Investigation
20

Evolution of Cellobiase

OVERVIEW

The kingdom Fungi contains the Basidiomycota or club fungi, a group that includes what we call mushrooms. Mushrooms are decomposers that have evolved to grow in diverse environments. The button mushroom (*Agaricus bisporus*), also known under various names including: common mushroom, table mushroom, and champignon mushroom, is native to grasslands. The oyster mushroom (*Pleurotus ostreatus*) is typically found on trees and wood. Other mushrooms are mycorhizal, which means that they have evolved symbiotic relationships with the roots of specific trees. The matsutake (*Tricholoma magnivelare)* and chanterelles *(Cantharellus sp.)* are examples of mycorhizal mushrooms. Morels, false morels, and truffles are also decomposers, but they are not true mushrooms, they are classified as cup fungi and belong to the group Ascomycota.

In this investigation, your students will be studying the cellobiase activity found in different types of club and/or cup fungi. *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 to produce glucose as a food source.

In the Preliminary Activity, your students will (1) produce a button mushroom extract, (2) react this cellobiase-containing extract with substrate, (3) collect samples after 1, 2, 3, 4, and 6 minutes of reaction, (4) use a Spectrometer to measure the absorbances of the colored samples, (5) plot a graph of absorbance *vs*. time, and (6) use the graph to determine the rate of the cellobiase catalyzed 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 mushrooms, cellobiase activity, and evolution using the course textbook, other available books, and the Internet. They will then generate and investigate researchable questions dealing with cellobiase and the evolution of mushrooms. (In the Guided Inquiry approach, students will plan and conduct investigations of the researchable question(s) assigned by you.)

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

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 cellobiase containing samples.
- Determine absorbances of the prepared samples.
- Determine the initial rates of the cellobiase catalyzed reactions.
- Compare the cellobiase activities of various fungi.

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	50 minutes	50 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*
goggles
button mushroom
balance
extraction buffer
mortar and pestle
20–200 µL micropipet**
100–1000 µL micropipet**
200 µL micropipet tips (1 box)
1000 µL micropipet tips (1 box)
1.5 mL microtube
six 1.5 mL cuvettes with lids
stop solution
1.5 mM substrate
15 mL conical tube
stopwatch
lint-free tissue
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 cellobiase activity.

Sample Results

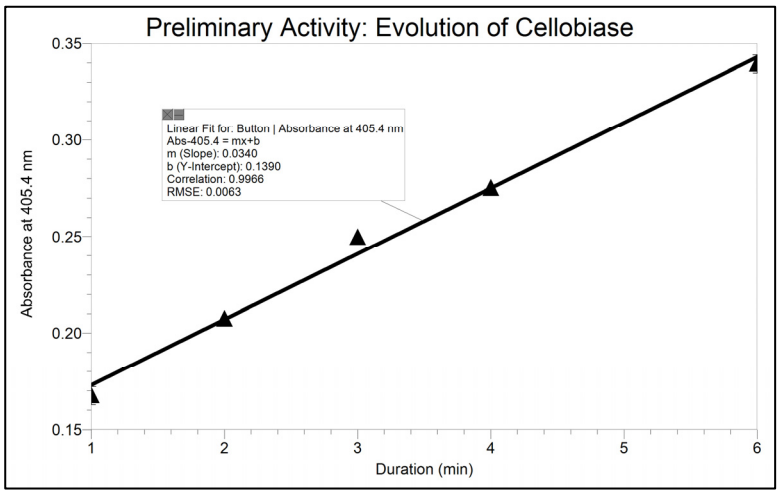

Figure 1 *Cellobiase activity of a button mushroom*

Answers to the Questions

1. What was the rate of your cellobiase catalyzed reaction?

   Answers will vary. The rate of reaction in the Sample Results above is 0.0340 Δ abs/min.

2. List three varieties of mushrooms available in your area.

   Answers will vary. Some commonly available edible mushrooms are Agaricus biporus (known variously as button mushroom, common mushroom, table mushroom, champignon mushroom, crimini mushroom, and white mushroom), shiitake, maitake, oyster, and enoki.

3. List two factors that could have affected cellobiase activity in different mushrooms as they evolved.

   Answers will vary. Some factors that might have affected cellobiase activity in different mushrooms are the availability of cellobiose, habitat, lifestyle, available nutrients, availability of wood, temperature, and substrate pH.

4. List at least one researchable question concerning cellobiase and the evolution of mushrooms.

   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 for this investigation are listed below:

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

- How do the cellobiase activities of various store-bought mushrooms compare?
- How do the cellobiase activities of store-bought shiitake, crimini, and chanterelle mushrooms compare?

- How do the cellobiase activities of store-bought oyster and button mushrooms compare?
- How do the cellobiase activities of various dried mushrooms compare?
- How do the cellobiase activities of dried shiitake, morel, and matsutake mushrooms compare?
- How do the cellobiase activities of different parts of the same mushroom compare?

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

- How do the cellobiase activities of various mushrooms of the Agaricus genus compare?

Recommended for Advanced Students (sample results provided)

- How do the cellobiase activities of store-bought shiitake, crimini, and chanterelle mushrooms compare? (advanced version)
- How do the protein concentrations of shiitake and button mushrooms extracts compare?
- How do the cellobiase activities of field collected fungi compare? (for college courses only)

Recommended for Advanced Students (sample results not provided)

- How do the cellobiase activities of various store-bought mushrooms compare? (advanced version)

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.

Comparing the Cellobiase Activities of Store-Bought Mushrooms

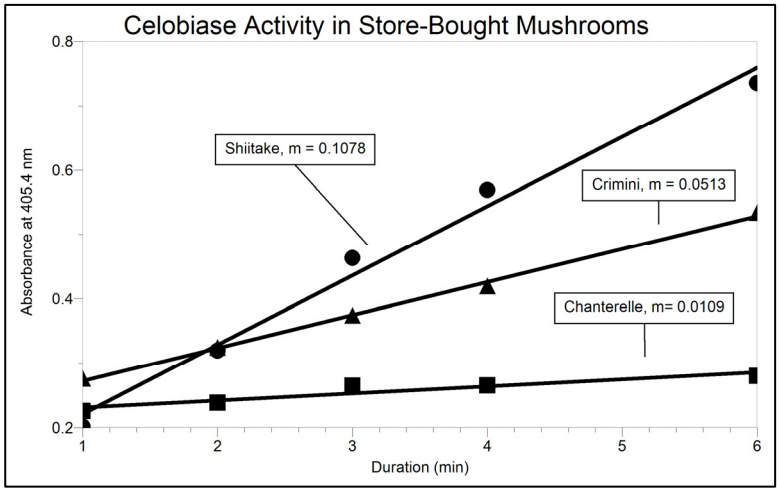

Figure 2 *Comparing cellobiase activities*

Table 1: Cellobiase Activity of Store-Bought Mushrooms		
Name	Where it grows	Cellobiase activity (Δ abs/min)
Shiitake (*Lentinula edodes*)	On dead wood from *Castanopis cuspidate* trees	0.1078
Crimini (*Agaricus bisporus)*	In fields and grassy areas	0.0513
Chanterelle (*Cantharellus sp.)*	In mixed woods under conifers and oaks; never on decaying wood or trees	0.0109

This investigation addresses the question, "How do the cellobiase activities of store-bought shiitake, crimini, and chanterelle mushrooms compare?" These data were collected using the Preliminary Activity procedure.

The cellobiase activities of the three mushroom types vary significantly. Shiitake mushrooms, which grow on dead wood, a rich source of cellulose, exhibit the highest cellobiase activity of the three. The cellobiase activity of the shiitake sample was 0.1078/0.109 or 9.9 times as great as that of the chanterelle sample.

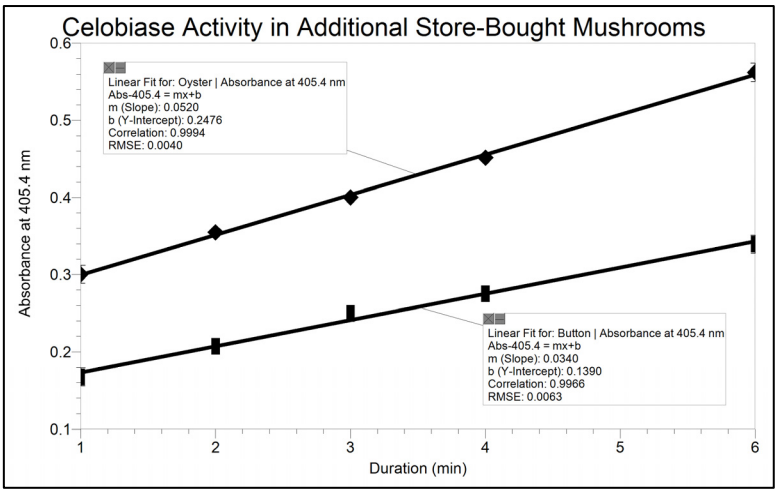

Figure 3 *Comparing cellobiase activities*

Table 2: Cellobiase Activity of Additional Store-Bought Mushrooms		
Name	Where it grows	Cellobiase activity (Δ abs/min)
Oyster (*Pleurotus ostreatus*)	On dead hardwood trees	0.0520
Button (*Agaricus bisporus)*	In fields and grassy areas	0.0340

This investigation addresses the question, "How do the cellobiase activities of store-bought oyster and button mushrooms compare?" These data were collected using the Preliminary Activity procedure.

Oyster mushrooms, which generally grow on dead wood, a rich source of cellulose, exhibit higher cellobiase activity than button mushrooms.

Comparing the Cellobiase Activities of Dried Mushrooms

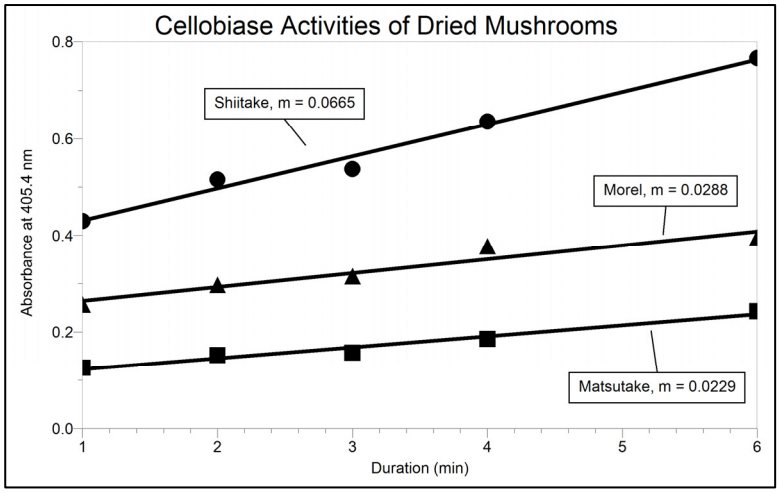

Figure 4 *Comparing cellobiase activities*

Table 3: Cellobiase Activity of Dried Mushrooms		
Name	Where it grows	Cellobiase activity (Δ abs/min)
Shiitake (*Lentinula edodes*)	On dead wood from *Castanopis cuspidate* trees	0.0665
Morel (*Morchella sp.)*	Under deciduous trees	0.0288
Matsutake (*Tricholoma magnivelare)*	On forest floor; forms symbiotic relationship with tree roots	0.0229

This investigation addresses the question, "How do the cellobiase activities of dried shiitake, morel, and matsutake mushrooms compare?" Mushroom extracts were made by rehydrating 2 grams of dry mushroom with 2 mL of distilled water. After an hour of hydration, the mixture was transferred to a mortar, 2 mL of extraction buffer were added, and a pestle was used to produce a slurry. The data were then collected using the Preliminary Activity procedure.

The cellobiase activities of morel and matsutake mushrooms, which grow in somewhat similar conditions, are similar. The cellobiase activity of shiitake mushrooms, which grow directly on decaying wood, is greater.

Comparing the Cellobiase Activities of Different Parts of a Mushroom

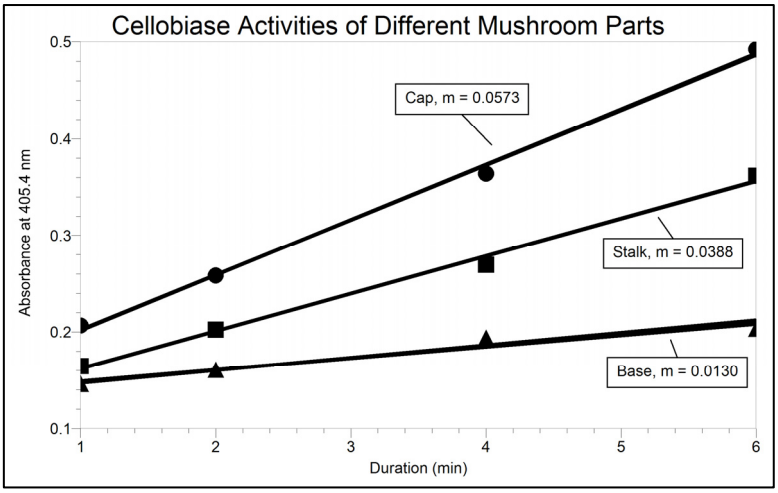

Figure 5 *Comparing cellobiase activities*

Table 4: Cellobiase Activity of Mushroom Parts	
Mushroom part	Cellobiase activity (Δ abs/min)
Cap	0.0573
Stalk	0.0388
Base	0.0130

This investigation addresses the question, "How do the cellobiase activities of different parts of the same mushroom compare?" These data were collected using the Preliminary Activity procedure.

As can be seen above, the mushroom cap exhibited the greatest cellobiase activity, and the base exhibited the least activity.

Comparisons Using Beer's Law and Concentrations (Advanced Topic)

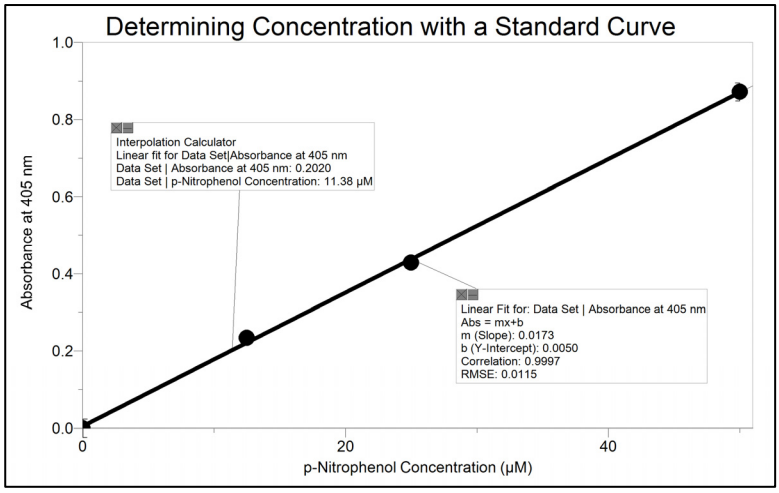

Figure 6 *Using a Beer's law standard curve to determine* p-*nitrophenol concentration*

Table 5: 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 6: Sample Concentration Determination		
Shiitake Mushroom	Absorbance (at 405 nm)	*p*-nitrophenol concentration (µM)
at time = 1 min	0.202	11.4

These data were collected using the Preliminary Activity procedure from Investigation 7, "Introduction to Biofuels: Enzyme Action." Figure 6 illustrates the use of a Beer's law standard curve to determine the concentration of *p*-nitrophenol in solution. Table 5 contains the concentration and absorbance data used to produce the Beer's law standard curve. Table 6 contains sample results for the use of Interpolation Calculator of Logger *Pro* to determine the *p*-nitrophenol concentration after 1 minute for a shiitake mushroom sample. Concentration values can similarly be determined using the Interpolate feature of Logger *Pro* or LabQuest App.

Note: A concentration of 11.4 µM = 11.4 µmol/L = 11.4 nmol/mL, and thus the 1 mL of sample in the cuvette mentioned in Table 6 above contains 11.4 nmol of *p*-nitrophenol.

Table 7: *p*-nitrophenol Concentrations Determined Using a Beer's Law Standard Curve						
	Shiitake		Crimini		Chanterelle	
Time (min)	Absorbance	Conc (μM)	Absorbance	Conc (μM)	Absorbance	Conc (μM)
1	0.202	11.4	0.275	15.6	0.226	12.8
2	0.320	18.2	0.324	18.4	0.238	13.5
3	0.463	26.5	0.374	21.3	0.265	15.0
4	0.569	32.6	0.419	23.9	0.265	15.0
6	0.736	42.2	0.533	30.5	0.280	15.9

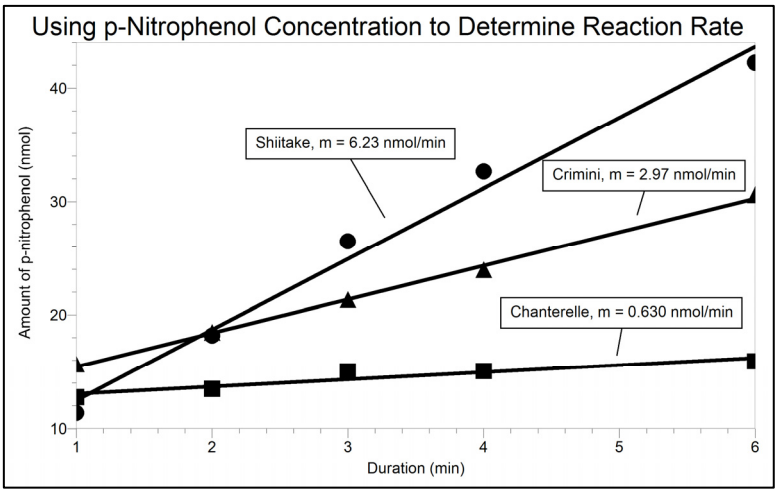

Figure 7 *Using concentration to determine initial reaction rate*

Table 8: Using Concentration Values to Determine Initial Reaction Rate						
Mushroom type	Time (min)					Reaction rate (nmol/min)
	1	2	3	4	6	
Shiitake	11.4	18.2	26.5	32.6	42.2	6.23
Crimini	15.6	18.4	21.3	23.9	30.5	2.97
Chanterelle	12.8	13.5	15.0	15.0	15.9	0.630

This investigation addresses the advanced version of the question, "How do the cellobiase activities of store-bought shiitake, crimini, and chanterelle mushrooms compare?" Initial reaction rates were determined with linear curve fits as is shown in Figure 7. See Investigation 7, "Introduction to Biofuels: Enzyme Action," for more details.

The initial *p*-nitrophenol production rates of the three mushroom types vary significantly. Shiitake mushrooms, which grow on dead wood, a rich source of cellulose, exhibit the highest initial reaction rate of the three. The rate of *p*-nitrophenol production rate by the shiitake sample was 6.23/0.630 or 9.9 times as great as that of the chanterelle sample.

Protein Content Store-Bought Mushroom Extract (Advanced Topic)

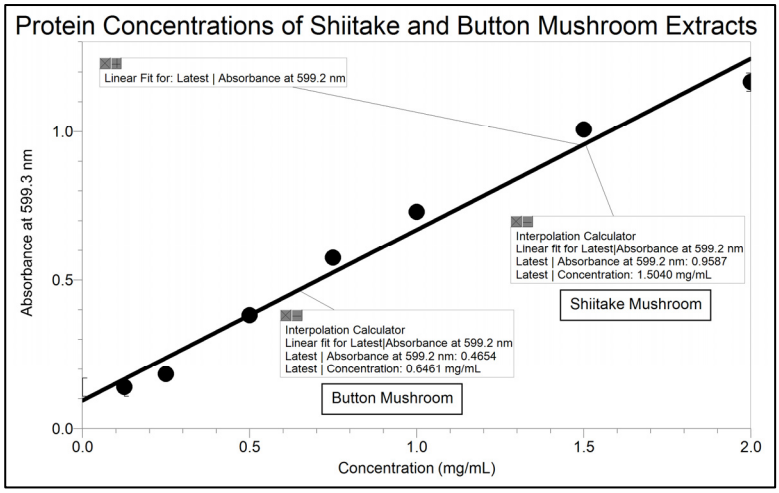

Figure 8 *Comparing protein concentration using the Bradford assay*

Table 9: Mushroom Extract Protein Concentrations			
Name	Where it grows	Observed concentration (mg/mL)	Actual concentration (mg/mL)
Shiitake (*Lentinula edodes*)	On dead wood from *Castanopis cuspidate* trees	1.50	6.00
Button (*Agaricus bisporus)*	In fields and grassy areas	0.65	2.60

This investigation addresses the question, "How do the protein concentrations of store-bought shiitake and button mushrooms compare?" The shiitake and button mushroom extracts were prepared as described in Preliminary Activity Steps 1–4 of this Investigation. The protein concentrations were then determined using a slight modification of the Preliminary Activity procedure of Investigation 5, "Experiments with Protein: The Bradford Assay." See the Tips section for details.

The protein concentration of the shiitake mushroom sample was found to be 6.00/2.60, or 2.31 times as great as that of the button mushroom sample.

Comparing the Cellobiase Activities of Wild Mushrooms (Advanced Topic)

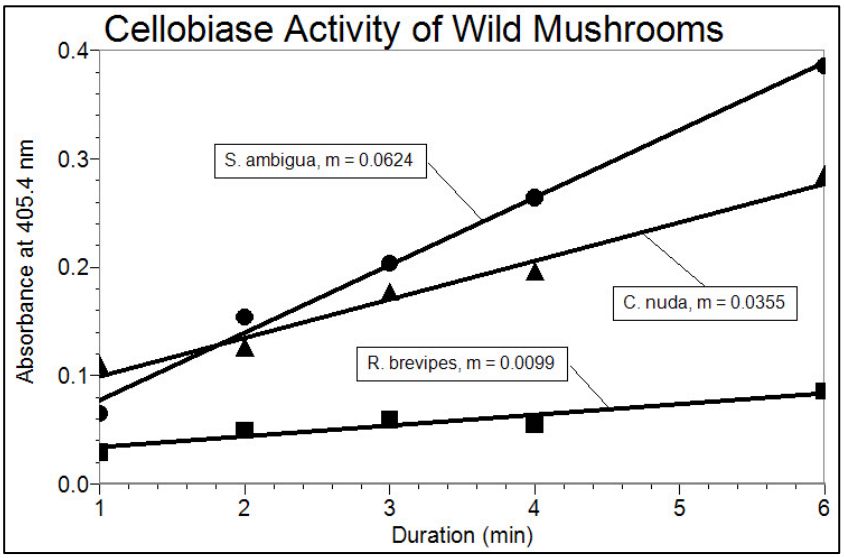

Figure 9 *Comparing cellobiase activities*

Table 11: Cellobiase Activity of Wild Fungi		
Name	Where it grows	Cellobiase activity (Δ abs/min)
Stropharia ambigua	In disturbed ground and among wood chips; was collected on decaying wood	0.0624
Clitocybe nuda (wood blewit)	Common on decaying organic matter, found on leaf litter under oak	0.0355
Russula brevipes	Found under leaf litter, mycorhizal	0.0099

This investigation addresses the question, "How do the cellobiase activities of field collected fungi compare?" Fungi in this activity were field collected and identified using the text *Mushrooms Demystified* by David Arora.[1] This activity is not recommended for high school students as some wild mushrooms are poisonous or toxic when eaten. This exercise is only recommended for college students that are closely supervised by an instructor who can properly identify different species of fungi. Mushrooms in the genus *Amanita* should never be used for this exercise. These data were collected using the Preliminary Activity procedure.

The cellobiase activities of the three mushroom types vary significantly. The *Stropharia sp.* which was found growing on dead wood, exhibited the highest cellobiase activity. The *Russula sp.* is mycorhizal and had the lowest activity.

[1] Arora, D. (1986). *Mushrooms demystified: A comprehensive guide to the fleshy fungi* (2nd ed.). Berkeley, CA: Ten Speed Press.

TIPS

1. The instructions provided assume that you have purchased the "Biofuel Enzyme Kit" from Bio-Rad Laboratories Inc. (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. Accordingly, be sure 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:
   - 3 mL of 1x stop solution.
   - 2 mL of extraction buffer
   - 3 mL of 1.5 mM substrate (*p*-nitrophenyl glucopyranoside).

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.

   1.5 mM substrate (*p*-nitrophenyl glucopyranoside): Label a bottle "1.5 mM substrate". Add 1 mL of 1x resuspension buffer to the vial of substrate and mix. Combine this 1 mL with the 198 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 the 1x resuspension buffer.

5. For the **protein concentration in mushrooms** investigation, we suggest these modifications of the Preliminary Activity of Investigation 5, "Experiments with Protein: The Bradford Assay."
   - Step 3 should be modified as follows: Place 60 µL of PBS (phosphate buffered saline) into a 1.5 mL microtube, and then add 20 µL of mushroom supernatant. (**Note**: The mushroom extract is thus being diluted by a factor of 4.)
   - Step 5 should be modified as follows: Place 1 mL of Bradford reagent into an empty cuvette, and then add 20 µL of the mushroom supernatant/PBS mixture from the 1.5 mL microtube. Cap the cuvette and gently invert the cuvette three times.
   - Steps 17 and 18 need no modification.
   - Multiply the observed protein concentration by 4 to get the actual protein concentration of the original mushroom extract.

6. **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.

7. 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.

8. 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 Got Protein? Kit. Text and figures are used with permission.