BIOCATALYTIC PERFORMANCE OF CARBONIC ANHYDRASE IMMOBILIZED WITHIN POLYURETHANE FOAM IN WATER-MISCIBLE ORGANIC SOLVENTS

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ABSTRACT

BIOCATALYTIC PERFORMANCE OF CARBONIC ANHYDRASE IMMOBILIZED WITHIN POLYURETHANE FOAM IN WATER-MISCIBLE ORGANIC SOLVENTS

The effects of water-miscible organic solvents such as acetonitrile and ethanol on the activity of free and immobilized bovine carbonic anhydrase (CA) were investigated. The CA was covalently immobilized within polyurethane (PU) foam by cross-linking. Although PU foam holds water almost 12 times of its weight, it was found that adsorption isotherm of moisture on PU foam was a Type III indicating that water and PU foam were non-interacting to each other. The activities for the free and immobilized CA were estimated using *para*-nitrophenyl acetate (*p*-NPA) as the substrate. The enzyme activities were estimated in increasing volume percents of organic solvent in Tris buffer (10-90%). *p*-NP, which is one of the products of the hydrolysis reaction of *p*-NPA, was characterized in the presence of organic solvents and it was observed that its aborptivities were decreased significantly as the organic solvent percentages were increased indicating that *p*-NP and the water-miscible organic solvent form a complex through mostly a hydrogen bonding. The free CA showed decreasing activity up to critical percentages of organic solvent (40-60%), and then exhibited an increasing activity. The immobilized CA showed decreasing activity in acetonitrile at percentages up to 50%, and then lost its total activity at higher acetonitrile percentages, however, the immobilized CA exhibited no activity in ethanol at percentages above 10%. Stability tests showed that the immobilized CA was dramatically inactivated in the organic solvents at percentages above 30% in shorter times. It was concluded that the water-miscible organic solvents severely perturbed the active site of the enzyme, thus denaturating the enzyme.

ÖZET

POLİÜRETAN SÜNGERE İMMOBİLİZE EDİLMİŞ KARBONİK ANHİDRAZIN SUYLA KARIŞABİLEN ORGANİK ÇÖZÜCÜLERDE BİYOKATALİZLEME PERFORMANSI

Asetonitril ve etanol gibi suyla karışabilen organik çözücülerin serbest ve immobilize edilmiş karbonik anhidraz enziminin aktivitesine etkileri araştırıldı. Karbonik anhidraz enzimi, poliüretan sünger içerisine kovalent çapraz bağlanma ile immobilize edildi. Poliüretan sünger kendi ağırlığının yaklaşık 12 katı kadar su tutabilmesine rağmen, poliüretan süngerin üzerindeki nemin adsorpsiyon izoterminin, Tip III adsorpsiyon izotermi olduğu bulundu ve bu durum, poliüretan sünger ile suyun birbirleriyle çok az etkileştiğini gösterdi. Serbest ve immobilize karbonik anhidraz enziminin aktiviteleri, substrat olarak *para*-nitrofenil asetat kullanılarak ölçüldü. Enzim aktiviteleri, Tris tampon çözeltisindeki organik çözücünün hacimsel yüzdesi arttırılarak hesaplandı (%10-90). *Para*-nitrofenil asetatın hidroliz reaksiyonu ürünlerinden biri olan *para*-nitrofenol, organik çözücülerde karakterize edildi. *Para*-nitrofenolün absorptivitesinin, karışımdaki organik çözücü yüzdeleri arttırıldığında önemli ölçüde düştüğü gözlemlendi. Bu durum, *para*-nitrofenol ve suyla karışabilen organik çözücülerin daha çok hidrojen bağları ile kompleks bir yapı oluşturduğunu gösterdi. Serbest enzim, kritik organik çözücü yüzdelerine (%40-60) kadar aktivite düşüşü ve daha sonra, yüksek organik çözücü yüzdelerinde aktivite artışı gösterdi. İmmobilize enzim, %50'ye kadarki asetonitril yüzdelerinde aktivite düşüşü gösterdi ve daha sonra, yüksek asetonitril yüzdelerinde bütün aktivitesini kaybetti. %10'dan yüksek etanol yüzdelerinde ise immobilize enzim hiçbir aktivite göstermedi. Stabilite testlerinde immobilize enzim, %30'tan yüksek organik çözücü yüzdelerinde, çarpıcı bir biçimde kısa zamanda inaktive oldu. Sonuç olarak, suyla karışabilen organik çözücüler, enzimin aktif bölgesinin yapısını ciddi bir biçimde bozarak enzimi denatüre etti.

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CHAPTER 1

INTRODUCTION

According to traditional concept, enzymes are active only in water. Historically, enzymatic catalysis has been performed mainly in aqueous systems. Water has the unique specificity for the enzymes that drew the interest of biochemists who were searching for highly selective catalytic agents.

At the end of the nineteenth century, scientists began to place enzymes in systems other than aqueous media (Krishna 2002). It has proven that the use of organic solvents as reaction media for biocatalytic reactions was an exceedingly useful approach and this approach extended the range and efficiency of practical applications of biocatalysis. Some advantages of using organic solvents can be listed as the increased solubility of hydrophobic substrates and favourable shifts of reaction equilibrium. Therefore, researchers increasingly turned their attention to the problems and potential of non-aqueous biocatalysis all over the world, and thus, the research in nonaqueous biocatalysis has made enormous progress in recent years. In particular, the main focus in non-aqueous enzyme research are the clarification of the enzyme structure, their properties in nonaqueous environments, improvement in the catalytic properties to use in organic solvents, the design of new types of the reaction environment, and finally, execution of these new developments for synthetic applications (Khmelnitsky and Rich 1999).

Native enzymes almost show low activities in organic solvents – often four or five orders of magnitude lower than in aqueous media. This is not surprising because enzymes naturally function in mainly aqueous environments (Serdakowski and Dordick 2008). Organic solvents, especially water-miscible organic solvents, may perturb enzyme molecules or may become competitive inhibitors through specific interactions with enzymes, which could alter the reaction kinetics and substrate specificity, thereby denaturating the enzyme (Ogino and Ishikawa 2001). Denaturation due to solvent-induced changes is expressed as the unfolding of the enzyme tertiary structure that leads to a disordered polypeptide. Due to the unfolding, key residues in polypeptide are no longer arrayed closely enough for functional or structure stabilizing interactions. According to the Lumry–Eyring approach, enzyme inactivation involve two steps: a

reversible unfolding of the native enzyme and then kinetically irreversible steps, which cause aggregation or covalent changes in the enzyme. The first step is due to the responsibility of the interactions that retain the native structure of the enzyme. In the second step, there is a natural evolution of the enzyme's native structure towards thermodynamically stable protein macromolecules. Stability of an enzyme is also regarded as being a crucial parameter for its industrial applications in non-aqueous enzymology. It is important to comprehend the mechanism of enzyme inactivation and the reversibility or irreversibility of the reactions thereby helping in enzyme stability characterization in the presence of organic solvents. Stability characterization provide better control over the deactivation process, stabilization approaches and catalytic properties of enzymes (Iyer and Ananthanarayan 2008). Despite the inactivation process in the presence of organic solvents, in many cases, enzymes can exhibit adequate catalytic activity and unique selectivity to be used synthetically in the nonaqueous environment. Besides, it is important to improve the enzyme function in the organic media in large-scale for the economically viable biotransformations (Serdakowski and Dordick 2008).

Today, different methods are utilized to improve the activity and/or stability of the enzymes in the presence of organic solvents. These methods contain the chemical modification of enzymes, the immobilization of enzymes on/in insoluble support matrices, the physical modification of enzymes with lipids or surfactants, the entrapment of enzymes in reversed micelles, and the molecular engineering of enzymes (Doukyu and Ogino 2010). Among these, immobilization of the enzyme in a stabilizing carrier is one of the most used method to protect enzymatic activity against denaturation in organic solvents. Enzyme immobilization also improves accessibility by molecular dispersion, i.e., preserve the enzymes against aggregation and gum formation (Odaly, Crumbliss et al. 1990). In this study, carbonic anhydrase (CA) was selected as a model enzyme and polyurethane (PU) foam was used as a carrier for the CA. Therefore, our objectives for this study were to immobilize CA within PU foam and characterize its enzymatic activity and stability in water-miscible organic solvents.

CHAPTER 2

LITERATURE SURVEY

2.1. Enzymes

Enzymes, also called biocatalysts, are striking molecular devices that determine the patterns of chemical transformations. Enzymes mediate the transformation of one form of energy into another and have two remarkable characteristics: catalytic power and specificity.

Enzymes increase the rate of chemical reactions by a factor of as much as a million or more. Reactions in biological systems do not occur at perceptible rates in the absence of enzymes mostly. Enzymes are highly effective catalysts in a wide range of chemical reactions because they specifically bind numerous molecules. Enzymes are highly specific to reactants, which are called substrates (Berg, Tymoczko et al. 2006).

2.1.1. Structural Components of Enzymes

Nearly all known enzymes are proteins. Proteins as a class of macromolecules have diverse biological functions in living organisms ranging from DNA replication, providing mechanical support, immune protection, to converting one molecule to another (Whitford 2005). Proteins typically have molecular weights of 6000 to several hundred thousand daltons. They are polymers that consist of amino acid monomers which are the building blocks of proteins (Shuler and Kargi 2002). As shown in Figure 2.1, an α-amino acid is composed of five different groups: amino (NH_3^+) and carboxyl (COO^-) groups attached to a central carbon atom, called the α carbon, a hydrogen atom, and a distinctive R group. The R group is also called the side chain.

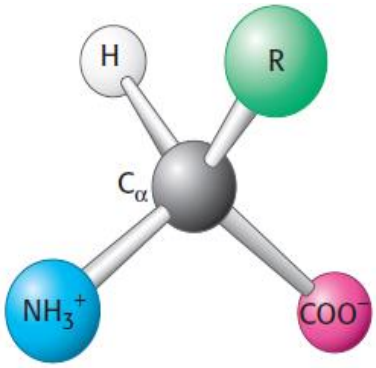

Figure 2.1. The 3-D model of an amino acid.
(Source: Berg, Tymoczko et al. 2006)

Proteins are composed mainly of 20 naturally occuring amino acids which are different from each other in the side chain groups. In living organisms, a wide range of biological functions mediated by proteins results from the diversity and versatility of these 20 building blocks (Berg, Tymoczko et al. 2006).

Amino acids are linked together by covalent amide bonds, called peptide bonds, to form the primary structure. A short chain of aminoacids is referred to as oligopeptide, longer amino acid chains are called polypeptides (Lodish and Zipursky 2001). Figure 2.2 illustrates the folding of a polypeptide chain. Polypeptide chains can fold into 3 regular structures: the alpha (α) helix, the beta (β) sheet, and turns. Secondary structures are three-dimensional arrangements of segments of a polypeptide chain which are joined together by hydrogen bonds between amide and carbonyl groups. α helices and β sheets comprise of 60 percent of the polypeptide chain in an average protein and 40 percent of the molecule is in coils and turns. Hence, α helices and β sheets are the main internal supportive elements in proteins. In the α helix, hydrogen-bonded amino acids keeps the backbone in a rodlike cylinder from which the side chains point outward. The characteristics of the side chains determine the relative hydrophobicity or hydrophilicity of a particular helix within a protein. The β sheet consists of laterally packed β strands which are almost fully extended rather than being tightly coiled as in the α helix. Unlike in the α helix, β sheets are stabilized by hydrogen bonding between β strands (Lodish and Zipursky 2001, Berg, Tymoczko et al. 2006).

Tertiary structure is a result of interactions between R groups and it refers to the arrangement of secondary structure elements and amino acid side chain interactions that determine the three-dimensional structure of the folded protein. Covalent, disulfide, or hydrogen bonds may be present among R groups. Two polypeptide chains can be

covalently linked by the disulfide bond, thus restricting the mobility of proteins and increasing the stability of their tertiary structures. In contrast with the secondary structures, stabilization of the tertiary structure is mainly obtained by hydrophobic interactions between nonpolar side chains, together with hydrogen bonds between polar side chains and peptide bonds. These forces compactly keep together elements of secondary structure. (Shuler and Kargi 2002, Copeland 2004, Harvey, Arnold et al. 2008).

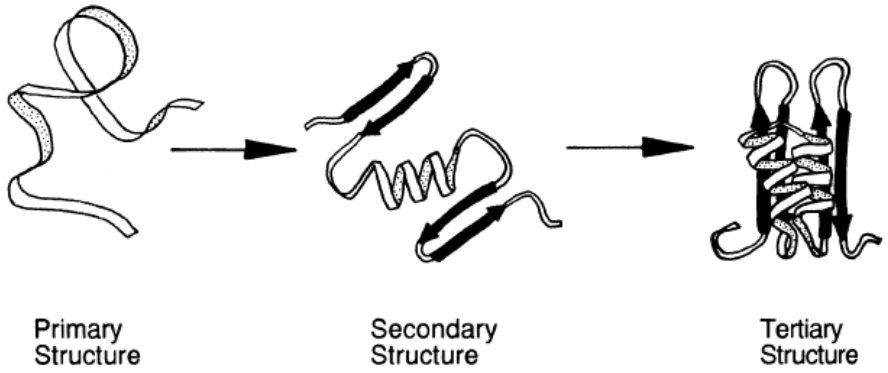

Figure 2.2. The folding of a polypeptide chain (the hierarchy of protein structure from primary structure through secondary structure and tertiary structure). (Source: Copeland 2004)

Most of the enzymes have nonprotein chemical groups in the structures of their active sites to facilitate rapid reaction. These nonprotein chemical groups are enzyme cofactors. They can be subdivided into two groups: coenzymes and metal ions (e.g., iron, zinc, copper, manganese). Coenzymes are small organic molecules often derived from vitamins such as NAD, FAD and Coenzyme A. Such an enzyme without its cofactor is called the apoenzyme; the active complex between the cofactor and the protein is called the holoenzyme (Copeland 2004, Berg, Tymoczko et al. 2006).

2.1.2. Enzyme Nomenclature

Enzymes are classified according to the types of reactions that they catalyze. Most of the enzymes are named by adding the suffix “ase” to the end of their substrates, such as ATPase or the reaction catalyzed such as ATP synthase. There are six broad classes of enzymatic reactions for a nomenclature of enzymes (Table 2.1). Each enzyme

has a designation with a four-digit number, for example, as EC 2.2.1.1., so that it is easy to identify all enzymes (Whitford 2005, Berg, Tymoczko et al. 2006).

Table 2.1. Six major classes of enzymes.
(Source: Berg, Tymoczko et al. 2006)

Class	Type of reaction	Example
1. Oxidoreductases	Oxidation-reduction	Lactate dehydrogenase
2. Transferases	Group transfer	Nucleoside monophosphate kinase (NMP kinase)
3. Hydrolases	Hydrolysis reactions (transfer of functional groups to water)	Chymotrypsin
4. Lyases	Addition or removal of groups to form double bonds	Fumarase
5. Isomerases	Isomerization (intramolecular group transfer)	Triose phosphate isomerase
6. Ligases	Ligation of two substrates at the expense of ATP hydrolysis	Aminoacyl-tRNA synthetase

2.1.3. Basic Concepts of Enzyme Catalysis and Kinetics

Enzymes accelerate the rate of a reaction by decreasing the free energy of activation without changing free energy change (ΔG), which is the difference in free energy between the reactants and the transition state, of the reaction. Hence, enzymes facilitate the formation of the transition state. There is a precise binding pocket specific to substrate within the enzyme molecule, known as the active site. Active sites consist of two regions: the substrate-binding site that binds the substrate and the catalytic site. Substrate molecules specifically bind to enzyme molecules at its active site, which is mediated by weak noncovalent interactions, to form enzyme-substrate (ES) complex. In lock and key model, as illustrated in Figure 2.3, the enzyme active site and substrate molecule are complementary to each other. In this model substrate molecule represents the key, and the active site represents the lock (Copeland 2004, Berg, Tymoczko et al. 2006, Harvey, Arnold et al. 2008).

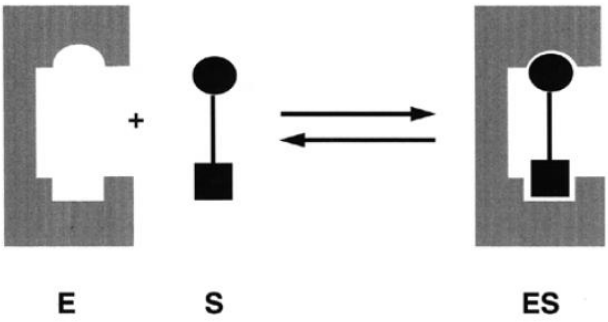

Figure 2.3. Schematic illustration of the lock and key model of enzyme—substrate interactions. (Source: Copeland 2004)

In enzymatic reactions, in the first step enzyme (E) binds to its substrate (S) to form an reversible enzyme-substrate (ES) complex with a rate constant k_1 and then in the second step ES complex irreversibly breaks down into free enzyme and product (P) with a rate constant k_2.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \qquad (2.1)$$

The rate of enzymatic reaction V_0, inreases as substrate concentration inreases and then approaches its maximal velocity V_{max} at higher substrate concentrations (Figure 2.4).

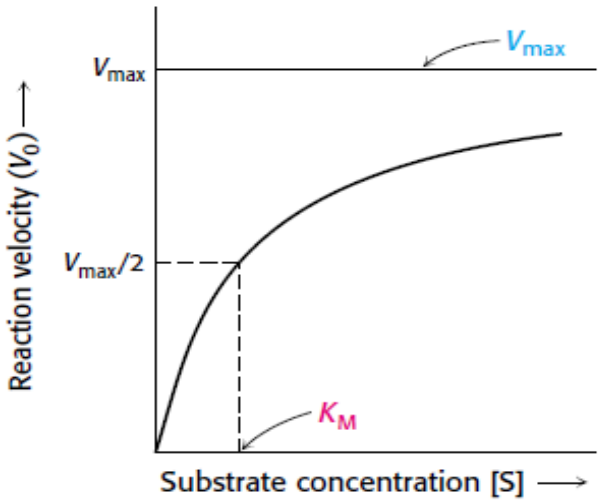

Figure 2.4. Plot of a typical enzymatic reaction.
(Source: Berg, Tymoczko et al. 2006)

Single-substrate enzyme-catalyzed reactions are represented by a Michealis-Menten Equation (eq 2.2).

$$V_0 = V_{\max} \frac{[S]}{[S] + K_M} \quad (2.2)$$

Here, K_m is the Michealis-Menten constant and a measure of affinity for the substrate. When [S] = K_m, then $V_0 = V_{max}/2$. Hence, K_m corresponds to substrate concentration at which the reaction velocity is half its maximal value (Berg, Tymoczko et al. 2006).

2.2. Enzymatic Reactions in Organic Media

Over the last twenty years, biocatalysis in organic solvents has emerged as an area of systematic research and industrial development due to chemical and pharmaceutical interest (Torres and Castro 2004). Enzymes are known to be denatured in the presence of organic solvents and their catalytic activity is significantly suppressed in comparison with aqueous media (Plou, Iborra et al. 1998). However, to date, in numerous studies it was well shown that enzymes could express catalytic activity in organic media. It is often possible to obtain catalytic activities as in the same order of magnitude as in aqueous media by selecting appropriate methods (Carrea and Riva 2008).

2.2.1. Solvent Effects on Enzymes

A hydration shell formed by water molecules which surrounds the protein molecule is essential for enzyme activation. Water molecules participate either directly or indirectly in noncovalent interactions, such as hydrogen bonding, hydrophobic and van der Waals interactions, to form the active center of the enzyme, along with its native conformation. Removal of bound water molecules from the hydration shell by organic solvent causes the disruption of the whole protein structure and denaturation of the enzyme (Gregory 1995, Plou, Iborra et al. 1998). Organic solvent can influence the enzyme by direct interaction which causes inhibition of the enzyme or conformational changes in the enzyme. Stability of the enzyme can be affected by direct interaction between solvent and enzyme as well as activity. Organic solvents can also influence the solvation of the substrates and products of the reaction catalyzed and the equilibrium

position of reactions. Another effect of organic solvents is lowering the free energy of the substrate and thereby its reactivity**.**

Enzyme activity in organic solvents depends very much on the nature of the solvent. Polarity of the solvent has a large influence on reaction rate, that is, hydrophobic solvents often provide higher reaction rates than more polar, hydrophilic solvents (Carrea and Riva 2008). Penetration of highly polar organic solvents into protein's interior reduce the local polarity near the active site, thereby deactivating the enzyme, whereas nonpolar solvents have lower capacity to remove (or 'strip away') tightly bound water molecules (Figure 2.5).

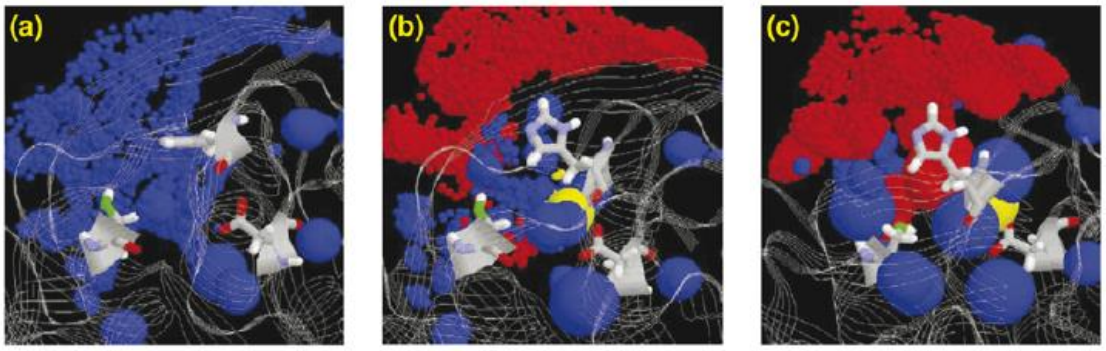

Figure 2.5. Molecular simulation of penetration of an organic solvent into an enzyme's active site. The water molecules are shown in blue and an organic solvent in red. In aqueous media (a), water molecules penetrate into the active site of the enzyme easily. In the nonpolar solvent (b), few solvent molecules penetrate into the active site, whereas, in the highly polar solvent (c), there is a significant solvent penetration into the active site as seen in the figure. (Source: Serdakowski and Dordick 2008)

Conformational flexibility af an enzyme is a crucial factor for protein function. Previous studies demonstrated that there is a correlation between conformational flexibility and increased water content of the protein. In nonpolar organic solvents, high enzyme stability has been obtained in many cases, but enzyme activity was reduced. This phenomenon was primarily attributed to the lack of water molecules, which presumably places enzymes in a restrained conformation, hence a more rigid protein molecule occurs. More rigidity provides resistance to thermal vibration, whereas enzyme-substrate interaction decreases. Therefore, rate of the reaction is reduced with conformational changes during the catalytic process. Removal of water by organic solvents can also result in dehydration of polar groups located at the protein surface

which reduces the ionic and charged form of these groups. (Torres and Castro 2004, Serdakowski and Dordick 2008).

In enzymatic reactions, the energy of binding between enzyme and the substrate is the main driving force. Binding occurs with desolvation of the substrate from the reaction media to the active center of the enzyme. When water is removed from the enzyme by an organic solvent, the substrate is no longer 'squeezed out' of the medium due to the hydrophobic effect. Hence the ground state of the substrate is stabilized in the organic solvent as compared to that in water and the activation energy of the reaction is increased. This results in lower reaction rates (Klibanov 1997).

2.2.2. Advantages and Disadvantages of Using Enzymes in Organic Media

Biocatalysis in organic solvents provides unique processing advantages as compared to that in aqueous media in some cases. There are also some disadvantages of enzymes working in organic solvents. These are listed in Table 2.2.

Table 2.2. Biocatalysts in non-aqueous media.
(Source: Castro et al. 2003, Serdakowski et al. 2008)

Advantages	Disadvantages
• Increased solubility of nonpolar substrates • Ability to catalyze certain reactions in the reverse direction as compared to that in water • Altered enantioselectivity as compared to that in aqueous media • Altered substrate specifity as compared to that in aqueous media • Lack of side reaction of hydrolysis in contrast to aqueous mileu • High efficieny of biocatalysts in water-organic mixtures • Ease of enzyme recovery by filtration or centrifugation	• Problems with recycling biocatalysts in non-covalently modified systems • Labour and cost-intensive preparation of biocatalysts in covalently modified systems • Limited activity in most pure organic solvents • Mass transfer limitations in the case of heterogeneous systems or viscous solvents • Mandatory low water content that has to be controlled

(cont. on next page)

Table 2.2 (cont.)

• Thermodynamic equilibria favors synthesis over hydrolysis • Often enhanced thermostability • Elimination of microbial contamination • Potential for enzymes to be used directly in a chemical process	

2.2.3. Organic Solvent Systems

There are three main types of organic solvent systems: (1) water + water-miscible organic solvent systems (organic cosolvent systems), (2) water + water-immiscible organic solvent systems (biphasic system), and (3) nearly dry organic solvent systems (Doukyu and Ogino 2010).

2.2.3.1. Water + Water-Miscible Organic Solvent System

Organic cosolvent systems are monophasic and have no interface between solvent and aqueous phase, thereby preventing high substrate and product concentrations around the enzyme and controlling the concentrations in the system easily. No diffusional resistance for substrates and products across the organic solvent-water interface occurs in monophasic systems, hence it can be an advantage in catalytic processes leading to high overall reaction rates. Water-miscible cosolvents are used to increase the solubility of substrates which have low solubility in aqueous medium. In this system mass-transfer limitations are substantially reduced, therefore more rapid reaction rates can be obtained for hydrophobic substrates. The disadvantage of these systems is direct contact of an organic solvent with an enzyme which causes dramatically disruption of the enzyme structure, hence rapid denaturation and inactivation. The decrease of enzyme activity in water/cosolvent mixtures can also be explained by the simultaneous occurrence of dielectric changes and competitive/noncompetitive inhibition.

2.2.3.2. Water + Water-Immiscible Organic Solvent System

This system is also called biphasic system which consists of two phases: an aqueous phase containing a dissolved enzyme and an immiscible (hydrophobic) organic solvent phase. In this system, there is an interface layer seperating the aqueous media and the solvent from each other. Enzymatic reactions occur in the aqueous phase dissolving enzyme, whereas the hydrophobic substrate is mostly located in solvent layer and partitioned in the aqueous media. The product formed in the reaction is extracted into the solvent phase during the process. There are several advantages of using enzymes in biphasic systems: shifting the reaction equilibria towards synthesis for the production of peptides and esters due to the low overall water content in the system, easy recovery of the enzyme, simple seperation of the products from the system and promoting stability of the enzyme due to preventing direct contact of the solvent with the enzyme. Enzymatic reaction rates in biphasic systems can be relatively low beacause of limited mass-transfer of molecules in the medium. Another disadvantage in these systems is denaturation and inactivation of the enzyme which sometimes occur at the interface between organic and aqueous phases.

2.2.3.3. Nearly Dry Organic Solvent Systems

In these systems, enzymes are not soluble. They can be solubilized with different methods such as lyophilization, immobilization or modification with amphipatic compounds, lipids, or suspended solid particles, or surfactants. Lyophilized enzymes often show high thermal stability, but more lower catalytic activity than in water, due to reversible disruption of the enzyme structure. Using additives such as carbohydrates, polymers, and salts in lyophilization can prevent this denaturation and activate the enzymes. Water content of the enzyme in the system is crucial in order to perform sufficient catalytic activity. Conformational flexibility of enzymes in these systems is restricted at such low water content, hence the enzymes have more rigidity in the system as compared to that in aqueous solution. This rigid structure results in unique substrate specificities and therefore some techniques can be used such as molecular imprinting (Ogino and Ishikawa 2001, Doukyu and Ogino 2010).

2.2.4. Stabilization of Enzymes in Non-Aqueous Media

It is important to understand the mechanism of enzyme inactivation for characterization of enzyme stability in organic solvents. Therefore, it will be easier to control over the deactivation process and stabilization approaches in solvent systems. In order to prevent processes in enzyme inactivation and improve enzyme activity and stability in non-aqueous media, different strategies were employed. These are: (a) isolation of naturally stable enzymes from extremophiles; (b) genetic manipulation to obtain stable enzymes; and (c) stabilizing existing enzymes by protein engineering (site--directed mutagenesis and directed evolution), covalent attachment of amphipathic compounds (PEG, aldehydes and imidoesters), non-covalent interaction with lipids or surfactants, entrapment in water-in-oil microemulsions or reverse micelles, utilization of solid enzymes (lyophilized enzyme powders and cross-linked crystals suspended in organic solvents), addition of additives and immobilization on appropriate insoluble supports (synthetic polyhydroxylic matrices, porous inorganic carriers, polymers and molecular sieves) (Torres and Castro 2004, Iyer and Ananthanarayan 2008).

Currently, immobilization of enzymes is one of the main industrial applications of non-aqueous enzymology. Immobilized enzymes are heterogeneous biocatalytic systems due to a visible seperation of phases. Immobilization is generally performed for optimum activity in non-aqueous media. Confinement of enzymes by immobilization method usually enhances accessibility by molecular dispersion and stability of the enzymes against denaturation by the organic solvents (Krieger, Bhatnagar et al. 2004, Torres and Castro 2004, Sheldon 2007).

2.3. Immobilization of Enzymes

Immobilization is a method of keeping the enzymes confined in a certain defined region of space to preserve their catalytic activity (Romaškevič, Budrienė et al. 2006). It is a useful technique to facilitate recovery and reuse of the biocatalysts. In industry, very stable enzymes can be prepared via immobilization for multiple reuses of them in catalytic reactions (Guisan 2006). Use of the immobilized enzymes also reduces production costs by efficient recycling and control of the process (Cao 2006).

Enzyme immobilization methods can be mainly subdivided into two groups: physical immobilization, where weak interactions between support and enzyme exist, and chemical immobilization, where covalent bonds with enzyme are formed (Krajewska 2004). Some immobilization techniques are listed in Table 2.3.

Table 2.3. Immobilization methods.
(Source: Krajewska 2004)

Physical Immobilization	**Chemical Immobilization**
Entrapment of the enzyme molecules	Enzyme attachment to the matrix by covalent bonds
Micro-encapsulation with a solid or liquid membrane	Cross-linking between enzyme and matrix
Adsorption on a water-insoluble matrix	Enzyme cross-linking by multifunctional substances

Enzyme attachment to the support material by covalent bonds is one of the most widely used methods in immobilization of enzymes. The functionality of the carrier and/or the enzyme must be activated before their use for an efficient binding. The covalent bond is created through the reaction between electrophilic (electron deficient) groups on the support and strong nucleophiles (electron donating) on the protein surface (e.g., NH_2 or OH groups). A strong interaction occurs between the enzyme and the carrier via covalent attachment, therefore the stability of the enzyme is increased. In some cases, this strong interaction may limit the conformational flexibility of the enzyme, thereby reducing catalytic activity (Cao 2006, Guisan 2006, Romaškevič, Budrienė et al. 2006).

2.4. Polyurethane Foam for Immobilization

Different structures can be used for enzyme immobilization and among these, polyurethane (PU) could be one of the best carrier for this purpose. PU foams are porous materials with microcellular structures. There are some advantages of using PU foams in immobilization: easy control of the pore size, efficient stability of enzymes and large-scale application at low price (Romaškevič, Budrienė et al. 2006). Wood et al. developed a method of covalent attachment of enzymes into isocyanate-capped PU in

1982 (Wood, Hartdegen et al. 1982). Today, their technique is still used in enzyme immobilization. In this method, a HYPOL prepolymer is mixed with the aqueous enzyme solution to achieve the immobilization (Ozdemir 2009). The polymerization of the HYPOL prepolymer in the presence of water and the mechanism of immobilization of enzymes into PU foam are presented in Figure 2.6. The polymerization is initiated by intimate contact of water, which is introduced with the enzyme solution, with isocyanate groups present within the HYPOL prepolymer. In the first step, prepolymer is exposed to a nucleophilic attack by an OH^- at the carbonyl group following a protonation and deprotonation to form the unstable intermediate. In the second step, the unstable intermediate degrades to an amine group yielding CO_2. The produced amine groups readily react with isocyanate groups, resulting in cross-linked prepolymer chains. Consequently, the produced CO_2 causes a porous and sponge-like matrix of the PU foam. Because an enzyme contains amine and/or hydroxyl groups, which can react with isocyanates, the enzyme becomes an integral part of the foam during polymerization at the same time. This process is relatively faster and higher activity retention could be achieved (LeJeune and Russell 1996, Bakker, van de Velde et al. 2000, Romaškevič, Budrienė et al. 2006, Ozdemir 2009).

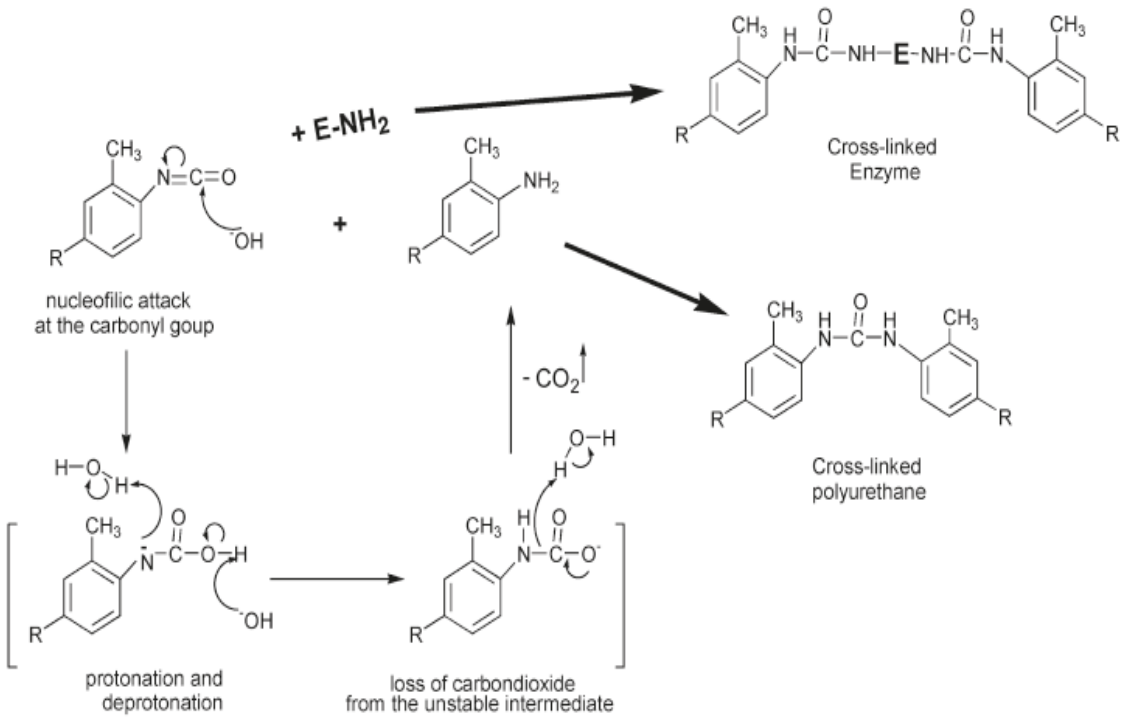

Figure 2.6. Enzyme immobilization by cross-linking within PU foam.
(Source: Ozdemir 2009)

2.5. Carbonic Anhydrase and Esterase Activity

Carbonic anhydrase (CA) was selected as the model enzyme for this thesis. Carbonic anhydrase is a zinc metalloenzyme that mainly catalyzes the reversible hydration of CO_2 to form bicarbonate (Zhang, Zhang et al. 2011). In living organisms, CA catalyzes the reaction of the interconversion of the carbon dioxide and the bicarbonate ion in physiological processes (Supuran, Scozzafava et al. 2004). However, the CAs can catalyze hydrolysis of esters and dehydration of various aldehydes (Whitney 1970).

There are at least five distinct classes of CA: α-CA, β-CA, γ-CA, Ɛ-CA and δ-CA (Savile and Lalonde 2011). CAs exist in a wide range of isoforms in animals, plants, bacteria and a variety of eukaryotic algae (Cox, McLendon et al. 2000). All mamalian cells have α-CA and 16 different isozymes of α-CA have been identified in mammals. At least ten of these isoenzymes belong to humans.

The three-dimensional structure of a bovine carbonic anhydrase (BCA), the isozyme used in the work for this thesis, is represented in Figure 2.7. BCA contains 259 amino acid residues forming a single polypeptide chain with a molecular weight of ~29000. BCA has mainly two structural components: a central twisted β-sheet consisting 13 β–strands and seven short α-helices surrounding β –sheets. The active site is situated in a large conical cleft with a Zn^{2+} ion as the reaction center. Zn^{2+} ion is coordinated by three histidine residues which are located in the middle of the β-sheet. Finally, the tetrahedral coordination geometry is completed with a H_2O molecule around the zinc ion. The amino acid sequence of CA has 18 lysine groups, mostly bound to the surface of the enzyme. Enzyme-efficient immobilization is achieved by these lysines containing amine groups (Ohta, Alam et al. 2004, Höst 2007, Ozdemir 2009).

There are some limitations to estimate accurately the enzymatic activity of CA in the hydration reaction of CO_2 (Ozdemir 2009). CA catalyses the hydrolysis of phenolic esters as well as reversible hydration of CO_2 and this activity has been utilized by several investigators in their studies. Phenolic esters offer some advantages as being substrates: more easily handled than CO_2 and the reaction rates can be measured by simple spectrophotometric methods.

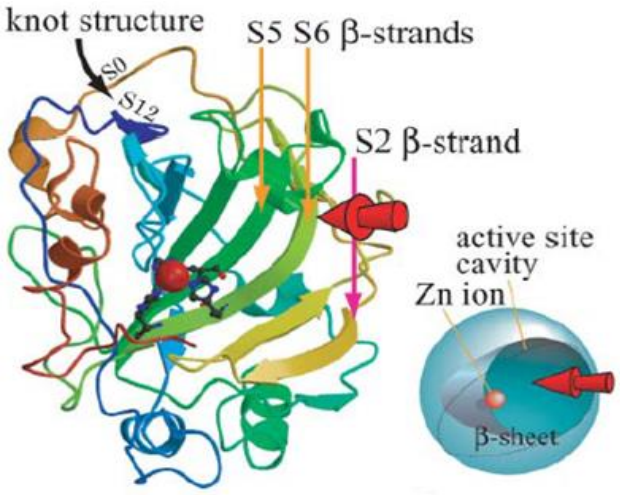

Figure 2.7. The three-dimensional structure of bovine carbonic anhydrase.
(Source: Ohta, Alam et al. 2004)

Hydrolysis rates of nitrophenyl esters, catalyzed by bovine CA, varies due to the position of the nitro group and the size of the acyl residue. Experimental studies exhibited that the most rapidly hydrolyzed phenolic substrate for Bovine CA is *para*-nitrophenyl acetate (*p*-NPA). In aqueous media, hydrolysis of *p*-NPA is initiated by the nucleophilic attack of water (or hydroxide ions) to the central atom of the substrate and a powerful activation of water by the zinc ion from the enzyme's active site cavity occurs due to the hydrophobic environment of the protein. Consequently, CA effectively hydrolyzes *p*-NPA. Figure 2.8 shows the enzyme-catalyzed hydrolysis of *p*-NPA, which yields *para*-nitrophenol (*p*-NP) and acetic acid (Thorslund and Lindskog 1967, Innocenti, Scozzafava et al. 2008).

For the reasons mentioned above, in this study, *p*-NPA was selected as the substrate instead of gaseous CO_2 for the free and immobilized CA enzyme activity determination in water/cosolvent mixtures.

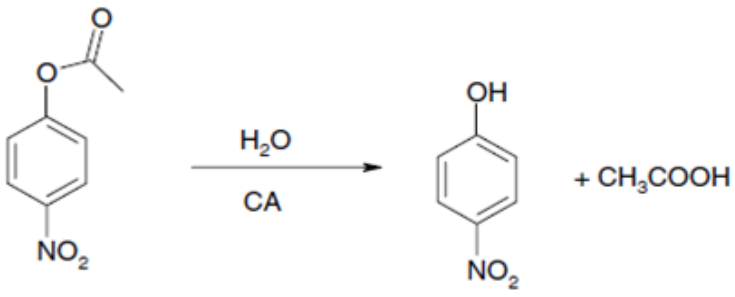

Figure 2.8. Hydrolytic reaction of *p*-NPA in the presence of CA.
(Source: Innocenti, Scozzafava et al. 2008)

2.6. Experimental Studies on Enzymes' Behaviour in Organic Solvents

To date, many studies have been conducted to investigate the biocatalytic behaviour and stability of different enzymes in organic solvents. Mozhaev et al. studied the catalytic performance and denaturation mechanism of α-chymotrypsin and laccase in different water/organic cosolvent mixtures (Mozhaev, Khmelnitsky et al. 1989). They have suggested that abrupt fall in enzyme activity at a critical concentration of organic solvent due to protein denaturation was a general phenomenon occuring in water-cosolvent mixtures. They indicated that conformational changes (denaturation) of the enzymes caused the inactivation. They also have shown that the loss of α-chymotrypsin activity due to the organic cosolvent penetration was completely reversible and a complete regeneration of the catalytic performance was achieved after dilution of a 70%, by vol. 1,4-butanediol solution with aqueous buffer to 60%, by vol. They reported that there was a linear correlation between the critical water residues on the enzyme's surface and the hydrophobicity of the organic cosolvents used in the system.

Verma and Ghosh studied the effects of different organic solvents (acetonitrile, dimethylformamide, methanol, ethanol, dimethyl sulfoxied, ethlyene gycol, propan-2-ol and *tert*-butanol) on the α-chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate (*p*-NPA) and *p*-nitrophenyl benzoate (*p*-NBA) at pH 7.75 using a cationic surfactant (Verma and Ghosh 2010). In their study, *p*-NPA and *p*-NBA were prepared in 14 % (v/v) organic solvents and all reactions were conducted spectrophotometrically by observing the appearance of *p*-nitrophenoxide ion at 400 nm. They indicated that enzyme activity was sensitive to type of organic solvents used. The results have shown that the enzymatic activity decreased dramatically as the polarity of the organic solvent was increased. They noted that the hydration water content that was available for solvation of an enzyme was an important factor in enzyme activity. It was suggested that the solvation water could affect catalytic activity of the enzyme by changing its conformational flexibility or by effecting its active site hydration. They also reported that the hydration water was stripped from the enzyme surface to different extents by organic solvents depending on the solvent polarity.

Yang et al. investigated the solvation of the enzyme subtilisin BPN′ in three different organic solvents (*n*-octane, tetrahydrofuran, and acetonitrile) and hydration of the enzyme and its active site (Yang, Dordick et al. 2004). They indicated that

acetonitrile molecules could penetrate the farthest into the enzyme, followed by tetrahydrofuran, and then *n*-octane, depending on the polarity of the solvents. They also noted the penetration of acetonitrile molecules was presumably aided by its relatively smaller size. They reported that the enzyme surface and its active site region were well hydrated in aqueous solution, however with increasing polarity of the organic solvent (octane ⟶ tetrahydrofuran ⟶ acetonitrile) the hydration water was removed from the enzyme surface by the penetration of the solvent molecules into the active site of the enzyme.

Micaelo and Soares studied the hydration mechanism of the enzyme protease cutinase in non-polar (hexane, di-isopropyl ether, 3-pentanone) and polar (ethanol, acetonitrile) organic solvents using molecular dynamics simulations (Micaelo and Soares 2007). In their study, it was clearly seen that in polar organic solvents, the amount of water bound at the enzyme surface was very low as compared to that in nonpolar solvents due to the nature of the organic solvent. They reported that nonpolar solvents enhanced the formation of large clusters of water that were tightly bound to the enzyme, whereas water in polar organic solvents was fragmented into single water molecules and small clusters of water molecules around the protein.

Griebenow and Klibanov investigated the secondary structure of lysozyme in various water-acetonitrile mixtures using Fourier-transform infrared (FTIR) spectroscopy (Griebenow and Klibanov 1996). In order to quantify the α-helices of the enzyme's secondary structure, amide I and amide III spectral regions were used in FTIR analysis. It was found that the α-helice content decreased as the percentage of acetonitrile in water increased up to 60% (v/v), but then increased at higher percentages of acetonitrile (beyond 60%). At 0-60% (v/v) acetonitrile, the enzyme was prepared in dissolved form, however, at higher acetonitrile contents in suspended form. It was demonstrated that the α-helix content of the protein increased at 60-90% (v/v) acetonitrile, because the fraction of the dissolved (and more denatured) protein decreased. They indicated that dissolved protein was more prone to denaturation than the suspended protein due to the loss of stabilizing protein-protein contacts. They finally noted that enzymes could be more catalytically active in neat organic solvents than in aqueous-organic mixtures due to their structural rigidity in such media (compared to water), resulting in high kinetic barriers preventing unfolding of the enzyme.

Castillo et al. studied the activity loss of various hydrolases in a range of organic solvents (Castillo, Pacheco et al. 2005). In their study, the results showed a roughly

exponential activity decrease for the enzymes which were used in different organic solvents. The enzymes studied also exhibited similar low storage stability on organic solvents. They suggested that there was only limited influence of the solvent, the nature of the enzyme itself, and the enzyme preparation method in deactivation process. They concluded that inactivation of enzymes in pure organic solvents was due to a breakdown of the catalytic machinery or a change in the pronotation state of active site residues.

Simon et al. examined the effects of different water-miscible organic solvents (ethanol, acetonitrile, 1,4-dioxane) on the conformational stabilities of various hydrolytic enzymes (trypsin, carboxypeptidase A, chymotrypsin and lipase) in buffered aqueous solution (Simon, Laszlo et al. 1998). These three organic solvents caused only slight decreases in the activites of trypsin and CPA. However, the activities of chymotrypsin and lipase decreased dramatically with increasing concentrations of organic solvents up to 40-50%, but at higher concentrations the enzymes exhibited increasing activity. They suggested that the ability of the water-miscible organic solvents to strip away the water molecules from the enzyme caused a reduction in activity at certain concentrations, but at higher solvent concentrations the properties and interactions of the solvents might contribute significantly to the preservation of the catalytic activities of the enzymes. They also noted that the alterations in stability of enzymes in organic solvents were greatly affected by the individual structures of the enzymes.

Partridge et al. studied the stability of α-chymotrypsin in aqueous-acetonitrile mixtures in order to determine whether the native enzyme was thermodynamically or kinetically stable under low water conditions (Partridge, Moore et al. 1999). In the experiment, high catalytic activities were obtained at high water levels, but in 50% acetonitrile, it lost all its activity after 10 minutes. However, at higher acetonitrile percentages the enzyme exhibited significant catalytic activity. After this assay, they investigated whether the denatured enzyme in 50% acetonitrile could be renatured by adding water or more acetonitrile. They observed full regeneration of catalytic activity of the fully denatured enzyme when a solution of α-chymotrypsin in 50% acetonitrile was diluted with aqueous buffer to 5% by volume. However, recovery of the enzyme's catalytic properties could not be attained on addition of acetonitrile (to 70% v/v) to the fully denatured enzyme in 50% acetonitrile. These results demonstrated that denaturation of α-chymotrypsin is thermodynamically reversible by addition of water. In contrast, it was thermodynamically irreversible in the case of adding more

acetonitrile. They also investigated the stability of the enzyme in 70% acetonitrile. It was found that 70% of the enzyme remained intact even after incubating for 3 h. They concluded that α-chymotrypsin is kinetically stable at low water content in acetonitrile.

Zhu et al. investigated the effects of acetonitrile on γ-chymotrypsin with the inclusion of crystal waters using combined molecular dynamics simulation with quantum mechanics calculation (Zhu, Yang et al. 2012). The results showed that the acetonitrile molecules penetrated into the enzyme's active site would give rise to a weakness in the strength of the catalytic H-bond networks. They also reported that the drop in the catalytic activity in the presence of acetonitrile might be associated with the lower flexibility and the increased proton-transfer barrier.

Sirotkin and his co-workers studied solubility and secondary structure of bovine pancreatic α-chymotrypsin in acetonitrile-water mixtures (Sirotkin, Zazybin et al. 2000). In the light of the results, they suggested that the changes in α-chymotrypsin solubility and secondary structure in water-acetonitrile mixtures could be explained as a result of two main factors: disruption of three dimensional hydrogen bond network of water molecules leading to weakening of hydrophobic interactions, and reduction of conformational motility of the protein molecule in water-poor media.

Safarian et al. investigated the effects of acetonitrile on the structure and function of bovine carbonic anhydrase II (Safarian, Saffarzadeh et al. 2006). The potential structural alterations in carbonic anhydrase was determined in the presence of different acetonitrile/buffer ratios . The results exhibited that the increase in acetonitrile content in the mixture was followed by a decrease in enzymatic rate, especially at 47.5% acetonitrile. They suggested that this could be due to the tertiary structural alterations of carbonic anhydrase and the reorientation of residues near the active site of the protein, thus resulting in a decrease in enzyme activity. In order to evaluate the possible structural changes of the enzyme, three critical points (0%, 17.5% and 47.5% v/v), which represented the sharp decline in the enzyme velocity, were selected from among the acetonitrile/buffer ratios. It was reported that the presence of acetonitrile in the medium had minimum effect on the secondary structure of carbonic anhydrase. However, thermal stability of the enzyme in the presence of acetonitrile was drastically decreased due to a rigorous decline in the melting temperature of the enzyme, especially at 47.5% acetonitrile, which was consistent with the results observed in the enzymatic rate changes. They suggested that the existence of acetonitrile in the medium caused a

considerable lowering of the dielectric constant of water and weakening of hydrophobic interactions, thus decreasing structural stability of the enzyme.

2.7. Immobilized Enzymes in Water-Miscible Organic Solvents

Costas et al. examined the effects of various water-miscible organic solvents on free and immobilized lipase in pectin microspheres (Costas, Bosio et al. 2008). Lipase was encapsulated into pectin hyrogel beads via cross-linking with calcium ions. Free lipase was tested at 0% to 90% concentrations of water-miscible organic solvents (diethylenglycol, glycerol, 1,2 propanediol and dimethylsulfoxide) for 1 to 12 h of incubation. After 12 h of incubation, stability of the enzyme decreased about 20% in all organic solvents, except DMF and DMSO. In the presence of these two solvents, the stability was reduced drastically at higher percentages. On the other hand, immobilized lipase was studied to test the water-miscible solvents at 50% concentration. It was demonstrated that in the immobilized system, the lipase activity was significantly enhanced or preserved even after 12 h of incubation.

Wan et al. studied the effects of organic solvents on the activity of free and immobilized laccase (Wan, Lu et al. 2010). Laccase was covalently immobilized onto chitosan by chemical derivatisation. Free and immobilized laccase activity was measured in triplicate spectrophotometrically using 2,6-dimethoxyphenol as substrate. In this study, the relative acitivities of free and immobilized laccase in a range of water/water-miscible organic solvent mixtures were examined as a function of increasing water content. It was demonstrated that with water-miscible organic solvents, in general a water content of ~20-50% (v/v) was required to achieve activity using free laccase, whereas with immobilized laccase less water was generally required to achieve enzyme activity. Hence, substantially higher enzyme activity was exhibited for immobilized laccase at lower water contents, compared with free laccase. They suggested that microenvironment of the enzyme immobilized on chitosan provided additional stability in preserving the active enzyme conformation.

Olofsson et al. examined the influence of a range of water-miscible organic solvents (methanol, ethanol, 1-propanol, 2-propanol, acetonitrile, *N,N′*-dimethylformamide and tetrahydrofuran) on the activity of α-chymotrypsin in solution and immobilized on Eupergit CM (Olofsson, Soderberg et al. 2006). The covalent attachment of the enzyme to solid matrice was achieved by mixing the enzyme in buffer

containing Eupergit CM (an epoxy-activated microporous acrylic microbead support). After 24 h of incubation, an effective obliteration of activity for free α-chymotrypsin was determined in all organic solvent/buffer mixtures (50%, v/v). However, in the case of immobilized α-chymotrypsin, enzymatic activities after 24 h exposure were significantly higher than for free enzyme in the corresponding solution, about 10- to 50-fold. They also studied the effect of the concentration (0-95%, v/v) of three organic solvents, acetonitrile, *N,N′*- dimethylformamide and ethanol, on both free and immobilized α-chymotrypsin using 6 h pre-incubation periods. It was demonstrated that in the case of free enzyme, activities diminished at 40-50% (v/v) concentrations of solvents, whereas the immobilized α-chymotrypsin achieved measurable activities in up to 90% (v/v) acetonitrile, 60% (v/v) *N,N′*- dimethylformamide and 60% (v/v) ethanol.

Azevedo et al. studied enzymatic activity and stability in aqueous–organic co-solvent mixtures, using horseradish peroxsidase (HRP) both free in solution and immobilised onto silica microparticles (Azevedo, Prazeres et al. 2001). Both free and immobilised HRP was tested at 50ºC in aqueous mixtures of 3.5, 20, 35 and 50% (v/v) DMSO. It was found that stability of free HRP was not affected by the presence of 3.5 and 20% DMSO, but a severe decrease in stability was observed for higher contents. The half-life of immobilised HRP increased more than 300% when changing from buffer to 20% DMSO, however, at higher organic solvent contents, the enzyme half-life was decreased. It was also demonstrated that the stability of immobilised HRP was higher than that of the free form in all aqueous mixtures of DMSO.

O'Daly et al. investigated the effects of different organic solvents (ethanediol, acetonitrile, and dichloromethane) on the activity and stability of free and immobilized bovine carbonic anhydrase (BCA) (Odaly, Crumbliss et al. 1990). In their study, BCA was immobilized on porous silica beads by covalent attachment via a spacer arm. The catalytic activity of the enzyme was determined by measuring the rate of hydrolysis of *p*-NPA. The results exhibited that catalytic activity of both free and immobilized BCA decreased as the solvent was changed from 1.0-mol fraction aqueous buffer to 1.0-mol fraction organic solution for all three organic solvents. It was shown that immobilized BCA retained its activity in aqueous/organic solvent mixtures to a greater extent than free BCA. In water/ethanediol mixtures, the immobilized enzyme maintained activity up to nearly anhydrous conditions, whereas the free enzyme lost all of its activity. In water/acetonitrile mixtures, free BCA had no activity in mole fractions above 0.34, while immobilized BCA had significant activity up to 1.0-mole fraction acetonitrile. It

was also found that immobilization on silica beads significantly enhanced the storage stability of BCA in organic solvents. After storage in ethanediol or acetonitrile for 24 h, free BCA regained only 15% of its original activity in water, while immobilized BCA showed 77% of its original activity. In the case of dichloromethane, immobilized BCA retained 22% of its original activity in water, while free BCA was totally inactivated after storage for 24 h.

In the present study, it was aimed to investigate the effects of water-miscible organic solvents on the activity and stability of the free and immobilized CA enzyme within PU foam.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Carbonic Anhydrase (CA) from bovine erythrocytes (MW, 29000; 89% pure in protein as dialyzed and lyophilized powder), *para*-nitrophenyl acetate (*p*-NPA), *para*-nitrophenol (*p*-NP) were all purchased from Sigma-Aldrich. Acetonitrile (99.9%, v/v), ethanol (99.9%, v/v), and hydrochloric acid (35%, v/v) were purchased from Merck. Polyurethane prepolymer, HYPOL-2060, was provided as a kind gift from Dow Chemical Co., Turkey.

Equipments used were UV/vis spectrophotometer (Perkin Elmer Lambda 45), humidity meter (COMET H3531P), magnetic stirrer (LABART SHT-5), and pH meter (Thermo Electron Corporation, Orion 5 Star).

3.2. Determination of the Water Holding Capacity of PU Foam

Hydrophilicity of immobilizing carriers is an important factor for enzyme immobilization and efficient enzymatic reactions in organic media. For this purpose, water holding capacity of the PU foam was determined by a simple experimental study. Firstly, PU foam was synthesized. Briefly, 3 mL of ultra pure water was poured onto about 3 g of HYPOL-2060 prepolymer in a 50 mL falcon tube. The two-phase system was mixed vigorously for 30 s by the help of a mixer drill to achieve a homogeneous mixture. When the mixing was settled, the level of the white polymeric solution started to rise as a result of CO_2 release. After the polymerization, 10 min were allowed for curing. After synthesis of the PU foam, different weights of foam pieces were cut from the whole product. The foam pieces were soaked and squeezed several times in ultra pure water and then the soaked foam pieces were weighed to determine their wet weight. Finally, the soaked foams were dried in an oven at 105˚C overnight to determine their dry weight.

3.3. Moisture Adsorption on PU Foam

In order to determine the adsorption isotherm of moisture on PU foam, different saturated salt solutions were prepared in glass jars with respect to their equilibrium relative humidities. Selected salts were: Potassium sulfate (97.6%), Potassium nitrate (94.62%), Potassium chloride (85.11%), Sodium chloride (75.47%), Magnesium nitrate hexahydrate (54.38%), Potassium carbonate (43.16%), Magnesium chloride hexahydrate (33.07%), Potassium acetate (23.11%), and Lithium chloride (11.31%). PU foam was synthesized and then five different weights of foam piece were cut from the middle of the whole product and assayed for each incubation in the presence of the saturated salt solution. Foam pieces were incubated in closed glass jars, which contain the saturated salt solution, for 4-7 days at ambient temperature. Foams were weighed at 1 h intervals for the first 4 hours, then at 24 h intervals for 4-7 days until the weight of foam pieces became constant. Finally, foams were dried in an oven at 105ºC overnight to determine their dry weight. The relative humidity of each closed system containing saturated salt solution was also measured with the humidity meter (COMET H3531P) for the calibration.

3.4. Characterization of *p*-NP

p-NP is one of the hydrolysis products of *p*-NPA. A 0.0146 g sample of *p*-NP was dissolved in 10 ml of Tris buffer (50 mM, pH 7.72) to establish a stock solution. *p*-NP solutions with concentrations of 0.08, 0.06, 0.04, 0.02, and 0.01 mM were prepared by dilution of the stock solution. Afterwards, these solutions were scanned between 200 nm and 600 nm in the UV/vis spectrophotometer to establish a plot of absorbance with respect to wavelength. The absorbance scanning for *p*-NP in Tris buffer (50 mM) with different pHs (8.86, 8.38, 7.82, 7.35, 6.84) was also performed in the UV/vis spectrophotometer keeping the *p*-NP concentration in the solutions constant.

3.5. Determination of the Absorbance Profiles for *p*-NP in the Presence of the Organic Solvents

Two water-miscible organic solvents, acetonitrile and ethanol, were used for the enzyme activity assays in this study. The absorbance profiles of *p*-NP in acetonitrile/buffer and ethanol/buffer mixtures were established, respectively, by scanning the absorbance of *p*-NP between 200 nm and 800 nm in the UV/vis spectrophotometer. The stock solution of *p*-NP was used for dilution. The final concentrations of *p*-NP in the acetonitrile/buffer and ethanol/buffer mixtures were 104.95 μM and 42.55 μM, respectively. The volume percents of the organic solvents in the mixtures ranged from 10% to 90%. These measurements were also carried out for acetonitrile/buffer mixtures keeping the Tris concentration (50 mM) in the mixtures constant.

3.6. Determination of pH Values for Different Concentrations of the Organic Solvent

The pH values of organic solvent/buffer mixtures were measured in the pH meter in order to determine whether pH changed with various organic solvent concentrations. The mixtures were prepared by mixing the organic solvents with Tris buffer (50 mM, pH 7.72) at various ratios ranging from 10% to 90% (v/v). These measurements were also carried out keeping the Tris concentration constant at 50 mM.

3.7. Self-Hydrolysis of *p*-NPA in the Presence of the Organic Solvents

The absorbance scanning for self-hydrolysis of *p*-NPA was conducted between 200 nm and 600 nm in the UV/vis spectrophotometer at different concentrations of the organic solvent while keeping the *p*-NPA concentration in the mixtures constant. A 0.0666 g of *p*-NPA was dissolved in 6 ml acetonitrile and a 0.0462 g of *p*-NPA was dissolved in 10 ml of ethanol. The final concentrations of *p*-NPA were 2.677 mM and 2.55 mM in the acetonitrile/buffer and ethanol/buffer mixtures, respectively. The self-hydrolysis of *p*-NPA was also estimated in Tris buffer containing 10% acetonitrile with

various pHs (8.86, 8.38, 7.82, 7.35, 6.84, 6.29, 5.78, 5.26) in order to determine the effect of pH change on the self-hydrolysis rate.

3.8. Calibration Curves

Calibration curves for *p*-NP were prepared in the presence of the organic solvents after the absorbance values of *p*-NP samples were measured in the UV/vis spectrophotometer at 400 nm and 404 nm for acetonitrile/buffer and ethanol/buffer mixtures, respectively. The absorbances were determined in various percent volume organic solvent/buffer mixtures (10-90% v/v). The stock solution of *p*-NP was diluted to five different concentrations for each organic solvent/buffer mixture. At the end of the measurements, the calibration curves were established as concentration versus absorbance.

3.9. Immobilization of Carbonic Anhydrase within PU Foam

A known amount of CA in powder was dissolved in 4 mL of ultra pure water and poured onto about 4 g of HYPOL-2060 prepolymer in a 50 mL falcon tube. The two-phase system was mixed vigorously for 30 s at room temperature by the help of a mixer drill to achieve a homogeneous mixture. When the mixing was settled, the level of the white polymeric solution started to rise as a result of CO_2 release. After the polymerization, 10 min were allowed for curing.

3.10. Enzyme Activity Assays

The activities of free and immobilized enzyme were measured at 25ºC by monitoring the changes in the concentration of *p*-NP. Enzyme activities for both free and immobilized enzyme were estimated in acetonitrile/buffer and ethanol/buffer mixtures of varying compositions (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% organic solvent v/v). For the free enzyme, the activity assay was carried out in a 1 ml UV quartz cuvette. A known amount of *p*-NPA was dissolved in the organic solvent similarly, the powder enzyme was dissolved in Tris buffer (50 mM, pH 7.72). The substrate (0.1 mL of *p*-NPA solution) and the enzyme concentration (0.1 mL of enzyme

solution) were kept constant in the reaction mixtures. Before the activity assays, blank run was monitored to estimate the self-hydrolysis of *p*-NPA in each mixture. The reaction mixture was mixed in the cuvette by the help of a micropipet. The enzyme activity was measured in the UV/vis spectrophotometer at 400 nm and 404 nm for 2 minutes in the acetonitrile/buffer and ethanol/buffer mixtures, respectively. Effect of substrate concentration on the enzymatic rate was also estimated in the presence of 10% acetonitrile v/v. For this purpose, a 0.0603 g sample of *p*-NPA was dissolved in 4ml of acetonitrile. Afterwards, a 0.0053 g sample of CA was dissolved in 3 ml of Tris buffer (50 mM, pH 7.72). *p*-NPA solution was diluted to different concentrations. After the solutions were prepared, the CA activity test was carried out. A 0.8 ml of tris buffer, 0.1 ml of CA solution, and 0.1 ml of *p*-NPA solution was mixed in the cuvette and absorbance of the solution was measured in the UV/vis spectrophotometer at 400 nm for 2 minutes. Consequently, different concentrations of *p*-NPA (8.321, 5.547, 4.165, 2.774, 2.08, 1.664, 1.04, and 0.832 mM) were tested in the assays.

The activity assays for the immobilized CA enzyme were performed in a small batch reactor containing 20 mL reaction mixture. Firstly, blank experiment was conducted by mixing a 0.5 mL of substrate solution (*p*-NPA dissolved in the organic solvent) with 19.5 mL of buffer/organic solvent mixture on a magnetic stirrer to estimate the self-dissociation of *p*-NPA in each reaction mixture. Afterwards, the reaction was initiated by adding the enzyme immobilized foam into the mixture. In every minute, 1 mL of sample was withdrawn from the reaction mixture and its absorbance was measured in the UV/vis spectrophotometer at 400 nm and 404 nm for acetonitrile/buffer and ethanol/buffer mixtures, respectively, and the sample was returned back into the reaction mixture in order to keep the reaction mixture's volume constant. This procedure was repeated for each buffer/organic solvent mixture at 1 min intervals for 20 min. At the end of the assays, the foam samples were dried in an oven at 105ºC over night to determine their dry weights. Seperate foam pieces were prepared for each buffer/organic solvent mixture to determine the immobilized enzyme activity. Before adding the foam into the substrate-containing mixture, the foam piece was squeezed several times in the organic solvent/buffer mixture containing the same concentration of the organic solvent in the reaction mixture in order to achieve an efficient mass transfer within the foam. After an assay was performed, a fresh reaction mixture was prepared for the next assay.

3.11. Stability Tests

Storage stabilities of the free and the immobilized CA enzyme within PU foam were determined in various percent volume organic solvent/buffer mixtures (10-90%). Stock solution of *p*-NPA was prepared in both acetonitrile and ethanol. In the case of free enzyme stability test, a 0.005 g sample of powder CA was dissolved in 5 ml of Tris buffer (50 mM, pH 7.72). Aliquots from the storage mixtures were used in the free enzyme activity assay at given time intervals. Activity assays were conducted in Tris buffer (50 mM, pH 7.72) containing 10% organic solvent. The concentration of *p*-NPA (~2.6 mM) was also kept constant in the reaction mixtures.

The stability of the immobilized CA was estimated using separate foam pieces stored in the buffer/organic solvent mixtures at room temperature. Before the activity tests, foam samples were washed by squeezing several times in the buffer/organic solvent mixtures containing the same concentration of the organic solvent in the reaction mixture. The activity was determined after returning the enzyme immobilized foam from the storage mixture to Tris buffer (50 mM, pH 7.72) containing 10% organic solvent. At the end of enzyme activity, foam samples were washed in ultra pure water and stored in the buffer/organic solvent mixtures until the next activity assay. Enzyme immobilized foams taken from the storage mixtures were used at given time intervals for the activity assays.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Water Holding Capacity of the PU Foam

It was expected that the PU foam had a hydrophilic nature and this property made the PU foam advantageous for enzyme immobilization, where the substrates or the products could easily diffuse in and out of the sponge containing the enzyme. High hydrophilicity also could provide a suitable environment for enzyme catalysis in the presence of organic solvents. Figure 4.1 shows the water holding capacity of the PU foam with respect to different weight of foam pieces. It was found that PU foams could hold up to about 12 times their weight in aqueous media and as seen in the figure, this capacity was nearly the same for different weights. This result indicated that the PU foam had a high water holding capacity, and hydrophilic.

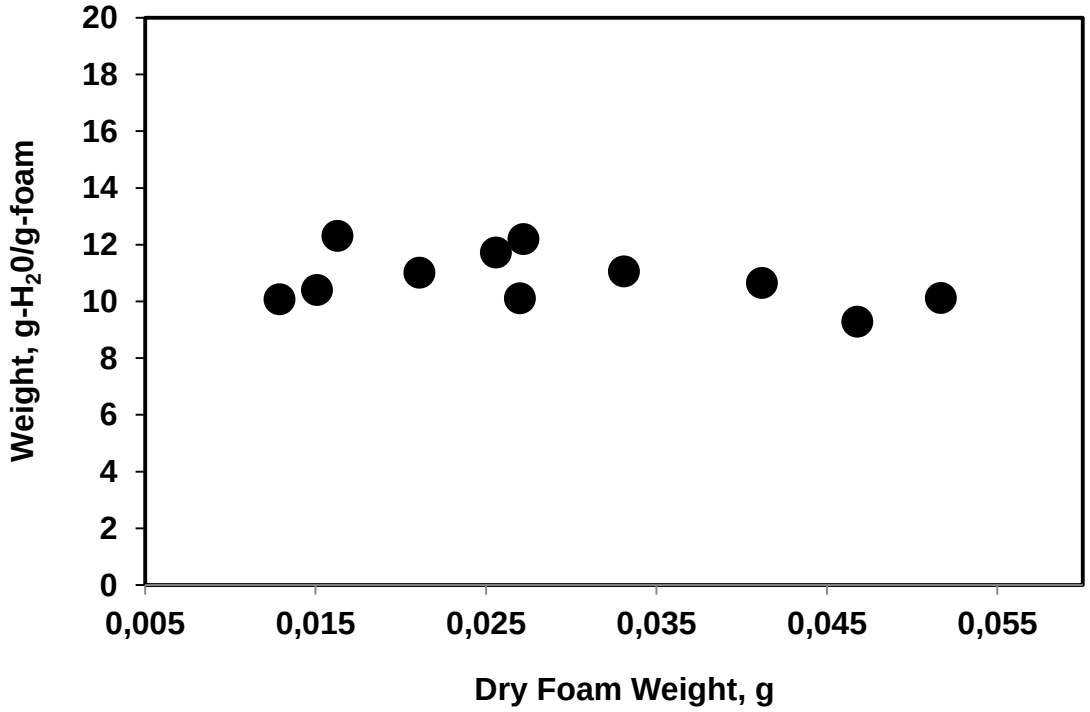

Figure 4.1. Water holding capacity of the PU foam.

4.2. Adsorption Isotherm of Moisture on the PU Foam

The phenomenon of adsorption is principally an attraction of adsorbate molecules (a gaseous or liquid component) to an adsorbent surface (a porous solid) (Crittenden and Thomas 1998). Moisture adsorption on PU foam was experimentally studied to determine the adsorption isotherm of moisture on the PU foam. Before the experimental study, the relative humidity of each closed system containing saturated salt solution was measured with the humidity meter for the calibration. Figure 4.2 shows the calibration curve between theoretical and measured relative humidities of saturated salt solutions. As shown in the figure, there is a good correlation between theoretical and measured relative humidity values.

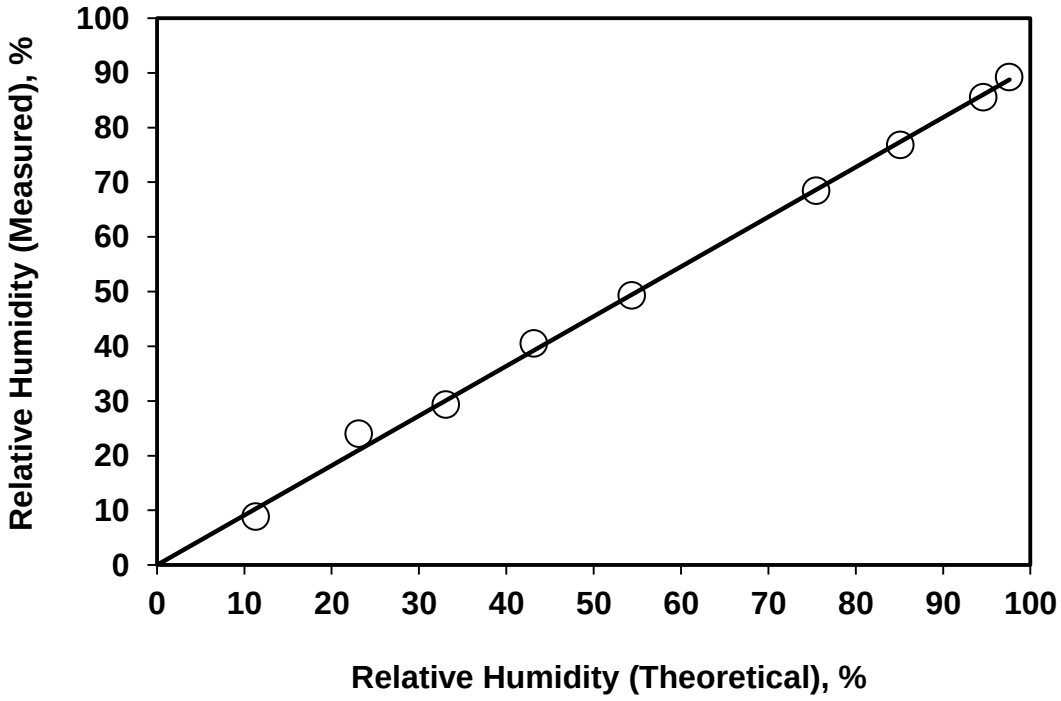

Figure 4.2. Calibration curve for the relative humidities.

The moisture adsorption capacity of PU foam was estimated by placing certain amount of PU foam in a jar containing saturated salt solution for certain relative humidity value. At certain intervals, foam samples were withdrawn from the jar and weighed to estimate their moisture content. Water amount adsorbed on the PU foam was calculated per gram of foam (eq 4.1).

$$Amount\ of\ adsorbed\ water = \frac{Weight\ of\ incubated\ foam - Dry\ weight}{Dry\ weight} \quad (4.1)$$

Figure 4.3 shows the moisture adsorption kinetics of different weights of foam pieces in the presence of potassium chloride with a 85.11% relative humidity. Because newly produced PU foam contained excess amount of water, the foam pieces lost their weight until the system reached an equilibrium in the presence of saturated salt solution. The equilibrium water amount was estimated at the very late stage of the kinetic data and calculated according to eq 4.1.

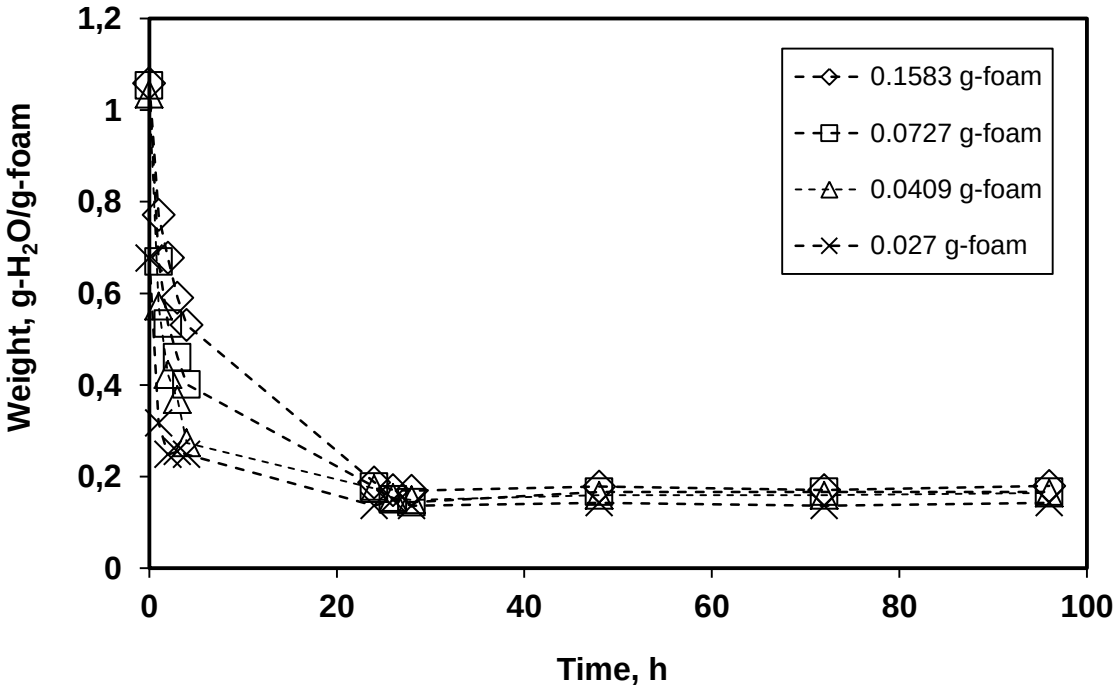

Figure 4.3. Moisture adsorption on PU foam.

Figure 4.4 shows the adsorption of water on PU foam. As shown in the figure, there is almost an exponential relationship between the water amount adsorbed on the PU foam and the relative humidity of saturated salt solution, as shown in Figure 4.5. This relationship corresponds to a Type III adsorption isotherm. Type III isotherm, which was continuously convex with respect to the relative humidity axis, showed a steady increase in adsorption capacity with increasing relative humidity (Crittenden and Thomas 1998). Type III isotherm include capillary condensation in addition to the multimolecular adsorption layer (Ng, El-Sharkawy et al. 2008). Therefore, the increase

in capacity at high relative humidities was due to capillary condensation occurring in pores if any, and condensation on the surface of the PU foam as the saturated vapor pressure was raised (Ruthven 1984). Type III also gives adsorption isotherms on macroporous adsorbents with weak affinities. As a result, while expecting a high water adsorption capacity, PU foam showed a type III adsorption isotherm, indicating that, indeed, there were very weak water-PU foam interactions.

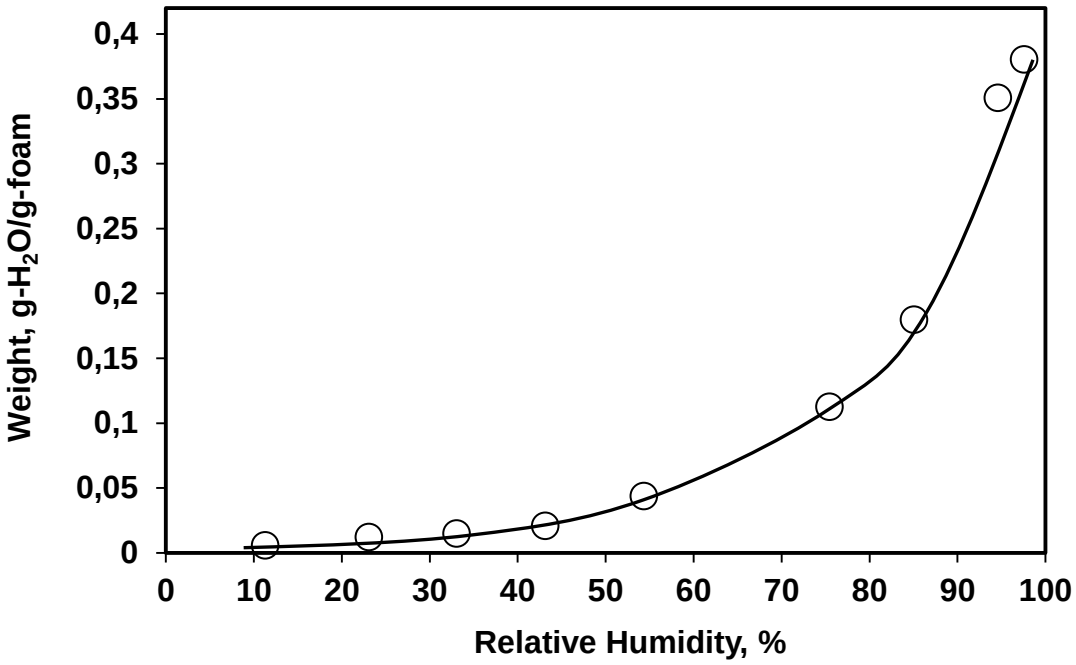

Figure 4.4. Adsorption isotherm of the PU foam.

At the beginning of our study, our objective was to use both water-miscible and water-immiscible organic solvents, for the CA activity. Figure 4.5 illustrates a small jacketed reactor system in which enzymatic reaction occured within the PU foam in the presence of an immiscible organic solvent. We know that, especially in non-polar organic solvent systems, a sufficient water content is crucial for the conformation of the enzyme and the enzyme activity. If there were sufficient water molecules adsorbed on the PU foam, these water molecules could provide a good microenvironment for the enzyme catalysis in the presence of immiscible organic solvent. Therefore, a presence of moisture was aimed in the PU foam containing the CA enzyme. However, when the immobilized enzyme activity assay was performed in the presence of toluene, a water-immiscible organic solvent, water was extracted by toluene creating droplets in the mixture, therefore UV/vis spectrophotometer could not be accurately detected the *p*-NP

as the product in the cuvette. As a result, effect of water-immiscible solvents on the immobilized CA activity could not be realised. Therefore, enzyme activity assays were only conducted in the presence of water-miscible organic solvents.

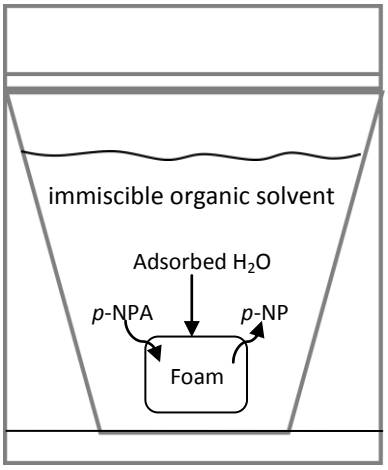

Figure 4.5. Schematic representation of a small jacketed reactor.

4.3. pH Effect on the Absorbance of *p*-NP

p-NP was dissolved in Tris buffer with various pHs and its absorbance was scanned in the UV/vis spectrophotometer. Figure 4.6 shows the change of absorbance for *p*-NP solutions with different pHs. It was found that the absorbance of *p*-NP increased as the pH value of the buffer solution was increased at constant *p*-NP concentration. This indicates that pH of the medium is a parameter effecting the absorbance of *p*-NP in buffer solution. Wavelengths of the solutions gave two peaks at 400 nm and 348 nm, as shown in the figure. These two peaks show the unprotonated species of *p*-NP and the total *p*-NP concentration, respectively. The absorbance value stayed unchanged at isosbestic point (348 nm) at constant *p*-NP concentration, at which spectras cross each other.

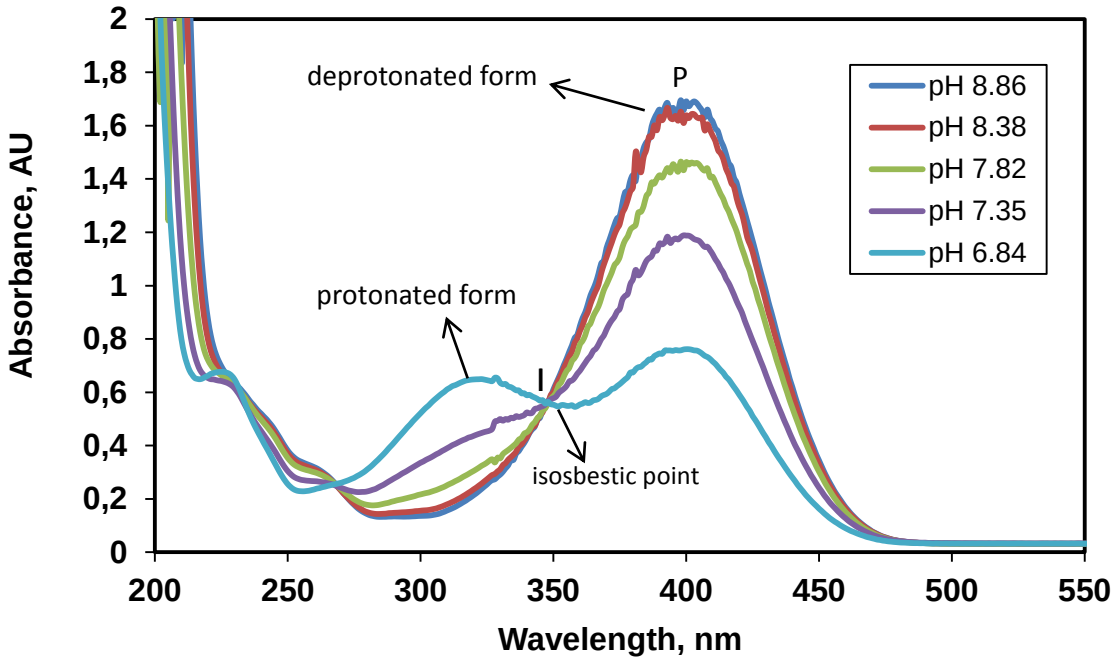

Figure 4.6. Absorbance values for *p*-NP solutions with respect to wavelength with different pHs.

Figure 4.7 shows the ratio of the absorbances at 400 nm to the absorbance at 348 nm. As can be seen in the figure, ratio of the *p*-NP absorbances ($\lambda_{400/348}$) increased with increasing pH value of the *p*-NP solutions. This indicated that the absorbance of *p*-NP is pH-dependent.

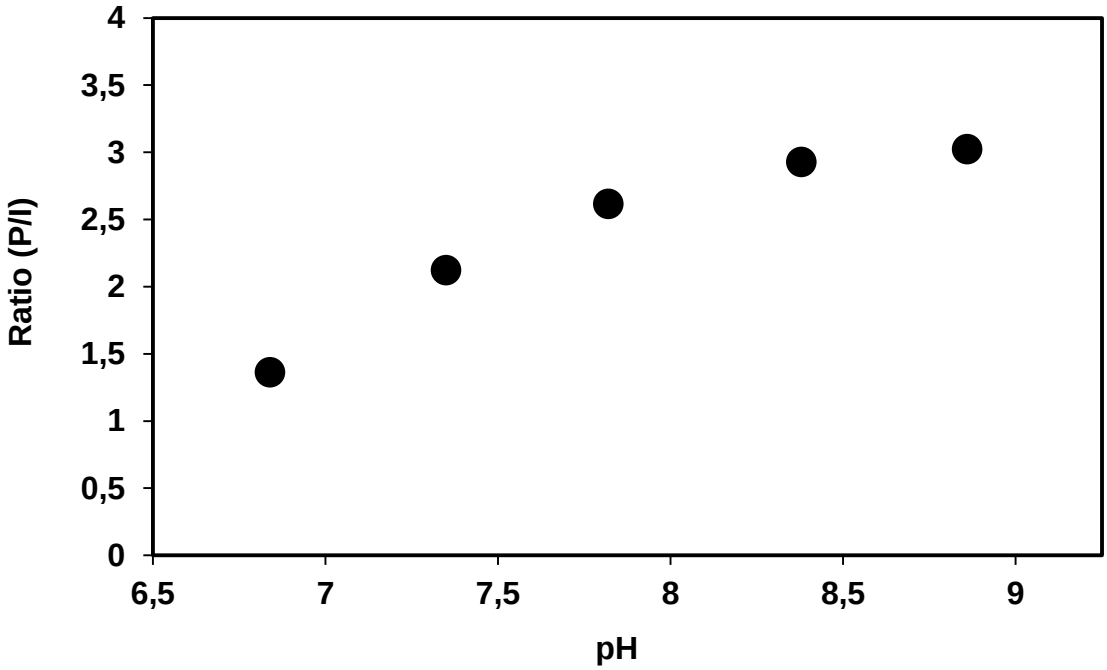

Figure 4.7. Ratio of the *p*-NP absorbances for $\lambda_{400/348}$ with respect to various pHs.

4.4. The Absorbance Scanning of *p*-NP in Tris Buffer

Different concentrations of *p*-NP in Tris buffer (50 mM, pH 7.72) were scanned between 200 nm and 600 nm in the UV/vis spectrophotometer at constant pH. It was observed that wavelengths of the solutions gave the peak at 400 nm, as seen in Figure 4.8. Increase in the *p*-NP concentration resulted in a significant increase in the absorbance value for the same wavelength.

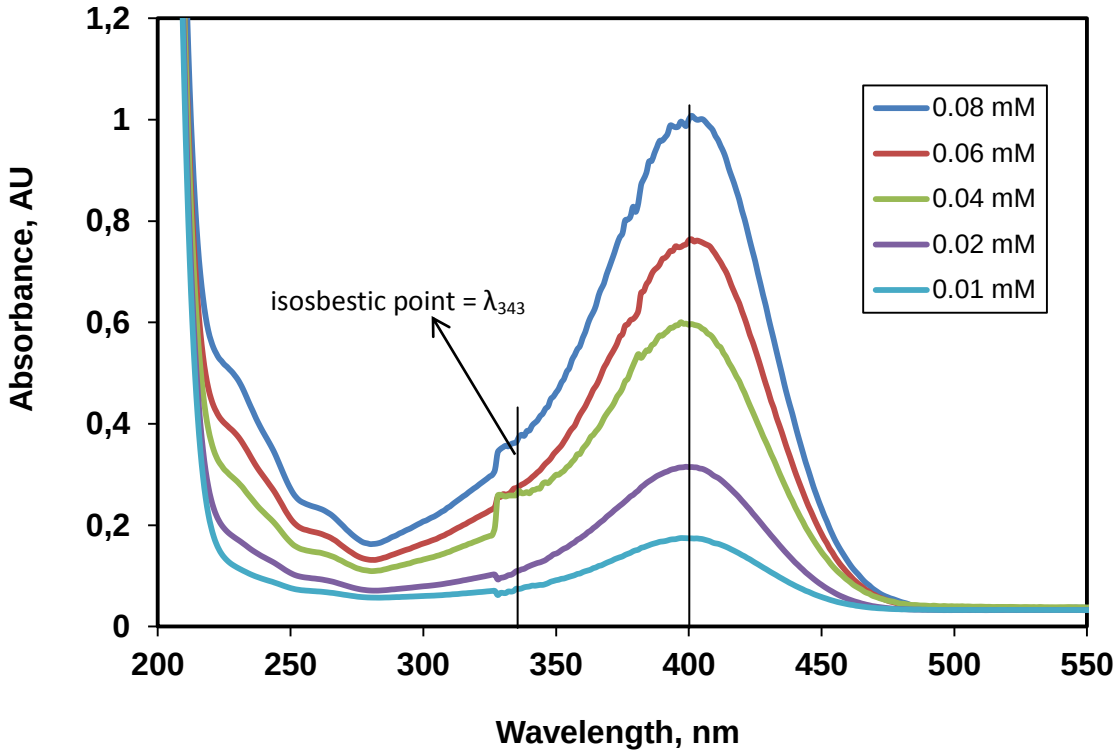

Figure 4.8. Change of the absorbance for different *p*-NP concentrations with wavelength.

Figure 4.9 shows the absorbance values for *p*-NP at 400 nm and at the isosbestic wavelength (λ_{343}) with *p*-NP concentration. This result demonstrated that the absorbance of *p*-NP in Tris buffer was also concentration-dependent.

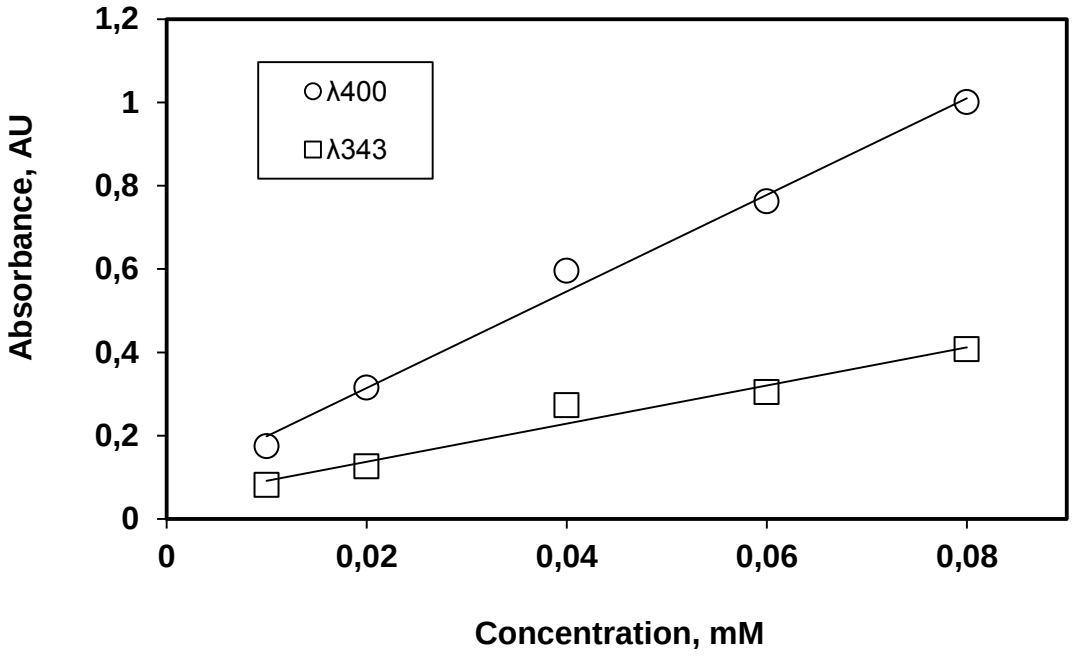

Figure 4.9. Absorbance changes for *p*-NP at the peak and isosbestic wavelength.

The ratio of the absorbances at these two wavelengths stayed nearly unchanged with respect to different *p*-NP concentrations, as seen in Figure 4.10. This result indicated that the ratio of the absorbances was independent of concentration at constant pH.

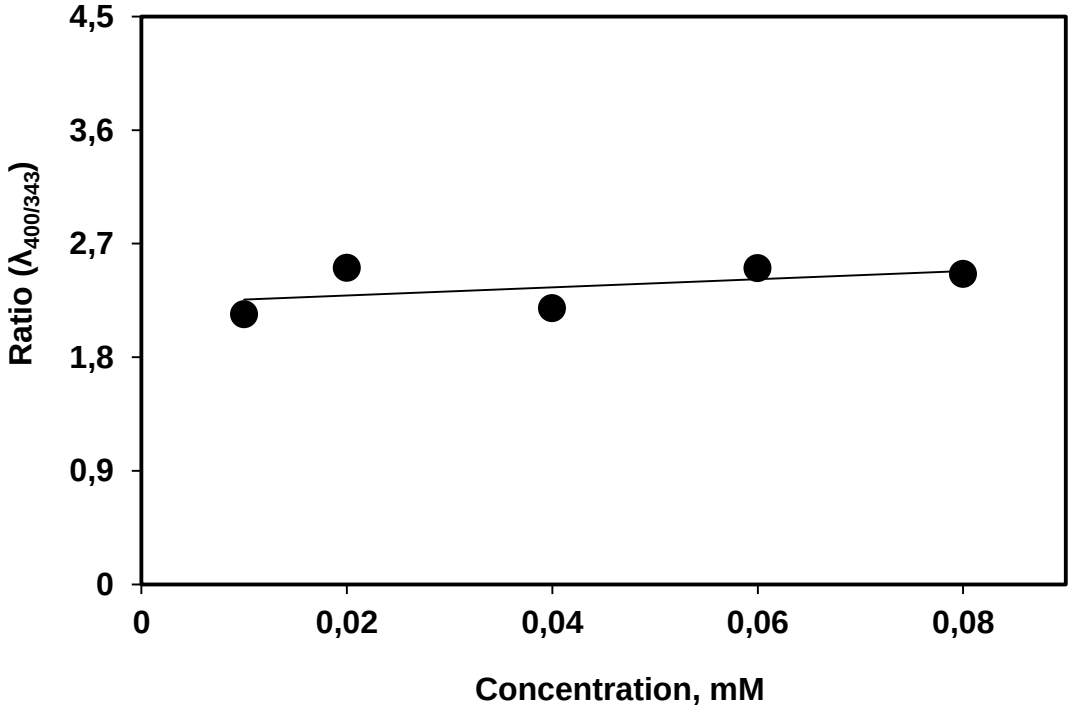

Figure 4.10. The ratio of *p*-NP absorbances with respect to various *p*-NP concentrations.

4.5. Acetonitrile As Water-Miscible Organic Solvent in CA Activity

4.5.1. The Absorbance Profile of *p*-NP in the Presence of Acetonitrile

The absorbance scanning of *p*-NP solutions was conducted in the presence of different volume percents of acetonitrile (10-90%) keeping the *p*-NP concentration constant in the mixtures. The absorbance of *p*-NP at 400 nm significantly decreased as the concentration of acetonitrile was increased. As shown in Figure 4.11, three peaks were observed in the absorption spectra of *p*-NP at 400 nm, 315 nm and 254 nm.

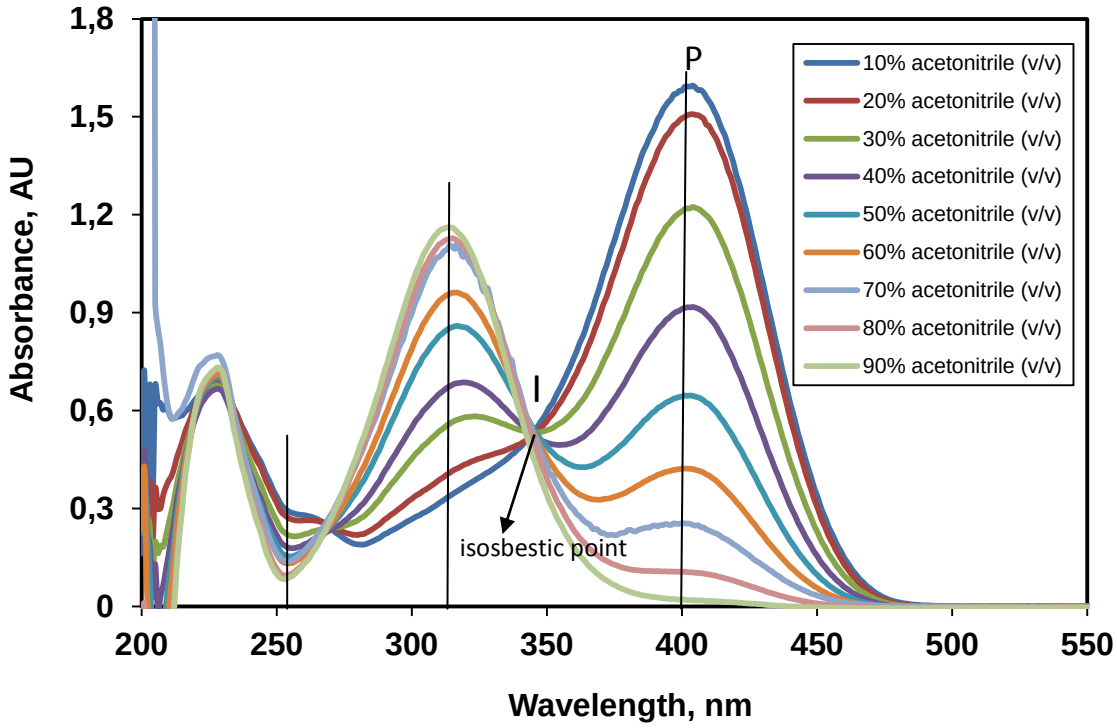

Figure 4.11. The spectrum profile for *p*-NP in various percent volume acetonitrile/buffer mixtures.

Figure 4.12 shows the ratio of the peak absorbances (400 nm) to the absorbance at the isosbestic point (343 nm) with increasing percentages of acetonitrile by volume. As can be seen from the figure, the ratio of the *p*-NP absorbances ($\lambda_{400/343}$) decreased with increasing volume percent of acetonitrile in the mixture.

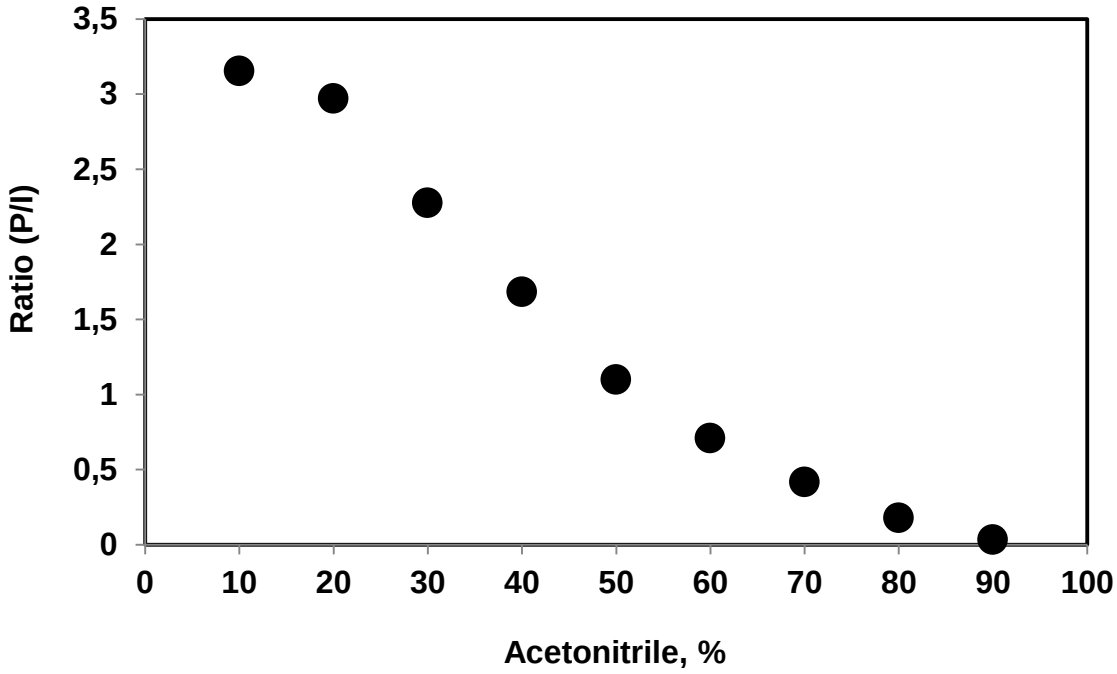

Figure 4.12. Ratio of the *p*-NP absorbances ($\lambda_{400/343}$) with respect to various percentages of acetonitrile in the mixture by volume.

As can be seen in Figure 4.6, a chemical shift could occur depending on pH change, thus resulting in the protonated form of *p*-NP. Therefore, the pH values of the acetonitrile/buffer mixtures were measured in order to determine whether pH was changed with increasing acetonitrile concentration. As shown in Figure 4.13, no significant pH change was seen when the acetonitrile content was increased in the mixture. This demonstrated that the decrease in *p*-NP absorbance at 400 nm in the presence of acetonitrile was not due to pH effect.

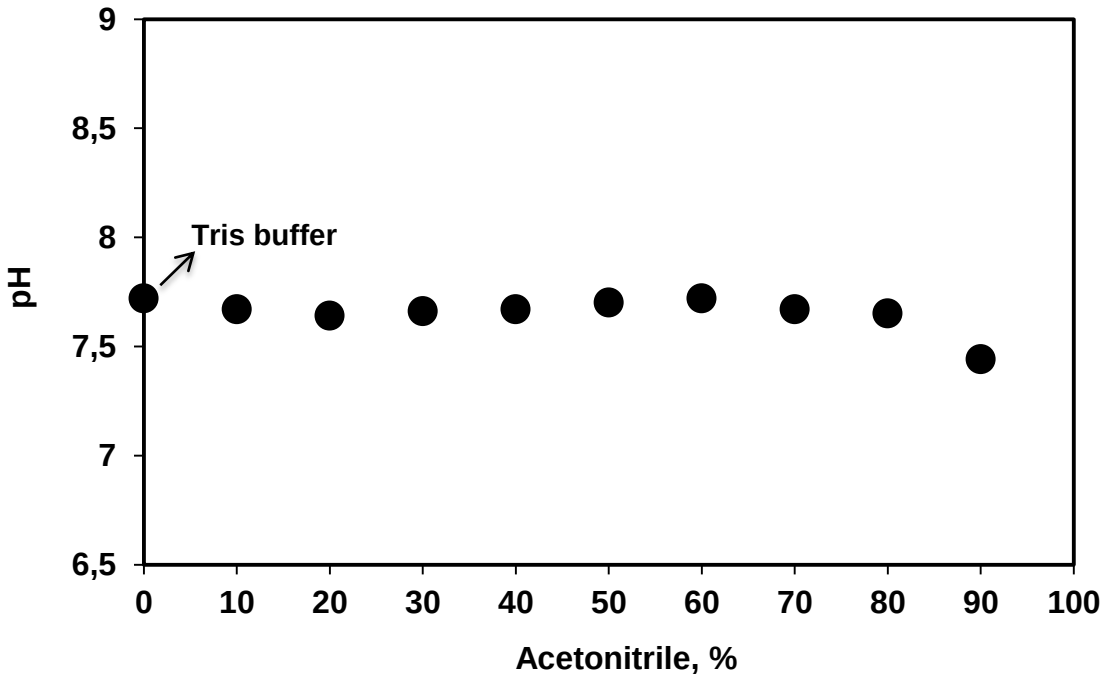

Figure 4.13. pH values of different percent volume acetonitrile/buffer mixtures.

When Tris buffer (50 mM, pH 7.72) was mixed with the organic solvent at different ratios, Tris concentration in the mixture was decreased when the concentration of the organic solvent was increased. In order to determine any possible effect of Tris concentration on the decline of *p*-NP absorbance, firstly, the pH values of the acetonitrile/buffer mixtures were measured keeping the Tris concentration constant. Afterwards, the absorbance scanning for *p*-NP solutions at various acetonitrile contents was performed in the UV/vis spectrophotometer keeping both Tris and *p*-NP concentration constant, when the Tris buffer concentration was constant in the acetonitrile-buffer solution at different ratios. As shown in Figure 4.14, no significant change was observed at pH values.

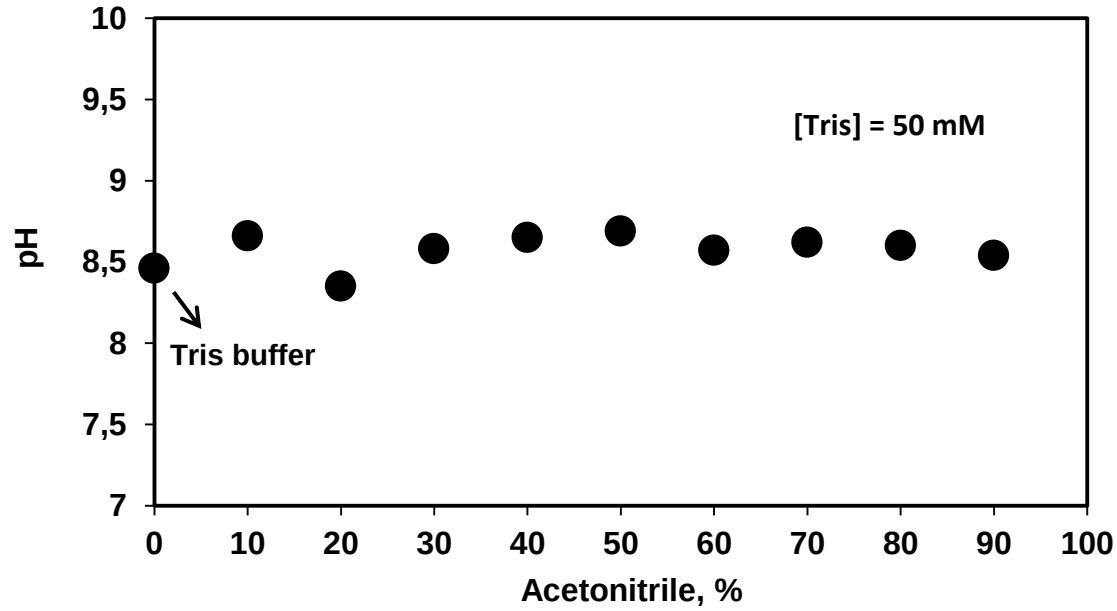

Figure 4.14. pH values with respect to various acetonitrile percentages in the mixture by volume at constant Tris concentration.

The *p*-NP absorbances at 400 nm also significantly decreased as the acetonitrile content was increased and an isobestic point occured at about 343 nm, as shown in Figure 4.15. As a result, the *p*-NP absorbance decline with increasing acetonitrile content was not due to pH effect or Tris concentration in the mixture.

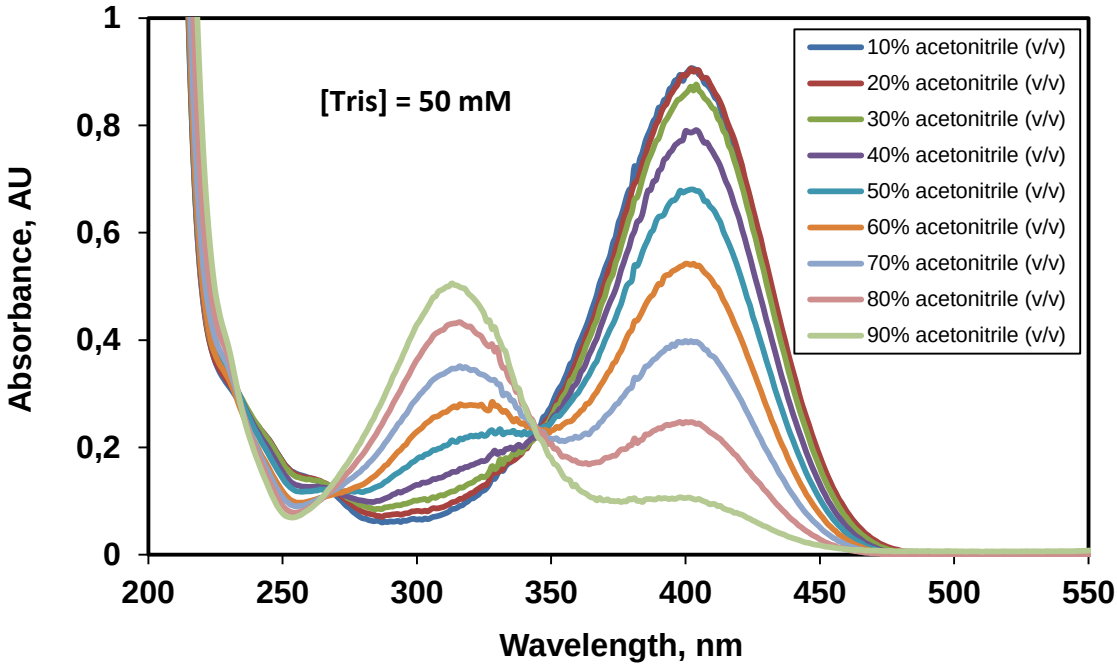

Figure 4.15. The spectrum profile of *p*-NP in the acetonitrile/buffer mixtures at constant Tris concentration.

If unprotonated form of *p*-NP was resulted in the peak at 400 nm, the peaks of 315 nm and 254 nm could result from the complex formation between acetonitrile and unprotonated or protonated form of *p*-NP (José, Sandra et al. 2008).

4.5.2. Calibration Curves for *p*-NP in the Presence of Acetonitrile

Calibration curves were prepared to determine the extinction coefficients (absorptivities) of *p*-NP at different volume percents of acetonitrile. The calibration curves for *p*-NP in the presence of acetonitrile is shown in Figure 4.16. R^2 values of all the lines established between the absorbance and concentration are nearly fit to data, as seen in the figure. The actual absorbance of a sample is dependent on the concentration and the path length via the Beer–Lambert law (eq 4.2).

$$A = \varepsilon \times c \times \ell \tag{4.2}$$

where ε is the extinction coefficient, c is the concentration of the sample, and ℓ is the path length defined as the distance that light travels through a sample in an analytical cell. The quartz cell used in this study was 1 cm in width. Therefore, the slope would indicate the absorptivity of *p*-NP at 400 nm.

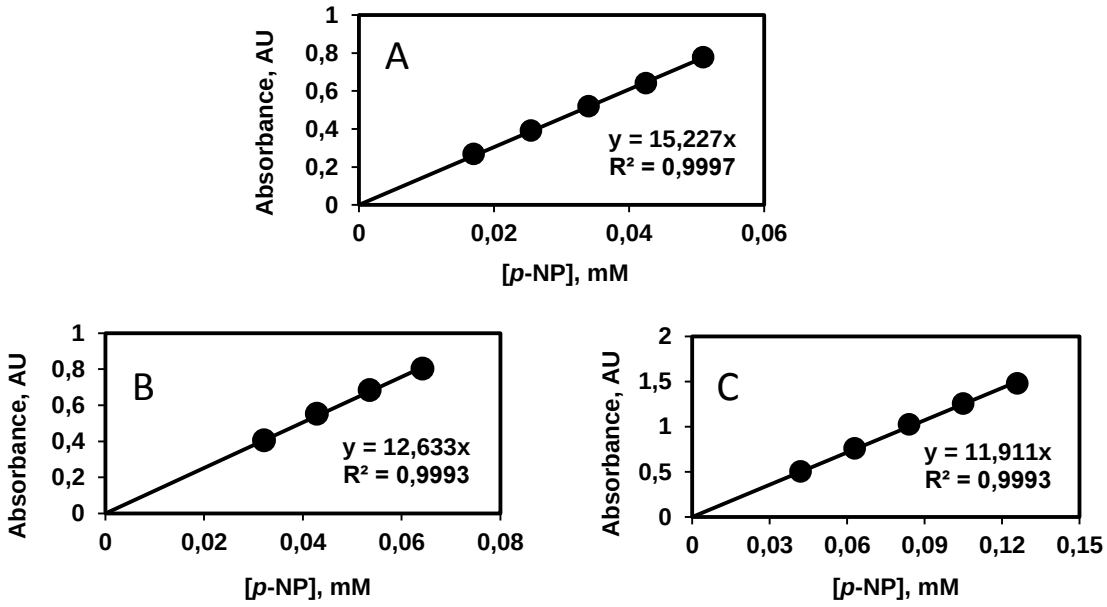

Figure 4.16. Calibration curves for *p*-NP in the presence of (A) 10%, (B) 20%, (C) 30%, (D) 40%, (E) 50%, (F) 60%, (G) 70%, (H) 80%, and (I) 90% acetonitrile v/v.

(cont. on next page)

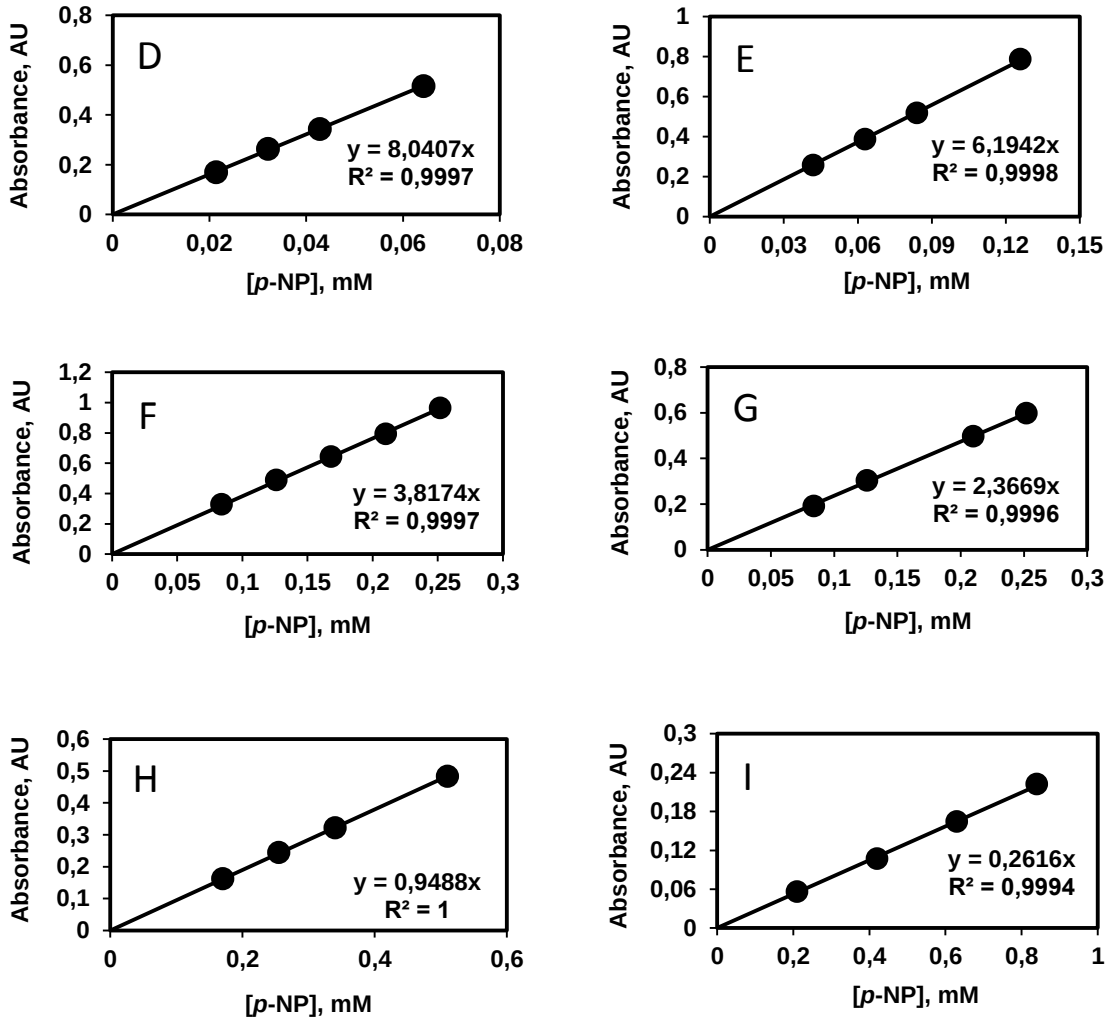

Figure 4.16 (cont.)

Both the absorptivity and absorbance values derived from spectrum profiles of *p*-NP are shown in Figure 4.17. As can be seen in the figure, the absorptivity and absorbance values for *p*-NP decreased with increasing organic solvent content and almost overlapped each other, which confirm the calibrations established for *p*-NP.

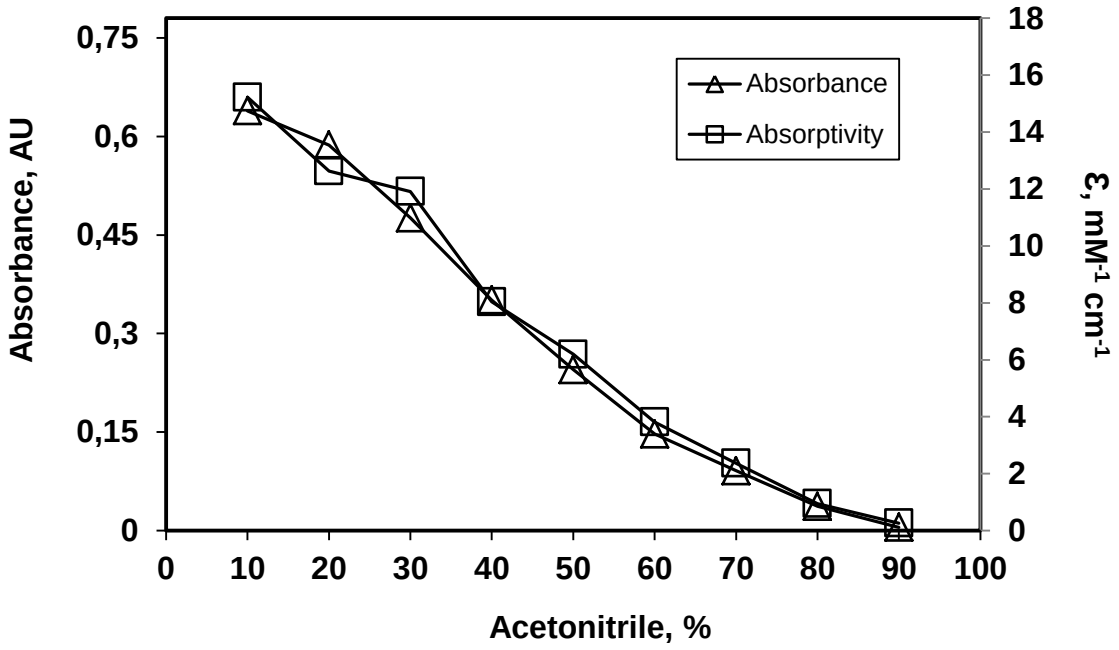

Figure 4.17. Change of the absorbance and absorptivity at 400 nm for *p*-NP in various percent volume acetonitrile/buffer mixtures.

The solvent effect on protonation-deprotonation equilibria, which resulted in the change of the absorptivities for *p*-NP, could be explained on the basis of three possible reasons: a) electron migration in the chemical structure of *p*-NP, b) H-bonding and c) change of the medium's dielectric constant. When *p*-NP dissolved in aqueous solution interacted with acetonitrile, the oxygen lone pair electrons could migrate with greater intensity towards the adjacent carbon atom, resulting in being more available to form stronger hydrogen bonds (Figure 4.18). This hydrogen bonding between acetonitrile and *p*-NP might generate a phenol-acetonitrile-water cluster (José, Sandra et al. 2008). As the acetonitrile concentration in the mixture was increased, in the UV/vis spectrophotometer, the visible light could be weakly absorbed by the deprotonated form of *p*-NP due to the cluster structure, hence the absorbance measured at 400 nm decreased with increasing acetonitrile concentration. Another possible reason might be the change of the medium's dielectric constant. Zekarias et al. indicated that chemical shift change towards protonation or change in free energy with the organic solvent content depended up on two factors: an electrostatic one, and a non-electrostatic one, which include specific solute-solvent interaction. They suggested that when the electrostatic effects predominated, the energy of electrostatic interaction was related inversely to dielectric constant (Zekarias, Hirpaye et al. 2011). In our study, the

dielectric constant of the medium presumably decreased as the organic solvent content increased. Therefore, the *p*-NP absorbance decline with increasing organic solvent content could be attributed to a possible dielectric constant change and the dominance of electrostatic forces in the protonation-deprotonation equilibria of *p*-NP.

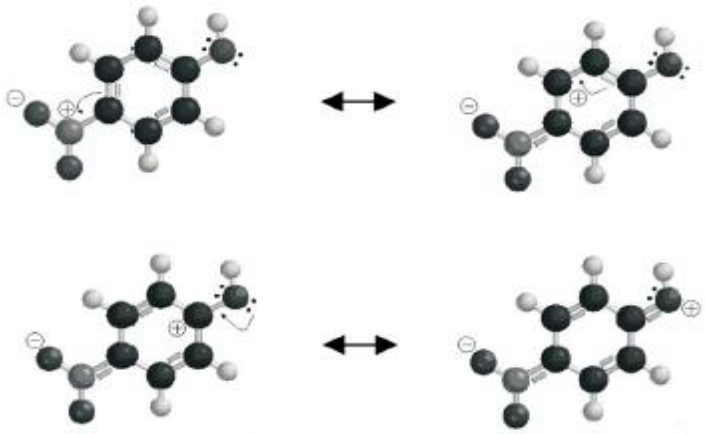

Figure 4.18. Mechanism of electron migration for *p*-NP.
(Source: José, Sandra et al. 2008)

4.5.3. Self-Hydrolysis of *p*-NPA in the Presence of Acetonitrile

The self-hydrolysis rate of *p*-NPA has to be obtained in order to subtract the background absorbance and to determine the actual activity rates for the free and immobilized enzyme. The self-hydrolysis of *p*-NPA was conducted in Tris buffer (50 mM, pH 7.72) containing 10% acetonitrile in order to determine the effect of pH change on the self-hydrolysis rate. No self-dissociation was observed under pH 7, however above pH 7, the self-dissociation rate increased exponentially with increasing pH of the medium, as shown in Figure 4.19. Because high self-hydrolysis rate was observed at high pHs, the pH value of Tris buffer was adjusted to about 7.72 for the enzyme activity tests.

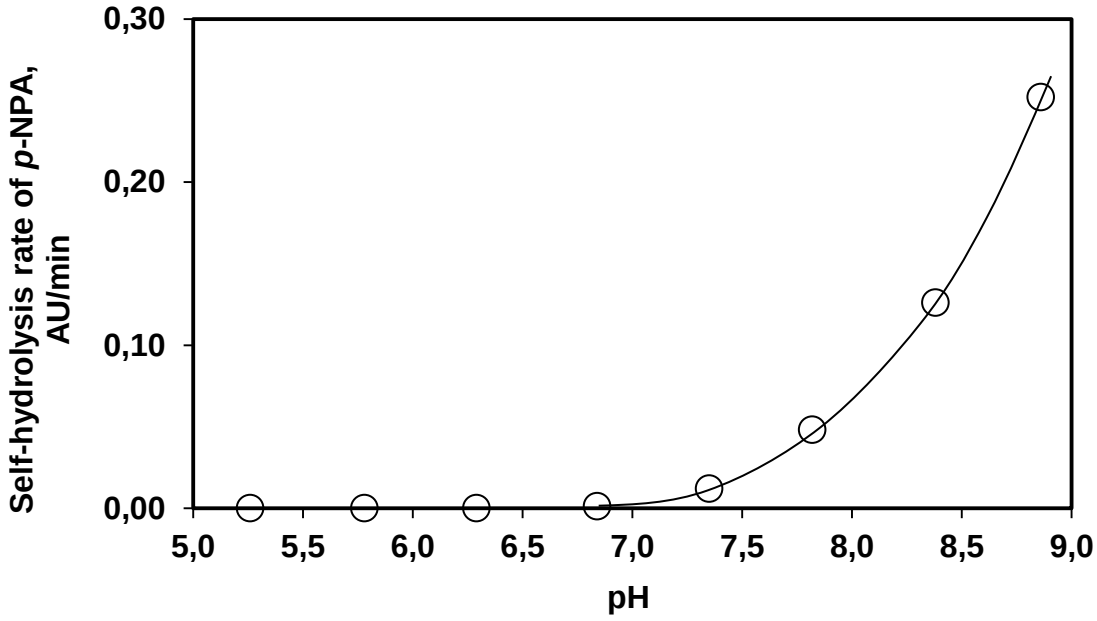

Figure 4.19. pH effect on the self-hydrolysis of *p*-NPA.

The absorbance scanning for the substrate, *p*-NPA, was performed at different acetonitrile concentrations while keeping the *p*-NPA concentration constant. Figure 4.20 shows the absorbance scan for the *p*-NPA at different volume percents of acetonitrile. As can be seen in the figure, no peak was seen at 400 nm.

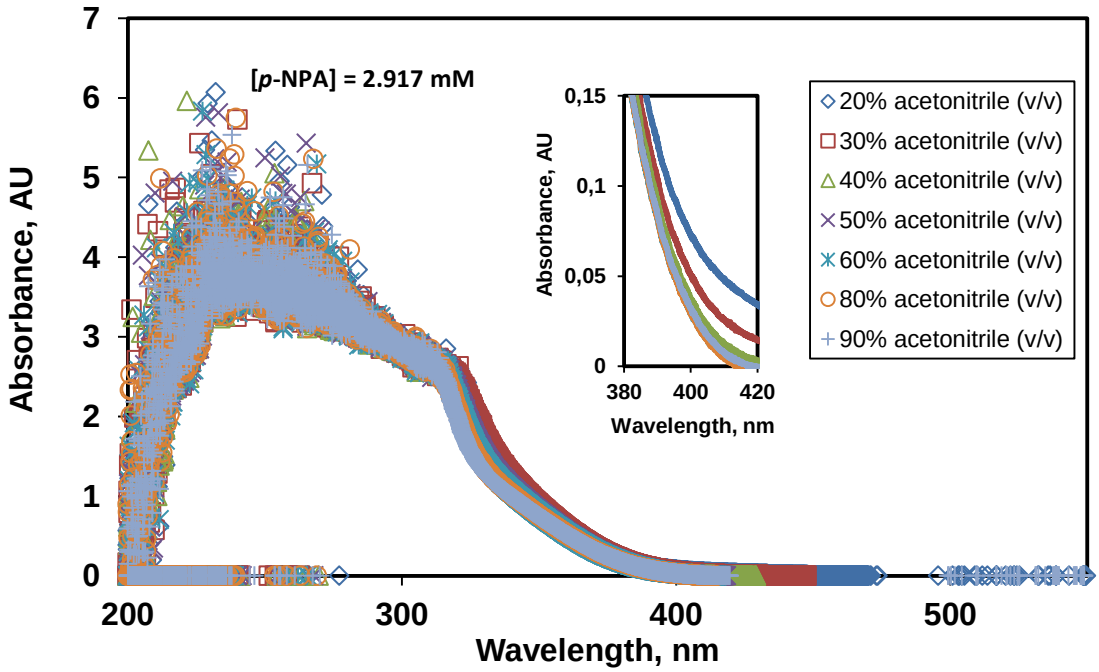

Figure 4.20. The spectrum profile for self-hydrolysis of *p*-NPA at different acetonitrile percentages by volume.

Figure 4.21 shows the absorbance values for the *p*-NP as a product of self-hydrolysis of *p*-NPA at 400 nm with increasing volume percents of acetonitrile. As the organic solvent concentration in the mixture was increased, the absorbance values of *p*-NP was decreased. This could be attributed to lower self-hydrolysis of *p*-NPA at higher acetonitrile concentrations. From the figure, it could be seen that the absorbance values of p-NP were very low which are negligible during the measurements for the enzyme activity assays.

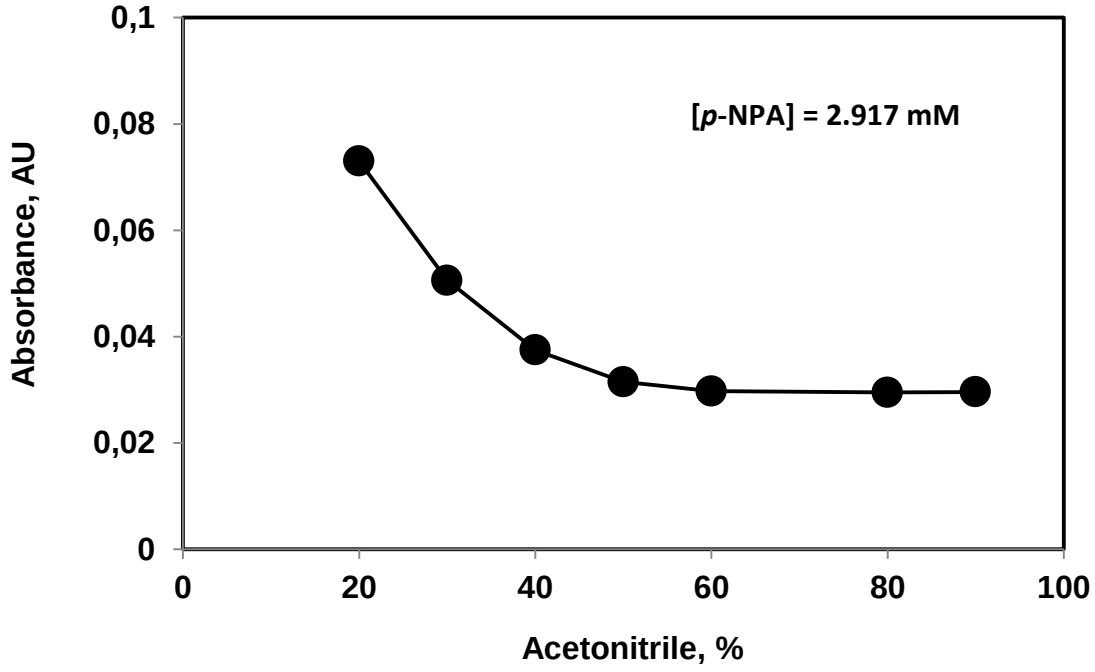

Figure 4.21. *p*-NP absorbance at 400 nm with increasing acetonitrile percentages in the mixture by volume.

4.5.4. Activitiy of Free CA in Acetonitrile

Before the activity tests for the free enzyme in the acetonitrile/buffer mixtures, effect of the substrate concentration on the free CA activity was examined keeping the enzyme concentration constant. Figure 4.22 shows the activity of the free CA in Tris buffer (50 mM, pH 7.72) containing 10% v/v acetonitrile at different substrate concentrations. As shown in the figure, the enzymatic rate increased as the concentration of *p*-NPA was increased up to 4.1 mM, but at higher substrate concentrations no activity increase was seen. There was also a linear-like trend at low substrate concentrations (0.83-2.77 mM), as seen in the figure. Therefore, the substrate

concentration of *p*-NPA could only be used up to ~3 mM due to the limited solubility of *p*-NPA in the aqueous phase. Higher p-NPA concentrations could be used if the acetonitrile concentration was increased.

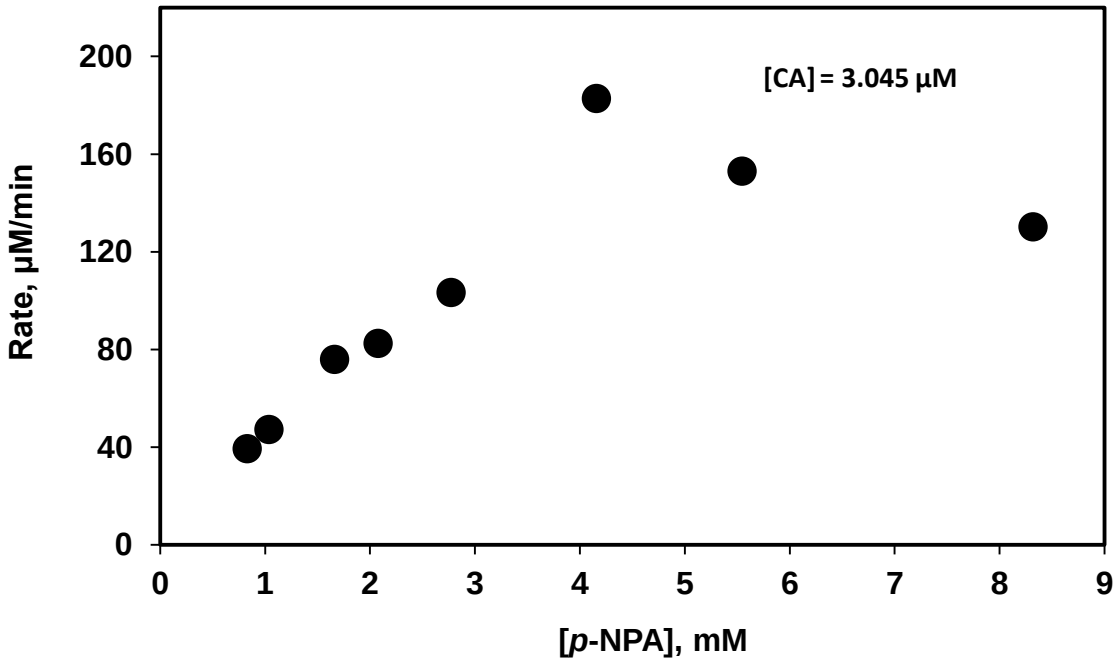

Figure 4.22. Effect of the substrate concentration on the free CA activity.

The acitivity of the free enzyme was estimated at different acetonitrile concentrations while keeping both substrate and enzyme concentration constant. The self-hydrolysis rate determined for each buffer/organic solvent mixture were subtracted from the raw data of the enzyme-catalyzed hydrolysis rate in order to determine the actual enzymatic rate. Figure 4.23 shows the enzymatic rate in different percent volume acetonitrile/buffer mixtures. The activity of the free CA decreased dramatically with increasing acetonitrile concentration up to 40-50%, but at higher concentrations the enzyme exhibited increasing activity. This result is in agreement with those reported in the literature for the media containing water-miscible organic solvent/water mixtures (Griebenow and Klibanov 1996, Simon, Laszlo et al. 1998).

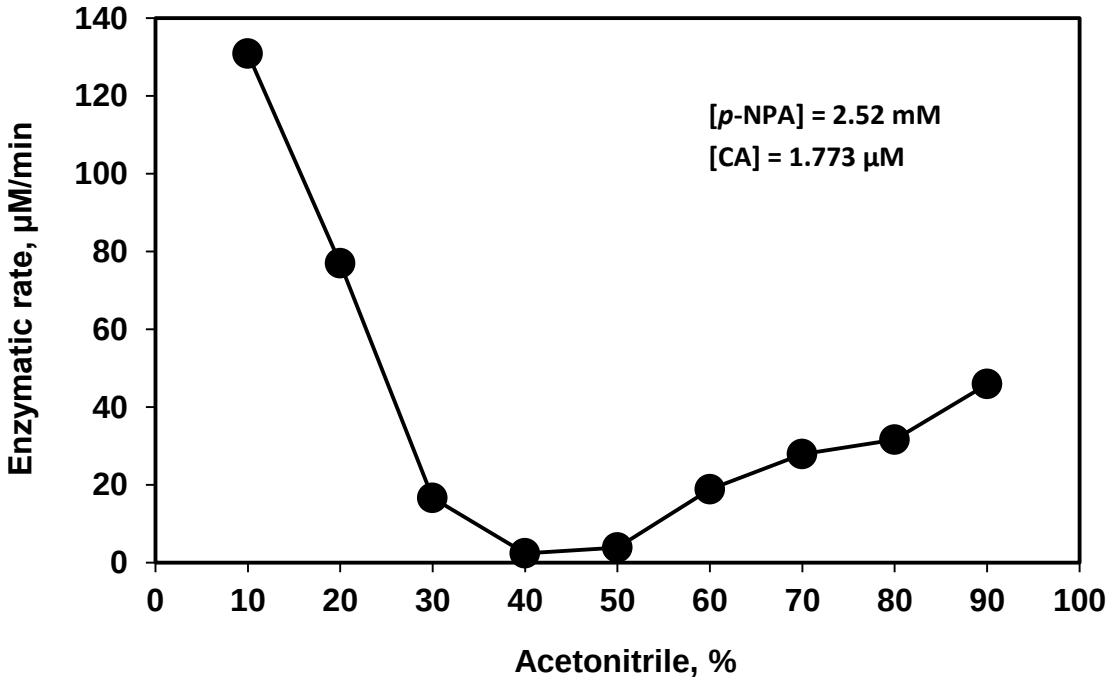

Figure 4.23. The enzymatic rates for free CA in different percent volume acetonitrile/buffer mixtures.

The hydration water content around the enzyme that is available for the solvation is an important factor in enzyme activity. Water-miscible organic solvents have the ability to strip away water molecules from the enzyme surface. Hence, the hydration water was presumably removed from the enzyme surface by the penetration of the solvent molecules into the active site of the enzyme. This could give rise to a weakness in the strength of the catalytic H-bond networks, thus resulting in a decrease in enzyme activity and denaturation of the enzyme at certain organic solvent concentrations. The drop in the activity in the presence of the organic solvents might be associated with the lower flexibility and the increased proton-transfer barrier (change in the pronotation state of active site residues). The activity of free CA diminished especially at 40-50% v/v acetonitrile, as seen in Figure 4.23. This is consistent with the results observed in a previous study (Safarian, Saffarzadeh et al. 2006). Safarian et al. indicated that the secondary structure of carbonic anhydrase was almost unaltered in three acetonitrile/buffer conditions (0%, 17,5% and 47,5% v/v). However, the decrease of enzymatic rate observed in the presence of acetonitrile was attributed to the tertiary structural alterations of carbonic anhydrase and the reorientation of residues near the active site of the protein.

Increasing activity observed at high concentrations of acetonitrile could be mainly attributed to stronger hydrogen bonding between the protein atoms and structural rigidity in such media (compared to water), resulting in high kinetic barriers (kinetic trapping) preventing unfolding of the enzyme (Griebenow and Klibanov 1996). The enzyme's capacity to actually undergo denaturation could be severely impaired in such conditions. Consequently, the properties and interactions of the organic solvents might contribute significantly to the retaining of the catalytic activitiy of CA. It was also observed that at 60-90% percentages of acetonitrile, the colour of the mixture turned into white as if a crystal flocks has occured and the solution became blurred when the free CA was added into the mixture. This colour change in water-poor media could be due to rigidity of the enzyme in acetonitrile.

4.5.5. Immobilization of CA within PU Foam

CA was immobilized within PU foam as described in the methods section. Carbon dioxide was released during the immobilization, when isocyanate groups were reacted with water, which resulted in gas bubbles in the foam. These bubbles generated large pores in the polymeric network, which made the PU foam a sponge-like material. The hydrophilic character of the PU foam and large porosity provided some advantages for enzyme immobilization. Substrates could easily access the PU foam and the products could easily diffuse in and out of the foam containing the enzyme (Ozdemir 2009).

4.5.6. Activitiy of Immobilized CA in Acetonitrile

The activity of the immobilized CA was estimated through the liberation of *p*-NP from the PU foam over time by the hydrolysis of *p*-NPA. The self-dissociation rates for the *p*-NPA was also subtracted from the enzyme-catalyzed hydrolysis rates. The immobilized enzyme assays were employed at various acetonitrile concentrations keeping the substrate concentration constant. The foam samples were cut at various weights and a piece of foam sample was assayed in each mixture, however all the enzymatic rates for the immobilized CA were calculated as per gram of foam. Figure 4.24 is a representative plot illustrating the activities of the immobilized CA in

increasing percentages of acetonitrile by volume. The activity of immobilized CA decreased as the acetonitrile concentration was increased up to 40-50% v/v, and then no activity was observed at higher concentrations. These results are noticeably different from those observed for the free CA in the presence of acetonitrile. In the case of free CA, above 50% v/v, the enzyme exhibited increasing activity. By considering only ~0.08-0.1g foam pieces of the whole PU foam were used in the assays, the activity loss at higher acetonitrile concentrations could be due to relatively very small change of the *p*-NP concentration produced in the reaction mixture, which probably could not be detected and measured by the spectrophotometer. Another possible reason could be the exposure of the enzyme in the PU foam to acetonitrile for 20 minutes during the activity assay which could resulted in enzyme denaturation. Another reason also could be entrapment of the product of *p*-NP within the foam which was too low to detect in the solution.

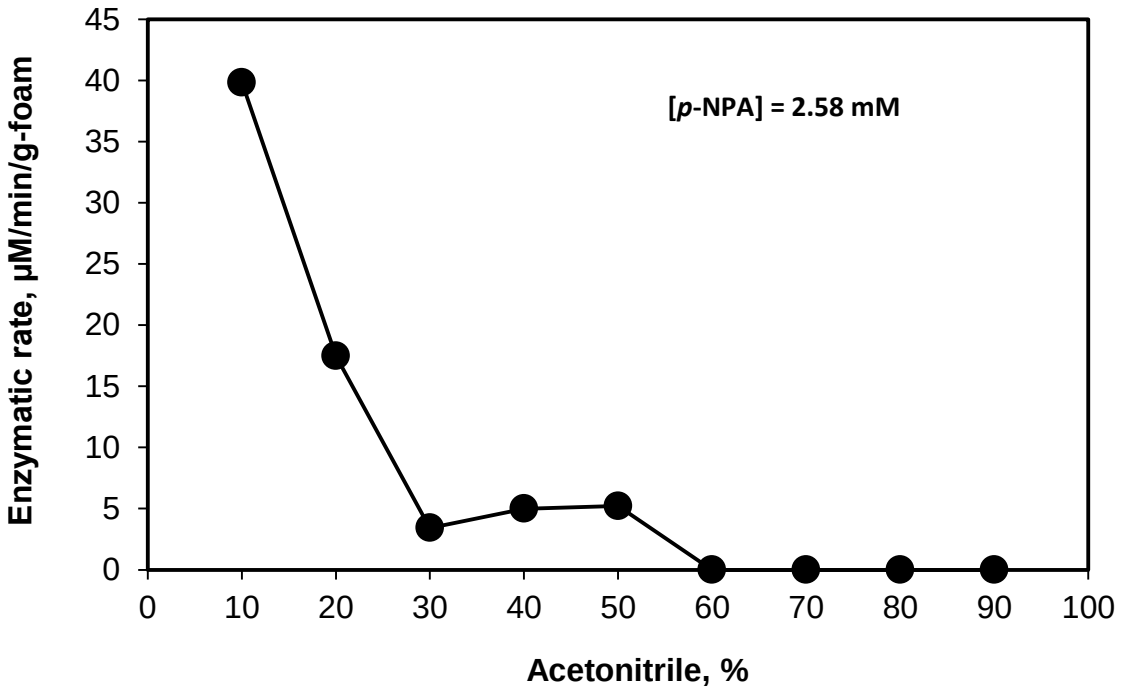

Figure 4.24. The activity of the immobilized CA in increasing percentages of acetonitrile by volume.

4.5.7. Stability of Free and Immobilized CA in Acetonitrile

The storage stabilities of the free and immobilized CA were estimated using the same substrate concentrations in various percent volume acetonitrile/buffer mixtures at ambient temperature in the laboratory. Figure 4.25 shows the relative activities of the free and immobilized CA over time. After storage in 10% v/v acetonitrile for 36 h, immobilized CA exhibited 100% of its original activity, while free CA regained 80% of its original activity. However, after storage in 20% v/v acetonitrile for 36h, immobilized CA exhibited 40% of its original activity, while free CA exhibited 70% of its original activity. After storage in 30-70% v/v and 80-90% v/v acetonitrile for 15 and 30 minutes, respectively, immobilized enzyme lost all of its activity. Free CA also lost all of its activity after storage for 30 minutes in the same range.

These results demonstrated that the reason why no enzymatic activity was seen above 50% v/v acetonitrile (Figure 4.24) could be attributed to the exposure of the enzyme in the PU foam to acetonitrile for 20 minutes during the activity assay, which inactivated the enzyme completely. As a result, the immobilized enzyme in the reactor lost its all of its activity at high acetonitrile concentrations during the activity assay, hence we could not measure any change for *p*-NP absorbance in the UV/vis spectrophotometer.

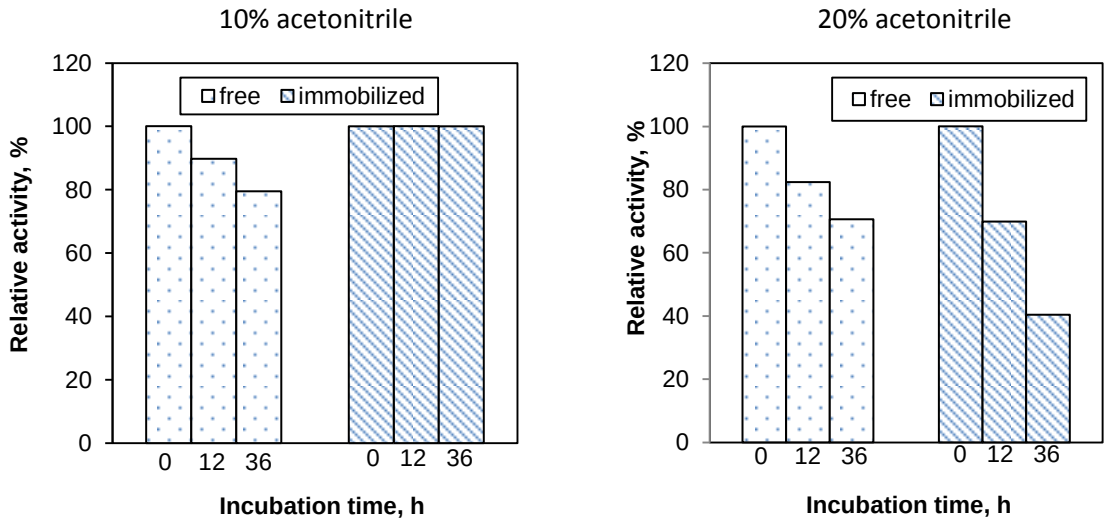

Figure 4.25. Stability of the free and immobilized CA in the presence of acetonitrile

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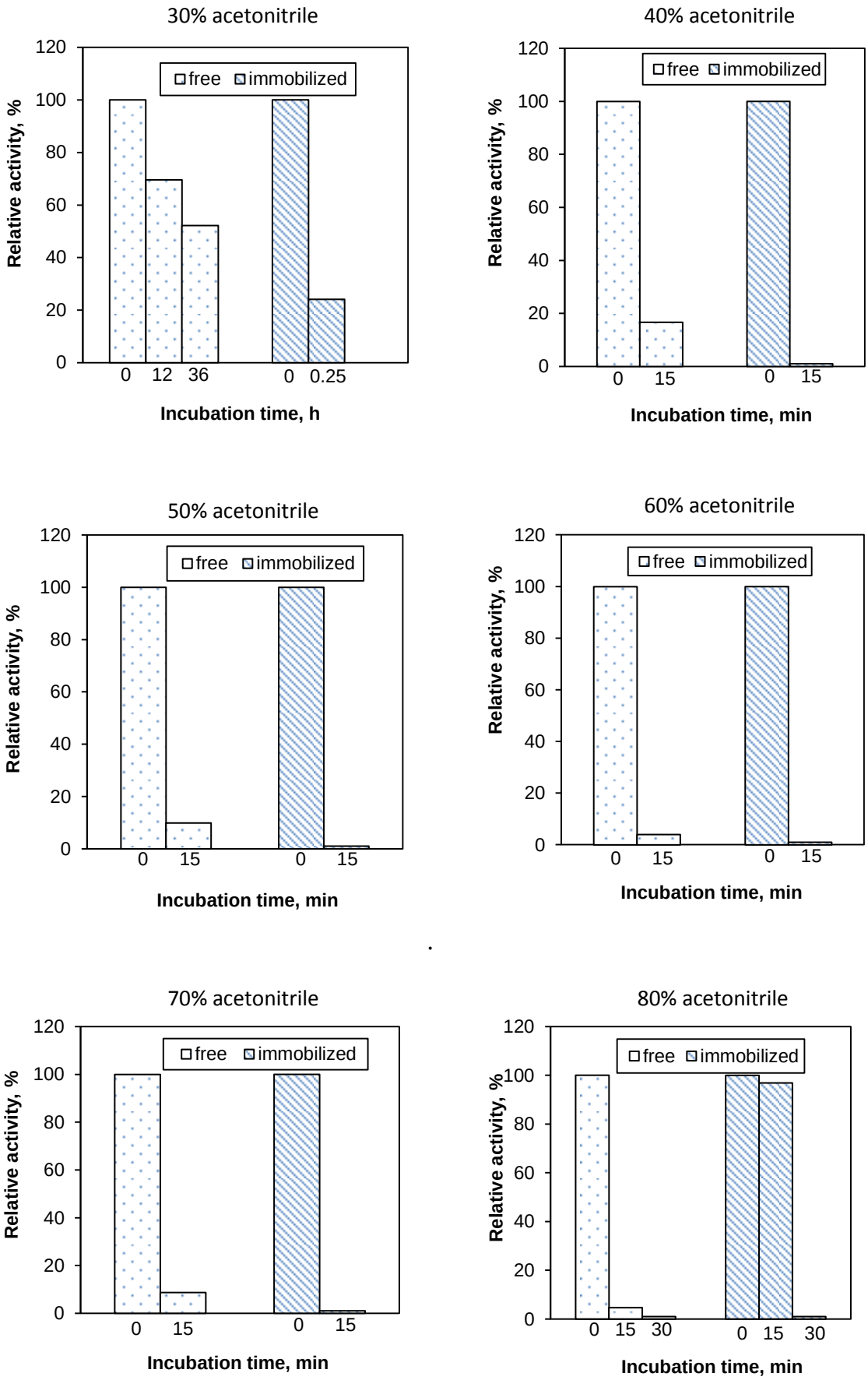

Figure 4.25 (cont.)

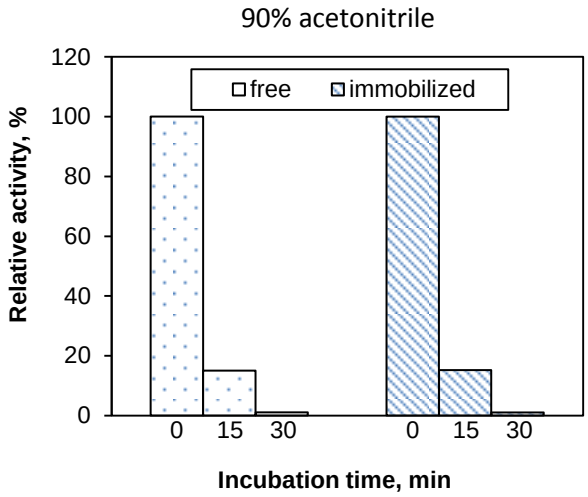

Figure 4.25 (cont.)

4.6. Ethanol As Water-Miscible Organic Solvent in CA Activity

4.6.1. The Absorbance Profile of *p*-NP in the Presence of Ethanol

The absorbance scanning of *p*-NP solutions was conducted in the presence of different percentages of ethanol by volume (10-90% v/v) keeping the *p*-NP concentration in the mixtures constant. Same results were observed in the presence of ethanol as well as in acetonitrile. The absorbance decreased significantly as the ethanol concentration was increased, as shown in Figure 4.26.

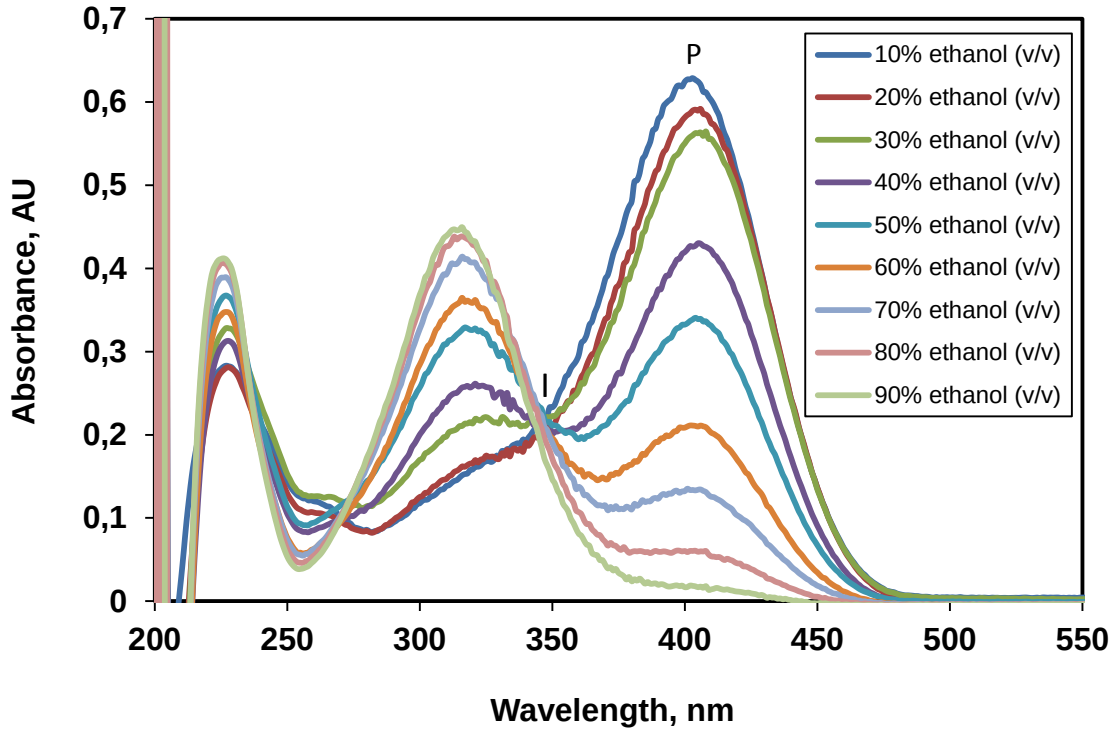

Figure 4.26. The spectrum profile of *p*-NP in different percent volume ethanol/buffer mixtures.

Figure 4.27 shows the ratio of the absorbances at 404 nm (the peak wavelength) to the absorbance at the isosbestic point (λ_{343}). As can be seen in the figure, the ratio of the *p*-NP absorbances ($\lambda_{404/343}$) decreased with increasing volume percents of ethanol in the mixture as well as in the presence of acetonitrile. The pH values of different percent volume ethanol/buffer mixtures were also measured in order to determine whether pH was changed with increasing organic solvent concentration. According to the results, no significant pH change was seen as the ethanol content was increased in the mixture as shown in Figure 4.28. This indicated that the protonation of *p*-NP was not due to pH effect in the presence of ethanol as well as in acetonitrile.

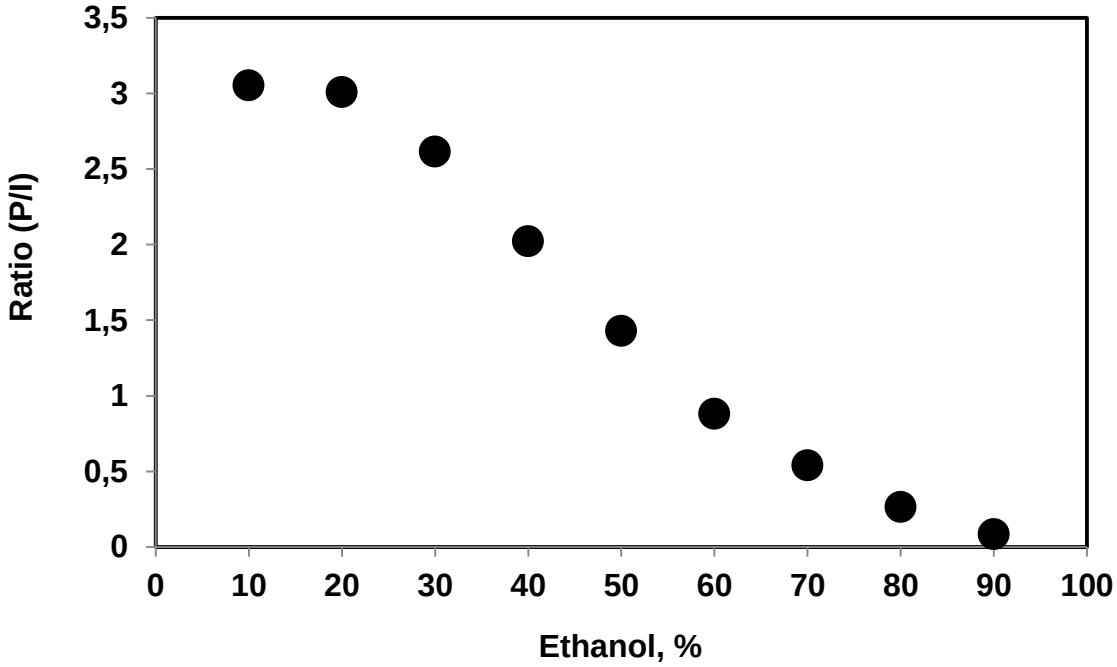

Figure 4.27. Change of the ratio of the *p*-NP absorbances for $\lambda_{404/343}$ with increasing percentages of ethanol by volume.

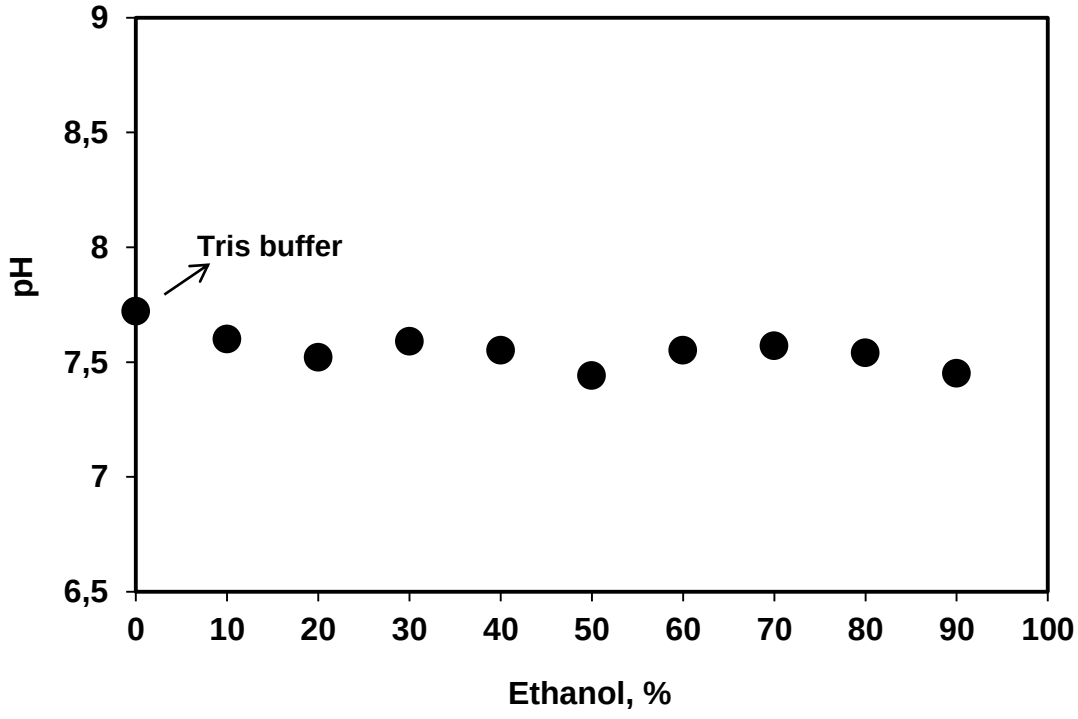

Figure 4.28. pH values for various percent volume ethanol/buffer mixtures.

4.6.2. Calibration Curves for *p*-NP in the Presence of Ethanol

Calibration curves were prepared to determine the extinction coefficients (absorptivities) of *p*-NP at different percent volume ethanol/buffer mixtures. The calibration curves for *p*-NP in the presence of ethanol is shown in Figure 4.29. R^2 values of all the lines established between the absorbance and concentration are nearly fit to data, as seen in the figure.

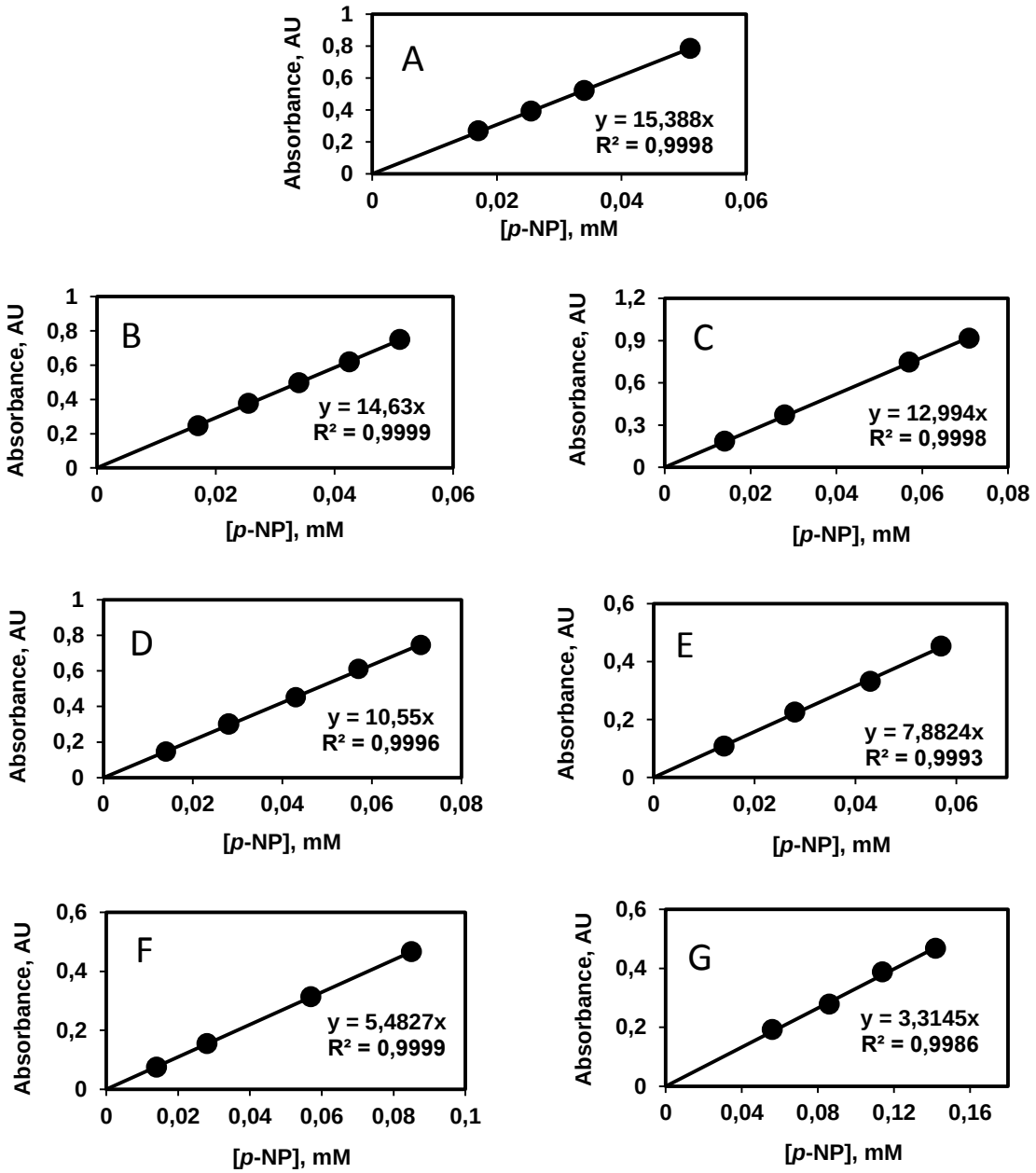

Figure 4.29. Calibration curves for *p*-NP in the presence of (A) 10%, (B) 20%, (C) 30%, (D) 40%, (E) 50%, (F) 60%, (G) 70%, (H) 80%, and (I) 90% ethanol v/v.

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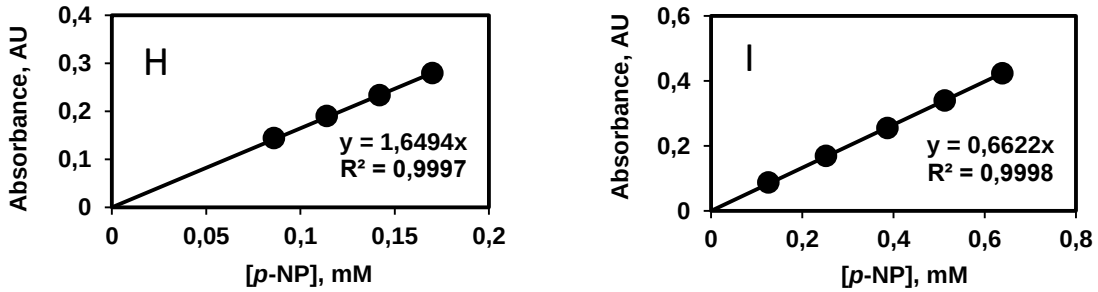

Figure 4.29 (cont.)

The absorptivities and absorbance values at 404 nm derived from the spectrum profile of *p*-NP are shown in Figure 4.30. As can be seen in the figure, the absorptivity and absorbance values for *p*-NP decreased with increasing organic solvent content and almost overlapped each other, which confirmed the calibrations established for *p*-NP. The same result was also seen in the presence of acetonitrile.

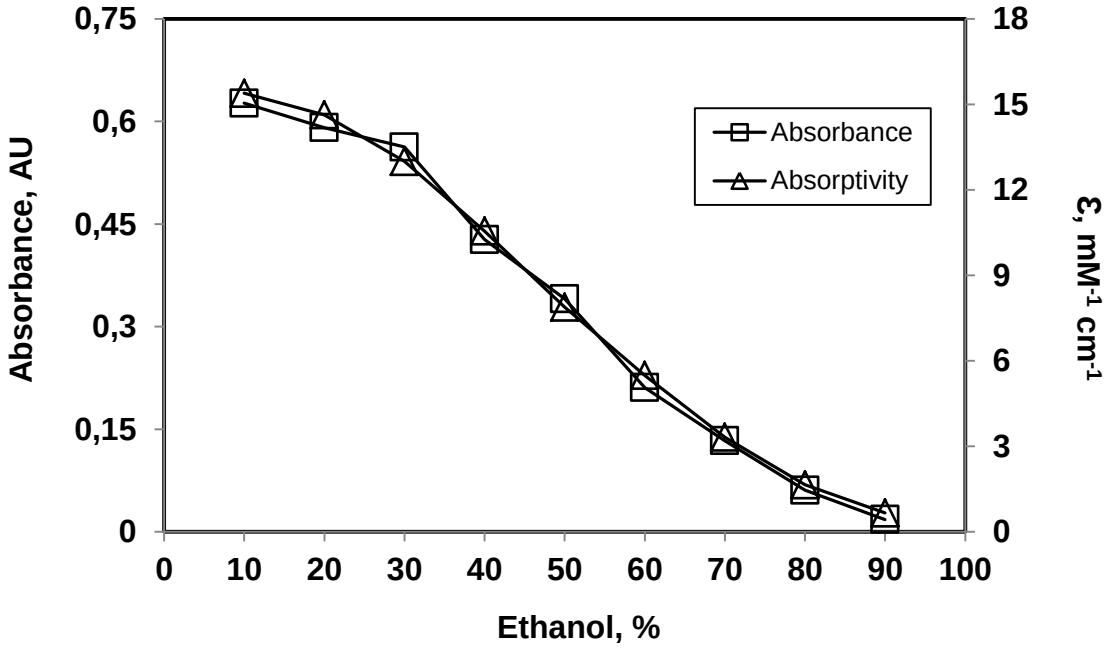

Figure 4.30. Change of the absorbance and absorptivity at 404 nm for *p*-NP with increasing ethanol percentages in the mixture (v/v).

4.6.3. Self-Hydrolysis of *p*-NPA in the Presence of Ethanol

The absorbance scanning for self-hydrolysis of *p*-NPA was performed at different ethanol concentrations while keeping the *p*-NPA concentration constant. Figure 4.31 shows the absorbance values of *p*-NP as a product of self-dissociation of *p*-NPA at different percentages of ethanol with respect to wavelength. No peak occurred due to ongoing hydrolysis process while measuring the absorbance of the samples. Figure 4.32 shows the absorbance values of *p*-NP as a product of the self-hydrolysis of *p*-NPA at 404 nm. As seen in the figure, as the ethanol concentration in the mixture was increased, the absorbance values of *p*-NP decreased. This could be attributed to the lower self-hydrolysis rate at higher organic solvent concentrations. From the figure, it could be seen that the absorbance values of p-NP were very low which could be negligible during the enzyme activity assays.

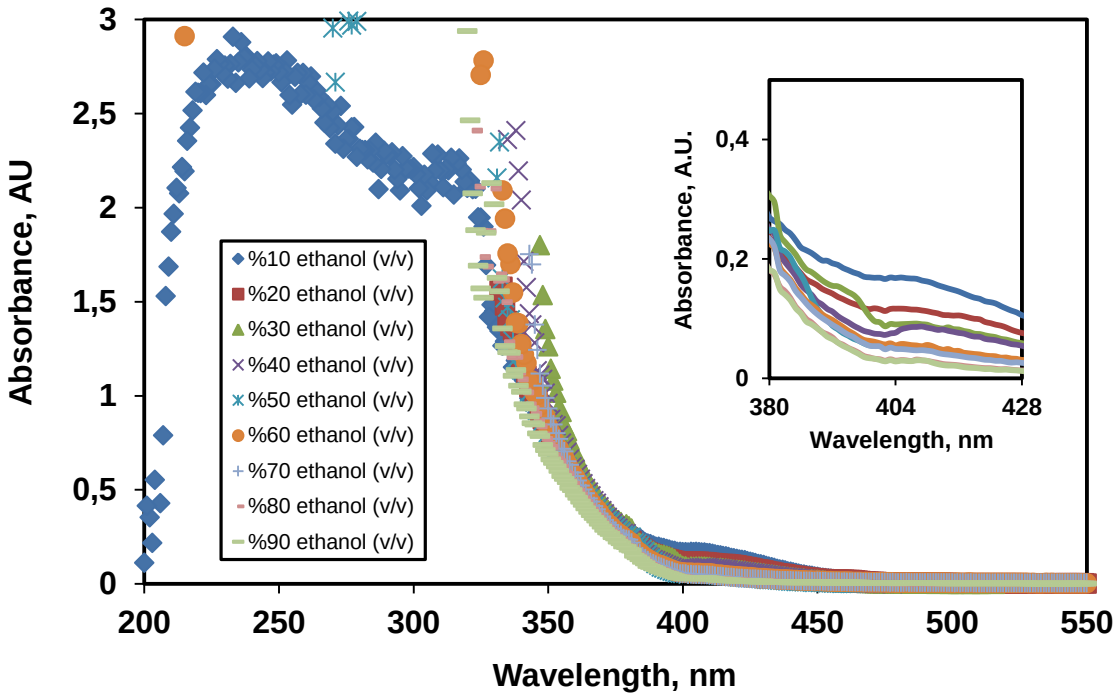

Figure 4.31. The spectrum profile for self-hydrolysis of *p*-NPA in various percent volume ethanol/buffer mixtures.

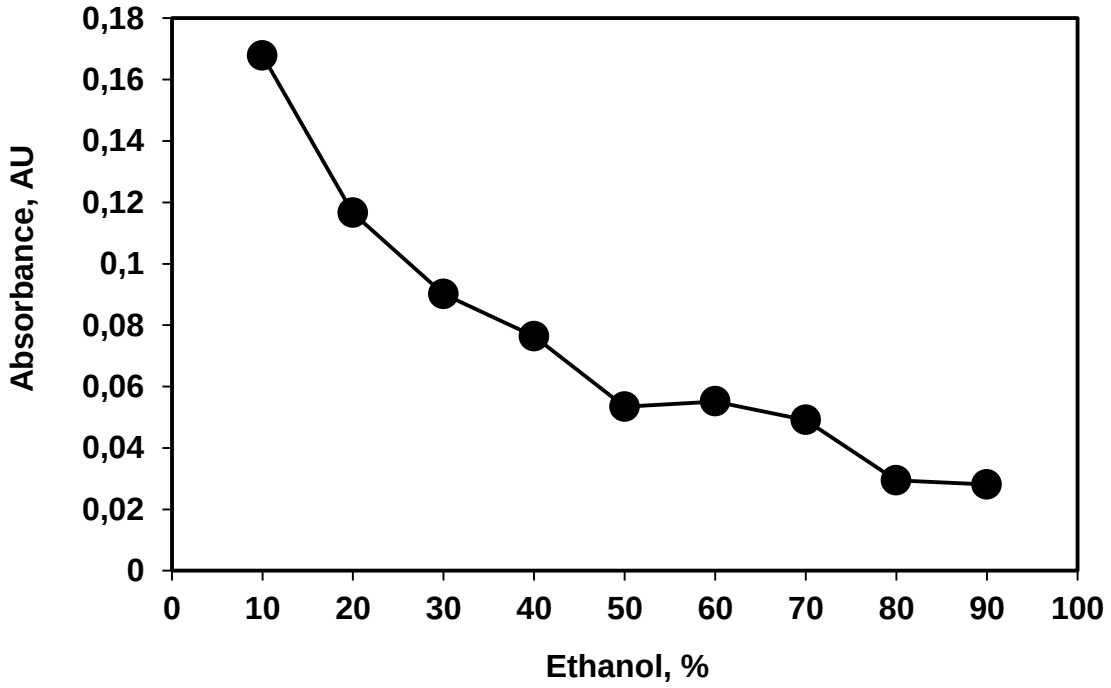

Figure 4.32. *p*-NP absorbance at 404 nm with increasing percentages of ethanol in the mixture by volume.

4.6.4. Activitiy of Free and Immobilized CA in Ethanol

The acitivity of the free enzyme was estimated at different ethanol concentrations while keeping both substrate and enzyme concentration constant. The self-hydrolysis rate determined for each buffer/organic solvent mixture were subtracted from the raw data of the enzyme-catalyzed hydrolysis rate in order to determine the actual enzymatic rate. Figure 4.33 shows the enzymatic rate in different percent volume ethanol/buffer mixtures. In the presence of ethanol, the free CA showed similar behaviour as well as in acetonitrile: the activity significantly decreased with increasing percentenages of ethanol up to 50-60% v/v, but at higher concentrations the enzyme exhibited increasing activity.

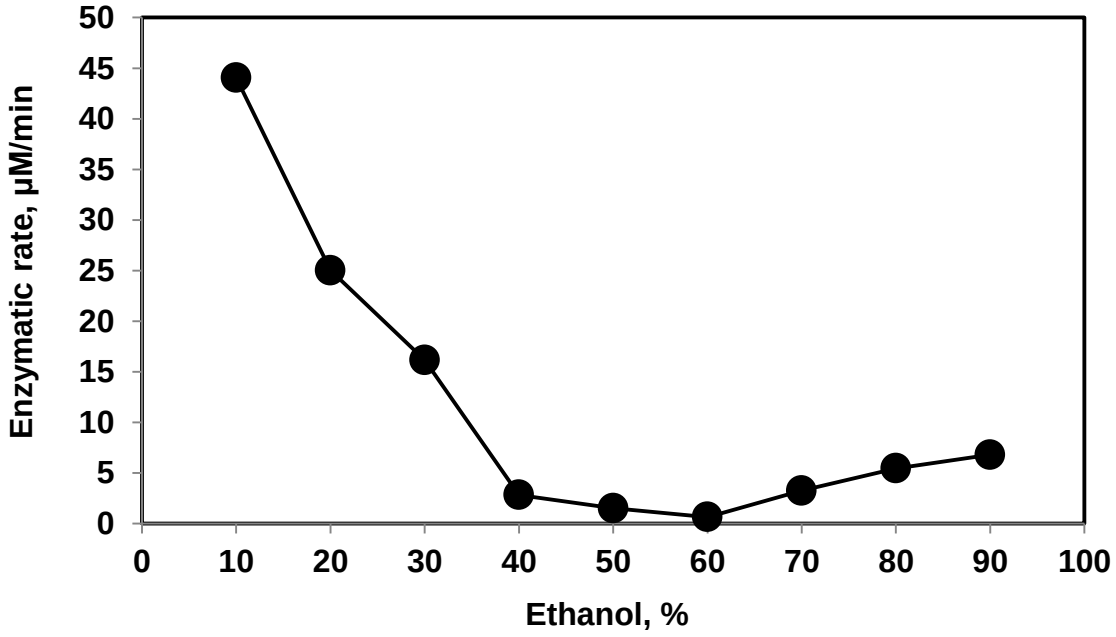

Figure 4.33. The enzymatic rates for free CA in different percent volume ethanol/buffer mixtures.

The activity of the immobilized CA was estimated through the liberation of *p*-NP from the PU foam over time by the hydrolysis of *p*-NPA. The immobilized enzyme assays were employed at various ethanol concentrations keeping the substrate concentration constant. Figure 4.34 shows the immobilized CA activity in increasing percentages of acetonitrile by volume. As seen in the figure, there was a drastic decrease in the activity up to 20% v/v and above 20% v/v, the enzyme lost all of its activity. This indicated that the effect of ethanol on the immobilized enzyme was to a greater extent than those on the immobilized enzyme exposed to acetonitrile.

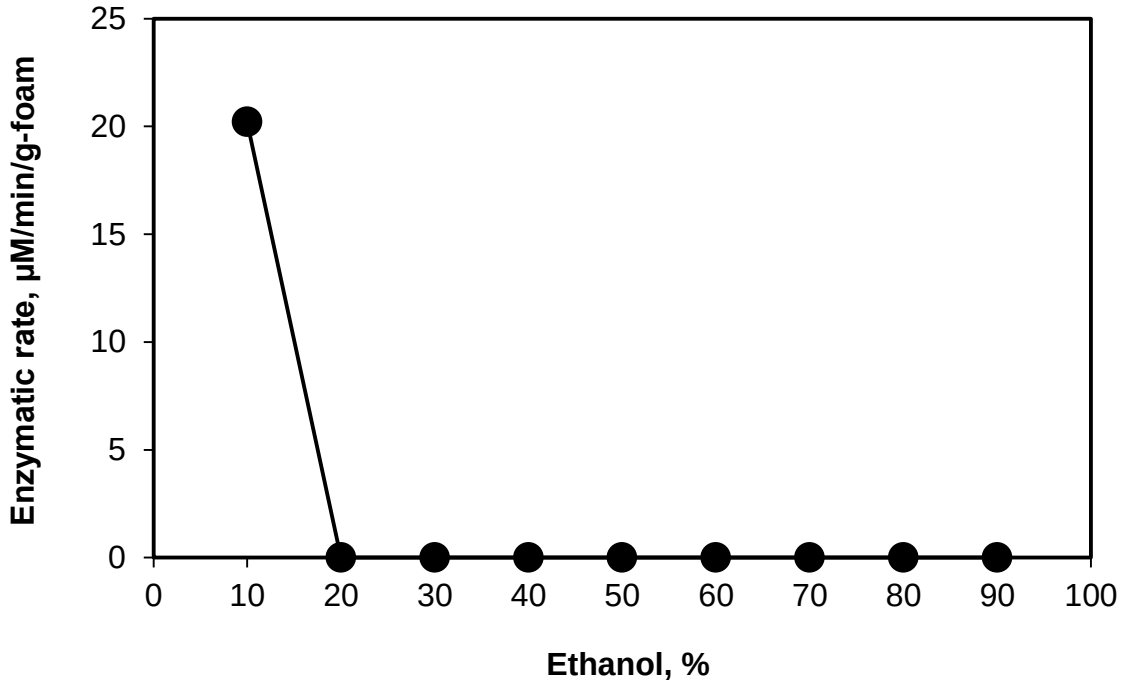

Figure 4.34. The activity of the immobilized CA in increasing percentages of ethanol by volume.

4.6.5. Stability of Free and Immobilized CA in Ethanol

In stability tests, the activities of the free and immobilized enzyme were estimated using the same substrate concentrations at various percent volume ethanol/buffer mixtures. Figure 4.35 shows the relative activities of the free and immobilized CA over time, respectively. After storage in 10% v/v ethanol for 36 h, immobilized CA exhibited 100% of its original activity, while free CA regained about 60% of its original activity. However, after storage in 20% and 30% v/v ethanol for 36h, immobilized CA exhibited 68% and 50% of its original activity, respectively, while free CA exhibited almost the same result. After storage in 40% and 50% v/v ethanol for 1.5 h, immobilized CA nearly lost all of its activity, while free CA still exhibited about 75% of its original activity. After storage in 60% and 70% v/v ethanol for 30 minutes, immobilized CA lost its all activity, while free enzyme showed some activities. After storage in 80% v/v ethanol for 1.5 h, immobilized CA lost all of its activity, while free CA exhibited some activities. Immobilized CA lost all of its activity after storage in 90% v/v ethanol for 15 minutes, while free CA showed surprisingly 45% of its original activity even after storage for 24 h.

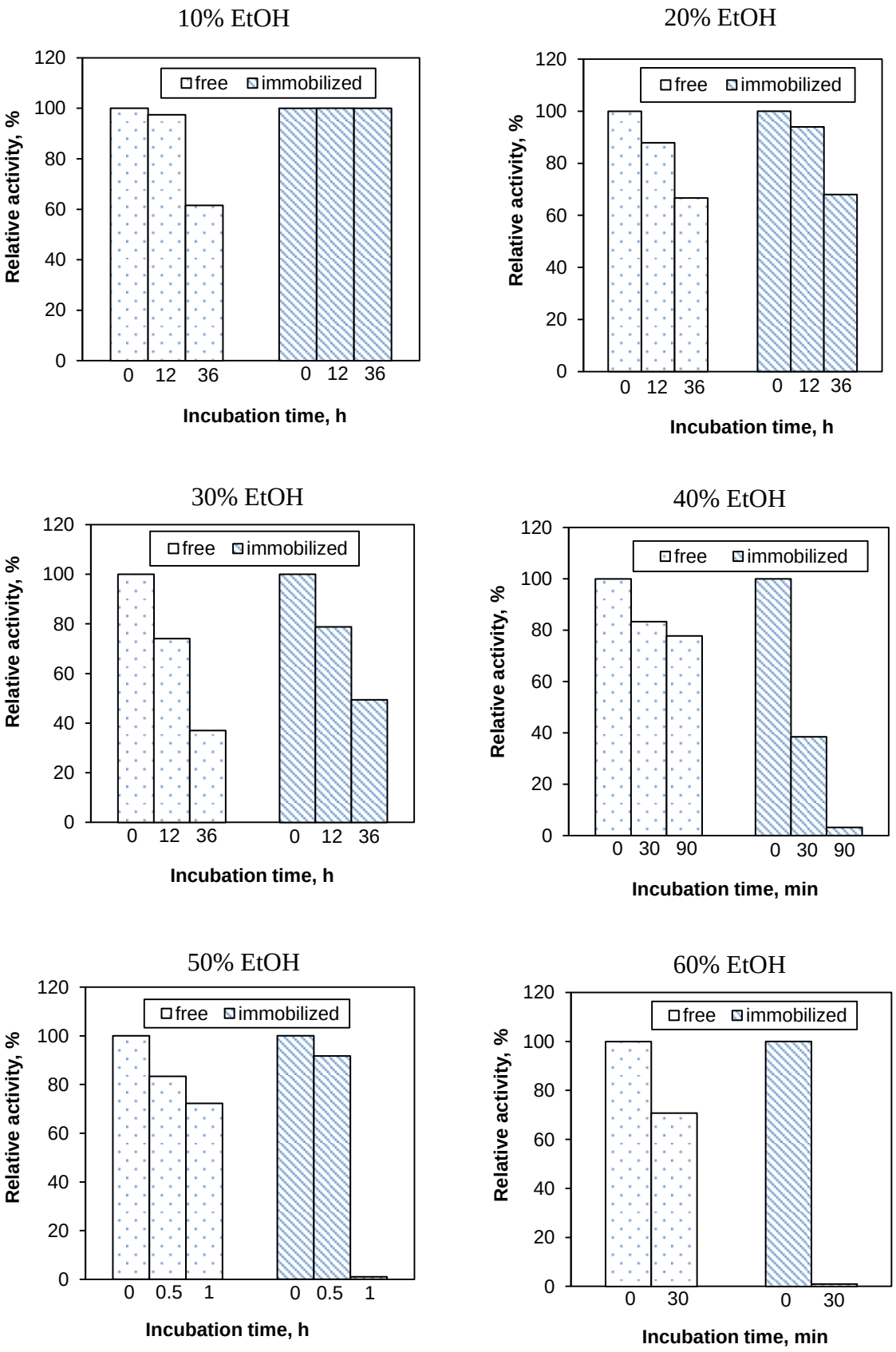

Figure 4.35. Stability of the free and immobilized CA in the presence of ethanol.

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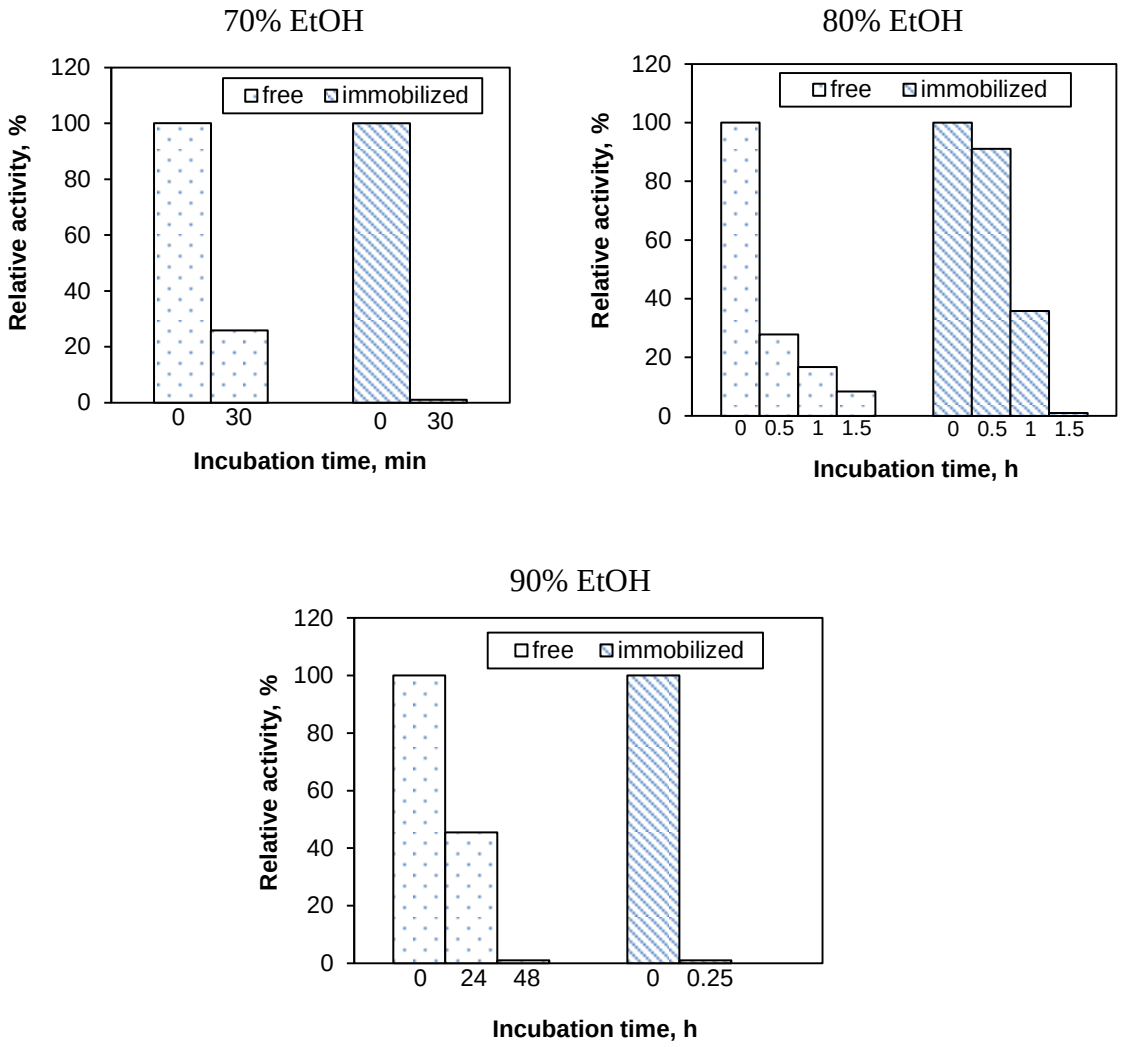

Figure 4.35 (cont.)

Immobilized enzyme showed significant activities after storage at low volume percents of ethanol and returning back to Tris buffer containing 10% ethanol. However, as seen in the activity assays (Figure 4.34), above 10% v/v ethanol the immobilized CA exhibited no activity. This could be attributed to the interactions between ethanol and active site of the enzyme immobilized within PU foam. Organic solvent molecules may perturb the hydrophobic shell on the surface of the active centre of the enzyme molecule, where hydrogen bonding and hydrophobic interactions are responsible for the balance of the enzyme's conformation, and the associated enzyme activity. Organic solvents also may remove water molecules from the enzyme surface. If the new solvent molecules could not maintain the hydrogen bonding/hydrophobic interactions, then the required enzyme conformation is lost, thus the enzyme activity. Some organic solvents can successfully maintain such interactions, however they may block to a greater or

lesser extent the active site of the enzyme and prevent access to the substrate, thereby decreasing enzyme activity (Wan, Lu et al. 2010). In our system, during the enzymatic reaction within the PU foam, the substrate or the product presumably could not diffuse in and out of the foam due to the blocking caused by ethanol, hence we could not detect any change for *p*-NP absorbance in the UV/vis spectrophotometer. In the presence of ethanol, free CA generally exhibited greater stability than the immobilized CA. Hydroxyl groups of water-miscible organic solvents are incompatible with the hydrophobic regions of enzymes, but can participate in hydrogen bonding to generate rigid intermolecular frameworks, resulting in high kinetic barriers (kinetic trapping) preventing unfolding of the enzyme (Wan, Lu et al. 2010). Thus, the free CA still exhibited some of its original activity in Tris buffer after storage at high volume percents of ethanol. On the contrary, immobilized enzyme was fixed by cross-linking within PU foam, therefore the enzyme presumably lost its conformational flexibility, and thus enzyme activity in the presence of the organic solvent.

CHAPTER 5

CONCLUSION

Biocatalytic performance of CA immobilized within PU foam was investigated in the presence of water-miscible organic solvents. It was experimentally found that the PU foam could hold up to 12 times of its weight in aqueous media. It was also demonstrated that the adsorption isotherm of moisture on the PU foam corresponded to a Type III adsorption isotherm indicating that water adsorbed on the PU foam either by capillary condensation and/or adsorption with a multimolecular layer on the PU foam. However, while expecting a high water adsorption capacity, PU foam showed a Type III adsorption isotherm, indicating that, indeed, there were very weak water-PU foam interactions. *p*-NP, one of the products of the hydrolysis reaction of *p*-NPA, was characterized in the presence of the organic solvents, acetonitrile and ethanol. The absorbance of *p*-NP in the UV/vis spectrophotometer decreased significantly as the concentration of the organic solvent in buffer solution was increased, while keeping *p*-NP concentration constant. During the absorbance scanning, it was observed that a chemical shift occured towards the protonated form of *p*-NP with increasing organic solvent content in the mixtures, resulting in a switching in absorption in the organic solvent/buffer mixtures. The absorbance decline for *p*-NP could be attributed to the possible interactions between the organic solvent and *p*-NP. The CA was successfully immobilized within PU foam. Because the PU foam was a highly porous polymeric material and immobilization of the enzyme was easy and fast, the PU foam was used as a stabilizing carrier. The activity of the free and immobilized CA was estimated in various percent volume organic solvent/buffer mixtures. In the case of acetonitrile, the activity of the free CA diminished as the organic solvent concentration was increased up to 50% v/v, and at higher acetonitrile concentrations the free CA exhibited increasing activity as if a u-shape. Immobilized CA exhibited decreasing activity in acetonitrile at percentages up to 50% v/v, and then no activity was seen at higher acetonitrile percentages. In the presence of ethanol, the immobilized CA exhibited no activity at organic solvent percentages above 10% v/v, while the free enzyme showed similar behaviour in the presence of acetonitrile. In stability tests, it was observed that the organic solvents at percentages above 30% v/v dramatically inactivate the immobilized

enzyme in shorter times. It was concluded that the water-miscible organic solvents severely perturbed the active site of the enzyme by removing the bound water molecules from its hydration shell, thus denaturating the enzyme.

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