THERMAL INACTIVATION STUDIES OF PROTEIN: A STEP TOWARD THEIR BIOSTABILIZATION

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

Bachelor of Technology
in
Chemical Engineering

By
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SRI BISWANATH JENA

Department of Chemical Engineering
National Institute of Technology
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Under the Guidance of
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Department of Chemical Engineering
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Rourkela

CERTIFICATE

This is to certify that the thesis entitled "Thermal Inactivation Studies of Protein: A step toward their biostabilization" submitted by Sri Shashi Kant Sharma and Sri Biswanath Jena in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Chemical Engineering at the National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by them under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

Date :
Place :

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CONTENTS

ABSTRACT

In this study the thermal inactivation of pure Yeast Alcohol Dehydrogenase was carried out both in presence and absence of carriers using the activity assay method. The enzyme activity was studied in a UV Spectrophotometer at 340 nm at room temperature and 8.8 pH using NAD as a co-enzyme. The objective of the study was to find out the change in activity or rather the increase in stability of the enzyme in the presence of salts and sugars of known concentrations and subsequent heat treatment in a constant temperature water bath at various temperatures.

The first step of the experiment was to choose appropriate inorganic salts and sugars which can provide protection to the enzymes when they are subjected to heat treatment. The next step was to measure the rate of change of absorbance of UV light in a UV Spectrophotometer when only the enzyme (i.e. pure yeast ADH with no other carrier such as salts and sugars) reacts with ethanol and NAD in the assay mixture. The enzyme activity (E) was calculated at various time intervals between 0-60 mins for different values of slopes obtained in the Absorbance-vs-Time curve. A graph of ln(E/Eo)-vs-Time was plotted and nature of the curve was studied and reported. In the next step suitable carriers (such as D-Mannitol, Ammonium Sulphate, Sucrose, etc) were added to the enzyme and similar procedures were followed to obtain the ln(E/Eo) graph.

The plot of ln(E/Eo)-vs-Time showed a nonlinear biphasic behavior of the enzyme when subjected to thermal inactivation. This nonlinear behaviour could be due to the formation of enzyme groups with different thermal stabilities or the presence of stable/ labile isoenzymes. It was also observed that with the increase in the percentage of the excipients in the final solution better stability was achieved, even at higher temperatures. It was also observed that when salts (Sodium Sulphate & Ammonium Sulphate) were used as excipients higher enzyme stability was achieved in comparison to sugars (Sucrose & Trehalose).

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Enzyme thermostability is an intrinsic property, determined by the primary structure of the protein. However, external environmental factors such as cations, substrates, co-enzymes, and proteins often increase enzyme thermostability. There are commercial advantages in carrying out enzymic reactions at higher temperatures, for example in food and pharmaceutical industries where we need to process enzymes at higher temperatures during sterilization, pasteurization, etc. Some industrial enzymes exhibit high thermostability. More stable forms of other industrial enzymes are eagerly being sought after. [1]

Proteins, including enzymes (which are just specialized proteins), can be segregated into those that are more heat stable (able to withstand a given degree of heat without the protein being denatured, or unfolded) and those that are more heat labile (unfold more easily upon exposure to extremes of heat). As this classification suggests, proteins normally exist in folded states in living systems. This means that proteins prefer to contort their amino acid chains into various curvatures that allow the proteins to achieve stable and functional conformations. These stable conformations can be disrupted or denatured by many environmental challenges, including heat extremes, acidity (pH changes), changes in the water or fat content of the solution in which the protein remains, and exposure to chemicals in solution. [15]

The degree of hydrogen bonding in a protein along with many other types of bond forces influences the stability of the conformation by allowing them to fold properly. A greater number of hydrogen bonds mean a more stable conformation and, therefore, less denaturing when exposed to heat (heat stable). Some ionic bonds can exist and influence folding stability. Noncovalent hydrophobic (van der Waals) bonding forces are quite important—the greater the number of these bonds, the greater the predicted heat stability. Further, proteins have unique amino acid sequences, and the types of amino acids in a sequence can determine whether a protein will be affected by acid denaturing (pH), for example. Many proteins have other partner proteins that bind—in other words, accompany or piggyback on—the main protein in living systems. These partner proteins play an important role in determining whether the main protein will be heat stable or heat labile. One such class of accompanying proteins is heat shock proteins.

1.2 Objective

- The main objective of this work is to study the thermal inactivation behaviour of the enzyme yeast alcohol dehydrogenase under the influence of external agents like inorganic salts and sugars.

- It is also intended to study the stabilizing effect of various salts and sugars on the enzyme when subjected to heat treatment.

- The effect of varying concentrations of these carriers at a constant temperature on the stability of the enzyme is to be found out.

- Further, the biphasic behaviour i.e. thermo stable and thermo labile nature of the enzyme is to be established by showing the actual experimental plots.

CHAPTER 2

THEORITICAL STUDIES

Need for protein Stabilization

Protein Structure

Protein Folding and Unfolding

Available Methods for protein satbilization

2.1 Need for Stabilization of protein :-

There are several reasons for making the protein molecules more stable. Few of them are:

- Many industrial processes require the protein molecules to pass through high temperatures and pressure without their properties being affected. Hence protein molecules have to be stabilized for such conditions.
- In the laboratory one needs to carry out experiments on normal meso stable proteins as well as mutated proteins. The mutated proteins are very unstable and hence need to be stabilized.
- In the pharmaceutical industries it is very essential to ensure that the drugs and medicines remain stable for a longer period of time under ordinary conditions. Since protein is most stable in dried state, protein formulations have to be stabilized by drying.

2.2 Protein Structure:-

Proteins are polymer of amino acids in which carbon atoms and peptide groups alternate to form a linear polypeptide chain, while specific groups- the amino acid side chains – project from the α-carbon atom. This linear sequence of amino acids is called the *primary structure* of protein which folds into unique three-dimensional protein structures. The shape into which a protein naturally folds is known as its *native state*, which is determined by its sequence of amino acids. The stretched out form of protein has no biological activity. Rather, protein function arises because polypeptides fold in complex ways to yield precisely three-dimensional structures.

Once linked in the protein chain, an individual amino acid is called a *residue* and the linked series of carbon, nitrogen, and oxygen atoms are known as the *main chain* or *protein backbone*.

Due to the chemical structure of the individual amino acids, the protein chain has directionality. The end of the protein with a free carboxyl group is known as *the C-terminus* or carboxy terminus, while the end with a free amino group is known as the *N-terminus* or amino terminus.

There is some ambiguity between the usage of the words protein, polypeptide, and peptide. Protein is generally used to refer to the complete biological molecule in a stable conformation, while peptide is generally reserved for short amino acid oligomers often lacking a stable 3-dimensional structure. Polypeptide can refer to any single linear chain of

amino acids, usually regardless of length, but often implies an absence of a single defined conformation.

Biochemists refer to four distinct aspects of a protein's structure:

- *Primary structure*: the linear amino acid sequence
- *Secondary structure*: It refers to spatial arrangement of amino acid residues that are near one another in the linear sequence. Some of these steric relationships manifest a regular repeat pattern, giving rise to periodic structures. The alpha-helices, beta-sheets, and random coils are elements of secondary structure.
- *Tertiary structure*: It refers to the overall three-dimensional architecture of the spatial arrangement of amino acid residues, including those that may be far apart in the linear sequence. This spatial arrangement is also referred to as the packing assembly of secondary structure units.
- *Quaternary structure*: the shape or structure that results from the union of more than one protein molecule, usually called protein subunits in this context, which function as part of the larger assembly or protein complex.

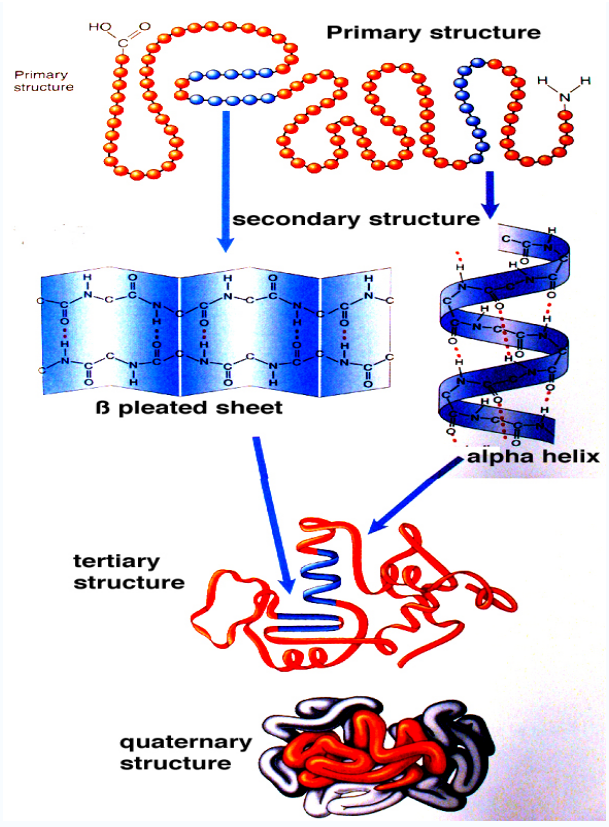

Fig 1.1

The primary structure is held together by covalent peptide bonds. The secondary structures are held together by hydrogen bonds. The tertiary structure is held together primarily by hydrophobic interactions but hydrogen bonds, ionic interactions, and are usually involved too.

The unique three-dimensional structure in case of proteins is possible due to the following non covalent interactions:

1 **The Hydrogen Bonding**:- In proteins intra strand hydrogen-bonding occurs between a hydrogen atom on a nitrogen atom adjacent to one peptide bond and an oxygen atom adjacent to a different peptide bond. This interaction gives rise to several particular polypeptide chain configurations.

2 **The Hydrophobic Bonding**:- A hydrophobic interaction is an interaction between two molecules (or portions of molecules) that are somewhat insoluble in water. These two molecules (which may be different) are poorly soluble in water, which tends to repulse them. In response to that natural repulsion, they tend to associate. Since the amino acids are not necessarily adjacent, hydrophobic interactions tend to bring distant hydrophobic parts of a polypeptide chain together.

3 **The Ionic Bonds**:- This is the result of attraction between unlike charges. At physiological pH amino acid chains are ionised as: negatively charged carboxyl groups (aspartic and glutamic acids) and positively charged amino groups (lysine, histidine and arginine). Ionic bonds bring together distant parts of a chain both by attraction (opposite charges) as well as repulsion (same charges).

4 **The Van der Waals Attraction**:- Although they are very weak they do exist for very nearby molecules and are easily overcome by thermal motion

2.3 PROTEIN FOLDING:-

Protein folding is the process by which a protein assumes its characteristic functional shape or tertiary structure, also known as the native state. Most proteins can only carry out their biological functions when their three-dimensional shape is at or near the native state because their geometry is critical to their function. For example, many enzymes have deep clefts or pockets on their surfaces that act as binding sites for substrates, and membrane proteins often have central pores through which they allow certain organic compounds or ions to pass. Most folded proteins have a *hydrophobic core* in which side chain packing stabilizes the folded state, and charged or polar side chains on the solvent-exposed surface where they form hydrogen bonds with surrounding water molecules.

The folded structure is stabilized by many non-covalent interactions (e.g., H-bonding, ionic interactions, van der Wall's attractions, etc). Physical properties of the peptide bond and the amino acids in the polypeptide chain govern the manner of this folding. There are two major types of non-covalent interactions contributing to the stability of a folded protein structure: hydrophobic interaction between apolar side chains that prefer to exclude water and electrostatic or polar interactions mediated by water. The latter includes hydrogen bonding and salt bridges.

2.4 PROTEIN UNFOLDING OR DENATURATION:-

The "reverse" of the folding process is called protein unfolding or denaturation, whereby the native structure of a protein is disrupted and a random coil ensemble of unfolded structures is formed instead. Denaturation can be carried out chemically by the addition of denaturants or thermally by heating (and sometimes cooling). Many denatured proteins precipitate into insoluble amorphous aggregates.A denatured protein is one which has lost its functional conformation. Once denatured, a protein loses most, if not all of its biological activity. A protein can be denatured through various means including exposure to extremes of heat, pH, salt concentration, denaturing agents like urea/guanidine chloride and use of detergents. During digestion, proteins are denatured by stomach acid allowing them to be degraded by proteolytic enzymes to their amino acid components. When a protein is denatured, the secondary and tertiary structures are altered but the peptide bonds between the amino acids are left intact. Since the structure of the protein determines its function, the protein can no longer perform its function once it has been denatured.

2.5 VARIOUS AVAILABLE METHODS FOR STABILIZING PROTEIN:

i. **FREEZE DRYING OR LYOPHILIZATION**: In the process of freeze drying, a liquid stream is atomized, frozen by contact with a liquid medium, for example, liquid nitrogen, and the frozen solvent is removed by sublimation. This technique has utility in protein processing, for instance in producing powders suitable for pulmonary delivery or for microencapsulation in poly (lactide-co-glycolide). For the latter application, the size and morphology of the protein powder is of interest because they affect the performance of the encapsulated protein. Also important is protein stability, which can be impacted by various stresses imposed by atomization, freezing, and drying.

ii. **MUTAGENESIS**: The process by which genetic material undergoes a detectable and heritable structural change. There are three categories of mutation: genome mutations, involving addition or subtraction of one or more whole chromosomes; chromosome mutations, which alter the structure of chromosomes; and gene mutations, where the structure of a gene is altered at the molecular level. Mutations are changes to the genetic material (either DNA or RNA). Mutations can be caused by copying errors in the genetic material. Sometimes mutating any one or all the three available tools can lead to stability.

iii. **SALT BRIDGES**: Salt bridges in proteins are bonds between oppositely charged residues that are sufficiently close to each other to experience electrostatic attraction. They contribute to the stability of the protein if the charges are 6 to 8 A away from each other. They contribute to protein structure and to the specificity of interaction of proteins with other biomolecules, but in doing so they need not necessarily increase a protein's free energy of unfolding. The net electrostatic free energy of a salt bridge can be partitioned into three components: charge-charge interactions, interactions of charges with permanent dipoles, and desolvation of charges. Energetically favorable Coulombic charge-charge interaction is opposed by often unfavorable desolvation of interacting charges. As a consequence, salt bridges may destabilize the structure of the folded protein. Salt bridges work better if we are able to combine a couple them. In

other words an array of charges like +-+-+- works better than two pair of charges +- that are far apart in the structure.

iv. **INORGANIC SOLVENTS**: Addition of some particular inorganic solvents makes the protein molecules more stable. Some common salts which are used are –

- Lithium Bromide
- Sodium Bromide
- Sodium sulphate
- Ammonium
- Potassium Thiocyanate (KSCN)

Some common sugars which are used are—

- Sucrose
- Trehalose
- Sugar alcohols

v. **REMOVING PROTEASES FROM THE MEDIUM**: Proteases (proteinases, peptidases, or proteolytic enzymes) are enzymes that break peptide bonds between amino acids of proteins. The process is called *proteolytic cleavage*, a common mechanism of activation or inactivation of enzymes, especially those involved in blood coagulation or digestion. They use a molecule of water for this and are thus classified as hydrolases.Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades for e.g., the blood clotting cascade. Proteases, being themselves proteins, are known to be cleaved by other protease molecules, sometimes of the same variety. This may be an important method of removing the protease molecules from the medium where protein reactions take place so that the protein can be stabilized.

2.6 PROPOSED EXPERIMENT

Proteins fold into their unique native structure, even in vitro. However, they tend to form undesirable and uncontrollable aggregates during the unfolding and refolding processes, both in the laboratory and even in their natural environment in living cells. Protein aggregation is a major problem in the large-scale production of recombinant proteins, as well as in living cells, where it may lead to the occurrence of fatal diseases. Various techniques have been developed to prevent the formation of protein aggregates.

One of the major approaches used to prevent protein aggregation is the addition of small molecules to the solution. Some of the inert chemicals (excipients) are needed during processes like drying of protein. These excipients help to form non covalent bonds with the proteins available in the medium. They prevent the degradation of proteins, due to extreme stresses of the process by offering preferential exclusion to the solute in the presence/absence of water. In absence of water the hydrophilic parts of protein molecules share partial bonds with the solute molecules which in this case are excipients. The hydrophobic part moves away from water and tend to sink more towards inside. In the presence of water protein molecules form partial bond with excipients like sugar, etc and stabilize the protein conformation. However, proper selection of excipients is very important for giving protection to thermolabile protein under stressful conditions. We would follow this method to find out most suitable molecular additives for preventing thermal inactivation and consequently stabilize the proteins under study.

CHAPTER 3

MATERIALS AND METHOD

FORMULAE AND CALCULATIONS

3.1 Materials:-

Pure yeast alcohol dehydrogenase was purchased from EMD Biosciences Inc. La Jolla; an affiliate of Merck, (Darmstadt, Germany). NAD, D-Mannitol, D-Trehalose, Ammonium Sulphate and Sodium Sulphate were purchased from SISCO Research Laboratories Pvt. Ltd, (Mumbai, India). Sodium dihydrogen phosphate, Di-sodium hydrogen phosphate and Sucrose were purchased from Merck Specialities Private Limited, (Mumbai, India). Tetra-sodium pyrophosphate was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Ethanol was purchased from Merck, (Darmstadt, Germany). All reagents were of analytical grade.

3.2 Apparatus:-

I. Constant Temperature Water Bath (Circulating type)

The Constant Temperature Water Bath , a kind of multi-functional thermostatical circulating type water bath, is comprised of thermostat with high-accuracy and compressor-cooling system through adopting PID control method. This kind of Water Bath is widely applied to such fields as petroleum, chemical industry, medicine, biochemistry, measuring, light industry, laboratory of the R&D institution, etc. It is very safe and reliable to use this kind of products. Laboratory scale, digital contol, constant temperature water bath & circulator is used to maintain steady temperature for laboratory analysis.

Fig 3.1 Constant Temperature Water Bath

II. UV Spectrophotometer

Principle

Many chemical species absorb light in either the visible or ultra violet region of the electromagnetic spectrum. This absorption is caused by the changing electron energy levels in the molecule and is therefore characteristic of the molecular structure. The technique is particularly useful for performing rapid quantitative concentration measurements of organic species in solution. The spectrophotometer measures quantitatively the fraction of light that passes through a given solution. In a spectrophotometer, a light from a lamp in a near-IR/VIS/UV spectrophotometer (typically a deuterium gas discharge lamp) is guided through a monochromator, which picks light of one particular wavelength out of the continuous spectrum. This light passes through the sample that is being measured. After the sample, the intensity of the remaining light is measured with a photodiode or other light sensor, and the transmittance for this wavelength is then calculated.

Applications of UV and Visible Spectrophotometer

- Spectrophotometry
- Suitable for colorimetric measurements
- Specular reflection measurements
- Trace organics analysis / monitoring

Fig 3.2 UV Spectrophotometer

Features of Laboratory's UV Spectrophotometer

- Wavelength range 190 to 1100nm and scanning facility from 200nm to 1000nm/min
- Software for Standard curve fitting, Absorbance ratio, Absorbance difference, Kinetics, fast survey scanning, multiple programmed wavelength measurement

3.3 Preparation of Assay Mixture:-

Components of assay mixture are

- NAD, to be maintained at 8.3mM
- Ethanol, concentration to be maintained at 0.7 M
- 10 mM Tetrasodium Pyrophosphate buffer, at 8.8 pH

We need approximately 25 ml of assay mixture for every run (11×2 ml + 1×2 ml = 24 ml). Therefore, for a 25 ml assay mixture 0.138 gm NAD was taken in a conical flask to which 1.02 ml of ethanol and 23.98 ml of 10mM pyrophosphate buffer were added.

3.4 Preparation of 10 mg/ml solution of ADH:-

A small amount (<10 mg) of ADH was dropped into an eppendorf tube whose empty weight had already been taken. The ADH was weighed and to it 10mM phosphate buffer pH 7.5 of volume 0.1 times the weight of ADH was added.

3.5 Thermal treatment of ADH without any carrier:-

Around 600 µL of enzyme mixture is needed (11×50 µL) for every run at a certain temperature in which ADH concentration should be 1 mg/ml. Thus 60 µL of 10 mg/ml ADH solution was drawn with a micropipette and mixed with 540 µL of 10 mM phosphate buffer of pH 7.5 to make the volume 600 µL in a blank eppendorf tube. It was capped and kept in its stand. The temperature of the water bath was set at 50 ºC and after the temperature is attained the epppendorf tube was allowed to float on the water surface for thermal treatment. At time intervals t=0, 1, 4, 7, 10, 13, 16, 20, 30, 40 and 60 minutes, 50 µL of the enzyme was drawn and pipetted out immediately into the sample cuvette of spectrophotometer containing 2 mL of assay mixture. The absorbance-vs-time curve was studied for 50 seconds.

3.6 Enzyme activity measurement

The activity of ADH was measured spectrophotometrically at 340 nm, 25 ºC in pH 8.8 tetrasodium pyrophosphate buffer. The procedure is based on progressive oxidation of ethanol, with NAD continuously getting reduced to NADH. 50 µL of diluted enzyme solution was introduced into a 1.5 mL assay mixture. NAD concentration was maintained at 8.3 mM and that at 0.7 M. The extinction coefficient value is 6.22 $mM^{-1}cm^{-1}$. Enzyme activity was calculated by linear regression of absorbance at 340 nm vs time data.

3.7 Heat treatment with D-Mannitol

Mannitol concentration was kept 20% (w/v) i.e. 20g/100 mL solution. So in 600 µL we add 0.12 g Mannitol. Thus 0.12 g of mannitol was taken in an eppendorf tube and to this 60 µL of 10 mg/mL ADH solution and 540 µL of 10 mM phosphate buffer pH 7.5 were added. All other steps in 3.3 and 3.4 are to be repeated for this sample also to calculate enzyme activity.

3.8 Heat treatment with Ammonium Sulphate and Sodium Sulphate

The procedure is same as used for D-Mannitol.

3.9 Heat treatment with Sucrose

Sucrose concentration was kept 40% (w/v) i.e 40g/100mL solution. So in 600 µL we add 0.24 g sucrose.Rest of the procedure were same.

3.10 Heat treatment with Trehalose

Here we have to carry out experiments with two concentrations i.e.20% and 40% .In first case we take 0.12g of trehalose and in second case we take 0.24g of trehalose for preparation of enzyme solution. Rest of the procedure were same.

3.11 Systems used for studying Thermal Inactivation

CARRIER	MOL. WT.	TEMPERATURE	CONCENTRATION OF CARRIER	
			in % (w/v)	in mM
D-Mannitol	182.17	60 ^{0}C, 65 ^{0}C	20%	1097.88
Ammonium Sulphate	132.13	60 ^{0}C , 65 ^{0}C	20%	1513.66
Sodium Sulphate	142.04	60 ^{0}C , 65 ^{0}C	20%	1408.05
Sucrose	342.30	60 ^{0}C , 65 ^{0}C	40%	1168.56
Trehalose	378.33	60 ^{0}C , 65 ^{0}C	20% 40%	528.63 1057.27

Table 3.1

3.12 Formulae and Calculations

I. Beer's Law

The **Beer-Lambert law**, also known as **Beer's law** or the **Lambert-Beer law** or the **Beer-Lambert-Bouguer law** is an empirical relationship that relates the absorption of light to the properties of the material through which the light is traveling.

Mathematically the Beer's law can be expressed as,

$$A = \alpha l c$$

where,

- A is absorbance
- l is the distance that the light travels through the material (the path length)
- c is the concentration of absorbing species in the material
- α is the absorption coefficient or the molar absorptivity or the molar extinction coefficient of the absorber

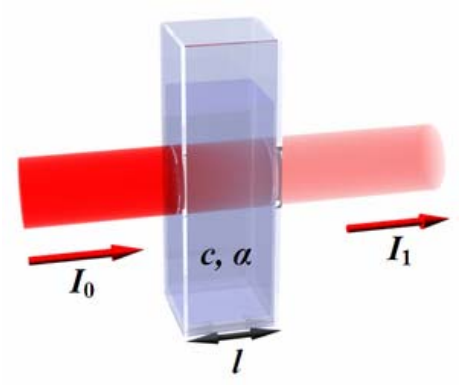

Fig 3.3 Diagram of Beer-Lambert absorption of a beam of light as it travels through a cuvette of size *l*

II. Enzyme activity

Enzyme activity =ΔA/Δt (at 340 nm)×Dilution factor ×1000/Extinction Coefficient

One unit of enzyme activity is the amount of enzyme which will catalyse the transformation of 1 micromole of the substrate per minute under standard conditions of temperature, optimal pH and optimal substrate concentration. It is represented by U.

Dilution factor = Total reaction volume/ Volume of the sample used.

CHAPTER 4

RESULTS
TABLES
GRAPHS
DISCUSSION

4.1 Results

The results obtained were tabulated for each case as shown subsequently.

4.1.1. Thermal treatment with no carrier at 50 ^{O}C

Without carrier, 50 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	2.122	14032.58065	1.000	0
1	1.849	12227.25806	0.871	-0.13771409
4	0.8607	5691.725806	0.406	-0.90236831
8	0.549	3630.483871	0.259	-1.35201588
12	0.453	2995.645161	0.213	-1.54422219
16	0.4138	2736.419355	0.195	-1.63473155
20	0.4082	2699.387097	0.192	-1.64835707
40	0.113	747.2580645	0.053	-2.9327265
60	0.055	363.7096774	0.026	-3.65278113

Table 4.1

4.1.2. Thermal treatment with no carrier at 60 ^{O}C

Without carrier, 60 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	2.126	14059.03226	1.000	0
1	1.087	7188.225806	0.511	-0.67082
4	0.537	3551.129032	0.253	-1.376
7	0.3074	2032.806452	0.145	-1.93385
10	0.1697	1122.209677	0.080	-2.52797
14	0.087	575.3225806	0.041	-3.19609
17.5	0.047	310.8064516	0.022	-3.81185
20	0.027	178.5483871	0.013	-4.36616
40	0.021	138.8709677	0.010	-4.61748
60	0.0073	48.27419355	0.003	-5.67412

Table 4.2

The following graph shows the plot of ln(E/Eo)-vs-Time for without any carrier :-

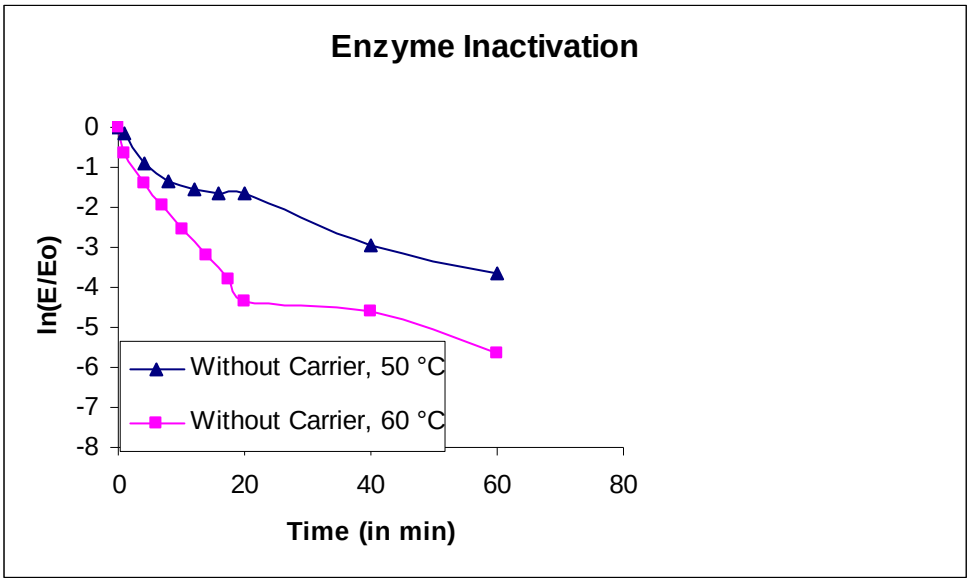

Fig 4.1

4.1.3. Thermal treatment with D-Mannitol at 60 ^{O}C

20% D-Mannitol, 60 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	1.476	9760.645161	1.000	2.97442E-11
1	1.755	11605.64516	1.189	0.173133131
4	0.808	5343.225806	0.547	-0.602528947
7	0.695	4595.967742	0.471	-0.75317916
10	0.493	3260.16129	0.334	-1.096581831
13	0.363	2400.483871	0.246	-1.402688171
16	0.287	1897.903226	0.194	-1.637608789
20	0.222	1468.064516	0.150	-1.894413623
30	0.112	740.6451613	0.076	-2.578592134
40	0.059	390.1612903	0.040	-3.219553561
60	0.021	138.8709677	0.014	-4.252568567

Table 4.3

4.1.4. Thermal treatment with D-Mannitol at 65 ^{O}C

20% D-Mannitol, 65 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	1.299	8590.16129	1.000	0
1	1.234	8160.322581	0.950	-0.051333812
4	0.254	1679.677419	0.196	-1.63201575
7	0.09	595.1612903	0.069	-2.669540346
10	0.0474	313.4516129	0.036	-3.310727788
13	0.024	158.7096774	0.018	-3.991296186
16	0.015	99.19354839	0.012	-4.461299816
20	0.0076	50.25806452	0.006	-5.141201769
30	0.0011	7.274193548	0.001	-7.074039837
40	0	0	0.000	#NUM!
60	0	0	0.000	#NUM!

Table 4.4

The following graph shows the plot of ln(E/Eo)-vs-Time for 20 % D-Mannitol :-

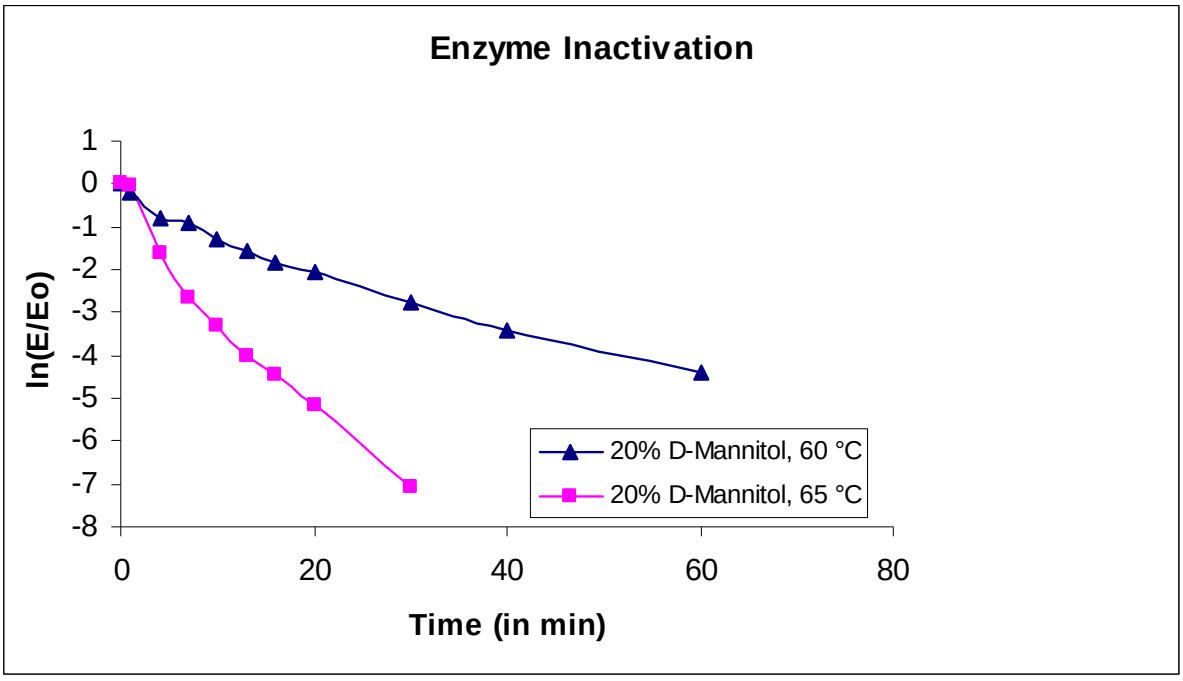

Fig 4.2

4.1.5. Thermal treatment with 20% Ammonium Sulphate at 60 ^{O}C

20% Ammonium Sulphate, 60 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	1.352	8940.645161	1.000	0
1	1.335	8828.225806	0.987	-0.01265369
4	1.149	7598.225806	0.850	-0.16269298
7	1.117	7386.612903	0.826	-0.19093846
10	1.107	7320.483871	0.819	-0.19993132
13	1.093	7227.903226	0.808	-0.21265877
16	0.992	6560	0.734	-0.30961715
20	0.987	6526.935484	0.730	-0.31467022
30	0.966	6388.064516	0.714	-0.33617642
40	0.955	6315.322581	0.706	-0.34762892
60	0.843	5574.677419	0.624	-0.4723733

Table 4.5

4.1.6. Thermal treatment with 20% Ammonium Sulphate at 65 ^{O}C

20% Ammonium Sulphate, 65 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	0.909	6011.129032	1.000	0
1	0.821	5429.193548	0.903	-0.101821985
4	0.799	5283.709677	0.879	-0.128984148
7	0.629	4159.516129	0.692	-0.368213837
10	0.609	4027.258065	0.670	-0.400526826
13	0.581	3842.096774	0.639	-0.447594337
16	0.492	3253.548387	0.541	-0.613866378
20	0.483	3194.032258	0.531	-0.63232844
30	0.343	2268.225806	0.377	-0.974614647
40	0.309	2043.387097	0.340	-1.079003817
60	0.175	1157.258065	0.193	-1.64755912

Table 4.6

The following graph shows the plot of ln(E/Eo)-vs-Time for 20% Ammonium Sulphate :-

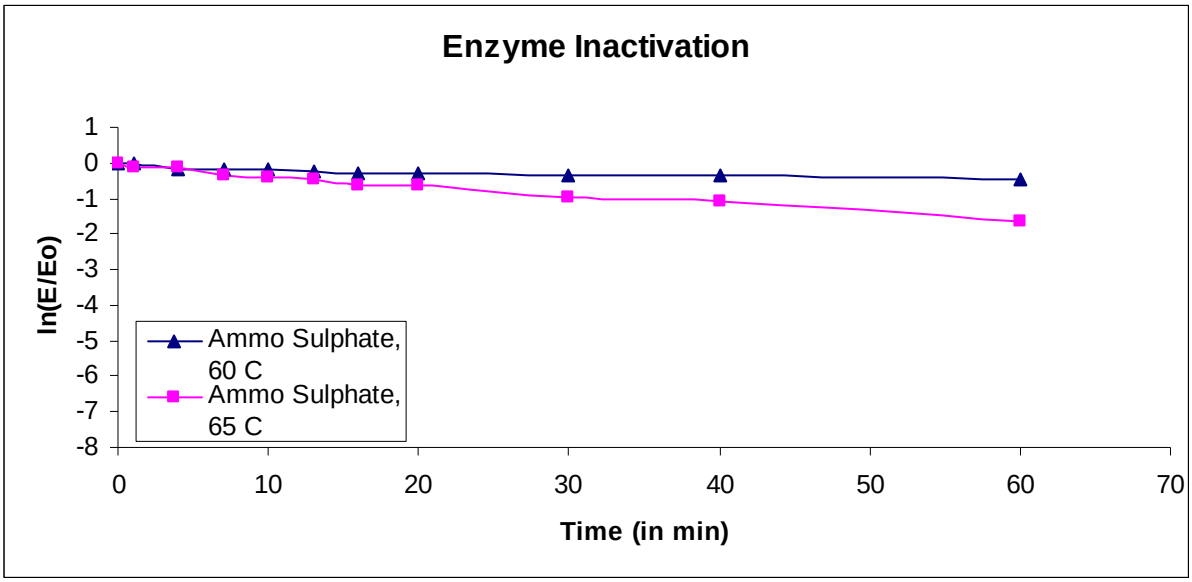

Fig 4.3

4.1.7. Thermal treatment with 20% Sodium Sulphate at 60 ^{O}C

20% Sodium Sulphate, 60 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	0.4	2645.16129	1.000	0
1	0.33	2182.258065	0.825	-0.192371893
4	0.298	1970.645161	0.745	-0.29437106
7	0.288	1904.516129	0.720	-0.328504067
10	0.264	1745.806452	0.660	-0.415515444
13	0.234	1547.419355	0.585	-0.536143432
16	0.206	1362.258065	0.515	-0.663588378
20	0.18	1190.322581	0.450	-0.798507696
30	0.174	1150.645161	0.435	-0.832409248
40	0.155	1025	0.388	-0.94803943
60	0.139	919.1935484	0.348	-1.056990614

Table 4.7

4.1.8. Thermal treatment with 20% Sodium Sulphate at 65 ^{O}C

20% Sodium Sulphate, 65 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	2.184	14442.58065	1.000	0
1	2.101	13893.70968	0.962	-0.038744636
4	1.453	9608.548387	0.665	-0.407527674
7	1.432	9469.677419	0.656	-0.42208599
10	1.325	8762.096774	0.607	-0.499745599
13	1.233	8153.709677	0.565	-0.571707834
16	1.115	7373.387097	0.511	-0.672303653
20	1.095	7241.129032	0.501	-0.690403695
30	1.003	6632.741935	0.459	-0.778162549
40	0.613	4053.709677	0.281	-1.270548401
60	0.436	2883.225806	0.200	-1.611271094

Table 4.8

The following graph shows the plot of ln(E/Eo)-vs-Time for 20% Sodium Sulphate :-

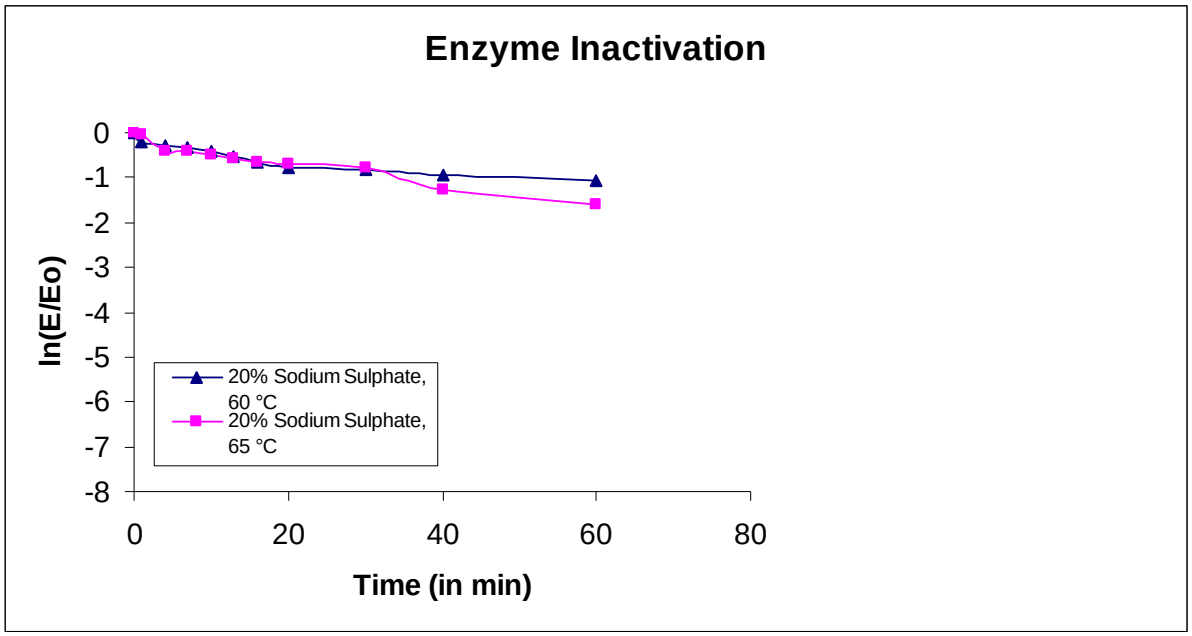

Fig 4.4

4.1.9. Thermal treatment with 40% Sucrose at 60 ^{O}C

40% Sucrose, 60 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	1.261	8338.870968	1.000	0
1	1.241	8206.612903	0.984	-0.015987551
4	0.724	4787.741935	0.574	-0.554868944
7	0.565	3736.290323	0.448	-0.802834605
10	0.489	3233.709677	0.388	-0.947297847
13	0.442	2922.903226	0.351	-1.048350454
16	0.398	2631.935484	0.316	-1.153208331
20	0.333	2202.096774	0.264	-1.331517846
30	0.243	1606.935484	0.193	-1.646598893
40	0.167	1104.354839	0.132	-2.021666524
60	0.098	648.0645161	0.078	-2.554692857

Table 4.9

4.1.10. Thermal treatment with 40% Sucrose at 65 ^{O}C

40% Sucrose, 65 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	1.341	8867.903226	1.000	0
1	1.286	8504.193548	0.959	-0.041878979
4	0.415	2744.354839	0.309	-1.172892363
7	0.287	1897.903226	0.214	-1.541688668
10	0.15	991.9354839	0.112	-2.190535589
13	0.145	958.8709677	0.108	-2.224437141
16	0.109	720.8064516	0.081	-2.509823001
20	0.062	410	0.046	-3.074036498
30	0.027	178.5483871	0.020	-3.905334017
40	0.014	92.58064516	0.010	-4.562113554
60	0.005	33.06451613	0.004	-5.591732971

Table 4.10

The following graph shows the plot of ln(E/Eo)-vs-Time for 40% Sucrose :-

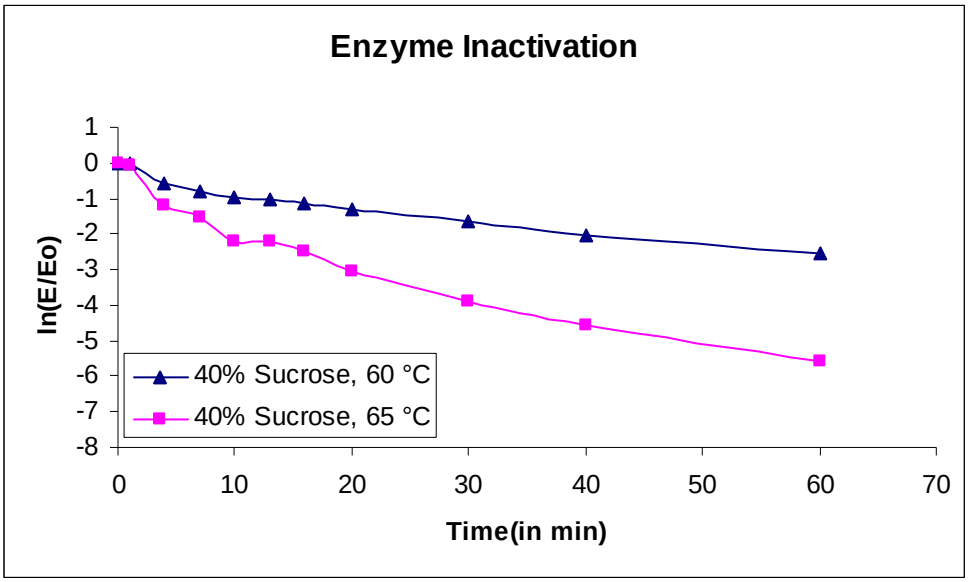

Fig 4.5

4.1.11. Thermal treatment with 20%Trehalose at 60 ^{O}C

Trehalos 20%, 60C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	0.97	6,393.9	1.000	0
1	0.906	5,972.0	0.934	-0.068258224
4	0.258	1,700.6	0.266	-1.324337945
7	0.162	1,067.8	0.167	-1.789701195
10	0.129	850.3	0.133	-2.017485126
13	0.087	573.5	0.090	-2.411389411
16	0.075	494.4	0.077	-2.559809416
20	0.053	349.4	0.055	-2.907005616
30	0.028	184.6	0.029	-3.54509302
40	0.02	131.8	0.021	-3.881565256
60	0.004	26.4	0.004	-5.491003169

Table 4.11

4.1.12. Thermal treatment with 40% Trehalose at 60 ^{O}C

Trehalos 40%, 60 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	2.493	16,433.0	1.000	0
1	1.993	13,137.1	0.799	-0.22385
4	1.342	8,846.0	0.538	-0.61933
7	1.12	7,382.6	0.449	-0.80016
10	1.075	7,086.0	0.431	-0.84117
13	0.894	5,892.9	0.359	-1.02554
16	0.84	5,537.0	0.337	-1.08784
20	0.739	4,871.2	0.296	-1.21595
30	0.55	3,625.4	0.221	-1.51133
40	0.431	2,841.0	0.173	-1.75514
60	0.253	1,667.7	0.101	-2.28786

Table 4.12

The following graph shows the plot of ln(E/Eo)-vs-Time for Trehalose at 60 ^{0}C:-

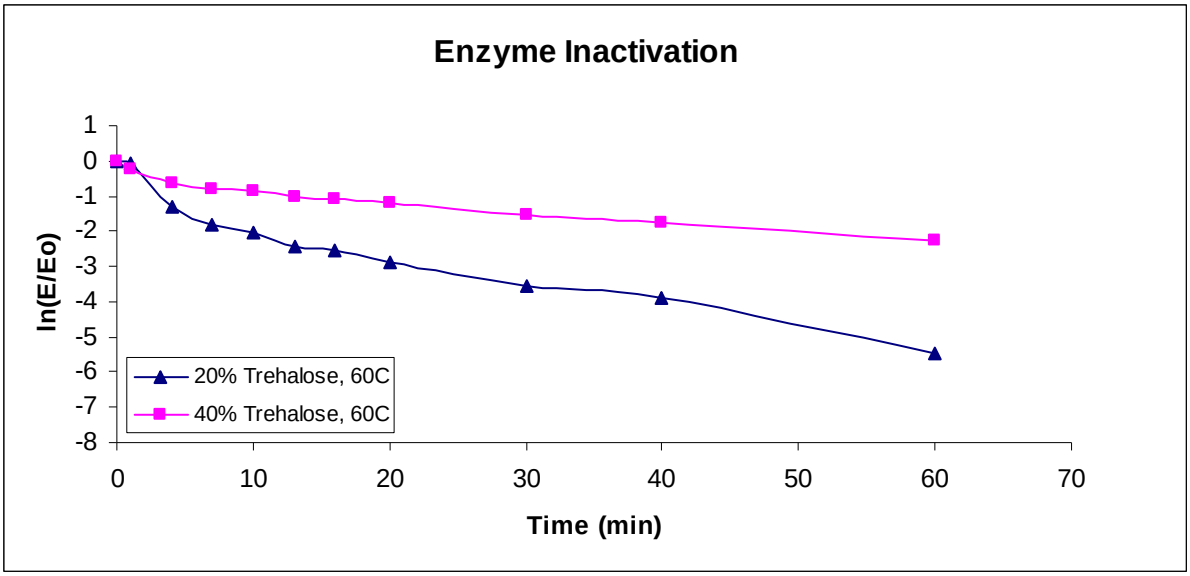

Fig 4.6

4.1.13. Thermal treatment with 20% Trehalose at 65 ^{O}C

Trehalose 20%, 65 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	0.829	5482.096774	1.000	0
1	0.589	3895	0.710	-0.341793971
4	0.083	548.8709677	0.100	-2.301379547
7	0.039	257.9032258	0.047	-3.056658509
10	0.019	125.6451613	0.023	-3.775781176
13	0.012	79.35483871	0.014	-4.235313505
16	0.007	46.29032258	0.008	-4.774310006
20	0.002	13.22580645	0.002	-6.027072975
30	0.002	13.22580645	0.002	-6.027072975
40	0.001	6.612903226	0.001	-6.720220155

Table 4.13

4.1.14. Thermal treatment with 40% Trehalose at 65 ^{O}C

Trehalose 40%, 65 °C				
Time	Slope	Enzyme Activity	E/Eo	ln(E/Eo)
0	2.457	16247.90323	1.000	0
1	2.145	14184.67742	0.873	-0.135801541
4	0.874	5779.677419	0.356	-1.033615997
7	0.514	3399.032258	0.209	-1.564473107
10	0.377	2493.064516	0.153	-1.874451185
13	0.296	1957.419355	0.120	-2.116336918
16	0.241	1593.709677	0.098	-2.321899439
20	0.235	1554.032258	0.096	-2.347110859
30	0.117	773.7096774	0.048	-3.044522438
40	0.076	502.5806452	0.031	-3.475963032
60	0.029	191.7741935	0.012	-4.439400543

Table 4.14

The following graph shows the plot of ln(E/Eo)-vs-Time for Trehalose at 65 ^{0}C:-

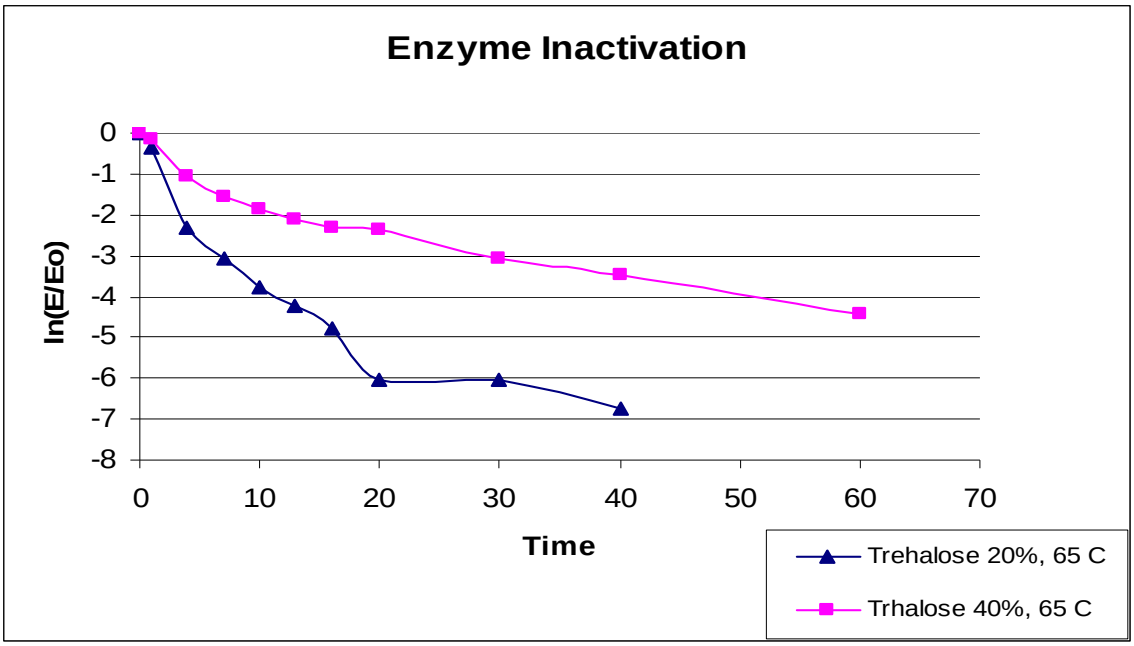

Fig 4.7

4.2 Discussion

The thermal inactivation of yeast alcohol dehydrogenase was carried out at 60 ^{O}C and 65 ^{O}C in the presence of the above mentioned carriers. The enzyme activity was calculated using the formula given in 3.11. The data obtained in each case was plotted in the ln(E/Eo)-vs-Time curve. It was observed that at a given temperature the protection capability of the carriers goes on increasing with increasing concentration i.e. at 60 °C the presence of 40% concentration of Trehalose in the enzyme mixture is more stabilizing in the comparision to 20% concentration. This can be explained by preferential formation of greater degree of H-bonds as well as other bonds with the enzyme.

CHAPTER 5

CONCLUSION

5.1 CONCLUSION:-

It is observed that the enzyme became more stable to thermal treatment for the presence of carriers than their absence. This is explained by the preferential formation of various bonds (H-bond, Ionic bond, Covalent bond, Van der Waal's bond, etc) by the carriers with the enzyme molecule over water. Their presence increases the chemical potential of the enzyme and this increase is proportional to the surface of the enzyme, so that the native folded state is favored over the denatured one. As a general rule, sucrose and other stabilizing solutes are preferentially excluded from the vicinity of the enzyme. This exclusion of sugars is attributed to their water surface tension increasing effect. The addition of sucrose depresses the water activity and that stabilization against denaturation arises from the increased degree of water organization induced by the solutes. The stabilization by sugars is due to the strengthening of hydrophobic interaction in the presence of solutes that reinforce the hydrogen-bonded organization of water.

Also from the graph of ln(E/Eo)-vs-Time we can clearly see a non-linear biphasic thermal inactivation behaviour with increase in time. Nonlinear biphasic ln(activity)-time curves would result when the enzyme system consists of two groups, heat-stable and heat-labile, with differing heat stability (enzyme aggregates formed during inactivation, each with its own thermostability or alternatively, heat-stable and heat-labile isoenzymes). There exists sufficient experimental (e.g., electrophoretic) evidence that this is true and heat-stable and heat-labile isoenzymes have even been isolated in several cases. [3] This nonlinear thermal inactivation behavior could be due to the formation of enzyme groups with different thermal stabilities or the presence of stable/labile isoenzymes. [2] [3]

It was also observed that inorganic salts are better stabilizers than the sugars. It is recommended that more data points be recorded at short times in enzyme inactivation experiments to permit accurate resolution of the faster decay.

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