ADARSH COLLEGE

OMERGA, DIST. OSMANABAD

DEPARTMENT OF MICROBIOLOGY

CERTIFICATE

Practical paper – II ***Bioenergetics and Enzymology***

Certified that Shri / Miss_____

has satisfactorily completed the course of practical work in M. Sc. I YEAR (SEMESTER I) prescribed by Dr. B. A. M. University, Aurangabad under my supervision in the Microbiology Laboratory during the academic year 20 – 20

Staff member incharge **Head of the department**

Date: Date:

Signature of the Examiner

Date:

Exam Seat No. _____

Paper –II **Bioenergetics and Enzymology**

Sr. No.	Name of Experiment	Page No.
1	Estimation of reducing sugar by Sumner's method	
2	Quantitative estimation of amino acids by Rosen's method	
3	Quantitative estimation of proteins by Biuret method	
4	Optimization of Temperature for α amylase	
5	Optimization of pH for α amylase	

Expt. No. 01 Date: -

Estimation of reducing sugar by Sumner's method

Aim: - To estimate concentration of reducing sugar in the given sample by Sumner's method.

Theory / Approach: -

In the hot alkaline solution, 3, 5 Dinitrosalicylic acid is reduced to 3 amino 5 nitro salicylic acid, which is a deep orange coloured compound. So produced colour is then determined colorimetrically. The intensity of the colour depends upon the concentration of the reducing sugar in the sample.

The concentration of the unknown sample can be calculated using standard graph plotted between the known concentration of sugars and the corresponding optical density measured on colorimeter.

Requirements: -

1) 3, 5 Di-nitro salicylic acid reagent (DNSA)

Preparation: - 1 gm of 3, 5 Di-nitro salicylic acid is moistened with few drops of water in 200 ml volumetric flask. 20 ml of 0.1 N NaOH is added slowly under constant shaking and finally 50 ml of water is added. When 3,5 Di-nitro salicylic acid dissolves completely, 30 gm of potassium sodium tartarate is added. The solution is made up to 200 ml with D / W. Shake well and filter.

2) Standard sugar solution (1 mg / ml)

Preparation: - Weigh accurately 100 mg of sugar and dissolve it in little quantity of D / W in 100 ml volumetric flask. Make the volume to the mark. This is a stock solution, which is 1 mg / ml. Using this stock solution different sugar dilutions are prepared.

3) Pipettes

4) Test tubes

5) Water bath

6) Colorimeter

7) Graph paper

Procedure: -

1. From the stock sugar solution the different dilutions of sugar are prepared and labelled accordingly (See protocol).
2. 3 ml of 3,5 Di-nitro salicylic acid reagent is then added to each tube. The contents are mixed.
3. The blank and the sample tubes are also prepared.
4. All the tubes are kept in boiling water bath fro 20 minutes till purple colour develops.
5. D/W is added to each tube to make the volume 10 ml.
6. OD is measured at 550 nm by using colorimeter.
7. Standard graph of OD versus concentration of sugar is plotted and concentration of sugar in sample is found out.

Protocol: -

Stand-ard sugar solution in ml	**D/W in ml**	**Conc. Of sugar mg/ml**	**DNS Reag-ent in ml**		**D/W in ml**	**OD at 550 nm**
0.2	0.8	0.2	3	**Keep in boiling water bath for 20 minutes**	6	
0.4	0.6	0.4	3		6	
0.6	0.4	0.6	3		6	
0.8	0.2	0.8	3		6	
1.0	--	1.0	3		6	
--	1.0	Blank	3		6	
--	Sample 1 ml	Un-known	3		6	

Observation: - *As per observation table*

Result: -

The concentration of sugar in given sample is _____mg / ml.

Conclusion: -

The reducing sugar reduces DNS reagent to 3 amino 5-nitro salicylic acid, which is a deep orange coloured compound. Intensity of colour is proportional to concentration of sugar. From the standard graph we can found out the concentration of sugar in a given sample.

Expt No. 02 Date

Quantitative estimation of amino acids by Rosen's method

Aim: To estimate the amount of amino acids

Theory

Amino acids are known as the building blocks of all proteins. There are 20 different amino acids commonly found in proteins. Amino acids are comprised of a carboxyl group and an amino group attached to the same carbon atom (the α carbon).

Alpha amino acids react with Ninhydrin involved in the development of color which is explained by the following steps.

α aminoacid + Ninhydrin ⟶ Reduced ninhydrin + Alpha amino acid + H_2O

This is an oxidative deamination reaction that elicits two hydrogen from the α amino acid to produce an alpha – imino acid. Also the ninhydrin reduced and loses an oxygen atom with the formation of water molecule.

α-amino acid + H_2O ⟶ α -keto acid +NH_3

The rapid hydrolysis of NH group in the alpha – imino acid will cause the formation of an α - keto acid with an ammonia molecule. This α -keto acid further involved in the decarboxylation reaction of step.

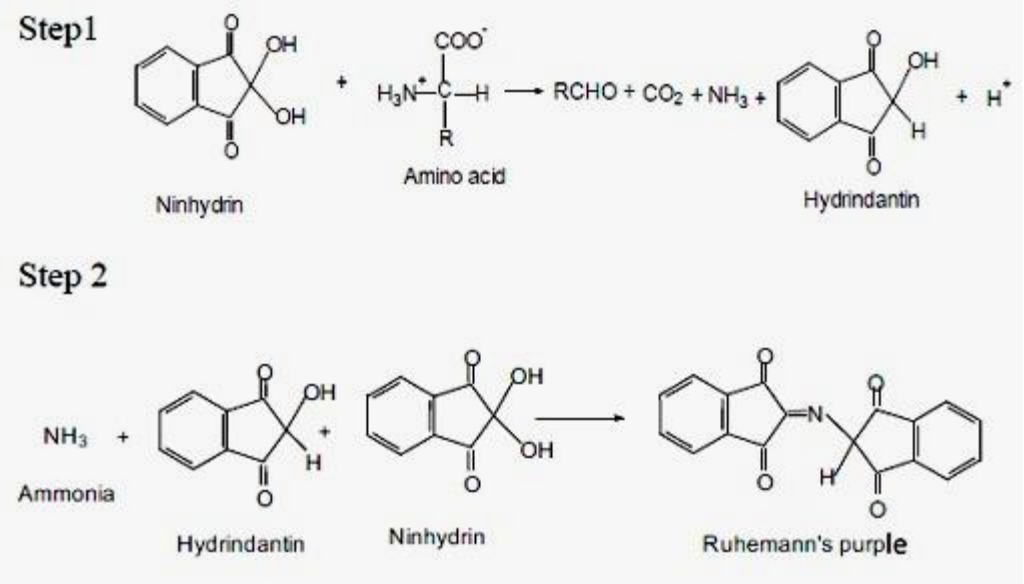
α -keto acid + NH_3 ⟶ aldehyde + CO_2

Under a heated condition to form an aldehyde that has one less carbon atom than the original amino acid. A carbon dioxide molecule is produced along with aldehyde. These first three steps produce the reduced ninhydrin and ammonia that are required for the production of color .The overall reaction for the above reactions is simply explained in Reaction as follows:

α -amino acid + 2 ninhydrin⟶CO_2 + aldehyde + final complex(purple) + 3H_2O

In summary, ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple called Ruhemann's purple. It is this purple color that is detected in this method. Ninhydrin will react with a free alpha-amino group, NH_2-C-COOH.

In the quantitative estimation of amino acid using Ninhydrin reagent, the absorbance of the purple colour formed by the reaction is measured at 570nm colorimetrically.



To determine the absolute concentration of a pure substance, a standard curve is constructed from the known concentrations and using that standard curve, the absorbance reading of the unknown concentration was determined. The determination of unknown concentration from the standard curve is done by drawing a line parallel to the X- axis from the point on the Y axis that corresponds to the absorbance of the unknown. This line will be made to intersect the standard curve drawn, and is extended vertically such that it meets the X-axis and the concentration of unknown is read from the X-axis.

Requirements

1. Standard amino acid stock solution (Standard amino acid stock solution (1mg/ml)).
2. Ninhydrin reagent 8% w/v [Preparation: 8g of ninhydrin dissolved in 100ml of acetone].
3. 50% v/v ethanol.
4. Distilled water.

Apparatus and Glasswares Required:

1. Test / Boiling tubes.
2. Pipettes [glass / micropipette].
3. Waterbath.
4. Colorimeter.

Procedure:

1. Label the test tubes as blank 0.2, 0.4, 0.6, 0.8, 1 and Unknown
2. Add 4ml of distilled water to the test tube labeled ‘Blank’ and 1ml unknown protein sample in test tube labeled ‘Unknown’.
3. Pipette out different volumes (0.2ml-1ml) of standard amino acid solution to the respective labelled test tubes.
4. Add distilled water in all the test tubes to make up the volume to 4ml.
5. Now add 1ml of ninhydrin reagent to all the test tubes including the test tubes labelled 'blank' and 'unknown'.
6. Mix the contents of the tubes by shaking the tubes.
7. Place all the test tubes in boiling water bath for 15 minutes.
8. Cool the test tubes in cold water and add 1ml of 50% ethanol to each test tube and mix well.
9. Fill the cuvette with the blank solution and set OD to zero then take readings for all other tubes.
10. Now record the absorbance at 570 nm of each solution using a colorimeter.
11. Plot the graph between OD and concentration of the amino acids and extrapolate the unknown OD value to find out the concentration of amino acids in unknown sample.

Protocol

Sr. No.	Stock 1 mg / ml	D/W in ml	Conc. of amino acid (µg/ml)	Ninhydrin reagent (in ml)	1 ml 50 % ethanol after 15 min boiling and cooling	OD at 570 nm
1	NIL	4	Blank	1	1	
2	0.2	3.8	50	1	1	
3	0.4	3.6	100	1	1	
4	0.6	3.4	150	1	1	
5	0.8	3.2	200	1	1	
6	1.0	3.0	250	1	1	
7	4 ml Sample	NIL	Unknown	1	1	

Result: The amount of amino acid in given sample is _____ **µg/ml.**

Expt. No. 03 Date

Quantitative estimation of proteins by Biuret method

Aim: To estimate the amount of protein in a given sample by Biuret method

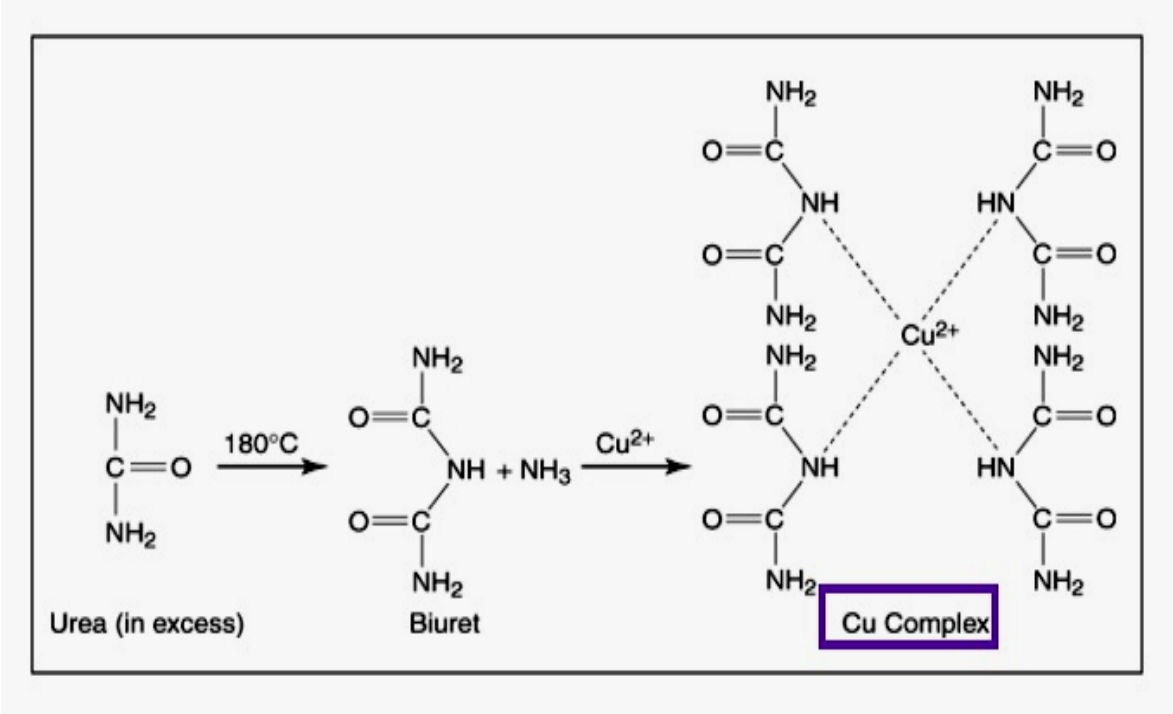
Theory:

The biuret reaction can be used for both qualitative and quantitative analysis of protein. The biuret method depends on the presence of peptides bonds in proteins. When a solution of proteins is treated with cupric ions (Cu2+) in a moderately alkaline medium, a purple colored Cu2+ - peptide complex is formed which can be measured quantitatively by spectrophotometer in the visible region. So, biuret reagent is alkaline copper sulfate solution.

The intensity of the color produced is proportional to the number of peptide bonds that are reacting, and therefore to the number of protein molecules present in the reaction system. The reaction don't occur with amino acids because the absence of peptide bonds, and also that with di-peptide because presence of only one peptide bond, but do with tri-, oligo-, and poly-peptides. Biuret reaction needs presence of at least two peptide bonds in a molecule. The reaction occurs with any compound containing at least two bonds of:

-HN-CO- , -HN-CH2- , -HN-CS-

The reaction takes its name "Biuret Reaction" from the fact that biuret itself, obtained by heating urea, gives a similar colored complex with cupric ions.



In the quantitative estimation of protein using Biuret reagent, the absorbance of the purple colour formed by the reaction is measured at 540 nm colorimetrically.

Biuret assay with various known concentrations of protein is carried out. Standard graph is plotted using concentration on the X axis, and the absorbance on the Y axis. Concentration of protein in given sample is determined by using standard graph.

Requirements

1. Protein sample of unknown concentration
2. Standard protein sample (5 mg/1 ml)
3. Distilled water
4. Biuret reagent

Distilled Water	100 ml
NaOH (0.2M)	800 mg
Sodium potassium tartarate	900 mg
Copper sulfate	300 mg
Potassium iodide	500 mg

5. Test tubes
6. Colorimeter

Procedure

1. Label the test tubes as blank 0.2, 0.4, 0.6, 0.8, 1 and Unknown
2. Add 1ml of distilled water to the test tube labeled ‘Blank’ and 1ml unknown protein sample in test tube labeled ‘Unknown’.
3. Pipette out different volumes (0.2ml-1ml) of standard protein solution to the respective labelled test tubes.
4. Add distilled water in all the test tubes to make up the volume to 1ml.
5. Now add 4 ml of Biuret reagent to all the test tubes including the test tubes labelled 'blank' and 'unknown'.
6. Mix the contents of the tubes by shaking the tubes.
7. Keep all the test tubes for 15 minutes.
8. Fill the cuvette with the blank solution and set OD to zero then take readings for all other tubes.
9. Now record the absorbance at 540 nm of each solution using a colorimeter.
10. Plot the graph between OD and concentration of the protein and extrapolate the unknown OD value to find out the concentration of protein in unknown sample.

Protocol

Sr. No.	**Stock 5 mg / ml**	**D/W in ml**	**Conc. of protein (mg/ml)**	**Biuret reagent (in ml)**	**OD at 540 nm**
1	NIL	1	Blank	4	
2	0.2	0.8	1	4	
3	0.4	0.6	2	4	
4	0.6	0.4	3	4	
5	0.8	0.2	4	4	
6	1.0	00	5	4	
7	1 ml Sample	NIL	Unknown	4	

Result: The amount of protein in given sample is _____ **mg/ml.**

Expt. No. 4 **Date: -**

Optimization of Temperature for α amylase

Aim: - To determine optimum temperature for α amylase enzyme activity

Theory/Approach: -

The rate of enzyme catalyzed reactions generally increases with the increase in temperature within a certain range. The enzyme and substrate concentrations are kept constant and only the temperature is changed like reaction at 10, 37, 50^0 C. OD is measured at 546 nm and graph is plotted between OD and temperature. From graph optimum temperature at which maximum enzyme activity takes place is found out.

Requirements: -

1. Test tubes
2. Starch solution 1 % (10 mg/ml)
3. Amylase Enzyme solution 1 % (10 mg/ml)
4. Distilled water
5. Pipettes
6. Spectrophotometer / Colorimeter
7. Freeze, Incubator
8. DNS reagent (3, 5 Di-Nitro Salycilic acid)

Water	100 ml
3,5 Di-Nitro salicylic acid	1 gm
Phenol	0.2 gm (200 mg)
Na_2CO_3	0.05 gm (50 mg)
NaOH	1 gm
Sodium potassium tartarate	20 gm

(1 gm of 3,5 Di-nitro salicylic acid is moistened with few drops of water in 200 ml volumetric flask. 20 ml of 0.1 N NaOH is added slowly under constant shaking and finally 50 ml of water is added. When 3,5 Di-nitro salicylic acid dissolves completely, 30 gm of potassium sodium tartarate is added. The solution is made up to 200 ml with D / W. Shake well and filter.)

9. Buffer of pH 7

Composition of buffer

A] 0.2 M solution of Monobasic Sodium dihydrogen phosphate (NaH_2PO_4)

127.8 gm in 1000 ml D/W

B] 0.2 M solution of Sodium Dibasic hydrogen phosphate (Na_2HPO_4) 53.65 gm in 1000 ml.

X ml of A + Y ml of B diluted to 200 ml with D/W

X ml of A	Y ml of B	pH
39.0 ml	61.0 ml	7

Procedure: -

1. 4 test tubes are taken and marked them as Blank, 10^0 C, 37^0 C and 50^0 c
2. 1 ml starch solution and 1 ml buffer of pH 7 is added to 10^0 C, 37^0 C and 50^0 C marked test tubes. Starch solution is not added to test tube marked as Blank, instead 2 ml buffer is added in it.
3. Tubes are incubated at different temperature ranging from 10^0 C, 37^0 C and 50^0 C for 15 minutes to reach appropriate temperature.
4. 1 ml amylase enzyme solution is added in all tubes.
5. Tubes are shaken well and incubated at 10^0 C, 37^0 C and 50^0 C for 15 minutes
6. 2 ml of DNS reagent is added in all tubes and tubes are kept in boiling water bath for 15 minutes.
7. Tubes are cooled and 6 ml D/W is added in each tube to make final volume 10 ml and shaken well.
8. OD is adjusted to zero by using Blank and OD of solution of other test tubes is measured at 546 nm.
9. A graph is plotted between OD and Varying temperature and optimum temperature is found out at which maximum enzyme activity has taken place.

Protocol: -

Test tube marked as	**1 % Starch solution in ml**	**Incubation for15 min at**	**Amylase sol^{n}**	**Incubation for 15 min at**	**DNS Reagent in ml**		**D/W in ml**	**OD at 550 nm**
Blank	NIL (Add 2 ml buffer)	Room temp	1 ml	Room temp	2 ml		5	
10^0 C	1 ml starch and 1 ml buffer of pH 7	10^0 C	1 ml	10^0 C	2 ml	**Keep in boiling water bath for 20 minutes**	5	
37^0 C	1 ml starch and 1 ml buffer of pH 7	37^0 C	1 ml	37^0 C	2 ml		5	
50^0 C	1 ml starch and 1 ml buffer of pH 7	50^0 C	1 ml	50^0 C	2 ml		5	

Observation: As per observation table and graph.

Result: - The optimum temperature for amylase is _____.

Conclusion: - The amylase showed maximum activity at 37^0 C and less activity at 10^0 C and 50^0 C indicating that temperature affects the activity of amylase.

Expt. No. 5 **Date: -**

Optimization of pH for α amylase

Aim: - To determine the optimum pH for α amylase enzyme activity

Theory/Approach: -

The nature of binding between enzyme and substrate is mainly electrostatic and depends upon the charge available on the substrate and the active site. Since active site is composed of amino acids, charges vary with pH and binding of enzyme with substrate is affected by pH of the medium.

The enzyme and substrate concentrations and temperature are kept constant and only the pH is changed like 6, 7, 8. OD is measured at 546 nm and graph is plotted between OD and pH. From graph optimum pH at which maximum enzyme activity takes place is found out.

Requirements: -

1. Test tubes
2. Starch solution 1 % (10 mg/ml)
3. Amylase Enzyme solution 1 % (10 mg/ml)
4. Distilled water
5. Pipettes
6. Spectrophotometer / Colorimeter
7. Freeze, Incubator
8. DNS reagent (3, 5 Di-Nitro Salycilic acid)

Water	100 ml
3,5 Di-Nitro salicylic acid	1 gm
Phenol	0.2 gm (200 mg)
Na_2CO_3	0.05 gm (50 mg)
NaOH	1 gm
Sodium potassium tartarate	20 gm

9. Phosphate buffer of pH 6, 7 and 8.

Composition of buffer

A] 0.2 M solution of Monobasic Sodium dihydrogen phosphate (NaH_2PO_4) 127.8 gm in 1000 ml D/W

B] 0.2 M solution of Sodium Dibasic hydrogen phosphate (Na_2HPO_4) 53.65 gm in 1000 ml.

X ml of A + Y ml of B diluted to 200 ml with D/W

X ml of A	Y ml of B	pH
87.7 ml	12.3 ml	6
39.0 ml	61.0 ml	7
5.3 ml	94.7 ml	8

Procedure: -

1. 4 test tubes are taken and marked them as Blank, pH 6, pH 7 and pH 8.
2. 1 ml starch solution and 1 ml buffer of respective pH is added to pH 6, pH 7 and pH 8 marked test tubes. Starch solution is not added to test tube marked as Blank; instead 2 ml D/W is added in it.
3. Tubes are incubated at 37^0 C temperatures for 15 minutes to reach appropriate temperature.
4. 1 ml amylase enzyme solution is added in all tubes.
5. Tubes are shaken well and incubated at 37^0 C for 15 minutes.
6. 2 ml of DNS reagent is added in all tubes and tubes are kept in boiling water bath for 15 minutes.
7. Tubes are cooled and 5 ml D/W is added in each tube to make final volume 10 ml and shaken well.
8. OD is adjusted to zero by using Blank and OD of solution of other test tubes is measured at 546 nm.
9. A graph is plotted between OD and Varying pH and optimum pH is found out at which maximum enzyme activity has taken place.

Protocol: -

Test tube marked as	**1 % Starch solution in ml and buffer solution**	**Incubation for15 min at**	**Amylase sol^{n}**	**Incubation for 15 min at**	**DNS Reagent in ml**		**D/W in ml**	**OD at 550 nm**
Blank	NIL (Add 2 ml d/w)	37^0 C	1 ml	37^0 C	2 ml		5	
pH 6	1 ml starch and 1 ml buffer of pH 6	37^0 C	1 ml	37^0 C	2 ml	**Keep in boiling water bath for 20 minutes**	5	
pH 7	1 ml starch and 1 ml buffer of pH 7	37^0 C	1 ml	37^0 C	2 ml		5	
pH 8	1 ml starch and 1 ml buffer of pH 8	37^0 C	1 ml	37^0 C	2 ml		5	

Observation: As per observation table and graph.

Result: - The optimum pH for amylase is _____.

Conclusion: - The amylase showed maximum activity at pH ___ and less activity at pH __ indicating that pH affects the activity of amylase.