ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IMMOBILIZATION OF LIPASE ON AN INORGANIC SUPPORT MATERIAL AND POLYCAPROLACTONE SYNTHESIS

M.Sc. THESIS

Cansu ÜLKER

Department of Chemical Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

LİPAZIN İNORGANİK TAŞIYICIDA İMMOBİLİZASYONU VE POLİKAPROLAKTON SENTEZİ

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HAZİRAN 2015

Cansu ÜLKER, **a M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 506131006, successfully defended the **thesis** entitled **"IMMOBILIZATION OF LIPASE ON AN INORGANIC SUPPORT MATERIAL AND POLYCAPROLACTONE"**, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 01 June 2015
Date of Defense : 16 June 2015

To my family,

FOREWORD

I would like to start with my deepest gratitude to my supervisor Prof. Dr. Yüksel Güvenilir for her guidance and support all the time. She always encouraged me throughout my thesis study. In addition, I would like to thank for her moral advices and presence behind me starting from my Bachelor's Degree period.

I would also like to thank Nazif Uğur Kaya for his guiding me in laboratory. I am very pleased to meet such an informative lab-mate. In addition, I also appreciate to my lab-mate Nurefşan Gökalp for her support and friendship during our long working periods. I wish both of them success in their academic lives.

I am grateful to Assoc. Prof. Dr. Didem Saloğlu for her kind support to my thesis by providing me FT-IR, DSC, and TGA analyses. In addition, many thanks to chemical engineers Esra Engin and Selin Özen for their helps during SEM analysis. Moreover, I am also thankful to Atilla Toplu for providing me rice husks from Edirne.

I would like to thank to İpek Akkaya, Didem Aycan, Özlem Haval Demirel, Pelin Güzel, Ezgi Bilgiç, Tuğçe İnan, and Ayşe Buse Özdabak for their friendship throughout my thesis period.

It is a pleasure to express my gratitude to my family and valuable parents Cüneyt Ülker and Ulpan Ülker for their great support, encouragement, understanding, and patience. I am also thankful to my dear Yağız Turan for his accompany and love.

JUNE 2015

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ABBREVIATIONS

°2 Th.	**:** °2 Theta
^{1}H-NMR	**:** Hydrogen nuclear magnetic resonance spectroscopy
3-APTES	**:** (3-aminopropyl)triethoxysilane
3-APTMS	**:** (3-aminopropyl)trimethoxysilane
3-GPTMS	**:** (3-glycidoxyptopyl)trimethoxysilane
3-TMSPEDA	**:** (3-trimethoxysilyl)propylethylendiamine
AD	**:** Physically adsorbed
Asp	**:** Aspartic acid amino acid
ca.	**:** Circa
CALB L	**:** Free form of *Candida antarctica* lipase B
CALB	**:** *Candida antarctica* lipase B
$CDCl_3$	**:** Deuterated chloroform
CR	**:** Cross-linked
DSC	**:** Differential scanning calorimetry
ε-CL	**:** ε-caprolactone
FDA	**:** Food and Drug Agency
FT-IR	**:** Fourier transform infrared spectroscopy
GPC	**:** Gel permeation chromatography
His	**:** Histidine amino acid
PCL	**:** Poly(ε-caprolactone)
PDI	**:** Polydispersity index
PGA	**:** Poly(glycolide)
pI	**:** Isoelectronic point
PLA	**:** Poly(lactide)
PLL	**:** Porcine pancreatic lipase
PLLA	**:** Poly(L-lactide)
RHA	**:** Rice husk ash
ROP	**:** Ring opening polymerization
SEM	**:** Scanning electron microscopy
Ser	**:** Serine amino acid
TEM	**:** Transmission electron microscopy
TGA	**:** Thermal gravimetric analysis
THF	**:** Tetrahydrofuran
U	**:** Unit
UV	**:** Ultraviolet
v/v	**:** Volume per volume ratio
w/w	**:** Weight per weight ratio
wt%	**:** Weight percent
XRD	**:** X-Ray diffraction

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$\mathbf{M_{n,NMR}}$	**:** Number average molecular weight calculated from ^{1}H-NMR
$\mathbf{M_n}$	**:** Number average molecular weight
$\mathbf{M_w}$	**:** Weight average molecular weight
$\mathbf{T_d}$	**:** Degradation temperature
$\mathbf{T_g}$	**:** Glass transition temperature
$\mathbf{T_m}$	**:** Melting temperature
$\mathbf{X_c}$	**:** Crystallinity percentage

IMMOBILIZATION OF LIPASE ON AN INORGANIC SUPPORT MATERIAL AND POLYCAPROLACTONE SYNTHESIS

SUMMARY

In recent years, aliphatic polyester synthesis via enzymatic ring opening polymerization (ROP) of lactones has become an attractive research area. There is a considerable interest on utilization of enzymatically synthesized aliphatic polyesters for biomedical applications due to their biodegradability, biocompatibility, and outstanding mechanical properties. In addition, catalysis with enzymes provides achievement of non-toxic materials which is essential for biomedical implementations such as drug delivery systems, tissue engineering, and medical devices.

Polycaprolactone (PCL) is an aliphatic polyester that can be synthesized via ROP of ε-caprolactone (ε-CL). Among other aliphatic polyesters, PCL has a relatively low melting temperature (T_m), between 59-64 ˚C, and low glass transition temperature (T_g), between -60-10 ˚C. Moreover its decomposition temperature is about 350 ˚C which provides high thermal stability. Besides good viscoelastic and rheological features, these thermal properties make PCL to be easily manufactured. In addition, PCL is highly soluble in variety of solvents such as tetrahydrofuran (THF), toluene, chloroform, benzene and dichloromethane and it is compatible with other polymers which makes possible to obtain blends. Furthermore, PCL is a suitable biopolymer for biomedical purposes due to its biocompatibility and biodegradability. When compared with the other biodegradable polymers, PCL degradation process is slower *in vivo*. This long-term biodegradation behavior provides advantage for drug delivery application of PCL. As a result of these advantageous properties, PCL has become an important subject for polymer science and achievement of enzymatic PCL synthesis has received attention for biotechnological applications.

ROP of ε-CL can be catalyzed by both biocatalysts (enzymes) and organometallic initiators. Organometallic initiators such as Zn, Al, Sn, and Ge, cannot be removed completely from polymer matrix at the end of the polymerization which may lead toxic effects when used as a biomaterial. On the other hand by using biocatalysts, this problem can be overcome since they can be removed easily at the end of the reaction and they are non-toxic and eco-friendly catalysts. Moreover, biocatalysts can catalyze polymerization at mild reaction conditions (relatively low temperatures and pressures). However, biocatalysts may lose their activities after long reaction periods and may have stability troubles. Thus, enzyme immobilization is the most widely used strategy to overcome utilization problems of enzymes such as low stability and lack of ability to be reused. By the immobilization of enzymes, enzyme activity and stability can be improved, enzyme can be recovered at the end of the reaction and reused which may provide a potential to enzyme to be used in a continuous process.

Candida antarctica lipase B (CALB) is one of the most efficient and selective lipase that can catalyze esterification and transesterification reactions. Its immobilized form is widely used as a biocatalyst and commercially available as Novozyme 435®. It is known with its effectivity on ROP of ε-CL. However, Novozyme 435® is immobilized on acrylic resin which is mechanically and thermally weak support. Therefore,

development of new carriers became an attractive research area to develop such an effective lipase that can catalyze polymerization reactions as efficiently as Novozyme 435®.

In this work, free CALB (CALB L, Lipozyme®) was immobilized on an inorganic support material, rice husk ash (RHA), since it is a cheap and plentiful (by-product of rice production) material. Furthermore, silica is a widely used support material for enzyme immobilization. Since RHA is rich with SiO_2 content (up to 95%), it is a promising support material. Therefore in this study it was aimed to show the efficiency of lipase immobilized on RHA with an application; catalysis of PCL synthesis. For this purpose, firstly support material was prepared and lipase was immobilized via two methods; physical adsorption and cross-linking. Then, PCL was synthesized with these new immobilized enzymes.

At support preparation and immobilization part, first of all RHA was obtained from the burning of rice husks at 600-650 °C for 6 hours. Then, by the use of a silanization agent 3-aminopropyl triethoxysilane (3-APTES), surface modification of RHA was achieved and functional amine ($-NH_2$) groups were added to the surface. Immobilization of lipase was performed by physical adsorption and cross-linking with glutaraldehyde. For the optimization of new immobilized lipases; different 3-APTES and glutaraldehyde concentrations and enzyme loading ratios were also tried. In addition, storage stability, operational stability, optimum temperature and pH were investigated. Moreover, fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA), and scanning electron microscopy (SEM) were used for the characterizations of RHA, surface-modified RHA, and immobilized lipases.

The second part of this study, which is another novel work, is PCL synthesis via immobilized lipases on surface-modified RHA with physical adsorption (AD) and cross-linking (CR) methods. Polymerization reactions were carried out in toluene which was shown before as an efficient solvent for ROP of ε-CL. For the determination of optimum polymerization conditions of immobilized lipases, polymerizations were performed for various reaction times (6, 24, 48, 72, and 120 hours) at different temperatures (30, 40, 60, and 80 °C). After obtaining the optimum polymerization conditions of immobilized lipases, effect of different enzyme concentrations were investigated and enzyme recycling studies were done. In addition, polymerizations via Lipozyme® and Novozyme 435® were also carried out at these conditions for the comparison of the performances of home-made and commercial enzymes. Molecular weight distributions and chain structures of the polymer samples were compared by gel permeation chromatography (GPC) and hydrogen nuclear magnetic resonance spectroscopy (^{1}H-NMR), respectively. In addition, for the characterization of chain structures, FT-IR was also used. Thermal properties of the polymer samples were obtained by TGA and differential scanning calorimetry (DSC) analysis. To record the crystal structure of polymer samples, X-Ray diffraction (XRD) analysis was carried out. Moreover, for determination of surface structures, SEM was used.

At the end of this study, surface-modified RHA was successfully obtained and addition of $–NH_2$ groups to the surface was showed by FT-IR and TGA analyses. Both AD and CR lipases were achieved with an immobilization efficiency of about 90%. In addition, the activities of AD and CR lipases were measured as 92.3% and 78.8% of the activity of free lipase, respectively. Furthermore, it was found that, specific activities became 1.8 and 2.0 folds greater than the specific activity of free lipase after immobilization via cross-linking and physical adsorption methods, respectively. Moreover, their specific activities were close to the specific activity of Novozyme 435® which has a

specific activity of 2.2 folds greater than free lipase. By using AD lipase, PCL (its structure was verified by FT-IR and ^{1}H-NMR) was synthesized with a molecular weight of 14000 g/mol at the end of 48 hours reaction period at 60˚C. Similarly by the use of CR lipase, 11580 g/mol molecular weight was reached at the end of 24 hours reaction period at 40˚C. Finally, immobilized lipases were recycled six times at their best reaction conditions. At the end of six reaction cycles, 34.1% and 35.7% of their initial activities were remained for AD and CR lipases, respectively. These results indicated that lipase immobilized by cross-linking and adsorption not only had good activity recovery, but also remarkable stability, better reusability and application adaptability than free lipase.

Moreover, in further studies, development of nonohybrids may be possible by grafting CALB immobilized onto inorganic support RHA into PCL matrix. There exists such an successful application with montmorillonite and sepiolite as inorganic support materials in literature.

LİPAZIN İNORGANİK TAŞIYICIDA İMMOBİLİZASYONU VE POLİKAPROLAKTON SENTEZİ

ÖZET

Son yıllarda, enzimatik halka açılması polimerizasyonu ile alifatik poliester sentezi oldukça ilgi gören çalışma alanlarından biri olmuştur. Bunun sebebi olarak da enzimatik olarak sentezlenen poliesterlerin biyomedikal uygulamalarda rahatlıkla kullanılabilmesidir. Biyobozunur, biyouyumlu ve üstün mekanik özelliklere sahip olan bu polimerler enzimatik olarak sentezlendiğinden toksik kimyasal katalizör kalıntısı içermemektedir. Bu da ilaç taşınım sistemleri, doku mühendisliği ve medikal araçlar (implant, protez, ameliyat ipliği, vb.) gibi biyomedikal uygulamalarda kullanımına imkan sağlamaktadır.

Alifatik poliesterlerden biri olan polikaprolakton (PKL), ε-kaprolakton (ε-KL) monomerinin halka açılması polimerizasyonu ile sentezlenebilmektedir. Diğer alifatik poliesterlere kıyasla PKL oldukça düşük erime (59-64˚C) ve camsı geçiş sıcaklığına (-60-10˚C) sahiptir. Bu sayede polimer düşük sıcaklıklarda şekillendirilebilmektedir. Öte yandan, bozunma sıcaklığı ise oldukça yüksek olup 350˚C civarındadır. Bu yüksek bozunma sıcaklığı sayesinde PKL'nin termal stabilitesi oldukça yüksektir. Üstün termal, viskoelastik ve reolojik özellikleri PKL'nin uygulamaları sırasındaki üretim proseslerini kolaylaştırmaktadır. Bunlara ek olarak, PKL birçok çözücüde (tetrahidrofuran, toluene, kloroform, benzen, diklorometan gibi) yüksek çözünürlüğe sahiptir. Ayrıca, diğer polimerlerle uyum gösterebilmesi kompozit eldesini mümkün kılmaktadır. Biyouyumlu ve biyobozunur yapıya sahip olan PKL biyomedikal amaçla kullanıma uygundur. Diğer biyobozunur polimerlere kıyasla vücut içinde polikaprolaktonun bozunma prosesi daha yavaştır. Bu uzun sürede bozunma davranışı PKL'nin ilaç taşınım sistemlerindeki kullanımı için avantaj sağlamaktadır. Tüm bu özellikleri sayesinde PKL polimer bilimi için önemli bir konu olmuş ve biyoteknolojik uygulamalar için enzimatik PKL sentezine olan ilgi artmıştır.

ε-KL'nin halka açılması polimerizasyonu hem biyokatalizörler (enzimler) ile hem de organometalik başlatıcılarla (Zn, AL, Sn, Ge gibi) katalizlenebilmektedir. Fakat organometalik başlatıcılar polimerizasyonun sonunda polimer matrisinden tamamen ayrılamamaktadır. Bu durum toksik etki oluşturacağından dolayı polimerin biyomalzeme olarak kullanımını sınırlandırmaktadır. Öte yandan, biyokatalizörler kullanıldığında reaksiyonun sonunda ortamdan kolaylıkla ayrılabilmekte, ayrılamayan kısım olursa da toksik etki yaratmamaktadır. Biyokatalizörlerin diğer bir avantajı da polimerizasyon reaksiyonunu ılımlı koşullarda (düşük sıcaklık ve basınç) katalizlemeleridir. Fakat biyokatalizörler uzun reaksiyon periyodları sonunda aktivite ve stabilitelerini kaybedebilmektedirler. Bu sorunu çözmek amacıyla enzimlerin immobilize halde kullanımı yaygınlaşmaya başlamıştır. Enzimlerin immobilizasyonu ile enzim aktivite ve stabilitesi arttırılmakta, enzimin tekrar tekrar kullanılması mümkün olmaktadır.

En verimli ve seçici lipazlardan biri olan *Candida antarctica* lipase B (CALB), esterifikasyon ve transesterifikasyon reaksiyonlarını katalizleyebilmektedir. Bu

enzimin immobilize formu olan Novozyme 435®, özellikle ε-KL'nin halka açılması polimerizasyonundaki etkinliği ile tanınmaktadır. Fakat Novozyme 435® mekanik ve termal açıdan dayanıksız bir taşıyıcı olan akrilik reçine üzerine immobilize haldedir. Bu sebeple, Novozyme 435® gibi özellikle polimerizasyon reaksiyonlarında etkili olabilecek bir lipaz eldesi için yeni taşıyıcıların geliştirilmesi son zamanlarda ilgi gören çalışma konularından olmuştur.

Bu çalışmada, serbest CALB (CALB L, Lipozyme®) literatürde daha önce hiç örneği olmayan bir inorganik taşıyıcıya, pirinç kabuğu külüne, immobilize edilmiştir. Pirinç kabuğu pirinç üretim prosesinin yan ürünü olarak açığa çıkan, oldukça bol ve diğer taşıyıcılara göre ucuz bir madde olduğundan lipaz immobilizasyonu için tercih edilmiştir. Silika enzim immobilizasyonunda yaygın olarak kullanıldığından ve pirinç kabuğu külünün de SiO_2 içeriği (% 95 'e kadar) oldukça yüksek olduğundan pirinç kabuğu külü lipaz immobilizasyonu için umut verici bir taşıyıcı olduğu sonucuna varılmıştır. Bu sebeple bu çalışmada pirinç kabuğu külüne immobilize lipazın verimliliği bir uygulama üzerinden gösterilmek istenmiştir. Bu amaçla, ilk olarak taşıyıcı madde hazırlanmış ve lipaz immobilizasyonu gerçekleştirilmiştir. Daha sonra ise, bu yeni immobilize lipazlar ile PKL sentezlenmiştir.

Taşıyıcının hazırlanması ve lipaz immobilizasyonu kısmında, ilk olarak pirinç kabukları 600-650 °C'de 6 saat yakılarak pirinç kabuğu külleri elde edilmiştir. Daha sonra, bir silanlama ajanı olan (3-aminopropil)trietoksisilan (3-APTES) kullanılarak pirinç kabuğu külünün yüzeyi modifiye edilmiştir. Bu modifikasyon sonunda yüzeyde fonksiyonel amin ($-NH_2$) grupları elde edilmiştir. Lipazın immobilizasyonu fiziksel adsorpsiyon ve glutaraldehit ile çapraz bağlama olmak üzere iki yöntem ile gerçekleştirilmiştir. Yeni immobilize lipazların optimizasyonu için; farklı 3-APTES ve glutaraldehit konsantrasyonları ve protein yükleme oranları denenmiştir. Bunlara ek olarai raf ömrü, tekrarlı kullanım stabilitesi, optimum sıcaklık ve pH değerleri araştırılmıştır. Ayrıca, pirinç kabuğu külünün, yüzeyi modifiye edilmiş pirinç kabuğu külünün ve immobilize lipazların karakterizasyonu için fourier transform kızılötesi spektroskopisi (FT-IR), termal gravimetrik analiz (TGA) ve taramalı elektron mikroskobu (SEM) kullanılmıştır.

Bu çalışmanın ikinci kısmında yeni immobilize enzimler ile PKL sentezlenmiştir. Polimerizasyon reaksiyonları daha önceden ε-KL'nin halka açılması polimerizasyonu için verimli olduğu gösterilmiş olan toluen ortamında gerçekleştirilmiştir. Enzimlerin optimum polimerizasyon koşullarının belirlenmesi amacıyla, polimerizasyon reaksiyonları farklı reaksiyon süreleri boyunca (6, 24, 48, 72 ve 120 saat) farklı polimerizasyon sıcaklıklarında (30, 40, 60, and 80 °C) gerçekleştirilmiştir. Her iki enzim için de en iyi polimerizasyon koşullarının belirlenmesinden sonra, farklı enzim konsantrasyonlarının etkisi ve enzimlerin tekrar kullanılabilirliği üzerine çalışmalar yapılmıştır. Ayrıca, yeni immobilize edilmiş olan lipazları ticari lipazlar ile karşılaştırmak amacı ile Lipozyme® ve Novozyme 435® ile de belirlenmiş olan en iyi reaksiyon koşullarında PKL sentezi gerçekleştirilmiştir. Polimer örneklerinin molekül ağırlığı dağılımları jel geçirgenlik kromatografisi (GPC) ile, zincir yapıları proton nükleer manyetik rezonans spektroskopisi (^{1}H-NMR) ve FT-IR ile, termal özellikleri TGA ve diferansiyel taramalı kalorimetri (DSC) analizi ile, yüzey yapıları ise SEM ile karakterize edilmiştir. Ayrıca polimer örneklerinin kristal yapısının belirlenmesi amacı ile X-ışını kırınımı (XRD) analizi uygulanmıştır.

Sonuç olarak, pirinç kabuğu küllerinin yüzey modifikasyonunun başarı ile sağlandığı ve yüzeyde $–NH_2$ gruplarının elde edildiği FT-IR ve TGA analizleri ile gösterilmiştir. Hem fiziksel adsorpsiyon hem de çapraz bağlama yöntemleri ile yaklaşık % 90'lık bir

immobilizasyon verimi elde edilmiştir. Buna ek olarak, fiziksel adsorpsiyon ile immobilizasyon sonucunda serbest lipazın % 92.3'lük, çapraz bağlama ile immobilizasyon sonucunda ise % 78.8'lik aktivitesi korunmuştur. Öte yandan, çapraz bağlama ile immobilizasyon sonucunda serbest lipazın spesifik aktivitesinin 1.8 katı, fiziksel adsorpsiyon ile immobilizasyon sonucunda ise 2 katı spesifik aktivite elde edilmiştir. Yeni immobilize edilmiş olan bu lipazların spesifik aktivitelerinin Novozyme 435®'in spesifik aktivitesine (serbest lipazın 2.2 katı) yakın olduğu sonucuna varılmıştır. Fiziksel adsorpsiyon yöntemi ile immobilize edilmiş olan lipaz kullanılarak sentezlenen PKL'nin (Polimer örneğinin PKL olduğu FT-IR ve ^{1}H-NMR ile gösterilmiştir.) molekül ağırlığı 60 $^{\circ}$C'de gerçekleştirilen 48 saatlik reaksiyon sonunda 14000 g/mol olarak elde edilmiştir. Aynı şekilde çapraz bağlama yöntemi ile elde edilen immobilize lipaz kullanıldığında ise 40 $^{\circ}$C'de gerçekleştirilen 24 saatlik reaksiyon sonunda 11580 g/mol molekül ağırlığına sahip PKL sentezlenmiştir. Son olarak, aynı enzimler arka arkaya altı kez PKL sentezinde kullanılmıştır. altıncı reaksiyon sonunda fiziksel adsorpsiyon yöntemi ile immobilize lipazın aktivitesinin % 34.1'i, çapraz bağlama yöntemi ile immobilize lipazın aktivitesinin ise %35.7'si korunmuştur. Elde edilen bu sonuçlar, her iki yöntem ile immobilize edilmiş olan lipazların kayda değer bir stabiliteye sahip olduğunu ve serbest lipaza göre uygulamalara adaptasyonunun daha iyi olduğunu göstermiştir.

Öte yandan, daha ileri bir çalışma olarak, inorganik bir taşıyıcı olan pirinç kabuğu külüne immobilize edilmiş olan lipaz enzimleri sentez sonrasında ortamdan ayrılmayarak PKL matrisi içerisine gömülerek nanohibritler elde edilebilir. Literatürde lipaz taşıyıcısı olarak montmorillonit ve sepiyolitin kullanılmış olduğu bir nanohibrit sistem başarıyla gerçekleştirilmiştir.

1. INTRODUCTION

Nowadays, biotechnology has become one of the most attractive topics. Biomaterial production is a leading application of biotechnology. Biomaterials can be used for; controlled drug release systems, tissue and organ transplantations, and orthopedic applications. Being biocompatible, non-toxic, and non-carcinogenic are the properties required for a fine biomaterial, besides having high chemical stability and mechanical resistance. In addition, biomaterials are biodegradable which makes them environmentally friendly materials [1].

Biopolymers are light and flexible biomaterials, which are more compatible to body when compared with other materials, such as biocompatible metals [1]. Biopolymers can be synthesized both chemically and enzymatically. In recent years, there exists an increasing interest on enzymatic polymerization, because of mild reaction conditions, high catalytic activity, and low toxicity of enzymes. Moreover, in enzymatic polymerization, there is no side reactions and no purification cost for undesired product [2, 3]. On the other hand, it is difficult to separate organometallic catalysts used in chemical synthesis from the product. It may cause toxic effect, if the biopolymer synthesized will be used in biomedical area [2, 3].

Poly (ε-caprolactone) (PCL) is a widely used in biomedical applications, since it is a biodegradable, biocompatible and permeable biopolymer [2, 3]. PCL can be synthesized via ring opening polymerization (ROP) by both chemical and enzymatic catalysis. In general, organometallic compounds are used for chemical synthesis and lipases are used for enzymatic synthesis. Lipase, which is responsible from hydrolysis of triglycerides and phospholipids, has also catalytic activity in esterification and transesterification reactions. Therefore, lipase is an appropriate biocatalyst for PCL synthesis [4]. In literature, Novozyme 435®, an immobilized form of *Candida antarctica* lipase B (CALB) is successfully used for PCL synthesis [5]. Immobilization of enzymes to a solid support materials has a great interest nowadays, due to their improved activity, stability, and selectivity. In addition, immobilized enzymes can be recovered at the end of the reaction and reused, which may overcome the high costs of

enzymes and increase their industrial usage [6, 7]. Therefore, it is important to improve new immobilized lipases for PCL synthesis as alternatives for Novozyme 435® by investigating new support materials.

In this study, rice husk ash (RHA) was used as an inorganic support material for the immobilization of free CALB (CALB L), with its commercial name Lipozyme®, since RHA is a cheap and plentiful material. Silica is a widely and successfully used support material for enzymes. Moreover, RHA has a high SiO_2 content (up to 95%), which makes RHA a promising support material for enzyme immobilization [8, 9]. The support material was achieved from burning of rice husks at 600-650 °C for 6 hours. After that, surface of RHA was modified by a silanization agent 3-aminopropyl triethoxysilane (3-APTES) and functional amine ($-NH_2$) groups were obtained. For the immobilization of lipase on surface-modified RHA, two immobilization methods were used; cross-linking and physical adsorption. Glutaraldehyde which is a common coupling agent was used as cross-linker. To optimize the newly synthesized immobilized lipase; different 3-APTES and glutaraldehyde concentrations and enzyme loading ratios were also tried. In addition, storage stability (shelf life), operational stability (recycling and reuse), and optimum temperature and pH were investigated. For characterizations of RHA, surface-modified RHA, and immobilized lipases; fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA), and scanning electron microscopy (SEM) were used.

In the second part of this study, PCL was synthesized via enzymes immobilized on surface-modified RHA by cross-linking and physical adsorption. Synthesis was carried out in toluene which was shown before as efficient for ring opening polymerization of ε-caprolactone (ε-CL) by Novozyme 435® [10]. Polymerizations were performed for various reaction times (6, 24, 48, 72, and 120 hours) at different temperatures (30, 40, 60, and 80 °C). By these serial reactions, optimum reaction time and temperatures were determined for both two type of immobilized enzymes. At these optimum reaction conditions, effect of different enzyme concentrations were investigated and enzyme recycling studies were done. In addition, polymerizations via Lipozyme® and Novozyme 435® were also carried out at the optimum reaction conditions of new enzymes to compare the performances of home-made and commercial enzymes. Molecular weight distributions and chain structures of the polymer samples were compared by gel permeation chromatography (GPC) and

hydrogen nuclear magnetic resonance spectroscopy (^{1}H-NMR), respectively. In addition, for the characterization of chain structures, FT-IR was also used. Thermal properties of the polymer samples were obtained by TGA and differential scanning calorimetry (DSC) analysis. To record the crystal structures of polymer samples, X-Ray diffraction (XRD) analysis was carried out. Moreover, for determination of surface structures, SEM was used.

2. THEORETICAL STUDY

2.1 Lipase Enzyme

Lipases (EC3.1.1.3), triacylglycerol acyl hydrolases, are enzymes which have hydrolytic activity on carboxylic ester bonds. They are responsible from hydrolysis of triglycerides into fatty acids and glycerol. However, they also have the ability of catalyzing esterification and transesterification reactions in non-aqueous media. Lipases have a α-helical oligopeptide chain which covers their active site (lid) and prevents the access of substrate. When there is no hydrophobic interface present, the active site of the enzyme is hidden from the reaction medium. This conformation of enzyme is called as "closed conformation". On the other hand, when there exists a hydrophobic interface, the enzyme goes on a conformational change to expose its catalytic triad to the hydrophobic phase. This conformational change of enzyme results in "open conformation". Thus, the activation process of lipase is named as "interfacial activation" [6]. The closed and open forms of lipase are shown in Figure 2.1 [11].

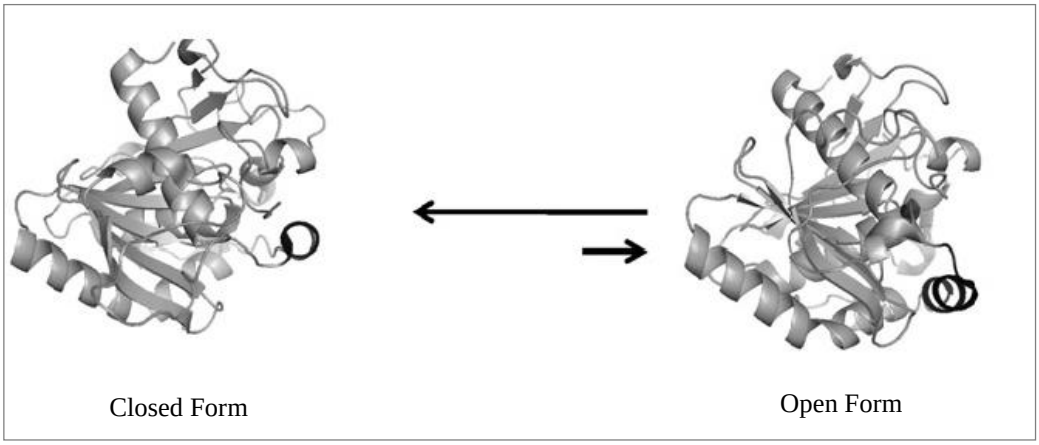

Figure 2.1: Closed and open forms of lipase.

Lipase has high selectivity (regio-, enantio-, stereo- and chemo-selectivities), the ability of catalysis in interface between aqueous and organic phases, and the ability of interacting with various substrates [12, 13]. As a result of this specific behavior of lipase, it is possible to develop lipase-catalyzed polymerization reactions. Aliphatic

polyesters or polycarbonates can be produced by lipases-catalyzed ROP of lactones, lactides, and cyclic carbonates [14].

Lipases obtained from different organisms and used for polyester synthesis can be seen from Table 2.1 [3]. CALB is the most effective one. Its molecular weight is 33 kDa with 317 amino acid residues and its isoelectric point (pI) is 6.0 [15, 16]. The enzyme has a size of 30x40x50˚A and its catalytic triad is composed of serine-histidine-aspartic acid (Ser-His-Asp) amino acids [17].

Table 2.1: Lipases used in polyester synthesis.

Organism	**Lipases**
Mammalian	Porcine pancreatic lipase (PLL)
Fungal	*Candida antarctica* (CALB), *Candida rugosa*, *Aspergillus niger, Penicillium roruefortic*, *Rhizopus delemar, Rhizomucor miehei*, *Candida cylindracea* lipases
Bacterial	*Pseudomonas cepacia*, *Pseudomonas flourescens* lipases

CALB is produced by submerged fermentation of a genetically modified *Aspergillus niger* microorganism. It is immobilized on macro porous acrylic resin and commercially available as Novozyme 435® [18]. In this study, free CALB was immobilized on a different support to obtain an alternative for Novozyme 435®.

2.2 Enzyme Immobilization

There is an increasing interest on enzymatic conversion and they are industrially used in various biotechnological applications such as production of specialty chemicals and enantiomerically pure pharmaceuticals and synthesis of some organics. But, utilization of enzymes are limited generally because of their low stability and lack of ability to be reused [19]. In addition, catalytic activity of enzymes have a tendency of decrement when they are used in organic solvents rather than aqueous environments [20]. To overcome these problems, there have been many studies including enzyme immobilization, enzyme modification, and genetic modification. Enzyme immobilization, which is defined as restriction of enzyme mobility in various matrices,

is the most widely used strategy since it has various advantages [19, 21]. By the immobilization of enzymes, enzyme activity and stability can be improved, enzyme can be recovered at the end of the reaction and reused which may provide a potential to enzyme to be used in a continuous process [7, 9]. However, enzyme and substrate mobility can be restricted by immobilization of enzymes [22]. Therefore, it is important to optimize the immobilization methods, conditions, and supports based on enzyme characteristics.

The methods used for enzyme immobilization can be divided into two main groups, which are chemical and physical methods. These methods can be seen in Figure 2.2 [22].

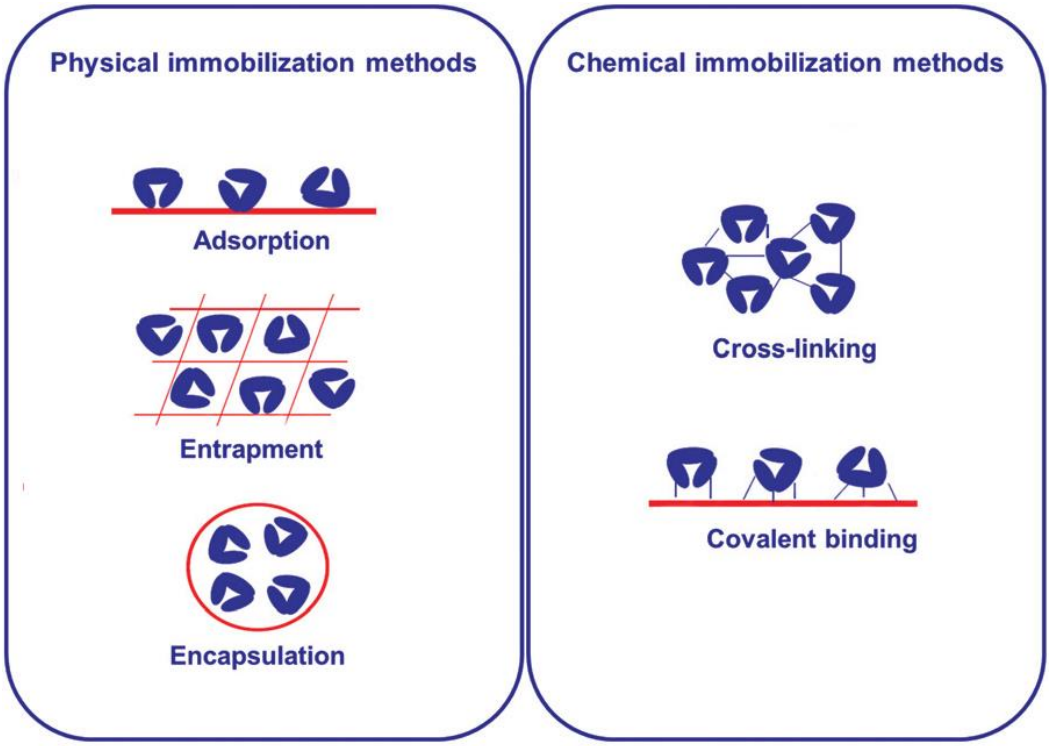

Figure 2.2: Immobilization methods of enzymes.

Physical immobilization is formed by weak interactions. The physical methods are; physical adsorption, enzyme entrapment, and encapsulation [22]. Physical adsorption method is simpler, more low-cost and retains higher enzyme activity when compared with other physical and chemical methods. Enzymes are not damaged during immobilization process since no chemicals are used. Therefore, it has a higher commercial potential. However, enzyme is bound to the support with relatively weak bonds such as Van der Waals forces, ionic and hydrogen bonding interactions [9, 23].

Enzymes may desorb easily from the support during washing process or reaction [9]. Moreover, non-specific binding, overloading on the support, and steric hindrance by the support may be the other problems encountered when the enzyme is immobilized by physical adsorption. Entrapment is another physical immobilization method in which free enzymes in a solution are surrounded by lattice structure of a gel. In this type of immobilization, leakage problem of enzymes is not observed and the lattice structure acts as a barrier to mass transfer by protecting enzymes from harmful substances. Encapsulation is similar to entrapment of enzymes since enzymes are free and surrounded by a support. Semipermeable membranes are used for the encapsulation of enzymes [23].

In chemical methods, covalent bonds are formed. The chemical methods are; covalent attachment and cross-linking. In covalent attachment, covalent bonds are formed between the functional groups of enzymes (with side chains of amino acids such as arginine, aspartic acid, and/or histidine) and the water-insoluble support [22, 24]. However, the amino acid residues included in the catalytic triad must not be covalently bound to the support in order to obtain high activity [25]. The reuse capacity of enzyme immobilized by covalent binding is higher than physically immobilized enzyme since it is strongly bound to the support [26]. This method is generally preferred when it is strictly necessary to obtain a pure product without any enzyme. Activation and addition of functional groups to the surface of the support material is an also important procedure for covalent bond formation [25]. Surface modification is going to be introduced in next sections. Lastly, in cross-linking method, a multifunctional reagent such as glutaraldehyde is used to form cross-linked enzyme aggregates [22, 24].

Lipases are generally immobilized on supports by adsorption through hydrophobic interactions since it is low-cost and enhances enzyme activity. For example, the most commonly used commercial enzyme Novozyme 435® is obtained by physical adsorption of CALB on porous acrylic resin [14, 21, 27].

2.2.1 Support materials for enzyme immobilization

Selection of appropriate support material for immobilization is as important as selection of immobilization method to obtain a high-performance immobilized enzyme. Inertness, physical strength, stability, reusability, regenerability, biocompatibility, resistance to microbial attacks, and availability with a low cost are

the properties needed for a support material to be ideal for enzyme immobilization. Moreover, it should has the ability of improving enzyme activity and specificity [22, 25]. Support materials are categorized into two groups as inorganic and organic based on their chemical composition. It can be seen from Table 2.2 that, there are two types of organic supports; natural and synthetic polymers. There are also examples of each type of supports are given in Table 2.2 [13, 24, 25].

Table 2.2: Classification of supports used for enzyme immobilization.

Chemical composition	**Example**
Organic	Natural polymers: Polysaccharides (cellulose, dextrans, agar, agarose, chitin, chitosan, alginate), proteins (collagen, albumin) Synthetic polymers: Polystyrene, polyacrylate, polymethacrylates, polyacrylamide, polyamides, vinyl, and allyl-polymers
Inorganic	Zeolites, ceramics, celite, silica, bentonite, clay, alumina, glass, activated carbon

Many of the supports given in Table 2.2 are used in lipase immobilization. Among them, silica is the widely used one for lipase immobilization since it has very high specific surface area, high biocompatibility, and it is low-cost material. In addition, its surface can be easily modified [9].

The most widely used commercial lipase Novozyme 435® is immobilized on acrylic resin which is mechanically and thermally weak support. Therefore, development of new carriers became an attractive research area to develop such a lipase that can catalyze polymerization reactions as efficiently as Novozyme 435®. For example, it was reported in a study that, CALB was immobilized on montmorillonite and sepiolite clays and then used as catalyst for ROP of ε-CL [14]. Organic supports such as chitin and chitosan have also been successfully used for immobilization of CALB. The resulted immobilized lipases have shown catalytic activity for PCL synthesis [28].

2.2.1.1 Rice husk ash (RHA) as a support material for lipase immobilization

Rice husk is generated from rice (23% of initial weight) as a by-product [8]. 20% of the rice husks is composed from inorganic substances and the rest is organic materials.

Rice husks contain; 24.3% hemicellulose, 34.4% cellulose, 19.2% lignin, 18.85% ash, and 3.25% else substances. The elemental composition of rice husks is (in weight); 37.05% C, 8.80% H, 11.06% N, 35.03% O, and 9.01% Si [29]. Rice husks can be used in agriculture as a fertilizer or in cement production as an additive. Moreover, elementary silicon and some silicon compounds such as silica, silicon carbide, and silicon nitride can be produced from rice husk as a result of its high silicon content. By burning rice husk at about 700˚C, RHA which has a color varying from grey to black is obtained. This color difference is resulted from inorganic impurities and unburned carbon amounts. The obtained RHA after burning process has a high specific surface area and high silica content (about 95%) [8, 30]. Since RHA is rich in silica content and shows amorphous silica properties based on X-ray diffraction and infrared spectrum results, it can be used as a porous support for lipase immobilization [31]. From left to right rice husks and rice husk ashes are shown in Figure 2.3.

Figure 2.3: Rice husks and rice husk ashes.

2.2.2 Addition of organofunctional groups to support surface

Silica is composed of siloxane (Si-O-Si) and silanol (Si-OH) groups. Silanol groups are distributed on the surface whereas siloxane groups are located in the inside parts. Chemical modifications are applied to the silanol groups on the surface. Modification of surfaces is accomplished with silanization by organosilane agents. Trifunctional silanes such as 3-aminopropyl trimethoxysilane (3-APTMS), 3-aminopropyl triethoxysilane (3-APTES), and 3-glycidoxyptopyl trimethoxysilane (3-GPTMS) are resulted in a more stable and uniform layer of immobilized enzyme [32, 33, 34]. This process is called as silanization if the organosilane agent reacts with –OH groups of silanol. However, these agents can react also with any other hydroxyl group included on the surface of another type of support different from silica. In this process, a spacer

arm is introduced between the enzyme and the support which enables the access of immobilized enzyme to its substrate [34].

By organofunctionalization process, depending on the type of agent, amine (-NH_2) or epoxy groups are obtained. For example, by using 3-APTMS or 3-APTES amine groups, and by using 3-GPTMS epoxy groups can be produced on the surface of the support. Silanization processes for a porous silica particle by 3-APTES and 3-GPTMS are given in Figure 2.4. The activated silica particles with added functional groups can be seen from this figure [35, 36].

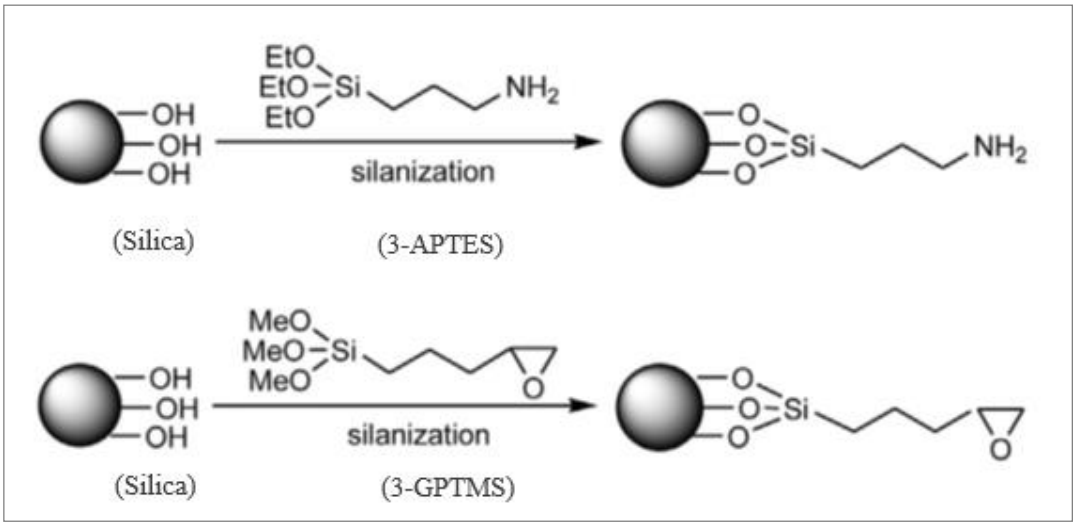

Figure 2.4: Silanization by 3-APTES and 3-GPTMS.

After activation of silica particles with organosilane agents, enzyme can be covalently immobilized on these particles. But, as a result of monolayer coverage of enzymes, enzyme loading capacity is limited by covalent binding. To overcome this problem and increase enzyme loading capacity of the support, a cross-linker such as glutaraldehyde is used to link enzyme aggregates in the solution to the activated silica particles. By this two-step immobilization method, higher enzyme activity and stability is achieved [37]. When the organofunctional group is –NH_2 rather than epoxy, one of the aldehyde groups of glutaraldehyde reacts with this –NH_2 group of activated silica. The other aldehyde group reacts with the –NH_2 group of the enzyme molecule and -CH = N- bond is formed. This reaction is called as "Schiff base reaction". At the end of this process, enzyme becomes immobilized on organofunctionilized silica particle by a cross-linking method [26, 34]. This process is summarized in Figure 2.5 with an example when 3-APTMS was used as silanization agent [34].

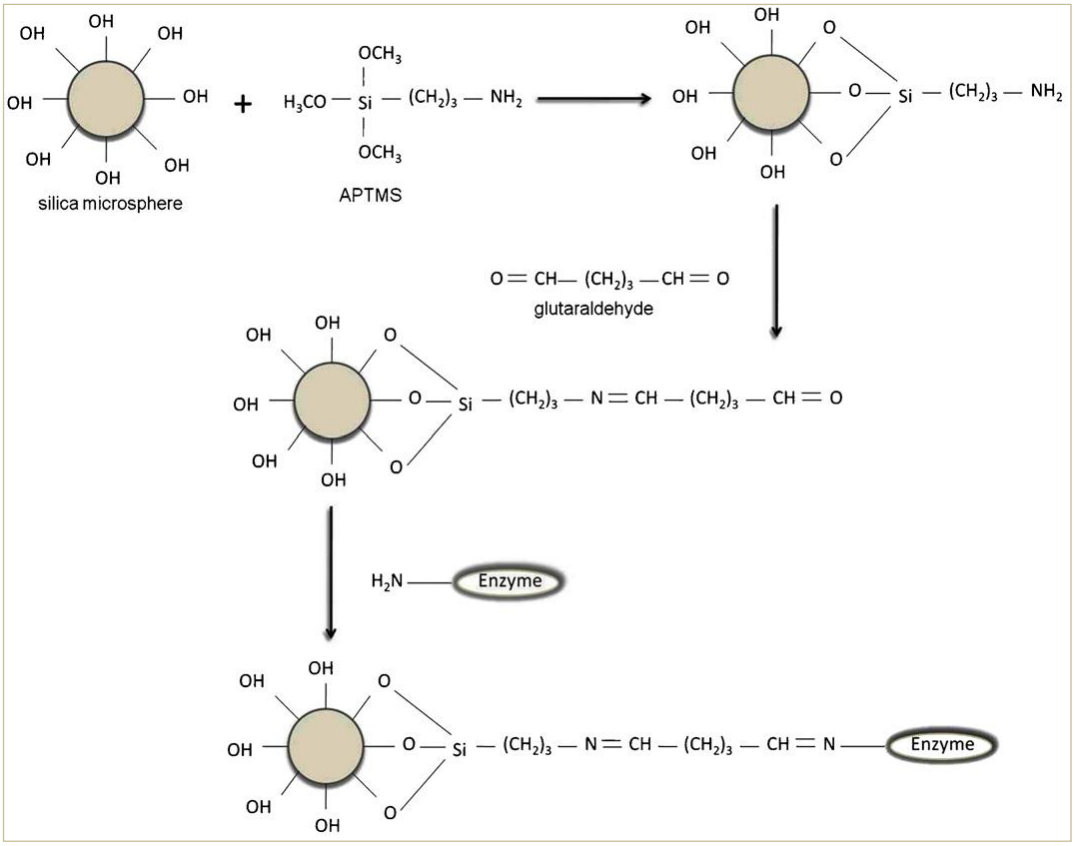

Figure 2.5: Two-step immobilization process by cross-linking.

Different from this procedure, in the case of epoxy-activated silica, nucleophilic groups on the surface of the enzyme can directly react with the epoxy groups on the surface of activated silica particle. As a result of this reaction, O-C and N-C bonds are formed between the enzyme and activated silica [33, 35]. Immobilization of enzyme by covalent attachment to the epoxy activated silica is given in Figure 2.6 [35].

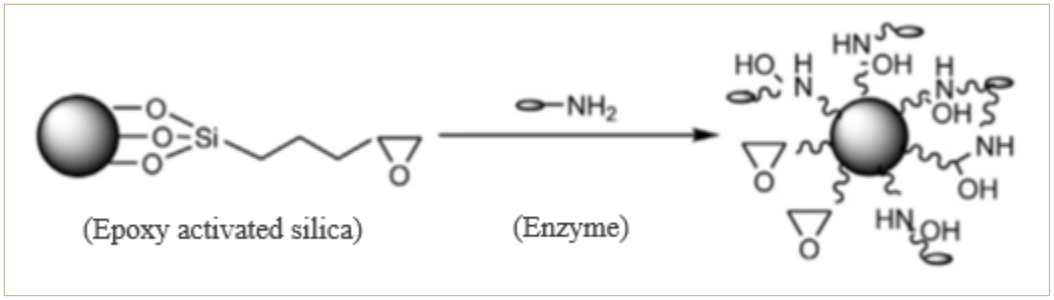

Figure 2.6: Immobilization on epoxy activated silica.

These methods can be applied to lipase immobilization. In a study, lipase from *Rhizopus oryzae* was immobilized on silica gel such a procedure introduced. Surface modification of silica gel was achieved by using three types of agents which were 3-APTES, 3-APTMS, and 3-trimethoxysilyl propylethylendiamine (3-TMSPEDA).

Glutaraldehde was used as a cross-linker. At the end, cross-linked lipase on surface-modified silica gel was obtained. It is reported that, 3-APTES resulted in the best results such as high protein binding yield and high immobilized enzyme activity [26].

2.3 Poly (ε-caprolactone)

PCL is a polyester which is a biomacromolecule and synthesized in living systems enzymatically. Based on this process, enzymatic synthesis of biopolymers has become a new trend in recent years since it is a green technique [3, 38]. PCL can be synthesized by ring opening polymerization of ε-CL with enzymes or organometallic catalysts. The structure of ε-CL monomer is given in Figure 2.7 [39].

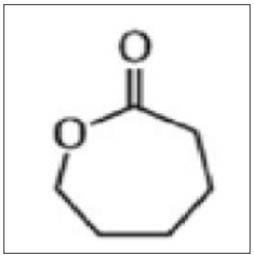

Figure 2.7: Structure of ε-caprolactone.

PCL was firstly synthesized by the Carothers Group in early 1930s [40]. PCL became commercially widespread after finding out its biodegradability in 1973 [41]. In addition, PCL attracted attention beginning from 1970s since it has advantages on other biopolymers, such as adjustable degradation kinetics and mechanical properties, easy to shape structure [40].

PCL is a hydrophobic and semi-crystalline linear aliphatic polyester. Its molecular weight varies between 3,000-100,000 g/mol and crystallinity is inversely proportional with molecular weight [1]. Depending on its crystal structure, melting point (T_m) and glass transition temperature (T_g) ranges between 59-64˚C and -60-10˚C, respectively. In addition, PCL has a decomposition temperature about 350˚C which provides high thermal stability [42]. It has a high solubility in tetrahydrofuran (THF), toluene, chloroform, benzene and dichloromethane at room temperature. PCL has a lower solubility in solvents such as, acetonitrile, ethyl acetate, and dimethylformamide. On the other hand, it is insoluble in alcohol, petroleum ether, and diethyl ether [41]. The repeating molecular structure of PCL homopolymers consist of five non-polar methylene groups and a polar ester group. High olefinic nature of PCL provides similar

mechanical properties as polyolefins have, whereas existence of unstable aliphatic ester linkages makes PCL biodegradable. This molecular structure of PCL makes it compatible with other polymers and possible to form polymer blends [43]. Low T_m and T_g values, good solubility and blending capacity with other polymers promoted researches about PCL on biomedical field [42]. Moreover, it can be easily manufactured because of its easy formability at low temperatures and good viscoelastic and rheological properties. Therefore, large scale production of PCL is possible for biomedical purposes [40, 44].

For the biomedical purposes, one of the important properties of PCL is biodegradable nature of the polymer. PCL can be degraded by living organisms such as bacteria and fungi in environment. In human and animal bodies, degradation process is slower. This long-term biodegradable behavior in human bodies provides advantage for PCL to be used in drug-delivery systems. As it is shown in Table 2.3, biopolymers such as, poly (lactide) (PLA), poly (glycolide) (PGA) and their copolymers' degradation is fast when compared with PCL. In Table 2.3, other properties such as, T_m and T_g values can also be seen. Another important property of PCL is its biocompatibility. Biocompatibility of PCL makes possible this biopolymer to be used as a medical device or as drug-delivery systems, since an appropriate host response can be obtained [40].

Table 2.3: Comparison of PCL and some other degradable polymer properties.

Polymer	**Tm (˚C)**	**Tg (˚C)**	**Degradation Time (months)**
PLA	173-178	60-65	6-12
PGA	225-230	35-40	>24
PCL	60-65	-60-10	>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

2.4 Poly (ε-caprolactone) Synthesis

PCL can be synthesized by ROP of ε-CL or by free radical polymerization of 2-methylene-1-3-dioxepane [40]. In this section, ROP of ε-CL will be introduced by classifying the catalysis type either chemical or enzymatic.

2.4.1 Chemically catalyzed ROP of ε-caprolactone

For the chemically catalyzed synthesis of PCL, metallic compounds such as metal alkoxide and alkylmetals (alkyl tin and alkyl lanthanides) are used generally. They are used as initiators and chain growth is provided by coordinative insertion of the monomer, which is cleaved from acyl-oxygen bond, into the metal-oxygen bond [45]. The mechanism of ROP of cyclic esters by metallic compounds can be seen from Figure 2.8, where I_0 represents initiator and M represents metal [46].

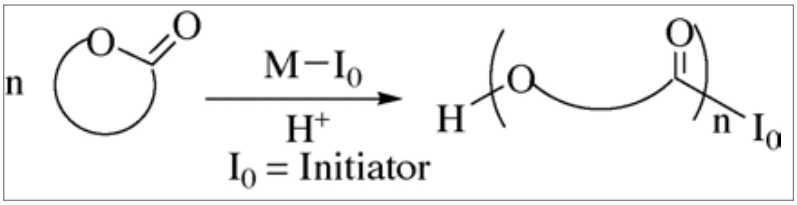

Figure 2.8: The mechanism of ROP of cyclic esters by metallic compounds.

Industrial polymerization of ε-CL is catalyzed by tin octoate, $Sn(Oct)_2$, in the presence of a higher alcohol which is used as an initiator. The industrially applied mechanism is given in Figure 2.9. As seen from the figure, 1-dodecanol is generally used as initiator alcohol [47]. Furthermore, $Sn(Oct)_2$ has been accepted as a food additive by the US Food and Drug Agency (FDA). Therefore, there is no need for purification of polymers when they are used for food applications, such as packaging [48].

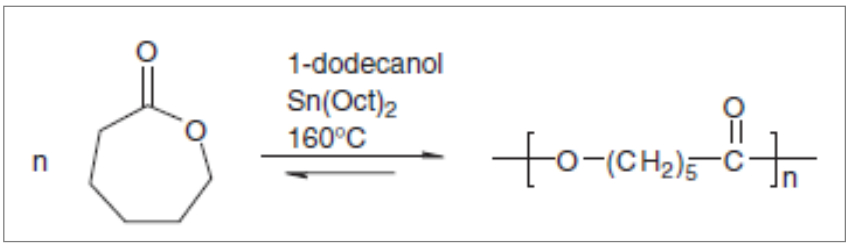

Figure 2.9: Industrial PCL synthesis mechanism.

Using bioresorbable salts, which are free from toxic metallic residues, for the ROP of cyclic lactones is another approach for aliphatic polyester production. These catalysts are including cations such as; Na^+, K^+, Mg^{+2}, Ca^{+2}, Zn^{+2}, and Fe^{+2}. These metals are

called as “friendly metals”. Various counter ions, such as chloride, ionide, oxide, hydroxide, carbonate, acetate, lactate, tartrate, citrate, etc., have been studied for these cations. Among them, zinc acetate was found as a friendly analogue of $Sn(Oct)_2$ for ROP of ε-CL [48].

Although some chemical catalysts provided efficient polymerization of lactones and can be used in food applications, they are not suitable for biomedical applications. Therefore, it is essential to improve enzymatic polymerization [48].

2.4.2 Enzymatically catalyzed ROP of ε-caprolactone

PCL is preferred for biomedical applications since it has properties such as high mechanical stability, biodegradability, and biocompatibility. However, organometallic catalysts such as Zn, Al, Sn, and Ge are used for PCL synthesis generally. These catalysts may be toxic for biomedical applications since they cannot be easily removed from the product. Furthermore, for a biomedical application it is necessary to obtain completely pure and catalyst-free product. Therefore, biocatalysts, which can be easily separated from the product and non-toxic, should be preferred in biomedical field [2, 3, 49]. Other advantages of enzyme catalyzed synthesis are [3, 4, 49]:

- Enzymatic reactions are performed under mild conditions (low temperature and pressure).
- By using enzymes, well-defined polymers can be obtained.
- Enzymes are recyclable catalysts since they can be separated from the product.
- Enzymes are natural catalysts and derived from renewable resources.
- Enzymes have high enantio- and regio- selectivity.
- Enzymatic reactions can be carried out in bulk, organic, or aqueous media.
- Large ring lactones can be polymerized efficiently by enzymes, whereas low molecular weights can be obtained when organometallic catalysts are used.
- Enzymes do not require strict precautions such as exclusion of water and air for the ROP of lactones.

ε-CL is the most extensively studied type of lactones which can be polymerized by lipase catalyzed ROP [48]. The purposed reaction mechanism for lipase catalyzed ROP of ε-CL is shown in Figure 2.10 [1].

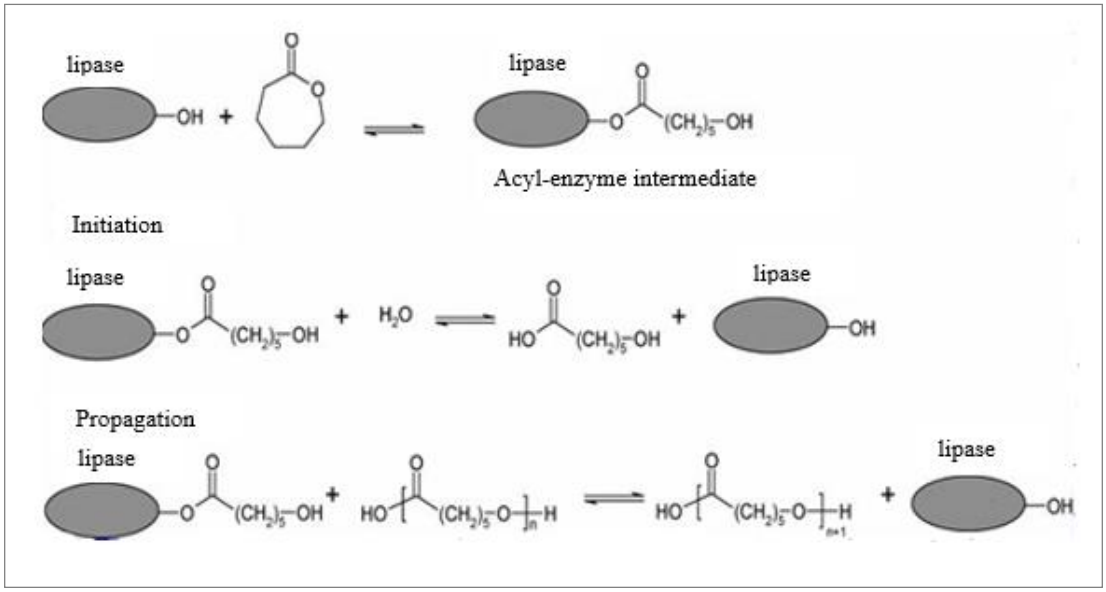

Figure 2.10: Reaction mechanism for ROP of ε-CL.

Firstly, ε-CL monomer reacts with the –OH group of serine amino acid which is included at the active site of the lipase and acyl-enzyme intermediate is obtained. This step, in which the lactone ring is opened, is rate-limiting. In the following step, initiation step, acyl-enzyme intermediate reacts with a nucleophile such as water, alcohol, or hydroxyl ended chain and first chain of the polymer is synthesized. The product obtained at the end of this step is named as ω-hydroxycarboxylic acid. Then, propagation step is proceeded by formation of additional polymer chain [5, 48].

ε-CL can be polymerized quickly by various types of lipases. Immobilized form of CALB on an acrylic resin (Novozyme 435®) is the most effective one among them. For example, polymerization by Novozyme 435® was performed at 70˚C for 24 hours with an enzyme/monomer ratio of 1% wt in toluene. At the end of this reaction, molecular weight (M_n) of PCL was reached to 25,000 with a polydispersity index (PDI) of 1.6 [5].

ROP of ε-CL via lipase can be carried out both in bulk or in an organic solvent such as toluene, heptane, 1,4-dioxane, diisopropyl ether or dibutyl ether [5]. Among these organic solvents, toluene has been found as the best solvent for ROP by Novozyme 435® since the enzyme is more stable in toluene and higher molecular weights are obtained [10]. Nowadays, supercritical carbon dioxide and ionic liquids, which are named as "green solvents", can also be used as solvents for lipase-catalyzed ROP [5].

2.5 Applications of Poly (ε-caprolactone)

PCL is widely used especially in biomedical field and food industry as a packaging material. In biomedical field researches are generally focused on drug-delivery systems, medical devices, and tissue engineering. Since it has good rheological and viscoelastic properties, PCL can be easily manufactured into a large range of implants and devices. Design and fabrication of long-term biodegradable implants, which has suitable degradation kinetics to be placed in a certain anatomic site, is promising as a result of relatively inexpensive production ways and FDA approval [40].

Moreover, PCL has also high permeability to many drugs besides its long-term biodegradation behavior. Thus, within last years, application of PCL in drug-delivery systems is increasing. In addition, PCL has a well blending capacity with other polymers. This ability provides PCL to be degraded with controlled kinetics [40]. To improve therapeutic efficiency of drug-delivery systems, PCL can be prepared as micro- and nanospheres [50].

Application of PCL for medical devices are; sutures, wound dressings, contraceptive devices, and fixation devices [40]. In dentistry, PCL is used with phosphate as filling material since PCL is a strength and resistant material. In addition, it does not give chance for bacterial growth [51]. Wound dressing application of PCL provides both covering and treatment of wounds. Treatment property of wounds can be provided by loading of antimicrobial drugs to PCL-based wounds by taking the advantage of porous structure of PCL [52].

On the other hand, another important biomedical application field of PCL is tissue engineering. For example, PCL can be used for artificial skin production and bone repair. In addition, PCL can also be used as a scaffold for tissue engineering [41, 53]. In tissue engineering, PCL is used to take the place of bone and cartilage tissues by the addition of scaffolds to the required places in the body. PCL scaffolds used as bone tissue is prepared by the addition of calcium phosphate. PCL-based bone and cartilage are suitable for attachment of cells, biocompatible, stable, and flexible. Moreover, PCL and poly-L-lactic acid (PLLA) copolymers have an application for nerve cell treatment and regeneration [1].

3. MATERIALS AND METHODS

3.1 Materials

Immobilized form of CALB (Novozyme 435®) and free form of CALB (CALB L, Lipozyme®) were purchased from Sigma Aldrich and Novozymes companies, respectively. Enzymes were used as received. Rice husks were provided from a rice production company in Edirne-Turkey. They were washed with distilled water and burned at 600-650˚C for 6 hours to obtain rice husk ashes (RHA). RHA was used as a support material for lipase immobilization. For the surface modification of RHA by silanization, a silanization agent, 3-aminopropyl triethoxysilane (3-APTES), was used. It was preferred for this study since it had been shown as the most efficient silanization agent in literature [26]. 3-APTES (99%, $C_9H_{23}NO_3Si$) was purchased from Merck. Acetone (99%, C_3H_6O) was used as a solvent for 3-APTES and provided from Riedel-de Häen. Glutaraldehyde (25% solution in water for synthesis), a coupling agent for immobilization process, was obtained from Merck. In order to prepare phosphate buffer for immobilization process, monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) were purchased from Carlo Erba and Merck companies, respectively. Olive oil, which was used for lipase activity measurement, was obtained from Komili. Ethanol (99%, C_2H_5OH) was used for the termination of hydrolysis reaction during lipase activity measurement and provided from Merck. Phenolphthalein (1% in ethanol) was used as an indicator in titration during lipase activity measurement and purchased from Merck.

In PCL synthesis part, ε-caprolactone (99%, $C_6H_{10}O_2$), monomer of the polymerization reaction, was provided from Alfa Aesar and stored over molecular sieves (3A˚) before polymerization reactions to avoid water. Molecular sieves were obtained from Sigma Aldrich. Toluene (99%, $C_6H_5CH_3$) was purchased from Merck and used as a solvent in polymerization reactions since it had been shown as the most efficient solvent for lipase-catalyzed ROP of ε-CL in previous studies [10]. Chloroform (99%, $CHCl_3$) was used for termination of the polymerization reaction and provided from Sigma Aldrich. For precipitation of the polymer at the end of the

reaction, methanol (99%, CH_3OH) was used and it was obtained from Merck. Tetrahydrofuran (THF, C_4H_8O) was used to solubilize polymer samples in GPC analysis and purchased from Carlo Erba with high purity (HPLC grade).

3.2 Methods

3.2.1 Preparation of the support material

Firstly, rice husks were washed with distilled water and dried in oven (Electro mag M3025P). Then, they were burned in furnace at 600-650˚C for 6 hours. Temperature of the furnace was increased with a rate of 10˚C/min until it reaches the burning temperature. Obtained RHA, which can be seen in Figure 3.1, was stored in a desiccator in order to avoid moisture.

Figure 3.1: Rice husk ashes (RHA) obtained after burning rice husks.

Before the immobilization step, the surface of the support material was modified by silanization with an agent 3-APTES. 250 mg RHA was mixed with 15% (v/v) 3-APTES in 5 mL acetone. The mixture was incubated in shaking water bath (Julabo SW22) at 50˚C and 160 rpm for 2 hours. The shaking water bath used in experiments is shown in Figure 3.2.

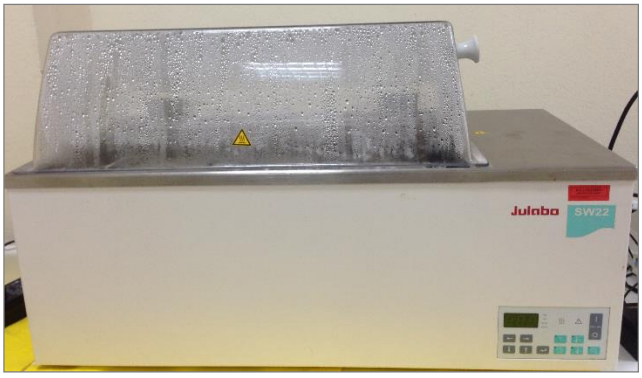

Figure 3.2: Shaking water bath used in experiments.

At the end of the reaction, surface-modified (activated) RHA was filtrated and washed with distilled water by using vacuum pump (Sartorius stedim 16612) to remove the unreacted compounds. Then, dried in oven at 60˚C for 2 hours. Activated RHA was mixed with 25 mL 0.2% (v/v) glutaraldehyde/phosphate buffer solution (pH 7, 0.015M) at room temperature for 2 hours on a magnetic stirrer (Heidolph MR3001). At the end of the reaction time, glutaraldehyde-treated activated RHA was washed with distilled water and filtrated by using vacuum pump. Then, directly used for immobilization process without drying. In addition, activated RHA was also used for lipase immobilization without glutaraldehyde treatment.

Parametric studies were also carried out for 3-APTES and glutaraldehyde concentrations. Activated RHA was obtained with 5%, 10%, and 20% (v/v) 3-APTES in 5 mL acetone. Moreover, different glutaraldehyde concentrations (0.02% and 2% (v/v)) were tried for the activated RHA with 15% 3-APTES.

0.015 M, pH 7 phosphate buffer was prepared by mixing 29.25 mL 1M $NaH_2PO_4.H_2O$ and 45.75 mL 1M $Na_2HPO_4.7H_2O$ with distilled water and completing to 1 L by distilled water. pH of the buffer was controlled by the pH meter (Inolab TWT).

3.2.2 Immobilization of lipase

The prepared supports were used for lipase immobilization. Two types of immobilization methods were applied: physical adsorption and cross-linking with a coupling agent. Glutaraldehyde was used as a coupling agent at the support preparation step. On the other hand, in physical adsorption method, activated RHA was not treated with glutaraldehyde at support preparation part. In both physical adsorption and cross-linking methods, prepared support materials were mixed with 500 mL free lipase (enzyme to support ratio is 2 µL/mg) and 25 mL phosphate buffer (pH 7, 0.015M) at

room temperature for 5 hours on a magnetic stirrer. At the end of the reaction time, immobilized enzymes were filtrated by using vacuum pump. The first filtrate was taken and reserved for protein determination. Then, immobilized enzymes were washed with the same phosphate buffer and dried in oven at 30˚C for 12 hours. The immobilized enzymes produced with both physical adsorption and cross-linking methods are shown in Figure 3.3 (from left to right).

Figure 3.3: Immobilized enzymes via physical adsorption and cross-linking.

Moreover, the same procedure was applied to the supports obtained with different 3-APTES and glutaraldehyde concentrations at support preparation step. As a further parametric study, enzyme loading capacity of the supports (activated with 15% 3-APTES) was investigated for both physical adsorption and cross-linking (0.2% glutaraldehyde) methods. Enzyme loading capacity experiments were carried out for enzyme to support ratios of 0.5 µL/mg, 1 µL/mg, and 3 µL/mg.

3.2.3 Protein determination of immobilized lipases

The amount of filtrate obtained at the end of the immobilization process was measured and its protein content was determined by using UV spectrophotometer (UV mini 1240 SHIMADZU spectrophotometer). 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. At this wavelength ultraviolet absorption depends on tyrosine and tryptophan amino acids. Protein concentration was determined from the standard curve prepared from lipase solution depending on the absorbance values recorded. Standard curve is given in Figure 3.4.

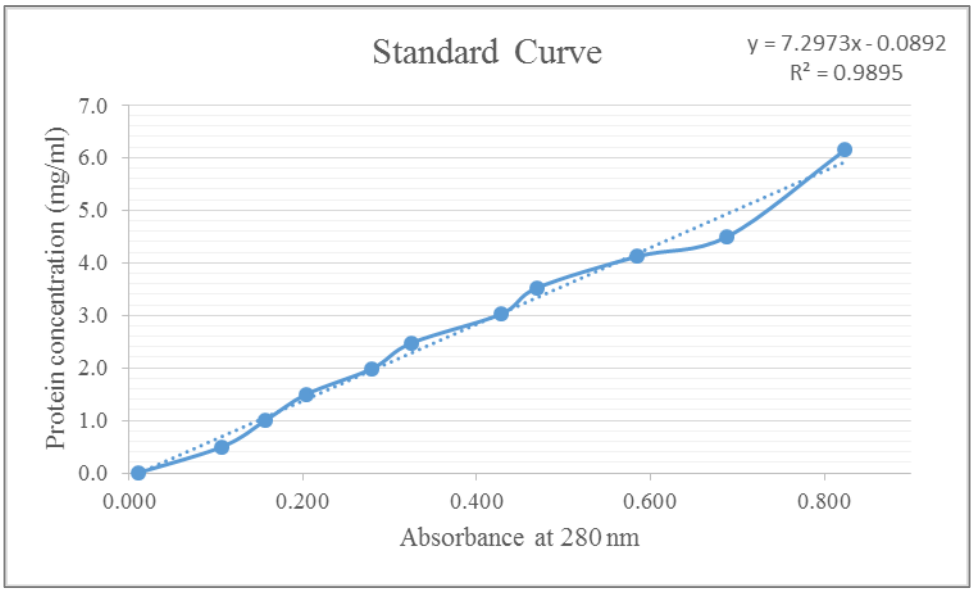

Figure 3.4: Standard curve used for protein determination.

Immobilization efficiency (amount of loaded protein) was calculated from Equation 3.1, in which the term amount of protein coupled (mg/mL) is the difference between protein loaded and protein in supernatant [28].

$$Immobilization\ efficiency\ (\%) = \frac{amount\ of\ protein\ coupled}{amount\ of\ protein\ loaded} \times 100 \qquad \textbf{(3.1)}$$

3.2.4 Activity measurement of immobilized lipases

Activity of immobilized lipases were measured by titration method. The enzyme sample was prepared with a concentration of 10 mg/mL in phosphate buffer (pH 7, 0.015M). Since lipases are responsible from the hydrolysis of oils, activity of the enzyme was determined from the hydrolysis rate of olive oil [6]. Therefore, 100 µL from 10 mg/mL enzyme solution and 500 µL olive oil were put together to a tube. To neutralize the pH of the reaction medium, 2.5 mL phosphate buffer (pH 7.2, 0.1M) was added to the tube and mixed. Tube was incubated in shaking water bath at 37˚C and 120 rpm for 30 minutes. At the end of the 30 minutes, reaction was terminated by the addition of 1.25 mL acetone, 1.25 mL ethanol, and 3-5 drops of phenolphthalein. After that, the mixture was titrated with 0.1M NaOH solution. The liberated fatty acid amount is equivalent to the NaOH spent in titration process. One unit of enzyme activity was defined as 1 µmol of fatty acids liberated per minute (1U = 1 µmol fatty acid/min). Specific activity (U/mg) is defined as the ratio of immobilized enzyme activity to amount of protein loaded [54].

Activity measurements were done for the determination of newly-immobilized enzyme, enzyme activity at recycle experiments, enzyme activity change in storage stability studies (activity change in 2 months when stored at +4˚C), optimum pH, and optimum temperature.

Phosphate buffer (pH 7.2, 0.1M) used in activity measurement was prepared by dissolving 0.4363 g $NaH_2PO_4.H_2O$ and 1.8326 g $Na_2HPO_4.7H_2O$ in 100 ml distilled water.

Each reported value was the mean of three experiments at least, and the standard deviation was within ca. ± 5 %. Also all results are reproducible.

3.2.5 ROP of ε-CL by immobilized lipases

ROP of ε-CL was carried out by the enzymes immobilized by both physical adsorption and cross-linking methods. Reactions were performed in 1000 mg of toluene (ε-CL to toluene ratio 1:2 (w/w)) under dry nitrogen. Enzyme concentration was 20% (w/w) (enzyme to monomer ratio). Reaction medium was stirred at 120 rpm with a magnetic stirrer. Reactions were carried out at 30˚C, 40˚C, 60˚C, and 80˚C for 6, 24, 48, 72, and 120 hours with both two immobilized enzymes. At the end of the reaction time, reaction was terminated by the addition of excess chloroform to the mixture. Enzyme was separated from the reaction mixture by filtration. Then, chloroform was evaporated in oven at 50˚C. After that, by the use of cold methanol, polymer was precipitated. The polymer sample was washed with methanol and filtrated. Polymer was dried in oven at 35˚C.

Moreover, at best reaction conditions of the two enzyme, effect of enzyme concentration was investigated. For this purpose, polymerization reactions were performed with 2.5%, 5%, and 10% (w/w) enzyme concentrations. In addition, to compare the performance of immobilized lipases on surface-modified RHA with commercial lipases, Novozyme 435® and Lipozyme®, polymerization reactions with these enzymes at best reaction conditions were also performed. The apparatus that was used in polymerization reactions is given in Figure 3.5.

Figure 3.5: Apparatus used for polymerization reactions.

3.2.5.1 Determination of reuse capacity of immobilized lipases for PCL synthesis

For the determination of reuse capacity of immobilized lipases, enzymes separated from the reaction mixture at the end of the polymerization were washed with phosphate buffer (pH 7, 0.015M) and dried in oven at 30˚ C. Then, they were used in polymerization reaction again with the same procedure at the best reaction conditions of each type of enzyme which was determined before. At the end of the each polymerization reaction, activity of the enzyme and molecular weight of the polymer was measured. This procedure was completed until the activity of enzyme decreases too much that cannot be resulted in a noticeable monomer conversion. In addition, molecular weight of the polymer also decreases with the decreased enzyme activity. At the end, enzyme activity and molecular weight of the polymer becomes very close to the previous cycle.

3.3 Characterization Techniques

3.3.1 Ultraviolet (UV) spectrophotometer

Lipase immobilization efficiency was evaluated by using UV mini 1240 SHIMADZU spectrophotometer. Absorbance of the samples were measured at 280 nm to determine the protein concentration. Phosphate buffer (pH 7, 0.015M) was used as a blank sample.

3.3.2 Fourier transform infrared spectroscopy (FT-IR)

Perkin Elmer FT-IR Spectrum One B Spectrometer was used for the determination of chemical structure and composition of RHA, surface-modified RHA, immobilized enzymes, and PCL samples. The differences occurred in functional groups after each step of enzyme immobilization could be observed by FT-IR analysis. Moreover, by using FT-IR, the synthesized polymer was verified to be PCL by comparing with characteristic functional groups and bonds of PCL. Characteristic infrared bands of PCL is given in Table 3.1 [44]. Each sample was analyzed by KBr pellet. The spectra were recorded by at least 32 scans with a resolution of 2 cm^{-1} .

Table 3.1: Characteristic infrared bands of PCL.

Wavelength (cm^{-1})	Structure of Bonds
2949	Asymmetric CH_2 stretching
2865	Symmetric CH_2 stretching
1727	Carbonyl stretching
1293	C-O and C-C stretching (in crystalline phase)
1240	Asymmetric C-O-C stretching
1170	Symmetric C-O-C stretching

3.3.3 Thermal gravimetric analysis (TGA)

Thermal characterization of surface-modified RHA and PCL samples were carried out by TGA. For this aim, 5-10 mg of samples were heated from room temperature to 1000˚C with a rate of 10˚C/min under air flow. The apparatus used is SEIKO TG/DTA 6300.

3.3.4 Differential scanning calorimetry (DSC)

For the determination of T_g and T_m values of synthesized polymer samples, SEIKO 7020 DSC was used. Under inert nitrogen atmosphere 10-15 mg samples were analyzed. The materials were exposed to thermal cycles (heat-cool-heat). The first heating scan allows to erase the thermal memory of the materials. Thermal characterization was carried out between -70 and 200˚C at 10˚C/min. By DSC analysis, crystallinity percentages of polymer samples can also be obtained besides T_g

and T_m values. For the calculation of crystallinity percentage (X_c), Equation 3.2 was used [55].

$$X_c = \left(\frac{\Delta H_m}{\Delta H°_m}\right) x100 \quad \textbf{(3.2)}$$

In Equation 3.2, $\Delta H°_m$ is the melting enthalpy of PCL where it has 100% crystalline structure and its value is 139.3 J/g [55]. ΔH_m is the enthalpy value at T_m temperature of the PCL sample.

3.3.5 Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was used for the determination of molecular weights and polydispersity indexes of PCL samples. Measurements were carried out by Agilent 1100 model GPC apparatus. The apparatus is consisted of a pump, refractive index and UV detectors, Zorbax PSM colons (1000-S, 300-S, 60-S). THF was used as an eluent with a flow rate of 1 mL/min. Analysis was carried out at 25˚C. Samples were prepared by dissolving 3 mg of PCL in 1 ml THF. Before injection, all samples were filtered via 0.45 μm filter syringe.

3.3.6 Hydrogen nuclear magnetic resonance spectroscopy (^{1}H-NMR)

Hydrogen nuclear magnetic resonance spectroscopy (^{1}H-NMR) was used for the determination of molecular weight and molecular structure of PCL. The apparatus used for analysis was Bruker Ultrashield 300 MHz. Deuterated chloroform ($CDCl_3$) was used as a solvent during analysis. ^{1}H-NMR spectra was obtained with respect to tetramethylsilane (TMS) standard. Molecular weight (M_n) value of PCL can be calculated based on the areas of peaks obtained at characteristic chemical shift (δ) values of 4.07 ppm (CH_2O) and 3.65 ppm (CH_2OH, end group). The formula used for $M_{n,NMR}$ calculation is given in Equation 3.3 [54, 56].

$$M_{n,NMR} = \frac{5xI_{4.07}}{2xI_{3.65}} xM_{\varepsilon-CL} \quad \textbf{(3.3)}$$

In this equation, $M_{\varepsilon\text{-CL}}$ is the molecular weight of ε-CL, $I_{4.07}$ and $I_{3.65}$ are the integrated peak areas [56]. Moreover, monomer conversion can be calculated from the ratio of integrated peak areas at 4.07 ppm and 3.65 ppm. This ratio is named as degree of polymerization (DP) value [1].

3.3.7 Scanning electron microscopy (SEM)

Surface morphologies of immobilized enzymes and PCL samples were observed by JEOL JSM-6390LV SEM. Samples were coated with platinum before observation. Analysis was performed at 5 kV with different magnifications.

3.3.8 X-Ray diffraction analysis (XRD)

For the investigation of crystal structures of PCL samples, X-Ray diffraction (XRD) patterns were recorded by PANalytical XPER'T PRO XRD apparatus with CuKα radiation source (λ=1.5406 Å). Analyses were carried out between 15 and 30 °2 Theta (°2 Th.) values with an increment rate of 0.026 °2 Th./s at 25˚C.

4. RESULTS AND DISCUSSION

4.1 Lipase (CALB) Immobilization on RHA

The free form of CALB was immobilized on RHA via physical adsorption and cross-linking methods. Before the immobilization process, surface of the RHA was modified by silanization with an organosilane named 3-APTES. With this process, amine ($-NH_2$) groups were aimed to be obtained. The presence of $-NH_2$ groups was checked with FT-IR analysis. Since the intensity of the peak which is investigated was so low, FT-IR spectrum of surface-modified RHA was given between 3700 and 2900 cm^{-1} in Figure 4.1.

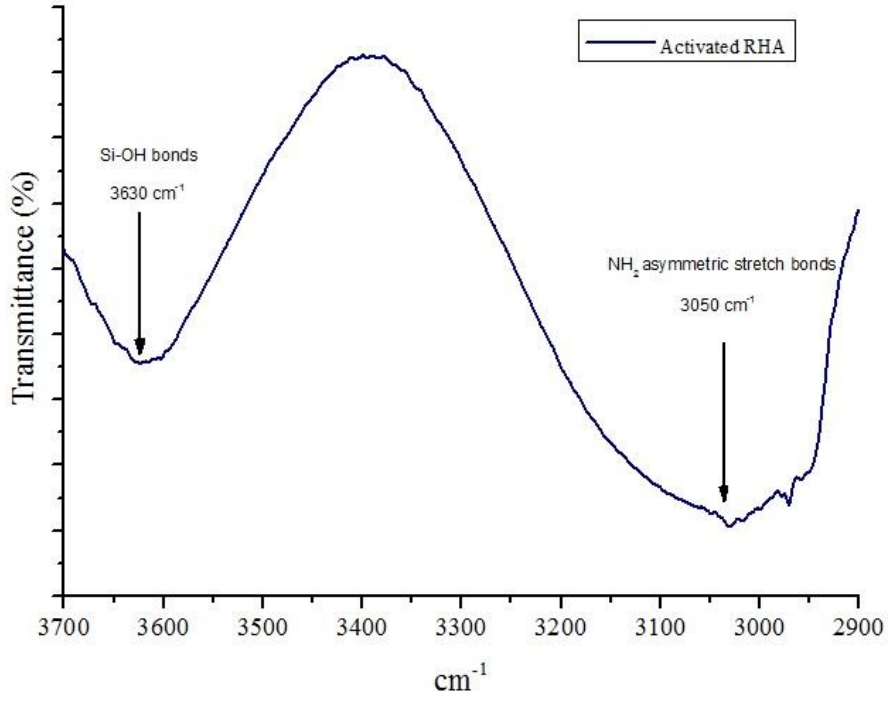

Figure 4.1: FT-IR spectrum of surface-modified RHA.

Absorptions between 3500 and 3100 cm^{-1} were as a result of the asymmetric stretching vibration of primary amines ($-NH_2$) [57, 58]. This region also overlaps with Si-OH and –OH bonds [57, 59]. However, in Figure 4.1, there was a shift in the maximum intensity to lower wavelengths (about 3100-3000 cm^{-1}) which was resulted from the

hydrogen bonding after introduction of amine groups. Therefore, it can be safely stated that, surface modification of RHA was successfully achieved.

Moreover, surface-modified RHA was further characterized by TGA in order to observe the contribution of organofunctional groups. TGA thermogram of surface-modified RHA was illustrated in Figure 4.2.

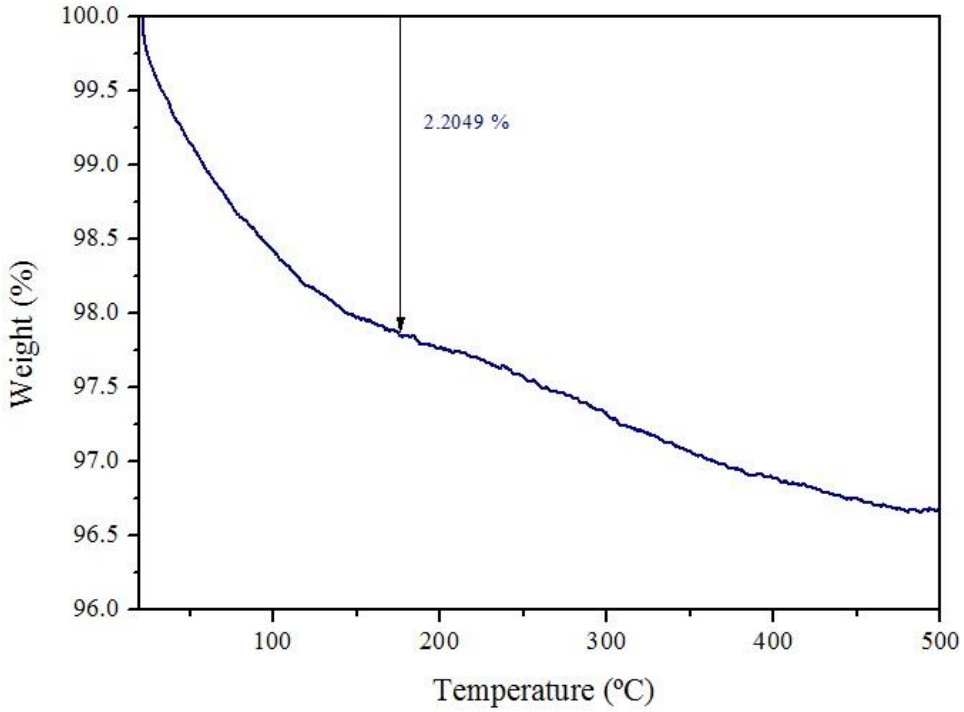

Figure 4.2: TGA thermogram of surface-modified RHA.

As seen from Figure 4.2, there was a cumulative weight loss as 2.2049% until the temperature reaches 180-190 ˚C. Based on the fact, pure 3-APTES was totally evaporated at 190˚C, a part of the observed weight loss belonged to 3-APTES [58]. When surface-modified ash TGA trace was compared with the thermogram of pure ash, it can be seen that pure ash loses 1.0260% of its initial weight until the temperature reaches 180-190˚C [60]. This means that the excess part (about 1.1789%) represents the amount of evaporated 3-APTES.

After preparation of the support material, lipase was immobilized via two methods to obtain physically adsorbed (AD) lipase and cross-linked (CR) lipase. FT-IR spectrums of all steps including bare RHA, surface-modified RHA, glutaraldehyde treated surface-modified RHA, and immobilized enzymes (via both two methods) were given between 2000 and 650 cm^{-1} in the same graph in Figure 4.3.

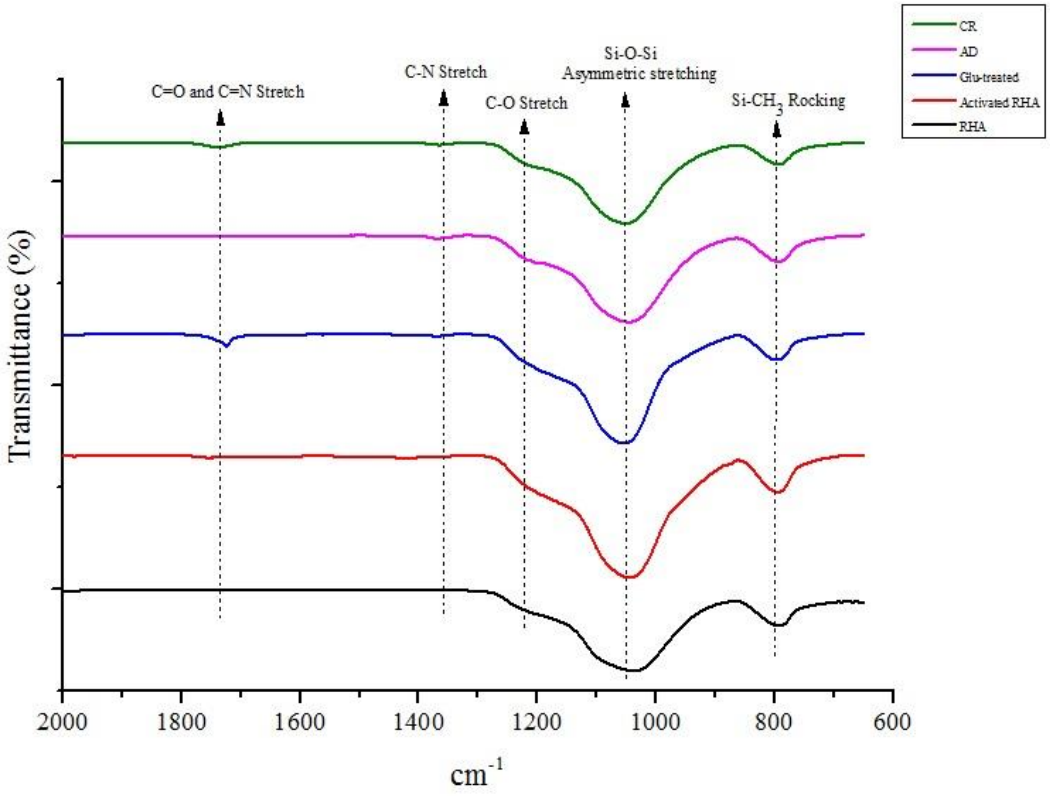

Figure 4.3: FT-IR spectra of immobilization steps.

In Figure 4.3, both spectra have a broad band between 1100 and 1000 cm^{-1} corresponds to asymmetric stretching vibrations of Si-O-Si which is characteristic for a silica-based material (RHA) [13, 57]. Moreover, there is an additional characteristic peak for RHA at about 800 cm^{-1} representing Si-CH_3 rocking [57]. At about 1720 cm^{-1}, strong C=O stretching can be seen as a result of contribution of aldehyde groups after glutaraldehyde treatment [57]. The intensity of this peak decreases after immobilization (for CR lipase) since one of the C=O groups of glutaraldehyde is consumed by interacting with the amino group of enzyme. On the other hand, other C=O group of glutaraldehyde interacts with the $–NH_2$ group of activated RHA (Schiff base reaction) and results in imine (C=N) formation [26, 34]. At the same region (about 1690 cm^{-1}) C=N (imine) stretching may partially overlap with C=O stretching in FT-IR spectra of glutaraldehyde treated activated RHA and CR lipase [57]. As an expected result, there is no peak formation at this region of AD lipase spectrum since it was immobilized without using glutaraldehyde. The weak peaks observed after immobilization (for both AD and CR lipases) at 1365 cm^{-1} and 1215 cm^{-1} may belong to N-terminus (C-N stretch) and C-terminus (C-O stretch) of lipase, respectively [61].

Immobilization efficiencies and activities of immobilized enzymes were given in Table 4.1 with standard deviations which have been calculated from triplet measurements.

Table 4.1: Immobilization efficiency of CALB immobilized via adsorption and cross-linking.

Immobilization Type	Efficiency (%)	Activity (U)	Relative Activity (%)	Specific Activity (U/mg)
Adsorption	90.7	2400	92.3	9.2
Cross-linking	89.8	2050	78.8	8.1

It can be seen clearly from Table 4.1, the activity of free CALB was 2600 U and the relative activities of CALB immobilized via adsorption and cross-linking were calculated as 92.3% and 78.8%, respectively. Immobilization efficiency was obtained at about 90% for both immobilization method. Lipase immobilized by adsorption onto the surface modified RHA showed the highest immobilization yield at 92.3%. On the other hand, the specific activity increased from 4.5 U/mg for the free enzyme to 8.1 U/mg and 9.2 U/mg after immobilization via the cross-linking and adsorption methods, respectively. Lipase immobilization efficiency as well as specific activity of the enzyme was clearly dependent on type of immobilization. According to the immobilization yield results, adsorption was very efficient and resulted in high activity.

Adsorption tends to be less disruptive to the enzyme than chemical means of attachment because the binding is mainly by hydrogen bonds, and Van der Waals forces. Because of the weak bonds involved, desorption of the enzyme resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Also lipase immobilized by cross-linking onto the surface modified rice husk ash showed the lower immobilization yield and the lowest activity per gram of carrier. This situation can be explained as active sites of enzyme can be blocked by cross-linking agent [25].

In addition, when the specific activities of both immobilized CALB were compared with Novozyme 435® (10 U/mg), which is known with its high catalytic activity for ROP of ε-CL, their specific activities were very close [14, 62]. Therefore, it can be

safely stated that, immobilized CALB on RHA via both methods were promising for PCL synthesis.

For a better comparison, fold changes of specific activities of CR, AD, and Novozyme 435® based on free enzyme (Lipozyme®) was given in Figure 4.4.

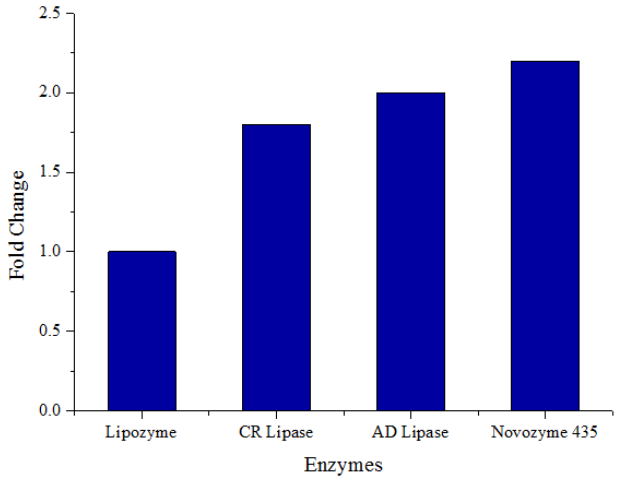

Figure 4.4: Fold changes of specific activities.

Figure 4.4 shows that, specific activities became 1.8 and 2.0 folds greater than the specific activity of free lipase after immobilization via cross-linking and physical adsorption methods, respectively. Moreover, their specific activities were close to the specific activity of Novozyme 435® which has a specific activity of 2.2 folds greater than free lipase.

Surface morphologies of immobilized lipases were observed by SEM in Figure 4.5. SEM images of RHA, AD, and CR were given at 1000x magnification.

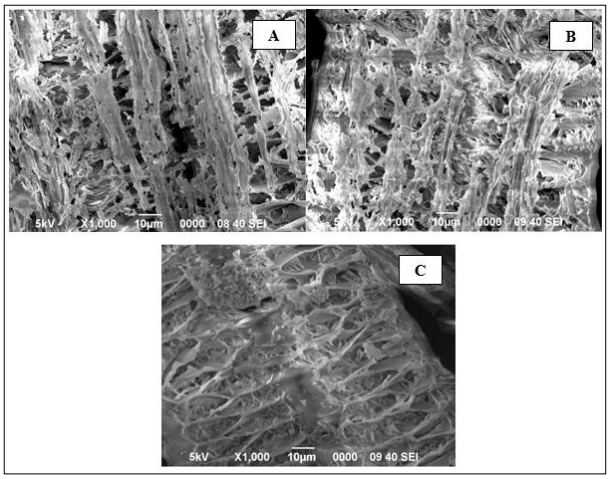

Figure 4.5: SEM images of RHA (A), AD (B), and CR (C).

As it can be seen clearly from Figure 4.5(A), rice husk ash (A) has a porous and mesh-like structure. It is quite clear that, the neat RHA exhibited a somewhat porous structure but mostly has closed cells with a few ca. 10 to 50 micrometer sized open cells between meshes. In the case of adsorption and cross-linking immobilization of CALB (Fig. 4.5(B) & 4.5(C)), a lipase agglomeration was occurred and lipase directs open cell structures dominated, with almost open-cells. Moreover, with enzyme immobilization, cell size decreased sharply from ca. 5 micrometers. The smaller sizes in open cells between meshes for both immobilization method may result from the formation of subnetworks having extended cross-linking chains by leaving much larger pores behind. Especially in Fig. 4.5(C), glutaraldehyde addition for cross-linking agent, number of dispersed and enzyme molecules directed mesh structures dominated, with almost meshes.

4.1.1 Storage stability of immobilized CALB

Storage stability is a major concern in biocatalysis. The storage stabilities of the lipase immobilized by cross-linking and adsorption methods were examined for 2 months at 4°C. In Figure 4.6, relative activity (the ratio of immobilized enzyme activity to free enzyme activity) changes of AD and CR enzymes within 2 months were given.

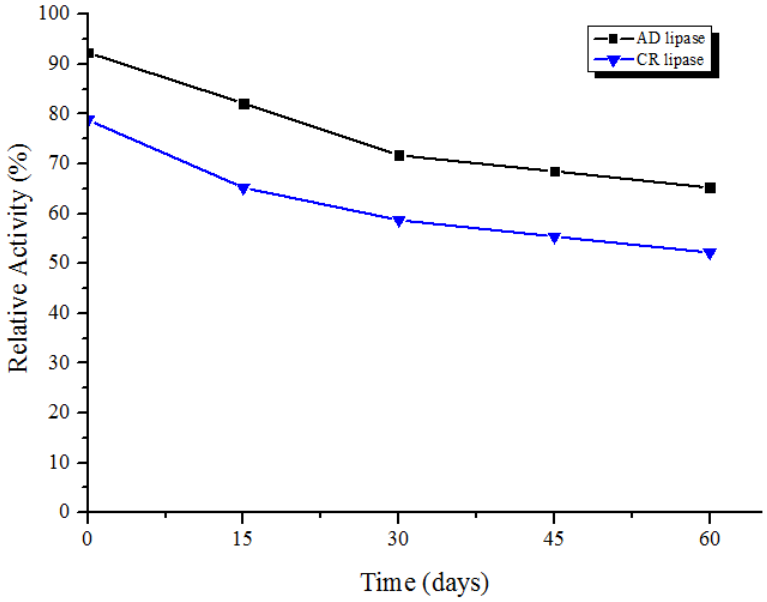

Figure 4.6: Storage stability of CALB immobilized via adsorption and cross-linking.

Relative activities of AD and CR lipases were decreased about 20% in first 30 days (Figure 4.6). Also it can be safely stated that, CALB immobilized via adsorption and

cross-linking maintained 65% and 55% of their activities, respectively. Having good storage stability is clearly an advantage for a biocatalyst and increases storage stability upon immobilization have previously been recorded [63].

4.1.2 Effect of pH on immobilized CALB activity

Optimum pH of immobilized CALB were determined by measuring the relative activities at different pH values with a range of 4.5-10.5. Immobilized lipases were dispersed in phosphate buffers with these pH values for the preparation of samples for activity measurements. Then the same procedure for activity measurement was applied. Relative activity changes of immobilized lipases with pH values were shown in Figure 4.7.

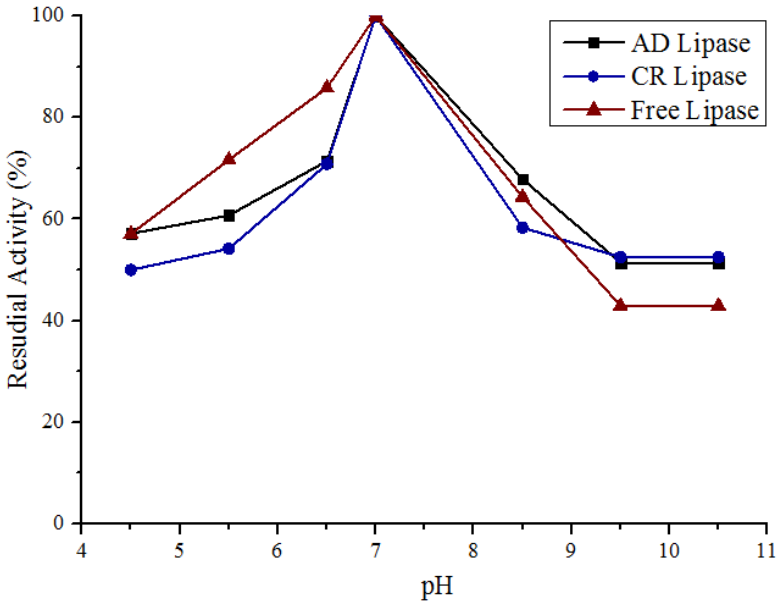

Figure 4.7: Effect of pH on immobilized CALB activity.

As given in Figure 4.7, both immobilized enzymes have an optimum pH of 7.0 which was parallel to literature [28]. At pH 4.0–5.5, immobilized lipases were active but at basic pH values immobilized lipases showed better activity than free CALB. Therefore, it can be safely stated that, activity of CALB were enhanced after immobilization via both methods.

4.1.3 Effect of temperature on immobilized CALB activity

Optimum temperatures of immobilized lipases were determined by measurement of their activities at different temperatures. For this aim, reactions were carried out at between 30 and 60˚C. Then the same procedure for activity measurement was applied.

Relative activity variations of immobilized lipases with temperature were shown in Figure 4.8.

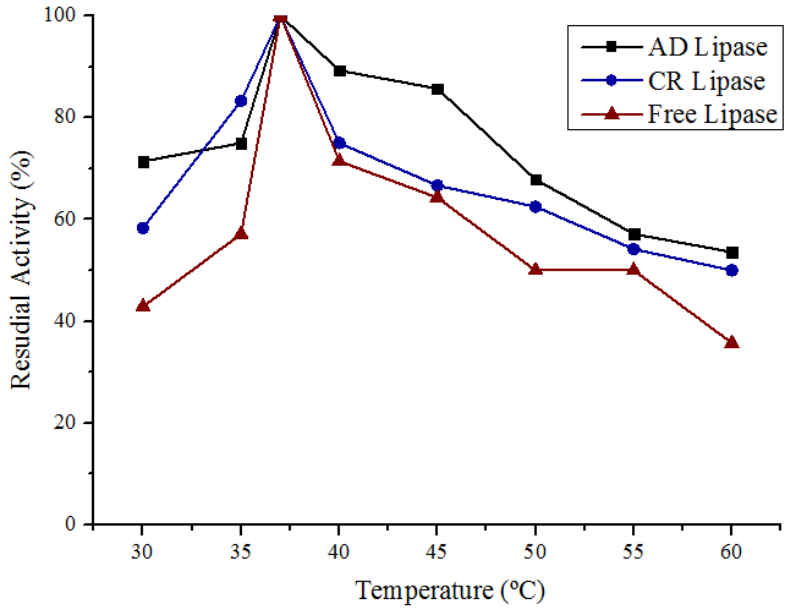

Figure 4.8: Effect of temperature on immobilized CALB activity.

The effect of temperature on the activity of immobilized lipase at pH 7.0 was shown in Figure 4.8. It is evident from Figure 4.8, optimum temperature of both of immobilized lipases were obtained at 37˚C which was parallel to literature [6, 7, 27]. The activities of immobilized lipases preparing both adsorption and cross-linking methods were adversely affected at temperatures over 37˚C. However, the activity of free lipase dropped significantly above 37˚C, decreasing just 45% and 50% from the maximum activities for adsorption and cross-linking methods, respectively. Also these results showed that, the amount of lipase bound on the surface modified rice husk ash resulted in enhanced thermal stability and less denaturation of enzyme at high temperatures.

4.2 Parametric Studies for CALB Immobilization on RHA

During support preparation and immobilization steps, there are crucial parameters which may effect on immobilization efficiency, relative activity, and specific activity. These parameters are; 3-APTES concentration and glutaraldehyde concentration at support preparation part and enzyme to support ratio at immobilization part.

4.2.1 Effect of 3-APTES concentration

CALB was immobilized onto RHA activated with 15% (v/v) 3-APTES. In order to determine the effect of 3-APTES concentration on immobilization efficiency, relative activity, and specific activity, additional 3-APTES concentrations (5%, 10%, and 20% (v/v) 3-APTES) were tried. The next steps (cross-linking and enzyme immobilization) were applied with the same procedure. Relative activity variations of immobilized lipases with 3-APTES concentration were given in Figure 4.9.

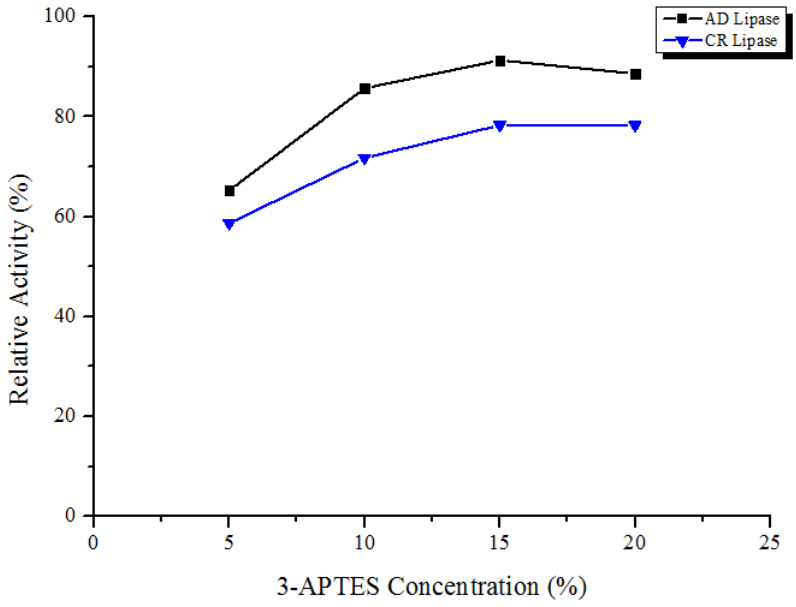

Figure 4.9: Effect of 3-APTES concentration on lipase immobilization.

As seen from Figure 4.9, for both types of immobilization methods, highest relative activity was obtained at 15% 3-APTES concentration.

In addition, immobilization efficiencies and specific activities of enzymes for different 3-APTES concentrations were given in Table 4.2. As seen, immobilization efficiencies were not affected so much from 3-APTES concentration, whereas highest specific activities for both types of immobilized lipases were obtained at 15% 3-APTES concentration (Figure 4.2). This situation can be explained by silanization reaction mechanism in which surface of the material is covered with organofunctional alkoxysilane molecules through self-assembly. Then, the organofunctional groups introduced to the surface was utilized by the enzyme molecules.

The results had shown that, 15% 3-APTES concentration was optimum for silanization reaction to proceed and provide immobilized CALB with higher activity. These results

were parallel to a study in literature in which lipase was immobilized on silica gel by cross-linking [26]. Therefore, it was found to be appropriate to study with 15% 3-APTES concentration.

Table 4.2: Effect of 3-APTES concentration on immobilization efficiency and specific activity.

Immobilization Type	3-APTES concentration (%)	Efficiency (%)	Specific Activity (U/mg)
Physical adsorption	5	92.0	6.4
	10	92.4	7.6
	15	91.3	9.0
	20	92.8	8.2
Cross-linking	5	92.7	5.7
	10	92.3	7.0
	15	92.0	7.7
	20	92.7	7.6

4.2.2 Effect of glutaraldehyde concentration

Glutaraldehyde concentration is an important parameter during immobilization process. Low concentrations may result in enzyme leakage to the reaction mixture, whereas high concentrations may cause improper enzyme agglomeration which may result in activity loss [25].

Immobilization of lipases with cross-linking method was performed with two additional glutaraldehyde concentrations: 0.02% and 2% (v/v). The effect of glutaraldehyde concentration on immobilization efficiency, relative activity, and specific activity was obtained. In Figure 4.10, relative activity change with glutaraldehyde concentration was given.

Relative activity of immobilized lipase decreases with the increased glutaraldehyde concentration as seen from Figure 4.10. Relative activity of non-cross linked CALB was decreased about 35% at 2% glutaraldehyde concentration. The main reason of this situation is, by increasing the concentration of cross-linking agent, amount of blocked active sites of enzyme might be increased which results in activity loss [25].

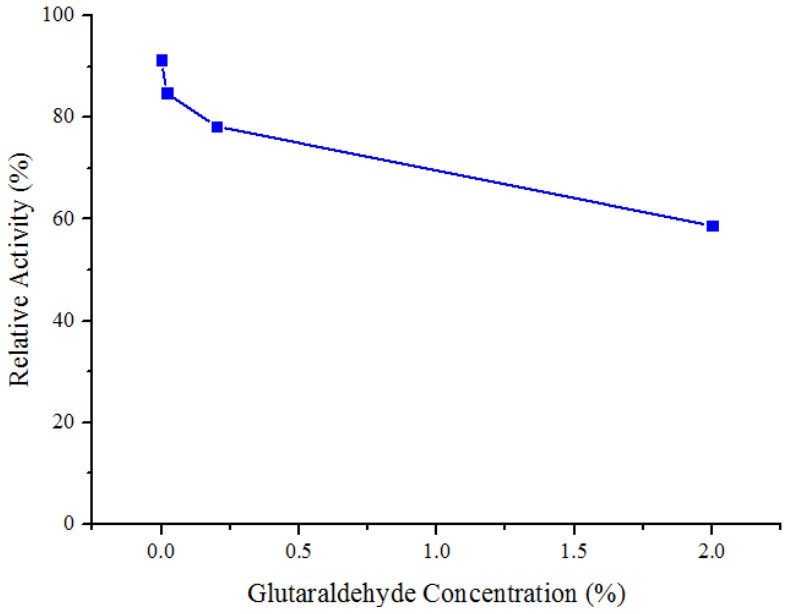

Figure 4.10: Effect of glutaraldehyde concentration on lipase immobilization.

In addition, immobilization efficiencies and specific activities obtained for each glutaraldehyde concentration was shown in Table 4.3.

Table 4.3: Effect of glutaraldehyde concentration on immobilization efficiency and specific activity.

Glutaraldehyde Concentration (%)	Efficiency (%)	Specific Activity (U/mg)
0[a]	91.3	9.0
0.02	92.4	8.3
0.2	92.0	7.7
2	92.3	5.7

[a]0% glutaraldehyde concentration means the immobilization method is physical adsorption

There existed no significant change in immobilization efficiencies, which were in the range of 91.3% and 92.4%. However specific activity of immobilized lipases decreased (from 9.0 U/mg to 5.7 U/mg) with the increased concentration of glutaraldehyde, based on the same reason with relative activity loss as seen from Table 4.3. On the other hand, cross-linking increases reuse capacity of immobilized enzyme [26]. Therefore, in this study a moderate concentration (0.2% (v/v)) of glutaraldehyde was used to obtain cross-linked (CR) lipase.

4.2.3 Effect of enzyme to support ratio

Capacity of enzyme loading is another important parameter for enzyme immobilization. Therefore, experiments for different enzyme to support ratios (0.5 µL/mg, 1 µL/mg, and 3 µL/mg) were carried out, additional to 2 µL/mg ratio. Relative activity changes of immobilized lipases with enzyme to support ratios were illustrated in Figure 4.11. As seen, relative activities of lipases were in the range of 75.0% and 92.3%. The relative activities of enzymes increased up to 2 µL/mg loading ratio and decreased after this point.

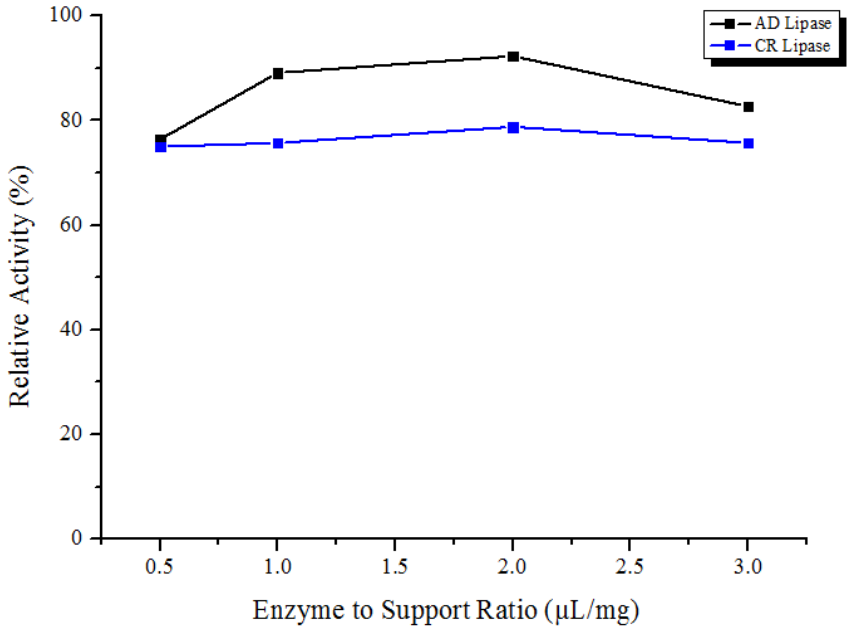

Figure 4.11: Effect of enzyme loading ratio on lipase immobilization.

Immobilized lipases with both two methods have the highest relative activities when enzyme to support ratio was 2 µL/mg. In addition, the variation of immobilization efficiencies and specific activities depending on the ratio of enzyme to support was given in Table 4.4.

Based on Table 4.4, it can be said for both type of immobilized enzymes that, immobilization efficiency and specific activity decreases with increased enzyme to support ratios. Experimental results are consistent with literature. In another study, it was seen that, immobilization efficiency decreases at high enzyme loadings. Moreover, enzymes form multiple layers on the support when they are immobilized. This structure results in inaccessible proportions of enzyme which may not contribute

to the activity. Therefore, decrement of specific activity with increased enzyme to support ratio is an expected result [64].

Table 4.4: Effect of enzyme to support ratio on immobilization efficiency and specific activity.

Immobilization Type	Enzyme to support ratio (μL/mg)	Efficiency (%)	Specific Activity (U/mg)
Physical adsorption	0.5	93.4	30.2
	1	94.6	17.4
	2	90.1	9.4
	3	85.4	6.0
Cross-linking	0.5	96.0	29.1
	1	94.4	14.9
	2	87.5	8.4
	3	85.0	5.5

4.3 ROP of ε-CL via Immobilized Lipases on RHA

PCL was synthesized via lipases immobilized on surface-modified RHA by physical adsorption and cross-linking methods. Polymerization reactions were carried out in toluene for various reaction times (6, 24, 48, 72, and 120 hours) at different reaction temperatures (30, 40, 60, and 80 ˚C). Optimum reaction time and temperatures were determined for both physically adsorbed (AD) and cross-linked (CR) lipases.

4.3.1 Synthesis via lipase immobilized by physical adsorption method

Monomer conversions, molecular weights (M_n), and polydispersity indexes (PDI) of PCL samples synthesized by AD lipase at 30 ˚C were given in Table 4.5 depending on reaction time.

M_n value increased with reaction time and reached 9000 g/mol at the end of 72 hours polymerization (Table 4.5). However, after 72 hours M_n started to decrease. CALB has also the ability of catalysis of PCL degradation, therefore M_n decrement after a certain reaction time was an expected result [65]. In addition, after 72 hours polymerization reached 85.0% monomer conversion and began to decrease after 72

hours. This result shows that monomer consumption could go further for longer reaction periods at these conditions, but after a while reaction shifts to degradation.

Table 4.5: Polymerization results obtained at 30˚C by the use of AD lipase.

Time (h)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
6	39.6	4000	1.1
24	43.1	6120	1.5
48	63.1	8670	1.5
72	85.0	9000	1.4
120	84.3	7300	1.4

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

Moreover, in Table 4.5 polydispersity values can also be seen. PDI shows how close the chain lengths of the polymer to each other are. If its value is close to 1.0, polymer has a monodisperse structure and for natural polymers PDI value is equal to 1.0 [66]. As given in Table 4.5, PDI values were in the range of 1.0-1.5. Since they were close to 1.0, polymer samples can be considered as monodisperse.

Polymerization results obtained at 40˚C by the use of AD lipase were given in Table 4.6 depending on reaction time.

Table 4.6: Polymerization results obtained at 40˚C by the use of AD lipase.

Time (h)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
6	32.7	4825	1.2
24	65.8	10050	1.5
48	91.5	11160	1.5
72	81.7	10010	1.5
120	77.0	10190	1.5

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

It can be clearly seen from Table 4.6 that, M_n reached to its highest value of 11160 g/mol at the end of 48 hours reaction. At longer reaction periods, M_n decreased only about 1000 g/mol. In addition, monomer conversion reached 91.5% at the end of 48

hours and started to decrease after this point. This may a result of degradation activity of enzyme [65]. In addition, PDI values were around 1.5, therefore it can be safely stated that polymer samples had a structure that was close to monodisperse [66].

Monomer conversions, M_n and PDI values obtained at 60˚C by the use of AD lipase were given in Table 4.7 depending on reaction time.

Table 4.7: Polymerization results obtained at 60˚C by the use of AD lipase.

Time (h)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
6	41.9	6750	1.3
24	82.2	9860	1.5
48	87.6	14000	1.5
72	85.3	9500	1.5
120	81.5	8790	1.5

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

As seen from Table 4.7, M_n value increased with reaction time and reached 14000 g/mol at the end of 48 hours reaction. However, after 48 hours M_n started to decrease which was a result of degradation activity of enzyme [65]. In addition, highest monomer conversion was 87.6% which had been obtained at the end of 48 hours reaction. After 48 hours, conversion started to decrease as a result of the beginning of polymer degradation. Polymer samples were close to monodispersity since the PDI values were again around 1.5 [66].

Polymerization results obtained at 80˚C by the use of AD lipase were given in Table 4.8 depending on reaction time.

Based on Table 4.8, highest M_n value was obtained at the end of 24 hours, which was 11820 g/mol. After 24 hours, reaction shifted from polymerization to degradation and molecular weight started to decrease. On the other hand, monomer conversion reached to its maximum value of 85.5% at the end of 24 hours and began to decrease after this point as a result of degradation activity of enzyme [65]. Based on PDI values, which were around 1.5, it can be said that polymer samples were close to monodispersity at these reaction conditions [66].

Table 4.8: Polymerization results obtained at 80˚C by the use of AD lipase.

Time (h)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
6	50.0	6300	1.3
24	85.5	11820	1.5
48	83.9	10230	1.5
72	79.3	9710	1.5
120	77.3	9960	1.5

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

In order to observe the effect of temperature on molecular weights of polymers obtained by AD lipase catalyzed polymerizations, Figure 4.12 was given.

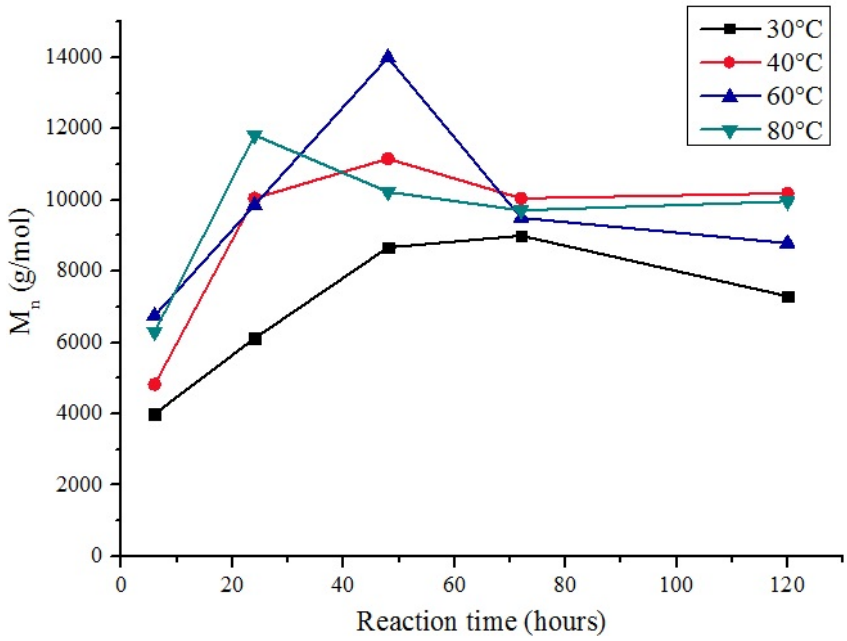

Figure 4.12: Effect of temperature on M_n of PCL synthesized by AD lipase.

To summarize based on Figure 4.12, highest molecular weight, which was 14000 g/mol, was obtained via AD lipase catalyzed polymerization at 60˚C at the end of 48 hours. In addition, as seen from the graph, molecular weights of polymers obtained at 30˚C, which was a very low temperature for polymerization, were considerably high. This situation makes the polymerization process energy saving.

For the characterization of chemical structure of polymer sample synthesized via AD lipase at 60˚C and 48 hours, FT-IR analysis was carried out. The FT-IR spectrum of polymer obtained from AD lipase-catalyzed ROP of ε-CL was shown in Figure 4.13.

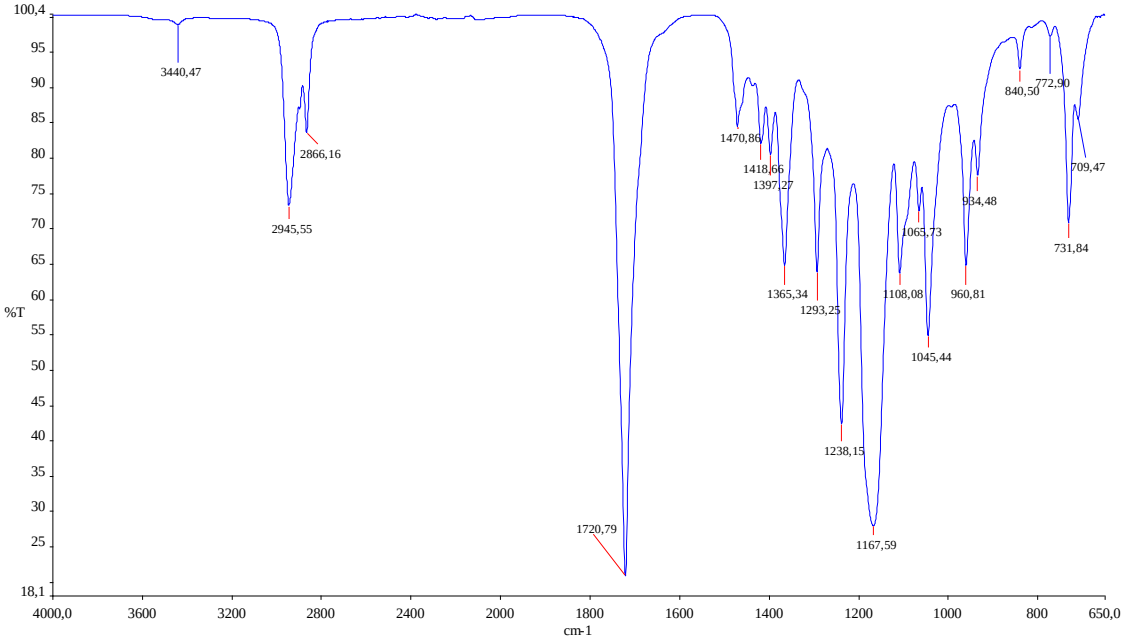

Figure 4.13: FT-IR spectrum of polymer sample synthesized via AD lipase.

The infrared bands of polymer sample synthesized by AD lipase was consistent with characteristic infrared bands of PCL, which are asymmetric CH_2 bonds at 2945 cm^{-1}, symmetric CH_2 bonds at 2866 cm^{-1}, carbonyl (C=O) bonds at 1720 cm^{-1}, C-O and C-C bonds seen in crystalline phase at 1293 cm^{-1}, asymmetric C-O-C bonds at 1238 cm^{-1}, and symmetric C-O-C bonds at 1167 cm^{-1} [44]. This situation proves that the synthesized polymer was PCL.

Chemical structure of polymer sample synthesized by AD lipase at 60˚C and 48 hours was further characterized by ^{1}H-NMR spectroscopy. The zoomed spectrum between 4.25 ppm and 3.5 ppm range, in which the characteristic peaks that were used for molecular weight calculation can be seen, was shown in Figure 4.14. The chemical shifts (ppm) between 4.25 ppm and 3.5 ppm were as follow: 4.07 ppm (t, CH_2O) and 3.65 ppm (t, CH_2OH, end group) which are characteristic for PCL [53].

Moreover, molecular weight of the PCL sample was also calculated from ^{1}H-NMR spectrum. $M_{n,\,NMR}$ value was obtained as 14880 g/mol by using Equation 3.3.

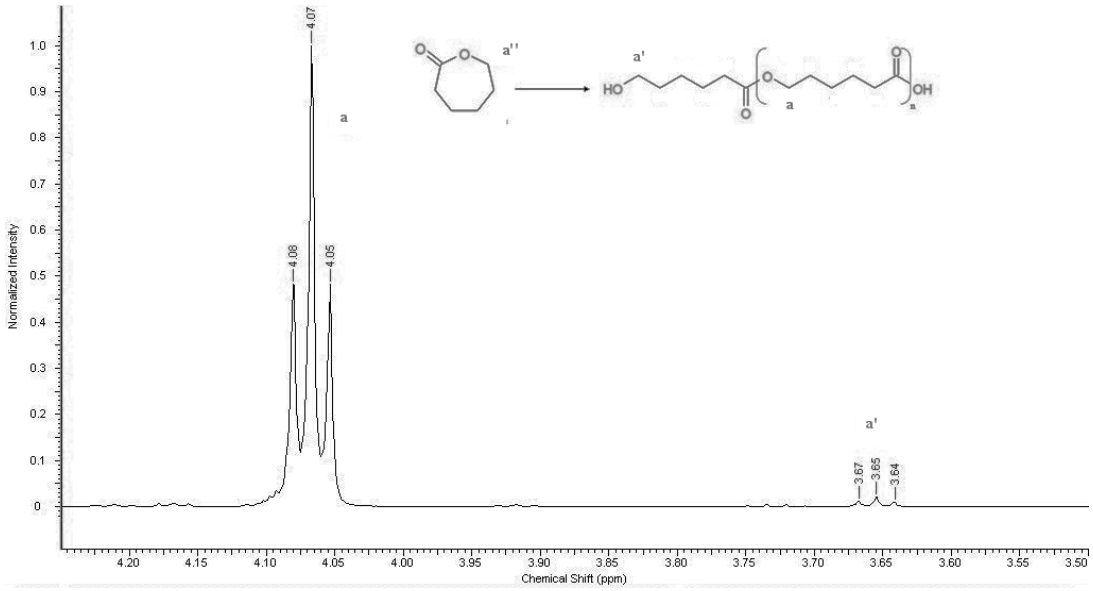

Figure 4.14: ^{1}H-NMR spectrum of polymer sample synthesized by AD lipase.

For thermal characterization of polymer sample synthesized by AD lipase at 60˚C and 48 hours, TGA and DSC analyses were applied. TGA curves of polymer obtained from AD lipase-catalyzed ROP of ε-CL was given in Figure 4.15.

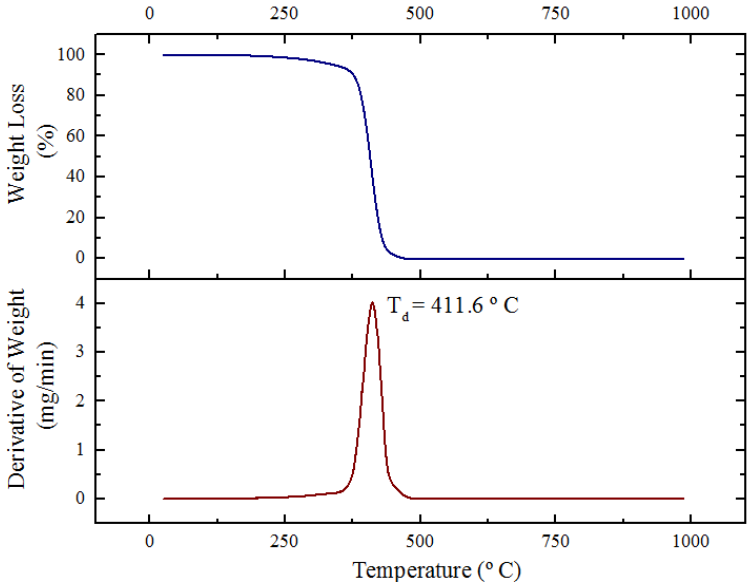

Figure 4.15: TGA curves (weight loss and derivative of weight) of polymer sample synthesized by AD lipase.

Ruseckaite and Jiménez has published that, maximum degradation temperature of PCL is about 415 ˚C under same thermal analysis conditions with the conditions applied in this study [67]. As seen from TGA curves given in Figure 4.15, degradation temperature (T_d) of PCL synthesized by AD lipase catalyzed polymerization reaction

was determined as 411.6˚C. This result has shown that, PCL synthesized with this new immobilized lipase had a high thermal stability.

Moreover, DSC was also applied for the determination of T_g, T_m, ΔH_m, and X_c values of PCL sample synthesized via AD lipase-catalyzed ROP of ε-CL at 60 ˚C and 48 hours. In Figure 4.16, DSC thermogram (endodown graphic) of this sample can be seen.

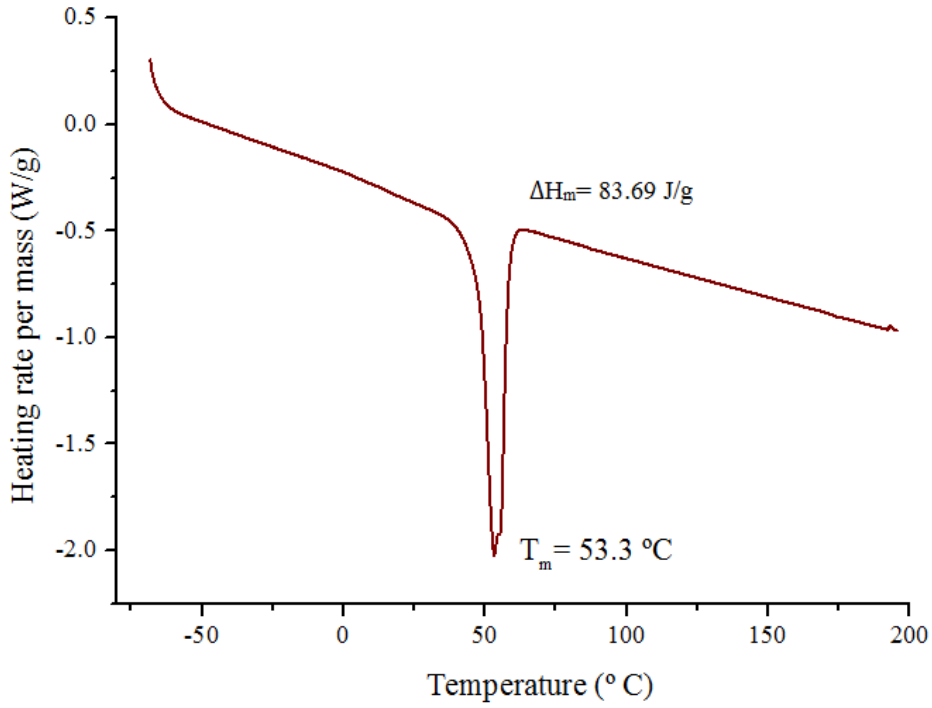

Figure 4.16: DSC thermogram of polymer sample synthesized by AD lipase.

From the DSC thermogram given, it was not possible to see T_g value since it had a very low value which was under -50˚C. It may be observed if the sample can be cooled down to a lower temperature by the use of liquid nitrogen. Moreover, T_m value of PCL sample was obtained as 53.3˚C. This low T_m value makes the polymer sample easily reshaped. From the area of melting peak, ΔH_m was computed as 83.69 J/g. By using Equation 3.2, crystallinity percentage (X_c) was calculated as 60%, which showed that polymer sample had a semi-crystalline structure.

Surface structure of polymer sample synthesized by AD lipase at 60˚C and 48 hours was observed by SEM analysis. The obtained SEM images were shown in Figure 4.17.

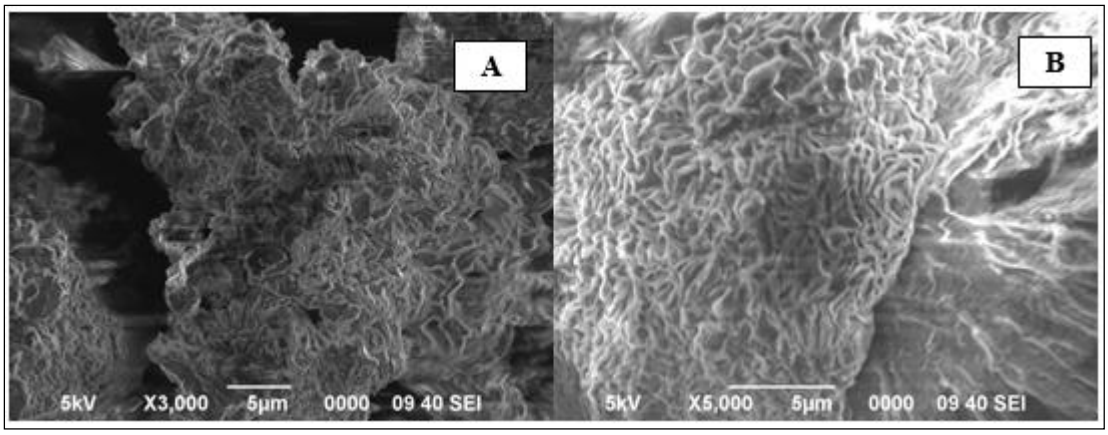

Figure 4.17: SEM images of polymer samples synthesized by AD lipase at 3000x (A) and 5000x (B) magnifications.

As seen from Figure 4.17, the polymer sample had a foam-like structure. There exists examples of PCL samples with such a structure. This type of surface makes PCL to be used as scaffolds for tissue engineering successfully [40].

The crystal structure of polymer sample synthesized via AD lipase at 60˚C and 48 hours was further investigated (in addition to DSC analysis) by XRD analysis. Its XRD pattern was given in Figure 4.18.

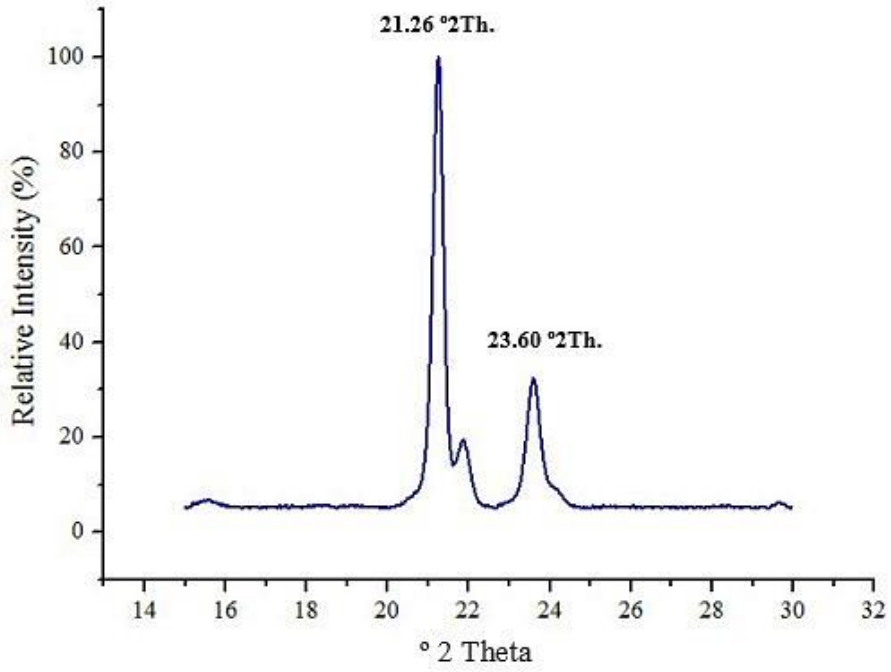

Figure 4.18: XRD pattern of PCL synthesized via AD lipase.

There existed strong diffraction peaks around 21.26° and 23.60° 2 Theta angles, which are characteristic for the semi-crystalline polymer PCL (Figure 4.18). Moreover, sharp and distinct peaks show that the polymer sample was highly crystalline [68].

4.3.2 Synthesis via lipase immobilized by cross-linking method

Monomer conversions, molecular weights (M_n), and polydispersity indexes (PDI) of PCL samples synthesized by CR lipase at 30˚C were given in Table 4.9 depending on reaction time.

Table 4.9: Polymerization results obtained at 30˚C by the use of CR lipase.

Time (h)	**Conversion[a] (%)**	**M_n[b] (g/mol)**	**PDI[b] (M_w/M_n)**
6	25.6	3350	1.3
24	71.2	7115	1.4
48	84.5	7480	1.4
72	88.5	11100	1.5
120	82.6	10180	1.5

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

It can be clearly seen from Table 4.9 that, M_n reached to its highest value of 11100 g/mol at the end of 72 hours reaction. At longer reaction periods, M_n decreased only about 1000 g/mol. In addition, monomer conversion reached 88.5% at the end of 72 hours and started to decrease after this point. This may a result of degradation activity of enzyme [65]. In addition, PDI values were around 1.5, therefore it can be stated that polymer samples had a structure that was close to monodisperse [66].

Polymerization results obtained at 40˚C by the use of CR lipase were given in Table 4.10 depending on reaction time.

M_n value increased with reaction time and reached 11580 g/mol at the end of 24 hours reaction period (Table 4.10). However, after 24 hours M_n started to decrease. In addition, at the end of 24 hours polymerization reached 83.8% monomer conversion and began to decrease after this point. These were the results of degradation activity of enzyme [65]. Moreover, PDI values of polymer samples had increased after 24 hours which may result in increased heterogeneity of polymer samples [28].

Table 4.10: Polymerization results obtained at 40˚C by the use of CR lipase.

Time (h)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
6	29.6	5020	1.2
12	63.2	5540	1.6
18	71.0	8085	1.4
24	83.8	11580	1.3
48	83.1	7865	1.7
72	82.5	7300	1.7
120	81.5	7840	1.7

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

Polymerization results obtained at 60 ˚C by the use of CR lipase were given in Table 4.11 depending on reaction time. Based on Table 4.11, it can be said that, highest M_n value, which was 10840 g/mol, was obtained at the end of 48 hours. After 48 hours, reaction shifted from polymerization to degradation and molecular weight started to decrease.

Table 4.11: Polymerization results obtained at 60˚C by the use of CR lipase.

Time (h)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
6	45.7	6230	1.5
24	80.7	8280	1.6
48	85.6	10840	1.4
72	71.5	8370	1.8
120	70.3	7645	1.7

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

On the other hand, as seen from Figure 4.11, monomer conversion reached to its maximum value of 85.6% at the end of 48 hours and began to decrease after this point as a result of degradation activity of enzyme [65]. PDI values had increased after 48 hours which may result in increased heterogeneity of polymer samples [28].

Polymerization results obtained at 80˚C by the use of CR lipase were given in Table 4.12 depending on reaction time.

Table 4.12: Polymerization results obtained at 80˚C by the use of CR lipase.

Time (h)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
6	62.0	6300	1.5
24	80.2	9910	1.5
48	84.0	10900	1.5
72	83.9	9190	1.5
120	83.2	8770	1.7

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

As clearly seen from Table 4.12, M_n value increased with reaction time and reached 10900 g/mol. However, after 48 hours M_n started to decrease which was a result of degradation activity of enzyme [65]. In addition, highest monomer conversion was 84.0% which had been obtained at the end of 48 hours reaction. After 48 hours, conversion started to decrease as a result of the beginning of polymer degradation. Polymer samples were close to monodispersity since the PDI values were 1.5 until 72 hours reaction period [66]. After this point, PDI increased with reaction time and reached 1.7. This means heterogeneity of the polymer samples had increased [28].

In order to observe the effect of temperature on molecular weights of polymers obtained by CR lipase catalyzed polymerizations, Figure 4.19 was given.

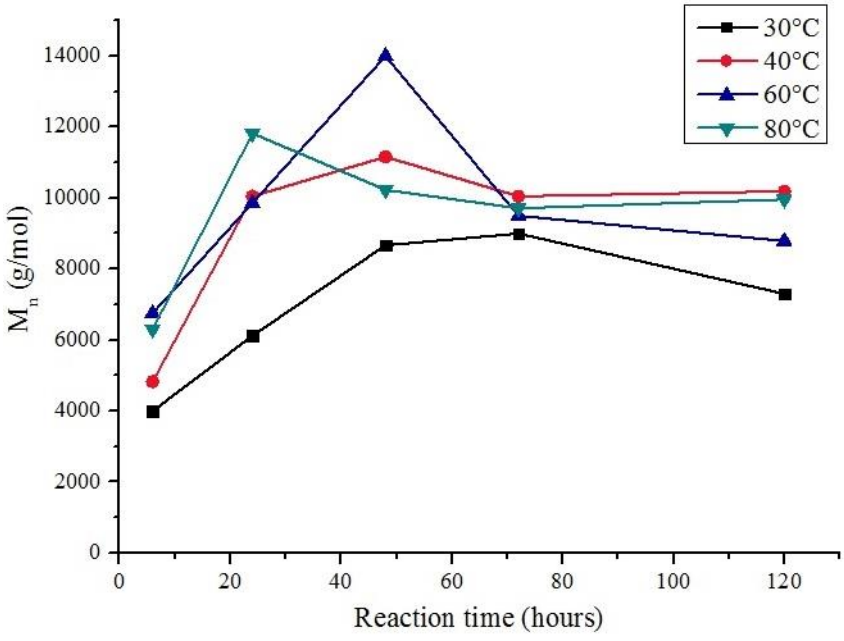

Figure 4.19: Effect of temperature on M_n of PCL synthesized by CR lipase.

To summarize based on Figure 4.19, highest molecular weight, which was 11580 g/mol, was obtained via CR lipase catalyzed polymerization at 40˚C at the end of 24 hours. In addition, as seen from the graph, molecular weights of polymers obtained at 30˚C were considerably high as in the case of AD lipase catalyzed reaction. For example, the highest molecular weight at 30˚C was obtained as 11100 g/mol at the end of 72 hours.

For the characterization of chemical structure of polymer sample synthesized via CR lipase, FT-IR analysis was carried out. The FT-IR spectrum of polymer obtained from CR lipase-catalyzed ROP of ε-CL at 40˚C and 24 hours was shown in Figure 4.20.

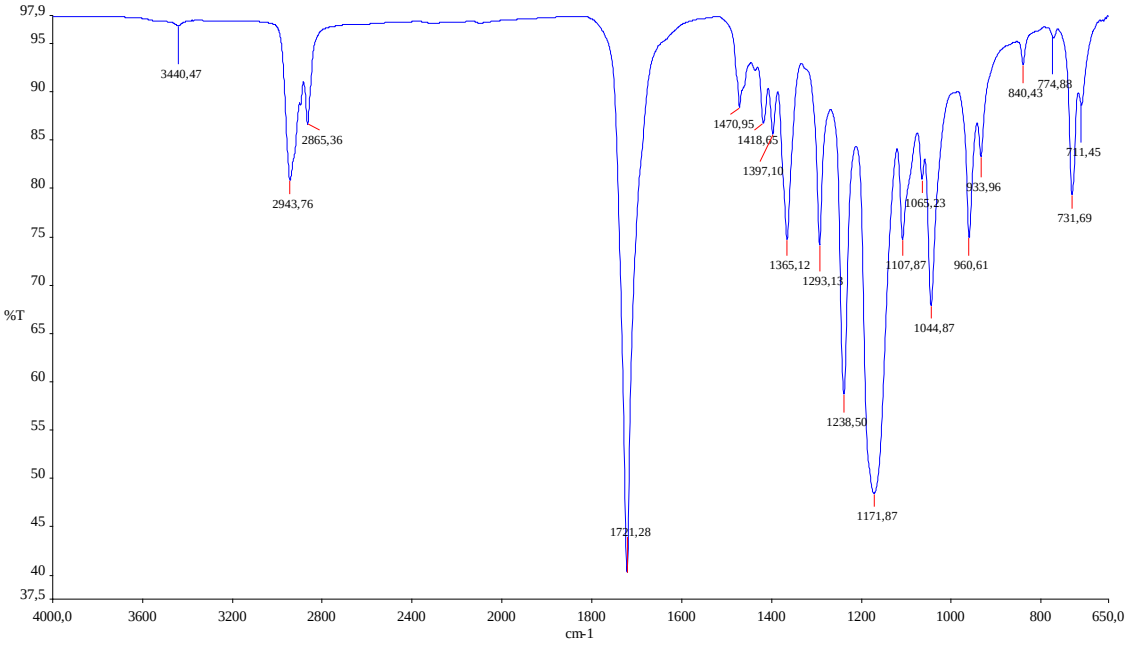

Figure 4.20: FT-IR spectrum of polymer sample synthesized via CR lipase.

The infrared bands of polymer sample synthesized by CR lipase was consistent with characteristic infrared bands of PCL, which are asymmetric CH_2 bonds at 2943 cm^{-1}, symmetric CH_2 bonds at 2865 cm^{-1}, carbonyl (C=O) bonds at 1721 cm^{-1}, C-O and C-C bonds seen in crystalline phase at 1293 cm^{-1}, asymmetric C-O-C bonds at 1238 cm^{-1}, and symmetric C-O-C bonds at 1171 cm^{-1} [44]. This situation proves that the synthesized polymer was PCL.

Chemical structure of polymer sample synthesized by CR lipase at 40˚C and 24 hours was further characterized by ^{1}H-NMR spectroscopy. The zoomed spectrum between 4.25 ppm and 3.5 ppm range, in which the characteristic peaks that were used for molecular weight calculation can be seen, was shown in Figure 4.21. The chemical

shifts (ppm) between 4.25 ppm and 3.5 ppm were as follow: 4.07 ppm (t, CH_2O) and 3.66 ppm (t, CH_2OH, end group) which are characteristic for PCL [53].

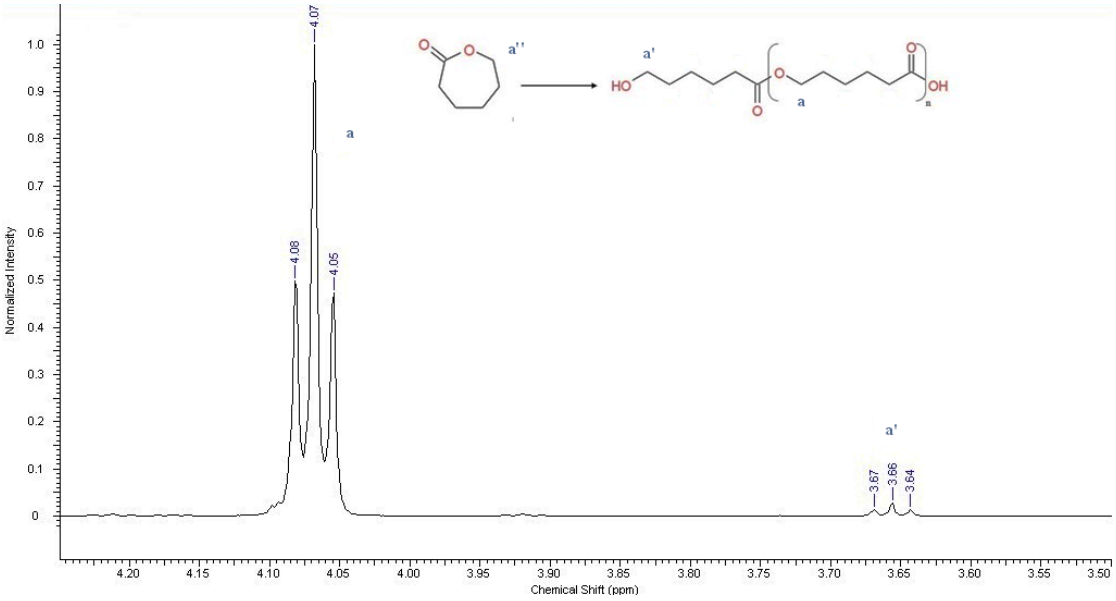

Figure 4.21: ^{1}H-NMR spectrum of polymer sample synthesized by CR lipase.

Moreover, molecular weight of the PCL sample was also calculated from ^{1}H-NMR spectrum. $M_{n,NMR}$ value was obtained as 11495 g/mol by using Equation 3.3.

For thermal characterization of polymer sample synthesized by CR lipase at 40˚C and 24 hours, TGA and DSC analyses were applied. TGA curves of polymer obtained from CR lipase-catalyzed ROP of ε-CL was given in Figure 4.22.

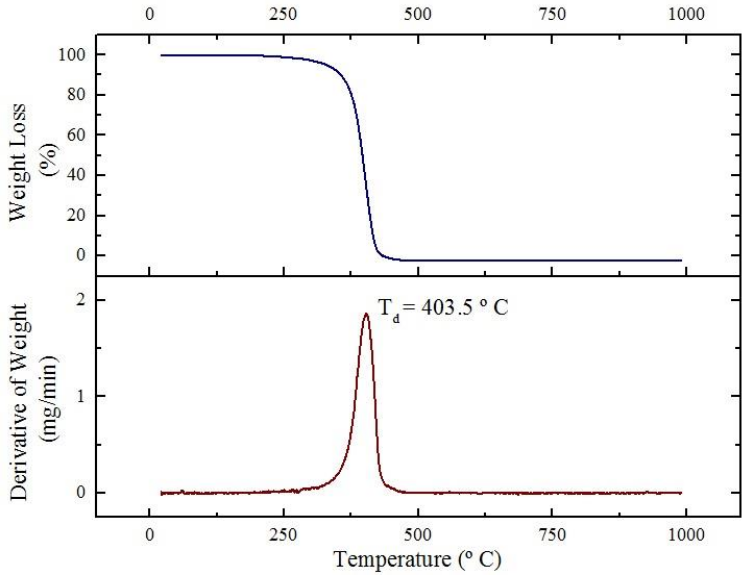

Figure 4.22: TGA curves (weight loss and derivative of weight) of polymer sample synthesized by CR lipase.

As seen from TGA curves given in Figure 4.22, degradation temperature (T_d) of PCL synthesized by CR lipase catalyzed polymerization reaction was determined as 403.5 ˚C, which makes the polymer highly thermal stable. This result was expected since the maximum degradation temperature of PCL has been given as 415˚C [67].

In addition to TGA, DSC was also applied for the determination of T_g, T_m, ΔH_m, and X_c values of PCL sample synthesized via CR lipase-catalyzed ROP of ε-CL at 40˚C and 24 hours. In Figure 4.23, DSC thermogram (endodown graphic) of this sample can be seen.

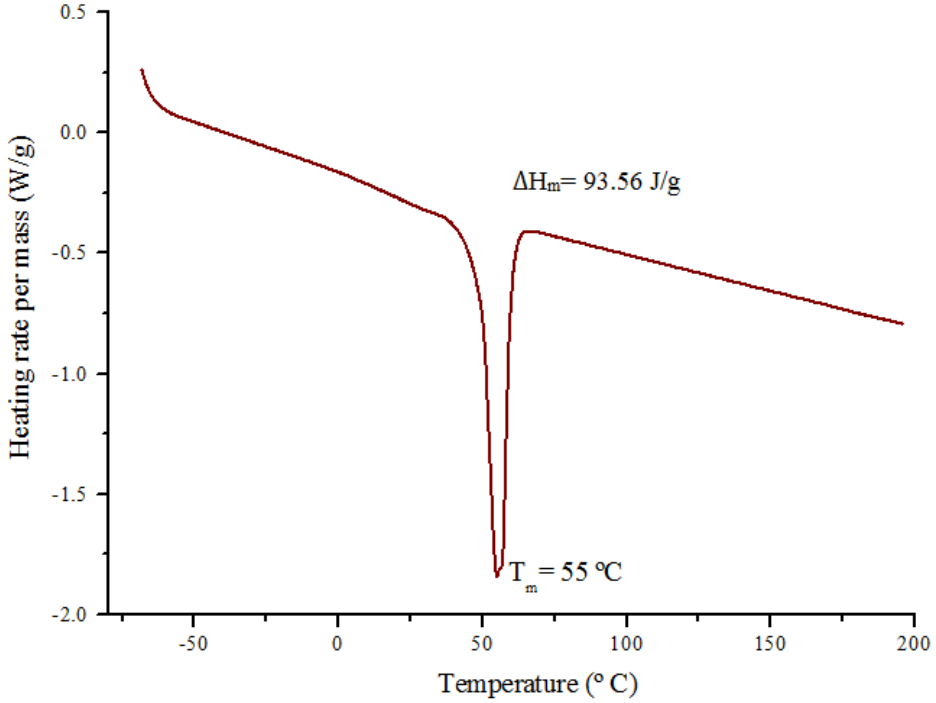

Figure 4.23: DSC thermogram of polymer sample synthesized by CR lipase.

From this DSC thermogram it was not possible again to see T_g value since it had a very low value which was under -50˚C. Moreover, T_m value of PCL sample was obtained as 55˚C. This low T_m value makes possible to be easily reshaped. From the area of melting peak, ΔH_m was computed as 93.56 J/g. By using Equation 3.2, crystallinity percentage (X_c) was calculated as 67%, which showed that the polymer sample had a semi-crystalline structure.

Surface structure of polymer sample synthesized by CR lipase at 40˚C and 24 hours was observed by SEM analysis. The obtained SEM images were shown in Figure 4.24.

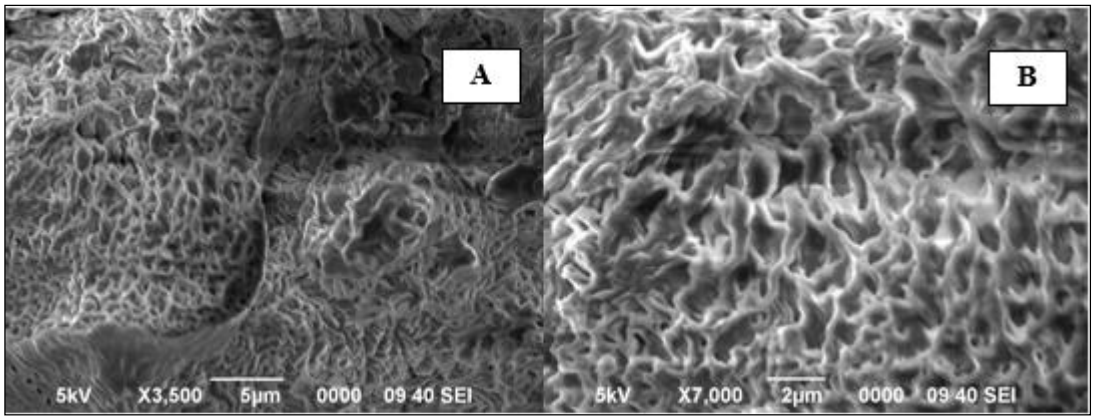

Figure 4.24: SEM images of polymer samples synthesized by CR lipase at 3500x (A) and 7000x (B) magnifications.

The polymer sample synthesized by CR lipase had also a foam-like structure (Figure 4.24). Therefore, PCL sample can be used as scaffold for tissue engineering successfully since its structure is suitable for cell attachment and growth, like the PCL sample synthesized by AD lipase [1, 40].

Furthermore, XRD analysis was carried out for the determination of crystal structure of polymer sample synthesized via CR lipase at 40˚C and 24 hours. XRD pattern of polymer sample was given in Figure 4.25.

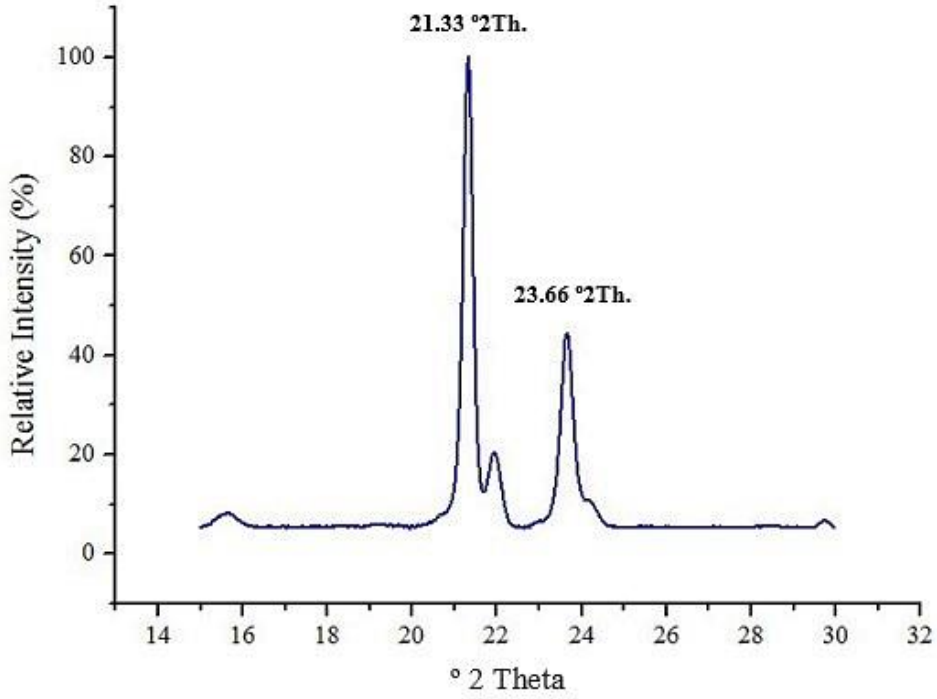

Figure 4.25: XRD pattern of PCL synthesized via CR lipase.

There existed strong diffraction peaks at 21.33° and 23° 2 Theta angles, as also observed for the polymer sample synthesized via AD lipase, which are characteristic for PCL (Figure 4.25). As seen from the XRD pattern, polymer sample was highly crystalline [68].

4.3.3 Synthesis via commercial lipases at best reaction conditions

At the best polymerization conditions of both AD and CR lipases, PCL synthesis was also carried out by using commercial lipases; Lipozyme® and Novozyme 435®. Commercial lipase-catalyzed polymerization results obtained at 60˚C for 48 hours and 40˚C for 24 hours reaction conditions were given in Table 4.13.

Table 4.13: Polymerization results obtained by the use of commercial lipases.

Reaction Conditions	Enzyme name	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
60˚C, 48 hours	Lipozyme®	52.0	3720	1.2
	Novozyme 435®	90.4	10630	1.5
	AD	87.6	14000	1.5
	CR	85.6	10840	1.4
40˚C, 24 hours	Lipozyme®	65.3	3380	1.2
	Novozyme 435®	83.4	12860	1.5
	AD	65.8	10050	1.5
	CR	83.8	11580	1.3

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

As seen from Table 4.13, M_n values obtained from the reaction carried out at 60˚C for 48 hours with Lipozyme® and Novozyme 435® enzymes were 3720 g/mol and 10630 g/mol, respectively. Lipozyme®, which is the free form of CALB enzyme, resulted in lower molecular weight and monomer conversion when compared with Novozyme 435®, which is the immobilized form of CALB. This may a result of enhanced enzyme activity and stability by immobilization [7, 9]. Moreover, PDI values of polymer samples were lower or equal to 1.5. Therefore, the resulted polymer samples had a monodisperse structure [66]. In addition, M_n values obtained from the reaction carried out at 40˚C for 24 hours with Lipozyme® and Novozyme 435® enzymes were 3380 g/mol and 12860 g/mol, respectively. The free lipase Lipozyme® resulted in lower

molecular weight and monomer conversion when compared with immobilized lipase Novozyme 435® as in the case of 60˚C and 48 hours reactions. Enhanced enzyme activity and stability after immobilization may the reason of this result [7, 9]. Moreover, at these conditions PDI values of polymer samples were lower or equal to 1.5, too. Therefore, it can be said that polymer samples have a structure that was close to monodisperse [66].

In Figure 4.26, comparison of performances of AD, CR, Lipozyme®, and Novozyme 435® enzymes were given based on molecular weights.

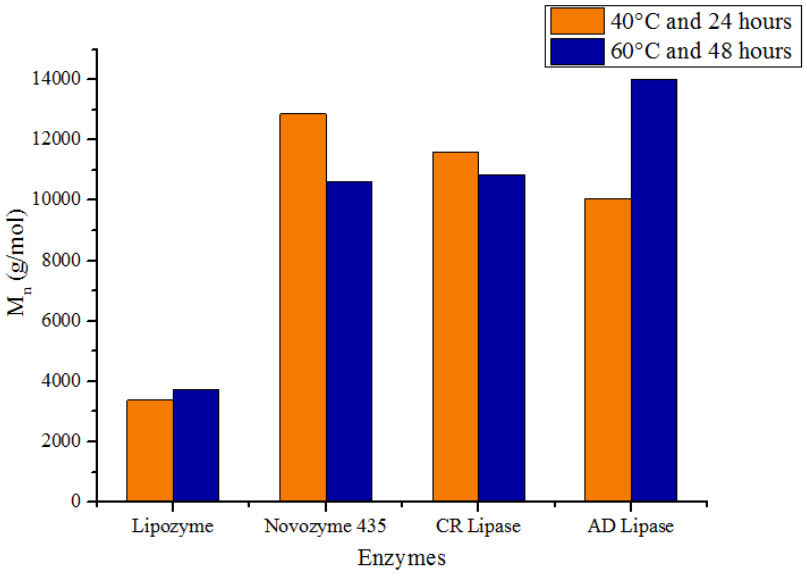

Figure 4.26: Performances of lipases based on molecular weights.

Based on Figure 4.26 it can be safely stated that, at 40˚C and 24 hours conditions highest molecular weight was obtained by using Novozyme 435®. However, CR lipase-catalyzed reaction resulted in a polymer with a very close molecular weight to that value. On the other hand, at 60˚C and 48 hours conditions AD lipase-catalyzed reaction resulted in a polymer sample with the highest molecular weight, which was even higher than Novozyme 435®. Moreover, at these conditions CR lipase-catalyzed reaction also resulted in higher molecular weight than Novozyme 435®-catalyzed reaction. As an expected result, Lipozyme®-catalyzed reactions was given rise to polymers with lowest molecular weights since it was not immobilized. As a result of being not immobilized, specific activity of Lipozyme® was the lowest of all (Figure 4.4). In addition, the molecular weight of polymer sample obtained by AD lipase-catalyzed reaction at its best conditions (60˚C and 48 hours) was higher than the molecular weight of polymer sample obtained by CR lipase-catalyzed reaction at its

best conditions (40˚C and 24 hours). This may a result of higher specific activity of AD lipase (Figure 4.2).

4.4 Parametric Study for ROP of ε-CL via Immobilized Lipases on RHA

Effect of enzyme concentration on polymerization was investigated for the best synthesis conditions of both two immobilized enzymes. In addition to 20% (w/w) enzyme concentration which was used in serial polymerization reactions, 2.5% (w/w), 5% (w/w), and 10% (w/w) concentrations were also tried for both two immobilized lipases. Since a higher enzyme concentration is not economic, polymerizations with a higher enzyme concentration were not performed.

Polymerization results obtained from AD lipase-catalyzed reaction at 60˚C for 48 hours with different enzyme concentrations was given in Table 4.14.

Table 4.14: Effect of enzyme concentration on polymerization reaction catalyzed by AD lipase.

Enzyme concentration (%) (w/w)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
2.5	23.1	6340	1.2
5	39.9	11020	1.6
10	78.1	11330	1.5
20	87.6	14000	1.5

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

As it was shown in Table 4.14, highest molecular weight and monomer conversion was obtained when AD lipase was used with a concentration of 20% (w/w). Monomer conversion and molecular weight decreased with decreasing the enzyme concentration.

Polymerization results obtained from CR lipase-catalyzed reaction at 40˚C for 24 hours with different enzyme concentrations was given in Table 4.15.

It can be clearly seen from Table 4.15 that, highest molecular weight and monomer conversion was obtained when CR lipase was used with a concentration of 20% (w/w). Monomer conversion and molecular weight decreased with decreasing the enzyme

concentration. Since the monomer conversion was too low for 2.5% (w/w) enzyme concentration, molecular weight could not be measured by GPC.

Table 4.15: Effect of enzyme concentration on polymerization reaction catalyzed by CR lipase.

Enzyme concentration (%) (w/w)	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
2.5	1.5	-	-
5	21.8	6230	1.3
10	48.8	7270	1.4
20	83.8	11580	1.3

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

4.5 Reuse performance of immobilized lipases for ROP of ε-CL

Recycle performances of immobilized lipases were determined by using them over again for ROP of ε-CL at the best polymerization conditions which were determined before for each enzyme. Polymerization results obtained from recycled AD lipase-catalyzed reaction at 60˚C for 48 hours were given in Table 4.16 depending on cycle numbers.

Table 4.16: Results obtained from recycled AD lipase-catalyzed polymerization.

Cycle No	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
1	87.6	14000	1.6
2	86.3	13830	1.5
3	76.9	11885	1.4
4	50.3	9570	1.4
5	18.0	4750	1.2
6	4.5	2550	1.1

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

As seen from Table 4.16, monomer conversions and molecular weights decrease with the further usage of recycled AD lipase. Based on this table it can be said that, AD

lipase can be reused four times to obtain polymer samples with about 50% monomer conversion and 9500 g/mol molecular weight. At the end of the sixth cycle, monomer conversion and molecular weight of polymer sample decreased to 4.5% and 2550 g/mol, respectively.

Moreover, activity of immobilized lipases were measured at the end of each polymerization reaction. Activity change of AD lipase with reaction cycles was shown in Figure 4.27.

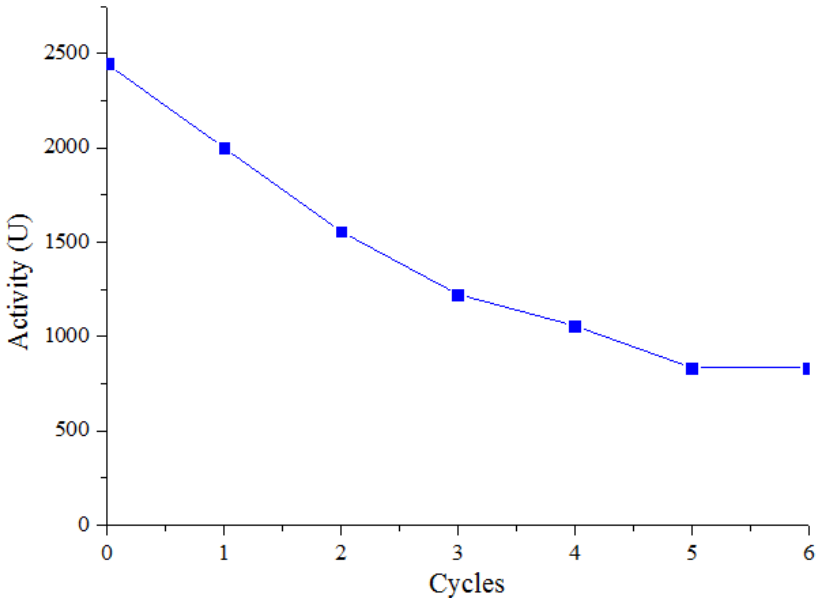

Figure 4.27: Reusability of AD lipase for PCL synthesis.

Activity of AD lipase decreased and became stable after fifth cycle. At the end of the sixth cycle, 34.1% of its initial activity was remained. Consequently, it can be safely stated that, AD lipase can be reused successfully for the catalysis of ROP of ε-CL about six times.

Polymerization results obtained from recycled CR lipase-catalyzed reaction at 40˚C for 24 hours were given in Table 4.17 depending on cycle numbers.

As shown in Table 4.17, further usage of recycled CR lipase for polymerization reactions resulted in decreased monomer conversions and molecular weights as in the case of further usage of recycled AD lipase. It can be said that, CR lipase can be reused three times to obtain polymer samples with over 50% monomer conversion and about 8500 g/mol molecular weight. At the end of the sixth cycle, monomer conversion and molecular weight of polymer sample decreased to 21.6% and 4950 g/mol, respectively.

Table 4.17: Results obtained from recycled CR lipase-catalyzed polymerization.

Cycle No	Conversion[a] (%)	M_n[b] (g/mol)	PDI[b] (M_w/M_n)
1	83.8	11580	1.4
2	63.0	8860	1.4
3	54.0	8500	1.5
4	40.4	6270	1.3
5	39.6	6500	1.3
6	21.6	4950	1.2

[a]Conversion was calculated gravimetrically
[b]M_n and PDI were obtained by GPC

Activity change of CR lipase with reaction cycles was shown in Figure 4.28.

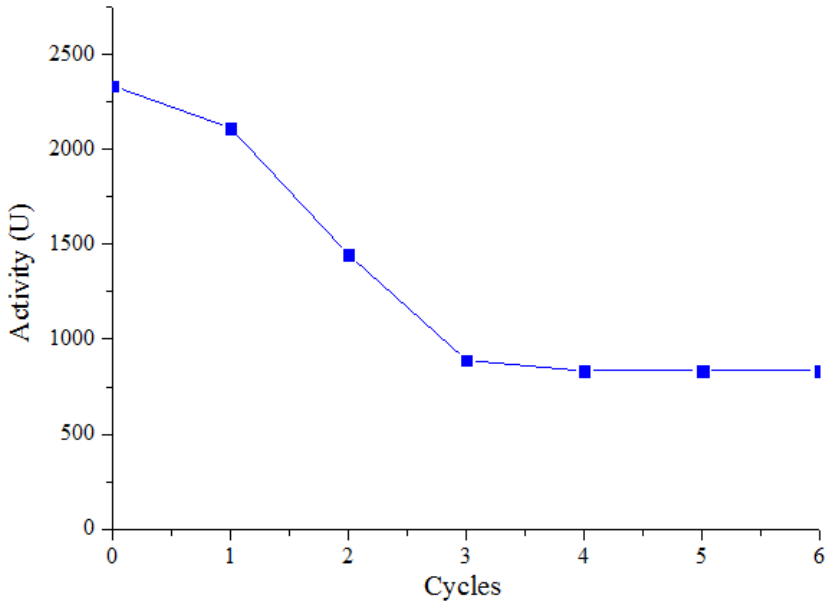

Figure 4.28: Reusability of CR lipase for PCL synthesis.

CR lipase lost its activity until fourth cycle and became stable after fourth cycle. 35.7% of its initial activity was maintained at the end of the sixth cycle. Based on these results it can be stated that, CR lipase can be reused successfully for the catalysis of ROP of ε-CL about six times.

If the reuse capacities of AD and CR lipases are compared, at the end of six polymerization cycles monomer conversion and molecular weight of polymer samples synthesized via recycled AD lipase were lower than polymer samples synthesized via

recycled CR lipase. Moreover, at the end of six cycles the remaining activity of CR lipase was a little higher than AD lipase. These results are consistent with literature since immobilization of enzymes by physical adsorption results from weak interactions. Therefore, enzymes may desorb easily from the support during reaction or washing processes which reduces reuse performance of immobilized enzymes. On the other hand, reuse capacity of cross-linked enzymes is higher since enzyme is strongly bound to the support [9, 23, 26].

5. CONCLUSIONS AND RECOMMENDATIONS

This study is composed from two parts; lipase immobilization on surface-modified RHA via physical adsorption and cross-linking methods and PCL synthesis by the use of this new immobilized lipases. For the addition of $–NH_2$ groups to the surface of the silica-based material RHA, 3-APTES was used and surface modification of RHA was achieved. The free form of CALB was immobilized after this step via physical adsorption and cross-linking with glutaraldehyde. For the evaluation of these new catalysts, their activities, immobilization efficiencies, storage stabilities, optimum activity pH and temperatures were determined. In addition, effect of some crucial parameters (3-APTES concentration, glutaraldehyde concentration, enzyme to support ratio) on immobilization efficiencies and activities were investigated. FT-IR and TGA analyses were applied to confirm the contribution of $–NH_2$ groups to the surface of RHA. Moreover, FT-IR was also used for the comparison of each immobilization step. In addition, the surface morphology of RHA and immobilized lipases were compared by using SEM.

ROP of ε-CL was catalyzed by AD and CR lipases to evaluate the performances of new enzymes. For this aim, polymerization reactions were carried out in toluene at different temperatures (30, 40, 60, and 80 °C) for different reaction periods (6, 24, 48, 72, and 120 hours). At optimum reaction conditions of each enzyme, effect of enzyme concentration, reuse stability of enzymes, and PCL synthesis with commercial lipases (Lipozyme® and Novozyme 435®) were performed. Characterizations were also applied to the polymer samples obtained at optimum reaction conditions of each immobilized lipase. Chemical structure of polymer samples were characterized by ^{1}H-NMR and FT-IR analyses. Thermal properties were obtained by DSC and TGA. Moreover, molecular weights and polydispersity indexes were achieved by the use of GPC. For the characterization of crystal structures of polymer samples, XRD was used. In addition, surface morphologies of polymer samples were observed with SEM.

In the lipase immobilization part of this study, firstly activated RHA was investigated for the $–NH_2$ group contribution by the use of FT-IR and TGA. According to FT-IR

spectrum, there exists a peak at 3050 cm^{-1} which belongs to NH_2 asymmetric stretch bonds [57, 58]. In addition, there is an additional weight loss (when compared with bare RHA) at about 180-190 $^{\circ}$C at which 3-APTES evaporates [58]. Based on this results, it can be said that, silanization of the support material was successfully achieved. After characterization of the activated-RHA, by performing FT-IR analysis contribution of glutaraldehyde and lipase to the structure were determined. There observed peaks at 1720 cm^{-1}, which belongs to C=O groups, after addition of glutaraldehyde and the intensity of peak decreased after lipase immobilization since one of the C=O groups of glutaraldehyde is consumed by interacting with the amino group of enzyme [34, 57]. On the other hand, other aldehyde group interacts with $–NH_2$ groups of the activated RHA and forms imine (C=N) structure, which gives peak at about 1690 cm^{-1} and may partially overlap with C=O stretching in the spectra of glutaraldehyde treated activated RHA and CR lipase [26, 34, 57]. Since in physical adsorption method glutaraldehyde was not used, there was no peak formation at this region of AD lipase spectrum. In addition, there observed weak peaks after immobilization (with both two methods) at 1365 cm^{-1} and 1215 cm^{-1} which may belong to N-terminus (C-N stretch) and C-terminus (C-O stretch) of lipase, respectively [61].

Immobilization efficiencies and activities of immobilized lipases were also determined. Free lipase was immobilized on surface-modified RHA via both two methods with high efficiency (about 90%). After immobilization of free lipase via physical adsorption and cross-linking methods, 92.3% and 78.8% of its activity was remained, respectively. As seen, the activity of AD lipase was higher than CR lipase since the active sites of enzyme may be blocked by cross-linking [25]. Moreover, specific activity of free enzyme was increased from 4.5 U/mg to 8.1 U/mg and 9.2 U/mg after immobilization via cross-linking and physical adsorption methods. Since Novozyme 435®, which is known with its high catalytic activity for PCL synthesis, has a specific activity of 10 U/mg, immobilized lipases on RHA is promising for PCL synthesis [14, 62].

For the determination of storage stability of immobilized lipases that kept at 4 $^{\circ}$C, activity measurements were carried out for 2 months. At the end of 2 months, AD and CR lipases maintained 65% and 55% of their activities, respectively. In addition, effects of pH and temperature on activity were also investigated. Activities of lipases

were measured in buffers between pH 4.5 and pH 10.5. Optimum pH value was obtained as 7.0 which is same with the optimum pH value of CALB [28]. Similarly, activity measurements were carried out at incubation temperatures between 30 °C and 60 °C. Optimum temperature of both two enzymes were determined at about 37 °C at which many type of lipases also reach their highest activities [6, 7, 27]. As parametric studies, additional 3-APTES (5%, 10%, and 20%), glutaraldehyde (0.02% and 2%) concentrations and enzyme to support ratios (0.5 μL/mg, 1 μL/mg, and 3 μL/mg) were also tried. At the end, highest relative and specific activities were obtained at 15% 3-APTES concentration which was used in this study. Moreover, it was seen that, relative and specific activities decreased with the increased glutaraldehyde concentration since the active site of enzyme may be blocked by cross-linking [25]. However, in this study a moderate concentration of 0.2% was used in order to obtain a balance between decreased activity and increased reuse stability [26]. Furthermore, the highest relative activity was obtained when the enzyme to support ratio was 2 μL/mg, which was used in this work. On the other hand, immobilization efficiency and specific activity were decreased with the increased loading ratio since multiple layers are formed on the support at higher loading ratios. This structure results in inaccessible proportions of enzyme which may not contribute to the activity [64].

In the PCL synthesis part of this study, molecular weight distributions of polymer samples (synthesized via both AD and CR lipase-catalyzed polymerizations) depending on reaction time were obtained for each reaction temperature. According to GPC results, AD lipase-catalyzed ROP of ε-CL gave rise to highest molecular weighted PCL (14000 g/mol) at 60 °C and 48 hours. On the other hand, highest molecular weight was obtained at 40 °C and 24 hours as 11580 g/mol when ROP of ε-CL was catalyzed by CR lipase. Furthermore, molecular weights obtained at 30 °C reactions were considerable for a polymerization reaction that performed at such a low temperature. Therefore, this process can be considered as energy-saving besides being environmentally friendly. Moreover, polydispersity indexes were also obtained from GPC. The values were around 1.5 which means the synthesized polymer samples were monodisperse [66].

Performances of new immobilized lipases were compared with commercial lipases (Lipozyme® and Novozyme 435®) and with each other based on molecular weights of polymer samples obtained at the best reaction conditions of both two immobilized

lipases. At 40 °C and 24 hours condition, highest molecular weight was reached when Novozyme 435® was used (12860 g/mol). However, molecular weight of CR-lipase catalyzed PCL was close to this value (11580 g/mol). At 60 °C and 48 hours condition, highest molecular weight was obtained when AD lipase was used (14000 g/mol) which is even higher than the polymer obtained from Novozyme 435® catalyzed reaction (10630 g/mol). At both two conditions, Lipozyme® catalyzed ROP of ε-CL resulted in the lowest molecular weights (3380 g/mol and 3720 g/mol, respectively) since free enzymes have lower activity and stability [7, 9]. Moreover, when AD and CR lipases were compared with each other at their best reaction conditions, AD lipase gave rise to a higher molecular weighted PCL since its specific activity was higher.

As a parametric study, effect of enzyme concentration on molecular weight was investigated. For this aim 2.5%, 5%, 10%, and 20% concentrations were tried. It was seen that, molecular weights were increased with the increased enzyme concentration. Therefore, 20% enzyme concentration, which was used in this study, was found to be suitable. Higher enzyme concentration was not tried since it may not economic.

For the determination of reuse stability of AD and CR lipases, six polymerization cycles were performed (at their best reaction conditions) by using enzymes over again. At the end of sixth cycle, monomer conversion and molecular weight of polymer sample synthesized via AD lipase decreased to 4.5% and 2550 g/mol, respectively. Similarly for CR lipase-catalysis, monomer conversion was 21.6% and molecular weight was 4950 g/mol after decrement through six cycles. In addition, 34.1% and 35.7% of initial activities of AD and CR lipases were remained at the end of sixth cycle. Since immobilization of enzymes by physical adsorption results from weak interactions rather than strong covalent linkages, enzymes may desorb easily during reaction or washing processes. Therefore, reuse performance of AD lipase was lower than CR lipase [9, 23, 26].

Characterizations were applied to highest molecular weighted polymers obtained from the best reaction conditions of AD and CR lipases. Molecular structure of polymer samples were determined by FT-IR and ^{1}H-NMR analyses. Since the results were consistent with literature, it was proved that obtained polymers were PCL [44, 53]. From TGA analysis T_d of polymer samples resulted from AD and CR lipase catalyzed reactions were determined as 411.6 °C and 403.5 °C, respectively. This result means that, obtained PCL samples were highly thermal stable. T_m values of PCL samples

were attained from DSC analysis as 53.3˚C (AD lipase catalyzed) and 55˚C (CR lipase catalyzed). In addition, X_c values of PCL samples were also calculated from DSC thermograms as 60% (AD lipase catalyzed) and 67% (CR lipase catalyzed). Based on these results it can be said that, PCL samples were semi-crystalline. In addition, crystal structure of PCL samples were further verified and characteristic crystalline peaks were observed by XRD analysis. Finally, surface morphologies of PCL samples were observed by SEM. SEM images showed that, surface of PCL samples had a foam-like structure which is suitable for cell attachment and growth when they are used for tissue engineering applications [1, 40].

In conclusion, new immobilized lipases were obtained with good storage and reuse stabilities in this study. Moreover, activity of free CALB was enhanced for extreme pH and temperature conditions after immobilization with both methods. In addition, rice husk ash, which is a by-product of rice production, was shown to be evaluated for immobilization of CALB since it acted as a porous silica. Therefore, rice husk ash was suggested to be a cheap and useful alternative support material for lipase immobilization. Moreover, it was also shown that, the newly immobilized lipases were successful for the catalysis of ROP of ε-caprolactone. Furthermore, it is possible to develop a new nonohybrid structure that can be applied to biomedical devices which was examined before for montmorillonite and sepiolite [54].

In further studies, amount of $–NH_2$ added after silanization can be calculated from elemental analysis and compared for different 3-APTES concentrations. In addition to 3-APTES, different silanization agents such as 3-APTMS and 3-GPTMS can be tried for the achievement of surface modification. Moreover, effect of immobilization time and temperature on immobilization efficiency and enzyme activity can be investigated.

In this study, monomer conversion was calculated gravimetrically after removing unreacted monomer. It would be more precise if sample was taken from the reaction medium at the end of each reaction to monitor monomer conversion by ^{1}H-NMR analysis. Moreover, T_g values could not be determined since it was not possible to cool the samples to a lower temperature during DSC analysis. By using a DSC apparatus that applies cooling with liquid nitrogen may overcome this problem. Finally, different toluene ratios can be tried during polymerization reactions to enhance monomer conversions and molecular weights.

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- **Ulker, C.,** Gokalp, N., and Guvenilir-Avcibasi, Y., 2015: Ring Opening Polymerization of ε-caprolactone by a Novel Enzymatic Catalyst *Candida antarctica* Lipase (CALB L) Immobilized on a Modified Silica-Based Material by Cross-linking. *EPF 2015*, June 21-26, 2015 Dresden, Germany. (Oral Presentation)
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