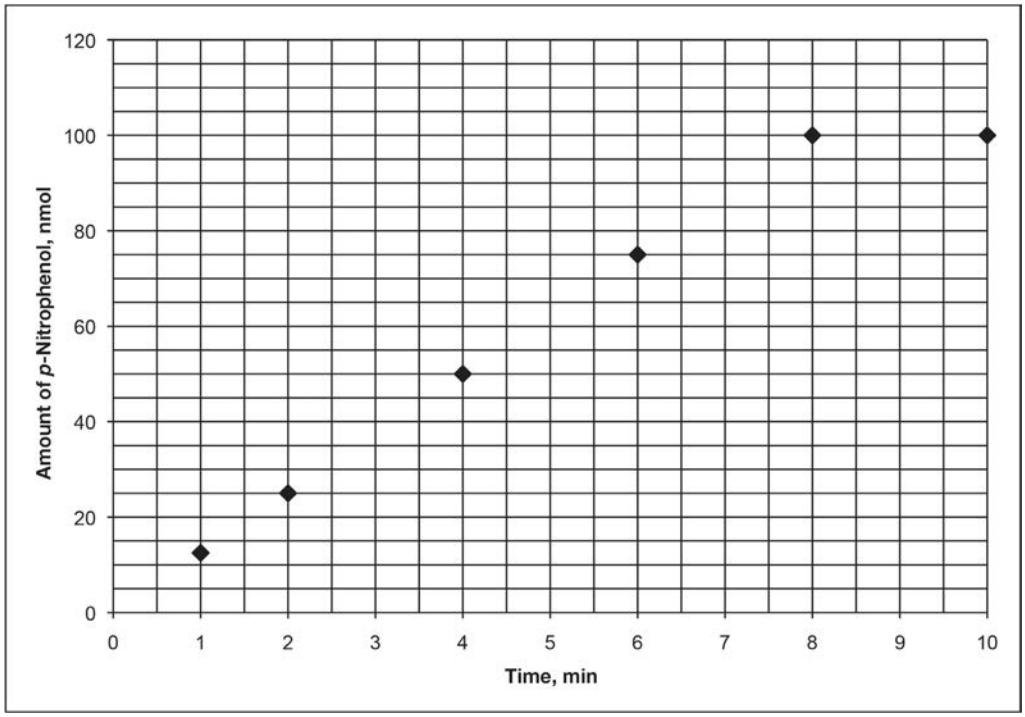


Fig. 10. Example of a rate curve for an enzyme reaction. The amount of product made is plotted against time to determine the initial rate of reaction.

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 0 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 illustrated in Figure 10, plot the amount of *p*-nitrophenol produced over time on Figure 11 using data from either Table 2 or Table 4. Draw a line that best fits through the data points.

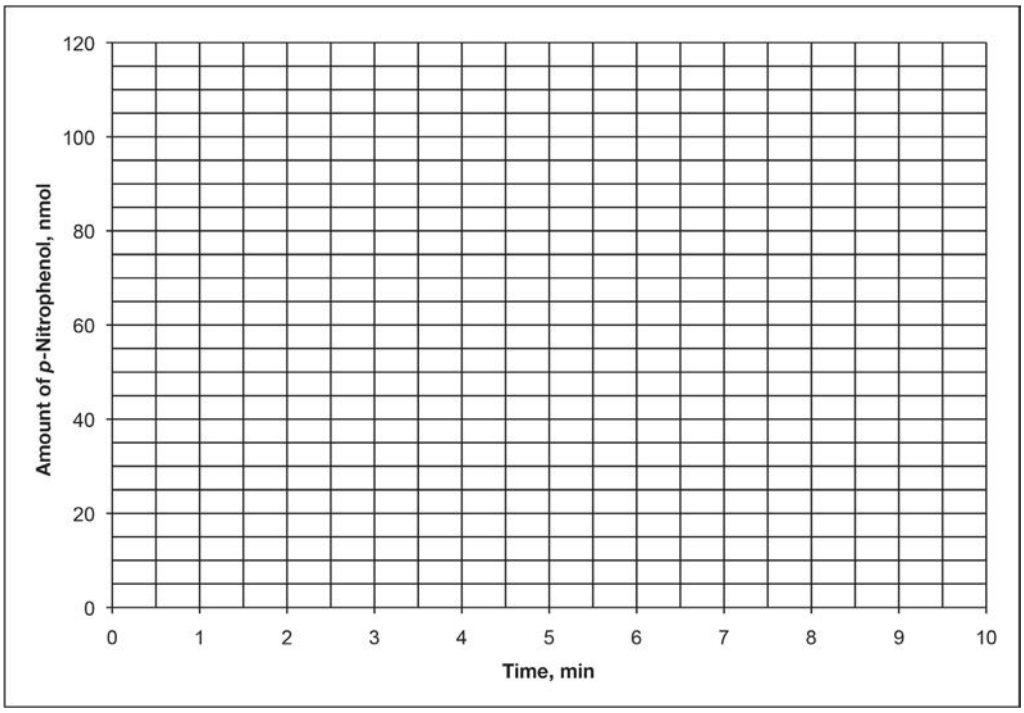


Fig. 11. Reaction rate curve for cellobiase. The amount of product made is plotted against time to determine the initial rate of reaction.

At the beginning of the reaction, there is plenty of substrate available for the enzyme to encounter and create product. Locate the linear 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 that the product is produced when there is plenty of substrate.

Perform these calculations for your data

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

Rate of product formation with no enzyme present = _____nmol/min

Activity 1 Analysis Questions

1. Did you observe any changes in the enzyme reaction and control reaction conical tubes during the time that the reaction was occurring?

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

3. Describe the chemical reaction that occurred in this experiment.

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

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

6. How did you estimate the amount of product (in nmol) produced by the enzyme?

7. Why is the amount of light absorbed by the sample proportional to the amount of product produced?

8. Determine the initial rate of product production from your absorbance measurements. **Hint**: The rate of product production is measured in absorbance units/min or nmol/min and it is the slope of the line between the zero and 1 minute time points.

9. Is the rate of product production constant over time? **Hint**: Is the slope of the line constant or does it change?

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

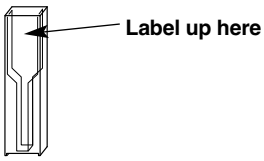
Temperature can affect the speed of the reaction. Heat can speed up the movement of the substrate and enzyme molecules, which would increase the number of collisions and therefore speed up the reaction. However, at some point, the forces that allow the enzyme to maintain its proper shape will be broken,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

Student Workstation	**Quantity**	**(✔)**
1.5 mM substrate	1	❒
Enzyme, low concentration	1	❒
Stop solution	1	❒
DPTPs	4	❒
Colorimetric standards (S1–S5) from Activity 1	1 of each	❒
Microcentrifuge tubes, 1.5 ml	6	❒
Cuvettes	3	❒
Marker	1	❒
Beaker with ice water	1	❒
Beaker with 37°C water	1	❒
Thermometer	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

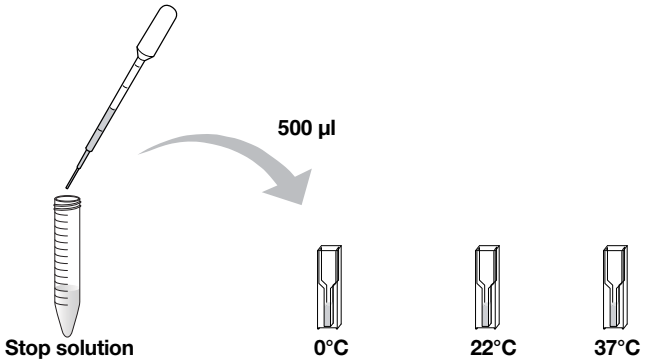
Instructor's Workstation (Optional)	**Quantity**	**(✔)**
Spectrophotometer	1	❒

Protocol

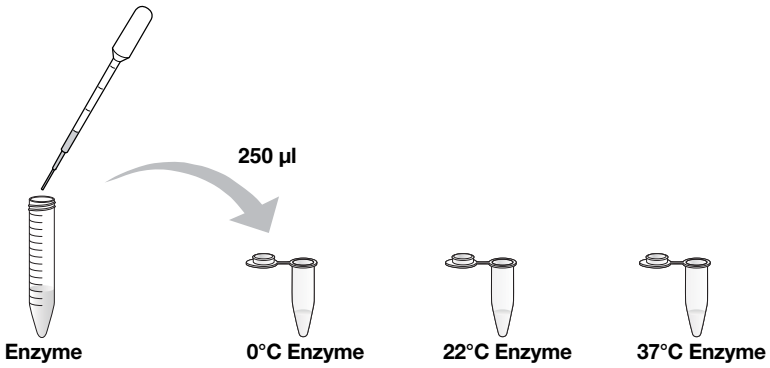
1. Label your cuvettes "0°C", "22°C", and "37°C". Only label on the upper part of the cuvette face.



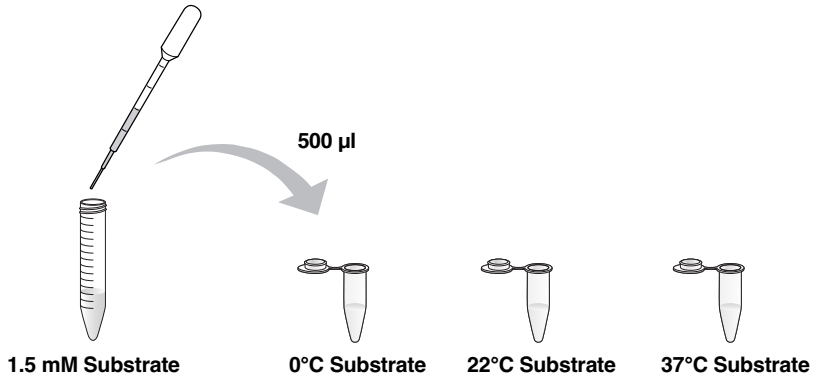
2. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Wash the DPTP out thoroughly with water and save.



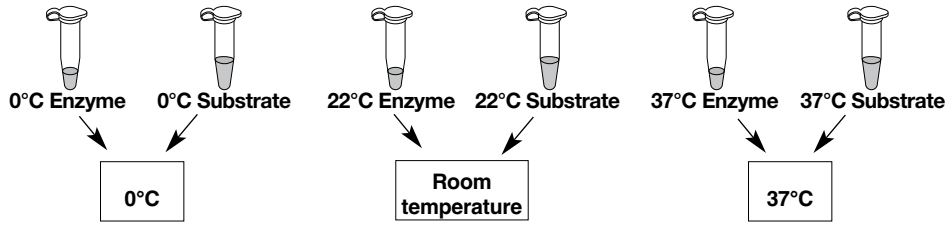
3. Label three 1.5 ml microcentrifuge tubes with “0°C Enzyme”, “22°C Enzyme”, and “37°C Enzyme”. Using a clean DPTP, pipet 250 µl of enzyme into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.



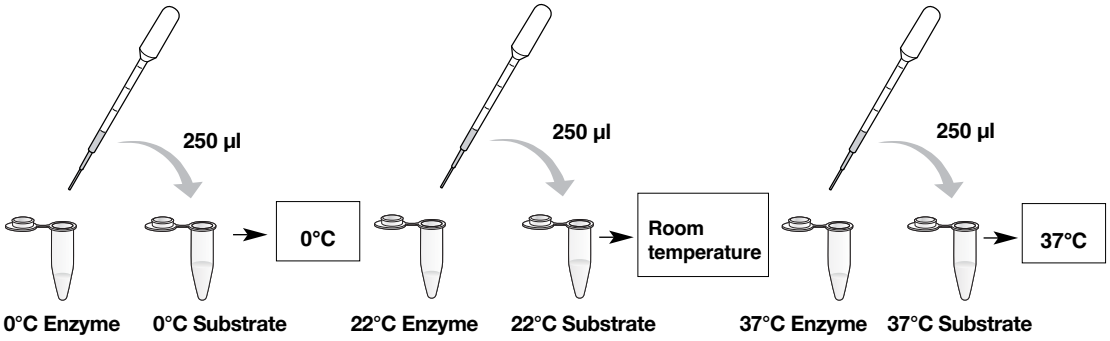
4. Label three 1.5 ml microcentrifuge tubes with “0°C Substrate”, “22°C Substrate”, and “37°C Substrate”. Using a clean DPTP, pipet 500 µl of the 1.5 mM substrate into each microcentrifuge tube. Rinse out the DPTP thoroughly with water.



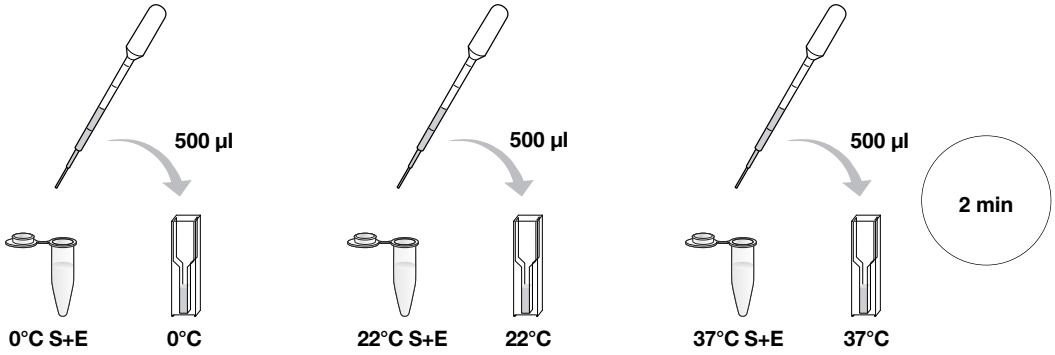
5. Place the microcentrifuge tubes labeled “0°C Enzyme” and “0°C Substrate” in the ice cup. Place the microcentrifuge tubes labeled “22°C Enzyme” and “22°C Substrate” on your lab bench. Place the microcentrifuge tubes labeled “37°C Enzyme” and “37°C Substrate” in the beaker of warm water at 37°C. Allow the tubes to equilibrate to their respective temperatures for at least 5 minutes.



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



7. After 2 minutes, use a clean DPTP for each temperature reaction to transfer 500 µl of your reactions to the appropriately labeled cuvettes containing stop solution. Allow all solutions in the cuvettes to reach room temperature for approximately 5 minutes.



8. After all of your samples have been analyzed, rinse out the DPTPs and cuvettes used in this activity with copious amounts of water and save them for future activities.

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

Qualitative Analysis of the Amount of Product Formed at Different Temperatures

1. You should have five cuvettes of standards labeled S1–S5 at your lab bench. Take your 0°C, 22°C, and 37°C cuvette samples, and make a note of the standard that best matches each temperature reaction cuvette. Write down the concentration of that standard (from Table 5 into Table 6) for each of your different temperature reactions.

Table 5. *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 mol

Table 6. Determination of *p*-nitrophenol produced at three different temperatures based on *p*-nitrophenol standards.

Temperature	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
0°C		
~22°C (room temperature)		
37°C		

2. If you do not have a spectrophotometer, please skip ahead to Analysis of Results.

Quantitative Analysis of the Amount of Product Formed at Different Temperatures

1. Blank your spectrophotometer with the S1 standard at 410 nm and then measure the absorbance values for your three cuvettes. Record the absorbance values in Table 7.

2. Following the same protocol and standard curve derived in Activity 1, convert the amount of product you have from units of absorbance measured on the spectrophotometer to nmol, and fill these values in the third column of Table 7.

Table 7. Determination of *p*-nitrophenol produced at three different temperatures based on a standard curve (similar to the one shown in Figure 8).

Temperature	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
0°C		
~22°C (room temperature)		
37°C		

Analysis of Results

1. Calculate the initial rate of reaction at each of the three different temperatures. Since you only measured the amount of *p*-nitrophenol at one time point (2 minutes), assume that the amount of *p*-nitrophenol at 0 minutes is 0 nmol.

Example: After 2 minutes, the 37°C sample gave an absorbance reading at 410 nm of 0.35, which looked most similar to standard S3. Qualitatively (using the standards), this means that you have ~25 nmol of *p*-nitrophenol. Quantitatively, you would use the standard curve you generated in Activity 1 to determine the amount of p-nitrophenol with an absorbance of 0.35 corresponds to ~22 nmol.

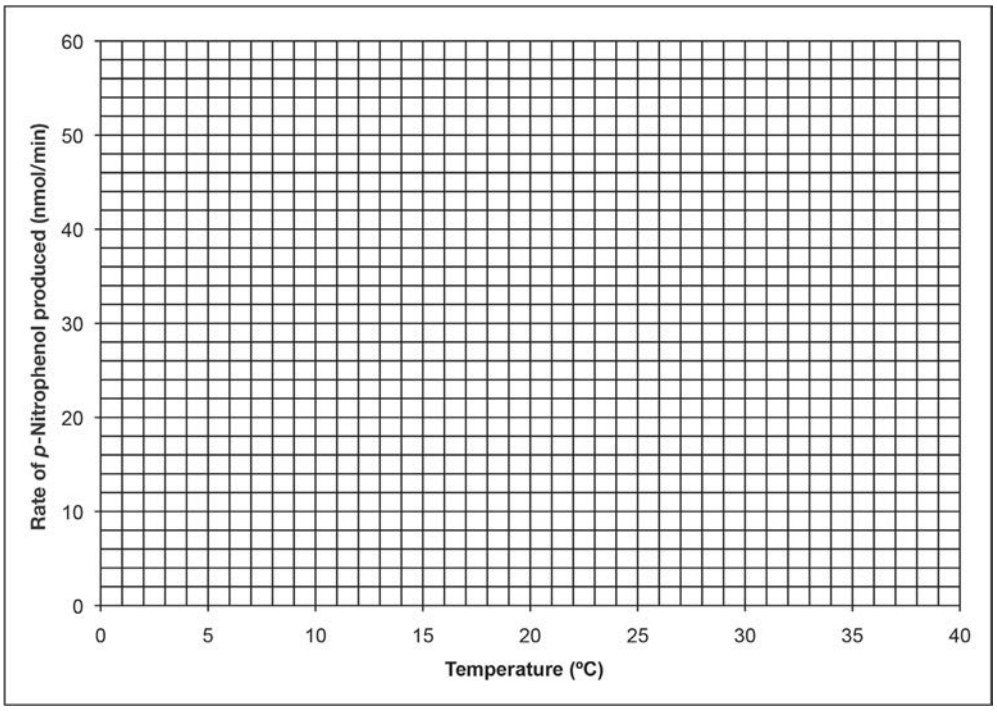
The initial rate for the qualitative data = (25 nmol – 0 nmol)/(2 min – 0 min) = 12.5 nmol/min.

The initial rate for the quantitative data = (22 nmol – 0 nmol)/(2 min – 0 min) = 11 nmol/min.

Perform these calculations for your data

Initial rate of product formation at 0°C =_____nmol/min
Initial rate of product formation at 22°C (room temp) =_____nmol/min
Initial rate of product formation at 37°C =_____nmol/min

2. Plot the effect of temperature on the rate of the enzymatic reaction.



Actvitiy 2 Analysis Questions

1. How can you determine the initial rate of the reaction for each temperature?

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

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

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

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

6. If you were a scientist who wanted to use this enzyme to produce glucose, at what temperature should you run the reaction?

7. In what type of environment might an organism that produces this enzyme live? Explain your reasoning.

Challenge questions

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

2. Covalent bonds between R groups occur between which amino acids?

3. What would be a disadvantage of using the highest temperature that yields the fastest rate of product formation?

Activity 3: Determine the Effect of pH on Reaction Rate

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

Student Workstation	**Quantity**	**(✔)**
3.0 mM substrate	1	❒
Enzyme, low concentration	1	❒
Stop solution	1	❒
Colorimetric standards (S1–S5) in cuvettes from Activity 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	❒

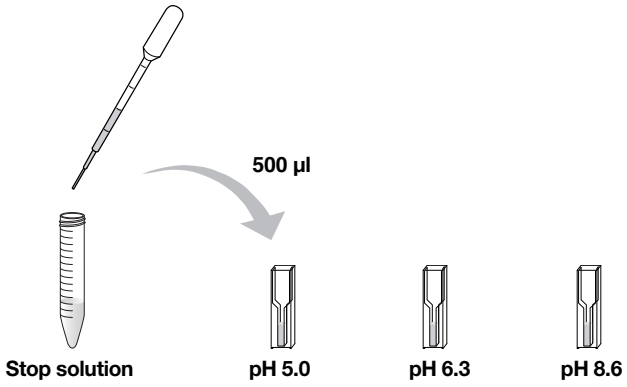
Instructor’s Workstation (Optional)	**Quantity**	**(✔)**
Spectrophotometer	1	❒

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

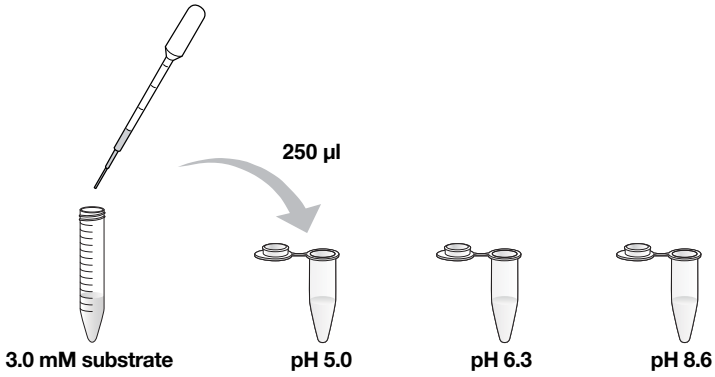
Protocol

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

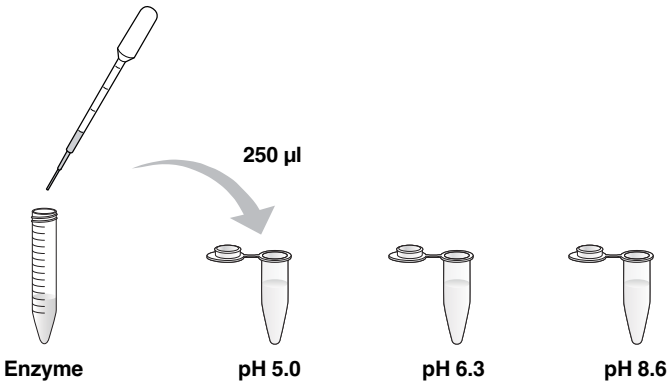
2. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Wash the DPTP out thoroughly with clean water.



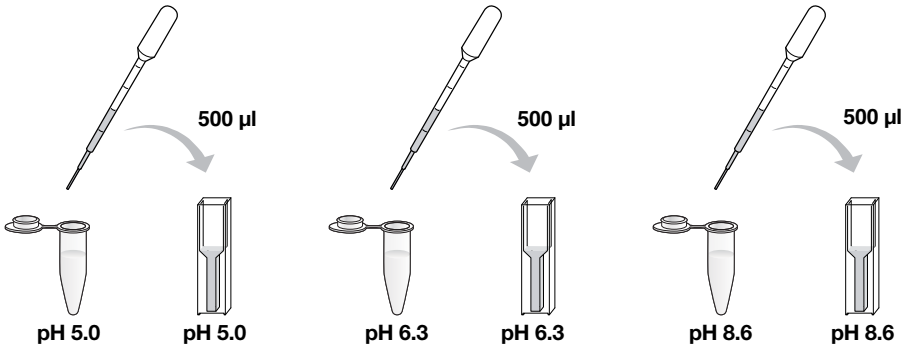
3. Using a clean DPTP, pipet 250 µl of 3.0 mM substrate into each microcentrifuge tube containing different pH adjustment buffers labeled “pH 5.0”, “pH 6.3” and “pH 8.6” by your instructor. Wash the DPTP out thoroughly with water.



4. Have a stopwatch ready. Using a clean DPTP, add 250 µl of enzyme to each of the labeled microcentrifuge tubes and start your stopwatch.



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



6. After all of your samples have been analyzed, rinse out the DPTPs and cuvettes used in this activity with copious amounts of water and save them for future activities.

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

Qualitative Analysis of the Amount of Product Formed at Different pH Levels

1. You should have five cuvettes of standards labeled S1–S5 at your lab bench. Take your pH 5.0, pH 6.3, and pH 8.6 cuvette samples and make a note of the standard number that each cuvette color matches the closest. Write down the concentration of that standard (from Table 8) in Table 9 for each of your pH reaction samples.

Table 8. *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 mol

Table 9. Determination of *p*-nitrophenol produced at three different pH values based on *p*-nitrophenol standards.

pH	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
pH 5.0		
pH 6.3		
pH 8.6		

2. If you do not have a spectrophotometer, please skip ahead to Analysis of Results.

Quantitative Analysis of the Amount of Product Formed at Different pH Levels

1. Blank your spectrophotometer with the S1 standard at 410 nm, and then measure the absorbance values for your three cuvettes. Record the absorbance values in Table 10.

2. Following the same protocol used in Activity 1, convert the amount of product you have from units of absorbance measured on the spectrophotometer to units of concentration (nmol). Fill these values in the third column on Table 10.

Table 10. Determination of *p*-nitrophenol produced at three different pH values based on a standard curve (similar to the one shown in Figure 8).

pH	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
pH 5.0		
pH 6.3		
pH 8.6		

Analysis of Results

1. Calculate the initial rate of reaction at each of the three different pH levels. Since you only measured the amount of *p*-nitrophenol at one time point (5 minutes), assume that the amount of *p*-nitrophenol at 0 minutes is 0.

Example: After 5 minutes, the pH 6.3 sample gave an absorbance reading at 410 nm of 0.14, which looked most similar to standard S2. Qualitatively (using the standards), this means that you have ~12.5 nmol of *p*-nitrophenol. Quantitatively, you would use the standard curve you generated in Activity 1 to determine the amount of *p*-nitrophenol with an absorbance of 0.14 corresponds to ~9 nmol.

The initial rate for the qualitative data = (12.5 nmol – 0 nmol)/(5 min – 0 min) = 2.5 nmol/min.

The initial rate for the quantitative data = (9 nmol – 0 nmol)/(5 min – 0 min) = 1.8 nmol/min.

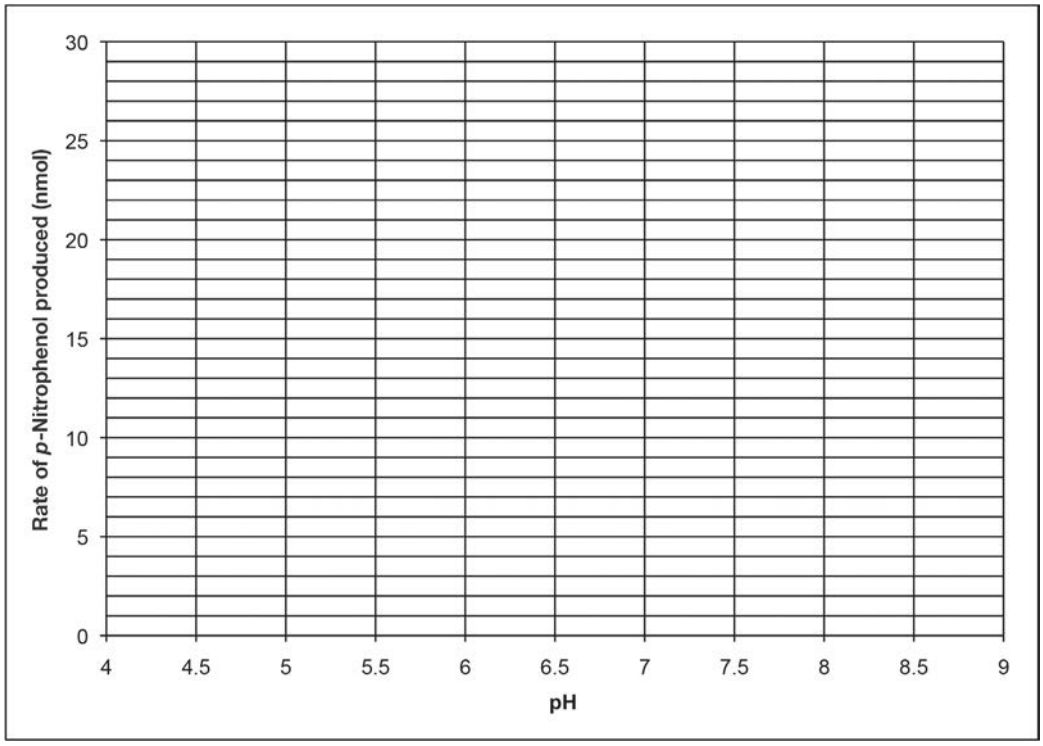
Perform these calculations for your data

Initial rate of product formation at pH 5.0 =_____nmol/min

Initial rate of product formation at pH 6.3 =_____nmol/min

Initial rate of product formation at pH 8.6 =_____nmol/min

2. Plot the effect of pH on the rate of the enzymatic reaction.



Activity 3 Analysis Questions

1. How can you determine the initial rate of the reaction for each pH?

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

3. Why do most enzymatic reactions slow down at extremely high or low pH values?

4. In what type of environment might an organism that produces this enzyme live? Explain your reasoning.

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

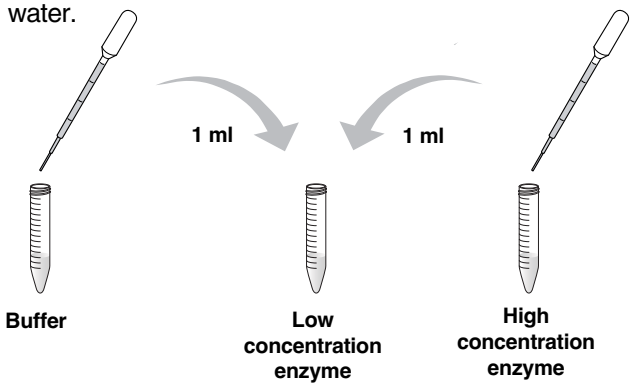
For this activity, you will determine the effect of changing the enzyme concentration on the initial rate of the reaction and on the amount of product formed. One simple way to think of the effect of enzyme concentration is to consider squirrels in a small forest with a known number of trees and a set number of nuts on those trees. The goal of the squirrels is to gather up all of the nuts. If there was just one squirrel, it would take it longer to move from tree to tree and gather up all the nuts, but given enough time, the squirrel would make it to all the trees and find and gather all the nuts. However, if there were many squirrels, they could gather up the nuts much more quickly, but in the end, they would have the same number of nuts as the single squirrel. Increasing enzyme concentration has an analogous effect. Initially, if there is plenty of substrate, the reaction will go faster in the presence of more enzyme. However, both a reaction with a lot of enzyme and a reaction with less enzyme have a set amount of substrate to work on (nuts to find), and if enough time is allowed, all the substrate will be converted to product. You can use the Toothpickase Assay, an activity created by Peggy Skinner as an example to demonstrate the effect of enzyme concentration on the rate of reaction (http://www.accessexcellence.org/AE/ATG/data/released/0166-PeggySkinner/).

Student Workstation	**Quantity**	**(✔)**
1.5 mM substrate	1	❒
High concentration enzyme (3 ml in a 15 ml conical tube)	1	❒
Buffer	1	❒
Stop solution	1	❒
Colorimetric standards (S1–S5) in cuvettes from Activity 1	1 of each	❒
DPTPs	4	❒
15 ml conical tube	1	❒
Cuvettes	6	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

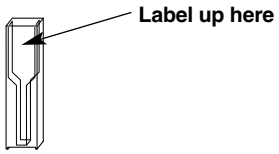
Instructor's Workstation (Optional)	**Quantity**	**(✔)**
Spectrophotometer	1	❒

Protocol

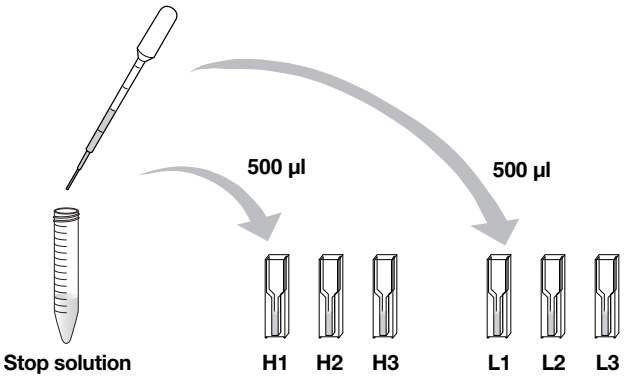
1. Label one 15 ml conical tube "Low Concentration Enzyme". Using a clean DPTP, pipet 1 ml of buffer into the tube. Wash out the DPTP with water. Pipet 1 ml of high concentration enzyme to your tube labeled "Low Concentration Enzyme" and mix. Wash out the DPTP thoroughly with water.



2. Label three cuvettes “H1–H3” (for high enzyme concentration time points) and the remaining three cuvettes “L1–L3” (for low enzyme concentration time points). Only label on the upper part of the cuvette face.



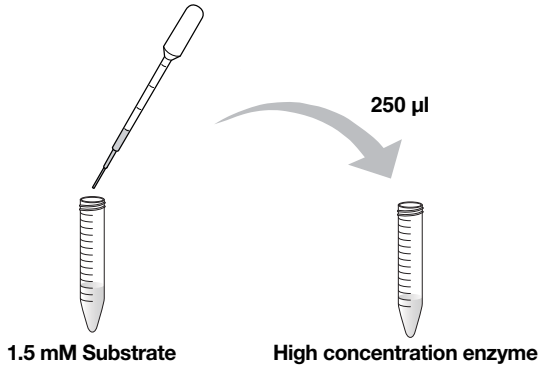
3. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Wash out the DPTP thoroughly with water.



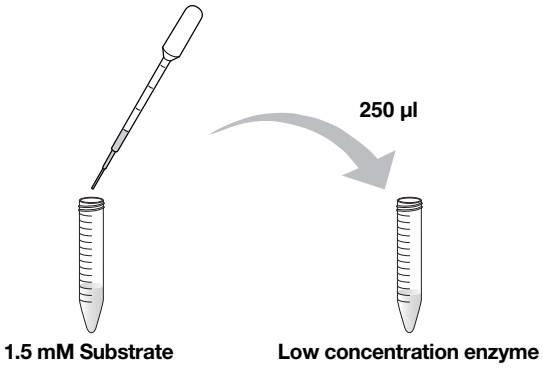
4. Label one clean DPTP with an “H” for high enzyme concentration and a second clean DPTP with an “L” for low enzyme concentration.

Please read steps 5–7 fully before proceeding. These steps are time sensitive!

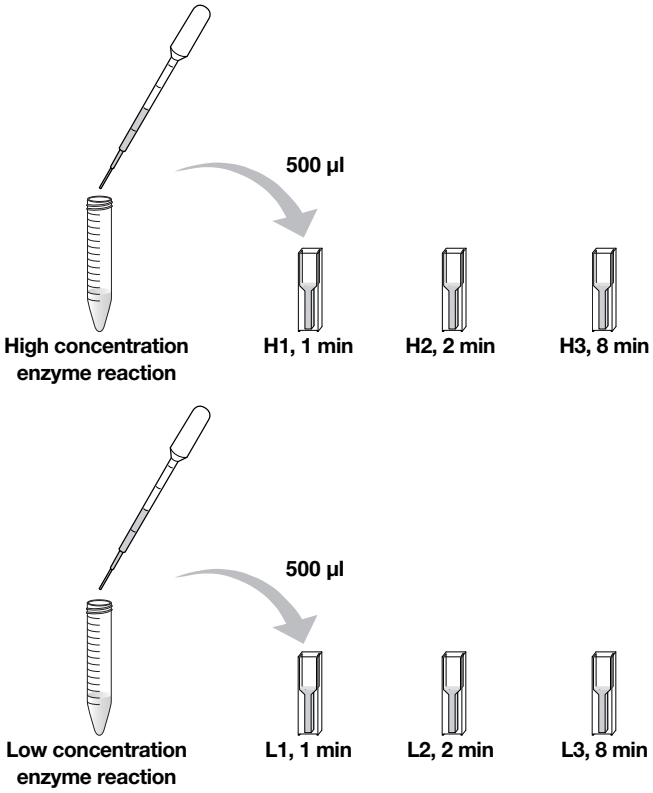
5. Using the DPTP labeled with an “H”, pipet 250 µl of 1.5 mM substrate into your 15 ml conical tube containing enzyme labeled “High Concentration Enzyme”.



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



7. At the times indicated in the table below, use the correctly labeled DPTP to remove 500 µl from the 15 ml conical tubes labeled "High Concentration Enzyme" and "Low Concentration Enzyme", and add it to the appropriately labeled cuvette that already contains the stop solution.



Make sure to use only the DPTP labeled "H" for the high enzyme concentration reaction tube and the DPTP labeled "L" for the low enzyme concentration reaction tube.

Time	Cuvette (High Concentration Enzyme)	Cuvette (Low Concentration Enzyme)
1 min	H1	L1
2 min	H2	L2
8 min	H3	L3

8. Rinse out all DPTPs with copious amounts of water and save them for later activities. After you have finished your analysis, rinse out your 2 reaction (conical) tubes and the 6 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.

Qualitative Analysis of the Amount of Product Formed at Different Enzyme Concentrations

1. You should have five cuvettes of standards labeled S1–S5 at your lab bench. Take your H1–H3 and L1–L3 cuvette samples, and make a note of the standard number that each cuvette color matches the closest. Write down the concentration of that standard (from Table 11) into Table 12 for each of your reaction time point samples.

Table 11. *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 mol

Table 12. Determination of *p*-nitrophenol produced using a high and a low enzyme concentration based on *p*-nitrophenol standards.

Cuvette	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
H1		
H2		
H3		
L1		
L2		
L3		

2. If you do not have a spectrophotometer, please skip ahead to Analysis of Results.

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

1. Blank your spectrophotometer with the S1 standard at 410 nm and then measure the absorbance values for your six cuvettes, and record the absorbance values in the second column of Table 13.

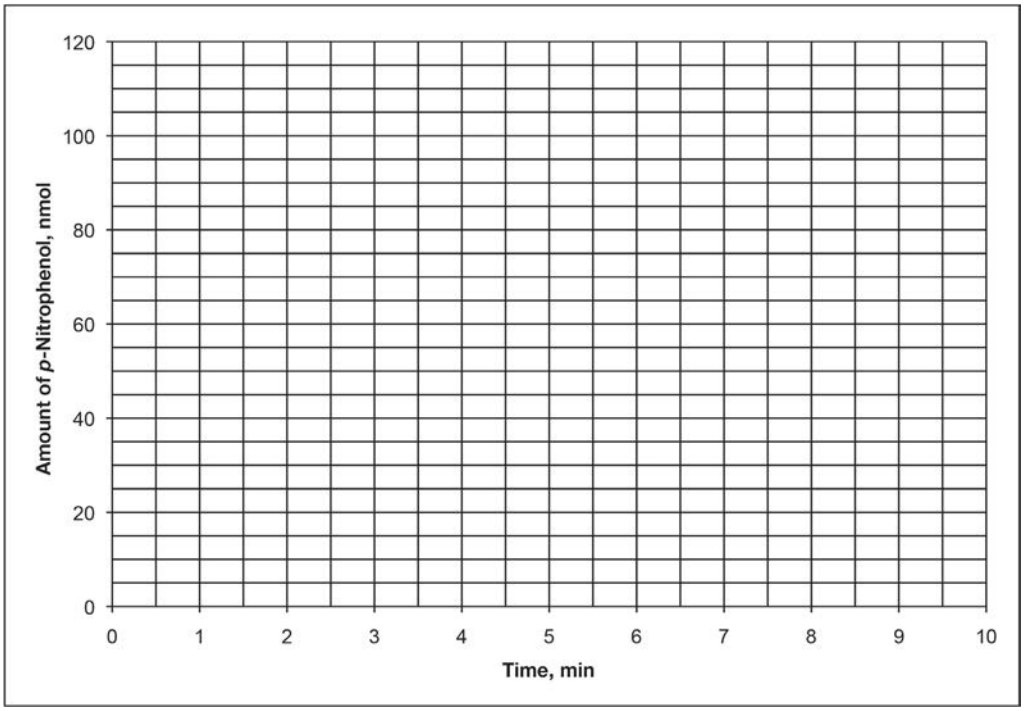
2. Using the protocols you learned in Activity 1, calculate the amount of *p*-nitrophenol formed in all of your samples and record it in the third column of Table 13.

Table 13. Determination of *p*-nitrophenol produced using a high and low enzyme concentration based on a standard curve (similar to the one shown in Figure 8).

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

Analysis of Results

1. Plot the amount of product produced versus time for both the high and low concentration enzyme reactions on the same graph below.



2. Calculate the initial rate of reaction for both the high and low enzyme concentration reactions. Assume that the amount of product at 0 minutes is 0 nmol. Use the amount of product at 1 minute to do your calculation of initial rate.

Initial rate of reaction for high enzyme concentration = Amount of product at 1 min/1 min = _____ nmol /min

Initial rate of reaction for low enzyme concentration = Amount of product at 1 min/1 min = _____ nmol /min

Activity 4 Analysis Questions

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

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

3. If you were a scientist who is responsible for determining the concentration of enzyme to use in the hydrolysis process of producing sugar from cellulose, what advantage would there be to using a high concentration of enzyme? What disadvantage would there be to using a high concentration of enzyme?

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

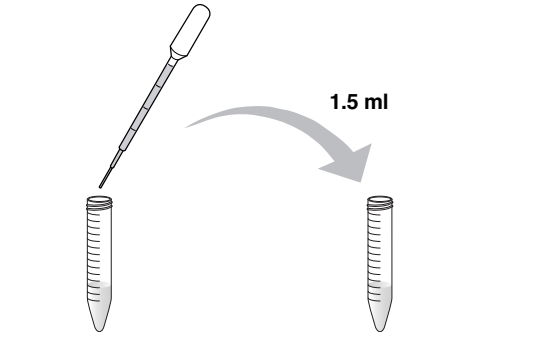
For this activity, you will determine the effect that changing the substrate concentration has on the initial rate of the reaction. We can again use the simple analogy of squirrels gathering nuts in the forest to understand how substrate concentration affects the initial rate of the reaction and the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

Student Workstation	**Quantity**	**(✔)**
1.5 mM substrate	1	❒
Enzyme	1	❒
Buffer	1	❒
Stop solution	1	❒
Colorimetric standards (S1–S5) in cuvettes from Activity 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	❒

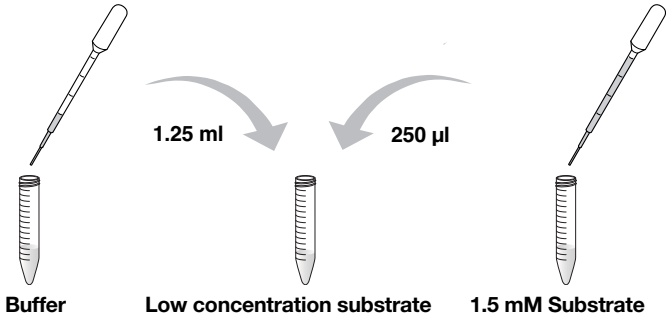
Instructor's Workstation (Optional)	**Quantity**	**(✔)**
Spectrophotomer	1	❒

Protocol

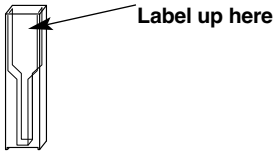
1. Label one clean 15 ml conical tube "Low Concentration Substrate" and one clean 15 ml conical tube "High Concentration Substrate".
2. Using a clean DPTP, pipet 1.5 ml of 1.5 mM substrate into the 15 ml conical tube labeled "High Concentration Substrate". Rinse the DPTP thoroughly with clean water.



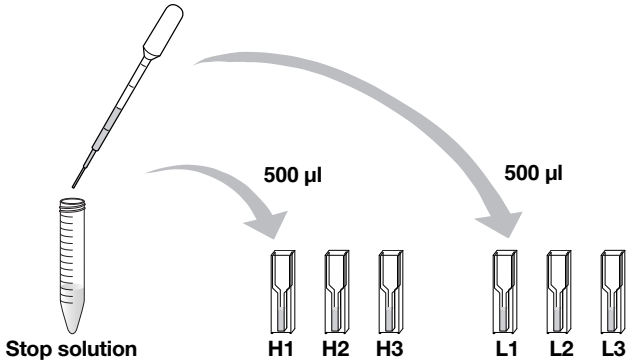
3. Using a clean DPTP, pipet 1.25 ml of buffer into the 15 ml conical tube labeled “Low Concentration Substrate”. Rinse the DPTP thoroughly with water and then pipet 250 µl of 1.5 mM substrate into the 15 ml conical tube labeled “Low Concentration Substrate” and mix. Rinse the DPTP thoroughly with water.



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



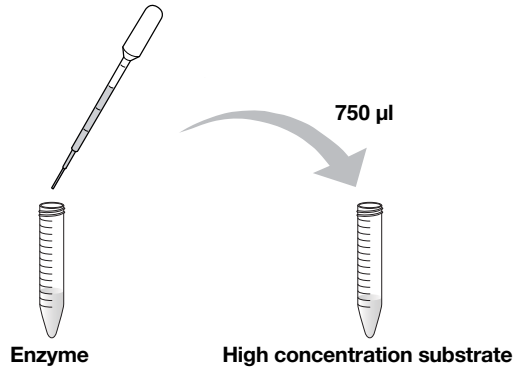
5. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Rinse the DPTP thoroughly with water.



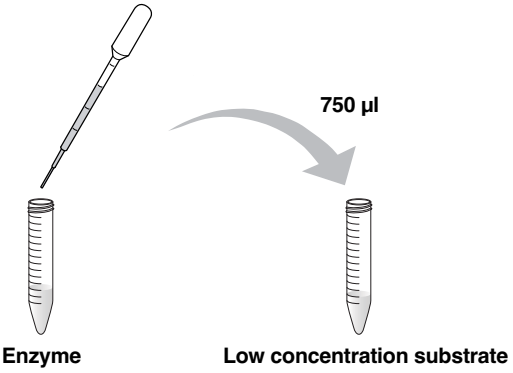
6. Label one DPTP as “H” for high concentration substrate and a second DPTP as “L” for low concentration substrate.

Please read and understand steps 7–9 fully before proceeding. These steps are time sensitive!

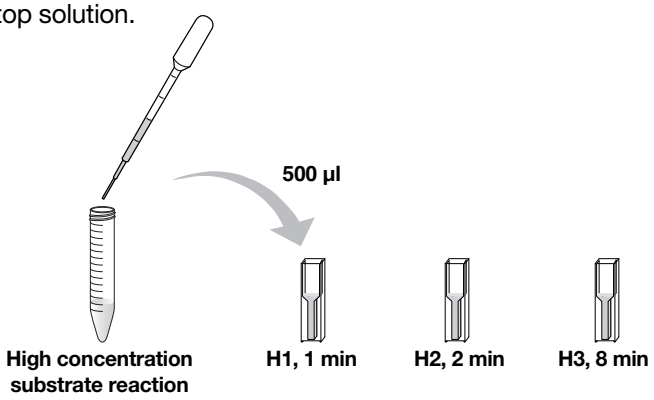
7. Using a clean DPTP, pipet 750 µl of enzyme into your 15 ml conical tube of substrate labeled “High Concentration Substrate”.

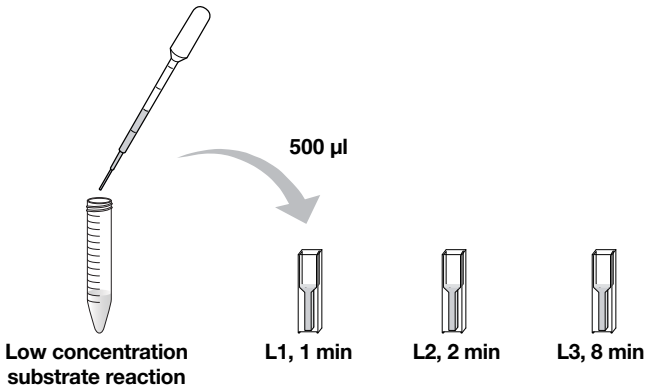


8. Using a clean DPTP, pipet 750 µl of enzyme into your 15 ml conical tube of substrate labeled “Low Concentration Substrate”. **START YOUR TIMER**.



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





Make sure to only use the DPTP labeled “H” for the high substrate concentration reaction tube and the DPTP labeled “L” for the low substrate concentration reaction tube.

Time	Cuvette (High Substrate)	Cuvette (Low Substrate)
1 min	H1	L1
2 min	H2	L2
8 min	H3	L3

10. Rinse out all DPTPs with copious amounts of water and save them for later activities. After you have finished your analysis, rinse out your 2 reaction (conical) tubes and 6 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.

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

1. You should have five cuvettes of standards labeled S1–S5 at your lab bench. Take your H1–H3 and L1–L3 cuvette samples and make a note of the standard that best matches each cuvette color. Write down the concentration of that standard (from Table 14) into Table 15 for each of your reaction time point samples.

Table 14. *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 mol

Table 15. Determination of *p*-nitrophenol produced using a high and a low substrate concentration based on *p*-nitrophenol standards.

Cuvette	Standard That Is Most Similar	Amount of *p*-Nitrophenol Produced (nmol)
H1		
H2		
H3		
L1		
L2		
L3		

2. If you do not have a spectrophotometer, skip to Analysis of Results.

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

1. Blank your spectrophotometer with the S1 standard at 410 nm. Measure the absorbance values for your six cuvettes and record the absorbance values in Table 16 in the second column.

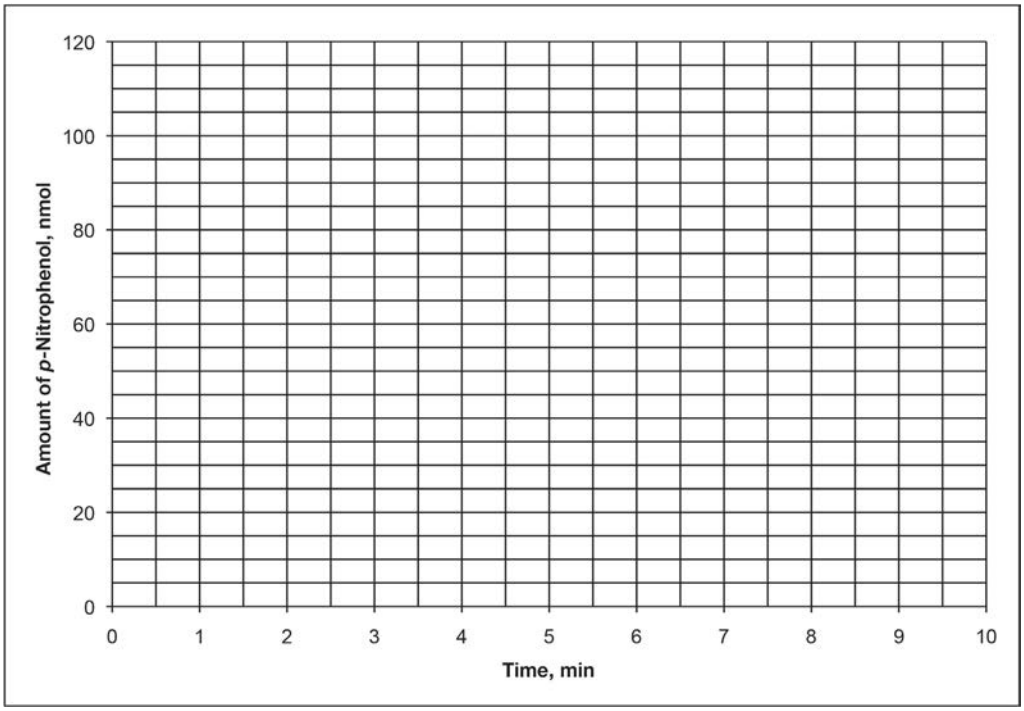
2. Using the protocols you learned in Activity 1, calculate the amount of *p*-nitrophenol formed in all of your samples and record it in Table 16 in the third column.

Table 16. Determination of *p*-nitrophenol produced using a high and low substrate concentration based on a standard curve (similar to the one shown in Figure 8).

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

Analysis of Results

1. Plot the amount of product produced vs. time for both the high substrate and the low substrate concentration reactions on the same graph below.



2. Calculate the initial rate of reaction for both the high substrate and low substrate reactions. Assume that the concentration of product at 0 minutes is 0 nmol. Use the amount of product at 1 minute to do your calculation of initial rate.

Initial rate of reaction for high substrate concentration =

(Amount of product at 1 min)/1 min = _____nmol /min

Initial rate of reaction for low substrate concentration =

(Amount of product at 1 min)/1 min = _____nmol /min

Activity 5 Analysis Questions

1. How does the amount of substrate present change the initial rate of reaction?

2. Come up with an analogy or draw a cartoon to explain how changing the concentration of substrate affects the rate of product formation.

3. Does the amount of substrate change the final amount of product, assuming that you let each reaction proceed for a really long time?

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 extracts of 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.

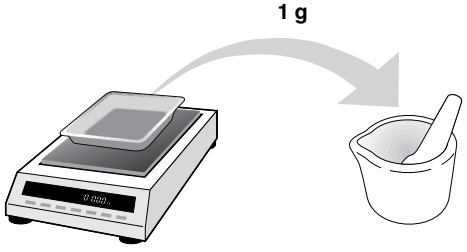
Student Workstation	**Quantity**	**(✔)**
Mushroom sample	1	❒
1.5 mM substrate	1	❒
Stop solution	1	❒
1x extraction buffer	1	❒
15 ml conical tube	1	❒
1.5 ml microcentrifuge tube	1	❒
DPTPs	4	❒
Cuvettes	6	❒
Marker	1	❒
Mortar and pestle	1	❒
Filter paper, cheese cloth, or strainer	1	❒
Beaker with deionized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

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

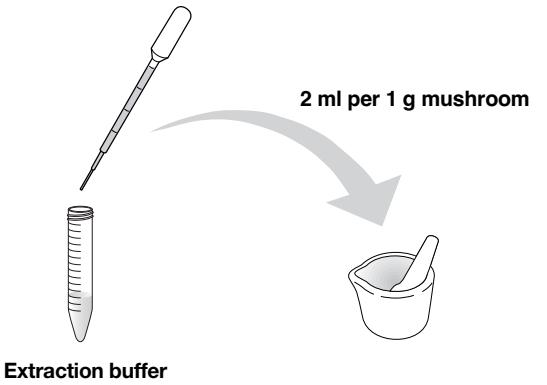
Protocol

1. Write down the name of your mushroom _____

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

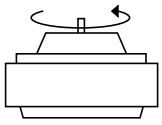


3. Add 2 ml of extraction buffer for every gram of mushroom into the mortar. _____ml



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

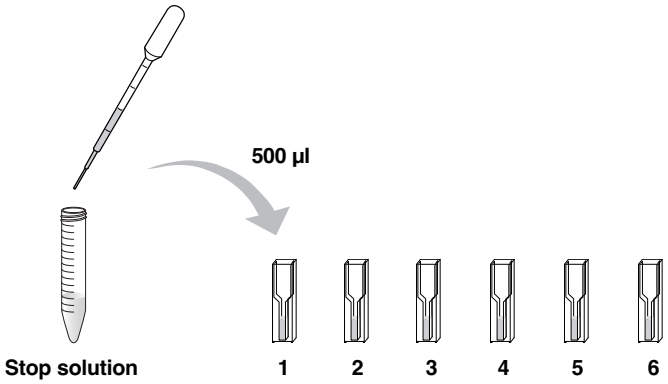
5. Strain the solid particles out of your slurry using a piece of filter paper or cheese cloth into a 1.5 ml microcentrifuge tube. Alternatively, if you have a centrifuge, scoop the slurry into a 1.5 ml microcentrifuge tube and then pellet the solid particles by spinning at top speed for 2 minutes.



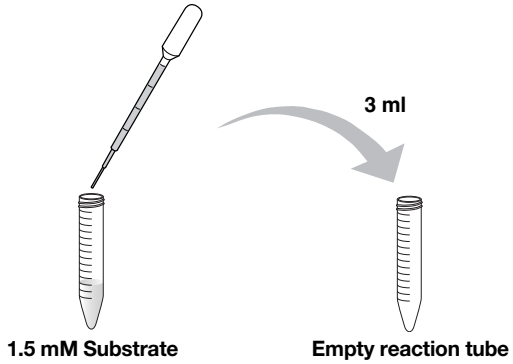
Note: You will need at least 250 µl of extract to perform the enzymatic reaction.

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.

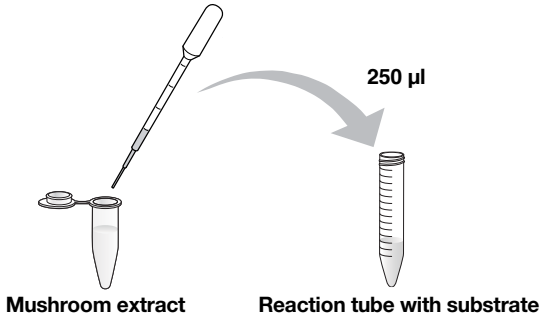


8. Label a 15 ml conical tube with the type of mushroom you are using. Using a clean DPTP, pipet 3 ml of substrate into the tube.

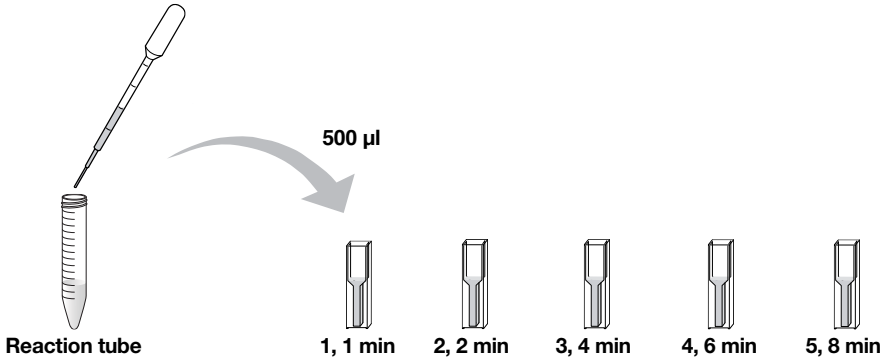


Please read and understand steps 10–11 fully before proceeding. These steps are time sensitive!

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**.

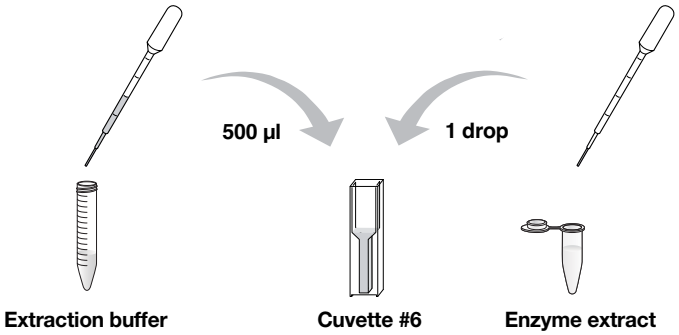


10. At the times indicated in the table below, remove 500 µl of mushroom extract/substrate mixture from the 15 ml conical tube, and add it to the appropriately labeled cuvette that already contains stop solution.



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.

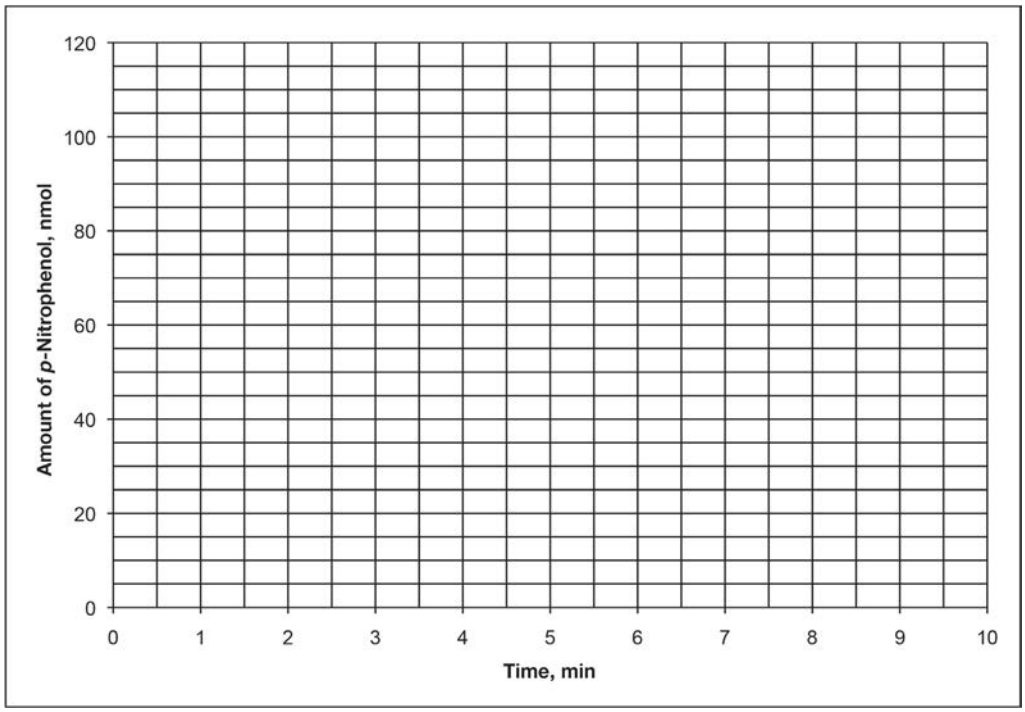
2. Using the protocols you learned in Activity 1, calculate the amount of *p*-nitrophenol formed in all of your samples and record it in column 3 of Table 17.

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?

4. Scientists are constantly looking for sources of enzymes that can be used in industrial processes. If you were going to pick a source of cellobiase for ethanol production for biofuels, what type of organism might you look for as a source of this enzyme? **Hint**: The production of glucose to be converted to ethanol in biofuel production requires the reactions to occur at high temperatures and low pH.

Appendix A
Enzyme Kinetics

How Enzymatic Rate Changes with Changing Substrate Concentration

If you were in charge of a cellulosic ethanol plant, you would want to ensure that the enzyme that you were using is the most efficient one available for the job. To compare how well an enzyme performs relative to another, you would want to compare the enzymatic activity of the different enzymes under a given set of conditions. Enzymatic activity, or how well an enzyme catalyzes a reaction, can be expressed in terms of the maximum rate of a reaction or 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).

An enzyme can work at its maximum rate (also known as V_{max} or maximum velocity) when its active sites are completely saturated with substrate. This occurs 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, since it can only occur when there is substrate 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 similar to Activity 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 12.

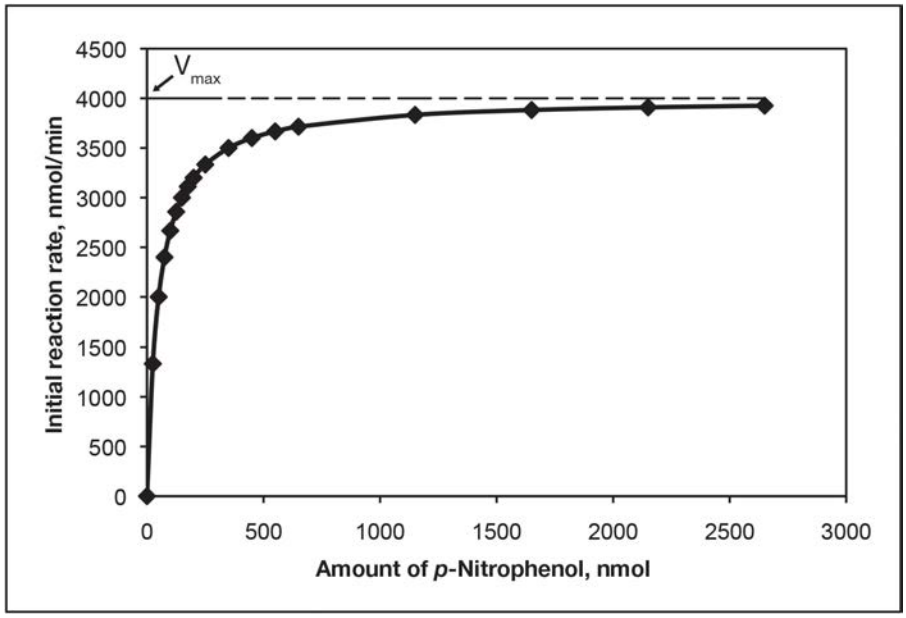


Fig. 12. The relationship between the initial velocity of an enzymatic reaction and the substrate concentration added at the beginning of the reaction. V_{max} is approached but never achieved at extremely high substrate concentrations.

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_o) and the starting substrate concentration, [S]. The relationship between the maximum velocity V_{max}, the initial velocity (V_o), 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.

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

Where

V_o = 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 where the reaction proceeds at half of the maximum rate. In the case where the initial rate of reaction, V_o, is equal to ½ the maximum rate, plugging into the Michaelis-Menten equation we get the following:

Equation 2: $$½V_{max} = \frac{V_{max} * [S]}{([K_m + [S])}$$

Solving for K_m, we get K_m= [S], when V_o= ½V_{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 the enzyme (E) or the ES complex can fall apart without the substrate being converted to product and a rate constant of k_{-1}.

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

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

In steady state equilibrium, the rate at which the enzyme-substrate complex 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 $k_{-1} + k_2$, 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 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: $V_o = V_{max} * F$

We can do one more substitution to get

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

Compare Equation 8 to the Michaelis-Menten equation (Equation 1). $$V_o = \frac{V_{max} * [S]}{(K_m + [S])}$$

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 12 is hyperbolic, it is difficult to determine exactly where V_{max} is. To solve that problem, it is best to take the inverse of both the Y values (initial rates) and the X values (substrate concentration). This will produce a linear graph called the **Lineweaver–Burk** plot or double reciprocal plot (Figure 13).

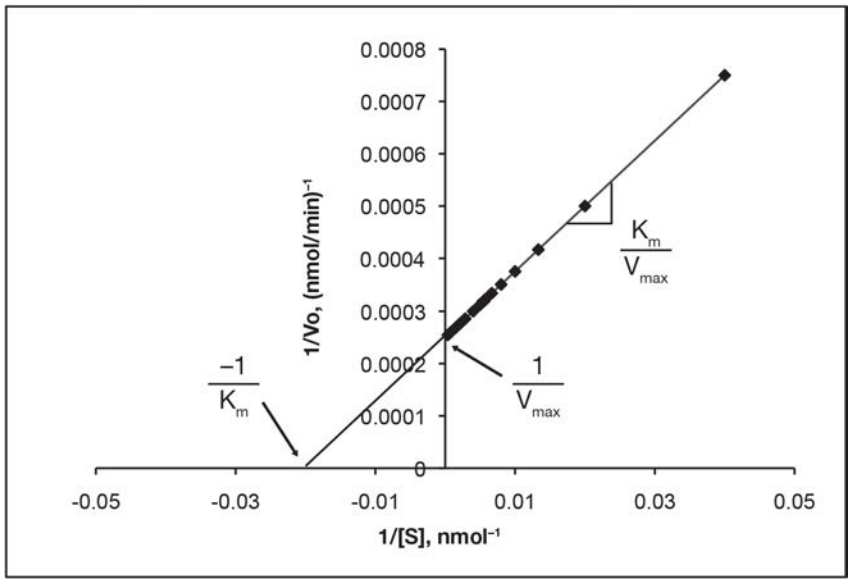


Fig. 13. A Lineweaver-Burk plot. The linear representation of the reaction rate data was used to calculate the Michaelis constant K_m and the maximum reaction rate V_{max}.

There are advantages of 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 Activity 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 that were created from biological sources long dead. Current biofuel technologies tend to fall into four main categories – cellulosic ethanol, ethanol production from sugar and starch sources, syngases, and biodiesels. 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 resulting 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 H.

First Generation Ethanol Production for Fuel

Currently, much of the ethanol used in the fuel industry is a result of conversion of starch, such as that found in corn kernels, to sugar for fermentation to ethanol. Conversion of starches to sugar has two primary 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 to produce 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 from 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 not adding or taking away any carbon dioxide from the biosphere. This is called a "carbon neutral" process. However, converting corn stalks, husks, grasses, or other non-food 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 13). If the world could use less petroleum and increase the usage of materials produced from plants in a sustainable manner, we could reduce greenhouse gas emissions.

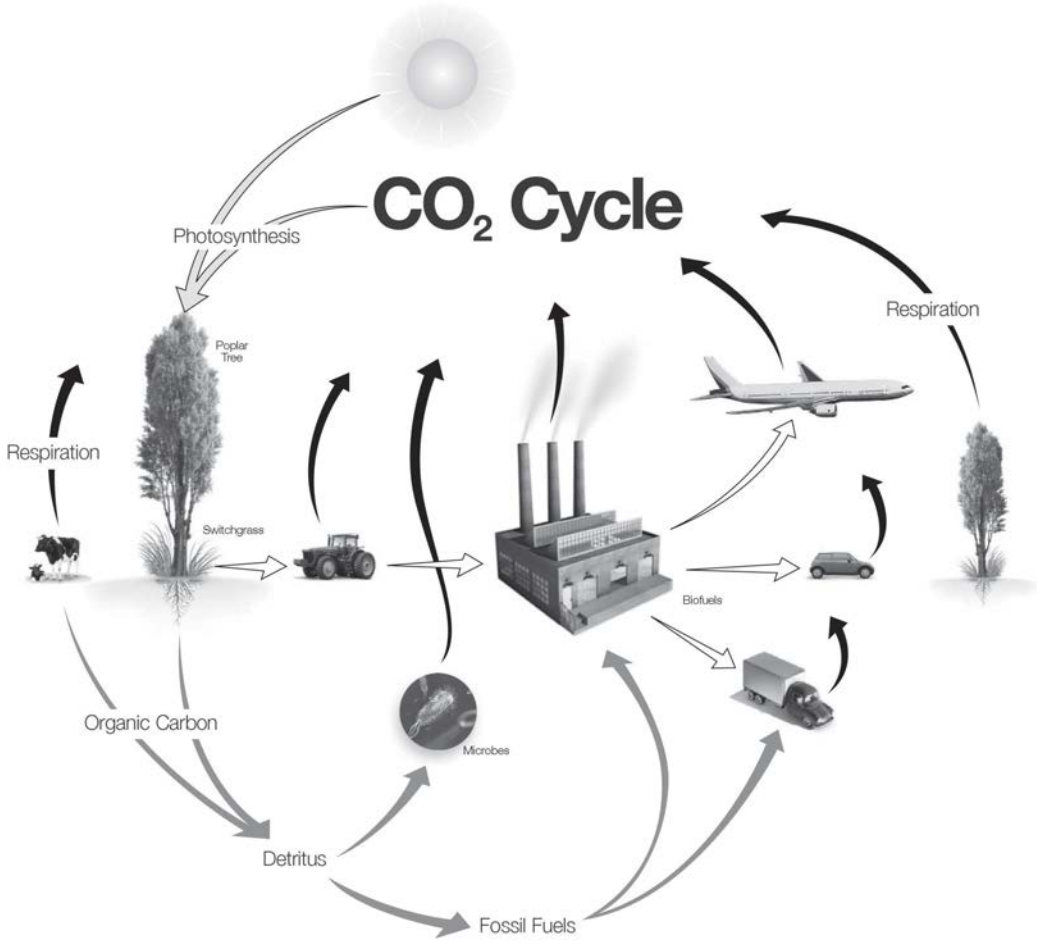


Fig. 13. A carbon cycle with the inclusion of biofuel production and use. Plants remove carbon dioxide from the atmosphere, and in the presence of sunlight they can convert it to complex polysaccharides including cellulose. Plants, such as switchgrasses and poplar trees, can then be harvested and processed 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 produced 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 only contribute 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, sugar cane and switch grass (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 quicker 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. If inorganic fertilizers are used to replace this loss, then there are two drawbacks. First, it takes fuel to produce inorganic fertilizer. Second, nitrogen-based green house 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 (Smith 2009). These problems can be solved by either using the wastewater and biomass from the cellulosic fuel plant to fertilize the fields (and thus return the nitrogen to the soil) or by using 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 in 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 cost input. 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 that 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, which is then copied by a technique called “error prone PCR”. 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. Bioengineered yeast strains that can metabolize all the sugars produced in the break down 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 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 the "λ" button.

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 substract 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.

Read 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.

   A410=0.250
   Samp #1

   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 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 for Activities 1–5. Cuvette #6 will serve as your blank for Activity 6. To correctly use cuvettes, remember to:

   - Wipe the outside of each cuvette with a lint-free tissue
   - Handle cuvettes only by the top edge of the ribbed sides
   - Dislodge any bubbles by gently tapping the cuvette on a hard surface
   - Always position the cuvette so the light passes through the clear sides

3. Calibrate the spectrophotometer.

   a. Place the S1 cuvette (Activities 1-5) or Cuvette #6 (Activity 6) 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 a peak absorbance. 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 the 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. Obtain the cuvette labeled S1. 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. Obtain the cuvette labeled S2. Wipe the outside of the cuvette and place it in the device. When the absorbance value stabilizes, click Keep, type **12.5** in the edit box, and press **ENTER**.

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

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

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

10. In the **Quantitative Determination 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 if 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 near or through 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 labeled E1–E5, Start and End, and record the absorbance values in Table 4. Use either the standard curve to graphically calculate the amount of *p*-nitrophenol in your reaction samples or do this calculation algebraically. See **Quantitative Determination of Amount of Product Formed** in Activity 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 for Activities 1–5. Cuvette #6 will serve as your blank for Activity 6. To correctly use cuvettes, remember to:

    - Wipe the outside of each cuvette with a lint-free tissue
    - Handle cuvettes only by the top edge of the ribbed sides
    - Dislodge any bubbles by gently tapping the cuvette on a hard surface
    - Always position the cuvette so the light passes through the clear sides

3. Calibrate the spectrophotometer.
    a. Place the S1 cuvette (for Activities 1–5) or Cuvette #6 (Activity 6) 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. Obtain the cuvette labeled S5 and place it into 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 (l 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. Obtain the cuvette labeled S1. Wipe the outside of the cuvette 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. Obtain the cuvette labeled S2. Wipe the outside of the cuvette and place it in the device. When the absorbance value stabilizes, tap **Keep**, enter **12.5** as the concentration, and select **OK**.
7. Obtain the cuvette labeled S3. Wipe the outside of the cuvette and place it in the device. When the absorbance value stabilizes, tap **Keep**, enter **25** as the concentration, and select **OK**.
8. Obtain the cuvette labeled S4. Wipe the outside of the cuvette and place it in the device. When the absorbance value stabilizes, tap **Keep**, enter **50** as the concentration, and select **OK**.
9. Obtain the cuvette labeled S5. Wipe the outside of the cuvette 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 the 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 near or through the data points.

    d. 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 your reaction time points. Measure the absorbance values of your cuvettes labeled E1–E5, Start and End, and record the absorbance values in Table 4. Use either your standard curve to graphically calculate the amount of p-nitrophenol in your reaction samples or do this calculation algebraically. See **Quantitative Determination of Amount of Product Formed** in Activity 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 together 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 together 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 enzyme is cellulase. See http://www.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 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 non-reducing ends (cellobiohydrolase II family) of cellulose polymeric chains. The accepted name for this family of enzymes is cellulose 1,4-β-cellobiosidase. For alternative names see http://www.expasy.org/enzyme/3.2.1.91.

Hemicellulose: A polymer that consists of chains of five-carbon 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 wall 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.

Appendix F
Biofuels Debate

Post-Lab Debate Activity

Many people object to the use of crops for biofuel production. They argue that by using crops for fuel, resources are reduced for the use of the crops or cropland for food for people or animals. Many are concerned with the removal of essential nutrients from the fields if non-food portions of crops are used for fuel and are not left fallow on fields as natural fertilizer. Proponents of cellulosic ethanol argue that the use of non-food portions of food crops such as corn stover and cover crops such as switchgrass 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, the largest amount of biomass in the world is present in the cellulosic portions of plants, and hence this would be a renewable fuel source, unlike petroleum. Here we include a debate activity to facilitate discussion of these issues.

Day 1: Set the Stage

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. Explain the format of the debate and have each team pick a captain.

Days 2–5: Student Research

- Students conduct research on the development and use of cellulosic ethanol technology for biofuel production using the pro/con data sheet on the next page (optional: assign for homework)
- Teams compile research from all members
- Teams write 4-minute opening statements and assign spokespersons

Day 6: The Debate

Debate Format
Opening Statement: Proponents of cellulosic ethanol technology for biofuel production present an opening statement outlining the benefits of cellulosic ethanol technology (4 minutes).
Break: Opponents assemble a list of questions they believe show holes in the proponents' argument (2 minutes).
Questions: Opponents present questions (2 minutes).
Opening Statement: Opponents of cellulosic ethanol technology for biofuel production present an opening statement outlining the reasons cellulosic ethanol technology should not be allowed (4 minutes).
Break: Proponents assemble a list of questions they believe show holes in the oppositions' argument (2 minutes).
Questions: Proponents present questions (2 minutes).
Rebuttal: Proponents present answers to opponents' questions (2 minutes).
Rebuttal: Opponents present answers to proponents' questions (2 minutes).
Closing arguments: Opposing view (3 minutes).
Closing arguments: Supporting view (3 minutes).

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, or not well presented.

1 = Questions were unrelated to the subject, did not raise legitimate concerns, or not well presented.

Rebuttal

4 = Students used research to directly refute the questions.

3 = Students used research to partially refute the questions.

2 = Students improperly used research 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 why we should use cellulosic ethanol technology for biofuel production (include references).

Make a list of why 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
Instructor's Answer Guide

Pre-lab Questions

1. What type of molecule is an enzyme?

   Enzymes are typically protein molecules that are made up of amino acids. There are some enzymes that are made of nucleic acids as well.

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

   An enzyme's active site needs to be the right shape so that the substrate molecule can fit into it properly. The primary structure of a protein determines its secondary, tertiary, and if applicable, quaternary structure. The chemical groups of the amino acids interact forming hydrogen, ionic, covalent, and Van der Waals interactions to create a specific shape. The R groups of the amino acids in the active site are the most important amino acids in an enzyme. This is because they are the chemical groups responsible for shifting electron densities to make or break bonds within the substrate. If these amino acids are not in the correct orientation, the active site will not be able to catalyze the reaction.

3. How does an enzyme speed up chemical reactions?

   Enzymes speed up chemical reactions by positioning the substrate molecule(s), which lowers the energy of activation. The energy of activation is the amount of energy that is required to bring the substrate molecule into the transition state (unstable intermediate). An enzyme stabilizes the transition state and lowers the amount of energy of the transition state molecule. This will cause the reaction to occur faster because less energy is required for each substrate molecule to be converted to product.

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

   Cellobiase (a type of cellulase) or beta-glucosidase

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

   Production of cellulosic ethanol for fuel

6. What is the natural product of this enzyme?

   Glucose

7. What is the natural substrate of this enzyme?

   Cellobiose (a dissacharide)

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

   When the artificial substrate is broken down by cellobiase, the product will turn yellow in the presence of a basic solution. The amount of yellow can be measured with a spectrophotometer or visually compared to standard samples with a known amount of product.

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

   The amount of product produced in a certain amount of time is calculated by determining the initial slope of the graph plotted with the product produced as a function of time (rise over run). To determine the reaction velocity at any point in time, take the difference in Y values (difference in amount of product produced between two time points) and divide it by the time interval. This will give you the velocity in nmoles/min.

Activity 1 Analysis Questions

1. Did you observe any changes in the enzyme reaction and control reaction conical tubes during the time that the reaction was occurring?

   No, both solutions remained clear with no visible changes.

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

   The solutions that contained enzyme turned yellow. The longer the time the enzyme and substrate were together, the darker the yellow color. The control cuvette without enzyme did not change color.

3. Describe the chemical reaction that occurred in this experiment.

   The artificial substrate is p-nitrophenol glucopyranoside. The enzyme breaks this molecule into glucose and p-nitrophenol.

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

   No detectable product was produced after 8 minutes in the reaction without enzyme. The reaction with enzyme produced about 100 nmol of product after 8 minutes or an absorbance at 410 nm of 1.6.

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

   The amount of product produced is increasing at each time interval. I predict that product will be produced at a constant rate until there is a limited amount of substrate available. Then the rate of product formation will eventually drop to zero.

6. How did you estimate the amount of product (in nmol) produced by the enzyme?

   We visually compared the amount of yellow color in our samples with a standard that contained a known concentration of product.

7. Why is the amount of light absorbed by the sample proportional to the amount of product produced?

   The product produced turns yellow in the presence of the stop solution. The more yellow the color, the more product there is. The spectrophotometer measures the amount of light (at the wavelength of 410 nm) that is absorbed by the sample. The deeper yellow the sample is, the more light that is absorbed by the sample (and the more product there is present).

8. Determine the initial rate of product production from your absorbance measurements. **Hint**: The rate of product production is measured in absorbance units/min or nmol/min and it is the slope of the line between the zero and 1 minute time points.

   Sample rates = 12.5 nmol/min or 0.2 absorbance units (AU)/min

9. Is the rate of product production constant over time? Hint: Is the slope of the line constant or does it change?

   The slope of the line is constant for earlier time points, which means that the rate of product production is constant during this time. At later time points, the rate of product production should decrease some. If the reaction is carried out to completion, then the rate will eventually go to zero.

Activity 2 Analysis Questions

1. How can you determine the initial rate of the reaction for each temperature?

   The initial rate is equal to the amount of absorbance between the 0 and 2 minute time point divided by 2 minutes. This is also the initial slope of the line of product produced vs. time.

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

   This enzyme seems to work best at 37°C. I know this because the rate of product production was the highest at this temperature. Note: if the rate continued to increase, then the student would not be able to guess the optimal temperature but only be able to predict that it would be at a somewhat higher temperature than what was tested.

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

   Molecules move more quickly (have more kinetic energy) which increases the number of collisions between molecules. The average kinetic energy of the substrates is higher and therefore more substrate molecules have the required activation energy in order to reach the transition state.

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

   Molecules are moving slower at lower temperatures. Therefore, there are fewer collisions and the molecules may not have enough energy to reach the transition state.

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

   Most enzymes are proteins. Proteins denature at high temperatures. This is because the weak bonds between amino acids break, and the protein can lose its three dimensional structure. At higher temperatures, bonds such as hydrogen, ionic, and Van der Waal's interactions can break. This will unravel the protein's tertiary and secondary structures. Any covalent bonds will remain. The only covalent bonds involved in tertiary structure are the disulfide bonds between cysteine amino acids.

6. If you were a scientist who wanted to use this enzyme to produce glucose, at what temperature should you run the reaction?

   For the experiments run, the enzyme works fastest at about 37°C. In order to save energy, however, it may be more efficient to run the reaction at lower temperatures.

7. In what type of environment might an organism that produces this enzyme live? Explain your reasoning.

   Because this enzyme is heat stable, it is possible that this organisms lives in a warm environment.

Challenge questions

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

   The bonds that will break at high temperature are hydrogen, ionic and Van der Waal's interactions. Covalent bonds will not break.

2. Covalent bonds between R groups occur between which amino acids?

   Cysteine

3. What would be a disadvantage of using the highest temperature that yields the fastest rate of product formation?

   It requires energy to heat up the reaction. Since one of the goals of producing cellulosic ethanol is to reduce energy, it would be more advantageous to have a longer, more energy efficient process.

Activity 3 Analysis Questions

1. How can you determine the initial rate of the reaction for each pH?

   The initial reaction rate is the amount of product produced during the first time interval divided by the amount of time of the first time interval. It is also the same as the initial slope of the line of product vs. time.

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

   The optimal pH for this enzyme is near pH 5. We determined that from our data that showed that pH 5.0 has the highest initial rate of product production.

3. Why do most enzymatic reactions slow down at extremely high or low pH values?

   Extreme pH changes can break the bonds that hold the enzyme in its critical three-dimensional structure. The active site will not be the same shape, and therefore will not be able to catalyze the reaction. Any pH changes on either side of the optimal pH could change the charge in the active site of the enzyme (basic pH will tend to produce a more negative charge, while acidic pH will produce a more positive charge). For instance, an R group with a carboxyl group will be in the form COOH when in an acidic environment and it will be in the form COO^- in a basic environment, while amino acids with NH_3 on their R group will be in the form of NH_4^+ in an acidic environment.

4. In what type of environment might an organism that produces this enzyme live? Explain your reasoning.

   An acidic environment such as a bog or the soil in a redwood forest or perhaps a compost pile should be a good environment for bacteria or fungi that produce enzymes active at this pH level.

Activity 4 Analysis Questions

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

   The initial rate will increase as the amount of enzyme is increased until there is excess enzyme.

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

   No, the amount of substrate will determine the overall amount of product produced. If there is a low concentration of enzyme, it will take longer to produce the product, but eventually the same amount of product will be made.

3. If you were a scientist who is responsible for determining the concentration of enzyme to use in the hydrolysis process of producing sugar from cellulose, what advantage would there be to using a high concentration of enzyme? What disadvantage would there be to using a high concentration of enzyme?

   Higher enzyme concentration will produce product quicker. However, enzymes are costly. The least amount of enzyme should be used to make product in a reasonable amount of time. Tests should be run to see what concentration of enzyme would yield product at the maximum rate while not being in excess. Excess enzyme would be wasteful, making the overall process less cost efficient.

Activity 5 Analysis Questions

1. How does the amount of substrate present change the initial rate of reaction?

   Increasing the amount of substrate will increase the rate of product production until there is excess substrate present. Once there is excess substrate present, then additional increases to the concentration of substrate will no longer make a difference in the rate of product production.

2. Come up with an analogy or draw a cartoon to explain how changing the concentration of substrate affects the rate of product formation.

   Imagine a factory that slices potatoes for frozen French fries. The potatoes are like the substrate and the French fries are the product. If there are very few potatoes and lots of chopping machines, the pace of product production will be slow. If you increase the number of potatoes you feed into the machines, you increase the amount of product you make. However, there is a point in which every chopping machine is busy chopping potatoes. Adding more potatoes will not increase the rate any further.

3. Does the amount of substrate change the final amount of product, assuming that you let each reaction proceed for a really long time?

   Yes, the amount of substrate has a direct effect on the amount of product produced. In the case of cellobiase breaking down cellobiose, the more cellobiose you add to the reaction, the more glucose you will produce as the end product.

Activity 6 Analysis Questions

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

   Answers will vary. Mushrooms that were found to work were shiitake, enoki, white button, crimimi, and oyster mushrooms. Treatment and age of the mushroom (for example, a pickled, dried, or fresh mushroom from the store vs. a slimy one that has been sitting around for a while) can affect results due to enzyme breakdown with heat, pH, or proteases.

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?

   The enzyme extract in this case was in a different buffer (the extraction buffer), while resuspension buffer was used for earlier experiments. The new blank needs to take into account any contribution to absorbance of this new buffer along with any color that the mushroom extract itself might have. This could be due to the yellow color of the mushroom itself.

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?

   Answers will vary. In cases where the initial rate of reaction is higher with the enzyme extract, some answers might mention that the higher initial rate could be due to more enzyme in the extract than used in the lab, the extracted enzyme works better at room temperature than the enzyme used in the lab, the extract works better at the set pH than the enzyme used in the lab, etc.

4. Scientists are constantly looking for sources of enzymes that can be used in industrial processes. If you were going to pick a source of cellobiase to be used for ethanol production for biofuels, what type of organism might you look for as a source of this enzyme? **Hint**: The production of glucose to be converted to ethanol in biofuel production requires the reactions to occur at high temperatures and low pH.

   I would look for an organism that naturally lives at a high temperature and low pH, such as a composting bacterium or a hot spring bacterium. Other organisms such as composting fungus or bacteria that live in the rumen of animals might also work well.

Appendix H
References and Additional Resources

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Additional Resources

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