İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IMMOBILIZATION OF LACCASE ONTO PTFE MEMBRANES USING VARIOUS METHODS

M.Sc. Thesis by
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Programme : **Molecular Biology-Genetics and Biotechnology**

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JANUARY 2010

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

LAKKAZ ENZİMİNİN PTFE MEMBRANLARA ÇEŞİTLİ YÖTEMLERLE TUTUKLANMASI

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FOREWORD

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Evren TAŞTAN

Molecular Biology, M Sc.

TABLE OF CONTENTS

Page

ABBREVIATIONS

ABTS	**:** 2, 2-azinobis-(3-ethylbenzthiazoline-6-sulfonate)
PTFE	**:** Polytetrafluoroethylene
NHS	**:** N-hydroxy-succinimide
EDC	**:** N-(3-dimethyaminopropyl)-n-ethyl-carbodiimide
GA	**:** Glutaraldehyde
MOBGAM	**:** Molecular biology and genetics research centre
FTIR	**:** Fourier transform infrared
pAAc-G-PTFE	**:** polyacrylic acid grafted polytetrafuoroethylene
pAAm-G-PTFE	**:** polyacrylamide grafted polytetrafuoroethylene
HPLC	**:** High performance liquid chromatography
W	**:** Watt
Pa	: Pascal

LIST OF TABLES

Page

LIST OF FIGURES

Page

IMMOBILIZATION OF *Trametes versicolor* LACCASE ONTO PTFE MEMBRANES BY DIFFERENT METHODS

SUMMARY

Phenolic compounds are byproducts of a number of industrial applications and discharged by wastewaters. Therefore detection of these compounds is very important for environmental protection and control. Conventional techniques for their detection are generally time consuming, expensive and do not allow to make measurements in the field. Biosensors are therefore could be designed as alternative tools for this purpose.

The aim of the study is to construct a biosensor from laccase enzyme isolated from *Trametes versicolor* as a portable, easy-to-use alternative to known analytical methods like HPLC. To do this, laccase was immobilized on PTFE using three different techniques and the most suitable technique was determined in terms of sensitivity, storage stability, reusability, etc. Enzyme activity was measured by using an oxygen electrode for each case. One of the immobilization methods, entrapment to gelatin, is a well-known and easy-to-prepare method so it was chosen to compare its performance with that of the new method that we proposed. For the other two immobilization methods, PTFE membranes were grafted with polyacrylamide and polyacrylic acid respectively using plasma discharge treatment. For polyacrylamide grafted PTFE, a two step polymerization process was used. The membranes were treated with hydrogen plasma (125 W, 13 Pa, 2 min) to increase surface wettability, thus allowing the acrylamide monomers (50% w/v in ethanol/acetone 50% v/v) to spread on the surface. Then argon plasma (50 W, 13 Pa, 1 min) was applied to initiate polymerization. Newly formed amide groups in polyacrylamide grafted PTFE (pAAm-g-PTFE) were detected by ATR-FTIR. For the third method, PTFE was treated with argon plasma (50 W, 13 Pa, 1 min) to generate peroxide groups required for polymerization. Acrylic acid was then grafted to the surface by heat treatment (70 ^{o} C, 6 h). The carboxyl groups in (pAAc-g-PTFE) were confirmed by ATR-FTIR. For both case, an optimized carbodiimide coupling reaction was used for enzyme immobilization. Then all three biosensor were characterized and compared in terms of optimum working conditions, storage stability and reusability.

Our study concluded that although the activity of the gelatin entrapped laccase is better, the mechanical instability and poor storage life of gelatin layer makes the gelatin biosensor unattractive for multiple usage and for field measurements compared to the other two biosensors. Our investigation suggests that the pAAc-g-PTFE biosensor is more stable and highly reusable than the other two biosensors and its sensitivity is suitable for field applications. Therefore, the pAAc-g-PTFE biosensor could be proposed as an alternative on-site detection tool for phenolic compound monitoring.

Trametes versicolor LAKKAZININ PTFE MEMBRANLARA ÇEŞİTLİ YÖNTEMLERLE TUTUKLANMASI

ÖZET

Fenolik bileşikler çeşitli sanayilerin yan ürünüdürler ve atıksularla birlikte atılırlar. Bu nedenle bu bileşiklerin tespit edilebilmesi çevrekoruma ve kontrolü için büyük önem taşır. Bu bileşikleri için kullanılan geleneleneksel tayin teknikleri genellikle çok zaman alır, pahalıdır ve yerinde tespite olanak tanımaz. Bu nedenle alternatif olarak biyosensörler dizayn edilebilir.

Bu çalışmanın amacı *Trametes versicolor*'dan izole edilmiş lakkaz enzimini kullanarak HPLC gibi analiz metodlarına alternatif, taşınabilir ve kullanımı kolay bir biyosensör inşa etmektir. Bu amaç için lakkaz enzimi üç farklı yötem kullanılarak PTFE membranlara tutuklanmıştır ve hassasiyet, raf ömrü, tekrar kullanılabilirlik vs, gibi kriterler karşılaştırılarak en uygun teknik belirlenmeye çalışılmıştır. Enzim aktivitesi ölçümleri için oksijen elektrodu kullanılmıştır. Kullanılan metodlardan biri olan jelatin içine hapsetme tekniği çok kullanılan ve kolay hazırlanan bir metoddur. Bu nedenle çalışmamızda kullandığımız yeni metodların performansını değerlendirebilmek için kullanılmıştır. Diğer iki tutuklama metodu için PTFE membranlar plazma uygulamaları ile poliakrilamid ve poliakrilik asit ile aşılanmıştır. Poliakrilamidin PTFE membranlara aşılanması için iki basamaklı bir polimerizasyon işlemi uygulanmıştır. Membranlar yüzey ıslanırlığını artırabilmek için once hidrojen plazması (125 W, 13 Pa, 2 dk) ile muamele edilmiştir. Böylece akrilamid monomer çözeltisinin (etanol/aseton %50 h/h içerisinde %50 ağırlık/hacim) yüzeye yayılabilmesi sağlanmıştır. Yüzeyine monomer yayılmış PTFE polimerizasyonu başlatmak için argon plazması (50 W, 13 Pa, 1 dk) ile muamele edilmiştir. Akrilamid aşılanması (pAAm-g-PTFE) ile yeni oluşan amin grupları ATR-FTIR kullanılarak tespit edilmiştir. Üçüncü metodda ise PTFE membranlar argon plazması (50 W, 13 Pa, 1 min) ile muamele edilerek yüzeylerinde polimerizasyon için gereken peroksit grupları oluşumu sağlanmıştır. Sonrasında ısı uygulaması ile (70 ^{o}C, 6 saat). akrilik asidin yüzeye aşılanması sağlanmıştır. Poliakrilik asit aşılanması ile oluşan karboksil grupları ATR-FTIR kullanılarak tespit edilmiştir. Bu iki metod içinde optimize edilmiş karbodiimid bağlama reaksiyonu kullanılarak enzimleri tutuklanmıştır. Yapılan üç biyosensör optmimum çalışma koşulları, Raf ömrü ve tekrar kullanılabilirlik bakımından karşılaştırılmıştır.

Yapılan deneyler sonucunda jelatin içerisine tutuklanmış lakkazın aktivitesinin yüksek olmasına rağmen, mekanik dayanıksızlığı ve jelatin tabakasının saklanmasındaki problemler nedeniyle çoklu kullanım ve saha ölçümleri için çok uygun olmadığı görülmüştür. Yaptığımız araştırmalara göre pAAc-g-PTFE biyosensörü diğer iki biyosensöre göre daha kararlı ve tekrar kullanılabilirliğinin daha yüksek olduğu ve saha kullanımı için yeterli aktiviteye sahip olduğu görülmüştür. Bu nedenle fenolik asitlerin tayin yöntemlerine bir alternatif olarak pAAc-g-PTFE biyosensörünü teklif ediyoruz.

1. INTRODUCTION

With new developments in biosensor technologies, biosensors started to play more important roles in the environmental surveillance of toxic contaminants. Phenols, which are the side products of industrial and agricultural processes, are one of the important contaminants in nature and many of them are resistant to biotic and abiotic degradation thus remains in the environment [Kulys *et al.*, 2002] Therefore sensitive, rapid and accurate on site determination of phenols is a growing interest. In this study the aim is to construct an effective, reliable and easy-to-use laccase biosensor for on-site detection of these toxic compounds.

1.1 Aim of the study

The aim of the study is to immobilize laccase enzyme on polymer grafted PTFE membranes and construct an effective biosensor for phenolic compound detection.

For this purpose we constructed three different biosensors for effective, reliable and easy-to-use laccase biosensor for on-site detection of these toxic compounds. First one is a conventional gelatin entrapment system. For the other two biosensors, PTFE membranes were subjected to plasma surface modification and amine and carboxyl groups necessary for covalent protein immobilization was grafted on PTFE. These amine and carboxyl groups acted as immobilization site for laccase enzymes. Laccase was immobilized on grafted PTFE and their optimum activities were investigated and compared.

2. PHENOLS AND BIOSENSORS

2.1 Phenolic compounds

Phenols are very harmful contaminats in industrial wastewaters. These compounds can originate from both industrial and agricultural sources, namely textile, pulp and paper industry, coal conversion, resins, metal coating, petrochemicals, olive processing plants, partial degradation of phenoxy based herbicides and wood preservatives. Phenolic compounds identified in these wastewaters are phenols, guaiacols, cresols and cathecols. Chemical structure of some phenolic componuds are given in figure 2.1. Since many of these compunds are toxic, their presence in drinking and irrigation water poses a health risk [Lante *et al*., 2000]. Some of these compounds have esterogenic and antiesterogenic activities that distrupt endocrine system [Georgieva *et al.*, 2008]. Phenolic compounds has one or more hydroxyl groups on the aromatic ring and/or rings. Phenolic compounds are one of the most widely distributed group of substances in the plant kingdom. Phenolic compunds are products of the secondary metabolism of plants.

Phenolic compounds like ferulic acid (3-methoxy4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), syringic acid (3,5-dimethoxy-4hydroxybenzoic acid) have antioxidant effects for metabolism. For example caffeic acid has antioxidant, antimutagenic, anticarcigenic, lipooxygenase inhibitory, antibacterial, antiinflamatory characteristics. Dehydrodimers of ferulic acid are structural components of plant cell wall and increase the structural integrity of the cell. Phenolic compounds are free radical scavengers. When used as a food preservative ferulic acid inhibits food spoilage due to oxidation [Odacı *et al*., 2006].

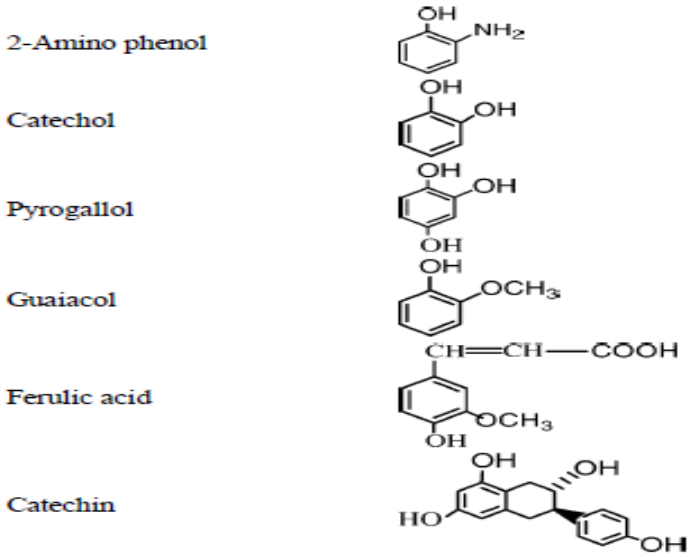

Figure 2.1: Chemical structures of some phenolic compounds [Odacı *et al*., 2006]

2.1.1 Toxic effects of phenolic compounds

Unwanted health effects of phenol exposure have been reported in literature. Repeated exposure to low levels of phenol has been linked with diarrhea and mouth sores. Ingesting high levels of phenols causes kidney problems, mouth and throat burns, abdominal pain, vomiting and effects blood, nervous system and in some cases death. Skin contact with high levels of phenols burns the skin and damages kidneys, liver, brain and lungs. Some phenolic compounds are suspect carcinogenic substances in longterm exposure in air or in water. Therefore discharge of industrial wastewater containing phenols may cause an ecological disaster [EPA, 1999].

2.1.2 Methods of phenolic compound detection

Phenolic compounds are widespread in nature both by organic sources and by industrial applications. Some of these compounds may have good effects on living organisms but most of them are toxic and above some levels lethal. Therefore rapid determination and degradation of these compounds are crucial in public health and environmental protection and control.

There are many reported detection methods for phenolic compounds like mass spectrophotometry, gas chromatography, liquid chromatography and capillary electrophoresis [Odaci *et al*., 2007]. But most these detection methods are expensive and may require several operations. These methods require large amounts of sample and reagents, require time for separation and may not always be environmentally friendly. Also the equipment required is expensive and generally not portable. To reduce the cost of detection and to allow on-site detection many biosensors were developed. Mostly these biosensors uses the catalytic activity of redox enzymes. These biosensors were constructed by using tyrosinase, laccase and peroxidase enzymes and using flow systems, various electrodes and sample treatment techniques [Roy *et al*., 2004]. The use of these biosensors are limited due to the fact that each enzyme used have different catalytic activity and may not always react with all phenolic compounds. For example while tyrosinase based biosensor can be used for detecting compounds with ortho-position free of substituent group, laccase biosensor can be used for compounds with para- and meta-position free of substituent group [Abdullah *et al*., 2007].

2.2 Laccase enzyme

Laccase enzymes (Figure 2.2) are multi-copper containing oxidaze enzymes (EC 1.10.3.2) that can be found in plants, fungi, insects and bacteria. Laccase has a molecular weight of 50-100 kDa [Kunamenni *et al*., 2007] and three copper bound sites, type 1, type 2 and type 3. Laccase catalyses the monoelectronic oxidation of substrates using molecular oxygen and the four copper atom core of the enzyme assists in the redox reaction (Figure 2.3). This four copper atom core also gives the enzyme a blue colour due to its electronic absorption of Cu-Cu bonds [Roy *et al*., 2004, Shleev *et al*., 2004].

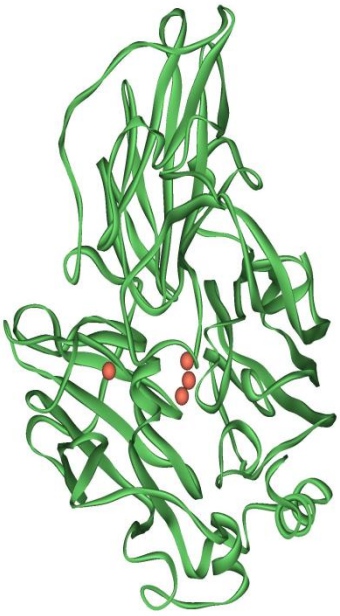

Figure 2.2: 3D structure of Laccase from *Trametes versicolor*

(The Armstrong Research Group 2005)

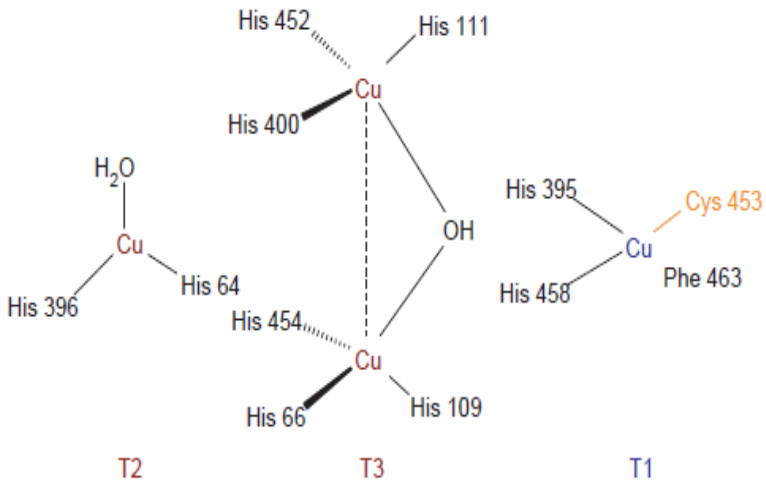

Figure 2.3: Core structure of laccase [Riva *et al.*, 2006]

Laccase enzyme reduces one molecule of oxygen to two molecules of water without the formation of hydrogen peroxide and by doing so oxidation of 4 substrate molecules to four radicals (Figure 2.4). After the reaction these products can form dimers, oligomers and polymers. Laccases have a wide substrate specificity. Laccases easily oxidize both para-, meta- and ortho-diphenols. Other than phenolic compounds laccases can oxidize aromatic compounds like diamines, aromatic amines, thiols and can olso oxidize inorganic compounds like iodine [Thurston *et al*., 1994].

In laccase phenolic compound reaction, a phenolic substrate is subjected to a one-electron oxidation and an aryloxyradical. This active species can be converted to a quinone in the second stage of the oxidation. The quinone as well as the free radical product, undergoes non-enzymatic coupling reactions leading to polymerization [Duran *et al.,* 2002, Minussi *et al*., 2002].

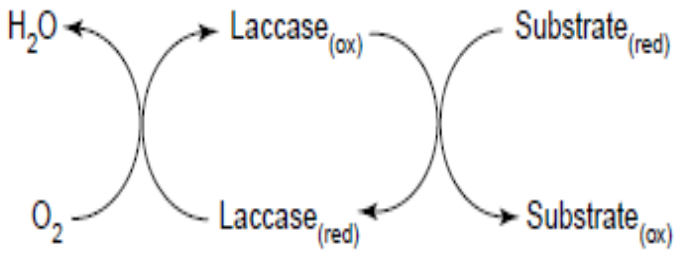

Figure 2.4: Typical laccase reaction [Riva *et al.*, 2006]

Laccases have low redox potential when compared to more powerful oxidases (lignin peroxidase, manganes peroxidase) and have the ability to oxidize compounds which 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 can be overcome by using mediators as electron shuttles. Mediators are compounds that are easily oxidized by laccases. These mediator can be used as electron shuttles. When laccases oxidise a mediator that mediator diffuses in to reaction chamber and oxidize the substrate that laccase cannot react directly (Figure 2.5) [Baiocco *et al*., 2002].

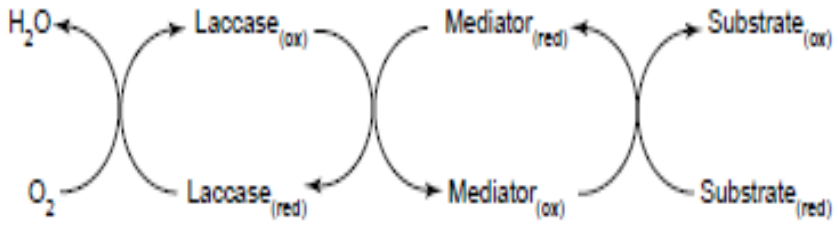

Figure 2.5: Laccase-mediator catalysed substrate reaction [Riva *et al.*, 2006]

2.2.1 Laccase enzyme in nature

Laccase enzyme is found widely in plant kingdom, almost every fungi, insects and prokaryotes and can be extracellular or intracellular depending on the organism. Laccases has different role in different organisms: pigmentation of fungal spores, tobacco plant protoplast regeneration, protection against some virulance factors (pyhtoalexin and tanins), cell wall lingification. White rot fungi utilizes laccase in delignification [Mayer *et al*., 2002].

Laccase is responsible for hardening and stabilizing the newly formed exoskeletons of insects [Kramer *et al*., 2001]. Laccase is also found in bacteria. *Azosipirillum lipoferum,* a plant root bacteria uses laccase in melanin synthesis also *Marinomonas mediterranea* a marine bacteria was found to be containing a laccase but the role of the enzyme is still undetermined [Sharma *et al*., 2008].

2.2.2 Laccase enzyme in technology

Due to laccases high nonspecific oxidation potential many biotechnological methods were developed to exploit the enzyme. Direct oxidation of phenolic compounds were exploited for industrial wastewater treatment. When laccase oxdises phenolic compounds they became polymerized and insolube in water. Thus simple filtration is enough to separate the polyphenolic compounds from wastewater [Alcalde *et al*., 2006].

Laccases use in environmental protection and control is the extensively studied. There have been many successful studies on the enzymatic treatment of wastewater [Couto *et al*., 2006, Zille *et al*., 2003, Nyanhongo *et al*., 2002] and laccase biosensors for phenolic compound detection [Kulys *et al*., 2002, Yaropolov *et al*., 2004, Roy *et al*., 2004, Odacı *et al*., 2006, Gomes *et al*., 2003].

Laccase is also used in the food industry. in stabilization of beverages (fruit juices, wine and beer) and on cork stoppers for wine bottles to diminish the cork taste from wine [Conrad *et al*., 2000]. Use of laccase in baking resulted in improvement on the properties of the dough and allowed easier machine handling [Minussi *et al*., 2002].

Main uses of laccase is textile industry, dye and painting industries in dye decolorisation, denim finishing, cotton bleaching, rove scouring, dye synthesis and anti-shrinking treatments and in pulp and paper industry for delignification of wood [Couto *et al*., 2006, Bajpai *et al*., 1999].

2.3 Polytetrafluoroethylene (PTFE)

PTFE is a synthetic fluoropolymer of tetrafluoroethylene that is used widely in both industry and daily life and is also known as it's Dupont brand name TEFLON^{tm}. PTFE consists of only carbon and fluorine atoms and there bonds are the strongest ones in organic chemistry. It's many useful properties that makes it an excellent tool for many fields. PTFE's low friction coefficient, high temperature, electric and chemical resistance, hydrophobicity, non-flamability and most impartantly unreactivity. PTFE has a melting point of 342 ^{o}C. Flourine atoms conformation around the carbon backbone (Figure 2.6) gives the non-polar state of PTFE by balancing the charge of the molecule. The high energy bonds and non-polar structure is the reason for PTFEs chemical inertness. (Plunkett., 1941).

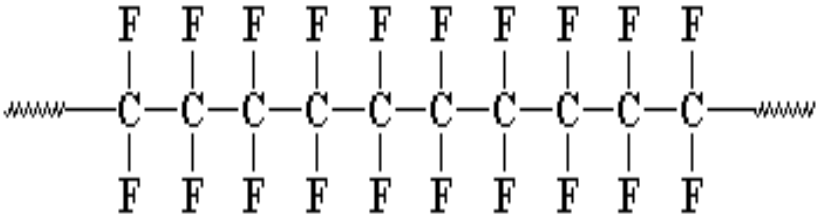

Figure 2.6: Structure of PTFE

PTFE was accidentally discovered while Roy Plunkett was trying to invent a new type of chlorofluorocarbon refrigerant. PTFE polymerized in the experiment container. Due to its excellent properties PTFE is widely used from mechanical joints to household applications, surgical tools to simple tapes. There two major ways to modify the surface and change the properties of PTFE like chemical and plasma discharge modifications. These processes allow the PTFE to be used in different areas requiring the bulk properties of PTFE but limited by its surface properties. PTFE modification is extensively studied for new areas of use. These studies were reviewed by [Hu *et al*., 2002]. As plasma discharge was the method of choice in this study, more detailed information about this technique is given in the following sections.

2.4 Glow discharge modification

Essentially plasma is an electrically neutral system composed of highly charged positive and negative ions it shows collective behavior in the presence of an electromagnetic field. Gases used to generate plasma exhibits very different chemical and physical properties that their original form. Plasma can be influenced by magnetic and electrical fields, it is conductive and shows a collective behavior. For these reasons plasma is considered to be the fourth state of matter [Gomathi *et al*., 2008]. Glow discharge is used in many fields to change the surface properties of a material without affecting the bulk characteristics of the material. These materials can be metals, polymers or organic compounds [König *et al*., 1999].

2.4.1 Plasma discharge modification

There are three main reactions in plasma generation. The gas serves as environment for the electrical discharge. When the high energy is applied the internal energy of gas atoms reaches a higher state with the reaction called excitation. When excited atoms starts colliding with other atoms causing loosely bound electrons to start to disperse from the atoms. This reaction is called ionization and is the source of charged ions present in plasma (Figure 2.7).

The third reaction is called dissociation and is the basis of plasma modification. When these positive or negative ions hit the surface of the substrate high energy results in bond breakage. These reactions makes the surface modification possible [Guerin *et al.*, 2002]. When these charged molecules collide with the surface the cause disruptions at the molecular level and change the surface properties [Kaplan *et al.*, 1993].

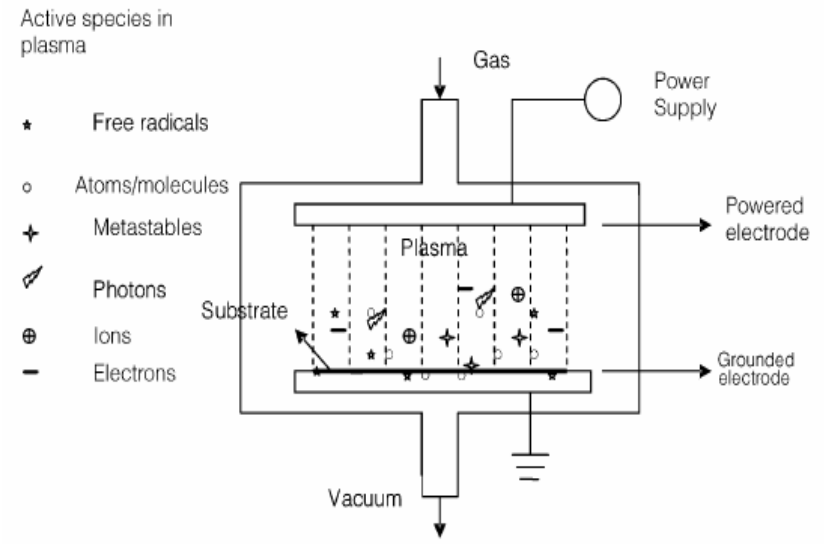

Figure 2.7: Plasma composition [Gomathi *et al.*, 2008]

2.4.2 Plasma surface modification

Energy generated by plasma is enough energy to break the covalent bonds on the exposed surface. Glow discharge can be achieved by using reducing, noble, active, fluorinated, polymerizing and oxidizing gases. Type of the surface depends on the surface composition and the gas used. Plasma modification is used in many fields to achieve different goals.

Table 2.1: Plasma gasses and their applications

Plasma gas	*Application*
Reducing gasses (H_2 and mixtures of H_2)	Removal of oxidation sensitive materials, Replacement of F or O on the surface
Oxidizing gases (O_2, air, H_2O, N_2O)	Organic contaminant removal and generating oxygen species
Active gases (NH_3)	To generate amino groups
Noble gases (Ar, He)	To generate free radical on the surface for crosslinking or to generate active sites for future modifications
Polymerizing gases (monomers)	To generate a layer of polymers on the surface
Fluorinated gases (CF_4, SF_6 and other perfluorinated gases)	To make surface inert and hydrophobic

Removal of surface contaminants

Plasma can be applied to remove surface contaminants like fingerprints, oxide layers and chemicals from air and microorganisms [Krüger *et al.,* 1999]. Different gases can be used to achieve different results. For removing simple contaminants noble gases are used but for organic contaminants oxygen can be used to oxidize the organic contaminants on the surface or hydrogen can be used to reduce the oxides and sulfides from the surface [Lee *et al*., 2007, Krüger *et al*., 1999]. Therefore choice of gas depends on the contaminant and the surface. But using plasma for surface decontamination leads to surface modification. The radicals generated on the surface reacts with plasma and create new properties.

Etching

Etching is used for remove a thin layer from the surface. Plasma selectively removes surface material by physical sputtering or chemical reactions. Etching increases the surface energy and roughness. Therefore increasing adhesive strength and surface area. This process allow liquids to wet or penetrate the surface of the material [Hu *et al*., 2002, Kim *et al*., 2000, Coates *et al*., 1996].

Chemical group substitution

By using the gas with desired chemical group the chemical groups on the surface of the material can be modified by plasma using active gases. The chemical groups on the surface is substituted with the chemical groups in plasma gas such as carbonyl, hydroxyl, amino, carboxylic or peroxyl groups [Pringle *et al*., 1996, Sarra-bournett *et al*., 2006, Trigwell *et al*., 2006, Kang *et al*., 1999, Wilson *et al*., 2001, Caro et al., 1999]. Functional group substitution increases the surface reactivity and energy.

Plasma induced grafting

Plasma induced grafting is achieved by using a different approach. First by incorporating reactive sites or functional groups on the surface of the material.

For this purpose inert gases are used to generate free radicals. When monomer is introduced to the material surface their reaction with with free radicals leads to grafting of material. Grafting of material yields increased adhesion and wettability [Kang *et al*., 1999, Matsuda *et al*., 2006, Jin *et al*., 2008, Sun *et al*., 2005]. This process incorporates new material to the surface rather than just modifying the surface [Coates *et al*., 1996].

Plasma polymerization

In this process the monomers are present at the plasma treatment of the material. When plasma is applied high energy of plasma also activates the monomers present. Free radicals initiate polymerization by incorporating themselves in to monomers. Increased molecular weight of the monomer leads to deposition onto surface of the material [Sarra-Bournett *et al*., 2006, Chen *et al*., 2008, Zettsu *et al*., 2007, Coates *et al*., 1996, Sun *et al*., 2005].

2.5 Biosensors

Biosensors are analytical devices that convert a biological signal into a quantifiable or processible signal. It has two important components: A biological component sensing the presence of the analyte and the transducer converting the biological signal into a readable signal. Basically the analyte that binds or reacts specifically with the biological elements which can be an enzyme, tissue, antibody, microorganism, cell or organelle There are lots of different transducers which could detect different changes occurring as a result of the action of biological element and these were summarized in section 2.5.1 [Vo-Dinh *et al*., 2000]. The interface between these two element allows the transducer to pick up the reaction or interaction signal (Figure 2.8). The first biosensor was constructed by Clark and Lyons in 1962. The enzyme glucose oxidaze (the biological element) was immobilized on to an oxygen electrode (transducer). This biosensor measured the current generated by the glucose oxidaze reaction at a constant potential.

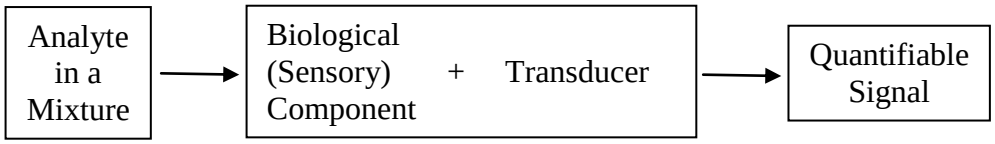

Figure 2.8: A schematic representation of biosensors

Biosensors are versatile tools for quantitative or qualitative analysis. But the succes 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].

The successful immobilization of the biological element is also very important to construct a biosensor. Immobilization is a method for binding the enzyme or bioelement onto the surface of the construct. There are number of ways to achieve immobilization; physical adsorption, covalent binding, encapsulation, entrapment and cross-linking. The immobilization method is depended on the nature of the bioelement, type of transducer, substrate and the conditions of the measurement [Mello *et al*., 2002].

Physical adsorbtion

Physical adsorbtion is the simplest of all immobilization methods. In this method the bioelement is simply adsorbed onto the surface material by weak wan der waals, ionic and hydrogen bonds. This type of immobilization is reversible. However bioelements are not subjected to activation or chemical modification. Therefore the structural integrity of the bioelement is intact.

The disadvantage of this method is the leakage of bioelement with time. Due to the interaction by weak bonds, bioelements may detach from the surface if a change occurs (pH, temperature, ionic strength, substrate concentration, rapid movement) in the reaction medium [Scheller *et al*., 1992].

Covalent Binding

In this method the bioelements are immobilized by forming covalent bonds between bioelement and the surface. Covalent bonds between surface and biomolecule can be created by activation functional groups on the amino acids. Amino groups or carboxyl groups can be used to form covalent bonds. Covalent bonds are strong and extends the bioelements stability. However to achieve covalent bonding the bioelement or the surface must be activated by chemical or physical treatment. [Scheller e*t al.*, 1992, Zhavnerko *et al., 2004*]. Disadvantage of this method is the possible loss of activity due to the chemical modification of the biomolecule.

Encapsulation

In encapsulation method the bioelements are enveloped in a membrane that allows diffusion of substrates. Diffusion is limited by the size of the substrates, the porosity of the membrane and chemical characteristics of both. Small substrates and the products can pass through the membrane more easily than the large substrates. Poor design may cause the accumulation of products or slow diffusion of the substrate. The advantage of this method is the ability to co-immobilize different bioelements [Vastarella e*t al*., 2002].

Entrapment

Entrapment method is similar to the encapsulation method but in this case the bioelements are confined in a matrix rather than a membrane [Vastarella e*t al.*, 2002].

Crosslinking

Cross linking method involves the formation of covalent bonds between the bioelements without the need for support material. When the bioelements form covalent bonds in-between, they become a large and complex structure. Cross linking can be achieved by physical and chemical methods. The bioelements can be immobilized physically by flocculation. This technique is widely used in industry. Chemical method involves the use of chemical agents to form covalent bond between bioelements. Cross linking method is a simple end effective method but since the covalent bond formation is random, the catalytic site of the bioelement may become inoperable or excessive cross linking may leads the loss of activity [Vastarella *et al.*, 2002].

2.5.1 Types of biosensors

The biosensors can be classified based on their bioelement or transducer. When bioelements are concerned, two categories can be given: catalytic biosensors and affinity biosensors. In catalytic biosensors, the bioelement which can be an enzyme, cell, etc, a catalytical activity takes place so there is a change in the concentration of some compounds. In affinity sensors, including oligonucleotide or antibody sensors, the analyte binds the bioelement based on complementarity or affinity, so there is no catalytic activity.

When it comes to transducers, the activity of the biological element can be monitored by several ways such as oxygen consumption, hydrogen peroxide formation, changes in NADH concentration, fluorescence, absorption, pH change, conductivity, temperature or mass. Biosensors can also be classified according to the mode of their transducers (Figure 2.9) [Mello *et al*., 2002].

Optical biosensors

In this type of biosensor, the signal of the reaction is light. Basically immobilized bioelement cause a change in the medium in the presence of the analyte and this changes the interaction of light with the solution. This change is measured and correlated to analyte amount. Optical detection biosensors can be based optical diffraction or electrochemiluminescence [Scheller *et al*., 1992].

Piezoelectric biosensors

In this type of biosensors, a crystal which can oscillate under electrical voltage is used. Physical adsorbtion on this crystal results in change in the mass and consequently in its vibration frequency. Piezoelectric sensors are used for the measurement of ammonia, nitrous oxides, carbon monoxide, hydrocarbons, hydrogen, methane, sulfur dioxide and certain organophosphate compounds as chemical sensors. To be able to construct a biosensor, and antibody, a receptor or a DNA molecule can be attached to the surface of the crystal and the binding of specific ligands or complementary strands can be detected [Tombelli *et al*., 2000].

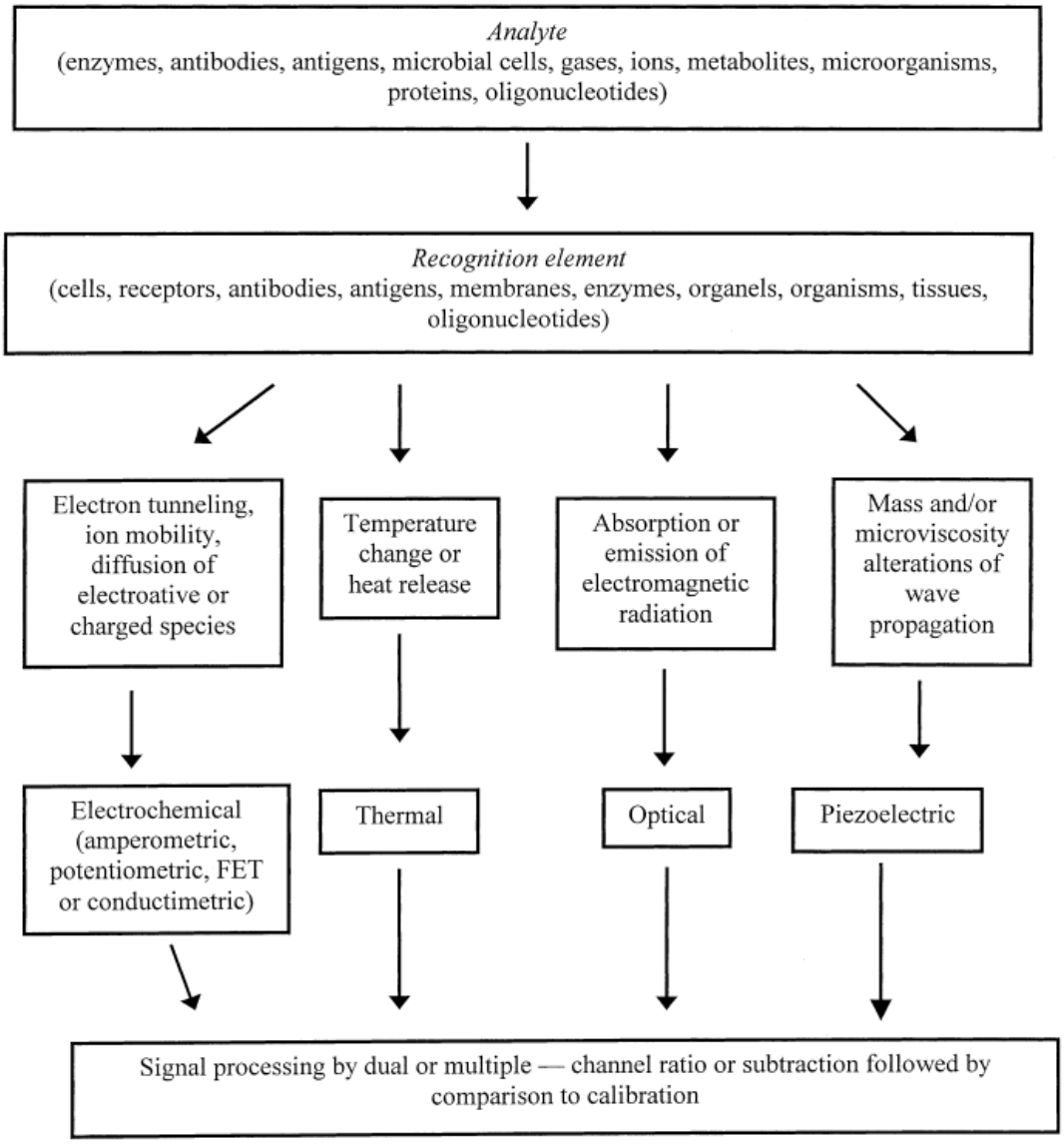

Figure 2.9: types of biosensors [Mello *et al*., 2002]

Thermal biosensors

Thermal biosensors are based upon the fact that all biological reactions produces or absorbs energy thus heat. When bioelement reacts with the substrate, a change in medium temperature occurs due to the heat released or absorbed and this can be quantified by thermostators [Buerk *et al*., 1993]. But these biosensors have low sensitivity because the heat generated wasted by irradiation and conduction [Mello *et al*., 2002]

Ion sensitive biosensors

in this case, the change in potential as a result of enzymatic activity is measured . Semiconductor transistors are used in this biosensor type. pH measurement is an example to this type of application [Buerk *et al*., 1993].

Electrochemical biosensors

Most chemical reaction consumes or generates ions or electrons, thus change the electrical properties of the medium. This fact is exploited by measuring these changes in the solution. There are three parameters, namely conductimetric, amperometric, potentiometric to measure for these changes and these parameters also give their names to the biosensors.

Conductiometric biosensors measure the electrical resistance or conductivity of the solution. The ions and electrons produced or consumed by the reaction changes the electric properties of the solution. Conductimetric biosensors are usually have a poor signal noise ratio [Mello *et al*., 2002]. Amperometric biosensors can detect electroactive species produced by the action of the bioelement. They are based on monitoring the current related with the oxidation or reduction of an electroactive species [Freire *et al*., 2001]. Potentiometric biosensors measures the oxidation or reduction potential of the electrochemical reaction. The information is gathered by converting the reaction process into a potential signal.

The most popular biosensors are amperometric and potentiometric biosensors. Amperometric biosensor are more sensitive and have a wider linear range than other types of biosensors. However, activity of these devices are limited to the redox potential of the substrate [Mello *et al*., 2002].

2.5.2 Laccase biosensors

Laccase biosensors are used for detection of phenolic compounds in wastewater, human plasma, wine and beverage samples. When laccases react with phenols, they convert molecular oxygen into water. Laccase biosensors are proposed to be an alternative for HPLC and spectrophotometric methods and have been extensively studied using various immobilization and co-immobilization methods [Freire *et al*., 2001] and many biosensors have been developed in the past for phenolic compound detection using gold surfaces, modified polymers and modified electrodes [Kulys *et al*., 2002, Yaropalov *et al*., 2004, Gupta *et al*., 2002, Gomes *et al.*, 2003, Odacı *et al.*, 2006, Vianello *et al.*, 2005, Mousty et al., 2007, Roy *et al.*, 2004, Abdullah *et al* 2007, Liu *et al.*, 2008, Fernandes *et al*., 2009, Li *et al*., 2005, Júnior et al., 2008, Kim et al., 2003].

3. MATERIAL AND METHODS

In order to achieve the objective of the study, four goals were set. First goal was the measurement of the free laccase activity and investigation of its optimum working conditions. The second goal was the construction of the conventional gelatin entrapment biosensor and it's the optimization. Third goal was to generate the functional groups on the inert surface of the PTFE by plasma treatment and the fourth and final goal of the study was the immobilization of the enzyme on grafted PTFE and the optimization of the immobilized systems activity.

3.1 Materials and equipment

3.1.1 Equipment

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

3.1.2 Buffers, reagents and enzymes

The compounds and enzymes used are listed in Appendix B with their suppliers. The recipes of the buffers used are given in appendix C.

3.2 Methods

3.2.1 Preparation of enzyme stocks

Laccase enzyme isolated from Trametes versicolor (Sigma 53739) was dissovled in (pH 7.0, 0.1 M, 1 ml) phosphate buffer. Prepared stock was aliquoted into 20 small eppendorf tubes (50 microliters each) and stored at -20 ^{o} C until use in order to minimize the enzyme denaturation which may be originated from freeze-thaw cycles, Each tube had 50 mg/ml (1120 units/ml) laccase enzyme.

3.2.2 Determination of free laccase enzyme activity

3.2.2.1 Activity measurement by using oxygen electrode unit

The oxygen electrode unit (Hansatech Instruments) was used to measure the immobilized and free enzyme activity (Figure 3.1). As shown in the figure, the unit has a water jacket to keep the temperature in the reaction vessel constant. The other advantage of the unit is that small volume of sample are enough (200-2000 μL) for measurements. Oxygen electrode measures the decrease in molecular oxygen content (nmol/ml). For each oxygen molecule consumed 4 phenolic molecules was oxidized. The device calibration was done before each experiment. To do this, first maximum molecular oxygen content in reaction chamber was measured after continuously aerating the reaction chamber with air and considered as 100 % oxygen saturation point. Then sodium dithionite which consumes the oxygen in the reaction chamber was added to the medium and the minimum point was assigned as zero oxygen concentration. The decrease in the oxygen amount that originated from the enzyme activity was given as percentage.

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

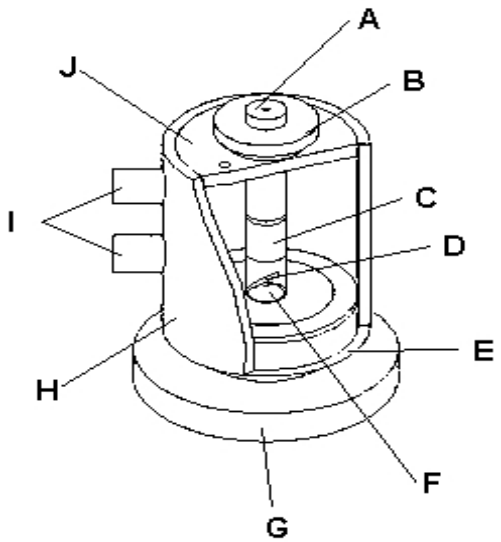

Figure 3.1: Oxygen electrode unit (Hansatech manuel)

To find the response of the biosensor to different substrates, immobilized enzyme system was treated with various substrates; Guaiacol (Sigma), chlorophenol (Aldrich) and cathecol (Sigma). Optimum working pH and temperature of the free enzyme was found by using these substrates.

3.2.2.2 Measurement by using 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) assay

Some of the activity measurements were done using ABTS as substrate. The product of the reaction can be measured spectrophotometricly at 420 nm. After the addition of ABTS to laccase solution, samples were taken in 30 s intervals for 5 minutes and their absorbance were measured at 420 nm. The measurements gave semi-quantitavie information about the reaction.

3.2.3 Plasma graft polymerization of PTFE

PTFE is s non-reactive polymer. Due to the high energy bonds between carbon and fluorine atoms PTFE is inert and hydrophobic so unsuiatable for biosensor construction without modification. In order to immobilize laccase on PTFE surface, PTFE membranes were cut into 1.5 x 2.0 cm pieces and treated with RF plasma. Two different polymers, polyacrylamide and polyacrylic acid, were grafted on the surface of the PTFE by plasma treatment and their potential in the construction of the biosensor was analyzed.

3.2.3.1 Plasma polymerization of polyacrylamide (pAAm) to PTFE

PTFE samples were grafted with polyacrylamide using a two step polymerization method. Samples were first washed with acetone for 5 min to clean the surface from the contaminants and then treated with hydrogen plasma (125 W, 13 Pa, 2 min) to increase surface wettability Hydrogen plasma treated samples were exposed to air for 2 hours to generate the necessary peroxide groups for plasma polymerization.

In the second step, 100 µL of acrylamide solutions (25 % w/v and 50 % w/v in 50 % (v/v) acetone/ethanol) was spread over the samples and allowed to dry at room temperature for 15 min. Dried samples were treated with inert argon plasma (50 W, 13 Pa, 1 min) to initiate grafting of polyacrylamide onto PTFE. Treated samples were taken out and washed in dH_2O in orbital shaker overnight (200 rpm) to remove unbound polyacrylamide from the surface and dried in vacuum for 2 hours at room temperature. Dried samples were analyzed with FTIR to confirm pAAm layer on the PTFE and new membranes were named as pAAm-g-PTFE.

3.2.3.2 Grafting of polyacrylic acid (pAAc) to PTFE

In order to graft polyacrylic acid, PTFE samples (1.5x2 cm), samples were washed with acetone for 5 min to remove any contaminants and were treated with inert argon plasma (50 W, 13 Pa, 1 min). The high energy generated by argon plasma results in bond breakage of C-F bonds. Since argon plasma is inert, the broken bonds do not reform with argon. The samples were then exposed to air to generate the hydroperoxides and peroxides which acts as a initiator and a binding site for acrylic acid monomers. After plasma treatment the samples were immersed in acrylic acid solutions (30 % in dH_2O and ethanol (v/v)). The effect of temperature, oxygen, solvent and initiators were investigated. To initiate polymerization of acrylic acid, samples were placed in a constant temperature water bath (50-80 ^{o}C) for 6 hours. After the polymerization was completed, samples were taken out of the bottle and washed with dH_2O overnight in orbital shaker (200 rpm) to remove weakly bound polyacrylic acid from the surface. After drying samples in vacuum for 2 hours at room temperature, the samples were analyzed with FTIR to confirm the pAAc layer on the surface and new membranes were named as pAAc-g-PTFE.

3.2.4 Biosensor construction

In this study, 3 different immobilization method was tried for biosensor construction. Gelatin entrapment is well-known, easy and cheap method for biosensor construction. Therefore the gelatin entrapped biosensor was chosen as a control group. The other two biosensors were the laccase immobilized pAAm-g-PTFE and the laccase immobilized pAAc-g-PTFE.

3.2.4.1 Gelatin entrapment

Gelatin (5, 7.5, 10 mg) was dissolved in 200 μL phosphate buffer (0.1M, pH 6.0) at 38 ^{o} C. Before gelatin hardened, laccase enzyme (5 and 1 units) was added. The solution was put (1 cm2) to the surface of the PTFE (1.5 x 2 cm) and stored at 4 ^{o} C for 1 hour. When the gelatin completely dried and hardened, it was immersed in glutaraldehyde (2.5, 5, 7.5 %) for 4 min to crosslink the gelatin matrix. The construct was washed twice with dH_2O for 5 min to remove glutaraldehyde and unbound enzymes and stored at 4 ^{o}C until measurements.

3.2.4.2 Immobilization of laccase on pAAm-g-PTFE membranes

To immobilize laccase on pAAm-g-PTFE, carbodiimide-succinimide reaction was used. Amine groups on PTFE generated by plasma polymerization was covelently bound to the carboxyl groups on the enzymes using N-(3-dimethyaminopropyl)-n-ethyl-carbodiimide (EDC) and N-hydroxy-succinimide (NHS).

For covalent immobilization, 10 units of laccase enzyme were used. First the amount of carboxyl groups on the enzyme was calculated and EDC concentration was adjusted to active 10 % of these carboxyl groups (0,735 μmol). Half of that amount (0,360 μmol) was used for NHS (10:1:0.5). Several activation percents were tested.The immobilization reaction was carried out in MES buffer (0.1 M, pH 5.5, 5 ml) overnight at 4 ^{o}C with continuous shaking (40 rpm). The samples were washed twice with dH_2O and stored at 4 ^{o}C until measurements.

3.2.4.3 Immobilization of laccase on pAAc-g-PTFE membranes

Acrylic acid is a carboxylic acid, thus pAAc-g-PTFE surface has free carboxyl groups. Therefore during the covalent immobilization of laccase on PTFE, first the membrane surface could be modified and then enzyme would be incubated with activated surface. In this case, EDC was dissolved in PBS (5mg/ml; 0.1 M, pH 7.4) and pAAc-g-PTFE was immersed in this solution at 30 ^{o}C for 1 hour to activate the carboxyl groups present on the surface.

After activation, the samples were washed twice with dH2O to remove unbound EDC from the surface. To immobilize laccase, samples were immersed in PBS (0.1 M, pH 7.4) and 10 units of laccase was added to the solution. The immobilization reaction was carried out at 4 ^{o}C overnight with continuous shaking (40 rpm). The samples were washed twice with PBS (0.1 M, pH 7.4) and stored at 4 ^{o}C until measurements.

3.2.5 Detection of enzyme amount

For immobilized systems the effect of enzyme amount was investigated. After immobilization reactions, the unbound enzyme amount in the solution of immobilization reaction can be quantitatively analyzed by Bradford protein assay. But for gelatin entrapped system and pAAm-g-PTFE immobilized system final reaction solutions contained Glutaraldehyde and EDC respectively. EDC and glutaraldehyde were interfering substances for Bradford protein assay [Bradford *et al.,* 1974]. Therefore gelatin entrapped system and pAAm-g-PTFE immobilized systems were unsuitable for conventional protein assays. To observe the effect of increased enzyme amount, the activity of the constructs was presumed to be the indicator for immobilized enzyme amount. pAAc-g-PTFE immobilized systems final reaction solution contained only the unbound enzymes, thus the immobilized enzyme amount were calculated by Bradford protein assay quantitatively.

3.2.6 Characterization of immobilized systems

The immobilized enzyme systems were characterized by observing the activity at different pH and temperature values and optimum working conditions were determined.

3.2.6.1 Determination of optimum working temperature

The optimum working temperatures of pAAm-g-PTFE and pAAc-g-PTFE immobilized systems were investigated by using oxygen electrode unit. For gelatin entrapped system ABTS assay was used (section 2.2.2.2). The immobilized systems' activity was measured at temperatures between 25 – 50 ^{o}C.

3.2.6.2 Determination of optimum working pH

For the detection of optimum working pH of the immobilized systems, ABTS and oxygen electrode unit was used. The activities of the constructs were measured between pH's 3.5-7.0 using 400 μM guaiacol as substrate.

3.2.6.3 Determination of reusability

In order to evaluate the reusability of the immobilized systems, the activities of the constructs were measured at optimum working conditions by using guaiacol (400 μM) as substrate repeatedly until the activity decreased to 50 % of the first activity.

3.2.6.4 Storage stability of immobilized systems

One of the main objectives of the immobilization is to extend the life of the enzymes. Therefore storage stability of the immobilized system is very important for a successful biosensor. To investigate the storage stability, thus the shelf life of the immobilized system, prepared constructs were stored at 4 ^{o}C in PBS. For each time point, two of these constructs were taken and their activity was measured at their optimum working conditions with 400 μM guaiacol.

4. RESULTS AND DISCUSSION

4.1 Measurement of free laccase activity

4.1.1 Activity of laccase with various substrates

Laccases are known to use different phenolic compounds as substrates. Three of these substrates, guaiacol, chlorophenol and cathecol, were chosen to determine the optimum working conditions and potential analytes for the enzyme in 2 different enzyme concentration, 1 and 5 units. All experiments were done in acetate buffer (0.1 M, pH 5.0) at 45 ^{o}C.

Free laccase activity in low enzyme concentration (1 unit) was measured using chlorophenol (Figure 4.1) and guaiacol (Figure 4.2) as substrates. Guaiacol and chlorophenol was injected into reaction chamber of the oxygen electrode unit at different concentrations to examine the change in reaction rates. The relevant working range is from 100 μM to 400 μM in our study due to the phenol discharge standards.

When Figure 4.1 and Figure 4.2 was compared, it can be seen that the reaction rate for guaiacol was considerably higher than that of chlorophenol.For 200 μM of substrate concentration, activity was found to be 80 nmol O_2 consumed/mL/min for guaiacol while it was 4.5 for chlorophenol, which is ca. 18 times more. This was believed to be the result of laccases ability to oxidize different substrates at different rates. Chlorophenol molecular structure is more complex and bigger than the guaiacol, thus chlorophenol is oxidized more slowly than guaiacol But the activities obtained by 1 unit of enzyme was very low so 5 units of enzyme was used in further studies.

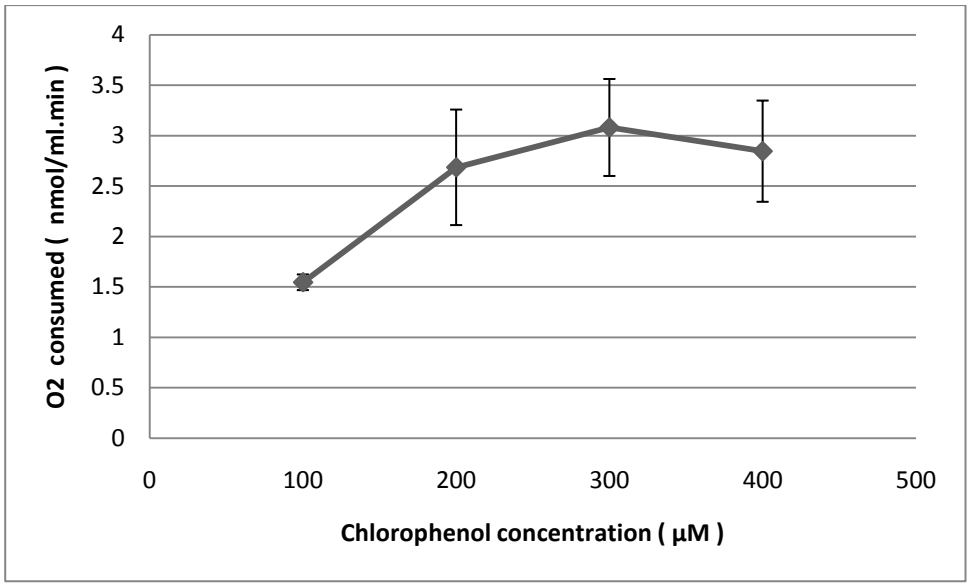

Figure 4.1: Free laccase activity (1 unit) for chlorophenol

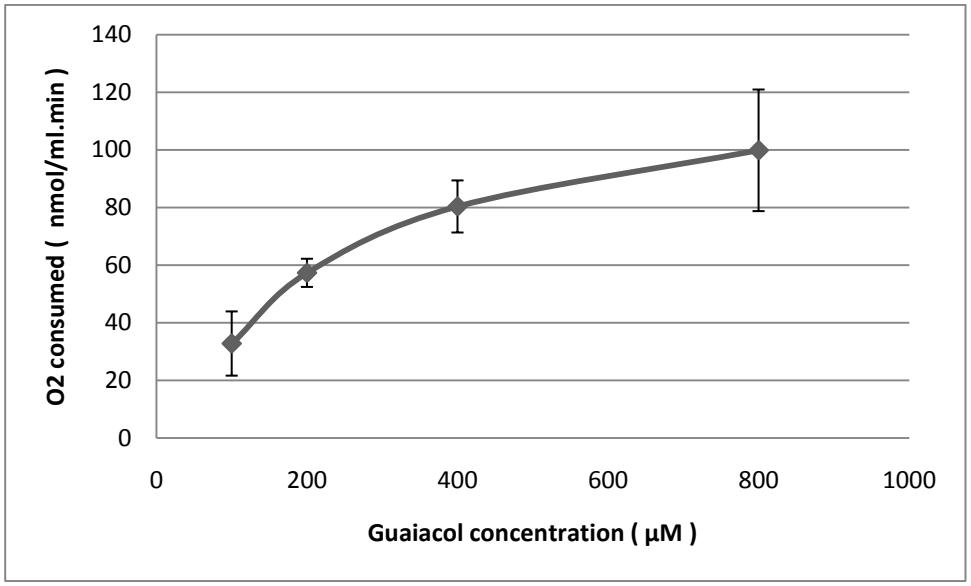

Figure 4.2: Free laccase activity (1 unit) for guaiacol

Cathecol was added as a new substrate in 5 units of free laccase studies and experiments were repeated using chlorophenol, guaiacol and cathecol. The results of the experiments were shown in Figures 4.3, 4.4 and 4.5 respectively.

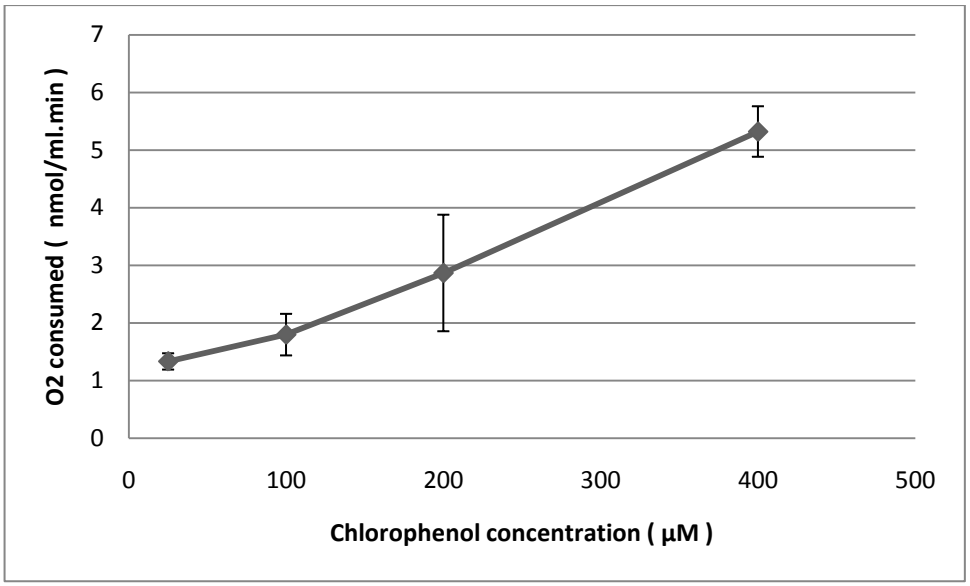

Figure 4.3: Free laccase activity (5 unit) for chlorophenol

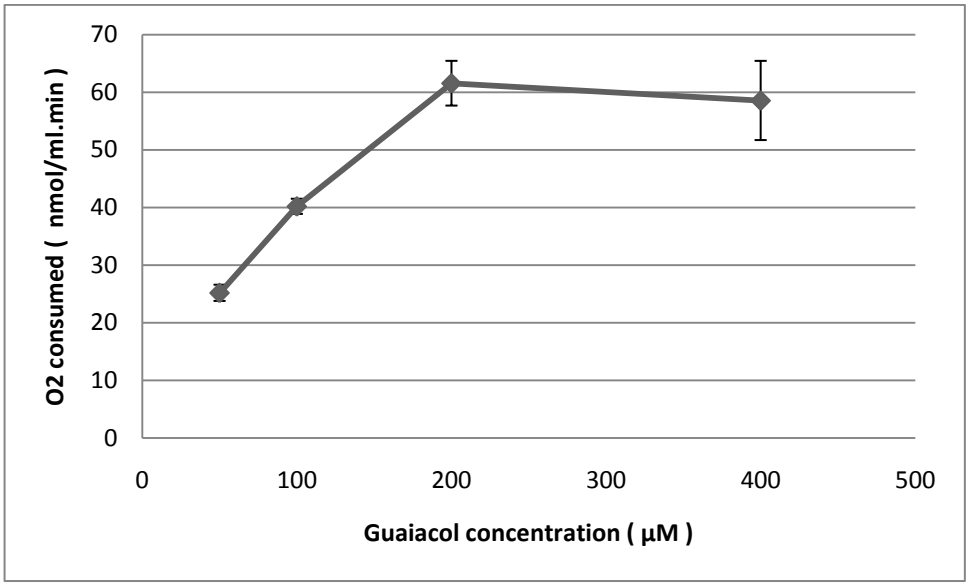

Figure 4.4: Free laccase activity (5 unit) for guaiacol

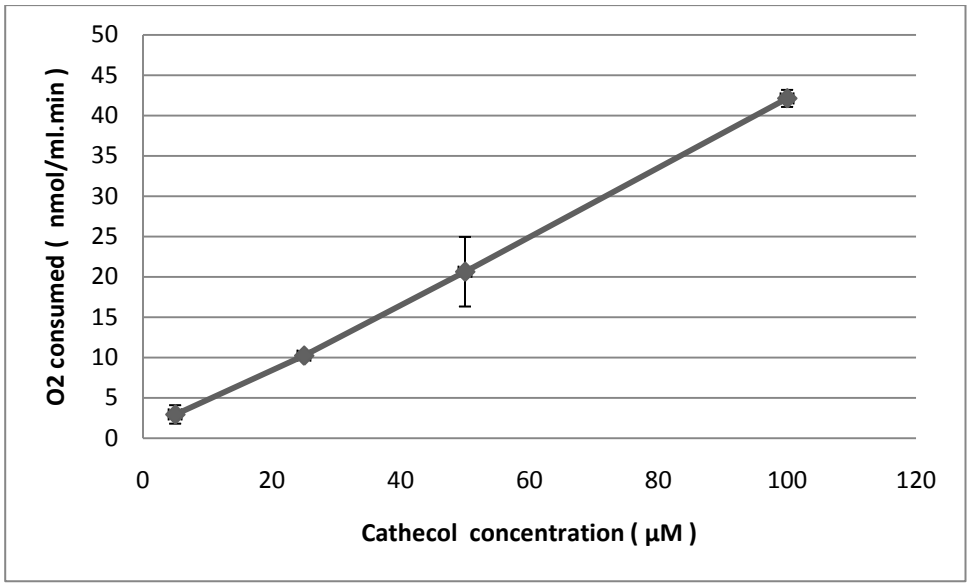

Figure 4.5: Free laccase activity (5 unit) for cathecol

Guaiacol was chosen as the model analyte for the characterization and optimization of the immobilization procedure. Chlorophenols' reaction rate is 18 times slower, that is too slow so it would be time consuming and cathecols reaction rate is 22.5 times faster than guaiacol and cathecols reaction rate is too fast for sensitive and accurate measurement due to technical difficulties. However in order to investigate the reaction rates of chlorophenol, guaiacol and cathecol with immobilized systems, the substrates were used with the gelatin entrapped system

4.2 Immobilization of laccase on PTFE membranes

To immobilize enzymes to PTFE surface, first reactive groups must be generated on the inert surface of the PTFE. An enzyme can be immobilized via a carboxyl-amine group bonding. So these reactive groups to be generated can either be amine or carboxyl groups. In our study, the surface of the PTFE was modified with two different polymers to determine the difference between amino or carboxyl group modified surface.

4.2.1 Activation of PTFE surface for enzyme immobilization

Plasma glow discharge was used to generate reactive groups by graft polymerization and plasma polymerization. Polyacrylic acid and polyacrylamide was grafted on PTFE by plasma treatment.

Polyacrylic acid is a carboxylic acid so the grafting yielded carboxyl groups on the surface while polyacrylamide grafting yielded amine groups. The presence of these groups was confirmed by using FTIR spectrophotometry.

4.2.1.1 Generation of polyacrylamide grafted PTFE membranes (pAAm-g-PTFE)

After following the modification steps mentioned in section 2.2.3.1, plasma polymerization of pAAm on PTFE was confirmed using FTIR spectrophotometry. The FTIR results are shown in Figure 4.6.

25 % (w/v) acrylamide was successfully grafted on PTFE but the grafting yield was found to be too low for laccase immobilization. Enzymes were immobilized on the grafted PTFE but the immobilized enzyme activity was too low. We believed that the grafted layer is too thin for enzyme immobilization and as a result the immobilization yield was also low (data not shown). But by using 50 % (w/v) acrylamide we achieved the thickness required for enzyme immobilization.

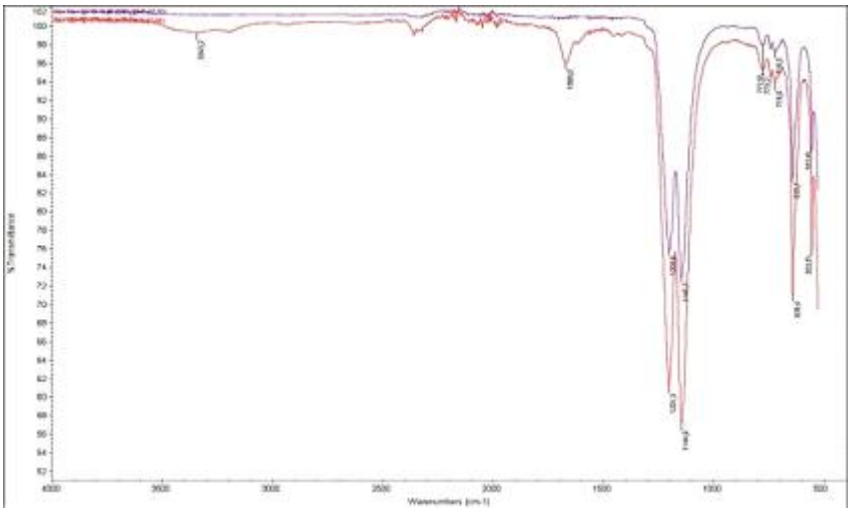

Figure 4.6: FTIR results of virgin PTFE (purple) and pAAm-g-PTFE (red)

pAAm-g-PTFE samples showed a new peak at about 1700 cm^{-1} corresponding to amine groups generated by pAAm grafting. The graph for the unmodified PTFE has peaks only prior to 1200 cm^{-1} corresponding to C-F bonds and C-C bonds.

4.2.1.2 Generation of polyacrylic acid grafted PTFE membranes (pAAc-g-PTFE)

Plasma graft polymerization procedure was mentioned in section 2.2.3.2. Before the optimization of immobilized enzyme systems, the grafting of pAAc needed to be optimized. After plasma treatments, grafting experiments were conducted. The effect of temperature, oxygen, solvent and initiators were investigated.

Firstly, oxygen is found to be an inhibitor of polymerization. Every experiment that contained even small amounts of oxygen resulted in failed grafting. The acrylic acid monomer solution needed to be vigorously degassed and held under nitrogen atmosphere.

The effect of temperature on grafting was also investigated. Between temperatures 50-80 ^{o}C. only at temperature between 60-70 ^{o}C grafting was achieved. Temperature is the initiator of grafting. We believed that 50 ^{o}C was too low for polymerization. Polymerization and grafting are competitive reactions to high temperatures results in homopolymerization rather than grafting due to increased viscosity [Wang *et al.*, 2007]. In the case of 80 ^{o}C we believed homopolymerization dominated the reaction mixture and grafting was unsuccessful.

Effect of solvent was also investigated by using dH_2O and ethanol as solvents. Experiments concluded that ethanol was not an effective solvent. Although ethanol was used successfully as a solvent for polymerization in previous studies [Njatawidjaja *et al*., 2006] our FTIR results didn't yield the expected results.

The effect of initiators was investigated by adding sodiumdithionit to the AAc monomer solution. Metal salt inhibits homopolymerization [Turmanova *et al.*, 2008] and oxygen is also inhibits polymerization. Sodiumdithionit is a metal salt, scavenges molecular oxygen. Both qualities were to our advantage. Although our grafting experiments with sodiumdithionit containing AAc solutions were successful, enzyme immobilization experiments failed. We believed that the grafted layer composition was not suitable for enzyme immobilization.

According to our experiments immersing argon treated PTFE in degassed 30 % (v/v) AAc monomer solution and placing the reaction mixture in constant temperature bath (70 ^{o}C) for 6 hours yield successful grafting. Plasma graft polymerization was confirmed by FTIR spectrophotometry and the results are presented in Figure 4.7.

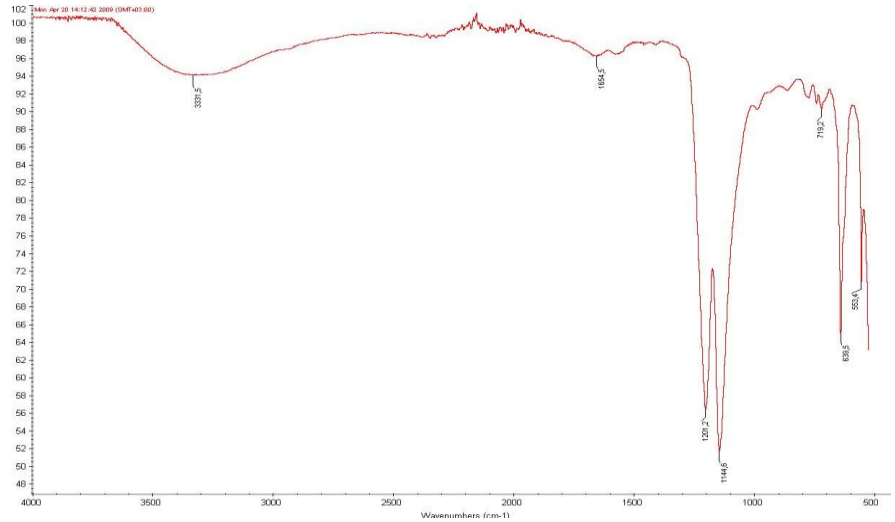

Figure 4.7: FTIR results for pAAc-g-PTFE

The pAAc-g-PTFE showed two new peaks, one at about 1700 cm^{-1} corresponding to C=O bonds and the other between 2900 cm^{-1} and 3500 cm^{-1} corresponding to O-H bonds. Acrylic acid is a carboxylic acid so this result was expected.

4.2.2 Optimization of immobilized laccase activity

4.2.2.1 Optimization of gelatin entrapment

Entrapment method is an easy and cheap method for protein immobilization. Laccase enzyme was entrapped in gelatin (section 2.2.4.1). The system was ready for use in two hours.

4.2.2.1.1 Effect of enzyme amount

The same procedure that was used for free enzyme activity measurement was followed. First 1 unit of laccase enzyme was entrapped in 10 mg of gelatin. The 1 unit laccase immobilized system showed insufficient activity for a successful immobilized system (data not shown). Low activity was believed to be the result of low enzyme amount or the small pore size of gelatin, or both. To understand what the real cause was, first the effect of enzyme amount was tested by increasing it to 5 units.

Chlorophenol, guaiacol and cathecol substrates were reacted with the (5 U, 10 mg gelatin) immobilized system (Figure 4.8, 4.9, 4.10).

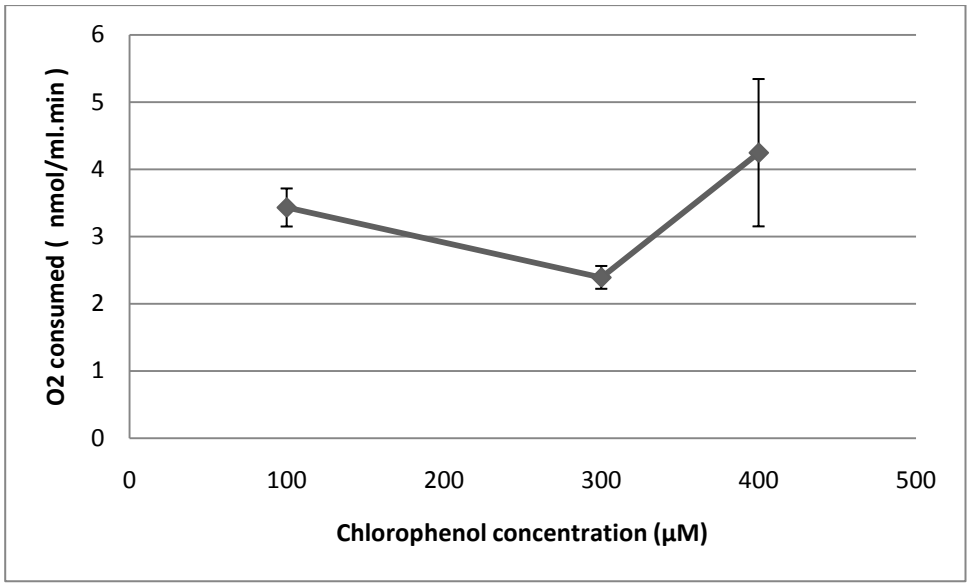

Figure 4.8: Gelatin (10 mg) entrapped systems activity for chlorophenol

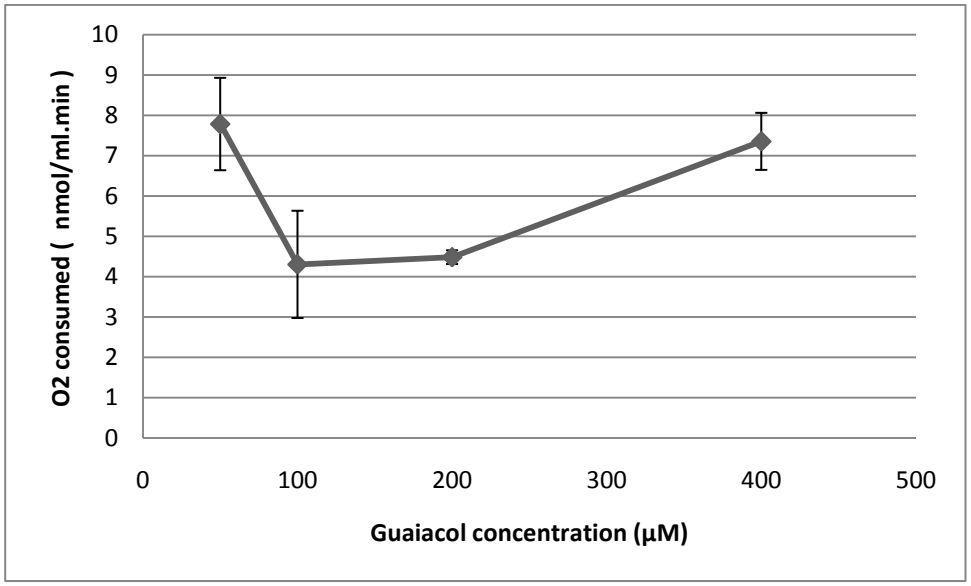

Figure 4.9: Gelatin (10 mg) entrapped systems activity for guaiacol

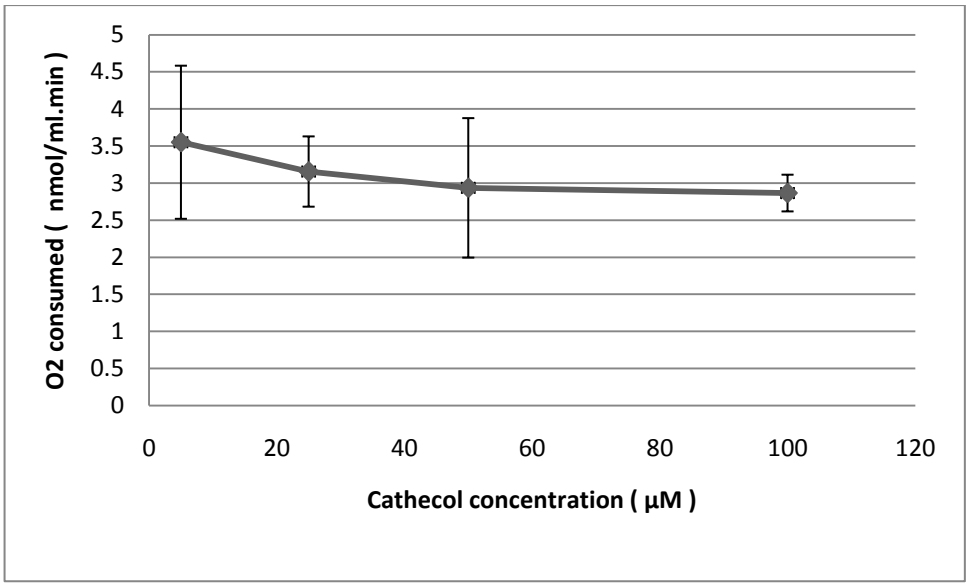

Figure 4.10: Gelatin (10 mg) entrapped systems activity for cathecol

The results concluded that the increased enzyme amount also increased the activity of the system but the system showed nearly same activity for all concentrations of the substrate used. This confirmed our assumption on the gelatin pore size: When high gelatin concentration was used, the density of the membrane increased which led to a decrease in the pore size. In this case, diffusion of substrates and products in and out of the system become limited. To solve this problem, the gelatin amount was optimized.

4.2.2.1.2 Effect of gelatin amount on immobilized system activity

To test if the gelatin amount is the limiting factor or not, 2 systems were constructed using different amounts of gelatin (5 and 7.5 mg). Lower amounts of gelatin were thought to result in bigger pore size. 5 mg of gelatin entrapped system lacked the mechanical stability and ruptured when inserted into the oxygen electrode reaction chamber.

Therefore, only 7.5 mg gelatin containing system could be tested by using chlorophenol, guaiacol and cathecol as substrates. The results of 7.5 mg of gelatin entrapped laccase experiments are shown in figures 4.11, 4.12, 4.13. The experiments were carried out in pH 4.0 at 35 ^{o}C.

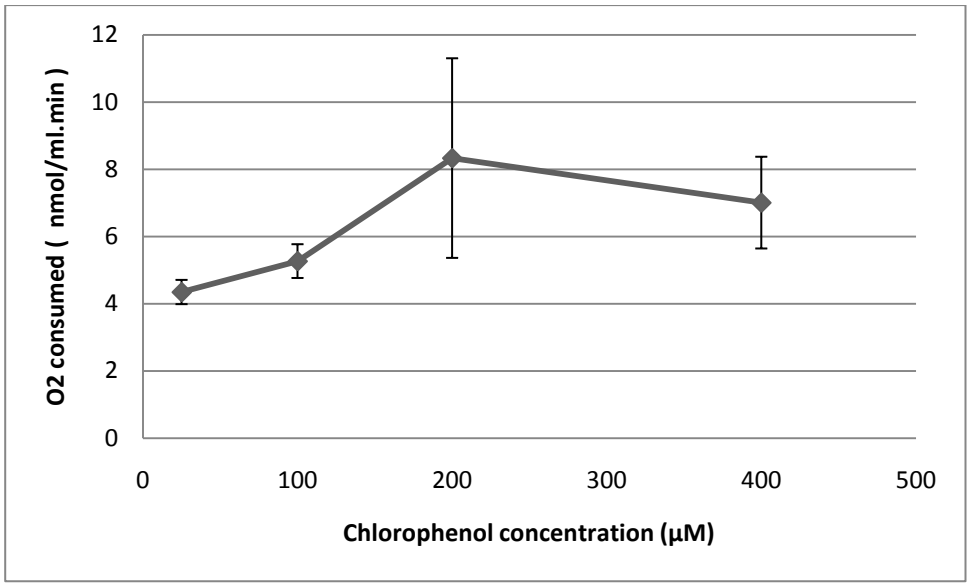

Figure 4.11: Gelatin (7.5 mg) entrapped systems activity for chlorophenol

Gelatin entrapped (5u, 7.5 mg) system reached maximum activity at 200 µM of chlorophenol. No increase was observed after this concentration.

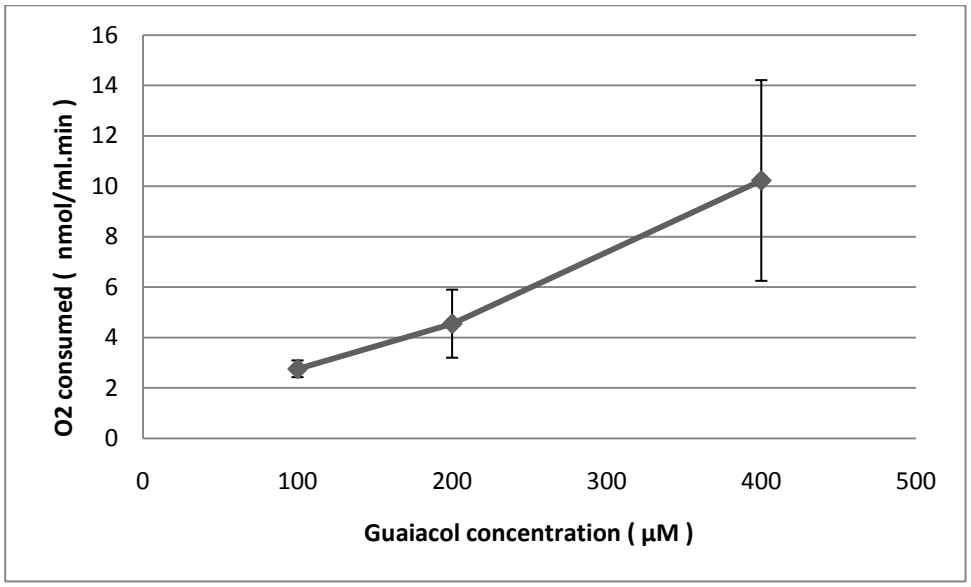

Figure 4.12: Gelatin (7.5 mg) entrapped systems activity for guaiacol

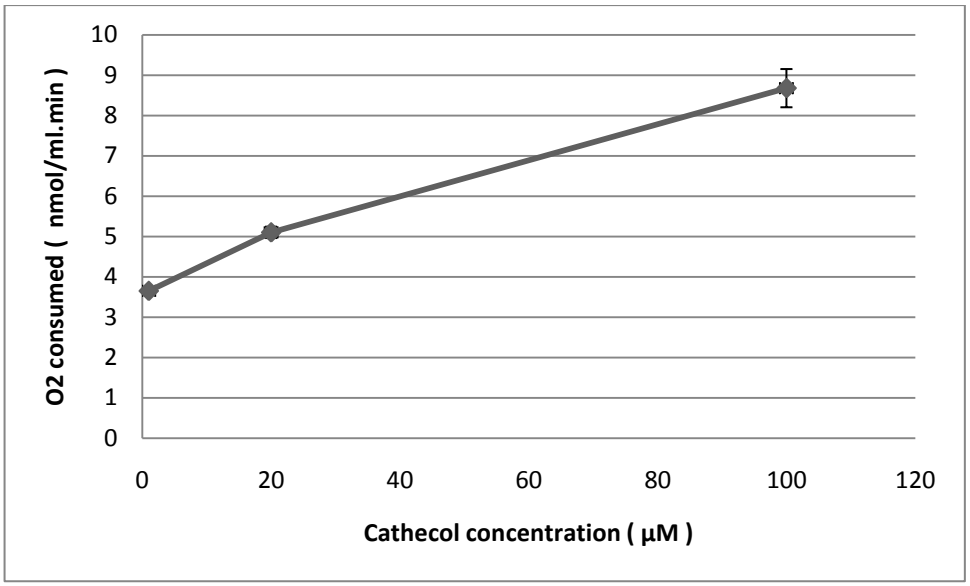

Figure 4.13: Gelatin (7.5 mg) entrapped systems activity for cathecol

The increased pore size allowed to obtain a linear increase in activity depending on increase in substrate concentration. For chlorophenol, gelatin entrapped (5u, 7.5 mg) system reached its maximum activity at 200 µM and no further increase was observed after this concentration.

With guaiacol linear activity was observed until 400 µM. Cathecol showed higher activity at very low concentrations but this high activity decreases the accuracy. According to these results, guaiacol was chosen to be the most suitable substrate for accurate and sensitive detection. After this point, characterization of immobilized enzyme systems were done only by guaiacol as substrate for reliable comparisons.

4.2.2.1.3 Effect of glutaraldehyde concentration on gelatin entrapped system

After optimizing the gelatin and enzyme amounts, the effect of glutaraldehyde concentration was also studied. For this purpose, different concentrations of glutaraldehyde (2.5 %, 5 %, 7.5 %) were used to crosslink the gelatin and enzymes. The experiment was carried out in pH 4.0 acetate buffer at 35 ^{o}C.

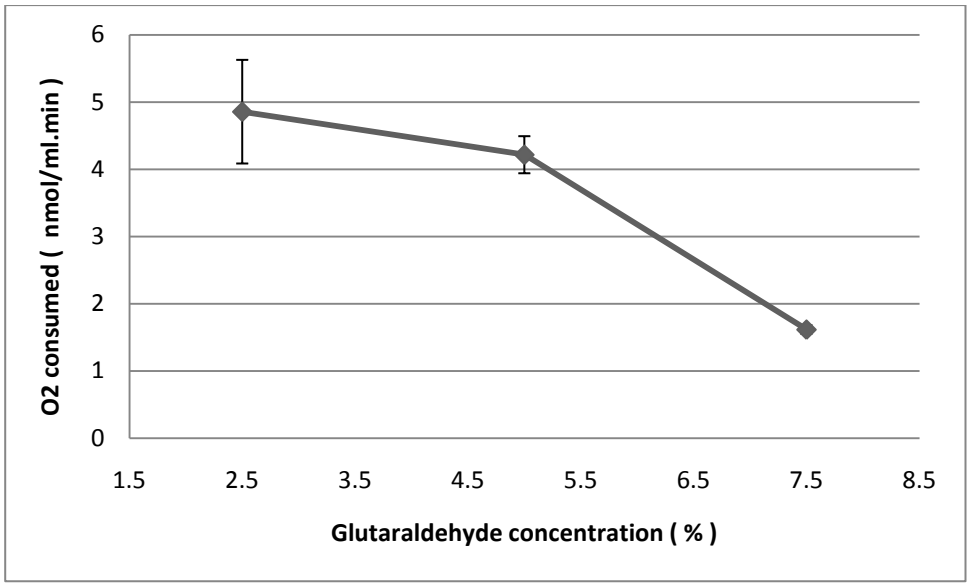

Figure 4.14: Effect of glutaraldehyde concentration on gelatin system

The results showed that (Figure 4.14) the activity of the entrapped system decreased with increasing glutaraldehyde concentration. This phenomenon was believed to be the result of the increased crosslinking of the enzyme. High crosslinking of the proteins may result in lower or loss of activity and this phenomenon is reported in literature [Freire *et al.*, 2001].

4.2.2.2 Optimization of pAAm-g-PTFE immobilized system construction

Before the optimum activity experiments, the construction of the system must be optimized. For this purpose different grafting conditions, NHS/EDC amounts and enzyme concentrations were tested.

4.2.2.2.1 Effect of NHS/EDC amount on pAAm-g-PTFE immobilized system activity

To covalently bind enzyme‘s carboxylic groups to the amine groups on the surface, NHS/EDC coupling reaction was used. NHS/EDC amount was calculated as explained in section 3.2.4.2 by first calculating the carboxyl groups on the enzyme. Several EDC/carboxylic group (mol/mol) ratios were tested (from 0.05-0.6) and the results in pH 6.0 PB at 35 ^{o}C using 400 µM guaiacol are shown in Figure 4.15.

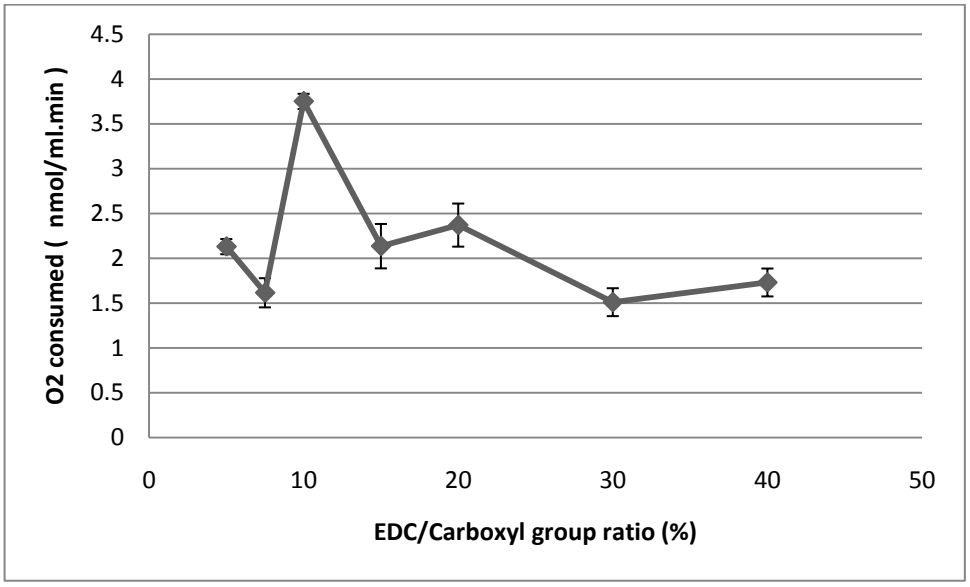

Figure 4.15: Effect of EDC/Carboxyl group ratio on pAAm-g-PTFE system

It can be seen that NHS/carboxyl group ratio of 0.1 gave the maximum activity for enzyme immobilized system. After 0.4-ratio, no activity was observed. Probably the enzyme activity was negatively affected in high reactive chemical concentrations. As a result, a NHS/carboxyl group ratio of 0.1 was chosen in further studies.

4.2.2.2.2 Effect of enzyme amount on pAAm-g-PTFE system activity

To investigate the effect of enzyme amount on pAAm-g-PTFE immobilized system 10, 20 and 30 units of laccase enzyme was immobilized on pAAm-g-PTFE (Figure 4.16).

In order to calculate the immobilized enzyme amount, remaining enzyme concentration in the medium needed to be determined as explained in section 3.2.5. But carbodiimide is an interfering substance for the protein assay and as the immobilization reaction mixture contained carbodiimide, the remaining enzyme amount could not be detected for this method. For this reason, enzyme amount given in Figure 4.16 is the starting enzyme amount.

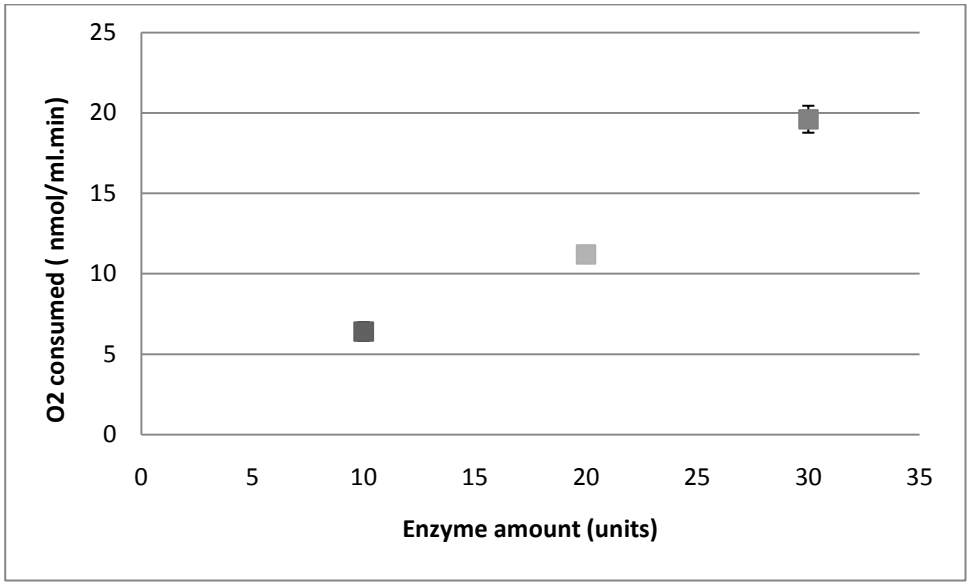

Figure 4.16: Effect of enzyme amount on pAAm-g-PTFE immobilized system

The results showed that there is a linear increase in the immobilized enzyme activity with increasing enzyme amount within the studied concentrations.

4.2.2.3 Optimization of pAAc-g-PTFE immobilized system

4.2.2.3.1 Effect of enzyme amount

Different starting enzyme concentrations (10, 20, 30 u) were tested and immobilized enzyme amount was determined by using bradford protein assay as explained in section 3.2.5. The immobilized enzyme amount and the yield are given in table 4.1.

It could be seen that tripling the enzyme concentration did not increased the immobilized enzyme amount in the same amount. The immobilization yield dropped from 37.5 % to 23.7 % and as a result only 2 fold increase in immobilized enzyme amount could be obtained. Increasing the starting enzyme amount increases the lost enzyme so for further studies, we used 10 U of enzyme.

Table 4.1: Effect of enzyme amount on pAAc-g-PTFE immobilized system

Starting enzyme amount (U)	Immobilized enzyme amount (U)	Immobilization yield
10	3,75	37,5
20	5,60	28,0
30	7,10	23,7

4.3 Characterization and comparison of immobilized systems

4.3.1 Effect of pH on free and immobilized laccase

To determine the optimum working pH for free and immobilized laccase, measurements were done at different pH values using 500 µM guaiacol. For gelatin system, pH dependence was determined by ABTS assay. The results can be seen in Figure 4.17.

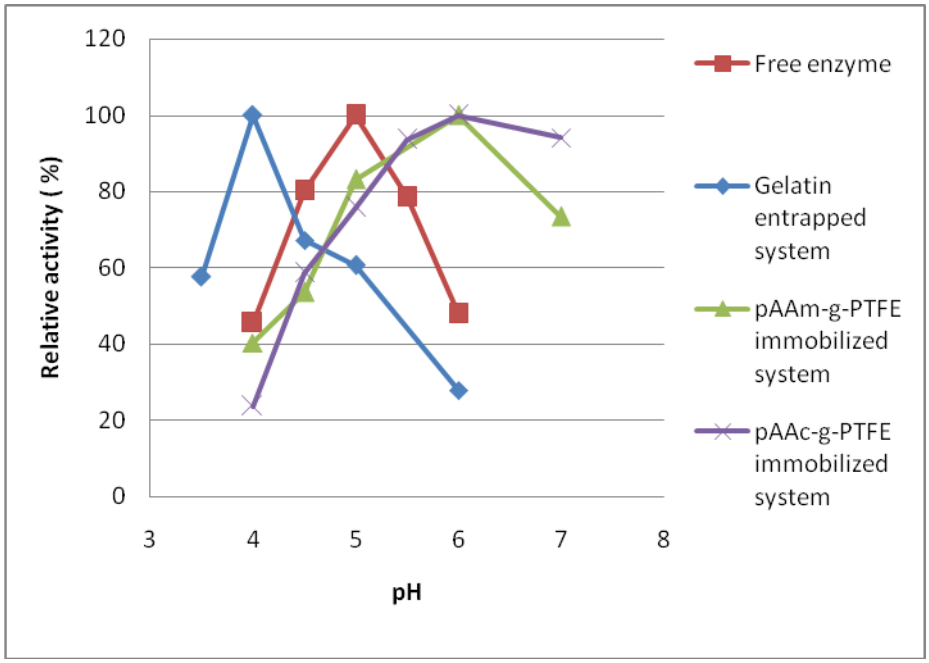

Figure 4.17: Effect of pH on free and immobilized laccase activity

The optimum pH of free laccase was found to be pH 5.0. But for immobilized systems optimum pH shift was observed as expected. For gelatin entrapped system optimum pH shifted to acidic values (pH 4.0) and for covalently immobilized laccase systems, optimum pH shifted to alkaline values (pH 6.0). The optimum pH shift of immobilized enzymes is generally the result 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 than the bulk solution. Due to their different pI values, the polymers may be charged positively or negatively on different pH values. Gelatin is a positively charged matrix and because of this, the pH of the microenvironment shifts to higher pH values. Therefore the pH of the microenvironment is higher than the solution pH, that is the measurable pH. That means that when we measure pH 4.0 in the bulk solution, the pH around the enzyme is about 5.0. For this reason, we see this shift in the results.

The optimum pH of the pAAm-g-PTFE and pAAc-g-PTFE immobilized systems were found to be pH 6.0. Unlike the gelatin entrapped system, optimum pH shifted towards higher values. In this case, polyacrylamide is an anionic (negatively charged) polymer, thus the microenvironment pH value is lower than bulk pH.

For both covalently immobilized enzymes, systems showed similar responses to pH changes. In pH 4.0 pAAm immobilized system showed 20 % more activity than pAAc immobilized system relative to its maximum activity. But in pH 7.0 the same this effect was reversed. The pAAc immobilized system retained 20 % more activity than pAAm immobilized system. Therefore pAAm system is more resistant to lower pH values then pAAc and pAAc immobilized system is more resistant to higher pH than pAAm Different buffering capacities of the polymers were believed to be the cause for this effect.

4.3.2 Effect of temperature on free and immobilized laccase activity

To determine the optimum working temperature of free and immobilized laccase, measurements were done at different temperatures using 500µM guaiacol. For gelatin system, temperature dependence was determined by ABTS assay. For every immobilized system, their optimum working pHs were chosen. The results are compared in Figure 4.18

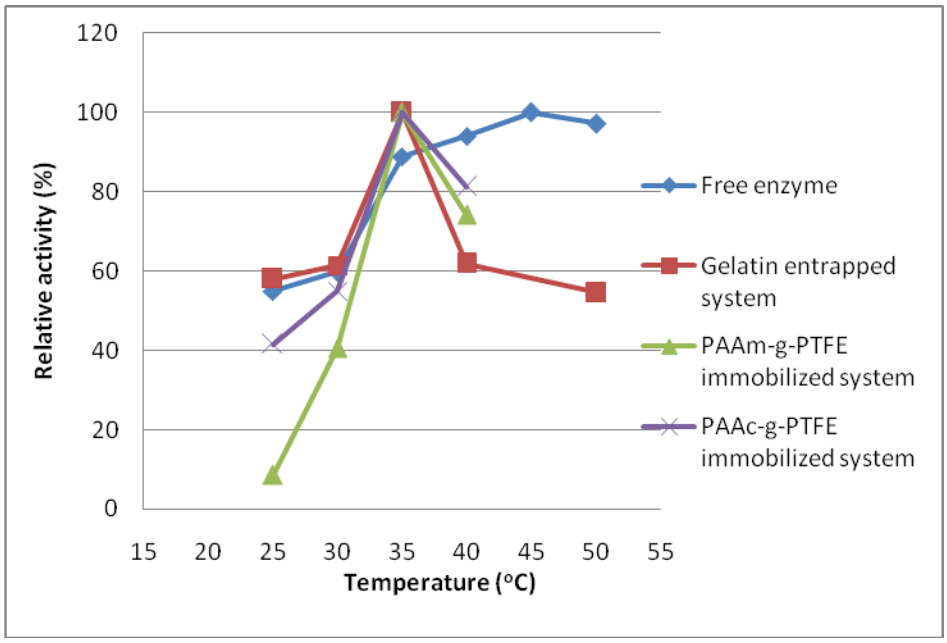

Figure 4.18: Effect of temperature on free and immobilized laccase

Experiments showed that the activity of free laccase is above 90 % in between temperatures 35-50 ^{o}C and the maximum activity was observed at 45 ^{o}C. However the impact of temperature change is stronger in immobilized systems. Maximum activity of the immobilized systems was observed at 35 ^{o}C. The pAAm-g-PTFE immobilized system was found to be the most temperature sensitive system. A 5 ^{o}C drop of temperature resulted in 40 % of its maximum activity. Temperature stability changes was observed and reported in literature [li *et al*., 2005, Cho *et al*., 2008]

Gelatin is sensitive to heat. Above 40 ^{o} C the gelatin matrix loses its stability and starts to melt and dissolve in to the reaction chamber. Another gelatin entrapment systems constructed by [Odaci et al., 2006] also reported activity loss after 35 ^{o} C. The most thermally stable system was found to be the pAAc-g-PTFE immobilized system.

4.3.3 Comparison of activity of immobilized systems

Enzyme immobilization may result in a decrease of activity. This decrease of activity could be acceptable if higher stability and/or reusability is obtained. Our experiments showed that the immobilization of laccase results in drastic loss of activity. Different immobilization methods yielded different activities and optimum working conditions. The comparison of activities were done in optimum conditions of each construct using 400 µM guaiacol as substrate and listed in table 4.2.

Table 4.2 : Activity comparison of the constructs

	Free laccase	Gelatin entrapped laccase	pAAm-g-PTFE immobilized laccase	pAAc-g-PTFE immobilized laccase
Activity (nmol/ml.min)	320.48	12.04	6.36	7.44

Our experiments showed that free laccase lost 96 % of its activity when immobilized in gelatin, 98 % of its activity when immobilized on pAAm-g-PTFE and 97 % of its activity when immobilized on pAAc-g-PTFE. Immobilization of laccase usually results in loss of activity [Duran *et al*., 2002]. To our knowledge previously studied laccase biosensors are amperometric and they are more sensitive than conductometric biosensors but lack the ability to quantitative measurement. Therefore our results couln't be compared with previous studies.

4.3.4 Reusability of the immobilized systems

The main advantage of immobilization is the ability to use the same system again with minimal or no loss of activity. Therefore a stable immobilized system is an important property of a biosensor. To determine the reusability of the constructs, the same immobilized enzyme system was repeatedly used and activities were determined (Figure 4.19). Since gelatin entrapped construct was not mechanically stable, the membrane ruptured after 3 or 4 uses. This resulted in enzyme leakage and rapid loss of activity, thus the results are not included in Figure 4.19.

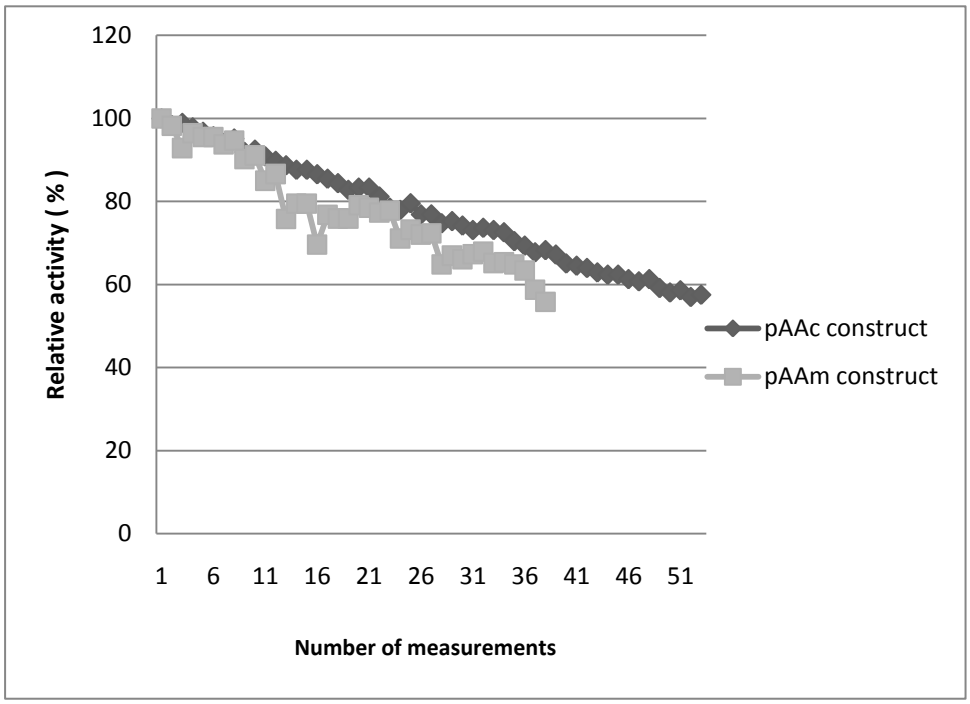

Figure 4.19: Reusability of the immobilized constructs

Covalent immobilization of laccase on pAAm and pAAc grafted PTFE resulted in a distinct advantage in terms of reusability. pAAc immobilized system was found to be more stable than the pAAm immobilized construct. The pAAm immobilized construct lost 40 % of its initial activity after 40 consecutive uses. The pAAc immobilized construct, on the other hand, reached the same percent of activity loss after 50 consecutive uses. Nearly all of the previous studies on laccase biosensors are amperometric and most of these biosensors are modified electrodes, thus disposable. When compared to non disposable biosensors, pAAc-g-PTFE biosensor is found to be more reusable than most of the previous amperometric biosensors [Roy *et al.*, 2005, Kochana et al., 2008].

4.3.5 Storage stability of the immobilized constructs

Another important property for biosensors is the long shelf life with no or minimal loss of activity. Therefore the storage stabilities of immobilized constructs were investigated. For each time point, a new immobilized system (stored in pH 6.0, 0.1 M PB) was taken from 4 ^{o}C and activity measurements were done. The results of the storage stability experiments are shown in Figure 4.20.

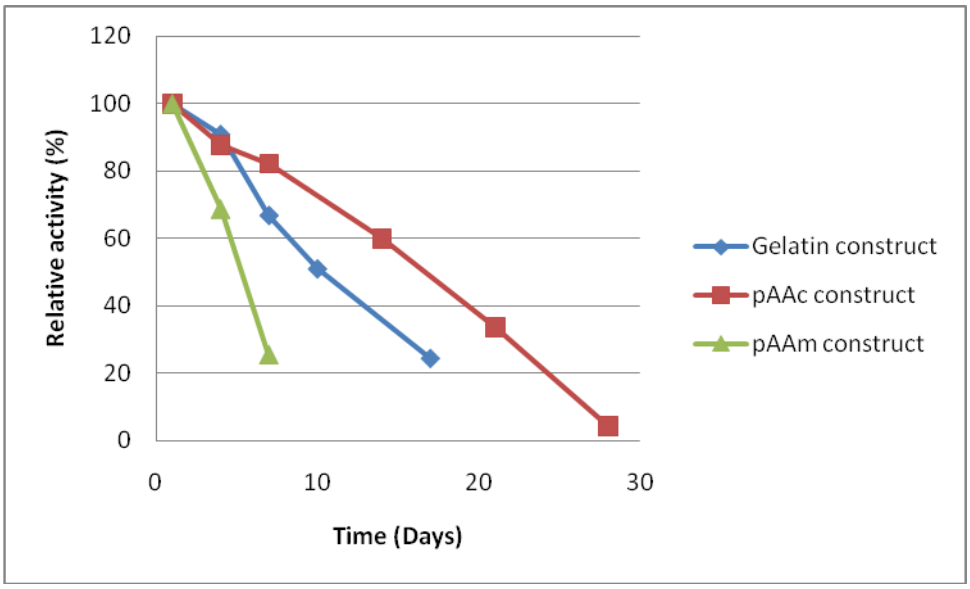

Figure 4.20: Storage stability of the immobilized constructs

Our experiments showed that the pAAm-g-PTFE immobilized system is the most unstable construct. The pAAm construct lost its initial activity rapidly and after 7 days 80 % of its activity was lost. The pAAc immobilized system showed the highest stability in these experiments, total loss in activity was resulted after 30 days. Most of the previous laccase biosensors are diposible. Therefore there is little information on storage stability. But the pAAc biosensors is more stable than most of previous laccase biosensors that are tested for storage stability. [Yaropolov *et al.*, 2005, Kim et al., 2003].

Gelatin entrapped system retained some of its activity for 14 days. Although the gelatin matrix is not mechanically stable, its storage stability is relatively high. These rapid decreases in activity is probably the result of the wet storage. A suitable drying technique, preferably something like freeze-drying could be a better alternative for the storage of the protein related systems to decrease the activity loss.

5. CONCLUSION

Biosensors are promising alternatives for on-site detection of analytes. PTFE is an attractive material for biosensor construction due to its oxygen permeability, high mechanical and electrical resistances and its unreactivity. PTFE can be modified with chemical or physical treatment to be used as support material for immobilization of enzymes or proteins. The aim of this thesis was to change the inert nature of PTFE to allow protein immobilization to its surface and investigate its performance as an immobilization support material for biosensor construction.

For this purpose grafting of PTFE with polyacrylic acid and polyacrylamide by using plasma discharge was optimized, then laccase enzyme isolated from *Trametes versicolor* was immobilized on the grafted PTFE. To our knowledge no previous study was made with this method for laccase biosensor. To be able to compare our biosensor performance, an easy and conventional gelatin entrapment biosensor was constructed and the activity of this biosensor was also optimized.

In a previous study of our research team, PTFE was grafted with polyacrylamide (25%) and a protein was successfully immobilized. In the previous study plasma treatment conditions of PTFE was optimized. But the same method was found to be not applicable for laccase enzyme and to increase the grafting yield of the pAAm, we used 50 % acrylamide mixture for plasma initiated polymerization. With subsequent plasma treatments of H_2 and Argon, the PTFE was successfully grafted with pAAm. For polyacrylic acid (pAAc), PTFE was first treated with the optimized argon plasma and then acrylic acid monomers were polymerized by heat application. Laccase enzyme was immobilized on the grafted PTFE and the characterization of the biosensors were done by using an oxygen electrode unit for the activity measurements

As a result, we modified inert PTFE surface and grafted with amine and carboxyl groups and used these groups as binding sites for enzyme immobilization. After the optimization of enzyme immobilization, the characterization of all three sensors was done. Our results concluded that pAAm and pAAc grafted PTFEs are a stable, resistant and portable alternative for conventional detection methods.

According to our research the pAAc biosensor is the best alternative among our biosensors. In our comparison of constructs pAAc biosensor showed better storage stability, reusability, pH and temperature stability.

The constructed pAAc biosensor is ready for further experiments with different substrates and live wastewater and environmental samples and dry storage experiments.

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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
Microsyringes	Hamilton co 10 µL.
pH meter	Mettler Toledo MP220
Pure water systems Labwater	USF Elga UHQ-PS-MK3, Elga
Shaker (37 ^{o} C)	Certomat S II
Orbital Shaker (4 ^{o} C)	Heidolph Duomax 1030
RF Power Generator	PFG COOE/Hüttinger
Vacuum Equipment	TSY HF450/PECVD350/MMPS20/C Genertec International Corporation
ATR-FTIR	NICOLET 6700
Balances	Precisa XB220A
	Precisa BJ610C
Oxygen Electrode	Helmut saur laborbedorf oxygraph
Water Bath	Huber

Appendix B

Chemicals

Ethanol	Sigma-Aldrich
Acetone	Sigma-Aldrich
Acrylamide	Sigma-Aldrich
Acrylic Acid	Fluka
Gelatin	Fluka
N-(3-Dimethylaminogrouply)-N-ethly-carbodiimide	Sigma-Aldrich
N-Hydroxysuccinimide	Fluka
Guaiacol	Sigma-Aldrich
4-chlorophenol	Sigma-Aldrich
Catecol	Sigma-Aldrich
2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS)	Sigma-Aldrich
Glutaraldehyde (70%)	Sigma-Aldrich
Sodium Dithionite	Merck
MES hydrate	Sigma-Aldrich
NaOH	Riedel-de Haën
Sodium Acetate Anhydrous	Sigma-Aldrich
Laccase (*Trametes versicolor*)	Sigma-Aldrich

Appendix C

Solutions and buffers

Guaiacol solution (0.1 M) 100ml

- 1.24 gr guaiacol
- 100 ml dH_2O

4-chlorophenol solution (0.1 M) 100 ml

- 1.28 gr 4-chlorophenol
- 100 ml dH_2O

Catecol solution (0.1 M)

- 1.10 gr Catecol
- 100 ml dH_2O

N-Hydroxysuccinimide NHS Solution (5 ml)

- 1ml dH_2O
- 2 mg NHS
- dilute to required amount

N-(3-Dimethylaminogrouply)-N-ethly-carbodiimide solution

- 1 µl EDC
- 1 ml dH_2O
- Dilute to required amount

Acrylamide Solution (25 % and 50 % w/v, 50 % v/v Ethanol/Acetone) (30 ml)

- 7.5 gr Acrylamide (25 %)
- 15 gr Acrylamide (50 %)
- 15 ml EtOH
- 15 ml Acetone

Acrylic acid solution (30 % v/v) (3 ml)

- 1 ml Acrylic acid monomer
- 2 ml dH_2O

Phosphate Buffer (1 M) (pH: 7.4) (100 ml)

- 13.6 gr KH_2PO_4
- 100 ml dH_2O
- Adjust pH to required value by adding NaOH

Acetate Buffer (0.1 M) (pH 5.0)

- -8.2 gr Sodium acetate anhydrous
- -300 µL Glacial acetic acid
- -100 ml dH_2O
- -Adjust pH

MES buffer (0.05 M) (pH 5.5) 400 ml

- 3.9 gr MES hydrate
- 400 ml dH_2O

Laccase enzyme stocks

- 50mg Laccase enzyme isolated from *Trametes versicolor*
- 1 ml Phosphate buffer (pH 6.0, 0.1 M)
- Alicoted to tubes as 5 µl

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