IMMOBILIZATION OF THERMOPHILIC ESTERASE ON MAGNETIC CORNSTARCH NANOPARTICLES FOR BIOLOGICAL APPLICATIONS

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ABSTRACT

IMMOBILIZATION OF THERMOPHILIC ESTERASE ON MAGNETIC CORNSTARCH NANOPARTICLES FOR BIOLOGICAL APPLICATIONS

In last three decades, even the role of enzymes for biological and industrial applications has become more worthy, enzymes also have some defects. The enzyme immobilization allows to overcome these defects by improving abilities of reusing of catalysts by multiple times, easier reactor operation and product separation. Due to its potential use in biological and industrial applications, isolated thermophilic esterase from *Geobacillus sp.* was immobilized on magnetic cornstarch nanoparticles.

In order to determine activity performance of immobilized enzyme, the effects of temperature, pH and some chemicals on enzyme activity were investigated. The results have shown that after immobilization, the relative activity of immobilized esterase has increased to 80% at 80 ^{0}C in comparison to free esterase. Therewithal, the reusability of immobilized esterase has increased fourfold in comparison to free esterase. The magnetic character of the support media has brought ease to separate biocatalysts from reaction media.

ÖZET

TERMOFİLİK ESTERAZIN BİYOLOJİK ÇALIŞMALAR İÇİN MANYETİK MISIR NİŞASTASI NANOPARÇACIKLARI ÜZERİNE BAĞLANMASI

Geçtiğimiz son otuz yılda, enzimlerin biyolojik ve endüstriyel uygulamalardaki rolü her ne kadar değerlendiysede, bazı kusurları da vardır. Enzim sabitlemesi, bu kusurların üstesinden gelinerek, katalizörün birçok defa kullanılmasına, reaksion sırasında daha kolay aktivleşmesine ve enzimlerin reaksiyon ortamından kolay ayrılmasına olanak sağlamaktadır. Biyolojik ve endüstriyel uygulamalarda olası kullanımından dolayı, *Geobacillus sp.* türünden izole edilen termofilik estaraz manyetik mısır nişastası nanoparçacıkları üzerine sabitlendi.

Sabitlenmiş enzimin aktivite performansına karar vermek için, sıcaklık, pH ve bazı kimyasal maddelerin bağlı enzim aktivitesi üzerindeki etkileri araştırıldı. Enzimlerin bağlanmasının ardından sonuçlar gösterdi ki, sabitlenmiş esterazın bağıl aktivitesi, serbest enzime kıyasla 80 ^{0}C sıcaklıkta %80 arttı. Ayrıca serbest esteraz ile kıyaslandığında, sabitlenmiş esterazın tekrar kullanılabilirliği dört kat arttı. Bağlanılan maddenin manyetik özelliği biyokatalizörleri ortamdan ayırmak için kolaylık sağladı.

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CHAPTER 1

INTRODUCTION

1.1. Enzymes

All chemical reactions play roles as part of an orchestra for the continuity of life. These actions of organic compounds in laboratory occur very slowly at low temperatures and low atmosphere pressure. This finding indicates the need of rapid occurrence of chemical reactions with high heat, high atmospheric pressure and extreme ranges of pH values with some chemicals that are called catalysts. However, these chemical reactions that needed for the maintenance of life should come forward fast generally at 37 - 38 ^{0}C, at low atmospheric pressure and under the neutral pH range. On account of these, nature has developed very special catalysts that can work at mild conditions. Nowadays, these biological catalysts are known as “enzymes”. The role of enzymes is very crucial for living organisms, from viruses to man. Hence many extracted enzymes from living organisms maintain their catalytic activity, this biomolecules have made a huge impact on industry. Even though enzymology and enzyme related applications show a great progress in last three decade, in fact, they are known over centuries.

Although the exact nature of enzymatic reactions had not been know, these reactions have been used long before the invention of writing. The souring of milk, alcohol obtaining by the fermentation of sugar, wine, vinegar, cheese making, and fermentation of the bread are some enzymatic reactions that have been known and used since ancient times.

For a long time, it had been thought that these reactions can only work with presence of some microorganisms. Payen and Persoz discovered the first enzyme “diastase” by extracting from malt solution in 1833 (Ercolini 1900). After three years Theodor Schwann discovered pepsin (Daroff 2003). The term of “enzyme” has first been proposed by Wilhelm Kühne in 1877 (Copeland 2000). Emil Fischer is the first

man who used an isolated enzyme for an assymetric synthesis by applying the cyanohydrin reaction to L-arabinose in 1894 (Fessner 2000). By 1920s varied of enzymes discovered and their potential for variety of commercial applications was discussed till early 1970s. Then enzymes are first used in food industry (corn, potato and starch) with carbohydrate processing enzymes (Fessner 2000). After that enzyme related applications show a great increase in industry.

Enzymes are bio macro molecules that produced in living cells with specific catalytic functions to catalyze the biochemical reactions to maintain life. In comparison to inorganic catalysts, enzymes shows more selectivity, specificity, high catalytic activity and also do not require extreme pressures and temperatures. For these reasons, enzymes have gained an excellent importance for commercial and industrial purposes.

How enzymes accelerate bio-reactions? These remarkable bio-macromolecules decrease the activation energy barrier of the reaction. In addition to this, the overall energy change was found invariant in between the reaction with or without enzyme. Figure1.1. shows the energy diagram of a bio-reaction with and without enzyme.

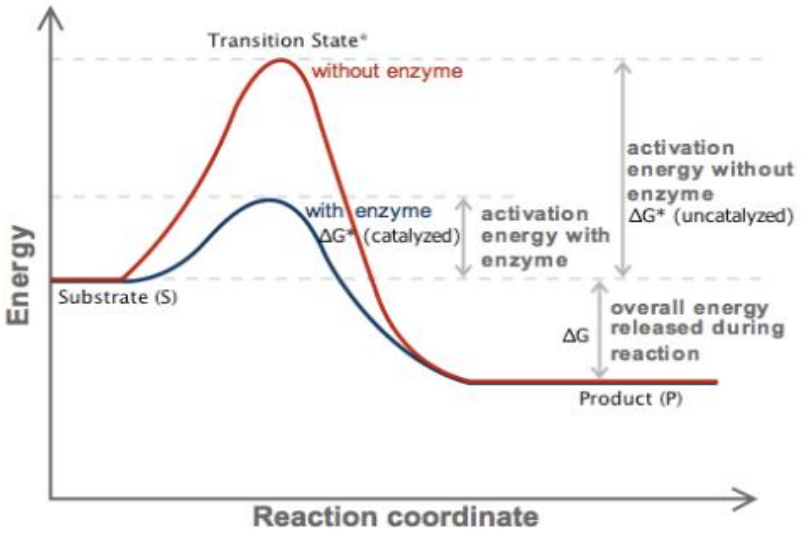

Figure1.1. Energy activation diagram of a reaction with/without enzyme (Source: http://www.felloweducators.com/brick/bio/images/bioi_activation_energy.gif)

1.1.1 Thermostable Enzymes

Microorganisms are divided into three main groups according to their optimal growth temperatures. Psychrophiles grows below 20 ^{0}C, mesophiles grows in moderate temperatures and thermophiles grows in temperatures that is above 55 ^{0}C. Some of the thermophilic bacteria grows above 80 ^{0}C and they are named as hyperthermoplic bacteria (Turner et al. 2007).

Thermostable enzymes are member of the enzyme classes known as extermophiles. Extremophilic microorganisms live in extreme conditions like high salt (halozymes), high alkaline conditions (alkolozymes), high pressure, and acidity and so on. Those thermostable enzymes are isolated from thermophilic organisms. Thermostable enzymes have both kinetic and thermodynamic stabilities at high temperatures. Characteristic feature of thermostable enzymes is having high unfolding temperature (Tm) (Vieille et al. 2001). Thermophiles are resistant to denaturation in high temperatures. Special proteins in the name of chaperonins provide quick refolding of proteins into their native conformation after denaturation. Cell membrane of thermophiles is also composed of saturated fatty acids that provide integrity and rigidity of cell membrane in higher temperatures. In the DNA level, reverse DNA gyrase provide positive supercoiling and increases melting point of DNA (Haki & Rakshit 2003).

Use and development of molecular biology techniques, genetic analysis and recombinant gene transfer methods activities of thermostable enzmes increased. Today use of thermophiles, their proteins and enzymes is dominantly increasing in industrial applications over the rest of microorganisms. Using thermostable enzymes in industry has many benefits than other microorganisms. Thermostable enzymes are better suitable for the harsh industrial conditions. These enzymes also have intrinsic thermostability that provides longer shelf life at room temperatures, reduced contamination risk by mesophiles, increased tolerance to organic solvents provides efficient bioremediation and loosing low activity in processing because they stays below transition temperature of enzymes (Haki & Rakshit 2003; Turner et al. 2007).

Usage areas of thermophiles are so wide. The most widely usage area of thermophiles is in starch industry as amylases. Also those thermostable enzymes are widely using in amino acid synthesis in food industry. In petroleum industry thermostable enzymes are using in the elimination of sulphur containing biodegradable materials (Haki & Rakshit 2003).

1.1.2. Thermophilic Esterase

Esterases and lipases are enzymes that catalyze the hydrolysis of ester bonds (Kademi et al. 2000). Lipases have hydrophobic domain on the active site of the enzyme. By this mean, lipases work well in water insoluble systems by hydrolyzing long chain trigicerides. On the other hand, esterases hydrolyze triglycerides with short chains in water soluble systems. Esterification reaction can be as esterification, transesterification and interesterification reactions (Ateşlier & Metin 2006). At the end of esterification reaction an acid and an alcohol are produced as metabolites.

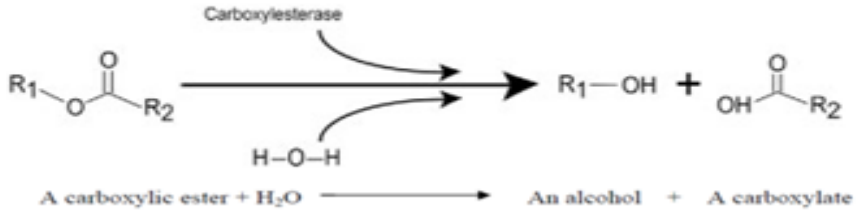

Figure 1.2. Hydrolyze reaction of esterase enzyme (Source: (Montella et al. 2012))

This reaction occurs in two steps. First nucleophilic attack by the catalytic serine hydroxyl on carbon of an ester bond and an alcohol produced as metabolite, by this way enzyme become acylated. As second reaction hydrolysis of water with nucleophilic attack of water on the acylated enzyme and release of carboxylic ester and free active enzyme occurs (Montella et al. 2012).

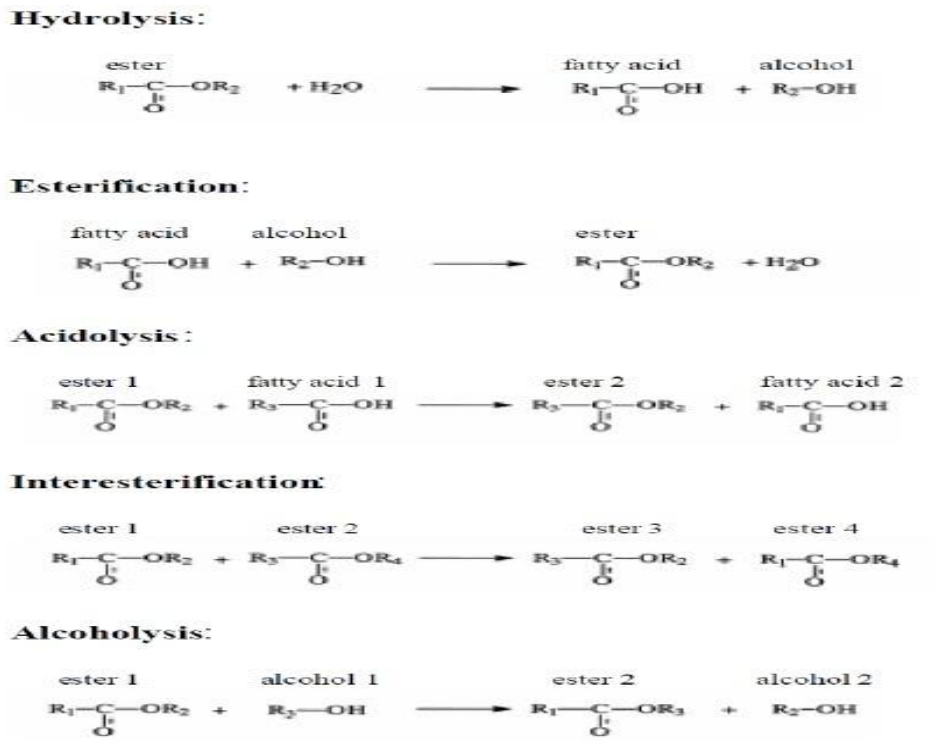

Figure1.3. Esterification reaction (Villeneuve et al. 2000)

Esterases can be produced from animals and plants other than bacteria. However, bacterial esterases have both physiological and physical advantages over animals and plants.

- Because mass production of bacteria is higher, there is large amount of purified esterase become available.
- Bacterial esterases are generally more stable than plants and animals esterase.
- Thermostable microorganisms and enzymes stable at higher temperatures and extreme chemical environments are of advantage in industrial uses.
- Due to specificity of enzymes, unwanted side products that normally appear in the waste stream are reduced or eliminated.
- The use of enzymes can decrease the side reactions and separation problems.
- Esterase catalyzed processes are cheaper, in comparison with traditional processing.
- Esterase remains active in organic solvents during industrial processes.

- Immobilized esterase's can be used under higher conditions that reaction temperatures are higher than 80 oC for prolonged periods (Hasan et al. 2006).

Esterases are widely using in biotechnology applications. Some of the using area of esterases are; as flavor and aroma in food industry especially in cheese production, lubricants and additives in cosmetics industry, hydrolysis of milk fat diary industry and for production of wine, fruit juices, beer and alcohol, chiral drug production in pharmaceutical industry, hydrolysis of methyl esters into valuable acids (Haki & Rakshit 2003). Also, most of the esterases can be used as organic solvents in detergent industry, food industry, oleochemical industry, pulp and paper industry and biodegradation of human-made pollutants such as plastics, polyesters, polyethyleneglycol. At the table 1.1 it is given a detailed presentation of application and source of esterases.

Table1.1. Applications of esterases (adapted from (Panda & Gowrishankar 2005))

FORM OF ESTERASE	APPLICATION	SOURCE
Acetylcholinesterase	Development of new drugs, Biomarker for organo-phosphates in marine environment, Assessment of poison due to pesticides and heavy metals	Blood of *Schistosoma sp.*, *Mytilus edulis*, *Drawida willsi*
Acetyl esterase, Methyl esterase, Acetylglucomannan esterase Acetyl xylan esterase	Release of acetyl and methyl residues from cell wall, Degradation of cellulose, acetic acid from O-acetyl-galactoglucomannan and O-acetyl-4-O-methylglucuronoxylan	*Aspergillus*, *Trichoderma sp*
Aryl esterase	For flavor development in food and alcoholic beverages	*Saccharomyces cerevisiae*
Cinnamoyl ester hydrolase	Plant cell wall degradation	*Piromyces equi*
Erythromycin esterase	Clinical medicine in human, poultry and fish	*Pseudomonas sp*
Sterol esterase, steryl esterase Cholesteryl esterase	Paper manufacturing, to reduce pitch problems during paper manufacture	*Ophiostoma piceae*, *Pseudomonas sp.*, *Chromobacterium*

(cont. on next page)

Table1.1. (cont.)

Carboxylesterases	Degradation of ethylene glycol dibenzoate ester, Lowering toxicity of malathion, Hydrolysis of aspirin and organophosphorous insecticides, Determining metabolic resistances to pyretheroid insecticides,	*Streptomyces lividans*, livers of rat and guinea pig, *Lucilia cuprina*, *Pediculus capitis*, *Bacillus coagulans*
Esterases	Production of isoamyl acetate and mannitol, detoxification of xenobiotics, Control of physiological process of hormone, Hydrolysis of diphthalates, ofloxacin, microbial activity of soil, flavor quality of sake, Fermented sausages, Improvement of aroma and flavor, Fatty acid production	*Locusta migratoria manilensis*, *Micrococcus sp*
Feruloyl esterase	Synthesis of pentylferulate ester used in cosmetics and perfumes industries, Decolorization of paper mill effluent	*A. niger*, *Streptomyces avermitilis*

1.1.3. Thermophilic *Bacillus* Species

Bacillus species produces much number of enzymes and some of them used in industrial applications. Some of the industrially important enzymes are; pyrophosphatase, cellulose, amylase, esterse, lipase and elastase and so on (Hasan et al. 2006).

Formerly it is assumed only *Bacillus stearothermophilus* is thermophilic species of *Bacillus* Genus. Later with the 16S rRNA analyses it is showed that in the genus of *Geobacilus* there is 56 different strains of bacteria is found. After that all those bacteria are classified in the as *Geobacillus. Geobacilus* is rod shaped, gram positive bacteria. It is thermostable and widely distributed in soil, hot springs and ocean sediments (Coorevits et al. 2012). In this study, *Geobacilus sp.* was previously isolated from Balçova (Agememnon) Geothermal region (Tekedar & Şanli-Mohamed 2011).

1.2. Enzyme Immobilization

Enzymes are widely accepted class of biomolecules that are important for production, substrate specify and non-harmful chemical applications (Datta et al. 2012).

“Immobilized enzymes” term is 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.” (Brena et al. 2013).

With the introduction of immobilized enzymes, there is great improvement in both technical performance and industrial processes. Introduction of immobilized enzyme systems allows reuse of catalysts by multiple times, easier reactor operation and product separation. By this mean it provides a wider choice of reactors used in reaction. Contamination risk of product by enzyme or other factors are minimized. Also, enzymes may be stabilized against heat or solvent effects.

On the other hand immobilized enzyme systems can cause loss or reduction in activity, diffusional limitations and additional costs in both production and maintenance of systems (Brena et al. 2013).

Immobilization techniques are not only used in biotechnological applications but also used in applications of diagnosis, pharmaceutics, bio-affinity chromatography and biosensors.

There are two major components of an immobilized enzyme: The enzyme and support (or carrier). To become immobilized enzyme, enzyme need to be in interaction with the carrier. During the enzyme immobilization protocol optimization there is some important factors that need to be considered. In Table 1.2, it is showed the important features of enzyme immobilization system.

Table1.2. Important points of the enzyme immobilization strategy (Source: (Hartmann & Kostrov 2013)).

COMPONENTS	PARAMETERS AND EXAMPLES
Carrier	Chemical parameters: Composition, functional groups, stability against solvents, swelling behavior, hydrophobicity.
	Physical parameters: Morphology, particle size, porosity (pore structure, pore size distribution) active surface area, surface charge, abrasion (for stirred tanks), flow resistance (for a fixed-bed reactor).
Enzyme	Biochemical properties: Availability, storage stability, toxicology, purity, conformational flexibility, molecular weight, size, active site, morphology, functional groups on the surface, surface charge (pI)
	Kinetic parameters: Specific activity, pH and temperature profiles, stability towards reaction conditions (pH, solvents, temperature), stabilizing or activating additives.

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Table1.2. (cont.)

Carrier+ Enzyme (Immobilization Process)	Immobilization strategy: Physical adsorption, encapsulation, covalent binding, cross-linking
	Immobilization conditions: PH, temperature, solvents, stabilizing agents.
	Mass transfer: Buffer effect, viscosity of reaction media, pore diffusion.
	Performance: Space-yield time, recycling, reusability, product removal, toxicity, enzyme inhibition, kinetics
	Cost: Costs of enzyme and support, waste disposal, energy

1.2.1. Choice of Support/Carrier

The most important issue in the determining the support is the performance of the immobilized enzyme system. The ideal support should be resistant to pressure, hydrophilic, biocompatible, resistant to microbial organisms and should be maintained at low cost. Supports are classified as their chemical composition as organic and inorganic. Organic supports are subdivided into two groups: natural and synthetic polymers (Brena et al. 2013).

Table1.3. Classification of supports (from (Brena et al. 2013; Datta et al. 2012))

Organic	
Natural Polymers	Polysaccharides; Cellulose; dextrans, agar, agorose, chitin, chitosan, starch Proteins; Collagen, albumin and Carbon
Synthetic Polymers	Polystyrene Other polymers; polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl, and allyl-polymers
Inorganic	Natural minerals bentonite, silica, celite
	Processed materials: glass (nonporous&controlled pore), metals, controlled pore metal oxides, zeolites, charcole

1.2.2. Immobilization Techniques

In enzyme immobilization there are two methods. In irreversible immobilization, after biocatalyst is attached to matrix surface it cannot be detached without destroying either biological activity of the enzyme or the surface. In reversible immobilization, reversibly immobilized enzyme can be detached from support. In reversible immobilization support can be changed with fresh one and new enzyme can be loaded.

There are several ways for enzyme immobilization and various factors that affect the performance of mobilized enzymes. It is important to determine which techniques and materials are appropriate to ongoing experiment. In this study it will be used crosslinking method by using glutaraldehyde as a crosslinking agent.

Table 1.4. Approaches to enzyme immobilization (Brena et al. 2013; Datta et al. 2012)

METHOD	ADVANTAGES	USED MATERIAL	EXAMPLES
IRREVERSIBLE METHODS			
Covalent Binding	Stable nature of bonds between enzyme and matrix-enzyme is not relased into solution	bi/multifunctional reagents	Glutaraldehyde Bisdiazobenzidine Hexamethylene diisocyanate
Entrapment (beads and fibers) / Microencapsulation	Enzyme is not bound to the support or membrane	Polymers	Collagen, Cellulose κ-car-rageenan
Cross Linking	intermolecular cross-linking of the protein, to functional groups on an insoluble matrix	bi/multifunctional reagents	Glutaraldehyde Bisdiazobenzidine Hexamethylene diisocyanate
REVERSIBLE METHODS			
Affinity Binding	Complementary biomolecules are applied	Affinity ligands	Antibody lectins

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Table 1.4 (cont.)

Adsorption	Enzymes are attached to matrix through hydrogen bonding mild, easy to perform process, and usually preserves the catalytic activity of the enzyme	Uses water-insoluble carriers	Polysaccharide derivatives Synthetic polymers Glass
Chelation or Metal Binding	Transition metal salts hydroxides found on the surface of organic carriers attacks nucleophilic groups on the matrix	Immobilized Metal-Ion Affinity adsorbents	EDTA
Disulfide Bonds	a stable covalent bond is formed between matrix and enzyme, it can be broken by reaction with a suitable agent	Thiol-reactive adsorbents	dithiothreitol (DTT)
Ionic Binding	Enzymes are attached to matrix through salt bridges Simple and reversible	Uses water-insoluble carriers-containing ion-exchange residues	polymeric-ionic ligands

1.2.2.1. Crosslinking Methods

As a disadvantage of solid support enzyme immobilization, it can be reduced the specific and volumetric activity of the biocatalysts. In the early 1960s, as an alternative of support binding immobilization, discovery of cross-linking of dissolved enzyme with a chemical cross-linker showed higher catalytic activity. Use of bi-functional cross linkers, reaction of surface NH2 groups, such as glutaraldehyde, binds to enzymes

together without a support (Brady & Jordaan 2009; Sheldon 2007). Crosslinking can be made using different methods like chemical, physical and irradiation cross-linking.

Table1.5. Different crosslinking methods in enzyme immobilization
Source: (Gulrez et al. 2003)

CROSSLINKING METHOD	CROSSLINKING REACTION	EXAMPLES
Physical Cross-Linking	Heating And Cooling Polymer Solution	Cooling Hot Solutions Of Gelatin Or Carrageen Polyethylene Oxidepolypropylene Oxidepolyethylenem Glycolpolylactic Acid Hydrogel
	Ionic Interaction	Chitosan, Chitosan-Glycerol Phosphate Salt, Chitosan-Dextran Hydrogels
	H-Bonding	Hydrogen-Bound CMC (Carboxymethyl Cellulose) Network Carboxymethylated Chitosan (CM-Chitosan) Hydrogels
	Heat Induced Aggregation	
	Freeze And Thawing	Freeze-Thawed Gels Of Polyvinyl Alcohol And Xanthan
Chemical Crosslinking	Chemical Crosslinkers	Glutaraldehyde Epichlorohydrin
	Grafting	Acrylic Acid By Using N-Vinyl-2-Pyrrolidone Irradiation And Gamma And Electron Beam
Radiation Cross-Linking		Irradiation Uv-Gamma Rays

Cross-linking enzyme immobilization method has many advantages over the other methods.

- By using cross-linking method multimeric enzymes can be stabilized.
- In harsh conditions stabilization can be achieved.
- It is not necessary to having high purity of enzyme.
- No adsorption from the support.
- In tandem systems, different enzymes can be immobilized at the same time
- Enzyme does not conformational changes.

On the other hand, using cross-linking method have some disadvantages.

- There is more than one experimental steps are necessary.
- Toxic chemicals are used like glutaraldehyde.
- According to reduced pore size diffusion rates can be decreased. (Hartmann & Kostrov 2013).

1.2.2.1.1. Glutaraldehyde

Glutaraldehyde, a linear, 5-carbon dialdehyde, is a colorless pungent oily liquid that is soluble in all proportions in water and alcohol and in organic solvents. Glutaraldehyde is mainly available in (pH 3.0–4.0) aqueous solutions. It is commercially available and cheap (Migneault et al. 2004).

Glutaraldehyde is mostly used as cross linker. Use of glutaraldehyde is simple and efficient. Glutaraldehyde modifies proteins by involving their primary amino acid structures by reacting with amine groups. In some instances of multi-subunit immobilization, it allows to improving of enzyme stability (Wang et al. 2014; Betancor et al. 2006).

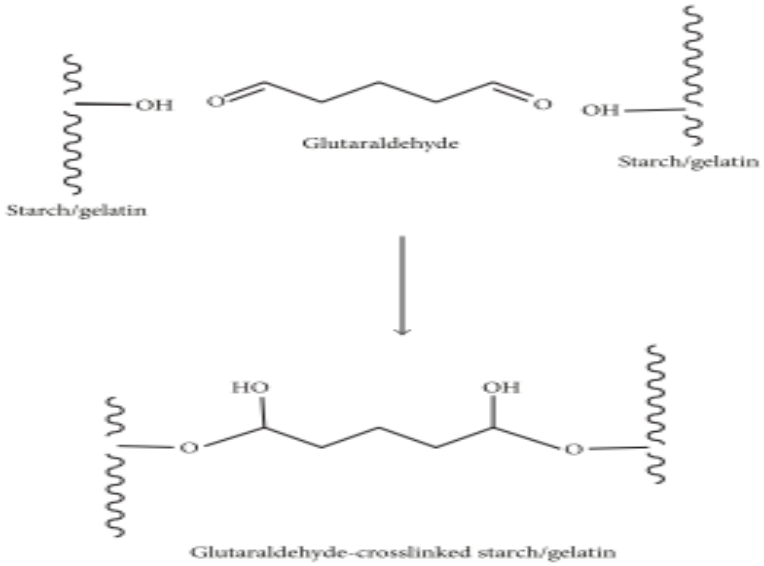

Figure 1.4. An illustration of crosslinking reaction of glutaraldehyde starch/gelatin (Phromsopha & Baimark 2014).

Immobilization by covalent attachment to water insoluble carriers using glutaraldehyde is one of the most used methods in enzyme immobilization. Glutaraldehyde is mostly using in enzyme immobilization because the reaction proceeds in aqueous buffer solution under conditions close to physiological pH, ionic strength, and temperature (Migneault et al. 2004).

1.3. Nanoparticles

- Nanoparticle are taken into consideration by their small size, customized surface, solubility in desired solvent and multiple functionality (Shi et al. 2011). According to **IUPAC;** nanoparticle is a particle of any shape with dimensions in the 1×10^{-9} and 1×10^{-7} m range.

Researchers prepared nanoparticles from various compounds like chitin, chitosan, dextran, albumin, alginate and starch and so on. Nanoparticles should maintain the features below:

- Biocompatible and degradable
- Maintain physical properties after modifications
- Chemically stable
- In therapeutic applications gives no harm to cells
- No formation of side products (Markides et al. 2012).

1.3.1. Corn Starch Nanoparticles

Starch is a natural, cheap, biodegradable and reproducible polymer. It is composed of many number of glucose that joined together by glycosidic bond. Because of these features, starch is used in food, chemical, textile, paper, medicine and many industries. It is produced by plants and used as an energy store. In the world, the main sources of starch are maize (corn), wheat, potato and cassava (Le Corre et al. 2010; Datta et al. 2012).

With the some degree modifications, functionality of starch can be increased. Starch nanoparticles are result of such modifications and they can be used in drug delivery systems. Starch is constituted from linear amylase and branched amylopectin units and widely used as an enzyme immobilizer. Starch powder is composed of microscopic granules, sizes changing between 2 to 100 um. With proper cross-linking agent, starch nanoparticles can be encapsulated and delivers hydrophobic drugs to cellular targets (Shi et al. 2011; Le Corre et al. 2010).

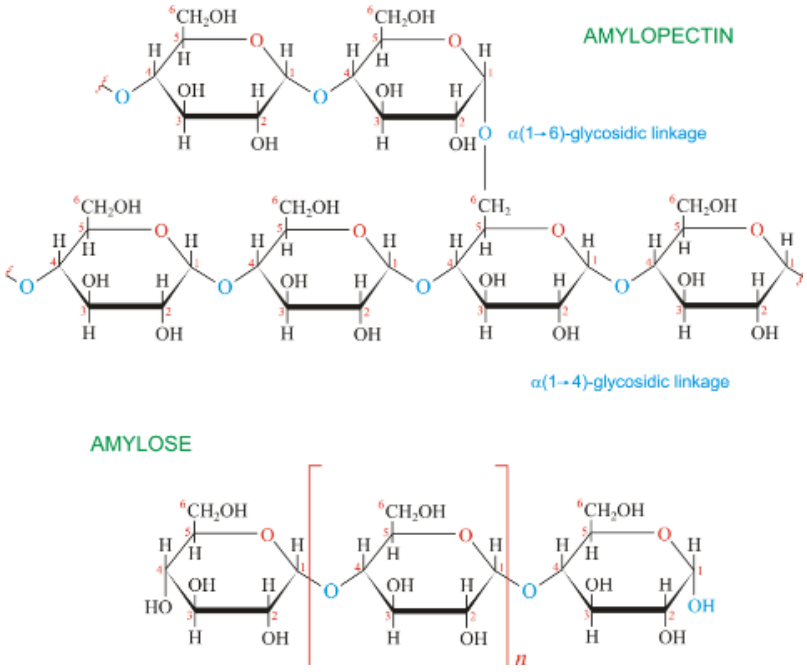

Figure1.5. Structure of starch, amylose and amylopectin

(Source:http://glossary.periodni.com/glossary.php?en=starch)

1.3.2. Magnetic Nanoparticles

Use of nanoparticles offers many advantages as mentioned before due to their sized and physiochemical properties. Magnetic nanoparticles are a class of nanoparticles that commonly contains magnetic elements such as nickel, cobalt, iron and so on. Magnetic nanoparticles can be manipulated by magnetic fields. Different type of magnetic nanoparticles are widely using in biotechnology, biomedical applications, engineering and environmental areas. In these area, specifically, magnetic nanoparticles are used in magnetic fluids, catalysis, magnetic resonance imaging, data storage and bioremediation (Lu et al. 2007; Akbarzadeh et al. 2012). As an advantage of magnetic nanoparticles, they can easily be separated from a reaction system using external magnetic field. By this way, thermal, operational and storage stabilities of enzyme improved (Jiang et al. 2009).

1.3.2.1. Magnetite

Magnetic nanoparticles gain their magnetic properties by the natural occurring forms of iron oxides. This exhibits super paramagnetic properties of nanoparticles and they typically composed of magnetite (Fe_3O_4) or maghemite (g-Fe_2O_3). Those magnetite and maghemite named as ferromagnetic compounds. However the most used form of magnetic nanoparticles is magnetite (Fe_3O_4) due to its high biocompability. On the other hand, the magnetite properties are highly dependent on its formation method. The most used and the simplest method to form Fe_3O_4 particles is co-precipitation method (Soares et al. 2016).

In biological applications usage of Iron oxide (Fe^{+2} and Fe^{+3}) based magnetic nanoparticles has advantages listed below:

- Super paramagnetic nanoparticles do not react with each other
- Risk of cluster formation minimized
- Fe is naturally found in human body and iron containing nanoparticles are biocompatible to human body
- Fe is metabolized in human body without toxicity
- Tagging and tracing Fe oxide in body is easy (Markides et al. 2012).

In this experiment magnetic corn starch nanoparticles will be bound to thermophilic esterase by the glutaraldehyde cross-linking reagent.

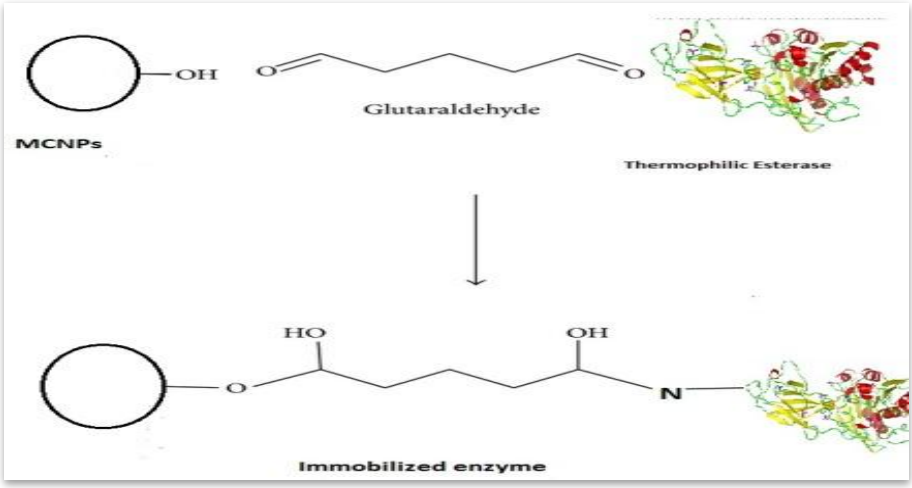

Figure1.6. Magnetic corn starch nanoparticles bounded to thermophilic esterase by the glutaraldehyde

1.4. Aim Of The Study

In this thesis, I studied on the immobilization of thermophilic esterase on paramagnetic cornstarch nanoparticles. The (multiple) aims of the study;

- To developed a cost effective, non-toxic and environmentally safe method for esterase immobilization.
- To investigate potential improvements of enzyme activities.
- To improve an easily removable enzyme carrier for industrial processes.
- To overcome limitations of reusability of enzymes.

CHAPTER 2

MATERIAL AND METHODS

2.1. Materials

All used chemicals, buffers, solutions and their compositions were listed detail in Appendix A and Appendix B.

2.2. Preparation of Magnetic Cornstarch Nanoparticles (MCSNPs)

In this study, 0,003 mole $FeCl_2.4H_2O$ (Sigma) and 0,0045 mole $FeCl_3.6H_2O$ (Sigma) (2:3 molar ratio) were dissolved in 90 ml distilled water under stirring. 1 g of cornstarch (pure commercial powder) was poured into 10 ml distilled water and the mixture was stirred until dissolved. The starch mixture was added into the Fe^{+2}/Fe^{+3} solution and heated (Wisestir MSH 20A) in water bath less than 65 ^{0}C in final volume 100 ml. After this step, the mixture was treated with 4 M NaOH (Sigma) solution to adjust pH value to 12-13. Then the mixture was treated for 20 minutes by ultrasonic wave (Elmasonic). Afterwards, another stirring were performed under 65^{0}C for 2 hours. Then, the mixture was cooled down to room temperature and neutralized by acetic acid (Merck). Thereafter, the formed precipitate was washed twice with 95% ethanol (Merck) and gathered by magnetic decantation. At last the collected magnetic precipitates were freeze dried (Hettich Rotina 38R) and were stored at +4^{o}C for further studies. These study was performed according to method of Wang B. and Cheng F. (Wang et al. 2013). Co-precipitation method of ferric and ferrous ions results in magnetite (Fe_3O_4) and magnetite gives the magnetic character to the particles (Kim et al. 2003).

Figure 2.1. Magnetic decantation of MCSNPs by magnets

2.3. Binding of Glutaraldehyde on MCSNPs

At first, 10 ml and 5% of glutaraldehyde (Sigma) solution in 0,01 M sodium phosphate (Na-P) buffer at pH 7 was prepared. Then 1 g of magnetic cornstarch nanoparticles was added into 10 ml of the glutaraldehyde solution. For crosslinking process, the mixture was incubated at 30^0C in rotary shaker (Ivymen system shaking incubator) at 300 rpm for 20 minutes. Increasing incubation time can lead double crosslinking of glutaraldehyde with starch. After that, the precipitates were collected magnetically and washed with 0,01 M sodium phosphate buffer at pH 7. Then, this precipitates were dried by vacuum freezing and stored at +4 ^{o}C.

2.4. Characterization of MCSNPs

In this section size distribution and SERS analysis of the magnetic corn starch nanoparticles were examined. Size determination is important to proof that the particles small enough to be at nanoscale. SERS analysis was also crucial to show the composition of the nanoparticles.

2.4.1. Size Determination of MCSNPs

The characterization studies of MCSNPs were carried out by measuring the size distribution with ZetaSizer (Malvern Zetasizer 3000). In order to obtain optimum size distribution, the nanoparticle synthesis was performed by changing the temperature, molar ratios of iron ions and amount of corn starch. The synthesis was carried out at room temperature, 50^0C, 65^0C and 80^0C. The used molar ratios of ferric ion and ferrous ion were 1:1, 2:1 and 2:3. Then the synthesis was performed by using 0,8 g, 1,0 g and 1,2 g of corn starch. After MCSNPs were synthesized, 100 μL of the separated precipitate was mixed with pure water and the samples were analyzed. All measurements were repeated three times.

2.4.2. SERS Analysis of MCSNPs

Each material has their own characteristic Raman peaks. Surface Enhanced Raman Spectroscopy (SERS) used to determine the composition of the MCSNPs. In order to proof the synthesized nanoparticle contains magnetite, starch and glutaraldehyde, their individual analyses were performed and these results were compared with glutaraldehyde modified magnetic corn starch nanoparticles results. The first examination was to find out the characteristic corn starch Raman peaks. For this

reason, a small amount of corn starch and the magnetic iron nanoparticles was analyzed by Raman microscopy (Horiba). In addition to analyze starch and magnetite, same procedure was followed with starch coated magnetite to see the peaks of them together. At last, modified magnetic starch nanoparticles with glutaraldehyde were analyzed and to proof the MCSNPs contains glutaraldehyde.

2.5. Immobilization of Esterase on MCSNPs

First, 1 g of MCNPs that modified with glutaraldehyde was dissolved in 10 ml sodium phosphate buffer (pH 8, 0,01 M) and heated to 30 ^{o}C for 30 minutes. Then 0.5 mg/ml of liquid esterase was slowly added in to the modified MCSNPs solution and mixture was incubated with continuous shaking at 100 rpm for 4 hours at 30 ^{o}C for complete equilibrium. Finally MCSNPs bounded esterase were collected by magnetic decantation, washed with sodium phosphate buffer at pH 7 and stored at 4 ^{0}C and used for activity assays according to procedure of (Wang et al. 2013).

2.6. Obtaining and Confirmation of Thermophilic Esterase

The esterase samples that used in this study were purified by (Tercan 2011) from *Geobacillus sp*. For the purpose of proving the thermophilic esterase samples were still active, the enzyme activity assay of (Tekedar & Şanli-Mohamed 2011) was followed.

2.6.1. Nanodrop Analysis of Esterase Samples

The enzyme samples in tubes that stored at -20 ^{0}C were incubated at room temperature. After the samples were melted, they were measured with Nano drop (Thermo Scientific) at 280 nm. The results of the measurements were presented at results section in Table 3.1.

2.6.2. SDS-PAGE Analysis of Esterase Samples

In previous studies, the weight of the thermophilic esterase that purified from *Geobacillus sp.* was found as 27 kDa (TERCAN 2011). SDS-PAGE assay was followed to proof the esterase samples have not been denatured over time. The studies were carried out according to Laemmli procedure (Laemmli 1970) at Biotechnology, Bioengineering Application and Research Center, IZTECH.

2.6.3. Esterase Activity Determination

In this part of the study, the activity of the thermophilic esterase was analyzed by spectrophotometric method. The *p*-nitrophenyl acetate (*p*-NPA) was used as substrate for the activities of both free and immobilized enzyme.

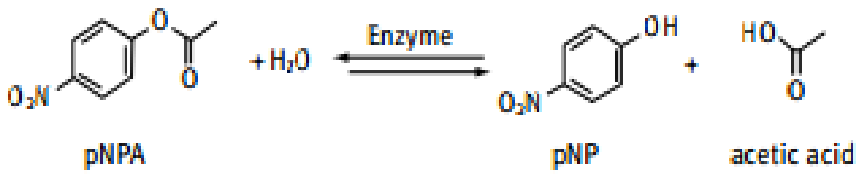

Figure 2.2. Scheme of the *p*-NPA assay (Ganske & Labtech 2009)

In this assay, the enzyme hydrolyzes the p-NPA by help of H_2O and the reaction products are p-nitrophenyl and acetic acid. The presence of the p-NP is captured by spectrophotometric detection (Ganske & Labtech 2009).

Given values in Table 2.1 were used in activity determination free enzyme.

Table 2.1. Followed Values for Esterase Activity Determination

	Sample	Blank
Tris-HCl buffer (0.1 M – pH 9.0)	990 µl	990 µl
Substrate (50 mM)	9.0 µl	------
Enzyme (1.0 mg/ml)	1.0 µl	1.0 µl
Water	------	9.0 µl

The assay was carried out by using values at Table 2.1. The activities of both free and immobilized enzyme were measured with Varian spectrophotometer (Cary 50 Scan) at 420 nm.

Activity calculations of both free and immobilized enzyme were calculated according to equation (2.1):

$$\text{Enzyme Activity} = {Units}/{ml}\ \text{enzyme} = \frac{\Delta A420\text{nm}}{\mathcal{E}\ X\ VX\ t} \qquad (2.1)$$

ΔA: Absorbance of the reaction sample
$\mathcal{E}$: Extinction coefficient (0.0148)
V : Volume of used enzyme
t : Incubation time (minutes)

During the reaction that was given at Figure 2.2., the amount of enzyme that had released 1.0 μM of p-NP from p-NPA per minute was defined as one unit of esterase activity at pH 7.2 at 55 ^{0}C. This procedure was repeated for different temperature (25, 35, 45, 50, 55, 60, 65, 70, 80 and 90 ^{0}C) and different pH values (pH 4, 5, 6, 7, 8, 9, 10, 11, 12) for both activity and stability tests. Activity assays from previous study (Tekedar & Şanli-Mohamed 2011) were directly followed.

A different activity determination assay was followed for immobilized esterase. During immobilization of the enzyme, the amount of esterase that used was 1.0 mg/ml. The activity determination assay was performed by preparing 3 ml mixture that contains immobilized enzyme, 2985 μl of buffer (Tris-HCl 0.1 M, pH 8.0) and 15 μl substrate (p-NPA). This mixture was incubated at 55 ^{0}C with slow shaking for 5 minutes and 1 ml of the solution was taken to determine the catalytic activity of the immobilized enzyme spectrophotometrically at 55 ^{0}C, 420 nm. Activity and stability determination of the immobilized esterase was determined according to previous immobilization study with esterase (Tercan 2011).

The supported enzyme activity and stability tests were carried out with same temperature and pH values as used with free esterase experiments.

Table 2.2. Followed Values for Activity Determination of Immobilized Esterase

	Tris-HCl (0.1 M, pH 8.0)	Immobilized enzyme solution	Water	Substrate p-NPA
Blank	980 μl	10 μl	10 μl	----------
Sample	980 μl	10 μl	----------	10 μl

In order to find out the enzyme recovery after immobilization, both free and immobilized enzyme activities were determined according to (Wang et al. 2013) and the rate of enzyme recovery (Er%) was calculated by Eq (2.2).

$$Er = {}^{Ui}/_{U0} \times 100\% \qquad (2.2)$$

Er : Enzyme recovery (%)

Ui : Total enzyme activity recovered on the support

Uo : Enzyme units offered for immobilization

CHAPTER 3

RESULTS

3.1. Characterization of Magnetic Cornstarch Nanoparticles (MCSNPs)

In order to characterization of MCSNPs, at first the effect of reaction condition of the particle formation is determined. Afterwards, SERS analysis showed us the particle character by raman peaks. Then thermophilic esterase was bounded to the MCSNPs and the enzyme kinetics studies performed.

3.1.1. Effects of Reaction Conditions on MCSNPs Size

There are so many parameters that can affect the particles sizes and their characters such as pH, temperature, chemical agents and chemical compositions. The characterization studies were performed over reaction temperature, iron ions composition and starch amount during the reaction. Each experiments were performed three times.

3.1.1.1. Effect of Reaction Temperature

The particle sizes are strongly affected by temperature. Since our support material is a natural organic polymer, it was a vital to find an optimum temperature for the reaction. In order to determine the most convenient temperature, the reaction was performed at room temperature, 50^0C, 65^0C and 80^0C respectively. All data were analyzed 3 times and the mean averages of different temperatures were used in the Figure 3.1.

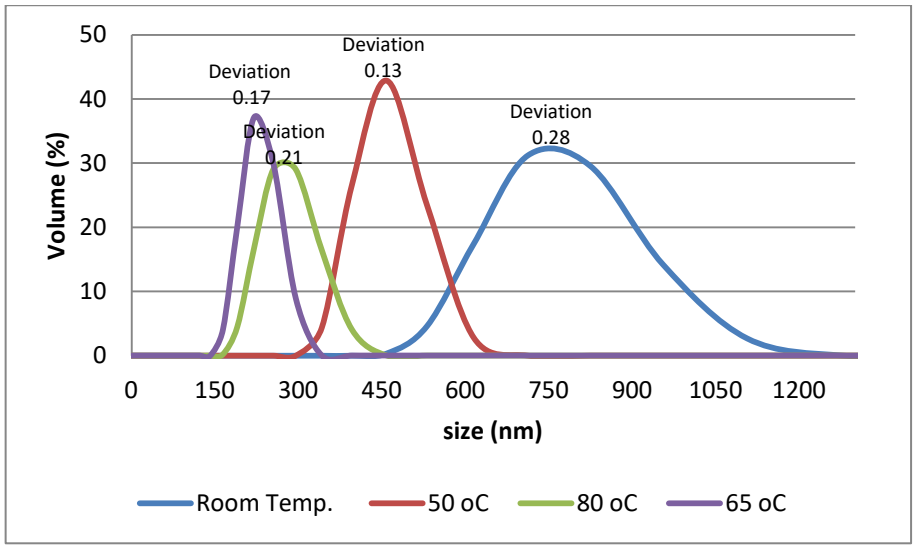

Figure 3.1. Effect of reaction temperature on particle size with 0,8 g starch in 10 ml of reaction mixture and Fe^{+2}/ Fe^{+3} molar ratio is 1:1

There has been a dramatic effect of temperature on particle size as can be seen in Figure 3.1. The dissolution of starch is so hard in water at room temperature, so it is understandable to encounter with high sized particles. In addition, it was observed that the 50 ^{0}C is not enough to break the chemical bounds (Hydrogen bonds, Wan der Waals force etc.) among starch molecules. Because the sizes of the synthesized particles were still not enough as small as be called “nanoparticles”. So, the reaction was repeated at

65^0C and 80 ^{0}C. At the end of the reactions, gelatinization of the starch was observed at 80 ^{0}C that disrupt the particle behavior in time. At 65 ^{0}C, the particles were at desired size level and also gelatinization was not observed.

3.1.1.2. Effect of Ferrous and Ferric Ions Molar Ratios

There are many procedures that explain the magnetic iron oxide nanoparticles synthesis and almost all procedures result in different size formations. In this study, since the iron oxide nanoparticles was designed as core shell for cornstarch, it was important to find out the most suitable molar ratios of iron ions during the reaction. Figure 3.2 shows the 2:1, 1:1 and 2:3 molar ratios of iron ions.

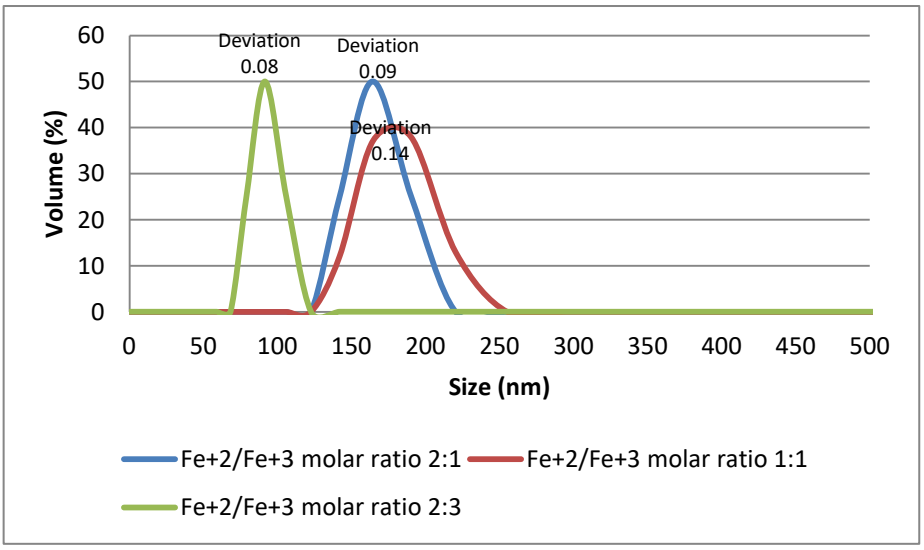

Figure 3.2. Effect of molar ratios of Fe^{+2} and Fe^{+3} ions on particle size with 1,0 g starch in 10 ml of reaction mixture.

In literature, many studies have shown that magnetite nanoparticles tend to agglomerate after co-precipitation reaction of ferric and ferrous ions. In order to prevent this phenomena, some stabilizers are used such as starch (An et al. 2011). Also, the

molar ratios of the ions affect the particle size. At this study, the particle size at 2:3 molar ratios of iron ions shows a good result below 100 nm. The reason may be the agglomeration of the magnetite increase or decrease in some molar ratios of ferric and ferrous ions.

3.1.1.3. Effect of Starch Amount

Cornstarch was used to coat the magnetic iron oxide nanoparticles. Therefore, the amount of the starch was vital for particle size.

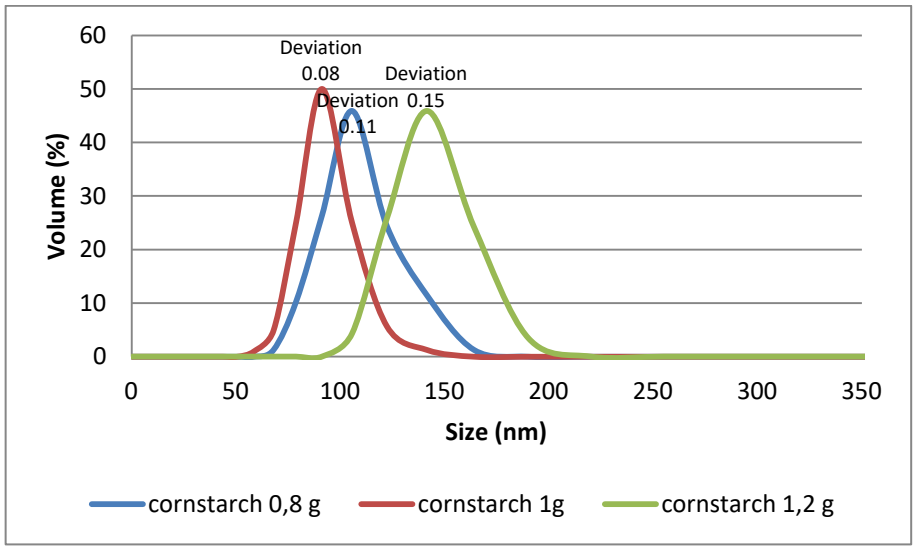

Figure 3.3. Effect of the used amount of starch (g) on particle size distribution (molar ratio of Fe^{+2}/ Fe^{+3} is 2:3)

As it mentioned above, starch is used to prevent agglomeration of the magnetite nanoparticles. By this information, we can say that the different amounts of starch in reaction media may result in different particle sizes. In this part, three different amount of starch in reaction was examined which are 0,8 g, 1,0 g and 1,2 g respectively. In

Figure 3.3, it is seen that 1,0 g of starch in reaction mixture gives a good result below 100 nm particle size. This mean size was small enough for our further studies.

3.2. Characterization of Modified CSNPs by SERS

Before the immobilization of the thermophilic esterase, MCNPs were analyzed by Surface Enhanced Raman Spectroscopy (SERS) technique. SERS was employed to determine the interactions between starch, magnetite and glutaraldehyde. In this step, analysis of starch, magnetite, starch-magnetite nanoparticles and modified magnetite cornstarch nanoparticles with glutaraldehyde were performed.

3.2.1. SERS Analysis of Starch

The SERS analysis of the cornstarch was investigated to see the characteristic raman peaks of the cornstarch. In Figure 3.4, there are strong peaks at 2917 cm^{-1} and 478 cm^{-1}.

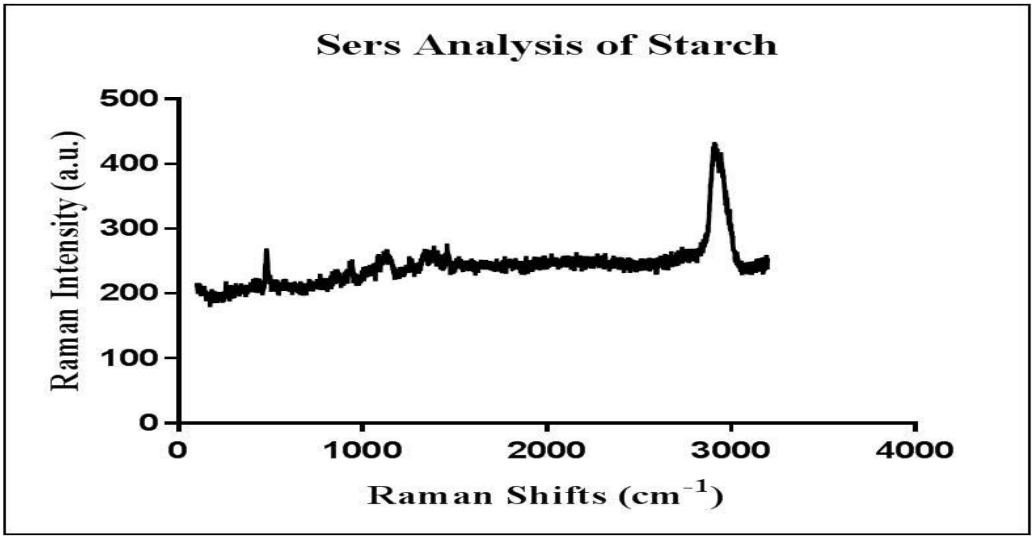

Figure3.4. Raman peaks of corn starch

3.2.2. SERS Analysis of Magnetite (FE_3O_4)

In order to determine the characteristic Raman peaks of magnetite in the MCSNPs, the SERS analysis of magnetite was performed. As can be seen in Figure 3.5, There is a strong peak at 668 cm^{-1}.

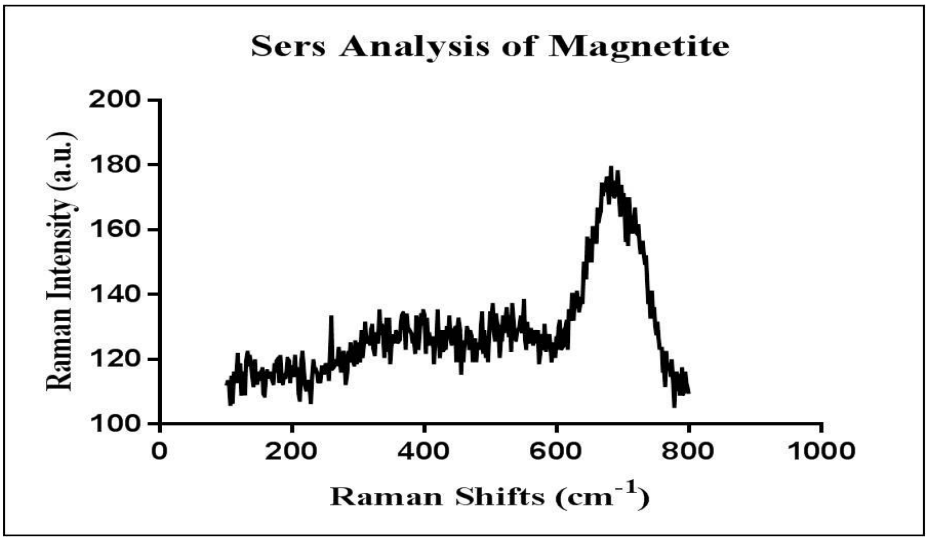

Figure 3.5. Raman peaks of magnetite (FE_3O_4)

3.2.3. SERS Analysis of Magnetic Corn Starch Nanoparticles

SERS analysis of starch and magnetite gave chance to see their characteristic peaks individually. In order to determine the reactions between starch and magnetite and also to proof that magnetic corn starch nanoparticles was really contains magnetite and starch, this analysis was performed. As can be seen, Figure 3.6 contains both magnetite and corn starch with their specific peaks.

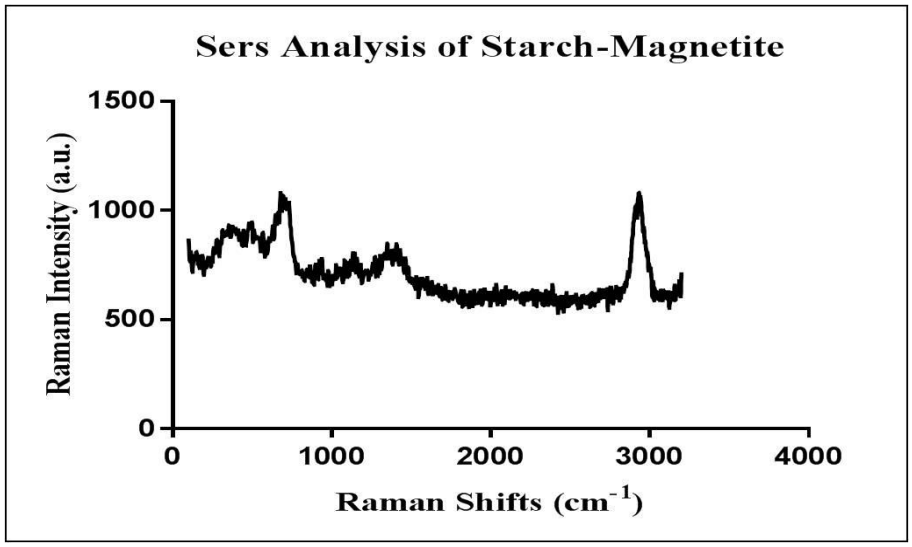

Figure 3.6. SERS analysis of MCSNPs

3.2.4. SERS Analysis of Glutaraldehyde Modified MCSNPs

After MCSNPs modified with glutaraldehyde, it was important to some changes on the nanoparticles Raman peaks. In Figure 3.7, the peaks number around 0-1000 cm^{-1} was increase. This can be proof that glutaraldehyde was bounded to the nanoparticles and the reactions between MCNPs - glutaraldehyde is also observed.

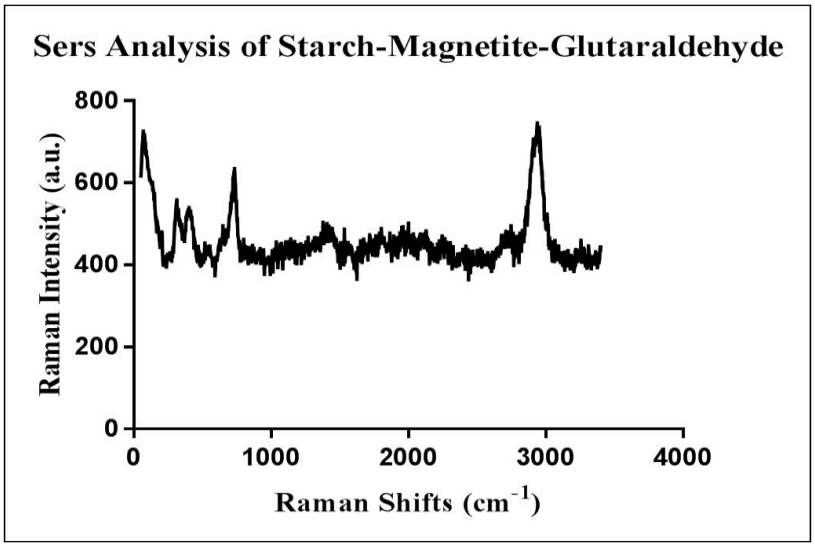

Figure 3.7. SERS analysis of Glutaraldehyde modified MCSNPs

3.3. Obtaining Thermophilic Esterase

The esterase enzyme samples that used in this study were purified at previous immobilization study of Tercan in 2011 (Tercan 2011).

3.3.1. Confirmation of Thermophilic Esterase

In order to understand whether if the enzyme samples still works or not, concentration determination by Nano drop, SDS-PAGE procedure and enzyme activity assays were performed with these enzyme samples. In previous studies, the molecular weight of purified thermophilic esterase from *Thermophilic Bacillus spp*. was found 27 kDa (Gülay, 2009).

3.3.1.1. Nanodrop Analysis of Enzyme Samples

The concentration of the enzyme samples were determined by using nanodrop spectrophotometer (Thermo Scientific).

Table 3.1. Protein concentration of sample 1, sample 2 and sample 3.

	Sample 1	Sample 2	Sample 3
Protein concentration (ng/ul)	0.85	1.2	1.1

3.3.1.2. SDS-PAGE Analysis of Enzyme Samples

After found out the protein concentration of the samples SDS-PAGE performed to investigate molecular weight of the samples. This examination was carried out at Biotechnology, Bioengineering Application and Research Center, IZTECH. In the Figure 3.8, it can be seen that the molecular weight of three esterase samples are 27 kDa.

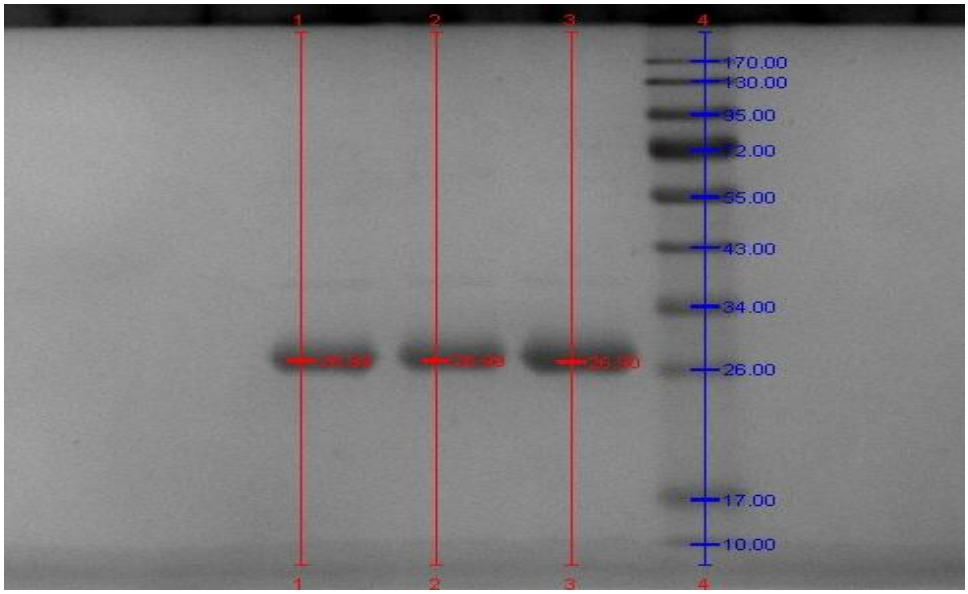

Figure 3.8. SDS-PAGE conformation of thermophilic esterase where the line 1, 2, 3 are the enzymes samples and line 4 is the protein ladder.

3.3.1.3. Enzyme Activity Tests of the Esterase Samples

In order to prove that the thermophilic esterase samples still work, enzyme activity assays that used from previous study were performed from Tercan, 2011.

3.3.1.3.1. Effect of Temperature on The Esterase Samples

Each enzyme has its unique optimum temperature that shows best activity. The optimum temperature for thermophilic esterase was investigated by Tekedar 2008, In their studies the best activity was at 65^0C. As can be seen in the Figure 3.9, the enzyme still works at optimum temperature conditions.

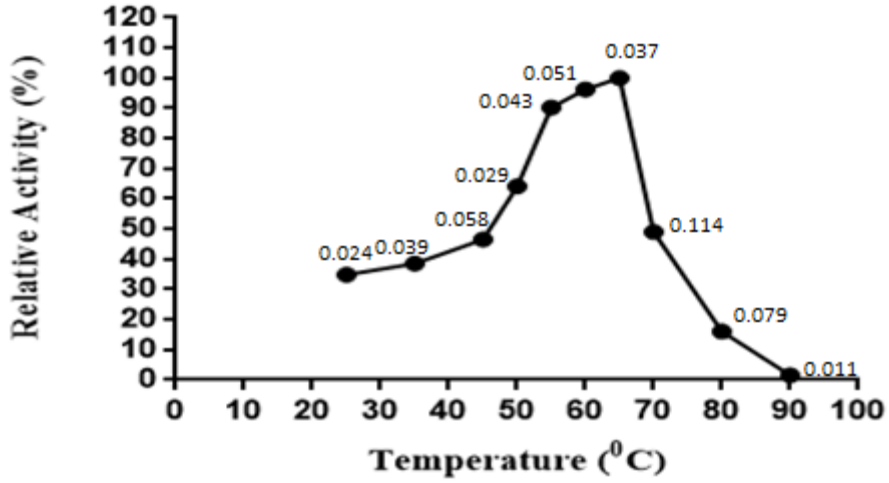

Figure 3.9. Effect of temperature on thermophilic esterase enzyme with 5 min incubation.

3.3.1.3.2. Effect of pH on The Esterase Samples

Another important parameter for enzyme activity is the pH value of the reaction media. The optimum pH value for the esterase was founded as pH 9, 5 at previous study Tekedar 2008. In Figure 3.10, the maximum activity is at 9 pH.

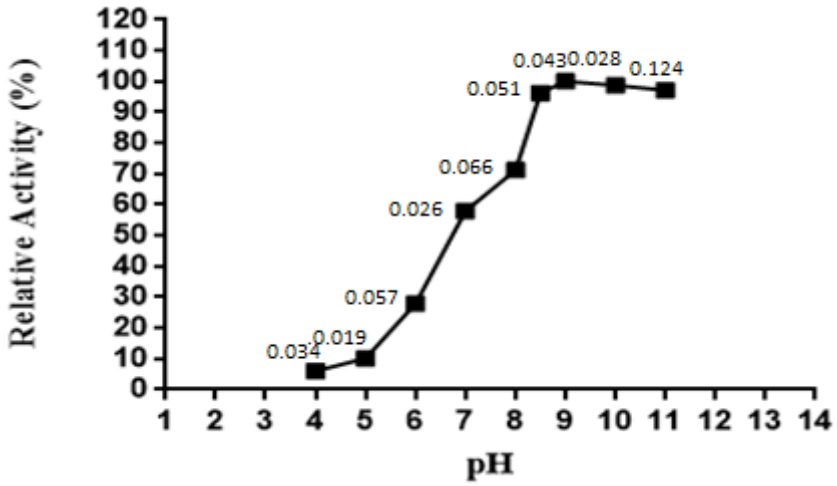

Figure 3.10. Effect of pH on thermophilic esterase enzyme (5 min incubation times)

3.4. Characterization of Immobilized Thermophilic Esterase on MCSNPs

Our main aim is to improve the activity performance and stability. In this part of the study, the immobilized enzyme was investigated by temperature and pH effect on its activity to compare with pure enzyme behavior on this conditions.

3.4.1. Effect of Temperature on Immobilized Enzyme Activity

As the pure enzyme activity, the immobilized enzyme activity was examined among 25 ^{0}C and 90 ^{0}C. In the figure 3.11, it can be observed that the maximum enzyme activation is at 65 ^{0}C.

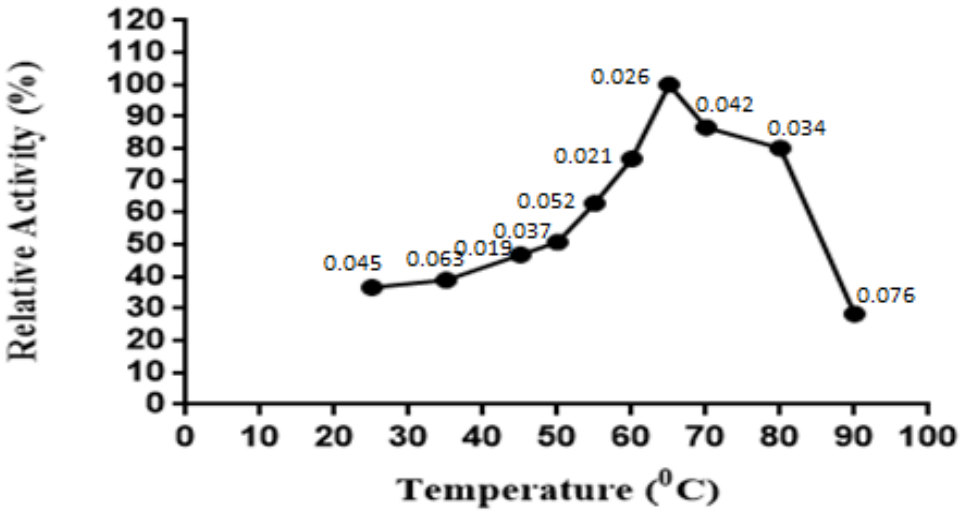

Figure 3.11. Effect of temperature on immobilized enzyme (5 min incubation time)

3.4.2. Temperature Stability of Immobilized Enzyme

Temperature stability of the immobilized enzyme is crucial as much as its activity change in temperature interval. In order to examine the effect of the temperature stability, the immobilized enzyme has been incubated for 1 hour duration for 10 different temperature values from 25°C to 90°C (25°C, 35°C, 45°C , 50°C, 55°C, 60°C, 65°C, 70°C, 80°C and 90°C) and then incubated again 5 more minutes after substrate has been added to the medium. The maximum activity was at 65 ^{0}C.

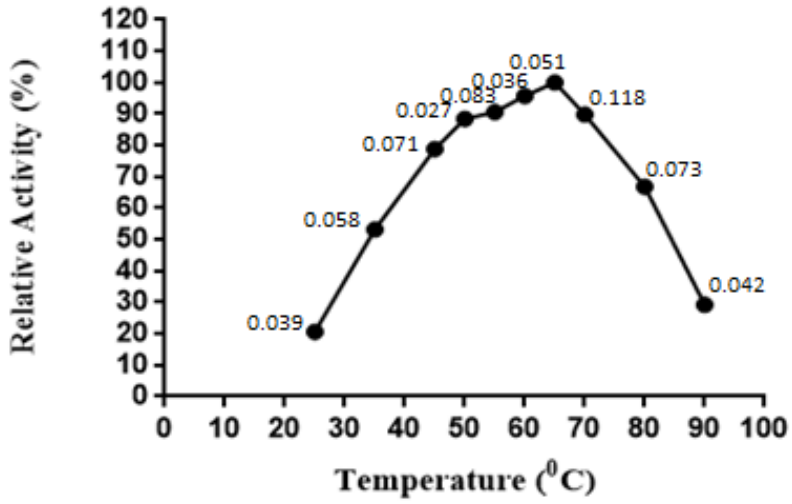

Figure 3.12. Temperature stability of immobilized enzyme after 1 hour incubation times at different temperatures.

3.4.3. Effect of pH on Immobilized Enzyme Activity

Changing in pH values are strongly affects the enzyme activity. After immobilization of the enzyme it's more crucial to find out the optimum pH activity and the behavior of the activity over pH range. In figure 3.13, it can be seen that the maximum activity at pH 9.

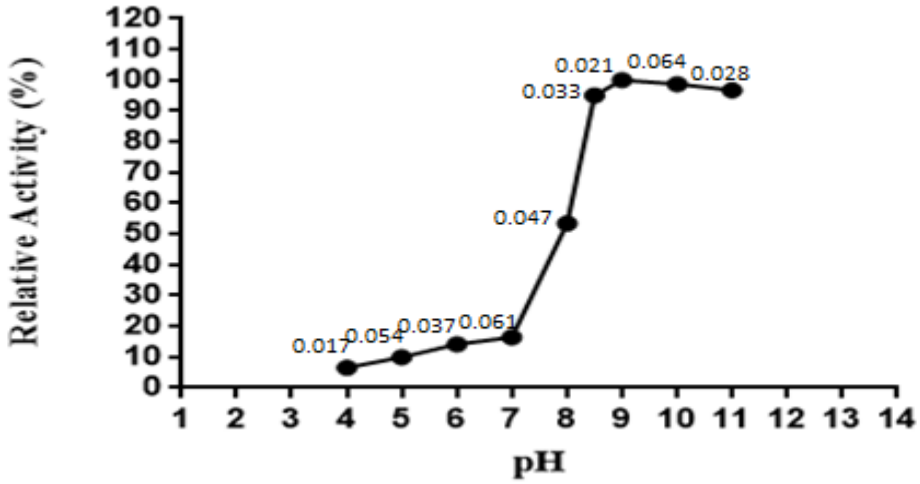

Figure 3.13 Effect of pH on immobilized enzyme activity after 5 min incubation time.

3.4.4. pH Stability of Immobilized Enzyme

Stability of the immobilized enzyme on pH range is also very important parameter that gives the characteristic of the immobilization. In order to determine the the pH stability, the immobilized enzyme has been incubated for 1 hour duration at 9 different pH values (4, 5, 6, 7, 8, 9, 10, 11 and pH 12) at 55^0C. As can be seen in the Figure 3.14, between the range of pH 8-12 the relative activity of the immobilized enzyme over %90. Maximum activity is at pH 9.

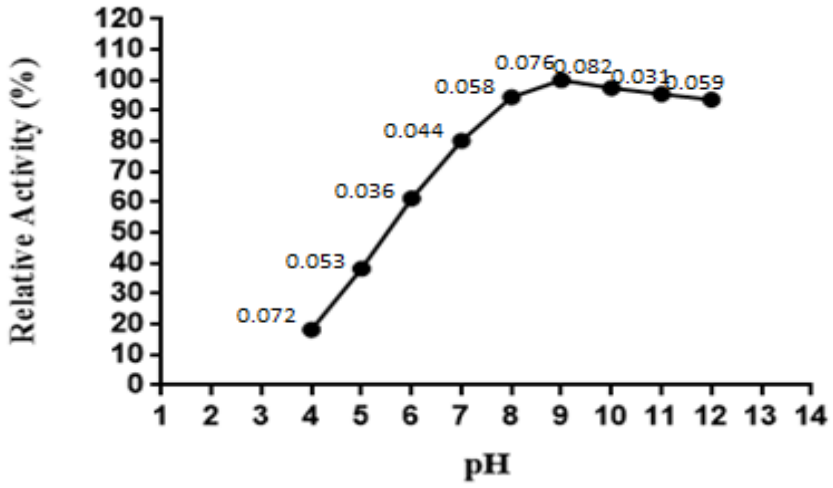

Figure 3.14. pH Stability of the immobilized enzyme with 1 h incubation time, at 55 ^{0}C.

3.4.5. Effect of Different Chemicals on Immobilized Enzyme Activity

This part of the study was performed to examine the effect of different chemicals over immobilized enzyme activity. In Figure 3.15, the effect of six metal ions and effect of SDS on immobilized enzyme was examined by comparison to no chemical condition.

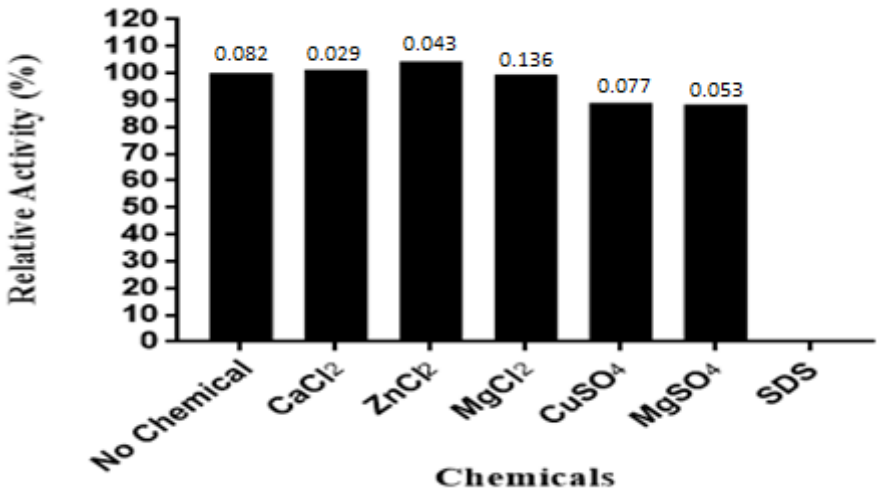

Figure 3.15. The effect of different chemicals on immobilized enzyme relative activity

3.4.6. Reusability of the Immobilized Enzyme

In order to find out reusability, the immobilized enzyme activity was measured seven times. At each replication the immobilized enzyme separated.

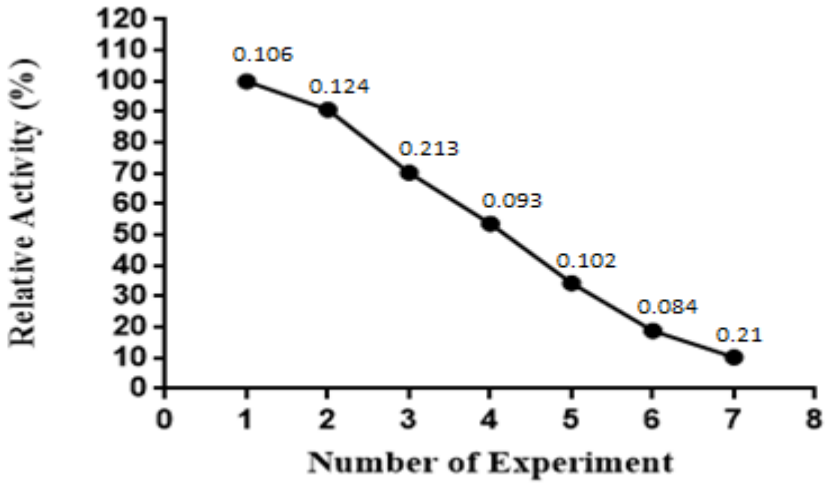

Figure 3.16. Effect of reuse of immobilized esterase enzyme on relative activity at 65^0C and with 5 min. incubation.

CHAPTER 4

DISCUSSION

In this study the thermophilic esterase that isolated from *Geobacillus sp.* was immobilized on magnetic cornstarch nanoparticles by crosslinking method. The support material was cornstarch coated magnetic iron nanoparticles and glutaraldehyde was used as crosslinking agent.

Before the immobilization process, the characterization of magnetic cornstarch was carried out. It was important to find out the optimum reaction condition for further studies. In order to the fact that different temperatures and variety of the starch, iron ions amounts were examined.

At first, the synthesis reaction was performed at room temperature, 50 ^{0}C, 65 ^{0}C and 80 ^{0}C respectively. According to previous studies (Saboktakin et al. 2009), the smaller size in support material results in larger surface area for immobilization. Actually, at the end of this experiment, it was expected that smaller size distribution would be observed at highest temperature. However, as can be seen in Figure 3.1., at 65 ^{0}C the particle size was the most suitable for immobilization. Because above this temperature, wider particle sizes were obtained. The reason of that could be the gelatinization of the starch above 65 ^{0}C.

In literature, there are varieties of magnetic iron nanoparticle formation assays. However at each study, the sizes of the synthesized particles were changes by used methods and support materials. In this study, the method of co-precipitation of ferrous and ferric ions was carried out and corn starch was used as support material.

Three different molar ratios of iron ions were examined and as can be seen in Figure 3.2. The best result was determined by using 2:3 molar ratio of Fe^{+2}/Fe^{+3}. May be the reason is different molecular magnetite structures are formed in different molar ratios of the iron ions. Also, the support materials prevent aggregation of magnetic

nanoparticles. The used amount of corn starch may succeed that at these molar ratios of the iron ions. So these structural conformations lead the changing in different size distribution of the nanoparticles.

Since starch a bulky natural polymer, the effect of amount corn starch was also important for particles size. 0.8 g, 1.0 g and 1.2 g of corn starch were used individually in the synthesis. As can be seen at Figure 3.3, with 1.0 g corns starch smaller particle size distribution was obtained.

After size distribution studies, the MCSNPs were analyzed by SERS. At Figure 3.4. it was seen that there is a characteristic strong starch Raman peak at 2917 cm^{-1} and a weak peak at 478 cm^{-1} (Pereira et al. 2014). Then magnetite particles were analyzed at same conditions and characteristic Raman peak were observed at 668 cm^{-1} (Slavov et al. 2010) from Figure 3.5. After individual analysis of starch and magnetite, the SERS analysis of magnetite corn starch was carried out. In Figure 3.6. both starch and magnetite peaks were observed. Another SERS analysis were performed for glutaraldehyde modified MCSNPs. Figure 3.7. shows not only corn starch, magnetite peaks but also there are some new peaks between 500-0 cm^{-1}, signal increasing around 1500-2000 cm^{-1} and 2700-3000 cm^{-1} . Aldehydes have moderate and weak peaks between 1700-1800 cm^{-1} and 2780-2830 cm^{-1}. These characteristic peaks can be seen at Figure 3.7.

There were many esterase samples in eppendorfs that purified from previous studies. In order to find out whether the samples still have worked or not, Nanodrop, SDS-PAGE and activity assays were performed. As can be seen at Table 3.1 nanodrop results of the enzyme samples concentrations were 0.85, 1.2 and 1.1 (ng/ul). Then the SDS-PAGE procedure was carried out for these samples. We know from previous studies, the molecular weight of the isolated thermophilic esterase from *Geobacillus sp.* was confirmed as 27 kDa (TERCAN 2011). At Figure 3.8. each three samples' band at 27 kDa by comparing with protein ladder.

Enzyme activity assays were carried out for both free and immobilized thermophilic esterase. The purpose of repeat the activity experiments for free esterase was to proof that the samples of stored enzymes are still active. Effect of temperature on free enzyme is at Figure 3.9 while the immobilized esterase's at Figure 3.11. At both

relative activity (%) – temperature (^{0}C) graph, the maximum activity was observed at 65 ^{0}C. Above this temperature the free enzyme activity decrease dramatically, however at 70 ^{0}C relative activity of immobilized enzyme was 85%, 80% at 80^{0}C and 28% at 90^{0}C respectively.

The temperature stability tests were performed for immobilized esterase. Figure 3.12 shows that the maximum activity was observed at 65 ^{0}C. Above this temperature, the relative activity of the immobilized enzyme decreased to 89 % at 70^{0}C, 67 % at 80^{0}C and 29 % at 90 ^{0}C respectively. However, the relative activity is 88 % at 50 ^{0}C, 90 % at 55 ^{0}C and 95 % at 60 ^{0}C.

The pH activity tests were also performed for both free and immobilize enzyme. The comparison of Figure 3.10 and Figure 3.13 says that the maximum relative activity is at pH 9. In addition to this, above pH 8 both free and immobilized esterase relative activities have high relative activity around 96 %. However, below pH 8, the relative activity of immobilized enzyme shows lower performance in comparison to free enzyme. The pH stability tests for immobilized enzyme shows us at Figure 3.14, the relative activity reaches 80 % at pH 7 increase gradually to maximum at pH 9. After this point relative activity remains above 90 % up to pH 12.

After temperature and pH assays, the effects of six chemicals on enzyme activity were examined. While $CaCl_2$ and $MgCl_2$ have not any effects on immobilized enzyme activity, there is a slight increase with $ZnCl_2$ to 105 %. However, the relative activity decreases to 88% with $CuSO_4$ and $MgSO_4$. At last, it was observed that SDS has a dramatic effect on activity. As it can be seen at Figure 3.15, immobilized esterase do not shows any activity with SDS. The reason this is the unfolding effect of SDS on protein species.

The reusability of immobilized esterase was also examined by carrying out activity assay seven times with same sample. At Figure 3.16, after four times usage of the immobilized enzyme, the relative activity still remains above 50%. After fourth usage, the activity decrease to 34 %, 18 % and 10% respectively. In comparison to the free enzyme reusability tests that performed at previous study (Tekedar & Şanli-Mohamed 2011), the immobilized enzyme shows great activity performance.

CHAPTER 5

CONCLUSION

In this study, isolated the thermophilic esterase from *Geobacillus sp.* was immobilized on magnetic corn starch nanoparticles (MCSNPs) by cross-linking method. During synthesis of MCSNPs, optimum conditions for synthesis were determined and the characterization studies were performed. After immobilization of the esterase, the effect of temperature, pH and some chemicals over enzyme activity were determined. Reusability of the immobilized enzyme is the most important goal of the immobilization process. After immobilized enzyme was used four times, the relative activity has still been above 50 %. The pH and temperature stability experiments have shown that immobilized thermophilic esterase on MCSNPs exhibits a dramatic increase in resistance against thermal and pH denaturation. According to experimental results, the maximum activities of immobilized esterase were observed at same pH (10) and temperature (65^0C) values with free esterase. However, the dramatic decreases in activity above these points for free enzyme have not been observed for immobilized esterase. The magnetic character of the support media gives the able to separate immobilized enzyme from reaction media easily. In chemical effect study for immobilized esterase, SDS has a strong inhibitory on activity among other chemicals. Over all, immobilized thermophilic esterase has shown great improvements at high pHs and temperatures that enable applicability of the esterase in industrial processes and for further scientific studies.

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APPENDIX A

MEDIA

BUFFERS**:**

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

1M Na2HPO4 --57.7ml
1M NaH2PO4--42.3ml diluet to 1L with 900ml distile water

B. 1.5M Tris-HCl,

18.15g Tris Base
~80ml distilled water
Dissolve Tris base in distilled water and adjust to find disered pH with HCl.
Make up to 100ml with
distillled water and store at 4ºC.

C. Neutralization Buffer

0.1M, pH 6.5 Tris-phosphate in deionized water

APPENDIX B

CHEMICALS, REAGENTS AND SOLUTIONS

Table.1. Chemicals and Reagents Used in Experiments

NO	CHEMICALS	COMPANY
1	Iron (III) Chloride Hexahydrate ($FeCl_3.6H_2O$)	Carlo Erba
2	Iron(II) Chloride Tetrahydrate ($FeCl_2.4H_2O$)	Sigma
3	Glutaraldehyde	Sigma
4	Corn Starch	Commercial
5	Sodium Chloride	Sigma
6	Acetic Acid	Merck
7	Absolute Ethanol	AppliChem
8	Calcium Chloride	Sigma
9	Zinc Chloride	Merck
10	Magnesium Chloride	Sigma
11	Copper Sulfate	Sigma
12	Magnesium Sulfate	Sigma
13	SDS	Sigma
14	Sodium Hydroxide	Sigma
15	*p*-NP Acetate	Sigma
16	Hydrochloric Acid (HCl)	Merck