



**ENZYME ELECTRODES FABRICATED BY  
DAD TYPE Poly (2,5-di(furan-2-yl)thiazolo[5,4-  
d]thiazole) CONDUCTING POLYMER**

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**THESIS OF MASTER OF SCIENCE  
DEPARTMENT OF CHEMISTRY**

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DAD TYPE Poly (2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazole)  
CONDUCTING POLYMER**

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**BY**

**Nadia Mohammed Salama KURZAMA**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR  
THE DEGREE OF MASTER OF SCIENCE IN  
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I certify that in my opinion the thesis submitted by Nadia Mohammed Salama KURZAMA titled “ENZYME ELECTRODES FABRICATED BY DAD TYPE Poly (2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazole) CONDUCTING POLYMER” is fully adequate in scope and in quality as a thesis for the degree of Master of Science.

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The degree of Master of Science by the thesis submitted is approved by the Administrative Board of the Graduate School of Natural and Applied Sciences, Karabük University.

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*“I declare that all the information within this thesis has been gathered and presented in accordance with academic regulations and ethical principles and I have according to the requirements of these regulations and principles cited all those which do not originate in this work as well.”*

Nadia Mohammed Salama KURZAMA

## **ABSTRACT**

**M. Sc. Thesis**

### **ENZYME ELECTRODES FABRICATED BY DAD TYPE Poly (2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazole) CONDUCTING POLYMER**

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The Department of Chemistry**

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**Assist. Prof. Dr. A. Elif BÖYÜKBAYRAM**

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In this study, DAD type polymer was used as a new electrode material and enzyme electrodes were prepared by immobilizing polyphenol oxidase enzyme in this matrix. 2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazole monomer was polymerized electrochemically and coated to platinum electrodes and polyphenol oxidase enzyme was immobilized on the polymer surface with cross-linker. Kinetic characterization and optimization of enzyme electrodes formed was made and the study was completed with real sample analysis. Kinetic parameters were assessed for  $V_{\max}$  (maximum reaction rate) and  $K_m$  (enzyme affinity of substrate) respectively as  $0.028 \pm 0.001 \text{ } \mu\text{mol min}^{-1} \text{ electrode}^{-1}$  and  $669.68 \pm 64.73 \text{ mM}$ . The effect of reaction conditions was examined and the highest enzyme activity was obtained at pH 7.5 and 45 °C. Linear operation interval determined in optimum conditions is between 1.0 – 90.0 mg mL<sup>-1</sup>. And LOQ value was found as 7.827 mg mL<sup>-1</sup>. The stability of enzyme electrodes was investigated with

consecutive activity measurements. After 50 consecutive measurements, it was observed that activity of enzyme electrode was at 70% value. Meanwhile, in the shelf-life study, it was determined that activity fell to 70% after 50 days. Enzyme electrodes application was designed in the form of analysis of polyphenolic substances in waste water samples. Total polyphenolic substance amount in sample was found as  $268.48 \pm 6.62 \text{ mg mL}^{-1}$  by enzyme electrodes. This result was verified using Folin-Ciocalteu analysis method as the control method with a result of  $262.86 \pm 19.54 \text{ mg mL}^{-1}$ .

**Key Words** : Electrochemical polymerization, conducting polymer, enzyme immobilization, polyphenol oxidase, enzyme electrode.

**Science Code** : 20115

## ÖZET

Yüksek Lisans Tezi

### DAD TİPİ Poli (2,5-di(furan-2-il)tiyazolo[5,4-*d*]tiyazol) İLETKEN POLİMERİYLE OLUŞTURULAN ENZİM ELEKTROTLARI

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Bu çalışmada yeni bir elektrot malzemesi olarak DAD tipi polimerde polifenol oksidaz enzimi tutuklanarak enzim elektrotları hazırlanmıştır. 2,5-di(furan-2-il)tiyazolo[5,4-*d*]tiyazol monomeri elektrokimyasal yolla polimerleştirilerek platin elektrotlara kaplanmış ve polifenol oksidaz enzimi çapraz bağlayıcı ile polimer yüzeyine yerleştirilmiştir. Oluşturulan enzim elektrotlarının kinetik karakterizasyonu ve optimizasyonları yapılmış ve numune analizi ile çalışma sonuçlandırılmıştır. Kinetik parametreler,  $V_{max}$  (maksimum reaksiyon hızı) ve  $K_m$  (substratın enzim ilgisi) tutuklanmış enzim için sırasıyla  $0.028 \pm 0.001$   $\mu\text{mol dak}^{-1}$  elektrot $^{-1}$  ve  $669.68 \pm 64.73$  mM olarak tayin edilmiştir. Reaksiyon koşullarının etkisi incelenmiş ve en yüksek enzim aktivitesi pH 7.5 ve 45 °C sıcaklıkta elde edilmiştir. Optimum koşullarda tespit edilen doğrusal çalışma aralığı 1.0 – 90.0 mg mL $^{-1}$  arasındadır. LOQ değeri ise 7.827 mg mL $^{-1}$  olarak bulunmuştur. Ardışık aktivite ölçümleri ve raf ömrü çalışmasıyla

enzim elektrotlarının stabilitesi incelenmiştir. 50 ardışık ölçümden sonra enzim elektrodunun aktivitesinin %70 değerinde olduğu gözlenmiştir. Raf ömrü çalışmasında ise 50 günün sonunda aktivitenin %70'e düştüğü saptanmıştır. Enzim elektrotlarının uygulaması atık su numunelerinde polifenolik maddelerin analizi şeklinde tasarlanmıştır. Toplam polifenolik madde miktarı enzim elektrotlarıyla  $268,48 \pm 6,62$  mg mL<sup>-1</sup> olarak saptanmıştır. Bu sonuç kontrol yöntemi olarak Folin-Ciocalteu analiz metodu kullanılarak doğrulanmış ve  $262,86 \pm 19,54$  mg mL<sup>-1</sup> bulunmuştur.

**Anahtar Kelimeler :** Elektrokimyasal polimerizasyon, iletken polimer, enzim tutuklaması, polifenol oksidaz, enzim elektrodu.

**Bilim Kodu** : 20115



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## ABBREVIATIONS

$K_m$	:	Michaelis-Menten constant
$V_{max}$	:	Maximum reaction rate
LOQ	:	Limit of Quantitation
mL	:	Mililiter
$\mu\text{m}$	:	Micrometer
$\mu\text{L}$	:	Microliter
$\mu\text{mol}$	:	Micromole
nm	:	Nanometer
$^{\circ}\text{C}$	:	Degree Celcius
g	:	Gram
mg	:	Miligram
min	:	Minute
Å	:	Ångström
A	:	Amper
V	:	Volt
VB	:	Valence Band
CB	:	Conductance Band
CV	:	Cyclic Voltamogram
SEM	:	Scanning Electron Microscope
UV	:	Ultraviolet
PA	:	Polyacetylene
PAN	:	Polyacrylonitrile
PPy	:	Polypyrrole
PPO	:	Polyphenole oxidase
MBTH	:	3-Methyl-2-benzodiazolinon hydrazon hydrochloride monohydrate
DAD	:	Donor-Acceptor-Donor
TTzFr	:	2,5-di(furan-2-yl)thiazolo[5,4- <i>d</i> ]thiazol monomer

- PTTzFr : poly(2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol) polymer
- Pt/Ppy/PPO : Enzyme electrode composed of polyphenol oxidase enzyme immobilized into PPy coated on platinum electrode
- Pt/PTTzFr/PPO : Enzyme electrode composed of polyphenol oxidase enzyme immobilized into PTTzFr coated on platinum electrode



## **PART 1**

### **INTRODUCTION**

It is an extensive research field that enzymes are immobilized in many natural or synthetic organic polymers and inorganic materials. Enzymes are materials that are both expensive and lose their activity easily. For this reason, it is important to immobilize and regain the enzyme back and use it more and more instead of using them one time in a free form. An immobilized enzyme can be received back from the reaction environment and this is an important factor in reducing cost. Besides, by receiving the enzyme back from the reaction environment, a green production can be obtained.

Enzymes are specific catalyzers and specific analysis of the enzyme's substrate can be possible by the entrapped enzyme. Beside the advantage of enzyme being specific to its substrate, the high sensitivity that is created by electrochemical methods resulted in more researches to be made with enzyme electrodes for analysis purposes. Enzyme electrodes are electrode structures where the enzyme is entrapped within or on the surface of a conductive matrix and it is the main component of enzymatic biosensors. Electrode materials are gold (plate or wire), platinum (plate or wire) and carbon-based materials (paper, rod, paste, metalized carbon, glassy carbon, carbon fiber). Enzyme electrodes are basically composed of the enzyme and a composite structure covered on the electrode material. The performance of the enzyme electrode is much more due to the used composite materials. These materials are conductive polymers, functional polymers, metal complexes, sol-gel materials and Nano-materials (carbon Nano-tube, Nano-particulates). Researches in the field of biosensors are in the direction to develop new matrices and composite materials and obtain more sensitive, easy-to-use and cheaper methods.

Electroactive conductive polymers are used widely in the field of bioanalysis with their charge carrying capacity and biocompatibility. Two important reasons that they are used in enzyme electrodes are their active enzyme binding capacity and helping electron exit from the enzyme and carrying the electrons to the surface of the electrode, which are important factors in amperometric biosensors. For this reason, conductive polymers have sensitivity increasing effects. Besides this, due to their stable structure, the enzyme lengthens the life of the electrode and increases its repeatability. With these advantages of them, conductive polymers have attracted more attention recently and they were coated to the surface of electrodes from many different materials. These coated electroactive films are used to entrap enzymes or proteins.

The lifetime of the entrapped biomolecule depends on both the composite material and the entrapment technique of the enzyme. Generally, enzyme immobilization methods are adsorption, covalent bonding, cross-linking and entrapment. As the adsorption method consists of weak bonds between the electrode and enzyme, it can't hold the enzyme in a manner that is strict enough. Entrapment method maintains a strict closeness between the enzyme and the matrix but it doesn't contain covalent bonds. In covalent bonding, a cross-linking material such as glutaraldehyde is used in order to bond the enzyme to polymer or enzyme molecules with each other. In this method, bonds forming between enzyme molecules give stability to the entrapped enzyme and it lengthens the life time of the enzyme electrode.

Polyphenol oxidase is a metalloprotein that contains copper and it is an enzyme used commonly in animals, plants and microorganisms. The substrates of polyphenol oxidase are phenolic materials and it is an important biomaterial that can be used in the analysis of phenolic compounds. Recent studies are focused on polyphenolic materials which exist in waste waters.

Phenolic compounds in nature are sourced from natural ways and industrial waste drained to rivers and these compounds in wastes cause contamination in the water and soil. Phenols are used commonly in the industry, the most important of which are plastics and textile production, drugs, petroleum and paper industries and insecticide and fungicide productions. Phenols in rivers poison water life and it is one of the

important problems of environmental chemistry since they persist longtime in the nature. Phenolic compounds are life-threatening even in small concentrations. Analysis and follow up of phenolic compounds which are among the most important contaminants of water is important to protect public health and environment. Traditional methods used for this purpose require complicated preparation processes and they are not suitable for analysis in the field. In spite of this, analysis method by enzyme electrodes is effective and simple. It attracts interest as an alternative method with the advantages to be more practical compared to conventional methods, receiving fast responses, not requiring long sample preparations, being easy-to-use and cheap.

In this study, polymerization was performed by cyclic voltammetry. The morphology of the polymer was examined with SEM images. After polymerization, polyphenol oxidase enzyme was immobilized on the polymer surface by cross-linking method. First, activity determination and optimization of the enzyme electrodes were performed. Optimum enzyme amount and glutaraldehyde amounts were determined and its optimization and then characterization of it was made by pH, temperature and strength tests. Then, following the examination of enzyme electrodes, the study was completed by sample analyses.

## PART 2

### THEORETICAL BACKGROUND AND LITERATURE SURVEY

#### 2.1. CONDUCTING POLYMERS

Polymers were previously known as insulating organic materials that have very low conductivity at room temperature. Therefore, they have been used as electrically insulating materials for long years. It has been understood first with studies made on polyacetylene (PA) that polymers can gain conductivity by transmitting electricity directly over electrons.

While polyacetylene which is also known as the first conductive polymer was produced as a black powder material, Natta et al. developed the system related to polymerization of acetylene by using a Ziegler type catalyzer. And in 1963, Karl Ziegler and Giulio Natta were jointly awarded the Nobel Prize due to their discoveries in the field of polymer chemistry and technology. In 1974, Hideki Shirakawa produced polyacetylene films in silver color which don't have the required conductivity by using  $\text{Ti}(\text{O}-n\text{-But})_4$  known as the Ziegler-Natta catalyzer. In 1977, H. Shirakawa, A.J. Heeger and A.G. MacDiarmid discovered that the conductivity of polyacetylene films increase  $10^9$  times when they are subjected to Iodine, Chlorine or Fluorine vapor, that is to say, the polymer is oxidized and its conductivity reaches to around  $10^5 \text{ S.m}^{-1}$  [1]. This discovery related to the electrical conductivity system of polymers led them be awarded with the Nobel Prize for chemistry in 2000.

##### 2.1.1. Conductivity Theory in Polymers

Conductive polymers have  $\pi$  conjugation which occurs as a result of ordering of single and double bonds in their chains sequentially. Due to the conjugated double bonds in the main chain of polymers electrons are carried along the chain and electrical conduc-

tivity is maintained. For a high conductivity, conjugation is not sufficient alone. For this reason, electrons that will maintain conductivity are given to or taken from the polymer structure and negatively or positively charged holes are formed in the polymer network. An electron which jumps to the positively charged hole from another place will also leave a positively charged hole in the place it came from. And the electrons are transmitted when this process is repeated sequentially along the polymers chain [2].

### 2.1.1.1. Band Theory

During bond formation, two new energy levels appear; the bonding orbital where there are two electrons and the anti-bonding orbital which is the anti-bonding energy level in empty condition. Electrons in the bonding orbital may ascend to the anti-bonding orbital with higher energy when they receive sufficient energy with the effect of heat or light. It is also possible to explain bond formation between complex molecules which have more than one electron. When a new atom joins a molecule, a new bonding and anti-bonding energy levels are formed in the electronic structure of the molecule. This condition is shown in Figure 2.1 for a medium-sized molecule.

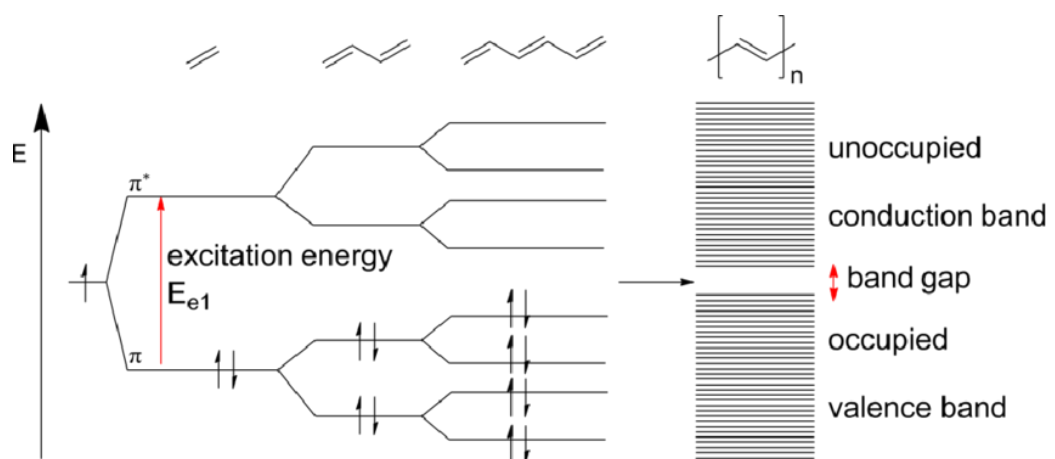


Figure 2.1. Formation of bonding and anti-bonding orbitals in polymer molecule [2].

When the size of molecule increases, number of bonding orbitals also increases. Therefore, the difference between orbital energy levels decreases. After a point, instead of energy levels that are separated clearly from each other, a continuous merged

energy band is formed. This band is called as valence band (Figure 2.2). Electrons within the valence band can easily change their places and move easily inside the band. And the anti-bonding orbitals having number close to infinity form another energy band with the approaching anti-bonding orbitals named as the conductivity band. The interval between the valence band and the conductivity band is named as band gap and the energy required to pass this interval is named as band gap energy. In grouping materials as insulator, semi-conductor, conductor, the magnitude of band gap energy is considered [2].

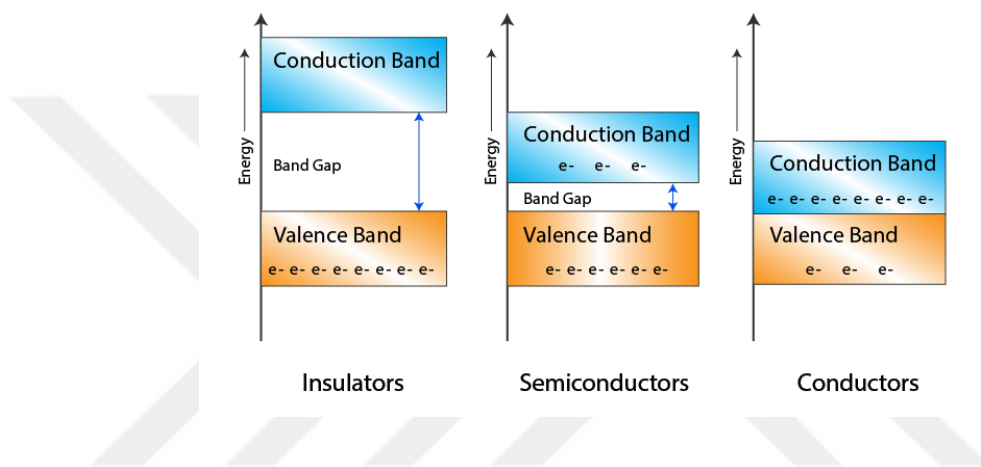


Figure 2.2. Band structures for insulators, semi-conductors and conductors.

The undoubled electrons are responsible from electrical conductivity which exists at the valence band, conduction band or at a new energy level in the band gap. This type of free electrons moves to the desired direction according to the applied potential. When the valence band energy levels are totally full of electrons, it is hard to maintain the flow of electrons to one direction. In such a system, free electrons may be formed with heat or light excitation. Electrons which have sufficient energy on the top level of valence band pass the band gap and they are situated to the energy level at the bottommost level of the conduction band and they maintain conductivity. In insulators, the band gap is wide enough not to let this pass [2].

In semi-conductors, band threshold gap is smaller than insulators and their conductivity varies between  $10^{-6}$  -  $10^2$  S.cm<sup>-1</sup>. Although the electrical conductivity seems to be low at this level, it is at sufficient size to maintain sufficient amount of electric current. Conjugated polymers that include single and double bonds



sequentially may exhibit semi-conductance. When the energy level in semi-conducting polymers between valence band and conduction band is sufficiently small, free electrons may pass to the lowest energy level of the conduction band. These electrons move within the conductance band and carry charges and they are directed towards positively charged direction by moving along the chain. Meanwhile, the positively charged hole remaining inside the valence band moves in opposite direction to the electron along the polymer chain. Their conductivity increases with an increase in temperature or light intensity [2].

Most metal atoms have a single electron and it can't make a covalent bond with another metal atom near it. So, valence band of metals are partially full and conductance band is empty. Metal electrons exist at the low energy orbitals of the valence band with a great possibility. There are always empty places with higher energy levels where they may pass within the same band or the conductance band matching the same band. They maintain transmission of their electrons over partially full valence or conductance band or by band gap transition [2].

### **2.1.1.2 Doping in Conducting Polymers**

If electrons in the valence band of the polymer are removed with an oxidizing agent the polymer is loaded with positive charge, it is called as p-type doping and if this process happens by giving one electron to the empty conductance band with a reducing agent, it is called as n-type doping. With this doping process, dopant molecules don't exchange places with polymer atoms, dopants only help electrons pass from energy shells. As the band theory isn't sufficient to explain conductivity of conducting polymers, it is made use of neutral polymer, polaron and bipolaron structures as it is seen in Figure 2.3. Electric charge given to make doping to the skeleton structure of the polymer causes a change in the electronic condition of the polymer [2].

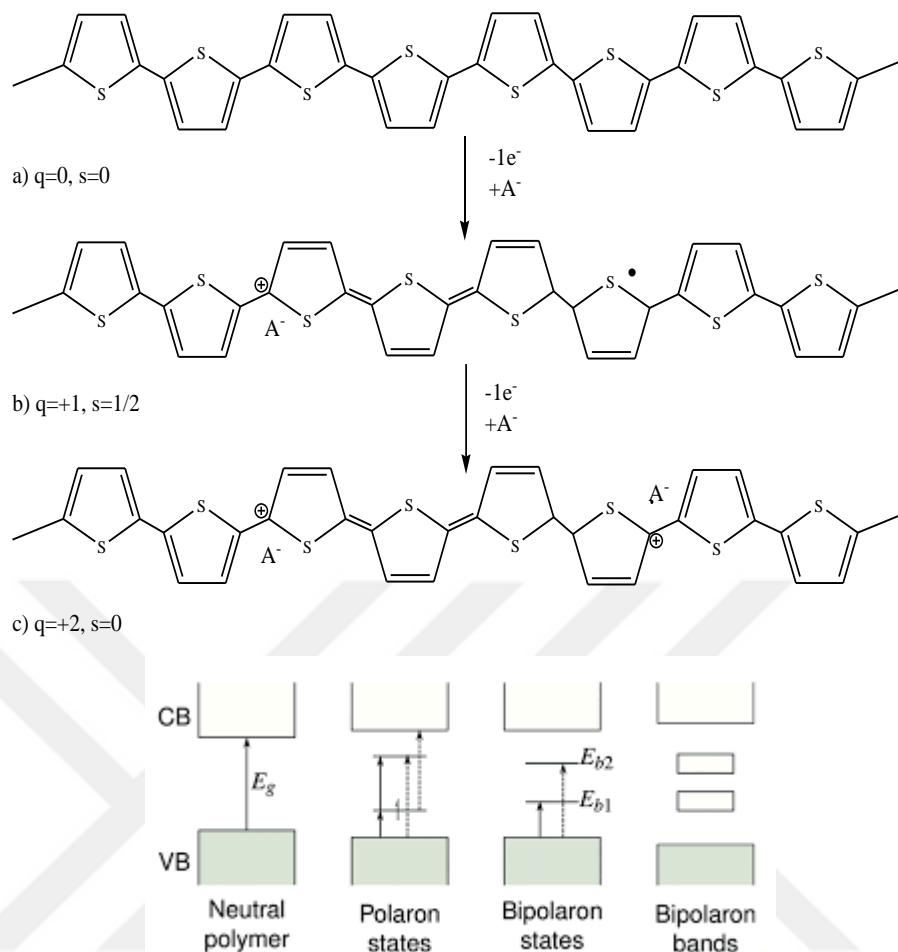


Figure 2.3. Polaron, bipolaron structures and band diagrams. a) neutral polymer b) polaron structure c) bipolaron structure.

By the oxidation of uncharged polymer having conjugated bonds (Figure 2.3 (a)), the double bond is destroyed and a positively charged radical is formed over the polymer chain (Figure 2.3 (b)). These charge carriers formed on the conducting polymer chain are named as ‘polaron’ or ‘radical cation’. Joining of two radicals coming from polaron creates a new  $\pi$  bond. And with the oxidation of the free radical of polaron, a new positive hole without spin is formed named as ‘bipolaron’ (Figure 2.3 (c)). Here, two radicals combine and form a new  $\pi$  bond. There are no undoubled electrons in bipolaron and by this way conductance is maintained without requiring free electrons [3]. Polarons and bipolarons can move along the chain according to the mobilization capability of counter ions. For the ions to be mobile enough, satisfactory amount of counter ions should be provided by doping. As it is shown in Figure 2.4, an electron is removed with controlled doping of polyacetylene and a neutral or charged ‘soliton’

is obtained. Soliton which is formed within the structures makes the charge distribution along the polymer chain and carbenium (carbocation) stable. In a similar way, if the polymer is processed with an electron-giving or n-doping material, one electron is added to the medium-level energy gaps and a negative soliton is formed [3].

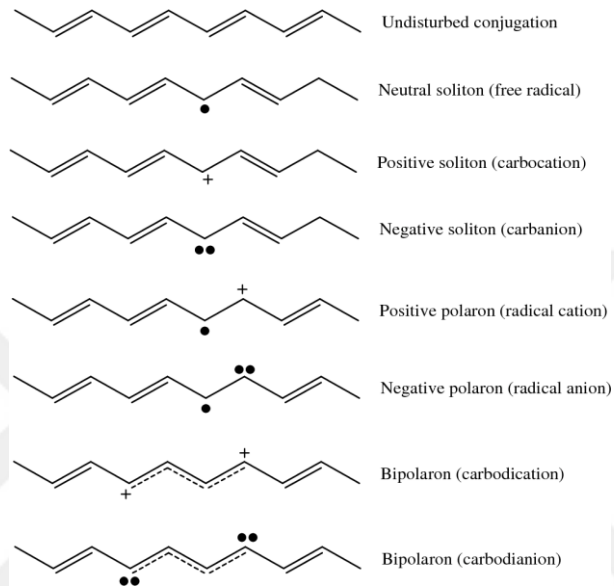


Figure 2.4. Polyacetylene's soliton, polaron and bipolaron structures.

### 2.1.1.3. Hopping in Conducting Polymers

Besides the charge carriers along the chain, transfer of electrical charge between different polymer chains is made by hopping. A neutral soliton interacts with the charged soliton in a chain close to itself and the electron of the charged soliton jumps to the neutral soliton. In the hopping rule, mobility of the electronic charge is maintained by transfer between chains and transfer between blocks along the polymer chain (Figure 2.5) [3].

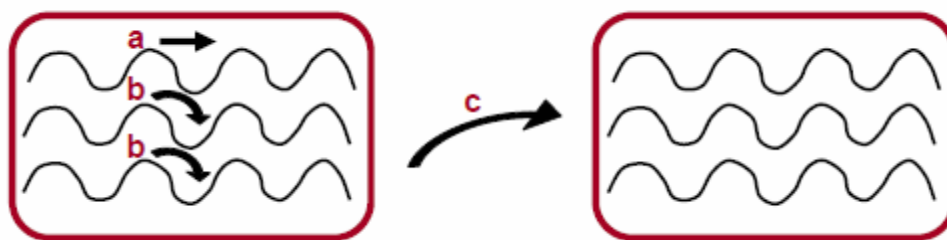


Figure 2.5. Transportation of charge, a) transportation of charge along the chain, b) transportation of charge between chains, c) transportation of charge between chain blocks [3].

## 2.1.2. Synthesis of Conducting Polymers

### 2.1.2.1. Chemical Synthesis

In the chemical method, a monomer that is dissolved in a suitable solvent is polymerized by interacting with a chemical substance used as an oxidation or reduction agent. This method has disadvantages like oxidation step can't be controlled and the product not being pure and advantages like obtaining products in the desired amount with a reasonable cost. The doping material and catalyst that will be used in the chemical method has an important effect on the electrical conductivity of the conducting polymer to be obtained [4]. In the study of synthesizing poly(p-phenylene) made by Toshima, the polymer which is obtained by using  $\text{CuCl}_2$  as doping material and  $\text{AlCl}_3$  as catalyzer didn't exhibit electrical conductivity. However, by using materials such as  $\text{AsF}_5$  or  $\text{Li}$ , conductivity that varies between  $0.3 \text{ S.cm}^{-1}$  -  $500 \text{ S.cm}^{-1}$  has been observed. All of conjugated polymers can be synthesized with the chemical method. In another study, the polymer of pyrrole was prepared by the chemical method using methanol as solvent and  $2.5 \text{ M FeCl}_3$  as doping material and it has been determined to reach a conductivity of  $190 \text{ S.cm}^{-1}$  [4].

### 2.1.2.2. Electrochemical synthesis

Electronically conductive polymers are obtained by electrochemical oxidation of aromatic molecules in resonance stability such as pyrrole, thiophene, aniline and furan.

Electropolymerization is made by putting monomers into solvent/electrolyte medium and applying a potential to this system. With the neutral polymer and radical cation reaction or radical cation/radical cation reaction, polymerization occurs. In figure 2.6, polymerization mechanism of heterocyclic compounds that happened in the anode is given [5].

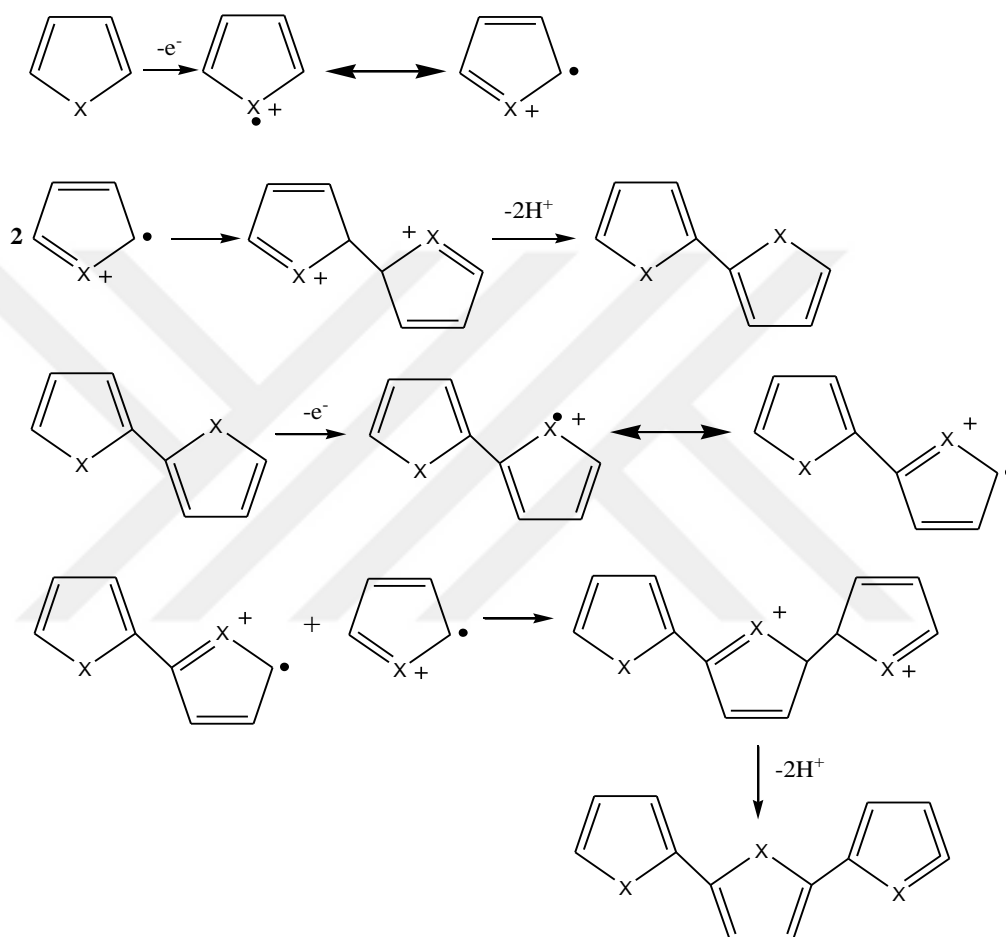


Figure 2.6. Electropolymerization mechanism of heterocyclics (X= N-H, S, O).

Electrochemical stage begins with the monomer to be oxidized over the electrode with a suitable potential and form a radical cation. Electron transfer reaction is faster than the diffusion of the monomer and the solution around the electrode surface has a high radical concentration. This first stage is followed by the coupling reaction that happens with two radical cations couple each other. In case of radical-radical coupling, dihydro dimer cation is formed by two hydrogens separating from one dimer. This anti-aromatic condition represents the chemical stage. As potential is applied, dimer is

oxidized and subjected to a coupling reaction again with a monomeric radical. From this coupling reaction, a trimer forms then it loses two protons. Dimers, trimers and oligomers continue to go to coupling reaction with the radical cation of the monomer and they lose their protons and become anti-aromatic. Electrochemical and chemical stages continue this way following one after another. Until oligomers remain undissolved within the electrolyte solution, this reaction continues on the electrode surface [6].

In electrochemical polymerization, cyclic voltammetry (CV) technique can be used. Cyclic voltammetry provides information related to the oxidation potential of the monomer, its growth on the film, redox behavior of the polymer and surface concentration (charge spent by the polymer). In the specific area, CV can be used about the interaction between the polymer and solvent molecules and ions, charge transportation process and the ratio of charge transporters. The potential of an electrode is used to shed light to electrode reactions as a result of examining current-voltage relationship by changing it linearly between the working electrode and the opposite electrode. In this technique, first scanning is made in a certain potential interval and then it is continued to scan in the opposite way (anodic-cathodic). When the potential applied to the working electrode reaches the oxidation or reduction potential of the electro-active material inside the electrolysis cell, the material on the surface of the electrode is consumed rapidly. Measured current between working and opposite electrodes increases. As a result of this, concentration difference occurring between electrode surface and the solution causes mass transfer from the solution to electrode surface. As mass transfer rate is lower than transfer rate of electrons, a current decrease is observed and a peak is obtained. The potential which corresponds to the peak point of this peak is called as 'oxidation or reduction peak potential' [2].

### **2.1.3. Usage Areas of Conducting Polymers**

Conductive polymers have extensive usage areas due to that they can be synthesized with electrochemical methods, show electrical conductivity and they can be used with insulator polymers. Conductive polymers are used as electrodes in batteries such as

heart batteries as they are rechargeable and long life due to that they produce low currents and they have reversible doping characteristics.

Chemical signals which are formed as a result of reduction-oxidation reactions of the polymer are converted into electric signals and electronic components such as diodes and transistors are made. Besides this, they are used in production of materials working with electrochromic principle like smart windows, high technology eye-glasses and military camouflage dresses. Besides, it is used in pH, gas and humidity sensor, corrosion inhibitor, photoelectrochemical cells, light emitting diodes (LED-OLED), field effective transistors, photovoltaic cells and supercapacitors [7,8].

Another important usage area of conducting polymers is biosensors and enzyme electrodes. Biosensors that are analytical instruments which are formed by a selective biological element and the detector that produces signals proportional to the concentration of the analyte have the potential to be used in analyzing many materials as enzyme sensor due to their high selectivity, quick response time, simple design and cheapness [9,10,11].

## **2.2. ENZYMES**

Enzymes are protein-structured biocatalyzers which increase the rate of almost all biochemical reactions in a ratio of about  $10^{10}$  and  $10^{12}$  like synthesis and degradation of organic molecules, which are not consumed during the reaction and which can be reused as they are not altered after the reaction [12].

Enzymes are only synthesized by living organisms and their difference from other chemical catalyzers is that they are specific. Their catalyzing the molecule entering the reaction, that is to say, its substrate expresses absolute specificity, the activity of an enzyme to a specific substrate group shows its group specificity and their activity to a certain bond shows its bond specificity. This property of specificity maintains all unwanted side reactions and products to be prevented and the selected reaction to be realized and to gain more efficiency. Therefore, as the desired product will be

produced with fewer steps and as energy requirement is low and it can be worked in mild conditions, cost is also reduced.

The minimum energy required for a reaction to start is called as activation energy. And an alternative path towards lowering the activation energy occurs by the help of enzymes as it is seen in Figure 2.7 [12].

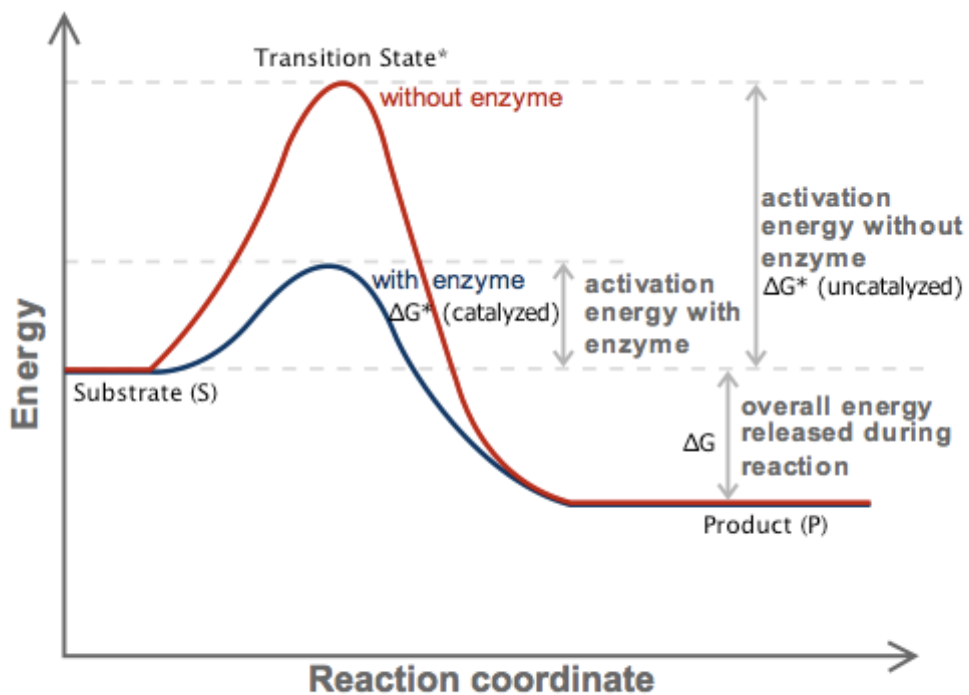


Figure 2.7. Effect of enzyme in activation energy

Enzymes stimulate their substrates in the appropriate direction and maintain them to bind to their active sites consisting of certain amino acid sequences. Thus, enzyme-substrate complex is formed that is named as active complex or transition complex. The first hypothesis related to this formation mechanism is the key-lock model proposed by Emil Fischer the first time (Figure 2.8).



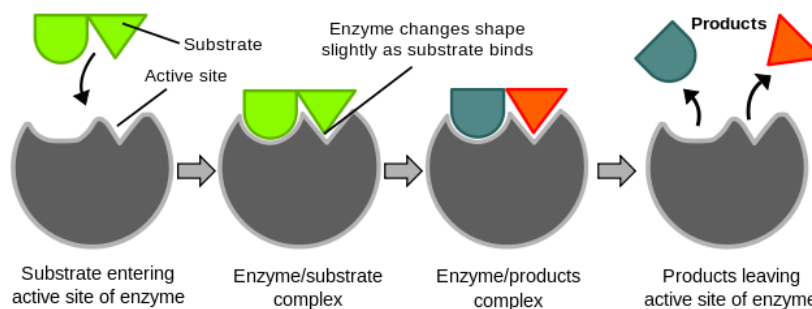


Figure 2.8. Key-lock model proposed for the enzyme-substrate complex.

### 2.2.1. Classification of Enzymes

Simple enzymes are enzymes formed only from protein. The part of simple enzymes that show catalytic effect is polypeptide chain of which pepsin and urease enzymes are examples.

On the other hand, complex enzymes are enzymes that contain its protein part and a much smaller organic molecule or metal with this. The part of enzymes that composes only from protein is named as ‘apoenzyme’ and the non protein group providing it to show catalytic effect is named as ‘cofactor’. Cofactors can be composed of a metal ion and they may also compose of an organic group named as “coenzyme” [12,13].

Today, enzymes are divided into 7 basic classes by the International Union of Biochemistry and Molecular Biology (IUBMB). These are: oxyreductases, transferases, hydrolases, liases, isomerases, ligases and translocases [12].

### 2.2.2. Enzyme Activity

The activity of an enzyme is defined according to the substrate amount spent in unit time and converted to product at optimum conditions. Its unit is IU. 1 IU enzyme activity is the enzyme amount that catalyzes 1  $\mu\text{mol}$  of substrate in 1 minute when optimum conditions are present. There are many factors that influence rate of enzymatic reactions. Some of these are substrate concentration, enzyme concentration, temperature, pH and inhibitor effect [12].

### 2.2.2.1. Effect of Substrate Concentration

In enzyme catalyzed reactions, reaction rate at optimum conditions increases proportionally with increase in substrate concentration. When the entire enzyme molecules form the enzyme-substrate complex, reaction rate reaches maximum ( $V_{max}$ ). Although substrate concentration continues increasing, no increase in reaction rate is observed anymore. This means that enzyme is saturated with substrate (Figure 2.9) [12].

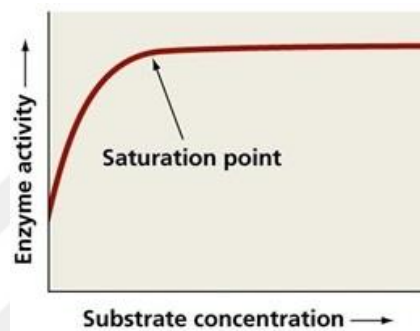


Figure 2.9. Effect of substrate concentration over reaction rate [12].

### 2.2.2.2. Effect of Enzyme Concentration

When unlimited substrate is present in the environment, reaction rate increases proportionally with enzyme concentration. The more enzyme amount is added, the more reaction rate increases (Figure 2.10) [12].

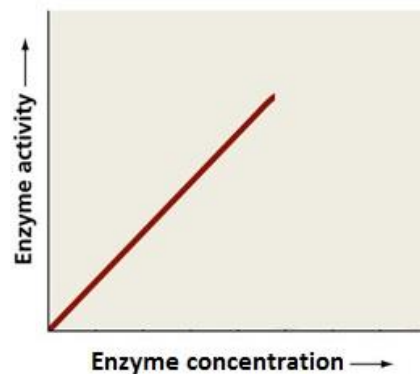


Figure 2.10. Effect of enzyme concentration over reaction rate [12].

### 2.2.2.3. Effect of Temperature

The collision rate of molecules that enter reaction increases when temperature increases. Rate of reactions show 1 to 3 times of increase with each 10 °C of temperature increase. But this increase continues up to a certain value. This value is the optimum temperature of that reaction as it is in Figure 2.11. Afterwards, some changes which occur in enzyme molecules either slow down the reaction or stop it completely. As enzymes are protein-structured, the temperature causes a deterioration in their three-dimensional structures, that is to say, denaturation. This denaturation can be reversed at low temperatures, that is to say, they may regain their activity by a decrease in temperature but they can't be reversed at high temperatures. In this condition, activity is totally lost [12].

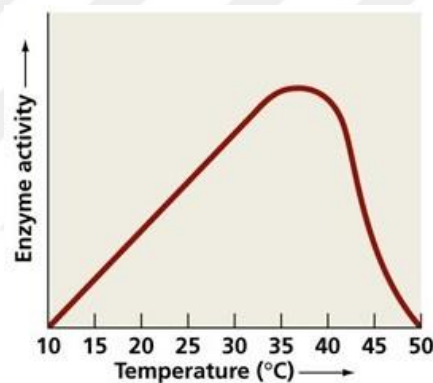


Figure 2.11. Effect of temperature over reaction rate [12].

### 2.2.2.4. pH Effect

Enzymes are amphoteric molecules which contain both acidic and basic groups. The pH of the environment changes the charge of these groups and affects the charge distribution on the surface of the enzyme and net charge. Therefore, a change occurs in the activity of the enzyme. The pH at which enzymes exhibit maximum activity is named as optimum pH as seen in Figure 2.12. This pH value is different for each enzyme [12,13].

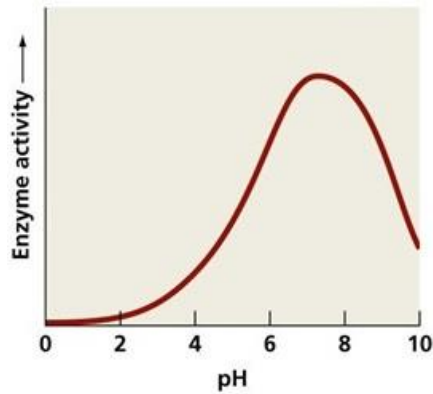


Figure 2.12. Effect of pH over reaction rate [12].

### 2.2.2.5. Inhibitor Effect

Materials which lower the reaction rate or stop it are named as inhibitors. As it is shown in Figure 2.13, some inhibitors form intermediate compounds with the enzyme and lower the reaction rate and some of them are bonded to the active region of enzyme and inhibit the enzyme activity [13].

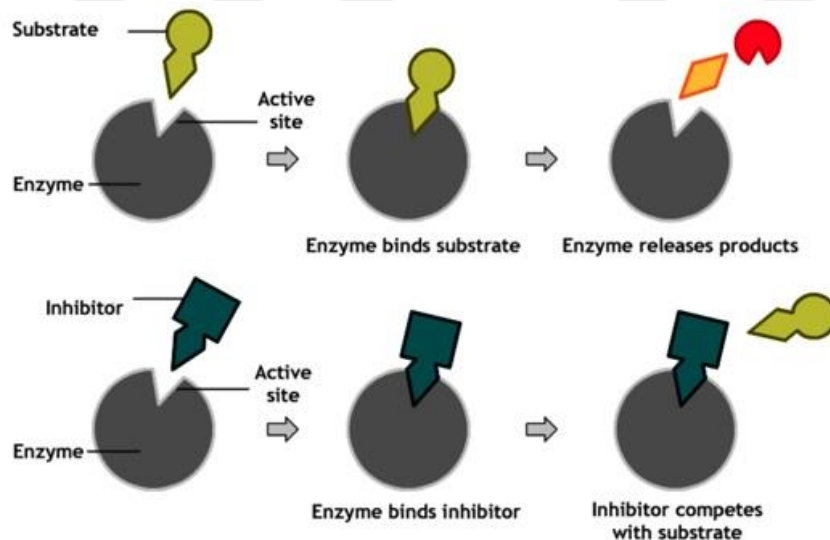
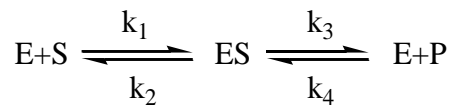


Figure 2.13. Enzyme-inhibitor relationship [23].

Other than these, there are many other factors that influence enzyme activity like water amount, product concentration and time.

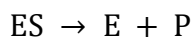
### 2.2.3. Enzyme Kinetics

The first studies related to enzymatic reactions were made by Michaelis-Menten in 1913 and they are known with the theory titled by their names. According to this rule, the enzyme (E) first combines with the substrate (S) and forms the enzyme-substrate (ES) complex. Afterwards, this complex is converted to the product (P) and free enzyme [14,15].



Here,  $k_1$ ,  $k_2$ ,  $k_3$ ,  $k_4$  shows the reaction rate constants that with  $k_1$  ES complex forms, with  $k_2$  ES complex is separated to the enzyme and substrate, with  $k_3$  ES complex forms the enzyme and product and  $k_4$  forms the ES complex from enzyme and product.

The rate of free enzyme and substrate to be converted into ES complex ( $k_4$ ) is very low and ignored and the total reaction is shown as below [14,15].



When the reaction rate is plotted versus substrate concentration, the hyperbolic curve in Figure 2.14 is obtained.

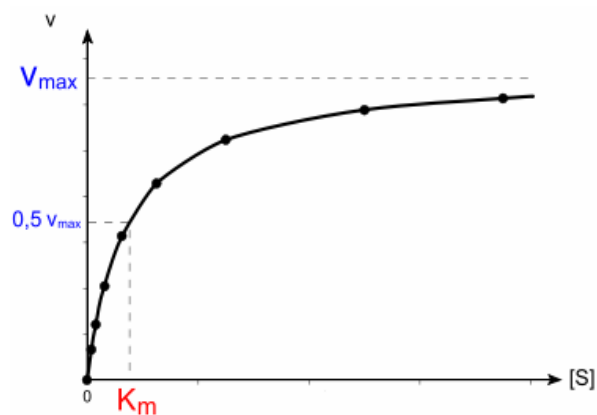


Figure 2.14. Substrate concentration versus reaction rate graph [13].

As it is also seen in the reaction rate vs. substrate concentration graph, rate is directly proportional with substrate concentration. The consumption of [ES] is two types here. A part of [ES] is separated to enzyme and substrate by  $k_2$  rate constant. And the other part of it is converted to enzyme and product with  $k_3$  rate constant [14,15].



The rate of formation of [ES] is given by Equation 2.1.

$$V = k_1 \cdot [E][S] \quad (2.1)$$

The consumption rate of [ES] is given in Equation 2.2.

$$V = k_2 \cdot [ES] + k_3 \cdot [ES] = [ES] (k_2 + k_3) \quad (2.2)$$

According to the steady state approach theory, as the formation rate of [ES] is equal to the consumption rate of [ES], equation 2.3 is obtained [14,15].

$$k_1 \cdot [E][S] = (k_2 + k_3) [ES] \quad (2.3)$$

If Equation 2.3 is solved, Equation 2.4 is obtained.

$$\frac{[E][S]}{[ES]} = K_m \quad (2.4)$$

When concentrations of substances E and S is divided by [ES], a standard value is obtained. As part of total enzymes in the environment ( $E_t$ ) is in free form and part of is in [ES] form, the equation below is obtained (Equation 2.5) [14,15].

$$[E] = [E_t] - [ES] \quad (2.5)$$

Equation 2.5 is written in its place at Equation 2.4 and the new equation is as below (Equation 2.6).

$$[ES] = \frac{[E_t][S]}{K_m + [S]} \quad (2.6)$$

As [ES] is equal to E+P in certain conditions, rate of enzymatic reaction is expressed by Equation 2.7.

$$V = k_3 [ES] \quad (2.7)$$

If Equation 2.6 is written in Equation 2.7, the new rate obtained is as shown below (Equation 2.8).

$$V = k_3 \frac{[E_t][S]}{K_m + [S]} \quad (2.8)$$

As a result of the reaction, as the entire enzyme will be binded with the substrate, maximum rate is reached and it is given by Equation 2.9.

$$V_{\max} = k_3 [E_t] \quad (2.9)$$

Equation 2.9 is written in its place at Equation 2.8 and Michaelis-Menten Equation is obtained (Equation 2.10).

$$V = \frac{V_{\max}[S]}{K_m + [S]} \quad (2.10)$$

$K_m$  shows the interest of the enzyme to the substrate and it is substrate concentration that makes half of the maximum rate. If the interest of the enzyme to substrate is high,  $K_m$  is small. It means that even in low [S]'s, enzyme makes [ES] complex with the substrate. If the interest of enzyme to the substrate is weak,  $K_m$  becomes high [14,15].

As it is seen in Michaelis-Menten equation, the rate of an enzymatic reaction depends on [S] and  $K_m$ .

When different conditions are evaluated;

- If  $[S]$  is too smaller than  $K_m$ , adding  $[S]$  to  $K_m$  changes its value very few anymore. So,  $[S]$  is removed from the denominator (Equation 2.11). In this condition, rate will depend on  $[S]$ .

$$V = \frac{V_{\max}[S]}{K_m+[S]} \cong \frac{V_{\max}[S]}{K_m} \cong K[S] \quad (2.11)$$

- If  $[S]$  is too bigger than  $K_m$ , adding  $K_m$  to  $[S]$  changes its value very few anymore. So,  $K_m$  is removed from the denominator (Equation 2.12). The beginning rate is equal to  $V_{\max}$ .

$$V = \frac{V_{\max}[S]}{K_m+[S]} \cong \frac{V_{\max}[S]}{[S]} = V_{\max} \quad (2.12)$$

- When  $[S]$  equals to  $K_m$ , Equation 2.13 is obtained. In this condition, beginning rate is half of maximum rate.

$$V = \frac{V_{\max}[S]}{K_m+[S]} \cong \frac{V_{\max}[S]}{[S]+[S]} = \frac{V_{\max}}{2} \quad (2.13)$$

If Michaelis-Menten Equation which is a hyperbolic curve is reversed to be converted into a linear equation for practicality and separated to its multiples, Lineweaver-Burk equation is obtained (Equality 2.14) [14,15].

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{[S]}{V_{\max}[S]} \quad (2.14)$$

The expression that gives a straight line equation is shown by Equation 2.15.

$$y = ax + b \quad (2.15)$$

By using the similarity given in Equation 2.14 as  $y = \frac{1}{V}$ ;  $a = \frac{K_m}{V_{\max}}$ ;  $x = \frac{1}{[S]}$  ;

$b = \frac{[S]}{V_{\max}[S]}$  when  $y = 0$ ,  $x = \frac{(-b)}{a}$  and therefore Equation 2.16 is obtained.

$$x = \frac{(-1)}{K_m} \quad (2.16)$$



Value of  $K_m$  is calculated from here. And from the b value of Lineweaver-Burk graph,  $V_{max}$  is obtained. And kinetic parameters are assigned in this manner (Figure 2.15).

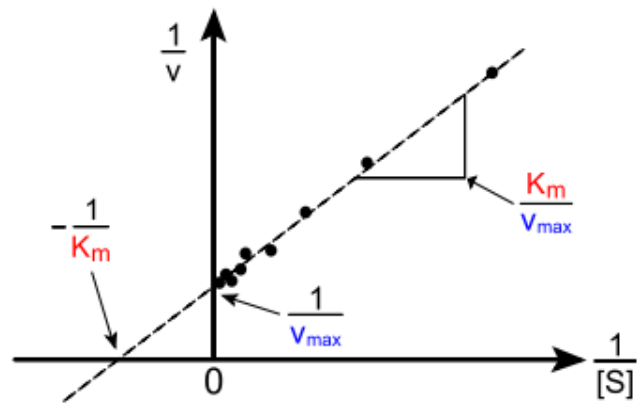


Figure 2.15. Lineweaver-Burk graph [13].

#### 2.2.4. Enzyme Immobilization Methods

Enzyme immobilization is the process of immobilization or bonding of the free enzyme to a carrying substance [9]. As a result of this process, enzymes can be easily separated from the product and reused. And this eliminates the processes applied to prevent contamination of the product and provides advantages such as saving from time and cost. Besides, they have many more advantages such as being more stable and having longer life time and enzymes in different optimum conditions can be used together in the same process [9].

Enzyme immobilization methods are divided into three as binding, cross-linking and encapsulation as it is seen in Figure 2.16.

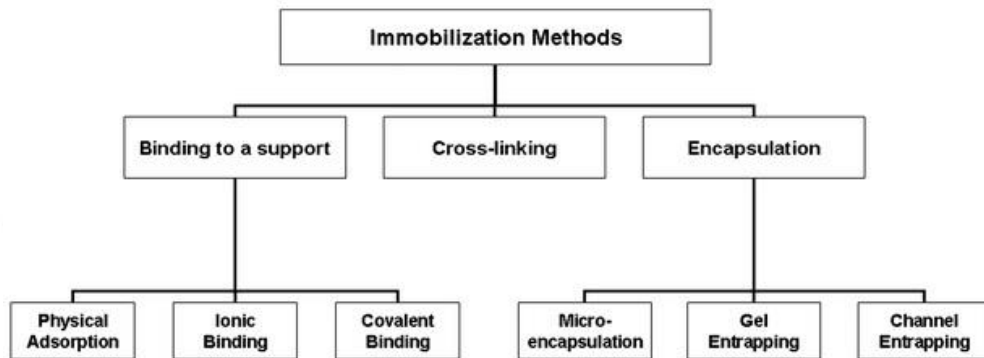


Figure 2.16. Enzyme immobilization methods.

##### 2.2.4.1. Binding to a Support

In this process, new bonds are established between the enzyme and functional groups of support and enzyme is modified. Binding to the transporter is made by enzymes to be binded to the supporting material with physical adsorption or making ionic or covalent bonds (Figure 2.17). These supports are inorganic materials such as silica and porous glass, organic supports such as polyacrylamide including weak activity –OH group or biological supports such as cellulose [16].

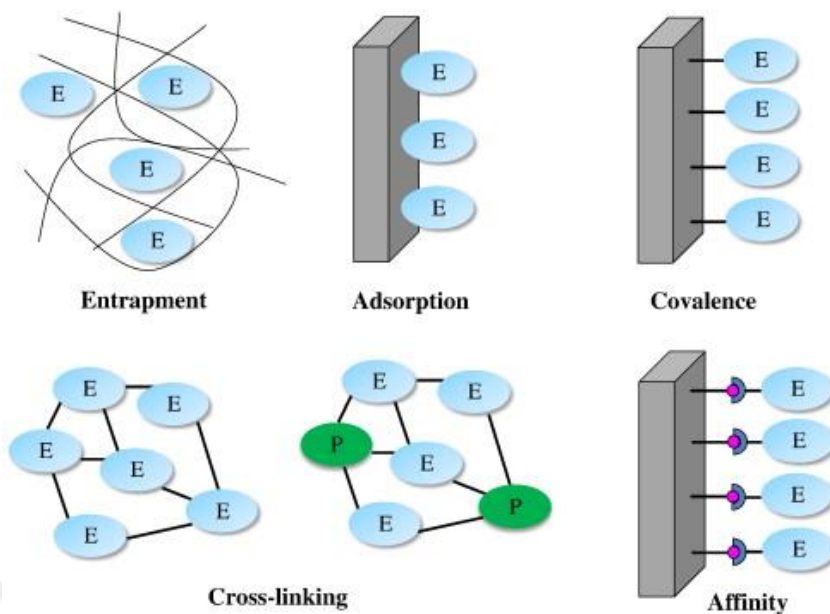


Figure 2.17. Enzyme immobilization methods.

#### 2.2.4.2 Cross-linking

It is a method that enzyme molecules form nonsoluble complexes in water by cross-linking with each other with multiple covalent bonds as a result of their reaction with a cross-linker that contains a functional group like glutaraldehyde (Figure 2.17) [17]. In this method, it can be seen a decrease in enzyme activity due to other structure be bonded to the enzyme structure with covalent bonds or active region can not reach to the substrate staying at the center of the cross-linked structure. On the other hand, cross-linking increases the stability of the enzyme and maintains immobilization to be long lasting.

#### 2.2.4.3. Entrapment (Encapsulation)

It is the placing of the enzyme in an appropriate porous polymer or a membrane for the diffusion of substrate in and diffusion of product out (Figure 2.17). Entrapment method is good for smaller substrate or product molecules compared to other methods. The difference of the entrapment method is that there are no bonds between the enzyme and matrix. As there is no bond between, enzyme losses through matrix pores can be

possible. Electrochemical polymerization method in physical immobilization of enzymes is an attractive one as it is faster, reliable, economical and simple. [18,19].

### 2.2.5. Polyphenol Oxidase

Enzymes have a distinguished place as biocatalysts. Many reactions that enzymes catalyze do not occur without these catalysts and many of these effective synthetic catalysts that will replace these biological catalysts couldn't be synthesized yet.

Polyphenol oxidase (PPO, also known as tyrosinase; EC 1.14.18.1) is an enzyme which was discovered by Schoenbein in 1856 in mushrooms. Polyphenol oxidases are oxidoreductases containing copper and in the presence of molecular oxygen, they catalyze hydroxylation and oxidation of phenolic compounds. Polyphenol oxidases are divided into three groups as tyrosinase, catechol oxidase and laccase. Tyrosinase catalyzes (EC. 1.14.18.1) hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity) (Figure 2.18). Catechol oxidase (EC. 1.10.3.1) catalyzes oxidation of only *o*-diphenols. And laccase (EC. 1.10.3.2) catalyze oxidation of both *o*-diphenols and *p*-diphenols to corresponding quinones. Laccase and catechol oxidases can't catalyze hydroxylation reactions [20,21].

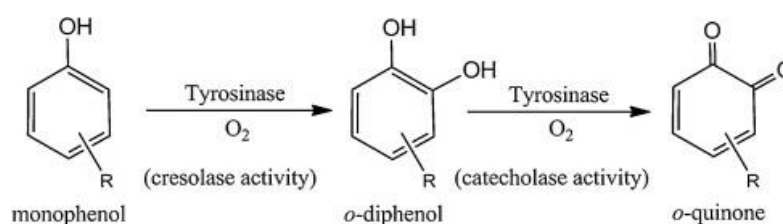


Figure 2.18. Polyphenol oxidase catalysis reaction.

Reaction catalyzed by polyphenol oxidase is a biotransformation that occurs in almost all organisms and it is only catalyzed by tyrosinase enzyme [22]. Tyrosinase enzyme is responsible from the formation of pigments in skin, hair and eye and the color darkenings occurring in fruits, vegetables and mushrooms as a result of contact with oxygen.

This enzyme that is commonly found in plants oxidizes its substrates to dark colored quinonoic compounds in the presence of oxygen. These compounds polymerize and form brown pigments and due to this, these reactions are named as browning reactions. These browning reactions that tyrosinase form are unwanted in the food industry [23]. Fruits and vegetables darkening when they are cut and peeled decreases the food quality and value of these products. This is known as enzymatic darkening in the food industry. On the other hand, this color change is something desired in some foods. For example, these browning products give taste and odor to foods such as tea, coffee, cacao, dry grapes and dry prune. The good color and taste of dry fig and date is as a result of enzymatic browning [24].

### 2.2.5.1. Working Mechanism of Polyphenol Oxidase

PPO does not need coenzyme for its activity. They are enzymes that use all phenolic compounds as substrate which show group specificity. Generally, PPO oxidizes phenolic substrates in the presence of oxygen. Active sites of PPO pass through transitions between metoxy, oxy and deoxy forms in a cyclical manner (Figure 2.19).

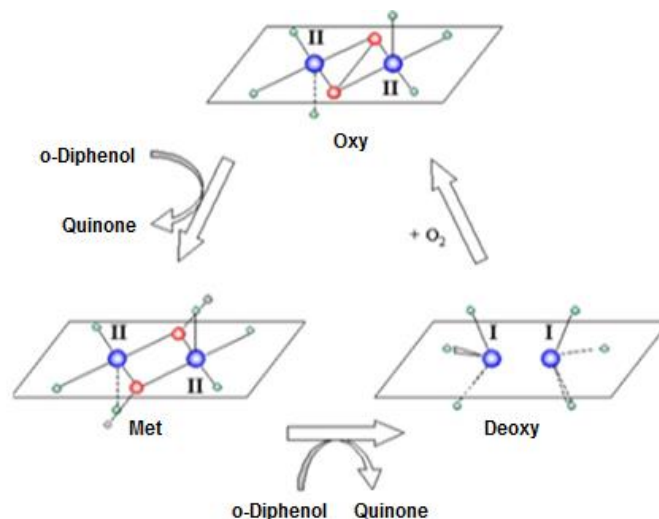


Figure 2.19. Cyclical transitions of PPO's active site between oxy and deoxy forms during catalysis. Blue, red and green circles represent copper, oxygen and histidine [26].

In each cycle, two catechol molecules are oxidized. One molecule oxygen is reduced to water and two quinone products are formed. Dioxygen ( $O_2$ ) replaces the solvent molecule ( $H_2O$ ) that is bonded to CuA in reduced enzyme form (reduced deoxy form) and it is bonded to the copper metal center of the enzyme [25].

UV spectroscopy results support the idea that molecular oxygen is first bonded as peroxide and then catechol is bonded. One of two hydroxyl groups of catechol molecule goes under deprotonation and catechol is bonded to CuB (oxy form) (Figure 2.20). After two electrons are transferred from the substrate to peroxide, peroxide group is protonated and O-O bond is broken [25].

The second hydroxyl group that didn't enter coordination gives one proton and maintains formation of water loss and formation of *o*-quinone product. Protoning of the bridge making group with solvent makes active site hydroxyl bridged with two coppers form (metoxy form). Another catechol molecule performs a co-substrate function and reduces hydroxyl bridged Cu (II) to Cu (I) form. This step of the proposed reaction path is supported by data about *o*-diphenol oxidase activity of tyrosinase (Figure 2.20). Cu(I)-Cu(I) form of the active site repeats catalytic cycle again [25].

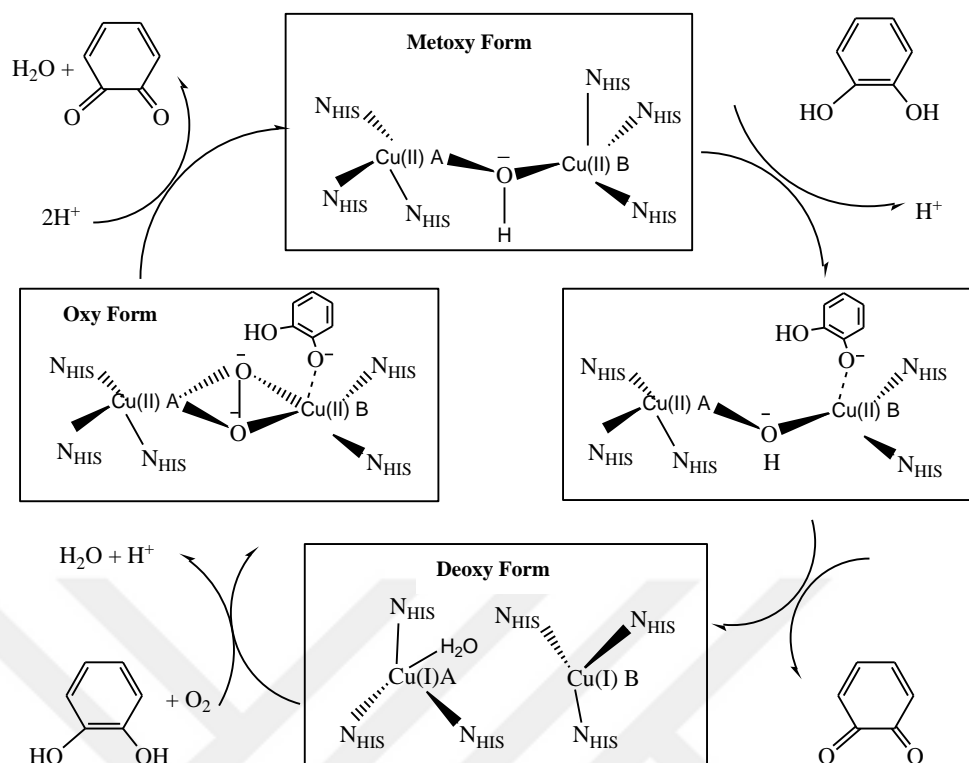


Figure 2.20. Reaction mechanism of PPO.

### 2.2.5.2. Usage Areas of Polyphenol Oxidase

Industrial use and applications of tyrosinase attract the interest of scientists and researchers more recently. Tyrosinase enzyme is used in health and cosmetics industry, in cleaning of waste waters and determination of phenolic materials. An important usage area of tyrosinase is the food industry. It is used in eliminating the sour and bitter taste in tea, coffee and cacao beans and developing the taste of these drinks. On the other hand, tyrosinase enzyme should be active for products like dry grape and dry prune to become desired in view and taste [26]. Another use of it is that it is an alternative material for transglutaminase enzyme used in enzymatic cross-linking of proteins. Besides this, tyrosinase is used in synthesis of some drug active materials and in the synthesis of melanin added to cosmetic products to prevent ultraviolet lights from the sun.

On the other hand, phenolic substances that contaminate environmental waters with industrial and domestic waste create pollution and it is hazardous for living species in

water. In order to prevent phenolic compounds that show toxicity to contaminate nature, it is better to give waste materials to the environment after being refined from phenolic compounds and for this purpose, enzymes are used as an efficient and easy way. For the use of tyrosinase to remove toxic phenolic compounds, studies continue in the field of engineering. When the enzyme is used in waste water, it oxidizes selectively phenolic substances and converts them to quinones. The reactive quinones that are formed precipitate by polymerizing and polyphenols are removed from water by this way.

### **2.2.5.3. Use of Polyphenol Oxidase in Biosensors**

Like all pollutants, it is also important to determine phenolics in environmental waters and industrial waste water. Besides this, it is required to analyze phenolic compounds in vegetables and fruits or determine these materials in industrial processes. But methods used for this purpose are expensive and require long processes. As an alternative method, biosensors lead in this area and another use of tyrosinase is that it exists as biomaterial in biosensors used in determining phenolic compounds. Biosensors which have sensitive and fast determining quality are obtained as a result of combination of sensor systems with biological materials and they are used for analysis purposes in many areas like medicine, defense, food industry etc. [27]. In enzymatic biosensors, the biological material are enzymes and selective determination of polyphenols can be made by tyrosinase based amperometric biosensors due to the enzyme being a catalyzer specific to phenolics [28-32].

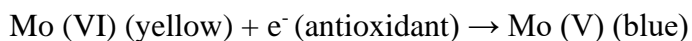
## **2.3. SAMPLE ANALYSIS**

In this study, total polyphenol material content of a waste water sample was measured using polyphenol oxidase enzyme electrodes. Folin-Ciocalteu method was also used as control method to validate the analysis by enzyme electrodes.

Folin-Ciocalteu method developed by Singleton et al is used to measure total phenolic content. This method is based on the fact that phenolic compound dissolved in water forms a colored compound with the Folin reactive in alkali environment [33] and



measurement of absorbance according to this color intensity. Folin-Ciocalteu reactive (FCR) which gives its name to the method is a molybdophosphotungstic heteropolyacid and its assumed active center is Mo (VI) ( $3\text{H}_2\text{O} \cdot \text{P}_2\text{O}_5 \cdot 13\text{WO}_3 \cdot 5\text{MoO}_3 \cdot 10\text{H}_2\text{O}$ ).



Phenolic compounds enter into reaction with FCR only under basic conditions and pH of the environment is set to 10 by sodium carbonate solution. Removal of a phenolic proton causes formation of a phenolate anion that has reducing capability of FCR and blue colored compounds are formed between phenolate and FCR. FCR can also be reduced by many non-phenolic compounds and it is not specific to phenolic compounds. In fact, this method measures the reduction capacity of a sample. It may interact with reductant materials in the environment such as ascorbic acid [34].

In spite of this disadvantage, this method is used very frequently due to its simplicity, repeatability and the correlation it shows with other methods. Method results are given as a standard phenolic material or usually as gallic acid equivalent.

## **PART 3**

### **MATERIALS AND METHODS**

#### **3.1. CHEMICAL MATERIALS**

Tyrosinase from mushroom (EC 1.14.18.1) and 25% glutaraldehyde solution was purchased from Sigma-Aldrich. Phosphate buffer components,  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  were from Merck. For MBTH solution, 3-Methyl-2-benzodiazolinon hydrazon hydrochloride monohydrate (Aldrich) was dissolved in ethanol (Carlo Erba). Acetone and catechol were provided from Sigma-Aldrich and sulfuric acid was obtained from Merck Company.

#### **3.2. INSTRUMENTS**

##### **3.2.1. Potentiostat**

Polymerization was made by cyclic voltammetry using GAMRY Instruments Interface 1000 Potentiostat/Galvanostat ZRA. The voltage was changed in the scanning interval cyclically and the current was measured. Polymerization was performed in 60 cycles.

##### **3.2.2. UV-Visible Spectrometry**

In measurements, to determine the activity of immobilized enzyme, Shimadzu UV-1201V model spectrophotometer was used. Colored complexes of polyphenol oxidase products formed after biotransformation were obtained using Besthorn's Hydrazone method and their absorbance in 495 nm were measured. Enzyme activities were determined with this method.

### **3.2.3. SEM**

SEM images were taken with Carl ZEISS ULTRA PLUS GEMINI FESEM scanning electron microscope in Karabük University Iron & Steel Institute MARGEM laboratories. Poly(2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol) conducting polymer was coated over platinum electrode. 5000 times and 10000 times magnified pictures of surface of polymer coated electrode were taken before and after enzyme immobilization.

### **3.2.4. pH meter**

Hanna Instruments HI 221 brand pH meter was used. This instrument uses an electrode that produces an electrical signal to provide a potentiometric measurement converting the signal into pH units. The signal is measured as potential. What is required to make pH measurement is the voltage that sensing electrode provides proportional to the logarithm of hydrogen ion activity within the product and the voltage that reference electrode provides ideally that is independent, fixed and continuous. Electrical signal is formed with the difference between these two voltages. Voltage difference between the reference and sensing electrode is measured by pH meter and converted into pH value.

### **3.2.5. Magnetic Mixer with Heater**

It was performed to mix prepared solutions with the help of a magnet and with the effect of magnetic field at the same number of cycles and temperature by a MTOPS MS300 HS brand magnetic mixer with heater.

### **3.2.6. Vortex Tube Mixer**

Dlab MX-S Brand mixer was used in constant speed. It was used for the purpose of providing an effective and efficient mixing vortex to the solutions in experiment tubes and obtaining a homogeneous mixing.

### 3.2.7. Shaking Water Bath

Nuve ST 30 brand shaking water bath was used for keeping solutions inside shaking water bath at 25 °C constant temperature and by maintaining homogeneous mix of them by shaking during the reaction of enzyme and substrate. In temperature optimization, water bath is brought to temperatures between 4 °C and 80 °C and activity measurements were made to determine the temperature at which enzyme electrode works best.

## 3.3. METHODS

### 3.3.1. Electrochemical Synthesis of Poly(2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol)

Polymerization of 2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol (TTzFr) monomer was performed electrochemically as it is seen in Figure 3.1. Monomer was synthesized by Söylemez et al. [35].

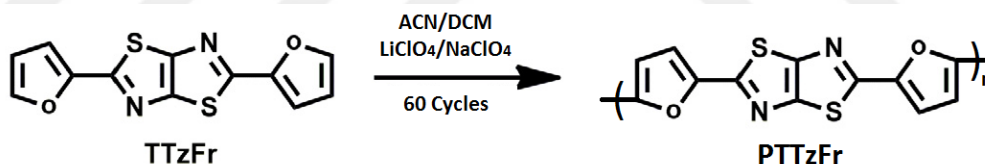


Figure 3.1. Polimerization of TTzFr.

Electropolymerization was made by cyclic voltammetry using a 3-electrode system (Figure 3.2) and poly(2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol) (PTTzFr) polymer was obtained. Platinum was used as working and counter electrode and silver (Ag/Ag<sup>+</sup>) electrode was used as reference electrode.

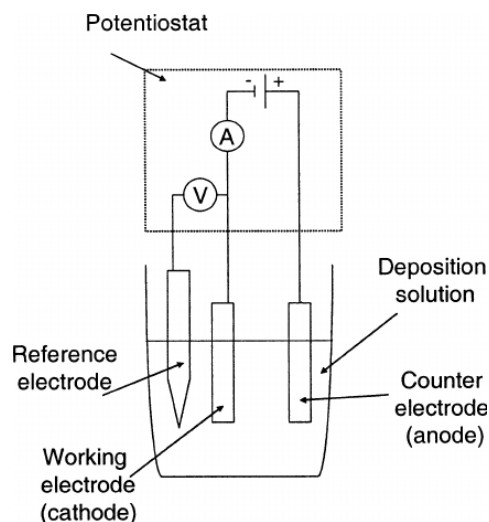


Figure 3.2. Three electrode system.

Electropolymerization cell was prepared by adding monomer and electrolyte to the solvent (deposition solution) (Figure 3.3). As solvent, 95:5 ratio acetonitrile (ACN) and dichloromethane (DCM) was used and as electrolyte, 0.1 M lithium perchlorate/sodium perchlorate was used. Monomer concentration was taken as  $10^{-3}$  M and polymerization window was determined by scanning with cyclic voltammetry in (-2) – (+2) V potential interval, then by using this interval, polymerization of the monomer was maintained [10]. After this operation, each enzyme electrode was washed a few times with distilled water to remove free supporting electrolyte remaining on the surface. When it is not used, it was kept at +4 °C in the pH 7.5 phosphate tampon.

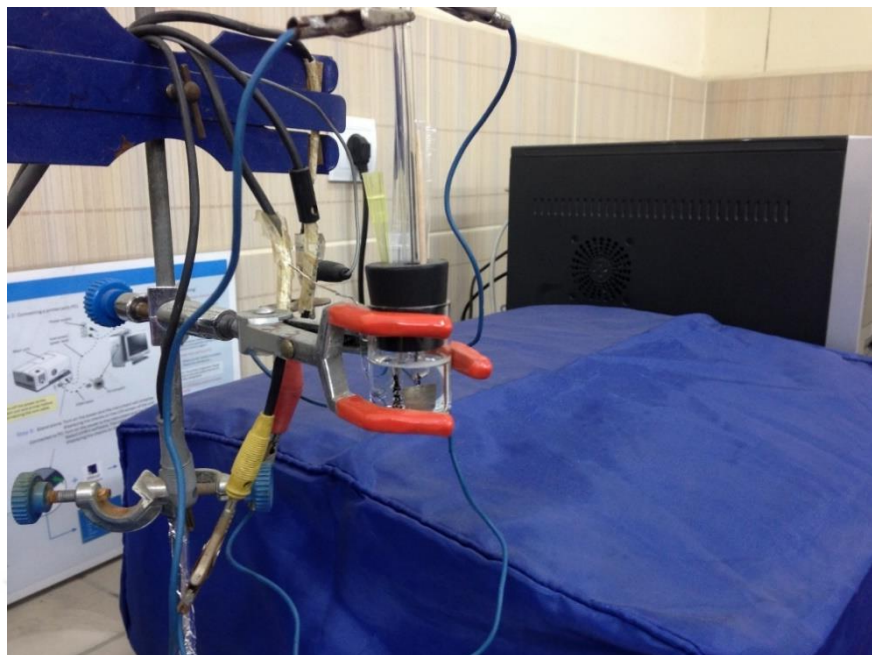


Figure 3.3. Electropolymerization cell.

### 3.3.2. Enzyme Immobilization in PTTzFr Matrix

In this study, enzyme immobilization was made with cross-linking method. In enzyme immobilization, adsorption, covalent bonding, cross-linking and entrapment methods are used. Adsorption method causes the enzyme flow to the environment faster and disappear and enzyme electrode loses its activity after a short time. Entrapment method is more advantageous than adsorption. It maintains the enzyme to remain stable for longer time. And in cross-linking and covalent bonding, stability of the enzyme and therefore the electrode increases remarkably. In this immobilization method, more enzyme is immobilized, enzyme loss is less and higher signals are received. In this study, enzyme solution was prepared at  $0.2 \text{ g mL}^{-1}$  concentration in pH 7.5 phosphate buffer (Enzyme Unit: 2687 U/mg solid). 6  $\mu\text{L}$  enzyme solution was dropped to the surface of polymer coated on platinum electrode and it was left to dry for 30 minutes at room temperature conditions. 6  $\mu\text{L}$  1% glutaraldehyde solution was added above and it was dried for 2 hours at room temperature and after this it was left for one night at  $+4 \text{ }^\circ\text{C}$ . Therefore, enzyme electrodes coated with polymer over platinum and which PPO enzyme was immobilized were prepared (Pt/PTTzFr/PPO).

### 3.3.3. Besthorn's Hydrazone Method

Catechol solutions are prepared in various concentrations and Besthorn's Hydrazone method is applied with its process steps in the literature [36]. Firstly, MBTH reactive is added to the solutions. Enzyme electrode is dipped for certain periods. After product formation, H<sub>2</sub>SO<sub>4</sub> and acetone is added. The colored complexes which are formed are measured with respect to their absorption in 495 nm and enzyme activities are determined (Figure 3.4.).

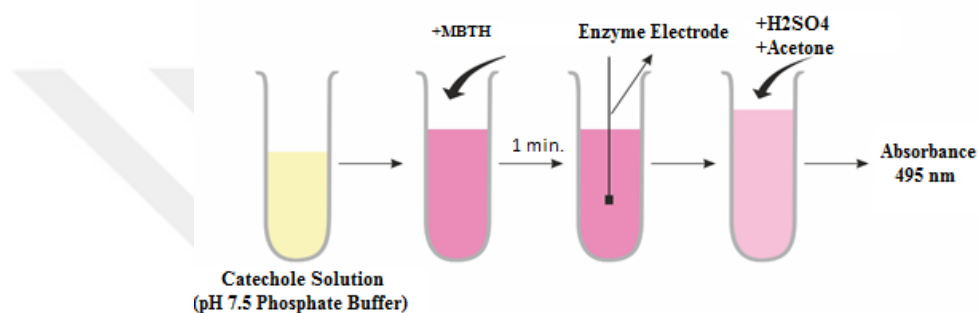


Figure 3.4. Besthorn's Hydrazone method.

MBTH solution used in this method was prepared as the concentration of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (C<sub>8</sub>H<sub>9</sub>N<sub>3</sub>S.HCl.H<sub>2</sub>O) was 3.0 mg mL<sup>-1</sup> dissolving in ethyl alcohol.

### 3.3.4. Determination of the Enzyme Activity

In the reaction where polyphenol oxidase enzyme is the catalyzer, the substrate of the enzyme is phenolic materials. In the substrate analysis, polyphenol oxidase enzyme procedure was applied taking as a basis Besthorn's Hydrazone method and pyrocatechol that is one of the substrates of the enzyme was used (Figure 3.5).

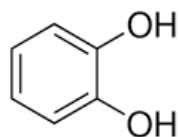


Figure 3.5. Pyrocatechol.

Solutions of pyrocatechol in different concentrations were prepared in the pH 7.5 buffer and after adding 1 mL MBTH reactive to each solution with different concentration, polyphenol oxidase enzyme electrode was dipped for 0, 5, 10, 15 minutes and transformation reaction of phenols to *o*-quinone was performed as it is seen in Figure 3.6.

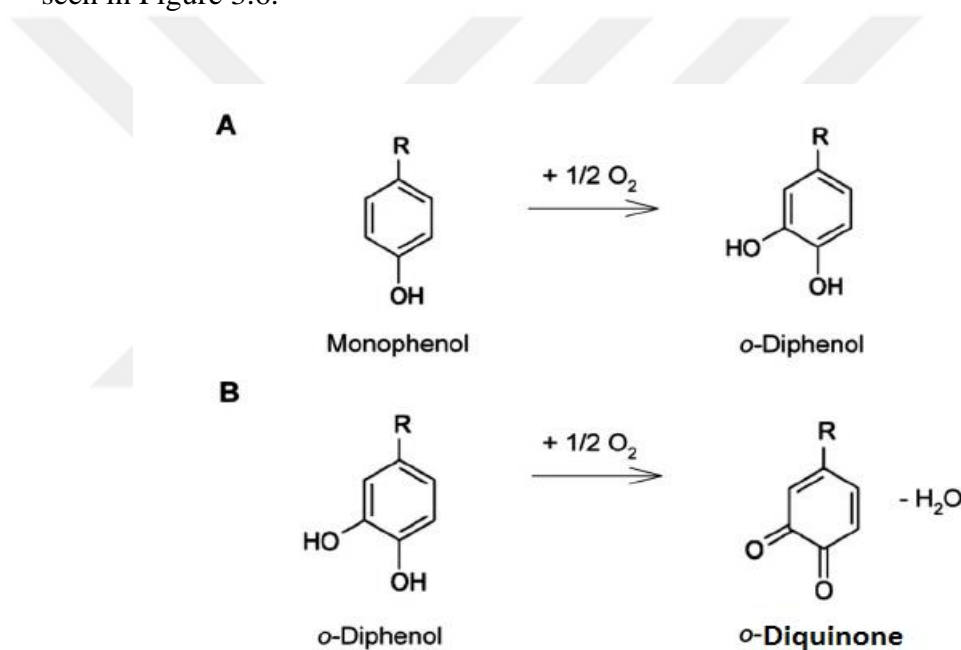


Figure 3.6. PPO's cresolase and catecholase activities: A) monophenol's *o*-hydroxylation, B) oxidation of *o*-diphenol to *o*-quinone.

Afterwards, complex formation of quinones with MBTH was occurred as it is seen in Figure 3.7. 1 mL sulfuric acid, 1 mL acetone solutions were added and mixed then absorbance measurements were performed at 495 nm. Absorbance versus time graphs were plotted and reaction rate was calculated from the slope of these graphs. Therefore, for each concentration, polyphenol oxidase enzyme electrode activities were determined.



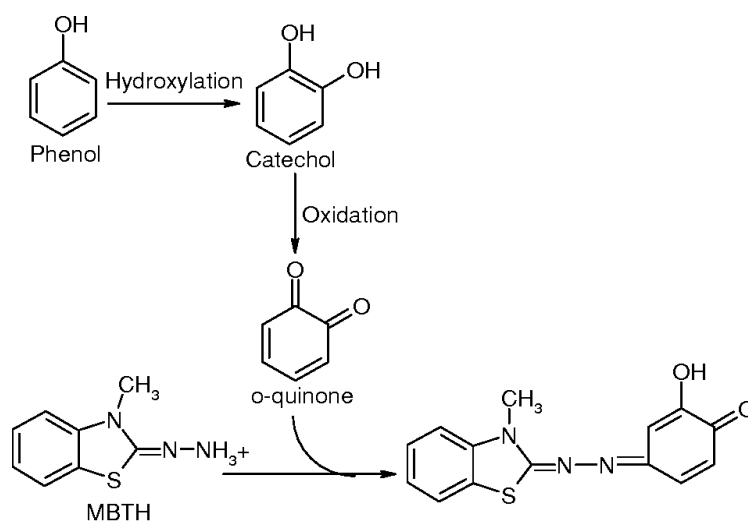


Figure 3.7. *o*-Quinone formed to establish colored compound with MBTH reactive [36].

### 3.3.5. Assessment of Kinetic Parameters

With measurements made, kinetic characterization of enzyme electrode was made for immobilized polyphenol oxidase enzyme. Enzyme activity  $V$  vs. substrate concentration  $[S]$  graph was plotted and from this graph, Lineweaver-Burk graph was obtained ( $1/V$  vs.  $1/[S]$ ) [38,39]. From Lineweaver-Burk graph, with Michaelis-Menten method [40], enzyme electrode's kinetic parameters maximum enzyme activity ( $V_{\max}$ ) and Michaelis-Menten constant ( $K_m$ ) were detected.

## 3.4. OPTIMIZATIONS

### 3.4.1. Optimum pH Determination

For immobilized polyphenol oxidase enzyme, pH optimization was made. For this purpose, activity measurements of enzyme electrodes were taken at pH values between 3.0 and 10.5 and the optimum pH value at the enzyme electrode that operates best was determined.

### **3.4.2. Optimum Temperature Determination**

Temperature optimization was made for immobilized polyphenol oxidase enzyme. For this purpose, activity measurements of enzyme electrode were made at temperatures between 4 °C ve 80 °C and the temperature at which enzyme electrode works best was determined.

### **3.4.3. Optimization of Immobilized Enzyme Amount**

By changing the immobilized enzyme amount, it was tried to determine the enzyme concentration that gives the best result. For this purpose, 4 different amounts of enzyme were applied to 4 different polymer coated electrode. They were dried by adding glutaraldehyde solution and enzyme activities were measured.

### **3.4.4. Optimization of Glutaraldehyde Amount**

In order to determine optimum glutaraldehyde concentration, enzyme solution in the same amount and concentration was dropped to polymer surfaces of 4 different electrodes. Glutaraldehyde solutions of four different amount were applied to these 4 electrodes and enzyme activities of electrodes were measured to determine the glutaraldehyde concentration which gives the best result.

### **3.4.5. Assessment of Stability**

It was aimed to determine stability of enzyme electrodes with measurements following each other. With activity measurements made one after another in the same day, it was checked whether activity changes or not when number of measurements increase. Therefore, stability of the enzyme electrode was determined.

### **3.4.6. Determination of Shelf Life**

For PPO electrode, activity measurements were made once in three days to observe how the stability of enzyme electrode changes and shelf life was determined.

### **3.5. PHENOLIC SUBSTANCE DETERMINATION IN WASTE WATER SAMPLE**

Some part of the studies made in Karabük University Department of Chemistry on “Bioenergy Production from Solid Wastes” is over Lignin. Ligneous wastes consist of 3 main components: cellulose, hemicellulose and lignin. In the paper industry, wood and lignocellulosic biomass is separated by delignification to produce paper. As a result of this process, lignin remains as a waste. However, lignin is a natural source of aromatic monomeric and dimeric phenol which contains phenyl and propane units. For this reason, by liquefying lignin, bio-oil is obtained that is rich in phenolic compounds. Studies to develop more efficient and economical methods in producing biofuels by liquefaction of lignin continue in our department. In the present study, we worked on determination of polyphenolics in the process water that appears as a waste during bioenergy production from lignin and the samples were taken from the process water which was formed as a result of the studies above.

In this study, waste water obtained from processing fir tree at 275 °C for 12 hours was analyzed for polyphenolic substances.

#### **3.5.1. Analysis with Pt/PTTzFr/PPO Enzyme Electrode**

Catechol standard solutions in buffer at different concentrations were prepared at concentrations between 0.1 M and 0.8 M and calibration curve was plotted with absorbance measurements of standards. First, Pt/PTTzFr/PPO electrode was dipped for 10 minutes to each solution and then Besthorn’s Hydrazone method was applied and their absorbance was measured at 495 nm. Waste water sample was diluted 1:10 and electrode was dipped for 10 minutes and the same method was applied. Results were placed in the calibration graph and total polyphenolic amount in sample was calculated as mg catechol equivalent.

### 3.5.2. Analysis with Folin-Ciocalteu Method

Folin-Ciocalteu method used as control method was adapted to catechol and calibration graph was prepared by catechol solutions. As it is seen in Figure 3.8, first catechol standards in different concentrations (between 0.01 and 0.4 M) were prepared. 40  $\mu\text{L}$  were taken from standards and 3.16 mL water and 200  $\mu\text{L}$  Folin-Ciocalteu reactive were added on them. It was mixed and after waiting 5 minutes, 600  $\mu\text{L}$  20%  $\text{Na}_2\text{CO}_3$  solution was added and mixed. After waiting for 2 hours, absorbance of solutions was measured at 765 nm and calibration graph was plotted. Same operations were applied for samples also by taking 40  $\mu\text{L}$  waste water sample (1:10 diluted) and absorbance values were measured and from the calibration curve, total polyphenol amount in sample was determined as mg catechol equivalent [33].

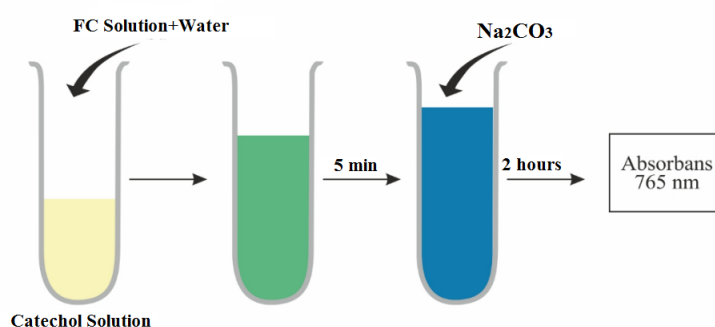


Figure 3.8. Process steps of Folin-Ciocalteu method.

## **PART 4**

### **RESULTS AND DISCUSSION**

In this study, formation of enzyme electrodes was performed by immobilization of polyphenol oxidase enzyme to conductive polymer poly(2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol) matrix. Optimum conditions for preparation of enzyme electrodes were determined, their kinetic characterization was performed and sequential stability of activity measurements and shelf life were determined. Calibration graph was plotted, linear range and LOQ was obtained and enzyme electrodes developed were applied to the analysis of polyphenolic materials in waste water sample.

#### **4.1. ELECTROPOLYMERIZATION OF TTzFr MONOMER**

Synthesis of poly(2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol) with cyclic voltametry was performed in ACN/DCM (95:5) solution that includes 0.1 M lithium perchlorate/sodium perchlorate and  $10^{-3}$  M TTzFr monomer. Scans were made for 60 cycles between 0.0 and +1.4 V [35]. This interval is the scanning interval that oxidation and reduction peaks of the polymer take place. As it is seen in Figure 4.1, when polymerization progresses in each cycle, peak current increases and shows that polymerization occurred.

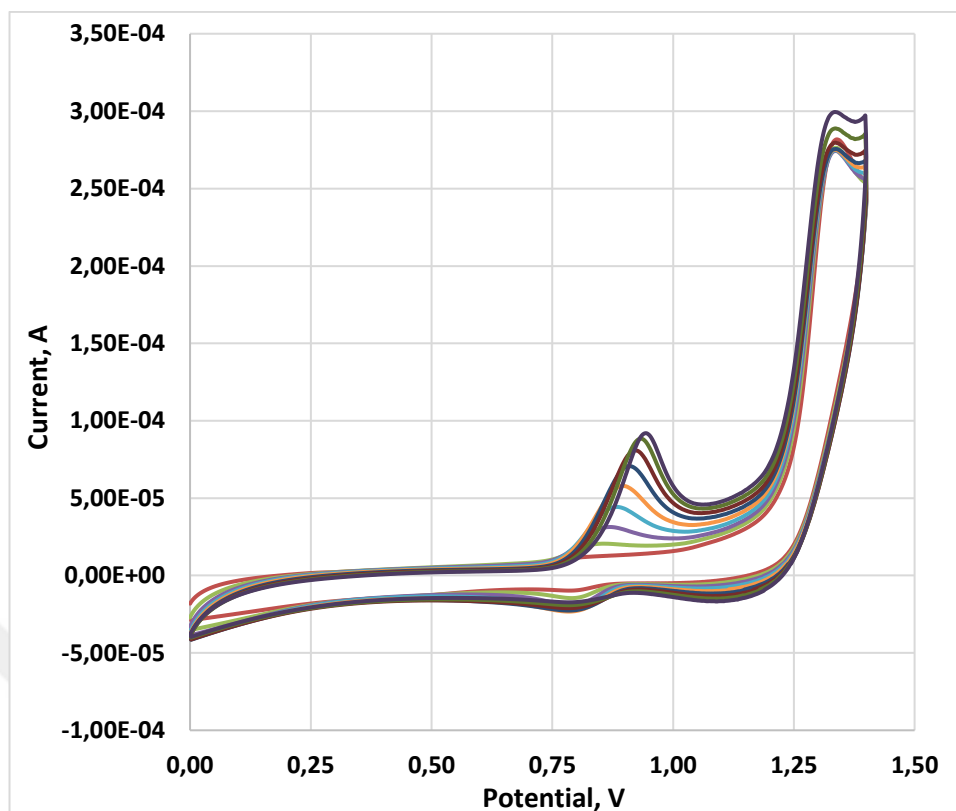


Figure 4.1. Polymerization of TTzFr monomer against Ag/AgCl with cyclic voltametry.

#### 4.2. SEM ANALYSIS

Platinum electrode was covered with poly(2,5-di(furan-2-yl)thiazolo[5,4-*d*]thiazol) and the enzyme was immobilized. SEM images of the polymer surface was taken at 5000 and 10000 magnification before and after enzyme immobilization. As it is seen in Figure 4.2 (a) and (b), electrode surface covered with PTTzFr has a uniform view of conducting polymer coating. After enzyme immobilization, SEM views of surface were taken again at 5000 and 10000 magnification. Surface morphology has changed a lot and it shows the enzyme molecules immobilized homogeneously (Figure 4.3 (a) and (b)). During the SEM analysis, enzyme molecules were observed as moving bodies when the electrons beat the enzyme molecules. Inorganic substances do not show such a behaviour but organic enzyme molecules show motions under the effect of electron beam.

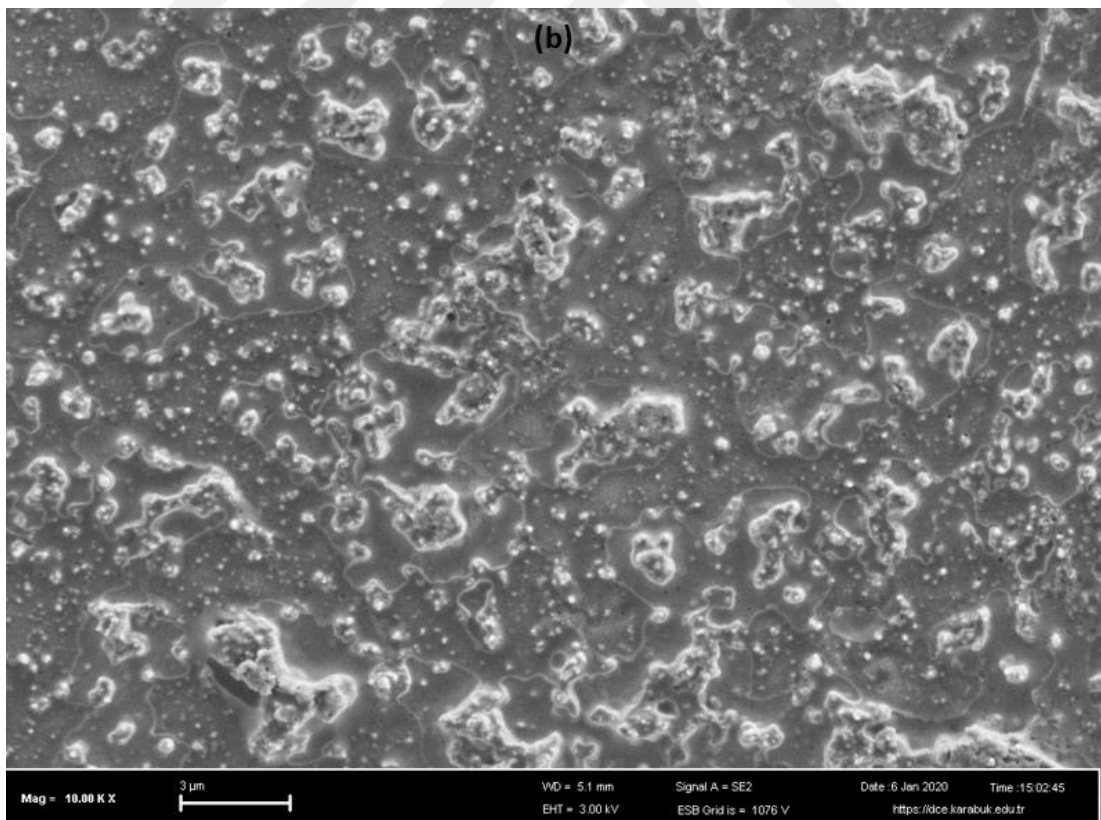
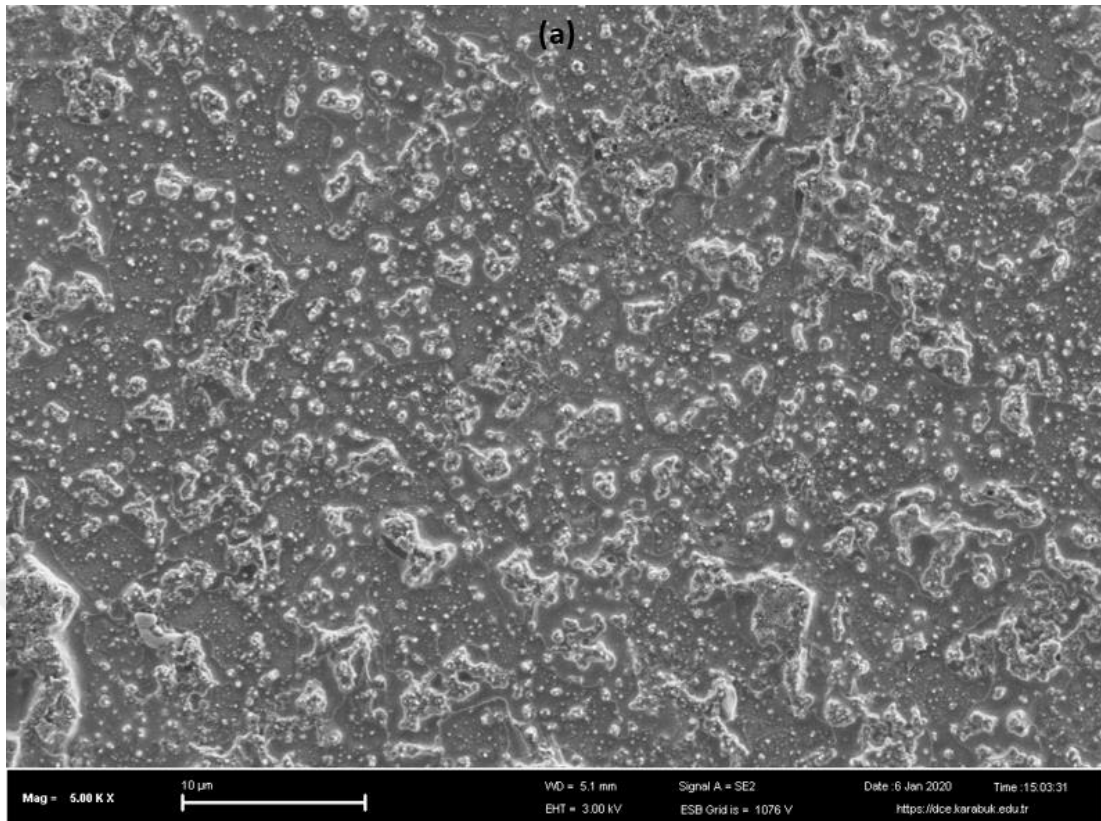


Figure 4.2. a) SEM, polymer surface (x5000), b) SEM, polymer surface (x10000).

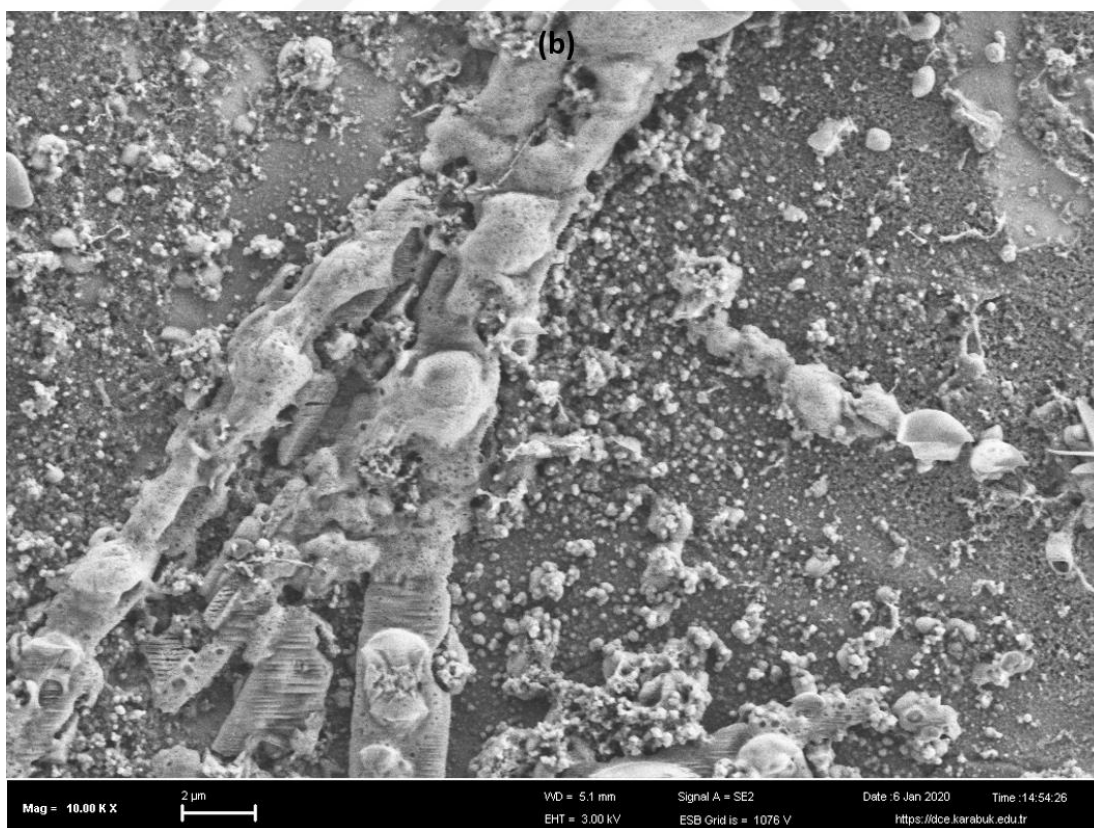
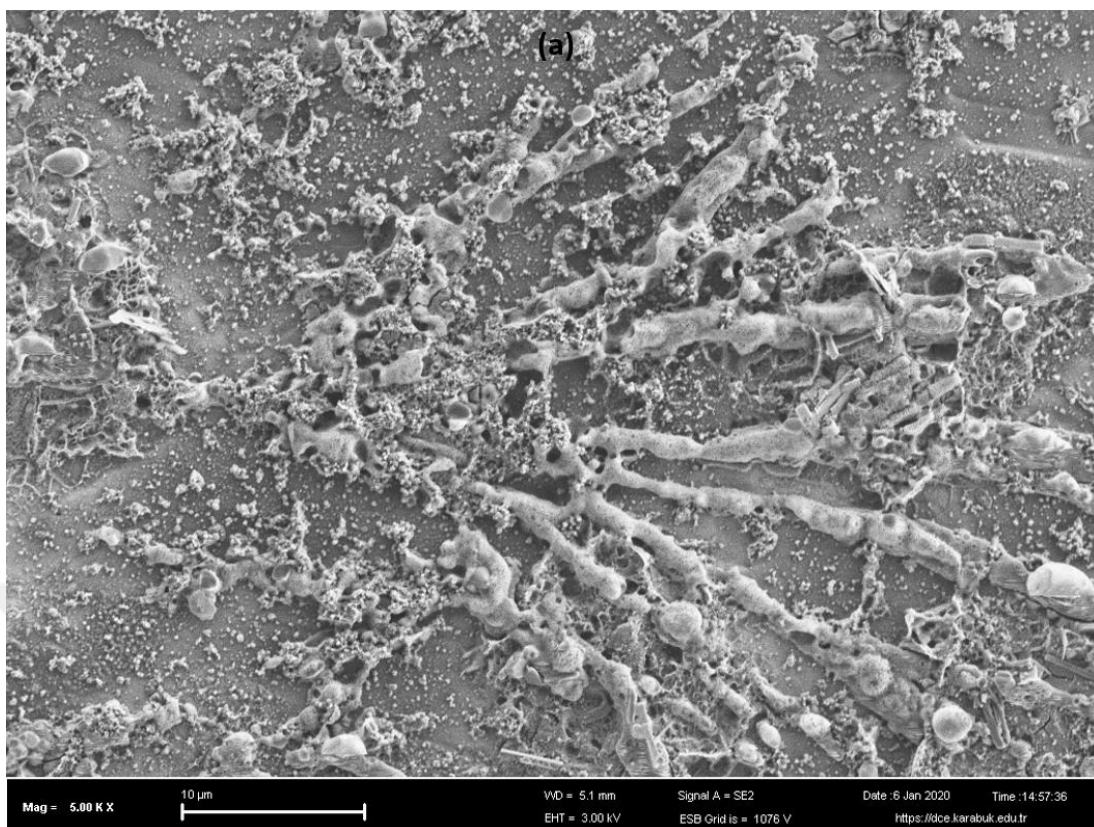


Figure 4.3. a) SEM, after enzyme immobilization (x5000), b) SEM, after enzyme immobilization (x10000).



### **4.3. OPTIMIZATION OF ENZYME ELECTRODES**

Optimization of enzyme electrodes was made with activity measurements of substrate solutions prepared in constant concentration. The parameter examined was changed and enzyme activity was measured. Electrode was dipped to substrate solution for different times and reaction rate was obtained and enzyme activities were calculated.

#### **4.3.1. Effect of pH on Enzyme Electrodes**

Behaviour of immobilized polyphenol oxidase against pH change was examined. Measurements were made with 1.0 M catechole solutions prepared in phosphate buffer at 25 °C. Buffer pH was set to pH values between 3.0 and 10.5 and electrode activity were measured for each pH. In studies before this, optimum pH of free enzyme and immobilized enzyme on platinum electrodes have been found as 5.0 and 7.0 respectively [41]. In this study, the optimum pH for PPO immobilized to PTTzFr polymer on platinum electrode was determined as 7.5. As it is seen in Figure 4.4, maximum activity is observed with pH 7.5 and enzyme activities obtained at pH 7.5 and pH 8.0 are very close to each other and both pH values can be used. In case poly(2,5-di(furan-2-yl) thiazolo[5,4-*d*] thiazol) is used as the matrix, it was observed that pH value at which the enzyme shows maximum activity increases relative to the free enzyme. This condition indicates that pH of the micro-environment of the enzyme is different from pH of the measured point, that is to say, pH of the micro-environment that the enzyme is in (pH 5) still keeps its value even though pH of the exterior is high. According to this, poly(2,5-di(furan-2-yl) thiazolo[5,4-*d*] thiazol) matrix contributes to protecting pH of the micro-environment that the enzyme is within up to a certain point. Thus pH 7.5 was used in optimizations, characterization, and sample analysis in this study.

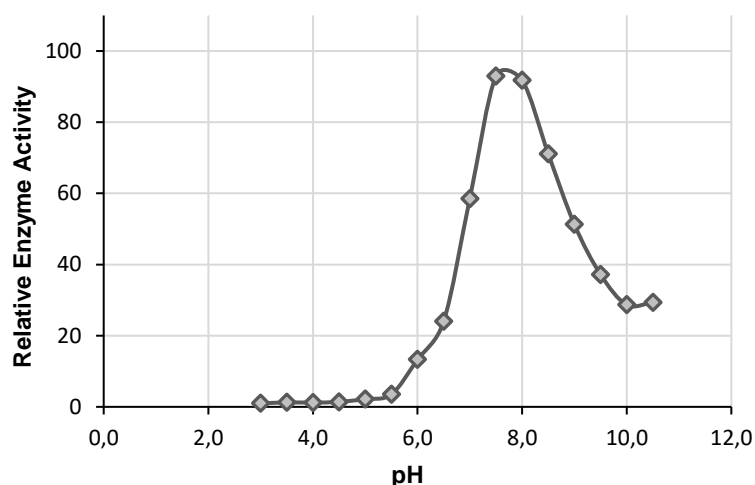


Figure 4.4. Effect of pH on Pt/PTTzFr/PPO electrode activity.

#### 4.3.2. Effect of Temperature on Enzyme Electrodes

The behaviour of enzyme that was immobilized to PTTzFr polymer matrix was examined against temperature change. Measurements were made with 1.0 M catechole solutions prepared in pH 7.5 phosphate buffer. The temperature was set to values between 4 °C and 80 °C and electrode activity measurements were made at each temperature. The effect of temperature change over the activity of Pt/PTTzFr/PPO electrode is given in Figure 4.5. Maximum activity of immobilized enzyme is at 45 °C and enzyme shows activity up to 75 °C. Enzyme activities between 45 °C and 75 °C are close to each other and enzyme electrode can be used in this interval with 60% activity or above. And for the free PPO, optimum temperature was indicated as 40 °C in previous studies [42]. Change of maximum operation temperature of the immobilized enzyme according to free PPO makes us think that there may be some conformational changes in the enzyme upon immobilization. Most probably, these are conformational changes that cross-linking cause and these changes may change the position and activity of active sites of the enzyme and then they may also change its optimum temperature.

As it is seen in the figure, PPO activity in the polymer matrix decreases after 45 °C when temperature increases. As it is in all chemical reactions, when temperature

increases reaction rate increases in enzymatic reactions also. But after a certain temperature value, activity falls due to the denaturation occurring in enzyme structure. When temperature increases, first the tertiary structure and then the secondary structure (alpha helix structure) of enzyme molecules are distorted. For this reason, active center of the enzyme is affected and enzyme activity decreases [43].

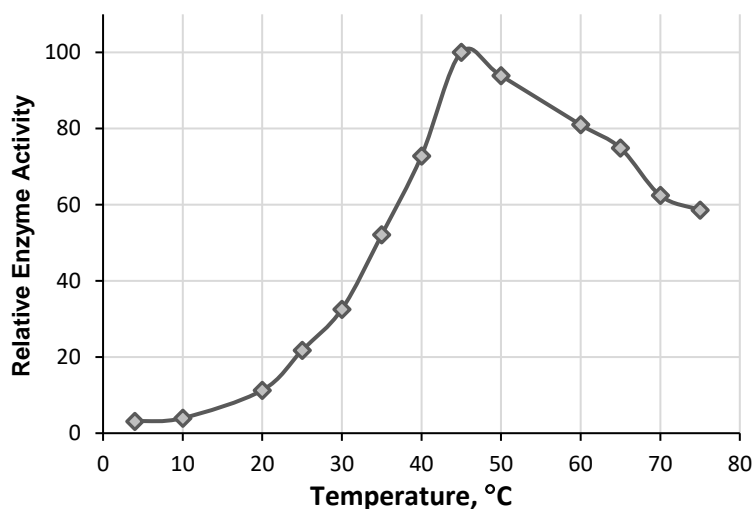


Figure 4.5. Effect of temperature on Pt/PTTzFr/PPO electrode activity.

### 4.3.3. Optimization of Immobilized Enzyme Amount

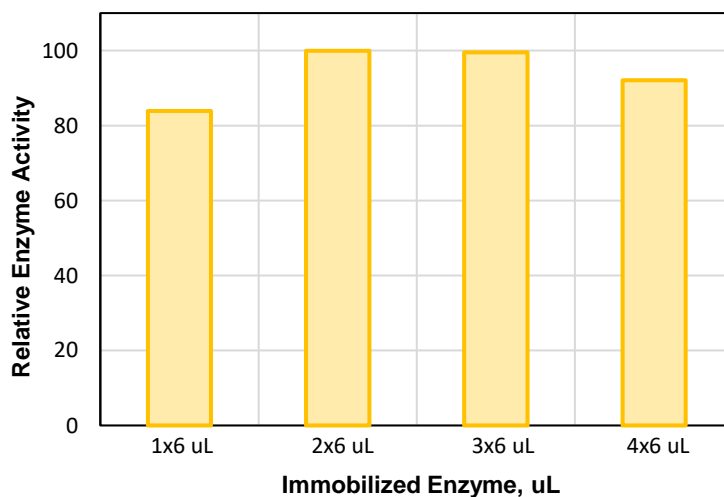


Figure 4.6. Effect of enzyme amount on activity.

Enzyme solution was prepared in  $0.02 \text{ g mL}^{-1}$  concentration and immobilized in 4 different electrodes at different amounts and enzyme activities were examined. 4 electrodes composed of polymer coated platinum plates were numbered as electrode no. 1, 2, 3 and 4 and  $1 \times 6 \text{ uL}$ ,  $2 \times 6 \text{ uL}$ ,  $3 \times 6 \text{ uL}$  and  $4 \times 6 \text{ uL}$  of enzyme were applied to each electrode respectively. After each  $6 \text{ uL}$  of enzyme application, electrode was dried for 30 minutes. After this operation, enzyme was immobilized by treating with GA. Relative enzyme activities taken from electrodes are seen in Figure 4.6. Maximum enzyme activity was observed in 2nd and 3rd electrodes and activity observed in 4th electrode was 90% and in other parts of this study, enzyme was applied as  $2 \times 6 \text{ uL}$ . It was expected an increase in maximum enzyme activity while entrapped enzyme amount increases. This increase wasn't observed and activities in 2nd, 3rd and 4th electrodes were very close to each other. This situation indicates that the total amount of immobilized enzyme didn't interact with substrate. Enzyme molecules placed at the surface performed a catalyzer function. Although immobilized enzyme amount increases, enzyme remaining below the surface layer doesn't perform its function and substrate molecules can't reach inside. For this reason, increase in enzyme amount doesn't make increase in enzyme activity.

#### 4.3.4. Optimization of GA Amount Applied

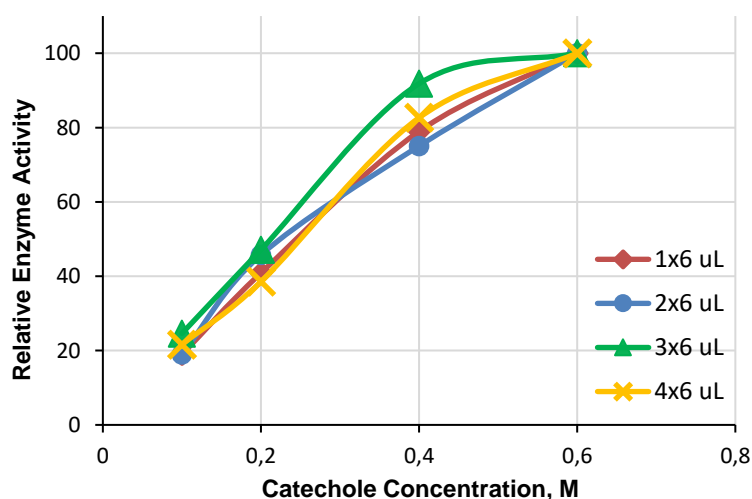


Figure 4.7. Effect of GA amount on activity.

GA solution was prepared in 2.5% concentration. 4 different electrodes were numbered and different amounts of GA were applied and their enzyme activities were checked. 4 platinum plates covered with polymer were first dropped 0.02 g mL<sup>-1</sup> enzyme solution as 2x6 uL, it was dried and GA was applied to 1st, 2nd, 3rd and 4th electrodes as 1x6 uL, 2x6 uL, 3x6 uL and 4x6 uL respectively. Activity of electrodes were examined in 4 different catechol concentrations (0.1 M, 0.2 M, 0.4 M, 0.6 M). For each electrode, catechol concentration vs. relative enzyme activity graphs were plotted as seen in Figure 4.7. It was observed that increasing GA amount didn't change activity at a reasonable amount. In the other parts of this study, GA was applied as 1x6 uL. This GA amount's cross-linking effect is enough and the enzyme is entrapped efficiently. For this reason, increased GA amount doesn't increase enzyme activity.

#### 4.4. DETERMINATION OF KINETIC PARAMETERS

0.5 cm square plate platinum electrodes were covered with PTTzFr by electropolymerization and polyphenol oxidase enzyme is immobilized. Immobilized enzyme's kinetic parameters; maximum enzyme activity ( $V_{max}$ ) and Michaelis-Menten constant ( $K_m$ ) were determined with different substrate concentrations at constant pH and temperature. Michaelis-Menten graph of Pt/PTTzFr/PPO electrodes is seen in Figure 4.8. From Michaelis-Menten graph, Lineweaver-Burk graph was obtained (Figure 4.9.). The point that the line in Lineweaver-Burk graph intersects y-axis gives  $\frac{1}{V_{max}}$  and that intersects x-axis gives  $-\frac{1}{K_m}$  [38]. From here,  $V_{max}$  and  $K_m$  were found as  $0.028 \pm 0.001 \text{ umol min}^{-1} \text{ electrode}^{-1}$  and  $669.68 \pm 64.73 \text{ mM}$  respectively.

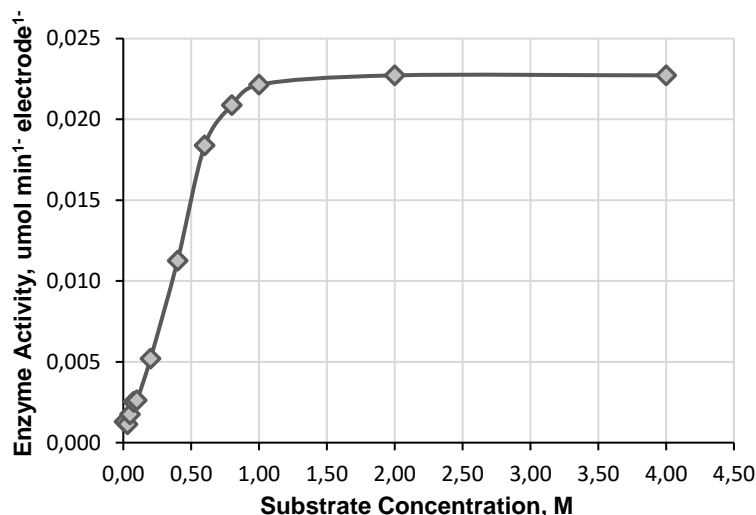


Figure 4.8. Michaelis-Menten graph of Pt/PTTzFr/PPO electrode.

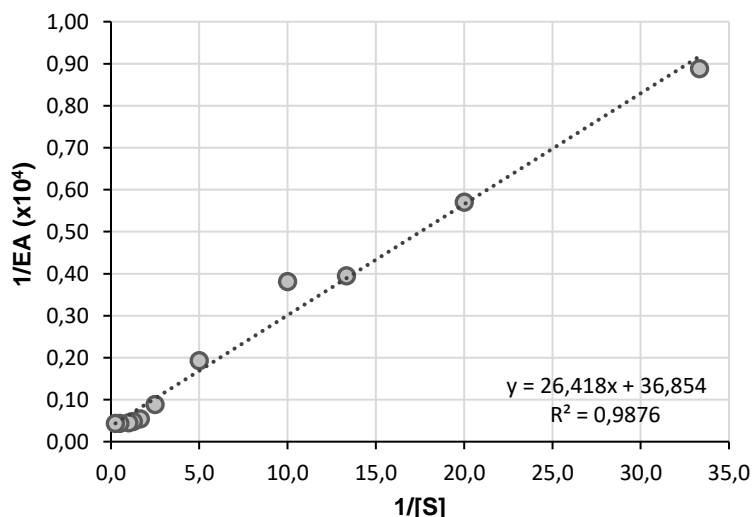


Figure 4.9. Lineweaver-Burk graph of Pt/PTTzFr/PPO electrode.

In Table 4.1, it is shown the data for two previous studies and kinetic parameters obtained in this study. For comparison,  $V_{\max}$  and  $K_m$  values of the free enzyme and Pt/PPy/PPO electrode (PPO entrapped with entrapment method to platinum electrode covered with PPy) were given. As it is seen in the table, free enzyme's  $V_{\max}$  value is considerably higher than immobilized enzyme's  $V_{\max}$  value and this is something expected. When an enzyme is immobilized in a matrix, its activity generally decreases.  $V_{\max}$  value decreases and  $K_m$  value increases. An increase in  $K_m$  which is inversely proportional with the affinity of enzyme to substrate means that it is difficult for the enzyme and substrate come together. This result is expected in immobilized enzymes.

The possibility of free enzyme in solution to come together with substrate is much higher compared to immobilized enzyme and this causes  $V_{\max}$  value expressing enzyme activity to be higher in free enzyme. On the other hand, when kinetic parameters of polyphenol oxidase immobilized in PPy and PTTzFr matrices are compared, it is seen that  $V_{\max}$  values are close to each other. In these two matrices, enzymes show similar activity and entrapped enzyme amounts are close. Pt/PPy/PPO electrode given in the chart was produced by coating PPy to 1.0 cm square platinum plate electrode and enzyme was entrapped by entrapment method. Total surface area of 2 surfaces of this electrode is 2 cm<sup>2</sup>. In this study, PTTzFr polymer was coated to 0.5 cm square platinum plate electrode and total surface area of 2 surfaces of this electrode is 0.5 cm<sup>2</sup>. Although surface area of electrode prepared by PPy is 4 times the surface area of electrode prepared by PTTzFr, both matrices show similar activities and this shows us that close amount of enzyme was entrapped in these two electrodes. According to this result, enzyme entrapment capacity of PTTzFr is higher and by cross-linking method, higher amount of enzyme can be immobilized compared to entrapment method. And this is a parameter which increases the sensitivity of the method.

Table 4.1. Kinetic parameters of free and immobilized PPO enzyme.

	$V_{\max}$	$K_m$
Free PPO [40]	0.073 $\mu\text{mol min}^{-1} \text{ mL}^{-1}$	4 mM
*Pt/PPy/PPO [40]	0.031 $\text{umol min}^{-1} \text{ electrode}^{-1}$	96 mM
**Pt/PTTzFr/PPO	0.028 $\pm$ 0.001 $\text{umol min}^{-1} \text{ electrode}^{-1}$	669.68 $\pm$ 64.73 mM

\*Surface Area: 2 cm<sup>2</sup>; \*\*surface area: 0.5 cm<sup>2</sup>.

$K_m$  value to be lower in Pt/PPy/PPO electrode compared to Pt/PTTzFr/PPO shows that in PPy matrix environment, the affinity between PPO and its substrate is higher. This interest shows that PPO enzyme accepts the substrate easier in the micro environment of the PPy matrix that it is inside and this points us the interaction between the PPy structure and substrate is higher. This interest is considerably less in PTTzFr matrix and  $K_m$  is much higher. PTTzFr reduces enzyme-substrate interaction and this implies that there may be repelling forces between PTTzFr and substrate.

## 4.5. STABILITY STUDIES

### 4.5.1. Operational Stability of Enzyme Electrodes

Operational stability of enzyme electrodes was determined by 50 measurements made one after another from Pt/PTTzFr/PPO electrodes prepared by immobilization of polyphenol oxidase using optimum enzyme and GA amounts. The measurements were made at 25 °C, pH 7.5 and 1.0 M catechol concentration. After 50 measurements, it was seen that the remaining relative enzyme activity was 70%. Free enzyme can only be used one time but immobilized enzyme can be used multiple times as it is seen in Figure 4.10.

In order to see the difference when enzyme is immobilized to PTTzFr polymer, PPO enzyme solution was dropped to platinum plate not covered with polymer, GA is applied over it and Pt/PPO was prepared. The same stability study was done by this electrode also and as it is seen in Figure 4.11, it was seen that relative enzyme activity was 40% after 50 measurements. A stability difference was observed between enzyme immobilized to PTTzFr matrix and bare platinum without polymer matrix and 70% relative activity went down to 40% when there is no polymer present after 50 measurements.

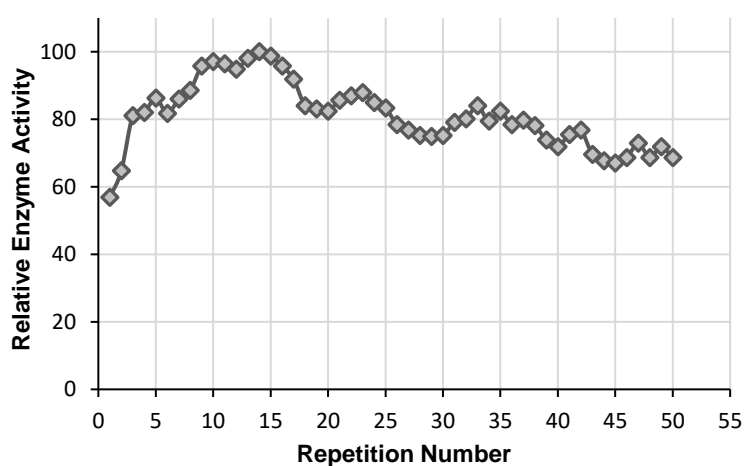


Figure 4.10. Operational stability of Pt/PTTzFr/PPO electrode.



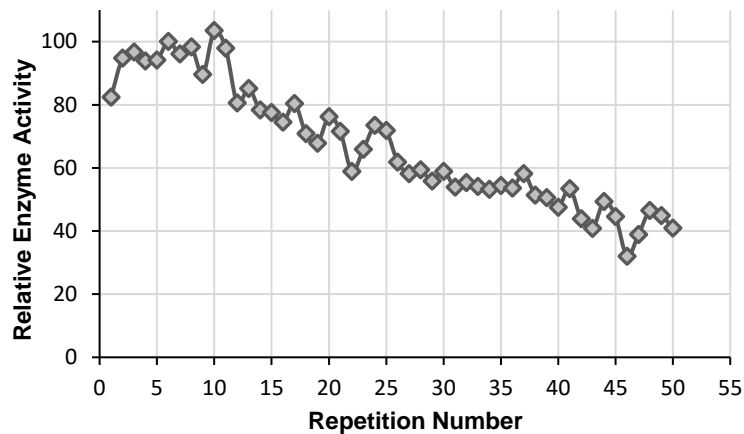


Figure 4.11. Operational stability of Pt/PPO electrode.

#### 4.5.2. Shelf Life of Enzyme Electrodes

In order to determine how much of their activity of enzyme electrodes lost in how much of a period, activity determination was made in two days of intervals for a period of 50 days. The measurements were made at 25 °C, pH 7.5 and 1.0 M catechol concentration.

Enzyme electrodes lost 30% of their initial activities at the end of 50 days and the remaining activity was determined as 70%. This fall in enzyme activities may result from desorption of the enzyme as time passes. It is known by previous studies that free enzyme loses its total activity within 8 days [19]. This decrease in the activity is a natural loss due to time but it was prevented at an important degree by the help of immobilization.

#### 4.6. ANALYSIS OF WASTE WATER SAMPLES

It was looked at the polyphenolic material amount in the process water used during obtaining biofuel from chips of fir tree. Total polyphenol content that was determined by using Pt/PTTzFr/PPO enzyme electrode and calibration graphs prepared by Folin-Ciocalteu method is given in Table 4.2 as its mg/mL catechol equivalent.

#### 4.6.1. Determination of Polyphenolics with Pt/PTTzFr/PPO Enzyme Electrode

For the calibration curve, 8 catechol solutions between 10.0 and 90.0 mg mL<sup>-1</sup> were prepared in pH 7.5 phosphate buffer. Pt/PTTzFr/PPO electrode was dipped to each solution for 10 minutes and the absorbance of solutions colored with Besthorn's Hydrazone method were measured and calibration curve seen in Figure 4.11 (a) was obtained. 15 times of blank measurements were made and LOQ was calculated as 7.827 mg mL<sup>-1</sup> making use of the standard deviation and calibration curve slope. In order to obtain the linear working range and Beer's Deviation point, 11 catechol solutions between 1.0 and 440.0 mg mL<sup>-1</sup> were prepared and the same procedure described above was applied. From the curve shown on Figure 4.12 (b), the linear range was determined as 1.0 – 90.0 mg mL<sup>-1</sup>.

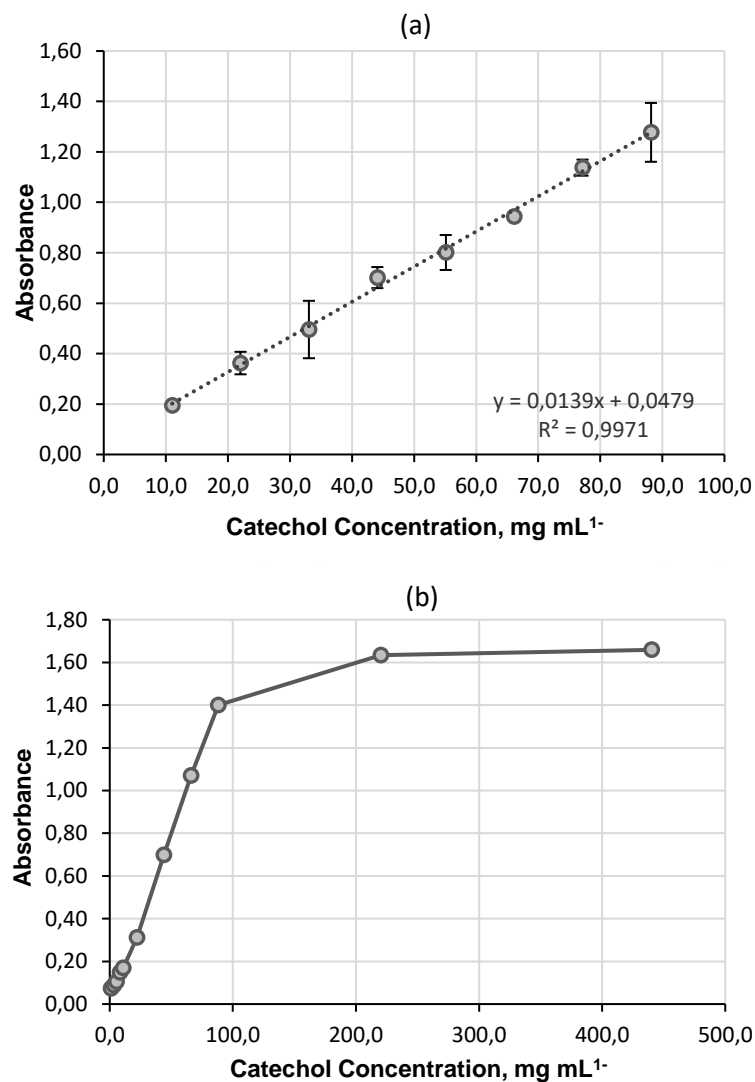


Figure 4.12. Calibration graph of Pt/PTTzFr/PPO electrode (a) 8 catechol solutions (b) 11 catechol solutions.

#### 4.6.2. Determination of Polyphenolics with Folin-Ciocalteu Method

In order to confirm polyphenolic material determination made by enzyme electrodes, Folin-Ciocalteu analysis method was used as the control method. The calibration graph prepared by Folin-Ciocalteu method is seen in Figure 4.13.

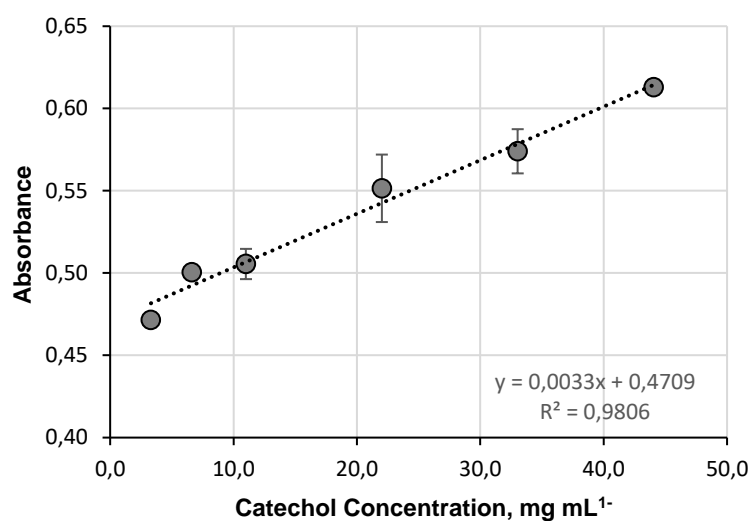


Figure 4.13. Calibration graph prepared by Folin-Ciocalteu method.

Table 4.2. Total polyphenolic material amounts determined as mg/mL catechol equivalent in waste water samples with Pt/PTTzFr/PPO enzyme electrode and Folin-Ciocalteu method.

Method	Total Polyphenol Amount in Process Water (mg.mL <sup>-1</sup> )
Enzyme Electrodes	268.48 ± 6.62
Folin-Ciocalteu Method	262.86 ± 19.54

## PART 5

### CONCLUSION AND SUGGESTIONS

In this study, poly(2,5-di(furan-2-yl) thiazolo[5,4-*d*] thiazol) polymer was coated over a platinum electrode and new enzyme electrodes were produced that are not found in the literature. The formation of polymerization was shown by cyclic voltammetry. Kinetic parameters of immobilized polyphenol oxidase enzyme were determined.  $V_{\max}$  and  $K_m$  were obtained as  $0.028 \pm 0.001 \text{ umol min}^{-1} \text{ electrode}^{-1}$  and  $669.68 \pm 64.73 \text{ mM}$  respectively. The effect of reaction conditions over enzyme activity were examined. The pH and temperature at which immobilized polyphenol oxidase enzyme showed the highest activity were found as pH 7.5 and 45 °C. Amount of enzyme and glutaraldehyde immobilized to the electrodes were changed and in the examination, it was detected that it is sufficient to add 2x6 uL enzyme and 1x6 uL glutaraldehyde to the electrode. The activity of Pt/PTTzFr/PPO electrode was followed by 50 consequential measurements. It has been seen after 50 measurements that immobilized enzyme activity is 70%. As a result of shelf life examinations, it was determined that Pt/PTTzFr/PPO electrode lost 30% of its initial activity at the end of the 50th day and the remaining activity was 70%. Calibration graph was plotted for Pt/PTTzFr/PPO electrode ( $y = 0.0139x + 0.0479$ ). LOQ value was calculated as  $7.827 \text{ mg mL}^{-1}$  and the working interval was found as 1.0 – 90.0  $\text{mg mL}^{-1}$ . In total phenolic material analyses made with this electrode, the results found in waste water samples have also been confirmed by Folin-Ciocalteu method.

The lifetime of enzyme electrodes constructed in this study might be possible to increase if some functional groups are added to the TTzFr monomer. Amine group or carboxylic acid groups can be added to the monomer structure. Glutaraldehyde and other crosslinking agents are used in such a case and enzyme molecules are connected covalently to the polymer chains by this way. This connection most probably makes the stability of enzyme molecules increases. Since the enzyme is covalently bonded, it

is not lost in time easily. Therefore, the lifetime of enzyme electrodes is made lengthens and their remaining relative activity also becomes much closer to 100%. This strong connection prevents the enzyme lost and serves for also a higher sensitivity. Since the degree of the signal thus the sensitivity is related with the immobilized enzyme amount, how much stable enzyme immobilization is provided, so much higher sensitivity is obtained.



## REFERENCES

1. Shirakawa, H., Louis, E. J., MacDiarmid, A. G., Chiang, C. K. and Heeger, A. J., "Synthesis of electrically conducting organic polymers: Halogen derivatives of polyacetylene", *J. Chem. Soc. Chem. Commun.*, 474: 578–580 (1977).
2. Chandrasekhar, P., "Conducting polymers, fundamentals and applications: A practical approach", *Kluwer Academic Publishers*, Boston, (1999).
3. Nalwa, H. S., "Handbook of organic conductive molecules and polymers", *John Wiley and Sons Ltd*, Chichester, United Kingdom (1997).
4. Toshima, N. and Hara, S., "Direct synthesis of conducting polymers from simple monomers", *Progress in Polymer Science*, 20 (1): 155–183 (1995).
5. Pron, A., Gawrys, P., Zagorska, M., Djurado, D. and Demadrille, R., "Electroactive materials for organic electronics: preparation strategies, structural aspects and characterization techniques", *Chemical Society Reviews*, 39 (7): 2577–2632 (2010).
6. Işık, S., Alkan, S., Toppare, L., Cianga, I. and Yağcı, Y., "Immobilization of invertase and glucose oxidase in poly 2-methylbutyl-2-(3-thienyl) acetate/polypyrrole matrices", *European Polymer Journal*, 39 (12): 2375–2381 (2003).
7. Sajid, I. and Sharif, A., "Recent development in hybrid conducting polymers: Synthesis, applications and future prospects", *Journal of Industrial and Engineering Chemistry*, 60: 53–84 (2018).
8. Gyorgy, I., "Conducting polymers: past, present, future", *Journal of Electrochemical Science and Engineering*, 8: 3–37 (2018).
9. Ekiz, F., "Bioactive surface design based on conducting polymers and applications to biosensors", Master Thesis, *Middle East Technical University Graduate School of Natural and Applied Sciences*, Ankara (2012).
10. Söylemez, S., "Functionalization and fabrication of polymer based device platform architectures for sensor applications", PhD Thesis, *Middle East Technical University Graduate School of Natural and Applied Sciences*, Ankara (2018).
11. Sarma, A. K., Vatsyayan, P., Goswami, P. and Minter, S. D., "Recent advances in material science for developing enzyme electrodes", *Biosensors and Bioelectronics*, 24 (8): 2313–2322 (2009).

12. Palmer, T., “Enzymes: Biochemistry, Biotechnology, Clinical Chemistry” *Elsevier*, (2007).
13. Price, N. C. and Stevens, L., “Fundamentals of Enzymology: The Cell and Molecular Biology of Catalytic Proteins, Chapter 4”, *Oxford University Press Inc.*, New York, 118-153 (1999).
14. Marangoni, A.G., “Enzyme Kinetics: A Modern Approach”, *John Wiley & Sons, Inc.*, New York, 244 (2003).
15. Bisswanger, H., “Enzyme Kinetics Principles and Methods 3<sup>rd</sup> ed.”, *WILEY-VCH Verlag GmbH.*, Germany Weinheim (2002).
16. Hartmeier, W., “Immobilized Biocatalysts: An Introduction”, *Springer Science & Business Media*, (2012).
17. Soylemez, S., Kanik, F.E., Nurioglu, A.G., Akpınar, H. and Toppare, L., “A novel conducting polymer: Investigation of its matrix properties for cholesterol biosensor applications”, *Sensors and Actuators B: Chemical*, 182: 322–329 (2013).
18. Gürsel, A., Alkan, S., Toppare, L. and Yağcı, Y., “Immobilization of invertase and glucose oxidase in conducting H-type polysiloxane/polypyrrole block copolymers”, *Reactive And Functional Polymers*, 57 (1): 57–65 (2003).
19. Kartal, M., Kayahan, S., Bozkurt, A. and Toppare, L., “Entrapment of invertase in an interpenetrated polymer network of alginate acid and poly (1-vinylimidazole)”, *Talanta*, 77 (2): 659–662 (2008).
20. Decker, H. and Tuzek F., “Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism”, *Trends in Biochemistry Science*, 25, 392– 397 (2000).
21. Smith, M.B. and March, J., “March’s advanced organic chemistry 5th ed.”, *Wiley-Interscience Publication*, New York (2001).
22. Plonka P.M. and Grabacka, M., “Melanin synthesis in microorganisms- biotechnological and medical aspects”, *Acta Biochimica Polonica*, 53, 429–443 (2006).
23. Claus, H. and Decker, H., “Bacterial tyrosinases”, *Systematic and Applied Microbiology*, 29, 3–14 (2006).
24. Dinçer, B. “Döngel Bitkisi (*Mespilus germanica* L., Rosaceae) Meyvelerindeki Polifenol Oksidaz Aktivitesinin Karakterizasyonu”, Yüksek Lisans Tezi, *Karadeniz Teknik Üniversitesi Fen Bilimleri Enstitüsü*, Trabzon (1999).
25. Tomas-Barberan, F. and Espin, J.C., “Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables”, *Journal of the Science of Food and Agriculture*, 81, 853–876 (2001).



26. Mishra, B.B., “Studies on polyphenol oxidase (PPO) in Brinjal (eggplant; solanum melongena)”, PhD Thesis, *Homi Bhabha National Institute*, Mumbai (2012).
27. Önez, Z., “Üzümünden (vitis vinifera L.) izole edilen polifenol oksidaz enziminin özelliklerinin belirlenmesi” Yüksek Lisans Tezi, *Ankara Üniversitesi Fen Bilimleri Enstitüsü*, Ankara (2016).
28. Polatoğlu, İ., “Elektrokimyasal Biyosensörler için Karbon Pasta Elektrot Tasarımı ve Karakterizasyonu”, *Süleyman Demirel Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, 22: 141–148 (2018).
29. Apetrei, C., Rodriguez-Mendez, M.L. and De Saja, J.A., “Amperometric tyrosinase based biosensor using an electropolymerized phosphate-doped polypyrrole film as an immobilization support. Application for detection of phenolic compounds”, *Electrochimica Acta*, 56, 8919–8925 (2011).
30. Rahman, S.F., Min, K., Park, S.H., Park, J.H., Yoo, J.C. and Park, D.H., “Selective determination of dopamine with an amperometric biosensor using electrochemically pretreated and activated carbon/tyrosinase/nafion–modified glassy carbon electrode”, *Biotechnology Bioprocess Engineering*, 21, 627–633 (2016).
31. Carvalho, J.O. and França Orlanda, J.F., “Heat stability and effect of pH on enzyme activity of polyphenol oxidase in buriti (mauritia flexuosa linnaeus f.) fruit extract”, *Food Chemistry*, 233, 159–163 (2017).
32. Sandeep, S., Santhosh, A.S., Swamy, N.K., Suresh, G.S., Melo J.S. and Nithin, K.S., “Electrochemical detection of L-dopa using crude polyhenol oxidase enzyme immobilized on electrochemically reduced RGO-Ag nanocomposite modified graphite electrode”, *Materials Science and Engineering B*, 232, 15–21 (2018).
33. Slinkard, K. and Singleton, V. L., “Total phenol analysis: automation and comparison with manual methods”, *American Journal of Enology and Viticulture*, 28: 49–55 (1977).
34. Huang, D. J., Ou, B. X. and Prior, R. L., “The chemistry behind antioxidant capacity assays”, *Journal of Agricultural and Food Chemistry*, 53 (6): 1841–1856 (2005).
35. Söylemez, S., Kaya, H.Z., Udum, Y.A., Toppare, L., “A multipurpose conjugated polymer: Electrochromic device and biosensor construction for glucose detection”, *Organic Electronics*, 65: 327–333 (2019).
36. Pifferi, P. G. and Baldassari, L., “A spectrophotometric method for the determination of the catecholase activity of tyrosinase by Besthorn’s hydrazone”, *Analytical Biochemistry*, 52 (2): 325–335 (1973).
37. Rodriguezlopez, J. N., Escribano, J. and Garciacanovas, F., “A Continuous Spectrophotometric Method for the Determination of Monophenolase Activity of

- Tyrosinase Using 3-Methyl-2-benzothiazolinone Hydrazone”, *Analytical Biochemistry*, 216 (1): 205–212 (1994).
38. Lineweaver, H. and Burk, D., “The Determination of Enzyme Dissociation Constants”, *Journal of The American Chemical Society*, 56 (3): 658–666 (1934).
39. Engel, P. C. and Ferdinand, W., “The significance of abrupt transitions in Lineweaver–Burk plots with particular reference to glutamate dehydrogenase. Negative and positive co-operativity in catalytic rate constants”, *Biochemical Journal*, 131 (1): 97–105 (1973).
40. Michaelis, L. and Menten, M. L., “The Kinetics of Invertase Action”, *Biochemische Zeitschrift*, 49: 333–369 (1913).
41. Böyükbayram, A.E., “Immobilization of invertase, polyphenol oxidase and glucose oxidase in conducting copolymers of thiophene capped polytetrahydrofuran and pyrrole”, PhD Thesis, *Middle East Technical University Graduate School of Natural and Applied Sciences*, Ankara (2005).
42. Kıralp, S., “Synthesis of conducting block copolymers and their use in the immobilization of invertase and polyphenol oxidase enzyme”, Ph. D. Thesis, *Middle East Technical University Graduate School of Natural and Applied Sciences*, Ankara (2004).
43. Aydar, S., “Tiyofen ve 3,4-etilendioksitiyofen donör gruplarının benzotiyadiazol akseptör grubuyla yaptığı dad tipi iletken polimerlerde enzim immobilizasyonu”, *Karabük Üniversitesi Fen Bilimleri Enstitüsü*, Karabük (2012).

## **RESUME**

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