DEVELOPMENT OF ACETYLCHOLINESTERASE BIOSENSOR FOR THE DETECTION OF PESTICIDES

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ABSTRACT

DEVELOPMENT OF ACETYLCHOLINESTERASE BIOSENSOR FOR THE DETECTION OF PESTICIDES

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Pesticides are natural or artificial molecules aimed to kill, or mitigate any harmful organism. Although their use in agriculture provides us with an increased crop yield, remains of chemicals on the products creates health concerns in society. Organophosphates and carbamates are two groups of insecticides. Although they are far more lethal against insects and small animals, they can also cause poisoning in humans through the inhibition of acetylcholinesterase enzyme (AChE) that plays an important role in human nervous system. Therefore, the detection of these compounds is crucial. The conventional methods for the detection of these compounds are expensive, time-consuming and need expertise. In this study, a fast, disposable, cheap and accurate acetylcholinesterase biosensor was developed to detect organophosphate and carbamate-based pesticide residues. By means of adsorption method, AChE, the chromophore 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) and artificial substrate acetylthiocholine (ATCh) were immobilized on the supporting material. In optimization studies; from 3 to 15U/mL concentrations were experimented for AChE, 1 to 5mM DTNB and 1 to 5mM ATCh concentration gradients were used.

As a result of the optimization studies 12U/mL ACHE concentration, 5mM DTNB concentration and 5mM ATCh concentration were determined for constructing a pesticide biosensor.

Detection limit of malathion, an organophosphate-based insecticide was found as 2.5ppm in 5% methanol solution. The biosensor conserved its integrity between pH 4 and 8, and gave false positive results after pH 10. Stability studies showed that, biosensor retained its activity for at least 60 days at 4°C to discrimnate between positive and negative controls.

Key words: Acetylcholinesterase, biosensor, organophosphates, carbamates, pesticides.

ÖZ

PESTİSİTLERİN BELİRLENMESİ İÇİN ASETİLKOLİNESTERAZ BİYOSENSÖRÜNÜN GELİŞTİRİLMESİ

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Pestisitler, zararlı etki gösteren organizmaları durdurmak ya da öldürmek amaçlı doğal ve yapay moleküllerdir. Tarımda kullanılmaları verim artışı sağlasa da, ürünler üzerindeki kimyasal kalıntılar toplumda sağlık endişesi yaratmaktadır. Organofosfatlar ve karbamatlar, iki pestisit grubudur. Böcekler ve küçük hayvanlara karşı daha ölümcül olmalarına rağmen, insanlarda sinir sisteminde önemli rol oynayan asetilkolinesteraz (AChE) enziminin inhibasyonu sonucunda zehirlenmelere sebep olabilirler. Dolayısıyla bu bileşiklerin tespiti önemlidir. Bu pestisit türlerinin tespiti için kullanılan geleneksel yöntemler pahalı, zaman alan ve uzmanlık istemektedir. Bu çalışmada organofosfat ve karbamat temelli pestisitlerin tespiti için; hızlı, atılabilir, ucuz ve tutarlı bir asetilkolinesteraz biyosensörü geliştirilmiştir. Emdirme metoduyla, enzim AChE, kromofor 5,5'-Ditio-bis(2-nitrobenzoik asit) DTNB ve yapay substrat astitiokolin (ATCh) destek materyalin üzerine sabitlenmiştir. Optimizasyon çalışmalarında; AChE için 3 ile 15U/mL arası yoğunluklar denenmiş, DTNB ve ATCh için ise 1 ile 5mM yoğunluk aralıkları kullanılmıştır.

Optimizasyon çalışmalarının sonucu olarak 12U/mL AChE yoğunluğu, 5mM DTNB yoğunluğu ve 5mM ATCh yoğunluğu pestisit biyosensörünün yapımı için uygun bulunmuştur.

Organofosfat temelli böcek öldürücü malatyonun tespit limiti %5 metanol çözeltisinin içinde 2.5ppm olarak bulunmuştur. Biyosensör pH 4 ve 8 arasında yapısını korumuş olup, pH 10'dan sonar yanlış pozitif sonuç vermiştir. Yapılan stabilite çalışmalarına göre, tasarlanan biyosensor aktivitesini 4°C'de 60 gün boyunca pozitif ve negative sonuçları ayırt edecek kadar muhafaza etmiştir.

Anahtar kelimeler: Asetilkolinesteraz, biyosensör, organofosfatlar, karbamatlar, pestisitler.

To My Mom..

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LIST OF ABBREVIATIONS

AChE- Acetylcholinesterase

DTNB- 5,5'-Dithio-bis(2-nitrobenzoic acid)

ATCh- acetylthiocholine

ATChI- acetylthiocholine iodide

CHAPTER 1

INTRODUCTION

1.1 Biosensors

1.1.1 Definition of Biosensor

"A biosensor is defined by The National Research Council (part of the U.S. National Academy of Sciences) as a detection device that incorporates a) a living organism or product derived from living systems (e.g., an enzyme or an antibody) and b) a transducer to provide an indication, signal, or other form of recognition of the presence of a specific substance in the environment." (Centers for Disease Control and Prevention ; Luong, Male et al. 2008) (Fig.1.1) (Lee and Mutharasan 2005)

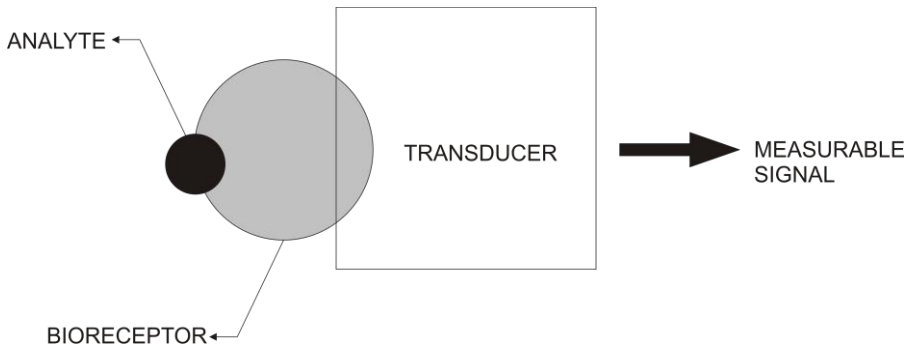

Figure 1.1: General scheme of a biosensor. (Lee and Mutharasan 2005)

There are two important parts of a biosensor; a bioreceptor and a transducer. (Luong, Male *et al*. 2008) In some biosensors, a data processing device is also incorporated due to the nature of data which transducer produces (Xavier, Moreno-Bondi *et al*. 2000; Li and Su 2006).

In the process of developing a biosensor, it is important to choose the right type of bioreceptor and transducer as the interaction between them is crucial for the biosensor. The immobilization of these parts on a platform basically determines receptor-transducer interaction and the general function of the biosensor. Therefore; immobilization of the parts is important for the efficiency of a biosensor. There are different immobilization methods with some advantages and disadvantages, which are listed in Table 1.1 (Mayes 2002).

Several requirements should be satisfied to develop an efficient biosensor. First of all, the construction of the biosensor platform should be easy and should result with a reliability and reproducibility. Second, transducer and method of detection (with or without a device) should be specific enough to help differentiating positive and negative results, should have high signal-to-noise ratio, should be cheap in terms of sensing and labeling materials, and should be used without a need of high technical background (Compton, Wildgoose *et al*. 2009). Chaplin tabulated the properties of a successful biosensor (Chaplin 2000), as Şenyurt described; "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 and stirring. The response of a biosensor should be rapid, accurate, reproducible and precise..." (Şenyurt 2008). To acquire these parameters, different types of biosensors have been developed.

Table 1.1: Different immobilization techniques. (Mayes 2002)

METHOD		ADVANTAGES	DISADVANTAGES
Adsorption		Simple, inexpensive Good for single-use applications	Relatively unstable Proteins denature on hydrophobic surfaces Adsorption highly pH, temperature, solvent, surface and biomolecule dependent – may need extensive optimization
Entrapment	Behind membrane	Simple universal approach for macromolecules Very mild conditions Large excess of protein can be trapped Long working life	Difficult to mass produce Diffusion barrier slows response time
	in polymer gel	Mass production potential	Protein denaturation by free radicals
Covalent coupling		Stable coupling Intimate contact with transducer Low diffusion barrier – rapid response	Complexity and cost of derivatization steps Limited sites for attachment leads shorter lifetime
Covalent coupling to surface immobilized polymer		Larger number of coupling sites Increased signal size May give lower steric hindrance to binding	More complex preparation More complex kinetics and diffusion
Use of 'capture system'		Generic surfaces where specificity can be switched Many options for regeneration Opportunities for antibody orientation	Expensive and complex multi-step derivatization procedures Multi-layer structure may reduce signal Non-specific binding to components of capture system

1.1.2 Types of Biosensors

Biosensors can be classified according to type of bioreceptor or type of transducer of the biosensor.

1.1.2.1 According to Bioreceptors

Bioreceptor is a crucial part of a biosensor since it determines the target and specificity of the biosensor. According to the interaction and mechanism with the target molecule or cell, bioreceptors can be classified into two different sub-categories; catalytic and non-catalytic.

1.1.2.1.1 Catalytic

The catalytic biosensors are based on the covalent modification of the substrate (or analyte). They detect the concentration decrease in the substrate or the increase in the product via different transducer mechanisms. The catalytic part can be made up of an enzyme (protein alone or whole cell), or nucleic acids (ribozymes and aptazymes).

Enzymes are the most commonly used catalytic bioreceptor molecules. In table 1.2 (Sharma, Sehgal *et al.* 2003), some of the enzymes for detection of different analytes were given. The classic example is the amperometric glucose biosensor used basically for blood glucose concentration. In addition, peroxidase and tyrosinase enzymes were used for the detection of phenolic compounds in water (Chang, Rawson *et al.* 2002; Şenyurt 2008). Various enzymatic biosensor examples can be found in the literature.

Table 1.2: Enzyme-based catalytic biosensors and their applications (Sharma, Sehgal *et al.* 2003)

Enzymes	Analyte	Applications
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
Formate dehydrogenase	Formic acid	Fermentation industry and healthcare
Glucose oxidase	Glucose	Diabetes, fermentation and food industry
Glutaminase	Glutamine	Myocardial and hepatic diseases
Glutamate 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

As D'Souza reviewed in his article comprehensively; microbial sensors are made up of transducer which is in relation with a viable or non-viable cells (D'Souza 2001). Whole cell biosensors are basically the enzymatic biosensors in which enzymes used in their *in vivo* state and more than one enzyme can participate in the process of sensing the analyte.

By using *Saccheromyces cerevisiae*, Ikebukuro *et al.* created a whole cell biosensor system that could detect cyanide using an oxygen electrode. Respiration activity of the microorganism was monitored and the inhibitory effect of cyanide on this system was determined (Ikebukuro, Honda *et al.* 1996).

Apart from classical protein-based enzymes, new type of, mostly artificial catalytic molecules have been emerged. These are ribozymes, catalytic single stranded RNA molecules, and aptazymes, catalytic aptamers made up of either DNA or RNA. Both mechanisms are at the stage of development and do not have any commercial applications yet. A ribozyme based assay was designed by Kossen *et. al*. for virus detection. Ribozyme became active and cleaves the virus genome when the base pairing occurs between a part of ribozyme and hepatitis C virus genome. Enzyme-linked immunosorbent assay (ELISA)-based studies were done by the same collaborators (Kossen, Vaish *et al.* 2004). In addition, Ogava and Maeda produced an artificial aptazyme-based riboswitch, which can catalyze the cleavage of its own part resulting a control at the level of translation according to the chosen analyte to its aptamer part (Ogawa and Maeda 2008).

1.1.2.1.2 Noncatalytic

Noncatalytic biosensors are based on the non-covalent interactions of the bioreceptor part of the sensor with the target analyte. Nucleic acids (NA), as short oligos for sequence recognition of DNA/RNA or aptamers for shape recognition of mostly proteins; and proteins, as antibodies for immunosensors or receptors for ligands are the bioreceptor elements of these sensors.

In NA-based biosensors, short DNA/RNA strands are used for recognition of their complementary strands. The transduction of recognition is an important field mostly related with lab-on-a-chip technologies and nanotechnology (Wang 2000). Genetic recognition is basically achieved with these biosensors. Aptamers, oligonucleotides specifically selected to an analyte with high binding affinity due to its tertiary

structure, have been used in biosensors. Song *et. al.* summarized the aptamer biosensors in their reviews (Isoda, Urushibara *et al.* 2007).

Protein-based biosensors are especially important with the immunosensor technology. With antibody-antigen interaction, array platforms were developed for pathogen detection (Isoda, Urushibara *et al.* 2007).

1.1.2.2 According to Transducers

The transducer part of the biosensor determines the sensitivity of the detection of the designed biosensor. There are various transducer systems; amperometric, potentiometric, conductometric, calorimetric, optical, piezoelectric, and even acoustic. However, as Luong stated in his report; "Although a variety of transducer methods have been feasible toward the development of biosensor technology, the most common methods are electrochemical and optical followed by piezoelectric." (Luong, Male *et al.* 2008)

1.1.2.2.1 Amperometric Biosensors

Amperometric biosensors basically measure the change in the electric current, which is occurred due to an oxidation-reduction reaction activity of the bioreceptor element with the analytes.

There is an anode and cathode part of the transducer. The change in the potential with respect to a reference electrode (mostly Pt cathode), gives a quantifiable data for the reaction. The first biosensor, developed for glucose monitoring, was an amperometric biosensor (Clark 1956). In this sensor study; a decrease in the oxygen level was determined as an indicator of the glucose concentration.

1.1.2.2.2 Potentiometric Biosensors

Potentiometric biosensors work on a principle of ion selective electrode. With the catalytic reaction of the bioreceptor part of the biosensor, some ions such as; H^+, F^-, I^-, CN^-, Na^+, K^+, Ca^{2+}, NH_4^+ emerged or consumed throughout the process (Chaplin 2000). The relative concentration difference between two sides of this electrode can be measured quantitavely since with the concentration, charge difference also occurs.

1.1.2.2.3 Optical Biosensors

Optical biosensors based on evanescent field phenomenon in order to detect the changes in the refractive index of light on the sensor surface(Leatherbarrow and Edwards 1999). The electromagnetic radiation, including "light" as we know, is used to generate signal. Optical sensors or optrodes, are based on different optical principles for detection of the analyte such as; absorbance, reflectance, luminescence, and fluorescence (Jerónimo, Araújo *et al.* 2007).

BIAcore (Uppsala, Sweden), Affinity Sensors (Cambridge, UK), and Artificial Sensing Instruments (ASI) (Zurich, Switzerland) are the main players in optical sensor fields and produce sensor systems (mainly assays) for medical sector via detecting the antibody-antigen interactions (Leatherbarrow and Edwards 1999).

1.1.2.2.4 Colorimetric Biosensors

Colorimetric biosensors are simple and fast systems, in which a chromogenic substance is used in order to create a color. Optical identification is done mostly by naked eye, instead of a mechanical transducer. There are some studies stating easy and fast environmental monitoring with a colorimetric-based transducer mechanism for phenolic compounds (Şenyurt 2008), and pesticides (No, Kim *et al.* 2007). In Turkey; METU Technopolis-based company, NANObiz Inc. firm commercialized the study of Şenyurt's phenol sensor (Şenyurt 2008) for environmental monitoring

and Karakaş's alkaline phosphatase sensor (Karakaş 2009) for milk pasteurization monitoring under the trademark of Sensobiz. Both of these are biostrip-type disposable sensors and alternatives of conventional time consuming and laboratory-based methods.

1.2 Applications of Biosensors

The concept of biosensor was created by Professor Leland C. Clark Jr. (Newman and Turner 2007). In 1956, Clark published a paper about the design of an oxygen monitoring electrode in human fluids (Clark Jr. and Lyons 1962). Later in his studies, he added the concept of "enzyme electrode" to his biosensor design. As seen in table 1.3 (Newman and Turner 2007) and other sources in literature (Kenar 2010), through the course of evolution of biosensor design, the main areas have been medical, environmental and military.

1.2.1 Biosensors in Medical Diagnostics

According to the statistics of 2005, the health industry is the biggest sector in the world by far, with an incredible $1.7 trillion volume. The pharmaceutical industry, with $180 billion volume, has not been included to this number (Uldrich and Newberry 2005). Some of the biggest companies such as; Abbott Point of Care, Inc., Affinity Sensors, Neosensors Limited, Siemens Healthcare Diagnostics Inc., Animas Corp, Lifescan, Inc., Medtronic Diabetes, Roche Diagnostics Ltd., AgaMatrix Inc., Cranfield Health, LifeSensors Inc., M-Biotech, and Nova Biomedical Corp. are in the sector of medical diagnostics.

Table 1.3: Some defining events in the history of biosensor development (Newman and Turner 2007)

Date	Event
1916	First report on the immobilization of proteins: adsorption of invertase on activated charcoal
1922	First glass pH electrode
1956	Invention of the oxygen electrode
1962	First description of a biosensor: an amperometric enzyme electrode for glucose
1969	First potentiometric biosensor: urease immobilized on an ammonia electrode to detect urea
1970	Invention of the ISFET
1972–1975	First commercial biosensor: Yellow Springs Instruments glucose biosensor
1975	First microbe-based biosensor
	First immunosensor: ovalbumin on a platinum wire
	Invention of the pO2/pCO2 optode
1976	First bedside artificial pancreas (Miles)
1980	First fiber-optic pH sensor for in vivo blood gases
1982	First fiber-optic-based biosensor for glucose
1983	First SPR immunosensor
1984	First mediated amperometric biosensor: ferrocene used with GOx for the detection of glucose
1987	Launch of the MediSense ExacTech blood glucose biosensor
1990	Launch of the Pharmacia BIACore SPR-based biosensor system
1992	i-STAT launches handheld blood analyzer
1996	Glucocard launched
1996	Abbott acquires MediSense for $867million
1998	Launch of LifeScan FastTake blood glucose biosensor
1998	Merger of Roche and Boehringer Mannheim to form Roche Diagnostics
2001	LifeScan purchases Inverness Medical's glucose testing business for $1.3 billion
2003	i-STAT acquired by Abbott for $392million
2004	Abbott acquires TheraSense for $1.2 billion

ISFET, ion-selective field-effect transistor; SPR, surface plasmon resonance; GOx, glucose oxidase.

Developing a biosensor system for human-oriented sectors has some extra challenges to cope with. The consistency of the results and the potential effects of the system on human health during the usage are the major problems. A report called "Biosensors: A Clearer View" constituted the possible budget, years of commercialization and needed workforce in order to develop a biosensor for medical diagnosis (Sadana 2006). The result of the survey, shown in the table 1.4, clarified why there is not too much company in the sector of health diagnosis. It takes roughly 10 years to commercialize a biosensor with a budget between $20 and $50 million. However; there are lots of studies in the literature in various fields of health sector.

Table 1.4: Needs for developing a biosensor for medical sector (Sadana 2006)

Number of scientists, administrators, employees	15 - 20
Number of years required to develop the biosensor	10 - 12
Number of man years required	150 - 240
Cost of man-year (scientist, administrator, employee)	$50,000-70,0000
Total personnel cost	$7.5-16.8 million
Overhead (includes cost of financing project, equipment, supplies) at 200% of personnel cost	$15-33.6 million
TOTAL	**$22.5-50.4 million**

An example study to an amperometric biosensor for medical sector was carried out by Stefan and his colleagues. They produced an amperometric biosensor for the detection of creatine and creatinine, both of which in clinical analyses since they show the muscle damage and kidney diseases (Stefan, Bokretsion *et al*. 2003). They

used creatinase and sarcosine oxidase enzymes together in the electrode and were able to detect nmol concentrations of these molecules. Since; with the kidney disease the concentrations reach mmol levels, the designed biosensor proved its usage in monitoring and potential early detection ability.

Point-of-care (POC) biosensor technologies, which are diagnostic devices being used near the site of patient care, are emerging recently. Transportable, portable, and handheld instruments are the center of this technological advancement. Apart from environmental, military, and food sectors, medical diagnosis is using this technology far more. Glucose monitoring, pregnancy testing, hepatitis testing, drugs of abuse screening testing, infectious disease testing, HIV, coagulation testing, and fertility testing are the most commonly used POC biosensor systems available (Sadana 2006).

1.2.2 Biosensors in Environmental Monitoring

"Biosensors are currently being considered for development for detection of environmental pollutants such as phenols, genotoxins, and pesticides such as organophosphates, 2,4-D, etc." (Rogers and Gerlach 1996). Since any contamination in the environment is both directly and indirectly related to the human health via water, food and disease and/or disease related compounds, the detection and monitoring of the hazardous materials is crucial. Thus, there are immense amount of scientific and technological studies completed and/or ongoing.

Rogers and Gerlach listed the general requirements for the environmental biosensors, which is tabulated in table 1.5 (Rogers and Gerlach 1996).

Table 1.5: General requirements for the environmental biosensors (Şenyurt 2008)

Requirement	Specification Range
Cost	$1-15 per analysis
Portability	Can be carried by one person; no external power
Assay time	1-60 minutes
Personnel training	Can be operated after 1-2 hour training period
Format	Reversible, continuous, in situ
Matrix	Minimal preparation for ground -water, soil extract, blood and urine
Sensitivity	Parts per million to parts per billion
Dynamic range	At least two orders of magnitude
Specificity	*Enzymes/receptors/nucleic acids:* specific to one or more groups of related compounds *Antibodies:* specific to one compound or closely related group of compounds

Şenyurt developed a simple and effective biosensor for environmental monitoring which can meet the properties mentioned in the table 1.5. Her colorimetric biosensor system monitors phenols and phenolic compounds in environmental samples with tyrosinase as an enzyme capable of catalyzing activity with phenolic compounds and 3-methyl-2-benzothiazolinone as a chromophore for the maroon color formation (Şenyurt 2008).

Other biosensors for environmental monitoring are mainly based upon the non-catalytic antibody recognition systems since most of the environmental biosensors have been develop in order to identify microbial cell-based analytes (Rodriguez-Mozaz, Alda *et al*. 2006). *Escherichia coli* (Koubová, Brynda *et al*. 2001), *Salmonella enteriditis* (Pathirana, Barbaree *et al*. 2000), *Salmonella typhymurium* (Ercole, Galloa *et al*. 2002) are three microbes that can be detected with antibodies. A similar non-catalytic mechanism was used in order to monitor other hazardous materials such as pesticides (Rodriguez-Mozaz, Reder *et al*. 2004). The antibody-

analyte interaction was determined via amperometric and potentiometric transducer mechanisms.

For chemical hazardous materials; catalytic, literally enzymatic, biorecognition elements were used. Pesticides such as paraoxon and carbofuran were monitored in wastewater via acetylcholinesterase enzyme inhibition mechanism in many studies. Horseradish peroxidase was used in a study by Degiuli and Blum instead of tyrosinase for detection of an important pollutant cholorophenol (Rodriguez-Mozaz, Alda *et al.* 2006).

Apart from being analytes, microbial cells can also be used as biorecognition elements for environmental pollution monitoring. Genetically modified Escherichia coli cells were used to determine heavy metals such as zinc, mercury, arsenite (Liao, Tseng *et al.* 2006). In addition to these; biological oxygen demand tests can be done with microbes in order to monitor the organic quality of samples, basically water (Chee, Nomura *et al*. 2000).

Within these studies, different commercialized biosensors have been emerged. In table 1.6, some of these environmental biosensors can be seen (Rodriguez-Mozaz, Alda *et al*. 2005).

1.2.3 Biosensors in Military Applications

The military applications of the biosensors are mainly based on the detection of the harmful biological and chemical warfare agents. Biological warfare agents can be explained as bacteria, virus, or fungi-classified microorganisms and the toxins being produced by them which are harmful to human and environment health. *Bacillus anthracis* causing anthrax, *Yersinia pestis* causing plague, and Evola virus hemorrhagic fever are three of these threads that can be classified biological warfare agents. A comprehensive list of these biological threads can be found online from Centers for Disease Control and Prevention website (Centers for Disease Control and

Prevention). Chemical warfare agents are organic and/or inorganic compounds, basically derived from agriculture sector.

Table 1.6: Commercialized biosensors (Rodriguez-Mozaz, Alda et al. 2005)

Instrument	Company	Transducing and recognition element
BIACORE	BIAcore AB (Uppsala, Sweden)	Optical BI
IBIS	Windsor Scientific Ltd. (Berks, UK)	Optical BI
SPR-CELLIA	Nippon Laser and Electronics Lab (Japan)	Optical whole cells or macromolecules
Spreeta	Texas Instruments Inc. (Dallas, USA)	Optical BI
BIOS-1	Artificial Sensing Instruments (Zurich, Switzerland)	Optical BI
	Amersham International	Optical immunoreagent
	XanTec Bioanalytics GmbH (M¨unster, Germany)	Optical BI
Kinomics PlasmoonTM	BioTul AG (Munich, Germany)	Optical BI
IASys plus TM–	Affinity Sensors, (UK)	Optical Antibody
REMEDIOS	Remedios (Aberdeen, Scotland)	Optical whole cell
Cellsense	Euroclon Ltd. (Yorkshire, UK)	Electrochemical *Escherichiacoli*
PZ 106 Immunobiosensor System	Universal Sensors, (Kinsale, IR)	Piezoelectric antibody
ARAS BOD	Dr. Bruno Lange GmbH (Duesseldorf, Germany)	Electrochemical whole cell
ToxSenTM	Abtech Scientific Inc., (Yardley, USA)	Electrochemical BI
	Universal Sensors Inc., (New Orleans, USA)	Electrochemical enzymes

BI: biomolecular interaction.

The kit-based commercial biosensor systems are the aim of the studies in military. Since there various agents, a main system incorporating into the different types of these agents with specific kits is the generally-accepted method.

One of the sensor systems for military applications was developed by ISIS Pharmaceuticals, which is called TIGER (acronym for Triangulation Identification Genetic Evaluation of Risks). The system is simultaneously monitoring the infectious agents. US governmental agencies in the table 1.7 are supporting the development and application of this sensor system.

Table 1.7: Governmental agencies in US supporting military biosensor (Sadana 2006)

Governmental agency	**Purpose**
Defense Advanced Research Projects Agency	Bioweapons defense
Centers for Disease Control and Prevention (CDC)	Epidemiological surveillance
Federal Bureau of Investigation (FBI)	Microbial agent database
National Institute of Allergy and Infectious Diseases (NIAID)	Biological products screening

Another biosensor example for military sector was developed by Biotrace (specializes in bioluminescence systems) and Smiths Group's Greaseby (specializes in detection systems). The set-up of this biosensor is similar to TIGER, in which the kit detects the harmful agents in a two-step process and under 2 minutes. The scientific background is a patented system of adenosine triphosphate (ATP) luminescence (Sadana 2006).

1.2.4 The Perspective of the Market

The study on biosensors occupies an important field in both scientific and technological areas. Luong explained the current situation with statistics as; "Annual worldwide investment in biosensor R&D is estimated to be $300 US million (Weetall, 1999; Alocilja and Radke, 2003; Spichiger-Keller, 1998). Both publications and patents issued are phenomenal in biosensor research. From 1984 to1990, there were about 3000 scientific publications and 200 patents on biosensors (Collings and Caruso 1997; Fuji-Keizai USA, Inc., 2004). The same number of publications (~3300 articles) but almost double the patent activity (400 patents) was noticed from 1991 to 1997. The explosion of nanobiotechnology from 1998 to 2004 had generated over 6000 articles and 1100 patents issued/pending (Fuji-Keizai USA, Inc., 2004)." (Luong, Male *et al*. 2008).

As an opposition to this scene; Lowe noted, "It is ironic that one of the few biosensors to be successfully introduced into the market place, and which accounts for over 85% of biosensor sales, has been an amperometric glucose sensor based loosely on the original concept of the enzyme electrode" (Lowe 2000). A similar conclusion can be drawn from the table 1.8 (Chaplin 2000)

The reasons, which Lowe stated, for this inadequate market achievement of biosensors, were mainly the mass production problem and inadequate storage stability.

However; the survey studies and forecasts showed that, the current market size of biosensors is between $6-7 billion (Newman and Turner 2007) (Global Industry Analysts 2008). And, "The biosensors market is expected to grow from $6.72 billion in 2009 to $14.42 billion in 2016." according to a report of 2010 (Frost & Sullivan Inc. 2010).

Table 1.8: The market share of biosensor application areas (Chaplin 2000)

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

1.3 Biosensors for Pesticide Detection

1.3.1 Pesticides

1.3.1.1 Definition of Pesticides

"A pesticide is any substance or mixture of substances intended for: preventing, destroying, repelling, or mitigating any pest. Though often misunderstood to refer only to insecticides, the term pesticide also applies to herbicides, fungicides, and various other substances used to control pests." (U.S. Environmental Protection Agency 2010) Pests are insects, weeds, fungi, microorganisms such as bacteria and viruses that cause damage to crops or humans or other animals.

1.3.1.2 Applications of Pesticides

The main reason of the pesticide formulation development and usage is for the agriculture and horticulture sectors. Mostly herbicides, fungicides, and insecticides have been using in these sectors. However, with the development of urban areas, especially insecticide consumption by costumers was increased. Even in 1980 there

more than 1000 types of pesticides, creating $1.1 billion for urban usage and $4.9 billion agriculture usage after 10 years just in USA (Larson, Capel *et al.* 1998).

During 1950's and 1960's, the pesticides, especially herbicides and fungicides were used in forestry in order to control the environment. However this usage was declined with following years. Another pesticide usage area is surface waters. The aim is to control algae, fish parasites and fishes (Larson, Capel *et al*. 1998).

The side effects and toxicity of these pesticides have been a crucial issue in agriculture sector. Ground water, surface waters, crops, animals, and finally humans are under thread of these dangerous chemicals. "Considerable costs of development are consumed by safety tests prescribed by regulatory authorities, and the costs of new long term and short term safety tests continually add to the developer's costs and delay the introduction of the product to the market. Establishment of the United States Environmental Protection Agency (EPA) in 1970 made the EPA responsible for registration of pesticides in the United States. Measures to safeguard the environment were introduced and environmental regulations continue to grow in complexity." (Plimmer 2001)

With the emergence of terrorism worldwide and becoming an issue at a global scale; bioterrorism is a topic of concern. Insecticides have a mechanism to inhibit acetylcholinesterase and block the nervous system. This enzyme, being responsible for nerve impulse control by degrading acetylcholine in the synapses, is also found in humans. So, nearly all insecticides are potential warfare agents.

Thus; the monitoring and the detection of pesticides, especially insecticides are a major concern for environmental, health and security reasons. There have been various methods for detection of insecticides.

1.3.2 Detection Methods

1.3.2.1 Conventional Methods

The traditional methods for determining the insecticide in a medium are gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with mass selective detectors (MSD) (Schulze, Scherbaum *et al.* 2002). There are some studies with these methods such as HPLC method coupled with UV detection (Martinez, Gonzalo *et al.* 1992) or dual electrochemical detection coupled with liquid chromatography (Martinez, Gonzalo *et al.* 1993). Some methods for different pesticides from different samples were listed in table 1.9 (Aprea, Colosio *et al.* 2002).

Table 1.9: Conventional methods for detection of pesticides (Aprea, Colosio *et al.* 2002)

Analytes	**Azinphos-ethyl**	**Malathion**	**Malathion**	**Fenitrothion**
Sample	Blood, urine, gastric lavage liquid	Blood	Blood, stomach contents, liver	Blood, gastric lavage liquid
Sample preparation	Extraction with benzene	HS-SPME	Extraction with solvents	Extraction with solvents after absorption on Extrelut
Apparatus	GLC–FPD or thermoionic detector	GC–MS-EI SIM *m*/*z* 173	GC–MS-EI SIM *m* /*z* 173	GC–FID GC–FPD and GC–MS
LOD	1 mg/l	1 mg/g	1 mg/g	–
Recovery (%)	92–102	86.466.3	–	–

The main disadvantage of these systems is the need for high price equipment and highly qualified personal to operate them. These methods are not only expensive but also time consuming and not portable. Thus; other methods have been developing for better biosensor platforms.

1.3.2.2 Amperometric Methods

In amperometric biosensor developments; butrylcholinesterase, organophosphate hydrolase, alkaline and acid phosphatase, ascorbate oxidase, acetolactate synthase and aldehyde dehydrogenase enzymes can also be used in addition to acetylcholinesterase, since all of these enzymes are inhibited by the pesticides (Trojanowicz 2002). Schulze and his colleagues used acetylcholinesterase to develop a disposable screen-printed biosensor and compared it with the traditional analytical methods for 26 food samples (Schulze, Scherbaum *et al.* 2002). They ended up with consistent results according to the traditional methods. Although this was an easier method than the conventional counterparts, need of a screen printer limits its usage as a portable biosensor.

Kröger and his team designed an amperometric biosensor for the detection of herbicide 2,4-D in methanolic soil extracts (Kröger, Setford *et al.* 1998). They compared their result with a commercial 2,4-D immunoassay test kit and got sufficient results according to it. However; the need for a device for sensor usage was still a problem. Kök and Hasırcı also developed an amperometric biosensor for binary pesticide detection such as aldicarb and carbofuran. They found that, combined inhibitory effect of the binary pesticides was lower than the individual ones (Kök and Hasırcı 2004).

1.3.2.3 Potentiometric Methods

Potentiometric measurements of pesticide detection are similar to amperometric studies since both are electrochemical sensors. In one potentiometric study,

Organophosphorus hydrolase (OPH), a biological catalyst, was used to effectively hydrolyze a range of organophosphate esters, pesticides such as parathion, coumaphos and acephate, and chemical warfare agents such as soman, sarin, VX, and tabun (Mulchandani, Mulchandani *et al.* 1998). In this study, potentiometric determination of the hydrolysis activity was measured. Instead of inhibition ability, degradation characteristics of the pesticides were used. However there was no study about the non-specific enzymatic activity of the OPH, thus in real sample usage of any kind, the consistency of the biosensor is unknown.

1.3.2.4 Colorimetric Methods

Development of colorimetric biosensors for pesticide detection is especially important field to study. Because, it is the only method that makes possible to produce transportable, portable and handheld biosensor platforms. The mobility and simplicity of the colorimetric biosensor with naked eye observation provides the ability to use in different fields from waste water environmental monitoring in agricultural areas to warfare agents monitoring in battlefields.

The colorimetric pesticide biosensors are basically based on the Ellman's method, which was developed by Ellman and his colleagues (Ellman, Courtney *et al.* 1961). In this method an artificial substrate acetylthiocholine was cleaved with acetylcholinesterase, target of insecticides for inhibition. After cleavage, free thiols of artificial substrate react with DTNB (Ellman's reagent) in order to produce a colorful TNB^- reagent. The concept is as follows; if there is no insecticide, the reaction will go as expected and yellow color formation occurs, however if there is insecticide in the sample, acetylcholinesterase will be inhibited and no reaction will occur.

There are some studies based upon this principle. Hossain and his colleagues, and Nagatani and his colleagues designed biosensor strips in this manner (Nagatani, Takeuchi *et al.* 2007; Hossain, Luckham *et al.* 2009). In the first study, the sol-gel

entrapment of the enzyme was experimented in order to increase the shelf-life of the biosensor. Although the preparation of these biosensors was more difficult, they got satisfactory results. Nagatani and his colleagues designed these biosensors on the physical adsorption of the enzyme. They detected DZN-oxon, a pesticide up to a concentration of 2 ppm but their shelf-life was insufficient.

Indophenylacetate, 2,6-dichloroindophenyl acetate, and indoxyl acetate molecules are also used for colorimetric studies. They are also the substrates of AChE but the products of their cleavages give color without a need of chromophore. No and his colleagues studied these molecules and developed a dipstick biosensor (No, Kim *et al.* 2007). Although their detection limits were acceptable, the consistent shelf-life was an inequate 6 weeks, after which a gradual decline of activity was observed.

Alternatively; Pohanka and his colleagues designed a biosensor, that pH indicator strips were used (Pohanka, Karasova *et al.* 2010). However; for a result, overnight incubation time was needed and this study is far from being commercialized.

1.4. Aim of the Study

Insecticides are hazardous to human health. There are various biosensor studies for pesticide monitoring but as a simple and fast commercial product, none of these studies meet the expectations of the field usage. Thus the aim of this study is to develop an insecticide biosensor which is disposable, easy to use, portable, reliable and fast pesticide biosensor, specifically for insecticides since they are the most hazardous ones for human health, which will be used colorimetric and results can be observed with naked eye.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

AChE, DTNB and ATCh (in the form of acetylthiocholine iodide salt) were purchased from Sigma-Aldrich Inc. The chemicals used in the preparation of buffers were purchased from Merck. The pesticide inhibitor malathion was kindly donated by Prof. Dr. Öner Koçak from Biology Department, Hacettepe University

2.1.2 Support Materials

Munktell filter discs quality no.1 was purchased from Munktell Company. The plastic ground material for the biosensor was purchased from common stationery. The filter paper used on the biosensor was purchased from Whatman Company.

2.2 Methods

2.2.1 Construction of Biosensor

The standard procedure of constructing a biosensor consists; a mixture of enzyme and chromophore solution was prepared with determined final concentrations.

This mixture was stabilized on a filter paper support via physical adsorption of the filter paper. After pouring, the filter paper was incubated in a desiccator at -600mmHg for 20 minutes. Other component of the biosensor; ATChI solution was prepared at desired concentration and used either adsorbed to another filter paper or as a solution by itself. The sample of interest (any solution of unknown insecticide content) was poured on the enzyme-chromophore mix adsorbed filter paper and incubated till complete drying. (20μL of sample and 20 minutes waiting is generally sufficient). After that the ATChI solution was poured on the biosensor and incubated for 3 to 5 minutes. For the quantification of the observation apart from naked eye, the biosensors were placed between two transparent acetate papers and scanned through a scanner. The images were analyzed with an image processor program.

This procedure can be accepted as a standard. There were some variations or changes throughout the experiments and the ones needed consideration was mentioned in the related sections of this study.

2.2.1.1 Preparation of Support Materials

For the optimization studies each Munktell paper disc was divided into 1x1 cm^2 pieces and autoclaved at 120°C for 25 minutes prior to use. For the construction of a pilot biosensor the Whatman filter paper was cut into pieces of 1.3 cm height strands. Two of these strands were stabilized via two-sided plaster on plastic grounds with a gap between them. Finally, the complete structure was cut into 0.75 x 9 cm pieces with a print cutter in order to create the biosensor platform as shown in the figure 2.1.

Figure 2.1: Strip-type biosensor design

2.2.1.2 Preparation of Solutions

0.1M sodium phosphate buffer was prepared by adding 3.1 g of NaH_2PO_4 and 10.9 g of Na_2HPO_4 into 1 L of sterile dH_2O. pH was adjusted with HCl and NaOH. For the shelf-life studies, glucose, trehalose and BSA was added to the prepared buffer with projected concentrations. 0.02% of sodium azide (NaAZ) was also added to provide sterility. The buffers being used in the pH study were prepared separately according to the Handerson-Hasselbalch equation.

Lyophilized enzyme AChE stock was dissolved in cold, 0.02 M phosphate buffer at pH 7.0 containing 1mg/mL BSA for stabilization, and stored at -20°C. For each use, necessary amount was diluted into a cold, sterile 0.1M phosphate buffer at pH 7.4.

For chromophore preparation, 40 mg of DTNB was dissolved in 1 ml of sterile 0.1M phosphate buffer at pH 7.4. For artificial substrate, ATCh preparation, 29 mg of ATChI salt was dissolved in 1 ml of sterile dH_2O. Both solutions were prepared fresh.

2.2.1.3 Immobilization of Solutions on Support Material

A mixed solution of AChE and DTNB were prepared accordingly and 15 µL of this mix was dropped on 1x1 cm Munktell filter papers. In control studies, 15 µL of DTNB solution was used.

15 µL of ATCh solution was either pipetted on the enzyme-chromophore saturated filter paper or was dropped on the other side of the biosensor platform. Loaded filter papers dried in a desiccator under vacuum at 600mmHg for 20 minutes at RT.

2.2.2 Concentration Studies of Molecules

2.2.2.1 Concentration Studies of AChE

In order to determine the optimum concentration of the enzyme for the best biosensor response, different dilutions of enzyme stock was used. The enzyme stock was prepared at a final concentration of 300U/mL. Five different and separate concentrations were prepared from this stock, which were 3, 6, 9, 12, and 15U/mL respectively. These were final concentrations in the enzyme-chromophore mix. The dilution was done with 0.1M phosphate buffer of pH 7.4.

2.2.2.2 Concentration Studies of DTNB

The concentration of the chromophore DTNB was studied for obtaining adequate color shift from white to yellow that can be observed with a naked eye. To optimize five different final concentration of DTNB was used; 1, 2, 3, 4, 5µL/mL in 0.1M phosphate buffer at pH 7.4.

2.2.2.3 Concentration Studies of ATChI

To determine the concentration for optimized yellow color formation, 5mL ATChI solution was prepared freshly before the experiments and diluted to 1, 2, 3, and 4mL concentrations. These concentrations, with the 5mL stock, were experimented with biosensor.

2.2.3 pH and Temperature Studies

2.2.3.1 Effect of pH on Biosensor

pH studies were carried out with 0.1M phosphate buffers having pH values between 1.0 and 13.0 with 1.0 increments. The enzyme-chromophore mixtures were prepared with buffer having the corresponding pH value. Then the standard procedure was carried out.

2.2.3.2 Effect of Temperature on Biosensor

Effect of temperature was investigated at 4ºC, RT (~25ºC), 37ºC, and 50ºC to determine optimum condition for the biosensor to work efficiently. In the shelf-life experiment, 4ºC and RT were assayed. These values were achieved with a refrigerator and a water bath. In all temperatures, biosensors were incubated at that specific temperature for 5 minutes after dropping of ATChI solution.

2.2.4 Sensitivity of the Biosensor to Inhibitors

Malathion was used as an inhibitor of enzyme and target molecule of the biosensor. 200, 100, 50, 25, 10, 5, 2.5, and 1.0 ppm concentrations were investigated to determine the detection limit of the biosensor.

2.2.5 Stability Studies of Biosensor

The stability studies of the biosensor were done with experimentation of the glucose (with a concentration gradient of 5%, 10%, and 15% w/v), trehalose (with a concentration gradient of 2%, 4%, 6%, 8% w/v), and BSA (with a concentration gradient of 1%, 2%, 3%, 4%, and 5% w/v) in order to improve stability of the enzyme AChE. The stabilizator giving the best result was used in the shelf-life studies.

2.2.6 Quantitative Analysis of the Biosensor Response and Function

The digital picture of the sensor measurements were obtained by putting the filter papers between two acetate paper and scanning the experiments via Canon Pixma MP610 multifunctional scanner and with time intervals if necessary.

The digital picture of the sensor measurements were obtained by putting the filter papers between two acetate papers and scanning them via Canon Pixma MP610 multifunctional scanner with time intervals, if necessary.

These digital pictures were converted into numerical values using OBİTEK® ColorMaster software as seen in the figure 2.2.

ColorMaster measures the RGB values of a user-determined area. Since the results were yellow and its tones, all the digital pictures of the results were inverted with digital photo editing programs such as Adobe® Photoshop®. OBİTEK® ColorMaster program works with a unit scale of 0 to 255. 0 represents pure black and 255 pure white. The readings decreased with increasing color intensity (Şenyurt 2008). However, since the colors of the digital pictures of the results were converted colorimetrically, increase in the number of blue data means also an increase in the intensity of the original yellow color.

Figure 2.2: Screenshot of OBİTEK® ColorMaster program

2.2.7 Statistical Analysis of Data

The statistical data were obtained using Minitab® 15.0 software. The parameters under investigation were means, standard error of the means, and the one way analysis of variance (ANOVA) at 95% confidence interval. The graphical representations of the data were carried out with Microsoft® Excel® 2007 software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization Studies

Optimization studies were conducted to determine the suitable concentrations of AChE as the enzyme, DTNB as the chromophore, and ATChI as the artificial substrate. Optimum concentrations were selected according to the quantified color production and the observation based on the naked eye, since the proposed biosensor use would be with naked eye.

3.1.1 Effect of AChE Concentration on Biosensor Response and Function

In order to determine the enzyme concentration, different concentrations of AChE (3 U/mL, 6 U/mL, 9 U/mL, 12 U/mL, and 15 U/mL) were used in the mixture of AChE/DTNB solutions. 4µg/mL final DTNB concentration was obtained in the solutions. 15µL of these mixtures were immobilized on support material as described previously. For the concentration of ATChI as the artificial substrate, 4µg/mL was used. As a control of enzyme, blank phosphate buffer was used and as the control of artificial substrate, dH_2O was used. As shown in figure 3.1, the density of the yellow color is more obvious as the enzyme concentration increases.

After the enzyme concentration of 6 U/mL, the yellow color formation was distinguishable from that of control. The similar result can be observed from figure 3.2. However; during the experiment, the filter papers with 12 U/mL enzyme concentration and above showed a more rapid color formation and more obvious differentiation from 6 and 9 U/mL enzyme concentrations.

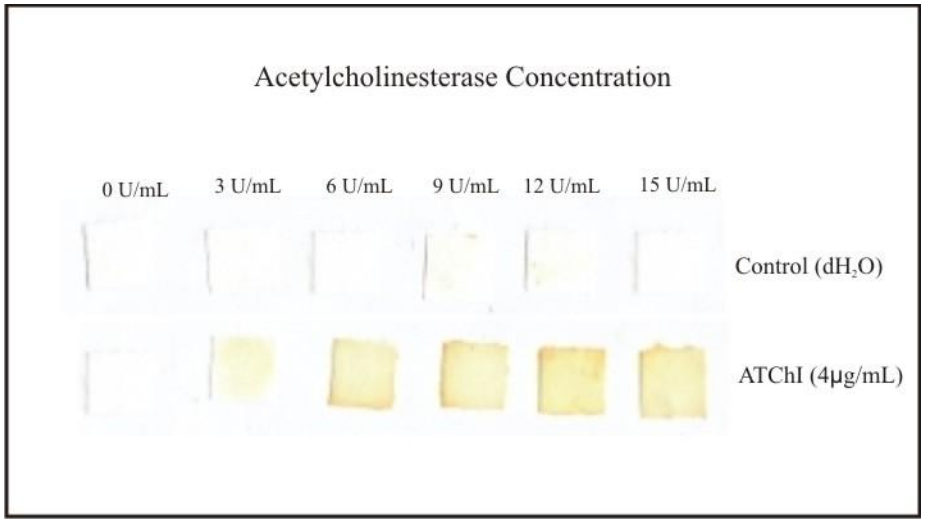

Figure 3.1: The response of biosensor at different enzyme concentrations for 4µg/mL DTNB and 4µg/mL ATChI.

Considering the possible activity loss during the storage of the biosensor; 12 U/mL enzyme concentration was chosen for further experiments. Although highest color formation was observed in 15 U/mL of AChE, reckoning with the final cost of the biosensor, 12 U/mL was appropriate since it was also observed with naked eye as 15 U/mL enzyme concentration.

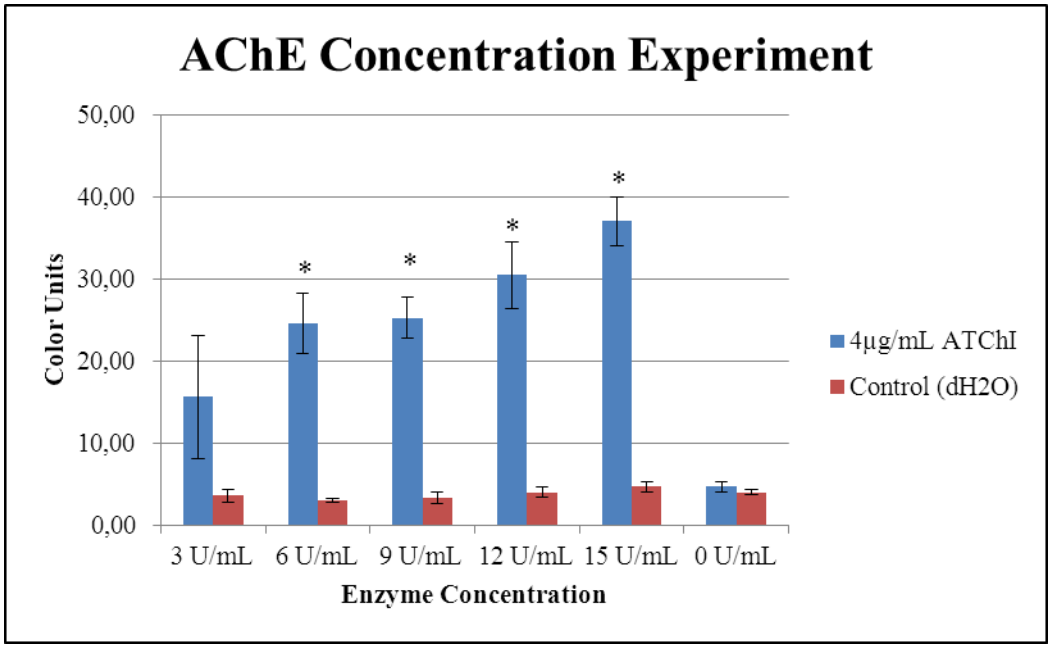

Figure 3.2: Effect of enzyme concentration on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to control (no Acetylcholinesterase), respectively. Mean values, SEM and significant values are tabulated Table B.1 in Appendix B.

3.1.2 Effect of DTNB Concentration on Biosensor Response and Function

5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) is a slightly yellow powder creating a white solution at low concentrations with hydrophilic solvents such as water and phosphate buffer. It is also known as Ellman's Reagent, after his paper on determining ACHE activity (Ellman, Courtney et al. 1961). It is a commonly used chromophore used to determine the free thiol groups. This aromatic disulfide reacts with aliphatic thiol groups to form a mixed disulfide of the protein and one mole of 2-nitro-5- thiobenzoate per mole of protein sulfhydryl group. DTNB has little if any absorbance, but when it reacts with -SH groups on proteins under mild alkaline

conditions (pH 7-8), the 2-nitro-5-thiobenzoate anion (TNB2-) gives an intense yellow color at 412 nm (Sigma-Aldrich 2002).

For the determination of the DTNB concentration; 1µg/mL, 2µg/mL, 3µg/mL, 4µg/mL, and 5µg/mL final concentrations of DTNB were experimented in the biosensor. 12U/mL enzyme concentration was used as determined previously. For ATChI concentration, 4µg/mL was used.

As shown in figure 3.3 for naked eye observation and figure 3.4 for the quantification of the data, a higher density in yellow color formation was observed with an increasing DTNB concentration. 5µg/mL DTNB concentration both gave a distinguishable yellow color and had a response time faster than the other concentrations. Thus, 5µg/mL of DTNB as the final concentration for the chromophore in the biosensor was chosen.

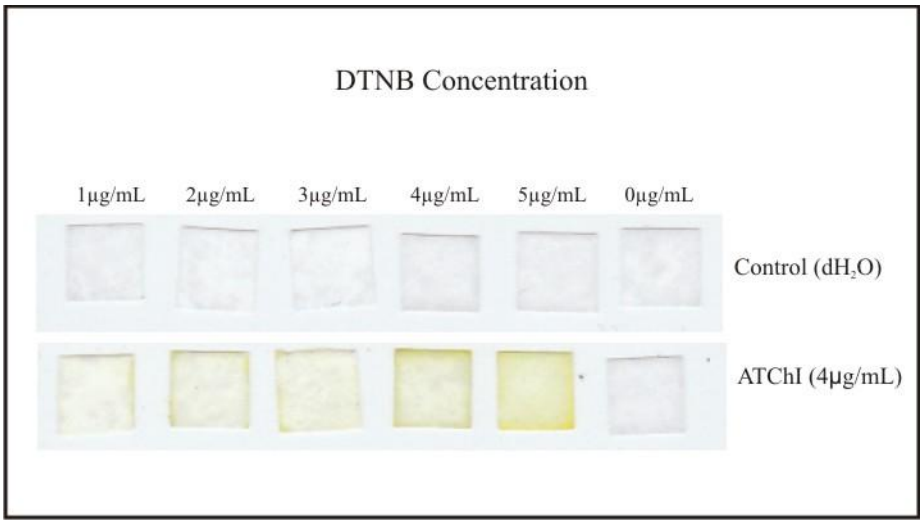

Figure 3.3: The response of biosensor at different DTNB concentrations for 12U/mL AChE and 4µg/mL ATChI

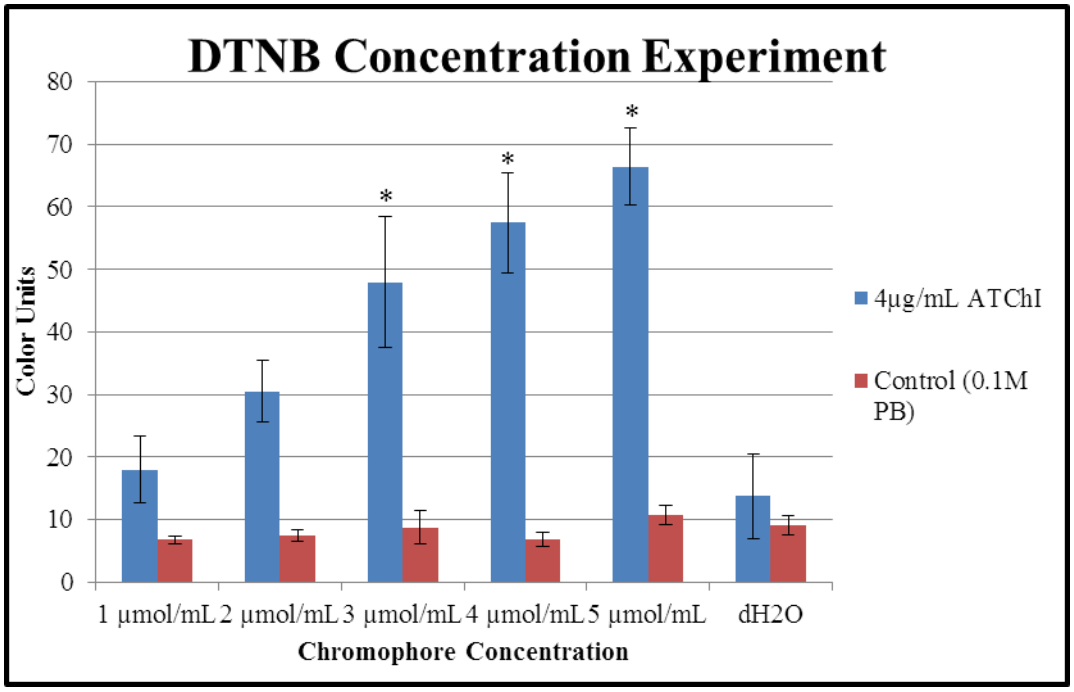

Figure 3.4: Effect of DTNB concentration on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to control (no DTNB), respectively. Mean values, SEM and significant values are tabulated Table B.2 in Appendix B.

3.1.3 Effect of ATCh Concentration on Biosensor Response and Function

The ATChI is an artificial substrate that can be cleaved by AChE to yield a free thiol group. This thiol group gives a reaction with DTNB. To determine the concentration of the ATChI for this reaction; 1µg/mL, 2µg/mL, 3µg/mL, 4µg/mL, and 5µg/mL of ATChI solutions were prepared with sterile dH_2O. 12U/mL enzyme concentration was used, which had been decided in section 3.1.1 and 5µg/mL chromophore concentration was used, which was determined in section 3.1.2.

As shown in figure 3.5 for naked eye observation and figure 3.6 for the quantification of the data, there was a linear correlation between the yellow color formation and the ATChI concentration. Since the response time and the density of the yellow color formation of 5µg/mL is significantly better than other concentrations, this concentration of the artificial substrate was chosen for the developing biosensor. Higher concentrations were not experimented since the response time (under 3 minutes) and yellow color intensity of the 5µg/mL concentration was homogeneous and bright enough for biosensor usage with naked eye.

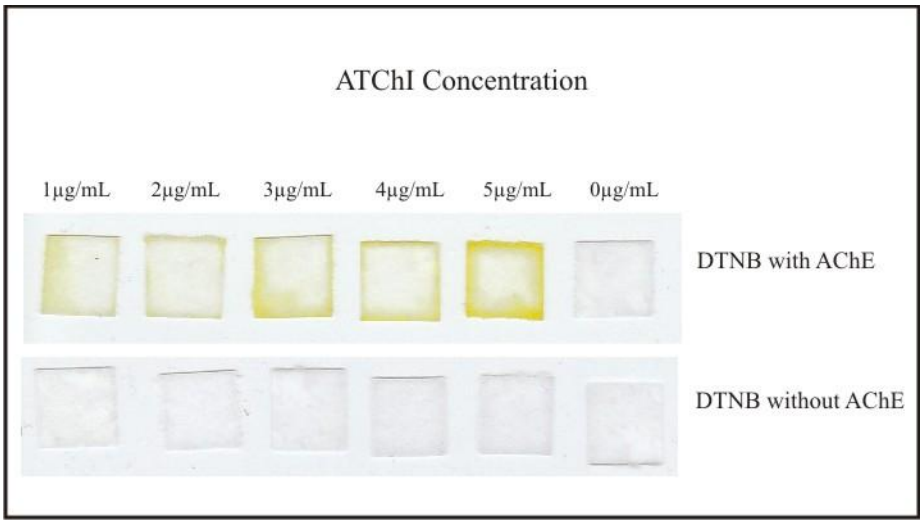

Figure 3.5: The response of biosensor at different ATChI concentrations for 12U/mL AChE and 5µg/mL DTNB

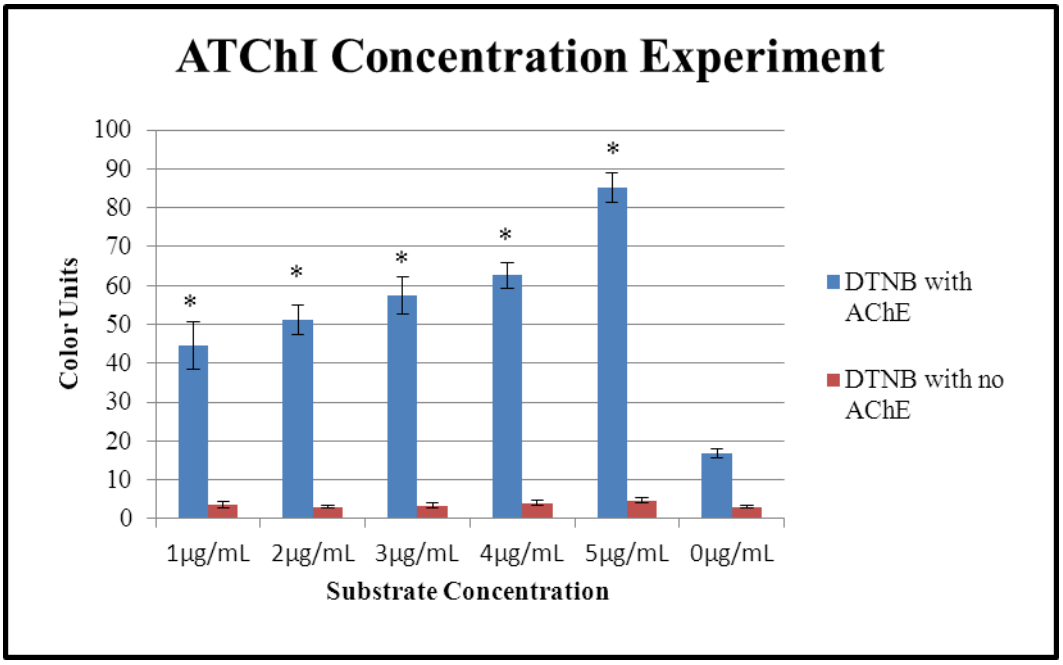

Figure 3.6: Effect of ACThI concentration on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to control (no ATChI), respectively. Mean values, SEM and significant values are tabulated Table B.3 in Appendix B.

3.2 Effect of pH on Biosensor Response and Function

The effect of pH on the biosensor platform was investigated between the pH ranges of 1 to 13. These values of pH were achieved with phosphate buffer as it has three pKa values (2.15, 7.20, 12.33) covering a wide range of pH values.

As shown in figure 3.7 for naked eye observation and figure 3.8 for the quantification of the data; there was a response from the biosensor between the values of pH 2 to pH 8. Due to a slight activity loss or the degradation of the enzyme, at pH 9, slightly less yellow color formation was obtained. At pH values more than 10, the yellow color formation was instant after pouring the ATChI on to

the enzyme-chromophore stabilized part, and the density of the yellow increased immensely. We concluded that either DTNB or the ATChI was cleaved or transformed chemically, therefore there was no need for the activity of AChE on ATChI and false positive results were obtained. For possible commercialization of the biosensor, this finding should be considered. The pH of the samples for analysis should be detected before biosensor usage.

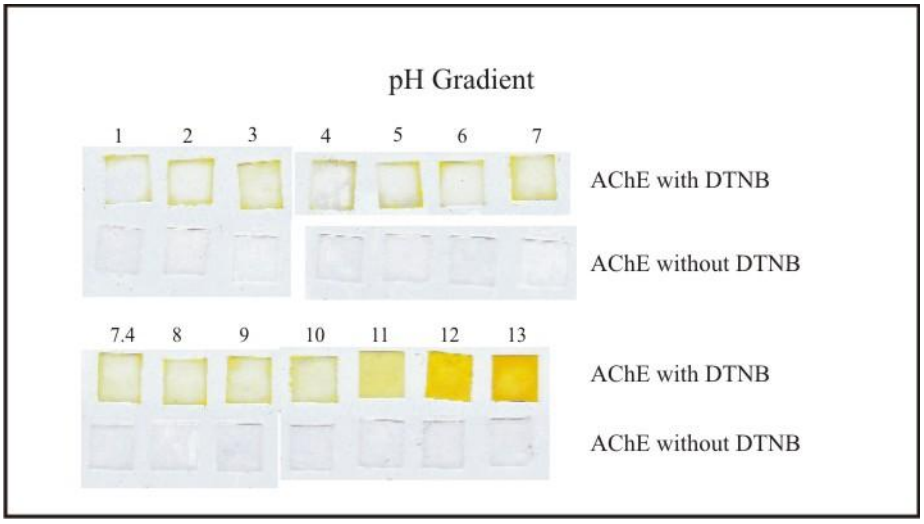

Figure 3.7: The response of biosensor at different pH values from 1 to 13

3.3 Effect of Temperature on Biosensor Response and Function

The effect of temperature on the biosensor platform was investigated at temperatures 4°C, room temperature (nearly 25°C), and 37°C. For all temperatures, the biosensors were incubated at that specific temperature for 5 minutes after the addition of ATChI solution.

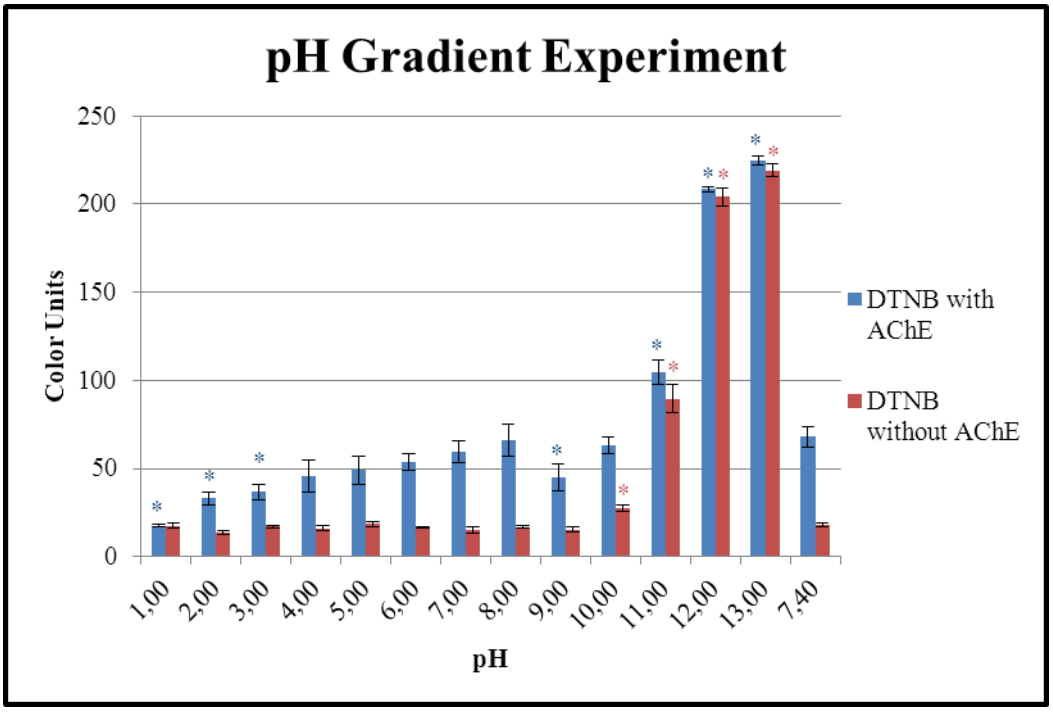

Figure 3.8: Effect of pH on biosensor response. Vertical bars and blue * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to control (no ATChI), respectively. Mean values, SEM and significant values are tabulated Table B.4 in Appendix B.

Temperature studies showed that, at 4°C the activity of the enzyme ceased and no color formation was observed. However, if same sensors incubated at room temperature for some time, the yellow color formation was observed after ATCh application. At room temperature and 37°C, the yellow color formation occurred with significantly different color units with respect to control sensors.

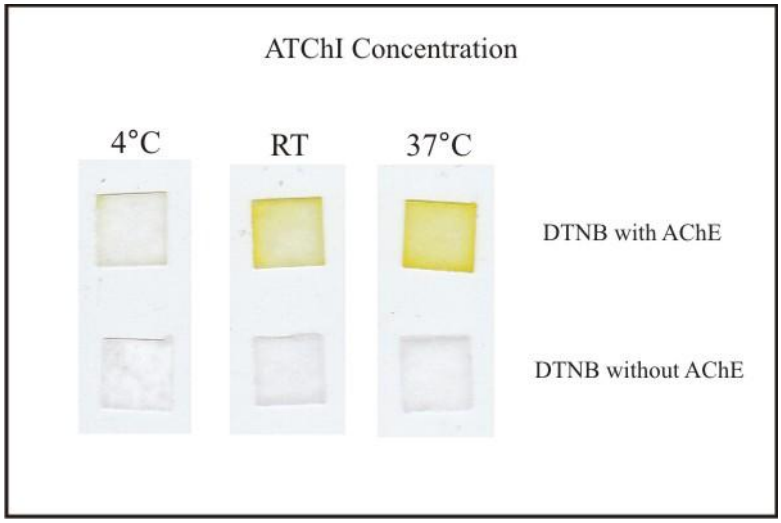

Figure 3.9: The response of biosensor at different temperature values.

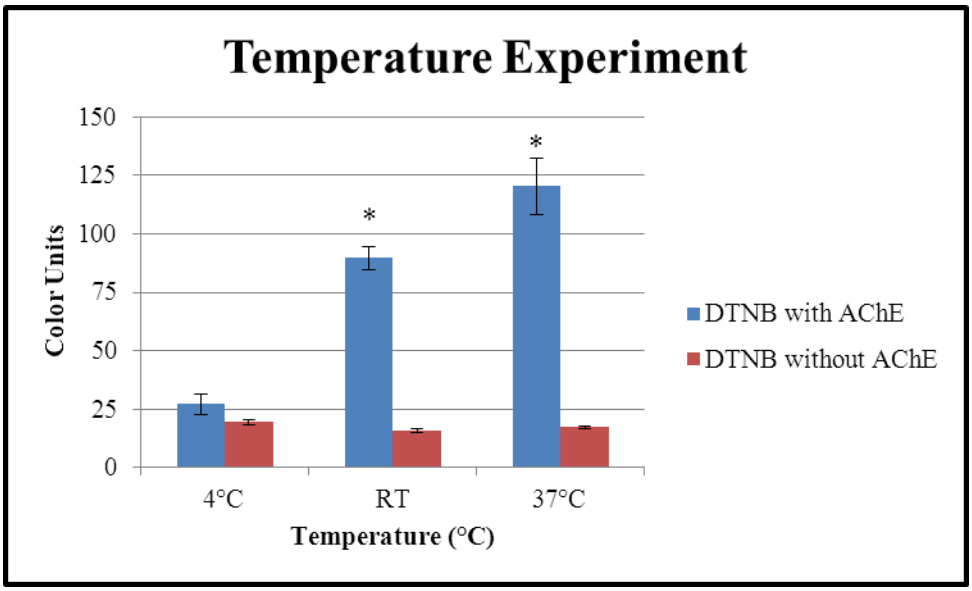

Figure 3.10: Effect of temperature on biosensor response. Vertical bars indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to control (no AChE), respectively. Mean values, SEM and significant values are tabulated Table B.5 in Appendix B

3.4 Stability of Biosensor

According to the studies (Estrada-Mondaca and Fournier 1998; Tümtürk, Şahin et al. 2007) the stability of the AChE is the most labile part of the biosensor. The activity loss of the enzyme should be prevented for the biosensor to be commercialized. Therefore, several of the known enzyme stabilizers (bovine serum albumin or BSA, glucose, and trehalose) were added to the phosphate buffer to investigate their effect on the enzyme activity. In addition, low concentration of sodium azide (NaAZ) was also used to maintain sterility as all these stabilizers can be used as a carbon source by microorganisms.

3.4.1 Effect of BSA on Stability of Biosensor

Effect of BSA on the stability of the biosensor was tested at 1%, 2%, 3%, 4%, and 5% (w/v) of BSA concentrations by adding to the phosphate buffer. After the standard procedure with optimized enzyme (12U/mL), chromophore (5µg/mL) and artificial substrate (5µg/mL) concentrations it was found that; the negative controls with no enzyme exhibited slight yellow color formation meaning false positive result. This observation can be seen in figure 3.11. Although BSA stabilized the enzyme and kept its activity very well (Sigma-Aldrich 2006); a non-specific background reaction was observed. This background color formation may be due to the protein nature and sulfide moieties of the BSA, DTNB reacted with them and gave a yellow color. Thus it was concluded that, BSA could not be used as an enzyme stabilizer in this biosensor development study.

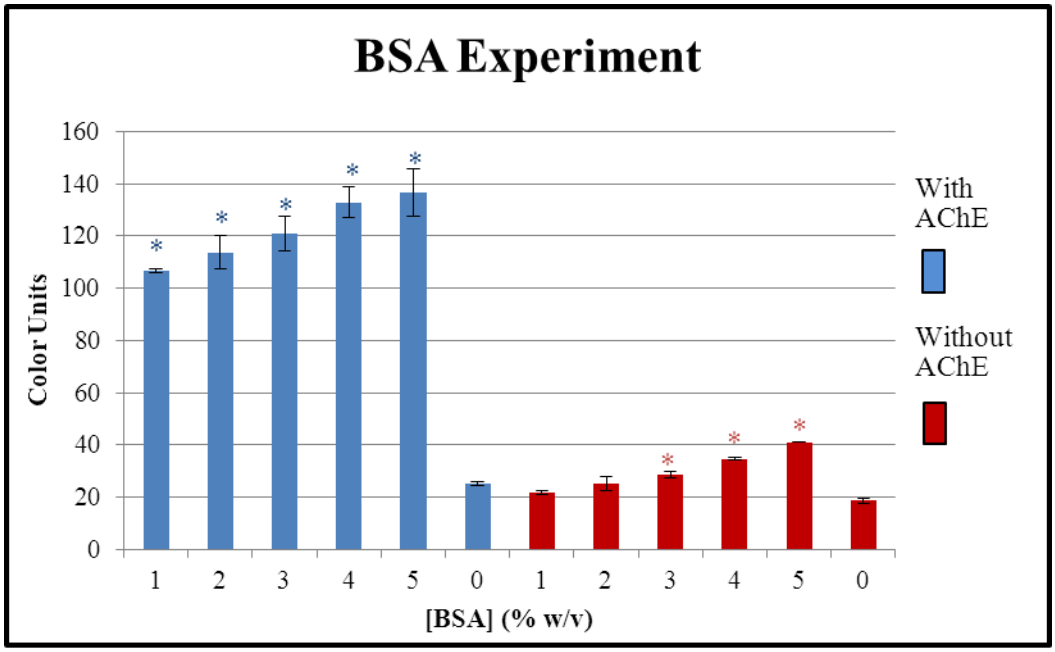

Figure 3.11: Effect of BSA concentration on biosensor response. Vertical bars and blue * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no BSA with or without AChE), respectively. Mean values, SEM and significant values are tabulated Table B.6 in Appendix B.

3.4.2 Effect of Glucose on Stability of Biosensor

Effect of glucose on the stability of the biosensor was tested at 5%, 10%, and 15% (w/v) glucose concentrations in the phosphate buffer. Standard procedure was applied with optimized enzyme, chromophore, and artificial substrate concentrations being determined in the previous studies. It was found that glucose stabilized the enzyme and kept its activity well enough for the biosensor. With the addition of glucose, faster and denser yellow color formation was obtained. The data in the figure 3.12 showed the response of the sensor 3 minutes after application of ATChI on the enzyme-chromophore site of the sensor. The difference between the no-glucose control (concentration 0 with AChE bar of the graph) and the glucose-added

biosensor responses were significantly different. It was concluded that glucose could be a good choice for the stabilization of the enzyme for longer shelf-life. Since 15% (w/v) glucose concentration gave the highest yellow color formation with significantly different colorimetric values from other two concentrations, it was chosen as glucose concentration to be used in further studies.

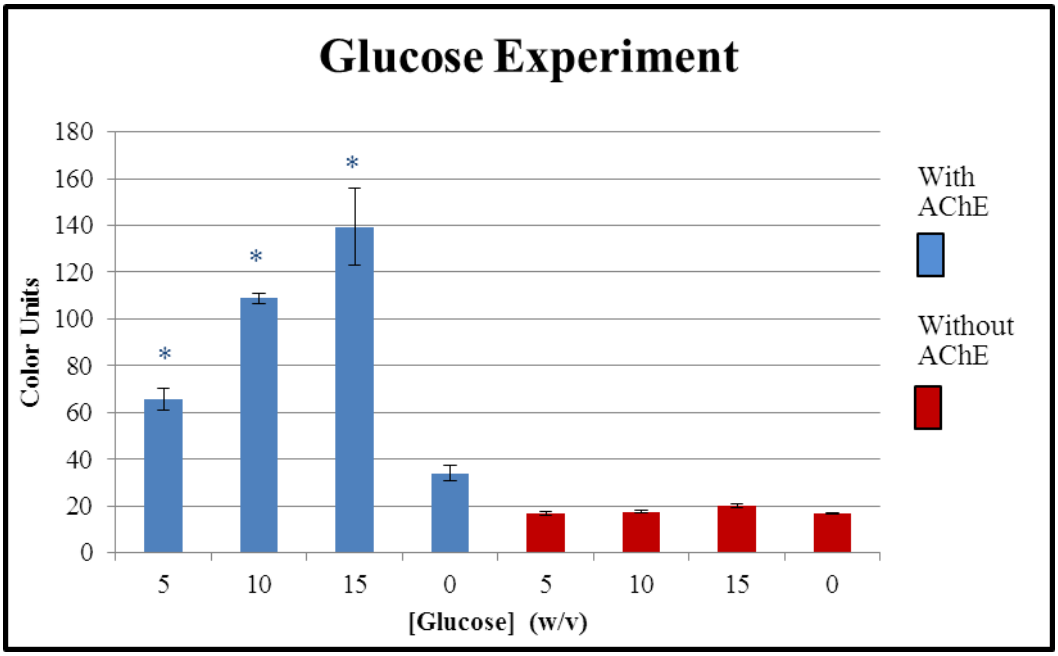

Figure 3.12: Effect of glucose on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no glucose with or without AChE), respectively. Mean values, SEM and significant values are tabulated Table B.7 in Appendix B.

3.4.3 Effect of Trehalose on Stability of Biosensor

Trehalose is a dimer of two glucose molecule. Effect of trehalose on the stability of the biosensor was tested at 2%, 4%, 6%, and 8% (w/v) of trehalose levels in the phosphate buffer. Standard procedure was applied with optimized enzyme, chromophore, and artificial substrate concentrations being determined in the previous studies. The result of the experiment, which were seen in the figure 3.13 also, showed that; higher the trehalose concentration, higher the yellow color formation. This means that, trehalose stabilized the enzyme and kept its activity.

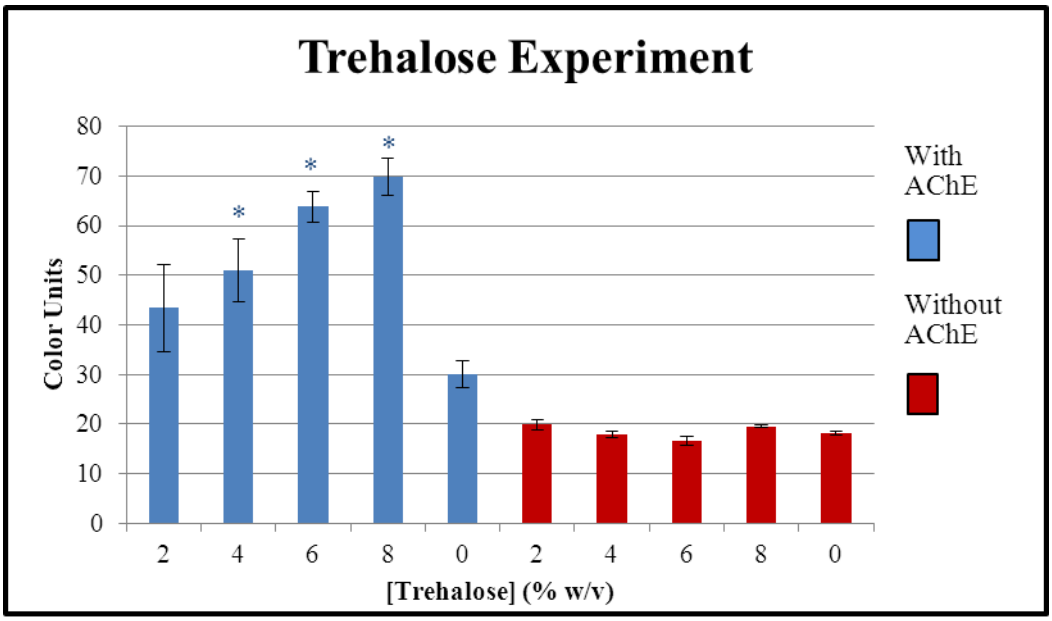

Figure 3.13: Effect of trehalose on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no trehalose with or without AChE), respectively. Mean values, SEM and significant values are tabulated Table B.8 in Appendix B.

However, its stabilization ability was not as good as glucose. This can be seen in the figure 3.13, and can be compared with figure 3.12. This difference of stability performance is most probably due to the size of the molecules. Since glucose monomer is smaller than the trehalose dimer, it occupied more grooves of the enzyme, thus stabilizes it more. From these findings it was concluded that; although trehalose stabilized the enzyme and improved the stability, glucose achieved this expectation more. Thus glucose was used for the rest of the studies

3.4.4 Effect of NaAZ on Stability of Biosensor

Sodium azide is an antibacterial agent, commonly used in the biosensor developments (Frébort, Skoupá et al. 1999; Wakamatsu, Okui et al. 2004). In this study; 0.02% (w/v) NaAZ was used for its antibacterial effect, and the possible effect on the activity of the enzyme was experimented. Standard procedure was applied with optimized enzyme, chromophore, and artificial substrate concentrations being determined in the previous studies. As observed in the figure 3.14, %0.02 NaAZ increased the activity of the biosensor. This result was most probably due to the similar reasons explained in the glucose (3.3.2) and trehalose (3.3.3) sections. Even NaAZ affected the stability of the enzyme by interacting with it and helped the enzyme to protect its active-state physical shape. According to these findings, it was concluded that; NaAZ did not have a negative effect on the activity of the biosensor, even had a positive effect as seen in the figure 3.14. Therefore it was concluded that NaAZ could be used as antibacterial agent in the biosensor.

3.5 Sensitivity of Biosensor to the Pesticides

Malathion is an organophosphate insecticide that irreversibly binds to acetylcholinesterase and inhibits its activity of cleaving acetylcholine in synapsis.

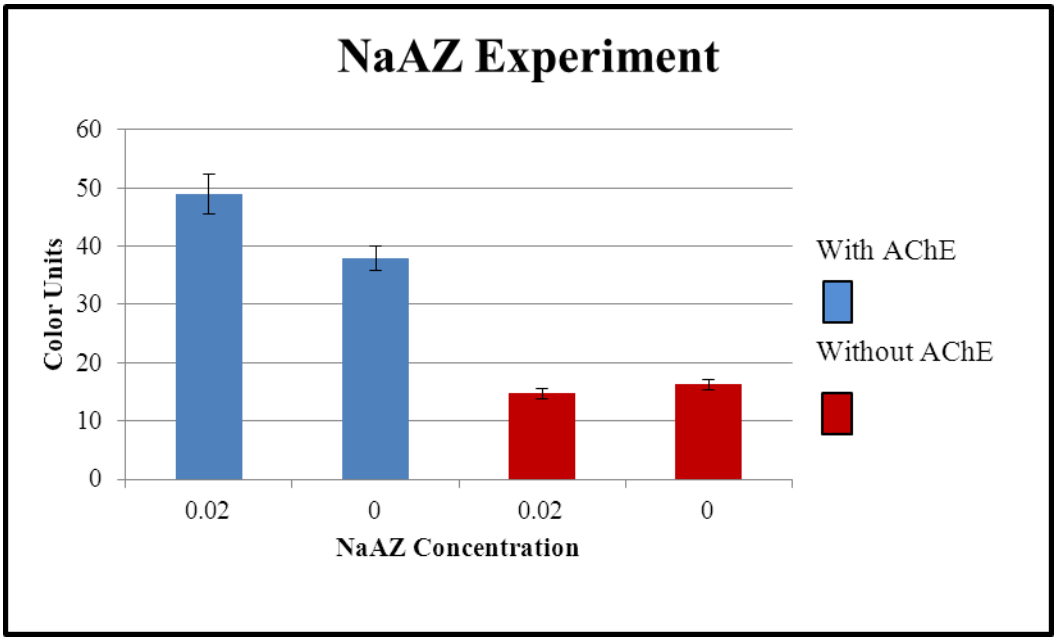

Figure 3.14: Effect of NaAZ on biosensor response. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no NaAZ with AChE), respectively. Mean values, SEM and significant values are tabulated Table B.9 in Appendix B.

The inhibitory effect of malathion on the designed biosensor was tested at different concentrations (200ppm, 100ppm, 50ppm, 25ppm, 10ppm, 5ppm, 2.5ppm, 1ppm). The inhibitor solutions were prepared and diluted in 0.1M phosphate buffer with 5% methanol. Standard procedure was applied with optimized enzyme, chromophore, and artificial substrate concentrations. All the inhibitor solutions were prepared freshly due to the fast degradation tendency of the malathion. 20µL of malathion solutions were applied to biosensors and the biosensors were incubated for 20 minutes. After incubation, as optimized previously, 15µL of 5µg/mL ATChI solutions were applied. The colorimetric results in the figure 3.15 were obtained after 5 minutes incubation of the biosensor with ATChI solutions.

As shown in the graph, above 2.5 ppm malathion concentrations, the biosensor response to ATChI was completely inhibited. However, at 1 ppm malathion concentration, the yellow color formation was observed with naked eye. Although it was lesser compared to the control sensor with 0 ppm, the naked eye observation was enough for excluding 1 ppm concentration from detection limit of the biosensor. Therefore it can be concluded that the sensitivity of the designed biosensor was 2.5 ppm for malathion insecticide.

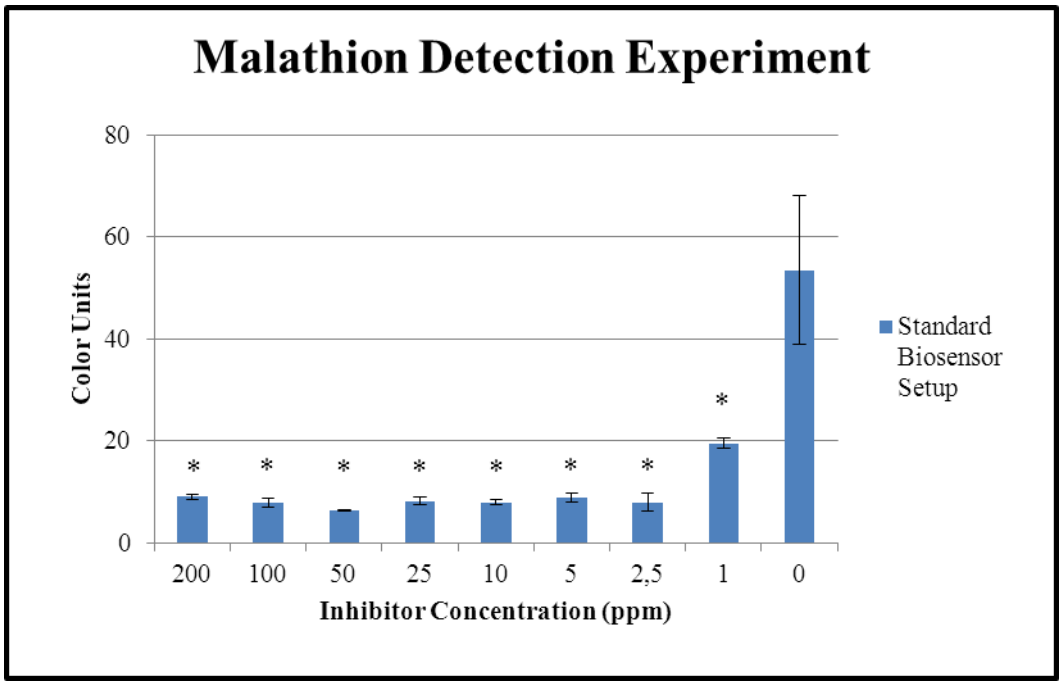

Figure 3.15: Detection of malathion. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no BSA with or without AChE), respectively. Mean values, SEM and significant values are tabulated Table B.10 in Appendix B.

3.6 Shelf-Life of the Biosensor

The shelf-life studies were experimented with the pilot biosensor whose design is explained in section 2.2.1.1. Both DTNB and ATCh are light sensitive molecules. In addition ATCh is a humidity sensitive substance having a storage temperature of -20°C. Thus; the prepared pilot biosensors were incubated in a non-transparent plastic bottles in order to prevent direct light on them. Silica-gel packets were added inside of bottles with biosensors for decreasing the humidity inside the bottles.

However, slight yellow color formation, meaning false positive result, was observed at the substrate side of the biosensors. This might be due to the degradation of ATChI or interaction of it with the filter paper. It was concluded that this system needed further investigation and remeaning experiments of shelf-life were done with a fresly prepared ATCh solutions.

The shelf-life experiments were done first with artificial substrate, ATChI only. Then inhibitor malathion was used for the observation of the biosensor detection ability through time. The shelf-life with ATChI was conducted only at +4°C and room temperature (RT). Biosensors at +4°C were incubated 10 minutes at room temperature before the eperiments. Standard procedure was applied with optimized enzyme, chromophore, and artificial substrate at optimum concentrations as determined previously. Data were taken on daily basis.

As seen in figure 3.16 for RT and figure 3.17 for 4°C; after the first day, the biosensor response to ATChI decreased to some extent. Biosensor kept at +4°C held its activity more than that of room temperature, as expected. Because the degradation of protein-based enzyme AChE would be faster under warmer conditions.

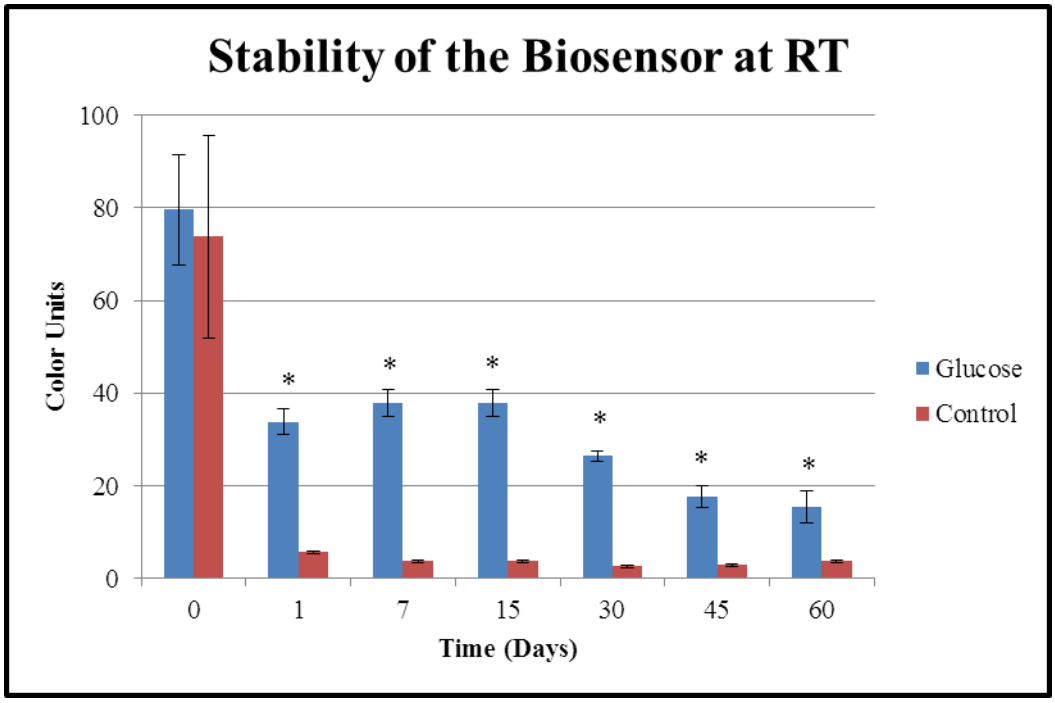

Figure 3.16: Stability of biosensor at room temperature. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no glucose), respectively. Mean values, SEM and significant values are tabulated Table B.11 in Appendix B.

It was also noticed that, control sensors without any glucose stabilization lost their activity even after first day and became completely useless after one week. Throughout the assayed time, biosensors at 4°C held their activity better than the ones kept at room temperature and gave similar results with statistically insignificant difference from first month to second month. From this finding, it can be concluded that the biosensor being developed in this study has a shelf-life of at least two months, which depending on the application, is an acceptable time for a commercial biosensor.

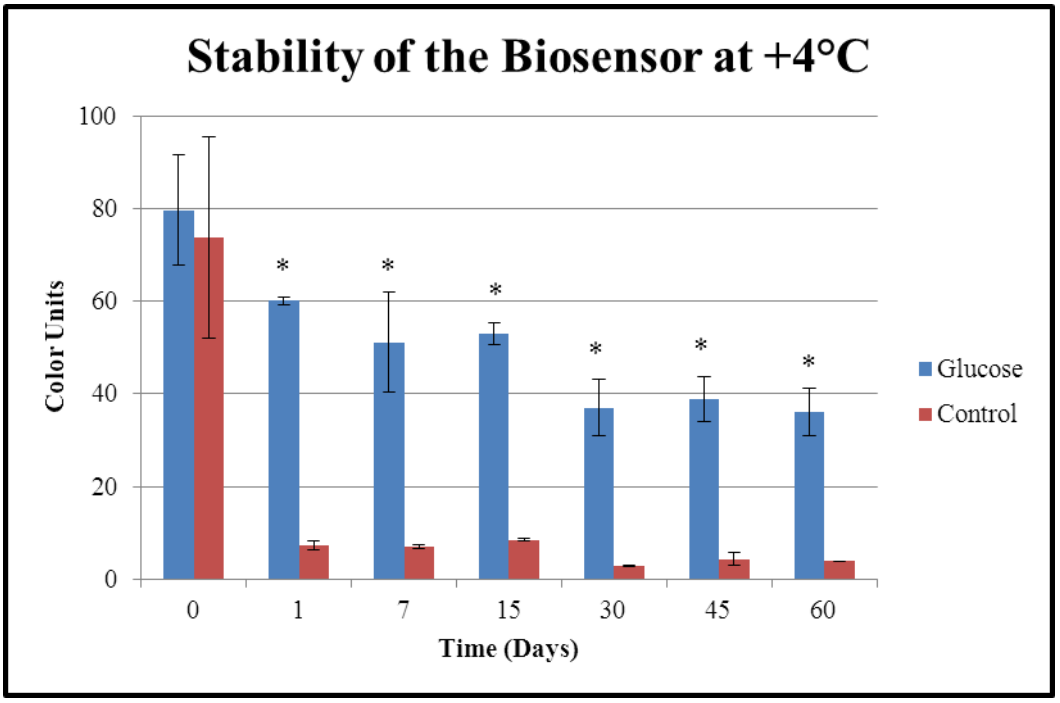

Figure 3.17: Stability of biosensor at 4°C. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no glucose), respectively. Mean values, SEM and significant values are tabulated Table B.12 in Appendix B.

Under same conditions, the designed sensor was experimented with malathion inhibition. According to the previous study, whose result was shown in figure 3.15, 2,5 ppm malathion concentration was used in the shelf-life experiments.

The results of the malathion experiments showed that, the designed biosensor retained its sensitivity to malathion and enabled to detect it. At room temperature after 60 day, the biosensor itself lost most of its activity. Thus it was concluded that, at room temperature shelving, the biosensor would have a tendency toward exploiting false positive result to malathion or other inhibitory molecules.

At 4°C, the biosensor retained its activity more and showed significantly different results between the presence and absence of inhibitor malathion throughout 60 days. Although, results were also significantly different at room temperature, with naked eye observation, the discrimination between the presence and absence of inhibitors was more obvious at 4°C. As a conclusion; at 4°C, the designed acetylcholinesterase biosensor could detect the presence of malathion for 60 days.

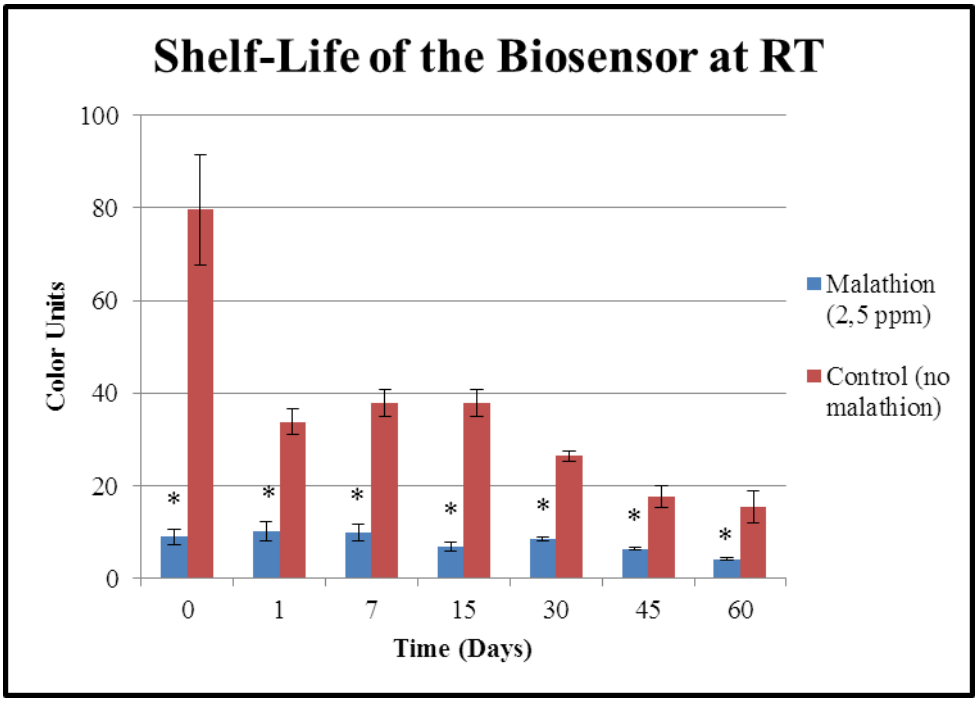

Figure 3.18: Shelf-life of biosensor at room temperature. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no glucose), respectively. Mean values, SEM and significant values are tabulated Table B.13 in Appendix B.

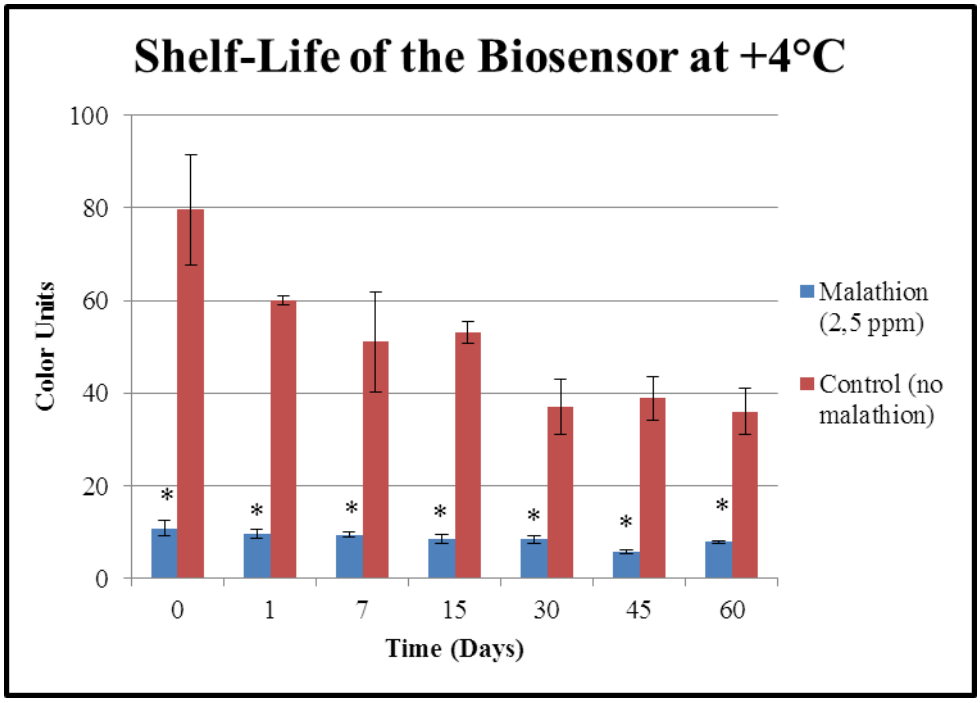

Figure 3.19: Shelf-life of biosensor at 4°C. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p<0,05$) compared to controls (no glucose), respectively. Mean values, SEM and significant values are tabulated Table B.14 in Appendix B.

CHAPTER 4

CONCLUSION

The aim of this study was to develop a disposable, transportable and easy to use paper type acetylcholinesterase biosensor for the detection of organophosphate and carbamate type pesticides. There have been various studies for pesticide detection biosensors. However, there is no commercialization of one of these studies for simple and fast environmental monitoring of pesticides in Turkey.

As a result of optimization studies; 12U/mL acetylcholinesterase, 5µg/mL DTNB, and 5µg/mL ATCh concentration were determined as optimum parameters for the chemicals used in biosensor. The effect of pH on the response of the biosensor studies revealed the sensitivity of the DTNB on basic conditions. The biosensor was found to be active between the ranges of pH 4 and 8.

Glucose, trehalose, and BSA were tested for their stability effects and glucose was chosen. NaAZ was used as an antimicrobial agent. The stability experiments showed that, the biosensor was stable for at least 30 days at room temperature, and 60 days at 4°C. These shelf-life results exhibited the commercialization potential of the developed biosensor.

The results documented in this study suggest that the developed acetylcholinesterase biosensor platform can be used for the detection of organophosphate and carbamate-based insecticides.

Malathion, used as a sample insecticide, could be detected up to 2.5 ppm concentration, which is an acceptable result according to other sensor development studies.

Further studies of the biosensor can be concentrated on increasing the shelf-life of the biosensor at ambient temperatures in order to widen its application areas such as long term field monitoring. The false positive result due to the degradation of the ATChI is another problem to be solved for a successful commercialization. The pH range is not very broad. Thus, different buffers and/ or concentrations can be experimented in order to increase working pH gap.

REFERENCES

Aprea, C., C. Colosio, et al. (2002). "Biological monitoring of pesticide exposure: a review of analytical methods." Journal of Chromatography B **769**: 191-219.

Centers for Disease Control and Prevention. "Bioterrorism Agents/Diseases." Retrieved 10.08.2010, 2010, from http://www.bt.cdc.gov/agent/agentlist.asp.

Chang, S. C., K. Rawson, et al. (2002). "Disposable tyrosinase-peroxidase bi-enzyme sensor for amperometric detection of phenols." Biosensors and Bioelectronics **17**(11-12): 1015-1023.

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

Chee, G.-J., Y. Nomura, et al. (2000). "Optical fiber biosensor for the determination of low biochemical oxygen demand." Biosensors and Bioelectronics **15**(7-8): 371-376.

Clark Jr., L. C. and C. Lyons (1962). "Electrode systems for continuous monitoring in cardiovascular surgery." Annals of the New York Academy of Sciences **102**: 29-45.

Clark, L. C., Jr. (1956). "Monitor and Control of Blood and Tissue Oxygen Tensions." Transactions - American Society for Artificial Internal Organs: **2**(1): 41-48.

Compton, R. G., G. G. Wildgoose, et al. (2009). Carbon Nanotube-Based Sensors and Biosensors. Biosensing Using Nanomaterials. A. Merkoçi**:** 3-37.

D'Souza, S. F. (2001). "Microbial biosensors." Biosensors & Bioelectronics **16**: 337-353.

Ellman, G. L., K. D. Courtney, et al. (1961). "A new and rapid colorimetric determination of acetylcholinesterase activity." Biochemical Pharmacology **7**(2): 88-90.

Ercole, C., M. D. Galloa, et al. (2002). "A biosensor for *Escherichia coli* based on a potentiometric alternating biosensing (PAB) transducer." Sensors and Actuators B: Chemical **83**(1-3): 48-52.

Estrada-Mondaca, S. and D. Fournier (1998). "Stabilization of Recombinant Drosophila Acetylcholinesterase." Protein Expression and Purification **12**: 166-172.

Frébort, I., L. Skoupá, et al. (1999). "Amine oxidase-based flow biosensor for the assessment of fish freshness." Food Control **11**(1): 13-18.

Frost & Sullivan Inc. (2010). Analytical Review of World Biosensors Market.

Global Industry Analysts, Inc., (2008). Biosensors in Medical Diagnostics.

Hossain, S. M. Z., R. E. Luckham, et al. (2009). "Reagentless Bidirectional Lateral Flow Bioactive Paper Sensors for Detection of Pesticides in Beverage and Food Samples." Analytical Chemistry **81**(21): 9055-9064.

Ikebukuro, K., M. Honda, et al. (1996). "Flow-Type Cyanide Sensor Using an Immobilized Microorganism." Electrounulysis **8**(10): 876-879.

Isoda, T., I. Urushibara, et al. (2007). "Development of a sensor-array chip with immobilized antibodies and the application of a wireless antigen-screening system." Sensors and Actuators B: Chemical **129**(2): 958-970.

Jerónimo, P. C. A., A. N. Araújo, et al. (2007). "Optical sensors and biosensors based on sol–gel films." Talanta **72**: 13-27.

Karakaş, C. (2009). The Development of Alkaline Phosphatase based paper bioreporter for evaluation of Milk Pasteurization. Biotechnology. Ankara, Middle East Technical University. **Master of Science:** 148.

Kenar, L. (2010). "The Use of Biosensors for the Detection of Chemical and Biological Weapons." Turkish Journal of Biochemistry **35**(1): 72-74.

Kossen, K., N. K. Vaish, et al. (2004). "High-Throughput Ribozyme-Based Assays for Detection of Viral Nucleic Acids." Chemsitry and Biology **11**(6): 807-815.

Koubová, V., E. Brynda, et al. (2001). "Detection of foodborne pathogens using surface plasmon resonance biosensors." Sensors and Actuators B: Chemical **74**(1-3): 100-105.

Kök, F. N. and V. Hasırcı (2004). "Determination of binary pesticide mixtures by an acetylcholinesterase–choline oxidase biosensor." Biosensors and Bioelectronics **19**: 661-665.

Kröger, S., S. J. Setford, et al. (1998). "Immunosensor for 2,4-dichlorophenoxyacetic acid in aqueous/organic solvent soil extracts." Analytical Chemistry **70**(23): 5047–5053.

Larson, S. J., P. D. Capel, et al. (1998). Pesticides in Surface Waters Distribution, Trends, and Governing Factors, CRC Press, LLC.

Leatherbarrow, R. J. and P. R. Edwards (1999). "Analysis of molecular recognition using optical biosensors." Current Opinion in Chemical Biology **3**.

Lee, Y. H. and R. Mutharasan (2005). Biosensors. Sensor Technology Handbook. J. S. Wilson, Newnes Inc.**:** 161-180.

Li, Y. and X.-L. Su (2006). "Microfluidics-Based Optical Biosensing Method for Rapid Detection of Esherichia Coli O157:H7." Journal of Rapid Methods and Automation in Microbiology **14**(1): 96-109.

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

Lowe, C. R. (2000). "Chemoselective biosensors." Current Opinion in Chemical Biology **3**(1): 106-111.

Luong, J. H. T., K. B. Male, et al. (2008). "Biosensor technology: Technology push versus market pull." Biotechnology Advances **26**(5): 492-500.

Martinez, R. C., E. R. Gonzalo, et al. (1993). "Automated high-performance liquid chromatographic method for the determination of organophosphorus pesticides in waters with dual electrochemical (reductive—oxidative) detection." Journal of Chromatography A **644**(1): 49-58.

Martinez, R. C., E. R. Gonzalo, et al. (1992). "Sensitive method for the determination of organophosphorus pesticides in fruits and surface waters by high-performance liquid chromatography with ultraviolet detection " Journal of Chromatography A **697**(1): 37-45.

Mayes, A. G. (2002). Immobilisation chemistry of biological recognition molecules. Biomolecular Sensors. E. Gizeli and G. R. Lowe, Taylor & Francis**:** 49-86.

Mulchandani, A., P. Mulchandani, et al. (1998). "Enzyme Biosensor for Determination of Organophosphates." Field Analytical Chemistry and Technology **2**(6): 363-369.

Nagatani, N., A. Takeuchi, et al. (2007). "Rapid and sensitive visual detection of residual pesticides in food using acetylcholinesterase-based disposable membrane chips." Food Control **18**: 914-920.

Newman, J. D. and A. P. F. Turner (2007). Historical Perspective of Biosensor and Biochip Development. Handbook of Biosensors and Biochips. R. S. Marks, C. R. Lowe, D. C. Cullen, H. H. Weetall and I. Karube, John Wiley & Sons, Inc. **1:** 1-16.

No, H.-Y., Y. A. Kim, et al. (2007). "Cholinesterase-based dipstick assay for the detection of organophosphate and carbamate pesticides." Analytical Chiica Acta **594**: 37-43.

Ogawa, A. and M. Maeda (2008). "An Artificial Aptazyme-Based Riboswitch and its Cascading System in E. coli." ChemBioChem **9**: 206-209.

Pathirana, S. T., J. Barbaree, et al. (2000). "Rapid and sensitive biosensor for Salmonella." Biosensors and Bioelectronics **15**(3-4): 135-141.

Plimmer, J. R. (2001). Chemistry of Pesticides. Handbook of Pesticide Toxicology. R. Krieger, Academic Press**:** 94-107.

Pohanka, M., J. Z. Karasova, et al. (2010). "Colorimetric dipstick for assay of organophosphate pesticides and nerve agents represented by paraoxon, sarin and VX." Talanta **81**: 621-624.

Rodriguez-Mozaz, S., M. J. L. d. Alda, et al. (2006). "Biosensors as useful tools for environmental analysis and monitoring." Analytical and Bioanalytical Chemistry **386**: 1025-1041.

Rodriguez-Mozaz, S., M. J. L. d. Alda, et al. (2005). "Biosensors for environmental monitoring A global perspective." Talanta **65**(65): 291-297.

Rodriguez-Mozaz, S., S. Reder, et al. (2004). "Simultaneous multi-analyte determination of estrone, isoproturon and atrazine in natural waters by the RIver ANAlyser (RIANA), an optical immunosensor." Biosensors and Bioelectronics **19**(7): 633-640.

Rogers, K. R. and C. L. Gerlach (1996). "Environmental Biosensors: A Status Report." Environmental Science and TEchnology **30**: 486A-491A.

Sadana, A. (2006). Binding and Dissociation Kinetics for Different Biosensor Applications Using Fractals, Elsevier B.V.

Schulze, H., E. Scherbaum, et al. (2002). "Development, validation, and application of an acetylcholinesterase-biosensor test for the direct detection of insecticide residues in infant food." Biosensors and Bioelectronics **17**: 1095-1105.

Sharma, S. K., N. Sehgal, et al. (2003). "Biomolecules for development of biosensors and their applications." Current Applied Physics **3**: 307-316.

Sigma-Aldrich, Inc, (2002). "5,5'-DITHIO-BIS(2-NITROBENZOIC ACID) - Product Information." Retrieved 15.07.2010, 2010, from http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/d8130pis.Par.0001.File.tmp/d8130pis.pdf.

Sigma-Aldrich, Inc, (2006). "Acetylcholinesterase From Electrophorus electricus (electric eel) - Product Information." Retrieved 10.05.2010, 2010, from http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Datasheet/3/c2888dat.Par.0001.File.tmp/c2888dat.pdf.

Stefan, R.-I., R. G. Bokretsion, et al. (2003). "Simultaneous determination of creatine and creatinine using amperometric biosensors." Talanta **60**: 1223-1228.

Şenyurt, Ö. (2008). Development of Paper Type Tyrosinase Biosensor. Biotechnology. Ankara, Middle East Technical University. **Master of Science:** 58.

Trojanowicz, M. (2002). "Determination of Pesticides Using Electrochemical Enzymatic Biosensors." Electroanalysis **14**(19-20): 1311-1328.

Tümtürk, H., F. Şahin, et al. (2007). "A new method for immobilization of acetylcholinesterase." Bioprocess and Biosystem Engineering **30**: 141-145.

U.S. Environmental Protection Agency (2010, 04.08.2010). "What is a Pesticide?". Retrieved 10.08.2010, 2010, from http://www.epa.gov/opp00001/about/index.htm.

Uldrich, J. and D. Newberry (2005). The Next Big Thing Is Really Small: How Nanotechnology Will Change the Future of Your Business, Crown Business.

Villatte, F., T. T. Bachman, et al. (2001). "Acetylcholinesterase Assay for Rapid Expression Screening in Liquid and Solid Media." BioTechniques **30**(1): 81-86.

Wakamatsu, J., J. Okui, et al. (2004). "Establishment of a model experiment system to elucidate the mechanism by which Zn–protoporphyrin IX is formed in nitrite-free dry-cured ham." Meat Science **62**(2): 313-317.

Wang, J. (2000). "From DNA biosensors to gene chips." Nucleic Acids Research **28**(16): 3011-3016.

Xavier, M. P., B. C. Moreno-Bondi, et al. (2000). "Fiber optic monitoring of carbamate pesticides using porous glass with covalently bound chlorphenol red." Biosensors & Bioelectronics **14**(12): 895-905.

Zhou, Y., S. Wang, et al. (2010). "Catalytic Reaction Mechanism of Acetylcholinesterase Determined by Born−Oppenheimer Ab Initio QM/MM Molecular Dynamics Simulations." TPhysical Chemistry B **114**(26): 8817-8825.

APPENDIX A

REACTION MECHANISMS

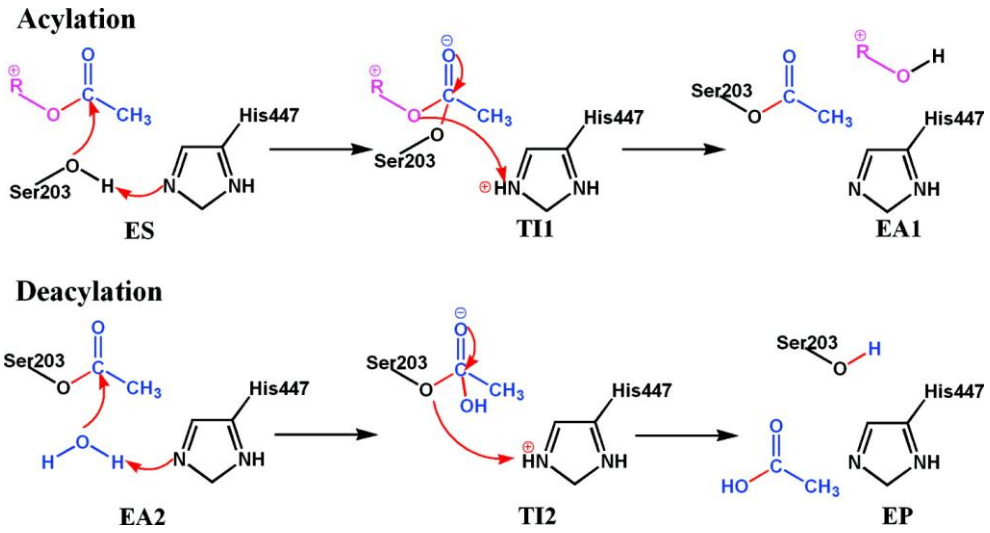

Figure A.1. Mechanism of acetylcholinesterase to substrates (Zhou, Wang et al. 2010)

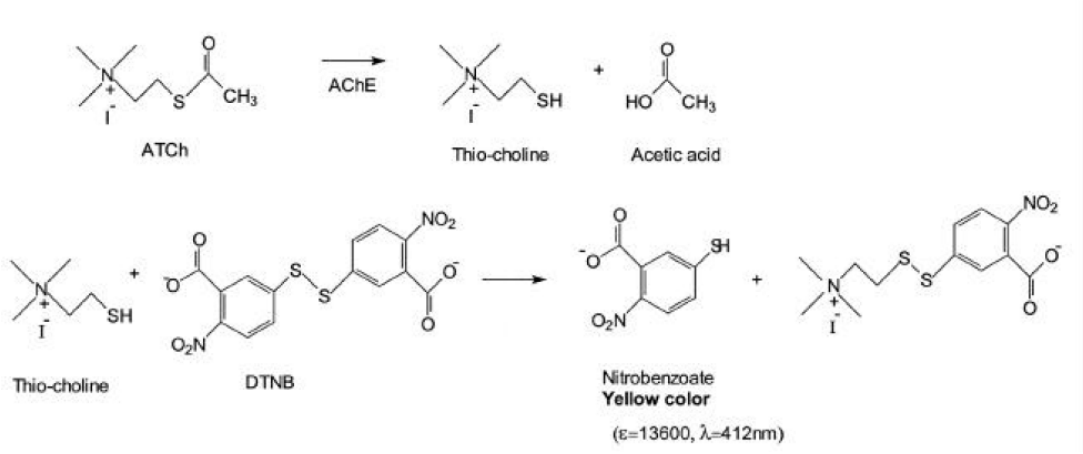

Figure A.2. Hydroxylation reaction of acetylthiocholine by acetylcholinesterase in the presence of DTNB, reacting with free thiol groups and giving yellow color. (Villatte, Bachman et al. 2001)

APPENDIX B

TABULATED VALUES OF GRAPHS

Table B.1. Mean values, SEM and significant values for Figure 3.2 (Effect of enzyme concentration on biosensor response.)

Enzyme concentration	**Color Units**	
	4µg/mL ATChI	Control (no ATChI)
Control	4,69 ± 0,57	4,02 ± 0,28
3U/mL	15,66 ± 7,51	3,59 ± 0,75
6 U/mL	24,63 ± 3,64*	3,02 ± 0,29
9 U/mL	25,23 ± 2,50*	3,39 ± 0,74
12 U/mL	30,46 ± 4,07*	4,03 ± 0,58
15 U/mL	37,05 ± 2,97*	4,69 ± 0,57

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

DTNB concentration	**Color Units**	
	4µg/mL ATChI	Control (no ATChI)
Control	13,71 ± 6,74	9,04 ± 1,55
1µg/mL	17,94 ± 5,30	6,72 ± 0,64
2µg/mL	20,50 ± 4,89	7,45 ± 0,88
3µg/mL	47,93 ± 10,50*	8,73 ± 2,71
4µg/mL	57,50 ± 8,01*	6,80 ± 1,16
5µg/mL	66,40 ± 6,16*	10,64 ± 1,52

Table B.3. Mean values, SEM and significant values for Figure 3.6 (Effect of ATChI concentration on biosensor response.)

ATChI concentration	Color Units	
	12U/mL AChE	Control (no AChE)
Control	16,78 ± 1,23	3,02 ± 0,29
1µg/mL	44,49 ± 6,01*	3,59 ± 0,75
2µg/mL	51,04 ± 3,86*	3,02 ± 0,29
3µg/mL	57,43 ± 4,89*	3,39 ± 0,58
4µg/mL	62,57 ± 3,23*	4,03 ± 0,58
5µg/mL	55,20 ± 3,79*	4,69 ± 0,57

Table B.4. Mean values, SEM and significant values for Figure 3.8 (Effect of pH on biosensor response.)

pH	Color Units	
	12U/mL AChE	Control (no AChE)
Control	67,89 ± 5,93*	17,96 ± 0,99
1	17,64 ± 0,67*	17,42 ± 1,33
2	32,99 ± 3,50*	13,70 ± 1,27
3	36,81 ± 4,46	17,11 ± 0,84
4	45,74 ± 9,04	16,10 ± 1,40
5	49,04 ± 7,89	18,44 ± 1,44
6	53,51 ± 4,82	16,47 ± 0,26
7	59,53 ± 6,31	14,99 ± 1,74
8	65,75 ± 9,08	16,82 ± 0,80
9	45,07 ± 7,49*	15,43 ± 1,53
10	63,20 ± 4,61	27,33 ± 1,62**
11	104,66 ± 7,00*	89,59 ± 8,11**
12	208,43 ± 1,22*	204,11 ± 5,26**
13	224,73 ± 2,79*	219,11 ± 3,67**

Table B.5. Mean values, SEM and significant values for Figure 3.10 (Effect of temperature on biosensor response.)

Temperature	Color Units	
	12U/mL AChE	Control (no AChE)
4°C	27,28 ± 4,40	19,49 ± 1,11
RT (~25°C)	89,56 ± 4,96*	15,74 ± 0,73
37°C	120,30 ± 12,20*	17,31 ± 0,44

Table B.6. Mean values, SEM and significant values for Figure 3.11 (Effect of BSA concentration on biosensor response.)

BSA concentration	Color Units	
	12U/mL AChE	Control (no AChE)
Control	25,10 ± 0,67	18,71 ± 0,90
1% (w/v)	106,68 ± 0,67*	21,94 ± 0,74
2% (w/v)	113,78 ± 6,41*	25,05 ± 2,67
3% (w/v)	120,94 ± 6,63*	28,79 ± 1,15**
4% (w/v)	132,95 ± 5,75*	34,76 ± 0,37**
5% (w/v)	136,87 ± 9,05*	40,96 ± 0,03**

Table B.7. Mean values, SEM and significant values for Figure 3.12 (Effect of glucose concentration on biosensor response.)

Glucose concentration	Color Units	
	12U/mL AChE	Control (no AChE)
Control	34,00 ± 3,94	16,73 ± 1,05
5% (w/v)	65,60 ± 11,3*	16,78 ± 0,65
10% (w/v)	108,82 ± 5,11*	17,55 ± 0,25
15% (w/v)	139,40 ± 11,6*	20,18 ± 3,66

Table B.8. Mean values, SEM and significant values for Figure 3.13 (Effect of trehalose concentration on biosensor response.)

Trehalose concentration	**Color Units**	
	12U/mL AChE	Control (no AChE)
Control	29,97 ± 2,65	18,07 ± 0,43
2% (w/v)	43,41 ± 8,78	19,85 ± 0,94
4% (w/v)	50,93 ± 6,42*	17,94 ± 0,70
6% (w/v)	63,81 ± 3,14*	16,51 ± 0,88
8% (w/v)	69,74 ± 3,73*	19,51 ± 0,20

Table B.9. Mean values, SEM and significant values for Figure 3.14 (Effect of NaAZ concentration on biosensor response.)

NaAZ concentration	**Color Units**	
	12U/mL AChE	Control (no AChE)
Control	37,90 ± 4,40	16,28 ± 0,87
0,02% (w/v)	48,90 ± 5,39	14,71 ± 0,78

Table B.10. Mean values, SEM and significant values for Figure 3.15 (Detection of malathion.)

Malathion concentration	**Color Units**
Control	53,40 ± 14,6
1 ppm	19,46 ± 1,01*
2,5 ppm	7,99 ± 1,84*
5 ppm	8,81 ± 0,82*
10 ppm	7,93 ± 0,52*
25 ppm	8,19 ± 0,70*
50 ppm	6,40 ± 0,21*
100 ppm	7,84 ± 0,91*
200 ppm	9,10 ± 0,48*

Table B.11. Mean values, SEM and significant values for Figure 3.16 (Stability of biosensor at room temperature.)

Time	Color Units	
	15% (w/v) Glucose	Control (no Glucose)
Control	79,60 ± 11,90	73,80 ± 21,80
1 Day	33,83 ± 2,71*	5,66 ± 0,19
7 Days	37,94 ± 2,91*	3,71 ± 0,33
15 Days	37,96 ± 2,89*	7,68 ± 0,34
30 Days	26,42 ± 1,09*	2,29 ± 0,27
45 Days	17,66 ± 2,35*	2,98 ± 0,29
60 Days	15,46 ± 3,53*	3,71 ± 0,23

Table B.12. Mean values, SEM and significant values for Figure 3.17 (Stability of biosensor at 4°C.)

Time	Color Units	
	15% (w/v) Glucose	Control (no Glucose)
Control	79,60 ± 11,90	73,80 ± 21,80
1 Day	60,09 ± 0,87*	7,36 ± 0,93
7 Days	51,10 ± 10,80*	7,03 ± 0,35
15 Days	53,05 ± 2,32*	8,52 ± 0,17
30 Days	37,05 ± 6,08*	2,94 ± 0,09
45 Days	38,88 ± 4,80*	4,28 ± 1,37
60 Days	36,08 ± 5,10*	3,86 ± 0,10

Table B.13. Mean values, SEM and significant values for Figure 3.18 (Shelf-life of biosensor at room temperature.)

Time	Color Units	
	2,5 ppm malathion	Control (no malathion)
Control	8,99 ± 1,60*	79,60 ± 11,90
1 Day	10,21 ± 2,01*	33,83 ± 2,71
7 Days	9,78 ± 1,80*	37,94 ± 2,91
15 Days	6,95 ± 1,02*	37,96 ± 2,89
30 Days	8,54 ± 0,53*	26,42 ± 1,09
45 Days	6,44 ± 0,26*	17,66 ± 2,35
60 Days	4,22 ± 0,25*	15,46 ± 3,53

Table B.14. Mean values, SEM and significant values for Figure 3.19 (Shelf-life of biosensor at 4°C.)

Time	Color Units	
	2,5 ppm malathion	Control (no malathion)
Control	10,80 ± 1,65*	79,60 ± 11,90
1 Day	60,09 ± 0,99*	60,09 ± 0,87
7 Days	51,10 ± 0,47*	51,10 ± 10,80
15 Days	53,05 ± 1,01*	53,05 ± 2,32
30 Days	37,05 ± 0,87*	37,05 ± 6,08
45 Days	38,88 ± 0,43*	38,88 ± 4,80
60 Days	36,08 ± 0,17*	36,08 ± 5,10