A STUDY ON THE IMMOBILIZATION OF ENZYMES ON POLYMERIC SUPPORTS

Thesis submitted to
Cochin University of Science and Technology
in partial fulfillment of the requirements
for the Degree of

DOCTOR OF PHILOSOPHY
in
CHEMISTRY

by

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April 2009

CERTIFICATE

This is to certify that the thesis entitled **"A STUDY ON THE IMMOBILIZATION OF ENZYMES ON POLYMERIC SUPPORTS"** submitted for the award of the Degree of Doctor of Philosophy of the Cochin University of Science and Technology is a record of original research work carried out by Ms. Ashly P. C. under my supervision and guidance in the Department of Applied Chemistry, and further that it has not formed the part of any other thesis previously.

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DECLARATION

I hereby declare that the thesis entitled **"A STUDY ON THE IMMOBILIZATION OF ENZYMES ON POLYMERIC SUPPORTS"** submitted for the award of Ph. D. Degree, is based on the original research work done by me under the guidance of Dr. P. V. Mohanan, Lecturer, Department of Applied Chemistry, Cochin University of Science and Technology and further that it has not previously formed the basis for the award of any other degree.

Kochi-22
06.04.2009

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ACKNOWLEDGEMENT

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

Dr. P. V. Mohanan - my supervising guide, for his stimulating suggestions, understanding and constant encouragement during my research

Dr. K. Girish Kumar - Head of the department of Applied Chemistry, for all helps especially in official formalities, made easy road in my research work

Dr. K. Sreekumar - for giving guidelines in my thesis writing

Dr. M. J. Joseph - for detailed and constructive ideas

All the other faculty members, administrative staff, librarian - for helping me run smoothly and for assisting me in many different ways

Navya Antony, Priya Rajan - my labmates for their companionship

Rajesh, Mahesh, Elizabeth, Mangala, Rema, Pearl, Beena, Sindhu, Litha, Resmi, Bolie, Roshini, Manju, Gisha - friends of Department of Applied Chemistry

Divya, Biji - friends of IIT, for their support and caring

Remya Varadarajan, Vanisree - my room mates in hostel for their emotional support, advice, care, and the wonderful moments shared with

Jisha, Sini - my hostelmates for every entertainments

Dr. C. P. Sebastian - my husband for his inspirational and moral support and great patience

My parents and sister - for rising, teaching and loving me

And my dear friends

PREFACE

Enzymes are very efficient catalysts for biochemical reactions produced by living organisms. Many of the biological processes such as conversion of food in to energy are governed by this classical biomaterial. From the beginning, enzymes were existed in the earth, and only 100 years back it was noticed by scientists. Even though enzymes showed its remarkable importance in controlling the life cycle of human being and other organisms, the instability, rather, the denaturation after a prolonged time limited their application in industry. Since then many people devoted their life in the field of enzymes in order to improve its catalytic activity and stability. During the past few decades the technique of enzyme or whole cell immobilization has revolutionized the prospects of enzyme application in industry. Immobilization of biocatalysts helps in their economic reuse and in the development of continuous bioprocesses. Immobilization often stabilizes structure of the enzymes, thereby allowing their applications even under harsh environmental conditions.

This work was focused to study the immobilization of enzymes on polymers. A large range of polymer matrices have been employed as supports for enzyme immobilization. Here polyaniline (PANI) and poly(*o*-toluidine) (POT) were used as supports. PANI and POT provides an excellent support for enzyme immobilization by virtue of its facile synthesis, superior chemical and physical stabilities, and large retention capacity. We selected industrially important starch hydrolyzing enzymes α-amylase and glucoamylase for the study. In this work the selected enzymes were immobilized via adsorption and covalent bonding methods.

To optimize the catalytic efficiency and stability of the resulting biocatalysts, the attempt was made to understand the immobilization effects on enzymatic properties. The effect of pH of the immobilization medium, time of immobilization on the immobilization efficiency was observed. The starch hydrolyzing activity of free α-amylase and glucoamylase were compared with immobilized forms. Immobilization on solid supports

changes the microenvironment of the enzyme there by influences the pH and temperature relationship on the enzymatic activity. Hence these parameters also optimized. The reusability and storage stability of immobilized enzymes an important aspect from an application standpoint, especially in industrial applications. Taking in to consideration of this, the reusability and the long term storage stability of the immobilized enzyme investigated.

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CONTENTS

CHAPTER 1
INTRODUCTION

1.1 Enzyme Catalysis

The catalyst is a substance which either increases or decreases the rate of a reaction without any net change in its structure, and the process is called catalysis. The concept of catalysis was developed during the early 19^{th} century with the discovery of powerful catalysts from biological source. They were called as enzymes and later found to be as proteins. Most of the synthetic and degradative reactions in the living organisms were mediated by the enzymes and were called as enzyme catalysis! Enzymes are often superior to the conventional catalysts and then find their significant part in the high-technological society. Their utilization has created a billion dollar business including a wide diversity of industrial processes, consumer products, and biosensors. Further applications are being discovered constantly.

Compared to the conventional chemical catalysts, enzymes have a lot of distinct advantages. First and most important among these is their specificity and selectivity for a selected reaction. Enzymes catalyze reactions with remarkable degrees of regiospecificity and stereospecifity. They catalyse selectively the reactions of very narrow ranges of reactants (substrates), which may consist of a small number of closely related classes of compounds, a single class of compounds, or a single compound. This clearly indicates that the selected reaction can be catalysed to the exclusion of side-reactions, eliminating undesirable by-products. This selectivity phenomenon creates the higher productivity and lower material cost. Generally, the product obtained via enzyme catalysis is in an uncontaminated state which reduces the further purification costs and environmentally sound good. Compared to the conventional catalysis, enzymes assist to produce the desired products within smaller number of steps. Another important advantage is that enzymes work under generally mild processing conditions of temperature, pressure and pH. Certain stereospecific reactions (e.g. the conversion of glucose into fructose) cannot be achieved by classical chemical methods without a large expenditure of time and effort. This decreases the energy requirements for enzymes again reduce the capital costs due to corrosion-resistant process equipment. The high reaction velocities and straightforward catalytic

regulation achieved in enzyme-catalysed reactions allow an increase in productivity with reduced manufacturing costs.

There are some notable disadvantages in the use of enzymes which cannot be ignored, but which are currently being addressed and overcome. In particular, the high cost of enzyme isolation and purification still discourages their use, especially in areas which currently have an established alternative procedure. The generally unstable nature of enzymes, when removed from their natural environment, is also a major drawback to their more extensive use.

1.2 Enzymes

Enzymes are delicate life-like substance found in all living cells, whether animal or vegetable. Enzymes are energized protein molecules necessary for life. They catalyze and regulate nearly all biochemical reactions that occur within the human body. In other words, enzymes turn the food we eat into energy and unlock this energy for use in the body. They are difficult to see even with the most powerful microscope, but their presence and strength have long been known to scientists based on improved blood and immune system functioning.

Enzymes act by attaching to the reaction molecules, which are called the substrates. Enzymes are highly specific, meaning that each catalyzes only a single reaction or a very limited class of reactions. The specific three-dimensional shape of an enzyme is such that only the substrates it acts upon can "fit" into its active site - the particular portion of the enzyme that binds the substrates. An enzyme functions by lowering the activation energy necessary to initiate a chemical reaction. After catalyzing the reaction, the enzyme releases the products of the reaction. The enzyme remains intact in the process and can immediately bind fresh substrate. Thus, an enzyme molecule can be used over and over again. Enzymes regulate nearly all metabolic activities and are responsible for the building of complex molecules (anabolism) as well as the breakdown of large molecules into smaller ones (catabolism).

1.2.1 Effect of pH and ionic strength on enzyme catalysis

Enzymes are proteins with amino acid side chains containing groups such as -COOR and $-NH_2$ that readily gain or lose H^+ ions. The charges on these groups will vary according to their acid dissociation constants with the pH of their environment. As the pH is lowered an enzyme will tend to gain H^+ ions, and eventually enough side chains will be affected so that the enzyme's shape is disrupted. Likewise, as the pH is raised, the enzyme will lose H^- ions and eventually lose its active shape. Many enzymes perform optimally in the neutral pH range and are denatured at either an extremely high or low pH. Similarly if the ionic concentration is close to zero, the charged amino acid side chains of the enzyme molecules will attract each other. The enzyme will denature and form an inactive precipitate. On the other hand, if the salt concentration is very high, normal interaction of charged groups will be blocked, new interactions will occur, and again the enzyme will precipitate. These effects are especially important in the neighborhood of the active site. An intermediate salt concentration such as that of human blood (0.9 %) or cytoplasm is the optimum for many enzymes. Taken together the change in charges with pH affects the activity, structural stability and solubility of the enzyme.

1.2.2 Effect of temperature

Generally, chemical reactions speed up as the temperature is raised. As the temperature increases, more of the reacting molecules have enough kinetic energy to undergo the reaction. Since enzymes are catalysts for chemical reactions, enzyme reactions also tend to go faster with increasing temperature. However, if the temperature of an enzyme-catalyzed reaction is raised still further, a temperature optimum is reached; above this value the kinetic energy of the enzyme and water molecules is so great that the conformation of the enzyme molecules is disrupted. The positive effect of speeding up the reaction is now more than offset by the negative effect of changing the conformation of more and more enzyme molecules. Many proteins are denatured by temperatures around 40-50 °C, but some are still active at 70-80 °C, and a few even withstand boiling.

1.2.3 Effect of activators and inhibitors

Many molecules other than the substrate may interact with an enzyme. If such a molecule increases the rate of the reaction it is an activator, and if it decreases the reaction rate it is an inhibitor. These molecules can regulate how fast the enzyme acts. Any substance that tends to unfold the enzyme, such as an organic solvent or detergent, will act as an inhibitor. Some inhibitors act by reducing the -S-S- bridges that stabilize the enzyme's structure. Many inhibitors act by reacting with side chains in or near the active site to change its shape or block it. Many well-known poisons such as potassium cyanide and curare are enzyme inhibitors that interfere with the active site of critical enzymes.

1.2.4 Enzyme kinetics

Enzyme kinetics and theories of enzyme action were pioneered by Michaelis and Menten, as well as other investigators. According to the Michaelis-Menten hypothesis, enzyme E and substrate S combine reversibly to form an enzyme-substrate complex ES, which then reversibly dissociates to form product P and the regenerated enzyme E as depicted below.

$$\mathbf{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathbf{ES} \xrightarrow{k_2} \mathbf{E + P} \qquad 1.1$$

Where k_1, k_{-1} and k_2 are rate constants for the individual steps.

Enzyme action can be modulated by a number of factors. One of the most fundamental factors affecting enzyme activity is substrate concentration. The effect of substrate concentration [S] on velocity [*V*] of reaction is usually expressed using a Michaelis-Menten plot such as the one shown below in *fig.1.1*, and enzymes which generate such a plot are said to obey Michaelis-Menten kinetics. Michaelis-Menten plots show three distinct regions which correspond to reaction order. At low [S], the reaction accelerates as more substrate is added, reflecting first-order kinetics. At high [S], the concentration of enzyme becomes limiting, and additional substrate cannot accelerate the

reaction. This situation is known as zero-order kinetics. Finally, there is a transition period between first order and zero order where kinetics is mixed.

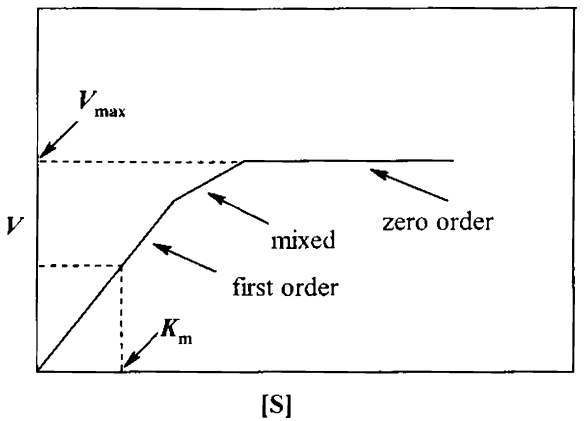


Fig. 1.1 Saturation curve for an enzyme showing the relation between the concentration of substrate and rate.

The point in Y-axis, V_{max}, is the maximum rate of reaction for a given concentration of enzyme. A second kinetic constant derived by drawing a line from the Y-axis at 1/2 V_{max} to the curve, and then down to the X-axis is known as the K_m, is the concentration of substrate needed to drive the reaction at half V_{max}. Each substrate will generate a unique K_m and V_{max} for a given enzymatic process.

A standard equation used to express the kinetic constants under the Michaelis-Menten hypothesis is aptly called the Michaelis-Menten equation, and is shown below. Later, two other investigators rearranged this equation to generate a second useful equation, the Lineweaver-Burk equation, is also given below.

The Michaelis-Menten equation

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad 1.2$$

The Lineweaver-Burk equation

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \qquad 1.3$$

The Lineweaver-Burk equation is in the form y = mx + b, and as such, a plot of this equation will generate a straight line for enzymes obeying simple Michaelis-Menten kinetics. In addition, the x and y values for the plot are both inverted, and as such, the plot is often referred to as the double reciprocal plot. The Lineweaver-Burk plot has two advantages over the Michaelis-Menten plot, in that it gives a more accurate estimate of V_{max}, and it gives more accurate information about inhibition. A typical Lineweaver-Burke plot is shown in *fig.1.2*. V_{max} is derived from the y-intercept, and K_m can be derived either from the slope, or from extrapolating the line to the negative X-axis.

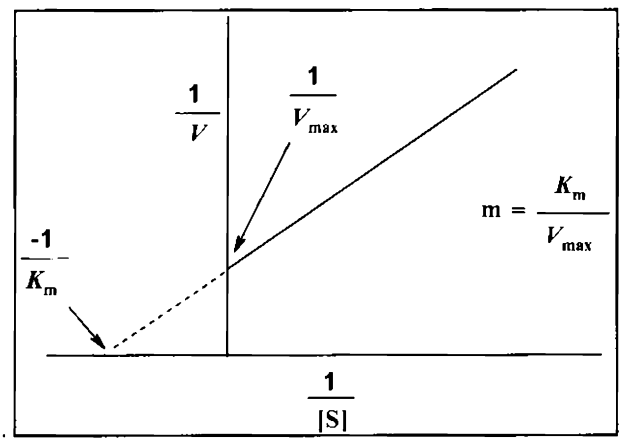


***Fig. 1.2** Lineweaver-Burk or double-reciprocal plot of kinetic data, showing the significance of the axis intercepts and gradient.*

Hanes-Woolf plot is also a graphical representation of enzyme kinetics in which the ratio of the initial substrate concentration [S] to the reaction velocity V is plotted against [S]. It is based on the rearrangement of the Michaelis-Menten equation shown below.

$$\frac{[S]}{V} = [S]\frac{1}{V_{max}} + \frac{K_m}{V_{max}} \qquad 1.4$$

It is clear from the equation, that perfect data will yield a straight line of slope $1/V_{max}$, a y-intercept of K_m/V_{max} and an x-intercept of $-K_m$.

Several other types of plots each with advantages and disadvantages can also be used to determine K_m and V_{max} from kinetic data. Nevertheless, Lineweaver-Burk plots are valuable for visual presentation of kinetic data.

1.3 Enzyme Immobilization

Since the second half of the last century, numerous efforts have been devoted to the development of insoluble immobilized enzymes for a variety of applications [1]; these applications can clearly benefit from the use of immobilized enzymes rather than the soluble counterparts, for instance as reusable heterogeneous biocatalysts, with the aim of reducing production costs by efficient recycling and control of the process [2, 3], as stable and reusable devices for analytical and medical applications [4–10], as selective adsorbents for purification of proteins and enzymes [11], as fundamental tools for solid-phase protein chemistry [12, 13] and as effective microdevices for controlled release of protein drugs [14].

Immobilized enzymes have been defined as enzymes that are physically confined or localized, with retention of their catalytic activity, and which can be used repeatedly and continuously [15-17]. This limitation of mobility may be achieved by widely different methods, ranging from covalent chemical bonding to physical entrapment. However an immobilized enzyme must comprises two essential functions, namely the non-catalytic functions that are designed to aid easy separation (e.g. isolation of catalysts from the application environment, reuse of the catalysts and control of the process) and the catalytic functions that are designed to convert the substrates within the time and space desired. Non-catalytic functions depend upon the physical and chemical nature of the non-catalytic part

of the immobilized enzymes, especially the geometric properties, e.g. the shape, size, thickness, and length of the selected carrier, whereas the catalytic functions are linked to the catalytic properties, for example activity, selectivity, and stability, pH and temperature profile.

1.3.1 Methods of immobilization

It is more important to choose the method of immobilization which will prevent the loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of enzyme. Considerable knowledge in the nature of the active site of the enzyme will be helpful to achieve this task. Alternatively, an active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity. In some cases, this protective function can be fulfilled by a substrate or a competitive inhibitor of the enzyme. There are four principal methods available for immobilizing enzymes as explained below.

1.3.1.1 Adsorption

Adsorption of enzymes onto insoluble supports is a very old and simple method of wide applicability and capable of high enzyme loading relative to other immobilization methods. Enzymes can be immobilized by simply mixing the enzymes with a suitable adsorbent, under appropriate conditions of pH and ionic strength. After washing off loosely bound and unbound enzyme, the immobilized enzyme is obtained in a directly usable form. Adsorption process is based on van der Waals forces, ionic and hydrogen bonding as well as hydrophobic interactions. These forces are very weak, but in large number, impart sufficient binding strength. The particular choice of adsorbent depends principally upon minimizing leakage of the enzyme during use. Existing surface properties of enzymes and support are utilized so that no chemical modification is required and little damage is done to enzyme. Although the physical links between the enzyme molecules and the support are often very strong, they may be reduced by many factors including the introduction of the substrate. Care must be taken that the binding forces are not weakened during use by inappropriate changes in pH or ionic strength. Physical adsorption generally leads to

dramatic changes in the protein microenvironment, and typically involves multipoint protein adsorption between a single protein molecule and a number of binding sites on the immobilization surface [18]. The main disadvantage of this method is that the biocatalyst is easily desorbed by factors like temperature fluctuations, changes in substrate and ionic concentrations [19] *etc.*, so that reaction conditions should be maintained constant throughout the reaction. Adsorbents can be selected from a wide variety of inorganic and organic substances as well as natural and synthetic polymers.

1.3.1.2 Covalent binding

The most intensely studied immobilization technique is the formation of covalent bonds between the enzyme and the support matrix. Enzymes are linked to the support through the functional groups in the enzymes which are not essential for the catalytic activity. The binding reaction must be performed under conditions that do not cause loss of enzymic activity, and the active site of the enzyme must be unaffected by the reagents used. The functional groups of proteins suitable for covalent binding under mild conditions include the alpha amino groups of the chain and the epsilon amino groups of lysine and arginine, the alpha carboxyl group of the chain end and the beta and gamma carboxyl groups of aspartic and glutamic acids, the phenol ring of tyrosine, the thiol group of cysteine, the hydroxyl groups of serine and threonine, the imidazile group of histidine, and the indole group of tryptophan. A small number of reactions have been designed to couple with functional groups on the protein other than the amino and phenolic residues. It is possible in some cases to increase the number of reactive residues of an enzyme in order to increase the yield of immobilized enzyme and to provide alternative reaction sites to those essential for enzymatic activity. Sometimes functional groups on the support material are activated by certain reagents and enzyme is then coupled to the support material via covalent linkage. Coupling agents used include cyanogen bromide, carbodiimide, reagents used for diazotization *etc.* The connection between the carrier and enzyme can be achieved either by direct linkage between the components or via an intercalated link of differing length, which is called spacer. The spacer molecule gives a greater degree of mobility to the coupled biocatalyst so that its activity can be enhanced when compared to that of direct

coupled biocatalyst. The wide variety of binding reactions, and insoluble carriers with functional groups capable of covalent coupling, or being activated to give such groups, makes this a generally applicable method of immobilization, even if very little is known about the protein structure or active site of the enzyme to be coupled.

1.3.1.3 Entrapment

Entrapment restricts the movement of enzymes in a porous gel, yet keeping them as free molecules in solution. Entrapment of enzymes within gels or fibers is a convenient method for use in processes involving low molecular weight substrates and products. However, the difficulty which large molecules have in approaching the catalytic sites of entrapped enzymes precludes the use of entrapped enzymes with high molecular weight substrates. The entrapment process may be a purely physical caging or involve covalent binding. Biocatalysts have been entrapped in natural polymers like agar, agarose and gelatine through thermo reverse polymerization, but in alginate and carrageenan by ionotropic gelation. A number of synthetic polymers like polyvinylalcohol hydrogel [20], polyacrylamide [21, 22] have also been investigated. Entrapment is the method of choice for the immobilization of microbial, animal and plant cells, where calcium alginate is widely used.

Membrane confinement or encapsulation is similar to entrapment in restricting movement of enzyme molecules, but this time in a semipermeable membrane. This membrane retains the high molecular weight enzymes, while allowing low molecular weight compounds to diffuse in and out. The commonly used membranes are nylon, cellulose, polysulfone and polyacrylate.

1.3.1.4 Cross-linking

This method involves attachment of biocatalysts to each other by bi- or multi-functional reagents or ligands. In this way, very high molecular weight typically insoluble aggregates are formed. Cross-linking is a relatively simple process. It is not a preferred method of immobilization as it does not use any support matrix. So they are usually

gelatinous and not particularly firm. Since it involves a bond of the covalent kind, biocatalyst immobilized in this way frequently undergoes changes in conformation with a resultant loss of activity. Still it finds good use in combination with other support dependent immobilization technologies, namely to minimize leakage of enzymes already immobilized by adsorption. The most commonly used bifunctional agent for cross-linking is glutaraldehyde. The reactive aldehyde groups at the two ends of glutaraldehyde react with free amino groups of enzymes through a base reaction and have been extensively used in view of its low cost, high efficiency, and stability. The enzymes or the cells have been normally cross-linked in the presence of an inert protein like gelatine, albumin, and collagen and can be applied to either enzymes or cells. The main disadvantages are the undesirable activity losses that can arise from the participation of catalytic groups in the interactions responsible for the immobilization. The cross-linking reaction is not easily controlled and so it is very difficult to obtain large enzyme aggregates with high activity retention. The gelatinous physical nature of the immobilized enzyme preparations is a great limitation in many applications.

Although the basic methods of enzyme immobilization can be categorized into a few different methods as mentioned above, hundreds of variations, based on combinations of these original methods, have been developed [23]. Correspondingly, many carriers of different physical and chemical nature or different occurrences have been designed for a variety of bio-immobilizations and bio-separations [24-26].

1.3.2 Choice of support for immobilization

The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization, although it is difficult to predict in advance which support will be most suitable for a particular enzyme. Important requirements for a support material are that it must be insoluble in water, have high capacity to bind enzyme, be chemically inert and be mechanically stable. The activity of the immobilized enzyme will also depend upon the bulk mass transfer and local diffusion properties of the system. The morphology of the support also plays an important role in the immobilization process

which affects enzyme's expressed activity and apparent kinetics considerably. Their form, shape, density, porosity, pore size distribution, operational stability and particle size distribution influence the reactor configuration in which the immobilized biocatalyst may be used. The surface density of binding sites together with the volumetric surface area sterically available to the enzyme, determine the maximum binding capacity. The binding capacity also depends on electrostatic charge distribution and surface polarity (*i.e.* the hydrophobic-hydrophilic balance) on both enzyme and support. A good support also increases the enzyme specificity whilst reducing product inhibitions, shift the pH optimum to the desired value for the process, and discourage microbial growth and non-specific adsorption. Sometimes matrices with special properties are selected, which are useful for particular purposes such as magnetism which enables transfer, stabilization and separation of the biocatalyst by means of magnetic fields [27-35], conducting polymers for the fabrication of biosensor [36-45], nano particles and fibers due to their large surface area for high enzyme loading [46-49], membrane surfaces for the operation in continuous systems due to low pressure drop, short residence time and low external and internal diffusional resistances [50, 51] or nonporous support to eliminate diffusion constrains [52-54]. And finally the overall process cost of the support is relevant to their use in the industrial process, either they should be cheap enough to discard or can be regenerated after the useful lifetime of the immobilized enzyme. Due to the often conflicting requirements of a good support, various materials have been used. At present there is not a universally recommended support, the final choice being a compromise for each particular enzyme and experimental system.

1.3.3 Support activation and enzyme attachment

An activated support is defined as a material having an enzyme reactive functional group covalently attached to an otherwise inert surface. The stability of the resulting bond between the enzyme and the support, the local environment of the enzyme and the potential loss of activity on immobilization must all be considered.

Activation with glutaraldehyde is the commonly used procedure since it introduces a spacer arm between support and enzyme. The spacer molecule gives a greater degree of mobility to the coupled biocatalyst so that its activity can under certain circumstances, be higher than if it is bound directly to the carrier. Other general schemes for support activation are diazotization or reaction with carbodiimides, thiophosgene, thionyl chloride, N-hydroxysuccinimide or transition metal salts such as titanium chloride. The activated support is then covalently linked to the enzyme, most commonly via direct reaction with available amino functions, e.g. on lysine residues, but also via thiol and phenol functions.

1.3.4 Properties of immobilized enzymes

It is important to understand the changes in physical and chemical properties which an enzyme would be expected to undergo upon immobilization. Changes have been observed in the stability, pH and temperature dependence and kinetic properties of enzymes because of the microenvironment imposed upon them by the supporting matrix and by the products of their own action. The extent of such changes depends upon the enzyme, carrier used, and method of immobilization.

1.3.4.1 Stability

The stability of a native enzyme (*i.e.* a non-immobilized or modified enzyme) is principally determined by its intrinsic structure whereas the stability of an immobilized enzyme is highly dependent on many factors, including the nature of its interaction with the carrier, binding position and number of bonds, the freedom of the conformation change in the matrix, the microenvironment in which the enzyme molecule is located, the chemical and physical structure of the carrier, the properties of the spacer (for example, charged or neutral, hydrophilic or hydrophobic, size, length) linking the enzyme molecules to the carrier, and the conditions under which the enzyme molecules were immobilized. Hence the stability of the immobilized enzymes with respect to time, temperature and other storage conditions and experimental variables might be expected to either increase or decrease on immobilization. The rate of immobilized enzyme inactivation is often found diminished in comparison with free enzyme. The steric hindrance of the immobilized enzymes towards

high molecular substrates can be useful for shielding them from the attack of microorganisms and exogenous proteases [55].

1.3.4.2 Activity

It has been found that many enzymes immobilized by different immobilization techniques have higher activity than the native enzymes. For instance, epoxy hydrolase adsorbed on DEAE-cellulose by ionic bonding was more than twice as active as the native enzyme [56], lipase–lipid complex entrapped in n-vinyl-2-pyrrolidone gel matrix was 50-fold more active than the native enzyme [57]. Activation by immobilization is, however, often regarded as an additional benefit rather than a rational goal of enzyme immobilization. Activity retention by carrier-bound immobilized enzymes is usually approximately 50 %. At high enzyme loading, especially, diffusion limitation might occur as a result of the unequal distribution of the enzyme within a porous carrier, leading to a reduction of apparent activity [58]. The conditions for high activity retention are often marginal, thus often requiring laborious screening of immobilization conditions such as enzyme loading, pH, carrier and binding chemistry [59]. The immobilized enzymes can be more active than the native enzymes, when the inhibiting effect of the substrate was reduced. For example, immobilization of invertase from *Candida utilis* on porous cellulose beads led to reduced substrate inhibition and increased activity [60]. A positive partition effect (enrichment of substrates in the proximity of the enzymes) might also enhance enzyme activity as was observed for kinetically controlled synthesis of ampicillin with penicillin G acylase immobilized on a positively charged carrier [61] or horse liver alcohol dehydrogenase immobilized on poly(methylacrylate-co-acrylamide) matrix [62, 63].

1.3.4.3 Kinetic properties

Changes in activity of enzymes due to the actual process of immobilization have not been studied very much. There is usually a decrease in specific activity of an enzyme upon immobilization, and this can be attributed to denaturation of the enzymic protein caused by the coupling process. Once an enzyme has been insolubilized, however, it finds itself in a microenvironment that may be drastically different from that existing in free solution. The

new microenvironment may be a result of the physical and chemical character of the support matrix alone, or it may result from interactions of the matrix with substrates or products involved in the enzymatic reaction.

The Michaelis constant has been found to decrease by more than one order of magnitude when substrate of opposite charge to the carrier matrix was used. Again, this only happened at low ionic strengths, and when neutral substrates were used. The diffusion of substrate from the bulk solution to the micro-environment of an immobilized enzyme can limit the rate of the enzyme reaction. The rate at which substrate passes over the insoluble particle affects the thickness of the diffusion film, which in turn determines the concentration of substrate in the vicinity of the enzyme and hence the rate of reaction. The effect of the molecular weight of the substrate can also be large. Diffusion of large molecules will obviously be limited by steric interactions with the matrix, and this is reflected in the fact that the relative activity of bound enzymes towards high molecular weight substrates has been generally found to be lower than towards low molecular weight substrates. This, however, may be an advantage in some cases, since the immobilized enzymes may be protected from attack by large inhibitor molecules.

1.3.5 The advantages of immobilized enzymes

The main objective of enzyme immobilization was to maximize the advantages of enzyme catalysis, which is possible by using a support with low synthesis cost and high binding capacity. The other important aspect to be considered was the possibilities of reaction interruption by removing the immobilized enzyme, conferring to these systems refine control over product formation, which was not possible when enzyme was free in solution. Some of the advantages of the immobilized enzymes over free enzymes are as follows.

- Easy separation from reaction mixture, providing the ability to control reaction times and minimize the enzymes lost in the product.
- Re-use of enzymes for many reaction cycles, lowering the total production cost of enzyme mediated reactions.

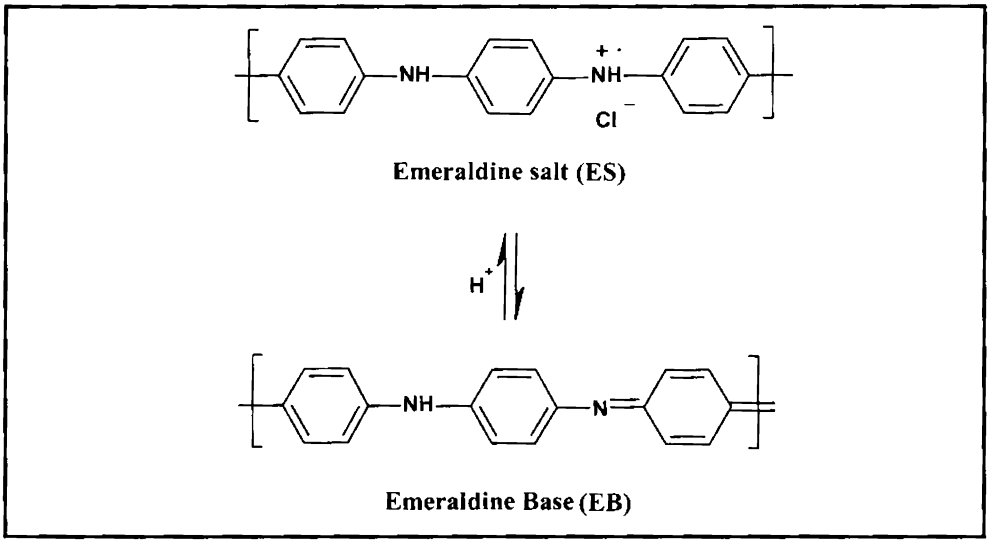
- ❖ Ability of enzymes to replace multiple standard chemical steps and provide enatomerically pure products.
- ❖ The ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or *vice versa*).
- ❖ Product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries).
- ❖ Enzymes are usually stabilized by immobilization.
- ❖ Analytical purposes - long 1/2-life, predictable decay rates, elimination of reagent preparation, *etc.*

1.4 Polyaniline and Poly(*o*-toluidine) as Supports for Enzyme Immobilization

Enzymes have been covalently bonded to a wide variety of water-insoluble polymeric supports. Synthetic polymers were already being employed for coupling biologically active materials in the 1950's. The use of synthetic polymers as supports for enzyme immobilization provides several advantages, e.g. inertness to micro-organism attack, higher chemical resistance, and the option to use complex buffer components. Recently, the class of organic polymers called 'conducting polymers' has attracted considerable attention and has been used as supports for enzyme immobilization due to direct electron transfer between enzyme and polymer and thus potential usability as sensing materials [64, 65]. Polyaniline (PANI) and substituted PANI occupy an important place among these, conducting polymers because of their unique properties, which include good environmental stability, the simplicity of their doping process and the excellent redox recyclability enabling polymers with significant differences to be constructed by means of simple acidic or basic treatments. Thus PANI and related polymers are potentially attractive materials for the applications in the field of biochemical engineering [66, 67]. Besides all these interesting properties, chemically synthesized PANI's and substituted PANI's are easy to prepare with high synthesis yield, they are highly stable to extremes of temperature and pH, resistant to attacks from micro-organisms, are stable in oxygen atmosphere and

contains amino groups, and are suitable polymeric supports for the immobilization of biomolecules [68] with and without the use of spacer molecules.

Chemically synthesized polyaniline and poly(*o*-toluidine) hydrochloride salts are green in color. They are known to exist in a variety of forms differing in color, some of which are not electrically conducting [69]. The most important form, protonated salt (doped) are green and electrically conducting; they are produced directly by the oxidative polymerization of the monomers, usually as the hydrochloride forms. On direct treatment with alkali, they are converted to the blue, non-conducting base (undoped). The interconversion between the salt and base forms is illustrated by the following scheme (*scheme 1.1*).

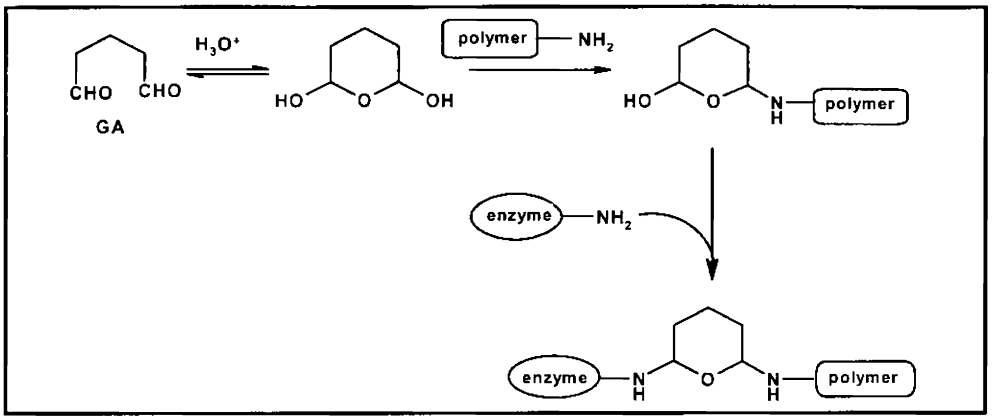


Scheme 1.1 *The interconversion between the salt and base forms of emeraldine.*

1.4.1 Activation of polymers with glutaraldehyde

Glutaraldehyde (GA) is one of the most popular bifunctional reagents to immobilize proteins on solid supports for use in enzyme reactors, affinity chromatography and in immunochemical research [70-72]. The glutaraldehyde activation is simple and efficient.

Even it allows improving enzyme stability by multipoint or multisubunit immobilization [73-75]. This invariably gives high yields of immobilized protein on the support. Glutaraldehyde has also been used to introduce intermolecular cross-linking in proteins [76-78].



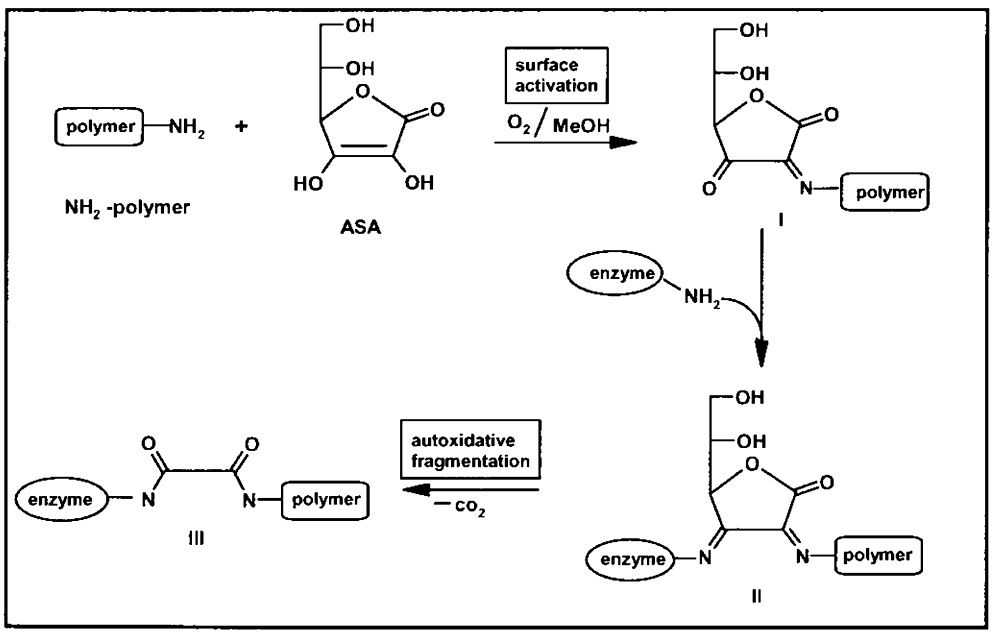
Scheme 1.2 *Proposed mechanism for the immobilization of enzyme on NH_2 polymer via glutaraldehyde as cross-linking agent.*

Bonding of enzyme with support by glutaraldehyde chemistry should provide stable, insolubilized enzyme derivatives that do not leach enzyme into the surrounding solution. Only mild conditions are required to activate aminated supports with glutaraldehyde, having between one or two molecules of glutaraldehyde per primary amino groups, and the use of more drastic conditions (pH over 8 and higher glutaraldehyde concentration above 15 %) promoted uncontrolled glutaraldehyde polymerization [79]. It was believed that the enzyme was immobilized via the covalent bond formation through glutaraldehyde on the support. Reaction of aldehyde with amino group of protein is expected to be the formation of Schiff's base structure, but according to *Walt et al.* [80] these bases are highly labile in acidic condition and they break down to regenerate aldehyde and amine. But as there were no signs of free amino and aldehyde group after immobilization, they suggested a different reaction mechanism. Under acidic and neutral solutions, glutaraldehyde exists as a monomer in its free aldehyde from, hydrate or

hemiacetal. According to them, cyclic hemiacetal form of glutaraldehyde in diluted acidic and neutral solution was responsible for the immobilization reaction. Hence for monomeric glutaraldehyde, they recommended the reaction depicted in *scheme 1.2*, but the exact mechanism of immobilization of protein via glutaraldehyde is still under discussion [81, 82].

1.4.2 Activation of polymers with ascorbic acid

The pathway of enzyme immobilization via ascorbic acid (ASA) was proposed by *Tiller et al.* [83]. It was based on the assumption that ASA in methanol reacts with amino group of NH_2 polymer and also enzyme protein forming dehydroascorbic acid derivatives with neighboring Schiff's base structures. These undergo subsequent fragmentation reaction and form stable oxalic acid diamide derivatives as coupling structures between enzyme and polymer. The proposed reaction scheme is shown below (*scheme 1.3*).



Scheme 1.3 *Proposed pathway for the immobilization of enzyme on an NH_2 polymer by bifunctional ASA reaction.*

In the activation step the NH_2 groups of the polymer react with methanolic ASA, to form dehydroascorbic acid derivative (**I**). It was then treated with enzyme in buffer for immobilization. The β-keto group of the ASA reacts with NH_2 group in the protein resulting neighboring Schiff's base structure (**II**). These are not stable and undergo (O_2-dependent) autoxidative fragmentation reaction forming oxalic acid diamide bridge structure (**III**).

1.5 Objectives of the Thesis

The primary goal of the present work was to incorporate enzymes into polymers and study the enzyme behavior once inserted in the polymer matrices. Two different starch converting enzymes were used: α-amylase and glucoamylase, because they are easy to handle in the laboratory atmosphere as well as are most economical industrial enzymes, especially for food and pharmaceutical purposes. Adsorption and covalent bonding methods are used as they are gentle and simple methods, which provide good immobilization efficiency. In order to design an effective immobilized enzyme matrix, the influence of several immobilization parameters in the immobilization were investigated such as enzyme to polymer weight ratio, processing time of immobilization and pH of the reaction medium during immobilization. The kinetic parameters, properties and stabilities of the immobilized enzyme under optimum conditions were determined. The main objectives of the thesis are summarized as follows;

- ***Preparation of polyaniline and poly(o-toluidine) in protonated salt and base form, and their activation by ascorbic acid and glutaraldehyde.***
- ***Immobilization of enzymes, α-amylase and glucoamylase on polyaniline and poly(o-toluidine) via adsorption and covalent bonding.***
- ***Optimization of immobilization conditions, pH of the immobilization medium and time for immobilization.***

- ***Characterization of polymers before and after immobilization via various physico-chemical techniques like FT-IR spectroscopy, TG-DTG analysis, surface area measurement and SEM.***
- ***Study on the efficiency of immobilization on various polymers.***
- ***Estimate the activity and efficiency of the immobilized enzyme on the hydrolysis of starch.***
- ***Study on the Influence of the parameters, pH and temperature on the activity and stability of free and immobilized enzymes, comparison of enzymatic properties with native enzyme.***
- ***Evaluation of kinetic constants K_m and V_{max}.***
- ***Study on the reusability and long term storage stability.***

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CHAPTER 2
EXPERIMENTAL TECHNIQUES

2.1 Materials Used

Amylglucosidase ex. *Rhizopus*	Sisco Research Laboratories Pvt. Ltd. Mumbai
Ammonium persulphate	s. d. fiNE cHEM LTd. Mumbai
Albumin Bovine	Sisco Research Laboratories Pvt. Ltd. Mumbai
L-ascorbic acid	s. d. fiNE cHEM LTd. Mumbai
Diastase α-amylase	HiMedia Laborotories Pvt.LTd Mumbai
Dextrose anhydrous	s. d. fiNE cHEM LTd. Mumbai
3,5-Dinitrosalicylic acid	s. d. fiNE cHEM LTd. Mumbai
Folin & Ciocalteu's Phenol Reagent	Sisco Research Laboratories Pvt. Ltd. Mumbai
Glutaraldehyde	LOBA CHEMIE PVT. LTD. Mumbai
Aniline	s. d. fiNE cHEM LTd. Mumbai
O-toluidine	SPECTROCHEM PVT. LTD. Mumbai
Starch soluble (potato)	s. d. fiNE cHEM LTd. Mumbai

2.2 Preparation of Polymers

2.2.1 Preparation of emeraldine salt (ES)

ES was prepared by chemical oxidation of 0.2 M of aniline hydrochloride with 0.25 M ammonium peroxydisulphate according to literature procedure [1, 2]. The efficient polymerization of aniline is achieved only in acidic medium; where aniline exist as anilinium cation. Here, hydrochloric acid was used in equimolecular proportion of aniline so that aniline hydrochloride formed *in situ* was used as the monomer. Peroxydisulphate is the most commonly used oxidant, and its ammonium salt was preferred to potassium counterpart because of its better solubility in water. The recommended stoichiometric ratio of peroxidisulphate to aniline was 1.25:1 [1]. The polymerization was completed within 1 hour at 0-2 °C, since the reaction was exothermic, low temperature was preferred.

Aniline (3.7 mL, 0.2 M) was dissolved in 0.2 M hydrochloric acid in a volumetric flask to 200 mL of solution. Ammonium peroxydisulfate (11.4 g, 0.25 M) was dissolved in distilled water to 200 mL as well. Both solutions were kept for 1 hour in an ice bath, mixed

in a beaker, stirred and left at rest to polymerize at room temperature for 1 hour. The green precipitate was collected on a filter, washed with 100 mL 1 M hydrochloric acid to remove the residual monomer, the oxidant and its decomposition products. The treatment with HCl provides more uniform protonation of ES. It was washed with distilled water and then 300 mL acetone to remove the low molecular weight organic intermediates and oligomers. It also prevents the aggregation of ES during drying, so that product was obtained as fine powder. The resulting protonated emeraldine was dried at room temperature for 3 hour and further in an oven at 70 °C for 3 hour.

2.2.2 Preparation of emeraldine base (EB)

The emeraldine base was prepared by deprotonation of emeraldine salt [3, 4]. For that ES was stirred with 0.1 M NaOH for 24 hour. It was then filtered, washed several times with distilled water and dried.

2.2.3 Preparation of poly(*o*-toluidine) salt (TS)

Poly(*o*-toluidine) was prepared in a manner analogous to polyaniline by oxidation of *o*-toluidine hydrochloride with ammonium persulphate in aqueous acidic media [5]. *O*-toluidine (4.4 mL, 0.2 M) was dissolved in 200 mL of 1 M HCl, and the solution was cooled to 0-5 °C in an ice bath. A few drops of $FeSO_4$ solution were added as catalyst. A pre-cooled solution of 4.6 g of $(NH_4)_2S_2O_8$ in 100 mL of distilled water was added drop wise with vigorous stirring during a period of 10 minutes. Ten to fifteen minutes after the reactants were mixed; the solution started to show a green tint and became intense green as a precipitate was formed. After ~2 hour, the precipitate was collected on a Buchner funnel. The blue-green powder was transferred into a beaker containing 200 mL of 1 M HCl to ensure complete protonation. After stirring at room temperature for 10 hour, the mixture was filtered, and the precipitate was washed with 500 mL of 1 M HCl until the filtrate became colorless. It was dried in air and stored.

2.2.4 Preparation of poly(*o*-toluidine) base (TB)

For the conversion of poly(*o*-toluidine) hydrochloride into poly(*o*-toluidine) base, the hydrochloride was suspended in aqueous NaOH (100 mL of 0.5 M) with stirring for 16 hour at room temperature [5]. The pH of the solution was periodically adjusted to ~10 (pH paper test) by the addition of drops of 1 M NaOH. The suspension was filtered, and the precipitate was washed with ~ 400 mL of 0.1 M NaOH followed by five 50 mL portions of a 1:1 mixture of methanol and 0.2 M NaOH and finally with distilled water and dried.

2.3 Activation of Polymers

2.3.1 Activation with glutaraldehyde

Activation of the support with glutaraldehyde followed by the immobilization of the enzyme is one of the most popular techniques to immobilize enzymes. The method is quite simple and even allows improving stability of immobilized enzyme [6]. All the polymers, emeraldine salt (ES), emeraldine base (EB), poly(*o*-toluidine) salt (TS) and poly(*o*-toluidine) base (TB) prepared were activated with glutaraldehyde for covalent immobilization of enzymes to obtain ES1, EB1, TS1 and TB1 respectively according to the methodology described by *Olsson* and *Örgen* [7]. 1 g each of the dried polymers was mixed with 2.5 % (v/v) glutaraldehyde solution prepared in 0.1 mol L^{-1} potassium phosphate buffer on pH 6. The mixture was allowed to react under reflux for 2 hour. All supports were washed with the same buffer to free of excess glutaraldehyde and dried.

2.3.2 Activation of polymers with ascorbic acid

The bifunctional reaction of ascorbic acid (ASA) with amino compounds can be adapted in to enzyme immobilization on support materials which contain NH_2 groups [8]. 1 g each of ES, EB, TS and TB was stirred with 1 g (5.7 mmol) of ASA dissolved in 50 mL methanol. The reaction mixture was warmed slightly for 30 minutes. It was then filtered, washed with distilled water and dried to obtain ES2, EB2, TS2, and TB2 respectively.

2.4 Preparation of Immobilized Enzymes

In order to immobilize the enzyme, the polymer powder was mixed with equal volume of enzyme solution and buffer of the desired pH. It was shaken in a water bath shaker at room temperature for one hour and centrifuged [9, 10]. It was washed several times with the same buffer to remove the unbound enzyme. The biocatalyst was filtered using a sintered glass filter and dried in airflow. It was stored in refrigerator at 4 °C and used for further studies. The supernatant and washings were tested for any unbound enzyme.

2.4.1 Optimization of immobilization procedure

The influence of pH, time of incubation, and enzyme concentration on immobilization were determined by carrying out immobilization at various pH (3-8), contact time (15-90 minutes) and enzyme concentration of 0.5-2.5 mg glucoamylase and 1-6 mg α-amylase. The immobilized polymers were analyzed for enzyme activity under constant condition. Sodium acetate-acetic acid (0.2 M, pH 3-5.5) and sodium phosphate (0.2 M, pH 5.6-8) were used as buffers for optimization studies.

2.4.2 Estimation of protein

The amount of protein present in the solution before immobilization, both in the filtrate and washings were determined by the method proposed by *Lowry et al.* [11] using Folin & Ciocalteus Phenol reagent and measuring absorption at 730 nm in Spectro UV-Vis Double Beam UVD-3500 spectrophotometer. The amount of protein on the support was calculated from the difference between the amount of protein initially present in the reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

2.4.3 Immobilization efficiency

Immobilization yield (**IY**) was calculated by measuring concentration of protein in the supernatants before and after immobilization, according to equation 2.1 below.

$$IY\ (\%) = \frac{C1 - C2}{C1} \times 100 \qquad 2.1$$

Where, C1 was the concentration of protein taken for immobilization and C2 was the concentration of protein present in supernatant after immobilization.

And the activity yield was determined by the equation 2.2.

$$AY\ (\%) = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of free enzyme}} \times 100 \qquad 2.2$$

The immobilization efficiency,

$$IE = \frac{AY}{IY} \qquad 2.3$$

2.5 Determination of Activity of Enzymes

2.5.1 Activity of α-amylase

Activities of free and immobilized α-amylase were estimated by the hydrolysis of starch with the enzyme and subsequent determination of the residual starch with iodine-potassium iodide reagent [12]. 1 g soluble potato starch was dissolved in 100 mL boiling buffer of suitable pH. The solution was then cooled to 40 °C. A known amount of the free or immobilized enzyme in buffer was mixed with 2 mL of starch solution and incubated at optimum temperature in a water bath with constant shaking. After the particular time interval, the reaction was arrested by the addition of 0.1 mL 1 N HCl. 1 mL of above solution was withdrawn, added with 0.1 mL of iodine-potassium iodide reagent and was diluted with distilled water. The blue color developed was measured at 650 nm in a UV-Vis spectrophotometer. The results were compared with the absorbance of standard solution and the amount of starch converted was calculated. All tests were performed in triplicate and results are presented in average and standard deviation.

One enzyme unit (EU) of α-amylase activity was defined as the amount of enzyme, which converts 1 mg mL^{-1} of starch in 1 minute at optimum temperature and pH. Specific activity was calculated by the equation 2.4.

$$\text{Specific activity} = \frac{\text{Enzymatic activity (mg mL}^{-1}\text{ min}^{-1})}{\text{Amount of protein } (\mu g)} \qquad 2.4$$

2.5.2 Activity of glucoamylase

The activity of glucoamylase (GMA) was determined by estimating the concentration of reducing sugar formed using DNS reagent [13]. 1 wt. % solution of soluble starch was prepared by dissolving starch in 0.2 M buffer of desired pH. In a test vial, a known amount of free enzyme or immobilized enzyme in buffer was placed. Subsequently, 1.0 mL starch solution in buffer was added and the system then incubated in a water bath with constant shaking at optimum temperature exactly for the particular time. The reaction was arrested by adding 0.5 mL of 3, 5-dinitrosalicylic acid reagent and with rapid cooling. It was placed in a boiling water bath for 10 minutes. The solution was cooled under running water and diluted with distilled water. The amount of reducing sugar produced was determined spectrophotometricaly at 500 nm. A standard calibration curve was constructed with dextrose solution and the reducing sugar content was estimated. One unit (EU) of glucoamylase activity was defined as the amount of enzyme required to liberate 1μmol of reducing sugar per minute at optimum pH and temperature. Each result was an average of three separate set of experiments. Specific activity of GMA was calculated from the following equation,

$$\text{Specific activity} = \frac{\text{Released reducing sugar } (\mu \text{ mol mL}^{-1}\text{ min}^{-1})}{\text{Amount of protein } (\mu g)} \qquad 2.5$$

2.6 Biochemical Characterization

2.6.1 Effect of pH on activity

The influence of pH on activity of free and immobilized α-amylase and glucoamylase was tested at different pH using 0.2 M acetate buffer (pH 4-5.5) and 0.2 M phosphate buffer (pH 5.6-8).

2.6.2 Effect of temperature on activity and stability

The effect of temperature on the activity of free and immobilized α-amylase and glucoamylase was tested by subjecting them to various temperatures ranging from 30-70 °C at optimum pH with 1 % starch solution. For investigating temperature stability, free and immobilized enzymes were placed in a water bath at various temperatures from 30-60 °C for 1 hour. After being cooled to optimum temperature, the enzymatic reaction was carried out. Thermal inactivation curves at optimum temperature with respect to time was obtained by incubating enzyme at optimum temperature and after definite time intervals, known amount of enzyme was withdrawn and tested for activity by standard assay procedure. All results are presented with the highest value of each assigned the value of 100 % activity.

2.6.3 Determination of kinetic parameters

The kinetic constants K_m and V_{max} values of free and immobilized enzymes were determined by measuring the rates of the reaction at various substrate concentrations at optimum temperature and pH. K_m and V_{max} were calculated from the Lineweaver-Burk plots, and Hanes-Woolf plots.

2.6.4 Storage stability of free and immobilized enzymes

The storage stability of soluble and immobilized enzymes was measured by calculating their activities after being stored at 4 °C for a required period. The measurement was conducted at regular intervals of time. The activity was compared with initial activity and was represented as percentage initial activity retained.

2.6.5 Reusability study of immobilized enzymes

The reusability of the immobilized enzyme was tested by repeated batch experiments by measuring the residual activity of the immobilized enzyme preparation at optimum conditions at intervals of 30 minutes. After each run, the immobilized enzyme was removed, washed with the buffer solution and mixed with fresh substrate solution.

2.7 Physico-Chemical Characterization

2.7.1 FT-IR spectroscopy

Fourier Transform Infrared Spectroscopy (FT-IR) is a popular tool for identifying and characterizing polymer materials and their additives. It can be applied to the analysis of solids, liquids, and gasses. The term Fourier Transform Infrared Spectroscopy (FT-IR) refers to a fairly recent development in the manner in which the data is collected and converted from an interference pattern to a spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. FT-IR spectra of pure compounds are generally so unique that they are like a molecular "fingerprint".

The FT-IR spectra of synthesized, activated and α-amylase immobilized polymers were obtained using a FT-IR spectrometer using KBr pellet method. Changes in the absorption bands were investigated in the 500-4000 cm^{-1} region and the resolution applied was 4 cm^{-1}. The spectra were corrected for the H_2O and CO_2 content in the optical path.

2.7.2 Surface area analysis

A Micromeritics Gemini 2360 Surface Area Analyzer was used to measure the nitrogen adsorption isotherms of the samples at liquid nitrogen temperature. From this specific surface area was determined. Prior to the measurement all samples were degassed at room temperature for 8-10 hours in nitrogen flow.

2.7.3 Thermogravimetric analysis

Thermogravimetric analysis (TGA) is an analytical technique used to determine a material's thermal stability and its fraction of volatile components by monitoring the weight change that occurs as a specimen is heated. The measurement is normally carried out in air or in an inert atmosphere, such as Helium or Argon, and the weight is recorded as a function of temperature. Sometimes, the measurement is performed in a lean oxygen atmosphere (1 to 5 % O_2 in N_2 or He) to slow down oxidation. TGA is used to determine polymer degradation temperatures, residual solvent levels, absorbed moisture content, and the amount of inorganic (non-combustible) filler in polymer or composite material. It can also assist in de-formulation of complex polymer products.

Thermal studies were performed on Perkin Elmer Pyris Diamond 6 Thermogravimetric Analyzer in nitrogen atmosphere in the temperature range of 40-600 °C and heating rate of 10 °C per minute. Powdered samples of about 3 mg were sealed in standard platinum pans. The instrument was calibrated using indium and tin as standards. Sample residual weight (TG curves) and its derivative (DTG curves) versus temperature were automatically generated by Pyris software.

2.7.4 Scanning electron microscopy

To determine the surface topography and morphology of samples SEM images were recorded using the LEICA 420 I scanning electron microscope in collaboration with Dr. C. P. Sebastian at Institute for Inorganic and Analytical Chemistry, Münster, Germany. SEM allows for studies of surface features at low nanometer scale. The study was performed on the synthesized polyaniline (ES and EB) and on poly(*o*-toluidine) (TS and TB). A sample for investigation was placed on a conducting polymer foil on an aluminum holder. The high magnification of the microscope allowed visualizing details of the crystal shape and taking photographs.

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CHAPTER 3
SYNTHESIS AND PHYSICO-CHEMICAL CHARACTERIZATION OF POLYMERS

3.1 Introduction

A comprehensive literature survey reveals an enormous number of publications and a variety of different physico-chemical characterization techniques ranging from spectroscopic to surface techniques were used to characterize the enzymes, substrates and the immobilization process. Of course, the chosen technique strongly depends on the particular problem to be solved. Within the scope of this contribution, focus has been given to FT-IR, surface area analysis, thermogravimetric analysis (TGA) and scanning electron microscopy (SEM). These techniques help to understand the systems explained in this thesis.

3.2 Synthesis of Polymers

PANI was synthesized by polymerization of aniline in presence of ammonium peroxydisulfate and HCl. The emeraldine hydrochloride salts (ES) obtained was green in color with more than 80 % yield. On treatment with NaOH it converted to blue emeraldine base (EB). Similarly poly(o-toluidine) hydrochlorides (TS) powders were obtained in good yield and its blue base (TB) form was obtained on treatment with NaOH. The activation with glutaraldehyde provides ES1, EB1, TS1 and TB1 and with ascorbic acid provides ES2, EB2, TS2 and TB2.

3.2 FT-IR Spectroscopy

3.2.1 Spectra of polyaniline (PANI)

The aniline polymers have the general formula $[(\text{-B-NH-B-NH-})_y(\text{B-N=Q=N-})_{1-y}]_x$, in which B and Q denote the phenyl rings in the benzoid and quinoid form, respectively. Thus the intrinsic oxidation state of the polymers can range from that of fully oxidized pernigraniline (y = 0) through that of 75 % intrinsically oxidized nigraniline (y = 0.25), 50 % intrinsically oxidized emeraldine (y = 0.5) to fully reduced leucoemeraldine(y = 1) [1, 2]. IR spectroscopy is one of the useful methods for determining functional groups present in a compound, and for polyaniline, it is most useful for obtaining qualitative information

regarding the average oxidation state. IR spectroscopy can distinguish between benzoid rings and quinoid rings in the 1300 to 1600 cm^{-1} region of the spectrum; this region of the spectrum is most useful for distinguishing between oxidation states in the undoped polymer, as the quinoid stretches disappear upon doping [3]. Hence the spectrum gives a clear structural indication of the polymer chain. It was suggested by *Genies* and *Laprowaski* [4], and *Harada et al.* [5] that the relative concentration of various N-moities: NH, N^+H_2, N=, ^+NH=, C–N^+– in polyaniline depends on the nature and percentage of doping.

The FT-IR spectrum of PANI samples [*figs. 3.2.1 & 3.2.2*] plotted with the percentage of transmittance as a function of wave number (cm^{-1}). The spectra of PANI samples show similar characteristics with literature reports [3, 6-10]. The main absorption peaks present in the spectra of samples are almost at the same positions, but their relative intensities are changed.

Fig. 3.2.1 shows the IR spectra recorded for emeraldine salt samples ES, ES1, ES2, ES1-Enzyme (enzyme attached ES1) and ES2-Enzyme (enzyme attached ES2). Various IR bands and their assignments are given in the *table 3.1.*

The N-H stretching region in PANI is located in 3500-3100 cm^{-1}. The absorbance of PANI in this region is rather weak. The broad band centered at 3440 cm^{-1} is the result of stretching vibration of free N-H group. The small band at 3224 cm^{-1} corresponds to the vibration of hydrogen bonded N-H group [3].

The C-H stretching region is 3100-2800 cm^{-1}, the absorption in this region is very weak, and showed by ES, ES1 and ES2 at 2979 cm^{-1}.

The 1600-1450 cm^{-1} is the aromatic ring breathing, the main peaks in this region are at 1570 cm^{-1} and at 1480 cm^{-1} due to the stretching deformation of C=C in quinoid (Q) ring and benzoid (B) ring respectively [6]. The analysis of infra-red spectra shows that quinoid to benzoid (intensity) ratio was varied for all the samples, showing indication of some structural modification by the activation and immobilization procedure.

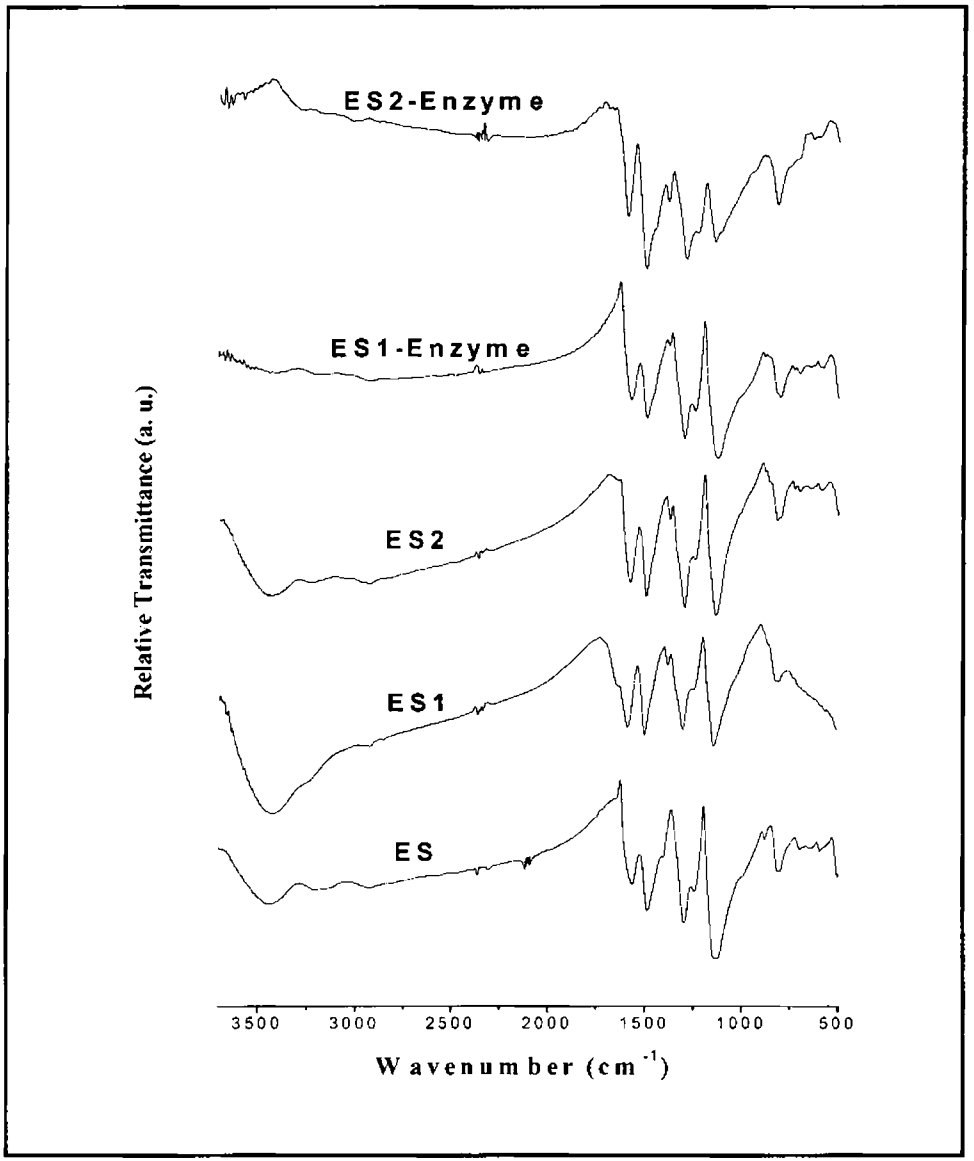

Fig. 3.2.1 FT-IR Spectra of emeraldine salts.

The C-N stretching region for aromatic amines is in the range of 1400-1240 cm^{-1}. The spectrum of PANI gives three peaks in this region around 1380 cm^{-1}, 1296 cm^{-1} and at

1240 cm^{-1} [6]. The weak peak at 1380 cm^{-1} can be attributed to the C-N stretching vibration in the quinoid imine units in $QB_{trans}Q$. A strong peak at 1296 cm^{-1} is because of the C-N stretching vibration in the alternative unit of $QB_{cis}Q$, QBB or BBQ. The weak peak at 1240 cm^{-1} can be ascribed to the C-N stretching in the B-B-B triad sequence.

Table 3.1 *Assignment of the main peaks in the FT-IR spectra of emeraldine salts.*

Peak assignment (cm^{-1})	**ES**	**ES1**	**ES2**	**ES1-Enzyme**	**ES2-Enzyme**
Stretching of N=Q=N	1568	1587	1580	1571	1590
Stretching of N-B-N	1487	1492	1484	1489	1490
C-N stretching in Quinoid imine unit (Q=N-B_t)	1395	1378	1374	1378	1380
Stretching of C-N in QBQ, QBB, BBQ	1299	1296	1293	1296	1289
Stretching of C-N in BBB	1241	1239	1239	1238	1228
Bending of C-H (in plane), vibrational mode of $Q=N^{-}H-B$ or $B-N^{+}H-B$	1140	1131	1125	1125	1135
Out of plane deformation of C-H in 1,4 disubstituted benzene ring	813	816	816	807	816

Aromatic C-H bending occurs in the region of 1220-500 cm^{-1}. Intrinsic PANI gives two main peaks in this region at 1145 and 830 cm^{-1}, corresponds to C-H in plane bending and C-H out of plane bending vibration of the 1,4-disubstituted benzene ring [7-9]. The band at 1145 cm^{-1} is weak, merged in to one band at 1140 cm^{-1} and grows as the main absorption band of doped PANI. This band is a vibrational mode of $B-N^{+}H=Q$ or $B-N^{+}H-B$ which is formed in doping reactions [6]. This band is very intense and broad, may be

attributed to the existence of positive charge on the polymer. Compared to the spectrum of ES all other spectra showed a decrease in intensity of this peak, which display the deprotonation or reduction of the polymer chain. Considerable reduction of intensity of this band is observed for ES2-Enzyme spectrum with the increase in the intensity of band at 1490 cm^{-1} proved the reduction of ES as the consequence of enzyme immobilization. It is reported that protonated emeraldine get reduced by acidic solution of ascorbic acid [11], and by glutaraldehyde [12].

Table 3.2 Assignments of main peaks in the spectra of emeraldine base.

Peak assignment (cm^{-1})	**EB**	**EB1**	**EB2**	**EB1-Enzyme**	**EB2-Enzyme**
Stretching of N=Q=N	1587	1590	1595	1581	1579
Stretching of N-B-N	1493	1498	1500	1492	1492
C-N stretching in Quinoid imine unit (Q=N-B$_i$Q)	1383	1380	1378	1372	1383
Stretching of C-N in QB$_c$Q, QBB, BBQ	1300	1301	1301	1306	1284
Stretching of C-N in BBB	1236	1237	1234	1237	1228
Bending of C-H (in plane), vibrational mode of Q=N$^+$H-B or B-N$^+$H-B	1140	1140	1140	1133	1135
Out of plane deformation of C-H in 1,4-disubstituted benzene ring	824	821	822	821	816

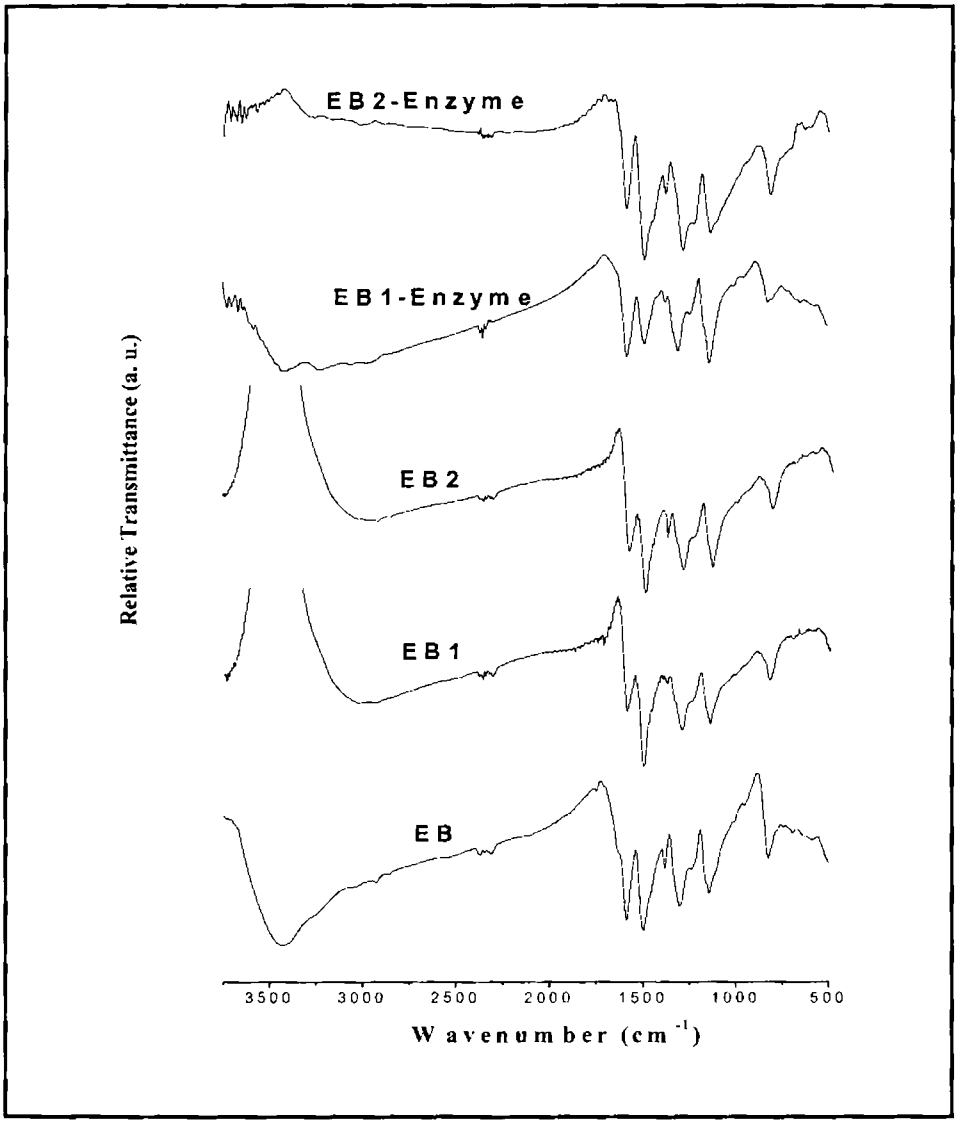

Fig. 3.2.2 FT-IR Spectra of emeraldine bases.

Emeraldine base gives almost the same spectrum that of emeraldine salt, with the difference in the relative intensity of benzoid and quinoid bands. All the peaks can be interpreted as that of emeraldine salts (*table 3.2*). It is observed from IR scan, (*fig 3.2.2*) the

relative intensity of band at 1493 cm^{-1} to band at 1587 cm^{-1} in the spectrum of EB is almost equal showing the presence of almost equal number of benzoid and quinoid ring in the polymer chain. But in other three samples EB1, EB2, and in EB2-Enzyme the intensity of quinoid band reduces and benzoid band increases, which shows presence of more reduced units in the polymer chain.

3.2.2 Spectra of poly(*o*-toluidine) (POT)

Fig. 3.2.3 and *fig. 3.2.4* show FT-IR spectra of poly(*o*-toluidine) as synthesized in the protonated salts and base forms, with their modified form prepared for immobilization. The spectra showed good agreement with reported works [13-17]. In the region of 3500-2000 cm^{-1} only weak bands are observed due to the high background absorption hiding partially or totally the polymer vibrational bands. The band at around 3220 cm^{-1} corresponds to N–H stretching mode of secondary amine. The weak characteristic bands at about 2918 cm^{-1} can be assigned to the stretching vibration of the methyl ($-CH_3$) group. The aromatic C-H stretching is present, at 3030-3050 cm^{-1}, whereas the methyl group shows, in the base forms, three bands at 2865, 2920 and 2962 cm^{-1}. In general it presents only two bands, but in this case the asymmetric stretching shows split into two bands due to steric hindrance and show a total of 3 bands. In the wavenumber region of 1610-1480 cm^{-1}, two bands of varying intensity are observed. *Colomban et al.* [13] have assigned these two sharp bands at 1585 and 1490 cm^{-1} to stretching of the C=C bonds of the quinoid and benzoid ring. The 1490 cm^{-1} band undergoes an important intensity decrease as the polymer is oxidized. This is consistent with the fact that quinones show no absorption at these wavenumbers. So the intensity diminishes with the number of benzoid units. Taking the base and salt forms, it is seen that both of them show an intensity decrease upon activation, confirmed the reduction in the polymer chain. The behavior is similar as observed in PANI.

The bands at 1375 cm^{-1} are due to the symmetric deformation of methyl group [14]. A number of bands appear in the range 1350 to 1200 cm^{-1}, which are assigned to the splitting of C-N stretching modes [13]. The bands at 1316 and 1210 cm^{-1} can be assigned to the C–N vibration. The three bands appearing at 1155, 1002, 960 are assigned to C-H in

plane bending vibration [15, 16] and at 875, 810 are assigned to C-H out of plane bending vibration [17] in 1,2,4-trisubstituted benzene ring.

Table 3.3 *Assignment of main peaks in the IR spectra of poly(o-toluidine) salts.*

Peak assignment (cm^{-1})	**TS**	**TS1**	**TS2**	**TS1-Enzyme**	**TS2-Enzyme**
Stretching of CH_3	2934	2924	2917	2937	2917
Stretching of N=Q=N ring	1592	1604	1588	1588	1592
Stretching of N-B-N ring	1492	1498	1495	1495	1491
Bending of CH_3	1381	1381	1371	1378	1370
Stretching of C-N	1305-1216	1305, 1228	1302, 1217	1311, 1217	1318, 1208
Bending of C-H (in plane)	1155, 1108, 1010	1160, 1114, 1007	1160, 1107, 1008	1152, 1107, 1007	1157, 1107, 1004
Out of plane deformation of C-H in 1,4-disubstituted benzene ring	881, 808	852, 802	880, 808	875, 802	880, 802

In PANI the peak at 832 cm^{-1} is assigned to an out-of-plane C-H vibration. In poly(*o*-toluidine), this band is observed at 810 cm^{-1}. Besides, as the polymer has a 1, 2, 4-trisubstitution in the benzoid rings, it shows a C-H vibration at approximately 860 to 880 cm^{-1}, which is not present in PANI. It corresponds to one isolated ring hydrogen atom

situated between two substituents. This band shifts from 860 to 880 cm^{-1} in the presence of quinoid groups [14]. No strong feature peaks of hydroxyl and carbonyl groups appear in the FT-IR spectrum, showing very low concentration of ascorbic acid and glutaraldehyde on the polymer surface.

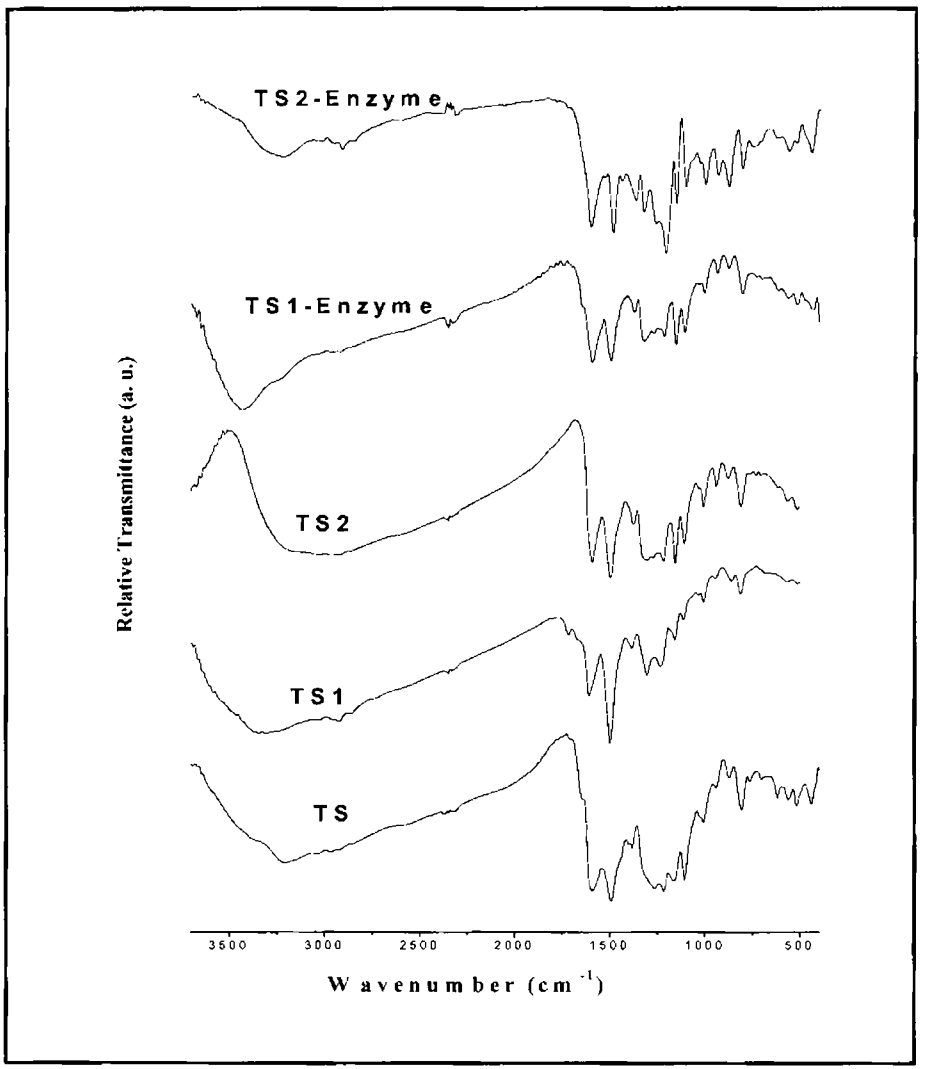

Fig. 3.2.3 *FT-IR spectra of poly(o-toluidine) salts.*

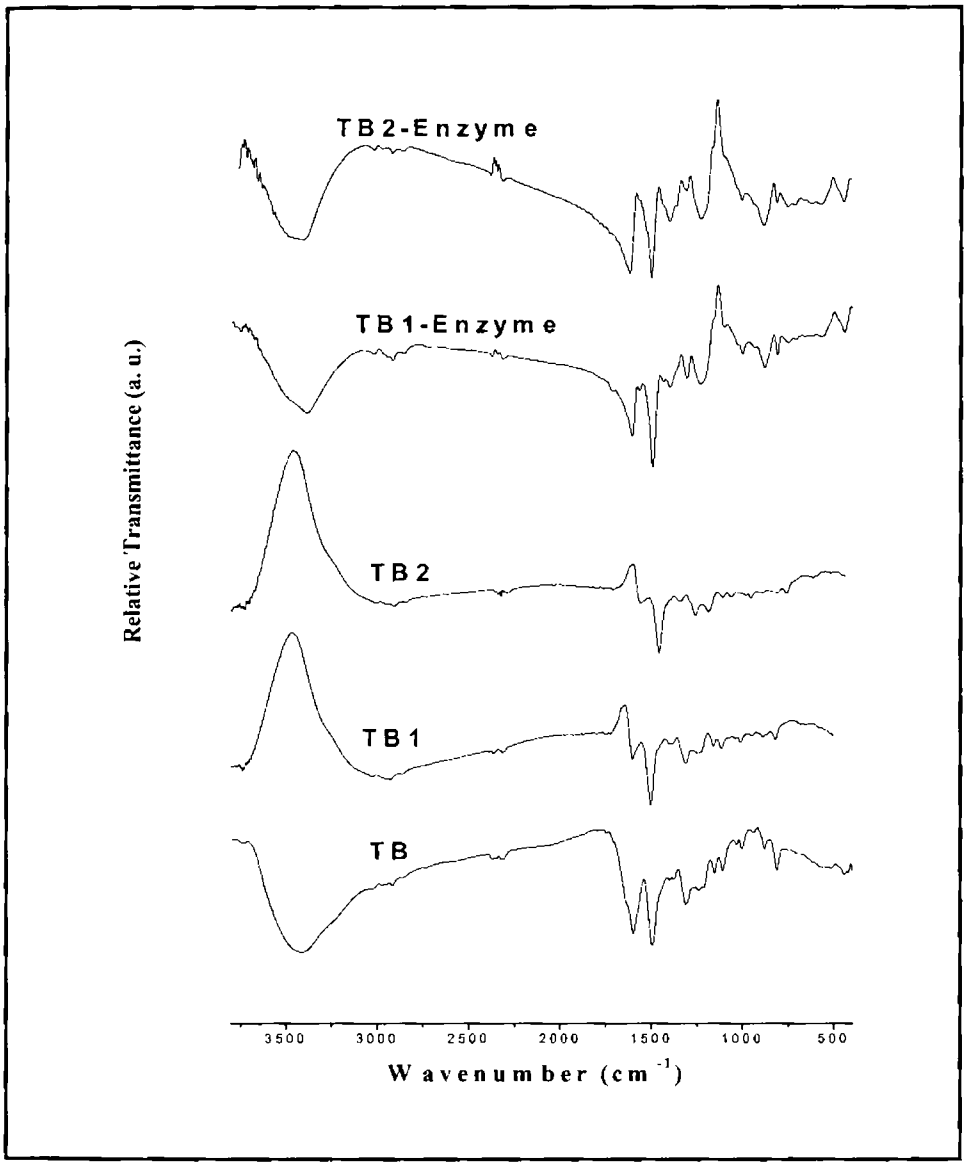

Fig. 3.2.4 FT-IR Spectra of poly(o-toluidine) base.

Table 3.4 Assignment of main peaks in the IR spectra of poly(o-toluidine)base.

Peak assignment (cm^{-1})	TB	TB1	TB2	TB1-Enzyme	TB2-Enzyme
Stretching of CH_3	2912	2915	2919	2917	2910
Stretching of N=Q=N ring	1592	1593	1594	1607	1609
Stretching of N-B-N ring	1490	1490	1496	1498	1495
Bending of CH_3	1376	1370	1379	1378	1387
Stretching of C-N	1305, 1216	1306, 1224	1305, 1232	1311, 1228	1305, 1208
Bending of C-H (in-pl.)	1155, 1106, 1002	1157, 1114, 1005	1160, 1112, 1008	1152, 1099, 1004	1154, 1106, 1007
Out of plane deformation of C-H in 1,4-disubstituted benzene ring	885, 808	888, 821	860, 815	885, 805	894, 808

3.3 Surface Area Analysis

BET surface area of the polymers are given in *table 3.5*.The highest surface area is shown by poly(*o*-toluidine) base, in its ascorbic acid activated form. In comparison, among the non activated forms also, poly(*o*-toluidine) gives highest surface area. It is evident that glutaraldehyde activation reduces the surface area, whereas ascorbic acid activation increases the surface area of the polymers. However, the surface area did not play an

important role on the activity yields of covalently bound enzymes, hence on the immobilization efficiency. In most cases enzymes immobilized via glutaraldehyde linkage showed maximum activity yield. Even though protein loading is increased with surface area, most of the immobilized proteins were unable to retain their original activity.

Table 3.5 BET surface area of the polymers.

Polymer	Surface area ($m^2 g^{-1}$)
ES	36.55
ES1	32.25
ES2	37.14
EB	52.60
EB1	42.31
EB2	53.88
TS	9.96
TS1	9.42
TS2	11.45
TB	56.87
TB1	45.58
TB2	58.07

3.4 Thermogravimetric Analysis

3.4.1 Thermogram of α-amylase

Fig. 3.4.1 shows the TG (Thermogravimetric) and DTG (Differential thermogravimetric) curves for free amylase which was characterized by several weight loss peaks. The first weight loss in TG was at temperature range from 90 to 150 °C, characterized by a low weight loss (4.3 %) due to the dehydration of the interstitial water containing in the free α-amylase sample. From 200 to 350 °C, continuous weight loss was

observed indicating a complete decomposition of the organic structure of the enzyme. The DTG curve shows the corresponding two major peaks at 144 °C, and at 304 °C and two minor peaks at 200 °C and at 225 °C. The peak at 144 °C may be corresponding to the water loss and latter peaks may be due to the decomposition of enzyme.

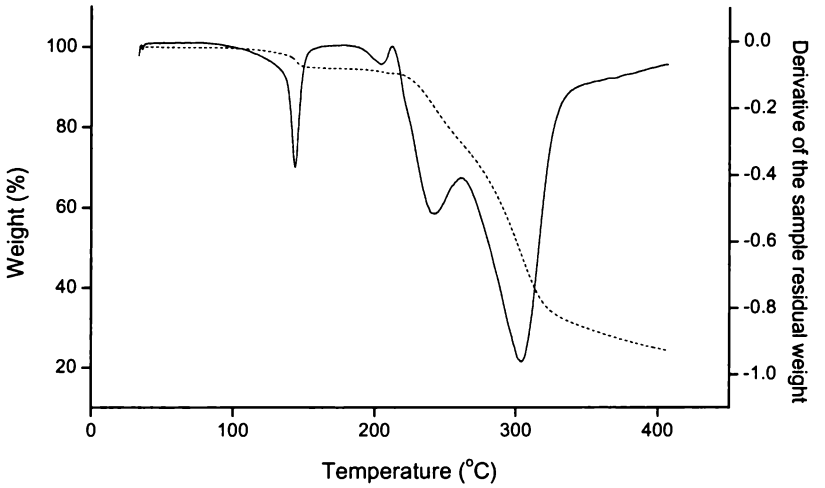

Fig. 3.4.1 TG (----) and DTG (—) curves of α-amylase.

3.4.2 Thermogram of polyaniline

The TG and DTG curves of ES under nitrogen atmosphere are shown in the *fig. 3.4.2.* The thermogram shows a typical three step weight loss behavior. The first weight loss of 23 % starts from room temperature to 180 °C. The second weight loss occurs between 200 and 320 °C and is followed by a final step starting at 420 °C. The first weight loss corresponds mainly to water and a small proportion of gaseous HCl originating from a slight deprotonation process [18]. The most of the HCl loss then occurred during the second weight loss together with water molecules which are more strongly bonded to the polymer in ES [19]. The thermal decomposition of polyaniline backbone gives the third weigh loss

starting at 420 °C. The DTG curves show corresponding peaks at 183, 219, and 555 °C.

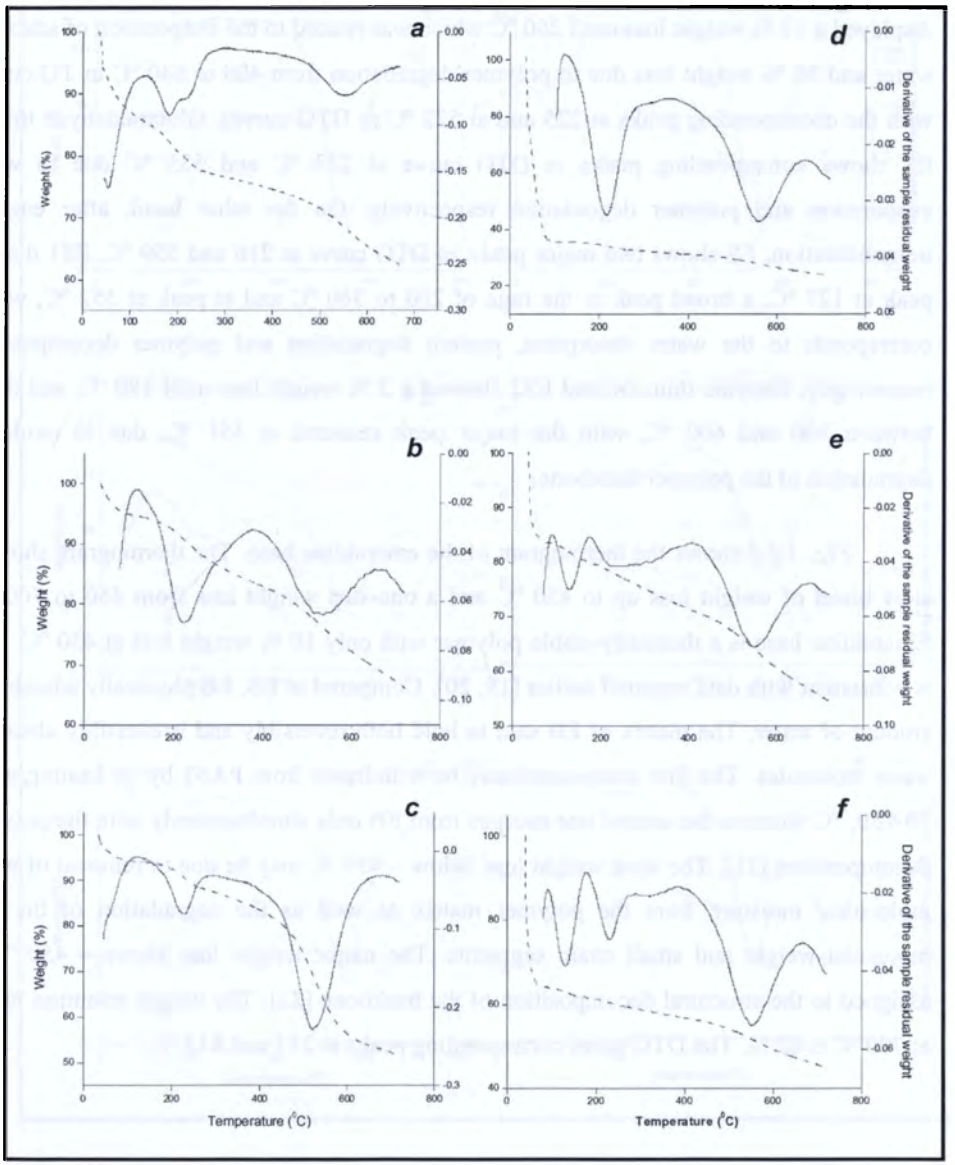

Fig. 3.4.2 *TG(----) and DTG(—) curves of ES* ***(a)*** *ES1* ***(b)*** *ES2* ***(c)*** *ES-Enzyme* ***(d)*** *ES1-Enzyme* ***(e)*** *ES2-Enzyme* ***(f)***.

The thermogram of activated EB also shows typical two step weight loss behavior as can be deduced from the maxima of the DTG curve. The first one extends from room temperature to 250 °C. It can be attributed to the desorption of water molecules physically adsorbed in the polymer matrix and also desorption of solvent molecules. The second weight loss starts typically around 425 °C. This has been attributed to thermal decomposition of the polyaniline backbone structure. Attachment of enzyme on EB, EB1 and EB2 display some small additional peaks in the DTG curves arising mainly from the degradation of enzyme molecules.

3.4.3 Thermogram of poly(*o*-toluidine)

For doped poly(*o*-toluidine), thermal degradation occurs in three major steps similar to that of ES. The first 13 % weight loss step, occurring between 110 and 270 °C, is assigned to the loss of moisture and residual HCl, whereas the next weight-loss step, between 280 and 390 °C, is due to the loss of dopants and side groups [23]. The degradation of the polymers occurs above 430 °C. The DTG curves of the polymers also show three-step degradation consistent with the TG curves. The corresponding peaks in DTG occur at 174, 348 and 575 °C.

For poly(*o*-toluidne) base, the first weight loss is slow, up to 400 °C, then it leads to the one step final weight loss at 450-600 °C. This can be explained to similar to emeraldine base as given above in the section 3.4.2.

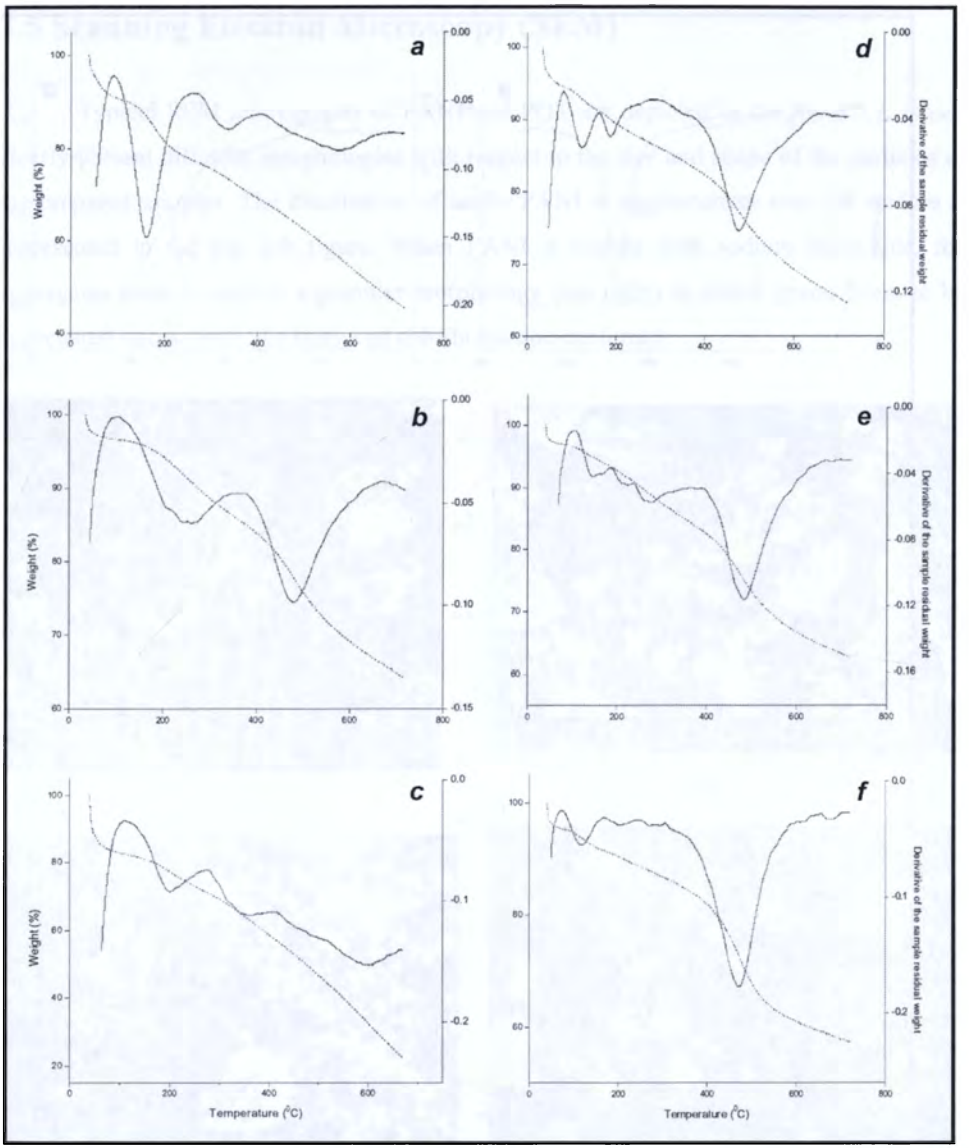

Fig. 3.4.4 *TG(---) and DTG (—) curves of TS* ***(a)*** *TS1* ***(b)*** *TS2* ***(c)*** *TS-Enzyme* ***(d)*** *TS1-Enzyme* ***(e)*** *TS2-Enzyme* ***(f)****.*

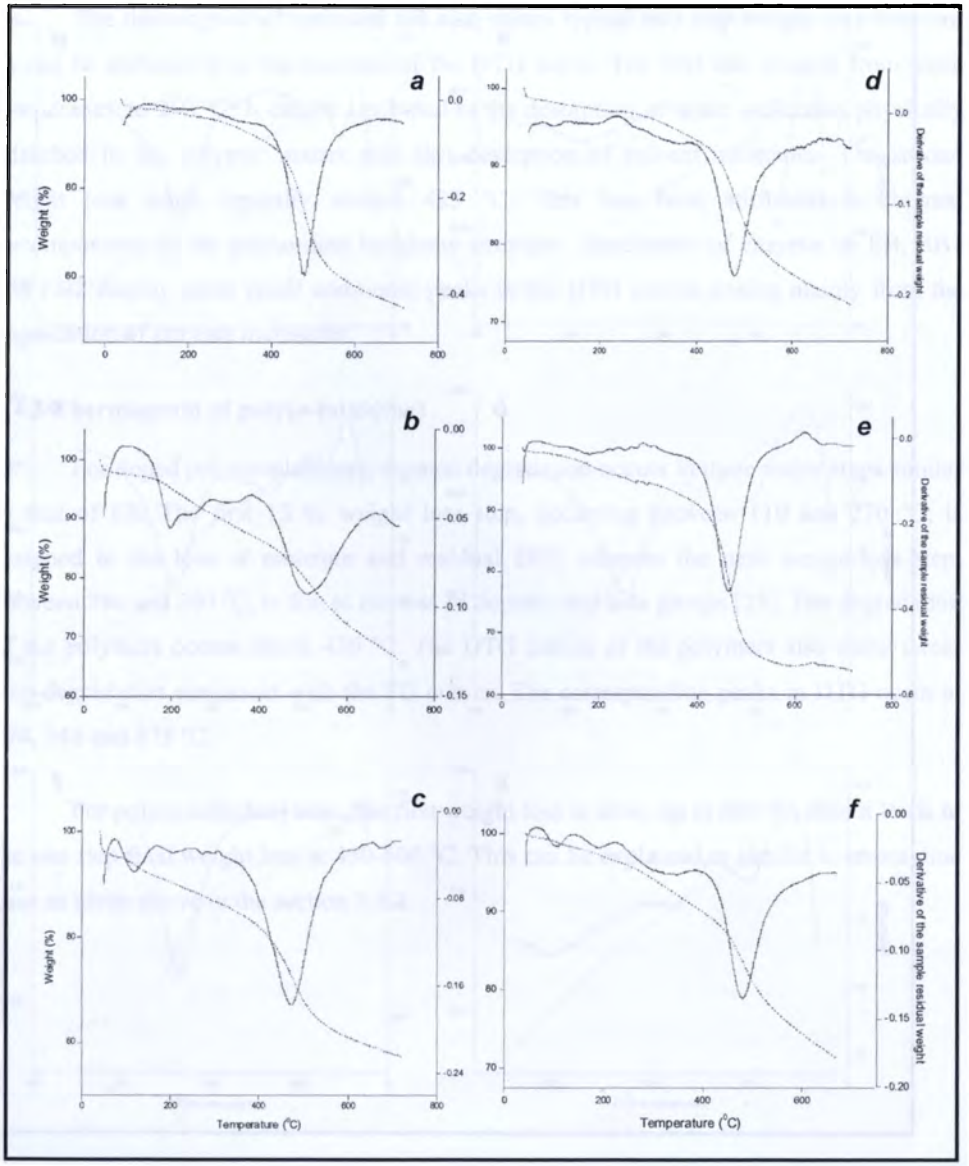

Fig. 3.4.5 *TG (---) and DTG (—) curves of TB* (***a***) *TB1* (***b***) *TB2* (***c***) *TB-Enzyme* (***d***) *TB1-Enzyme* (***e***) *TB2-Enzyme* (***f***).

3.5 Scanning Electron Microscopy (SEM)

Typical SEM micrographs of PANI and POT are depicted in the *fig. 3.5.1*, which clearly present different morphologies with respect to the size and shape of the particles of the prepared samples. The distribution of acidic PANI as agglomerates over the surface is represented in the top left figure. When PANI is treated with sodium hydroxide, the aggregates seem to exhibit a granular morphology (top right) in which grains seem to be aggregated into a stone like body and distributed non-uniformly.

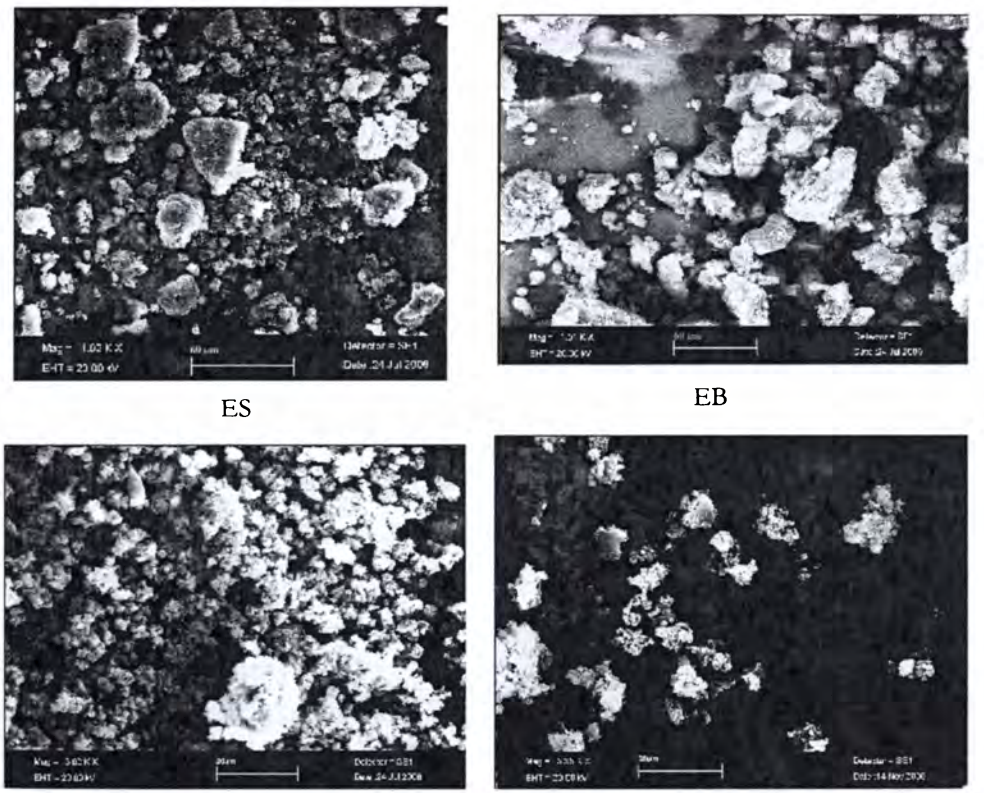

***Fig 3.5.1** SEM images of ES, EB, TS and TB.*

Similar kind of images are observed for the poly(*o*-toluidine) salt and base form. The treatment of PANI and POT with acid appears to lead to a granular morphology but the grains are collected into a coral-like body.

3.6 Conclusion

FT-IR spectra, TG-DTG, surface area and SEM analysis of PANI and POT used in this work revealed features characteristic of these polymers.

- ***Polymers showed differences in the FT-IR spectra, more specifically in the relative intensities of the benzoid and quinoid related bands, characterizing differences in the oxidation states. This analysis allowed the classification of polymers, from a reduced state (EB and TB) to protonated state (ES and TS), passing through intermediary protonated and deprotonated forms by activation methods.***

- ***ES, EB, TS, and TB polymers are thermally-stable polymer with thermal stability to temperatures in excess of 420 °C. The activation with GA and ASA and immobilization of enzyme did not affect the thermal stability of these polymers.***

- ***Surface area analysis showed that treatment with GA reduces the surface area of the polymers where as treatment with ASA increases the surface area of PANI and POT.***

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CHAPTER 4
IMMOBILIZATION OF DIASTASE α-AMYLASE

4.1 Introduction

Diastase (from Greek *διαστασις*, "separation") is any member of a class of enzymes occuring in the seed of grains and malt which catalyses the breakdown of starch into smaller carbohydrate molecules such as maltose. It was the first type of enzymes discovered, in 1833, by *Anselme Payen* [1], who found it in malt solution. Today, diastase means any α-, β-, or γ-amylase (all of them hydrolases which differ in the way they attack the bonds of the starch molecules) that can break down carbohydrates.

α-amylase (EC 3.2.1.1; 1, 4 α-d-glucan glucanohydrolase) is a widespread enzyme occurring in mammals as salivary and pancreatic α-amylase; in the seeds of plants, it is produced by large numbers of microorganisms. The α-amylases are calcium metalloenzymes, completely unable to function in the absence of calcium. This enzyme catalyses the hydrolysis of α-1, 4-glucosidic linkages in amylose, amylopectin and glycogen in an endo-fashion [2, 3]. It does not hydrolyse the α-1, 6 linkages or any other branch points and so produces maltose and limit dextrins; the precise action pattern depends on the source of the α-amylase [4]. Amylases, which are starch hydrolyzing enzymes, are the most important industrial enzymes and are used in different processes in food, textile, pharmaceutical industries, beverage industries *etc.* [2]. *Bacillus subtilis, Bacillus amyloliquefacines, Bacillus licheniformis* and *Aspergillus oriyzae* are very important α-amylase sources [5-8] because their enzymes are highly thermostable. α-Amylase is known to attack both insoluble starch and starch granules held in aqueous suspension [9].

To improve their economic feasibility in food, pharmaceutical, medical, industrial and technological processes, soluble enzymes are usually immobilized onto a solid support. In order to achieve the desired catalytic activity, support material can have a critical role in the process of immobilization. Several attempts have been carried out in the past to immobilize the enzyme α-amylase on different supports [7, 8, 10-33]. Some examples involve zirconium dynamic membrane [7], glass beads and glutaraldehyde fixation [10],

covalent cross-linking onto silica gel [11], coconut fiber [12], and adsorption on zirconia [13]. Many of the immobilization processes were done by using polymer based supports [14-19]. *Tümtürk et al.* [14] covalently immobilized α-amylase on poly(2-hydroxyethyl methacrylate) and poly(styrene-2-hydroxyethyl methacrylate) micro spheres, which were activated using epichlorohydrin. *Varlan et al.* had reported [15] paramagnetic polyacrolein beads as a support for immobilization. *Bajpai et al.* used [17] semi-interpenetrating polymer network of poly(ethylene glycol), poly(vinyl alcohol) and polyacrylamide as support for immobilization of α-amylase.

This chapter describes the immobilization of diastase α-amylase under very mild conditions on polyaniline and poly(*o*-toluidine), both were prepared as acidic and basic forms. Immobilization efficiency and enzyme activity of α-amylase were examined at various pH and temperature and discussed in detail.

4.2 Immobilization of α-amylase on Emeraldine Salt

4.2.1 Coupling of α-amylase and immobilization efficiency

The uptake of enzyme varied significantly according to the support, method chosen, immobilization medium, enzyme concentration and contact time of enzyme to support. Therefore these parameters were tested to optimize enzyme immobilization on emeraldine salt (ES), glutaraldehyde activated emeraldine salt (ES1), and ascorbic acid activated emeraldine salt (ES2). The effect of solution pH during the immobilization step on enzyme activity is shown in the *fig. 4.2.1*. The best results were obtained in the pH range from 4.5 to 5.5, with maximum activity retention at pH 5.0. The optimum pH is that which favors the bonding between support and enzyme, and the pH which favors the enzyme to retain its activity. The pH stability of the enzyme also has to be considered. Selecting an optimum solution pH for enzyme immobilization is therefore important. Even if the enzyme loading is achieved maximum at any other pH, it is not worth if the enzyme shows less activity. Hence taking into consideration of all the above facts, we have measured the remaining

activity of immobilized enzyme after immobilization, and it found that pH 5 was best for immobilization of α-amylase on all emeraldine salts.

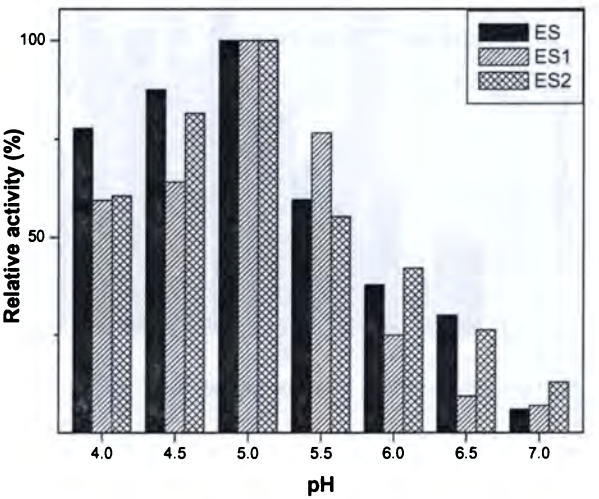

Fig. 4.2.1 *Effect of pH of the immobilization medium on the relative activity of immobilized α-amylase.*

The time necessary for complete immobilization of α-amylase is shown in *fig. 4.2.2.* For operational reasons, 90 minutes of immobilization was chosen. Increasing the immobilization time increased the immobilized enzyme activity up to 60 minutes for both ES and ES2 and 30 minutes for ES1. After this, there was a decrease in activity observed due to lower accessibility of substrate by multilayer adsorption of enzyme. Similar decline in activity was observed when different forms of PANI were used for the immobilization of enzyme horseradish peroxidase [34].

The amount of protein bonded to PANI, after optimization of immobilization conditions, is given in *fig. 4.2.3.* The figure shows the effect of protein added to 1 g of support.

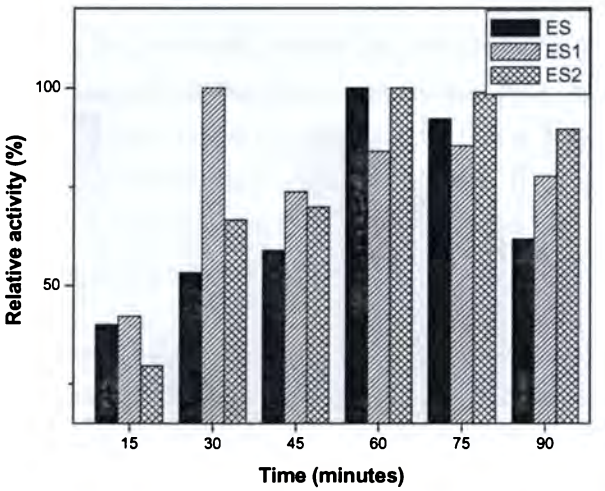

Fig.4.2.2 Effect of time of immobilization on the recovered activity of immobilized α-amylase.

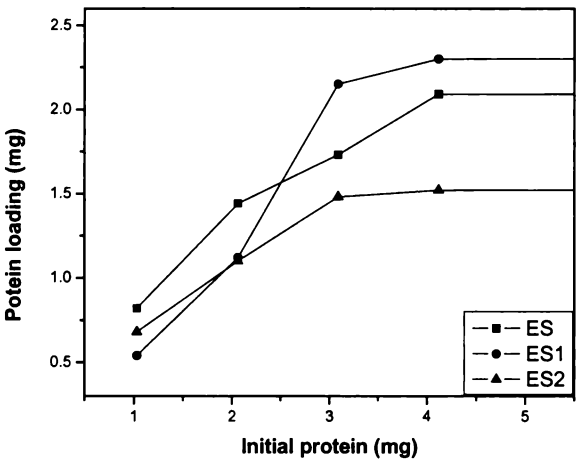

Fig. 4.2.3 Effect of initial protein concentration on protein loading.

On the addition of protein to the support, immobilization amount increases and reaches a saturation point. Further addition did not make any remarkable increase in protein loading. The saturation point depends upon the properties of the support and the methods of immobilization. Immobilization via adsorption depends mainly on the surface area available for immobilization. It incorporated almost 2 mg protein per 1 g ES at the plateau. For ES1 it was above 2.3 mg and for ES2 it was nearly 1.5 mg when initial enzyme taken was 3.1mg.

However, the protein content is not the only parameter that characterizes an immobilized enzyme. The coupling procedure may have an adverse effect on enzymatic activity and so activity yield should also have to be determined.

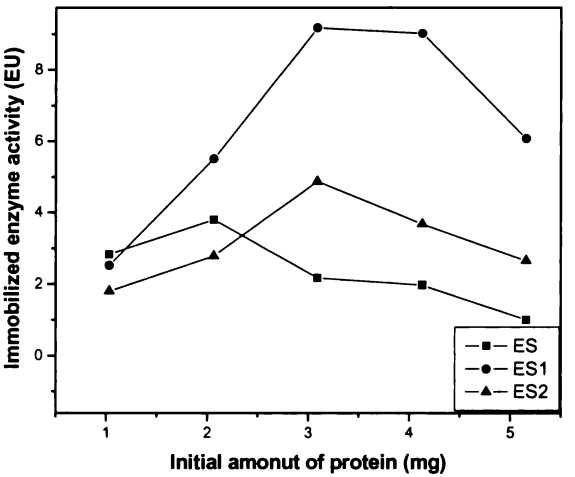

Fig. 4.2.4 Effect of initial protein concentration on immobilized α-amylase activity.

It can be noted from the *fig. 4.2.4* that the maximum activity showed by ES1 and ES2 are when the ratio of protein added to them is up to 3 mg protein g^{-1} polymer support, and for ES it is 2 mg protein g^{-1} ES. During the addition of protein to PANI, the amount of protein immobilized is increased gradually and finally get saturated.

Table 4.1 *Efficiency of immobilization of α-amylase per g of emeraldine salts.*

Polymer	Initial protein (mg)	Immobilized protein (mg g^{-1} support)	Immobilization Yield (%) IY	Initial activity (EU)	Immobilized enzyme activity (EU g^{-1} support)	Activity Yield (%) AY	Immobilization Efficiency (%) IE= AY/IY
ES	2.1	1.5	71.4	16.4	3.8	23.2	32.5
ES1	3.1	2.2	71	24.6	9.2	37.4	52.7
ES2	3.1	1.7	54.8	24.6	5.9	24	43.8

Beyond this saturation level there was a slow increase in protein loading but the activity was found to be decreasing. Covalently coupled enzyme showed maximum activity at this saturation level, but adsorbed enzymes followed a different trend. Here in the case of ES the enzyme adsorbed at the saturation level is 2.1 mg g^{-1} ES, but the activity was very low, and showed maximum activity when the protein loaded was 1.5 mg g^{-1} ES. The decrease in activity of enzyme (*fig. 4.2.4*) before (for adsorbed) or beyond (for covalently bound) this saturation level may be attributed to multilayer adsorption on the single layered adsorbed or covalently bonded enzyme, so that a considerable amount of enzymes are less accessible for starch. Similar conclusions were put forward for the decrease in enzyme activity when glucoamylase was covalently immobilized onto surface grafted polyaniline [35].

Immobilization yields, activity yields, and the efficiency of immobilization are shown in *table 4.1*. Maximum activity yield obtained was 37.4 % which represents the amount of actively bound enzyme. Immobilization efficiency is defined as the ratio of activity yield to immobilization yield. In all cases immobilization yield is higher than activity yield, and hence efficiency of immobilization is low. *Tanyolac et al.* [36] immobilized α-amylase onto nitrocellulose membrane and the maximum enzyme yield was around 21 % and *Sun et al.* reported [37] about 33 % recovery of activity when thermal composite hydrogel membranes were used as support. This fact is usually explained with difficult accessibility of the substrate to the immobilized enzyme due to steric restrictions or unfavorable orientation of the immobilized enzyme molecules [12]. As a result of these effects interaction between the substrate and the active site of the immobilized enzyme is blocked.

4.2.2 Effect of pH on enzyme activity

Enzymes are very fragile molecules when removed from their natural medium. They are amphoteric molecules containing a large number of acidic and basic groups, situated mainly on the surface. The charges on these groups will vary with the pH of their environment. This will affect the total net charge of the enzyme and the distribution of charge on its exterior surface, in addition to the reactivity of the catalytically active groups.

Hence a change in pH or ionic media may cause denaturation of the protein structure and complete loss of the activity of the enzyme. So the pH value has considerable influence on the efficiency and stability of all enzymes.

The pH effect on the activity of the free and immobilized forms of α-amylase has been studied in buffer solution at different pH values (pH 4-8) and the results are presented in *fig. 4.2.5.* Activity under optimum conditions was assigned a value of 100 %. The pH values for optimum activity of the free amylase are 5 and 5.5. The present result is comparable to earlier reports in the literature [8, 12].

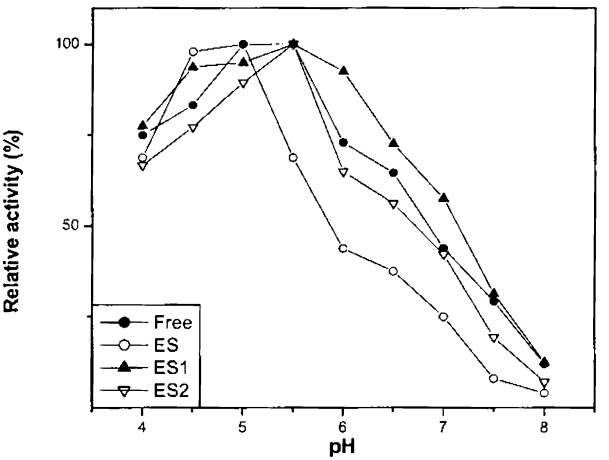

Fig. 4.2.5 *Effect of pH on activity of free and immobilized α-amylase.*

Upon immobilization there was a slight change in the nature of pH profile depending upon the method of immobilization, and spacer used. The adsorbed enzyme showed its maximum activity at pH 5 and for covalently bound enzymes, the value was at 5.5. The shift of optimum pH towards acidic region was reported when α-amylase covalently immobilized to functionalized glass beads [26] and zirconium dynamic membrane [7]. The slight positive charge on the surface of ES has the effect that more ions of opposite charges are drawn in to the vicinity of the bound enzyme, so enzyme will

encounter this pH value at its micro environment but the bulk of this solution has higher H^+ ions. Hence the optimum for the ES shifted towards the lower value. In activated ES1, glutaraldehyde spacer enabled the attached molecule to act like free enzyme but in ES2 the spacer not long so the property of the surface somewhat affected the action of attached enzyme. Hence the pH-dependent activity profile for the immobilized α-amylase on ES and ES2 is considerably narrowed to acidic region. But for ES1 it is broadened and showing less susceptibility to pH change. Similar observations, with resistance to pH change were reported by other researchers [29, 30]. Thus, the immobilized amylase on ES1 displays significantly improved stability to pH changes over the free form and other immobilized forms.

4.2.3 Effect of temperature on the activity

The effect of temperature on the activity of free and immobilized α-amylase was studied by subjecting them to various temperatures ranging from 30-60 °C and is given in *fig. 4.2.6.*

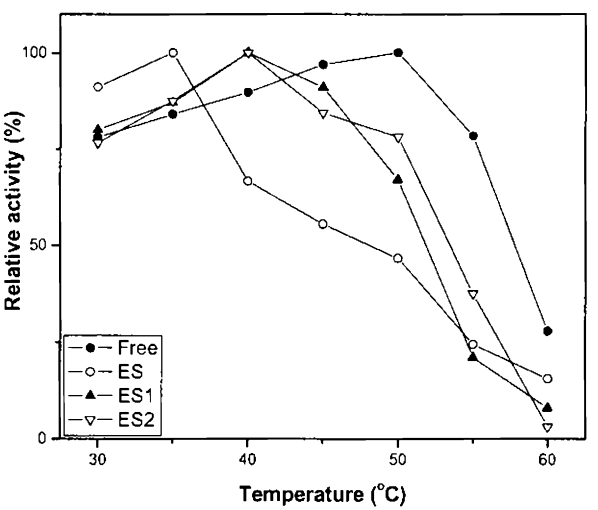

***Fig. 4.2.6** Effect of temperature on relative activity of free and immobilized α-amylase.*

The optimum temperature of native enzyme was 50 °C which decreased to 35-40 °C when it immobilized. *Handa et al.* reported [8] a 5 °C decrease in optimum temperature when α-amylase was immobilized on to polyacrylonitrile. The decrease in the optimum temperature might arise from changing the conformational integrity of the enzyme structure by immobilization [5, 38] which favored amylase activity below 50 °C. Otherwise the attachment on the support might have affected the three dimensional protein structure which either caused the denaturation of the protein structure or affected the conformation of protein which resulted in an alteration of enzyme substrate affinity [12] and hence showed a decrease in the immobilized enzyme catalytic activity at higher temperature.

4.2.4 Thermal stability of the free and immobilized enzymes

Thermal stability experiments were carried out with free and immobilized enzymes, which were incubated for 1 hour in the absence of substrate at various temperatures. *Fig. 4.2.7* shows the thermal inactivation curves between 30 and 60 °C for the free and immobilized enzymes.

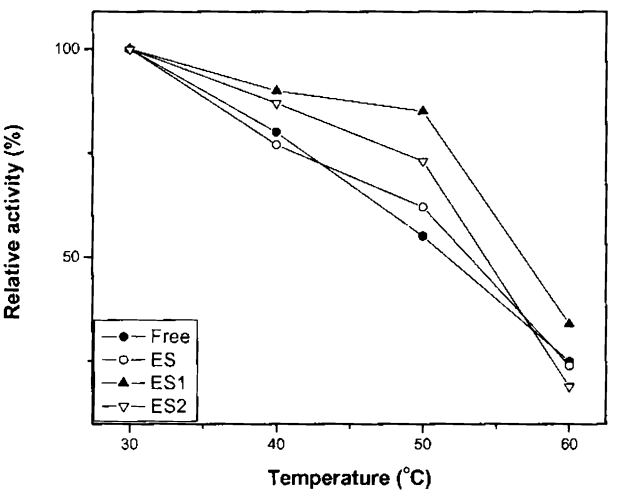

Fig. 4.2.7 *Effect of temperature on stability of free and immobilized α-amylase.*

There was no change in the activity of free and immobilized enzyme for 1 hour at 30 °C. As the temperature rises, the stability dropped significantly for both free and immobilized amylase. At 40 °C, the immobilized and free enzymes retained their activity about to a level of 80-90 %. At higher temperature the immobilized amylase was inactivated at a much slower rate than that of the free form. The free and immobilized enzyme lost 60-80 % their activity at 60 °C after 1 hour heat treatment. *Fig. 4.2.8* shows the effect of incubation time at optimum temperature on the activity of each immobilized enzyme. Almost 90 % of the immobilized enzymes were active even after 120 minutes of incubation at their respective optimum temperatures. These results suggest that the thermal stability of α-amylase increased considerably as a result of immobilization onto emeraldine salts, and is suitable for long term process. Improved thermal stability of the immobilized amylase was reported by *Bryjak* [39] when using acrylic carriers as the support.

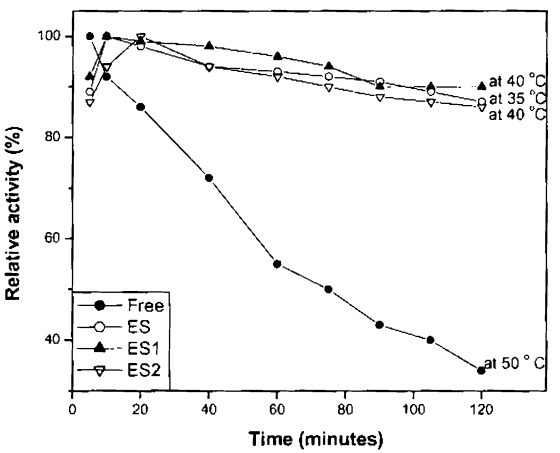

Fig. 4.2.8 *Effect of pre-incubation time on stability of free and immobilized α-amylase.*

The immobilized enzyme, especially in the activated ES bound system, was more resistant than that of the soluble form against heat. The strong interaction with the support material is supposed to preserve the tertiary structure of the enzyme which enables the enzyme being protected from conformational changes imposed by heat [26]. It has been

reported [40] that the multipoint attachment to a complementary surface of a relatively rigid support drastically increased the thermal stability of the enzyme.

4.2.5 Determination of Michaelis constant

It seems interesting to analyze the enzymatic hydrolysis with immobilized α-amylase in the framework of the Michaelis-Menten mechanism. The kinetic parameters, the Michaelis constant K_m and maximum velocity V_{max} for free and immobilized α-amylase, were determined by varying the concentration of starch in the reaction medium. Linewcaver-Burk plots and Hanes-Woolf plots for free and immobilized α-amylase are shown in *fig. 4.2.9*. The kinetic parameters of free and immobilized enzymes are summarized in *table 4.2*. The apparent K_m values for the immobilized α-amylase were higher than those of free α-amylase. In general K_m of an immobilized enzyme is different from that of free enzyme due to the alteration in enzyme conformation, micro environmental effects of the carrier, and bulk and diffusional effects [44]. The decrease in affinity towards substrate is also caused by the structural changes introduced in the enzyme by immobilization procedure and hence resulted in the lower the accessibility of the substrate to the active sites of the immobilized enzyme [42]. On the other hand, the V_{max} values followed the opposite trend, suggesting the residual activity of the immobilized α-amylase decreased. Such observations have been generally reported [43-45] during immobilization of enzymes.

Table 4.2 *Kinetic parameters of the free and immobilized α-amylase.*

	Free enzyme	**ES**	**ES1**	**ES2**
K_m **(mg mL^{-1})**	0.46 ± 0.09	2.15 ± 0.11	0.83 ± 0.08	2.89 ± 0.03
V_{max} **(EU μg^{-1} protein)**	7.95 ± 0.05	2.71 ± 0.07	4.73 ± 0.04	3.89 ± 0.13

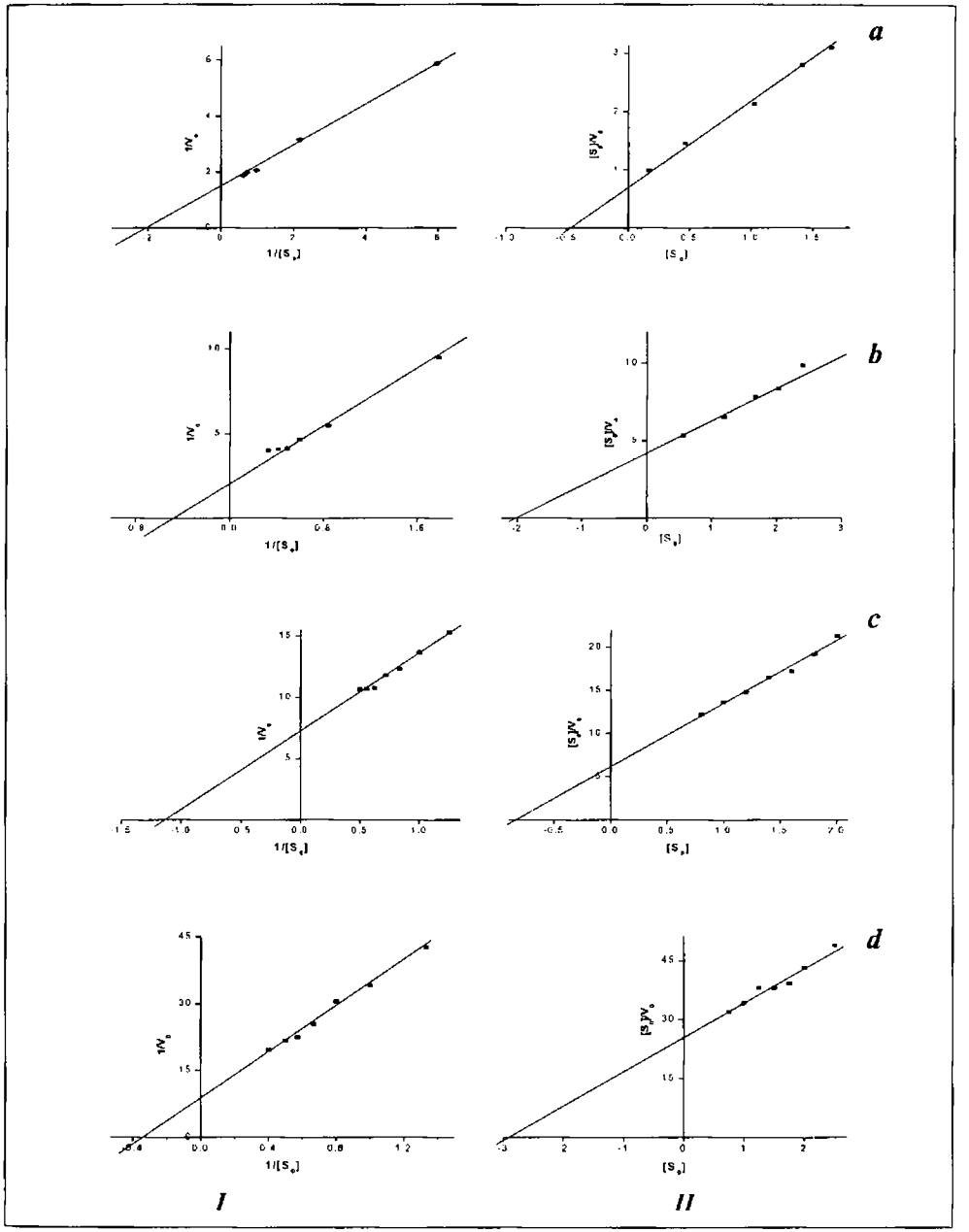

Fig. 4.2.9 Lineweaver-Burk plots (I) and Hanes-Woolf plots (II) of free α-amylase (a) and immobilized on ES (b) on ES1 (c) and on ES2 (d).

4.2.6 Storage stability of the immobilized α-amylase

One of the most important parameters to be considered in enzyme immobilization is storage stability. The stability for long term storage of the immobilized α-amylase on emeraldine salt powders was examined by storing them at 4 °C in semidry condition for a period of time. Free α-amylase in buffer solution was also prepared and stored under the same conditions. The free enzyme in buffer solution lost all of its activity within 2 days. Under the storage conditions, less reduction in activity for the immobilized enzymes was observed.

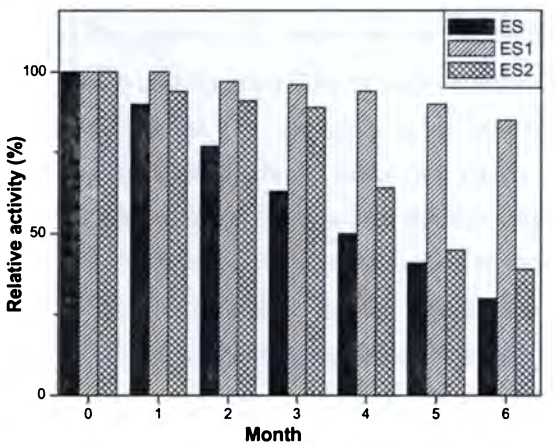

Fig. 4.2.10 *Effect of storage time on the relative activity of immobilized α-amylase stored for 6 months.*

As shown in *fig. 4.2.10*, the adsorbed α-amylase retained more than 50 % of their original activity over a period of four months, and the activity of the covalently bonded enzyme on ES1 was more than 80 % of its original activity after 6 months while that on ES2 retained only 40 % of its original activity over the same period of time. This result readily suggests that the immobilized amylase exhibits improved storage stability over the free enzyme. *Kahraman et al.* reported [26] 80 % activity retention after 25 days of storage for α-amylase immobilized on glass beads and *Lim et al.* reported [18] a 10 % loss of

activity in every 12 days of storage for amylase on silanized silica particles. The stability of the immobilized α-amylase can be attributed to its improved resistance to heat and denaturing agents [46], as a result of adsorption and covalent fixation on the surface of the emeraldine salt powders.

4.2.7 Reusability

In order to improve enzymatic process economically, the enzyme could be used in continuous process over a long period of time to exploit it completely. Inactivation is the most prominent problem when exposed to inadequate ambient conditions, such as extreme pH or temperature [47], organic solvent and surfactant [48]. On this account, improvement in the reusability of immobilized enzymes is of great importance. The excellent reusability will prolong the half-life of enzymes. The easy separation of catalyst from the product mixture followed by reuse is one of the most important advantages of heterogeneous catalysis.

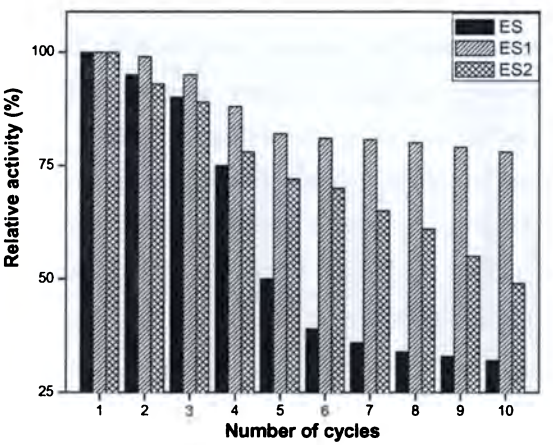

Fig 4.2.11 Reusability of immobilized α-amylase.

In our study activity for 10 cycles of use for the immobilized enzymes was monitored and the results are shown in the *fig. 4.2.11*. It was examined in a batch wise manner by using the same condition repeatedly 10 times within 8 hours. It was observed

that the immobilized enzyme activity decreased when recycling number was increased. Both of the covalently bonded enzymes retained 80 % activity after 4 runs and about 50-70 % activity even after 10 cycles of use and further washing. But the adsorbed enzyme was leached out during repeated use and could retain only 30 % activity after 10 runs. This excellent reusability could be explained by improved resistance to denaturation and conformational changes caused by the surrounding conditions such as buffer solution, as a result of covalent immobilization on the support [26]. This agreed well with the reported results, the reuse capabilities of α-amylase was in the range of 74-88 % when immobilized on UV curable support [16] and in another study, reuse capabilities of α-amylase on poly(dimer acid-co-alkyl polyamine) particles [49] demonstrated more than 99 % activity after 20 runs and 91.3 % activity after 40 runs.

4.3 Immobilization of α-Amylase on Emeraldine Base

4.3.1 Optimization of immobilization conditions

The effects of enzyme coupling time and pH on the enzyme activity were studied with glutaraldehyde activated EB1, ascorbic acid activated EB2 and non-activated EB at room temperature and at different reaction coupling pH and time. The effect of immobilization pH on the activity of immobilized amylase is shown in *fig. 4.3.1.* The best activity showed after immobilization was found in the pH region of 5-5.5. The possible reasons are lower loading and enzyme conformational change due to unfavorable charge distribution of amino acid residues by the medium which may reduce the activity of the enzyme [13]. Since the free α-amylase used in this experiment was stable at narrow pH range (4.5-5.5) the possible denaturation of enzyme in alkaline solution was also expected.

The activity yield of the immobilized α-amylase coupled at different coupling reaction time is presented in *fig. 4.3.2.* An increase in the coupling duration time up to 30-45 minutes led to an increase in the activity of the immobilized enzyme on EB1 and EB2. The maximum enzyme activity of adsorbed enzyme on EB powders was observed after 75

minutes of reaction coupling time. Both activated and non activated EB showed a decrease in activity yield after prolonged incubation time.

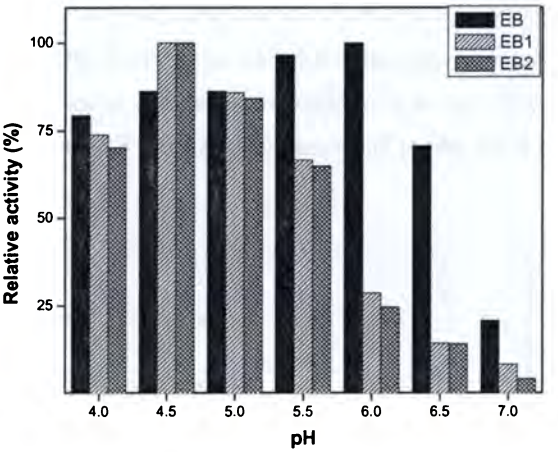

Fig. 4.3.1 Effect of pH of immobilization medium on the activity of bound α-amylase.

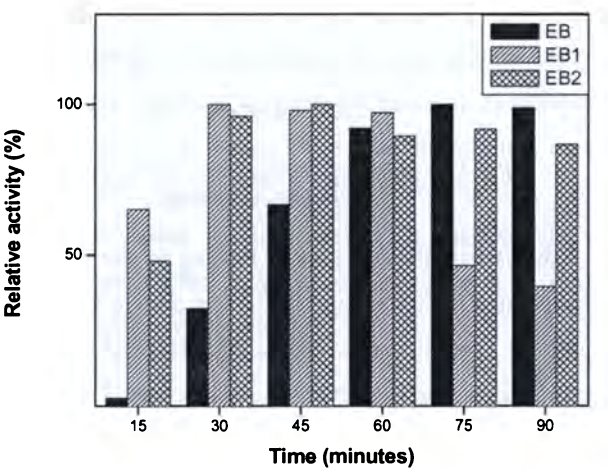

Fig. 4.3.2 Effect of contact time for immobilization on the activity of bound α-amylase.

On the other hand, as time prolonged a large amount of amylase was immobilized to EB, on the basis of protein determinations, but the specific activity of these preparations was low. The decrease in the immobilized enzyme activity can be due to the multipoint attachment of the enzyme molecules to the activated EB1 and EB2 or overcrowding of the immobilized amylase on non-activated EB, as a result of which substrate diffusion limitations occur [50]. In view of these observations, coupling time of 30, 45 and 75 minutes were used in the rest of the experiment for EB1, EB2, and EB respectively.

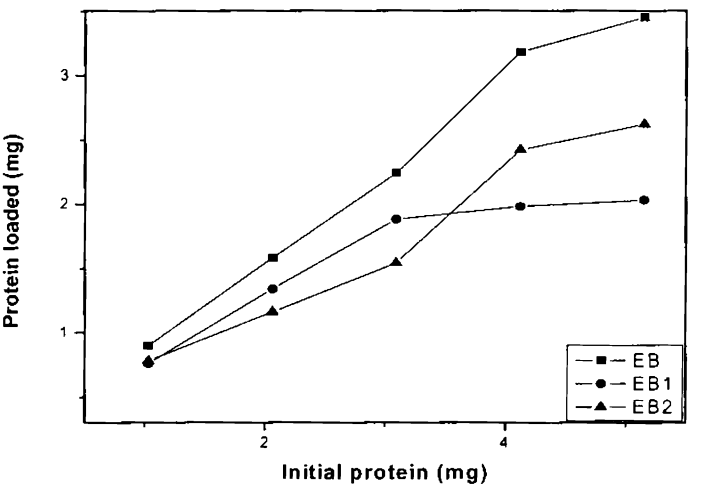

Fig 4.3.3 *Influence of initial protein concentration on the protein loading.*

Fig. 4.3.3 shows the effect of protein loading on the immobilization yields of the supports. EB incorporated higher amount of 3.5 mg protein g^{-1} EB, which was 66.9 % of the enzyme offered for immobilization. EB2 coupled almost 50 % of the offered enzyme. On EB1 a saturation level reached when the loading was 2 mg protein g^{-1} EB1. Further addition only made a negligible change in the protein loading. This nonlinear relationship after a particular interval of protein loading was reported by *Fernandes et al.* [34] and *Chen et al.* [35].

Fig. 4.3.4 shows the recovery of the enzyme activity upon the addition of enzyme to support. The maximum activity is shown by enzyme immobilized on EB1with activity of more than 6 EU.

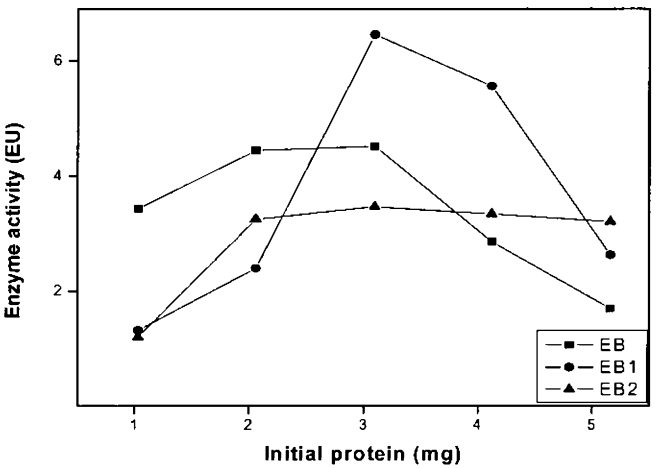

Fig. 4.3.4 *Effect of initial protein amount on the activity of immobilized enzyme.*

Table 4.3 also shows this immobilization yield, activity yield and immobilization efficiency for the enzymes and it can be observed that high immobilization efficiency was obtained for EB1 (42.4%). These values show that the covalent coupling of amylase to glutaraldehyde linked EB1 through their free amino groups, provided immobilized derivatives with good enzymatic activities. But in all cases activity yield less than one, which was expected as usual after enzyme immobilization as a result of conformational changes, shear and non productive binding [11].

Table 4.3 *Immobilization efficiency for α-amylase per g of emeraldine base.*

Polymer	Initial protein (mg)	Immobilized protein (mg g^{-1} support)	Immobilization Yield (%) IY	Initial activity (EU)	Activity of immobilized enzyme (EU g^{-1} support)	Activity Yield (%) AY	Immobilization Efficiency (%) IE = AY/IY
EB	3.1	2.2	71	24.6	4.5	18.3	25.8
EB1	3.1	1.9	61.3	24.6	6.4	26	42.4
EB2	3.1	1.5	48.4	24.6	3.5	14.2	29.3

4.3.2 Effect of pH on enzyme activity

As a result of immobilization the pH optimum and the activity curve for enzyme may change depend upon the ionic conditions of its immediate environment. Hence, the pH of the assay medium was altered from 4 to 8 and the resulting effect on the enzyme activities were measured (*fig.4.3.5*).

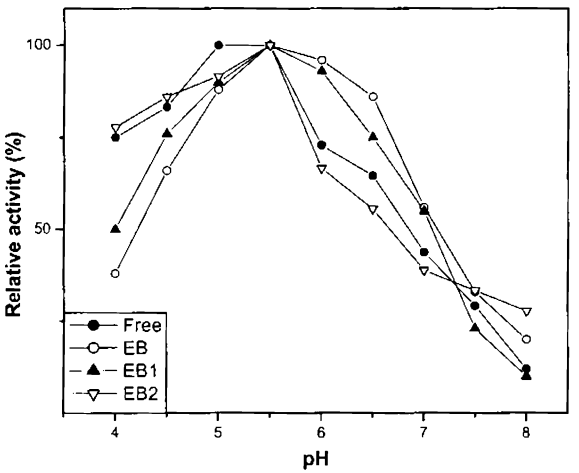

Fig. 4.3.5 *Effect of pH on the activity of free and immobilized α-amylase.*

Immobilized enzymes showed its optimum activities at pH 5.5. Within the range of pH 4.0-7.5, the pH profile for both free and immobilized on EB2 α-amylase were very similar. Compared to them amylase immobilized on EB and EB1 exhibited higher activity above pH 6.0 and lower activity below pH 5.0. This indicated that the immobilization procedure improved the stability of α-amylase appreciably particularly in the alkaline region. A possible explanation of the results obtained is that immobilization on these supports may be created some secondary interactions like hydrogen bonding, ionic and polar interactions between enzyme and polymeric matrix which cause conformational changes in the enzyme molecules resulting in changes of protein's charge [17, 41].

4.3.3 Effect of temperature on activity

Enzymes are sensitive to temperature changes. The effect of temperature on relative enzyme activity is illustrated in *fig. 4.3.6.* Upon immobilization on EB the optimum temperature for the activity of amylase is decreased from 50 °C to 40 °C. This indicates that less energy of activation required for immobilized enzyme, or higher temperatures favor the formation of some multiple linkages between support and enzyme leading to a decrease in the activity of the enzyme [51].

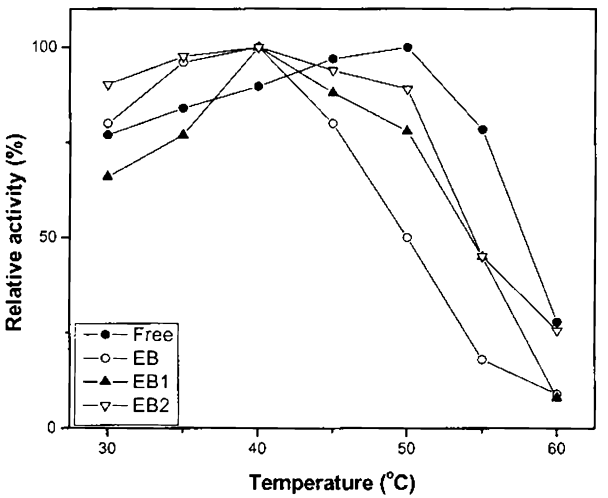

Fig. 4.3.6 *Effect of temperature on the activity of free and immobilized α-amylase.*

4.3.4 Effect of temperature on stability

The effect of temperature on the stability of the free and immobilized α-amylase is shown in *fig. 4.3.7.* The stability of the α-amylase is strongly dependent on temperature and the activity of the α-amylase shows a more critical temperature dependence at temperatures above the optimum temperature. The free and immobilized amylases retain only less than 30 % of their optimum activities at temperature above 60 °C. The temperature stability of

both free and immobilized enzyme with respect to the preincubation time at their optimum temperature is shown in the *fig.4.3.8.*

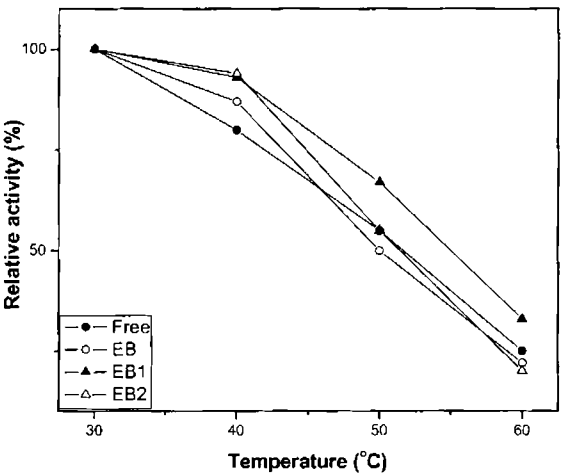

Fig. 4.3.7 *Effect of temperature on the stability of free and immobilized α-amylase.*

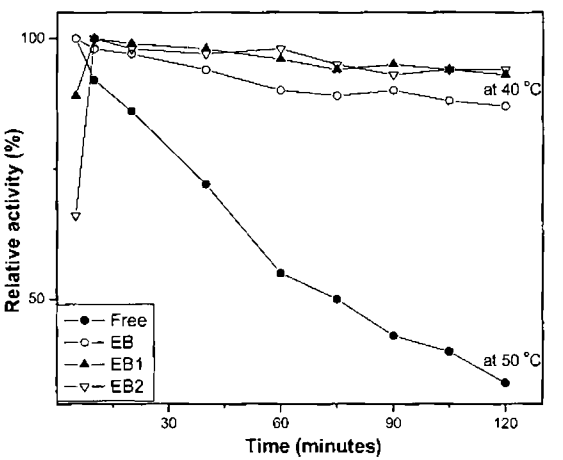

Fig. 4.3.8 *Effect of preincubation time on the activity of free and immobilized α-amylase at optimum temperature.*

The results suggested that the immobilized amylase holds much more excellent heat resistance than that of the free enzyme. Almost 90 % of immobilized enzymes retained their activity after 120 minutes of heat treatment, while free enzyme could retains only 30 % of its initial activity.

An explanation for the difference in temperature activity profiles between the free and immobilized amylase is that the latter is less susceptible to temperature-induced conformational changes after immobilization on EB powders. The decrease of relative activity of the free enzyme was probably due to its thermal denaturation, while the relative activity of the immobilized α-amylase decreased slowly because of some protections of the carrier for the immobilized enzyme [26]. It was also suggested that [11] immobilization can help to distribute the thermal energy imposed to the protein at higher temperatures.

4.3.5 Kinetic constants

Kinetic parameters, the Michaelis constant K_m and the V_{max} for free and immobilized α-amylases were determined using soluble starch as substrate and are summarized in *table 4.4.* In the free and immobilized enzyme preparations, Michaelis-Menten kinetic behavior was observed. The Lineweaver-Burk plots and Hanes-Woolf plots for the immobilized α-amylases are presented in *fig. 4.3.9.* For the free enzyme, K_m was found to be 0.46 mg mL^{-1} whereas V_{max} was calculated as 7.95 EU μg^{-1} proteins. Kinetic constants of the immobilized amylase were also determined in the batch system. The K_m values were found to be as 4.58, 1.99, 1.03 mg mL^{-1} for adsorbed, glutaraldehyde coupled and ascorbic acid coupled enzymes respectively. The V_{max} values of immobilized enzyme for adsorbed, glutaraldehyde and ascorbic acid activated preparations were estimated from the data as 2.23, 3.8 and 2.48 EU μg^{-1} protein of immobilized enzyme onto the emeraldine base, respectively. In this study, as expected, the K_m and V_{max} values were significantly affected after immobilization of amylase onto emeraldine base. These effects were more pronounced when enzyme physically adsorbed on the support. The change in the affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure and by lower accessibility of the large

substrate to the active site of the immobilized enzyme. Since immobilization process does not control proper orientation of the enzyme on the support, this may change the property of the active site, which might hinder the active site for binding the substrates [52].

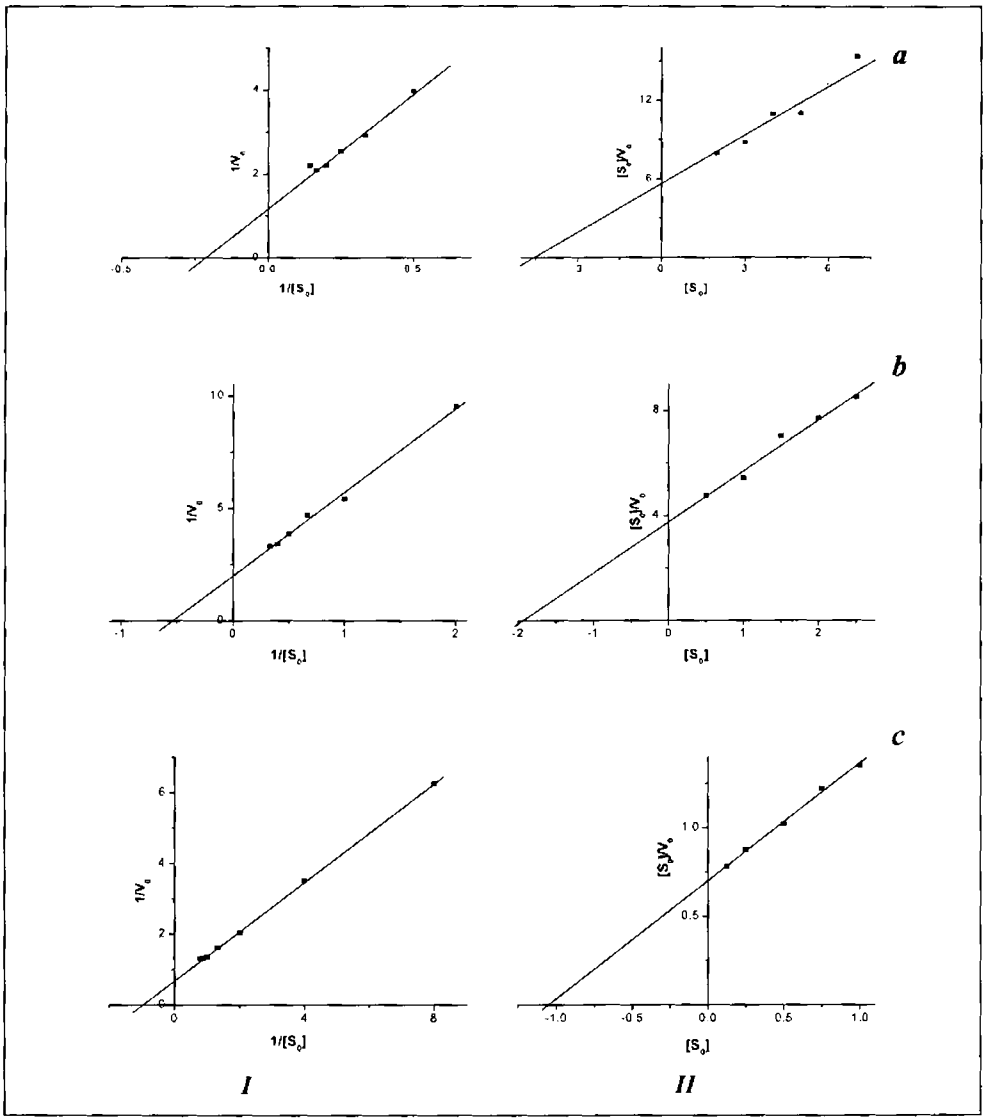

Fig. 4.3.9 *Lineweaver-Burk plots (**I**) and Hanes-Woolf plots (**II**) for α-amylase immobilized on EB (**a**), EB1 (**b**) and EB2 (**c**).*

Table 4.4 *Kinetic constants for the α-amylase immobilized on emeraldine base.*

	Free enzyme	EB	EB1	EB2
K_m (mg mL^{-1})	0.46 ± 0.09	4.58 ± 0.05	1.99 ± 0.04	1.03 ± 0.06
V_{max} (EU μg^{-1} protein)	7.95 ± 0.05	2.23 ± 0.03	3.8 ± 0.05	2.48 ± 0.09

4.3.6 Storage stability

Stability for long term storage is one of the important parameters to be considered in enzyme immobilization. The immobilization of enzyme to a support often limits its freedom to undergo drastic conformational changes, and hence results in increased stability towards denaturation. In general the enzymes solutions are not stable during storage. Storage stabilities of free and immobilized enzymes were studied at 4 °C is depicted in *fig. 4.3.10.*

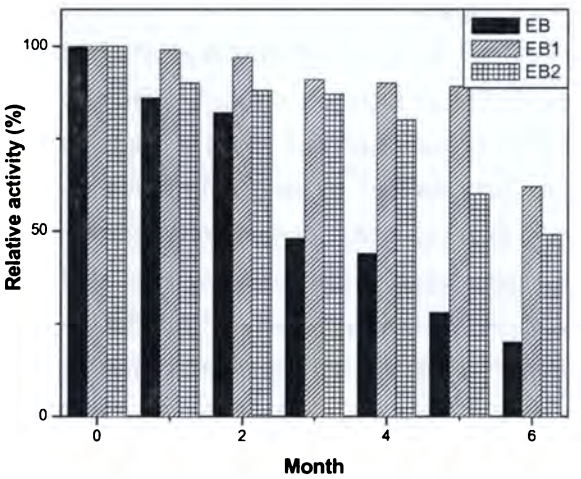

Fig. 4.3.10 *Storage stabilities of bound α-amylase stored at 4 °C.*

Free amylase solution stored at 4 °C lost its original activity within 2 days, while immobilized enzymes stored in semi-dry condition at 4 °C retained above 60 % of its activity after a period of 3 months. The covalently coupled enzymes stored at 4 °C lost nearly 50 % of their activity, but the adsorbed enzyme stored at 4 °C lost about 80 % of its activity after 6 months storage. *Bayramoğlu et al.* reported [29] the enhanced stability of α-amylase when immobilized on reactive polymer membranes. Hence it is clear that immobilization definitely held the enzyme in a stable position in comparison to the free counterpart.

4.3.7 Activity after various cycles

In order to check the reusability of the immobilized enzymes, the system was submitted to ten consecutive reaction cycles. The results (*fig. 4.3.11*) indicate that the catalytic activity of the immobilized enzyme is durable under repeated use. Thus, the immobilized enzyme is able to maintain good starch hydrolysis above 60 % even after five runs.

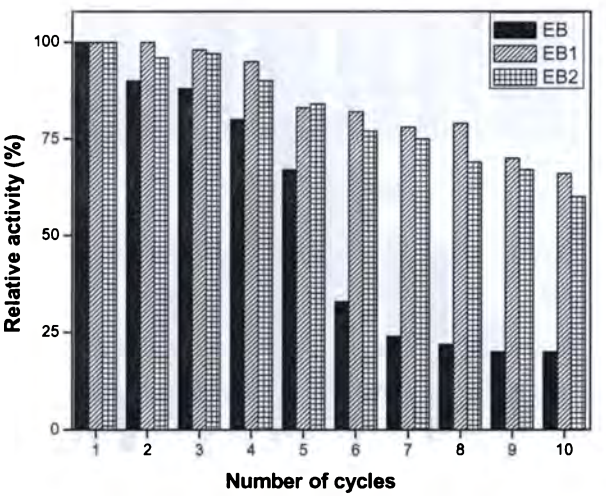

Fig. 4.3.11 *Relative activity of the immobilized α-amylase after repeated use.*

There was no drastic decrease in percent hydrolysis by covalently coupled enzymes, especially coupled with glutaraldehyde even after ten uses, due to reduction of enzyme leakage from the support. This result suggests that the covalently immobilized amylase is more stable on the polyaniline surface. Similar to this, *Sadhukhan et al.* [53] reported that the activity loss of immobilized α-amylase was 40 % after the sixth cycle, whereas *Bayramoğlu et al.* reported [54] that α-amylase film strip hardened with chromium sulphate was able to retain 41.2 % of activity after 20 runs. It should be noted that this high operational stability could significantly reduce the operation cost in practical applications. A big advantage gained with this immobilization is the washing, reusing possibility and long term storage in semi-dry condition without much activity loss.

4.4 Immobilization of α-amylase on Poly(*o*-toluidine) Salt

4.4.1 Enzyme immobilization parameters

The effect of pH of immobilization medium on the enzyme activity is shown in *fig. 4.4.1*. The best pH for immobilization was observed in the range of 5-5.5, since most of free amylase taken for immobilization is less stable above and below this pH range.

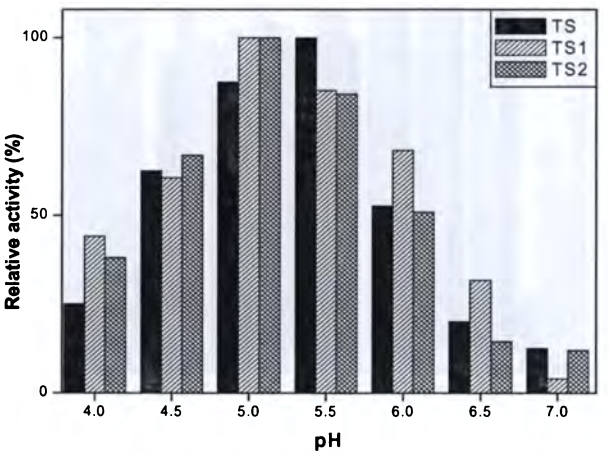

Fig. 4.4.1 *Influence of immobilization pH on the activity of α-amylase.*

The time necessary for immobilization of α-amylase is shown in *fig. 4.4.2*. The figure shows significant differences in the immobilized enzyme activity up to 30-60 minutes. After this point, a decrease in activity retention was observed. Apparently, the immobilization reaction occurred in the first 30-60 minutes. Contact with the free enzyme in the solution and those immobilized after a particular time may produce layers of adsorbed enzyme that presents lower activity than those covalently immobilized in the first minutes. Another possibility was pointed out that increasing the contact of enzyme and support might promote multiple bonds and result in enzyme deformations, compromising the active site integrity [55].

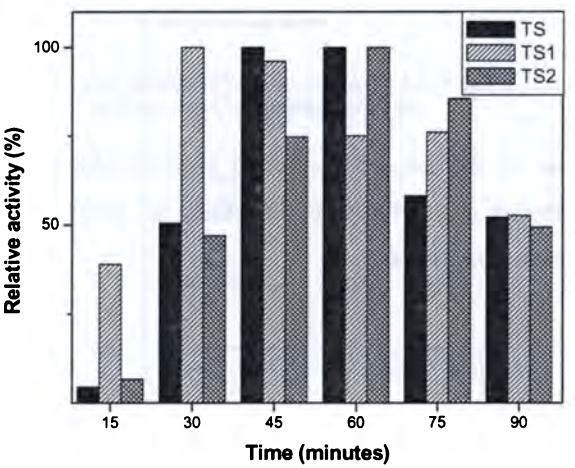

***Fig 4.4.2** Influence of coupling time on the activity of immobilized α-amylase.*

The best protein loading was obtained (1.7mg g^{-1} of support) with activating and cross-linking by glutaraldehyde (TS1), as shown in *fig. 4.4.3*. This immobilized enzyme has an activity of 4.9 EU, which in terms of relative activity, was only 14.9 % (*table 4.5*). The relative activity is the effective activity of the immobilized enzyme compared with the activity in solution. This low relative activity reflects that much of the enzyme was immobilized in an inactive form owing to the instability towards immobilization process.

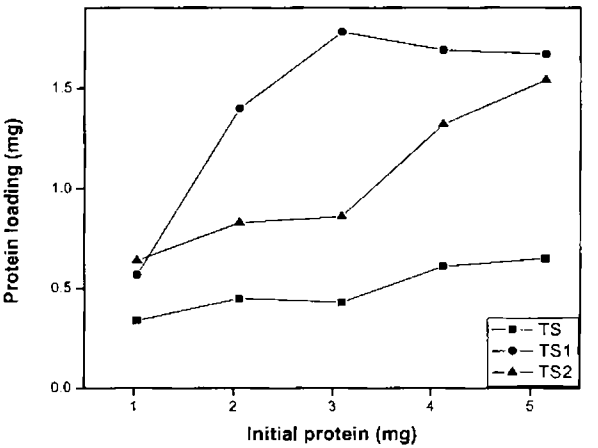

Fig. 4.4.3 Variation of protein loading relative to the initial amount of protein taken.

In the case of ascorbic acid activated poly(*o*-toluidine) the relative activity of immobilized α-amylase was increased by increasing the amount of protein in the solution up to 4 mg, as shown in *fig. 4.4.4.*

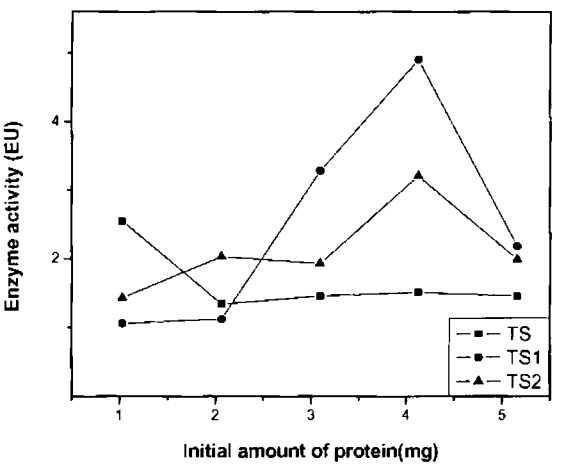

Fig. 4.4.4 Influence of initial protein concentration on the activity of immobilized enzyme.

Table 4.5 Immobilization efficiency of α-amylase on TS.

Polymer	Initial protein (mg)	Immobilized protein (mg g^{-1} support)	Immobilization Yield (%) IY	Initial activity (EU)	Immobilized enzyme activity (EU g^{-1} support)	Activity Yield (%) AY	Immobilization efficiency (%) IE = AY/IY
TS	1.0	0.3	30	8.2	1.3	15.9	53
TS1	4.1	1.7	41.5	32.8	4.9	14.9	35.9
TS2	4.1	1.3	31.7	32.8	3.2	9.8	30.9

When more enzymes were added, the amount of protein bound on the TS2 surface increased, but the activity per mg of protein did not increase any further. This suggests that a higher coverage of protein could either hide or damage some active sites of the immobilized enzymes and thus lead to a decline in total activity. The adsorbed enzyme on TS could incorporate only a small amount of enzyme around 0.3 mg in the saturation level. The maximum activity yield was 15.9, which has highest value for immobilization efficiency of 53 % indicated that most of the immobilized enzymes retained their original activity.

In the literature there are many different loading values available for different supports with various levels of activity retention for α-amylase immobilization. For example coupling capacities were reported as 3 and 29 mg^{-1} g respectively, on polystyrene and silica based supports [21], 25-90 mg g^{-1} for celluloic supports [56] and 94 mg g^{-1} for UV curable polymeric materials [57].

4.4.2 Effect of pH on the activity of free and immobilized α-amylase

The effect of pH on the activity of free and immobilized α-amylase in starch hydrolysis was determined in the pH range 4.0-7.0 and the results are presented in *fig. 4.4.5*. The optimum activity for free enzyme was observed for both at pH 5 and 5.5. Similar to our previous results of immobilization on PANI, the optimum pH value of α-amylase narrowed to a single value of 5 and 5.5, after immobilization via adsorption and covalent coupling on the poly(*o*-toluidine), respectively. It is said that polyionic matrices cause the partitioning of protons between the bulk phase and the enzyme microenvironment causing a shift in the optimum pH value, and shift depends on the method of immobilization as well as on the structure and charge of the matrix [29, 41, 58, 59]. Usually the shift of pH optimum to the acidic side is explained with increase of positive charge. As a result of that the concentration of H^+ ions in the microenvironment of the immobilized enzyme decreases which means that the pH of the immobilized enzyme region is more alkaline than that of the external solution. Since the effect of pH on the enzyme activity is expressed based on

the pH of the external solution, which in this case decreases, the pH optimum shifts to the acidic side [7].

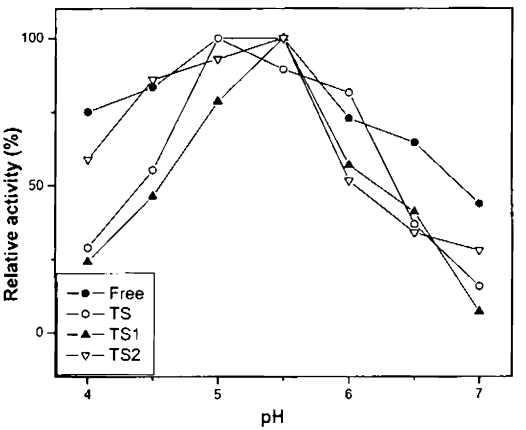

Fig. 4.4.5 Effect of pH on the activity of free and immobilized α-amylase.

4.4.3 Effect of temperature on activity

Optimum activity for free and both immobilized preparations were observed at 50 °C and 40 °C, respectively (*fig. 4.4.6*). Such significant change of temperature optimum after immobilization of amylase has also been reported by other authors [26, 30, 41]. Compared with the free α-amylase immobilized one exhibited lower activity at temperature above 40 °C. Probably upon immobilization conformational changes in the enzyme molecules occurred that favored amylase activity at temperature 40 °C. On the other hand, these changes caused decreasing of the immobilized enzyme catalytic activity in the interval 45-60 °C compared to the free enzyme. In general, the effect of changes in temperature on the rates of enzyme-catalyzed reactions does not provide much information on the mechanism of biocatalysts. However, these effects can be important in indicating structural changes in enzyme.

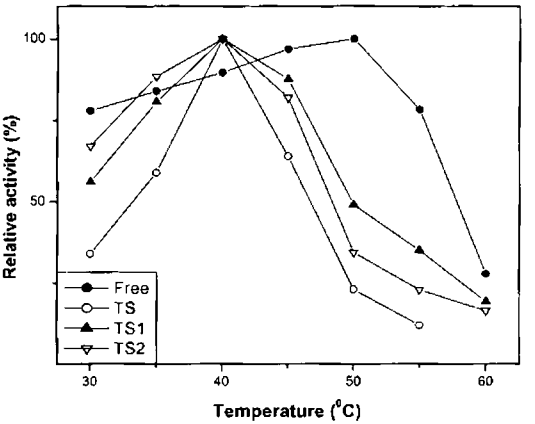

Fig. 4.4.6 Effect of temperature on the activity of free and immobilized α-amylase.

4.4.4 Thermal stability

Fig. 4.4.7 represents the stability of free and immobilized enzymes at elevated temperatures, which is recorded by measuring enzyme activity after incubation of enzyme in buffer for 1 h at different temperatures.

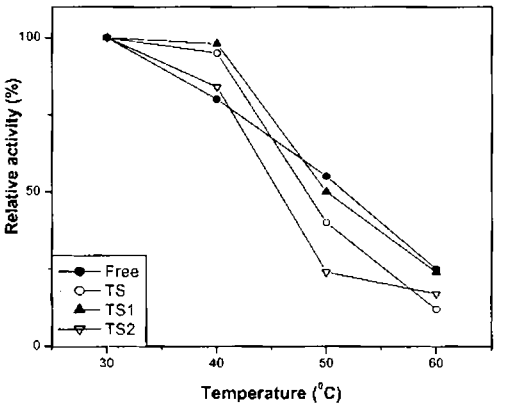

Fig. 4.4.7 Effect of temperature on the stability of the free and immobilized α-amylase.

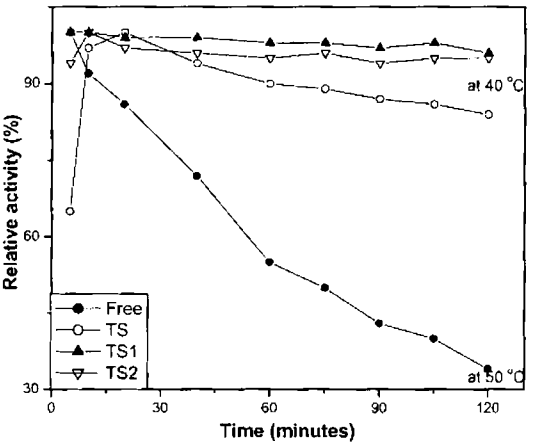

Fig. 4.4.8 Variation of activity of enzymes relative to preincubation time at their optimum temperature.

At 40 °C immobilized enzymes showed good stability hence good activity than free enzyme, after that their stability decreased. From the *fig. 4.4.8* it is clear that immobilized enzymes are retaining more than 90 % activity at their optimum temperature even after 120 minutes of incubation. As it is evident from the *fig. 4.4.7* and *fig. 4.4.8* the immobilized enzyme possessed a better heat-resistance than free enzyme. Probably this can also be explained by the fact that the immobilization procedure could protect the enzyme active conformation from distortion or damage by heat exchange [60].

4.4.5 Kinetic parameters of the amylase immobilized on TS

The values of K_m and V_{max} were calculated from Lineweaver-Burk and Hanes-Woolf plots (*fig. 4.4.9*). As seen from the *table 4.6*, the calculated K_m for the immobilized enzymes were 6.35, 1.57 and 2.95 mg mL^{-1} on TS, TS1 and TS2 respectively, which are higher than that of free enzyme (0.46 mg mL^{-1}); whereas the V_{max} of immobilized enzyme on the toluidine were 4.2, 3.08 and 2.68 EU μg^{-1} protein on TS, TS1 and TS2 which are smaller than that of the free enzyme (7.95 EU μg^{-1} protein). These data indicated that

affinity between immobilized enzyme and substrate decreased in comparison with free enzyme.

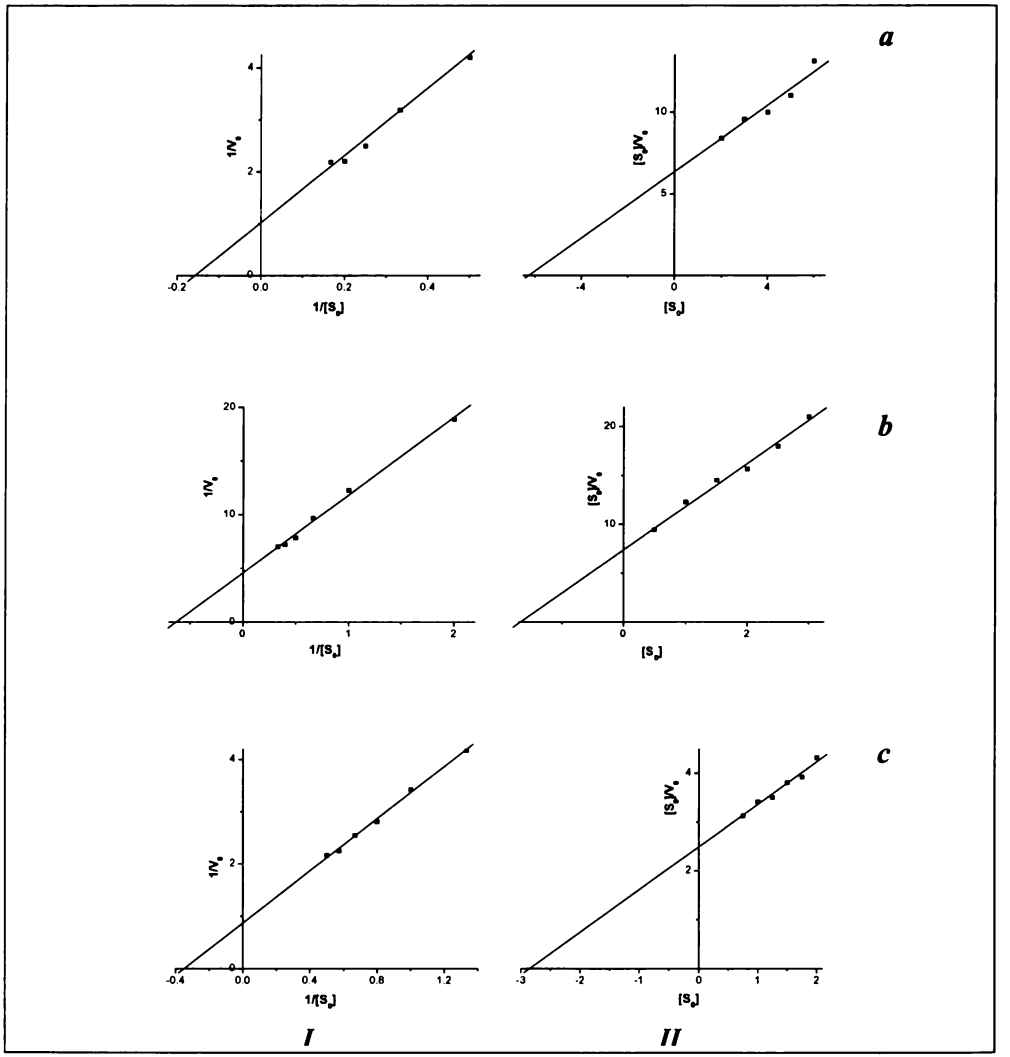

Fig. 4.4.9 Lineweaver-Burk plots (I) and Hanes-Woolf plots (II) for α-amylase immobilized on TS (a) TS1 (b) and TS2 (c).

This might be related to diffusion barriers and steric effects, conformational changes, or the new microenvironment effects [42, 61]. On the other hand, the change in

affinity could be also owing to the structural changes caused by enzyme immobilization onto the supports, and hence resulted in the lower accessibility of the substrate to the active sites of the immobilized enzyme [62].

Table 4.6 *Kinetic constants for free and immobilized α-amylase.*

	Free enzyme	**TS**	**TS1**	**TS2**
K_m **(mg mL^{-1})**	0.46 ± 0.01	6.35 ± 0.05	1.57 ± 0.04	2.95 ± 0.09
V_{max} **(EU μg^{-1} protein)**	7.95 ± 0.05	4.2 ± 0.03	3.08 ± 0.08	2.68 ± 0.08

4.4.6 Storage stability

The stability of an immobilized enzyme is dictated by many factors such as the number of bonds formed between the enzyme and support, the nature of the bonds, the degree of confinement of enzyme molecules in the matrix, and the immobilization conditions.

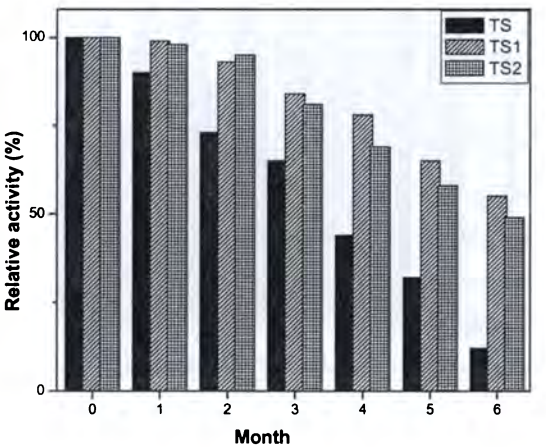

Fig. 4.4.10 *Storage stability of immobilized α-amylase.*

Under the semi dry storage conditions, the starch hydrolyzing activity of the α-amylase immobilized via covalent coupling decreased at a slower rate than that of the adsorbed enzyme (*fig. 4.4.10*). Upon 5 months of storage, the adsorbed enzyme preserved only 32 % of its original activity, while the covalently bound enzyme had about 60 % retention in the activity.

4.4.7 Reuse of immobilized enzymes

The recovery and recycling of enzymes could lower the overall cost of the enzyme immobilization process. However, the applications of immobilization are often challenged by difficulties that arise with regard to enzyme recovery and recycling. In this study, the batch reaction repetitions, the activity of the immobilized enzyme remained stable throughout the experiment, and above 70 % activity were retained for covalently bound enzyme after consecutively repeating the reactions 10 times. This value is higher than that reported by *Laska et al.* [63] for urease immobilized on polyaniline, which exhibited an 80 % loss in activity after seven batch reactions.

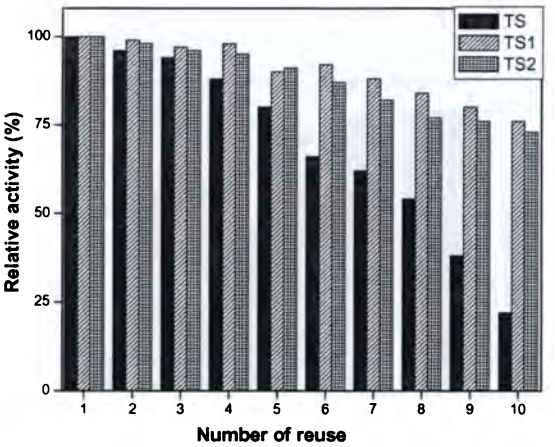

Fig. 4.4.11 Reusability of α-amylase immobilized on TS.

4.5 Immobilization of α-amylase on Poly(*o*-toluidine) Base

4.5.1 Optimal parameters for α-amylase immobilization

The effects of the following parameters on the immobilization process were investigated: pH of the solution, reaction time and concentrations of enzyme. As expected from our previous studies, it was found that the immobilized enzymes presented a relatively high activity when the pH value of the enzyme solution was 5-5.5 (*fig. 4.5.1*).

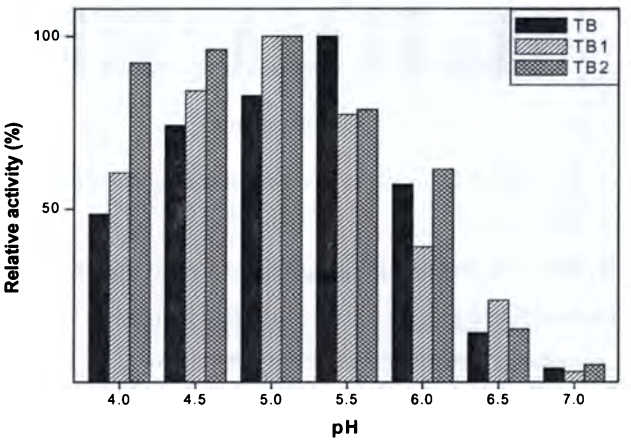

Fig. 4.5.1 *Influence of immobilization pH on the retained activity of α-amylase.*

As shown in *fig. 4.5.2* prolonging the immobilization time more than 45, 30 and 60 minutes did not improve the activity of α-amylase immobilized on TB, TB1, and TB2 respectively. In this case, a possible interpretation is that the reactive functional groups on the support were saturated by the enzyme molecules after duration of 45-60 minutes, hence extending the duration time further could not improve immobilized enzyme activity. Instead, the activity may be reduced owing to the spatial repulsion of overcrowded enzyme molecules immobilized onto supports. It has been reported [29] that the coupling time is leveled after 18 hour of immobilization of α-amylase on reactive membranes with maximum loading 83 $\mu g\ cm^{-2}$ of support.

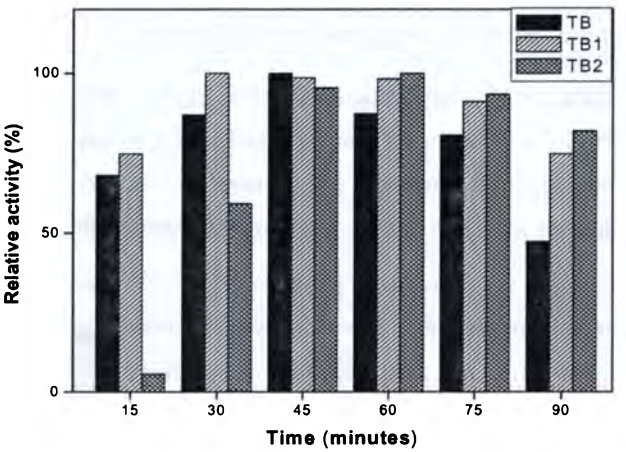

Fig. 4.5.2 *Influence of coupling time on the activity of bound α-amylase.*

The activities of α-amylase preparations related to the protein loading on supports, the amounts of immobilized protein per g of different carriers as well as the immobilization efficiency are summarized in *table 4.7*. The data shows that, all types of the prepared support particles are suitable for enzyme immobilization, especially enzyme on TB1 obtained with glutaraldehyde spacer exhibiting the highest activity. Although highest amount of protein bound on the TB2 particles, the highest activity was obtained by using the TB1 support activated with glutaraldehyde. Considerably less activity was reached after α-amylase immobilization on TB2 than on TB1 (*table 4.7*). As resulted from the enzyme immobilization experiments, the highest amount of protein was bound by TB2, but the lowest specific activity of the immobilized enzyme was obtained by this support. Considering the rather high specific surface area of TB2, compared to other supports the reason for the low activity can be due to the steric hindrance caused by the enzyme molecules forming protein multilayer on the supports' surface. The figure shown below (*fig. 4.5.3*) is the influence of initial enzyme concentration on the loading of enzyme on different supports. *Fig. 4.5.4* shows the effect of amylase concentration (1-5 mg) on the activity of enzyme immobilized per g of supports. The activity was attained maximum when the

concentrations of amylase were 4.1 mg g^{-1} of activated TB and 2.1 mg g^{-1} for non activated TB. When the concentration of enzyme was increased further, the relative activity decreased. This may be caused by the jostling of too many enzymes on the supports as described earlier.

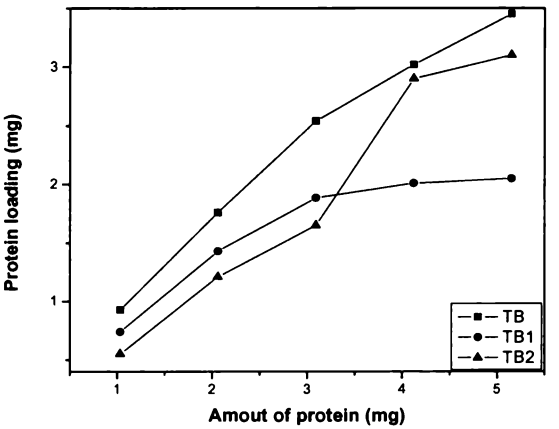

Fig. 4.5.3 Variation of protein loading with respect to the initial protein amount.

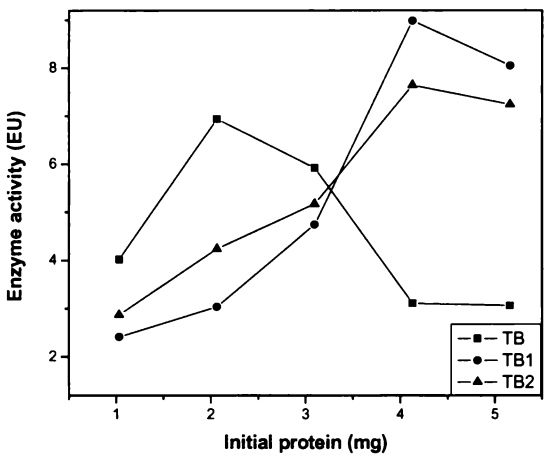

Fig. 4.5.4 Variation of activity of immobilized enzyme relative to the initial amount of protein.

Table 4.7 Immobilization efficiency for α-amylase on TB.

Polymer	Initial protein (mg)	Bound protein (mg)	Immobilization Yield (%) IY	Initial activity (EU)	Immobilized enzyme activity (EU)	Activity Yield (%) AY	Immobilization Efficiency (%) IE = AY/IY
TB	2.1	1.8	85.7	16.4	6.9	42.1	49.1
TB1	4.1	2.0	48.8	32.8	9.0	27.4	56.1
TB2	4.1	2.6	63.4	32.8	7.6	23.2	36.6

4.5.2 Effect of pH on activity

The initial activities of free and immobilized α-amylase were determined for different pH values in the range of 4-7. Individual relative activities for both states are presented in *fig. 4.5.5*. Maximum activities were obtained at pH 5.5 for all immobilized enzymes. As already evident, α-amylase is very stable at pH 5.5. The close similarity of pH profiles for both states may show that no important conformational change occurs in the immobilization of the amylase. The only difference was that for the glutaraldehyde coupled amylase pH profile more broadened in the alkaline range whereas for the enzyme in solution, the alkaline pH not favored the activity of enzyme.

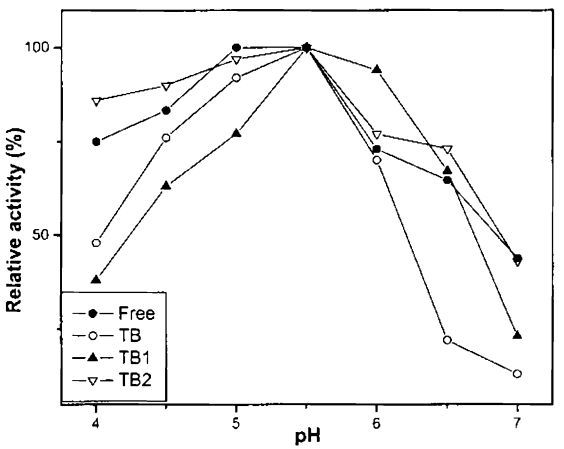

Fig. 4.5.5 Variation of activity with pH for free and immobilized α-amylase.

4.5.3 Effect of temperature on the activity

Immobilization produced remarkable change on the temperature dependence on the activity of the enzyme. It is evident from the *fig. 4.5.6* that the optimum temperature for starch hydrolysis decreased by 10 °C compared to free enzyme. After 40 °C, a sharp decrease in activity of immobilized enzymes was observed. The decline in activity of α-

amylase at higher temperature as a result of desorption of enzyme from support was reported [12] by *Dey et al.*

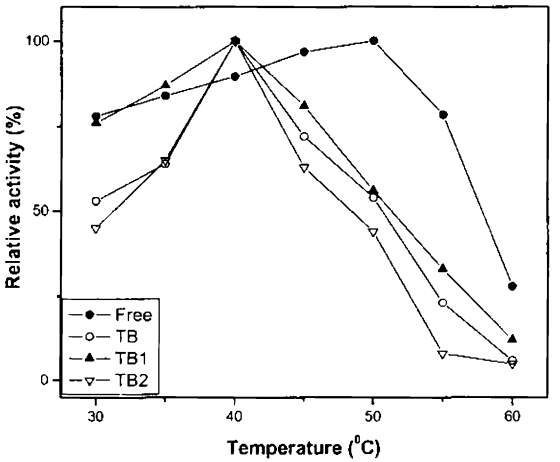

Fig. 4.5.6 *Influence of temperature on the activity of free and bound α-amylase.*

4.5.4 Thermal stability

Utilization of enzymes in processes often encounters the problem of thermal inactivation of enzyme. At high temperature, enzyme undergoes partial unfolding by heat-induced destruction of non-covalent interaction [28, 29]. The thermal stability of free and immobilized enzyme was investigated with in the temperature range of 30-60 °C (*fig.4.5.7*) and thermal inactivation checked at definite time intervals (*fig. 4.5.8*) within 2 hour, by measuring residual activity. It is clear from the graph that the enzyme on TB is less stabile than other forms. In TB1 and TB2 immobilization improved the thermal stability of the protein structure through a protection against heat. In all forms immobilized α–amylase showed better thermal stability than free form even after prolonged incubation in the absence of substrate. The reason may be that the polymer matrix is supposed to preserve the enzyme structure. It has been reported that polymeric carriers such as poly(2-hydroxyethyl methacrylate) [5] poly(methyl methacrylate-acrylic acid) [41] and poly(allyl glycidyl ether

co-ethylene glycol dimethacrylate) [50] protect enzyme from thermal inactivation and yield higher thermal stabilities.

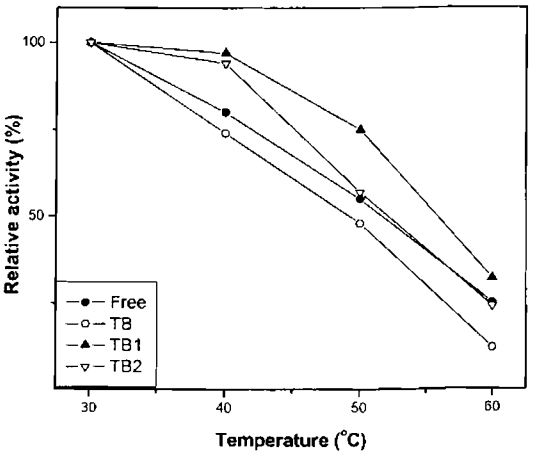

Fig. 4.5.7 Thermal stability of free and immobilized α-amylase.

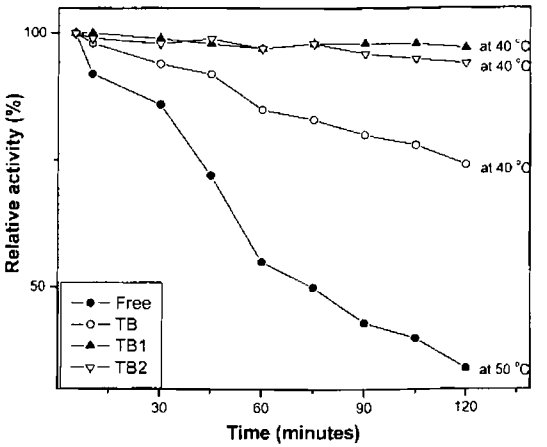

Fig. 4.5.8 Variation of enzyme activity relative to the preincubation time.

4.5.5 Kinetic parameters

The effect of immobilization on the kinetic parameters K_m and V_{max} was also studied. Results obtained using starch as substrate is shown in *table 4.8*. The relationship between the velocity of product formation and substrate concentration described a typical quadratic hyperbole that was converted in the double reciprocal plot of Lineweaver-Burk and Hanes-Woolf plots (*fig. 4.5.9*).

Table 4.8 *Kinetic parameters for the free and immobilized α-amylase.*

	Free enzyme	TB	TB1	TB2
K_m (mg mL^{-1})	0.46 ± 0.01	7.74 ± 0.13	5.09 ± 0.06	4.11 ± 0.04
V_{max} (EU μg^{-1} protein)	7.95 ± 0.05	4.33 ± 0.07	4.82 ± 0.08	3.28 ± 0.09

There was 9-17 fold increase in K_m values presented for the immobilized enzymes. Similar to this observations *Aksoy et al.* observed [41] a 12-fold increase in K_m on immobilization to poly(methyl methacrylate acrylic acid) microspheres and noted that immobilized form possessed between 68-80 % of the activity of the soluble enzyme depending on the coupling method used. The restricted mobility of the enzyme caused by the immobilization process, as well as the presence of factors such as the limited rate of mass transfer of the substrate from the bulk of the solution to the surface of the support material, were expected to affect the reaction rate as well the affinity of the enzyme [64]. Hence the V_{max} is found to be always low.

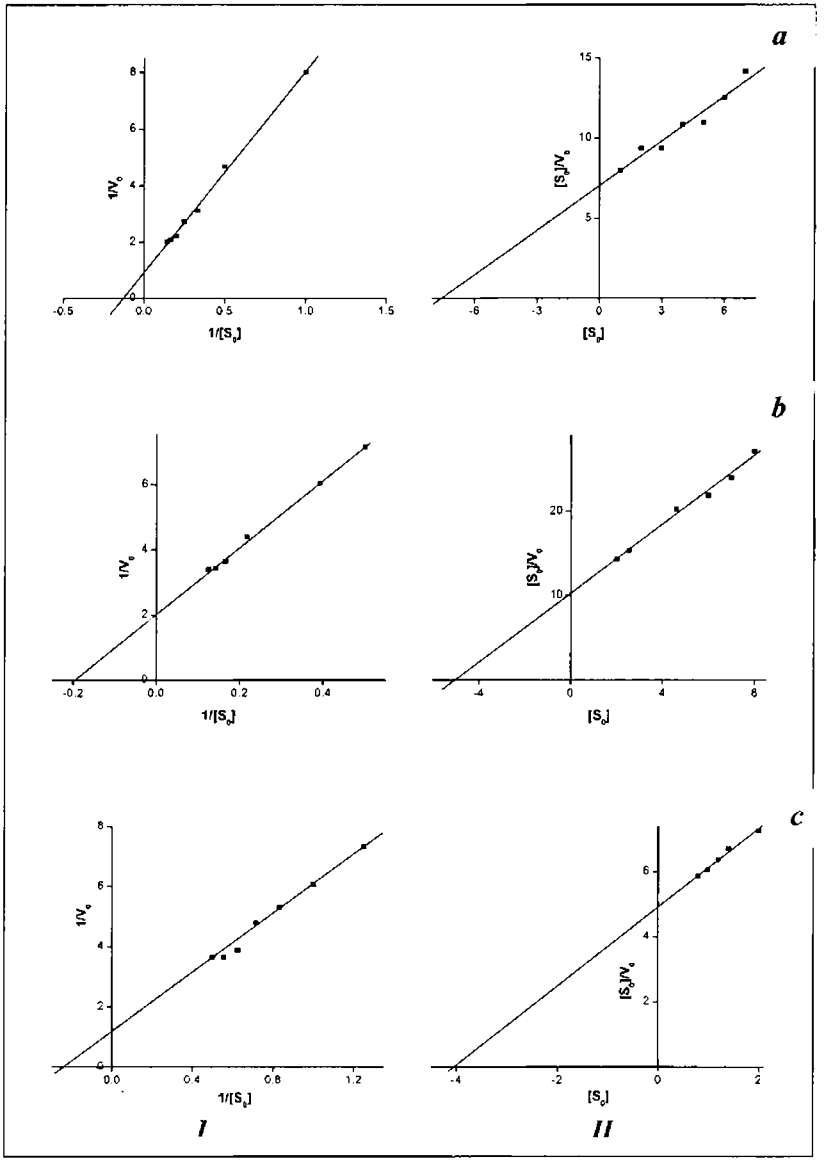

Fig. 4.5.9 Lineweaver-Burk plots (I) and Hanes-Woolf plots (II) for α-amylase immobilized on TB (a) on TB1 (b) and on TB2 (c).

4.5.6 Storage stability

Long term storage for 6 months of immobilized amylase on poly(*o*-toluidine) is shown in *fig. 4.5.10*. As expected, immobilization improved the storage stability of the enzyme. Highest stability was showed by covalently bonded enzyme using glutaraldehyde spacer. Adsorbed enzyme showed least stability but better than free enzyme when free enzyme lost all its activity within 2 days. Immobilized enzyme retained more than 50 % of its initial activity during the storage period of 4 months. This decrease in activity was explained as the time-dependent natural loss in enzyme activity.

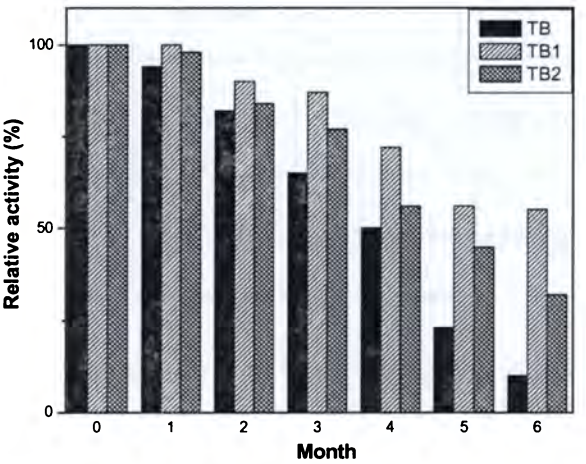

Fig. 4.5.10 *Influence of storage time on percentage residual activity of α-amylase immobilized on TB.*

4.5.7 Reusability

The reusability of the immobilized enzyme is another very important factor from the point of view of reducing the cost of the enzyme, which is an important aspect when taking into account its suitability for commercial applications. It was observed that the covalently immobilized enzymes retained 70 % of its initial activity even after the reaction was repeated nine times (*fig. 4.5.11*).

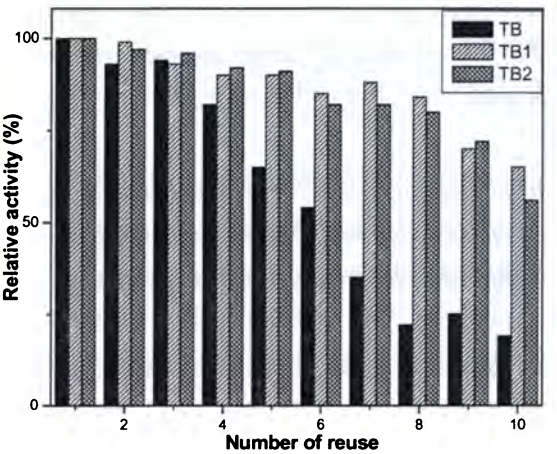

Fig. 4.5.11 Reusability of α-amylase immobilized on TB.

4.6 Conclusion

We have studied various carriers polyaniline and poly(*o*-toluidine) in acidic and basic form that are applicable for immobilization of α-amylase and determined several parameters essential for the practical application of the immobilized enzyme. Initial work using amylase evaluated the binding parameters for the various carriers tested. Taking into account the main parameters, such as immobilization efficiency, pH and thermal stability, reusability, and storage stability we found that covalent binding with glutaraldehyde spacer are the most efficient methods of immobilization for all of the supports selected. The tendency to form dimeric form structure by this activator results in creation of long spacer between matrix and protein surfaces, which keep the enzyme not so close to the polymers surface and the access of large starch molecules to α-amylase not so restricted [65]. The main observations of the study on immobilized α-amylase are:

- ***Free diastase α-amylase hydrolyzed starch when the optimum conditions of pH 5-5.5 and temperature 50 °C. The Michaelis-Menten constant for starch hydrolysis was evaluated as 0.46 mg mL^{-1}.***

- *The enzyme α-amylase immobilized on various supports in the pH range of 4.5-5.5 within the duration of 30-60 minutes irrespective of the supports and methods used.*

- *After immobilization the pH for optimum enzyme activity was changed to either 5 or 5.5, depends upon supports and method used. The glutaraldehyde activated supports showed better resistance to pH changes.*

- *The optimum temperature for starch hydrolysis was lowered by 10-15 °C after immobilization. The immobilized enzymes showed better thermostability and retained 80-90 % activity after 2 hour incubation at their optimum temperature.*

- *The kinetic parameter K_m showed a 2-17 fold increase after immobilization and V_{max} was found to be low for immobilized enzyme.*

- *The immobilized enzyme could be reused efficiently in batch reactor. Covalently immobilized retained more than 50% activity even after 10 cycles of use.*

- *The immobilization led to an improvement in storage stability. Enzyme could be stored for 6 months with very little change in activity.*

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CHAPTER 5
IMMOBILIZATION OF GLUCOAMYLASE

5.1 Introduction

Glucoamylase (GMA) (1,4-*a*-D-glucan-glucohydrolase; (EC 3.2.1.3) (alternative names: Glucan 1,4-*a*-glucosidase; amyloglucosidase; Exo-1,4-*a*-glucosidase; *γ*-Amylase; lysosomal *a*-glucosidase; 1,4-*a*-D-glucan glucohydrolase), an exosplitting enzyme splits off *β*-D-glucose units from the nonreducing end of the chains of amylose, amylopectin, and other *a*-glucocans by hydrolyzing the *a*-1,4- and *a*-1,6-glucosidic linkages of raw or soluble starches and related oligosaccharides [1]. GMA has been used in industrial processes such as the production of glucose syrup and other food-processing applications [2-6]. The immobilization of GMA is an important development in modern technology for the production of sweeteners from renewable vegetable feedstock, because it can generate a heterogeneous biocatalyst which can design for one of the key stages of starch conversion [7-9].

Although many fungal species are capable of producing GMA under different growth conditions [7], the industrial development of GMA has focused only on GMA from *Aspergillus niger* and *Rhizopus oryzae* because of their stability and high activity [3, 10, 11]. In industrial conventional enzymatic reactions, a mixture of the substrate and soluble enzyme is incubated. During sacharification, glucoamylase gets inactivated partially. Secondly, saccharification is time consuming process which necessitates operation at higher temperature (60 °C). As mentioned in the previous chapters the process would be more economical if the enzyme could be reused, especially, by immobilization on a solid support. The combination of unique biocatalytic properties of enzymes on one side, and heterogeneity of the support on the other, makes it possible to achieve the main aim of immobilization, which is reusing the enzymes. It also possible to reduce the conversion time and maintain lower inactivation rates at higher temperatures employed. To achieve this GMA is immobilized on various insoluble carriers with the retention of its catalytic properties which can be used repeatedly and continuously [12-27]. Although much research has been carried out to develop immobilized GMA systems, there has been little success in satisfying industrial requirements. Among all of the methods used for immobilizing glucoamylase, covalent bonding is most widely investigated because of its obvious

advantages (e.g., stable, wide range of supports and binding methods, *etc.*) compared with the other methods [24, 28]. To effectively immobilize glucoamylase onto supports, many quite different support materials, such as artificial polymer materials [12-17], magnetic microparticles [18–22], alginate fiber [23] macroporous hydrophilic beads [24], gelatin [25] activated charcoal [26], carbon support sibunit [27] were employed. Compared with other supports, recently conducting polymer materials like polyaniline and other derived compounds showed relatively good properties owing to the possibility of designing the supports structure through polymerization or chemical and physical modification and has been used for immobilization of many enzymes [29-37]. After the enzyme was immobilized onto these supports, it's operational, thermal, and storage stabilities were found to be increased.

In this chapter, we are discussing the immobilization studies of glucoamylase on polymers; polyaniline and poly(*o*-toludine). Immobilization may leads to change in pH and temperature stabilities as well as activity of the enzyme. It also sometime shows better storage and operational stability along with excellent reusability. So the investigation of optimum pH, optimum temperature and kinetic constants are very important.

5.2 Immobilization of GMA on Emeraldine Salts (ES)

The GMA immobilization on non-activated emeraldine salt (ES), glutaraldehyde activated emeraldine salt (ES1) and ascorbic acid activated emeraldine salt (ES2) is first discussing here in this section.

5.2.1 Optimum parameters for immobilization of GMA

To find the optimum binding condition necessary for the enzyme, we measured the immobilized glucoamylase activity under the conditions of different pH value. Immobilization of an enzyme is affected by pH. The changes in pH bring about apparent changes in the enzyme structure, which influence its activity. The effect of pH of the medium on the relative activity of the immobilized enzyme is shown in *fig. 5.2.1*. From the figure it is evident that the pH on immobilization is different for both types of supports

used. pH 4-6 is suitable for activated polyaniline, with maximum at 5 and pH 6 for non-activated polyaniline. Below pH 4 and above 6, the relative activity of the immobilized enzyme decreased sharply. The decline in immobilization efficiency beyond pH 6 and below 4 may owe to desorption of the enzyme from the carrier or the instability of the pure enzyme in that pH.

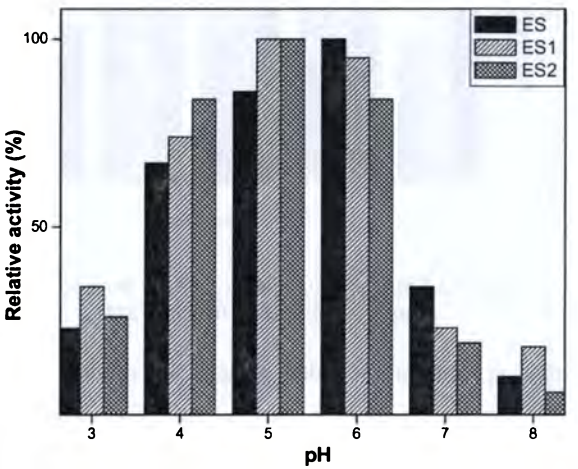

Fig. 5.2.1 *Effect of pH of the immobilization medium on the activity of immobilized GMA.*

Fig. 5.2.2 shows the effect of contact time on immobilization of GMA. It was obtained by measuring activity of the immobilized enzyme, (after incubating enzyme with supports at different time intervals). The maximum activity was taken as 100 %, and was shown by GMA on ES1 after 30 minutes (contact time) of immobilization, whereas the enzyme coupled on ES and ES2 required 45 and 60 minutes respectively in order to obtain the maximum enzyme loading and hence activity.

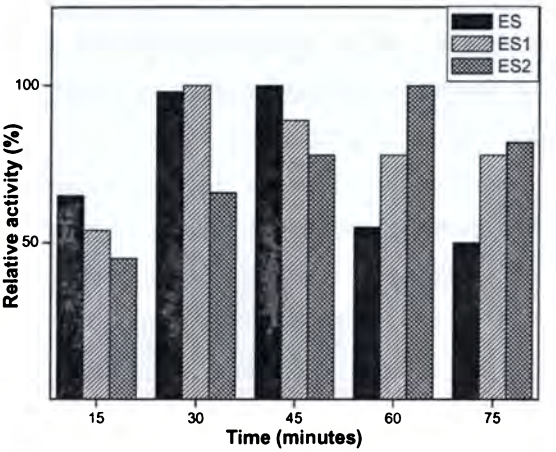

Fig. 5.2.2 Influence of contact time for immobilization on the retained activity of GMA.

With increasing amount of added protein per gram of polymer, the amount of proteins loaded and the specific activity initially increased, and reached a plateau as shown in the *fig. 5.2.3* and *fig. 5.2.4*.

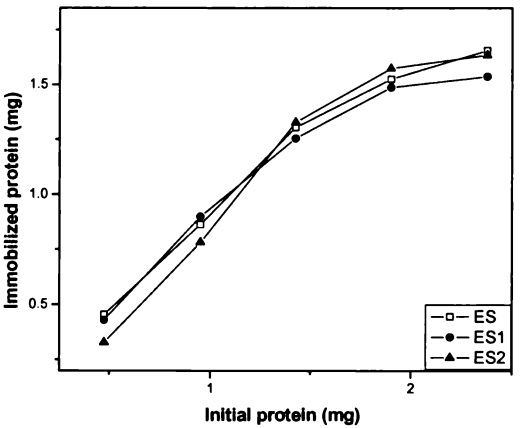

Fig. 5.2.3 Effect of initial protein concentration on the uptake of protein.

The plateau in the *fig. 5.2.4* was the result of diffusional limitation caused by the overloading of polymer which reduces the access of the substrate to the active sites of the bound enzyme, as described in the previous chapter. Immobilization efficiency (*table 5.1*) indicated that the lowest value (70.2 %) was found for GMA physically adsorbed on ES. The low efficiency indicates that much of the protein was apparently immobilized in a non-active form. Enzyme immobilized by covalent binding through the glutaraldehyde spacer group showed the highest efficiency in ES1 (94.1 %) with highest activity yield of 54.3 EU g^{-1} carrier.

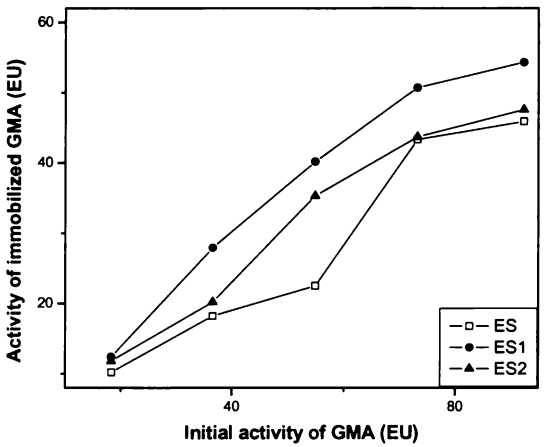

Fig. 5.2.4 *Activity of immobilized enzyme as a function of initial enzyme activity.*

1 EU (Enzyme Unit) is defined as the amount of enzyme that liberates 1 μmol of glucose per mL per minute. ES2 showed considerable bound (47.6 EU g^{-1}) and immobilization yield (66.7 %). This good loading efficiency for the immobilization by covalent binding might have been due to the formation of stable cross linking between the carrier and the enzyme through a spacer group.

Table 5.1 Immobilization efficiency for the Glucoamylase on emeraldine salt.

Polymer	Initial protein (mg)	Immobilized protein (mg g^{-1} polymer)	Immobilization Yield (%) IY	Initial activity (EU)	Immobilized enzyme activity (EU g^{-1} polymer)	Activity Yield (%) AY	Immobilization efficiency (%) IE
ES	2.4	1.7	70.8	92.4	45.9	49.7	70.2
ES1	2.4	1.5	62.5	92.4	54.3	58.8	94.1
ES2	2.4	1.6	66.7	92.4	47.6	51.5	77.2

5.2.2 Influence of pH on the activity of free and immobilized enzyme

The effect of pH on the enzyme activity of free and bound forms were examined within the pH range of 4-7. The enzyme activity is presented here as the relative activity with respect to the maximum value at pH optimum. As can be seen from *fig. 5.2.5*, the pH optimum of the ES-GMA, ES2-GMA was shifted to the acidic region relative to free glucoamylase.

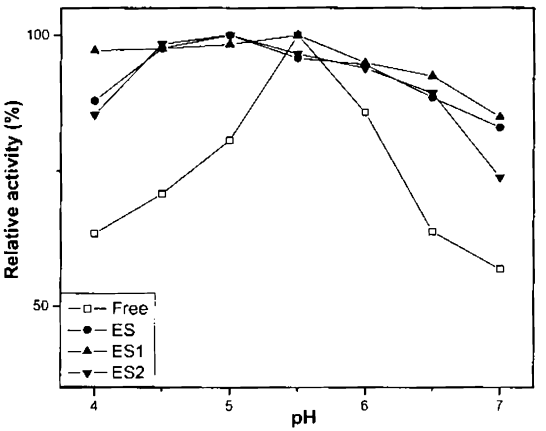

Fig. 5.2.5 pH profiles of free and immobilized GMA.

The activity of free enzyme was more dependent on pH than the activity of immobilized enzyme. The maximum activity was observed at 5.5 for free enzyme and with a change of 1 pH unit, the free enzyme lost 40-50 % of its original activity at the optimal pH. A change in pH could cause an expansion or shrinkage of the protein structure leading to conformational changes that will affect the activity of the enzyme. On the other hand, immobilized enzyme showed good activity within the range of pH (4-7) and was less susceptible to pH change. The excellent adaptability in a wide range of pH for immobilized enzyme was well reported in the literature [24, 26]. Particularly for PANI, these effects have been noticed during immobilization of peroxidase and trypsin [29, 38]. The immobilization of enzymes to charged supports often leads to displacements in the pH

activity profile, ascribable to unequal partitioning of H^+ and OH^- between the microenvironment of the immobilized enzyme and the bulk phase due the electrostatic interactions with the matrix [24].

5.2.3 Influence of temperature on activity

Temperature plays an important role in determining activity of enzymes. The results presented in *fig. 5.2.6*, shows that all of the immobilized enzymes were optimally active at a higher temperature (60-65 °C) than the free enzyme (55 °C).GMA on ES and ES2 showed maximum activity at 60 °C and on ES1 at 65 °C.

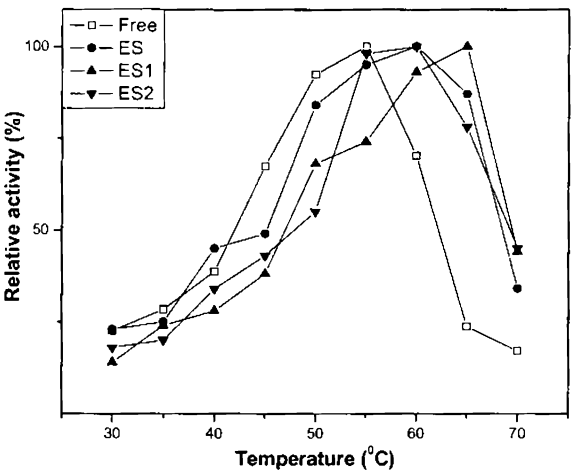

Fig. 5.2.6 *Temperature profiles for free and immobilized glucoamylase.*

Similar to these results, *Bai et al.* reported [24] that the optimum temperature for *Aspergillus niger* glucoamylase was increased from 45 °C to 55 °C after immobilization on hydrophilic carrier. *Huo et al.* reported [17] a 5 °C rise in optimum temperature when GMA was covalently immobilized onto polymer supports of vinylene carbonate and 2-hydroxyethyl methacrylate [17]. These facts suggested that the conformational rigidity of the GMA was increased after the attachment to the ES matrices then requiring more

temperature to express its maximum catalytic activity. Since immobilization and cross-linking provided more rigid external backbone for enzyme molecules, the effect of higher temperature in breaking the interactions that are responsible for the proper globular, catalytic active structures became less prominent, thus increasing the thermal stability also.

5.2.4 Thermal stability

The thermostability of free and immobilized GMA was studied with respect to temperature and time in the temperature range 30 to 70 °C. The data are presented *figs. 5.2.7* and *5.2.8.*

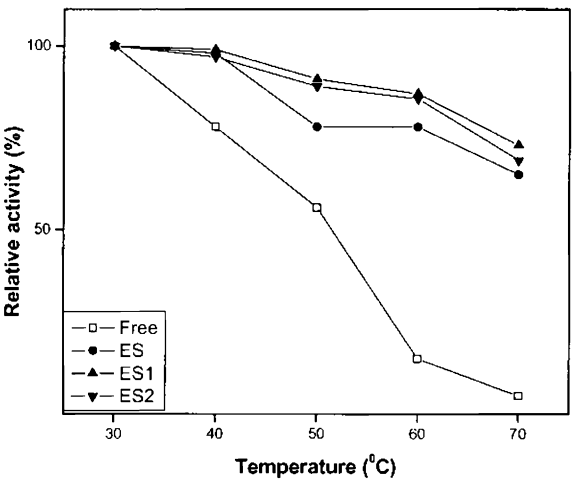

***Fig. 5.2.7** Influence of temperature on the stability of free and immobilized GMA.*

The immobilized glucoamylase retained 80-90 % of its initial activity at 50 °C, while free enzyme lost 50 % its initial activity about during a 60 minutes incubation period. At 60 °C the immobilized and free enzymes retained their activities around at levels of 80 and 30 %, respectively. It also indicated that, the immobilized enzymes retained more than 75 % of their initial activity even after 60 minutes of incubation at optimum temperature which indicates that the immobilized form was inactivated at a much slower rate than the

free form. These results suggest that the thermostability of immobilized GMA increased considerably as a result of immobilization onto ES polymers. The activity of the immobilized enzyme, especially in a covalently bound system, is more resistant against heat and denaturing agents than that of the soluble form. The improvement in denaturation resistance of the covalently immobilized *Aspergillus niger* glucoamylase due to multipoint attachments on PANI was already reported [14].

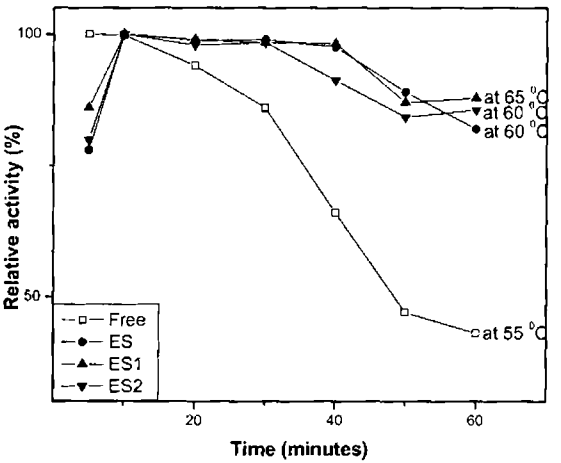

Fig. 5.2.8 Influence of preincubation time on the activity of free and immobilized GMA at their optimum temperature.

5.2.5 Kinetic parameters

Kinetic parameters, K_m and V_{max} for the free and immobilized GMA were, determined by varying concentration soluble starch in the reaction medium. Lineweaver-Burk plots and Hanes-Woolf plots for free and immobilized enzyme are shown in *fig. 5.2.9*. The kinetic parameters for free and immobilized enzymes are summarized in *table 5.2*. The apparent K_m values of the immobilized enzymes were higher and V_{max} was lower than those of free enzyme. The increase in K_m values for the immobilized GMA has been reported by various authors [7, 19, 22].

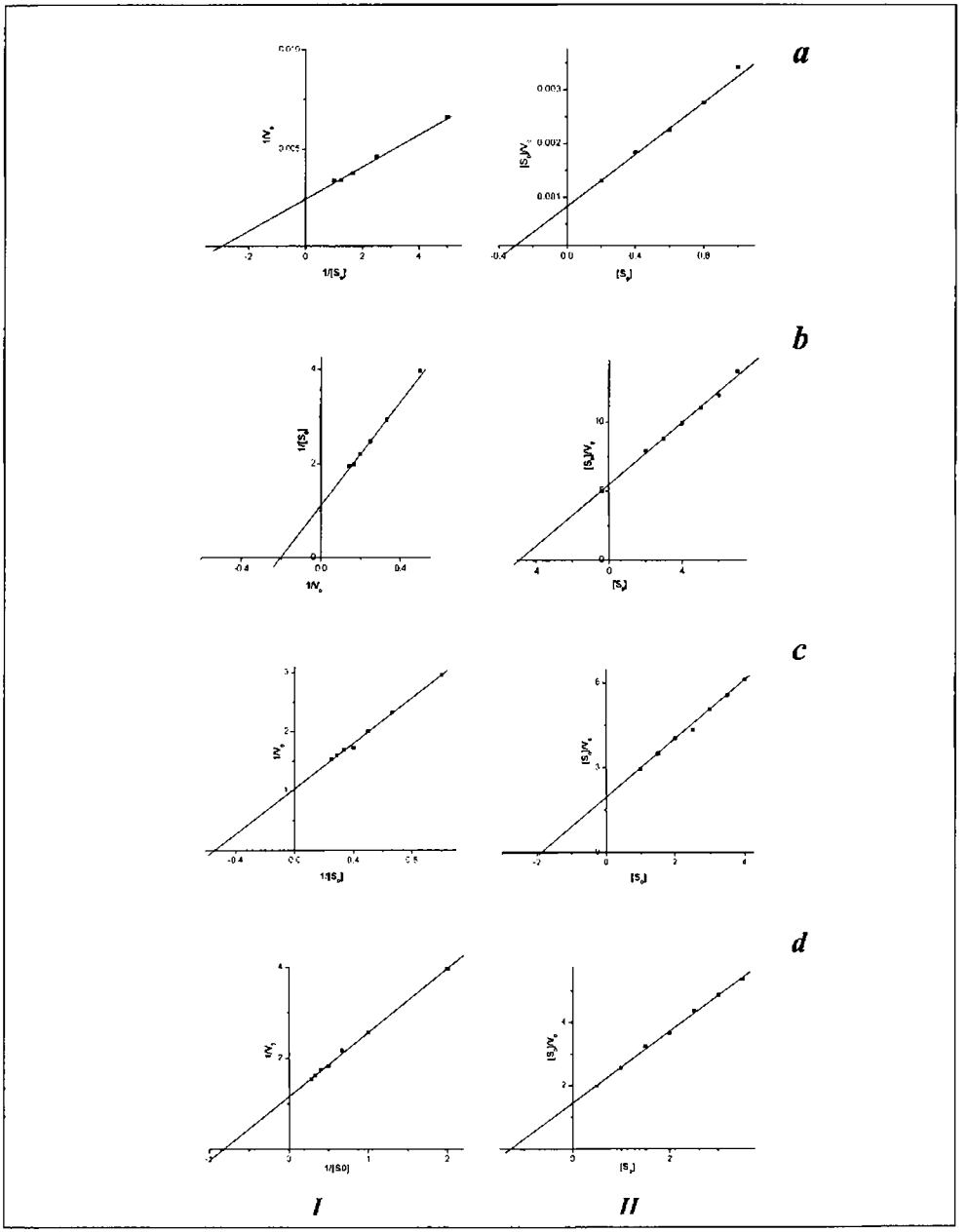

Fig. 5.2.9 (I) Lineweaver-Burk plots (II) Hanes-Woolf plots of (a) free GMA and immobilized on (b) ES (c) ES1 (d) on ES2.

These changes in kinetic parameters may be a consequence of either structural change in the enzyme occurring upon immobilization or lower accessibility of substrate to the active sites of the immobilized enzyme, as well described in the literature [21].

Table 5.2 *Kinetic constants of free and immobilized GMA.*

	Free GMA	ES-GMA	ES1-GMA	ES2-GMA
K_m (mg mL^{-1})	0.32 ± 0.04	4.84 ± 0.12	1.85 ± 0.03	1.24 ± 0.05
V_{max} (EU μg^{-1} protein)	1.65 ± 0.03	1.15 ± 0.06	1.55 ± 0.08	1.22 ± 0.07

5.2.6 Storage stability

For successful commercialization of the heterogeneous process of starch saccharification, a biocatalyst should have an appreciable life time, which corresponds to a long term operation of the reactor without reloading. If an enzyme is in buffer solution, it is not stable during storage, and the activity is gradually reduced.

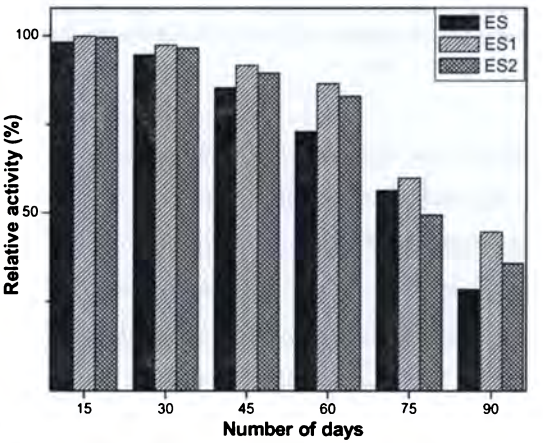

Fig. 5.2.10 *Storage stability of immobilized GMA.*

Fig. 5.2.10 shows the stability of immobilized glucoamylase stored in semi-dry condition 4 °C. As can be seen from the figure, the immobilization of GMA increased its storage stability, while the free GMA in buffer solution lost all its activity within 10 hours. It was observed here that the immobilized GMA retained almost 90 % of its initial activity during the storage period of 45 days. This gradual decrease in activity is explained as the time dependent natural loss in enzyme activity. *Huo et al.* reported [17] an 84 % of activity recovery after 26 days of storage when glucoamylase immobilized on porous polymer.

5.2.7 Reusability

Reusability of immobilized GMA was tested in subsequent cycles of starch hydrolysis. It can be observed that ES-GMA could retain only less than 75 % of its initial hydrolytic activity after the eighth cycle of reaction whereas ES1-GMA retained almost 90 % after the eighth cycle (*fig. 5.2.11*). Other authors [24] observed that GMA immobilized on hydrophilic beads retained more than 88 % of its initial activity after the 20th cycle of starch hydrolysis. The worse operational stability of ES-GMA, when compared to GMA immobilized on activated ES1 and to ES2, may be due to enzyme desorption during reaction, and by the low amount of enzyme adsorbed.

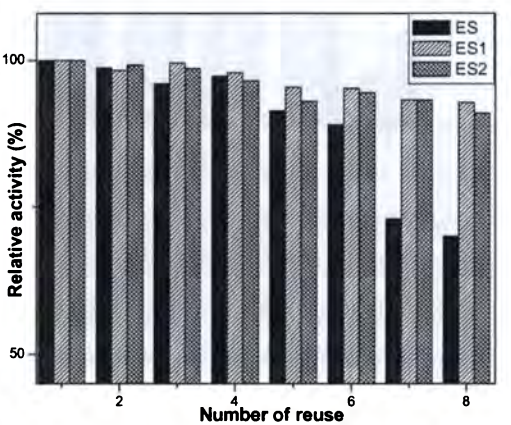

Fig. 5.2.11 *Reusability of immobilized GMA in subsequent batches of starch hydrolysis.*

5.3 Immobilization of GMA on Emeraldine Base (EB)

5.3.1 Optimum preparation conditions of immobilized GMA

As it was explained earlier, the properties of immobilized enzyme preparations depend to a great extend on the pH of the medium from which the enzyme bonding is affected. It is evident from *fig. 5.3.1* that the immobilized enzyme presented relatively high activity when the pH values of the immobilization medium are in the range 5-6. EB and EB2 (non activated and ascorbic acid activated emeraldine base) worked well with both of pH 5 and 6 and EB1 (glutaraldehyde activated emeraldine base) preferred pH 6 only. A pronounced decline in the enzymatic activity of glucoamylase immobilized outside the stable pH range between 4 and 6 in the *fig. 5.3.1* can be explained with these observations.

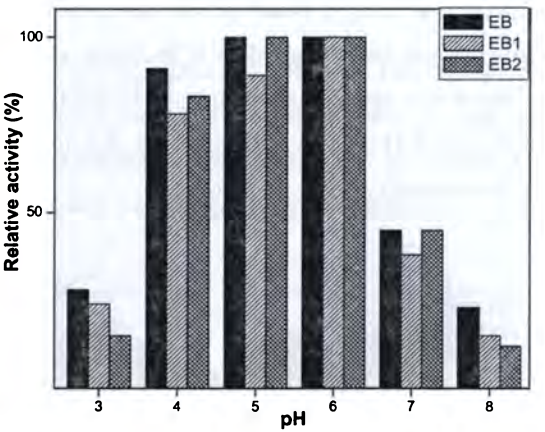

Fig. 5.3.1 *Effect of pH of the immobilization medium on the activity of immobilized GMA.*

Fig. 5.3.2 illustrates that the activity of the immobilized glucoamylase increased with incubation time and that the highest activity was obtained under immobilization was allowed to proceed for 30, 15 and 45 minutes for EB, EB1 and EB2 respectively. However, the activity decreased if incubation time is prolonged further because of the formation of disordered multilayer

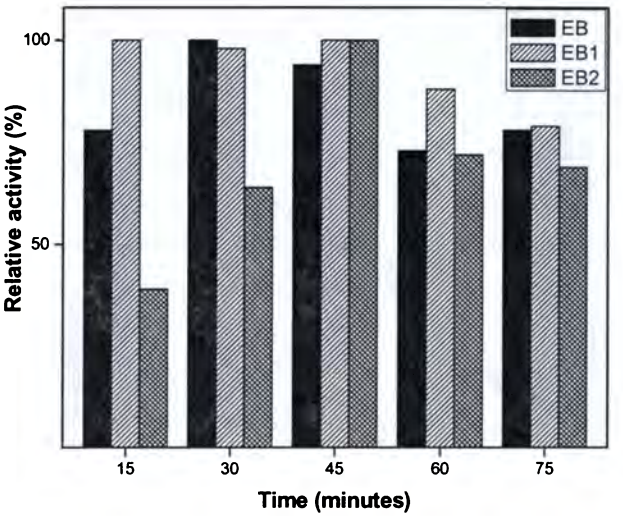

Fig. 5.3.2 *Effect of incubation time on relative activity of immobilized GMA.*

In order to evaluate the binding capacity, EB polymer (the supports) particles were incubated with different concentrations of GMA and analyzed for GMA activity and protein loading. The results are shown in the *fig. 5.2.3*, *fig. 5.2.4* and *table 5.3*. The activity and amount of protein, immobilized have increased with increase in enzyme concentration from 0.4 to 2.4 mg g^{-1} of support. However, the activity of immobilized GMA was found to decrease beyond 54.1, 60.4, and 52.3 EU g^{-1} for EB, EB1, and EB2 respectively. The decrease in enzyme loading beyond this probably might have resulted in embedding of active sites of enzyme molecules during the immobilization process by increased stacking and also by increased diffusion limitations [39]. In general, the specific activity of free enzyme is higher than the immobilized enzyme and this may be due to the rigidification of the enzyme protein conformation and decrease in the flexibility of the enzyme molecule [40].

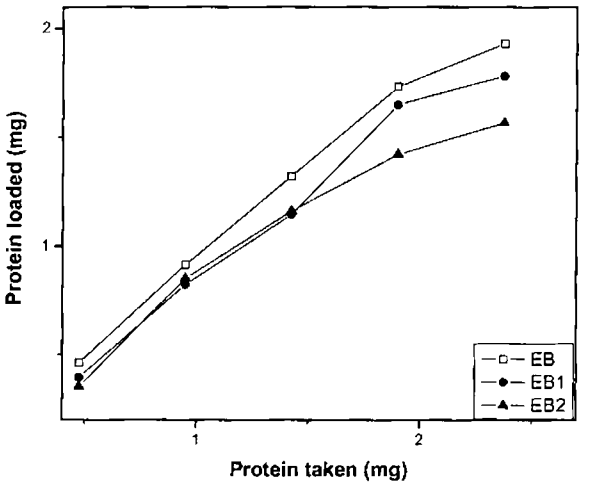

Fig. 5.3.3 Influence of initial protein amount on the uptake of protein.

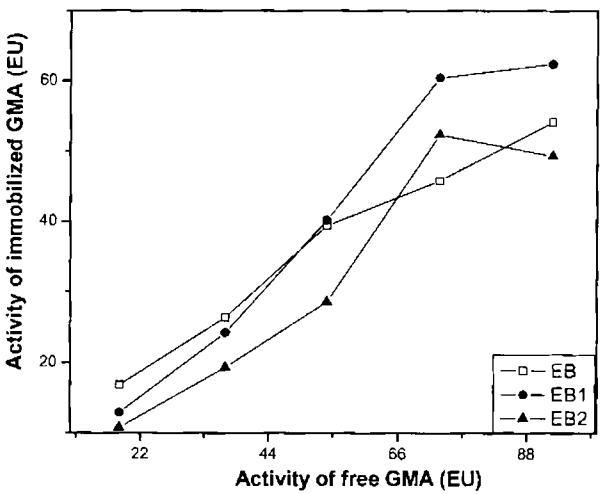

Fig. 5.3.4 Activity of immobilized enzyme as a function activity of free enzyme.

Table 5.3 *Immobilization efficiency for GMA on emeraldine base.*

Polymer	Initial amount of protein (mg)	Immobilized protein (mg g^{-1} polymer)	Immobilization Yield (%) IY	Initial activity of enzyme (EU)	Activity of Immobilized enzyme (EU g^{-1} of polymer)	Activity Yield (%) AY	Immobilization Efficiency (%) IE
EB	2.4	1.9	79.2	92.4	54.1	58.5	74
EB1	1.9	1.7	89.5	73.2	60.4	82.5	92.2
EB2	1.9	1.4	73.7	73.2	52.3	71.4	97

5.3.2 Effect of pH on activity

Fig. 5.3.5 gives an account of the variation of glucoamylase activity with respect to pH for free and immobilized forms of the enzyme. The optimum pH for both free and immobilized enzyme is 5.5. The maintenance of optimum pH for the free and immobilized glucoamylase could be attributed to the fact that the concentration of charged species (e.g., hydrogen ions) in the domain of the immobilized enzyme was similar to that in the bulk solution.

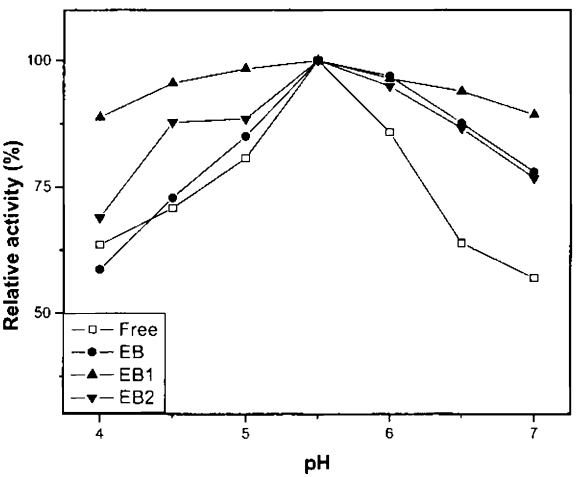

Fig. 5.3.5 *Influence of pH on the activity of free and immobilized GMA.*

From this figure we can also see that the EB1-GMA was stable in the entire pH range studied, whereas the residual activity of the free and other two of the immobilized forms, *i.e.*, EB-GMA and EB2-GMA diminished to about 70-75 % as the pH varied from 5.5 to 7. At pH<5.5 the residual activity varied slightly for EB and EB2 compared to the free GMA (60-70 %), but EB1 shows higher activity of 85 %.

5.3.3 Effect of temperature on activity

The determination of optimum temperature is very important since immobilization can bring about a change in the optimum value. *Fig. 5.3.6* shows the effect of temperature on the activity of the free, adsorbed and covalently linked GMA. The activities obtained in the temperature range of 30-70 °C were expressed as a percentage of the maximum activity. In the resultant bell-shaped curve, the maximum activity for the free enzyme was obtained at 55 °C. But for the immobilized GMA the maximum activity was at 60 °C and the temperature profiles were slightly broader than that of the free enzyme.

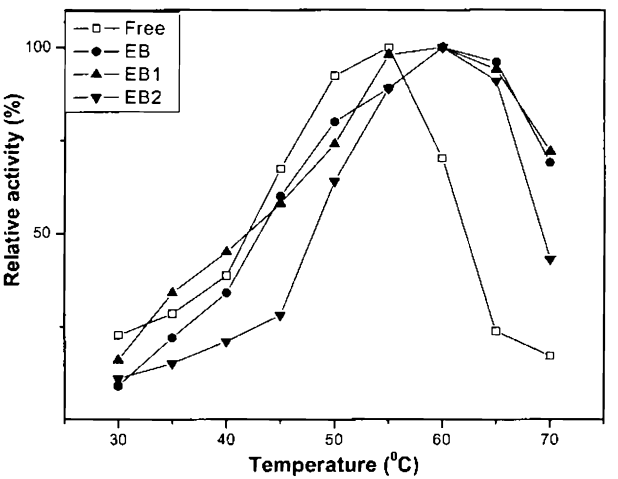

Fig. 5.3.6 Influence of temperature on the activity of free and immobilized GMA.

The increases in optimum temperature and hence activation energy are caused by the changing physical and chemical properties of the enzyme. Similar increase in optimum temperature was reported by *Arica et al.*, when glucoamylase was immobilized onto magnetic poly(methyl methacrylate) microspheres [12] and they speculated that hydrophobic interaction and other secondary interactions of the immobilized enzyme might impair conformational flexibility necessitating higher temperature for the enzyme

molecules to reorganize and attain a proper conformation for its functioning and binding of the substrate.

5.3.4 Thermal stability of immobilized GMA

One of the main reasons for enzyme immobilization is the anticipated, increase in its stability to various deactivating forces, due to restricted conformational mobility of the molecules following immobilization [41, 42]. However, in principle, the thermal stability of an immobilized enzyme can be enhanced, diminished, or unchanged relative to free counterparts, and several examples of each kind have been previously reported [41, 43, 44]. Normally, rate of reaction increases with an increase in temperature; so it is always advantageous to perform reactions at higher temperature in order to increase productivity. Because the rapid inactivation of enzyme protein at high temperature, high temperatures are not favored in enzyme catalysis. If the thermal stability of an enzyme is enhanced by immobilization, the potential utilization of such enzymes would be extensive.

We studied the effect of temperature on the stability of free and both immobilized forms enzymes (*fig. 5.3.7 & fig. 5.3.8.*). Compared to the free enzyme the immobilized enzyme exhibited higher thermal stability. This thermal stability behavior might be due to the protection of the immobilized enzyme from conformational changes and lower flexibility of immobilized form, due to the multipoint attachments to the support. The free glucoamylase retained about 43 and 15 % of its initial activity at 55 and 60 °C after 60 minutes incubation. After 60 minutes treatment at 60 °C, immobilized glucoamylase via glutaraldehyde and ascorbic acid coupling retained about 88 and 73 % of their initial activities respectively, while adsorbed enzyme retained 69 % of its initial activity. At 70 °C, these were 65, 68 and 53 % for EB1, EB2 and EB, respectively. Similarly, from the *fig.* 5.3.8, it is clear that more than 90 % of immobilized enzymes are active up to 40 minutes of incubation at their optimum temperature. After that their activity reduced considerably. But free enzyme lost 20 % of its activity after 20-30 minutes of incubation at its optimum temperature. These results suggested that the thermostability of immobilized glucoamylase became significantly higher than that of the free enzyme at high temperature.

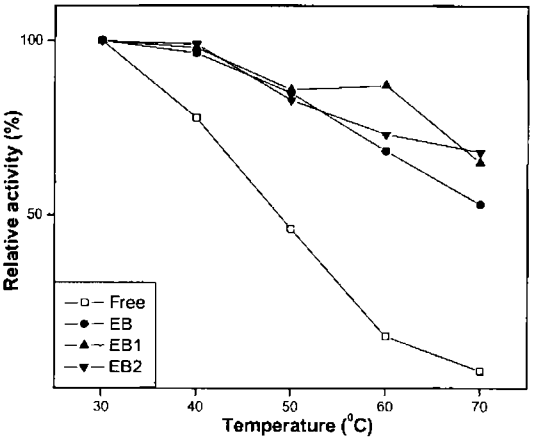

Fig. 5.3.7 Influence of temperatures on the stability of free and immobilized GMA.

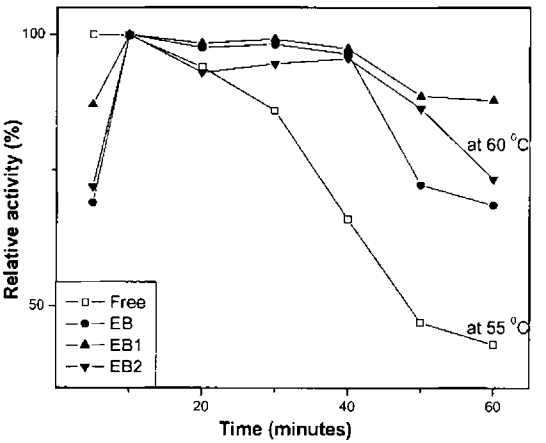

Fig. 5.3.8 Influence of incubation time at optimum temperature on the activity of free and immobilized GMA.

5.3.5 Kinetic parameters of immobilized enzymes

The maximum reaction rate, V_{max} and Michaelis-Menten constant, K_m were obtained from Lineweaver-Burk plots and Hanes-Woolf plots (*fig. 5.3.9*).

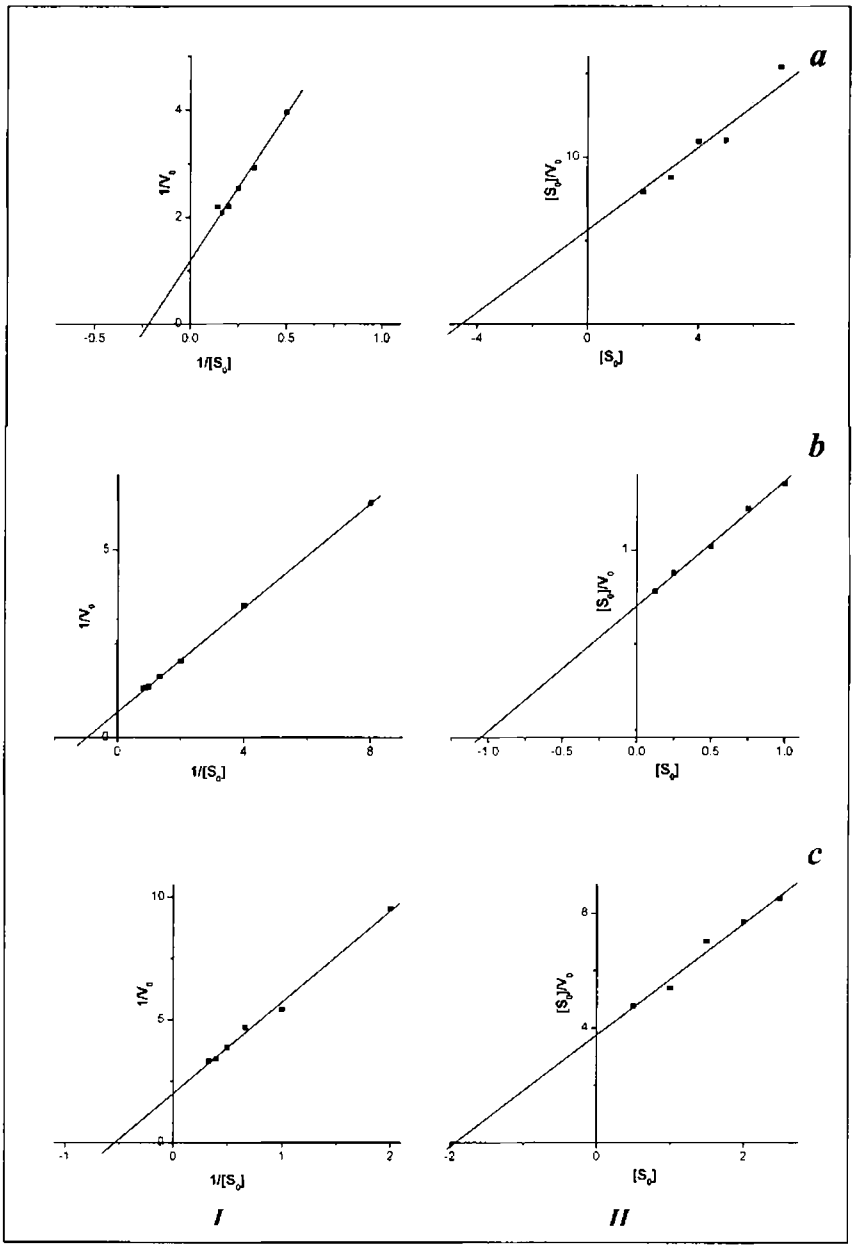

Fig. 5.3.9 (I) Lineweaver-Burk plots and (II) Hanes-Woolf plots of GMA immobilized on (a) EB (b) EB1 and (c) EB2.

The kinetic behavior for glucoamylase was significantly changed via immobilization on emeraldine base polymers. Kinetic constant, K_m values for the immobilized glucoamylase are found to be higher and V_{max} is lower than that of the free GMA (*table 5.4*). *Bahar* and *Celebi* reported [19] the immobilization GMA on magnetic polystyrene particles and found the K_m value of the bound enzyme was three times greater than that of the free enzyme and the V_{max} of the immobilized enzyme was about 70 % less than that of the free enzyme. The increase in K_m and decrease in V_{max} may have resulted from the change in the affinity of the enzyme to its substrate which was probably caused by the structural change of the enzyme upon attachment to the support consisting polymer chains [45] and restricted diffusion of substrate to the enzyme [46].

Table 5.4 *Kinetic parameters of free and immobilized GMA.*

	Free GMA	**EB-GMA**	**EB1-GMA**	**EB2-GMA**
K_m **(mg mL^{-1})**	0.32 ± 0.04	4.58 ± 0.15	1.03 ± 0.08	1.94 ± 0.11
V_{max} **(EU μg^{-1} protein)**	1.65 ± 0.03	1.21 ± 0.16	1.5 ± 0.06	1.59 ± 0.07

5.3.6 Storage stability

The storage stabilities of glucoamylase at 4 °C were investigated by measuring the enzyme activities at certain time intervals and the results are given in *fig. 5.3.10.* The free enzyme in buffer solution lost its activity completely within 10 hour. The retained activities were found to be 98, 90 and 91 % for EB1, EB2, and EB immobilized enzymes respectively, upon 30 days of storage at 4 °C. From these results, it was seen that immobilization definitely put the enzyme into a more stable position in comparison to free enzyme and, stability of enzymes are substantially increased by covalent coupling.

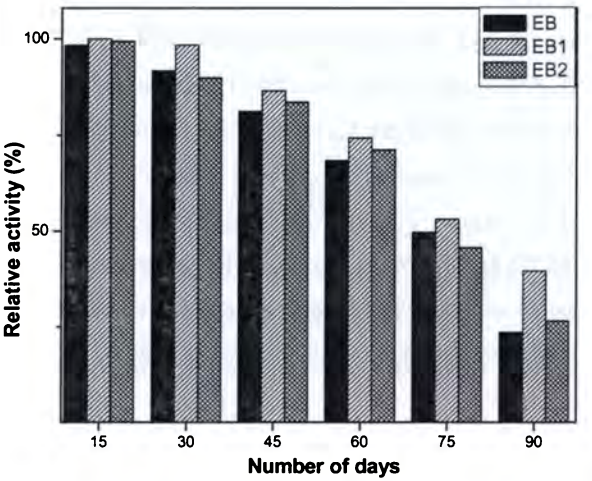

Fig. 5.3.10 Storage stability of immobilized GMA.

5.3.7 Reusability

Immobilization is carried out mainly to facilitate easy separation of the biocatalyst from the reaction mixture and to aid reuse of the enzyme. Operational stability of an immobilized enzyme preparation is one of the most important factors affecting its utilization in a bioconversion process whereby the reactor can be operated continuously. So evaluation of the reusability and operational stability is very much essential for immobilized enzyme preparations. *Fig. 5.3.11* shows the reusability and operational stability of immobilized enzyme. It is found that even after eight cycles of use, all forms of immobilized enzyme retained more than 85 % of its original activity. The GMA immobilized on activated emeraldine base retained more than 90 % of initial activity. The change of protein content before and after repeated use was small, indicating that the amount of enzyme leaching from the support was low since the enzyme molecule was tightly bound to the support by covalent attachment. The results proved that GMA covalently bound to polyaniline can be used as a potential immobilized bioreactor for continuous process.

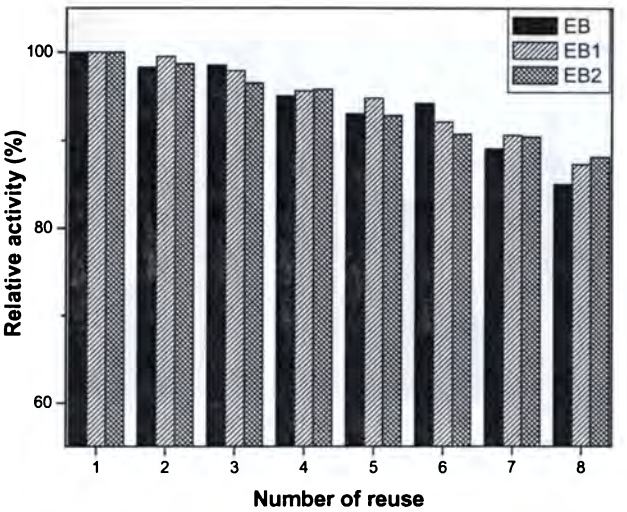

Fig. 5.3.11 Reusability of GMA immobilized on emeraldine base.

5.4 Immobilization of GMA on Poly(*o*-toluidine) Salts (TS)

5.4.1 Determination of immobilization parameters

The effect of pH and time on immobilization of GMA on TS is shown in *fig. 5.4.1* and *fig. 5.4.2*. The highest protein and maximum activity were shown at pH 5-6 with a time interval of 30-60 minutes. While at lower and higher pH, protein loading and enzyme activity was found to decrease sharply. The change in pH causes change in the conformation of the enzyme due disruption of intramolecular hydrogen bonding. At the same time bonding with the support causes intense chemical modification with multi linkages that also leads to changes in the conformation of the enzyme. Both these effects are minimized or cancelled each other at a particular pH, leading to an immobilized enzyme, which retains its original conformation almost completely. This is the optimum pH of immobilization at particular enzyme.

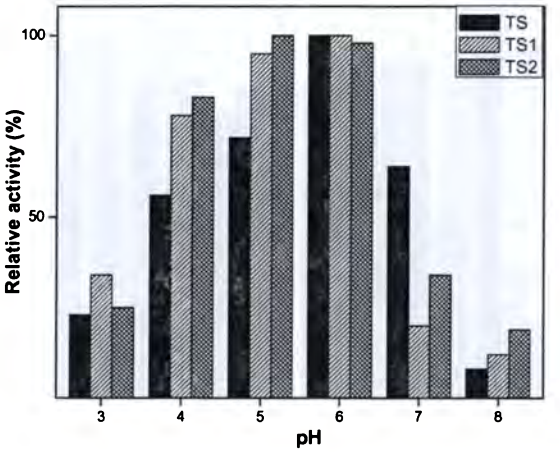

Fig. 5.4.1 *Effect of pH of the medium on the activity of immobilized GMA.*

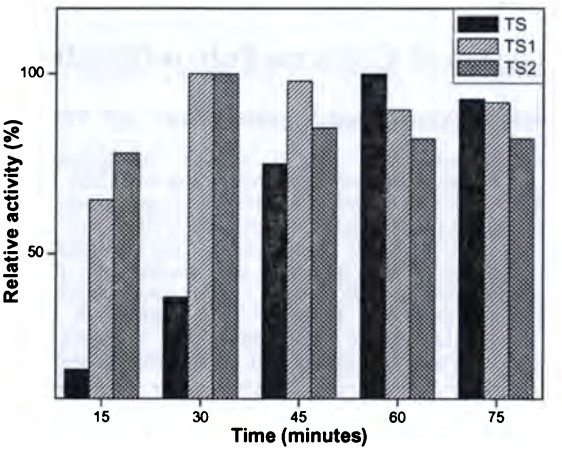

Fig. 5.4.2 *Effect of immobilization time on the activity of the immobilized enzyme.*

The time required for maximum protein loading and activity is of great importance in enzyme immobilization studies. The results are presented in *fig. 5.4.2*. The time needed

for maximum immobilization is 30 minutes on TS1, TS2 and 60 minutes on TS. Above this incubation time in all supports, the immobilization yield appeared to increases slightly, but the specific activity decreases indicating the enzyme active sites were deformed and/or partially blocked in a dense absorption layer.

The amount of GMA immobilized on carrier was limited because the binding sites of the carrier were saturated. In order to find optimal amount, the immobilization of various amounts of GMA per g TS supports was analyzed. The effect of enzyme to support ratio on protein loading and efficiency of immobilization is shown in *figs. 5.4.3, 5.4.4* and *table 5.5* below.

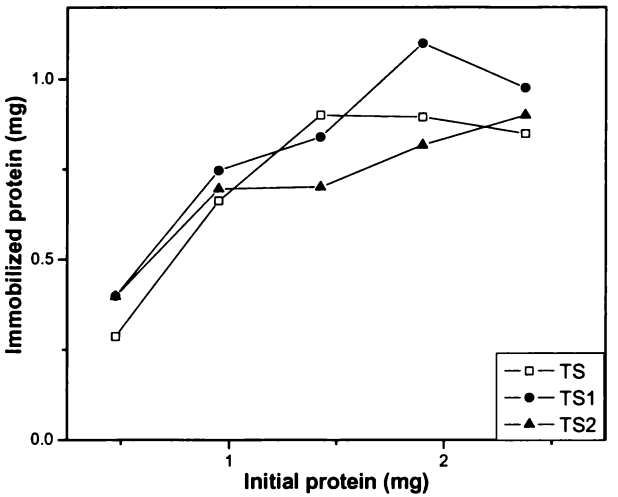

Fig. 5.4.3 *Uptake of protein with respect to amount of initial protein.*

The results shows maximum activity yield and maximum immobilization efficiency was obtained with GMA covalently immobilized on TS1. The activity of the immobilized enzyme increased as the amount of enzyme loaded increased up to 73.2 EU of initial enzyme concentration (*fig. 5.4.4*). However, an increase in the enzyme loading beyond this showed saturated reaction rate due to the increase in substrate diffusion inhibition by

enzyme aggregation. The highest recovery yield of GMA activity was about 51.7 % right after immobilization on TS1. The amount of covalently bound GMA on TS1 was 1.1 mg g^{-1} of polymer. TS and TS2 incorporated 0.9 mg of GMA per g of polymer with the immobilization yield of 70.9 and 69.9 %. The specific activity reduction in immobilized enzymes with respect to their native forms has been widely reported [7, 14, 21]. Structural changes by covalent attachment and diffusional limitations could be the reasons for these activity losses.

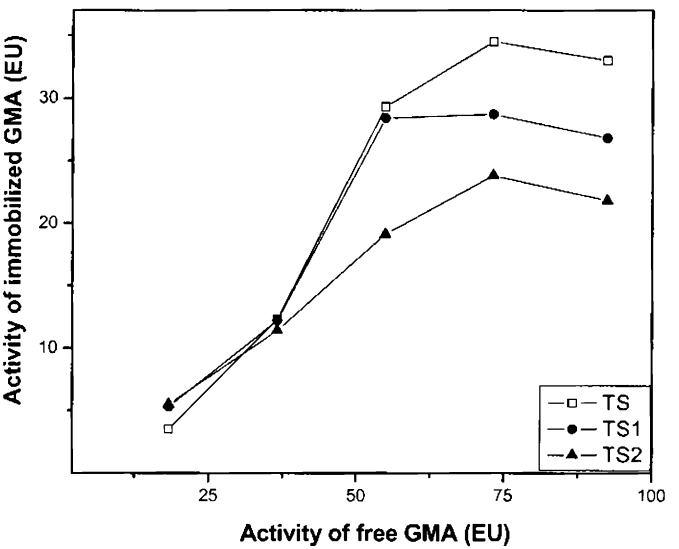

Fig. 5.4.4 Activity of immobilized enzyme as a function of activity of free enzyme.

Table 5.5 Immobilization efficiency for GMA on poly(o-toluidine) salts.

Polymer	Initial protein (mg)	Immobilized protein (mg g^{-1} polymer)	Immobilization Yield (%) IY	Initial activity (EU)	Activity of immobilized GMA (EU g^{-1} of polymer)	Activity Yield (%) AY	Immobilization Efficiency (%) IE
TS	1.4	.9	70.9	73.2	34.5	47.1	73.3
TS1	1.9	1.1	76.8	54.9	28.4	51.7	89.3
TS2	2.4	0.9	69.9	73.2	23.8	32.5	86.7

5.4.2 Effect of pH on activity

Figure 5.4.5 shows the variation of relative activity for free and immobilized glucoamylase with respect to pH of the substrate solution. The optimum pH for the free enzyme was observed at 5.5. Adsorbed glucoamylase showed pH optimum at 5 and the activity decreases after pH 5.5. For the covalently bound enzyme there was no shift in optimum pH value, and TS1-GMA was active over wider pH range, indicating that the sensitivity to pH is reduced as a result of immobilization. In general, for the immobilized enzyme, the optimum pH value depends on the properties of the supports. Adsorbed enzyme showed higher activity at lower pH and a sharp drop of its activity in the basic solution compared with the free enzyme. This can be attributed to the charge effects of the matrix [14, 24]. Covalently bound glucoamylase showed no change in the optimum value because of the presence of spacer molecules. It is also known that the secondary interactions such as ionic and polar interactions between the enzymes and ionic matrices are the major factors for the shifts in optimum pH [21].

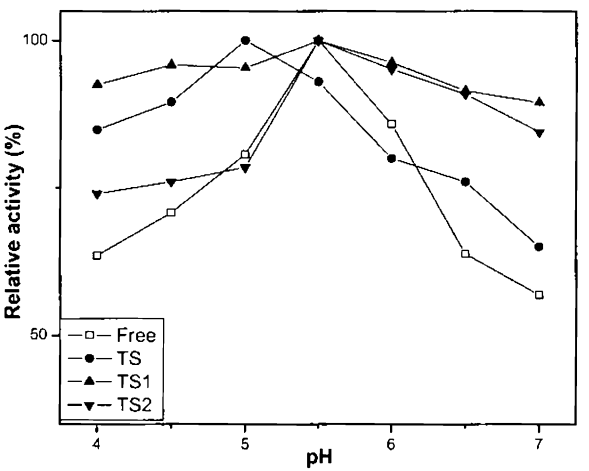

Fig. 5.4.5 Effect of pH on the activity of free and immobilized GMA.

5.4.3 Effect of temperature on activity

The temperature dependence on activity of free and immobilized glucoamylase was studied in the temperature range of 30-70 °C. The results obtained are depicted in *fig. 5.4.6.* Optimum catalytic activity was observed at 60-65 °C, for immobilized glucoamylase; however the free enzyme had a lower optimum temperature of 55 °C. The increase in optimum temperature by immobilization is caused by the physical and chemical properties of the enzyme is changing, and is explained by the creation of conformational limitations on the movement of the enzyme as a consequence of the formation of multiple bonds between the enzyme and the support.

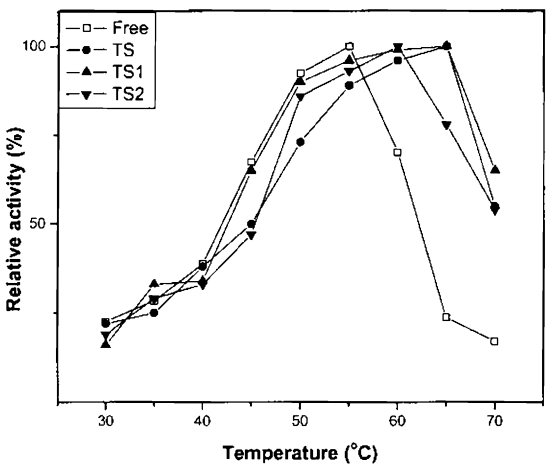

Fig. 5.4.6 Effect of temperature on the activity of free and immobilized GMA.

5.4.4 Thermal stability

The stability of free and immobilized glucoamylase was studied at various temperatures (30-70 °C) and the results are shown in the *fig 5.4.7.* The activity of enzyme relative to preincubation time in the absence of substrate is shown in *fig. 5.4.8.* It is evident from the figure that all forms of immobilized glucoamylase required around 5-10 minutes to regain its activity and was exhibited better thermal stability than the free enzyme at their

optimum temperatures. The results in the *fig. 5.4.6* showed that the maximum catalytic activity is obtained at 55 °C for free glucoamylase and 60-65 °C for immobilized glucoamylase; however, as the incubation time and temperature increases, (*figs. 5.4.7* and *5.4.8*) the stability of free enzyme reduced rapidly compared to immobilized form.

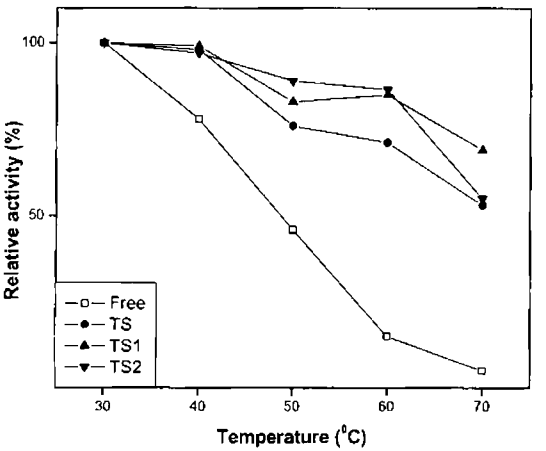

Fig. 5.4.7 *Effect of temperature on the stability of free and immobilized GMA.*

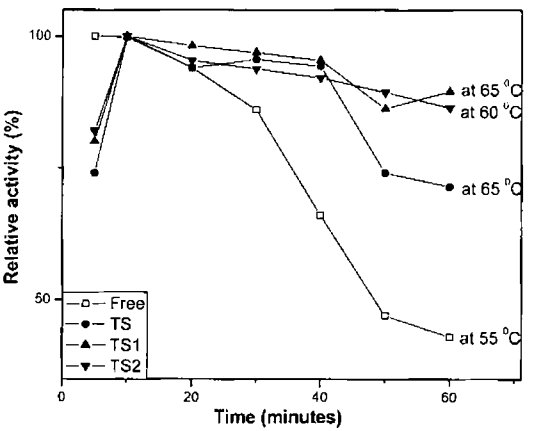

Fig. 5.4.8 *Influence of incubation time on the relative activity of free and immobilized GMA.*

At 40 °C, the immobilized enzyme preserved 99 % its initial activity whereas the free enzyme lost about 21 % of its initial activity and at 55 °C, 43 % free enzyme remained active where as 70-80 % immobilized enzyme remained active during 60 minutes incubation. The increased thermal stability of the immobilized enzyme may be ascribed to the stabilizing effects of immobilization [47]. Also many research workers have been reported [48-50] that immobilization lead to the thermal stabilization in the case of glucoamylase from different sources.

5.4.5 Kinetic constants

The Michaelis constants K_m and V_{max} for free and immobilized GMA were determined by varying the concentration of starch in the reaction medium. Lineweaver-Burk plot and Hanes-Woolf plot for free and immobilized are shown in *fig. 5.4.9*. The kinetic parameters of free and immobilized enzymes are summarized in *table 5.6*.

Table 5.6 *Kinetic parameters for free and immobilized GMA.*

	Free GMA	TS-GMA	TS1-GMA	TS2-GMA
K_m (mg mL^{-1})	0.32 ± 0.04	3.64 ± 0.1	1.35 ± 0.09	2.04 ± 0.09
V_{max} (EU μg^{-1} protein)	1.65 ± 0.03	1.21 ± 0.06	1.47 ± 0.08	1.43 ± 0.07

The K_m value of free glucoamylase was 0.32 mg mL^{-1} of starch. The K_m value estimated for TS, TS1 and TS2 are 3.64, 1.35 and 2.04 mg mL^{-1} respectively *(table 5.6)*. The apparent K_m value of for TS1-GMA was approximately 4.5-fold higher than that of the free enzyme. The comparison with the results obtained by *Sanjay et al.* [51] for GMA immobilized on the montmorillonite clay showed that the K_m values of covalently bound enzyme are about 5 times larger than that of the free enzyme. This may be caused by

structural changes in the enzyme introduced by the immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme [41].

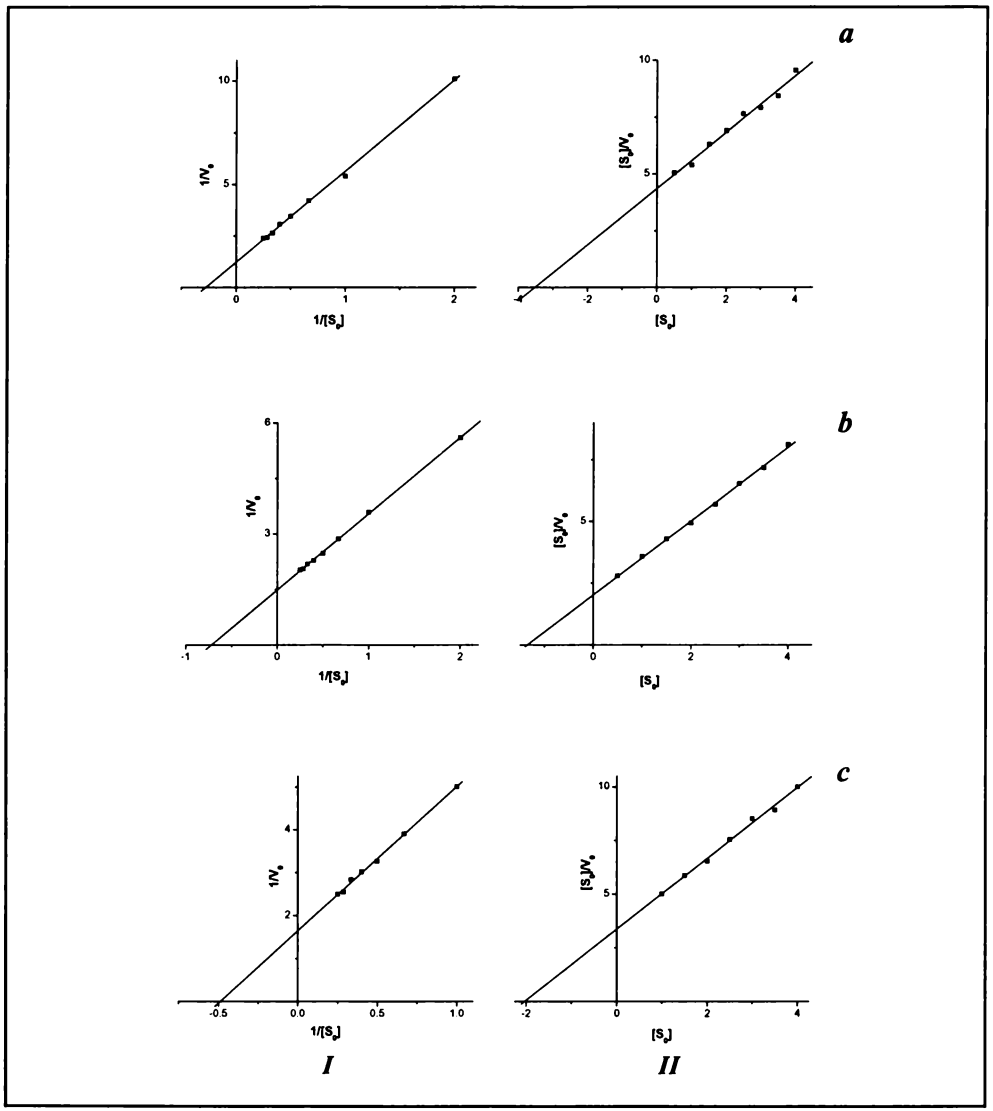

Fig. 5.4.9 (I) Lineweaver-Burk plots and (II) Hanes-Woolf plots for (a) free GMA and immobilized on (b) TS (c) TS1 and (d) TS2.

On the other hand, the V_{max} values of immobilized GMA were estimated from the data as 1.21, 1.47 and 1.43 EU μg^{-1} of protein on TS, TS1 and TS2 respectively, suggesting the residual activity of immobilized enzyme decreased in course of immobilization.

5.4.6 Storage stability

Storage stability measurements were conducted in semi-dry form at 4 °C for a period of 90 days (*fig. 5.4.10*). It is clear from the figure that all forms of immobilized enzymes shows appreciable amount of activity up to 60 days of storage. After 90 days, the catalytic activity of the adsorbed enzyme retained was only 13.6 % of its original activity, where as covalently bonded TS1-GMA and TS2-GMA retained 38 % and 29 % of their initial activity respectively. GMA immobilized on TS1 exhibited higher stability than that of the free and other two forms immobilized enzyme.

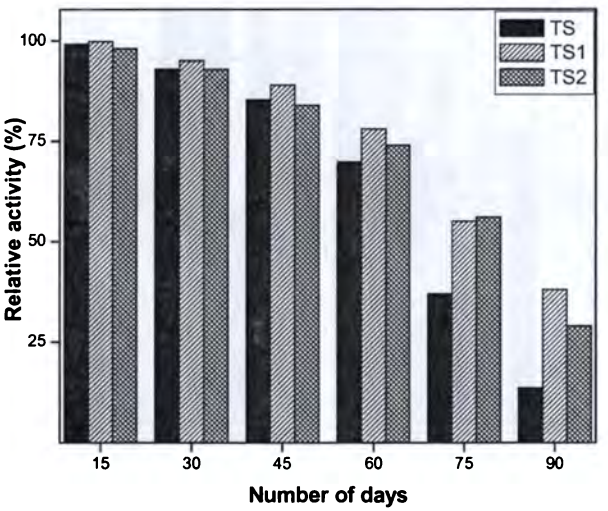

Fig. 5.4.10 *Storage stability of GMA immobilized on poly(o-toluidine) salts.*

On the basis of these observations, it was concluded that these supports provide better stabilization effect, minimizing possible distortion effects, which might be imposed

from aqueous medium on the active site of the immobilized enzyme. The generated multi-point ionic interactions between enzyme and matrixes also conveyed a higher conformational stability to the immobilized enzyme [52].

5.4.7 Multiple use of the immobilized GMA

Reusability of the immobilized GMA samples was examined by using the same conditions repeatedly 8 times within 6 hour and the measured activities are shown in *fig. 5.4.11*. It was observed that the covalently bound enzyme on TS1 demonstrated more than 95 % activity after 4 runs and 85 % activity after 8 runs. Adsorbed enzyme on TS could retain only 55 % and GMA coupled on TS2 were 80.7 % active after 8 runs. 85 % activity recovery after seven repeated use was reported [17] when GMA was immobilized on porous vinyl polymer. The immobilization of GMA on TS1 proved to be advantageous for the continuous use of the enzyme for starch conversion in biotechnological process.

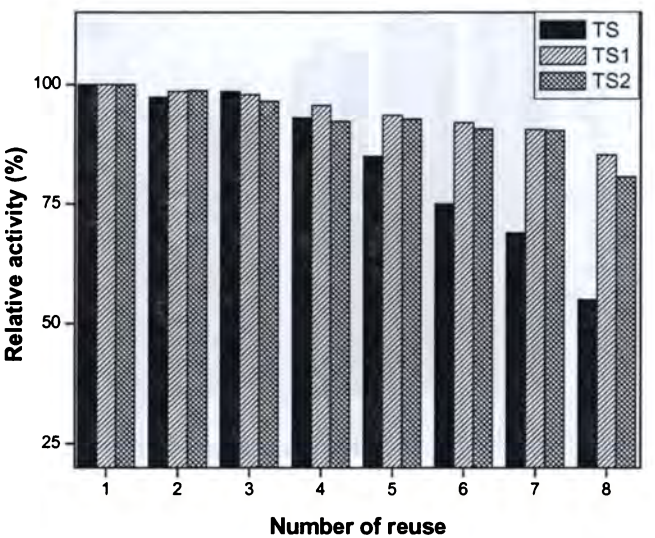

Fig. 5.4.11 *Reusability of immobilized GMA*

5.5 Immobilization of GMA on Poly(*o*-toluidine) Base (TB)

5.5.1 Optimized immobilization conditions

Fig. 5.5.1 shows the influence of pH on immobilization of GMA. As expected from the previous studies, the pH for immobilization was found best in the range of 4-6. The highest activities were obtained at pH 5 in sodium acetate-acetic acid buffer. Lower activity recoveries were obtained at both lower and higher pH values.

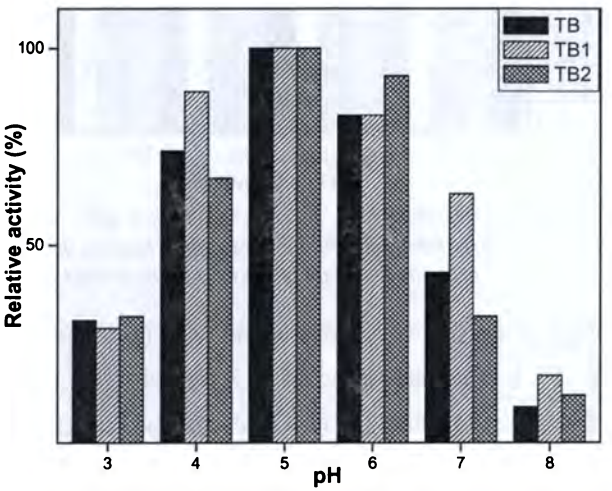

Fig. 5.5.1 *Influence of pH of the immobilization medium on the recovered activity.*

The recovered activity vs. time of immobilization is given in the *fig. 5.5.2*. TB-GMA showed the maximum activity after 15 minutes, while GMA on TB1 and TB2 showed highest activity after 30 and 45 minutes respectively. The highest activity favored short time, as the stirring time extent, the protein conformation of GMA might be denatured to lead the limitation of immobilization process. Thus optimum immobilization time considered was up to 75 minutes.

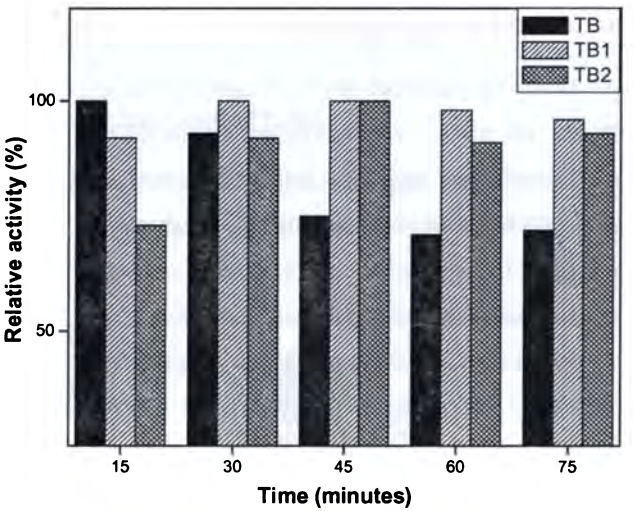

Fig. 5.5.2 *Effect of immobilization time on the recovered activity of the immobilized GMA.*

The amount of enzyme which was bound onto TB and the resultant activities are presented in *fig. 5.5.3, 5.5.4* and in the *table 5.7* below. The maximum immobilization efficiency of 96.7 % was obtained for TB-GMA system. TB1-GMA and TB2-GMA also showed comparatively good immobilization efficiency; 94.7 and 94.6 % respectively.

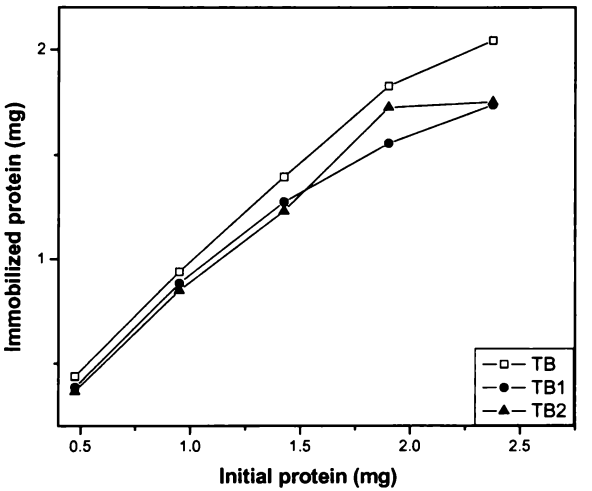

Fig. 5.5.3 Effect of initial protein amount on the immobilization of protein.

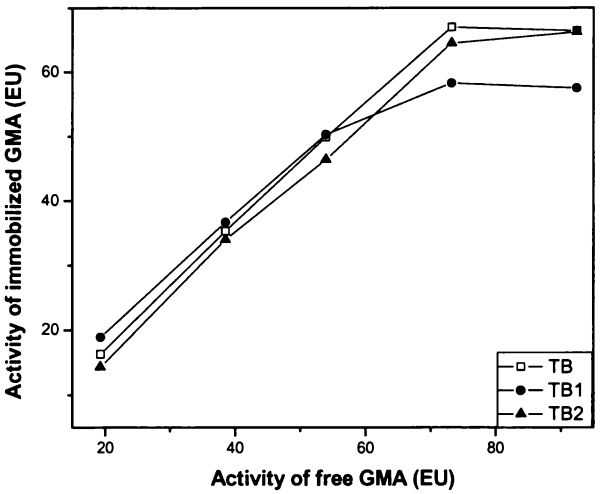

Fig. 5.5.4 Activity of immobilized GMA as a function of initial activity.

Table. 5.7 *Immobilization efficiency for the GMA on poly(o-toluidine) base.*

Polymer	Initial amount of protein (mg)	Immobilized protein (mg g^{-1} polymer)	Immobilization Yield (%) IY	Initial activity of GMA (EU)	Activity after immobilization (EU g^{-1} polymer)	Activity Yield (%) (AY)	Immobilization efficiency (%) IE
TB	1.9	1.8	94.7	73.2	67	91.5	96.7
TB1	1.9	1.6	84.1	73.2	58.3	79.6	94.7
TB2	2.4	1.8	75.7	92.4	66.2	71.6	94.6

5.5.2 Effect of pH on enzyme activity

The behavior of an enzyme molecule may be modified by its immediate microenvironment. An enzyme in solution can have a different or same optimal pH from the same enzyme immobilized on a solid matrix depending on the surface and residual charges on the solid matrix and the nature of the enzyme-bound pH value in the immediate vicinity of the enzyme environment. All previous results underline this behavior. As can be seen from these curves below (*fig. 5.5.5*), the starch hydrolysis reaction for both free and immobilized enzymes have the same pH optimum at 5.5. However, the pH profiles for the immobilized GMA display slightly improved stability on both sides of the optimum pH value, in comparison to that of the free form, which means that the immobilization method preserved the enzyme activity in different pH values. This result might probably be attributed to the stabilization of GMA molecules resulting from multipoint immobilization especially on the glutaraldehyde activated support [52].

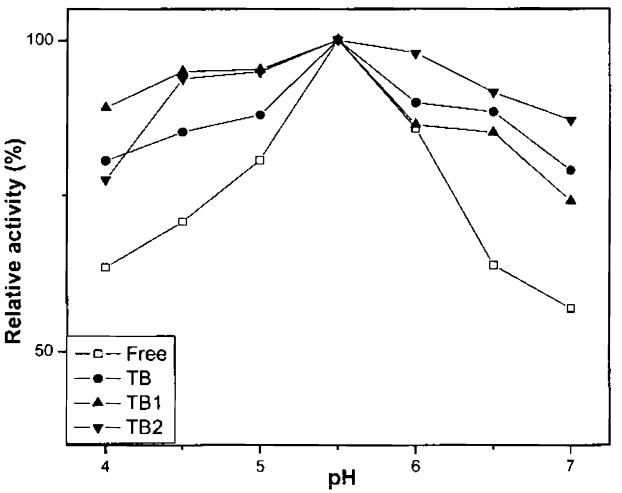

Fig. 5.5.5 *Effect of pH on the activity of free and immobilized GMA.*

5.5.3 Effect of Temperature on the activity of GMA

The variation of glucoamylase activity with temperature was investigated and presented in *fig. 5.5.6.* As mentioned earlier the soluble enzyme had an optimum temperature of 55 °C, whereas the temperature optimum for covalently immobilized enzyme was shifted to 60 °C. This indicated that the covalently coupled GMA required higher activation energy and also could resist denaturation due to temperature rise. However, adsorbed GMA preparations showed the same temperature optimum that of free enzyme at 55 °C, and the immobilized enzyme retained greater fraction of catalytic activity at higher temperatures as compared to free enzyme.

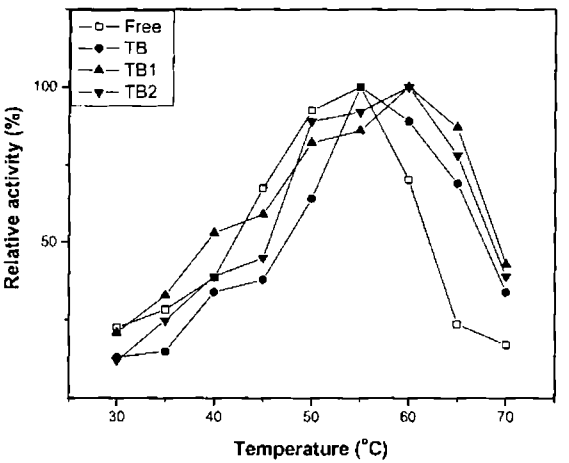

Fig. 5.5.6 Effect of temperature on the activity of free and immobilized GMA.

5.5.4 Thermal stability

The effect of immobilization on thermal stability was evaluated by incubating free and immobilized GMA for 60 minutes at temperatures ranging from 30 to 70 °C. The results presented in *fig. 5.5.7* indicate that free GMA was stable up to 60 °C, and lost all of its activity when incubated 60 minutes at 70 °C.

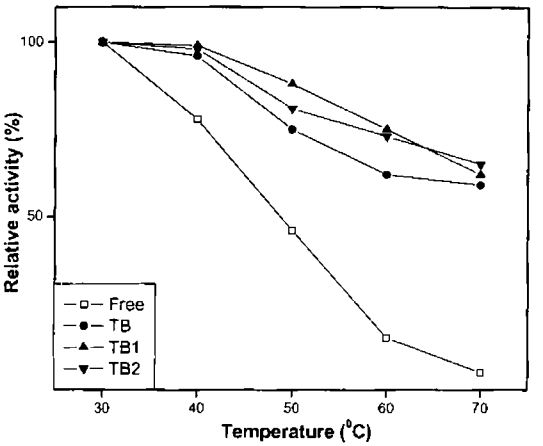

Fig. 5.4.7 Effect of temperature on the stability of free and immobilized GMA.

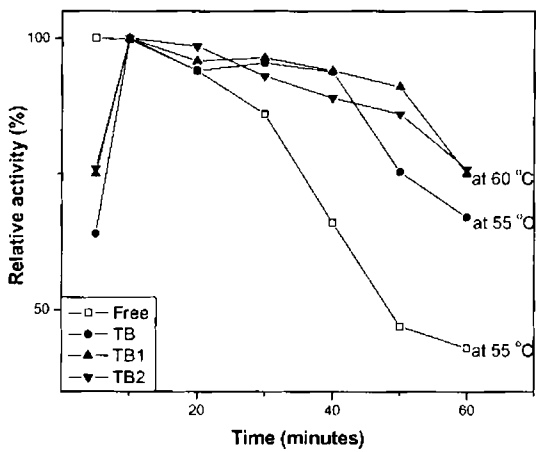

Fig. 5.5.8 Influence of incubation time on the activity of GMA at their optimum temperature.

The immobilized enzymes retained 65-75 % of their initial activity after 60 minutes incubation at their optimum temperature indicating that the immobilized enzymes were inactivated at a much slower rate than those of the free enzyme. Therefore, the

immobilization led to a considerable increase of thermal stability. It is often found that immobilized enzyme has a higher thermal stability than free enzyme because of restriction of conformational flexibility in immobilized enzyme. Similar to these *Storey et al.* [53] immobilized GMA in polyurethane polymers and observed greater thermostability and improved stability under various denaturating conditions.

5.5.5 Kinetics parameters

Kinetic parameters, K_m and V_{max} for the free and immobilized GMA, were assayed at starch concentration from 0.5 to 10 mg mL^{-1} (*fig. 5.5.9* and *table 5.8*). This parameter reflects the effective characteristics of the enzyme and depends upon both partition and diffusion effects. The K_m value for free enzyme was 0.32 mg mL^{-1}, whereas V_{max} value was 1.65 EU μg^{-1} proteins. K_m values of immobilized enzymes were found as 7.7 mg mL^{-1}, 5.01 mg mL^{-1} and 4.1 mg mL^{-1} and V_{max} value for immobilized enzymes were found as 1.60, 1.56, 1.54 EU μg^{-1} proteins (*fig. 5.5.9*) for TB, TB1 and TB2 respectively. For the immobilized GMA, as noted earlier there was an increase in K_m and decrease in V_{max} values compared to the free enzyme. This increase in the K_m values is either due to the conformational changes of the enzyme resulting in a lower possibility of forming a substrate-enzyme complex, or to the lower accessibility of the substrate to the active sites of the immobilized enzyme caused by the increased diffusion limitation [23, 24].

Table 5.8 *Kinetic parameters for the free and immobilized GMA.*

	Free GMA	**TB-GMA**	**TB1-GMA**	**TB2-GMA**
K_m (mg mL^{-1})	0.32 ± 0.07	7.7 ± 0.12	5.01 ± 0.18	4.10 ± 0.05
V_{max} (EU μg^{-1} proteins)	1.65 ± 0.03	1.60 ± 0.06	1.56 ± 0.05	1.54 ± 0.09

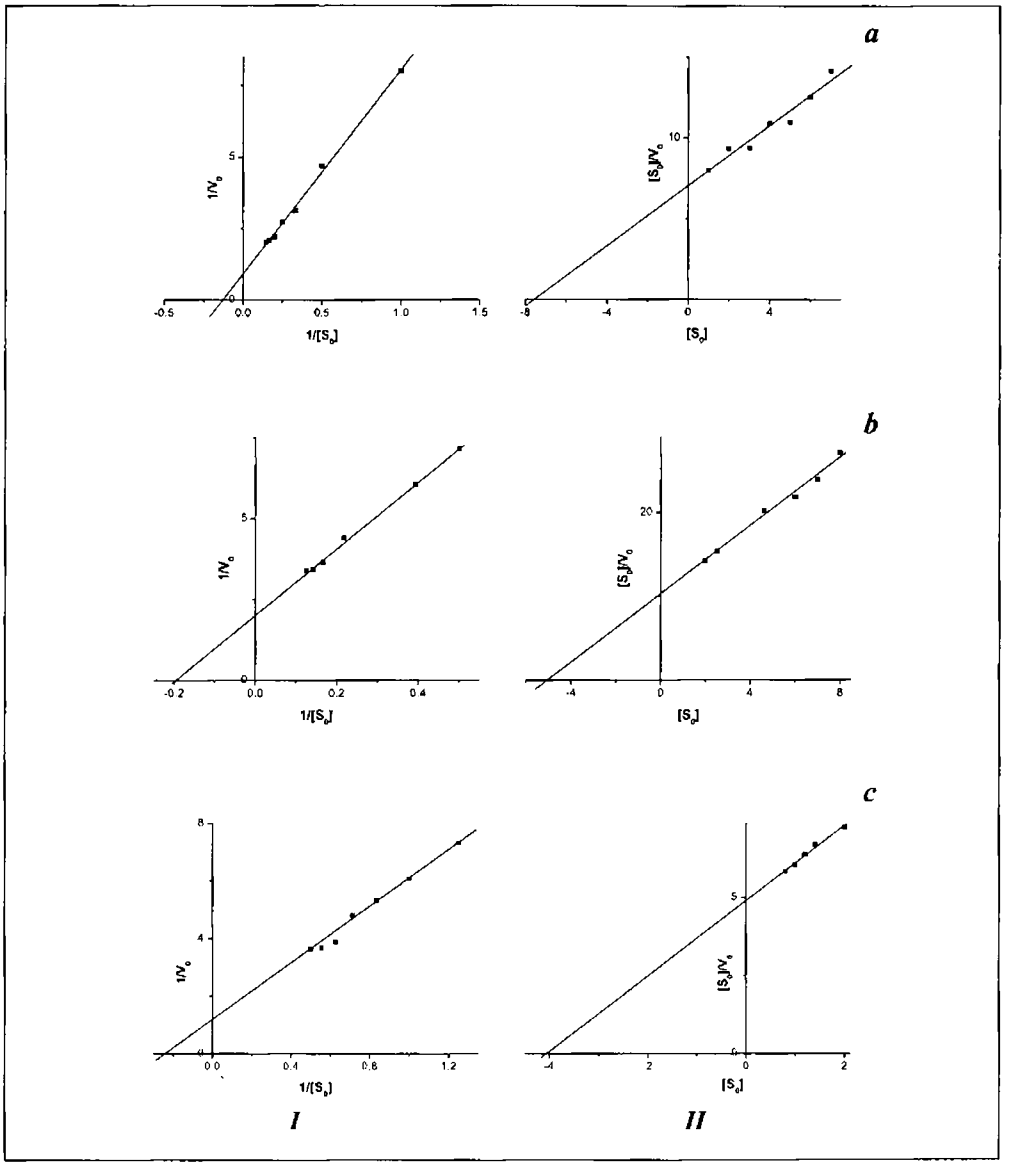

Fig. 5.5.9 (I) Lineweaver-Burk plots and (II) Hanes-Woolf plots of (a) TB-GMA (b) TB1-GMA and (c) TB2-GMA.

5.5.6 Storage stability

In order to investigate the industrial applicability of an immobilized enzyme process, one of the most important parameters to be considered in enzyme immobilization is storage stability. The stability of immobilized glucoamylase, stored at 4 °C in semidry condition was determined by periodically removing samples over a 3 months period and testing for enzymatic activity. As shown in *fig. 5.5.10*, the immobilized glucoamylase molecules still retain more than 90 % of their original activity in all forms even after 30 days of storage. The immobilized enzyme TB, TB1 and TB2 retained 23, 36 and 30 % of their initial activity after 90 days of storage. These results are similar to that obtained for GMA immobilized on polyaniline previously discussed in this chapter.

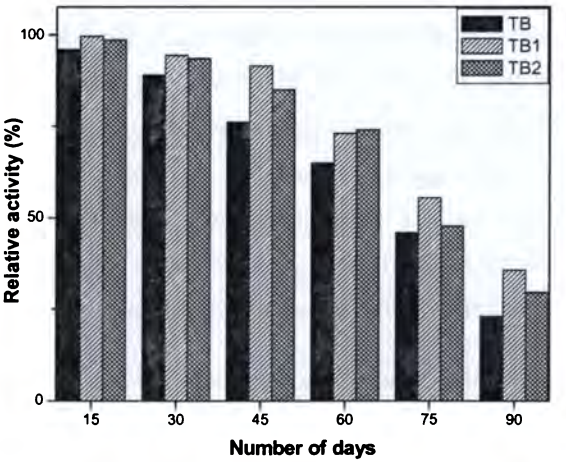

Fig. 5.5.10 *Activity of immobilized GMA stored for 90 days.*

5.5.7 Repeated batch reaction

The long-term operational stability of immobilized biocatalysts is one of the most important aspects of the contemporary biotechnological processes, which make the enzyme-based processes cost-effective. The operational stability of the immobilized glucoamylase was evaluated in a repeated batch process. The results (*fig. 5.5.11*) indicated

that the catalytic activity of the immobilized enzyme was retained under repeated use. Percent hydrolysis was determined to be 98 % for TB1-GMA after 4 cycles. On subsequent uses, i.e. after 5th, 6th 7th and 8th cycles the percent hydrolysis is found to be 95, 93, 89 and 86 %, respectively. GMA on TS and TS2 also retained more that 80 % of starch hydrolysis even after 8 runs. There was no drastic decrease in percent hydrolysis even after eight cycles, which could be due to glutaraldehyde treatment, which prevented the leakage of enzyme.

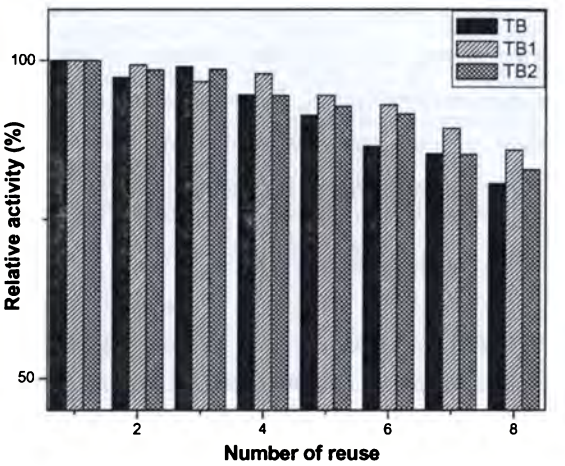

Fig. 5.5.11 Relative activity after repeated batch reaction of immobilized GMA.

5.6 Conclusion

In this study, GMA originating from *Rhizopus* was efficiently immobilized onto polyaniline and poly(*o*-toluidine), both in their salt and base forms. For the preparation of immobilized GMA, which has a high activity and high stability to repeated use, the optimum conditions for the preparation reaction were investigated in detail. The optimum conditions for the preparation reaction are quantified on the basis of the enzymatic activity and the protein content on the support. Furthermore, enzyme properties of immobilized GMA prepared at optimum conditions are compared with the free enzyme. The optimum

temperature and pH for the free enzyme for starch hydrolysis reaction are 55 °C and 5.5, respectively. Immobilized GMA via covalent bond formation at the optimum conditions was very stable against the repeated use and had more than 80 % of relative activity after the eighth procedure. In addition, after five operational cycles, it was observed that the entire immobilized enzyme retained more 80 % of its activity. The mild conditions for immobilization could help the enzyme in retaining its activity. The immobilized enzyme remained stable for longer periods of time and also at higher temperatures compared to the free enzyme in solution. pH studies indicated that the immobilized enzyme remained significantly active over a broader pH range compared to that in solution. Such a stable immobilized enzyme system is suitable for a number of applications that would not be feasible with a solution enzyme system. The process described here is a mild method used for the development of new materials, which may provide the basis for the future development of efficient biosensors and enzymatic reactors.

The main observations can be summarized as;

- ***The optimum pH for starch hydrolysis for Rhizopus glucoamylase was observed at pH 5.5. The temperature for maximum activity was 55 °C. K_m and V_{max} for starch hydrolysis were calculated as 0.32 mg mL^{-1} and 1.65 EU μg^{-1} proteins respectively.***
- ***The enzyme immobilized within short contact time and yielded good immobilization efficiency.***
- ***The immobilized enzyme showed improved pH and thermal stability.***
- ***After immobilization there was a shift in optimum pH and temperature observed.***
- ***The kinetic constants are varied after immobilization. K_m values showed an increase and V_{max} values showed a decrease after immobilization.***
- ***Immobilized enzyme showed better storage stability and reusability.***

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CHAPTER 6
SUMMARY AND CONCLUSION

6.1 Introduction

With the increased emphasis on process economics and improved efficiency, the use of immobilized enzymes will continue to grow in the food, pharmaceutical, and chemical industries. It allows for the reduction of process steps, increases productivity, and decreases waste. The wide variety of immobilization supports provides suitable options for different enzymes, process systems, and cost requirements and thus emphasizing the immobilization as an excellent process choice. There are many technical challenges in this field that has to be addressed and solved. Many researchers put tremendous effort to solve those challenges via immobilization of enzymes. This method is becoming increasingly important in the field of biotechnology. In this thesis we have addressed the main advantages and the drawbacks of the enzymes and tried to solve those problems via immobilization method. α-Amylase and glucoamylase are the enzymes used for our studies and were efficiently immobilized into polyaniline and poly(*o*-toluidine) both in their salt and base form by adsorption and covalent bonding.

6.2 Summary

The thesis work is presented in six chapters. A chapter-wise summary is given below.

Chapter 1. A general introduction about the enzymes and enzyme catalysis is provided. The factors affecting enzyme catalysis and kinetics of enzyme reaction are described in detail. The methods of immobilization of enzymes and their advantages and disadvantages over others are explained as well. A description of polymeric supports used for the immobilization process, their activation and finally the objectives of the present work are given in this chapter.

Chapter 2. This chapter provides the experimental procedures adopted for this research work. It gives the method of preparation of polymers PANI and POT followed by the techniques used for the activation of polymers. The immobilization of enzymes by adsorption and covalent bonding are described in the next section. Later it describes

enzyme activity studies, biochemical characterization techniques used for free and immobilized enzymes and techniques used to characterize the polymers.

Chapter 3. The results of various physico-chemical methods used to characterize the polymeric supports before and after immobilization are illustrated in this chapter. Characterization of the polymers via FT-IR, Surface area measurement, TG-DTG, and SEM were explained in detail.

Chapter 4. This chapter has outlined the results of immobilization of α-amylase on PANI and POT. The polymers were activated with bifunctional reagents GA and ASA for covalent bonding. The activity of the free and immobilized enzymes was compared. The effects of pH of the immobilization medium, time of incubation, and the dependence of protein concentration on the activity of the immobilized enzymes were studied. The conditions such as pH and temperature for the optimum activity followed by the thermal stability of free and immobilized enzymes were investigated. A detailed kinetic study was performed and kinetic parameters were evaluated using Lineweaver-Burk plot and Hanes-Woolf plot. Stability studies with respect to long term storage and repeated use were performed.

Chapter 5. The immobilization studies of glucoamylase on PANI and POT by adsorption and covalent bonding are presented in this chapter. The immobilization parameters such as pH of the medium, contact time, and protein concentration are optimized. The immobilization efficiency was evaluated. The optimum conditions such as pH and temperature for the starch hydrolysis for free and immobilized GMA are compared. The kinetic parameters are evaluated. The thermal stability, storage stability and reusability of immobilized GMA were studied.

Chapter 6. Provides the summary of the work along with major outcomes.

6.3 Conclusions

The major outcome of this thesis is outlined below:

- *This study has shown that PANI polymers are suitable for immobilization techniques. Its advantages include the ease of polymeric support synthesis in a minimum time, high yield, and ease of handling and excellent stability.*

- *FT-IR spectra showed all the characteristic peaks for PANI and POT. Protonation and deprotonation behavior reflected in the intensity of the corresponding peaks.*

- *TG-DTG analysis showed a three step weight loss behavior for ES and TS and two step weight losses for EB and TB. All the polymers showed good thermal stability.*

For α-amylase

- *For free α-amylase the optimum conditions for starch hydrolysis were of pH 5-5.5 and temperature 50 °C. The Michaelis-Menten constant for starch hydrolysis was evaluated as 0.46 mg mL^{-1}.*

- *The enzyme α-amylase immobilized on ES, EB, TS and TB by adsorption and covalent bonding on GA activated ES1, EB1, TS1 and TB1 and ASA activated ES2, EB2, TS2 and TB2 in the pH range 4.5-6 within the duration of 30-75 minutes. The immobilized enzymes showed good activity yield.*

- *After immobilization, the pH for maximum enzyme activity was slightly shifted depending upon supports and methods used.*

- *The optimum temperature for starch hydrolysis was lowered by 10-15 °C after immobilization. The immobilized α-amylase showed enhanced thermal stability.*

- *All enzyme system obeyed Michaelis-Menten kinetics. The kinetic parameter K_m showed a 2-17 fold increase after immobilization and V_{max} was found to be decreasing after immobilization of α-amylase.*

- *The immobilized α-amylase showed excellent stability for storage and reuse; most of them retained more than 50 % activity even after 6 months of storage and recyclable for more than 10 cycles.*

For Glucoamylase (GMA)

- *The optimum conditions for starch hydrolysis for free Rhizopus glucoamylase were observed at pH 5.5 and temperature 55 °C. The kinetic constants K_m and V_{max} for starch hydrolysis were evaluated as 0.32 mg mL^{-1} and 1.65 EU μg^{-1} proteins respectively.*

- *GMA was immobilized by adsorption and covalent bonding with GA and ASA spacer on PANI and POT. This was used to prepare heterogeneous biocatalysts for the hydrolysis of starch.*

- *Immobilization time, pH of immobilization medium, and protein concentration of coupling mixture affected immobilization yield.*

- *The optimum pH for starch hydrolysis was found at 5 or 5.5. The pH stability of the immobilized GMA was higher than that of the free enzyme.*

- *The immobilized GMA had a wider catalytic reaction temperature profile than free enzyme.*

- *The covalently linked enzyme has shown excellent stability on repeated reuse for 8 cycles and storage for 60 days without any significant loss in the enzyme activity.*

Curriculum Vitae

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Other Research Experience

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'***Design & synthesis of a melamine-based Adenine Mimetic as a novel nucleobase for Peptide nucleic acids***'.

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Hands on experience

Surface area analyzer, UV-VISIBLE spectrophotometer, FT-IR Spectrometer, TG- DTA.

Techniques familiar with

NMR and MASS Spectroscopy, Powder and Single crystal X-Ray Diffraction, SEM.

Conference attended

Frontiers in Chemistry, *National Conference on Chemistry at Cochin University of Science and Technology in March 2006.*

MatCon 2007 *International Conference on Materials for the Millennium at Cochin University of Science and Technology in January 2007.*

ICAM-2008 International Conference on Advanced Materials at M. G. University, Kottayam in February 2008.

List of Publications

1. **Immobilization of α- Amylase on Polyaniline; P.C. Ashly**, M. J. Joseph and P.V Mohanan; *Presented at MatCon 2007, the International Conference on Materials for the Millennium at Cochin University of Science and Technology in January 2007.*
2. **Activity of Diastase α-amylase Immobilized on Emeraldine; P.C. Ashly**, M. J. Joseph and P.V. Mohanan. *Communicated with the journal 'Enzyme and Microbial Technology'.*