DEVELOPMENT OF PAPER TYPE TYROSINASE BIOSENSOR

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÖZGE ŞENYURT

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

MAY 2008

Approval of the thesis

DEVELOPMENT OF PAPER TYPE TYROSINASE BIOSENSOR

submitted by **ÖZGE ŞENYURT** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Gülay Özcengiz
Head of Department, **Biotechnology** _____

Prof. Dr. Hüseyin Avni Öktem
Supervisor, **Biology Dept., METU** _____

Prof. Dr.M.Yakup Arıca
Co-Supervisor, **Biology Dept., Gazi University** _____

Examining Committee Members:

Prof. Dr. Meral Yücel
Biology Dept., METU _____

Prof. Dr. Hüseyin Avni Öktem
Biology Dept., METU _____

Prof. Dr. Haluk Hamamcı
Food Engineering Dept., METU _____

Prof. Dr. Cumhur Çökmüş
Biology Dept., Ankara University _____

Assist. Prof. Dr. Füsun İnci Eyidoğan
Education Faculty, Başkent University _____

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Özge Şenyurt

Signature:

ABSTRACT

DEVELOPMENT OF PAPER TYPE TYROSINASE BIOSENSOR

Şenyurt, Özge
M.S., Department of Biotechnology
Supervisor: Prof. Dr. Hüseyin Avni Öktem
Co-supervisor: Prof.Dr. Yakup Arıca

May 2008, 58 pages

Phenolic compounds are the chemicals which are used by many different industries and as a result of this spread to the environment. These compounds can be absorbed easily through the human and animal skin and through the mucosal membrane, mix in to the blood circulation and thus create a toxic effect on several tissue and organs including, liver, lung and kidneys. For this reason, determination of phenolic compounds emitted to environment is a very important issue. In fact, there are standard methods for the determination of these compounds like HPLC, Spectrophotometric and calorimetric methods however, these are time consuming methods and requires to be expertise. On the other hand, there are also different types of biosensors developed for the phenolic compound detection. In this study, a new, disposable, cheap and convenient tyrosinase biosensor was developed for the phenolic compound detection. By means of absorption method, the enzyme tyrosinase and the chromophore MBTH were immobilized on the support material and as a model substrate L- dopa was used.

As a result of optimization studies 1mg/ml tyrosinase concentration and 1.5mM MBTH concentration were determined for using in biosensor construction.

Detection limit of l-dopa, model substrate, found as 0,064 mM and for other phenolic compounds, 4-chlorophenol, catechol, m-cresol and p-cresol, detection limit was obtained 0.032 mM, 0.032 mM, 0.128 mM, 0.128 mM, respectively. In addition, we found that the biosensor response was not affected by pH changes ranging from 3 to 11. The stability of biosensor which is one of the important parameter for commercialization was not change through 70 days at room temperature and 4°C when compared to at the beginning response.

Key words: Tyrosinase, biosensor, phenolic compounds, adsorbed enzyme

ÖZ

KAĞIT TİPİ TİROSİNAZ BİYOSENSÖRÜNÜN GELİŞTİRİLMESİ

Şenyurt, Özge
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem
Yardımcı Tez Yöneticisi : Prof.Dr.Yakup Arıca

Mayıs 2008, 58 sayfa

Fenolik bileşikler, bir çok farklı endüstri tarafından kullanılan ve çevreye salınan bunun sonucunda da çevre kirliliğine yol açan kimyasallardır. Hayvan ve insan derisinden, mukoza zarından kolayca emilerek vücuda karışmakta ve karaciğer, akciger, böbrek olmak üzere birçok organ ve dokuda toksik etki yaratmaktadır. Çevrede yarattıkları toksik etki nedeniyle bu bileşiklerin belirlenmesi büyük önem taşımaktadır. Fenolik bileşiklerin belirlenmesi için standart yöntemler (HPLC, spektrofotometrik, kalorimetrik) mevcuttur fakat bu metodlar zaman alan ve deneyimli kişi gerektiren yöntemlerdir. Bunun yanında fenolik bileşikleri belirlemek için farklı türde biyosensörler de geliştirilmiştir. Bu çalışmada endüstride fenolik bileşikleri belirlemek için tek kullanımlık, ucuz ve kolay kullanım özelliği olan tirosinaz biyosensörü geliştirilmiştir. Tirosinaz enzimi ve kromofor olarak kullanılan MBTH adsorpsiyon yoluyla kağıtlara bağlanmış ve model substrat olarak L-Dopa kullanılmıştır.

Yapılan optimizasyon çalışmaları sonucunda 1mg/ml tirosinaz konsantrasyonu ve 1.5mM MBTH konsantrasyonu biyosensor oluşturulması için uygun bulunmuştur.

Model substrat olan L-dopa için belirleme konsantrasyonları 0.064 mM ve diğer fenolik bileşikler için belirleme konsantrasyonu 4-klorofenol, katechol, m-kresol and p-kresol için sırasıyla 0.032 mM, 0.032 mM, 0.128 mM, 0.128 mM olarak belirlenmiştir. Geliştirilmiş olan biyosensörün cevabında pH 3-11 aralığında bir değişim olmadığı gözlenmiştir.

Ticarileşme için önemli bir parameter olan kararlılık çalışmalarında oda sıcaklığında ve 4°C'de biyosensörün cevabında yetmiş gün boyunca biyosensör cevabında ilk güne göre önemli bir değişim olmadığı gözlemlenmiştir.

Anahtar kelimeler: Tirosinaz, biyosensör, fenolik bileşikler.

To my family,

ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor Prof. Dr. Hüseyin Avni Öktem for his guidance, valuable comments and endless supports I would also like to express my gratitude to Prof. Dr. Meral Yücel for her encouragement, valuable supports and precious remarks. I am pride of being a member of their laboratory.

I would also like to thank my co-supervisor Prof.Dr.Yakup Arıca for insight, helps and comments.

I would like to thank to all my thesis committee for their suggestions and criticism.

I would also to thank my lab mates Ufuk Çelikkol Akçay, Hamdi Kamçı, Beray Gençsoy, Tahir Bayraç, Musa Kavas, Gülsüm Kalemtaş, Cengiz Baloğlu, Taner Tuncer, Ceyhun Kayıhan, Emre Aksoy, Nilüfer Afşar, Murat Kavruk, Sena Cansız, Fatma Kılbacak and Ceren Karakaş one by one for their splendid technical and moral supports, valuable comments and sincere friendships.

I appreciate Oya Ercan and Tufan Öz for their advices in experiments , their critical views throughout my study and writing the thesis; endless patience and valuable friendships whenever I needed.

I wish to thank Zeynep Öktem and Fatma Doğu for always being helpful and support throughout writing the thesis.

I am also thankful to my friends, Esra Terzi, Günçe Çetin, Filiz Morova and Oya Cingöz for their endless love and support throughout writing the thesis.

Finally I am thankful and giving all my love and appreciation to my mother Türkan Şenyurt ,my father Sami Şenyurt and my brother Özgür Şenyurt for their endless love ,thrust , encouragement and support at all stages of my life. I also would like to thank Gülce Şenyurt for her friendship and support as a member of my family.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

LIST OF ABBREVIATIONS

MBTH- 3-methyl-2-benzothiazolinone

L-dopa- L-3,4-dihydroxyphenylalanine

CHAPTER 1

INTRODUCTION

1.1 Biosensor

Biosensor is an analytical device incorporating a biological material (e.g enzyme, antibody, receptor, cell etc) in an intimate contact with a suitable transducer device (e.g optic, electrochemical, piezoelectric, thermometric, magnetic etc) that converts the biochemical signal into quantifiable electric signals (Marco *et al.*, 1996; Sharma *et al.*, 2003) (Fig.1) (Gerard *et al.*, 2002). Digital electronic signal generated by biosensors is proportional to the concentration of a specific analyte (Sharma *et al.*, 2003).

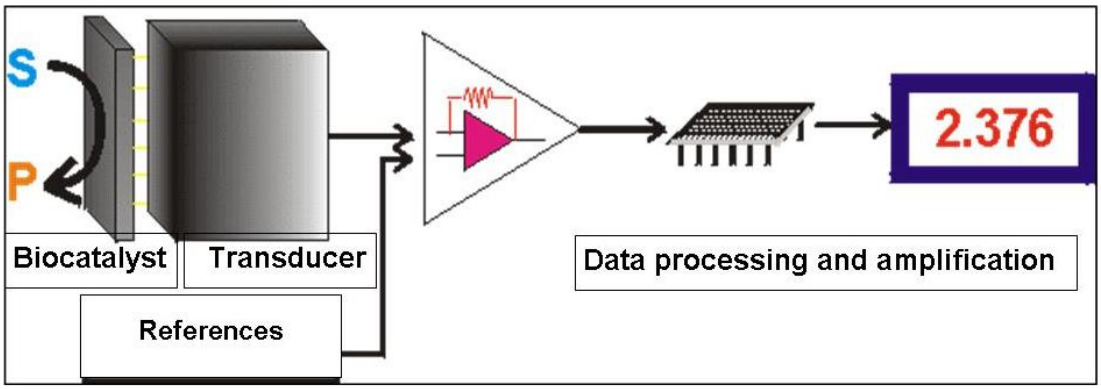


Figure 1.1: Schematic representation of biosensor (Chaplin, 2004)

Biosensors are used in different area such as in medical diagnostics, environmental monitoring, defense, genetics and food processing industries (Malhotra *et al.*, 2005).

While clinical uses of biosensors are mainly in area of monitoring clinical metabolites, especially blood glucose, the environmental uses are in pollution control.

In defense industry biosensors are used for detection of explosives, nerve gases, microbial toxins and in food, cosmetics and in fermentation industry it is used during process control, quality control and monitoring. When the market share of application areas is analyzed, clinical diagnostics area has the biggest market share, especially glucose biosensors. Market share of biosensor application area shown in Table 1.1 (Chaplin M.F,2000).

Table 1.1: The market share of biosensor application areas (Chaplin M.F, 2000).

Application area	**Market share(%)**
Clinical diagnostics	
Glucose	85
Lactate and others	4
Research	4
Pharmaceuticals	3
Environmental	2
Food	2
Robotics, defence and others	<1

A successful biosensor must have at least some of the properties listed in Table 1.2 (Chaplin M.F, 2000). The biological component used in biosensor construction should be highly specific for the analyte and the reaction should be independent of physical parameters such as pH, temperature, stirring. The response of biosensor should be rapid, accurate, reproducible and precise. If the biosensor is used for clinical diagnosis, the sensor probe must be biocompatible, small and having no any toxic effect. In addition to all features a successful biosensor should be miniature

size, low cost and portable, capable of using semi-trained operators (Chaplin M.F, 2000; Chaplin M, 2004).

Table 1.2: The properties required of a successful biosensor (Chaplin, 2000)

Required property	**Achievable with ease**
Specificity	Yes
Discrimination	Yes
Repeatability	Yes
Precision	Yes
Safe	Yes
Accuracy	Yes, as easily calibrated
Appropriate sensitivity	Yes, except in trace analysis
Fast response	Yes, usually
Miniaturizable	Yes, generally
Small sample volumes	Yes, generally
Temperature independence	Yes, may be electronically compensated
Low production costs	Yes, if mass-produced
Reliability	Yes, generally
Marketable	Difficult, due to competing methodology
Drift-free	Difficult but possible
Continous use	Yes, for short periods(days)
Robust	No, generally need careful handling
Stability	No, expect on storage(months)or in the short term(weeks)
Sterilizable	No, except on initial storage
Autoclavable	Not presently achievable

Biosensors can be divided into two distinct group according to biological component i.e. catalytic and noncatalytic (affinity) biosensors. The biological component of catalytic biosensors are enzymes, microorganisms while the affinity based biosensors consist of antibodies, nucleic acid, receptors, cell etc. Different

types of transducers can be used for detection of analytes which are electrochemical (amperometric, potentiometric and conductometric), optical, calorimetric, piezoelectric, and acustic etc. (Sharma *et al*., 2003). Figure 1.2 shows biomolecules and transducer types used in biosensor construction (Adhikari & Majumdar, 2004).

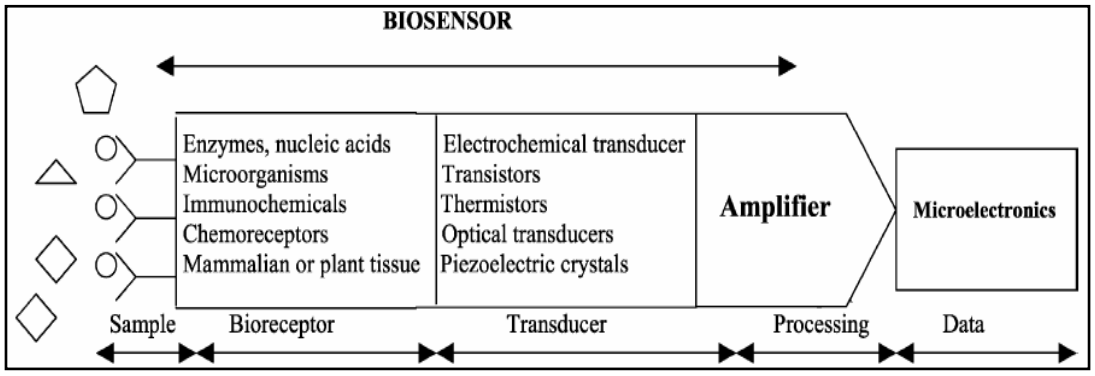


Figure 1.2: Types of biomolecules and transducer used in biosensor development

1.1.1 Types of Biosensors according to Transducer

1.1.1.1 Electrochemical Biosensors

"An electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element" (Thévenot *et al.,* 2001). The electrochemical biosensors are divided into groups of three which are amperometric, conductometric and potentiometric according to measurement type of transducer (Thévenot *et al.,* 2001; Mello *et al.,* 2002).

1.1.1.1.1 Amperometric Biosensors

The working principle of amperometric biosensors relies on measuring the current resulting from the oxidation or reduction of a product or reactant (Thévenot *et al.,* 2001; Gerard *et al.*, 2002). The first amperometric biosensor was developed for blood glucose measurement by Clark and Lyon in 1962.

Glucose oxidase was immobilized on oxygen electrode via entrapment and measurement of glucose level was done by the oxygen consumption monitoring. In order to measure the reduction of oxygen consumption a negative potential was applied to the platinum cathode (Wang J., 2008)**.**

Anode reaction: $\text{glucose} + O_2 \longrightarrow \text{gluconic acid} + H_2O_2$ (1.1)

Cathode reaction: $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$ (1.2)

1.1.1.1.2 Potentiometric Biosensors

Potentiometric biosensors using ion selective electrode (ISE) determine the potential difference between two reference electrodes in order to measure concentration changes of selected ions. The most common ion selective electrodes used in potentiometiric biosensor construction are pH electrodes, different ion electrodes (F^-, I^-, CN^-, Na^+, K^+, Ca^{2+}, Na^+, NH_4^+) and gas selective electorodes (CO_2, NH_3) (Thévenot *et al.,* 2001; Chaplin, 2000).

1.1.1.1.3 Conductometric Biosensors

The conductivity change resulting from ion concentration changes of the reaction medium is detected by conductometric biosensors (Mello *et al.,* 2002; Chaplin, 2000).

Urea biosensor is an example of conductometric biosensor in which enzymatic urea hydrolysis leads to conductivity increase via ammonium and bicarbonate ions production (Sheppard *et al., 1996;* Chaplin, 2000).

1.1.1.2 Calorimetric Biosensors

Enthalpy change during biochemical reaction forms the working principle of calorimetric biosensors also called enzyme thermistors or thermometric or thermal biosensors (Gerard *et al*., 2002; Chaplin, 2000). A number of analyte such as glucose, ethanol, cholesterol, ascorbic acid, lactate, penicillin, antigens etc. can be detected via thermal biosensors. When optical biosensors compared to other biosensor types, it has advantages that calorimetric biosensors do not affected by environmental changes and a number of analyte can be detected with one calorimetric biosensors via co-immobilization of different enzyme (Chaplin, 2000).

1.1.1.3 Optical Biosensors

Optical biosensors also called optrodes are based on the measurement of light output during the interaction of the biocomponent with the analyte or a light absorbance difference between the reactants and products (Chaplin, 2004; Mello *et al.,* 2002). Optrodes advantage is that there is no need for a reference electrode during the process (Chaplin, 2000).

1.1.1.4 Piezoelectronic Biosensors

The basic principle of piezoelectronic biosensors is that change of frequency of oscillation because of the crystal mass change resulting from adsorption of analyte (Gerard *et al*., 2002; Chaplin, 2000). OCM based biosensors are the examples of

piezoelectronic biosensor (Bunde *et al*., 1998). Piezoelectronic type biosensors are used for the detection of ammonia, hydrogen, methane, carbon monoxide, nitrousoxide and other organophosphorous compounds (Gerard *et al*., 2002).

1.1.2 Types of Biosensors according to Biological Component

Biological components one of the key part biosensors functions as biological transducers which are enzymes, antibodies, aptamers, receptors, cells, nucleic acids, biomimetic materials and plant or animal tissues (Lei *et al.,* 2006; Vo-Dinh & Cullum, 2000; Gerard *et al*., 2002). According to biocomponent recognition properties biosensors can be classified as biocatalytic biosensors and bioaffinity biosensors (Sharma *et al*., 2003; Marazuela & Moreno-Bondi, 2002; Vo-Dinh & Cullum, 2000).

1.1.2.1 Biocatalytic biosensors

Enzymes, microorganisms and tissues (plant or animal) are used as biological recognition elements in biocatalytic biosensors (Thévenot *et al.,* 2001). In biocatalytic biosensors the concentration of detected analyte can be determined by measurement of the formation rate of product, the degradation of reactant or reaction inhibition (Marazuela & Moreno-Bondi, 2002).

Enzymes are most commonly used biocomponent in biosensor technology because of specificity, selectivity and efficiency (Thévenot *et al.,* 2001, Marazuela & Moreno-Bondi, 2002). As mentioned before the first enzyme biosensors was amperometric glucose biosensor developed by Clark and Lyon in 1962 (Wang, 2008). Many of the enzyme based biosensors were developed for detection of different analytes, for example; penicillin, urea, alcohol, nitrate, fish freshness, glutamine, choline, pesticides summarized in Table 1.3 (Marazuela & Moreno-Bondi, 2002; Sharma *et al*., 2003).

Table 1.3: Enzymes used for development biosensors and their applications (Sharma *et al.*, 2003).

Enzymes	Analyte	Application
Alcohol oxidase	Acetic acid	Blood and saliva alcohol test and fermentation industries
Alcohol oxidase	Alcohol	Alcohol test and fermentation industries
Cholesterol oxidase and esterase	Cholesterol	Cardiovascular diseases ,Cholesterol sensor for healthcare and food industry
Formate dehydrogenase	Formic acid	Fermentation industry and health care
Glucose oxidase	Glucose	Diabetes, fermentation and food industry
Glutaminase	Glutamine	Myocardial and hepatic diseases
Glutmate dehydrogenase	Glutamic acid	Myocardial and hepatic diseases
Lactate dehydrogenase	Lactic acid	Liver and heart diseases
Lactate oxidase	Lactate	Human healthcare
Malate dehydrogenase	Malate	Fermentation industry
Nitrate reductase	Nitrate	Environmental and industrial processes
Nitrite reductase	Nitrite	Environmental and industrial applications
Oxalate oxidase	Oxalate	Diagnosis of hyper-oxaluria in urine (kidney disease)
Penicillinase	Penicillin	Pharmaceutical industry
Succinate dehydrogenase	Succinic acid	Fermentation industry
Tyrosine dehydrogenase	Tyrosine	Human healthcare
Urease	Urea	Kidney function test
Uricase	Uric acid	Kidney function test
Acetylcholine esterase	Choline	Choline sensor for healthcare
Alcohol dehydrogenase	Alcohol	Alcohol sensor for blood, saliva and fermentation
Alcohol oxidase	Alcohol	Alcohol sensor for blood and fermentation process
Cytochrome B2	Lactate	Used in bienzyme lactate sensor (LO/LDH)
Cytochrome C	Formic acid	Formic acid sensor used in urine, blood and gastric juices
Glutamate decarboxylase	Glutamate	Glutamate sensor for healthcare
Lactate dehydrogenase and oxidase	Lactate	Lactate sensor for liver and heart diseases
Nitrate reductase	Nitrate	Nitrate sensor for environment and industrial application
Urease	Urea	Urea sensor for kidney function test and industrial application
Uricase	Uric Acid	Uric acid sensor for hematology disorder
Xanthine oxidase	Xanthine	Fish freshness sensor

Whole cell (microorganisms, plant or animal cells, tissues) biosensors play an important role in biosensors technology since they posses wide detection range, tolerance of temperature and pH changes, easy biosensor regeneration (Marco & Barcelo, 1996; Marazuela & Moreno-Bondi, 2002; D'Souza, 2001). The basis of whole sensors is that genetically engineered reporter gene inserted into the cell is expressed in the presence of monitored analyte which can be quantitatively measured or alternatively cell metabolism, cell respiration can be analyzed as an indication of monitored analyte (Badahi-Mossberg *et al.*, 2007; Vo-Dinh & Cullum, 2000).

Whole cell biosensors based on microorganisms can be constructed by using luciferase, β-galactosidase or alkaline phosphatase reporter systems emitting visible light and by this way in the presence of monitored analyte produced reporter protein is detected and quantified (Ramanathan *et al.*, 1997; Köhler *et al.*, 2000; Daunert *et al.,* 2000).

In addition to microorganisms, enzymes, plant or animal cells can function as biosensing element in whole cell biosensor type. Some examples include, algal biosensors for herbicides detection relies on chlorophyll fluorescence measurement related to concentration of herbicides (Frense *et al.,* 1998; Naessens *et al.,* 2000).

1.1.2.2 Bioaffinity Biosensors

Bioaffinity biosensors relies on the measurement of physicochemical changes resulting from the irreversible and noncatalytic interaction between analyte and biocomponent (Marazuela & Moreno-Bondi, 2002).

The most developed examples of bioaffinity biosensors are antibody based biosensors called also immunosensor whose functions depends on the antigen-

antibody interaction. The important factor using antibody as a biological component is specific binding capacity of antibody to interested analyte (Vo-Dinh & Cullum, 2000; Marazuela & Moreno-Bondi, 2002; Badahi-Mossberg *et al*., 2007). Different immunosensors were developed for environmental and clinical analysis for instance; for the pathogenic bacteria (*L.monocytes & Bacillus cereus*) detection (Susmel *et al.*, 2003), for the analysis of insecticides (Brummel *et al.*, 1997) and for herbicide detection during food control quality (Yulaev *et al.*, 2001).

Another biorecognition element used in bioaffinity biosensor design is nucleic acids. Hybridization of target molecule which is complementary DNA target sequence with single stranded DNA or RNA probe immobilized onto transducer surface is the underlying principle of DNA biosensors (also named genosensors) which are used for genetic and infectious diseases and for the detection of DNA interaction and damage. Alternatively, instead of DNA peptide nucleic acids (PNAs), artificial oligo amide capable of stable complex with complementary nucleic acid sequence can be used as a biocomponent of biosensor (Junhui *et al*., 1997; Palecek *et al.,* 1998; Sassolas *et al.,* 2008; Nakamura *et al.,* 2003).

1.2 Biosensors for environmental monitoring

The quantity of compounds released as a result of industrial and agricultural process into the environment increases in everyday and the released compounds because of their toxicity may affect adversely the ecosystem and public healthcare. Therefore, biosensors for environmental monitoring are an important issue (Marco and Barcelo, 1996).

Biosensors for environmental monitoring can measure toxicity effect (cytotoxicity, genotoxicity, mutagenity, carcinogenity) or can detect compounds causing environmental pollution (Rodriguez-Mozaz *et al.,* 2006).

Different biosensors have been developed for the detection of more than 30 different environmental relevant compound, such as ammonia, nitrate, urea, sulfur dioxide, phenol, xenobiotics (pesticides, herbicides) etc. (Riedel, 1998).

In the field of environmental monitoring most of the developed biosensors are microbial biosensors since they have long-term stability and no need a cofactor (Riedel, 1998). A different kind of microbial biosensor have been developed for detection of environmental pollutant by quantifying light, fluorescence, color or electric current (Badahi-Mossberg *et al.,* 2007). The principle on the basis of microbial biosensor for environmental monitoring is that generally genetically engineered bacteria are used as a biological transducer of biosensor (Rodriguez-Mozaz *et al.,* 2006). For example recombinant *Escherichia coli* based biosensors are used for the determination heavy metals (Liao *et al*., 2006) and nitrate (Taylor *et al*., 2004). In addition to these examples BOD (biochemical oxygen demand) biosensors which are whole cell biosensor have been developed for environmental monitoring. Biochemical oxygen demand (BOD) is an indication of the amount biodegradable organic material in water and since its detection is time consuming and not suitable for online process monitoring, BOD biosensors based on the bacterial respiration rate measurement have been developed for fast determination (Rodriguez-Mozaz *et al.,* 2006).

Besides microbial cell based biosensor, different types of enzyme biosensor have been developed for environmental monitoring. Detection of environmental pollutant relies on enzymatic transformation or enzyme inhibition (Badahi-Mossberg *et al.*, 2007). Some examples of biosensors for environmental monitoring includes, detection of organophosphorus compounds, widely used as insecticides, by organophosphorus hydrolase based biosensor (Wang *et al.,* 1999; Mulchandani *et al.,* 2001) or by acethylcholinesterase based biosensor relied on inhibition of acethylcholinesterase activity (Neufeld *et al.,* 2000); detection of formaldehyde, one

of the hazardous air pollutant, by formaldehyde dehydrogenase based biosensors (Herschkovitz *et al.,* 2000).

Also, different immunosensor are used for detection of pollutants, such as polycyclic aromatic hydrocarbons which is a widespread organic pollutants (Fȁhnrich *et al.,* 2003) and food pathogens (*L.monocytes* & *Bacillus cereus*) (Susmel *et al.*, 2003).

1.3. Tyrosinase Biosensors

Tyrosinase (E.C 1.14.18.1), also called polyphenol oxidase, phenolase or catecholase, is a copper - containing monooxygenase enzyme that catalyzes the orthohydroxylation of monophenols to form o-diphenols (cresolase activity) (1) and the oxidation of o-diphenols to o-quinines (catecholase activity) (2) which are molecular oxygen dependent reaction (See also appendix A.1) (Abdullah *et al.*, 2006a; Marco & Barcelo, 1996; Karam & Nicell, 1997; Duran *et al.*, 2006):

phenol + tyrosinase (O_2) → catechol (1)

catechol + tyrosinase (O_2) → *o*-quinone + H_2O (2)

Tyrosinase is found widespread in nature such as it is present in plants, fungi, microorganism and mammals with different function (Streffer *et al.*, 2001; Halaouli *et al.*, 2006). The skin and hair color of mammals is determined by melanin biosynthesis catalyzed by tyrosinase in the presence of the tyrosine aminoacid (Kim & Uyama, 2005). While the function of tyrosinase in plants is melanin scab formation that provides protection of plants against the attacks of insects and microorganisms, in vegetables and fruits tyrosinase is responsible for enzymatic browning, following bruising which are results form cutting or other damage to the cell (Van Gelder *et al.*, 1997).

The ability of tyrosinase to convert monophenols into diphenols and diphenols into o-quinines provides using this enzyme in different application area such as food industry, pharmaceutical industry and environmental pollution control (Streffer *et al.*, 2001).

In food industry, tyrosinases are used for the production of antioxidant food additives. For example ; caffeic acid and hydroxytyrosol production were performed by tyrosinase. Also; in food technology enzymatic protein-protein cross-linking is an important process and tyrosinases are used for polymerization of different enzymes, such as casein , lysozyme and ribonuclease. In pharmaceutical industry tyrosinases are used for biosynthesis of L-dopa, a useful drug for the treatment of Parkinson's and myocardium disease. For environmental pollution aqueous phenols and some pesticides are removed by tyrosinases (Halaouli *et al.*, 2001). In addition to these, immobilized tyrosinases are used for measurement of polyphenol content of wines, tea and amount of phenolic compounds in waste water.

Immobilization of tyrosinase in polysiloxane/ polypyrolle copolymer matrices by Arslan *et al*. (2005) provides measurement of polyphenol in tea which is one of the source of phenolic compounds. Also, Kıralp and Toppare(2006) were analyzed polyphenol content of Turkish wines by tyrosinase electrode which prepared via entrapment of tyrosinase on conducting polymers. In addition to these studies, in order to detect the tea polyphenols Abhijith *et al*. (2007) developed amperometric tyrosinase biosensor which is constructed by immobilization of tyrosinase on cellephane membrane via cross-linking and combining enzyme membrane with Clark-type amperometric electrode.

Chuang *et al*. (2006) achieved development of an electrochemical tyrosinase biosensor for the measurement of albumin and total protein in human serum. This developed biosensor based on tyrosine catalysis by tyrosinase immobilized on a

modified carbon sensing electrode. As a result of tyrosine residue catalysis the protein concentration were quantified by measuring the reduction current.

Environmental pollution control is another application area of tyrosinase in order to detect phenolic compounds or remove phenolic compounds from wastewater (Karam & Nicell, 1997; Marco & Barcelo, 1996). The study of using tyrosinase for the determination of phenolic compounds, based on electrochemical analysis, started in the 1970's. In addition to phenolic compounds tyrosinase is used for detection of herbicides and pesticides (Anh *et al.,* 2004; Tanimoto de Albuquerque and Ferreira, 2007).

Conductometric tyrosinase based biosensor was develepod by Anh *et al*.(2004) for the detection of diuron, atrazine and its main metabolites wich are common pollutants found in the enviroment. Another study was reported by Tanimoto de Albuquerque and Ferreira (2007) that they achieved construction of amperometric tyrosinase on the composite electrode surface by cross-linking .The study of Anh *et al*.(2004) and Tanimoto de Albuquerque and Ferreira (2007) relied on determination of toxic compunds, such as diuron, atrazine, by the principle of tyrosinase inhibition.

Phenolic compounds are widely used by large number of industries such as resin, plastic, wood preservation, petroleum refining, dyes and textile industry and as a result of process they are released into the environment. Because of their high toxicity towards living organism and easily absorbed by animals and humans through the skin and mucous membranes, determination of phenolic compounds in the environments constitutes a crucial role in environmental monitoring (Abdullah *et al.*, 2006a). Phenolic compounds determination can be achieved by traditional analytical methods such as HPLC, capillary electrophoresis, gas chromotography, calorimetric and spectrophotometric methods (Abdullah *et al.*, 2006a; Nistor *et al.*, 2002).

However, these traditional methods are time-consuming, slow and need well-trained operators and expensive instrumentation (Abdullah *et al.*, 2006a, Abdullah *et al.*, 2006b). Therefore, for the determination of phenolic compounds different types of tyrosinase based biosensors, posses many advantages such as simple, fast and portable, have been developed.

An optical tyrosinase based biosensor, consist of immobilized tyrosinase in chitosan film on microscope glass slide was reported by Abdullah *et al.* (2006a). Phenol determination was performed by maroon color adduct, formed as a result of reaction between quinone produced from enzymatic oxidation of phenol and 3-methyl- 2-benzothiazolinone (MBTH).

Another study for the detection of phenols was performed by Wang et al (2002). They achieved development of tyrosinase biosensor which offers a high-sensitivity towards phenolic compounds by immobilization of tyrosinase in a positively charged chitosan film on a glassy carbon electrode.

Perez et al (2006) developed an amperometric tyrosinase biosensor designed by putting polyacrylamide microgels in which tyrosinase was entrapped, on the glassy carbon electrode surface.

Paranjpe *et al.* (2001) achieved developed a disposable optrode relied on immobilization of tyrosinase in a composite biopolymer matrix composed of jellose and agarose.

Single walled carbon nanotubes (Zhao *et al*., 2005) and multiwalled carbon nanotubes (Tsai and Chiu, 2007) were used for the determination of phenolic compounds. Zhao *et al*., 2004 achieved development of tyrosinase based single walled carbon nanotubes sensor by immobilization of tyrosinase on single walled

carbon nanotubes modified glassy carbon electrode. Tsai and Chiu (2007) constructed an amperometric tyrosinase biosensor based on coating of glassy carbon electrode with multiwalled carbon nanotubes-nafion- tyrosinase mix. Both biosensor are amperometric and have high sensitivity towards phenolic compounds.

Development of another biosensor consists of immobilized tyrosinase to core-shell magnetic nanoparticles was attached to the surface carbon paste electrode with a permanent magnet was reported by Liu *et al.* (2005).

Cosnier *et al*. (1999) developed a polyhenoloxidase – poly (amphilic pyrrole) electrode for on site monitoring of phenol in aqueous effluents with a low detection limit, 10nM and high storage time.

Anh *et al*.(2002) achieved the development of potentiometric tyrosinase pH-sensitive field effect transistor(pH-FET) for 4-chlorophenol detection in water solutions. The principle of biosensor is measuring the pH change, resulting from the tyrosinase action, inside the sensitive membrane found on the FET gate.

Wang and Chen (1995) developed a submersible probe, tyrosinase based carbon paste electrode, for remote on site monitoring of phenolic compounds by measuring electrochemically.

Also, another study conducted by Freire *et al.* (2002) for remote on site monitoring of phenolic compounds .They developed highly sensitive phenol biosensor by co-immobilization of laccase and tyrosinase on carbon fiber microelectrodes.

Russell and Burton (1999) achieved construction of a portable, disposable bioprobe by immobilization of mushroom polyphenol oxidase on a synthetic membrane for detection and semi-quantification of phenolic substances in water.

1.4 Aim of the Study

Although many different types of tyrosinase biosensors have been reported in the literature, still they are far from commercialization for routine and remote on-site sensing of environmental pollutants. Thus, the aim of this study is to develop a cheap, single-use and easy to use paper type tyrosinase biosensor for the detection of phenolics compounds.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Tyrosinase from mushroom, 3-methyl-2-benzothiazolinone (MBTH), L-dopa were purchased from Sigma Chemical Company. Pyrocatechol, 4-chlorophenol, m-cresol and p-cresol were purchased from Aldrich. All other chemicals were purchased from Merck.

2.1.2 Support Material

Whatman filter paper no .1 was used as support material and was purchased from Whatman.

2.2 Methods

2.2.1 Construction of Biosensor

2.2.1.1 Preparation of Support Material

Whatman filter paper was cut into 1x1 cm pieces which were autoclaved prior to use.

2.2.1.2 Preparation of Enzyme Solution, MBTH Solution and Substrate

For optimization of enzyme concentration 8 mg Tyrosinase was dissolved in 1mL cold 0.05 M PBS at pH 7.0 and 0.0161mg MBTH solutions was dissolved in 1.5 mL distilled water for preparing stock solutions. Both solutions were prepared freshly.

As a model substrate L-dopa was used. Stock 5mM L-dopa solution was prepared by dissolving 0,0015 g in 1.5mL 0.05 M PBS at pH 7.0 and then diluted with buffer to a final concentration of 0.256 mM.

2.2.1.3 Immobilization of Enzyme/MBTH Solution on Support Material

For preparation of biosensor, tyrosinase and MBTH solution were mixed at different concentration and then 25 µL was transferred on Whatman paper (1×1cm). For control, only 25 µL MBTH was loaded on paper. The loaded papers were dried in vacuum desiccator at 680 mmHg for 15 min.

2.2.1.4 Effect of Enzyme Concentration on Biosensor Response

In order to determine the effect of enzyme concentration on biosensor response, different enzyme/MBTH mixture having different enzyme concentration were prepared and transferred to the paper. For preparation of these mixture , 8mg /ml stock tyrosinase enzyme solution and 50 mM MBTH stock chromophore solution were used. Four different concentration of enzyme/MBTH mixture were prepared w between 0.5 - 4 mg/mL enzyme concentration and 24 mM MBTH concentration. For control 24 mM MBTH solution was used.

2.2.1.5 Effect of MBTH Concentration on Biosensor Response

To determine the effect of MBTH concentration eight different enzyme/MBTH mixture were used which have 0.187mM, 0.375mM, 0.750mM, 1.5mM, 3mM, 6mM, 12mM, 24mM MBTH concentration and 1mg/mL tyrosinase concentration.

2.2.2 Sensitivity towards Different Substrates

Besides model substrate L-dopa for determination the effect of different substrate concentration pyrocatechol, p-cresol, m-cresol and 4-chlorophenol on the enzyme activity were determined. These substrates were prepared as 5mM stock solutions in distilled water and then were diluted with water to different concentrations 0.001mM-0.512 mM.

2.2.3 Effect of pH on Biosensor Response

The effect of pH on biosensor response was investigated by observing the color change produced by adding 0.256 mM L-dopa solution having different pH values (3-11). 5 mM stock L-dopa solution was prepared in 0.05 M PBS at pH 7.0 and then for preparation 0.256 mM L-dopa solution 0.05 M PBS at different pH (1-13) were used.

2.2.4 Stability of biosensor

In order to determine the stability of biosensor (enzyme/MBTH solution : 1mg/mL Tyrosinase /1.5mM MBTH) it was stored in plastic boxes sealed with parafilm and covered with aluminium foil at 4°C and at room temperature. The response of biosensor towards 0.256mM L-dopa were measured in 10 days intervals for 2 months

2.2.5 Quantitative Analysis of the biosensor response

Numerical measurement of the colours were obtained after drying for 1.5 hours by scanning the coloured paper-type biosensor using Hewlett-Packard Photosmart C3180 and measuring the levels of red colour with OBİTEK® ObiColorMaster software (Fig.1).

ObiColorMaster color units are on a scale of 0 to 255, where 0 is pure black and 255 is pure white. The readings decreased with increasing color intensity, in order to obtain better demonstration sample readings were subtracted from 255 and by this way increasing signal was obtained with increasing substrate concentration. All of the experiments were carried out in triplet.

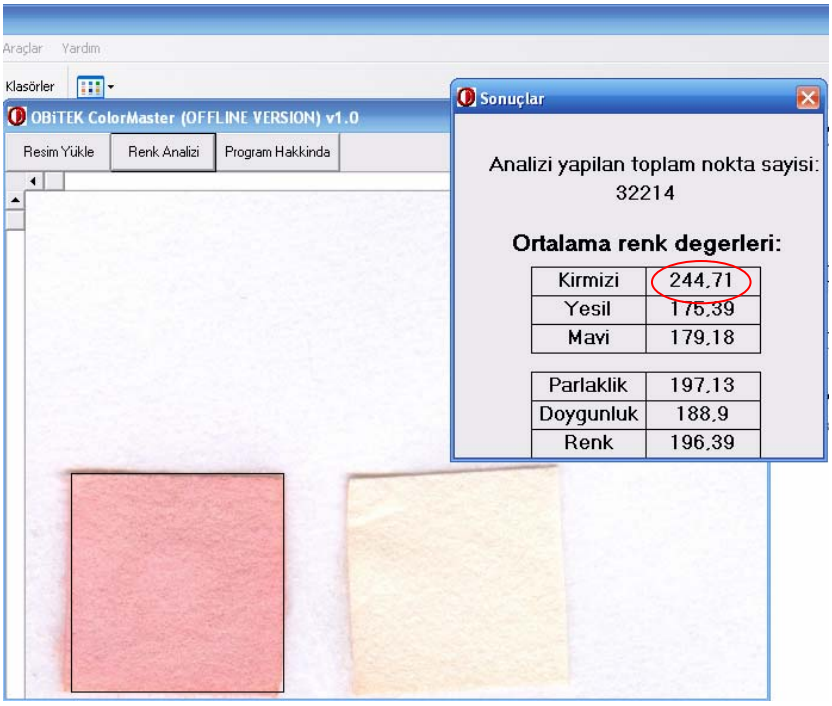


Figure 2.1: Colour analysis via OBİTEK ObiColor Master Software

2.2.7 Statistical Analysis

All of the statistical analyses were carried out by using Minitab 14.0 software. Means and standard error of means (SEM) were calculated by using Minitab 14. One way analysis of variance (ANOVA) at 95% confidence interval was used to determine variances in means.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization Studies

A series of experiments were performed in order to determine the enzyme (Tyrosinase) and the chromophore (MBTH) concentration used in biosensor construction. In these studies, L-dopa was used as model substrate at 0.256 mM concentration and the response was monitored by determining color produced in relation to the different concentration of Tyrosinase and MBTH.

3.1.1 Effect of Enzyme Concentration on Biosensor Response

In this study, different Tyrosinase/MBTH mixture having different enzyme concentration (0.5 mg/ml, 1mg/ml, 2mg/ml and 4mg/ml) was used for the determination of tyrosinase concentration. Prepared Tyrosinase/MBTH mixes were immobilized on support material via physical adsorption as described earlier (section 2.2.1.3). As shown in figure 3.1, color units increases while the enzyme concentration increases and the highest value was obtained at 4mg/ml. All values obtained with different enzyme concentration, except 0.5mg/ml, were significantly different from control (without Tyrosinase) (Figure 3.2). Although the highest color unit value was obtained at 4 mg/ml , we preferred 1mg/mL enzyme concentration for further studies in order to reduce production cost of biosensor.

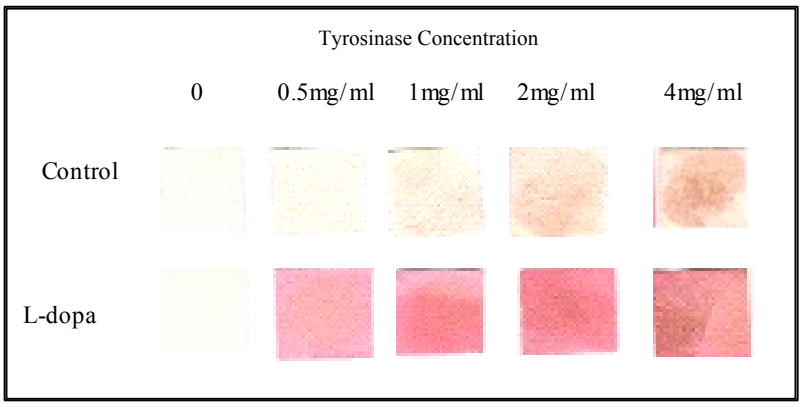


Figure 3.1: The response of biosensor for 0.256 mM L-dopa at different enzyme concentration (controls on the first row without L-dopa)

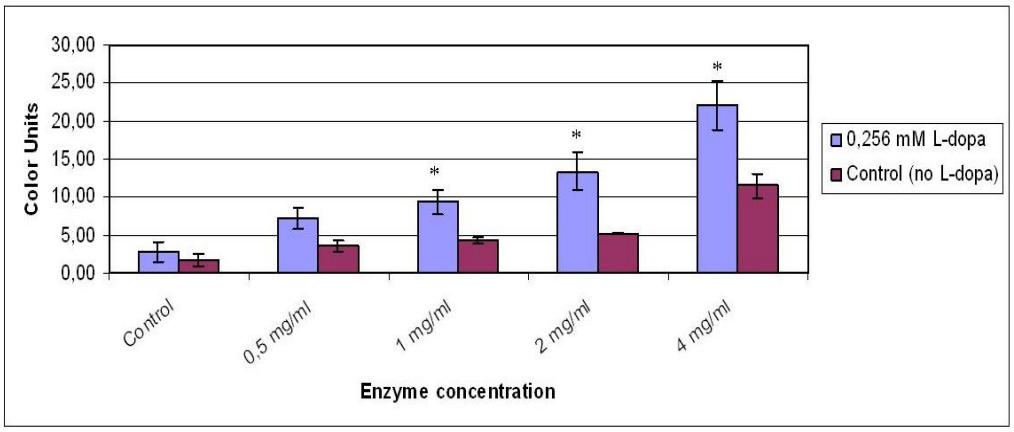


Figure 3.2: Effect of enzyme concentration on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to control (no Tyrosinase), respectively. Mean values, SEM and significant values are tabulated Table B.1 in Appendix B.

3.1.2 Effect of Chromophore Concentration on Biosensor Response

MBTH is commonly used as a colour reagent which reacts with o-quinones produced by Tyrosinase to form red color product instead of brown colored product in the absence of MBTH (1).

o-quinones + MBTH (colorless) ⟶ o-quinones-MBTH (marron color) (1)

This red color product formation constitutes the basis of optical tyrosinase biosensors. For the determination of MBTH concentration seven different Tyrosinase/MBTH mixture having different MBTH concentration (0.375mM, 0.75mM, 1.5mM, 3mM, 6mM, 12mM, 24mM) were immobilized on the Whatmann paper. It is found that the response of biosensors containing 0.75mM and 1.5mM MBTH concentrations were significantly different from control (no MBTH) which results no color formation (Figure 3.4) and also there was no significant difference between 0.75mM and 1.5mM MBTH concentrations. Increasing the MBTH concentration (3mM, 6mM, 12mM, 24mM) did not increase the color unit. In our study, we continued with 1.5mM MBTH concentration for further experimental studies.

In the literature different MBTH concentrations were used for the marron color adduct formation according to biosensor response measurement technique. Abdullah *et al*. (2006) was found that the optimum MBTH concentration is 0.20 mM for the construction of optical biosensor and Rusell *et al.* (1999) used 5nM MBTH concentration in order to develop tyrosinase bioprobe for detection of phenolic pollutants. Parajpe *et al*. (2001) were used 3.3 mM MBTH concentration for their disposable optrodes. So, in the literature different MBTH concetration were reported according to different techniques used for measurement and we decided to use 1.5 mM MBTH concentration according to our experimental design.

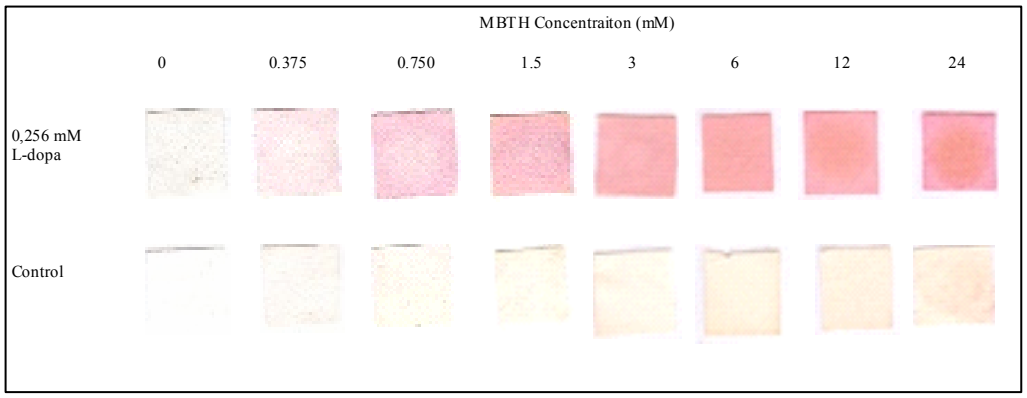


Figure 3.3: The change of biosensor response at different MBTH concentrations (controls on the second row are no L-dopa).

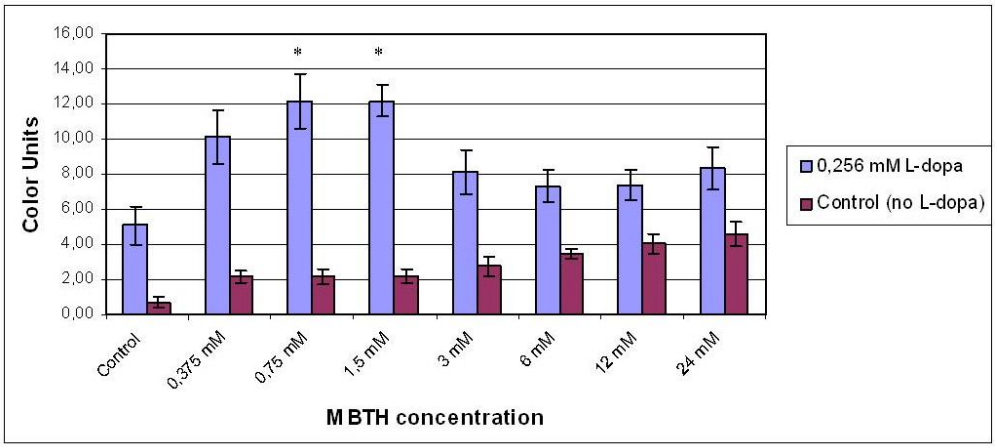


Figure 3.4: Effect of MBTH concentration on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to control (no MBTH), respectively. Mean values, SEM and significant values are tabulated Table B.2 in Appendix B.

3.1.3 Detection Limit of Biosensor

As a model substrate L-dopa was used through the optimization studies. In order to determine the detection limit of biosensor different L-dopa concentration, ranging from 0.001 mM to 0.512 mM , was used which is displayed in Figure 3.5.

When the results of different L-dopa concentration are compared to control (no L-dopa) only the color units formed by adding 0.064, 0.128, 0.256 and 0.512 mM L-dopa are significantly different from control. Therefore; a detection limit of 0.064 mM were achieved by biosensor. Paranjpe *et al*. (2001) was developed a disposable fiber-optic tyrosinase biosensor that they found a concentration dependent linear response in the concentration range from 0.05 mM to 0.4mM.

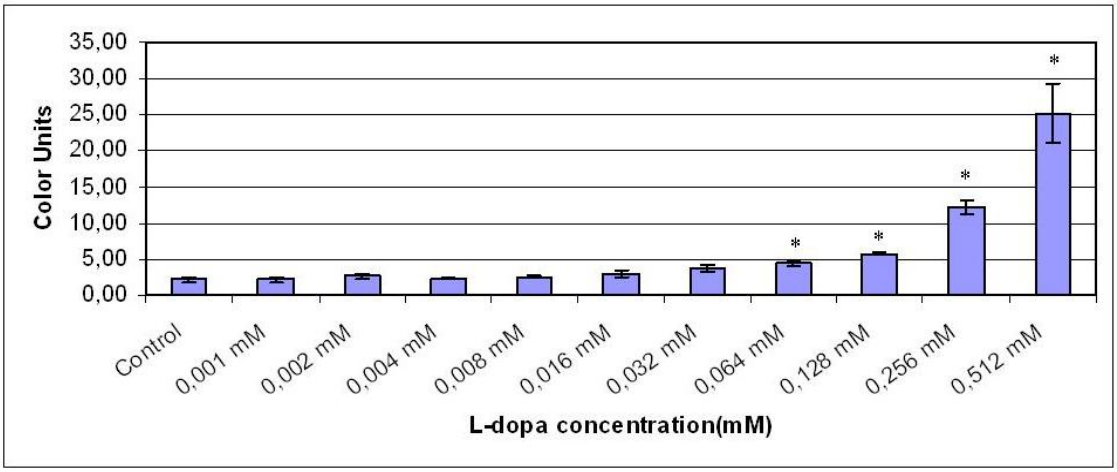


Figure 3.5: Detection limit of L-dopa .Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to control (without L-dopa), respectively. Mean values, SEM and significant values are tabulated Table B.3 in Appendix B.

3.1 Sensitivity of Biosensor towards Phenolic Compounds

Sensitivity is one of the important parameter of biosensors. In order to determine the sensitivity of biosensors towards different phenolic compounds, four different phenolic compound, 4-chlorophenol, catechol, m-cresol and p-cresol were used as substrate in the concentration range from 0.001mM to 0.512 mM. The results of 4-chlorophenol, catechol, m-cresol and p-cresol are displayed in Figure 3.7, 3.9, 3.11 and 3.13, respectively. The graphical representations of data are shown in Figure 3.6, 3.8, 3.10, 3.12.

The detection limit of biosensor for 4-chlorophenol and catechol was obtained 0.032 mM and it was 0.128 mM for m-cresol and p-cresol. In fact these detection limits are high when compared to other detection limits of developed biosensors which are optical or electrochemical. Wang G. *et al*. (2002) developed highly sensitive biosensor based on the immobilization of tyrosinase in chitosan on glassy carbon electrode, containing detection limits 50 nm. Also another electrochemical biosensor has been developed for remote on-site monitoring of phenolic compound and its detection limits for cresol and phenol were 0.2 μM and 0.3 μM ,respectively (Wang J.*et al*, 2002). Freire *et al. (*2002) were able to detect 8.5 mM chloropenol, chloroguaiacol, concentration by developed mixed/ enzyme (laccase/tyrosinase) based remote electrochemical biosensor. The detection limits of optical biosensor, developed by Abdullah *et al*.(2005), were 0.9 μM for 4-chlorophenol, 1 μM for m-cresol, 3 μM for p-cresol. Russell *et al*. (1999) developed an immobilized-polyphenol oxidase bioprobe having 0.05mg/L detection limits for phenol and p-cresol. The European Community directive (80/778/EEC) determined the maximum concentration permitted for all phenols in aquatic environments 0.5 μg/L and 0.1 μg/L for individual phenols while The United States Environmental Protection Agency sets the limit at 3.5 μg/L. In Turkey, the limit of phenolic compounds for

water resource is between 0.002mg/mL and 0.1mg/mL and for waste water it is between 0.5 mg/mL and 1mg/mL. Therefore; very sensitive biosensor is needed for detection of phenolic compounds.

Parellada *et al.* (1998) stated that biosensors has an important role in field monitoring when they are reliable and cheap. Biosensors can not be compared with classical analytical methods however they can be used for routine analysis and screening of analytes and showing the analyte present or not.

Actually, paper-type tyrosinase biosensor developed in this study is selective but is not very sensitive when compared other biosensor described above. However; it has advantage that there is no need any instrument and measurement can be performed by non-expert and it has also low cost. This biosensor provides remote on -site monitoring of phenolic compounds with minimum detection limit of 64μM.

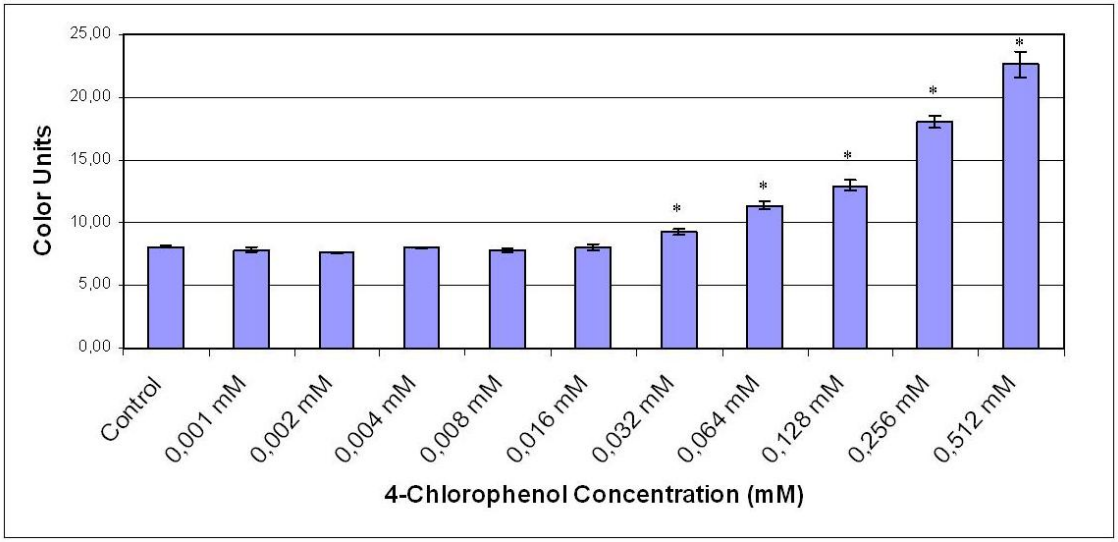


Figure 3.6 : Detection limit of 4-chlorophenol .Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to control (no 4-chlorophenol), respectively. Mean values, SEM and significant values are tabulated Table B.4 in Appendix B.

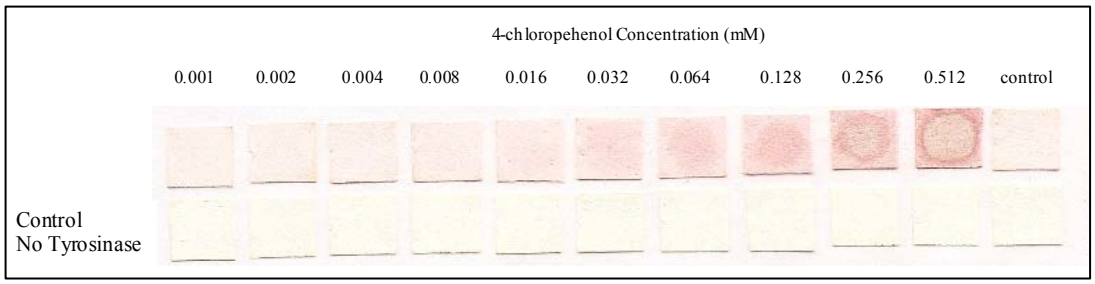


Figure 3.7: Biosensor response to different 4-chlorophenol concentration (control on the right column is no 4-chlorophenol).

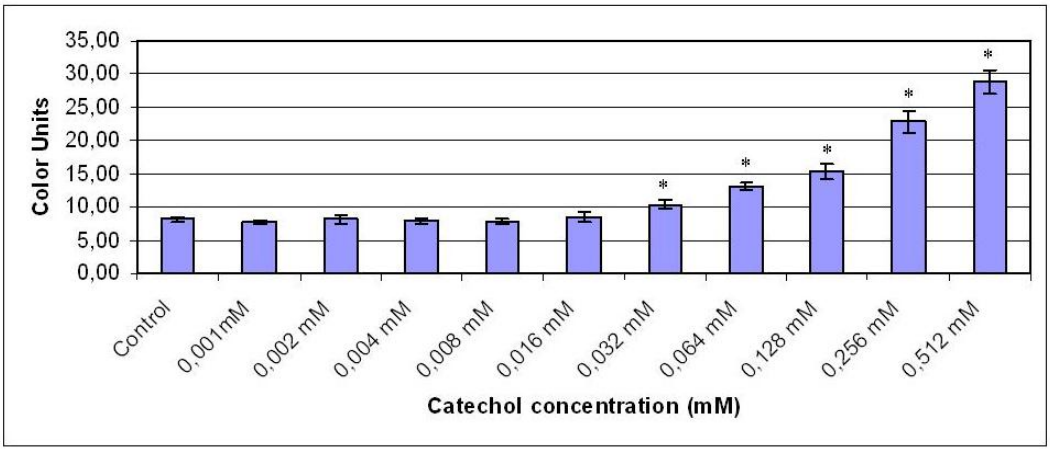


Figure 3.8 : Detection limit of catechol .Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to control (no catechol), respectively. Mean values, SEM and significant values are tabulated Table B.5 in Appendix B

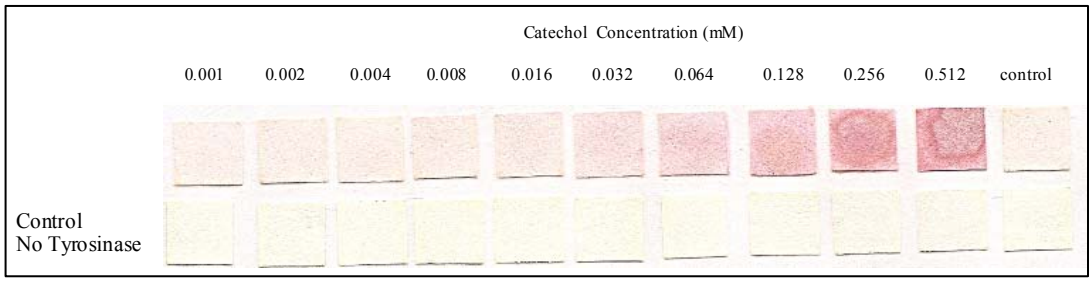


Figure 3.9: Biosensor response to different catechol concentration (control on the right column is no catechol).

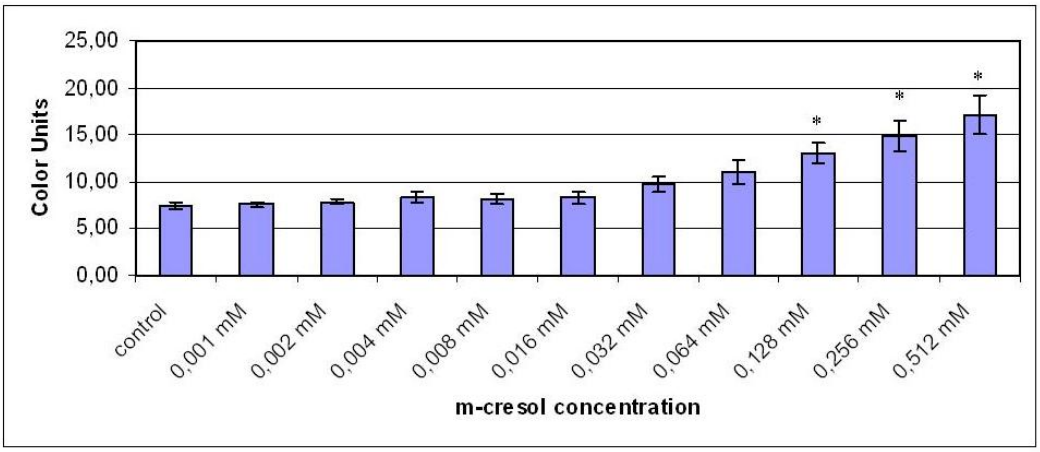


Figure 3.10: Detection limit of m-cresol .Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to control (no m-cresol), respectively. Mean values, SEM and significant values are tabulated Table B.6 in Appendix B**.**

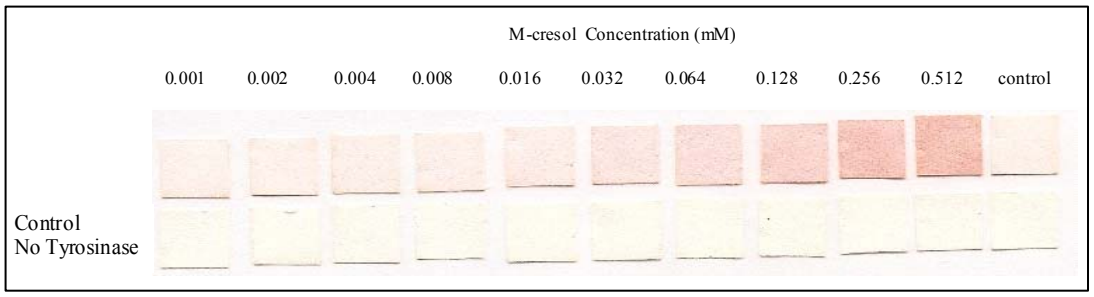


Figure 3.11: Biosensor response to different m-cresol concentration (control on the right column is no m-cresol).

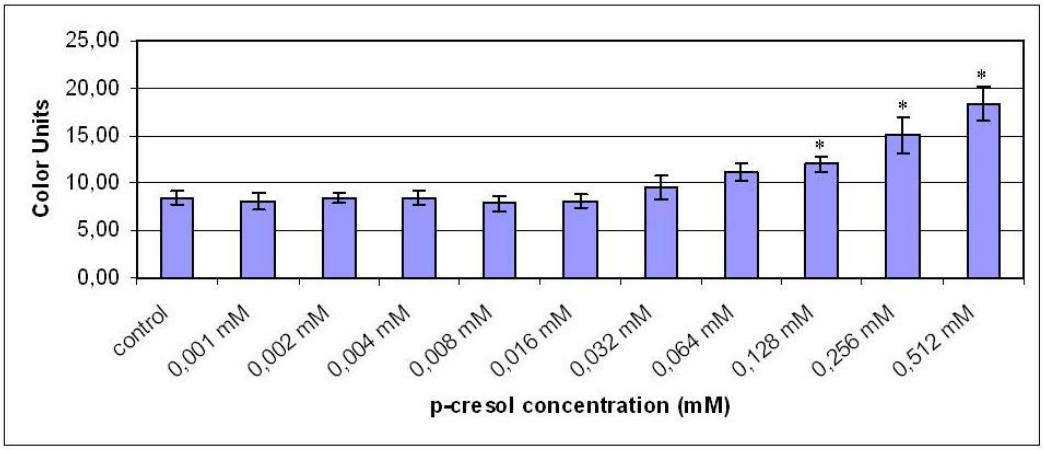


Figure 3.12 : Detection limit of p-cresol .Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to control (no p-cresol), respectively. Mean values, SEM and significant values are tabulated Table B.7 in Appendix B.

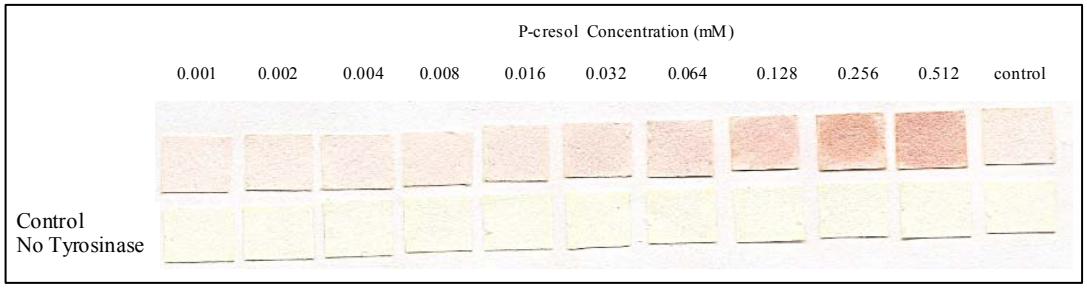


Figure 3.13: Biosensor response to different p-cresol concentration (control on the right column is no the p-cresol).

3.3 The Effect of pH on Biosensor Response

Another important parameter in biosensors for remote on-site monitoring is that there should be no change on biosensor response according to environmental changes. Therefore, the influence of pH upon the response of biosensor was investigated. Biosensor response was examined at 0,256 mM L- dopa solution at different pH (3-11). While the maximum red color product formation related to biosensor activity was observed at pH 7 and 9 , there is no significantly differences in color intensities produced in the pH range from 3 to 11 which reveals that biosensor activity was not affected in this pH range (Figure 3.15). The optimum biosensor response of optical biosensor for detection of biosensor developed by Abdullah *et al.* (2006) is pH 6-7 and the the polyphenol oxidase probe developed by Russell *et al* (1999) is effective in a pH range from 4 to 10.

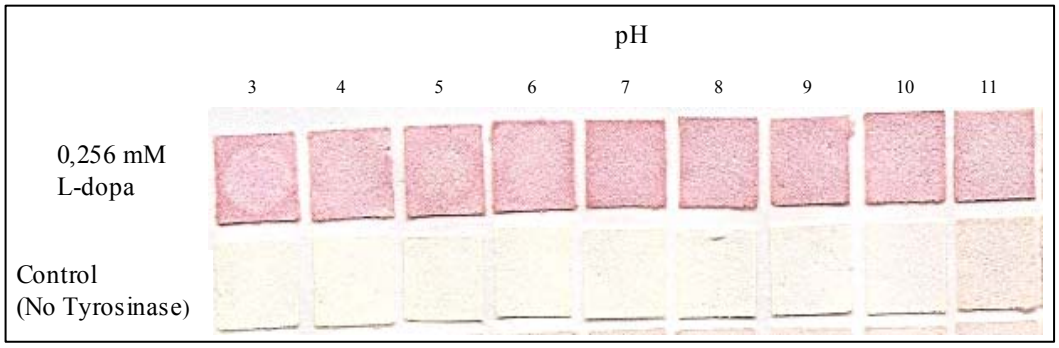


Figure 3.14: The biosensor response at different pH ranging from 3 to 11.

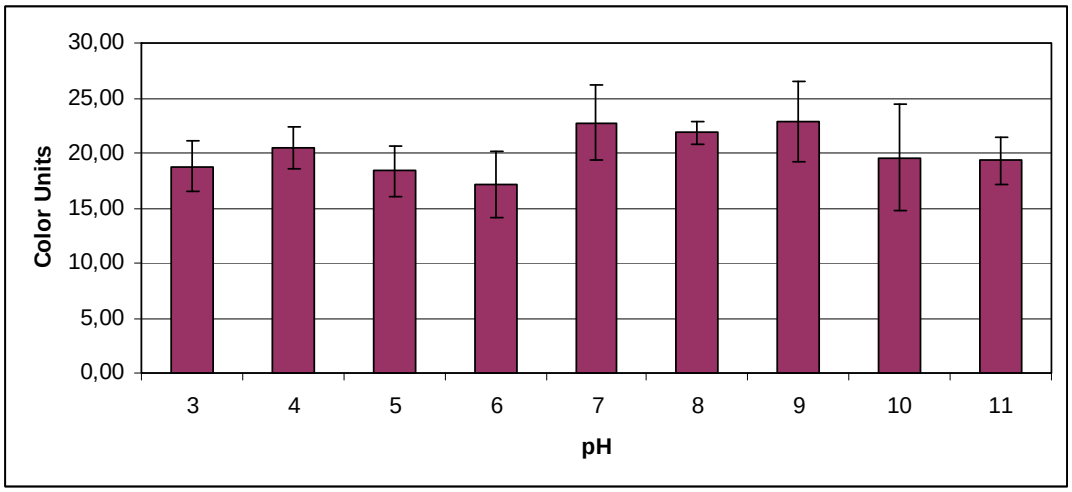


Figure 3.15: Effect of pH on biosensor response.Vertical bars indicate SEM (standard error of mean). Mean values, SEM and significant values are tabulated Table B.8 in Appendix B.

3.4 Stability of Biosensor

Stability studies are important for the commercialization of biosensor. It would have to be stored for a long time without loss of activity. Stability studies were performed at two different temperatures (Room Temperature and 4°C) and the stored biosensor response were measured at ten days intervals.

At room temperature, there is no significant decrease of biosensor activity through two months storage period, represented in Figure 3.20. Throughout the storage period, the biosensor response was significantly different than the control no substrate, which is L-dopa, and no tyrosinase (Figure 3.18) at each ten days interval and also stored biosensors showed nearly same colors when compared to color produced at the beginning of storage period (Figure 3.20). The biosensors stored at

4°C showed good stability for at least 70 days (Figure 3.21). Although it gives significantly different response when compared to control at each measurement, displayed in Figure 3.19, through the storage time it shows a significant decrease of biosensors activity at 60th day but most probably this decrease results from experimental error. So, as a result of stability studies the commercialization of biosensor seems feasible.

The stability of optical biosensor developed by Abdullah *et al.* (2006) is two months, while Russell *et al. (*1999) found the storage time of developed polyphenols oxidase as one month.

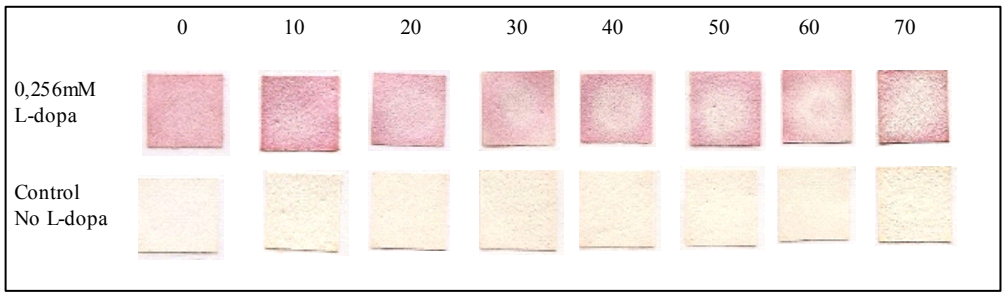


Figure 3.16: Stability studies at between ten days intervals at room temperature

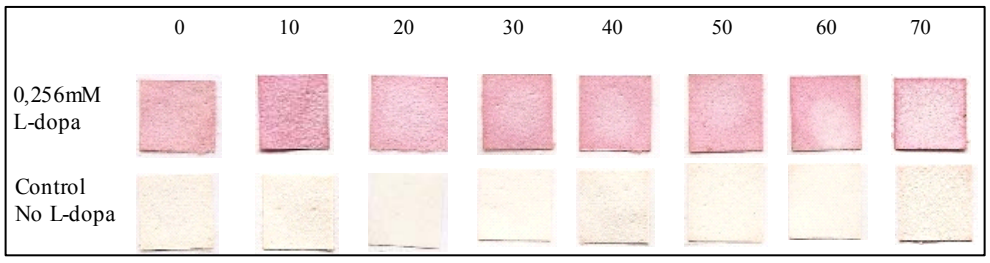


Figure 3.17: Stability studies at between ten days intervals at 4°C

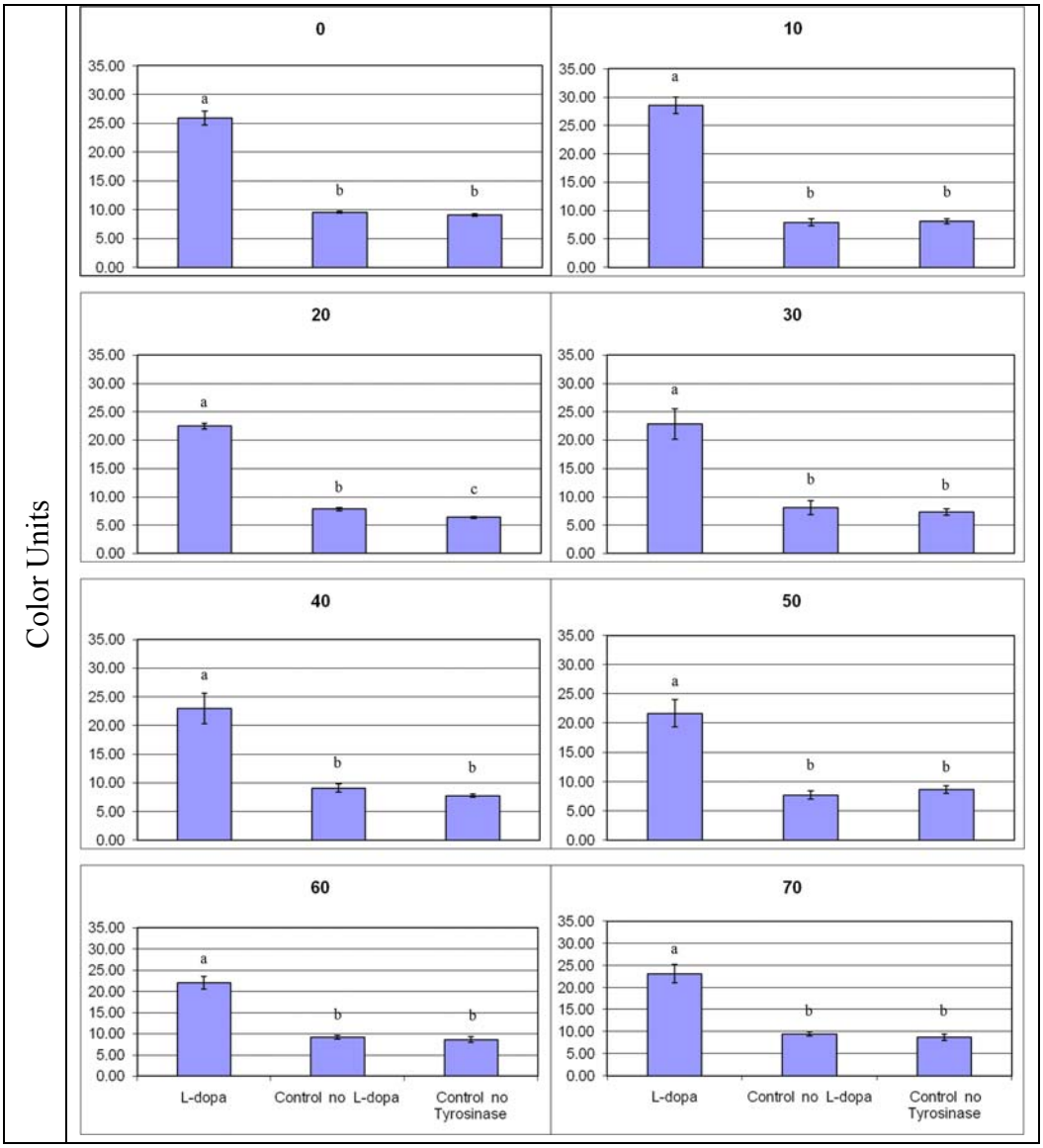


Figure 3.18: Stability studies of biosensor at room temperature at different storage time. Vertical bars indicate SEM (standard error of mean) .Values marked with same letter are not significantly different ($p < 0,05$) compared to control (no tyrosinase). Mean values, SEM and significant values are tabulated Table B.9 in Appendix B.

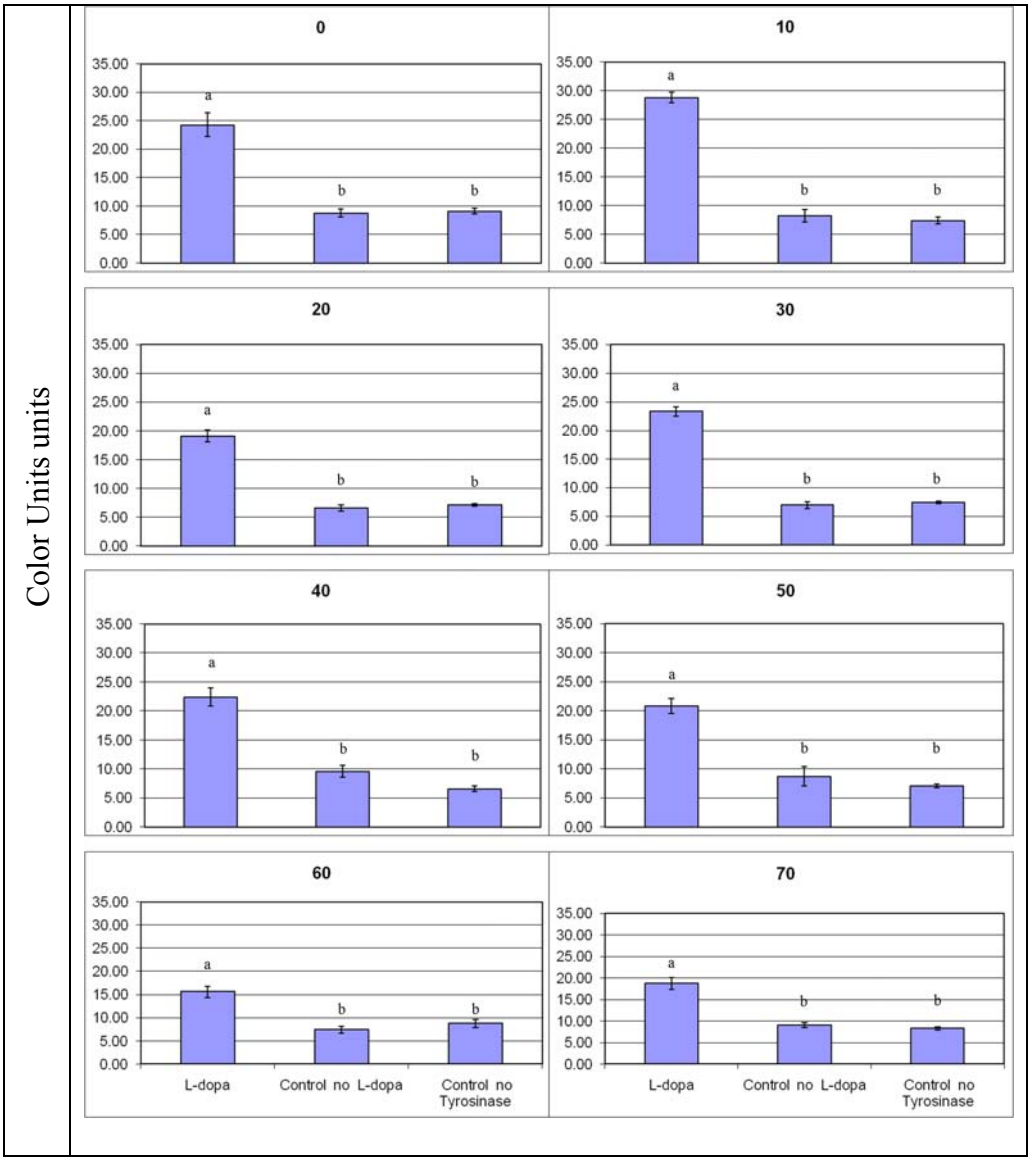


Figure 3.19: Stability studies of biosensor at 4°C at different storage time. Vertical bars indicate SEM (standard error of mean) .Values marked with same letter are not significantly different ($p < 0,05$) compared to control (no tyrosinase). Mean values, SEM and significant values are tabulated Table B.10 in Appendix B

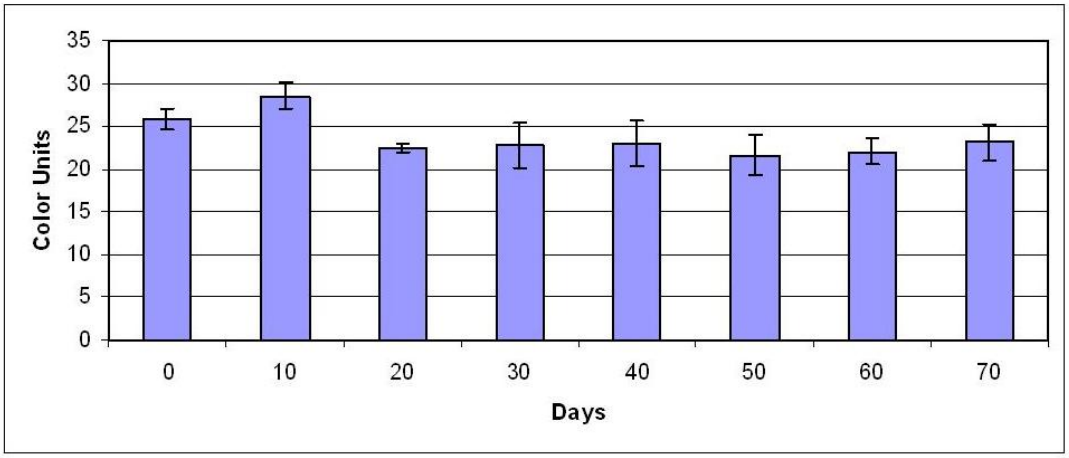


Figure 3.20: Stability studies of biosensor at room temperature. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to beginning (0)

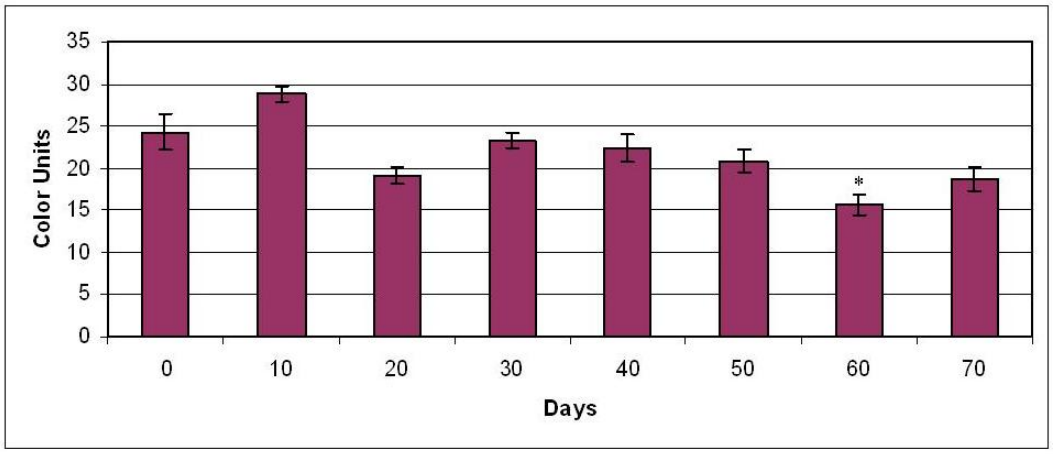


Figure 3.21: Stability studies of biosensor at 4°C. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0,05$) compared to beginning (0)

CHAPTER 4

CONCLUSION

The aim of this study was to develop a cheap, disposable and easy to use paper type tyrosinase biosensor for the detection of phenolics compounds. Different types of biosensors were developed for the determination of phenolic compounds but none of them were commercialized and also their uses need experts.

As a result of optimization studies 1mg/ml tyrosinase concentration and 1.5mM MBTH concentration were determined as optimum for using in biosensor construction. Detection limit of L-dopa, model substrate, was found as 0.064 mM and for other phenolic compounds, 4-chlorophenol, catechol, m-cresol and p-cresol, the detection limit was obtained as 0.032 mM, 0.032 mM, 0.128 mM and 0.128 mM, respectively. Although the sensitivity of biosensor is not higher than the previous developed tyrosinase biosensor, it has advantage that it can be used by non-experts, there is no need any instrument, it is cheap and portable. In addition to these we found that the biosensor is operational at a wide pH range (3-11) which exhibits an important advantage for monitoring environmental samples.

The stability of biosensor is one of the most important parameter for commercialization. The developed sensor was stable for 70 days at room temperature and 4°C.

These results represented here suggest that the developed biosensor can be used for detection of phenolic compounds found at concentration up to 32 μM in the waste water samples. Actually, this biosensor gives preliminary information of phenolic compound determination with easy uses, low cost and short operation time.

To our knowledge such type of a paper tyrosinase biosensor has not been reported previously in the literature.

For further studies in order to increase the sensitivity of biosensor, in addition to tyrosinase immobilization also laccase will be co-immobilized with tyrosinase on paper. In addition to this, different storage conditions will be examined for longer shelf life.

REFERENCES

Abdullah J., Ahmad M., Heng L.Y, Karuppiah N., Sidek H., 2006(b). Chitosan-based tyrosinase optical phenol biosensor employing hybrid nafion/sol-gel silicate for MBTH immobilization, Talanta, 70, 527-532.

Abdullah J. , Ahmad M., Karuppiah N., Heng L.Y, Sidek H. 2006(a), Immobilization of tyrosinase in chitosan film for an optical detection of phenol, Sensors and Actuators B, 114, 604–609

Abhijith K.S., Sujith Kumar P.V., Kumar M.A., Thakur M.S., 2007, Immobilized tyrosinase-based biosensor for the detection of tea polyphenols , Anal.Bioanal.Chem., 389, 2227-2234.

Adhikari B., Majumdar S., 2004. Polymers in sensor applications, Progress in Polymer Science, 29, 699–766.

Ambient Water Quality Criteria for Phenol; U.S. EPA 440/5-80-066; U.S. EPA: Washington, DC, 1980.

Anh T.M., Dzyadevych S.V., Van M.C., Renault N.J., Duc C.N., Chovelon J.-M., 2004, Conductometric tyrosinase biosensor for the detection of diuron, atrazine, and its main metabolites.Talanta, 63, 365-370.

Anh T.M., Dzyadevych S.V., Soldatkin A.P., Duc Chien N., Jaffrezic-Renault N., Chovelon J.-M., 2002, Development of tyrosinase biosensor based on pH-sensitive field effect transistors for phenols determination in water solutions, Talanta, 56, 627-634.

Arslan A., Kıralp S., Toppare L., Yağcı Y., 2005, Immobilization of tyrosinase in polysiloxane/plypyrole copolymer matrices. International Journal of Biological Macromolecules, 35, 163-167

Badihi-Mossberg M., Buchner V., Rishpon J., 2007, Electrochemical Biosensors for Pollutants in the Enviroment, Electroanalysis,19, 2015-2028

Brummel,K.E., Wright,J., Eldefrawi M.E., 1997, Fiber Optic Biosensor for Cyclodiene Insecticides, Journal of Agricultural and Food Chemistry, 45 (8), 3292-3298

Bunde R.L.,. Jarvi E.J., Rosentreter J.J., 1998, Piezoelectric quartz crystal biosensors, Talanta, 46 ,1223–1236

Chaplin M.F. (2000). Biosensors. In J.M.Walker, R.Rapley (Eds.), Molecular Biotechology and Biotechnology (pp: 521-555). Cambridge: Royal Society of Chemistry.

Chaplin M. (2004). What are biosensors? Retrieved December 20, 2004, from London South Bank University website: http://www.lsbu.ac.uk/biology/enztech/biosensors.html

Chuang M.-C., Liu C.-C., Yang M.-C., 2006, An electrochemical tyrosinase-immobilized biosensor for albumin- toward a potential total protein measurement, Sensors and Actuators B, 14(1), 357-363.

Cosnier S., Fombon J.-J., Labbe P., Limosin D., 1999, Development of a PPO-poly(amphiphilic pyrolle) electrode for on site monitoring of phenol in aqueous effluents, Sensors and Actuators B, 59, 134- 139.

Daunert S., Barrett G., Feliciano J.S., Shetty R.S., Shrestha S., and Smith-Spence W.,2000, Genetically Engineered Whole-Cell Sensing Systems: Coupling Biological Recognition with Reporter Genes Chemical Reviews,100(7), 2705-2738

D'Souza S.F., 2001, Microbial biosensors, Biosensors & Bioelectronics, 16, 337–353

Durán N.,. Rosa M.A., D'Annibale A., Gianfreda L., 2002. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review, Enzyme and Microbial Technology, 31, 907–931

Fȁhnrich K.A, Pravda M., Guilbault G.G, 2003, Disposable amperometric immunosensor for the detection of polycyclic aromatic hydrocarbons (PAHs) using screen-printed electrodes, Biosensors and Bioelectronics, 18 (1), 73-82

Freire R.S., Thongngamdee S. , Durán D., , Wang J. , Kubota L.T., 2002, Mixed enzyme (laccase/tyrosinase)-based remote electrochemical biosensor for monitoring phenolic compounds, Analyst, 127, 258–261

Frense D., Müller A. and Beckmann D., 1998, Detection of environmental pollutants using optical biosensor with immobilized algae cells , Sensors and Actuators B: Chemical, 51(1-3) , 256-260

Gerard M., Chaubey A., Malhotra B.D., 2002, Application of conducting polymers to biosensors, Biosensors & Bioelectronics, 17: 345-359.

Halaouli, S., Asther, M., Sigoillot, J.-C., Hamdi, M., Lomascolo, A. 2006.Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. Journal of Applied Microbiology, 100, 219-232.

Herschkovitz Y., Eshkenazi I., Campbell C.E., Rishpon J., 2000, Electrochemical biosensor for formaldehyde, Journal of Electroanalytical Chemistry, 491 (1-2), 182-187.

Junhui Z., Hong C. and Ruifu Y.,1997, DNA based biosensors, Biotechnology Advances,15(1), 43-58

Karam J., Nicell J.A, 1997, Potential applicaiton of Enzymes in Waste Treatment , J.Chem. Tech. Biotechnol., 69, 141-153

Kim Y.-J., Uyama H., 2005. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future, Cellular and Molecular Life Sciences, 62, 1707-1723

Kıralp S., Toppare L., 2006, Polyphenol content in selected Turkish wines, an alternative method of detection of phenolics. Process Biochemistry, 41, 236-239.

Köhler S., · Belkin S, · Schmid R.D, 2000, Reporter gene bioassays in environmental analysis, Fresenius J Anal Chem, 366,769-779

Lei Y., Chen W.Mulchandi A., 2006, Microbial Biosensors, Analytica Chimica Acta, 568, 200-210

Liao V.H.-C., Chien M.-T., Tseng Y.-Y., Ou K.-L., 2006, Assessment of heavy metal bioavailability in contaminated sediments and soils using green fluorescent protein-based bacterial biosensors , Environmental Pollution 142 (1),17-23

Liu Z., Liu Y., Yang H., Yang Y., Shen G., Yu R., 2005. A phenol biosensor based on immobilizing tyrosinase to motified core-shell magnetic nanoparticles supported at a carbon paste electrode, Analytica Chimica Acta, 533, 3-9

Malhotra B. D., Singhal R., Chaubey A., Sharma S.K., Kumar A., 2005, Recent trends in biosensors, Current Applied Physics, 5: 92-97

Marazuela M.D., Moreno-Bondi M.C., 2002, Fiber-optic biosensors-an overview, Anal Bioanal Chem, 372, 664-682

Marco M. & Barcelo D., 1996, Enviromental applications of analytical biosensors, Meas,Sci.Technol., 7: 1547-1562

Mello L.D. & Kubota L.T , 2002, Review of the use of biosensors as analytical tools in he food and drink industry, Food Chemistry, 77: 237-256

Mulchandani A., Chen W., Mulchandani P., Wang J., Rogers K.R., 2001, Biosensors for direct determination of organophosphate pesticides, Biosensors and Bioelectronics, 16 (4-5),225-230.

Naessens M., Leclerc J.C.and Tran-Minh C.,2000, Fiber Optic Biosensor Using *Chlorella vulgaris* for Determination of Toxic Compounds , Ecotoxicology and Environmental Safety, 46(2), 181-185

Nakamura H., Karube I., 2003,Current research activity in biosensors, Anal.Bioanal. Chemistry, 377, 446-468

Neufeld T., Eshkenazi I., Cohen E., Rishpon J., 2000, A micro flow injection electrochemical biosensor for organophosphorus pesticides, Biosensors and Bioelectronics, 15 (5-6), 323-329.

Nistor C., Rose A., Farré M., Stoica L., Wollenberger U., Ruzgas T., Pfeiffer D., Barcelo D., Gorton L., Emnués J., 2002. In-field monitoring of cleaning efficiency in waste water treatment plants using two phenol-sensitive biosensors. Analtica Chimica Acta ,456, 3-17.

Palecek E., Fojta[a] M., Tomschik M.,Wang[c] J., 1998, Electrochemical biosensors for DNA hybridization and DNA damage, Biosensors and Bioelectronics, 13(6), 621-628

Paranjpe P., Dutta S.; Karve M., Padhye S., Narayanaswamy R., 2001, A disposable Optrode using immobilized tyrosinase films, Analytical Biochemisrty, 294, 102-107

Parellada J., Narveaz A., Lopez M.A., Dominguez E., Fernandez J.J, Pavlov V., Katakio I., 1998, Amperometric İmmunosensors and enzyme electrodes for environmental applications, Analytica Chimica Acta, 362, 47-57.

Perez H.J.P., Lopes M.S.-P., Lopes-Cabarcos E., Lopes-Ruiz B., 2006, Amperometric tyrosinase biosensor based on polyacrylamide microgels, Biosensors and Bioelectronics, 22, 429-439.

Ramanathan S., Ensor M. and Daunert S., 1997, Bacterial biosensors for monitoring toxic metals, Trends Biotechnology,15, 500-506

Riedel K. ,1998. Application of Biosensors to Enviromental Samples.In Ramsay G., Commercial Biosensors :Applicaiton to Clinical, Bioprocess and Enviromental Samples (pp: 267-294). United States of America: John Wiley & Sonc.Inc.

Rodriguez-Lopez J., Escribano J., Garcia-Canovaz F., 1994, A continous Spectrophotometric Method for the determination of Monophenolase Activity of Tyrosinase using 3-Methyl-2- benzothiazolinone Hydrazone, Anaytical Biochemistry , 216, 205-212.

Rodriguez-Mozaz S., Lopez de Alda M. J., 2006, Barcélo Damia, Biosensors as useful tools for enviromental analysis and monitoring, Anal.Bioanal. Chemistry, 386, 1025-1041

Russell I.M., Burton S.G., 1999, Development and demonstration of an immobilized-polyphenol oxidase for the detection of phenolic pollutants in water, Anayltica Chimaca Acta, 389, 161-170.

Sassolas A., Leca-Bouvier B.D., and Blum L.J.,2008, DNA Biosensors and Microarrays, Chem. Rev. , 108, 109-139

Sharma S. K., Sehgal N., Kumar A., 2003, Biomolecules for development of biosensors and their applications, Current Applied Physics, 3: 307-316

Sheppard N.F, Mears D.J, Guiseppielie A.,1996, Model of an immobilized enzyme conductimetric urea biosensor, Biosensors Bioelectronics, 11(10), 967- 979

Sheppard N.F, Mears D.J, Guiseppielie A.,1996, Model of an immobilized enzyme conductimetric urea biosensor, Biosensors Bioelectronics, 11(10), 967- 979

Streffer K.,Vijgenboom E., TepperA.W.J.W., MakowerA., Scheller F. W., Canters G. W. , Wollenberger U., 2001. Determination of phenolic compounds using recombinant tyrosinase from *Streptomyces antibioticus*, Analytica Chimica Acta, 427, 201–210

Susmel S., Guilbault G.G., O'Sullivan C.K., 2003, Demonstration of labeless detection of food pathogens using electrochemical redox probe and screen printed gold electrodes, Biosensors and Bioelectronics, 18 (7), 881-889

Taylor C. J., Bain L. A. , Richardson D. J., Spiro S. and Russell D. A.,2004, Construction of a whole-cell gene reporter for the fluorescent bioassay of nitrate. Analytical Biochemistry , 328 (1), 60-66

Tanimoto de Albuquerque Y.D., Ferreira L.F., 2007, Amperometric biosensing of carbamate and organophosphate pesticides utilizing screen-printed tyrosinase-modified electrodes, Analytica Chimica Acta, 596, 210-221.

Thévenot D.R., Toth K., Durst R.A., Wilson G.S., 2001, Electrochemical biosensors:recomended definitions and classification, Biosensors & Bioelectronics, 16: 121-131.

Tsai Y.-C., Chiu C.-C., 2007, Amperometric biosensors based on multiwalled carbon nanotube-Nafion- tyrosinase nanobiocomposites for the determination of phenolic compounds, Sensors and Actuators B, 125, 10-16.

Wang G., Xu J.-J., Ye L.-H., Zhu J.-J., Chen H.-Y, 2002. Highly sensitive sensors based on the immoblization of tyrosinase in chitosan, Bioelectrochemistry, 57, 33-38

Wang J., 2008, Electrochemical Glucose Biosensors, Chemical Reviews, 108:814-825

Wang J., Chen Q., 1995, Remote electrochemical biosensor for field monitoring of phenolic compounds, Analytica Chimica Acta, 312 (1), 39-44.

Wang J., Chen L., Mulchandani A., MulchandaniP., ChenW., 1999, Remote biosensor for in-situ monitoring of organophosphate nerve agents, Electroanalysis, 11 (12), 866-869.

Van Gelder, C. W. G., Flurkey W. H., Wichers, H. J. (1997). Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry*, 45 (7), 1309-1323

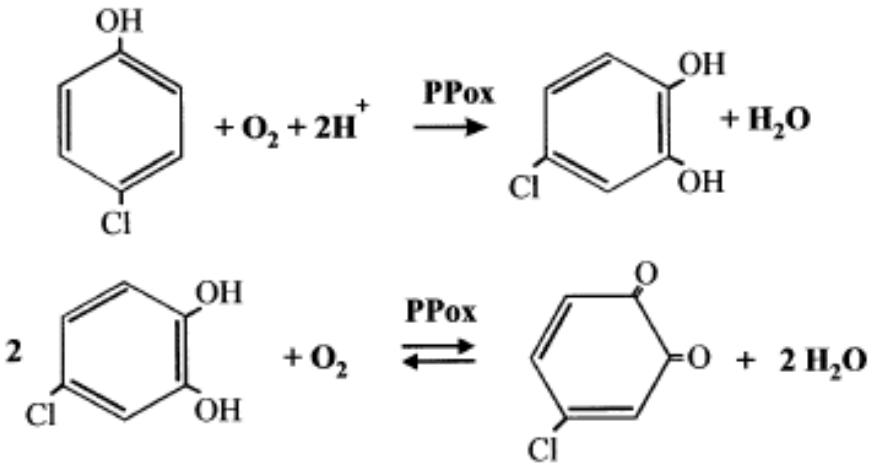
Vo-Dinh T., Cullum B., 2000, Biosensors and biochip : advances in biological and medical diagnostics, Fresenius J Anal Chem, 366, 540-551

Yulaev,M.F., Sitdikov R.A.,Dmitrieva N.M.,Yazynina E.V.,Zherdev A.V., Dzantiev B.B., 2001, Development of a potentiometric immunosensor for herbicide simazine and its application for food testing, Sensors and Actuators, B: Chemical, 75 (1-2),129-135

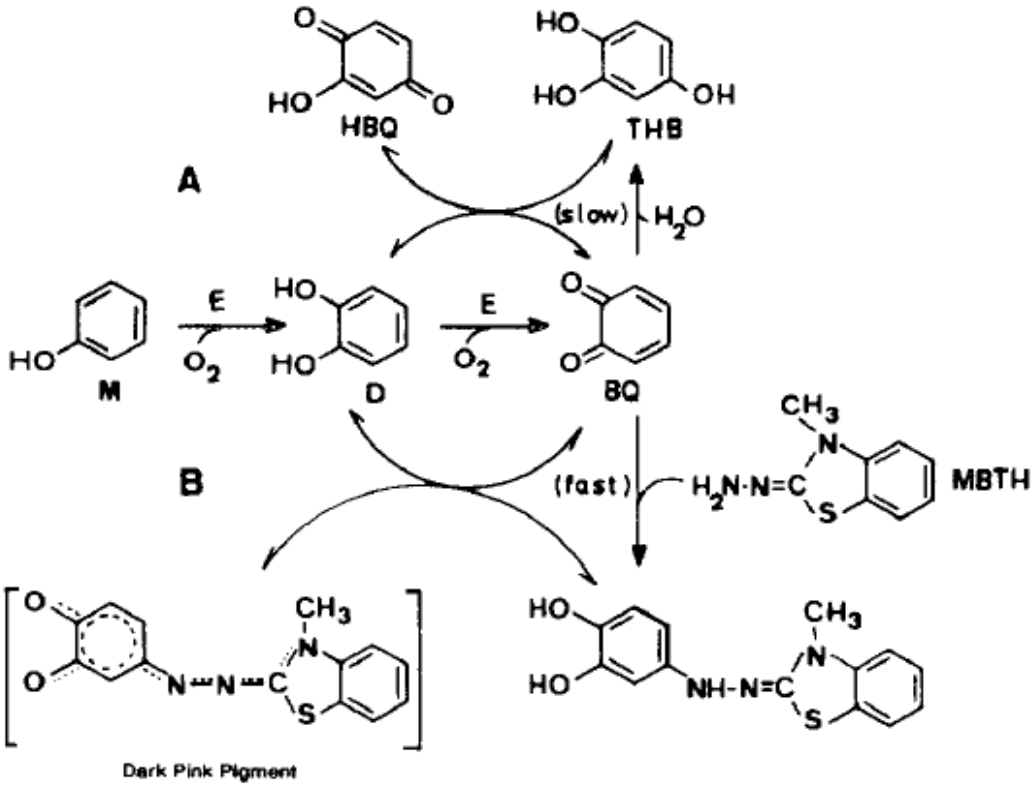
Zhao Q., Guan L., Gu Z., Zhuang Q., 2005, Determination of phenolic compounds based on the tyrosinase – single walled carbon nanotubes sensors, Electroanalysis, 17(1), 85-8

APPENDIX A

REACTION MECHANISMS



A.1 Enzyme activity mechanism of tyrosinase (Anh *et al.*, 2002)



A.2 Hydroxylation of phenol catalyzed by tyrosinase, in the absence (A) and presence of MBTH (B).E: tyrosinase, M:phenol D: pyrocatachol; BQ: o-bezoquinone; THB: 1,2,4- trihydroxybenzene; HBQ: Hydroxybenzoqunone. (Rodriguez-Lopez *et al.*, 1994)

APPENDIX B

TABULATED VALUES OF GRAPHS

B.1. Mean values, SEM and significant values for Figure 3.4 (Effect of enzyme concentration on biosensor response.)

Enzyme concentration	**Color Units**	
	0,256mM L-dopa	Control (no L-dopa)
Control	2,80 ± 1,2	1,74 ± 0,892
0,5 mg/ml	7,22 ± 1,45	3,56 ± 0,791
1 mg/ml	9,35 ± 1,57*	4,32 ± 0,465
2 mg/ml	13,33 ± 2,43*	5,21 ± 0, 05
4 mg/ml	22,07 ± 3,2*	11,49 ± 1,58

B.2. Mean values, SEM and significant values for Figure 3.4 (Effect of MBTH concentration on biosensor response.)

MBTH Concentration	**Color Units**	
	0,256 mM L-dopa	No L-dopa
Control	5,1± 1,110	0,73 ± 0,318
0,375 mM	10,13 ± 1,540	2,16 ± 0,338
0,75 mM	12,18 ± 1,540*	2,19 ± 0,439
1,5 mM	12,19 ± 0,918*	2,18± 0,387
3 mM	8,16 ± 1,270	2,77 ± 0,572
6 mM	7,35 ± 0,890	3,48 ± 0,295
12 mM	7,37 ± 0,852	4,05 ± 0,561
24 mM	8,34 ± 1,210	4,60 ± 0,669

B.3. Mean values, SEM and significant values for Figure 3.5 (Detection limit of L-dopa)

L-dopa concentration	**Color Units**
Control	2,18± 0,387
0,001 mM	2,12± 0,332
0,002 mM	2,63± 0,325
0,004 mM	2,35± 0,191
0,008 mM	2,6± 0,203
0,016 mM	3± 0,404
0,032 mM	3,71±0,534
0,064 mM	4,45±0,387*
0,128 mM	5,82±0,101*
0,256 mM	12,19±0,918*
0,512 mM	25,19±4,03*

B.4. Mean values, SEM, and significant values for Figure 3.6 (Detection limit of 4-chlorophenol)

4-chlorophenol concentration(mM)	**Color Units**
Control	8,06 ± 0,055
0,001 mM	7,83 ± 0,181
0,002 mM	7,64 ± 0,072
0,004 mM	7,98 ± 0,044
0,008 mM	7,82 ± 0,131
0,016 mM	8,05 ± 0,290
0,032 mM	9,29 ± 0,267*
0,064 mM	11,37 ± 0,321*
0,128 mM	12,95 ± 0,443*
0,256 mM	18,06 ± 0,497*
0,512 mM	22,62 ± 1,070*

B.5. Mean values, SEM and significant values for Figure 3.8 (Detection limit of catechol)

Catechol concentration	**Color units**
Control	8,12 ± 0,385
0,001mM	7,77 ± 0,284
0,002 mM	8,11 ± 0,682
0,004 mM	7,95 ± 0,390
0,008 mM	7,81 ± 0,434
0,016 mM	8,41 ± 0,731*
0,032 mM	10,40 ± 0,704*
0,064 mM	13,05 ± 0,556*
0,128 mM	15,33 ± 1,070*
0,256 mM	22,78 ± 1,680*
0,512 mM	28,8 ± 1,830*

B.6. Mean values, SEM and significant values for Figure 3.10 (Detection limit of m-cresol)

m-cresol Concentration	**Color Units**
control	7,38 ± 0,390
0,001 mM	7,52 ± 0,332
0,002 mM	7,83 ± 0,249
0,004 mM	8,27 ± 0,574
0,008 mM	8,17 ± 0,590
0,016 mM	8,26 ± 0,576
0,032 mM	9,69 ± 0,808
0,064 mM	11,10 ± 1,300
0,128 mM	13,08 ± 1,080*
0,256 mM	14,90 ± 1,650*
0,512 mM	17,11 ± 2,100*

B.7. Mean values SEM and significant values for Figure 3.12 (Detection limit of p-cresol)

p-cresol Concentration(mM)	**Color Units**
control	8,41 ± 0,729
0,001 mM	8,13 ± 0,873
0,002 mM	8,47 ± 0,596
0,004 mM	8,51 ± 0,737
0,008 mM	7,86 ± 0,835
0,016 mM	8,11 ± 0,750
0,032 mM	9,53 ± 1,330
0,064 mM	11,17 ± 0,848
0,128 mM	11,97 ± 0,745
0,256 mM	15,04 ± 1,830
0,512 mM	18,29 ± 1,790

B.8 Mean values, SEM and significant values for Figure 3.14 **(**The biosensor response at different pH ranging from 3 to 11)

pH	**Color Units**
3	18,75 ± 2,3
4	20,50 ± 1,87
5	18,38 ± 2,31
6	17,12 ± 3,06
7	22,75 ± 3,38
8	21,86 ± 1,04
9	22,90 ± 3,68
10	19,59 ± 4,9
11	19,30 ± 2,19

B.9. Mean values, SEM and significant values for Figure 3.18 (Stability studies of biosensor at room temperature at different storage time at RT)

Days	Color Units		
	L-dopa	Control no L-dopa	Control no Tyrosinase
0	25,88± 1,2	9,55± 0,157	9,1± 0,243
10	28,54± 1,48	7,89± 0,61	8,15± 0,442
20	22,44± 0,489	7,78± 0,286	6,34± 0,195
30	22,83± 2,7	8,07± 1,2	7,29± 0,594
40	22,95± 2,62	9,09± 0,708	7,76± 0,305
50	21,67± 2,35	7,67± 0,713	8,63± 0,611
60	22,02± 1,55	9,19± 0,421	8,56± 0,678
70	23,11± 2,07	9,51± 0,477	8,68± 0,745

B.10. Mean values, SEM, and significant values for Figure 3.19 (Stability studies of biosensor at room temperature at different storage time at 4°C)

Days	**Color Units**		
	L-dopa	**Control no L-dopa**	**Control no Tyrosinase**
0	24,29±2,11	8,77±0,665	9,05±0,49
10	28,81±0,954	8,23±1,06	7,38±0,58
20	19,10±1,03	6,58±0,584	7,15±0,58
30	23,28±0,857	6,95±0,621	7,44±0,227
40	22,39±1,57	9,59±1,02	6,59±0,465
50	20,85±1,29	8,72±1,65	7,09±0,305
60	15,62±1,22	7,41±0,794	8,74±0,841
70	18,67±1,38	9,07±0,561	8,28±0,367

B.11. Mean values, SEM and significant values for Figure 3.20 (Stability studies of biosensor at room temperature)

Days	Color units
0	25,88±1,2
10	28,54±1,48
20	22,44±0,489
30	22,83±2,7
40	22,95±2,62
50	21,67±2,35
60	22,02±1,55
70	23,11±2,07

B.12. Mean values, SEM and significant values for Figure 3.20 (Stability studies of biosensor at 4°C)

Days	Color units
0	24,29±2,11
10	28,81±0,954
20	19,1±1,03
30	23,28±0,857
40	22,39±1,57
50	20,85±1,29
60	15,62±1,22
70	18,67±1,38