Introduction to Biofuels: Enzyme Action

OVERVIEW

See the student handout for an introduction to this investigation. For more thorough background information, see the Background for Instructors section on pages 5–9 of the Instruction Manual accompanying the Biofuel Enzyme Kit (available from Bio-Rad Laboratories, Inc., #166-5035EDU).

In the Preliminary Activity, your students will use a spectrophotometer to determine *p*-nitrophenol concentration. They will first produce a *p*-nitrophenol containing sample. Next, they will determine the absorbance values for a set of *p*-nitrophenol solutions with known concentrations in order to create a Beer's law standard curve. They will then determine the *p*-nitrophenol concentration of their prepared sample by measuring its absorbance and then finding the corresponding concentration on the standard curve using the Interpolation Calculator feature or the Interpolate feature of the data-collection program. They will also determine the initial rate of their *p*-nitrophenol producing reaction. A student handout for the Open Inquiry version of the Preliminary Activity can be found at the end of this investigation. A Guided Inquiry version can be found on the CD accompanying this book.

During the subsequent Inquiry Process, your students will first find out more about biofuels and the cellobiase catalyzed reaction that produces *p*-nitrophenol using the course textbook, other available books, and the Internet. They will then generate and investigate researchable questions. (In the Guided Inquiry approach, students will plan and conduct investigations of the researchable question(s) assigned by you.)

LEARNING OUTCOMES

In this inquiry investigation, students will

- Identify variables, design and perform the investigation, collect data, analyze data, draw a conclusion, and formulate a knowledge claim based on evidence from the investigation.
- Produce a *p*-nitrophenol containing sample.
- Create a Beer's law standard curve.
- Determine the *p*-nitrophenol concentration of the prepared sample.
- Determine the initial rate of the *p*-nitrophenol producing reaction.

THE INQUIRY PROCESS

Suggested Time to Complete the Investigation

See page xiii in the Doing Inquiry Investigations section for more information on carrying out each phase of an inquiry experiment.

	Inquiry Phase	Open Inquiry	Guided Inquiry
I	Preliminary Activity	40 minutes	40 minutes
II	Generating Researchable Questions (Omitted in Guided Inquiry Approach)	10 minutes	0 minutes
III	Planning	10 minutes	10 minutes
IV	Carrying Out the Plan	40 minutes	40 minutes
V	Organizing the Data	10 minutes	10 minutes
VI	Communicating the Results	10 minutes	10 minutes
VII	Conclusion	5 minutes	5 minutes

MATERIALS

Make the following materials available for student use. Items in bold are needed for the Preliminary Activity.

data-collection program
SpectroVis Plus spectrophotometer*
1.5 mL cuvette with lid
20–200 µL micropipet**
100–1000 µL micropipet**
200 µL micropipet tips (1 box)
1000 µL micropipet tips (1 box)
1.5 mL microtube
goggles
lint-free tissue
1x stop solution
1.5 mM substrate
low concentration enzyme
***p*-nitrophenol standards set S1–S4**
stopwatch
others as requested by students

* Other Vernier-compatible spectrophotometers can be used (e.g., Vernier Spectrometer, Ocean Optics Red Tide)
** Appropriate graduated transfer pipets (1 and 5 mL) may be substituted.

I Preliminary Activity

This inquiry begins with an activity to reinforce prior knowledge of the use of Vernier data-collection technology and to introduce a method for determining *p*-nitrophenol concentration.

Sample Results

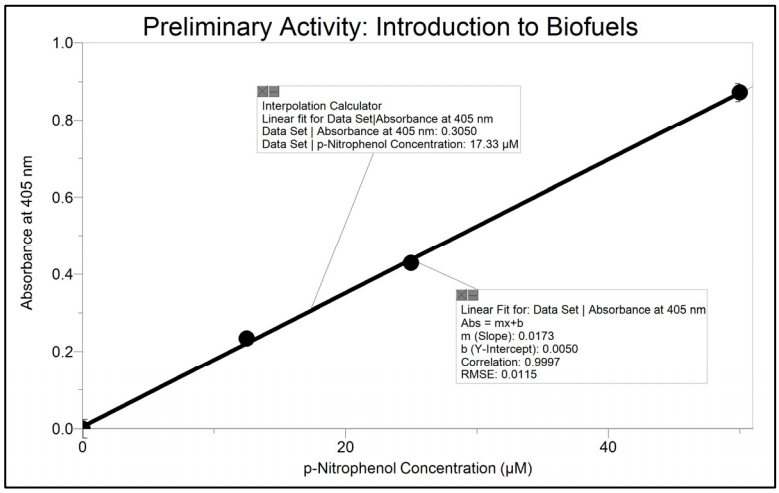

Figure 1 *Using a Beer's law standard curve to determine* p-*nitrophenol concentration*

Table 1: Data for Determination of the Beer's Law Standard Curve		
Standard	*p*-Nitrophenol concentration (µM)	Absorbance (at 405 nm)
S1	0	0
S2	12.5	0.234
S3	25	0.429
S4	50	0.872

Table 2: Determining the *p*-Nitrophenol Concentration from a Standard Curve		
Time (min)	Absorbance (at 405 nm)	*p*-Nitrophenol concentration (µM)
2	0.305	17.3

Answers to the Questions

1. Calculate the initial rate of *p*-nitrophenol production. Since you only measured the amount of *p*-nitrophenol at one time point, 2 minutes, assume the amount of *p*-nitrophenol at 0 minutes is 0 nmol. Note: The amount of *p*-nitrophenol present in the cuvette, measured in nmol, has the same numerical value as the concentration expressed in µM. For example, 42.8 nmol corresponds to 42.8 µM.

   Answers will vary. In the Sample Results above, a concentration of 17.3 µM corresponds to an absorbance of 0.305 after 2 minutes of reaction. Thus the initial rate can be determined as follows.

   (17.3 nmol – 0 nmol)/(2 min – 0 min) = 8.65 nmol/min

   Note: 17.3 µM = 17.3 µmol/L and 17.3 µmol/L = 17.3 nmol/mL, thus the 1 mL (1000 µL) of product solution contained 17.3 nmol of *p*-nitrophenol after 2 minutes of reaction.

2. What is the name of the enzyme studied in this investigation?

   The enzyme studied in this investigation is cellobiase (a type of cellulase or a beta-glucosidase).

3. What is one practical industrial application of this enzyme?

   One practical application of this enzyme is the production of cellulosic ethanol for fuel.

4. Identify the natural product of this enzyme.

   Glucose is the natural product.

5. Identify the natural substrate of this enzyme.

   Cellobiose, a disaccharide, is the natural substrate for cellobiase.

6. List at least three factors that might influence the rate of *p*-nitrophenol production for the reaction being studied in this investigation.

   Some factors that might influence the rate of *p*-nitrophenol production are temperature, pH, enzyme concentration, substrate concentration, enzyme source, ethanol concentration, and salinity.

7. List at least one researchable question concerning the rate of *p*-nitrophenol production.

   Answers will vary. See the Researchable Questions list below for some possible answers.

II Generating Researchable Questions

Note: Researchable questions are assigned by the instructor in the Guided Inquiry approach. See page xiii in the Doing Inquiry Investigations section for a list of suggestions for generating researchable questions. Some possible researchable questions are listed below:

Recommended for Open Inquiry or Guided Inquiry (sample results provided)

- How does temperature affect the rate of *p*-nitrophenol production?
- How does pH affect the rate of *p*-nitrophenol production?
- How does substrate concentration affect the rate of p-nitrophenol production?
- How does the rate of yeast metabolism of cellobiose in the presence of cellobiase compare to the rate without cellobiase present?

Recommended for Open Inquiry or Guided Inquiry (sample results not provided)

- How does cellobiase concentration affect the rate of p-nitrophenol production?
- How does salinity affect the rate of p-nitrophenol production?

Recommended for Advanced Students (sample results provided)

- How do the reaction rates in the presence and absence of enzyme compare?
- How does temperature affect the rate of p-nitrophenol production? (advanced version)
- How does pH affect the rate of p-nitrophenol production? (advanced version)

Recommended for Advanced Students (sample results not provided)

- How does enzyme source affect the rate of *p*-nitrophenol production?
- How does ethanol concentration affect the rate of *p*-nitrophenol production?

There are many more possible researchable questions. Students should choose a researchable question that addresses the learning outcomes of your specific standards. Be sure to emphasize experimental control and variables. (Instructors using the Guided Inquiry approach select the researchable questions to be investigated by their students. We encourage you to assign multiple researchable questions because this strategy enhances student interaction and learning during phases IV–VII.)

III Planning

During this phase students should formulate a hypothesis, determine the experimental design and setup, and write a method they will use to collect data. The plan should list laboratory safety concerns and specify how they will be addressed during the investigation. Circulate among the student groups asking questions and making helpful suggestions.

IV Carrying Out the Plan

During this phase, students use their plan to carry out the investigation and collect data. Circulate among the student groups asking questions and making helpful suggestions.

V Organizing the Data

See page xv in the Doing Inquiry Investigations section for suggestions concerning how students can organize their data for their inquiry presentations.

VI Communicating the Results

See page xv in the Doing Inquiry Investigations section for a list of inquiry-presentation strategies.

VII Conclusion

Using your notes recorded during the Communicating the Results phase, summarize the group results for the experiment and tell how they will fit into the upcoming instruction.

VIII Assessment

See page xv in the Doing Inquiry Investigations section for ideas on assessment strategies.

SAMPLE RESULTS

Student results will vary depending on experimental design.

The Effect of Temperature on Reaction Rate

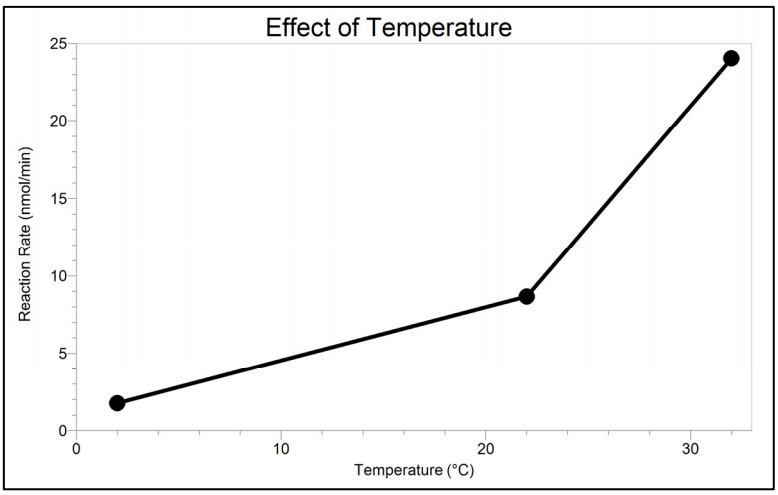

Figure 2 *Initial rate of the cellobiase catalyzed reaction as a function of temperature*

Table 3: The Effect of Temperature			
Temperature (°C)	Absorbance (at 405 nm)	Concentration (μM)	Reaction rate (nmol/min)
2	0.066	3.52	1.76
22	0.305	17.3	8.67
32	0.837	48.0	24.0

This investigation addresses the question, "How does temperature affect the rate of *p*-nitrophenol production?" These data were collected using a modification of the Preliminary Activity procedure. See the Tips section for details.

The reaction is very responsive to temperature change. The rate of reaction increases with increasing temperature.

The Effect of pH on Reaction Rate

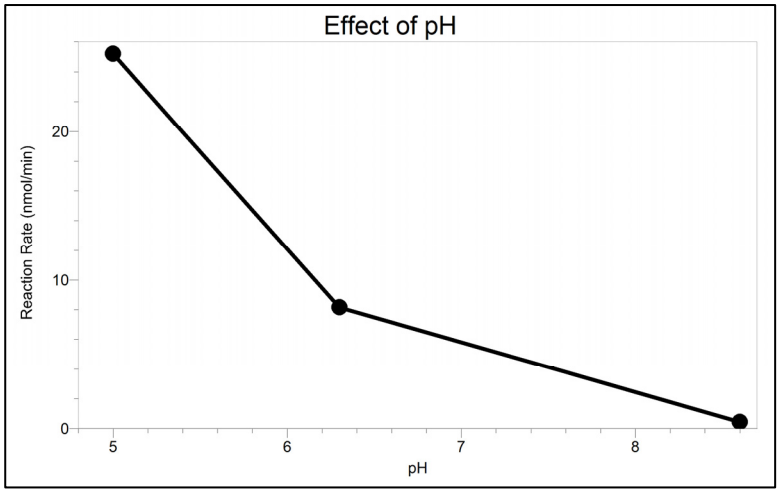

Figure 3 *Initial rate of the cellobiase catalyzed reaction as a function of pH*

Table 4: The Effect of pH			
pH	Absorbance (at 405 nm)	Concentration (μM)	Reaction rate (nmol/min)
5.0	0.877	50.4	25.2
6.3	0.287	16.3	8.15
8.6	0.020	0.87	0.44

This investigation addresses the question, "How does pH affect the rate of *p*-nitrophenol production?" These data were collected using a modification of the Preliminary Activity procedure. See the Tips section for details.

The reaction is also very responsive to pH. The rate of reaction decreased with increasing pH over the range of pH values tested. These results explain the use of pH 9.5 stop solution to stop the reaction.

The Effect of Substrate Concentration on Reaction Rate

Table 5: The Effect of Substrate Concentration				
Original concentration (mM)	Concentration after mixing (mM)	Absorbance (at 405 nm)	*p*-Nitrophenol concentration (μM)	Reaction rate (nmol/min)
1.5 (High)	0.75	0.892	51.2	25.6
0.25 (Low)	0.13	0.352	20.0	10.0

This investigation addresses the question, "How does substrate concentration affect the rate of *p*-nitrophenol production?" These data were collected using a slight modification of the procedure from the Preliminary Activity. See the Tips section for details.

Decreasing the substrate concentration decreased the initial reaction rate as shown above.

Comparing Yeast Metabolism Rates of Cellobiose

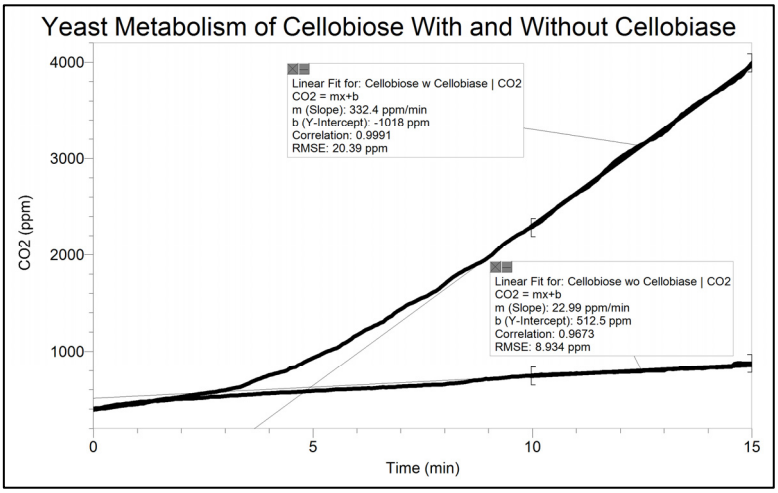

Figure 4 Y*east metabolism rates of cellobiose with and without cellobiase present*

Table 6: Comparing Rates of Yeast Metabolism of Cellobiose	
Conditions	Reaction rate (ppm CO_2/min)
With yeast and cellobiase present	332
With yeast, but without cellobiase present	23.0

This investigation addresses the question, "How does the rate of yeast metabolism of cellobiose in the presence of cellobiase compare to the rate without cellobiase present?" These data were collected using a CO_2 Gas Sensor and the procedure from Investigation 10, Sugar Metabolism with Yeast. See the Tips section for more details.

The metabolism rate of cellobiose by yeast with cellobiase present was found to be 14.4 times as great as the rate without cellobiase present.

Determining Reaction Rate in the Presence and Absence of Enzyme

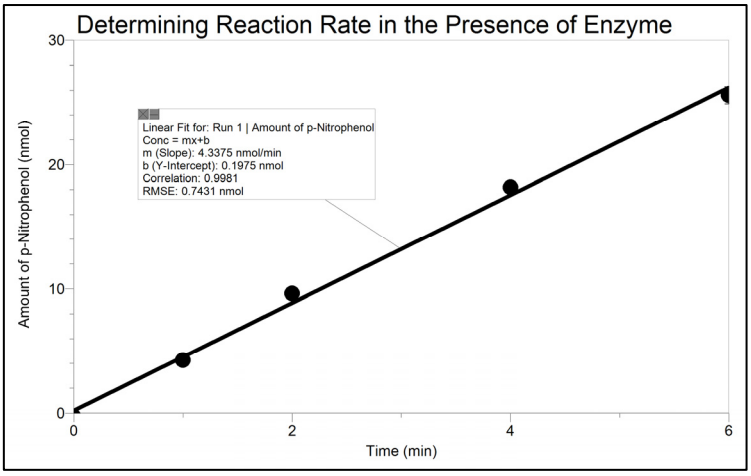

Figure 5 *Using concentration to determine reaction rate in the presence of enzyme*

Table 7: Data With and Without Enzyme Present			
Time (min)	Cuvette	Absorbance at 405 nm	Amount of *p*-Nitrophenol (nmol) from the Beer's Law Standard Curve
0	Start	0	0
6	End	0	0
1	E1	0.079	4.27
2	E2	0.171	9.62
4	E3	0.320	18.2
6	E4	0.448	25.6

Table 8: Data With and Without Enzyme Present	
Rate of product formation with enzyme present	4.34 nmol/min
Rate of product formation with no enzyme present	0 nmol/min

This investigation addresses the question, "How do the reaction rates in the presence and absence of enzyme compare?" This is a slight modification of Activity 1 found in the Instruction Manual accompanying the Biofuel Enzyme Kit. See the Tips section for details.

The rate of production of *p*-nitrophenol with cellobiase present was found to be 4.34 nmol/min, whereas the rate with cellobiase absent was 0 nmol/min.

A Closer Look at the Effect of Temperature on Rate of Reaction

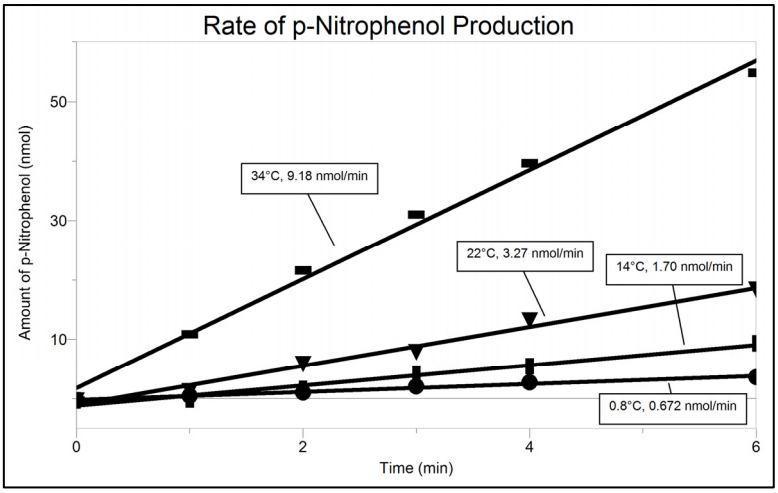

Figure 6 *Rate as a function of temperature*

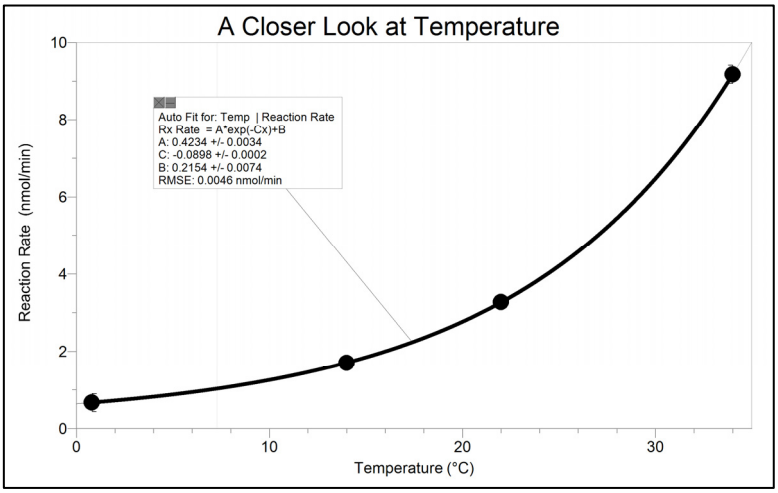

Figure 7 *A natural exponent curve fit of temperature data*

Table 9: Concentration Data for a Closer Look at the Effect of Temperature							
Temp (°C)	Time (min)						Rate (nmol/min)
	0	1	2	3	4	6	
0.8	0	0.461	1.01	2.05	2.71	3.62	0.672
14	0	-	1.63	4.17	5.46	9.34	1.70
22	0	1.28	5.88	7.93	13.2	18.4	3.27
34	0	10.9	21.6	31.0	39.7	54.9	9.18

This investigation addresses the question, "How does temperature affect the rate of *p*-nitrophenol production? (advanced version)" These data were collected using a modification of the Preliminary Activity procedure. See the Tips section for more details.

The reaction is very responsive to temperature change. The rate of reaction increases with increasing temperature, and natural exponent curve fit of the data produces a well-fitting exponential curve.

A Closer Look at the Effect of pH on Rate of Reaction

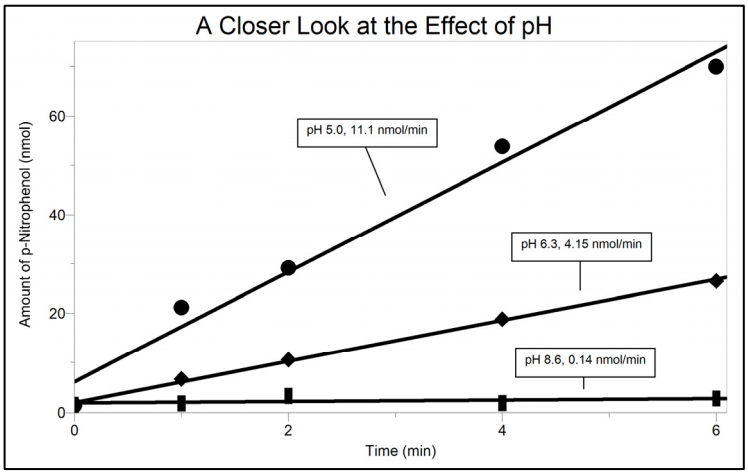

Figure 8 *Rate of reaction determined with pH data at multiple points*

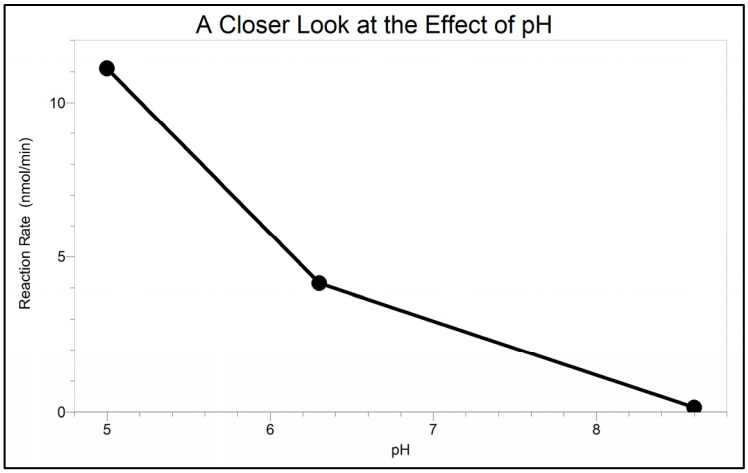

Figure 9 *Reaction rate as a function of pH*

Table 10: Looking at the Effect of pH Using Concentration Data						
pH	Time (min)					Rate (nmol/min)
	0	1	2	4	6	
5.0	1.41	21.1	29.1	53.8	79.8	11.1
6.3	1.18	6.71	10.6	18.8	26.5	4.15
8.6	1.58	1.80	3.30	1.87	2.80	0.14

This investigation addresses the question, "How does pH affect the rate of *p*-nitrophenol production? (advanced version)" These data were collected using a modification of the Preliminary Activity procedure. See the Tips section for more details.

The reaction is also very responsive to pH change. The rate of reaction decreased with increasing pH over the range of pH values tested. The reaction was nearly stopped at pH 8.6.

TIPS

1. The instructions provided assume that you have purchased the "Biofuel Enzyme Kit" from Bio-Rad Laboratories, Inc. (explorer.bio-rad.com, Catalog # 166-5035EDU).

2. The reaction being studied in this investigation, the cellobiase catalyzed breakdown of cellobiose into *p*-nitrophenol and glucose, is very temperature sensitive. **Important**: As such, be sure to to bring all reagents to room temperature before use.

3. Each student group will require the following solutions in the specified amounts for the Preliminary Activity:
   - 500 µL of 1x stop solution
   - 500 µL of 1.5 mM substrate (*p*-nitrophenyl glucopyranoside)
   - 250 µL of low concentration enzyme (cellobiase)
   - One set of colorimetric standards, S1–S4. Each of the three cuvettes S2–S4 is to contain 1 mL of *p*-nitrophenol solution with its specified concentration. Cuvette S1 is to contains1 mL of 1x resuspension buffer. Place lids on the cuvettes. Cuvette lids are available from Vernier (order code: CUV-LID).

   Note: The colorimetric standards sets can be used by successive classes. If students save the Beer's law standard curves produced during the Preliminary Activity, they will be able to access and use them during subsequent lab sessions. **Important**: Students switching spectrophotometers will need to prepare new standard curves.

4. Directions for preparation of the solutions are below. **Important: Store the prepared solutions at 4°C.**

   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.

   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.

3.0 mM substrate (p-nitrophenyl glucopyranoside): Label a bottle "3.0 mM substrate". Add 1 mL of 1x resuspension buffer to the vial of substrate and mix. Combine this 1 mL with the 98 mL of 1x resuspension buffer in the bottle. Add another 1 mL of 1x resuspension buffer to the vial and mix. Transfer this 1 mL into the same bottle containing the substrate solution. Mix until are 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.

1.5 mM substrate (p-nitrophenyl glucopyranoside): 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 by shaking.

High concentration enzyme (cellobiase): Label a bottle "High Concentration Enzyme". Add 600 µL of 1x resuspension buffer to the 400 µL of enzyme in the vial and mix. Combine this 1 mL of diluted enzyme with 63 mL of 1x resuspension buffer in the bottle and mix by shaking.

Low concentration enzyme (cellobiase): Label a bottle "Low Concentration Enzyme". **Note**: This is the enzyme used in the Preliminary Activity. Combine 20 mL of high concentration enzyme with 60 mL of 1x resuspension buffer in the bottle and mix by shaking.

5. To prepare **the colorimetric standard solutions (*p*-nitrophenol)**:
   a. Label four 50 mL bottles S1–S4.
   b. Put 10 mL of deionized water into each bottle.
   c. Add 2 mL of standard + 8 mL of deionized or distilled water to bottle labeled S4 and mix.
   d. Transfer 10 mL of standard from bottle S4 to bottle S3 and mix.
   e. Transfer 10 mL of standard from bottle S3 to bottle S2 and mix.
   f. Remove 10 mL of standard from bottle S2 and discard.
   g. Add 10 mL of 1x stop solution to all four bottles and mix.

6. Many spectrophotometers, including the SpectroVis Plus, begin to lose linearity above an absorbance of 1.0. For this reason, we exclude the Biofuel Enzyme Kit *p*-nitrophenol standard labeled S5, with a concentration of 100 µM, from the Preliminary Activity materials list. We suggest that you avoid the use of solutions that produce absorbance values greater than 1.0, unless your spectrophotometers maintain linearity well above 1.0.

7. The Preliminary Activity directs students to use the Interpolation Calculator to determine the concentration. If you wish to include more mathematics in this investigation, your students could calculate the concentration using the equation for the linear fit

$$y = mx + b$$

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

For example, suppose the data-collection program displays the following information for the linear fit: $y = 0.429$, $m = 0.0173$, $b = 0.0050$, and that the concentration units are µM. Upon substituting into the equation $y = mx + b$ you obtain the following solution.

$$0.429 = (0.0173\ \mu M^{-1})(x\ \mu M) + 0.0050$$

$$x = (0.429 - 0.0050)/(0.0173\ \mu M^{-1}) = 24.5\ \mu M$$

8. Tips for **determining the effect of temperature on reaction rate**:
   - The 500 µL of 1.5 mM substrate and 250 µL of enzyme should be pipeted into separate 1.5 mL microcentrifuge tubes and in turn be placed into the appropriate water bath for at least five minutes prior to the initiation of the reaction.
   - After the 250 µL of enzyme have been pipeted into the microcentrifuge tube containing the substrate to initiate the reaction, the microcentrifuge tube containing the substrate and enzyme mixture should be returned to the water bath for two minutes.
   - When the 2-minute period has elapsed, 500 µL of the mixture should be pipeted into the cuvette containing the stop solution. Allow the contents of the cuvette to warm (or cool) to room temperature for at least five minutes before determining absorbance.
   - **Note**: One of the temperatures may be room temperature, for which no water bath is necessary.

9. Tips for determining the effect of pH on reaction rate.

   Prepare the pH 5.0, 6.3, and 8.6 solutions.
   - The pH 5.0 solution is 1x resuspension buffer.
   - The pH 6.3 is a mixture that is 1 part 1x stop solution and 3 parts distilled water.
   - The pH 8.6 solution is 1x stop solution.

   Change Step 2 of the student Procedure to read "Pipet 250 µL of 3.0 mM substrate into a 1.5 mL microcentrifuge tube, and then pipet 250 µL of the appropriate pH solution (pH 5.0, 6.3, or 8.6) into the same microcentrifuge tube. Proceed as in the Preliminary Activity, using your original Beer's law standard curve.

10. Tips for **determining the effect of substrate concentration on reaction rate**:
    - Pipet 500 µL of 1.5 mM substrate into a 1.5 mL microcentrifuge tube labeled "High Concentration Substrate".
    - Pipet 100 µL of 1.5 mM substrate and 500 µL of 1x resuspension buffer into a 1.5 mL microcentrifuge tube labeled "Low Concentration Substrate". Mix, then remove and discard 100 µL of the solution.
    - For each sample, proceed as in the Preliminary Activity.

11. Tips for **comparing the yeast metabolism rates of cellobiose with and without cellobiase**:
    - Prepare the yeast suspension by adding 5 grams of yeast and 5 grams of glucose to 100 mL of warm distilled water. Stir overnight on a magnetic stirrer.
    - Prepare the cellobiose solution by adding 0.2 g of cellobiose to 20 mL of distilled water. **Note:** Cellobiose is not provided in the Biofuel Enzyme Kit.
    - Prepare the experimental (with cellobiase) sample by combining 2 mL of high concentration enzyme and 6 mL of cellobiose solution in a 15 mL conical tube.
    - Prepare the control (without cellobiase) sample by combining 2 mL of resuspension buffer and 6 mL of cellobiose solution in a 15 mL conical tube.
    - Place both samples in a water bath at 31–33°C for one hour.
    - Collect data using a CO_2 Gas Sensor and the procedure from Investigation 10, Sugar Metabolism with Yeast.

12. Tips for **determining the effect of the presence and absence of enzyme (advanced topic)**:
    - To allow for the multiple trials, pipet 3 mL of 1.5 mM substrate into a 15 mL conical tube.
    - To initiate the reaction, pipet 200 µL of low concentration enzyme into the substrate.
    - At the specified time, pipet 500 µL of the reaction mixture into the prepared cuvette containing 500 µL of stop solution.

13. Tips for **a closer look at the effect of temperature (advanced topic)**:
    - To allow for the multiple trials, pipet 3 mL of 1.5 mM substrate into a 15 mL conical tube.
    - Pipet 250 µL of low concentration enzyme into a 1.5 mL microcentrifuge tube.
    - Place both into a water bath at the desired temperature for at least five minutes.
    - To initiate the reaction, pipet 200 µL of low concentration enzyme into the substrate.
    - At the specified times, pipet 500 µL of the reaction mixture into the prepared cuvette containing 500 µL of stop solution.
    - It is best to use a blank consisting of 500 µL of substrate and 500 µL of stop solution.

14. Tips for **a closer look at the effect of pH (advanced topic).**

    Prepare the pH 5.0, 6.3, and 8.6 solutions.

    - The pH 5.0 solution is 1x resuspension buffer.
    - The pH 6.3 is a mixture that is 1 part 1x stop solution and 3 parts distilled water.
    - The pH 8.6 solution is 1x stop solution.

    Collect data for each pH solution (pH 5.0, 6.3, or 8.6) as follows:

    - To allow for the multiple trials, pipet 1 mL of the appropriate solution (pH 5.0, 6.3, or 8.6) into a 15 mL conical tube.
    - Pipet 1 mL of 1.5 mM substrate into the tube.
    - To initiate the reaction, pipet 1 mL of low concentration enzyme into the tube.
    - At the specified times, pipet 500 µL of the reaction mixture into the prepared cuvette containing 500 µL of stop solution.

15. **HAZARD ALERT:**

    There is a strong base solution (stop solution, pH 9.5) used in this investigation, so safety protocols should be observed. If any solution gets into a student's eyes, flush with water for 15 minutes. Lab coats, goggles, or other protective clothing should be worn to avoid any injury caused by spilled base.

    The hazard information reference is: Bio-Rad Laboratories Inc, MSDS for product 166-5035, 1-800-424-6723, www.bio-rad.com.

16. More information about the sensor used in this Investigation, as well as tips for optimal performance, can be found in the sensor's user manual available for download from the Vernier web site, www.vernier.com/sensors.

17. The plans that your students submit for approval should list laboratory safety concerns, including chemical safety concerns, and specify how they will address these safety concerns during their investigations.

This investigation was adapted from Bio-Rad Laboratory's Biofuel Enzyme Kit. Text and figures are used with permission.