IMMOBILIZATION OF THERMOPHILIC RECOMBINANT ESTERASE ENZYME BY MICROENCAPSULATION IN ALGINATE-CHITOSAN/$CaCl_2$ POLYELECTROLYTE BEADS

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

MASTER OF SCIENCE

in Chemistry

by
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November 2011
İZMİR

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ACKNOWLEDGEMENTS

First of all, I would like to state my special thanks to my supervisor Assist. Prof. Dr. Gülşah ŞANLI for her guidance, support, her smiling face, providing me all the opportunities, patience, understanding not only for this study but also for all other situations.

I would like to thank to Prof. Dr. Ahmet E. EROĞLU for his kind dedication of his valuable time, his everytime open door, his professional supervision and beneficial suggestions for this study. I am also thankful to Mustafa M. DEMİR for his helps and valuable comments on this study.

I would like to thank to Dr. Hüseyin ÖZGENER for providing me his technical help. I would like to thank sincerely to Ezel BOYACI and Nesrin H.POLAT for their helps and scientific supports.

Also, I would like to share my special thanks to my lab mates Seden GÜRACAR, Hüseyin İLGÜ, Melda GÜRAY, Taylan TURAN, Erhan BAL, Tülin BURHANOĞLU, Yusuf SÜRMELİ, Ayça ZEYBEK and school mates, Deniz BÖLEK, Işıl ESMER, Cenk DAĞLIOĞLU, Çağdaş GÖKTAŞ, Merve DEMİRKURT and Esen DÖNERTAŞ. Thanks for their good friendship, sincere helps and technical supports during my experiments. I also wish to express my thanks to all my other friends working in the Chemistry Department and Molecular Genetic Laboratory.

Finally, I am grateful to my parents, Mercan, Hamdi, and to my lovely sister, Yağmur, for their endless support, love and understanding throughout my thesis study as in all stages of my life. And my special thanks for Tamer ÖZKAYNAK for his endless love, limitless support and encouragement. Without these people, I was not able to finish this thesis.

ABSTRACT

IMMOBILIZATION OF THERMOPHILIC RECOMBINANT ESTERASE ENZYME BY MICROENCAPSULATION IN ALGINATE-CHITOSAN/$CaCl_2$ POLYELECTROLYTE BEADS

In recent years, enzyme immobilization has gained importance for design of artificial organs, drug delivery systems, and several biosensors. Polysaccharide based natural biopolymers used in enzyme or cell immobilization represent a major class of biomaterials which includes agarose, alginate, dextran, and chitosan. Especially, chitosan has used many biomedical applications, including tissue engineering, because of its biodegradability and biocompatibility, non-toxicity and degradation in the body.

In this research, Recombinant esterase enzyme was purified from Thermophilic *Bacillus sp.* That was isolated from Balçova (Agamemnon) Geothermal region in İzmir by using one-step affinity purification chromatography.

In the second step, purified enzyme encapsulated in alginate-chitosan/$CaCl_2$ polyelectrolyte beads that were prepared by adding dropwise a protein-containing sodium alginate mixture into a chitosan-$CaCl_2$ crosslinker solution. And then the polyelectrolyte beads were stabilized in at the same crosslinker solution 30 minutes more.

In the third step, the effect of different conditions were tested such as temperature and pH, bead diameter, reuse of beads. Also the effects of inhibition of $CaCl_2$, $ZnCl_2$, $MgCl_2$, $CuSO_4$, $MgSO_4$, Sodium dodecyl sulfate (SDS) and Triton X-100 onto the immobilized and free enzyme activity were studied.

In the last step, analysis of surface morphologies of polyelectrolyte beads were determined and examined by means of Scanning Electron Microscope.

ÖZET

TERMOFİLİK REKOMBİNANT ESTERAZ ENZİMİNİN MİKROENKAPSULASYON YÖNTEMİ İLE ALJİNAT-KİTOSAN/KALSİYUMKLORÜR POLİELEKTROLİT BONCUKLAR İÇERİSİNDE HAREKETSİZLEŞTİRİLMESİ

Son yıllarda enzim immobilizasyonu protez organların, ilaç salınım sistemlerinin ve çeşitli biyosensörlerin dizaynı açısından büyük önem kazanmıştır. Enzim ya da hücre immobilizasyonunda kullanılan polisakkarit yapılı doğal polimerler agaroz, aljinat, dekstran ve kitosanın da içerisine dahil olduğu biyomateryallerin temel bir sınıfını oluştururlar. Özellikle kitosan biyolojik olarak uyumlu, zehirsiz ve vücut içerisinde paraçalanabilmesinden dolayı, doku mühendisliğinin de içerisine dahil olduğu pek çok biyomedikal uygulama alanına sahiptir. Bu araştırma sırasında, rekombinant esteraz enzimi tek basamaklı afinite kromatografisi kullanılarak, İzmir Balçova jeotermal tesislerinden izole edilen Termofilik *Basilus sp.* türünden izole edilmiştir.

İkinci basamak da saflaştırılmış enzim, proteinimizi ihtiva eden sodyumaljinat karışımının kitosan/kalsiyumklorür çözeltisi içerisine damlatılması yolu ile hazırlanmış aljinat-kitosan/kalsiyum klorür polielektrolit boncukları içerisine hapsedilmiştir. Ve daha sonra bu polielektrolit küreler aynı bağlayıcı çözelti içerisinde 30 dakika daha bekletilmek sureti ile daha dayanıklı hale getirilmiştir.

Üçüncü aşamada karakterizasyon çalışmaları gerçekleştirilmiştir. Sıcaklık ve pH'ın immobilize esteraz enzimi üzerine etkisi incelendi. Ve boncuk çapı ve boncukların yeniden kullanılması ile ilgili deneyler gerçekleştirildi. Ayrıca kalsiyum klorür, çinko klorür, magnezyum klorür, bakır sülfat, sodyum dodesil sülfat ve triton gibi kimyasalların immobilize ve serbest enzim üzerine inhibisyon etkileri incelendi. Son basamak da ise polielektrolit boncukların yüzey morfolojileri elektron mikroskobu ile belirlenip incelendi.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

PRE-INTRODUCTION

1.1. Overview

Biocatalysis has emerged as an important tool in the industrial and biotechnological synthesis of bulk chemicals, pharmaceutical and agrochemical intermediates, active pharmaceuticals, and food ingredients. Biocatalysts have excellent properties such as stability and selectivity. Despite of these properties, they also have some disadvantages. For example, generally many enzymes are soluble in reaction media so it is difficult to recover them from the reaction effluents. As a result of this problem, some properties of enyzmes should be improved before their implementation in industry in order to reduce the cost of the chemical process. The operational stability of enzymes applied in chemical processes has been improved over the years through the use of genetic engineering, immobilization or process alterations.

Enzyme immobilization method is the most efficient and suitable way to impart the desirable features of conventional biocatalysts.

1.2. Aim of the Study

Proton conducting biopolymers have potential use for enzyme immobilization. The cost effective, non-toxic and environmentally safe biopolymers such as chitosan and alginate have a great importance in development of new immobilization matrixes.

In our study, alginate-chitosan/$CaCl_2$ polyelectrolyte beads were prepared in order to develop a biocompatible matrix for enzyme immobilization where the protein is retained in a solid core and the bead allows permeability control over substrates and products.

Esterase from *E.coli* was microencapsulated by drop-wise addition of an aqueous mixture of sodium alginate and the biocatalyst to a hardening (crosslinker) solution of chitosan and $CaCl_2$. Then, firstly the catalytic activity and the stability of immobilized esterase was examined at different conditions. After this step, operational

stability of polyelectrolyte beads was tested. The effect of different beads and different metal ions on relative activity of immobilized enzyme were measured. Lastly, the surfaces of the polyelectrolyte beads were studied with Scanning Electron Microscope.

CHAPTER 2

INTRODUCTION

2.1. Enzymes

During the last three decades, enzymology and enzyme technology have make progressed considerably. In fact, the enzyme molecules are known to have existed over a century. Many applications such as the production of some foods and beverages, leather and polished plates or polishing clothes in ancient times, although even at that time it is unknown, are important applications of enzymes. In France, Anselme Payen and Jean - François Persoz in 1833 announced the isolation of barley sprouts amylolytic components. Shortly after that the Swedish chemist Jons Jacob Berzelius in 1835, defined the components those accelerating chemical reactions as catalyst. In Germany, physiologist Theodor Schwann in 1836 has defined the digestive enzyme pepsin. In 1877 Wilhelm Kühne has proposed the use of the term of enzyme. In 1897 Hans and Eduard Buchner showed that the conversion of glucose into ethanol in the extract of the yeast cell is executed by chemical catalysts (enzymes). In the 1870's the Danish chemist Christian Hansen achieved to obtain the cheese yield in pure form which results construction of cheese and improves the quality and quantity of the cheese (Polaina and MacCabe 2007). The enzyme studies which began in 19th century have accelerated in 20th century and the first enzyme in ure form was obtained in Cornell University successfully. Isolation and crystallization of the urease enzyme from a male rabbit has succeeded by Sumner (Bilen 2009).

Enzymes are biomacromolecules or in other words complex protein molecules with specific catalytic functions that are produced by all living cells to catalyse the biochemical reactions required for life. Enzymes have some excellent properties (high catalytic activity, selectivity, and specificity). Thanks to these behaviours, compared with inorganic catalysts, enzymes do not require the extremes of temperature and pressure. Because of their enormous catalytic power in aqueous solution at normal temperatures and pressures, enzymes are of great commercial and industrial importance.

Enzymes speed up bioreactions by lowering the activation energy barrier without being consumed during a chemical reaction.

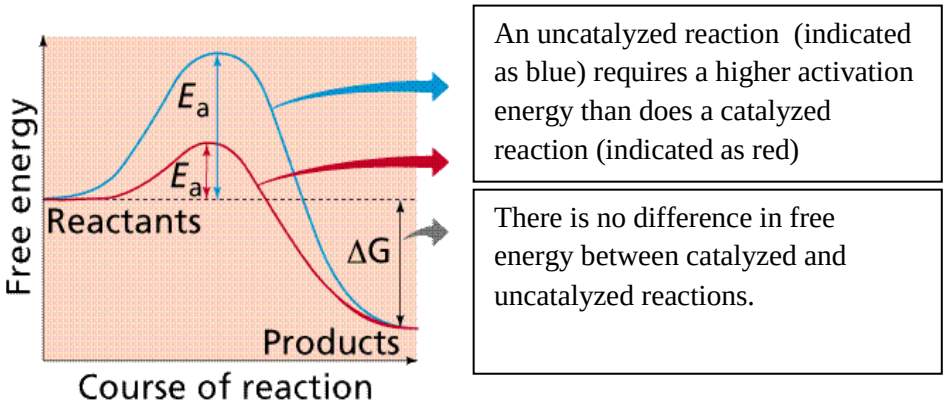

Figure 2.1. Reaction coordinate diagram for a chemical reaction.

Unlike chemical catalysts that display only limited selectivity most enzymes are specific. The active site of an enzyme typically consists of 3-12 amino acid residues organised into a precise three-dimensional arrangement. The active site is an enzyme's catalytic center and this site show strong affinity for the substrate that shown in Figure 2.2. because the chemical nature of these amino acid residues and their three-dimensional arrangement form a region that complements certain groupings on the substrate molecule. The enzyme must bind its specific substrate in the correct orientation otherwise there would be no reaction. When a substrate or substrates binds to an enzyme, the enzyme catalyzes the conversion of the substrate to the product (Stryer et al. 2005).

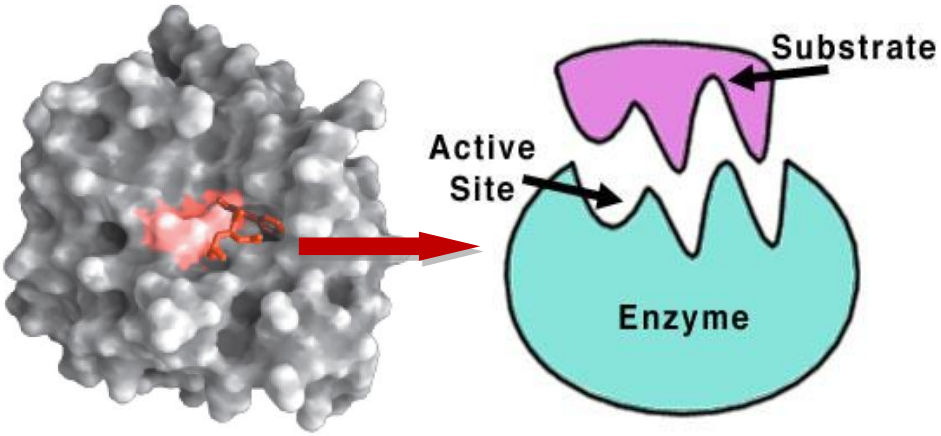

Figure 2.2. Binding of a substrate to an enzyme on the active site.

Active site is an enzyme's catalytic center and typically a three-dimensional pocket or groove on the surface of the enzyme into which the substrate fits.

Enzymes can be classified to six major classes according to the chemical reactions they catalyzed.

Table 2.1. Classifications of Enzymes
(Source: Kara 2006)

Class	Name of Enzyme	Type of chemical rection that enzyme catalyzes
1	Oxidoreductases	Oxidation / reduction reactions
2	Transferases	Group or atom transfer between two molecules
3	Hydrolases	Hydrolysis reactions
4	Lyases	Seperation of a group from substrate by any way except hydrolysis
5	Isomerases	Isomerisation reactions
6	Ligases	Synthesis of a new bond by demolition of ATP and other nucleoside tri phospat

Enzymes are found in small amounts at biological systems. As a result, the amount of activity shown by biological systems, rather than the amount of protein is measured.

One unit of enzyme: The amount of the enzyme which converts 1 micromolar (μmol) substrate to the product is considered as a unit.

Factors affecting the rate of reactions catalyzed by enzymes can be listed as follows;

- pH of the media.
- Temperature.
- Concentration of enzyme.
- Concentration of substrate.
- Time.
- Product of the reaction.
- Chracteristics and concentration of ions.
- Effect of light and other physical factors.

In order to measure the activity of enzyme, it is necessary to measure the amount of substrate lost or the amount of product formed per unit time.

Advantages of the enzymes for use in industry:

- They are of natural origin and nontoxic.
- They have great specificity of action.
- They work best under mild conditions of moderate temperature and near neutral pH.
- They have rapidly at relatively low concentrations and the rate of reaction can be readily controlled by adjusting temperature, pH and amount of enzyme employed.
- They are easily inactivated when reaction has gone as far as desired.
- They enable higher product quality, lower manufacturing cost, and less waste.

Enzymes are currently used in the following areas, fermenting of wine, the paper industry, starch industry, leather industry, baking industry and beer brewing industry, washing detergent industry, toxic wastes removal, diagnostic industry and production of pharmaceuticals.

Table 2.2. Enzymes in industry
(Source: Brady and Jordaan 2009)

Laundry Detergents	Proteinase (91%)	Used in pre-soaks to remove protein-based stains
	Lipase (6%)	Now commonly included todigest oils and fats
	Amylase (2%)	Removes resistant starch residues
	Cellulase (1%)	Digests the cotton 'fuzz' which acumulates with excessive washing
Starch Industry	Amylases, amyloglucosiadases and glucoamylases	Converts starch to glucose and other sugar syrups
	Glucose Isomerase	Converts glucose syrups into fructose syrups
Dairy Industry	Rennin from the stomachs of young ruminant animals	Manufacture of cheese
	Lipases	Enhances ripening of blue-mold cheese
	Lactases	Break down lactose to glucose and galactose

(cont. on next page)

Table 2.2 (cont.)

Textile Industry	Amylase	Now widely usd to remove starch from woven fabrics. Starch is used as an adhesive (or size) on the threads of many fabrics to prevent damage during weaving. Traditionally, chemicals were favoured but now bacterial amylases are commonly used.
Brewing Industry	Amylases, glucanases, proteinases	Splits polysaccharides and proteins in the malt.
	Proteinases	Reduces clouding of beers
	Amyloglucosidase	Low Calorie beer production
	β-glucanese	Improves filtration characteristics
Baking Industry	α-amylase	Catalyses the breakdown of starch in flours. Used in the manufaccture of bread.
	β-xylanase	Improves the characteristics and rising of bread
	Proteinases	Reduces the protein in flour. Used in biscuits manufacture
Leather Industry	Proteinase (trypsin)	The process known as 'bating' treats the leather with proteinases to make it more pliable. Trypsin isolated from both slaughterhouses and micro-organisms replaces the old method of using dog and pigeon faeces.
Pulp and Paper Industry	β-xylanases	Emerging technology for enhancing pulp-bleaching
	Lipases	Reduces 'pitch' which causes paper to stick to rollers and tear.

Table 2.3. Advantages and disadvantages of enzymes as biocatalysts in comparison with chemical catalysts.

ADVANTAGES	DISADVANTAGES
Stereo-and regioselective	Unstable at high temperatures
Low temperatures required	Unstable at extreme pH values
Low energy consumption	Unstable in aggressive solvents
Active at Ph 2-12	Inhibited by some metal ions
Non-toxic when correctly used	Very expensive
Can be reused	Require expensive cosubstrates
Can be biologically degraded	
Can be produced in unlimited quantities	

2.2. Why Recombinant Enyzme?

Today, enzymes are used in the industrial field generally is obtained from micro-organisms. However, very little portion is provided as a part of the vegetable and animal origin. Reasons for the choice of microorganisms as a source of enzyme, formation of byproduct is less, activity is high, is more economical, to have stability and producability at high levels of purity (Gümüşel 2002 and Wiseman 1987).

Recent developments in microbial genetics have created a new potential for enzyme production. The enzyme industry is expected to expand as genetic engineering (recombinant DNA technology) is applied to the microbial production of enzymes. The techniques of genetic engineering can be used to manipulate DNA such that multiple copies of a particular gene encoding an enzyme of commercial value can be made. Via this technique large amounts of the desired protein that resistant to hard conditions can be produced by using recombinant microorganisms.

2.3. Enzyme Stability and Immobilization

2.3.1. Importance of Enzyme Stability

Enzyme instability is an important factor that prevents their wider use in industry. Enzymes may be exposed to unnatural non physiological environments. Chemicals like organic solvents, elevated temperatures and pH values outside their normal in vivo values can denature the enzyme with consequent loss of activity. In such a case, thermophilic enzymes and enzyme immobilization draw attention to increase enzyme durability. Thermophilic enzymes are more stable under hard reaction conditions such as high temperatures and pressures when compared with other enzymes. So they have great importance in industrial development (Kumar and Nussinov 2001, Sterner and Liebl 2001).

2.4. Thermophiles

Microorganisms can be grouped into broad categories, according to their temperature ranges for growth. The temperature in many natural environments changes

drastically over the seasons and microorganisms are easily adapted to these changes. Every organism has an optimium temperature for growth. According to their optimium growing conditions microorganisms separate four main goups such as Psychrophiles, Mesophiles, and Thermophiles, Hyperthermophiles (Gomes and Steiner 2004). (The word of Phile has the meaning of "love" and the word of extreme has the meaning of 'in excess') Psychrophiles (cold loving) can grow at 0°C and some even as low as -10°C; their upper limit is often about 25°C.

- Mesophiles grow in the moderate temperature range, from about 20°C (or lower) to 45°C.
- Thermophiles are heat-loving, with an optimum growth temperature of 50°C or more, a maximum of up to 70°C or more, and a minimum of about 20°C.
- Hyperthermophiles have an optimum temperature above 75°C and thus can grow at the highest temperatures tolerated by an organism (Baker et al. 2001).

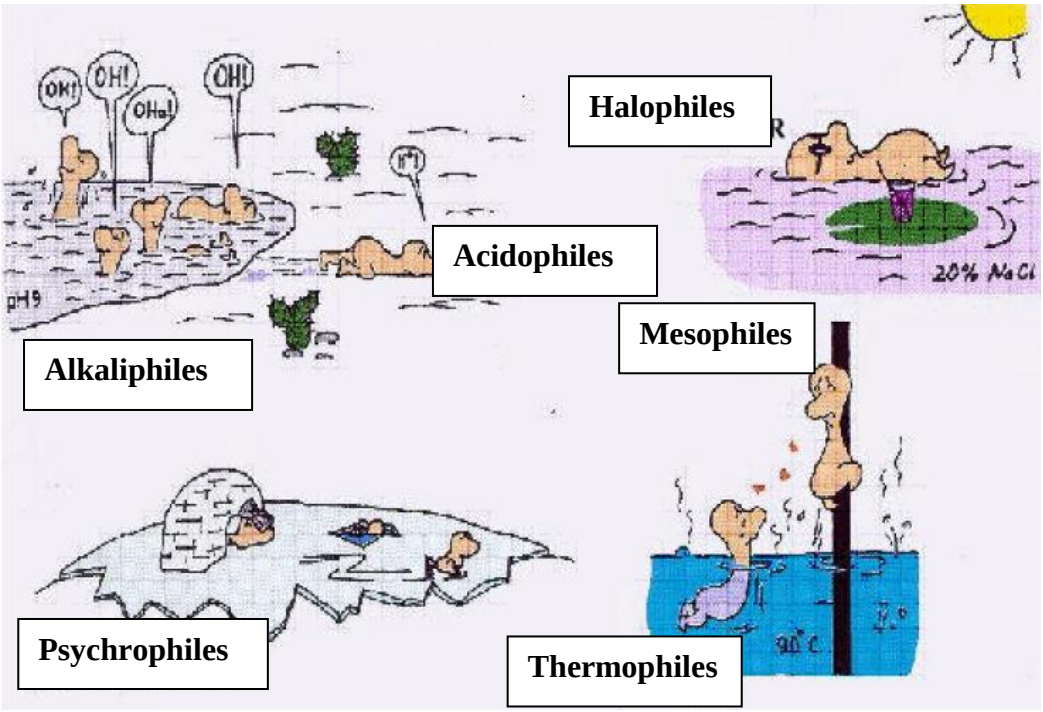

Figure 2.3. Classification of extreme thermophiles according to environments (Source: Baker et al. 2001).

The most suitable habitats for thermophilic organisms are geothermally and volcanically heated hydrothermal systems such as solfataric fields, neutral hot springs and submarine saline hot vents (Horikoshi 1998).

Thermophilic microorganisms have some special characteristics when compared with mesophiles. They have several advantages as high reproductive rates, easily winning the final product, high process stability and yielding, they can directly ferment natural polymers such as starch, cellulose. Thermophiles are found in various geothermally heated regions of the earth. Also thermophiles can be subclassified as moderately thermophiles (grow at 50-60°C) and extreme thermophiles (grow at 60-80°C). Cellular components of thermophilic organisms (enzymes, proteins and nucleic acids) are also thermostable. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications (Kumar and Nussinov 2001).

2.4.1. Thermophilic Bacillus

Bacillus is an aerobic or facultatively anaerobic, gram positive, rod-shaped bacteria that differentiate in to heat-resistant endospores under aerobic conditions are placed in the genus *Bacillus* (Rainey and Oren 2006). Many kinds of species which have thermophilic, psychrophilic, acidophilic, alkalophilic and halophilic properties are included in the genus *Bacillus* (Nazina et al. 2001). Thermophilic bacteria belonging to genus *Bacillus* show optimal growth temperatures in the range of 45-70ºC (Maugeri et al. 2001, Rainey et al. 1993). The importance of thermophilic *Bacillus* increased because of their biotechnological importance as sources of thermostable enzymes (proteases, amylases, pullunases, glucose isomerases, lipases, xylanases, cellulases and DNA restriction endonucleases) (Maugeri et al. 2001). In our experiment thermophilic *Bacillus* are isolated from Balçova (Agamemnon) Geothermal region.

2.4.2. Thermophilic Enzymes

Thermostable enzymes, which have been isolated mainly from thermophilic organisms and these enzymes which are thermostable, resist denaturation and proteolysis (Kumar and Nussinov 2001). It has been demonstrated in studies that thermophilic microorganisms make the cells durable in order to survive at high temperature environments, and have high ratio of saturated fatty acids in the cell membranes. And fatty acids created a hydrophobic environment for the cell (Herbert and Sharp 1992). It has been determined that thermophiles with disulfide bonds and

hydrophobic interactions have become resistant to different temperature values. Thermostable proteins contain many charged residues and hydrophobic residues (Fujiwara 2002). In addition, it has been defined that their DNA's contain revese gyrase that make up positive super-coils. That structure raises the melting point of DNA and therefore makes the microorganisms more resistant to high temperatures (Robb et al. 2007). Also additional intermolecular interactions such as hydrophobic interactions, disulfide bonds, electrostatic interactions metal binding and hydrogen bonds that are not exist in mesophilic enzymes get thermophilic enzymes more stable (Steel and Walker 1991).

Table 2.4. Main advantages of thermostable enzymes
(Source: Haki and Rakshit 2003)

Property	Advantages
Thermostability	The half life of the enzymes increases. The purification of the enzymes is easier.
Resistance against various chemical agents	They can tolerate hard conditions including important amounts of organic solvents, diverse pH level frequently neccessaries during industrial process.
High optimal temperature	Low activity at room temperature. It does not require active cooling in fermentation. High diffusion rates of substrates and products
Solubility	At high temperatures the concentrations of substrates can be increased, with the exception of gases.
Viscosity	Decreases. Mixing and pumping can be also increased.
Microbial contamination	The probability of contamination decreases as the temperature rises. Contaminant enzymes are inactivated at high temperature.

The ability of thermophilic enzymes to work at high temperatures implies many advantages for their applications in industrial reactors or fermenters.

However, despite the many economically important advantages of thermophilic enzymes, there are also disadvantages for specific applications.

Table 2.5. Main problems of the application of thermophilic enzymes in industry.

Property	Main Problem Observed
Thermal sensivity	There are many substrates, products or enzyme cofactors unstable at high temperature
Solubility of gases	Decrease. The diffusion of gases limits some reactions.
Enzyme stability	The inactivation of the enzyme results extremely difficult.
Equipment Stress	All the materials are damages in a short time, unless especially designed

2.4.2.1. Applications of Enzymes from Thermophiles

As shown in Table 2.6 there are many applications of enzymes from thermophiles. For each application the temperature range and microorganism type were indicated.

Table 2.6. Main applications of thermostable enzymes at present.

Enzyme	T(◦C)*	Application	Origin**
Enz. Acting on Carbohydrates			
α-amylase	60-90	Starch hydrolysis	*Bacillus licheniformis*
Pullulanase	50-60	Starch hydrolysis	*Klebsiella aerogenes*
Xylose isomerase	50-55	Sweetening of corn syrups	*Actinoplanes missouriensis*
Cellulase	55-65	Hydrolysis of cellulose, Ethanol production, paper bleaching	*Clostridium thermocellum*
Proteases			
Neutral protease	40-80	Food prcessing	*Bacillus stearothermophilus*
Alkaline protease	40-80	Detergents	*Bacillus licheniformis*
Molecular Biology			
Taq polymerase	45-95	DNA amplification (PCR)	*Thermus spp.*
Vent DNA polymerase	50-98	DNA amplification (PCR)	*Thermococcus litoralis*
Pfu DNA polymerase	50-98	DNA amplification (PCR)	*Pyrococcus furiosus*
Tth polymerase	45-95	Reverse transcription of RNA	*Thermus thermophilus HB8*

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Table 2.6 (cont.)

RNA polymerase	65-75	RNA synthesis	*Thermus spp.*
Restriction Enzymes	65-75	DNA specific digestion	*Thermus spp. Bacillus sulfolobus*
Aneaerobic treatment of residual waters	50-60	Organic compounds elimination	*Methanogenic bacteria Metanobacterium Metanosarcina*
*Range temperature at which the enzyme is used **Microorganisms from which the enzym has been obtained			

2.5. Esterases

Esterases are hydrolases that catalyse the cleavage of ester linkages by the addition of a water molecule (Kontkanen 2006, Carey and Sundberg 2007). Substrate specificity and interfacial activation distinguish esterases from lipases (Panda and Gowrishankar 2005). Lipases are esterases that preferentially catalyze hydrolysis of water-insoluble substrates such as long-chain triglycerides at the interface between the substrate and water. By contrast, typical esterases such as carboxylesterase are restricted to water-soluble esters of short chain carboxylic acids. Esterases cannot be classified solely based on their substrate specificities; analyses of their sequence and structure are also needed. Esterases split esters in to an acid and an alcohol in a chemical reaction with addition a water molecule (Kontkanen 2006).

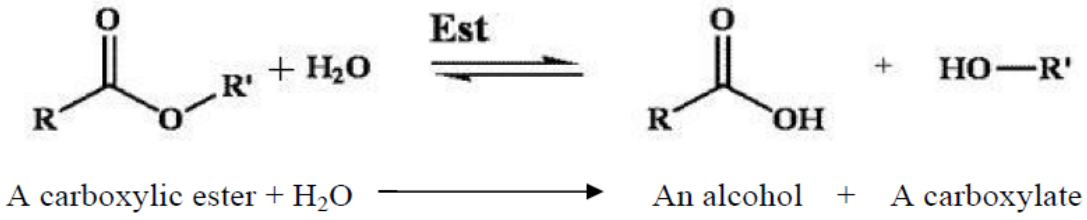

Figure 2.4. Hydrolase reaction of a typical esterase enzyme
(Source: Carey and Sundberg 2007)

2.5.1. The Chemical Reactions of Esterases

There are some different reactions catalysed by lipases and esterases. Those reactions are listed in Figure 2.5.

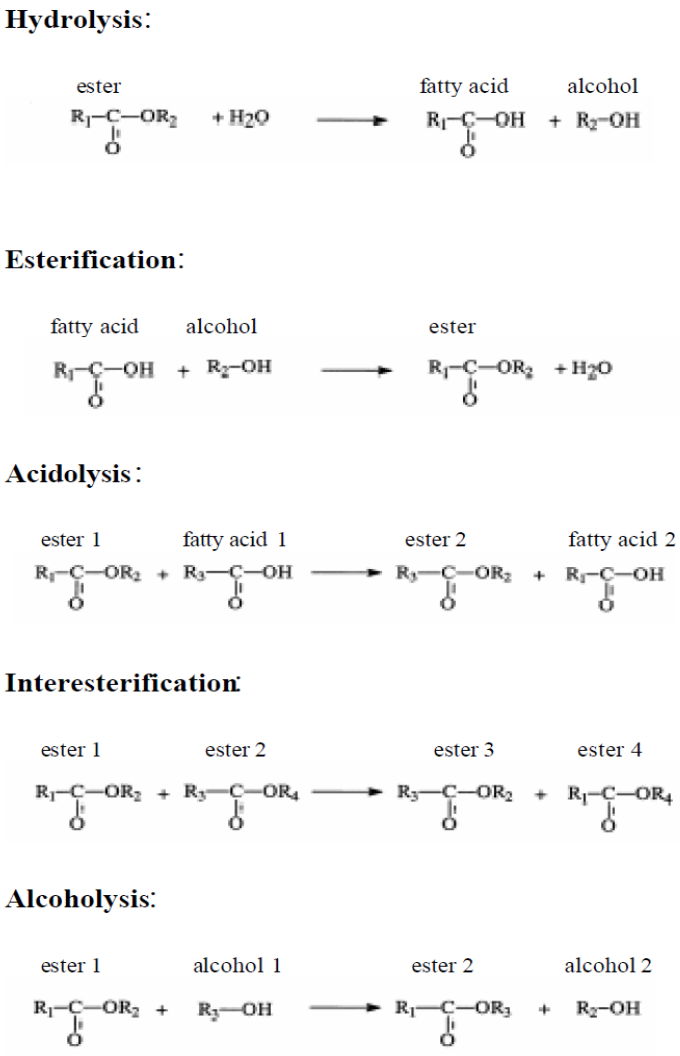

Figure 2.5. Different reactions catalysed by lipases/esterases in aqueous and non-aqueous solution (Source: Villeneuve et al. 2000)

Some properties of esterases are shown at below (Kim et al. 2008):

• They do not require cofactors.

• They have broad substrate specificity.

• They show enzymatic activity in both aqueous and nonaqueous solvents.

• They are enantioselective that catalyzes the reaction of only one of a pair of enantiomer. As a result of this optically pure compounds are produced.

• They have the characteristic α/β hydrolase fold and a similar catalytic triad consisting of the imidazole ring from a histidine.

There are several sources to produce esterases; from *Streptomyces sp.* (Nishmura and Inovye 2000), *Pseudomonas sp.* (Kim et al. 2002), *Bacillus sp.* (Kim et al. 2004), *Lactobacillus sp.* (Choi and Lee 2001), *Pencillium sp.* (Horne et al. 2002), *Saccharomyces sp.* (Lomolino et al. 2003) etc., from animal sources (Finer et al. 2004), from plants (Pringle and Dickstein 2004) for important applications in bioprocesses.

Different sources yield different esterases. Thus, a large and varied nomenclature is reported in the literature, e.g., carboxylesterase, cholinesterase, acetylxylan esterase, aryl esterase, phosphotriesterase, phenolic esterase, pig liver esterase, tanin esterase.

2.5.2. Applications of Esterases

- Hydrolysis of some important methyl esters by esterase and product of this reaction acid produced. Acid = Ferulic / Sinapic / Caffeic / p-Coumaric acid. They are widely used in the food, beverage, and perfume industries (Chaabouni et al. 1996).
- An esterase from Fusarium oxysporum plays a significant role in producing flavoring and fragrance compounds from geraniol and fatty acids (Christakopoulos et al. 1998, Chaabouni et al. 1996).
- Esterases are employed in dairies, and for the production of wine, fruit juices, beer, and alcohol. In order to transform low value fats and oils in to more valuable ones.
- Esterases and lipases from Lactobacillus casei are used significantly for hydrolysis of milk fat fort he purpose of flavor enhancement in the manufacture of cheese-related products (Choi and Lee 2001).
- An esterase from yeast plays a significant role in determining the final ester level in products such as membrane filtered beer and bottle re-fermented beer (Dufour and Bing 2001).
- To degrade some man-made pollutants, such as plactics, polyurethane, polyesters, polyethylene glycol adipate, etc., cholesterol esterase and polurethanase are widely used (Jahangir et al. 2003).

- Esterase plays a major role in the synthesis of chiral drugs (Bornscheuer 2002).
- Sterol esterase from Ophiostoma piceae is applied in paper manufacture, as it efficiently hydrolyzes both triglycerides and sterol esters. Further, steryl esterase and cholesteryl esterase from Pseudomonas sp. also play a significant role in reducing pitch problems during paper manufacture (Kontkanen et al. 2004).

Table 2.7. Applications of esterases
(Source: Panda and Gowrishankar 2005)

SI #	Form of esterase	Nature of application	Source	Reference
1	Acetylcholinesterase	Development of new drugs for schistosomiasis, biomarker for organo-phosphates in marine environment, assessment of poison due to pesticides and heavy metals	Blood of *Schistosoma sp*. , *Mytilus edulis*	Bentley et al. 2003; Brown et al. 2004; Panda and Sahu 2004
2	Acetyl esterase, methyl esterase, acetylglucomannan esterase and acetyl xylan esterase	Release of acetyl and methyl residues from cell wall, degradation of celulose, acetic acid from O-acetyl-galactoglucomannan and O-acetyl-4-O-metyhl-glucuronoxylan	*Aspergillus, Trichoderma sp*	De Vries Visser 1999; Poutanen et al. 1990; Puls et al 2001; Tenkanen et al. 1995
3	Aryl esterease	For flavor development in food and alcoholic beverages	*Saccharomyces cerevisia*	Lomolino et al.2003
4	Carboxylesterases	Degradation of ethylene glycol dibenzoate ester, lowering, toxicity, of malathion, hydolysis, of aspirin, and organophosphorous insecticides, D-acetylthioisobutyric acid, synthesis of racemates of esters of 1,2-O-isopropylideneglycerol, PHA depolymerase	*Streptomyces lividans*, ivers of rat and guinea pig, *Lucillia cuprina*, *Pediculus capitis, Bacillus* coagulans	Biely et al. 1996; Vincent and Lagreu 1981; Heidari et al. 2004; Picollo et al. 2000; Ozaki and Sakashita 1997; Monavi et al. 1996; Riegels et al. 1997
5	Cephalosporin acetyl esterase	Detecting actyl groups from cephalosporin dervatives	Burkholderia gladioli	Peterson et al. 2001
6	Cholesterol esterase and pseudocholinesteras e, cholinesterase	Degradation of poly (ether-urethane), prerequisite for working	Rat liver and other sources	Jahangir et al. 2003; Wheeler et al. 1972
7	Cinnamoyl ester hydrolase	Plant cell wall degradation	Piromyces equi	Fillingham et al. 1999
8	Erythromyc in esrterase	Clinical medicine in human, poultry and fish	*Pseudomonas sp*	Kim et al. 2002

(cont. on next page)

Table 2.7 (cont.)

9	Esterases	Transesterification reactions in organic solvents, resolution of (R, S)-β-acetyl-mercaptoisobutyrate, conversion of(R,S)-ketoprofen ethyl ester and linalyl acetate, food processing and dairy industries, hydrolisis of esters of tertiary alcohol, cefditoren pivoxil (a prodrug) – (UD Patent 4839350), fluorecein diacetate and 5-(6)- carboxyfluorescein diacetate to detect yeast in food, production of isoamyl acetate and mannitol, detoxification of xenobiotics,, control of physchological process of hormone, hydrolysis of diphthalates, ofloxacin, microbial activity of soil, flavor quality of sake, fermented sausages, detection methyl-parathion resistance and malathion susceptibility, improvement of aroma and flavor, fatty acid production	*Fusarium oxysporum, recomb Escherichia coli, Pseudomonas sp., Burkholderia gladioli, Lactobacillus casei, Rhodococcus sp., Saccharomyces cerevisia, Bacillus sp., Pedicoccus pentosaceus, Diabrotica virgifera, Locusta migratoria manilensis, Micrococcus sp*	Christakopoulos et al. 1998; Gokul 1999, Kim et al. 2002a,b; Peterson et al. 2001, Kermasha et al. 2000; Gudelj et al. 1998; Breeuwer et al. 1995; Fakuda et al. 1998; Costenoble et al. 2003; Laranja et al. 2003; Wezel et al. 2000; Kim et al. 2004; Valarini et al. 2003; Ostdal et al. 1996, Zhou et al. 2004; He et al. 2004; Fernandez et al. 2004; Jung et al. 2003
10	Ferulic acid esterase	Release of ferulic acid	*Aspergillus niger, Pencillium sp*	Asther et al. 2002; Kroon et al. 2000
11	Feruloyl esterase	Synthesis of pentylferulate ester used in cosmetics and perfumes indusries, decolorization of paper mill effluent	*A niger, Streptomyces ov ermitilis*	Giuliani et al. 2001; Garcia et al. 1998
12	Esterases from human system	Retinyl palmitate to retinol, resistant against inflammatory cells lysosomal enzymes, biodegradation of dental composites, metabolism of aspirin and non-narcoticanalgesics, conversion of proparacetamol, hydrolysis of acetylsalicylate to salycylic acid in plasma, conversion of oseltamirvir phosphate to oseltamirvir carboxylate, activation of etoposide prodrugs, hydrolysis of succinylcholine and procaine		Fu et al. 2002, Tang et al. 1997, Finer et al. 2004, Chavkin 2004, coppens et al. 2002, de Ruiter 2002, Abbott 2001, Wrasidlo et al. 2002, Henderson 2003

(cont. on next page)

Table 2.7 (cont.)

13	Methyl jasmonate esterase	Hydrolyzing methyl esters of abscisic acid, indole-3-acetic acid and fatty acids	*Lycopersicon esculetum*	Stuhlfelder et al. 2002
14	Phosphotriesterase	Hydrolyzed product of coumaphos and coroxon	*Psedomonas monteilli*	Kermasha et al. 2000
15	Pig liver esterase, porcine liver esterase and recombinant pig liver esterase	Desymmetrization of a centrosymmetric cyclo hexanediacetate, Enanrioselective production of levofloxacin from ofloxacin butyl ester, kinetic resolution of (R,S)-1-phenyl-3-butyl acetate and (R,S)-1-phenyl-2-pentyl acetate		Bohm et al. 2003; Choi and Lee 2001; Musidlowska-Persson and Bornscheuer 2003
16	Polyurethanase	Degradation of polyester polyurethane and polyether polyurethane	*Comamonas acidovorans, Pseudomonas chlororaphis*	Howard et al. 2001
17	Recombinant esterase (PFI-K)	Preparation of (S)-flurbiprofen	*Pseudomonas sp.*	Baronet al. 1980
18	Sterol esterase, steryl esterase and Cholesteryl esterase	Paper manufacturing, to reduce pitch problems during paper manufacture	*Ophiostoma piceae, Pseudomonas sp., Chromobacterium*	Calero-Rueda et al. 2002; Kontkanen et al. 2004

2.6. Immobilization

Enzymes are proteins that catalyse the chemical reactions. Compared with other inorganic and organic catalysts, enzymes are more fragile molecules. So enzyme properties such as stability, re-usability and activity are to be usually improved before their implementation at industrial scale. And another problem with enzymes is the solubility of enzymes in process media during the chemical process. Economical usage of enzymes for example the use of a relatively expensive catalyst as an enzyme requires its recovery and reuse to make an economically feasible process in industry. As a result of these factors, immobilization is the most powerful tool to improve almost all enzyme properties and reduce the cost (Mateo et al. 2007). The use of an immobilized enzyme permits to simplify the design of the reactor, continuous process and the control of the reaction.

2.6.1. Immobilization of Enzymes

In general the term ‘immobilization’ refers to the act of the limiting movement or making incapable of movement. The term ‘immobilized enzymes’ refers to enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously. Immobilization means associating the biocatalysts with an insoluble matrix or immobilized proteins and cells to an insoluble support. Practically, the procedure consists of mixing together the enzyme and the support material under appropriate conditions and following a period of incubation, separating the insoluble material from the soluble material by centrifugation or filtration. Today, a large number of immobilized enzymes are used in industry (Karadağ 2001).

In general, immobilization applications are commonly used at appropriate support materials, pharmaceutical, protein, microorganism, plant and animal cells, biosensor and bioreactor applications and controlled drug delivery systems except enzyme system (Aksoy 2003).

2.6.2. Advantages of Enzyme Immobilization

There are a number of advantages to immobilize enzymes from free solutions to insoluble supports via immobilization technique. Some of them are listed below:

- Immobilized enzyme is more robust and stable compared with soluble one.
- Immobilized enzyme generally shows greater pH and thermal stability.
- Thanks to immobilization enzymes can easily be added to or removed from reaction media, it enables greater control of the reaction time and rate.
- Problems of separating the catalyst from the products are practically eliminated.
- Product is not contaminated with the enzyme (especially useful in food and pharmaceuticals industries).
- Low downstream processing cost.
- Continuous processes using columns of immobilized enzyme become more practical and automation is possible.
- Enzymes may be stabilized against heat or solvent effects.

- Immobilized enzyme easily reused multiple times for the same reaction with longer half-lives (Brady and Jordaan 2009).

In spite of the advantages, the immobilization process has some disadvantages that are shortly listed below (Guisan 2006):

- Loss of enzymatic activity due to the immobilization process.
- The cost of carriers and immobilization method.
- Mass transfer limitations.
- Changes in enzyme properties such as selectivity.

It is also possible to immobilize whole cells rather than individual enzymes or some organelles.

2.6.2.1. The Major Components of an Immobilized Enzyme

An immobilized enzyme has some major components and those major components are listed as follows:

- The enzyme,
- The carrier or support,
- Mode of interaction of the enzyme with the carrier.

2.6.2.2. The Requirements of an Ideal Immobilization Support

There are a variety of insoluble materials to bind enzymes and several techniques to achieve immobilization.

The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization. It is difficult to predict in advance which support will be most suitable. However, in general an enzyme carrier should have some properties that listed as follows (Sandwick and Schray 1988):

- It should present large surface to have good geometrical congruence with the enzyme surface.
- It should include physical resistance to compression.
- It should be biocompatible and show inertness toward enzymes ease of derivatization.
- It should show resistance to microbial attack.
- It should be available at low cost and biodegradable.
- It should be simple non-toxic and sterile.
- It should have a character like easy separation of carrier from reaction media.
- Suitable shape and particle size for conventional reactor systems.
- Should have mechanical strenght.
- Should have high enzyme-mass loading capacity.

Carrier materials can be divided in to two group, first inorganic carriers and the second is organic origin carriers. The advantage of inorganic materials, they are not susceptible to microbial attack, and have a greater structural and operational stability. Common organic supports are cellulose derivatives that have free hydroxyl or amino groups. The goups can participate to link with covalent coupling the groups on the enzyme molecule (Kara 2006).

Table 2.8. Examples of Carriers Used for Enzyme Immobilization (Source: Kennedy and White 1985)(Source: Guisan 2006).

Organic
Natural polymers • Polysaccharides: Cellulose, agar, agarose, chitin, alginate dextrans. • Proteins: Collagen, albumin • Carbon **Synthetic polymers** • Polystyrene • Other polymers: Polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl, and allyl-polymers
Inorganic
Natural minerals: Bentonite, silica, sand. Processed materials: Glass(nonporous and controlled pore), metals, controlled pore Metal oxides(e.g. ZrO2, TiO2, Al2O3)

The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) will be of major importance for the performance of the immobilized systems and will determine the type of reactor used under technical conditions (i.e., stirred tank, fluidized, fixed beads). In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show few diffusional limitations but have a low loading capacity. Therefore, porous supports are generally preferred because the high surface area allows for a higher enzyme loading and the immobilized enzyme receieves greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices.

2.6.3. Methods for Enzyme Immobilization

There is a variety of way to immobilize enzymes. Three main principle methods exist to immobilize enzymes as shown in Figure 2.6, carrier binding, cross-linking, entrapment (Tanaka and Kawamoto 1999). Each has its own advantages and disadvantages. And no one method is ideal for all immobilization situations. In some processes, two or more methods can be combined to increase efficiency of process, enzyme activity and stability.

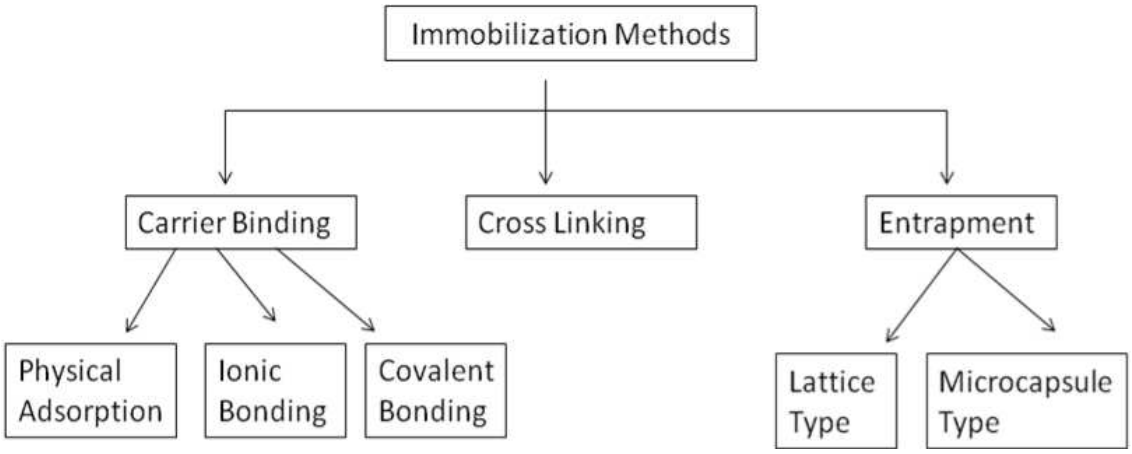

Figure 2.6. Various immobilization methods
(Source: Telefoncu 1997)

2.6.3.1. Carrier Binding

The carrier binding method is the oldest immobilization technique for enzymes. The selection of the carrier depends on the nature of the enzyme itself, as well as the following items:

- Particle size
- Surface area
- Molar ratio of hydrophilic to hydrophobic groups
- Chemical composition (Dumitriu et al 1988).

In general, an increase in the ratio of hydrophilic groups and in the concentration of bound enzymes, results in a higher activity of the immobilized enzymes. Some of the most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel. According to the binding mode of the enzyme, the carrier-binding method can be further sub-classified into (Cao 2006):

- Physical adsorption
- Ionic binding
- Covalent binding.

2.6.3.1.1. Physical Adsorption

Adsorption method is the oldest and simplest method of immobilization (Glick 1979). This method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. During physical adsorption, the hyrogen bonds, van der Waals forces and hydrophobic interactions are the responsible forces for immobilization (Chen et al. 1996).

Hence, the method causes little or no conformational change of the enzyme or destruction of its active center. This method is reversible, and this provides reuse of support material and enzymes again for different usages (Zaborsky 1973). If a suitable carrier is found, this method can be both simple and cheap. However, it has the disadvantage that the adsorbed enzyme may leak from the carrier during use due to a weak binding force between the enzyme and the carrier.

2.6.3.1.2. Ionic Binding

The ionic binding method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues (Brena and Batista 2008). Advantages of the ionic binding, first, the conditions are much milder than those needed for the covalent binding method. Second is, little changes in the conformation and the active site of the enzyme enable high activity in most cases. Addition to the advantages, the disadvantage is leakage of enzymes from the carrier may ocur in substrate solution of high ionic strength or upon variation of pH.

2.6.3.1.3. Covalent Binding

The most intensely studied of the immobilization techniques is the formation of covalent bonds between the enzyme and the support matrix. This technique allows the derivatives of enzyme to be stable and prevents enzymes penetration into solution (Carr and Bowers 1980). Covalent binding is used generally when the structure of enzyme and functional groups are known. When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics: (1) the binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and (2) the active site of the enzyme must be unaffected by the reagents used. Enzymes are covalently bound to the insoluble matrix through the functional groups on the enzyme. The functional groups that may take part in this binding are listed in Table 2.9.

Table 2.9. Functional groups used in covalent binding.

Amino group	Carboxyl group	Sulfhydryl group,
Hydroxyl group	Imidazole group	Phenolic group
Thiol group	Threonine group	Indole group

Immobilization by covalent binding is performed in two stages. First stage is activation of support material and second stage is covalent binding of enzyme. It is shown in Figure 2.7. Also depending on the nature of these functional groups, some

various activating materials such as cyanogen bromide, epichloridrin, glutaraldehyde, carbodiimit, cyanuric chloride can be (Srere and Uyeda 1976) used.

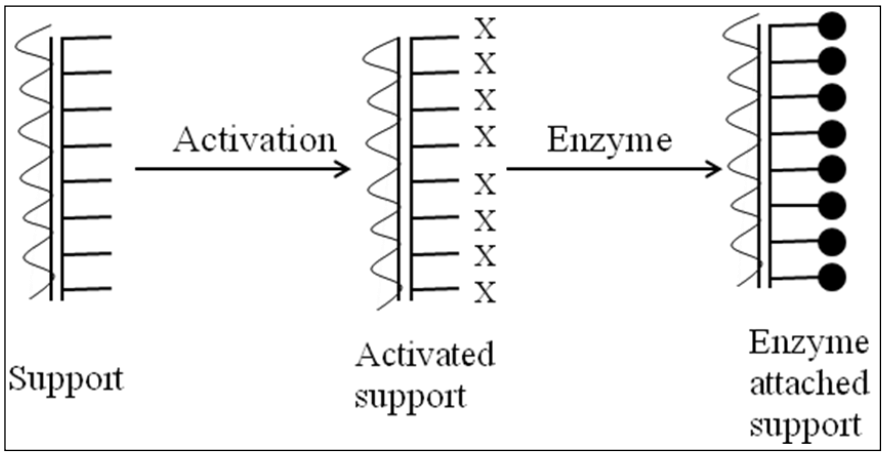

Figure 2.7. Immobilization by covalent binding.

It is possible in some cases to increase the number of reactive residues of an enzyme in order to increase the yield of the immobilized enzyme. This provides alternative reaction sites to those essential for enzymatic activity.

2.6.3.2. Crosslinking

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble matrix. Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support. This will result in relatively low enzymatic activity. Generally, cross-linking is best used in conjunction with one of the other methods. It is used mostly as a means of stabilizing adsorbed enzymes and also for preventing leakage from polyacrylamide gels.

Enzyme activity depends on some factors such as reaction time, temperature, ionic strength, pH, cross-linker material, enzyme concentration and balance between those factor. The most important advantage of this method is using two or multifunctional materials in order to immobilization of enzymes. The disadvantage of this method is the difficulty in controlling intermolecular cross-linking reaction for obtaining immobilized enzyme which shows high activity.

The most common reagent used in this method is glutaraldehyde which establishes intermolecular cross-linking with amino groups of enzyme. The structure of glutaraldehyde is shown in Figure 2.8.

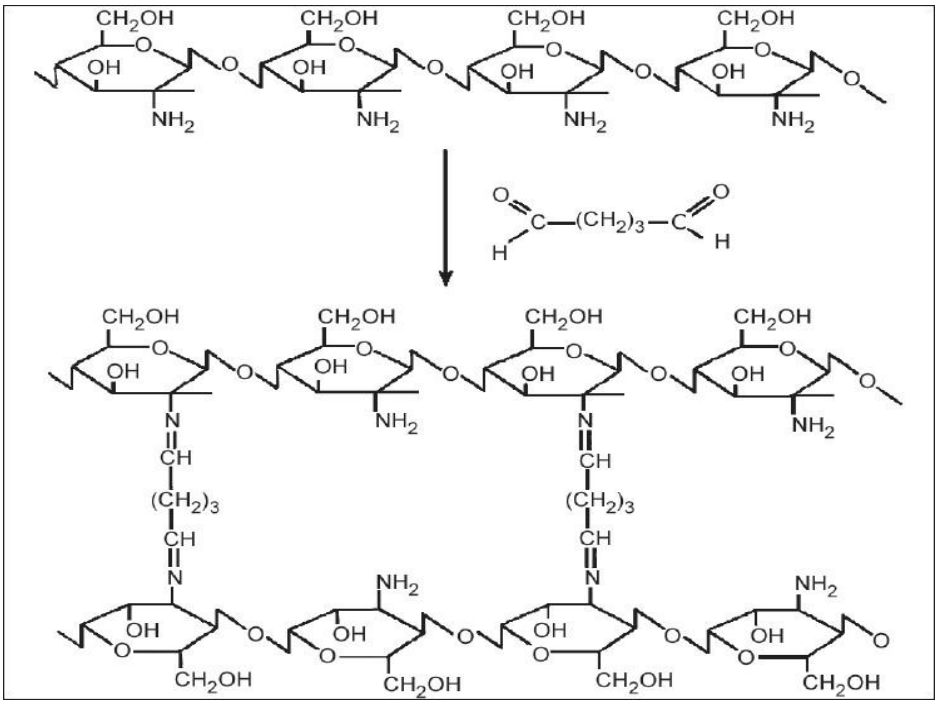

Figure 2.8. The crosslinking agent glutaraldeyhde
(Source: Migneault et al. 2004).

2.6.3.3. Entrapping Enzymes

In this method, enzyme is physically confined in an polymeric environment or lattice where substrate and product is able to pass whereas enzyme retain (Arıca and Hasırcı 1987). Enzyme entrapment is typically achieved using a polymer network such as an organic polymer or sol-gel (Sheldon 2006). Entrapment protects enzyme by preventing direct contact with the environment. Polymeric matrix structure should be rigorous enough to prevent diffusion of the protein while allowing diffusion of substrate and product. Alginate, carrageenan, agarose, polyacrylamide, pectin, gelatin, chitin or chitosan can be used as polymeric matrix (Wadiack and Carbonell 1975).

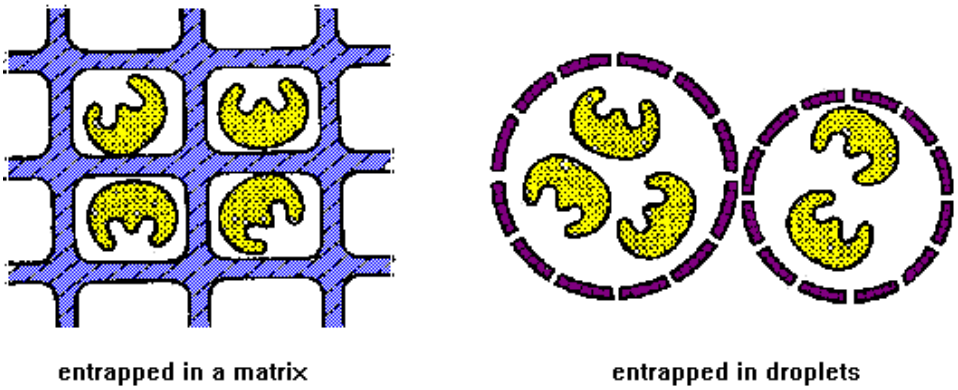

Figure 2.9. The illustration of entrapment in a matrix and other is in droplets (Source: Costa et al. 2004).

Also entrapment method can be separated into five major types as lattice, microcapsule, liposome, membrane, and reverse micelle.

2.6.3.4. Microencapsulation

This method is a type of entrapment. It refers to the process of spherical particle formation where in a liquid or suspension (the core) is surrounded or coated with a continuous film of polymeric material (the shell) to produce capsules in the micrometer to millimetre range, known as microcapsules (Bansode et al. 2010). Microencapsulation method largely used in pharmaceutical applications for controlled drug delivery systems. In this method, the enzyme is entrapped within a semipermeable membrane. The activity of enzyme is not affected by the microencapsulation method. But the movement of the substrate to the active site may be restricted by the diffusional limitations.

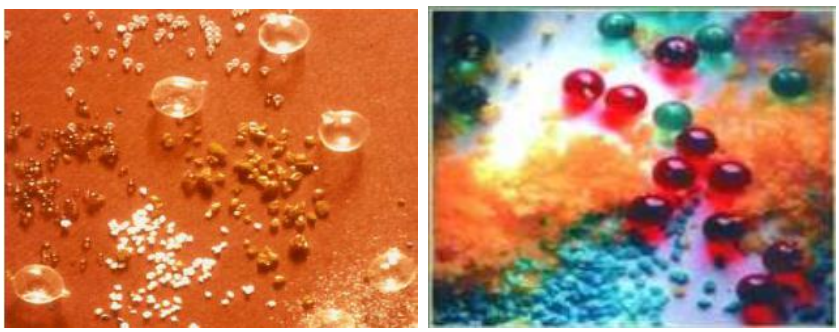

Figure 2.10. Illustration of beads that formed by microencapsulating.

For example: to mask taste and odor of many drugs to improve patient compliance, to convert liquid drugs in a free flowing powder, to stabilize or preserving drugs which are sensitive to oxygen, moisture or light, to prevent vaporization of volatile drugs (Bansode et al. 2010).

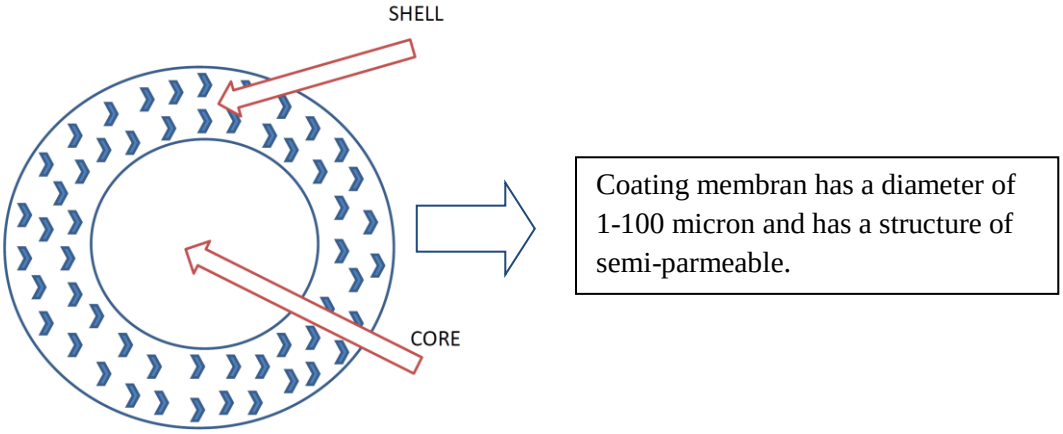

Figure 2.11. The structure of a microencapsulated bead.

While this membrane prevents big proteins and enzymes get out of microcapsules, let little substrates and products get in and out freely. The enzyme acticity is very close to the free enzyme activity since there is no modification during this confinement mthod by microcapsul. By this method a big surface-volume ratio has been obtained. This high surface- volume rate causes an increase on the enzyme substrat reaction which occurs in microcapsules. The disadvantages of this method are the requirement of high protein concentration during creation of microcapsules and being limited for high molecule weight substrates and products.

Microencapsulation methods are further subclassified as:

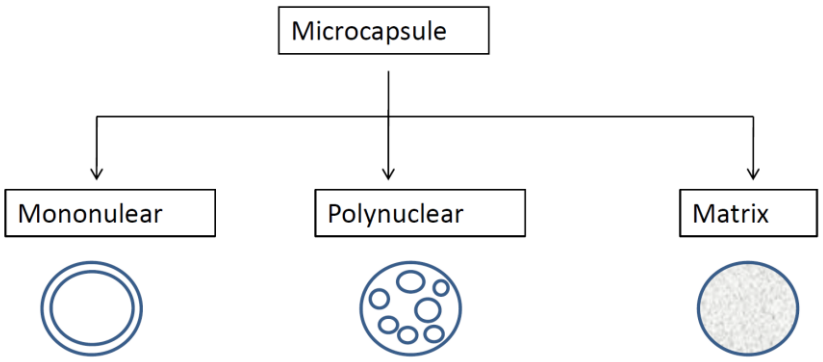

Figure 2.12. The sub-classification of microencapsulation method.

The most commonly used microencapsulation coating materials are Gums (gum arabic, sodium alginate, and carrageenan), Carbohydrates (starch, dextran, sucrose), Celluloses (carboxymethylcellulose, methycellulose), Lipids (bees wax, stearic acid, phospholipids). Selection of the most convenient coating material for core is the primary important factor for application of this method.

Suitable coating material should have the features listed below:

- Capable of forming a film that is cohesive with the core material.
- Chemically compatible and nonreactive with the core material.
- Film-forming, pliable, tasteless, stable.
- Controlled release under specific conditions.
- Inert toward active ingredients.

Immobilization methods are compared in Table 2.10.

Table 2.10. Comparison of the Immobilization Methods
(Source: Guisan 2006).

Characteristics	Entrapment	Covalent Binding	Ionic Binding	Adsorption	Cross linking
Preparation	Difficult	Difficult	Easy	Easy	Intermediate
Cost	Intermediate	Intermediate	Low	Low	Intermediate
Binding force	-	High	Medium	Low	High
Enzyme activity	Low	High	High	Medium	Low
Applicability	Yes	No	Yes	Yes	No
Stability	High	High	Intermediate	Low	High
Reusability	Impossible	Rare	Possible	Possible	Impossible

2.6.4. Choosen of Suitable Immobilization Method

The following factors should be considered for a successful immobilization (Mosbach 1976):

1. The mechanical properties especially the physical form and mechanical stability of support material should be considered.
2. Enzyme must be stable at reaction conditions.

3. Crosslinker reagents should not react with the enzyme's active ends or they should be big enough in order not to penetrate the active end of enzyme.
4. If possible, the active end of enzyme should be protected. For example, sulfhydryl enzymes can be protected by reaction with glutathione or cysteine. Afterwards, enzyme can be reactivated.
5. The washing process for removing the unbounded enzyme during immobilization should not affect the enzyme.
6. If immobilized enzyme will be used as continuous catalyst in some chemical reactions, teh nature of the reaction should be considered before choosing the method of immobilization.

In our study, we tried to immobilize of esterase enzyme into Alginate-Chitosan / $CaCl_2$ polymeric beads. Alginate and chitosan are both natural biopolymers and have several advantages such as availability from replenishable agricultural or marine food resources, biocompatibility, and biodegradability.

2.7. Natural Polymers

Natural polymers arepolymers those can be produced biologically and have unique functional properties. Proteins such as collagen, gelatin, elastin, actin, etc.), polysaccharides (cellulose, starch, dextran, chitin, etc.) and polynucleotide (DNA and RNA) are the main natural polymers. Natural polymers have different fields of use due to thier functional properties. They can be used as thickener, gel-maker, linker, distributing agent, lubricant, adhesive and biomaterial. Natural polymers are indispensable sources of field of biomaterials. They do not give adverse reactions such as inflammation and toxic effect when in contact with a live body since they are similar or identical to macromolecules in the biological environment. However, their main disadvantages are having difficulty when being shaped and being immunogenic in order to give rise to an immune response.

Table 2.11. Requirements for natural polymers
(Source: Park and Lakes 1992).

Property	Description
Biocompatibility	Noncarcinogenesis, nonpyrogenicity, nontoxicity, and nonallergic response
Sterilizability	Autoclave, dry heating, ethylenoxide gas, and radiation
Physical property	Strength, elasticity, and durability
Manufacturability	Machining, molding, extruding, and fiber forming

2.7.1. Alginate

Alginate is a naturally occuring biopolymer, quite abundant in nature. Alginates consist of (1-4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues of widely varying composition and sequence (Kierstan and Bucke 1997).

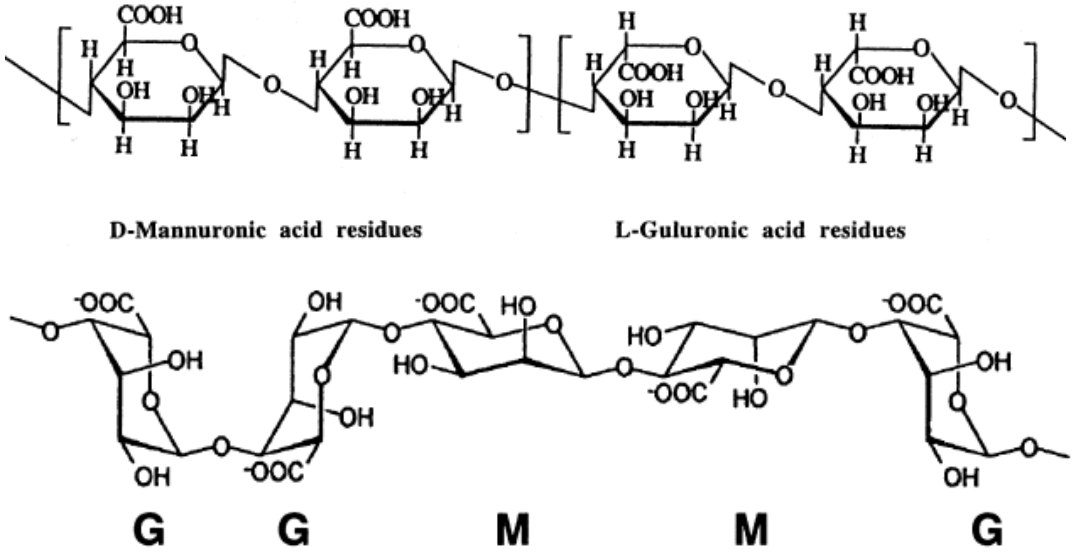

Figure 2.13. Chemical structures of mannuronic (M) and guluronic (G) acid monomers and alginate chain conformation (Source: Painter et al. 1968).

In general, if G block ratio is high, alginates create more resistant gels against pressure but those gels are brittle. If G block ratio is low, alginates create less resistant gels against pressure but those gels are flexible (Kara 2006).

All commercially available alginates are extracted from brown Algae Laminaria, Macrocystis and Ascophyllum. Comprise up to 40% of the dry weight. Bacterial alginates have also been isolated form Azotobacter vinelandii and several Pseudomonas

species. The structure and molecular weight of an alginate depends on the type of alg, age of alg, sections and extraction processes (Tanaka, Matsumura and Veliku 1984).

Alginate exists in the algs as calcium salt. It is put on the market generally in the form of sodium alginat. Sodium alginate is widely used in food sector as gel maker, stabilizator and thickener. In addition, alginate gels are used as matrix for protein, medicines, releasing or holding of cells, maintenance of grain and organs (Kara 2006).

Although alginic acid and sodium, potassium, ammonium salts, derivatives of propylene glycol ester can be easily dissolved in water, the calcium salt and alginic acid solubility in water is extremely limited. Sodium alginate is an odorless, tasteless powder and forms a viscous colloidal solution when dissolved in water (Rousseau et al. 2004).

Alginate as a matrix is drawing increasing interest on account of its biocompatibility, low toxicity and easy bead formation by ionotropic gelation. In addition, it is soluble and degradable under normal physiological conditions.

An important feature of alginate is its ionotropic gelation, alginate is a negatively charged polymer and induced by divalent cations (i.e.Ca^{2+}).

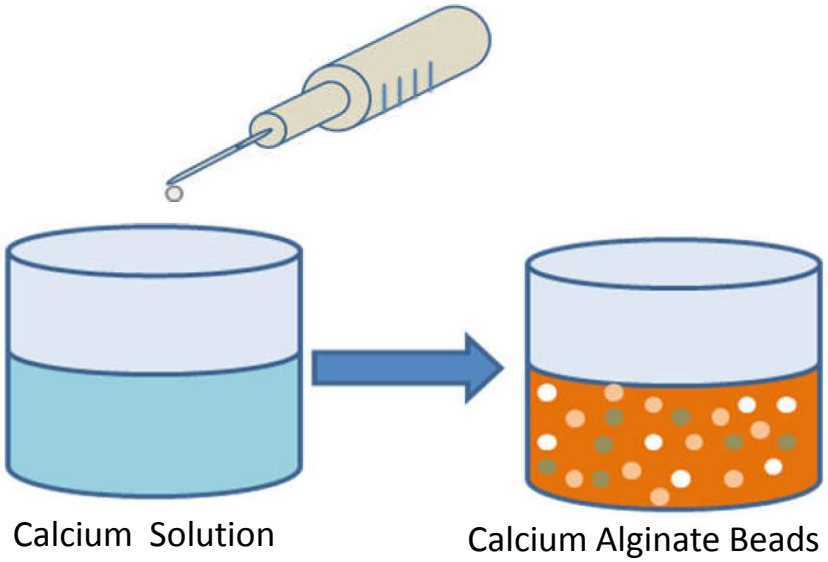

Figure 2.14. Enzyme immobilization with Ca-alginate beads

Increasing the concentration of alginate solution and $CaCl_2$ is the cause of developing tighter cross bonded gels.

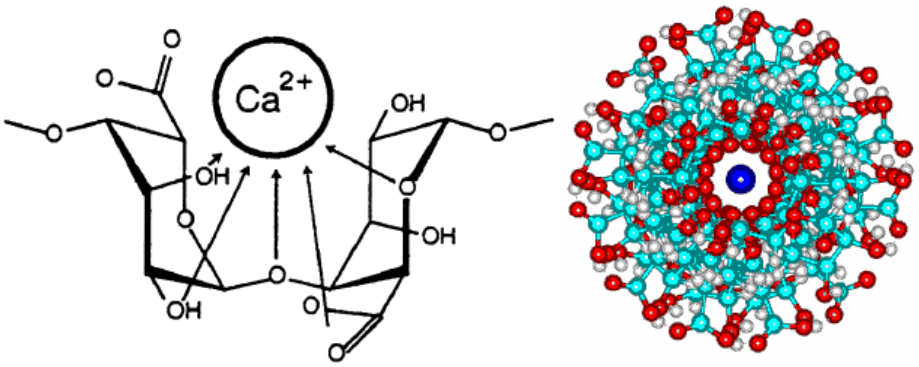

Figure 2.15. The first structure is ‘’egg-box’’ model for binding of divalent cations to homopolymeric blocks of α-L-guluronate residues (Source: Rousseau et al. 2004).

Regions of the alginate polymers rich in “G” residues display higher selectivity for divalent ions over mannuronic rich regions.

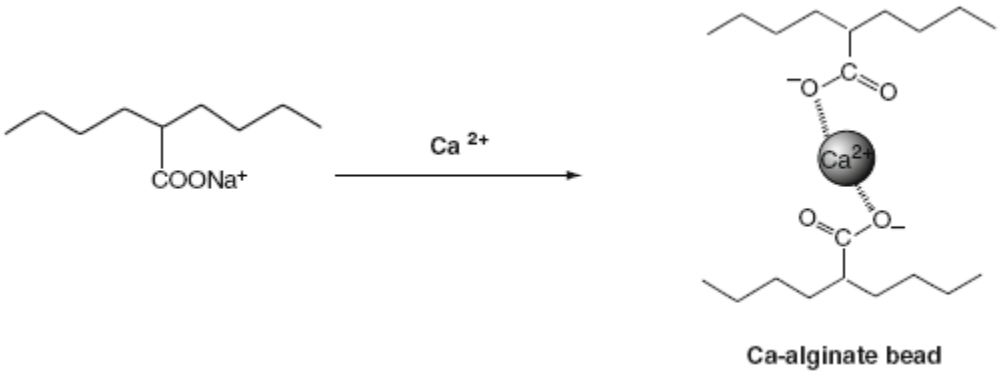

Figure 2.16. Basic mechanism for the formation Ca-alginate beads (Source: KHYMO 2009) .

Alginate beads are formed when a solution of sodium alginate and the desired substance is extruded as droplets in to a divalent solution to encourage cross-linking of the polymers.

Such cross-linking solutions may include cations such as Ca^{2+},Sr^{2+}, or Ba^{2+}, while monovalent cations and Mg^{2+} do not induce gelation, and Ba^{2+} and Sr^{2+} ions produce very strong alginate gels (Clark and Ross-Murphy 1987). Numerous other cations including Pb^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Mn^{2+} will induce gelation, but due to their toxicity they are rarely used.

Due to high water content of alginate beads, which is around 95%, the microenvironment of alginate is usually inert to protein drugs and cells. In addition, alginate matrices are very biodegradable and can be broken down under normal physiological conditions (Gombotz and Wee 1998). All these advantages make

alginates very useful materials for biomedical applications, especially for controlled drug delivery and other biologically active compounds and for the encapsulation of cells.

2.7.2. Chitin and Chitosan

Chitin is a naturally most abundant mucopolysaccharide. Chitin can be isolated from a variety of sources such as the shells of several crustaceans, krill, but it also forms part of the exoskeleton of insects and is present in the cell walls of fungi. It is consist of 2-acetamido-2-deoxy-β-D-glucose through a β(1-4) linkage. Chitin can be degraded by chitinase. It is a highly insoluble material resembling cellulose in its solubility and chemical reactivity. Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas (Zikakis 1984). Chitosan is the N-deacetylated form of chitin which is produced by thermochemical alkaline treatment of chitin. It has a high nitrogen content (7%) which makes it as a useful chelating agent (Kurita 2006, Tolaimate et al. 2000). Chitosan is a linear polysaccharide composed of randomly distributed β(1-4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitin and chitosan are attractive materials with unique properties of non-toxicity, film and fiber forming properties, adsorption of metal ions, coagulation of suspensions or solutes, and distinctive biological activities (Kurita 2006).

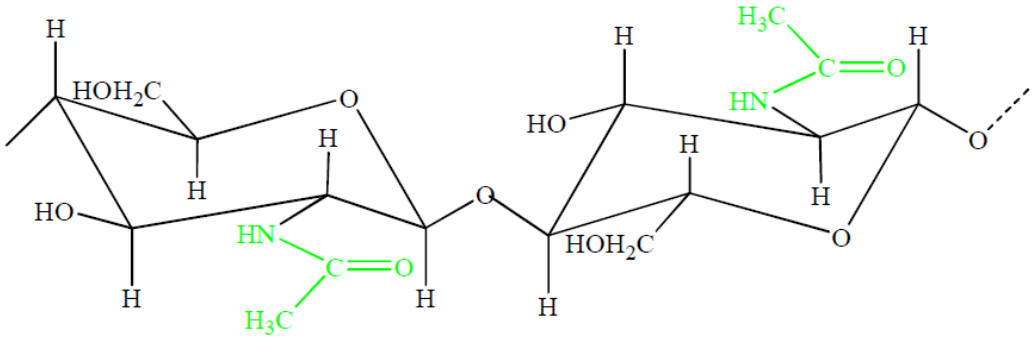

Figure 2.17. Structure of repeated units of chitin
(Source: Dutta et al. 2004).

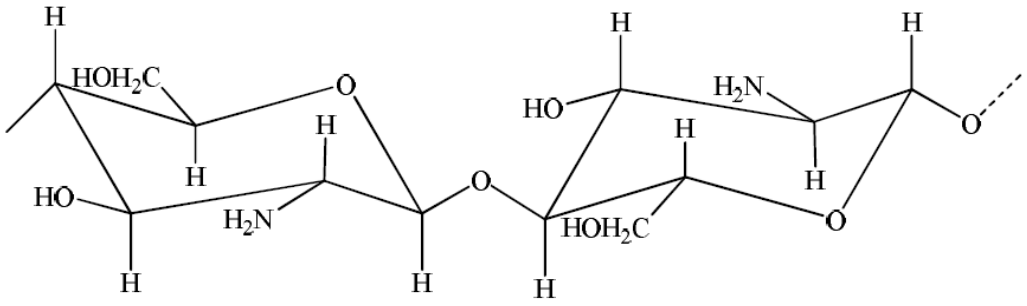

Figure 2.18. Structure of repeated units of chitosan
(Source: Kumar 2000).

The word chitosan refers to a large number of polymers which differ in their degree of N-deacetylation (65-95%) and molecular weight (3800-2.000.000 daltons). These two characteristics are very important to the physicochemical properties of the chitosans and hence they have a major effect on the biological properties (Kas 1997).

Chitosan is characterised by the degree of acetylation which is the ratio of two structural units called 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose. This ratio has a striking effect on chitosan solubility and solubility properties. Chitosan is insoluble at neutral and alkaline pH values but it is soluble in acidic solutions, under pH of 6.3. However, its solubility is much dependent on the degree of deacetylation. The degree of deacetylation necessary to obtain a soluble product is being 80-85% or higher. Chitosan is not soluble at neutral pHs. Its solubility is enhanced through the protonation of amino groups on deacetylated units. The solubilization occurs by protonation of the $–NH_2$ function on the C-2 position of the D-glucosamine repeat unit (Mukoma et al. 2004). Additionaly, amino groups make chitosan a cationic polyelectrolyte (pKa = 6.5), one of the few found in nature (charge on –NH3 groups) (Wang et al. 2003).

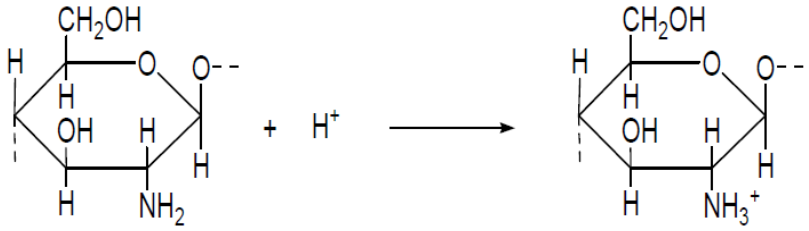

Figure 2.19. Protonation of chitosan
(Source: Günbaş 2007).

Since chitosan is biodegradable, non-toxic, non-immunogenic and biocompatible in animal tissues, much research has been directed toward its use in medical applications such as drug delivery, artificial skin, and blood anticoagulants. Chitosan has also been suggested for use as flocculant, food thickener, paper and textile adhesive, membrane and chelating agent for metals. It is also has an antibacterial activity. Thanks to it's bioactivity, facilitates wound healing, reduce blood cholesterol levels, and stimulate the immune system.

Cationic properties of chitosan:

- Linear Polyelectrolyte
- High charge density
- Excellent flocculant
- Adheres to negatively charged surfaces
- Substantive to hair, skin
- Chelates metal ions
    - Iron (Fe), Copper (Cu)
    - Toxic metals (Cd, Hg, Pb, Cr, Ni)
    - Radionucleids (Pu, U)

Chitosan is a linear polyelectrolyte at acidic pH's. It has a high charge density, one charge per glucosamine unit. Since many materials carry negative charges (e.g. protein, aionic polysaccharides, nucleic acids, etc.), the positive charge of chitosan interacts strongly with negative surfaces to give an electric neutrality. Chitosan adheres easily to natural polymers such as hair and skin, which are composed of negatively charged mucopolysaccharides and proteins. Also chitosan used for removing of toxic heavy metal ions such as silver, cadmium.

Table 2.12. Principal applications for chitosan

Agriculture	-	Defensive mechanisms in plants
	-	Stimulation of plant growth
	-	Seed coating
	-	Time release of fertilizers and nutrients into the soil
Water&waste treatment	-	Flocculant to clarify water (drinking water, pools)
	-	Removal of metal ions
	-	Ecological polymer (eliminate synthetic polymers)
	-	Reduce odors
Food & beverages	-	Not digestible by human (dietary fiber)
	-	Bind lipids (reduce cholesterol)
	-	Thickener and stabilizer for sauces
	-	Protective, fungistatic, antibaterial coating for fruit
Cosmetics&toiletries	-	Maintain skin moisture
	-	Treat acne
	-	Improve suppleness of hair
	-	Tone skin
	-	Oral care (toothpaste, chewing gum)
Biopharmaceutics	-	Immunologic, antitumoral
	-	Hemostatic and anticoagulant
	-	Healing, bacteriostatic

Nowadays, chitosan-alginate polyelectrolyte complex (PEC) systems take great attention. Compared with the constituent polymers, the PEC has asdvantages when applied as coating membranes and controlled release delivery systems. Oppositely charged polysaccharides in aqueous solutions interact spontaneously to form polyelectrolyte complexes (PECs) when they are mixed. Polyelectrolyte complexes of chitosan and NaAlg are mainly formed via coulombic interactions between positively charged amino groups of chitosan and negatively charged carboxylate groups of NaAlg. The properties of the PEC are mainly determined by the degree of interaction between the polymers. This depends essentially on their global charge densities and determines their relative proportion in the PEC (Berger et al. 2004).

Since both chitosan and NaAlg are weak polyelectrolytes some factors have to be controlled to determine the properties of PEC. The most important factor that has to be controlled is the pH of the solution, but temperature, ionic strength (Chavasit et al. 1988, Argüelles-Monal et al. 1993, Park and Ha 1993, Lee et al. 1997) and the order of mixing are also important.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Detailed list of used chemicals, buffers, solutions and their compositions are presented in Appendix A, Appendix B and Appendix C.

3.2. Methods

3.2.1. Preparation of Protein Sample

The thermophilic esterase enzyme purified in six steps in this study. These steps are shown at below:

Table 3.1. Experiment steps of preparation of protein sample.

Number	Steps
1	Cell Source
2	Cell disruption
3	Debris Removal
4	Initial Purification (Affinity Chromatography)
5	High Resolution Purification (Gel Filtration)
6	Drying under Vacuum
7	Concentrated Purified Enzyme

3.2.1.1. Escherichia coli Growth

In this study, the source of protein was choosen a microorganism Thermophilic *Bacillus sp*. that was isolated from Balçova (Agamemnon) Geothermal region in Izmir,

have used in our studies (Tekedar 2008). Also it belongs to prokaryotes as host because of rapid growth and simple medium components.

The bacteria isolate that expression vector including esterase gene transformed *E.Coli* was taken from -80°C. The isolate was named as Est33 in Pet28 BL21. The isolate was spread on agar plates with Kanamycin and incubated overnight in an incubator at 37°C. Our bacteria was inserted a gene that resistant to Kanamycin and grown under these conditions.

Another day the agar plates were controlled if there was any bacterial growth on it or not. And a single colony was choosen and inoculated in LB ^{kan} media of 50 ml and incubated one day more overnight at 37°C in a incubator shaker for 15-16 hours.

3.2.1.2. Expression of the Transformed Genes

After nearly 15-16 hours the 50 ml of bacteria culture was taken from shaker and the volume was diluted into total 500 ml and grown at 37°C to an optical density of A_{600}=1,0 which is half-stationary phase. At that point expression of the esterase genes was induced by the addition of 1 mM IPTG.

IPTG (isopropyl-β-D thiogalactopyranoside) induces the expression of the esterase genes which is a lactose analog. It represses the lac operator and allows the expression of T7 RNA polymerase, which in turn transcribes the target gene. The cells were allowed to grow for an additional 4.0 h after addition of IPTG and were then harvested by centrifugation (8,000 X g for 10 min). The harvested cell pastes were stored frozen at -20 °C until ready for use of purification.

3.2.1.3. Total Protein Extraction

The cell pellets were taken from –20°C and dissolved nearly in 10 ml of 50 mM sodium phosphate buffer, pH 7.0 and disrupted by a sonicator (Sim-Aminco, Spectronic Instruments) for 10 min. Cells were agitated by ultrasound energy, cell membranes were disrupted and cellular contents released. This sonication step was done at +4°C to preserve protein from denaturation. After sonication cell content was harvested by high-speed centrifugation at 10000 rpm, 10 min, +4°C. Then the cell debris was thrown away

and the supernatant kept for the next step. The supernatant that includes all proteins of cells was ready for purification.

3.2.1.4. Protein Purification and Determination

3.2.1.4.1. Affinity Chromatography

The purification procedure was carried out on ice using His-taq Nicel Affinity column (2.5 cm x 10 cm His-taq Nickel Affinity (Sigma)) chromatography system previously equilibrated with phosphate buffer. The supernatant was slowly loaded to the column. Then the column was washed with phosphate buffer including 0.3M NaCl, and the bound proteins eluted with a step elution of 250 mM immidazole in phosphate buffer including 0.1M NaCl. The eluted proteins were collected slowly into tubes as 20 drops in each. At the end of affinity chromatography, column was washed with phosphate buffer for cleaning.

3.2.1.4.2. Nanodrop

Protein concentrations of all samples in the collected tubes were measured with nanodrop (Thermo Scientific). The absorbance of protein in each samples were measured at 280 nm and the sample that give high absorbance in which tubes was collected and loaded to size-exclusion column to get rid of imidazole coming from elution buffer of affinity column.

3.2.1.4.3. Size-exclusion Chromatography

This technique is also known as gel filtration that separates molecules based on molecular size. This chromatography can be applied using resin or membrane. Basis of this technique depends on the shape of the molecules. The larger molecules pass through the resin and are collected first while the smaller molecules take longer to pass because these smaller particles get hold up within the pores of the resins.

The collected fractions in a tube after affinity chromatography were loaded to the gel filtration column (Sephadex G-75 (Sigma)). (The fraction ranges of sephadex G-

75 is 3K-80K). The fractions which show high absorption were collected according to the programe of device 40 droplets in each tube. After that the SDS-PAGE method was used to determine the homogeneity and molecular weight of the esterase between the choosen fractions.

In this study, size exclusion chromatography was used to send away the imidazole that arised during the one-step purification of affinity chromatography. Imidazole is an organic compound with the formula $C_3H_4N_2$. Imidazole is a smaller molecule as compared with esterase protein. As a result of this, esterase protein molecules passed through the column before the imidazole.

3.2.1.4.4. SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis is a technique to separate proteins to their size and no other physical feature. SDS that is an anionic detergent was used to convert all proteins to the same shape.

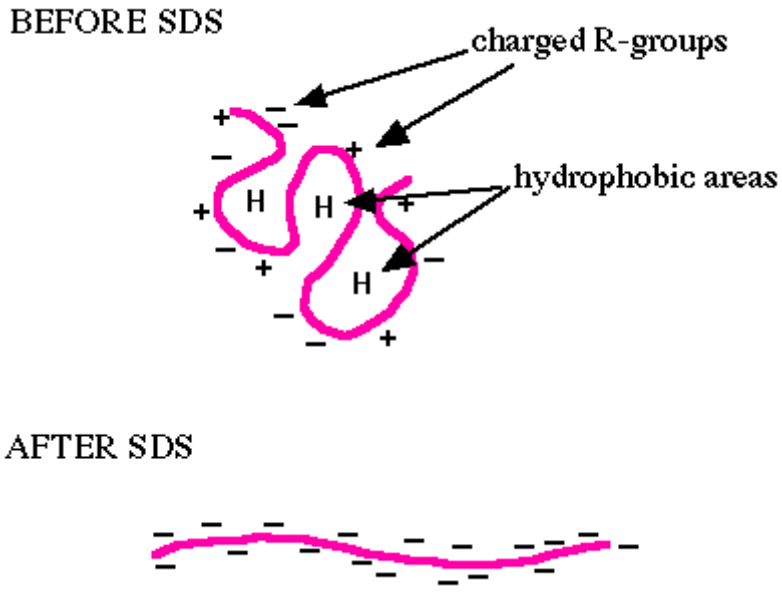

Figure 3.1. The illustration of interaction SDS with a protein molecule.

As a result of this application all proteins were became at same shape and negatively charged. When electric current impacted, the negatively charged protein samples migrated to the bottom of the polyacrylamide gel.

In this study, the molecular weight of esterase protein was determined by following the Laemmli method (Laemmli 1970). Thermo Scientific Electrophoresis was used as separating device. Firstly all equipments and glass plates were washed with deionized water and after this all cleaned with ethanol. First, separating gel was prepared according to the table in Appendix B, polymerization began as soon as the TEMED had been added to mixture. Without delay, the mixture rapidly was poured into the gap between the glass plates. The top of the gel was covered by deionized water to avoid drying. After 30 minutes the polymerization finished. The distilled water was poured off and the top of the gel was washed two times with deionized water to remove any unpolymerized acrylamide. Secondly, the stacking gel was prepared according to the table in appendix B. The components was mixed in the order shown. Polymerization began as soon as the TEMED had been added. Without delay, the mixture was poured slowly into gap over the separating gel. This process has been performed in a gentle way so that the creation of foam could be prevented. Instantly a clean Teflon comb was inserted into the stacking gel solution and more stacking gel was added to fill the spaces of the comb completely. During the stacking gel polymerization, the protein samples were mixed with sample buffer with the ratio of 1 : 4 (v/v). Then samples were heated to 100°C for 5 minutes to denature proteins. After nearly 30 minutes the polymerization of stacking gel was completed. The teflon comb was removed carefully and the 10 spaces were washed with deionized water two times to remove any unpolymerized acrylamide. The broad range protein marker and protein samples were loaded to the gel in buffer tank in the presence of running buffer. Electrophoresis was run at 65 volts for 30 minutes and followed by voltage at 100 volts for 2 hour until the blue dye reached at the bottom of the gel.

Finally when the run completed, the gel was removed from between the glass plates by using a spatula. And the gel was washed with deionized water, stained with Coomassie Brilliant Blue for 24 hours with slow shaking. Another day, the gel was washed first deionized water and then treated with destaining solution for 1 hour. When destaining completed gel washed with deionized water very carefully and photographed using the gel photo system.

3.2.1.4.5. Protein Concentration Determination

After purification and determination of esterase protein, it was concentrated under vacuum to increase durability without loss activity at -20°C. Memmert Vacuum Oven V0400 was used. Total esterase concentration was measured spectrophotometrically according to the Bradford protein assay method (Bradford 1976). In this method, Coomassie Brilliant Blue G-250 dye was used. The dye bound to our esterase protein and gave absorption at 595nm. Bradford method also known as colorimetric protein assay. When the protein concentration increases, the color of the sample becomes darker. Bovine Serum Albumin (BSA) was used as Standard protein. After formed the immobilized beads, the protein concentration determination was applied to the Chitosan/$CaCl_2$ filtrate that crosslinking solution to measure immobilization yield and enzyme leakage. All characterization studies were done also with Bradford protein concentration determination method Appendix C.

3.2.2. Esterase Activity Determination

The esterase activity was assayed spectrophotometrically using p-nitrophenyl acetate as a substrate having one of the best hydrolytic activity among the variety of pnitrophenyl (p-NP) esters with different acyl chain lengths (C2-C16) in previous studies (Tekadar 2008, Gülay 2009).

The enzyme hydrolyzes the acetate ester with the help of water. The products are acetic acid and p-nitrophenol (pNP), the latter showing an absorption maximum at about 420 nm.

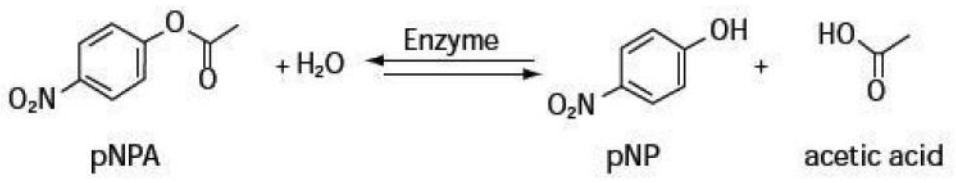

Figure 3.2. The Scheme of the pNPA assay
(Source: Gülay 2009)

Shimadzu spectrophotometer (UV-2450-2550) device was used to measure the esterase enzyme activity in this study.

Figure 3.3 Spectrophotometer Illustration

If the enzyme concentration is high, enzyme is used in low amounts but if the enzyme concentration is low, enzyme is used in high amounts according to the esterase activity measurement procedure. Measurement of free esterase enzyme and immobilized esterase enzyme differ from each other in technique. Free esterase enzyme activity was measured with the amounts were shown in table.

Table 3.2. Esterase activity determination.

	Sample	Blank
Tris-HCl buffer (0.1 M – pH: 8.0)	990 µl	990 µl
Enzyme(2.4 mg/mL)	0.5 µl	0.5 µl
Substrat(50 mM)	9.5 µl	-
Water	-	9.5 µl

The assay was performed using a suitably thermostatted spectrophotometer (Perkin Elmer) at 55°C observed to be optimum temperature for esterase enzyme activity in our previous studies. One unit of esterase activity was defined as the amount of enzyme releasing 1.0 nanomole of p-nitrophenol per minute at pH 7.2 at 55 °C using p-nitrophenyl acetate as a substrate.

We assayed a different activity determination method for immobilized esterase enzyme. The assay mixture (3ml) was prepared that included 2985 µl Tris-HCl buffer

(0.1M pH 8.0), 15 µl substrate (50 mM pNPA) and 10 silicate coated beads with enzyme. After 5 minutes of incubation time at 55°C with slow shake, 1 ml solution taken from the assay mixture to determine the catalytic activity and leakage of enzyme if any. The absorbance was measured at 55°C at 420nm. with spectrophotometrically.

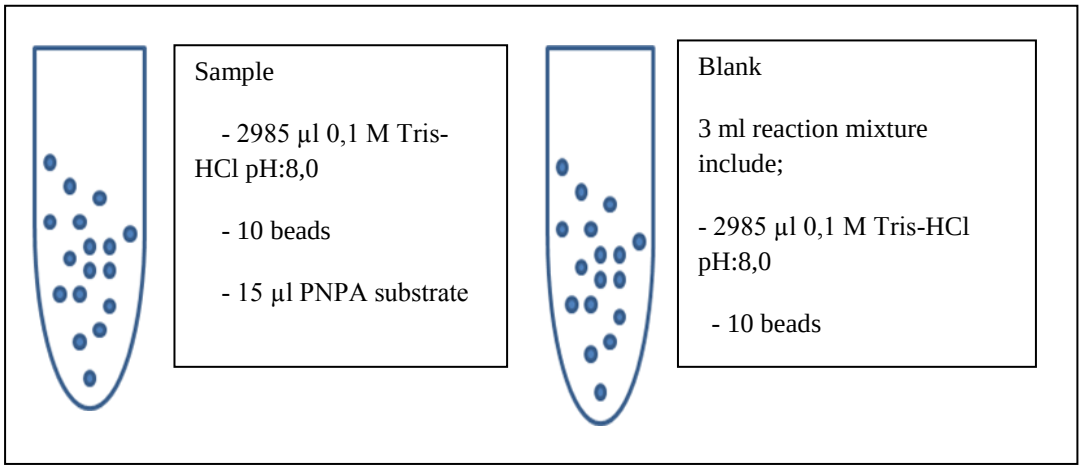

Figure 3.4. Immobilized Esterase Activity Determination.

The proportions of tris HCl buffer, solution, water and substrates for the experiments of activity determination are given in Table 3.3.

Table 3.3. The Proportions for Activity Measurement of Immobilized Polyelectrolyte Beads.

	Tris HCl buffer (0,1 M, pH:8,0)	15 µl solution (protein)	Water	Substrate PNPA
Blank	980 µl	10 µl	10 µl	-
Sample	980 µl	10 µl	-	10 µl

Activity calculation of free and immobilized enzyme is the same. Units were calculated according to the equation (3.1):

$$\text{Enzyme Activity} = {}^{Units}/_{ml}\ enzyme = \frac{\Delta A420nm}{\mathcal{E}\ x\ E\ x\ t} \qquad (3.1)$$

ΔA: Absorbance of the reaction sample

$\mathcal{E}$: Extinction coefficient (0.0148)

E: Volume of enzyme used in ten beads

t: Incubation time (minutes)

The volume of enzyme used in ten beads which was described as E; can be found as follows: At the beginning 2,5 mg enzyme which is 1 ml used to crosslink with 4 ml of alginate solution. Therefore we obtain 5ml of solution which includes enzyme of 0.5mg/ml concentration. However; 0.098 mg of enzyme was obtained as excess amount without being crosslinked. This excess amount of 0.098 mg can be substracted from the initial amount 0.5mg so that net used amount enzyme can be found as 0.402 mg. Thus, the volume of net used enzyme can be determined as 0.804 ml. Since from 0.804 ml enzyme approximately 500 beads can be formed; 10 beads can be formed from 0.016 ml enzyme. During all experiments for determination of enzyme activity 10 beads were used as constant enzyme amount. Using the equation 3.1 the terms in the denominator can be multiplied and in this study that denominator value used as constant value for all calculations. Denominator value can be found by multiplication of $\mathcal{E}$, E and t. The nominator value which is absorbance can be read from the spectrophotometer at 420 nm. By putting these values in equation 3.1 the enzyme activity can be found.

The specific enzyme activity (Units/mg protein) was defined as the ratio of enzyme activity (U/ml) to the mg of protein per ml (mg/ml). Therefore,

$$\text{Specific enzyme activity} = \frac{\text{Enzyme activity}}{\text{Mg of enzyme/10 beads}} \qquad (3.2)$$

The calculated value from equation 3.1 can be put into nominator of equation of equation 3.2. The denominator of this equation means that the amount enzyme in 10 beads. Since 10 beads makes 0.016 ml and we know that 1 ml of volume makes 0.5 mg of enzyme; therefore it is calculated that there are 0.008 mg of enzyme in 10 beads. However, during all experiments for determination of activity there are some portions of enzyme leaking from bead to outside. For that reason, for each experiment that leaking amount of enzyme must be calculated by Bradford protein assay. Net amount of

enzyme in the beads can found by substracting the Bradford number (leaking amount) from the initial value 0.008 mg of enzmye in 10 beads.

3.2.3. Preparation of Suitable Immobilization Polymer

3.2.3.1. Chitosan Synthesis

Various methods for chitosan synthesis were given in literature; all of them are based on the alkaline treatment of chitin under high temperature. This study was performed according to the method of Rigby and Wolfrom given in the monograph by Muzzarelli 1973. Different degree of deacetylation can be obtained depending on the treatment conditions. In our study, 15.0 g of chitin were treated with 720 mL of 40.0% (w/w) aqueous NaOH solution in a one liter three-necked round bottomed flask with reflux condenser connected to its middle neck. A thermometer was connected to control the temperature during reaction and N_2 gas was bubbled through the solution from the side arm to provide inert atmosphere in the reaction medium. Constant reflux was obtained at 115℃ and continued for 6 hours. After cooling the alkaline mixture to room temperature, chitosan flakes were washed with distilled water until a neutral filtrate was obtained. Resulting, chitosan flakes were dried at 60℃ for 2 hours before use. The washed chitosan particles were incubated in a drying oven for two days until the all water molecules removed away (Boyacı 2008). In order to determine of deacetylation degree of chitosan, elemental analysis performed. As a result of these studies we named our chitosan as nearly % 87,3 deacetylated and middle molecular weight. For characterization the following formula was used:

$$\text{Degree of Deacetylation; DD} = \left(1 - \frac{{}^{C}/_{N} - 5.145}{6.816 - 5.145}\right) \text{x } 100 \qquad (3.3)$$

Figure 3.5. The Scheme of the Reflux System

3.2.4. Immobilization of Thermophilic Esterase Enzyme in to Alginate-Chitosan/CaCl2 Polyelectrolyte Beads

Firstly, the most suitable Alginate-Chitosan/$CaCl_2$ ratio and reaction conditions were determined with several optimization studies. In order to compare our results suitably the same reaction conditions and same amounts were used as the previous immobilization study of Gülay 2009.

3.2.4.1. Preparation of Alginic acid-Esterase Enzyme Solution

The purified thermophilic recombinant esterase enzyme and alginate is soluble in water. Firstly, alginate (w/v, 2%) was dissolved in 10 ml Tris-HCl buffer (0.1M pH 8.0) by heating at 30°C for 30 minutes. When the dissolving of all alginate particles completed, the liquid esterase enzyme (0.5 mg/ml) was slowly added by stirring into the alginate solution. This mixture was stirred for 1 hour for complete homogenation of enzyme and alginate.

3.2.4.2. Preparation of Chitosan/$CaCl_2$ Solution

Chitosan flakes (w/v, 1%) were dissolved in dilute acedic acid media (v/v, 2%) by heating at 50°C. As a result of the chemical structrure of chitosan, a viscose mixture was carried out during this dissolution step.

Figure 3.6. Chitosan flakes

After obtained a transparent-clear solution, $CaCl_2$ (0,7 M) was added to the chitosan solution and then the mixture was stirred thoroughly to ensure complete mixing at 50°C for a 1 hour more.

Figure 3.7. Calcium chloride

3.2.4.3. Microencapsulation of Esterase in to Alginate-Chitosan/$CaCl_2$ Polyelectrolyte Beads

The two phases one of them is alginate-enzyme and the other one is chitosan/$CaCl_2$ were put together to get enzyme entrapped polyelectrolyte bead (EEPEB). First prepared phase alginate-esterase was dripped into 100 ml of chitosan/$CaCl_2$ solution with a syringe in small droplets by slow stirring until the syringe is empty. Result of that nearly numerically 500 white opaque beads were formed. The bead size was changed by using syringes with different needle diameters. After 30 min of hardening, the beads were separated from the calcium chloride solution by vacuum filtration. Lastly, they were washed two times with distilled water and kept in Tris-HCl buffer (0.1M pH 8.0) at 4°C to use in further characterization studies.

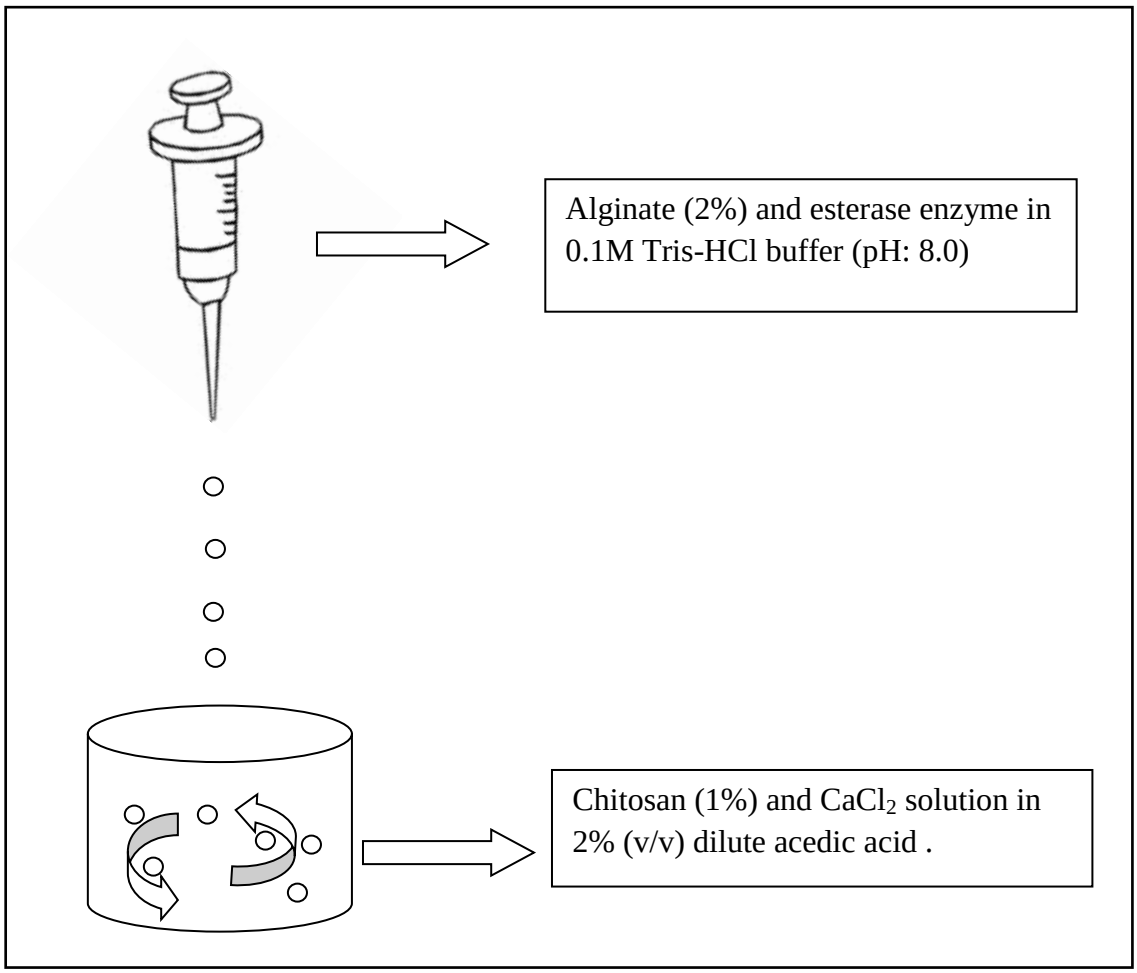

Figure 3.8. The scheme of alginate-chitosan/$CaCl_2$ polyelectrolyte beads formation.

3.2.5. Characterization of Immobilized Thermophilic Esterase Enzyme

Firstly, the sufficient incubation time of enzyme with its substrate pNPA was determined according to the below mentioned method.

Five solutions were prepared that includes each one 2985 µl Tris-HCl buffer, 15 µl substrate (50 mM pNPA) and 10 coated beads with enzyme. (Total solution volume is 3 mL). These 3 ml solutions were shaken slowly for 60, 120, 180, 240 and 300 seconds at 55°C in a incubator shaker at 90 rpm. After incubation, 1 ml solution was taken from each of them and absorbance of this solutions mesured spectrophotometrically.

In order to find the best working conditions for immobilized esterase enzyme, effect of some parameters were investigated. These parameters include temperature, pH, metal ion, organic solvents and detergent effect, reuse of enzyme, bead diameter and surface morphology of coated beads. Also during all these studies, leakage of enzyme concentration determination was done with using Bovine Serum Albumin (BSA) as a standard protein. Then the results were compared with results of thesis study of Seçkin Gülay.

3.2.5.1. Effect of Different Temperatures on Immobilization

Effect of temperatures on immobilized esterase activity was studied by testing a wide range of temperature (4°C-100°C). Ten solutions including 2985 µL Tris-HCl buffer (0.1 M pH:8.0), 10 polyelectrolyte beads and 15 µL substrate (50 mM pNPA) were incubated for 5 minutes at 4, 25, 35, 45, 50, 55, 60, 70, 80°C in incubator shaker at 90 rpm. Then 1 ml solution was taken from each of them to measure activity. The results were expressed as relative activities (%). Similarly, to determine temperature stability, ten solutions including 2985 µL Tris-HCl buffer (0.1 M pH:8.0), 10 polyelectrolyte beads were incubated for 1 hour by order of at 4, 25, 35, 45, 50, 55, 60, 70, 80°C in incubator shaker at 90 rpm. Then 15 µL substrate (50 mM pNPA) was added to each of them and reincubated in incubator shaker at 90 rpm. And 1 ml solution was taken from each of them to measure activity.

3.2.5.2. Effect of Different pH Values on Immobilization

Secondly to find effect of pH on immobilized esterase activity was studied by testing a wide range of pH values (4, 5, 6, 7, 7.5, 8, 8.5, 9, 10 and 11). 10 polyelectrolyte beads and 15 µL substrate (50 mM pNPA) were incubated in 4, 5, 6, 7, 7.5, 8, 8.5, 9, 10 and 11 pH Tris-HCl buffer (0.1M) for 5 minutes in incubator shaker at 55°C at 90 rpm. Then 1 ml solution was taken from each of them to measure activity. Determination of pH stability for 1 hour was investigated among pH values of 4, 5, 6, 7, 7.5, 8, 8.5, 9, 10 and 11. And after waiting 1 hour in different pH values, 15 µl substrate (pNPA) was added and solution was shaken for 5 minutes more. Then 1 ml solution was taken from each of them to measure activity.

3.2.5.3. Effect of Chemicals on Immobilized Esterase

To investigate effect of different chemicals on enzyme activity $CaCl_2$, $ZnCl_2$, $MgCl_2$, $CuSO_4$, $MgSO_4$, SDS, Triton X-100 were choosen. The experiment was done with the incubation of 10 coated beads in 2000 µl pH: 8.0 Tris-HCl buffer (0.1M) includes 985 µl chemical (1mM) at 55°C for 5 minutes with 15 µl substrate (50 mM pNPA). Then 1 ml solution was taken from each of them to observe activity.

3.2.5.4. Effect of Reuse of Immobilized Esterase

Such as general enzyme activity method, firstly 10 polyelectrolyte beads were incubated with 2985 µl Tris-HCl buffer and15 µL substrate (50 mM pNPA) for 5 minutes at 55°C in incubator shaker at 90 rpm. Then 1 ml solution was taken from each of them to measure activity. After absorbance measurement, ten polyelectrolyte beads washed three times with deionized water and lastly 2985 µl Tris-HCl buffer. And this experiment was replicated 6 times more.

3.2.5.5. Effect of Bead Diameter

Alginate beads of three different sizes were generated by changing the size of a needle. The diameter of beads was determined with the formula as shown below.

$$\text{Increment in Volume} = 4/3\ \pi\ r^3\ \text{Number of beads} \qquad (3.4)$$

3.2.5.6. Scanning Electron Microscope (SEM)

The surface morphology and internal structure of the beads are examined using a scanning electron microscope (Phillips XL-30S FEG). Polyelectrolyte beads were dried for 1 hour at 50^{o}C to get rid of wetness. Then, samples were mounted on carbon stubs, and gold coated under vacuum, and then examined.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Expression and Purification of the Recombinant Esterases in E.coli

4.1.1. Expression

To obtain the wanted *E.coli* colonies, the glycerol stock before prepared by our labmates was taken from -80°C and spread on LB^{kan} plates. One colony was choosen and inoculated into the LB^{kan} media to express the transformated genes. We get high level of esterases thanks to the addition of IPTG to the LB^{kan} media. The expression reached maximum levels within 4 hours after induction by IPTG.

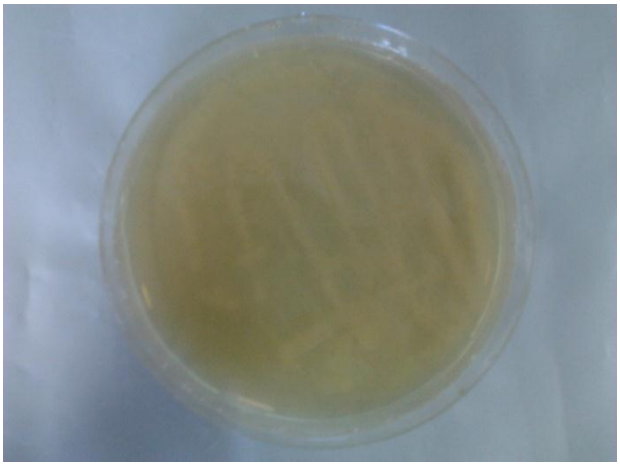

Figure 4.1. The growth colonies on LB^{kan} plate.

4.1.2. Purification of Esterase Protein by Affinity Chromatography

After expression of the wanted specific genes in the pET-28a (+) vector which carries the codons encoding both C-terminal and N-terminal His-taq region (Tekedar 2008), the cellular fragments were distrupted suitably by sonication and the clarified supernatant part was separated for one-step purification. In this study affinity chromatography was choosen to purify protein sample because the Ni-NTA affinity

resin (Sigma) of affinity chromatography is specific to the His taq regions of our esterase protein. This property of resin enables ease to purification step.

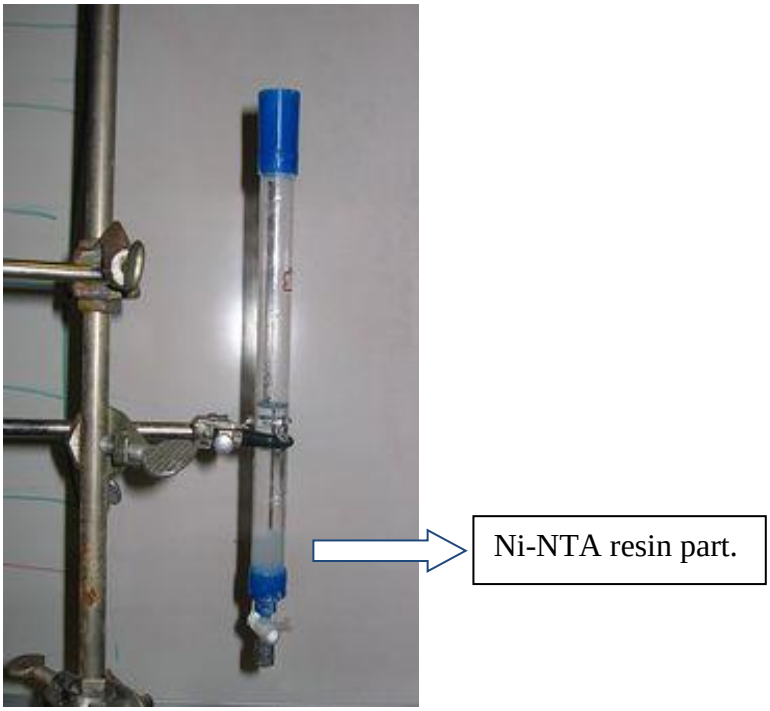

Figure 4.2. Affinity Chromatography (Sigma).

Elution of the protein from the column was accomplished by addition of elution buffer which included imidazole in phosphate buffer. The fractions that each one includes 20 drops of buffer were collected from 1 to 36 tubes. The absorbance of the fractions was measured by nanodrop (Thermo Scientific) at 280 nm. The fractions 10 to 23 which show high absorbance were collected in a one tube. And the high purified sample was loaded to gel filtration column to get rid of imidazol. After gel fitration the selected fractions were analyzed on 15% SDS-PAGE (Figure 4.3). The protein marker was loaded to lane 1 and 2, from lane 3 to lane 8 the protein samples that obtained after gel filtration were loaded, lastly the protein sample that obtained after affinity chromatography was loaded to the lane 9. The purified recombinant esterases migrated as a single band, with a relative molecular mass of 28 kDa on SDS-PAGE.

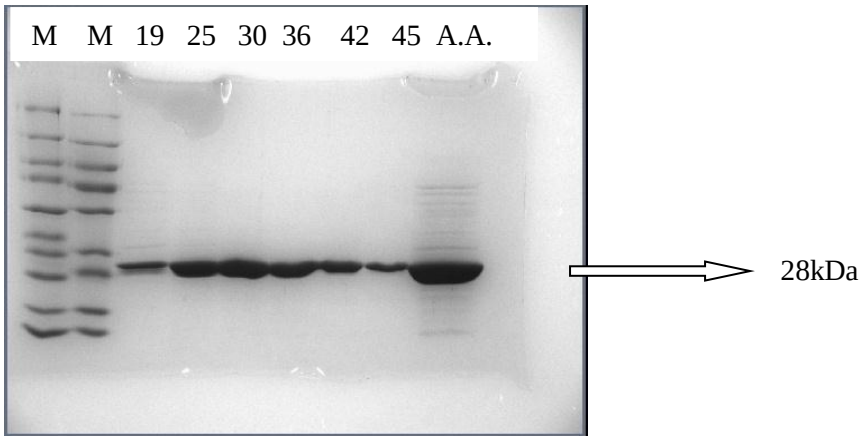

Figure 4.3. 15% SDS-PAGE analysis of selected fractions. (M: molecular mass markers from the top to bottom 200, 116, 68, 43, 29, 14.4 and 6.5 kDa. 19, 25, 30, 36, 42, 45 (Fractions after size exclusion step), A.A (fraction after affinity) selected fractions.)

One-step purification of the esterase proteins using Ni-NTA affinity chromatography has resulted in efficient purification to continue our studies with that much pure enzyme. Size-exclusion chromatography was applied after affinity column against phosphate buffer (pH 7.0) to get rid of imidazole from esterase enzyme. After size-exclusion chromatography, enzyme was aliquoted in to eppendorf tubes as 1 ml volume and kept it at -20^{o}C until use.

4.2. Immobilization of Thermophilic Esterase Enzyme in Alginate-Chitosan/$CaCl_2$ Polyelectrolyte Beads

4.2.1. Immobilization Yield

In order to calculate immobilization yield, the total enzyme leakage was measured in filtrate and the crosslinking solution that composed of Chitosan/$CaCl_2$. The initial concentration of esterase was 0.5 mg/ml and 0.098 mg/ml esterase enzyme has been determined from washing steps. As a result of this, immobilization yield was calculated as 80.40 %.

4.3. Characterization of Immobilized Thermophilic Esterase Enzyme

During characterization studies, we investigated effects of different parameters such as pH, temperature, chemical agent, bead size on enzyme acvitiy, also we

compared the our results with the results of Gülay 2009. In addition to these experiments, surface morphology and reusability of immobilized enzyme studied. At the same time all experiments were done together with enzyme leakage tests. For all characterization studies, specific activities were calculated with using entrapment efficiency (amount of enzyme in the beads) and changed in absorbance values. The reason for why we did not perform optimization studies before characterization studies is that we selected the same working conditions of thesis study of one of our lab colleagues Seçkin Gülay in order to make a comparison with her study and examine the results under same conditions.

Below are the graphs of activity change according to different parameters such as temperature, pH, reuse, chemicals used, bead size. Specific activity was calculated according to the equation of (3.2) and then for demonstrating the activity value the relative activity values were used. For each different temperature, pH, chemical, bead size, reuse number values two measurements were performed by spectrophotometer and therefore two activity values have been obtained. From these two activity values the mean activity was calculated for each point. The maximum measured specific activity has been regarded as 100 and the other points are calculated relatively.

4.3.1. Effect of Temperature

In general, high temperature increases the rate of an enzyme's activity, because at high temperatures, molecules move around faster, so an enzyme is likely to come in contact with a substrate very quickly. Temperature stability of an enzyme is an important parameter, especially for industrial usage of enzymes. Generally industrial chemical reactions perform at high temperatures and it can be damage the structure of enzyme or denature. But enzymes obtained from thermophilic organisms that live in high temperatures and also they are more resistant to heat denaturation than those from mesophiles, organisms that live in moderate temperatures. These thermophilic enzymes are useful for the industry because of their superior stability.

The esterase enzyme used in our studies was purified from Thermophilic *Bacillus sp.* It has been believed that the enzyme can be durable at high temperature conditions. Depending on this decision, both the activity behaviours and the enzyme leakage rates have been observed firstly for 5 minutes incubation and then for 1 hour

long incubation from 4°C upto 80°C temperature. Finding the optimum temperature conditions is at great importance in order to get benefit effectively.

Table 4.1 indicates the change in relative activity with respect to different temperature values after 5 minutes duration of incubation time.

Table 4.1. Temperature effect on activity for 5 minutes

Temperature (°C)	**Absorbance #1**	**Absorbance #2**	**Calculated Activity # 1**	**Calculated Activity # 2**	**Mean Activity Value**	**Relative Activity**
4	0,215	0,216	25940,000	26061,400	26000,700	19,77
25	0,230	0,228	28035,700	27508,570	27772,130	21,11
35	0,275	0,277	34155,880	34404,410	34280,140	26,06
45	0,300	0,298	37260,290	37011,760	37136,020	28,230
50	0,370	0,371	47348,480	47475,750	47412,110	36,050
55	0,450	0,455	58096,960	58225,750	58161,350	44,220
60	0,523	0,525	66927,270	67183,300	67055,280	50,000
70	0,980	0,981	129328,120	129459,370	129393,740	98,000
80	0,980	0,982	131380,950	131649,200	131515,070	100,000

*All measurements were done @420 nm with 90 rpm shake.

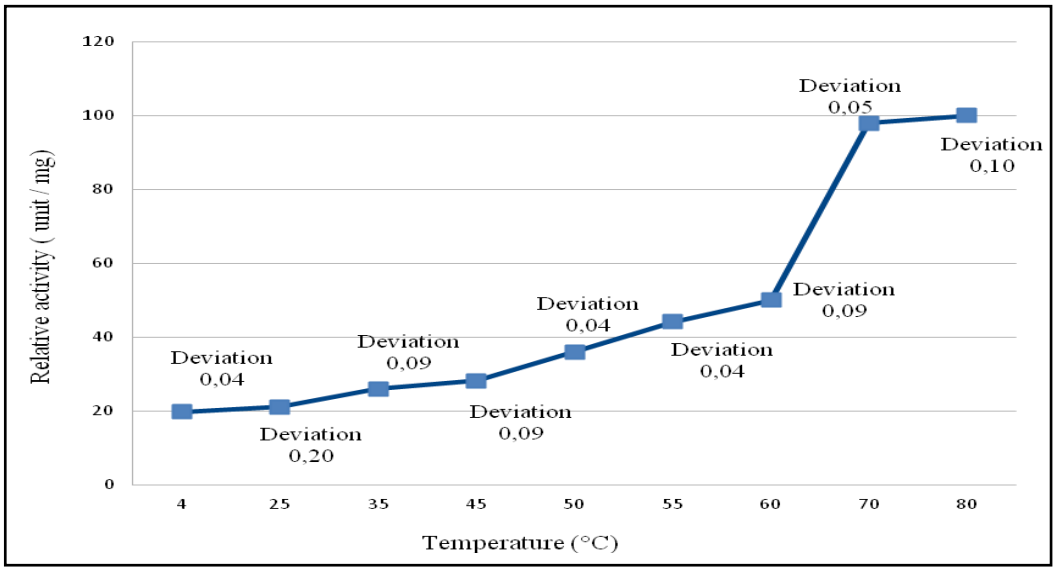

Figure 4.4. Effect of temperature for 5 minutes on relative activity of immobilized esterase enzyme.

As it can be seen from Figure 4.4, immobilized enzyme shows low relative activity at low temperature values. The relative activity of the enzyme is incresing directly as the temperature rises. The highest value of activity has been reached as

temperature is 80°C. In Gülay's study, for 5 minutes, maximum specific activity was observed at 45°C. So we can easily say that our new formed alginate/chitosan membrane is more stable from alginate/calcium chloride matrix.

4.3.1.1. Entrapment Efficiency for 5 Minutes

In addition, after 5 minutes of experiment the remaining enzyme amount in the beads were calculated for each temperature values. Those amounts in per cent are given in Table 4.2.

Table 4.2. Percent amount of enzyme in beads during pH effect test for 5 minutes

Temperature (°C)	**% amount of enzyme in beads**
4	87,5
25	87,5
35	85
45	85
50	82,5
55	82,5
60	82,5
70	80
80	78,75

According to the values in Table 4.2 the graph in Figure 4.5 shows the amounts of enzyme in beads as percent for each measure temperature values.

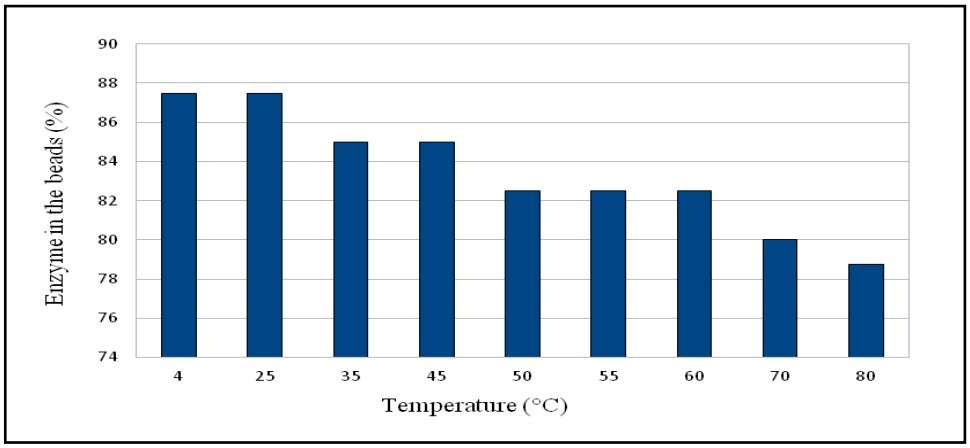

Figure 4.5. Entrapment efficiency at different temperatures for 5 minutes

As it can be seen from Figure 4.5, the amount of enzyme in the beads decreases when the temperature rises from 4°C to 80°C. This result is caused by the deformation of the beads when temperature rises. The ratio of enzyme in the beads decreases to 78.75% at 80°C while it was 87.5% at 4 °C.

4.3.2. Temperature Stability

Beside the effect of temperature on the activity of the enzyme, temperature stability is also another important parameter. In order to examine the effect of the temperature stability, enzyme has been incubated for 1 hour duration for different temperature values from 4°C to 80°C and then incubated again 5 more minutes after substrate has been added to the medium.

Table 4.3. The data for temperature stability test for 1 hour.

Temperature (°C)	**Absorbance #1**	**Absorbance #2**	**Calculated Activity # 1**	**Calculated Activity # 2**	**Mean Activity Value**	**Relative Activity**
4	0,689	0,688	84336,230	84214,490	84275,360	18
25	1,840	1,838	235454,540	235206,060	235330,300	51,47
35	2,560	2,561	322710,400	322835,820	322773,110	70,6
45	2,820	2,821	378055,550	378190,470	378123,010	82,710
50	2,830	2,833	379396,820	379798,410	379597,615	83,030
55	2,835	2,836	399070,000	399211,660	399140,830	87,310
60	3,033	3,029	457437,500	456833,920	457135,710	100,000
70	2,800	2,790	429974,540	428438,180	429206,360	93,890
80	2,420	2,421	371620,000	365135,710	368377,855	80,580

*All measurements were done @ 420 nm and @ 90 rpm shake.

The measurements have been performed and Figure 4.6 has been obtained according to the values in Table 4.3.

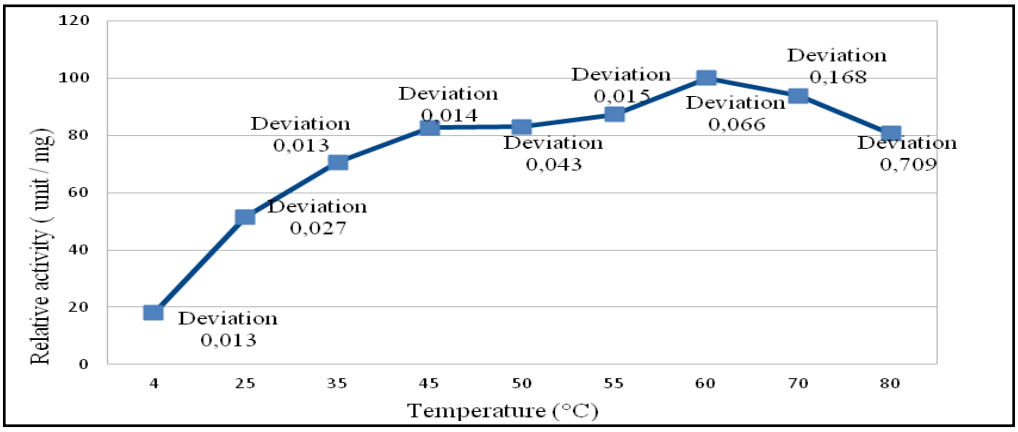

Figure 4.6. Effect of temperature stability on immobilized esterase enzyme relative activity for 1 hour.

The highest relative activity has been obtained at 60°C. However, above this temperature value at 70°C and 80°C a drop has been observed in relative activity. When these results are compared to the similar study of our friend Seçkin Gülay, it has been observed that the immobilization matrix of alginate-chitosan/$CaCl_2$ is more durable to the increasing temperature values. The most convenient temperature value is 55°C for free enzyme, 80°C for our immobilize enzyme and 45°C for Seçkin Gülay's enzyme (Gülay 2009).

4.3.2.1. Entrapment Efficiency

After 5 minutes of incubation time, the enzyme amount in the polyelectrolit beads is %78.75 at the thighest temperature level of 80°C. After 1 hour incubation time this value at 80°C is %68.75 and it is believed that this is caused by the increasing of incubation time. At high temperatures, if the incubation time of polyelectrolit beads increases, deformation rate also increases as directly proportional with temperature.

Table 4.4. Percent amount of enzyme in beads in temperature stability test for 1 h.

Temperature (°C)	4	25	35	45	50	55	60	70	80
% amount of enzyme in beads	86,25	82,5	83,75	78,75	78,75	75	70	68,75	68,75

Temperature stability effect analysis is documented in Table 4.4. As shown in Figure 4.7 enzyme amount slightly decreases as temperature rises.

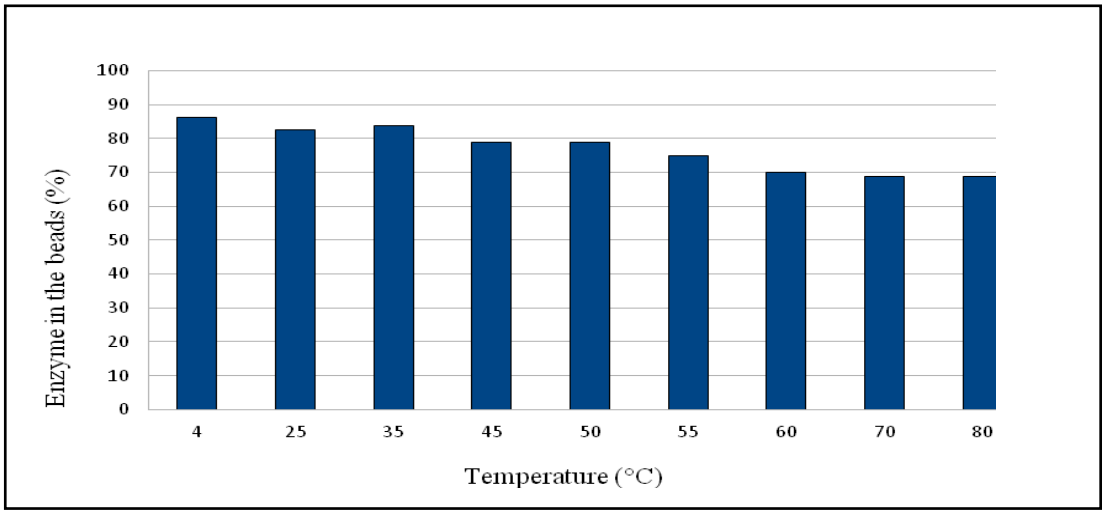

Figure 4.7. Entrapment efficiency at different temperatures for 1 hour.

4.3.3. Effect of pH on Thermophilic Esterase Activity

The activity of enzymes is strongly affected by changes in pH and each enzyme works best at a certain pH value, its activity decreases above and below that point. If the pH changes much from the optimum, the chemical nature of the amino acids can change. This may result in a change in the bonds and so the tertiary structure may break down. The active site will be disrupted and the enzyme will be denatured.

Investigation of pH effect and pH stability effect on enzyme activity were done at pHs ranging from 4.0 to 9.0. Specific activity results were calculated with using entrapment efficiency and change in absorbance values for 5 min and 1 hour.

For free enzyme maximum activity was observed in alkali pHs that means after pH: 8.0 (Tekedar 2008) and for immobilized enzyme of Seçkin Gülay this value was pH: 8.0 for the same conditions.

When compared our study to these two previous studies, as it can be seen from the chart below, our immobilized enzyme shows better activity at basic pH values. So it can be conclude that in this study, immobilized enzyme is resisted to alkaline pH changes.

Table 4.5. The data for pH effect test for 5 minutes

pH	Absorbance #1	Absorbance #2	Calculated Activity # 1	Calculated Activity # 2	Mean Activity Value	Relative Activity
4	0,115	0,116	13874,280	13995,710	13934,995	9,85
5	0,117	0,115	14115,710	13874,280	13994,995	9,89
6	0,114	0,118	13754,280	14237,140	13995,710	9,89
7	0,324	0,325	40241,170	40366,170	40303,670	28,500
7,5	0,458	0,460	59510,760	59770,760	59640,760	42,127
8	0,470	0,475	61069,230	61720,000	61394,615	43,410
8,5	0,712	0,710	92515,380	92255,380	92385,380	65,330
9	1,072	1,071	141468,750	141337,500	141403,125	100,000
10	0,822	0,821	108476,560	108345,310	108410,935	76,660
11	0,732	0,734	98133,33	98401,58	98267,455	69,49

*All measurements were done @55ºC @90 rpm @420 nm.

The measurements have been performed and Figure 4.8 has been obtained according to the values in Table 4.5.

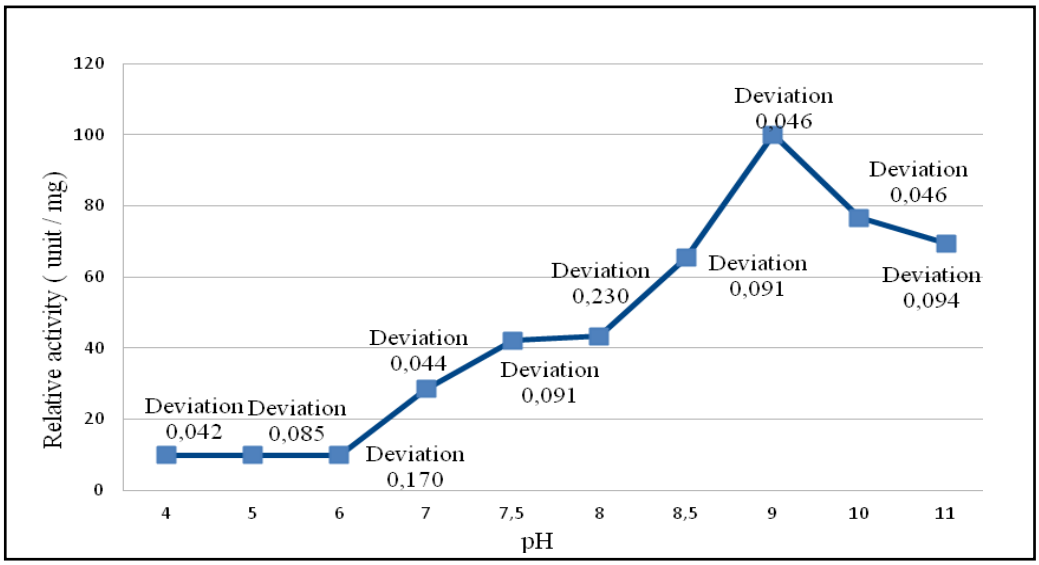

Figure 4.8. Effect of different pH values on immobilized esterase enzyme relative activity for 5 minutes.

The highest activity of enzyme has been observed at pH value of 9,0. This result is different from previous two studies and it may be caused by the ionic and chemical structure of the environment around the enzyme is different.

4.3.3.1. Entrapment Efficiency

As pH value becomes closer to the base pH, the amount of enzyme in the polyelectrolyte beads decreases. It can be stated that base medium causes deformation of beads. When pH stability of the enzyme after one hour is considered for the same values of pH; we see that activity of enzyme has become durable until pH value of 10.

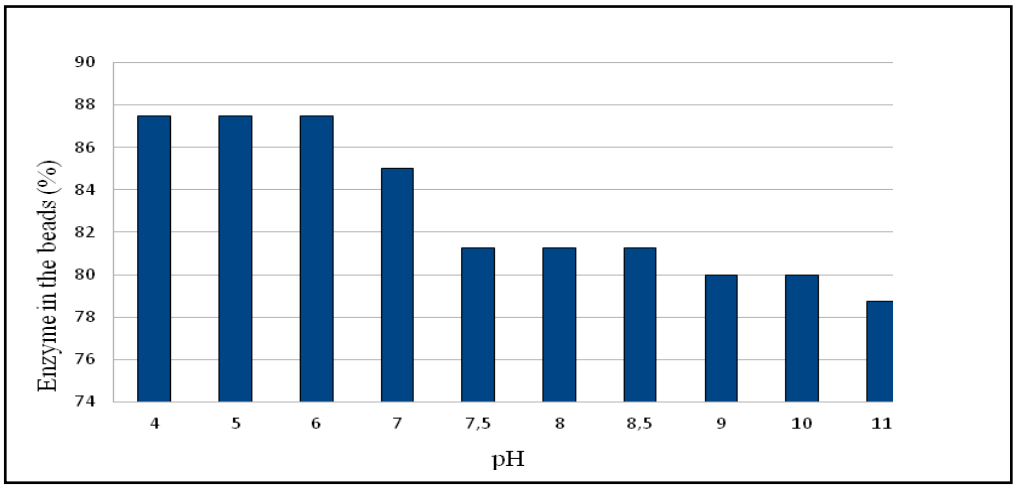

Figure 4.9. Entrapment efficiency at different pH values for 5 minutes

Figure 4.9 shows the percent enzyme amount in the beads according to pH change and for different pH values the amount of enzyme is shown in Table 4.6.

Table 4.6. Percent amount of enzyme in beads during pH effect test for 5 minutes

pH	% amount of enzyme in beads
4	87,5
5	87,5
6	87,5
7	85
7,5	81,25
8	81,25
8,5	81,25
9	80
10	80
11	78,75

4.3.4. pH Stability of Esterase Enzyme

Effect of different pH values on the activity of esterase enzyme was investigated and data is shown in Table 4.7.

Table 4.7. The data for pH stability test for 1 hour.

pH	Absorbance #1	Absorbance #2	Calculated Activity # 1	Calculated Activity # 2	Mean Activity Value	Relative Activity
4	0,059	0,060	7327,940	7451,470	7389,705	6,5
5	0,090	0,088	11178,450	10930,040	11054,245	9,73
6	0,132	0,133	16639,770	16765,830	16702,800	14,71
7	0,232	0,234	28815,580	29063,990	28939,785	25,490
7,5	0,267	0,269	33162,750	33411,160	33286,955	29,320
8	0,524	0,525	66054,860	66180,910	66117,885	58,240
8,5	0,880	0,881	110931,820	111057,880	110994,850	97,770
9	0,900	0,901	113453,000	113579,060	113516,030	100,000
10	0,891	0,892	112318,470	112444,530	112381,500	99,000
11	0,797	0,798	100468,93	100594,99	100531,960	88,56

*All measurements were done @55ºC @420 nm @90 rpm.

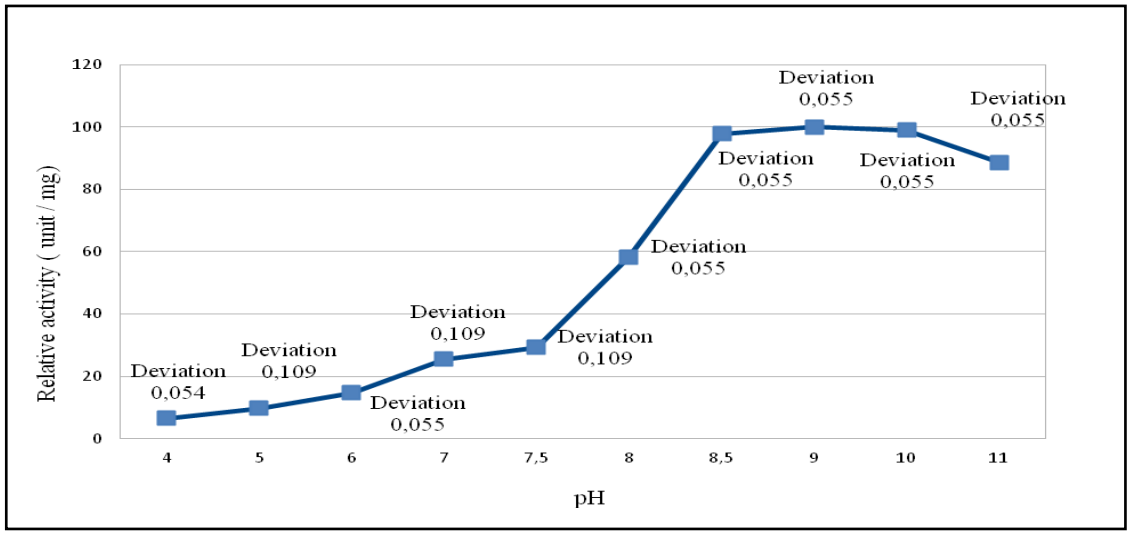

Figure 4.10. Effect of pH on immobilized esterase enzyme activity for 1 hour.

As shown in Figure 4.10 the highest activity of enzyme has been observed at pH value of 9,0 again like as the pH test for 5 minutes.

4.3.4.1. Entrapment Efficiency

Determination of pH stability for 1 hour was investigated among the pH of: 4.0-6.0-7.0-7.5-8.0-8.5-9.0-10.0 and 11.0. After incubating immobilized enzyme for one hour in different pH values, half of specific activity was still protected at pH: 10.0.

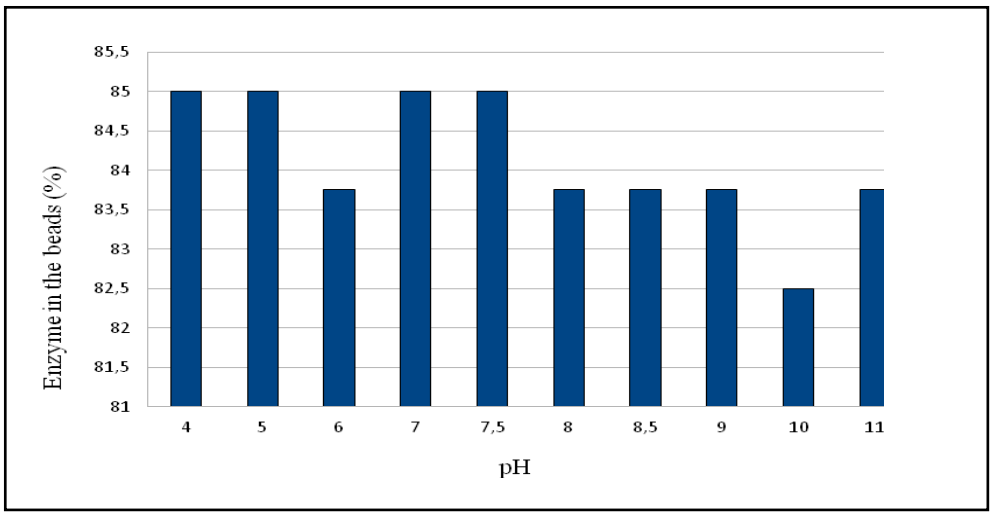

Figure 4.11. Entrapment efficiency at different pH values for 1 hour.

As a result, immobilized esterase enzyme for one hour was still displaying maximum activity at pH: 10.0. Specific activity of immobilized esterase enzyme versus pH values were shown in Figure 4.10 and entrapment efficiency of enzyme were shown in Figure 4.11 after one hour incubation time.

Table 4.8. Percent amount of enzyme in beads during pH stability test for 1 hour.

pH	**% amount of enzyme in beads**
4	85
5	85
6	83,75
7	85
7,5	85
8	83,75
8,5	83,75
9	83,75
10	82,5
11	83,75

4.3.5. Effect of Different Chemicals on Enzyme Activity

In this part, the effects of different chemicals on the immobilized enzyme activity were examined. The effect of chemicals is shown in Table 4.9.

Table 4.9. The experiment data of different chemical effects on immobilized enzyme.

Chemical Name	Time	Absorbance #1	Absorbance #2	Calculated Activity # 1	Calculated Activity # 2	Mean Activity Value	Relative Activity
No Chemical	5 min	0,451	0,454	56016,490	56389,100	56202,790	100
$CaCl_2$	5 min	0,455	0,457	56513,310	56761,720	56637,510	100,77
$ZnCl_2$	5 min	0,467	0,471	58003,770	58500,590	58252,180	103,64
$MgCl_2$	5 min	0,337	0,336	42481,840	42355,780	42418,810	75,47
$CuSO_4$	5 min	0,395	0,392	49061,000	48688,390	48874,690	86,96
$MgSO_4$	5 min	0,420	0,422	53746,920	54002,860	53874,890	95,85
SDS	5 min	0,000	0,000	0,000	0,000	0,000	0
Triton	5 min	0,221	0,219	32746,560	32450,210	32598,380	58

*Reaction conditions ; Temperature = 55 °C / pH=8,0 / Stirring speed = 90 rpm

The concentrations of the metal ions used were all 1 mM. The sample named as 'No Chemical' that did not contain any chemical was the control and we compared effects of other chemicals according to this control.

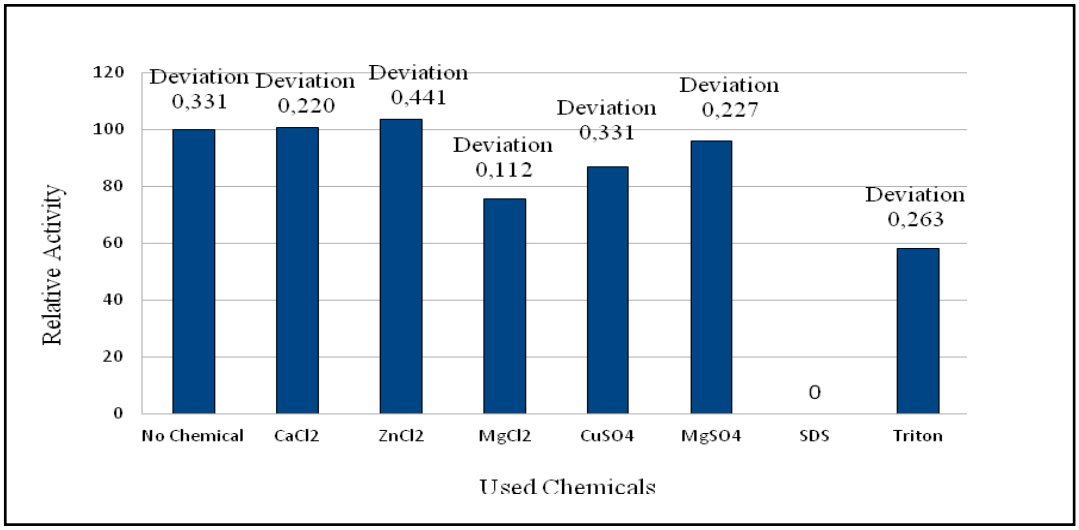

Figure 4.12. The effect of different chemicals on immobilized enzyme relative activity.

The results are shown that as in Figure 4.12, the activities of esterase were enhanced by $CaCl^{2+}$, $ZnCl^{2+}$ but slightly inhibited by $MgCl^{2+}$, $CuSO_4$, Triton X-100 and SDS showed strong inhibitory effect on our immobilized enzyme.

4.3.6. Reuse of Immobilized Enzyme

The experiment conditions: 55°C, 90 rpm, 5 min. incubation time, p-NPA as substrate.

Table 4.10. The experiment data for reuse of beads.

# of experiments	Absorbance #1	Absorbance #2	Calculated Activity # 1	Calculated Activity # 2	Mean Activity Value	Relative Activity
1	0,452	0,451	56140,690	56016,490	56078,590	100
2	0,423	0,419	52538,750	52041,930	52290,340	93,2
3	0,327	0,326	41221,250	41095,190	41158,220	73,39
4	0,220	0,219	28586,270	28456,340	28521,305	50,850
5	0,118	0,119	14656,200	14780,400	14718,300	26,240
6	0,107	0,110	13488,300	13866,470	13677,385	24,380
7	0,062	0,062	7934,070	8317,970	8126,020	14,490

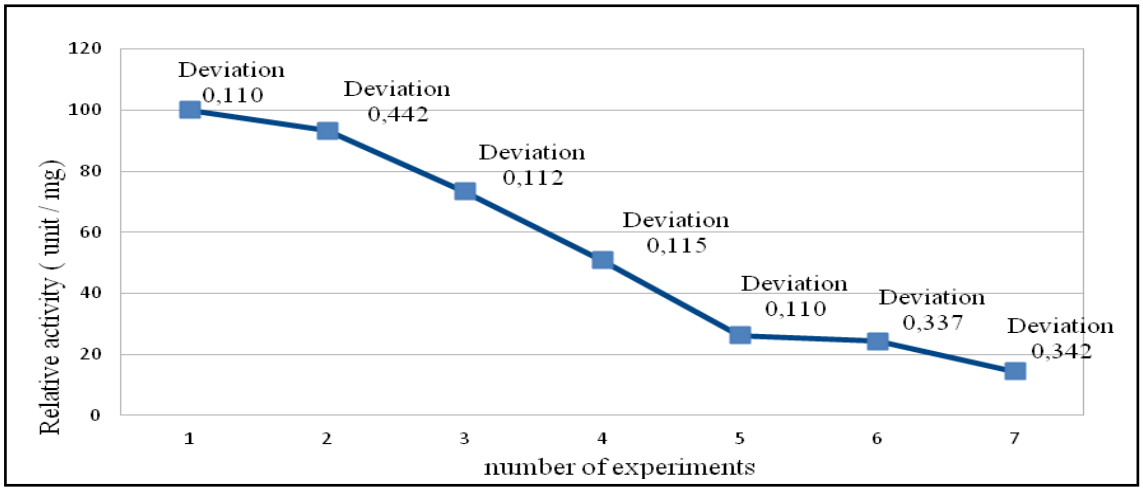

Figure 4.13. Effect of reuse of immobilized esterase enzyme on relative activity.

In order to test the stability of esterase microencapsulation into polyelectrolyte beads, the beads were used seven times for the hydrolysis reaction. Each run lasted the

beads were separated and washed with firs distilled water. The reaction medium was then changed with fresh medium. As a result of this test, the activity measurement was determined as 100% for the first run. During other trials, acticity decreased more and more. During 7th trial relative activity was almost zero.

4.3.7. Effect of Bead Size on Immobilized Esterase Enzyme

The size of the beads was measured by Scanning Electron Microscope. The diameter of each bead was measured at three different angles and averaged. 12 beads were used to give an average bead size. The average bead size measured by an optical microscope was 2.6±0.2, 1.8±0.2 and 3.9±0.3 mm. Specific activities of esterase entrapped in the beads decreased as the bead size increased.

Table 4.11. Activity values according to the radius of the beads.

Radius of the bead (mm)	Absorbance #1	Absorbance #2	Calculated Activity # 1	Calculated Activity # 2	Mean Activity Value	Relative Activity
2,6	0,428	0,431	55613,300	56003,110	55808,200	84,6
1,8	525,000	0,521	66180,910	65676,680	65928,790	100
3,9	0,396	0,394	52259,290	51995,350	52127,320	79

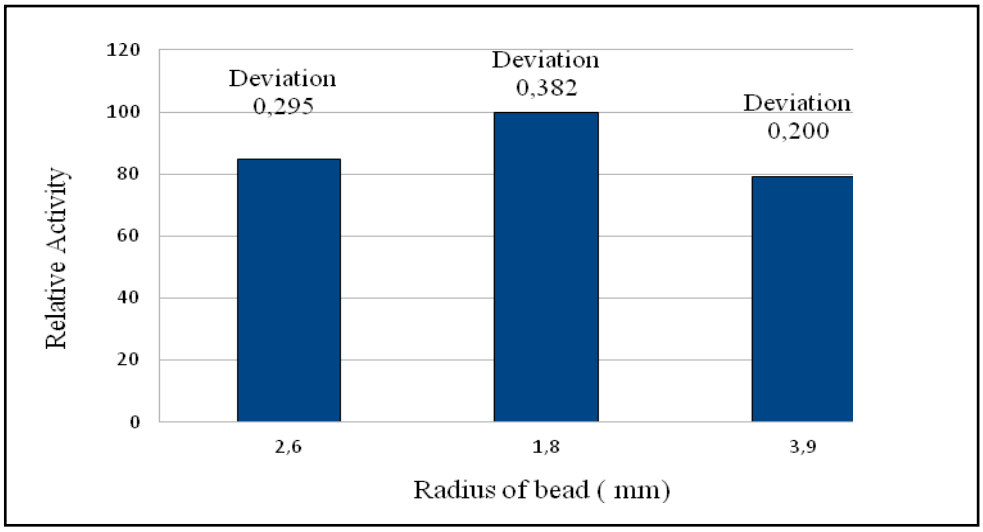

Figure 4.14. The effect of bead size on immobilized enzyme mean activity.

4.3.8. Scanning Electron Microscope (SEM)

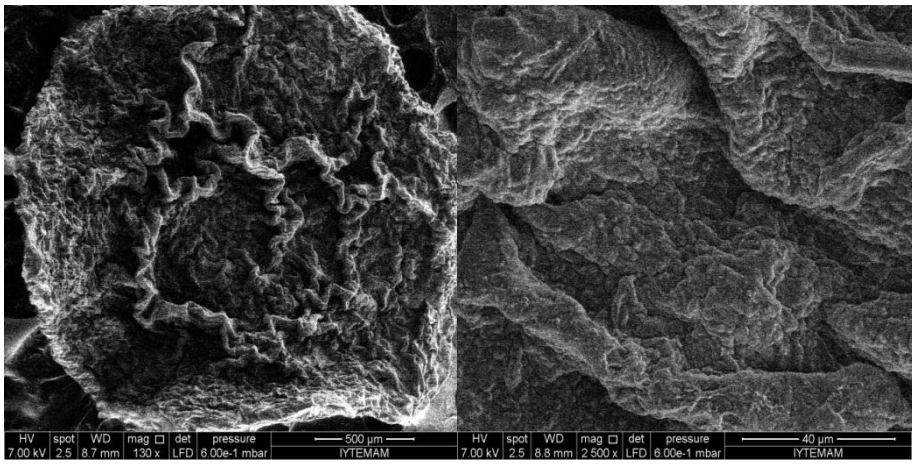

Figure 4.15. SEM photos of the surface morphology of the chitosan-alginate beads.

The surface morphology of dried beads was studied using scanning electron microscopy (SEM). All samples were coated with gold prior to observation. As shown in Figure 4.15 the surface of the coated beads has looked like a mesh, and has very compact structures.

CHAPTER 5

CONCLUSION

In this study, firstly the recombinant thermophilic esterase enzyme expressed and purified from *E.Coli* strain. Purification of esterase protein was performed via one-step affinity chromatography method. Then during immobilization procedure, purified protein successfully microencapsulated into the Alginate-Chitosan/$CaCl_2$ polyelectrolyte beads. Immobilization yield was 80.40%. After purification, the characterization studies were done such as effect of temperature and pH, temperature and pH stability, effect of bead size on immobilized enzyme, effect of chemical agents; re-use of immobilized enzyme and lastly SEM (Scanning Electron Microscopy) analyses of beads. Our experiments have shown that the esterase immobilized alginate-chitosan/$CaCl_2$ polyelectrolyte beads exhibits an improved resistance against thermal and pH denaturation when compared with Gülay's immobilized beads (Gülay 2009). According to the pH stability results the optimum pH of esterase enyzme was investigated that relatively alkaline pH values. Also especially we can say that SDS shows strong inhibitory effect on activity of our immobilized esterase. And this temparature and pH improvement enables applicability for use this enzyme in industrial processes and other further laboratory studies.

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APPENDIX A

CHEMICALS, SOLUTIONS AND BUFFERS

CHEMICALS:

Isopropyl-β-D thiogalactopyranoside (IPTG)

4-Nitrophenyl Acetate (pNPA)

Alginate

NaCl

Immidazole

Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$)

SOLUTIONS:

Luria Bertani (LB) broth, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl and dH_2O up to 1 L.

Luria Bertani (LB) agar, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar and dH_2O up to 1 L.

BUFFERS:

Na-P phosphate buffer: for 1 L 0.1M stock buffer ph=7

1M Na_2HPO_4 --57.7ml

1M NaH_2PO_4--42.3ml diluet to 1L with 900ml distile water.

APPENDIX B

REAGENTS AND GEL PREPARATION FOR SDS-PAGE

Stock Solutions

A. 30% Acrylamide Mixture

• 29.2g acrylamide

• 0.8g N'N'-bis-methylene-acrylamide

Make up to 100 ml with distilled water. Filter and store at 4ºC in the dark for at least one month.

B. 1.5M Tris-HCl, pH 8.8

• 18.15g Tris Base

• ~80ml distilled water

Dissolve Tris base in distilled water; adjust to pH 8.8 with HCl. Make up to 100ml with distillled water and store at 4ºC.

C. 0.5M Tris-HCl, pH 6.8

• 6g Tris Base

• ~80ml distilled water

Dissolve Tris base in distilled water, adjust top H 6.8 with HCl. Make up to 100ml with distilled water and store at 4ºC.

D. 10% SDS

Dissolve 10g SDS in 90ml water with gentle stirring and bring to 100 ml with distilled water.

E. Sample Buffer

• 3.8 ml deionized water

• 1.0ml 0.5M Tris-HCl, pH 6.8

• 0.8ml Glycerol

• 1.6ml 10% (w/v) SDS

• 0.4ml 2-mercaptoethanol
• 0.4ml 1% (w/v) bromophenol blue

F. 5X Running Buffer
• 15g Tris Base
• 72g Glycine
• 5g SDS

Dissolve Tris base, glycine and SDS in ~800ml deionized water and make uo tp 1L with water. Store at 4ºC. For electrophoretic run, dilute 5X stock solution to 1X with deionized water.

G. 10% Ammonium persulfate (APS)
Dissolve 0.1g APS in 1ml deionized water. This solution should be prepared fresh daily.

H. Colloidal Coomassie Staining Solution
Dissolve 40g ammonium sulfate in ~300ml water, add 8ml 85% o-phosphoric acid and add 0.5g Coomassie Brilliant Blue G-250. Make up to 400ml with water, add 100ml methanol to 500ml total volume. Store at 4ºC.

I. Neutralization Buffer
0.1M, pH 6.5 Tris-phosphate in deionized water.

J. Destaining Solution
25% (v/v) methanol solution.

K. Fixation Solution
20% (w/v) Ammonium sulfate in deionized water.

Seperating Gel Preparation (1.2%)

Deionized water	3,35 ml
1,5 M Tris-HCL, pH:8,0	2,5 ml
10%(W/V) SDS stock	100 µl
30% Acryamide / Bis	4.0 ml
10% daily prepared Ammonium Persulfate	50 µl
TEMED	5µl
Total monomer	10 ml

Stacking Gel Preparation (4%)

Deionized water	3,05 ml
0,5 M Tris-HCl, pH:6,8	1.25 ml
10% (W/V) SDS	50 µl
30 % Acrylamide / Bis	665 µl
10 % ammonium persulfate daily prepared	25 µl
TEMED	
Total stock manomer	5 ml

APPENDIX C

PREPARATION OF BRADFORD REAGENT

• 10.0mg Coomassie Brilliant Blue G-250 (CBB G-250)
• 5ml 95% ethanol
• 10ml 85% phosphoric acid

Dissolve CBB G-250 in ethanol, add 10ml phosphoric acid. Bring to 100 ml with ultra pure water and when the dye has completely dissolved, filter through Whatman No. 1 paper. Store at 4ºC.

Preparation of BSA standards from 0,2 mg/ml BSA and Test Sample for the Bradford Protein Assay:

Test Sample	Sample Volume, µl	Water Volume, µl	Coomassic Reagent Volume, µl
Blank	0	800	200
BSA Standard – 1 µg/ml	5	795	200
BSA Standard – 2 µg/ml	10	790	200
BSA Standard – 4 µg/ml	20	780	200
BSA Standard – 6 µg/ml	30	770	200
BSA Standard – 8 µg/ml	40	760	200
Protein Sample	2	798	200

Bradford Protein Concentration Assay Experiment Table:

	Water	Sample Protein	Bradford Mixture
Blank	800 µl	-	200 µl
Sample	795 µl	5 µl	200 µl