EXERCISE 6 - Lab Procedures

I. Determine the effect of substrate concentration on enzyme activity.

⇒ **Be sure you do not confuse the enzyme (glucose oxidase) with the substrate (glucose)!**

1. Turn on the Spec-20 and set the wavelength to 510 nm. The Spec-20 must warm up for at least 15 minutes before it will give accurate measurements.

2. Examine the solutions you have been given. One should be labeled "ENZYME: Glucose Oxidase." This is the ENZYME that will catalyze the reaction you will observe. Another solution should be labeled "GLUCOSE: Stock Solution (60 mg/dL)." This is one of the substrates for the reaction you will observe. Be very careful to use the correct solution for each part of the lab exercise.

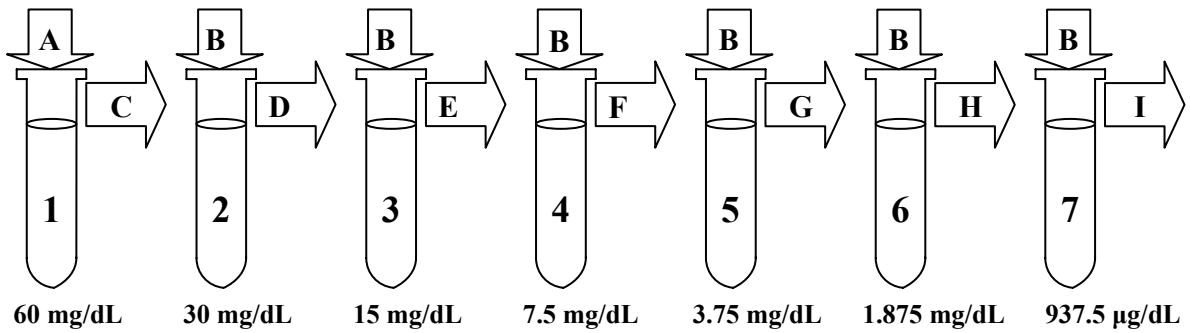
3. Using labeling tape and a Sharpie, label one 5 mL pipette "dH_2O" and another 5 mL pipette "Glucose." Be very careful to use the "dH_2O" pipette only with distilled water and the "Glucose" pipette only with the glucose stock solution. Do not cross-contaminate any of your solutions.

⇒ **It is critical that the enzyme solution not be contaminated with glucose and that the glucose solution not be contaminated with enzyme! Contamination usually occurs because the same pipette has been used to measure both solutions or the tip of the pipettor has touched the other solution while dispensing. If you have accidentally cross-contaminated your solutions, or if either solution appears pink while in its original container, give the contaminated solution to your instructor and obtain uncontaminated solutions before you continue the activity. To avoid contaminating your enzyme solution with glucose, use only a micropipettor and clean tips to dispense the enzyme solution.**

4. Using labeling tape and a Sharpie, label 8 clean Spec-20 cuvettes as follows: label one cuvette "B" (for "blank") and label the remaining 7 cuvettes with the numbers 1-7. When labeling Spec-20 cuvettes, never write on the cuvette! Use a small piece of label tape affixed to the top of the cuvette (so that it will not interfere with the spectrophotometer reading).

5. Add 3.0 mL of dH_20 (using the "dH_2O" pipet) and 2.0 mL of the enzyme (using a 1000 µL micropipettor with a clean tip) to the Spec-20 cuvette labeled "B" and set it aside. Later, this cuvette will be used to calibrate the Spec-20. (If this cuvette turns pink, it probably means that your blank cuvette has been contaminated with glucose and must be prepared again. You must locate the source of your contamination immediately to avoid making the same mistake again before continuing with the experiment.)

6. Starting with the stock solution containing 60 mg/dL of glucose, and using dH_2O as your diluting solution, you need to make 3 mL of each of the following glucose solutions: 30 mg/dL, 15 mg/dL, 7.5 mg/dL, 3.75 mg/dL, 1.875 mg/dL, and 937.5 µg/dL. As part of the Prelab, you were supposed to label the diagram below to show how you would prepare these solutions using the serial dilution technique. Ask your instructor to check your diagram before you proceed



7. Prepare your serial dilution of glucose in the Spec-20 cuvettes numbered 1 – 7 as follows:

   A. Use the pipette labeled "Glucose" to place the correct amount (v1 + v2) of stock solution in tube 1.
   B. Use the pipette labeled "dH_2O" to place the correct amount (v2) of dH_2O in tubes 2 - 7.
   C. Use the pipette labeled "Glucose" to transfer the correct amount (v1) of solution from tube 1 to tube 2.
   D. Mix the contents of tube 2 and then use a clean pipette to transfer the correct amount (v1) of solution from tube 2 to tube 3.
   E. Mix the contents of tube 3 and then use a clean pipette to transfer the correct amount (v1) of solution from tube 3 to tube 4.
   F. Mix the contents of tube 4 and then use a clean pipette to transfer the correct amount (v1) of solution from tube 4 to tube 5.
   G. Mix the contents of tube 5 and then use a clean pipette to transfer the correct amount (v1) of solution from tube 5 to tube 6.
   H. Mix the contents of tube 6 and then use a clean pipette to transfer the correct amount (v1) of solution from tube 6 to tube 7.
   I. Mix the contents of tube 7 and then use a clean pipette to discard the correct amount (v1) of solution from tube 7.

8. Decide which student in your group will be the timekeeper, which student will write down the absorbance levels measured (the note-taker), which student will add glucose oxidase enzyme to the Spec-20 cuvettes, and which student will operate the Spec-20. As you watch the readout while each tube is in the Spec-20, it will be constantly changing, so careful coordination in your group is necessary. The timekeeper will call out the times, the operator of the Spec-20 will call out the readings, and the note-taker will record the data. The note-taker must prepare an appropriately labeled table in his lab notebook to record the data, and this data must be transferred to every lab partner's notebook before starting Part II of the procedures.

9. Zero the Spec-20 following the instructions for the Spec-20 or the Spec-20D. Use the cuvette labeled "B", which you prepared in step 5, as your blank.

   Note: In the next step, the timekeeper should note the exact time that the glucose oxidase enzyme is added to the glucose. This is time zero. The first reading is at 15 seconds, so you have 15 seconds to mix the solutions, wipe off the cuvette with a Kimwipe, and place the cuvette in the Spec-20. You will be adding enzyme only to the diluted glucose solutions in cuvettes 2 – 7, not to the stock solution in cuvette # 1.

10. Add 2.0 mL of glucose oxidase enzyme to cuvette # 2 ONLY, and note the time. DO NOT add enzyme to more than one cuvette at a time! Quickly mix the glucose and enzyme solutions by sealing the end of the cuvette with Parafilm and gently inverting it 2-3 times.

11. After cleaning the cuvette with a Kimwipe to remove any fingerprints or spills, immediately place the cuvette into the Spec-20. Record the A_{510} values in your lab notebook every 15 seconds for 2 minutes, using a table such as the one below:

A_{510} Readings after Adding 2 mL of Glucose Oxidase to Varying Concentrations of Glucose

Cuvette	Glucose Concentration (mg/dL)	15 s	30s	45s	60 s	75 s	90 s	105 s	120 s
2									
3									
4									
5									
6									
7									

12. Repeat the procedure with the remaining 5 cuvettes (# 3 - 7).

13. Before disposing of your solutions, ask your instructor to check your absorbance measurements for plausibility.

14. Thoroughly clean your cuvettes by rinsing them several times with tap water and then with dH_2O. Place the cuvettes upside down in a test tube rack for later use.
    ⇒ **Remember: Never use abrasive cleansers or brushes when cleaning Spec-20 cuvettes. Rinse them with tap water and dH_20 only!!!!**

II. Determine the effect of temperature on enzyme activity.

1. Label four clean cuvettes 1 - 4 with labeling tape and a Sharpie. Add 2.0 mL dH20 and 1.0 mL of stock glucose solution to each cuvette. Place cuvette #1 in the ice bucket, #2 in the rack on your bench, #3 in the 37°C water bath, and #4 in the 65°C water bath to equilibrate the temperatures.

2. Check the thermometer in the lab to determine the lab room temperature for the cuvette left on your lab bench. Enter the temperature of each of the cuvettes in a clearly labeled table.

3. Label four test tubes (**not** Spec-20 cuvettes!) as follows: "ice", "room temperature", "37°C", "65°C". Add 2.0 mL of glucose oxidase enzyme solution to each of the four test tubes. Place the appropriate tube in the ice bucket, in a test tube rack on your lab bench, in a 37°C water bath, and in a 65°C water bath. Note the time and be ready to mix the enzyme tubes and the substrate tubes together after about 5 minutes.

4. Re-zero the Spec-20 using your "B" cuvette. Before proceeding, make sure all of the solutions have been in the ice bath/test tube rack/water baths for at least 5 minutes to allow them to reach the temperature of their surroundings. While waiting, you can be labeling beakers for Part III and/or planning and preparing for your experiment for Part IV. The note-taker can also prepare appropriately labeled tables for recording the data from this procedure. Also, don't forget that this data must be transferred to every lab partner's notebook before starting Part III of the procedures!

5. Pour the 2.0 mL of the cold glucose oxidase enzyme from the tube in the ice bucket into the cuvette # 1, which is also in the ice bucket. Cover with Parafilm, gently invert the cuvette several times, and return it to the ice. Note the time when the glucose oxidase enzyme was added and prepare to take absorbance readings every 15 seconds.

6. Keep cuvette #1 in the ice until about 10 sec. have elapsed since the enzyme was added. Then remove the cuvette and wipe it thoroughly with a Kimwipe (this will require more care than usual because condensation will tend to form on the outside of the cuvette). Quickly insert it into the Spec-20 and measure the absorbance at 15 sec. after the enzyme was added, as you did in the enzyme assay in Part I, above. (If you miss the 15 sec. mark, record the exact time with your absorbance and continue.)

7. Return the cuvette to the ice bucket. Remove the cuvette, dry it thoroughly, and measure the absorbance at 30, 45, 60, 75, 90, 105, and 120 seconds. Try to keep the cuvette on ice as much as is practical between measurements. Hold the cuvette by the top to avoid warming it with your hands.

8. Repeat this process with the other 3 cuvettes. The room temperature cuvette may remain in the Spec-20 until all of its measurements are made. Return the other two cuvettes to their respective water baths between readings. (If this is awkward, fill a beaker with the incubation water and bring the beaker to the Spec-20 during your measurements---the temperature will not deviate significantly over the time period that you will be taking your measurements.) Make sure you dry the cuvettes thoroughly before placing them in the Spec-20!

9. Thoroughly clean your cuvettes by rinsing them several times with tap water and then with dH_2O. Place the cuvettes upside down in a test tube rack for later use.
    ⇒ **Remember! Never use abrasive cleansers or brushes when cleaning Spec-20 cuvettes. Rinse them with tap water and dH_20 only!!!!**

III. Determine the effect of pH on enzyme activity

1. You have been provided with **4 unknown buffers labeled #1 - 4**. Label 4 clean cuvettes #1 - 4 and put 2.0 mL of the appropriate buffer in each cuvette using clean pipets to avoid cross-contamination.

2. Add 1.0 mL of glucose stock solution to each of the 4 cuvettes, seal with Parafilm, and mix by inverting the cuvette several times.

3. Use your reagent blank in cuvette "B" to re-zero the Spec-20.

4. After zeroing the Spec-20 with the "B" cuvette, add 2.0 mL of glucose oxidase enzyme to one of the 4 cuvettes containing buffered glucose solution, and note the time. Quickly seal the cuvette with Parafilm and mix the buffered glucose and enzyme solutions by covering the cuvette with Parafilm and inverting it 2-3 times. Wipe off the cuvette with a Kimwipe and place it into the Spec-20. Record the absorbance at 15, 30, 45, 60, 75, 90, 105, and 120 seconds in an appropriately labeled table in your lab notebook.

5. Repeat step 4 with the 3 remaining cuvettes and record your data.

6. Calibrate a pH meter and measure the pH of the reaction mixtures in cuvettes #1-4. If your pH probe will not fit into the cuvettes, pour some of each reaction mixture into a small beaker and take your readings. Record the pH values in your notebook.

7. Thoroughly clean your cuvettes by rinsing them several times with tap water and then with dH_2O. Place the cuvettes upside down in a test tube rack for later use.
   ⇒ **Remember! Never use abrasive cleansers or brushes when cleaning Spec-20 cuvettes. Rinse them with tap water and dH_20 only!!!!**

IV. Determine the specificity of the enzyme-substrate interaction

Galactose and mannose are isomers of glucose. All three of these monosaccharides have the formula $C_6H_{12}0_6$, but the arrangement of the atoms in each sugar differs.

So far, you have used glucose as the substrate for the reaction catalyzed by glucose oxidase. For this part of the lab, you have been provided with mannose and galactose solutions in the same concentration as the glucose stock solution. Design and carry out an experiment to determine if mannose and/or galactose can also act as substrates for the glucose oxidase enzyme.

V. Identify the cofactors required by the enzyme catacholase

In the first 4 parts of this lab, you examined how glucose oxidase activity is affected by 4 different variables: substrate concentration, temperature, pH, and substrate identity. Another variable that affects the activity of some enzymes is the presence of cofactors. A cofactor is a nonprotein substance that must be present in order for an enzyme to function normally. For example, some metal ions function as cofactors by helping to draw electrons away from substrate molecules, thereby making it easier for the reaction to proceed. When a cofactor is a nonprotein organic molecule, it is called a coenzyme. Many vitamins are parts of coenzymes.

Because plants are unable to physically escape from attacking pathogens, many rely on "chemical warfare" for protection. **Catecholase** is an enzyme that acts to protect many plants from pathogens. Catecholase catalyzes a reaction between catechol and O_2 (the substrates of this reaction.) As a result of the reaction, benzoquinone and water (the products of the reaction) are formed. Both catechol and catecholase are found in many plant cells. However, the reaction occurs only if the plant cells are damaged, which releases the catechol and catecholase and brings them into contact with the oxygen in the air. The benzoquinone that is formed during this reaction works as an antiseptic, protecting the plant against pathogens.

In this part of the lab, you will study the reaction catalyzed by catecholase in a test tube. As benzoquinone molecules are produced, they will react with each other to form long-chain brown pigments. The faster the reaction occurs, the faster benzoquinone will be produced, and the faster the reaction mixture will turn brown.

The source of catecholase in this experiment is a crude potato extract. Included in this extract are all the solutes of the potato cell, including cations such as Ca^{2+} , Mg^{2+} , and Cu^{2+} . These cations frequently serve as cofactors for a variety of enzymes. Chelators such as **ethylene diamine tetraacetic acid (EDTA)** and **phenylthiourea (PTU)** will be added to the reaction mixture in some test tubes to remove specific cations from solution. EDTA preferentially removes Ca^{2+} and Mg^{2+}, while PTU preferentially removes Cu^{2+}.

This part of the lab activity will be prepared as a demo by the instructor.

Instructor:

Label five test tubes and add:

					Add Last:
tube #	dH_2O (mL)	potato juice (mL)	EDTA (mL)	PTU (mL)	1% catechol (mL)
1	2.0	1.0	--	--	--
2	1.0	1.0	--	--	1.0
3	2.0	--	--	--	1.0
4	--	1.0	1.0	--	1.0
5	--	1.0	--	1.0	1.0.

Cover the tubes with Parafilm and mix by holding the Parafilm firmly in place with your thumb and inverting the tube a few times. This will ensure that the potato juice comes in contact with both oxygen and catechol.

Students:

Observe the 5 demonstration tubes on display in lab. Make an appropriately labeled table in your lab notebook to record your observations. Your table should show the contents of each tube, as well as the relative intensity of the brown color in each tube.

Clean up

Cleaning the Spec-20 cuvettes: Spec-20 cuvettes are not ordinary test tubes. They are expensive, and great care must be taken to avoid scratching them. Scratches interfere with the passage of light through the tube and that can lead to inaccurate results. Rinse the cuvettes thoroughly with tap water and then with dH_2O.

Clean your glassware.

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Ask your instructor to check your work area before you leave.