

**PREPARATION OF TYROSINASE IMMOBILIZED
CHITOSAN AND GELATIN FILMS FOR
DETECTION OF PARATHION-METHYL**

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ABSTRACT

PREPARATION OF TYROSINASE IMMOBILIZED CHITOSAN AND GELATIN FILMS FOR DETECTION OF PARATHION-METHYL

In this work, tyrosinase immobilized chitosan and gelatin films as optical enzyme sensors which were characterized and optimized by UV spectrophotometer, were developed for detection of the pesticide parathion-methyl. The results show that the best sensor was the chitosan supported ones (TCA) prepared by adsorption method due to higher responsiveness and higher catalytic activity. The prepared sensors used for 10 times without significant loss in activity indicate good operational stability. Immobilization of the enzyme to chitosan support increases the pH, temperature and storage stability with respect to free enzyme due to providing structural rigidity and appropriate flexibility by immobilization. The other indication is that, isopropyl alcohol is the most appropriate organic solvent to solve the pesticide due to showing higher solubility for parathion-methyl and lowest inhibition (decrease in activity) for the enzyme. TCA has wide linear detection range and it can detect the parathion methyl concentration below the Maximum Residue Limit (MRL). The decrease in enzyme activity was obtained up to 10 mg/l (ppm) parathion-methyl concentration. The other result is that, kinetics of the immobilized enzyme was reaction controlled for higher substrate concentrations as understood from effectiveness factor, η . Atomic force microscopy (AFM) and FTIR analysis show that tyrosinase enzyme was successfully coated on the surface of chitosan and gelatin films. After using the optical enzyme sensor the binding of product o-quinone to chitosan and gelatin films were observed which was characterized by AFM results and the UV visible absorption peaks.

ÖZET

PARATİON-METİL TAYİNİ İÇİN TİROZİNAZ TUTTURULMUŞ KİTOSAN VE JELATİN FİLMLEİNİN HAZIRLANMASI

Bu çalışmada, organofosforlu tarım ilaçlarını tayin etmek için UV spektrofotometre kullanılarak karakterize ve optimize edilen tirozinaz enzimi tutturulmuş kitosan ve jelatin filmleri optik enzim sensörü adı altında geliştirilmiştir. Sonuçlar göstermiştir ki, en iyi sensör yüksek hassasiyet ve yüksek katalitik aktivite gösterdiğinden dolayı adsorpsiyon metodu ile hazırlanan kitosan destekli olanıdır (TCA). Hazırlanan sensörlerin aktivitede önemli kayıplar olmaksızın 10 defa kullanılması kullanım kararlılığının iyi olduğunun göstergesidir. Enzimin kitosan desteğine tutturulması yapısal sertlik ve uygun esneklik sağladığından enzimin pH, sıcaklık ve depolama kararlılığını serbest enzime göre arttırmıştır. Diğer bir bulgu şudur ki, izopropil alkol paratyon-metil için yüksek çözünürlüğe sahip olması ve enzim için asgari engelleme (aktivitede düşüş) göstermesinden dolayı tarım ilaçlarının çözünmesinde kullanılacak en uygun organik çözücüdür. TCA geniş bir aralıkta doğrusallığa sahiptir ve sensör paratyon-metil konsantrasyonunu izin verilen Azami Miktardaki Kalıntı Limitlerinin (MRL) altında tespit edebilmektedir. Enzim aktivitesindeki düşüş 10 mg/l (ppm) paratyon-metil konsantrasyonuna kadar gözlemlenebilmektedir. Diğer bir sonuç şudur ki, tutturulan enzimlerin kinetiği yüksek substrat konsantrasyonları için verimlilik katsayısından, η anlaşılacağı üzere reaksiyon kontrollüdür. Atomik Güç Mikroskopisi (AFM) ve FTIR analizi göstermiştir ki, tirozinaz enzimi kitosan ve jelatin filmlerinin üzerine başarılı bir şekilde kaplanmıştır. Optik enzim sensörlerinin kullanımından sonra oluşan o-kuinonların kitosan ve jelatin filmlerinin üzerine bağlandığı AFM sonuçları ve UV görünür absorpsiyon pikleri ile karakterize edilerek gözlemlenmiştir.

TABLE OF CONTENTS

LIST OF FIGURES	viii
LIST OF TABLES	xi
CHAPTER 1. INTRODUCTION	1
CHAPTER 2. ENZYMES	5
2.1. Tyrosinase Enzyme	10
2.2. Enzymatic Biosensor	14
2.3. Enzyme Immobilization Strategies	18
2.3.1. Chemical Methods	20
2.3.2. Physical Methods	21
2.4. Properties of Immobilized Enzyme	23
2.5. Selection of Support Material	24
2.5.1. Chitosan as Support	25
2.5.2. Gelatin as Support	26
CHAPTER 3. PESTICIDES	28
3.1. Detection of Pesticide Residue by Enzymatic Biosensor	32
CHAPTER 4. THEORETICAL ESTIMATION OF SOME KINETIC PROPERTIES FOR ENZYME IMMOBILIZED FILMS	35
4.1. Characterization of Kinetic Parameters	35
4.2. Characterization of Mass Transfer Resistance	38
CHAPTER 5. EXPERIMENTAL	42
5.1. Materials	42
5.2. Methods	42
5.2.1. Preparation of the Solutions	42
5.2.2. Preparation of Optical Enzyme Sensor	43
5.2.2. Enzyme Assay	44

CHAPTER 6. RESULTS AND DISCUSSIONS	47
6.1. Free Enzyme Studies	47
6.1.1. The Effect of pH and Temperature on Tyrosinase Activity	47
6.1.2. The Effect of Organic Solvents on Tyrosinase Activity.....	48
6.1.3. Kinetic Analysis of Free Tyrosinase.....	51
6.2. Immobilized Enzyme Studies	52
6.2.1. The Effect of Immobilization Methods on Tyrosinase Activity....	52
6.2.2. The Development of the Optic Enzyme Sensors.....	54
6.2.3. Kinetic Analysis of Optical Enzyme Sensor	58
6.3. Chitosan Supported Optic Enzyme Sensor (TCA)	59
6.3.1. Storage Stability of TCA	59
6.3.2. The Effect of pH and Temperature on The Activity of TCA	60
6.3.3. The Effect of Organic Solvents on The Activity of TCA.....	62
6.3.4. Kinetic Analysis of TCA in Organic Solvent Media.....	62
6.4. Determination of Parathion-Methyl by TCA.....	66
6.5. Characterization of The Films and Optical Enzyme Sensors	68
6.5.1. Chitosan film (C) and TCA	68
6.5.2. Gelatin film (G) and TGE.....	74
6.6. Mechanism of enzymatic reaction	77
CHAPTER 7. CONCLUSIONS	79
REFERENCES	81
APPENDIX A. DETERMINATION OF MAXIMUM ABSORBANCE PEAK FOR THE PRODUCT O-QUINONE.....	88
APPENDIX B. DETERMINATION OF RELEASE ENZYME AMOUNT FROM THE FILM DURING THE WASHING STEP.....	91
APPENDIX C. DETERMINATION OF SOLUBLE AMOUNT OF PARATHION METHYL IN ORGANIC SOLVENTS	92

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
Figure 2.1. Formation of peptide between two adjacent α -amino acids.....	5
Figure 2.2. Hierarchy of protein structure; primary structure (a), secondary structure (b), tertiary structure (c).....	7
Figure 2.3. Subunit structures of <i>S. castaneoglobisporus</i> tyrosinase (in red: the mature tyrosinase protein and in blue: the carrier protein	11
Figure 2.4. Schematic representation of binuclear copper site, C:Cu ion, O:oxygen, and H:His-N	11
Figure 2.5. Basic reaction mechanism of tyrosinase. E _{met} -D: mettyrosinase o-diphenol binding complex, E _{oxy} -D: oxytyrosinase o-diphenol binding complex	13
Figure 2.6. Calibration curve for an enzymatic biosensor with a linear range showing lower detection limit	18
Figure 2.7. Classification of immobilization method	20
Figure 2.8. Structure of chitin and chitosan.....	26
Figure 2.9. Soluble and insoluble form of chitosan.....	26
Figure 2.10. Chemical structure of the gelatin.....	27
Figure 3.1. Three examples of organophosphates (a) Malathion (b) Parathion-methyl (c) Dichlorvos	30
Figure 4.1. Substrate and product profiles in the immobilized enzyme system as a consequence of partition and mass transfer limitations under static condition	40
Figure 6.1. Change in free enzyme activity with pH at 25 °C	47
Figure 6.2. Change in free enzyme activity with temperature at pH 6.5.	48
Figure 6.3. Influence of organic solvent on free tyrosinase activity.....	49
Figure 6.4. The effect of substrate concentration (catechol) on free enzyme activity. Points: experimental, line: model. (T:26 °C and pH:6.5)	51
Figure 6.5. The enzyme released from (a) TCA (b) TGE E _i (μ g loaded/ μ g support): 0.1765 (x), 0.1175 (\blacktriangle), 0.0785 (\blacklozenge), 0.0387 (+), 0.0295 (-), 0.0282 (\bullet), 0.0118 (---), 0.0031 (\blacksquare).	54

Figure 6.6. Change in the amount of immobilized enzyme with loaded enzyme for (a) TCA (b) TGE.	55
Figure 6.7. Change in the activity with immobilized enzyme amount for (a) TCA (b) TGE.....	56
Figure 6.8. Operational stability of TCA and TGE	57
Figure 6.9. The effect of substrate concentration (catechol) on TCA and TGE Points: experimental, line: model (T:27 °C and pH:6.5).....	58
Figure 6.10. Storage stability of free enzyme (◆) and TCA (■)	59
Figure 6.11. The effect of pH on the activity of free enzyme (◆) and TCA (■) (T:25 °C)	60
Figure 6.12. The effect of temperature on the activity of free enzyme (◆) and TCA (■) (pH:6.5).....	61
Figure 6.13. Influence of organic solvents on immobilized tyrosinase activity	62
Figure 6.14. The effect of substrate concentration on the activity of TCA in solvent free media (a) and organicsolvent containing (10% v/v) media ethanol (b), methanol (c), isopropyl alcohol (d), acetone (e), acetonitrile (f). Points: experimental, line: model (T:27 °C and pH:6.5)	64
Figure 6.15. Solubility of parathion-methyl in organic solvent-water mixture (T=27 °C)	66
Figure 6.16. Relative activity of TCA in the presence of parathion-methyl. Inset is the linear range of the curve	66
Figure 6.17. The effect of substrate (catechol) concentration on TCA in the presence of 15 ppm parathion-methyl. Points: experimental, line: model (T:27 °C and pH:6.5)	67
Figure 6.18. AFM images of (a) C, (b) TCA (c) TCA after the enzymatic reaction.....	69
Figure 6.19. The images of TCA (a) before and (b) after the enzymatic reaction	70
Figure 6.20. FTIR spectra of chitosan powder (- - -) and chitosan film (—) in the range of 4000-400 cm ⁻¹	70
Figure 6.21. FTIR spectra of chitosan powder (- - -) and chitosan film (—)	71
Figure 6.22. FTIR spectra of chitosan film (—) and TCA (.....).....	72
Figure 6.23. FTIR spectra of the chitosan film (—), TCA before use (.....) and TCA after uses (-••-)	73
Figure 6.24. Reaction pathways for enzymatically generated o-quinone and nonenzymatic crosslinking reaction with chitosan	73

Figure 6.25. UV visible spectra of TCA before and after the enzymatic reaction	74
Figure 6.26. AFM images of (a) G, (b) TGE (c) TGE after the enzymatic reaction	75
Figure 6.27. The section TGE images (a) before and (b) after the enzymatic reaction..	76
Figure 6.28. Reaction pathways for enzymatically generated o-quinone and nonenzymatic reaction with gelatin	76
Figure 6.29. UV visible spectra of TGE before and after the enzymatic reaction.....	76
Figure 6.30. Variation of effectiveness factors as a function substrate concentration for TCA (■) and TGE (◆).....	78

LIST OF TABLES

<u>Table</u>	<u>Page</u>
Table 2.1. Structure and properties of amino acid side chains	6
Table 2.2. Advantage and drawbacks of enzymes as catalysts	9
Table 2.3. Classification of enzyme according to type of reaction catalyzed	10
Table 2.4. Commonly used transducers	15
Table 2.5. Some studies about the tyrosinase based optical enzyme sensors	16
Table 2.6. Comparison of immobilization methods	23
Table 2.7. Type of support materials	25
Table 3.1. Main side groups on different classes of organophosphate compounds.....	29
Table 3.2. Common Organophosphorus Pesticides, their formulas and synonyms	31
Table 3.3. Maximum residue limits for three different OP compounds and for three different crops	32
Table 4.1. Summary of the effect of reversible inhibitors on enzyme catalytic parameters V_m and K_m	38
Table 5.1. The solutions used in this study.....	43
Table 6.1. Properties of organic solvents used in this study.....	50
Table 6.2. Kinetic parameters of free tyrosinase in organic solvent (10% v/v) containing media.....	51
Table 6.3. Response characteristic of free tyrosinase enzyme	52
Table 6.4. The effect of immobilization method on the activity of optical enzyme sensor	53
Table 6.5. Comparison of the kinetic parameters for the free and immobilized tyrosinase	59
Table 6.6. Comparison of pH and temperature of free tyrosinase with TCA.....	61
Table 6.7. Response characteristic of TCA in solvent free and organic solvent containing media (10% v/v).....	65
Table 6.8. Sensing performance of TCA in parathion-methyl containing media.....	67
Table 6.9. Response characteristic of TCA in free and parathion-methyl containing media	68
Table 6.10. FTIR spectrums and their assignments.....	71

CHAPTER 1

INTRODUCTION

One of the most important problems in agricultural industry is to protect the food products from pest and herb. For this reason the use of pesticides become inevitable. Among pesticides, insecticides with high toxicity level such as azinphos-methyl, dichlorvos (DDVP), endosulfan, methamidophos, methyl-bromide and parathion-methyl are highly used (Delen et al. 2005). However the toxicity of pesticides comes out some problems. Their residues harmful for human health and ecological balance (Rekha et al. 2000). In Turkey the use of pesticides has been increasing at 270% from 1979 to 2007 (Durmusoglu et al. 2010). Hazardous foods have been launched due to the lack of pesticide residue controls (Çopur et al. 2010). Besides Turkey becomes second country among the 125 countries that are exported inconvenient food to European Union (Durmusoglu et al. 2010). In this manner the extensive and insensible uses of pesticides in agriculture increase the requirement for fast methods in residue analysis.

Chromatographic techniques such as GC, HPLC, or coupled techniques (GC-MS) currently used as reference methods in order to detect and measure pesticide residues at and below maximum residue limit (MRL) levels (Bicchi et al. 1996; Babicka et al. 1986). Besides the some advantages such as very high sensitivity, reliability and precision, these techniques require expensive instrumentation and highly trained personal, are time consuming, and are not easily adapted for in field analysis (Solna et al. 2005). Biosensors could be a reliable and promising alternative to the standard methods applied for detection of pesticide residue. They allow both real time and in situ monitoring (Lee et al. 2002; Ramirez et al. 2008).

Biosensors are usually designed by considering appropriate conditions for enzyme loading to ensure that sufficient biocatalyst activity is available. The biosensor should be easy to use and most important one is cost of the analysis mostly depends on the sensitivity of the enzyme electrode. (Watson 2004). Most of the studies have recently focused on the development of ideal low cost enzyme electrode which influenced by choice of sensor, the number of units to be manufactured, immobilization techniques and the nature of the enzyme (Nade et al. 2003).

Most of enzymes like tyrosinase and acetylcholinesterase are affected by several factors such as pH, which controls the distribution of charges in the active site, is especially critical for enzyme-catalyzed reactions, temperature that can cause inactivation of the enzyme and also organic solvents. Most enzymes are water soluble and a certain amount of water is necessary for the solubilization of them. In the case of low solubility of substrates or other reactant, organic solvents which influence activity of the enzymes have to be used. Adverse conditions of these factors can reduce or destroy the biological activity of the enzyme. (Illanes 2008; Yoruk and Marshall 2003; Drauz and Waldmann 2002). Therefore a great deal of research has been conducted towards the development of enzyme immobilization to extend shelf life and improved length of useful measurement times (Buerk 1993). Enzymes could be immobilized to the supports with entrapment, micro encapsulation, adsorption, covalent cross-linking and covalent binding methods. Wide range of supports such as organic (polyamides, nylon, polyacrylamide, polyethylene, silk, starch, agarose) and inorganic materials (zeolite, clays and metals) present for the immobilization of enzymes by using these methods (Drauz and Waldmann 2002). Among these supports chitosan has been widely used for enzyme immobilization which contains large groups of $-NH_2$ and $-OH$. This material is also preferable due to its excellent biocompatibility, nontoxicity, cheapness, easy-handling and high mechanical strength (Krajewska 2004; Dung et al. 2009; Bhatt et al. 2010). Gelatin is also another attractive supports for enzyme immobilization due to its high hydrophilicity, nontoxicity, requires only simple equipment and being relatively inexpensive (Seo et al. 2003; Peña et al. 2010). Both supports are transparent and available to study with most optical detection methods for pesticide determination. In this study chitosan and gelatin were used as supports materials due to their distinctive properties.

The detection of pesticide is possible by inhibiting the enzyme such as organophosphorous hydrolase, alkaline phosphatase, ascorbate oxidase, tyrosinase, acid phosphatase, butyrylcholinesterase and acetylcholinesterase (Du et al. 2007). The decline in the amount of enzymatically produced product is defined as inhibition of enzyme activity (Watson 2004). The conversion of substrate to product causes a change in optical, electrical or mechanical properties of the reaction system. Most of the studies were conducted by using electrochemical detection as technique and acetylcholinesterase as enzyme for pesticide detection. In the study of Du et al. (2007) this enzyme was immobilized by using glutaraldehyde as cross-linker to multiwall

carbon nanotubes–chitosan composite for determination of pesticide (triazophos). Vastarella et al. (2007) also prepared electrochemical biosensors by covalently immobilizing the acetylcholinesterase enzyme on different nylon pre-activated membranes by means of glutaraldehyde for the determination of pesticide (paraoxon). Different immobilization methods for this enzyme such as prussian blue modified screen-printed electrodes by cross-linking with glutaraldehyde (Suprun et al. 2005), immobilization in photocrosslinkable poly(vinyl alcohol) photopolymer, encapsulation in a sol–gel matrix or immobilization by metal–chelate affinity (Andreescu et al. 2002) were used to detect the pesticide in the literature. For these studies the inhibition of pesticides on enzyme was monitored by measuring the decrease in oxidation current of product (thiocholine). However these enzyme sensors show irreversible inhibition on enzyme activity leads to a rapid decrease in the sensitivity and performance (Nade et al. 2003). If the reactivation of the enzyme is impossible in this case disposable biosensors are designed. Tyrosinase (TYR) can be an alternative enzyme for the detection of pesticides. Vidal et al. (2008) reversibly immobilized the tyrosinase to 1,2-naphthoquinone by electropolymerization method to determine the dichlorvos and atrazine.

However electrochemical enzyme sensor is not a hydrodynamic technique. In the other words the solution is generally stagnant and hence process is limited by the diffusion of substrate. Cyclic voltammetry is popular methods among the electrochemical biosensor and has the disadvantage such that processes occurs at a given potential can depend on preceding potentials (Koryta et al. 1993). These techniques are also relatively expensive. Photometric enzyme assay has the advantage to be fast, easily manageable, convenient, capable of rapidly providing accurate and reproducible results, provide controlling the mass transfer by stirring the solution and moreover cheap in performance with respect to the electrochemical methods. Enzyme assays based on changes in the absorbed light are more frequently used among the photometric methods (Walz and Schwack 2007; Eisenthal and Danson 2002). Optical biosensors prepared by tyrosinase immobilized on a nylon membrane (Russell and Burton 1999) jellose/agarose membrane (Paranjpe et al. 2001) and pyrrole matrix (Narlı et al. 2006) have been reported. In these studies phenol was detected based on the reaction between the enzymatically produced *o*-quinone and 3-methyl-2-benzothiazolinone hydrazone (MBTH) result in maroon color product. The use of MBTH or other nucleophilic compounds provide stable stoichiometric adduct with the

o-quinones. Because o-quinones are very unstable and undergo to a sequence of non-enzymatic reactions within the short time (Fiorentino et al. 2010). However, some MBTH-quinone adducts shows solubility problems. Methods rely on spectrophotometric measurement of o-quinone formation (do not include any nucleophilic compounds) are rapid, practical and perhaps more accurate (Yoruk and Marshall 2003). In this respect it is significant issue to measure the initial reaction rate which is linear for only a short period in the absence of nucleophilic compounds. In this study the change in the absorbance of enzymatically produced product was monitored without using any nucleophilic compounds. The reaction was initiated by stirring the reaction media while the spectrophotometric measurement was started (in situ analysis).

Based on all of these considerations the aims of this study are;

- To prepare tyrosinase immobilized chitosan and gelatin films as optical enzyme sensors for detection of Parathion-methyl (by utilizing the enzyme inhibition ability of pesticide), a model pesticide.
- To investigate the performance of the sensors by measuring their activities with respect to pH, temperature, organic solvent tolerance, storage and operational stabilities without using any nucleophilic compounds.
- To investigate the inhibition mechanism exerted by organic solvents and parathion-methyl by characterizing the apparent kinetic parameters of the enzyme catalyzed reaction.

CHAPTER 2

ENZYMES

Enzymes are proteins composed by a number of amino acid residues. Peptide bond formation occurs between carbon atom of the carboxyl group of one amino acid and the nitrogen atom of the α -amino group of the following as depicted in Figure 2.1.

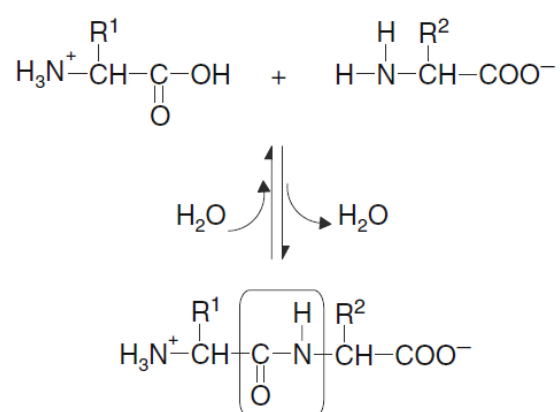
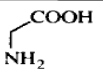
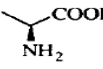
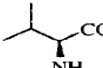
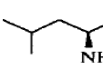
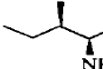
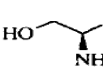
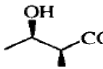
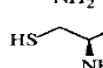
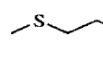
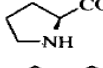
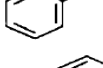
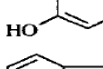
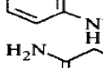
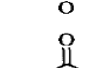
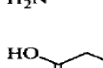
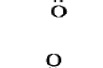
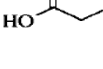
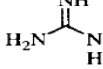
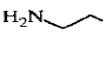
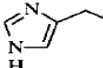


Figure 2.1. Formation of peptide between two adjacent α -amino acids

The distribution of amino acids (can be non-polar (hydrophobic) or polar (charged or uncharged) depending on the nature of the R group) along the protein molecule determines its behavior. Table 2.1 shows the general structure of amino acids. Primary structure is related with the amino acid sequence of protein while secondary three-dimensional structure is the result of interactions of amino acid residues proximate in the primary structure (for enzymes, these interactions dictate a predominantly ribbon-like coiled configuration termed α -helix). The interactions of amino acid residues located apart in the primary structure formed the tertiary three-dimensional structure which is essential for the biological functionality of the protein. Figure 2.2 shows Hierarchy of protein structure.

Table 2.1. Structure and properties of amino acid side chains
(Source: Drauz and Waldmann 2002).

Name	Symbol	Structure	pK _a of ionizing side chain ^a
Glycine	Gly (G)		
Alanine	Ala (A)		
Valine	Val (V)		
Leucine	Leu (L)		
Isoleucine	Ile (I)		
Serine	Ser (S)		
Threonine	Thr (T)		
Cysteine	Cys (C)		9.1–9.5
Methionine	Met (M)		
Proline	Pro (P)		
Phenylalanine	Phe (F)		
Tyrosine	Tyr (Y)		9.7
Tryptophane	Trp (W)		
Asparagine	Asn (N)		
Glutamine	Gln (Q)		
Aspartate	Asp (D)		4.5
Glutamate	Glu (E)		4.6
Arginine	Arg (R)		~ 12.0
Lysine	Lys (K)		10.4
Histidine	His (H)		6.2
α-amino group			6.8–7.9
α-carboxyl group			3.5–4.3

^a: The pK_a values depend on temperature, ionic strength and, especially on the microenvironment of the ionizable group

The main types of interactions responsible for the three-dimensional structure are

- Hydrogen bonds which is main determinant of the helical secondary structure and plays a significant role in tertiary structure as well
- Apolar interactions which is rather weak however, contribute to the stabilization of the three dimensional structure.
- Disulphide bridges relevant in the stabilization of the three-dimensional structure
- Ionic bonds between charged amino acid residues which contribute to the stabilization of the three-dimensional structure
- Other weak type interactions, like van der Waals forces, whose contribution to three-dimensional structure is not considered significant.

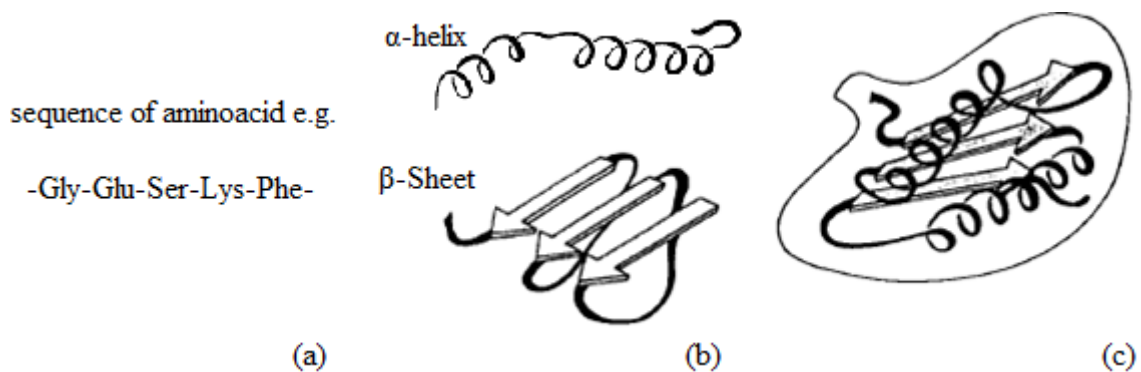
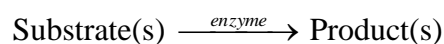


Figure 2.2. Hierarchy of protein structure; primary structure (a), secondary structure (b), tertiary structure (c).

The only difference between enzymes and proteins is that enzymes possess catalytic activity. Enzymes are biological catalysts that facilitate the conversion of substrates into products by providing favorable conditions that lower the activation energy of the reaction. The regions of the enzyme that are directly involved in the catalytic process are called the active sites. This small part is usually hydrophilic cleft or cavity formed by very few amino acid residues. Substrate is bound to the active site of enzyme and changes in the distribution of electrons in its chemical bonds cause the reactions that lead to the formation of products. Then products are released from the enzyme and therefore enzyme is ready for the next catalytic cycle.



Enzyme activity defined as the capacity of an enzyme to catalyze a chemical reaction is strictly dependent on its molecular structure. Some compounds can decrease (inhibitors) or increase (activators) the enzyme activity. The change in enzyme activity can be irreversible, reversible or partially reversible. For instance irreversible inhibitors (poisons) completely inactivate the enzyme (process called inactivation). On the other hand reversible inhibitors reduce enzyme activity and when the inhibitor is removed enzyme activity is restored (no chemical changes occur in the protein molecule). Partial reversibility occurs if some of enzyme activity is restored when the inhibitor is removed. The term “inhibition” is used for fully reversible or partially reversible processes. Factors that affect enzyme activity are also

- The nature of the enzyme and substrate
- The concentrations of enzyme and substrate
- pH
- Temperature
- Organic solvents
- Heavy metals and chelating agents
- External factors such as irradiation and shear stress.

Adverse conditions of these factors can reduce or destroy its biological activity by distorting the proper active site configuration. (Illanes 2008; Missen et al. 1999)

Enzymes contain many polar amino acids which may be protonated or unprotonated depending on medium pH. Change in pH can change the distribution of charges in the active site which may prevent proper conformation of the active site, binding of substrates, and/or catalysis of the reaction. Temperature also affects both enzyme activity and stability. At low temperatures slower reaction observed due to decrease in kinetic energy of the reactant molecules. An increase in temperature increases the rate of the chemical reaction. At higher temperatures the concentration of active enzyme decreases. Thermal inactivation can occur as a consequence of the weakening of the intermolecular forces responsible of the preservation of its three-dimensional structure. Inactivation may involve covalent or non-covalent bond disruption with subsequent molecular aggregation or improper folding. At extremes pH or temperature values the enzyme activity tends to decrease (Illanes 2008; Yoruk and Marshall 2003). Mechanical forces such as shear and surface tension can disturb the the

shape of the enzyme molecule and this may drastically affect enzyme activity (Missen et al. 1999).

Water levels also have important effects on enzyme behavior. In the case of too little water the catalytic activity significantly decreases while enzyme stability increases. At high water levels catalytic activity also falls. This is because water result in mass transfer limitation by promoting agglomeration of catalyst particles, or may act as a competitive inhibitor for any of the substrates. It is difficult to prepare desired water content. Sometimes organic solvents have to be used. Because substrates may not be sufficiently soluble in water or desired reaction may be favorable in the media containing organic solvent which influence activity and stability of enzymes. In recent years, researcher shows that the majority of bulk water in a reaction system may be replaced by organic solvents which influence dielectric properties of the reaction medium responsible for the ordered water structures. (Drauz and Waldmann 2002).

Consequently the medium pH, temperature, solvent concentration (if necessary), water content, flow rates and stirrer speeds must be controlled and optimize to obtain high enzyme productivity.

Enzymes display distinct properties when compared with chemical catalysts due to their complex molecular structure. The advantages and drawbacks of enzymes as catalysts are summarized in Table 2.2.

Table 2.2. Advantage and drawbacks of enzymes as catalysts
(Source: Illanes 2008).

Advantages	Drawbacks
High specificity	High molecular complexity
High activity under moderate conditions	High production costs
High turnover number	Intrinsic fragility
Highly biodegradable	
Generally considered as natural products	

Enzymes can be classified into six main classes according to the type of reaction catalyzed as depicted Table 2.3.

Table 2.3. Classification of enzyme according to type of reaction catalyzed
(Source: Missen et al. 1999; Drauz and Waldmann 2002)

Class	Type of reaction catalyzed
Oxidoreductases	Various types of oxidation-reduction reactions (dehydrogenases, oxidases, oxygenases and peroxidases)
Transferases	Transfer of functional groups, such as aldehydic or acyl groups
Hydrolases	Hydrolysis reactions (proteases, amylases, acylases, lipases, and esterases)
Isomerases	Isomerization and transfer reaction within one molecule (glucose isomerase)
Ligases	ATP (adenosine triphosphate) as a cofactor, lead to the formation of bonds between carbon and other atoms, including carbon, oxygen, nitrogen, and sulfur (glycine ligase)
Lyases	Addition of chemical groups onto double bonds (fumarase, aspartase, decarboxylases, dehydratases, and aldolases)

Among them oxidoreductases have several of technological significance. This class of enzymes catalyze the oxidation/reduction reactions that involve the transfer of electrons, hydrogen or oxygen atoms. Recently electrochemical study of redox enzymes has attracted due to the significance of bioscience and their potential applications in biosensors and biocatalysts. Polyphenol oxidases are immobilized to several supports to construct the enzymatic biosensors. (Mohammadi et al. 2009; Fiorentino et al. 2010)

2.1. Tyrosinase Enzyme

Tyrosinase enzyme is a binuclear copper cluster containing monooxygenase having monophenolase activity (catalyzes o-hydroxylation of monophenols) and diphenolase activity (subsequent oxidation of the formed o-diphenols into o-quinones by consuming the molecular oxygen). Later on, the enzymatic product quinones may undergo nonenzymatic autopolymerization to produce colored compounds melanin (Aytar and Bakir 2008; Xue et al. 2008; Khan et al. 2007; Munoz et al. 2008).

Tyrosinases have been characterized according to its sources, including bacteria, fungi, plants and mammals. The exact structure of tyrosinase is still unknown. However

two related proteins (hemocyanin and catechol oxidase) containing the same type-3 copper according to the spectroscopic similarities. Figure 2.3 shows three-dimensional structures of tyrosinase from *S. castaneoglobisporus* (type 3 copper proteins). The central domain with the active site consists of the α -helical structure which surrounds the catalytic binuclear copper centre. Binuclear copper site serve as substrate binding centre is located in the bottom of the large concavity according to tertiary structures of tyrosinase (Selinheimo 2008).

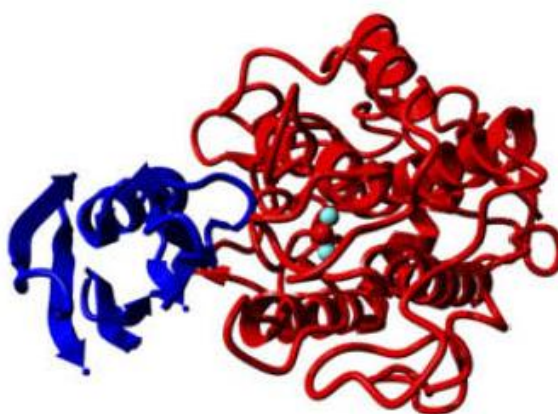


Figure 2.3. Subunit structures of *S. castaneoglobisporus* tyrosinase (in red: the mature tyrosinase protein and in blue: the carrier protein)

Figure 2.4 shows the binuclear copper active site of tyrosinase enzyme. The active site contains a dicopper core. Six nitrogen donor atoms from histidine residues (His-N) bind a pair of copper ions which interacts with both molecular oxygens (O_2). (Garcia-Molina et al. 2007; Koval et al. 2006).

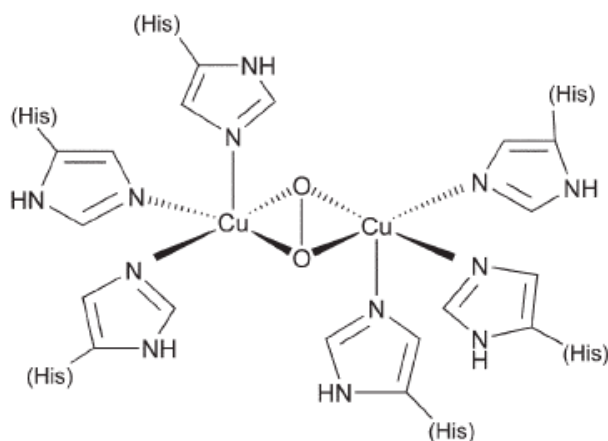


Figure 2.4. Schematic representation of binuclear copper site, C:Cu ion, O:oxygen, and H:His-N

Three forms can exist in the active site. These are met-tyrosinase, oxy-tyrosinase, and deoxy-tyrosinase. In the met-form hydroxyl ion is bound to the active site in which the coppers are in the form of $\text{Cu}^{2+}\text{-Cu}^{2+}$. In the oxy-form molecular oxygen is bound to the active site and oxygen is bridged as peroxide between the copper atoms. The enzyme is deoxy-form in instable state which immediately binds molecular oxygen to give the oxy-form. In nature the enzyme usually consists of 85-90 % of a met-form, and the rest is an oxy-form (Selinheimo E. 2008). Mushroom tyrosinase has a molecular mass of 120 kD and isoelectric point value of 4.5. This enzyme takes a negative charge at $\text{pH} > 4.5$ in aqueous solution. The pH range from 4-8 enzyme is more active and activity significantly decrease at a higher acid environment (Mohammadi et al. 2009; Xie et al. 2003; Yoruk and Marshall 2003).

Tyrosinase activity can be measured by several methods (Munoz et al. 2008), including

- Using nucleophilic reagents that trap the o-quinones and generate chromophoric adducts
- Using reducing agents such as ascorbic acid, measuring their disappearance as a result of oxidation by the quinones
- Measuring the consumption of oxygen
- Directly measuring the formation of o-quinones or their products

O-quinone can be enzymatically produced from mono-, di- and trihydroxyphenols as substrate by using tyrosinase (Munoz et al. 2008). However this enzyme shows greater affinity for dihydroxyphenols. In addition to this, it was also reported that catechol result in maximum activity as a substrate among the monohydroxyphenols (*p*-cresol and tyrosine), dihydroxyphenols (catechol, L-dopa, D-dopa, catechin, and chlorogenic acid), and trihydroxyphenols (pyrogallol). This suggests that enzyme exhibits highest activity when catechol (o-diphenol) was used as substrate (Seo et al. 2003).

Figure 2.5 shows the basic reaction mechanism of tyrosinase on *o*-diphenols. Firstly *o*-diphenol molecule (D) binds the native form of the enzyme (met-tyrosinase) to form the complex ($\text{E}_{\text{met}}\text{-D}$). The oxidized form of D is released as product (*o*-quinone) and the enzyme is reduced to the deoxy form (deoxy-tyrosinase). This form binds to an oxygen molecule (oxy-tyrosinase), which reacts again with another D and produces the complex $\text{E}_{\text{oxy}}\text{-D}$. Then *o*-quinone is to be produced by the oxidation of D and the

enzyme returns to its native form (E_{met}) (Mohammadi et al. 2009, Garcia-Molina et al. 2007).

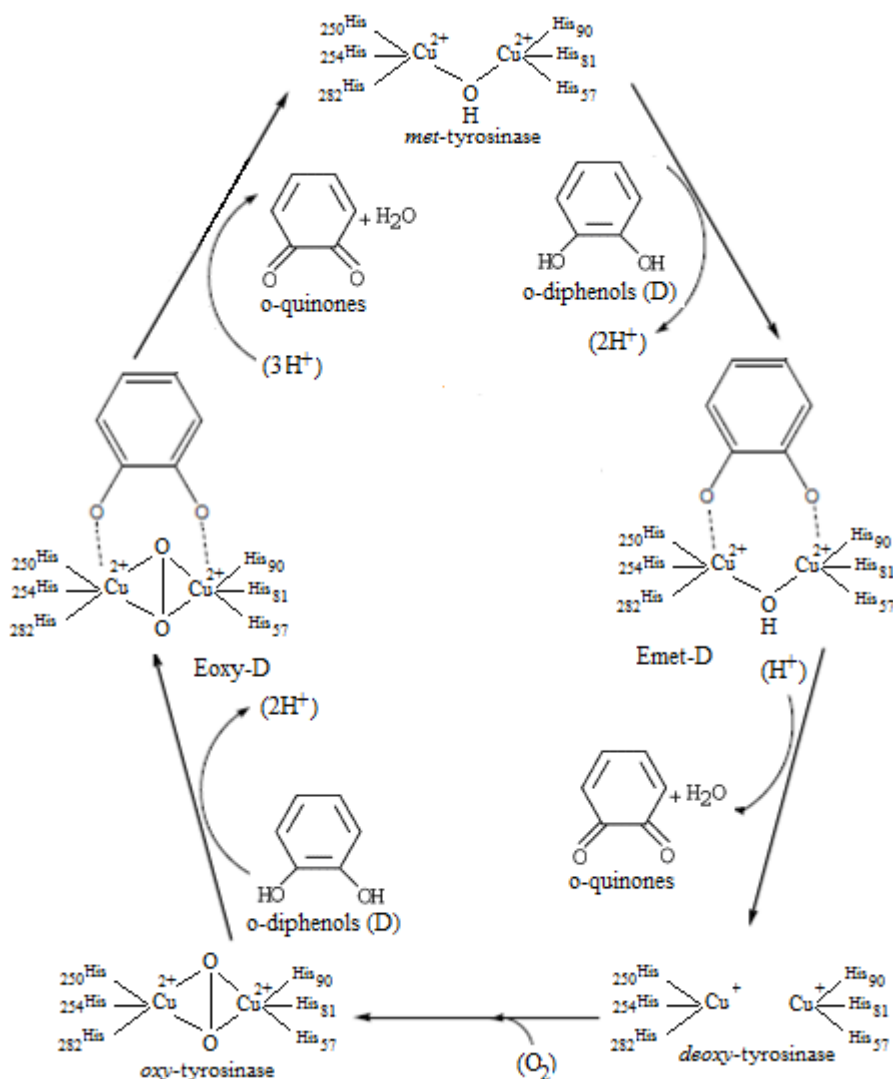


Figure 2.5. Basic reaction mechanism of tyrosinase. E_{met} -D: mettyrosinase o-diphenol binding complex, E_{oxy} -D: oxytyrosinase o-diphenol binding complex

The initial reaction rate is linear for only a short period when o-diphenols is used as substrate. This may be because quick reaction inactivation (suicide inactivation: o-semibenzoquinone free radicals and intermediate products disintegrate the active site's histidine residue(s) consequently tyrosinase enzyme becomes inactive) and irreversible binding of products to the active site of tyrosinase through a Michael reaction (Yoruk and Marshall 2003).

Tyrosinase enzyme have a wide application area including pharmaceutical drug production such as L-DOPA used in the treatment of Parkinson's disease, prodrug

therapy for melanoma, potential tools in treating melanoma patients, Tailoring properties of polymers, using as antibody microarrays, detoxification of phenolic compounds and the detection of phenols by biosensor preparation (Selinheimo 2008; Aytar and Bakir 2008; Xue et al. 2008).

In animals, plants and lower organisms tyrosinases are involved in the melanin pathway starting from l-tyrosine or l-DOPA as substrate leading to the formation of l-dopachrome. In animals, tyrosinase is responsible for melanization (for instance pigmentation in skin, eye, and hair). In some vegetables and fruits, tyrosinase activity is also responsible for browning reactions which cause undesired darkening results in a loss of nutritional and market values (Fiorentino et al. 2010; Selinheimo 2008; Xie et al. 2003).

2.2. Enzymatic Biosensor

Enzymatic biosensors are analytical devices that use a biological element fixed on sensor surface to interact with the analyte leading to produce a measurable output that can be detected by a transducer. Measurable signal generated by transducer can be monitored by several ways such as hydrogen peroxide formation, changes in NADH concentration, oxygen consumption, fluorescence, absorption, conductivity, pH change, temperature or mass. Biosensor can be classified in several types according to its transduction element outlined in Table 2.4.

Among them, two common forms electrochemical and optical transducers used for pesticide analysis. Electrochemical transducers generate a current or voltage measured proportional to the concentration of analyte in the sample. Optical ones generate a signal measured as a light intensity in proportion to the analyte. Both of them may be an inverse relationship. The change in optical properties (such as UV/vis absorption, reflectance and fluorescence) of analyte is monitored in biosensors based on optical methods

Table 2.4. Commonly used transducers
(Source: Mulchandani and Rogers 1998).

Transducer	Output
Electrochemical	
Amperometric	Applied current
Potentiometric	Voltage
Conductimetric	Impedance
Optical	
Colorimetric	Color
Luminescence	Light intensity
Fluorescence	Light intensity
Calorimetric	
Thermistor	Temperature

Enzymes are frequently used as sensing elements in biosensors due to their specificity and catalytic properties. The first enzyme-based sensor was described by Clark and Lyons, who developed amperometric enzyme electrode for glucose. Enzymatic biosensors have been constructed by integrating the variety of enzymes belonging to classes of oxido-reductases, hydrolases, and lyases to different transducers for applications in health care, veterinary medicine, food industry and environmental monitoring (Watson 2004; Mulchandani and Rogers 1998). Recently studies with optical methods to detect polyphenol oxidase (PPO) activity attract attention. Spectrophotometric measurement of o-quinone formation from o-diphenolic compounds by using tyrosinase enzyme is most commonly assayed (Mohammadi et al. 2009; Yoruk and Marshall 2003). In the literature there are some studies about the use of optical biosensor constructed by immobilization of tyrosinase enzyme on different supports as summarized in Table 2.5.

Table 2.5. Some studies about the tyrosinase based optical enzyme sensors

Reference	Enzyme	Support	Application	Measuring techniques
Kıralp et al. 2003	Tyrosinase	Entrapment of enzyme to copolymers of thiophene functionalized menthyl monomer (MM) with pyrrole by electrochemical polymerization coated platinum electrode	Determination of phenolic compounds in wines	UV at 495 nm (Besthorn's Hydrazone method). Optical biosensor immersed in citrate buffer + MBTH + catechol with different concentrations
Rajesh and Kaneto, 2005	Tyrosinase	Covalent attachment of enzyme to the poly-pyrrole film on a stainless steel	Development of novel amine functional poly-pyrrole based biosensor	UV at 280 nm in PBS (pH:6.5)
Abdullah et al. 2006	Tyrosinase	Bi-layer which consist of immobilized MBTH in composite nafion/sol-gel silicate film and immobilized enzyme in chitosan film on glass slide	Detection of phenol	UV at 490 nm. (Besthorn's Hydrazone method) Optical biosensor immersed in 3 ml PBS and phenol
Narlı et al. 2006	Tyrosinase	Entrapment of enzyme to poly (terephthalic acid bis-(2-thiophen-3-yl ethyl) ester) (PTATE) with pyrrole as the matrix by electropolymerization coated platinum electrode	Determination of phenolic compounds in red wines	UV at 495 nm (Besthorn's Hydrazone method). Optical biosensor immersed in citrate buffer + MBTH + catechol with different concentrations.
Abdullah et al. 2006	Tyrosinase	Physical adsorption of enzyme to chitosan film coated on glass slide via spin coating	Detection of phenol	UV at 490 nm. (Besthorn's Hydrazone method) Optical biosensor immersed in 3 ml PBS (pH:6.7) and 0.2 mM MBTH (color reagent) and phenol
Ayter and Bakır, 2008	Tyrosinase	Cross-linked enzyme aggregate (CLEA) via precipitation with ammonium sulfate and crosslinking with glutaraldehyde.	Immobilization of enzyme	UV at 420 nm . 0.1 M catechol solution
Fiorentio et al. 2010	Tyrosinase	Cationic polymer poly (dimethyldiallylammonium chloride) (PDDA) on quartz slide by LBL method which provide electrostatic interactions with enzyme	Detection of phenol	UV at 475 nm over first 2 min. Optical biosensor immersed in 2.5 ml PBS (pH:6.7) including substrate without exogenous reagent

Tyrosinase-based optical biosensors have been developed and their performances were investigated by the researchers as outlined in Table 2.2.2. The aim is to obtain the fast, sensitive and inexpensive enzyme biosensor. Therefore the optimum design of enzyme sensor is very important to detect and measure the analyte at and below maximum residue limit (MRL) levels. Optimization of enzyme sensor can be defined as finding the best design or elite designs by taking into consideration the several basic physical properties of the measuring system summarized as; (Cuyper and Bulte 2002; Newman and Setford 2006).

- *Sensitivity*; defined as the magnitude of the enzyme sensor output signal at final steady state change with respect to change in concentration of analyte (slope of the linear range: $\Delta S / \Delta C$) as illustrated in Figure 2.6.
- *Linearity*; an enzyme sensor has to be wide linear range to be practically useful. Hence, linear biosensor will have a constant sensitivity over the substrate concentration range from zero to maximum.
- *Detection limit*; the analyte concentrations must be detectable for a desired application. Therefore, Ideal enzyme sensor can detect the lowest concentration of analyte
- *Stability*; the length of the time that biosensor remain sensitive under normal operational conditions must be long term (storage stability). Also biosensor has to be used for several times without a significant loss of its function (operational stability). The life time may be dependent on the total measurements number.
- *Selectivity and quick response*; the ideal enzyme sensor should have high selectivity and quick response which depend on diffusion of the chemical species to the active surface of the transducer. The changes in analyte concentrations have to be sensed in a short time, and biosensor also should not be influenced by the other chemical species.
- *Hysteresis*; Enzyme sensor would have zero hysteresis or in the other words it should not be affected by its past history of measurements
- *Easy to use and cost effectiveness*; an ideal biosensor is easily designed and being cheaper. It should not require expensive instrumentation and highly

trained personnel, are not time consuming, and are easily adapted for in field analysis.

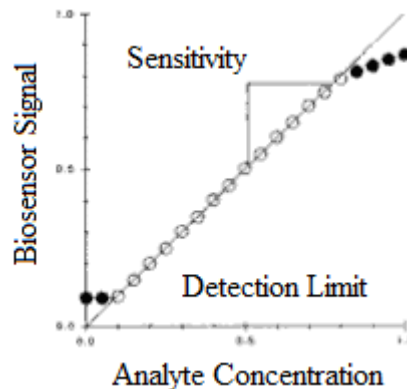


Figure 2.6. Calibration curve for an enzymatic biosensor with a linear range showing lower detection limit.

The success and performances of enzyme sensors are largely depend on the enzyme immobilization techniques. Many different strategies have been used to stabilize the enzyme molecules depending on the nature of the support and the biological molecule (Watson 2004).

2.3. Enzyme Immobilization Strategies

Immobilization is the most commonly used strategy to provide desirable features for enzymes. Enzyme immobilization can be defined as the conversion of an enzyme to a form restricted mobility (allows for containment and recovery of the enzyme) and retention of catalytic function.

The advantages of immobilizations are:

- It is possible to use enzyme repeatedly or continuously.
- Enzymes can be easily separated from reaction media which prevent contamination of the final product.
- pH, temperature, storage stability and catalytic properties can be enhanced.
- Control of microbial contamination becomes simpler.

However there are some practical limitations for immobilized enzymes such as;

- The yield of protein binding is rarely quantitative.
- The cost of the support may exceed the cost of the enzyme.

- Decrease in enzyme activity observed in immobilized form due to chemical modification of the protein, steric hindrance and mass transfer limitations.
- The proportion of active enzyme rarely exceeds 5-10 % w/w in immobilized form, which reduces catalytic activity per weight of solid.

One of the most important issue in immobilization is to enable the substrate accommodation to the active site of the tyrosinase which depends on structural rigidity (allowing the retention of a specific 3D conformation) and flexibility (allowing the protein to perform its catalytic function) of the enzyme. Therefore the choice of immobilization technique becomes significant and important considerations (reducing the limitations, proper balance between structural rigidity and flexibility) have to be taken into account when enzyme is immobilized to the support (Drauz and Waldmann 2002; Cuyper and Bulte 2002).

The immobilization methods can be classified as;

- Chemical methods
 - ✓ Covalent binding
 - ✓ Covalent cross-linking
- Physical methods
 - ✓ Entrapment
 - ✓ Encapsulation
 - ✓ Adsorption

Figure 2.7. illustrates the types of immobilization. In Chemical methods, a covalent bond is established between the functional groups in the support (also called the matrix or the carrier) and functional groups in the enzyme. Physical immobilization methods do not involve covalent bond formation with the enzyme. Figure 2.7 shows the classification of immobilization methods.

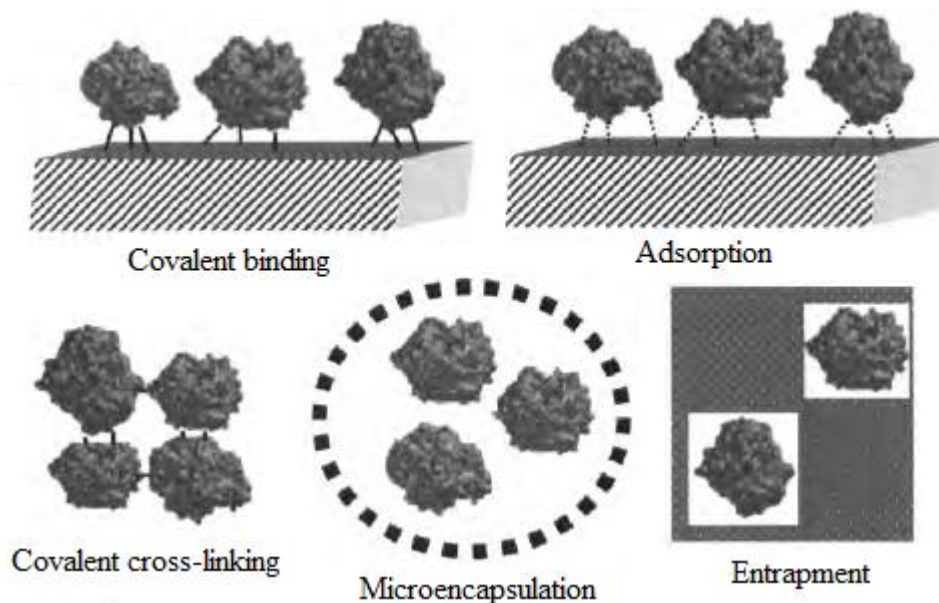


Figure 2.7. Classification of immobilization method
(Source: Drauz and Waldmann 2002).

2.3.1. Chemical Methods

This method includes covalent binding and cross-linking using multifunctional reagents, such as glutaraldehyde and cyanuric chloride and etc. The enzyme can be covalently attached or crosslinked to supports.

Covalent binding: The immobilization of enzymes by covalent attachment to support involves formation of a covalent bond between the functional groups of supports with amino acid residues of the enzyme like OH, SH, NH₂, and COOH. Activation of the support, enzyme coupling, and removal of loosely bound enzymes are three main steps. Silanes, carbodiimide or glutaraldehyde are the chemicals used for covalent attachment of enzyme to the support. In this technique rational control of immobilized enzyme is possible, the enzyme value and operational stability is high and enzyme release from support is minimal. The establishment of the stronger carrier-protein linkage also provides a more stable immobilized enzyme. However significant decrease in (or even loss of) activity occurs. Glyoxyl agarose and amino-epoxy are interesting supports for enzyme immobilization by covalent attachment.

Covalent cross-linking: Enzymes can be insolubilized by cross-linking with bifunctional reagents (like glutaraldehyde or toluene diisocyanate) without the addition of an inert carrier. The obvious advantage is that the specific activity of the enzyme is

very high. This is because the enzyme is auto-immobilized in its own protein mass (no support is involved). This technique can be performed over the soluble enzyme protein (cross-linked enzyme, CLE), over a crystallized enzyme protein (crosslinked enzyme crystal: CLEC) or over a protein enzyme aggregate (cross-linked enzyme aggregate: CLEA). Direct cross-linking of the enzyme followed by precipitation results in low activity and poor mechanical properties. CLECs are produced by cross-linking of purified enzyme crystals which exhibit excellent properties such as high stability under adverse conditions (high temperatures, extreme pHs, organic solvents), excellent mechanical properties and simple biocatalyst recovery. However, a main drawback is their high cost arises from the requirement of a high degree of enzyme purity to crystallize. CLEAs are obtained by cross-linking of protein aggregates produced by conventional protein precipitation techniques which provide insoluble and stable cross-linked enzyme aggregates. In this technique purified enzyme is not required and therefore the cost of production is low. On the other hand mechanical properties and control of particle size are the main drawbacks.

2.3.2. Physical Methods

This immobilization method mainly entrapment, micro encapsulation and adsorption do not involve covalent bond formation and therefore the native composition of the enzyme does not change.

Entrapment: In this method enzyme is held by the support and physically restricted within a confined space or network to keep them from diffusing away. There is no direct interaction between enzyme and support, therefore, its conformational structure and hence activity can be preserved. The enzymes can be entrapped by the supports such as polymeric matrices, gelation of polyanionic or polycationic polymers, hollow-fiber ultrafiltration, membranes (cellophane, cellulose acetate or nitrate, poly (vinyl alcohol) and polyurethane), entrapment gels (agarose, alginate, κ -carrageenan, gelatin, polyacrylate and polyacrilamide) liposomes, cross-linked arrays and cross-linked whole cells. The pore structure of the support used for entrapment should enable to diffuse small molecules (substrates and products) while keep the macromolecular enzyme within the network. Mass transfer limitations are an issue due to impossible control of pore size and leakage of enzyme is often a problem in these systems. The

retention of enzyme and mechanical strength can be improved by using glutaraldehyde or other covalent cross-linking reagents.

Encapsulation: In this technique there is no covalent attachment of the enzyme to the medium. Enzymes can be encapsulated within the microscopic semi-permeable membranes (microencapsulation) or within the macroscopic hollow-fiber membranes. Reverse micelles and liposomes (composed by a double layer of surfactant) are common microscopic encapsulation technique. Microenvironment is adequate for the enzyme and mass transfer limitations are negligible for enzyme reverse micelles. On the other hand mechanical weakness is the main drawback.

Adsorption: The interactions between the enzyme and the support including non covalent bonds like van der Waals forces (short-range interactions), and also hydrophobic interactions, electrostatic and ionic bonds (stronger ones) are termed as adsorption. Hydrophobic bonds are formed between hydrophobic surfaces and hydrophobic domains of enzymes whereas electrostatic bonds can be formed between charges on the support surface and charged groups on the enzyme. It is the simplest method where the carrier can be easily recovered. Adsorption is frequently used in industrial processes because of the low cost, and high immobilization yields. This type of immobilization does not require any chemical modification and protein structure remains usually unaltered. However, the main problem is that the enzyme can be easily desorbed from the support which can be prevented by cross-linking. Randomly orientated biological molecule is the other drawback for this technique (Illanes 2008; Drauz and Waldmann 2002; Mulchandani and Rogers 1998; Buerk 1993; Lojou and Bianco 2006; Mikkelsen and Corton 2004; Watson 2004).

Table 2.6 summarizes the advantage and disadvantage of immobilization methods.

Table 2.6. Comparison of immobilization methods
(Source: Drauz and Waldmann 2002).

Immobilization Methods	Advantages	Disadvantages
Covalent binding	<ul style="list-style-type: none"> • Tight binding • Wide variety of supports and linkers available • Rational control of enzyme loading, distribution and microenvironment 	<ul style="list-style-type: none"> • Chemical modification of enzyme • Often expensive • Activity diluted by support • May limit mass transfer
Covalent cross-linking	<ul style="list-style-type: none"> • High volumetric activity • Compatible with elevated temperature and organic solvents • No carrier required • Tight binding • Efficient for whole cells 	<ul style="list-style-type: none"> • Chemical modification of enzyme • Little control of particle properties (especially for precipitate and whole cell) • Requires crystallization of enzyme (for CLEC) • May limit mass-transfer
Entrapment and Encapsulation	<ul style="list-style-type: none"> • No chemical modification of enzyme • Can be simple • Efficient for whole cells 	<ul style="list-style-type: none"> • Little or no stabilization • Environmental changes can disrupt network and cause leakage • Often limits mass transfer
Adsorption	<ul style="list-style-type: none"> • Simple • No chemical modification of enzyme • Reversible • Often inexpensive 	<ul style="list-style-type: none"> • Weak binding, leaching of enzyme • Little or no stabilization • Non-specific binding • May limit mass transfer

2.4. Properties of Immobilized Enzyme

The catalytic behavior of immobilized enzymes may differ from that of soluble ones. Reduction of the overall enzyme activity can be observed due to enzyme inactivation, steric hindrance or mass transfer limitations. Reaction kinetics of enzyme can also be affected by conformational (native three-dimensional structure of the

enzyme protein) and micro-environmental (refers to partition and mass transfer limitations) effects caused by immobilization. Mass transfer limitations are usually expressed as diffusional restrictions. Because substrate transportation from the bulk to the enzyme and transportation of products away from the enzyme is governed by molecular diffusion. Diffusional restrictions are usually divided into two categories external and internal. Reaction rate (strongly depend on the stirring in the batch reactor or on the flow rate of a substrate in the column reactor) should not depend on pH or enzyme concentration if a reaction is completely limited by external diffusion. The partitioning of substrates, products, inhibitors, metal and hydrogen ions between the bulk solution and support is another important phenomenon for immobilized enzymes. Mostly, partition occurs with respect to protons due to the redistribution of hydrogen ions between a bulk solution and support. Negatively charged supports result in a shift of pH profile to a basic pH while positively charged supports result in a shift of pH profile to an acidic pH. These electrostatic effects exist in solutions with low ionic strength and the shift in pH displacement is reduced by increasing the ionic strength of the medium (Illanes 2008; Drauz and Waldmann 2002).

2.5. Selection of Support Material

Some desirable characteristics should be common to any supports even there is no universal ones for all enzymes and their applications. These include: high affinity to proteins, availability of reactive functional groups, hydrophilicity, susceptibility, mechanical and chemical stability and rigidity, regenerability and excellent film forming ability. High protein binding capacity, compatibility, insolubility in the reaction medium, recoverability after use and conformational flexibility are the other desired characteristics for the supports. The materials should also be nontoxic, biocompatible biodegradable, and cost-effectiveness (Krajewska 2004; Dung et al. 2009; Bhatt et al. 2010; Peña et al. 2010)

There are lots of supports available for the immobilization of enzymes by using the wide range of methods. Table 2.7 shows the type of supports used for enzyme immobilization (Drauz and Waldmann 2002).

Table 2.7. Type of support materials.

Organic - synthetic polymer	Organic - biopolymer	Inorganic
	Polysaccharide	Minerals
Polyamides		
Nylon	Cellulose	Sand
Poly alkylene	Starch	Pumice
Polystyrene	Agarose	Met al oxides
Polyacrylates	Dextran	Diatomaceous earth
Polyacrylamide	Chitin	Clays
Polyethylene	Polyalginate	
Polypropylene	Carrageenan	
Polyvinyl alcohol	Proteinaceous	Synthetic
Polyvinyl acetate	Gelatin	Glass, controlled pore
Polyvinyl chloride	Collagen	glass
Polyethylene glycol	Silk	Zeolites
Polyester	Albumin	Silica
Polycarbonate	Bone	Sol-gel
Polyurethane		Alumina
Polysiloxane		Met al Oxides
Phenol-formaldehyde		Met als

Special care should be taken in choosing a representative support for enzyme immobilization. Among the many carriers that have been considered and studied for immobilizing enzymes, chitosan and gelatin offer most of desired characteristics.

2.5.1. Chitosan as Support

Chitin and chitosan are natural polyaminosaccharides (Dung et al. 2009; Li et al. 2008; Bhatt et al. 2010). Chemically, chitin which is a long chain linear polymer composed by $\beta(1 \rightarrow 4)$ linked 2-acetamido-2-deoxy- β -d-glucose units (or *N*-acetyl-d-glucosamine), as shown in Figure 2.8. It is insoluble in most solvents. Chitosan is a copolymer of *N*-acetyl-d-glucosamine and d-glucosamine (the principal derivative of chitin), and characterized by the degree of deacetylation. It is insoluble in water, but below the pH value about 6.5 (in acidic solutions) the presence of amino groups provides solubility (Krajewska 2004). Chitosan is a promising matrix for enzyme immobilization due to having reactive amino and hydroxyl functional groups. The surfaces of this material are also transparent in the UV and visible regions, therefore should have little effect on most optical detection methods (Abdullah et al. 2006).

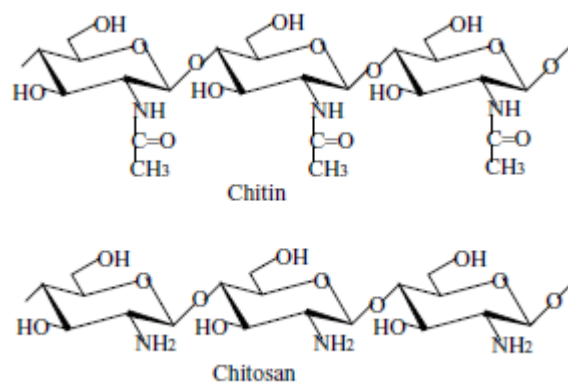


Figure 2.8. Structure of chitin and chitosan

Chitosan properties are largely depends on the pH which substantially alters the charged state of this material. The amines in chitosan are protonated and positively charged at low pH, and chitosan becomes a water-soluble cationic polyelectrolyte. In the case of high pH condition, the polymer loses its charge due to deprotonation of chitosan's amines and the material becomes insoluble as depicted in Figure 2.9 (Yi et al. 2005). The pKa value of chitosan is near neutral and the soluble-insoluble transition occurs at pH between 6 and 6.5 which is a particularly convenient range for biological applications.

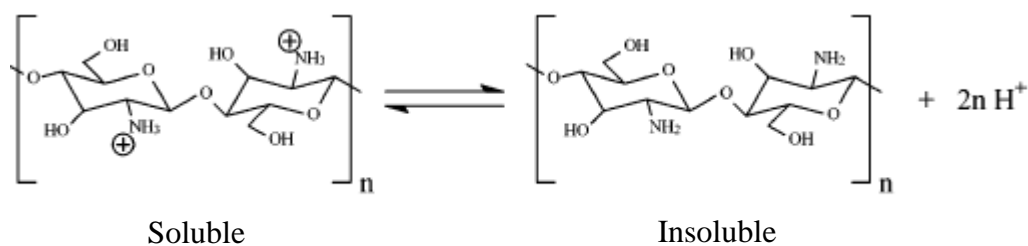


Figure 2.9. Soluble and insoluble from of chitosan

2.5.2. Gelatin as Support

Gelatin is a heterogeneous mixture of water-soluble proteins (on dry weight basis, consists of 98 to 99% protein) with high molecular weight. Peptide bonds keep together the coils of amino acids. The unique protein structure provides wide range of functional properties which are still being discussed today despite it is one of the polymers recognized for millennia. These proteins derived from the chemical

degradation of collagen, with average molecular weights from 65,000 to 300,000 g/mol, depending on the grade of hydrolysis and also form a compound (triple) helix in aqueous solution. Gelatin contains mainly glycine, proline and 4-hydroxyproline (for pigskin gelatin 33%, 13% and 9%, respectively). Chemical structure of gelatin is shown in Figure 2.10.

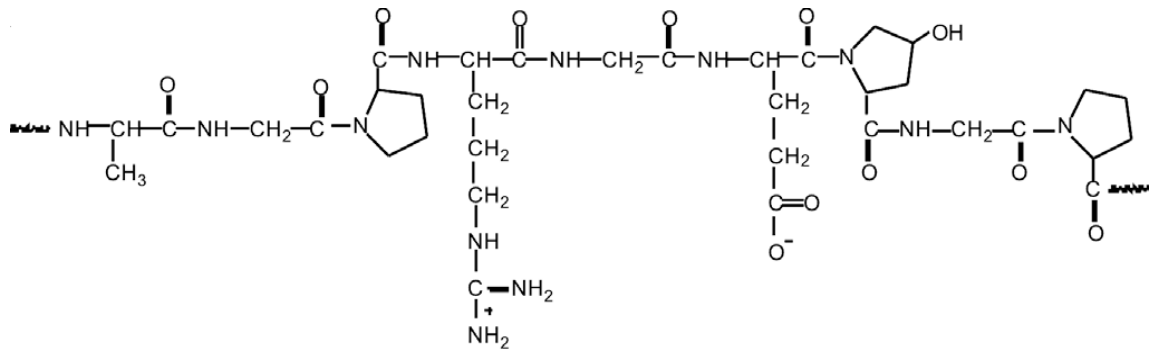


Figure 2.10. Chemical structure of the gelatin

This material is brittle in dry state with high moisture absorption arise from the tightly bounds (hydrogen bonds and hydrophobic interactions) present in its structure and the polar groups of amino acids. These gels are insoluble in organic solvents and soluble in hot water, glycerol, and acetic acid. Their viscosity increases under stress (thixotropic) and are thermally reversible. Gelatin is neither acidic nor alkali (amphoteric) depending on the nature of the solution. The isoelectric point of gelatin (the pH at which gelatin's charge in solution is neutral) ranges between 4.8 and 9.4. The protein gelatin is also colorless, brittle when dry, odorless slightly yellow and tasteless. Gelatin has wide application area such as photography, pharmaceutical, tissue engineering, food industries and also glue since ancient times. (Peña et al. 2010; Omri 2002). These materials can be successively used for most optical detection methods due to having transparent surfaces.

CHAPTER 3

PESTICIDES

The science dealing with poisons or toxicants is defined as toxicology (Klassen et al. 1996). Although nutritional toxicology and food toxicology are related with each other and they can be considered as a parallel branch, they are not synonymous. Overall, the effect of natural and synthetic compounds present in food on human health have been investigated taking into account dangerous effects (carcinogenicity, allergic reactions, immunotoxicity, neurotoxicity, and the potential for the prevention of disease) by the researchers working in the area of food toxicology. Pests are living organisms where they are not wanted or that cause damage to crops, humans, or other animals. Pesticides are type of toxicant used in agriculture and play an important role in the protection food plants. They assist in ensuring substantial yield by controlling insects, weeds, organisms responsible for plant diseases, and other pests and provide a wide variety of foods at inexpensive prices to the consumers. They are inherently designed to kill but must be selective with regard to target species.

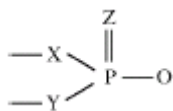
However the use of pesticides results in some degree of risk to human health, animals and ecological balance. It should be considered the potential health risks of pesticides in the human diet. Because pesticides are toxic chemicals and many of them may potentially leave residues on foods available for human consumption. The possible risks are available caused by pesticide residue such as acute poisoning (exposure to large amounts of pesticides consumed in a short duration) and chronic risks (exposure to low levels of pesticide residues over extended periods of time). Agricultural workers involved in the mixing, loading, or application of pesticides and to those working in fields treated with pesticides are also at risk in addition to consumer concerns. Some epidemiological evidence caused by specific pesticides and occupational illnesses and injuries resulting from pesticide use have been reported frequently. These materials are also present environmental concerns such as air pollution, destruction of natural vegetation, reductions in natural pest populations, water and soil contamination. They can also effect the non-target organisms including fish, wildlife, and livestock, creation of secondary pest problems, and the evolution of pesticide resistance.

Some pesticides including the organophosphate, carbamate, and pyrethroid pesticides disrupt the movement of nerve impulses. The time for harvesting is very important because some of ones are residual in action and continue to be effective for months after their application. For example the triazine herbicides persist in the soil and kill emerging weeds over the lifetime of a crop (Watson et al. 2004). Many common pesticides and herbicides are categorized as; organochlorine pesticides, organophosphorus pesticides, carbamate pesticides, triazine herbicides, chlorophenoxy acid herbicides and urea-type herbicides. In many countries, the application of organochlorine pesticides in agriculture has been banned while other classes of pesticides such as organophosphates and carbamates have become popular.

Organophosphorus (OP) pesticides are small molecules. They are derived from phosphoric acid with the oxygen atoms being either replaced by other atoms, such as sulphur, and/or linked to aliphatic, aromatic, anhydrides or heterocyclic groups. Table 3.1 lists the more important categories of OP compounds with their particular side chains, and Figure 3.1 shows the structure of three examples of common OPs.

Table 3.1. Main side groups on different classes of organophosphate compounds
(Source: Watson et al. 2004)

Class of organophosphorus	X	Y	Z
Phosphate	-O	-O	O
Phosphorothionate	-O	-O	S
Phosphorothiolate	-S	-O	O
Phosphorodithionothiolate	-S	-O	S
Phosphorodithiolate	-S	-S	O
Phosphoramidate	=N	-O	O
Phosphordiamidate	=N	=N	O
Phosphoramidothionate	=N	-O	S
Phosphoramidothiolate	=N	-S	O
Phosphonate	≡C	-O	O
Phosphonothionate	≡C	-O	S
Phosphonothionothiolate	≡C	-S	S



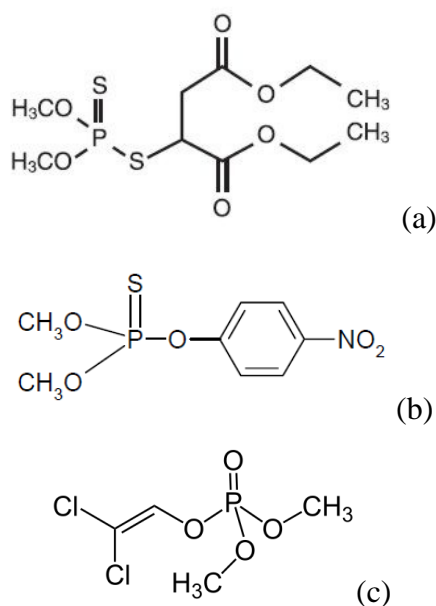


Figure 3.1. Three examples of organophosphates (a) Malathion (b) Parathion-methyl (c) Dichlorvos

Some common pesticides of OP and their alternative names are also listed in Table 3.2 (Patnaik et al. 2004).

OPs have been extensively used as insecticides, and are also used as nematocides, helminthicides and have fungicidal and herbicidal properties. These compounds exert their toxic effects by tendency to inhibit a number of important enzymes such as acetylcholine esterase. The importance of this enzyme is related with the inactivation of the fast-acting neurotransmitter acetylcholine found in the nerve synapses of the neuromuscular junction and brain nicotinic junctions. The use of OP is strictly regulated to prevent harmful effects to the population and the maximum permissible level of OP residue, known as the maximum residue limit (MRL) in ppm is measured.

Table 3.2. Common Organophosphorus Pesticides, their formulas and synonyms

Pesticide	Molecular formula	Alternative Names
Abate	$C_{16}H_{20}O_6P_2S_3$	Difenphos, Temefos, Bithion
Acephate	$C_4H_{10}NO_3PS$	Acetamidophos, Orthene
Akton*	$C_{12}H_{14}Cl_3O_3PS$	Axiom
Aspon	$C_{12}H_{28}O_5P_2S_2$	ASP 51, Propyl thiopyrophosphate
Azinphos methyl	$C_{10}H_{12}N_3O_3PS_2$	Gusathion, Guthion, Carfene
Azinphos ethyl	$C_{12}H_{16}N_3O_3PS_2$	Gusathion ethyl, Ethyl guthion, Bionex
Bolstar sulfone	$C_{12}H_{19}O_4PS_3$	—
Bomyl	$CHOP$	GC 3707
Carbofenthion	$C_{11}H_{16}ClO_3PS_3$	Trithion, Hexathion, Acarithion
Chlorfenvinphos	$C_{12}H_{14}Cl_3O_4P$	Birlane, Enolofos, Sapecron
Chlorofos	$C_4H_8Cl_3O_4P$	Metrifonate, Anthon, Trichlorfon
Coumaphos	$C_{14}H_{16}ClO_3PS$	Muscatox, Asuntol, Baymix
Crotox yphos	$C_{14}H_{19}O_6P$	Ciodrin, Decrotox
Demeton-O	$C_8H_{19}O_3PS_2$	Mercaptophos, Thiodemeton
Demeton-S	$C_8H_{19}O_3PS_2$	Isosystox, Thioldemeton
Diazinon	$C_{12}H_{21}N_2O_3PS$	Basudin, Dimpylate, Neocidol
Dicapthion	$C_8H_9ClNO_3PS$	Isochlorthion, Chlorthion, Dicaptan
Dichlofenthion	$C_{10}H_{13}Cl_2O_3PS$	Nemacide, Mobilawn, Bromex
Dichlorvos	$C_4H_7Cl_2O_4P$	Chlorvinphos, Cyanophos, Atgard
Dicrotophos	$C_8H_{16}NO_3P$	Carbicron, Ektafos, Bidrin
Dimethoate	$C_5H_{12}NO_3PS_2$	Fosfotox, Cygon, Phosphamid
Dioxathion	$C_{12}H_{26}O_6P_2S_4$	Navadel, Ruphos, Delnatex
Disulfoton	$C_8H_{19}O_2PS_3$	Dithiodemeton, Glebofos, Dithiosystox
Dursban	$C_9H_{11}Cl_3NO_3PS$	Chlorpyrifos, Lorsban, Eradex
EPN	$C_{14}H_{14}NO_4PS$	Santox, EPN 300
Ethion	$C_9H_{22}O_4P_2S_4$	Rodocide, Phosphotox E, Ethanox
Ethephon	$C_2H_6ClO_3P$	Camposan, Ethrel
Famaphos	$C_{10}H_{16}NO_5PS_2$	Famphur, Warbex, Cyflee
Fenamiphos	$C_{13}H_{22}NO_3PS$	Nemacur, Phenamiphos
Fensulfothion	$C_{11}H_{17}O_4PS_2$	Terracur P, Desanit, Hexazir
Fenthion	$C_{10}H_{15}O_3PS_2$	Baycid, Mercaptophos, Baytex
Folithion	$C_9H_{12}NO_3PS$	Fenitrothion, Metathion, Nitrophos
Fonofos	$C_{10}H_{15}OPS_2$	Dyphonate, Difonate
Isazophos	$C_9H_{17}ClN_3O_3PS$	Miral
Isofenphos	$C_{15}H_{24}NO_4PS$	Oftanol, Amaze
Leptophos	$C_{13}H_{10}BrCl_2O_2PS$	Phosvel, Abar, MBCP
Malathion	$C_{10}H_{19}O_6PS_2$	Fosfothion, Carbofos, Malafos, Cythion
Merphos	$C_{12}H_{27}PS_3$	Folex, Tributyl trithiophosphite
Methamidophos	$C_5H_8NO_2PS$	ENT, Tamaron, Monitor
Methidathion	$C_6H_{11}N_2O_4PS_3$	Supracide, Medathion, Ultracide
Mevinphos	$C_7H_{13}O_6P$	Phosdrin, Duraphos, Mevinex
Monocrotophos	$C_7H_{14}NO_5P$	Azodrin, Bilobran
Montrel	$C_{12}H_{19}ClNO_3P$	Crufomate, Amidophos, Ruelene
Naled	$C_4H_7Br_2Cl_2O_4P$	Dibrom, Bromex, Dibromfos
Oxydemeton-methyl	$C_6H_{15}O_4PS_2$	Metasystox R, Metaisosystox sulfoxide
Oxydemetonmethyl sulfone	$C_6H_{15}O_5PS_2$	Metasystox R sulfone
Parathion-ethyl	$C_{10}H_{14}NO_5PS$	Parathion, Thiophos, Foliodol
Parathion-methyl	$C_8H_{10}NO_5PS$	Metaphos, Azofos, Metron, Nitrox
Paraoxon	$C_{10}H_{14}NO_6P$	Phosphacol, Diethyl paraoxon
Phenamiphos	$C_{13}H_{22}NO_3PS$	Nemacur
Phorate	$C_7H_{17}O_2PS_3$	Thimate, Aastar, Granutox
Phosalone	$C_{12}H_{15}ClNO_4PS$	Rubitox, Zolone, Benzophosphate
Phosfolan	$C_7H_{14}NO_3PS_2$	Cyolane, Cylan

(Continued in next page)

Table 3.2. (cont.)

Pesticide	Molecular formula	Alternate Names
Phosphamidon	$C_{10}H_{19}ClNO_5P$	Famfos, Dimecron, Apamidon
Phosmet	$C_{11}H_{12}NO_4PS$	Imidan, Decemthion, Safidon
Profenofos	$C_{11}H_{15}BrClO_3PS$	Polycron, Seleccion
Prophos	$C_8H_{19}O_2PS_2$	Ethoprophos, Mocap, Ethoprop
Sulfotepp	$C_8H_{20}O_5P_2S_2$	Dithiophos, Thiolepp, Pirofos
TEPP	$C_8H_{20}O_7P_2$	Fosvex, Nifos, Hexamite
Terbufos	$C_9H_{21}O_2PS_3$	Counter, ST-100
Tetrachlorvinphos	$C_{10}H_9Cl_4O_4P$	Stirofos, Rabon, Gardona
Tokuthion	$C_{11}H_{15}Cl_2O_2PS_2$	Protothiophos
Trichloronate	$C_{10}H_{12}Cl_3O_2PS$	Agritox, Phytosol, Phenophosphon
Zinophos	$C_8H_{13}N_2O_3PS$	Cyanophos, Menafos, Thionazine

MRL levels are set for different pesticides used for varied crops and additional variation can also be seen between the different regulating authorities. In addition, only certain OPs are licensed, with many OPs being banned. Table 3.3 gives examples of MRLs for three different OP compounds for three different foods.

Table 3.3. Maximum residue limits for three different OP compounds and for three different crops (Source: Budak 2001)

Organophosphate compound	Crop	Maximum Residue Limit (MRL in ppm)
Malathion	Peach	2
	Tomato	8
	Corn	0.5
Parathion-methyl	Peach	0.05
	Tomato	0.1
	Corn	0.2
Dichlorvos	Peach	0.1
	Tomato	0.2
	Corn	1

3.1. Detection of Pesticide Residue by Enzymatic Biosensor

Traditionally, separation techniques such as chromatography or electrophoresis have been used in order to determine the OPs by exploiting their chemical and

physicochemical properties. Quantification is achieved using gas chromatography (GC) or high performance liquid chromatography (HPLC). One of the sensitive and relatively quick techniques GC has the disadvantage of being expensive equipment that must have a gas supply, so is limited to laboratory use. The other preferred method for OP analysis is HPLC which does not have the disadvantage of thermal degradation of the sample as occurs in GC. However there are a number of limitations for this technique on its application such as expensive equipment, uses solvents and requires trained operators and as such is limited to laboratory use. The identification of OP can be achieved using spectral methods such as NMR and mass spectroscopy. NMR can identify a single pesticide residue whereas mass spectroscopy, when interfaced to either GC or HPLC, allows identification of more than one pesticide residues. Being highly specialised, expensive and limited to laboratories where trained personnel perform the analysis has some disadvantages for these techniques.

In recent years, immunoassay and enzyme inhibition techniques developed by using the biochemical and immunological properties of pesticide residues are now widely used. These methods depend on the interaction between the pesticide residue and biological molecule differs from the techniques described in the previous methods. This interaction may be specific or non-specific for a particular pesticide as in the interaction with an antibody or as in the way a number of different pesticides interfere with an enzyme reaction respectively, (Watson 2004). The popularity of these methods comes from their advantages such as they are relatively simple, require little or no sample pretreatment, and do not require expensive instrumentation and provide rapid detection of pesticide residues (Mikkelsen and Corton 2004).

First known enzymatic assay was reported by Osann in 1845: hydrogen peroxide (H_2O_2) was quantified using the enzyme peroxidase. In enzymatic methods OP compounds inhibit the biological activity of particular enzymes and prevent to form their products from given substrates. Measuring the activity of the enzyme in the absence and presence of the sample gives an indication of total OP concentration. There will be a decrease in enzyme activity (measured by monitoring the disappearance of substrate or the accumulation of product) in the presence of organophosphate residues. The most commonly used enzymes in these methods are acetylcholine esterase (AChE), butyrylcholine-esterase, organophosphorus hydrolase and ascorbate oxidase, alkaline phosphatase, tyrosinase and acid phosphatase (Du et al. 2007; Solna et al. 2005). The detection of pesticide by tyrosinase enzyme sensor rely on the fact that the pesticide

inhibit the activity of tyrosinase enzyme by preventing the formation of enzymatically produced o-quinones (cause decrease in product amount) while catechol is consumed.

The conversion of analyte to another chemical species by the enzyme through a biochemical reaction causes a change in optical, electrical or mechanical properties of the analyte. Recently optical detection becomes mostly preferred ones among these detection techniques. Because they are convenient methods and rapidly providing accurate and reproducible results can be obtained. Sometimes incorporation of appropriate additional reagents such as 3-methyl-2-benzothiazolinone hydrazone (MBTH) allows the reaction to be monitored photometrically. Among the photometric methods enzyme assays based on changes in the light absorbed as the reaction proceeds are more frequently used one (Eisenthal and Danson 2002).

Detection of pesticide residue is generally performed in aqueous solutions. Besides this, water solubility of most pesticides is very low with respect to organic solvents which are used for the extraction of their residues from food before analysis. However enzymes show limited tolerance for the solvents. Therefore it is necessary to be considered the influence of different solvents on enzyme activity and applied solvent has to be carefully chosen, as well. The effects of organic solvents differ according to the hydrophobicity, solvating ability, molecular geometry, concentration of the solvents and the nature of the enzyme (Turdean and Turdean 2008). Their effects on enzymatic reaction rates are two types. One of them is distortion of enzymes arise from displacement of water molecules both in the hydration layer surrounds the protein molecules in aqueous solution and in the interior of the protein. The flexibility of the enzyme is restricted in the case of too low water content (Krishna 2002). Besides this polar organic solvents can interact with the essential water layer of enzyme which is required for maintaining the proper polypeptide conformation (Micaelo and Soares 2007). Therefore they disturb the non-covalent and hydrophobic interactions, which hold the enzyme in the proper folded state. The other type of effect is that, organic solvents behave as probable inhibitors or activators through specific interactions with enzymes, which cause to changes in the reaction kinetics and substrate specificity (Pazhang et al.2006). In the case of high solvent concentrations a partition effect of the solvent between the bulk solution and the enzyme microenvironment is produced (Illanes 2008).

CHAPTER 4

THEORETICAL ESTIMATION OF SOME KINETIC PROPERTIES FOR ENZYME IMMOBILIZED FILMS

The immobilized enzyme activity in operational modes could be reduced. Because the stagnant film surrounded around the non-soluble enzyme molecules prevent the transportation of substrate and products into or from the catalytic micro-environment. In general the mass transfer resistance tries to be minimized by adjusting the operating parameters. In this chapter, some theoretical approach for the estimation of some important kinetic parameters is discussed for enzyme immobilized films.

4.1. Characterization of Kinetic Parameters

The interaction between the enzyme (E) and substrate (S) is usually weak forces (van der Waals force and hydrogen bonding). The substrates can bind to the enzyme solution which has a fixed number of active site. At high substrate concentration the enzyme is saturated. In the other words all these sites may be occupied by substrates. Saturation kinetics can be explained by a simple reaction scheme which involves a reversible step for enzyme-substrate complex formation and dissociation step for the ES complex.



It is assumed that the rate of the reverse reaction of the second step is negligible and the ES complex is established rather rapidly . The rate of product (P) formation is;

$$V = \frac{d[P]}{dt} = k_2[ES] \quad (4.2)$$

In the development of quasi-steady state approach, the rate expression suggests that initial substrate concentration greatly exceeds the initial enzyme concentration $[E_0]$. Since $[E_0]$ was small and $d[ES]/dt \approx 0$. This can be written as;

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \quad (4.3)$$

By arranging the equation $[ES] = \frac{k_1[E][S]}{k_{-1} + k_2} = \frac{[E][S]}{K_m}$ (4.4)

Where $K_m = \frac{k_{-1} + k_2}{k_1}$ corresponds to the substrate concentration at the half-maximal reaction velocity. The meaning of K_m is complicated. For a two step reaction with $k_2 \ll k_{-1}$, K_m reduces to (k_{-1}/k_1) . This is the dissociation constant for the ES complex, and K_m also measures the affinity of the enzyme for substrate. Low K_m value indicates greater affinity of the enzyme for the substrate that the enzyme requires little amount of substrate to achieve saturated conditions.

In the system the total enzyme is $[E]_0 = [E] + [ES]$ (4.5)

Multiplying the total enzyme to rate of product formation;

$$\frac{V}{[E]_0} = \frac{k_2[ES]}{[E] + [ES]} \quad (4.6)$$

Where, V_m is the maximal velocity. When all of the $[E_0]$ enzyme is in the ES complex represented as;

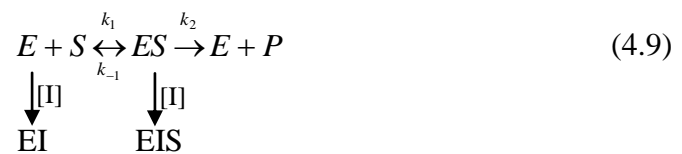
$$V_m = k_2[E_0] \text{ or } [E_0] = \frac{V_m}{k_2} \quad (4.7)$$

By applying these assumptions and substitution of equation 4.7 ($[E_0]$) and equation 4.4 ($[ES]$) to the equation 4.6, the rate equation becomes;

$$v = \frac{V_m[S]}{K_m + [S]} \quad (4.8)$$

Therefore the reaction kinetics is expressed with Michaelis-Menten equation (Equation 4.8) (Marangoni 2003; Shuler and Kargi 2002).

The enzyme activity is influenced in the presence of any inhibitor (such as organic solvents and pesticides) which decreases the rate of an enzyme-substrate reaction. The enzyme inhibition can be irreversible or reversible. In the case of reversible inhibition competitive, uncompetitive and noncompetitive ones are most commonly types. The values of V_m and K_m can be changed for each types of reversible enzyme inhibition as shown in Table 4.1.



Competitive inhibitors are usually substrate analogs and compete with substrate for the active site of the enzyme. The net effect of competitive inhibition is an increased value of K_m and therefore reduction reaction rate. Uncompetitive inhibitors bind to ES complex and have no affinity for the enzyme itself. The net effect of uncompetitive inhibition is a reduction in both V_m and K_m values and net result is a reduction in reaction rate. Noncompetitive inhibitors are not substrate analogs, bind at the enzyme (E) or at the ES complex. This type of inhibitor has no effect on the K_m value but lowers the V_m value. This is because the inhibitor binds all enzyme species with the same affinity.

Table 4.1. Summary of the effect of reversible inhibitors on enzyme catalytic parameters V_m and K_m (Source: Marangoni 2003).

	Competitive	Uncompetitive	Noncompetitive
V_m^*	No effect (-) V_m	Decrease (\downarrow) V_m/α	Decrease (\downarrow) V_m/α
K_m^*	Increase (\uparrow) αK_m	Decrease (\downarrow) K_m/α	No effect (-) K_m

Where, V_m^* and K_m^* correspond to the enzyme maximum velocity and enzyme–substrate dissociation constant in the presence of an inhibitor respectively.

$$\alpha = 1 + \frac{[I]}{K_i} \quad (4.10)$$

Where $[I]$ is the inhibitor concentration and K_i is the enzyme-inhibitor dissociation constant.

4.2. Characterization of Mass Transfer Resistance

During enzymatic processes the external and internal mass transfer resistances should be minimized to apply the Michaelis-Menten kinetics which assumes that;

- The substrate-binding step and formation of ES complex are fast with respect to the breakdown rate which leads to the approximation that the substrate binding reaction is at equilibrium.
- The substrate concentration remains essentially constant during the reaction ($[S_0] \approx [S_t]$) due partly to the fact that initial velocities are used.
- The conversion of product to the substrate is negligible. Because very little product has had time to accumulate during the reaction time.
- The system does not involve any mass transfer limitations during the enzymatic process.

These assumptions are based on the following conditions:

- The enzyme is stable during the measurements time used for determination of the reaction velocities.
- Reaction velocities are calculated from the initial rates of the reaction
- The reaction velocity is directly proportional to the total concentration of the enzyme (Marangoni 2003).

In the case of immobilized enzyme to the surface of the chitosan and gelatin film, external mass transfer resistance might be expected. Because homogenous catalytic reaction becomes heterogeneous which leads to hindrance of substrate transportation by the stagnant liquid surrounding solid enzyme. Figure 4.1 shows concentration profiles of substrate and products at the interface of optical biosensor under static condition.

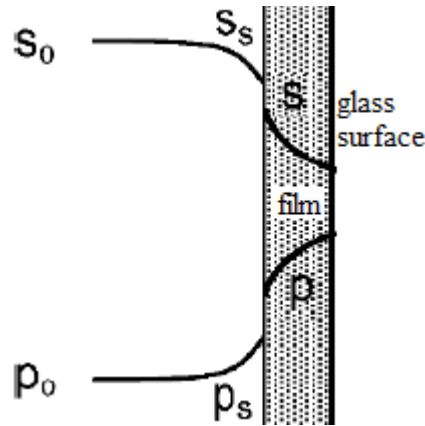


Figure 4.1. Substrate and product profiles in the immobilized enzyme system as a consequence of partition and mass transfer limitations under static condition

As can be seen from the figure substrate conversion occurs in three steps

- i) Substrate transport from the bulk medium to the biocatalyst surface
- ii) Enzymatic conversion of substrate to product
- iii) Product transport from the biocatalyst surface to the bulk medium.

At steady-state, the average rate of substrate transport from the bulk fluid to the surface of the support is balanced by the enzymatic reaction rate.

$$r = k_s A (S_0 - S_s) = \frac{V_{max} S_0}{K_m + S_0} \quad (4.11)$$

Where S_s and S_0 are the substrate concentrations at the interface and in the bulk fluid respectively. If we arrange the equation (Engasser 1978);

$$S_s = S_0 - \frac{v}{k_s A} \quad (4.12)$$

The substrate or product diffusion can limit the enzyme catalytic efficiency. Any of these steps is the rate-limiting. The influence of external mass transfer resistance on the overall enzymatic reaction can be represented by effectiveness factor, η , which is defined physically by

$$\eta = \frac{\text{(observed reaction rate)}}{\text{(rate in the case of no mass transfer resistance } S_s = S_0)} \quad (4.13)$$

If η is equal to 1, in this case there is no external mass transfer resistance. Mathematically, η is described by the following equation

$$\eta = \frac{\frac{V_{max}S_s}{K_m+S_s}}{\frac{V_{max}S_0}{K_m+S_0}} = \frac{\beta_s(1+\beta_0)}{\beta_0(1+\beta_s)} \quad (4.14)$$

Where

$$\beta_0 = S_0/K_m \quad (4.15)$$

$$\beta_s = \frac{-(1+\lambda-\beta_0) \pm \sqrt{[(1+\lambda-\beta_0)^2 + 4\beta_0]}}{2} \quad (4.16)$$

$$\lambda = V_{max}/k_s K_m \quad (4.17)$$

Only the positive root will give the positive value of β_s . λ can be calculated by **maximum velocity** (V_{max}) and Michaelis constant (K_m). These values are determined from the Michaelis-Menten equation. The mass transfer coefficient k_s during the enzymatic reaction can be calculated by empirical correlation developed by Cussler (1984).

$$\frac{k_s \cdot r_{film}}{D_{i,\infty}} = \alpha Re^{0.5} .Sc^{0.33} \quad (4.18)$$

$$Re = \frac{D_p^2 N \rho_w}{\mu_w} \quad (4.19)$$

$$Sc = \frac{\mu_w}{\rho_w D_{i,\infty}} \quad (4.20)$$

Where r_{film} is the radius of the film, α is the device geometry (dimensionless: 0.323), D_p is the propeller diameter (m), N is the rotational speed (rate/s), $D_{i,\infty}$ is the

diffusion ($1.13 \times 10^{-9} \text{ m}^2/\text{s}$) coefficient of the solute (m^2/s), μ_w is the viscosity of water and ρ_w is the density of water (Miyabe and Okada 2002).

CHAPTER 5

EXPERIMENTAL

5.1. Materials

In this study, enzyme: tyrosinase from mushroom (T3824, Merck) with 25 KU, substrate: catechol (C9510, $\geq 99\%$, Merck), supports: chitosan (448869, Sigma-Aldrich) and gelatin from porcine skin (Merck), chemicals: di-sodium hydrogen phosphate (Na_2HPO_4 , 98%, Merck) and sodium-dihydrogen phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 98%, Merck), organic solvents: ethanol (99.2%, Merck), methanol (99.8%, Merck), isopropyl alcohol (99.8%, Merck), acetone (99.8%, Merck) and acetonitrile (99.9%, Sigma-Aldrich), acid: HCl (37.% Merck), and pesticide: parathion-methyl (36187, Fluka) were used. All aqueous solutions were prepared with ultra pure water (18.2 M Ω cm).

5.2. Methods

5.2.1. Preparation of the Solutions

The phosphate buffer (pH: 6.5), tyrosinase (activity: 5370 U/mg) chitosan, gelatin, HCl, catechol, organic solvent and pesticide solutions were used in this study as outlined in Table 5.1. 50 mM phosphate buffer solution was used for the preparation of tyrosinase, catechol, chitosan, gelatin solutions and organic solvents. Chitosan or gelatin dissolved in HCl at 25 °C for 2 hours was used to prepare the immobilized tyrosinase film via adsorption method on the glass rod. Chitosan stock solutions were also used tyrosinase immobilization via entrapment method. Gelatin stock solution prepared in PBS was used to prepare the immobilized tyrosinase film via entrapment method on the glass rod. Organic solvents were used to determine their effect on enzyme activity and to solve the parathion methyl (in organic solvent) which was used for its detection by developed optic enzyme sensor.

Table 5.1. The solutions used in this study

Solutions	Concentrations	Preparation
Phosphate buffer at pH: 6.5(PBS)	50 mM	Using Na ₂ HPO ₄ and NaH ₂ PO ₄ H ₂ O
Tyrosinase (activity:5370 U/mg)	4.7 mg /ml	In PBS
Catechol	0.01 -2 mM	In PBS
Chitosan or gelatin	20 mg/ml	In HCl (0.3 M)
Gelatin	100 mg/ml	In PBS at 35-40 °C
Organic solvents (ethanol, methanol, isopropyl alcohol, acetone and acetonitrile)	1-20 %v/v	In PBS
Pesticide (parathion methyl)	25 mg/l	In Isopropyl alcohol solution (5%v/v)

5.2.2. Preparation of Optical Enzyme Sensor

The optic sensors are obtained by coating the glass rods with the films. Initially the glass rods were ultrasonically cleaned with ethanol-water mixture and then rinsed with ultra pure water before the coatings. The films for the sensors are prepared by immobilization of tyrosinase on chitosan or gelatin via adsorption and entrapment method as represented below.

Immobilization via adsorption;

10 µl-30µl chitosan solution or 30µl gelatin solution (prepared in HCl) dropped on the area (0.8 cm x 0.8 cm) of the cleaned glass rods (0.8 cm x 4 cm) and then dried at 60 °C and at 25 °C for 1h to prepare the chitosan (C) or gelatin (G) supports, respectively. In preparation of the films tyrosinase solutions (1-10 µl) were dropped on the support C or on the support G (2 µl tyrosinase dropped for G) and then dried for 2 h at room temperature (25 °C). After this TCA and TGA sensor will be used for the film prepared with support C and G, respectively.

Immobilization via entrapment;

The tyrosinase (2 µl)- chitosan (30 µl) and tyrosinase (1-10 µl) –gelatin (10-30 µl) mixtures were dropped on the area (0.8 cm x 0.8 cm) of the cleaned glass rods (0.8 cm x 4 cm) and then dried for 2 h at (25 °C) to obtain TCE and TGE sensors, respectively.

Prior to the characterization the optic enzyme sensors were immersed in PBS (3 ml) for 30 min to wash out the non-immobilized components from the film surface. The amount of enzyme in the solution (E_w) was determined by using the calibration curve presented in the Appendix B. The enzyme immobilized on the film is;

$$E_{im}=E_i-E_w \quad (5.1)$$

where E_{im} and E_i is the immobilized amount and initial (loaded) amount of enzyme in the film.

The films of the sensors were characterized by using atomic force microscopy (AFM) using tapping mode (MMSPM Nanoscope IV), FTIR spectrophotometer (SHIMADZU 8400S) recorded in the range of 4000–400 cm^{-1} on KBr discs (2 mg sample and 148 mg KBr) and also UV spectrophotometer before and after the enzymatic reactions.

5.2.3. Enzyme Assay

In the assay UV-visible spectrophotometer (Perkin Elmer Lambda 45) was used. As explained in the appendix in situ analysis was performed for the measurement of enzymatic activity by following the formation of o-quinone (wave length: 390 nm) at pH 6.5 and at 25 °C during 1 min by stirring the reaction media at 1000 rpm. Beer–Lambert relationship was used to calculate the activity;

$$\frac{dP}{dt} = \frac{dA}{dt} \frac{1}{\epsilon l} \quad (5.2)$$

where ϵ is extinction coefficient of o-quinone ($\epsilon_{390} = 1417 \text{ M}^{-1} \text{ cm}^{-1}$) as stated by Aytar and Bakir (2008), l is path-length (1 cm), dA/dt is the change in absorbance at 390 nm with time for 1 minute.

The effect of the pH (in the range between 5-8), temperature (in the range between 15-40 °C), organic solvents (in the range between 1-20 % v/v) and parathion-methyl as pesticide (0.01-15 mg/l) on enzyme activity were investigated by means of enzyme activity.

The relative enzyme activity of tyrosinase in the presence of solvents or parathion-methyl (A_s)

$$A_s(\%) = \frac{(dP/dt)_s}{(dP/dt)_c} \times 100 \quad \% \quad (5.3)$$

was determined from the ratio of the activity of solution with $((dP/dt)_s)$ and without $((dP/dt)_c)$ solvent or parathion-methyl (control sample) at 0.4 mM catechol concentration.

The storage stabilities of the free enzyme and developed sensor were determined by measuring the relative activities after a given time of storage. They were stored in PBS at 4°C when not in use. The operational stability of prepared sensors was also determined by measuring the activity of the sensor for 10 uses.

Reaction kinetics;

The kinetics of the reaction between the tyrosinase (E) and catechol solution (S):



conducted in a 3 mL quartz cuvette is expressed with Michaelis-Menten equation:

$$v = \frac{V_m[S]}{K_m + [S]} \quad (5.5)$$

where v and V_m are the production rate of o-quinone at any and maximum substrate concentrations and K_m is the Michaelis constant which corresponds to the substrate concentration at half-maximal reaction velocity ($V_m/2$). The experimental data was obtained with three replicates by using the different catechol concentrations (0.01–2 mM) to form reaction velocity vs. substrate concentration curve. Then the data were fitted to this model equation by minimizing the error.

$$\text{Error: } \left[\sum (v_{\text{exp}} - v_{\text{cal}}) \right] \quad (5.6)$$

By this way the effect of the organic solvents and pesticide (parathion-methyl) on the reaction kinetics (V_m and K_m) were investigated and then possible inhibition models were studied.

The sensing performance such as linearity, sensitivity (the slope of the linear range) and detection limit were determined for the sensor. The limit of detection (LOD) was also calculated by using the equation:

$$LOD = \frac{(3.S_{y/x})}{a} \quad (5.7)$$

Where a is the sensitivity of the method and $S_{y/x}$ is the standard deviation of the fit.

CHAPTER 6

RESULTS AND DISCUSSIONS

In this section firstly the results for free enzyme were given. Then the results for immobilized enzyme in terms of development of optic enzyme sensors were discussed. Finally the best sensor was decided to use for determination of parathion-methyl.

6.1. Free enzyme Studies

The effects of pH, temperature and organic solvents on free tyrosinase activity were investigated in order to optimize the reaction conditions. Beside this kinetic parameters for free tyrosinase were also evaluated by using the Michaelis-Menten equation.

6.1.1. The Effect of pH and Temperature on Tyrosinase Activity

The effect of pH on free tyrosinase activity was investigated by varying the pH of the solution from 5.5 to 8 at 25 °C. As shown in Figure 6.1 the maximum activity observed at neutral pH, 6.57.

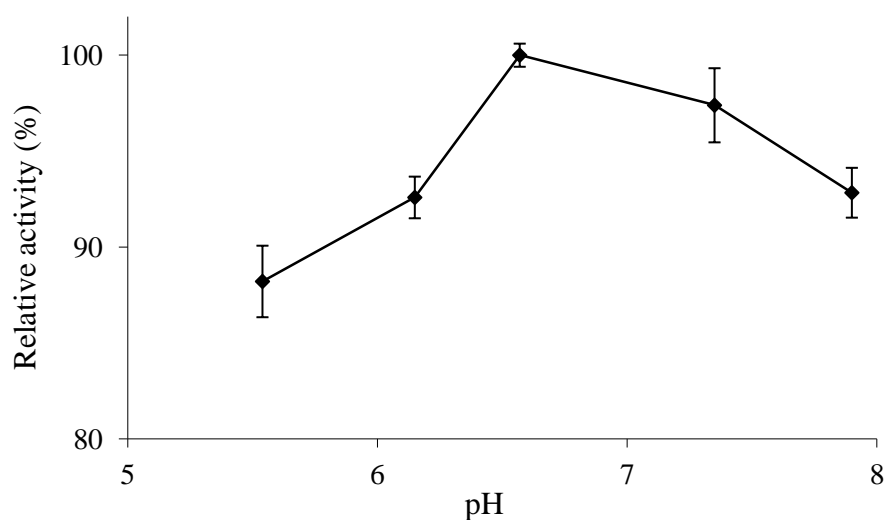


Figure 6.1. Change in free enzyme activity with pH at 25 °C.

Towards acidic medium the activity decreases due to protonation. The protonation of catalytic groups essential for catalysis diminishes the strong binding of the enzyme to its active copper site. On the other hand in basic medium the deactivation was observed due to conformational changes in the enzyme active site as stated by Yoruk and Marshall (2003).

The experiment for the effect of temperature on free enzyme activity was also conducted. As can be seen from Figure 6.2 the highest activity was observed at 26 °C. The decrease in activity at lower or higher temperature than 26 °C arise from decrease in the kinetic energy of the reactant molecules or thermal denaturation, respectively, as stated by Yoruk and Marshall (2003) and Suzuki et al. (2010).

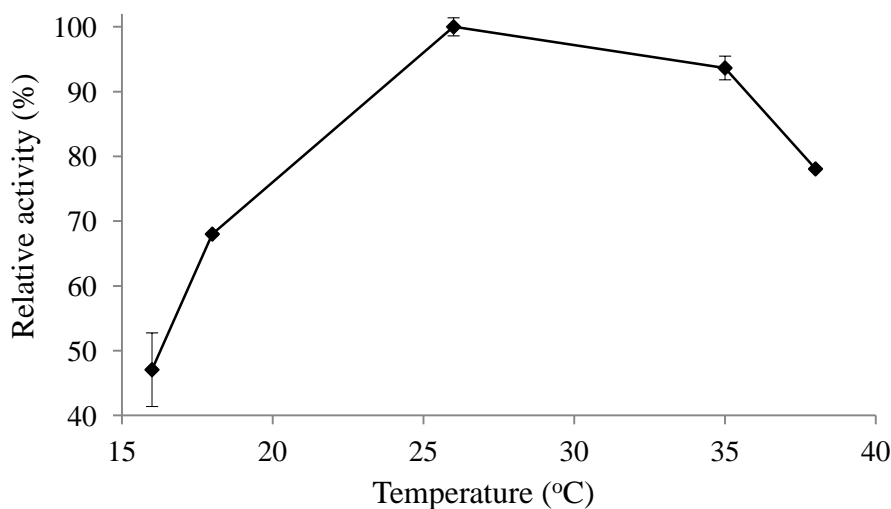


Figure 6.2. Change in free enzyme activity with temperature at pH 6.5.

The activity obtained at pH 6.57 and temperature 26 °C were used as maximum activity for the free enzyme studies which is compatible with the literature. The maximum activity was observed at pH 7 and temperature 30 °C in the study of Chuanga et al. (2005) and Akyilmaz et al. (2010).

6.1.2. The Effect of Organic Solvents on Tyrosinase Activity

Solubility of parathion-methyl in water is very low (37-60 ppm) (Mackay et al. 2006). As an alternative to water, organic solvents can be used as solvents for parathion-methyl. However the influence of different organic solvents on enzyme

activity is stated (Doukyu and Ogino 2010). Therefore the effect of organic solvents (ethanol, methanol, isopropyl alcohol, acetone and acetonitrile) solution on free tyrosinase activity was investigated. In the presence of ethanol, methanol and isopropyl alcohol the increase in the relative enzyme activity was observed (Figure 6.3). This may be due to binding of solvent to the active sites in the enzyme molecule which may provide more favorable orientation of a substrate in the enzyme (Shipovskov and Levashov 2003; Kudryashova et al. 1997). However acetone and acetonitrile decreases the enzyme activity with increasing solvent concentration.

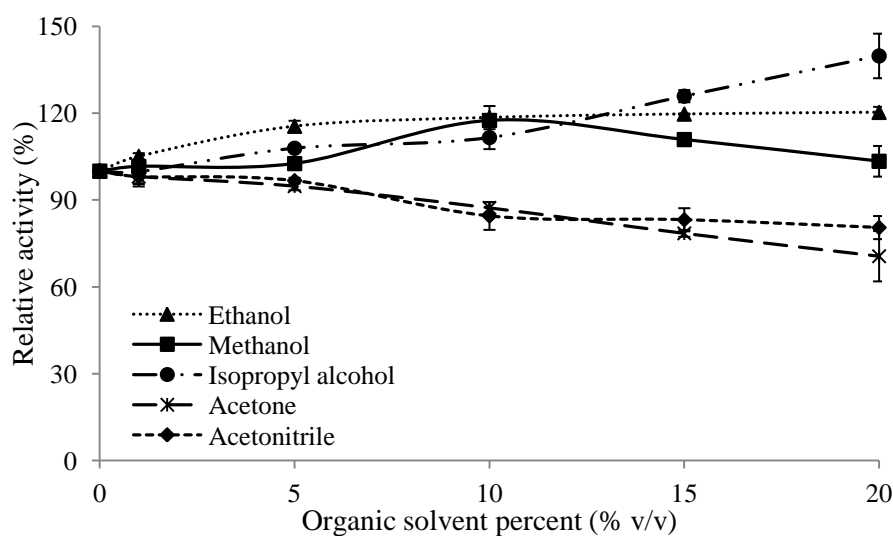


Figure 6.3. Influence of organic solvent on free tyrosinase activity

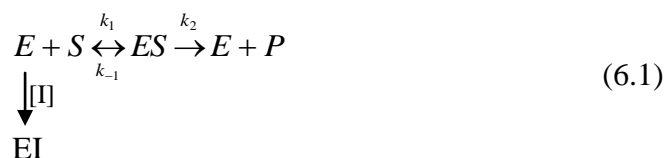
This can be related with the polarity of organic solvents. A measure of molecular polarity is a quantity called the dipole moment (D). The polarity in other words, the solubility in water increases with increasing the dipole moment (Jinde 1994). This quantity is high for aprotic solvents (acetone and acetonitrile) with respect to protic ones as depicted in Table 6.1. In other words acetone and acetonitrile have higher solubility in water leading to higher ability to strip essential water from the enzyme molecules. Therefore the enzyme activity decreases in the presence of acetone and acetonitrile due to change in protein structure arise from displacement of bound water molecules by organic solvents (Walz and Schwack 2007; Doukyu and Ogino 2010).

Table 6.1. Properties of organic solvents used in this study.

Organic solvents	Classification	Chemical Formula	Dipole moment (D)*
Methanol	Polar protic	CH ₃ -OH	1.70
Ethanol	Polar protic	CH ₃ -CH ₂ -OH	1.69
Isopropyl-alcohol	Polar protic	CH ₃ -CH(-OH)-CH ₃	1.66
Acetone	Polar aprotic	CH ₃ -C(=O)-CH ₃	2.69
Acetonitrile	Polar aprotic	CH ₃ -C≡N	3.44

*: (Source: Koenhen and Smolders 1975).

The Michaelis-Menten kinetic parameters were evaluated for the solvents and the inhibition mechanism exerted by acetone and acetonitrile were investigated. Slight decrease in K_m value in ethanol, methanol, isopropyl alcohol containing media exhibit that catechol had higher affinity for the enzyme. However an increase in the value of K_m ($(k_{-1}+k_2)/k_1$) shows that catechol had lower affinity for the enzyme in the presence of acetone and acetonitrile. Both solvent showed competitive inhibition due to increase in K_m value while V_m is constant (Table 6.2).



The catalytic efficiency which indicates the ability of tyrosinase to convert catechol into the production of o- quinone in the selected organic solvent media expressed as (Kermasha et al. 2001);

$$\text{Catalytic efficiency} = V_m/K_m \tag{6.2}$$

was also used. Slight increase in catalytic efficiency of tyrosinase was observed in the presence of ethanol, methanol and isopropyl alcohol. Valuable decrease occurred in the case of acetone and acetonitrile due to their inhibition type (V_m constant while K_m increases).

Table 6.2. Kinetic parameters of free tyrosinase in organic solvent (10% v/v) containing media.

Solvent	V_m ($\mu\text{mol}/\text{min}$)	K_m (mM)	Catalytic efficiency
Free	0.67	0.56	1.21
Ethanol	0.67	0.53	1.27
Methanol	0.68	0.54	1.25
Isopropyl alcohol	0.65	0.50	1.29
Acetone	0.68	1.07	0.64
Acetonitrile	0.66	0.76	0.87

6.1.3. Kinetic Analysis of Free Tyrosinase

Michaelis-Menten constant (K_m) and maximum reaction rate of free enzymes were evaluated by fitting the experimental data to Michaelis-Menten equation. Figure 6.4 shows the change in the activity with the catechol concentration at 26 °C and pH 6.5. The curve is linear up to catechol concentration approximately 0.4 mM. The experimental data fitted by minimizing the error to model equation gives high correlation coefficient.

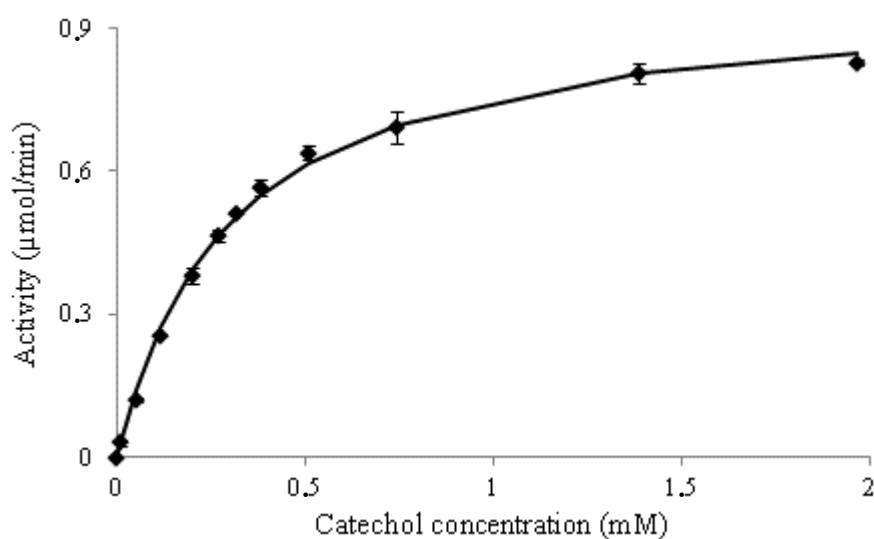


Figure 6.4. The effect of substrate concentration (catechol) on free enzyme activity. Points: experimental, line: model. (T:26 °C and pH:6.5)

The apparent Michaelis-Menten constant (K_m) (gives the information about the enzyme-substrate kinetics) and maximum velocity were calculated as 0.300 mM and 0.980 $\mu\text{mol}/\text{min}$ as depicted in Table 6.3.

Table 6.3. Response characteristic of free tyrosinase enzyme

K_m (mM)	V_m ($\mu\text{mol}/\text{min}$)	R^2	Error
0.300	0.980	0.997	0.127

This result is concord with the literature data. K_m value was found as 0.22 mM and 0.47 mM for mushroom tyrosinase at pH 7 when catechol was used as substrate in the study of Aytar and Bakir (2008) and Duckworth and Coleman (1970) respectively.

6.2. Immobilized Enzyme Studies

Tyrosinase enzyme was immobilized to the chitosan and gelatin supports by using adsorption and entrapment methods. The effect of immobilization methods on tyrosinase activity was investigated and the methods which give maximum activity were decided to use. Then optic enzyme sensors were developed for selected sensors by taking into consideration the effect of washing, effect of immobilized enzyme amount on the enzyme activity and also operational stability. Kinetic parameters for selected sensor were evaluated and the best sensor was found out depending on initial slope of the curve (responsiveness) and V_m value. Storage stability, the effect of pH, temperature and organic solvent on the activity of best sensor was studied in order to optimize the reaction conditions. Then the sensor was used for determination of parathion methyl.

6.2.1 The Effect of Immobilization Methods on Tyrosinase Activity

9.4 μg Tyrosinase enzyme was immobilized on 30 μl chitosan or gelatin via adsorption and entrapment method. After the washing step the activity of the optical enzyme sensors were measured. As seen from Table 6.4 the highest activity was obtained for the chitosan and gelatin supported sensors prepared by adsorption and entrapment methods, respectively. This result shows that gelatin is smart matrix for

entrapment method with respect to adsorption method. This may be due to the randomly oriented enzyme molecules on gelatin supports arise from its chemical structure when adsorption method was used.

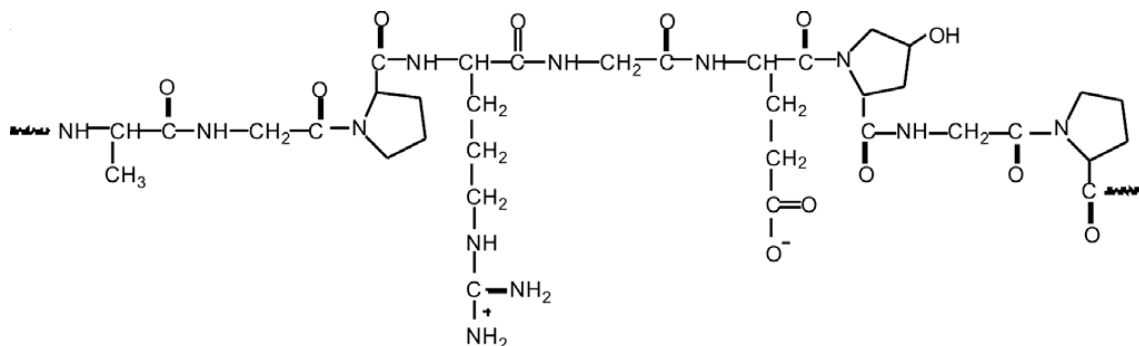


Figure 2.10. Chemical structure of the gelatin

Besides chitosan is smart matrix for adsorption method due to having large quantity of NH_3^+ groups result in ability to adsorb enzyme molecule with electrostatic interactions.

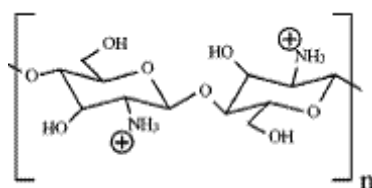


Figure 2.9. Soluble form of chitosan. Therefore the enzyme molecules are not restricted by supports result in easier substrate transportation.

Table 6.4. The effect of immobilization method on the activity of optical enzyme sensor.

Sensor codes	Support	Immobilization method	Enzyme immobilized	Activity
			(E_{im}) (μg)	($\mu\text{mol}/\text{min mg enzyme}$)
TCA	Chitosan	Adsorption	8.77	$11.97^{\pm 0.89}$
TCE		Entrapment	8.50	$1.99^{\pm 0.23}$
TGA	Gelatin	Adsorption	8.65	$0.82^{\pm 0.05}$
TGE		Entrapment	6.38	$5.80^{\pm 0.63}$

Therefore the chitosan supported optical enzyme sensor via adsorption (TCA) and the gelatin supported optical enzyme sensor via entrapment (TGE) obtained highest activity were used for further experiments.

6.2.2. The Development of the Optical Enzyme Sensors

In development of the optical enzyme sensor, washing period to remove non-immobilized constituents were studied in chitosan supported optical enzyme sensor (TCA) and gelatin supported optical enzyme sensor (TGE). The effect of enzyme amount on the enzyme activity, operational stability of the sensors and also kinetic parameters were investigated.

The effect of washing time;

Figures 6.5a and 6.5b show the amount of enzyme released from the TCA and TGE to the PBS.

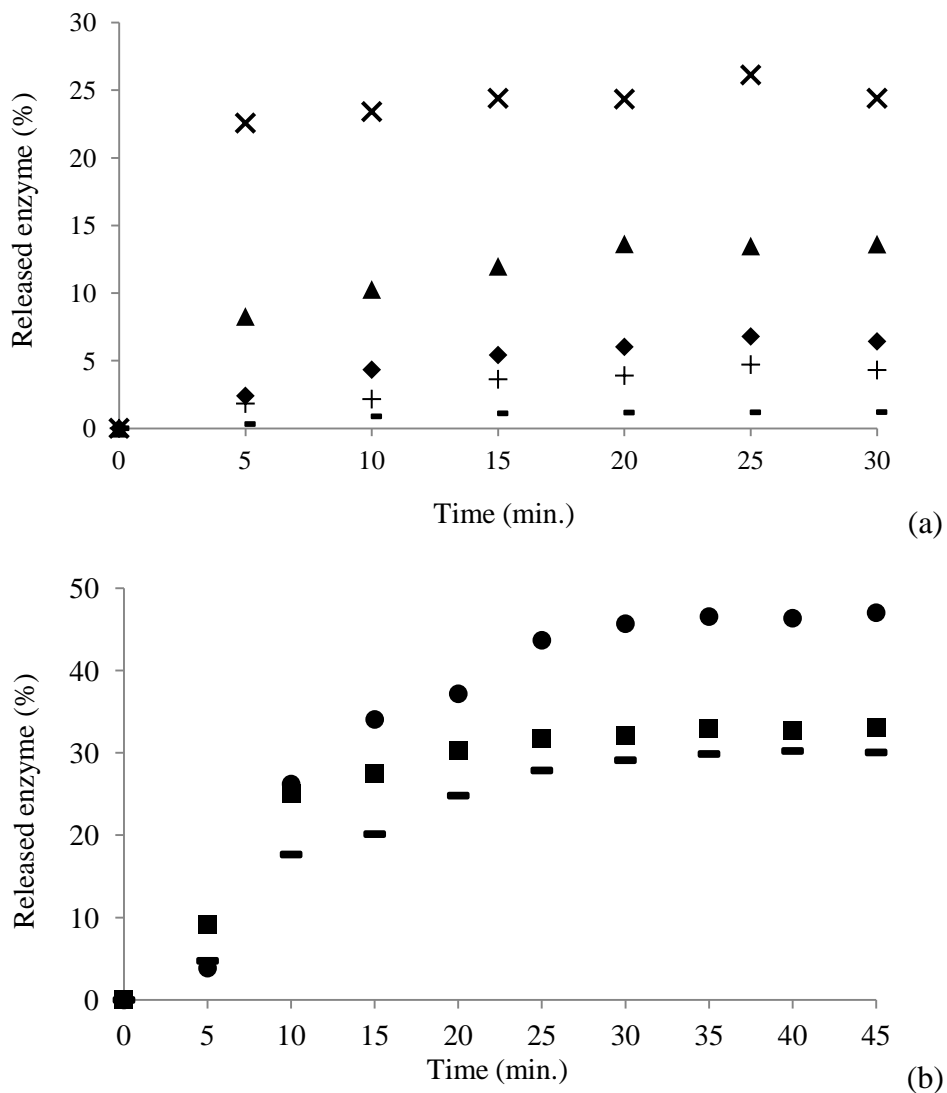


Figure 6.5. The enzyme released from (a) TCA (b) TGE E_i (μg loaded/ μg support): 0.1765 (x), 0.1175 (▲), 0.0785 (◆), 0.0387 (+), 0.0295 (-), 0.0282 (●), 0.0118 (—), 0.0031 (■).

The experiments performed by the sensors loaded with different amount of enzyme shows that the released enzyme amount from the sensors increased with time. According to the Figure 6.5, 30 min is sufficient to remove all of non-immobilized enzyme. The other indication is that, the released enzyme amount was higher for gelatin supported optical enzyme sensor (TGE). Because the leakage of enzyme is often a problem in entrapment methods (Drauz and Waldmann 2002).

The effect of loaded enzyme amount on immobilized amount of enzyme;

Figure 6.6a and 6.6b shows the effect of loaded enzyme amount on immobilized amount of enzyme for chitosan and gelatin films, respectively.

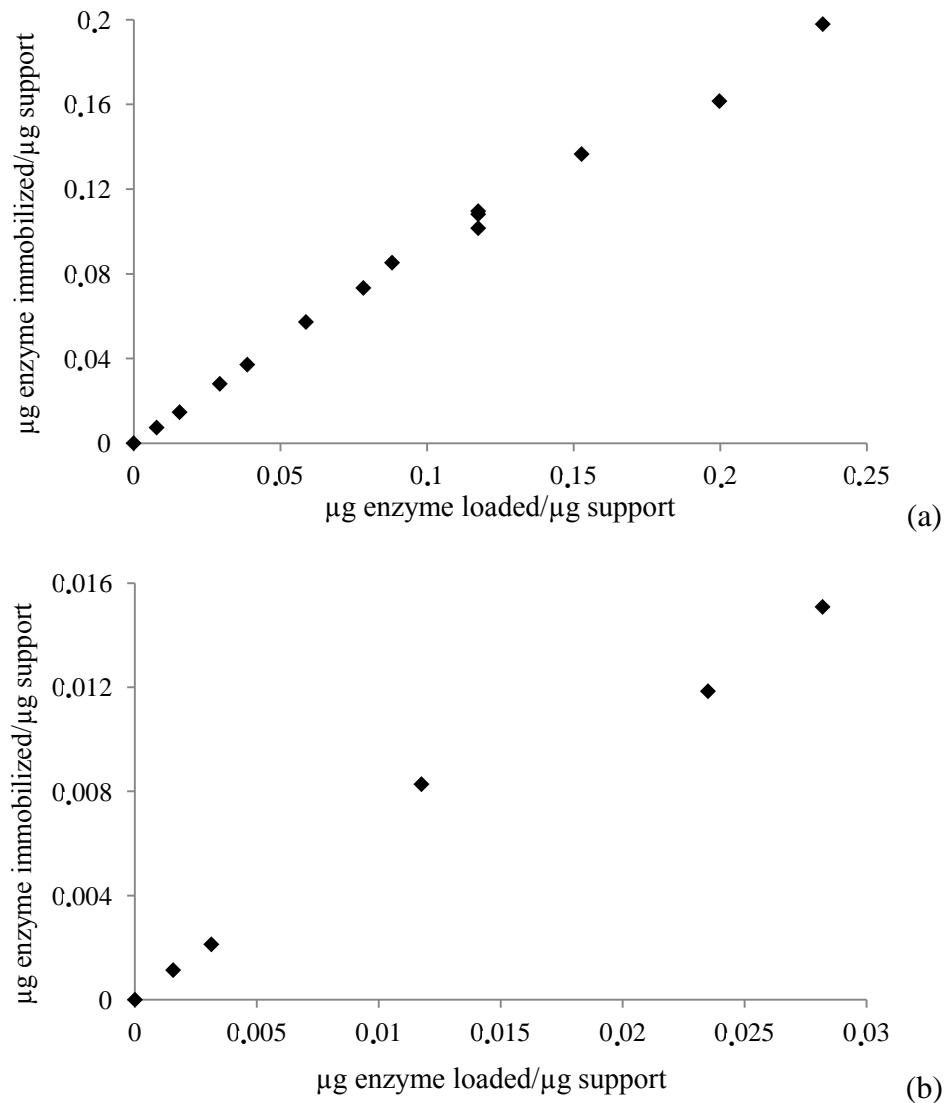


Figure 6.6. Change in the amount of immobilized enzyme with loaded enzyme for (a) TCA (b) TGE.

As seen from the figures, increase in the loaded enzyme amount linearly increased the immobilized enzyme amount for both type of the sensors. However chitosan support immobilized higher amount of enzyme with respect to gelatin ones due to lower enzyme release.

The effect of immobilized enzyme amount on the activity of optic enzyme sensor;

The activity of the optical enzyme sensors was not significantly affected by the amount of immobilized enzyme on supports as shown in Figure 6.7a and Figure 6.7b respectively.

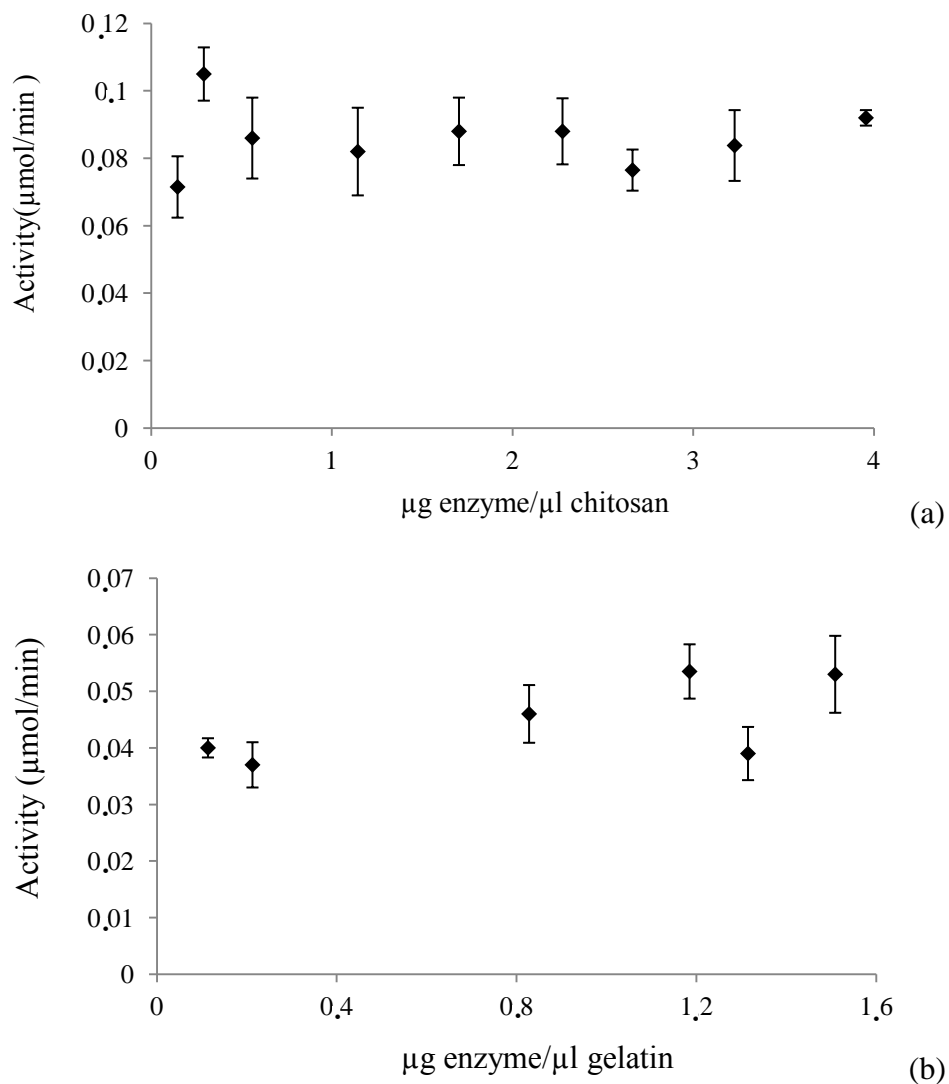


Figure 6.7. Change in the activity with immobilized enzyme amount for (a) TCA (b) TGE.

The highest enzyme activity ($\mu\text{mol}/\text{min } \mu\text{g enzyme}$) was found when lowest amount of enzyme was used for the preparation of TCA and TGE (1 $\mu\text{mol enzyme}/30 \mu\text{l support}$). Therefore there is no need to use higher amount of enzyme. After that the minimum amount of enzyme was used in development of the optical enzyme sensor.

The Operational Stability of Optic Enzyme Sensors:

The repetitive use is a very significant parameter when developing new enzyme sensor. Figure 6.8 shows activity change with the number of use (the operational stability) for TCA and TGE. The sensors show good operational stability and there was no significant decrease in activity after even 10th use.

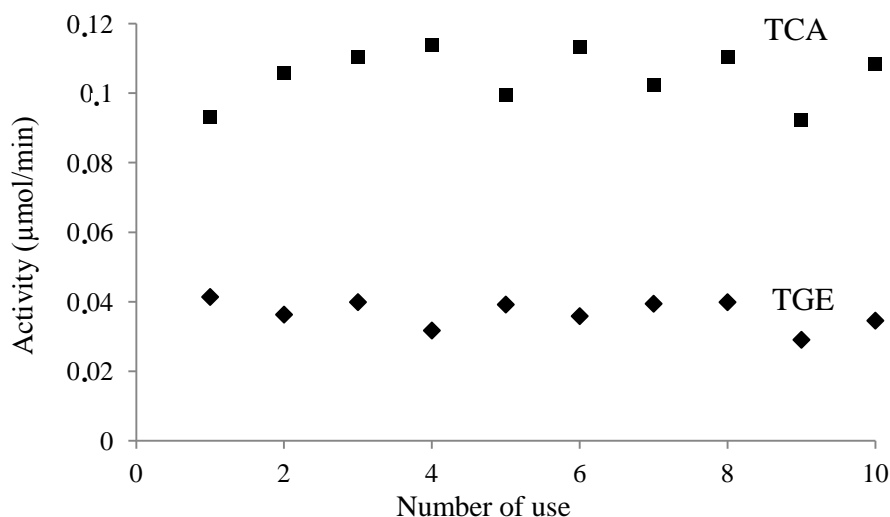


Figure 6.8. Operational stability of TCA and TGE

The average activity of chitosan supported optical enzyme sensor (TCA) is higher than gelatin supported ones (TGE). This could be attributed to the immobilization type of the enzyme: in entrapment method there may be an additional mass transfer resistance for the substrate (or product) transport to (or from) the film. In adsorption method the substrate can reach the enzyme molecules without additional mass transfer resistance. Therefore the position of the enzyme in chitosan supported sensor is more appropriate and substrate can easily reach the enzyme so product formation can occur rapidly. As a result the sensors prepared in which the enzyme immobilized on the support by means of electrostatic bonds, give higher activity. The electrostatic bonds can be formed between protonated groups on the support surface (NH_3^+) and charged groups in the enzyme (carboxylic group COOH).

6.2.3. Kinetic Analysis of Optical Enzymes Sensors

Kinetic parameters such as Michaelis-Menten constant (K_m) and maximum velocity of TCA chitosan and TGE were evaluated by fitting the experimental data to Michaelis-Menten model equation. Figure 6.9 shows the change in activity of the sensors with catechol.

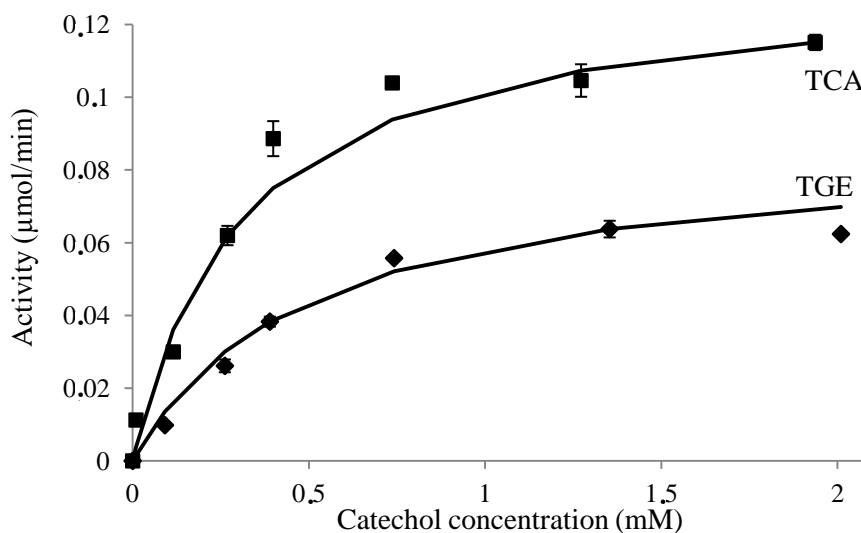


Figure 6.9. The effect of substrate (catechol) concentration on TCA and TGE. Points: experimental, line: model (T:27 °C and pH:6.5).

The initial slope of the curve was higher when TCA was used as a sensor. Both sensors have wide linear response (up to nearly 0.4 mM catechol concentration) as depicted in Figure 6.9.

The value of K_m gives information about the affinity of enzyme to the substrate that is inversely proportional (Kıralp et al. 2003). In the case of large K_m value substrate and enzyme do not prefer to be close for a long time. TCA exhibit nearly the same K_m with free enzyme but TGE shows higher K_m than that of free enzyme. The enzyme substrate complexes [ES] form the product and immediately leave each other to give way for the next substrate when TGE was use. On the other hand V_m values decreased for both type of the sensor when compared with the free enzyme as shown in Table 6.5. This could be attributed to the changing in enzyme structure by immobilization and increasing diffusional resistance for the substrate to reach the enzyme (Drauz and Waldmann 2002; Illanes 2008)

Table 6.5. Comparison of the kinetic parameters for the free and immobilized tyrosinase

Type of enzyme	K _m (mM)	V _m (μmol/min)	R ²	Error
Free	0.300	0.980	0.997	0.127
TCA	0.311	0.134	0.978	0.040
TGE	0,497	0.087	0.980	0.019

As a result TCA was used for determination of parathion-methyl due to having higher initial slope of the curve (higher responsiveness) and V_m value to compared TGE. Therefore storage stability, the effect of pH, temperature and organic solvent on TCA was studied and the results were given in the next section.

6.3. Chitosan Supported Optical Enzyme Sensor (TCA)

6.3.1. Storage Stability of TCA

Figure 6.10 shows the storage stability of free enzyme and TCA at 4 °C in PBS. As can be seen from the figure, significant decrease in free enzyme activity was observed while activity of TCA remains unaltered approximately after 45 days under the same storage conditions. So, the immobilized tyrosinase exhibits higher stability than that of the free enzyme.

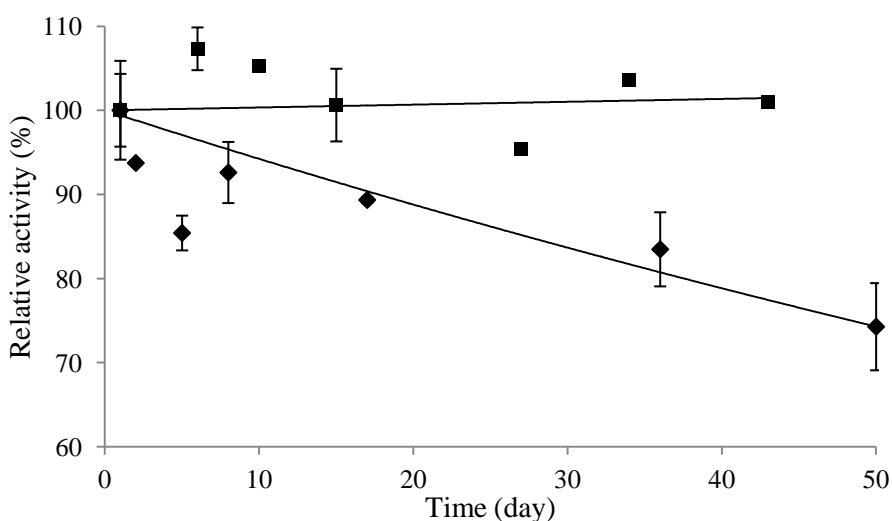


Figure 6.10. Storage stability of free enzyme (◆) and TCA (■)

This result shows that immobilization enhance the storage stability of the enzyme which could be attributed to the higher conformational stability. Immobilization of the enzyme with electrostatic interactions conserves its structure by hindering of protein unfolding, aggregation or dissociation (Drauz and Waldmann 2002).

6.3.2. The Effect of pH and Temperature on The Activity of TCA

Chitosan supported optical enzyme sensor (TCA) was selected and compared with free enzyme. The response characteristic of TCA was measured at the pH values between 4.5 and 7.9 as shown in Figure 6.11. The results were presented in a normalized form with the highest value for free enzyme and TCA being assigned the value of 100% activity. The maximum response for the sensor was obtained at pH 6.02. Besides this, optical sensor shows good catalytic response for all pH values.

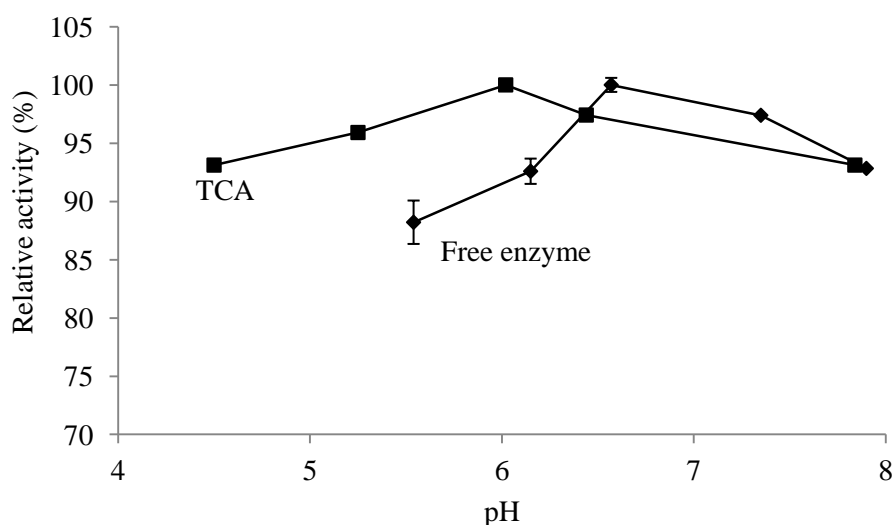


Figure 6.11. The effect of pH on the activity of free enzyme (◆) and TCA (■) (T:25 °C).

Comparison of the pH of TCA with free enzyme shows that the slight shift in maximum pH to the acidic region was observed. This shift could be caused by newly formed interactions between enzyme and support due to the change in acidic and basic amino acid side chain ionizations in the microenvironment around the active site (Aytar and Bakir 2008). The shift toward a acidic pH for the tyrosinase can be explained by the presence of positively charged amino groups on chitosan. The other indication is that

the activity of the enzymes slowly decreases through the acidic and basic regions for free and immobilized enzyme as depicted in Figure 6.11.

The influence of the reaction temperature on the activity of TCA is shown in Figure 6.12.

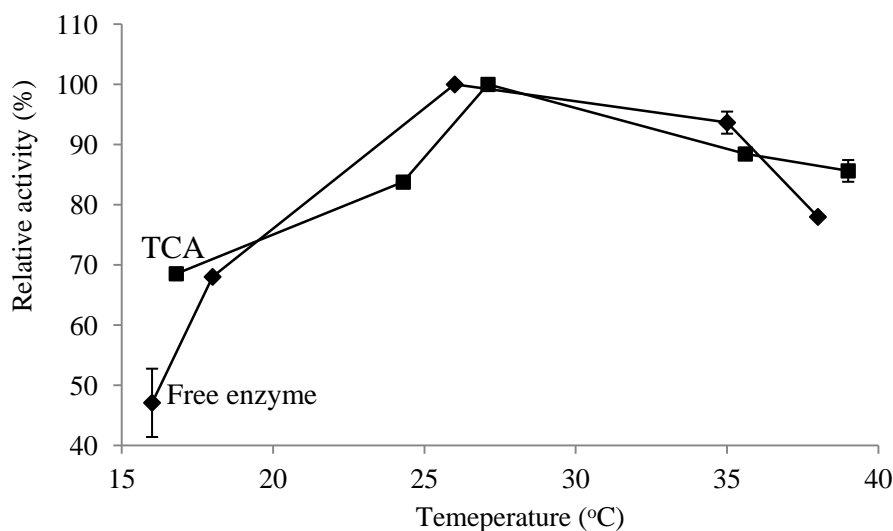


Figure 6.12. The effect of temperature on the activity of free enzyme (♦) and TCA (■) (pH:6.5).

There was no shift in temperature occurs for TCA with respect to free enzyme. As seen from Figure 6.12 the optimum temperature for TCA is 27 °C. Above or especially below the optimum temperature, decrease in enzyme activity was observed for both free and immobilized enzyme.

Simply enzymes show higher resistance for the change in pH with respect to change in temperature as depicted in Table 6.6. On the other hand immobilization increases the pH and temperature stability of the enzyme especially at lowest pH and temperature value.

Table 6.6. Comparison of pH and temperature of free tyrosinase with TCA

Position of enzyme	pH at max activity	T (°C) at max activity	Relative activity at pH:5.5 and 7.9	Relative activity at T (°C):16 and 38
Free	6.57	26	88 % - 93 %	47 % - 78 %
Immobilized	6.02	27	96 % - 93 %	69 % - 86 %

6.3.3. The Effect of Organic Solvents on The Activity of TCA

Water-soluble organic solvents show remarkable influence on tyrosinase activity, especially at higher concentrations (Figure 6.13). As opposed to the free enzyme the enzyme activity decreases for ethanol, methanol and isopropyl alcohol containing reaction media. This might be because immobilization effect the conformation of enzyme and therefore flexibility of enzyme is restricted. Beside this, support material retains the water molecule which is necessary for enzymes to exhibit catalytic activity. Reduction observed in the tyrosinase activity especially at higher solvent concentration is also likely due to destruction of active enzyme conformation by displacement of bound water molecules with organic solvent as stated by Walz and Schwack (2007). Similar to the free enzyme the decrease in enzyme activity is higher when acetone and acetonitrile were used.

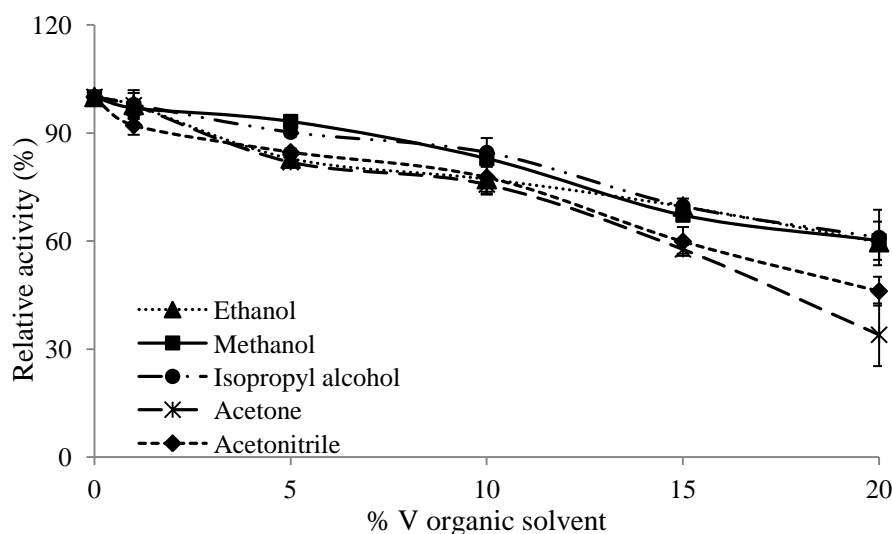


Figure 6.13. Influence of organic solvents on immobilized tyrosinase activity.

6.3.4. Kinetic Analysis of TCA in Organic Solvent Media

Figure 6.14 shows the change in activity of TCA with various catechol concentrations (0.01-2 mM) in organic solvent containing media (10% v/v). As seen from the figures the organic solvents used decreased the enzyme activity. Kinetic parameters such as Michaelis-Menten constant (K_m) and maximum velocity of the TCA

in the selected organic solvent media were evaluated by fitting the experimental data (activity of TCA) to Michaelis-Menten model equation.

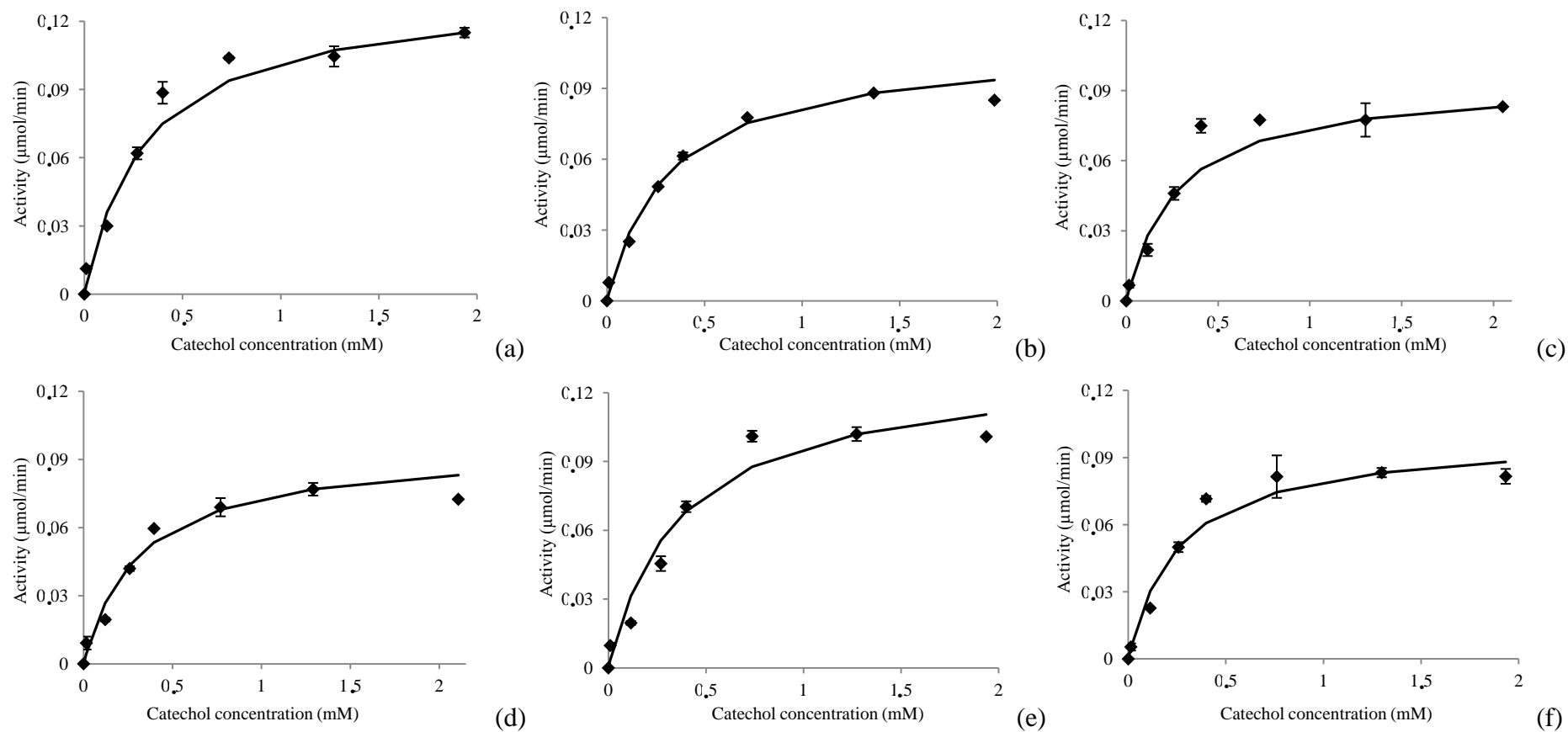


Figure 6.14. The effect of substrate concentration on the activity of TCA in solvent free media(a) and organic solvent containing (10% v/v) media, ethanol (b), methanol (c), isopropyl alcohol (d), acetone (e), acetonitrile (f). Points: experimental, line: model (T:27 °C and pH:6.5).

Type of inhibition exerted by the organic solvents depending on the K_m and V_m value were also characterized as depicted in Table 6.7. The net effect of competitive inhibition is an increased value of K_m while V_m is constant, for noncompetitive inhibition decrease in V_m value while no effect on the K_m and for uncompetitive inhibition is a reduction in both V_m and K_m values (Marangoni 2003).

Table 6.7. Response characteristic of TCA in solvent free and organic solvent containing media (10% v/v).

Medium	K_m (mM)	V_m ($\mu\text{mol}/\text{min}$)	R^2	Error	Inhibition type	Relative Activity (%)
Solvent free	0.311	0.134	0.978	0.040	-	-
Ethanol	0.314	0.108	0.989	0.021	Noncompetitive	77.2 ^{±8.0}
Methanol	0.273	0.094	0.955	0.036	Uncompetitive	82.9 ^{±3.2}
Isopropyl alcohol	0.311	0.095	0.969	0.031	Noncompetitive	84.6 ^{±8.3}
Acetone	0.369	0.132	0.958	0.053	Competitive	75.7 ^{±8.4}
Acetonitrile	0.256	0.099	0.971	0.032	Uncompetitive	77.7 ^{±7.7}

The inhibition mechanism exerted by acetone is competitive, whereas ethanol and isopropyl alcohol are noncompetitive, and methanol and acetonitrile are uncompetitive. Decrease in activity was highest (relative activity) in the case of acetone while isopropyl alcohol and methanol show smaller effect on enzyme activity with respect to other ones.

In the reaction mixture it is necessary to adjust the solvent level by taking into consideration both the lowest loss of enzyme activity and also highest solubility of pesticide (calculated by using the regular solution model explained in Appendix C). According to the Figure 6.15, parathion methyl is mostly soluble in acetone-water and less soluble in methanol-water mixtures. However the experiment conducted to investigate the influence of different organic solvents concentrations on tyrosinase activity shows that inhibition of enzyme activity is higher in the presence of acetone and acetonitrile as depicted in Table 6.7. Therefore isopropyl-alcohol is an appropriate solvent for parathion-methyl with the high solubility for parathion-methyl (pesticide) and low inhibition for the enzyme.

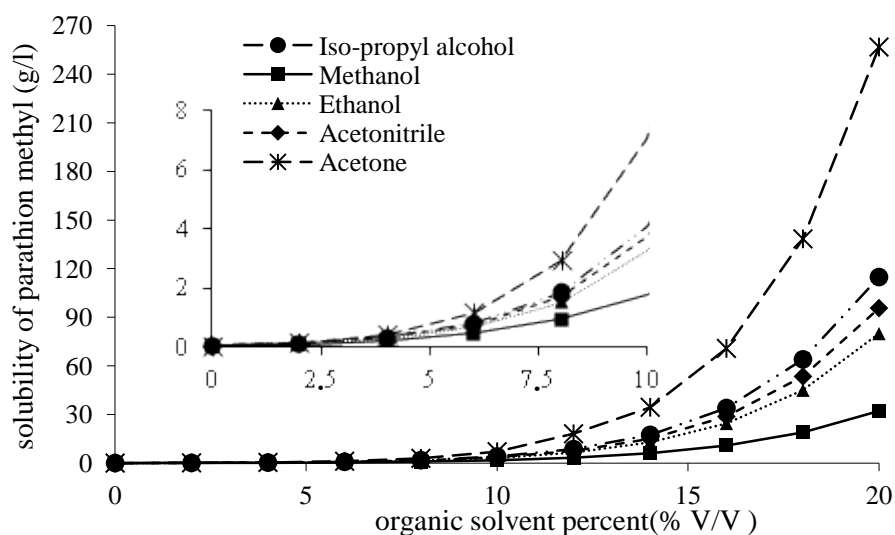


Figure 6.15. Solubility of parathion-methyl in organic solvent-water mixture (T=27 °C).

6.4. Determination of Parathion-Methyl by TCA

In order to determine the parathion-methyl residue, chitosan supported optic enzyme sensor (TCA) was preferred due to their advantages (the highest activity and responsiveness) as mentioned in section 6.2. Parathion-methyl solutions (0.01-15 mg/l) were prepared by dissolving different amount of parathion-methyl in isopropyl alcohol. The activity measurements were performed in the solutions by using TCA at 27 °C and pH 6.5. Figure 6.16 shows relative activity of TCA in pesticide containing media.

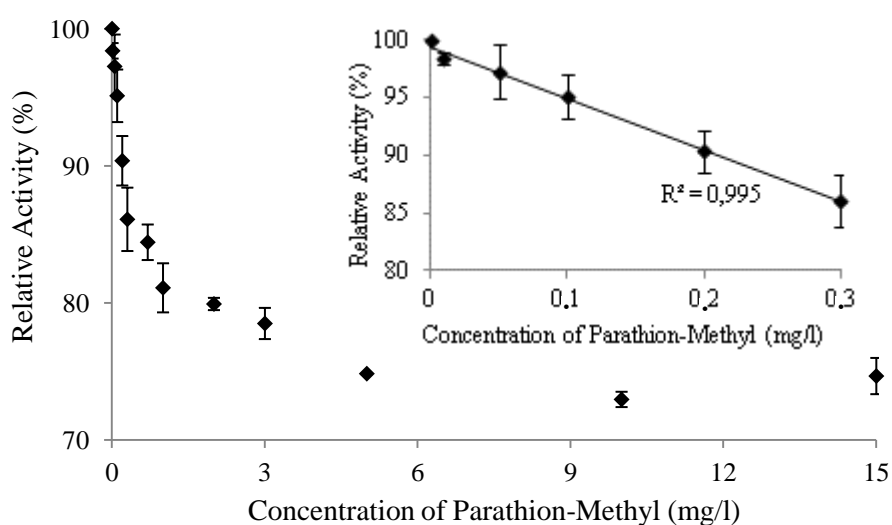


Figure 6.16. Relative activity of TCA in the presence of parathion-methyl. Inset is the linear range of the curve.

Relative activity decreases nearly to 73% at 10 mg/l pesticide concentration. After this value decrease in activity was not observed as seen from Figure 6.16. The activity of TCA linearly decreases up to 0.3 mg/l pesticide concentration. The developed optic enzyme sensor (TCA) can detect the parathion-methyl in the concentration range of 0.06-0.3 mg/l (ppm) with high sensitivity as depicted in Table 6.8. According to the regulations of Turkish Food Codex, maximum residue limits of parathion-methyl are the range of 0.05-0.2 mg/l (ppm) as shown in Table 3.3 (Budak 2001). Therefore the developed sensor can detect the parathion-methyl residue within the maximum residue limits.

Table 6.8. Sensing performance of TCA in parathion-methyl containing media.

Medium	Sensitivity (act/ppm)	R ²	Detection limit (ppm)	Linearity (ppm)
Parathion-methyl	0.043	0.995	0.059	0.06-0.300

The inhibition of enzyme activity exerted by parathion methyl was also investigated by fitting the experimental data to the Michaelis-Menten model equation as depicted in Figure 6.17.

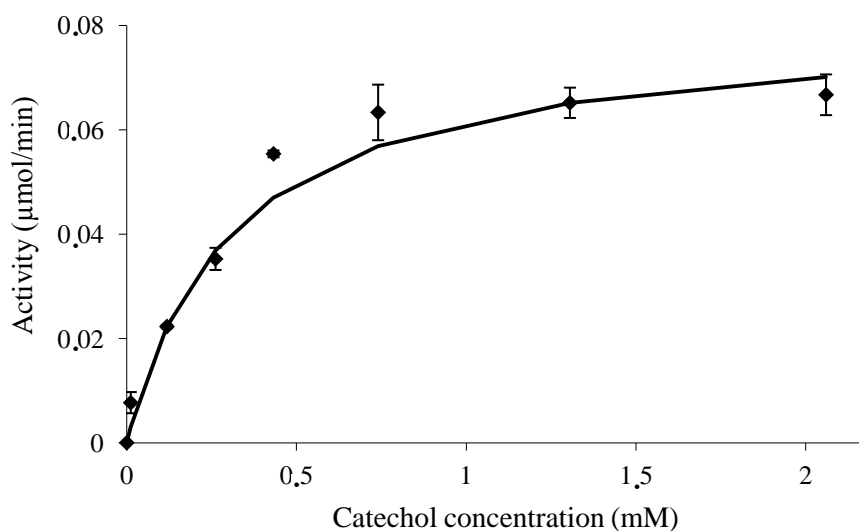


Figure 6.17. The effect of substrate (catechol) concentration on TCA in the presence of 15 ppm parathion-methyl. Points: experimental, line: model (T:27 °C and pH:6.5)

Parathion-methyl decreased the activity by inhibiting the enzyme noncompetitively due to decrease in V_m while K_m is constant as seen from the Table 6.9.

Table 6.9. Response characteristic of TCA in free and parathion-methyl containing media.

Medium	K_m (mM)	V_m ($\mu\text{mol}/\text{min}$)	R^2	Error	Inhibition type	Relative Activity (%)
Pesticide free	0.311	0.095	0.969	0.031	-	100
Parathion-methyl	0.310	0.081	0.976	0.025	Noncompetitive	74.7 \pm 1.3

Parathion methyl solutions with the known concentrations (0.050 ppm and 0.300 ppm) were also prepared by using tap water in order to examine the accuracy of the developed sensor (TCA). The results show that the sensor measured the 0.050 and 0.300 ppm parathion methyl concentration as 0.047 and 0.301 ppm with deviation of ± 0.004 ppm and ± 0.016 ppm respectively.

6.5. Characterization of the Films and the Optical Enzyme Sensors

6.5.1 Chitosan Film (C) and TCA

The morphology of chitosan film (C) and optical enzyme sensor by enzyme immobilized form via adsorption (TCA) were characterized by using Atomic Force Microscopy (AFM). A typical AFM picture of the film C displays uniform porous network structure to provide a significant amount of enzyme loading as depicted in Figure 6.18a. When tyrosinase was immobilized on film C, the aggregates of the enzyme molecules exhibit island-like structures (Figure 6.18b), as stated in the study of Cabaj and Soloduch (2011) which may facilitate the specific reaction between the substrate and the enzyme, resulting in a good response for the biosensor applications. The change in the morphology of TCA with the enzymatic reaction was also characterized by comparing AFM images. Island-like structures for TCA before the reaction were disappeared indicating possible product adsorption to the optical enzyme sensor (Figure 6.18c).

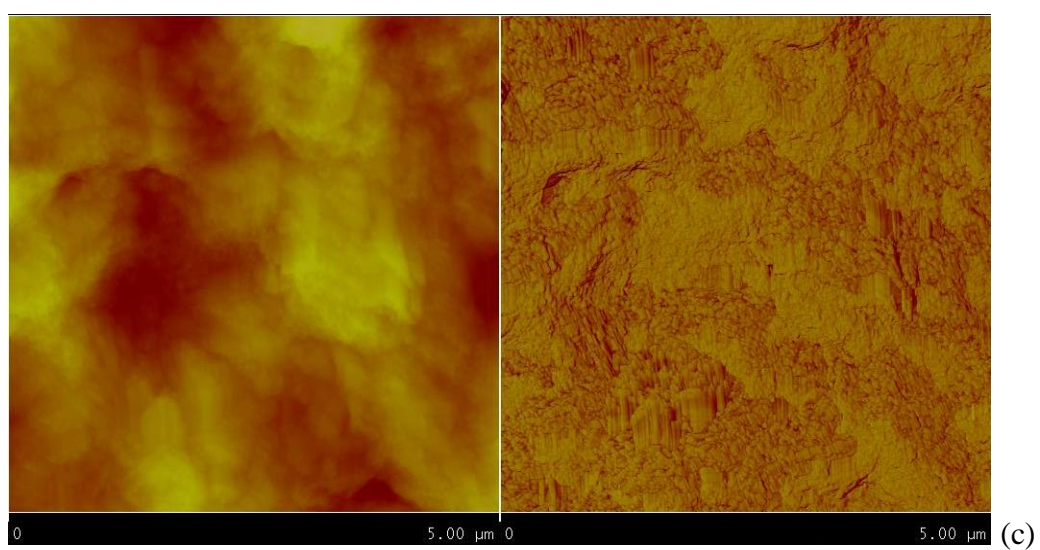
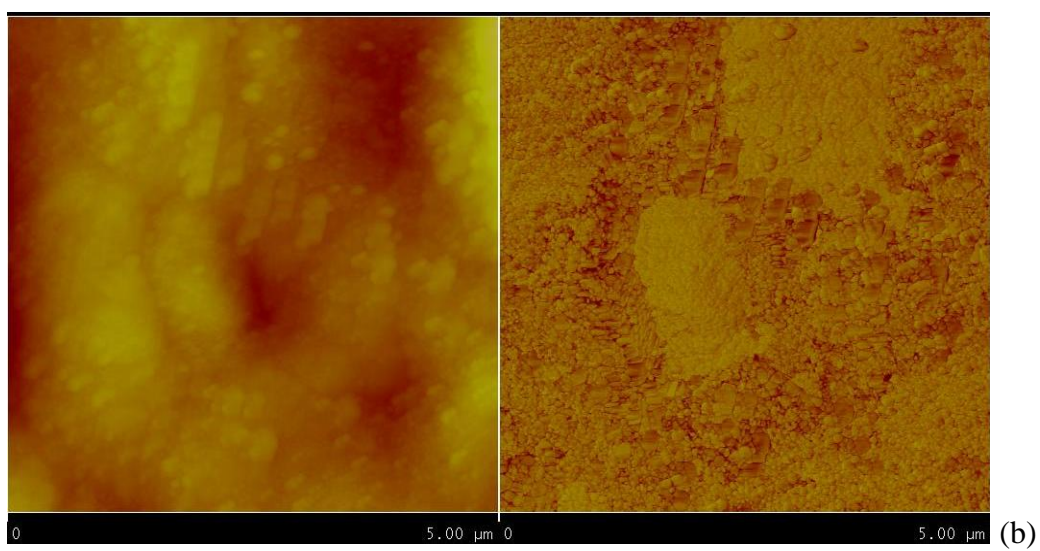
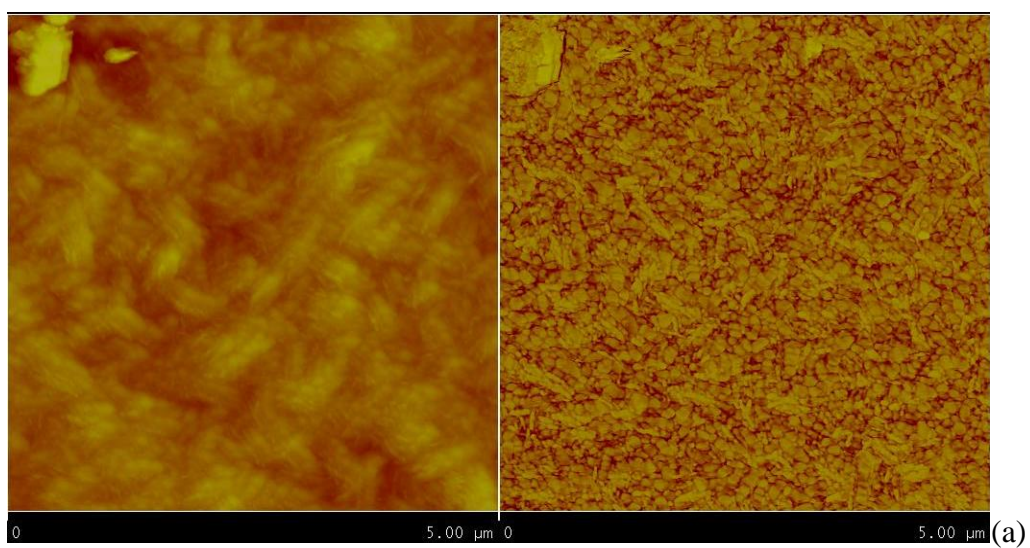


Figure 6.18. AFM images of (a) C, (b) TCA (c) TCA after the enzymatic reaction.

The roughness analysis which gives idea about the texture of the film was performed by using AFM software. The average roughness (Rms) was calculated as 45.9 nm and 64.2 nm for optical enzyme sensor before and after reaction respectively, which may arise from o-quinine adsorption on the surface of optical enzyme sensor.

Figure 6.19 shows the section of TCA images (a) before and (b) after reaction. As can be seen from the figure, the color of the film becomes brown with use. It was deduced that o-quinine produced with the enzymatic reaction was bound on the TCA sensor as stated in the literature (Kumar et al. 1999).

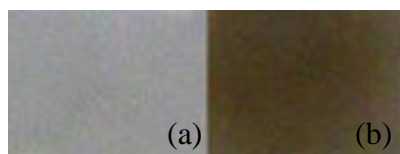


Figure 6.19. The images of the TCA (a) before and (b) after the enzymatic reaction.

Fourier Transform Infrared (FTIR) spectra of the powder chitosan, chitosan film (C) and TCA before and after the enzymatic reactions were examined to realize the bounded sites. Figure 6.20 shows the spectra of powder chitosan and C in the range of 4000-400 cm^{-1}

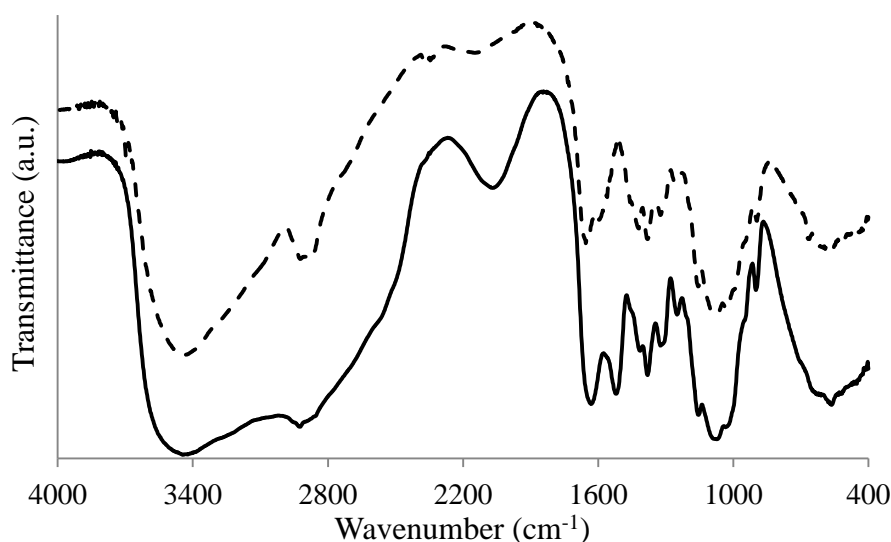


Figure 6.20. FTIR spectra of chitosan powder (- - -) and chitosan film (—) in the range of 4000-400 cm^{-1}

As seen from Figure 6.20 the vibrations at 1650, 1595, 1420 and 1370 cm^{-1} were assigned to amide I (C=O), amide II (N-H bending and C-N stretching), symmetric NH_3^+ bending and CH_3 deformation for powder chitosan, respectively (Table 6.10.) as stated by Wang et al. (2008), Kim et al. (2006), Kumar et al. (1999) and Zhou et al. (2006).

Table 6.10. FTIR spectrums and their assignments

Vibration (cm^{-1})	Assignment
3290-3450	O-H and N-H stretch
2864-2915	C-H stretch
1700-1600	Amide I (C=O)
1620-1500	Amide II (N-H bending and C-N stretching)
1420	Symmetric NH_3^+ bending
1375	CH_3 symmetrical deformation
1073-1031	C-O group or C-C group of amino acid side

When this powder was dissolved in dilute HCl solution, hydrogen ions bind to amide I and amide II groups in the molecular chains of chitosan. The attachment of hydrogen ions to amide I and antisymmetric NH_3^+ deformation is observed with the shift of 1650 cm^{-1} to 1630 cm^{-1} as seen from Figure 6.21.

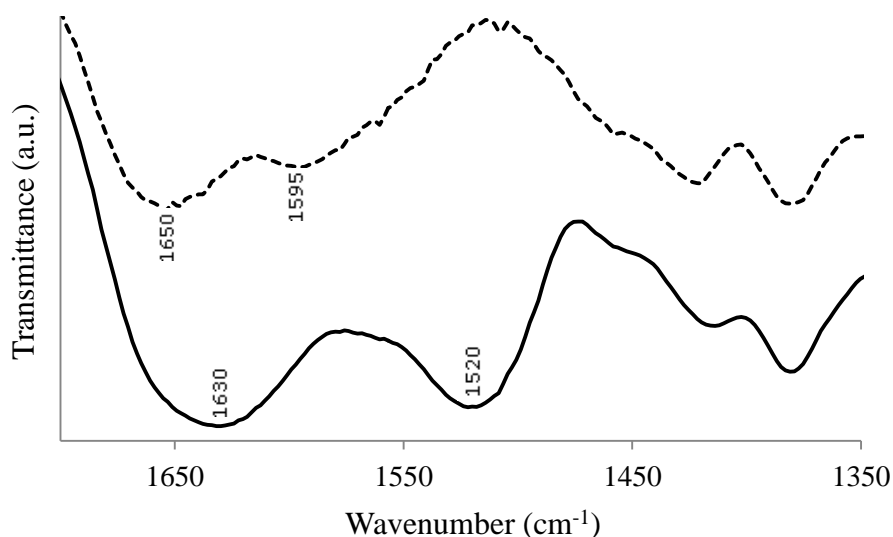


Figure 6.21. FTIR spectra of chitosan powder (- - -) and chitosan film (—)

The disappearance of the weak band at 1595 cm^{-1} and formation of the strong band at 1520 cm^{-1} can be attributed to the attachment of hydrogen ions to amide II site (Kim et al. 2006). The band at 1520 cm^{-1} contains the amide II, N-H bending vibration as well as the symmetric -NH_3^+ deformation as observed in the study of Lawrie et al. (2007).

Figure 6.22 shows the change in the spectrum of chitosan film (C) with enzyme immobilization. The band at 1420 cm^{-1} , representing symmetric -NH_3^+ bending, associated with protonated residues of the chitosan film (Kumar et al. 1999) became weaker with immobilization. This is consistent with the formation of an electrostatic bond between enzyme and amino group of the chitosan film. The occurrence of the bands at 1064 cm^{-1} , attributable to various C-C modes of amino acid side chains (Sampaio et al. 2005) is distinctive.

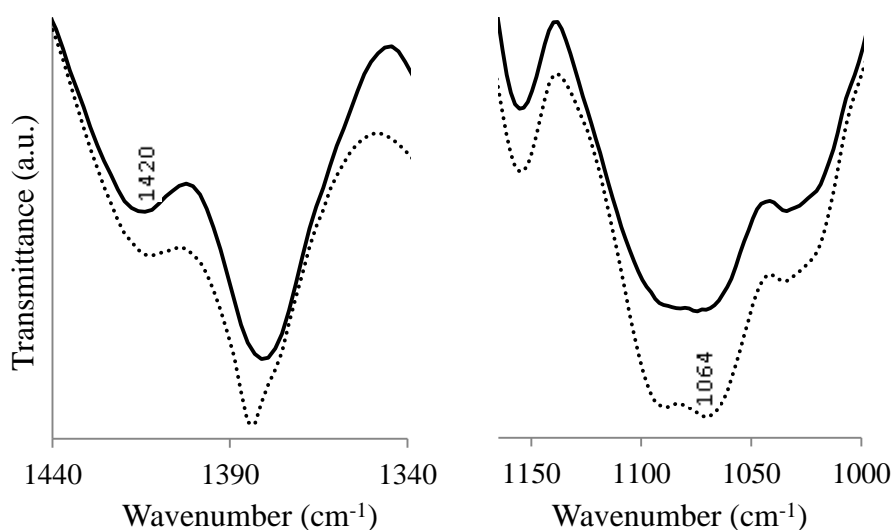


Figure 6.22. FTIR spectra of the chitosan film (—) and TCA (.....)

Figure 6.23 compares the spectra of chitosan film with the TCA (before reaction); the amide II band (at 1520 cm^{-1}) shifted to 1527 cm^{-1} due to the amides or aromatic residues present in the enzyme which physically adsorbs onto the film. With the enzymatic reaction the amide I and II bands shifted to higher values (1641 cm^{-1} and 1545 cm^{-1}) supporting the occurrence of a Schiff-base reaction (Sampaio et al. 2005).

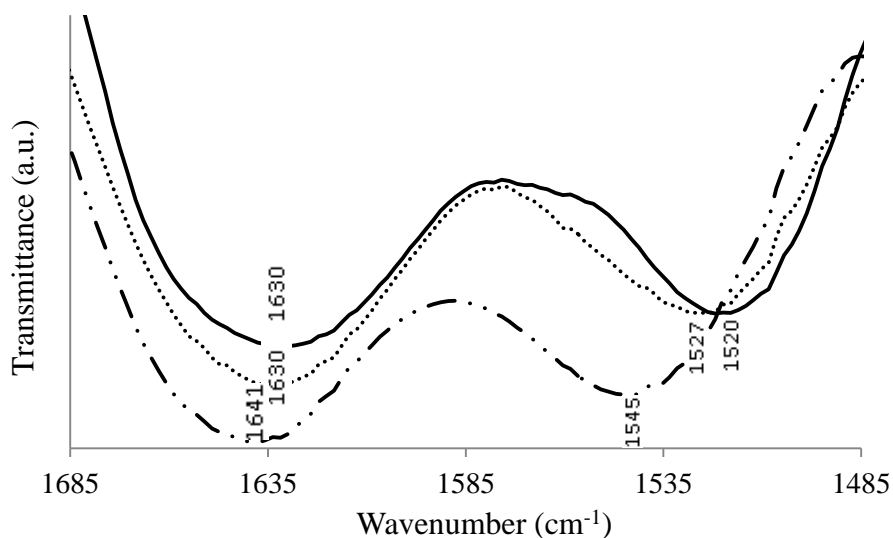


Figure 6.23. FTIR spectra of the chitosan film (—), TCA before use (.....) and TCA after uses (-•-)

Although the chemistry has not been completely established, it appears that the amine in the chitosan structure reacts with compounds containing carbonyl groups to form covalent bonds (Sun et al. 1992). Therefore the enzymatically generated o-quinone can be nonenzymatically reacted with amino groups in chitosan as can be seen from the Figure 6.24. Payne and Sun (1994) showed that the produced quinones from reaction of tyrosinase with phenols bounded to chitosan.

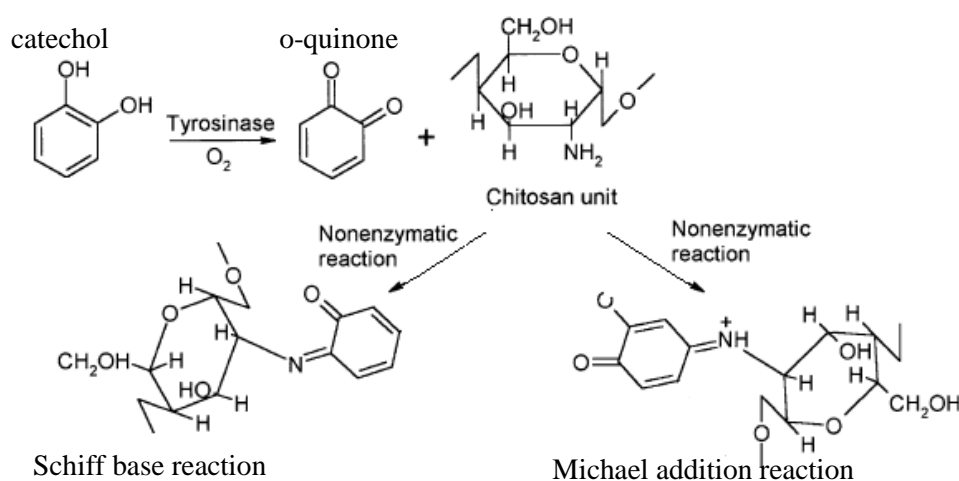


Figure 6.24. Reaction pathways for enzymatically generated o-quinone and nonenzymatic crosslinking reaction with chitosan.

Figure 6.25 shows the UV visible spectra of TCA before and after reaction. Before the reaction, there is no absorbance peak. After the reaction, strong shoulder

peak at around 360 and progressive increase in the absorbance at 470 nm was observed as shown in the figure. These two peaks were assigned to the binding of quinone to amino groups in chitosan (Yamada et al. 2000; Kang et al. 2004).

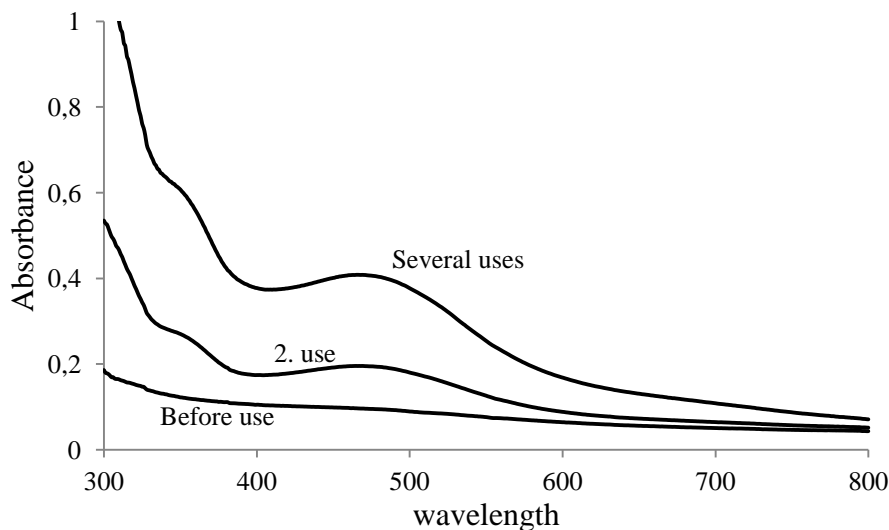


Figure 6.25. UV visible spectra of TCA before and after the enzymatic reaction.

6.5.2 Gelatin Film (G) and TGE

The morphologies of gelatin film (G) and gelatin supported optical enzyme sensor (TGE) before and after the enzymatic reaction were characterized by using AFM. A typical AFM picture of the gelatin aggregates show spherical structure with different diameters as depicted in Figure 6.26a. When tyrosinase was immobilized on gelatin film the surface becomes smooth. After the reaction, big spherical structure observed indicating possible product adsorption to the optical enzyme sensor (Figure 6.26c). The roughness analysis was performed by using AFM software. The average roughness (Rms) which gives idea about the texture of the film was calculated as 0.87 nm and 13.1 nm for TGE before and after the enzymatic reaction respectively, which may arise from o-quinone sorption on the surface of sensor.

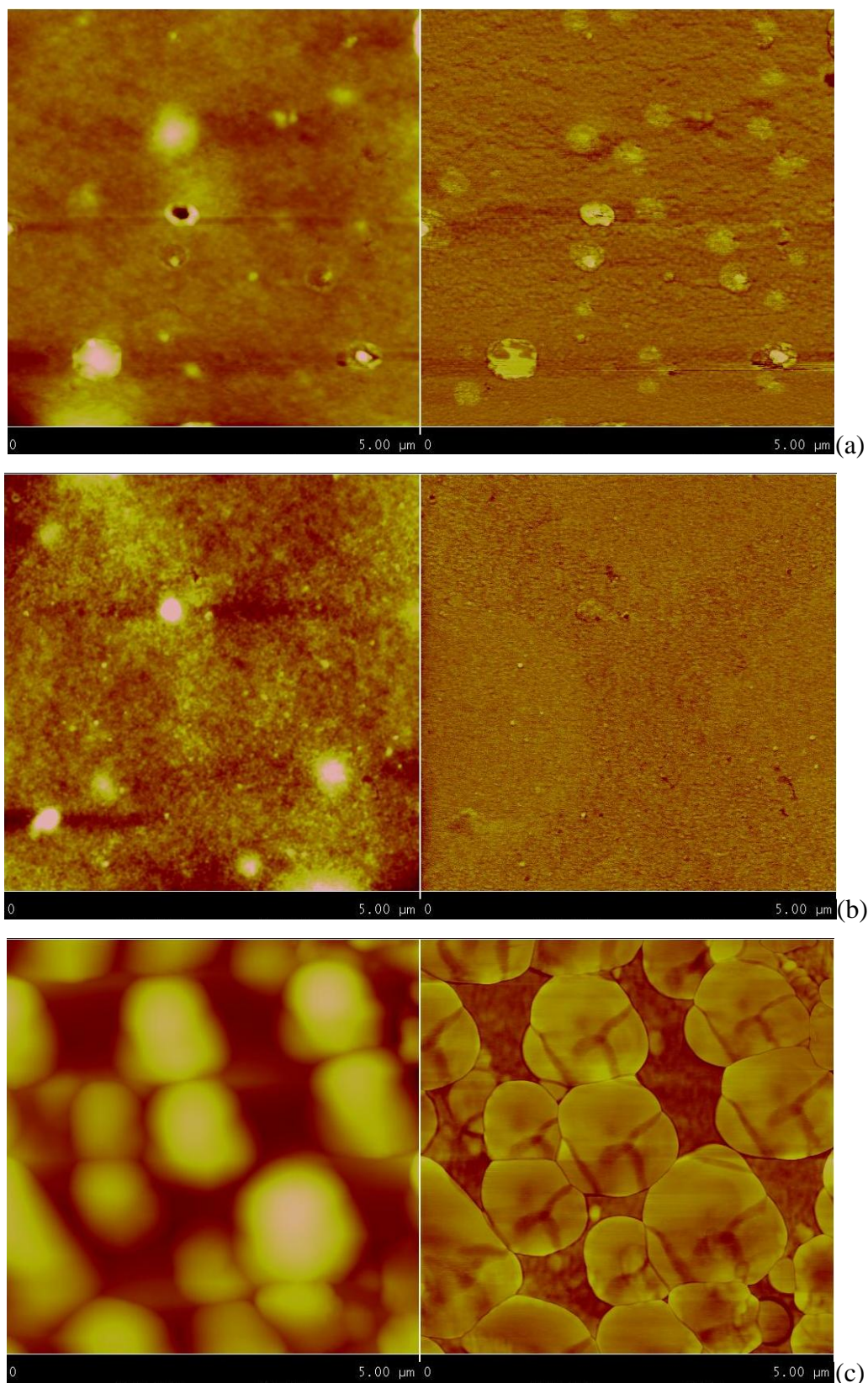


Figure 6.26. AFM images of (a) G, (b) TGE (c) TGE after the enzymatic reaction

Figure 6.27 shows the image of TGE before and after the reaction. The color of the film becomes brown with use as can be seen from the Figure 6.27. This can be

explained with the adsorption of the tyrosinase-generated quinones on gelatin based sensor.

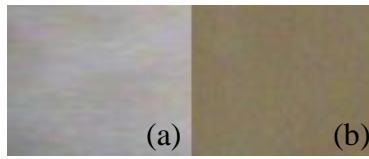


Figure 6.27. The section of TGE images (a) before and (b) after the enzymatic reaction.

Tyrosinase-generated quinones are known to undergo nonenzymatic reactions with various amino groups (Sampaio et al. 2005). Spectroscopic examination of the gelatin film suggested that the protein were modified by the quinone with the possible reaction as represented in Figure 6.28.

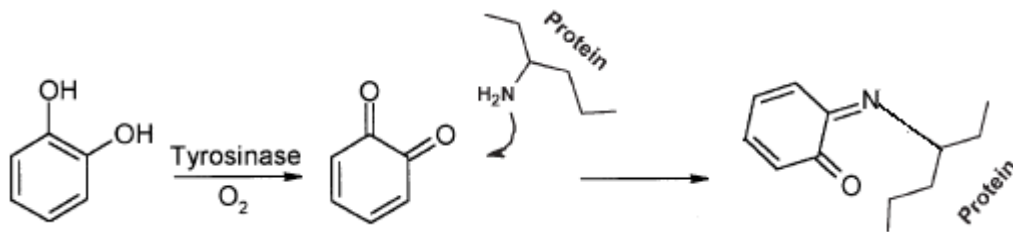


Figure 6.28. Reaction pathways for enzymatically generated o-quinone and nonenzymatic reaction with gelatin

Figure 6.29 shows the change in the absorbance at 470 nm with use of TGE.

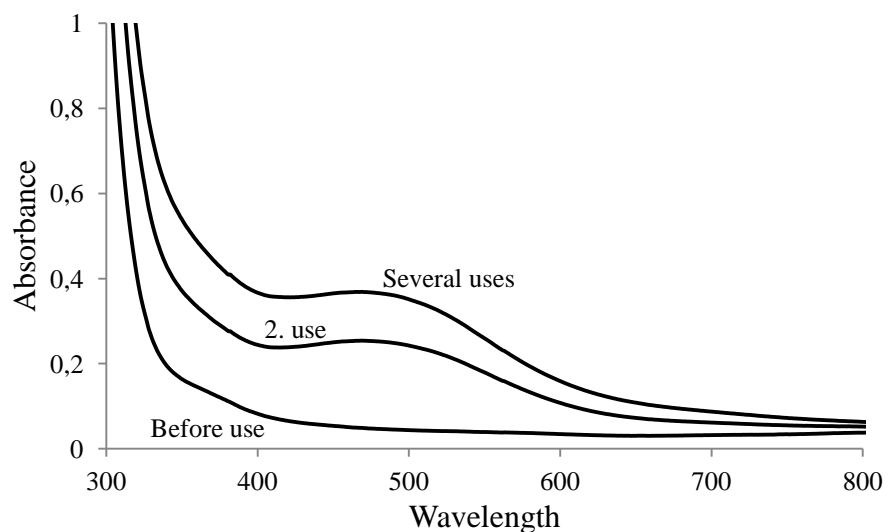


Figure 6.29. UV visible spectra of TGE before and after the enzymatic reaction

The increase in intensity of the peak at 470 nm reveals that tyrosinase catalyzes the oxidation of gelatin and amino groups are involved in quinone binding as stated by Chen et al. (2002).

6.6. Mechanism of Enzymatic Reaction

Mechanism of enzymatic reaction on TGE and TCA sensors were also studied. There are three possible steps for overall enzymatic reaction; substrate transportation from the bulk medium to the surface of the film (external mass transfer), enzymatic conversion into product and product transportation back from the surface to the bulk medium. One or more than one of these steps is the rate-limiting. The influence of external mass transfer resistance on the overall enzymatic reaction is represented using the effectiveness factor;

$$\eta = \frac{\text{(observed reaction rate)}}{\text{(rate in the case of no mass transfer resistance } S_s=S_0)} \quad (4.13)$$

Where S_s and S_0 represent the substrate concentrations at the interface and in the bulk fluid respectively. As explained in section 4.2. mathematically, η

$$\eta = \frac{\frac{V_{max}S_s}{K_m+S_s}}{\frac{V_{max}S_0}{K_m+S_0}} = \frac{\beta_s(1+\beta_0)}{\beta_0(1+\beta_s)} \quad (4.14)$$

Where

$$\beta_0 = S_0/K_m \quad (4.15)$$

$$\beta_s = \frac{-(1+\lambda-\beta_0) \pm \sqrt{[(1+\lambda-\beta_0)^2 + 4\beta_0]}}{2} \quad (4.16)$$

$$\lambda = V_{max}/k_s K_m \quad (4.17)$$

Mass transfer coefficient (k_s) during the reaction was calculated using the empirical correlation (given below) developed by Cussler (1984) for laminar flow and flat plate;

$$\frac{k_s \cdot r_{film}}{D_{i,\infty}} = 0.323 \text{Re}^{0.5} \cdot \text{Sc}^{0.33} \quad (4.18)$$

Figure 6.30 shows variation of effectiveness factors as a function of substrate concentration for TCA and TGE (immobilized enzyme on chitosan and gelatin films, respectively). The effectiveness factors approach to 1 at high substrate concentration which confirms that the kinetics of the immobilized enzyme was observed in the absence of external mass transfer limitations for higher concentrations (0.25 mM <).

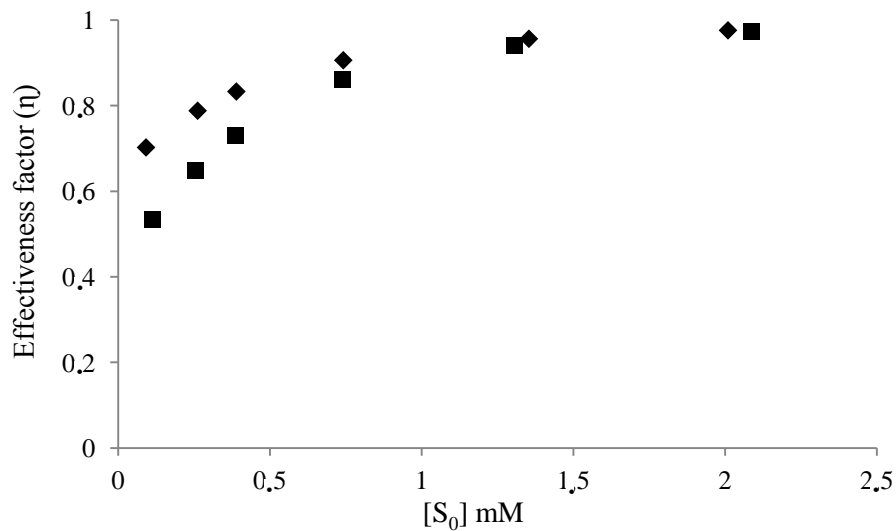


Figure 6.30. Variation of effectiveness factors as a function substrate concentration for TCA (■) and TGE (◆)

CHAPTER 7

CONCLUSIONS

In this study, tyrosinase immobilized chitosan and gelatin films via adsorption and entrapment methods, respectively were prepared as optical enzyme sensors (TCA, and TGE respectively) in order to determine the pesticide (parathion-methyl) residue.

In preparation of the optical sensors, non-immobilized enzyme was removed by washing the films with PBS for 30 min. It was found that 1 μl enzyme for 30 μl support, the lowest enzyme to support ratio was sufficient to observe the maximum activity for preparation of optical enzyme sensor. According to the AFM and FTIR results chitosan and gelatin films were successfully coated with tyrosinase enzyme. It was understood that o-quinone, the product of the enzyme and substrate reaction bind the chitosan and gelatin films.

The activity of both sensors did not significantly change after 10 uses indicating good operational stability (the average activity after 10 uses; TCA: $0.105^{\pm 0.008}$ $\mu\text{mol}/\text{min}$ and TGE: $0.037^{\pm 0.004}$ $\mu\text{mol}/\text{min}$). According to the kinetic analysis, approximately 7 fold and 11 fold decreases in maximum activity for TCA and TGE, respectively were observed with respect to free enzyme. The reaction kinetics between the substrate and enzyme is the reaction-limited and the mass-transfer limited at high and low substrate concentration, respectively for both sensors. Nevertheless TCA was used for parathion-methyl detection due to having higher responsiveness and higher catalytic activity with respect to TGE. pH, temperature and storage stability of the enzyme were enhanced by immobilizing the enzyme to chitosan support due to providing structural rigidity and appropriate flexibility. However organic solvent (ethanol, methanol, isopropyl alcohol, acetone and acetonitrile) tolerance decreased with immobilization. Acetone and acetonitrile decreased both free and immobilized enzyme activity. Whereas ethanol, methanol and isopropyl alcohol caused to increase in free enzyme activity but decrease in immobilized enzyme activity. This arises from the change in enzyme structure and the diffusion resistance to substrate. Isopropyl alcohol seems to be most appropriate organic solvent for parathion-methyl detection due to high solubility for parathion-methyl and low activity loss for the enzyme.

It was observed that parathion methyl inhibited the enzyme and the activity of the sensor decreased nearly from 100% to 73%, when 10 mg parathion-methyl in one liter aqueous solution was determined. TCA sensor detected the parathion-methyl concentration with the linear range of 0.06-0.3 mg/l (ppm) which is in the range of maximum residue limits (0.05-0.2 ppm for parathion methyl).

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APPENDIX A

DETERMINATION OF MAXIMUM ABSORBANCE PEAK FOR THE PRODUCT O-QUINONE

It is necessary to find the maximum peak for catechol and enzymatic product quinone in order to observe the increase in absorbance versus time. For this aim 0.1 ml tyrosinase (2.35 μg) and 0.2 mM catechol at pH 6.5 was put in the cuvette and wavelength scanning was performed before and after the reaction (2. min) to see the maximum absorption peak for catechol and product *o*-quinone. Figure A.1 shows the spectrophotometric scanning of catechol and colored compound *o*-quinone. The results show that before the reaction only catechol gave absorbance peak at 275 nm. After 2 min, the tyrosinase-catechol biocatalysis gave increasing absorbance peaks with time at the same maximum absorption of 390 nm as reported in the literature (Lee et al. 2002, Mason 1949, Ibrahim and Ramadan 2006) for the colored compounds *o*-quinones in the reaction media.

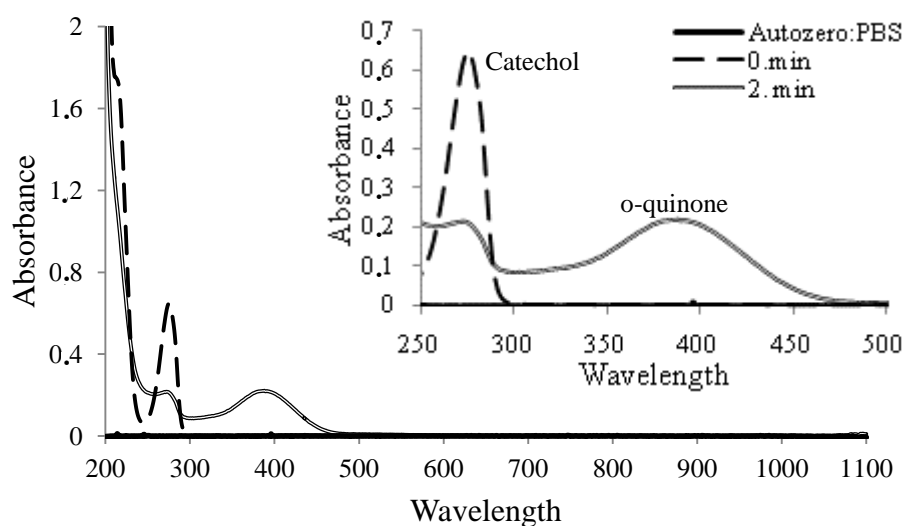


Figure A.1. Scanning profiles of catechol and enzymatic product *o*-quinones.

The enzymatic product *o*-quinones are very unstable and undergo to a sequence of non-enzymatic reactions that preclude any isolation of an end product. To use the end-point approach one is forced to add MBTH or other nucleophilic compounds able to produce a stable stoichiometric adduct with the *o*-quinones (Fiorentino et al. 2010).

Figure A.2 shows the scanning profiles of tyrosinase-catechol reaction with time. As can be seen from the Figure, there was not any other peak and any shift seen indicates that the product o-quinone did not undergo to a sequence of non-enzymatic reactions even at 4. min. This is a time short enough to avoid the occurrence of non-enzymatic reactions

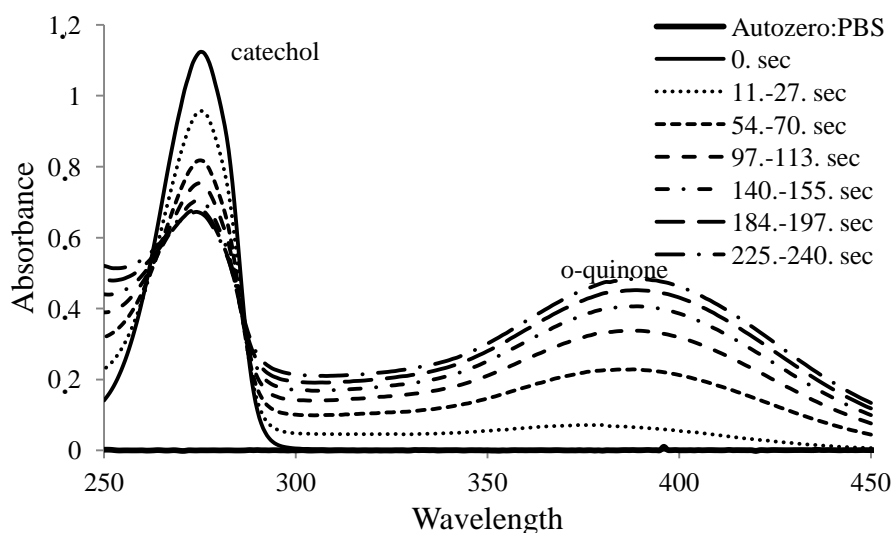


Figure A.2. Scanning profiles of tyrosinase-catechol reaction with time.

Figure A.3 shows the consumption of catechol (at 275 nm) and formation of o-quinone (at 390 nm) with time. The absorption peak for catechol decreases (at 275 nm) while the peak for product (390 nm) increases with time.

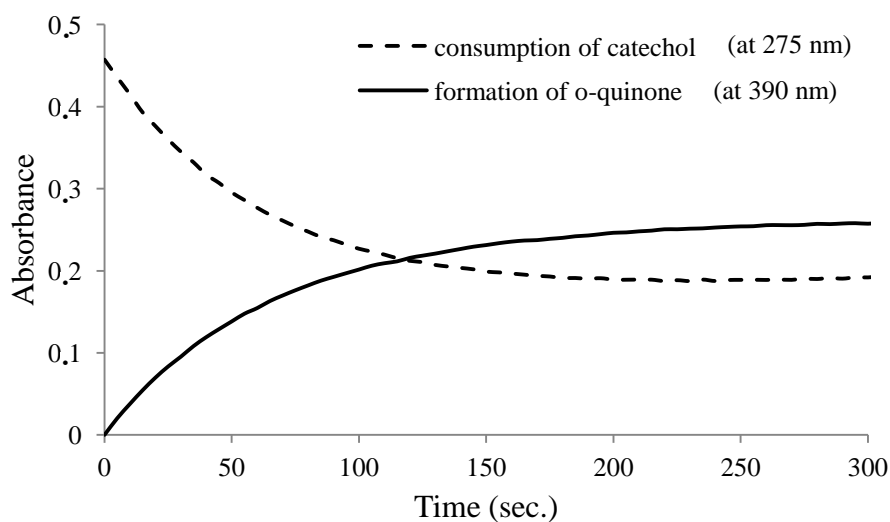


Figure A.3. Consumption of catechol (at 275 nm) and formation of o-quinone (at 390 nm) with time.

Therefore the enzymatic activity for free and immobilized enzyme can be measured by consumption of catechol and formation of o-quinone. In this work all of the enzymatic activity measurements were conducted by means of absorbance profile at λ : 390 nm (the maximum peak for enzymatically produced o-quinone) in UV- visible spectrophotometer during 1 min without using the any nucleophilic compounds. The change of absorbance ΔA over the time interval Δt : 1min (slope of the reaction: dA/dt), gives the initial steady-state condition of the reaction with the quasi linear shape as shown in Figure A.4.

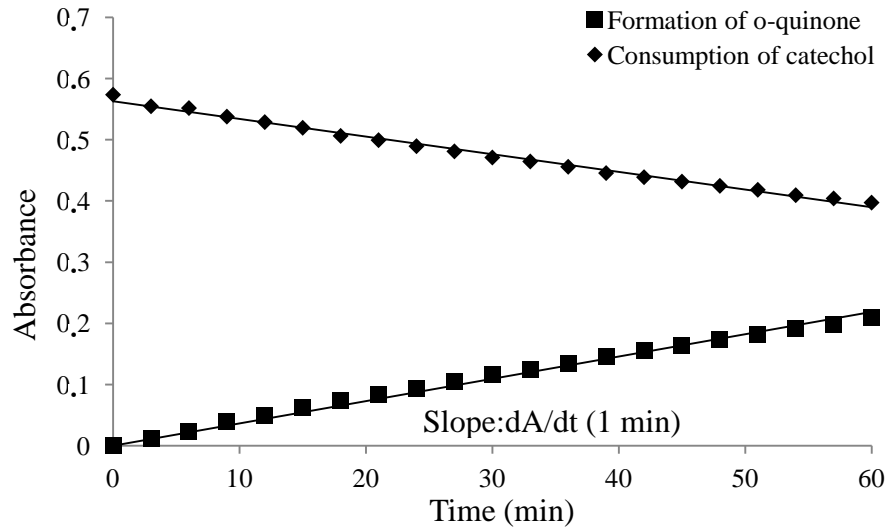


Figure A.4. The change of absorbance due to product formation with time.

Using the Beer–Lambert relationship relating absorbance (A) to concentration (c) extinction coefficient (ϵ) and path-length ($l=1\text{ cm}$) (Eisenthal and Danson 2002).

$$A = \epsilon cl \quad (\text{A.1})$$

In our case the concentration is the product concentration ($c = P$). The slope of the kinetic curves was converted to the tyrosinase activity expressed as $\mu\text{mol}/\text{min}$ using formula by taking the extinction coefficient for o-quinone formation from catechol at 390 nm as $\epsilon_{390} = 1417\text{ M}^{-1}\text{ cm}^{-1}$ (Aytar and Bakir 2008).

$$\frac{dP}{dt} = \frac{dA}{dt} \frac{1}{\epsilon l} \quad (\text{5.2})$$

APPENDIX B

DETERMINATION OF RELEASE ENZYME AMOUNT FROM THE FILM DURING THE WASHING STEP

Before use the optical enzyme sensors, they were immersed in PBS (3 ml) for 30 min to wash out the non-immobilized components from the film surface. The amount of enzyme release from the film to the solution (E_w) was determined by using the calibration curve represented in Figure A.5.

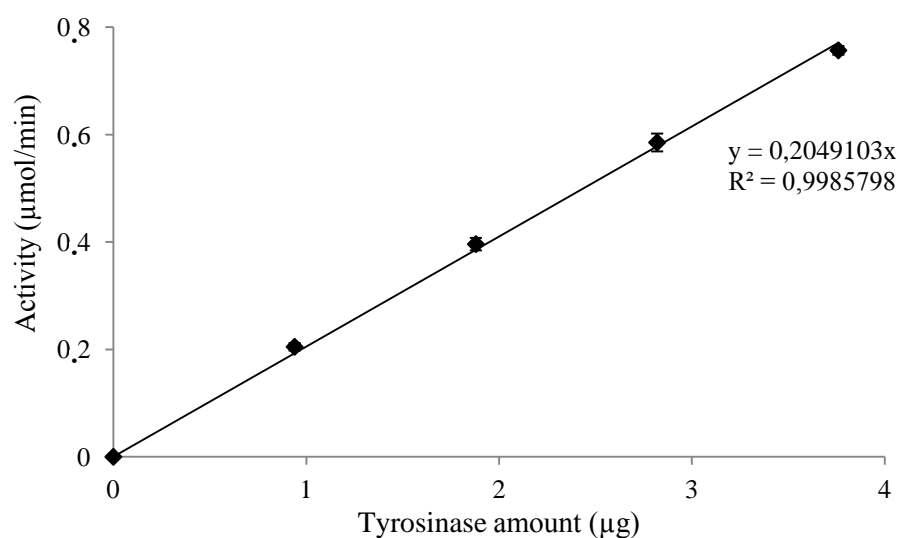


Figure B.1. Calibration curve of tyrosinase amount with respect to tyrosinase activity.

APPENDIX C

DETERMINATION OF SOLUBLE AMOUNT OF PARATHION METHYL IN ORGANIC SOLVENTS

Step 1. Estimation of solubility parameter of organophosphates

$$\delta = \left(\frac{\Delta \text{vap} \underline{U}}{\underline{V}^L} \right)^{1/2} = \left(\frac{\Delta \text{vap} \underline{H} - RT}{\underline{V}^L} \right)^{1/2} \quad (\text{C.1})$$

δ : Solubility parameter of organophosphate (cal/cc)^{1/2}

$\Delta \text{vap} \underline{U}$: Vaporization internal energy of organophosphate (J)

\underline{V}^L : Molar volume of organophosphate pesticide (cc/mol)

$\Delta \text{vap} \underline{H}$: Vaporization enthalpy of organophosphate (J)

R : Universal gas constant (J/molK): 8.314

T : Temperature (K)

$\Delta \text{vap} \underline{H}$ from Clausius-Clapeyron Equation

$$\frac{\ln P^{\text{vap}}(T_2)}{\ln P^{\text{vap}}(T_1)} = -\frac{\Delta \text{vap} \underline{H}}{R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right) \quad (\text{C.2})$$

$$\underline{V}^L : \text{Molar volume of liquid pesticide (cc/mol)} \quad \underline{V}^L = \frac{M_{w(\text{pesticide})}}{\rho_{(\text{pesticide})}}$$

$$M_w : \text{Molecular weight (g/mol)} \quad \rho : \text{density (g/cc)}$$

Table C.1. Solubility parameters of organophosphates.

Organophosphate	M_w	ρ (g/cc)	\underline{V} (cc/mol)	$\Delta \underline{H}$ vap(J/mol)	δ (cal/cc) ^{1/2}
Parathion-methyl	263,21	1,358	193,822	245254	17.302

Table C.2. Solubility parameters of water and organic solvents.

Organophosphate	M_w	ρ (g/cc)	<u>V</u>(cc/mol)	Δ<u>H</u>vap(J/mol)	δ (cal/cc)^{1/2}
Water	18.02	0.997	18.069	43990	23.433
Methanol	32.04	0.792	40.465	35278	13.919
Ethanol	46.07	0.789	58.390	38560	12.153
Acetonitrile	41.05	0.777	52.831	33225	11.794
Acetone	58.08	0.790	73.519	31300	9.680
Isopropyl-alcohol	60.10	0.786	76.463	44000	11.392

Step 2. Estimation of $\Delta fus H(T_m)$

$$\ln\left(\frac{S_1}{S_2}\right) = -\frac{\Delta fus H}{R} \left(\frac{T_2 - T_1}{T_1 T_2}\right) \quad (C.3)$$

S_1 : Solubility of organophosphate in water at T_1

S_2 : Solubility of organophosphate in water at T_2

Table C.3. Organophosphate's fusion enthalpies.

Organophosphate	T_m	T₁	T₂	S₁ (mg/L)	S₂ (mg/L)	R (J/molK)	Δ<u>H</u>fus (J/mol)
Parathion-methyl	308.55	293,15	298,15	55	60	8,314	12645,639

Step 3. Estimation of activity coefficient of organophosphate in organic solvent-water mixture

$$RT \ln \gamma_1 = \underline{V}_1^L (\delta_1 - \bar{\delta})^2 \quad (C.4)$$

$$\bar{\delta} = \sum_j \Theta_j \delta_j \quad (C.5)$$

$$\Theta_j = \frac{x_j \underline{V}_j}{\sum_k x_k \underline{V}_k} \quad (C.6)$$

γ_1 : Activity coefficient of organophosphate in water-organic solvent mixture

δ_1 : Solubility parameter of organophosphate (cal/cc)^{1/2}

$\bar{\delta}$: Solubility parameter of water-organic solvent mixture (cal/cc)^{1/2}

Step 4. Estimation of solubility of organophosphate in organic solvent-water mixture

$$\ln x_1 = -\ln \gamma_1 - \left[\frac{\Delta fus H(T_m)}{RT} \left(1 - \frac{T}{T_m} \right) \right] \quad (C.7)$$

x_1 : solubility of organophosphate in organic solvent-water mixture.

VITA

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Immobilization of enzyme to chitosan and gelatin supports
Solubility of pesticides in organic solvents and the effect of organic solvents on the enzyme activity
Development of optic enzyme sensor and detection of pesticide by optic enzyme sensor

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SCI expanded Journal Papers

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- Polatoğlu İ., Çakıcıoğlu-Özkan, F., “Aqueous interactions of zeolitic material in acidic and basic Solutions ,Microporous and Mesoporous solutions”, 132(1-2), 219-225 (2010).
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Publications in International Congress

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 - Polatoğlu İ., Özkan F., “Tyrosinase-Fe₃O₄-Chitosan nanobiocomposite film for biosensor applications, NANOTR6, İzmir-Turkey, 2010
 - Polatoğlu İ., Özkan F., “The Buffering Effect of Natural Zeolite on Gastric Disturbance”, EMCC6, Antalya-Turkey,2010
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Publications in National Congress

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- Polatoğlu İ., Özkan F., “Holandit-Klinoptilolit Doğal Zeolitin Ortamındaki Proton Transfer Mekanizmalar”, 9. National Chemical Engineering Congress, Gazi University, Ankara, 2010.
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