REPUBLIC OF TURKEY
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PREPARATION AND CHARACTERIZATION OF POLYMER-COATED MESOPOROUS SILICA NANOPARTICLES AND THEIR APPLICATION IN ENZYME IMMOBILIZATION

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TABLE OF CONTENTS

Page

LIST OF SYMBOLS viii

LIST OF ABBREVIATIONS ix

LIST OF FIGURES x

LIST OF TABLES xii

ABSTRACT xiii

ÖZET xv

CHAPTER 1 1

INTRODUCTION 1

1.1 Literature Review 1

1.2 Objectives of the Thesis 3

1.3 Hypothesis 3

CHAPTER 2 5

GENERAL INFORMATION 5

2.1 Enzymes 5

2.1.1 General Properties of Enzymes 6

2.1.2 Mechanism of Enzyme Catalyzed Reactions 6

2.1.3 Parameters Affecting Enzyme Activity 8

2.1.4 Nomenclature and Classification of Enzymes 8

2.1.5 Protease 12

2.1.5.1 Subtilisin 13

2.2 Enzyme Immobilization 14

2.2.1 Chemical Methods used for Enzyme Immobilization 16

2.2.2 Immobilization via Containment 18
2.3 Carriers Used for Immobilization 20
2.4 Nanobiocatalysis 21
2.5 Mesoporous Silica Nanoparticles (MSNs) 22
2.6 Chitosan (CS) 26
2.7 Poly(acrylic) Acid (PAA) 29
CHAPTER 3 32
LITERATURE SURVEY 32
3.1 Studies on Polymer Blends for the Coating Layer on MSNs 32
3.2 Studies on Enzyme Immobilization 37
CHAPTER 4 44
MATERIALS AND METHODS 44
4.1 Materials 44
4.2 Preparation of Mesoporous Silica Nanoparticles 44
4.3 Preparation of Polymer-coated Mesoporous Silica Nanoparticles 45
4.4 Characterization of MSNs and CS-PAA/MSNs 47
4.5 Enzyme Immobilization Studies 47
4.5.1 Analytical Methods 47
4.5.1.1 Protein Determination by Folin-Lowry Method 47
4.5.1.2 Enzyme activity assay 49
4.5.2 Experimental Procedure of Enzyme Immobilization 51
4.5.2.1 Preparation of Immobilized Enzyme onto Polymer-coated MSNs . 51
4.5.2.2 Effect of pH on Immobilized Enzyme Activity 52
4.5.2.3 Effect of Temperature on Immobilized Enzyme Activity 52
4.5.2.4 Effect of Repeated Use on Immobilized Enzyme Activity 52
4.5.2.5 Effect of Storage Time on Immobilized Enzyme Activity 52
CHAPTER 5 53
RESULTS AND DISCUSSION 53
5.1 Characterization of MSNs 54

5.1.1 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis 54
5.1.2 Scanning Electron Microscope (SEM) Analysis 55
5.1.3 Transmission Electron Microscopy (TEM) Analysis 55
5.2 Characterization of CS-PAA/MSNs 56
5.2.1 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis 56
5.2.2 Transmission Electron Microscopy (TEM) Analysis 58
5.2.3 Thermogravimetric Analysis (TGA) 58
5.2.4 Zeta Potential Analysis 59
5.3 Enzyme Immobilization Studies 62
5.3.1 Effect of Matrix Amount to Percent Enzyme Loading 62
5.3.2 Effect of Incubation Time to Percent Enzyme Loading 62
5.3.3 Effect of Immobilization pH to Percent Enzyme Loading 64
5.4 Immobilized Enzyme Activity Studies 65
5.4.1 Effect of pH on Immobilized Enzyme Activity 65
5.4.2 Effect of Temperature on Immobilized Enzyme Activity 65
5.4.3 Repeated Use Capability of Immobilized Enzyme 67
5.4.5 Storage Stability of Immobilized Enzyme 67
CONCLUSIONS 71
REFERENCES 74
APPENDIX 82
CURRICULUM VITAE 87

LIST OF SYMBOLS

σ	Standart deviation
Au	Enzyme activity unit
C	Concentration
^{o}C	Degree Celcius
E	Enzyme
ES	Enzyme/substrate complex
h	Hour
mg	Milligram
ml	Milliliter
mV	Millivolt
µl	Microliter
P	Product
pKa	Acid dissociation constant
R^2	regression coefficient
S	Substrate
$	American dollar
U	Enzyme unit

LIST OF ABBREVIATIONS

AA	Acrylic acid
APTES	3-aminopropyl)triethoxysilane
BSA	Bovine serum albumin
CLE	Cross-linked enzyme
CLEA	Cross-linked enzyme aggregate
CS	Chitosan
CS-PAA/MSNs	Chitosan-Poly(acrylic) acid-coated MSNs
CTAB	Cetyltrimethylammonium bromide
DOX	Doxorubicin
FESEM	Field emission scanning electron microscope
FTIR	Fourier transform infrared spectroscopy
GLA	Glutaraldeyde
HRTEM	High resolution transmission electron microscope
MSNs	Mesoporous silica nanoparticles
KPS	Potassium persulfate
PAA	Poly(acrylic) acid
PMAA	Poly(methacrylic acid)
SEM	Scanning electron microscope
TG/DTA	Thermogravimetric/differential thermal analysis
UV	Ulltraviolet

LIST OF FIGURES

		Page
Figure 2.1	Structure of haloenzyme [18]…….....	6
Figure 2.2	Scheme of an enzyme-catalyzed reaction [19]…….	7
Figure 2.3	Comparison of the activation energies of enzyme catalyzed and uncatalyzed reactions (Eu<Ec) [20]…….	7
Figure 2.4	Parameters affecting enzyme activity a) Temperature, b) pH, c) Substrate concentration [22]…….	8
Figure 2.5	Three-dimensional illustration of Subtilisin focused to catalytic triad [27]…….....	14
Figure 2.6	The algoritm that could be used in the study of an immobilized enzyme biocatalyst [28] ……..	15
Figure 2.7	The major methods used for enzyme immobilization [20] …….	16
Figure 2.8	Functionalization of mesoporous silica materials with different reactive groups [36] …….	23
Figure 2.9	Field emission scanning electron microscopy (FESEM) images of functionlized MSNs [41] …….	24
Figure 2.10	Formation of CLEAs a) in the solution and b) in the pores of a convenient carrier [35] …….	25
Figure 2.11	Chemical structure of chitosan [53] …….	27
Figure 2.12	Effect of pH on chemical structure of PAA [64] …….	29
Figure 2.13	Schematic illustration of pH-sensitive polymer-coated mesoporous silica spheres [66] ……	30
Figure 2.14	SEM micrographs of (a) chitosan-graft-poly(acrylic acid) and (b) chitosan-graft-poly(acrylic) acid/cellulose nanofibrils hydrogels composites and scheme of chitosan-graft-poly(acrylic acid)/cellulose nanofibrils hydrogel composite formation [70] …..	31
Figure 3.1	The synthesis of pH-responsive nano-carrier MSNs–PAA [72]…..	33
Figure 3.2	Illustration of the preparation of PAA-MSN particles [75]……..	36
Figure 3.3	Scheme of the preparation of PAA-CS polymer magnetic microspheres [76] …….	37
Figure 4.1	The glass reactor system used for MSN synthesis and polymer coating……..	45
Figure 4.2	The steps of the coating process of MSNs a) MSNs, b) After mixing AA+CS+MSNs via sonic treatment (milk white color), c) After polymerization reaction (light yellow color), d) Cross-linking before washing step, e) Dried PAA/CS-MSNs……	46
Figure 4.3	The calibration curve for Folin-Lowry method……	49

Figure 4.4	The calibration curve for enzyme activity assay……..	51
Figure 5.1	FTIR spectra of MSN and MSN-CTAB……...…...	54
Figure 5.2	SEM image of MSNs……	55
Figure 5.3	TEM image of MSNs……	55
Figure 5.4	FTIR spectra of MSN-CTAB, MSN, CS-PAA coated MSN, and CS. …….....	57
Figure 5.5	FTIR spectra of CS-PAA gel and CS-PAA coated-MSN……	57
Figure 5.6	TEM micrographs of a) MSNs, and b) CS-PAA coated-MSNs.....	58
Figure 5.7	TGA curves of MSN, CS-PAA coated-MSN, and CS…….	59
Figure 5.8	The effect of PAA/[NH2] ratio to the zeta potential at various pH values (ratio 0 nominates MSN without coating)…….	60
Figure 5.9	The effect of PAA/[NH2] ratio to the hydrodynamic diameter of CS-PAA/MSNs at different pH values…….	62
Figure 5.10	The effect of matrix amount to percent enzyme loading…….	63
Figure 5.11	Percent enzyme loading versus incubation time……...	63
Figure 5.12	The effect of immobilization pH on percent enzyme loading…….	64
Figure 5.13	pH effect on immobilized enzyme activity……..	66
Figure 5.14	Temperature effect on immobilized enzyme activity……..	66
Figure 5.15	Effect of repeated use (operational stability) on immobilized enzyme activity……	67
Figure 5.16	Storage stability of subtilisin enzyme immobilized onto CS-PAA/MSNs……..	68

LIST OF TABLES

		Page
		Page
Table 2.1	Main application areas of enzymes and the industries [24]…….	10
Table 2.2	The advantages and disadvantages of the most common used immobilization methods[11]…….……..	19
Table 2.3	The parameters to be considered for the optimization of immobilization [36] …….…….…….……	21
Table 2.4	Main applications of chitosan [56], [63] …….…….……..	28
Table 3.1	Literature survey for the immobilization of enzymes onto polymer-coated MSNs…….…….…….……	42
Table 5.1	The findings of various studies compared with the present study	69
Table A.1	Calibration curve data for Folin-Lowry Method…….……	83
Table A.2	Calibration curve data for enzyme activity assay…….……..	84
Table A.3	The effect of PAA/[NH2] ratio to the zeta potential at different pH values (ratio 0 nominates MSN without coating) …….……..	84
Table A.4	The effect of PAA/[NH2] ratio to the hydrodynamic diameter of CS-PAA/MSNs at different pH values…….…….……..	84
Table A.5	The effect of matrix amount to percent enzyme loading…….	85
Table A.6	The change of percent enzyme loading with incubation time……..	85
Table A.7	The effect of immobilization pH on percent enzyme loading……	85
Table A.8	pH effect on the immobilized enzyme activity…….……..	86
Table A.9	Temperature effect on the immobilized enzyme activity……	86
Table A.10	Effect of repeated use on the immobilized enzyme activity…….	87
Table A.11	Storage stability of immobilized enzyme…….…….……..	87

ABSTRACT

PREPARATION AND CHARACTERIZATION OF POLYMER-COATED MESOPOROUS SILICA NANOPARTICLES AND THEIR APPLICATION IN ENZYME IMMOBILIZATION

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MSc. Thesis

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The increasing application areas of enzymes made essential of production of the enzymes by more economical and efficient techniques. Subtilisin (also known as Alcalase) is a protease that one of the enzymes used for production of the cosmetic goods and detergents. The lack of the operational stability and high cost of the enzymes are resulted in low efficiency in the industrial applications. High temperatures or non-convenient pH values may lower enzyme activity which decrease reaction rate. Since its advantages like flexibility for the process conditions and increased stability of enzyme, immobilization is the key solution to overcome these obstacles [1].

Mesoporous silica nanoparticles (MSNs) have great potential as nanocarriers for biomolecules because of their biocompatibility, low toxicity, large surface area, high porosity, modifiable pore diameter, and adjustable surface properties. Chitosan (CS) has great potential as enzyme immobilization support because it is non-toxic, inexpensive and, user-friendly. The main reason of this great potential is the amino and hydroxyl groups in the chemical structure of chitosan [2]. For immobilization process, chitosan has the disadvantage of having a highly hydrophilic and swelling structure in aqueous media since high water content causes the polymer structure to lose its mechanical strength. To eliminate this obstacle, chitosan has been studied as a component of a polymer blend structure to obtain more strong and efficient matrices [3]. It is known that blending poly(acrylic acid) (PAA) and chitosan provides increased mechanical strength and pH stability. Also, linear polymer chains (such as poly(acrylic acid: PAA) grafted onto the surface of mesoporous silica nanoparticles (MSNs) show that pH-dependent behaviour [4]. Therefore, in the present study, acrylic acid and chitosan have blended onto mesoporous silica nanoparticles via in-situ polymerization technique. About this type of application, to the best of our knowledge, no study was reported in the literature about this type of polymer blend as a coating layer of mesoporous silica nanoparticles.

On the other hand, nanobiocatalysis eliminates the activity-lowering effect of traditional immobilization methods by means of the stabilization of enzymes with nanoparticles which provides higher enzyme load per unit weight of the carrier since the nanostructured materials have the advantage of higher surface area. Therefore, subtilisin immobilization onto polymer-coated mesoporous silica nanoparticles (CS-PAA/MSNs) which were obtained via in-situ polymerization was investigated. Then, the optimal conditions for immobilization process were determined at pH 7 and 4 h of immobilization time at temperature of 25^{o}C. Finally, the activity of enzyme immobilized at optimum conditions was compared with that of free enzyme, at different pH and temperature. Beside of this, the operational and storage stability of immobilized enzyme were also determined. The activity of immobilized enzyme reduced from 100% to 40% after 5 repeated use. According to storage stability experiments, the activity of immobilized enzyme reduced from 100% to 60% after 4 weeks of the storage time.

Keywords: Nanobiocatalysis, Silica Nanoparticles, Chitosan, Enzyme Immobilization, Subtilisin, Optimal conditions

ÖZET

POLİMER KAPLI MEZO GÖZENEKLİ SİLİKA NANOPARÇACIK SENTEZİ, KARAKTERİZASYONU VE ENZİM İMMOBİLİZASYONUNDA KULLANIMI

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Enzimler tepkimenin hızını ve özgüllüğünü belirleyen olan organik katalizörlerdir. Enzimlerin uygulama alanlarının her geçen gün artması, enzimlerin daha ekonomik ve etkin tekniklerle üretilmesini kaçınılmaz hale getirmiştir. Subtilisin (Alkalaz) enzimi, kozmetik ve temizlik ürünleri bileşeni olarak kullanılan bir enzimdir. Bu ürünlerin uygulamaları enzimin yüksek maliyetli olması ve operasyonel kararlılığının düşük olması sebebiyle düşük verimle gerçekleşmektedir. Yüksek sıcaklıklar ve uygun olmayan pH değerleri enzimin aktivitesini olumsuz yönde etkileyerek reaksiyon hızını düşürmektedir. İmmobilizasyon proses şartlarında esneklik sağladığı ve enzim kararlılığını arttırdığı için bu dezavantajları aşmak için kilit bir yöntemdir [1].

Mezogözenekli silika nanoparçacıklar (MSNs) biyouyumlulukları, düşük toksisiteleri, geniş yüzey alanları, yüksek gözenekli yapıları, ayarlanabilir gözenekleri, değiştirilebilir yüzey özellikleri sebebiyle biyomolekül taşıyıcısı olarak büyük bir potansiyele sahiplerdir. Kitosan toksik olmaması, kullanıcı dostu ve maliyeti düşük olması sebebiyle enzim immobilizasyonu için büyük bir potansiyele sahiptir. Kitosanın (CS) yapısında bulunan amino ve hidroksil grupları, enzim immobilizasyonu için büyük avantaj sağlar [2]. Kitosanının ve sulu ortamda şişen bir yapıya sahip olması immobilizasyon prosesinde kullanımı için bir dezavantajdır çünkü, yüksek su içeriği polimerik yapısının mekanik dayanımını yitirmesine sebep olur. Bu problemin üstesinden gelebilmek için kitosan bir polimerik karışımın bileşeni olarak kullanılmıştır [3]. Poliakrilik asit (PAA) ve kitosanın karıştırılması sonucu elde edilen polimerik yapının mekanik dayanımının ve pH kararlılığının yüksek olduğu bilinmektedir. Ayrıca, poliakrilik asit gibi lineer polimer zincirlerin MSNlerin yüzeyine kaplanmaları ile pH'a bağlı davranış gösterdikleri tespit edilmiştir [4]. Bu bilgiler doğrultusunda, kitosan ve akrilik asitin çözelti polimerizasyonu reaksiyonu ile MSNlerin yüzeyine kaplanması çalışılmıştır. Yapılan literatür

taramasında, mezogözenekli silica nanoparçacıklara bu polimer karışımı ile daha önce kaplama yapılmadığı görülmüştür.

Nanobiyokataliz geleneksel immobilizasyon yöntemlerinin aktiviteyi düşürücü etkisini nanoparçacıklarla enzimlerin kararlı hale getirerek yok eder. Nanoyapıdaki parçacıklar geniş yüzey alanına sahip olduklarından birim taşıyıcı başına enzim yükleme miktarı yüksektir. Bu amaçla, poliakrilik asit (PAA) ve kitosanın (CS) karıştırılmasıyla elde edilen polimer tabaka ile kaplanmış mezo gözenekli silica nanoparçacıkların (MSNs) yüzeyine subtilisin enzimi immobilize edilmiştir. Yapılan çalışmalar sonucunda, immobilizasyon prosesi ve enzim aktivitesi için optimum koşullar 25^{o}C sıcaklıkta: pH 7 ve 4 saat immobilizasyon süresi olarak belirlenmiştir. Optimum koşullarda immobilize edilen enzimin aktivitesi serbest enzimin aktivitesi ile farklı pH ve sıcaklıklarda karşılaştırılmıştır. Ayrıca, immobilize enzimin operasyonel ve depo kararlılığı incelenmiş ve immobilize enzimin aktivitesinin 5 kez tekrar kullanımdan sonra %40'ına ve depolama kararlılığı deneylerine göre 4 hafta sonra %60'ına düştüğü gözlemlenmiştir.

Anahtar Kelimeler: Nanobiyokataliz, Silika Nanoparçacık, Kitosan, Enzim Immobilizasyonu, Subtilisin, Optimum Koşullar

CHAPTER 1

INTRODUCTION

1.1 Literature Review

The industrial enzyme market is estimated about $2.5–3 billion size per year, which is made up of enzymes used in food processing, animal feed, and nonfood/feed enzyme applications. As global market, it is estimated to rise 7 percent to $8.0 billion in 2015 [5, 6]. Proteases are assumed to have 60% of the total enzyme market with $1.5–1.8 billion annual sale per year. Looking the sales of alkaline protease, the most wide use is in cleaning industry [6, 7]. Subtilisin is the key player as a detergent additive to remove protein deposits and stains. They are used in dish washer detergents in powder and liquid laundry detergents and also in laundry bleaching agent additive. Subtilisins (<10%) are also catalyzers in the processes such as protein hydrolysation, leather treatment, and in the textile and cosmetics industry [8].

Enzyme immobilization is a common interest of researchers studied for the optimization and economical rehabilitation of these processes. Since immobilization process increases the stability and activity of enzymes, many researchers use immobilization as an accelerator for the reactions. To increase the efficiency of enzymatic reaction is a priority for researchers because of the extending global market. Immobilization provides reuse of enzyme that can reduce over-all cost, better control of the reaction with easier recovery and purification of the biocatalyst, stabilization against the changes of microenvironment (i.e. temperature, pH, ion concentration and etc.), ease of operation and retained activity [9].

Enzymes can bind to carriers by covalent or noncovalent interactions. Covalent bonds may be formed between functional groups in the activated carrier and functional groups in the catalytic part of the enzyme [10, 11]. Noncovalent immobilization to solid matrices includes all types of interactions between the enzyme and the support except covalent bonds; hydrophobic interactions, ionic bonds, and weaker interactions like van der Waals forces; these methods are named as adsorption. Most of the carrier materials have sufficient surface-charge properties appropriate for immobilization by adsorption [12].

For the maximum immobilization efficieny, carrier should provide some basic properties. These properties are biocompatibility with the enzyme, functional groups on its surface or to be easily functionalized, stability with the changes in pH, ionic strength, and temperature, eliminating enzyme leaching, permitting diffusion of substrates and products, selective and sensitive for determined species, easy to prepare, inexpensive for minimizing the economic impact of the process [11, 13].

The nanoparticles which on enzymes immobilized showed an extended working range of pH and temperature and better thermal stability than the soluble form. The benefits of using nanoscale structures as matrices are reduced diffusion limitations and increased enzyme loading by means of maximized functional surface area [14].

Mesoporous silica nanoparticles provide controllable pore structure, standart pore-size distribution, high specific pore volumes, and large internal surface area. Physical adsorption is the simplest method of enzyme immobilization on mesoporous silica naoparticles. However, the method is simple; enzyme leakage out from the mesoporous particles is the main limitation for adsorption. For an efficient enzyme immobilization onto mesoporous silica nanoparticles, presence of the functional groups on the surface of the nanoparticles is essential. These surface functionalized particles with reactive groups will increase the enzyme load and stability.

Natural and synthetic polymers are commonly used for the functionalization of mesoporous nanoparticles. Chitosan (CS) is a natural polyaminosaccharide, based on its favorable biological (biodegradable and bioactive) and chemical characteristics (polycationic, reactive groups such as -OH and -NH_2), chitosan have attracted great attention [15]. On the other hand, poly(acrylic acid) (PAA) is a type of water soluble polymers, containing a carboxyl group in each repeated unit and shows anionic

polyelectrolyte features, which provide favorable or attractive electrostatic interaction [16].

1.2 Objectives of the Thesis

The main objectives of the present study were given as below:

- To functionalize the mesoporous silica nanoparticles via in situ polymerization technique with the polymer blend of chitosan and poly(acrylic) acid. To the best of our knowledge, this polymer blend has not been reported as a coating layer of mesoporous silica nanoparticles.
- To obtain the optimal process conditions for the synthesis of polymer-coated mesoporous silica nanoparticles (CS-PAA/MSNs) and to investigate the important properties such as morphologic pattern, the mean size, size distribution, surface charge of the particles, thermogravimetric stability with SEM, TEM, Zetasizer and TGA analyses.
- To investigate the efficiency of the polymer-coated mesoporous silica nanoparticles as immobilization matrix with Subtilisin enzyme chosen as model because of being the key player as a detergent additive to remove protein deposits and stains, and to investigate the process conditions to maximize the percent enzyme loading to the polymer-coated mesoporous silica nanoparticles.
- To deteremine the effects of the process parameters such as temperature, pH, processing time and repeated-use and storage stability on the enzyme activity.

1.3 Hypothesis

The major disadvantage of enzymatic processes is the high cost of the enzymes. This economical obstacle can be eliminated by increasing the stability of biocatalysts. In this study, the increment of the stability of Subtilisin enzyme which is a key player as a detergent additive to remove protein deposits and stains was aimed using polymer-coated mesoporous silica nanoparticles as matrix. First, the synthesis of the core-shell of immobilization matrix was achieved. Then, coating mesoporous silica nanoparticles via polymerization and cross-linking reactions was carried out. The important properties such as morphologic pattern, the mean size, size distribution, and surface charge of the

particles, thermogravimetric stability were analysed at various parameters of the synthesis process.

It has been revealed that this study can also be an initial step for the development of a drug delivery system since a pH-sensitive polymer layer was used. The various process conditions were studied to optimize the immobilization conditions and the remained enzyme activity.

CHAPTER 2

GENERAL INFORMATION

The industrial enzyme market is estimated about $2.5–3 billion size per year, which is made up of enzymes used in food processing (~$800 million), enzymes for animal feed (~$400 million), and nonfood/feed enzyme applications ($1.4–1.7 billion). As global market, it is estimated to rise 7 percent to $8.0 billion in 2015.

Companies reported to have the largest share of the enzyme market are Novozymes, Genencor International and DSM with respective approximate market shares are 41–44%, 21%, and 8% [5, 6].

Enzyme immobilization is a common interest of researchers who study food, pharmaceutical, biomedical, and chemical processes. Since immobilization process increases the stability and activity of enzymes, many researchers use immobilization as an accelerator for the reactions. To increase the efficiency of enzymatic reactions is a priority for researchers because of the extending global market.

2.1 Enzymes

Enzymes are protein molecules functioning as catalysts that are secreted by living cells. These molecules are essential for many biological reactions like DNA replication, protein synthesis [5]. They can also catalyze specific chemical reactions very efficient and do not require extreme conditions compared with inorganic catalysts [9]. To date, many biocatalytic processes have been implemented on an industrial scale, including the syntheses of acrylamide, nicotinamide and lactam antibiotics [17].

2.1.1 General Properties of Enzymes

Enzymes are selective catalysts with an active site that has a strong affinity for the substrate coming from the complemantation between the amino acid arrangement of enzyme and the chemical structure of substrate. Generally, enzymes are protein structures, some of them require nonprotein structure which is called cofactors which are inorganic ions, coenzymes or prosthetic groups. Coenzymes are functional organic substances that many have vitamin molecule. Cofactor forms holoenzyme with this inactive protein structure which is known as apoenzyme [9]. The structure of haloenzyme was given in Figure 2.1.

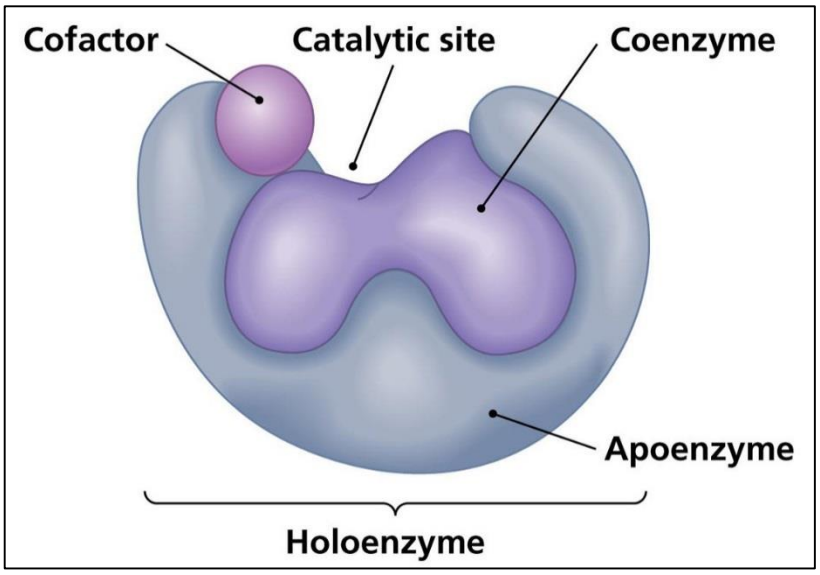


Figure 2.1 Structure of haloenzyme [18]

2.1.2 Mechanism of Enzyme Catalyzed Reactions

Enzyme catalyzed reactions occur by the formation of enzyme-substrate complex which can be seen from Figure 2.2. Firstly, substrate binds to enzyme's active site, enzyme/substrate complex forms, then it turns out to enzyme/product complex and finally products leave active site of the enzyme.

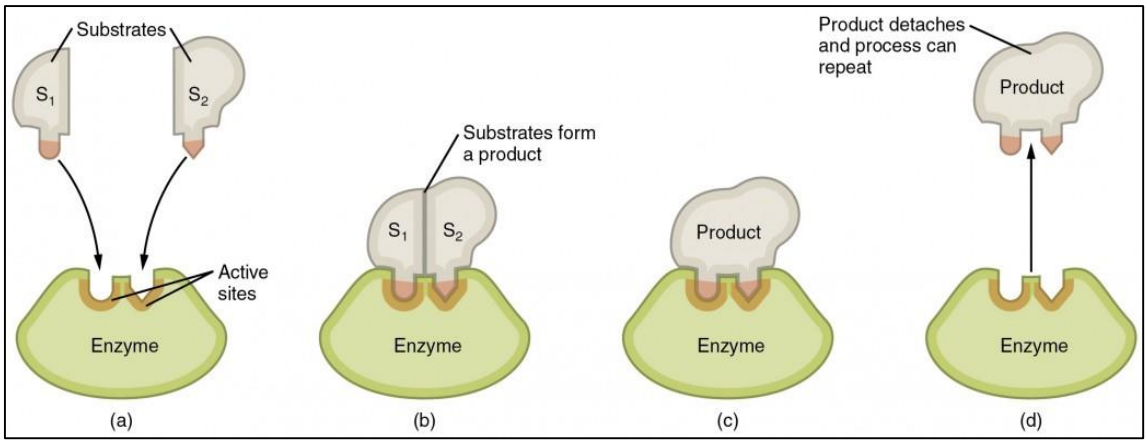


Figure 2.2 Scheme of an enzyme-catalyzed reaction [19]

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} P$$

Where E = enzyme, S = substrate, ES = enzyme/substrate complex and P = product. The rates of forward and backward reactions are k_1 and k_2 respectively. The rate of the reaction which ES turns to P is k_{cat} [9]. Enzyme lowers the activation energy of the reaction by binding substrate and forming enzyme/substrate complex. Enzyme does not affect the free-energy change or the equilibrium constant (Figure 2.3).

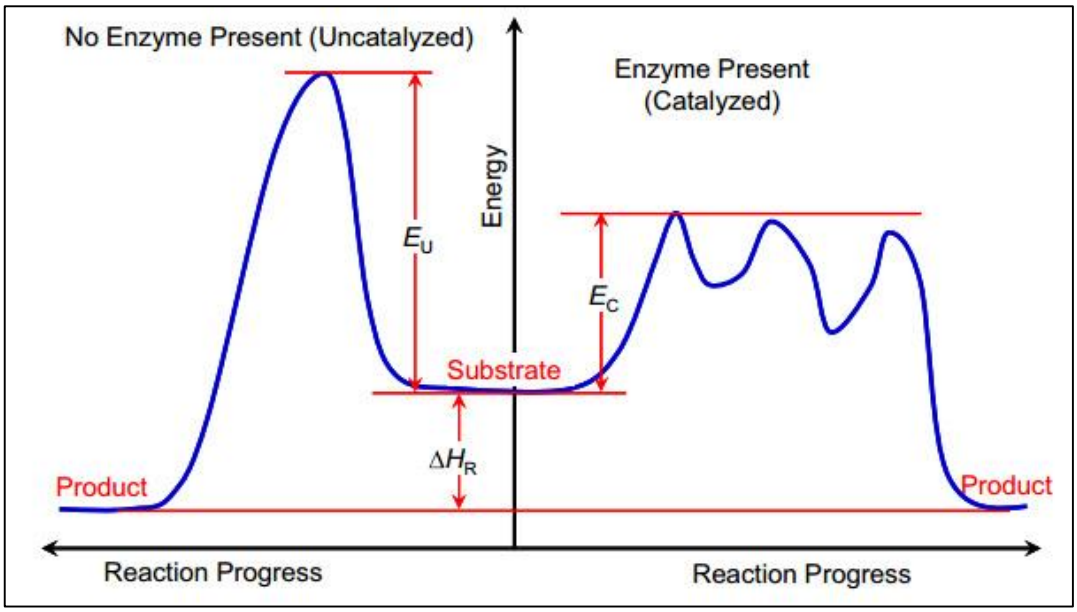


Figure 2.3 Comparison of the activation energies of enzyme catalyzed and uncatalyzed reactions (Eu<Ec) [20]

2.1.3 Parameters Affecting Enzyme Activity

Enzymes have evolved to work under the conditions of their nature, activity and stability are a result of that nature [21]. A change in the ion concentration or temperature can simply affect the rate of catalysis reaction. Changing pH alters the level of protonation of amino acid arrangement of the active site, altering its conformation. It can be seen in Figure 2.4 that each enzyme has an optimal pH value to reach maximum catalysis rate [22].

Enzyme activity increases with increasing temperatures; however, as temperature gets higher, enzyme begins to denature. Except thermophilic enzymes, enzymes are most active at 36-37°C. If there are enough enzymes with avaliable active site, activity increases as substrate concentration increase. Also, enzymes can be affected by inhibitors which are the substances reduce activity of an enzyme by altering the structure of active site [9].

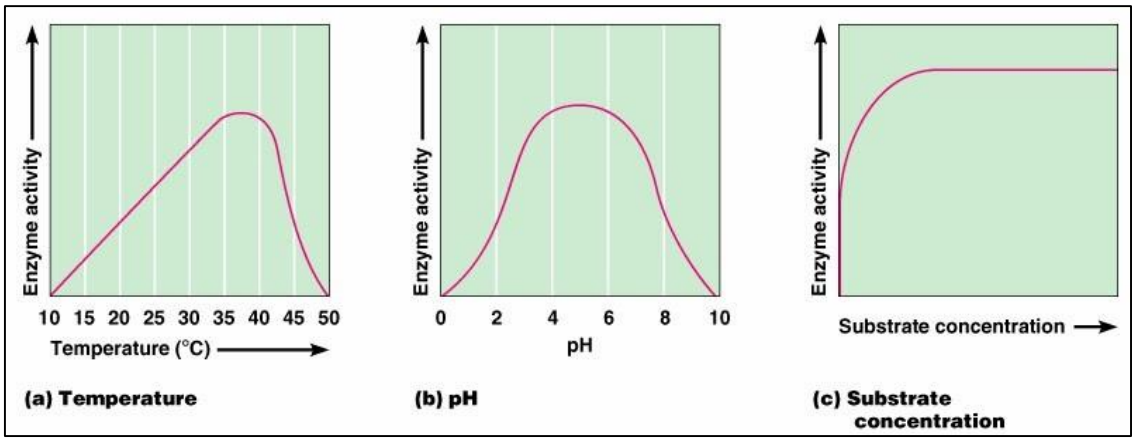


Figure 2.4 Parameters affecting enzyme activity a) Temperature, b) pH, c) Substrate concentration [22]

2.1.4 Nomenclature and Classification of Enzymes

Most of the enzymes are named by adding the suffix "-ase" to the end of the name of substrate (e.g. amylase, hydrolyses, amylose and etc.). Enzymes were classified in six main groups according to the reactions they catalyze in the report of Enzyme Commision which had gathered in 1961 [23]:

1. **Oxidoreductases** catalyze oxidation-reduction reactions providing H^+, O_2 and e^- transfer from one substrate to another.

2. **Transferases** catalyze the transfer of functional groups (e.g amino and phosphate groups)
3. **Hydrolases** catalyze hydrolysis reactions (Protease is a member of this group).
4. **Lyases** catalyze the addition of a group across a double bound.
5. **Isomerases** catalyze rearrangement reactions within a molecule.
6. **Ligases** catalyze bond formation between two molecules coupled with the hydrolysis of pyrophosphate bond [9].

Main application areas of enzymes and the industries were given in Table 2.1 [23].

Table 2.1 Main application areas of enzymes and the industries [24]

Industry	**Enzyme class**	**Application**
Detergent	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-	Cyclodextrin production
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition – fuel)
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties

Table 2.1 (continues)

Industry	**Enzyme class**	**Application**
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning
	Phospholipase	Dough stability and conditioning (in situ emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestibility – phosphorus release
	Xylanase	Digestibility
	β-Glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal

Table 2.1 (continues)

Industry	Enzyme class	Application
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	De-pickling
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial

2.1.5 Protease

There are too many different proteases have been categorized in the literature. Animal, plant, and, mostly, microbial proteases have the most common commercial use in detergent, food (brewing and cereal processing, cheese making, baking), animal-feed, leather and fabric processing, protein hydrolysate production, waste treatment, pharmaceutical industry, and organic synthesis.

Proteases are assumed to have 60% of the total enzyme market with $1.5–1.8 billion annual sale per year. Looking the sales of alkaline protease, the most wide use is in the cleaning industry [6, 7]. Subtilisin is the key player as a detergent additive to remove protein deposits and stains. They are used in dish washer detergents in powder and liquid laundry detergents and also in laundry bleaching agent additive. The subtilisin is used in very low level in cleaning agents, concentration ranges between 0.007-0.1%. Subtilisins (<10%) are also catalyzers in the processes such as protein hydrolysation, leather treatment and in textile and cosmetic industry [8].

Subtilisin serine proteases including *Subtilisin Carlsberg* are the first generation of detergent proteases with an optimal pH value of 9-10. The second-generation detergent proteases, having a higher optimal pH value of 10-11 and better temperature stability produced from *Bacillus* species. The third generation of detergent proteases are engineered via using site-directed mutagenesis and protein engineering tools aiming to extend their stability [25].

2.1.5.1 Subtilisin

The name Subtilisin comes from *Bacillus subtilis*, which is the bacterial species enzyme was first isolated from. The subtilisins (EC 3.4.21.62) are a subgroup of serine proteases secreted by *Bacillus* species. The first discovery of this enzyme was with subtilisin carlsberg from *Bacillus licheniformis* (also known as subtilisin A, subtilopeptidase A, alcalase Novo) by Linderstrøm-Lang and Ottensen. This enzyme was also the starting point of production of the third generation of detergent enzymes which is resistant to oxidation by bleach. This generation involves substitution of an oxidation-sensitive amino acid next to the enzyme's active site with an anti-oxidant amino acid [6, 26]. In Figure 2.5, three-dimensional illustration of subtilisin enzyme and its catalytic region (focused version) can be seen [26].

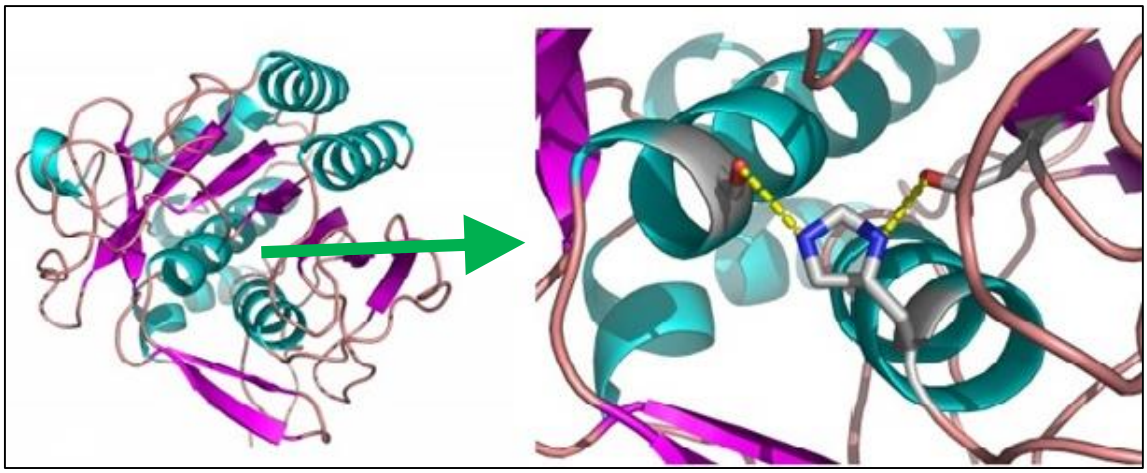


Figure 2.5 Three-dimensional illustration of Subtilisin focused to catalytic triad [27]

2.2 Enzyme Immobilization

Improving proteases performance can be possible with managing proteases efficiency via: chemical modification, immobilization of enzyme, ultrasound treatment, using proteases from thermophilic microorganisms and tailoring enzyme [7]. Immobilization will be investigated for this case. Advantages of enzyme immobilization can be listed as below [9]:

- Reuse of the enzyme that can reduce over-all cost.
- Better control of the reaction with easier recovery and purification of the biocatalyst.
- Stabilization against the changes of microenvironment (i.e. temperature, pH, ion concentration).
- Ease of operation for continuous systems via use of column immobilized enzyme.
- Retained activity.

The algoritm used for the enzyme immobilization process was given in Figure 2.6 [28]. On the other hand, the methods of enzyme immobilization can be divided as chemical and physical according to interactions of enzyme molecules with the carrier surface or each other. Figure 2.7 shows the illustrations of most common immobilization methods used [20].

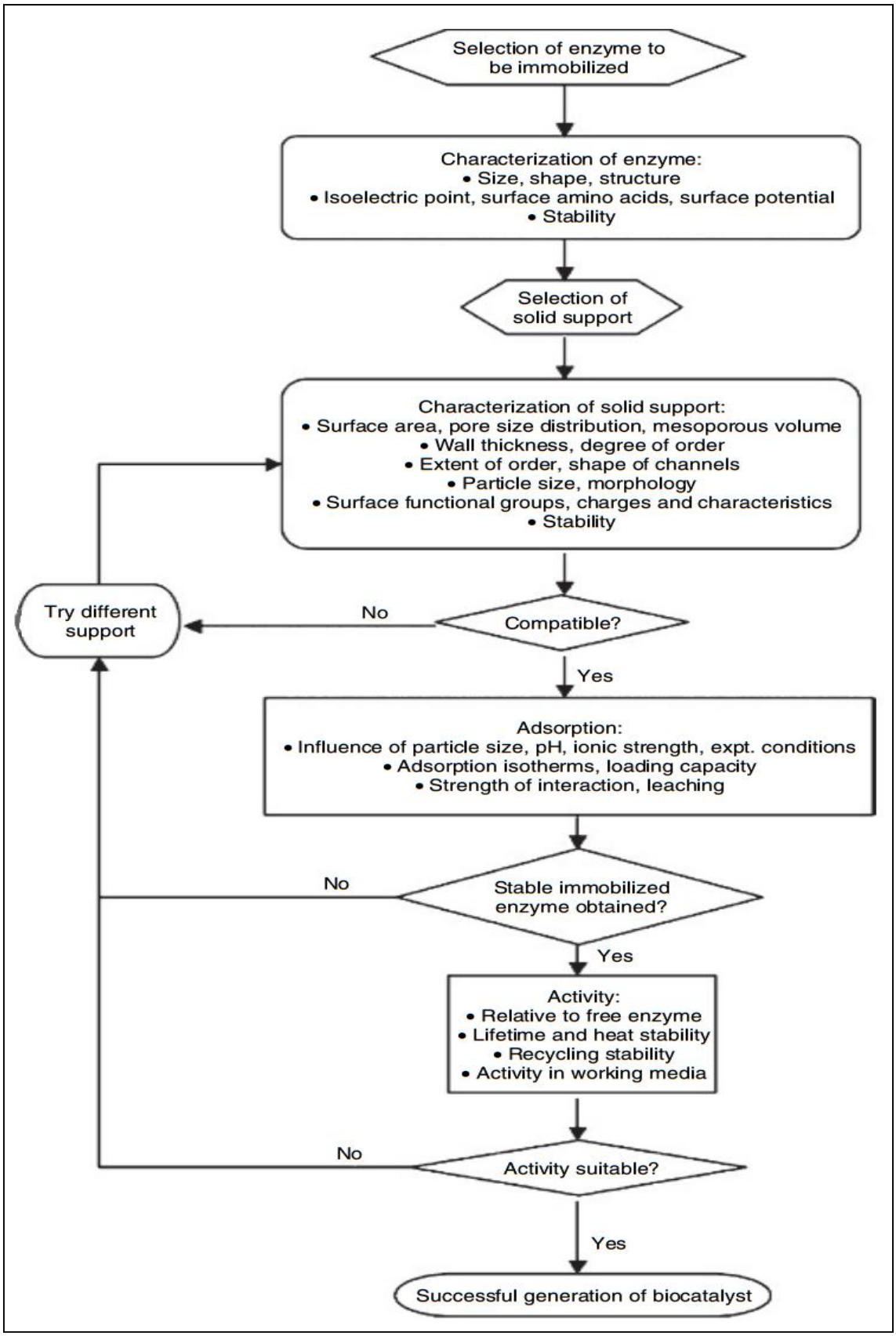


Figure 2.6 The algoritm that could be used in the study of an immobilized enzyme biocatalyst [28]

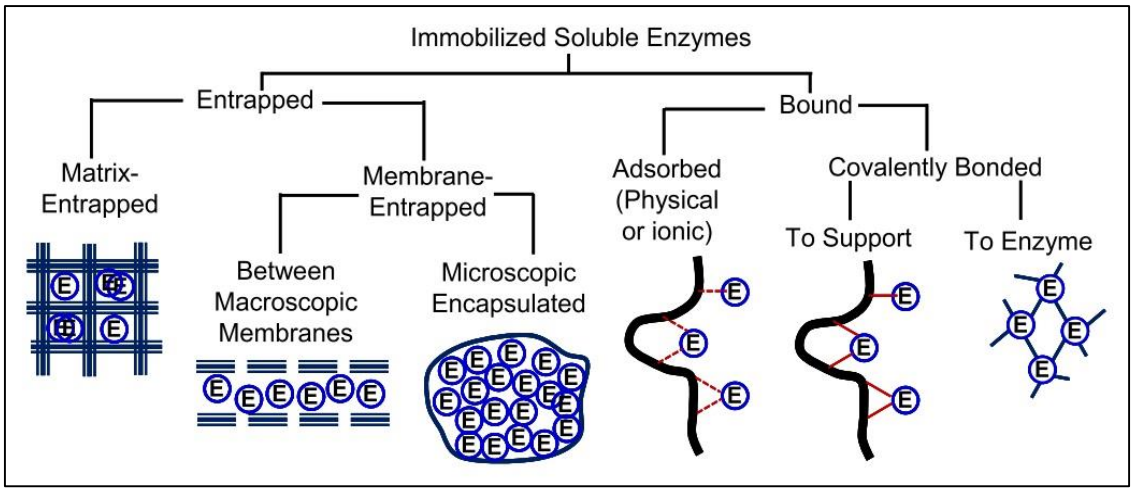


Figure 2.7 The major methods used for enzyme immobilization [20]

2.2.1 Chemical Methods used for Enzyme Immobilization

The enzyme molecules may bind to an inert carrier by covalent or noncovalent bonds in which case a carrier-bound biocatalyst is obtained. If the enzyme molecules are chemically bound among themselves, generally through a bifunctional reagent, without an inert carrier the method used is termed as 'carrier free'.

2.2.1.1 Carrier-Binding Immobilization

Carriers for enzyme immobilization can be divided into nonporous and porous. Nonporous carrier-enzyme complex, in which the enzyme is attached to an impenetrable surface, inferior to minimum mass transfer limitations while enzyme loading is rather low. Porous network-enzyme complex, suffers from significant mass transfer constraints but it has a high enzyme loading capacity. In recent years, great advances have been held in carrier design studies. Nanotechnology has provided significant tools to develop nanostructures as enzyme carriers where the above limitations can be solved [10].

Enzymes can be binded to carriers by covalent or noncovalent interactions. Covalent bonds may be formed between functional groups in the activated carrier and functional groups in the catalytic part of the enzyme, such as –OH, –SH, $–NH_2$, and –COOH. Following functionalization, activation of supports with specific activating agents (i.e. organic/inorganic halides, glutaraldehyde, carbodiimides, and various bifunctional molecules) is necessary for efficient immobilization. Covalent immobilization is a rather

complicated method, the carrier is hardly recoverable, and immobilization yield is comparatively low [10, 11].

Noncovalent immobilization to solid matrixs includes all types of interactions between the enzyme and the support except covalent bonds; hydrophobic interactions, ionic bonds, and weaker interactions like van der Waals forces; these methods are named as adsorption. Most of the carrier materials have sufficient surface-charge properties appropriate for immobilization by adsorption. They include inorganic carriers (ceramic, alumina, activated carbon, kaolinite, bentonite, porous glass) [12], organic synthetic carriers (nylon, polystyrene) [29], and natural organic carriers (chitosan, dextran, gelatin, cellulose, starch) [30]. The procedure simply includes mixing an aqueous solution of enzyme with the support material for an interval of time, and then free enzyme is removed via washing the support. Enzyme leakage from the mesoporous material is a main problem that can be solved by initiating covalent or ionic interactions, but different methods of immobilization can also be used to eliminate this problem like entrapping agents that partially close the inlet of the mesopores aiding enzyme retention. Stability can also increased by cross-linking of the adsorbed enzyme onto the carrier surface [10, 31].

2.2.1.2 Carrier-Free Immobilization

In the beginning of 1960s, solid phase protein chemistry studies led to the discovery that cross-linking of dissolved enzymes via chemical reaction of surface NH_2 groups with bifunctional reagents, such as glutaraldehyde, yielded insoluble cross-linked enzymes (CLEs) with retained catalytic activity. This method is low in cost and contains biocatalysts of higher specific activity. The structure is named as cross-linked enzyme: CLE when a soluble protein is cross-linked; as CLE crystal: CLEC when a crystallized enzyme is cross-linked or CLE aggregate: CLEA when a precipitated enzyme aggregate synthesized followed by cross-linking. CLEs are obsolete because of their poor mechanical properties, low activity and low reproducibility, on the other hand, CLECs, are high-quality biocatalysts in terms of activity, stability, and reusability, but their cost is high because of the purification process from crystals. Limitations of CLEAs in industrial use are mainly poor mechanical properties and small and nonstandart size that affects their handling in process negatively [10, 32]. Generally, CLECs present higher

activity and in organic solvents than they do in water. For example, the activity of CLECs of subtilisin in decane was 780 times greater than that in trimethylamine [33, 34].

2.2.2 Immobilization via Containment

Immobilization via containment can be applied in two ways: matrix entrapment and membrane retention.

2.2.2.1 Matrix Entrapment

The enzyme is entrapped within usually polymeric materials like Ca-alginate, agar, k-carrageenan, polyacrylamide, and collagen. Some solid matrixs can also be used such as activated carbon, porous ceramic, and diatomaceous earth. The matrix can be in the form of a particle, a membrane, or a fibre. When immobilizing in a polymer matrix, enzyme solution is mixed with polymer solution before polymerization reaction occurs. Polymerized gel-containing enzyme is either formed or a template is used to manipulate the structure of the particles from a liquid polymer-enzyme mixture. Both entrapment and surface attachment may be used in some cases [20].

2.2.2.2 Membrane Retention

In contrast with the other immobilization methods using carrier, enzyme is not bound to a carrier or aggregated, but restricted to a certain space by a semipermeable membrane that physically separates the enzyme from the other units of the reaction system [10]. Membranes made of nylon, polysulfone, cellulose, and polyacrylate are mostly used. Various configurations are possible including hollow fibers. A semipermeable membrane which can provide retaining of high molecular weight compounds (enzymes), while allowing small molecular weight compounds (substrates or products) access to the enzyme [20].

A special form of membrane entrapment is microencapsulation which is produced by initiating a polymerization reaction in the surface of droplets of enzyme aqueous solution dispersed (with the aid of a surfactant) in a water-immiscible organic solvent [10]. The advantages and disadvantages of the major methods used for immobilization were listed in Table 2.2 [11].

Table 2.2 The advantages and disadvantages of the most common used immobilization methods [11]

Method of Immobilization	**Advantages**	**Disadvantages**
Encapsulation /entrapment	Enzyme is not chemically modified	Enzyme leakage
	Enzyme should retain catalytic activity under the conditions of polymerization/transition of the support	Mass transfer constraints
Enzyme cross-linking	Support is unnecessary	Enzyme is chemically modified
	Stabilization of the enzyme	Complex procedure
	Minimal enzyme leakage	Mass transfer constraints
Adsorption	Enzyme is not chemically modified	Enzyme leakage
	Easy and economic to use	Poor specificity of the reaction (i.e., adsorption and ion-exchange could cover each other)
Electrostatic interaction	Enzyme is not chemically modified	Enzyme leakage
	Easy to use	Low specificity of the reaction (i.e., adsorption and ion-exchange could cover each other)
Affinity	Highly specific the the reaction	Expensive and complex to be designed
		The presence of functional groups on the enzyme is required
Covalent binding	Strong binding	Possible decrease in enzymatic activity
	Minimal enzyme leakage	Chemical modifications of the support are necessary
	Stable enzyme	Usually irreversible attachment, inhibitssupport reuse
		Possibility of sterical modifications of the enzyme

2.3 Carriers Used for Immobilization

The properties of immobilized enzymes depend on some parameters as can be seen on Table 2.3 [35]. Some of these parameters are the reaction conditions, the immobilization method, and some properties of immobilization matrix like surface area, porosity, stability, ionic structure. Basic properties required for an ideal enzyme carrier [11, 13]:

- biocompatible with the enzyme,
- includes functional groups necessary for enzyme attachment or easily functionalized,
- stable with the changes in pH, ionic strength, and temperature,
- provides enzyme stability,
- eliminates enzyme leaching,
- permits diffusion of substrates and products,
- selective and sensitive for determined species,
- easy to prepare,
- inexpensive for minimizing the economic impact of the process,
- minimum hydrophobic surface, since it favors undesired protein,
- adsorption and denaturation.

Carriers can be classified according to their chemical structure (organic or inorganic), and the former can be further classified into natural or synthetic carriers [31]. A basic classification can be made as three main categories [35]:

1. Hydrophilic biopolymers based on natural polysaccharides such as agarose, dextran and cellulose.
2. Lipophilic synthetic organic polymers such as polyacrylamide, polystyrene and nylon.
3. Inorganic materials such as controlled pore glass and iron oxide.

Table 2.3 The parameters to be considered for the optimization of immobilization [36]

	Parameters	**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
Immobilization process	Immobilization strategy	Physical adsorption, encapsulation, covalent binding, cross-linking
	Immobilization conditions	pH, temperature, solvents, stabilizing agents.
	Diffusion/mass transfer	Buffer effect, viscosity of reaction media, pore diffusion
	Performance	Space-yield time, recycling, reusability, product removal, toxicity, enzyme inhibition, kinetics.
	Process cost	Costs of enzyme and support, waste disposal, energy.

2.4 Nanobiocatalysis

Nanotechnology has led to the development of nanobiocatalysis in which enzymes are embodied with nanomaterials include nanoporous media, nanofibers, carbon nanotubes and nanoparticles. Nanobiocatalysis systems have showed great efficiency in the manipulation of the nanoscale structure of the enzyme by developing activity and stability of enzymes [37].

Enzymes immobilized on nanoparticles showed an extended working range of pH and temperature and better thermal stability than the soluble form. Nanoparticle-matrix immobilization provides three important features compared with the conventional immobilization methods;

1. Nanoparticle-enzyme complex is easy to synthesize in high solid content without toxic reagents and surfactants.
2. Homogeneous and standart core–shell nanoparticles with a thick enzyme layer as shell can be obtained.
3. Particle size can be manipulated due to use within limits.

The benefits of using nanoscale structures as matrixs are reduced diffusion limitations and increased enzyme loading by means of maximized functional surface area. Besides, the physical properties of nanoparticles such as increased diffusion and mobility of particles can affect natural catalytic activity of binded enzymes. Irradiation resistance, thermal stability, and increased surface area can provide use for nanobiocatalysts in solar cells, photodetectors, nano-generators, ceramics, and biosensors. In the recent studies, nanoparticles have been configured as various nanostructures like nanorods, nanotubes, nanowires, nanorings etc. [14].

Conventional methods of immobilization such as adsorption, covalent coupling, entrapment and encapsulation are also sustainable with nanomaterials. Two basic methods have been used: enzyme adsorption onto the matrix surface with or without covalent binding, and entrapment in the matrix generally via self-assembly [38]. The encapsulation of a single enzyme molecule in a single nanoparticle (SEN), provides a new way of preservation of enzyme activity and increased enzyme stability [17].

2.5 Mesoporous Silica Nanoparticles (MSNs)

Mesoporous silica materials synthesized from silane precursors using a surfactant template provide controllable pore structure, standart pore-size distribution, high specific pore volumes (up to nearly 1.5 cm^3g^{-1}), large internal surface area (up to 1500 m^2g^{-1}). Their pore size can be arranged in between 1.5-30 nm by choosing a convenient surfactant or changing the conditions of synthesis [32]. These supports promise high enzyme load

and are water-insoluble, thermally, mechanically and chemically stable and resistant towards microbiological concerns. Besides, silica supports include sufficient functional groups on their surface for enzyme attachment or convenient for modification [32, 39]. All these advantages make them great candidates for the immobilization enzymes. Recent studies have shown that the immobilization of enzymes onto mesoporous silica particles may increase catalytic activity and stability of the enzymes [36].

Physical adsorption is the simplest method of enzyme immobilization on mesoporous silica, the active site of the enzyme is generally unaffected, and nearly all of the activity is protected. However, the limitation of this method is the enzyme leakage out from the mesoporous material, which means poor stability. The common solution to eliminate the enzyme leakage is functionalizing of the mesoporous silica support [40]. Some of the functionalized-silica structures can be seen at Figure 2.8 [35]. On the other hand, field emission scanning electron microscopy (FESEM) images of functionalized mesoporous silica nanoparticles can be seen at Figure 2.9 [40].

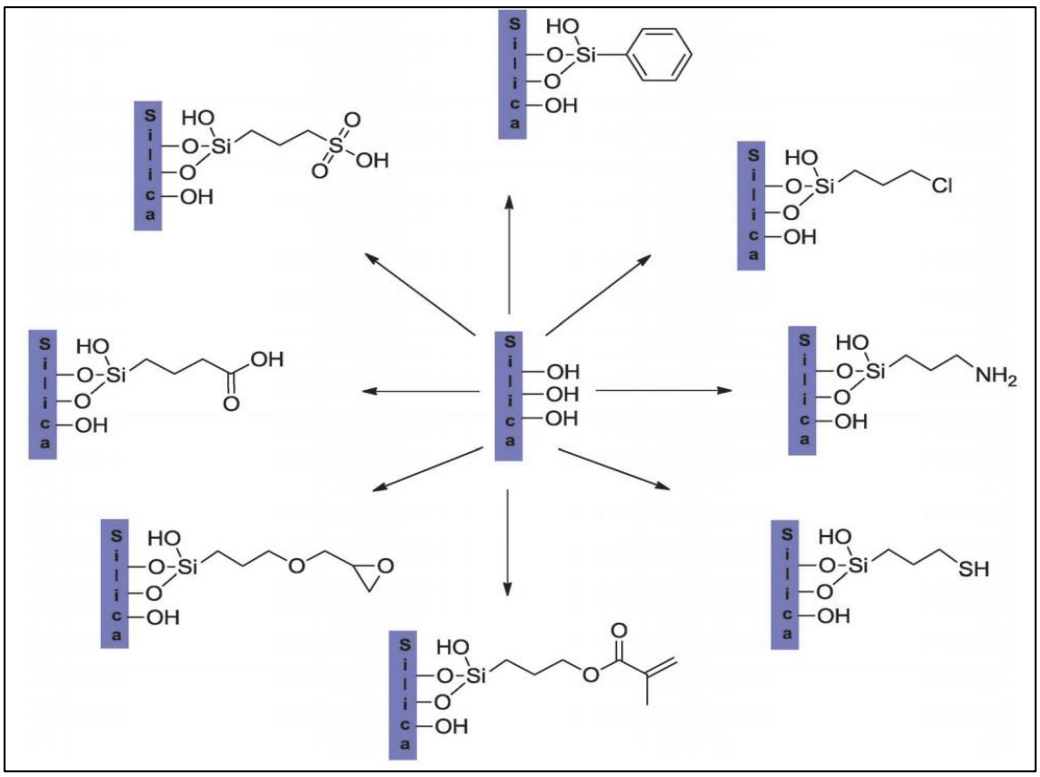


Figure 2.8 Functionalization of mesoporous silica materials with different reactive groups [36]

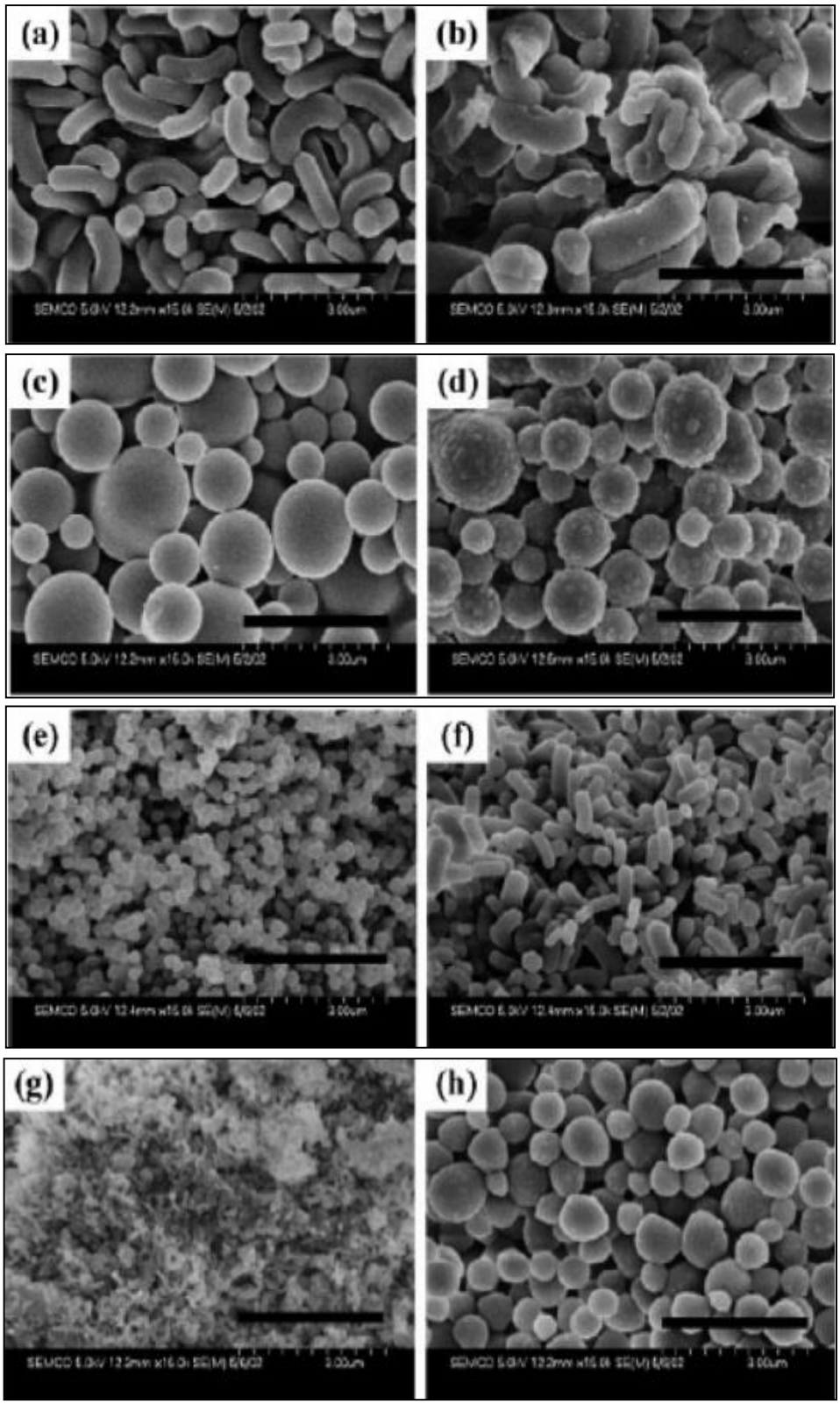


Figure 2.9 Field emission scanning electron microscopy (FESEM) images of (a) 3-aminopropyl-MSN, (b) *N*-(2-aminoethyl)-3-aminopropylMSN, (c) 3-[2-(2-aminoethylamino)ethylaminopropyl-MSN, (d) 3-ureidopropyl-MSN, (e) 3-isocyanatopropyl-MSN, (f) 3-cyanopropyl-MSN, (g) allyl-MSN and (h) nonfunctionalized MSN [41]

The most utilized functional parts of the enzymes are the amino groups of arginine, lysine and histidine residues, the carboxylic groups of glutaric acid or the sulfhydryl groups of cysteine residues [42]. The common method for covalent immobilization on porous silica supports is the functionalization of the material surface with amino groups (mostly using (3-aminopropyl)triethoxysilane, APTES) followed by activation with glutaraldehyde (GA) to obtain a convenient linker. The amino groups react with the aldehyde groups on the surface of the silica support under formation of imine bindings. As cross-linking agent, bifunctional spacers such as glutaric anhydride, succinimido-3-maleimidopropanoate, cyanuric or bulky polyaldehydes, chlorides can be also used [43]. Other organosilanes used for functionalization of the surface with epoxy groups or thiol groups (respectively) are 3-(glycidoxypropyl)-trimethoxysilane [44] and 3-(mercaptopropyl)-triethoxysilane [45], respectively. Figure 2.10 illustrates the formation of CLEAs via cross-linking by glutaraldehyde [35].

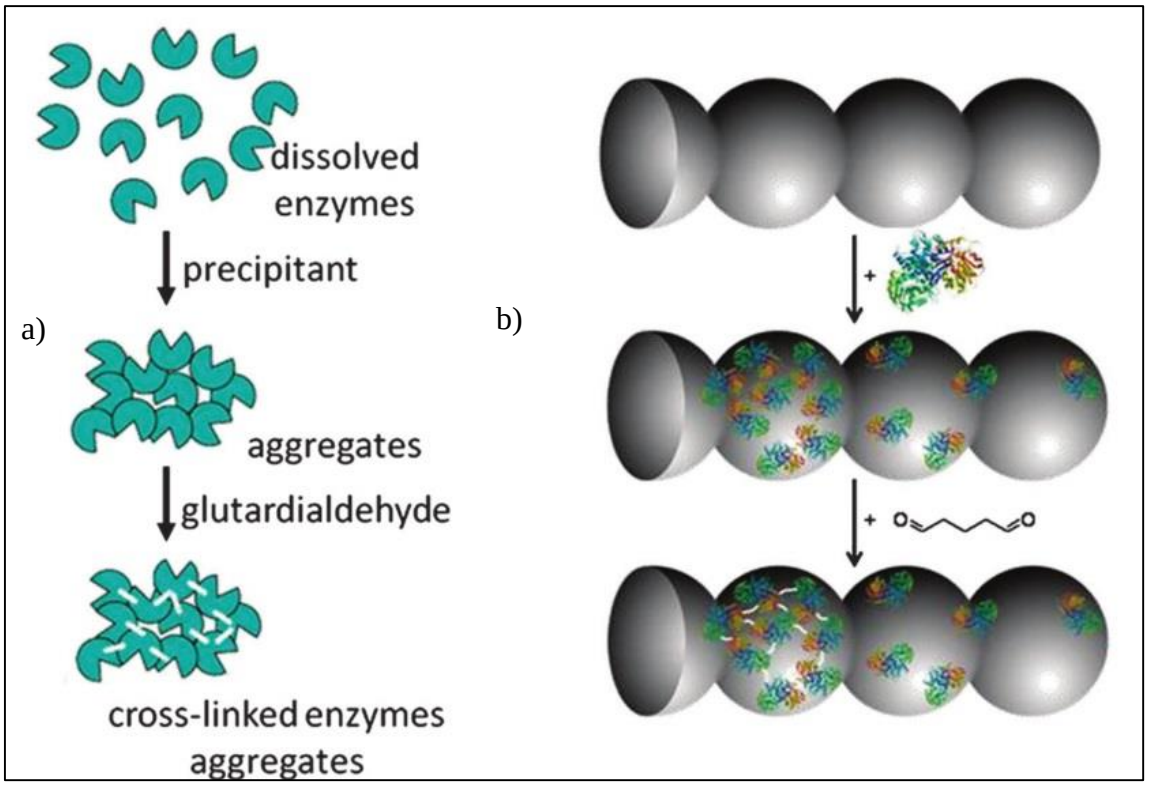


Figure 2.10 Formation of CLEAs a) in the solution and b) in the pores of a convenient carrier [35]

Covalently bound enzyme-silica carrier complexes can also be used in polar solvents such as water, while physically adsorbed enzymes can only be used in organic solvents or with pure hydrophobic reactants in order to avoid enzyme leakage [36, 46]. For an efficient enzyme immobilization onto mesoporous silica nanoparticles, the presence of functional groups on their surface is essential. The surface functionalized particles with reactive groups are required to increase the enzyme load and stability. Natural and synthetic polymers are commonly used for the functionalization of mesoporous nanoparticles. The polymers usually used in the coating silica-based matrices include agarose [47], dextran [48], poly(vinyl alcohol) [49] and chitosan [15, 50–52]. For this study, chitosan and poly(acrylic) acid were used as coating layer for the functionalization of the mesoporous silica nanoparticles.

2.6 Chitosan (CS)

Chitosan is a natural polyaminosaccharide, and obtained by the partial deacetylation of chitin which is the second most abundant natural polymers in the world. Based on its favorable biological (biodegradable and bioactive) and chemical characteristics (polycationic, reactive groups such as -OH and -NH_2), chitosan has attracted great attention [15]. The chemical structure of chitosan [53] was given in Figure 2.11.

Chitosan-based supports are used in various forms such as powder, flake, and gel [15]. Chitosan gel can be by prepared via solvent evaporation method, neutralization method, cross-linking method and ionotropic gelation method. Chitosan gel is generally manufactured in form of membranes, beads, capsules, coatings, fibers, hollow fibers and etc. Variable modifications have been studied to improve the gel strength, stability and adsorption capacity [54]. For immobilization of enzymes on chitosan supports, glutaraldehyde is the most commonly used activating agent via cross-linking reaction with amino groups. Main application areas of chitosan and the advantage of chitosan for the usage in these applications were presented in Table 2.4 [54], [55].

Micro and nanoparticles can be developed if the degree of deacetylation and molecular weight of chitosan is optimized. Chitosan nanoparticles has been given results as one of the most promising bio-based polymeric carriers for enzyme immobilization because they increase the stability of immobilized enzymes and get easier the separation of enzymes from the reaction medium.

Chitosan has ability to control the release of active agents and there is no need to use hazardous organic solvents in production of the particles since it is soluble in aqueous acidic solution. Chitosan has a mucoadhesive (adheres to a mucose membrane) structure, which has an increased residual time at the site of absorption.

Several methods have been studied to synthesize chitosan nanoparticles. The choice of the method depends on the requirements based on the shape and particle size. Ionotropic gelation, emulsion cross linking, coalescence, emulsion-droplet, reverse micelles, coacervation/precipitation, template polymerization and molecular self-assembly are the main preparation methods of chitosan nanoparticles [55–60].

Chitosan can be coated on the surface of silica particles via putting the particles into a chitosan solution (in acetic acid) then precipitating the polymer (either alone or as a blend with polyelectrolytes, such as polymethacrylic, polyacrylic, or poly(ethylenemine)) using a solvent (i.e. acetone) [61].

In the present study, in-situ polymerization method used for coating mesoporous silica nanoparticles, chitosan was firstly dissolved in an acrylic acid solution under magnetic stirring. Due to the electrostatic interaction, the negatively charged acrylic monomers align along the chitosan molecules. After complete dissolution of chitosan, the polymerization was initiated by $K_2S_2O_8$ at 80°C. The complete polymerization leads to the appearance of an opalescent solution, indicating the nanoparticles formation [60, 62].

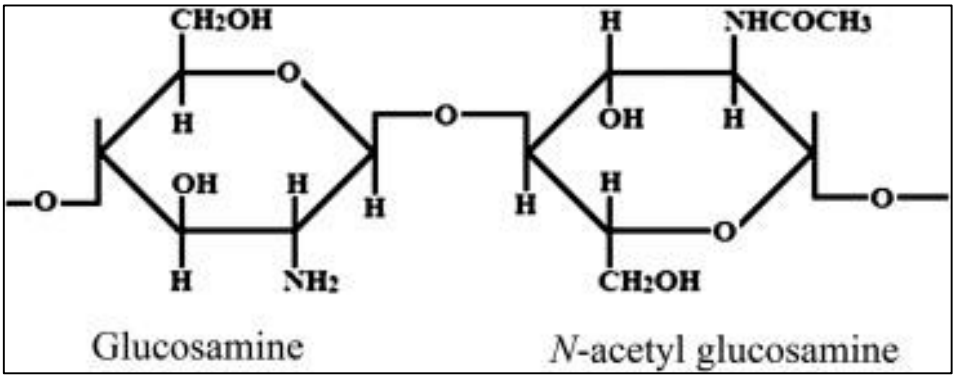


Figure 2.11 Chemical structure of chitosan [53]

Table 2.4 Main applications of chitosan [56, 63]

Industry	**The role of chitosan**	**Process**
waste water treatment	high adhesive and insolubility properties	membrane purification processes and removal of heavy metal ions
food	good emulsifying, superior thickening, gelling agent	preservative, packaging material, anticholesterol and fat binding, and animal feed additive
agriculture	fixing agent	seed and fertilizer coating, controlled agrochemical release
pulp and paper	wet strength to paper	surface treatment, photographic paper
cosmetics and toiletries	fungicidal and fungistatic properties	moisturizer, body creams, bath lotion
absorbable sutures and wound dressing materials	wound healing acceleration and immune system stimulation	water-absorbent, biocompatible films
photography	resistance to abrasion, optical characteristics and film forming ability	
dye removal	affinity for many classes of dyes, including disperse, direct, reactive, acid, vat, sulfur and naphthol dyes	
drug delivery	biocompatibility, biodegradability and ecological safety	
tissue engineering	porous structure, gel forming properties, ease of chemical modification, high affinity to in vivo macromolecules	
enzyme immobilization	excellent membrane forming ability, good adhesion, low cost, low immunogenicity and nontoxicity, high mechanical strength and hydrophilicity as well as the improvement of stability	

2.7 Poly(acrylic) Acid (PAA)

pH-sensitive hydrogels are the polymers that can response to the pH of their environment and change their properties according to this signal. The reason of this response is the presence of basic/ionisable weak acidic moieties binded to a hydrophobic backbone. These functional groups include ionisable acidic groups such as sulfonic and carboxylic acids or basic groups like amine that can donate or accept protons according to the change in pH of the solution. pK_a value determines the rate of change in pH via affecting the degree of ionization. PAA becomes negatively charged when the pH of the solvent is above its pKa (~4) due to the ionization of its -COOH groups and it has poor solubility at low pH values, it gets better at high pH values (pH ≥ 8) (Figure 2.12) [64].

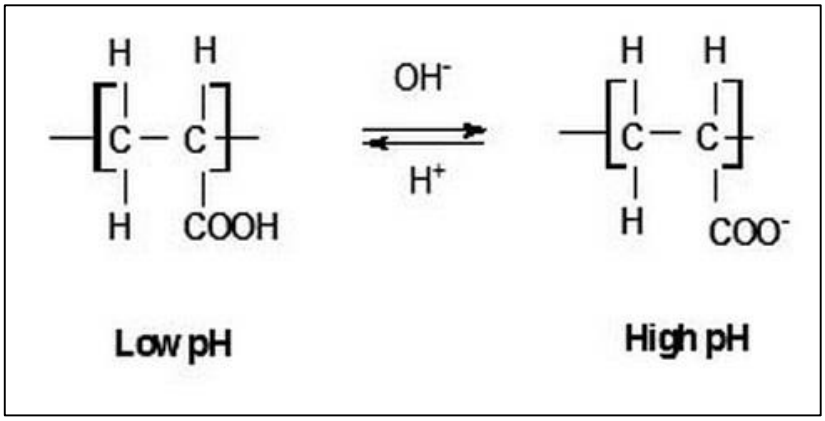


Figure 2.12 Effect of pH on chemical structure of PAA [64]

Linear polymer chains such as PAA [65] and poly(methacrylic acid) (PMAA) [66] were grafted onto the surface of MSNs to perform as pH-sensitive gatekeeper, which is open at high pH value by means of extending polymer chain, thus molecules can be released while in acidic medium, the chain is narrowed and the release of molecules are blocked [51]. The mechanism can be seen in the Figure 2.13.

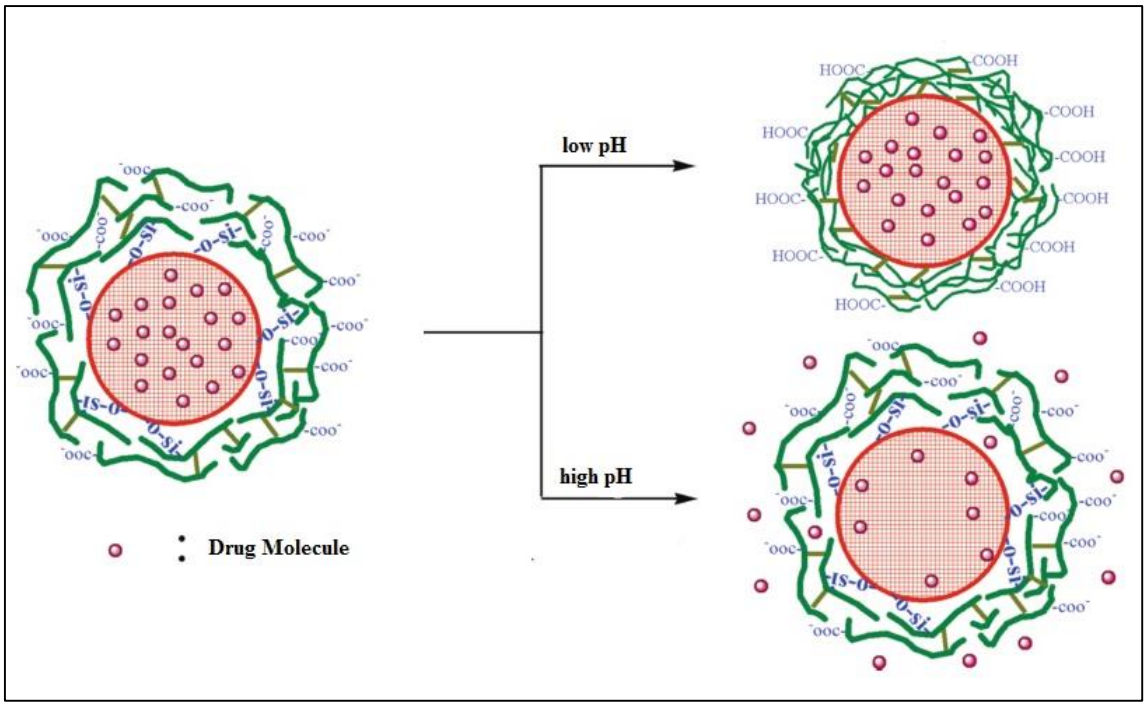


Figure 2.13 Schematic illustration of pH-sensitive polymer-coated mesoporous silica spheres [66]

Because of its features mentioned above, MSNs have been modified with PAA by three methods [51]:

a) **"layer-by-layer adsorption technique"**, building a multilayered structure with polyelectrolytes on the surface of MSN via electrostatic attraction [2];

b) **"graft from" strategy**, like free radical polymerization [67];

c) **"graft to" strategy**, surface-initiated polymerization reactions initiated by the functional groups on cores (i.e. –R–Br, $–NH_2$, –OH,$–CHCH_2$) [68].

There are some disadvantages of these methods that could not have been eliminated yet. These are multiple-step synthesis, use of suitable surfactants, decreased efficieny of surface grafting or encapsulation and etc. [69].

PAA is a type of water soluble polymers, containing a carboxyl group in each repeated unit and shows anionic polyelectrolyte features, which provide favorable or attractive electrostatic interaction. The application of the surface grafting method of PAA onto chitosan beads has also been studied by Dai et al. (2010) [16] to extend their selectivity or adsorption capacity due to high content of carboxyl groups and anionic polyelectrolyte feature of chitosan. The research group has also examined that glutaraldehyde (GLA) cross-linked CS/PAA beads had increased stability in lower pH conditions and higher

mechanical strength than CS-GLA beads without PAA. The long chains of PAA grafted onto the chitosan granules can also be expected to significantly increase the adsorption capacity via enhancing the number of adsorption sites [16].

In the study of Spagnol et al. (2012) [69], chitosan-graft-poly(acrylic acid) matrix was studied. As can be seen in Figure 2.14a, chitosan-graft-poly(acrylic acid) hydrogel has an interlocked network and highly porous structure. The pores show small and standart size, distributed into the hydrogel matrix. The chitosan-graft-poly(acrylic acid)/cellulose nanofibrils hydrogel composite morphology seems to be more irregular with large pores and leaflike shape. Chitosan-graft-poly(acrylic acid) and its blend with cellulose was reported as responsive to pH and to ion concentration that approves high potential of application as devices for controlled release of solutes and drugs.

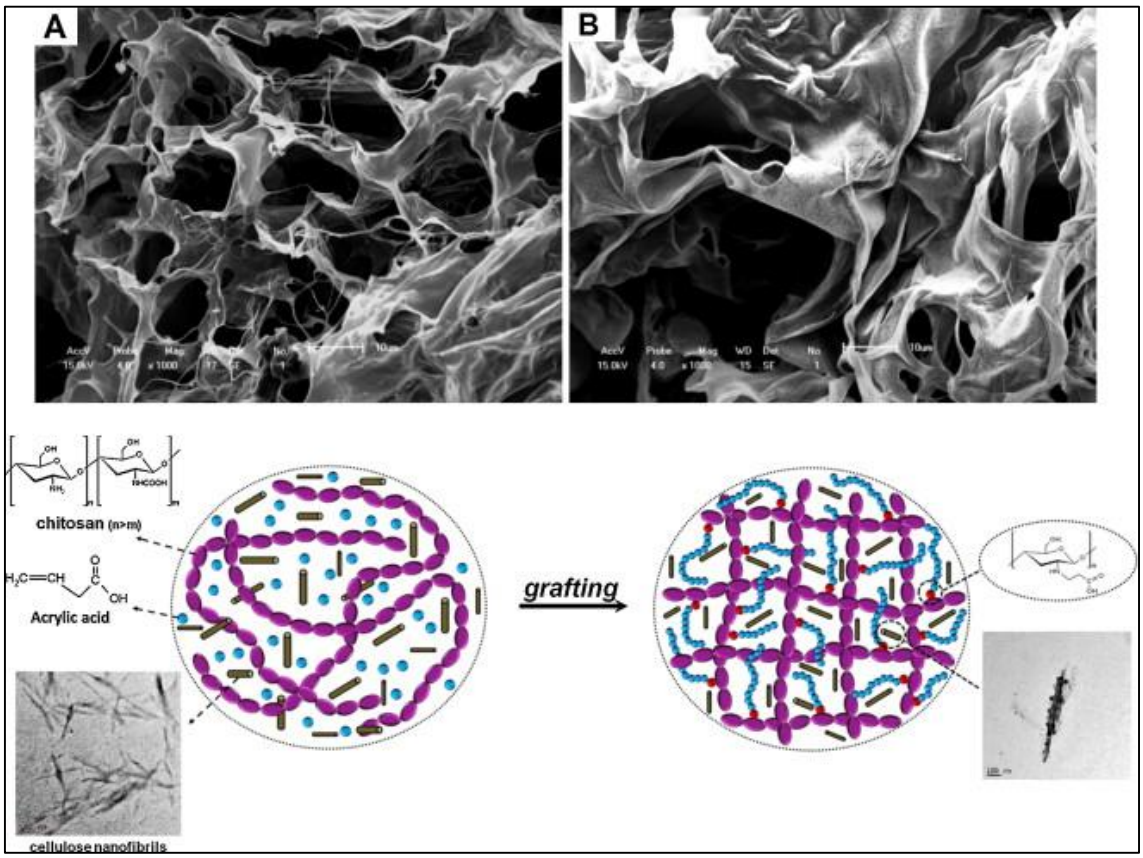


Figure 2.14 SEM micrographs of (a) chitosan-graft-poly(acrylic acid) and (b) chitosan-graft-poly(acrylic acid)/cellulose nanofibrils hydrogels composites (Mag 1000×, scale 10 μm) and scheme of chitosan-graft-poly(acrylic acid)/cellulose nanofibrils hydrogel composite formation [70]

CHAPTER 3

LITERATURE SURVEY

3.1 Studies on Polymer Blends for the Coating Layer on MSNs

For an efficient enzyme immobilization onto mesoporous silica nanoparticles, the presence of functional groups on their surface is essential. The surface functionalized particles with reactive groups are required to increase the percent enzyme loading and stability. Natural and synthetic polymers are commonly used for the functionalization of mesoporous nanoparticles. In this section, the studies demonstrated in the literature about the synthesis of well-functionalized polymer surfaces were summarized.

Feng et al. (2014) [71] have proposed a simple layer-by-layer self-assembly technique to construct mesoporous silica nanoparticles (MSNs) as an agent for a pH-responsive drug delivery system with extended efficiency and biocompatibility. In this system, biocompatible polyelectrolyte multilayers of alginate/chitosan were coated on MSN's surface to obtain pH-responsive nanocarriers. The functionalized-MSNs were tested for blood compatibility and showed good results over the raw-MSNs in terms of cytotoxic activity and low hemolytic against human red blood cells. As model drug, the anticancer drug doxorubicin (DOX) was loaded into nanocarriers to examine their use for the pH-responsive drug release. The DOX release from nanocarriers showed pH dependent behaviour, and the release rate also changed with pH, release faster at lower pH than that of at higher pH.

Peng et al. (2013) [72] have studied the synthesis of pH-responsive nano-carrier MSNs–PAA, possessing mesoporous silica nanoparticles (MSNs) cores and poly(acrylic acid) (PAA) shell-layers. The algorithm of the synthesis was illustrated in Figure 3.1; vinyl

double bonds modified MSNs were synthesized by using cetyltrimethylammonium bromide as organic template, tetraethyl orthosilicate as silicon ancestor, and 3-(trimethoxylsilyl) propyl methacrylate as surface modification agent. The pH-sensitive layers of PAA were grafted onto the vinyl double bonds of the MSNs via precipitation polymerization, producing the MSNs–PAA with a hollow cubic core and mesoporous shell with penetrating pore channels. The MSNs–PAA was investigated as drug carrier in different pH mediums. The results showed that the PAA layers on the surface of MSNs–PAA achieved opened-closed states at different pH values, which can provide controlled drug delivery.

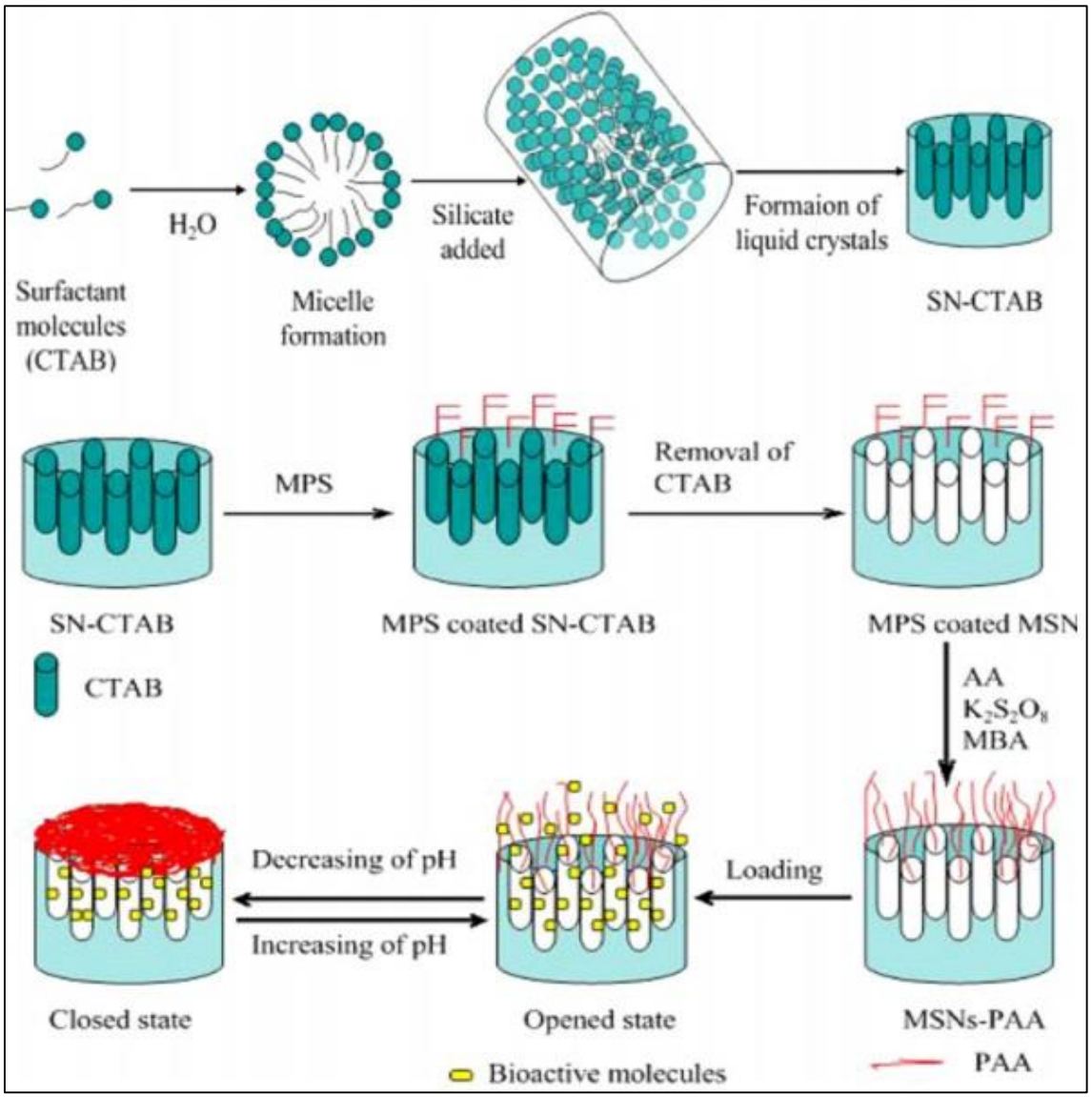


Figure 3.1 The synthesis of pH-responsive nano-carrier MSNs–PAA [72]

Haghighizadeh et al. (2012) [73] have investigated the immobilization of noble metal nanoparticles on silica microspheres treated by chitosan. The dual support system is includes organic-inorganic materials synthesized via core-shell method. Chitosan/silica nanocomposites were prepared with different chitosan concentrations in order to adjust shell thickness. When high concentration of chitosan was added, it was revealed that the shell completely covers the silica layer. The silver nanoparticles were then immobilized on the shell of the support via sol-gel method. Increased chitosan concentration caused amorphous structure and the shell became unclear. Higher metal loading was also observed when concentration of CS is lower. The catalyst was used in the catalytic hydrogenation of cyclohexene resulting with high yield.

Popat et al. (2012) [74] have proposed an effective and facile method for coating chitosan onto phosphonate-functionalised MSNs to generate pH sensitive nanocarriers. Controlled-release of ibuprofen was studied and it was seen that ibuprofen releases under mild acidic conditions because of dissolution of chitosan layer and a decreased release was achieved at pH above the isoelectric point of chitosan. This work approves the potential of chitosan based inorganic–polymer blends as next generation delivery agents for biomedical applications.

Tang et al. (2011) [51] have studied pH-responsive polymer (chitosan/poly(methacrylic acid) (CS-PMAA))-coated mesoporous silica nanoparticles through "the facile in situ polymerization method". The prepared composite microspheres revealed a flexible control over surface charges, hydrodynamic size, shell thickness by changing the feed ratio of MSNs and the molar ratio of [–NH2]/MAA. The well defined core–shell composite microspheres were confirmed by the results from TEM images, DLS, FTIR spectra, BET and TGA measurements. The release of a cancer treatment drug has been studied and the results have showed pH-responsive release characteristics.

Kalkan et al. (2011) [15] have been studied preparation of chitosan-coated magnetite nanoparticles and usage of these particles for immobilization of laccase enzyme. Reversed phase suspension technique has been used for coating of Fe_3O_4 nanoparticles with CS. GLA has been used as a cross-linking agent. Laccase enzyme has been immobilized on these magnetically separable chitosan-coated magnetite nanoparticles via adsorption and covalent binding methods. Immobilized laccase activity had a better stability against pH and temperature compared with soluble enzyme.

Pastor et al. (2011) [50] have studied protein delivery via uncoated and chitosan-coated mesoporous silicon microparticles. The synthesis and characterization of two new mesoporous silicon particles prepared by means of electrochemistry. Both carriers were studied for the capacity to load a therapeutic protein (insulin) and a model antigen (bovine serum albumin, BSA) by adsorption method. The results have shown that mesoporous silicon microparticles provide great affinity for insulin and albumin. The formation of chitosan coating on the microparticles surface was approved both qualitatively by atomic force microscopy and quantitatively by a colorimetric method. Studies of insulin release from the particles had shown that the release from the prepared devices was fast (>80% insulin released at 45 min) but controlled (leakage< 20%).

Yuan et al. (2011) [75] have studied the preparation of poly(acrylic acid) grafted mesoporous silica nanoparticles (PAA-MSNs) through a facile "graft onto" strategy (Figure 3.2), i.e., the amidation between PAA homopolymer and amino-functionalized MSNs. The resultant PAA-MSNs had uniform spherical shape and a mean diameter of ~150 nm. By means of the covalent graft of hydrophilic and pH-sensitive PAA, the particles are well-dispersed in aqueous solution, which is a favorable property of drug carriers to build a pH-responsive and adjustable drug delivery system. N_2 adsorption-desorption isotherm resulted that, a well-known anticancer drug, doxorubicin hydrochloride (DOX) could be effectively loaded into the channels of PAA-MSNs via electrostatic interaction. The loading content and the entrapment efficiency of the drug could reach up to 48% and 95%, respectively.

Dai et al. (2010) [16] have studied preparation of poly(acrylic acid) blended chitosan (CS/PAA) hydrogel beads by one-step method. It was found that GLA cross-linked CS/PAA beads had higher mechanical strength and better stability in lower pH solutions than GLA cross-linked CS beads. Results obtained from removal of copper (Cu) ions from aqueous solutions also showed that the adsorption capacity of CS/PAA beads was greater than that of CS beads.

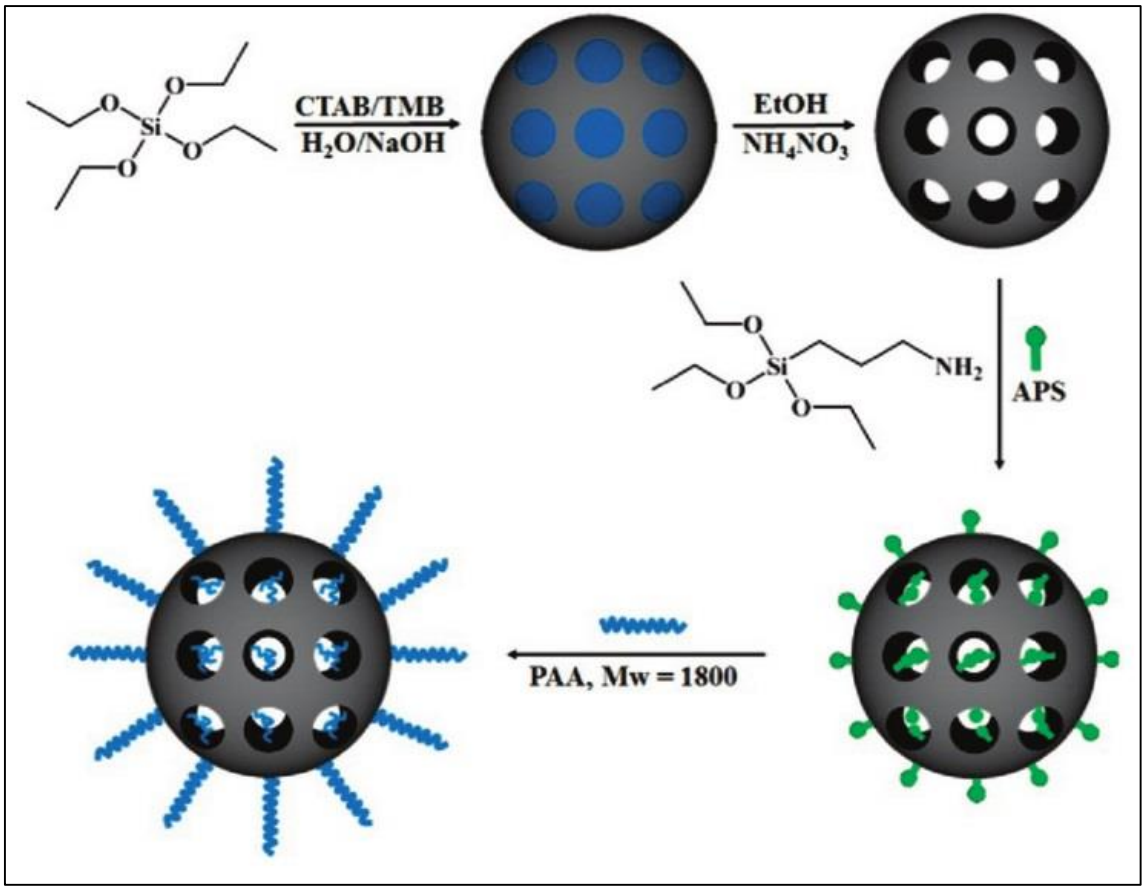


Figure 3.2 Illustration of the preparation of PAA-MSN particles [75]

Lei and Bei (2007) [2] have studied a strategy for the preparation of the silica-coated chitosan support using layer by layer method. Their strategy was confirmed by the successful synthesis of the silica-coated chitosan support to immobilized pectinase. The silica-coated chitosan supports were cross-linked with glutaraldehyde for better stability in both alkaline and acidic media. The results have shown that the pectinase immobilized on the silica-coated chitosan support showed better stability against thermal and pH denaturation.

Wu et al. (2006) [76] have studied a preparation method of CS–PAA polymer magnetic microspheres with high Fe_3O_4. Acrylic acid was polymerized by the initiation of potassium persulfate onto the CS–Fe_3O_4 cores, which were self-assembled by the electrostatic interaction between negatively charged Fe_3O_4 nanoparticles and protonated CS (Figure 3.3). The magnetic microspheres reported as pH-sensitive and superparamagnetic. The study of release of the entrapped ammonium glycyrrhizinate in

such polymer magnetic microspheres had showed that the potential applications of these microspheres for drug delivery.

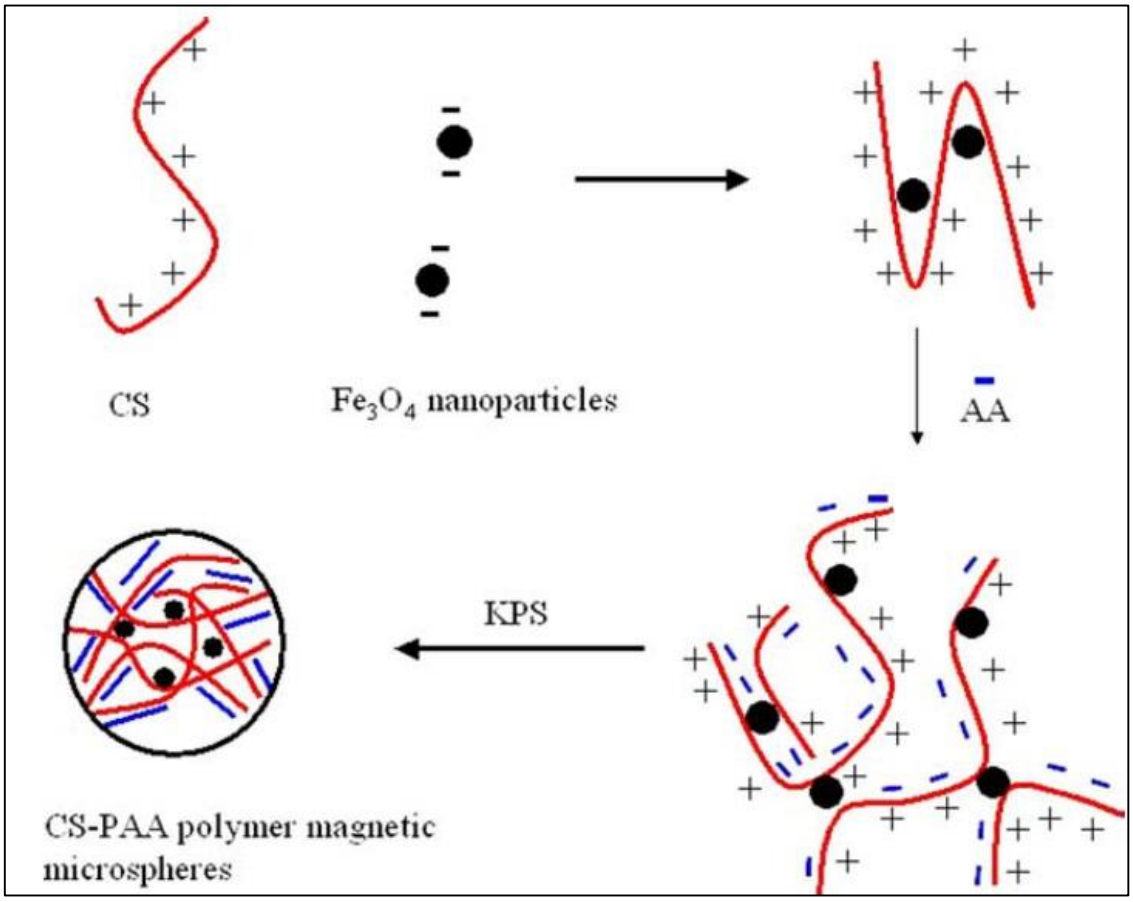


Figure 3.3 Scheme of the preparation of PAA-CS polymer magnetic microspheres [76]

3.2 Studies on Enzyme Immobilization

In this section, the studies demonstrated in the literature about the investigation of the parameters of the immobilization of Subtilisin and other enzymes onto various carriers were summarized.

Lee et al. (2015) [77] have investigated the immobilization of β-glucanase on silica, and the effects of the enzyme-silica size, temperature, time and amount of enzyme-silica on the hydrolysis of polysaccharides in the Chamaecyparis obtusa residue were studied. The enzyme-silica size, time range, temperature, and amount of enzyme immobilized silica ranges were 10-63 mm, 2-12 h, 25-45ºC, and 0.05-0.25 g, respectively. The optimum values were found to be 10-25 mm silica, 10 h, 40ºC, and 0.15 g, respectively. Denaturation of the enzyme could not be eliminated at temperatures higher than 40ºC.

Junoi et al. (2015) [78] have investigated an immobilized enzyme process for deproteinization natural rubber latex containing allergenic proteins. The procedure has been developed with cellulose-chitosan composite beads for immobilization of protease enzyme. Optimal conditions were found as 15% cellulose in beads, immobilization period of 24 h and pH value of 9. The enzyme loaded beads were tested as operationally stable and reusable for at least five times.

Soleimani et al. (2012) [79] aimed to examine the effect of protease immobilization on silica nanoparticles and the effects of immobilization to protease performance as catalysis for improving the removal process of protein soils. Detergent products contain many components that may affect activity and stability of the free soluble enzyme. The effect of enzyme immobilization on the removal of protein based soil has been examined on cotton fabrics. The effect of temperature and humidity on the stability of free and immobilized enzyme has been studied. It was resulted that the immobilized enzyme increased its cleaning efficiency toward protein soil removal on cotton fabrics. Moreover, thermal and humidity stability of the immobilized enzyme was higher than that of soluble enzyme.

Murai et al. (2011) [80] have synthesized mesoporous silica materials (MPSs) and studied the immobilization of Subtilisin from *Bacillus licheniformis* to MPSs. After the functionalization of the surface of MPS, Subtilisin from *Bacillus licheniformis* was immobilized by a direct one-step immobilization process. MPS modified with ethyl group has shown the maximum activity of subtilisin. Denaturation temperature of immobilized subtilisin has been shifted to a higher temperature than that of free-enzyme. Immobilized enzymes conserved ~30% of its original activity after 5 times reuse.

Singh et al. (2011) [81] have studied the immobilization of procerain B, a novel cysteine protease, on glutaraldehyde-activated chitosan beads via covalent attachment. Glutaraldehyde has been reported as not only a cross-linking agent but also linker of the procerain B on the surface of bead through primary amine group by Schiff base linkage. Immobilized enzyme was tested for optimal range and stability with respect to pH and temperature. The chitosan-immobilized procerain B has been reported as with extented pH and thermal optima. The effects of substrate concentration and operational stability of immobilized beads were also studied and it was seen that nearly 50% of the original activity was retained until the 10th use.

Zhao et al. (2010) [82] have synthesized three new ether-functionalized hydrophobic liquids (ILs) and examined the immobilization of two proteases (subtilisin and α-chymotrypsin) to chitosan. Both subtilisin and α-chymotrypsin showed high synthetic activity and selectivity in ether-functionalized ILs, covalent immobilization was seen to improve the stability of enzymes. The high selectivity and protease activity in ether-functionalized ILs were explained by means of the cations and chitosan helping to control the water activity of reactions and, the protective role of the ether chain in cations to reduce the cation- protein interaction.

Xiao et al. (2008) [83] have studied the immobilization of lysozyme onto morphologically different silica materials: hollowsilica nanotubes, hollowsilica nanospheres and solid silica nanoparticles. The prepared silica materials were characterized by TEM, FTIR and BET. The amount of immobilized lysozyme on solid silica nanoparticles, within hollowsilica nanotubes and hollow silica nanospheres was 186 mg/g silica, 351 mg/g silica and 385 mg/g silica, respectively. The hollow silica nanospheres achieved the highest immobilization percent among the three types of silica supports. It was observed that large pore size and good hollow structure of silica materials resulted in the increase of the percent immobilization of large molecules, such as lysozyme, within silica materials. The airtight structure of hollow silica nanospheres is an important phenomenon which has two different effects on enzymatic immobilization. However, the relatively airtight structure of hollow silica nanospheres is more useful for the molecules of lysozyme to be captured and restrained into the void space of silica nanospheres, this structure affected the immobilized lysozyme activity negatively via limiting diffusion/mass-transfer processes upon substrates entering in and products flowing out through the shell of hollow silica nanospheres.

Tang et al. (2006) [58] have studied the immobilization of neutral proteinase on chitosan nano-particles prepared by ionization gelation method. The highest enzyme activity of immobilized enzyme was achieved at pH 7.5, identical to that of free enzyme, but the range of the optimum pH was extended. The optimal temperature of immobilized enzyme was 55°C, and the range of the optimum temperature was also extended. The thermal, operational and storage stabilities of immobilized enzyme were better for immobilized enzymes on chitosan nanoparticles.

Kasavi and Güvenilir (2006) [76] studied the immobilization of protease enzyme on chitin and chitosan by covalent binding method at 30ºC and also on alginate and zeolite by physical adsorption method at 40ºC. Chitin and chitosan were treated with glutaraldehyde for cross-linking. In both methods, changes in the optimum conditions and stabilities of the enzyme, effects of immobilization time, reusability and the compatibility with detergents were investigated. It was observed that the immobilization yield was directly affected with the immobilization time. The highest immobilization yield was found with chitin by covalent binding method. The immobilized enzyme retained 80% of total activity compared with the soluble enzyme. Immobilized protease was compared of the pH and temperature activity profiles with the soluble enzyme and it was found that the pH or temperature optima of immobilization of the enzyme are different from that of soluble enzyme. The optimum temperature for catalytic activity of the immobilized protease on chitin and alginate 60ºC and 40ºC, respectively, while that of free protease 50ºC. Relative activities of the immobilized and free protease were studied and optimum pH of the immobilized enzyme on chitosan was found. The activities of immobilized enzyme for each pH values studied were higher than the free enzyme. Optimum pH values of the immobilized enzymes on alginate, chitin and zeolite were found as 10.0, 11.5 and 8.4, respectively.

Wang and Caruso (2005) [84] examined mesoporous silica spheres as support matrix for enzyme immobilization. A variety of enzymes with isoelectric points (pI) and different molecular sizes (e.g., lysozyme, peroxidase, cytochrome C and catalase) were examined in mesoporous silica particles with different pore sizes. The results of MS spheres with a bimodal mesoporous structure (BMS, 2-3 nm and 10-40 nm) shown that higher immobilization rates and significantly extended enzyme immobilization capacity than similar particles with smaller mesopores. Rapid uptake (several minutes) and high percent enzyme loading values (20-40 wt %) were observed in BMS spheres for enzymes with pI $\geq$ 10 and a molecular size $\leq$ 3 nm (e.g., cytochrome C, lysozyme and protease) while ~8 wt % for larger enzymes such as catalase.

Pandya et al. (2005) [85] have studied the immobilization of α-amylase in ordered mesoporous silicas for hydrolysis of starch. For meso-cellular foams (MCF) which have higher pore diameter than mesoporous silica particles, enzyme seemed to be immobilized inside the pores and high specific activity increase was observed. In fact, MCF-335 shows

specific activity equal to 80% of the specific activity of free enzyme. Thermal and pH stability of the immobilized enzyme was examined as higher compared to free α-amylase.

Ferreira et al. (2003) [86] studied covalent immobilization of Alcalase 2T, a commercial preparation of Subtilisin Carlsberg, onto physiochemically characterized-silica supports. The effects of surface chemistry and mean pore diameter on catalytic activity with casein substrate were studied. Two sets of chemically functionalized silica supports were tested presenting terminal amino (S_{APTES}) or hydroxyl groups ($S_{TESPM\text{-}pHEMA}$). The percent of protein loading was smaller in S_{APTES} (31–39%) than that of $S_{TESPM\text{-}pHEMA}$ (62–71%), but higher catalytic activity was observed for S_{APTES} than $S_{TESPM\text{-}pHEMA}$. Large pores provided higher specific activities to silica matrices than smaller pore sizes. Intraparticle diffusion limitations have not been observed for the immobilized enzyme. The effects of pH, temperature, storage time and reuse of immobilized enzyme and free enzyme were examined.

The main findings and the experimental conditions of the studies mentioned above about the immobilization process of subtilisin onto various matrices or other enzymes immobilized onto silica-based matrices were summurized in Table 3.1.

Table 3.1 Literature survey for the immobilization of enzymes onto polymer-coated MSNs

Reference	**Enzyme**	**Enzyme Amount**	**Matrix**	**Matrix Amount (mg)**	**Duration (h)**	**$T_{immobilization}$ (°C)**	**$pH_{immobilization}$**	**$T_{optimal}$ (°C)**	**$pH_{optimal}$**
Lee et al. (2015) [77]	β-glucanase	1 g	silica	1000	24	25	4	40	not examined
Soleimani et al. (2012) [79]	Protease	1.5 mg/mL	silica nanoparticles	7.5	1	25	4.5	40-50	not examined
Murai et al. (2011) [80]	Subtilisin	1 mg / 900 µl	functionalized-MSNs	3	12	4	7	not examined	not examined
Singh et al. (2011) [81]	Procerain B	0.2 mg/mL	chitosan beads	not specified	20,24	4	8.5	55	3 and 6
Zhao et al. (2010) [82]	Subtilisin Carlsberg	0.1 g	chitosan	1000	3	25	7.4	not examined	not examined
Kasavi and Güvenilir (2006) [87]	Protease	400 U	chitosan	400	2	30	7.2	60	9.5
		400 U	chitin	400	2	30	7.2	60	9.5
		100 U	alginate	100	16, 20, 24	4	7.2	40	10
Tang et al. (2006) [58]	Alcalase 2T	1 mg	chitosan nanoparticles	1	2	40	7.5	55	7.5

Table 3.5 continues

Reference	**Enzyme**	**Enzyme Amount**	**Matrix**	**Matrix Amount (mg)**	**Duration (h)**	**$T_{immobilization}$ (°C)**	**$pH_{immobilization}$**	**$T_{optimal}$ (°C)**	**$pH_{optimal}$**
Wang and Caruso (2005) [84]	Protease	0.5 mg/mL	siliceous particles	10	not specified	25	7	not examined	7.5
Pandya et al. (2005) [85]	α-amilase	0.5 mg	functionalized-MSNs	1000	5	25	6.6	30	6
Ferreiara et al. (2003) [86]	Subtilisin Carlsberg	0.449 mg	silica	50	18	25	8	60	8

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

Chitosan (Coarse ground flakes and powder) with the deacetylation degree >75% was purchased from Sigma-Aldrich. Acrylic acid (AA) (Sigma-Aldrich) was distilled under reduced pressure before use. Tetraethyoxysilane (TEOS), cetyltrimethylammonium bromide (CTAB), sodium hydroxide (NaOH) and glutaraldehyde were obtained from Sigma-Aldrich. Subtilisin (Alcalase®) enzyme produced from *Bacillus licheniformis* was obtained from Novozymes.

4.2 Preparation of Mesoporous Silica Nanoparticles

Mesoporous silica nanoparticles (MSNs) were prepared according to the sol–gel method [88, 89]. Briefly, 2 g CTAB and 0.56 g NaOH were mixed in 960 mL deionized water. The solution was stirred vigorously at 80°C for 30 minute, and 10 mL TEOS was added to be stirred for another 2 h. Then the as-prepared product (MSN-CTAB) was washed with ethanol for 3 times and redispersed in ethanol solution of NH_4NO_3 (800 mL, 10 mg/mL) at 80°C to remove the organic template of CTAB. The mixture was refluxed for 6 h, collected by centrifugation and washed with ethanol repeatedly. Finally, the obtained MSN sample was dried and white powder was obtained. Figure 4.1 shows the glass reactor system used for the synthesis reaction.

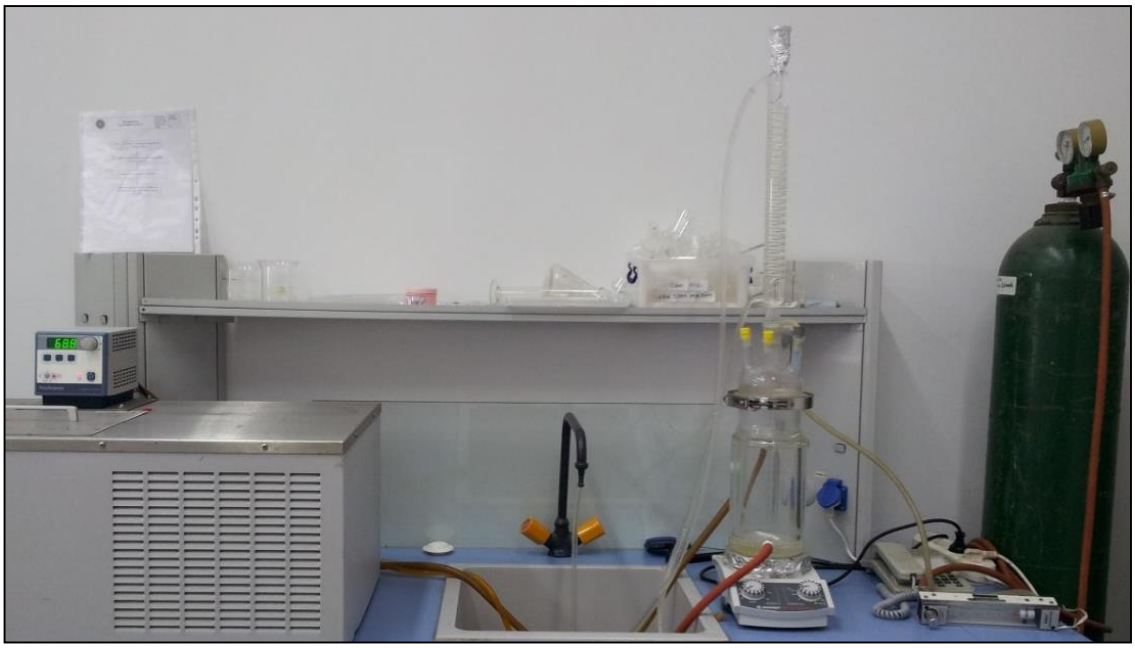


Figure 4.1 The glass reactor system used for MSN synthesis and polymer coating

4.3 Preparation of Polymer-coated Mesoporous Silica Nanoparticles

For the methodology, the study of Tang and First (2011) [51] was taken as a reference. They used a chitosan/poly (methacrylic acid) as polymer coating layer for MSNs. 0.215 g CS was dissolved in 30 mL aqueous solution of 190 µl AA (the molar ratio of 1:2; [glucosamine unit]:[AA]). Then, 0.16 g MSN was added to the mixture and sonicated for 20 min by ultrasonic probe. The solution was stirred under nitrogen environment for 0.5 h while being heated to 80°C. When the temperature became constant, the initiator of the polymerization reaction (Potassium persulfate: $K_2S_2O_8$) was added to the reaction mixture (concentration of KPS in the mixture system was about 4.0×10^{-3} mol/L).

The reaction was continued at 80ºC under a nitrogen atmosphere for another 2 h. During polymerization reaction, white color of the reaction mixture turned to light yellow. At the end of 2 h, the temperature of the system was lowered to 50ºC. After the system reached a stable condition, 0.1 mL glutaraldehyde (0.25 wt %) was added to the system to start crosslinking reaction. The crosslinking reaction was continued for 2 h, and the color of the reaction solution became dark yellow. The sample was separated from solution by centrifugation. Finally, obtained sample was washed by deionized water for three times.

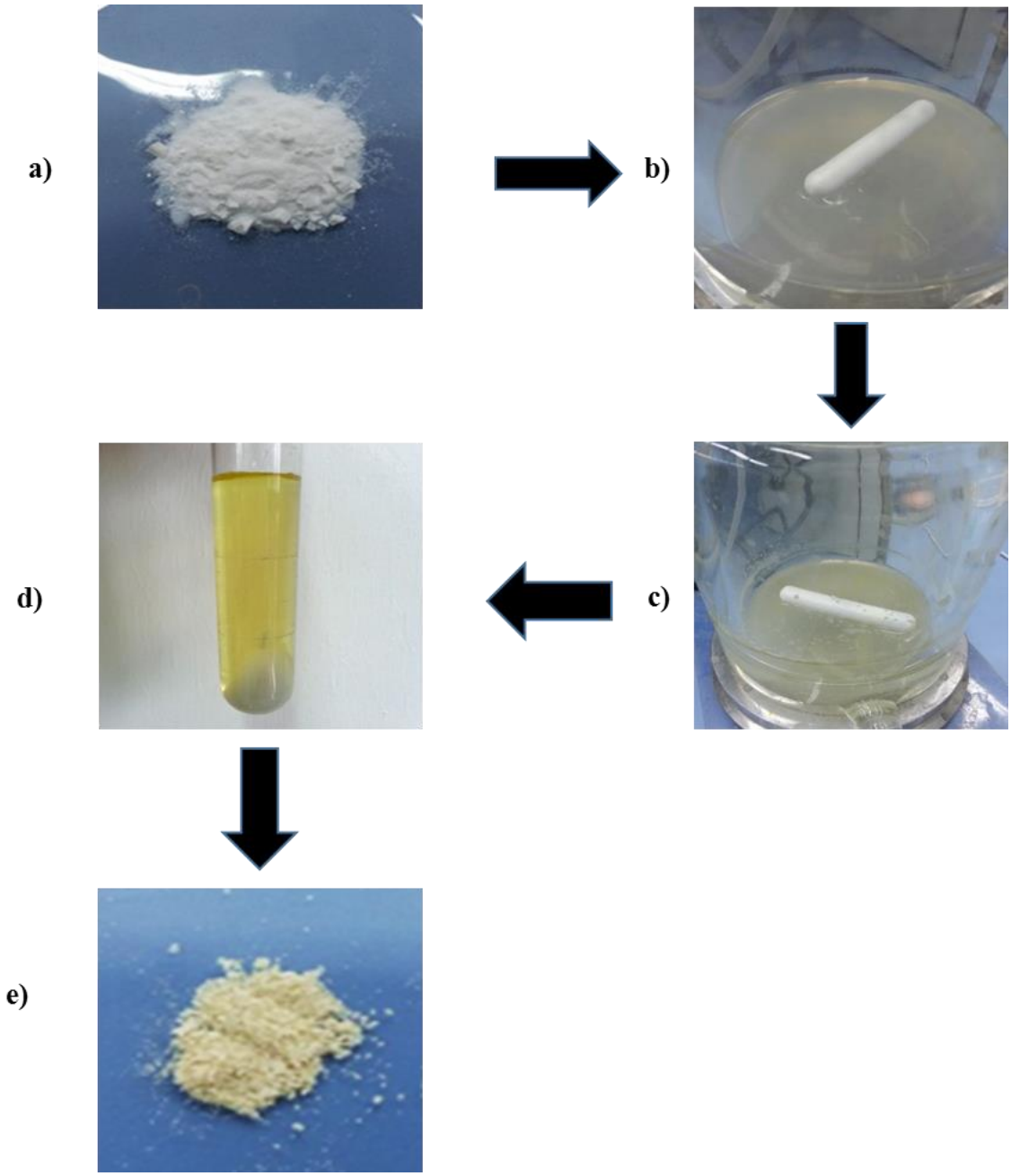


Figure 4.2 The steps of the coating process of MSNs a) MSNs, b) After mixing AA+CS+MSNs via sonic treatment (milk white color), c) After polymerization reaction (light yellow color), d) Cross-linking before washing step, e) Dried PAA/CS-MSNs

4.4 Characterization of MSNs and CS-PAA/MSNs

For the characterization of the uncoated and polymer-coated mesoporous silica nanoparticles, the following devices were used;

- Transmission electron microscopy (TEM) images were obtained using a device called as JEOL 2100 JEM HRTEM (High resolution transmission electron microscope) at TUBITAK (The Scientific and Technological Research Council of Turkey) Materials Institute.
- The mean size and size distribution of the particles were measured via dynamic light scattering by zetasizer (Malvern, Zen 3600) in aqueous solution at pH 4.5 and 7.4.
- The zeta potential of the particles was measured on a Zetasizer Nano-ZS (Malvern) at 25^0C.
- FTIR spectra were measured on a Perkin Elmer-Spectrum 100.
- Thermogravimetric analysis of the particles was measured on SII Nanotechnology-SII6000 Exstar TG/DTA 6300 with a heating rate of 20^0C/min at a nitrogen environment.

4.5 Enzyme Immobilization Studies

4.5.1 Analytical Methods

4.5.1.1 Protein Determination by Folin-Lowry Method

Folin-Lowry analytical method, firstly defined by Lowry [90] in 1951, is often used for the determination of protein concentration. This method based on the color change involves two steps:

1. Reduction with Cu^+ (copper) in alkali via the complex formed between copper ion and amide.

2. Folin-Ciocalteu reagent reducted with tyrosin and triptophane reagents. Folin-Ciocalteu gives blue color.

Protein amount is dedected by reading UV absorption values of color strength of reducted Folin-Ciocalteu reagent at 750 nm wavelength by Shimadzu UV-1800 spectrophotometer.

i. Materials

A reagent: 20 g Na_2CO_3 was dissolved in 1 L of 0.1 N NaOH.

B reagent: 0.5 g $CuSO_4.5H_2O$ was dissolved in 100 mL of 1% (w/v) sodium tartrate.

C reagent: Just before analyse, prepared by mixing 100 mL of A reagent and 2 mL of B reagent.

D reagent: Prepared by diluting Folin-Ciocalteu Lowry reagent with the ratio of 1:1 using deionized water. It is kept in a dark colored bottle at +4ºC.

Potassium phosphate buffer: For 25 mM phosphate buffer at pH 7, 2.105 g KH_2PO_4 and 2.174 g $K_2HPO_4.3H_2O$ were dissolved in 900 mL pure water. Then, pH value was adjusted with 5 N KOH and completed by adding distilled water to 1000 mL.

ii. Procedure

After immobilization process, the immobilized enzymes were separated by centrifuge from free enzymes. Lowry method was used to determine the amount of free enzymes. The sample was taken from supernatant and diluted with phosphate buffer. Then, 5 mL of C reagent was added to 1 mL sample and incubated for 10 minutes at 25°C. After 10 minutes, 0.5 mL of D reagent was added and incubated for another 30 minutes at 25°C. Finally, absorbance value was read at 750 nm by Shimadzu UV-1800 spectrophotometer. Blank sample was prepared by using 1 mL buffer instead of 1 mL sample.

iii. Calculations

For determination of the enzyme concentrations using the absorbance values, the calibration curve was prepared using Bovine Serum Albumin (BSA). BSA was dissolved in phosphate buffer at different concentrations in order to use as standart sample. The absorbance values versus BSA concentrations were read at 750 nm by Shimadzu UV-1800 spectrophotometer. Then, the data obtained were used to draw a graph which was

shown in Figure 3.3 (Table A.1 in the Appendix). The equation obtained for the calculation of protein concentration was given as below:

C (g/L) = 0.4212 x (Absorbance value)

The statistical values, regression coefficient (R^2) and standart deviation (σ) were calculated as 0.9867 and 0.0427, respectively.

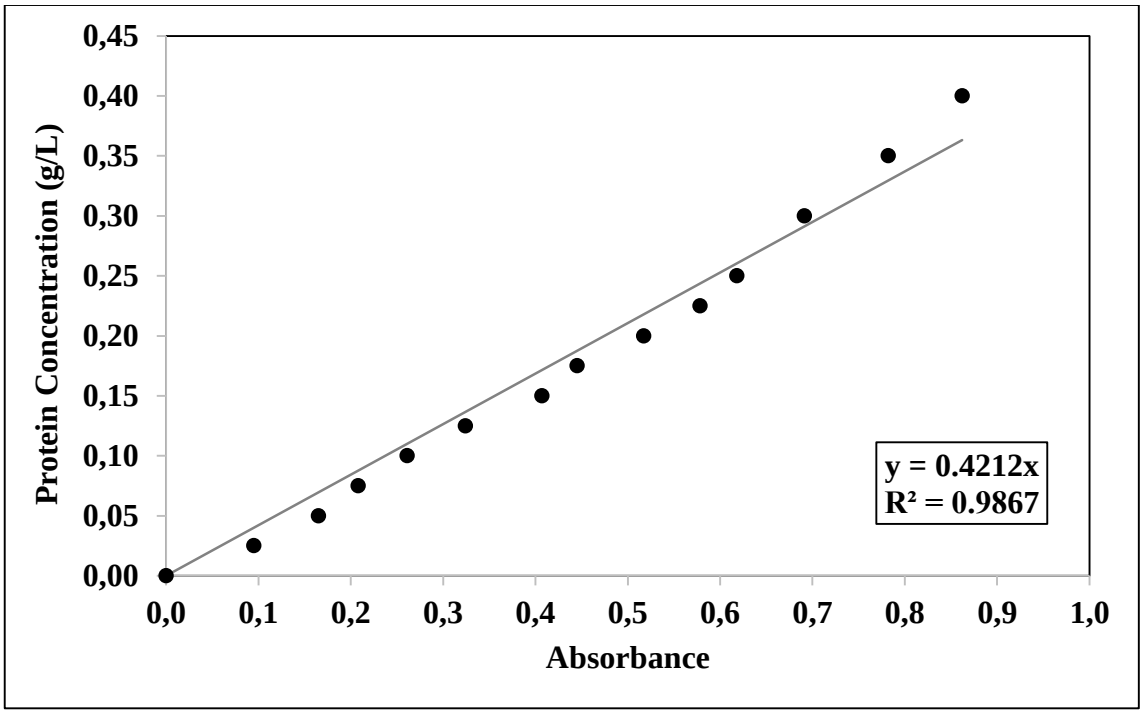


Figure 4.3 The calibration curve for Folin-Lowry method

4.5.1.2 Enzyme activity assay

Azocasein is a modified protein with orange colored sulfanilamide groups. Enzymatic activity of protease was determined via spectrophotometric analysis by means of the density of orange color existing as a result of the reaction which breaks azocasein into sulfanilamide groups at the end of the incubation. The procedure modified by Tomarelli et al. (1949) [91] was used for the enzyme activity assay.

i. Materials

- Sodium phosphate buffer

For preparation of 0.1 M pH 7.4 buffer, 1.56% (w/v) $NaH_2PO4.2H_2O$ solution was added to 1.78% (w/v) $Na_2HPO_4.2H_2O$ (v:v; 1:4) solution slowly by controlling pH.

- Azocasein solution

Azocasein was dissolved in 0.1 M phosphate buffer at 7.4 pH.

- TCA (Trichloroacetic acid) solution

10% (w/v) solution was prepared by dissolving TCA crystals in distilled water.

- NaOH solution

1M solution was prepared by dissolving 4% (w/v) NaOH in distilled water.

ii. Procedure

Immobilized enzyme and free enzyme (both of them were kept at same protein content) were diluted with phosphate buffer (pH 7.4). Then, 1.5 mL of 1% azocasein solution (substrate) was added to enzyme solution to start the reaction at 40°C. Incubation was ended with the addition of 2 mL of 10% TCA solution after 10 minutes. Samples were kept at room temperature for 10 minutes before centrifuge. Then, 2 mL sample were diluted with 9 mL of NAOH solution. The absorbance values were read at 440 nm by Shimadzu UV-1800 spectrophotometer. The blank sample was prepared by the same method, but only TCA solution was added before azocasein. The measurements were repeated for 3 times, and the average of the data was taken.

iii. Calculations

The calibration curve was prepared by reading absorbance values at different enzyme units. The samples with different enzyme concentrations were reacted with azocasein. The same procedure with enzyme activity assay was followed. The absorbance values were read at 440 nm by Shimadzu UV-1800 spectrophotometer. The blank sample was prepared by the same method, but only TCA solution was added before azocasein. The measurements were repeated for 3 times, and the average of the data was taken. Then, the data obtained were used to draw a graph which was shown in Figure 3.4 (Table A.2 in the Appendix). The equation obtained for the calculation of enzyme activity versus absorbance value was given as below:

Enzyme activity (Au/L) = 3.2894 x (Absorbance value)

The statistical values, regression coefficient (R^2) and standart deviation (σ) were calculated as 0.9885 and 0.0399, respectively.

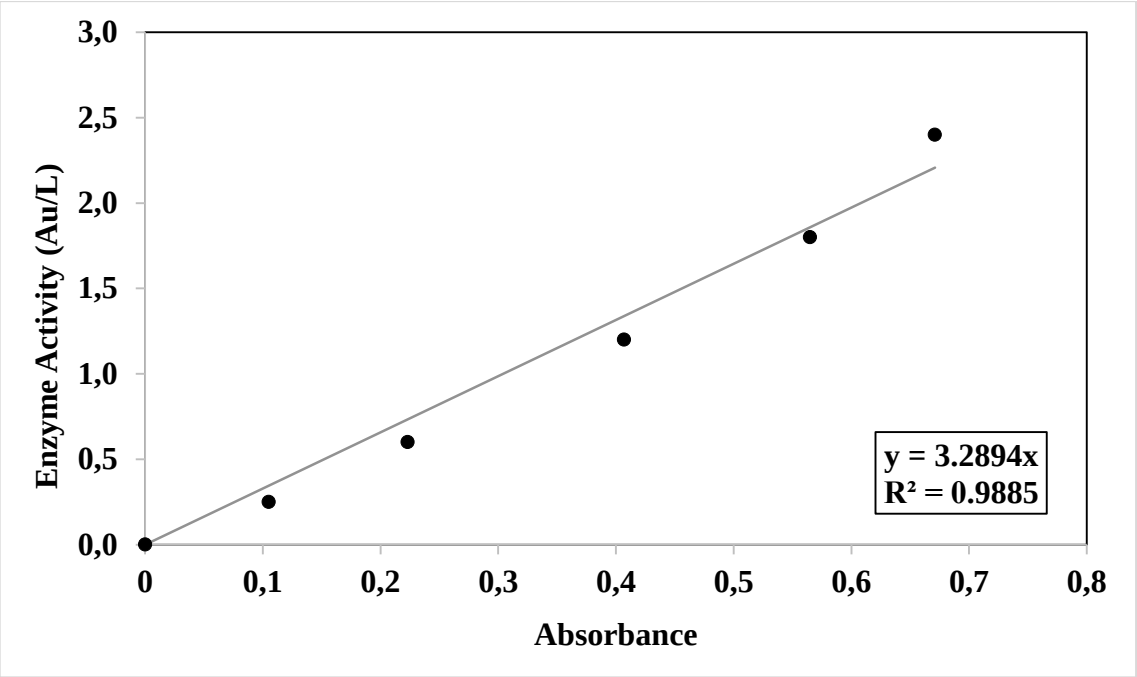


Figure 4.4 The calibration curve for enzyme activity assay

4.5.2 Experimental Procedure of Enzyme Immobilization

4.5.2.1 Preparation of Immobilized Enzyme onto Polymer-coated MSNs

Subtilisin is the key player as a detergent additive to remove protein deposits and stains. Since the cleaning industry is a big market, this enzyme was chosen as model enzyme to investigate the immobilization to polymer-coated MSNs.

Subtilisin immobilized polymer-coated MSNs were prepared by dissolving subtilisin in 2 mL of buffer solution, and then well-dispersed particles (10 mg) in 2 mL buffer solution were added to enzyme solution (0.2 mg/mL). The final volume of the immobilization solution was 4 mL. The experiments were carried out at various pH values for different time intervals at the temperature of 25°C and a stirring rate of 250 rpm. In order to optimize the immobilization process, the effects of pH, matrix amount and immobilization time to the percent enzyme loading were investigated.

After the immobilization step, the particles containing the enzyme molecules were washed with distilled water, and then used for the further studies. Then, the effects of pH, temperature, storage time and repeated use on immobilized enzyme activity were investigated.

4.5.2.2 Effect of pH on Immobilized Enzyme Activity

Influence of pH on activity was determined by carrying out the reaction at various pH values in the range of 5-10 and keeping all other reaction conditions constant.

4.5.2.3 Effect of Temperature on Immobilized Enzyme Activity

Influence of temperature on activity was determined by carrying out the reaction at various temperature values in the range of 30-80^{o}C and keeping all other reaction conditions constant.

4.5.2.4 Effect of Repeated Use on Immobilized Enzyme Activity

To investigate the operational stability of subtilisin immobilized on CS-PAA/MSNs, the activities were examined after each repeated use (operational stability). Then, the relationship between the enzyme activity and the number of repeated use were obtained.

4.5.2.5 Effect of Storage Time on Immobilized Enzyme Activity

For determination of the storage stability of the subtilisin, immobilized enzyme samples were kept at 4ºC for 30 days. The activity of immobilized enzyme was measured every week, and then the relative activity values were calculated to examine the activity loss of stored enzyme.

CHAPTER 5

RESULTS AND DISCUSSION

For an efficient enzyme immobilization, the surface functionalized particles with reactive groups are required to increase the enzyme load and stability. Natural and synthetic polymers are commonly used for the functionalization of mesoporous nanoparticles. In the present study, in-situ polymerization method used for coating mesoporous silica nanoparticles, chitosan was firstly dissolved in an acrylic acid solution under magnetic stirring. Due to the electrostatic interaction, the negatively charged acrylic monomers align along the chitosan molecules. After complete dissolution of chitosan, the polymerization was initiated by $K_2S_2O_8$ at 80°C. The complete polymerization leads to the appearance of an opalescent solution, indicating that the nanoparticles formation [60, 62].

The structure and chemistry of MSNs were examined as well as the changes occurred after the coating process of MSNs with the polymer blend of poly(acrylic) acid and chitosan. The important properties such as morphologic pattern, mean size, size distribution, surface charge of the nanoparticles, and thermogravimetric stability were characterized by using SEM, TEM, Zetasizer and TG-DTA analyses.

Subtilisin was chosen as a model enzyme to test the particles as immobilization matrix. Becaues subtilisin is a key player as a detergent additive to obtain main characteristics, such as stability and activity in alkaline pH range and extreme temperatures, compatibility with detergent components such as surfactants, perfumes, and bleaches [25]. Immobilization parameters have been optimized to maximize the percent enzyme loading to the particles. The effects of the parameters such as temperature, pH, processing time,

repeated use (operational stability) and storage time on the enzyme activity were investigated.

5.1 Characterization of MSNs

Mesoporous silica nanoparticles (MSNs) were prepared according to the sol–gel method and chemical structure was determined via FTIR spectra, porous structure and molecular size was analysed with SEM and TEM micrographs.

5.1.1 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

As can be seen in Figure 5.1, the characteristic absorption band was found to appear at 1060 cm−1, which could be assigned to the absorption of Si–O in the spectrum of MSN. C–H stretch (2854 cm^{-1} and 2923 cm^{-1}) peaks from CTAB surfactant disappeared after the CTAB removal (reflux section) process, which showed that no residual CTAB existance in the structure of MSNs, so the effect of the toxicity from organic template was eliminated as pointed in the study Tang et al.(2011) [51].

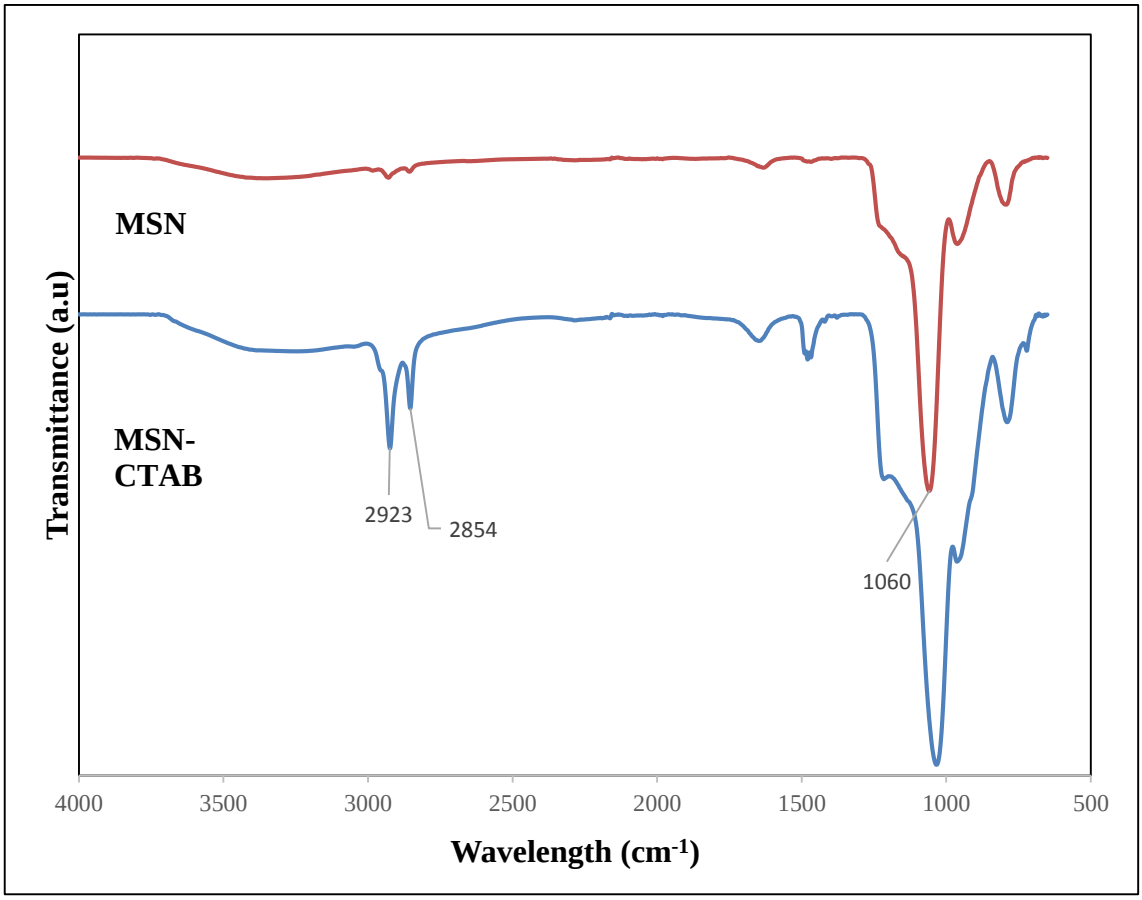


Figure 5.1 FTIR spectra of MSN and MSN-CTAB

5.1.2 Scanning Electron Microscope (SEM) Analysis

Figure 5.2 shows that uniform-mesoporous silica nanoparticles were synthesized in the range 60-100 nm diameter with the mean value 80±10 nm.

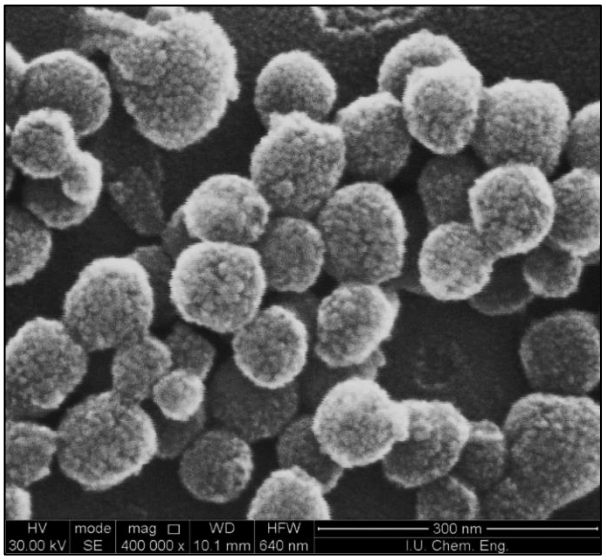


Figure 5.2 SEM image of MSNs

5.1.3 Transmission Electron Microscopy (TEM) Analysis

The porous structure of the nanoparticles can also be seen clearly from the micrograph given in Figure 5.3.

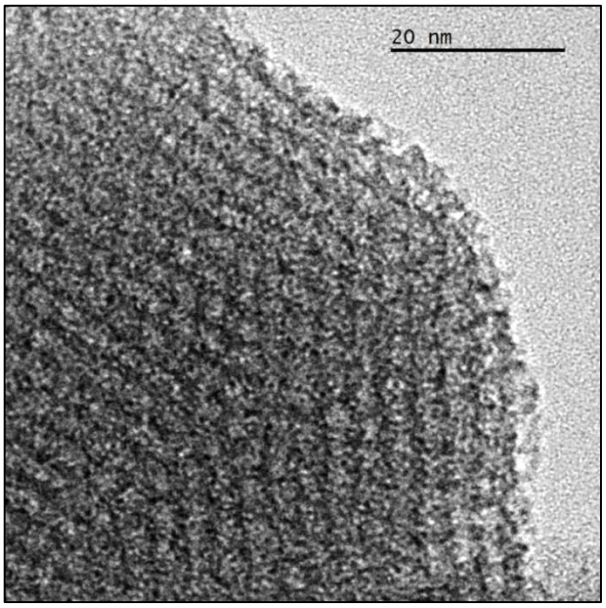


Figure 5.3 TEM image of MSNs

5.2 Characterization of CS-PAA/MSNs

5.2.1 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

As can be seen from Figure 5.4, chitosan revealed typical peaks of 1595 cm^{-1} (amide II) and 1375 cm^{-1} (amide III) [92]. In the spectrum of CS-PAA/MSNs, when compared with those of CS and MSN, the characteristic absorption bands were found to appear at 1710 cm^{-1} , 1629 cm^{-1} and 1060 cm^{-1}, respectively, which could be assigned to the absorption of carboxyl groups of PAA [93], protonated amino groups of CS [94], and Si–O of MSN [95].

The bands at 1537 and 1406 cm^{-1} could be assigned to asymmetric and symmetric stretching vibrations of COO– anion groups, which verified that PAA were dissociated into COO– groups and complexed with CS through electrostatic interaction to form polyelectrolyte complexes during the polymerization. Similar reaction was achieved by Tang et al. (2011) [51] with chitosan and poly(methacrylic) acid.

To investigate the structural difference between CS-PAA and CS-PAA coated MSNs, CS and AA was mixed without MSNs and gel formation was observed. FTIR spectrum of this gel structure has lack of the peak at 1060 cm^{-1} (absorption of Si–O of MSN) in contrast to that of CS-PAA/MSN (Figure 5.5).

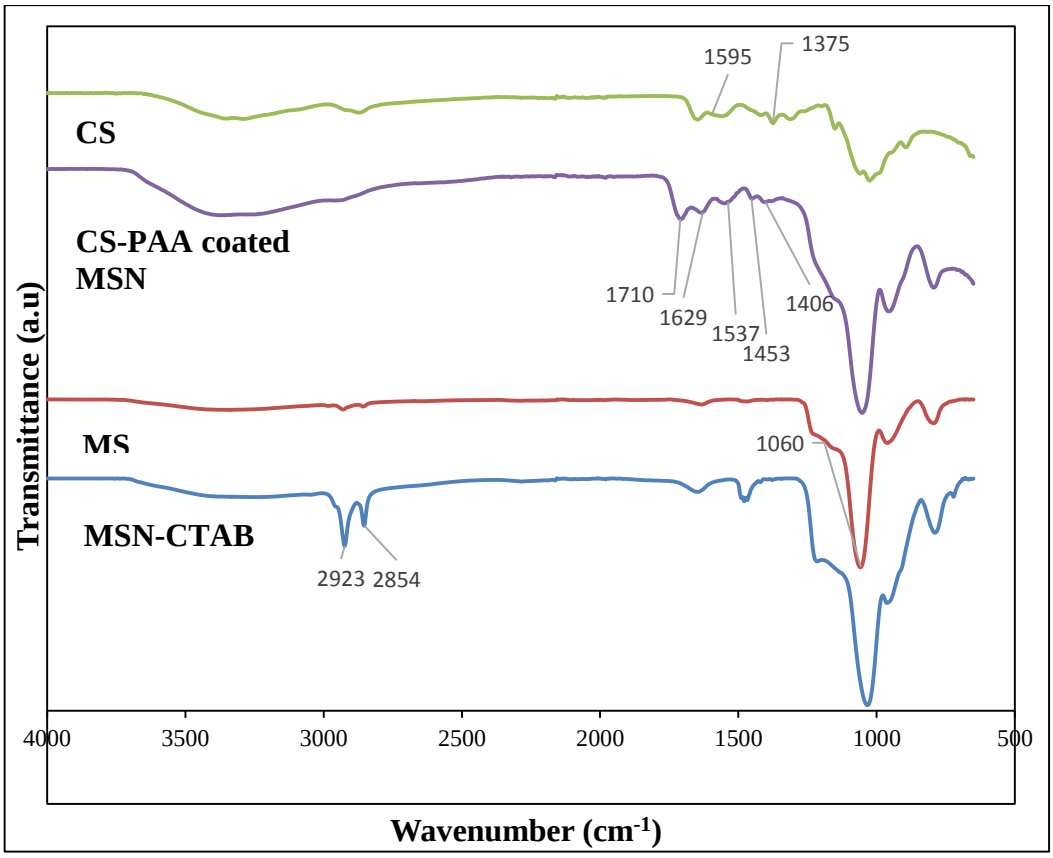


Figure 5.4 FTIR spectra of MSN-CTAB, MSN, CS-PAA/MSN, and CS

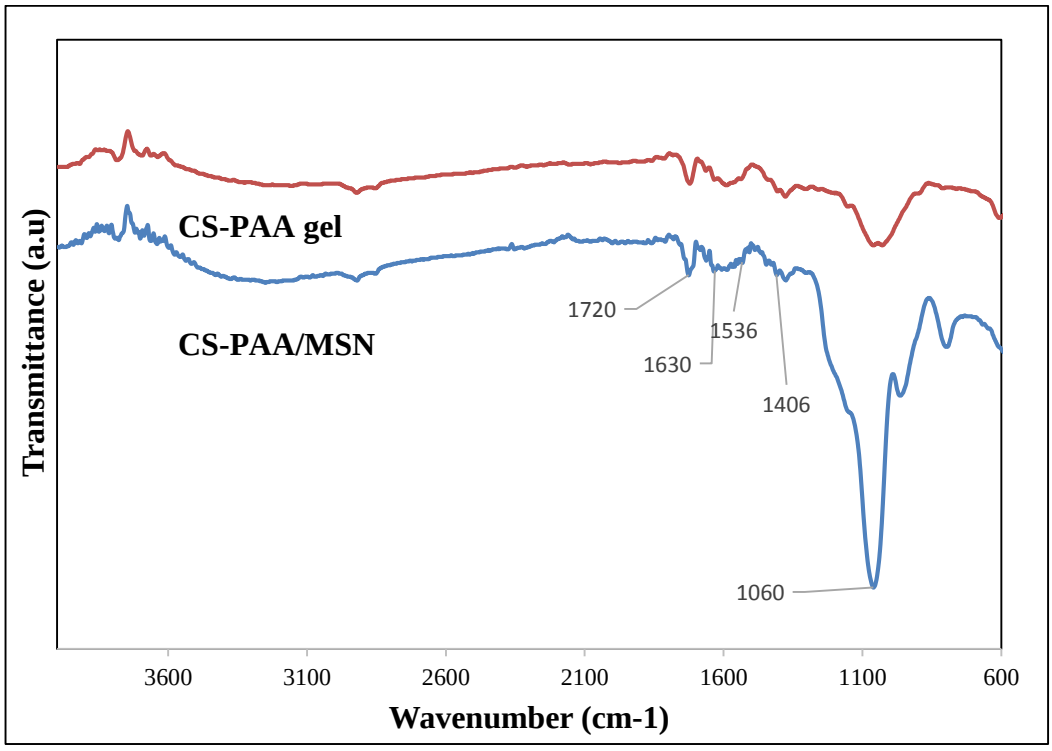


Figure 5.5 FTIR spectra of CS-PAA gel and CS-PAA/MSN

5.2.2 Transmission Electron Microscopy (TEM) Analysis

Figure 5.6 shows the TEM images of MSNs and CS-PAA/MSNs. It can be seen from Figure 5.6a that MSNs have uniform-porous-spherical structure with a diameter of ~100 nm. When the polymerization occurred on the surface of MSNs, porous surface was covered by a smooth-gray outer layer which proving that the presence of organic moieties and, the diameter was increased to the range of 100-200 nm. The shell layer seemed to cause the uniform spheres to lose their standart shape and size, as can be seen from Figure 5.6b, the particles stick together with irregular shape. Pore size can be read as 4-5 nm from TEM micrographs.

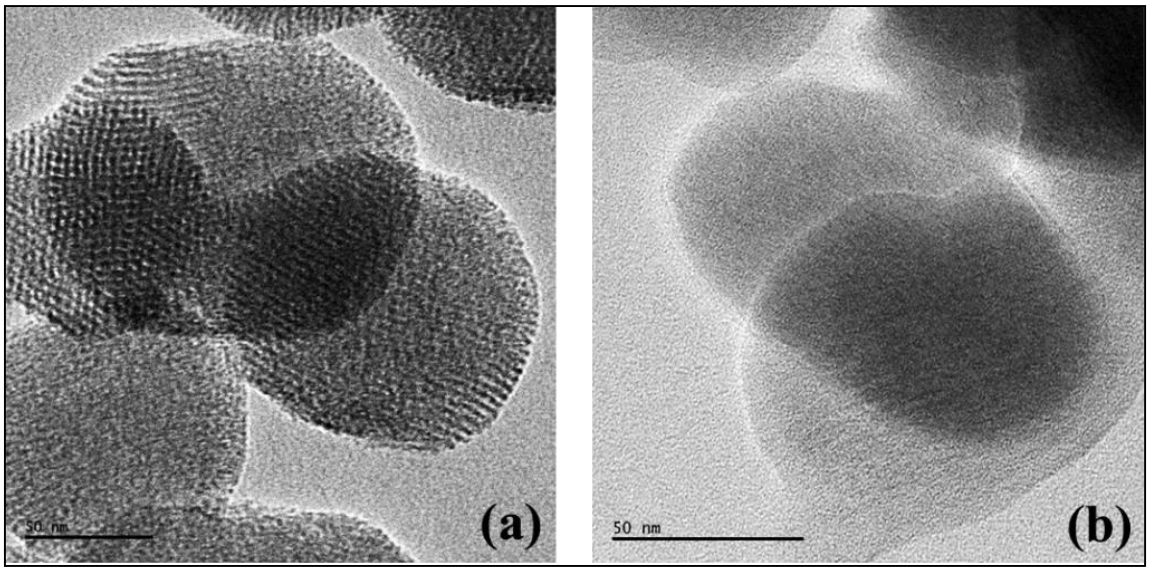


Figure 5.6 TEM micrographs of a) MSNs, and b) CS-PAA/MSNs

5.2.3 Thermogravimetric Analysis (TGA)

CS-PAA coated MSN composite particles were measured through the TGA runs in the condition of nitrogen atmosphere at the heating rate of 20°C/min. The TGA curves at Figure 5.6 shows that the weight loss of MSN was 16.6%, while that of CS-PAA/MSN was 28.8%. The weight loss of pure chitosan is 68.9%. Thus, the content of CS-PAA blend was about 23.4 wt% in CS-PAA/MSNs. For CS-PAA/MSNs, the weight loss below 200°C is quite small (7%) because of the removal of absorbed physical and chemical water. It was seen that the structure of CS-PAA begin to decomposite from ~230 to 500°C, and there is no significant weight loss from 500 to 900°C as also found in the study of Guo et al. (2010) [96].

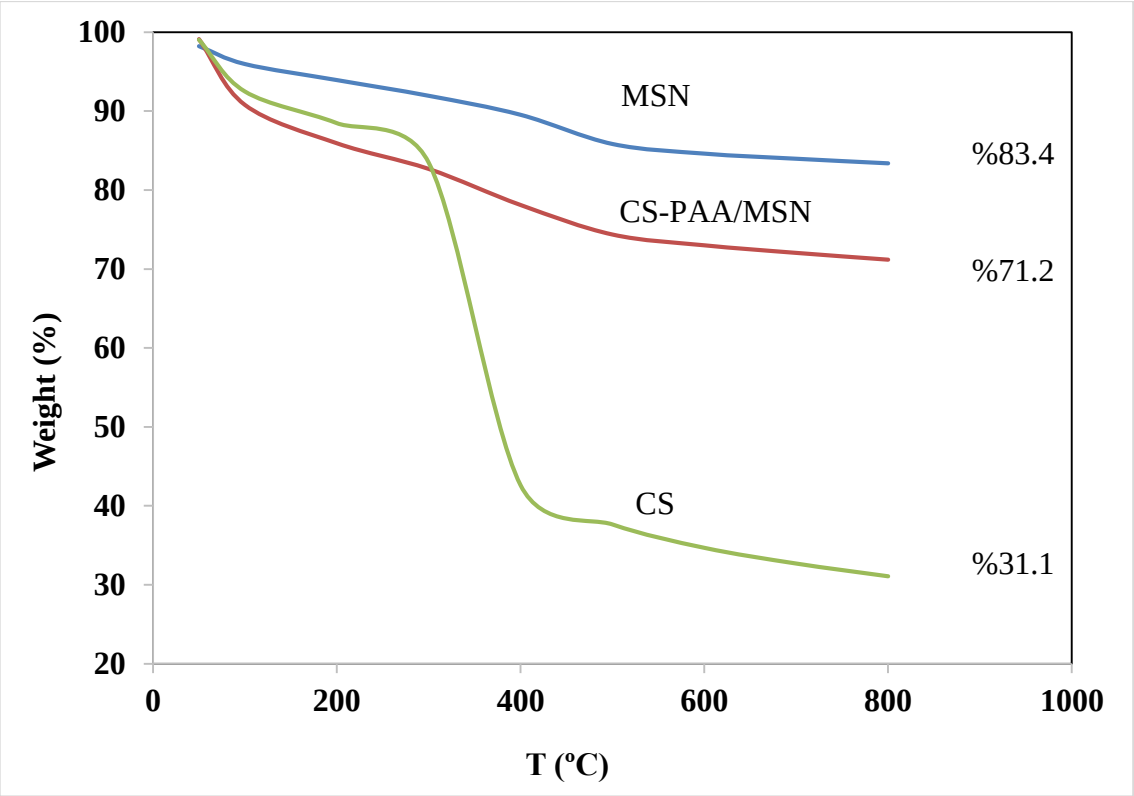


Figure 5.7 TGA curves of MSN, CS-PAA/MSN, and CS

5.2.4 Zeta Potential Analysis

Zeta Potential analysis is a technique for determining the surface charge of nanoparticles dispersed in solutions (colloid). Nanoparticles have a surface charge that pulls toward a thin layer of ions of opposite charge to the nanoparticle surface. This attraction causes the formation of a double-layer of ions on the surface. This double layer moves with the nanoparticle as it spreads throughout the solution. The electric potential at the boundary of the double layer is known as the Zeta potential of the particles and has values that typically between +100 mV and -100 mV [97].

As pointed in the thesis study of Malkoç (2011) [98], silica nanoparticles have high colloidal stability in aqueous medium. Thus, it can be seen that the hydroxyl groups in silica shifts zeta potential of MSNs to negative region at neutral pH.

The effect of pH values on the zeta potential of the CS–PAA/MSNs was plotted in Figure 5.8 (Table A.3 in the Appendix). The changes in the zeta potential values with pH change of the medium are an image of the charge density of the particles. The zeta potential of CS–PAA/MSNs decreased as the pH value increased from 4.5 to 7.4 from -11.3 to -26.4

mV for 2:1 ratio of PAA:[NH_2] because of the decrease of CS ionization and increase of PAA ionization. When PAA/[NH_2] ratio was increased, zeta potential decreased at both pH values due to the increasing PAA amount in the polymer layer. The more PAA amount means the more ionization and the more negative charge density.

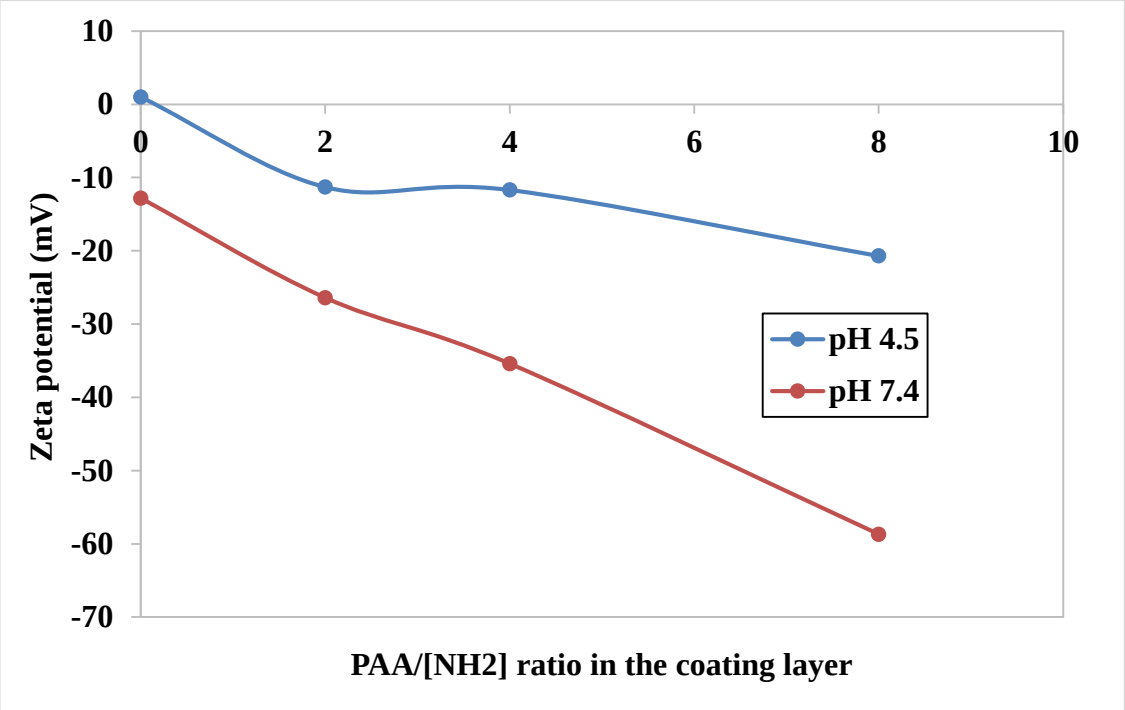


Figure 5.8 The effect of PAA/[NH_2] ratio to the zeta potential at various pH values (ratio 0 nominates MSN without coating)

The pKa value determines the effect of change in pH to the structure of polymer via affecting the degree of ionization. PAA becomes negatively charged when the pH of the solvent is above its pKa 4.7 due to the ionization of its -COOH groups and it has poor solubility at low pH values. The intrinsic pKa of the CS has been reported as 6.5 in the study of Ahn et al. (2001) [99].

Above pH 6.5, –COOH from PAA dissociates and –NH_2 from chitosan deprotonates, increase of pH leads to an increase of the ionization degree and charge density of the PAA molecules, which would reduce the electrostatic interactions and molecular linkages including hydrogen bounds between the CS and PAA chains, resulting in the increase of hydrodynamic size and the decrease of zeta potential simultaneously. Below pH 6.5, –NH_2 from chitosan would be protonated. With the decrease of pH value, the electrostatic repulsion of the protonated amino groups of the CS chains extends, the polymer layer

swells and the zeta potential increases which is consistent with the foundings of the study of Tang et al. (2011) [51].

Hydrodynamic diameter values are greater than that of the determined values from TEM images because of the hydrate layer formation on the surface of the particles in aqueous environment as also pointed in the study of Yuan et al. (2011) [75].

The effect of pH values on the particle size of the CS–PAA/MSNs was shown in Figure 5.9 (Table A.4 in the Appendix). As can be seen from this figure, the particle size increased from 550 to 1578 nm for 2:1 ratio of PAA:[NH_2] with decreasing the pH value from 7.4 to 4.5 due to the extending polymer layer. Linear polymer chains of PAA is known to perform as pH-sensitive gatekeeper when grafted onto the surface of MSNs [65].

The polymer chain is open at high pH value by means of extending polymer chain. As mentioned above, the polymer layer below pH 6.5 swells progressively due to electrostatic repulsion of the protonated amino groups of the CS chains. Thus, since 4.5 is closer value than 7.4 to the pKa value of 6.5, the diameter values are bigger at 4.5 due to swelling CS. It can be estimated that if pH would be higher than 9-10, the values of hydrodynamic diameter were close to those of at pH 4.5. When PAA/[NH_2] ratio was increased, hydrodynamic diameter was also increased at both pH values by means of increasing PAA amount in the layer.

According to the findings mentioned above, CS–PAA/MSNs were pH-sensitive which means the surface of MSNs could have positive or negative charges due to the change of the medium pH.

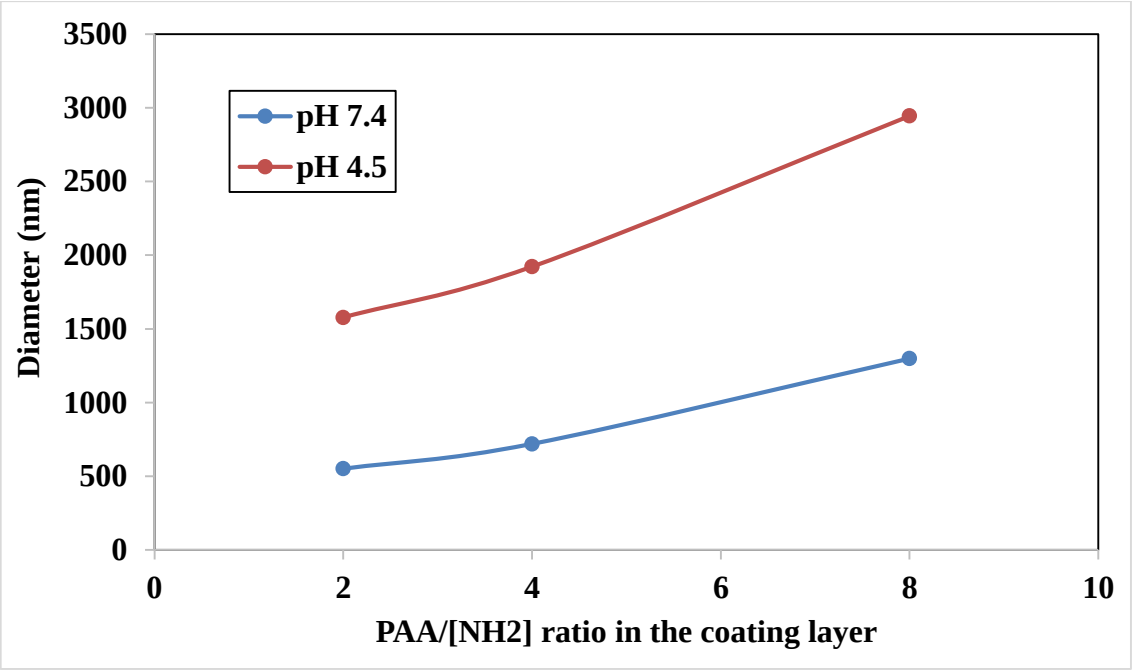


Figure 5.9 The effect of PAA/[NH_2] ratio to the hydrodynamic diameter of CS-PAA/MSNs at different pH values

5.3 Enzyme Immobilization Studies

The activities of free and immobilized subtilisin were calculated by measuring the absorbance values of the azocasein solutions at 440 nm. The reactions were carried out at various pH and temperature values at a mixing rate of 250 rpm, the effects of these parameters were examined.

5.3.1 Effect of Matrix Amount to Percent Enzyme Loading

One of the parameters affecting percent loading was found as matrix amount. Matrix amount was examined as 10, 15, 20 mg based on the studies which had used polymer-composites as immobilization matrix as given in Table 3.1 in the Section 3.2. According to the data obtained (Figure 5.10) (Table A.5 in the Appendix). 10 mg was chosen as optimum matrix amount since percent enzyme loading/mg matrix is higher for 10 mg matrix.

5.3.2 Effect of Incubation Time to Percent Enzyme Loading

Another parameter affecting percent loading is the incubation time. As can be seen in Figure 5.11 (Table A.6 in The Appendix), maximum loading occurs in first 15-20 minutes

but then, leaching starts immediately. However, there is no significant difference between 4 and 20 h. Thus, it was assumed that the system reaches equilibrium in 4 h and the incubation time was determined as 4 h.

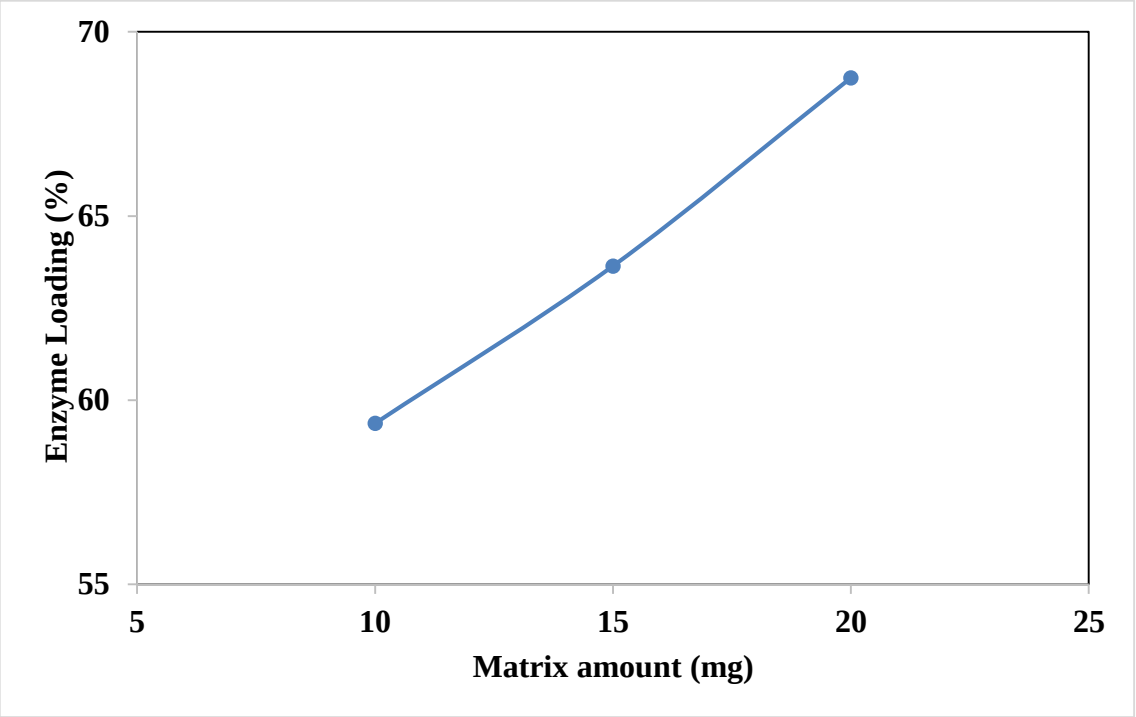


Figure 5.10 The effect of matrix amount to percent enzyme loading

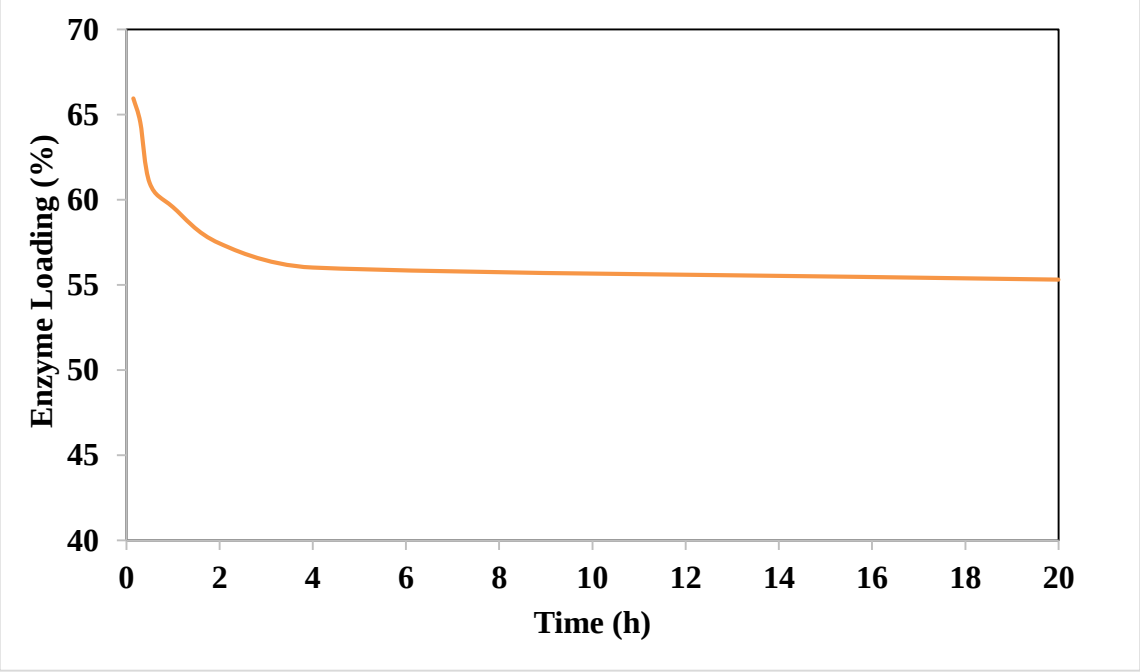


Figure 5.11 Percent enzyme loading versus incubation time

5.3.3 Effect of Immobilization pH to Percent Enzyme Loading

As can be seen in Figure 5.12 (Table A.7 in the Appendix). the effect of pH of immobilization solution to percent enzyme loading was examined taking the optimal pH interval of subtilisin published in the literature by Novozymes (2014) into consideration [100]. At pH 7, percent enzyme loading equals to two times that of pH 8 and 9. As can be seen in Table 3.1 in the Section 3.2, most of the studies had been achieved at the range of pH values between 7 and 8.

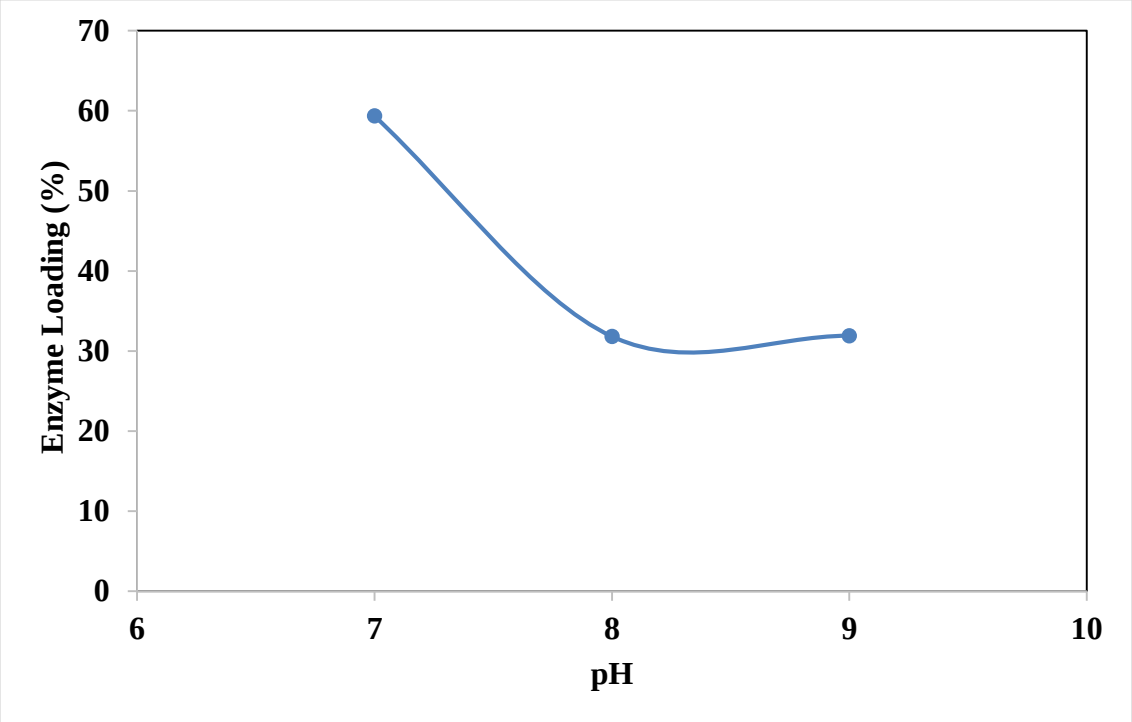


Figure 5.12 The effect of immobilization pH on percent enzyme loading

The denaturation of the enzyme is higher when the lower pH was applied. The isoelectric point (pI) of serine protease is ~6 as pointed in the study of Mehrnoush et al. (2012), thus the enzyme has negative charge above pH 7–9 [101]. pKa of PAA is 4.7, when pH of the medium is increased, ionization of PAA increases. pKa value of CS is 6.5 as said in the study of Wu et al. (2006), above this value CS is neutral [76]. Above pH 7, the sharp decrease in the percent loading was observed. This can be explained with the tendency of the particles to repel each other since both of the matrix and enzyme loaded with a negative charge.

5.4 Immobilized Enzyme Activity Studies

The optimal conditions for immobilization process were determined at pH 7, temperature of 25^{o}C, immobilization time of 4 h and mixing rate of 250 rpm. After the optimization was obtained for the maximum percent enzyme loading, the effects of temperature, pH, repeated use, and storage time on immobilized and free enzyme activity were investigated.

5.4.1 Effect of pH on Immobilized Enzyme Activity

The effect of pH on free and immobilized subtilisin enzyme activity was studied for azocasein hydrolysis at temperature of 40^{o}C. The effect of pH on free and immobilized enzyme preparations were investigated in the pH range of 5.0 and 10.0 in phosphate buffer, and the results were presented in Figure 5.13 (Table A.8 in the Appendix).

The maximum enzyme activity was achieved at pH 9.0 for free subtilisin and immobilized subtilisin. The optimum pH value for free enzyme is consistent with that of in the literature published by Novozymes (2014) [100]. The trends of the curves are similar for both cases. After immobilization process, the optimum pH interval for azocasein hydrolysis did not change. As pointed in thesis study of Erdem (2007) [102] at pH values lower than 8, enzyme activity is inhibited for two reasons: i) the relative-narrow structure of pH-sensitive polymer chains at low-pH values, and ii) a possible change of the enzyme conformation due to an unfavorable charge distribution of the amino acid moeities that cause a further activity decrease.

It can be concluded that the change in pH of the reaction solution affected immobilized enzyme activity since the surface charge of the polymer layer of CS-PAA/MSNs is pH-dependent, and the structure of free enzyme is not stable at the values other than 6-10.

5.4.2 Effect of Temperature on Immobilized Enzyme Activity

The activities of free and immobilized subtilisin were calculated by measuring the absorbances of the azocasein solutions at 440 nm. The temperature effect on the activity of free and immobilized enzymes was shown in Figure 5.14 (Table A.9 in the Appendix) by means of relative activities. Free enzyme exhibited its highest activity at around 70 ºC and this interval shifted to 70-80 ºC for immobilized enzyme. It can be said that

immobilized enzyme is more stable at higher temperatures compared with the free enzyme.

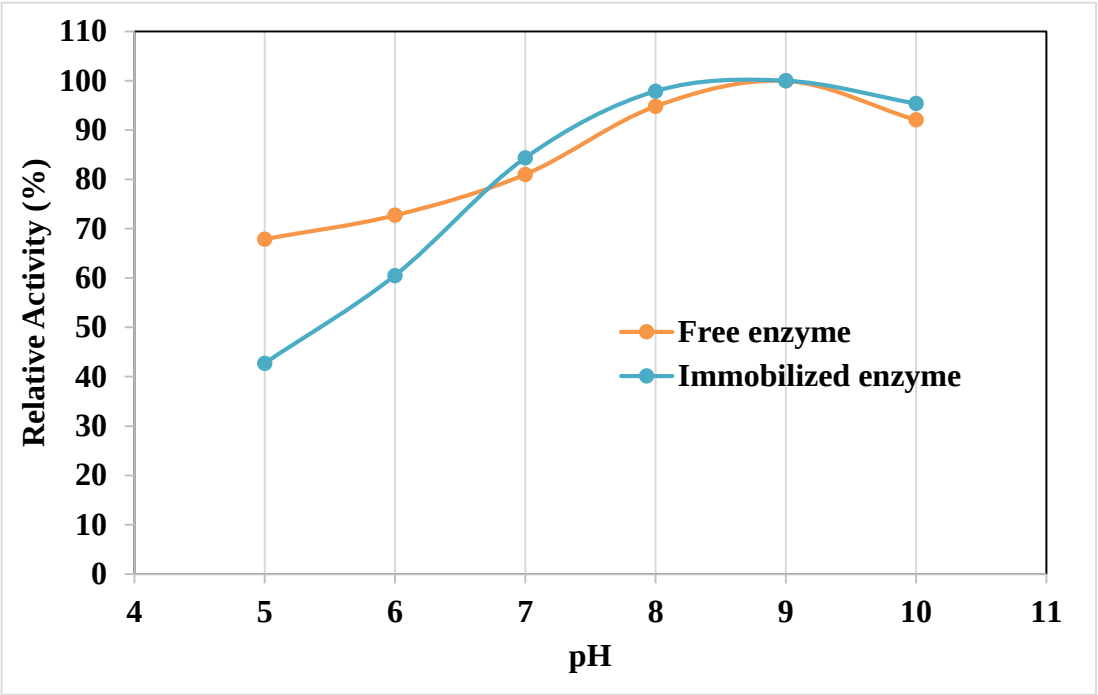


Figure 5.13 pH effect on immobilized enzyme activity

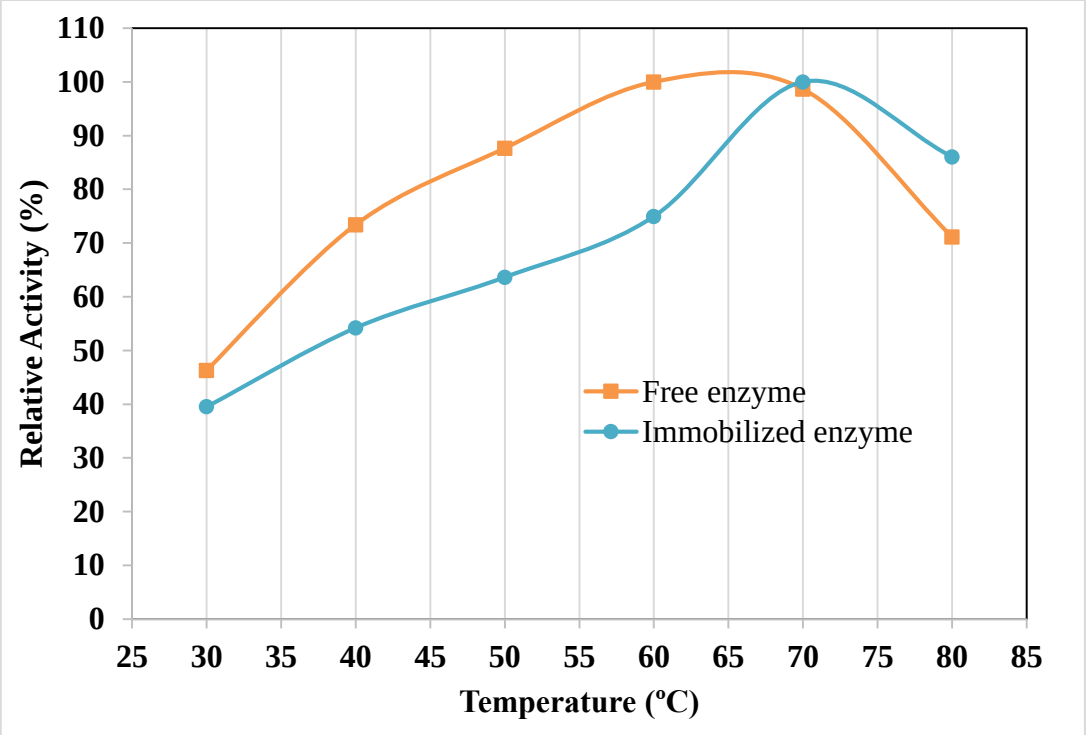


Figure 5.14 Temperature effect on immobilized enzyme activity

5.4.3 Repeated Use Capability of Immobilized Enzyme

The activity of subtilisin immobilized on CS-PAA/MSNs was determined after repeated use (operational stability) at the experimental conditions; pH 7, immobilization time of 4 h, mixing rate of 250 rpm. Then, the relationship between immobized enzyme activity and number of repeated use was obtained. Immobilized enzyme activity reduced from 100% to 45.8% after 5 times repeated use as seen in Figure 5.15 (Table A.10 in the Appendix). The reason of the decrease of the activity can be explained by the enzyme leakage from CS-PAA/MSNs after each repeated use.

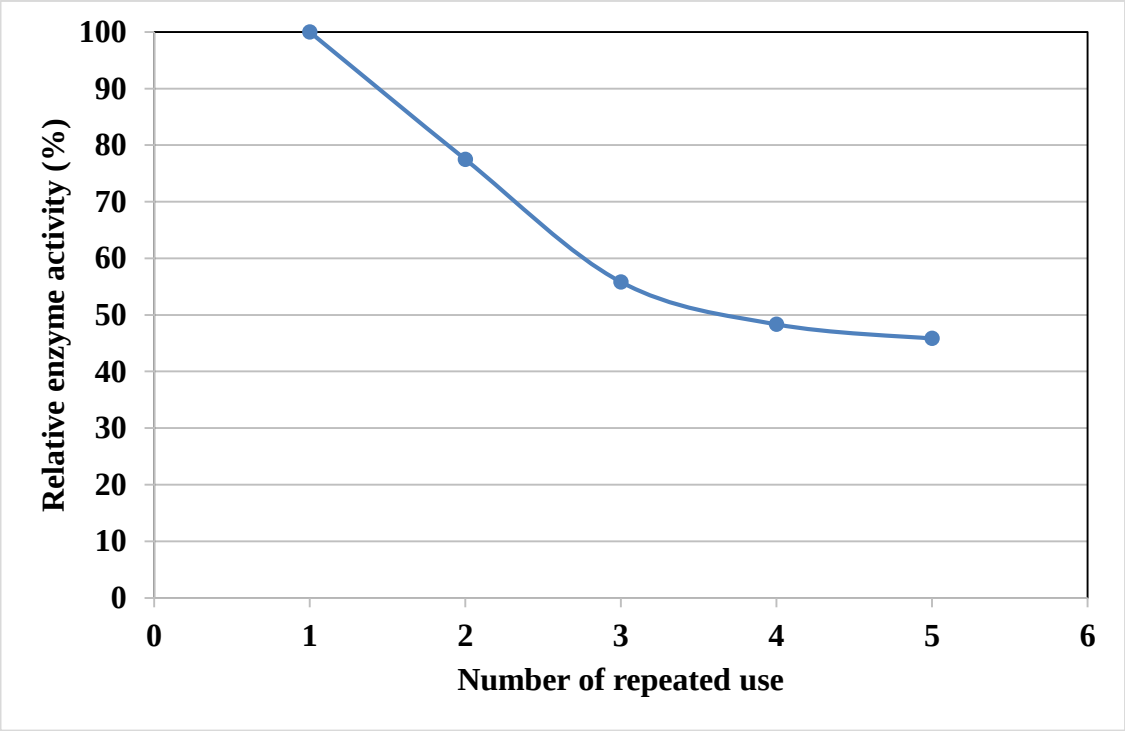


Figure 5.15 Effect of repeated use (operational stability) on immobilized enzyme activity

5.4.5 Storage Stability of Immobilized Enzyme

Time stability of immobilised subtilisin was examined after stored in buffer at 4°C for 28 days. The activity of immobilised enzyme was measured every week and the residual activity was determined in comparison with the activity at the beginning of storage. Activity of immobilized enzyme reduced from 100% to 60% after 4 weeks as seen in Figure 5.16 (Table A.11 in the Appendix).

The reason of the reduction of the activity is the increase in the enzyme leakage from CS-PAA/MSNs depending on time. The covalent-bounding can preserve activity via eliminating the leakage of the enzyme from the support surface. The decrease in immobilized enzyme activity was fitted in a linear model with the statistical values of regression coefficient (R^2) and standart deviation (σ) were calculated as 0.9941 and 1.2182, respectively.

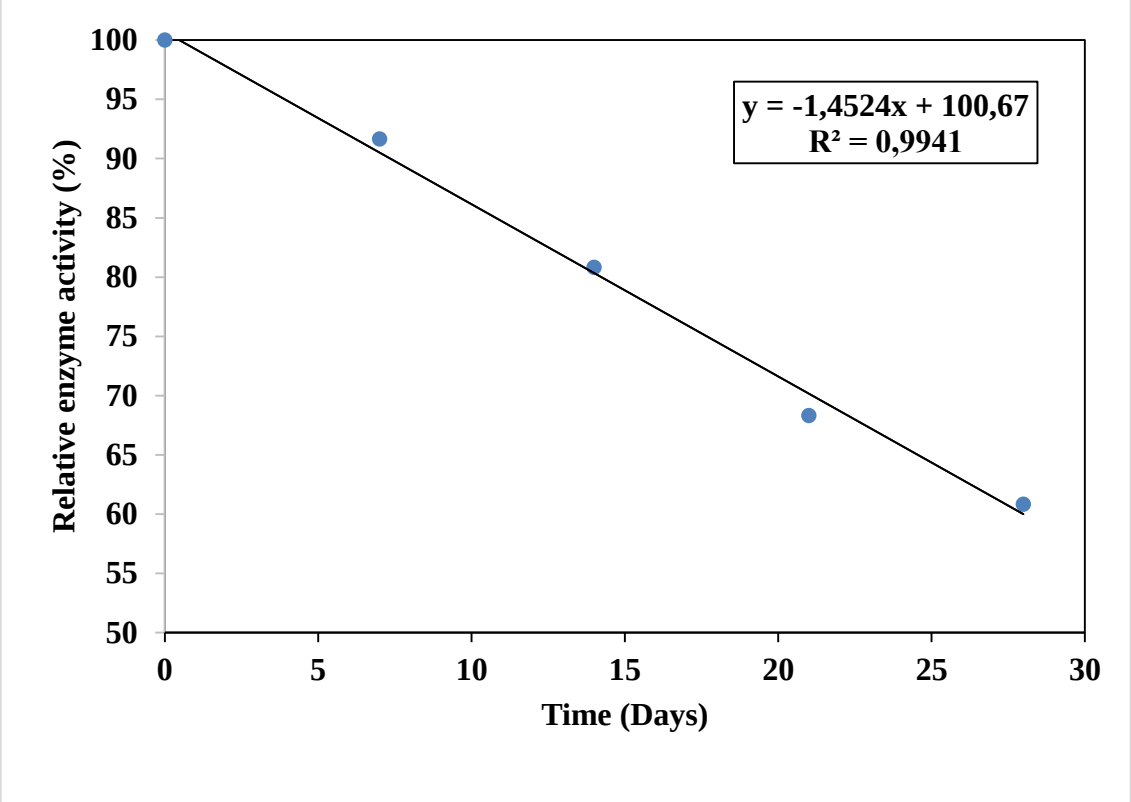


Figure 5.16 Storage stability of subtilisin enzyme immobilized onto CS-PAA/MSNs

5.5 The findings of various studies compared with the present study

The main findings and the experimental conditions of the present study and the studies in the literature demonstrated about the immobilization process of subtilisin onto various matrices or other enzymes immobilized onto silica-based matrices were compared in Table 5.1.

Table 5.1 The findings of various studies compared with the present study

Reference	**Enzyme**	**Enzyme Amount**	**Matrix**	**Matrix Amount (mg)**	**Duration (h)**	**$T_{immobilization}$ (°C)**	**$pH_{immobilization}$**	**$T_{optimal}$ (°C)**	**$pH_{optimal}$**
Present study (2015)	Alcalase (Subtilisin)	0.1 mg/mL	CS-PAA/MSNs	10 mg	4	25	7	70	8
Lee et al. (2015) [77]	β-glucanase	1 g	silica	1000	24	25	4	40	not examined
Soleimani et al. (2012) [79]	Protease	1.5 mg/mL	silica nanoparticles	7.5	1	25	4.5	40-50	not examined
Murai et al. (2011) [80]	Subtilisin	1 mg / 900 µl	functionalized-MSNs	3	12	4	7	not examined	not examined
Singh et al. (2011) [81]	Procerain B	0.2 mg/mL	chitosan beads	not specified	20, 24	4	8.5	55	3 and 6
Zhao et al. (2010) [82]	Subtilisin Carlsberg	0.1 g	chitosan	1000	3	25	7.4	not examined	not examined
Kasavi and Güvenilir (2006) [87]	Protease	400 U	chitosan	400	2	30	7.2	60	9.5
		400 U	chitin	400	2	30	7.2	60	9.5
		100 U	alginate	100	16, 20, 24	4	7.2	40	10

Table 5.1 continues

Reference	**Enzyme**	**Enzyme Amount**	**Matrix**	**Matrix Amount (mg)**	**Duration (h)**	**$T_{immobilization}$ (°C)**	**$pH_{immobilization}$**	**$T_{optimal}$ (°C)**	**$pH_{optimal}$**
Tang et al. (2006) [58]	Alcalase 2T	1 mg	chitosan nanoparticles	1	2	40	7.5	55	7.5
Wang and Caruso (2005) [84]	Protease	0.5 mg/mL	siliceous particles	10	not specified	25	7	not examined	7.5
Pandya et al. (2005) [85]	α-amilase	0.5 mg	functionalized-MSNs	1000	5	25	6.6	30	6
Ferreiara et al. (2003) [86]	Subtilisin Carlsberg	0.449 mg	silica	50	18	25	8	60	8

CONCLUSIONS

In the present study, the polymer-coated mesoporous silica nanoparticles were prepared by in-situ polymerization technique for subtilisin immobilization. In the preparation of poymer-coated, chitosan and poly(acrylic) acid were used to form a blend-coating layer. After preparation of the particles, they were characterized based on their morphology, chemical structure, size-size distribution. Then, the polymer-coated mesoporous silica particles obtained at optimum conditions were used for subtilisin enzyme immobilization studies. Finally, the activities of the enzyme immobilized onto polymer-coated mesoporous silica nanoparticles were investigated at various process conditions.

Firstly, for the preparation of mesoporous silica nanoparticles, the steps were followed as;

1. FTIR studies showed the characteristic absorption band of Si–O in the spectrum of MSN and used to examine if mesoporous silica nanoparticles have cleaned from organic template or not.
2. Morphological investigations were made by SEM and TEM. Uniform-mesoporous silica nanoparticles seem to have been synthesized in the range 60-100 nm diameter with the mean value 80±10 nm. The porous structure of the nanoparticles can also be seen clearly from TEM micrograph.

Secondly, for the preparation of polymer-coated mesoporous silica nanoparticles, the steps were followed as;

1. After approving successful reflux process of MSN synthesis, coating was achieved and FTIR spectra used to show that PAA was dissociated into COO- groups and there was a formation of chitosan-PAA polymer composite on the surface of MSNs.

2. Morphological investigations were made by SEM and TEM. CS-PAA/MSNs have diameter in the range 100-200 nm. Uniform shape and size could have not been obtained for the polymer-coated particles As can be seen from the micrographs, the beads have a spherical form and textured-porous surface and the uniform shape has lost its regularity after coating layer formed which can be seen as thin-gray layer at the outer surface of MSNs.
3. Size-size distribution of the particles were evaluated based on PAA/[NH_2] ratio in the polymer blend of coating layer and pH. Size of the particles increased with increment of the PAA/[NH_2] ratio, and decreased with increment of pH. Zeta potential of the particles have also investigated based on PAA/[NH_2] ratio in the polymer blend of coating layer and pH. It was seen that the surface charge density increases with increment of the PAA/[NH_2] ratio and decreases with increment of pH value due to the ionization of PAA into–COOH groups.

After preparation of polymer-coated mesoporous silica nanoparticles, the enzyme immobilization process conditions were investigated by taking into account the percent enzyme loading. The experiments were performed at constant temperature and stirring rate such as 25^{o}C and 250 rpm, respectively. According to the evaluation of the experimental data, the optimum enzyme immobilization conditions were found as pH 7, 10 mg of matrix amount and 4 h of immobilization time.

Next, the effects of pH, temperature, storage time and repeated-use on immobilized enzyme activity were investigated. The major findings of the present study were given as follows;

1. Free and immobilized enzyme preparations were investigated at the pH range between 5.0 and 10.0. Maximum enzyme activity was achieved at pH 9.0 for both free and immobilized subtilisin.
2. Free and immobilized enzyme preparations were investigated at the temperature range between 30 and 80^{o}C. Free enzyme exhibited a temperature optimum around 70 ^{o}C and this interval shifted to 70-80^{o}C for immobilized enzyme. It can be concluded that immobilized enzyme is more stable at higher temperatures compared with the free enzyme.

3. The repeated use (operational stability) of immobilized enzyme was also investigated via analyzing the activity of immobilized enzyme after each repeated use. The activity of immobilized enzyme reduced from 100% to 40% after 5 repeted use.
4. According to the experiments, the activity of immobilized enzyme reduced from 100% to 60% after 4 weeks of the storage time. The reason of the decrease of the enzyme activity is the increase in enzyme leakage from CS-PAA/MSNs depending on the storage time. The covalent-bounding can preserve activity via eliminating leaking of the enzyme from the support surface.

To sum up, the findings showed that CS-PAA/MSNs were suitable agent for enzyme immobilization. But, covalent immobilization should be investigated for increased stability and efficient use of the enzyme.

The synthesis part of this study also could have an application on the drug delivery systems since the particles provided pH-sensitive behaviour due to the zeta potential and hydrodynamic size analysis. Polymer coated MSNs have widely studied in the development of drug-delivery systems, because polymers can provide adjustable properties of structure, size, stability, biocompatibility, and functionality. Polymer-based drug delivery systems have the advantages of higher loads, improved drug solubility and targeting, and controlled release of the drug.

REFERENCES

[1] Gupta, M. N., Kaloti, M., Kapoor, M., and Solanki, K., (2011). "Nanomaterials as matrices for enzyme immobilization.", Artificial cells, blood substitutes, and immobilization biotechnology, 39(2):98–109.

[2] Lei, Z., and Bi, S., (2007). "The silica-coated chitosan particle from a layer-by-layer approach for pectinase immobilization", Enzyme and Microbial Technology, 40(5):1442–1447.

[3] Zhao, H., Xu, J., Lan, W., Wang, T., and Luo, G., (2013). "Microfluidic production of porous chitosan/silica hybrid microspheres and its Cu(II) adsorption performance", Chemical Engineering Journal, 229:82–89.

[4] Kim, J., Grate, J. W., and Wang, P., (2008). "Nanobiocatalysis and its potential applications.", Trends in biotechnology, 26(11):639–46.

[5] Li, S., Yang, X., Yang, S., Zhu, M., and Wang, X., (2012). "Technology Prospecting on Enzymes : Application , Marketing and Engineering", (September).

[6] Ward, O. P., (2011). Comprehensive Biotechnology, Comprehensive Biotechnology. Elsevier.

[7] Tavano, O. L., (2013). "Protein hydrolysis using proteases: An important tool for food biotechnology", Journal of Molecular Catalysis B: Enzymatic, 90:1–11.

[8] Zhu, D., Wu, Q., Wang, N., and Academy, C., (2012). "Industrial Enzymes", Comprehensive Biotechnology, 3–13.

[9] Teal, A. R., and P.E.O., W., (1994). "Enzymes and their Role in Biotechnology".

[10] Illanes, A., (2011). Immobilized Biocatalysts, Comprehensive Biotechnology (Second Edi., Vol. 1). Elsevier B.V.

[11] Zucca, P., and Sanjust, E., (2014). "Inorganic Materials as Supports for Covalent Enzyme Immobilization: Methods and Mechanisms", Molecules, 19(9):14139–14194.

[12] Springer (1988). "Immobilized Biocatalysts - An Introduction", http://www.springer.com/gp/book/9783540188087, 20.05.2015.

[13] Erokhin, Viktor Ram Kumar, Manoj Yavuz, O., (2008). The New Frontiers of Organic and Composite Nanotechology.

[14] Ansari, S. A., and Husain, Q., (2012). "Potential applications of enzymes immobilized on/in nano materials: A review.", Biotechnology advances, 30(3):512–23.

[15] Kalkan, N. A., Aksoy, S., Aksoy, E. A., and Hasirci, N., (2011). "Preparation of Chitosan-Coated Magnetite Nanoparticles and Application for Immobilization of Laccase".

[16] Dai, J., Yan, H., Yang, H., and Cheng, R., (2010). "Simple method for preparation of chitosan/poly(acrylic acid) blending hydrogel beads and adsorption of copper(II) from aqueous solutions", Chemical Engineering Journal, 165(1):240–249.

[17] Ge, J., Yang, C., Zhu, J., Lu, D., and Liu, Z., (2012). "Nanobiocatalysis in Organic Media: Opportunities for Enzymes in Nanostructures", Topics in Catalysis, 55(16-18):1070–1080.

[18] "Enzymes - BIOChemReview", http://biochemreview.weebly.com/enzymes.html, 20.05.2015.

[19] "Organic Compounds Essential to Human Functioning|Anatomy and Physiology", https://courses.candelalearning.com/ap2x1/chapter/organic-compounds-essential-to-human-functioning/, 20.05.2015.

[20] Kinetics, E., Assumption, S., Hypothesis, S., Kinetics, E., Effects, S., Reactions, E., Works, E., (2013). Bioprocess Engineering.

[21] Talbert, J. N., and Goddard, J. M., (2012). "Colloids and Surfaces B : Biointerfaces Enzymes on material surfaces", Colloids and Surfaces B: Biointerfaces, 93:8–19.

[22] MidlandsTech Courses, "Enzymes", http://classes.midlandstech.com/carterp/Courses/bio225/chap05/ss2.htm, 20.05.2015.

[23] Behnke, U., (1986). "International Union of Biochemistry: Enzyme Nomenclature 1984. Recommendations of the Nomenclature Committee of the International Union of Biochemistry on the Nomenclature and Classification of Enzyme-Catalysed Reactions. 646 Seiten. Academic Press, Inc.,", Food / Nahrung, 30(7):738–738.

[24] Kirk, O., Borchert, T. V., and Fuglsang, C. C., (2002). "Industrial enzyme applications", Current Opinion in Biotechnology, 13(4):345–351.

[25] Hirata et al. (2013). "Enzymatic activity of a subtilisin homolog, Tk-SP, from Thermococcus kodakarensis in detergents and its ability to degrade the abnormal prion protein.", BMC biotechnology, 13(1):19.

[26] Graycar, T. P., Bott, R. R., Power, S. D., and Estell, D. a., (2013). "Subtilisins", Handbook of Proteolytic Enzymes, (1999):3148–3155.

[27] Gutteridge, A., (2005). "Understanding the Relationship Between Enzyme Structure and Catalysis".

[28] Hudson, S., Magner, E., Cooney, J., and Kieran, B., (2005). "Methodology for the immobilization of enzymes onto mesoporous materials", Journal of Physical Chemistry B, 109(41):19496–19506.

[29] Crook, E. M., (1989). "Immobilised enzymes and cells. A. Roseveare, J. F. Kennedy, and J. M. S. Cabral. Adam Hilger: Bristol and Philadelphia, (1987).", Cell Biochemistry and Function, 7(1):77–77.

[30] Poznansky, M. J., (1983). "Enzyme-protein conjugates: new possibilities for enzyme therapy", Pharmacology & Therapeutics, 21(1):53–76.

[31] Costa, S. A., Azevedo, H. S., and Reis, R. L., (2005). "Immobilization in 17 Enzyme Biodegradable Polymers for Biomedical Applications", 301–324.

[32] Sheldon, R. a., (2007). "Enzyme immobilization: The quest for optimum performance", Advanced Synthesis and Catalysis, 349(8-9):1289–1307.

[33] Xie, T., Wang, A., Huang, L., Li, H., Chen, Z., and Wang, Q., (2009). "Recent advance in the support and technology used in enzyme immobilization", 8(19):4724–4733.

[34] Noritomi, H., Sasanuma, A., Kato, S., and Nagahama, K., (2007). "Catalytic properties of cross-linked enzyme crystals in organic media", Biochemical Engineering Journal, 33(3):228–231.

[35] Worsfold, P. J., (1995). "Classification and chemical characteristics of immobilized enzymes (Technical Report)", Pure and Applied Chemistry, 67(4):597–600.

[36] Hartmann, M., and Kostrov, X., (2013). "Immobilization of enzymes on porous silicas--benefits and challenges.", Chemical Society reviews, 42(15):6277–89.

[37] Kim, J., (2012). "Nanobiocatalysis and Its Potential Applications", Topics in Catalysis, 55(16-18):1055–1056.

[38] Illanes, A., Cauerhff, A., Wilson, L., and Castro, G. R., (2012). "Recent trends in biocatalysis engineering", Bioresource Technology, 115:48–57.

[39] Hartmann, M., (2005). "Re V iews Ordered Mesoporous Materials for Bioadsorption and Biocatalysis", Chem. Mater., 17:4577–4593.

[40] Hwang, E. T., and Gu, M. B., (2013). "Enzyme stabilization by nano/microsized hybrid materials", Engineering in Life Sciences, 13(1):49–61.

[41] Huh, S., Wiench, J. W., Yoo, J., Pruski, M., and Lin, V. S., (2003). "Article Organic Functionalization and Morphology Control of Mesoporous Silicas via a Co-Condensation Synthesis Method Organic Functionalization and Morphology Control of Mesoporous Silicas via a Co-Condensation Synthesis Method", Chemistry of Materials, (10):4247–4256.

[42] Faber, K., (2011). Biotransformations aid organic chemists, ChemInform.

[43] Nestl, B. M., Nebel, B. a., and Hauer, B., (2011). "Recent progress in industrial biocatalysis", Current Opinion in Chemical Biology, 15(2):187–193.

[44] Jung, D., Streb, C., and Hartmann, M., (2010). "Covalent anchoring of chloroperoxidase and glucose oxidase on the mesoporous Molecular Sieve SBA-15", International Journal of Molecular Sciences, 11(2):762–778.

[45] Petkova, G. A., Záruba, K., and Král, V., (2012). "Synthesis of silica particles and their application as supports for alcohol dehydrogenases and cofactor

immobilizations: conformational changes that lead to switch in enzyme stereoselectivity.", Biochimica et biophysica acta, 1824(6):792–801.

[46] Hanefeld, U., Gardossi, L., and Magner, E., (2009). "Understanding enzyme immobilisation.", Chemical Society reviews, 38(2):453–68.

[47] Gaberc-Porekar, V., and Menart, V., (2001). "Perspectives of immobilized-metal affinity chromatography.", Journal of biochemical and biophysical methods, 49(1-3):335–60.

[48] Petroapb, M., Gemeiner, P., and Berek, D., (1994). "Dextran-grafted silica gel for high-performance chromatography of proteins", 665:37–45.

[49] Nash, D. C., McCreath, G. E., and Chase, H. A., (1997). "Modification of polystyrenic matrices for the purification of proteins. Effect of the adsorption of poly(vinyl alcohol) on the characteristics of poly(styrene-divinylbenzene) beads for use in affinity chromatography.", Journal of chromatography. A, 758(1):53–64.

[50] Pastor, E., Matveeva, E., Valle-Gallego, A., Goycoolea, F. M., and Garcia-Fuentes, M., (2011). "Protein delivery based on uncoated and chitosan-coated mesoporous silicon microparticles", Colloids and Surfaces B: Biointerfaces, 88(2):601–609.

[51] Tang, H., Guo, J., Sun, Y., Chang, B., Ren, Q., and Yang, W., (2011). "Facile synthesis of pH sensitive polymer-coated mesoporous silica nanoparticles and their application in drug delivery", International Journal of Pharmaceutics, 421(2):388–396.

[52] Zhu, M., Zhu, Y., Zhang, L., and Shi, J., (2013). "Preparation of chitosan/mesoporous silica nanoparticle composite hydrogels for sustained co-delivery of biomacromolecules and small chemical drugs", Science and Technology of Advanced Materials, 14(4):045005.

[53] Giri, T. K., Thakur, A., Alexander, A., Ajazuddin, Badwaik, H., and Tripathi, D. K., (2012). "Modified chitosan hydrogels as drug delivery and tissue engineering systems: present status and applications", Acta Pharmaceutica Sinica B, 2(5):439–449.

[54] Krajewska, B., (2004). "Application of chitin- and chitosan-based materials for enzyme immobilizations: a review", Enzyme and Microbial Technology, 35(2-3):126–139.

[55] Agnihotri, S. A., Mallikarjuna, N. N., and Aminabhavi, T. M., (2004). "Recent advances on chitosan-based micro- and nanoparticles in drug delivery.", Journal of controlled release : official journal of the Controlled Release Society, 100(1):5–28.

[56] Malmiri, H. J., Ali, M., Jahanian, G., and Berenjian, A., (2012). "Potential Applications of Chitosan Nanoparticles As Novel Support in Enzyme Immobilization", American Journal of Biochemistry and Biotechnology, 8(4):203–219.

[57] López-León, T., Carvalho, E. L. S., Seijo, B., Ortega-Vinuesa, J. L., and Bastos-González, D., (2005). "Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior.", Journal of colloid and interface science, 283(2):344–51.

[58] Tang, Z.-X., Qian, J.-Q., and Shi, L.-E., (2006). "Characterizations of immobilized neutral proteinase on chitosan nano-particles", Process Biochemistry, 41(5):1193–1197.

[59] Biró, E., Németh, A. S., Sisak, C., Feczkó, T., and Gyenis, J., (2008). "Preparation of chitosan particles suitable for enzyme immobilization.", Journal of biochemical and biophysical methods, 70(6):1240–6.

[60] Shi, L., Fang, X., Xing, L., Chen, M. I. N., and Zhu, D., (2011). "Chitosan Nanoparticles as Drug Delivery Carriers for Biomedical Engineering", 33(6):43028051.

[61] Jin, J.-J., Chen, G.-C., Huang, M.-Y., and Jiang, Y.-Y., (1994). "Catalytic hydrogenation behaviours of palladium complexes of chitosan-polyacrylic acid and chitosan-polymethacrylic acid", Reactive Polymers, 23(2-3):95–100.

[62] Fang, H., Huang, J., Ding, L., Li, M., and Chen, Z., (2009). "Preparation of magnetic chitosan nanoparticles and immobilization of laccase", Journal of Wuhan University of Technology-Mater. Sci. Ed., 24(1):42–47.

[63] Dutta, P. K., Duta, J., and Tripathi, V. S., (2004). "Chitin and Chitosan: Chemistry, properties and applications", Journal of Scientific and Industrial Research, 63(1):20–31.

[64] "NPTEL :: Textile Engineering - High Performance and Specialty Fibres", http://www.nptel.ac.in/courses/116102006/13, 22.05.2015.

[65] Hong, C.-Y., Li, X., and Pan, C.-Y., (2009). "Fabrication of smart nanocontainers with a mesoporous core and a pH-responsive shell for controlled uptake and release", Journal of Materials Chemistry, 19(29):5155.

[66] Gao, Q., Xu, Y., Wu, D., Sun, Y., and Li, X., (2009). "pH-responsive drug release from polymer-coated mesoporous silica spheres", Journal of Physical Chemistry C, 113(29):12753–12758.

[67] Carniato, F., Bisio, C., Paul, G., Gatti, G., Bertinetti, L., Coluccia, S., and Marchese, L., (2010). "On the hydrothermal stability of MCM-41 mesoporous silica nanoparticles and the preparation of luminescent materials", Journal of Materials Chemistry, 20(26):5504.

[68] Liu, R., Liao, P., Liu, J., and Feng, P., (2011). "Responsive polymer-coated mesoporous silica as a pH-sensitive nanocarrier for controlled release", Langmuir, 27(6):3095–3099.

[69] Fleming, M. S., Mandal, T. K., and Walt, D. R., (2001). "Nanosphere−Microsphere Assembly: Methods for Core−Shell Materials Preparation", Chemistry of Materials, 13(6):2210–2216.

[70] Spagnol, C., Rodrigues, F. H. a, Pereira, A. G. B., Fajardo, A. R., Rubira, A. F., and Muniz, E. C., (2012). "Superabsorbent hydrogel composite made of cellulose nanofibrils and chitosan-graft-poly(acrylic acid)", Carbohydrate Polymers, 87(3):2038–2045.

[71] Feng et al. (2014). "Effect of pH-responsive alginate/chitosan multilayers coating on delivery efficiency, cellular uptake and biodistribution of mesoporous silica

nanoparticles based nanocarriers", ACS Applied Materials and Interfaces, 6(11):8447–8460.

[72] Peng et al. (2013). "A pH-responsive nano-carrier with mesoporous silica nanoparticles cores and poly (acrylic acid) shell-layers : Fabrication , characterization and properties for controlled release of salidroside", International Journal of Pharmaceutics, 446(1-2):153–159.

[73] Haghighizadeh, A., Tan, W. L., Bakar, M. A., and Ghani, A., (2012). "Synthesis and Properties of Nanosized Silver Catalyst Supported on Chitosan-Silica Nanocomposites", 271(2012):255–271.

[74] Popat, A., Liu, J., Lu, G. Q. (Max). and Qiao, S. Z., (2012). "A pH-responsive drug delivery system based on chitosan coated mesoporous silica nanoparticles", Journal of Materials Chemistry, 22(22):11173.

[75] Yuan, L., Tang, Q., Yang, D., Zhang, J. Z., Zhang, F., and Hu, J., (2011). "Preparation of pH-Responsive Mesoporous Silica Nanoparticles and Their Application in Controlled Drug Delivery", The Journal of Physical Chemistry C, 115(20):9926–9932.

[76] Wu, Y., Guo, J., Yang, W., Wang, C., and Fu, S., (2006). "Preparation and characterization of chitosan–poly(acrylic acid) polymer magnetic microspheres", Polymer, 47(15):5287–5294.

[77] Lee, Y. R., Tang, B., Zhang, H., Bi, W., and Row, K. H., (2015). "Effects of β-glucanase-Immobilized Silica on Hydrolysis of Polysaccharides in *Chamaecyparis obtusa* Residues", Journal of Liquid Chromatography & Related Technologies, 38(5):613–618.

[78] Junoi, S., Chisti, Y., and Hansupalak, N., (2015). "Optimal conditions for deproteinizing natural rubber using immobilized alkaline protease", Journal of Chemical Technology & Biotechnology, 90(1):185–193.

[79] Soleimani, M., Khani, A., Dalali, N., and Behbehani, G. R., (2012). "Improvement in the Cleaning Performance Towards Protein Soils in Laundry Detergents by Protease Immobilization on the Silica Nanoparticles", Journal of Surfactants and Detergents, 1–6.

[80] Murai, K., Nonoyama, T., Ando, F., and Kato, K., (2011). "Catalytic performance of subtilisin immobilized without covalently attachment on surface-functionalized mesoporous silica materials", IOP Conference Series: Materials Science and Engineering, 18(19):192007.

[81] Singh, A. N., Singh, S., Suthar, N., and Dubey, V. K., (2011). "Glutaraldehyde-activated chitosan matrix for immobilization of a novel cysteine protease, procerain B.", Journal of agricultural and food chemistry, 59(11):6256–62.

[82] Zhao, H., Song, Z., and Olubajo, O., (2010). "High transesterification activities of immobilized proteases in new ether-functionalized ionic liquids", Biotechnology Letters, 32(8):1109–1116.

[83] Xiao, Q.-G., Tao, X., Zou, H.-K., and Chen, J.-F., (2008). "Comparative study of solid silica nanoparticles and hollow silica nanoparticles for the immobilization of lysozyme", Chemical Engineering Journal, 137(1):38–44.

[84] Wang, Y., and Caruso, F., (2005). "Mesoporous Silica Spheres as Supports for Enzyme Immobilization and Encapsulation", Chemistry of Materials, 17(5):953–961.

[85] Pandya, P. H., Jasra, R. V., Newalkar, B. L., and Bhatt, P. N., (2005). "Studies on the activity and stability of immobilized amylase in ordered mesoporous silicas", Microporous and Mesoporous Materials, 77(1):67–77.

[86] Ferreira, L., Ramos, M. a., Dordick, J. S., and Gil, M. H., (2003). "Influence of different silica derivatives in the immobilization and stabilization of a Bacillus licheniformis protease (Subtilisin Carlsberg)", Journal of Molecular Catalysis B: Enzymatic, 21(4-6):189–199.

[87] Kasavi, C., and Günevilir, Y. (Avcıbaşı). (2006). "Kovalent bağlanma ve fiziksel adsorpsiyon metotları ile proteaz enziminin immobilizasyonu", Istanbul Technical University, Gradute School of Science Engineering and Technology, MSc Thesis.

[88] Slowing, I. I., Trewyn, B. G., Giri, S., and Lin, V. S. Y., (2007). "Mesoporous Silica Nanoparticles for Drug Delivery and Biosensing Applications", Advanced Functional Materials, 17(8):1225–1236.

[89] Tang, H., Guo, J., Sun, Y., Chang, B., Ren, Q., and Yang, W., (2011). "Facile synthesis of pH sensitive polymer-coated mesoporous silica nanoparticles and their application in drug delivery.", International journal of pharmaceutics, 421(2):388–96.

[90] Randall, R. J., and Lewis, a, (1951). "The folin by oliver", Readings, 193(1):265–275.

[91] Tomarelli, R. M., Charney, J., And Harding, M. L., (1949). "The use of azoalbumin as a substrate in the colorimetric determination or peptic and tryptic activity.", The Journal of laboratory and clinical medicine, 34(3):428–33.

[92] Schiffman, J. D., and Schauer, C. L., (2007). "Cross-linking chitosan nanofibers.", Biomacromolecules, 8(2):594–601.

[93] Lin Peng, Feng Jian-Jun, Zou Zhi-Hua, Wang Shu-Hong, Yang Qian, W. Y.-L., (2008). "Determination of Outer Membrane Protein of Aeromonas Hydrophila by pH Controlled Phase Separation Fluoroimmunoassay", Chinese Journal of Analytical Chemistry.

[94] Hu, Y., Jiang, X., Ding, Y., Chen, Q., and Yang, C. Z., (2004). "Core-Template-Free Strategy for Preparing Hollow Nanospheres", Advanced Materials, 16(11):933–937.

[95] Morey, M. S., Bryan, J. D., Schwarz, S., and Stucky, G. D., (2000). "Pore Surface Functionalization of MCM-48 Mesoporous Silica with Tungsten and Molybdenum Metal Centers: Perspectives on Catalytic Peroxide Activation", Chemistry of Materials, 12(11):3435–3444.

[96] Guo, L., Liu, G., Hong, R. Y., and Li, H. Z., (2010). "Preparation and characterization of chitosan poly(acrylic acid) magnetic microspheres", Marine Drugs, 8(7):2212–2222.

[97] Nanocomposix, (2012). "Zeta Potential Analysis of Nanoparticles", Nanocomposix Publications, 1–6.

[98] Malkoç, A., (2011). "Polimer modifiyeli silika nanopartiküllerin enzim immobilizasyonunda kullanımı".

[99] Ahn, J.-S., Choi, H.-K., and Cho, C.-S., (2001). "A novel mucoadhesive polymer prepared by template polymerization of acrylic acid in the presence of chitosan", Biomaterials, 22(9):923–928.

[100] Novozymes, (2014). "Enzymes for biocatalysis for smarter chemical synthesis".

[101] Mehrnoush, A., Mustafa, S., Sarker, M. Z. I., and Yazid, A. M. M., (2012). "Optimization of serine protease purification from mango (Mangifera indica cv. Chokanan) peel in polyethylene glycol/dextran aqueous two phase system.", International journal of molecular sciences, 13(3):3636–49.

[102] Çetin Erdem, F. B., (2007). "Preparation and characterization of magnetic polymeric carriers for enzyme immobilization", MSc Thesis, Hacettepe University Graduate School of Science and Engineering.

APPENDIX

Table A.1 Calibration curve data for Folin-Lowry Method

BSA concentration (g/L)	Absorbance Trial 1	Absorbance Trial 2	Absorbance Trial 3	Mean Absorbance
0	0	0	0	0
0.025	0.000	0.000	0.000	0.000
0.050	0.106	0.091	0.089	0.095
0.075	0.202	0.159	0.135	0.165
0.100	0.219	0.199	0.206	0.208
0.125	0.275	0.279	0.230	0.261
0.150	0.322	0.333	0.318	0.324
0.175	0.430	0.418	0.373	0.407
0.200	0.444	0.464	0.428	0.445
0.225	0.528	0.519	0.504	0.517
0.250	0.612	0.579	0.544	0.578
0.300	0.614	0.637	0.603	0.618
0.350	0.650	0.695	0.728	0.691
0.400	0.723	0.784	0.840	0.782

Table A.2 Calibration curve data for enzyme activity assay

Enzyme Activity, Au/L	Absorbance Trial-1	Absorbance Trial-2	Absorbance Trial-3	Mean Absorbance
0.00	0.000	0.000	0.000	0.000
0.25	0.100	0.102	0.113	0.105
0.60	0.210	0.240	0.219	0.223
1.20	0.370	0.400	0.451	0.407
1.80	0.570	0.560	0.565	0.565
2.40	0.668	0.670	0.675	0.671

Table A.3 The effect of PAA/[NH_2] ratio to the zeta potential at different pH values (ratio 0 nominates MSN without coating)

PAA/[NH2] ratio	Zeta potential (pH 4.5)	Zeta potential (pH 7.4)
0	1.0	-12.8
2	-11.3	-26.4
4	-11.7	-35.4
8	-20.7	-58.7

Table A.4 The effect of PAA/[NH_2] ratio to the hydrodynamic diameter of CS-PAA/MSNs at different pH values

PAA/[NH2] ratio	Hydrodynamic Size (pH 4.5)	Hydrodynamic Size (pH 7.4)
2	1578	551
4	1922	720
8	2945	1298

Table A.5 The effect of matrix amount to percent enzyme loading

Matrix amount (mg)	**Enzyme loading (%)**
10	59.38
15	63.65
20	68.75

Table A.6 The change of percent enzyme loading with incubation time

Time (h)	**Enzyme loading (%)**
0.0	0.0
0.15	65.96
0.3	64.54
0.5	60.99
1	59.57
2	57.45
4	56.03
20	55.32

Table A.7 The effect of immobilization pH on percent enzyme loading

pH	**Enzyme Loading (%)**
7	59.38
8	31.82
9	31.91

Table A.8 pH effect on immobilized enzyme activity

pH	**Relative activity of free enzyme (%)**	**Relative activity of immobilized enzyme (%)**
5	67.91	42.70
6	72.72	60.50
7	81.01	84.34
8	94.83	97.86
9	100.00	100.00
10	92.07	95.37

Table A.9 Temperature effect on immobilized enzyme activity

T(°C)	**Relative activity of free enzyme (%)**	**Relative activity of immobilized enzyme (%)**
30	46.30	39.55
40	73.43	54.24
50	87.67	63.65
60	100.0	74.95
70	98.67	100.0
80	71.16	86.06

Table A.10 Effect of repeated use on immobilized enzyme activity

Repeated use (times)	**Relative Immobilized Enzyme Activity (%)**
1	100.0
2	77.50
3	55.83
4	48.33
5	45.83

Table A.11 Storage stability of immobilized enzyme

Time (days)	**Relative Immobilized Enzyme Activity (%)**
0	100.0
7	91.67
14	80.83
21	68.33
28	58.33

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Degree	Department	University	Date of Graduation
Undergraduate	Chemical Engineering	Hacettepe University	06.2012
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PUBLISHMENTS

Conference Papers

1. Ünal, Ş. and Özbek, B., 2015, "An experimental study to optimize subtilisin immobilization using polymer-coated mesoporous silica nanoparticles", *10th European Congress of Chemical Engineering (ECCE10).* 27th September-1st October, Acropolis Convention Center, Nice, France, Submission No-1182.

2. Özbek, B. and Ünal Ş., 2015, "An experimental study on hydrolysis of lactose in an ultrasonic bath", *10th European Congress of Chemical Engineering*

(ECCE10). 27th September-1st October, Acropolis Convention Center, Nice, France, Submission No-1577.

3. Şimşek, S., Ünal, Ş. and Özbek, B., 2015, "Biosorption of aluminium ions from aqueous solutions by using brewery waste", *10th European Congress of Chemical Engineering (ECCE10).* A 27th September-1st October, cropolis Convention Center, Nice, France, Submission-1703.

4. Ünal, Ş. and Özbek, B., 2015, "Effect of blending ratio of polymer layer on structural properties of polymer-coated mesoporous silica nanoparticles", *International Nanotechnology Conference (NANOTECH FRANCE 2015). 15*-17 June 2015, Pole Universitaire Leonard de Vinci, La Defense, Paris, France, pp. 18.

5. Ünal, Ş. and Özbek, B., 2014, "Synthesis optimization and characterization of polymer-coated mesoporous silica nanoparticles", *21st International Congress of Chemical and Process Engineering (CHISA 2014).* 23-27 August 2014, Prague, Czech Technical University, Czech Republic, P5.43.

6. Ünal, Ş. and Özbek, B., 2013, "Adsorption Characteristics of Reactive Blue 19 from Aqueous Solution onto Sesame and Safflower Seed Cakes", *1st International Porous and Powder Materials Symposium and Exhibition (PPM 2013).* 3-6 September, İzmir, Turkey, pp. 92.

7. Ünal, Ş., Özbek, S. and Özbek, B., 2013, "Adsorption Characteristics of Reactive Blue 19 from Aqueous Solution onto Modified Commercial Activated Carbon", *1st International Porous and Powder Materials Symposium and Exhibition (PPM 2013).* 3-6 September, İzmir, Turkey, pp. 108.

8. Bal, H., Balikcioglu, A, Karacakol, M., Ünal, Ş., 2012, "Atık Portakal ve Limon Kabuklarından, Düşük Maliyetli, Çevre Dostu, Biyobozunur, Yenilebilir, Güçlü Antimikrobiyal Özellikli Yeni Bir Gıda Ambalaj Filmi Eldesi", *Bilim ve Bahar Şenliği Ispartek Proje Pazarı,* 16-18 Mayıs 2012, Süleyman Demirel Üniversitesi, Isparta, Türkiye, pp. 075.

Projects

1 Özbek, B., Kılıç, D.K., Demirhan, E., and Ünal, Ş., 2015, **"**α-Amilaz, alkalaz ve β-galaktozidaz enzimlerinin çeşitli adsorban yüzeylerine immobilizasyonunun optimizasyonu ve immobilize enzimlerin sıcaklık, pH ve zaman stabilitelerinin incelenmesi", BAP, Yıldız Teknik Üniversitesi, 2012-07-01-KAP02.

2 Özbek, B. and Ünal, S., 2015, "Polimer Kaplı Mezo Gözenekli Silika Nanoparçacık Sentezi, Karakterizasyonu ve Enzim İmmobilizasyonunda Kullanımı", BAP, Yıldız Teknik Üniversitesi, 2014-07-01-YL02.