B.Sc. Biotechnology 2019-2020

SEMESTER - I

19BTU111 BIOCHEMISTRY AND METABOLISM PRACTICAL 4H - 2C

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

Course Objectives

- To train the students of the subject on handling various experimental methods and techniques on order to analyze the given biological samples from biochemical stand points.

Course Outcomes (CO's)

1. Students of the subject will acquire skills to quantitatively estimate various biomolecules and as well to carryout enzyme kinetics.

Practical

1. Preparation of buffers.
2. Qualitative tests for Carbohydrates, lipids and proteins
3. Principles of Colorimetry: (i) Beer's law, estimation of protein. (ii) To study relation between absorbance and % transmission.
4. Separation of Amino acids by paper chromatography.
5. Estimation of blood glucose by glucose oxidase method.
6. To study activity of any enzyme under optimum conditions.
7. Determination of - pH optima, temperature optima, Km value, V_{max}, Effect of inhibitor (Inorganic phosphate) on the enzyme activity.
8. To study the effect of pH, temperature on the activity of salivary amylase enzyme.

SUGGESTED READINGS

1. Buchanan B, Gruissem W, and Jones, R. (2015). Biochemistry and Molecular Biology of Plants. 2nd edition. American Society of Plant Biologists.
2. Nelson DL. and Cox, M.M. (2013). Lehninger: Principles of Biochemistry. 6th edition. W.H. Freeman and Company. New York.
3. Murray RK, Bender DA, Botham KM, and Kennelly PJ. (2012). Harper's illustrated Biochemistry. 29th edition. McGraw-Hill Medical. London.
4. Berg JM, Tymoczko JL, and Stryer L. (2015). Biochemistry. 8th edition. W.H. Freeman & Company. New York.
5. Hopkins WG, and Huner P.A. (2008). Introduction to Plant Physiology. 4nd edition. John Wiley & Sons.

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1. Preparation of buffer solutions

Aim: To prepare buffer solutions at various pH values.

Principle: A buffer solution is consisting of a mixture of a weak acid and its conjugate base, or *vice versa*. Its pH changes very little when a small amount of strong acid or base is added to it. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications. However, resistance of a buffer system is depending on its capacity, which is directly proportional to the buffer concentration.

Buffer solutions achieve their resistance to pH change because of the presence of an equilibrium between the acid HA and its conjugate base A^-. When some strong acid is added to an equilibrium mixture of the weak acid and its conjugate base, the equilibrium is shifted such a way the hydrogen ion concentration increases by less than the amount expected for the quantity of strong acid added. Similarly, if strong alkali is added to the mixture the hydrogen ion concentration decreases by less than the amount expected for the quantity of alkali added.

Procedure: Preparation of Acetate buffer

Stock solutions:

A: 0.2 M solution of acetic acid (1.55 ml in 100 ml)

B: 0.2 M solution of sodium acetate (1.64 g in 100 ml)

X ml of A and Y ml of B diluted to a total of 10 ml.

X	Y	pH
4.6	0.4	3.6
4.1	0.9	4.0
2.5	2.5	4.6
1.5	3.5	5.0
0.5	4.5	5.6

Preparation of phosphate buffer

Stock solutions:

A: 0.2 M solution of monobasic sodium phosphate (2.78 g in 100 ml)

B: 0.2 M solution of dibasic sodium phosphate (5.4 g in 100 ml)

X ml of A and Y ml of B diluted to a total of 10 ml.

X	Y	pH
9.2	0.8	5.8
5.1	4.9	6.8
3.9	6.1	7.0
1.6	8.4	7.5
0.5	9.5	8.0

Preparation of Carbonate-bicarbonate buffer

Stock solutions:

A: 0.2 M solution of anhydrous sodium carbonate (2.1 g in 100 ml)

B: 0.2 M solution of sodium bicarbonate (1.7 g in 100 ml)

X ml of A and Y ml of B diluted to a total of 10 ml.

X	Y	pH
0.4	4.6	9.2
2.2	2.8	9.8
3.3	1.7	10.2
3.9	1.1	10.4
4.5	0.5	10.7

The pH values of the buffer solutions were measured using pH meter, which was calibrated before the pH measurements.

Result: Three different buffer solutions were prepared and their pH values were measured using pH meter.

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2. Qualitative Tests for Carbohydrates, Lipids and Proteins

Aim: to qualitatively detect the presence of carbohydrate, lipids and proteins in the given test solution.

Methods

Test for Amino acids and proteins

1. Ninhydrin Test: To one ml test solution, 1 ml of freshly prepared 2% Ninhydrin reagent was added and heated. Appearance of blue colour is an indication for the presence of proteins (Blue colour for amino acids/proteins and yellow color for proline).

   Preparation of Ninhydrin reagent: Mix the solution A and B just prior to your experiments.

   Solution A: 200 mg stannous chloride in 100 ml of 0.1 M citrate buffer.

   Solution B: 4 gm Ninhydrin in 100 ml of 2-methoxy ethanol (or methyl cellulose)

2. Biuret Test: The test solution was treated with 1.0 ml of 40% sodium hydroxide solution and 2 drops of 2% copper sulphate reagent. Appearance of violet colour is an indication for the presence of proteins.

Test for Carbohydrates

1. Fehling's test

The test solution 1 ml was treated with 1.0 ml of Fehling's solution A & B and the resultant mixture was kept at boiling water bath. Formation of reddish brown precipitate indicates the presence of reducing sugars.

Fehling's Reagent A: 7 g $CuSO_4$. $5H_2O$ in 100 ml water (make up).

Fehling's Reagent B: 24 g KOH and 34.6 g Sodium Potassium tartrate in 100 ml water (make up).

2. Benedict's test

To 1 ml the test solution, 5 ml of benedict's solution was added and kept at boiling water bath. Reddish orange precipitate indicates the presence of reducing sugars.

17.3 g Sodium citrate and 10 g Sodium carbonate in 75 ml water.

1.7 g CuSO4.5H2O in 25 ml water.

Thoroughly mix the above solutions to prepare Benedict's reagent.

Test for fixed oils and fats

Spot Test

Press a small quantity of extract between two-filter papers. Oil stains on the filter paper indicates the presence of fixed oil.

Solubility test

1 ml of test solution was treated with 2 ml of water/ 2 ml of ethanol/ 2 ml of choloroform and solubility of the test samples were noted and interpreted accordingly.

Test samples

Weigh 10 g of the given sample and grind well with a pestle and mortar in 10 ml of distilled water. Centrifuge and use the supernatant for proteins/carbohydrates/lipids estimation.

Results:

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3. Principles of colorimeter and its application on estimating proteins

Aim: To understand the principles of calorimeter and to use the calorimeter to quantitatively estimate proteins present in the given test solution.

Principle of colorimeter

In physical and analytical chemistry, colorimetry or colourimetry is a technique "used to determine the concentration of colored compounds in solution." A colorimeter is a device used to test the concentration of a solution by measuring its absorbance of a specific wavelength of light. To use the colorimeter, different solutions must be made, including a control or reference of known concentration.The concentration of a sample can be calculated from the intensity of light before and after it passes through the sample by using the Beer–Lambert law.

The color or wavelength of the filter chosen for the colorimeter is extremely important, as the wavelength of light that is transmitted by the colorimeter has to be the same as that absorbed by the substance being measured. For example, the filter on a colorimeter might be set to red if the liquid is blue.

Beer – Lamberts Law

The **Beer–Lambert law**, also known as **Beer's law** relates the attenuation of light to the properties of the material through which the light is travelling. Lambert's law stated that absorbance of a material sample is directly proportional to its thickness (path length). Much later, August Beer discovered another attenuation relation in 1852. Beer's law stated that absorbance is proportional to the concentrations of the attenuating species in the material sample. The modern derivation of the Beer–Lambert law combines the two laws and correlates the absorbance to both the concentrations of the attenuating species as well as the thickness of the material sample.

According to the law, the mathematical expression can be written as shown below herein.

$$A = \varepsilon c l$$

Wherein, ‘A’ is optical density or absorbance; ‘ε’ is extinction co-efficient;‘c’ is the concentration of test solution; ‘l’ is path length.

The relationship between the absorbance (A) and transmittance (T) can be understood from the following mathematical expression.

$$T = 10^{(2-A)}$$

Moreover, there are at least six conditions that need to be fulfilled in order for Beer–Lambert law to be valid. These are:

1. The attenuators must act independently of each other.
2. The attenuating medium must be homogeneous in the interaction volume.
3. The attenuating medium must not scatter the radiation — no turbidity.
4. The incident radiation must consist of parallel rays, each traversing the same length in the absorbing medium.
5. The incident radiation should preferably be monochromatic, or have at least a width that is narrower than that of the attenuating transition.
6. The incident flux must not influence the atoms or molecules; it should only act as a non-invasive probe of the species under study.

Protein estimation by Lowry method

The **Lowry protein assay** is a biochemical assay for determining the total level of protein in a solution. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques.

The method combines the reactions of copper ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of aromatic protein residues. The Lowry method is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction). The result of this reaction is an intense blue molecule known as heteropolymolybdenum Blue.[5] The concentration of the reduced Folin reagent (heteropolymolybdenum Blue) is measured by absorbance at 660 nm. As a result, the total concentration of protein in the sample can be deduced from the concentration of tryptophan and tyrosine residues that reduce the Folin–Ciocalteu reagent.

Materials Required

Reagent A: 2% Sodium Carbonate in 0.1 N Sodium Hydroxide.

Reagent B: 0.5 % Copper Sulphate ($CuSO_4.5H_2O$) in 1%potassium sodium tartrate.

Reagent C: Mix 50 ml of A and 1 ml of B prior to use

Reagent D: Folin – Ciocalteau Reagent

Protein Solution (Stock Standard)

Weigh accurately 100 mg of bovine serum albumin and dissolve in distilled water and make up to 100 ml in a standard flask (1mg/ml).

Working Standard

Dilute 20 ml of the stock to 100 ml with distilled water in a standard flask (200 mg/ml).

Test samples

Weigh 500 mg of the given sample and grind well with a pestle and mortar in 10 ml of the buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of protein

1. Pipette out 0.2. 0.4, 0.6, 0.8 and 1 ml of the working standard into series of test tubes.
2. Pipette out 0.1 ml and 0.2 ml of the test sample in two other test tubes.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1ml of water serves as the blank
4. Add 5 ml of reagent C to each tube including the blank. Mix well and allow standing for 10 min.
5. Then add 0.5 ml of reagent D, mix well and incubate at room temp in the dark for 30 min. Blue colour is developed.
6. Take the reading at 660 nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

Calculation

Express the amount of protein mg/g or 100 g sample.

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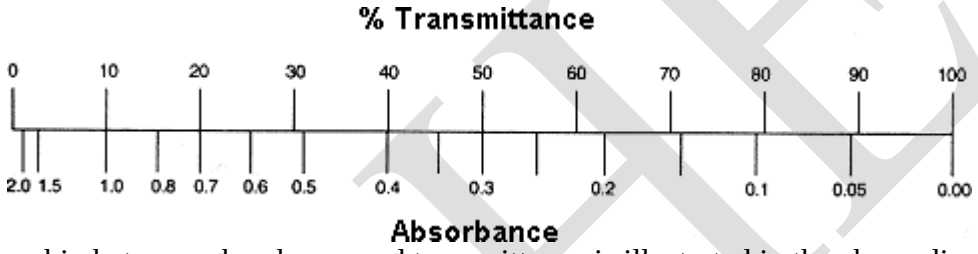
Studying the relation between absorbance and transmission

The relationship between the absorbance (A) and transmittance (T) can be understood from the following mathematical expression.

$$T = 10^{(2-A)}$$

Or

$$A = 2 - \log_{10} I_T$$



The relationship between absorbance and transmittance is illustrated in the above diagram.

So, if all the light passes through a solution *without* any absorption, then absorbance is zero, and percent transmittance is 100%. If all the light is absorbed, then percent transmittance is zero, and absorption is infinite.

Result:

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4. Separation of Amino acids by paper chromatography

Aim: to separate and to identify amino acids by using paper chromatography method.

Principle:

Chromatography is the most powerful technique to separate chemically closely related substances into the individual components on the basis of their physiochemical properties. The components are separated on the basis of their partial coefficients between two immiscible phases. The static phase may be a solid or liquid while the mobile phase may be a solid, liquid or gas. Depending on the static and the mobile phases, a variety of chromatographic techniques are available. Paper chromatography are still easy technique, can be set up even in laboratory without much expenditure.

The separation of the solutes (amino acids) is based on the liquid –liquid partitioning of amino acids in paper chromatography. The partitioning takes place between the water molecule (static phase) adsorbed to the cellulosic matter of the paper and the organic (mobile phase).

Materials Required

Whatman No.1 filter paper
Chromatography chamber
Hair- dryer or Spot lamp Atomizer
Micro syringe or Micropipette

Mobile phase

Mix n-butanol, glacial acetic acid and water in the ratio 4:1:5 in a separating funnel and stand to equilibrate for 30 min. drain off the lower aqueous phase into a beaker and place it inside to saturate the chromatography chamber. Save the upper organic phase and use it for developing the chromatogram.

Sample preparation

Dissolve different individual amino acids in distilled water at a concentration of 1

mg/ml. and use 0.05N HCl to dissolve the free amino acids tyrosine and phenylalanine and use 0.05N NaOH to dissolve phenylalanine, tyrosine and tryptophan.

Extraction of Sample

Grind a known quantity of the sample material (dry/ wet) in a pestle and mortar with 10 – fold volume of 70% ethanol. Shake the contents at 55^{o}C for 30 min. centrifuge the contents at 10,000 rpm for 10 min. collect the supernatant. Repeat the extraction of the pellet at 55^{o}C at least twice. Pool the supernatant (for leaf extracts, treat with equal volume of petroleum ether 40 -60^{o}C) and shake vigorously. Discard the petroleum ether layer containing chlorophyll. Evaporate the alcohol fraction to dryness under vacuum using either a water – pump or rotary evaporator at 40-50oc. Dissolve the residue in a known volume of absolute ethanol or water for analysis.

Ninhydrin Reagent: Dissolve 100 mg Ninhydrin in 100 ml acetone.

Procedure

1. Cut the chromatography sheet carefully to a convenient size (40 X 24cm). Draw a line with pencil across the sheet about 5cm away from one end. Mark a number of points at intervals of 3cm.
2. Apply a small volume (25µl) of each amino acid as a separate small spot using a micro syringe. A stream of hot air from a hair – dryer facilitates fast drying of spot. The spot should be as small as possible for better resolution.
3. Similarly spot different known aliquots of sample extract
4. After spotting, place the sheet in a steel trough in the chromatography chamber, firmly hold it by placing a long steel rod over the sheet. The spot- end of the sheet should be in the trough (decending chromatography). Otherwise, the sheet may be rolled as a cylinder, tied together with fine thread and placed upright with the spots as the bottom in a large petridish for upward movement of solvent (ascending chromatography)
5. Add the organic (phase) solvent to the trough/Petri dish and close the chamber airtight. Develop the chromatogram, preferably overnight or longer, until the solvent moves almost to the other end.

6. Note the solvent front and dry the chromatogram free of solvent in a fume chamber.

7. Spray the chromatogram with the Ninhydrin reagent using automizer. Dry the paper for about 5 min at room temp followed by at 100oc in an oven for 2- 3min.

8. Amino acid appears as purple spots; hydroxyproline and praline give yellow coloured spots. Mark all the spots and calculate their R_f values by the formula

Distance (cm) moved by the solute from the origin/
Distance (cm) moved by the solvent from the origin

The amino acids present in the sample are then identified by comparing the R_f values with that of the authentic amino acids, co- chromatographed.

9. For quantitative estimation, cut each spot into several small bits and transfer to the bottom of the test tubes. Add 3ml of elution mixture. Shake the tubes vigorously for 15 min. Decant the liquid and elute the pieces with another 2ml of elution mixture. Repeat the elution with small aliquots until the bits are colourless. Combine and clear the elute by centrifugation at 10,000 rpm for 10min. Read the intensity of purple color at 570nm in a colorimeter. Use the spot of leucine (50µg) run as standard for comparison.

Result: The number of amino acids in the given mixture and their R_f values were found out. On the basis of the R_f values, the presence of amino acids in the given mixture were also identified. The identified amino acids and their R_f values are as given below herein:

Amino acids	**R_f value**

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5. Estimation of blood glucose by glucose oxidation method.

Aim: To estimate blood glucose quantitatively by using dinitro salicylic acid.

Principle:

3,5-Dinitrosalicylic acid (DNS or 2-hydroxy-3,5-dinitrobenzoic acid) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which strongly absorbs light at 540 nm. It is mainly used in assay of alpha-amylase. However, enzymatic methods are usually preferred due to DNS lack of specificity.

Requirement:

Test tubes,

Pipette

Colorimeter/Spectrophotometer

DNS

Reagent preparation:

Reagent A: 5% DNS in 2M NaOH (100 ml)

Reagent B: 60% Sodium Potassium Tartarate (300 ml)

Mix 100 ml of 'A' and 250 ml of 'B' and make up to 500 ml with distilled water.

Procedure:

Prepare standard glucose solution by dissolving 1 g in 100 ml of distilled water (10 mg/ml).

Aliquot glucose concentration ranging from 2 to 10 mg/ml (0.2, 0.4, 0.6, 0.8, 1.0 ml).

Add 2 ml of the DNS reagent to the aliquots and keep the mixture at 60 ^{o}C for 10 minutes.

Cooling down the reaction mixture at room temperature and measure the absorbance at 540 nm.

Plot a standard graph for absorbance vs. glucose concentration and using the plot, amount of glucose present in the given test sample can be calculated.

Result: The amount of glucose present in the given test sample was found to be mg/ml.

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6. Studying enzymatic activity of amylase under optimum conditions

Aim: To examine enzymatic activity of amylase at optimum pH and temperature

Principle

Amylase is the hydrolytic enzyme which breakdown many polysaccharide like starch, dextrins and yields a disaccharide (maltose)

Reagent requirement

Substrate (starch)

Mix 1/2/5/10/20 g of soluble starch in 100 ml of 0.1M of phosphate buffer (pH 6.8), boil for 3 min and cool down at room temperature.

Enzyme

Collect few ml of saliva in a breaker and dilute it 1:20 in distilled water

(200 mg amylase in 100 ml phosphate buffer – pH 6.8)

1% of sodium chloride - It is necessary for enzyme activity

DNS (Dinitro salicyclic acid)

Dissolve 1.6 g of NaOH in 20 ml of distilled water

Take 1 gm of DNS in the NaOH solution .In other beaker take 30 g of Sodium potassium phosphate and dissolve in 50 ml of distilled water.

Make up the volume to 100 ml (if necessary the solution may be boiled)

Standard solution of maltose: dissolve 200 mg maltose in 100 ml (2mg/ml)

PROCEDURE:

Take 0.5 ml of substance and 0.2 ml of 1% NaCl in a test tube

Incubate at 37 C for 1 min

Add 0.3 ml of diluted saliva/enzyme stock solution and incubate for 15 min at 37 C

Stop the reaction by addition of 1 ml of DNS reagent.

Mix well and keep the test tube in boiling water bath for 10 min

Cool down at room temperature and record the absorbance at 520 nm

RESULT

The amount of maltose present in the given test samples are

The amount of maltose produced from the starch by amylase enzyme under the defined experimental condition is mg/ml

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7. Effect of pH and temperature on the activity of salivary amylase enzyme

AIM

To study the effect of pH and temperature on the enzymatic activity of salivary amylase

PRINCIPLE

Optimal activity for most of the enzyme is generally observed between PH 5.0 and 9.0.However a few enzymes eg,.pepsin are active at PH,values well outside this range . Above and below this range, the reaction rate reduces as the enzymes get denatured.

REQUIREMENT

GLASSWARES:

Test tubes ,beakers ,dropper ,funnel

CHEMICALS:

NaCl,

Na_2HPO_4 and KH_2PO_4,

Iodine Crystals,

Starch

DNS, Sodium Potassium tartrate

Buffer solution of PH 4, 6.8 and 9

EQUIPMENTS:

Water bath or oven, Thermometer

MISCELLANEOUS:

Cotton, Rubber, Distilled water

A) PROCEDURE FOR pH EFFECT ON SALIVARY AMYLASE ENZYME:

1. Buffer solution of pH 4, 6.8 and 9 can be prepared by dissolving buffer tablets in 50 ml of distilled water.

2. Prepare three sets of indicator test tube (8 to 10) in each set in three separate test tube stands. Label test tube stand as A (for 6.8 pH), B (for 4 pH) and C (for 9 pH). In each test tube, take 0.5 ml of iodine solution.

3. In a test tube take 5 ml of 1% starch solution; 1ml of 1% NaCl solution; and 1 ml of PH 6.8 Buffer solution, mark it as control tube or A.

In a second test tube, take 5 ml of 1% starch solution; 1 ml of 1% Nacl solution; and 1 ml of PH 4 buffer solution. Mark it as experimental tube B.

In a third test tube, take 5 ml of 1% starch solution; 1 ml of 1% NaCl solution and 1 ml of 9 PH buffer solution and mark it as experimental tube C.

4. Transfer 1 ml of dilute saliva/enzyme stock solution into each test tube and mix the them thoroughly and place all three test tubes in water bath set at 37 C.

5. Take a drop of dropper and add to the corresponding indicator tubes containing iodine solution

6. Note the color change.

B) PROCEDURE FOR TEMPERATURE EFFECT ON SALIVARY AMYLASE ENZYME:

1) Buffer solutions of PH 4, 6.8 and 9 can be prepared by dissolving buffer tablets in 50 ml of distilled water

2) Prepare three sets of indicator test tubes (8 to 10 in each set) in three separate test tube stands. Label test tube stands as A1 (for 6.8 pH), B1 (for 4 pH) and C1 (for 9 pH) and add 0.5 ml of iodine solution in each test tube (step 1).

3) Duplicate step1 and label test tube into A2 (for 6.8 pH), B2 (for 4 pH) and C2 (for 9 pH)

4) Prepare experimental test tube take 5 ml of 1% starch solution; 1 ml of NaCl solution and 1 ml of pH 6.8 buffer solution and mark it as A2.

In a second test tube, take 5 ml of 1% starch solution; 1ml of 1% NaCl solution and 1ml of pH 4 buffer solution and mark it as experimental tube B2.

In a third test tube, take 5 ml 1% starch solution; 1ml of 1% NaCl solution and 1 ml of pH 9 buffer solution and mark it as experimental tube C2 (step2).

5) Transfer 1 ml of dilute saliva into each test tube and mix them thoroughly and place the experimental test tubes (A2, B2 & C2) in water bath at 37 C.

7) Take a drop down from each of the experimental tubes with the help of dropper and add to the corresponding indicator tubes containing iodine solution.

8) Note the color change.

RESULT:

A) Color of the iodine changed yellow to blue in tubes B (for 4 pH) and C (for 9 pH). No color change found in test A (for 6.8 pH)

B) Color of the iodine changed yellow to blue in tubes B1 and C1 tubes. No color change found in tube A1 implying temperature play a crucial role on influencing the activity of salivary enzyme in breaking down the starch.

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8. Determination of pH optima, temperature optima, K_M value, V_{max} value and Effect of inhibitor on the enzyme activity

Aim: To examine effect of inhibitors on the activities of amylase and as well to determine K_M value for the enzyme at optimum pH and temperature.

Principle:

Enzymes are the catalysts of biological systems and are extremely efficient and specific as catalysts . In fact, typically an enzyme accelerates the rate of a reaction by factors of at least a million compared to the rate of the same reaction in the absence of the enzyme. Most biological reactions catalyzed by the enzyme in the hydration of CO_2.The catalyst in this reaction is carbonic anhydrase .This reaction is part of the respiration cycle which expels CO_2 from the body. Carbonic anhydrase is a highly efficient enzyme and the enzyme molecule can catalyze the hydration of 10 CO_2 molecule per second

pH:

Each enzyme works within quite a small pH range. There is a pH at which its activity is greatest (optimal pH). This is because changes in pH can make and break intra and intermolecular bonds changing the shape of the enzyme and therefore its effectiveness.

Temperature:

As the temperature rises reacting molecules have more and more kinetic energy. This increase the chances of a successful collision and so the rate increases. There is a certain temperature at which an enzyme catalytic activity is greatest. This optimal temperature is usually around human body temperature (37 C) for the enzymes in human cells.

Above the temperature the enzyme structure begin to break down (denature), since at higher temperature intra and intermolecular bonds are broken as the enzyme molecules gain even more kinetic energy.

Result: