ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CONSTRUCTION OF OXYGEN DETECTION BASED LACCASE BIOSENSORS

M.Sc. THESIS

Kadir BİLİR

Department of Advance Technologies

Molecular Biology-Genetics and Biotechnology Programme

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ABBREVIATIONS

ABTS	**:** 2, 2-azinobis-(3-ethylbenzthiazoline-6-sulfonate)
AMC	**:** Amino modified carboxycellulose
APST	**:** 3-Aminopropylsilantriol
BSA	**:** Bovine serum albumin
CMC	**:** Carboxycellulose (CMC)
DMF	**:** Dimethylformamide
DMSO	**:** Dimethyl sulfoxide
EDC	**:** Carbodiimide
EDD	**:** Ethylenediamine-dihydrochloride
FIBOX 3	**:** Fiber-optic oxygen meter
FRET	**:** Fluorescence resonance energy transfer
FTC-PSt3	**:** Flow-Through Cell with Integrated Planar Oxygen Sensor
GA	**:** Glutaraldehyde
HPLC	**:** High performance liquid chromatography
MOBGAM	**:** Molecular biology and genetics research center
OXY-4	**:** 4-Channel Fiber-Optic Oxygen Meter
PBS	**:** Phosphate buffer saline
pI	**:** Isoelectric point
PTFE	**:** Polytetrafluoroethylene
PVA	**:** Polyvinyl alcohol, Mowiol
SDR	**:** SensorDish® Reader
SP-PSt3	**:** Planar Oxygen-Sensitive Foils
TEOS	**:** Tetraethyl orthosilicate
TMOS	**:** Tetramethyl orthosilicate
Tri-MOS	**:** Trimethoxymethtlsilane

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CONSTRUCTION OF OXYGEN DETECTION BASED LACCASE BIOSENSORS

SUMMARY

Industrial developments have led to environmental pollution by many varieties of industrial by-products and phenolic compounds are one of the most important members of these by-products which are being released to the environment as a result of uncontrolled discharge or leakage of wastewaters. Rapid identification and quantification of phenolic compounds, is very important for efficient environmental protection and control. Traditional chromatographic methods used for determination of phenolic compounds are usually time consuming and expensive. Moreover, these methods do not allow on-site detection and need trained personnel. To remove these disadvantages, alternative ways, mainly enzyme biosensors, have been investigated in the determination of phenolic compounds.

The aim of this study is to construct a biosensor from laccase enzyme, which was isolated from *Trametes versicolor* or *Pleurotus ostreatus* (mushroom), as a portable, cheap, easy-to-use alternative to known traditional methods for detecting toxic phenolic compounds. For this purpose, two different biosensors were constructed. The first one was based on a classical oxygen electrode unit (oxygraph) as a transducer and constructed according to a conventional gelatin entrapment method. Laccase from *Trametes versicolor* was entrapped in gelatin by glutaraldehyde (GA) crosslinking and immobilized on PTFE membranes. Optimum cross linker concentration, temperature and pH values for biosensor were determined using catechol and chlorophenol as substrate. Optimum glutaraldehyde concentration was found as 2.5% and optimum temperature and pH was established at 35 °C and 5, respectively for both catechol and chlorophenol. Another finding was that 10-20 consecutive measurements under optimum conditions could be done with the biosensor. It was observed that catechol was a better substrate than chlorophenol for laccase biosensor because higher laccase activity was determined in a shorter reaction time. Therefore, only catechol was used as a model substrate while working with the second biosensor.

Second biosensor was based on a fiber optic system and was completely different from previous biosensor in terms of immobilization method and chemicals used. In this biosensor, laccase from *Pleurotus ostreatus* was immobilized on fiber optic oxygen sensor spots with a simple method that uses 3-aminopropylsilantriol (APST) for surface activation; GA as cross-linker and amino modified carboxycellulose (AMC) to form a mechanically stable matrix. Thereafter, biosensor's optimum construction and working conditions (enzyme amount, diffusion layer quantity, pH value) and performance factors (reproducibility, response time, measurement period, repeatability of biosensor response, dynamic working range, storage stability and applicability to different systems such as flow through systems or sensor dishes)

were investigated while catechol was used as a substrate. Moreover, phenolic compound concentration in real samples (apple juice and different sort of tea) was investigated.

For the optical biosensor, optimum enzyme amount, diffusion layer number and pH value was determined as 1.5 mg, 1 and 6.9, respectively. Dynamic working range of biosensor was 0.04-0.6 mM (for catechol), response time was within seconds and measurement period was ca. 13 min. In addition, storage stability of the sensors was at least 85 days and reproducibility of sensors was very high. Our sensors were applicable to flow through systems and sensor dishes. Finally, sensors were shown to be effectively used for phenolic compound detection of real samples like fruit juices and tea.

OKSİJEN DETEKSİYONU TEMELLİ LAKKAZ BİYOSENSÖRLERİ YAPIMI

ÖZET

Sanayinin son yıllarda hızlı bir şekilde gelişmesi, çeşitli sanayi yan ürünlerinden kaynaklanan çevre kirliliğine neden olmaktadır ve bunların en önemlilerinden biri olan fenolik bileşikler atıksuların kontrolsüz deşarjı ve sızıntılar sonucunda çevreye salınmaktadır. Fenol bileşiklerin hızlı biçimde tespit edilebilmesi ve miktarının belirlenmesi, çevrenin korunması ve kontrol edilebilmesi için büyük önem taşır. Fenolik bileşiklerin tayininde kullanılan geleneksel tayin teknikleri genellikle çok zaman alır ve işlem süreci pahalıdır. Ayrıca bu yöntemler ile bileşiğin tespiti yerinde yapılamamakta ve analizi yapacak olan kişinin eğitim almış olması gerekmektedir. Bu dezavantajları kaldırmak için fenolik bileşiklerin tespitinde alternatif yollar araştırılmaktadır. Bu alternatif yolların en önemlisini de enzim biyosensörlerdir.

Bu çalışmanın amacı toksik fenolik bileşiklerin tespit edilmesinde geleneksel yollara alternatif, *Trametes versicolor* veya *Pleurotus ostreatus* (mantar)'dan izole edilmiş lakkaz enzimini kullanarak ucuz, taşınabilir ve kullanımı kolay bir biyosensör inşa etmektir. Bu amaç doğrultusunda iki farklı biyosensor düzenlendi. İlk biyosensor çevirici olarak klasik bir oksijen elektrod ünitesi (oxygraph) kullanılarak ve klasik jelatin hapsetme metoduna göre inşa edildi. Bunun için, *Trametes versicolor*'dan elde edilen lakkaz, sıcak su banyosu içerisinde eritilmiş olan jelatin içerisine koyuldu ve bu karışım PTFE yüzeyine aktarıldı. Bu sayede enzimin jelatin yapı içerisinde hapsedilmesi sağlandı. Hapsetme işleminden sonra yapının donması beklendi ve ardından üzerinde enzim ve jelatin bulunan PTFE örnekleri içerisinde gluteraldehit (GA) bulunan tüplerde tutuldu. Böylelikle jelatinin PTFE yüzeyine çapraz bağlanması yani immobilizasyonu sağlandı. Ardından, PTFE yapıları ölçümler yapılıncaya kadar $^{o}4$ C'de bekletildi. Ölçümler sırasında katekol ve klorofenol substratları kullanılarak biyosensörün çalışabildiği optimum çapraz bağlayıcı derişimi, sıcaklık ve pH değerleri belirlendi. Optimum gluteraldehit yüzdesini bulmak için bazı biyosensör örnekleri PTFE yüzeyine tutturulduktan sonra 2.5% gluteraldehit ile reaksiyona sokulurken; bazıları da 5% gluteraldehit ile reaksiyona sokuldu. Optimum gluteraldehit yüzdesi tespitinden sonra biyosensörün çalıştığı en iyi pH değeri belirlendi. Bu aşamada 2.5% gluteraldehit kullanılarak immobilize edilmiş örnekler kullanıldı. Daha sonra tek bir örneğin sırasıyla 4.0-4.5-5.0-5.5-6.0-6.5-7.0 pH değerlerine sahip tampon çözeltilerindeki aktiviteleri hesaplandı. Optimum gluteraldehit yüzdesi ve pH'nın belirlenmesinden sonra biyosensörün çalıştığı en iyi sıcaklık değeri belirlendi. Deneyin bu aşamasında ilk olarak sensörler 2.5% gluteraldehit kullanılarak immobilize edildi. Daha sonra tek bir örneğin sırasıyla 25-30-35-40-45-50 ^{o}C sıcaklığa sahip tampon çözeltisindeki aktivitesi belirlendi. Elde edilen aktivite değerlerinin karşılaştırılması sonucunda biyosensörün katekol ve klorofenol substratlarına karşı çalıştığı en iyi sıcaklık ve pH değeri

belirlendi. Yapılan deneyler sonucunda jelatin içerisine tutuklanmış lakkaz yapısından oluşan biyosensörün çalışabildiği optimum gluteraldehit yüzdesi 2.5% olarak bulundu. Bu sonuca elde edilen aktivite değerleri, biyosensörün tepki süresi ve jelatinin PTFE membranından ayrılma süresi incelenerek ulaşıldı. Deneyler sonucunda elde edilen diğer bir veri ise; 2.5% gluteraldehit solüsyonunda hazırlanmış lakkaz biyosensörünün katekol ve klorofenol substratları için aynı sonuçları vermiş olmasıdır. Her iki substratta da biyosensörün çalışabildiği optimum sıcaklık derecesi 35 ^{o}C ve optimum pH değeri 5 olarak tespit edildi. Deneyin bir diğer sonucu ise, biyosensör ile optimum koşullar altında üst üste 10- 20 ölçümün yapılabilmesidir. Ayrıca, daha yüksek aktivite değerleri tespit edildiğinden ve reaksiyonun daha kısa sürede gerçekleşmesinden dolayı lakkaz biyosensörünün karakterizasyonu için katekolün klorofenole göre daha uygun substrat olduğu gözlemlenmiştir. Bu nedenle fiber optik biyosensör ile çalışılırken substrat olarak sadece katekol kullanılmıştır.

İkinci biyosensör fiberoptik bir sistemdir ve ilk biyosensörden immobilizasyon yöntemi ve kullanılan kimyasallar bakımından farklıdır. Fiber optik biyosensörde, *Pleurotus ostreatus*'dan elde edilen lakkaz enzimi kullanıldı ve bu enzim, içerisinde florofor bulunan fiberoptik oksijen sensorleri üzerine kovalent bağlanma yöntemiyle immobilize edildi. İmmobilizasyon sırasında 3-aminopropilsilantriol (APST) yüzey aktifleyici, gluteraldehit (GA) çapraz bağlayıcı ve amino modifiye edilmiş karboksisellüloz (AMC) mekanik sabit matriks oluşturucu olarak kullanıldı. Enzimin immobilizasyonundan sonra, enzim tabakasının üzerine koruyucu görev sağlaması için difüzyon tabakası ilave edildi. Bu tabaka sayesinde enzimin dış etkenlerden korunması, enzime düzenli bir şekilde substrat ulaşması ve reaksiyon sonucu oluşan ürünlerin düzgün biçimde ortamdan ayrılabilmesi sağlandı. Difüzyon tabakasının esnek olması ve enzim tabakasıyla bağ yapabilmesi için TMOS ve Tri-MOS kimyasalları kullanıldı. Oluşturulan biyosensörler ölçümler yapılıncaya kadar ^{o}4 C'de bekletildi. Ölçümler sırasında ilk olarak biyosensörün optimum hazırlanma ve çalışma koşulları (enzim miktarı, difüzyon tabakası sayısı, pH) incelendi. Optimum enzim miktarının tespiti için üç farklı enzim miktarı içeren üç farklı sensör hazırlandı. Faz açısı değerlerinin ve sensörlerin tepki zamanların karşılaştırılması sonucunda optimum enzim miktarı belirlendi. Optimum enzim miktarı belirlendikten sonra optimum difüzyon tabakası sayısı araştırıldı. Bu amaç doğrultusunda bazı sensörler difüzyon tabakasısız, bazıları bir difüzyon tabakalı, bazıları da iki difüzyon tabakalı şekilde hazırlandı ve ölçümler yapıldı. Optimum hazırlanma ve çalışma koşullarının belirlenmesinde son adım olarak optimum pH değeri incelendi. Hazırlanan sensörlerin farklı pH değerine sahip tampon çözeltileri içerisinde gösterdikleri aktivite değerleri ve tepki süreleri karşılaştırılarak sensörlerin çalıştığı optimum pH değeri belirlendi. Optimum çalışma koşulları belirlendikten sonra biyosensörün performans değerleri araştırıldı. Bu doğrultuda biyosensörün tekrarlanabilirliği, tepki süresi, ölçüm süresi, dinamik aralığı, saklama stabilitesi araştırıldı. Tüm bu parametrelerin belirlenmesi sırasında kullanılan sensörlerin yapımında optimum enzim miktarı ve difüzyon tabakası sayısı kullanıldı. Ayrıca ölçümler optimum pH değerinde gerçekleştirildi. Performans değerleri belirlendikten sonra sensörlerin farklı sistemlere uygulanabilirliği araştırıldı. Bu doğrultuda ilk olarak biyosensör devirdaim yapan sistemlere uygulandı. Devirdaim yapan sistemler sayesinde biyosensörün aralıksız şekilde ölçüm yapması hedeflendi. Diğer bir uygulama alanı olarak da 24 kuyucuklu plateler kullanıldı. Bu plateler sayesinde her bir plate kuyucuğu ufak bir biyoreaktör olarak düşünüldü ve aynı anda pek çok farklı parametrenin bu sistemde incelenebilirliği araştırıldı. Karakterizasyonu ve

optimizasyonu yapılan biyosensör ile son olarak gerçek örneklerin (elma suyu ve farklı çeşitlerde çay) fenolik bileşik konsantrasyonları incelendi. Fiber opitk biyosensör ile yapılan tüm deneylerde substrat olarak katekol kullanıldı.

Yapılan deneyler sonucunda optik biyosensörün optimum enzim miktarı, difüzyon tabakası sayısı ve pH değeri sırasıyla 1.5 mg, 1 ve 6.9 olarak belirlendi. Bu değerler biyosensörün optimum çalışma koşulları olarak kaydedildi ve ardından, sensörün performans değerleri araştırıldı Bu doğrultuda biyosensörün dinamik aralığı 0.04-0.6 mM, tepki süresi birkaç saniye ve ölçüm süresi ortalama 13 dakika bulundu. Yapılan ölçümlerden sonra sistemin yenilenme süresi de ortalama 12 dakika olarak hesaplandı. Buna ilaveten, sensörlerin saklama stabilitesi en az 85 gün ve sensörlerin yeniden üretilebilirliği oldukça yüksek olarak tespit edildi. Ayrıca sensörlerin tepkilerinin tekrarlanabilirliği de oldukça yüksek şekilde gözlemlendi. Sensörlerin uygulanabilirliği ilk olarak devirdaim yapan sistemde denendi ve deney sonuçlarına göre sensörlerin devirdaim yapan sistemlere uygulanabilir olduğu gözlemlendi. Diğer bir uygulama alanı olarak da 24 kuyucuklu plate kullanıldı ve sensörlerin bu sisteme uygulanabilirliğinin düşük olduğu görüldü. Elde edilen sonuçların her defasında farklı olması ve sensörlerin kısa sürede parçalanması bu sonuca neden olmuştur. Son olarak, sensörlerin meyve suları ve çay gibi gerçek örneklerde bulunan fenolik bileşik konsantrasyonun tespitinde etkili biçimde kullanılabildiği gösterildi.

İki biyosensör sistemi de, özellikle fiber optik sistem, fenolik bileşik tespiti için ideal performans göstermiştir. Biyosensölerle yapılan deneyler sonucunda elde edilen verilere göre biyosensörlerin tekrarlanbilirlik sonuçları, tepki süreleri ve farklı konsantrasyondaki fenolik bileşikleri ayırt edebilme özellikleri biyosensörlerin fenolik bileşik tayininde kullanılabilirliğini göstermektedir. Fiber optik sistemin oksijen elektrod ünitesi ile kıyaslandığında daha kesin sonuç vermesi ve daha düşük konsantrasyondaki fenol bileşiklerini tespit edebilmesi nedeniyle daha sonraki deneylerde sadece fiber optik sistemin kullanılması düşünülmektedir. Bu doğrultuda ilk olarak fiber optik biyosensörün katekol dışındaki diğer fenolik bileşiklere olan tepkisinin incelenmesi planlanmaktadır. Böylelikle biyosensörün birden fazla fenolik bileşiğe tepkisi anlaşılabilecektir ve doğada kompleks halde bulunan fenolik bileşiklerin tayini daha kesin biçimde yapılabilecektir. Fiber optik biyosensör ile daha sonra yapılması planlanan deneylerde farklı gerçek örneklerle çalışılması düşünülmektedir. Böylelikle biyosensörün sebze ve meyvelerin içerisinde bulunan fenolik bileşiklerinin tayininde ve bu değerlerin ideal aralıkta olup olmadığının tespitinde kullanılması düşünülmektedir. Bu duruma ilaveten, çevre atık suların ve toprak kirliliği gerçekleşmiş yerlerin fenolik bileşik oranının tespitinde de kullanılması düşünülmektedir. Bu sayede çevre kirliliğine neden olan fenolik bileşiklerin kısa sürede yerinde tespiti sağlanabilecektir. Tüm bu deneylerin dışında ayrıca fiber optik sensörlerin uygulanabilirliğinin geliştirilmesi planlanmaktadır. Bunun için ilk olarak devirdaim yapan sistemin karakterizasyonu üzerine çalışılmalıdır. Bunun için devirdiam yapan sistemin dinamik aralığı, reaksiyon süresi ve saklama stabilitesi gibi performans faktörleri incelenmelidir. Öte yandan, diğer bir uygulama alanı olarak düşünülen 24 kuyucuklu platelerin ilk olarak optimizasyonu yapılmalıdır. Bu amaç doğrultusunda bu sistemlerin çalışabildiği optimum enzim miktarı, difüzyon tabakası sayısı ve pH değeri belirlenmelidir. Bu değerlerin belirlenmesinden sonra sistemin performans faktörleri araştırılacaktır.

1. INTRODUCTION

Scientists have recognized the power of incorporating biological principles and molecules into the design of analytical devices for many decades. Biosensors, a merge of sensitive recognition bio-element, physio-chemical transducer and signal processing system, play a significant role in medicine, food and processing technologies. Compactness, rapidity, accuracy, portability, high specificity and sensitivity represent some reasons why biosensors have a high potential for replacing current analytical practices [Yoo et al., 2010]. With the help of new developments in biosensor technologies, biosensors started to play important roles in the environmental control of toxic contaminants such as polyphenolic compounds. Phenols, resistant compounds to biotic and abiotic degradation, are the side products of industrial and agricultural processes and an environmental pollution so, on site determination of phenols with accurate, sensitive and rapid biosensors is a growing interest [Kulys et al., 2002].

1.1 Aim of the Study

In this study, the aim is to immobilize laccase enzyme on different matrices like oxygen sensor spots and PTFE membranes to construct an applicable, reliable and easy-to-use laccase biosensor for on-site detection of toxic phenolic compounds.

For this purpose, two different biosensors were constructed. First biosensor is a conventional gelatin entrapment system in which laccase was entrapped in gelatin and immobilized on PTFE membranes. After that, biosensor's optimum activities under different conditions (temperature, pH, glutaraldehyde concentration, etc.) were investigated and compared. The other biosensor was based on a fiber optic system. Possibility of immobilizing an enzyme on fluorescence and fiber optic oxygen sensor spots was shown with the successful acquisition of the project "Enzyme Immobilization on Commercial Optical Oxygen Sensor Membranes" [Justice et al., 2007]. Thus, laccase was immobilized on oxygen sensor spots with a simple method that uses 3-aminopropylsilantriol (APST) for surface activation, glutaraldehyde (GA)

as a cross-linker and amino modified carboxycellulose (AMC) to form a mechanical stable matrix. Thereafter, biosensor's optimum working conditions, reproducibility, stability, applicability etc. were investigated.

2. LITERATURE REVIEW AND THEORY

2.1 Phenolic Compounds

Phenols, sometimes called phenolics or carbolic acid, are organic compounds that consist a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon (figure 2.1) and has a chemical formula of C_6H_5OH. Some phenolic compounds, such as threebenzenediols, can have one or more hydroxyl groups on the aromatic ring and/or rings.

Although they have similar structure with alcohols, they are not classified as alcohols because of their chemical reaction (showing acidic feature in the water while alcohols are not). In addition, their aqueous solution gives a purple color and it helps to separate them from the alcohols [Silva, 2009].

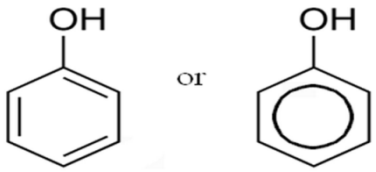

Figure 2.1 : The simplest of the phenols.

Phenolic compounds were divided into 2 groups as phenolic acids and flavonoids. Flavonoids have diphenilpropan structure (C_6-C_3-C_6) and contain 15-carbon atom (figure 2.2). They are poly phenolic antioxidants and exist in herbal tea, vegetables and fruits [Balasundram *et al.*, 2006].

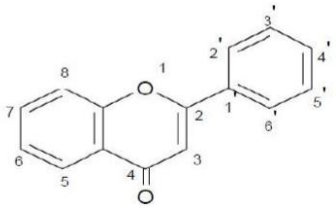

Figure 2.2 : Flavonoid structure.

On the other hand, phenolic acids have 2 different sub groups called as hydroxybenzoic acid and hydroxysinamic acid whose structure are C_6-C_1 (phenyl-methane) and C_6-C_3 (phenyl propane), respectively (figure 2.3).

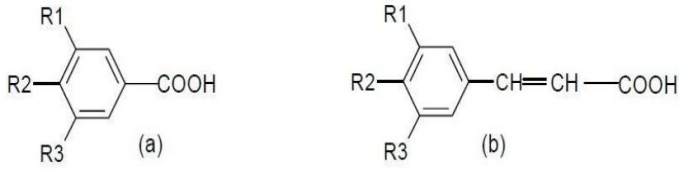

Figure 2.3 : Structure of benzoic acid (a) and sinamic acid (b) [Shahidi *et al*., 1995].

In the plant kingdom, phenolic compounds are one of the most widely distributed groups of substances as a secondary metabolism product. These compounds give the acrid taste of fruit and vegetables, which are specific to them and cause the browning. Furthermore, they can be used as a disinfectant and they are important for human health even in trace amounts as antimicrobial and anti-oxidative agents [Karakaya *et al.*, 2001].

Phenolic compounds do not have only advantages but also disadvantages like being toxic for human health and ecosystem, especially in high concentrations.

2.1.1 Toxic effects of phenolic compounds

In literature, it has been reported that some phenols have unwanted health effects. Repeated exposure to low levels of phenol may cause diarrhea and mouth sores. Ingesting high levels of phenols causes kidney problems, mouth and throat burns and in some cases death. Additionally, it effects nervous and blood system, cause skin burns, damage kidneys, liver, brain and lungs [EPA, 1999]. On the other hand, the relationship of phenols with cancer has not been established yet [Warner, 1985].

Phenols have environmental effects beside the human health. Their leakage from the industrial wastewaters to drinking or irrigation waters may cause an ecological disaster. Phenols, guaiacol, cresol and catechol are the phenolic compounds that were identified in the wastewaters. They can be originated from both industrial and agricultural sources such as metal coating, petrochemicals, olive processing plants, textile, paper industry and wood preservatives [Hanscha, 2000].

2.1.2 Detection methods of phenolic compounds

Most of phenolic compounds are toxic for living organisms. Therefore, rapid determination and degradation of them are important for public health and environmental control and protection.

Many detection methods like mass spectroscopy, gas chromatography, liquid chromatography and capillary electrophoresis was reported for phenolic compound detection [Yi *et al.*, 2001]. However, the common problem of these methods is being expensive, non-portable and requiring several operation and sample preparation steps. Moreover, they are not 100 % ecofriendly, need long time for separation and require large amounts of sample and reagents. To remove these disadvantages, many biosensors have been developed that utilize catalytic activity of redox enzymes. Laccase, tyrosinase, and peroxidase enzymes, flow systems, various electrodes and sample treatment techniques have been used in biosensor construction [Roy *et al*., 2004]. Although use of biosensors are limited due to different catalytic activity and substrate range of each enzyme, they are the most advantageous, useful and common method for phenolic compound detection [Abdullah *et al*., 2007].

2.1.3 Catechol

Catechol, which is also called as 1,2-dihidroxybenzene, is a member of phenolic compounds and its molecular formula is $C_6H_4(OH)_2$. Its chemical structure is given at figure 2.4. Catechol is rapidly soluble in the water and cause typical toxic effects as other phenolic compounds [Gaber *et al.*, 2009]. In the nature, fruit and vegetables contain trace amount of catechol. Higher concentration of catechol may spread to nature from plants and may mix with drinking water, underground water, ground and wastewater.

Half of the synthetic catechol is used for agricultural drug production and other half is used as a precursor material of chemicals (perfume, drug etc.). Moreover, catechol is used in industry for the production of nice scents and tastes [Barner, 2004]. Beside these fields, catechol can be used as an analytical reagent and paint material.

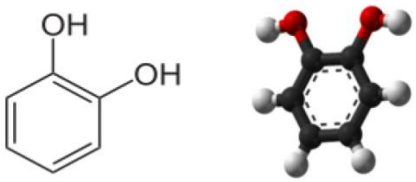

Figure 2.4 : Structure of catechol.

2.1.4 Chlorophenol

Chlorophenol, a type of organocloride phenol, is produced from phenol with electrophilic halogenation method. It has one or more chlorine atoms attached to its

aromatic ring. Its molecular formula is C_6H_5ClO and it is less irritant and bactericidal than phenols [Chang *et al.*, 1999].

Chlorophenolic compounds exist in wastewater of petrochemical, plastic, pesticide and refinery industries. They have mutagenic toxicity, so they show their effect by causing mutagenesis and damage ecological system. They can pollute underground and overground waters, if they are used in high concentration [Ha *et al.*, 2000].

Chlorophenol is widespreadly used at high concentrations in forest industry, and at low concentrations in insecticide production. Furthermore, it is used at low concentrations in agriculture, oil paint, textile, drug production and as a binding agent [Koontongkaevv *et al.*, 1988].

2.2 Laccase

Laccase, whose EC number is 1.10.3.2, is a multi-copper containing oxidase enzyme and has four copper atom core (figure 2.5). This core gives the enzyme a blue colour and assists the redox reaction. Moreover, laccase uses molecular oxygen to catalyze the monoelectronic oxidation of substrates [Roy *et al.*, 2004, Shleev *et al.*, 2004].

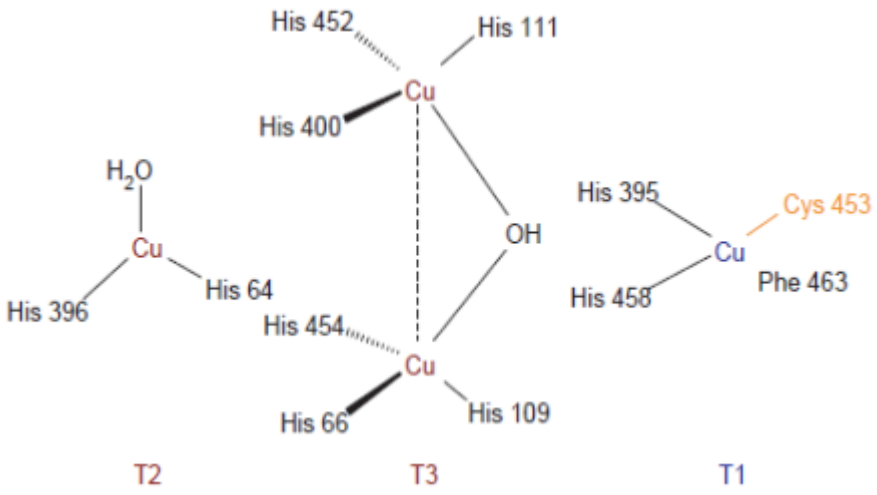

Figure 2.5 : Laccase structure.

Laccase reduces one molecule of oxygen to two molecules of water without the formation of hydrogen peroxide. In addition, four substrate molecules are oxidized to four radicals during the reaction (figure 2.6). At the end of reaction, products can form oligomers, dimers, and polymers.

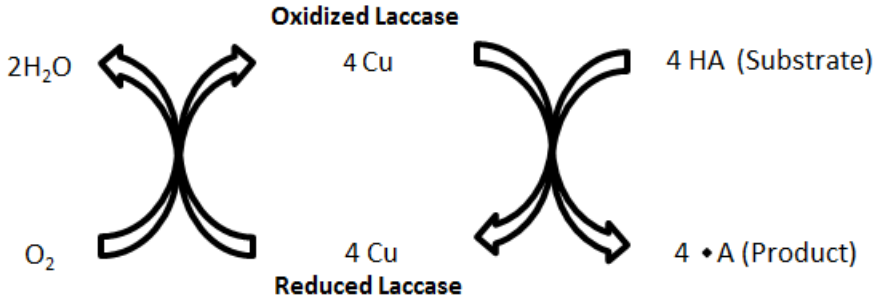

Figure 2.6 : Reaction of laccase.

Substrate specificity of laccase is wide. They can oxidize para-, meta- and ortho-diphenols, aromatic compounds such as aromatic amines, thiols and inorganic compounds like iodine [Thurston *et al*., 1994].

Laccase enzyme is found widely in plant kingdom, almost every fungi, insects and prokaryotes. They have different role in different organisms like pigmentation of fungal spores, regeneration of tobacco plant protoplast, lignification of cell wall, formation of insect's exoskeletons and synthesizing of melanin in *Azosipirillum lipoferum* bacteria [Mayer *et al*., 2002].

Laccase is used extensively for environmental protection and control. Many successful studies were reported about the enzymatic treatment of wastewater and laccase biosensors for the detection of phenolic compound [Kulys *et al*., 2002, Roy *et al*., 2004]. Furthermore, laccase is used in the food industry as a stabilizer of beverages and a cork stoppers for wine bottles. In baking, it is used to improve the properties of the dough and allow easier machine handling. Additionally, pulp and paper industry, textile, dye and painting industries use the laccase [Couto *et al*., 2006].

2.3 Biosensors

Biosensor is a compact analytical device or unit that connects a biological or biologically derived sensitive recognition element with a physiochemical transducer. It converts a biological signal into a quantifiable or processable signal and has three components: The biological recognition elements that differentiate the target molecules in the presence of various chemicals, a transducer that converts the bio-recognition event into a measurable signal and a signal processing system that converts the signal into a readable form [Yoo *et al.*, 2010]. Basically the biological

recognition elements such as enzyme, tissue, antibody, microorganism, cell or organelle reacts specifically with the analyte; then, different type of transducers, which were mentioned in section 2.3.1.2, detect the differences occurring as a result of the reaction between of biological recognition element and analyte [Vo-Dinh *et al*., 2000]. The schematic representation of biosensors is seen in figure 2.7. In 1962, Clark and Lyons constructed the first biosensor that contains immobilized glucose oxidase (biological recognition element) on to an oxygen electrode (transducer) [Clark *et al*., 1962].

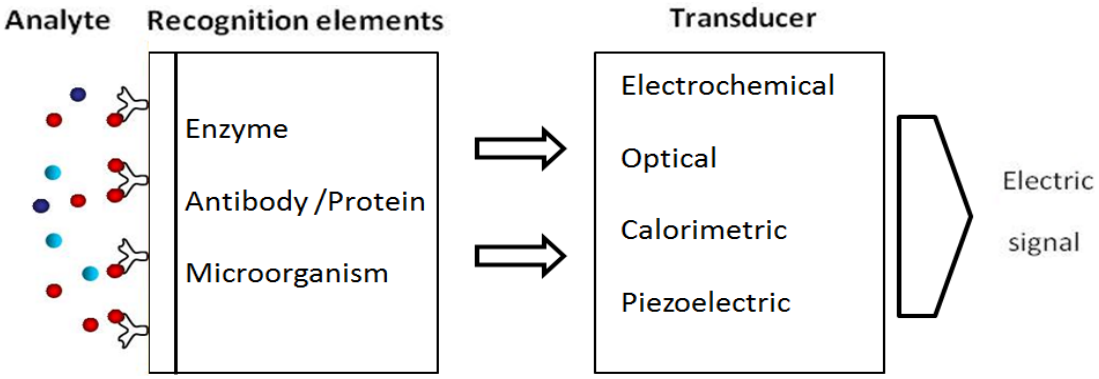

Figure 2.7 : Schematic representation of biosensor.

Biosensors are versatile tools for quantitative or qualitative analysis; however, the success of the biosensor depends on a number of properties. The bio element must be highly specific for the compound that needs to be analyzed and the signal should be reproducible. Minimal sample pre-treatment would be an advantage. If the co-enzymes and co-factors are involved, they could be co-immobilized with the bioelement [Kochana *et al*., 2008]. In addition, the results must be accurate and linear over a wide range of analyte concentrations. To be able to use the biosensor in the field, it should be small, portable and easy-to-use. When used in clinical applications the biosensor should be biocompatible and should resist inactivation or proteolysis [Grieshaber *et al*., 2008].

2.3.1 Types of biosensors

Biosensors are classified according to their bioelement or transducer.

2.3.1.1 According to bioelement

Enzymes, tissues, antibodies, microorganisms, cells and organelles are all different type of bioelements and they show different activity. According to the mode of

detection, biosensors can be classified as catalytic or affinity biosensors. Catalytic biosensors could be constructed from enzymes, tissues, microorganisms and organelles that have biocatalytic effect. These elements can react specifically with analyte, generate chemical reaction and produce a product at the end of this reaction. Transducers can detect the difference in concentration of compound and make the measurement [Kim, 2006].

Affinity biosensors make use of immuno agents, nucleic acids and receptor molecules as bioelement. These molecules have biospecificity and bind specifically to analyte instead of chemically reacting with them. After that, transducers can detect the difference and measure it [Telefoncu, 1999].

2.3.1.2 According to transducer

The activity of the biological element can be monitored with the help of oxygen consumption, hydrogen peroxide formation, pH change, differences in NADH concentration, fluorescence, conductivity, temperature or mass. For each parameter, biosensors use different type of transducer. The main transducer classes are optical, electrochemical, thermometric and piezoelectric (figure 2.8).

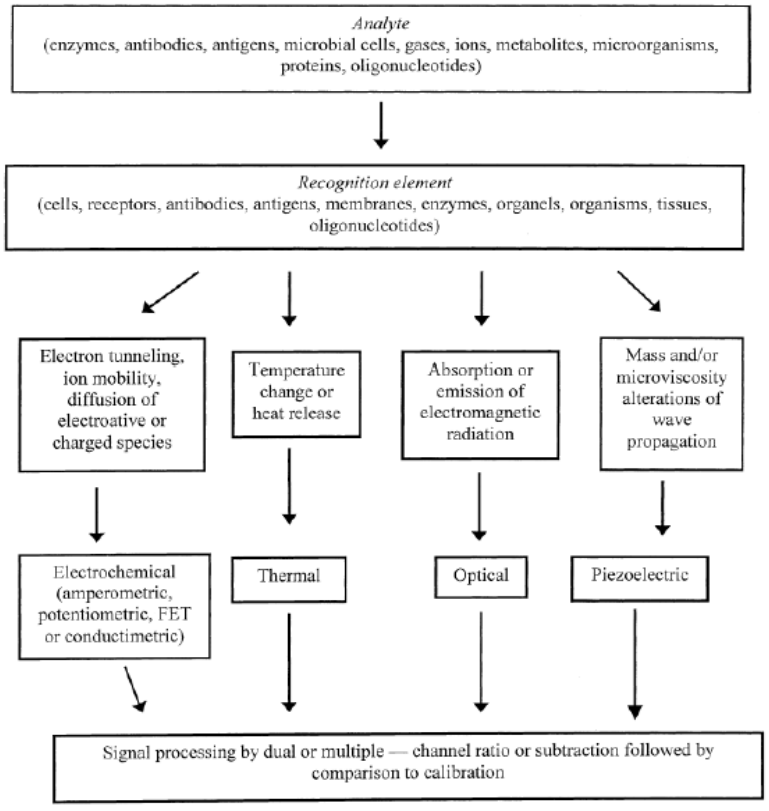

Figure 2.8 : Main types of biosensors [Mello *et al*., 2002].

2.3.1.3 Electrochemical biosensors

Electrical property of the solution usually changes because of the consumption or generation of the ions and electrons. This fact can be detected by electrochemical biosensors. According to electrical parameter, they are classified in three groups; conductiometric, amperometric and potentiometric biosensors [Sethi *et al*., 1990].

Amperometric biosensors are based on monitoring the current related with the oxidation or reduction of an electroactive species. Amperometric biosensors are more sensitive than other type electrochemical biosensors and have wider linear range. Therefore, they are the one of the most popular biosensor type. On the other hand, their activity is limited with the redox potential of the substrate.

After the chemical reaction between bioelement and analyte, ion or electron concentration of the solution changes and this affects the solution's electrical resistance or conductivity. In brief, conductiometric biosensor's measurement principal is based on the electrical resistance or conductivity of the solution. However, they have low sensitivity [Mello *et al*., 2002].

Potentiometric biosensor measures the electrochemical reaction's reduction or oxidation potential. The potential is unique for each reaction, so analyte can be easily detected [Sethi *et al*., 1990].

2.3.1.4 Optical biosensors

In optical biosensors, is the change in optical properties of the medium caused by the bioelement in the presence of the analyte is detected. With the help of transducer, this change can be measured and correlated to analyte amount. Optical detection biosensors are based on optical diffraction or electrochemiluminescence [Scheller et al., 1992].

2.3.1.5 Piezoelectric biosensors

A crystal, oscillating under constant electrical voltage, is used in piezoelectric biosensor. The mass accumulated on the crystal surface cause the change in vibration frequency. For the measurement of ammonia, nitrous oxides, carbon monoxide, hydrocarbons, hydrogen, methane and certain organophosphate compounds piezoelectric sensors are used as chemical sensors. For the construction of a biosensor, some molecules such as antibody, receptor or DNA could be attached to

the surface of the crystal. Thus, the binding of specific ligands or complementary strands can be detected [Tombelli et al., 2000].

2.3.1.6 Thermal biosensors

Thermal biosensors are constructed from combination of enzymes and heat sensors. When the analyte reacts with bioelement, change in medium temperature occurs because of the heat release or absorption and then, thermostators can quantify it. After that, the amount of heat could be related with the concentration of analyte. However, thermal biosensors have low sensitivity because during the reaction heat loss occurs because of irradiation and conduction [Buerk et al., 1993].

2.4 Factors Affecting Biosensor's Performance

2.4.1 Performance factors

Biosensor selectivity: This is one of the most important characteristic of biosensor parameters and it shows the biosensor's ability to discriminate between different substrates. Mainly it is a function of the biological component, however sometimes the transducer type contributes to selectivity [Eggins *et al*., 1996].

Response time: Biosensors usually have longer response time than chemical sensors, i.e. 30s or more. Biosensor's response time can be determined from response-time graphs.

The recovery time: Minimum time needed before a biosensor is ready to analyze the next sample.

The working lifetime and storage stability: These are usually dictated by the instability of the biological material. This factor also affects the calibration rate and reproducibility of the biosensor. The working lifetime can vary from a few days to a few months.

Calibration requirement: Ideal biosensor does not need a calibration or need few calibrations in theory. In practice, however, biosensor must be calibrated periodically.

Reproducibility: Ideally, under the same conditions, a biosensor must give almost the same results for consecutive measurements. In practice, reproducibility will not be 100 %, and a deviation of ± 5 % could be considered as good reproducibility.

Sensitivity: Its detection range usually needs to be in sub-millimolar range, however, in special cases it may go down to femtomolar (10^{-15}M) range.

Simplicity and cheapness: A simple and inexpensive design, convenient usage is important for ideal biosensors. This will allow the rapid commercialization of the biosensor.

Linear working range: Response vs. analyte concentration curve should be linear in a wide concentration range for ideal biosensors.

Size and sterilizability: These two features are especially important for implantable biosensors.

2.4.2 Buffer

Precise control of the pH of the test solution is necessary to obtain best results, so a buffer solution, commonly a phosphate buffer, is used. Optimum pH value for different biosensors varies depending on the biosensing element and immobilization method used.

2.4.3 Enzyme amount

Enzyme loading is of the parameters that can strongly influence the sensor signal. As catalyst, they are not consumed during the reaction and their amount is almost the same, so concentration of enzyme is not crucial for the operation of biosensor. However, enzyme concentration is one of the limiting factors. Michaelis-Menten equation also shows that enzyme amount is directly proportional to the reaction rate [Jusoh et al., 2012].

$$v = v_{max}\frac{[S]}{K_m+[S]} = k_{cat}[E]_0\frac{[S]}{K_m+[S]} \qquad \textbf{(2.1)}$$

Where v is reaction rate, v_{max} is maximum rate, $[S]$ is substrate concentration, K_m is Michaelis constant, k_{cat} is turnover number and $[E]_0$ is enzyme concentration.

Reaction rate changes according to enzyme concentration when a sufficient active enzyme present in the solution. However, when there is too much enzyme or the quality of the enzyme preparation is poor; excess of material can affect the rate of mass transport (diffusion) to the transducer. This problem is rarely mentioned in manuscripts.

2.4.4 Immobilization

The attachment of enzymes or bioelements to a solid support matrix (immobilization) is another important parameter to construct a biosensor. There are five main ways to achieve immobilization: covalent binding, physical adsorption, encapsulation, cross-linking and entrapment. These methods are used depending on the nature of the bioelement, transducer type, substrate and the measurement conditions [Mello *et al*., 2002].

2.4.4.1 Covalent binding

Covalent bonds are formed between bioelement (functional groups on the amino acids) and the surface for the immobilization. To achieve covalent binding the surface or the bioelement must be activated by chemical or physical treatment.

The possible loss of activity can be seen in this method due to the chemical modification of the biomolecule [Zhavnerko *et al., 2004*].

2.4.4.2 Physical adsorption

Weak Wan der Waals force, ionic and hydrogen bonds are used to bind the bioelement onto surface material in this method. This reversible immobilization type has a little effect on the structural integrity of bioelement.

Due to the interaction by weak bonds, bioelements may detach from the surface and leak with and this is the disadvantage of physical adsorption [Scheller *et al*., 1992].

2.4.4.3 Encapsulation

A membrane, allowing the diffusion of analytes, is used to envelope bioelements in encapsulation method. The porosity of the membrane, size of the analyte and chemical characteristics of both of them are the limitations. Moreover, poor design may cause the product accumulation or slow analyte diffusion. The advantage of encapsulation is the ability of co-immobilize different bioelements [Vastarella et al., 2002].

2.4.4.4 Cross linking

Cross-linking involves the formation of covalent bonds between the bioelements without any support material by physical and chemical methods. Then, bioelements

become a large and complex structure. Cross-linking is a simple end effective method. On the other hand, bioelement's catalytic site may become inoperable or leads activity loss because of the random covalent bond formation and excessive cross-linking [Vastarella, 2002].

2.4.4.5 Entrapment

This method is similar with encapsulation; however, the bioelements are confined in a matrix rather than a membrane. A covalent bonding between bioelement and polymeric matrix is not necessary in entrapment. In this case, enzyme can be entrapped into molecular network [Kovacs, 1998].

2.4.5 Diffusion

A prerequisite for any reaction is the fact that the partners must find each other. In a free space, a particle moves in a straight direction with a kinetic energy of $k_BT/2$, T is absolute temperature and k_B the Boltzmann constant. Moreover, according to Einstein's kinetic energy theory, a particle moves in a straight direction with mass m and with velocity v is $mv^2/2$. When both relationships are combined, it can be easily seen that velocity of the particle depends on the particle mass (equation 2.2).

$$v^2 = \frac{k_B T}{m} \qquad \textbf{(2.2)}$$

In the dense fluid of a cell, the moving particles are permanently obstructed and deflected from linear movement by countless molecules such as water, ions, metabolites, macromolecules and membranes. Thus, the molecule moves more like a staggering drunkard than a straight movement. Moreover, this situation increases the collision frequency and the probability of distinct molecules meeting each other.

The distance x covered by a molecule in solution within the time t in one direction depends on the diffusion coefficient D according to equation 2.3.

$$x^2=2Dt \qquad \textbf{(2.3)}$$

The diffusion coefficient is a function of the concentration of the diffusing compound that means concentration affects diffusion. It also depends on the particle size, the consistency of the fluid and the temperature. At the same time in dilute solutions, diffusion coefficient can be considered constant.

2.5 Laccase Biosensor

For detection of phenolic compounds in food and beverages, wastewater, ground and human plasma laccase biosensors are used. They have been proposed to be an alternative for HPLC and spectrophotometric methods and extensively studied by using various immobilization and co-immobilization methods [Freire *et al*., 2001]. Many laccase biosensors have been developed in the past for phenolic compound detection using gold surfaces, modified polymers and modified electrodes [Kulys et al., 2002, Jusoh *et al*., 2012, Gupta *et al*., 2003].

Laccase has low redox potential than some oxidases such as lignin peroxidase, manganese and peroxidase. Thus, laccase has the ability to oxidize compounds that are relatively easier to be oxidized. Other substrates can be too large to fit in the active site or they may have high redox potential for laccase. This obstacle could be overcome by using mediators, which are easily oxidized by laccase, as electron shuttles. When laccase oxidize a mediator that mediator diffuses in to reaction chamber and oxidize the substrate that laccase cannot react directly. These types of biosensors are called as mediated laccase biosensors [Baiocco *et al*., 2002].

Most used laccase biosensors are electrochemical type because of their good sensitivity, reproducibility, low cost and easy handling.

2.6 PTFE

Polytetrafloroetylene (PTFE) is a synthetic fluoropolymer of tetrafluoroethylene that was produced by Du Pont firm in 1938 by trade name known as TEFLONtm. It was launched in 1945 and in short time, it has started to be used widely both at industry and daily life.

PTFE's chemical structure contains only carbon and fluorine atoms and these atoms have the strongest bonds existing in organic chemistry; because of that, structure of PTFE is rigid (figure 2.9).

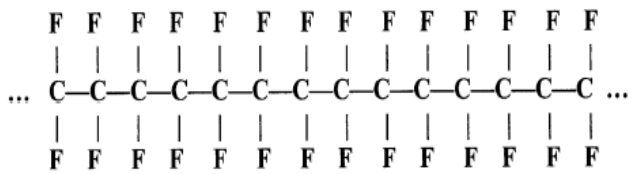

Figure 2.9 : Structure of PTFE.

PTFE has high temperature, electric and chemical resistance, low friction coefficient, high hydrophobicity, non-flammability and most importantly unreactivity. These properties give PTFE nonstick properties [Blumenthal, 1990].

2.7 Sol-gel Technology

The sol-gel process is a wet chemical technique that is used in the materials science and ceramic engineering. In this technique, the "sol" (solution) gradually evolves towards the gel-like diphasic formation containing a liquid and solid phase. The sol-gel material's chemical and physical properties can be altered with precursor composition and polymerization conditions like pH, time, temperature, molar ratio (R) between the water and precursor. The precursor sol can be used for powder synthesis and deposited on a substrate for film forming. Furthermore, it can be cast into a suitable container with the desired shape [Cajlakovic *et al*., 2002].

The sol-gel approach is a low-temperature and cheap technique, and the control of the chemical composition of product is easy. It can be used in ceramic processing or in the production of very thin films for different purposes. Moreover, sol-gel derived materials are used in optics, energy, space, electronics, biosensors, medicine and separation technology.

The observation of the tetraethyl orthosilicate (TEOS) hydrolysis under acidic conditions had led to the formation of SiO2 in fibers and monoliths in the middle of 1880s, and the interest in sol-gel processing was started. Sol-gel research has grown to be so important that in the 1990s, more than 35,000 papers were published worldwide about this process.

Optical transparency, mechanical stability and high porosity of the obtained structures make sol-gel materials suitable for optical sensor devices [Brinker et al., 1990].

2.8 Fluorescence

Photoluminescence is luminescence generation through molecule excitation by visible or ultraviolet light photons and divided into two categories as fluorescence and phosphorescence. Fluorescent molecules emit light from electronically excited states that are created by a physical (absorption of light), mechanical (friction) or

chemical mechanism. Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and after a brief interval to emit longer wavelength light.

The fluorescence process is controlled by three important events. In the first process, susceptible molecule is excited by an incoming photon and it happens in femtoseconds (10-15 seconds). Vibrational relaxation of excited state electrons to the lowest energy level is the second process and measured in picoseconds (10-12 seconds). The final process is emission of a photon with a longer wavelength photon and return of the molecule to the ground state. This process occurs in the relatively long period of nanoseconds (10-9 seconds). Eventually, the entire molecular fluorescence lifetime, from excitation to emission, is measured in only billionths of a second.

2.8.1 Fluorescence quenching

Quenching is defined as any process that decreases the fluorescence intensity of a given substance and often heavily dependent on pressure and temperature. After quenching, different reactions such as excited state reactions, energy transfer, complex formation and collisional quenching can happen. Two type of quenching, dynamic and static, require molecular contact between fluorophore and quenchers such as molecular oxygen, iodide ions and acrylamide. In both cases, the measured fluorescence intensity is changed according to the quencher concentration. A differentiation between dynamic and static quenching is not possible while the fluorescent intensity is measured. However, the measurement of fluorescent decay time is used to distinguish between the dynamic and static quenching (Blum et al., 2009].

Quenching is used in optode sensors because the quenching effect of oxygen on certain ruthenium complexes allows the measurement of oxygen saturation of solution. Moreover, it is the basis for fluorescence resonance energy transfer (FRET) assays and used for molecular imagining [Huber, 1999].

2.8.1.1 Dynamic quenching

The principle of dynamic quenching of luminescence by quencher (oxygen molecule) is seen in figure 2.10.

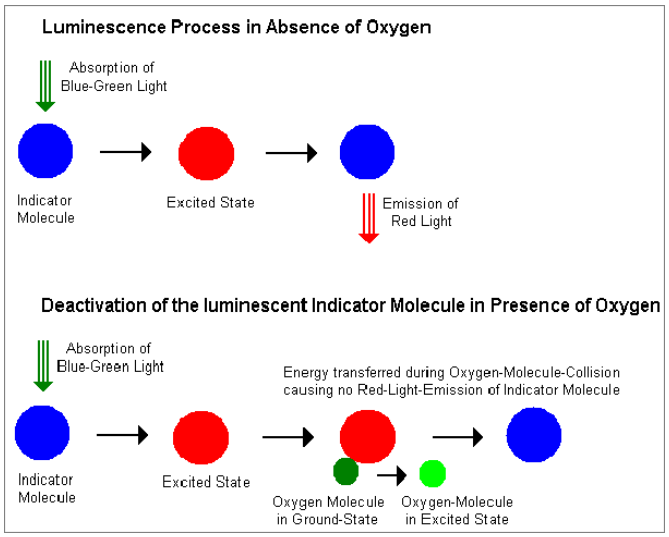

Figure 2.10 : The principle of dynamic quenching [Centec, n.d.]

The collision between the luminophore (in excited state) and the quencher (oxygen) results in radiationless deactivation, which is also called as dynamic quenching. After the collision, energy is transferred from the excited indicator molecule to quencher. Therefore, it transfers quencher from ground state (triplet state) to excited singlet state. As a result, the indicator molecule does not emit luminescence and the measurable luminescence signal decreases.

The Stern–Volmer relationship allows exploring the kinetics of a photophysical intermolecular deactivation (quenching) process. In general, this process is represented in equation 2.4. Moreover, the kinetics of this process is seen in equation 2.5.

$$A^* + Q \rightarrow A + Q^* \qquad \textbf{(2.4)}$$

$$\frac{I_f^0}{I_f} = 1 + k_q \tau_0 [Q] \qquad \textbf{(2.5)}$$

Where I_f^0 is the intensity (rate of fluorescence) without a quencher, I_f is the intensity with a quencher, k_q is the quencher rate coefficient, τ_0 is the fluorescence lifetime of A without a quencher present and [Q] is the concentration of the quencher.

2.8.1.2 Static quenching

The formation of a non-fluorescent ground state complex of the fluorophore with the quencher is described as static quenching.

The association constant K_S for the complex formation is given in equation 2.6.

$$K_s = \frac{[F-Q]}{[F][Q]} \quad \textbf{(2.6)}$$

Where [F-Q] is the concentration of the complex, [F] is the uncomplexed fluorophores concentration and [Q] is the concentration of quencher. The total concentration of fluorophores [F_0] is the sum of complexed and uncomplexed fluorophores. When the fluorophore concentrations are substituted with the fluorescence intensities, a Stern-Volmer similar equation is obtained (equation 2.7).

$$[F_0] = [F] + [F\text{-}Q] \qquad K_s = \frac{[F-Q]}{[F][Q]}$$

$$K_s = \frac{[F_0]-[F]}{[F][Q]} \qquad \frac{F_0}{F} = 1 + K_s[Q] \quad \textbf{(2.7)}$$

In brief, the system will apparently follow the Stern-Volmer quenching law; however, the quenching constant (K_S) is the equilibrium constant of complex formation. Moreover, static quenching has no effect in the fluorophore's fluorescent lifetime, because complex formation takes place in the ground state [Instruction Manual of OXY-4, 2005].

2.9 Instrumentation by PreSens Regensburg

2.9.1 FIBOX 3 and OXY-4

The sensor system provided by PreSens, which is based on 2 mm polymer optical fibers, is a single channel (FIBOX 3) or multi-channel (OXY-4) fiber optic oxygen meter for minisensors that is temperature compensating. The robust design and low power consumption makes the system useful for indoor and outdoor applications. The FIBOX 3 and OXY-4 are controlled with user-friendly software, which saves and visualizes the measured values.

In optical chemical sensors, the analyte interacts with an indicator and changes its optical properties such as the color (absorbance or spectral distribution) or luminescence properties (intensity, lifetime and polarization). In addition, in these sensors light acts as an information carrier.

A typical fiber-optical sensing system have four major components; a light source to illuminate the sensor, an optical fiber as a signal transducer, a photo detector and the

optical sensor (indicator immobilized in a solid matrix).The principle of the sensor operation is based on luminescence quenching caused by the collision of molecular oxygen and luminescent dye molecules (in excited state). Thus, sensor can detects oxygen in solution as well as in gaseous phase.

Figure 2.11 shows a typical response curve of an oxygen sensitive sensor. In the presence of oxygen, the signal (in our case the phase angle Φ) decreases.

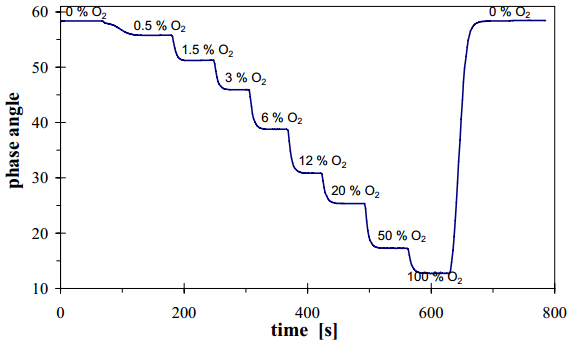

Figure 2.11 : Typical response curve of an oxygen sensitive sensor.

The technology is inert to interferences caused by pH, ammonia, carbon dioxide, ionic species like sulfide, sulfate, chloride or salinity. Turbidity and different stirring rates have also no influence on the measurement.

2.9.1.1 Planar oxygen sensitive foils (SP-PSt3)

Planar oxygen sensor SP-PSt3 is a matrix of polymers with an oxygen quenchable fluorophore and an additional overcoat of medical grade silicone. They can be easily cut into small round pieces, whose diameter is 3mm (figure 2.12). The optical isolation is recommended while working with whole blood, urine or chlorophyll containing samples. The planar sensors can be glued onto different supports such as glass or polyester and inside glass vials such as cell culture flasks, bags, and disposables. Thus, the oxygen concentration is measured non-invasively and non-destructively from outside through a transparent and non-fluorescent wall.

Figure 2.12 : Sensor spot PSt3.

2.9.1.2 Flow through cell with integrated planar oxygen sensor (FTC-PSt3)

The flow-through oxygen minisensor (FTC-PSt3) is a miniaturized fiber-optic chemical sensor that is integrated in a T-shape flow-through cell (figure 2.13). Moreover, the flow-through cell is connected to the FIBOX oxygen meter by a polymer optical fiber with 2 mm diameter as a light guide. A glass tube, which has 2 mm inner and 4 mm outer diameter, is coated with oxygen-sensitive dye at its inner wall. The capacity of FTC cell is 100 (± 10) µL. T-shape flow cell can be easily connected to external tubing via Luer-Lock adapters and sample solutions such as water, blood or fruit juices can be pumped through the cell.

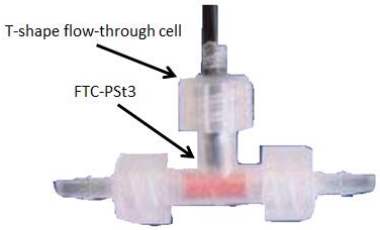

Figure 2.13 : Flow through cell with integrated planar oxygen sensor.

2.9.2 SensorDish® Reader (SDR)

The SensorDish® Reader is controlled by user-friendly software that stores and visualizes the measured data. Up to 10 SensorDish® Readers can be combined in parallel to a multi-instrument set-up (figure 2.14). The SDR is placed below the SensorDish® in the incubator and 24 channel SDR measures dissolved oxygen or pH of samples in an OxoDish® or HydroDish®, respectively. A splitter is connected to the first SDR, to the PC and to a suited power supply (100 – 240 V). Subsequent SDR are joined in series by cables.

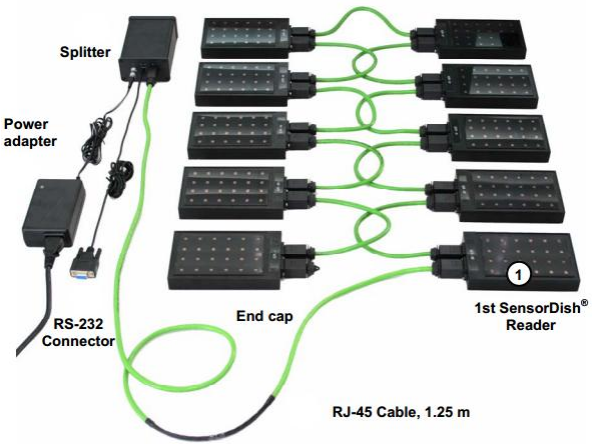

Figure 2.14 : SDR system.

2.9.2.1 SensorDish®

OxoDish® is a 24-well multidish with integrated optical-chemical sensors for oxygen (figure 2.15). It is disposable and is not stable in most of the organic solvents such as ethanol, DMF, DMSO or acetone.

Figure 2.15 : 24-wells OxoDish® with integrated sensor spots.

2.9.3 Luminescence decay time

The measurement principle of FIBOX 3, OXY-4 and SDR is based on the effect of dynamic luminescence quenching by molecular oxygen. They measure the luminescence decay time of the immobilized luminophore as the oxygen-dependent parameter by using the phase modulation technique. When the luminophore is excited with light, its decay time delays in the emitted time signal. This delay is called as the phase angle, which is shifted as a function of the oxygen concentration. Relation between decay time and phase angle is shown in the equation 3.1.

$$\tau = \frac{\tan \Phi}{2\pi . f_{mod}} \qquad \textbf{(3.1)}$$

τ is the luminescence decay time, Φ is the phase angle and f_{mod} is the modulation frequency.

The measurement of the luminescence decay time has advantages when compared to the conventional intensity measurement:

- The decay time does not depend on fluctuations in the intensity of the light source and the sensitivity of the detector,
- Signal loss because of fiber bending or intensity changes because of changes in the geometry of the sensor is not influential,

- Variations in the optical properties of the sample such as turbidity, refractive index and coloration is not influential in the results,
- Photo bleaching and bleaching of the indicator dye has no influence on the measuring signal [Instruction Manual of OXY-4, 2005].

2.10 Instrumentation by Hansatech

2.10.1 Oxygen electrode unit (oxygraph)

The oxygen electrode unit, whose working principle is based on the amperometric detection of molecular oxygen amount (nmol/ml) is seen in figure 2.16. After the detection of oxygen content, values are processed by the software and an oxygen concentration vs. time graph is constituted. The advantage of the unit is that it allows measurement in small volumes (200-2000 μL). Unit has a water jacket to keep the temperature in the reaction vessel constant.

A: Plunger screw

B: Plunger nut

C: Reaction vessel

D: Magnetic follower

E: Base plate O-ring

F: Electrode disc

G: Base ring

H: Water jacket and bottom plate

I: Water jacket connectors

J: Top plate

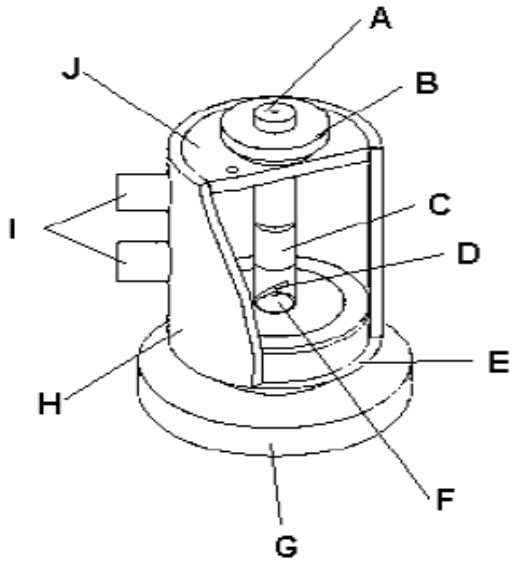

Figure 2.16 : Oxygen electrode unit [Hansatech manual].

3. MATERIAL AND METHODS

3.1 Materials and Equipment

3.1.1 Equipment

The laboratory equipment used during this study was listed in Appendix A.

3.1.2 Buffers, reagents and enzyme

Chemicals were listed in Appendix B with their suppliers. The recipes of the buffers and stock enzyme were given in Appendix C.

3.1.3 Amino-modified carboxycellulose

Ten gram of carboxycellulose (CMC) was washed for one hour with 200 ml Millipore filtered deionized water containing 5g sodium chloride. After that, CMC was vacuum filtered and a second wash was carried out with 200 ml deionized water (Millipore) for another hour. After the vacuum filtration, a last wash was done using 70 % ethanol for 1h. Then, the filtered CMC was resuspended in 200 ml deionized water.

To obtain amino-modified CMC, 4 gram of ethylenediamine-dihydrochloride (EDD) was added into CMC solution in small portions under continuous stirring. Ten minutes later, 350 mg carbodiimide (EDC) was added and the pH was set to 4.6 by the addition of 0.1M hydrochloride acid. The solution was stirred for 4h and the cellulose was filtered off.

The cellulose was then resuspended in 200 ml water, stirred for 15 minutes and vacuum filtered. This step was repeated five times. In the next step, the cellulose was successively washed with NaOH solution (100 ml, 1M) and water. Finally, the powder was washed with 100 ml ether and dried over silica gel in a desiccator at room temperature over the weekend.

3.1.4 Mowiol solution

Mowiol 4-88 (0.24 g) and glycerol (6 g) were put into a 50ml beaker and mixed well with a stir bar. After an hour, water (6 ml) was added and mixture was stirred for 3-4 hours at room temperature. Tris-HCl (tris(hydroxymethyl)aminomethane) solution (12ml, 0.2M) was then added and all mixture was stirred overnight. It is important to note that the mixture must be constantly mixed until the Mowiol 4-88 is dissolved; otherwise, the Mowiol would start to precipitate and trap the stir bar.

3.2 Methods

The methods were given in three major parts: the first one is the basic information about the enzyme stocks, the determination of the free and immobilized laccase (*Pleurotus ostreatus*) activity according to ABTS and catechol assays and calibration of both systems (Oxygraph and PreSens) used in biosensor design (Section 3.2.1 to 3.2.6). The second one was the methods used in the laccase immobilized on PTFE surface (Oxygraph, Section 3.2.7). The third one was methods used in the production of fiber optic laccase biosensors (PreSens, Section 3.2.8-3.2.10).

3.2.1 Preparation of *Trametes versicolor* laccase stocks

Laccase enzyme was dissolved in (pH 7.0, 0.1 M, 1 ml) phosphate buffer and prepared stock was aliquoted into 20 small eppendorf tubes (50 microliters each). Each tube had 50 mg/ml laccase enzyme. Eppendorfs were stored at -20 oC until use in order to minimize the enzyme denaturation that may be originated from freeze-thaw cycles.

3.2.2 Activity determination of *Pleurotus ostreatus* laccase

First of all, the absorption spectrum between 300-500 nm was measured to determine the absorption maxima of ABTS (5 mM) and catechol (50 mM) separately.

Then, the free and immobilized enzyme's (Pleurotus ostreatus) activity was determined with ABTS and catechol as substrate. The product of the reactions was measured with Perkin-Elmer Lambda UV-VIS spectrophotometer at room temperature by monitoring the absorbance increase at 414 nm (for ABTS) or 404 nm (for catechol). Sodium acetate buffer (0.1 M, pH 5), ABTS (5 mM), catechol (50

mM) and laccase (1 mg/ml) were used at activity assays. For each assay, all measurements were repeated thrice.

For the immobilized enzyme activity measurements, laccase was immobilized on the sensor spots according to experimental protocol at section 3.2.8. After that, sensor spots were fixed into cuvettes with RS 692-542 (silicone glue). Test samples contained laccase (1mg per spot) in their enzyme layer while blank samples did not.

3.2.2.1 Determination of free laccase activity (ABTS assay)

Some of the activity measurements were done with ABTS as substrate. The absorbance increase was monitored spectrophotometrically at 414 nm (light path 1 cm). The assay mixture for the test and blank cuvettes were shown below (table 3.1).

Table 3.1 : Content of the test and blank cuvettes (free laccase-ABTS assay).

	Test (ml)	**Blank (ml)**
Deionized Water	-	0.05
Buffer	2.90	2.90
Enzyme	0.05	-
Substrate	0.05	0.05

After the addition of ABTS to laccase + buffer + deionized water mixture, cuvettes were immediately put into spectrophotometer and the increase in absorbance was measured in 10 seconds intervals for 5 minutes.

3.2.2.2 Determination of free laccase activity (catechol assay)

Some of the activity measurements were done spectrophotometrically at 404 nm with catechol as substrate (light path 1 cm). The assay mixture for the test and blank cuvette were shown in Table 3.2.

Table 3.2 : Content of the test and blank cuvettes (free laccase-catechol assay).

	Test (ml)	**Blank (ml)**
Deionized Water	-	0.3
Buffer	2.1	2.1
Enzyme	0.3	-
Substrate	0.6	0.6

After the addition of catechol to laccase + buffer + deionized water mixture, cuvettes were immediately put into spectrophotometer and the increase in absorbance was measured in 30 seconds intervals for 15 minutes.

3.2.2.3 Determination of immobilized laccase activity (ABTS assay)

ABTS and buffer were added into cuvettes and cuvettes were immediately put into spectrophotometer. The increase in absorbance was measured in 1 minute intervals for 75 minutes at 414 nm (light path 1 cm). The assay mixture for the test and blank cuvettes were shown below (Table 3.3).

Table 3.3 : Test and blank cuvettes content (immobilized laccase-ABTS assay).

	Test (ml)	**Blank (ml)**
Buffer	2.90	2.90
Immobilized Enzyme	+	-
Substrate	0.05	0.05

3.2.2.4 Determination of immobilized laccase activity (catechol assay)

Catechol and buffer were added into cuvettes and cuvettes were immediately put into spectrophotometer. The increase in absorbance was measured in 5 minutes intervals for 235 minutes at 404 nm (light path 1 cm). The assay mixture for the test and blank cuvette were shown in Table 3.4.

Table 3.4 : Test and blank cuvettes content (immobilized laccase-catechol assay).

	Test (ml)	**Blank (ml)**
Buffer	2.4	2.4
Immobilized Enzyme	+	-
Substrate	0.6	0.6

3.2.3 Calibration of the untreated oxygen sensor (PreSens)

Calibration could be done either manually or automatically before the measurement.

3.2.3.1 Manual calibration

In some experiments, calibration of the PSt3 and FTC-PSt3 sensor spots was done by a two-point calibration. First, oxygen saturated distillated water was measured and set as maximum value. The zero oxygen point was set with nitrogen-saturated water.

For each case, 100 ml distilled water was purged with gas (air or nitrogen) for an hour.

3.2.3.2 Automatic calibration

In most experiments with PSt3 and FTC-PSt3 sensor spots and in all experiments with sensor dishes, calibration of the sensors was done automatically. Specific phase angle values for 100 % and 0 % oxygen points which were send by the manufacturer were directly written to the software for the calibration before the measurement.

3.2.4 Software by PreSens for measurement

The software that was used for OxyView-PSt3- V5.32, Oxy4v2_19FBV3 and SDRv38, was user-friendly and it allowed the storage of phase angle, amplitude, log time and the oxygen concentration (in the unit of choice %air saturation, µmol/L, hPa (mbar), mg/L (ppm), Torr) data. Details about the software are available at the instruction manual page of PreSens GmbH, Regensburg.

A representative image of the software interface was given in figure 3.1. After the "Start" button was clicked, the sampling rate, temperature compensation, logging the data could be decided by the user. Moreover, from the "Calibration" tab, calibration of the system could be done.

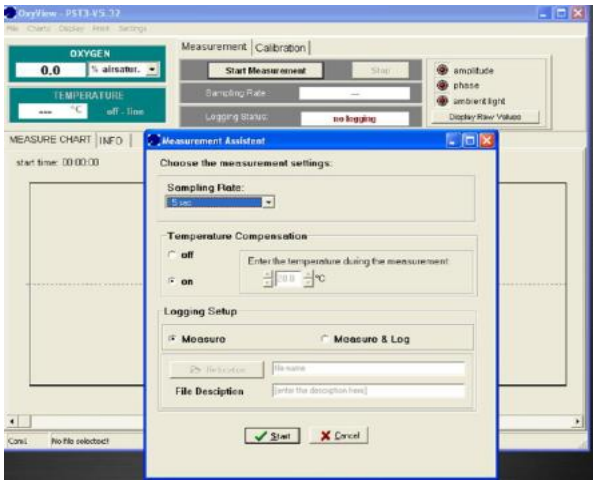

Figure 3.1 : OxyView-PSt3-V5.32 software

3.2.5 Calibration of the oxygraph (Hansatech)

The device calibration was repeated before each experiment by a two-point calibration. To do this, first maximum molecular oxygen content in reaction chamber

was measured after continuously aerating the reaction chamber with air. This point was considered as 100 % oxygen saturation point. After that, sodium dithionite ($Na_2S_2O_4$) which consumes the oxygen in the reaction chamber was added to the medium and the minimum point was assigned as zero oxygen concentration.

3.2.6 Software of oxygraph for measurement

The software that was used with oxygen electrode unit was user-friendly and allowed the storage of time vs. the oxygen concentration (in the unit of choice % air saturation, nmol/ml) data. Details about the software are available at the instruction manual page of Hansatech.

A representative image of the software interface was given in figure 3.2. Before the "Start" button was clicked, the stirrer must be opened by clicking "stirrer→on". Then, sampling rate and logging the data could be decided by the user. Moreover, from the "Calibration" tab, calibration of the system could be done.

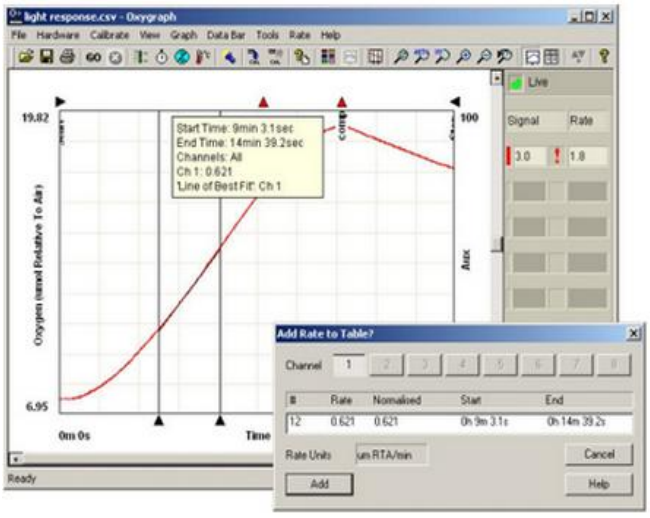

Figure 3.2 : Oxygraph software

3.2.7 Production of the laccase immobilized on PTFE

3.2.7.1 Construction of biosensor

Gelatin entrapment method was used for biosensor construction. First, gelatin (7.5 mg) was dissolved in 200 µL phosphate buffer (0.1M, pH 6.0) at 45 $^{\circ}$C for 10 min. Before gelatin hardened, 5 units of laccase enzyme (from *Trametes versicolor*) was added and then the solution was put to the surface of the PTFE membrane (1.5 x 2

cm). The construct was stored at 4 °C for 1.5 hour. When the gelatin completely dried and hardened, enzyme containing membrane was immersed in glutaraldehyde (2.5 mL, 5 % (v/v)) for 4 min to crosslink the gelatin matrix. After that, the construct was washed twice with dH2O for 5 min to remove glutaraldehyde and unbound enzymes. Finally, construct was stored at 4 °C till measurements.

3.2.7.2 Measurement

After the calibration of system, measurements were done at Oxygraph. First, 2000 µL buffer (may have different temperature and pH) was added into reaction vessel. After that, the construct, which was prepared according to section 3.2.7.1, was carefully placed into reaction vessel. Then, the stirrer was opened and "Go" button was clicked. When the signal had reached the equilibrium, 10 µL of substrate (0.1M catechol or chlorophenol) was added. Finally, when the graph had constant slope measurement was stopped and the slope was noted.

3.2.7.3 Characterization of immobilized laccase

The immobilized enzyme systems were characterized by observing the activity at different pH, temperature and glutaraldehyde values while catechol and chlorophenol were used as a substrate.

Two different glutaraldehyde concentrations (2.5 and 5.0 % (v/v)) were tested to obtain optimum glutaraldehyde amount. Catechol (10 µL, 0.1M) was used as a substrate and measurements were done at 35 °C in phosphate buffer (0.1M, pH 5.0).

Optimum pH value was investigated using sensors that are prepared with 2.5 % glutaraldehyde for both catechol (10 µL, 0.1M) and chlorophenol (10 µL, 0.1M). During the reactions, phosphate buffer with pH values varying between 4.0-7.0was used. All measurements were done at 35 °C.

Finally, the immobilized systems' activity was measured at different temperatures between 25-50 °C in 5 °C increments for both catechol (10 µL, 0.1M) and chlorophenol (10 µL, 0.1M).

3.2.8 Production of the laccase immobilized PSt3 sensor spots

The PSt3 sensors were used at the OXY-4 multi-channel fiber optic oxygen transmitter. Different treatments and modifications were carried out during the

immobilization and the effect of these treatments on sensor response, stability and reproducibility was studied.

3.2.8.1 Activation

All sensors were first washed with 75 % 2-propanol (1 ml) and with distilled water (1ml) twice. Then, 3-Aminopropylsilantriol (APST, 20 %) was poured into porcelain dishes and sensor's non-shiny parts, which is without sensor support such as glass or polyester foil, were laid on the solution overnight at room temperature under light protection for the activation. The next day, the sensors were washed twice in water for 3-5 minutes.

3.2.8.2 Immobilization

Different amounts of enzyme (1-1.5-3 mg) were dissolved in sodium phosphate buffer (22 µL, 0.1 M, pH 6.9). The cross linker (glutaraldehyde, 25 %, 3µL) and a matrix stabilizer (AMC, 25 µL) was then added. After that, 50µL of the well mixed suspension (pipette up and down slowly) were transferred onto the upside (non-shiny) surface of the sensor. Enzyme layer dried overnight at room temperature, under light protection. Sensors were covered with aluminum folio to prevent rapid drying.

3.2.8.3 Diffusion layer (protective film)

Diffusion layer was prepared according to the procedure optimized in Dr. Werner's Group using TMOS:PVA:HCl:Tri-MOS in a volumetric ratio of 2:8:5:1, respectively. After preparation, this mixture (40 µL) was pipetted onto the laccase layer and left to dry overnight at room temperature under light protection. The chemical reactions that led to the formation of diffusion layer were shown in figure 3.3.

To evaluate the effect of diffusion thickness to the sensor performance, diffusion layer were added on the sensor spots twice. In this case, after the addition of first diffusion layer, the sensor surface was left to dry for 2 hours to pipette the second layer.

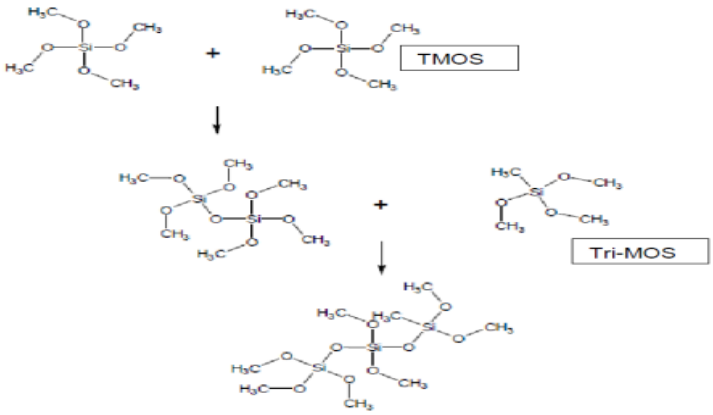

Figure 3.3 : Cross linking of the diffusion layer.

3.2.8.4 Fixation of the sensor spots

After the diffusion layer dried out, sensor spots were glued into the vials that were rinsed prior to use with acetone to remove all fatty residues. The glue RS 692-542 (silicone glue) was used and sensors were left to dry for 24h at room temperature and under light protection.

3.2.8.5 Measurement

PBS (20 ml, pH 6.9) was added to each vial after the glue had dried. Then, vials were connected to OXY-4 transmitter with fiber optic cables and placed on the magnetic stirrer (figure 3.4). Their calibration values (for each sensor spot, the readings for 0 % and 100 % oxygen was given in the batch number) were inserted to the software and measurement was started. When the background signal was constant, a known volume of catechol solution was added into vials. After the signal was stabilized and remains constant, the solution was thrown away and vials were washed with distilled H_2O. After that, PBS (20 ml, pH 6.9) was again added to each vial. Measurements were done at room temperature and measurement interval was 15 seconds.

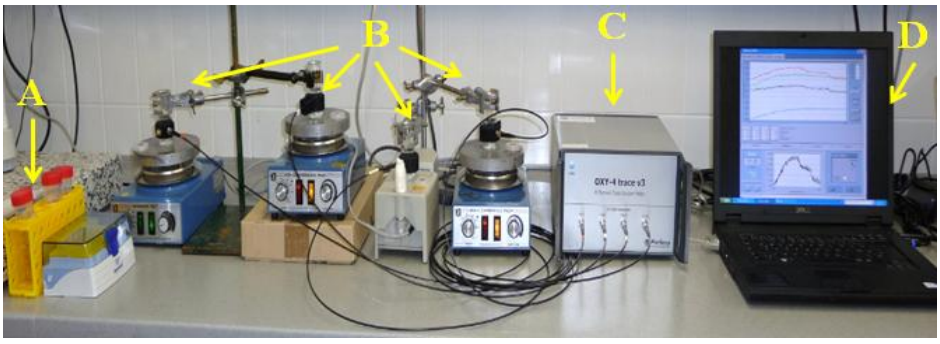

Figure 3.4 : PSt3 sensor spot measurement set up (A: Catechol solutions, B: Vials with PSt3 sensors, C: OXY-4 transmitter, D: Computer).

The flow-through oxygen minisensors (FTC-PSt3) were prepared to develop a stable, reproducible laccase sensor for continuous detection of phenolic compounds. These sensors were used at the FIBOX 3 fiber optical oxygen transmitter.

3.2.8.6 Biosensor storage

The prepared biosensors were stored in dark at 4°C without PBS before the first measurement. After the measurements, sensors were stored in phosphate buffer (pH 6.9) and in the refrigerator (4°C) under light protection to prevent crack formation because of drying. In order to evaluate the storage stability of the biosensors, the sensor spots were stored in phosphate buffer (pH 6.9) and in the refrigerator (4°C). Their activity was tested at different days (1st-30th-85th).

3.2.8.7 Biosensor performance in real samples

Commercial apple juice and two different tea samples were used as real samples. Apple juice was used directly without any preprocess. On the other hand, to prepare the tea samples, one bag of tea was put into cup that contained 300 ml distilled hot water and waited for 15 minutes. After that, the liquid was taken and put into fridge until the measurements.

3.2.9 Production of the laccase immobilized FTC-PSt3 sensor spots

The flow-through oxygen minisensors (FTC-PSt3) were prepared to develop a stable, reproducible laccase sensor for continuous detection of phenolic compounds. These sensors were used at the FIBOX 3 fiber optical oxygen transmitter.

3.2.9.1 Activation

All sensors were washed with 75 % 2-propanol (1 ml) and distilled water (1ml) twice. After that, activation was carried out as described in section 3.2.8.2.

3.2.9.2 Immobilization

Laccase (10 mg), BSA (10 mg), glycerin (Glycerol, 6 mg) was mixed thoroughly in 100 µL dH_2O. Glutaraldehyde (25 µL, 2.5 %) was then added to this solution and mixed for 10 seconds with vortex. After that, 2.6 µL of the mixture was pipetted on the surface of each sensor. Enzyme layer was dried overnight at room temperature in the dark and with the aluminum folio on to prevent rapid drying.

3.2.9.3 Diffusion layer (protective film)

Diffusion layer was prepared according to the procedure optimized in Dr. Werner's Group using ethylcellulose:D4:ethanol solution (90 %) with a mass ratio of 2:1:27, respectively. After overnight stirring at room temperature, 1.6 µL of this mixture was pipetted once on each sensor surface and left to dry overnight at room temperature in the dark.

3.2.9.4 Fixation of the sensor spots

Sensor spots were glued to the head of plastic tube with the help of a metal apparatus (figure 3.5). The glue RS 692-542 (silicone glue) was used and sensors were left to dry for 24h at room temperature in the dark.

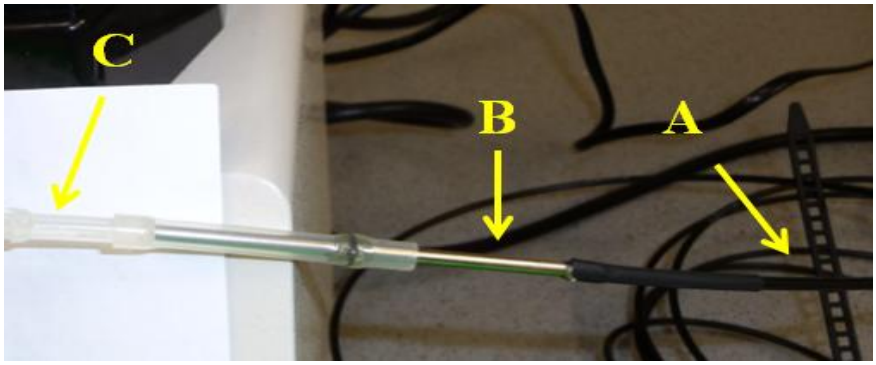

Figure 3.5 : Fixation of the sensor spot (A: Fiber optic cable, B: Metal apparatus, C: Sensor fixation point).

3.2.9.5 Sensor equilibration

Plastic tubes containing sensor spots at the end was incubated in PBS (0.1 M, pH 6.9) overnight in order to humidify the matrix of the sensors.

3.2.9.6 Measurement

Sensors were connected to FIBOX 3 transmitter with fiber optic cables and to peristaltic pump with Fluran peristaltic tubing (figure 3.6). Their calibration values obtained from the supplier were written to software and measurement was started. When the background signal was stabilized, the catechol solution with known concentration was introduced the measurement chamber through the tubing. The end of the peristaltic tubing was changed in the measurement of different catechol concentrations. At the end of measurement, system was washed with water for 10

minutes and with PBS (pH 6.9) for 3-5 minutes. Measurements were done at room temperature and measurement interval was 15 seconds.

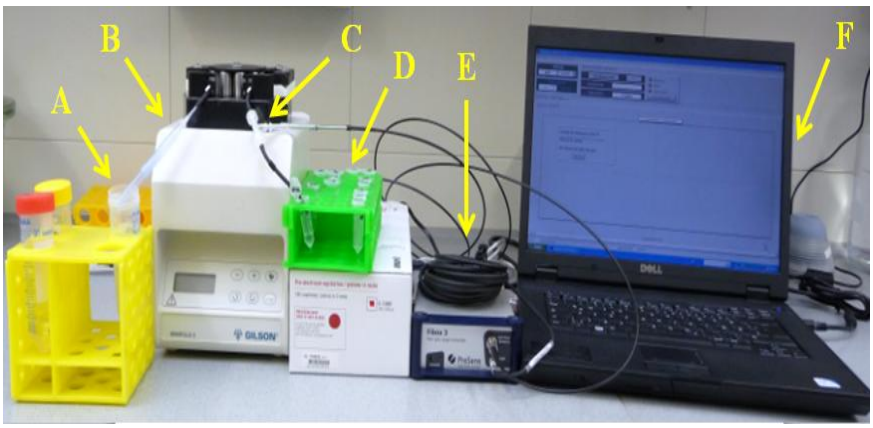

Figure 3.6 : FTC-PSt3 sensor spot measurement set up (A: Waste, B: Peristaltic pump, C: Sensor spot, D: Catechol solutions, E: FIBOX 3, F:Computer)

3.2.9.7 Biosensor storage

The prepared biosensors were stored in dark at 4°C without PBS before the first measurement. After the measurements, sensors were stored in phosphate buffer (pH 6.9) and in the refrigerator (4°C) under light protection.

3.2.10 Production of the laccase coated sensor dishes

The 24-well plates (sensor dishes) were used to develop a stable, reproducible laccase sensor for cell culture experiments. A sensor spot (PSt5) was placed at the bottom of each well and the response of the sensors were monitored using SDR SensorDish Reader.

3.2.10.1 Immobilization

Laccase (10 mg), BSA (10 mg), glycerin (glycerol, 6 mg) was mixed thoroughly in 100 µL dH_2O. Then, 25 µL glutaraldehyde (2.5 %) was added and mixed for 10 seconds with vortex. Resulting mixture was pipetted on each sensor surface (2 µL).Enzyme layer was dried overnight at room temperature in the dark and with lid on to prevent rapid drying.

3.2.10.2 Diffusion layer (protective film)

Diffusion layer was prepared according to the procedure optimized in Dr. Werner's group using ethylcellulose:D4:ethanol solution (90 %) with a mass ratio of 2:1:27,

respectively. The mixture was stirred overnight at room temperature and 4 µL of mixture was then pipetted on each sensor and left to dry overnight at room temperature in the dark.

To investigate the effect of diffusion layer on the sensor performance, the prepared mixture was added on the sensor spots twice. In this case, it was waited for 3 hours between two successive additions.

3.2.10.3 Sensor equilibration

For the sensor equilibration, 1 ml PBS (0.1 M, pH 6.9) was added to each well and left overnight in order to homogeneously humidify the matrix of the sensors.

3.2.10.4 Measurement

PBS was taken out from all wells and fresh PBS (1 ml) was added to each well again. After that, set up of the SDR was adjusted as shown in figure 2.14. Three sensor dish readers (SDR) were put into incubator which was set to 37°C and 5 % CO_2 concentration, connected to each other and to splitter. Then, sensor dishes were placed on the readers and their calibration values provided by the supplier were written to the software. The system was set to send a signal to sensor dishes in every 30 seconds and the measurement was started. When the background signal was stabilized, known concentrations of catechol solution was added to each well. After the highest reading was reached, the solution was thrown away and wells were washed with distilled H_2O. After that, 1 ml of PBS was added to each well again.

3.2.10.5 Biosensor storage

The prepared sensor dishes were stored in dark at 4°C with PBS (pH 6.9) before the first measurement and after each measurement.

4. RESULTS AND DISCUSSION

4.1 Activity Assay of *Trametes versicolor* Laccase

Enzyme from *Trametes versicolor* was used from stocks that were described in section 3.2.1 and activity of stocks was measured with oxygen electrode unit in our previous work. Free enzyme's specific activity had been determined as 22.4 U/mg.

4.2 Activity Assay of *Pleurotus ostreatus* Laccase

After the measurements were done according to section 3.2.2, activity of laccase solution [U/ml] was calculated from equation 4.1. Moreover, equation 4.2 was used to determination of specific enzyme activity [U/mg].

$$A = \frac{\Delta c}{\Delta t} = \frac{\Delta E}{\Delta t} \cdot \frac{V}{\varepsilon \cdot d \cdot v} \cdot 10^6 \quad \textbf{(4.1)}$$

$$specific\ A = \frac{\Delta E}{\Delta t} \cdot \frac{V}{\varepsilon \cdot d \cdot v . c_E} \cdot 10^6 \quad \textbf{(4.2)}$$

In equations 4.1 and 4.2, V is the total volume of the cuvettes (ml); v is the volume of the added enzyme; c_E is the protein concentration of the used enzyme solution (mg/ml); d is the layer thickness of the cuvette (cm); Ɛ is the molar absorption coefficient (cm^2/mol) and E is the extinction.

4.2.1 Free laccase (ABTS assay)

Firstly, free enzyme activity was determined using ABTS as a substrate. The measurements were carried out three times and the average ΔE/Δt was found to be 1.26 Abs/min. Moreover, at the ABTS assay V=3 ml; v= 0.05 ml; Ɛ= 36.800.000 cm^2/mol; d= 1 cm and c_E= 1 mg/ml. When these values were used in equation 4.2, specific activity of free enzyme (ABTS assay) was found as 2.06 U/mg.

4.2.2 Free laccase (catechol assay)

Free enzyme activity was also determined using catechol as substrate.

$\Delta E/\Delta t$ was averaged from three measurements and found as 0.33 Abs/min. At the catechol assay, V=3 ml; v= 0.3 ml; ε=740.000 cm^2/mol; d=1 cm and c_E= 1.33 mg/ml values were inserted into equation 4.2 and specific activity for free enzyme (catechol assay) was found as 3.34 U/mg.

4.2.3 Immobilized laccase on planar oxygen sensitive foils (ABTS assay)

Activity of immobilized laccase with ABTS was determined and average $\Delta E/\Delta t$ for three measurements was found as 0.08 Abs/min. Moreover, for the ABTS assay, V=3 ml; v= 0.05 ml; ε= 36.800.000 cm^2/mol; d= 1 cm and c_E= 1 mg/ml values were inserted into equation 4.2 and specific activity for immobilized enzyme (ABTS assay) was found as 0.12 U/mg.

4.2.4 Immobilized laccase on planar oxygen sensitive foils (catechol assay)

Finally, activity of immobilized laccase with catechol was determined.

Average $\Delta E/\Delta t$ was found as 0.0034 Abs/min based on three measurements. At the catechol assay, V= 3 ml; v= 0.05 ml; ε = 740.000 cm^2/mol; d=1 cm and c_E= 1 mg/ml values were used to calculate the specific activity as 0.28 U/mg.

4.2.5 Comparison of activity of free and immobilized laccase

ABTS is a common chemical used for activity measurement of some enzymes and catechol, on the other hand, is our model analyte, so they were used during activity assays. It was clearly seen that activity loss happened during immobilization as expected (table 4.1). Moreover, while immobilized enzyme reached to equilibrium point in 70 minutes, free enzyme reached to the same point in 3 min (figure 4.1). In brief, immobilization affected the enzyme activity, probably due to enzyme denaturation by chemical and physical effects and also by diffusion limitations.

Table 4.1 : Specific activity results for free and immobilized laccase

Substrate	**Specific activity [U/mg]**	
	Free Enzyme	**Immobilized Enzyme**
ABTS	2.06	0.12
Catechol	3.34	0.28

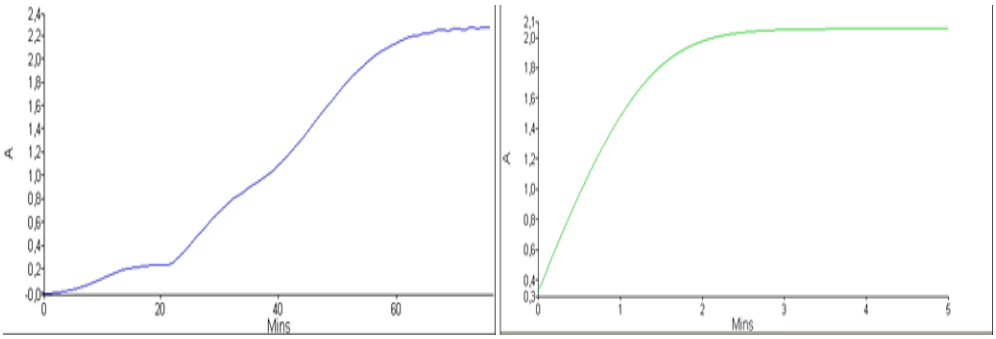

Figure 4.1 : Comparison of immobilized (left) and free (right) laccase activity using catechol assay.

4.3 Optimization of Immobilized Laccase Sensor (Oxygraph)

4.3.1 Effect of glutaraldehyde concentration

Cross linker amount, which was glutaraldehyde in this study, is an important parameter for biosensor construction. Therefore, two different concentrations of glutaraldehyde (2.5 and 5 % v/v) were used to crosslink the gelatin to entrap the enzyme. The experiment was carried out in different pH buffers at 35 °C. Catechol was used as substrate. Activity results, response time and stability of gelatin layer were compared to find optimum cross linker concentration.

After the measurements, activity results were compared. At more basic pH values (5.5-7.0), enzyme's activity was higher when 2.5 % glutaraldehyde concentration was used (Figure 4.2). One of the reasons may be the negative effect of high cross linker amount on protein structure due to excessive bond formation [Freire et al., 2001]. In addition, high crosslinking will reduce the pore size of the gelatin membrane and decrease the mass transfer rate.

Another observation was that while working with 5 % glutaraldehyde, gelatin layer stability was lower and it was easily separated from PTFE. Maximum 3 or 5 measurements was done with sensors that were prepared with 5 % glutaraldehyde. On the other hand, at least 10 measurements were done with sensors that were prepared with 2.5 % glutaraldehyde. Thus, enzyme's reaction surface extended and 5 % glutaraldehyde concentration had higher activity at pH 4.5 and both concentrations had almost the same activity at pH 5. Furthermore, except pH 4.0 response time of sensors was shorter at 2.5 % glutaraldehyde concentration (figure 4.3).

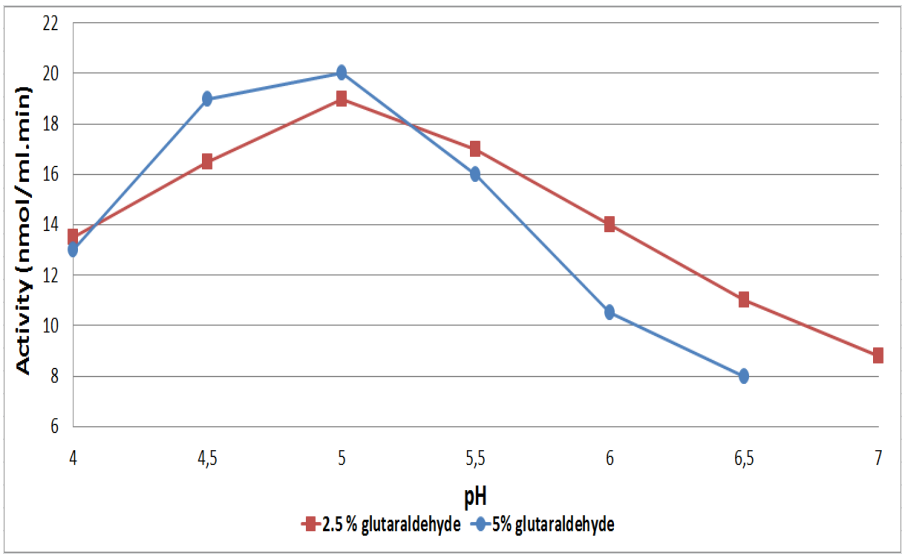

Figure 4.2 : Activity comparison of 2.5 % and 5 % (v/v) glutaraldehyde cross linked gelatin membranes.

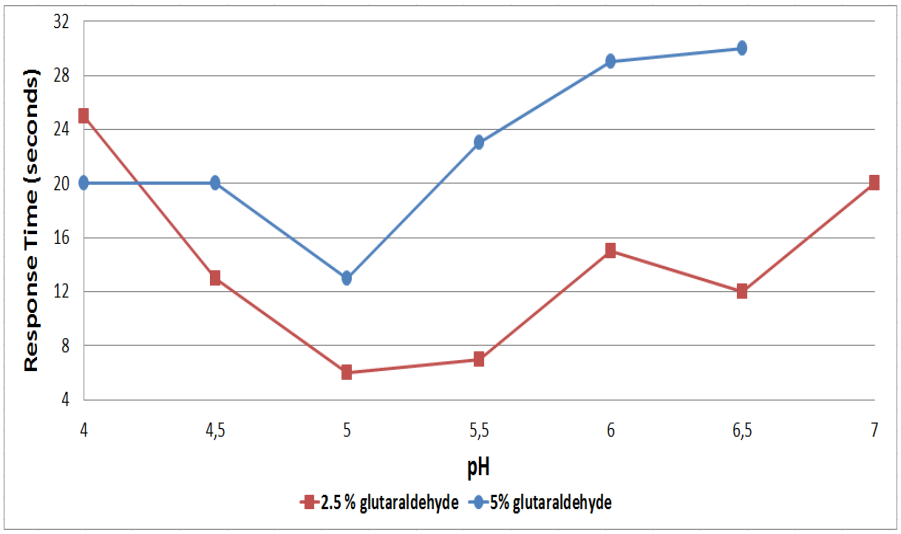

Figure 4.3 : Response time comparison of 2.5 % and 5 % (v/v) glutaraldehyde cross linked gelatin membranes.

In conclusion, 2.5 % glutaraldehyde concentration was chosen for further experiments because of higher activity, more stability and shorter response time.

4.4 Characterization of Immobilized Laccase Sensor (Oxygraph)

4.4.1 Optimum pH

The experiments were carried out in different pH buffers (4.0-7.0) at 35 °C using 2.5 % glutaraldehyde. Catechol and chlorophenol were used separately as a substrate. At

the end of experiments, activities (figure 4.4) and response times (figure 4.5) for each substrate were compared to find the optimum pH.

There may be different factors affecting the dependence of immobilized enzyme activity on pH. One of the reasons may be the negative effect of high cross linker amount. Moreover, another reason may be the protonation state of the immobilization matrix, in this case gelatin, at different pH values. Gelatin has a pKa value at around 4.7 so it would be positively charged below this pH and negatively charged above [Prideaux, 1931]. This will affect the microenvironment of the enzyme and both enzyme activity and substrate diffusion rate (so substrate availability) could change consequently.

The reason of pH shift of immobilized enzymes is generally a consequence of the proton exchange properties of the immobilization matrix [Cho et al., 2008]. Immobilization matrix creates a microenvironment around the enzymes and this microenvironment has different characteristics from the bulk solution. Moreover, the polymers may be charged positively or negatively on different pH values because of their different pI values and different buffering capacities of the polymers were believed to be the cause pH shifting.

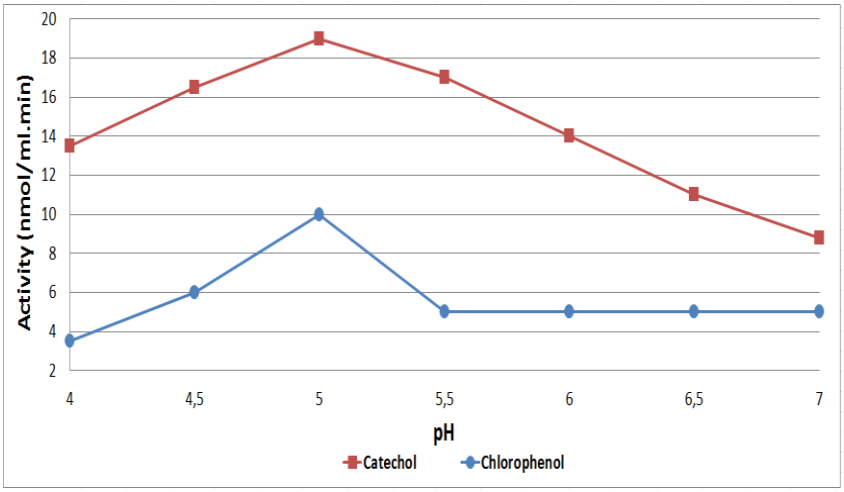

Figure 4.4 : Optimum pH determination of gelatin entrapped laccase for the detection of catechol and chlorophenol.

Optimum pH value was found to be as 5 for both substrates. The highest activity was recorded 19.0 nmol/ml.min for catechol at this pH whereas chlorophenol gave a lower activity 10 nmol/ml.min. Moreover, the quickest response time (6 seconds for catechol and 9 seconds for chlorophenol) was seen at pH 5. Eventually, pH 5 was

determined as an optimum pH value for biosensor and further experiments were done at pH 5.

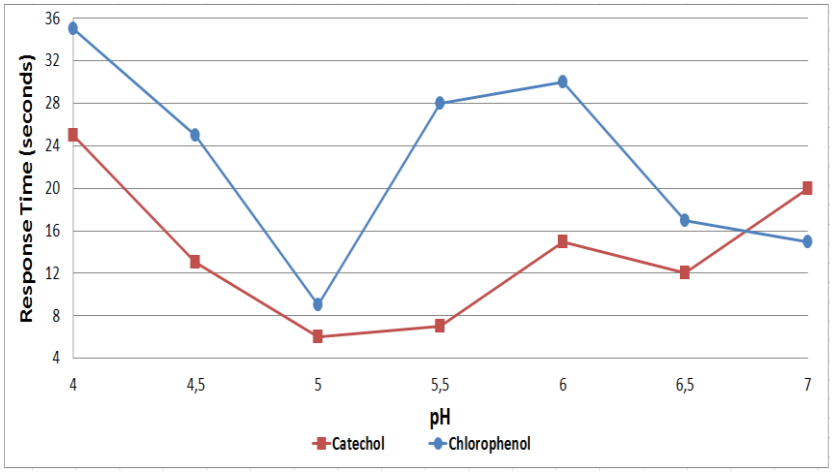

Figure 4.5 : Response time comparison of gelatin entrapped laccase at different pH values.

4.4.2 Optimum temperature

The experiments were carried out in different temperatures (25-50 °C) at pH 5 and the sensors were prepared at 2.5 % glutaraldehyde concentration. At the end of experiments, activities (figure 4.6) and response times (figure 4.7) for each substrate were compared to find the optimum temperature.

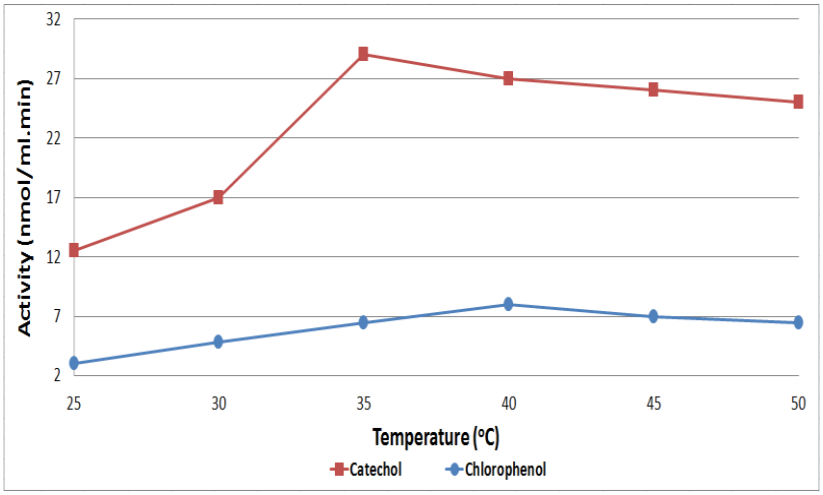

Figure 4.6 : Optimum temperature determination of gelatin entrapped laccase for the detection of catechol and chlorophenol.

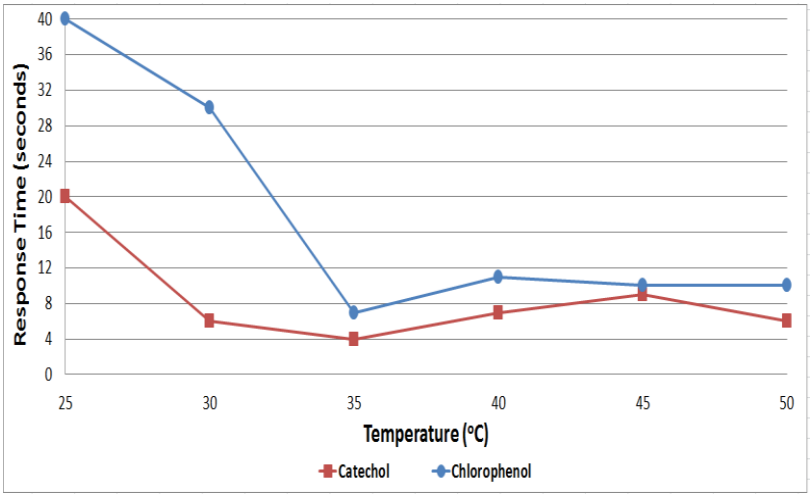

Figure 4.7 : Response time comparison of gelatin entrapped laccase at different temperatures.

It was seen that at high temperatures (above 40 °C) gelatin lost its stability, started to melt and dissolved into the reaction chamber. Because of the dissolution and separation of gelatin layer from PTFE membrane, the reusability of the sensors drastically reduced in these temperatures. Besides these temperatures, the highest activity values and the quickest response time were observed at 35 ^{o}C for both catechol and chlorophenol. Eventually, 35 ^{o}C was determined as the optimum temperature for biosensor and further experiments were done at pH 5 and 35 ^{o}C.

4.4.3 Effect of substrate

After the identification of optimum pH and temperature, response of biosensor to catechol and chlorophenol was compared. At the optimum conditions activity value of biosensor was 29.0 and 6.5 nmol/ml.min for catechol and chlorophenol, respectively. Moreover, the response time was 4 second for catechol and 7 seconds for chlorophenol. Consequently, biosensor's activity was higher and its response time was shorter when catechol was used, so it is obviously a better substrate for laccase than chlorophenol. Thus, only catechol was used as the model substrate at the fiber optic sensor system.

4.5 Optimization of Immobilized Laccase Fiber Optic Sensor (SP-PSt3)

During the experiments, catechol was used as a substrate. Main solution (0.1 M) was prepared and different amounts were added into glass tubes to see the response of the biosensor. Moreover, measurements were done at least thrice and their average

values were used on the tables and graphs. Amounts on the graphs (eg. 0,1 mM, 0,2 mM, 0,4 mM etc.) show the final molarity values of the added substrate.

4.5.1 Effect of enzyme amount

For the construction of biosensor, different layers (enzyme and diffusion) were prepared on the PSt3 sensors. Enzyme layer was the first one and different amounts of laccase (1, 1.5 and 3 mg/sensor) from *Pleurotus ostreatus* was used at the beginning of optimization. Sensors were prepared according to section 3.2.8 without any diffusion layer. The measurements were made using increasing concentrations of catechol for each enzyme amount (figure 4.8) and the change in the phase angles based on catechol concentration was plotted figure 4.9).

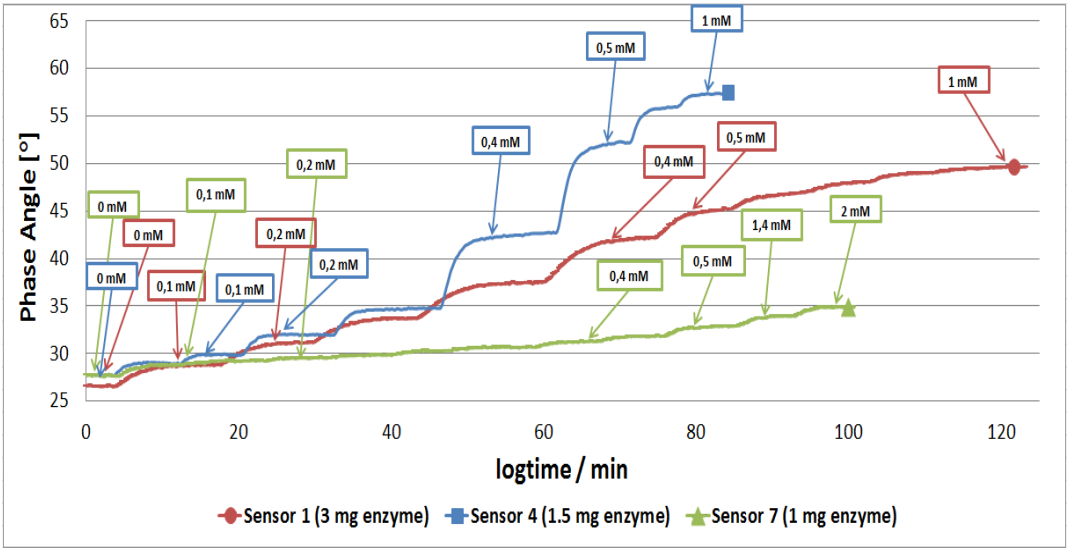

Figure 4.8 : Optimum enzyme amount determination for the fiber optic sensor.

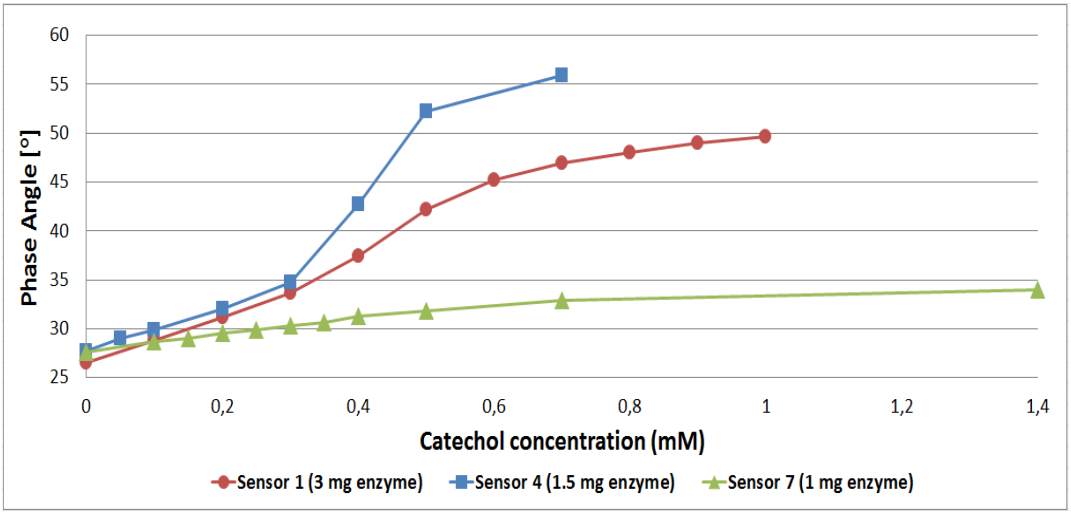

Figure 4.9 : Optimum enzyme amount determination based on comparison of sensors' phase angles against different catechol concentrations.

It could be seen that 1 mg enzyme sensors reach lower phase angles than other sensors, which means that this amount was not enough to construct a biosensor that could respond effectively to the changes in catechol concentration (figure 4.9). On the other hand, 3 mg enzyme sensors did not give highest phase angles as expected; their values were less than other sensor that contained 1.5 mg laccase. Excessive amount of enzyme might cause overcrowding and this dense enzyme layer could cause limitations in enzyme flexibility and diffusion of the substrate needed for optimum activity. In brief, the optimum laccase amount was determined as 1.5 mg per sensor and used in further experiments.

4.5.2 Effect of diffusion layer (protective film) quantity

After the optimization of enzyme amount, the effect of diffusion layer quantity on sensor response was checked. At this step, sensors with (one or two layer) or without diffusion layer were prepared according to section 3.2.8. After that, measurements were done and the results were given in figure 4.10.

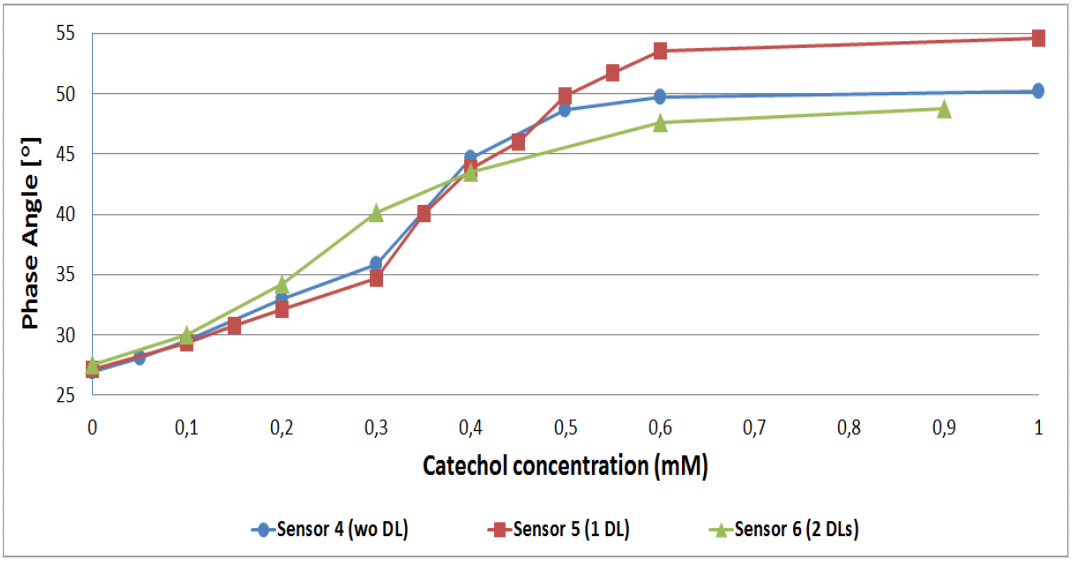

Figure 4.10 : Optimum diffusion layer quantity determination of the fiber optic sensor.

It was seen that presence of the diffusion layer affected the maximum phase angle reached by the system; Biosensor with two diffusion layers reached to lower phase angles (ca. 48^{O}), while biosensor without diffusion layer reached to the highest one (ca. 55^{O}) as expected. Without diffusion layer, on the other hand, maximum two measurements could be done which means that the reusability of these sensors were limited. Because of external factors such as stirring, buffer changes or washing,

enzyme layer was separated from sensor surface easily. Thus, biosensor with one diffusion layer was chosen for further experiments.

4.6 Characterization of Immobilized Laccase Fiber Optic Sensor (SP-PSt3)

4.6.1 Effect of pH

After the determination of optimum enzyme amount and diffusion layer quantity, pH effect was checked with the optimized sensor (1.5 mg enzyme and 1 diffusion layer). For this, measurements were done in PBS buffers with different pH values as 5, 6.9 and 9. Phase angle values for each pH values were determined at increasing catechol concentrations (figure 4.11) and corresponding working range graphs were plotted (figure 4.12).

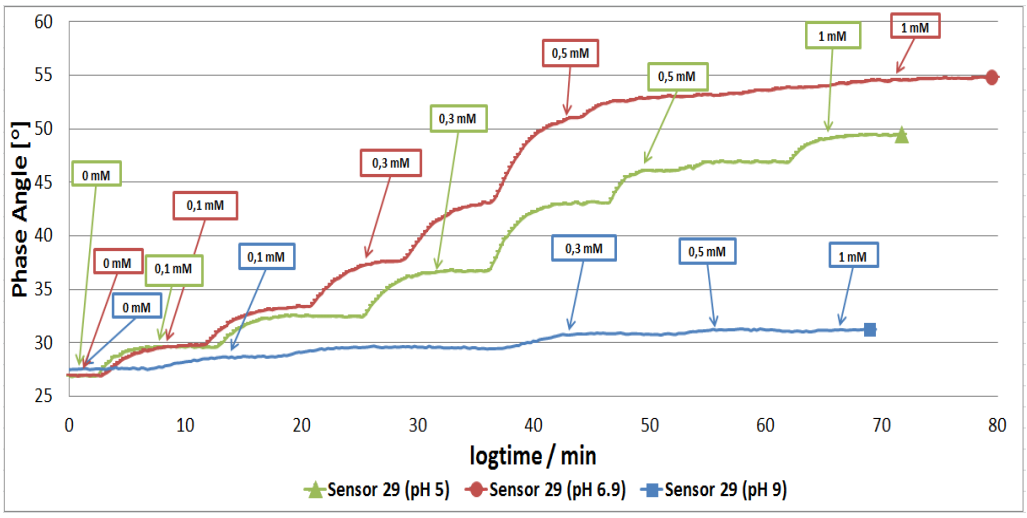

Figure 4.11 : Optimum pH determination for the fiber optic sensor.

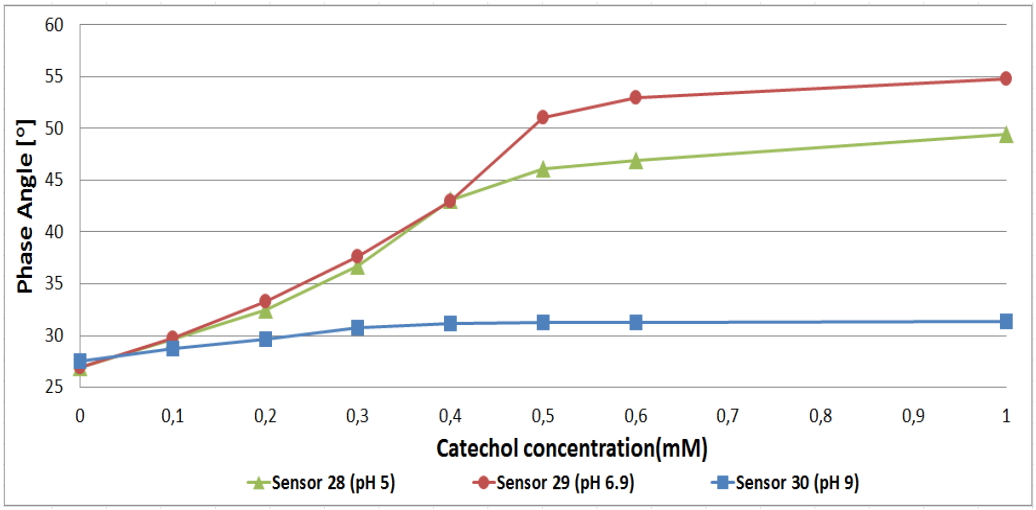

Figure 4.12 : Phase angle change vs catechol concentration at different pH values.

The optimum pH of free laccase was 4.5 (Sigma, 2012). However, a shift in optimum pH was observed at immobilized systems as expected. When the results were examined, optimum pH of the covalently immobilized systems was found as pH 6.9. Furthermore, biosensor could work at pH 5 without any problem, but its activity almost completely hindered at pH 9 (figure 4.12).

Unlike the gelatin entrapped system, whose optimum pH was 5, optimum pH shifted towards higher alkaline values when compared with free laccase optimum pH. In the fiber optic system different chemicals such as TMOS, PVA and Tri-MOS, which have different pI from gelatin, from gelatin entrapped system. Thus, optimum pH value shifted towards higher alkaline values.

Moreover, sensors were used successively in each pH value during the measurements and it was noticed that different pH values did not disrupt sensor structure permanently. Although a sensor showed lower activity at pH 9, it could reached to higher activity when it was replaced in pH 6.9. However, before each measurement, sensors must be washed very well with distilled water and PBS to remove the effect of previous pH value to next measurement.

4.6.2 Dynamic working range of biosensor

Each sample may contain different phenolic compound content and this difference may be very wide. Thus, biosensors' dynamic working range must be as wide as possible while measuring the minimum possible amount of phenol. For this purpose, the highest and lowest phenol detection limit of biosensor was investigated. During the measurements optimum enzyme amount (1.5mg), diffusion layer number (1) and PBS buffer (pH 6.9) was used.

For the highest detection limit, catechol concentrations between 0.1-5 mM were used. In these experiments, catechol was added up to 0.6 mM final concentration step by step (0.1 mM at each step) and then buffer was refreshed. Sensors were washed with distilled water and new PBS was added before measurements. This procedure was performed before each measurement for 1 mM, 2mM and 5 mM catechol. The results were seen in figure 4.13.

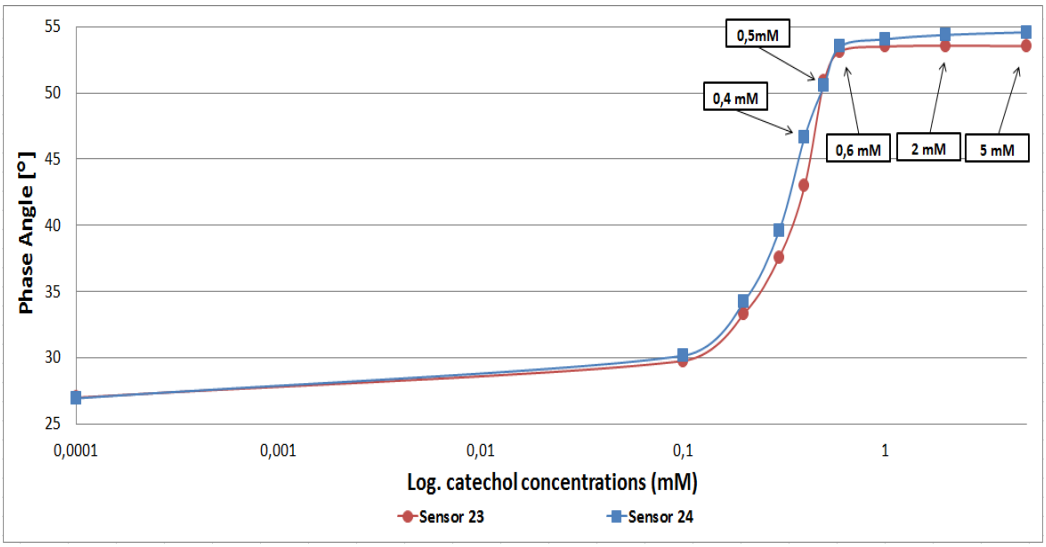

Figure 4.13 : Dynamic working range of fiber optic biosensor.

Biosensor's highest detection limit was found to be 0.6 mM as seen in figure 4.13, because above this concentration, phase angle values did not change significantly so biosensor's response remained same.

After that, catechol samples with concentrations between 0.01-0.1 mM were used as a substrate to find the limit of detection and linear working range of biosensor. Results were plotted in figure 4.14.

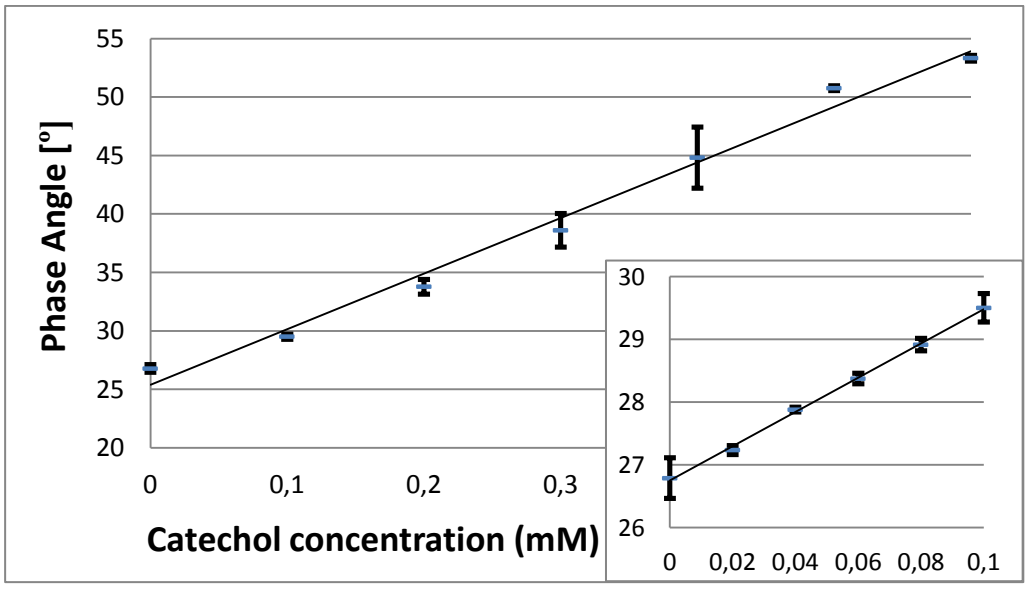

Figure 4.14 : Lineer working range of fiber optic sensor. Insert shows the behavior of the sensor at low cathecol concentration range.

The lowest detection phase value was found according to equation 4.1.

3 x (Standard deviation value of 0 mM) + (Phase angle value of 0 mM) **(4.1)**

According to equation 4.1, the lowest detection phase value was 27.761. Thus, 0.04 mM, whose phase number was 27.878 (± 0.039) can be noted as the LOD of the biosensor for catechol.

As a conclusion, biosensor's dynamic working range was detected as 0.04–0.6 mM.

4.6.3 Reproducibility of sensor spots

Sensors produced according to the same protocol must give compatible results to produce a reliable biosensor. To determine the reproducibility of sensor spots, five spots were produced simultaneously according to section 3.2.8. After that, different concentrations of catechol were used to evaluate the response of each sensor. Phase angle vs. catechol concentration graphs were plotted and the slope of trend lines was compared.

Slopes of 5 sensors produced with the same conditions were found as 46.59, 47.53, 47.18, 46.95 and 45.96, respectively. The trend lines and their slopes were very close to each other which shows the reproducibility of biosensor.

4.6.4 Repeatability of biosensor response

The main advantage of immobilization is the ability to use the same system again with minimal or no loss of activity. To determine the repeatability of biosensor, first different concentrations of catechol were added gradually up to 0.6 mM (1st measurement). Then, buffer was refreshed and waited until the signal reached to the equilibrium. After that, measurements were done in the back order with gradually decreasing catechol concentration. During this step, buffer was changed and a specific buffer + catechol mixture was added before each measurement (2nd measurement). Finally, 0.6 mM catechol solution was added one more time into the vessel and then buffer was refreshed again (figure 4.15). Results were also compared in figure 4.16.

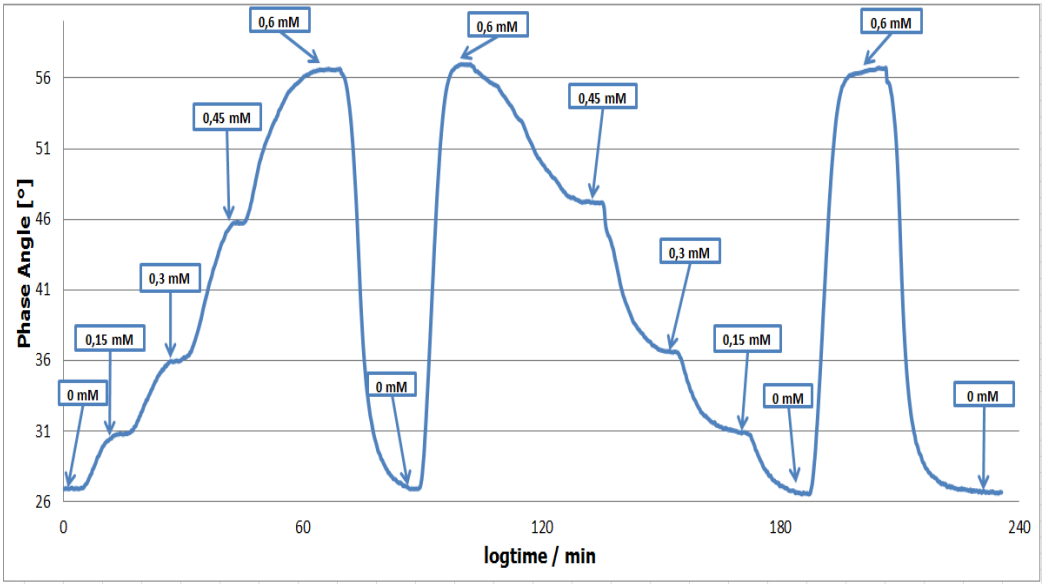

Figure 4.15 : Repeatability of fiber optic biosensor response.

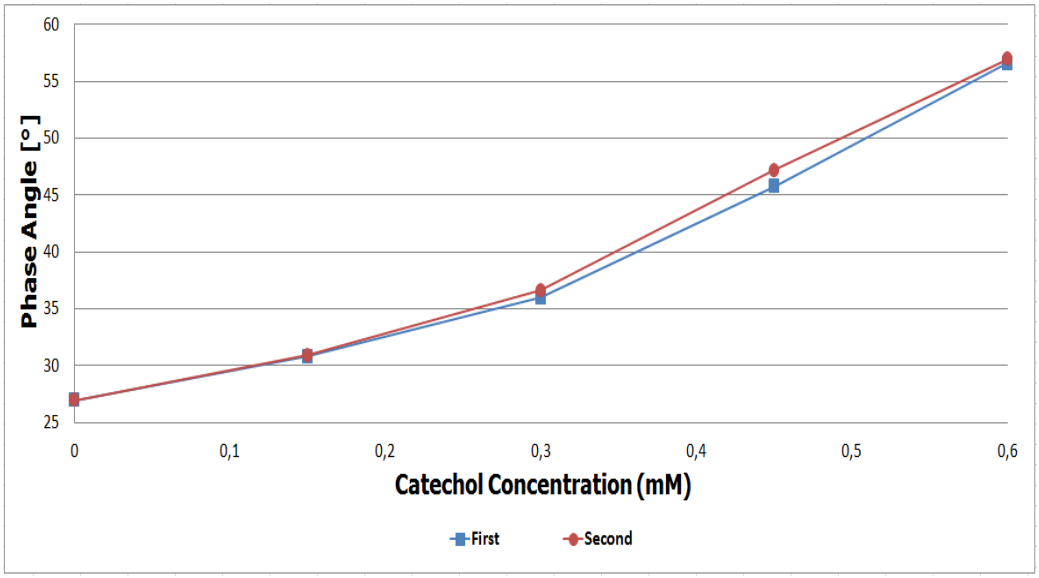

Figure 4.16 : Comparison of first and second measurements' phase angles to determine repeatability of biosensor.

Biosensor showed almost the same response against the same catechol concentration whether catechol's concentration was decreased or increased gradually. The phase angle values of each measurement were very similar (figure 4.16). Thus, repeatability of the biosensor was very high. However, to get better results, system must be washed very well with distilled water and PBS before each sample addition.

4.6.5 Storage stability of biosensor

Biosensor's storage life is an important parameter to preserve its activity over long periods of time. Thus, storage stability of immobilized system were investigated in

pH 6.9 PBS buffer at 4 °C. Activity measurements were done at different time intervals. The results were shown in figure 4.17.

When the slopes of 1st, 30th and 85th day measurements, which are in order of 43.96, 43.47 and 41.72, were compared, it was seen that almost 95-98% of biosensor's initial activity was retained after 85 days. Moreover, biosensor has better storage stability when it was compared with other biosensors that appeared in literature [Abdullah *et al.*, 2007, Gomes *et al.*, 2003]. In brief, biosensor's storage stability was very high if it was stored according to section 3.2.8.6.

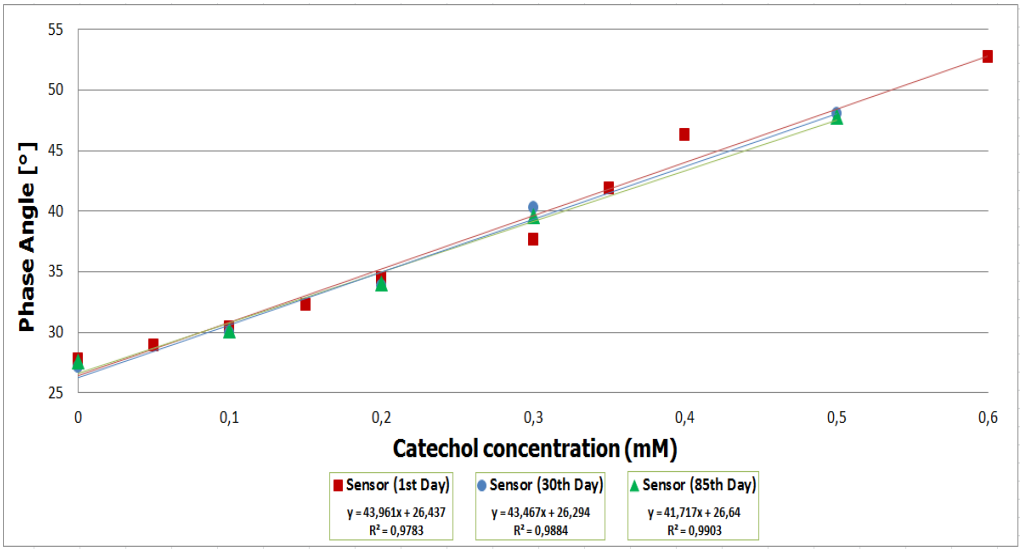

Figure 4.17 : Comparison of 1st, 30th and 85th day measurements according to their phase angles for storage stability.

4.6.6 Response time and measurement period of biosensor

Other important parameters for biosensors are response time and measurement period. Both parameters must be as short as possible to analyze more samples in shorter period. Response time is the time necessary for the sensor to start detection for a given sample. Furthermore, the time pass from the addition of sample to the equilibration of the signal can be called as measurement period. To understand the response time and measurement period of biosensor, different concentration catechol samples, whose final concentrations were 0.1- 1.5 mM, were used.

At the beginning, 0.1 mM catechol was added into glass tube and then waited until a stable signal was obtained. After that, PBS was refreshed and catechol concentrations were increased gradually. Between each catechol sample addition, the system was refreshed with PBS. In figure 4.18 response of the biosensor can be followed.

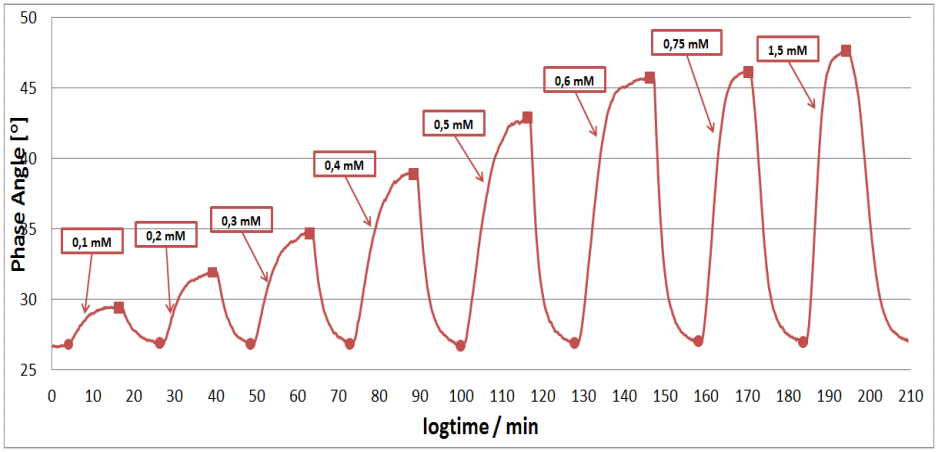

Figure 4.18 : Determination of fiber optic sensor's response time and measurement period.

In figure 4.18, dots represent the substrate addition time and squares represent the time when the signal is equilibrated. After the addition of substrate, increase at the phase angle was started immediately for each concentration as seen in figure 4.18.

Moreover, time between the addition of sample and equilibration of the signal was listed in table 4.2 for different catechol concentrations.

Table 4.2 : Measurement period of biosensor for different catechol concentrations.

Molarity (mM)	Measurement period (min.)
0.1	13
0.2	13
0.3	14
0.4	13.5
0.5	13.5
0.6	13.5
0.75	11
1.5	10

The measurement period against different catechol concentrations was similar, so average measurement period of biosensor can be stated as 13 minutes.

Moreover, after the PBS was changed, system's recovery time lasts approximately 15 minutes. In brief, at least 25 minutes was needed between successive sample measurements.

4.6.7 Applicability to other systems

Flow through system and sensor dishes were used to enlarge the application field of the biosensor.

Flow through system is important for continuous measurements. With the help of this system, any type or concentration of analyte can be added one after another. The washing step between successive measurement will be easier so more analyte can be examined in a shorter time with flow through system.

For this reason, FTC-PSt3 sensor spots were prepared according to section 3.2.9. Optimum parameters of the previous sensor (SP-PSt3) were used during the FTC-PSt3 sensor construction. After the construction, measurements were done with different catechol concentrations to determine the response and reliability of the sensor. Most concentrations were measured twice and the results were presented in figure 4.19.

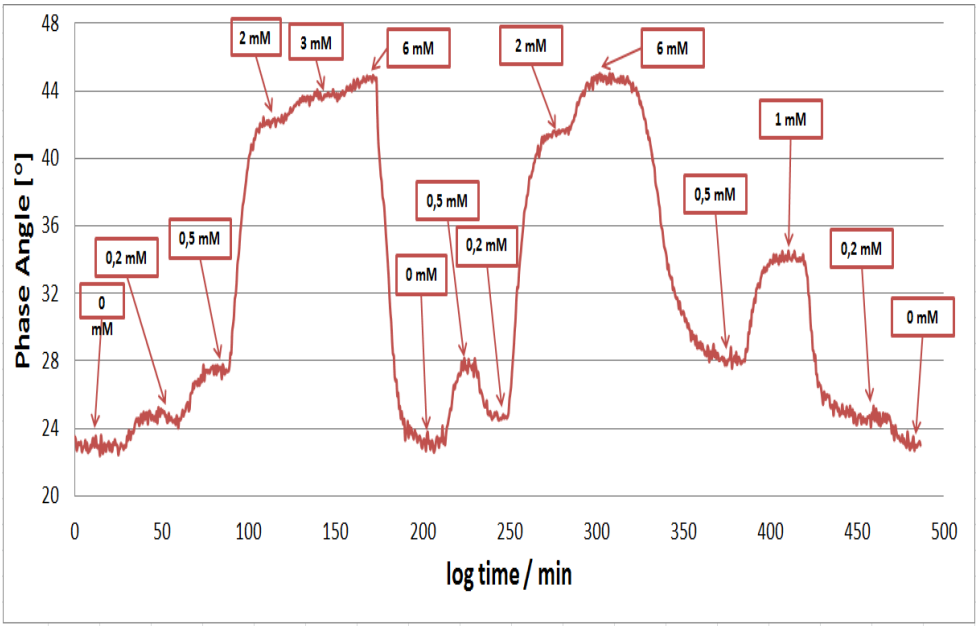

Figure 4.19 : Determination of sensor applicability to flow through system according to phase angle.

Figure 4.19 shows that the signal could be reproduced at each attempt so it can be said that FTC-PSt3 sensors work properly on flow through system when they were produced according to our protocol.

Sensor dishes, which are usually used in cell culture experiments, were used as a second application for our biosensor. For this purpose, a sensor dish was prepared according to section 3.2.10. To understand if our sensors and protocol were applicable to sensor dishes, only one parameter, diffusion layer number, was changed at each well during the construction. Content of each well was given in table 4.3.

Table 4.3 : Layer content of sensor dish's each well.

	1	2	3	4	5	6
A	2 DL[a], E[b]	2 DL, E	1 DL, E	1 DL, E	2 DL, nE[c]	wDL[d], wE[e]
B	2 DL, E	2 DL, E	1 DL, E	1 DL, E	2 DL, nE	wDL, wE
C	2 DL, E	2 DL, E	1 DL, E	1 DL, E	1 DL, nE	wDL, wE
D	2 DL, E	2 DL, E	1 DL, E	1 DL, E	1 DL, nE	wDL, wE

[a] DL= diffusion layer, [b] E=sensor has enzyme layer, [c] nE=sensor has enzyme layer without enzyme, [d] wDL= sensor without diffusion layer, [e] wE= sensor without enzyme layer.

Wells from A1 to D4 (group 1) had 2 diffusion layers while wells from A3 to D4 (group 2) had only 1. Moreover, wells from A5 to D6 were used as a control group, thus they did not have any laccase.

After the preparation of plate, catechol, whose final concentrations vary from 0.1 to 2.1 mM, were added to each well and response of the sensors were displayed in figure 4.20.

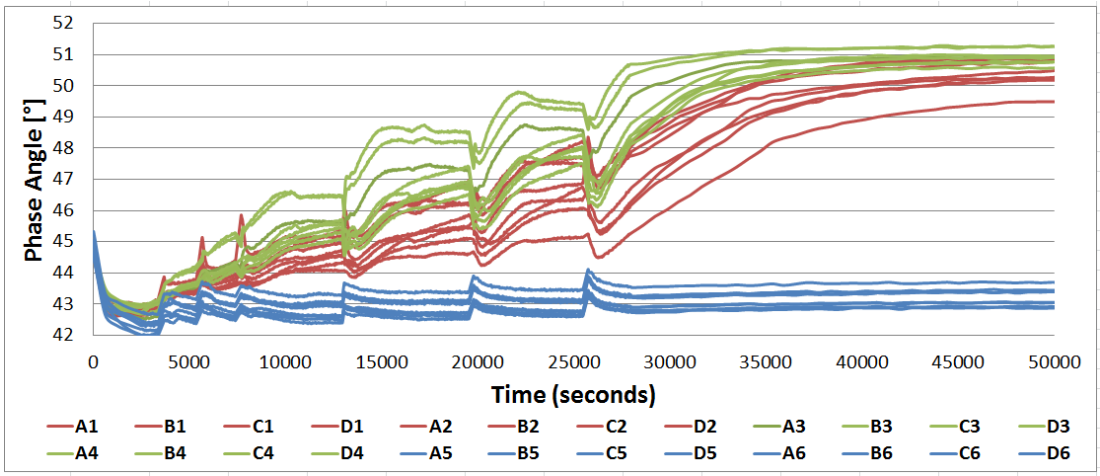

Figure 4.20 : Effect of diffusion layer quantity to sensor spots at SDR system.

During the measurement, the plate was removed from incubator to add catechol. This step lasted approximately two minutes and at this time measurement was paused. However, when the measurement was restarted system made a peak at phase angle values. The reason could be the temperature and CO_2 concentration change in the incubator during catechol addition.

All control group wells had almost stabile phase angle values during the measurement that means the system worked without any problem. Moreover, sensors with 1 diffusion layer (green lines) reached to higher phase angle values than sensors with 2 diffusion layers (red lines) as expected; sensors responded to catechol concentration according to their diffusion layer quantity. On the other hand, reproducibility of the system was not good when the response of the different wells, which had same layers content, to the same catechol concentration was observed (figure 4.20). Photos taken at Biozero 8000K compact fluorescence microscope, also supported this problem (figure 4.21). Because of small size of sensors, pipetting enzyme and diffusion layers on the sensors was very hard. During the layer addition, diffusion or enzyme layers spread out of the sensors, so thickness of the layers differed at each sensor (eg. Sensors 1A, 2B and 3C). Moreover, because of handling problem some sensors had bubbles above. In brief, all sensors did not have exactly the same layer thickness or structure and as a result of that reproducibility was low.

Although low reproducibility, it can be said that sensors are still useful at sensor dishes. Furthermore, if more optimization experiments will be done on this system, better results can be obtained.

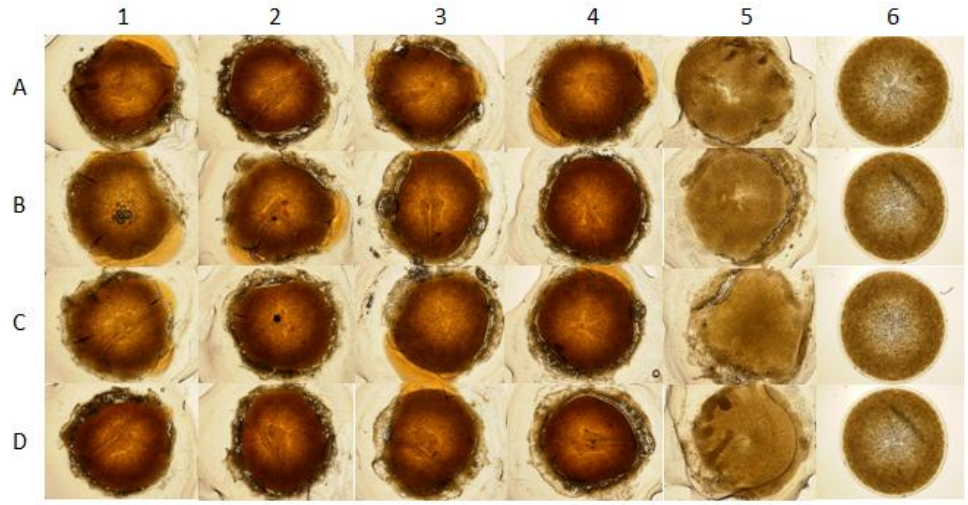

Figure 4.21 : Fluorescence microscope images of each sensor after construciton.

In brief, according to our results, sensor spots and our protocol could be applied to flow through system effectively but more optimization is needed for sensor dish systems.

4.7 Real Sample Measurements

The response of the biosensor to real samples is very important since real samples may show matrix effect and several of their constituents may interfere with the results. Thus, real samples were used with the optimized fiber optic sensors (SP-PSt3) to evaluate its effectiveness. Two different teas, black tea and honey tea, and an apple juice were used for this purpose. Tea samples were prepared according to section 3.2.12. On the other hand, apple juice was bought from supermarket and used directly without any process. Tea and apple juice samples (2500 µl) were diluted in 20 ml PBS (pH 6.9) and added into glass tubes containing sensors calibrated with different concentration of catechol (0.1- 0.5 mM). Final phase angle values of each sample were used to find the concentration of phenolic compounds for each sample.

In figure 4.22, the first part showing the gradual increase in the signal corresponds to the calibration part. The decrease shows the refreshing of sensors by PBS wash. The final part of the graphs represents the response of the sensor against the sample.

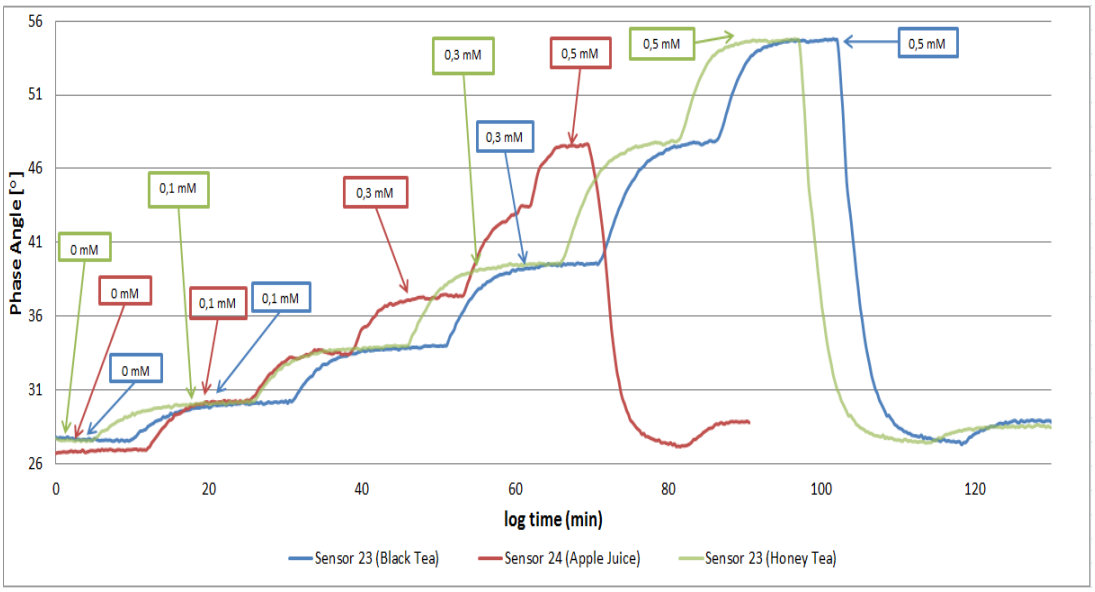

Figure 4.22 : Phenolic compound concentration determination of tea and apple juice samples.

According to phase angle against different catechol concentration, calibration curve for each sensor was plotted (figure 4.23).

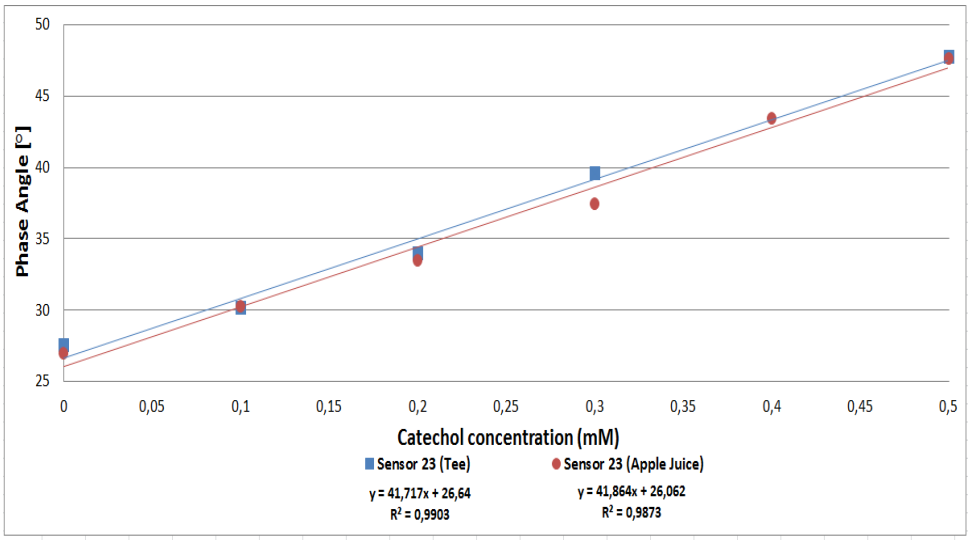

Figure 4.23 : Calibration curve of tea and apple juice samples for phenolic compound determination.

The concentration of analyte in diluted apple juice sample was found as 0.067 mM. To find the main phenolic compound concentration, equation 4.2 was used.

$$\text{Molarity}_1 \times \text{Volume}_1 = \text{Molarity}_2 \times \text{Volume}_2 \qquad \textbf{(4.2)}$$

Actual phenolic compound concentration of apple juice was found ca. 0.6 mM.

Same calculations were done for tea samples. Phase angle value of black tea and honey tea analyte was 28.93 and 28.56, respectively. Moreover, the trend line equation of tea analytes was $y = 41.717x + 26.64$ ($R^2 = 0{,}9903$). Analyte concentration of black tea and honey tea was found ca. 0.5 and 0.4 mM, respectively. In the case, one bag of tea, which contain 1.75 g tea, was used in sample preparation. Thus, phenol content of black and honey tea was 0.28 and 0.24 mM per gram, respectively.

In literature, many experiments have been done to identify the phenolic compound of fruit juices and different sort of tea [Mullen *et al.*, 2007; Papkovsky *et al.*, 1993]. When our results were compared with theirs, similar phenol concentrations were detected for tea and apple juice analytes. However, it must be considered that only catechol was used as a substrate in our research. To obtain 100 % reliable results different sort of phenolic compounds or mixture of phenols must be used as a substrate and the concentrations of phenolic compounds should be confirmed with other methods like HPLC.

5. CONCLUSION

Biosensors have very important role for the detection of phenolic compounds and they are promising alternatives for on-site detection of analytes. The aim of this thesis is construction and optimization of laccase biosensor for phenolic compound detection using two different approaches.

For this purpose, first laccase enzyme isolated from *Trametes versicolor* was entrapped with gelatin and immobilized on the PTFE surface. After the construction of biosensor with an easy and conventional gelatin entrapment method, optimum glutaraldehyde percentage, temperature and pH of biosensor was examined. Gelatin entrapped biosensor's optimum glutaraldehyde concentration was found as 2.5% and biosensors' optimum temperature and pH was established at 35 °C and 5, respectively for both catechol and chlorophenol. Another result was that minimum 10 or maximum 20 consecutive measurements under optimum conditions could be done with the biosensor. Furthermore, catechol showed better activity and shorter response time, thus only catechol was used as a substrate in the fiber optic system.

After that, a different immobilization method and chemicals were used to construct a fiber optic system. Laccase from *Pleurotus ostreatus* was immobilized on fiber optic oxygen sensor spots with the help of APST, glutaraldehyde and AMC to form a mechanical stable matrix. Thereafter, biosensor's optimization, characterization, applicability to other systems and performance with real samples (apple juice and tea) were investigated.

For the optical biosensor, optimum enzyme amount, diffusion layer quantity and pH value was determined as 1.5 mg, 1 and 6.9, respectively. Its response time was short and measurement period was approximately 13 minutes against different catechol concentrations. In addition, storage stability of the sensors was at least 85 days. Dynamic working range of biosensor was 0.04-0.6 mM and storage stability was at least 85 days. Repeatability of biosensor response and reproducibility of sensors was

very high. Our sensors were applicable to flow through systems and sensor dishes. Finally, sensors were shown to be effectively used for phenolic compound detection of real samples like fruit juices and tea. On the other hand, it must be considered that in our research only catechol was used as a phenolic compound during the measurements.

Both biosensor systems, and especially fiber optic biosensors, showed a good performance in terms of reproducibility, dynamic working range and storage life. They should now be tested for different substrates and real samples, like wastewater and environmental samples. Moreover, optimization of other systems such as flow-through and sensor dishes should be done as a next step.

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APPENDICES

APPENDIX A: Laboratory Equipment
APPENDIX B: Chemicals
APPENDIX C: Solutions and buffers

APPENDIX A

Laboratory Equipment

Pipettes	**:** Eppendorf 10 µL 100 µl, 1000 µl, 2500 µl
Microsyringes	**:** Hamilton co 10 µL.
pH meter	**:** Mettler Toledo MP220
	: WTW pHmeter
Pure water systems	**:** USF Elga UHQ-PS-MK3, Elga Labwater
	: Millipore (Milli-Q), Millipak express
Computer	**:** Dell Latitude E5500
Peristaltic pump	**:** Minipuls 3, GIBSON
Desiccator	**:** Vakuumfest
Vacuum system	**:** 589 colorful ribbonS&S Filter Paper Circles
Vacuum equipment	**:** TSY HF450/PECVD350/MMPS20/C Genertec International Corporation
Stirrer	**:** IKA-COMBIMAG RCH
Shaker	**:** Certomat S II
Orbital Shaker	**:** Heidolph Duomax 1030
Balances	**:** Precisa XB220A and Precisa BJ610C
Water bath	**:** Huber
Oxygen electrode	**:** Helmut saur laborbedorf oxygraph
Fibox 3	**:** Fiber optic oxygen transmitter, PreSens
Oxy 4	**:** 4 channel trace oxygen meter, PreSens
Microscope	**:** Biozero 8000K compact fluorescence microscope, KEYENCE
Incubator	**:** Hermo Scientific Heracell 240i CO_2 incubator
Spectrophotometer	**:** PerkilElmer Lambda 35 UV/VIS Specktrometer

APPENDIX B

Chemicals

Sodium acetate	**:** Merck
Acetic acid (0.1 M)	**:** Merck
Ethanol (100 %)	**:** Merck
Sodyum Dithionite	**:** Merck
Tri-MOS	**:** Fluka
TMOS	**:** Fluka
APST	**:** Degussa AG Aerosil & Silanes (AS)
2-Propanol	**:** Fluka
Carboxycellulose	**:** Serva
Ethylenediamine-dihydrochloride	**:** Fluka
EDC	**:** Merck
Mowiol	**:** Kuraray
Glycerol	**:** Carl Roth
Acetone	**:** Sigma-Aldrich
Carboxycellulose	**:** Serva
Chlrophenol	**:** Sigma-Aldrich
Catechol	**:** Sigma-Aldrich
ABTS	**:** Calbio Chem
HCl (0.1 M) and KCl	**:** VWR BDH Prolab **:** Riedel-de Haën
NaOH (0.25 M)	**:** Merck
MES hydride	**:** Sigma-Aldrich
Gelatin	**:** Sigma-Aldrich
KCl	**:** NormaPur.

Dinatriumhidrogenphosphat **:** Grüssing

NaCl **:** Ph. Eur.

Kaliumdihidrogenphosphat **:** Riedel-de Uaen

D4 **:** AdvanceSource Biomaterials

Glutaraldehyde (70 %) **:** Sigma-Aldrich

Glutaraldehyde (25 %) **:** Carl Roth GmBH

Laccase *(Trametes versicolor)* **:** Sigma-Aldrich

Laccase *(Pleurotus ostreatus)* **:** Sigma-Aldrich

Black Tea **:** Messmer Darjeeling

Honey Tea **:** Bad heilbrunner markası

APPENDIX C

Solutions and buffers

Chlorophenol solution (0.1 M) 100 ml

- 1.28 gr Chlorophenol
- 100 ml dH_2O

Catechol solution (0.1 M) 100 ml

- 1.10 gr Catechol
- 100 ml dH_2O

PBS (pH: 6.9) (1000 ml)

- 8 gr NaCl
- 0.2 ml KCl
- 1.44 gr Na_2HPO_4
- 0.24 gr KH_2PO_4
- 1000 ml dH_2O

Glutaraldehyde solution (2.5 %)

- 175 µL Glutaraldehyde (70 %)
- 4825 µL Phosphate buffer (pH 6.0, 0.1 M)

Glutaraldehyde solution (5 %)

- 357 µL Glutaraldehyde (70 %)
- 4643 µL Phosphate buffer (pH 6.0, 0.1 M)

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