ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IMMOBILIZATION OF LIPASE ENZYME ONTO PRECIPITATED SILICA AND SYNTHESIS OF POLYCAPROLACTONE

M.Sc. THESIS

Nurefşan GÖKALP

Department of Chemical Engineering

Chemical Engineering Programme

DECEMBER 2015

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IMMOBILIZATION OF LIPASE ENZYME ONTO PRECIPITATED SILICA AND SYNTHESIS OF POLYCAPROLACTONE

M.Sc. THESIS

Nurefşan GÖKALP
(506131026)

Department of Chemical Engineering

Chemical Engineering Programme

Thesis Advisor: Prof. Dr. Yüksel GÜVENİLİR

DECEMBER 2015

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

LİPAZ ENZİMİNİN ÇÖKTÜRÜLMÜŞ SİLİKA ÜZERİNE İMMOBİLİZASYONU VE POLİKAPROLAKTON SENTEZİ

YÜKSEK LİSANS TEZİ

Nurefşan GÖKALP
(506131026)

Kimya Mühendisliği Anabilim Dalı

Kimya Mühendisliği Programı

Tez Danışmanı: Prof. Dr. Yüksel GÜVENİLİR

ARALIK 2015

Nurefşan GÖKALP, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID 506131026, successfully defended the thesis entitled "IMMOBILIZATION OF LIPASE ENZYME ONTO PRECIPITATED SILICA AND SYNTHESIS OF POLYCAPROLACTONE", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Yüksel GÜVENİLİR** Istanbul Technical University	.....
Jury Members :	**Prof. Dr. Nuran DEVECİ AKSOY** Istanbul Technical University	.....
	Assoc. Prof. Dr. Didem SALOĞLU Yalova University	.....

Date of Submission : 27 November 2015
Date of Defense : 24 December 2015

To my grandmother and family,

FOREWORD

To start, I would like to thank to my supervisor Prof. Dr. Yüksel Güvenilir for her suggestions, encouragement, support and guidance in writing the thesis. She was always behind me with her technical and moral advices during my thesis study. I feel myself lucky due to that I met with her and was a part of ITU-family.

I would also like to say that I am very grateful to my lab-mate Cansu Ülker for her support, encouragement and friendship throughout my thesis study. She was always helpful, and beside me with her suggestion and advice all the time. I would also like to thank Nazif Uğur Kaya for his guidance in my laboratory studies. He was always very patient and helpful, and supported me with his moral and technical advices.

I would like to acknowledge 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.

I would like to thank to my dear friends Özlem Keleş and Damla Gizem Arslan for their friendship, moral advice and support during my thesis period and all my life. In addition, I am so grateful to Masoud Teymourfamianasl, İlayda Oksal and Elvan Aydın who supported me with their moral advices and kindly friendship.

It is a pleasure to express my gratitude to all my family, my dear father Erdin Gökalp and mother Zübeyde Gökalp for their endless love, great support, encouragement, understanding, patience and presense in any condition beside me. I am also thankful to my dear sister Betül Gökalp for her support, endless mirth and smile. Words would be insufficient to describe the feelings that I grow for them in my heart.

December 2015

Nurefşan GÖKALP
(Chemical Engineer)

TABLE OF CONTENTS

Page

ABBREVIATIONS

APTES **:** Aminopropyltriethoxysilane
BC : *Burkholderia cepacia*
CA : *Candida antarctica*
CALB : *Candida antarctica* lipase B
CLEAs : Crosslinked Enzyme Aggregates
CLECs : Crosslinked Enzyme Crystals
CRL : *Candida rugosa* lipase
DSC : Differential Scanning Calorimetry
EC : Enzyme Class
ε-CL : ε-caprolactone
ε-KL : ε-kaprolakton
EM : Enzyme-activated Monomer
FTIR : Fourier Transform Infrared Spectroscopy
GPC : Gel Permeation Chromatography
^{1}H NMR : Proton Nuclear Magnetic Resonance Spectroscopy
IEc : Immobilized enzyme by crosslinking
IEa : Immobilized enzyme by physical adsorption
N-435 : Novozym® 435
PCL : Polycaprolactone
PDI : Polydispersity
PDLA : Poly(D,L-lactide)
PF : *Pseudomonas fluorescens*
PGA : Polyglycolide
PHAs : Poly(α-hydroxy acids)
PHB : Poly(β-hydroxybutyrate)
PKL : Polikaprolakton
PLA : Polylactide
PPL : Porcine Pancreatic Lipase
ROP : Ring opening polymerization
SEM : Scanning Electron Microscope
TGA : Thermal Gravimetric Analysis
THF : Tetrahydrofuran
UV : Ultraviolet

LIST OF SYMBOLS

E	**:** Yang modulus
$\mathbf{M_{n,NMR}}$	**:** Number average molecular weight calculated from ^{1}H-NMR
Mn	**:** Number average molecular weight
Mw	**:** Weight average molecular weight
Td	**:** Degradation temperature
Tg	**:** Glass transition temperature
Tm	**:** Melting temperature
ΔH	**:** Enthalpy
ε	**:** Elongation
η_{inh}	**:** Inherent viscosity
η	**:** Intrinsic viscosity
σ	**:** Tensile strength
χ	**:** Crystallinity percentage

LIST OF TABLES

Page

LIST OF FIGURES

Page

IMMOBILIZATION OF LIPASE ENZYME ONTO PRECIPITATED SILICA AND SYNTHESIS OF POLYCAPROLACTONE

SUMMARY

Well-defined aliphatic polyesters with end-functionalities, such as poly(e-caprolactone) (PCL) has been known as an important material in a range of biomedical applications because of its fascinating characteristics, like biodegradability and permeability. In the PCL synthesis, organometallic catalysts with stress on tin carboxylates and aluminium alkoxides were generally used as catalysts. However, residues of organometallic catalysts cannot tolerate in biomedical applications by reason of their toxicity. Lately, non-metal catalysts from natural sources like lipases were progressively more employed as biocatalysts for PCL synthesis. Furthermore, mild reaction conditions, high enantio- and regioselectivity, and reusability of enzyme provided it an superiority over conventional chemical polymerization.

Among lipases, the lipase obtained from *Candida antarctica* fraction B (CALB) is one of the most commonly utilized lipases. It is commercially available in both free and immobilized form, and has high degree of selectivity in a wide range of synthetic applications of industry importance, comprising kinetic resolutions, aminolysis, esterification and transesterification. By enzyme immobilization, catalysts can be advanced with important advantages compared to the free enzyme. Many literature reports define the high benefit of immobilized CALB for chemical transformations of low-molar-mass compounds and polymerization reactions.

In the first part of this master thesis, an inorganic support material precipitated silica was used as a carrier to immobilize lipase B from *Candida antarctica* by both crosslinking and physical adsorption methods. Precipitated silica with mesoporous structure was chosen because of its suitability for CALB immobilization over other inorganic and organic carriers, and it did not use before to immobilize CALB. Accordingly, it is aimed to determine the performance of precipitated silica on CALB immobilization process. By performing two different immobilization techniques, the more effective method was also determined for the immobilization reactions. The aminosilane (3-aminopropyl)triethoxysilane (APTES) and the crosslinker glutaraldehyde were used to modify CALB. The ratios of 3-APTES/acetone solution were adjusted to 5 %, 10 %, 15 %, 20 % (w/v), whereas the ratios of glutaraldehyde/phosphate buffer solution were regulated as 0.02 %, 0.2 % and 2 % (v/v), respectively. After that, these modified support materials were used for immobilization reactions by adjusting the ratios of enzyme/silica as 0.5 (w/w), 1 (w/w), 2 (w/w), 3 (w/w). In this way, the effect of 3-APTES, glutaraldehyde and lipase concentrations on immobilization processes were researched to define the most effective amounts of these materials for immobilization reactions. The activity and protein content of immobilized lipases were calculated by alkalimetric final titration method and ultraviolet spectrophotometer (UV). The results showed that

adsorbed lipase possessed higher activity than crosslinked lipase, which were about 4330 and 2830 U, respectively. The highest activity for both immobilized enzymes were obtained at the 3-APTES/acetone solution ratio of 15 % (w/v), without glutaraldehyde solotion (by physical adsorption) and enzyme/silica ratio of 2 (w/w). Both crosslinked and adsorbed immobilized lipases were very stable, protecting 59 and 50.1 % of their initial activity after 4 months storage at 4 ºC. In addition, the activities of immobilized lipases were calculated at pH values of 4.5, 5.5, 6.5, 7, 7.5, 8.5, 9.5 and 10.5 and temperature values of 30, 35, 37, 40, 45, 50, 55 and 60 ºC to determine the optimum pH and optimum temperature. The highest activity for adsorbed lipase was obtained at 37 ºC and a pH value between 7-8, while the optimum results was determined as around 35-37 ºC and a pH value of 7 for crosslinked lipase. Besides, the activities of immobilized enzymes were compared to the activities of free and commercial immobilized CALB (Novozym® 435). According to the results, it is shown that the activities of both immobilized lipases were higher than the free CALB, whereas only the activity of adsorbed lipase could approach the activity of Novozym® 435. Finally, precipitated silica and immobilized enzymes were successfully characterized by fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA), scanning electron microscopy (SEM) analyses.

In the second part of this study, the immobilized lipases were used as catalysts to polymerize ε-caprolactone (ε-CL), 7-membered lactone, via ring opening polymerization. Reactions were carried out at 40, 60, 80 ºC and 6, 24, 48, 120, 150 and 170 hour to investigate the optimum temperature and time period for PCL synthesis. Moreover, the ratio of enzyme to ε-caprolactone was adjusted to 2.5 %, 5 %, 10 % and 20 % (w/w), and the optimum enzyme concentration was determined for polymerization reactions catalyzed by both adsorbed and crosslinked enzymes. Molecular weights of PCLs were determined by using gel permeation chromatography (GPC) and hydrogen nuclear magnetic resonance (^{1}H-NMR) analysis. The results showed that high molecular weights and monomer conversions of PCL catalyzed by immobilized enzyme by physical adsorption were achieved about 14300 g/mol, and 90 %, respectively. On the other hand, high molecular weights and monomer conversions of PCL catalysed by using immobilized enzyme by crosslinking were obtained about 9000 g/mol and 90 %, respectively. Furthermore, the highest molecular weights were obtained in a reaction conducted at 20 % (w/w) enzyme concentration. Reusability of both immobilized lipases were determined also by reaction cycles of polycaprolactone synthesis, and after 5 cycles uses, about 32 and 42 % of the initial activity could be retained for immobilized enzyme by physical adsorption and crosslinking methods, respectively. Additionally, the results obtained by using both immobilized lipases were compared to the molecular weights noted by using free CALB and Novozym® 435. According to this comparison study, both immobilized lipases provided higher molecular weights than the values acquired by using free CALB. Adsorbed lipase exceeded the performance of Novozym® 435, while crosslinked lipase approached about 70 % to the molecular weight obtained by using Novozym® 435. Finally, the surface morphologies of PCLs were analyzed by scanning electron microscopy. Besides, PCLs were successfully characterized by fourier transform infrared spectroscopy, thermal gravimetric analysis and differential scanning calorimetry (DSC) analysis. The characteristic peaks of PCL were shown on the graphs of these analyses.

All in all, this study demonstrated immobilization of lipase improves many properties like reusability, activity and stability of free lipase. The activity of lipases increased dramatically after immobilization for both immobilized lipases compared to the activity of free CALB. Further, both immobilized enzymes was used repeatedly without a significant amount loss of their initial activity. Thus, usage of lipases in industrial applications can become more economical, and this work can contribute to “eco-friendly processes” used enzymes. Lastly, this study showed also that polycaprolactone can be synthesized via green friendly methods with high molecular weights and monomer conversion rates, and it can be definitely said that this master thesis will be contributed green polymer chemistry.

LİPAZ ENZİMİNİN ÇÖKTÜRÜLMÜŞ SİLİKA ÜZERİNE İMMOBİLİZASYONU VE POLİKAPROLAKTON SENTEZİ

ÖZET

Polikaprolakton (PKL), alifatik polyersterler sınıfından olan sentetik bir polimerdir ve sentetik özellikte olmasına rağmen biyouyumluluk ve geçirgenlik gibi özellikleri sayesinde biyomedikal uygulamalar başta olmak üzere oldukça geniş bir uygulama alanına sahiptir. PKL sentezi için genel olarak kalay karboksilatlar ve alüminyum alkoksitler gibi organometalik katalizörler kullanılmaktadır; ancak sentez sonrasında bu katalizörlerin kalıntılarını ortamdan tamamen ayırmak mümkün değildir. Bu kalıntılar zehirli içerikleri nedeniyle, özellikle biyomedikal uygulamalarda tolere edilemeyecek miktardadırlar. Son yıllarda, metal katalizörler yerine lipazlar gibi birçok biyokatalizör PKL sentezi için kullanılmaya başlanmıştır. Lipaz katalizörlüğünde gerçekleştirilen enzimatik polimerizasyon reaksiyonları, yüksek enantio ve regioseçiçilik, enzimin tekrar tekrar kullanılabilir özellikte olması ve ılımlı reaksiyon koşulları gibi özellikleri sayesinde geleneksel kimyasal polimerizasyon reaksiyonlarına göre daha avantajlı bir seçenek haline gelmiştir.

Candida antarctica lipaz B, hem serbest formda hem de immobilize formda en fazla kullanılan lipazlardan biridir. Yüksek seçicilik özelliği ile aminoliz, esterifikasyon ve transesterifikasyon gibi birçok reaksiyonu katalizleyebilme özelliğine sahiptir. Diper enzimler gibi lipazların da destek bir malzemeye immobilize edilmesi ile, serbest formdaki lipazın stabilite, tekrar kullanılabilirlik ve seçicilik gibi özellikleri geliştirilebilmektedir. Yapılan birçok çalışma, immobilize formdaki CALB'nin polimerizasyon reksiyonlarında daha etkin olduğunu göstermiştir.

Bu çalışmanın ilk aşamasında; *Candida antarctica* lipaz B, çapraz bağlama ve fiziksel adsorpsiyon yöntemleri ile immobilize edilmiştir. İki farklı immobilizasyon tekniğinin kullanılmasıyla, daha etkin olan yöntemi belirlemek hedeflenmiştir. Daha önce yapılan çalışmalar incelenerek; silika bazlı malzemelerin CALB immobilizasyonu için daha uygun olduğu görülmüş ve bundan dolayı mezopor yapıya sahip olan çöktürülmüş silika, bu çalışmada taşıyıcı malzeme olarak seçilmiştir. Ayrıca bu çalışmada çöktürülmüş silika, ilk defa CALB immobilizasyonunda kullanılmıştır. Çalışmada 3-aminopropiltrietoksisilan ve glutaraldehit, sırasıyla silanlama ajanı ve çapraz bağlayıcı olarak kullanılmıştır. 3-APTES/aseton oranı % 5, % 10, % 15 ve % 20'ye; glutaraldehit/fosfat tampon çözeltisi oranı da % 0.02, % 0.2 ve % 2'ye ayarlanarak; optimum 3-APTES ve glutaraldehit oranları belirlenmiştir. Daha sonra CALB, silanlanmış ve çapraz bağlanmış taşıyıcı malzemeler üzerine, enzim/silika oranı 0.5, 1, 2 ve 3'e ayarlanarak immobilize edilmiştir. Böylelikle, optimum lipaz konsantrasyonu da belirlenmiştir. Lipaz aktivitesi ve protein tayini, alkalimetrik titrasyon ve ultraviyole spektrofotometre ile hesaplanmıştır. Elde edilen sonuçlara gore, fiziksel adsorpsiyon ile immobilize edilen enzimin çapraz bağlama ile immobilize edilen enzyme gore daha aktif olduğu söylenebilir. Aktivite değerleri adsorplanan ve çapraz bağlanan

enzimler için sırasıyla: yaklaşık olarak 4330 ve 2830 U olarak hesaplanmıştır. Her iki immobilize enzim için de, optimum 3-APTES/aseton ve lipaz konsantrasyonu, % 15 ve 2 olarak belirlenmiştir. Kullanılan glutaraldehit miktarı arttıkça ise, enzim aktivitesinin düştüğü görülmüştür. Buna göre glutaraldehit kullanılmadan, yani fiziksel adsorpsiyon yöntemi ile yapılan immobilizasyon işlemi sonucu, daha aktif enzimler elde edilmiştir. İmmobilize edilen enzimlerin raf ömrünü belirlemek için 4 ay boyunca düzenli olarak aktivite ölçümü yapılmıştır. Elde edilen sonuçlara göre, çapraz bağlanan ve adsorplanan lipazlar sırasıyla: % 59 ve % 50.1 oranında başlangıç aktivitelerini korumuşlardır. Ek olarak, lipaz aktiviteleri 4.5, 5.5, 6.5, 7, 7.5, 8.5, 9.5 and 10.5 pH değerlerinde ve 30, 35, 37, 40, 45, 50, 55 ve 60 ºC'de ölçülmüş ve optimum pH ve sıcaklık değerleri belirlenmiştir. Fiziksel adsorpsiyon ile immobilize edilen enzim için en yüksek aktivite yaklaşık 7-8 pH değerlerinde ve 37 ºC'de elde edilirken; çapraz bağlama ile immobilize edilen lipaz için 7 pH değeri ve yaklaşık 35-37 ºC optimum değerler olarak belirlenmiştir. Bunun yanında immobilize edilen enzimlerin aktiviteleri, CALB'nin serbest ve ticari immobilize formu (Novozym® 435)'nun aktiviteleri ile de karşılaştırılmıştır. Sonuçlara gore, her iki immobilize enzimin de aktivitesi serbest CALB'den yüksek iken; sadece adsorpsiyon ile immobilize edilen enzim aktivitesi Novozym® 435'in aktivitesine yaklaşabilmiştir. Son olarak, çötürülmüş silika ve immobilize enzimler fourier dönüşümlü kızılötesi spektroskopisi, termogravimetrik analiz ve taramalı elektron mikroskobu ile karakterize edilmiştir.

Çalışmanın ikinci aşamasında, immobilize edilen enzimler ε-kaprolaktonun (ε-KL) halka açılma polimerizasyonu yöntemi ile polimerize edilmesi için katalizör olarak kullanılmıştır. Reaksiyonlar 40, 60, 80 ºC sıcaklık değerleri ve 6, 24, 48, 120, 150 and 170 saatlik zaman periyotlarında gerçekleştirilmiştir. Enzim/ε-KL oranı % 2.5, % 5, % 10 ve % 20 olarak ayarlanarak; PKL'nin immobilize edilen enzimlerle sentezi için, optimum enzim konsantrasyonları belirlenmiştir. Sentezlenen PKL'lerin molekül ağırlıkları, jel geçirgenlik kromatografisi ve proton nükleer manyetik rezonans spektroskopisi ile belirlenmiştir. Sonuçlara göre, fiziksel adsorpsiyon ile immobilize edilen lipazın katalizörlüğünde sentezlenen PKL'ler için en yüksek molekül ağırlığı ve monomer dönüşüm oranı 14300 g/mol ve % 90 olarak hesaplanmıştır. Çapraz bağlı immobilize enzim kullanılarak sentezlenen PKL'ler için ise; en yüksek molekül ağırlığı 9000 g/mol ve % 90 olarak belirlenmiştir. Ayrıca, en yüksek molekül ağırlıkları her iki immobilize enzim için de % 20'lik enzim/ε-KL oranında elde edilmiştir. İmmobilize enzimlerin tekrar kullanılabilirliklerinin tayini için, her iki immobilize lipaz da seri halinde 5 defa PKL sentezi için kullanılmış ve 5. reaksiyon sonunda adsorplanan enzim yaklaşık % 32, çapraz bağlanan enzim ise yaklaşık % 42 oranında başlangıç aktivitesini korumuştur. Ek olarak, immobilize enzimlerin kullanıldığı sentez sonuçları, serbest CALB ve Novozym® 435 kullanılarak sentezlenen PKL'lerin molekül ağırlıkları ile kıyaslanmıştır. Buna göre, her iki immobilize enzim ile elde edilen sonuçlar, serbest CALB ile elde edilene oranla daha yüksek olmuştur. Ayrıca, adsorplanan CALB ile elde edilen sonuç Novozym® 435 ile elde edilene göre daha yüksek olurken; çapraz bağlı enzim bu değere % 70 oranında yaklaşabilmiştir. Son olarak, sentezlenen PKL'lerin yüzey morfolojileri taramalı elektron miksroskobu ile analiz edilmiştir. Bunun yanında, fourier dönüşümlü kızılötesi spektroskopisi, termogravimetrik analiz ve diferansiyel tarama kalorimetrisi ile katarizasyon yapılmıştır. PKL'nin karakteristik pikleri bu analizler sonucunda elde edilen diyagramlarda gösterilmiştir.

Sonuç olarak bu çalışma; immobilizasyon ile, lipazın aktivite, stabilite ve tekrar kullanılabilirlik gibi birçok özelliğinin geliştiğini göstermiştir. Lipaz aktivitesi immobilizasyon sonunda beklenmedik şekilde artmış ve her iki immobilize enzim de çok ciddi bir aktivite kaybı olmadan defalarca kullanılabilmiştir. Böylelikle, lipazın endüstriyel uygulamalarda kullanımı daha ekonomik hale gelmiş ve çalışma bu yönüyle, enzimlerin kullanıldığı "çevredostu proses"lere katkı sağlamıştır. Son olarak, çalışmada enzimatik polimerizasyon yöntemi uygulanarak; polikaprolakton sentezlenmiş ve diğer polimerizasyon yöntemleriyle elde edilen sonuçlara paralel olarak yüksek molekül ağırlığı ve monomer dönüşüm oranlarına ulaşılmıştır. Bu sayede mevcut kirlilik sorunlarını ortadan kaldırmayı hedefleyen "yeşil polimer kimyası"na da katkı sağlanmıştır.

1. INTRODUCTION

Over the last two decades, "the green friendly method" enzymatic polymerization was greatly developed and became an important technique for biodegradable and biocompatible polymeric materials. Enzymatic polymerization has many benefits over chemical polymerization methods such as mild reaction conditions, high enantio-, chemo- and regioselectivity, high activity and few by-products. Furthermore, this polymerization method can help to overcome problems related to residues of metallic catalysts which have high toxicity and cannot completely remove from the resulted polymer. Therefore, enzymatic polymerization has been become a good alternative to conventional chemical methods.

There are two enzymatic polymerization types for enzyme catalyzed synthesis of aliphatic polyesters: ring opening polymerization of lactones, and polycondensation, containing polycondensation of diacids and hydroxyl acids or their activated esters (Zhang et al., 2014). In particular, several studies have showed that small, medium and large ring sized lactones could be successfully polymerized in enzyme catalyzed ring opening polymerization. The medium sized monomer ε-caprolactone (ε-CL) is the most widely studied lactone in comparison to the other lactones because it has high activity in enzymatic polymerization reactions (Yang et al., 2011). Polycaprolactone (PCL) from Mn = 500 to 50000 g/mol is produced with polymerization of ε-CL and it is an important polyester used in different biomedical applications because of its eco-friendly properties such as biodegradability, biocompatibility and permeability (Li et al., 2011).

On the other hand, the use of lipases for the enzyme catalyzed synthesis of polymers has been rapidly increased over the last years (Santos et al., 2012). Lipases are the most widely used group of enzymes that they can catalyze not only hydrolysis reactions of mono-, di- and triacylglycerols, but also esterification or transesterification reactions with very high efficiency. Lipases can begeneratedin almost every living cell, however microbial lipases take place on the top because of theirvarious advantages. *Rhizomucor miehei, Thermomyces lanuginosus, Candida*

rugosa, and lately *Candida antarctica* (type A and B) are most widespreadly used lipases.

Lipases can be used for many industrial applications such as chemical, pharmaceutical and cosmetic industry. However, the free enzymes have very high costs and complicated downstream processing. Therefore, several immobilization techniques apply to make enzymes more useful in commercial applications by improving enzyme properties. Immobilization techniques provide stable performance and possibility of repeated use for many reaction cycles, and application in a continuous process. Physical adsorption, entrapment in polymer matrices and covalent binding were mostly used as immobilization techniques. In addition, they can be immobilized onto different support materials that can be divided in organic or inorganic and natural or synthetic. Specifically, organic particles such as polystyrene resins, octyl agarose and inorganic particles such as fumed silica, carbon materials and titania can be used to immobilize *Candida antarctica* lipase B (CALB), which is the most commonly used lipase (Mihailović et al., 2014).

In this study, an inorganic support precipitated silica was prefered because of their more advantageous properties over organic carriers (Silva et al., 2013). An another reason to choose precipitated silica is effectivity of its mesoporous structure for immobilization processes (Serra et al., 2010). Both physical adsorption and crosslinking methods are performed for immobilization to compare the immobilization efficiency, stability and activity of the resulted immobilized enzymes. The immobilization conditions were changed also to optimize the processes. Secondly, the immobilized enzymes were used to polymerize the monomer ε-CL at three different temperatures (40, 60, 80 ºC) within a determined time range. The free CALB and Novozym® 435 were also used for ring opening polymerization (ROP) of ε-CL to compare the best results by using immobilized enzymes. Moreover polymerization conditions were optimized, and immobilized enzymes were repeatedly used for PCL synthesis to determine reusability of immobilized lipases. Finally, the resulted polymers and immobilized enzymes were characterized by fourier transform infrared spectroscopy, thermal gravimetric analysis and scanning electron microscope. The polymers additionally characterized by proton nuclear magnetic resonance spectroscopy, gel permeation chromatography, and differential scanning calorimetry.

2. LITERATURE REVIEW

2.1 Introduction on Enzyme Immobilization

Enzymes are biological catalysts that are widely used in diverse bioprocess technologies under environmentally conditions (Datta et al., 2013). These molecules, occuring thousands of atoms, are able to catalyze the variety of different chemical reactions (Brena and Batista-Viera, 2013). They have superior properties like regio-, enantio- and stereo- selectivity, specificity and activity under mild conditions made biocatalysts better alternative to the classical chemical modifications (Miletić et al., 2010). Especially at the beginning of the 20th century, enzymes were used for fermentation processes and after that they were started to use widespread processes such as the textile, pharmaceutical, food, and chemical industries. However, using catalysts in reactions has also some disadvantages. First of all maintenance of enzyme stability is relatively challenging during biochemical reactions, their costs of isolation are very high and recovery of enzymes from the reaction mixture and separation of enzymes from substrates and products is mostly very difficult (Brena and Batista-Viera, 2013). Therefore for commercialization of these biocatalysts, reusability of enzymes becomes very essential factor, otherwise would not be economic (Datta et al., 2013). There are some techniques to overcome these problems such as 'enzyme immobilization' which is an important approach for improving enzyme properties and performances (Miletić et al., 2010).

Table 2.1 : Steps in the development of immobilized enzymes.

Step	Date	Use
First	1815	Empirical use in processes such as acetic acid and waste water treatment.
Second	1960s	Single enzyme immobilization: production of L-aminoacids, isomerization of glucose.
Third	1985-1995	Multiple-enzyme immobilization including co-factor regeneration and cell immobilization.

Enzyme immobilization means that enzymes physically were localized in a certain defined zone of area without loss of catalytic activities. The first step of modern history of enzyme immobilization started to the late 1940s, and the first industrial use of immobilized enzymes was reported in 1967 by Chibata and coworkers. They immobilized *Aspergillus oryzae* aminoacylase for the resolution of synthetic racemic D-L amino acids. In the following step, only immobilized single enzymes were usedbut more complicated systems were developed by the 1970s. In the past three or four decades, improving of enzyme immobilization technology has increased rapidly (Table 2.1).

Immobilized biocatalysts greatly improved not only technical performance but also economy of the industrial processes (Brena and Batista-Viera, 2013). To improve all these properties of immobilized enzymes must have some ability: high activity, high selectivity (ability to decrease side reactions), high stability (can be effectively reused many times), cost-effective (economically attractive since enzymes can be recycled), safe to use and novel to improve properties of free enzymes used in industrial applications (Idris and Bukhari, 2012).

Firstly, immobilized enzymes can be easily separated from product or reaction mixture, and separability of enzyme provides minimizing protein contamination of the product. Immobilized enzymes can be recovery and reuse also repeatedly through this property. Reusability of immobilized enzymes make more economically using enzymes in biochemical processes (Sheldon, 2007). Besides, the use of an immobilized enzyme enable to greatly facilitate the design of the reactor and the control of the reaction (Mateo et al., 2007). Secondly, many of the enzyme molecules cannot attain to substrate molecules, while immobilized enzymes can achieve easily. This property provides better attainability and stability (Sheldon, 2007). An another advantage of immobilization is that enzymes become more stable after immobilization processes. Immobilization improve operational, thermal and storage stability of enzymes which are very important properties for all reactions used biocatalysts (Mateo et al., 2007).

Many immobilized enzymes have been developed and their performances reported: Mendes et al. (2013) used also epoxy–chitosan/alginate support to immobilize *Thermomyces lanuginosus* and *Pseudomonas fluorescens* lipases by covalent attachment. They reported that the enzymes retained about 95 % of the initial activity

after 5 cycles uses. Khoobi et al. (2014) immobilized *Thermomyces lanuginosa* lipase by using different immobilization methods. The results has demonstrated that about 83 % of the initial activity could be retained after 12 cycles of uses.

2.1.1 Enzyme immobilization methods

There are several enzyme immobilization methods, and three of them are most common techniques: adsorption, entrapment, and crosslinking or covalently binding to a carrier (Spahn and Minteer, 2008).

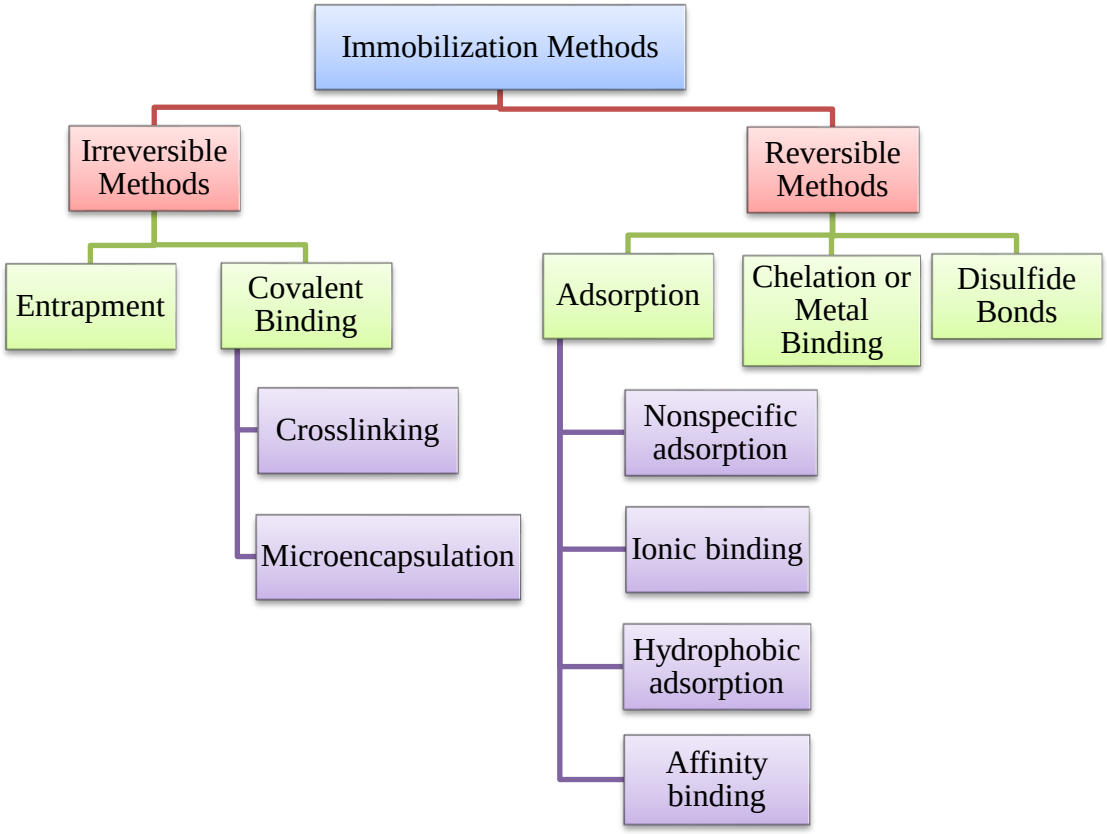


Figure 2.1: Enzyme immobilization methods.

Enzyme immobilization techniques can be classified by different approaches. Irreversible and reversible techniques are one of them, and these categories based on the strength of the binding (Figure 2.1). The strength of the binding is generally inversely, thereby it can be reversed. Immobilization methods aim to make the bond as strong as possible and decrease reversibility.

2.1.1.1 Methods of irreversible enzyme immobilization

The most common irreversible enzyme immobilization methods are covalent coupling, entrapment or microencapsulation, and crosslinking (Figure 2.2). In irreversible immobilization techniques, enzyme is attached to the support, and it overthrow either the biological activity of the enzyme or the support when it separated.

Covalently binding or crosslinking**,** is one of the most widespread technique, has advantages such as stability of the bond between enzyme and matrix. In this method it is necessary to reach high levels of bound activity that the amino acid residues for catalytic activity must not be included in the covalent linkage to the support (Brena and Batista-Viera, 2013).

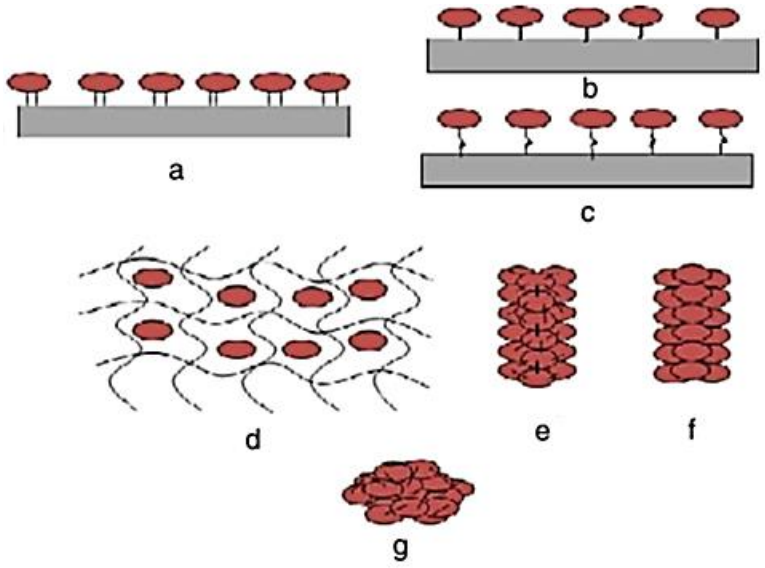


Figure 2.2 : Different immobilization techniques (enzyme and supports are presented in red and gray color respectively): Multiple covalent attachment (a), single covalent attachment (b), covalent attachment via spacer arm (c), entrapment in polymeric gel in membrane (d), crosslinked enzyme crystals (e), crosslinked enzyme aggregates (f), crosslinked enzyme crystals (g) (Idris and Bukhari, 2012).

Covalent binding immobilization methods are used when there is a strict necessity for the absence of the enzyme in the product. There are many reactions depending on the functional groups available on the matrix, and they can be classified two main categories: (1) activation of the matrix by putting to a reactive function to a polymer

and (2) modification of the polymer backbone to obtain an activated group (Table 2.2).

In activation processes of croslinking methods, electrophilic groups are produced on the carrier which react is produced that strong nucleophiles on the proteins. Lysine (ε-amino group), cysteine (thiol group), and aspartic and glutamic acids (carboxylic group) are mostly used for the following side chains of the amino acids.

Table 2.2 : Covalently coupling or crosslinking methods of enzymes.

Activation Method	**Group that reacts**
Tresyl chloride, sulfonyl chloride	Thiol, amines
Cyanogen bromide	Amine
Bisoxiranes (epoxides)	Thiol, amine
Epichlorohydrin	Thiol, amine
Glutaraaldehyde	Amine
Glycidol-Glyoxyl	Amine
N-Hydroxy-succinimidyl	Amine

Enzymes generally contacted to the carrier through either amide, ether, thioether, or carbamate bonds in the covalent reactions. For this reason, the enzyme is strongly bound to the matrix and, it makes enzymes also more stable. Nevertheless, enzymatic activity and immobilization yield decrease due to this bond. Because the matrix are destroyed with enzyme together (Brena and Batista-Viera, 2013).

An another immobilization method is crosslinking of enzyme aggregates or crystals, using a bifunctional reagent, to prepare carrierless particles. In this way, reducing of enzymatic activity can be prevented successfully. Moreover high stability and low production costs can be obtained in carrier-free immobilized enzymes like crosslinked enzyme crystals (CLECs) and crosslinked enzyme aggregates (CLEAs) (Sheldon, 2007).

Glutaraldehyde is one of the most effective enzyme immobilizing agents used widely to immobilize biocatalysts by crosslinking method (Table 2.2). There is some strategies for reaction between the glutaraldehyde and the carrier. In the method used glutaraldehyde is generally accepted that reactions between the carbonyl group of glutaraldehyde and the amino groups of the enzyme occur giving a Schiff base. However stability of Schiff bases is not high and activity of enzymes with glutaraldehyde is also low. Therefore alkaline-treated glutaraldehyde can be used to increase activity of enzymes (Park et al., 2002).

An another strategy is that all primary amino groups of the support and the enzyme are activated by treating with one glutaraldehyde molecule to adsorb the enzyme on the support. In this way, it takes place crosslinking between the enzyme and the support. In some cases, it can be also took place that multipoint covalent attachment between enzyme and support, as an enzyme-amino-glutaraldehyde or support-amino-glutaraldehyde reaction. Furthermore, the whole enzyme molecules can be modified with glutaraldehyde, and this modification can impact stability and activity of the enzyme negatively or positively because of the modification of the all amino groups of the carrier surface.

According to the other possibility, two glutaraldehyde molecules are used to activate the support. This activation causes that the carrier change to an ionic exchanger because of coating of carrier surface. On the other hand, glutaraldehyde can be used not only to crosslink enzyme molecules in many type of carriers, but also it is used to generate CLECs and CLEAs (Barbosa et al., 2012).

Yang et al. (2010) used glutaraldehyde to immobilize *Arthrobacter sp*. lipase by enzyme aggregate coating method which compared to the conventional covalent attachment and covalent attachment plus crosslinking methods. They synthesized the glutaraldehyde activated amino silica gel to use as the carrier. According their results, the immobilized enzyme by enzyme aggregate coating had both higher activity and stability than those by other methods. The lowest activity yield was exhibited by covalent attachment plus crosslinking method.

Entrapment of enzymes in a polymeric network like organic polymer, silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule is used for low molecular weight substrates and products. It is necessary extra covalent attachment because of preventing enzyme leakage. Moreover enzyme should be present during synthesis of the polymeric network the enzyme is entrapped (Sheldon, 2007). The entrapment process can be only physical caging or include covalent binding. The practical use of entrapment methods is restricted by mass transfer limitations through membranes or sol-gels (Brena and Batista-Viera, 2013). As an example of this method, a new kind of poly(methylmethacrylate-co-divinylbenzene) porous copolymer was encapsulated Fe_3O_4 magnetic carrier has been developed for *Mucor javanicus* lipase immobilization by Meng et al. (2013).

2.1.1.2 Methods of reversible enzyme immobilization

The most common reversible enzyme immobilization methods are adsorption, chelation or metal binding and disulfide bonds (Figure 2.3). Reversible methods are prefered mostly because of economical reasons. Furthermore, carrier can be regenerated and reloaded with free enzyme when enzyme activity decays.

In physical adsorption method, the enzymes are attached to the matrix via hydrogen bonding, van der waals forces, or hydrophobic interactions. However bondings in this process can be reversed because of the weakness of the noncovalent bonds and changing of conditions such as ionic strength, temperature affect interactions between support and enzyme. On the other hand, this method is used to immobilize enzymes due to that it provides generally retention of catalytic activity of enzyme. Moreover adsorption is mild and easy to perform to process (Brena and Batista-Viera, 2013).

As an example of immobilization by physical adsorption, Zou et al. (2010) immobilized porcine pancreatic lipase (PPL) on ionic liquid modified mesoporous silica SBA-15 by physical adsorption method. They reported that the enzymatic properties have improved, especially the activity increased from 594 to 975 U/g PPL after immobilization. Moreover, Kharrat et al. (2011) immobilized *Rhizopus oryzae* lipase on silica aerogels by physical adsorption, and they obtained a novel immobilized enzyme which can be used 12 cycles without a significant loss of its catalytic activity. In the study, storage stability of this immobilized enzyme is also reported that its activity is stable after 4 months.

In ionic binding method, immobilization occur via protein ligand interactions which based on chromatographic principles. Ion exchangers are used to immobilize the enzymes in this simple reversible method. Moreover polyethyleneimine is used mostly as a polymeric ionic ligands which has a number of patents. However, there is some disadvantages that the strength of bond and the activity are not high enough. Besides, enzyme properties such as pH stability can changed due to that the kinetics are destroyed because of diffusion phenomena. But it can be an advantage under more alkaline or acidic conditions, because the optimum conditions of enzyme can change successfully.

Hydrophobic adsorption is an another reversible method which is used as a chromatographic principle. It depends on different experimental parameters such as pH, salt concentration, and temperature. In this method, there are hydrophobic interactions between adsorbent and protein instead of chemical bonds. The degree of substitution of the carrier and the size of the hydrophobic ligand molecule are responsible for controlling of the hydrophobicity of the adsorbent (Brena and Batista-Viera, 2013). An example of hydrophobic adsorption is that Kurtovic et al., (2011) immobilized a bile salt activated lipase from Chinook salmon on two hydrophobic supports. According to this study, this immobilized enzyme had 10-fold higher activity than *Candida antarctica* lipase B immobilized on Lewatit (Novozym® 435).

Affinity binding is one of the reversible method which provides specificity of enzyme to carrier under various physiological conditions. In this method, the matrix is attached to an affinity ligand such as lectin or affinity ligand and the matrix compose a structure, and the enzyme is conjugated to this structure (Datta et al., 2013). However, costly affinity ligand requirement is the disadvantage of this method.

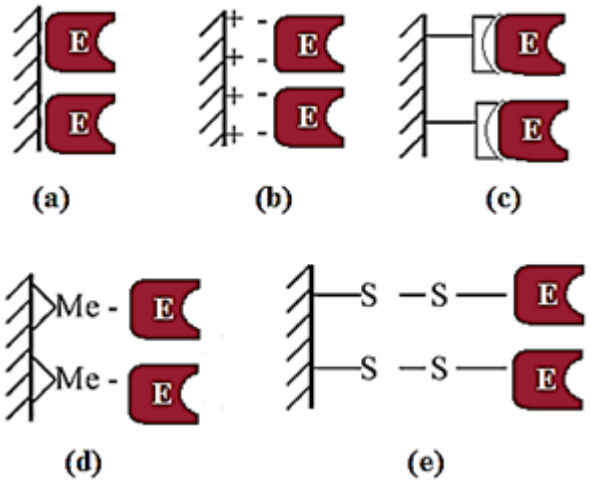


Figure 2.3 : Different reversible immobilization techniques: Physical adsorption (a), ionic binding (b), affinity binding (c), chelation or metal binding (d), disulfide bond (e).

In metal link immobilization method, metal salts or hydroxides existed on the surface of support attached to nucleophilic groups on the matrix. As metal salts are used mostly titanium and zirconium salts. The metal salts or hydroxides are precipitated on the organic carrier via heating or neutralization processes. Specific activity of

enzyme can be high enough with this immobilization method, nevertheless operational stabilities are changeable. Present of nonuniform adsorption sites cause change of operational stability. In order to overcome this problem, chelator ligands can be immobilized onto the carrier. In this way stable covalent bonds are obtained which attached to the metal ions. The carrier is washed with a chelator like ethylenediamine tetraacetic acid, and it is named Immobilized Metal-Ion Affinity adsorbents.

In the last reversible immobilization method disulfide bonds, stable covalent bonds are generated between matrix and enzyme which can be broken by reaction with anagent such as dithiothreitol. The reactivity of the thiol group can be controlled by pH changing due to obtaining high specificity of thiol-reactive adsorbent prefered in this method (Brena and Batista-Viera, 2013).

2.1.2 Choice of enzyme supports for immobilization

Supports are used in most of the immobilization methods, and they have an important role to determine properties of the immobilized enzymes (Miletić et al., 2012). Chemical characteristics and some mechanical properties such as mean particle diameter, mechanical strength, and pressing behavior of supports influence performance of the immobilized enzymes (Figure 2.4). Therefore, selecting support is a key parameter for an immobilization process.

Optimum carrier features contain physical resistance to compression, hydrophilicity, immobility toward enzymes ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost. In addition, particle size and pore diameters define the total surface area which is also effective for binding of enzymes. Firstly, loading capacity of nonporous carriers is low, although they have few diffusional limitations. On the other hand, porous carriers have generally high surface area enables the high enzyme loading ratios. Porous carriers should have also a controlled pore distribution because of optimization capacity and flow features.

There are different materials can be used as immobilization carrier which can be divided as inorganic and organic depending on their chemical composition (Brena and Batista-Viera, 2013).

2.1.2.1 Organic supports

Many materials can be used as organic carriers divided in natural and synthetic polymers (Table 2.3). Alginate is one of the natural polymer used as an organic carrier that it is obtained from cell walls of brown algae are sodium, calcium and magnesium salts of alginic acid. Alginates are used generally as xanthan alginate beads, calcium alginate beads and alginate polyacrylamide gels improved activity and reusability of biocatalysts (Datta et al., 2013).

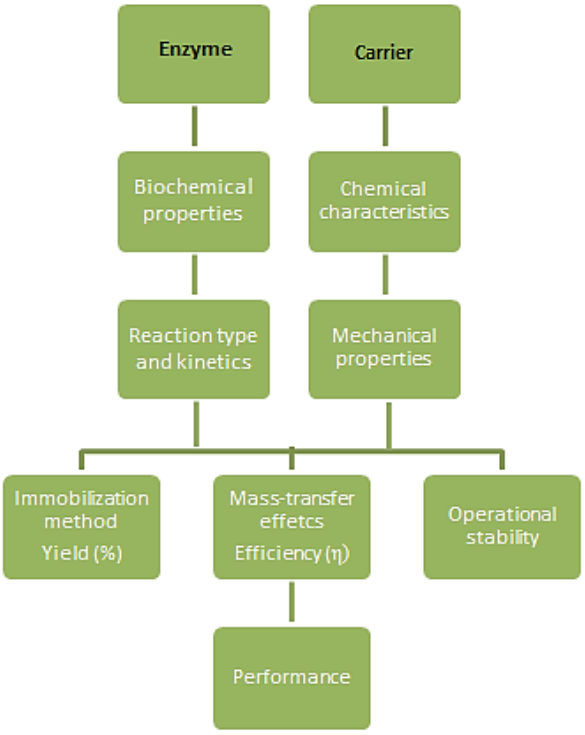


Figure 2.4 : Properties of enzyme and support determine the properties of the immobilized enzyme (Miletić et al., 2012).

Chitin and chitosan are used also as natural organic supports that chitosan is mostly used in combination with alginate. Silva et al. (2012) studied *Candida antarctica* lipase B immobilization by covalent attachment onto chitosan and onto chitosan–alginate complex previously activated by different methods. They reported that thermal stability of immobilized enzymes was 33 times higher than the soluble enzyme, and all initial activity of enzyme retained after 8 cycles of butyl oleate synthesis.

Cellulose is an another natural polymer widely used for immobilization such as glucoamylase, α-amylase, tyrosinase, lipase and β-galactosidase immobilization. Singh and Kayastha (2014) reported that α-amylase is immobilized on

diethylaminoethyl-modified cellulose with 86 % immobilization yield. Besides, collagen, carrageenan, gelatin, starch, pectin and sepharose are natural polymers used as organic support materials (Datta et al., 2013).

Synthetic polymers such as polyvinyl chloride and polystyrene can be used as carriers for immobilization processes. Furthermore in many immobilization studies, enzymes are immobilized onto some polymers such as polyacrylate, polymethacrylate, polyacrylamide, polyamide, vinyl, and allyl-polymers (Datta et al., 2013). Vaidya et al. (2008) immobilized *Candida rugosa* lipase on poly(allyl glycidyl ether-co-ethylene glycol dimethacrylate) macroporous polymer particles, and they reached about 78 % activity yield. An another study was performed by Cui et al. (2013) who immobilized *Yarrowia lipolytica* lipase Lip2 onto polyethyleneimine-coated polyurethane foam to improve activity and stability of lipase. They reported that the free enzyme lost most of its initial activity, while about 70 % of its initial activity was retained in immobilized enzyme at the same conditions.

2.1.2.2 Inorganic supports

Inorganic materials can be used to immobilize the enzymes because of their superior properties like good mechanical properties, high thermal stability, and highly strength against microbial attack and organic solvents (Park et al., 2002). They also have high specific surface area, pore volume and mesoporous diameter which are important properties for supports in immobilization processes (Zaidan et al., 2010). Inorganic supports are more advantageous than organic supports because of these excellent properties (Silva et al., 2013). There are several inorganic solids such as alumina, bentonite and silica used as inorganic carrier (Sheldon, 2007). Inorganic carriers can be subdivided in natural minerals and processed minerals (Brena and Batista-Viera, 2013).

One of the inorganic supports, bentonite is derived from montmorillonite, has natural abundance, electrostatic interactions, easily derivable and availability at low cost. Furthermore, silanol groups provided structural and functional adjustment for immobilization exist in bentonites. Dong et al. (2013) studied lipase immobilization onto three different functionalized bentonites. These immobilized enzymes showed

better resistance to heating inactivation, storage stability and reusability than free lipase (Brena and Batista-Viera, 2013).

Table 2.3 : Classification of carriers (Brena and Batista-Viera, 2013).

Classification of carriers	
Organic carriers	
Natural polymers	• Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate • Proteins: collagen, albümin • Carbon
Synthetic polymers	• Polystyrene
Other polymers	• Polyacrylate, • Polymethacrylates, • Polyacrylamide, • Polyamides, • Vinyl, and allyl-polymers etc.
Inorganiccarriers	
Natural minerals	• Bentonite, silica etc.
Processed materials	• Glass, metals, controlled pore metal oxides etc.

Celite is a diatomaceous and bioaffinity material consisted of highly porous diatomaceous beads. It is available to immobilize biocatalysts by physical adsorption due to their chemical inertness and perfect interconnected pore structure. Moreover, diatomaceous beads composed of silica and some inorganic oxides improve reaction rates because of enabling a good distribution of the enzyme. Chang et al. (2007) easily immobilized *Candida rugosa* lipase on celite by adsorption. They determined the optimum parameters such as immobilization time and temperature for this immobilization process.

Various enzymes are immobilized onto activated silica used mostly for different immobilization processes (Datta et al., 2013). Porous silica gels, especially mesoporous silica gels are available supports for enzyme immobilization because of their large surface area, good mechanical properties, adjustable porosity and low cytotoxicity (Boros et al., 2013). Moreover, the macropores of supports can enable wide space for enzyme molecules, thus there will be little resistance for immobilization. Depending on this property of carriers, silica aerogels can be successfully used as biocatalyst carrier (Gao et al., 2009). The performance of different hydrophobic mesoporous silicas for lipase immobilization are compared by

Serra et al. (2010). They immobilized *Candida antarctica* lipase B onto silicas activated by octyltriethoxysilane, octyltrimethoxysilane, tetraethoxysilane and methyltriethoxysilane. The results showed that silica activated by octyltriethoxysilane had higher catalytic activity than other silicas. In addition, glass, activated carbon, charcoal, zeolite and ceramic can be also used for biocatalyst immobilization (Datta et al., 2013).

2.1.3 Precipitated silica as a carrier for immobilization

Silica types can be divided based on their some properties such as particle size and production processes in silica gels and sols, micro-silica, fumed silica and precipitated silica (Table 2.4). It is assumed that precipitated silica will show the most rapid growth over the next decade. It is generally generated by mixing of aqueous solutions of sodium metasilicate and a mineral acid in multiple steps (Figure 2.5). Moreover, it is possible 'green production' of precipitated silica using either batch or continuous processing.

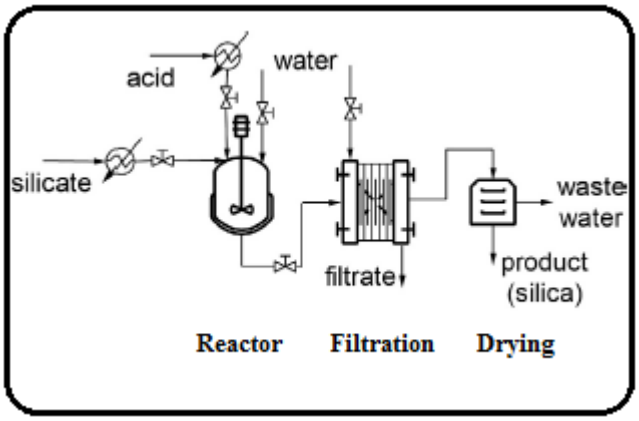


Figure 2.5 : Typical process flow diagram for current industrial silica production process (Drummond et al., 2014).

Drummond et al. (2014) studied biologically production of precipitated silica provided decreasing both operational costs and carbon dioxide emissions because of no heat requirements. The synthesis temperature, precipitation time, pH, addition of coagulant and conditions of washing and drying are effective on the properties of precipitated SiO_2. This parameters determine the size of the SiO_2 molecules, their specific and total surface area (Musić et al., 2011).

Precipitated silica can have mesoporous or macroporous structure based on their particle morphology (Table 2.4). It is explained in inorganic supports that the high pore size offer a good diffusivity between substrate and/or products. Therefore amorphous mesoporous silicas are used in many enzyme immobilization studies (Serra et al., 2010). Precipitated silica is one of the amorphous silica based materials and it can be successfully used as a support because of its advantageous properties.

Table 2.4 : Silica types and particle sizes (Öney-Kıroğlu, 2014).

	Tanecik boyutu (µm)
Natural (Crystalline)	
Quartz	1-10
Diatomite	1-5
Synthetic (Amorphous)	
Fumed silica	0.005 – 0.02
Precipitated silica	0.01 – 0.03
	0.04 – 0.08
Ferro-silicon by-products	0.10

2.1.4 Modification of supports by silanization

Surface of supports are modified usually by silanization using different organosilane agents. Silanization process is usually applied in two steps: firstly at room temperature and after that at reflux temperature (Zhang et al., 2013). Furthermore, different parameters such as pH, reaction time and temperature are effective on silanization reaction (Flesch et al., 2005).

Organosilanes are used to make better the chemical, physical and mechanical features of carriers such as metal, plastic, glass, rubber and silica. Moreover, these environment friendly chemicals provide increasing adhesion and resistance against corrosion. They are monomeric silicon chemicals included minimum one carbon-silicon bond. The nonpolar carbon-silicon bond included alkyl group shows low surface energy, high stability and hydrophobic effects.

Organo functional silanes combine the inorganic functionality of an alkyl silicate and the functionality of a reactive organic group in one molecule. Through this unique property, they can be used as a molecular bridge between inorganic materials and organic substrates of silanes. The organic group of organosilanes can be both reactive or nonreactive which can be either hydrophilic or hydrophobic (Table 2.5).

They can be categorized as chlorosilanes, silazanes, alkoxysilanes and acyloxysilanes based on the nature of the hydrolysable groups. Reactive groups are used mostly as a coupling agents, while nonreactive groups are used as hydrophobing or dispersing agents. In addition, concentration and type of surface hydroxyl groups, physical sizes of the substrate and the hydrolytic stability of the bond are effective parameters on surface modification (Kregiel, 2014).

Tablo 2.5 : Examples of functional organosilanes (Kregiel, 2014).

Organo group X	Alkoxy group OR	Chemical name
Nonreactive silanes		
Methyl	methoxy	methyltrimethoxysilane
Methyl	Ethoxy	methyltrimethoxysilane
Methyl	methoxy	dimethyldimethoxysilane
Propyl	methoxy	propyltrimethoxysilane
i-butyl	methoxy	isobutyltrimethoxysilane
Phenyl	methoxy	phenyltrimethoxysilane
n-octyl	Ethoxy	n-octyltriethoxysilane
Reactive silanes		
Amino	methoxy	aminopropyltrimethoxysilane
Amino	Ethoxy	aminopropyltriethoxysilane
Chloropropyl	methoxy	γ-chloropropyltrimethoxysilane
Epoxy	methoxy	γ- glycidoxypropyltrimethoxysilane
Vinyl	methoxy	vinyltrimethoxysilane
Vinyl	acethoxy	vinyltriacethoxysilane
Mercapto	Ethoxy	mercaptopropyltriethoxysilane

Zaidan et al. (2010) used aminopropyl-, octyl-, vinyl-, mercapto- and glycidoxy-triethoxysilanes to modificate mica for lipase immobilization. Lipases immobilized micas have higher activity than the free lipase following the order; Amino-*Candida rugosa* lipase (CRL) > Glu-Amino-CRL > Octyl-CRL > Vinyl-CRL > Glycidoxy-CRL > Mercapto-CRL > Mica-CRL.

2.2 Introduction on Lipases

Lipases (EC 3.1.1.3) are a class of biocatalysts that can be easily found from plant, fungi, microbial, and animal world (Forde et al., 2010). They catalyze the hydrolysis of esters to glycerol and long chain fatty acids by cleavage of ester bonds (Zaidan et al., 2010). Depending on reaction conditions, lipases can also catalyze aminolysis, ammoniolysis, esterification and transesterification reactions with high activity, regio and enantio selectivity (Yang et al., 2010).

Lipases belong to hydrolases class of enzymes which are the most commonly used biocatalysts, and lipases have an important role in this class (Magnusson, 2005). They have a wide range of industrial applications such as chemical industries, biomedical sciences and food technology (Hwang et al., 2004). Detergent industry takes the first place with using 30 % of the total enzyme market. Mostly *Thermomyces lanuginosa* lipase is used to produce detergents. Besides, lipases are used for fat and oil processes, for instance in the production of cocoa butter. It is produced via acyl transfer reactions by using *Rhizopus niveus* lipase. They are also used in the pulp and paper, cosmetics, and pharmaceuticals industry (Magnusson, 2005). However, like most of enzymes, lipases have higher prices due to their short lifetimes which restricted industrial applications of lipases. There are many methods to overcome this problem such as enzyme immobilization, enzyme modification, medium engineering and protein engineering (Yang et al., 2010). Enzyme immobilization is the most widespread method to provide improving enzyme stability, selectivity, increasing number of uses and reducing operational costs (Yıldırım et al., 2014).

2.2.1 Immobilization of lipases

There are many techniques to immobilize lipases like other enzymes, and lipases can be immobilize on different supports such as cellulose, chitin, chitosan, silica, clay and alumina. Choosing of support and immobilization methods are important to determine thermal stability and both chemical and mechanical properties of the resulting immobilized lipase. Furthermore, immobilization cost is an another parameter for a lipase immobilization process. Inorganic supports are cheaper than organic supports, and they have also superior mechanical and thermal properties (Silva et al., 2013).

As an example of lipase immobilization, Zaidan et al. (2010) immobilized *Candida rugosa* lipase onto the different micas. Mica modification and lipase immobilization methods can be seen on Figure 2.6. They reached to 78 % immobilization yield and high protein loading ratios.

2.2.1.1 CALB immobilization and Novozym® 435 commercial catalyst

Lipase B from *Candida antarctica* is the most used lipase because of its superior properties such as high selectivity and efficiency (Idris and Bukhari, 2012). It is produced from the yeast *Candida antarctica* which is isolated in Antarctica. CALB is consist of 317 amino acids and has a molecular weight of 33000 g/mol and it is a part of α/β-hydrolase-fold family (Magnusson, 2005).

Most of lipases show interfacial activation which takes place by the opening of a flap structure of the enzyme. This opening flap structure is the active form of the enzyme, that provide the connectivity between substrate and enzyme, and increase enzyme activity. In contrast, CALB does not show typical interfacial activation like other lipases, because the active site of CALB is not exist or very small (Magnusson, 2005). This property provides that CALB can perform a wide variety of reactions (Forde et al., 2010). The structure of CALB was found in 1994 (Figure 2.7).

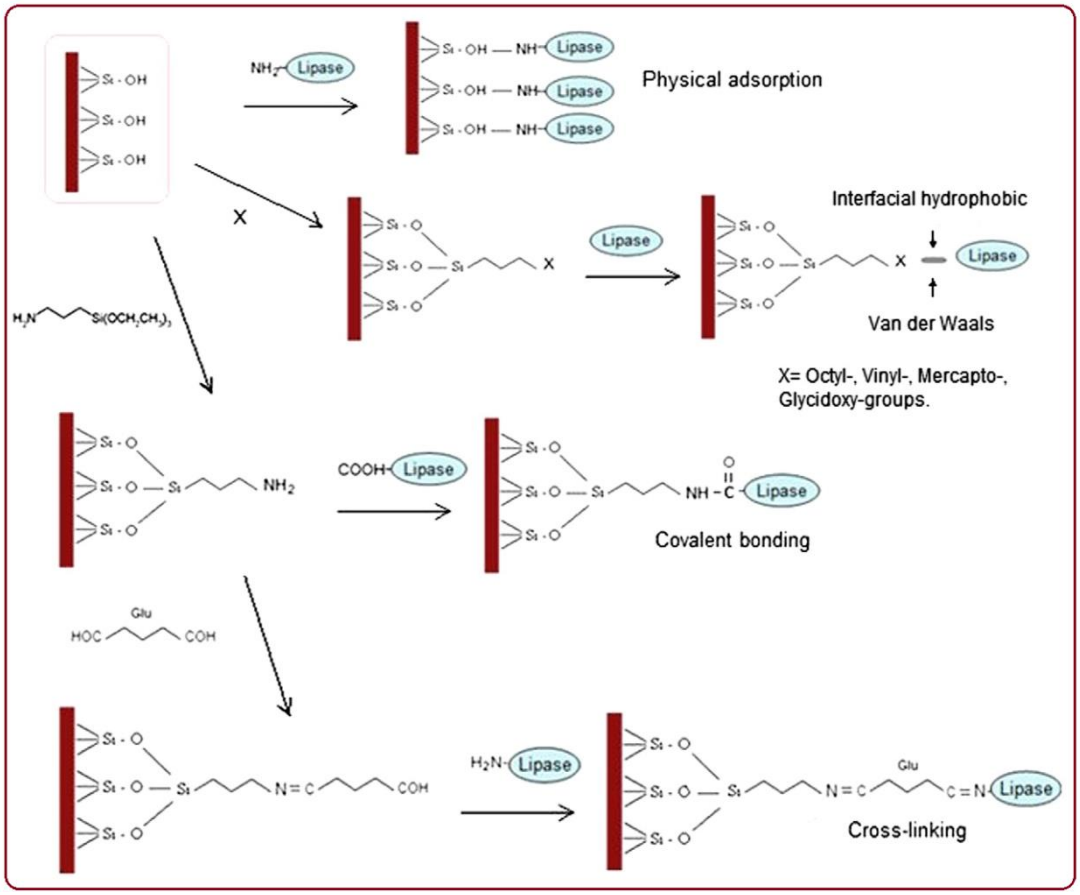


Figure 2.6 : Schematic explanations of mica modification and lipase immobilization via different methods (Zaidan et al., 2010).

The active site of CALB have a stereospecificity pocket and oxyanion hole. The stereospecificity pocket provide a high enantioselectivity against chiral secondary alcohols, while oxyanion hole stabilizes the transition site. The steric requirement of stereospecificity pocket is defined the enantioselectivity of CALB against chiral secondary alcohols (Magnusson, 2005).

CALB can be used in many applications like in the resolution of chiral secondary alcohols, in the production of polylactones and polyesters (Magnusson, 2005), hydrolysis in water, esterification in organic solvents, enantio- and regioselective transformations of many low molar mass and polymer substrates (Idris and Bukhari, 2012).

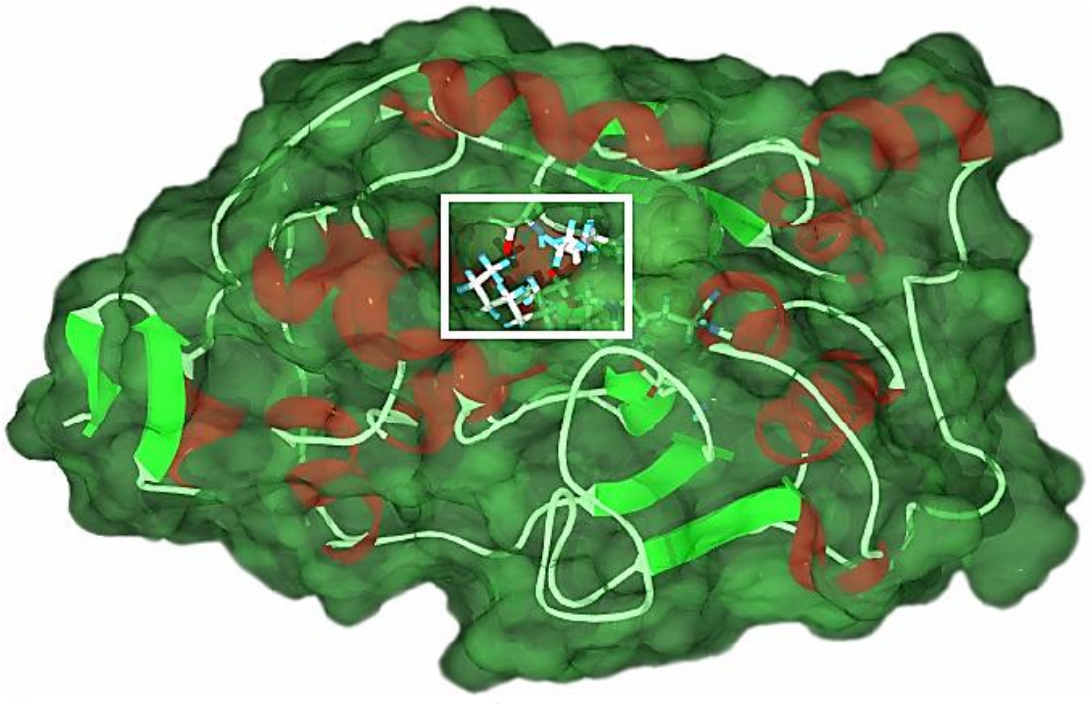


Figure 2.7 : Structure of *Candida antarctica* lipase B. The α-helices are shown in red, the β-sheets in pale green, the active site of lipase in white rectangular, and the enzyme surface is in dark green. The ends of the acyl and alcohol chains of the substrate are visible in the narrow entrance of the active site. The catalytic amino acids and the other parts of the substrate are buried in the active site (Magnusson, 2005).

Novozym® 435 (N-435) is physically immobilized form of CALB which is a commercially available biocatalyst (Öztürk-Düşkonkorur et al., 2014). It is produced by CALB immobilization onto a macroporous resin poly(methylmethacrylate-co-divinylbenzene). N-435 shows an extra regio- and enantioselectivity during

esterification, transesterification and hydrolysis reactions. Moreover, it is effective for polymer synthesis reactions from different lactones monomers (Poojari and Clarson, 2013). However, its support material acrylic resin does not have enough mechanical and thermal properties for some polymerization reactions (Öztürk-Düşkonkorur et al., 2014).

There are many studies used CALB and N-435 and their results are reported: Tzialla et al. (2010) studied immobilization of CALB onto smectite group nanoclays. According to the results, 90 % and 60 % of initial activity retained after immobilization of CALB and four reaction cycles uses, respectively. Poppe et al. (2013) immobilized CALB on aldehyde-activated Immobead 150 support by multipoint covalent attachment. They reached very high immobilization efficient and yield which are about 96 % and 86 %, respectively. Furthermore, N-435 is successfully used for different processes: Deng and Gross (1999) studied ring opening bulk polymerization of ε-caprolactone and trimethylene carbonate by using N-435. Cruz et al. (2009) immobilized CALB on fumed silica and compared their immobilized lipases results to the results of experiments used N-435.

2.3 Introduction on Biodegradable Polyesters

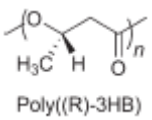
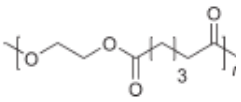
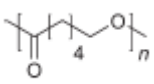
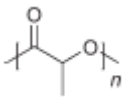
Biodegradable polyesters took attention in the past decade because of obtainable from renewable resources. These materials contribute "green and eco-friendly technologies" (Heiny et al., 2014) due to that they exist during a little period of time in natüre before becoming waste material (Agarwal, 2012). There are many biodegradable polyesters (Table 2.6) which are poly(α-hydroxy acids) (PHAs), especially polylactic acid (PLA) and polylactide, PCL and other polyesters depended on lactones such as β-propiolactone, γ-butyrolactone, δ-valerolactone, or pivalolactone (Heiny et al., 2014).

Biodegradation process occurs that the polymer chain breaks by the division of bonds between the monomers in the polymer. It is like an enzymatically catalyzed hydrolysis process. Firstly, enzymes cannot penetrate in the polymer bulk which names the surface erosion process. After that enzymes are absorbed by microbial cells and according to the European Committee for Standardization a polymer degradation process takes place from this action of microorganisms with final

conversion to water, carbon dioxide and/or methane, and biomass. This process can happen in soil, natural water, human beings, and animals.

Biodegradable polyesters are used in different applications such as agriculture, medicine, pharmacy, and biomedicine. They can be used as drug carriers for the delivery of drugs, and also to repair tissue and organs. Moreover, these polyesters especially PLA are used to produce coffee cups, garbage bag, and other household items (Agarwal, 2012).

Table 2.6 : Classification of Biodegradable Polyesters (Agarwal, 2012).

B.P.	Type	Representative example	Chemical structure
N.P.	poly(3-hydroxyalkanoate)	poly(3-hydroxybutyrate)	 Poly((R)-3HB)
S.P.	Poly(alkylenedicarboxylates)	Poly(ethylene adipate)	
	Lactones	Poly(ε-caprolactone)	
	Lactides	Poly(lactide)	

*B.P. is biodegradable polyesters, N.P. is natural polyesters and S.P. is synthetic polyesters

2.3.1 Properties of polycaprolactone

Polycaprolactone is one of the biodegradable aliphatic polyesters which has a hydrophobicity and semi-crystallinity. Its crystallinity degree can increase about 69 % (Labet and Thielemans, 2009) which reduces with increasing molecular weight of PCL (Woodruff and Hutmacher, 2010). The molecular weight and crystallinity degree of PCL are effective on the physical, thermal and mechanical properties of PCL (Table 2.7).

PCL has good solubility and low melting point (59-64 ºC) that it dissolves extremely in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane; slightly in acetone, 2-butanone, dimethylformamide, ethyl acetate and acetonitrile. However it cannot dissolve in alcohols, petroleum ether, diethyl ether and water. Additionally, PCL can be mixed with many polymers like poly(vinyl chloride), poly(styrene–acrylonitrile), poly(acrylonitrile butadiene styrene), poly(bisphenol-A) and other polycarbonates.

Biodegradation of PCL is completed within several months to several years by many microbes in nature. It relates to the conditions of degradation, the molecular weight and the crystallinity of PCL. In biodegradation process of PCL, firstly the amorphous phase degrades, the degree of crystallinity increases and there is no change of molecular weight. After this step, ester bonds cleaves and the polymer degrades by end polymer chain division. It can be enzymatically degraded in the environment, in contrast PCL degradation cannot enzymatically happen in the body (Labet and Thielemans, 2009).

Table 2.7 : Properties of PCL (Labet and Thielemans, 2009).

Properties	**Range**
Number average molecular weight (Mn/gmol^{-1})	530 – 630000
Density (ρ/gcm^{-3})	1.071 – 1.200
Glass transition temperature (Tg/ºC)	(-65) - (-60)
Melting temperature (Tm/ºC)	56–65
Decomposition temperature (/ºC)	350
Inherent viscosity (η_{inh}/cm^{3}g^{-1})	100–130
Intrinsic viscosity (η/ cm^{3}g^{-1})	0.9
Tensile strength (σ/MPa)	4–785
Young modulus (E/GPa)	0.21–0.44
Elongation at break (ε/%)	20–1000

2.3.2 Application of polycaprolactone

The return of PCL in the 21st provided a new approach in biomaterials arena and this trend increased linearly day by day, it can be seen on Figure 2.8 (Woodruff and Hutmacher, 2010). There are many biomaterials made by PCL which are used in some industrial applications such as scaffolds in tissue engineering, in long term drug delivery systems, in microelectronics, as adhesives, biomedical applications and in packaging (Figure 2.9). Their feasible and excellent properties such as controlled degradability, copolymerization with other polymers, biocompatibility and

possibility of generation from monomer obtained from renewable sources affect usability of PCL. It is also an inexpensive polymer that offers a suitable alternative for industry (Labet and Thielemans, 2009).

PCL and its copolymers can be used in a number of drug delivery devices because of their mechanical properties and degradation kinetics. PCL degrades slower than polyglycolide (PGA), poly(D,L-lactide) (PDLA) and its copolymers, for this reason the drug delivery devices made by PCL can remain active for over 1 year. Furthermore, it can easily take shape, enable appropriate pore sizes conducive to tissue in growth and provide controlled delivery of drugs.

PCL can be used in the form of microspheres and nanospheres in drug delivery systems. First of all, biodegradable microspheres can be ingested, injected and also degraded in a suitable time period without decomposing into toxic and high molecular weight. Secondly, PCL nanospheres are used as transport support materials for drugs or other active molecules in colloidal drug delivery systems. On the other hand, PCL is not suitable for replacing metal devices by using biodegradable implants due to their insufficient mechanical properties in high load bearing applications of medical device industry. However, it is mostly used to produce sutures, wound dressings, contraceptive devices, fixation devices and filling materials which are the other applications of biomedical device industry.

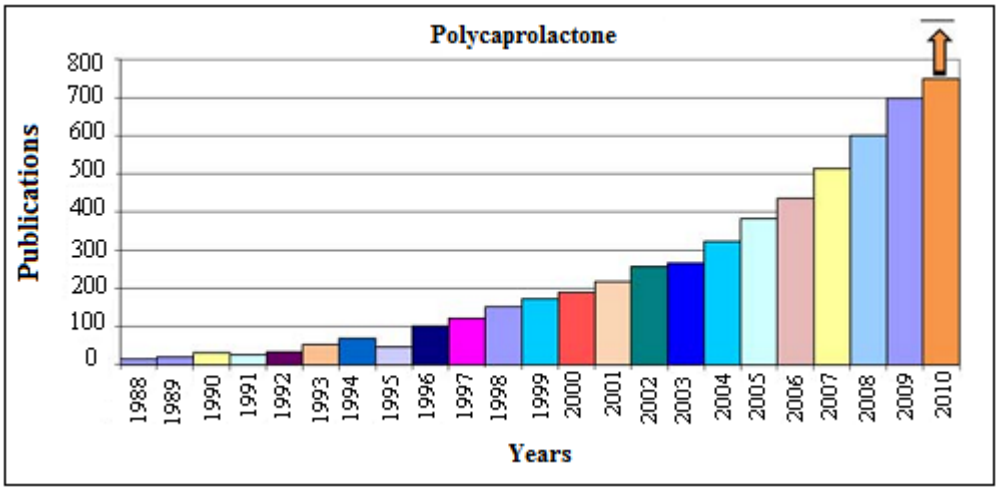


Figure 2.8 : Publications using PCL in the field of Biomaterials or Tissue Engineering during the last 20 years, until April 2010.

Beside of these, there are some studies reported: PCL was used in bone engineering, cartilage engineering, tendon and ligament engineering, cardiovascular engineering,

blood vessel engineering and skin engineering applications which are parts of tissue engineering. All in all, PCL and its copolymers constituted a new area for drug delivery systems, tissue engineering applications and long term degradable implants for the future (Woodruff and Hutmacher, 2010).

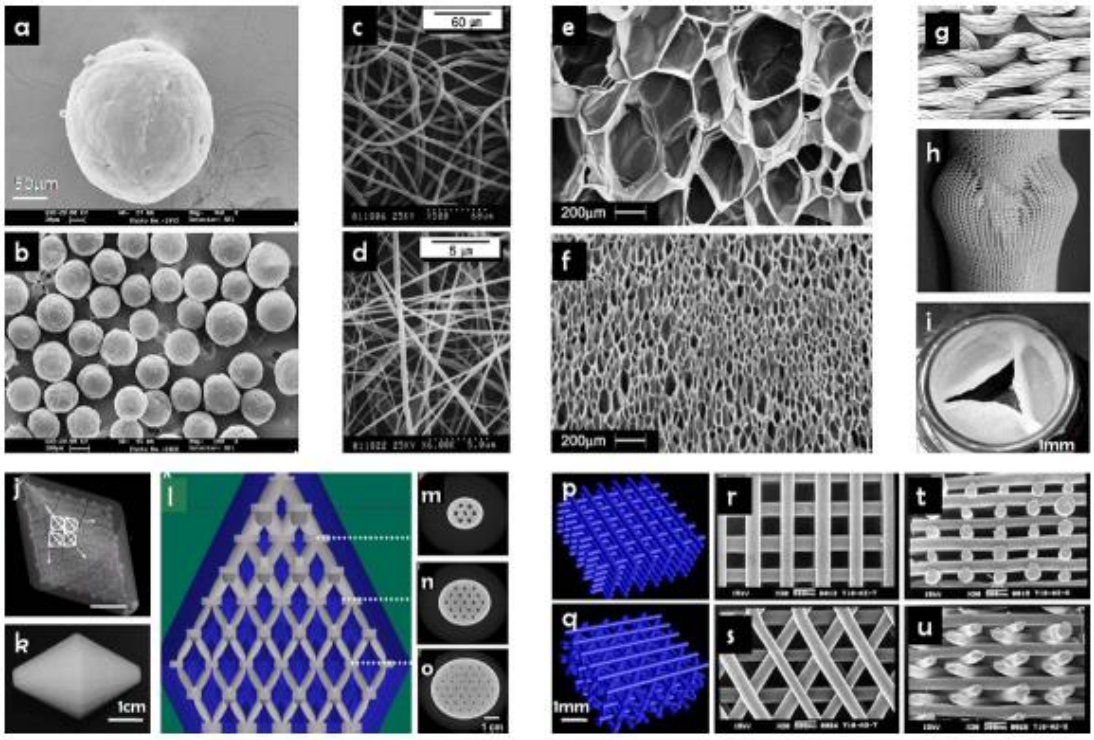


Figure 2.9 : Structures made from PCL: Nanospheres (a,b), nanofibres (c,d), foams (e,f), knitted textiles (g,h,i), selective laser sintered scaffold (j-o), fused deposition modeled scaffolds (p–u), (Woodruff and Hutmacher, 2010).

2.3.3 Methods of polycaprolactone synthesis

PCL can be synthesized by polycondensation of 6-hydroxycaproic (6-hydroxyhexanoic) acid and the ring opening polymerization of ε-CL. There are many studies that synthesizes PCL by polycondensation of hydroxycarboxylic acids. The polycondensation reactions can be performed without/with the addition of catalyst. *Candida antarctica* and *Pseudomonas sp.* were used as catalyst in some studies, and in these studies were reached that an average molecular weight of 9000 $gmol^{-1}$ and 5400 $gmol^{-1}$, respectively. However, PCL synthesized by ring opening polymerization have higher molecular weight and lower polydispersity polymerization (Labet and Thielemans, 2009).

Ring opening polymerization of lactones can be divided in transesterification side reactions and general mechanisms. General mechanisms can be also subdivided in four mechanisms based on the type of catalyst that are anionic, cationic, monomer-activated and coordination-insertion ROP.

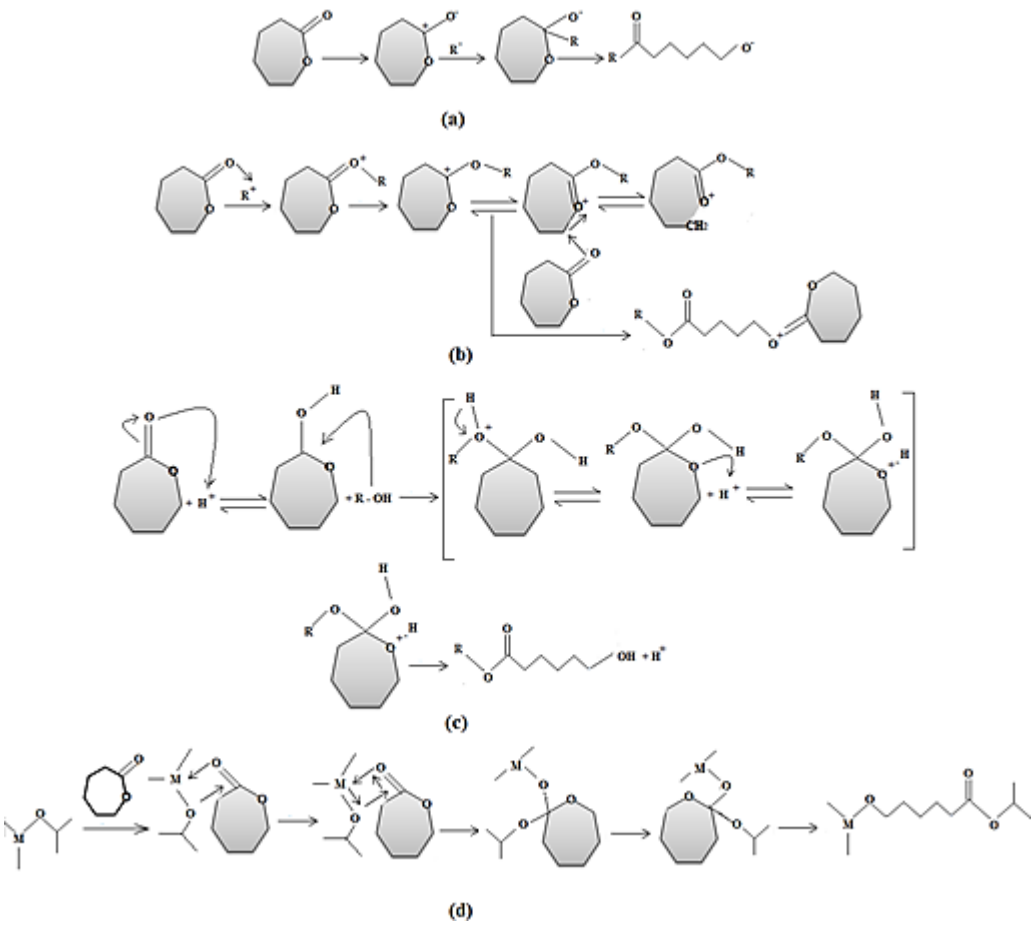


Figure 2.10 : The initiation steps of ring-opening polymerization mechanisms: (a) anionic ROP, (b) cationic ROP, (c) monomer-activated ROP, (d) coordination–insertion ROP.

In anionic ROP method, anionic species attack the carbonyl carbon of the monomer. The acyl-oxygen bond opens and then an alkoxide occurs. However, there is a disadvantage of this technique from which low molecular weight polymers are produced because of intramolecular transesterification reactions occured in the following stages of polymerization. Secondly, a bimolecular nucleophilic substitution reaction occurs in cationic ROP method in which cationic species is attacked by the carbonyl oxygen of the monomer (Figure 2.10).

In monomer activated ROP, the monomer molecules are activated by using a catalyst, and after that the activated monomer attacked on the polymer chain end. An

another mechanism used commonly is coordination–insertion ROP. In other words, in this pseudo-anionic ROP mechanism, monomer is inserted inside a metal-oxygen bond of the catalyst. Polymer chain grow up and is attached to the metal via alkoxide bond.

In transesterification side reactions, intermolecular and intramolecular transesterification can occur as side reactions. An initiator and a catalyst are used in this type of ROP. These reactions occur usually in the following stages of polymerization. Therefore it is difficult to provide control of polymerization (Labet and Thielemans, 2009).

2.4 Enzyme Catalyzed Polymerization of Biodegradable Polyesters

Biodegradable polyesters can be synthesized either by fermentation and chemical processes or by enzyme catalyzed polymerization. Enzymatic reactions are generally accepted as virtually reversible, and therefore the equilibrium can be kept under control by properly choosing the reaction conditions. Many of the hydrolases are catalyzed the enzymatic polymerization of polymers via a reversible bond-forming reaction.

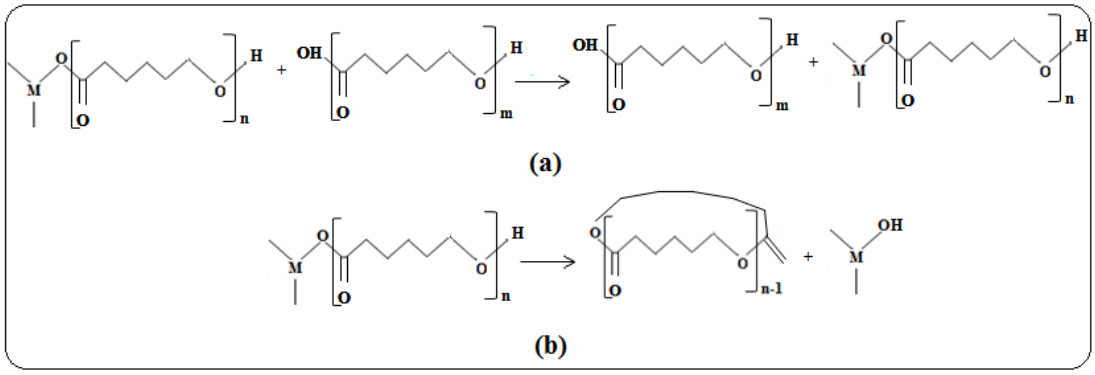


Figure 2.11 : Transesterification side reactions: (a) intermolecular transesterification reaction during the polymerisation of PCL, (b) intramolecular transesterification reaction during the polymerisation of PCL.

Lipases, which are parts of hydrolases class of enzymes, are used mostly as catalyst for enzymatic polymerization of polyesters. They can be used for esterification, transesterification and the hydrolysis of fatty acid esters reactions with different reaction types of lipase catalyzed polymerization that are shown in Figure 2.12.

2.4.1 Lipase catalyzed ring opening polymerization of lactones

Lipase catalyzed ring opening polymerization have been used for reactions used different cyclic esters (Figure 2.13). They can catalyze the ring opening polymerization of nonsubstituted lactones (4- to 17- membered). The first lipase catalyzed polymerization was studied in 1993: *Candida cylindracea, Burkholderia cepacia* (lipase BC), *Pseudomonas fluorescens* (lipase PF), and porcine pancreas lipases (PPL) catalyzed the polymerization of medium-size lactones, δ-valerolactone (δ-VL, 6-membered) and ε-caprolactone (ε-CL, 7-membered).

An another lactone, β-propiolactone (β-PL, 4-membered) was polymerized by using *Pseudomonas* family lipases and *Candida rugosa* lipase. Polymers with high molecular weights were produced end of these polymerization reactions. Moreover, the substituted 4-membered lactone, β-butyrolactone (β-BL), was polymerized by using PPL and CRL to produce poly(β-hydroxybutyrate).

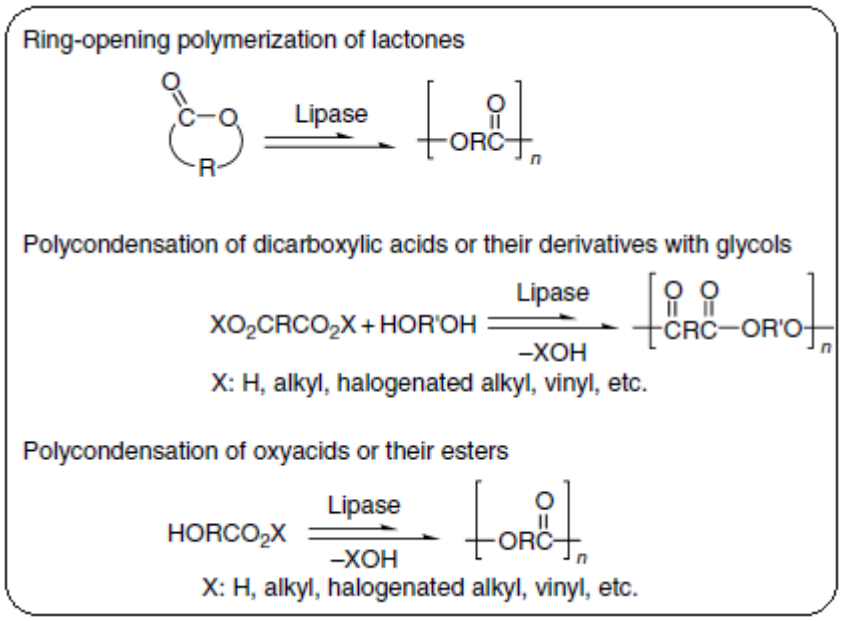


Figure 2.12 : Reaction types of lipase catalyzed polymerization.

γ-butyrolactone (γ-BL, 5-membered) was converted the oligomer form by using PPL or *Pseudomonas* sp. lipase as catalyst. The 6-membered lactones, δ-VL and 1,4-dioxan-2-one were polymerized by using different lipases, however the molecular weight of polymer produced by δ-VL as catalyst was less than 2000.

ε-CL, 7-membered lactone, is industrially produced and a widely used biodegradable polymer polycaprolactone is obtained by polymerization of ε-CL. Polycaprolactone

degradation happened also throughout ε-CL polymerization. There are a great number of catalysts such as PPL, CRL, lipase BC and lipase PF are used mostly for the ε-CL polymerization. Beside of these, lipase CA is more advantageous option for the ε-CL polymerization because of its high catalytic activity. On the other hand the sufficient amount of lipase CA is about 1 wt % for ε-CL, whereas more than 40 wt % is enough to polymerize ε-CL by using other lipases. Additionally, lipase CA can be reused for the polymerization (Uyama, 2007).

In the bulk polymerization of ε-CL, the linear polymer was produced, while the main product was generated in organic solvents. Lately, microwave-assisted lipase catalyzed polymerization of ε-CL was also reported. Different organic solvents were used in the polymerization of ε-CL catalyzed by lipase CA and toluene revealed the best performance with the high molecular weight polycaprolactone. Furthermore, the best molecular weight and monomer conversion of polycaprolactone was obtained toluene ε-CL ratio almost 2:1 in the reaction.

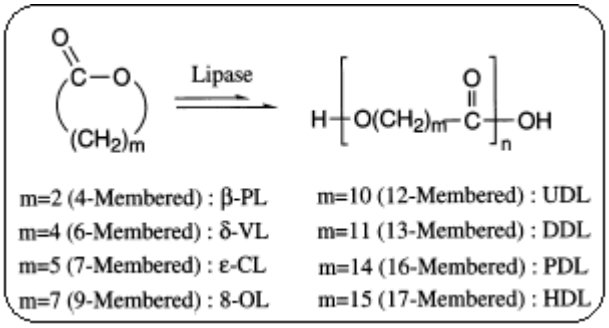


Figure 2.13 : Lipase-catalyzed ring-opening polymerization of lactones (Namekawa et al., 1999).

An another lactone, 8-octanolide (8-OL, 9-membered) was polymerized by using lipase BC and lipase CA as catalyst. One of the unsubstituted macrolides, 12-decanolide (13-membered, DDL) can be enzymatically polymerized by using lipase BC, lipase PF, CRL and PPL. The highest catalitic activity belongs to lipase BC, whereas PPL shows the smallest activity. Lipase CA catalyzed also the unsubstituted macrolide 15-pentadecanolide (16-membered, PDL) polymerization in toluene and the molecular weight of polymer reached about 8×10^4. The other unsubstituted macrolides, 11-undecanolide (12-membered, UDL) and 16-hexadecanolide (17-membered) can be also polymerized enzymatically.

Reactivity of cyclic esters change depend on their ring sizes. The large ring size esters have small ring opening reactivity, whereas the small and intermediate ring size esters have higher reactivity than the others. The dipol moment determines also ring strain of lactones. δ-VL and ε-CL have higher dipole moment than the macrolides. Furthermore, the rate constant of δ-VL and ε-CL in ring opening polymerization is higher than macrolides. As is also understood from these informations, the macrolides have less reactivity and and polymerization ability than the medium size lactones.

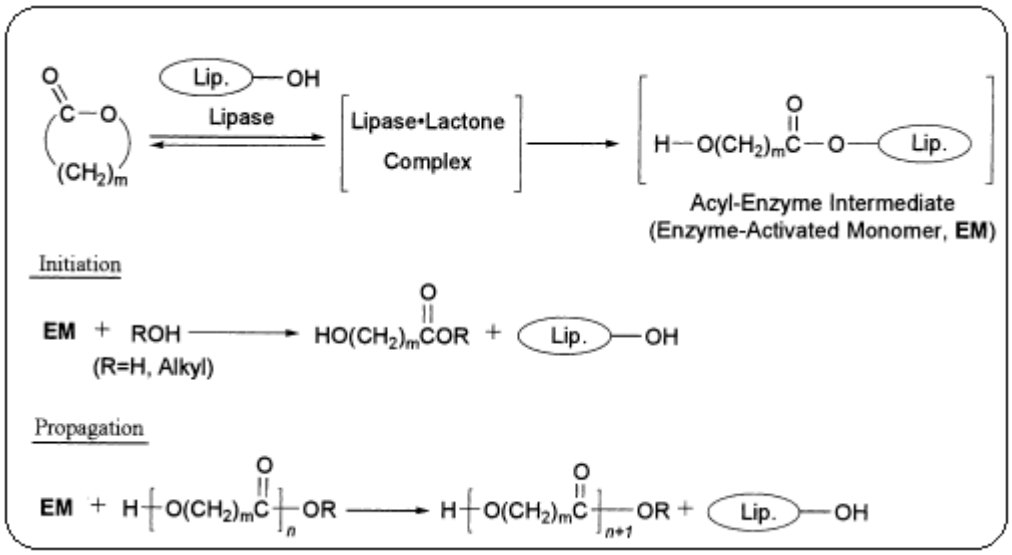


Figure 2.14 : Mechanism of enzymatic ring opening polymerization.

In the mechanism of lipase catalyzed ring opening polymerization of lactones, the catalytic site of lipase (a serine residue) catalyzes the reaction (Figure 2.14). The reaction continues via an acyl–enzyme intermediate called "enzyme-activated monomer", EM which is the most important step of this reaction mechanism. The enzyme includes partially water carried out a nucleophilic attack on the acyl carbon of the intermediate and in this way the initiation step takes place to produce ω-hydroxycarboxylic acid. In the propagation step, the intermediate is nucleophilically attacked by the terminal hydroxyl group of a propagating polymer to elongate one unit-more the polymer chain. According to the kinetics of the polymerization, the obtaining of the enzyme-activated monomer is the rate determining step of the polymerization. Therefore, the activated-monomer mechanism is the key stage for ring opening polymerization which provides to continue whole polymerization reaction (Uyama, 2007).

There are many lipase catalyzed ring opening polymerization studies and the results reported: Yu et al. (2004) used immobilized PPL as biocatalyst in enzymatic ring opening polymerization of 2,2-dimethyltrimethylene. The highest molecular weight (41500 g/mol) was obtained by using the second recycling immobilized PPL with about 92 % yield. An another example, Li et al. (2011) synthesized polycaprolactone by using *Fervidobacterium nodosum* lipase as catalyst in ring opening polymerization. A number average molecular weight of 2340 g/mol and almost 100 % monomer conversion were obtained at 90 ºC for 72 h.

2.4.1.1 CALB catalyzed ring opening polymerization of lactones

Thefree CALB catalyzed ring opening polymerization of lactones resulted in biodegradable and biorenewable polyesters of low molecular weight because of the heat which caused inactivation of free CALB. The improved thermal stability and repetitive use of immobilized CALB can maintain the ring opening polymerization of lactones more attractively (Idris and Bukhari, 2012).

There are many studies used CALB to catalyze the ring opening polymerization of lactones: Córdova et al. (1998) used CALB as catalyst to polymerize ε-CL and the ring opening polymerization reactions were carried out in organic solvents as well as without solvent at 60 ºC. They reached the highest weight average molecular weight (Mw) of PCL 4701 g/mol without solvents, while 2984, 1297 and 1862 g/mol were respectively obtained in dioxane, acetonitrile and tetrahydrofuran (THF) after 24 h reaction time. In another study, Córdova et al. (1999) were used CALB as catalyst for ring opening polymerization of ε-CL by initiation and termination reactions. They reached high monomer conversions about 99 % and between 1960-2400 g/mol Mw of PCL.

2.4.2 Advantages of enzyme catalyzed polymerization over chemical polymerization for the synthesis of biopolyesters

Enzyme catalyzed polymerization is an eco-friendly technique contributed “green chemistry”. This method have various benefits over conventional chemical polymerization methods. In chemical polymerization methods, metallic catalysts such as Zn, Al and Sn are mostly used in ring opening polymerization of lactones. However, it is not possible to remove completely this metallic catalysts and its residues from the polymers. These polymers are not suitable to use biomedical

applications because of the high toxicity of these metallic catalysts. On the other hand, in the conventional polycondensation reactions, strong acidic catalysts are used and a mixture included diol and dicarboxylic acid or a hydroxy carboxylic acid is heated to high temperatures. These strong catalysts can cause discoloration of polymers and the stoichiometry of the reaction can disturb at high temperatures. However, in enzyme catalyzed polymerization, polymerization reactions can be proceed at low temperatures by using biocatalysts.

The advantages of enzyme catalyzed polymerization are:

- Enzymes catalyze the polymerization reactions with high regio- and enantio-selectivity.
- Enzyme catalyzed polymerization can take place under mild reaction conditions such as low temperature and pressure etc.
- Enzymes can be used in bulk media, in organic solvents and at different interfaces.
- Enzymes are reusable and non-toxic materials.
- Enzymes are eco-friendly materials obtained from renewable sources and they can be easily removed from polymers.
- In enzyme catalyzed polymerization, polymers with well-defined structures can be obtained easily.
- The elimination of water and air is not necessary in lipase catalyzed polyester synthesis. Because, water is used as initiator in contrast to conventional chemical methods.
- Cyclic lactones (especially the small and intermediate ring size lactones) can be easily polymerized by enzymatic polymerization (Varma et al., 2005).

3. MATERIALS AND METHODS

3.1 Materials

The free form of *Candida antarctica* lipase B and Novozym® 435 were purchased from Sigma Aldrich Company (Germany). Precipitated silica was provided from a company in Istanbul and it was used as a support to immobilize CALB. Acetone was supplied Sigma Aldrich Company (Germany) and used as solvent in silanization. The organosilane agent 3-APTES was purchased from Merck Company (Germany). Glutaraldehyde solution was purchased from BDH Chemicals Company (England) and used for immobilization by crosslinking method.

Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) and sodium monohydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$) were used to prepare phosphate buffer solution and purchased from Carlo Erba Reagenti Company (Italy) and Merck Company (Germany), respectively. Sodium hydroxide was used to prepare NaOH solution and purchased from Carlo Erba Reagenti Company (Italy). In addition, ethanol was used to stop the reaction in titration and were supplied from Merck Company (Germany).

Table 3.1 : Chemical properties of ε-caprolactone.

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

ε-caprolactone (99 %) was used as monomer of the polymerization and supplied from Alfa Aesar Company (Germany). Chemical properties of ε-caprolactone are demonstrated in Table 3.1. Before polymerization reactions, ε-caprolactone was dried by using molecular sieves. Toluene and methanol were purchased from Merck Company (Germany) with more than 99 % purity. Chloroform was supplied from Sigma Aldrich Company (Germany), and was used without applying any

pretreatment process. Tetrahydrofuran was used to dissolve the polymer in GPC Analysis and purchased from Labkim Company (Istanbul).

3.2 Equipments

The following equipments were used in this study:

- ✓ Shaking Water Bath, Julabo SW22
- ✓ UV mini 1240 SHIMADZU spectrophotometer
- ✓ Fourier transform infrared spectroscopy (FT-IR), Pelkin Elmer
- ✓ Gel Permeation Chromatography (GPC), Agilent 1100 Series
- ✓ Scanning Electron Microscopy (SEM), JEOL JSM-6390LV
- ✓ Differential Scanning Calorimetry (DSC), SEIKO 7020
- ✓ Thermal Gravimetric and Differential Thermal Analysis (TG/DTA), SEIKO 6300
- ✓ Proton Nuclear Magnetic Resonance (^{1}H-NMR), Agilent VNMRS 500 MHz
- ✓ Drying Oven, BINDER and Elektro-Mag
- ✓ pH meter, Inolab, TWT

3.3 Methods

3.3.1 Immobilization of CALB on precipitated silica

CALB was immobilized on precipitated silica by using 2 different immobilization methods which were physical adsorption and crosslinking methods. Silanization was the first step of immobilization by both methods (Figure 3.1). 3-aminopropyltriethoxysilane (3-APTES) is a most common used reactive silane for silanization because its amino groups are sensitive to the coupling reaction (Park et al., 2002). Therefore 3-APTES was used as an organosilane agent to modificate the precipitate silica. Furthermore, acetone was used as a solvent for silanization step of immobilization (Lee et al., 2006). During the silanization, firstly precipitated silica was activated by adding different amount of 3-APTES/acetone solution. 250 mg precipitated silica was mixed with 0.25 mL, 0.5 mL, 0.75 mL and 1 mL 3-APTES to obtain the 5 %, 10 %, 15 % and 20 % (w/v) ratios of 3-APTES/acetone solution, respectively (Park et al., 2002). These suspensions were stirred (160 rpm) at 50 ºC for 2 hour in shaking water bath. The activated precipitated silicas were washed with water and dried at 60 ºC for 2 hour in drying oven (Lee et al., 2006).

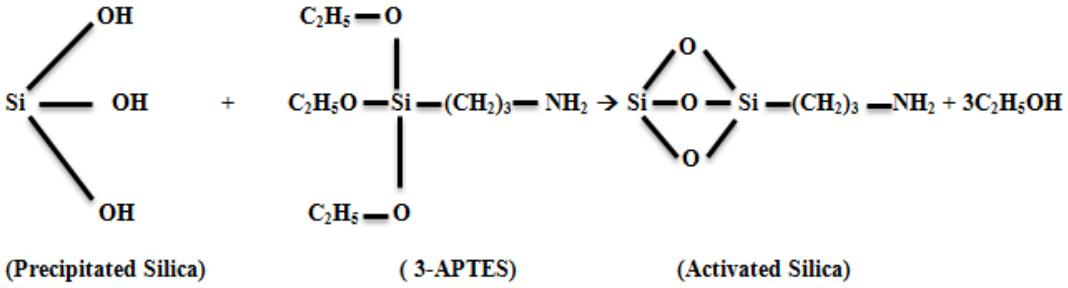


Figure 3.1 : Modification of precipitated silica by using 3-APTES (Miletić et al., 2010).

The activated silicas were mixed with different amounts of CALB to immobilize the lipase by physical adsorption. 0.125 mL, 0.250 mL, 0.5 mL and 0.75 mL of CALB were stirred with the activated silicas in 25 mL of phosphate buffer solution (pH 7, 0.015M) to obtain the 0.5 (w/w), 1 (w/w), 2 (w/w) and 3 (w/w) ratios of enzyme/silica, respectively (at 25 ºC for 5 hour). After stirring for 5 hour, the immobilized enzymes were filtered under vacuum and the filtrates were separated to determine the amount of protein loaded. Immobilized enzymes then were washed with phosphate buffer solution (pH 7, 0.015M) and dried at 30 ºC for 12-24 hour in drying oven. Finally, the immobilized enzymes by physical adsorption were stored at 4 ºC.

As a crosslinking agent, glutaraldehyde solution was used to immobilize CALB by crosslinking method (Figure 3.2). The activated silicas were stirred with 0.02 %, 0.2 % and 2 % (v/v) glutaraldehyde/phosphate buffer solution (pH 7, 0.015M), 0.005 mL, 0.05 mL and 0.5 mL glutaraldehyde solutions were used respectively for crosslinking the activated silicas at 25 ºC for 2 hour. After that, the silicas were filtered under vacuum by washing with water (Lee et al., 2006).

After crosslinking step of immobilization by crosslinking method, CALB was immobilized on precipitated silicas modified with 3-APTES and glutaraldehyde solution (Figure 3.3). The same procedure was applied to immobilize CALB by crosslinking method in which the precipitated silicas and different amounts of CALB were mixed in 25 mL of phosphate buffer solution (pH 7, 0.015M) at 25 ºC for 5 hour. Then the immobilized enzymes were filtered under vacuum and the filtrates were separated to determine the amount of protein loaded. Eventually, immobilized

enzymes were washed with phosphate buffer solution (pH 7, 0.015M) and dried at 30 ºC for 12-24 hour in drying oven (Lee et al., 2006).

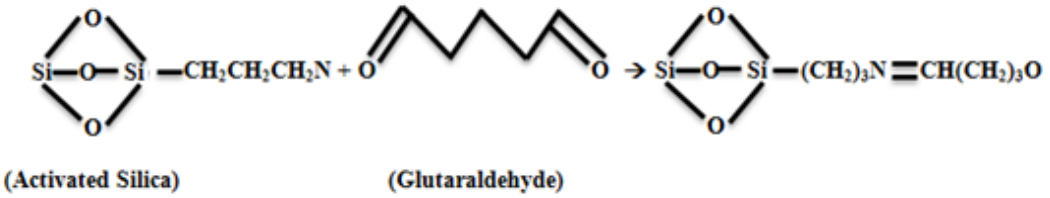


Figure 3.2 : Crosslinking of the activated silica by glutaraldehyde solution (Miletić et al., 2010).

Phosphate buffer solution was prepared by adding 45.75 mL $Na_2HPO_4.7H_2O$ and 29.25 mL $NaH_2PO_4.H_2O$ in 1000 mL deionized. The ionic groups of glutaraldehyde reacts with supports as an anionic exchanger. Therefore pH value of phosphate buffer solution was adjusted to neutral condition (pH:7) for preventing the ionic strength (İyisan, 2011).

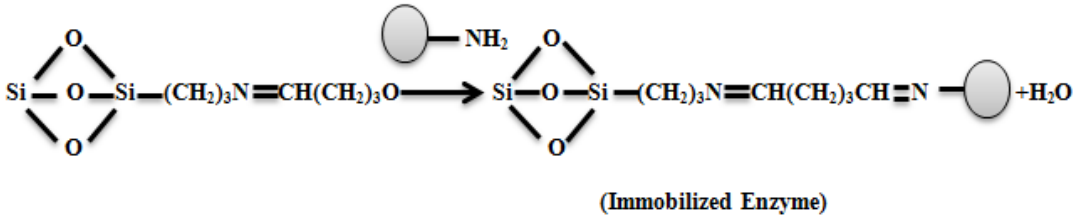


Figure 3.3 : Immobilization of CALB by crosslinking method (Miletić et al., 2010).

3.3.2 Lipase activity determination

Activity of immobilized lipases was determined by alkalimetric final titration. For preparing the sample, 10 mg immobilized enzyme was dissolved in 1 mL phosphate buffer solution (pH 7, 0.015M). 100 µL sample and 500 µL olive oil, which was used as the substrat, were added in 2.5 mL of phosphate buffer solution (pH 7.2, 0.1 M). This mixture was stirred (160 rpm) at 37 ºC for 30 minutes in shaking water bath. The reaction was stopped with 1.25 mL acetone/1.25 mL ethanol mixture. After that, the reaction mixture was titrated with 0.1 M NaOH by adding 2-3 drops phenolphthalein as indicator (Cernia et al., 2002). Immobilized lipase activities were calculated by using Equation 3.1 (Öztürk-Düşkünkorur, 2012), where V_t and V_f mean the titrant volume for immobilized enzyme and free lipase, respectively.

$$\text{Lipase activity } (\mu L/\text{min}) = [(Vt - Vf) \,/\, 30 \text{ min }] \times 10^6 \qquad \textbf{(3.1)}$$

The optimum pH and the optimum temperature were determined also to observe the effect of temperature and pH on lipase activity. First of all, phosphate buffer solutions had different pH values (4.5, 5.5, 6.5, 8.5, 9.5 and 10.5) were prepared to determine the optimum pH. The assay samples were mixed with 500 µL olive oil in these different phosphate buffer solutions (2.5 mL). Secondly, the incubation of the mixture included the sample, the olive oil and the phosphate buffer solution was carried out at different temperatures which were 30, 35, 40, 45, 50, 55 and 60 º C. Titration was made by following the same procedure for both determination of the optimum temparature and the optimum pH.

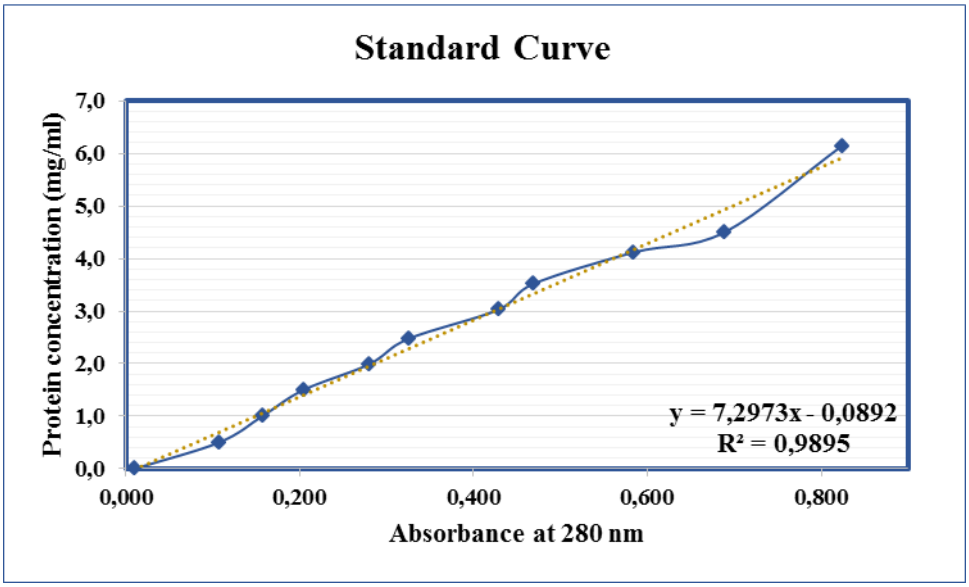


Figure 3.4 : Standard curve for lipase protein determination (İyisan, 2011).

Lipase activities were measured periodically to define storage stability of immobilized enzymes. Finally, the residual activity was calculated by using Equation 3.2, where A_1 and A_2 mean that initial activity of immobilized lipase and activity of immobilized lipase at a specified time, respectively.

$$Residual\ activity\ (\%) = \frac{A2}{A1} \times 100 \qquad \textbf{(3.2)}$$

3.3.3 Lipase protein determination

UV spectroscopy was chosen as protein determination method among various methods. Standart curve was formed by using lipase enzyme solution (Figure 3.4). The filtrates separated during filtering the immobilized enzymes were diluted (10 %) in phosphate buffer solution (pH 7, 0.015M). Absorbance measurements were noted at 280 nm and amounts of protein loaded (mg/mL) were calculated by using these absorbance values. Specific activity was calculated also by using Equation 3.3 which is the ratio of lipase activity to amount of protein loaded.

$$\text{Specific activity (U/mg)} = \text{Lipase activity / Amount of protein loaded} \qquad \textbf{(3.3)}$$

Protein loading was determined by using Equation 3.4 (Zou et al., 2010). C_i and C_f mean the concentrations of the enzyme protein initial and final in the immobilization medium, respectively.

$$Protein\ loading\ (\%) = \frac{Ci - Cf}{Ci} \times 100 \qquad \textbf{(3.4)}$$

3.3.4 Synthesis of polycaprolactone by immobilized enzymes

Polymerization reactions were carried out by using immobilized enzymes, the free form of *Candida antarctica* lipase B and Novozym® 435. Toluene was used as organic media because of their excellent performance over other organic solvents used in polymerization reactions (İyisan, 2011). The polymerization reactions were performed in 1000 mg toluene (toluene to ε-caprolactone, 2:1 (v/v)) by using 100 mg enzyme as catalyst (Uyama, 2007). Inert nitrogen was supplied in the reaction medium for 1 minutes. The reaction medium was stirred at 120 rpm with a magnetic stirrer during a specified time. After the specified time, the reaction was stopped by adding chloroform and enzyme was separated from the reaction medium by filtering with chloroform. Then, enzyme was washed also with phosphate buffer solution (pH 7, 0.015M) and dried at 50 ºC for 24 hour. On the other hand, the filtrate included the reaction medium in chloroform was dried to evaporate the chloroform at 50 ºC in drying oven. After evaporation of a large amount of chloroform, the solution was precipitated in cold methanol. Finally, polymer was filtered under vacuum and dried at 30 ºC for 12-24 hour. The dried polymers were stored to characterize in the desiccator. The experimental setup is shown in Figure 3.5.

$$Monomer\ conversion\ (\%) = \frac{Wp}{Wm} \times 100 \qquad \textbf{(3.5)}$$

The monomer conversions of polycaprolactones were gravimetrically calculated by using Equation 3.5 in which Wp and Wm mean the weight of the resulted polymer and monomer, respectively.

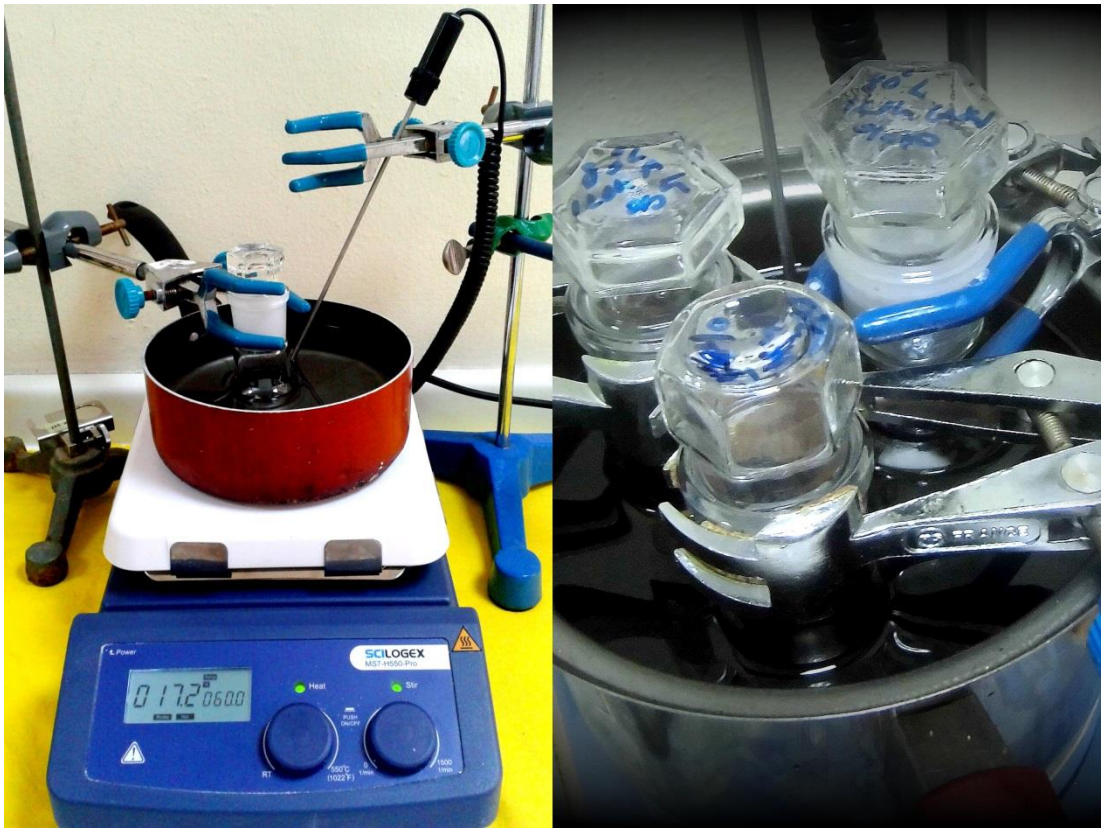


Figure 3.5 : The experimental setup.

The polymerization reactions were carried out at 40 ºC, 60 ºC and 80 ºC temperatures during the different time periods which were 6, 24, 48, 72, 96 and 120 hour. During 150 h and 170 h were also studied to observe the stability of the number average molecular weights (Mn) for some polymers. In addition, the ratios of enzyme to monomer were changed (enzyme to ε-caprolactone, % 2.5, % 5, % 10 and % 20 (w/w)) and it was obtained the optimum temperature, time period and enzyme concentration. Finally, immobilized enzyme reusability was demonstrated by reaction cycles of polycaprolactone synthesis. The retention of initial enzyme activity was determined after reaction cycles.

3.3.5 Characterization techniques of immobilized enzymes and synthesized polycaprolactones

3.3.5.1 Ultraviolet spectrophotometer (UV)

Efficiency of lipase immobilization was determined by UV mini 1240 SHIMADZU spectrophotometer (Figure 3.6). Phosphate buffer solution was used as a blank sample and absorbance measured at 280 nm. The samples were prepared by diluting to 10 % and 100 % with phosphate buffer solution (pH 7, 0.015M).

Figure 3.6 : UV mini 1240 SHIMADZU spectrophemeter.

3.3.5.2 Thermogravimetric analysis (TGA)

Thermal characterization of the precipitated silica, activated silica and polycaprolactones were carried out by thermogravimetric analysis (TGA) on 10-20 mg samples by heating from room temperature to 900 ºC at 10 ºC/min under nitrogen or air flow using a TG/DTA6300 apparatus (Öztürk-Düşkünkorur, 2012).

3.3.5.3 Scanning electron microscopy (SEM)

The surface morphology of the polycaprolactones, precipitated silica and immobilized enzymes were characterized by Scanning Electron Microscopy (SEM). The assay samples were initially coated with a layer of platinum to provide conductivity between the samples and electrodes. Analyses were performed on a

JEOL JSM-6390LV SEM apparatus with accelerating voltage of 5 kV and images were recorded at different magnifications.

3.3.5.4 Fourier transform infrared spectroscopy (FTIR)

The bond structures of polycaprolactones and immobilized enzymes were characterized by using a Perkin Elmer Fourier Transform Infrared Spectroscopy (FTIR) apparatus. The analysis was performed by using the ATR kit of FTIR apparatus. Through FTIR analysis, the synthesized polymers possessed the highest Mn were analyzed whether the resulting polymer has the characteristic properties of PCL or not (Özsağıroğlu, 2011). Moreover, the amount of deposited enzyme per area of the Si surface was determined by FTIR analysis (Miletić et al., 2010).

3.3.5.5 Differential scanning calorimetry (DSC)

Differential Scanning Calorimetry (DSC) was conducted on a Seiko 7020 DSC (Sensitivity, 0.2 μW) under inert atmosphere on 7-9 mg samples placed in aluminium pans. The materials were exposed to consecutive thermal cycles (heat-cool-heat) with the first heating scan allowing to obliterate the thermal or thermomechanical history of the materials. Thermal characterization was performed between -80 and 100 ºC for PCL at 10 ºC/min. DSC was used to determine the thermal properties of polymers such as glass transition temperature (Tg), crystallization temperature (Tc), the melting enthalpy (ΔH_e), the fusion enthalpy (ΔH_f) and melting temperature (Tm).

Crystallinity percentages were calculated by taking the ratio of fusion enthalpy of the sample to the fusion enthalpy of purely crystalline polymer ($\Delta H^0 f$) showed in the Equation 3.6.

$$\chi\,(\%) = [\Delta H_{fsample}\,(J/g) / \Delta H^0_f\,(J/g)] \times 100 \qquad \textbf{(3.6)}$$

where ΔH^0_f PCL= 139 J/g (theoretical value for 100% of crystallinity) and χ is crystallinity (Öztürk-Düşkünkorur, 2012).

3.3.5.7 Gel permeation chromatography (GPC)

An Agilent 1100 Series Gel Permeation Chromatography (GPC) was used to determine the number average molecular weight (Mn), the weight average molecular weight (Mw) and polydispersity (PDI) of the synthesized polycaprolactones (Figure 3.7). The samples were previously prepared by dissolving into tetrahydrofuran. The

dissolved samples were filtered then by using 0.45 µm pore size membrane. The analyses were performed during 20 or 45 minutes.

Figure 3.7 : Gel permeation chromatography (GPC).

On the other hand, the obtained results by using GPC are not completely clear, although GPC is one of the most common apparatus to determine molecular weight of polymers. The molecular weights can change depending on used solvents (Özsağıroğlu, 2011). Therefore, the determined Mn values were compared to the determined Mn measurements by using ^{1}H NMR.

3.3.5.6 Nuclear magnetic resonance (NMR)

Proton nuclear magnetic resonance (^{1}H NMR) was used to determine the reaction conversion rate, calculating of Mn values of polymers and the detection of the polymer structure. The ^{1}H NMR spectrums were recorded on an Agilent VNMRS 500 MHz ^{1}H NMR apparatus.

The samples were prepared by adding sufficient amount of a deuterium solvent (deuterated chloroform, 0.6 - 0.75 mL) in 10 mg sample. The Mn values of polymers were calculated by using the Equation 3.7. In this equation, the areas of two peaks on ^{1}H NMR spectrum were used for calculation (Sha et al., 2005).

$$Mn,_{NMR} = [(5 \times I_{4.07}) / (2 \times I_{3.65})] \times M,_{\varepsilon\text{-CL}} \qquad \textbf{(3.7)}$$

The polymerization degree is calculated by the ratio of 4.07 ppm peak area to 3.65 ppm peak area. The reaction conversion rates are calculated also by using these polymerization degrees.

^{1}H NMR was performed for the synthesized polycaprolactones had the highest Mn and the results were compared to the determined Mn values by using GPC (Özsağıroğlu, 2011).

4.RESULTS AND DISCUSSION

4.1 Immobilization of CALB on Precipitated Silica by Physical Adsorption and Crosslinking Methods

Candida antarctica lipase B was successfully immobilized onto precipitated silica by physical adsorption and crosslinking methods (Figure 4.1). The effect of 3-APTES concentration, glutaraldehyde concentration and enzyme loading on immobilization were investigated, and also storage stability of immobilized enzymes, the optimum pH and the optimum temperature were researched in this study.

Figure 4.1 : CALB immobilization steps on precipitated silica.

4.1.1 The effect of 3-APTES concentration on immobilization

Firstly, the effect of 3-APTES concentration on immobilization processes was observed by calculating the activity (U), protein loading (%) and specific activity (U/mg) of immobilized enzymes, which can be seen in Table 4.1.

Table 4.1 : Different amounts of 3-APTES concentration.

Immobilization Method	3-APTES conc. (%)	Protein loading (%)	Activity (U)	Specific Activity (U/mg)
Physical Adsorption	**5**	87	2500	10.1
	10	90	2670	10.4
	15	88	3330	13.4
	20	62	2670	15.1
Crosslinking	**5**	95	2500	9.2
	10	94	2670	10.0
	15	90	2670	10.6
	20	94	2500	9.4

From Table 4.1, a remarkable difference was not observed in activity of immobilized enzymes by crosslinking method (IEc), while the best specific activity was obtained at 15 % (w/v) concentration of 3-APTES in acetone solution. Moreover, protein loading percentages of crosslinked enzymes were changing between 90 % and 95 % showed that CALB was successfully immobilized with high percentages. In contrast, the protein loading percentage decreased to 62 % from 88-90 % values at 20 % (w/v) concentration of 3-APTES in acetone solution for immobilized enzymes by physical adsorption (Figure 4.2). Besides, the best specific activity 15.1 was obtained at 20 % (w/v) concentration of 3-APTES, whilethe highest activity of immobilized enzymes by physical adsorption (IEa) was obtained at 15 % (w/v) concentration of 3-APTES in acetone solution.

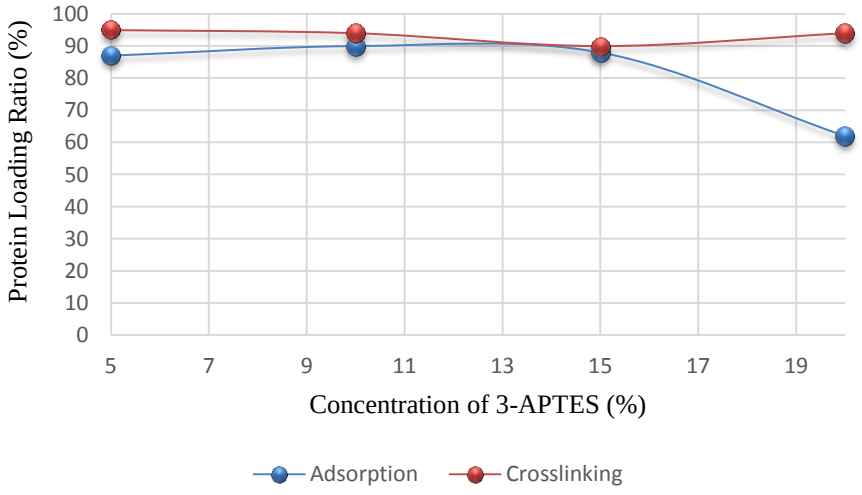


Figure 4.2 : The effect of 3-APTES concentration on protein loading ratio.

When the best results of activity, protein loading and specific activity were considered, it can be said that the optimum 3-APTES concentration is 15 % for both

immobilized enzymes by physical adsorption and crosslinking. Lee et al. (2006) researched the effect of 3-APTES concentration on lipase loading and activity. They reported similarly that the most effective concentration of 3-APTES was % 15.

4.1.2 The effect of glutaraldehyde concentration on immobilization

The effect of glutaraldehyde concentrationwas determined for immobilized enzymes by crosslinking method. It is shown in Table 4.2 that protein loading percentages did not significantly change, while the highest activity was obtained without adding glutaraldehyde solution (by physical adsorption).

Table 4.2 : Different amounts of glutaraldehyde concentration.

Glutaraldehyde concentration (%)	Protein loading (%)	Activity (U)	Specific Activity (U/mg)
0.0	**88**	3330	13.4
0.02	**88**	3000	12.0
0.2	**90**	2670	10.6
2.0	**90**	2170	8.5

It can be said from Figure 4.3 that the specific activity of immobilized enzymes reduced by increasing glutaraldehyde concentration (%). The negative effect of glutaraldehyde can be explained that a different enzyme orientation versus the support and a different lipase form (in support the enzyme can move the lid and this movement may be changed by the chemical modification).

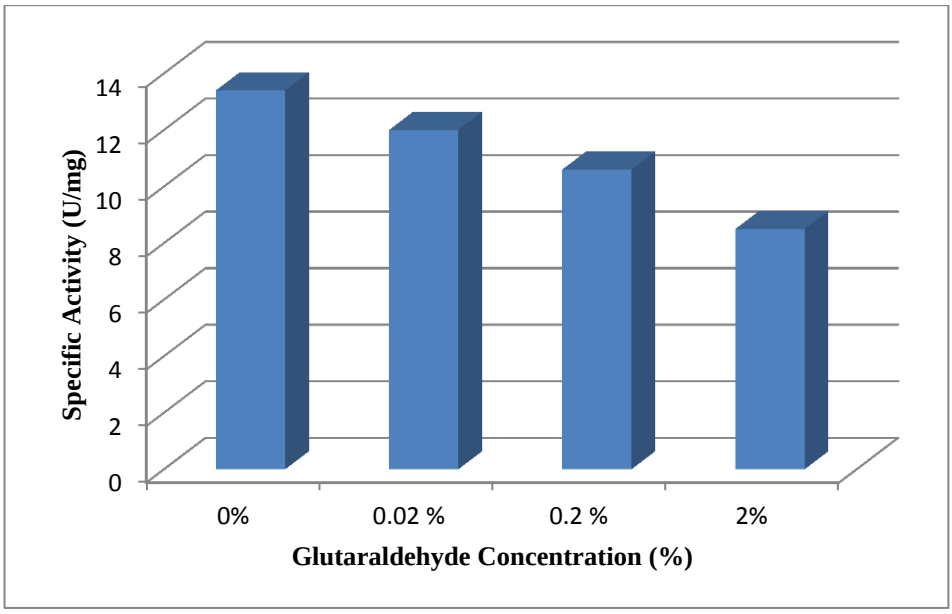


Figure 4.3 : Change of specific activity for different amounts of glutaraldehyde.

Moreover, there are some possibility for modification of CALB by glutaraldehyde, and the modification in this study could be cause a decline in the enzyme activity. Barbosa et al. (2012) reported the effect of glutaraldehyde concentration on enzyme activity. They used CALB as catalyst to immobilize on pre-activated agarose beads, and the specific activity of immobilized enzymes was lower than free lipase.

4.1.3 The effect of enzyme loading on immobilization

The activated silicas with and without modified by glutaraldehyde were used as carrier to immobilize CALB, and the effect of CALB amount were determined by calculating protein loading percentages which mean also immobilization efficiency. The activity (U) and specific activity (U/mg) of immobilized enzymes were also calculated at different ratio of enzyme to silica, which are shown in Table 4.3. The highest activity of immobilized enzyme was obtained at 2 (w/w) ratio of enzyme to silica for both physical adsorption and crosslinking methods.

Table 4.3 : Protein loading (%) for different amount of enzyme/silica (w/w).

Immobilization Method	Enzyme/Silica (w/w)	Protein loading (%)	Activity (U)	Specific Activity (U/mg)
Physical Adsorption	**0.5**	95	3000	47.5
	1.0	88	2670	21.5
	2.0	**88**	**3330**	**13.4**
	3.0	83	2400	6.7
Crosslinking	**0.5**	98	2330	33.7
	1.0	96	2100	15.5
	2.0	**90**	**2670**	**10.6**
	3.0	85	1700	4.6

From Figure 4.4, it is clear that the protein loading ratios did not substantially change which were very large quantities changing between 83 and 98 %. It means that CALB was successfully immobilized on precipitated silica, because protein loading ratio equals also immobilization efficiency (%). The highest immobilization efficiency was 98 % at 0.5 (w/w) ratio of enzyme to silica for IEc, while 95 % immobilization efficiency was obtained for IEa.

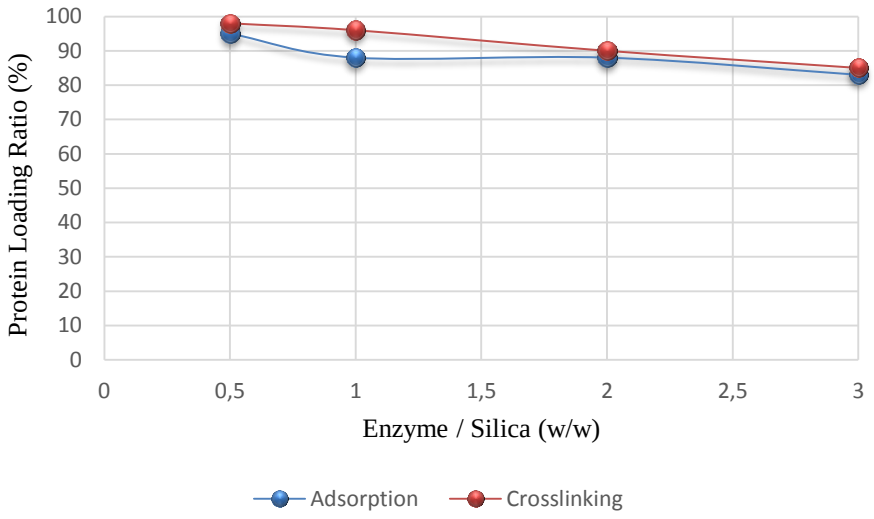


Figure 4.4 : Change of protein loading percentages of immobilized enzymes at the different ratio of enzyme to silica.

From Figure 4.5, it is seen that the specific activity of immobilized enzymes were increased by decreasing the ratio of enzyme to silica. It can be explained that when amount of enzyme loading increased, too many layers of enzymes filled the pores of the modified precipitated silica (Gao et al.,2009). Thus, enzyme molecules may deactivate by strong interaction with silica at high available surface of silica. Therefore, increasing enzyme concentration can be affect negatively specific activity of immobilized enzymes.

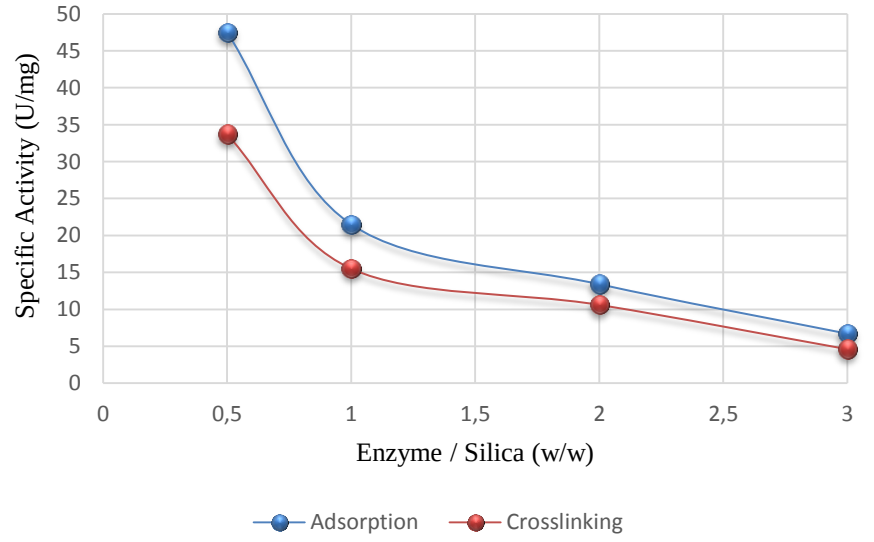


Figure 4.5 : Change of specific activity of immobilized enzymes at the different ratio of enzyme to silica.

Cruz et al. (2009) noted the activity of CALB immobilized on fumed silica (U/mg), and they observed also an adverse effect on specific activity when the ratio of enzyme to fumed silica increase. In another study, Gao et al. (2009) studied *Candida rugosa* lipase immobilization on silica by physical adsorption. They researched the effect of enzyme amount on the lipase activity, and observed similarly that increasing enzyme loading ratio caused reducing the specific activity of free and immobilized lipase.

4.1.4 Storage stability

The activity of immobilized enzymes were periodically measured to determine storage stability of enzymes (Table 4.4). The residual activity (%) was also calculated to determine retention of the initial activity of enzymes during specified time period.

Table 4.4 : Storage stability of immobilized enzymes.

Immobilization Method	Time (days)	Activity (U)	Residual activity (%)
Physical Adsorption	**0**	4330	-
	30	4000	92.0
	60	2300	53.0
	80	2170	50.1
	100	2170	50.1
	120	2170	50.1
Crosslinking	**0**	2830	-
	30	2070	73.15
	60	1670	59.0
	80	1670	59.0
	100	1670	59.0
	120	1670	59.0

From Figure 4.6, it is seen that immobilized enzyme by crosslinking was more stable than by physical adsorption because of the strength of crosslinked bonds between support and enzyme. It is clear that there is no a very big amount of activity changing for IEc after 2 months, while the enzyme activity reduced from 4330 U from 2170 U for enzyme by physical adsorption. On the other hand, the activity of enzyme by physical adsorption is higher than the immobilized enzyme by crosslinking. In addition, the enzyme activity became stable for both physical adsorption and crosslinking. After 4 months storage the immobilized CALB by physical adsorption and crosslinking protected 50.1 and 59 % of their initial activity at 4 ºC, respectively.

Based on these datas, it can be said that usage of CALB in industry became more feasible with immobilization on precipitated silica.

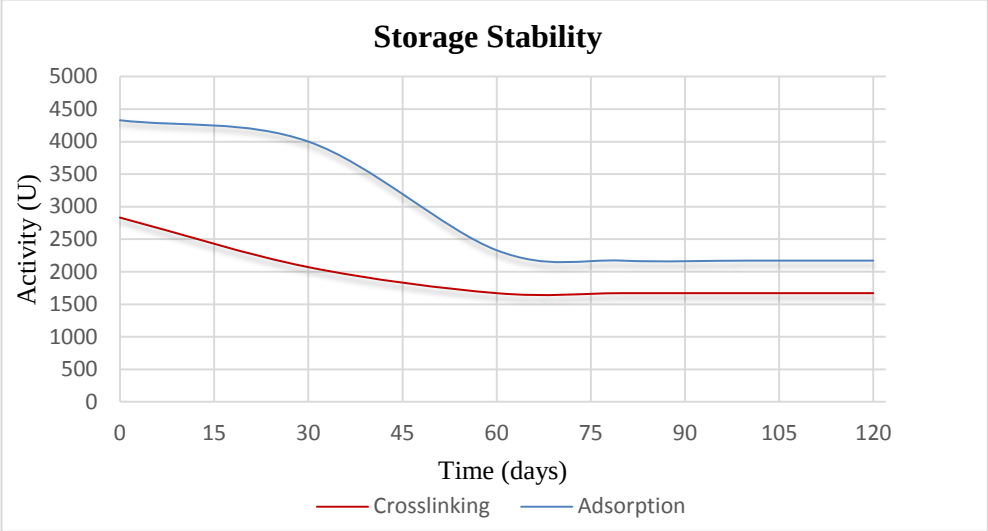


Figure 4.6 : Change of activity at specified time.

There are some studies: Cruz et al. (2009) investigated the long term stability of immobilized CALB at 4 ºC. They observed similar to the immobilized enzyme by crosslinking that there is no a very big change of activity of immobilized lipase after 6 months. Yıldırım et al. (2014) immobilized *Mucor miehei* lipase on Florisil which was a silica based material, and they obtained that after 30 days storage the immobilized lipase protected 43 % of its initial activity at 5 ºC. All in all, it can be said that both immobilized enzymes in our study are effective about the long term storage stability.

4.1.5 The optimum pH

The optimum pH was determined for free CALB and both immobilized lipases, and the highest activity (U) and specific activity (U/mg) were obtained at pH 7 for both immobilized enzymes by physical adsorption and crosslinking methods. Yang et al. (2010) determined the optimum pH for a free lipase from *Candida antarctica* and immobilized enzyme on silica. They reported that the optimum pH was 7.5, 7 and 8 for free lipase and two types of immobilized enzyme, respectively. Forde et al. (2010) noted that CALB has an optimum pH of pH 7 – 8, at which pH CALB has a appropriate molecular conformation and is four times more stable. According to this studies, it can be said that the determined optimum pH complied with the literature datas.

Table 4.5 : The optimum pH.

Immobilization Method	pH	Activity (U)	Specific Activity (U/mg)
Physical Adsorption	**4.5**	2000	8.0
	5.5	2670	11.0
	6.5	3000	12.0
	7.0	**3330**	**13.4**
	8.5	3170	13.0
	9.5	3170	13.0
	10.5	2330	9.4
Crosslinking	**4.5**	1330	5.3
	5.5	1670	6.6
	6.5	2000	7.9
	7.0	**2670**	**10.6**
	8.5	2000	7.9
	9.5	1670	6.6
	10.5	1670	6.6

From Figure 4.7, it can be more clearly seen that the highest activity for immobilized enzyme by crosslinking was obtained at a pH value of 7, while this value changing between at a pH value between 7-8 for immobilized enzyme by physical adsorption. Activites for free CALB were also calculated at different pH values, and optimum pH was similarly determined at pH 7 for free form of CALB.

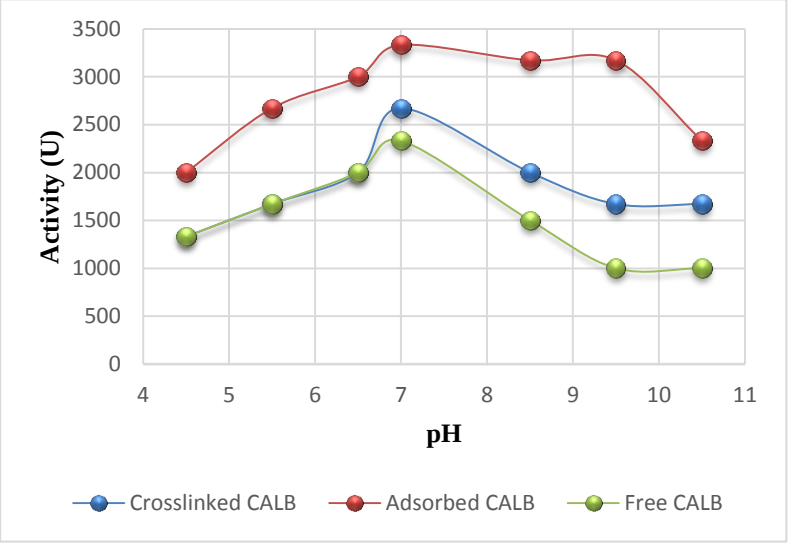


Figure 4.7 : The optimum pH.

4.1.6 The optimum temperature

The optimum temperature for both immobilized CALB was investigated that was about 35-40 ºC which can be seen in Table 4.6. The specific activity of immobilized enzymes reached to 13.4 and 10.6 U/mg for physical adsorption and crosslinking,

respectively. From Figure 4.8, it is shown that the highest activity for adsorbed lipase was obtained at 37 ºC, while the optimum temperature was determined at around 35-37 ºC for crosslinked lipase. Activities of free CALB was also calculated at different temperatures, and the optimum temperature was parallelly defined 37 ºC can be seen on Figure 4.8. In addition, both immobilized lipases and free CALB were deactivated at the higher temperatures. Cruz et al. (2009) investigated the effect of temperature on the activity of CALB immobilized on fumed silica at different fumed silica contents. The best results were obtained between 35 – 45 ºC in this study. According to this study, it can be said that the determined optimum temperature was seemed to be literature datas.

Table 4.6 : The optimum temperature.

Immobilization Method	Temperature (ºC)	Activity (U)	Specific Activity (U/mg)
Physical Adsorption	**30**	2670	10.7
	35	3000	12.0
	37	**3330**	**13.4**
	40	2670	10.7
	45	2000	8.0
	50	1800	7.4
	55	1670	6.7
	60	1300	5.4
Crosslinking	**30**	2000	8.0
	35	2670	10.6
	37	**2670**	**10.6**
	40	2000	8.0
	45	1800	7.3
	50	1000	4.0
	55	1000	4.0
	60	1000	4.0

After determination of the optimum 3-APTES concentration, glutaraldehyde concentration, enzyme amount, temperature and pH, immobilization of CALB was performed by using 15 % 3-APTES (w/v) in 20 mL acetone solvent, and 2 % (v/v) glutaraldehyde was added for immobilization by crosslinking. The ratio of lipase to silica adjusted as 2 (w/w) due to that the best lipase activity was obtained at this value. Immobilization of CALB was repeated for many times by physical adsorption and crosslinking, and lipase activity was measured at the optimum pH and temperature which are 7 and 37 ºC respectively.

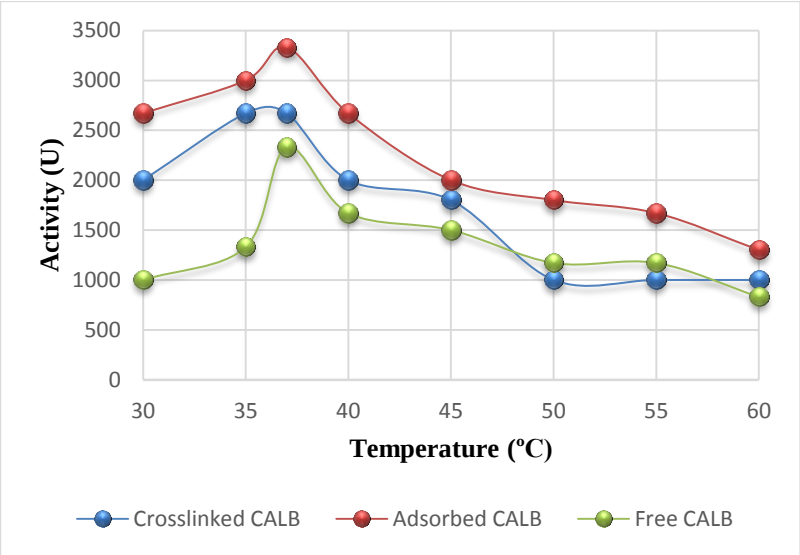


Figure 4.8 : The optimum temperature.

According to the results of the series of experiments, the maximum activity of immobilized enzyme by physical adsorption was 4330 U, while 2830 U was obtained for immobilized CALB by crosslinking method. Both of these values were higher than the activity of free CALB (Figure 4.9). The active site structure of CALB is not like other lipases, shows increased activity when immobilized on carriers, especially hydrophobic carriers (Mihailović et al., 2014). It can be said that useful structure change in immobilized lipases could be formed due to hydrophobic interactions and intermolecular force between precipitated silica, 3-APTES and glutaraldehyde solution (Zou et al., 2010).

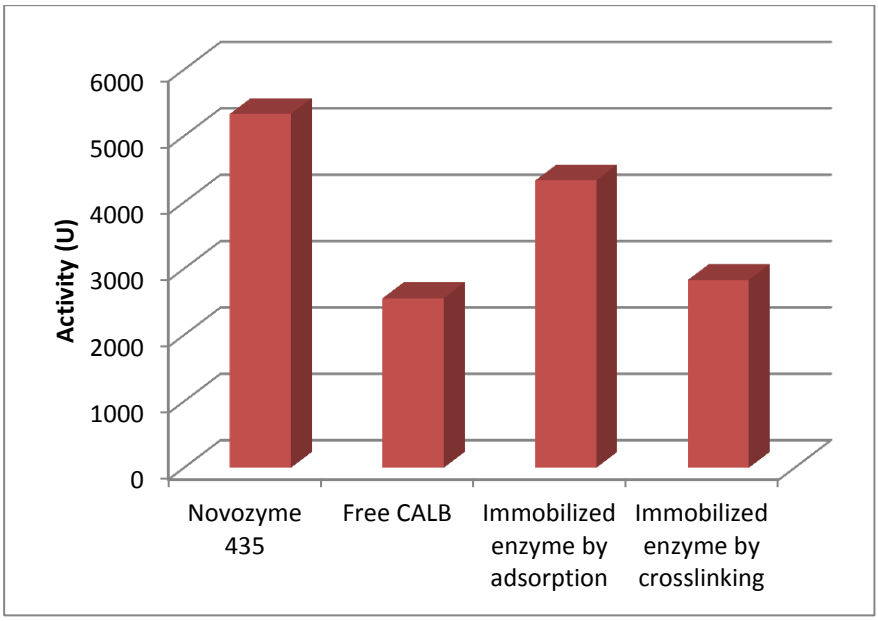


Figure 4.9 : Comparison of enzyme activities.

Similarly, Zou et al. (2010) reported a positive effect on lipase activity via immobilization. They immobilized porcine pancreas lipase on mesoporous silica, and the activity of lipase increased from 594 U to 975 U.

4.2 Characterization of Immobilized Enzymes

SEM was used to determine surface morphology of precipitated silica and immobilized enzymes which can be seen at Figure 4.10 and Figure 4.11, respectively. Figure 4.10 shows SEM image of the amorphus SiO_2 particles at 5000x magnification. SiO_2 particles showed a tendency to from bigger particles. Additionally, SEM image of precipitated silica used in this study was similar to SEM image of precipitated silica synthesized by Musić et al. (2011).

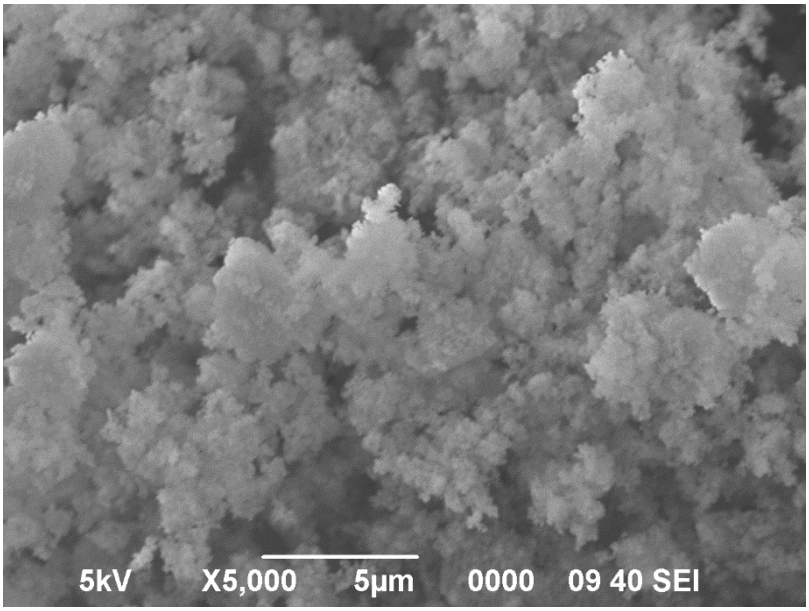


Figure 4.10 : SEM image of precipitated silica.

Figure 4.11 shows SEM images of immobilized enzymes at 5000x magnification. SEM image of immobilized enzyme by crosslinking method looks more ordered than image of immobilized enzyme by physical adsorption. The crosslinked bonds between precipitated silica and lipase could be caused these ordered structure.

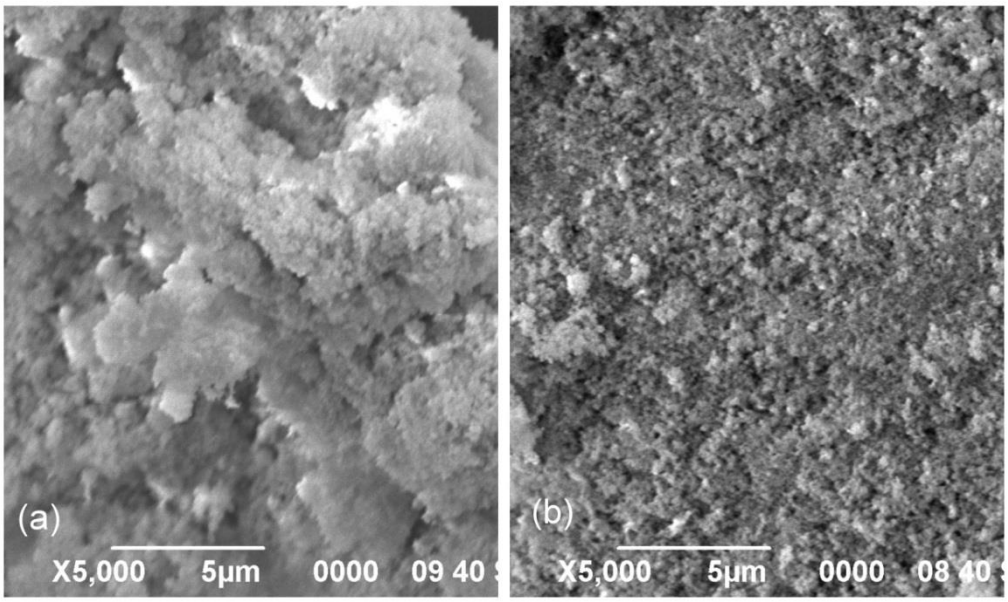


Figure 4.11 : SEM images of immobilized enyzmes: (a) crosslinking, (b) physical adsorption.

TGA analysis was applied to determine the thermal stability and chemical composition of the initial sample, and on the products formed (Silva et. al, 2013). It was showed that the results of TGA analysis for native precipitated silica and activated silica in Table 4.7. The total evaporation of 3-APTES takes place at 190 ºC (Valentini et. al, 2006). Therefore, in Table 4.7 was noted the values at 190 ºC, and the bonding ratio of 3-APTES was calculated by using this values. From Table 4.7, precipitated silica loses about 93 % of initial mass, while activated silica loses about 70 % of initial mass at 190 ºC. It means that precipitated silica was successfully modified by using 3-APTES with 23.18 % bounding ratio.

Table 4.7 : The results of TGA analysis for native precipitated and activated silica.

	Time (minute)	**Temperature (ºC)**	**TG**	**DTG**	**Mass loss (%)**
Precipitated silica	18.8	190	2663	-0.23	93.04
Activated silica	19.0	190	6288	3.90	69.87

The thermal stability of activated silica can be seen more clearly in Figure 4.12. The TGA curve of activated silica showed that degradation occurs in single stage, because there is an excess mass loss in this stage at temperatures in the range from 25 ºC to 180-190 ºC, with 30.13 % mass loss belonged to 3-APTES (Valentini et. al, 2006).

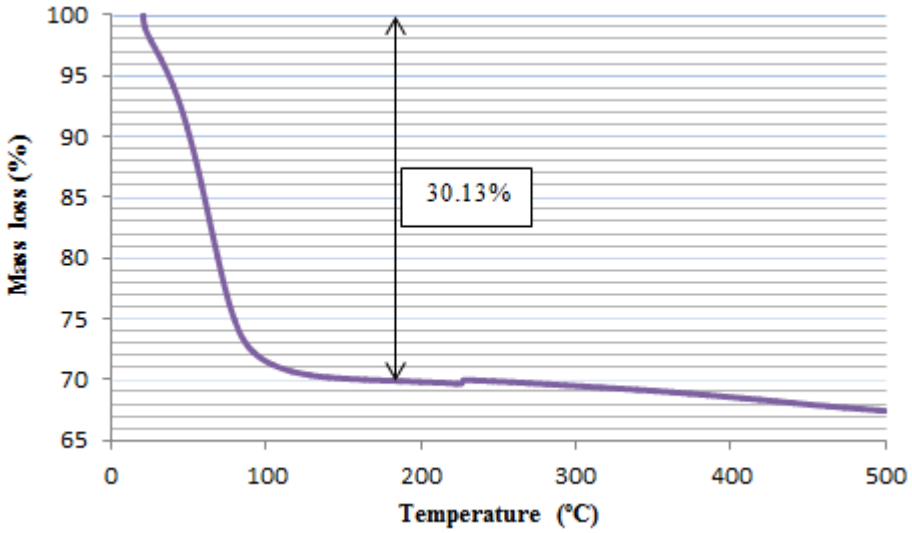


Figure 4.12 : TGA curve for activated silica.

FTIR spectrums were used to determine enzyme-support bond which can be seen in Figure 4.13. The FTIR spectrums of IEa and IEc have all bands characteristic for presence of lipase and silica support. From Figure 4.13, the IR bands at 1072.43 and 1074.95 cm^{-1} (for IEa and IEc, respectively) were appeared characteristic asymmetric SiO_2 bands, which is seen in precipitated silica. Moreover, the IR band at 800 cm^{-1} can be assigned to Si-O-Si symmetric stretching vibrations in precipitated silica, and the IR band at about 1600 cm^{-1} is due to the bending vibration of H_2O molecules. (Musić et. al, 2011).

For immobilized enzyme by crosslinking, there should be C=N and C=O stretching in the IR bands at 1700 cm^{-1} because of glutaraldehyde modification. However, it cannot be seen in spectrums due to that it may be overlap characteristic band of precipitated silica the IR band at 1600 cm^{-1}. The IR bands at 1800-1200 cm^{-1} include amino groups (N-H bending vibration at 1570 cm^{-1}) and C–H because of 3-APTES modification. However amino groups are more dominated than C-H peaks, and it could be that these bands overlap on the spectrum (Miletić et. al, 2010).

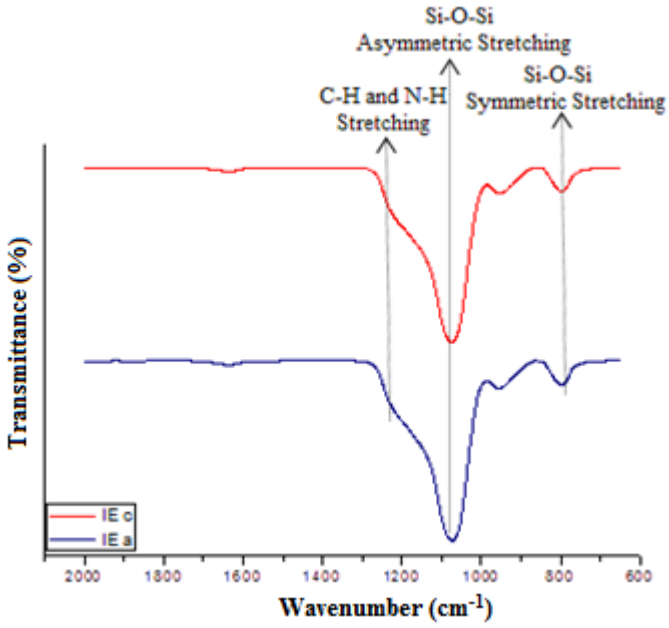


Figure 4.13 : FTIR spectra of immobilized enzymes.

The FTIR spectrums were also sketched in the wave length range between 2800 and 4000 cm^{-1}. From Figure 4.14, C-H stretching can be seen more clearly between 2800-3000 cm^{-1}, which was marked on spectrum. Similarly, Miletić et al. (2010) observed that the FTIR spectra of APTES films showed similar properties in the wave length range between 3000 and 2800 cm^{-1} which belong to C–H stretching modes of the APTES backbone and ethoxy groups.

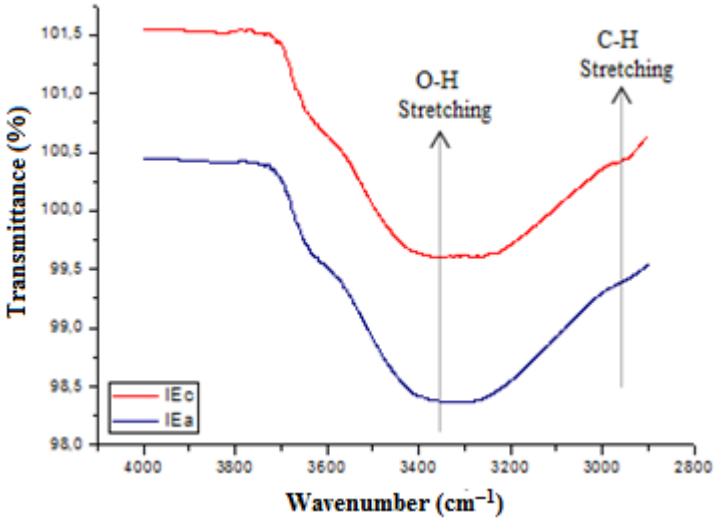


Figure 4.14 : The IR bands at 2800-4000 cm^{-1}.

Furthermore, there is a decrease in the intensity of the absorption approximately 3500 cm^{-1} (O-H stretching) is observed, demonstrating the successful modification of precipitated silica with 3-APTES (Silva et. al, 2013).

4.3 Synthesis of Polycaprolactone via Ring Opening Polymerization of ε-CL

Polymerization reactions were carried out at different time periods and temperatures to determine optimum time and temperature for PCL polymerization catalyzed by IEa and IEc. Mn values were calculated by GPC and ^{1}H NMR analysis, and the GPC traces of PCLs synthesized by IEa and IEc was given in Appendix A. Monomer conversion (%) was gravimetrically calculated.

Figure 4.15 : Polycaprolactone synthesized by immobilized enzymes.

Polycaprolactone was successfully synthesized via ring opening polymerization of ε-CL by both immobilized enzymes, was demonstrated in Figure 4.15. The effect of time, temperature and enzyme concentration were investigated in this study. Additionally, comparison of enzyme performances and reusability of immobilized enzymes for PCL polymerization were also noted regularly.

4.3.1 The effect of time and temperature on PCL polymerization catalyzed by IEc

First of all, the optimum time and temperature were determined to define best conditions for PCL polymerization. The results of PCL polymerization catalyzed by immobilized CALB by crosslinking method at 40 ºC can be seen in Table 4.8. In Table 4.8, Mn and polydispersity values of polycaprolactones obtained from GPC analysis were given respectively. Monomer conversion rates, which also were noted in Table 4.8, were measured by using Equation 3.6.

Table 4.8 : The effect of time on polymerization catalyzed by IEc at T = 40 ºC.

Temperature (ºC)	Time (h)	Mn (g/mol)	PDI (Mw/Mn)	Monomer Conversion (%)
	6	-	-	-
	24	3700	1.4	24
40	48	4100	1.5	45
	72	**4500**	**1.6**	**54**
	120	4000	1.6	47

From Table 4.8, the highest Mn and monomer conversion rate were obtained at the end of 72 h at 40 ºC, while any polymer could not be obtained for 6 h. It can be said that 6 h are not enough to polymerize ε-CL at 40 ºC. Furthermore, Mn values did not linearly increase or decrease with changing time period, in contrast a fluctuation was observed on Mn values at 40 ºC. Besides, changing between 1.4 and 1.6 PDI values were obtained at 40 ºC.

Table 4.9 : The effect of time on polymerization catalyzed by IEc at T = 60 ºC.

Temperature (ºC)	Time (h)	Mn (g/mol)	PDI (Mw/Mn)	Monomer Conversion (%)
	6	2900	1.2	8
	24	**5400**	**1.2**	**21**
60	48	3400	1.6	42
	72	5000	1.8	88
	120	5100	1.4	90

The results at 60 ºC were demonstrated in Table 4.9. From Table 4.9, the highest Mn of PCL, 5400 g/mol was obtained at 24 h, while the highest monomer conversion rate 90 % was obtained at 120 h.For Mn values of PCLs were observed a fluctuation at 60 ºC where the Mn values reduced from 5400 g/mol to 3400 g/mol reducedfor 48

h, and 5100 g/mol was finally obtained at 120 h. The reason of that, simultaneous degradation reactions could be in PCL polymerization. Moreover, PDI values were obtained changing between 1.2 and 1.8 at 60 ºC. At the end of 6 and 24 h reaction times was obtained a dispersity of 1.2 which was the most approximate value to monodispersity.

Table 4.10 : The effect of time on polymerization catalyzed by IEc T = 80 ºC.

Temperature (ºC)	Time (h)	Mn (g/mol)	PDI (Mw/Mn)	Monomer Conversion (%)
80	6	3500	1.2	7
	24	4300	1.5	45
	48	2900	1.3	23
	72	2500	1.4	58
	120	4700	1.6	63
	150	9000	1.4	67
	170	**9400**	**1.5**	**69**

ε-CL polymerization was performed for seven different times at 80 ºC (Table 4.10), because Mn values of PCLs continued to increase after 120 h. The highest Mn and monomer conversion rate were obtained at 170 h, at which was achieved similar results to values measured at 150 h. Therefore, it was accepted that Mn values were become stable, and the serie of experiment was completed for 80 ºC. Additionally, PDI values were obtained changing between 1.2 and 1.6 at 80 ºC. A dispersity of almost 1 was achieved at 6 h.

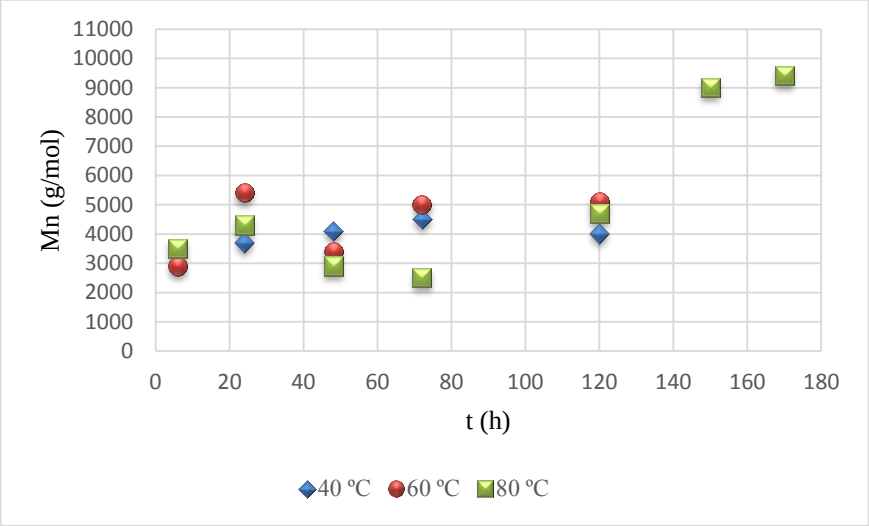


Figure 4.16 : Comparison of Mn values for synthesized polycaprolactones via IEc catalyzed polymerization.

The comparison of Mn values is shown in Figure 4.16. As it can be seen in Figure 4.16, the highest Mn value, 9400 g/mol was achieved at 80 ºC-170 h. At 40 ºC, Mn values of PCL increased until 72 h, decreased during 120 h, and became stable about 4000 g/mol. At 60 ºC, Mn value became stable between 72-120 h after fluctuation of values which is as similar as the results obtained at 40 ºC. ε-CL polymerization reaction is a reversible reaction during which degradation reaction can simultaneously take place (Uyama, 2007). Because of this the fluctuation may be happen in PCL polymerization at 40 and 60 ºC.

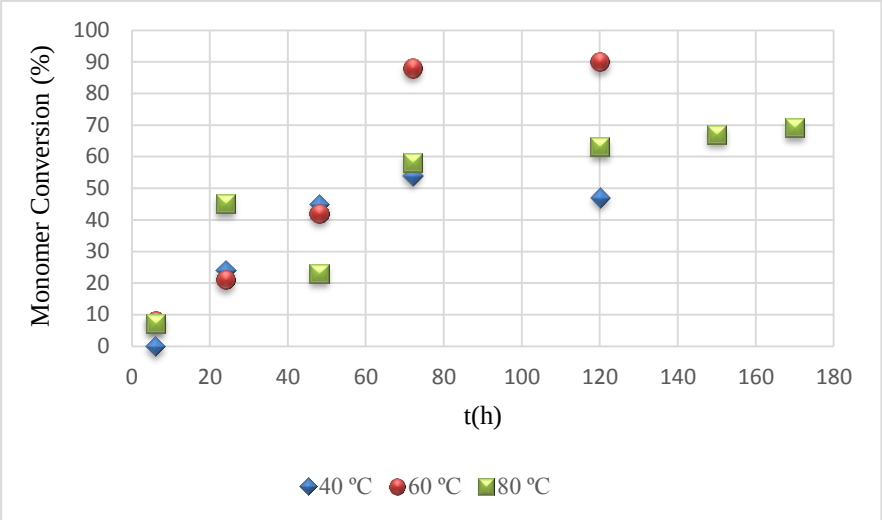


Figure 4.17 : Comparison of monomer conversion rates for synthesized polycaprolactones via IEc catalyzed polymerization.

The comparison of monomer conversion rates is shown in Figure 4.17. The highest monomer conversions were observed at 60 ºC that 88 % and 90 % were obtained at 72 and 120 h, respectively. Although Mn values are high at 80 ºC, the best monomer conversion is 69 % which is not enough compared the results obtained at 60 ºC. At 40 ºC, monomer conversion rates are generally lower than the rates measured at 60-80 ºC. The highest monomer conversion rate is 54 % at 40 ºC.

Mn of PCL is the most important parameter to optimize the reaction conditions for polymerization. Because it defines mechanical strength of PCLs, andalso mechanical strength is an effective factor on usage of PCL in industrial application, especially biomedical applications (Özsağıroğlu, 2011). Polydispersity is an another parameter which define mechanical properties of biopolymers. It means heterogeneity index which should be almost 1 for monodisperse systems, biopolymers. During the series of experiments, low values of disperty were obtained generally at short time period.

Time period is also a significant factor to characterize polymerization reactions. As it can be seen in Table 4.10, the highest Mn values of PCL obtained at 150 and 170 h are too similar which are 9000 and 9400 g/mol, respectively. However, polymerization at 150 h is more advantageous due to that short time period is more suitable for industrial applications. Conversely, monomer conversion rates are not high at 80 ºC like the reactions at 60 ºC, but the values are higher than the monomer conversion rates measured at 40 ºC. About 70 % monomer conversion rate, which is good enough for polymerization, was obtained at 80 ºC-150 h. All in all, 80 ºC and 150 h were determined as the optimum temperature and time period for PCL polymerization via immobilized CALB by crosslinking method.

4.3.2 The effect of time and temperature on PCL polymerization catalyzed by immobilized enzymes by physical adsorption

The reactions were performed at 6, 24, 48, 72, 120 and 150 h for PCL polymerization catalyzed by IEa. The results at 40 ºC are shown in Table 4.11. Following 120 h reaction, the highest molecular weight and monomer conversion were obtained 7900 g/mol and 77 %, respectively. The Mn values of PCLs started to reduce after 120 h, while they increased between 6-120 h reaction time. At long time periods, monomer conversion rates were higher than the conversion values obtained at short time periods. Moreover, PDI values ranged from 1.4 to 1.7 were noted in Table 4.11.

Table 4.11 : The effect of time on polymerization catalyzed by IEa at T = 40 ºC.

Temperature (ºC)	Time (h)	Mn (g/mol)	PDI (Mw/Mn)	Monomer Conversion (%)
40	6	2400	1.4	16
	24	3700	1.6	38
	48	5100	1.6	70
	72	5400	1.7	68
	120	**7900**	**1.4**	**77**
	150	5100	1.7	75

In Table 4.12, Mn, PDI and monomer conversion rates of PCLs at 60 ºC are demonstrated by changing time period. The highest molecular weight was acquired 8100 g/mol at 48 h, while the highest monomer conversion rate was obtained about 90 % at 72 h. Monomer conversion rates were changing between 83-89 %, which were high enough, after 72 h. It was also observed that there isa molecular weight

fluctuation of PCLs at 60 ºC because of simultaneous degradation reactions. Molecular weight of PCL reduced from 8100 to 3500 g/mol between 48-72 h. Furthermore, PDI values of PCLs were achieved in the range of 1.3-1.6. The lowest dispersity was achieved at 6 h.

Table 4.12 : The effect of time on polymerization catalyzed by IEa at T = 60 ºC.

Temperature (ºC)	Time (h)	Mn (g/mol)	PDI (Mw/Mn)	Monomer Conversion (%)
	6	2500	1.3	11
	24	4400	1.6	60
60	**48**	**8100**	**1.5**	**64**
	72	3500	1.6	89
	120	7400	1.4	84
	150	5800	1.8	83

From Table 4.13, the best result was a polymer chain size of 14300 g/mol, obtained in a reaction conducted at 120 h and 80 ºC. There was an increase of molecular weights of PCLs till 120 h, after that molecular weight reduced from 14300 to 8800 g/mol. It can be said that polymer chain could be degraded between 120-150 h. Additionally, the highest monomer conversion was 86 %, achieved at 72 h-80 ºC. At short time period, monomer conversion rates were 1 and 21 % at 6 and 24 h, respectively. PDI values were also obtained in the range of 1.2 and 1.7. The lowest dispersity was obtained 1.2 at 6 h.

Table 4.13 : The effect of time on polymerization catalyzed by IEa at T = 80 ºC.

Temperature (ºC)	Time (h)	Mn (g/mol)	PDI (Mw/Mn)	Monomer Conversion (%)
	6	1800	1.2	1
	24	2900	1.3	21
80	48	5300	1.3	63
	72	5800	1.7	86
	120	**14300**	**1.6**	**77**
	150	8800	1.5	69

The comparison of molecular weights was shown in Figure 4.18. It is clear that the best result for the all experiments were performed by IEa was 14300 g/mol, obtained at 80 ºC. As can be clearly seen in Figure 4.18, molecular weights of PCLs were continuously changed at 60 ºC. The degradation reactions could be the reason, which is similar to the results obtained by crosslinked enzymes at 40-60 ºC. When the

results compared to the Mn values obtained at 60-80 ºC, the measured molecular weights at 40 ºC were not high enough.

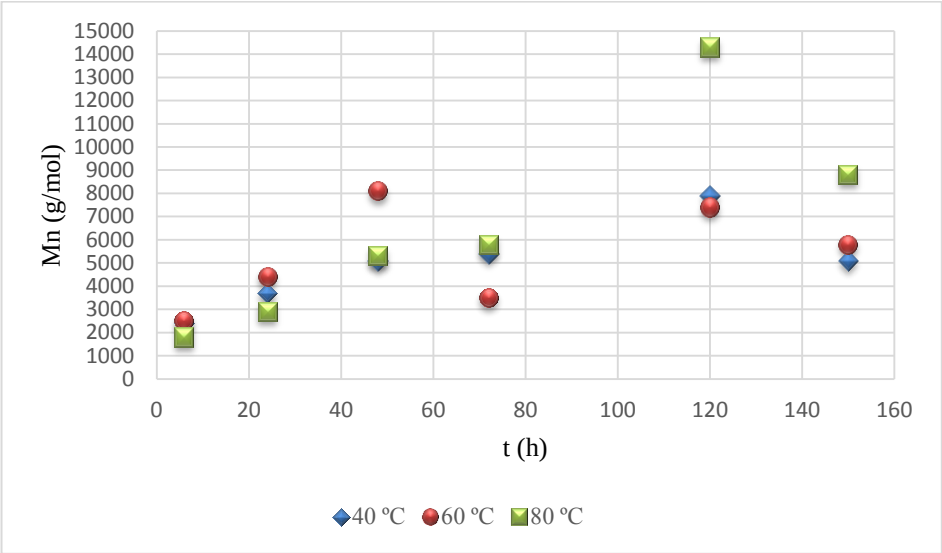


Figure 4.18 : Comparison of Mn values for synthesized polycaprolactones via IEa catalyzed polymerization.

The comparison of monomer conversion rates were demonstrated in Figure 4.19. For all three temperatures, sufficient conversion rates were obtained in the range of 75-90 % at different time period. On the other hand, very low conversion rates were observed at 80 ºC. However, the lowest monomer conversion rate 1 % was successfully increased to 86 % by increasing time period. Generally, the best monomer conversion rates were noted at long time period. Therefore, it can be said that formation of long polymer chain size became more possible with long reaction time in PCL polymerization. Because long time period provides always long interaction between the monomers.

In conclusion, it can be said that the optimum temperature and time for PCL polymerized by IEa are 80 ºC and 120 h respectively, with 14300 g/mol molecular weight and 77 % monomer conversion rate**.** Although a high polydispersity index of 1.6 was obtained at 80 ºC-120 h, number average molecular weight was primarily considered for determination.

As expected, this result is the best of polymerization reactions catalyzed by immobilized enzymes both crosslinking and physical adsorption. Because the activity of immobilized enzymes by physical adsorption were higher than the activity of immobilized enzymes by crosslinking. Parallelly, the best molecular weight was

obtained by immobilized enzymes by physical adsorption possessed the highest catalytic activity. These results showed that both immobilized enzymes were more effective at high temperatures, because the best results were obtained at 80 ºC. The results obtained at 60 ºC were also high enough for both immobilized enzymes compared to the results achieved at 40 ºC. Furthermore, very low values of dispersity were obtained via ROP catalyzed both immobilized enzymes of ε-CL such as 1.2 and 1.3. The lowest dispersity values were obtained at short time period for both immobilized enzymes. It is a very successful result for enzymatic ring opening polymerization, because low dispersity was generally obtained with controlled polymerization methods (Yuan et al., 2015).

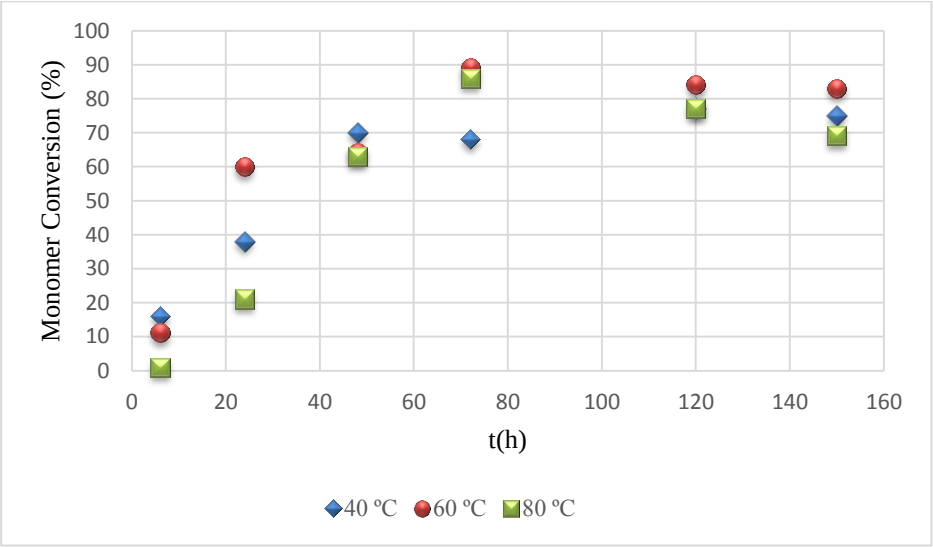


Figure 4.19: Comparison of monomer conversion rates for synthesized polycaprolactones via IEa catalyzed polymerization.

There is some similar studies and their results were reported: Li et al. (2011) studied ring opening polymerization of ε-Cl catalyzed by a novel lipase from *Fervidobacterium nodosum*. They investigated catalytic activity of enzyme for polycaprolactone synthesis, and reported that lipase from *Fervidobacterium nodosum* showed the highest activity towards the polymerization of ε-caprolactone at high temperature 90 ºC. Furthermore, Zhu et al. (2014) studied ROP catalyzed *Candida* sp. 99-125 of ε-CL, and they reported that number average molecular weight of resulted polycaprolactones ranged from 3000 to 4700 g/mol which were lower than the obtained molecular weights in our study.

4.3.3 The effect of enzyme concentration on PCL synthesis

The effect of enzyme concentration on PCL polymerization reactions was researched to determine the most effective enzyme amount for synthesis of PCL. The reactions were carried out under determined conditions, which were 80 ºC-150 h and 80 ºC-120 h for immobilized enzyme by crosslinking and physical adsorption, respectively. Mn, PDI and monomer conversion rates of PCLs were shown in Table 4.14. From Table 4.14, the monomer conversion rates of PCLs increased by increasing enzyme concentration for both immobilized enzymes. However, conversion rates at 2.5 % (w/w) were obtained 1.4 and 3.3 %, were very low. PDI values obtained also in the range of 1.2-1.6. Deng and Gross (1999) studied ring opening polymerization of ε-Cl catalyzed by Novozym® 435, and they reported similarly that monomer conversion rates increased with increasing enzyme concentration. In their study, a monomer conversion rate of 80 % was obtained in a 4 h time period for an enzyme concentration of 9.8 mg lipase per mmol monomer, while for an enzyme concentration of 1.8 mg lipase per mmol monomer, 48 h was required to achieve 80 %. When all parameters were considered that a high monomer conversion rate was obtained with high enzyme content at short time. According to this study, the effect of enzyme content can be also investigated at different time periods.

Table 4.14 : The results at different enzyme loading.

Immobilization Method	Enzyme Concentration (%)	Mn (g/mol)	PDI (Mn/Mw)	Monomer Conversion (%)
Physical Adsorption	**2.5**	2500	1.2	1.4
	5.0	4400	1.6	22
	10.0	8300	1.4	78
	20.0	**14300**	**1.6**	**77**
Crosslinking	**2.5**	2300	1.3	3.3
	5.0	4300	1.6	27
	10.0	5300	1.7	42
	20.0	**9000**	**1.4**	**67**

The comparison of number average molecular weights of PCLs can be seen more clearly in Figure 4.20. The molecular weights and monomer conversion rates of PCLs went up with increasing enzyme concentration during reactions catalyzed by immobilized enzymes. The best results were 14300 and 9000 g/mol for IEa and IEc respectively, obtained in a reaction conducted at 20 % (w/w) enzyme concentration. According to these results, it can be said that immobilized enzymes content presented

a positive effect on PCL polymerization regarding the molecular weights and also monomer conversions.

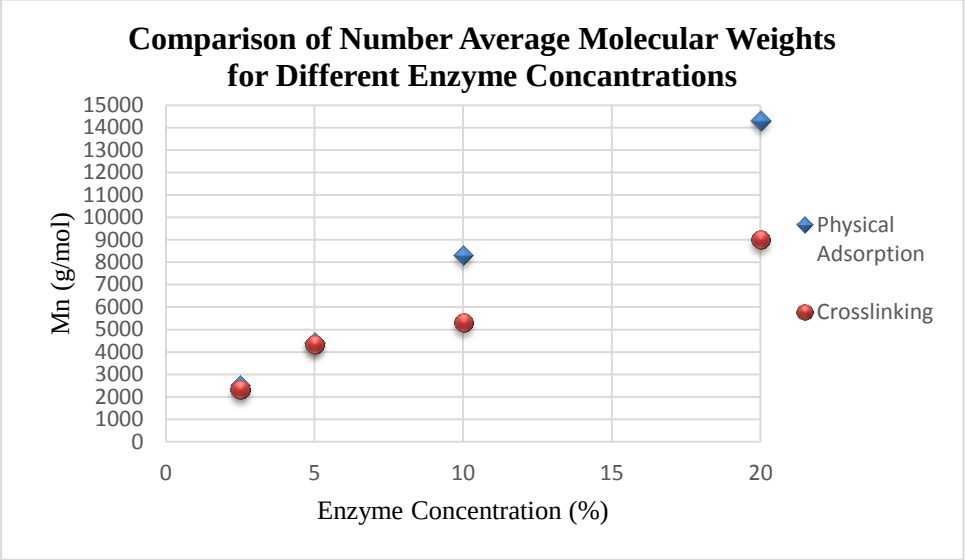


Figure 4.20 : The effect of enzyme concentration on number average molecular weights of PCLs.

Parallelly, Rosso et al. (2013) studied enzymatic synthesis of polycaprolactone polymerization catalyzed by Novozym®-435 in supercritical carbon dioxide medium, and they investigated the effect of enzyme concentration on PCL polymerization. They reported that the enzyme content showed a positive effect on polymerization. In their study, the highest molecular weights were obtained Mn of 9000 g/mol and Mw of 16500 g/mol at 15 wt % enzyme concentration.

4.3.4 Comparison of enzyme performances

In this study, ε-CL was polymerized by free CALB and Novozym® 435 to compare the results obtained under best conditions which were determined by researching effect of time and temperature on polymerization. Reactions were carried out at 80 ºC-120 h to compare the best results of PCL polymerization catalyzed by immobilized enzyme by physical adsorption. From Table 4.15, the highest monomer conversion rate was obtained 95 % by Novozym® 435. On the other hand, 80 and 77 % were achieved for polymerization by free CALB and immobilized enzyme by physical adsorption, respectively.

Table 4.15 : The results of polymerization catalyzed by different enzyme types.

	Enzyme Type	Mn (g/mol)	PDI (Mn/Mw)	Monomer Conversion (%)
Physical Adsorption	**Free CALB**	2500	1.5	80
	Novozym® 435	12700	1.7	95
	Immobilized Enzyme	14300	1.6	77
Crosslinking	**Free CALB**	2900	1.5	78
	Novozym® 435	12700	1.7	95
	Immobilized Enzyme	9000	1.4	68

The comparison of the molecular weights obtained at 80 ºC-120 h was shown in Figure 4.21. It is clear that the highest molecular weight was dramatically obtained by immobilized enzyme by physical adsorption. Although Novozym® 435 is a commercial enzyme with high activity (≥ 5000 U/g), 12700 g/mol was noted after polymerization catalyzed by Novozym® 435, were lower than the molecular weight obtained by IEa. On the other hand, the lowest molecular weight was obtained 2500 g/mol by using free CALB. It is about 6 times lower than the result obtained by IEa.

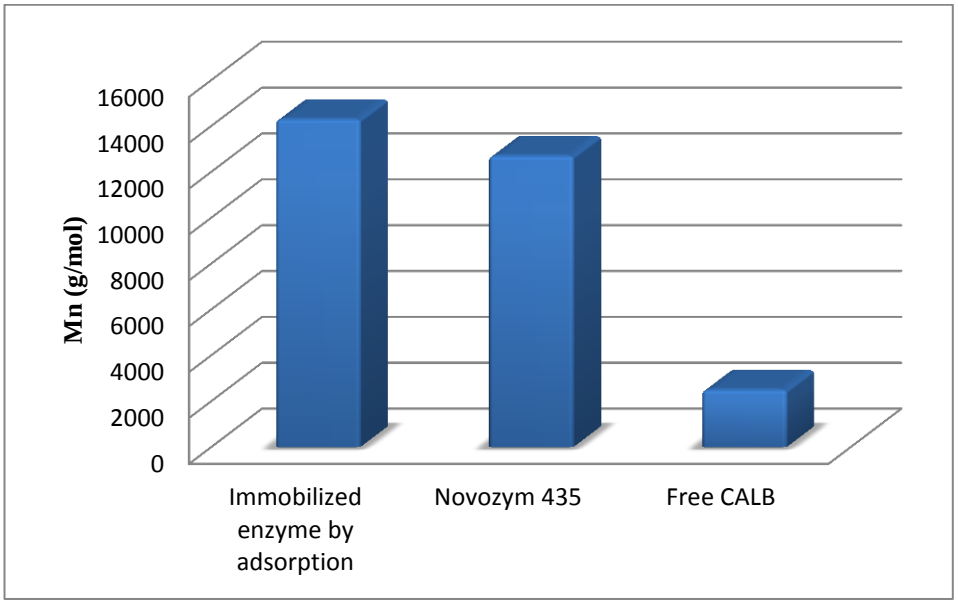


Figure 4.21 : Comparison of the results obtained at 80 ºC-120 h.

The results obtained at 80 ºC-150 h can be also seen in Table 4.15. To compare these results, the results obtained at 80 ºC-120 h were used for polymerization catalyzed by Novozym® 435. Because the reaction catalyzed by Novozym® 435 stopped after 120 h. From Table 4.15, the best results were achieved by Novozym® 435 compared to performance of free CALB and immobilized enzyme by crosslinking. The highest

monomer conversion rate was obtained 95 %, while 78 and 68 % were obtained for reaction catalyzed by free CALB and immobilized enzyme by crosslinking, respectively. Moreover, the highest molecular weight was obtained by Novozym® 435, which can be seen more comprehensively in Figure 4.22.

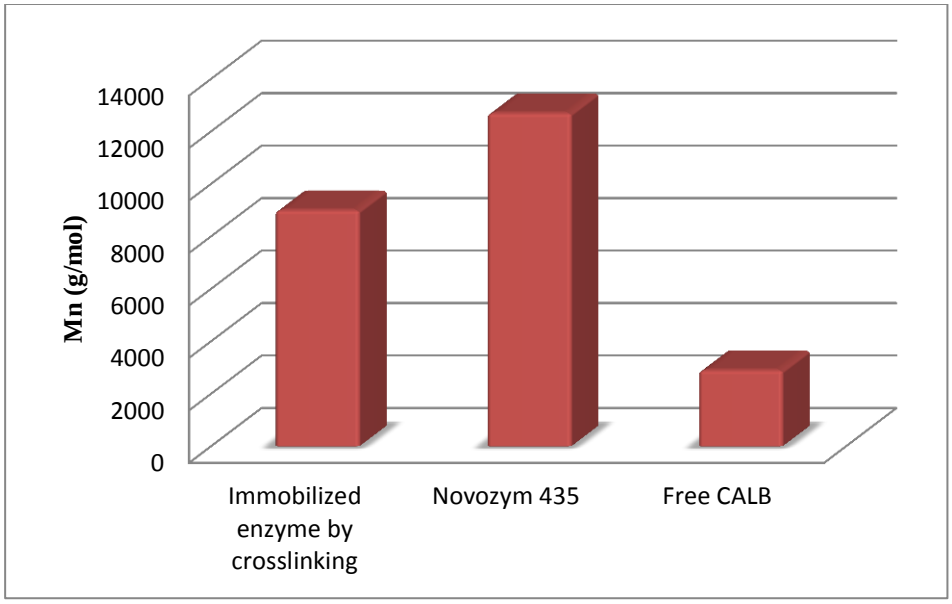


Figure 4.22 : Comparison of the results obtained at 80 ºC-150 h.

From Figure 4.22, a molecular weight of 2900 g/mol was obtained by free CALB, while 3 times better molecular weight was obtained by immobilized enzyme by crosslinking. Although effectivity of Novozym® 435 was better than immobilized enzyme by crosslinking, immobilized enzyme was reached 9000 g/mol a high molecular weight with a monomer conversion of about 70 %.

4.3.5 Reusability of immobilized enzymes for PCL polymerization

To determine reusability of immobilized enzymes, the retention rate of initial lipase activity after reaction cycles was observed for both immobilized lipases. The results were noted in Table 4.16. From Table 4.16, monomer conversion rates of PCLs were rapidly reduced after 2 cycles usage of both immobilized enzymes. At fourth cycle, formation of PCL did not observe for polymerization catalyzed by IEa, while only a monomer conversion of 1.4 % was obtained for ROP reaction catalyzed by IEc. Moreover, PCL could not be obtained after 5 cycles uses for both immobilized enzymes.

Table 4.16 : The results after 5 cycles usage of immobilized enzymes.

Immobilization Method	Number of Reaction	Activity (U)	Mn (g/mol)	Monomer Conversion (%)
Physical Adsorption	**0**	4170	-	-
	1	3300	7400	79
	2	3300	5600	46
	3	2670	3100	12
	4	1670	-	-
	5	1300	-	-
Crosslinking	**0**	2800	-	-
	1	2000	4800	64
	2	1300	4000	20
	3	1300	2800	7
	4	1300	2600	1.4
	5	1170	-	-

Molecular weights of PCLs decreased with increasing reaction cycles for both immobilized enzymes. However, Mn values of PCLs were more stable for immobilized enzyme by crosslinking, while a rapid reduction in Mn values was observed for immobilized enzyme by physical adsorption.

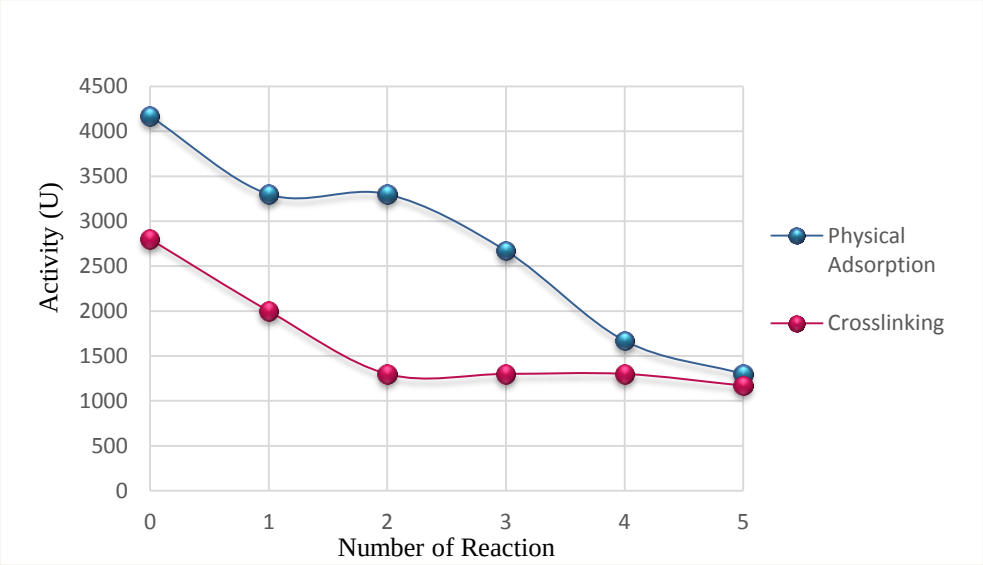


Figure 4.23 : Change of immobilized enzymes activity with reaction cycles.

Change of immobilized enzymes activity was shown in Figure 4.23. From Figure 4.23, immobilized enzyme by physical adsorption lost higher amount of its initial activity than immobilized enzyme by crosslinking, although activity of immobilized enzyme by physical adsorption was very high (4170 U) in beginning. After 5 cycles uses, about 32 and 42 % of the initial activity could be retained for immobilized enzyme by physical adsorption and crosslinking methods, respectively. It could be

said that crosslinking of support provided stronger bond between enzyme and support, thus immobilized enzyme by crosslinking showed more stable performance after 5 cycles uses.

4.4 Characterization of Polycaprolactones

SEM was used to determine surface morphology of polycaprolactones which can be seen at Figure 4.24 and Figure 4.25, respectively. Figure 4.24 (a) and Figure 4.24 (b) show that SEM images of PCL catalyzed by IEa at 2000x and 3000x magnifications. Structure of polycaprolactone catalyzed by IEa looks like a foam which is similar to SEM image of PCLs showed in Woodruff and Hutmacher (2010) study.

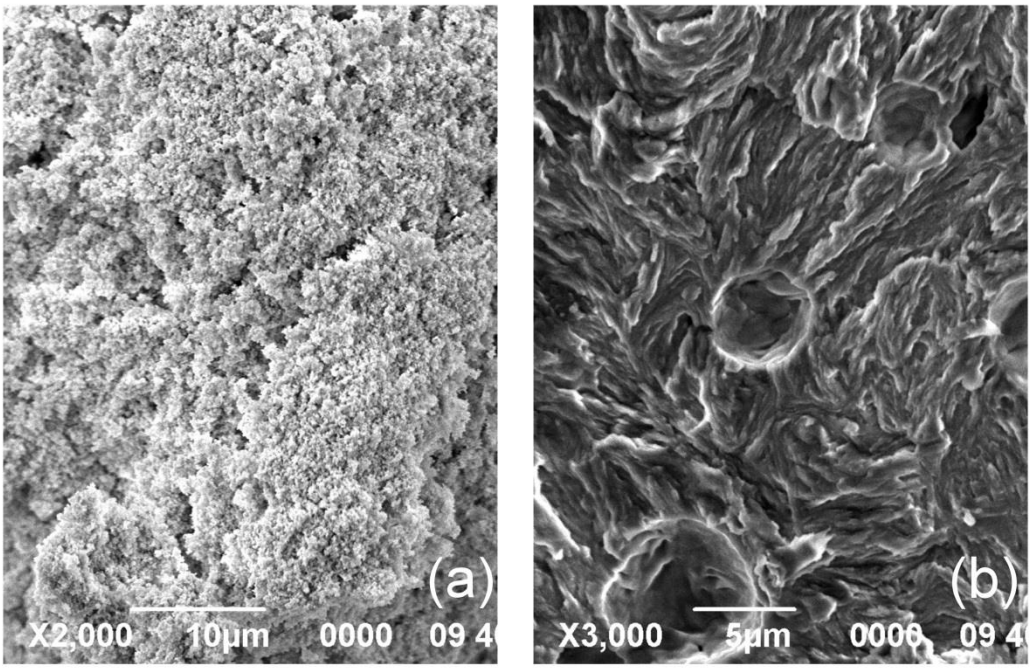


Figure 4.24 : SEM images of PCL catalyzed by IEa at 2000x (a) and 3000x (b) magnifications.

Figure 4.25 (a) and Figure 4.25 (b) show that SEM images of PCL catalyzed by IEc at 2000x and 3000x magnifications. Parallelly the image of PCL looks a foam, but holes on the surface are more wide and long than PCL catalyzed by IEa. According to the study belonged to Woodruff and Hutmacher (2010), both PCLs have foam structure and can be used in a wide range of scaffold fabrication technologies, drug delivery and tissue engineering arena.

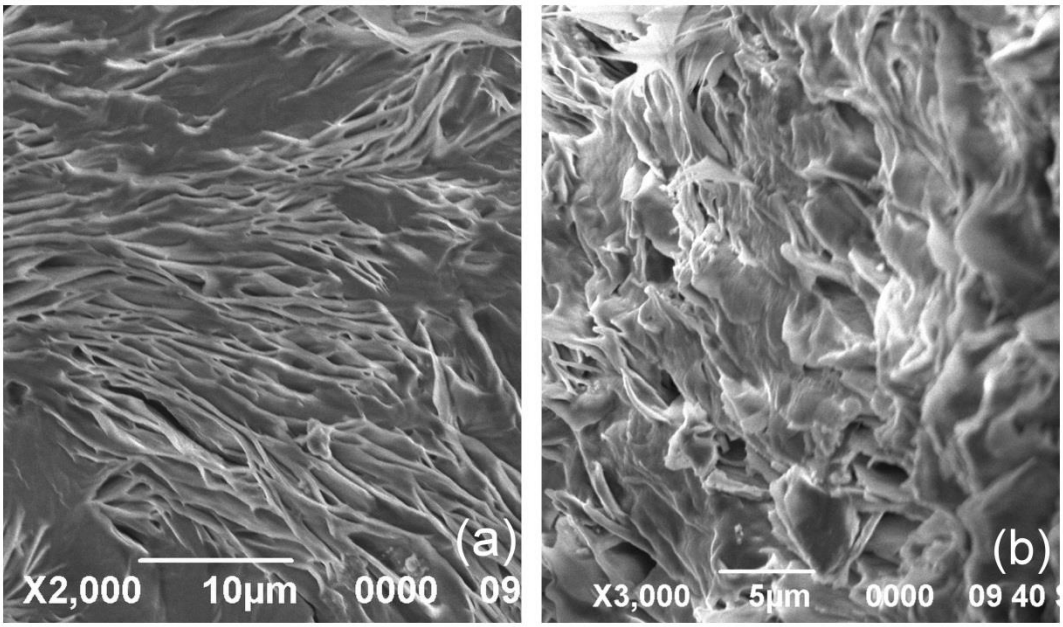


Figure 4.25 : SEM images of PCL catalyzed by IEc at 2000x (a), 3000x (b) magnifications.

^{1}H NMR analysis was applied for PCLs possessed the highest molecular weight, which were 14300 and 9000 g/mol for PCL catalyzed IEa and IEc, respectively. Figure 4.26 shows that ^{1}H NMR spectra of PCL catalyzed by IEa and IEc. It was noted that the structural groups of PCL on Figure 4.26: (δ, ppm): 4.07 (t, CH_2O, main chain) and 3.65 (t, CH_2OH, chain-end). The other groups are 2.32 (t, CH_2CO), 1.6-1.7 (m, CH_2) and 1.37 (m, CH_2) (Özsağıroğlu, 2011). According to this result, the result polymers have the characteristic peaks of PCL, and it can be definetly said that PCL was polymerized successfully. Additionally, the number average molecular weights of PCL were calculated by using Equation 3.7. Mn of PCL catalyzed by IEa and IEc are 10600 and 7500, respectively. These results are lower than the results obtained by GPC. However, it is possible obtaining different values by using GPC and ^{1}H NMR because of measuring based on different standarts.

PCLs synthesized by IEa and IEc were characterized by TGA analysis. Their degradation behavior is very similar occuring one main degradation process with an inflection point that can be seen in Figure 4.27. Thus, it can be said that molecular weight of PCL is not effective on degradation process of PCL. PCL degradation was recorded at 408.9 and 408.5 ºC for PCL catalyzed IEc and IEa, respectively.

Furthermore, Tmax values were recorded from TGA curves (Figure 4.28), Tmax values are 426 and 435.7 ºC for PCL catalyzed IEa and IEc, respectively.

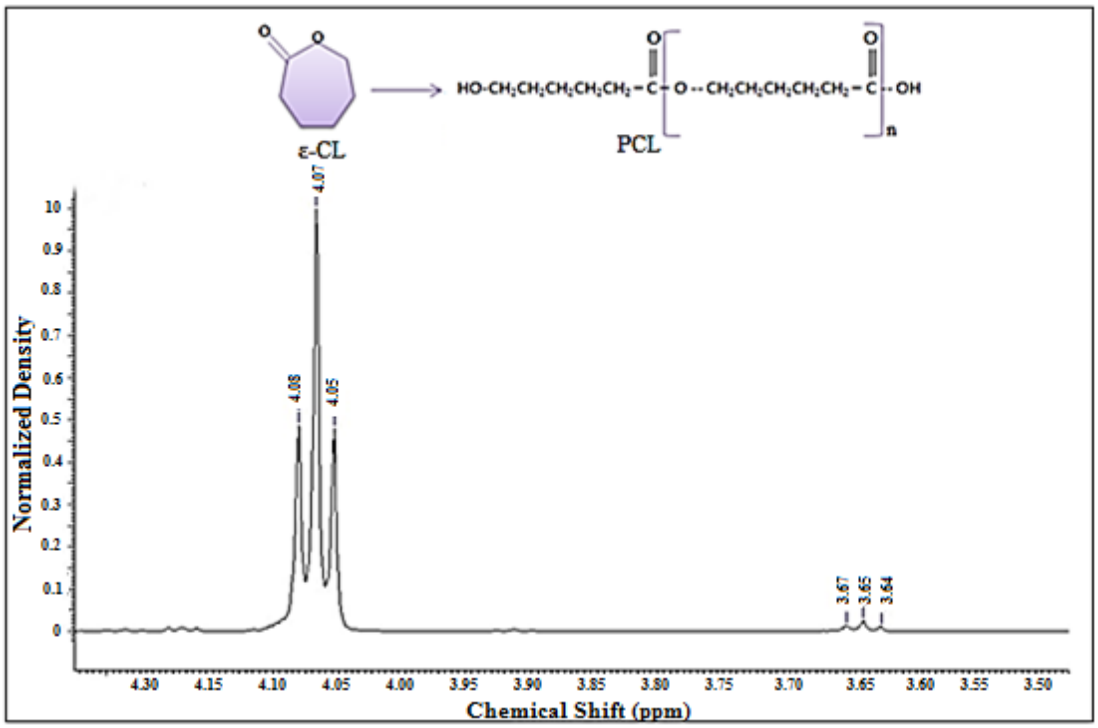


Figure 4.26 : ^{1}H NMR spectra of PCL catalyzed by IEa and IEc.

Persenaire et al. (2001) studied thermal degradation of polycaprolactone, and they noted similarly degradation of PCL at 420 ºC. Moreover, DTG curves of PCL polymerized in their study are very similar to the curves stayed in Figure 4.27 and Figure 4.28. Parallelly, Öztürk-Düşkünkorur et al. (2014) studied lipase catalyzed synthesis of polycaprolactone and clay-based nanohybrids. They obtained similarly one main degradation for PCL, and degradation process occured at 400-420 ºC.

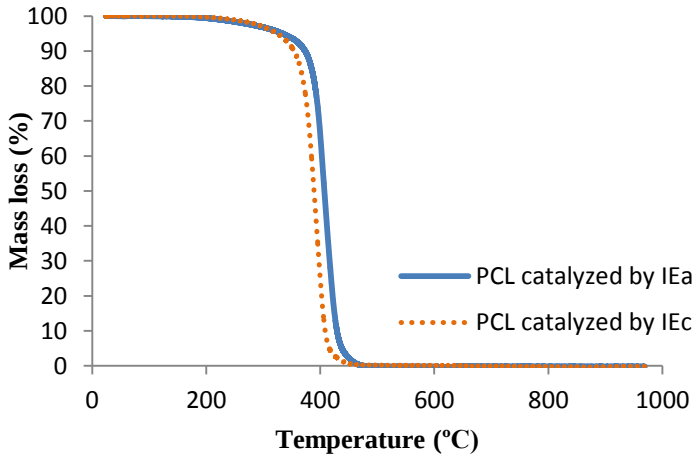


Figure 4.27 : TGA curves for PCL catalyzed by IEc and IEa.

Bajsić et al. (2014) studied characterization of biodegradable polycaprolactone containing titanium dioxide micro and nanoparticles. They noted Tmax, Tend and ΔHm (%) values measured by TGA curves. They obtained Tmax values between 410-414 ºC, Tend values between 426-443 ºC and ΔHm (%) values between 98-99 % which are similar the results obtained in this study. According to these datas, it can be said that the result polymers polymerized by IEa and IEc have characteristic thermal behavior of PCL.

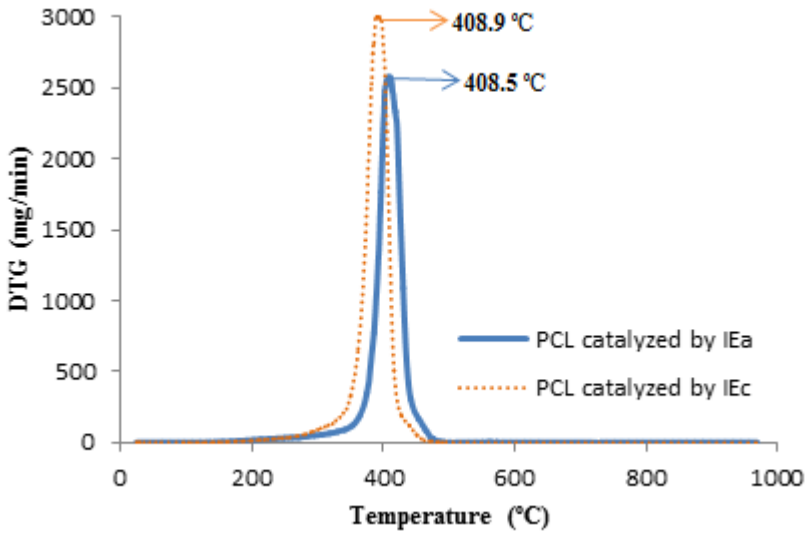


Figure 4.28 : DTG spectra for PCL catalyzed by IEc and IEa.

FTIR spectra of PCL catalyzed by IEa is showed in Figure 4.29, which belonged to PCL polymerized at 80 ºC 120 h with the highest molecular weight 14300 g/mol. The characteristic peaks of PCL are asymmetric CH_2 bonds, symmetric CH_2 bonds and carbonyl bonds (C=O), which can be seen in Figure 4.29 the IR band at 2945.32, 2865.41 and 1720.78 cm^{-1}, respectively. Moreover C-O, C-C, asymmetric and symmetric C-O-C bands are seen for a FTIR spectra of PCL in crystalline phase (Özsağıroğlu, 2011). Parallelly, the C-O and C-C bands can be seen the IR band at 1293.38 cm^{-1}, asymmetric symmetric C-O-C bands can be also seen the IR band at 1238.73 and 1155.36 cm^{-1}, respectively. Additionally, the IR band at 3440.47 cm^{-1} show the stretching of OH group of carboxylic acid, while the peaks at 1470.87 and 1364.91 cm^{-1} are related to to stretching of CH_2 and OH group, respectively (Zhang et al., 1994).

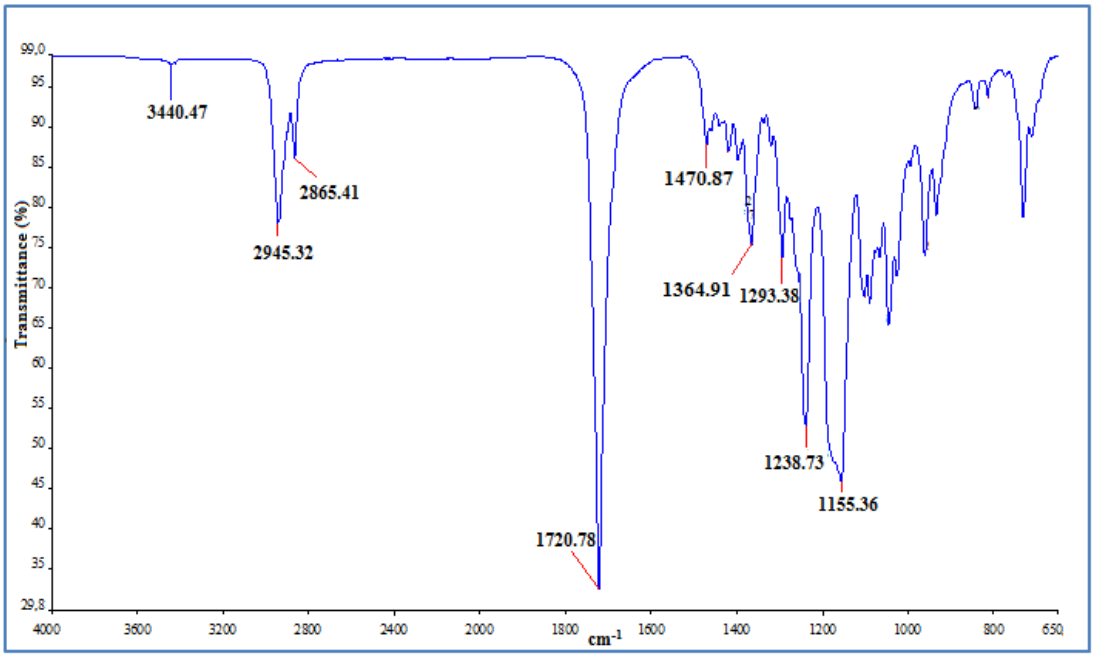


Figure 4.29: FTIR spectra of polycaprolactone polymerized by IEa.

FTIR spectra of PCL catalyzed by IEc is showed in Figure 4.30, which belonged to PCL polymerized at 80 ºC 150 h with a molecular weight of 9000 g/mol. The characteristic peaks of PCL are asymmetric CH_2 bonds, symmetric CH_2 bonds and carbonyl bonds (C=O), which can be seen in Figure 4.30 the IR band at 2943.98, 2865.31 and 1721.12 cm^{-1}, respectively. Furthermore, the C-O and C-C bands can be seen the IR band at 1292.96 cm^{-1}, asymmetric symmetric C-O-C bands can be also seen the IR band at 1238.34 and 1168.22 cm^{-1}, respectively (Özsağıroğlu, 2011). Additionally, the IR band at 1470.89 and 1365.08 cm^{-1} are related to to stretching of CH_2 and OH group, respectively (Ranjha et al., 2011).

Ranjha et al. (2011) studied synthesis and characterization of polycaprolactone/acrylic acid hydrogel for controlled drug delivery, and they characterized the result polymers by using FTIR. The FTIR spectra in their study showed the peak at 1752 cm^{-1} and 3575 cm^{-1}, which belong to carbonyl bonds and the stretching of OH group of carboxylic acid, respectively. Moreover, they obtained that the peaks at 1460-1375 cm^{-1} are because of stretching of CH_2 and OH group, respectively. Wu (2005) studied thermal properties and biodegradability of polycaprolactone/chitosan and acrylic acid grafted polycaprolactone/chitosan, and characterized these polymers by using FTIR. In this study is obtained that the characteristic peaks of PCL in the wave length at 3200-3700, 1710-1720 cm^{-1}, which belong to OH and carbonyl stretching, respectively. Based on these studies, it can be

said that the FTIR spectra of PCL catalyzed by IEa and IEc have all characteristic bands of PCL. It can be concluded that PCL was succesfully polymerized in this study.

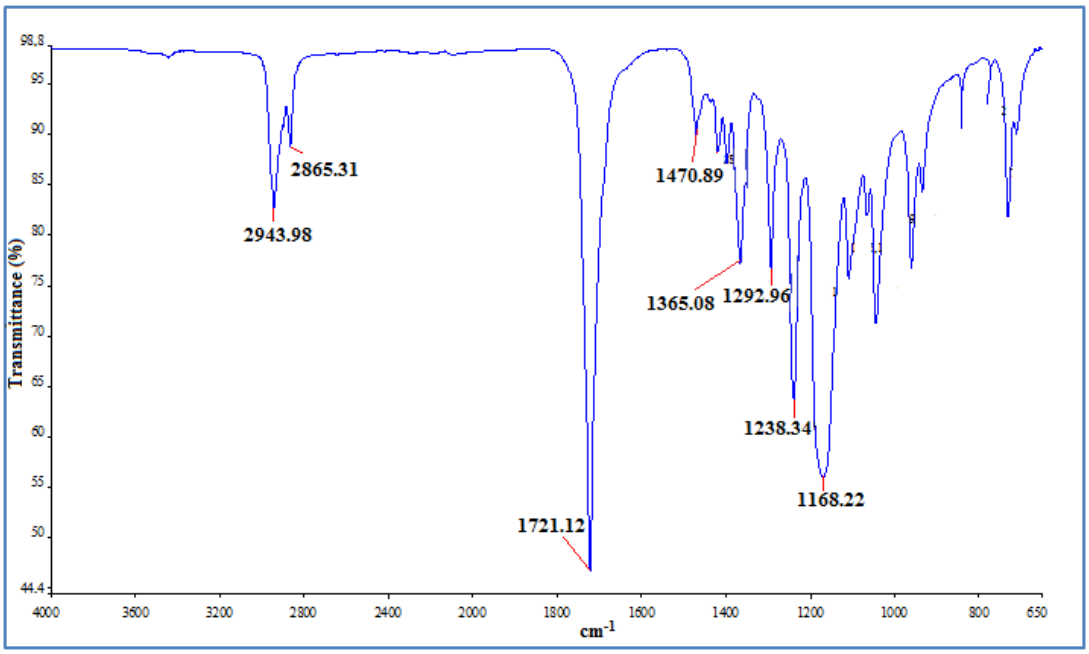


Figure 4.30 : FTIR spectra of polycaprolactone polymerized by IEc.

DSC scans of PCL catalyzed by IEa and IEc can be seen in Figure 4.31 and 4.32. From DSC thermogram of PCL catalyzed by IEa, Tm and ΔH_f values were calculated, which are 55.8 ºC and 79.4 J/g. For PCL catalyzed by IEc, 54.2 ºC of Tm and 83.1 J/g of ΔH_f were determined from Figure 4.32. Moreover, crystallinity percentages of polycaprolactones were measured by using Equation 3.6, which are 57 and 59.6 % for PCL catalyzed by IEa and IEc, respectively.

PCL is a semi crystalline polyester and crystallinity degree of this polymer can reach 69 % (Öztürk-Düşkünkorur, 2012). According to these results, it can be said that the crystallinity degrees of the result products matched with characteristic properties of PCL. Öztürk-Düşkünkorur (2012) synthesized PCL/clay nanohybrids, and it was obtained similar Tm and crystallinity degrees which were approximately 57 ºC and 50 %, respectively.

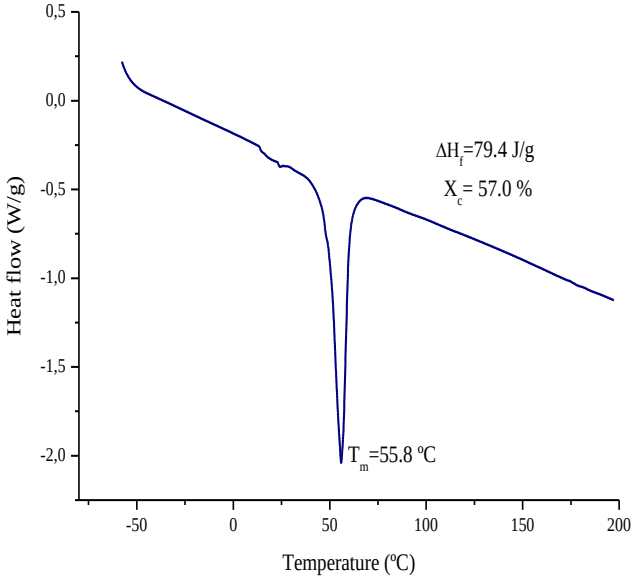


Figure 4.31 : DSC scan of PCL catalyzed by IEa.

Kuo et. al (2001) has studied hydrogen-bonding strength in polycaprolactone blends by DSC and FTIR. They obtained similar DSC thermograms of PCL included between 60-70 ºC of Tm values. Lastly, Özsağıroğlu (2011) synthesized polycaprolactone and characterized by DSC. In this study was obtained a Tm value of 56 ºC and a crystallinity degree of 50 %, which are similar to the obtained value in this master thesis.

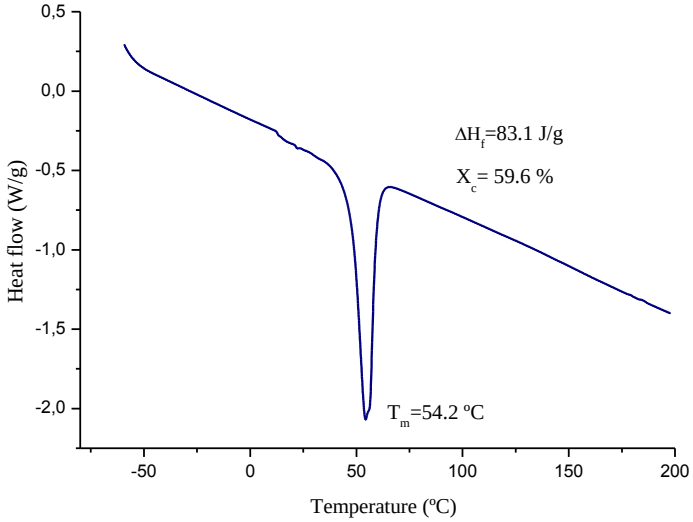


Figure 4.32 : DSC scan of PCL catalyzed by IEc.

5. CONCLUSION AND RECOMMENDATIONS

In this master thesis, immobilization of CALB onto precipitated silica and enzymatic ring opening polymerization of ε-CL were studied via using immobilized enzymes produced in the first step of this study. Both physical adsorption and crosslinking methods were used to immobilize precipitated silica, and immobilization and polymerization conditions were optimized successfully. Furthermore, Novozym® 435 and free CALB were used for ROP of ε-CL, and the results were compared to the best results obtained by using immobilized enzymes. Finally, characterization of precipitated silica, immobilized enzymes and polycaprolactones were made via TGA, FTIR and SEM analysis, while DSC, GPC and ^{1}H NMR were also used to characterize the obtained polycaprolactones.

The effect of 3-APTES concentration, glutaraldehyde concentration and enzyme amount on both immobilization processes were investigated, and the best results were obtained at 15 % (w/v) of 3-APTES concentration and 20 % (w/w) of enzyme amount. Glutaraldehyde concentration showed a negative effect on immobilization of CALB. Furthermore, the optimum pH and the optimum temperature were researched for both immobilized enzymes, 7 and 35-37 ºC, respectively. Storage stability of IEa and IEc was inverstigated by measuring periodically immobilized lipase activities. After 4 months storage the immobilized CALB by physical adsorption and crosslinking protected 50.1 and 59 % of its initial activity at 4 ºC, respectively. According to the results, immobilized enzyme by physical adsorption had higher activity than immobilized enzyme by crosslinking, while IEc showed higher stability. Both immobilized enzymes were characterized, and TGA, SEM and FTIR spectrums of immobilized lipases were proved that immobilization was achieved successfully.

The immobilized enzymes were used to polymerize ε-CL via ring opening polymerization at 40, 60 and 80 ºC for 6, 24, 48, 72, 120, 150 and 170 hours. Different immobilized lipase concentration for synthesis of PCL was investigated to determine the most effective enzyme concentration. The highest molecular weights were obtained at 80 ºC-120 h and 80 ºC-170 h for PCL catalyzed by IEa and IEc,

respectively. 20 % (w/w) of enzyme concentration was determined also the optimum enzyme concentration for synthesis of PCL catalyzed by both immobilized enzymes. Furthermore, Novozym® 435 and free CALB were used to polymerize ε-CL, and Mn values were compared. Immobilized lipase by physical adsorption showed higher activity than Novozym® 435 and free CALB. Immobilized enzyme by crosslinking showed higher activity than free CALB, while the number average molecular weight of polycaprolactone was not higher than the PCL polymerized by Novozym® 435. However, PCL was synthesized by IEc with a high enough Mn, 9000 g/mol. Moreover, the retention rate of initial lipase activity after reaction cycles was observed to determine reusability of both immobilized enzymes. After 5 cycles of uses, about 32 and 42 % of the initial activity could be retained for immobilized enzyme by physical adsorption and crosslinking methods, respectively. Finally, polycaprolactones possessed the highest molecular weight were characterized, and TGA, FTIR, DSC and ^{1}H NMR spectrums were demonstrated that the result polymers have all characteristic peaks of polycaprolactone. SEM analysis supported also that surface morphology of the result polymer has a foam structure, which is one of the classical surface view of polycaprolactone.

Additionally, the effect of time and temperature on immobilization can be investigated to improve immobilization processes. There are some studies that lipase was immobilized at different immobilization time and temperature (Chang et al., 2007; Silva et al., 2013). Moreover, different organosilane agents can be used to determine silanation effect on immobilization. There are various organosilane agents were used to immobilize different lipases (Netto et al., 2009; Oh et al., 2006). On the other hand, monomer conversion rates of polycaprolactones were calculated gravimetrically (about 90 %), which is not precision enough method. Therefore, monomer conversion rates can be calculated by observing polymerization reactions via ^{1}H NMR. Otherwise, to obtain higher molecular weight of polycaprolactone, the immobilized lipases by physical adsorption and crosslinking methods can be stirred, and more time period can be tested for synthesis. All in all, immobilized lipase onto precipitated silica was not used for PCL synthesis before this work. Thus, this master thesis can be the beginning and contribute studies related immobilization and enzymatic polymerization processes.

REFERENCES

Agarwal, S. (2012). Biodegradable Polyesters. *Polymer Science: A Comprehensive Reference,* (Vol. 5, pp. 333-359). Philipps-Universität, Marburg, Germany.

Bajsić, E. G., Bulatović, V. O., Slouf, M. and Šitum, A. (2014). Characterization of biodegradable polycaprolactone containing titanium dioxide micro and nanoparticles. *World Academy of Science, Engineering and Technology International Journal of Chemical, Nuclear, Materials and Metallurgical Engineering*, Vol.8, no.7. Date retrieved: 25.04.2015, adress: http://waset.org/publications/9998694/

Barbosa, O., Torres, R., Ortiz C. and Fernandez-Lafuente, R. (2012). Versatility of glutaraldehyde to immobilize lipases: Effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. *Process Biochemistry, 47,* 1220–1227.

Boros, Z., Weiser, D., Márkus, M., Abaháziová, E., Magyar, Á., Tomin, A., Koczka, B., Kovács, P. and Poppe, L. (2013). Hydrophobic adsorption and covalent immobilization of *Candida antarctica* lipase B on mixed-function-grafted silica gel supports for continuous-flow biotransformations. *Process Biochemistry, 48,* 1039–1047.

Brena, B. M. and Batista-Viera, F. (2006). Immobilization of enzymes. *Methods in Biotechnology: Immobilization of Enzymes and Cells* (2nd ed.). Totowa, NJ.

Cernia, E., Delfini, M., Cocco, E. D., Palocci, C. and Soro, S. (2002). Investigation of lipase-catalysed hydrolysis of naproxen methyl ester: use of NMR spectroscopy methods to study substrate–enzyme interaction. *Bioorganic Chemistry, 30,* 276–284.

Chang, S., Chang, S., Yen, Y. and Shieh, C. (2007). Optimum immobilization of *Candida rugosa* lipase on Celite by RSM. *Applied Clay Science, 37*, 67–73.

Córdova, A., Iversen, T., Hult, K. and Martinelle, M. (1998). Lipase-catalysed formation of macrocycles by ring-opening polymerisation of ε-caprolactone. *Polymer, 39,* 6519–6524.

Córdova, A., Iversen, T. and Hult, K. (1999). Lipase-catalyzed formation of end-functionalized poly(ε-caprolactone) by initiation and termination reactions. *Polymer, 40,* 6709–6721.

Cruz, J. C., Pfromm, P. H. and Rezac, M. E. (2009). Immobilization of *Candida antarctica* Lipase B on fumed silica. *Process Biochemistry, 44,* 62–69.

Cui, C., Tao, Y., Li, L., Chen, B. and Tan, T. (2013). Improving the activity and stability of *Yarrowia lipolytica* lipase Lip2 by immobilization on polyethyleneimine-coated polyurethane foam. *Journal of Molecular Catalysis B: Enzymatic, 91,* 59– 66.

Datta, S., Christena, L. R. and Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials.*Biotechnology,3,* 1–9.

Deng, F. and Gross, R. A. (1999). Ring-opening bulk polymerization of ε-caprolactone and trimethylene carbonate catalyzed by lipase Novozym 435. *International Journal of Biological Macromolecules,* 25, 153–159.

Dong, H., Li, Y. Sheng, G. and Hu, L. (2013). The study on effective immobilization of lipase on functionalized bentonites and their properties. *Journal of Molecular Catalysis B: Enzymatic, 95,* 9– 15.

Drummond, C., McCann, R. and Patwardhan, S. V. (2014). A feasibility study of the biologically inspired green manufacturing of precipitated silica. *Chemical Engineering Journal, 244,* 483–492.

Flesch, C., Joubert, M., Bourgeat-Lami, E., Mornet, S., Duguet, E., Delaite, C. and Dumas, P. (2005). Organosilane-modified maghemite nanoparticles and their use as co-initiator in the ring-opening polymerization of ε-caprolactone. *Colloids and Surfaces A: Physicochem. Eng. Aspects, 262,* 150–157.

Forde, J., Vakurov, A., Gibson, T. D., Millner, P., Whelehan, M., Marison, I. W. and Ó'Fágáin, C. (2010). Chemical modification and immobilisation of lipase B from *Candida antarctica* onto mesoporous silicates. *Journal of Molecular Catalysis B: Enzymatic, 66,* 203–209.

Gao, S., Wang, Y., Wang, T., Luo, G. and Dai, Y. (2009). Immobilization of lipase on methyl-modified silica aerogels by physical adsorption. *Bioresource Technology, 100,* 996–999.

Hwang, S., Lee, K., Park, J., Min, B., Haam, S., Ahn, I. and Jung, J. (2004). Stability analysis of *Bacillus stearothermophilus* L1 lipaseimmobilized on surface-modified silica gels.*Biochemical Engineering Journal, 17,* 85–90.

Heiny, M., Wurth, J. J. and Shastri, V. P. (2014). Progress in functionalized biodegradable polyesters. *Natural and Synthetic Biomedical Polymers*(pp. 167-180). University of Freiburg, Freiburg, Germany.

Idris, A. and Bukhari, A. (2012). Immobilized *Candida antarctica* lipase B: Hydration, stripping off and application in ring opening polyester synthesis. *Biotechnology Advances, 30,* 550–563.

İyisan, B. (2011). Synthesis of polycaprolactone via enzymatic ring opening polymerization. *M.Sc Thesis,* ITU, Institue of Science and Technology, Istanbul.

Kharrat, N., Ali, Y. B., Marzouk, S., Gargouri, Y. and Karra-Châabouni, M. (2011). Immobilization of *Rhizopus oryzae* lipase on silica aerogels by adsorption: Comparison with the free enzyme. *Process Biochemistry, 46,* 1083–1089.

Khoobi, M., Motevalizadeh, S. F., Asadgol, Z., Forootanfar, H., Shafiee, A. and Faramarzi, M. A. (2014). Synthesis of functionalized polyethylenimine-grafted mesoporous silica spheres and the effect of side arms on lipase immobilization and application. *Biochemical Engineering Journal, 88,* 131–141.

Kuo, S. W., Huang, C. F. and Chang, F. C. (2001). Study of hydrogen-bonding strength in poly(e-caprolactone) blends by DSC and FTIR. *Journal of Polymer Science: Part B: Polymer Physics, 39,* 1348–1359.

Kurtovic, I., Marshall, S. N. and X. Zhao X. (2011). Hydrophobic immobilization of a bile salt activated lipase from Chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Molecular Catalysis B: Enzymatic, 72,* 168–174.

Labet, M. and Thielemans, W. (2009). Synthesis of polycaprolactone: a review. *Chemical Society Reviews,38,* 3484–3504.

Lee, D.H., Park, C.H., Yeo, J. M. and Kim, S.W. (2006). Lipase immobilization on silica gel using a cross-linking method. *Journal of Industrial and Engineering Chemistry, 12,* 777-782.

Li, Q., Li, G., Yu, S., Zhang, Z., Ma, F. and Feng, Y. (2011). Ring-opening polymerization of ε -caprolactone catalyzed by a novel thermophilic lipase from *Fervidobacterium nodosum. Process Biochemistry, 46,* 253–257.

Magnusson, A. (2005). Rational redesign of *Candida antarctica* lipase B. Royal Institute of Technology, Stockholm, May 2005. Date retrived: 25.04.2015. adress: http://www.diva-portal.org/

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M. and Fernandez-Lafuente R. (2007).Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology, 40,* 1451–1463.

Mihailović, M., Stojanović, M., Banjanac, K., Carević, M., Prlainović, N., Milosavić, N., and Bezbradica, D. (2014). Immobilization of lipase on epoxy-activated Purolite® A109 and its post-immobilization stabilization. *Process Biochemistry, 49,* 637-646.

Miletić, N., Fahriansyah, Nguyen, L.T. and Loos, K. (2010). Formation, topography and reactivity of *Candida antarctica* lipase B immobilized on silicon surface. *Biocatalysis and Biotransformation, 28,* 357–369.

Miletic´, N., Nastasovic´, A. and Loos, K. (2012). Immobilization of biocatalysts for enzymatic polymerizations: Possibilities, advantages, applications. *Bioresource Technology 115,* 126–135.

Mendes, A. A., Castro, H. F., Andrade, G. S.S., Tardioli, P. W. and Giordano R. L.C. (2013). Preparation and application of epoxy–chitosan/alginate support in the immobilization of microbial lipases by covalent attachment. *Reactive & Functional Polymers, 73,* 160–167.

Meng, X., Xu, G., Zhou, Q. L. Wu, J. P and Yang L. R. (2013). Improvements of lipase performance in high-viscosity system by immobilization onto a novel kind of poly(methylmethacrylate-co-divinylbenzene) encapsulated porous magnetic microsphere carrier. *Journal of Molecular Catalysis B: Enzymatic, 89,* 86– 92.

Musić, S., Filipović-Vinceković, N. and Sekovanić, L. (2011).Precipitation of amorphous SiO_2 particles and their properties. *Brazilian Journal of Chemical Engineering, 28,* 89-94.

Namekawa, S., Suda, S., Uyama, H. and Kobayashi, S. (1999). Lipase-catalyzed ring-opening polymerization of lactones to polyesters and its mechanistic aspects. *International Journal of Biological Macromolecules, 25,* 145–151.

Netto, C. G. C. M., Andrade, L. H. and Toma, H. E. (2009). Enantioselective transesterification catalysis by *Candida antarctica* lipase immobilized on superparamagnetic nanoparticles. *Tetrahedron: Asymmetry, 20,* 2299–2304.

Oh, C., Lee, J. H., Lee, Y. G., Lee, Y. H., Kim, J. W., Kang, H. H. and Oh, S. G. (2006). New approach to the immobilization of glucose oxidase on non-porous silica microspheres functionalized by (3-aminopropyl)trimethoxysilane (APTMS). *Colloids and Surfaces B: Biointerfaces, 53,* 225–232.

Öney-Kıroğlu, C. (2014). Development and characterization of silica based süper insulation materials. *M.Sc. Thesis,* ITU, Institue of Science and Technology, Istanbul.

Özsağıroğlu, E. (2011). Enzimatik polimerizasyon ile sentezlenen polikaprolaktona reaksiyon koşullarının etkisinin ve polikaprolaktonun biyobozunurluğunun incelenmesi. *M.Sc. Thesis,* ITU, , Institue of Science and Technology, Istanbul.

Öztürk-Düşkünkorur, H. (2012). Biopolyester synthesis by enzymatic catalysis and development of nanohybrid systems. *PhD Thesis,* ITU, Istanbul, Turkey.

Öztürk-Düşkünkorur, H., Pollet, E., Phalip, V., Güvenilir, Y. and Avérous, L. (2014). Lipase catalyzed synthesis of polycaprolactone and clay-based nanohybrids. *Polymer, 55,* 1648-1655.

Park, S. W., Kim, Y. I., Chung, K. H., Hong, S. I. and Kim, S. W. (2002). Covalent immobilization of GL-7-ACA acylase on silica gel through silanization. *Reactive & Functional Polymers, 51,* 79–92.

Persenaire, O., Alexandre, M., Dege´, P. and Dubois, P. (2001). Mechanisms and kinetics of thermal degradation of poly(ε-caprolactone). *Biomacromolecules, 2,* 288-294.

Poojari, Y. and Clarson, S. J. (2013). Thermal stability of *Candida antarctica* lipase B immobilized on macroporous acrylic resin particles in organic media. *Biocatalysis and Agricultural Biotechnology, 2*, 7–11

Poppe, J. K., Costa, A. P. O., Brasil, M. C., Rodrigues, R. C. and Ayub, M. A. Z. (2013). Multipoint covalent immobilization of lipases on aldehyde-activated support: Characterization and application in transesterification reaction. *Journal of Molecular Catalysis B: Enzymatic, 94*, 57-62.

Ranjha, N. M., Mudassır, J. and Majeed, S. (2011). Synthesis and characterization of polycaprolactone/acrylic acid (PCL/AA) hydrogel for controlled drug delivery. *Bulletin of Materials Science, 34*, 1537–1547.

Rosso, S. R. C., Bianchin, E., Oliveira, D., Oliveira, J. V. and Ferreira, S. R. S. (2013). Enzymatic synthesis of poly(ε -caprolactone) in supercritical carbon dioxide medium by means of a variable-volume view reactor. *Journal of Supercritical Fluids, 79*, 133–141.

Santos, R. D., Comim, S. R. R., Oliveira, D., Treichel, H., Luccio, M. D., Ferreira, S. R. S. and Oliveira, J. V. (2012).Lipase-catalyzed synthesis of poly(ε-caprolactone) in supercritical carbon dioxide. *Biocatalysis and Agricultural Biotechnology, 1*, 280–283.

Serra, E., Díez, E., Díaz, I. and Blanco, R. M. (2010). A comparative study of periodic mesoporous organosilica and different hydrophobic mesoporous silicas for lipase immobilization. *Microporous and Mesoporous Materials, 132*, 487–493.

Sha, K., Qin, L., Li, D., Liu, X. and Wang, J. (2005). Synthesis and characterization of diblock and triblock copolymer by enzymatic ring-opening polymerization of ε-caprolactone and ATRP of styrene. *Polymer Bulletin, 54*, 1–9.

Sheldon, R. A. (2007). Enzyme immobilization: the quest for optimum performance. *Advanced Synthesis & Catalysis, 349*, 1289 – 1307.

Singh, K. and Kayastha A. M. (2014). Optimal immobilization of α-amylase from wheat (*Triticum aestivum*) onto DEAE-cellulose using response surface methodology and its characterization. *Journal of Molecular Catalysis B: Enzymatic, 104*, 75–81.

Silva, A. L. P., Nascimento, R. G., Arakaki, L. N. H., Arakaki, T., Espínola, J. G. P. and Fonseca, M. G. (2013). Organofunctionalized silica gel as a support for lipase. *Journal of Non-Crystalline Solids, 376*, 139–144.

Silva, J.A., Macedo, G.P., Rodrigues, D.S., Giordano, R.L.C. and Gonçalves, L.R.B. (2012). Immobilization of *Candida antarctica* lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies. *Biochemical Engineering Journa,l 60*, 16-24.

Spahn, C. and Minteer, S. D. (2008). Enzyme immobilization in biotechnology. *Recent Patents on Engineering, 2*, 195-200.

Tzialla, A. A., Pavlidis, I. V., Felicissimo, M. P., Rudolf, P., Gournis, D. and Stamatis, H. (2010). Lipase immobilization on smectite nanoclays: Characterization and application to the epoxidation of α-pinene. *Bioresource Technology, 101*, 1587–1594.

Uyama, H. (2007). Enzymatic polymerization. *Future Directions in Biocatalysis* (pp. 205-251). Osaka University, Suita, Japan.

Vaidya, B. K., Ingavle, G. C., Ponrathnam, S., Kulkarni, B.D. and Nene, S. N. (2008).Immobilization of *Candida rugosa* lipase on poly(allyl glycidyl ether-co-ethylene glycol dimethacrylate) macroporous polymer particles. *Bioresource Technology, 99*, 3623–3629.

Varma, I. K., Albertsson, A. C., Rajkhowa, R. and Srivastava, R. K. (2005). Enzyme catalyzed synthesis of polyesters. *Progress in Polymer Science, 30,* 949–981.

Valentini, L., Macan, J., Armentano, I., Mengoni, F. and Kenny, J. M. (2006). Modification of fluorinated single-walled carbon nanotubes with aminosilane molecules. *Carbon, 44*, 2196–2201.

Woodruff, M. A. and Hutmacher, D. W. (2010).The return of a forgotten polymer -Polycaprolactone in the 21st century. *Progress in Polymer Science, 35*, 1217-1256.

Wu, C. S. (2005). A comparison of the structure, thermal properties, and biodegradability of polycaprolactone/chitosan and acrylic acid grafted polycaprolactone/chitosan. *Polymer, 46*, 147–155.

Yang, G., Wu, J., Xu, G. and Yang, L. (2010). Comparative study of properties of immobilized lipase onto glutaraldehyde-activated amino-silica gel via different methods. *Colloids and Surfaces B: Biointerfaces, 78,* 351–356.

Yang, J., Ma, X., Zhang, Z., Chen, B., Li, S. and Wang, G. (2010). Lipase immobilized by modification-coupled and adsorption–cross-linking methods: A comparative study. *Biotechnology Advances, 28,* 644–650.

Yang, Y., Yu, Y., Zhang, Y., Liu, C., Shi, W. and Li, Q. (2011). Lipase/esterase-catalyzed ring-opening polymerization: A green polyester synthesis technique. *Process Biochemistry, 46,* 1900–1908.

Yıldırım, D., Tükel, S. S., Alptekin, Ö. and Alagöz, D. (2014). Optimization of immobilization conditions of *Mucor miehei* lipase onto Florisil via polysuccinimide spacer arm using response surface methodology and application of immobilized lipase in asymmetric acylation of 2-amino-1-phenylethanols. *Journal of Molecular Catalysis B: Enzymatic, 100,* 91– 103.

Yu, X. H., Zhuo, R. X., Feng, J. and Liao J. (2004). Enzymatic ring-opening polymerization of 2,2-dimethyltrimethylene carbonate catalyzed by PPL immobilized on silica nanoparticles. *European Polymer Journal, 40*, 2445–2450.

Yuan, L., Hamidi, N., Smith, S., Clemons, F., Hamidi, A. and Tang, C. (2015). Molecular characterization of biodegradable natural resin acid-substituted polycaprolactone. *European Polymer Journal, 62,* 43–50.

Zaidan, U. H., Rahman, M. B. A., Basri, M., Othman, S. S., Rahman, R. N. Z. R. A. and Salleh, A. B. (2010). Silylation of mica for lipase immobilization as biocatalysts in esterification. *Applied Clay Science, 47,* 276–282.

Zhang, J., Shi, H., Wu, D., Xing, Z., Zhang, A., Yang, Y. and Li, Q. (2014). Recent developments in lipase-catalyzed synthesis of polymeric materials. *Process Biochemistry, 49,* 797–806.

Zhang, Q., Xu, W. and Wang, Z. (1994). Synthesis of polycaprolactone with two carboxyl end groups. *Journal of Materials Science & Technology,* Vol.10.Date retrieved: 25.04.2015,
adress: http://www.jmst.org/fileup/PDF/pass441.pdf

Zhang, Z., He, F. and Zhuo, R. (2013). Immobilized lipase on porous silica particles: Preparation and application for biodegradable polymer syntheses in ionic liquid at higher temperature. *Journal of Molecular Catalysis B: Enzymatic, 94,* 129– 135.

Zhu, N., Zhang, Z., He, W., Geng, X., Fang, Z., Li, X., Li, Z. and Guo, K. (in pres). Highly chemoselective lipase from *Candida* sp. 99-125 catalyzed ring-opening polymerization for direct synthesis of thiol-terminated poly(e-caprolactone). *Chinese Chemical Letters.*

Zou, B., Hu, Y., Yu, D., Xia, J., Tang, S., Liu, W. and Huang, H. (2010). Immobilization of porcine pancreatic lipase onto ionic liquid modified mesoporous silica SBA-15. *Biochemical Engineering Journal, 53,* 150-153.

APPENDICES

APPENDIX A: GPC traces of PCLs synthesized by IEc and IEa

APPENDIX A

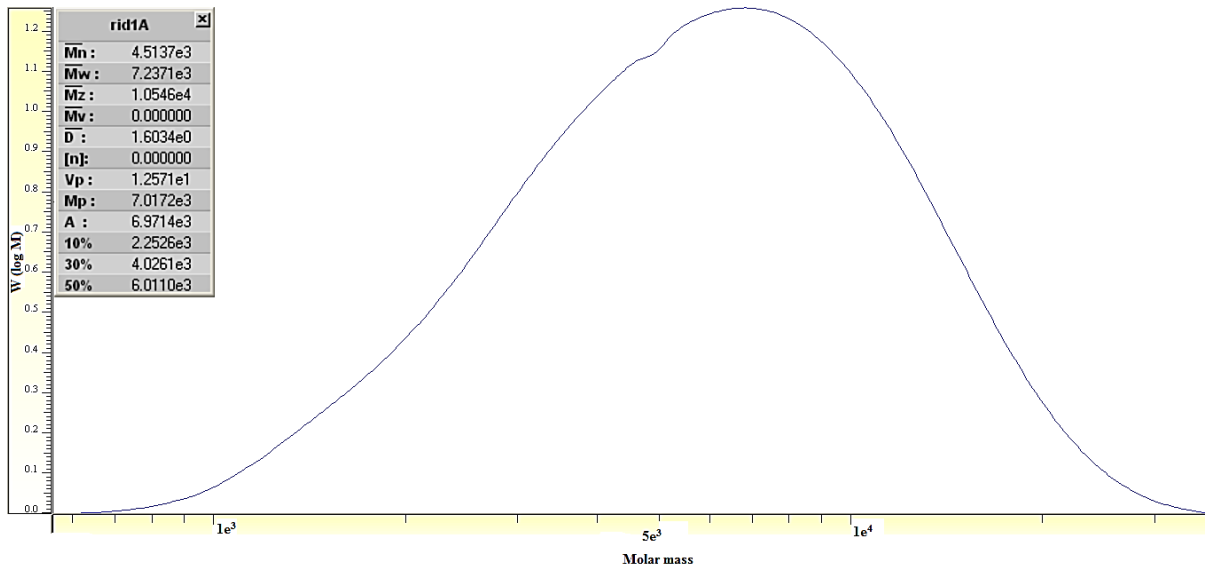


Figure A.1 : GPC trace of PCL synthesized by IEc at 40 ºC – 72 h.

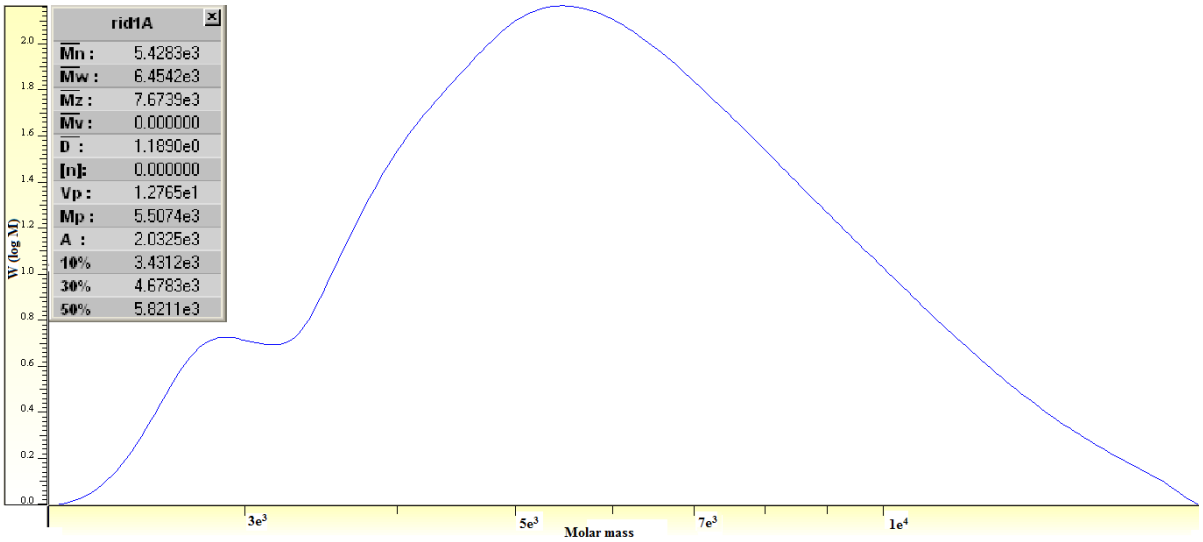


Figure A.2 : GPC trace of PCL synthesized by IEc at 60 ºC – 24 h.

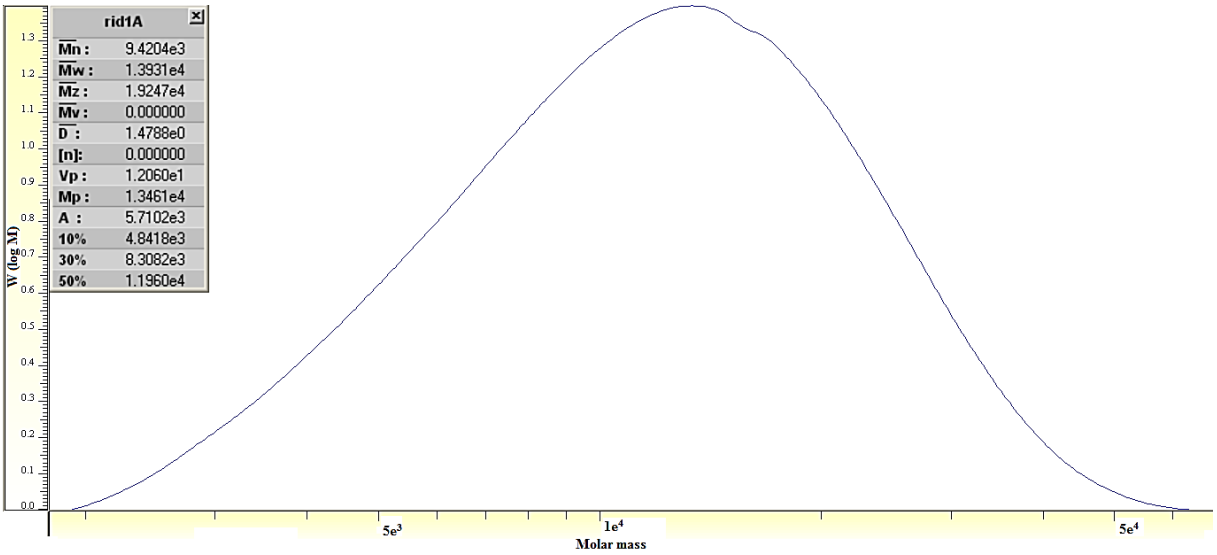


Figure A.3 : GPC trace of PCL synthesized by IEc at 80 ºC – 170 h.

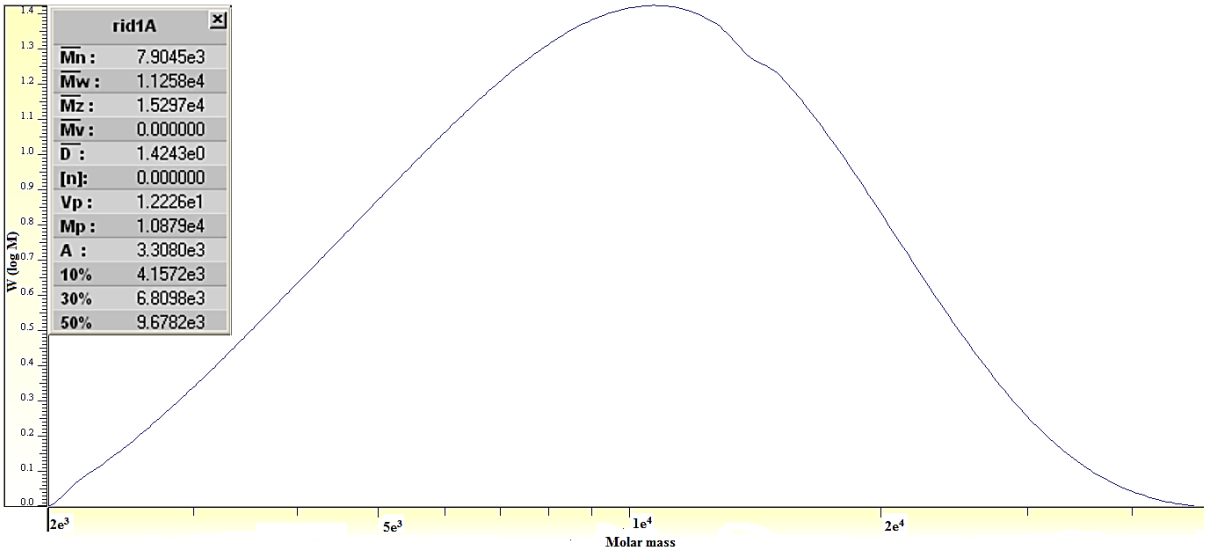


Figure A.4: GPC trace of PCL synthesized by IEa at 40 ºC – 120 h.

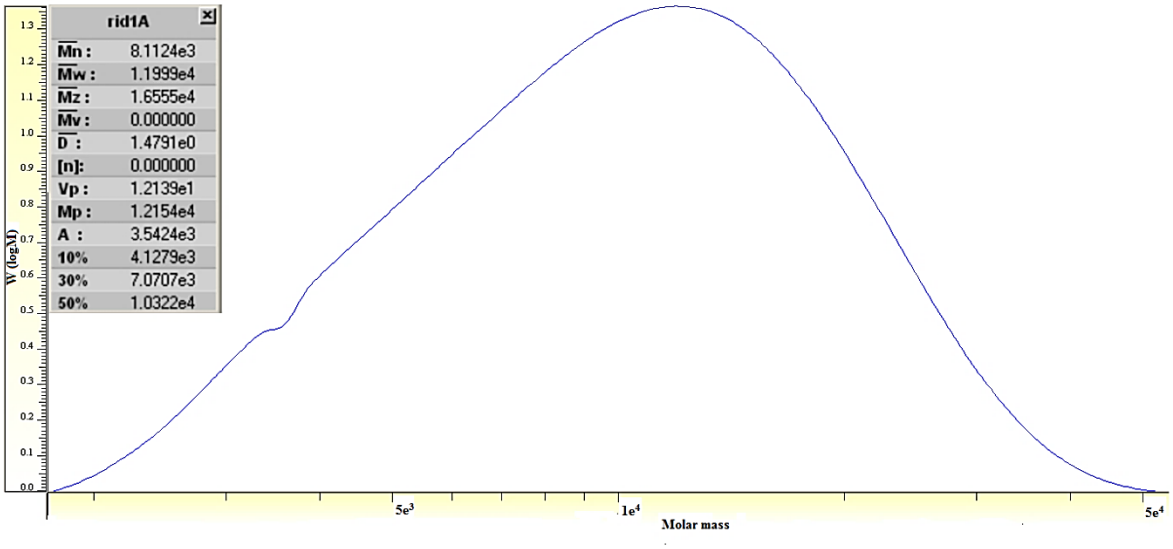


Figure A.5: GPC trace of PCL synthesized by IEa at 60 ºC – 48 h.

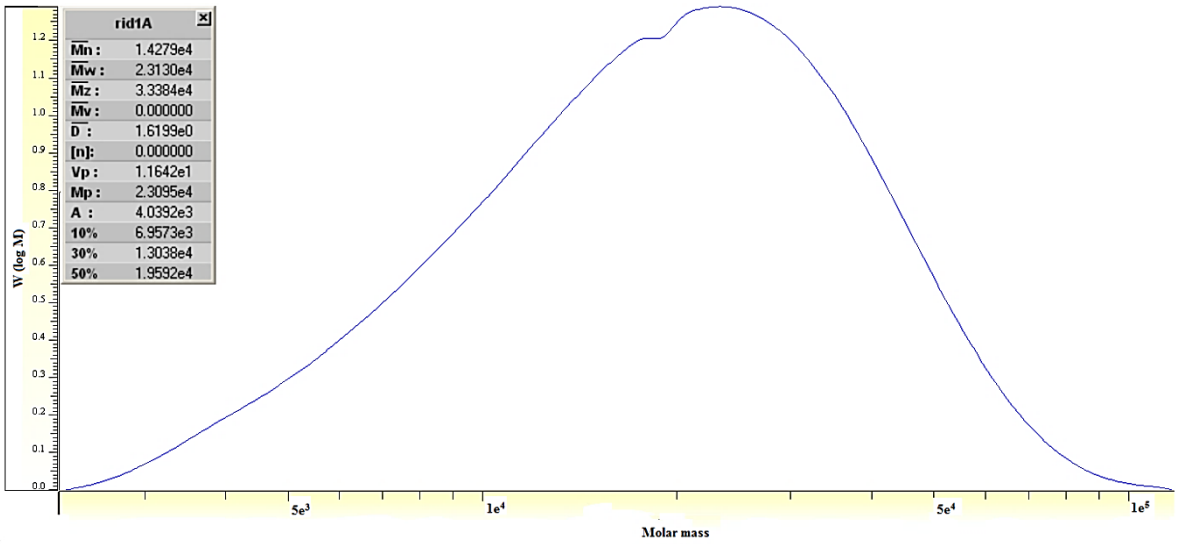


Figure A.6: GPC trace of PCL synthesized by IEa at 80 ºC – 120 h.

CURRICULUM VITAE

Name Surname: Nurefşan GÖKALP

Place and Date of Birth: Istanbul/30.12.1989

E-Mail: nurefsan.gokalp89@gmail.com

EDUCATION:

B.Sc.: Department of Chemical Engineering, Yıldız Technical University, 2013

PRESENTATIONS ON THE THESIS:

- ✓ **Gokalp N.,** Ulker C., Guvenilir, Y., 2015. Immobilization of *Candida antarctica* lipase onto an amorphous silica support by physical adsorption method for ring-opening polymerization of ε-caprolactone. *European Polymer Congress*, June 21-26, 2015 Dresden, Germany. (Poster Presentation)
- ✓ **Gokalp N.,** Ulker, C., Guvenilir, Y., 2015. Immobilization of *Candida antarctica* lipase onto an amorphous silica support by cross-linking method for ring-opening polymerization of ε-caprolactone. *Advanced Materials World Congress*, August 23-26, 2015 Stockholm, Sweden.(Oral Presentation)
- ✓ **Gokalp N.,** Ulker, C., Saloglu, D., Guvenilir, Y., 2015. Enzymatic ring opening polymerization of ε-caprolactone by using lipases immobilized on rice husk ash and precipitated silica by crosslinking method. *Biopol,* October 6-9, 2015 Donostia-San Sebastian,Spain. (Poster Presentation)

SUBMITTED MANUSCRIPTS ON THE THESIS:

- ✓ **Gokalp, N.,** Ulker, C., Guvenilir, Y., 2015. Synthesis of polycaprolactone via ring opening polymerization catalyzed by *Candida antarctica* lipase B immobilized onto an amorphous silica support, *Journal of Polymer Materials.* (in pres)
- ✓ **Gokalp, N.,** Ulker, C., Guvenilir, Y., 2015. Enzymatic ring opening polymerization of ε-caprolactone by using a novel immobilized biocatalyst, *Advanced Materials Letters*. (in pres)
- ✓ **Gokalp, N.,** Ulker, C., Guvenilir, Y., 2015. A comparative study of properties of immobilized lipase on precipitated silica via physical adsorption and crosslinking methods, *Applied Biochemistry and Microbiology.* (Submitted)

OTHER SUBMITTED MANUSCRIPTS:

- ✓ Ulker, C., **Gokalp, N.,** Guvenilir-Avcibasi, Y., 2015. Immobilization of Candida *antarctica lipase B* (CALB) on surface-modified rice husk ashes (RHA) via physical adsorption and cross-linking methods, *Biocatalysis and Biotransformation.* (in pres)
- ✓ Ulker, C., **Gokalp, N.,** Guvenilir-Avcibasi, Y., 2015. Poly (ε-caprolactone) synthesis by a novel enzymatic catalyst: *Candida antarctica* lipase B (CALB) immobilized on a modified silica-based material by physical adsorption, *Advanced Materials Letters*. (in pres)
- ✓ Ulker, C., **Gokalp, N.,** Guvenilir-Avcibasi, Y., 2015. Enzymatic synthesis and characterization of polycaprolactone by using free and immobilized lipase onto a renewable carrier, *Journal of Chemical Sciences.* (Submitted)
- ✓ Ulker, C., **Gokalp, N.,** Guvenilir-Avcibasi, Y., 2015. Biocatalysis and characterization of polycaprolactone via recently immobilized lipases with different strategies, *Polymer International.* (Submitted)

OTHER PRESENTATIONS:

- ✓ Ulker C., **Gokalp N.,** Guvenilir, 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. *European Polymer Congress*, June 21-26, 2015 Dresden, Germany. (Oral Presentation)
- ✓ Ulker, C., **Gokalp, N.,** Guvenilir, Y., 2015. Poly (ε-caprolactone) synthesis by a novel enzymatic catalyst: *Candida antarctica* lipase (CALB L) immobilized on a modified silica-based material by physical adsorption. *Advanced Materials World Congress*, August 23-26, 2015 Stockholm, Sweden. (Oral Presentation)
- ✓ Ulker, C., **Gokalp, N.,** Saloglu, D., Guvenilir, Y., 2015. A comparative study of properties of lipases immobilized onto different silica-based materials via physical adsorption to polymerize polycaprolactone. *Biopol*, October 6-9, 2015 Donostia-San Sebastian, Spain. (Poster Presentation)