The Neotia University

Paper: Enzymology Practical
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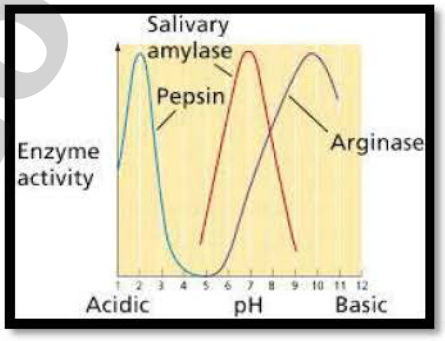
CONTENT

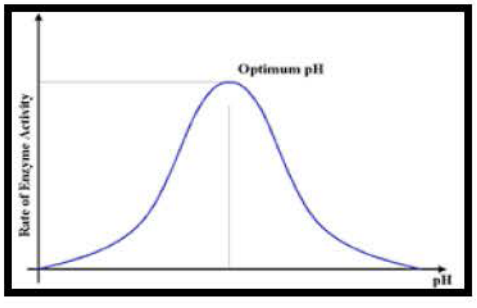
Practical 1 (Effect of pH on the rate of an enzyme catalyzed reaction)

Aim: To establish the relationship between pH and the rate of an enzyme catalyzed reaction and to determine the optimum pH for such a reaction.

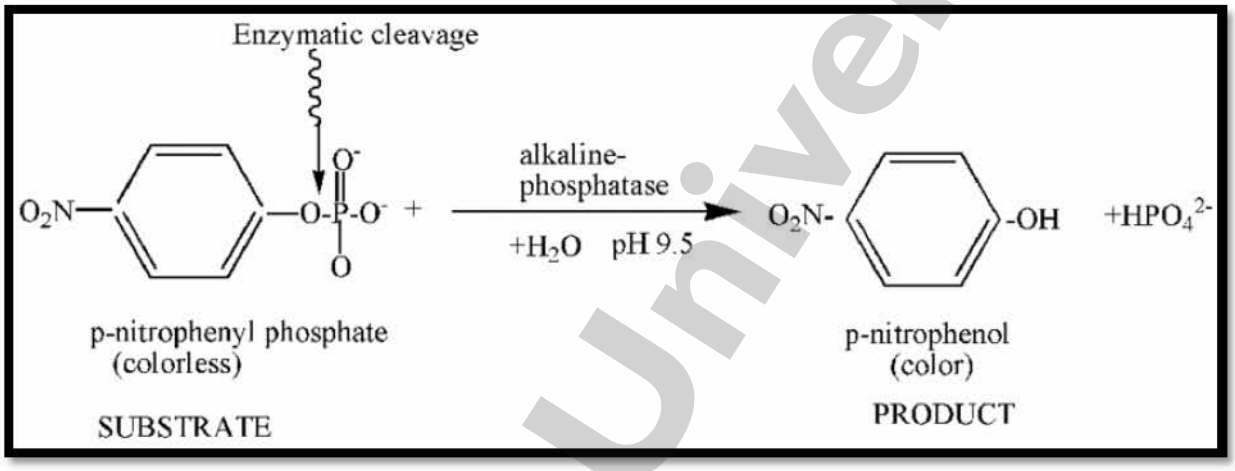
Principle: Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the total net charge of the enzymes and the distribution of charge on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighbourhood of the active sites. Taken together, the changes in charges with pH affect the activity, structural stability and solubility of the enzyme. However, the rate of enzymatic reaction depends on pH of the medium. Each enzyme has an optimumpH, where the rate of enzymatic reaction shows its maximum value. At higher or lower pH, the rate of an enzymatic reaction decreases.For most enzymes, the optimum pH lies in the range from pH 5 to pH 9.With some exceptions, pepsin's optimum pH is extremely acidic and arginase's optimum pH is extremely basic.



The relationship between the rate of an enzymatic reaction and pH takes form of a bell-shape.



Many phosphatases are not very specific with respect to the compounds they use as substrates and any one phosphatase will often be able to hydrolyse various organo-phosphates. The low specificity will allow us to utilize only one substrate to test the activity of two different phosphatases in this experiment. The test reaction that will be used to measure (assay) enzyme activity is hydrolysis of para-nitrophenylphosphate.



Materials required:

- Alkaline phosphatase
- Paranitrophenyl phosphate
- Test tubes
- Glycine-NaOH buffer
- Conical flasks
- Micro pipette
- pH meter
- Sterile distilled water
- Sodium hydroxide (NaOH)
- Spectrophotometer

Procedure:

1. Obtain the enzyme solution and keep it that on ice.

2. Prepare nine cuvettes as indicated in Table (mentioned below).

3. Place the cuvettes in the 37°C water bath and allow them to warm to 37°C for about 5 min.

4. Note the time accordingly. Now add enzyme to each of the numbered cuvettes and mix.

5. After 30 min, remove the cuvettes from the water bath and stop the reaction by adding 2 ml NaOH per cuvette. Mix as soon as possible after adding the NaOH.

6. Using tube 0 as the reference cuvette, read A405 of each reaction mixture.

Tube	Buffer pH (2 ml each)	6 mM ? -NPP	Enzyme	0.2 M NaOH (at 30 or 40 min.)
0 (Blank)*	None	1 ml	None	2 ml
3	3	1 ml	1 ml	2 ml
4	4	1 ml	1 ml	2 ml
5	5	1 ml	1 ml	2 ml
6	6	1 ml	1 ml	2 ml
7	7	1 ml	1 ml	2 ml
8	8	1 ml	1 ml	2 ml
9	9	1 ml	1 ml	2 ml
10	10	1 ml	1 ml	2 ml

***Add 3ml of the MILLIQ-water in tube 0(Blank).**

(6 Mm?-NPP should be replaced by 6 mM PNPP)

Result: The OD generated in each reaction set shall be converted to enzyme activity accordingly and would be plotted against different pH on a graph paper/excel.

Discussion: Based on the result obtained, the enzyme optimum pH may be determined. The result may indicate the nature of the enzyme in the context of pH sensitivity (whether acidic, neutral or alkaline).

Safety precautions: Basic laboratory manual displayed at the laboratory is to be practiced while conducting the experiment. No radioactivity or any other hazards would be practiced here.

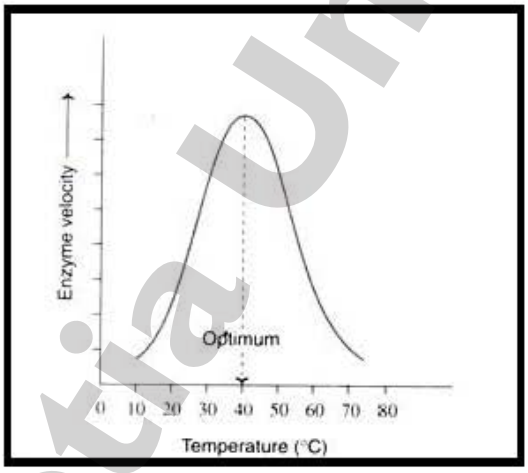
Practical 2 (Effect of Temperature on the rate of enzyme catalysed reaction)

Aim: To establish the relationship between temperature and the rate of an enzyme catalysed reaction and to determine the optimum temperature for such a reaction.

Principle: The single most important property of enzymes is the ability to increase the rates of reactions occurring in living organisms, a property known as catalytic activity. Because most enzymes are proteins, their activity is affected by factors that disrupt protein structure, as well as by factors that affect catalysts in general. Factors that disrupt protein structure include temperature and pH; factors that affect catalysts in general include reactant or substrate concentration and catalyst or enzyme concentration. The activity of an enzyme can be measured by monitoring either the rate at which a substrate disappears or the rate at which a product forms.A general rule of thumb for most chemical reactions is that a temperature rise of 10°C approximately doubles the reaction rate. To some extent, this rule holds for all enzymatic reactions. After a certain point, however, an increase in temperature causes a decrease in the reaction rate, due to denaturation of the protein structure and disruption of the active site. For many proteins, denaturation occurs between 45°C and 55°C. Furthermore, even though an enzyme may appear to have a maximum reaction rate between 40°C and 50°C, most biochemical reactions are carried out at lower temperatures because enzymes are not stable at these higher temperatures and will denature after a few minutes. At 0°C and 100°C, the rate of enzyme-catalysed reactions is nearly zero. This fact has several practical applications. We sterilize objects by placing them in boiling water, which denatures the enzymes of any bacteria that may be in or on them. We preserve our food by refrigerating or freezing it, which slows enzyme activity. When animals go into hibernation in winter, their body temperature drops, decreasing the rates of their metabolic processes to levels that can be maintained by the amount of energy stored in the fat reserves in the animals' tissues. Protein enzymes play an important role in many physiological

processes, such as growth and reproduction.

The addition of phosphates activates many proteins, and enzymes called phosphatases remove these phosphates when the activated protein has finished its work. Phosphatases operate best at their optimal temperature.In the human body, the optimum temperature of alkaline phosphatase does not range far from 37 degrees Celsius, the normal body temperature. For example, the optimum temperature of alkaline phosphatase taken from a mole caused by tapeworm cysts proved to be 40 degrees Celsius.The relationship between the rate of an enzymatic reaction and temperature takes form of a bell-shape.



Materials required:

- Alkaline phosphatase
- Paranitrophenyl phosphate
- Test tubes
- Glycine-NaOH buffer
- Micro pipette
- Sterile distilled water
- Waterbath
- Sodium hydroxide (NaOH)

- Spectrophotometer

Procedure:

l. Obtain an enzyme solution and keep that on ice.

2. Prepare seven cuvettes as indicated in Table (mentioned below)

3. Place the cuvettes in the 37°C water bath and allow them to warm to 37°C for about 5 min.

4. Note the time. Now add enzyme to each of the numbered cuvettes and mix.

5. After that every test tubes should be warmed according to the following table

6. Remove the cuvettes from the water bath and stop the reaction by adding 2 mlNaOH per cuvette. Mix as soon as possible after adding the NaOH.

6. Using tube 0 as the reference cuvette, read A405 of each reaction mixture.

Test Tube	**Buffer (mL)**	**Enzyme (mL)**	**Substrate (mg/ml) (mL)**	**Temperature (°C)**	**NaOH (mL)**
Blank 1	0.8	-	0.2	37	2
Blank 2	0.8	0.2	-	37	2
2	0.6	0.2	0.2	4	2
3	0.6	0.2	0.2	37	2
4	0.6	0.2	0.2	40	2
5	0.6	0.2	0.2	60	2
6	0.6	0.2	0.2	80	2

Result: The OD generated in each reaction set shall be converted accordingly to the enzyme activity and the same would be plotted against different temperatures on a graph paper/excel.

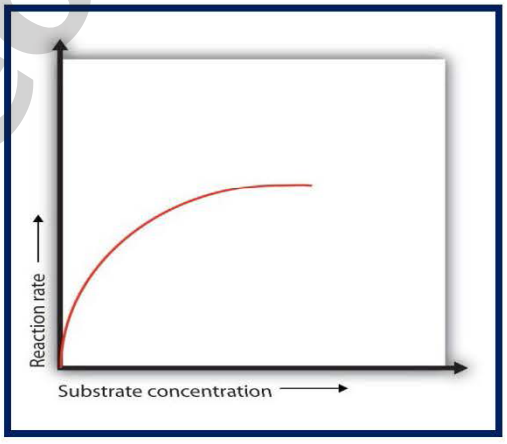
Discussion: Based on the result obtained, the enzyme optimum temperature shall be determined. The result may indicate the nature of the enzyme (whether heat stable, neutral or heat labile) in the context of temperature sensitivity.

Safety precautions: Basic laboratory manual displayed at the laboratory is to be practiced while conducting the experiment. No radioactivity or any other hazards would be practiced here.

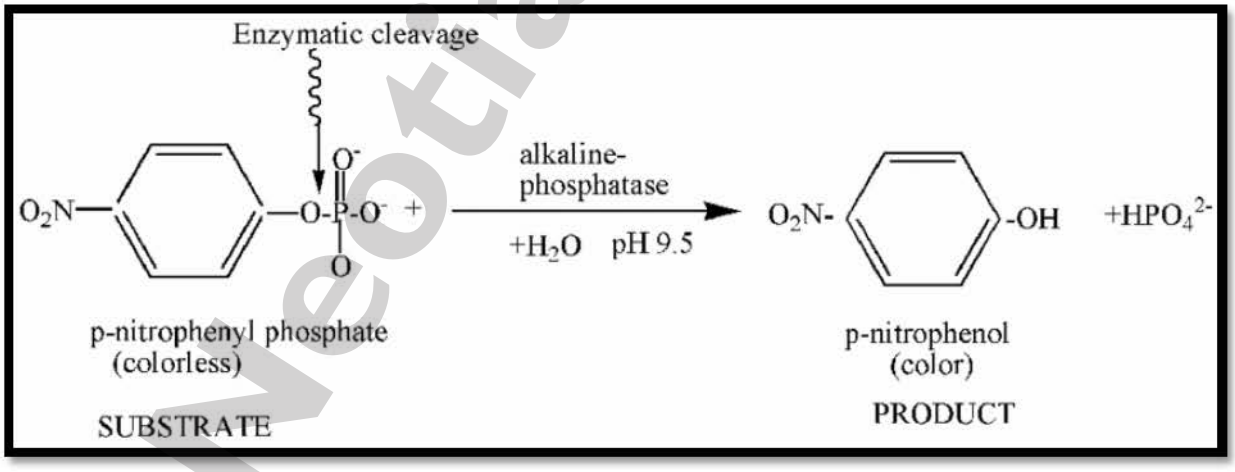
Practical 3: Effect of Substrate concentration on the rate of enzyme catalysed reaction

Objectives: To establish the relationship between substrate concentration and the rate of an enzyme catalysed reaction.

Principle: The single most important property of enzymes is the ability to increase the rates of reactions occurring in living organisms, a property known as catalytic activity. Because most enzymes are proteins, their activity is affected by factors that disrupt protein structure, as well as by factors that affect catalysts in general. Factors that disrupt protein structure include temperature and pH; factors that affect catalysts in general include reactant or substrate concentration and catalyst or enzyme concentration. The activity of an enzyme can be measured by monitoring either the rate at which a substrate disappears or the rate at which a product forms. In the presence of a given amount of enzyme, the rate of an enzymatic reaction increases as the substrate concentration increases until a limiting rate is reached, after which further increase in the substrate concentration produces no significant change in the reaction rate. At this point, so much substrate is present that essentially all of the enzyme active sites have substrate bound to them. In other words, the enzyme molecules are saturated with substrate. The excess substrate molecules cannot react until the substrate already bound to the enzymes has reacted and been released (or been released without reacting).The graph below represents the effect of substrate concentration on the rate of a reaction that is catalysed by a fixed amount of enzyme.



Phosphatases serve a variety of functions in living organisms. Some help to digest food so that the smaller products can be absorbed or metabolized. Such phosphatases occur as secreted enzymes, as in the mammalian intestine. They also occur as dormant enzymes in seeds; during germination, they become active and mobilize stored food to be used by the emerging seedling. Other phosphatases occur in the lysosomes of phagocytic cells and help to digest particulate matter captured during phagocytosis. Still others are active in the cytoplasm and serve to recycle phosphorus in metabolism or to remove phosphate groups from proteins whose activities are regulated by the addition and removal of phosphate groups. Many phosphatases are not very specific with respect to the compounds they use as substrates and any one phosphatase will often be able to hydrolyse various organo-phosphates. The low specificity will allow us to utilize only one substrate to test the activity of two different phosphatases in this experiment. The test reaction that will be used to measure (assay) enzyme activity is hydrolysis of para-nitrophenylphosphate:



Materials Required:

- Alkaline phosphatase
- Paranitrophenyl phosphate
- Test tubes
- Glycine-NaOH buffer

- Micro pipette
- Sterile distilled water
- Waterbath
- Sodium hydroxide (NaOH)
- Spectrophotometer

Procedure:

l. Obtain an enzyme solution and keep it in ice until you are ready to add it to your reaction cuvettes.

2. Using the buffer appropriate to your enzyme, prepare 10 cuvettes as indicated in Table below but do not add enzyme. (Be careful not to introduce any enzyme into cuvettes S0-S4; any para-nitro phenol formed in these tubes should arise by spontaneous (i.e. uncatalysed) hydrolysis of para-nitrophenylphosphate, and a little catalyst can have a significant effect.) Mix the contents of cuvettes S0 - S4 by closing each cuvette with Para film and inverting the cuvette two or three times

3. Place your cuvettes in the 37ºC water bath and allow them to warm to 37ºC for about 5 min

4. Note the time. Now add enzyme to cuvettes E0 - E4 and mix the contents of each cuvette. The time at which you begin to add enzyme is "OT."

5. At l0 min. intervals, remove two cuvettes (one S and one E cuvette), add 2 ml NaOH per cuvette, and mix the contents

NOTE: If you do not add the NaOH at the precise time intended, record the time at which addition was made. The results are equally useful and valid.

Uncatalyzed Reaction

Tube	Buffer	6 mM ? -NPP	Water	Time of .2M NaOH addition
S0	2 ml	1 ml	1 ml	OT
S1	2 ml	1 ml	1 ml	10 min
S2	2 ml	1 ml	1 ml	20 min
S3	2 ml	1 ml	1 ml	30 min
S4	2 ml	1 ml	1 ml	40 min

Catalyzed Reaction

Tube	Buffer	6 mM ? -NPP	Enzyme	Time of .2M NaOH addition
E0	2 ml	1 ml	1 ml	OT
E1	2 ml	1 ml	1 ml	10 min
E2	2 ml	1 ml	1 ml	20 min
E3	2 ml	1 ml	1 ml	30 min
E4	2 ml	1 ml	1 ml	40 min

(6 Mm?-NPP should be replaced by 6 mM PNPP)

Result: The OD generated in each reaction set would be converted to enzyme activity and would be plotted accordingly against the substrate concentrations on a graph paper/excel. The graph would help us to determine the Km and Vmax of an enzyme against a particular substrate using the Michaelis-Menten equation or Line-Weaver Burk equation.

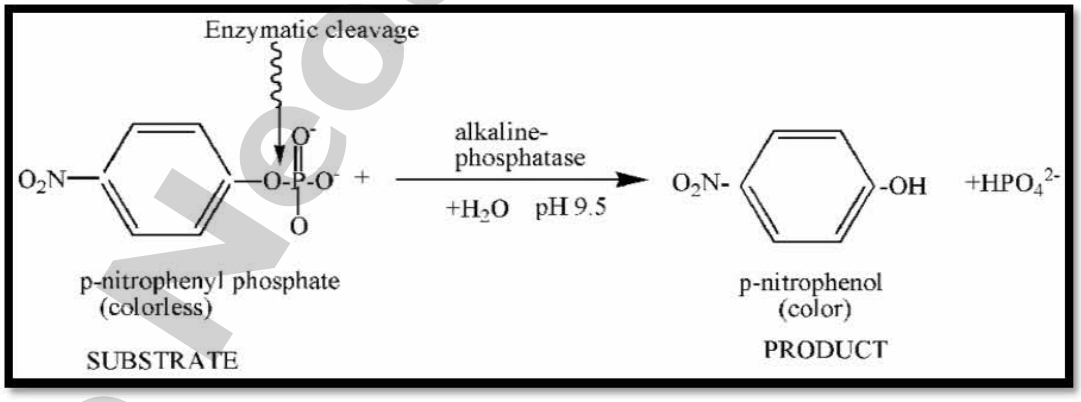
Discussion: Based on the result obtained, theaffinity of an enzyme towards a substrate can be measured accordingly.

Safety precautions: Basic laboratory manual displayed at the laboratory is to be practiced while conducting the experiment. No radioactivity or any other hazards would be practiced here.

Practical 4 (Effect of inhibitor and activator on the rate of enzyme catalysed reaction)

Aim: To study the enzyme kinetics in presence and absence of enzyme inhibitor as well as activator.

Principle: Phosphatases are enzymes that catalyse the hydrolysisof esters of phosphoric acid. They occur in the cells andextracellular fluids of a wide range of organisms. This largeand complex group of enzymes get influenced under the exposure of different agents including some metals. Phospho-monoesterase react with abroad range of substrates, which share common structural motifs. The phospho-monoesterase that lack substratespecificity are classified as acid or alkaline phosphatasesbased on their pH optima. Acid phosphatases function best at around pH 5.0 and are inhibited by fluoride ion butnot by divalent cation-chelating agents. The alkaline phosphataseshave pH optima of about 9.0 and are not generallysensitive to fluoride ion but are inhibited by divalentcation-chelating agents like EDTA (ethylene diamine tetraaceticacid, disodium salt). Many phosphatases are not very specific with respect to the compounds they use as substrates and any one phosphatase will often be able to hydrolyse various organo-phosphates. The low specificity will allow us to utilize only one substrate to test the activity of two different phosphatases in this experiment. The test reaction that will be used to measure (assay) enzyme activity is hydrolysis of para-nitrophenylphosphate:



Materials Required:

- Alkaline phosphatase
- Paranitrophenyl phosphate
- Test tubes

- Glycine-NaOH buffer
- Magnesium chloride
- EDTA
- Calcium chloride
- Monobasic sodium phosphate
- Micro pipette
- Sterile distilled water
- Waterbath
- Sodium hydroxide (NaOH)
- Spectrophotometer

Procedure:

- An enzyme source (alkaline phosphatase) would be taken in a fresh sterile container and would be kept in ice for further use.
- Different experimental set would be prepared as mentioned in the previous experiments in which the enzyme and substrate concentration would be identical. However, different concentrations of activator and inhibitor would be applied separately to the experimental set in order to examine the change in enzyme activity in contrast to control in which the enzyme would be allowed to react with the substrate in absence of any activator or inhibitor.
- All the experimental set would be incubated under similar condition for similar period of time.
- All the reaction would be stopped by adding NaOH at the same time.
- The extent of OD generated in each set would be recorded accordingly.

Result: The OD generated in each reaction set would be converted to enzyme activity and would be plotted against different concentration of modulators (activator/inhibitor) on a

graph paper/excel. Km and Vmax would be determined in each experimental set to understand the specific influence of the modulator (activator/inhibitor) on enzyme kinetics.

Discussion: The susceptibility/sensitivity of an enzymeagainst various agents (activators/inhibitors) would be determined accordingly.

Safety precautions: Basic laboratory manual displayed at the laboratory is to be practiced while conducting the experiment. No radioactivity or any other hazards would be practiced here.