

Chapter 6

A Quantitative Enzyme Study Using Simple Equipment

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Reprinted from: Nichols, B. A. D. and L. B. Cholewiak 1991. A quantitative enzyme study using simple equipment. Pages 89-99, *in* Tested studies for laboratory teaching. Volume 12. (C. A. Goldman, Editor). Proceedings of the 12th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 218 pages.

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Introduction

This enzyme study can be comfortably completed in one 3-hour laboratory period. There is little equipment needed, and the techniques can be mastered easily by science majors and non-majors alike. If time is short, the instructor may choose to prepare the catalase extract before class, although we feel that the exercise is demystified when students do their own preparation. The data is easily represented in graph form, either as the rate of reaction versus x , or if you prefer, simply as the time to float versus x .

Lehninger (1982) lists the optimum pH for this reaction as 7.6. In previous years, we have sometimes obtained a bimodal response. Generally, we obtain very good reaction rates between 6 and 9. This might make a good comparison with a more pH-specific enzyme, and lead to a discussion on the ubiquity of this enzyme.

Notes for the Instructor

1. Consult your department safety officer about the need for goggles or other protective equipment.
2. This study could easily be adapted to a more investigative format by allowing students to choose what concentrations, temperatures, and pHs they investigate.
3. For shorter laboratory periods, assign Tests 1 to 5 to different students, or prepare the enzyme extract just before class, and keep in covered urine cups on ice until needed.
4. If filters break due to strong suction, use two filters at a time.
5. This lab can be made more precise by using a measured amount of enzyme extract on each disc, applied with a capillary tube or a micropipet.
6. If the reaction rate for the 100% enzyme is the same as the rate for the 75% enzyme, further dilution may be needed. Try 30 g potato in 100 ml water.
7. Potato extract must be swirled before taking aliquots for an even suspension of catalase.
8. Gloves should be worn to handle hydroxylamine. Discard according to your safety officers' instructions.
9. Strainers at the sink will keep glass filters and potato pieces from going down the drain.
10. We use small vials or jars for enzyme in water baths; small beakers float.

Materials (*per 10 pairs of students*)

Aluminum cake pans for ice baths (10)
Aspirators and hoses on each sink (3–4)
Balances and plastic lids to hold potatoes (3)
Beakers, 50-ml, or 10 of the same size/pair (150)
Bulbs for Pasteur pipets, in small culture bowl (10)
Black cap vials or jars, instead of beakers in baths (20)
Blender containers, keep in refrigerator until used (3)
Blenders to homogenize potatoes (3)
Boiled Enzyme, can be made ahead if rushed
Brushes, at sinks with sign to “wash blender!” (3)
Culture bowl for potatoes, any container will do (1)
Cups for enzyme, urine cups with covers work well (10)
Cups for scooping ice (10)
Droppers for distilled water (10)
Filter paper, 5.5 cm, for suction devices (2 boxes)
Filter units, Nalgene (150-ml capacity with cellulose nitrate membrane removed) or Buchners and sidearms (10)
Filters, Whatman 2.1-cm glass fiber, in petri dishes (10)
Flasks filled with distilled water at room temperature (2)
Flasks, 250-ml, for distilled water (10)
Forceps, fine, to handle discs (10)
Gloves, S/M/L, advisable for use with hydroxylamine (1 box)
Graduated cylinders, 100-ml and 10-ml, or 10-ml pipets (10)
Hot plate to boil enzyme (1)
Hydrogen peroxide, 1%, cold, fresh, in brown bottles (10)
Hydrogen peroxide, 10%, cold, fresh, in brown bottles (10)
Hydroxylamine, 10%, with droppers (10)
Ice bucket or tub, students scoop own ice (1)
Jugs for cold distilled water, keep in ice baths to stay cold (2)
Knives, paring, or scalpels, peelers (10)
Pan, large, with ice for cold distilled water (1)
Paper towels (near benches)
Parafilm and scissors (1)
Pipet pump, green (5-ml to 10-ml) and pi-pump (10)
Pipets, 1-ml, non-sterile are acceptable
Pipets, 10-ml, non-sterile are acceptable
Pipets, Pasteur, 5" or 145-mm (1 box)
Pipetter, blue (1-ml) and pi-pump (10)
Potatoes (10 small)
Stop watches or classroom clock (10)
Strainers to keep filters out of sink (2)
Thermometers, 6" (less fragile) (10)
Water bath, 30°C or use warm tap water (1)
Water bath, 37°C or use warm tap water (1)
Wax pencils or markers (10)

Keep an extra supply of distilled water in the refrigerator.

Student Outline

Introduction

Enzymes are **biological catalysts** that carry out the thousands of chemical reactions that occur in living cells. They are generally large proteins made up of several hundred amino acids, and often contain a non-proteinaceous group called the prosthetic group that is important in the actual catalysis.

In an enzyme-catalyzed reaction, the substance to be acted upon, or **substrate**, binds to the active site of the enzyme. The enzyme and substrate are held together in an **enzyme-substrate** complex by hydrophobic bonds, hydrogen bonds, and ionic bonds.

The enzyme then converts the substrate to the reaction products in a process that often requires several chemical steps, and may involve covalent bonds. Finally, the products are released into solution and the enzyme is ready to form another enzyme-substrate complex. As is true of any catalyst, the enzyme is not used up as it carries out the reaction but is recycled again and again. One enzyme molecule can carry out thousands of reaction cycles every minute.

Each enzyme is specific for a certain reaction because its amino acid sequence is unique and causes it to have a unique three-dimensional structure. The “business” end of the enzyme molecule, the **active site**, also has a specific shape so that only one or few of the thousands of compounds present in the cell can interact with it. If there is a **prosthetic group** on the enzyme, it will form part of the active site. Any substance that blocks or changes the shape of the active site will interfere with the activity and efficiency of the enzyme.

If these changes are large enough, the enzyme can no longer act at all, and is said to be **denatured**. There are several factors that are especially important in determining the enzyme's shape, and these are closely regulated both in the living organism and in laboratory experiments to give the optimum or most efficient enzyme activity:

Salt concentration: If the salt concentration is very low or zero, the charged amino acid side-chains of the enzyme will stick together. The enzyme will denature and form an inactive precipitate. If, on the other hand, the salt concentration is very high, normal interaction of charged groups will be blocked, new interactions occur, and again the enzyme will precipitate. An intermediate salt concentration such as that of blood (0.9%) or cytoplasm is optimum for most enzymes.

pH: pH is a logarithmic scale that measures the acidity or H^+ concentration in a solution. The scale runs from 0 to 14, with 0 being the highest in acidity and 14 the lowest. Neutral solutions have a pH of 7. Acid solutions have a pH less than 7; basic solutions have a pH greater than 7. Enzyme amino acid side chains contain groups such as $-COOH$ and $-NH_2$ that readily gain or lose H^+ ions. As the pH is lowered, an enzyme will tend to gain H^+ ions, and eventually enough side chains will be affected so that the enzyme's shape is disrupted. Likewise, as the pH is raised, the enzyme will lose H^+ ions and eventually lose its active shape. Many enzymes have an optimum in the neutral pH range and are denatured at either extremely high or low pH. Some enzymes, such as those which act in the human stomach where the pH is very low, will have an appropriately low pH optimum. A **buffer** is a compound that will gain or lose H^+ ions so that the pH changes very little.

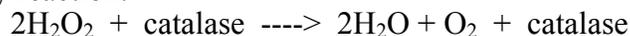
Temperature: All chemical reactions speed up as the temperature is raised. As the temperature increases, more of the reacting molecules have enough kinetic energy to undergo the reaction. Since enzymes are catalysts for chemical reactions, enzyme reactions also tend to go faster with increasing temperature. However, if the temperature of an enzyme catalyzed reaction is raised still further, an **optimum** is reached: above this point the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecules starts to be disrupted. The positive effect of speeding up the reaction is now more than offset by the negative effect of denaturing more

and more enzyme molecules. Many proteins are denatured by temperatures around 40–50°C, but some are still active at 70–80°C, and a few even withstand being boiled.

Small molecules: Many molecules other than the substrate may interact with an enzyme. If such a molecule increases the rate of the reaction it is an **activator**, and if it decreased the reaction rate it is an **inhibitor**. The cell can use these molecules to regulate how fast the enzyme acts. Any substance that tends to unfold the enzyme, such as an organic solvent or detergent, will act as an inhibitor. Some inhibitors act by reducing the –S–S– bridges that stabilize the enzyme's structure. Many inhibitors act by reacting with side chains in or near the active site to change or block it. Others many damage or remove the prosthetic group. Many well known poisons such as potassium cyanide and curare are enzyme inhibitors which interfere with the active site of a critical enzyme. For further information see Alberts et al. (1989), Keeton and Gould (1986), or Lehninger (1982).

Objectives

In this exercise you will study the enzyme **catalase**, which accelerates the breakdown of hydrogen peroxide (a common end product of oxidative metabolism) into water and oxygen, according to the summary reaction:



This catalase-mediated reaction is extremely important in the cell because it prevents the accumulation of hydrogen peroxide, a strong oxidizing agent which tends to disrupt the delicate balance of cell chemistry.

Catalase is found in animal and plant tissues, and is especially abundant in plant storage organs such as potato tubers, corms, and in the fleshy parts of fruits. You will isolate catalase from potato tubers and measure its rate of activity under different conditions. A glass, fiber filter will be immersed in the enzyme solution, then placed in the hydrogen peroxide substrate. The oxygen produced from the subsequent reaction will become trapped in the disc and will give it buoyancy. The time measured from the moment the disc touches the substrate to the time it reaches the surface of the solution is a measure of the rate of the enzyme activity.

Extraction of Catalase

1. Peel a fresh potato tuber and cut the tissue into small cubes. Weigh out 50 g of tissue.
2. Place the tissue, 50 ml of cold distilled water, and a small amount of crushed ice in a pre-chilled blender.
3. Homogenize for 30 seconds at high speed.

From this point on, the enzyme preparation must be carried out in an ice bath.

4. Filter the potato extract, then pour the filtrate into a 100-ml graduated cylinder. Add cold distilled water to bring up the final volume to 100 ml. Mix well. This extract will be arbitrarily labelled 100 units of enzyme per ml (100 units/ml) and will be used in Tests 1 to 4. Repeat the extraction procedure for Test 5 (*optional*).

Test 1: Effect of Enzyme Concentration

Before considering the factors which affect enzyme reactions, it is important to demonstrate that the enzyme assay shows that the enzyme actually follows accepted chemical principles. One way to demonstrate this is by determining the effect of enzyme concentration on the rate of activity, while using a substrate concentration which is in excess.

Label four 50-ml beakers as follows: 100, 75, 50, and 0 units/per ml. Prepare 40 ml of enzyme for each of the above concentrations.

enzyme (ml) +	cold distilled water (ml) =	units/ml
40*	0	100
30	10	75
20	20	50
0	40	0

* savethis undiluted enzyme for Tests 2 to 4

Keep your catalase preparations in the ice bath. Label an identical set of beakers for the substrate. Into each of these beakers, measure out 40 ml of a 1% hydrogen peroxide solution.

Using forceps, immerse a 2.1-cm glass, fiber filter disc to one-half its diameter in the catalase solution you have prepared. Allow the disc to absorb the enzyme solution for 5 seconds, remove and drain by touching the edge to a paper towel for 10 seconds. Drop the disc into the first substrate solution. The disc will sink rapidly into the solution. The oxygen produced from the breakdown of the hydrogen peroxide by catalase becomes trapped in the fibers of the disc causing the disc to float to the surface of the solution. The time (t) in seconds, from the second the disc touches the solution to the time it again reaches the surface is determined to be the rate R of enzyme activity where $R = 1/t$. Repeat the procedure twice for each enzyme concentration and average the results. Record your results in Table 6.1. Plot your results in Figure 6.1 and label the graph.

How does enzyme activity vary with enzyme concentration?

Table 6.1. Effect of enzyme concentration on rate of activity (Test 1).

Enzyme concentration (units/ml)	Time to float disc (seconds)				Rate
	Trial 1	Trial 2	Σ	Mean	

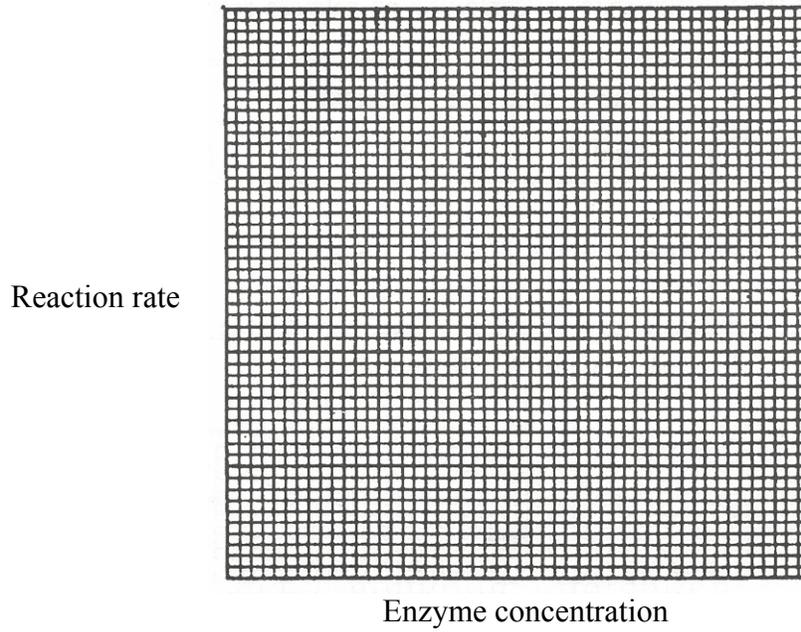


Figure 6.1. Effect of enzyme concentration on rate of activity (Test 1).

Test 2: Effect of Substrate Concentration

To determine the effect of substrate concentration on enzyme activity obtain eight 50-ml beakers and label them as follows: 0%, 0.1%, 0.2%, 0.5%, 0.8%, 1%, 5%, and 10% H₂O₂.

Add 40 ml of the proper (as outlined above) H₂O₂ solution to each beaker. Make sure that the substrate solutions reach room temperature before beginning your assay. Using the filter disc procedure described above, and the undiluted enzyme from Test 1, determine the rate of the reaction at the various substrate concentrations. Record your results in Table 6.2. Repeat the procedure twice for each substrate concentration and average the results. Plot your results in Figure 6.2 and label the graph.

How is the rate of enzyme activity affected by increasing the concentration of the substrate? What do you think would happen if you increased the substrate concentration to 20% H₂O₂? Does changing the substrate concentration exhibit the same effect as changing the enzyme concentration?

Table 6.2. Effect of substrate concentration on catalase activity (Test 2).

Substrate concentration	Time to float disc (seconds)				Rate
	Trial 1	Trial 2	Σ	Mean	

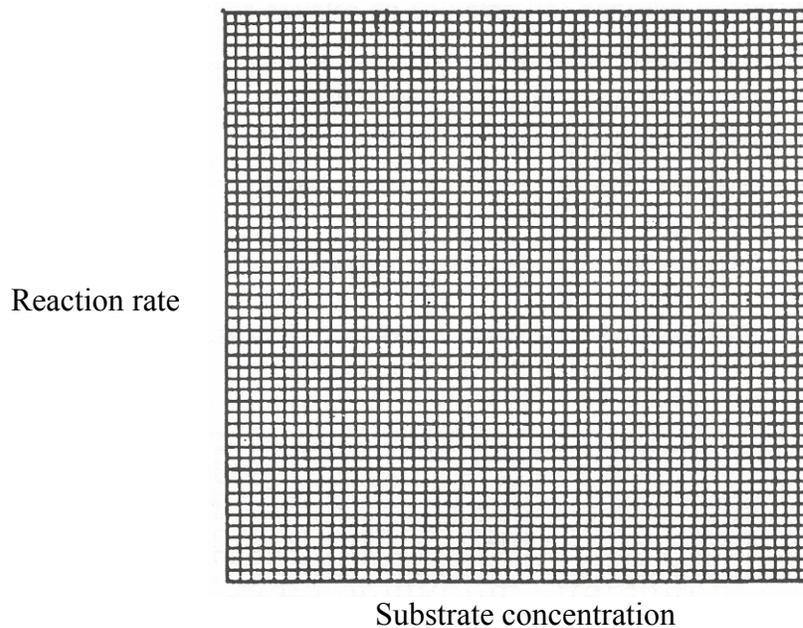


Figure 6.2. Effect of substrate concentration on catalase activity (Test 2).

Test 3: Effect of Enzyme Inhibitor

Hydroxylamine attaches to the iron atom (a part of the catalase molecule) and thereby interferes with the formation of enzyme-substrate complex. Add 5 drops of 10% hydroxylamine to 1 ml of enzyme extract and let it stand for 1 minute. Prepare a control solution to test. Then measure the activity of each solution. Use 40 ml of 1% H_2O_2 for the substrate. Record data in Table 6.3. Explain the results.

Table 6.3. Effect of inhibitor on catalase activity (Test 3).

Treatment	Time to float disc (seconds)				Rate
	Trial 1	Trial 2	Σ	Mean	

Test 4: Effect of Temperature

Using 40 ml of a 1% hydrogen peroxide solution as the substrate, and 5-ml aliquots of the 100 units/ml enzyme solution, measure the enzyme activity in the usual manner. Run the reactions in the water baths at different temperatures, such as 4°, 15°, room temperature which is about 22°, 30°, and 37°C. The catalase and substrate should be brought to the testing temperature *before* they are used. Record the exact temperature and your data in Table 6.4 and plot the results in Figure 6.3. Also test the activity of enzyme that has been boiled. (Do not boil the H_2O_2 .)

From these data, what can you conclude about how temperature affects enzyme activity? How would you explain the results?

Table 6.4. Effect of temperature on catalase activity (Test 4).

Temperature	Time to float disc (seconds)				Rate
	Trial 1	Trial 2	Σ	Mean	

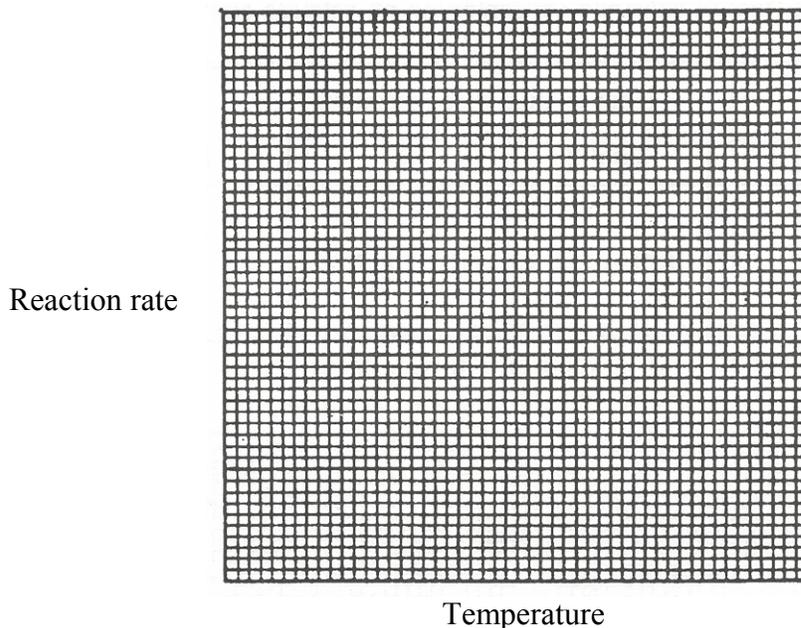


Figure 6.3. Effect of temperature on catalase activity (Test 4).

Test 5: Effect of pH (*optional*)

Obtain 5 50-ml beakers, and label them as follows: control, pH 4, pH 6, pH 8, and pH 10. Into each beaker, pour 10 ml of enzyme preparation and 30 ml of buffer solution at the appropriate pH. (Use 30 ml dH₂O for the control.) Using 40 ml of a 1% hydrogen peroxide solution as the substrate, measure the enzyme activity in the usual manner. Record your data in Table 6.5 and plot your results in Figure 6.4.

How does pH affect enzyme activity? Would you expect similar results with salivary amylase? With pepsin?

Table 6.5. Effect of pH on rate of activity (Test 5).

pH	Time to float disc (seconds)				Rate
	Trial 1	Trial 2	Σ	Mean	

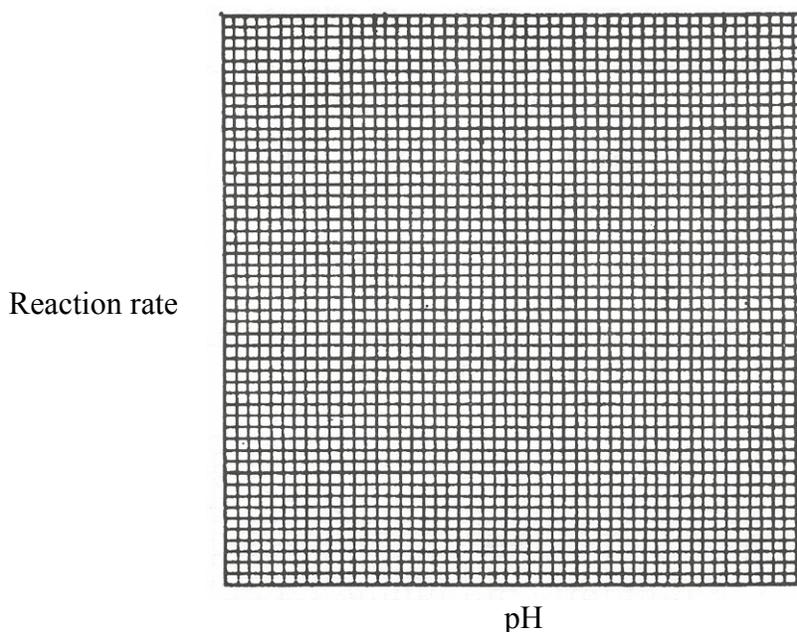


Figure 6.4. Effect of pH on rate of activity (Test 5).

Acknowledgements

This enzyme study laboratory is modified from one which has been in use at Princeton for many years and thus we regret that we are unable to cite the original author. Dr. Mary J. Philpott made some of the modifications.

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