KARPAGAM ACADEMY OF HIGHER EDUCATION
(*Deemed to be University Established Under Section 3 of UGC Act 1956*)
Pollachi Main Road, Eachanari Post, Coimbatore – 641 021. INDIA
Phone: 0422-6471113-5, 6453777; Fax No: 0422-2980022-3
Email: info@karpagam.com; Web: www.kahedu.edu.in

19MBU113 BIOCHEMISTRY PRACTICAL -I (4H –2C)

Semester – I
Instruction Hours / week: L: 0 T: 0P:4 Marks: Internal: 40 External: 60 Total:100
End Semester Exam: 3 Hours

COURSE OBJECTIVE

To familiarize the students with the basic cellular processes at molecular level

- **To make students familiar with practical techniques used for studying biochemical structure and analysis of biochemical methods.**

COURSE OUTCOME

1. **Students will get practical knowledge about various techniques used in Biochemistry.**

EXPERIMENTS

1. **Properties of water, concept of pH and buffers, preparation of buffers and numerical problems to explain the concepts.**
2. **Numerical problems on calculations of standard free energy change and equilibrium constant.**
3. **Standard free energy change of coupled reactions.**
4. **Qualitative/Quantitative tests for carbohydrates, reducingsugars ,and non-reducing sugars.**
5. **Qualitative/Quantitative tests for lipids and proteins.**
6. **Study of protein secondary and tertiary structures with the help of models.**
7. **Study of enzyme kinetics–calculation of Vmax, Km, Kcat values.**
8. **Study effect of temperature, pH and heavy metalsonenzyme activity.**
9. **Estimation of any one vitamin.**

SUGGESTED READING

1. **Campbell., M.K.(2012) Biochemistry,7th edition. Published by Cengage Learning.**
2. **Campbell,P.N., and Smith,A.D.(2011) Biochemistry Illustrated, 4th edition. Published by Churchill Livingstone.**
3. **Tymoczko,J.L. ,Berg,J.M., andStryer,L.(2012) Biochemistry: A short course, 2nd edition. W.H.Freeman**
4. **Berg, J.M., Tymoczko, J.L., and Stryer,L. (2011) Biochemistry, W.H. Freeman and Company.**
5. **Nelson, D.L., and Cox, MM..(2008) Lehninger Principles of Biochemistry, 5th Edition.W.H .Freeman and Company.**

6. **Willey, M.J.herwood, L.M.& Woolverton, C.J.(2013) Prescott, Harley and Klein's Microbiology 9th Edition. McGraw Hill**
7. **Voet, D,.and Voet, J.G. (2004) Biochemistry 3rd edition, John Wiley and Sons.**

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Details of the Experiment:

Exp. No.	Name of the Experiment	Support Materials
1.	Properties of water, concept of pH and buffers, preparation of buffer and numerical problems to explain the concepts	S1
2.	Numerical problems on calculations of standard free energy change and equilibrium constant	S1
3.	Standard free energy change of coupled reactions	S1
4.	Qualitative /Quantitative test for carbohydrate, reducing and non reducing sugar	R1: 15-22, 33-36 R2: 33-34
5.	Qualitative/Quantitative test for carbohydrate lipids and proteins	R1: 15-22, 33-36 R2: 33-34
6.	Study of protein secondary and tertiary structures with the help of models	
7.	Study of enzyme kinetics calculation of Vmax, Km K cat values	
8.	Study the effect of temperature pH and heavy metals on enzyme activity	
9.	Estimation of any one vitamin	

Referenes Book:

S1: David L Nelson and Michael M Cox (2008). Lehninger Principles of Biochemistry (5th Ed) freeman and company

R1: Sadasivam S and Manickam A. (2009). Biochemical methods, New age International Publishers, New Delhi

R2: Singh S.R. (2014) Introductory practical Biochemistry, Narosa publishing house, New Delhi

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DEPARTMENT OF BIOCHEMISTRY

Subject	**:**	**Biochemistry Practical - I**	**Semester**	**:**	**I**
Subject code	**:**	**19MBU103**	**Class**	**:**	**I B.Sc Microbiology**

Experiment No: 1

Properties of water, concept of pH and buffers, preparation of buffers and numerical problems to explain the concepts

Properties of water

Water is a major, unique, ubiquitous substance that is a major component of all living things. Its nature and properties have intrigued philosophers, naturalists and scientists since antiquity. Water continues to engage the attention of scientists today as it remains incompletely understood inspite of the intense study over many years. This is primarily because water is anomalous in many of its physical and chemical properties. Some of water's unique properties are literally essential for life, while others have profound effects on the size and shape of living organisms, how they work, and the physical limits or constraints within they must operate. This was recognized by Lawrence Henderson in 1913 in his classic and still very readable book, *The fitness of the environment*: *An Inquiry into the Biological Significance of the Properties of Matter*. Since then more has been learned about the structure and properties of water at the molecular level, much of its through spectroscopic and thermodynamic experiments. The more recent discipline of computer simulation has also played a role, having achieved a level of sophistication in the study of water in which it can be used to interpret experiment. Many of water's basic physical properties can now be explained, at least semi-quantitatively in molecular and structural terms.

Selected physical properties of water

Formula	Water (H_2O)
Molecular weight (g mol^{-1})	18
Density (kg L^{-1})	0.998
Boiling point (K)	373
Molecular volume (nm^3)	0.0299
Volume of fusion (nm^3)	0.0027
Liquid density maximum (K)	277
Specific heat (Jk^{-1} g^{-1})	4.18
(JK^{-1} mol^{-1})	75.2
Heat of vaporization (kJ g^{-1})	2.3
(kJ mol^{-1})	41.4
Surface tension (mN m^{-1})	72.8
Viscosity (µPa s)	1002
Dielectric constant	78.6
Dipole moment (Cm x 10^{30})[a]	6.01

Biological relevance of water's physical properties

Water, owing to its high boiling point, exists predominantly in its liquid form in the range of environment where life flow flourishes, although the other two phases, ice and vapour, play an essential role in shaping the environment. The high specific heat and heat of vaporization of water have important consequences for organisms at the cellular and physiological level, in particular for the efficiency of processes involving heat transfer temperature regulation, cooling etc. Viscosity is the major parameter of water that determines how fast molecules and ions can be transported and have rapidly they diffuse in aqueous solution. It thus, provides a physical upper limit to the rates of many molecular level events, within which organisms must live and evolve. These include the rates of ion channel conductance, association of substrates with enzymes, binding rates, and rates of macromolecular assembly.

Dielectric constant

The dielectric constant is a measure of how easily a material is polarized by an electric field relative to vacuum. It is defined by the magnitude of the dielectric polarization induced by a unit field. Water has nearly 80 times the dielectric constant of vacuum and it is an order of magnitude more polarizable than most organic solvents.

Ionization

Because the O-H bond of water is strongly polarized, the electron density around the hydrogen atom is very low and the O-H bond is rather weak compared with most covalent bonds. Thermal fluctuation in the liquid often (every 20 μs or so) result in sufficient further polarization of the O-H bond that the hydrogen nucleus can dissociate as a proton, or H^+ ion. Water being an excellent solvent for ions, it can solvate the resulting OH^- and H^+ ions, the latter primarily as H_3O^+. As a consequence dissociated water has a relatively long diffusion lifetime of about 100 μs in pure water before recombination. The spontaneous ionization of water is characterized by a dissociation constant, derived using equation.

$$\frac{[H^+][OH^-]}{[H_2O]} = 1.82 \times 10^{-16} \text{ mol L}^{-1}$$

Hydrogen bonding

Hydrogen bonding between water molecules explains many of its physical and chemical properties. The strong hydrogen bonding in water explains why water has such a high melting and boiling point, high latent heat condensation and crystallization and low vapor pressure.

Structure models

Several models for the structure of water have been proposed each attempting too much either experimental evidence from spectroscopy or modeling results.

Ice I

- Tetrahedral coordinated regular structure requiring 6 units to return to the origin.
- O·····O distances are equal at 2.76 Å.
- The protons are exactly in the O·····O plane.

Flickering cluster model

Flickering clusters are short-lived groups of water molecules that are interlinked by hydrogen bonds in liquid water. These clusters are representative of the fact that hydrogen bonds are easily broken and reformed.

Broken ice model

After ice melts, basic structure is retained and each other is bonded to 4 others.

Network structure

Most probable structure for cold water.

Concept of pH

pH

The negative logarithm of the hydrogen ion concentration, the pH is expressed on follows:

$$pH = -\log [H^+]$$

The pH scale is a measure of hydrogen ion concentration that eliminates dealing with large powers of 10 and compresses a large range of concentrations onto a more convenient scale, for aqueous solution between 1 and 14. At a high concentration of H^+, (10^{-1} M), the pH value is low, pH =1 while at low concentration (10^{-12} M), the pH is high= 12. Hydrogen ions in solution arise from the dissociation of acids. (HA represents undissociated acid, H^+ hydronium ion and A^- conjugate base).

$$HA \rightarrow H^+ + A^-$$

Strong acids (or bases) are considered to be completely dissociated into ions in dilute solution. However, weak acids (or bases) are only partially dissociated in solution, and thus equilibrium is established between ions and the undissociated molecules.

$$HA \leftrightarrow H^+ + A^-$$

The equilibrium constant K_a, is defined as follows.

$$K_a = [H^+][A^-] / [HA]$$

From this expression, we can observe the Henderson-Hasselbach equation, commonly called the “buffer equation” which relates the pH of solution to the pK_a of the acid and the relative concentration of the undissociated acid and the conjugate base forms. Solving first for the hydrogen ion concentration:

$$[H^+] = K_a\ [HA] / [A^-]$$

Converting to the logarithmic form and multiplying by -1, we obtain:

$$-\log [H^+] = -\log K_a - \log ([HA]/[A^-])$$

Defining operator 'p = - log' we have:

$$pH = pK_a - \log ([HA]/[A^-])$$

Finally, by inverting the log ([HA]/[A^-]) term, we obtain:

$$pH = pK_a + \log([A^-] / [HA])$$

or

$$pH = pK_a + \log([\text{conjugate base}] / [\text{acid}])$$

This is the Henderson-Hasselbach equation. It is most useful in the preparation of buffer and in understanding how the concentration of the acid and conjugate base forms of a weak acid effect the pH.

Concept of buffer

A buffer, by definition, resists changes in the pH of the solution. A buffer must contain the chemical species for "neutralizing" added amounts of acid or base. Generally, a buffer is a solution of a weak acid and its conjugate base or a weak base and its conjugate acid. A buffer is selected on the basics of the pK_a value and its chemical nature.

The Handerson-Hasselbach equation gives the relationship between pH, pK_a's and the ratio of the concentration of the salt and acid forms of the buffer. As shown by this equation, when the concentration of the conjugate base and the undissociated acid are equal, [Conjugate base] = [acid], the pH of the solution equals the pK_a of the buffer. When [conjugate base] = 10x[acid], then log ([conjugate base]/[acid]) = log 10 = 1 and therefore:

$$pH = pK_a + 1$$

When [conjugate base] = 1/10x[acid], then log ([conjugate base]/[acid]) = log 1/10 =-1 and therefore:

$$pH = pK_a - 1$$

The buffers are most effective in the range pH = pk_a ± 1. Outside that range, the concentration of either the acid of the conjugate base is too small to effectively resist the effect of added hydrogen or hydroxide ion.

Once the desired pH range for an experiment has been decided, one can select a buffer on a basic of pK value. Since temperature affects the dissociation of some weak acids and bases the pK value for buffers is temperature dependent. Therefore, pH of buffers should always be adjusted for the temperature at which they will be used.

Experiment No. 2

Numerical problems on calculations of standard free energy change and equilibrium constant

Calculate the standard free energy changes of the following metabolically important enzyme-catalyzed reactions at 25°C and pH 7.0 from the equilibrium constants given.

(a) Glutamate + oxaloacetate ↔ asparate + α-ketoglutarate K'eq = 6.8
(b) Dihyroxyacetone phosphate ↔ glyceraldehydes-3-phosphate K'eq = 0.00475
(c) Fructose-6-phosphate + ATP ↔ F-1,6-biphosphate + ADP K'eq = 254

$\Delta G^{\circ\prime} = -RT \ln K'eq$ R=8.315 J/mol°K T= 298°K

a. -8.315 (298) (In 6.8)
-4.757 J/mol = -4.76 kJ/mol

b. -8.315 (298) (In0.0475)
7.5kJ/mol

c. -8.315 (298) (In 254)
-13.7 kJ/mol

Calculate the equilibrium constants K'eq for each of the following reactions at pH 7.0 and 25°C using the $\Delta G^{\circ\prime}$ values given:

(a). Glucose-6-phosphate + H_2O ⇢ glucose + P_I $\Delta G^{\circ\prime} = -13.8$ kJ/mol
(b). Lactose + H2O ⇢ Glucose + Galactose $\Delta G^{\circ\prime} = -15.9$ kJ/mol
(c). Malate ⇢ fumarate + H_2O $\Delta G^{\circ\prime} = +3.1$ kJ/mol

(a). -13,800 = -8.315 (298) In K'eq
K'eq = 262

(b) -15,900 = -8.315(298) In K'eq
K'eq = 612

(c) +3,100 = -8.315 (298) In K'eq
K'eq = 0.286

Experiment No. 3

Standard free energy change of coupled reactions

This problem explores the consequences of coupling ATP hydrolysis under physiological conditions to a thermodynamically unfavorable biochemical reaction. Because we want to explore these consequences in stages, we shall consider the hypothetical transformation, X ⇢ Y, a reaction for which ΔG°' = 20 kJ/mol.

(a) what is the ration [Y]/[X] at equilibrium?

(b) Suppose X and Y participate in a sequence of reactions during which ATP is hydrolyzed to ADP and Pi. The overall reaction is

$X + ATP + H_2O \dashrightarrow Y + ADP + Pi$

Calculate [Y]/[X] for this reaction at equilibrium. Assume for the purposes of this calculation that the concentrations of ATP, ADP and Pi are all 1 M when the reaction is at equilibrium.

(c) We know that [ATP], [ADP], and [Pi] are not 1 M under physiological conditions. Calculate theration [Y]/[X] for the ATP-coupled reaction when the values of [ATP], [ADP], and [Pi] are 8.05 mM, 0.93 mM, and 8.05 mM, respectively.

a. 20,000 = -8.315 (298) in Keq

Keq = $3.2\ 10^{-4}$ M

b. $ATP + H_2O \dashrightarrow ADP + Pi$ ΔG°' = -30.5 kJ/mol when coupled to the reaction gives

ΔG°' = -10.5 kJ/mol

-10.500 = -8.315 (298) in K

K = 69.2

c. $Keq = \frac{[ADP]\ [Pi]\ [Y]}{[ATP]\ [X]}$ $69.2 = \frac{[0.93\ 10^{-3}]\ [8.05\ 10^{-3}]\ [Y]}{[8.05\ 10^{-3}]\ [X]}$

$\frac{[Y]}{[X]} = 7.44\ 10^{4}$

Experiment No. 4

Qualitative/Quantitative tests for carbohydrates, reducing and non-reducing sugars

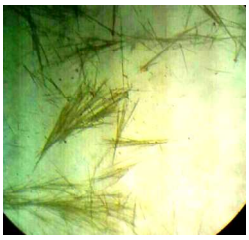
S. NO	EXPERIMENT	OBSERVATION	INFERENCE	REACTION
1	**Solubility Test** To a little amount of the given substance in a test tube added few drops of distilled water and shook well.	(a) Soluble (b) Insoluble	It indicates the presence of monosaccharides and disaccharides. It indicates the presence of polysaccharides.	
2	**Molisch's Test** To 2.0ml of the sugar solution added 2-3 drops of Molisch's reagent, mixed well and added 2.0ml of Conc. H_2SO_4 along the sides of the test tube. So that the acid forms a layer beneath the test solution.	(a) A deep violet coloured ring was got at the junction of the two layers. (b) No deep violet coloured ring was got at the junction of the two layers.	It indicates the presence of carbohydrates. It indicates the absence of carbohydrates.	Sugar is dehydrated to hydroxyl methyl furfural by concentrated sulphuric acid. The hydroxyl methyl furfurol then condenses with α - naphthol to give a violet coloured ring shows the presence of carbohydrates.
3.	**Iodine test** To 1.0ml of the test solution added 4-5 drops of iodine solution and contents are mixed gently.	(a) Blue coloured solution was got. (b) No change in colour	Presence of polysaccharide. Absence of polysaccharide	Iodine forms coloured adsorption complexes with polysaccharides.
4.	**Fehling's test** To 2.0ml of Fehling's reagent added 0.5ml of the given sugar solution mixed and heated in a boiling water bath for two minutes.	(a) A reddish brown precipitate is formed. (b) No characteristic precipitate was got.	It indicates the presence of reducing sugar. It indicates the absence of reducing sugar	Fehling's solution contains blue alkaline cupric hydroxide solution, heated with reducing sugars gets reduced to yellow or red cuprous oxide and is precipitated.

5.	**Benedict's test** To 2.0ml of Benedict's reagent, added 8 drops of the test solution and heated in a boiling water bath for 2 minutes and allowed to cool. spontaneously.	(a) The solution first turned to green and then reddish brown precipitate was formed. (b) No characteristic reddish brown precipitate was formed.	It indicates the presence of reducing sugar It indicates the absence of reducing sugar	free aldehyde or keto group in the reducing sugars reduce cupric hydroxide in alkaline medium to red colored cuprous oxide. Depending on the concentration of sugars, yellow to green color is developed
6.	**Picric acid test** To 2.0ml of sugar solution added 0.5ml of saturated picric acid and 0.5ml of 10% sodium carbonate and heated in a water bath.	(a) A mahagony red coloured solution was got. (b) No characteristic red coloured solution was got.	It confirms the presence of reducing sugar. It shows the absence of reducing sugar and confirms the presence of sucrose which is a non reducing sugar	It is due to the reducing action of the sugar.
7.	**Barfoed's test** To 2.0ml of freshly prepared Barfoed's reagent added 1.0ml of sugar solution and heated in a boiling water bath for 3 minutes. Allow to cool.	(a) A brick red precipitate is formed and settling down at the bottom or sides of the test tube. (b) No characteristic red precipitate was got.	It indicates the presence of monosaccharides. It shows the absence of monosaccharides.	Barfoed's test is used to detect the presence of monosaccharide (reducing) sugars in solution. Barfoed's reagent, a mixture of ethanoic (acetic) acid and copper(II) acetate, is combined with the test solution and boiled. A red copper(II) oxide precipitate is formed will indicates the presence of reducing sugar. This test is specific for monosaccharides
8.	**Seliwanoff's test** To 3.0ml of Seliwanoff's reagent added 4 drops of	(a) A cherry red colour was got.	It indicates the presence of keto sugar sucrose and	It indicates the presence of keto sugar sucrose and

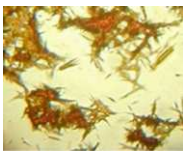
	sugar solution and heated in a water bath for 3 minutes and cooled the solution at room temperature.	(b) No characteristic cherry red colour was got.	fructose. It indicates the absence of keto sugar sucrose and fructose.	fructose which on treatment with HCl acid forms 5 hydroxy methyl furfural which then condences with resorcinol to give cherry red coloured complex.
9.	**Tollen's test** To equal volume of sugar solution and concentrated hydrochloric acid added a pinch of phloroglucinol and heated in a boiling water bath for 2 minutes.	(a) A red coloured solution was got. (b) No characteristic red coloured solution was got.	It confirms the presence of pentose, lactose and galactose. It shows the absence of pentose, lactose and galactose.	
10.	**Bial's test** To 2.0ml of Bial's reagent added 0.5ml of the test solution and heated in a boiling water bath for 3 minutes.	(a) A greenish blue coloured solution was got. (b) No characteristic greenish blue coloured solution was got.	It indicates the presence of pentoses. It indicates the presence of hexoses.	Bial's test is used to distinguish between pentoses and hexoses. They react with Bial's reagent and are converted to furfural. Orcinol and furfural condense in the presence of ferric ion to form a colored product. Appearance of green colour or precipitate indicates the presence of pentoses and formation of muddy brown precipitate shows the presence of hexoses.
11.	**Mucic acid test** To 50mg of sugar in a test tube added 1.0ml of concentrated nitric acid and	(a) A white precipitate was got and a colourless rod shaped crystals were	It shows the presence of lactose or galactose.	

	1.0ml of distilled water, heated in a boiling water bath for one and a half an hour and left it stand overnight and viewed the crystals under the microscope.	seen under the microscope. (b) No characteristic white precipitate was formed.	It shows the absence of lactose or galactose.	
12.	**Phenyl hydrazine Test** Took equal volume of sugar solution and phenyl hydrazine mixed, filtered and kept in a boiling water bath for half an hour and cooled at room temperature viewed the crystals under the microscope.	Formation of beautiful yellow crystals of osazone was formed (a) A yellow **needle** shaped crystals like sheeves of corn was observed. (b) A **flower** shaped crystals were observed (c) A **cotton ball or puff** shaped crystals were observed. (d) A small **sunflower** shaped crystals were observed. (e) A **rod** shaped crystals were observed.	It indicates the presence of **glucosazone and fructosazone** It indicates the presence of **galactosazone** It indicates the presence of **lactosazone** It indicates the presence of **maltosazone** It indicates the presence of **xylosazone**	The ketoses and aldoses react with phenylhydrazine to produce a phenylhydrazone which further reacts with another two molecules of phenylhydrazine to yield osazone.

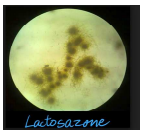
Shapes of different crystals



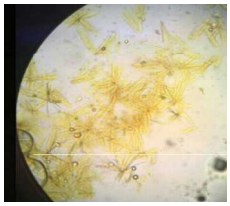
Glucosazone and Fructosazone



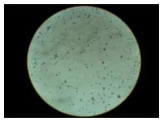
Galactosazone



Lactosazone



Maltosazone



Xylosazone

Experiment No. 5

Qualitative/Quantitative tests for lipids and proteins

S. NO	EXPERIMENT	OBSERVATION	INFERENCE
1	**Solubility Test:** a. Cold water b. Hot water c. C. Dilute potassium hydroxide d. Dilute ammonium hydroxide e. Dilute hydrochloric acid f. Alcohol	 a. Insoluble b. Soluble Soluble Soluble Soluble Soluble a. Soluble **b.** Insoluble	 The amino acid may be tyrosine The amino acid may be Arginine, Methionine, Cysteine, Tryptophan or Histidine. The amino acid may be Arginine, Methionine, Cysteine, Tryptophan, Tyrosine or Histidine. The amino acid may be Tryptophan or Histidine. The amino acid may be Arginine, Methionine, Cysteine or Tyrosine
2	**Ninhydrin Test:** To 5ml of amino acid solution taken in a test tube, add 0.5ml of 1% ninhydrin reagent. Place the test tube in a boiling water bath for 1-2 minutes and cool to room temperature.	**A violet colouration was got.**	This is due to the formation of Rheumann's purple which indicates the presence of amino acid.
3	**Pauly's diazo Test:** To 2ml of amino acid solution add 1ml of 1% sulphanilic acid in 10% hydro chloric acid and then cool in ice. Add 1ml of 5% sodium nitrite solution and leave in cold water for 3 minutes. Make the solution alkaline by the addition of 2ml of 1% sodium carbonate solution and note the colour formed.	a. A red colouration was got. b. No characteristic red colouration	The amino acid with diazotized sulphanilic acid in alkaline medium forms the highly coloured azo compound. This indicates the presence of aromatic amino acid tyrosine, tryptophan or histidine. It shows the absence of aromatic amino acids

		was got.	tyrosine, tryptophan or histidine.
4	**Acetic Acid Test:** To 2ml of amino acid solution add 1% of bromine in 33% acetic acid until an yellow colour was got. After 10 minutes add 5 to 10 drops of 5% ammonium carbonate solution.	a. A dark blue or violet coloured solution was got. b. No characteristic blue coloured solution was got.	This confirms the presence of Histidine. This shows the absence of histidine.
5	**Hopkin's Cole Test:** To 1ml of the amino acid solution add 2ml of glacial acetic acid (exposed to sun light) and add 2ml of concentrated sulphuric acid along the sides of the test tube.	a. A violet coloured ring was got at the junction of the 2 liquids. b. No characteristic violet coloured ring was got.	It is due to the condensation of aldehyde group with indole group in the presence of concentrated sulphuric acid. This test confirms the presence of tryptophan. It shows the absence of tryptophan.
6	**Voisent Rhode Test:** Add 1ml of amino acid solution to 1ml of 5% para dimethyl amino benzaldehyde in concentrated sulphuric acid.	a. A red violet coloured ring was got. b. No characteristic red violet coloured ring was got.	It is due to the condensation of aldehyde group with indole group in the presence of concentrated sulphuric acid. This test confirms the presence of tryptophan. It shows the absence of tryptophan.
7	**Aldehyde Test:** To 1ml of amino acid solution add one drop of 1 in 500 formalin and 1 drop of 15% mercuric sulphate in 10% sulphuric acid and add 3-4 drops of concentrated sulphuric acid.	a. A violet coloured ring was got at the junction of two liquids. b. No characteristic violet coloured ring was got.	It is due to the condensation of aldehyde group with an indole group in presence of concentrated sulphuric acid. This test confirms the presence of tryptophan. It shows the absence of tryptophan.
8	**Ehrlich's Test:** Add 2ml of Ehrlich's reagent to 2ml of the amino acid solution.	a. A red coloured solution was got.	It is due to the condensation of aldehyde group with indole ring in the presence of hydrochloric acid. It confirms the presence of tryptophan.

		b. No characteristic red coloured solution was got.	It shows the absence of tryptophan.
9	**Hoffmann's Test (or) Millon's Test:** To 1ml of amino acid solution add 1 drop of Millon's reagent and place it in a water bath for 30 seconds.	a. A deep red colouration was got. b. No red colouration was got.	It is due to the presence of phenolic hydroxyl group. This test confirms the presence of tyrosine. It shows the absence of tyrosine.
10	**Folin's Phenol Test:** To 1ml of amino acid add an equal volume of Folin's phenol reagent and treat with 6 drops of saturated sodium carbonate.	a. A blue colouration was got. b. No characteristic blue colouration was got.	It is due to the presence of phenolic hydroxyl group. This test confirms the presence of tyrosine. It shows the absence of tyrosine.
11	**Xanthoproteic acid Test:** To 2ml of amino acid solution add an equal volume of concentrated nitric acid cool and then sufficient amount of 40% sodium hydroxide to make the solution strongly alkaline..	a. An yellow colour was got in acid solution which turned to bright orange with alkaline solution. b. No orange colour was got.	It is due to nitration of amino acid to form yellow nitro derivative. This confirms the presence of tyrosine which has the phenolic group. It shows the absence of tyrosine.
12	**Ferric Chloride Test:** To the amino acid solution add one drop of dilute (0.05%) ferric chloride solution and a drop of 0.5% copper sulphate solution.	a. A transitory violet colour appeared on the addition of copper sulphate. b. No characteristic violet colour was got.	It is due to the presence of thiol group. This test confirms the presence of cysteine. It shows the absence of cysteine.
13	**Nitroprusside Test:** Treat2ml of amino acid solution with 0.5ml of 2% dilute sodium nitroprusside solution and add a drop of dilute solution of sodium hydroxide(1%).	a. A red coloured solution was got which faded away after 2 minutes.	It is due to the presence of thiol group. This indicates the presence of cysteine.
14	**Lead Acetate Test:** To 2ml of the amino acid solution add 1ml of 10% aqueous lead acetate and	a. A black precipitate was got.	It is due to the presence of thiol group which indicates the presence of cysteine.

	1ml of 40% sodium hydroxide. Heat in a water bath for 5 minutes.	b. No characteristic black precipitate was got.	It shows the absence of cysteine
15	**Boiling's Modification (or) Mc Carthy Sullivan Test:** To 1ml of the amino acid solution, add the following reagents in order and mix after each addition. Add 1.5ml of 0.5N sodium hydroxide, 1.5ml of 1% glycine, 0.3ml of 10% sodium nitro prusside solution and place it in a incubator at 37-40°C for 15 minutes and then added hydrochloric acid and let it stand at room temperature for 10 minutes.	A red colouration was got.	The methyl group is split to form homocysteine which gives a red colour with sodium nitroprusside solution. It confirms the presence of methionine.

Lipids

S .No	EXPERIMENT	OBSERVATION	INFERENCE
1.	**Greese spot test:** Take a small amount of oil on a piece of paper	A greesy spot penetrating the paper will be formed. No greesy spot was formed	Indicates the presence of lipids Absence of lipids
2.	**Test for free fatty acids:** Take a few drops of phenolphthalein solution in a test tube and in it one to two drops of very dilute alkaline solution just sufficient to give the solution a pink colour. Now add a few drops of oil and shake	Disappearance of colour No disappearance of colour	Presence of fatty acids since the alkali is neutralized Absence of fatty acids
3.	**Emulsification:** Take a clean dry test tube add 2 ml of dilute bile salt solution and few drops of unknown solution is added and shaken vigorously for 1 minutes.	Minutes droplets suspended in the liquid No droplets suspended in the luquid	Presence of oil or lipid Absence of oil or lipid
4.	**Saponification test:** Take 1 ml of unknown solution in a test	Soap formation	Indicates the presence of fatty

	tube and add an equal amount of alcoholic KOH solution, mix them thoroughly and keep the mixture during the colour of warming and shake up gently with a little distilled water	No soap formation	acids Absence of fatty acids
5.	**Test for unsaturated fatty acids:** A clean test tube containing 5 ml of CCl_4 and add a drop of lipid or oil, now bromine water is added drop by drop and number of drops added is recorded	Appearance of permanent yellowish red colour No characteristic colour formation	Indicates the presence of unsaturated fatty acids. Absence of unsaturated fatty acids
6.	**Acrolein test:** For a few drops of oil or lipid, a few crystals of potassium hydrogen sulphate warm gently ta mix and then heated strongly	A pungent colour of acrolein is produced No pungent colour is produced	Indicates the presence of glycerol Absence of glycerol
7.	**Dichromate test:** Take a dry test tube, 3 or 4 ml of oil or lipid, a few drops of 5% potassium dichromate solution and 5 ml of conc. H_2SO_4 mix well	Brown colour is changed to blue No colour change	Indicates the presence of glycerol Absence of glycerol

Experiment No. 6

Study the protein secondary and tertiary structures with the help of models

Aim:

To study the secondary and tertiary structures of proteins with the help of models.

3D structures are proteins sources of information:

- Shape and domain structures.
- Protein classification.
- Prediction of function for uncharacterized proteins.
- Interactions with other macromolecules.
- Interaction with small ligands, metal ions, nucleotides, substrates, cofactors and inhibitors.
- Evidence for enzyme mechanism.
- Structure based drug development.
- Post translational modification: disulfide bonds, N-glycosylations.
- Experimental evidence for transmembrane domains.

Basics of protein structures

- Primary structure
- Secondary structure
- Tertiary structure
- Quaternary structure

Secondary Structure

a) Helices

b) Strands

c) Turns and loops

Alpha-helix

Characteristics

Helical structures have negative phi and psi angles in typical values being -60° and -50°. Every main chain C=O and N-H group is hydrogen bonded to a peptide bond 4

residues away (i.e O to N, +4). This gives a very regular stable arrangement 3.6 residues per turn 5.4 Å repeat along the helix axis. Each residue corresponds to a rise of ca 1.5 Å.

Beta structure (strands):

Characteristics: Positive psi angles typically ca 130° and negative phi values typically ca -140°. No hydrogen bonds amongst backbone atoms from the same strand.

Beta strand can form parallel or anti-parallel beta sheets:

Characteristics: Stabilized by hydrogen bonds between backbone atoms from adjacent chains. The axial distance between adjacent residues is 3.5 Å. These are two residues per repeats unit which gives the beta strand a 7 Å pitch.

Turns and Loops:

Loops:

General name for a motile part of the polypeptide with no fined secondary structure.

Turns:

Several types, defined structure, requirement for specific or α a at key position meaning they can be predicted. The polypeptide chains it makes a u-turn over 2-5 residues.

Super secondary structures:

Compounds of 2-3 secondary structure elements.

Example: Helix turn helix motils, frequent in DNA binding proteins, coiled coils. Eg. From myosin.

Folding pattern of polypeptide chain:

- The folding pattern of a polypeptide chain can be described in turns of the angles of rotation around the main chain bonds.
- Phi and psi describe the main chain conformation omega corresponds to the trans (omega-180) or cis (omega = 0) conformation.
- Except pro, trans is the more stable conformation.

Key facts about a polypeptide chain:

- Chemical bonds have characteristic lengths.
- The peptide bond has partial double bond character, meaning it is shorter and rigid.
- Other bonds are single bonds (but restriction of rotation due to steric hindrance).

Ramachandran plot (1):

Each type of secondary structure has a characteristic combination of phi and psi angles.

Ramachandran plot (2):

For each positive conformation, the structure is examined for close contacts between atoms. Atoms are treated as hard spheres with dimensions corresponding to their van der Waals radii angles, which causes spheres to collides corresponds to sterically disallowed conformation of the polypeptide backbone.

Tertiary structures:

- Domains
- Repeats
- Zinc fingers

Domain:

Independently folded part of a protein, arrange size, about 150 α a residues lower limit (α 50 residues).

Repeats:

- Several types: LRR, ANK, Heat ……. Compose of view
- Secondary structure elements, sterilized by interactions between repeatesion form large structures.

Zinc fingers:

- Several types of structure is sterilized by bound zinc ion.

E7 hands:

- Structure is sterilized by bound calcium.

Experiment No. 7

Study of enzyme kinetics – calculation of Vmax, Km, Kcat values

Catalase

Catalase represents a special type of peroxidase which has a specific property of very rapidly catalyzing the decomposition of hydrogen peroxide.

$$2H_2O_2 \longleftrightarrow 2H_2O+O_2$$

It may be considered that one molecule of hydrogen peroxide acts as a substrate and electron donor and the other molecule serves as a electron acceptor or oxidizing agent. One molecule of water is reduced to OH ions giving a red product of two molecules of water and one molecule of oxygen.

Aim:

To prepare the crude enzyme from the given source and to study the effects of varying pH, temperature, enzyme and substrate concentration on the activity of catalase.

Principle:

Sodium perborate activity may serve as a suitable for catalase. Sodium perborate ($NaBO_3.4H_2O$) in aqueous solution is acted acted upon by catalase, the remaining H_2O_2 is determined by titration with $KMno_4$ in the presence of H_2SO_4.

$$2KMno_4+H_2O_2+2H_2SO_4 \longrightarrow 2KHSO_4+2Mno4+8H_2O+3O_2$$

Reagents:

- 1.54% sodium perborate solution:

  Molecular weight of sodium perboric acid is 154. The pH is adjusted to 6.8 with concentrated HCL (1,54g in 100ml of water).
- 2N H_2SO_4
- 0.01N $KMno_4$ prepared by diluting in $KMno_4$ (0.1N $KMno_4$ = 3.16g of $KMno_4$/litre)
- M/15 phosphate buffer
- Solution A: M/15 disodium hydrogen phosphate (11.877g/ litre)
- Solution B: M/15 potassium dihydrogen phosphate (9.072g /litre)

X ml of the solution A was mixed with Y ml of the solution B as shown in the tabular column to prepare phosphate buffer of different pH.

Enzyme preparation:

The cucumber or chow chow was cut into pieces and weighed 10g accurately. It was then homogenized in a mixie by adding 100ml of water and filtered the filtrate was kept in ice then used for the experiment.

Preparation of buffer:

S. No	pH	X ml of NA_2HPO4	Y ml of KH_2PO4
1.	6.0	12.0	88.0
2.	6.4	26.5	73.5
3.	6.8	50.0	50.0
4.	7.2	71.5	28.5
5.	7.6	86.8	13.2

Effect of Substrate Concentration on Catalase Activity

Aim:

To prepare the crude enzyme from the given source and to study the effects of varying substrate concentration on the activity of catalase.

Principle:

Sodium perborate activity may serve as a substrate for catalase. Sodium perborate (NaBo3.4H2O) in aqueous solution is acted upon by catalase, the remaining H2O2 is determined by titration with KMno4 in the presence of sulphuric acid.

$$2KMno_4+7H_2O_2+2H_2SO_4 \longrightarrow 2KHSO_4+2MnO_4+8H_2O+3O_2$$

Reagents:

1. 1.54% sodium perborate (substrate)
2. 2N sulphuric acid
3. 0.01N KMnO4
4. M/15 phosphate buffer
5. Enzyme (source)

Procedure:

Into a series of clean conical flasks pipetted out varying volume of substrate (0.0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0) and made up the total volume of each to 9ml with M/15 phosphate buffer of optimum pH 6.8. Then added 1.0ml of the enzyme to the experimental flask alone and mixed well. Incubated all the tubes at room temperature for 15mts. Stopped the reaction by adding 10.0ml of 2N H2SO4 at the end of incubation period. Controls were also conducted along with the experiments by adding the enzyme at the end of incubation period. The contents of the flask were then titrated against 0.01N KMno4 in the burette. The end point is the appearance of pale pink colour. The difference between the controls and experiments gives the volume of 0.01N KMno4 equivalent to the enzyme activity.

A graph was then drawn by plotting substrate concentration on X axis and volume of 0.01N KMno4 on y axis. From the graph Km value ie, the substrate concentration at half of the maximal velocity (Vmax) is determined. A line weaver burk plot was drawn by plotting 1/v on y axis and 1/[s] on x axis. From which the Km value is determined.

RESULT:

The Km values for the given enzyme catalase at room temperature at pH 6.8 for 15mts by Michaleis menton plot Km= ----------

Line weaver burk plot Km= ----------

Effect of substrate concentration on catalase activity:

S. No	S	1/[S]	V	1/V
1.	0.5	2.0	0.1	10.00
2.	1.0	1.0	0.2	5.00
3.	2.0	0.5	0.3	3.33
4.	3.0	0.33	0.4	2.50
5.	4.0	0.25	0.5	3.33
6.	5.0	0.20	0.6	1.67

Effect of substrate concentration on catalase activity:

S. No	Volume of substrate (ml)	Volume of buffer (ml)	Volume of enzyme (ml)	Volume of $2NH_2SO_4$	Volume of 0.01N KMno4						Difference	
					control			Experiment				
					Initial	Final	Titre value	Initial	Final	Titre value		
1.	0.5	8.5	1.0	10.0								self
2.	1.0	8.0	1.0	10.0								
3.	2.0	7.0	1.0	10.0								
4.	3.0	6.0	1.0	10.0								
5.	4.0	5.0	1.0	10.0								
6.	5.0	4.0	1.0	10.0								

Experiment No. 8

Effect of pH on Catalase Activity

Aim:

To prepare the crude enzyme from the given source and to study the effects of varying pH on the activity of catalase.

Principle:

Sodium perborate activity may serve as a substrate for catalase. Sodium perborate (NaBo3.4H2O) in aquous solution is acted upon by catalase, the remaining H2O2 is determined by titration with KMno4 in the presence of sulphuric acid.

$2KMno_4+7H_2O_2+2H_2SO_4 \longrightarrow 2KHSO_4+2MnO_4+8H_2O+3O_2$

Reagents:

1. 1.54% sodium perborate(substrate)
2. 2N sulphuric acid
3. 0.01N KMnO4
4. M/15 phosphate buffer
5. Enzyme (source)

Procedure:

Into a series of clean conical flask, 5ml of M/15 phosphate buffer of varying pH 6.0, 6.4, 6.8, 7.2, 7.6) was pipette out. Add 4.0ml of the substrate (1.54% sodium perborate) into each of the flask and then 1.0ml of enzyme to the experimental flask alone and mixed well. Incubated all the tubes at room temperature for 15mts. Stopped the reaction by adding 10.0ml of 2N H2SO4 at the end of incubation period. Controls were also conducted along with the experiments by adding the enzyme at the end of incubation period. The contents of the flask were then titrated against 0.01N KMno4 in the burette. The end point between the controls and experiments gives the volume of 0.01N KMno4 equivalent to the enzyme activity.

A graph was then draw by blotting pH variations on X axis and volume of 0.01N KMno4 on y axis, from which the optimum pH is calculated.

RESULT:

The optimum pH for catalase activity was found to be ---------- at room temperature for an incubation period for 15mts.

Effect of pH on catalase activity:

pH	Volume of buffer (ml)	Volume Of substrate (ml)	Volume of enzyme (ml)	Incubate at room temperature for 15mts	Volume of $2NH_2SO_4$	Add enzyme to the controls of each 1ml	Volume of 0.01N $KMnO_4$						Difference	self
							Control			Experiment				
							Initial	Final	Titre value	Initial	Final	Titre value		
6.0	5.0	4.0	1.0		10.0									
6.4	5.0	4.0	1.0		10.0									
6.8	5.0	4.0	1.0		10.0									
7.2	5.0	4.0	1.0		10.0									
7.6	5.0	4.0	1.0		10.0									

Effect of Temperature on Catalase Activity

Aim:

To prepare the crude enzyme from the given source and to study the effects of varying temperature on the activity of catalase.

Principle:

Sodium perborate activity may serve as a substrate for catalase. Sodium perborate (NaBo3.4H2O) in aqueous solution is acted upon by catalase, the remaining H2O2 is determined by titration with KMno4 in the presence of sulphuric acid.

$$2KMno_4+7H_2O_2+2H_2SO_4 \longrightarrow 2KHSO_4+2MnO_4+8H_2O+3O_2$$

Reagents:

1. 1.54% sodium perborate(substrate)
2. 2N sulphuric acid
3. 0.01N KMnO4
4. M/15 phosphate buffer
5. Enzyme (source)

Procedure:

Into a series of clean conical flask, 5ml of M/15 phosphate buffer of optimum pH 6.8was pipette out. Add 4.0ml of the substrate (1.54% sodium perborate) into each of the flask and then 1.0ml of enzyme to the experimental flask alone and mixed well. Incubated at varying temperature (10°, 20°, 28°, 37° and 40°C) for 15mts. Stopped the reaction by adding 10.0ml of 2N H2SO4 at the end of incubation period. The contents of the flask were then titrated against 0.01N KMno4 in the burette. The end point is the appearance of pale pink colour. The difference between the controls and experiments gives the volume of 0.01N KMno4 equivalent to the enzyme activity.

A graph was then drawn by plotting temperature variations on X axis and volume of 0.01N KMno4 on y axis, from which the optimum temperature is calculated.

Result:

The optimum temperature for catalase activity was found to be ---------- for an incubation period for 15mts at the pH 6.8.

Effect of temperature on catalase activity:

S. No	Temp °C	Volume of buffer (ml)	Volume of substrate (ml)	Volume of enzyme (ml)	Incubate at room temperature for 15mts	Volume of $2NH_2SO_4$	Add enzyme to the controls of each 1ml	Volume of 0.01N KMno4						Difference	self
								Control			Experiment				
								Initial	Final	Titre value	Initial	Final	Titre value		
1	10^0	5.0	4.0	1.0		10.0									
2	20^0	5.0	4.0	1.0		10.0									
3	28^0	5.0	4.0	1.0		10.0									
4	37^0	5.0	4.0	1.0		10.0									
5	45^0	5.0	4.0	1.0		10.0									

Experiment No: 9

Estimation of Ascorbic Acid in Citrus Fruits (Vitamin C)

Aim:

To estimate the amount of ascorbic acid present in the given unknown sample solution

Principle:

Ascorbic acid is first dehydrogenated by bromination. The dehydroascorbate then reacts with 2, 4-dinitrophenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophotometrically at 540 nm.

Reagents:

1. 4% Oxalic acid
2. 0.5N Sulphuric acid
3. 10% Thiourea
4. 2% DNPH (2g-2,4 Dinitro phenyl hydrazine in 100ml of 0.5N sulphuric acid filtered and used)
5. 80% Sulphuric acid.
6. Bromine water: 1 – 2 drops in 100ml of cold water.
7. Ascorbic acid: Stock standard 100mg of ascorbic acid in 100ml of 4% oxalic acid in a standard flask.
8. Working standard

Dilute 10ml of stock standard solution with 4% oxalic acid solution after bromination to concentration of working standard in 100mg/100ml.

Sample extraction:

2g of amla sample is taken in 25-50ml of 4% Oxalic acid solution. Centrifuged at 1000 rpm for 2 minutes, filtered and collected the liquid transformed and aliquote of 20ml to a conical flask and added bromine water dropwise with constant shaking. The phenolic hydrogen atoms in the extract turn orange yellow to the extent of bromine. Expel it by blowing in air made up to the known with 4% oxalic acid solution. Similarly converted 10ml of stock ascorbic acid in hydro form by bromination.

Procedure:

Pipetted out 0.2-1ml of ascorbic acid solution corresponding to µg value of 20-100. Similarly pipetted out 0.5ml of brominated sample extract and made up the volume in all tubes to 3ml by adding distilled water and add 1ml of DNPH reagent followed by 1-2 drops of thiourea into each tube. A blank was set as above but with distilled water.

Mix the contents of the tube thoroughly and incubated at 37°C for 3hours. After incubated the tubes were kept in the ice bath and dissolve the orange red colour osazone crystals formed by adding 7ml of 50% Sulphuric acid dropwise while tube in the ice bath. Then the tubes were removed from ice and allowed to stand for 30mts at room temperature and optical density is taken at 540nm.

A standard graph was drawn by taking the concentration of ascorbic acid on x-axis and optical density on y-axis. From the graph the concentration of the ascorbic acid in the unknown and sample was calculated.

Result:

1. a) The amount of ascorbic acid 2gm of amla (sample) = ----------mg of ascorbic acid.

b) The amount of ascorbic acid 2gm of green leaves (sample) = ----------mg of ascorbic acid.The amount of ascorbic acid in 100ml of unknown sample = ---------- mg of ascorbic acid.

Estimation of Ascorbic Acid in Citrus Fruits (Vitamin C)

S.No	Solution	Volume of solution (ml)	Concentration (µg)	Volume of water (ml)	Volume of DNPH (ml)	Volume of Thiourea (ml)	Incubate at 37 ° C for 3 hours	Volume of H_2So_4 (ml)	Allow to standard for 30 minute at room temperature	Optical Density at 630 nm
1	Blank	-	-	3.0	1.0	1-2 drops		7.0		
2	Standard									
	S1	0.2	20	2.8						
	S2	0.4	40	2.6						
	S3	0.6	60	2.4						
	S4	0.8	80	2.2						
	S5	1.0	100	2.0						
3	Sample	0.5	-	2.5						
		0.5	-	2.5						
4	Unknown	1.0	-	-						
		1.0	-	2.5						