**Introduction:** The catalytic activity of enzymes is attributed to a decrease in the activation energy for a chemical reaction. In this experiment, the Arrhenius activation energies, \( E_a \), for hydrolysis of the glycoside salicin, as catalyzed by an enzyme (β-D-glucosidase) and by acid will be compared. The progress of the reaction is monitored by production of salicyl alcohol. The reaction is stopped by addition of excess alkali, which also convert the alcohol to its anion which absorbs in the uv range (\( \lambda_{\text{max}} = 290 \text{ nm} \))

\[
\text{salicin} \quad \text{CH}_2\text{OH} \quad \text{O} \quad \text{O} \quad \text{CH}_2\text{OH} + \text{H}_2\text{O}
\]

\[
\text{salicyl alcohol; 2-hydroxybenzyl alcohol}
\]

In the **enzyme-catalyzed** reaction, salicin is in excess of the enzyme. Essentially all of the enzyme is present as the enzyme-substrate complex and the rate is independent of the salicin concentration. It therefore follows zero-order kinetics.

In the **acid-catalyzed** reaction, the concentration of the catalyst is greater than that of salicin. The rate of reaction depends on the decomposition of salicin protonated at the glycosidic oxygen. This reaction follows first-order kinetics.

See the appendix for the derivation of the appropriate kinetic equations to use for calculations.

**Procedure Overview:** For each type of catalysis, each group will determine the rate of reaction at one temperature and will provide their data to the rest of the class. To determine the rate of reaction, an incubation will be prepared that contains substrate and catalyst. Aliquots will be removed at specific time points. The reaction will be stopped in each aliquot by addition to base and the extent of product formation at each time point (in each aliquot) determined.

**Reagents:** The reagents below will be available

- 0.1M phosphate buffer, pH 6.0, 13.8 gm NaH\(_2\)PO\(_4\).H\(_2\)O in 1L water
- 25mg β-glucosidase (5 units per mg) in 70 mL buffer
- 0.67g salicin in 100 mL buffer
- 2M NaOH, 40gm NaOH in 500 mL water
- conc. HCl
- 0.67g salicin in 100 mL water/n-propanol (80:20)

For each incubation, prepare for the removal of 7 samples. One incubation will be prepared for each catalyst.
Procedure Detail:

a) Acid-catalyzed reaction:

In water baths set at 55° C, 60° C, 65° C, 70° C, 75° C, incubate the salicin solution in water/1-propanol (80:20) for temperature equilibration. Record the actual temperature of the bath. Prepare a set of 7 tubes with 5 mL 2 M NaOH in each tube. Start the reaction by mixing 6.0 mL pre-warmed salicin solution and 2.0 mL concentrated HCl in a separate tube and incubate at the appropriate temperature. **Note the time the reaction was started.** Remove a 0.15 mL sample as soon as possible after mixing salicin and acid and place it into a test tube with NaOH. Remove 0.15 mL samples at timed intervals (see below for suggested time intervals) and place them in the tubes with NaOH. Obtain a completely hydrolyzed sample by placing a 0.15 mL aliquot of the reaction mixture in a labeled clean test tube and boiling in a water bath for 15 min. Add 10.0 mL 2 M NaOH after cooling.

Measure the absorbance of all the samples at 290 nm.

b) Enzyme-catalyzed reaction:

In water baths set for 25° C, 30° C, 35° C, 40° C and 45° C place portions of the enzyme solution and the salicin to temperature equilibrate. Record the actual temperature of the bath. Prepare a set of 7 tubes with 5 mL of 2 M NaOH in each tube. Start the enzyme reaction by mixing 6.0 mL of pre-warmed salicin and 2.0 mL pre-warmed enzyme in another tube and incubate at the appropriate temperature. **Note the time that the reaction was started.** Remove a 0.15 mL sample as soon as possible and place it into a test tube with NaOH. Remove 0.15 mL samples at three minute intervals until a total of seven samples have been removed.

Measure the absorbance of all the samples at 290 nm.

Notes:

*** Please obtain data carefully. All data will be shared. While 5 data points for the determination of an enthalpy of activation is not what would be used in "real life", it should provide an idea of how this data is manipulated.

Note 1. In the acid catalyzed reaction use 5 min intervals for sample removal for temperatures 60-66°C, 3 min intervals between 67-75°C, and 2 min intervals between 76-80°C. In the enzyme catalyzed reaction 3 min intervals between samples should be used for all temperatures. Since enzymes are sensitive to heat denaturation, the temperatures for the enzyme catalyzed reactions should not be extreme.

Note 2. The volumes of the reaction mixture, the sampling aliquots and the stop solution can be adjusted to fit the amounts of reagents and available equipment. The ratio of salicin solution to catalyst is 3:1 so volumes of 6 and 2 mL or 3 and 1 mL could be used. The ratio of sampling aliquot to stop solution is 0.03 to 1 and any volumes that maintain this ratio are suitable.

Note 3. Air displacement pipetters, such as Pipettman, do not give good results at high temperatures. The hot solution heats the air in the pipet tip and forces the solution out of the tip. A positive displacement pipetter should work well in this situation. Alternatively, 0.2 or 0.5 mL graduated glass pipets give good results. Since the bore of these low volume pipets is small, the temperature of the aliquot is quickly lowered as the solution is drawn into the pipet and the rate of the reaction of the solution in the pipet is lowered.

Note 4. When the salicin reaction mixture is totally hydrolyzed, a gummy precipitate is formed and it is difficult to obtain a representative sample. The procedure employed here removes a representative sample of a homogeneous solution and then hydrolyses it. The entire gummy precipitate that is formed is dissolved in the NaOH.
Calculations:

a) Acid-catalyzed reaction:
To determine a first-order rate constant, subtract the absorbances of the timed samples from twice the absorbance of the fully reacted sample and plot the logarithm of the resulting values against time. Calculate the activation energy for the acid-catalyzed reaction. (Twice the absorbance of the fully reacted sample is used in the final calculations because a 0.15 mL aliquot was hydrolyzed and diluted with 10 mL NaOH whereas the other measurements were made on the same-sized aliquots diluted into 5 mL NaOH.)

b) Enzyme-catalyzed reaction:
Read the absorbance at 290 nm against a blank of 5 mL 2 M NaOH and 0.15 mL water. Determine the rate constants for each temperature from a plot of the absorbance (or molarity) vs. time. Calculate the activation energy for the enzyme-catalyzed reaction.

Questions:

1. Compare the activation energies for the enzyme- and acid-catalyzed hydrolysis of salicin.

2. Compare the activation energies you have determined with the values for other catalyzed and uncatalyzed reactions. Look in the literature, use Medline and Ohiolink.

3. Amylase is another enzyme that catalyzes the hydrolysis of glycosidic linkages. What are the differences in the substrates and products produced between amylase and β-glucosidase?