

EDCI 786 Topics: Science Research in the Classroom Activity

pH and Enzyme Function Lab Activity

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Background about laccases

Laccases are enzymes that catalyze oxidation of substrates and use the electrons they acquire to reduce molecular oxygen, O₂, to water. Laccases contain 4 coppers and can oxidize a variety of substrates, especially phenolic compounds¹. Laccase was first discovered in the Japanese tree *Rhus venicifera* in 1883². Currently we know laccases are present in plants, fungi, bacteria, and insects where they are involved in many different processes including detoxification, wound healing, cell wall synthesis, and lignin synthesis and degradation. People are interested in using laccases for a variety of purposes. In the food industry, laccases are used to reduce phenolic compounds for improving the clarity of fruit juice, beer and wine³. Laccases can be used for delignification in the paper and pulp industry and textile industries use laccases to remove dyes from waste water⁴. The Kanost Lab at Kansas State University studies the function of laccases in the mosquito *Anopheles gambiae* as well as the role of laccase in insect cuticle sclerotization (hardening). This activity introduces high school students to an enzyme activity assay in which enzyme activity can be detected by a color change. The substrate ABTS is clear in its reduced form and turns green after it is oxidized by laccase.

Instructor Notes

Time Required

Approximately two 50-min lab periods will be needed. Lab period one includes pre-lab instructions and Part 1, buffer preparation, of the lab activity. The second lab period includes all steps of Part 2, pH and Enzyme Function, of the lab activity.

Group Size

Students should work in teams of two or four. The total number of students working in the lab will depend on your lab facilities (8-24 students).

Materials Needed

Each group will prepare one buffer solution to be used by the entire class in part 2. Plan to have at least 4 student groups.

per student group:

Chemicals:

Part 1-

- citric acid, sodium phosphate (dibasic, Na₂HPO₄), 5 M NaOH, 5 M HCl, distilled water

NOTE: monobasic sodium phosphate may be used but it will require more solution to adjust pH.

¹ Piontek K. et al. (2002) *JBC* **277**, 37663-37669.

² Yoshida, H. (1883) *J. Chem. Soc. (Tokyo)* **43**, 472-486.

³ Couto S.R. and Herrera J.L.T. (2006) *Biotech Adv* **24**, 500-513.

⁴ Couto S.R. and Herrera J.L.T. (2006) *Biotech Adv* **24**, 500-513.

Part 2-

- *Rhus verificera* laccase (Sigma L2157) OR other commercially available laccase like *Tremetes versicolor* laccase (Sigma 53739); ABTS (Sigma A1888), buffer solutions from part 1

Equipment:

Part 1-

- Safety glasses, gloves and lab apron
- labeling tape and marking pencils or sharpies
- magnetic stirrers (or stirring rods)
- spatulas
- weigh boats or weigh paper
- 1 100 or 200-mL beaker
- 100 ml Graduated cylinder
- 100 ml glass storage bottle with lid
- Tongs (to remove magnetic stirrer from buffer solution)
- balances which can measure to the nearest 0.1 or 0.01 gram
- pH meter

Part 2-

- Safety glasses, gloves and lab apron
- 6 1-mL disposable transfer pipettes
- 8 small test tubes, stoppers, and rack
- 1.5 ml microcentrifuge tubes (so each group has their own supply of ABTS and laccase)
- Aluminum foil
- 4 small beakers if you want groups to have their own containers of the 100 mM Citrate/100 mM sodium phosphate buffers prepared in part 1. Each group will need 6 ml of each buffer.
- Optional: 8 microcentrifuge tubes for storing sample overnight.

Instructor preparation for part 2, laccase activity assay

Supplies:

- 1.5 ml microcentrifuge tubes so each group has their own supply of ABTS and laccase; 2 per group
- Aluminum foil
- Small beakers if you want groups to have their own containers of the 100 mM Citrate/100 mM sodium phosphate buffers prepared in part 1. Each group will need 6 ml of each buffer.

A. Prepare 0.25 unit(U)/ul *Rhus verificera* laccase

The *Rhus verificera* laccase you order from Sigma may come in different units than the bottle we have been using. Be sure to adjust the values accordingly.

$$0.250 \text{ U/ul} = 250 \text{ U/ml} \quad 250\text{U/ml} \times 1 \text{ mg}/120 \text{ U} = 2.08 \text{ mg laccase/ml buffer.}$$

We observed that transfer pipettes typically make drops that are ~30 ul. Each group will need 4 drops of laccase. Giving each group a microcentrifuge tube with 0.5 ml will give each group over three times what they need.

To make enough laccase for 20 groups, dissolve ~25 mg of laccase in 12 ml of 100 mM citrate 100 mM sodium phosphate buffer, pH either 4 or 5.5. Shake vigorously and for a few minutes, but know IT WILL NOT ALL DISSOLVE. This is normal, the laccase from Sigma is a crude preparation. You and your students can avoid using the chunks but it will be okay if a little insoluble material gets into the reactions. Once you are done shaking, aliquot 0.5 ml into 20 microcentrifuge tubes.

Laccase should be kept in the refrigerator or on ice until it is used. For longer term storage, add 50% glycerol and store at -20 C (freezer).

B. Prepare 25 mM ABTS

For 1 ml: $1 \text{ ml} \times 25 \text{ mmol}/1000 \text{ ml} = 0.025 \text{ mmol} \times 548.68 \text{ mg}/\text{mmol} = 13.7 \text{ mg ABTS}$

Each group will use 8 drops of ABTS. Giving each group 0.5 ml will give them twice as much ABTS as they'll need. We think it might be good to make up some extra, just in case of spilling. If you weigh out 200 mg of ABTS, dissolve it in 14.58 ml of buffer (pH 4 or 5.5) to make 25 mM. Store ABTS in the dark (perhaps covered with aluminum foil) until it is used. If you aliquot the ABTS into 0.5 ml in individual tubes cover those with foil, too. Plan to use the ABTS soon after dissolving it in buffer, certainly no longer than a week. The ABTS dissolves easily and will be a pale green color.

You might want your students to practice making drops from transfer pipettes on the same day they make buffer. It takes a little practice to consistently release just one drop, and that will be important for the enzyme assays they'll run in part 2.

Additional notes to the instructor:

If you have a spectrophotometer, students can take absorbance readings of their samples after a set length of time, perhaps 20 or 30 minutes, using light with a wavelength of 414 nm.

The detergent SDS can denature and thereby inhibit laccase. This can be worked into the experiment by having each group of students run 4 more tubes, adding buffer, ABTS, 5 drops of 20% SDS, and then enzyme. The tubes containing SDS are expected to stay clear, like the control. The instructor would need to prepare the 20% SDS (20g/100 ml); take care, SDS becomes suspended in the air and you don't want to breathe it in!

You can also compare the effect of concentration by adding an additional drop of substrate or enzyme or half as much substrate or enzyme; (fine tipped transfer pipettes tend to have drops of ~15 ul).

Finally, you and your class can use a computer to look at the three dimensional structure of *Tremetes versicolor* laccase or some other laccases for which there are crystal structures. PDB files are available from the Protein Data Bank at www.rcsb.org. One PDB file for Tremetes versicolor laccase is 1GYC. Entering this code into the space at the Protein Data Bank will take you to a page with more information. On the left you can click on “Display molecule”. One option is to use Jmol Viewer to move the protein around while you look at it. There are 4 small round balls representing the 4 bound copper molecules and the backbone of the protein is in gray.

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Procedure:

Part 1-buffer preparation

1. Write teacher assigned pH value here. _____
2. Make the 100 mM Citrate (citric acid) 100 mM Sodium Phosphate buffer:
--For 100 ml buffer
 - a. Measure 2.1 g citric acid and place in a large beaker.
(10 mmol citric acid 0.010 mol x 210.14 g/mol = 2.10 g citric acid)
 - b. Measure 1.38 g sodium phosphate and place in beaker with citrate.
(10 mmol Sodium Phosphate 0.010 x 138 g/mol = 1.38 g Sodium Phosphate)
3. Add 90 ml water and dissolve on magnetic stir plate. Pour into graduated cylinder and add water to make 100 ml.
4. Check pH.
5. Adjust pH to desired value (2.5, 4, 5.5, and 7.5)
 - a. Use NaOH solution to raise pH
 - b. Use HCl solution to lower pH.
6. Pour into storage bottle.
7. Label with buffer name, pH and date. Seal and store for use in Part 2.
8. Clean up lab area.
9. Use remainder of class to practice making single drops with the micropipette.

Part 2-Enzyme activity assay

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1. Label 8 test tubes, 2 for each pH (2.5, 4, 5.5, 7.5). Four of your tubes, one of each pH, will be a control. The control will have buffer and the substrate (ABTS). You may want to label the control tubes with a C. The other tube at each pH will have buffer, substrate, and enzyme (laccase). Label these tubes so you know they contain enzyme.
2. Add 3 ml of **appropriate** pH 100 mM Citrate/100 mM Sodium phosphate buffer to both the control and enzyme labeled tubes. **MAKE SURE THE BUFFER YOU PUT IN EACH TUBE HAS THE CORRECT pH!**
3. Add 1 drop 25 mM ABTS to every tube.
4. To the enzyme labeled tubes **ONLY**, add 1 drop of 0.25 U/ul tree laccase (*Rhus verificera*).
5. Cap your tubes and invert them to mix (turn the tubes upside down then right side up again). Try to start all the reactions (adding ABTS and laccase) at close to the same time. Compare the intensity of color change over the next half hour.
6. If you like, leave the tubes overnight and check their color the next day. Your instructor might like to put some of your sample in a microcentrifuge tube for the overnight storage. Rinse the rest of your sample down the sink and clean up your supplies and your bench space.

Enzyme Assay Pre-lab worksheet

name _____

Vocabulary list:

Enzyme/Substrate

Buffer

pH – acid, base

Molarity

Qualitative data

Quantitative data

Go to <http://people.ku.edu/~jbrown/enzyme.html> and answer questions 1-5.

Enzyme review

1. To which category of biomolecules do enzymes belong?
2. What is an enzyme? ...substrate?
3. How can enzymes affect a reaction?
4. What holds an enzyme in its conformation (shape)?
5. What environmental factors affect enzyme activity? Explain how they do so.

Scientific Calculations Practice

6. Use the definition of molarity (moles solute/liter solution) to calculate the amount (grams solute) of citric acid ($C_6H_8O_7$) and sodium phosphate (Na_2HPO_4) needed to prepare 100 ml of .1 M citric acid .1 M sodium phosphate buffer.