ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

SYNTHESIS OF POLYCAPROLACTONE BY LIPASE ENZYME IMMOBILIZED ON AN INORGANIC MATERIAL

M.Sc. THESIS

Şeyda Ercan Korkan

Department of Polymer Science and Technology
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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

İNORGANİK MALZEME ÜZERİNE İMMOBİLİZE EDİLMİŞ LİPAZ ENZİMİ İLE POLİKAPROLAKTON ELDESİ

YÜKSEK LİSANS TEZİ

Şeyda Ercan Korkan
(515161020)

Polimer Bilim ve Teknolojisi Anabilim Dalı

Polimer Bilim ve Teknolojisi Programı

Tez Danışmanı: Prof. Dr. Yüksel GÜVENİLİR

MAYIS 2019

Şeyda Ercan Korkan, a M.Sc. student of İTU Graduate School of Science Engineering and Technology student ID 515161020, successfully defended the thesis/dissertation entitled “Synthesis of Polycaprolactone by Lipase Enzyme Immobilized on an Inorganic Material”, which he/she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Name SURNAME** İstanbul Technical University	Yüksel Güvenilir
Jury Members :	**Prof. Dr. Name SURNAME** Istanbul Technical University	.....
	Prof. Dr. Name SURNAME ..... University	.....
	Prof. Dr. Name SURNAME ..... University	.....
(If exists)	**Prof. Dr. Name SURNAME** ..... University	.....
(If exists)	**Prof. Dr. Name SURNAME** ..... University	.....

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To my spouse and family,

FOREWORD

To begin with, I would like to record my gratitude to my supervisor Prof. Dr. Yüksel Güvenilir for encouraging and trusting me along my thesis study. I am grateful for her support during both my bachelor and master degree.
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April 2019

Şeyda ERCAN KORKAN
(Chemical Engineer)

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ABBREVIATIONS

°2 Th.	**:** °2 Theta
1H-NMR	**:** Hydrogen nuclear magnetic resonance spectroscopy
3-APTES	**:** (3-aminopropyl)triethoxysilane
3-APTMS	**:** (3-aminopropyl)trimethoxysilane
3-GPTMS	**:** (3-glycidoxyptopyl)trimethoxysilane
3-TMSPDA	**:** (3-trimethoxysilyl)propylethylendiamine
CALB	**:** Candida antarctica lipase
CR	**:** Cross-linked
DSC	**:** Differential scanning calorimetry
ε-CL	**:** ε-caprolactone
FDA	**:** Food and Drug Agency
FT-IR	**:** Fourier transform infrared spectroscopy
GPC	**:** Gel permeation chromatography
PCL	**:** Poly(ε-caprolactone)
PDI	**:** Polydispersity index
PLA	**:** Poly(lactide
RHA	**:** Rice husk ash
ROP	**:** Ring opening polymerization
SEM	**:** Scanning electron microscopy
TEM	**:** Transmission electron microscopy
TGA	**:** Thermal gravimetric analysis
THF	**:** Tetrahydrofuran
U	**:** Unit
UV	**:** Ultraviolet
v/v	**:** Volume per volume ratio
w/w	**:** Weight per weight ratio
wt%	**:** Weight percent
XRD	**:** X-Ray diffraction

SYMBOLS

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

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SYNTHESIS OF POLYCAPROLACTONE BY LIPASE ENZYME IMMOBILIZED ON AN INORGANIC MATERIAL

SUMMARY

Looking at the recent developments and events in the world, it can easily be seen that environmental pollution is real and increasing rapidly. Depending on these events and developments manufacturing non-hazardous biopolymer material has become attractive. Biopolymers are flexible and light. Because of that they are compatible with the human body. They can be synthesized enzymatically and chemically. Synthesis of biopolymers with enzymatic ring opening polymerization method is preferred because of its compatibility with the environment. Polymers as they have biodegradable, biocompatible and highly mechanical characteristic, they can be used in biomedical field such as in tissue engineering, in medical equipment and in drug systems.

Enzymatic ring opening is preferred in polycaprolactone synthesis thanks to its mild reaction conditions and non-toxic effect. In ring opening polymerization of ε-CL enzymes that have biocatalyzer feature and organometal materials can be used as catalyzer. However if the organmetals like Zn, Al, Sn and Ge are starters of a polymerization, they can't be seperated from the matrix. This situtation is not preferred because it can show toxic effect. On the other hand enzymes can be seperated rather easily and also there is no dangerous threat if not. Yet because of long reaction times biocatalyzer activities and stabilities may decrease. Immobilization method is used To increase the enzyme effects, to longer and re-usability. There are four main types of immobilization: Physical absorption, covalent binding, cross-linking and entrapment. Physical absorption method is rather easy but it is a weak binding method. If an enzyme is closed to a polymer matrix it is called entrapment. If an enzyme functions brings out strong covalent binds immobilization is performed by covalent binding method. Cross-linking can be provided binds among molecules in between protein-protein or protein-support material.

The Candida antarctica lipase B (CALB) which can work in the most selective and the mildest conditions is known as Novozym® 435 (NOV-435) commercially. To increase the effectiveness in lipase enzyme polymerization there are organic, inorganic or polymer based carriers. In this study for surface modification silanization was used. In silanization process rice husk ash which contains high silica rate was used as support material. RHA is easy to find in nature and is a cheap by-product. In their process immobilized enzymes were used to synthesize ROP and PCL from ε-CL.

PCL, by its nature, is a semi-crystal and hydrophobic polymer. Also it is biocompatible and biodegradable material. PCL's molecule weight and crystallinity effects thermal, physical and mechanical properties. It is preferred in this study because its availability in tissue engineering, medical material production, and producing of biomaterials from

many kind. It has -60 °C glass transition temperature, 60 °C melting point and -350 °C decomposition temperature.

If we handle this study in two parts, in first part RHA was obtained by burning rice husk at 600 ° C for 6 hours. As a result of the surface modification, to obtain functional (-NH2) groups 3-trimethoxysilyl propyl ethylenediamine (TMSPDA) was used as a silanization agent at the surface. Lipase immobilization was conducted by crosslinking with glutaraldehit. Optimum temperature, PH value, shelf life, usage stability was evaluated.

In the second part, policaprolactone was obtained. For ε-CL ring opening polymerization toluen was used. To evaluate the optimum polymerization conditions, different times 6, 24, 48, 72 and 120 hours and different temperatures 30, 40, 60, 80 °C was studied. Effect or damage of temperature and time to polymerization was observed. As a result optimum reaction time and temperature was picked. Gel Permeability Chromatography(GPC) was used for molecule weight distribution of polymer samples; for chain structure, proton nuclear magnetic resonance spektroscopy; for thermal characteristics TGA and DSC; for surface structure SEM equipment was used. Additionally XRD was preferred for crystal structure of polymer samples.

From the results, surface modification that was performed with RHA and TMSPDA was successful by the TGA data. In addition enzyme immobilization was performed with %98 productivity. When lipase which immobilized with crosslinking method was used according to the GPC results 60°C and 72 hour, at the end of the reaction time 12300 g/mole PCL synthesized. For the obtained PCL monomer change was performed with a very high % 90 rate. As the product that was obtained with TMSPDA has medium low % 47 crystallinity it is safe to say that it can be used in drug convection systems. Physical absorption method for immobilization can be performed for furher study. In addition, to extend the study lipase enzymes that are immobilized with rice husk ash can be used for producing nanohybrids by burying into the PCL matrix without leaving the environment after the synthesis.

İNORGANİK MALZEME ÜZERİNE İMMOBİLİZE EDİLMİŞ LİPAZ ENZİMİ İLE POLİKAPROLAKTON ELDESİ

ÖZET

Dünyadaki son gelişmelere bakıldığında çevre kirliliğinin giderek arttığı görülmektedir. Bu duruma bağlı olarak çevreye zararı olmayan biyopolimer malzeme üretimleri ilgi çekmeye başlamıştır. Biyopolimerler hafif ve esnek olmaları sebebiyle insan vücuduna da uyumlu malzemelerdir. Enzimatik ve kimyasal olarak sentezlenebilirler. Enzimatik halka açılımıyla biyopolimer sentezi çevreye olan uyumu sebebiyle tercih edilmektedir. Polimerler biyobozunur, biyouyumlu özellikleri sayesinde doku mühendisliği, medical malzemeler ve ilaç sistemleri gibi biyomedikal alanlarda kullanılabilirler. Bu çalışmaları kalp ameliyatlarında kullanılan malzemelerde, diş ipleri gibi malzemelerde görmek oldukça mümkündür.

Enzimatik halka açılımı, toksik etki yaratmaması ve ılımlı reaksiyon koşulları sayesinde policaprolactone sentezinde tercih edilmektedir. ε-KL'nin halka açılması polimerizasyonunda katalizör olarak biyokatalizör olan enzimler ve organometalik malzemeler kullanılabilir. Ancak organometalik katalizör olan Zn, AL, Sn, Ge gibi malzemeler polimer sentezinde başlatıcı olarak bulunduğunda matristen tamamen ayrılmamaktadır. Bu durum özellikle biyomalzemelerin yapımında toksik etki gösterebileceğinden tercih edilmemektedir. Enzimler ise matristen daha kolay ayrılabilir, ayrılmama durumunda ise herhangi bir tehdit oluşturmamaktadır. Ancak biyokatalizörlerin aktiviteleri ve stabiliteleri uzun reaksiyon sürelerinde veya farklı reaksiyon koşullarında azalabilmektedir. Enzimlerin etkilerinin arttırılması, daha uzun süre ve tekrar kullanılabilmeleri için immobilizasyon yöntemi kullanılır. Genel olarak, enzimin belirli bir bölgede sınırlandırılması için enzim ile katı destek materyali arasında kurulan etkileşimler istenir. Katalitik aktivite korunmasının yanı sıra artırılır. Buna ek olarak, immobilizasyon enzimin geri kazanma yoluyla tekrar kullanılmasına olanak sağlar. Bu durum, endüstriyel uygulamalar için sürekli ve ekonomik prosesleri mümkün kılar. Ayrıca, enzimin reaksiyon ortamından daha kolay uzaklaştırılmasını sağladığı için ürünün protein kontaminasyonuna da engel olur. Fiziksel adsorpsiyon, kovalent bağlama, tutuklama ve çapraz bağlama dört ana immobilizasyon yöntemi vardır. Fiziksel adsorpsiyon daha basit bir yöntem olmasına karşın zayıf bir bağlanma yöntemidir. Enzim eğer bir polimer matriksine kapatılıyorsa bu tutuklama olarak adlandırılmaktadır. Eğer enzim fonksiyonları güçlü kovalent bağlar ortaya çıkarıyorsa immobilizasyon kovaent bağlanma yöntemi ile gerçekleştirilmiştir. Çapraz bağlanma protein-protein arasında veya proteinler - destek materyali arasında moleküller arası bağlarla sağlanır.

Lipaz enzimi farklı endüstrilerde ve değişik uygulama alanlarında kullanılmaktadır. Bu farklı alanlardan biri temizlik endüstirisidir. Yağların hidroliz tepkimesini katalizledikleri için lipazlar deterjanlarda katkı maddesi olarak kullanılır ve yağ lekelerinin temizlenmesinden sorumludurlar. Diğer bir endüstriye bakıldığında ise kağıt üretiminde de lipazları görebilemmiz mümkündür. Kağıt üretiminde lipazlar biriktiğinde kağıt kalitesini düşüren zift maddelerinin arıtılmasında kullanılır. Ek

olarak gıda endüstrisinde, hem kullanılan emülgatörler yerine hem de tatlandırıcı olarak kullanılırlar. Enerji endüstrisinde, lipazlar fosil yakıtları yenilenebilir enerji kaynakları ile değiştirmek için başlıca biodizel üretiminde kullanılır. En önemli çalışma alanlarından biri ise biyomedikal alanlarda da kullanıllan biyouyumlu polimer üretimidir. Lipaz enzimi biyouyumlu bir polimer olan polikaprolaktonun sentez tepkimesi olan halka açılması polimerizasyonunu katalizler. Polikaproklakton biyouyumlu olmasında dolayı özellikle medikal uygulamalar için oldukça sık çalışılan bir malzemedir.

En sık kullanılan yüzey modifikasyonu yöntemlerinden biri silanlamadır. Yüzeylerinde hidroksil grupları bulunan materyaller söz konusu olduğunda, yüzey modifikasyonu bu hidroksil grupları aracılığıyla gerçekleşir. Genellikle, organosilanlar silanlama ajanı olarak kullanılır.

En seçici ve ılımlı koşullarda çalışılabilen Candida antarctica lipase B (CALB) lipase enzimi, ticari olarak Novozym® 435 (NOV-435) olarak bilinmektedir. Lipase enzimlerinin polimerleşmede etkinliğinin arttırılması için organik, inorganik yada polimer bazlı taşıyıcılar bulunmaktadır. Bu çalışmada yüzey moidifikasyonu olarak en yaygın olan silanizasyon yöntemi uygulanmıştır. Silanlama prosesinde destek materyali olarak içerisinde yüksek oranda silika taşıyan pirinç kabuğu külü kullanılabilir. Pirinç kabuğu külü pirinç üretiminde yan ürün olarak elde edilen, doğada daha kolay bulunabilen ucuz ve bol bir malzemedir. Bu çalışmada da lipazın pirinçe kabuğu külüyle immobilizasyonu gerçekleşmiştir. Proseslerinde devamında immobilize edilmiş enzimler de kullanılarak ε-KL'dan ROP ile PKL sentezlenir.

PKL, doğası gereği yarı kristal ve hidrofobik olan bir polimerdir. Aynı zamanda biyolojik olarak uyumlu ve biyobozunur olarak kabul edilen bir malzemedir. PKL'nin molekül ağırlığı ve kristalliği termal, fiziksel ve mekanik özelliklerini etkilemektedir. Doku mühendsiliği, medikal malzeme üretimlerinde ve bir çok biyomalzeme üretiminde kullanılabilirliği sebebiyle bu çalışmada da tercih edilmiştir. -60 °C'lik düşük bir cam geçiş sıcaklığına, 60 °C'lik bir erime noktasına ve ~ 350 ° C'lik yüksek bir ayrışma sıcaklığına sahiptir.

Bu çalışmada kullanılan CALB pirinç kabuğu külü üzerine immobilize edildi. Pirinç kabuğu doğada çok yaygın bulunabilen adete bir silika deposudur. Pirinç üretim prosesinin yan ürünü olarak açığa çıkan, bol ve bu bolluğuna bağlı olarak diğer taşıyıcılara göre ucuz bir madde olduğundan lipaz immobilizasyonu için tercih edilmiştir. Silika enzim immobilizasyonunda yaygın olarak kullanılır ve pirinç kabuğu külünün SiO^2 içeriği % 95 'e kadar varabilen oldukça yüksek potansiyele sahiptir. Bu çzelliklerine bağlı olarak pirinç kabuğu külünün lipaz immobilizasyonu için umut verici bir taşıyıcı olduğu sonucuna varılmıştır. Benzer çalışmalara da bakıldığında pirinç kabuğu külü ile başarılı sonuçlar elde edilmiştir. Bu çalışmada pirinç kabuğu külüne immobilize lipazın verimliliği bir uygulama üzerinden gösterilmek istenmiştir. İlk olarak taşıyıcı madde hazırlanmış ve lipaz immobilizasyonu gerçekleştirilmiştir. Daha sonra ise, bu yeni immobilize lipazlar ile PKL sentezlenmiştir.

Çalışmayı iki kısımda ele alırsak, İlk kısım için enzimlerin başarılı bir şekilde çapraz bağlama yöntemi ile immobilizasyonudur. Bu amaçla öncelikle ilk kısımda pirinç kabuğu külleri, pirinç kabuklarının 600 °C' de 6 saat boyunca yakılması ile elde edildi. Yüzey modifikasyonu ile yüzeyde fonksiyonel ($-NH_2$) grupları elde etmek için

3-trimethoxysilyl propyl ethylenediamine (TMSPDA) ise silanlama ajanı olarak kullanıldı. Lipaz immobilizasyonu glutaraldehit ile çapraz bağlama sağlanarak gerçekleştirilmiştir. Gluteraldehit, silanlama ajanı ve enzim konsantrasyonunun aktivite üzerindeki etkileri de karşılaştırmalı olarak incelenmiştir. Optimum sıcaklık, PH değerleri, raf ömrü, kullanım stabilitesi gibi değerlere bakılmıştır.

Çalışmanın ikinci kısmında ise enzimatik halka açılımı yöntemi ile polikaprolakton eldesi gerçekleştirilmiştir. ε-KL'nin halka açılması polimerizasyonu için toluen ortamı kullanılmıştır. Polimer üretiminde farklı reaksiyon süreleri 6, 24, 48, 72 ve 120 saat ve farklı polimerizasyon sıcaklıkları 30, 40, 60, and 80 °C çalışılmıştır. Sıcaklık ve reaksiyon sürelerinin polimerizasyon üzerine etkisi incelenmiştir. Buna bağlı oarak optimum sıcaklık ve reaskiyon süresi seçilmiştir. Polimerlerin moleküllerinin ağırlık dağılımları için jel geçirgenlik kromatografisi (GPC), termal özellikleri, stabiliteleri için termogravimetrik analiz TGA ve DSC, yüzey yapıları için taramalı elektron mikroskobu SEM ekipmanları kullanılmıştır. Bunlara ek olarak polimer örneklerinin kristal yapısı için X-ışını kırınımı (XRD) tercih edilmiştir.

Sonuçlara bakıldığında RHA ve 3-TMSPDA ile gerçekleştirilen yüzey modifikasyonu TGA verilerine göre başarılı olmuştur. Bununla beraber enzim immobilizasyonu çapraz bağlama ile %98 verimlilikle gerçekleştirilmiştir. GPC sonuçlarına göre 60°C ve 72 saatte gerçekleştirilen reaksiyon sonunda 12300 g/mol molekül ağırlığında PKL sentezlenmiştir. Elde edilen PKL için monomer dönüşümü %90 gibi yüksek bir oranda gerçekleşmiştir. 3-TMPSDA ile elde edilen ürünün %47 gibi orta düşük kristaliniteye sahip olması ilaç taşınım sistemleri için kullanılabilirliğini desteklemektedir. Diğer silanlama ajanları ve immobilşzasyon yöntemleri ile elde edilen polimerlere bakıldığında başarılı sonuçlar elde edildiği gözlemlenmiştir. Elde edilen her polimerin kendi özelliklerine bağşı olarak tercih edilebilir çalışma alanları bulunmktadır. Diğer çalışma örneklerine bakıldığında çalışmanın devamı olarak fiziksel absorbsiyon yöntemi ile de immobilizasyon gerçekleştirilebilir. Ç.zücü olarak kullanılan toluen yerine farklı malzemeler denenebilir. Dha gelişmiş sıcaklık ve reaksiyon saatleri gözlemlenebilir. Kristalinite özelliği kullanılarak nanohibrit çalışmalarında kullanımı desteklenebilir.

1. INTRODUCTION

Environmental problems have started to be noticed by everyone all around the world recently. There have been studies about how these problems can be solved. Generally, these problems are global warming, environmental pollution, depletion of natural resources. To prevent these problems, it is needed to use nature by recycling strategies and minimize the harm to nature.

The increasing amount of plastic waste in the last years, has alarmed the society as it effects the environment seriously. The possible remedy for this problem is to use biodegradable polymers instead of raw synthetic polymers as biodegradable polymers are more susceptible to microbial action. Because of its similar characteristic with commodity plastics such as PE, PP and PS; polylactic acid (PLA) has come forward among other biobased and biodegradable polymers [1].

If classified by their preparation method, there are two different kinds of polymers. The first-class polymers can be synthesized from renewable assets. The other class polymers are synthetic, that are extracted from mineral oil mostly. These derived polymers are biodegradable. The high impact of using renewable material in industrial field has been acknowledged in the last few years. This is also because of the extreme concern about environmental problems such as waste disposal and depleting resources. By using renewable resources bio-based polymers can replace existing petroleum-based polymers by their cost performance and their unharmful characteristic to the nature. In order to reduce the demand for fossil fuel bio-based products and other innovative and environmentally friendly technologies needed to be made conventional and developed. This will also help enhancing national security, reducing the environmental pollution and improving the economy [2].

In comparison with biocompatible metals; biopolymers are much better for the human body in terms of flexibility and lightness. Biopolymers can be synthesized enzymatically and chemically. Enzymatic polymerization has become attractive in the last few years since its soft reaction conditions, high catalytic activities and low toxicity of enzymes. Besides , there is no prufication cost for undesired product and

no side reaction. Enzymatic polymerization advances with the substrate monomer ant the enzyme combination. Method developed for the useful conditions of polymers came up thanks to enzyme catalysis, whereas in absence of this strategy it would be very difficult to produce by using conventional chemical catalysts. This also jumped to other synthesis of biodegradable polyemer for example; , polycarbonates and polyphospates aliphatic types of polyesters and furthermore to polyaromatics. The examples for usage of biodegradable polymers in biomedical industry are surgical sutures, plasma substitutes, hygiene products and drug delivery vehicles [3].

Additionally, in pharmaceutical industry, natural and synthetic polymers are both have a large scale of usage in different application fields which resulted as the development and generalizing of cosmetics, traditional dosage forms and drug delivery systems. As for example; some polymers are used as fillers, lubricants, disintegrates, binders, glidants, solubilizers and also stabilizers in tablets capsules suspensions or creams. Furthermore, especially for controlled drug release, biodegradable and bioadhesive polymers may have significant part for development of novel drug delivery systems [4].

Polycaprolactone (PCL) is the most important biodegradable polymer in the biomedical field. It is widely used because it is nontoxic, highly permeable and perfectly biocompatible. Furthermore PCL is bioresorbable material because it is completely cleared off from the human body with degradations products and by-products(low molecular weight compounds) which leaves no residual side effects. PCL as rubbery polymer degrades by hydrolytic or enzymatic pathways and has low Tg [5].

PCL is aliphatic polyester and furthermore PCL can be synthesize via enzymatic ROP of ε-caprolactone. Candida antarctica lipase B (CALB) is a most popular enzyme for synthesizing PCL . It is widely used by its mobilized form as a biocatalyst and goes by the name Novozyme 435® Very good results were obtained with this enzyme. [6]. Enyme's transfer or confinement to a spesific area without losing its kinetic activity is called "Immobilized enzyme" [7].

Rice husk ash (RHA) was provided as an inorganic assist material in order to immobilize the loose CALB throughout this study as RHA is an easy to obtain and cheap material. Since RHA has very high SiO^2 within (up to 95%), it is very suitable for being support material for enzyme immobilization. After burning, the support material is obtained. Then the RHA surface is altered by *N*-(Trimethoxysilylpropyl)

ethylenediamine (silanization agent). Thus, functional amine (-NH2) groups are acquired [8].

Secondly cross-linking method is used to immobilize the lipase on RHA. For cross-linker role Glutaraldehyde is preferred as it is a very prevalent coupling agent. Since it is highly efficient for ring opening polymerization of ε-caprolactone (ε-CL) by Novozyme 435 synthesis are performed in toluene. In order to make the most effective immobilized lipase various actions are held such as playing with the amount and the ratios of TMSPDA and glutaraldehyde concentrations and enzymes. Furthermore, the inquiry is continued with the storage stability (shelf life), optimum temperature, pH and operational stability(recycle).

Likewise, in enzymatic polymerization there are some standards which are needed to be changed in order to optimize the outcome. For example, monomer/solvent ratio, temperature, polymerization time etc. these variations and combinations thereof are observed by the study of surface modifications. [9].

The investigation resumed with different temperatures and different reaction times. To be precise polymerizations are carried out for (6, 24, 48, 72, 120 hours) with the temperature of (30, 40, 60, 80 °C) accordingly. After these reactions the most efficient reaction time and temperature for immobilized enzymes are detected. Optimum reaction condition and the impact of different enzyme concentration is studied.

After the actions above polymerizations containing Novozyme 435® are performed. This performance went along with the optimal reaction conditions of new enzymes since the need for the comparison of homemade and commercial enzyme has arisen. Gel Permeation Chromatography (GPC) are respectively used the polymer samples and their chain structures and molecular weight distributions. By using TGA and differential scanning calorimetry analysis thermal properties are obtained from the polymer samples. The analysis of the crystal structures of polymer samples are recorded by x-ray diffraction (XRD).

2. THEORETICAL STUDY

2.1 Polyesters

Widespread plastics left its place to biodegradable polyesters due to the growing interest to them because of the environmental concerns. Presumably some subdivision of the polyesters is environmentally degradable because ester linkages can be found in nature. To control the lifetime expectancy of biodegradable polymers while improving their mechanical properties, blends, random and block copolymers are inquired thoroughly. Instead of using the derived plastics from petrochemical material which are causing environmental pollution, alternative environmentally friendly methods have been searched to synthesize plastics that can be degradable on demand [10].
Polyesters could be created with the reaction of a diacid or acid anhydride and a diol and also elimination of water, or by ring-opening polymerization of cyclic (di-)esters. Referring to Figure 2.1, polyesters are classified by the composition of main chain as aliphatic, semi-aromatic and aromatic.

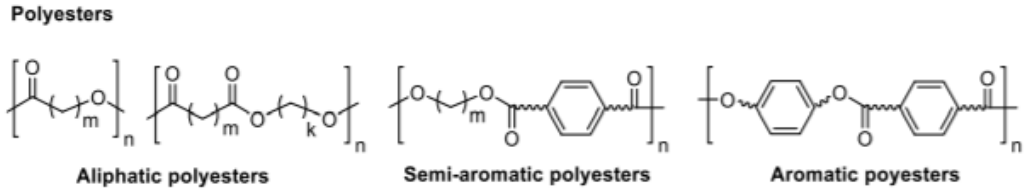

Figure 2.1: General chemical structures of aliphatic, semi-aromatic and aromatic polyesters**.**

Aromatic reactants make harder and more rigid while improving the heat resistance, as oppose to that aliphatic acids and diols increase flexibility, decrease the melting and softening point as well as improving the processability. Aliphatic diacid and aliphatic diol components make full aliphatic polyesters in a much smaller scale. Their melting and glass transition temperatures are low and also, they have poor hydrolytic stability. Some other aliphatic polyesters are common with their biocompatibility and

biodegradability. Furthermore, they are capable of blending with other commercial polymers [11,12].

In recent years a few biodegradable polymers have been improved. If we look at these sources, it can be seen that they are classified in two groups:

1) Can be obtained from renewable sources; For example, polymers based on microbiological origin or polymers derived from renewable sources
2) Obtained from non-renewable sources. For instance; fossil sources
More particularly, each aliphatic polyester is not known to be biopolymers such as polycondensation of hydroxy acids or diacids, some heterocycles polymers. All around the biodegradable polymers aliphatic polyesters are very dominant. There are two reasons for this. First one is they are potentially hydrolysable ester bonds and they have shorter chains in the macromolecules. Next reason is their exemplification for eco-friendliness. On the other hand they suffer lackness mechanically and physically. The solution for this handicap is upgrading the copolymer structures like poly(ester amid)s.
In Figure 2.2 is some bio based polyesters that have started to be used commercially and studied to be used commercially;
polylacticacid (PLA), polyglycolicacid (PGA), poly-ε-caprolactone(PCL), polyhydroxybutyrate (PHB), and poly(3-hydroxy valerate) [12].

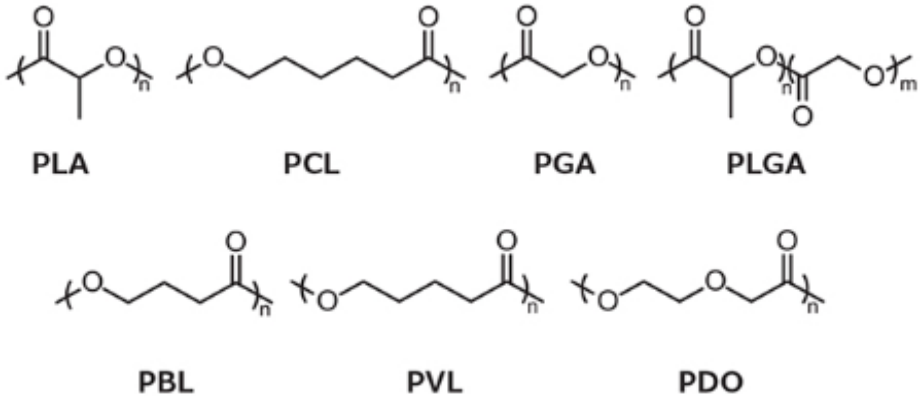

Figure 2.2 : Types of polyesters.

Poly(ε-caprolactones) (PCLs) is first generation synthetic aliphatic polyester. They have been explored thoroughly because of their biodegradability as they are

resorbable. Since shorter chain polyglycolides and derivatives become popular PCL lost interest. Besides for ε-caprolactone derived components for copolymer systems a significantly growing interest came up with further actions in recent years [13].

2.1.1 Polycaprolactone

Third Poly(ε-caprolactone) (PCL) is a significant degradable polymer. That is because it is widely studied for so long time since the first synthetic polyesters in 1930. The first big affinity to PCL came up with the discovery of PCL materials being degradable by bacterial and fungal enzymes as this is a important breakthrough in biodegradable material applications [13]. In some of the PCL appreciably films, synthetic fibers, plastics, coatings and plasticizers synthesis seemed noticeable too. In nature PCL is a crystalline and hydrophobic polymer [6, 9].

Among of all aliphatic polyesters, PCL has more rheological and viscoelastic structure. Thus, manufacturing and manipulating into a three-dimensional platform becomes rather simple [9,13].

PCL as aliphatic polyester is saturated with hexanoate repeat units, besides with the crystallinity up to 70% it is classed as semicrystalline depending on weight average molecular weights (M_w) typically ranging from 3000 to 800,000 g mol^{-1}. When molecular weights get higher crystallinity reduces for example it is charted that at M_w = 200,000 g mol^{-1}, crystallinity is 33% [13].

Because of its regular structure, PCL is semi crystalline polymer, so its melting temperature is above body temperature (59-64 °C), on the other hand its T_g is -60 °C, thus in the body PCL's semi crystalline structure gets too tough because the amorphous domains are in rubbery state. PCL has bright future ahead as biodegradable polymer with rather long degradation time. Besides it has been used in vivo and vitro widely. That's why PCL-based material is suitable for using in sutures, tendons, cartilage, bone and in other biomedical field actions where mostly mechanical strength is required. What's more this field contains bone and vascular graft. Since PCL can be degradable by microorganisms, it is used as biodegradable packaging material. Subsequently it was verified that PCL can also be degraded under physiological conditions by a hydrolytic mechanism. Of all the polyesters, PCL degrades the slowest. Because it has five hydropho-CH2 moieties in its repeating units, hence it limits its application to commercial sutures and delivery devices [14].

For PCL synthesizing operation with ring opening polymerization, *Candida Antartica Lipase B* (CALB), *Candida cylindracea* (lipase CC), *Pseudomonas fluorescens* (lipase PF), PPL (porcine pancreas lipase), *Pseudomonascepacian* (lipase PC), and *Rhizopusjaponicuslipase* (lipase RJ) are the enzymes that were used. In addition to these enzymes there are a few non-lipase enzymes. *Humicola Insolens Cutinase* (HIC) is one example for these non-lipase enzymes. CALB overpower the other enzymes for producing high molecular weighted PCL effectively [9].

2.2 Enzymatic ROP of ε-caprolactone

Biomedical materials or applications may require advanced features. These properties are targeted as different structural developments from other materials. One of the various structural developments are easily biodegradable architecture. The properties of initiators and catalysts are important in the polymerization processes. Their properties can provide the desired polymer structures. As a result, certain combination studies have been carried out for biodegradable polymers. Metal-based, organometallic or inorganic materials can be used as initiators and catalysts in the realization of the ring-opening polymerization process [15]. One of these methods is the use of caprolactone monomer and enzymes in the ring opening of PCL. Unlike other methods, it can be said that a more environmentally sensitive method has been developed by the enzymatic method [3]. ε-CL is the most extensively studied type of lactones which can be polymerized by lipase catalyzed ROP.

The application of enzymatic ring-opening polymerization has certain advantages. These are examined;

• This substance can be said to be the most significant advantage. The enzymes used are derived from renewable sources and do not leave harmful by-products. Lack of harmful deposits means it is environmentally friendly.

• High enantio- and regio-selectivity with unsimilar pH, temperature, pressure values are suitable for moderate enzymatic polymerization.

• Enzymes can be used in very different environments such as bulk or at multiple interfaces. The fact that enzymes can be easily separated from the products also increases their preference.

• When the polymerization results of this environmental application are examined, it is seen that the strong polymerization of characterization is obtained.

• In processes where lipase enzymes are used as catalysts, unlike other catalysts, water or air filters are not needed. Some forms of cyclic carboxylic esters can be polymerized by organometallic catalyzer.

On the other hand, organo-metallic processes are compared with enzyme-catalyzed processes, it can be observed that the molecular weight of the polymers is less, and the system progresses more slowly.

Natural proportions of enzymes in this proportional growth also make a great contribution [3, 15].

The environmentally friendly catalysts in polymer synthesis enzymes became significantly popular [6]. In the last decade depending on the advantages mentioned above, enzyme catalyzed polymerizations show exponential growth. Enzymes are high potential in various industrial areas also biologically catalysts as their superior selectivity, specificity and actions under mild conditions.

In other words, there are potential uses of enzymes in chemical industries as unique as medium-low size production of fine chemicals and pharmaceuticals, to huge size energy or food sectors [16]. ε-CL is one of the lactone types which has been investigated thoroughly among the others can be polymerized by lipase catalyzed ROP.

2.2.1 Lipase enzyme

Lipases are the enzymes which are very common in biocatalysts, and also vast variety of fields such as oil and margarine industry, in medicine [17]. Chemo selectivity, enantioselectivity and regioselectivity are their significant characteristics. If It is necessary to look at the reactions catalyzed by lipases, these reactions can be listed as hydrolysis, synthesis (esterification, transesterification), peroxidation, aminolysis, alcoholysis and epoxidation [17,18].

In 1990, the 3-dimensional form of lipase was first described. As can be seen from Figure 2.3 there are opened and closed form for lipases. It has been contemplated that a surface activation event may occur from a ring of amphiphilic peptide surrounding the active center of the enzyme, such as a lid. If there is no suitable interface, the lipase enzyme remains in the closed form and cannot access the substrate. however, if it encounters an active interface, it becomes open and becomes active [19]. Lipases obtained from different organisms and used for polyester synthesis.

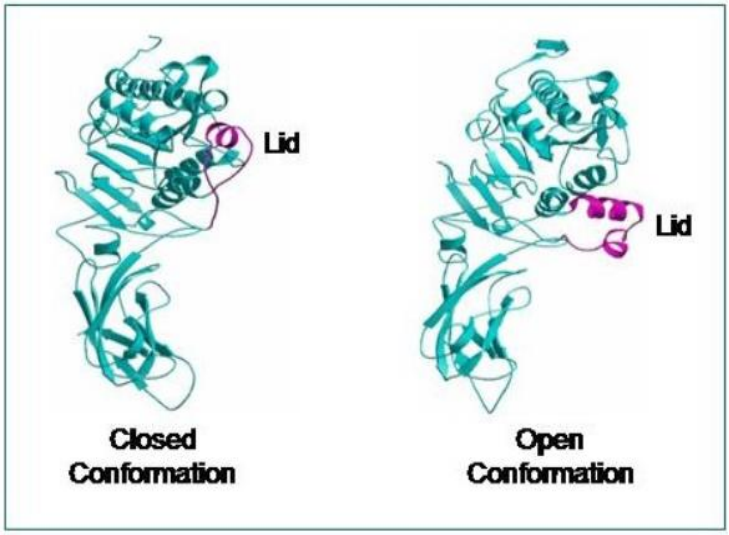

Figure 2.3 : (a) open and (b) closed forms of lipase [5].

According to previous studies, it can be said that lipase catalyzed polymerization processes become widespread. Although CALB B is the most suitable, different lipase enzymes are used such as Porcine pancreaticlipase, Candida rugose lipase, Pseudomonas flourescens lipase.

Enzymes' starting point has respectable effect on not only monomer conversions but also molecular weights of polymers. Additionally, it effects polydispersity indexes (PDI). CALB has very wide usage field in both ROP of ε-CL and organic solvents. In ionic liquid Yuan et al defined that the ROP of ε-CL catalyzed by an immobilized CALB, Novozyme-435 lipase. They found out that in %85 monomer conversion PCL was produced. This PCL had molecular weight (Mn) of 5942g·mol−1 in 1butyl-3-methylimidazolium hexafluorophosphate ([Bmim]PF6) at 60°C for 48h [6].

2.2.1.1 Lipase-catalyzed mechanism in ring-opening polymerization

Lipase is a hydrolases family. Certain conditions need to be carried out to catalyze the hydrolysis reaction. The first condition is the activation of the structure called the catalytic triad by hydrogen bonding. This nucleophilic residue is known to be responsible for polymerization [3, 17]. Based on Figure 2.4;

Initiation: to obtain ω-hydroxy carboxylic acid; the enzyme-activated monomer makes a nucleophilic attack with acyl carbon.

Propagation: to extend polymer chain; enzyme-activated monomer with terminal hydroxyl group of ω-hydroxy carboxylic acid attack to polymer structure.

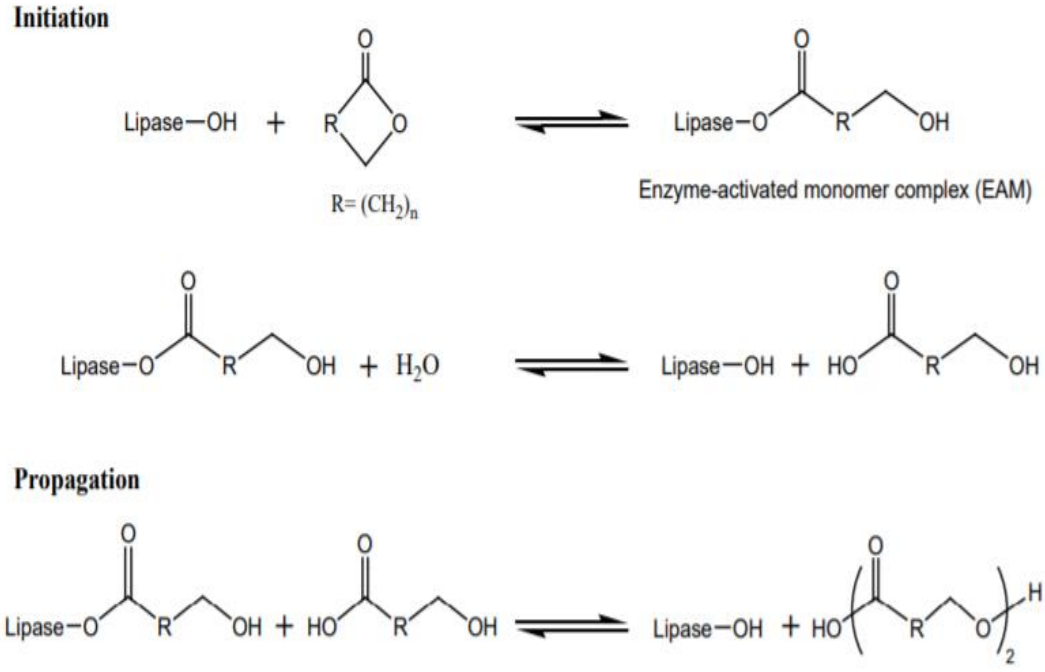

Figure 2.4 : General ROP mechanism of lipases [3].

2.3 Enzyme Immobilization

Free lipases on the market may have resolution problems with low activity. This low-resolution problem can be solved by immobilization. What's more basic characteristics like activity, stability, selectivity, specificity, resistance to inhibitors and purity can be developed after immobilization if correct protocols may apply [18].

The performance of immobilized enzymes is affected by several methods and various factors. They can be grouped into four categories; adsorption/carrier-binding, in cross-linking, covalent method, entrapment and occlusion which benefits from multifunctional reagents to link enzyme molecules to each other. When the adsorption process is considered, it is based on hydrophobic connections. To sustain support material adsorption, there are different way. One of them washed with enzyme [20]. Because of its simplicity, low cost and the ability to retain high enzyme activity, physical absorption is expected to possess much high commercial potential. Though the stability of physically absorbed enzymes is generally poor. That is because enzymes and applied carriers interacts weakly which causes enzymes to leak [21]. This leakage certainly limits the usage in industry. Besides it may cause a complication while judging the measured activity as it is whether immobilized lipase or the free one.

Covalent association of enzymes for supports happens thanks to their side chain amino acids such as arginine, aspartic acid, histidine and their degree of reactivity based on different functional groups such as imidazole, indolyl, phenolic hydroxyl etc. peptide-modified surfaces with controlled protein guidance when used for enzyme linkage ends with higher specific activity and stability with controlled protein orientation. The structures obtained from covalent bond silanized enzymes were found to be more stable [20]. As can be seen in the Figure 2.5 , many factors affect the activity and immobilization.

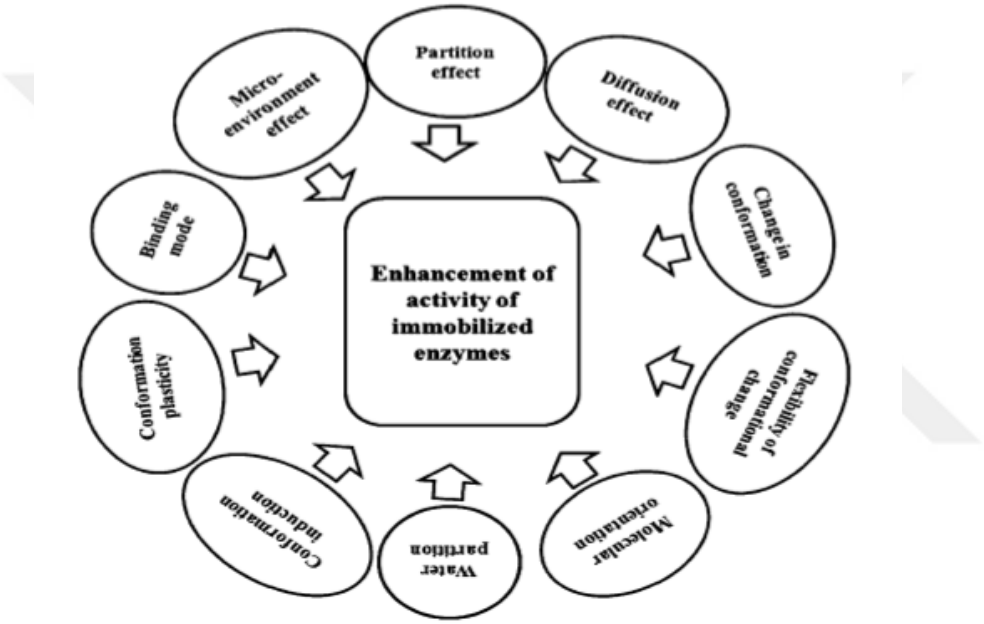

Figure 2.5 : Factors affecting enzyme immobilization and activity [22].

Cross-linkage during immobilization is achieved by the use of solvents. Stronger bonds are expected from these solvents while maintaining the structure of the enzyme. Glutaraldehyde is the most popular among these solvents, because it is soluble in aqueous solutions and stabilizes covalent bonds [20-22].

2.3.1 Support material

The support material should be selected according to the type of immobilization as indicated in the previous steps. Or, the type of immobilization is determined according to the support material. In addition, a number of variations, such as the types of

enzymes, the conditions of the process, affect the type and the result of the immobilization [23].

A wide variety of support materials can be seen in the Table 2.1 below. If it is necessary to classify these materials more generally; composites, inorganic, organic materials are observed. Harmonious selection of enzyme and support material is important to maintain the structure of the enzyme. The selected support should be able to eliminate conditions such as difficult reaction conditions and side effects. What's more for hydrophobic carriers in lipase immobilization, using a suitable material can affect increasing the activity of the biocatalyst [23,24].

Table 2.1: Iorganic/organic support material for enzymes immobilization [23].

Support Material	Binding Groups	Cross-Linking Agent	Immobilization Type	Immobilized Enzyme
Inorganic Materials				
Sol-gel silica	–OH	–	adsorption	lipase from *Aspergillus niger*
Silica gel	–OH, C=O	glutaraldehyde	covalent binding	commercial lipase
γAl_2O_3	–OH	–	adsorption	cysteine proteinases from *Solanum granuloso-leprosum*
ZrO_2	–OH	–	adsorption	α-amylase from *Bacillus subtilis*
Montmorillonite	–OH	3-aminopropyl-triethoxysilane	covalent binding	glucoamylase from *Aspergillus niger*
Hydroxyapatite	–OH	–	adsorption	glucose oxidase from *Aspergillus niger*
Bentonite	–OH, $–NH_2$	tetramethyl ammonium hydroxide	covalent binding	glucose oxidase from *Aspergillus niger*
Commercial activated carbon	–OH, C=O	–	adsorption	cellulose from *Aspergillus niger*
Activated charcoal	–OH, C=O, COOH	–	adsorption	papain
Activated charcoal	–OH, C=O, COOH	–	adsorption	amyloglucosidase
Organic materials				
polyaniline	–N–H, C=O	glutaraldehyde	covalent binding	α-amylase
polystyrene	C=O, epoxy groups	poly(glycidyl methacrylate)	covalent binding	lipase
poly(vinyl alcohol)	–OH, C=O	glutaraldehyde	covalent binding	laccase from *Trametes versicolor*
polypropylene	–OH	plasma activated	covalent binding	Glucose oxidase
Cellulose nanocrystals	–OH	–	adsorption	lipase from *Candida rugosa*
Luffa cylindrica sponges	–OH, C=O, COOH	–	adsorption	lipase from *Aspergillus niger*
chitosan	–OH, $–NH_2$	–	entrapment	lipase from *Candida rugosa*
agarose	–OH	–	entrapment	α-amylase

High thermal and chemical resistance, good mechanical properties are expected to be in the support materials. There are many inorganic support materials such as bentonite, commercial activated carbon. Considering the porosity and surface properties, silica is a blessing that is not involved in enzyme immobilization.

2.3.1.1 Rice husk ash (RHA)

Rice husks are cheap and common by product of human food processing, serves as fiber source that is considered a filler ingredient in cheap pet foods. Rice husks are a Class A insulating material. Since they are difficult to burn out and less likely to allow moisture to propagate mold or fungi. It has been discovered that when burned, rice husk produces important amounts of silica. Thus, it provides perfect thermal insulation [25].

From the milling process of rice grains, agricultural residue comes up. This residue is known as rice husk. There is approximately more than 700 million tons of rice production recently. The components that forms the rice husk are 50% cellulose, 25-30% lignin and 15-20% silica roughly. Figure 2.6 shows examples of ash. With the burning process of the mentioned rice husk, its ash occurs. Rice husk ash (RHA) has silica ash. Cellulose and lignin go away with the burning process. The temperature and the environment effect the characteristic of the RHA such as particle size.

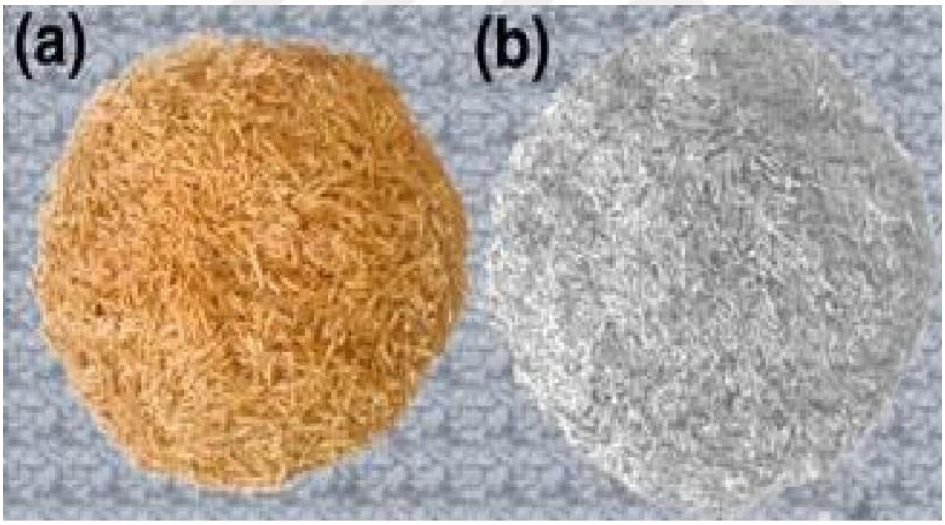

Figure 2.6 : Rice husk samples.

Reactivity of the RHA depends on its wide surface area formed by particles and additionally its silica content which is very high [26].

Furthermore, because of its high silicon substance basic silicon and some silicon compounds like silica, silicon carbide, and silicon nitride can be produced from rice husk. It also can be used as a support for lipase immobilization seen that RHA is significantly rich in silica content and demonstrates amorphous silica properties on X-ray diffraction and infrared spectrum results [27].

2.3.1.2 Organofunctional groups

During the surface functionalization processes, the use of organosilanes as cross linker reagent for the immobilization of bioprobes onto glass or silica substrates is thoroughly recorded in the literature [28,29]

The common approach is considering silanization reactions to alter the surface of the silica particles. The surface silanol groups of the silica react with a silane molecule, often a chlorosilane, which contains the chemical functionality wanted. The consequence is a silica surface containing desired functional group attached through a siloxane bond [30].

Organosilane agents are used for silanization to modify the surface. The outcome of the trifunctional silanes such as 3-aminopropyl trimethoxysilane (3-APTMS), 3-aminopropyl triethoxysilane (3-APTES), 3-trimethoxysilyl propyl ethylenediamine (TMSPDA) is a more stable and uniform layer of immobilized enzyme [29].

For some occasions it can be said that bonding and compatibility of the silane molecule is improvable by significant organofunctional groups. Certain functional silane combines conjunction with some primers. These primers by photo-polymerizing to calvarial bone had been measured as adhesion promoters so that finds its usage field in composite repair material. This indicates that silanes can upgrade the bonding strength of the bone tissue [31].

As can be seen from Figure 2.7 N-[3-(trimethoxysilyl)propylethylenediamine] has a unique chemical structure with both diamine and siloxane groups. If the structure of this silanization agent should be examined: functional end-groups, primary and secondary amino groups [32].

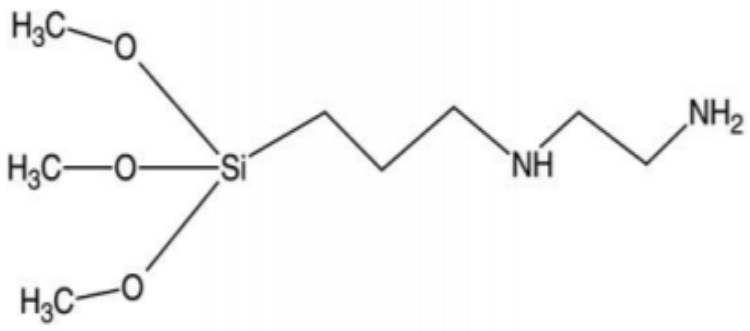

Figure 2.7: A non-functional cross-linker silane:
N-[3-(trimethoxysilyl)propyl] ethylenediamine.

Surface modification of silica gel was achieved by using TMSPDA. The diamine group enables TMSPDA to react with an amine monomer, and the siloxane group can be hydrolyzed into hydrophilic Si-OH groups in the aqueous solution. Besides, it has a high solubility in the organic phase which may further promote the interfacial polymerization [31].

3. MATERIALS AND METHOD

3.1 Materials

Free form of CALB (CALB L, Lipozym®) bought from Novozymes Co. Enzymes used as obtained. Rice husk supply is a rice production company in Edirne-Turkey. For preparing ash husks were cleared with distilled water and then these husks burned at 650 °C for 6 hours. Rice Husk Ashes (RHA) were used as a support material for lipase immobilization. For alteration of surface of the RHA by silanization, TMSPDA(3-[trimethoxysilyl)propylethylenediamine]) was used. It was bought from Merck. Riedelde Häen product Acetone (99%) was used as a solvent for TMSPDA. Glutaraldehyde (25% solution in water for synthesis) was purchased from Merck as a coupling agent for immobilization processes. Phosphate buffer solution used along the immobilization processes. These solutions prepared with monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) salts which were purchased from Carlo Erba and Merck, respectively. Olive oil that is used to measure lipase activity purchased from Komili. Ethanol (99%, C_2H_5OH) was used to terminate hydrolysis reaction throughout lipase activity measurement and also Phenolphthalein (1% in ethanol) that is used to indicate in titration during lipase activity measurement are provided from Merck.

As for PCL synthesis part, ε-caprolactone (99%, $C_6H_{10}O_2$), polymerization monomers need to preserve from water therefore these monomers were kept with sieves (3A˚). Molecular sieves were provided from Sigma Aldrich. Toluene (99%, $C_6H_5CH_3$) that had shown as the most efficient solvent for lipase-catalyzed ROP ε-CL in previous studies [27], was purchased from Merck. ROP of Chloroform (99%, $CHCl_3$) was used to terminate the reaction. he material used for this process was taken from Sigma Aldrich. For precipitation of the polymer at the end of the reaction, methanol (99%, CH_3OH) was used and it was provided from Merck. Tetrahydrofuran (THF, C_4H_8O) was used to solubilize polymer samples in GPC analysis and purchased from Carlo Erba with high purity (HPLC grade).

3.2 Methods

3.2.1 Support material

Rice husks washed with distilled water and dried in an oven for 24 h period. Oven was Electro mag M3025P. After that to create RHA dry rice husks burned in a furnace at 600 °C for 6 h. The temperature of the furnace was increased simultaneously (10 C/min) until burning temperature was reached. Finally, RHA was stored in a desiccator for further use.

Support material's surface was altered by silanization with agent TMSPDA. 250 mg RHA, 15% (v/v) 3-TMSPDA, 5 mL acetone were mixed and shaken. The conditions for this agitation was 50˚C,160 rpm and 2 hours. The shaking water bath used in experiments is shown in Figure 3.1.

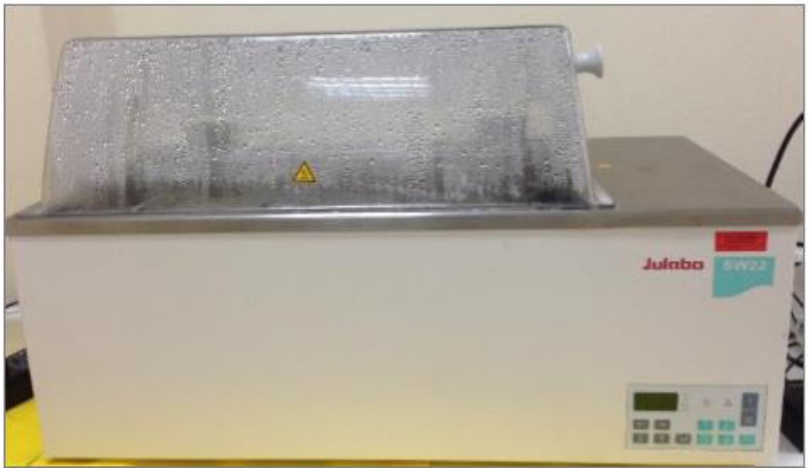

Figure 3.1: Shaking water bath.

As finalizing the reaction, surface-modified RHA washed and filtrated by using distilled water through vacuum pump to remove the unreacted compounds. After that, dried in oven at 60˚C for 2 hours. Activated RHA was mixed with 25 mL 0.2% (v/v) glutaraldehyde/phosphate buffer solution (pH 7, 0.015M) at 25°C for 2 hours on a magnetic stirrer (Heidolph MR3001). When reaching the end of the reaction time, glutaraldehyde-treated activated RHA was cleared with distilled water before vacuuming the pump. Then, directly used for immobilization process without drying.

Additionally, activated RHA was used for lipase immobilization without glutaraldehyde treatment.

3.2.2 Lipase immobilization

Lipase enzyme was immobilized on surface-modified RHA by cross-linking with a coupling agent. This coupling agent is Glutaraldehyde at the support preparation step. In crosslinking methods, prepared support materials were mixed with 500 mL free lipase (enzyme to support ratio is 2 µL/mg) and 25 mL phosphate buffer (pH 7, 0.015M) on a magnetic stirrer at room temperature for 5 hours. When the reaction time ended immobilized enzymes were filtrated by using vacuum pump. The first filter is reserved for protein determination. After that most recently acquired product were washed by solution used in previous steps and dried in oven at 30°C for 12 h.

3.2.3 UV spectrophotometer

To determine the amount of lipase enzyme that on the support the protein content of the first filtrate that obtained from the immobilization process was measured. It is conducted with UV spectrophotometric method at 280 nm. This way depends on the presence of tyrosine and tryptophan amino acids which absorb UV light strongly at 280 nm. The blank solution was phosphate buffer solution (0.015 M, pH 7.0) in which the samples were prepared. A curve was drawn before to determine the protein concentration of samples. This standard curve is given in Appendix part.

Immobilization efficiency (amount of loaded protein) was calculated from Equation 3.1, in which the term amount of protein coupled (mg/mL) is the difference between protein loaded and protein in supernatant [33].

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

3.2.4 Activity measurement of immobilized lipases

Titration method was used to measure immobilized lipase activities. The enzyme sample was arranged with a 10 mg/mL concentration in phosphate buffer (pH 7, 0.015M). As lipases are responsible from the hydrolysis of oils, enzyme activity was prompted by the olive oil's hydrolysis rate. Thus, 100 µL of 10 mg/mL enzyme solution and 500 µL olive oil were put together to a tube. 2.5 mL phosphate buffer (pH

7.2, 0.1M) was added in to the tube and mixed to neutralize the reaction medium pH. With Shaking water bath, mixture was shaked at 37˚C and 120 rpm for 30 minutes. After 30 minutes 1.25 mL acetone, 1.25 mL ethanol and 3-5 drops of phenolphthalein was added as the reaction was terminated. Consequently, mixture was titrated with 0.1M NaOH solution. NaOH spent in titration process is equal to the liberated fatty acid amount. One unit of enzyme activity was specified as 1 µmol of fatty acids liberated per minute (1U=1 µmol fatty acid/min). Specific activity(U/mg) is pointed out as the immobilized enzyme activity ratio to loaded protein amount [33].

3.2.5. Storage and operational stability

Immobilized enzyme activity was controlled every 15 days for 3 months to determine storage stability. Enzyme samples were kept approximately at 4 °C.

For operational stability, 10 mg of immobilized enzyme, 1000 µl phosphate buffer solution (0.015 M, pH 7.0) and 5000 µl olive oil was mixed and these mixtures were incubated at 37 °C and 120 rpm for 30 min. Last mixtures were centrifuged at 6000 rpm for 15 min. After centrifuge process, two different phases occurred as liquid phase and solid phase. Liquid phase was used to measure activity. Solid phase was used to repeat these operations.

3.2.6. Optimum temperature and pH

Different pH buffer solutions were prepared to determine optimum pH (pH 4.5, 5.5, 6.5, 8.5, 9.5, 10.5). 10 mg of enzyme samples were dispersed in 1 ml solutions for each various pH value. Each 100 µl of these dispersed solutions and 500 µl olive oil were mixed and these products were incubated 120 rpm and at 37 °C. This process was done for 30 minutes and after that 2.5 ml phosphate solution was put to these mixtures.

To determine optimum temperature, 30 °C, 4 °C, 45 °C, 50 °C, 55 °C and 60 °C temperatures were used.

3.2.7. PH and temperature stability

pH stability was measured with pH 4.5, 5.5, 6.5, 7.2, 8.5, 9.5, 10.5 values. The preparation of 10 mg/ml enzyme solution was performed by pH 7.0 phosphate buffer solution. 1 ml enzyme solution and 1ml buffer solution with different pH values were combined in test tubes and storaged at 37 °C for 24 h. Squence continues with 100 µl

of these solutions were mixed with 500 µl olive oil. All the other experiments were performed as rendered in the previous section.

Temperature stability measurements were continued at different temperatures (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C). At first, 10 mg/ml enzyme solution was prepared in 1ml pH 7.0 phosphate buffer solution. 500 µl of these solutions were separated as sample into test tubes and stored at the experiment temperatures for 15 minutes. After that, the samples were kept at room temperature for 5 min. Consequently, the same activity measurement procedure was performed as in pH stability experiment.

3.2.8 ROP of ε-CL by immobilized lipases

Enzyme immobilized by crosslinking was used for polymerization of ε-CL. Concentration enzyme was 20% (w/w) and 1000 mg toluene was applied for reactions. In addition, these studies were carried out under dry nitrogen. Reactions were performed with immobilized enzymes at 30˚C, 40℃, 60˚C, and 80˚C for 6, 24, 48, 72, and 120 hours and with magnetic stirrer at 120 rpm. When reaction comes to its end reaction was finished by excess chloroform addition. Necessary to separate the reaction mixture with the enzyme therefore filtration method was realized. After that, chloroform was evaporated in oven at 50˚C. Lastly polymer was precipitated by using cold methanol. Again, it was necessary to separate the polymer with methanol via filtration. Polymer was made dry at 35˚C.

3.3 Characterization Equipment

UV mini 1240 SHIMADZU spectrophotometer was used for immobilization activity measurements. Determination of protein concentrates was carried out at 280 nm phosphate buffer (pH 7, 0.015M).

Thermal characterizations were identified by TGA and DSC. SEIKO TG/DTA 6300 was TGA equipment model and SEIKO 7020 DSC was DSC equipment model. With thermal gravimetric analysis, RHA and PCL samples heated to 1000˚C degrees at a rate of 10˚C/min were analyzed. Synthesized polymer samples T_g and T_m values were analyzed between -70 and 200˚C at 10˚C/min. The degree of crystallinity was calculated according to the Formula 3.2.

$$X_c = \frac{\Delta Hm}{\Delta H°m} \, x \, 100 \qquad \textbf{(3.2)}$$

$\Delta H°_m$: melting enthalpy

ΔH_m: enthalpy value at T_m temperature

The scanning electron microscope (SEM) was used to scan the surface with a focused beam of electrons. FEI- Quanta GEF 250 equipment model was used for immobilized enzymes and PCL samples surface morphologies analysis. After the samples were covered with platinum, analyzes were performed at 5 kV.

X-ray diffraction (XRD analysis or XRPD analysis) is a unique method in determination of crystallinity of a compound. PANalytical XPER'T PRO XRD apparatus with CuK_α radiation source (λ=1.5406 Å) was used to analyze the samples. Operation range was between 15 and 30 °2 Theta (°2 Th.)

4. RESULTS AND DISCUSSIONS

4.1 Surface Modification of RHA

The surface of RHA was activated before immobilization procedure by silanization. For this purpose, 3-TMSDPA was used as the activating agent. Surfaced-modified RHA samples were characterized by TGA in order to observe the successful activation of the material. The cumulative weight loss of the TMSPDA-modified sample is calculated from the thermogram (Figure 4.1) as 11.8717 % up to 650 °C. It was reported in an earlier study that the cumulative weight loss of pure RHA up to this temperature is 1.7531 [34]. The difference between the two values, 10.1186 %, gives the amount of 3-TMSDPA that was successfully added to the surface of RHA.

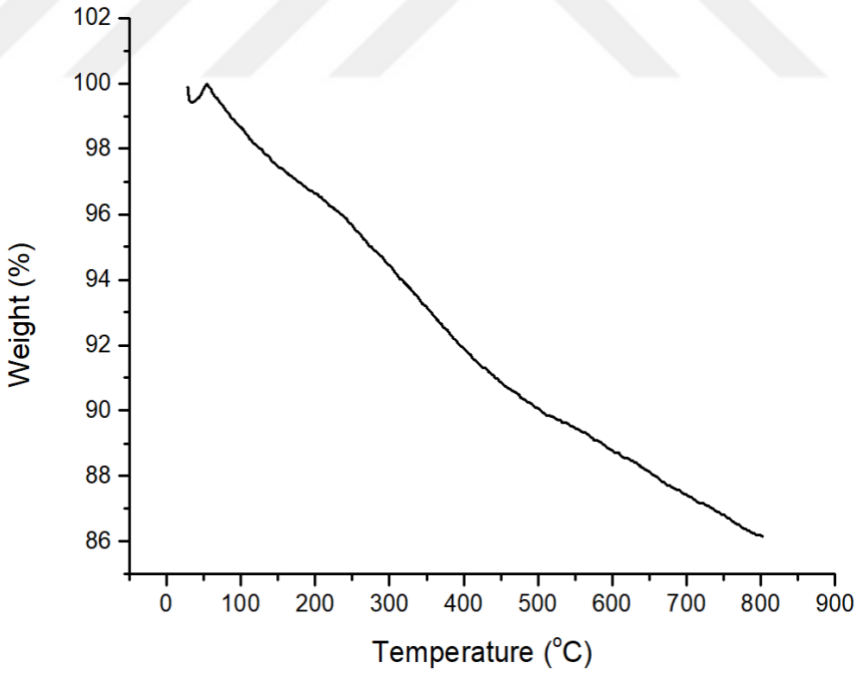

Figure 4.1: TGA analysis of TMSPDA-modified RHA.

Surface morphology of 3-TMSPDA-modified RHA was observed by SEM. The images given in Figure 4.2, reveal the porous surface structure of modified RHA. This

porous surface structure creates appropriate conditions for successful enzyme immobilization.

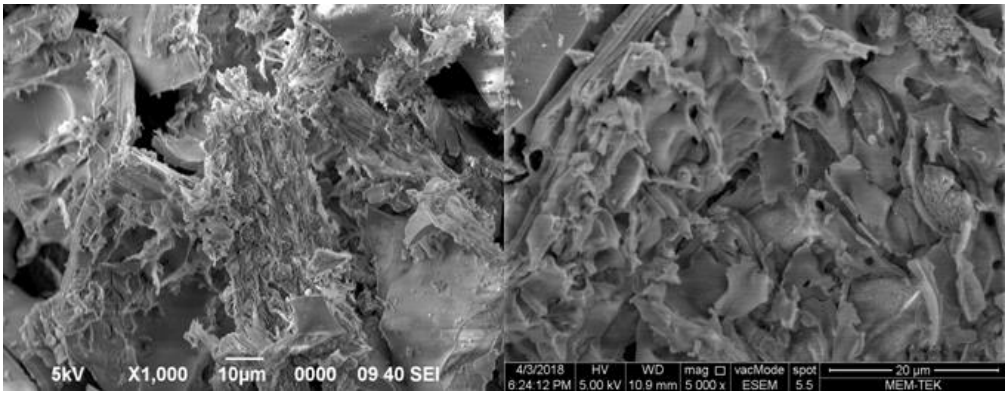

Figure 4.2: SEM images of pure RHA (left) [33] and 3-TMSPDA-modified RHA (right).

4.2 Immobilization of Lipase

Lipase enzyme was immobilized onto 3-TMSPDA-modified RHA by cross-linking. The performance of the immobilized enzyme was evaluated in terms of immobilization efficiency and catalytic activity. The results were summarized in Table 4.1 and also compared to the results that were obtained in earlier studies. Immobilization efficiency was found as high as the results given in aforementioned studies. However, loss of catalytic activity was calculated to be approximately 38 %. It is known that high activity values can be achieved by using different silanization agents.

Table 4.1: Results of immobilization experiments.

Silanization Agent	Immobilization Efficiency (%)	Relative Activity (%)	Specific Activity (U/mg)
3-GPTMS [33] (Physical adsroption)	95	80	3.7
3-APTMS [33] (Physical adsroption)	96	78	3.6
3-APTMS [35] (Cross-linking)	98	73	3.4

3-APTES [27] (Physical adsroption)	98	92	4.4
3-APTES [27] (Cross- linking)	98	79	3.7
3-TMSPDA (Cross-linking)	98	62	2.8

Agglomeration of lipase enzyme can be clearly seen in the SEM image of the immobilized CALB onto 3-TMSPDA-modified RHA. Figure 4.3, This structure proves that the lipase immobilization was successfully achieved.

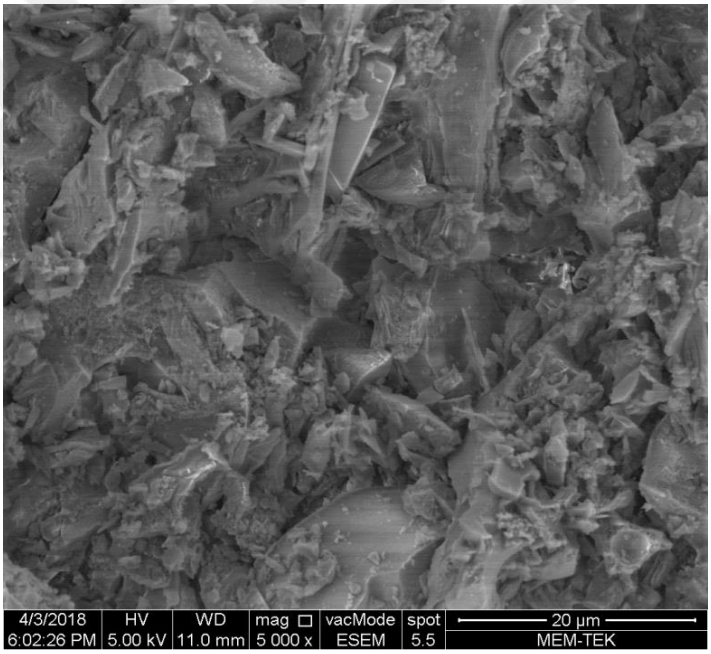

Figure 4.3: SEM image of immobilized lipase.

4.3 Storage Stability

Storage stability is a key parameter of immobilized enzymes that affects the potetial uses in industry. For this reason, storage stability of the immobilized lipase was also studied. The relative activity change in 3 month period were compared with the results in literature and are given in Figure 4.4. Although, the initial relative activity of the CALB immobilized onto 3-TMSPDA-modified RHA is lower than the values

given for other silanization agents, the activity loss of 3-TMSPDA sample over time was lower, meaning 3-TMSPDA sample was more stable. Such difference is expected since cross-linking as an immobilization methods creates chemical bonds rather than physical interactions [36].

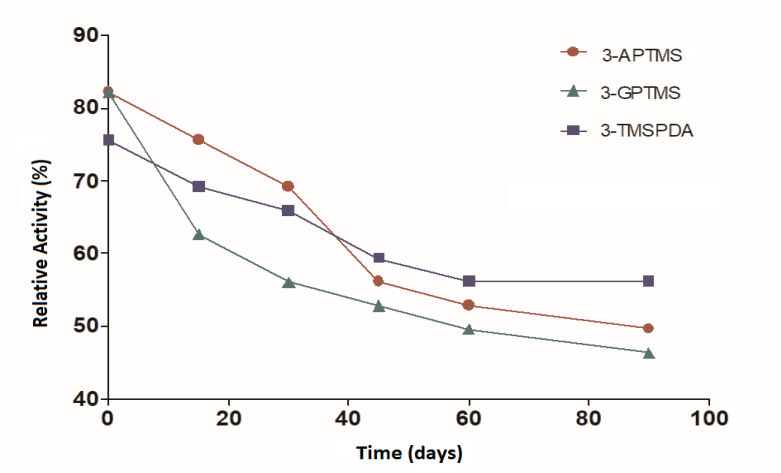

Figure 4.4: Storage stability (3-APTMS and 3-GPTMS results [33]).

4.4 Operational Stability

Reusability of the immobilized CALB samples are given in Figure 4.5. Reusability or operational stability is an important feature of enzymes for industrial applications because repeated use of enzymes can reduce the operational cost significantly. Immobilized CALB sample retained approximately 50 % of its initial activity after 8-repeated use. However, it is easily observed that the activity loss after the first cycle is very steep.

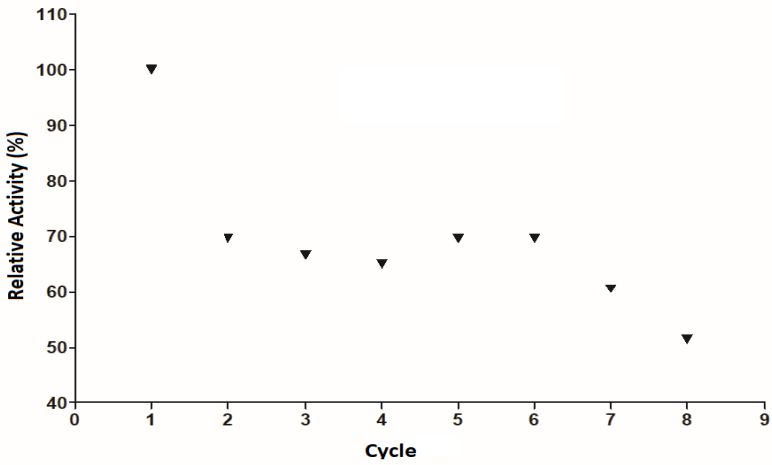

Figure 4.5: Operational stability.

4.5 The Effect of Temperature on Activity

Optimum temperature of the immobilized CALB was determined by measuring activities at different temperatures. The change of relative activities (ratio of activity of the sample to the activity of the sample with the highest value) is calculated and compared to that of free CALB and commercially available immobilized CALB, Novozyme 435®. The optimum operating temperature of 3-TMSPDA sample is found as 45 °C. It was also observed that at higher temperatures, activity of 3-TMSPDA was better than both free and immobilized forms.

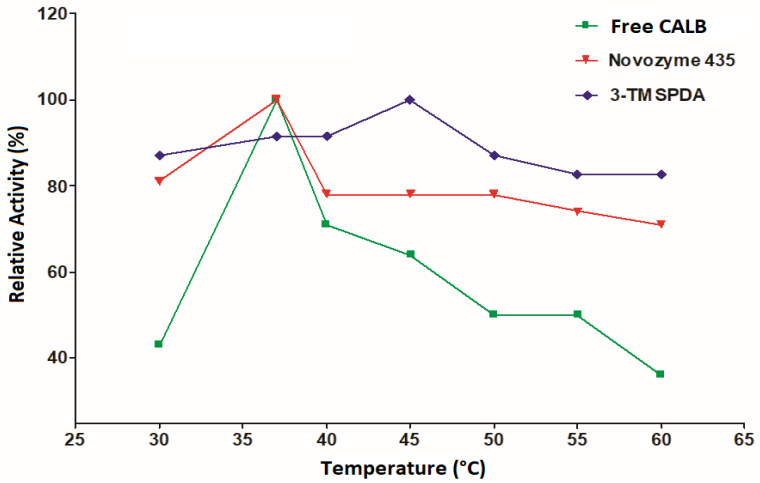

Figure 4.6: Optimum temperature.

Temperature stability curves of the 3-TMSPDA sample is given and compared with free eznyme and Novozyme 435® in Figure 4.7. Temperature stability range of 3-TMSPDA sample is narrower than the commercial samples.

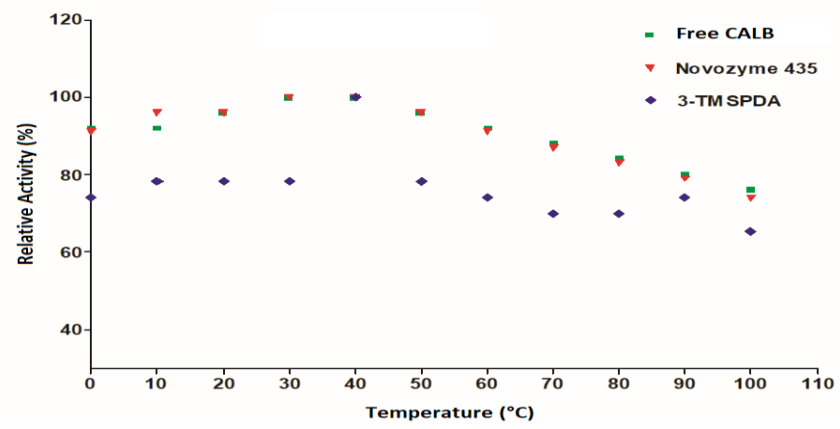

Figure 4.7: Temperature stability curves.

4.6 Effect of pH on Activity

Optimum pH of immobilized CALB were determined by measuring the relative activities at different pH with a range of 4.5-10.5. Immobilized lipases were dispersed in phosphate buffers with these pH values for the preparation of samples for activity measurements. Then the same procedure for activity measurement was applied. As can be seen in Figure 4.8, immobilized CALB showed best relative activity value at pH 7.0. It can be deduced that 3-TMSPDA sample has higher activity than free enzyme at both acidic and basic regions. In addition, 3-TMSPDA enzyme sample seems to work better at acidic medium.

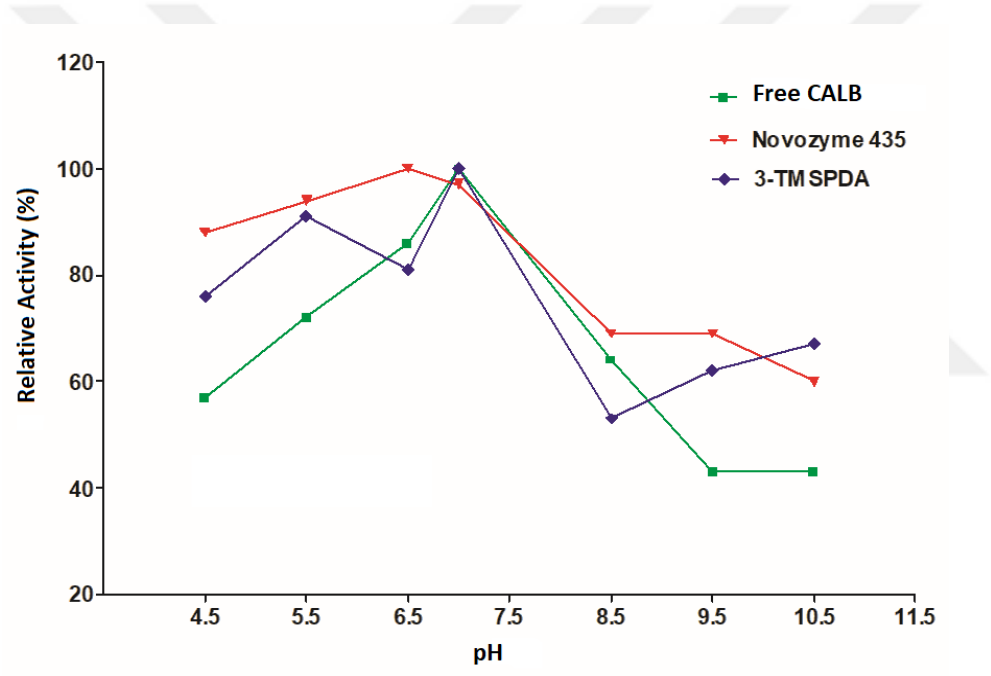

Figure 4.8: Optimum pH.

Stability curve of the immobilized CALB at a range of pH 4.5 – 10.5 is given in Figure 4.9. The change in relative activities at the same pH range were compared to those of free enzyme and Novozyme 435®. The 3-TMSPDA lipase sample was found to be stable at neutral pH range narrower than that of both free enzyme and commercial immobilized lipase, Novozyme 435®. Therefore, it can be safely stated that, activity of CALB were enhanced after immobilization method.

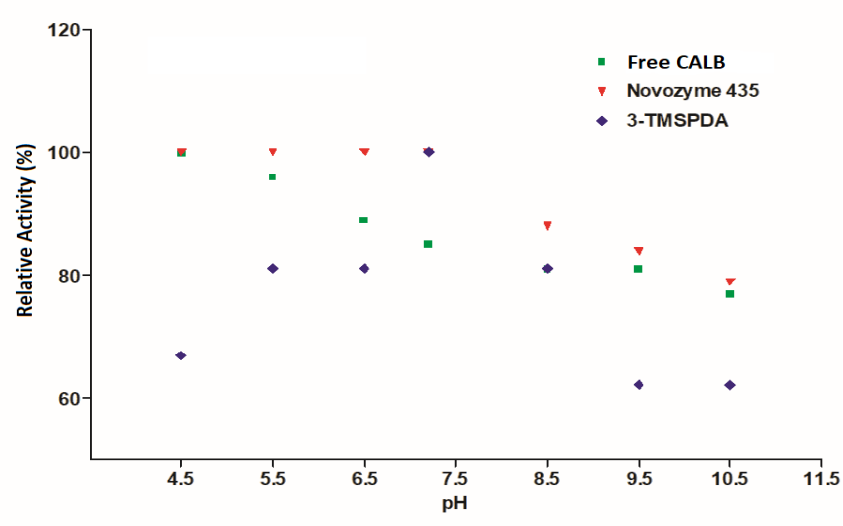

Figure 4.9: pH stability curves.

4.7 Effect of Silanization Agent, Enzyme and Glutaraldehyde on Activity

Silanization agent concentration is one of the parameters that affect the activity of the immobilized enzymes. The effect of silanization agent concentration on the catalytic activity of immobilized CALB onto 3-TMSPDA-modified RHA is given in Figure 4.10. High silanization agent concentration (20 %) obviously has an adverse effect on activity. This is thought to be caused by the interactions between excess silanization agent and the active sites of the lipase enzyme. Generally, it is not preferred to consume excess amounts of raw material due to economic reasons. For this reason, the optimum silanization agent concentration is decided to be 15 %.

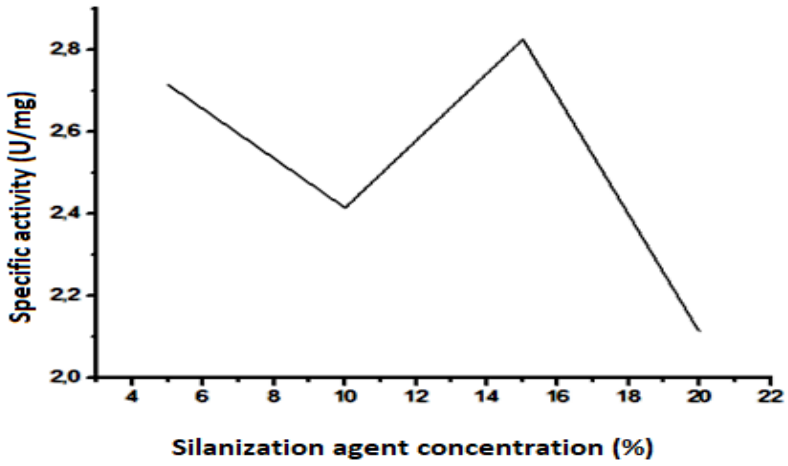

Figure 4.10: Effect of silanization agent concentration on activity.

Enzyme loading ratio is an important parameter related to the activity of immobilized enzymes. For this reason, different enzyme loading ratios were evaluated in terms of the effect on activity of immobilized CALB. The effect of enzyme loading ratio on catalytic activity of immobilized CALB onto 3-TMSPDA-modified RHA is given in Figure 4.11. As can be seen in the figure. speciifc activty of the immobilized CALB increases with with increasing enzyme loading. However, after a point, increasing enzyme loading had a negative effect on activity. The reason of such situation may be the hindrance of active sites of the enzymes due to the excess amount of enzyme and multi-layered immobilization.

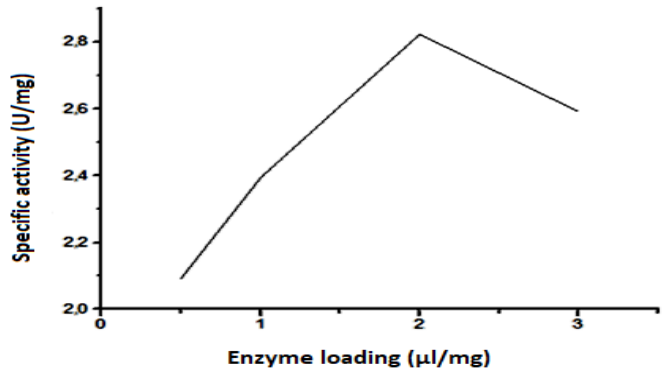

Figure 4.11: Effect of enzyme loading on activity.

Glutaraldehyde concentration is also an important parameter concerning immobilization process. High concentrations of glutaraldehyde cause enzyme agglomeration on the surface thus may lead to activity loss [37]. For this reason, low glutaraldehyde concentrations were investigated. Given in Figure 4.12, 0.2 % of glutaraldehyde concentration yields the highest specific activity value.

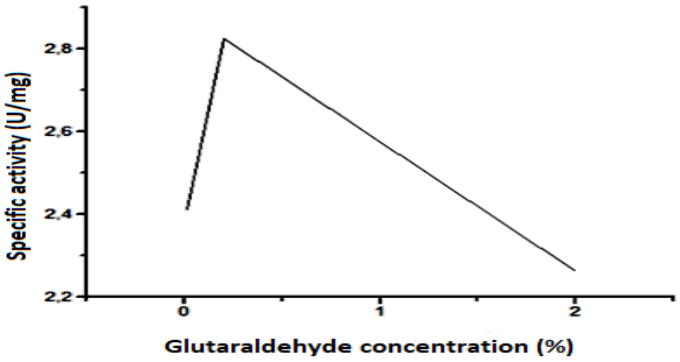

Figure 4.12: Effect of glutaraldehyde concentration on activity.

4.8 e-ROP of ε-caprolactone

Polycaprolactone (PCL) was synthesized by enzymatic ring opening polymerization (e-ROP) reaction catalyzed by CALB immobilized onto 3-TMSPDA-modified RHA by cross-linking. Polymerization reactions were carried out in the presence of toluene with these 6, 24, 48, 72, and 120 hours times and at these reaction temperatures 30, 40, 60, and 80˚C. Optimum reaction time and temperatures were determined considering molecular weight, monomer conversion and polydispersity index (PDI). The PDI values of the samples were more or less the same, changing insignificantly between the range of 1.3-1.5. This means the polymer samples were close to monodispersity. It is seen in Table 4.2 that high reaction times yield high molecular weight PCL. Similar effect can be observed in terms of monomer conversion. Optimum polymerization reaction conditions were concluded to be 72 h and 60 °C.

Table 4.2: Polymerization results.

Reaction Time (h)	Reaction Temperature (°C)	Polymerization Results		
		M_n (g/mol)	Monomer Conversion (%)	PDI
6	30	4500	14	1.23
	40	5300	15	1.24
	60	9400	70	1.29
	80	4500	24	1.17
24	30	6600	77	1.50
	40	6200	86	1.55

	60	7200	89	1.30
	80	9400	90	1.58
	30	9000	71	1.38
	40	8100	61	1.6
48				
	60	8600	61	1.51
	80	9400	69	1.57
	30	9800	81	1.58
	40	9700	78	1.59
72				
	60	12300	90	1.55
	80	13300	90	1.40
	30	9000	94	1.42
	40	11000	96	1.27
120				
	60	12000	91	1.23
	80	10300	46	1.35

Polymerization results at optimum reaction conditions were compared to the values found in literature and summarized in Table 4.3. It is seen that even though the

molecular weights were similar with the data from the literature, longer time and higher temperature was needed in order to reach high molecular weight in case of 3-TMSPDA modification.

Table 4.3*:* Comparison of polymerization results.

Silanization Agent		**Reaction Conditions**	**Molecular Weight (g/mol)**	**Monomer Conversion (%)**	**PDI**
3-GPTMS (Physical adsorption)	[38]	48 h, 40 °C	10300	85	1.47
3-APTMS (Physical adsorption)	[39]	48 h, 40 °C	12000	73	1.40
3-APTMS (Cross-linking)	[39]	24 h, 60 °C	13700	92	1.50
3-APTES (Physical adsorption)	[27]	48 h, 60 °C	14000	88	1.50
3-APTES (Cross-linking)	[27]	24 h, 40 °C	11600	84	1.30
3-TMSPDA (Cross-linking)		72 h, 60 °C	12300	90	1.55

The characterization of polymers was conducted by using samples synthesized at the optimum reaction conditions, 72 h and 60 °C. Chemical structure of polymer sample was examined by ^{1}H-NMR spectroscopy. The chemical shifts observed in Figure 4.13 were as follows: 4.1 (t, CH_2O), 2.30 (t, CH_2CO), 1.6–1.7 (m, 2xCH_2), 1.2-1.37 (m, CH_2), which are characteristic for PCL [40]. The peak observed at around 7.2 ppm belongs to deuterated chloroform which was used for preparation of samples for analysis [41].

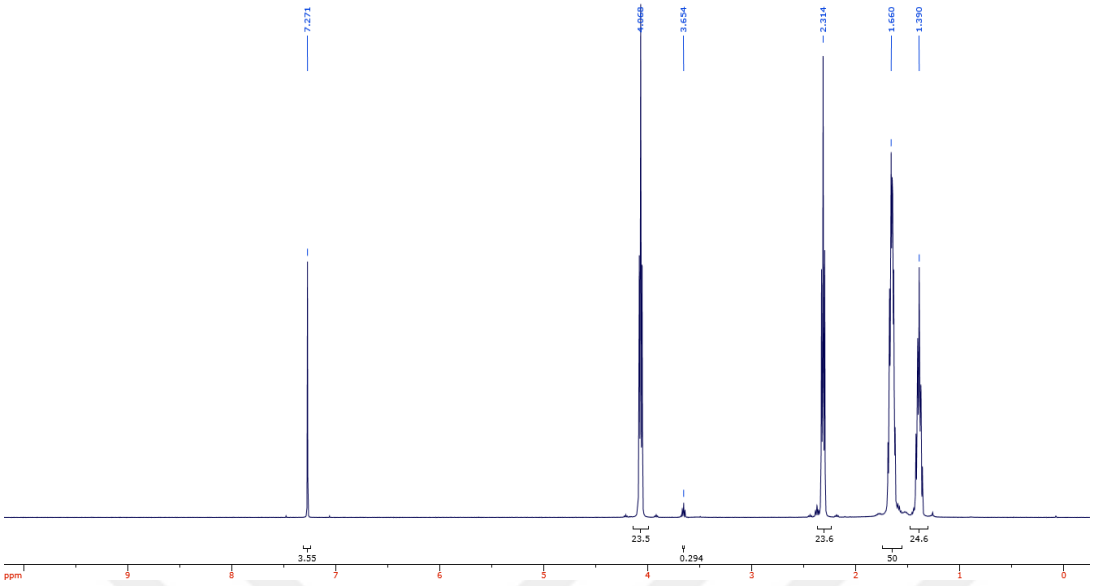

Figure 4.13: ^{1}H-NMR spectrum of PCL sample.

Polymers thermal structures was characterized by TGA and DSC analyses. TGA curve of polymer was given in Figure 4.14. TGA revealed that the polymer sample has high thermal stability. The degradation temperature (T_d) of PCL was determined to be around 400 °C.

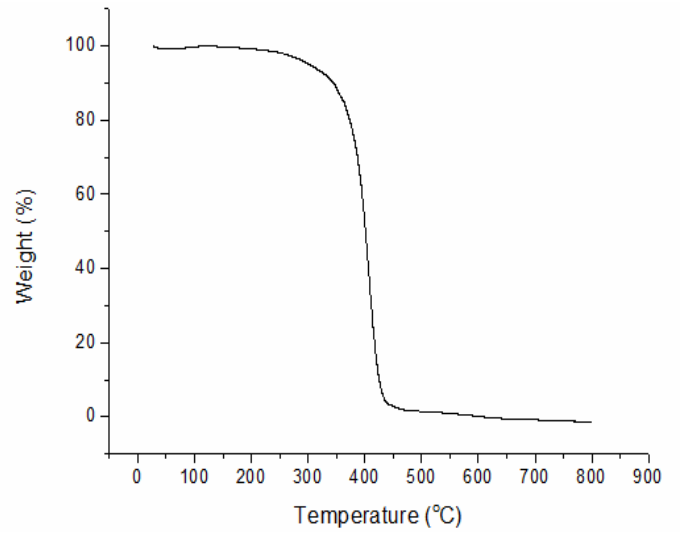

Figure 4.14: TGA curve of polymer sample.

Thermal properties of polymer sample were determined by DSC analysis. The melting temperature (T_m) was determined as 54 °C, as seen in Figure 4.15. In addition, crystallization temperature (T_c) and crystallinity degree were calculated and found to be 34 °C and 47 %, respectively. Glass transition temperature (T_g) could not be

observed since the machinery was not able to reach the temperature range in which characteristic T_g of PCL is observed [27].

Table 4.4: Comparison of Crystallinity percentage.

Silanization Agent	**Crystallinity Percentage (%)**
3-APTES [27] (Physical adsroption)	60
3-APTES [27] (Cross-linking)	67
3-TMSPDA (Cross-linking)	47

As seen in the Table 4.4 above the cristallinity of TMSPDA and the PCL that was obtained by using cross-linking method is lower than other polymers. Thus it can be used in drug oscillation systems due to its middle level crystallinity. PCLs that have high crystallinity may prevent controlled oscillation by localizing drug on its surface [42].

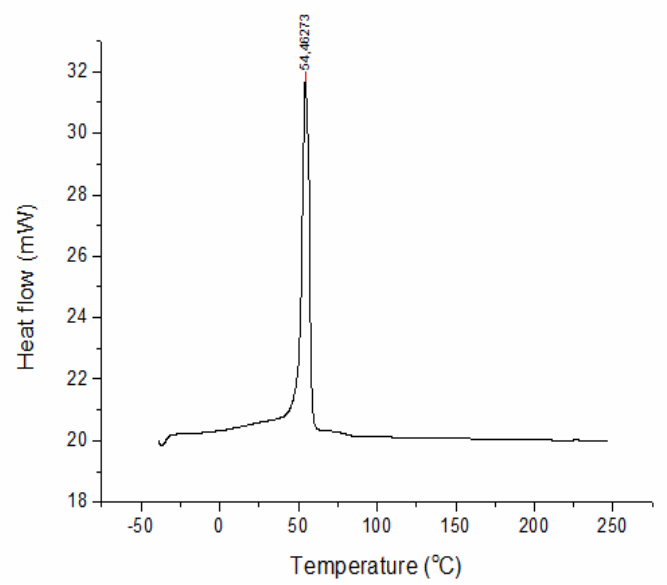

Figure 4.15: DSC thermogram of polymer sample.

XRD analysis was carried out for the determination of crystal structure of polymer sample synthesized by immobilized lipase. XRD pattern of polymer sample was given in Figure 4.16. The diffraction peaks observed at 21.33° and 23° 2 Theta angles are characteristic for PCL.

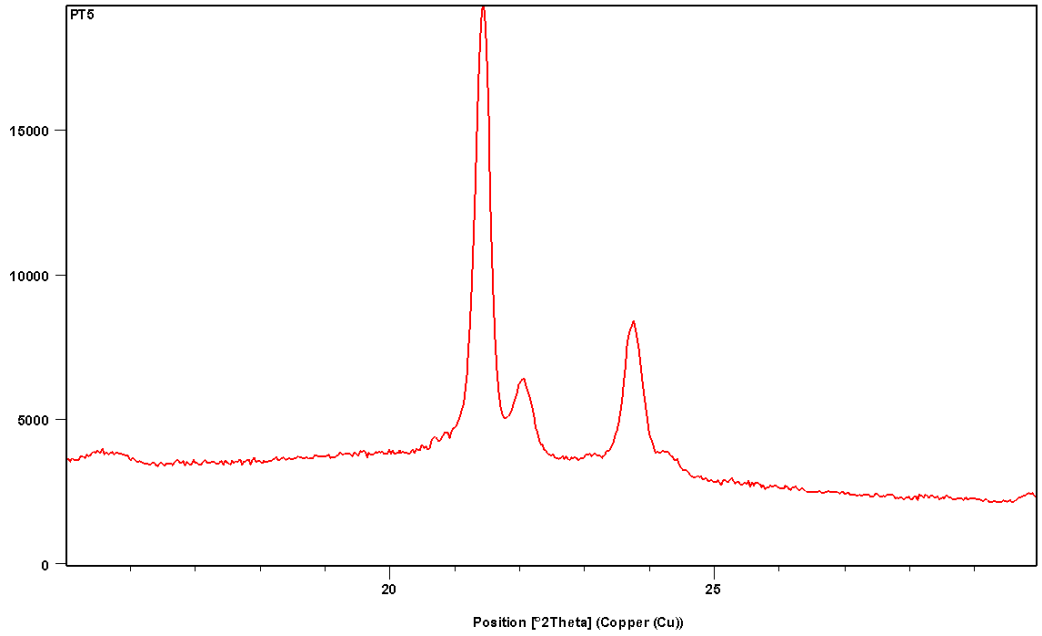

Figure 4.16: XRD pattern of polymer sample.

Surface morphology of polymer sample was monitored by SEM. The image, given in Figure 4.17, revealed a lamellar surface structure rather than a porous surface. PCL's surface is showing different morphological properties. This difference may originate from lipase catalyzed surface degradation. The material that has this surface properties can support cell binding [43].

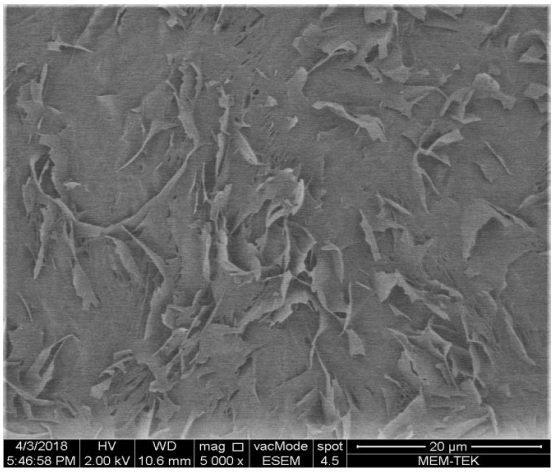

Figure 4.17: SEM image of polymer sample.

5. CONCLUSIONS AND RECOMMENDATIONS

In this thesis, immobilization of CALB onto surface-modified ash by was studied. Surface modification of RHA was achieved by using 3-TMSPDA which is a silanization agent. After surface modification, free CALB was immobilized by cross-linking during which glutaraldehyde was used as cross-linking agent. Properties of the immobilized enzymes were determined to discuss value of these novel catalysts in terms of their future use. Immobilization efficiencies of the immobilized CALB were measured by UV spectrophotometer. Activities of the immobilized enzymes were determined depending on hydrolytic effect on fat. The effects of pH and temperature on the activity of the immobilized enzymes were also investigated. Additionally, shelf life and reusability of the immobilized enzymes were determined. The effect of silanization agent concentration, enzyme loading ratio and glutaraldehyde concentration on activity of the immobilized CALB were studied. Firstly, surface-modified RHA was characterized by TGA analysis to the successful modification of RHA. TGA analysis of modified-RHA showed a weight loss of 11.8717 % up to 650 °C. The difference between this value and the weight loss of pure RHA up to the same temperature was calculated as 10.1186 %, which was attributed to the presence of 3-TMSPDA. According to these results, it was safely asserted that surface modification was successful. Surface morphologies of RHA and surface- modified RHA samples were scanned by SEM. SEM images revealed porous surface structure of surface-modified RHA which indicates the successful surface modification of RHA. Immobilization efficiencies and activities of the immobilized CALB were calculated. Free lipase was successfully immobilized onto surface-modified RHA, with efficiency of the immobilized enzyme 98 %. The activity loss of the immobilized lipase was calculated as 38 %. These values were also compared to the values given in earlier studies conducted with different silanization agents. The SEM images revealed an agglomerated structure of lipase. This structure proves that the lipase immobilization was successfully achieved.

Storage stability of the immobilized CALB was determined in order to find out the shelf life. The relative activity change in 3-month period was compared with the results

in literature. Although, the initial relative activity of the CALB immobilized onto 3-TMSPDA-modified RHA is lower than the values given for other silanization agents, the activity loss over time was lower, meaning 3-TMSPDA sample was more stable. Such difference is expected since cross-linking as an immobilization method creates chemical bonds rather than physical interactions [36]. Additionally, it was found that immobilized CALB retained approximately 50 % of its initial activity after 8-repeated use. The effects of temperature and pH on the activity of the immobilized enzyme were determined and compared to free CALB and commercially available immobilized CALB, Novozyme 435®. The optimum temperature of immobilized CALB onto 3-TMSPDA-modified RHA was found to be 45 °C. The optimum temperature was determined as 37 °C for Novozyme 435® and free lipase. At higher temperatures, activity of immobilized CALB onto 3-TMSPDA-modified RHA decreases very little, dropping to 80 % of the highest value at 45 °C. CALB immobilized on 3-TMSPDA-modified RHA was determined to be thermally stable between a narrower interval with respect to free lipase and Novozyme 435®. Optimum pH immobilized CALB was pH 7.0. CALB immobilized onto 3-TMSPDA-modified RHA showed better activity at acidic region than basic region. The 3-TMSPDA lipase was found to be stable at neutral pH range narrower than that of both free enzyme and commercial immobilized lipase, Novozyme 435®.

Silanization agent concentration, enzyme loading ratio and glutaraldehyde concentrations were investigated in terms of their effects on activity. It was decided that high silanization agent concentration had an adverse effect on activity. This is thought to be caused by the interactions between excess silanization agent and the active sites of the lipase enzyme. Generally, it is not preferred to consume excess amounts of raw material due to economic concerns. The optimum silanization agent concentration is decided to be 15 %. The studies on the effect of enzyme loading ratio on activity of immobilized CALB onto 3-TMSPDA-modified RHA showed that specific activty of the immobilized CALB increases with increasing enzyme loading. However, after reaching a specific point, increasing enzyme loading had a negative effect on activity. Such situation was attributed to the hindrance of active sites of the enzymes due to the excess amount of enzyme and multi-layered immobilization. Thus, optimum enzyme loading was decided to be 2 µl/mg. It was already known that high concentrations of glutaraldehyde cause enzyme agglomeration on the surface thus may lead to activity loss [37]. Thus, low glutaraldehyde concentrations were investigated.

The optimum glutaraldehyde concentration was found to be 0.2 % which yields the highest specific activity value.

Second part of this study, PCL synthesis, molecular weight distributions were studied based on both reaction temperature and reaction time. The resulting polymer samples were examined in GPC. In the light of this information, CR lipase catalyzed ring opening polymerization of ε-CL showed highest molecular weight with 13300 g/mol 70°C and 72h. However, in the study at a temperature of 60°C and 72 hours, 12300 g/mol PCL was obtained. Energy saving and the small weight difference of results at different temperatures were effective in selecting low temperature results. PDI was calculated by the results obtained from GPC. The PDI value of around 1.5 indicates that the polymers were monodispers. When looking at weight values and PDI of polymers obtained at other temperature, PCL can be obtained with low temperature and less reaction time. In addition, the weight of these polymers was undeniably high. At the same time, the monomer conversion rate is 90% which may indicate that the polymerization has been successful.

Characterization studies were conducted for 72h and 60°C as the optimum condition. ^{1}H-NMR spectroscopy is used for structure of polymer. Thermal characterization of the polymer sample was executed by TGA and DSC analyses. According to the TGA study, the degradation temperature of the resulting polymer was 400°C. Depending on this, PCL has high thermal stability. Looking at the results obtained from DSC, T_m was 54 °C and T_c was 34 °C. The calculated crystallinity was 47%. These results can be reported that PCL has semi-crystalline structure. In addition to that, this degree of crystallinity it is a good alternative for drug delivery systems. XRD was performed for crystal structure of polymer sample and two diffraction peaks came to exist. The PCL surface showed different morphological characteristics and was determined by SEM. Cell binding can be attained with these morphological properties [27,43].

Last of all, A successful immobilization was performed using 3-TMSPDA and RHA. PKL synthesis has also been successful with the immobilized enzyme. High molecular weights were obtained at each temperature and reaction time. The RHA used will increase its interest due to its easy availability and cheapness.

In addition to this work, the same work can be repeated with different silanizing agents such as 3-APTES, 3-GPTMS and different immobilization methods should be used to obtain better results. Apart from these, different enzyme concentrates can also be tried.

According to the result of the obtained crystallinity, this study can be developed for drug delivery systems. Crystallinity values can be referenced in composite studies.

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APPENDICES

APPENDIX A: Standard Curve

APPENDIX A

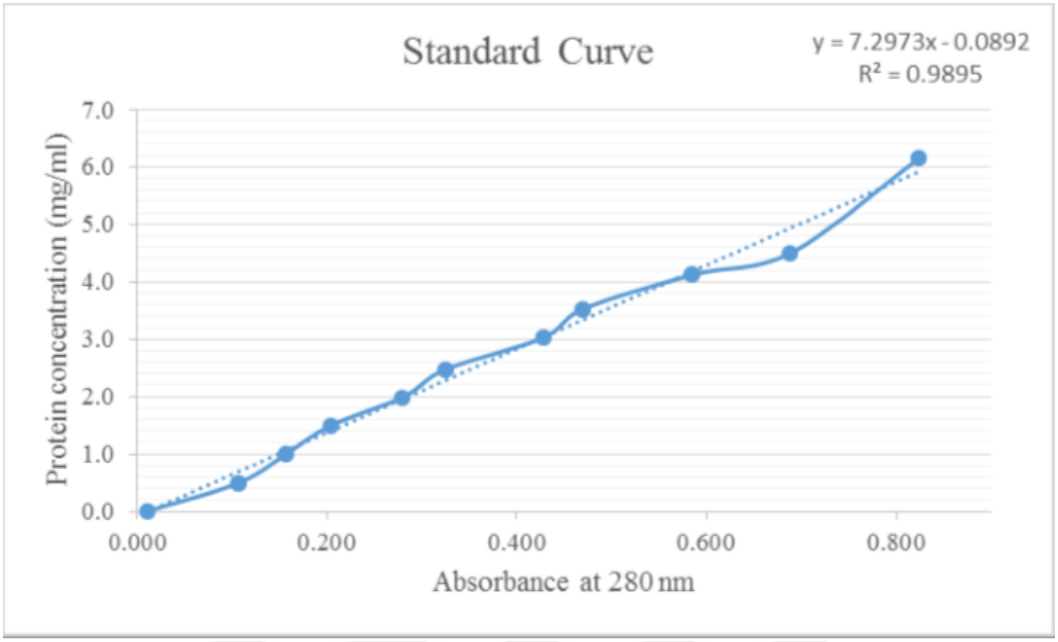

Figure A.1: UV calculation standard curve

CURRICULUM VITAE

Name Surname **:** Şeyda ERCAN KORKAN

Place and Date of Birth **:** EDIRNE / 22.05.1992

E-Mail **:** seydaaercan@gmail.com

EDUCATION **:**

- **B.Sc. :** Department of Chemical Engineering, Istanbul Technical University, 2016.

PROFESSIONAL EXPERIENCE AND REWARDS:

- **MES Engineer** NOVARTIS
  2017 September – 2018 December
- **Junior Manufacturing Systems Engineer** ESP
  2018 December-