ENZYMATIC CO_2 SEQUESTRATION BY CARBONIC ANHYDRASE

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
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December, 2008

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ACKNOWLEDGMENT

I would like to express my gratitude to my supervisor Ekrem ÖZDEMİR for his valuable advice, help, and support. Without him, this thesis could not be realized. I also express my gratitude to my co-adviser Ahmet YEMENİCİOĞLU for his valuable advice.

I also would like to thank to my labmates Murat MOLVA and Mehmet GÖNEN for their valuable help and friendship.

Finally, but not the least, I want to extend my appreciation to my family, my love Pınar GELGEÇ, and my friends for their endless support and motivation during all my school years.

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ABSTRACT

ENZYMATIC CO_2 SEQUESTRATION BY CARBONIC ANHYDRASE

Carbonic Anhydrase (CA) was immobilized within Polyurethane (PU) Foam and characterized for CO_2 sequestration purposes. The catalytic activities for the free and immobilized CA were estimated by using p-Nitrophenyl Acetate (p-NPA) as the substrate. The activities were estimated in tris buffer containing 10% Acetonitrile. Because, the p-NPA is only soluble up to 4mM in aqueous phase. Lineweaver-Burk relationship was employed to estimate the Michaelis-Menten kinetic parameters for the free and immobilized CA. The k_{cat}, K_M, and k_{cat}/K_M values for free enzyme were found to be $1.21s^{-1}$, 12.2mM, and $148.1M^{-1}s^{-1}$, respectively. The K_M value for immobilized BCA was estimated to be 9.59mM in tris buffer (50mM, pH=7.5), in the presence of 10% acetonitrile at the same conditions. The immobilized CA was stable and did not loose any activity over seven consecutive washings and activity tests. In addition, while the free CA lost its activity in 45 days stored at 4^oC in fridge, the immobilized CA was stable and did not loose any activity over 45 day period. The optimum temperature for the immobilized BCA was found to be between 35^oC and 45^oC. No activity was observed for the immobilized CA at 60^oC. Thermal deactivation energies for the free and immobilized CA were found to be 29kcal/mol and 86kcal/mol, respectively. The immobilized CA was employed in $CaCO_3$ precipitation. It was found that the $CaCO_3$ particles were less than 100nm and more evenly dispersed. It was concluded that the immobilized CA could be used in CO_2 sequestration.

ÖZET

KARBONİK ANHİDRAZ ENZİMİ İLE CO_2 DEPOLAMA

Karbonik Anhidraz (CA) enzimi karbon dioksit depolamak amacı ile poliüretan (PU) sünger içerisine immobilize edildi ve karakterizasyonu yapıldı. Serbest ve immobilize edilmiş enzimin katalatik aktiviteleri substrat olarak p-nitrofenol asetat (p-NPA) kullanılarak belirlendi. Sulu ortamda p-NPA 4mM'a kadar çözünebildiğinden aktiviteler %10 asetonitril içeren tris tampon çözeltisi içerisinde ölçüldü. Serbest ve immobilize enzimin Michaelis-Menten sabitlerini hesaplamak için Lineweaver-Burk denklemi kullanıldı. Serbest enzimin k_{cat}, K_M, ve k_{cat}/K_M değerleri sırasıyla $1.21s^{-1}$, 12.2mM ve $148.1M^{-1}s^{-1}$ olarak hesaplandı. İmmobilize enzimin K_M değeri ise $9.59s^{-1}$ olarak bulundu. İmmobilize edilmiş CA ard arda 7 defa yıkama ve aktivite testinden sonra dahi hala herhangi bir aktivite kaybı olmamaktadır. Buna ilave olarak, immobilize enzimin tris tampon çözeltisi içerisinde saklandığında 45 gün boyunca aktivitesini koruduğu, serbest enzimin ise 4^oC'de buzdolabında saklandığında aynı süre içinde neredeyse bütün aktivitesini kaybettiği görüldü. İmmobilize enzimin en yüksek aktiviteyi 35^oC ile 45^oC arasında gösterdiği bulundu; ancak 60^oC sıcaklıkta immobilize CA'da herhangi bir aktivite gözlemlenmedi. Serbest ve immobilize enzimin ısısal bozunma enerjisi sırasıyla 29kcal/mol ve 86kcal/mol olarak hesaplandı. İmmobilize enzim $CaCO_3$ çöktürme deneylerinde kullanıldı. $CaCO_3$ taneciklerinin boyutunun 100nm'den düşük olduğu ve boyut dağılımının daha az aralıkta olduğu bulundu. Poliüretan sünger içerisine immobilize edilmiş CA enzimi CO_2 depolanmasında kullanılabileceği değerlendirilmektedir.

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CHAPTER 1

INTRODUCTION

Atmospheric concentration of carbon dioxide (CO_2) has been increasing due to human induce (anthropogenic) activities. There is a general consensus among the scientists that high concentrations of CO_2 in the atmosphere cause global warming (NASA 1998). Future predictions show that the atmospheric concentrations of CO_2 will continue to increase if the production and release of CO_2 to the atmosphere is not controlled (Ramanathan 2006).

The Kyoto Protocol signed in 1997 requires that the industrialized nations need to reduce their CO_2 emissions to 95% of their 1990 levels by 2012 (Bolin 1998). Thus, many researches have focused on the development of methods for the mitigation of the CO_2. One of the strategies to control the CO_2 concentration in the atmosphere is to reduce its production and release into the atmosphere. For this purpose, less carbon intensive energy sources such as wind energy, solar energy, and nuclear energy, could be used. The other strategy could be to increase the efficiency of the energy use, from production to its end use (Reichle, et al. 1999). However, these two options are not practically possible. Therefore, a third option is required which is to capture and dispose the produced CO_2 in a safe manner (sequestration) (Reichle, et al. 1999, Herzog and Drake 1996)

A number of CO_2 sequestration options have been proposed. These are placement in the deep oceans; placement in geologic formations such as deep saline aquifers, abandoned oil or gas reservoirs, and unmineable coal seams, and consumption via advanced chemical and biological processes (Reichle, et al, 1999). However, none of these options has been proven to be ideal for CO_2 sequestration and are still under investigation. Nevertheless, the CO_2 has to be pure in these sequestration processes (Reichle et al, 1999, Herzog and Drake 1996, Bachu 2000, Gentzis 2000). That is, the CO_2 has to be captured from flue gases, to be compressed, to be transported, and finally, to be injected into the desired sequestration sites, which all together requires increasing the electric bill to the customers.

We propose that the sequestration cost could be reduced if the CO_2 is captured and sequestered directly at the production sites. Furthermore, the production of valuable

products from the CO_2 could be possible such as the nano-sized precipitated $CaCO_3$, which make the sequestration process economically desirable, and even profitable.

In order to capture CO_2 from the flue gases, the CO_2 should undergo a number of transformations such as the dissolution in an aqueous phase, hydration by water, ionization, and carbonate formation. Among these, the hydration of the CO_2 is the slowest step. It has been shown that the hydration of CO_2 can be enhanced by Carbonic Anhydrase (CA) (Bond, et al. 2001). However, there are some disadvantages using the free enzyme in solution such that the stability of the enzyme is low, its repeatable usage is limited, and recovery from the reaction environment generally won't be possible. These disadvantages can be eliminated by immobilizing the enzyme. There are some enzyme immobilization techniques such as entrapment in matrices, adsorption on the solid surfaces, covalent bonding, and cross-linking within polymeric networks (Shuler et al., 2002). Carbonic anhydrase has been immobilized on some solid and polymeric supports such as acrylamide, chitosan, chitosan-alginate bead (Liu, et al. 2005), porous silica and graphite bead, within the poly(acrylic acid-co-acryamide) hydrogel (Cheng et al., 2008), and on a hydrophobic adsorbent of Sepharose 4B via hexyl, octyl, dedocyl, and palmitive glycidyl ethers couplings (Hosseinkhani and Gorgani 2003). However, to our knowledge, the Carbonic Anhydrase has not been immobilized within polyurethane foam. Therefore, our aim in this study is to immobilize CA within polyurethane foam, to characterize the immobilized CA, and to employ the immobilized CA in $CaCO_3$ precipitation for CO_2 sequestration purposes.

CHAPTER 2

LITERATURE REVIEW

2.1. The Problem of CO_2 and Global Warming

Along with the industrialization, the atmospheric concentrations of greenhouse gases (GHGs) such as carbon dioxide (CO_2), methane (CH_4), chlorofluorocarbons (CFCs), and nitrous oxides (NO_x) are increasing due to human induced (anthropogenic) activities (Hansen, et al. 1997). CO_2 is the most abundant greenhouse gas, which is produced mainly by the burning of fossil fuels such as coal, oil, and natural gas. The CO_2 concentration in the atmosphere has increased from 280 ppm during the preindustrial era to about 380 ppm in 2007 with an accumulation rate of about 1.5 ppmv per year.(Halman and Steinberg 1999). The predictions indicate that the CO_2 emissions will continue to increase in the future as the fossil fuels will continue to be the major energy sources. (IPCC 1996)

In 1896, Svante Arrhenius, Swedish chemist, introduced that industrial revolation would increase the CO_2 concentration in the atmosphere because of the world's consumption of fossil fuels, particularly coal (Ramanathan 2006). He also reported that overall temperature of world would increase several degrees. This increase causes "Global warming" which is the increase in the average temperature of the Earth's near-surface air and oceans. (NASA 1998)

The CO_2 sequestration requires a number of cost effective processes. For instance, first, the CO_2 is to be captured from its production sources such as flue gases from coal firing power plants. Second, the CO_2 is compressed to the injection pressures and for the liquification. Third, the compressed CO_2 is transported to the injection site. And, finally, the CO_2 is injected into the sequestration site. The approximate costs for these processes are summarized in Table 2.1. As shown in the table, the total cost is about \$41-\$72 per tone of CO_2 sequestered, which adds up about \$0.45/kW to the customer's electric bills (Wong and Bioletti 2002, McCoy and Rubin 2008, Abadie and Chamorro 2008).

Table 2.1. Estimated Sequestration cost for CO_2 .
(Source: Wong and Bioletti 2002)

Sequestration Step	**Cost**
Seperation (amine process) ($/tone CO_2)	30-50
Compression ($/tone CO_2)	8-10
Transportation (pipelining) ($/tone/100 km)	0,7-4
Injection ($/tone CO_2)	2-8
Total	41-72

The sequestration cost could be reduced if the CO_2 is captured and directly converted into stable products at the production sites without further compression, transportation, and injection processes. For instance, the CO_2 readily reacts with the Ca^{2+} ions in solution at pH higher than 8.0 to produce $CaCO_3$. However, as shown in equation (2.1-2.5), the gaseous CO_2 needs to dissolve in water to produce loosely hydrated aqueous form. The hydration of CO_2 (equation (2.2)) is reported as the slowest step (Bond et al., 2001, Mirjafari, et al. 2007).

$$CO_{2(g)} \leftrightarrow CO_{2(aq)} \qquad \text{Dissolution} \qquad (2.1)$$

$$CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \qquad \text{Hydration} \qquad (2.2)$$

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \qquad \text{Dissociation} \qquad (2.3)$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \qquad \text{Carbonate Formation} \qquad (2.4)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \downarrow \qquad \text{Precipitation} \qquad (2.5)$$

The hydration of CO_2 can be accelerated by the Carbonic Anhydrase enzyme. As shown in equation (2.6-2.8), the biological catalyst, the enzyme carbonic anhydrase (CA), is able to accelerate the interconversion between CO_2 and carbonate form (Khalifaht, et al. 1972), and then carbonate ion can be precipitate in presence of the calcium cation to get valuable material. Therefore, the on-site capturing process will

serve not only for the CO_2 sequestration but also the production of nano-sized $CaCO_3$ particles which could even make the process profitable.

$$E \bullet ZnH_2O \leftrightarrow EZnOH^- + H^+ \quad \text{Activation} \quad (2.6)$$

$$E \bullet ZnOH^- + CO_2 \leftrightarrow EZnHCO_3^- \quad \text{Association} \quad (2.7)$$

$$E \bullet ZnHCO_3^- + H_2O \leftrightarrow EZnH_2O + HCO_3^- \quad \text{Hydration} \quad (2.8)$$

2.2. The Enzymes are Proteins

Proteins are linear polymeric molecules; basic building blocks of proteins are peptide residues. Peptide residues consist of amino acids, which are connected together by peptide bonds that link the carboxyl group of one amino acid to the amino group of the next. In nature, there are 20 different amino acids which are different from each other in the side chain group, which is connected to the α-carbon atom between the amino group and the carboxyl group (Mathews, et al. 2000). Amino acid sequence and length of protein determine the diversity of proteins (Host 2007).

Enzymes are proteins which have high molecular weight between 15000 and several million daltons that acts as a catalyst. Enzymes are specific, versatile, and very effective biological catalyst, resulting in much higher reaction rates as compared to chemically catalyzed reactions under ambient conditions. Enzymes are named by adding suffix –ase to the end of the substrate, such as urase, or the reaction catalyzed, such as alcohol dehydrogenase. Like a folded polypeptide chain, some enzymes have a simple structure. But, most of the enzymes have more than one subunit. These are divided into two groups. The first one is cofactors which are metal ions, Mg, Zn, Mn and Fe. The second group is coenzymes which are complex organic molecule such as NAD, FAD, CoA and some vitamins. An enzyme containing nonprotein group is called a holoenzyme. The protein part of the holoenzyme is the apoenzyme. Although, some enzymes have different molecular structures, they can catalyze the same reaction; these types of enzymes are called as isoenzymes. Enzymes are substrate specific and they are also classified according to the reaction that they catalyze (Lodish, et al. 1986, Whitford 2005).

Similar to the inorganic catalysis, enzymes decrease the activation energy of the reaction catalyzed by binding the substrate and forming an enzyme-substrate complex. The free-energy change or the equilibrium constant is not affected by enzymes. For

example, the activation energy for the uncatalyzed decomposition of hydrogen peroxide is 18 kcal/mol at 20°C. But the activation energy for the enzymatically catalyzed decomposition is 7 kcal/mol. In other words, the enzyme accelerates the rate of reaction by a factor of about 10^8. Small change in activation energy cause huge change in the reaction rate because of the exponential dependence of the rate on the activation energy. (Lodish, et al. 1986, Branden and Tooze 1991)

2.2.1. The Catalytic properties of Enzymes

There are weak forces between the enzyme and its substrate when they are in interaction. Usually, van der Waals forces and hydrogen bonding take places in formation of an enzyme-substrate (ES) complex. The enzyme is much larger molecule than substrate which fits into a certain region on the enzyme molecule. This certain region is called as active site of the enzyme. As shown in Figure 2.1, lock-and-key model is the simplest model which describes this interaction, in which the enzyme represents the lock, and the substrate represents the key. (Branden and Tooze 1991, Whitford 2005)

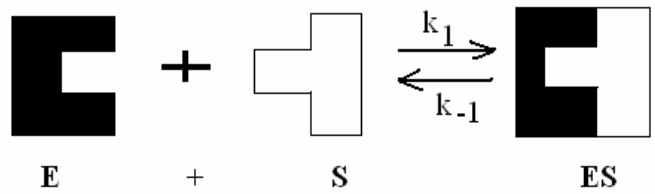

Figure 2.1. Schematic of the lock-and-key model of enzyme.
(Source: Branden and Tooze 1991)

In catalytic processes of enzymes, firstly, the enzyme and its substrate build reversible enzyme-substrate (ES) complex. In second step, chemical reaction occurs with a rate constant k_{cat} called turnover number. The k_{cat} is the maximum number of substrate molecules converted into product molecules per active site of enzyme per unit time (Branden and Tooze 1991, Whitford 2005).

$$E + S \xleftrightarrow{K_M} ES \xrightarrow{k_{cat}} E + P \qquad (2.9)$$

A typical enzymatic reaction curve is shown in Figure 2.2 The reaction curve can be represented by a Michealis-Menten Equation, equation (2.10).

$$R = \frac{k_{cat}\,[E]\,[S]}{K_M + [S]} \tag{2.10}$$

When substrate concentration is high, all the enzyme is saturated with the substrate and the reaction rate approaches its maximum, R_{max}. When the rate of the enzymatic reaction is the half of the maximum rate, at that time, the value of the substrate concentration is called as K_M, which describes dissociation constant of the ES complex and the affinity of the enzyme for the substrate. (Branden and Tooze 1991, Whitford 2005)

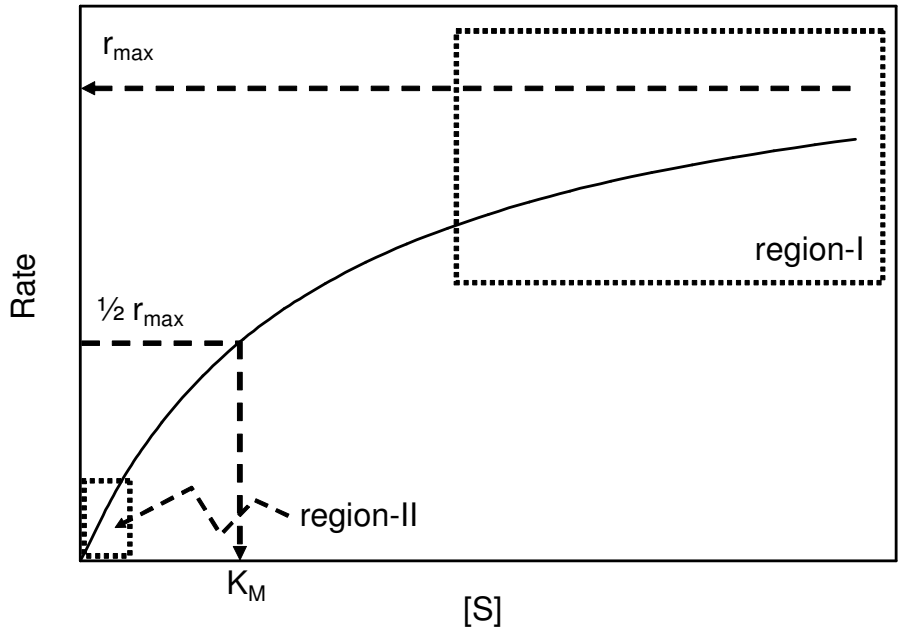

Figure 2.2. Enzymatic Reaction Curve.

Kinetic constants can be estimated by either fitting equation (2.10) to the experimental data or by using the double-reciprocal Lineweaver-Burk form:

$$\frac{1}{R} = \frac{1}{R_m} + \frac{K_M}{R_m}\frac{1}{[S]} \tag{2.11}$$

A plot of 1/R versus 1/S yields a linear line with a slope of K_M/R_m and intercept of $1/R_m$ as shown in Figure 2.3.

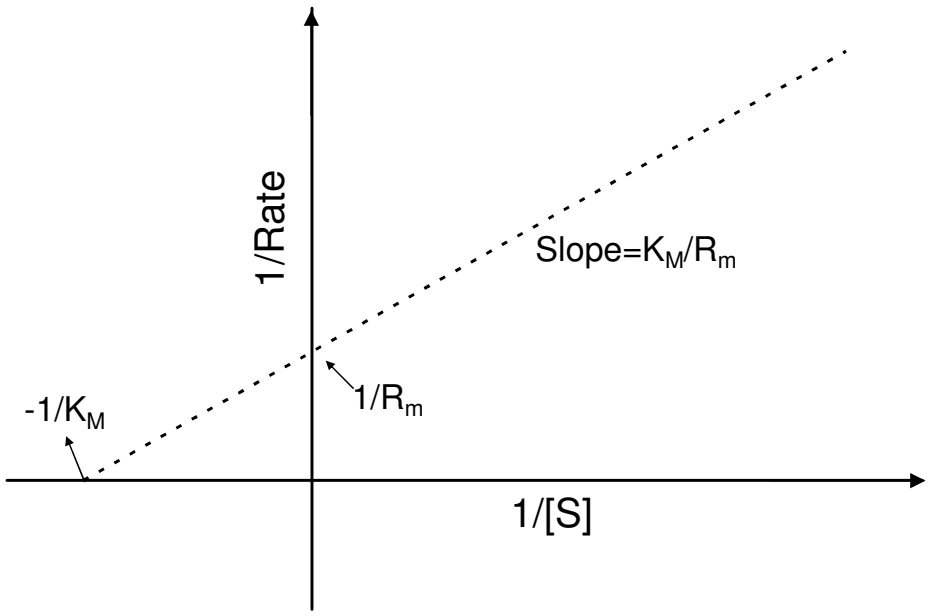

Figure 2.3. Double-reciprocal (Lineweaver-Burk) Plot.

2.2.2. Advantages and Disadvantages of Using Enzymes

There are some practical problems in the use of enzyme in the industrial applications. These are the high cost of isolation and purification of enzymes, the instability of their structures once they are isolated from their natural environments, and their sensitivity both to process conditions, and to trace levels of substances that can act as inhibitors. These problems result in short operational lifetime of enzyme. Most of the enzymes work in dissolved water for a homogeneous catalysis system. Therefore, they contaminate the product and, as a rule, cannot be recovered in the active form from reaction mixtures for reuse (Krajewska 2004).

The most successful method to overcome these limitations is to immobilize the enzymes. Immobilization is achieved by fixing enzymes to or within solid supports. As a result, heterogeneously immobilized enzyme systems are obtained. In nature, generally, enzymes are attached to the cellular membranes to stabilize their structure and activity. Moreover, immobilization causes a heterogeneous system which allows easy recovery of both enzyme and the product, multiple reuses of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and greater variety of bioreactor designs (Krajewska 2004).

2.3. Enzyme Immobilization

Generally, types of immobilization of enzymes are divided into two main groups. The first one is physical immobilization, where weak interactions exist between support and the enzyme, the second one is the chemical immobilization, where covalent bonds play role. Both physical and chemical immobilizations have several advantages and disadvantages. In the chemical immobilization, the covalent attachment of an enzyme to a support causes some activity loss. Such covalent bonds provide strong and stable enzyme attachment and in most cases can reduce the enzyme deactivation rates. In the physical immobilization, the attachment of the enzyme to the carrier is weak (Romaškevič, et al. 2006). Some chemical and physical immobilization techniques are summarized in Table 2.2 (Krajewska 2004).

Table 2.2. Immobilization techniques.
(Source: Krajewska 2004)

<table>
<tr><th>Physical immobilization methods</th><th>Chemical immobilization methods</th></tr>
<tr><td>Containment of an enzyme within a membrane reactor,</td><td rowspan="2">Covalent attachment to a water-insoluble matrix,</td></tr>
<tr><td>Adsorption (physical, ionic) on a water-insoluble matrix,</td></tr>
<tr><td>Inclusion or gel entrapment,</td><td rowspan="2">Crosslinking with use of a multifunctional, low molecular weight reagent,</td></tr>
<tr><td>Microencapsulation with a solid membrane</td></tr>
<tr><td>Microencapsulation with a liquid membrane</td><td rowspan="2">Co-crosslinking with other neutral substances.</td></tr>
<tr><td>Formation of enzymatic Langmuir-Blodgett films</td></tr>
</table>

The Covalent attachment of anenzyme is based on the binding of enzymes to water- insoluble carriers by covalent bonds. Some functional groups play role in this process. These are amino group, carboxyl group, sulfhydryl group, hydroxyl group, imidazole group, phenolic group, thiol group, threonine group and indole group (Romaškevič, et al. 2006).

2.3.1. Polyurethane Foam (PU) for Immobilization

Polyurethanes are widely used supports for immobilization of enzymes and cells. Researchers, who pay attention to immobilization of enzymes to PU, have noticed that PU is a good carrier for enzymes. The PU foam is an environmentally friendly material. Wood et al., in 1982, have patented a method of irreversibly immobilizing enzymes within polyurethanes (US Patent 4342834). The enzyme-containing polyurethane could reach the degree of immobilization of the enzyme to approximately 100% (US Patent 4342834). Today, their method is still used in immobilization assays and includes mixing of prepolymer HYPOL and aqueous enzyme solution together. As shown in Figure 2.4, isocyanates groups of polyurethanes play important role in immobilization. During the polymerization, water reacts with the isocyanates group and produces an amine group (NH_2) and cause to release of carbon dioxide. . The produced CO_2 causes porous and spongelike matrix of PU foam. At the same time the isocyanate groups also react with amine and/or hydroxyl group of the enzyme. The advantage of this assay is that no unreacted isocyanates groups are left. Therefore, enzyme can not be inhibited from the unreacted group. (Bakker, et al. 2000, Romaškevič, et al. 2006)

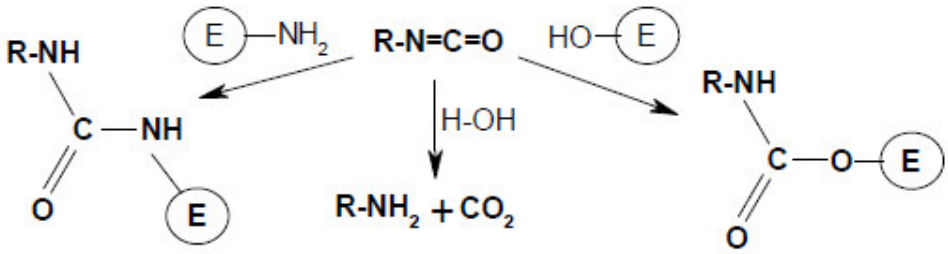

Figure 2.4. Covalent attachment of enzymes into polyurethane foams (R is the prepolymer based on toluene-2,6-diisocyanate). (Source: Romaškevič, et al. 2006)

The enzymes and whole cell can be immobilized within PU foam. Table 2.3 summarizes some of enzymes immobilized within PU foam.

Table 2.3. Some enzymes immobilized within PU foam.
(Source: Romaškevič, et al. 2006)

Enzyme (EC)	Application	Immobilization Method & Notes	References
Amyloglucoside (3.2.1.3)	Immobilization studies	Covalent Attachment	Wood, et al. 1982
Human butyrylcholinestarase (3.1.1.8)	Scavenger of organophosphorus pesticides and chemical warfare agent	Entrapment; starch was used as protective additive	Mehrani, et al. 2004
Fetal bovine serum acetylcholinesterase (3.1.1.7)	Detoxification and decontamination of organophosphates (OP) and as abiosensor to long-term OP determination	Covalent Attachment	Gordon, et al., (1999)
Aspergillus niger glucose oxidase (1.1.3.4)	Biocatalyst in enantioselective sulfoxidation	Entrapment and Covalent Attachment	Velde, et al., 2000
Candida rugosa lipase (3.1.1.3)	Oils and fats hydrolysis, esterification and interesterification	Entrapment and Covalent Attachment	Ferreira-Diaset, et al., 1999
Penicillium canescens β-galactosidase	Immobilization studies, lactose hydrolysis in whey	Covalent Attachment	Budriene, et al. 2005
Cellulose (3.2.1.4)	Immobilization studies	Covalent Attachment	Wood, et al., 1982)
Pectinase (3.2.1.15)	Immobilization studies	Covalent Attachment	Wood, et al. 1982
Urease (3.5.1.5)	Immobilization studies	Covalent Attachment	LeJeune, et al. 1997
Creatinine Amidohydrolase (3.5.3.3)	Immobilization studies for amperometric biosensor construction	Covalent Attachment	Berberich, et al. 2005
Escherichia Coli phosphotriesterase (3.1.8.1)	Immobilization studies	Covalent Attachment	LeJeune, et al. 1997
Pepsin (3.4.4.1)	Immobilization studies	Pepsin-nano gold-PU conjugates	Phadtare, et al. 2003

Bang et al. immobilized the whole cell of *Bacillus pasteurii* into PU foam (Bang, et al. 2001). They used HYPOL 2000 as a prepolymer of polyurethane foam. The cells were immobilized by mixing 0.5ml cell solution in saline and 0.5g prepolymer HYPOL 2000, and then stirred this mixture at 100 strokes with a sterile wood stick. As a result of immobilization, they determined that immobilization of cell did not cause any morphological damage to the cells as indicated by the scanning electron microscopy (SEM) pictures. They reported that both immobilized and free cell completed calcite precipitation at the same time (4hr). They also indicated that ammonia production rates were the same for immobilized and free cells.

Awang and his coworkers immobilized lipase from *Candida rugosa* into palm-based polyurethane foam (Awang, et al. 2007). They immobilized lipase by mixing the support, lipase solution and dried overnight at room temperature. They determined immobilized lipase activity by the esterification reaction of oleic acid and oleyl alcohol in hexane. In temperature tests, they stated that 40^{o}C gave the highest conversion within the temperature range between 30^{o}C and 70^{o}C. While they determined the storage period of immobilized lipase, they stored the lipase at -5^{o}C for 9 days and they monitored that %80 of activity was avoided. In repeated use of immobilized lipase tests, they establish that the enzyme still preserved %70 of its activity for four cycles, but free enzyme lost its activity dramatically.

Berberich et al. used enzyme creatinine amidohydrolase in their immobilization study into PU foam (Berberich, et al. 2005). They mixed vigorously prepolymer 0.4g HYPOL 2060G and buffered enzyme solution (0–150 units enzyme per gram of prepolymer) by spatula. They determined immobilized enzyme activity by using the oxygen electrodeThey reported that rate of immobilized enzyme was not diffusionally limited. The slope between activity and enzyme concentration in hydrogel were linear. The immobilized enzyme preserved its activity after 8 cycles. Most of the activity of immobilized enzyme was preserved during 90 days. But free enzyme activity was lost in 30-40 days. They finally noted that immobilization of the enzyme into polyurethane improved the enzymes stability in buffer at 37^{o}C increasing the half-life from six days to greater than 80 days.

Bakker et al. in 2000 immobilized glycosylated enzymes into PU foam (Bakker, et al. 2000). In addition, they used prepolymer HYPOL 3000 and emulsifier Brij 52 to improve the distribution of the enzyme in the polymerization mixture. They used Cobalt(II) chloride as a stabilizer for aminoacylase and as an initiator for the

polymerization reaction. Reusability tests were also done and it has been shown that immobilized enzyme kept its all activity after 10 cycles.

Although various enzymes have been immobilized into PU foam covalently, to our knowledge; the carbonic Anhydrase has not been immobilized within PU. Our aim in this thesis is therefore to immobilize the carbonic Anhydrase within PU covalently for the CO_2 sequestration purposes to decelerate the global warming.

2.4. Carbonic Anhydrase

The carbonic anhydrase is a zinc containing enzyme which catalyzes the reversible hydration of carbon dioxide. The carbonic anhydrase can also catalyze the dehydration of various aldehydes and the hydrolysis of esters which are very useful for activity assays (Host 2007, Whitney 1970).

The physiological function of CA is to catalyze the interconversion between carbon dioxide and bicarbonate. The waste CO_2 released from cells into the capillary blood diffuses across the erythrocyte membrane. In its gaseous form, CO_2 dissolves poorly in aqueous solution, such as blood plasma, and the carbonic anhydrase inside the erythrocyte converts the CO_2 to water-soluble bi carbonate (HCO_3^-) anion. The overall reaction of carbonic anhydrase can be written as (Lodish, et al. 1986),

$$H_2O + CO_2 \leftrightarrow HCO_3^- + H^+ \qquad (2.12)$$

Figure 2.5 shows the general structure of bovine carbonic anhydrase. Thick arrows represent β-strands. The catalytically active site consists of a cone-shaped cavity having a Zn^{2+} ion as a reaction center of the reversible hydration of carbon dioxide. Three histidine residues are located in the middle of the β-sheet, and their nitrogen atoms form coordination bonds with the zinc ion (web-1). There are 18 lysine groups in the amino acid sequence of carbonic anhydrase and most of them are at the surface of the enzyme. These lysines including amine groups provide the enzyme efficient immobilization. (Host 2007)

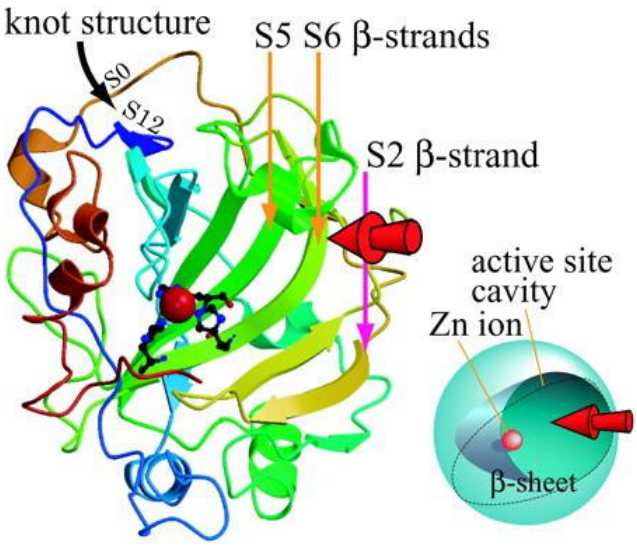

Figure 2.5. The 3-D structure of BCA.
(Source: Ohta. et al. 2004)

There are five independent Carbonic Anhydrase gene families such as α-CA, β-CA, γ-CA, ε-CA, and δ-CA. α-CA belongs to mammals and divided into four subgroups (cytosolic, mitochondrial, secreted and membrane bound) (Esbaugh and Tufts 2006). To date, 16 different CA isozymes have been identified in mammals All mammals, including humans, have α-CA (Hewett-Emmett and Tashian 1996). There are 16 different isozymes in mammals, and at least ten of them are human isozymes. Among them, four are cytoplasmic, two are mitochondrial, and four are membrane bound. β-CAs belong prokaryotic and plant chloroplast. γ-CAs is from methane-producing bacteria that grow in hot springs. (Esbaugh et al., 2006) Recently, δ-CAs have also been identified. (Host 2007)

The mechanism of action of CA on the CO_2 hydration is that hydroxyl ion that is coordinated by zinc of the enzyme; attack CO_2 to form bicarbonate at the active site of the enzyme. Then, bicarbonate of the enzyme displaces with the water molecule (Mirjafari, et al. 2007). The k_{cat}/K_M value for the Human Carbonic Anhydrase II (HCAII) which is the fastest isozyme is $1,5*10^8 M^{-1}s^{-1}$ for CO_2 . (Host 2007)

Carbonic anhydrase has many isozymes which have different kinetic parameters with different substrates. Kinetic constants of CA isozymes are shown in Table 2.4.

Table 2.4. Kinetic constants of CA isonzymes with different substrates.

Carbonic Anhydrase	**Substrate**	**Kinetic Constants**		**Referance**
		K_M (mM)	**k_{cat}/K_M ($M^{-1}s^{-1}$)**	
Bovine CA II	m-nitrophenyl acetate	13	240	Thobslund, et al. 1967
	p-nitrophenyl acetate	76.9	960	
	o-nitrophenyl acetate	-	100	
	p-nitrophenyl propionate	-	68	
Bovine CA II	p-nitrophenyl propionate	-	61	Drevon, et al. 2003
Human CA III	CO_2	-	$3*10^5$	Duda, et al., 2005
Bovine CA I, II	p-nitrophenyl acetate	-	266.6	Pocker, et al., 1966
Bovine CA	p-nitrophenyl acetate	-	153.3	Pocker, et al. 1968
Human CA I	p-nitrophenyl acetate	3.025	753	Innocenti, et al. 2008
Human CA II		30.53	2607	
Human CA I	p-nitrophenyl phosphate	0.935	65.55	
Human CA II		2.195	14.89	
Human CA I	CO_2	4	$5*10^7$	Supuran, et al. 2008
Human CA II		9.3	$1.5*10^8$	
Human CA III		52	$2.5*10^5$	
Bovine CA	CO_2	0.65	36.31	Mirjafari, et al. 2007
Carbonic Anhydrase	CO_2	12	$8.3*10^7$	Whitford 2005

2.4.1 Immobilization of Carbonic Anhydrase

Various forms of CA have been immobilized on various supports shown in Figure 2.4. For example, Drevon et al. immobilized carbonic anhydrase on coatings by covalent attachment; Liu et al. immobilized CA to chitosan alganite beads by encapsulation; Jovica and Kostic in 1999 immobilized CA within silica monoliths by Sol gel method (Drevon, et al. 2003, Liu, et al. 2005, Jovica and Kostic 1999)

Hosseinkhani and Gorgani reported that partial unfolding of carbonic anhydrase resulted in immobilization on hydrophobic adsorbents and protected it against irreversible thermoinactivation (Hosseinkhani and Gorgani 2003). They prepared immobilized enzyme by mixing enzyme solution (150g/ml in 50mM Tris-sulfate pH 7.5) and Sepharose-lipid at specific temperature for 30 minutes and then cooled at $4^{o}C$ for 30 minutes. To determine activity of carbonic anhydrase they used p-NPA as substrate and measured enzyme's esterase activity at 400nm. They immobilized carbonic ahydrase at 3 different temperature (58, 60, $62^{o}C$). Most efficent immobilization occured at $62^{o}C$ to octyl sepherose.

Ray purified and immobilized Human erythrocyte carbonic anhydrase in polyacrylamide gel (Ray 1977). As compared to the soluble enzyme, the immobilized enzyme was considerably more resistant to heat and sulphanilamide action. (Ray, 1977)

Bond et al. proposed that bacterial overexpression was the suitable way for the carbonic anhydrase production and they proposed three different supports for carbonic anhydrase immobilization (acrylamide, alginate, and chitosan-alginate) (Bond, et al. 2001). They noted that alginate and chitosan-alginate supports were desirable because these were cheap and easy to produce, non-toxic, biodegradable and environmentally friendly.

Table 2.5. Immobilization studies with enzyme Carbonic Anhydrase.

Enzyme	Support	Immobilization Method	Reference
Bovine Carbonic Anhydrase	octyl Sepharose-lipid	Hydrophobic Adsorption	Hosseinkhani and Gorgani 2003
	dodecyl Sepharose-lipid		
	palmityl Sepharose-lipid		
Carbonic Anhydrase	N-vinylformamide-based-water-soluble polymer	Covalent Attachment	Drevon, et al. 2003
Carbonic Anhydrase	Chitosan Alganite Beads	Encapsulation	Liu, et al. 2005
Carbonic Anhydrase	poly(acrylic acid-co-acrylamide)	Covalent Attachment	Cheng, et al. 2008
Bovine Carbonic Anhydrase	Silica monoliths	Encapsulation (Sol-gel method)	Jovica and Kostic 1999

2.5. Biomimetic CO_2 Sequestration

Mirjafari et al. studied the effect of free bovine carbonic anhydrase on the hydration of CO_2, and its precipitation in the form of calcium carbonate (Mirjafari, et al. 2007). In their study, enzyme solution was prepared in phospate buffer at different specific concentrations and then this solution mixed with a solution which was prepared by bubbling deionized water with gaseous CO_2. They noted that the rate of hydration reaction increased with both the enzyme concentration and temperature. They also determined that calcium carbonate precipitation was increased in the presence of enzyme carbonic anhydrase, but the concentration of the enyzme did not effect the precipitation. They indicated that temperature incerase caused increase in calcium carbonate formation and also the enzyme activity was not influenced by the pH of the reaction mixture. Finally, they found the k_{cat}/K_M and activation energy value of carbonic anhydrase for CO_2 substrate as $36.313 M^{-1}s^{-1}$ and 700.91cal/mol, respectively.

Bond et al. aimed to develop an enzymatic CO_2 scrubber which can be used to reduce CO_2 emissions from fossil fuel burning power plants and used bovine carbonic anhydrase as a catalyst to accelerate the rate of CO_2 hydration (Bond, et al. 2008). They

also investigated the effect of other chemical species (NO_x, SO_x) on the bovine carbonic anhydrase activity. NO_x and SO_x is very important in the flue gases. According to their work, high concentration level of NO_x (>0.05M) and SO_x (>0.005M) inhibited the enzyme.

Liu et al. studied the effect of cations in the produced water from the Permian and San Juan Basins to enzymatic CO_2 scrubber (Liu, et al. 2005). Bovine carbonic anhydrase was used as enzyme to accelerate CO_2 hydration by them. They reported that precipitation of calcium carbonate occurred much more quickly in presence of bovine carbonic anhydrase. They also investigated the effect of temperature on precipitation time. They have shown that the increases in temperature accelerated the precipitation for both enzymatic and control.

2.6. Esterase activity of Carbonic Anhydrase

It is hard to measure the activity of free or immobilized enzyme with gas phase substrate such as CO_2. For this reason, in this thesis carbonic anhydrase activity could also be measured in aqueous phase by using nitrophenyl esters.

CA catalyses the hydrolysis of nitrophenyl esters with different efficiencies depending on the structure of the acyl part of substrate. But, as shown in Table 2.6, most efficient ester substrate for Bovine CA and Human CAII is para-nitrophenyl acetate (p-NPA) because of short and few acyl groups. p-NPA is bound as neutral species to the CA active site, allowing the strong nucleophilic ($Zn^{2+}(OH)^-$) attack, without any electrostatic repulsions, thus effectively hydrolyze it (Innocenti, et al. 2008). Table 2.6 shows the kinetic constants of CA for different ester form of substrates.

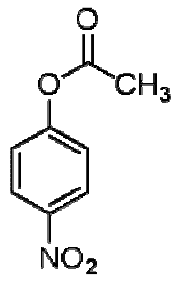

Figure 2.6. para-Nitrophenyl Acetate.

Table 2.6. The apparent rate constants for the CA-catalyzed hydrolysis of various esters. (Source: Thorslund, et al. 1967)

Substrate	**Rate Constant (k_{cat}/K_M, $M^{-1}s^{-1}$)**
Phenyl acetate	3
o-nitrophenyl acetate	100
m-nitrophenyl acetate	240
p-nitrohenyl acetate	960
p-nitrohenyl propionate	68
p-nitrophenyl butyrate	6
p-nitrophenyl caproate	2

2.7. Effect of Temperature on Carbonic Anhydrase activity and structure

Enzymes loose their activity at higher temperatures. After a specific temperature enzymes are irreversibly denaturated, and undergo a conformational alteration entailing a loss of biological activity. Denaturation may be due to covalent change such as removal of an amine group from a molecule, deamination of asparagine or rearrangement of the protein chain. Inactivation by heat denaturation has a profound effect on the enzymes productivity (Sheldon, 2007).

The activation energy of an enzyme is always lower than the activation energy for denaturation. For this reason, the enzyme denaturation by temperature is much faster than enzyme activation (Shuler and Kargi 2002), the enzyme denaturation kinetic can be given as;

$$-\frac{d[E]}{dt} = k_d[E] \tag{2.13}$$

where [E] is enzyme concentration and k_d is enzyme denaturation rate constant. If enzyme concentration is directly proportional to rate of reaction;

$$\ln\left(\frac{\text{Rate}}{\text{Rate}_o}\right) = -k_d t \tag{2.14}$$

Slope of ln(Rate/Rate$_o$) versus time plot yields denaturation constant, k_d. The rate of inactivation of the enzymes and the denaturation constant (E_d) can be estimated from typical Arrhenius equation shown in equation (2.15). The Arrhenius equation for activation energy for denaturation;

$$k_d = A_d e^{-E_d/RT} \tag{2.15}$$

or

$$\ln(k_d) = \ln(A_d) + \frac{-E_d}{R}\frac{1}{T} \tag{2.16}$$

Protein unfolding mechanism is a thermodynamically favorable research area for determining the maximum temperature at which the protein (enzyme) is active. For unfolding mechanism, the change in free energy must be positive ($G_{unfolding}$ - $G_{folding}$). For industrial applications, temperature limitations of enzymes are important parameter. When an enzyme is active at high temperatures, productivity of process may be increased. It is generally known that; the reaction rate of the most chemical reactions increases twice per 10^{o}C increase in temperature (Fogler 1999). There are two explanations for unfolding mechanism of enzymes. First one is the decreasing in the number of hydrogen bonds and salt links which are responsible for 3-D conformation of the structure. The second explanation is worse internal van der Waal's packing. These two reasons of thermal unfolding mechanism cover the 90% of mechanism. The number of hydrogen bonds and salt links, which decreases during the heating of enzyme, is more effective for denaturation. Decrease in internal van der Waal's packing and density play less effective role in denaturation (Vogt and Patrick 1997).

Sarraf et al. studied the temperature dependence of the activity and structure of the enzyme bovine carbonic anhydrase (Sarraf, et al. 2004). Firstly, they noted that the amino-acid sequences of human and bovine carbonic anhydrase are almost 87 percent identical. They used the esterification reaction for activity tests. It has been shown that there is a decrease in the amount of β-structures and a decrease in the amount of random coil, from 25 to 40^{o}C. But from 40 to 52^{o}C, the amount of helix is slightly decreased

and there is an increase in the percentage of β-structures. They reported that the initial decrease may be due to the lower stability of β-structures comparing to helices and enzyme became looser at higher temperatures. The reason of this could be either aminoacid may be liberated from their revelent hydrogen bond in protein structure or the establishment of β-structures may become more favorable.

Thermal behavior of bovine carbonic anhydrase was also carried out by Lavecchia and Zugaro. (Lavecchia and Zugaro 1991). They heated the enzyme solution from 40^{o}C to 70^{o}C and measured the esterase activity of carbonic anhydrase in UV spectrometer at 400nm. Degree of protein unfolding was determined by Ultraviolet Difference spectroscopy. They noted that carbonic anhydrase was active under 60^{o}C, but it lost its activity between 60-65^{o}C. They explained irreversible denaturation as the structural deformation of carbonic anhydrase and it's caused unfolding. X-Ray Diffraction study showed that 9 water molecule which is located in the active site region should have play key role in the irreversible thermal denaturation of carbonic anhydrase.

2.8. Calcium Carbonates Synthesis

Presence of organic compounds causes different calcium carbonate polymorphs. The proteins also affect the chemical or physical properties of precipitate calcium carbonates. The most stable polymorphs of calcium carbonate are calcite and aragonite. Biologic precipitations offer new possibilities in the synthesis of high performance nano-particles. (Sondi and Matijevi 2001)

Sondi et al. used urease and urea to precipitate and modify the properties of calcite (Sondi and Matijevi 2001). They determined that concentration of $CaCl_2$ and urea did not affect the properties of precipitates but urease concentration was effective on the rate of precipitation and on the properties of calcium carbonates polymorphs. They used Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD) to determine the $CaCO_3$ and the size of the precipitates. pH of the reaction solution changed from 9 to 6.7 at the end of the reaction. Reaction was stopped when pH 6.7 because at lower pH, precipitated $CaCO_3$ begins to dissolve in the solution. They showed that precipitates were nanosized particles at about 100nm in diameter and the XRD results showed that precipitates were calcite and with a calcite peak of $2\theta=29^{o}$.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Carbonic Anyhydrase from Bovine Erythrocytes (CA-I), para-Nitrophenyl Acetate (p-NPA), para-nitrophenol (p-NP) were purchased from Sigma-Aldrich. Acetonitrile (99.9%v/v), Sodium Hydroxide (97%w/w), Hydrochloric Acid (35%v/v), Calcium Hydroxide (96%w/w) were purchased from Merck. Polyurethane prepolymer, HYPOL-2060, was a kind gift from Dow Chemical Co., Istanbul,Turkey.

Equipments used were UV/VIS Spectrometer (Perkin Elmer Lambda 25/45), pH and Conductivity meter (Thermo Electron Corporation, Orion 5 Star), Centrifuge (Hettich-Zentrifugen, Universal 320), Scanning Electron Microscopy (Philips XL30 SFEG), X-Ray Differaction (Philips Xpert-Pro), Thermo Regulator (Velf Scientifica), Thermal Circulator (P-Selectra, Firigiterm).

3.2. Enzyme Activity Assay

Enzyme activity is a measure of the catalytic effect of active enzyme in the medium; therefore, it is dependent on conditions, which should be specified. In order to determine the activity of free and immobilized enzyme two similar procedures were followed at 25^{o}C. For the free enzyme, the activity assay was performed in 1 ml UV cuvette. Reaction mixture was prepared with 0.8 ml tris buffer solution (pH = 7.5, 50mM), 0.1 ml substrate solution (p-NPA dissolved in acetonitrile) and 0.1 ml enzyme solution (CA in tris buffer). This solution was mixed in the cuvette by the help of the micropipette, measured the enzyme activity with “time drive” function of Perkin Elmer software at 400nm according to p-nitrophenol (p-NP) production during 3 minutes.

To determine activity of immobilized enzyme, a 10 ml of reaction mixture was used. 9 ml of tris buffer (pH = 7.5, 50mM) and 1 ml of p-NPA (dissolved in acetonitrile) were mixed in 30 ml vial by the help of the magnetic stirrer. Then, the the enzyme immobilized foam was added to this solution. 1 ml of sample from this solution was taken and measured its absorbance in UV/VIS spectrometer at 400nm, and poured

back to the reaction mixture in order to hold the volume constant for the reaction mixture. The absorbance was measured in every minute for 25-30 minutes to estimate the amount of the p-NP production.

3.3. Calibration Curve

One of the products of p-NPA degradation is the p-NP. A concentration of 5, 10, 30, 50, 80 µM of p-NP were dissolved in tris buffer. Then absorbance values of these samples were measured in UV/VIS spectrometer at 400nm. At the end, the calibration curve was established as concentration versus absorbance. Also samples were scanned between 300 and 800nm to be sure that maximum value of absorbance is at 400nm.

3.4. Determination of Kinetic Constants for Free BCA

A 0.0225 g ($1.24*10^{-4}$ mol) sample of p-NPA was dissolved in 4 ml of acetonitrile in 5 ml vial and mixed with magnetic stirrer until p-NPA was completely dissolved. Afterwards, a 0.0028 g sample of Bovine Carbonic Anhydrase was dissolved in 3 ml of tris buffer (50mM and 7.5pH) in 5 ml vial mixing with magnetic stirrer until BCA was completely dissolved. This solution was diluted to five different concentrations (1.609 µM, 1.073 µM, 0.805 µM, 0.537 µM, 0.268 µM). After solutions were prepared, activity test was carried out. In this test, Lambda 45 UV/VIS Spectrometer was used. Hydrolysis reaction of p-NPA was performed in 1 ml cuvette. A 0.9 ml of tris buffer, 0.1 ml of CA solution, and 0.1 ml of p-NPA solution was mixed in the cuvette and UV absorbance of the solution were measured in every seconds for 3 minutes. Different concentrations of p-NPA (2.329 mM, 1.5525 mM, 0.9315 mM, 0.7763 mM) were tested to prepare a graph of the rate versus the substrate concentration.

3.5. Immobilization of Bovine Carbonic Anhydrase within Polyurethane Foam

Immobilization of bovine carbonic anhydrase within polyurethane foam was carried out in 50 ml falcon tube. BCA (sample of 0.0025-0.011g) was dissolved in 3ml of DI water and 15 µl of the tris buffer. This enzyme solution was mixed vigorously for 30 seconds with about 3.45 g of prepolymer, Hypol-2060, by the help of a drill mixer to achieve a homogeneous distribution of the enzyme within the prepolymer. Polymerization took place at room temperature. After polymerization foam was synthesized and stored for at least 2 h before use. Foam was washed with ultra pure water and soaked with tris buffer.

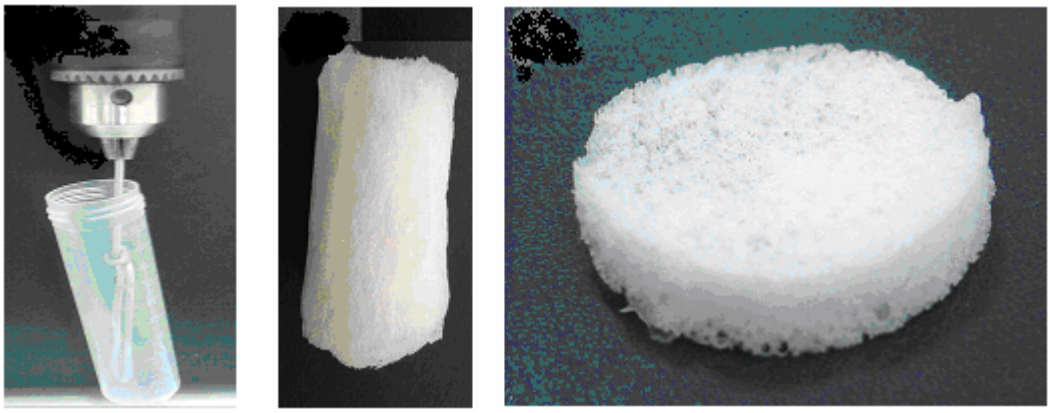

Figure 3.1. Immobilization of Carbonic Anhydrase within polyurethane foam.

3.6. Determining the Kinetic Constants for Immobilized CA

The kinetic constants for immobilized CA were estimated from Michealis-Menten plot of the enzyme activity data. The activity of immobilized enzyme was measured by the help of the hydrolysis reaction of different concentrations of p-NPA (2.52, 1.89, 1.26 and, 0.63mM). A 9 ml of Tris Buffer (50mM, pH=7.5) was taken in a glass vial. Then, a 1 ml of p-NPA in acetonitrile was added. A piece of foam was soaked in tris buffer, and squeezed several times. The reaction was started upon addition of the enzyme containing foam into the substrate solution. This procedure was repeated for different concentrations of substrate and different amount of foam pieces. After

assays, foams were dried in vacuum oven at 80^{o}C and at -0.8atm for 2.5hours to determine their dry weight.

3.7. Reusebility and Stability Tests

Firstly, a 10 ml of 2.52*10^{-4} M p-NPA solution was prepared. A slice of enzyme immobilized foam was washed with DI water for five times and soaked in tris buffer. Then, reaction mixture which involved 9 ml of tris buffer and 1 ml of p-NPA solution were prepared. Washed and soaked slice foam was added to the reaction mixture and absorbance of solution was measured at 400nm in Lambda 45 UV/VIS Spectrometer in every minute during 25-30 minutes. This procedure was repeated for 7 times with the same slice of the foam sample.

In stability tests, the activities of the same samples of free and immobilized CA were recorded during the 45-day period. Free enzyme was stored at 4^{o}C in tris buffer while the immobilized enzyme was stored at ambient temperature in tris buffer. Activity tests were repeated in every 3-4 days with the same concentration of p-NPA (≈2.5mM).

3.8. CA Loading to PU Foam

Various amounts of bovine carbonic anhydrase are immobilized for about nearly the same amount of HYPOL. Activity assays for each reaction were carried out in 10 ml reaction mixture and with the same concentration of p-NPA of 2.5mM. After activity assay, samples were dried in oven under vacuum at -0.8 atm and 80^{o}C. Weights of samples were measured after they were completely dried.

3.9. Thermal stability of the Free and Immobilized CA

First, the bovine Carbonic Anhydrase was dissolved in tris buffer (50mM and 7.5pH). The CA solution was stirred and incubated in the jacketed glass reactor at constant specified temperature for up to 120 minutes. To prevent the evaporation of the enzyme solution, the reactor was closed with a rubber stopper. Approximately, in every 20 minutes, 0.1 ml of enzyme solution was taken from the reactor and measured its

activity at 25°C with free enzyme activity test. This procedure was repeated for other incubation temperatures.

For the thermal stability of the immobilized enzyme, a piece of foam containing carbonic anhydrase was incubated at 40°C, 50°C, 53°C, 57 °C, and, 60°C for 2 hours in glass jacket reactor. Temperature of reactor was altered by the help of the thermal circulator. Different foams were incubated for every temperature assay. The foam samples were taken out at various intervals for enzyme activity test using the same p-NPA concentration of 2.5mM at 25°C. The samples were then returned back to the thermal stability tests after the activity assay was complete. This procedure was repeated for each temperature specified. At the end of each test, foam samples were dried in vacuum oven at 80°C and -0.8atm for 150 minutes to measure their dry weight.

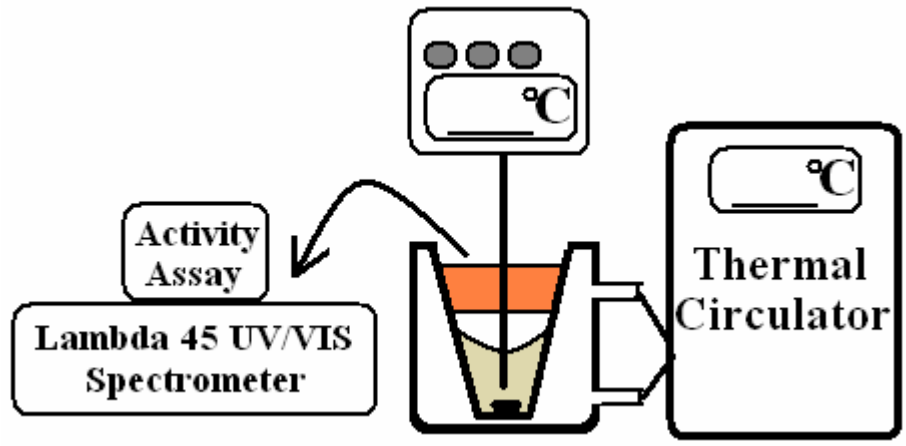

Figure 3.2. Thermal Stability Experiment for the free enzyme.

3.10. Effect of Reaction Temperature on Immobilized CA

In this experiment, the effect of reaction temperature on the activity of the immobilized enzyme was investigated. Activity test of the immobilized enzyme was performed in a glass jacket reactor at constant specific temperatures (18, 30, 40, 50, 60°C). Before the experiment, the buffer solution was heated to the specified temperature. A 20 ml of total reaction mixture, 90% of buffer and 10% of p-NPA solution, were used. Reaction temperature was hold constant by the help of the thermal circulator. A 1 ml of reaction mixture was taken into cuvette and measured its absorbance at 25°C in every minute for 25-30 minutes.

3.11. Effect of Immobilized CA on $CaCO_3$ Precipitation

The effect of immobilized bovine carbonic anhydrase on $CaCO_3$ precipitation was investigated. A three neck round bottom flask was used as reactor. A pH probe and a conductivity probe were attached to the two different necks. Third neck was used in order to inject CO_2 with a glass pipe. The leakage was prevented by rubber stoppers. Precipitation reaction was carried out at room temperature and CO_2 injection rate was not controlled. Firstly, a 200 ml, 30mM of $Ca(OH)_2$ solution was prepared by mixing at 600rpm for 10 minutes. Then, tiny sliced foam pieces containing carbonic anhydrase with a diameter of about 1-5mm were added to this solution. At the same time, CO_2 was begun to be injected. During the precipitation reaction, the pH and conductivity values were recorded at every 15 seconds. The experimental scheme was shown in Figure 3.3. Precipitation reaction was stopped when pH decreased to 7. Afterwards, the solution was centrifuged and dried at 103°C to handle precipitates. Dried precipitates were examined with the SEM and XRD.

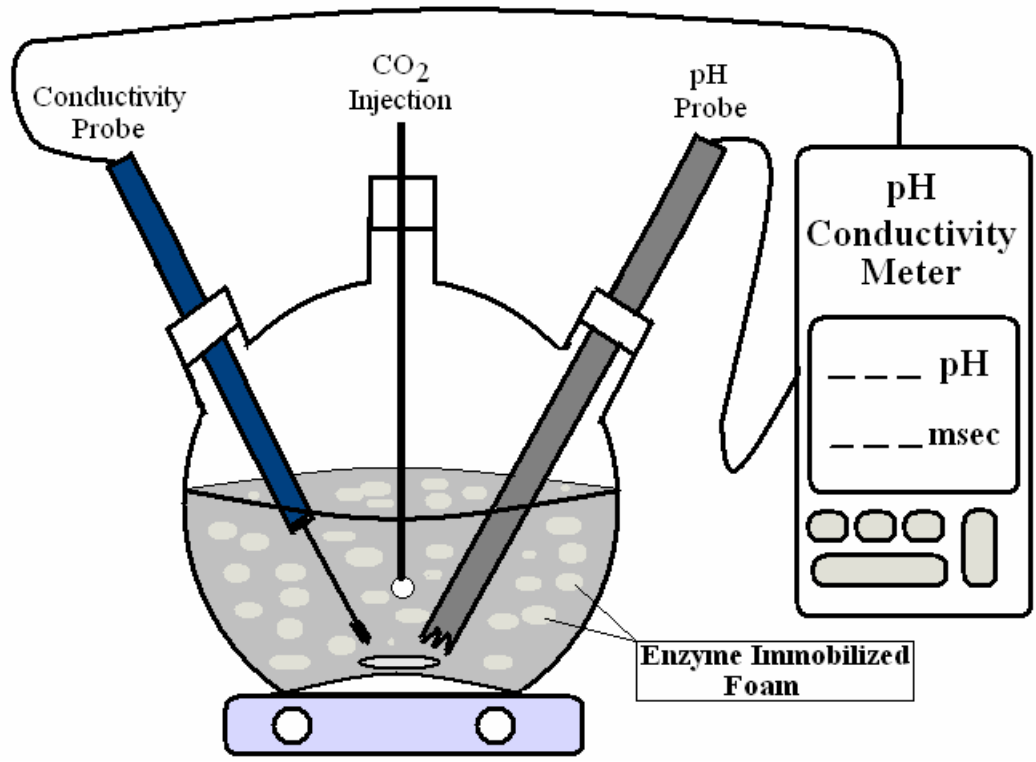

Figure 3.3. Precipitation of $CaCO_3$ in the presence of immobilized BCA.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Calibration Curve

To convert UV/VIS spectrometer data into concentration values, a calibration curve was prepared. The powder p-nitrophenol (p-NP) was dissolved in tris buffer (50mM, pH=7.5). The blank solution was also chosen as the same tris buffer. Figure 4.1 shows the calibration curve between p-NP concentration and the absorbance. R^2 value of the line is 0.9995 and nearly perfect fit to data. Slope of the calibration curve was calculated as 12.509Au/mM.

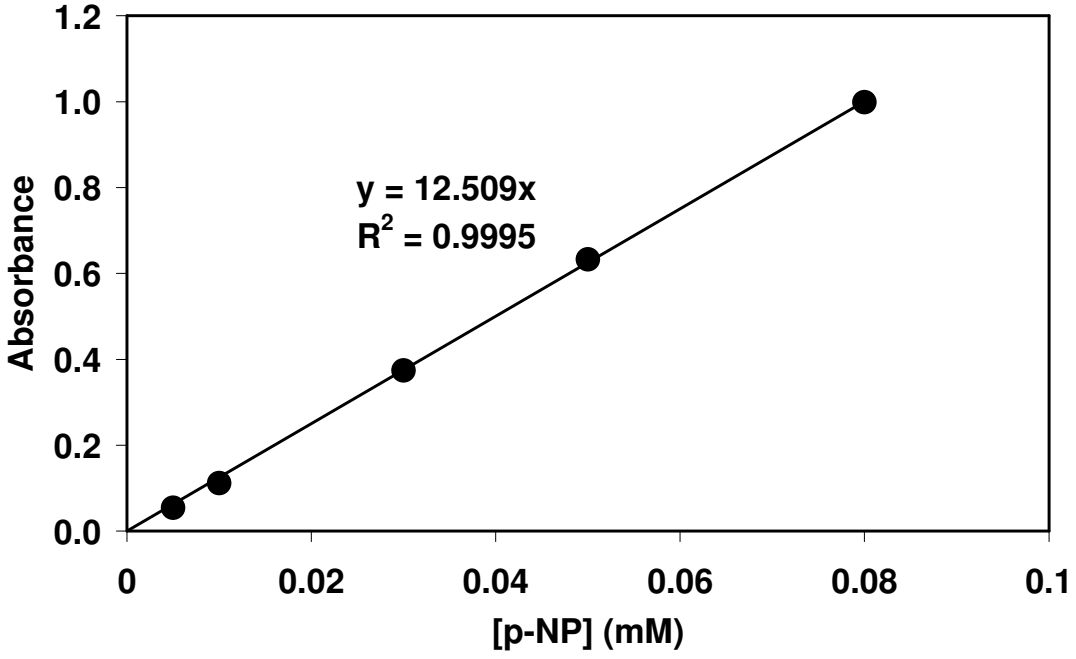

Figure 4.1. Calibration Curve for p-NP in Tris buffer (pH 7.5, 50mM).

4.2. Self Dissociation of p-nitrophenyl Acetate

Self dissociation of p-nitrophenyl acetate has to be determined in order to subtract the background absorbance and to obtain the absolute rate for the free and immobilized enzyme. The self dissociation of p-NPA for various concentrations was estimated. The self dissociation rate was seemed to be linear with the p-NPA concentration at 25°C as shown in Figure 4.2. From the data, the self dissociation obeyed the first order kinetic such that;

$$\text{Rate} = \frac{\partial(\text{p}-\text{NPA})}{\partial \text{t}} = \text{k}_\text{d}\ [\text{p}-\text{NPA}] \qquad (4.1)$$

where k_d is the dissociation constant.

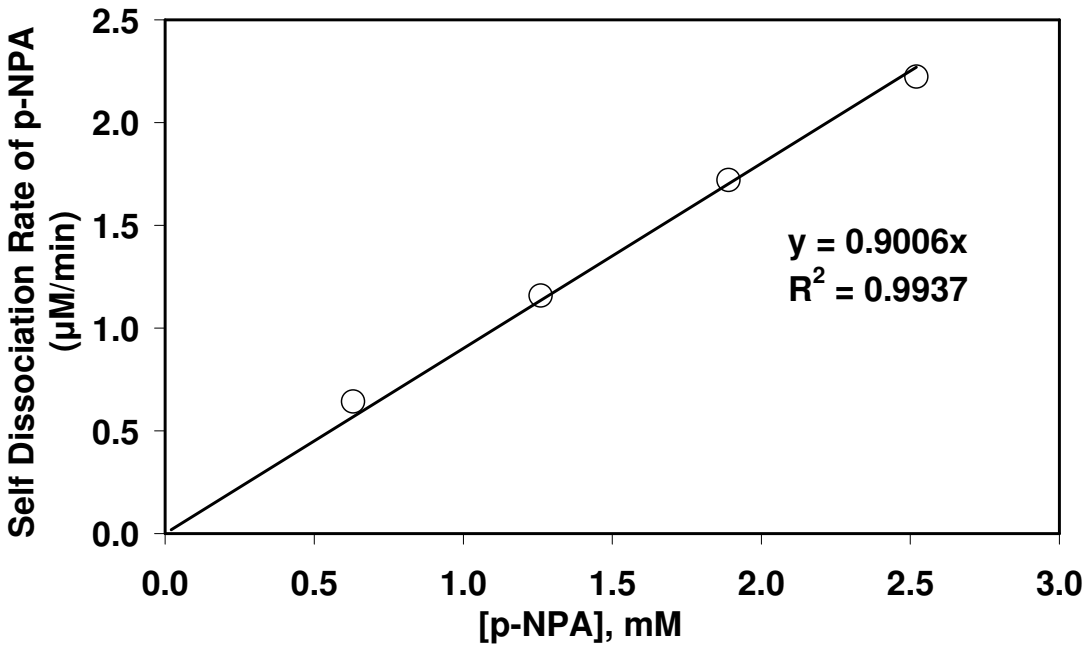

Figure 4.2. Effect of p-NPA concentration to self dissociation rate at 25°C.

The self dissociation of p-NPA was also estimated at various temperatures. As shown in Figure 4.3, the self dissociation rate increases exponentially with temperature. These dissociation rates of p-NPA were subtracted from the enzymatic reactions in

order to eliminate the background signal from the experimental data at each concentration and temperature.

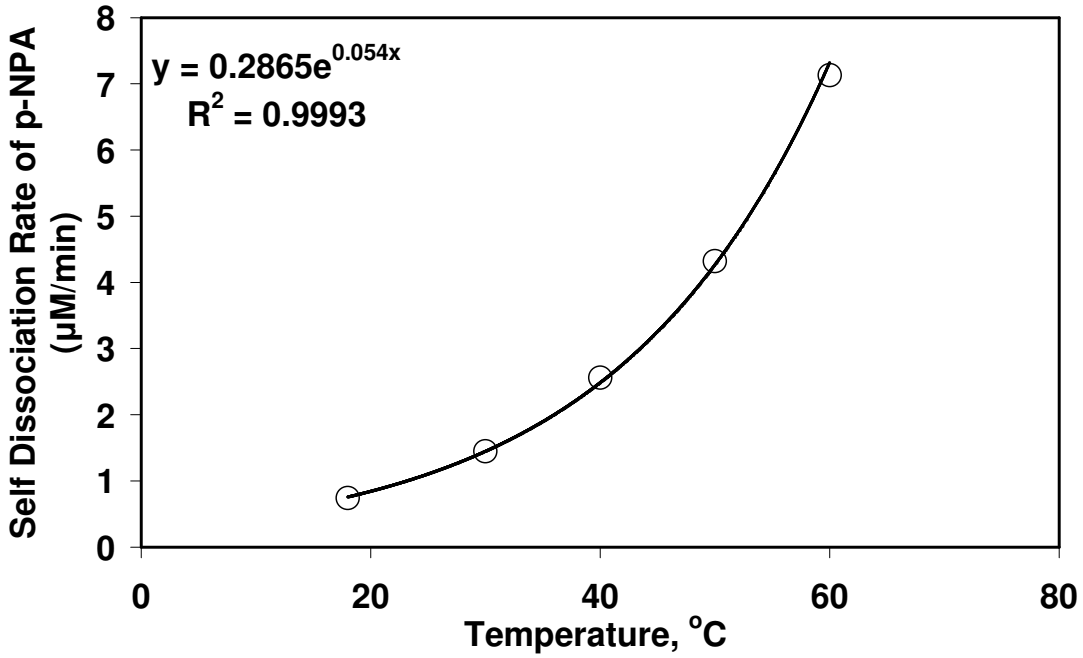

Figure 4.3. Effect of temperature to self dissociation rate of p-NPA.

4.3. Determining of Kinetic Constants for Free CA

The activity of the free carbonic anhydrase was estimated at different concentrations of substrate while keeping the enzyme concentration constant. The substrate concentration of p-NPA could only be used up to 3.1mM due to insolubility of p-NPA in aqueous phase. Figure 4.4 shows the activity of CA in tris buffer (50mM, pH=7.5) in the presence of 10% acetonitrile. The line is the curve fit of experimental data to Michealis-Menten Equation (equation (2.10)). The kinetic constants were obtained from the Lineweaver-Burk equation (equation (2.11)) as shown in Figure 4.5. The kinetic constants were calculated for the free CA as k_{cat}/K_M = 148.12$M^{-1}s^{-1}$, turnover number, k_{cat} = 1.21s^{-1}, and K_M = 12.2mM. These values are in good agreement with the literature data (Pocker and Stone 1968).

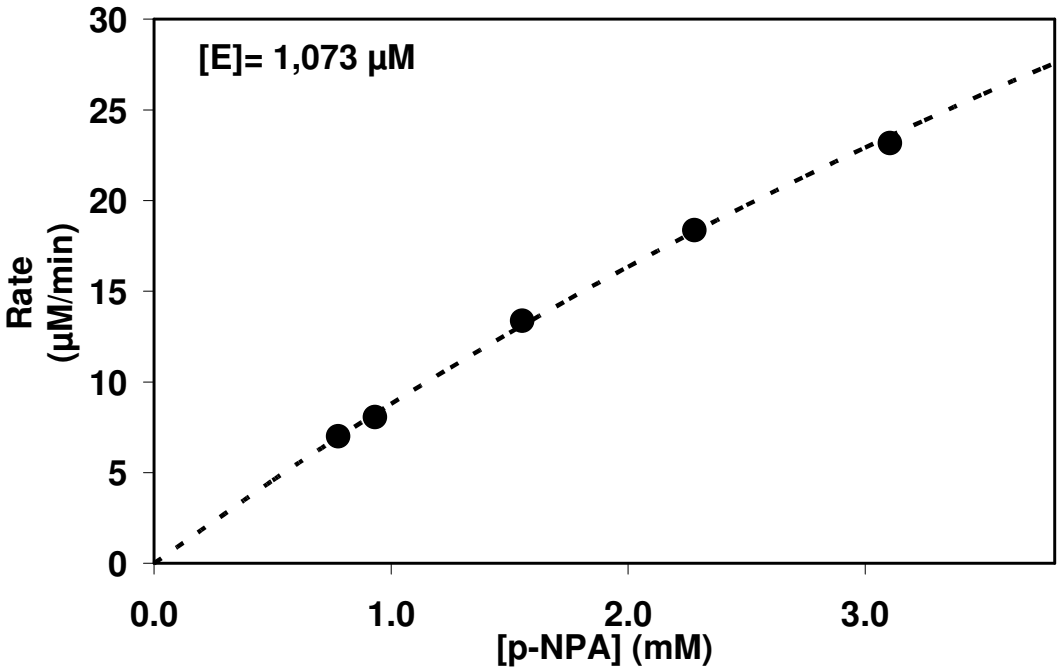

Figure 4.4. Effect of substrate concentration on enzymatic rate.

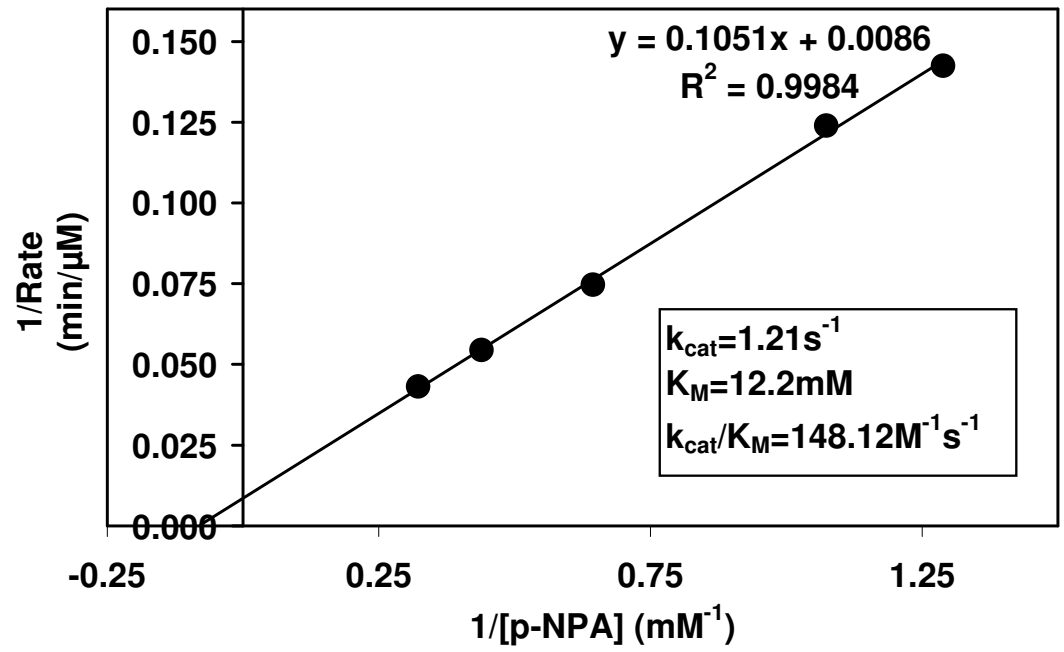

Figure 4.5. Double-Reciprocal (Lineweaver-Burk) plot for free enzyme.

4.4. Immobilization of Carbonic Anhydrase within Polyurethane Foam

The Bovine Carbonic Anhydrase enzyme was immobilized as described in the experimental section. The powder of the BCA was dissolved in DI water and poured onto a sample of the HYPOL prepolymer within falcon tube. A hand-made mixer was used to mix the mixture for 30 seconds. The enzyme bound covalently to isocynate group of polyurethane through its amine and/or hydroxyl groups during the polymerization.When the mixing was settled, the level has started to rise by the evaluation of CO_2 gas. The gas also helped to generate porous within the foam. Bovine carbonic anhydrase could be immobilized within polyurethane foam. A picture of the PU foam is shown in Figure 4.6.

Figure 4.6. A picture of PU foam containing CA.

4.5. Determining of Kinetic Constants for Immobilized Bovine Carbonic Anhydrase

The activity of the immobilized enzyme was estimated through the liberation of p-NP over time. There are two regions in the progress curves as shown in Figure 4.7. The first region is the non-linear region where adsorption and diffusion play role. The second region is the linear region where the adsorption and diffusion play almost negligible role. For instance, the progress curve for the self dissociation of p-NPA is higher comparing to that of for PU foam without enzyme. The effect of adsorption and diffusion on the estimation of the enzyme activity could be evaluated in a separate study. Here, the activity estimation was made from Region-II in the progress curves.

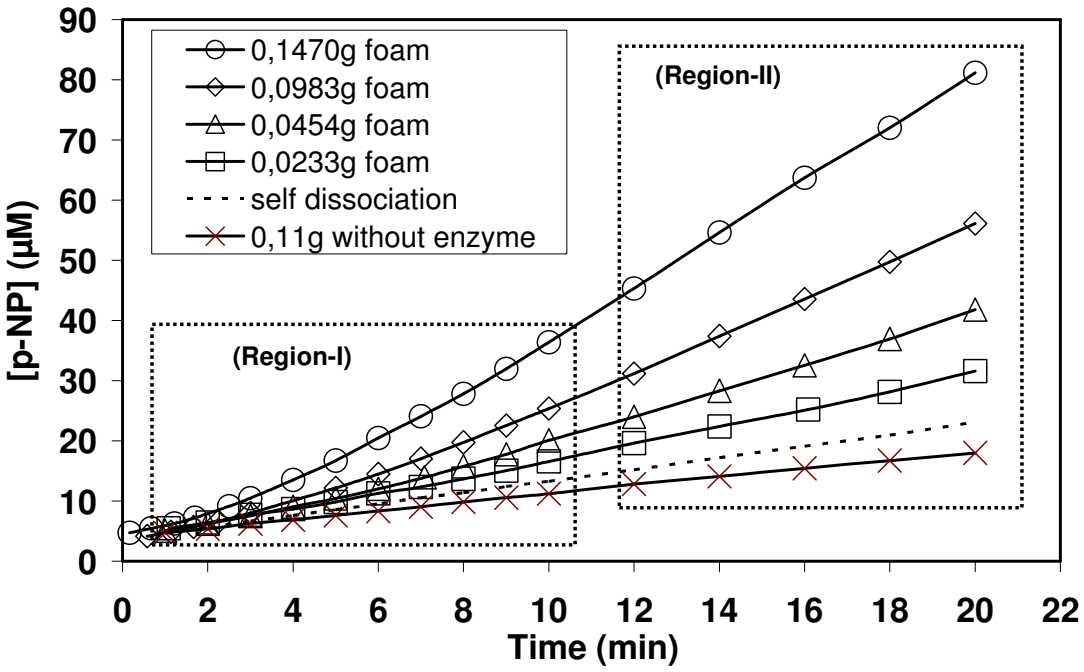

Figure 4.7. Activity of different size of enzyme immobilized foams.

The kinetic constants for the immobilized CA were estimated from the rate versus the substrate concentration as shown in Figure 4.8. Here, a piece of foam was soaked in tris buffer and assayed for activity at defined p-NPA concentration. The same foam was employed for the remaining of the substrate concentrations up to 2.5mM. Again, higher concentration of p-NPA could not be achieved due to lower stability of p-NPA in water. Here, the line is the curve fit to the Michelis-Menten equation, where the

kinetic constants were estimated by the help of the Lineweaver-Burk plot as shown in Figure 4.9.

From the data, the K_M value for the immobilized CA was found to be 9.59mM for p-NPA in tris buffer (50mM, pH=7.5) in the presence of the 10% acetonitrile. This value is very close to the K_M value of the free BCA. By considering a 5.6mg BCA was immobilized onto 3.09g HYPOL and only 0.032g-foam was used in the assay, and assuming all enzyme is active in the foam, the value of k_{cat} was estimated as $1.06s^{-1}$ and the value of k_{cat}/K_M was $111.2M^{-1}s^{-1}$. These values are somewhat lower than the free enzyme. The difference may indicate the loss of enzyme activity or loss of enzyme during immobilization. By comparing the kinetic data, 70% of enzyme is active within the PU foam.

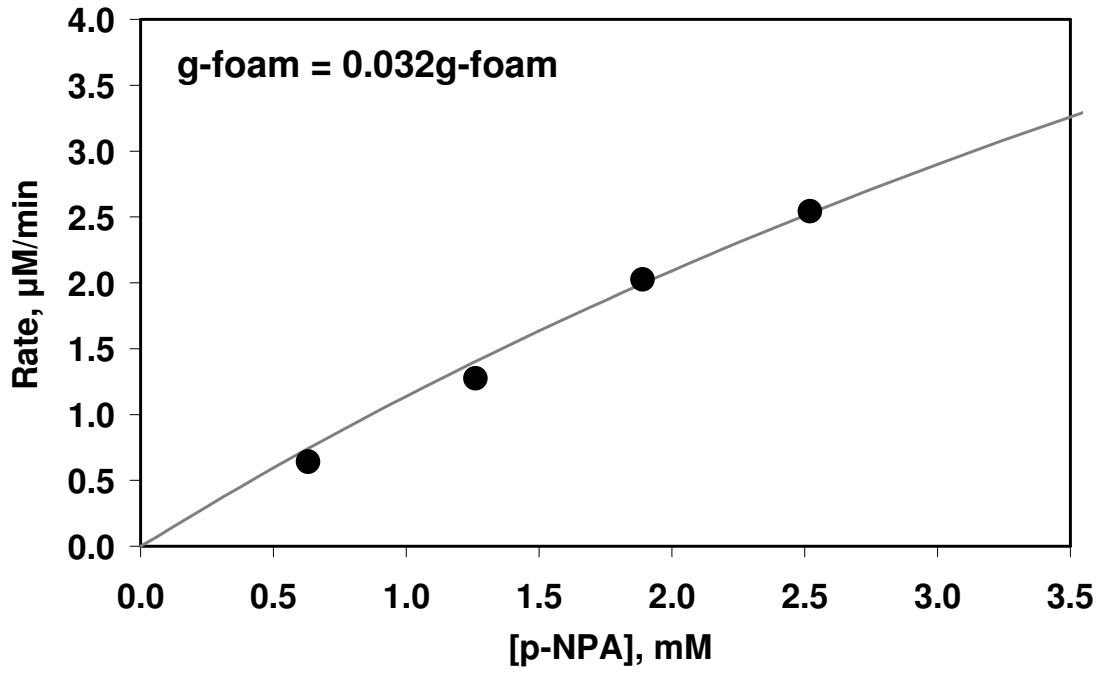

Figure 4.8. Effect of substrate concentration on immobilized enzyme activity.

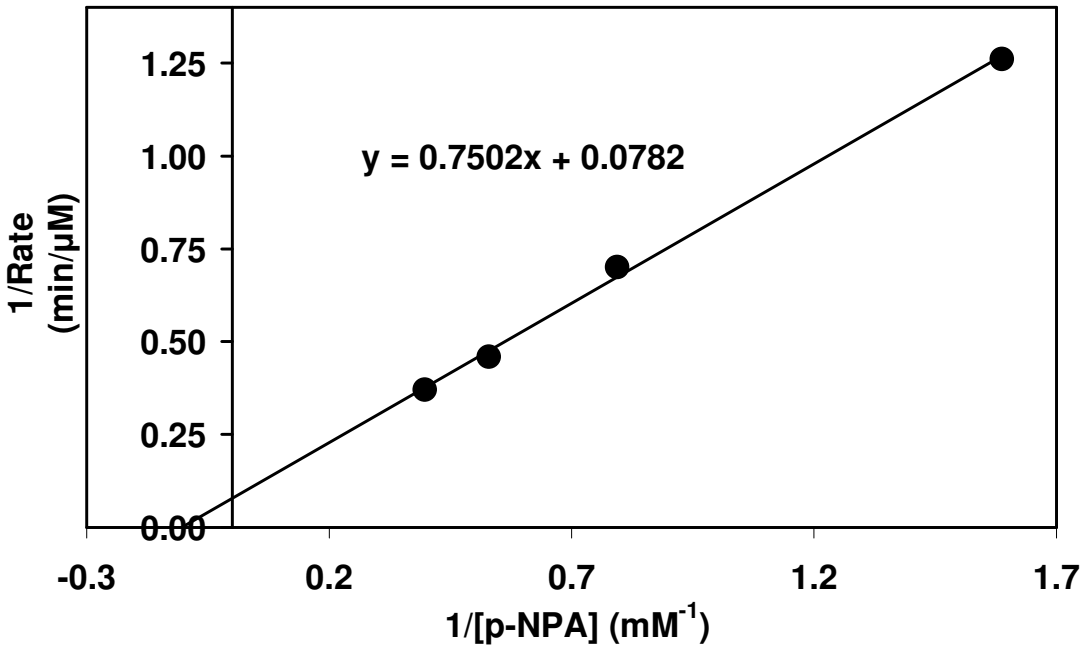

Figure 4.9. Double-Reciprocal (Lineweaver-Burk) plot for immobilized enzyme.

4.6. Reuse Ability of Immobilized BCA

Most important property of covalent attachment of an enzyme is that immobilized enzyme should hold a constant activity when it was used again and again. High reuse capacity is also necessary for industrial applications because extraction and production of native enzyme are expensive. Figure 4.10 shows the reuse capacity of BCA enzyme immobilized within polyurethane foam for 7 cycles. The immobilized enzyme maintained its activity during the whole cycles. The fact that the same immobilized enzyme foam was used and washed after every cycle, the biocatalytic foam kept its activity constant.

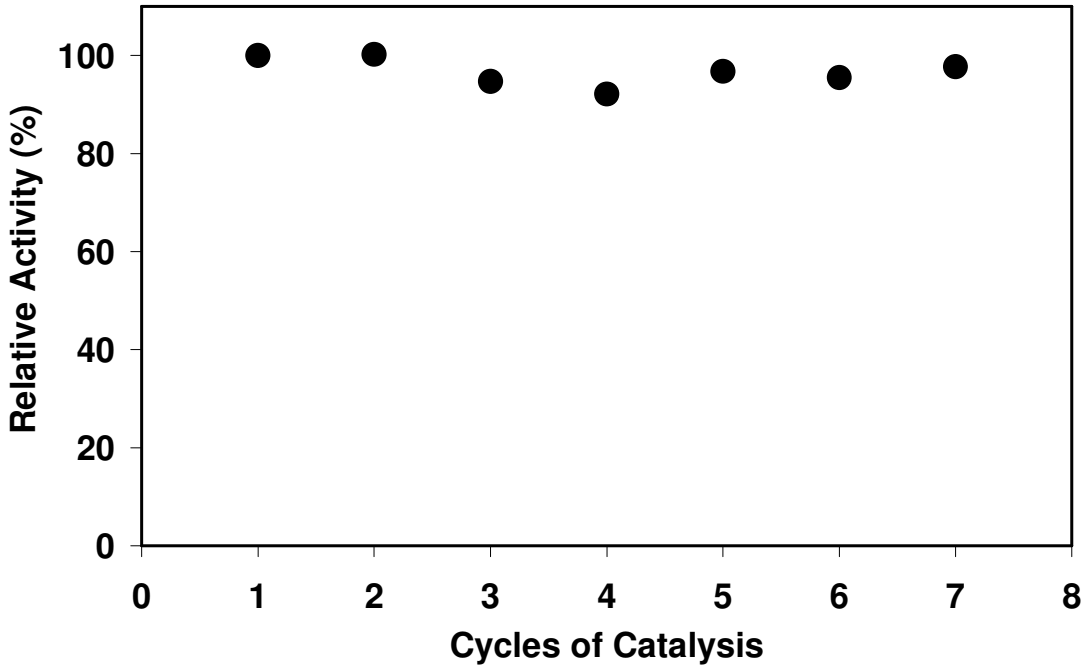

Figure 4.10. Reuse capacity of polyurethane foam that involves CA.

4.7. Different Amount of Loading of BCA within PU Foam

The aim of this experiment was to determine the optimum loading capacity of the enzyme. Different enzyme concentrations are immobilized to nearly the same amount of polyurethane prepolymer. The activity of each loading was estimated by using the enzyme assay with a piece of the foam and a 2.5mM of p-NPA in the presence of 10% acetonitrile. Figure 4.11 shows that there is a linear trend between the enzyme loading and the activity. The linear trend indicates that the immobilization efficiency is the same up to 3.5mg-BCA per g-HYPOL. Higher loadings could not be studied because of high cost of the enzyme.

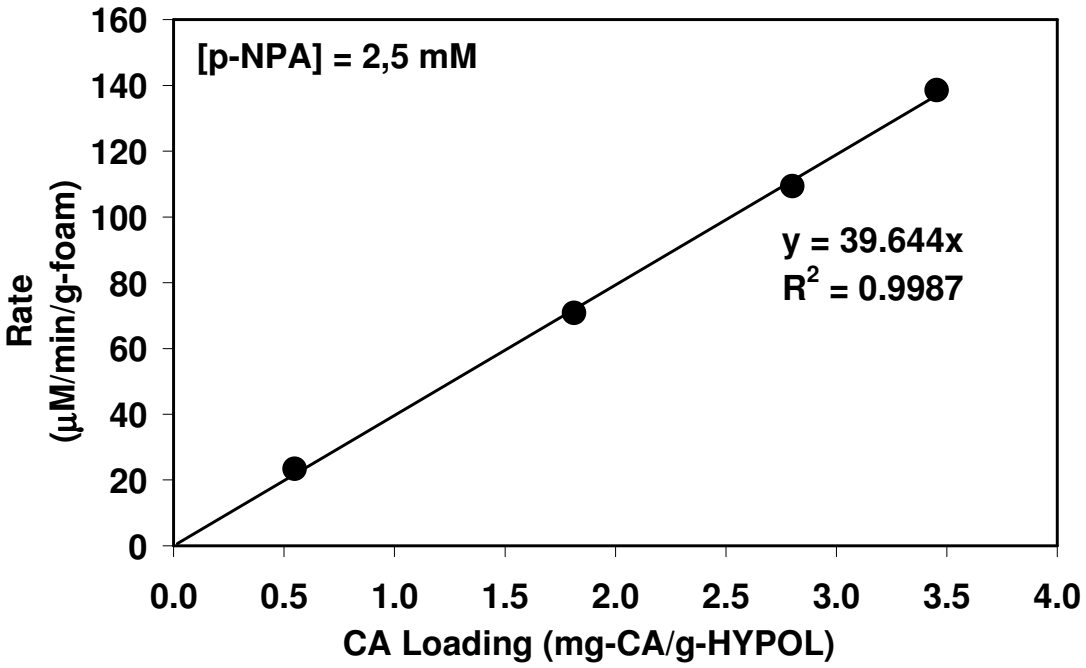

Figure 4.11. Effect of amount of BCA in PU foam to p-NP production rate.

4.8. Stability of Free and Immobilized CA

The stability of the free and immobilized BCA have been estimated. The free carbonic anhydrase was dissolved in tris buffer and stored at 4-5^{o}C in the fridge. The PU foam immobilized with bovine carbonic anhydrase was also stored in tris buffer at ambient temperature in the laboratory. In Figure 4.12, rates were normalized to the rate of p-NP production after the first day. As shown in the figure, free carbonic anhydrase has lost its activity during 45 days. However, the immobilized carbonic anhydrase was exhibited still a 100% activity. Improvement of the protein stability is important for enzymes for industrial processes, since it will prolong the half life of the protein. Figure 4.12 proves that half life of carbonic anhydrase was prolonged by immobilization within PU foam and this foam containing enzyme is convenient for industrial applications.

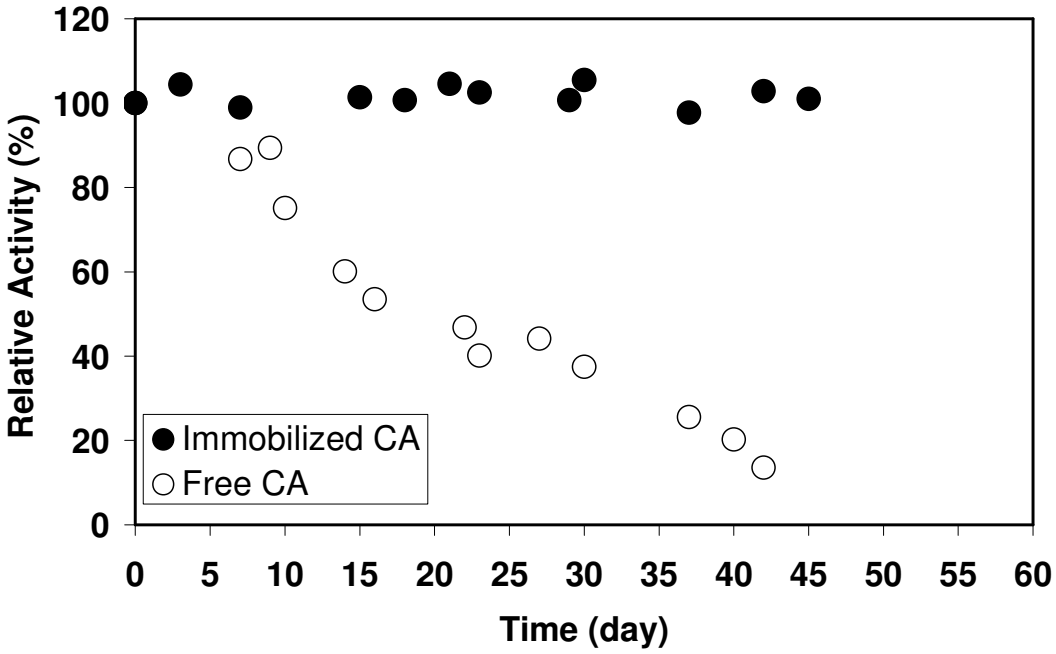

Figure 4.12. Stability of the free and immobilized CA.

4.9. Activity of Immobilized CA with Temperature

Reaction temperature is one of the most important parameter for enzymatic reactions. Optimum reaction temperature has to be determined to get a high productivity. Figure 4.13 shows the activity of the bovine carbonic anhydrase with temperature. As shown in the figure, the activity of immobilized BCA increases with temperature up to 40^{o}C. This is expected since the reaction rate increases with temperature for a chemical reaction. However, the activity began to decrease above 40^{o}C and no activity was observed at 60^{o}C for the immobilized BCA. It can be deduced from the figure that the optimum temperature for the immobilized BCA is in between 35-45^{o}C and the enzyme lost its activity at 60^{o}C

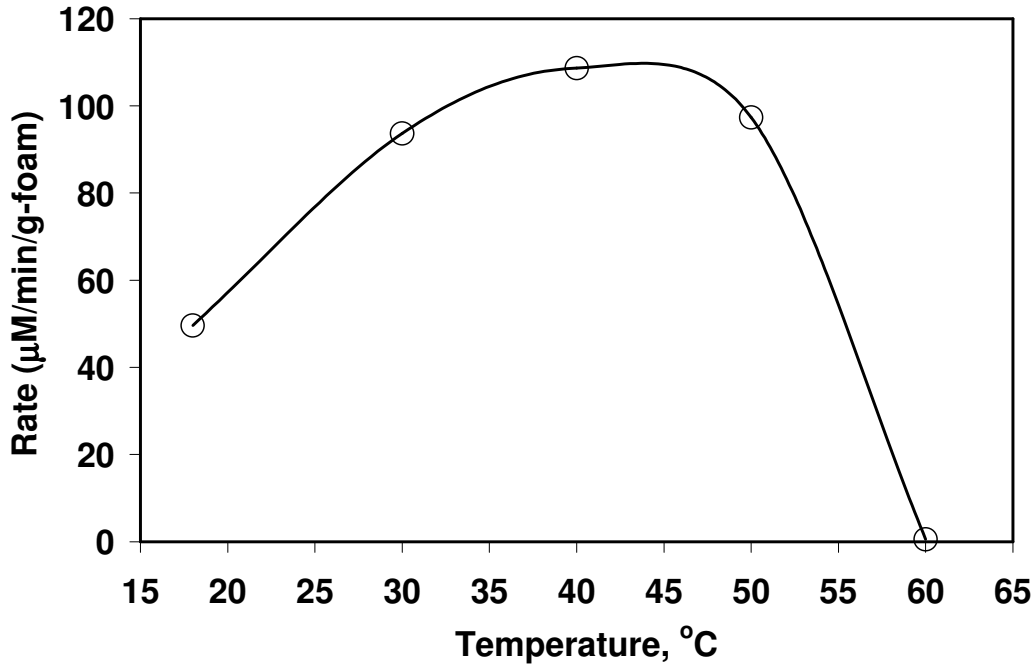

Figure 4.13. Temperature profile of BCA activity.

4.10. Thermal Stability of Free and Immobilized CA

In industrial reaction, stability is an important parameter. Especially, when elevated temperatures were used, the stability of the enzyme becomes more important. Here, the free and immobilized BCA were incubated at the desired temperatures and its activity was determined at various time intervals at 25°C with activity assay.

4.10.1. Thermal Stability of Free CA

Figure 4.14 shows the effect of incubation temperature on the free bovine carbonic anhydrase activity. In the figure, points show the experiment data and lines show the model fit of equation (2.14) which was used to calculate denaturation constants. As seen in the figure, the free bovine carbonic anhydrase preserved most of its stability for 120 minutes at 40°C. But when the incubation temperature was increased, the free enzyme began to lose its activity dramatically. Above 63°C, the free bovine carbonic anhydrase lost its activity completely in a very short time. The loss of enzyme activity at higher temperatures could be due to the breakage of the hydrogen

bonds and salt link which resulted in the conformational change of the bovine carbonic anhydrase from the folded structure to an unfolded structure.

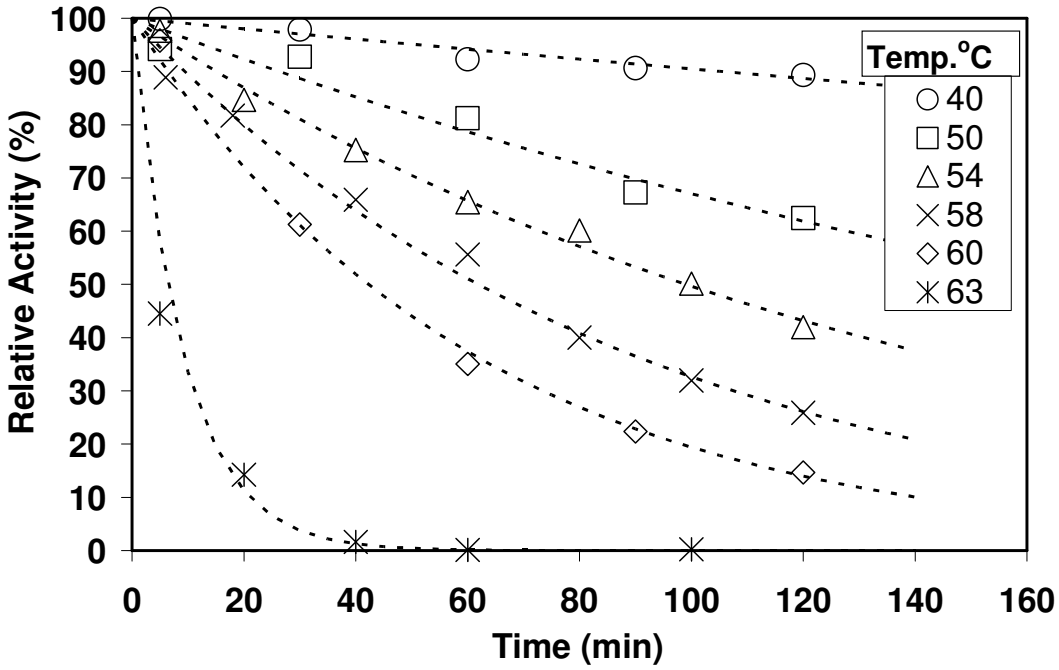

Figure 4.14**.** Heat inactivation curve of the free CA.

The denaturation constants were estimated from the evaluation of the experimental data at different temperatures and they are shown in Table **4.1**. As shown in the table, the denaturation constants are increasing with increasing temperature.

Table 4.1. Denatuıration constants (k_d) for free CA.

T (^{o}C)	**k_d (min^{-1})**
40	0.001
50	0.004
54	0.007
58	0.0112
60	0.0164
63	0.1143

The activation energy of the denaturation (E_d) was estimated by the help of the Arrhenius equation shown in equation (2.16). Figure 4.15 shows the relationship between $\ln k_d$ and 1/T. The slope of this figure was resulted in E_d/R and activation energy for denaturation (E_d) was calculated as 29 kcal/mol for the free BCA.

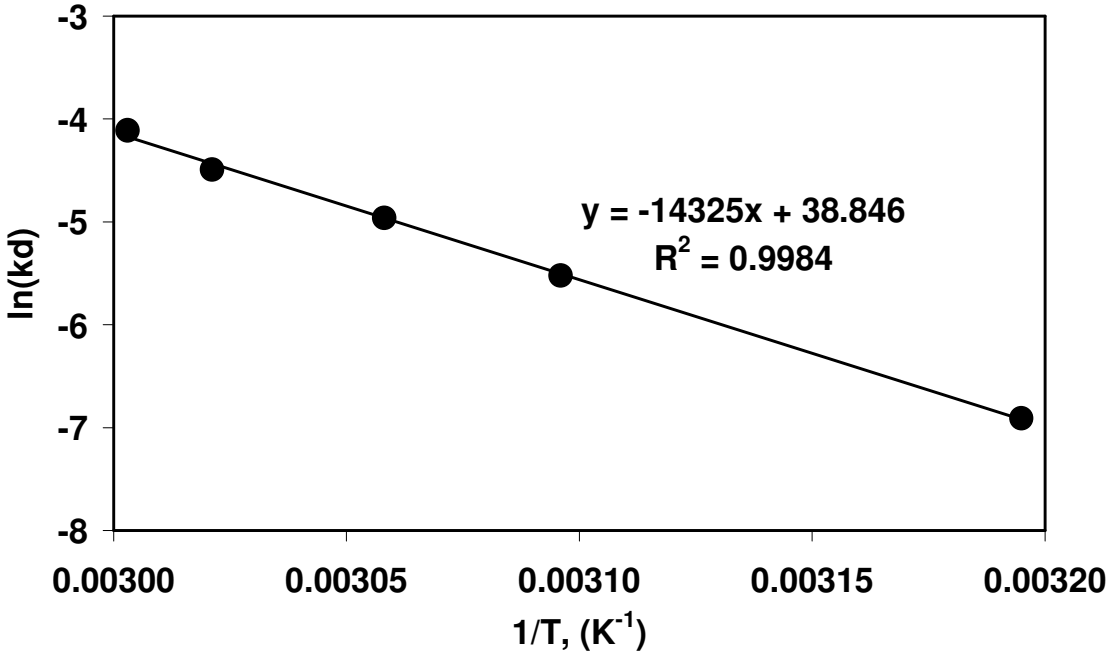

Figure 4.15. Logaritmic plot used for the determination of E_d.

4.10.2. Thermal Stability of Immobilized CA

The thermal stability of the the immobilized CA was also estimated. A piece of foam containing BCA was incubated at desired temperature over time. The activity of the foam was estimated with the enzyme assay at 25°C with various time intervals. Figure 4.16 shows the heat inactivation curves for the immobilized bovine carbonic anhydrase at different incubation temperatures. The lines are the curve fit for the denaturation kinetic of equation (2.14). The immobilized carbonic anhydrase was very stable at 40 and 50°C. Above 50°C, immobilized enzyme began to lose its activity. At incubation temperature of 60°C, the immobilized BCA lost its activity in 10-20 minutes.

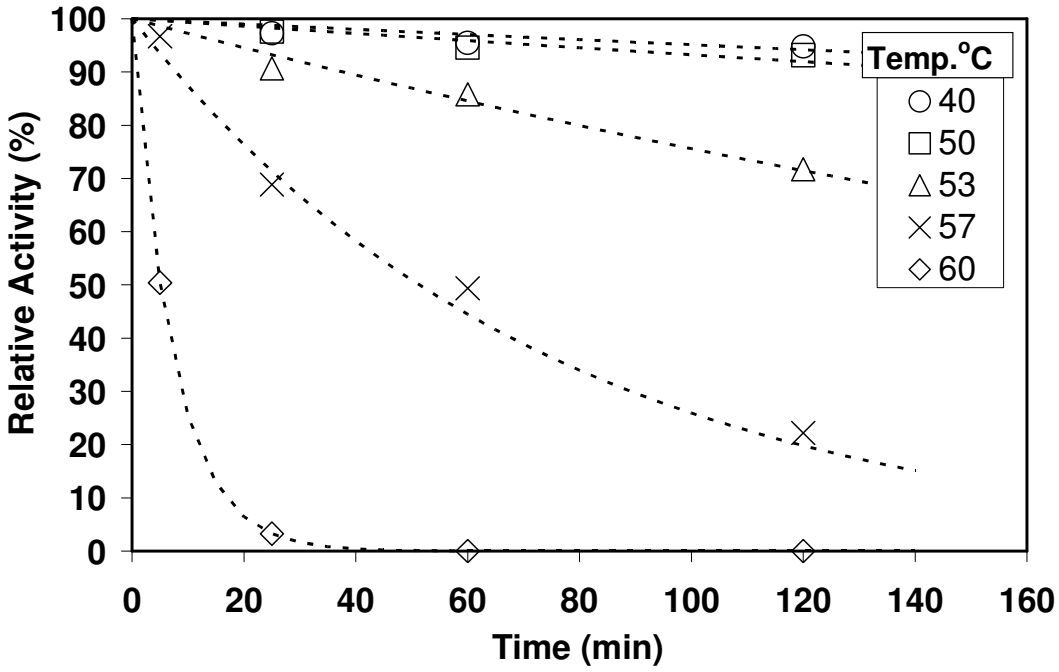

Figure 4.16**.** Heat inactivation curve of theimmobilized CA.

The denaturation constants, k_d, could be estimated from the evaluation of the data. Table 4.2 shows the k_d values for the immobilized BCA. As shown in the table the denaturation constant is increasing with temperature. However, these values are much smaller at lower temperatures comparing to the k_d values for the free BCA.

Table 4.2. Denaturation constants (k_d) for immobilized CA.

T (^{o}C)	**k_d (min^{-1})**
40	0.0005
50	0.0007
53	0.0028
57	0.0135
60	0.1231

The activation energy of denaturation for the immobilized BCA was also calculated from the slope $\ln k_d$ versus 1/T as shown in Figure 4.17. For the immobilized BCA, the E_d was calculated as 86kcal/mol. This value is almost three times higher than the value of the activation energy for denaturation for the free BCA.

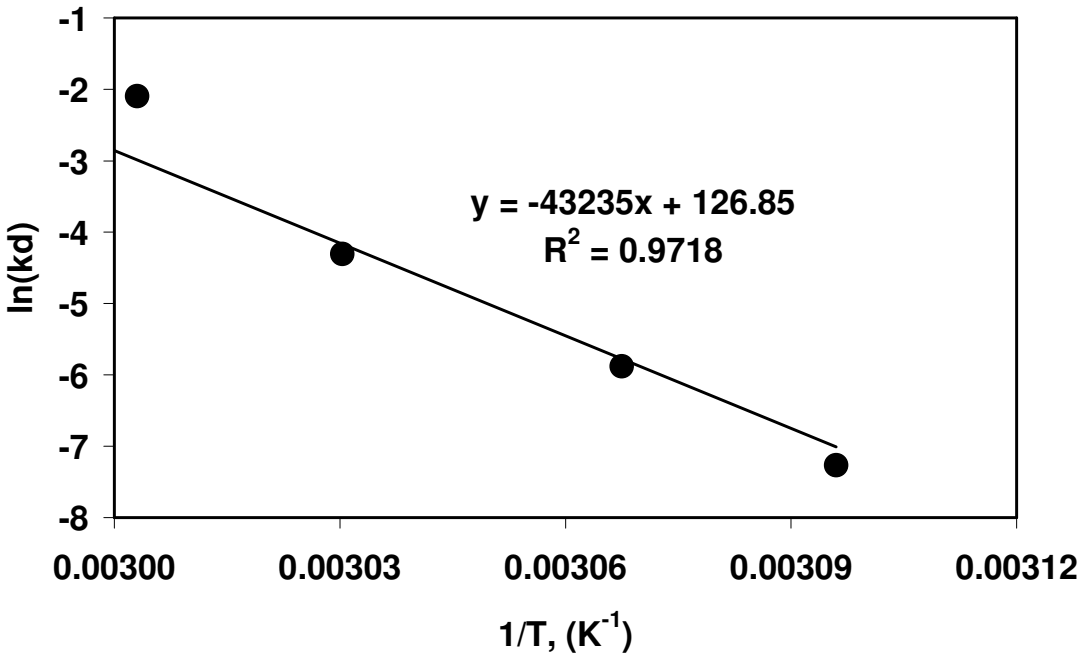

Figure 4.17. Logarithmic plot used for the determination of E_a.

Figure 4.18 compared the thermal stability for the free and immobilized bovine carbonic anhydrase at the end of the 1 hour of incubation at various temperatures. As shown in the figure, the activity for the free enzyme decreased monotonically. However, the activity for the immobilized BCA was stable up to 50°C and started to decrease as the temperature was increased. While the free BCA has lost its activity at 63°C, the immobilized BCA has lost its activity at 60°C. On the other hand, while the immobilized enzyme was more stable between 40 and 56 °C, the free enzyme was more stable between temperature of 56-63°C. This indicates that the BCA has an intermediate state. That is, the enzyme may undergo some unfolding at higher temperatures and loose some of its activity. However, when the enzyme was put into the assay solution at 25°C, it can refold to some degree and regain its activity. This figure shows that compared the free BCA, the immobilized BCA is more active at temperature lower than 56°C and less active at temperature greater than 56°C. This could indicate that the immobilized enzyme is more stable at low temperatures and could recover its activity at the assay temperature of 25°C. However, once unfolded at high temperature, the immobilized BCA could not recover its activity at the assay temperature due primarily to possible association of some groups on the enzyme with some groups on the PU foam backbone polymeric chains. Therefore, while immobilized BCA totally lost its activity at 60°C, the free enzyme could recover some of its

activity at the same temperature when temperature was reduced to the assay temperature of 25°C. As a result the immobilized BCA is therefore more stable up to temperature of 50°C, and it could be employed in the CO_2 sequestration.

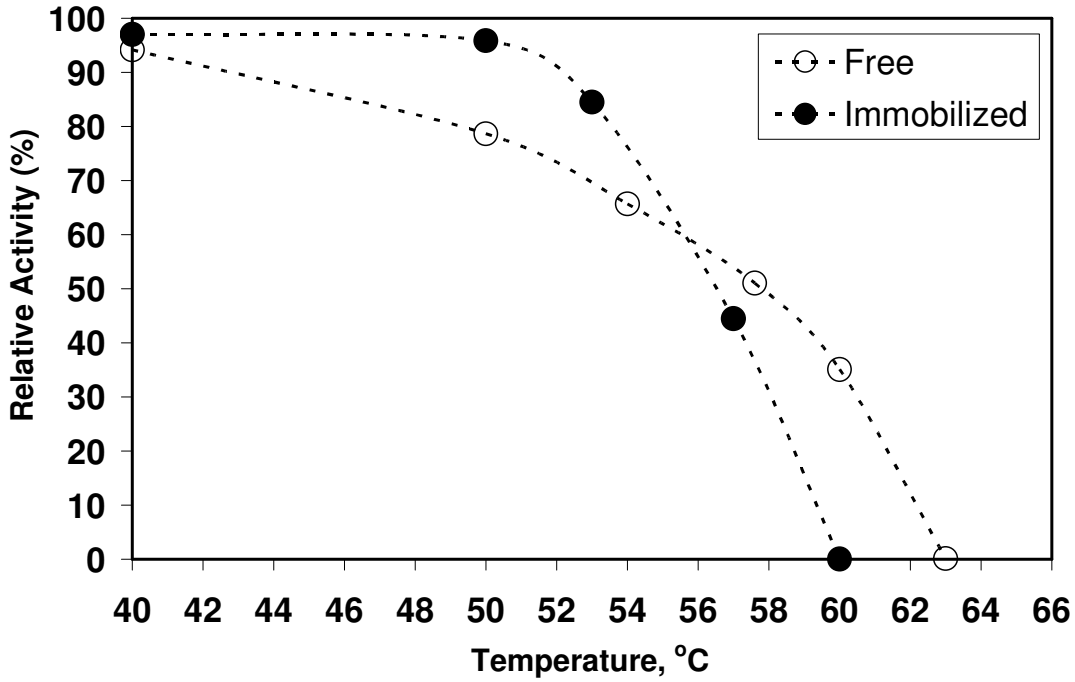

Figure 4.18. Temperature profile of CA after 1 hour incubation at constant temperature; free enzyme (open circles); immobilized enzyme (closed circles).

4.11. Immobilized Carbonic Anhydrase and Calcium Carbonate Precipitation

The immobilized BCA has been used in $CaCO_3$ precipitation studies. A 30mM of $Ca(OH)_2$ solution was prepared in a three-neck flask by stirring at 600rpm. CO_2 was injected through one-neck and the others were isolated by a pH probe and conductivity probe. The surface of the solution was filled with CO_2 in order to dissolve it in the solution. The pH and conductivity were recorded continuously to follow the precipitation process. Reaction was stopped when pH reached about 7 because precipitated $CaCO_3$ is to dissolve at lower pHs. Figure 4.19 shows the pH change during the $CaCO_3$ precipitation. The precipitation experiments were run with a control experiment containing no foam and no CA, a comparison experiment containing PU foam without CA, and precipitation experiment containing PU foam including

immobilized CA. As shown in Figure 4.19, the pH was stable over 20 minutes for the control experiments and precipitation was completed in 40 minutes. The pH profile switched a little to the right for the comparison experiment, where it was stable up to 30 minutes and the precipitation was completed in 55 minutes. On the other hand, the pH was stable up to 40 minutes for the precipitation experiment including PU foam containing CA and it gradually decreased up to 65 minutes and the precipitation was completed in 90 minutes.

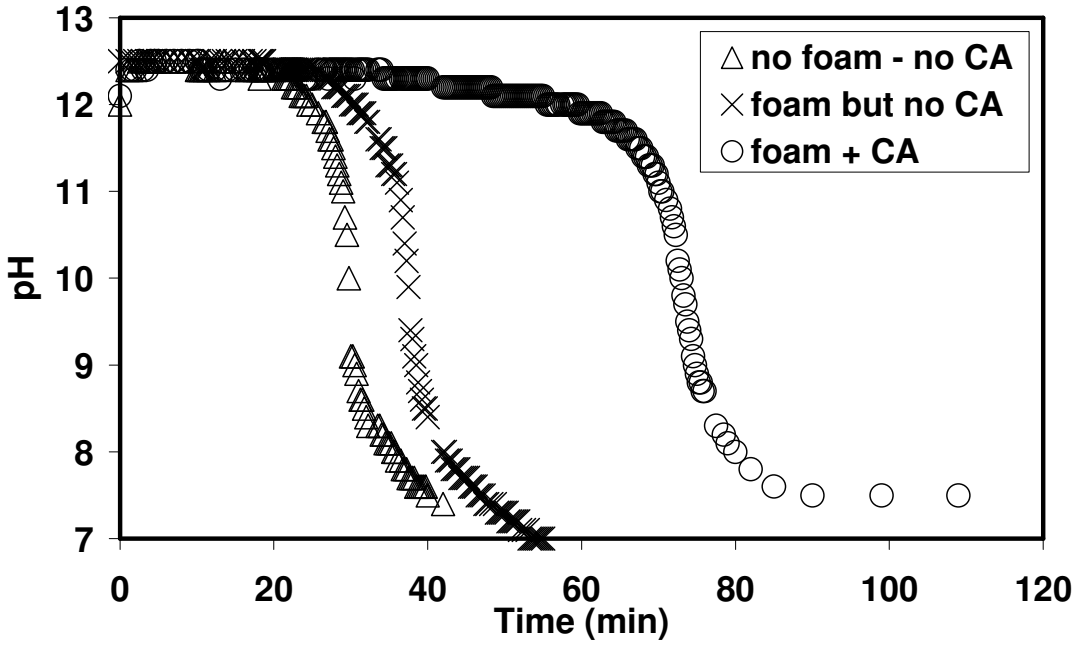

Figure 4.19. Effect of Foam containing bovine carbonic anhydrase to pH**.**

As shown in Figure 4.20, the conductivity change followed a similar trend with the pH trend. The conductivity for control experiment stayed up to 15 minutes and steadily decreased for completion. The conductivity experiment has also steadily decreased for the precipitation. However, the conductivity showed a similar trend up to 25 minutes for the comparison experiment and followed the completion of the precipitation in 40 minutes. Since the conductivity indicates the presence of ions in the solution, these trends were probably affected the crystallization kinetic and the particle formation.

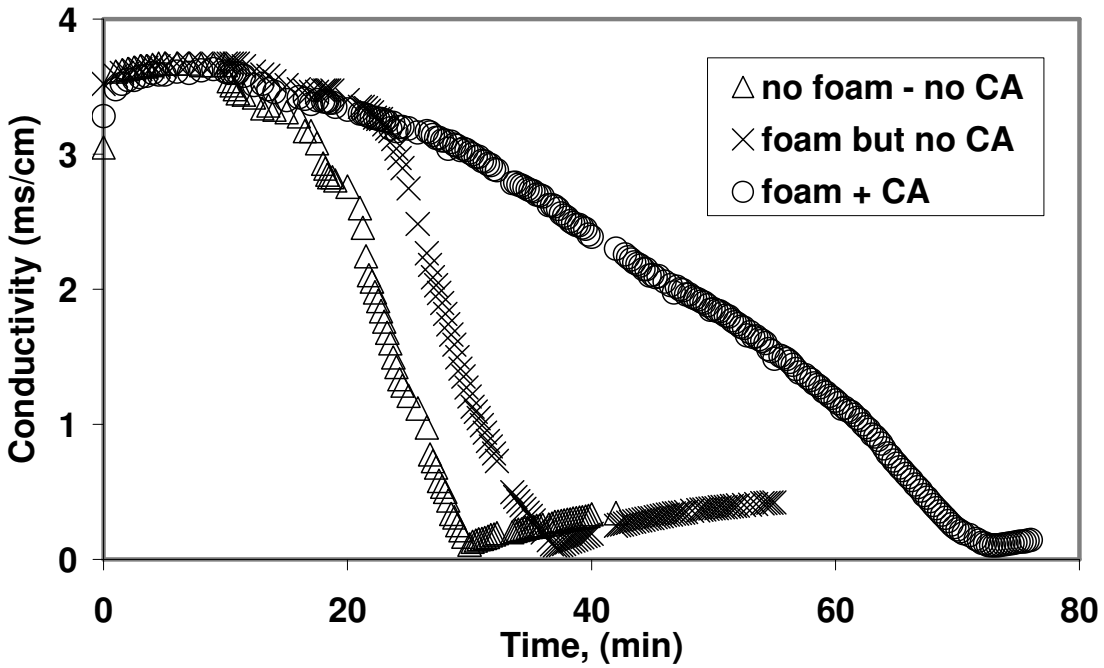

Figure 4.20. Conductivity change during the $CaCO_3$ precipitation.

As shown in Figure 4.21, the XRD pattern of precipitated products indicated that all three precipitated $CaCO_3$ are indeed calcite. The regions of peaks show that atomic arrangements are the same for the three precipitated $CaCO_3$.

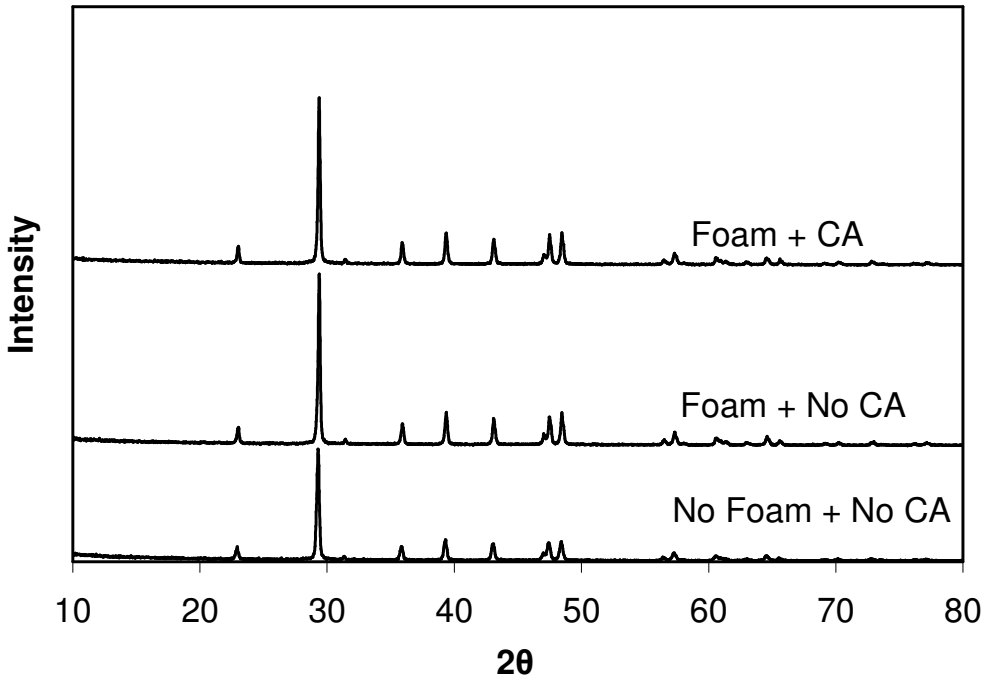

Figure 4.21. XRD patterns of $CaCO_3$.

The SEM images of the three precipitated $CaCO_3$ were shown in Figure 4.22 for control experiment, Figure 4.23 for the comparison experiment, and Figure 4.24 for the enzymatic precipitation experiment. As shown in Figure 4.22, the $CaCO_3$ particles for the control experiment are between 40-800nm and about 200nm in average. The particles are mostly round and oval shapes. The particles for the comparison experiment are distributed into two dimensions. One is big, larger than 200nm and the others are small, less than 200nm. On the other hand, the particle sizes of the enzymatic precipitation experiment are all less than 100nm, generally about 70nm, and show fine distrubution.

Although there seems to be a difference in employing BCA immobilized PU foams in $CaCO_3$ precipitation, the PU foam may also affect the precipitation by it self. This topic is currently under investigation and more discussion could be made in the future due to time constraints.

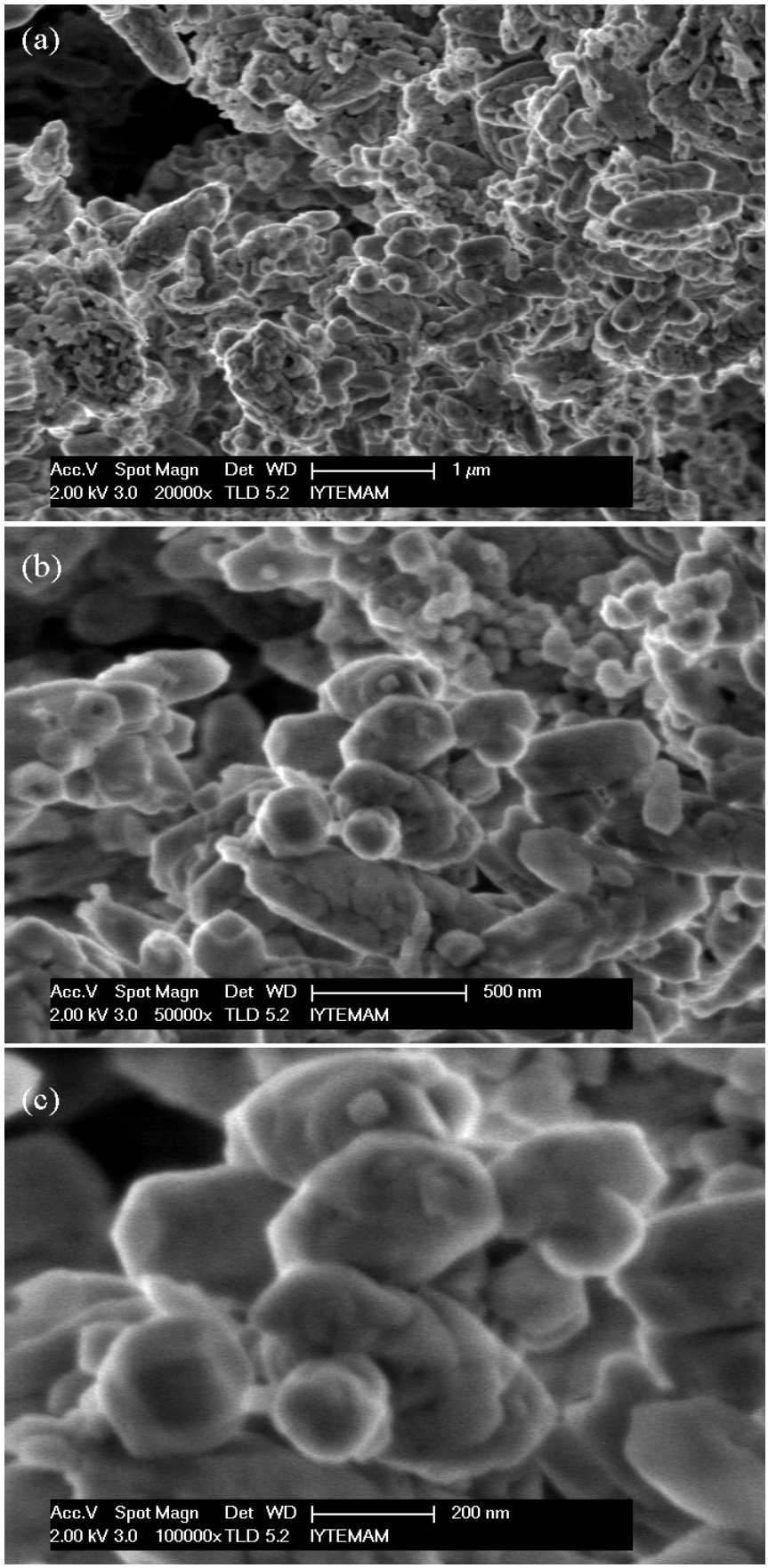

Figure 4.22. SEM images of $CaCO_3$ from control experiment (Reaction mixture includes no PU foam and no BCA).

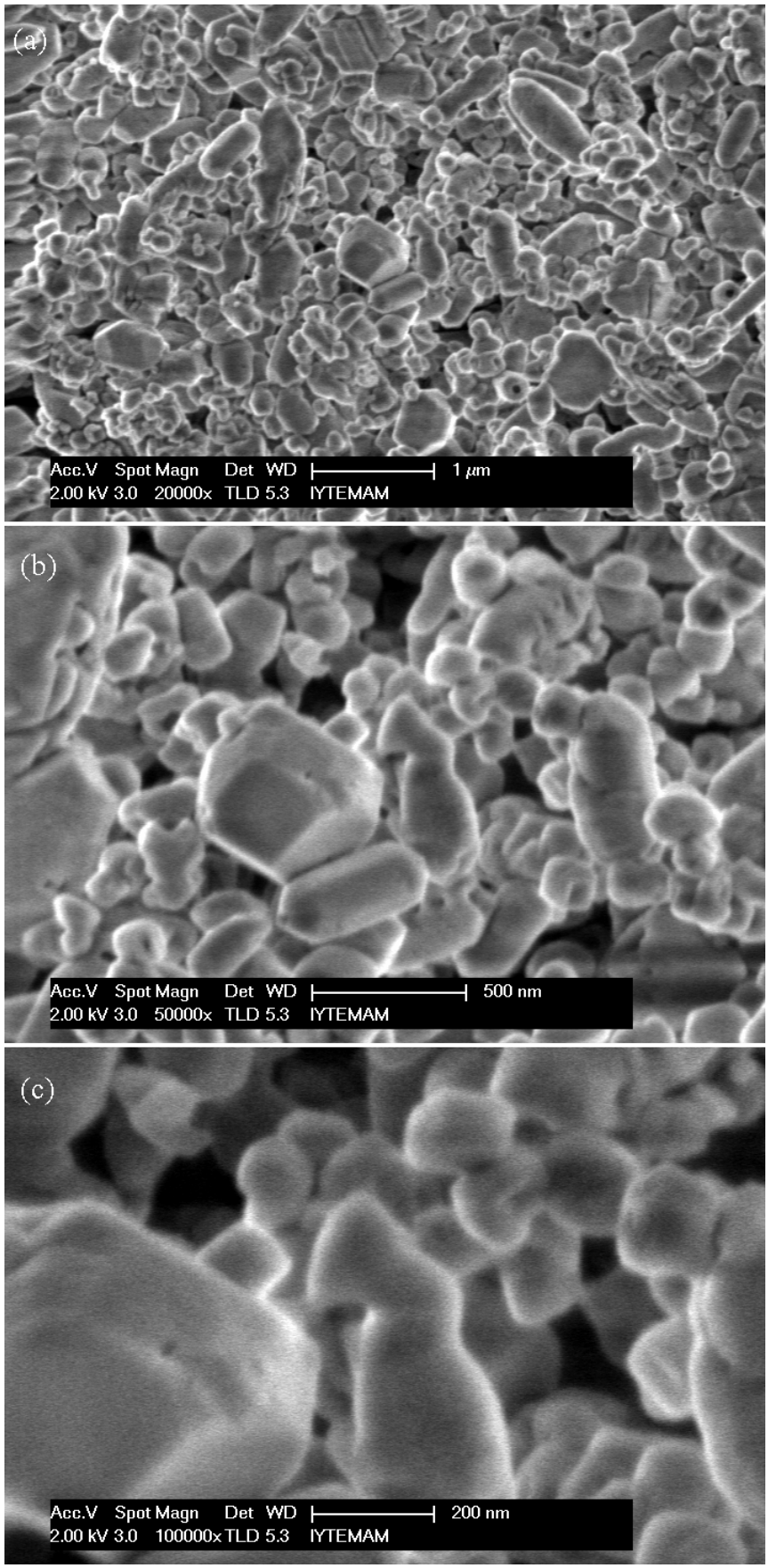

Figure 4.23. SEM images of $CaCO_3$ from comparison experiment (Reaction mixture includes PU foam but no BCA).

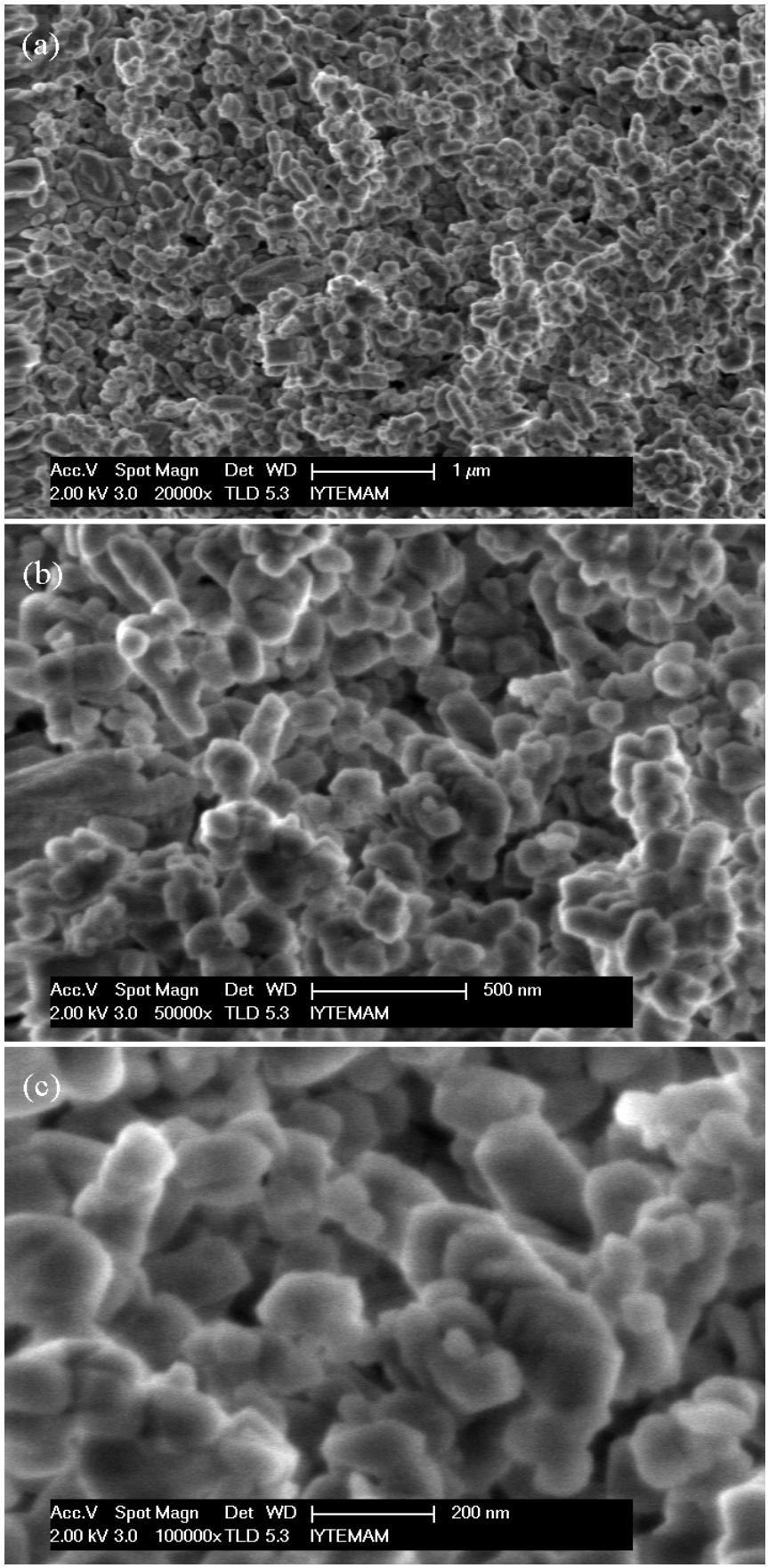

Figure 4.24. SEM images of $CaCO_3$ precipitation experiment (Reaction mixture includes PU foam including immobilized BCA).

CHAPTER 5

CONCLUSION

The Bovine Carbonic Anhydrase (BCA) enzyme was immobilized within polyurethane foam successfully. The immobilized BCA showed excellent stability and reuse capacity in aqueous solution. The ideal temperature range of operation was found to be 35^{o}C and 45^{o}C. When temperature was increased, the immobilized enzyme activity started to decrease and finally lost its activity at 60^{o}C. The immobilized CA was employed in $CaCO_3$ precipitation and found that the precipitated $CaCO_3$ was all calcite and more evenly distributed with particle sizes less than 100nm. Therefore, the immobilized CA could be used not only to sequester CO_2 but also to produce a valuable product. These nano-particles could be used in other industrial processes such as paper, ink, paint, or coating production plants.

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