IMMOBILIZATION OF THERMOPHILIC RECOMBINANT ESTERASE ENZYME BY ENTRAPMENT IN COATED Ca-ALGINATE BEADS

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ABSTRACT

IMMOBILIZATION OF THERMOPHILIC RECOMBINANT ESTERASE ENZYME BY ENTRAPMENT IN COATED Ca-ALGINATE BEADS

Recently, industrial enzymes produced by micro-organisms are being utilized widely, especially from thermophilic ones due to their ability to withstand intense heat. Esterase enzymes from thermophilic micro-organisms are special interest in a variety of biotechnological applications because of their many useful properties. Due to potential use as biocatalysts in variety of biotechnological applications, esterase enzyme isolated from Balçova (Agamemnon) geothermal site were aimed to be immobilized via a cost-effective protocol, in order to be re-used over very long periods of time**.**

Previously, the gene encoding thermophilic esterase from the thermal environmental samples, isolated from Balçova (Agamemnon) geothermal site, was cloned and respective protein was expressed in *Escherichia coli* in our group. In this study using that recombinant esterase enzyme, expression, purification and immobilization studies were carried out. The esterase enzyme was immobilized in the Ca-alginate beads which were coated with silica and the effects of the temperature and pH on the immobilized enzyme was determined and diameter of the beads, reuse and surface of the beads were analyzed. Immobilization yield for coated beads was determined as 71.27% and compared with non-coated ones which were 45.80%. Analysis of surface morphologies of beads was compared with Scanning Electron Microscope.

ÖZET

TERMOFİLİK REKOMBİNANT ESTERAZ ENZİMİNİN KAPLI Ca-ALGİNATE BONCUKLARINA IMMOBİLİZASYONU

Günümüzde mikroorganizmalar tarafından üretilen endüstriyel enzimlerden, özellikle yüksek sıcağa dayanıklı termofiliklerden, büyük oranda yararlanılmaktadır. Termofilik organizmalardaki esteraz enzimleri, birçok kullanışlı özelliklerinden dolayı, biyoteknolojik uygulamalarda büyük öneme sahiptir. Balçova jeotermal tesislerinden izole edilen esteraz enzimini biyoteknolojik uygulamalarda potansiyel biyokatalizör olarak kullanılabilmek için uygun maliyetli bir yöntemle immobilize edip tekrar kullanımını amaçladık.

Kodlanması yapılmış olan, Balçova termal bölgeden elde edilmiş esteraz enzimi, grubumuz çalışmalarıyla klonlanıp, ilgili protein *Escherichia coli*'ye ekspres edilmiştir. Bu çalışmada rekombinant esteraz enziminin üretimi, saflaştırılması ve immobilizasyonu yapılmıştır. Üretilen esteraz enzimi silikayla kaplı Ca-alginat boncuklarda immobilize edilip, sıcaklık ve pH'ın immobilize esteraz enzim üzerindeki etkisi ve boncuk çapı, tekrar kullanılabilirlik, boncukların yüzey yapısı incelenmiştir. İmmobilizasyon verimi kaplı ve kapsız boncuklarda karşılaştırılmıştır, %71.27 verimle immobilize edilmiş kaplı boncukların kapsızlara oranla %45.80 verimle enzim tutukladığı gözlemlenmiştir. Boncukların yüzey yapıları taramalı elektron mikroskobuyla incelenmiş ve karşılaştırılmıştır.

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CHAPTER 1

INTRODUCTION

1.1. Immobilization

Enzymes are macromolecules that act as a catalyst to induce chemical changes in other substances, while their self remaining apparently unchanged by the process. They are three-dimensional biological catalysts, only produced in living organisms.

The variety of chemical transformations catalyzed by enzymes has made these molecules a prime target of exploitation by the emerging biotechnology industries such as fine chemistry, food chemistry, therapeutics applications, decontamination processes, protein engineering. The use of enzymes in these applications has been limited by some factors, mainly their stability, high cost of production, availability in small amounts. It's difficult and expensive to recover them from reactor effluents at the end of the catalytic process because of their solubility. From this point of view, the transformation of chemical processes that from the laboratory to industry catalyzed by enzymes one of the most complex and exciting goals in biotechnology (Bugg 2001).

Over last few decades, intense research in the area of enzyme technology has provided many techniques that make easy their practical applications. Some of these techniques are protein engineering of existing enzymes, chemical modification of enzymes, addition of additives and immobilization (Iyer and Ananthanarayan 2008). Among them, the newer technological development in the field of immobilized biocatalysts can offer the possibility of a wider and more economical exploitation of biocatalysts.

Immobilized biocatalysts are not only enzymes, but also cells or organelles (or combinations of these). For many industrial applications, enzymes and cells have to be immobilized, via very simple and cost-effective protocols, in order to be re-used over very long periods of time (Meena and Raja 2006).

1.1.1. Advantages of Immobilization

Enzymes, in addition to their excellent functional properties (activity, selectivity, specificity, able to catalyze the most complex chemical processes under the most benign experimental and environmental conditions), also have some characteristics that are not very suitable for industrial applications:

- They are soluble catalysts, they are usually very unstable
- They may be strongly inhibited by substrates and products
- They only work well on natural substrates and under physiological conditions (Bugg 2001).

Enzyme immobilization technology can be an effective way to reuse enzymes and to improve their functional properties with these advantages that are given below:

1- Processes can be readily controlled and operated continuously.

- Facilitated process control
- Low residence time
- High volumetric activity
- Optimization of product yield.

2- Provides higher purity and product yields.

3- Greater pH and thermal stability.

4- Product inhibition is less apparent.

5- Effluent problems are minimized

6- Products are easily separated.

7- Greater flexibility in reactor design.

8- Catalyst can be reused.

9- Needs no enzyme isolation and purification (For microorganism and cells).

Immobilization increased enzymatic stability in extreme conditions of temperature, pH and organic solvents; recovery and reuse of the enzymes. Despite these advantages, industrial application is still limited by the cost of carriers, mass transfer limitations, traditional attitudes, changes in properties (selectivity), activity loss during immobilization (Guisan 2006).

1.1.2. Immobilization Methots

The main components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment or entrapment.

Ideal matrix properties include physical resistance to compression, hydrophilicity, stability under the conditions to be used, biocompatibility, resistance to microbial attack, and availability at low cost. Supports can be classified as inorganic and organic according to their chemical composition. The organic supports can be subdivided into natural and synthetic polymers (Kennedy and White 1985).

Table 1.1. Examples of Carriers Used for Enzyme Immobilization
(Source: Kennedy and White 1985).

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. ZrO_2, TiO_2, Al_2O_3)

Chemical and physical qualities of the support material including porosity, particle size and functional group on the surface have to be considered when making a choice for the immobilization technique (Guisan 2006).

The selection of the immobilization technique (the mode of attachment or entrapment) is based on the process specifications for the enzyme used because it

affects the kinetic parameters of the immobilized enzyme. The most commonly used immobilization methods are shown in Figure 1.1.

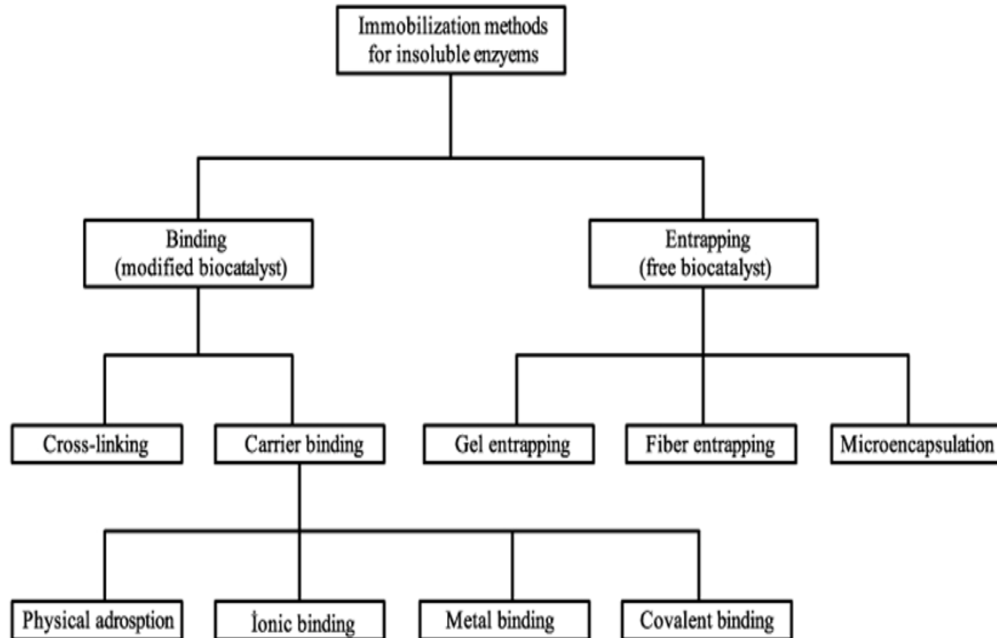


Figure 1.1. Immobilization methods for insoluble enzymes.

These immobilization techniques are sorted into as chemical and physical methods. Chemical methods involve the formation of covalent bonds between the functional group on the enzyme and functional groups on the support material while physical methods do not involve covalent bonding with the enzyme. Methods for enzyme immobilization can be classified into three main categories (Guisan 2006):

- Binding (modified biocatalyst)
- Entrapment (free biocatalyst)

1.1.2.1. Binding

This method can be sub-classified into cross-linking and carrier binding.

1.1.2.2. Cross-linking

Cross-linking is based on the formation of chemical bonds, as in the covalent binding method, but water-insoluble carriers are not used. It can be explained as the intermolecular cross-linking of enzymes by biofunctional or multifunctional reagents. Glutaraldehyde is the most common reagent that used for cross-linking.

Crosslink is a simple and rapid procedure to immobilized enzymes. It can be usefully employed in conjunction with other methods, namely to minimize leakage of enzymes already immobilized by adsorption. However the cross linking reaction is not easily controlled and so it is very difficult to obtain large enzyme aggregates with high activity retention. Cross-linking reactions are carried out under relatively severe conditions. Figure 1.2 These rough conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity (Sheldon et al. 2006).

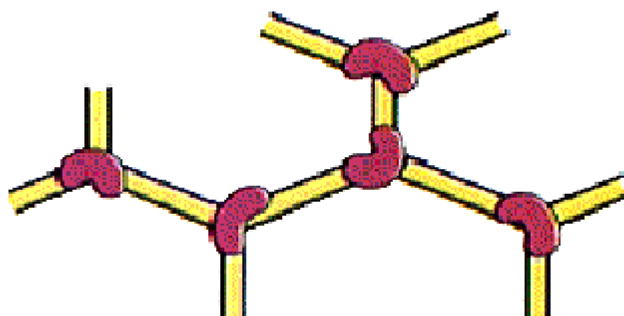


Figure 1.2. Schematic illustration of cross-linking method.
(Source: UFL 2009)

1.1.2.3. Carrier Binding

Carrier binding method is described as the binding of enzymes to water-insoluble carriers. It’s the oldest immobilization method for enzymes.

In this method shown in Figure 1.3, the amount of enzyme bound to the carrier and the activity depends on the nature of the carrier. The most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, agarose,

dextran, and polyacrylamide gel. The important factors of choosing carrier are (Cao 2006):

- High enzyme capacity and retained activity.
- Chemical inertness.
- Physical stability.
- Mechanical strength.
- Low cost, possibility of regeneration.
- Food grade (for use in food applications).

And the selection of the carrier depends on the particle size, surface area and molar ratio of hydrophilic to hydrophobic groups, chemical composition, and the characteristic properties of the enzyme (Cao 2006).

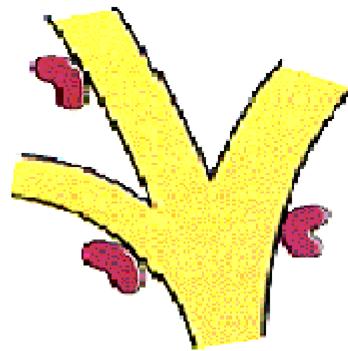


Figure 1.3. Schematic illustration of carrier binding method.
(Source: UFL 2009)

According to the binding mode of the enzyme, carrier-binding method can sub-classified into (Cao 2006):

1. Physical Adsorption
2. Ionic Binding
3. Metal Binding
4. Covalent Binding

1.1.2.2. Entrapment

The entrapment method is based on the sequestering of an enzyme (or a cell or a multi enzyme) within a polymeric network that allows the substrate and products to pass through but retains the enzyme. This method differs from the binding, in that the enzyme is not bound to the matrix or membrane. Enzyme molecules are free in solution, but entrapped by lattice or membrane. This membrane can be alginate, carrageenan, agarose, polyacrylamide, pectin, gelatin, chitin or chitosan (Wadiack and Carbonell 1975). There are some advantages of entrapment (Guisan 2006):

- Enzymes are immobilized without a chemical or structural modification that prevents hindrance of active side of the enzyme so there is no loss of activity because of the intermolecular linking. Denaturation is usually avoided.
- Entrapment is a general technique which has a wide applicability.
- Possibility of simultaneous immobilization of different enzymes in a single step and combination of other methods such as cross linking.
- Large specific area for the effective contact of the substrate and the enzyme.
- Biocompatibility. For example calcium alginate gels, are reported to be nontoxic for cells and hence suitable for cell immobilization.
- Easy, simply and low cost.

Despite these advantages, this method also has limitation such as possible leakage of enzyme during repeated use because of semi-permeable surface of matrix. In my experiment, crosslink with TEOS and hexane is used to prevent leakage. Next disadvantage is diffusion limitations. The practical use of entrapment methods is limited by mass transfer limitations through membranes or gels. The diffusion rate is sometimes the limiting parameter according as molecular weight of the substrate and the product. Generally, high substrate concentrations are necessary in order to limit its influence. Entrapped enzymes are better used with small substrates since larger ones may not be able to pass the membrane and reach the active site of the biocatalyst (Won, et al. 2005). The fundamental drawback of gel immobilization is the low mechanical strength of beads, which limits the duration of the working period. Trivalent ions have been used with good results as hardening agents with the aim of improving the mechanical

strength. On the other hand, high gel concentrations also fine down bead stability, even if diffusion limitations appear (Dominguez 1999).

1.1.2.2.1. Entrapment with Ca-Alginate

Alginates are naturally occurring polysaccharides extracted from the brown seaweeds (*Phaeophycea*) found in the shoal waters of temperate zones. In the 1880s, the first preparation of alginate was made by Stanford and a pure isolation was afterwards achieved by Krefting, then near 1930s that alginate was first exploited commercially. Different species of brown seaweeds (such as *Laminaria hyperborea* and *Macrocystis pyrifera)* and some bacteria (such as *Pseudomonas and Azotobacter)* responsible for producing alginates with different chemical composition. These algaes are prone to compositional variability due to seasonal and environmental changes (Remminghorst and Rehm 2006). Alginates do not have any regular repeating unit. They are unbranched binary copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G). The alginate monomers occur as regions made up exclusively of one unit of M blocks or G blocks or as alternating structures (MG blocks).

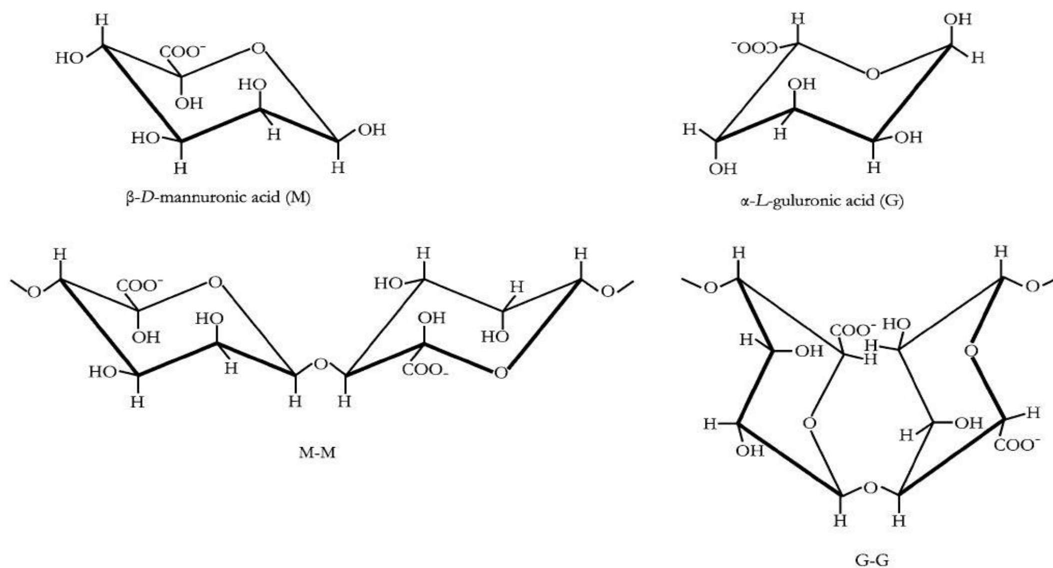


Figure 1.4. Chemical structures of mannuronic (M) and guluronic (G) acid monomers and their secondary structure as blocks (MM or GG) in the alginate chain. (Source: Remminghorst and Rehm 2006)

Alginate is a negatively charged polymer, imparting material properties ranging from viscous solutions to gel. It will form gels with a large number of divalent cations under controlled conditions.

The rigidity of the ionic alginate gels increases generally with the affinity of the ion in the order: Mn>Co>Zn>Cd>Ni>Cu>Pb>Ca>Sr>Ba. However, most of these ions cannot be used for immobilization. In general, Ca^{+2} is the most frequently employed ion for such purposes because it's low toxic (Smidsrød 1973).

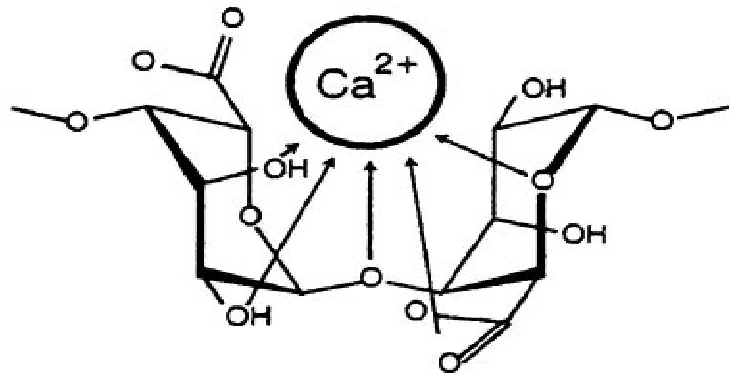


Figure 1.5. Ca and alginate interactions

Na (Alginate) + Ca^{++} ⟹ Ca $(Alginate)_2$ + 2 Na^+

(Source: KHYMO 2009)

When dripped sodium alginate into a solution containing calcium ions, each calcium ion (which holds a charge of +2) knocks away two sodium ions (each holding a charge of +1). The alginate molecule contains loads of hydroxyl groups (OH's) in its M and G blocks that can be coordinated to cations.

The gel-forming ability of the alginate is mainly related to molecular size and the composition of the alginates and its content of guluronic acid. Diaxially linked G blocks create a cavity that acts as a binding site for the cation (Mizuno, et al. 1983).

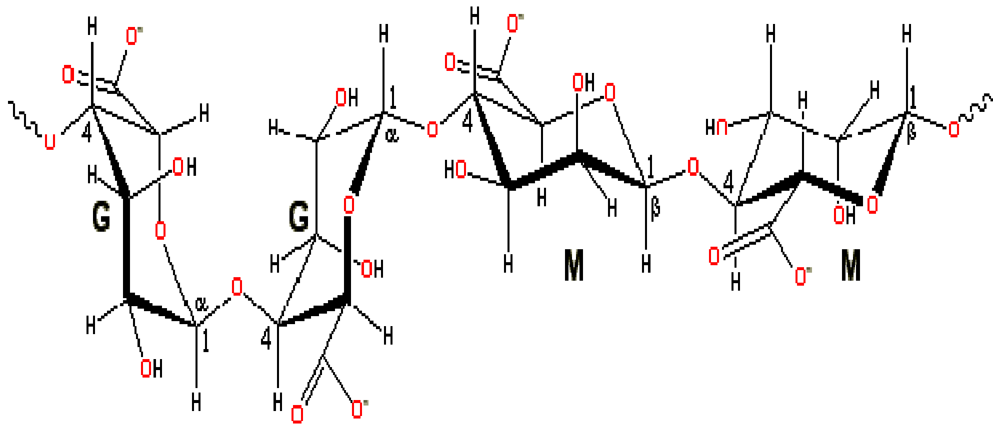


Figure 1.6. Alginate polymer
(Source: FREEWEBS 2009)

High-G alginates, with their long G-blocks and short elastic segments, create more open static networks and bind the divalent ions making gels with in general highest mechanical strength, more stability to chelating compounds, and higher porosity (Smidsrød 1973). When preparing alginate beads other important factors are pH, temperature, source, alginate type and viscosity, presence of sequestrates in the cross-linking solution (such as TEOS-hexane), concentration of calcium ions, and microencapsulation method.

1.1.2.2.1.1. Entrapped in droplets

They are three types of entrap in droplets with using alginate. Immobilization with bead, alginate-polylysine capsule, Michigan University process.

Figure 1.7. Entrapped in droplets
(Source: UFL 2009)

- Immobilization with beads: Entrapment of catalyst to a semi-permeable polymer. The preparation of enzyme microcapsules requires extremely well controlled conditions. Enzyme is mixed with sodium alginate solution and then the mixture is stirred thoroughly to ensure complete mixing. As soon as the mixed solution is dripped into $CaCl_2$ solution with a syringe, Ca-alginate beads are formed. After hardening, the beads are separated from the calcium chloride solution by vacuum filtration (Won, et al. 2005).

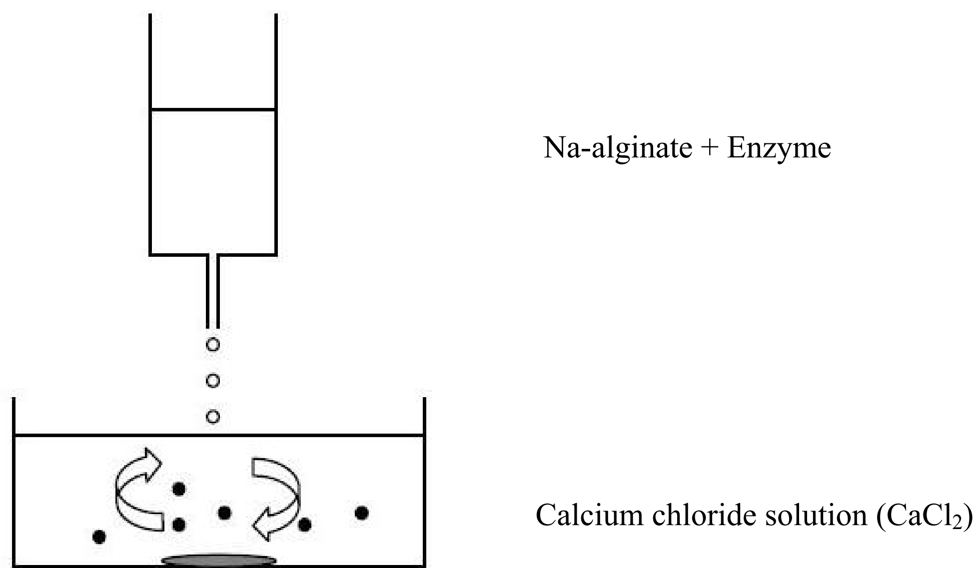


Figure 1.8. Enzyme immobilization with Ca-alginate beads.

- Alginate-polylysine capsule: Firstly enzymes or cells are immobilized in alginate beads, and then form a semipermeable alginate-poly-L-lysine membrane with coating the surface of the beads with poly-L-lysine. After injection citrate into the beads, capsules are obtained. Small molecules such as oxygen can easily diffuse into the capsules, although catalyst (cell, enzyme or multi enzymes) could not get out of the membrane (Strand, et al. 2002).

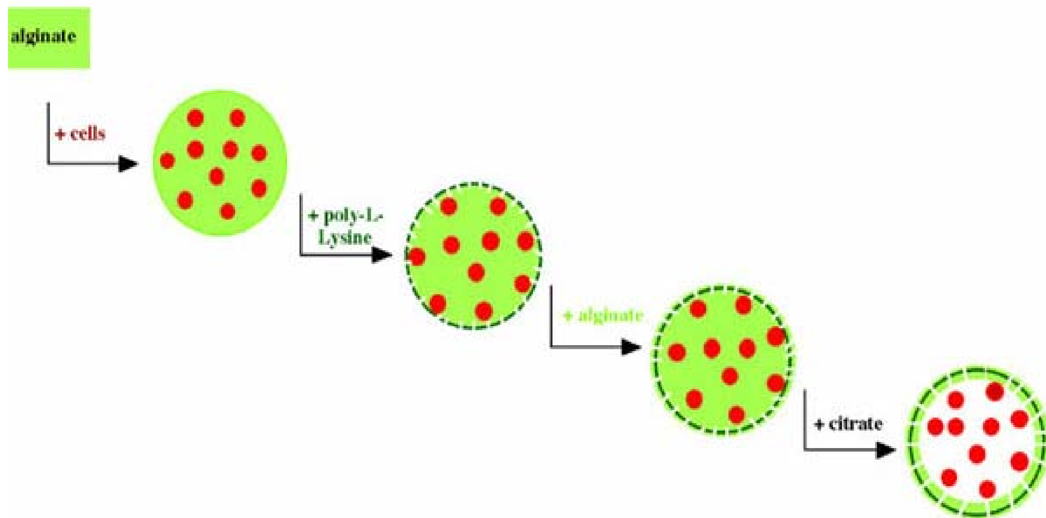


Figure 1.9. Formation of capsule with using polylysine

- Michigan University process: Enzyme is mixed with calcium chloride solution and then the mixture is stirred. Mixed solution is dripped into Na-alginate solution with a syringe and capsules are formed. Then capsules are kept waiting in $CaCl_2$ solution to make them more stern. Some chemicals (such as Xantham gum) are included enzyme and calcium chloride solution to have more strong and well shape capsules. The inner part of the capsules is liquid in Michigan University process and alginate-polylysine capsule method so enzymes are more active than immobilization with beads method (Chang, et al. 1998).

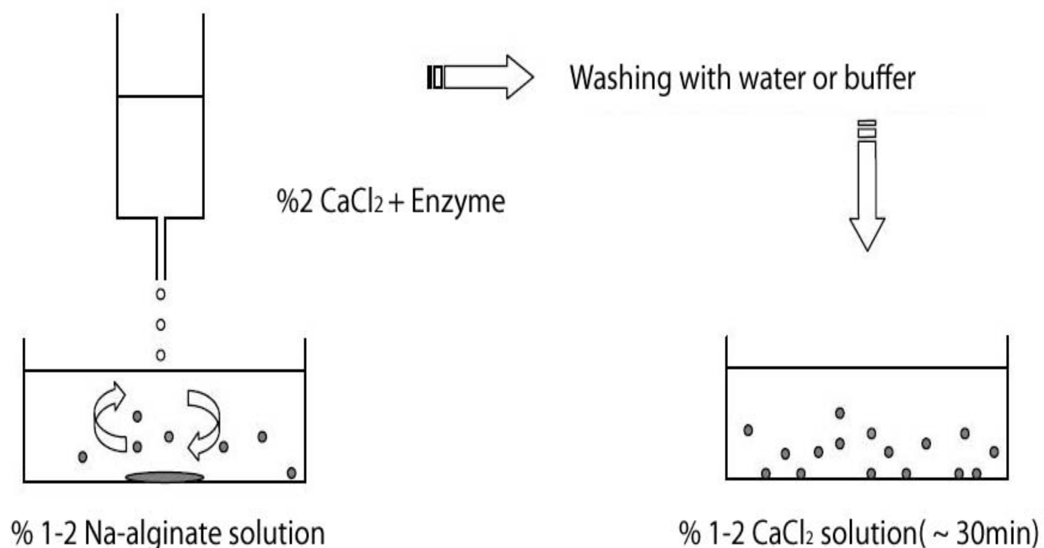


Figure 1.10. Michigan University process.

The immobilization of cells by entrapment in calcium alginate is a well-known technique. However, entrapment of enzymes in gels is not very efficient because the entrapped enzymes leak out during the course of time as a result of the large pores of the matrix.(Won, et al. 2005). To overcome this problem, various methods have been attempted, some of which are: bead can be coated with a membrane (hexane-TEOS), molecular weight of the enzymes can be increased with the help of polymer molecules, enzymes can binded to alginate covalently using carbodiimid, beads can strengthened with the help of colloidal silica, dried to reduce pores of beads (Guisan 2006).

1.1.2.2.1.2. Entrapped in a matrix

Some synthetic polymers such as polyacrylamide, polyvinyl alcohol, etc. and natural polymer have been used to immobilize enzymes using this technique that called lattice-type entrapment. Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. This method offers several advantages as resistant to weak acids and alkalis, high ionic strength, some organic solvents. However their use is limited to low-molecular weight substrates due to steric hindrance and inactivation of the enzyme may occur as a result of the use of precipitating agents, polymer solvents and water immiscible liquids (Guisan 2006).

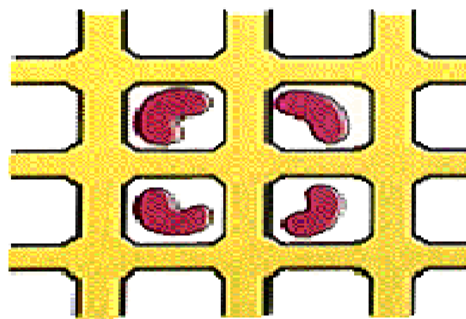


Figure 1.11. Enzyme entrapped in a matrix
(Source: UFL 2009)

1.1.3. Comparison of immobilization methods

Enzymes can be immobilized in different places for different purposes. In general, preparation, binding force, enzyme activity, applicability, and stability reusability properties of immobilization are important when working at the laboratory scale. It can be assumed that simplicity and the cost-effectiveness of immobilization are not very essential when working in a small scale but they would be critical for industrial applications. All of these points should be considered when an enzyme needs to be immobilized. There is a comparison of immobilization methods, which can be seen in Table 1.2.

Table 1.2. Comparison of immobilization methods
(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

1.2. Extremophiles

Extremophiles are organisms that thrive in and even may require physically or geochemically extreme conditions. They are classified, according to the conditions in which they exist, such as halophiles, acidophiles, alkaliphiles, psychrophiles, and barophiles. The discovery of extremophiles is very important because it points out the extraordinary adaptability of primitive life-forms. And also further raises the prospect of finding at least microbial life elsewhere in the solar system and beyond. There is also growing support for the idea that extremophiles were among the earliest living things in

the world. Extremophiles are a combination of the suffix 'phile', meaning "lover of" and a prefix specific to their environment. For example, thermophiles are organisms that love (phile) thermo (Rainey and Oren 2006).

1.2.1. Thermophiles

Temperature is an important environmental factor and contributing cause for all living organisms. For this reason classification of living organisms according to their relation to temperature is essential for biological systematic. Classification and examples of organisms according to their thermal tolerance can be seen in table 1.3.

Table 1.3. Classification of organisms according to their thermal tolerance (Baker, et al. 2001)

Environmental parameter	Type	Definition	Examples
Temperature	Hyperthermophile Thermophile Mesophile Psychrophlie	Growth > 80^0C Growth 60-80^0C 15-60^0C <15^0C	Pyrolobusfumarii,113C Synechococcus livids Homo sapiens (human) Psychobacter.

Thermophiles, the microorganisms that love heat, are further subdivided into three categories in accordance with their minimal and maximal growth temperatures as follows: moderate thermophiles (35-70ºC), extreme thermophiles (55-85ºC) and hyperthermophiles (75-113ºC) (Baker, et al. 2001).

1.2.1.1. Thermophilic Bacillus

Bacillus is a genus of rod-shaped, aerobic and facultatively anaerobic, gram-positive, endospore-forming, high phylogenetic heterogeneity bacteria. Bacillus genus consists of extremophiles such as acidophilic, alkalophilic, thermophilic, psychrophilic, and halophilic bacteria (Rainey and Oren 2006).Bacillus spores are very important for medical, biotechnological and industrial applications. In addition to medical uses, bacillus spores, due to their extreme tolerance to both heat and disinfectants, are used to

test heat sterilization techniques and chemical disinfectants. Bacillus are also used in the detergent manufacturing industry for their ability to synthesize important enzymes. (Henstra 1996) Forexample esterase, lipases, proteases, xylanases, glucose-isomerases, amylases and DNA restriction endonucleases can be synthesized from thermophilic bacillus. They grow optimally at high temperatures ranging from 50 to 85ºC and can be isolated from not only thermophilic but also mesophilic environments (Khalil 2002).

In this experiment thermophilic bacillus are isolated from Balçova (Agamemnon) Geothermal region.

1.2.1.2. Thermophilic Enzymes

Thermophilic microorganisms are able to survive in hot temperatures because of their ability to produce thermophilic enzymes. However most mesophilic enzymes, being proteins, begin to denature around 40 degrees, and are generally completely inactive beyond 50-60 degrees. What makes this difference? Although there is an evolutionary relationship (such as respiration, anatomy and metabolic processes) between mesophilic and thermophilic microorganism, there are lots of important discrepancies (Steel and Walker 1991).

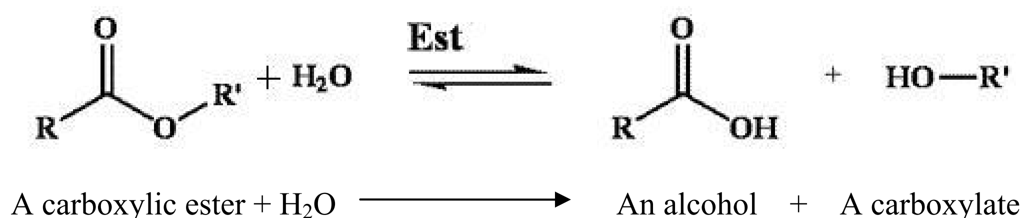
1) Adaptational difference: At high temperatures, thermophiles conserve their stability without transforming into spores, where as mesophiles forms spores in order to survive in harsh environmental conditions. An evolutionary explanation of this differences based on physical concepts of protein design ability (Berezovsky and Shakhnovich 2005).
2) Structural difference: In thermophiles, the constitution of proteins are compact and flexible. These properties are essential for stability in elevated temperatures (Steel and Walker 1991). Actually homologous mesophilic and thermophilic enzymes are very similar with their amino acid sequences, catalytic mechanisms and superimposable three-dimensional structures (Vieille and Zeikus 2001). But the thermophilic enzymes are not only more strong with their good general conformational structure (compact packing) and additional intermolecular interactions (hydrophobic interactions, disulfide bonds, electrostatic interactions metal binding and hydrogen bonds that are not exist in mesophilic enzymes) but

also more stretchy with their conformational strain release and specific amino acid compositions (Steel and Walker 1991).

3) Higher Hydrophobicity: Thermophilic enzymes mostly included charged residues such as Glu, Lys, Arg, Ser, Ala, Thr, Val and Ile that have the ability to provide an increase in the formation of ion pairs and their networks (Berezovsky 2006). These charges residues cause increase in hydrophobicity and decrease in flexibility. And also Gly→Ala and Lys→Arg exchanges leads to increase the frequency of helix forming residues while lowering the rate of helix breaking residues. It's known that higher alanine content in thermophilic proteins was supposed to reflect the fact that Ala was the best helix-forming residue (Vieille and Zeikus 2001). Proposed explanations offer that organisms evolved as mesophiles but later re-colonized a hot environment relied in their evolutionary strategy of thermophilic adaptation on "sequence-based" mechanism of thermostability (Berezovsky and Shakhnovich 2005). But hydrophobicity not only based on sequence but also relevant to cell membrane composition. The cell membranes of thermophiles are formed saturated fatty acids. This construction responsible for the rigidity of the membrane and provides a hydrophobic environment for the cell at high temperatures.
4) Chaperons and DNA gyrase: Thermophilic organisms produce specialized proteins for a repair mechanism, called chaperones. Chaperones help to refold the proteins that are misfolded or lost proper 3D structures to their native form after denaturation to maintain their functions (Robb, et al. 2007). Reverse DNA gyrase is a a unique type I DNA topoisomerase help stability at elevated temperatures with producing positive supercoils in the DNA by increasing the melting point of DNA (Robb, et al. 2007).

1.3. Esterase

An esterase is a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction with water called hydrolysis that shown in figure1.12.



A carboxylic ester + H_2O ⟶ An alcohol + A carboxylate

Figure 1.12. Hydrolysis

The present invention relates to an isolated or modified esterase describes having excellent thermo stable properties which can be utilized for ester hydrolysis reaction, ester synthesis reaction, transesterification, stereospecific hydrolysis, catalyze short chain substrates (Zhang, et al. 2003). These properties are (Kim, et al. 2008):

- Do not require cofactors.
- Broad substrate specificity.
- Enzymatic activity in both aqueous and nonaqueous solvents.
- Enantioselective that catalyzes the reaction of only one of a pair of enantiomers.
- Stable in organic solvents. It is proportional to its stability in high temperatures in an aqueous solution
- Stereoselectivity that the preferential formation in a chemical reaction of one stereoisomer over another.

There are several kinds of esterase. Three factors set each type of esterase apart from the others: substrate specificity, biological function, and protein structure (Kademi, et al. 2000). Lipases (EC 3.1.1.3) comprise a subclass of the esterases because it's a soluble enzyme that catalyzes the hydrolysis of ester bonds in water–insoluble, lipid substrates (>10 carbon atoms). Esterase hydrolyze small ester containing molecules (<10 carbon atoms) for example the specificity pattern showed that a marked substrate specificity for mid-chain-length fatty acids (3-8 carbon atoms) classified the enzyme as a carboxylesterase (EC 3.1.1.1) (Kademi, et al. 2000).

1.3.1. Biotechnological and Industrial Applications of Esterases

By producing more stable esterase enzymes and their special properties, they have numerous biotechnological, industrial and environmental applications in fine

chemistry, food industry (especially development of flavour in cheese), oleochemical and detergent industry (especially thermostable forms of these enzymes are important for laundry for cleaning of oil), cosmetics, pharmaceutical industries, biodiesel production and in sewage treatment (Kim, et al. 2008, Khanna, et al. 2009).

Esterases used for modify the physicochemical properties of triglycerides for organic synthesis reactions (Kademi, et al. 2000) and as a chiral biocatalyst useful for production of pharmaceuticals through the reactions involving various amines and primary or secondary alcohols. Stereospecific nature of enzyme is also used for resolution of the racemic mixtures in numerous aspects of organic chemistry (Panda and Gowrishankar 2005). Esterases are also used in the production perfumes, antioxidants and synthesis of optically pure compounds. And also with their enantioselective properties provide opportunity to synthesis of agriculturally important chemicals and pharmaceuticals anticancer drugs (Panda and Gowrishankar 2005).

Despite its attractive properties, isolation of esterases in high amounts is not an easy task unlike the lipases so enzyme stability should be increased to resist harsh conditions with using immobilization methods (Kademi, et al. 2000).

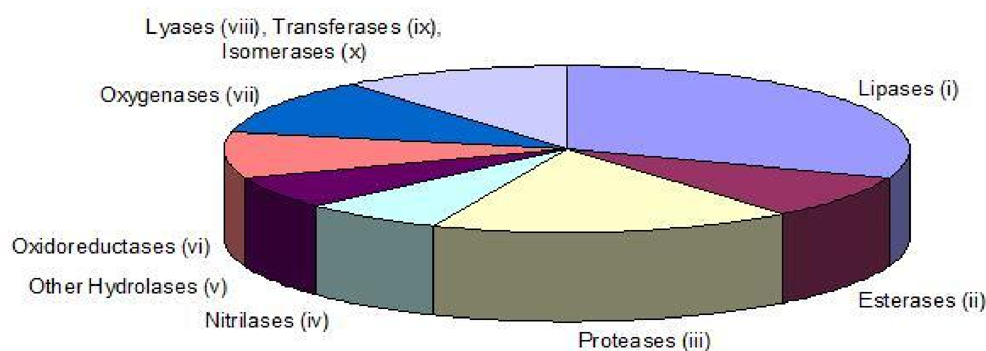


Figure 1.13. Biotransformation of Organic Compounds
(Source: Biocatalysis.Uni-Graz, 2009)

1.4. Previous Immobilization Studies with Esterase

1-Esterase: Hexa-arginine-tagged esterase (Arg6-esterase).

Immobilization method: Gold-coated magnetic nanoparticles were used as a carrier.

The enzymatic activity of the immobilized Arg (6)-esterase was investigated by monitoring the dissociation rate of its colorimetric substrate, p-nitrophenol butyrate (pNPB). The immobilized enzyme exhibited a lower activity compared to the free one, however its original activity was found to be retained even after seven times repetitive uses by magnetic decantation (Jeong, et al. 2006).

2- Esterase: Hexa-arginine-tagged esterase.

Immobilization method: Gold nanoparticles using mixed self-assembled.

In this study, the formation of MSAMs on gold nanoparticles (AuNPs) and the immobilization of hexa-arginine-tagged esterase (Arg(6)-esterase) on the surfaces of the resulting particles were examined. Arg(6)-esterase was immobilized in a highly specific manner onto AuNPs treated with mixed SAMs (MSAM-AuNPs) by providing a shielding ligand which reduce the non-specific adsorption of enzymes caused by hydrophobic interaction compared to AuNPs treated with single-component SAMs (SSAM-AuNPs). Moreover, Arg(6)-esterase immobilized on MSAM-AuNPs showed substantially enhanced catalytic activity up to an original activity compared to that on SSAM-AuNPs (Jeong, et al. 2010).

3- Esterase: Recombinant thermostable esterase (Pf2001) from Pyrococcus furiosus.

Immobilization method: Immobilization on microporous polypropylene.

A recombinant thermostable esterase (Pf2001 Delta 60) from the hyperthermophilic archaea Pyrococcus furiosus was immobilized on microporous polypropylene (Accurel MP 1000). The adsorption was rapid, with 90% total protein and esterase activity being retained in the first 15 min. No desorption of the enzyme was detected in a time course of 180 min after immobilization, which indicates an intense enzyme-support interaction. Effect of enzyme immobilization on activity was evaluated by retention activity parameter. This parameter was dependent of the protein/support ratio at the beginning of the immobilization process (Almeida, et al. 2008).

4- Esterase: Cholesterol esterase.

Immobilization method: Immobilization on conducting polypyrrole films.

Fabrication of an amperometric cholesterol biosensor by co-immobilization of cholesterol esterase (ChEt) and cholesterol oxidase (ChOx) onto conducting polypyrrole (PPY) films using electrochemical entrapment technique was described. Characterization of resulting amperometric biosensor for the estimation of cholesterol had been experimentally determined in terms of linear response range, optimum pH, applied potential, temperature, and shelf-life (Singh, et al. 2004).

5- Esterase: Carboxylic ester hydrolase, EC 3.1.1.3 from Bacillus subtilis
Immobilization method: Covalent crosslinking Cross-Linked Enzyme.

Immobilize enzymes as Cross-Linked Enzyme. Aggregates (CLEAs) consists of covalent cross linking of precipitated enzymes. This efficient and economically good method yields immobilized biocatalysts that do not include support material and therefore have a high activity per unit volume (CLEA Technologies 2007)

6- Esterase: Esterase enzyme
Immobilization method: Covalently bound to a solid support insoluble in water.

Lovastatin esterase enzyme immobilized on a solid support insoluble in water, the enzyme being covalently bound to a solid support activated with an at least difunctional coupling reagent, the immobilized lovastatin esterase exhibiting at least 5 times higher the hydrolytic activity towards lovastatin and salts thereof, in the presence of simvastatin and/or salts thereof, than towards simvastatin and salts thereof. Invention relates to a biocatalytic flow reactor with a bed, comprising a body of the reactor with an inner space connected to the fluid inlet and connected to the fluid outlet, in which inner space there is a bed containing the lovastatin esterase enzyme immobilized on a solid support insoluble in water (Ostaszewski, et al. 2009)

7- Esterase: Pregastric esterase
Immobilization method: Immobilized in a continuous flow, hollow-fiber reactor.

A kid goat pregastric esterase immobilized in a-continuous flow, hollow-fiber reactor was employed to hydrolyze anhydrous milkfat, producing lipolyzed butter oil. The effects' of temperature and pH on the rate of lipolysis were monitored by titration of the effluent stream for total acidity. Nonlinear regression analyses indicated that the

experimental data were consistent with the form of the ping pong bi mechanism that assumes that the rate-controlling step is acylation of the enzyme (Hill, et.al. 2002).

8- Esterase: Sterol esterase

Immobilization method: Immobilized on polyacrylate epoxy-activated carriers.

The sterol esterase from the ascomycete Ophiostoma piceae was immobilized on novel polyacrylate-based epoxy-activated carriers (Dilbeads(TM)). The distribution of the enzyme in the support was studied by fluorescence confocal microscopy. The immobilized esterase on Dilbeads(TM) TA showed a significant pH and thermal stability and was assayed in the continuous hydrolysis of cholesteryl esters -present in the pulp industry process waters (Torres, et al. 2008)

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Recombinant esterase enzyme from *Thermophilic Bacillus sp*. that was isolated from Balçova (Agamemnon) Geothermal region in Izmir have used in our studies (Tekedar 2008). Expression of the enzyme in *E.coli* and purification using one step affinity chromatography were carried out according to Tekedar (2008) procedures.

Some important properties of Thermophilic Bacillus esterase can be seen in Table 2.1. (Tekedar 2008).

Table 2.1 Some properties of Thermophilic Bacillus esterase

Molecular Weight (kDa)	27 kDa
Substrats	PNPA,PNPB
Optimum Temperature (^{0}C)	$55^{0}C$
pH Optimum	7-11 with Na-P Buffer

Throughout this study, distilled and deionized water was used in all preparations. All the analyses were carried out at least duplicate and mean values are reported. Detailed list of used chemicals, buffers, solutions and their compositions are presented in Appendix A and Appendix B.

2.2. Methods

2.2.1. Protein Sample Preparation

2.2.1.1. Escherichia Coli Growth

Expression vector including esterase gene transformed *E. Coli* that have been stored at -90 °C, was spread on a LB^{kan} agar plate and incubated overnight in incubator

at 37°C. A single colony was chosen from the incubated plate and inoculated in LB^{kan} media of 50ml continuing incubation at 37^0C for 15-16 hours in a shaker.

2.2.1.2. Expression of the Transformated Genes

Kanamycin was included in all media to maintain selective pressure for the vector because expression vector contains a kanamycin resistant marker. After 15 hours samples were diluted into 450ml fresh LB^{kan} media, total sample was 500 ml. The incubation was kept with a shaker at 37^0C and 198 rpm until observing the wanted 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. Sample was incubated in the shaker for an additional 4 h to allowed cells growth after adding IPTG. Recombinant esterase's cells were harvested by centrifugation **(**Optima Max, 150000 rpm, Beckman-Coulter) at 5000 rpm for 20 min. At the end, supernatant parts were removed away and pellets were obtained which contain bacterial cells. The harvested cells pastes were stored frozen at –20 °C until use for purification.

2.2.1.3. Total Protein Extraction

Cell pellets were dissolved in 50mM sodium phosphate buffer, pH 7.0 (phosphate buffer) and disrupted by a sonicator (Sim-Aminco, Spectronic Instruments) for 10 min. to break cells into pieces. Centrifugation was done at 5000 rpm for 20 min to remove cell debris. The supernatant that includes all proteins of cells was ready for purification.

2.2.1.4. Protein Purification and Determination

2.2.1.4.1. Affinity Chromatography

His-taq Nicel Affinity column (2.5 cm x 10 cm His-taq Nicel Affinity (Sigma)) were washed with phosphate buffer (50mM pH 7.0) to equilibration. After column equilibration supernatant was loaded onto it. 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 purpose at least five column volume.

2.2.1.4.2. 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 dialysed against phosphate buffer (50mM pH 7.0) to get rid of the imidazole coming from elution buffer of affinity column.

2.2.1.4.3. Dialysis

Previously prepared dialysis bag according to manufacturer's direction was washed with distilled water then collected samples were place into it. Top and bottom sides of the bag were closed with pincers and place into phosphate buffer pH 7.0 to get rid of the imidazole that comes from elution buffer. The bag was stirred at $+4^{0}$C for one night then the final dialyzed sample was collected and stored at -20^{0}C. SDS-PAGE method was used to determine the homogeneity and molecular weight of the esterase.

2.2.1.4.4. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) studies were carried out according to Laemmli, U.K. (Laemmli 1970). Firstly all supplies were cleaned with alcohol. Separating gel was prepared in reference to Appendix B. and components were mixed in the order shown. Polimerization began as soon as the TEMED was added. So without any delay, mixture was swirled and poured into gap between the glass plates. In order to avoid drying, the gel was covered by distilled water in each steps of SDS-PAGE. Polimerization completed after 30 min. then overlay was poured off and the top of the gel was washed several times with deionized water to remove any unpolimerized acrylamide. The top of the gel was drain thoroughly. This step was followed with the preparation of stacking gel using the values given in Appendix B. Stacking gel solution was poured directly onto the surface of the polimerized resolving gel. Instantly a comb was inserted into the stacking gel solution. After waiting 30 min for polimerization, the comb was removed carefully and gel was placed into the buffer. Reservoirs were filled until it reached the filling line level with 1X Tris-Glycine-SDS running buffer. Sample buffer was used for mixing with the protein sample that shown in Appendix B. and heated at 95°C in water bath for 4-5 minutes.

Comb had 10 holes placed on the gel, 4 of them were use in this study. After each of samples (10mikroliter) and marker (5mikroliter) were loaded the gel in buffer tank, electrophoresis run at constant voltage at 65 volts for 30 minutes and followed by constant voltage at 100 volts for 2 hours until the blue dye reached at the bottom of the gel. After electrophoresis, the gel was taken out from two glass plates with the help of a spatula and placed into the shallow staining container. Colloidal Coomassie Staining Solution that shown in Appendix B was applied on gel for 24 hours with 25 rpm shaking. The gel was washed with water after the coloration process and photo of gel was taken using the gel photo system.

2.2.1.4.5. Protein Concentration Determination

After determination of homogeneity and molecular weight, enzyme was concentrated with vacuum (Memmert, VO400) to use efficiently. Protein concentration

determination was spectrophotometrically measured at 595 nm according to Bradford's method (Bradford 1976) with using Bovine Serum Albumin (BSA) as a standard protein. Bradford Assay Calibration Graph was prepared in the range of 1µg/ml and 60 µg/ml. To determine immobilized enzyme concentration all beads were washed 3 times on a filter with water to remove the enzyme that wasn't entraped. The filtered $CaCl_2$ solution and washings were collected and tested for enzyme concentration to determine the entrapment efficiency. The amount of capsulated protein was determined indirectly from the difference between the amount of protein introduced into the reaction mixture and the amount of protein in the filtrates and also in washings after immobilization. This value was also given immobilizaiton yield that explained in section 3.2.2.

2.2.1.4.6. 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 *p*nitrophenyl (*p*-NP) esters with different acyl chain lengths (C2-C16) in previous studies (Tekedar 2008). In this study, one unit was defined as that quantity of enzyme that would liberate 1 µM of p-nitrophenol per minute under the conditions of assay (Ganske 2009).

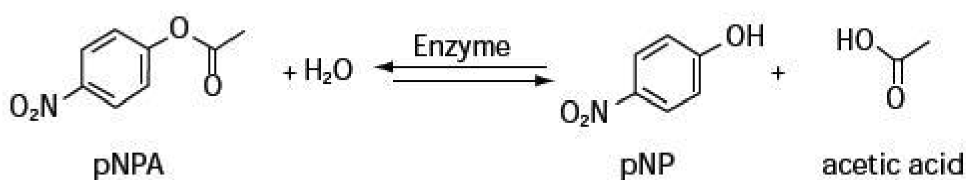


Figure 2.1. Scheme of the pNPA assay

The measurements of enzyme activity in this study (both free and immobilized enzyme) were done with the Shimadzu spectrophotometer (UV-2450-2550) at 420 nm. Free esterase enzyme activity determination was done with the values were given in Table 2.2. Enzyme was used in very low among because of high concentration.

Table 2.2 Esterase Activity Determination

	Sample	Blank
Tris-HCl buffer (0.1M - pH:9.0)	990 µl	990 µl
Enzyme	0.5 µl	0.5 µl
Substrat	9.5 µl	-
Water	-	9.5 µl

For the activity of immobilized enzyme ten solutions were prepared that included each one 2985 ml Tris-HCl buffer (0.1M pH 8.0), 15 ml substrate (50 mM pNPA) and 10 silicate coated beads with enzyme. These 3 ml solutions were shaken slowly for 15-30-45-60-90-120-150-180-240 and 300 seconds at 55^0C and taken 1 ml each of them to determine the enzyme activity and leakage of enzyme if any.

Activity calculation is same as the free enzyme. Units were calculated using the equation 2. 1.

$$\text{Units/ml enzyme} = \frac{\Delta A_{420nm}}{\varepsilon \times E} \qquad (2.1)$$

ΔA: Absorbance of the reaction sample.

ε : Extinction coefficient (0.0148)

E : Volume of enzyme used in a milliliter.

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,

$$\text{Specific enzyme activity} = \frac{\text{Enzyme activity}}{\text{Mg of protein/ml}} \qquad (2.2)$$

2.2.2. Immobilization of Thermophilic Esterase Enzyme in Coated Ca-Alginate Beads

2.2.2.1. Optimization of Thermophilic Esterase Enzyme in Ca-Alginate Beads

Firstly qualitative alginate beads determination was done with blank beads (beads with no enzyme). The best shaped ones were chosen with observation and waiting for 2 months in $CaCl_2$ and water at 55^0C, the best stable ones were chosen for further studies. For quantitative determination of the beads variety of % alginate concentration, $CaCl_2$ concentration, buffer conditions (both concentration and pH) and enzyme concentration were studied. Alginate concentration analyzes were done between %1 - %4 and $CaCl_2$ concentration were investigated for 0.05 M, 0.3 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, 1.0 M and 1.3 M. Convenient solutions to solve alginate were determined with 0.1 M- 0.5 M- 0.05 M Tris-HCl buffer and water. For solution 0.1 Molar Tris-HCl buffer was chosen and investigated in between pH: 5. 5, 7. 4, 8. 0, 8. 4, 9. 0, 9. 4, 10 and 11. Esterase solution with the concentrations of 0.01 U/ml, 0.1 U/ml and 0.5 U/ml were applied in Ca-alginate gel. The most efficient condition for immobilization of esterase was identified with spectrophotometrically using *p*-nitrophenyl acetate (50mM) as a substrate.

2.2.2.2. Immobilization of Thermophilic Esterase Enzyme in Ca-Alginate Beads

Alginate (w/v %2) was mixed with 0.1M-pH 8.0 Tris-HCl buffer for 30 min. at 50^0C. The solution was waited in $+4^0$C for 10 min. to eliminate bubbles. Enzyme (0.5 mg/ml) was mixed with alginate solution (total 10 ml) then the mixture was stirred thoroughly to ensure complete mixing for 10 min. As soon as the mixed solution was dripped into 75 ml of $CaCl_2$ solution (0.7 M) with a syringe, Ca-alginate beads were formed. The bead size was changed by using syringes with different needle diameters. After 30 min of hardening, the beads (more or less 500 beads) were separated from the calcium chloride solution by vacuum filtration. They were washed on a filter 3 times

with distilled water. Immobilization of thermophilic esterase enzymes in Ca-alginate was shown in figure 2.2.

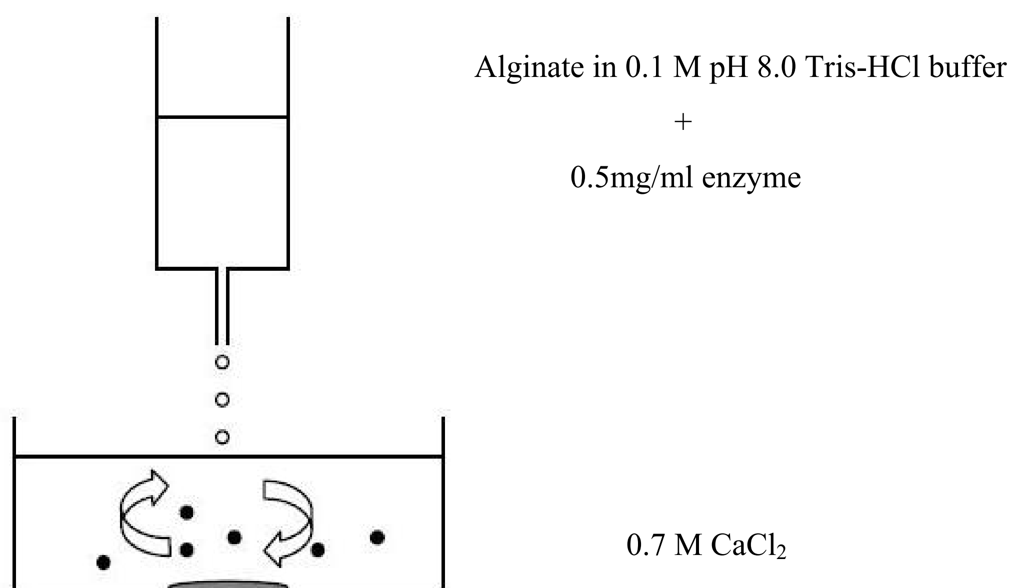


Figure 2.2. Thermophilic Esterase immobilization conditions in Ca-alginate.

2.2.2.3. Coating Ca-Alginate Beads with Silicate

After immobilization of esterase enzyme in Ca-alginate beads, in order to prevent enzyme from leaking out of the gel beads, beads were coated with silicate. Beads were waited in 4 ml n-hexane and 6 ml TEOS (tetraethyl orthosilicate) for 24 hours (Won, et al. 2005). Then they were washed on a filter 3 times with distilled water. Determination of leakage was calculated with Bradford assay at 595 nm and hexane-TEOS-water interaction absorbance was eliminated with using blank includes hexane-TEOS-water solution. Leakage in $CaCl_2$ solution was observed at 280 nm and calculated with a standard curve because it gives reaction with Bradford reagent.

2.2.3. Characterization of Immobilized Thermophilic Esterase Enzyme

The coated alginate beads were prepared according to the method described earlier. Firstly, time-absorbance graphics were examined to determine proper term for

characterization. Ten solutions were prepared that includes each one 2985 ml Tris-HCl buffer, 15 ml substrate (50 mM pNPA) and 10 coated beads with enzyme. These 3 ml solutions were shaken slowly for 15-30-45-60-90-120-150-180-240 and 300 seconds at 55^0C and taken 1 ml each of them to observe activity. All of characterization studies were done according to this proposal.

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/non-coated beads. Studies were compared with free thermophilic esterase enzyme. All leakage enzyme concentration determination was done with using Bovine Serum Albumin (BSA) as a standard protein.

2.2.3.1. Effect of Temperature and pH

In order to find the effect of temperature for 5 min. and 1 hour on immobilization of thermophilic esterase, temperatures were investigated with the incubation (with slow shake) of 10 coated beads at 4, 25, 35, 45, 50, 55, 60, 70 and 80^0C in 2985 ml Tris-HCl buffer (0.1 M pH 8.0) for 5 min. with 15 ml substrate (50 mM pNPA). Then 1 ml solution was taken from each of them to observe activity. Determination of thermal stability for 1 hour was investigated incubation at 4-25-35-45 and 50^0C. After 1 hour 15 ml substrate (50 mM pNPA) was added and solution was shaken for 5 min. more in 55^0C. Then 1 ml solution was taken from each of them to observe activity. The procedure indicated in section 2.2.1.4.6. was followed for determination of specific activity.

And in order to find effect of pH on immobilization of thermophilic esterase, pH values were investigated with the incubation (with slow shake) of 10 coated beads in 4, 5, 6, 7, 7.5, 8, 8.5, 9, 10 and 11 pH, 2985 ml Tris-HCl buffer (0.1M) and water at 55^0C for 5 min. with 15 ml substrate (50 mM pNPA). Then 1 ml solution was taken from each of them to observe activity. Determination of pH stability for 1 hour was investigated among pH: 4.0-6.0-7.0-8.0 and 8.5 and after waiting 1 hour in different Ph values 15ml substrate (pNPA) was added and solution was shaken for 5 min. more. Then 1 ml solution was taken from each of them to observe activity. The procedure

indicated in section 2.2.1.4.6. was followed for determination of specific activity. These values were compared with effect of temperature and pH on free esterase enzyme.

2.2.3.2. Effect of chemicals on immobilized esterase

In order to understand how different agents were effect the leakage of enzyme from beads and immobilized enzyme activity, $CaCl_2$, NaCl, isopropanol, EDTA and SDS were used. The experiment was done with the incubation of 10 coated beads in 2000 ml pH: 8.0 Tris-HCl buffer (0.1M) includes 985 ml chemical at 55^0C for 5 min. with 15 ml substrate (50 mM pNPA). Then 1 ml solution was taken from each of them to observe activity.

2.2.3.3. Reuse of immobilized enzyme

Firstly, 10 coated beads were incubated (with slow shaking mode) in 2985 ml Tris-HCl buffer (0.1 M pH 8.0) with 15 ml substrate (50mM pNPA) at 55^0C for 5min. Then 1 ml solution was taken to observe activity changes. Then beads were washed with 5 ml distilled water for 2.5 min and the same incubation condition was repeated for 7 times. The procedure indicated in section 2.2.1.4.6. was followed for determination of specific activity. The results of coated and non-coated beads were compared in section 3.2.3.5 and 3.2.3.6.

2.2.3.4. Bead Diameter

Alginate beads of three different sizes were generated by changing the size of a needle. The average bead size was measured 0.5 ± 0.2, 1.1 ± 0.2 and 3.4 ± 0.2 mm. The diameter of beads was determined with the formula as shown below.

Increment in Volume = $4/3\ \pi\ r^3$ Number of beads

2.2.3.5. Scanning Electron Microscope (SEM)

Surface analysis of films was done by using Scanning Electron Microscope (Phillips XL-30S FEG). Coated and non-coated beads were dried for 1 hour at 50^0C to get rid of wetness because it would be a problem for the machining operation.

CHAPTER 3

RESULTS AND DISCUSSION

In this study thermophilic recombinant esterase enzyme were used for immobilization studies by entrapment in coated Ca-alginate beads. Experimental results of the immobilization of esterase enzyme in silicate coated and non-coated beads were compared. After determination of the optimum immobilization conditions of esterase enzyme in silicate coated Ca-alginate beads, the effect of temperature, pH, some chemicals ($CaCl_2$, EDTA, ZnCl, isopropanol and SDS), reuse of enzyme and bead size were investigated on immobilization process and these studies were compare with free termophilic esterase enzyme. All experiments were done in duplicate at least.

3.1. Expression and Purification of the Recombinant Esterases in *E.coli*

3.1.1. Expression

In order to have high-level heterologous expression of esterase enzyme in E.coli, previously prepared glycerol stocks of esterase enzyme in expression vector were used (Tekedar, 2008). Glycerol stock of esterase was spread on LB plate containing kanamycin as a selectable marker in order to initiate expression (Figure 3.1).

Figure 3.1. LB agar plate with growth colonies
(Sciencephoto, 2009)

The cloned esterase gene in to pET-28a (+) expression vector have demonstrated high level expression in *E.coli* upon derepression of the *lac* operator by the lactose analog IPTG. Induction of expression by IPTG in the pET-28(+) expression vector in the BL21 (λDE3) *E. coli* host resulted in the production of a ~27 kDa protein for esterase gene (Figure 3.2). The control cells with no added IPTG showed no such proteins (Tekedar 2008). This level of expression indicates that esterase represent the major proteins in the induced cells. The expression reached maximum levels within 4 hours after induction by IPTG.

3.1.2. Purification

The main step of the purification procedure is the use of Ni-NTA affinity resin (Sigma). The matrix gel specifically binds His-taq regions which locate both N-terminal and C-terminal of pET-28a (+) expression vector. Elution of the protein from the resin is accomplished by a step elution with the buffer containing imidazole. His-taq affinity column was used at the very beginning of the purification scheme for esterase gene. The protein was approximately more than 95% pure after the initial affinity column. As shown in figure 3.2 (M: Marker, 1: Dilute protein sample after purification, 2: Protein sample after purification, 3: Protein sample before purification.) protein homogeneity was evaluated using Coomassie Blue visualized SDS PAGE. Yields from this purification protocol were typically 30-50 mg protein per liter of cells.

One step His-taq nicel affinity chromatography was good enough to purify esterase enzyme. 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. Because the elution of enzyme from column was accomplished with imidazole, dialysis was applied after affinity column against phosphate buffer (pH 7.0) for overnight to get rid of imidazole from esterase enzyme. After dialysis, enzyme was aliquoted in to eppendorf tubes as 1 ml volume and kept it at -20 C until use.

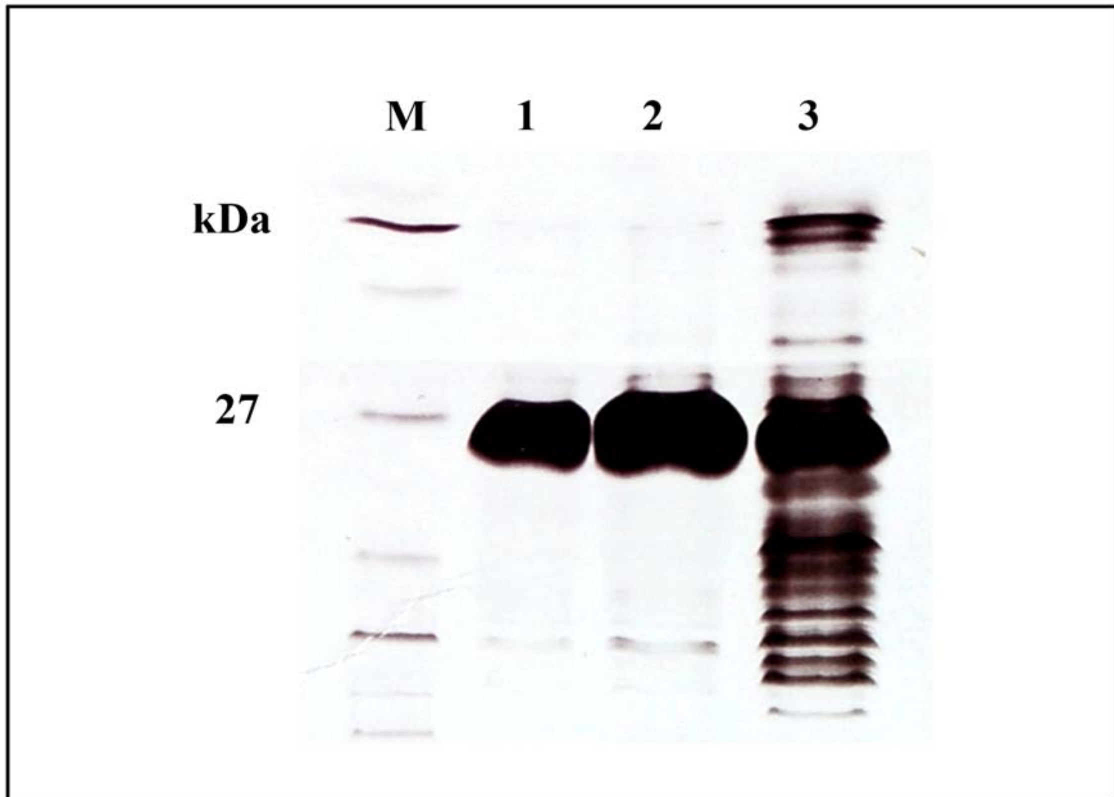


Figure 3.2. SDS-PAGE analysis of selected fractions.

3.2. Immobilization of Thermophilic Esterase Enzyme in Silicate Coated Ca-Alginate Beads

3.2.1. Optimization of Thermophilic Esterase Enzyme in Ca-Alginate Beads

Bead shapes and stability is very important for immobilization. First of all, the best stable beads were chosen qualitatively. Then solvent for alginate, $CaCl_2$ concentration and alginate concentration were investigated quantitively. To choose best solvent alginate was mixed with water and 0.5 M, 0.1 M, 0.05 M Tris-HCl buffer (pH: 5. 5, 7. 4, 8. 0, 8. 4, 9. 0, 9. 4, 10 and 11). separately. Beads formation was not occurred with using water so that tris buffer was chosen to continue in our works. Using different concentration and pH conditions of tris buffer has resulted in almost all condition beads shapes were in good looking. Therefore, the results were evaluated according to activity

measurements of the enzyme. The best activity was observed with 0.1 M Tris-HCl buffer at pH: 8.0.

Alginate concentration was also investigated to get stable and good bead shape. 1-2-3-4% of alginate concentration was mixed with 0.1 M Tris-HCl buffer at pH: 8.0 and the best beads were formed with 2% alginate concentration. 3% and 4% were too concentrated and %1 was not enough concentrated to form gel. As a result, for 1% concentration of alginate, the beads were too squash and not stable and for %3-4 concentration of alginate, diffusion limitation of entrapped enzyme may occur. Previous study has demonstrated that more than 2 % alginate concentration has decreased loading efficiency of enzyme due to diffusion limitation (Won, et al. 2001). However reuse of immobilized enzyme was increased with high alginate concentration (Won, et al. 2001). In this study 2% alginate concentration was chosen to have optimum values of both loading efficiency and reuse of enzyme immobilized beads.

Optimization of $CaCl_2$ concentration was done with %2 alginate concentrations and variety amount of $CaCl_2$: 0.05 M, 0.3 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, 1.0 M and 1.3 M. From 0.05 M to 0.5 M concentration of $CaCl_2$, the beads were not stable and their shapes were not good, either. Also beads with higher than 0.9 M $CaCl_2$ concentration were formed but dispersion were observed after 3 min. So the best condition for $CaCl_2$ concentration was determined according to enzyme activity measurements. According to that 0.6 M, 0.7 M, 0.8 M $CaCl_2$ were used to investigate the optimum concentration of $CaCl_2$ on beads stability and shape for immobilization studies.

Esterase solution with the concentrations of 0.1 mg/ml was enough to investigate optimization studies but it was not enough for the determination of leakage of the enzyme form the beads so 0.5 mg/ml enzyme were used for chacterization studies.

At the end of optimization, 0.7 M $CaCl_2$, 2% alginate and Tris-HCl buffer 0.1 M pH: 8.0 values were decided to use for immobilization of thermophilic esterase in Ca-alginate beads.

3.2.2. Immobilization Yield in coated beads

Immobilization was done with the chemical ratio of components described in the section 3.2.1. and then beads were coated with hexane and TEOS. They were washed on

a filter 3 times with 5 ml distilled water to determine leakage of enzyme if any. 0.456 mg/ml enzyme has been used for immobilization and 0.131 mg/ml enzyme has been obtained from washing steps. As a result immobilization yield were calculated as 71.27%.

When non-coated beads were used for the same immobilization condition 0.286 mg/ml leakage has been observed. This means that coated beads can have 45.80% more enzyme than non-coated ones.

3.2.3. Characterization of Immobilized Thermophilic Esterase Enzyme

In this chapter effect of bead size, temperature, pH, metal ion, organic solvents, and some detergent on immobilized thermophilic esterase enzyme were investigated and compared with free esterase enzyme. Besides that, reuse of immobilized enzyme and surface morphology were presented for coated and non-coated beads.

3.2.3.1. Effect of Temperature

In order to effectively facilitate the immobilization reaction and prevent enzyme deactivation at higher or lower reaction temperatures, it is very major to investigate thermal stability of immobilized enzyme. As observed in most of the chemical reactions, an increase in temperature increases the rate of reactions but it's different for proteins because the stability of a protein decreases due to the thermal inactivation.

Esterase was isolated from *Thermophilies Bacillus* so it is a thermophilic enzyme which's stability much higher than mesophile or psychrophlie ones can see Table 1.3. However beads can deform in high temperature. Determination of thermal stability is very important in finding the optimum operating conditions for immobilization in Ca alginate beads. In this study, the influence of temperature on immobilization system was investigated in the range of 4-80^{0}C for 5 min. and 1 hour. For 5 min. maximum specific activity was observed at 45^{0}C. Figure 3.3 shows specific activity of enzyme in the beads which means concentration of enzyme in the beads that interact with substrate (PNP-acetate) in a minute.

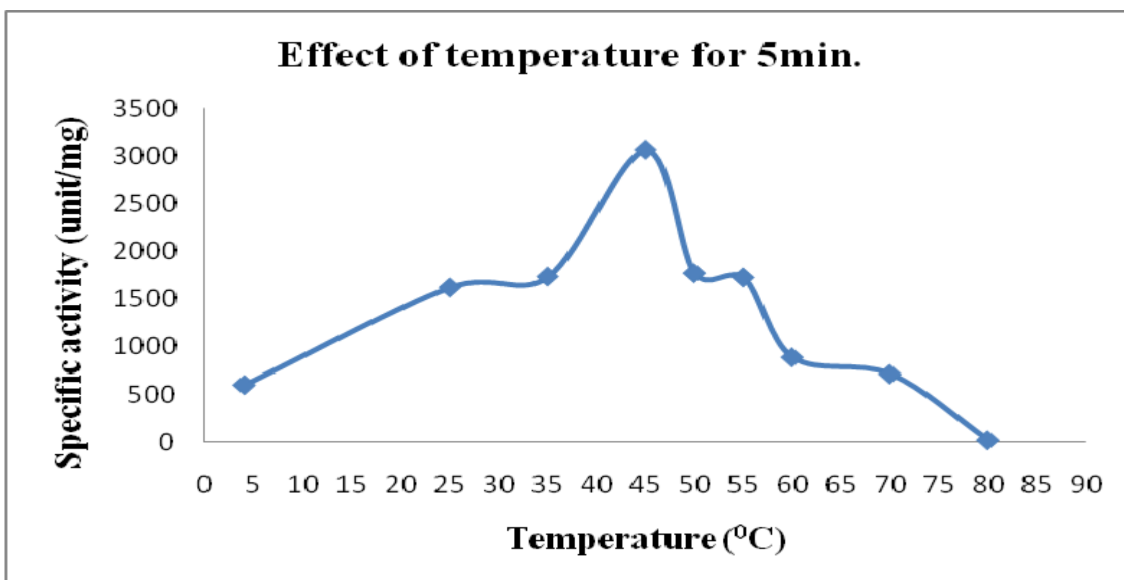


Figure 3.3. Effect of temperature on immobilized esterase enzyme specific activity.

For all characterization studies, specific activities were calculated with using entrapment efficiency (amount of enzyme in the beads) and changed in absorbance values. Figure 3.4 shows the enzyme concentrations in the beads which mean entrapment efficiency. They were found out by subtracting leakage enzyme concentration from total enzyme enzyme concentration. Figure 3.5 shows change in absorbance for the total enzyme which includes both leakage enzyme and the enzyme in the beads at different temperatures for 5 min.

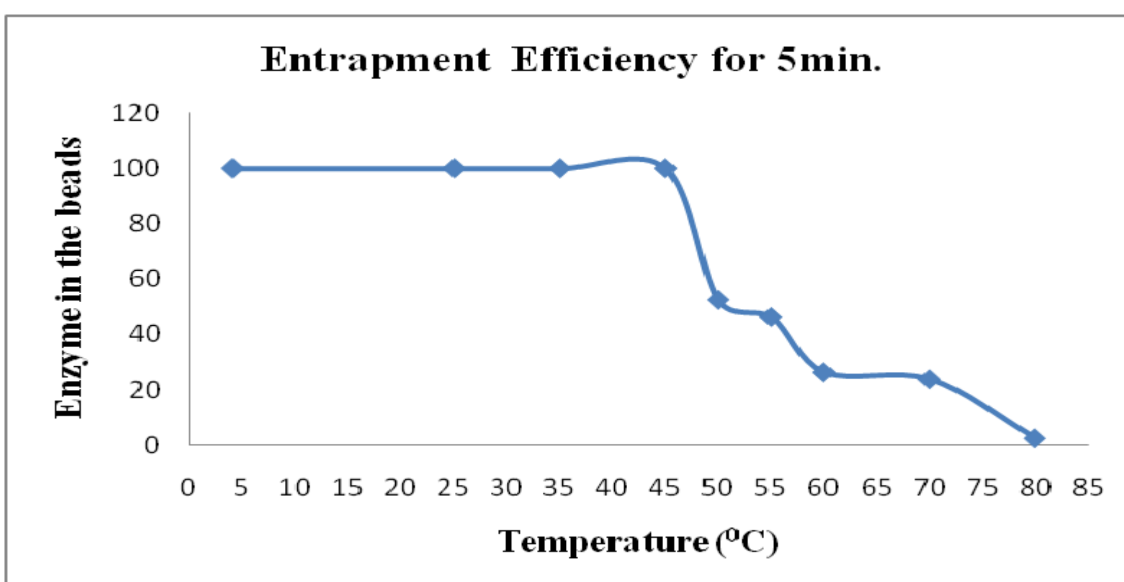


Figure 3.4. Entrapment efficiency at different temperatures for 5 min.

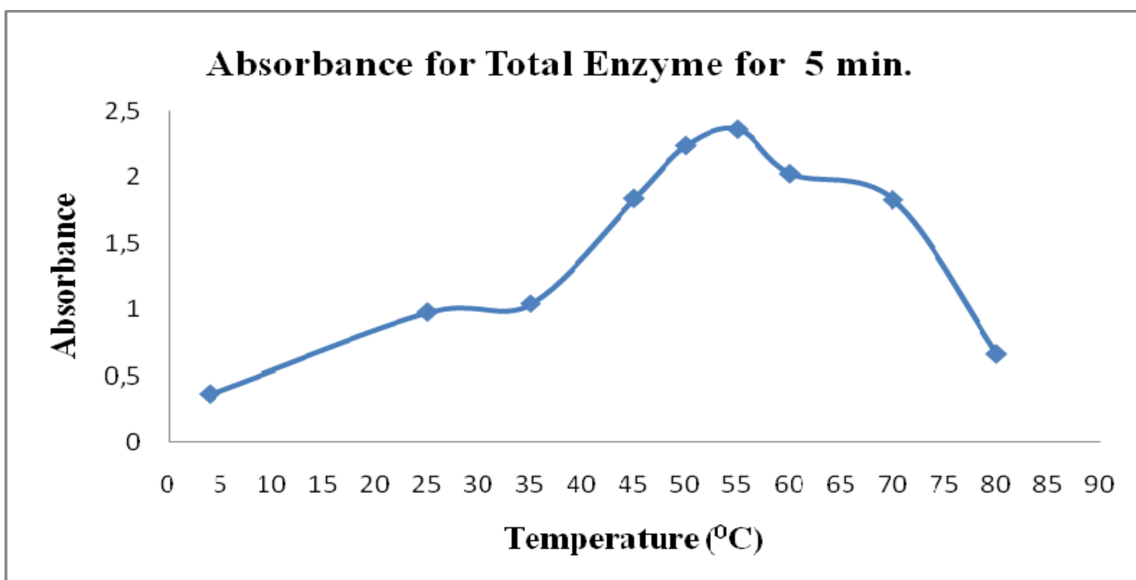


Figure 3.5. Total enzyme absorbance at different temperatures for 5 min.

As shown in figure 3.3 after 50 ^{0}C there is a decrease in specific activity of immobilized esterase so that there is no need to examine higher degrees for 1 hour. Determination of thermal stability for 1 hour was investigated at 4-25-35-45 and 50^{0}C and after waiting 1 hour in different temperatures half of specific activity was still protected at 25^{0}C. As a result, maximum specific activity after 1 hour maximum specific activity was observed at 25^{0}C that shown in figure 3.6 and entrapment efficiency shown in figure 3.7.

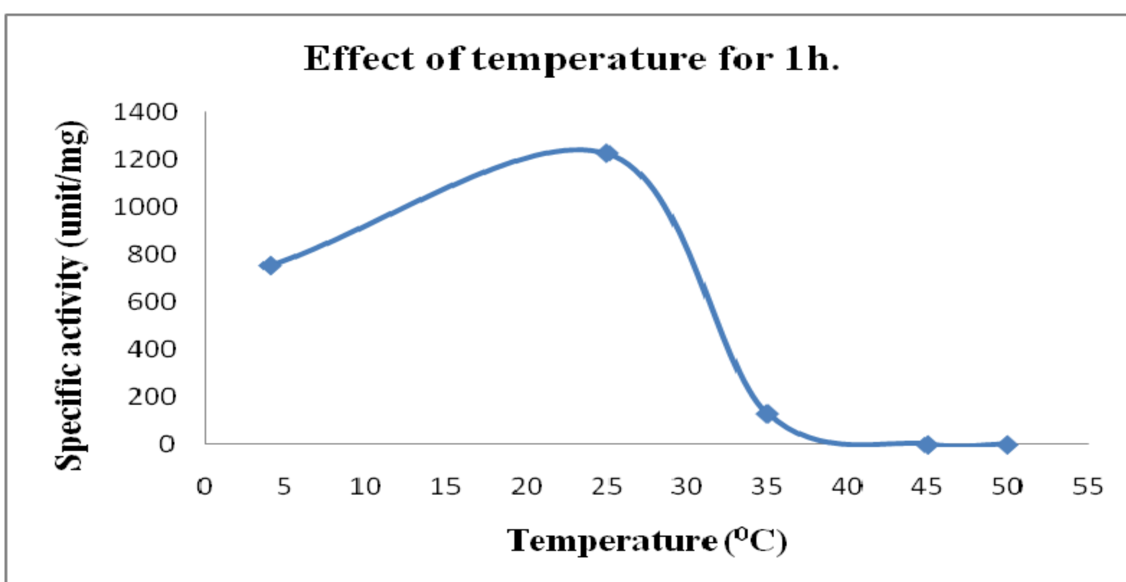


Figure 3.6. Effect of temperature on immobilized esterase enzyme activity for 1 hour.

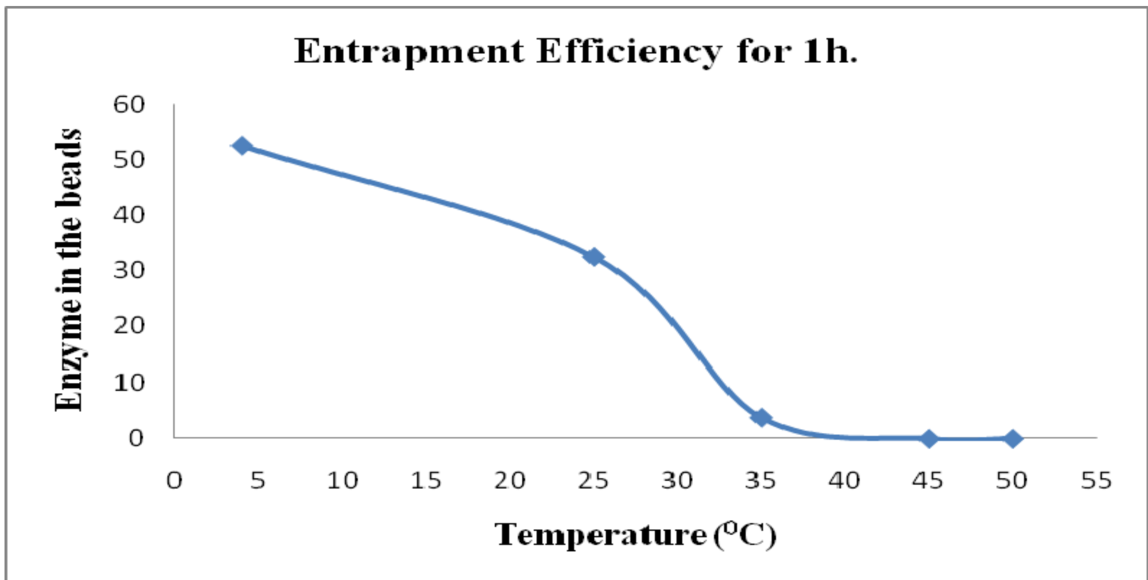


Figure 3.7. Entrapment efficiency at different temperatures for 1 hour.

The best activity was observed at 45^{0}C for the immobilized enzyme however free enzyme maximum activity was observed at 55^{0}C at the same conditions (Tekedar 2008) shown in figure 3.8. This might be due to effect of temperature on immobilization conditions.

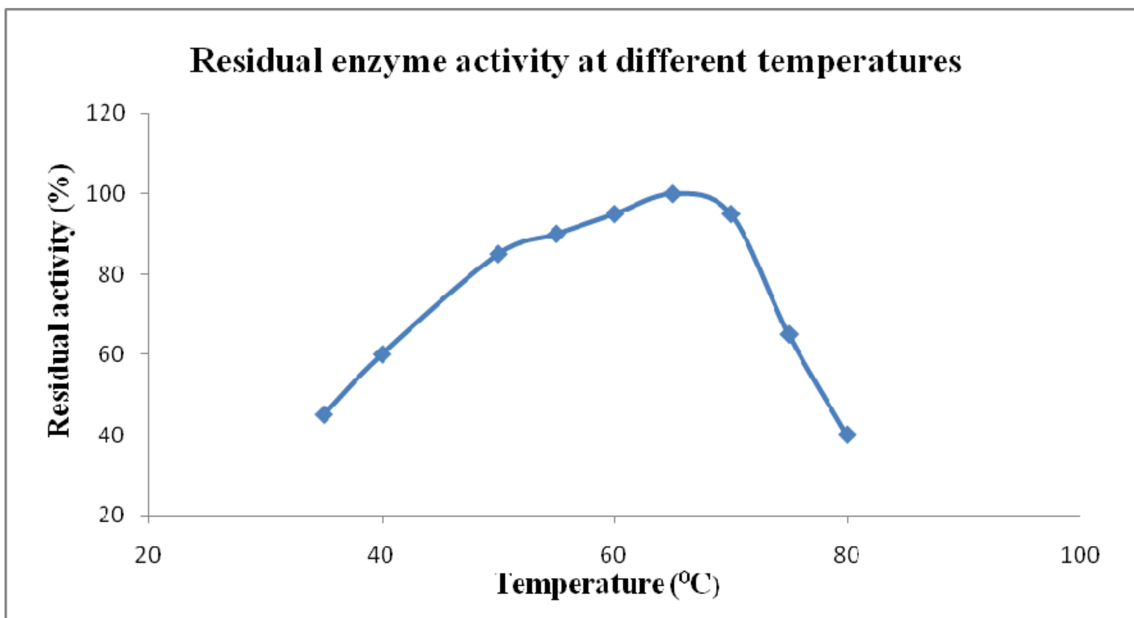


Figure 3.8. Residual free enzyme activity values at different temperatures.

3.2.3.2. Effect of pH

Each enzyme has an optimal pH range that help maintenance of its native conformation in an environment, which it operates. A change in pH can shift the ionization of interactions in the tertiary structure of a protein side chains such as hydrogen bonding between R groups can disrupted the native conformation and in some case denature the enzyme. Effect of pH on immobilized thermophilic esterase was investigated in the range of pH: 4.0-9.0 and specific activity results were calculated with using entrapment efficiency and change in absorbance values for 5 min.

For free enzyme max activity was observed in alkali pHs that means after pH: 8.0 (Tekedar 2008) and for immobilized enzyme this value was pH: 8.0 for the same conditions shown in Figure 3.9. This might be due to different pH effect for microenvironment of enzyme, substrate and product in terms of ionic conditions.

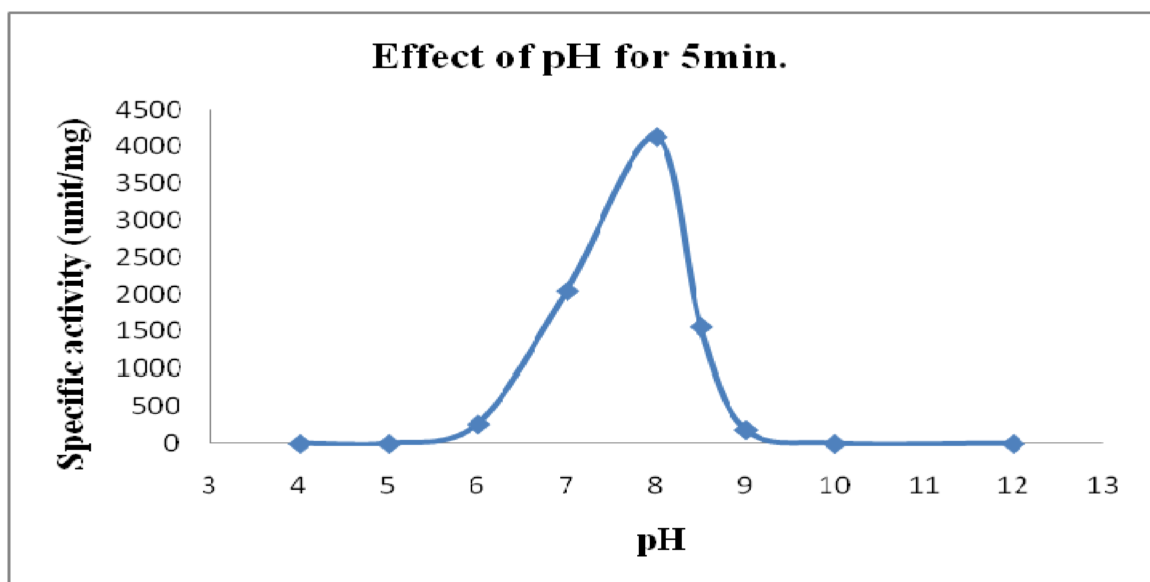


Figure 3.9. Effect of pH on immobilized esterase enzyme specific activity.

Figure 3.10 shows the enzyme concentrations in the beads which means entrapment efficiency and figure 3.11 gives total enzyme absorbance. It includes both leakage enzyme and rest of the enzyme in the beads at different pH values after waiting for 5 min.

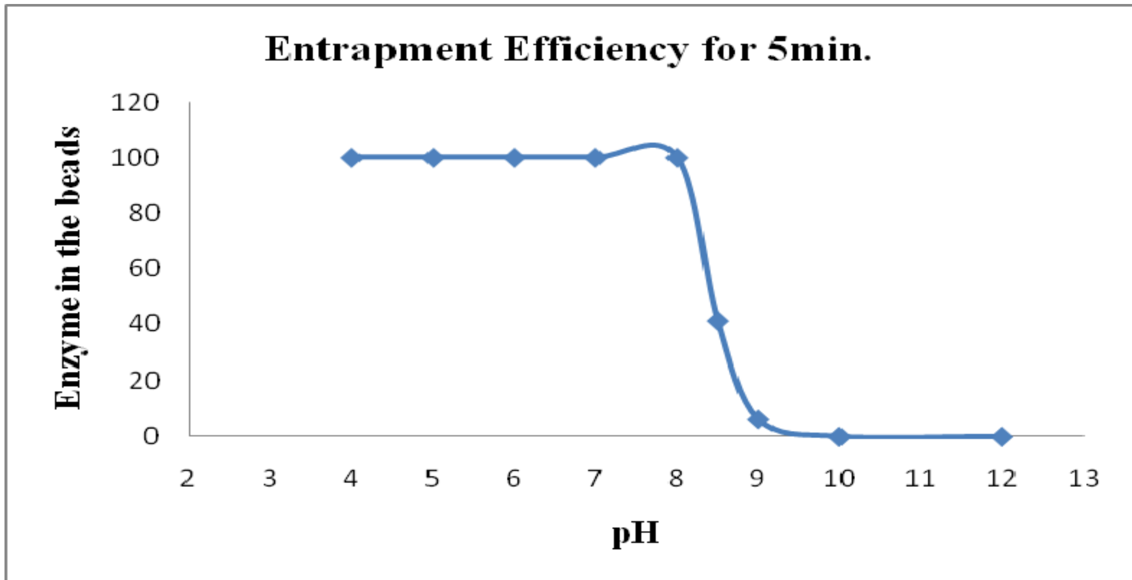


Figure 3.10. Entrapment efficiency at different pH values for 5 min.

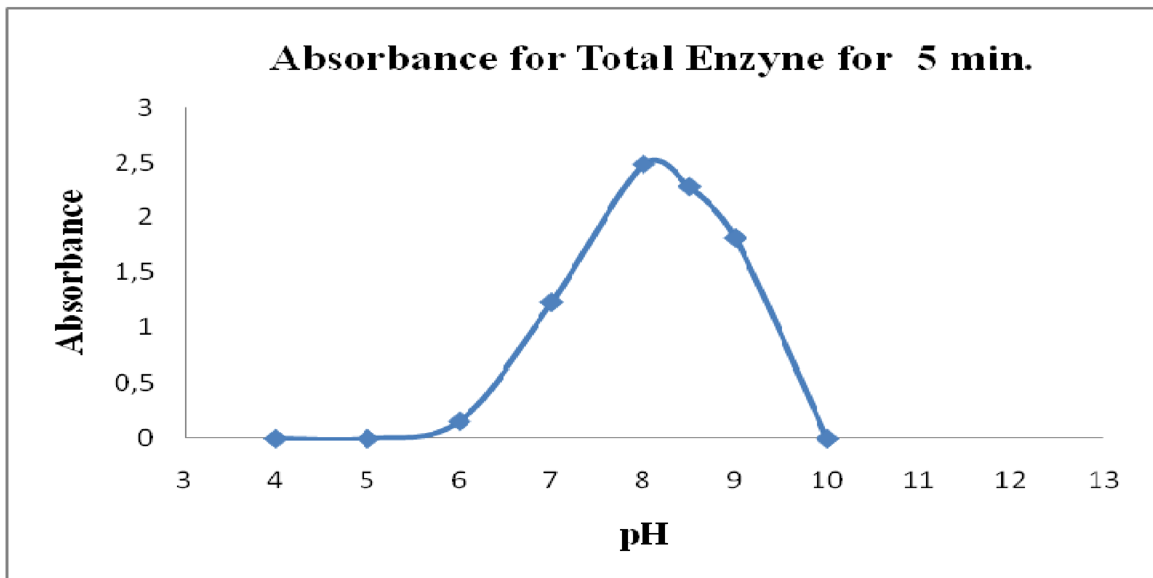


Figure 3.11. Total enzyme absorbance at different pH values for 5 min.

As shown in figure 3.9, after pH: 8.5 there is a decrease in specific activity of immobilized esterase so that higher pH values for one hour were not examined. Determination of pH stability for 1 hour was investigated among the pH of: 4.0-6.0-7.0-8.0 and 8.5. After incubating immobilized enzyme for one hour in different pH values, half of specific activity was still protected at pH: 7.0. As a result, immobilized esterase enzyme for one hour was still displaying maximum activity at pH: 7.0. Specific activity of immobilized esterase enzyme versus pH values were shown in figure 3.12 and entrapment efficiency of enzyme were shown in figure 3.13 after one hour incubation time.

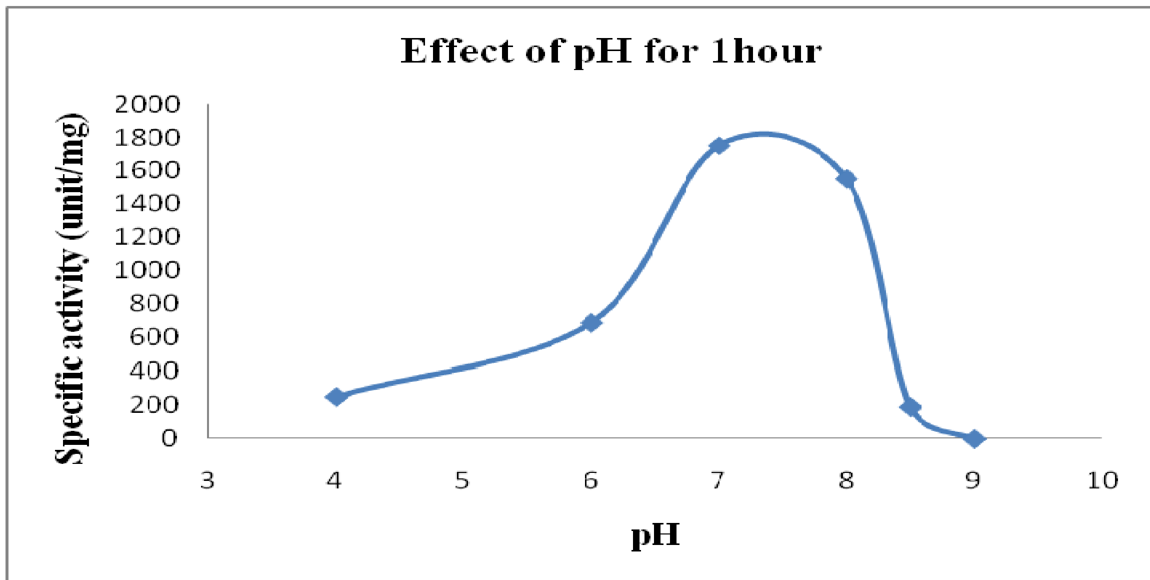


Figure 3.12. Effect of pH on immobilized esterase enzyme activity for 1 hour.

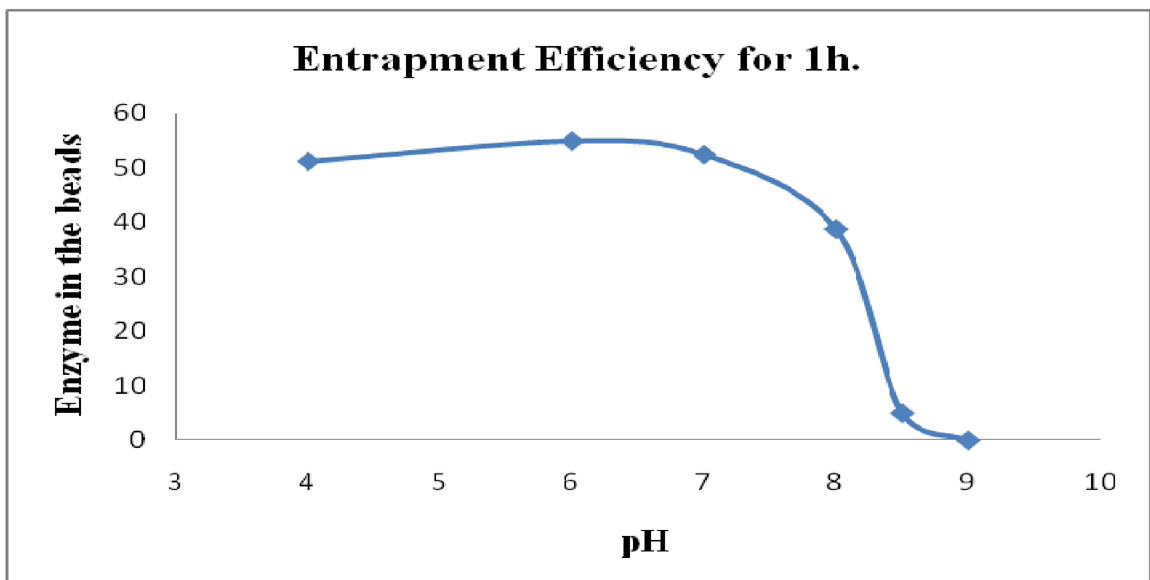


Figure 3.13. Entrapment efficiency at different pH values for 1 hour.

3.2.3.3. Effect of bead size on immobilized esterase enzyme

It was studied that enzymes in smaller beads show higher catalytic activity due to reduced substrate transfer resistance (Bhushan, et al. 2008 / Won, et al. 2004).

As expected, specific activities of esterase entrapped in the beads decreased as the bead size increased. As a result, 0,5 mm diameter beads was used in our studies.

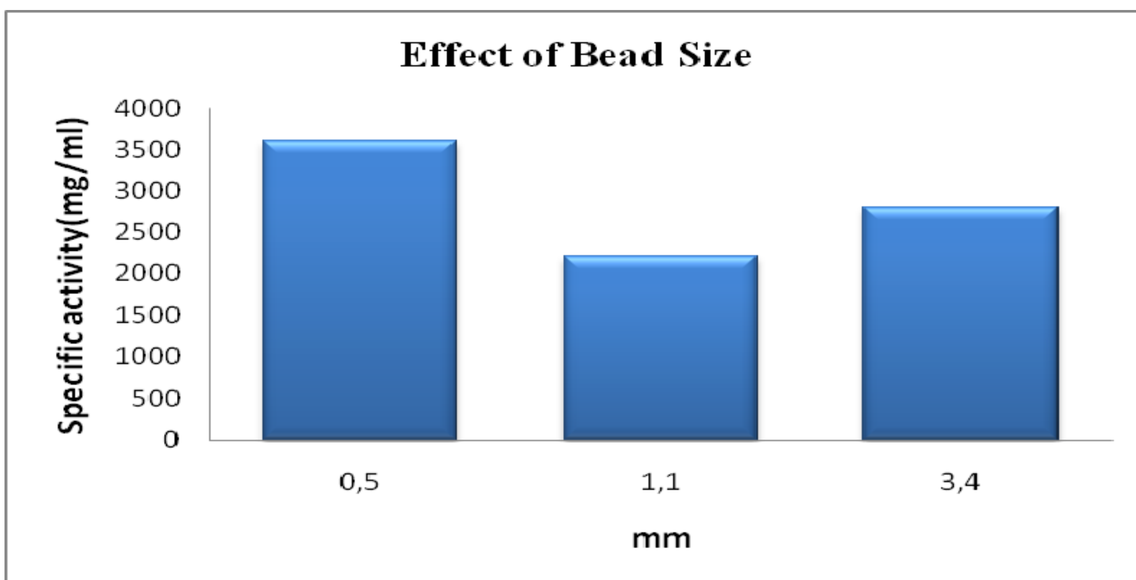


Figure 3.14. Effect of different bead sizes on specific activity.

3.2.3.4. Effect of chemicals on immobilized esterase

Chemicals, ions and detergents effect not only enzyme activity but also surface of the beads. To investigate these effects $CaCl_2$, NaCl, isopropanol, EDTA and SDS were used. Effect of chemicals on enzyme and surface of the beads were calculated with using change in absorbance values and entrapment efficiency, respectively.

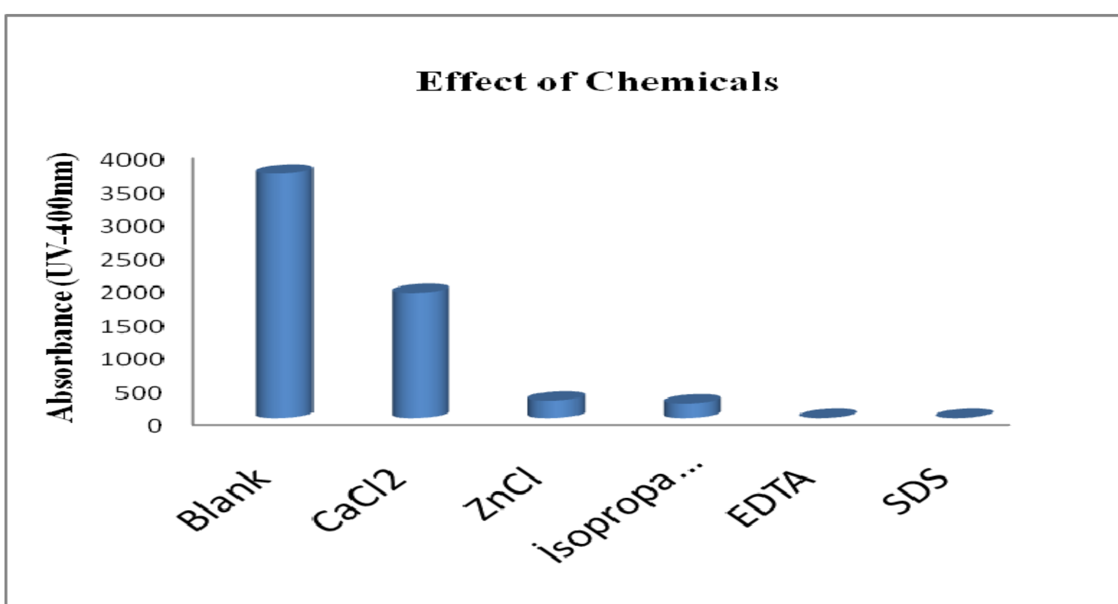


Figure 3.15. Total enzyme absorbance with different chemicals.

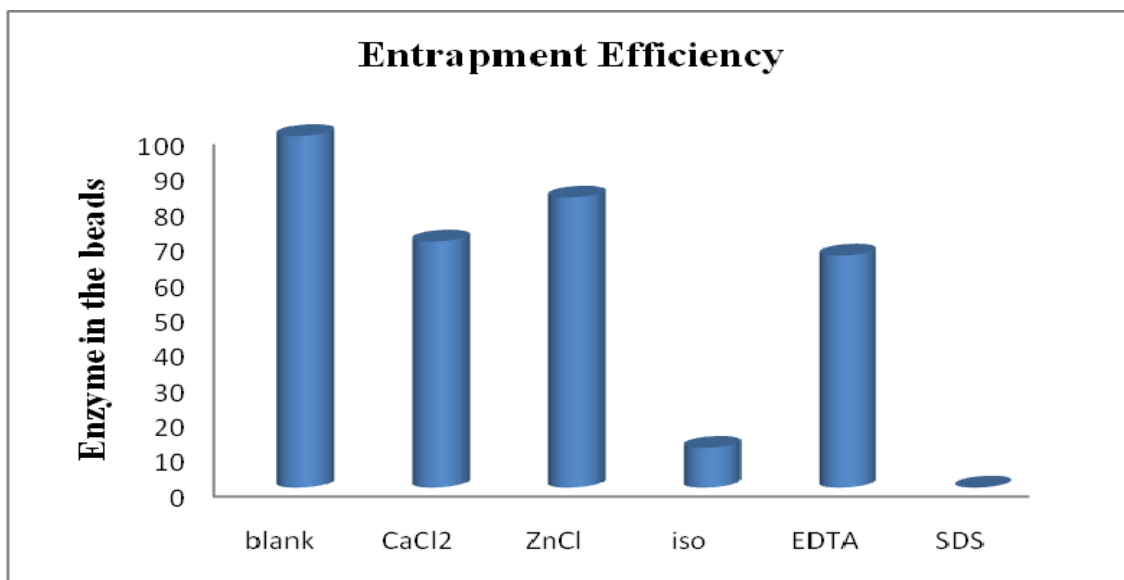


Figure 3.16. Entrapment efficiency at different pH values for 5 min.

Specific activities were calculated with using entrapment efficiency of the enzyme in beads and change in absorbance values for investigation of chemicals on immobilization;

$CaCl_2$: Both enzyme and surface of the beads were not effect very much with $CaCl_2$ so specific activity value was still half protected as shown in figure 3.17.

ZnCl: Surface of the beads almost had no effect with ZnCl as shown in figure 3.16 but enzyme was inhibited with a high percentage. This results shows that enzyme was entrapped in the beads, but ZnCl has inhibited the enzyme activity. As shown in figure 3.15 esterase has very low activity due to inhibition so that very low specific activity was calculated.

Isopropanol: Effect of isopropanol was very high both for enzyme and surface of the beads. It has disturbed beads surface and inhibited enzyme highly. As a result, very low specific activity was calculated shown in figure 3.17.

EDTA: Surface of the beads was not affected very much with EDTA although it has inhibited the activity of esterase enzyme totally as shown in figure 3.15. Enzyme was entrapped in the beads but the activity was diminished because of inhibition so that specific activity was calculated as zero.

SDS: Sodium dodecyl sulfate is a very strong detergent that can dissolve hydrophobic molecules. It has inhibited enzyme and disturbed beads totally in 5 min. There was no specific activity could be calculated after addition of SDS as shown in figure 3.17.

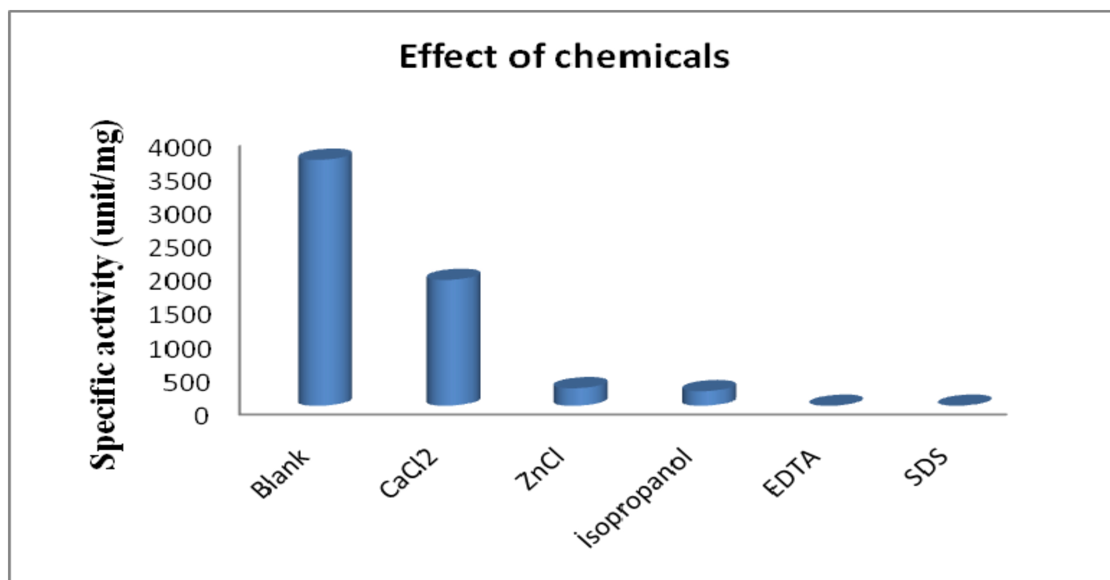


Figure 3.17. Effect of chemicals on immobilized esterase enzyme specific activity.

3.2.3.5. Reuse of immobilized enzyme

For commercial application the reuse of the immobilized enzyme is very important from the point of view of reducing the cost of the enzyme. Specific activity of the silicate coated and non-coated immobilized enzyme on reuse of the enzyme is shown in Figure 3.18. For coated beads, when specific activity of the first batch was taken as 100%, after using four times half of activity was still protected. The results confirmed that there was no significant loss of original enzymatic activity after three subsequent cycles. The stability of immobilized enzyme for repeated uses was effective and could be used more than six times effectively for the further studies.

When compare silicate coated and non-coated beads, non-coated ones were reused only three times. These results are shown in the figure 3.18. The reason might be that due to soft surface of non-coated beads. As a result, coating with silicate not only prevents leakage of enzyme from the beads but also increase reuse of beads.

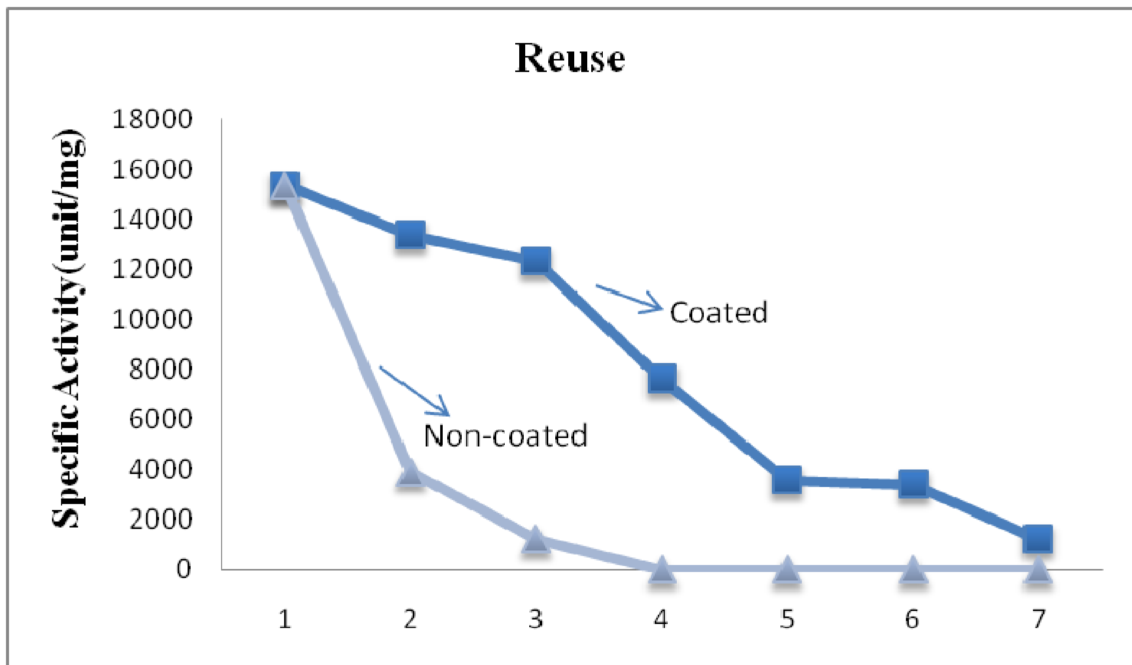
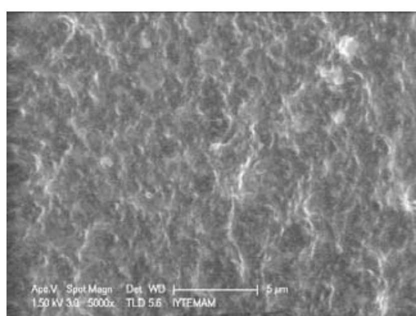


Figure 3.18 Reuse of coated and non-coated immobilized esterase enzyme.

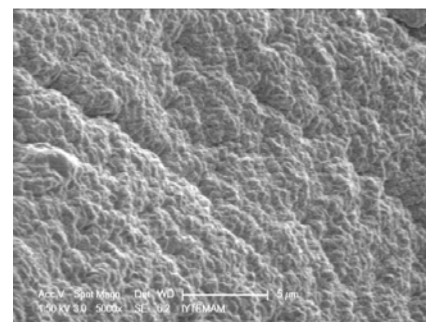
For non-coated Ca-alginate immobilization, similar results were observed by immobilizing porcine liver esterase (Lee, et.al. 2001). Entrapment of lipase in Ca-alginate gel beads let reuse, but activity loss was observed (Won, et al. 2005).

3.2.3.6. Scanning Electron Microscope (SEM)

Analysis of morphologies of films was investigated by using Scanning Electron Microscope. The formation of beads was described in section 2.2.2.2 and procedure was described in section 2.2.3.5. SEM micrographs were shown for silicate coated and non-coated beads in Figure 3.19. Surface morphologies of enzyme immobilized in coated beads were completely different when compared with non-coated one.



(a) Non-Coated bead



(b) Coated bead

Figure 3.19. Comparison of coated and non-coated beads surface

As shown in Fig. 3.19.(b), the surface of the coated beads has looked like a mesh, and has very compact structures. Furthermore, a comparison of Fig. 3.19.(a) and 3.19.(b) indicates that the coating material, silicate, has affected the surface morphology of the alginate beads.

CHAPTER 4

CONCLUSION

Immobilization of enzymes is very important not only for reuse of them but also for use them more efficient. In this study purification, extraction and immobilization of the thermophilic esterase enzyme was done with using Recombinat DNA technology applied *Thermophilic basillus*. As a conclusion, silicate coated and non-coated beads were compared. After determination of the optimum immobilization conditions of esterase enzyme in silicate coated Ca-alginate beads, the effect of temperature, pH, some chemicals ($CaCl_2$, EDTA, ZnCl, Isopropanol and SDS), reuse of enzyme and bead size were investigated on immobilization process and these studies were compare with free termophilic esterase enzyme. Immobilization of enzyme was done with Ca-alginate and silicate for coating with yield of %71.27 and compared with non-coated ones with the yield rate %45.80. Surfaces were compared with Scanning Electron Microscope.

REFERENCES

Almeida, R. V., Branco, R. V., Peixoto, B. 2008. Immobilization of a recombinant thermostable esterase (Pf2001) from Pyrococcus furiosus on microporous polypropylene: Isotherms, hyperactivation and purification. *Biochemical Engineering Journal* 39., 3:531-537

Baker, G. C., Gaffar, S., Cowan, D. A., Suharto, A. R. 2001. Bacterial community analysis of Indonesian hot springs. FEMS *Microbiology Letters*. 200:103-109.

Berezovsky, I. N. and Shakhnovich, E. I. 2005. Physics and evolution of thermophilic adaptation. *PNAS*. 102., 36:12742-12747.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem., 72, 248–252.

Bugg, D. H. T. 2001. The development of mechanistic enzymology in the 20th century. *Nat. Prod. Rep.,* 18, 465–493

Cao, L. 2006. Carrier-bound Immobilized Enzymes *Wiley-vch*

CLEA Technologies, 2007. Esterase BS2 Cross-Linked Enzyme. Aggregates (CLEAs). PS-CLEA401-81010

Dominiguez, E., Nisson, M., and Hahn-Hagerdal, B. 1988. Carbodiimide coupling of galactosidase from Aspergillus oryzae to alginate, **Enzyme Microb. Technol**. 10:606-610.

Dominguez, J. M., Cruz, J. M., Roca, E., Dominguez, H., Parajo, J. C. 2006. "Xylitol Production from Wood Hydrolyzates by Entrapped *Debaryomyces hansenii* and *Candida guilliermondii* Cells". *Biochemistry and Biotechnology*. 81., 2:119-130.

Guisan, M. J. 2006. Immobilization of Enzymes and Cells Second Edition. Institute of Catalysis, *CSIC*

Henstra, S. A., Tolner, B., Ten Hoeve Duurkens, R. H., Konings, W. N., Robillard, G. T. 1996. Cloning, Expression, and Isolation of the Mannitol Transport Protein from the Thermophilic Bacterium *Bacillus stearothermophilus*. *Journal of Bacteriology*. 178., 19:5586-5591.

Hill, C. G., Ghannouchi, S., Garcia, H. S. 2002. Lipolysis of Butter Oil by an Immobilized Kid Goat Pregastric Esterase *Journal of Food Science*. 67., 8:3069-3078

Iyer, P. V. and Ananthanarayan, L. 2008. Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochemistry*. 43., 10:1019-1032.

Jeong, J., Ha, T. H., Chung, B. H. 2006. Enhance reuseability of hexa-arginine-tagged esterase immobilized on gold-coated-magnetic nanoparticles. *Analytica Chimica acta*. 569., 1-2:203-209.

Jeong, J., Lee, C. S., Chung, S. J. 2010. Enhanced immobilization of hexa-arginine-tagged esterase on gold nanoparticles using mixed self-assembled monolayers *Bioprocess and Biosystems Engineering*. 33., 1:165-169

Kademi, A., Ait-Abdelkader, N., Fakhreddine, L., Baratti, J. 2000. Purification and Characterization thermostable estarese from moderate thermophile Bacillus circulans. *Microbiol Biotechnol.* 54:173-179.

Kennedy, J.F. and White, C.A. 1985. Principles of immobilization of enzymes. In: Handbook of Enzyme Biotechnology,Chichester, NewYork: *Halsted Press*.

Khalil, A. B. 2002. Isolation and Characterization of Thermopilic Bacillus Species from Thermal Ponds in Jordan. Pakistan *Journal of Biological Sciences*. 5:1272-1273.

Khanna, S., Sekhon, K. K., Prakash, N. T. 2009. Cloning and Expression of a Biosurfactant Gene from Endosulfan Degrading *Bacillus sp*: Correlation Between Esterase Activity and Biosurfactant Production. *Biotechnology*. 8., 2:235-241.

Koszelewski, D., Ostaszewski, R., Kurek, W. 2009. Lovastatin esterase enzyme immobilized on solid support, process for enzyme immobilization, use of immobilized enzyme, biocatalytic flow reactor and process for preparation and/or purification of simvastatin. *Instytut Chemii Organicznej*. 44/52, PL-01-224

Lee, S. Y., Min, B. H., Hwang, S. H., Koo, Y. M., Lee, C. K., Song, S. W., Oh, S. Y., Lim, S. M., Kim, S. L., Kim, D. 2001. Enantioselective production of levofloxacin by immobilized porcine liver esterase. *Biotechnology Letters* 23: 1033–1037.

Kim, S. B., Lee, W., Ryu, Y. W. 2008. Cloning and Characterization of Thermostable Estarese from Archaeoglobus fulgidus. *The Journal of Microbiology*. 46., 1:100-107.

Meena, K. and Raja, T. K. 2006. Immobilization of Saccharomyces cerevisiae cells by gel entrapment using various metal alginates. *World Journal of Microbiology & Biotechnology*. 22:651–652

Mizuno, H., Saito, T., Iso, N., Onda, N., Noda, K., Takada, K. 1983. Mannuronic to Guluronic Acid Rations of Alginic Acid Prepared from Various Brown Seaweeds. *Bulletin of the Japanese Society of Scientific Fisheries.* 40:1591-1593.

Panda, T. and Gowrishankar, B. S. 2005. Production and applications of esterases. *Appl Microbiol Biotechnol.* 67:160-169.

Rainey, F. and Oren, A. 2006. Extremophiles, Volume 35 (Methods in Microbiology) *Academic Press*: 821

Remminghorst, U. And Rehm, B. H. A. 2006. Bacterial alginates: from biosynthesis to applications. *Biotechnol Lett.* 28:1701-1712.

Robb, F., Antranikian, G., Grogan, D., Driessen, A. 2007. Thermophiles Biology and Technology at High Temperatures CRC, *Press,* 368

Sheldon, R. A., Schoevaart, R., Langen, L. M. V. 2006. Cross-Linked Enzyme Aggregates. *Methods in Biotechnology*. 22., 31-45

Singh S, Chaubey A, Malhotra BD. 2004. Amperometric cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase on conducting polypyrrole films.: *Analytica Chimica Acta* 502., 2:229-234.

Smidsrod, O. 1974. Molecular Basis for some Physical Properties of Alginates in the Gel State. *Chem. Soc.* 57:263-274.

Steel, D.M. and Walker, J.M. 1991. Thermostable Proteins. *Life Chemistry Reports Series*. 8,pp.49-96

Strand, B. L., Gaserod, O., Kulseng, B., Espevik, T., and Skjåk-Bræk, G. 2002. Alginate- polylysine-alginate microcapsules effect of size reduction on capsule properties. *J. Microencap.* 19:615–630.

Tekedar, H., C., 2009. Moleculer cloning overexpression and characterization of thermostable esterase and lipase from Thermophilic Bacillus sp. (Thesis)

Torres, P., Datla, A., Rajasekar, V.,W. 2008. Characterization and application of a sterol esterase immobilized on polyacrylate epoxy-activated carriers (Dilbeads™) *Catalysis Communications*. 9., 4:539-545

Timothy, D. H. B. 2001. 2001 The development of mechanistic enzymology in the 20th century. *Nat. Prod. Rep*., 18:465–493

Vieille, C. and Zeikus, G. J. 2001. Hyperthermophilic Enzymes: Sources, Uses, and MolecularMechanisms for Thermostability. *Microbiology and Molecular Biology Reviews.* 65., 1:1-43.

Wadiack, D. T. and Carbonell, R. G. 1975. Kinetic behavior of microencapsulated-galactosidase. *Biotechnol. Bioeng.* 17:1157–1181

Won, K., Kim, S., Kim, K., Park, H. W., Moon, S. J. 2005. Optimization of lipase entrapment in Ca-alginate gel beads. *Process Biochemistry*. 40:2149–2154.

Yadav, G.D. and Jadhav, S.R. 2005. Synthesis of Reusable Lipases by Immobilization on Hexagonal Mesoporous Silica and Encapsulation in Calcium Alignate: Transesterification in Non Aqueous Medium, *Microporous and Mesoporous Mater.*, 86: 215–222.

Zhang, J., Liu, J., Zhou, J., Ren, Y., Dai, X., Xiang, H. 2003.Thermostable esterase from *Thermoanaerobacter tengcongensis*: high-level expression, purification and characterization. *Biotechnology Letters.* 25: 1463–1467.

http://www.abe.ufl.edu/~chyn/age4660/lect/lect_21/FG12_017.GIF, (Figure 1.2)

http://www.abe.ufl.edu/~chyn/age4660/lect/lect_21/FG12_017.GIF, (Figure 1.3)

http://blog.khymos.org/wp-content/2006/09/calcium-alginate.jpg (Figure 1.5)

http://www.freewebs.com/amtomson/hyalg.gif, (Figure 1.6)

http://www.abe.ufl.edu/~chyn/age4660/lect/lect_21/FG12_017.GIF, (Figure 1.7)

http://www.abe.ufl.edu/~chyn/age4660/lect/lect_21/FG12_017.GIF, (Figure 1.11)

http://biocatalysis.uni-graz.at/Photos/biokat_2.gif, (Figure 1.13)

http://www.sciencephoto.com 11, 2009 (Figure 3.1)

APPENDIX A

CHEMICALS, SOLUTIONS AND BUFFERS

CHEMICALS:

Isopropyl-β-D thiogalactopyranoside (IPTG)

4-Nitrophenyl Acetate (pNPA)

Alginate

NaCl

Immidazole

Tetraethyl orthosilicate (TEOS)

Hexane

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

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.