ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

SYNTHESIS OF POLYCAPROLACTONE VIA ENZYMATIC RING OPENING POLYMERIZATION

Msc. Thesis by
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Department : Chemical Engineering

Programme : Chemical Engineering

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<u>İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ</u>

ENZİMATİK HALKA AÇILMASI POLİMERİZASYONU İLE POLİKAPROLAKTON SENTEZİ

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FOREWORD

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June 2011   Banu İYİSAN

                                                                                                          Chemical Engineer

To my parents and Umut,

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ABBREVIATIONS

PCL	: Poly(ε-caprolactone)
Ɛ-CL	: Epsilon caprolactone
^{1}H-NMR	: Proton Nuclear Magnetic Resonance Spectroscopy
GPC	: Gel Permeation Chromatography
DSC	: Differential Scanning Calorimetry
FTIR	: Fourier Transform Infrared Spectrophotometer
SEM	: Scanning Electron Microscopy
ROP	: Ring Opening Polymerization
PGA	: Poly (glycolide)
PLA	: Poly (lactide)
UV	: Ultraviolet
TEM	: Transmission Electron Microscopy
CALB	: Candida Antarctica Lipase B
CALA	: Candida Antarctica Lipase A
HPLC	: High Performance Liquid Chromatography
THF	: Tetrahydrofuran
GLT	: Gluteraldehyde
K_1	: Chitin immobilized lipase via cross-linking (0.02% GLT) GLT)
K_2	: Chitin immobilized lipase via cross-linking (0.2%) GLT)
K_3	: Chitin immobilized lipase via cross-linking (2% GLT)
K_4	: Chitin immobilized lipase via physical adsorption
$Immob_1$	: Chitosan immobilized lipase via cross-linking (0.02% GLT)
$Immob_2$	: Chitosan immobilized lipase via cross-linking (0.02% GLT)
$Immob_3$	: Chitosan immobilized lipase via cross-linking (2% GLT)
$Immob_4$	: Chitosan immobilized lipase via physical adsorption

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SYNTHESIS OF POLYCAPROLACTONE VIA ENZYMATIC RING OPENING POLYMERIZATION

SUMMARY

Recently, aliphatic polyesters obtained with enzymatic ring opening polymerization of lactones are of considerable interest due to its biocompatibility, biodegradability and superior mechanical properties. Biomedical applications of these polymers such as drug delivery systems and biomaterial area has been increasing nowadays because of their biodegradability and non-toxic effect in living systems.

Among polyesters, PCL has been receiving increased attention as an important tool for biomedical field. Since it has relatively low melting points (~60 ^{o}C) and blending ability with numerous polymers, design and manufacturing of desired structures can be obtained at lower temperatures. Also, it is a hydrophobic, semi-crystalline linear polymer and its glass transition temperature is -60 ^{o}C. Due to its superior rheological and viscoelastic properties comparing with other polyesters, PCL can be easily produced and large range of biomaterials can be manufactured. In comparison with other polyesters used in tissue engineering applications such as PLA and PGA, PCL can be degraded slower in living cells. This property is very important for drug delivery systems since drug can stay in cells longer time and therapeutic effect of the drugs are increasing. Due to these advantageous properties for biomedical applications, PCL has become an important tool for polymer science and biotechnological applications.

PCL is synthesized via ring opening polymerization of ε-caprolactone which is a cyclic ester. ROP can be carried out with organometallic initiators such as Zn, Al, Sn, Ge or chemical catalysts. However, it is impossible to remove entirely these toxic metallic compounds from polymer matrix. This is of concern for biomedical applications since residues of these compounds can cause inflammatory response when the polymer used as a biomaterial. Therefore, application of biocatalysts to polymer synthesis is one of the most promising trends nowadays. Since biocatalysts are derived from renewable resources, they are referred as eco-friendly materials. Another advantage of biocatalysts is mild reaction conditions: enzymes can catalyze reactions at relatively low temperatures and pressures comparing with chemical catalysts. Also enzymes can be active in different organic media and due to high entio- and regio-selectivity of enzymes, well-defined polymers can be synthesized. On the other hand, biocatalysts may have a stability problem and lose its activity for long reaction time. Thus, immobilization of enzymes has been receiving great attention since this process enhances enzyme stability, activity and reusability in reaction medium.

This study focused on PCL synthesis via enzymatic ring opening polymerization of ε-CL. It was aimed to develop different immobilized enzymes for PCL synthesis.

For this purpose, firstly polymerization reaction was performed with *candida antarctica lipase B (CALB)* enzyme immobilized on acrylic resin. This enzyme is available commercially (trade name: novozym 435) and often used for polyester synthesis in literature. Although it can catalyze the reaction efficiently, high costs and enzyme leakage problem leads scientists to solve these negative sides of novozym 435. Due to this, in the second part of the study, different supports are used for *CALB* immobilization in order to obtain efficient catalysts for PCL synthesis. As support material for immobilization process, chitin and chitosan was chosen in this study. The reason why chitin and chitosan was chosen as a support material is that they are cheap, ubiquitous and nontoxic materials; also they have high protein affinity.

Furthermore, immobilization methods were optimized by using two different techniques: physical adsorption and cross-linking with gluteraldehyde. Resulting immobilized catalysts were evaluated in polymerization reactions and immobilized lipases via cross-linking with gluteraldehyde were more efficient than physically adsorbed enzymes. Also, optimization of coupling agent amount (gluteraldehyde) was carried out. Thus, moderate gluteraldehyde ratio (0.2% v/v) provided most efficient catalysts either chitin or chitosan.

This study was concluded with evaluation of immobilized enzymes (novozym 435, K_2, Immob$_2$) at three different temperatures (60, 70 ve 80 ^{o}C) within a time range for ROP of ε-CL. Obtained polymers were characterized by ^{1}H NMR and FTIR analysis. Furthermore, DSC analysis was applied in order to observe thermal behaviors and crystallinity of polymers. Molecular weights and polydispersities of obtained polymers were determined with GPC.

In conclusion, performance of chitin and chitosan immobilized lipases were compared with novozym 435. Thus, it was seen that novozym 435 can catalyze reaction faster than chitin and chitosan immobilized lipases. However, polydispersity of polycaprolactones obtained with this enzyme was higher than the polymers synthesized via prepared enzymes in this study. Also, performance of chitin immobilized lipases was higher than chitosan immobilized lipases.

ENZİMATİK HALKA AÇILMASI POLİMERİZASYONU İLE POLİKAPROLAKTON SENTEZİ

ÖZET

Laktonların halka açılımı polimerizasyonu ile elde edilen alifatik poliesterler, biyobozunur olmaları, biyouyumlulukları, toksik özellik göstermemeleri ve üstün mekanik özellikleri ile biyomedikal ve ilaç endüstrisinde gittikçe artan bir oranda yer almaktadır. Biyobozunur özellikleri, bu polimerlerin implant malzeme olarak ve kontrollü ilaç salınım sistemlerinde önemli bir araç olmasını sağlamıştır.

Poliesterler içerisinde yer alan polikaprolakton, sahip olduğu birtakım özellikleri ile son yıllarda ön plana çıkmaktadır. Polikaprolakton (PCL), üstün mekanik özelliklere sahip, hidrofobik, yarı kristalin lineer bir polimerdir. Camsı geçiş sıcaklığı (Tg) -60 ^{o}C, erime noktası ise 60 ^{o}C'dir. Düşük erime noktası ve diğer polimerler ile kolaylıkla karışarak kompozit oluşturabilme kapasitesi, PCL ile ilgili araştırmaların özellikle biyomedikal alanda odaklanmasına neden olmaktadır. Bu sayede, polimerin şekillendirilmesi düşük sıcaklıklarda gerçekleştirilebilmekte ve istenilen özellikteki malzemeler kolaylıkla üretilebilmektedir. Buna ilaveten, büyük çapta üretimlerde PCL'nin üstün viskoelastik ve akış özellikleri üretim prosesini kolaylaştırmaktadır. PCL'nin kendisi gibi doku mühendisliği çalışmalarında kullanılan polilaktik asit ve poliglikolit gibi diğer poliesterlere kıyasla canlı ortamlarda daha uzun sürede biyobozunur olması, ilaç salım sistemlerinde kullanımı için önemli bir üstünlük sağlamaktadır. Tüm bu özellikler, araştırma dünyasında ve biyoteknoloji araştırmalarında polikaprolaktonu odak noktası haline getirmiştir.

PCL, halkalı bir ester olan ε-kaprolakton monomerinden halka açılımı polimerizasyonu ile sentezlenmektedir. Halka açılımı polimerizasyonu Zn, Al, Sn, Ge gibi organometalik başlatıcılarla veya kimyasal katalizörlerle gerçekleştirilebilir. Ancak toksik özellik gösteren bu kimyasalların polimerden tamamen uzaklaştırılması mümkün olmamaktadır. Bu nedenle biyokatalizörlerin kullanımı ile polimerizasyon işlemini yürütmek son yıllarda yeni bir eğilim olarak gündemdedir. Biyokatalizörler, yenilenebilir kaynaklardan elde edildiklerinden dolayı çevre dostu malzemeler olarak görülmektedir. Biyokatalizörlerin bir diğer avantajı ise; reaksiyonun daha ılıman koşullarda gerçekleştirilebilmesidir. Enzimler, kimyasal katalizörlere kıyasla daha düşük sıcaklık ve basınçlarda, farklı organik ortamlarda etkili olabilmektedir. Ayrıca enzim katalizli proseslerde, enzimlerin enantio ve regio seçicilikleri nedeniyle istenilen özellikte polimer sentezi gerçekleştirilebilmektedir. Bunlara paralel olarak enzimlerin immobilizasyonu ile oluşturulan farklı biyokatalizörlerin poliester sentezinde kullanımı son yıllarda dikkat çekmektedir.

Bu çalışma, halkalı bir ester olan ε-kaprolakton monomerinden enzimatik halka açılması polimerizasyonu ile polikaprolakton eldesi üzerine odaklanmıştır. PCL sentezi, farklı taşıyıcılar üzerine immobilize edile enzimlerin katalizörlüğünde gerçekleştirilmiş, ve poliester sentezinde yeni biyokatalizörlerin geliştirilmesi üzerine yoğunlaşılmıştır.

Bu amaçla, öncelikle polimerizasyon işlemi literatürde poliester sentez işlemlerinde sıklıkla kullanılan akrilik rezin üzerine immobilize edilmiş candida antarctica lipase B (CALB) enzimi kullanılarak gerçekleştirilmiştir. Ticari ismi 'Novozym 435' olan bu enzimin reaksiyonda yüksek performans göstermesine rağmen, pahalı olması ve taşıyıcı ile arasındaki bağın zayıflığı çözülmesi gereken bir sorun olarak göze çarpmaktadır. Bu nedenle çalışmanın ikinci kısmında, poliester sentezinde kullanılmak üzere lipaz enzimi farklı taşıyıcılar üzerine immobilize edilmiştir. Çalışmada, taşıyıcı malzeme olarak kitin ve kitosan doğal polimerleri seçilmiştir. Bunun nedeni kitin ve kitosanın ucuz, doğada bol bulunan ve toksik etki yaratmayan yapısıdır.

Deneysel çalışmanın devamında lipaz enziminin kitin ve kitosan polimerleri üzerine immobilizasyon işlemi optimize edilmiştir. Fiziksel adsorpsiyon ve çapraz bağlanma olmak üzere iki farklı immobilizasyon yöntemi uygulanarak elde edilen enzimlerin performansı polimerizasyon işleminde değerlendirilmiştir. Sonuçta, çapraz bağlanma yöntemi ile immobilizasyon fiziksel adsorpsiyona göre daha etkili olmuştur. Bu aşamada çapraz bağlanma ajanı olarak kullanılan gluteraldehit miktarı değiştirilerek, en uygun immobilizasyon işlemine karar verilmiştir. Deneysel çalışmanın bu kısmında, hem kitin hem de kitosan taşıyıcısı için hacimsel olarak 0.2% gluteraldehit kullanılarak elde edilen immobilize lipazların verimli olduğu görülmüştür.

Çalışmanın son kısmında elde edilen immobilize enzimler 60, 70 ve 80 ^{o}C olmak üzere üç farklı sıcaklıkta halka açılımı polimerizasyonunda değerlendirilmiştir. Elde edilen polimerlerin NMR ve FTIR analizleri ile yapısal karakterizasyonu yapılmış, DSC analizi ile ısıl özellikleri incelenmiştir. Ayrıca, sıcaklığın etkisi üretilen polimerlerin molekül ağırlıkları GPC analizi ile belirlenerek gözlenmiştir. Sonuçta kitin ve kitosan üzerine immobilize edilmiş lipaz enzimleri ile novoyzm 435 enzimi karşılaştırılmıştır. Novozym 435 enzimi polimerizasyon reaksiyonunu daha hızlı katalizlemesine rağmen, bu enzimle elde edilen polikaprolaktonların polimerizasyon derecesi kitin ve kitosan immobilizasyonu ile elde edilen lipazlara göre yüksektir. Kitin immobilizasyonu ile elde edilen lipaz enziminin performansı kitosan ile elde edilene göre daha yüksek olmuştur.

1. INTRODUCTION

Today, polymeric materials are indispensable part of a modern society and used in many applications such as electronics, communications, transportations, drug delivery systems, tissue engineering and medical devices [1]. Among them, biomedical applications are of considerable interest for last two decades. Therefore, development of biopolymers which are biodegradable, biocompatible and non-toxic has been an ongoing study field of polymer chemistry recently.

On the other hand, environmental aspects should also be concerned during polymer production. Since synthetic polymers are generally obtained from petroleum based resources, they have many harmless effects to the environment. Therefore, new trend 'green' chemistry has been rising in order to prevent negative effects of chemical synthetic methods. Energy minimization, using recyclable resources and moderate reaction conditions are aimed with these environmentally benign synthetic routes. Application of enzymes to polymer synthesis is the most promising eco-friendly tool for green chemistry [2].

Among biopolymers, aliphatic polyesters synthesized by ring opening polymerization (ROP) of lactones have extensively studied since their superior mechanical properties, biodegradable and biocompatible behaviors [3]. Polyesters are very important materials since they are in fourth place in living systems followed nucleic acids, proteins and polysaccharides. They can be synthesized by polycondensation or ring opening polymerization [1]. Among them, polycaprolactone is one of the important polyester which has the ability of blending with numerous polymers. This polymer can be produced easily for large scale because it has superior viscoelastic and rheological properties. On the other hand, long-term biodegradability leads PCL to drug-delivery systems. Therefore, it is a promising area for polymer technology.

Synthesis of PCL consisted of chemical and enzymatic modes of polymerization. Chemical polymerization can be performed with organometallic initiators or catalysts such as Zn, Al, Sn and Ge by ring opening polymerization of ε-caprolactone.

However, metallic compounds cannot be removed entirely from PCL and for biomedical applications they cause high toxicity. Also, chemical polymerizations proceed at high temperatures and residues of metallic compounds can effect environment negatively. On the other hand, enzymatic polymerization provides many advantages such as moderate reaction conditions, pure resulting polymer and recyclable biocatalyst. Also, well defined polymer structure can be obtained with enzymatic catalysis [4].

Enzymatic ring opening polymerization of ε-CL is performed by using lipase enzyme, a kind of hydrolyses that catalyze the hydrolysis of fats in living cells. Their hydrolysis effect can be reversed into ester synthesis in non-aqueous media. In literature, novozym 435 which is an immobilized form of *candida antarctica lipase B* is used for this polymerization mode. Immobilization is applied since enzyme activity, stability, selectivity and reusability is improved with this process. Although novozym 435 can catalyze the ROP reaction efficiently, because of the cost reasons and enzyme leakage problem, new supports for immobilization of lipases are investigated in literature [5-8].

In this study, chitin ad chitosan are used as supports for lipase immobilization since they are cheap, ubiquitous and nontoxic material [9, 10]. Firstly, reference polymerization reactions were performed with novozym 435 at three different temperatures (60, 70, 80 ^{o}C) within a determined time range. Secondly, chitin and chitosan were immobilized to lipase by two different methods: physical adsorption and cross-linking with glutaraldehyde which is a common coupling agent. Obtained immobilized enzymes were evaluated by polymerization reactions and the highest performance was determined. In the following steps of the study, chosen immobilization method was applied to lipases and these enzymes are used in further polymerization reactions. In this step, like novozym 435 catalyzed polymerizations, ROP of ε-CL was performed at three different temperatures (60, 70, and 80 ^{o}C) within a determined time range in order to optimize polymerization reactions. Thus, obtained polymers with different catalysts were characterized by FTIR, ^{1}H-NMR, GPC and DSC.

2. THEORETICAL STUDY

2.1 Poly (ε-caprolactone) (PCL)

PCL is a part of polyester family that is an important class of biomacromolecules in living systems. Polyesters and other biomacromolecules such as polysaccharides and proteins are synthesized by enzymes in living cells. By the use of this natural process, production of biopolymers with enzymes is a demanding trend for the last years since it is an eco-friendly technique. In comparison to annual synthetic polymer production, natural polymers are produced four to five magnitudes bigger than those derived from petroleum stock [1, 4].

PCL was synthesized by the Carothers Group in the early 1930s. It can be synthesized by ring opening polymerization of ε-caprolactone with enzymes or organometallic catalysts. It is hydrophobic, semi-crystalline linear aliphatic polyester. Its crystallinity is inversely proportional with its molecular weight. Researches about PCL are mainly focused on biomedical field because of its low melting point (59-64 ^{o}C), good solubility and blending capacity with other polymers. Solubility behavior of PCL in some solvents is seen in Table 2.1. It has also good mechanical properties, biodegradable and biocompatible structure. Its glass transition temperature (Tg) is -60 ^{o}C and molecular weight is in the range of 3000-80000 g/mol. Its easy formability at low temperatures and good viscoelastic and rheological properties provides easy manufacturing for PCL. Therefore, PCL can be easily produced in large scales as implants and devices. Among the polymers, which are used in biomedical field, these properties make PCL advantageously [11, 12].

Table 2.1 : Solubility of PCL

Solvent	Solubility Behavior of PCL
Chloroform, Dichloromethane, Benzene	Soluble at room temperature
Carbon Tetrachloride, Toluene	Soluble at room temperature
Acetone, Ethyl Acetate, Acetonitrile	Low solubility
2-butanone, Dimethylformamide	Low solubility
Alcohol, petroleum ether, diethyl ether	Insoluble

One of the important properties of PCL is biodegradable nature of the polymer. It can be said that although PCL can be degraded by outdoor living organisms such as bacteria and fungi, the degradation process is slower in human and animal bodies. Degradation studies in literature shows that degradation process is consisted of two steps: in the first step; ester linkages are cleaved by non-enzymatic hydrolysis, then in the second step; when the molecular weight decreases to less than 3000 g/mole intracellular degradation is shown. In this step, PCL is completely resorbed by the activity of macrophages and giant cells. Bioresorbability is another important property for PCL when it is used as a biomedical device or in drug delivery systems. Long-term biodegradable behavior in human beings provides PCL advantage for the usage in drug-delivery systems. As it is shown in Table 2.2, comparing with PCL degradation, poly (lactide) (PLA), Poly (glycolide) (PGA) and their copolymers' degradation is fast. Also, other properties such as melting point and glass transition temperature of these polymers are seen in Table 2.2. Another important characteristic of PCL is biocompatible nature of the polymer. Biocompatibility means that in a specific application, appropriate host response is obtained by the use of related material. In order to use the polymer as a medical device or in drug delivery systems, polymer has to be biocompatible. Therefore, PCL becomes important element for tissue engineering applications with its important properties [11].

Table 2.2: Comparison of PCL and some degradable polymer properties

Polymer type	Melting Point (^{o}C)	Glass Transition Point (^{o}C)	DegradationTime (months)
PLA	173-178	60-65	6-12
PGA	225-230	35-40	>24
PCL	65-60	-65-60	>24
Poly(D,L-lactide-co-glycolide)	Amorphous	50-55	5-6
Poly(L-lactide-co-glycolide)	Amorphous	50-55	5-6
Poly(L-lactide-co-D,L-lactide)	Amorphous	55-60	12-16

2.2 Enzymatic Polymerization

In the recent decades, enzymatic polymerization has become an important field in polymer chemistry. Enzymes provide a new 'green' synthetic strategy for well-defined and useful polymers. Enzymatic polymerization is an environmentally benign process since it uses renewable resources, performed under mild reaction conditions and it does not produce nontoxic compounds. Therefore, it is an ongoing field of study in polymer synthesis [13].

According to enzyme commission, enzymes are classified into six groups as shown in Table 2.3. Also, typical polymers synthesized by these enzymes can be seen from Table 2.3. Some of these enzymes are commercially available and used for industrial applications. Oxidoreductates, hydrolases, and isomerases are relatively stable and therefore among them some of enzymes are used in industry such as chemical, food and pharmaceutical industries. On the other hand, living cells contain ligases and lyases less amount than other enzymes. Additionally, they are less stable for separation from living organisms. Therefore, these classes of enzymes are not used in enzymatic polymerization [1].

Table 2.3: Classifications of enzymes, their examples and synthesized polymers

Enzymes	Example Enzymes	Synthesized Polymers
Oxidoreductases	Peroxidase, Laccase, Tyrosinase, Glucose Oxidase	Polyphenols, Polyanilines, Vinyl Polymers
Transferases	Glycosyltransferase, Acyltransferase	Polysaccharides, Polyesters, Cyclic Oligosaccharides
Hydrolases	Cellulase, lipase, Chitinase, Peptidase, Protease	Polysaccharides, Polyesters, Polycarbonates, Polyamides
Lyases	Decarboxylase, Aldolase, Dehydratase	
Isomerases	Recemase, Epimerase, Isomerase	
Ligases	Ligase, Acyl CoA Synthethatase	

Among the polymers synthesized by enzymatic polymerization, polyesters are one of the most important materials. They are in the fourth place in living systems following nucleic acids, proteins and polysaccharides. Some of significant polyesters are poly(ethylene terephthalate) (PET), poly(butylene succinate), PCL and poly(lactic acid) (PLA). In order to synthesize polyester, a kind of hydrolase called lipase enzyme (EC3.1.1.3) is used for polymerization. In living systems, lipases catalyze the hydrolysis of fatty acid esters in an aqueous environment. However, they show stable structure in organic solvents and hydrolytic effect of lipase in water can be changed into ester synthesis in non-aqueous media. By utilizing this special behavior, lipases are used to synthesize functional aliphatic polyesters by different polymerization routes [1, 3, 14]. Major polymerization types to synthesize polyesters by lipase are divided into two groups: polycondensation and ring opening polymerization. Polycondensation is consisted from two different polymerization modes: polycondensation of oxyacids or their esters and polycondensation of carboxylic acids or their esters with alcohols. In 1985, the first study was appeared which showed polycondensation of oxyacid monomer, 10-hydroxydecanoic acid in benzene with lipase enzyme. In this study, the degree of polymerization (DP) value was more than five. Another study for this type of polymerization was obtaining polyesters from 10-hydroxydecanoic acid and 11-hydroxyundecanoic acid by candida rugosa lipase. O'Hagan et.al. synthesized polyester with molecular weight of 22000 in the presence of activated molecular sieves from 11-hydroxyundecanoic in this study. The latter polycondensation type was seen firstly in 1984. Okumura et.al. reported dehydration polycondensation of several free dicarboxylic acids in the presence of an excess amount of a diol by *Aspergillus niger lipase*. For instance, a polyester with a degree of polymerization (DP) 20 was synthesized by polycondensation of adipic acid and 1,4-butanediol in diisopropyl ether [1].Typical polycondensation reactions to obtain aliphatic polyesters are seen in Figure 2.1 [13].
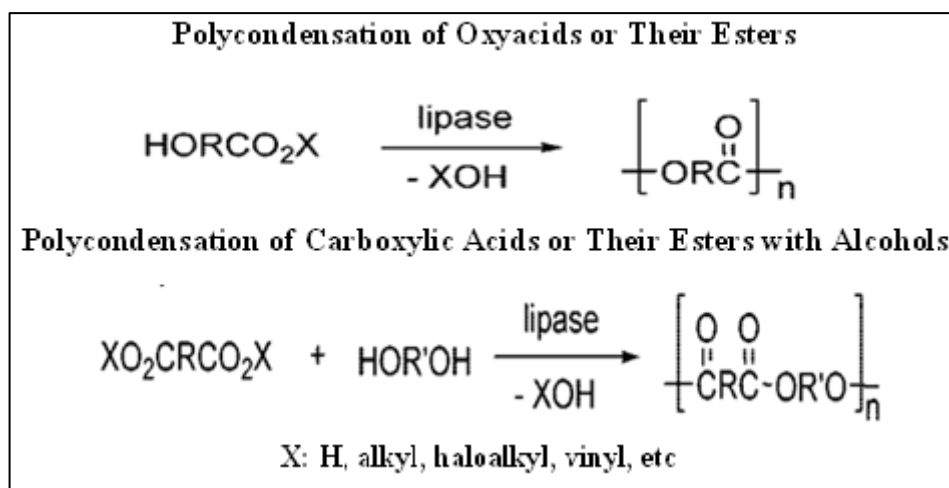
Figure 2.1: Typical Polycondensation Reactions Catalyzed by Lipase

Second type of polymerization for polyester synthesis is ring opening polymerization of cyclic esters. Among polymerization reactions ROP has been most extensively studied for polyester synthesis. Cyclic esters (lactones) used as a monomer for ROP reactions is shown in Figure 2.2. As it is seen from Figure, ring size changed as m values varies between 2-15 [4].
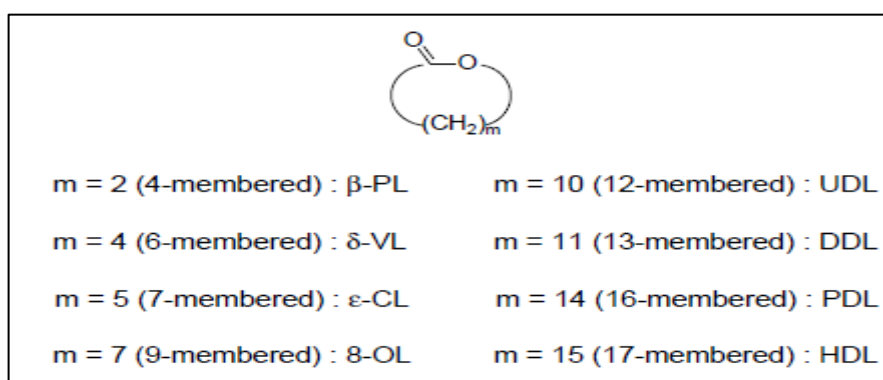
Figure 2.2: Monomers for Enzymatic ROP

There are many studies for every type of lactone in the literature. For example, ε-caprolactone (ε-CL, 7-membered lactone) and δ-valerolactone (δ-VL, 6-membered lactone) were polymerized by Uyama and Kobayashi in 1993. Polymerization was performed by lipase PF (*Pseudomonas fluorescens*), lipase CC (*Candida cylindracea*) and lipase PPL (*Porcine pancreatic lipase*) in bulk at 75 ^{o}C for 10 days. The highest monomer conversion was obtained by lipase PF (92%). Molecular weight Mn, and polydispersity of synthesized poly (ε-CL) in this study was 7700 and 2.4 respectively [15]. Another study by Knai et al. was ROP of ε-CL by crude PPL with methanol as nucleophile in n-hexane solution [16]. Solution polymerization was

also investigated by different independent groups. One of them is Gross et al. that they applied polymerization by using different solvents such as dioxane, toluene and heptane. As a monomer, enzyme and initiator, it was used ε-CL, PPL and butanol respectively. After polymerization, poly (ε-CL) with molecular weight (Mn) of 2700 was obtained [4]. Matsumura et al. investigated polymerization of different types of monomers such as cyclic diester-D, L-lactide and β-propiolactone. Polymerization reaction was carried out at 80-130 ^{o}C to obtain poly (lactic acid) with molecular masses up to 12600 [17]. Nobes et al. was first discovered lipase catalyzed polymerization of β-butyrolactone (β-BL, 4-membered lactone). Polymerization was performed by the use of equal weights of lipase and monomer for several weeks. After the reaction, only low molecular weight of polymers (Mw, 256-1045) was synthesized [18]. On the other hand, Matsumura et al. synthesized polymers with higher molecular weights (up to 7300) with PPL and lipase CC. Polymerization time was ranging from 12 to 20 hours and temperature was varies between 60 and 100 ^{o}C. Because of the intramolecular cyclisation, cyclic oligomers were formed during this polymerization process [19, 20].There are also studies comparing chemical and enzymatic polymerization modes. Albertsson and co-workers have studied extensively for polymerization of 1,5-dioxepan-2-one (DXO) [4]. The bulk polymerization of DXO by the use of organometallic initiators was performed at 110 ^{o}C and monomer is fully consumed after 20 hours whereas using novozym 435 (immobilized form of lipase, 10 wt. %) provided 97% conversion only after 4 hours at 60 ^{o}C. It is obvious from this result; enzymatic polymerization could be performed at mild reaction conditions [21]. On the other hand, long reaction time, high cost of enzymes and synthesized of low molecular weight polymers are some problems of enzymatic polymerization for large scale production in industry. In order to solve these negative sides, protein engineering has been studying to improve enzyme activity, stability and efficiency by different methods such as immobilization and covalent modification of enzymes [4].

2.3 Chemical Polymerization

Polyesters could be synthesized by using metal-based initiators or catalysts such as aluminum alkoxides, tin octoate and Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+} , Fe^{2+}which are referred as 'friendly metals'. Aliminum alkoxides are chosen because of their high

selectivity since well-defined polyesters with well-controlled molecular weight could be obtained in ring opening polymerization of lactones. Among these chemical initiators or catalysts, stannous octoate ($Sn(Oct)_2$) is the popular one since US Food and Drug Agency (FDA) accepted this compound as a food additive. Therefore, polymers obtained by this type of catalysts could be used for packaging without any further purification. However, even if they could be used in food applications, this toleration could not be employed for biomedical applications. Another way of chemical catalysis is using bioresorbable salts referred as 'friendly metals 'for the ROP of polyesters [22]. Zinc alkoxides were prepared and used for ROP of ε-CL. Also, zinc dichloride initiated ROP of ε-CL according to a coordination-insertion mechanism [23, 24]. Comparing with enzymatic polymerization, although some chemical initiators or catalysts polymerized lactones efficiently and can be used in food applications, there are some problems observed for biomedical applications [22].

2.4 Advantages of Enzyme Catalyzed Polymerization

Enzymatic polymerization offers an eco-friendly biosynthetic pathway for polymer production. Chemical catalysts or initiators such as Zn, Al, and S cannot be removed entirely from the polymer when they are used in the synthesis. Remnants of these metal compounds may be toxic and cause problem in biomedical applications. Therefore, enzymatic polymerization provides advantages when polymer is used in biomedical field. Other advantages of enzyme catalyzed polymerization are as follows [3, 4]:

- Enzymes are biocatalysts; therefore enzymatic reactions are performed under mild conditions such as lower temperature and pressure.
- It is possible to obtain well-defined polymers by use of enzymes.
- Separation of enzymes from synthesized polymer is easy and they can be used more than one application. Therefore, enzymes are recyclable eco-friendly materials.
- Enzymes are derived from renewable resources.
- ROP of lactones by enzymes can be performed easily since enzymes do not require strict precautions such as exclusion of water and air. On the other

hand, metal catalysts are very sensitive and water has to be removed from the polymerization media.

- Enzymes have high entio- and regio-selectivity and can be used in bulk or organic media.

- Enzymes have the ability of polymerization of large ring lactones (higher 7 member-macrolides) in normal reaction conditions. However, low molecular weights can be obtained with organometallic catalysts.

2.5 Synthesis of Poly(ε-caprolactone) by Enzymatic Ring Opening Polymerization

PCL is synthesized by ring opening polymerization of ε-caprolactone (ε-CL, 7-membered) which is a cyclic ester as shown in Figure 2.2. The most extensively studied type of lactone for lipase catalyzed ROP is ε-CL and this monomer could be rapidly polymerized by different lipases originated from different organism: microbial, plant and animal kingdom [22, 25].These lipases and organisms from which they were isolated are seen in Table 2.4 [4]. Among them, *lipase CA* was the most effective one for ROP of ε-CL [26, 27].

Table 2.4: Lipases used in polyester synthesis

Organism	Lipases
Mammalian	*Porchine pancreatic lipase (PPL)*
Fungal	*CALB, Candida Rugosa (CR), Aspergillus Niger (AN) Penicillium roruefortic (PR), Rhizopus delemar (RD), Rhizomucor miehei (RM), candida cylindracea (CC)*
Bacterial	*Pseudomonas cepacia (PC), Pseudomonas flourescens (PF), Pseudomonas species (PS)*

The proposed reaction mechanism for poly (ε-caprolactone) synthesis by lipase is shown in Figure 2.3. Firstly, ring opening of the ε-CL is performed to form acyl-

enzyme intermediate (enzyme-activated monomer, EM). In the following step, initiation is carried out in which first chain of polymer is synthesized by a compound containing hydroxyl group. Generally, this nucleophilic attack is performed by water which is probably contained in the enzyme. Other nucleophiles such as alcohol or amine can also be used in this polymerization [2, 28]. Gross et.al.used butanol and butylamine as an initiator for ROP of ε-CL by PPL catalyst. In this study, they showed rate of initiation by butanol and water was slower than by butylamine. There were also differences in molecular weight of resulting polymers obtained by using these nucleophiles. When water was used alone, resulting Mn of PCL was 7600 g/mol whereas butanol and butylamine usage resulted with 1900 and 1200 g/mol respectively [29]. After initiation step, ω-hydroxycarboxylic acid is formed and propagation is proceeded by formation of additional polymer chain. It was found that the rate determining step of the overall polymerization reaction is the formation of enzyme-activated monomer from kinetic studies [2].
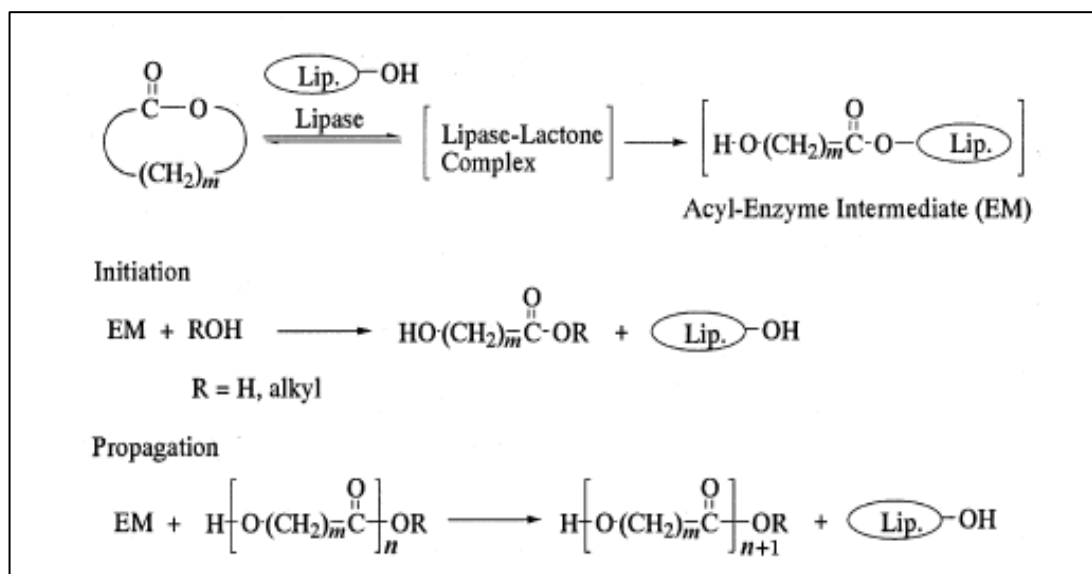
Figure 2.3: Reaction mechanism of ring opening polymerization of ε-caprolactone

Matsumura et. al. synthesized PCL with immobilized form of lipase CA (novozym 435). In this study, both bulk polymerization and solvent polymerization was applied by using toluene as a solvent. Polymerization was performed at 70 $^{\circ}$C for 24 hours. Enzyme/monomer ratio was 1% wt and by using toluene, resulting polymer has reached molecular weight (Mn) of 25000 with polydispersity of 1.6 whereas by bulk polymerization under the same conditions, molecular weight of PCL was 16000 with polydispersity of 1.6. In addition to that, monomer conversion was 99% and 41%

respectively by solvent and bulk polymerization of ε-CL [30]. Kumar and Gross showed *lipase CA* was stabilized by the help of toluene and reported polymerization could be performed effectively at 90 ^{o}C. In this study, reaction parameters such as temperature, solvent effect and monomer concentration were investigated. As a solvent, acetonitrile, dioxane, tetrahydrofuran, chloroform, butyl ether, isopropyl ether, isooctane and toluene were used. Among them, isooctane and toluene gave the highest percent monomer conversion as it is shown in Figure 2.4. Comparing with isooctane, toluene was more advantageous since both monomer and polymer dissolve in toluene and enzyme could be easily separated from the solution. However, ε-CL could not dissolve in isooctane. Therefore, 2 immiscible phase and enzyme were obtained when isooctane was used in polymerization.
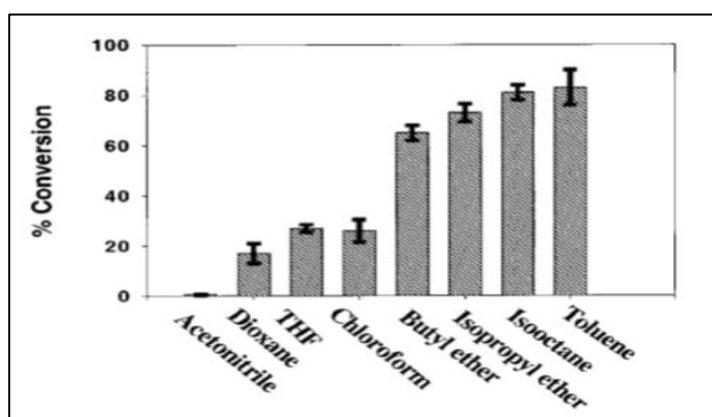
Figure 2.4: Different solvents used in PCL synthesis

Gross et.al also investigated ratio of toluene amount with respect to monomer on polymerization reaction. They changed toluene/ε-CL ratio (vol/vol) (0:1, 1:1 ,1.5:1, 2:1, 2.5:1, 3:1,4:1, 5:1, 10:1) and examined molecular weight of resulting polymers. Optimum results were seen when the ratio was 2:1 since molecular weight of PCL and monomer conversion were 17200 g/mol and 85% respectively. When bulk polymerization was applied, molecular weight and conversion was low comparing with solvent polymerization. Monomer concentration was another important reaction parameter for ROP of ε-CL. When Gross et.al. scaled up the polymerization process and used 10 g monomer and 20 ml toluene with 1 g novozym 435, within only 4 hours at 70 ^{o}C molecular weight was increased to 44800 g/mol. Additionally, polydispersity of resulted polymer was 1.7. They also investigated temperature effects on reaction rate. It was shown that when temperature was 100 and 105 ^{o}C, monomer conversion was low comparing with 90, 80, 70 and 60 ^{o}C. Monomer

conversion was lower than 5% after 3 hours at 105 ^{o}C since protein denaturation and deactivation were happened at these temperatures [31].

Water content of polymerization media is another important reaction parameter. Gross et.al examined effect of water content varies between 0.6-2.8% on molecular weight of polymer. They found that molecular weight was depended on water content not temperature [32]. Although molecular weight was decreased because of increasing water content, monomer conversion was favored by water. Since concentration of propagating chains has increased with water, monomer conversion was increased but molecular weight was decreased. Therefore, water content has to be chosen in optimum amounts [4]. For example, immobilized lipase catalyzed polymerization of 1,4-dioxan-2-one was performed with different water amount and it was shown that an increase in water content (up to 100 ppm) increased the rate of polymerization whereas excess water depressed the rate (app.224 ppm) [33].

Lipase concentration also effects PCL synthesis since different catalyst amount resulted with different monomer conversion and molecular weight of the polymer. Gross and Deng investigated ROP bulk polymerization of ε-CL using different amounts of catalyst: 9.77, 1.80 and 0.50 mg catalyst/ mmol ε-CL. The higher amount of lipase catalyzed polymerization rapidly within only 4 hours, conversion has reached 78% whereas the same conversion was gained within 48 hours using 1.80 mg enzyme. When 0.50 mg lipase was used, after 50 hours only 18% monomer conversion was seen. They concluded the study with comparing molecular weight of resulting polymers in which lower catalyst amount gave higher molecular weight [34].

Kind of lipase was a significant parameter on ROP of ε-CL. The rate of polymerization with different lipases (lipase CA, PC and PF) was investigated by using another lactone (8-octanolide). Comparing with lipase PF, lipase CA and PC was more effective on polymerization rate [35].

Studies on PCL synthesis has been focused on recycling of enzymes, improving enzyme activity and using green solvents such as supercritical carbon dioxide [36, 37]. Matsumura et.al and Kobayashi et.al. have studied on sustainable polymer recycling by degradation of polymer into oligomers and synthesized polymer from these resulted oligomers with same enzyme as it is shown in Figure 2.5 [30, 38].
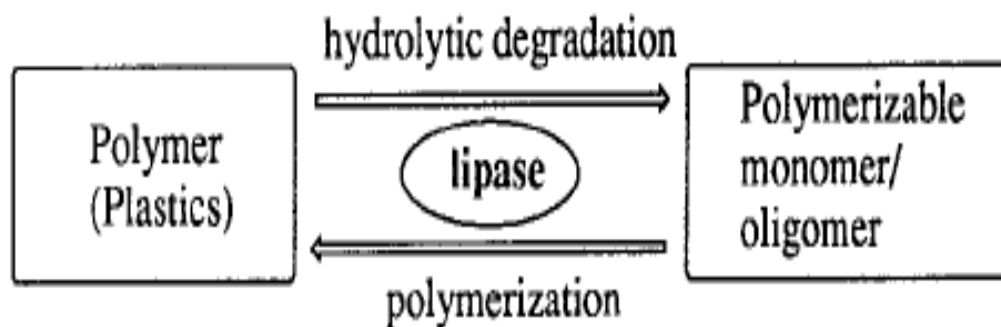
Figure 2.5: Sustainable polymer recycling with enzymes

2.5.1 Lipases for polymerization reactions

Lipase (EC3.1.1.3) is a kind of hydrolyse that catalyze the hydrolysis of fats in living cells. They have a widely usage area as important drugs for digestive diseases and pancreas disorders [25]. Also, they are used in organic synthesis as a catalyst to produce organic compounds since lipases can catalyze diverse amount of substrates comparing to other enzymes. In addition to that, they show stable structure in organic solvents and hydrolytic effect of lipase in water can be changed into ester synthesis in non-aqueous media [3]. By utilizing this special behavior, lipases are used to catalyze ring opening polymerization of lactones. All lipase types have similar structure and functionalities. However, when the origin of enzyme is changed, small variations in substrate binding site is seen. These small variations may have a significant effect on catalytic activities and stability of the catalyst [5].

Lipases used for polyester synthesis is shown in Table 2.4. Among them, *CALB* is the most effective one. Two different lipases A and B were produced by *candida antarctica* which was originally isolated in Antarctica as it is indicated by the name of the yeast. These two types of lipases show very different properties. *CALA* is highly thermostable whereas CALB is less thermostable. Additionally, these two types of lipases have distinct substrate specificity. Although CALB is less active toward large triglycerides, it is very active towards most of other esters, amides, thioesters, etc. On the other hand, CALA shows low activity towards simple esters. Molecular weight of CALB is 33 kD with 317 amino acid residues and it has an isoelectric point (pI) of 6.0 [5, 46]. Uppenberg et al. has solved amino acid sequence and 3 dimensional structure of CALB. Size of enzyme is 30x40x50 ^{o}A and Ser-His-Asp triad is responsible for the catalysis as seen in Figure 2.6 [39].
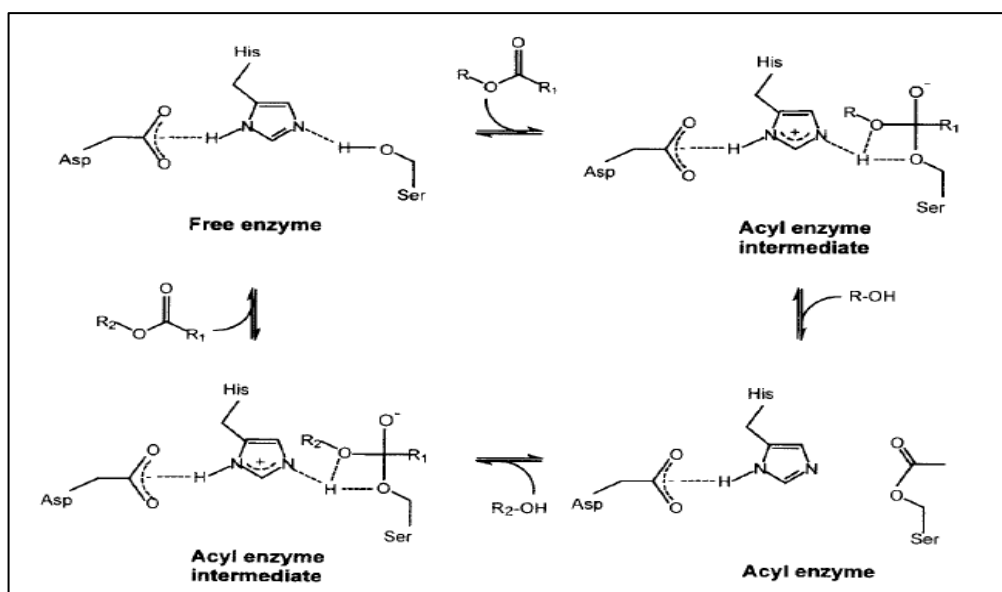
Figure 2.6: Ser-His-Asp triad for the catalytic mechanism of CALB

Optimum CALB catalysis is seen in pH 7.0 and this enzyme is stable in the range of pH 3.5-9.5 in aqueous media. Depending on pH of the media, denaturation temperature is in the range of 50-60 $^{\circ}$C. When the pH value decreased, higher denaturation temperature is observed. In order to reach more thermostable enzymes, immobilization was applied to CALB and it can be used continuously at 60-80 $^{\circ}$C without any significant activity loss. Also, substrate specificity of this enzyme is because of deep and narrow positioned active site [5, 39].

Industrially, CALB is produced by submerged fermentation of a genetically modified *Aspergillus Niger* microorganism and commercially available CALB used for organic synthesis is novozym 435 which is immobilized on macro porous acrylic resin. Immobilization of CALB on acrylic resin was performed by physical to reach novozym 435. Therefore, enzyme leaching is a known problem pointed out by various researchers. Although performance of novozym 435 is satisfactory, there were studies to strengthen linkage between enzyme and support and applied different immobilization procedures. For example, coating the immobilized enzyme enhanced the stability of novozym 435 [5-7].

2.6 Applications of Poly (ε-caprolactone)

PCL has wide application areas especially in biomedical field and food industry as a packaging material. It has numerous advantages such as easy manufacturing,

biodegradable and biocompatible structure. Since its degradation products are non-toxic and it has FDA approval, researches of PCL applications are focused on drug-delivery systems, medical devices and tissue engineering.

In addition to long-term biodegradation behavior, PCL has also high permeability to many drugs. Therefore, its usage in drug-delivery systems is increasing within last years. Blending capacity of PCL with other polymers is another important property since degradation kinetics can be controlled through this ability [11]. PCL have also been of interest to deliver peptide or protein drugs that have unusual physicochemical properties compared to other drugs. PCL can be prepared as microspheres or nanospheres in order to improve therapeutic efficiency for drug delivery systems [40].

Application of PCL for medical devices can be grouped as sutures, wound dressings, contraceptive devices, fixation devices and dentistry. For example, a block copolymer of PCL with glycolide provides reduced stiffness compared with other sutures obtained with different polymers [11]. On the other hand, tissue engineering applications of PCL is another important area of biomedical industry. For instance, bone repair is one of the usage areas of PCL for tissue engineering applications. For example, PCL networks were obtained by the reaction of PCL diol with acryloyl chloride as a scaffold for tissue engineering [41].

Moreover, PCL is also used in synthetic leather, fabrics, fibers and bags. For example, bags originated from PCL has been produced in Sweden, however they degraded before reaching the customers [42].

2.7 Immobilization of Enzymes

Immobilization of enzymes provides significant benefits such as increasing stability and improving catalytic activity of the catalyst. Recycling of enzymes is an important topic because of cost reasons and by immobilization process, activity of enzymes enhanced and they can be used several times in reactions [43].

Different immobilization methods can be applied to enzymes which are adsorption, covalent binding, entrapment, encapsulation and cross-linking methods as shown in Figure 2.7 [44].
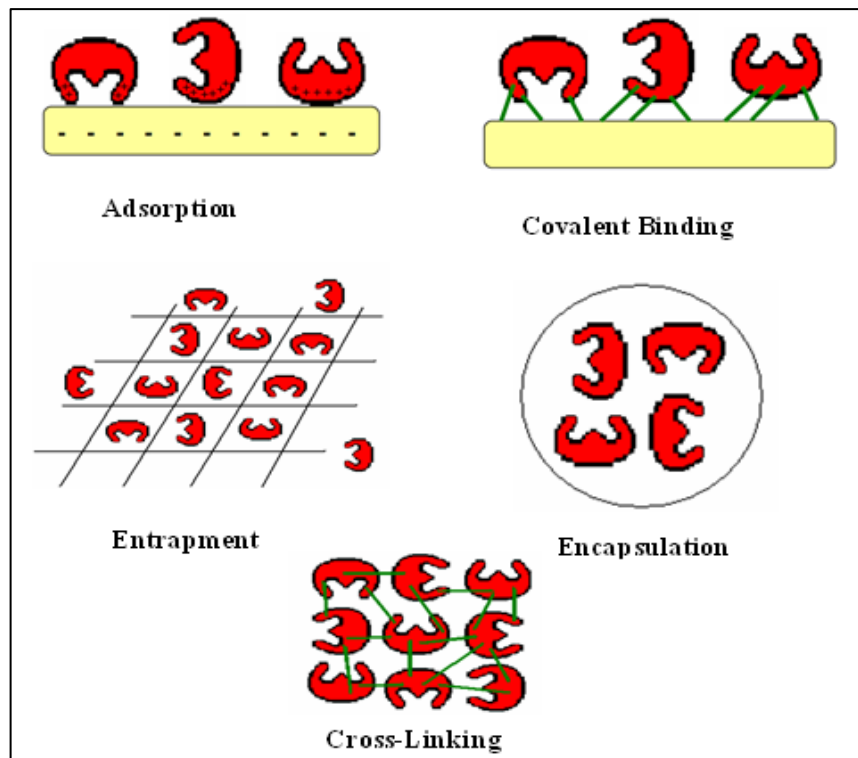
Figure 2.7: Immobilization methods of enzymes

The simplest method is immobilization by adsorption. Enzyme and support material may show reversible surface interactions in this type of immobilization method since forces of bonds are relatively weak. The major forces observed in adsorption are van der Waals, ionic and hydrogen bonding interactions. Advantages of adsorption are in the following:

- It is a cheap and simple process, thus it can be applied easily.
- Enzymes are not damaged during immobilization procedure.
- It is only physical process; there are no chemical changes in support or enzyme.
- Since it is a reversible process, enzymes can be regenerated.

Although it has significant advantageous, there are some problems including nonspecific binding, overloading on the support, steric hindrance by the support and leakage of enzymes from the support. The last problem may cause contamination of products by unwanted compounds. Also overloading the support may increase activity of enzyme and not having enough space between support and enzyme may lead to steric hindrance.

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Second immobilization method is covalent binding which involves covalent bond between enzyme and support. Functional groups on the surface of the support and functional groups on the surface of the enzymes (from amino acid residues) are cleaved to form covalent bonding. There are diverse amount of support materials for covalent bonding. On order to use appropriate support, both advantages and disadvantages must be taken into account. The most significant factor for a support is hydrophilicity since it maintains enzyme activity. Therefore, one of the important support materials is polysaccharides such as starch, cellulose, dextran. Porous silica and porous glass are other popular supports for enzyme immobilization by covalent bonding.

Entrapment is another immobilization method for enzymes which differs from adsorption and covalent binding. As it is seen from Figure 2.7, enzyme molecules are not bounded to the support. They are free in solution and surrounded by lattice structure of a gel. Leakage of enzymes is not observed in this type of immobilization. Also in order to protect enzymes from harmful substances like unwanted cells, proteins or enzymes, support act as a barrier to mass transfer.

The last two methods are encapsulation and cross-linking. The former is similar to entrapment of enzymes in which enzymes are free and restricted by the support. As a support material, semipermeable membranes are used. The most important benefit of encapsulation is coimmobilization that can construct desired immobilization for particular applications. The last immobilization method is cross-linking divided into physical and chemical routes. Chemical route of cross-linking method is consisted of covalent bond formation between enzyme and multifunctional reagent such as glutaraldehyde and toluene diisocyanate. Physical route of cross-linking method is cross-linking of enzymes by flocculation. Polyamines and polystyrene sulfonates are important flocculation agents used in physical cross-linking. Generally, cross-linking method is used to strengthen other immobilization methods in order to prevent enzyme leakage [45].

2.7.1 Immobilization of lipases for polymerization reactions

Immobilization of CALB favored ring opening polymerization reaction since catalytic activity and stability of enzyme was improved with this process. As it was indicated previous sections, novozym 435 is one of immobilized form of CALB that

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was physically adsorbed on macroporous acrylic resin. This enzyme was very effective for ROP of lactones since only small amount could be catalyzed the reaction. In different kinds of support materials were investigated by independent study groups [5, 7, 46, 47]. Uyama et.al.investigated the performance of supports such as ceramic, polypropylene, polystyrene and acrylic resin for CALB immobilization. Immobilization procedure for these supports was carried out as follows: firstly, support was washed with ethanol, ethanol-water mixture (equivolume) and water respectively. Washed support, lipase CA solution and phosphate buffer (pH 7.0) mixed and stirred at 4 ^{o}C for 4 hours. After immobilization process, the support was separated from aliquot and lyophilized. In the second part of the study, immobilized CALB were evaluated by ROP of ε-CL. Polypropylene immobilized CALB was the most effective one that catalyzed reaction with 88% monomer conversion. The other immobilized lipases by polystyrene and ceramic gave 51% and 57% respectively. Also, acrylic resin immobilized CALB were not able to catalyze the reaction. Molecular weights (Mn) of the resulting polymers were 7200, 4100 and 3100 g/mol by using polypropylene, polystyrene and ceramic as supports [7].

In another study, CALB was immobilized on silica particles by cross-linking method via the carriers PEI and GA. This method has prevented enzyme leakage whereas physically immobilized enzyme and carrier had weak bonds and enzyme was leached from the carrier in leakage test. After preparation immobilized enzymes, catalytic activity of the enzyme was evaluated by ring opening of polymerization. Activity of prepared enzyme was lower than novozym 435. Therefore, to reach same activity higher amount of enzyme was used in polymerization. Comparing with novozym 435, synthesized polymer by prepared enzyme has reached lower molecular weight (Mn) [5].

CALB was also covalently immobilized on epozy-activated macroporous poly (methyl methacrylate amberzyme beads and nanoparticles. In order to evaluate activity of enzymes, they used in ROP of lactones and step condensation polymerizations.In this study, nanoparticle immobilized enzyme gave the highest ε-CL conversion (65%) for 20 min whereas monomer conversion was 16% by novozym 435 catalysts [6].

2.7.2 Supports for lipase immobilization

Supports for enzyme immobilization has to be chosen according to the needs since there is no universal support suitable for all type of enzymes. However, there are some common properties that a support must have [10]:

- It must have high protein affinity and loading capacity.
- There must be reactive functional groups in support structure in order to react directly with enzymes.
- It must have mechanical stability, rigity and feasibility of regeneration.

Also, depending on the application such as food, biomedical and agricultural, nontoxicity and biodegradability can be required. There were many supports such as acrylic resins, polypropylene, amberlite, fumed silica, polystyrene, ceramic, rice husk and rice straw, chitin and chitosan used for lipase immobilization [6, 7, 9, 10, 48, 49]. Among them, chitin and chitosan is an ongoing study of enzyme immobilization since they are cheap, ubiquitous and nontoxic material [9, 10]. Also, their some other excellent properties such as biocompatibility, biodegradability to harmless products, physiological inertness, hydrophlicity, gel forming properties and high affinity for proteins make these supports significant topic for biological systems [50].

Chitin and chitosan are natural polyaminosaccaharides and chitosan is a derivative of chitin. Chitin is the most abundant renewable organic resources and exoskeletons of insects, cell walls of fungi and shells of crustaceans are consisted of chitin to supply stability and strength to the organism. Therefore, currently crab and shrimp shells are used in chitin and chitosan production. Now, India, Japan, Poland, Norway and Australia are produced these biopolymers. Structure of chitin and chitosan is shown in Figure 2.8. Chitin is formed from 2-acetamido-2-deoxy-β-D-glucose through β (1-4) linkage. Chitosan is formed by N-deacetylation of chitin that this process s is almost never complete. Dilute acids such as acetic acid and formic acid can dissolve chitosan whereas chitin is insoluble in most organic solvents. Also it is highly hydrophobic and insoluble in water [50, 51].
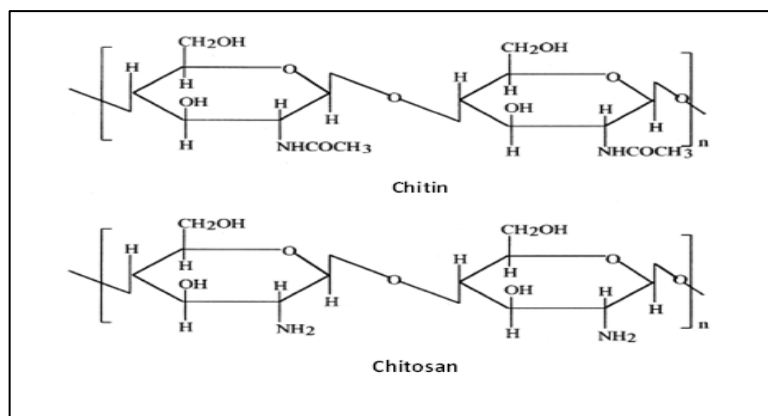
Figure 2.8: Structure of chitin and chitosan

Due to properties mentioned in above, chitin and chitosan is a valuable support for enzymes. There are studies about immobilization of enzymes on chitin and chitosan by different methods [9, 10, 48]. Foresti et.al. investigated chitosan immobilized lipases for the catalysis of fatty acid esterifications. Two different immobilization procedures were applied to *CALB* and 2 other kinds of lipases (*Candida Rugosa lipase, P.fluorescens)*: physical adsorption, gluteraldehyde pretreated lipases. In the latter process, gluteraldehyde was used as a coupling agent. Among lipases, immobilized *CALB* on chitosan was the most active one for esterification reactions in this study [10]. Also, binary immobilization of *candida rugosa* on chitosan was examined. In this study different immobilization methods were applied. The best method was as follows: firstly, immobilization of lipase to the hydroxyl groups of chitosan was performed by activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Then, by using gluteraldehyde, more lipases were immobilized through its amino groups. Figure 2.9 shows this immobilization method [48].
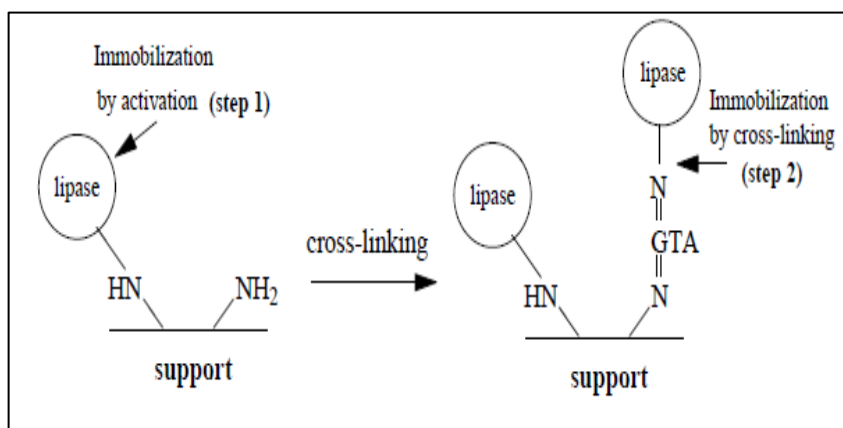
Figure 2.9: Binary immobilization of lipase

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3. EXPERIMENTAL PART

3.1 Materials and Chemicals

Novozym 435, immobilized form of *CALB* and free form of lipase enzyme (*Novozymes CALB L*) was purchased from Sigma Aldrich Company. Enzymes were used as received. Monomer of the polymerization reaction ε-caprolactone (99%) was provided from Alfa Aesar Company and stored under dry nitrogen over molecular sieves ($3A^o$) to decrease the water content. Molecular sieves $3 A^o$ is obtained from Sigma Aldrich and it was chosen according to literature [52]. Chemical properties of ε-caprolactone are shown in Table 3.1.

Table 3.1: Chemical properties of ε-caprolactone

Formula	$C_6H_{10}O_2$
Molecular weight (g/mol)	114.14
Melting Point (^{o}C)	-2
Boiling Point (^{o}C)	235-236
Flash Point (^{o}C)	109
Density	1.078

Chloroform and the supports chitin and chitosan were provided from Sigma Aldrich Company. Chitosan and chitin were from crab shells. Toluene and methanol was supplied from Merck Company with a high purity and used as received. Gluteraldehyde solution, a coupling agent for immobilization process was obtained from Sigma Aldrich Company. Tetrahydrofuran (THF) used in GPC analysis (HPLC grade) obtained from Sigma Aldrich Company.

In order to prepare phosphate buffer for immobilization process, monobasic sodium phosphate ($NaH_2PO_4.H_2O$) was obtained from Carlo Erba Company and dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) was purchased from Merck Company.

3.2 Equipment

- Digital Round-Top Stirring Hot Plate, IKA$^®$ RCT Basic IKAMAG
- pH meter, Inolab, TWT
- Shaking Water Bath, YUMATO
- Precision Scale, And, *Gr-200*
- Fourier transform infrared spectroscopy (FT-IR),Perkin Elmer FT-IR Spectrum One B Spectrometer
- Gel Permeation Chromatography (GPC), Agilent 1100 Serisi
- Scanning Electron Microscopy (SEM), Jeol, JSM-6390LV
- UV mini 1240 SHIMADZU spectrophotometer
- Differential Scanning Calorimetry, Perkin Elmer, Diamond DSC
- Proton Nuclear Magnetic Resonance (^{1}H-NMR), Bruker NMR Spectrometer
- Drying Oven, Binder
- Rotary Evaporator, Heidolph

3.3 Methods

3.3.1 Lipase immobilization

Immobilization of lipase was carried out with 2 organic supports (chitin, chitosan) by applying 2 different procedures: physical adsorption and cross-linking with a coupling agent. As a coupling agent, gluteraldehyde was chosen since it has been used widely in literature for immobilization of enzymes on chitosan [10, 53]. During the experiments, firstly gluteraldehyde pretreated supports (chitin, chitosan) were prepared by adding corresponding support (400 mg) into 50 ml 0.02%(v/v), 0.2%(v/v), 2% (v/v) gluteraldehyde/phosphate buffer solution (ph 7, 0.015M). Then this suspension was stirred (160 rpm) for 1 hour at 25 ^{o}C in shaking water bath. The gluteraldehyde pretreated supports were filtered and washed with distilled water for 3 times. Secondly, 1 mL *CALB* enzyme solution (*Novozyms CALB L*) was diluted by 50 mL phosphate buffer (ph 7, 0.015 M) and gluteraldehyde-pretreated supports were suspended in prepared diluted enzyme solutions. In order to perform physical adsorption method, supports without any pretreatment were also suspended into diluted enzyme solutions and enzyme-support suspensions were stirred (160 rpm) for

5 hours at 25 $^{\circ}$C. After this step, immobilized enzymes were filtered and dried in an oven at 30 $^{\circ}$C for 12 hours. From this procedure, 8 immobilized lipases were obtained which were K_1, K_2, K_3, K_4, $Immob_1$, $Immob_2$, $Immob_3$ and $Immob_4$. Immobilizations of enzymes were characterized by SEM. Apparatus that immobilization process was performed is seen in Figure 3.1.

Figure 3.1: Apparatus for immobilization process

Phosphate buffer was prepared by adding 29.25 mL $NaH_2PO_4.H_2O$ and 45.75 mL $Na_2HPO_4.7H_2O$ into 1 L deionized water and pH was adjusted to neutral condition. The reason why pH 7 was chosen for immobilization medium was based on gluteraldehyde structure. There are 2 amino groups (ionic groups) under GLT and it leads supports to act as an anionic exchanger. Because of this, changing the ionic strength during immobilization can affect the immobilization rate [10].

3.3.1.1 Lipase protein determination

Among several protein determination techniques, UV spectroscopy was chosen since it is a simple and quick method. Lipase enzyme solution was used as a standard in order to construct standard curve seen in Figure A.1. All samples were prepared in the same buffer solution which was used in immobilization procedure since UV detection is sensitive to pH and ionic strength. Absorbance measurements were recorded at 280 nm in which ultraviolet absorption usually depends on tyrosine and tryptophan amino acids at this wavelength. Immobilization efficiency of chitin and chitosan immobilized lipases were evaluated as follows [48].

$$\text{Immobilization efficiency (\%)} = \frac{amount\ of\ protein\ coupled}{amount\ of\ protein\ loaded} x100 \qquad \textbf{(3.1)}$$

Amount of protein coupled (mg/mL) = *(protein loaded-protein in supernatant)* **(3.2)**

3.3.2 Characterization methods for lipase immobilization

3.3.2.1 Scanning electron microscopy (SEM)

Immobilization of enzymes on chitin and chitosan supports were observed by JEOL JSM-6390LV SEM. Analysis was performed at 10 kV with appropriate magnification rates.

3.3.4.2 Ultraviolet spectrophotometer (UV)

Efficiency of lipase immobilization was evaluated by UV mini 1240 SHIMADZU spectrophotometer in this study. Absorbance was measured at 280 nm and as a blank sample, phosphate buffer was used.

3.3.3. Polymerization reactions

Polymerization reactions were performed with prepared enzymes by immobilization and commercially available *CALB* 'novozym 435'. Firstly, reaction was carried out in 2 mL toluene (toluene to ε-caprolactone ratio, 2:1(v/v)) under dry nitrogen at 70 ^{o}C with 4 different chitin immobilized *CALB*. Reaction medium was stirred at 200 rpm with a magnetic stirrer and enzyme concentration was 10% (w/w) (enzyme/monomer ratio). At a specified time, chloroform was added to reaction mixture and enzyme was separated by filtration. There is a conflict between different independent groups about the aim of adding chloroform or THF after reaction. Some researchers believe that it denatures enzyme and terminates the reaction. However, it is known that polymerization reaction can occur in these solvents [5, 31]. Therefore, in this study chloroform was added to the reaction mixture in order to dilute viscous solution. After this step, chloroform in the filtrate was largely evaporated by rotary evaporator at 40 ^{o}C under vacuum and resulted solution was precipitated in methanol. Polymer was filtered and dried at 35 ^{o}C in an oven.

Secondly, the results were evaluated with respect to molecular weight (Mn) of the PCL and percent monomer conversion of reaction. The best result (K_2) was chosen and for chitin immobilized lipases, further reactions were performed by this enzyme. This procedure was also applied to chitosan immobilized lipases and again best result was chosen (immob$_2$). Lastly, in order to optimize the polymerization process, reactions were carried out at 3 different temperatures (60 ^{o}C, 70 ^{o}C, 80 ^{o}C) with novozym 435, immob$_2$ and K_2. Also enzyme concentration was changed in order to

determine optimum amount of enzyme in this study (enzyme to ε-caprolactone ratio, 5%, 10%, 15%, 20%(w/w)). In Figure 3.2, apparatus that polymerization reaction was performed can be seen.

Figure 3.2: Apparatus for polymerization reactions

3.3.4 Characterization methods for poly (ε-caprolactone)

3.3.4.1 Fourier transform infrared spectroscopy (FTIR)

In order to define chemical structure and composition of PCL, Perkin Elmer FT-IR Spectrum One B Spectrometer was used in this study. To demonstrate that resulting polymer was PCL, characteristic functional groups and bonds were defined from FTIR spectra. Also spectrum of PCL was compared with ε-CL spectra and showed differences in bond length. From FTIR study of PCL, characteristic infrared bands were defined as shown in Table 3.1. In this Table, the band corresponding to 1727 cm^{-1} has been the major transmission peak of PCL which belongs to carbonyl stretching (COO) [12, 54].

Table 3.2: Major infrared bands of PCL

Wavenumber (cm^{-1})	Assignment
2949	Asymmetric CH$_2$ stretching
2865	Symmetric CH$_2$ stretching
1727	Carbonyl stretching (C=O)
1293	C-O and C-C stretching in the crystalline phase
1240	Asymmetric COC stretching
1190	OC-O stretching
1170	Symmetric COC stretching
1157	C-O and C-C stretching in the amorphous

27

3.3.4.2 Gel permeation chromatography (GPC)

Molecular weight and polydispersity of PCL was determined by GPC with an Agilent 1100 HPLC system consisting of a pump, refractive index and UV detectors and Zorbax PSM columns (1000-S, 300-S, 60-S).Calibration was performed with polystyrene standards ranging from 580 to 504.500 g/mol. As an eluent, THF was used with a flow rate of 0.5 mL/min. Analysis was carried out at 25 $^{\circ}$C. Sample concentration was 0.5% wt/vol and injections were 20 µL. All samples were filtered via 0.45 µm filter syringe to prevent columns of GPC from impurities.

3.3.4.3 Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR)

Molecular structure of PCL was determined by using Bruker NMR spectrometer at 300 MHz. ^{1}H spectra was obtained with respect to tetramethylsilane (TMS) as an internal standart. In the analysis, polymer was dissolved in chloroform-d (CDCl$_3$).

3.3.4.4 Differential scanning calorimetry (DSC)

Thermal analysis of PCL was performed by Perkin Elmer, Diamond DSC with 10 $^{\circ}$C/min heating rate under nitrogen. 3.6 mg sample was heated from -100 $^{\circ}$C to 150 $^{\circ}$C in order to observe glass transition temperature (T_g) and melting point (T_m). Heating range (-100-150) was chosen according to expected T_g and T_m values of PCL. The melting point was determined at the maximum of the melting endotherms and the glass transition temperature was calculated as the midpoint of heat capacity increase [55-57]. The crystallinity (X_c) of polymers were computed by determining heat of fusion (ΔH_f) of each PCL obtained with three different lipases (novozym 435, Immob$_2$, K$_2$). Percent crystallinity was determined with the following equation [58].

$$\% \: crystallinity \: (Xc) = (\Delta Ha - \Delta H)/(\Delta Ha - \Delta Hc) \times 100 \qquad \textbf{(3.3)}$$

In above equation, ΔH_a is the enthalpy change of pure amorphous standard where ΔH_c and ΔH is the enthalpy change of pure crystalline standard and unknown sample respectively. This equation is simplified by assuming ΔH_a=0 and it becomes as follows [58].

$$\% \: crystallinity \: (Xc) = (\Delta H/\Delta Hc) \times 100 \qquad \textbf{(3.4)}$$

As a reference enthalpy change of pure crystalline standard of PCL was used as $\Delta Hc = 142 \: J/g$ and crystallinity was determined by using above equation [12].

4. RESULTS AND DISCUSSION

4.1 Synthesis of PCL by Novozym 435

PCL was synthesized by commercially available CALB (novozym 435) at three different temperatures (60 ^{o}C, 70 ^{o}C, 80 ^{o}C) in toluene (toluene to ε-caprolactone ratio, 2:1(v/v)) with 10% (w/w) enzyme concentrations (enzyme to ε-CL ratio). Table 4.1 shows molecular weight (Mn) and polydispersity of resulted polymer at T=60 ^{o}C with respect to time.

Table 4.1: Effect of time on polymerization reaction catalyzed by Novozym 435 at T=60 ^{o}C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
2	52	10492	1,49
5	42	12069	1,63
7	35	12086	1,66
13	39	11290	1,39
48	33	14959	1,74
72	38	15836	1,71

[a]Conversion was calculated by gravimetrically
[b]Mn and polydispersity was obtained by GPC

From Table 4.1, it is seen that the highest molecular weight (Mn) was obtained after 72 hours. It was an expected result since studies showed that novozym 435 catalyzed ROP reactions showed living polymerization behaviors [31, 34]. Gross et.al studied kinetics of novozym 435 catalyzed polymerizations and reported that there was no chain termination and monomer consumption during polymerization at 60, 70, 80 and 85 ^{o}C. Also in this study, rate constant of initiation was larger than the rate constant of propagation which was an evidence of immortal characteristic of polymerization reactions [31]. On the other hand, increasing polydispersity with respect to time showed that heterogeneity of polymer samples was increasing [59].

Results of experiments carried out at 70 $^{\circ}$C with the same conditions are shown in Table 4.2. Again, polydispersity and molecular weight (Mn) of PCL was investigated with respect to time. Similar trend was observed with results obtained at 60 $^{\circ}$C. Again, molecular weight was increased with time. However, there was a different condition seen in this experiment. Polydispersity of PCL was decreased with time unlike the polymers obtained at 60 $^{\circ}$C.

Table 4.2: Effect of time on polymerization reaction catalyzed by Novozym 435 at T=70 $^{\circ}$C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
2	46	16485	1,71
5	56	14540	2,31
7	45	16444	1,63
13	34	16227	1,67
24	38	14126	1,58
48	41	17708	1,18
72	58	20402	1,30

[a]Conversion was calculated by gravimetrically
[b]Mn and polydispersity was obtained by GPC

Novozym 435 catalyzed polymerization was concluded by performing the reaction at 80 $^{\circ}$C. The highest molecular weight (Mn) of the resulting polymer was obtained after 72 hours which was 21571 g/mol. Polydispersities were changing between 1.79 and1.97. Also, within only 2 hours, PCL with a molecular weight 18408 g/mol was obtained. This result is consistent with studies in literature [31].

Table 4.3: Effect of time on polymerization reaction catalyzed by Novozym 435 at T=80 $^{\circ}$C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
2	23	18408	1,79
5	41	16455	1,63
7	40	18785	1,62
24	36	14979	1,94
48	45	14453	1,97
72	38	21571	1,81

[a]Conversion was calculated by gravimetrically
[b]Mn and polydispersity was obtained by GPC

Plots of molecular weight (Mn) versus time for three different temperatures (60, 70, 80 °C) were seen in Figure 4.1. From this graph, it is obvious the highest molecular weight was obtained at 80 °C followed by 70 and 60 °C. It can be said that activity of enzyme was decreased with increasing temperature and Mn was favored from this condition. At 60 °C, Mn of resulting polymer was 15836 g/mol while 20042 and 21571 g/mol at 70 and 80 °C respectively. Another important point was for 70 and 80 °C, at short periods such as 2 and 5hours, polymerization was resulted with a high value of molecular weight. This result is significant since if enzyme catalyzed polymerizations are adapted to large scale productions, it will help to decrease the costs by time saving.
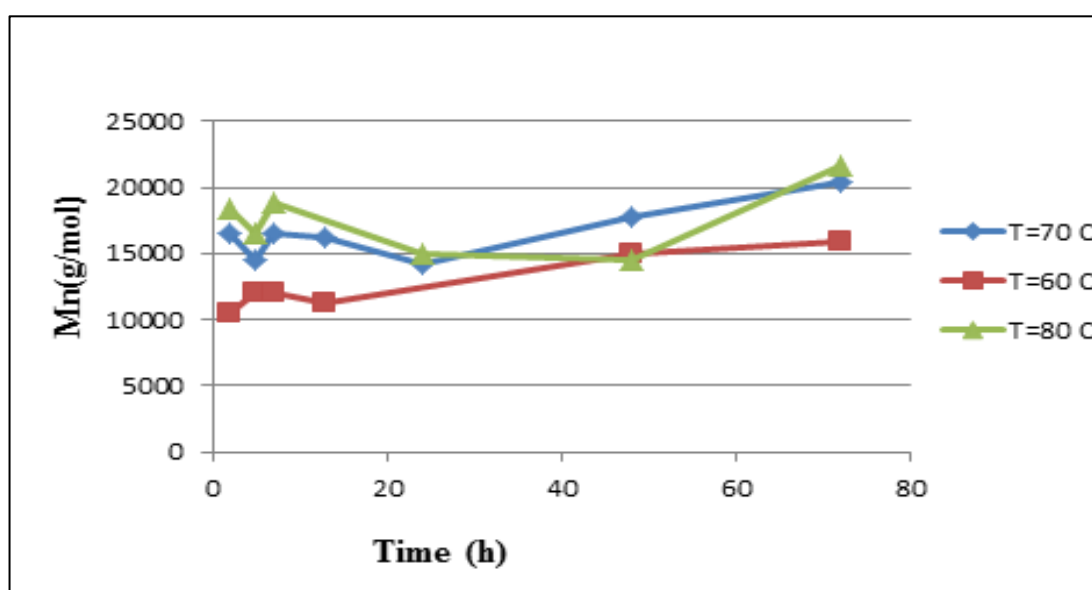

Figure 4.1: Effect of temperature on molecular weight of PCL synthesized by Novozym 435

FTIR spectrum of PCL (Figure 4.2) synthesized at 70 °C within 24 hours was evaluated according to major infrared bands of the polymer represented in Table 3.1.From spectrum, major transmission peak is seen clearly at 1721 cm^{-1}wave length which corresponds to carbonyl stretching (C=O) of the polymer. Other characteristic infrared bands of PCL are 2943, 2864, 1293, 1239 and 1176 cm^{-1} wave lengths corresponding to asymmetric CH_2 stretching, symmetric CH_2 stretching, C-O and C-C stretching in the crystalline phase, asymmetric COC stretching and symmetric COC stretching respectively. Among them, the band at 1293 cm^{-1} also showed crystalline structure of PCL [12]. This spectrum is consistent with the studies in literature and showed that synthesized polymer was PCL [12, 54].
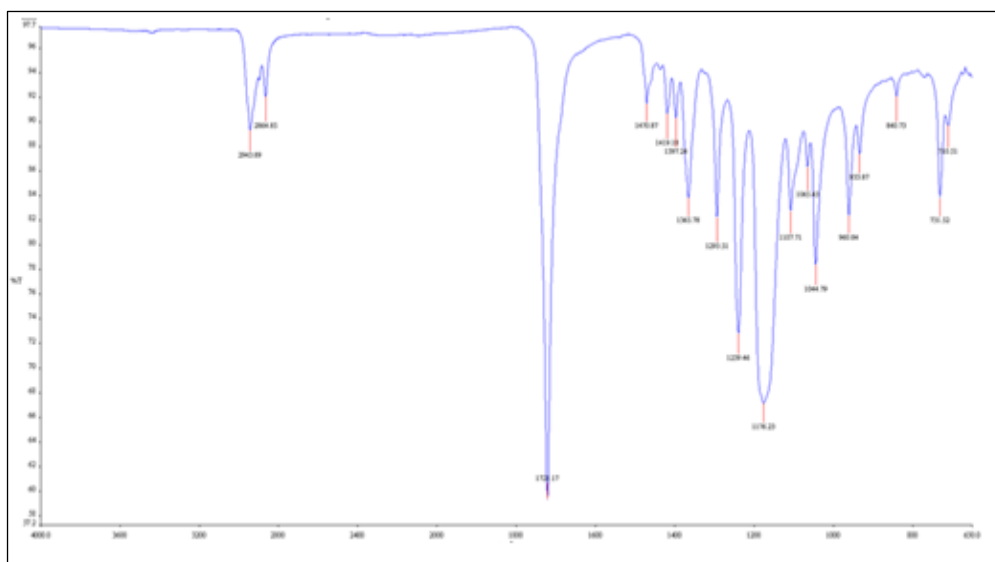
Figure 4.2: FTIR Spectrum of PCL synthesized with Novozym 435

In order to confirm molecular structure of PCL, ^{1}H NMR analysis was applied (Figure 4.3). From spectrum, chemical shifts (ppm) of PCL are as follows: 4.05 ppm (t, OCH2), 3.65 ppm (t, CH$_2$OH, end group), 2.3 ppm (t, CH$_2$CO), 1.6-1.7 ppm (m, 2xCH$_2$), 1.30-1.45 ppm (m, CH2). Structure of PCL was justified from these results and it is consistent with literature [31].
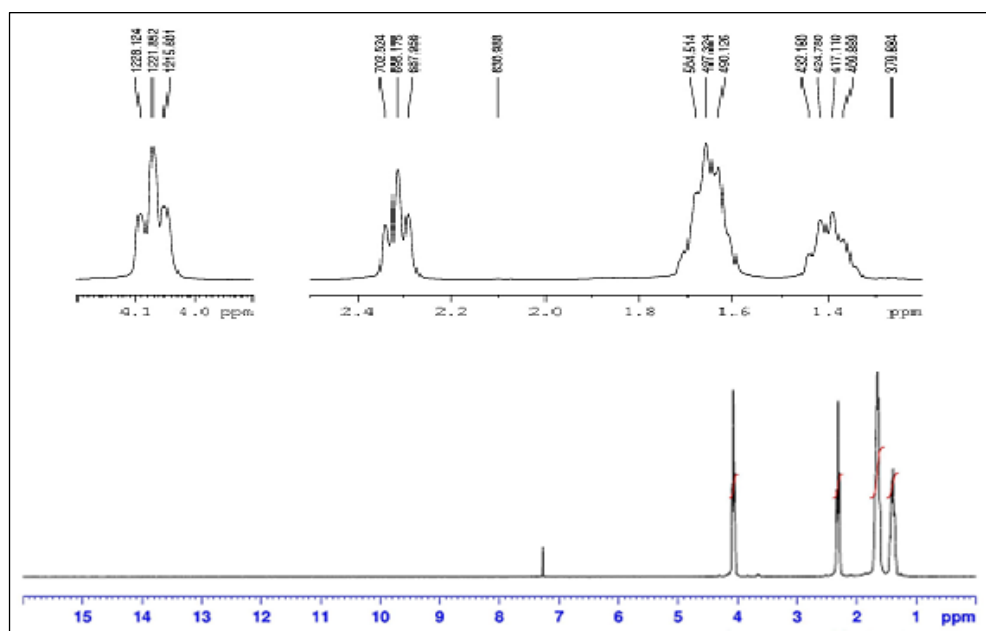

Figure 4.3: ^{1}H NMR spectrum of PCL synthesized with Novozym 435

In addition to NMR and FTIR analysis, DSC analysis was also applied to PCL synthesized at 70 °C within 24 hours (Figure 4.4). It was aimed to observe thermal behaviors and weight percent crystallinity of obtained PCL. DSC thermogram

showed glass transition temperature (Tg) and melting point (Tm) of PCL were -56.9 and 61.5 ^{o}C respectively. Also enthalpy of fusion (ΔH_f) was computed as 114 J/g from the area of melting peak. By using equation 3.2 and reference enthalphy change of total crystalline PCL, percent crystallinity was calculated as 80%. This result showed that obtained PCL was highly crystalline and the band at 1293 cm^{-1} in FTIR spectrum also justified this result [12].
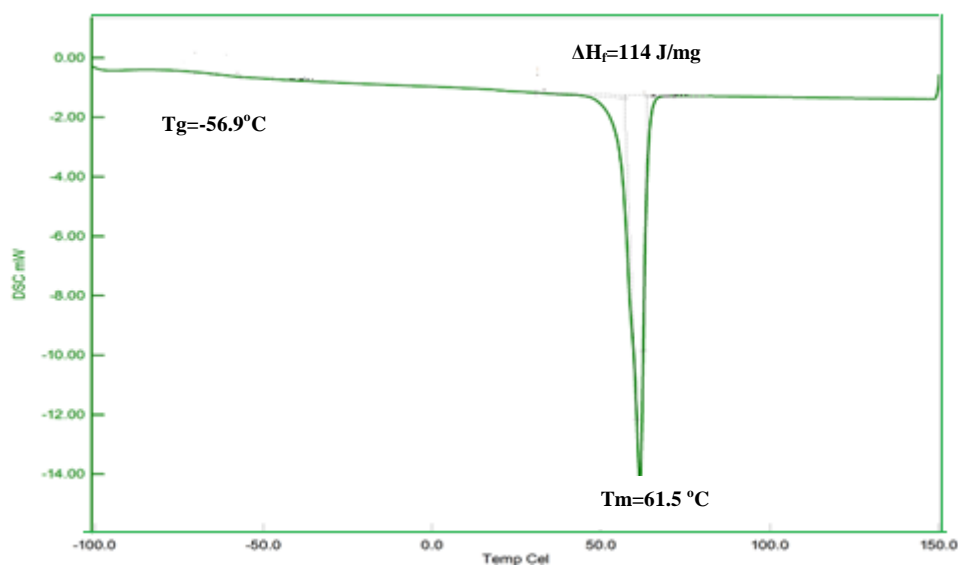

Figure 4.4: DSC thermogram of PCL synthesized with Novozym 435

4.2 Synthesis of PCL by Chitin Immobilized CALB

4.2.1 Optimization of immobilization methods for polymerization reaction

Lipase immobilization on chitin was performed with two different methods: physical adsorption and cross-linking with a coupling agent. It is known that amount of coupling agent could affect immobilization conditions significantly [10, 60]. Therefore, 3 different concentrations of gluteraldehyde were also applied to immobilization procedure. After immobilization process, obtained catalysts were evaluated with polymerization reactions in order to reach most efficient enzyme for PCL synthesis. Figure 4.5 shows comparison of immobilized catalysts with respect to percent monomer conversion of polymerization reaction. In this Figure, K_1, K_2 and K_3 was obtained by cross-linking with 0.02%, 0.2% and 2% (v/v) gluteraldehyde concentrations in 50 ml phosphate buffer solution respectively. K_4 was obtained by physical adsorption of lipase on chitin. Polymerization was performed at the same

conditions for all kinds of catalyst: (T=70 $^{\circ}$C, toluen/e-cl ratio=2:1 (v/v), stirring=200 rpm, enzyme/e-cl ratio=10% (w/w), polymerization time: 24 h).
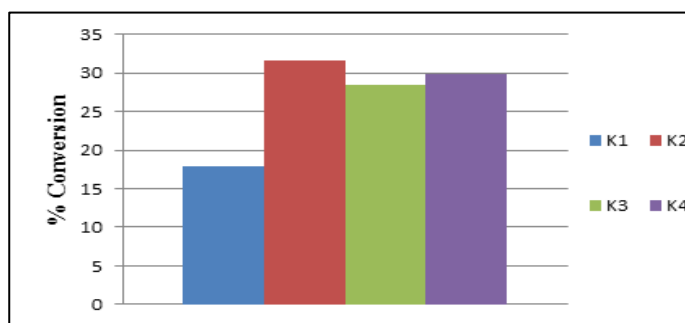

Figure 4.5: Evaluation of different immobilization methods for chitin immobilized CALB with respect to monomer conversion

It was observed that K_2 catalyzed ring opening polymerization of ε-CL was more rapidly than the other catalysts. After 24 hours, conversion of ε-CL into PCL was 32% with K_2 while the closer competitive K_4 catalyzed reaction with 30% conversion (Figure 4.5). This result showed that both physical adsorption and cross-linking method resulted with similar trend. However, in order to evaluate the obtained catalysts, molecular weight of resulting polymer was also an important factor since properties of polymeric materials are largely dependent on average molecular weights. For instance, low molecular weight polymers and oligomers are not useful for applications in which high strength is required [58, 59]. Figure 4.6 shows comparison of molecular weights of resulting polymers catalyzed by K_1, K_2, K_3 and K_4 catalyst. Again polymerization reaction conditions were the same for all kind of immobilized enzymes (T=70 $^{\circ}$C, toluen/e-cl ratio=2:1 (v/v), stirring=200 rpm, enzyme/e-cl ratio=10% (w/w), polymerization time: 24 h).
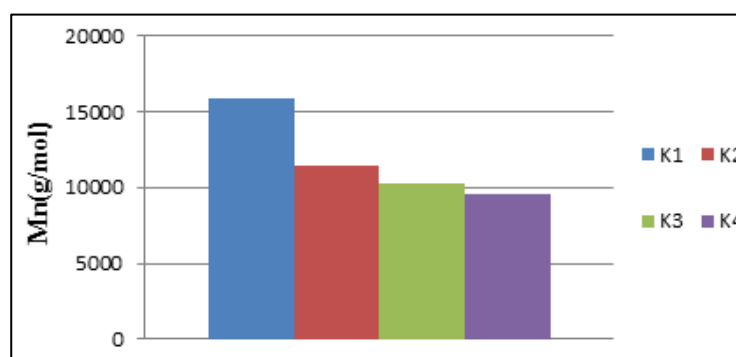

Figure 4.6: Evaluation of different immobilization methods for chitin immobilized CALB with respect to molecular weight (Mn)

In this part of the study, Mn was increased with the following order of catalysts: $K_1>K_2>K_3>K_4$. Thus, although K_1 gave the highest molecular weight, it catalyzed reaction with only 18% monomer conversion after 24 hours. On the other hand, K_2 was the most rapid catalyst as explained in above (Figure 4.6). Also, it was the second effective catalyst with respect to Mn results that synthesized PCL with a molecular weight of 11319 g/mol. Eventually, this was an acceptable result since different immobilized CALB rather than novozym 435 has been investigating by different study groups and molecular weight of obtained PCL's were in the range of 600-12000 g/mol [5, 7]. Therefore, K_2 was used for further reactions in order to optimize polymerization.

Characterization of chosen chitin immobilized lipase (K_2) was performed with SEM analysis. Figure 4.7 shows two SEM images of (a) chitin powder and (b) K_2. Lipases on SEM image were not seen clearly since it was scarce on the surface. Agglomerates of lipases on support could not be obtained from SEM images because lipases could be linked to inner places of chitin. This result was also seen in other studies and agglomerates of enzyme may not be positioned in the surface of support material [10]. TEM analysis can be performed in order to see if the enzymes were linked to inner places. Also, in literature imaging of protein distribution by infrared microscopy was used to observe bonded enzymes on support [6].

(a)   **(b)**
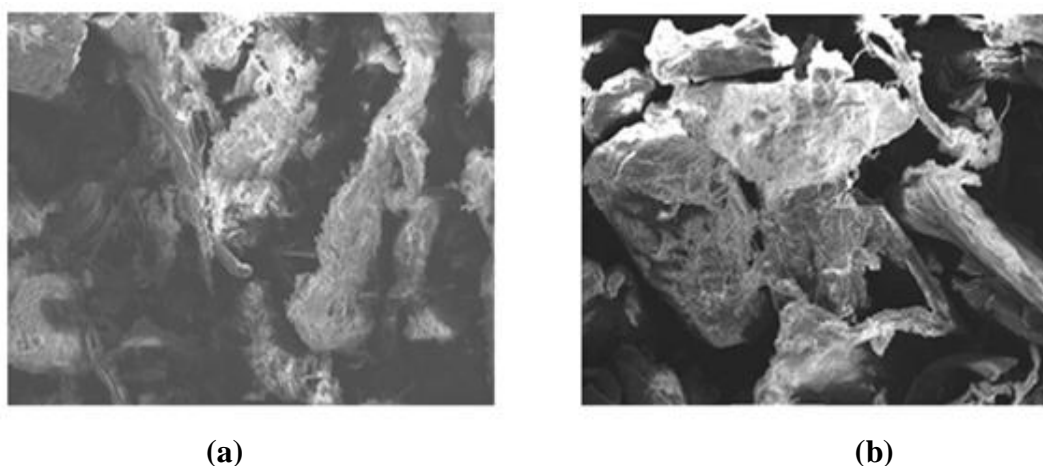
Figure 4.7: SEM images (a) Chitin powder (250 x), (b) K_2 (250 x)

In order to determine immobilization efficiency and see the differences between physical adsorption and cross-linking method, UV spectroscopy was also used. By the use of equation 3.1 and 3.2 calculated immobilization efficiencies are 15.50 % and 6.33 % for K_2 and K_4 enzyme respectively.

4.2.2 Parametric study of polymerization reactions

After K_2 was chosen, firstly drying effect of immobilized catalyst on polymerization reaction was investigated. In trial 1, K_2 was dried in an oven for 12 hours at 30 ^{o}C while in trial 2, it was not dried after immobilization procedure. The conditions of polymerization as follows: T=70 ^{o}C, toluen/e-cl ratio=2:1 (v/v), stirring=200 rpm, enzyme/e-cl ratio=10% (w/w), polymerization time: 24 h (Figure 4.8).
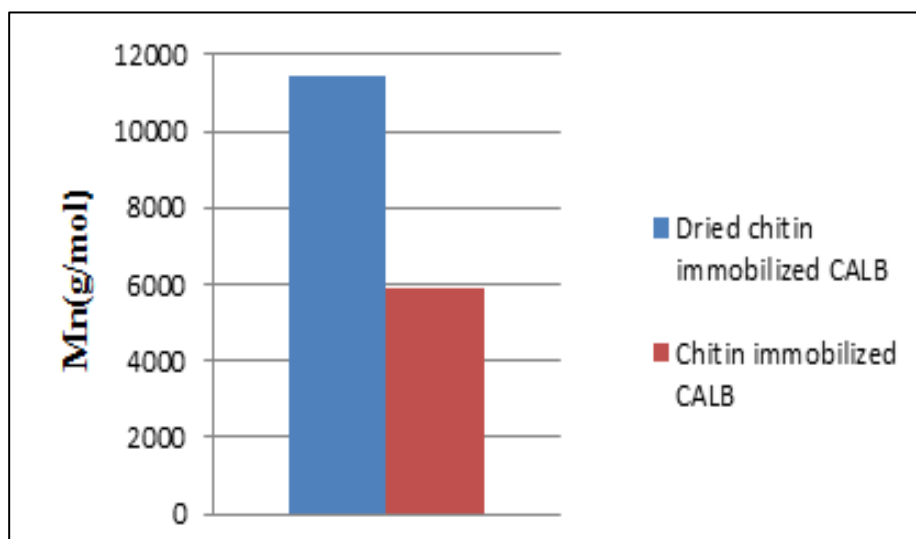
Figure 4.8: Drying effect on polymerization reaction for K_2

As it is shown in Figure 4.8, molecular weight (Mn) of the resulting polymers was changed significantly after drying. Polymerization mechanism of lactones showed that water acts as the initiator when no other nucleophiles are present in polymerization system by different study groups [14, 28, 61]. However, it was reported if water amount was high, molecular weight of the resulting polymers were decreased. Therefore water amount should be optimized [4, 33]. It is believed that high concentration of water leads to generate lots of polymer chains and this condition caused to obtain low molecular weights of polymer [4, 5, 31]. Thus, it was decided to dry immobilized catalysts prior to polymerization.

Second parametric study of K_2 catalyzed polymerization was performed to observe temperature and time effect on molecular weight of the resulting polymer and percent monomer conversion. Table 4.4 shows effect of time on polymerization reaction carried out at 60 ^{o}C. Reaction conditions were as follows: 2 mL toluene, 1 mL ε-CL, stirring=200 rpm, enzyme/e-cl ratio=10% (w/w).

Table 4.4: Effect of time on polymerization reaction catalyzed by K_2 at T=60 ^{o}C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
7	23	6488	1,26
24	38	9245	1,40
48	45	10664	1,50
72	40	11413	1,53
96	50	11224	1,54

[a]Conversion was calculated by gravimetrically

[b]Mn and polydispersity was obtained by GPC

It was observed from Table 4.4, molecular weight of the polymer was rising as the polymerization time increased. The highest molecular weights were 11413 g/mol and 11224 g/mol obtained after 72 and 96 hours respectively. Also polydispersities of polymers were increased with respect to time. Comparing with novozym 435, polymerization was slower and molecular weights were lower. However, polydispersity of the resulting polymers were decreased by this type of catalyst.

Parametric study was proceede with 70 and 80 ^{o}C respectively. Table 4.5 shows the results of polymerization at 70 ^{o}C. Polymerization conditions were the same as explained in above. Similar trend was seen in this polymerization either. Again, molecular weights were rising with increased time. Also, polydispersity was increasing with the time range of 2 to 96 hours since Mw was increased more rapidly than Mn with respect to time. On the other hand, after 96 hours polymerization has reached 45% monomer conversion, this showed that for longer periods monomer consumption could go further.

Table 4.5: Effect of time on polymerization reaction catalyzed by K_2 at T=70 ^{o}C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
2	1,2	3132	1,15
13	33	9317	1,33
24	32	11319	1,27
48	32	10170	1,49
72	32	10657	1,46
96	45	13261	1,54

[a]Conversion was calculated by gravimetrically

[b]Mn and polydispersity was obtained by GPC

Polymerization reactions catalyzed by K_2 were concluded with 80 $^{\circ}$C. From Table 4.6, it is obvious that like the previous reactions, molecular weight was increased with time. However, a deviation was seen in conversion at this temperature. With increased time, lower conversion was obtained. This result can be explained by denaturation of proteins in enzyme structure. It is known that high temperatures can cause deactivation of enzymes [31].

Table 4.6: Effect of time on polymerization reaction catalyzed by K_2 at T=80 $^{\circ}$C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
7	15	5192	1,19
24	26	9289	1,38
48	58	9643	1,52
72	46	11643	1,48
96	37	11586	1,60

[a]Conversion was calculated by gravimetrically
[b]Mn and polydispersity was obtained by GPC

In order to observe the effect of temperature on K_2 catalyzed polymerizations, Figure 4.9 is given. To summarize, reactions performed 70 $^{\circ}$C gave higher molecular weights with short time period. Within 24 hours, polymers with Mn of 9245, 11319 and 9289 g/mol were obtained at 60, 70, 80 $^{\circ}$C respectively. On the other hand, results were close to each other since change of temperatures were not in a wide range.
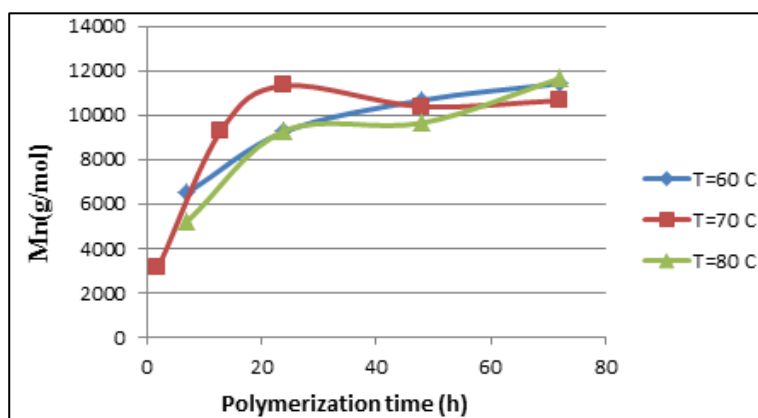

Figure 4.9: Effect of temperature on molecular weight of PCL synthesized by K_2

Also, monomer conversions with respect to time were plotted on the same graph as shown in Figure 4.10. After 48 hours, obtained PCL amount was decreased for all temperatures as seen in Figure 4.10. An explanation for this condition is the depolymerization effect of enzymes since lipases also catalyzes hydrolysis reactions and for long polymerization processes, reaction can be reversed.
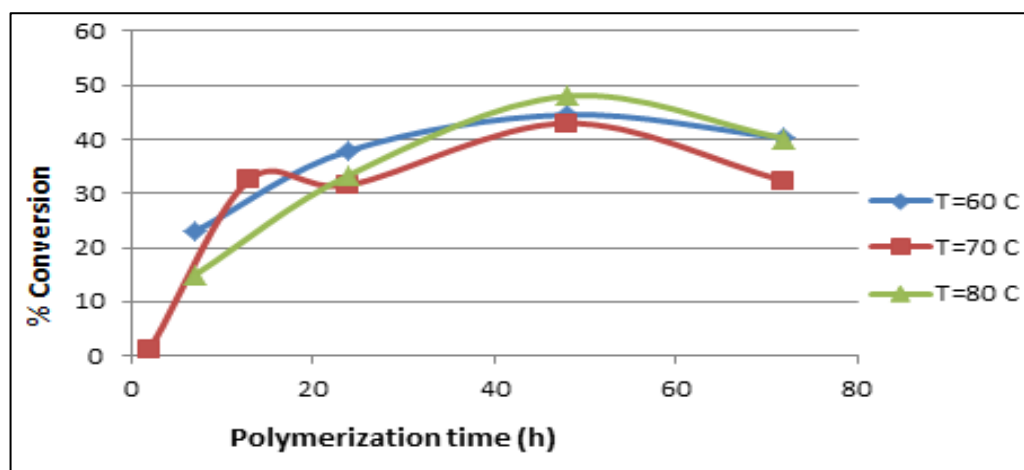

Figure 4.10: Effect of temperature on monomer conversion for polymerization reaction catalyzed by K_2

In this study, effect of enzyme concentration on polymerization reaction and molecular weight of synthesized polymer was also investigated. 4 different enzyme concentration was evaluated which were 5%, 10%, 15%, 20% (w/w)(enzyme to ε-caprolactone ratio). Table 4.7 shows the results of polymerization by different enzyme concentrations. Reactions were performed at 70 °C, with a toluen/e-cl ratio=2:1 (v/v) ratio for 24 hours. It is obvious that optimum result was 10% enzyme concentration for PCL synthesis via chitin-immobilized lipases.

Table 4.7: Effect of enzyme concentration on polymerization reaction catalyzed by K2.

Ratio of enzyme/ε-cl (%)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
5	5	6487	1,22
10	32	11319	1,27
15	39	9521	1,41
20	35	8095	1,33

[a]Conversion was calculated by gravimetrically

[b]Mn and polydispersity was obtained by GPC

Structure of polycaprolactone obtained with chitin immobilized lipases was analyzed by FTIR and NMR methods. FTIR spectrum of PCL obtained at 70 ^{o}C after 24 hours is seen in Figure 4.11.Like novozym 435 catalyzed polymerization, PCL synthesized using K_2 was proven with FTIR spectrum. Characteristic infrared bands of PCL (2943, 2864, 1293, 1239 and 1176 cm^{-1}wave lengths) corresponding to asymmetric CH_2 stretching, symmetric CH_2 stretching, C-O and C-C stretching in the crystalline phase, asymmetric COC stretching and symmetric COC stretching can be seen from spectrum and again PCL structure is proven [12, 54].
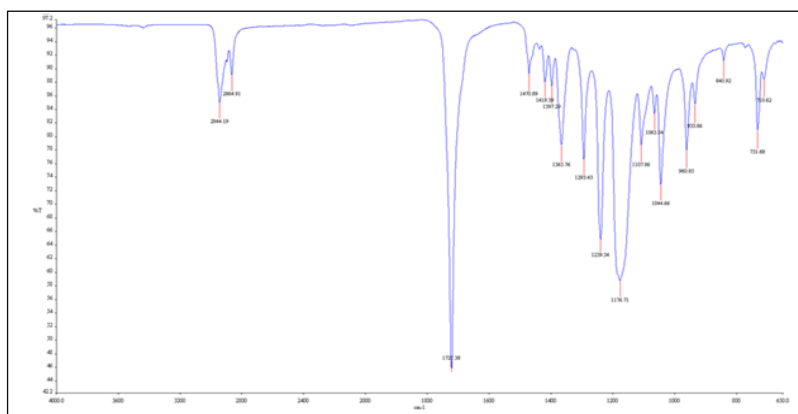
Figure 4.11: FTIR Spectrum of PCL synthesized using K_2

^{1}H NMR spectrum of PCL obtained at 70 ^{o}C after 24 hours is seen in Figure 4.12. This spectrum justified the FTIR results that synthesized polymer was PCL. Chemical shifts (ppm) of PCL seen in Figure are in the following: 4.05 ppm (t, OCH2), 3.65 ppm (t, CH_2OH, end group), 2.3 ppm (t, CH_2CO), 1.6-1.7 ppm (m, $2xCH_2$), 1.30-1.45 ppm (m,CH_2).This molecular structure corresponds to PCL and also consistent with literature [31].

Figure 4.12: ^{1}H NMR spectrum of PCL synthesized using K_2

Moreover, DSC analysis was also applied to PCL synthesized at 70 $^{\circ}$C within 24 hours (Figure 4.13) in order to see thermal behaviors and crystallinity of obtained PCL. It is shown in DSC thermogram,Tg and Tm of PCL were -60.3 and 62.1°C respectively. Also enthalpy of fusion (ΔH_f) was 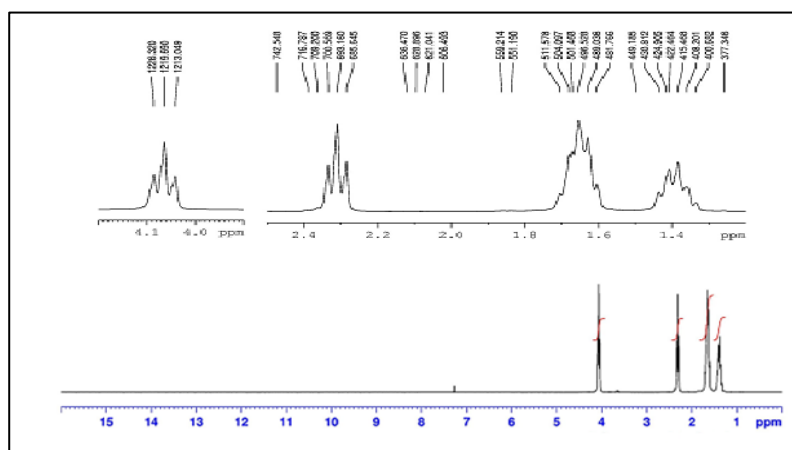computed as 99.5 J/g from the area of melting peak. By using equation 3.4 and reference enthalphy change of total crystalline PCL, percent crystallinity was calculated as 70%. Again, degree of crystallinity of PCL was relatively high however; it was lower than the polymer obtained from novozym 435. Crystalline structure of PCL was also confirmed with the band at 1293 cm^{-1} in FTIR spectrum [12].
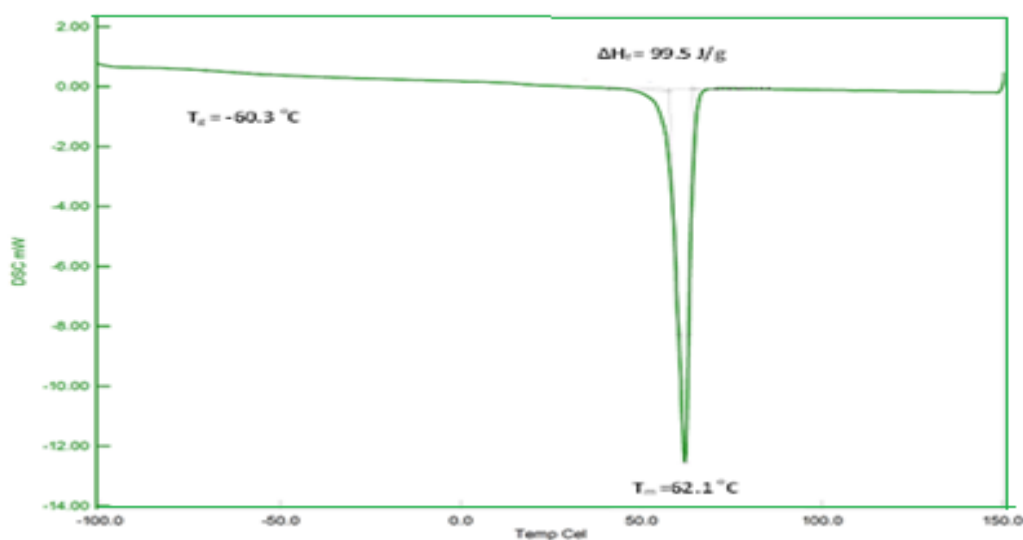
Figure 4.13: DSC thermogram of PCL synthesized using K$_2$

4.3 Synthesis of PCL by Chitosan Immobilized CALB

4.3.1 Optimization of immobilization methods for polymerization reactions

As explained in previous section, lipase immobilization on chitosan was performed with two different methods: physical adsorption and cross-linking with a coupling agent. Again 3 different concentrations of gluteraldehyde were applied to immobilization procedure since it is known that amount of coupling agent could affect immobilization conditions significantly [10, 60].

In order to evaluate obtained catalysts with different immobilization methods, same procedure was followed with chitin immobilized lipases. Polycaprolactone synthesis was carried out by these immobilized enzymes and the most efficient catalyst was

chosen for further reactions. Figure 4.14 shows comparison of immobilized catalysts with respect to percent monomer conversion of polymerization reaction. In this Figure; Immob$_1$, Immob$_2$ and Immob$_3$ were obtained by cross-linking with 0.02%, 0.2% and 2% (v/v) gluteraldehyde concentrations in 50 ml phosphate buffer solution respectively. Immob$_4$ was obtained by physical adsorption of lipase on chitosan. Polymerization was performed at the same condition with the other catalysts examined in previous sections in order to compare support efficiency (T=70 ^{o}C, toluen/e-cl ratio=2:1 (v/v), stirring=200 rpm, enzyme/e-cl ratio=10% (w/w), polymerization time:24 h).
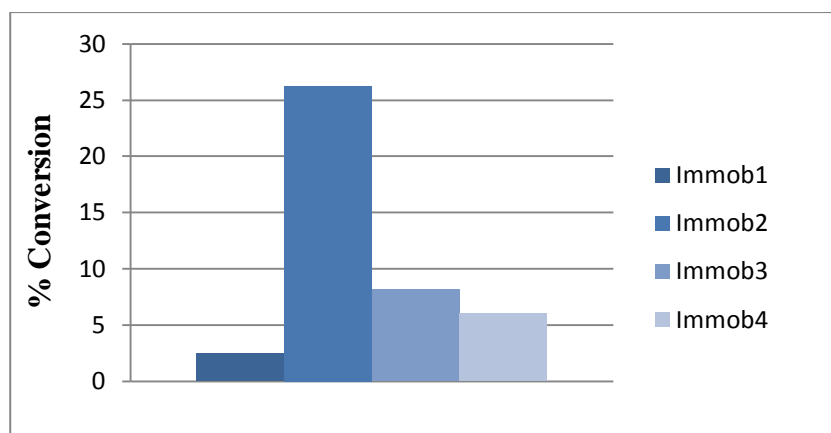

Figure 4.14: Evaluation of different immobilization methods for chitosan immobilized CALB with respect to conversion

It was clearly seen from Figure 4.14, immob$_2$ catalyzed ring opening polymerization of ε-CL more rapidly than the other catalysts (immob$_1$, immob3, immob4). Monomer conversion by immob$_2$ catalysis was 26% while conversion was 2%, 8% and 6% with the other immobilized enzymes corresponding in the following order: immob$_1$, immob3, immob4. This result showed that both immobilization procedure and gluteraldehyde concentration affected efficiency of prepared catalysts. Unlike chitin immobilized lipases, physical adsorption was resulted with a very low efficiency after 24 hours for PCL synthesis. It can be resulted by enzyme leakage during polymerization reactions. It is known that physical adsorption of enzymes on supports can be resulted with enzyme leakage during polymerization reactions. Also, bounds of the gluteraldehyde concentration caused inactive catalyst formation in this study. Optimum result was obtained by 0.2% (v/v) contained buffer solution since higher amount of gluteraldehyde could not cleaved to enzymes. It was consistent with other studies in which when amout of gluteraldehyde has reached 15%, it could

not cleaved to the enzymes [60]. Also, Foresti et.al. prepared chitosan immobilized lipases with cross-linking 0.025% and 0.25% (v/v) gluteraldehyde concentrations and they used these lipases in fatty acid esterification reactions. Again, the former one was more active than the latter catalyst [10].

It was obviously seen that immob$_2$ was the most efficient catalyst among chitosan immobilized lipases. However, molecular weights of the resulting polymers were also compared to see the behavior of this catalyst with respect to Mn results. Figure 4.15 shows comparison of molecular weights of resulting polymers catalyzed by Immob$_1$, Immob$_2$, Immob$_3$ and Immob$_4$ catalysts. Polymerization conditions were as follows: T=70 ^{o}C, toluen/e-cl ratio=2:1 (v/v), stirring=200 rpm, enzyme/e-cl ratio=10% (w/w), polymerization time: 24 hours.
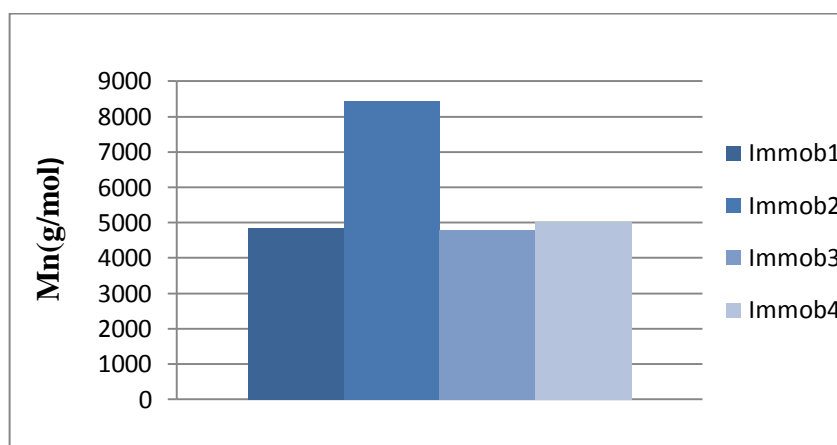
Figure 4.15: Evaluation of different immobilization methods for chitosan immobilized CALB with respect to molecular weight (Mn)

From Mn results (Figure 4.15), similar trend was obtained as explained in conversion comparison. Mn was increased with the following order of catalysts: Immob2>Immob1>Immob3>Immob4. Among them, again Immob$_2$was the most effective one according to polycaprolactone synthesis. Molecular weight (Mn) of PCL obtained by this catalyst was 8460 g/mol while the others were 4850 (Immob$_1$), 4784 (Immob$_3$) and 5024 (Immob$_4$) g/mol. Results showed that, except Immob1, by other chitosan immobilized lipases only mezopolymers with a very slow reactions could be obtained.Therefore, Immob$_2$ was used for further reactions in order to optimize polymerization and obtain high molecular weights of PCL.

Characterization of chosen chitosan immobilized lipase (Immob$_2$) was performed with SEM analysis. Figure 4.16 shows two SEM images of (a) chitosan powder and

(b) Immob$_2$. Compared with chitin immobilized lipases, chitosan immobilized lipases are seen distinctly from SEM image. Again agglomerates of lipases on support could not be obtained but lipases cleaved on surfaces of chitosan are shown in the microphotograph. In order to support that lipases are immobilized on chitosan, characterization methods that were indicated in chitin immobilized lipases part, can be applied because lipases could be linked to inner places of chitosan as indicated previously [6].

| **(a)** | **(b)** |
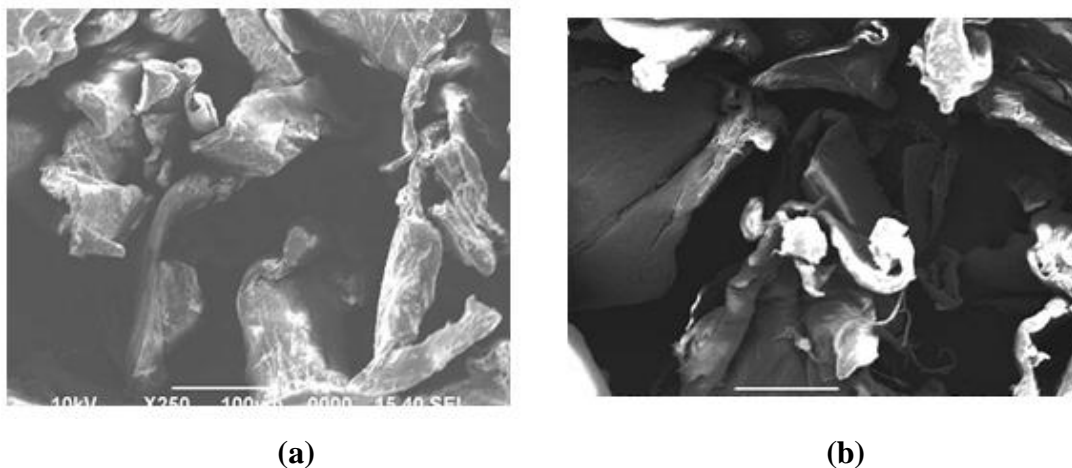
Figure 4.16: SEM images (a) Chitosan powder (250 x), (b) Immob$_2$ (250 x)

In order to determine immobilization efficiency and observe the differences between physical adsorption and cross-linking method, also UV spectroscopy was used. By using equation 3.1 and 3.2 immobilization efficiencies are calculated as 23.0% and 12.2% for Immob$_2$ and Immob$_4$ enzyme respectively.

4.3.2 Parametric study of polymerization reactions

Polymerization reactions were performed at three different temperatures (60, 70, 80 $^{\circ}$C) within a determined time range by chosen chitosan immobilized lipase (Immob$_2$). Previously, drying effect of immobilized catalyst (K$_2$) on polymerization reaction was investigated. The study showed that drying has played an important role on polymerization reaction. When the water amount was high, molecular weight of the resulting polymer decreases significantly. Therefore, Immob$_2$ was dried like K$_2$ and used to see temperature and time effect. Table 4.8 shows effect of time on polymerization reaction carried out at 60 $^{\circ}$C. Reaction conditions were as follows: 2 mL toluene, 1 mL ε-CL, stirring=200 rpm, enzyme/e-cl ratio=10% (w/w).

Table 4.8: Effect of time on polymerization reaction catalyzed by Immob$_2$ at T=60 ^{o}C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
7	0,9	4880	1,11
24	25	6768	1,26
48	36	7420	1,33
72	47	7082	1,38
96	44	13626	1,55

[a]Conversion was calculated by gravimetrically

[b]Mn and polydispersity was obtained by GPC

It is clearly seen from Table 4.8, increasing time favored the polymerization reaction. After 7 hours, only 0.9% of ε-CL was converted into PCL; however conversion has reached 47% within 72 hours. Also the highest molecular weight (Mn) was obtained after 96 hours at this temperature.

Table 4.9 shows the results of polymerization at 70 ^{o}C. Again, molecular weights were increasing with time. Also, comparing with 60 ^{o}C, increasing temperature caused to synthesize higher molecular weights of polymer up to 72 hours.

Table 4.9: Effect of time on polymerization reaction catalyzed by Immob$_2$ at T=70 ^{o}C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
13	14	6207,2	1,20
24	26	8450,4	1,33
48	37	7772	1,39
72	36	12771	1,45
96	40	12912	1,29

[a]Conversion was calculated by gravimetrically

[b]Mn and polydispersity was obtained by GPC

In order to see, upper limit of temperature for PCL synthesis with Immob$_2$, temperature increased to 80 ^{o}C and the results are seen in Table 4.10. Polymerization reaction was performed within 13-96 hours. Although Mn was increased with time up to 72 hours, higher molecular weights were obtained at 70 ^{o}C. Also, after 72 and 96 hours, Mn was decreased from 11058 to 10051 and then 9191 g/mol respectively

since reversible reaction could be occurred because of the hydrolysis ability of lipase at this temperature.

Table 4.10: Effect of time on polymerization reaction catalyzed by Immob$_2$ at T=80 ^{o}C

Time (h)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
13	25	7879	1,22
24	42	7438	1,42
48	40	11058	1,38
72	40	10051	1,39
96	44	9191	1,41

[a]Conversion was calculated by gravimetrically

[b]Mn and polydispersity was obtained by GPC

Plots of molecular weight (Mn) versus time for three different temperatures (60, 70, 80 ^{o}C) were given in Figure 4.17 in order to show effect of temperature on polymerization reaction. Immob$_2$ catalyzed polymerization did not show a similar trend for all temperatures. As it is seen from Figure, Mn was increasing up to 96 hours at 60 and 70 ^{o}C while Mn started to decrease after 48 hours at 80 ^{o}C. This can be explained by reversible reaction due to hydrolysis ability of enzyme at this temperature.

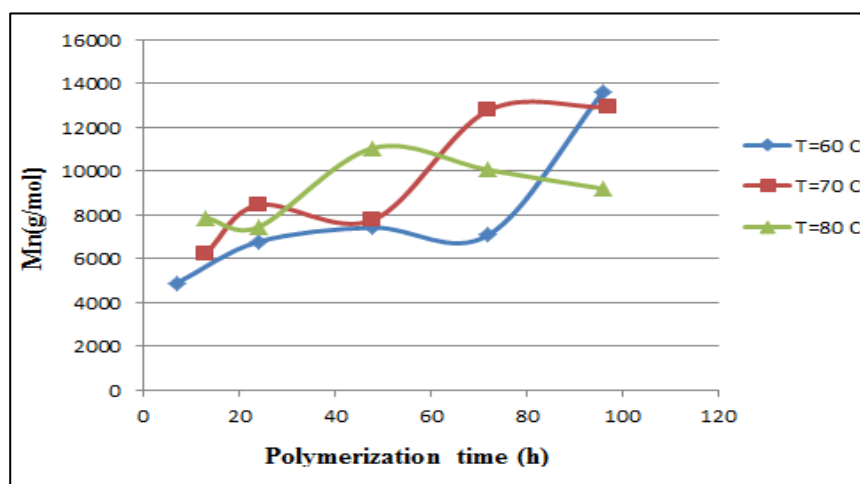**Figure 4.17**: Effect of temperature on molecular weight of PCL synthesized by Immob$_2$

Figure 4.18 showed the result of increasing the temperature from 60 to 70 and 80 ^{o}C was more rapid conversion of monomer up to 48 hours. However, interestingly after 48 hours conversion become more rapid at 60 ^{o}C than the other temperatures. It is known that stability of enzymes can change with increasing temperature. Because of

this, when enzyme stayed at relatively high temperatures (70, 80 ^{o}C) for long time, its stability has been decreasing. This result is consistent with Figure 4.17 since Mn of PCL at 60 ^{o}C was lower than the other temperatures. This showed that at high temperatures for long time, activity of enzyme was lower than it was at 60 ^{o}C since more polymer chains were formed and resulted with low molecular weight at this temperature.
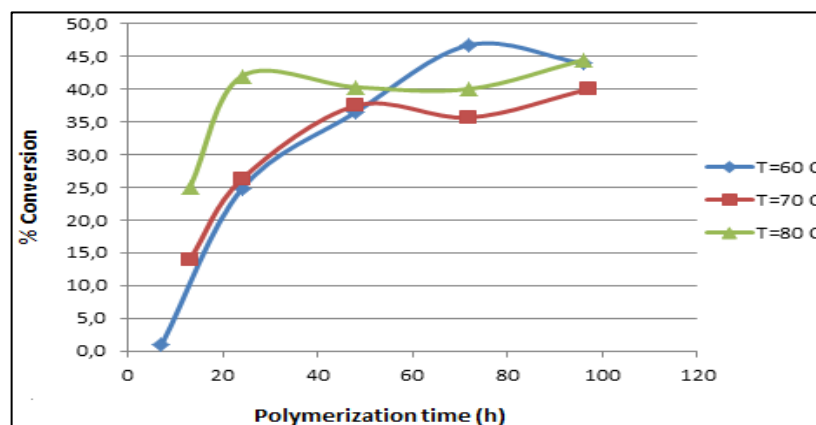

Figure 4.18: Effect of temperature on monomer conversion for polymerization reaction catalyzed by Immob$_2$

Lastly, effect of enzyme concentration on polymerization reaction and molecular weight of synthesized polymer was investigated. 4 different enzyme concentration were evaluated (5%, 10%, 15%, 20% (w/w) (enzyme to ε-caprolactone ratio)) in this study. Results are shown in table 4.11. Reactions were performed at 70 ^{o}C for 24 hours (toluene/ε-Cl=2:1 v/v).

Table 4.11: Effect of enzyme concentration on polymerization reaction catalyzed by Immob$_2$.

Ratio of enzyme/ε-cl (%)	Conversion[a] (%)	Mn[b] (g/mol)	Polydispersity (Mw/Mn)
5	26	6972	1,26
10	26	8450	1,33
15	31	7157	1,34
20	31	8753	1,37

[a]Conversion was calculated by gravimetrically

[b]Mn and polydispersity was obtained by GPC

FTIR and NMR methods were used to define structure of polycaprolactone obtained with chitosan immobilized lipases. FTIR spectrum of PCL synthesized using immob$_2$

is shown in Figure 4.19. Characteristic infrared bands of PCL (2943, 2864, 1293, 1239 and 1176 cm^{-1} wave lengths)assigned to asymmetric CH$_2$ stretching, symmetric CH$_2$ stretching, C-O and C-C stretching in the crystalline phase, asymmetric COC stretching and symmetric COC stretching are clearly seen in spectrum. Additionally, major transmission peak (1721 cm^{-1}wave length) seen in spectrum corresponds to carbonyl stretching (C=O) of the polymer. As it was indicated in previous sections, this spectrum is consistent with the studies in literature too and showed that synthesized polymer was PCL [13, 54].
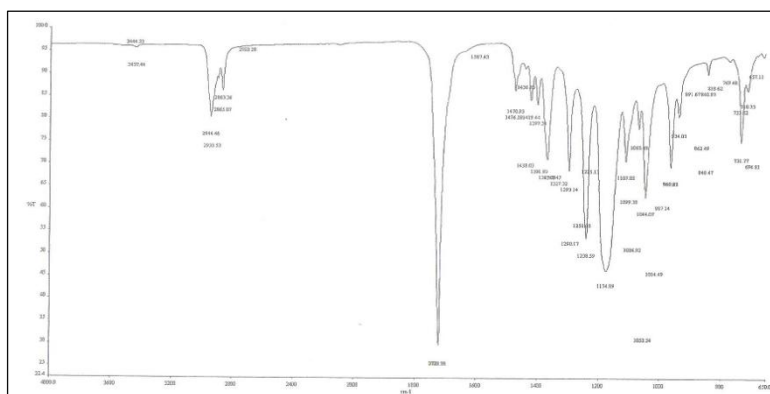
Figure 4.19: FTIR spectrum of PCL synthesized using Immob$_2$

As it was indicated in previous sections, to confirm PCL structure ^{1}H NMR analysis was peformed (Figure 4.20). Polymer was synthesized at 70 $^{\circ}$C after 72 hours. Similar spectrum was obtained with previous sections. Chemical shifts of PCL shown in Figure are as follows: 4.05 ppm (t, OCH$_2$), 3.65 ppm (t, CH$_2$OH, end group), 2.3 ppm (t, CH$_2$CO), 1.6-1.7 ppm (m, 2xCH$_2$), 1.30-1.45 ppm (m,CH$_2$). Like K$_2$ and novozym 435 catalyzed polymerizations, structure of PCL synthesized with Immob$_2$ was also confirmed from these results [31].

Figure 4.20: ^{1}H NMR spectrum of PCL synthesized using Immob$_2$

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In addition to structure characterization, thermal behaviors of PCL synthesized at 70 ^{o}C within 72 hours were investigated with DSC analysis [Figure 4.21]. DS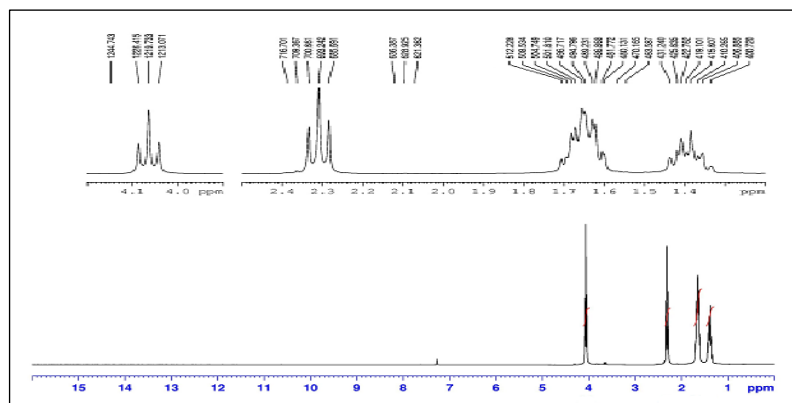C thermogram showed that Tg and Tm of PCL were -58 and 61.6 ^{o}C respectively. Also, crystallinity of PCL was computed according to equation 3.4 and reference enthalpy change of total crystalline PCL. Enthalpy of fusion (ΔH_f) was calculated as 126 J/g from the area of melting peak. Thus, percent crystallinity was calculated as 88%. Comparing with the above DSC results, crystallinity of this PCL was the highest. It is known that crystallinity increases tensile strength of the polymer and it can be said that PCL obtained using Immob$_2$can be used in applications which need high tensile strength. On the other hand, of course to justify this result mechanical behaviors of polymer must be investigated.
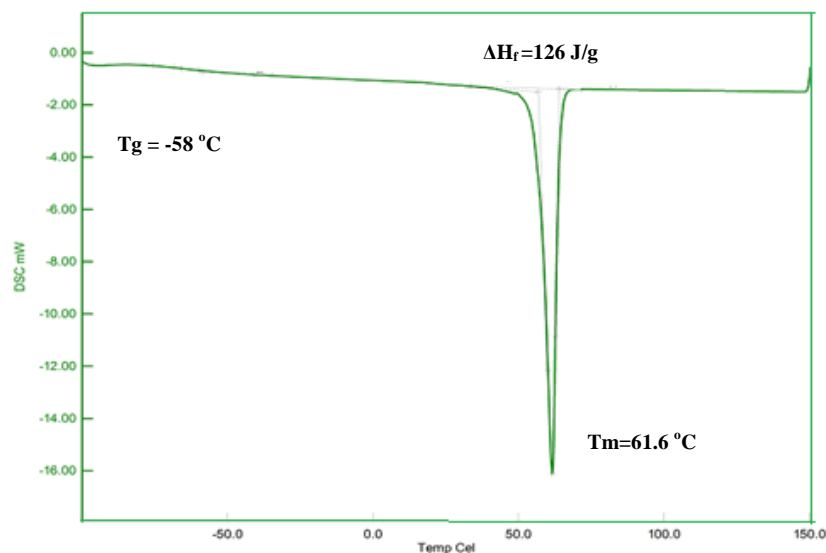

Figure 4.21: DSC thermogram of PCL synthesized using Immob$_2$

5. CONCLUSIONS AND RECOMMENDATIONS

In this master thesis, enzymatic ring opening polymerization of ε-caprolactone was investigated. As biocatalysts, chitin and chitosan immobilized lipases were prepared via different immobilization methods and evaluated in polymerization reactions. Also, polymerization reactions were performed with novozym 435 which is often used in polyester synthesis in literature in order to compare the efficiencies of prepared catalysts in this study. Furthermore, obtained polymers were characterized with ^{1}H NMR and FTIR analysis. Thermal behaviors and crystallinity of polymers were determined by DSC analysis whereas molecular weights and polydispersities of resulting polymeric were obtained from GPC analyses. According to indicated experiments and analysis, following results was observed in this study.

1. According to novozym 435 catalyzed polymerization results, the highest molecular weight of PCL was obtained at 80 ^{o}C (Mn=21571 g/mol) within 72 hours. Also Mn wasincreasing with time at this temperature. Furthermore, similar trend was observed in polymerization reactions performed at 70 and 60 ^{o}C via novozym 435. Molecular weight was increasing with time and within 72 hours; Mn of obtained PCL has reached to 20402 and 15836 g/mol at 70 and 60 ^{o}C respectively.

2. Another result of novozym 435 catalyzed polymerization was at short periods such as 2 and 5 hours, polymerization was resulted with a high value of molecular weight (within 2 hours, Mn=10492, 16485, 18408 g/mol at 60, 70 and 80 ^{o}C). This result is significant since if enzyme catalyzed polymerizations are adapted to large scale productions, it will help to decrease the costs by time saving.

3. The effect of reaction temperature on novozym 435 catalyzed reactions showed that Mn was increasing following order: 80 ^{o}C > 70 ^{o}C > 60 ^{o}C. On the other hand, monomer conversion was decreased when temperature rises from 60 to 80 ^{o}C (within 2 hours, monomer conversion= 52%, 46%, 23% at 60, 70 and 80 ^{o}C). This can be explained by change of enzyme activity during reaction. It can be

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said that increasing temperature lowered the activity of novozym 435. Therefore, less polymeric chains were obtained at high temperatures and Mn was favored from this condition.

4. Optimization of immobilization methods for chitin immobilized lipases showed that cross-linking with gluteraldehyde was the most efficient prepared catalyst among other methods. Also, coupling agent ratio significantly affected efficiency of prepared catalyst in polymerization reaction. Moderate gluteraldehyde ratio (2% v/v) was the best result since the use of drastic conditions such as high ratio of coupling agent resulted with uncontrolled reaction condition and coupling agent cannot be bonded to the enzyme [60].

5. Furthermore, optimization of immobilization methods for chitosan immobilized lipases showed similar trend with chitin immobilization. Again, the most efficient catalyst was prepared by cross-linking via gluteraldehyde at moderate ratios (0.2% v/v). This is an expected result since chitosan is derived from chitin by acetylation and their molecular structure was similar.

6. Among immobilization methods, physical adsorption of CALB on chitin and chitosan was not effective as the ones obtained via cross-linking methods. According to results of polymerization reactions; prepared immobilized catalysts via cross-linking gave a molecular weight (Mn) of 11319 and 8460 g/mol PCL with supports chitin and chitosan respectively (T=70 ^{o}C within 24 hours).On the other hand, physically immobilized lipases gave relatively low molecular weights of polymer (9581 g/mol and 5025 g/mol for chitin and chitosan respectively). From these results, it was obvious chitosan immobilized lipases via physical adsorption could only synthesize mezopolymer.

7. In addition to above results, it was seen that polymerization was more rapid with lipases obtained via cross-linking methods: 32% of monomer was consumed with chitin immobilized lipases via cross-linking whereas 30% of monomer was consumed with physically adsorbed lipase on chitin after 24 hours at 70 ^{o}C. These results were too close however, molecular weight of obtained PCL was higher with cross-linking method as it was explained in above, and for further polymerization reactions this enzyme was used (K_2).

8. Similar results were obtained for chitosan immobilized lipases as it was indicated in above. Again, polymerization was more rapid with lipases obtained via cross-

linking methods: 24% of monomer was consumed with chitosan immobilized lipases via cross-linking whereas 6% of monomer was consumed with physically adsorbed lipase on chitosan after 24 hours at 70 ^{o}C. Therefore, chitosan immobilized lipase with cross-linking method was used for further polymerization reactions (Immob$_2$).

9. The reason why cross-linking methods enhanced efficiency of catalyst for PCL synthesis can be explained by enzyme leakage problem seen in physical adsorption methods. Due to the weak bonds between enzyme and supports, enzyme could easily leak from support in physically adsorbed enzymes [5]. Immobilization efficiency results in this study also supported this approach since lipases bounded with a lower percentage to both chitin and chitosan with physical adsorption immobilization method.

10. It was also shown in this study that water content should be optimized since higher amount of water decreased molecular weight of resulting polymer. In one trial, chitin immobilized lipase with cross-linking method was dried at 30 ^{o}C for 12 hours and in second trial it was not dried. Both dried and the other one was used in polymerization (T=70 ^{o}C within 24 hours). Mn of obtained polymers was 11319 g/mol and 5917 g/mol respectively. This result was an excepted result since high concentration of water leads to generate lots of polymer chains and this condition caused to obtain low molecular weights of polymer [4, 31, 33].

11. According to optimization of polymerization reactions by using chitin immobilized lipases (K$_2$), temperature and time effect on monomer conversion and molecular weight of resulting polymer was investigated. It was seen that Mn was increasing with time like novozym 435 catalyzed polymerizations for all temperatures. After 96 hours, Mn of PCL was 11224, 13261 and 11586 g/mol at 60, 70 and 80 ^{o}C respectively.

12. It was also observed from chitin immobilized catalysis; reactions performed 70 ^{o}C gave higher molecular weights with short time period. Within 24 hours, polymers with Mn of 9245, 11319 and 9289 g/mol were obtained at 60, 70, 80 ^{o}C respectively. On the other hand, results were close to each other since change of temperatures were not in a wide range.

13. In addition to molecular weight comparison, monomer conversions with respect to time were observed for different temperatures (chitin immobilized lipases).

After 48 hours, obtained PCL amount was decreased for all temperatures. An explanation for this condition is the depolymerization effect of catalysts since lipases also catalyzes hydrolysis reactions and for long polymerization processes, reaction can be reversed.

14. According to optimization of polymerization reactions by using chitosan immobilized lipases (Immob$_2$), the highest Mn of polymer were 13626 and 12912 g/mol after 96 hours at 60 and 70 ^{o}C. For these two temperatures Mn was increasing up to 96 hours. However when the temperature has rised to 80 ^{o}C, Mn was increasing up to 48 hours and has reached 11058 g/mol. After 48 hours, Mn started to decreased to 10051 and 9191 g/mol after 72 and 96 hours respectively. This can be explained by reversible reaction due to hydrolysis ability of enzyme at this temperature.

15. Furthermore, monomer conversion of chitosan immobilized catalysis was increased with increase of temperature up to 48 hours. However, interestingly after 48 hours conversion become more rapid at 60 ^{o}C than the other temperatures. It is known that stability of enzymes can change with increasing temperature. Because of this, when enzyme stayed at relatively high temperatures (70, 80 ^{o}C) for long time, its stability has been decreasing. This result was also consistent with Mn results in which Mn of PCL at 60 ^{o}C was lower than the other temperatures. It can be said that activity of enzyme at high temperatures for long time was lower than it was at 60 ^{o}C since more polymer chains were formed and resulted with low molecular weight at this temperature.

16. In order to optimize enzyme concentration, also amount of enzyme was changed in 4 different values (5%, 10%, 15%, 20%) for both chitin and chitosan immobilized lipases. Eventually, it was observed 10% was optimum and increasing enzyme concentration was not effective.

17. This study also compared efficiency of prepared catalysts and novozym 435 for PCL synthesis. As shown from Figure 5.1, novozym 435 catalyzed reactions was more rapid and higher molecular weights of polymer was obtained with this enzyme (reaction conditions: T=70 ^{o}C within 24 hours) Also, chitin immobilized lipases were more effective than chitosan immobilized lipases as shown in Figure. However, comparing polydispersities of resulting polymer, chitin and

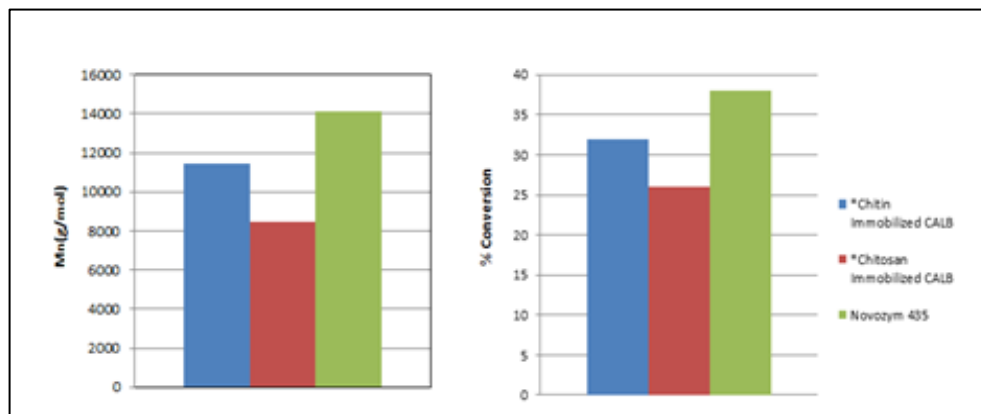chitosan immobilized lipases gave lower polydispersities (1.27 and 1.3 respectively) than novozym 435 (1.58).

Figure 5.1: Comparison of prepared catalysts and Novozym 435

18. Thus, it can be concluded that although novozym 435 was an efficient catalyst for PCL synthesis, chitin and chitosan could be alternative since they are cheap and most abundant biopolymers in nature. Chitin and chitosan immobilized lipases were not used in PCL synthesis before this study. Therefore, this study can be the beginning and in future studies immobilization methods can be improved in order to obtain more rapid polymerization reactions and higher molecular weights of PCL.

19. In addition to above results, DSC analysis showed that obtained polycaprolactones were highly crystalline. Crystallinity of polymers obtained via novozym 435, chitin and chitosan immobilized lipases were calculated as 80%, 70% and 88% respectively. These results were also consistent with FTIR spectrum of corresponding polymers in which the band at 1293 cm^{-1}justified highly crystalline structure [12].

20. DSC analyses also showed that glass transition temperature of polcaprolactones obtained via novozym 435, chitin and chitosan immobilized lipases were -56.9, -60.3 and -58 ^{o}C. Tg of polycaprolactone was reported as approximately -60 ^{o}C in literature however Tg also depends on molecular weights of the polymer. Increasing molecular weight of the polymer provides increased glass transition point [58]. Thus, this information justified the above result since molecular weight (Mn) of the polymers obtained via novozym 435, chitin and chitosan immobilized lipase were 14126, 11319 and 12771 g/mol. Also, melting points of

obtained polymers were 61.5, 62.1 and 61.6 $^{\circ}$C. This was consistent with the data in literature [55, 56].

21. Molecular structure of PCL was determined by ^{1}H NMR analysis and FTIR spectrum. Results were consistent with literature and proved that obtained polymers were PCL [12, 31].

22. It was reported that lipase catalyzed ring opening polymerizations has the characteristics of living polymerization [31, 34]. In order to asses this behavior, plot of Mn versus conversion is one of the criteria. If this plot is linear, then it can be said polymerization showed immortal characteristics. Also monomer consumption should follow first order rate law. [31, 34, 62]. Another criterion for living polymerization was increasing molecular weight with time and narrow molecular weight distribution. In this study, it was seen Mn was increasing with time and polydispersities were not higher from 2.0. These results can be characteristics of living polymerization. However in order to provide better understanding, above criteria must be applied in further studies.

23. In this study, monomer conversion was calculated gravimetrically. However, to obtain more precision data, it is recommended to monitor polymerization reactions with ^{1}H NMR since monomer conversion and degree of polymerization can be calculated from reaction mixture taken at determined time.

24. In further studies, crystallinity of resulting polymers can be justified with XRD analysis in addition to DSC results.

25. In order to observe stability of prepared catalysts and test their recyclability, they can be used more than one time and evaluated in polymerization reaction.

26. Lastly, to obtain higher molecular weights of polymer and more rapid polymerization reactions via chitin and chitosan immobilized lipases, toluene ratio can be changed for further studies.

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APPENDICES

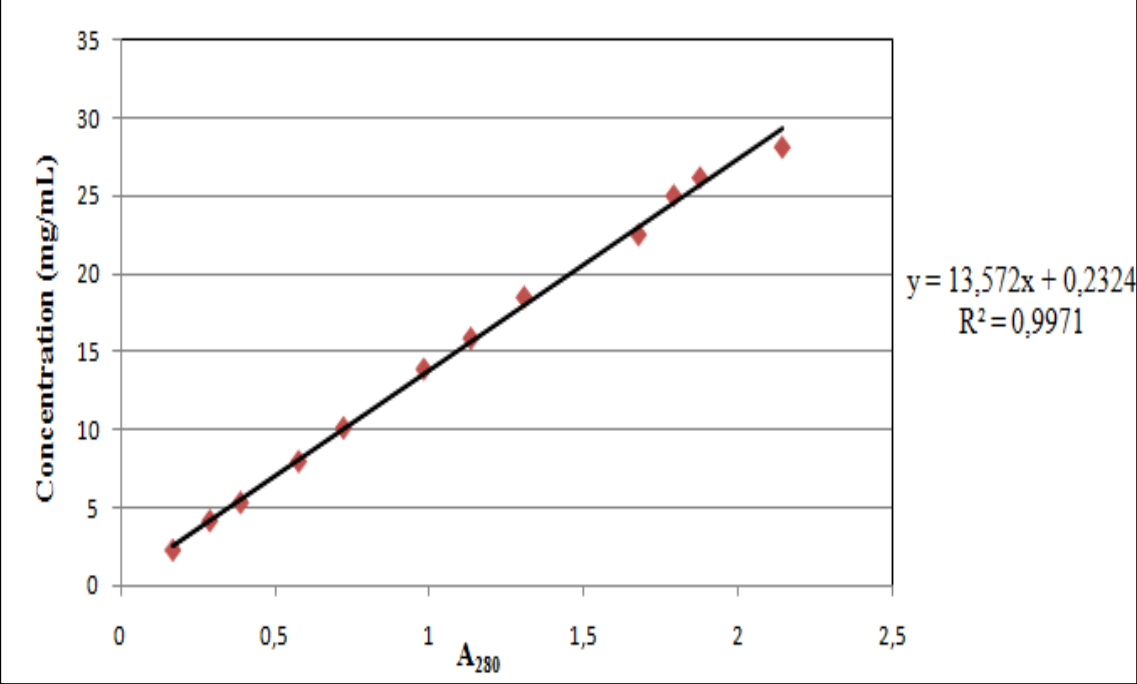**Appendix A.1:** Standard Curve for Lipase Protein Determination

APPENDIX A.1

Figure A.1: Standard curve for protein assay

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