Enzyme Activity

Learning Objectives

Upon completion of this lab activity, the student will be able to:

- Describe the components of an enzymatic reaction.
- Communicate the function of an enzyme's active site and its relationship to the substrate.
- Describe the relationship between an enzyme's structure and its function.
- Explain the relationship between maltose produced and enzymatic (amylase) activity.
- Analyze the effect of environmental factors on enzymatic activity.
- Define absorbance and transmittance and utilize data obtained with a spectrophotometer.
- Describe the relationship between absorbance and concentration.
- Construct a standard curve using known amounts of maltose.
- Utilize a standard curve to determine enzyme activity of the experimental amylase.

Background

Adapted from https://www.oercommons.org/courseware/lesson/56959

How Enzymes Function

A substance that helps a chemical reaction to occur is a **catalyst**, and the special molecules that catalyze biochemical reactions are called **enzymes**. Almost all enzymes are proteins, made up of chains of amino acids, and they perform the critical task of lowering the activation energies of chemical reactions inside the cell. Enzymes do this by binding to the reactant molecules, and holding them in such a way as to make the chemical bond-breaking and bond-forming processes take place more readily. It is important to remember that enzymes don't change the ΔG of a reaction. In other words, they don't change whether a reaction is **exergonic** (spontaneous) or **endergonic**. This is because they don't change the free energy of the reactants or products. They only reduce the **activation energy** required to reach the transition state (Figure 1).

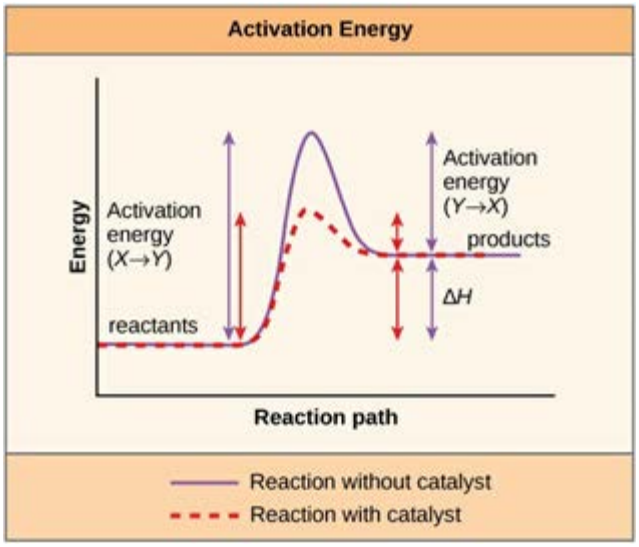

Figure 1: Enzymes lower the activation energy of the reaction but do not change the free energy of the reaction

Link to a video Enzymes and Activation Energy

Enzyme Active Site and Substrate Specificity

The chemical reactants to which an enzyme binds are the enzyme's **substrates**. There may be one or more substrates, depending on the particular chemical reaction. In some reactions, a single-reactant substrate is broken down into multiple products. In others, two substrates may come together to create one larger product molecule. Two reactants might also enter a reaction, both become modified, and leave the reaction as two products.

The location within the enzyme where the substrate binds is called the enzyme's **active site**. The active site is where the "action" happens, so to speak. Since enzymes are proteins, there is a unique combination of amino acid residues (also called side chains, or R groups) within the active site. Each residue is characterized by different properties. Residues can be large or small, weakly acidic or basic, hydrophilic or hydrophobic, positively or negatively charged, or neutral. The unique combination of amino acid residues, their positions, sequences, structures, and properties, creates a very specific chemical environment within the active site. This specific environment is suited to bind, albeit briefly, to a specific chemical substrate (or substrates).

Due to this jigsaw puzzle-like match between an enzyme and its substrates (which adapts to find the best fit between the transition state and the active site), enzymes are known for their specificity. The "best fit" results from the shape and the amino acid

functional group's attraction to the substrate. There is a specifically matched enzyme for each substrate and, thus, for each chemical reaction; however, there is flexibility as well.

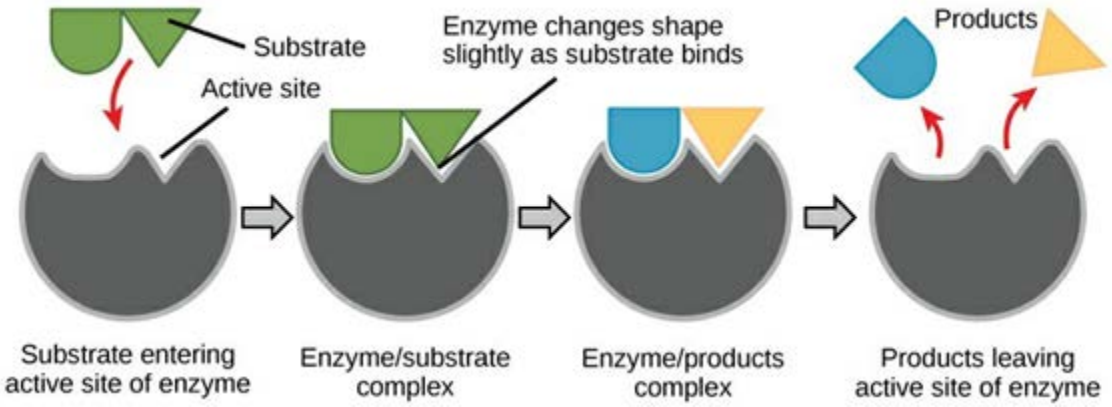

Figure 2: Induced-fit model of enzyme function
Created by TimVickers, vectorized by Fvasconcellos/Public domain

According to the **induced-fit model**, both enzyme and substrate undergo dynamic conformational changes upon binding (Figure 2). The enzyme contorts the substrate into its transition state, thereby increasing the rate of the reaction. The molecule(s) produced at the conclusion of the reaction is called a product(s).

Link to a video Enzymes the Induced Fit Model

Factors that affect the rate of enzyme activity:
Adapted from Amylase Activity Lab, Montgomery College, Rockville, MD (doi:10.25334/VKGJ-VT46).
1. **Temperature** affects enzyme activity in two ways. As the temperature rises, molecular motion (kinetic energy) increases and the rate of random collision between enzyme and substrate molecules increases, forming more products. After a certain point, increasing the temperature strains the non-covalent bonds, altering the shape of the active site, and the overall shape of the enzyme, the enzyme is then **denatured**. This decreases the rate of product formation. The temperature at which enzyme activity is the highest is called the **optimum temperature**. At high temperature, an enzyme will most likely unfold and denature.

2. **Changes in pH** (H+ concentration) and salt concentration primarily affect the stability of secondary and tertiary structures maintained by hydrogen bonds and disrupt salt bridges held by ionic bonds. As a result, enzymes denature at extreme pH and high salt

concentrations. In addition, substrates and/or enzyme active site groups may ionize, which further affects enzyme substrate binding. The pH at which enzyme activity is the highest is called the **optimum pH**.

3. **Substrate and enzyme concentration** also affect the rate of enzyme reaction. Increasing the concentration of substrate and/or enzyme increases the rate of reaction up to a certain point. As the reaction continues and the substrate molecules are used up, the rate of reaction will decrease regardless of any changes in enzyme concentration. By controlling enzyme and substrate concentration, organisms can regulate their metabolism.

Link to an interactive on Effect of pH on Enzyme Function

Link to an interactive on Effect of Temperature on Enzyme Function

Amylase

Enzyme Activity: Amylase

The enzyme being studied in this experiment is **amylase**, an enzyme which cleaves complex sugars (polysaccharides) into simple sugars (disaccharides). Amylase catalyzes the **hydrolysis** (splitting) of α-1,4 glycosidic linkages in polysaccharides (like starch) which breaks the large molecules into smaller molecules of sugar like maltose. Maltose is a reducing sugar that consists of two molecules of glucose bonded together. In this reaction, **starch** is the **substrate** and the **product** is **maltose** (Figure 3).

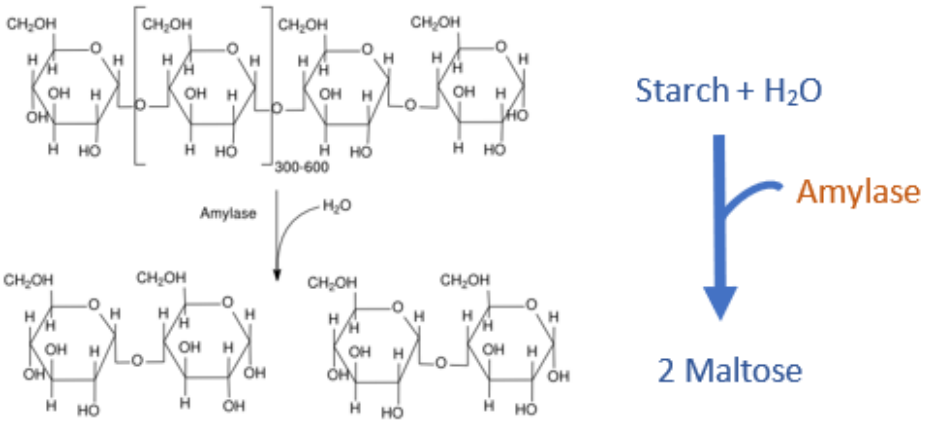

Figure 3: Hydrolysis of starch into maltose by amylase

From https://commons.wikimedia.org/wiki/Category:Maltose#/media/File:Amylase_reaction.png

Alpha-amylase is produced by a wide variety of organisms from all three domains of life: Bacteria, Archaea, and Eukarya. It serves to breakdown starch which is the most widely available polysaccharide on the planet. When digested, starch produces simple sugars that are readily available energy for organisms. In humans, alpha-amylase is a component of saliva produced by the salivary glands and of pancreatic secretions produced by the pancreas.

To measure **enzymatic activity**, it is necessary to measure the amount of substrate remaining or the amount of product produced through an enzyme-catalyzed reaction. In the reaction catalyzed by amylase, the product, maltose, can be measured utilizing a **colorimetric assay**. Colorimetric assays utilize a reagent that changes color in the presence of a compound of interest. The reagent that will be utilized is **3,5-dinitrosalicylic acid** (**DNS**) which changes color in the presence of maltose (the product) from yellow to a red/orange.

The DNS assay utilizes an oxidation-reduction reaction that occurs between a reducing sugar (like maltose) and DNS. The reducing sugar forms free aldehyde or ketone groups which enables the molecule to reduce the DNS reagent (Figure 4). In this oxidation-reduction process maltose is oxidized into maltonic acid and DNS is reduced to **ANSA** (**3-amino-5-nitrosalicylic acid**).

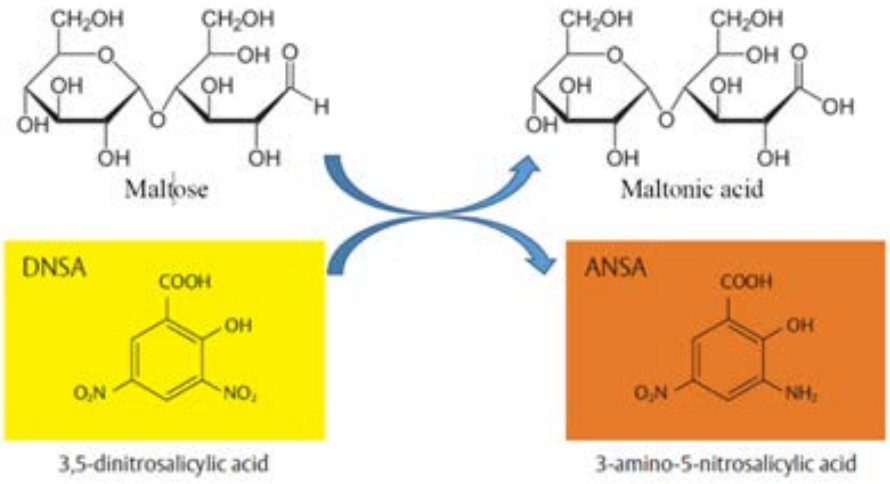

Figure 4: Reduction of DNS (yellow) by maltose into ANSA (red/orange)

From Department for Education (April 2014) GCE AS and A level subject content for biology, chemistry, physics and psychology.
https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/446829/A_level_science_subject_content.pdf

DNS is **yellow** in color. As maltose is produced through the hydrolysis of starch by amylase, DNS is reduced by the maltose, forming **ANSA** which is **orange/red** in color. The reduction of DNS will be catalyzed by heat in a boiling water bath. More maltose produced results in a darker orange/red color (Figure 5).

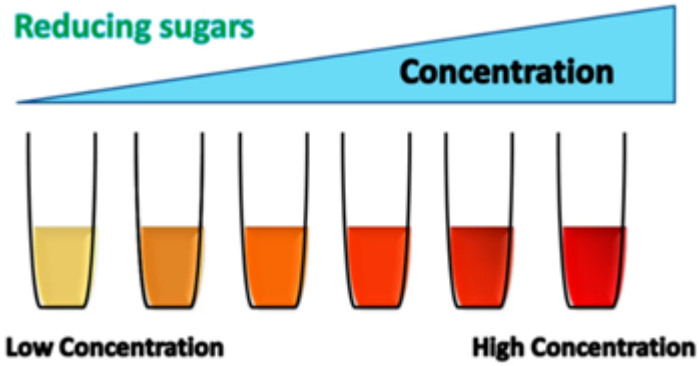

Figure 5: Reducing sugar concentration indicated by DNS assay
From https://youtu.be/NtqsWKRW7N8

Link to video on Reducing sugar by DNS method

This a colorimetric assay, which produces a colored product (ANSA) that will be used to measure how much maltose is produced. To measure the amount of a colored product, a **spectrophotometer** will be utilized. The spectrophotometer allows for the quantitative measurement of **light transmitted** through a sample. This transmittance can then be used to determine the amount of light absorbed (**absorbance**) by that same sample. Figure 6 shows a single wavelength spectrophotometer, the wavelength is set to a particular frequency of light (in this case 540nm) and the light is then passed through the sample.

Figure 6: Spectrophotometer

From https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps

In the diagram below (Figure 7) the internal function of the spectrophotometer can be seen. The light source sends light at the set wavelength through the sample in the cuvette and the amount of light transmitted through the sample is measured by the photocell. The machine then calculates absorbance based on the amount of transmitted light. **Absorbance is directly related to the concentration** of the sample being tested, which makes spectrophotometry an excellent tool for calculating concentration in solutions where concentration is unknown.

In this assay the concentration of maltose will be determined using DNS, this concentration will be directly related to the enzymatic activity of amylase.

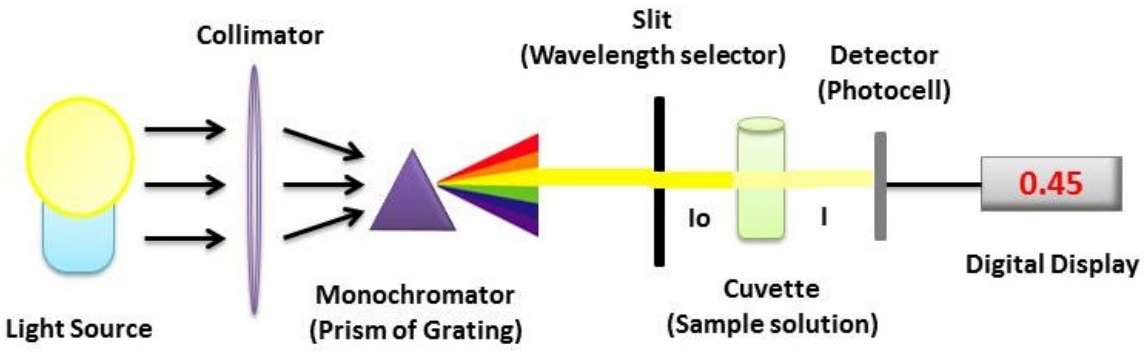

Figure 7: Spectrophotometer function

From Chem libre texts illustrated by Heesung Shim
https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps

Link to video on How does a spectrophotometer work?

Experimental Activity Part 1: Introduction to the DNS Assay as a Measurement Tool for Amylase Activity

The hydrolysis of **starch** is catalyzed by the enzyme **amylase** with the resulting product being **maltose**. The **DNS assay** allows us to determine the amount of maltose produced using **absorbance** data found with a spectrophotometer. The absorbance increases with the amount of maltose produced. **The amount of maltose produced is directly correlated to how active the amylase enzyme is.**

1. To introduce the assay, two tubes are prepared containing the contents listed in the table below. The contents are added to the tube using a micropipette.

Link to video on Micropipetting Basics

Table 1: Introduction to the Assay

Tube	Water (µl)	5% Starch (µl)	Amylase (µl)	DNS (µl)	H_2O (µl)	Total Vol (ml)	Absorbance	Maltose Concentration (mg/ml)
A	450	450	100	1000	8000		**0.358**	
B	550	450	0	1000	8000		**BLANK**	

2. Both tubes are incubated for 10 minutes at 25°C (room temperature)

3. 1000 µl (1 ml) of DNS is added and the tubes are placed in boiling water for 5 minutes.

4. 8 ml of dH_2O (deionized water) is added to both samples for dilution.

5. Both solutions are placed in a spectrophotometer and the absorbance is measured at 540nm.

Link to video on Using a Spectrophotometer

Tube B is used as a "**blank**" which is a sample created to calibrate the machine prior to measuring our samples.

What treatment is tube B in the experiment? _____

What treatment is tube A in the experiment? _____

6. The absorbance of Tube A (filled into Table 1) is **0.358**

7. After completing Part II and constructing your maltose standard curve, utilize the standard curve to determine the **concentration of maltose in Tube A** _____

Experimental Activity Part 2: Development of the Standard Curve for Estimation of Maltose Concentration

We use a **standard curve** to determine how much of a particular material is present in a sample.

- A standard curve is a graph of the relationship between the concentration of a material of interest and the response of a particular instrument (Figure 8).
- We will use known **concentrations of maltose** that will produce a color product when heated with the colorimetric DNS reagent.
- We will use a spectrophotometer to measure the **absorbance at 540nm** which is a measure of concentration of maltose (higher absorbance units, higher concentration of maltose).
- **Maltose is a measure of the amylase activity**. Maltose is a disaccharide of glucose. When amylase hydrolyzes starch, maltose is released.

The X-axis represents the known concentrations of maltose and the Y-axis represents the absorbance units at 540nm. The R2 value of a linear regression is 1.0. The readings of your experimental values are dependent on the validity of the standard curve, therefore, as close to an R2 value of 1.0 is important.

In Part III of this lab exercise, you will **use the standard curve to estimate the amount of maltose produced** during hydrolysis of starch, the reaction catalyzed by amylase. The **data on the amount of maltose produced will then be used to calculate amylase activity.**

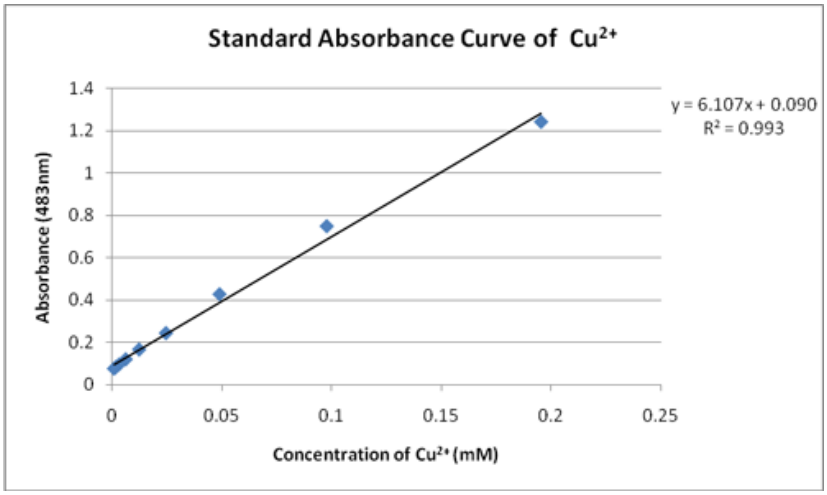

Figure 8: Example Standard Curve
From BioLibreTexts https://bio.libretexts.org/

To prepare the standard curve, the concentration of maltose (mg/ml) in each tube has to be determined first.

Please watch this video Graphing Sample Concentration v. Absorbance first to understand the steps involved in the calculations, and to construct the standard curve.

The **initial maltose concentration** (C_i) used is **2.5 mg/ml**. The **initial volume** (V_i) added of the maltose stock solution is indicated for you in column 1 of Table 1. After adding 1 ml DNS to the tubes, all samples are diluted with 8 ml of water bringing the **final volume** (V_f) to 10ml in each tube and the samples are boiled.

The boiling water catalyzes the reduction of DNS by maltose, causing the color change from yellow to orange/red if maltose is present (Figure 9). The intensity of the color is correlated to the amount of maltose present. The absorbance is determined in the spectrophotometer and indicated in the table.

1. **Complete the table below (Table 1)** and show a sample of your calculations. You will be using the formula $C_iV_i = C_fV_f$ **(equivalent to** $C_1V_1 = C_2V_2$**)** where **i** indicates initial and **f i**ndicates final.

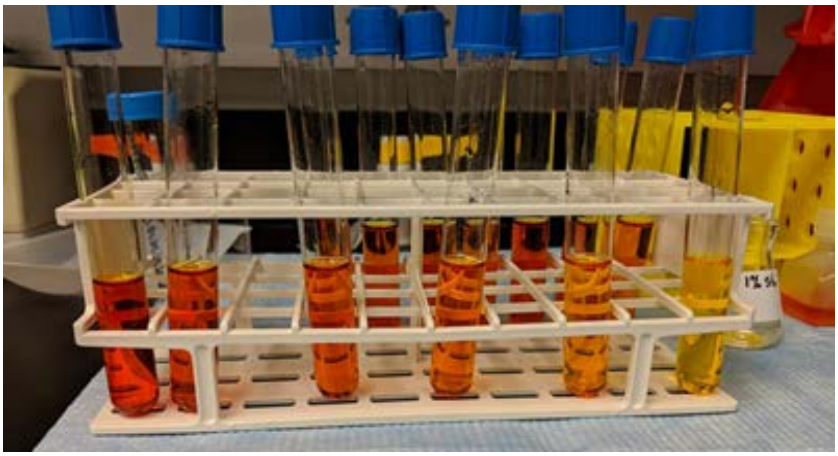

Figure 9: Example Standards with Color Produced by Maltose Color Reagent and Heat

From M. Lenahan Raritan Valley Community College

2. **Graph 1: graph the Absorbance vs. Concentration of maltose from Table 1.** There are two ways to do it, a) manually, on graph paper, OR b) in Microsoft Office Excel, depending on what your instructor has asked you to do. Remember to use titles and insert your graph below. If you are not sure how to do that, watch the video on Graphing Sample Concentration v. Absorbance for help.

   Choose one of these two ways to present your graph:

   a. Construct your graph **manually, on graph paper**. Draw a line of best fit through your points, making sure that line passes closer to as many data points as possible. Embed a picture below. This graph will be used in Part III, to determine the amount of maltose produced in your experimental tubes.

   OR

   b. Construct your **graph on Excel**, according to the instructions in the video provided. Draw a 'line of best fit' through your data points by clicking on a point and selecting "add trendline". In the menu that opens, select "display equation of line", to get the equation of the line to appear on your graph. Embed your excel graph below. You will use this equation of the line in Part III, to determine maltose produced in your experimental tubes.

Table 2: Final Concentration of Maltose

Volume of initial maltose solution V_i (µl)	Volume of initial maltose solution V_i (ml)	Volume of H_2O added to get 1ml (ml)	Final Maltose Concentration C_f (mg/ml)	Absorbance (540nm)
0	0	1		**0.000**
200	0.2	0.8		**0.228**
400				**0.487**
600				**0.751**
800				**1.031**
1000				**1.275**

Experimental Activity Part 3: Choose your Independent Variable and Test your Hypothesis

In this part of the activity, you will **determine how amylase activity is affected by environmental conditions** like pH and temperature. These conditions will be the **independent variables** for your experiment. You will have the choice of using one of two different types of amylase from two different organisms, *Homo sapiens* (human) or *Geobacillus stearothermophilus* (thermophilic bacteria).

In this activity you will utilize the steps of the scientific method for your inquiry.

The steps of the **scientific method** are:

1. **Question**: What are you asking? Be as specific as possible to help in your experimental design.

2. **Hypothesis**: What is a possible explanation? You should **research the organism that is the source of your amylase first**. This will allow your hypothesis to be an educated guess. The hypothesis should be written as a testable statement.
3. **Prediction**: What will you see if you test the hypothesis and it is correct? Write your prediction in the **If**_____ **then**_____ format.
4. **Experiment**: Test the hypothesis
    a. **Independent variable**: what you are testing
    b. **Dependent variable**: what you are measuring
    c. **Controls**: provide a comparison for experimental treatments to verify changes seen.
5. **Conclusion**: Summary and explanation of what occurred in the experiment. Directs future research.

Choose your Independent Variable

Select which independent variable you will assess and your amylase source by selecting a group from the table below (Table 3).

Now that you have selected the source for the amylase you will use and the environmental condition (independent variable) you are testing, do some research about your organism source before completing the next section. Your hypothesis and prediction will be based on this research and you will need the research for your paper. Be sure to cite your sources in **APA format**.

Table 3: Test your Hypothesis

Group	Amylase	Independent Variable
1	*Geobacillus stearothermophilus (*Bacterial)	Temperature
2	*Homo sapiens* (Human)	Temperature
3	*Geobacillus stearothermophilus (*Bacterial)	pH
4	*Homo sapiens* (Human)	pH

Fill in the following:

1. What is the question:

2. What is your hypothesis?

3. The prediction is:

Experimental Design

If you chose **temperature** as your independent variable **Table 4A** shows how the tubes are set up to conduct the experiment. If you chose **pH** as your independent variable **Table 4B** shows how the tubes are set up to conduct the experiment. Remember, you are assessing how these independent variables affect amylase activity.

The contents are added to the tubes and mixed. The tubes are then incubated at the appropriate temperatures for 10 minutes. 1 ml of DNS is added to all tubes prior to incubating the tubes in a boiling water bath for 5 minutes. 8 ml of water is then added to all tubes for dilution prior to measuring the absorbance of the resulting solutions at 540nm using a spectrophotometer.

Table 4A: Experimental Setup for Temperature

Tube	Water (µl)	5% Starch (µl)	Amylase (µl)	Temperature (°C)
1	450	450	100	0
2	450	450	100	25
3	450	450	100	37
4	450	450	100	45
5	450	450	100	65
6	450	450	100	85

Table 4B: Experimental Setup for pH

Tube	Water (µl)	5% Starch (µl)	Amylase (µl)	pH
1	450	450	100	1
2	450	450	100	3
3	450	450	100	5
4	450	450	100	7
5	450	450	100	9

6	450	450	100	12

The tubes listed in Table 4A and 4B are your **experimental treatment** tubes. You will also have a **negative control** tube. What will that tube contain?

Data

From the tables located in the Appendix, choose the one that contains the data for your independent variable and organism.

Please watch this video Temperature Effects on Enzyme Activity first.

1. Using either the **manual graph** or the **Excel graph** you created of the maltose standard curve:
    a. Determine the **maltose produced** in your experimental tubes and fill in that data in the table. Show an example calculation below the table.
    b. Determine the **amylase activity** in each tube and fill in that data in the table. Show an example calculation below the table.
2. **Graph** the resulting **amylase activity data vs. your independent variable** on Excel or manually on graph paper, this will be the second graph contained in the lab report you will write. The first was your standard curve.
3. **Synthesize the conclusion**. A conclusion should begin with a restatement of the hypothesis followed by a summary of the results based on your graph showing amylase activity vs temperature or pH in your organism of choice. Explain whether or not the data supports your hypothesis. Comment on what you think occurred with amylase's structure and function at points where your data indicated low activity. What do areas of high activity tell you about the organism you selected?

Write the Lab Report

You will construct a lab report using the findings from Experimental Activities Part 2 & 3. Your report must contain the following components:

1. Introduction
2. Experimental design
3. Materials and Methods
4. Results
5. Conclusion
6. References

Please refer to your instructor's specific guidelines for constructing a lab report.

Appendix: Data Tables

Table 5: Effect of Temperature on Human Amylase Data

Temperature (°C)	Absorbance (540nm)	Maltose Produced (mg/ml)	Amylase Activity (mg/ml/min)
0	0.107		
25	0.568		
37	0.874		
45	0.342		
65	0.031		
85	0.008		

Table 6: Effect of Temperature on Bacterial Amylase Data

Temperature (°C)	Absorbance (540nm)	Maltose Produced (mg/ml)	Amylase Activity (mg/ml/min)
0	0.116		
25	0.237		
37	0.309		
45	0.391		
65	0.895		
85	1.029		

Table 7: Effect of pH on Human Amylase Data

pH	Absorbance (540nm)	Maltose Produced (mg/ml)	Amylase Activity (mg/ml/min)
1	0.029		
3	0.103		
5	0.267		
7	0.945		

9	0.112		
12	0.015		

Table 8: Effect of pH on Bacterial Amylase Data

pH	Absorbance (540nm)	Maltose Produced (mg/ml)	Amylase Activity (mg/ml/min)
1	0.107		
3	0.209		
5	0.637		
7	0.995		
9	0.048		
12	0.037		

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