Enzymes: Amylase Activity in Starch-degrading Soil Isolates

Introduction

This week you will continue our theme of industrial microbiologist by characterizing how differing reaction conditions affect enzyme reaction rates of the amylase produced by your starch degrading isolates from last week. Even though they represent a rich source of energy for microorganisms (and us!), starch molecules are much too large to get into the cell. To overcome this and take advantage of this potential energy source, cells secrete amylase into the environment. The amylase breaks the starch into smaller, disaccharide, maltose molecules that can then be easily transported into the cell. Because amylase is an extra-cellular protein, we can grow starch degraders in broth culture, remove the cell mass by centrifugation and then simply use the resulting supernatant broth containing the extra-cellular enzyme to characterize our amylase without need for further purification (which we will also do later in the semester).

Learning Objectives

You should:

Conceptual

• understand that enzymes are protein catalysts necessary for cellular function.
• understand that environmental (and cytoplasmic) conditions (temperature, pH, etc.) affect enzyme performance
• understand how and why that performance is affected

Practical

• gain experience in experimental design
• gain experience at mixing reaction cocktails
• gain experience running a colorometric assay and the use of standard curves in analyzing data

Underlying Science

Amylases are a family of enzymes that degrade starch (polymers of glucose) into smaller disaccharides (maltose). A molecule of water is also split during this reaction and the OH\(^-\) and H\(^+\) ions bind to the exposed ends of the broken starch polymer. This type of reaction is called hydrolysis (water splitting). Hydrolysis is a common mechanism used by enzymes to break chemical bonds.

The hydrolysis of starch can be measured through the use of an enzyme test or assay. An enzyme assay will test for the simple presence of enzyme activity but can also be used to measure the reaction rate of an enzyme-catalyzed reaction. The assay can measure either the appearance of one of the products or the disappearance of one of the substrates over time.
To measure your amylase activity, you will monitor the disappearance of amylase’s substrate, starch. Starch reacts with iodine (which is yellow) to form a blue compound ($A_{\text{max}}$ 620 nm). This reaction is the basis of a colorimetric assay for amylase activity. Broth culture supernatant (which contains the secreted amylase) will be incubated with starch. After the incubation period, a portion (an aliquot) of this mixture is combined with acidic iodine. The acid stops the enzymatic reaction and the iodine reacts with the starch to produce the blue color. Any starch that has not yet been hydrolyzed by the amylase will turn blue, with the intensity of the blue color being proportional to the amount of starch remaining. The intensity of the blue color can be quantified spectrophotometrically by measuring its absorbance at 620 nm. The greater the change in absorbance between a sample containing the initial amount of starch (without enzyme) and the hydrolyzed mixture containing the enzyme, the greater the amount of starch degraded by the enzyme, therefore the greater the activity of the enzyme being measured.

Enzyme activity (reaction rates) is dependent upon the environmental conditions either in nature or in the laboratory (e.g. temperature, pH, etc.). This is because these conditions can alter the amino acid side chains in a protein, affecting protein structure and folding and sometimes the enzyme's active site. The effects of some of those conditions will be explored in this exercise.

Just as in any chemical reaction, the concentration of reactants (substrates) will affect enzymatic reaction rates. In regards to substrate concentration, enzyme kinetics follow the Michaelis-Menton Model:

$$V_0 = V_{\text{max}} \times [s]/K_m + [s]$$

Where: $K_m = \text{Michaelis constant}$  

$[s] = \text{substrate concentration}$

This will be discussed in detail in class.
Exercises

Part A. Determination of amylase activity in microbial isolates

Since your isolates secrete amylase outside of the cell, we will assay the supernatant of centrifuged broth cultures of your isolates for amylase activity. You must come in the day before your section meets to start cultures of your isolates. We will have 37°C and 50°C waterbaths ready for you.

Materials

- 2 culture tubes containing 5 ml of LB broth for growing your isolates
- 1 tube of LB broth to use for your "I" tubes
- Micropipettors and tips
- Phosphate buffer, pH 7 (200 mM phosphate)
- Starch solution (0.2% w/v)
- Acidic iodine solution
- 5 ml pipets
- Vortex
- Spectrophotometer and cuvettes
- Test tubes
- Test tube racks
- 2ml microcentrifuge tubes

Protocol

Part A. Determination of amylase activity from microbial isolates

1.) The day before your section meets you must come in to inoculate LB broth tubes with your starch-degrading isolates. Use an inoculating loop to inoculate the tubes and incubate the appropriate isolate overnight in the 37°C or 50°C shaking waterbaths.

2.) Using a P-1000 micropipettor, transfer 1500 µl of each isolate to 2 ml microcentrifuge tubes. Label the tubes with your team number and "37" for the 37°C isolate and "50" for the 50°C isolate and spin them in the microcentrifuge at 10,000 RPM for 5 minutes to pellet the bacterial cells.

3.) While your tubes are spinning, turn on your spectrophotometers to warm up. When they are warmed up, set the wavelength at 620 nm.

4.) Gently pipet the supernatant (containing the amylase) to a fresh microcentrifuge tube without disturbing the pellet (don't worry about leaving some of the supernatant behind). Put the supernatants on ice and dispose of the cell pellets in the orange biohazard bags ("kill buckets" in "Urbance-speak").

5.) Obtain three clean test tubes and label them “I” (for initial), "37" and "50".

6.) Use a P1000 to add 500 µl of pH 7 buffer to each tube; this buffer will keep the pH constant during the reaction.

7.) Add 500 µl of starch solution to each tube -- Mix by vortexing gently. It is important that each tube has exactly the same amount of starch solution since starch is the substrate for the reaction and the initial amount of starch affects the blue color ultimately formed when you add the iodine (step 10 below). Pipet carefully.

8.) To the "I" tube, add 100 µl of distilled water.
9.) Add 100 $\mu l$ of the 37°C supernatant to the tube labeled "37". Quickly vortex and note the time (time=0).

10.) Time your reaction carefully and after exactly 5 min add 500 $\mu l$ of iodine solution-- the solutions will turn blue from the starch-iodine complex.

11.) Quickly add 2.5 ml of water to the “37” tube to stop (slow) the reaction and get the color to an OD between 0.1 and 1 (linear range for the spectrophotometer).

12.) Use a blank tube consisting of 0.5 ml of buffer, 0.5 ml of starch solution and 2.5 ml of water to zero the spectrophotometer at 620 nm.

13.) Immediately read the I and “37” tubes in the spectrophotometer at 620 nm. Record your results.

You are measuring the enzyme rate of the amylase in your supernatant. That is why accuracy of your results is dependent upon precision timing. The "I" tube has all the ingredients used in the assay but should have no amylase because you add water as your sample. Since there is no amylase, all the starch you added should still remain after the 5 min incubation. This serves as a measure of how much starch you added -- your starting point! If your supernatants contained amylase, some of the starch would have been digested and the intensity of the blue color will be lessened. The difference in blue intensity between your "I" tube and your supernatant tube is a measure of how much starch was degraded. Combined with a fixed incubation time, this provides a relative measure of your amylase enzyme reaction rate.

14.) If your measured absorbance is not in the 0.1 to 1.0 range, you may need to repeat your assay by increasing or decreasing the amount of supernatant you add and/or changing the incubation time. Ask your instructors.

15) Determine the change ($\Delta$) Abs per ml of the assay. Divide the $\Delta$ Abs by the total volume of the assay in ml. To figure out the total volume, add up all the components (starch, buffer, supernatant, iodine and water) that you added to the assay. Record the $\Delta$ Abs/ml in your notebook.

16) Repeate steps 9 through 15 for the “50” tube.

Part B. The effect of environmental conditions on enzyme activity

In this part of the lab, you are going to determine what effect changing assay conditions (such as temperature, pH or substrate concentration) have on the efficiency of the amylase you have successfully produced from your environmental isolates.

1) You will construct a testable hypothesis about the effect of a factor (temperature, pH or substrate concentration) on amylase activity in your cell supernatants.

2) You will then design an experiment (using the assay you used in Part A) to test your hypothesis that should include the positive and negative controls needed to ensure that your results are valid. You must turn in your hypothesis and experimental design ahead of time for us to critique and approve. Deadlines for this were given in class.

3) Ideally, you would want to perform replicates of your analyses as part of good experimental design but you may not have time to do this within our allotted lab time. If you have time and would like to do this, you are welcome to do so.

4) Record your results in your lab notebook and calculate values as you did in Part A.

5) You will then present your data and your subsequent analyses to the class in an oral report two weeks from this lab period. More information about the oral report will be provided next week.
Suggested variables to examine:

We will have materials available for you to test the following parameters:

A. Effect of temperature:

   Ice and water baths will be available at six different temperatures: 0°C, room temperature (about 23°C), 37°C, 50°C, 70°C and 100°C. Enzyme assays should be conducted at each of the different temperatures.

B. Effect of pH:

   In part A, the buffer that you did your assays in was pH 7. Buffers with six different pH’s (4, 5, 6, 7, 8, 9, and 10) will be available.

C. Effect of substrate concentration:

   In part A, the concentration of starch used in the assay was 0.2%. Determine the effect that starch concentration has on activity by performing assays on solutions of 0.005%, 0.01%, 0.02%, 0.05%, 0.1% and 0.2%. Dilute the 0.2% starch to these concentrations using distilled water. Each tube should contain 500 µl of diluted starch solution.

You are welcome to design an experiment around a different parameter of your own design but you must run your idea by us as soon as possible to determine its feasibility.

You must turn in your hypothesis and experimental design ahead of time for us to critique and approve. Deadlines for this will be given in class.