# ENZYME ELECTRODES PREPARED BY ELECTROCHEMICAL POLYMERIZATION OF EDOT AND HYDROXYMETHYL EDOT

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# ENZYME ELECTRODES PREPARED BY ELECTROCHEMICAL POLYMERIZATION OF EDOT AND HYDROXYMETHYL EDOT

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Saseeyah Miftah Abdulsalam ALREEBAA

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I certify that in my opinion the thesis submitted by Saseeyah Miftah Abdulsalam ALREEBAA titled "ENZYME ELECTRODES PREPARED BY ELECTROCHEMICAL POLYMERIZATION OF EDOT AND HYDROXYMETHYL EDOT" is fully adequate in scope and in quality as a thesis for the degree of Master of Science.

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This thesis is accepted by the examining committee with an unanimous vote in the Department of Chemistry as a master thesis. February 19, 2021


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Saseeyah Miftah Abdulsalam ALREEBAA

# ABSTRACT

## M. Sc. Thesis

## ENZYME ELECTRODES PREPARED BY ELECTROCHEMICAL POLYMERIZATION OF EDOT AND HYDROXYMETHYL EDOT

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Poly(3,4-ethylenedioxythiophene) (PEDOT) and poly((2,3-dihydrothieno[3,4-*b*][1,4]dioxin-2-yl)methanol) (hydoxymethyl-PEDOT, MeOH-PEDOT) were used as new electrode materials and enzyme electrodes were prepared by immobilizing polyphenol oxidase enzyme in this two matrices. 3,4-Ethylenedioxythiophene (EDOT) and ((2,3-dihydrothieno[3,4-*b*][1,4]dioxin-2-yl)methanol) (MeOH-EDOT) monomers were polymerized electrochemically and coated to platinum electrodes and polyphenol oxidase enzyme was immobilized by codeposition of enzyme together with polymer. Kinetic characterization and optimization of enzyme electrodes were made and the study was completed with real sample analysis. Kinetic parameters, $V_{max}$ (maximum reaction rate) and $K_m$ (enzyme affinity of substrate) were respectively obtained as 0.00236 µmol $min^{1-}$ $electrode^{1-}$ and 17.722 mM for PEDOT, 0.00458 µmol $min^{1-}$ $electrode^{1-}$ and 4.962 mM for MeOH-PEDOT. The effect of reaction conditions

was examined and the highest enzyme activity was obtained at pH 7.0 and 8.0 and at 60 ℃ and 50 ℃ temperature. Linear operation interval determined in optimum conditions is between 1.0 and 110.0 mg mL$^{1-}$ and LOD values were found as 1.241 mg mL$^{1-}$ and 1.038 mg mL$^{1-}$ for PEDOT and MeOH-PEDOT respectively. The stability of enzyme electrodes was investigated with consecutive activity measurements. After 40 measurements, it was seen that the remaining relative enzyme activity was around 60% and 75% for Pt/PEDOT/PPO and Pt/MeOH-PEDOT/PPO electrodes, respectively. Meanwhile, in the shelf-life study, it was determined that activity fell to 70% after 50 days. Enzyme electrodes were applied to analysis of polyphenolic substances in peach extracts. Total polyphenolic substance amounts in sample were found as 69.43 ± 2.41 mg mL$^{1-}$ and 92.62 ± 3.43 mg mL$^{1-}$ catechol equivalent by PEDOT and MeOH-PEDOT electrodes. For verification, Folin-Ciocalteau analysis was used as control method and result of 84.45 ± 5.21 mg mL$^{1-}$ catechol equivalent was obtained.

# ÖZET

**Yüksek Lisans Tezi**

**EDOT VE HİDROKSİMETİL EDOT'UN ELEKTROKİMYASAL POLİMERİZASYONUYLA HAZIRLANAN ENZİM ELEKTROTLARI**

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**Şubat 2021, 77 sayfa**

Bu çalışmada yeni elektrot malzemesi olarak poli(3,4-etilendioksitiyofen) (PEDOT) ve poli((2,3-dihidrotiyeno[3,4-*b*][1,4]diyoksin-2-il)metanol) (hidroksimetil-PEDOT, MeOH-PEDOT) polimerleri kullanılmış ve bu iki matrise polifenol oksidaz enzimi tutuklanarak enzim elektrotları hazırlanmıştır. 3,4-Etilendioksitiyofen ve (2,3-dihidrotiyeno[3,4-*b*][1,4]diyoksin-2-il)metanol monomerleri elektrokimyasal yolla polimerleştirilerek platin elektrotlara kaplanmış ve polifenol oksidaz enzimi polimerle kodepozisyon yoluyla tutuklanmıştır. 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) PEDOT'da tutuklanmış enzim için 0.00236 µmol dak$^{-1}$ elektrot$^{-1}$ ve 17.722 mM olarak, MeOH-PEDOT'da tutuklanmış enzim için 0.00458 µmol dak$^{-1}$ elektrot$^{-1}$ ve 4.962 mM olarak tayin edilmiştir. Reaksiyon koşullarının etkisi incelenmiş ve en yüksek enzim aktivitesi PEDOT için pH 7.0 ve 60 ℃'ta,

MeOH-PEDOT için pH 8.0'de ve 50 ℃ sıcaklıkta elde edilmiştir. Optimum koşullarda tespit edilen doğrusal çalışma aralığı 1.0 – 110.0 mg mL$^{1-}$ arasındadır. LOD değeri ise 1.241 mg mL$^{-1}$ ve 1.038 mg mL$^{-1}$ olarak bulunmuştur. Ardışık aktivite ölçümleri ve raf ömrü çalışmasıyla enzim elektrotlarının stabilitesi incelenmiştir. 40 ardışık ölçümden sonra enzim elektrodunun aktivitesinin PEDOT için %60 MeOH-PEDOT içinse %75 değerinde olduğu gözlenmiştir. Raf ömrü çalışmasında ise 50 günün sonunda aktivitenin %70'e düştüğü saptanmıştır. Enzim elektrotlarının uygulaması şeftali ekstraktında polifenolik maddelerin analizi şeklinde tasarlanmıştır. Toplam polifenolik madde miktarı PEDOT enzim elektrotlarıyla 69.43 ± 2.41 mg mL$^{1-}$ olarak MeOH-PEDOT enzim elektrotlarıyla ise 92.62 ± 3.43 mg mL$^{1-}$ katekol eşdeğeri olarak saptanmıştır. Doğrulama amacıyla kontrol yöntemi olarak Folin-Ciocalteau analiz metodu kullanılmış ve şeftali ekstraktındaki polifenol miktarı 84.45 ± 5.21 mg mL$^{1-}$ katekol eşdeğeri olarak bulunmuştur.

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

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# ABBREVIATIONS

| | | |
|---|---|---|
| $K_m$ | : | Michaelis-Menten constant |
| $V_{max}$ | : | Maximum reaction rate |
| LOD | : | Limit of Detection |
| mL | : | Mililiter |
| μm | : | Micrometer |
| μL | : | Microliter |
| μmol | : | Micromole |
| nm | : | Nanometer |
| ℃ | : | Degree Celcius |
| g | : | Gram |
| mg | : | Miligram |
| min | : | Minute |
| A | : | Amper |
| V | : | Volt |
| VB | : | Valence Band |
| CB | : | Conductance Band |
| CV | : | Cyclic Voltamogram |
| CP | : | Conducting Polymer |
| UV | : | Ultraviolet |
| RCF | : | Relative centrifuge force |
| PA | : | Polyacetylene |
| PAN | : | Polyacrylonitrile |
| PPy | : | Polypyrrole |
| PPO | : | Polyphenole oxidase |
| MBTH | : | 3-Methyl-2-benzothiazolinon hydrazon hydrochloride |
| FCR | : | Folin-Ciocalteau reagent |
| EDOT | : | 3,4-Ethylenedioxythiophene |
| MeOH-EDOT | : | (2,3-Dihydrothieno[3,4-*b*][1,4]dioxin-2-yl)methanol |

|  |  | (Hydroxymethyl EDOT) |
| --- | --- | --- |
| Pt | : | Platinum |
| PEDOT | : | Poly(3,4-ethylenedioxythiophene) |
| MeOH-PEDOT | : | Hydroxymethyl PEDOT |
| Pt/PEDOT/PPO | : | Enzyme electrode that is polyphenol oxidase enzyme immobilized into PEDOT coated platinum electrode |
| Pt/MeOH-PEDOT/PPO | : | Enzyme electrode that is polyphenol oxidase enzyme immobilized to MeOH-PEDOT coated platinum electrode |

## INTRODUCTION

It is an extensive research field that enzymes are immobilized in several natural or synthetic organic polymers and inorganic materials. Enzymes are materials that are both expensive as well as which lose their activity easily. For this reason, it is important to do immobilization and regain the enzyme back which is associated with the usage of it more and more instead of utilizing one time in a free form. An immobilized enzyme can be received back from the reaction environment as well as this is an important factor in reducing cost. Besides, via receiving the enzyme back from the reaction environment, a cleaner production is obtained.

The utilization of enzymes has some drawbacks such as the enzyme sensitivity towards processing conditions, the low stability resulting from inhibition of enzyme by the high amounts of reaction products. Additionally, most of the enzymes are so unstable that manufacturing process is prevented because of the lack of shelf-life stability and the recovery process thus reutilization to be difficult.

The analysis of the enzyme's substrate can be possible via the entrapped enzyme. Enzyme-based biosensors provide high sensitivity and specificity, high stability and possibility for reusage of enzyme in the determination of enzyme's substrate. Food safety control, clinical analysis and routine disease testings are the most common purposes of research focusing on enzymes utilization biosensors which has attractivities like portability, to be cost-effective, possibility of miniaturization and point-of-care diagnosis.

The enzyme electrode remains a miniature chemical transducer which functions via combining a measurement process with immobilized enzyme activity. This might be a combination of an electrochemical probe or other types of detections like a UV-Vis

procedure with a thin layer of immobilized enzyme. The enzymes function here is to supply selectivity via its affinity towards the substrate substances. Therefore, the enzymes with their natural selectivity are generally utilized in construction of enzyme electrodes. The enzyme bearing electrode is the main component of biosensors. Efficiency of an electrode which means its sensitivity, reproducibility and stability depends on the selection of appropriate combination of materials in the fabrication of enzyme-modified electrodes. Type of enzyme immobilization matrix, conductive support as well as solid support must be combined properly.

Enzyme electrodes are electrode structures where the enzyme is entrapped within or on the surface of a conductive matrix. However, the major problem while fabricating enzyme-based biosensor is the immobilization of enzymes onto electrode matrix. Most of the time fabrication fails for the reason that losing binding, chemical modification, denaturation as well as loss of enzyme activity apart from instability of matrix. These failures can be minimized or avoided through the proper deposition of conducting polymer matrix as well as via adopting proper enzyme immobilization techniques.

The enzyme electrode has a composite structure (matrix and enzyme) covered on the electrode material. Electrode materials are metals and other substances utilized as the makeup of electrical components. Matrix materials are conductive polymers, functional polymers, metal complexes, sol-gel materials as well as Nano-materials (carbon Nano-tube, Nano-particulates). The performance of the enzyme electrode is much more for the reason that the utilized composite materials. Electroactive conductive polymers are used widely in the field of bioanalysis with their charge carrying capacity and biocompatibility. They have sensitivity increasing effects. Additionally, attributable to their stabile structure, they lengthen the life of the electrode as well as increases its repeatability. Likewise, conductive polymers have attracted more attention recently as well as they were coated to the surface of electrodes from several diverse materials. These coated electro active films are utilized to entrap enzymes or proteins.

Polyphenol oxidase (PPO) is extracted and purified from many agricultural products especially from mushroom. It is a protein structure with a molecular weight of around

50 kilodaltons. Substrates of this enzyme are polyphenolic substances with a wide variety. Last researches are focused on determination of polyphenolic materials which exist in waste-water [6]. Phenolic compounds in nature are sourced from natural also utilized in the industry. The most important of these are plastics and textile production, drugs, petroleum and paper industries and insecticide and fungicide productions. Furthermore, phenols in rivers poison aquatic life as well as it is one of the important problems of environmental chemistry that they persist in the nature. Therefore, monitoring this problem in environmental waters, there have been many studies among which biosensor approaches are concerned as a large area of research. In this study, enzyme electrodes have been fabricated by immobilization of PPO enzyme into conducting polymers poly(3,4-ethylenedioxythiophene) and poly((2,3-dihydrothieno[3,4-b][1,4]dioxin-2-yl)methanol). Properties of electrodes have been investigated and characterization studies have been performed.

## PART 2

## THEORETICAL BACKGROUND AND LITERATURE SURVEY

## 2.1. CONDUCTING POLYMERS

Conducting electro-active, biocompatible polymers (CPs) are widely utilized for their dual advantages of both binding the active enzyme which is concerned with allowing for appropriate electron transport in advanced enzyme electrodes. Conducting polymers give various advantages over metals, for instance, low weight, low cost, which is associated with lower manufacturing energy. Some examples of CPs are seen on Figure 2.1. Electrical conductivity which is invented for these polymers gives high attention to the utilization of organic compounds in microelectronics. Polymers can gain conductivity via transmitting electricity directly over electrons [1].

Polyacetylene (PA) was accidentally founded by Shirakawa, the first polymer allowed to conduct electricity in the mid of 1970s. What is more, it was first produced last decade with dynamic, self-contained films prepared via Ziegler-Natta polymerization. The polymers obtained via these procedures were, however, nonprocessable. Natta et al. developed the framework related to polymerization of acetylene via utilizing a Ziegler type catalyzer. During 1963, Ziegler and Natta have been awarded the Nobel Prize via reason of their documented research on polymer chemistry. Shirakawa has presented that polyacetylene films in silver color which do not have the required conductivity based on Ti(O-n-But)$_4$ usage which was termed as the Ziegler-Natta catalyzer. Likewise, Shirakawa et al. has detected on 1977 that the conductivity of polyacetylene films increases 109 times, when the polymer is oxidized and its conductivity reaches to around 105 S m$^{1-}$. As an outcome, the electrical conductivity of polymers led them be awarded with the Nobel Prize for chemistry domain during the 2000 [2].

Both fundamental and practical approaches about conductive polymers have been widely studied since their electrical and electrochemical features are very close to those of both metals and semiconductors. Furthermore, CP's have some well characteristics such as their synthesis to be in mild conditions, easy processing as well as structural diversity, conductance to be tunable, flexibility in structure. Moreover, the production of CP nanomaterials has been allowed by the progress in nanotechnology and they have started to be used with improved performance in a variety of applications such as electronics, optoelectronics, energy devices and sensors [1,3].
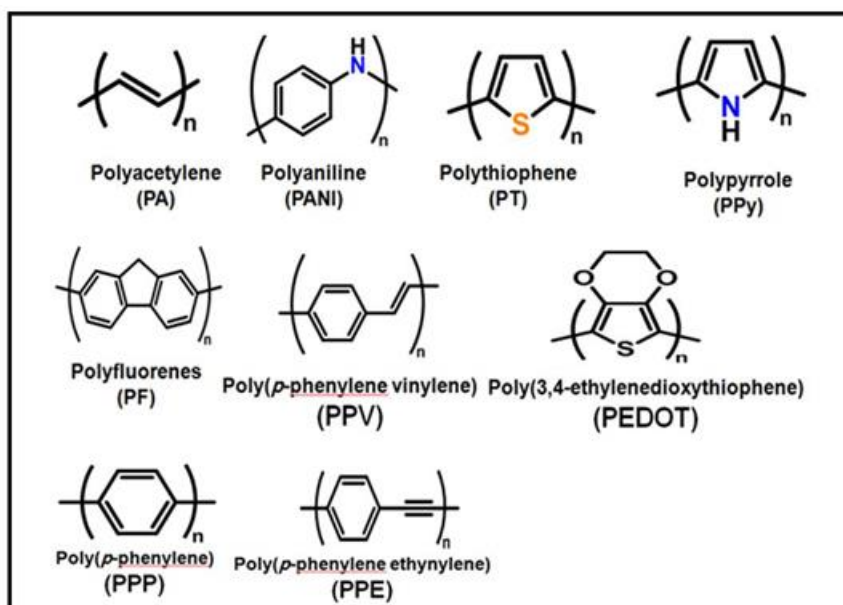


Figure 2.1. Some examples of conducting polymers.

### 2.1.1. Conductivity Theory in Polymers

Conductive polymers have $\pi$ conjugation in polyconjugated frameworks as seen on Figure 2.2, the $\pi$-electrons behaviours, for instance, their delocalized and polarized nature has an important effect on the determination of electrical structure of the framework.
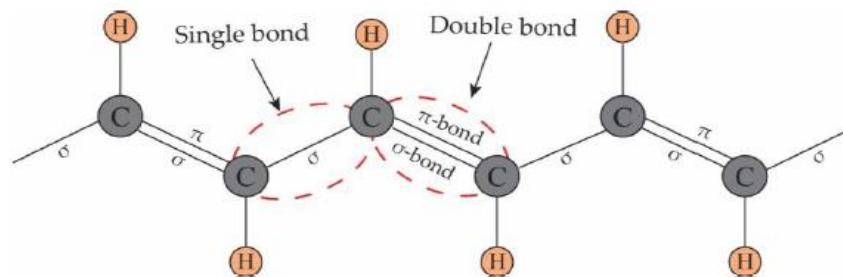
Figure 2.2. The structure of polyacetylene and the backbone containing conjuncted double bonds.

The maximum conductivity of first-generation CPs was low for the reason that they had significant disorder in the polymer structure which prevents π-electron delocalization, in consequence, is resulted in a retard in charge transport. Accordingly, metallic conductivity of first-generation CPs was rather low. Structural and morphological disorder decreases upon the doping of π-conjugated structure. Furthermore, a new generation of CPs is created where the undoped polymer conductivity can increase from the insulating to the metallic character. Charges produced with doping process generate solitons, polarons and bipolarons that usually exist with lattice distortion (Figure 2.3). Additionally, undoped polymers have conductivity around $10^{-6} – 10^{-10}$ S cm$^{-1}$, which lies in a region between semiconductor and insulator. The undoped polymers conductivity can be raised 10 or more orders of magnitude upon doping. For instance, Tsukamoto et al. documented the doping of polyacetylene with iodine as well as achieved conductivity [3,4].
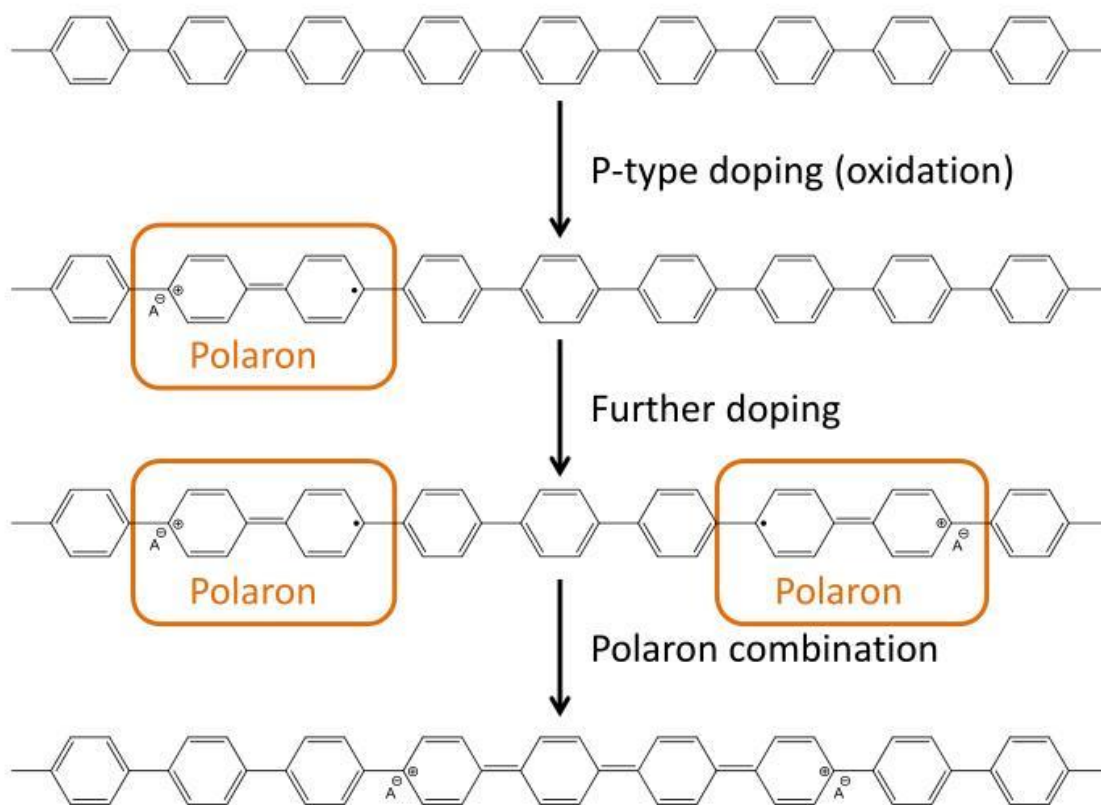
Figure 2.3. Chemical structures illustrating undoped form, polaron and bipolaron.

## 2.1.1.1. Band Theory

During bond formation, shared electrons located in the space amongst the two nuclei are called bonding electrons. The bonded pair is the "glue" that holds the atoms with each other in the molecular unit. Bond formation amongst complex molecules, a new bonding and anti-bonding energy levels are formed in the electronic structure of the molecule.

When the size of molecule increases, number of bonding orbitals increases as well. Therefore, the variance amongst orbital energy levels also decreases. At one point, instead of energy levels that are separated clearly from each other, a continuous merged energy band is formed. This band is termed as valence band as presented in Figure. 2.4. Electrons within the valence band can easily change their places and move easily inside the band. Besides, the anti-bonding orbitals having number close to

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infinity form energy band with the approaching anti-bonding orbitals named as the conductivity band [3,5].
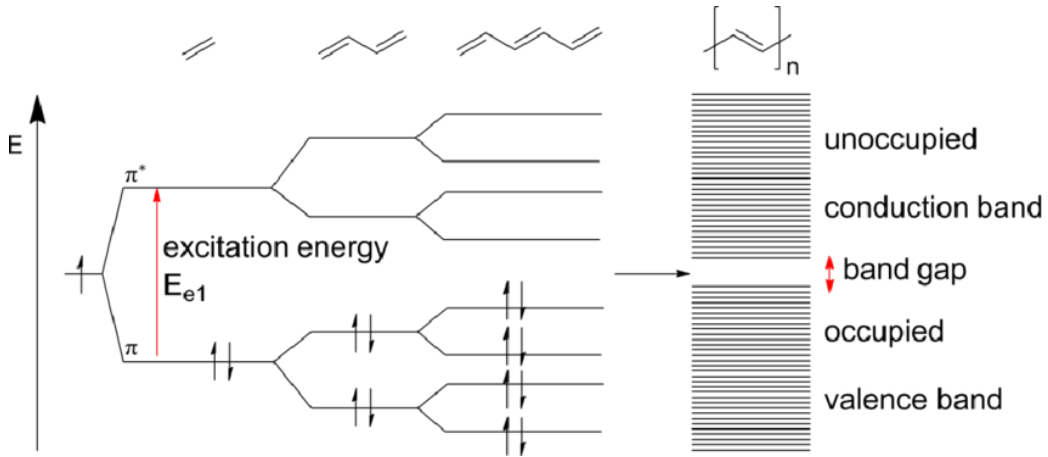


Figure 2.4. Formation of bonding and anti-bonding orbitals in polymer molecule.

A band gap is a space between a valence band and a conduction band as shown on Figure 2.5. The band gap essentially defines the minimum energy required to arouse an electron to a state in the conductive band where it can participate in conduction. The valence band energy level is lower than the conduction band and in semiconductors, this band is usually full. Upon heating, valence band electrons pass through the band gap and jump into the conduction band which creates conductivity in the material [3,5].
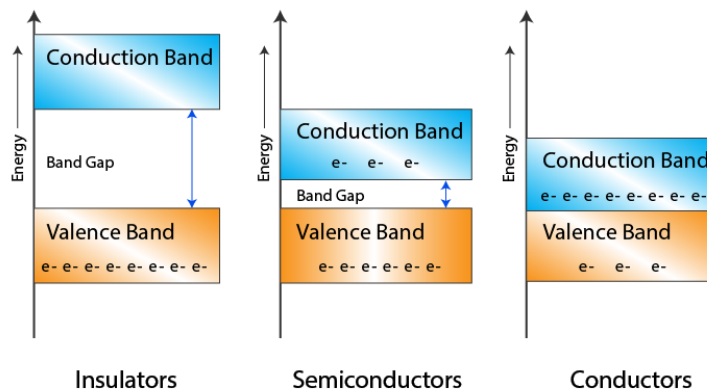


Figure 2.5. Band structures for insulators, semi-conductors and conductors.

The undoubled electrons are responsible from electrical conductivity. Furthermore, 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. Moreover, free electrons might be formed with heat or light excitation. Electrons which reach to 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. Likewise, in semiconductors, band threshold gap is smaller than insulators and their conductivity varies amongst $10^{-6} - 10^{2}$ S cm$^{1-}$. In metals, the valence band and the conduction band merge without bandgap [3,6].

Conjugated polymers and organic materials with low molecular weight bearing semi-conducting properties are a very rich set of materials that have properties giving the possibility of producing a large number of chemical structures with electronic and optical properties [3,6].

Most metal atoms have a single electron and it can't make a covalent bond with another metal atom near it. 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 might 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 via band gap transition [3,6].

**2.1.1.2 Doping in Conducting Polymers**

Chemical or electrochemical "p-doping" (oxidation) or "n-doping" (reduction), can increase the conductivity of certain organic polymers to metallic levels, the primary process of improving the electrical properties of the CPs to those of a metal as seen on Figure 2.6. Rather high-density charge carriers are introduced into the polymer upon doping, which can move through the 3D structure. The conductivity of CPs is maintained by the type of dopant, doping level and doping strength. Degree of polymer protonation means doping level and it provides conductivity to the polymer [3,6,7].
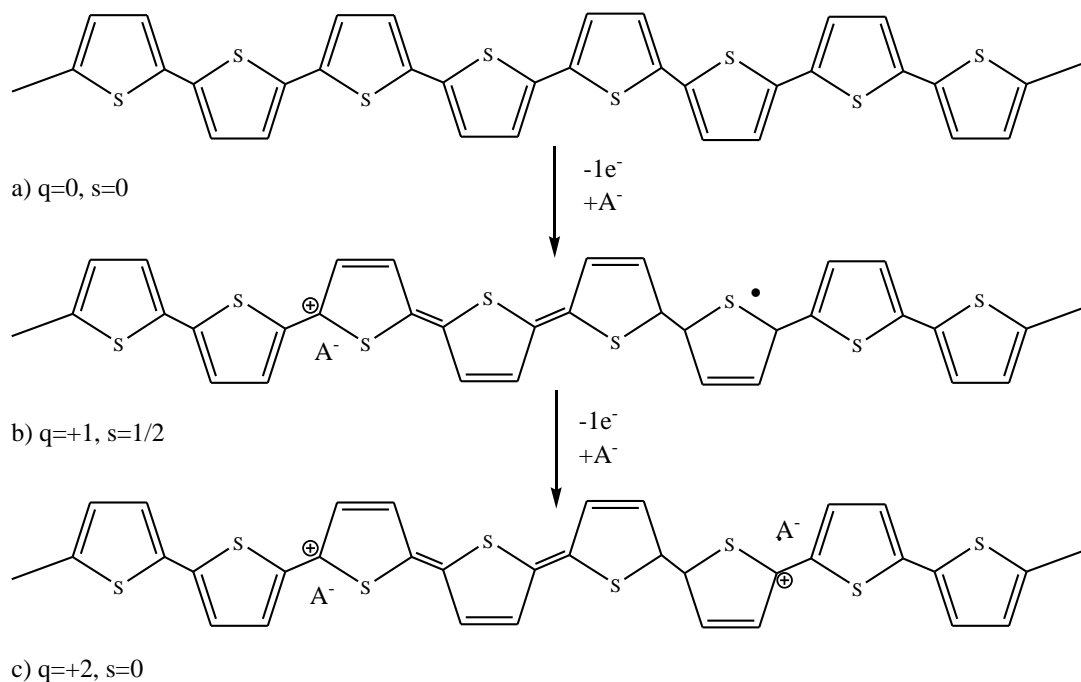
Figure 2.6. Oxidative doping process of polythiophene.

As the band theory is not sufficient to explain conductivity of conducting polymers, it is made the utilization of neutral polymer, polaron and bipolaron structures as presented in Figure.2.7 [8]. Electrical charge given to make doping to the skeleton structure of the polymer causes a change in the electronic condition of the polymer. Reversible acid-base doping as well as dedoping process allows for an effective control of the insulator-metal transition amongst CPs. Novel sensing functions are introduced by this easy and convertable transition in conductance, which has applications in sensors [6,7].
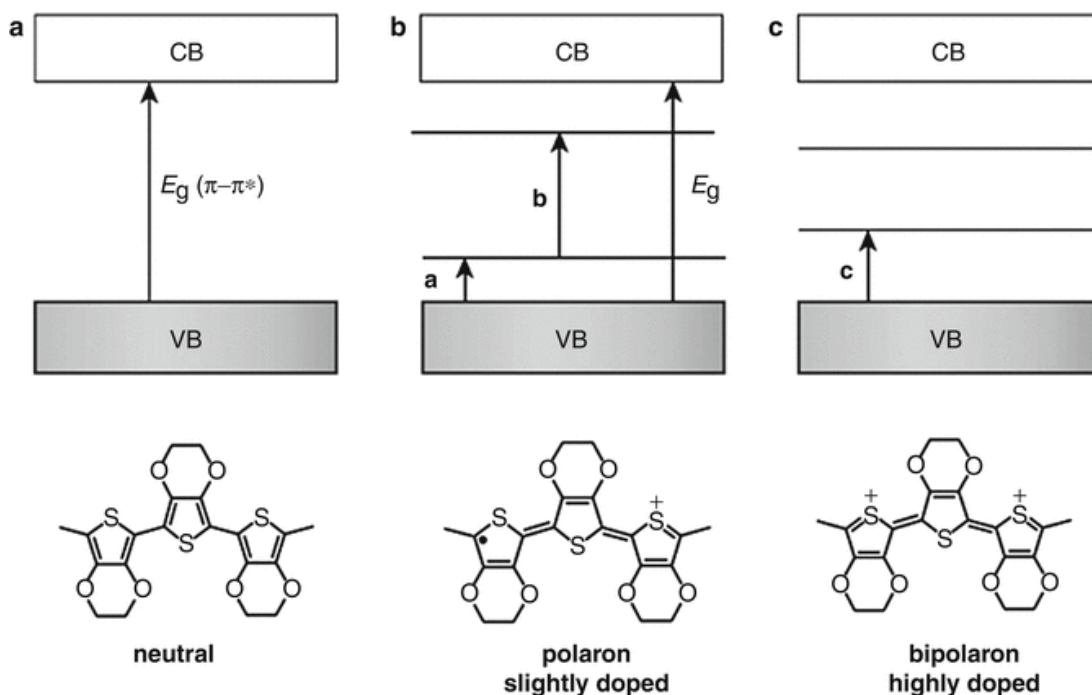
Figure 2.7.  p-doping: polaron, bipolaron structures and band diagrams, a) neutral
polymer b) polaron structure c) bipolaron structure.

Uncharged polymer having conjugated bonds is presented in Figure 2.7a. Upon oxidation, the double bond is destroyed and a positively charged radical is formed over the polymer chain as presented in Figure 2.7b [8]. 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.  Likewise, the oxidation of the free radical of polaron, a new positive hole without spin is formed named as "bipolaron" as presented in Figure 2.7c. At this point, two radicals combine and form a new $\pi$ bond. In the same way, there are no undoubled electrons in bipolaron which is linked to this way conductance is maintained without requiring free electrons [8]. Similarly, polarons as well as 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 via doping [9-11].

### 2.1.1.3. Hopping in Conducting Polymers

The charge carriers move through the chain and transfer of electrical charge amongst diverse polymer chains remains created via hopping (Figure 2.8).  In the hopping rule,

mobility of the electronic charge is maintained via transfer through the chain (Figure 2.8a) which is associated with transfer between chains (Figure 2.8b) and amongst blocks of polymer chains (Figure 2.8c) [1,8].
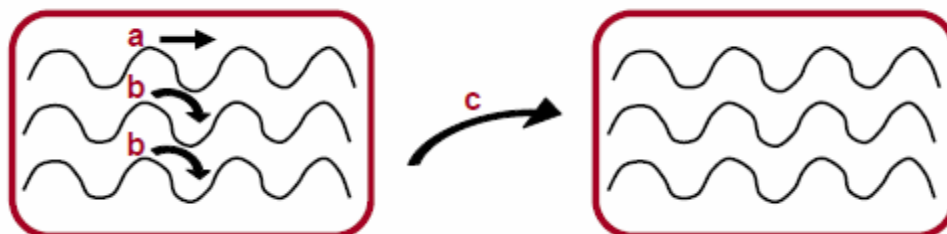


Figure 2.8. Transportation of charge, a) transportation of charge along the chain, b) transportation of charge between chains, c) transportation of charge between chain blocks [8].

What is more, a neutral chain interacts with the charged unit in a chain close to itself as well as the electron of the charged unit jumps towards the neutral chain [1,8].

### 2.1.2. Synthesis of Conducting Polymers

### 2.1.2.1. Chemical Synthesis

A monomer that is dissolved in a suitable solvent is polymerized via interacting with a chemical substance utilized as an oxidation or reduction agent. The method has several disadvantages, for instance, oxidation step can not be controlled, the product not being pure and the desired amount with a reasonable cost. The doping material as well as catalyst utilized in the chemical method has an important effect on the electrical conductivity of the conducting polymer to be obtained. For instance, in synthesizing poly(p-phenylene), the polymer that has been obtained via utilizing $CuCl_2$ as doping material which is associated with $AlCl_3$ as catalyzer didn't exhibit electrical conductivity. On the other hand, via utilizing materials, for instance, $AsF_5$ or Li, conductivity varies amongst 0.3 S $cm^{-1}$ – 500 S $cm^{-1}$ [12,13].

## 2.1.2.2. Electrochemical synthesis

Electropolymerization of conducting polymers is performed in a three-electrode cell consisting of a platinum operating electrode, an auxiliary platinum wire electrode, and the reference electrode $Ag/Ag^+$. Solutions consist of monomer and dopant ion dissolved in anhydrous $CH_3CN$ or $CH_2Cl_2$ [14].

Polymerization mechanism of heterocyclic compounds that happened in the anode is given in Figure 2.9. Polymerized heterocyclic compounds can find a wide range of utilization in the chemical field as polymers having thermal stability of at least 400 ºC. They can therefore be utilized in places where the material is subject towards a relatively large or extreme heat level [14,15].

Electrochemistry has been a powerful tool, not only for replicating of polymer films but also for in situ characterization of semiconducting materials. The electrochemical generation of intrinsically conductive polymers is based primarily on the oxidative condensation of various aromatic heterocycles and derivatives via radical cation formation [14,15].
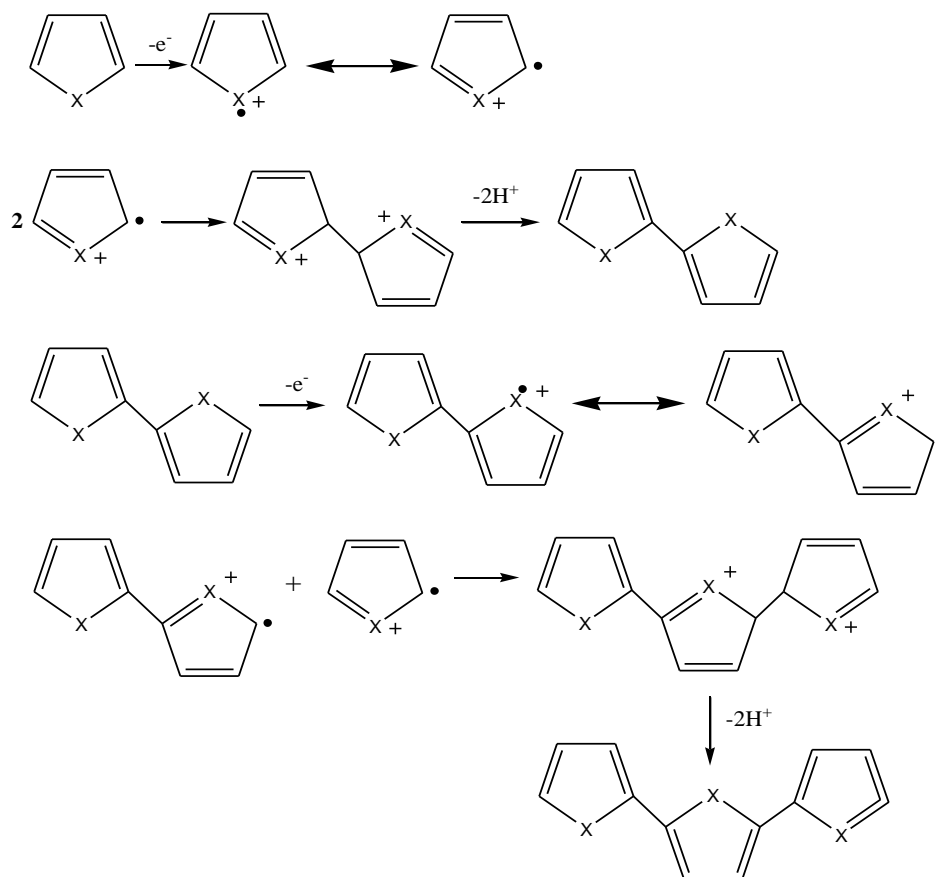
Figure 2.9. Electropolymerization mechanism of heterocyclic compounds (X= N-H, S, O).

Electron transfer reaction has a high radical concentration also, it is faster than the monomer diffusion and the solution around the electrode surface has a high radical concentration. This first stage is followed via the coupling reaction that happens with two radical cations couple each other. Upon radical-radical coupling, dihydro dimer cation is formed via two hydrogens separating from one dimer. Likewise, this anti-aromatic condition represents the chemical stage. As potential is applied, dimer is subjected to a coupling reaction again with a monomeric radical. Similarly, from the radical-monomer coupling reaction, a neutral trimer occurs which loses two protons and its other electron. In addition, dimers and oligomers enter into a coupling reaction with the radical cation of the monomer and they lose their protons and become anti-aromatic [1,16].

In electrochemical polymerization, cyclic voltammetry (CV) techniques can be utilized. Cyclic voltammetry is a standard tool in electrochemistry and has regularly

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been used for studying the electrodes performances and for characterization of transfer of electrons including microbial cells and biofilms. Generally, three electrodes are used for CV to determine accurate results. The working and counter electrodes are anode and cathode, for instance, in cathode, a platinum wire is used preferably. Cyclic voltammetry is a potent and a common technique usually utilized to examine oxidation and reduction processes of molecular substances. It is also valuable for the study of chemical reactions, which include catalysis, initiated via electron transfer [17].

Once the potential applied to the working electrode the material on the surface of the electrode is consumed rapidly. Moreover, measured current amongst working and opposite electrodes increases. Therefore, concentration variance occurring amongst electrode surface and the solution causes mass transfer from the solution to electrode surface. Consequently, as mass transfer rate remains lower than transfer rate of electrons, a decrease is evaluated and a peak is obtained. The potential which corresponds to the peak point of this peak is termed as "reduction peak potential" [17].

### 2.1.3. Usage Areas of Conducting Polymers

The interest in such material derives from the polymer component's low cost, light weight, mechanical durability and ease of process ability in conjunction with reasonably good bulk conductivity. Conductive polymers are utilized as electrodes in batteries, for instance, heart batteries as they are rechargeable and long life for the reason that they produce low currents and have reversible doping characteristics [18].

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 [19-21].
An additional important utilization of conducting polymers is biosensors as well as enzyme electrodes. Furthermore, biosensors that are analytical instruments which are

formed with the joining of the selectivity of the biological element which is concerned with the detector that produces signals proportional to the concentration of the analyte. They have the potential to be utilized in analyzing several materials as enzyme sensor as an outcome of their high selectivity, quick response time, simple design as well as cheapness [22,23].

Additionally, several CPs, for instance, polyacetylene (PA), Polyaniline (PANI), polypyrrole (PPy), poly(phenylene)s (PPs), Poly(p-phenylene) (PPP), poly(p-phenylenevinylene) (PPV), poly (3,4-ethylene dioxythiophene) (PEDOT), polyfuran (PF) as well as other polythiophene (PTh) derivatives have drawn special interest in the field of nanoscience and nano-technology [18,24].

## 2.2. ENZYMES

Enzymes in living organisms are biocatalysts that increase the biochemical reactions speed and can be isolated from plants to be utilized to catalyze a variety of important commercial processes. For instance, enzymes have significant place in the production of sweetening agents, they are used in cleaning products and washing chemicals and they have an outstanding importance in clinical, forensic and environmental applications [25].

Properties of enzymes are given in following items.
- Almost all of the enzymes are proteins, although some catalytically active RNA molecules were identified.
- Reaction catalysed by enzymes typically occur in milder circumstances, for instance, temperatures lower than 100 ℃, atmospheric pressure and pHs close to neutral value, compared to the uncatalyzed chemical reactions.
- A chemical reaction speed is increased by enzyme catalyzation however without changing itself [26].

The minimum energy required for a reaction to start is called as activation energy. An alternative path towards lowering the activation energy occurs via the help of enzymes as presented in Figure 2.10 [27].
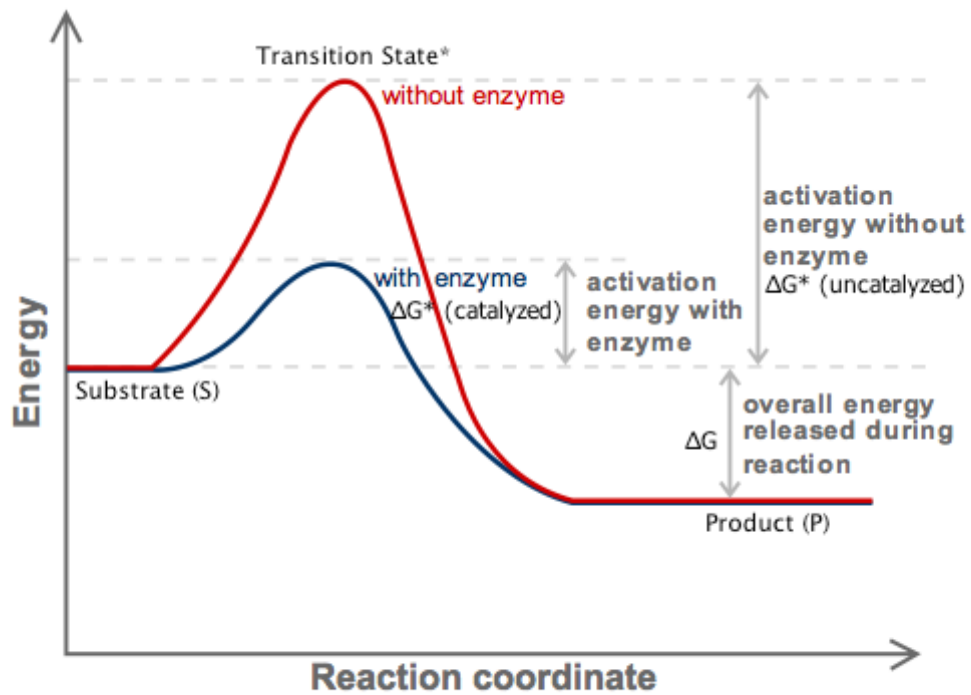


Figure 2.10. Effect of enzyme on activation energy.

Enzymes stimulate their substrates as the first hypothesis related to this formation mechanism is the key-lock model proposed via Emil Fischer the first time as presented in Figure 2.11 [27].
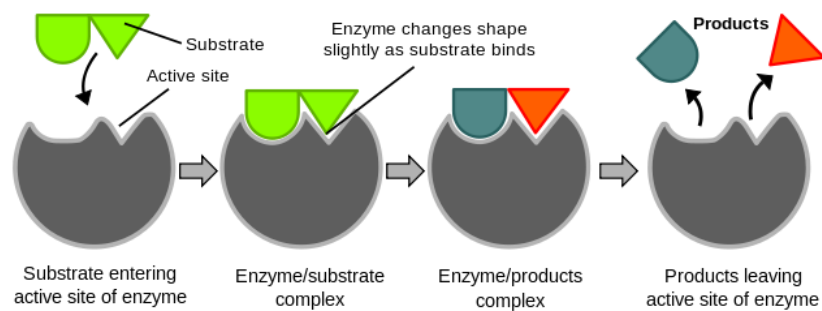


Figure 2.11. Key-lock model proposed for the enzyme-substrate complex.

Simple enzymes are biological catalysts which makes the biochemical reactions faster in living organisms. These can also be removed from cells, and then utilized to catalyze a wide variety of processes that are of commercial interest. Furthermore, complex enzymes are multienzyme frameworks which consist of a number of enzymes. Multifunctional enzymes also exist in the same molecule, with several diverse catalytic sites [28].

Meanwhile, complex enzymes are enzymes that contain a part which is concerned as a much smaller organic molecule. Moreover, the part of enzymes that composes only from protein is named as 'apoenzyme' as well as the group not in protein structure providing it to appearance catalytic effect is named as "cofactor". Cofactors are mostly metals that are needed in about two-thirds of all enzymes. They can be composed of a metal ion and they might compose of an organic group named as "coenzyme" as shown on Figure 2.12. An apoenzyme is an inactive enzyme that it is activated upon a cofactor binding. Apoenzyme and its cofactor together is a complete structure which has catalytical activity and it is called as Holoenzyme [28].
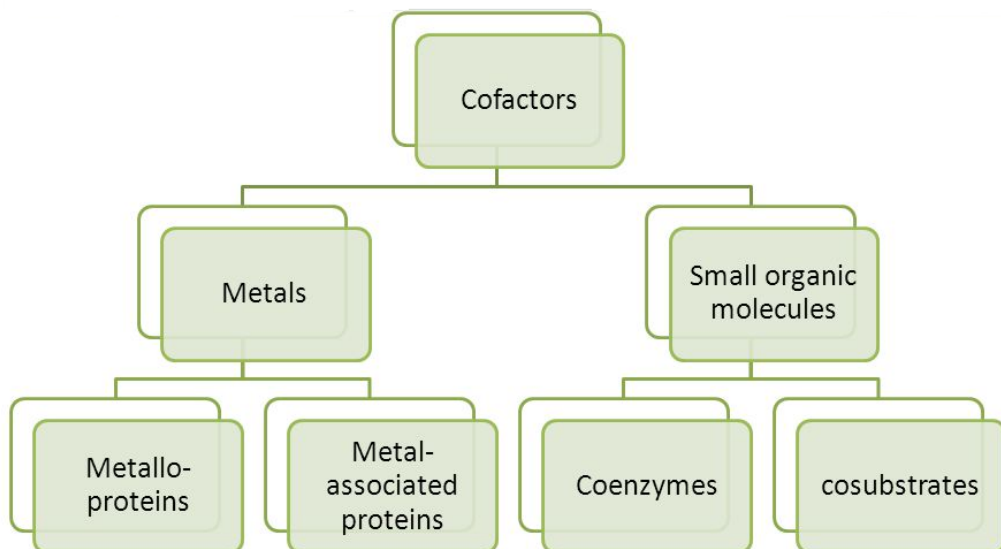


Figure 2.12. Types of Cofactor.

## 2.2.1. Classification of Enzymes

In the present day, enzymes are divided into 6 basic classes via the International Union of Biochemistry and Molecular Biology (IUBMB) as presented in Table 2.1 below. Enzymes are categorized into 6 categories according to the form of reactions catalyzed via the enzymes, which are oxidoreductases, transferases, hydrolases, isomerases, ligases. The most abundant sorts of enzymes are oxidoreductases, transferases, and hydrolases [29].

Table 2.1. Classification of enzymes.

| Number | Classification | Biochemical Properties |
|--------|----------------|------------------------|
| 1 | Oxidoreductases | Act on many chemical groupings to add or remove hydrogen atoms |
| 2 | Transferases | Transfer functional groups between donor and acceptor molecules. Kinases are specialized transferases that regulate metabolism by transferring phosphate from ATP to other molecules |
| 3 | Hydrolases | Add water across a bond, hydrolyzing it |
| 4 | Lyases | Add water, ammonia, or carbon dioxide across double bonds, or remove these elements to produce double bonds |
| 5 | Isomerases | Carry out many kinds of isomerization: L to D isomerizations, mutase reactions (shifts of chemical groups), and others |
| 6 | Ligases | Catalyze reactions in which 2 chemical groups are joined (or ligated) with the use of energy from ATP |

**2.2.2. Enzyme Activity**

The activity of an enzyme is defined according to the substrate amount spent. When enzymes are utilized in manufacturing processes as well as analytical procedures it becomes important towards accurately evaluate the enzyme activity. Furthermore, the activity of an enzyme is defined according to the substrate amount spent in unit time as well as converted to product at optimum conditions. Moreover, its unit is IU. Likewise, 1 IU enzyme activity is the enzyme amount that catalyzes 1 μmol of substrate in 1 minute when optimum conditions are present. There are several factors that influence rate of enzymatic reactions. Some of these are substrate concentration, enzyme concentration, temperature, pH as well as inhibitor effect. What is more, the manufacturing scale, the decision towards utilizing as well as not to utilize an enzyme in a process must be based on issues, for instance, the amount of enzyme needed to carry out the process properly, the length of the reaction, the amount of substrate to be transformed, the conditions under which the reaction occurs as well as the total cost of the process [27,30].

## 2.2.2.1. Effect of Substrate Concentration

In enzyme catalyzed reactions, reaction rate at optimum conditions increases proportionally with increase in substrate concentration. Furthermore, when the entire enzyme molecules form the enzyme-substrate complex, reaction rate reaches a maximum ($V_{max}$) [27,28,30]. In addition, this means that enzyme is saturated with substrate as presented in Figure 2.13.
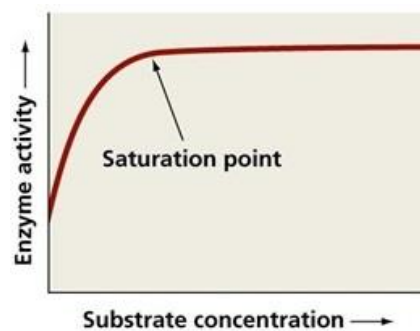


Figure 2.13. Effect of substrate concentration over reaction rate.

## 2.2.2.2. Effect of Enzyme Concentration

When there is plenty of substrate in the environment, reaction rate increases proportionally with enzyme concentration [27,28,30]. The enzyme amount is, reaction rate increases that much as presented in Figure 2.14.
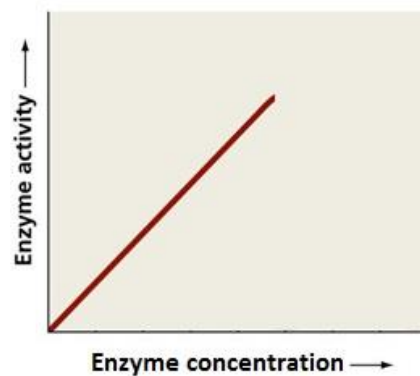


Figure 2.14. Effect of enzyme concentration over reaction rate.

### 2.2.2.3. Effect of Temperature

The collision rate of molecules that enter reaction increases when temperature increases. Rate of reactions demonstrate 1 to 3 times of increase with each 10 ℃ of temperature increase. On the other hand, this increase continues up to a certain value. Moreover, this value is the optimum temperature of that reaction as presented in Figure 2.15. Subsequently, particular changes which occur in molecules either slow down the reaction or stop it completely. As enzymes are protein-structured, this causes a deterioration in their 3-dimensional structures, called denaturation. In addition, this denaturation can be reversed at low temperatures, might regain their activity via reducing in temperature, on the other hand, they can not be reversed at high temperatures. In this circumstance, activity is completely lost [27,28,30].
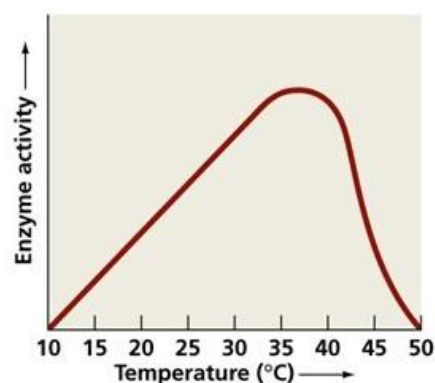


Figure 2.15. Effect of temperature over reaction rate.

### 2.2.2.4. pH Effect

Enzymes are amphoteric molecules which include acidic which is associated with basic groups. When the pH of the environment changes, the charge of these groups changes and this affects the surface charge distribution of the enzyme and net charge. For that reason, a change occurs in the activity of the enzyme. The pH which enzymes exhibit maximum activity is termed as optimum pH. In addition, it is presented in Figure 2.16 below, this pH value is diverse for each enzyme [27,28,30].
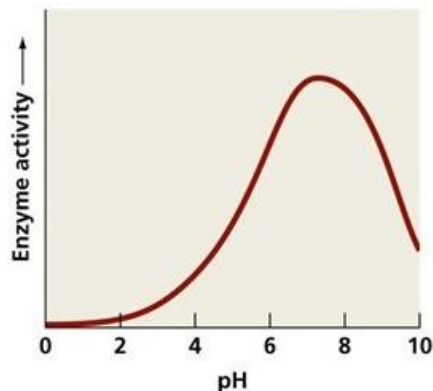
Figure 2.16. Effect of pH over reaction rate.

**2.2.2.5. Inhibitor Effect**

Materials which lower the reaction rate as presented in Figure 2.17 are called inhibitor. 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 [31]. Furthermore, inhibitors of enzyme are molecular species which makes interaction with the enzyme in other ways so that it does not operate normally. They can alter the enzyme's catalytic activity and thus slow down, or even avoid catalysis. While interacting with the enzyme, inhibitors might function in diverse ways: reversible or irreversible inhibition, covalent binding or non-covalent binding, and specific or non-specific inhibition [31].

Classification of inhibitors is made according to being reversible or irreversible. The enzyme forms a stable complex with an irreversible inhibitor. Consequently, permanent inactivation of enzyme occurs, and it can be reactivated very slowly taking hours or days. Usually, a covalent bond forms between enzyme and inhibitor if it is irreversible [31].

23
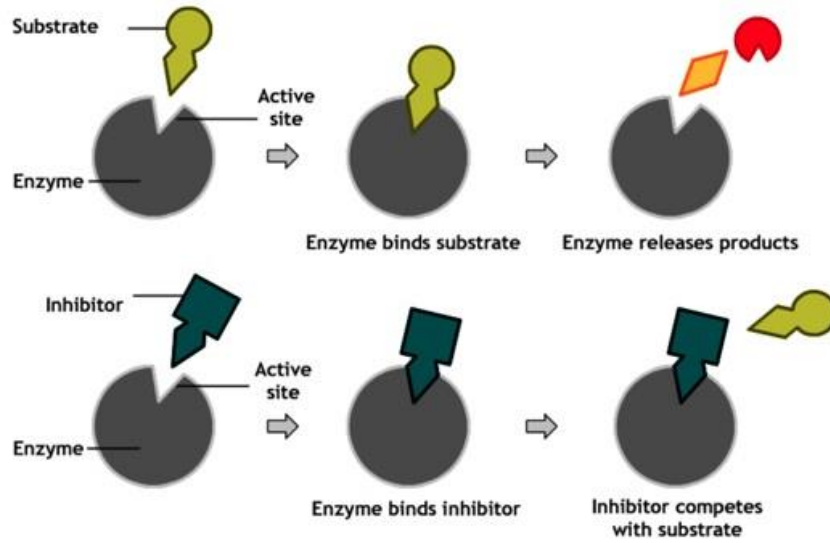
Figure 2.17. Enzyme-inhibitor relationship [23].

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

### 2.2.3. Enzyme Kinetics

Enzyme kinetics remains the biochemistry division that deals primarily with a quantitative explanation of this method, how experimental variables influence reaction rates. 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. For the duration of an enzyme catalyzed reaction, the enzyme (E) combines with the substrate (S) towards generating an enzyme-substrate complex (ES), as well as then the product is formed which is associated with dissociation from the enzyme (E+P). In addition, the kinetic scheme for an enzyme catalyzed reaction can be formulated as below [32,33].

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E+P$$
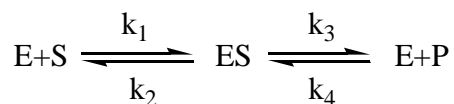
Here, $k_1$, $k_2$, $k_3$, $k_4$ illustrate the reaction rate constants that with $k_1$ ES complex forms, with $k_2$ E+S. The rate of ES complex conversion into free enzyme ($k_4$) is very low which is concerned with ignored as well as the reaction is as presented below [32,33].

24

$$ES \rightarrow E + P$$
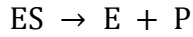
When the reaction rate is plotted versus substrate concentration, the hyperbolic curve in Figure 2.18 is obtained.
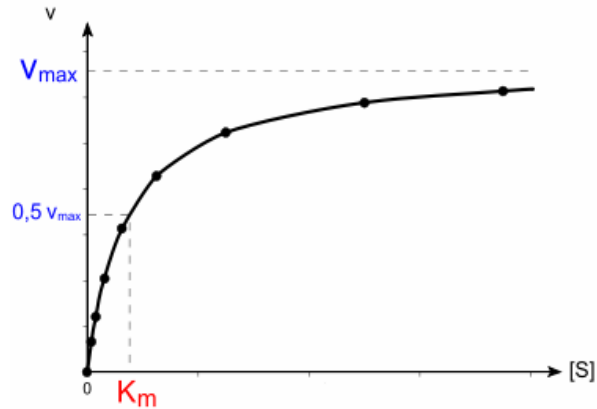


Figure 2.18. Substrate concentration versus reaction rate graph [34].

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 [32,33].

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

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

$$V = k_1.[E][S] \tag{2.1}$$

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

$$V = k_2.[ES] + k_3.[ES] = [ES]\,(k_2 + k_3) \tag{2.2}$$

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 [32,33].

$$k_1.[E][S] = (k_2 + k_3)[ES] \tag{2.3}$$

If Equation 2.3 is solved, Equation 2.4 is obtained.

$$\frac{[E][S]}{[ES]} = K_m \tag{2.4}$$

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) [32,33].

$$[E] = [E_t] - [ES] \tag{2.5}$$

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

$$[ES] = \frac{[E_t][S]}{K_m + [S]} \tag{2.6}$$

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

$$V = k_3[ES] \tag{2.7}$$

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

$$V = k_3 \frac{[E_t][S]}{K_m + [S]} \tag{2.8}$$

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.

$$V_{\max} = k_3 [E_t] \tag{2.9}$$

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

$$V = \frac{V_{\max}[S]}{K_m+[S]} \tag{2.10}$$

$K_m$ has presented the interest of the enzyme to the substrate as well as it is substrate concentration that makes half of the maximum rate. Consequently, the interest of the enzyme towards substrate remains high, $K_m$ is small. Therefore, it means that even in low [S]'s, enzyme makes [ES] complex with the substrate. In addition, if the interest of enzyme towards the substrate is weak, $K_m$ turn out to be high [32,33].

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:

- The condition [S] is too smaller than $K_m$, adding [S] towards $K_m$ changes its value very few anymore. Subsequently, [S] remains eliminated from the Equation 2.11 as presented below. In this condition, rate will depend on [S] value.

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

- In the condition, [S] remains too bigger than $K_m$, adding $K_m$ towards [S] changes its value very few anymore. Therefore, $K_m$ remains eliminated from denominator of the Equation. 2.12 below. In addition, the beginning rate remains equal to $V_{\max}$.

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

27

- Once [S] equals to $K_m$, Equation 2.13 is as presented below. In this condition, the rate remains half of maximum rate.

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

In this condition, Michaelis-Menten equation which is associated with a hyperbolic curve is reversed towards being converted into a linear equation for practicality as well as separated towards its multiples, Lineweaver-Burk equation is presented in Equality 2.14.

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

A straight-line equation is presented in the Equation 2.15 below.

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

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.

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

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 2.19).
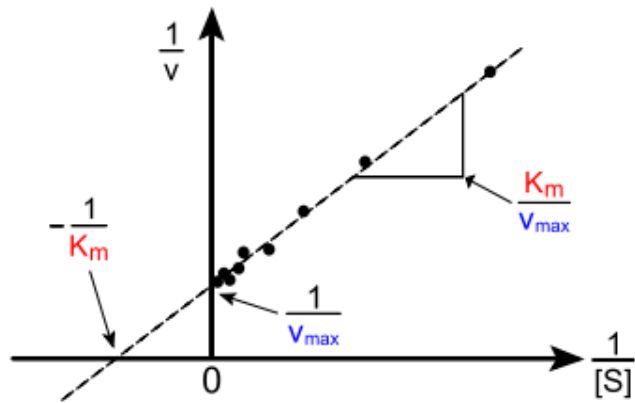
Figure 2.19. Lineweaver-Burk graph [34].

## 2.2.4. Enzyme Immobilization Methods

Enzyme immobilization is an approach developed explicitly for binding of the free enzyme to a carrying material. Enzyme immobilization is a process, mainly in order towards minimizing operating costs via allowing the enzyme being reutilized several times. Immobilization technique can significantly reduce the enzyme amount spent, process time as well as product cost. Moreover, enzymes can be easily separated from the product and be reused. Immobilization process provides some more advantages such as enzyme being more stable and having life-time longer than free enzyme. Enzyme immobilization approaches were divided into three as binding, cross-linking as well as encapsulation [35] as presented in Figure 2.20 below.
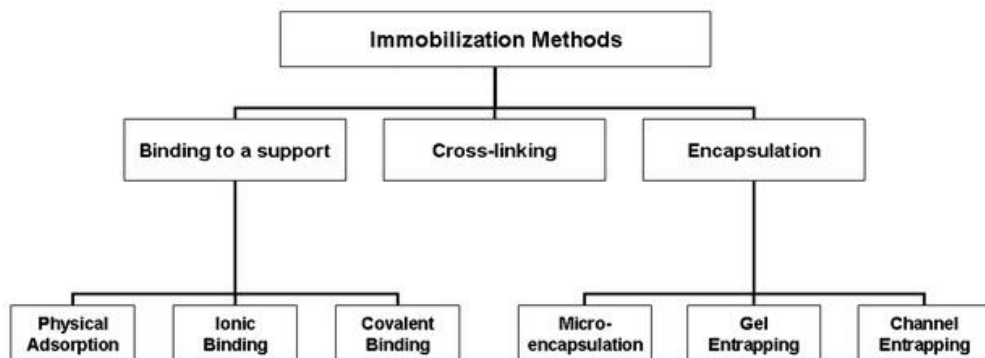


Figure 2.20. Enzyme immobilization methods.

### 2.2.4.1. Binding to a Support

In this process development of new bonds are conventional amongst the enzyme which is associated with functional groups of support as well as enzyme remains reformed. Furthermore, binding to the transporter is made via enzymes to be binded towards the supporting material with physical adsorption, ionic bonds as well as covalent bonds as presented in Figure 2.21 below. In addition, these supports developments are inorganic materials, for instance, silica as well as porous glass, organic supports, for instance, polyacrylamide including weak activity –OH group or biological supports, for instance, cellulose [35].
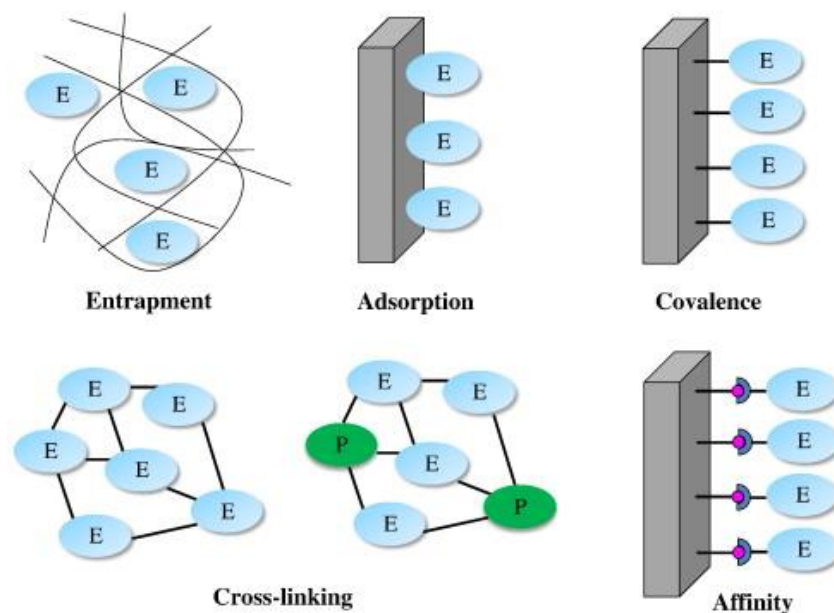
Figure 2.21. Enzyme immobilization methods.

### 2.2.4.2 Cross-linking

The approach techniques of enzyme molecules form complexes that do not dissolve in water via cross-linking with multiple covalent bonds as an outcome of their reaction with a cross-linker that contains a functional group like glutaraldehyde as presented in Figure. 2.21 above [36]. In this approach techniques, it is a decrease in enzyme activity for the reason that 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 [36].

### 2.2.4.3. Entrapment (Encapsulation)

It is the placing of the enzyme in an appropriate porous polymer for the diffusion of substrate in which is concerned with diffusion of product out as presented in Figure 2.21 above. Furthermore, entrapment approach is good for smaller substrate or product molecules compared to other approaches. Moreover, the variance of the entrapment approach is that there is no bonding amongst the enzyme and matrix. Since there is no bond amongst, enzyme losses through matrix pores can be possible. On the other hand, there is no activity losses which is a disadvantage of covalent binding. Additionally, electrochemical polymerization approach in physical immobilization of enzymes which is an attractive one as it is faster, reliable, economical as well as simple [37].

### 2.2.5. Poliphenol Oxidase

Polyphenol oxidase is termed as tyrosinase (EC 1.14.18.1) which is an enzyme has been discovered by Schoenbein in mushrooms during 1856. Streptomyces glaucescens which is concerned with the fungi Neurospora crassa as well as Agaricus bisporus derive the best characterized tyrosinases. In addition, the enzyme isolated from the Agaricus bisporus champignon mushroom is extremely homologous to mammalian ones, making it suitable as a model for melanogenesis studies. This enzyme has not been named consistently and several times it has been named according to an individual substrate that it has worked on, for instance, tyrosinase, phenolase, catechol oxidase, catecholase, $o$-diphenol oxidase, monophenol oxidase, as well as cresolase. There are two copper ions in the structure of enzymes active site. They are bound to six listidine species which associated with a cysteine specy. The enzyme has a distribution almost universal in animals, fungi, plants which is linked with bacteria. This enzyme in plants is necessary to make defence against herbivores and insects. Its function in fungi and bacteria, however, has not been understand yet. 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 as well as laccase. Moreover, tyrosinase catalyzes (EC. 1.14.18.1) hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) as well as oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity) as presented in Figure 2.22 below. Catechol oxidase (EC. 1.10.3.1) catalyzes oxidation of only *o*-diphenols. In addition, laccase (EC. 1.10.3.2) catalyze oxidation of both *o*-diphenols and quinones corresponding to *p*-diphenols. Laccase and catechol oxidases can not catalyze hydroxylation reactions [38-40].
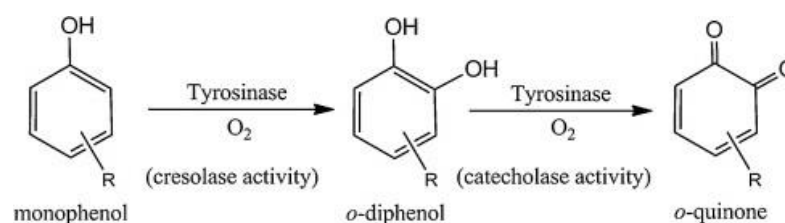


Figure 2.22. Polyphenol oxidase catalysis reaction.

The reaction that polyphenol oxidase catalyzes is called biotransformation which takes place in almost all living bodies and this biotransformation is only possible via tyrosinase activity. Formation of pigments in skin, hair as well as eye besides the color darkenings occuring in fruits, vegetables and mushrooms are the outcomes of tyrosinase activity in the presence of oxygen. Plant tyrosinase oxidizes phenolic compounds to brown colored quinonoic substances through the contact with oxygen. Polymerization of these quinonoic substances results in brown pigments formation and owing to this, these processes are named as browning reactions. It is an important reaction that take place during food manufacturing and storage. Either enzymatic and nonenzymatic browning might have a positive or negative effect on the consistency of the food, depending on the type of food. These browning reactions that tyrosinase form mostly unwanted in the food manufacturing. Likewise, tyrosinase catalyzes the initial step in pigment melanin forming tyrosine in the fungi and vertebrates. In plants, the physiological substrates are a number of phenolics which is associated with tyrosinase. Enzyme oxidizes them when tissues are damaged and the browning process starts. Fruits and vegetables darkening when they are cut and peeled decreases the food

quality and value of these products. On the other hand, tyrosinase has also been utilized in food industry to generate and improve food quality and diversity since browning is something desired in some foods. For instance, products of this color change provide taste and odor to some foods such as tea, coffee, cacao, dry grapes and dry prune [41,42].

**2.2.5.1. Working Mechanism of Polyphenol Oxidase**

Phenolics are oxidized by PPO with the existence of oxygen. This enzyme has group specificity and uses all pyhenolics as substrate. Polyphenol oxidases has a dinuclear copper center with aromatic rings that allow molecular oxygen to split into the ortho-hydroxyl group followed via monophenol hydroxylation to *o*-diphenol (catechol oxidase) which is associated with quinone oxidation [43]. Active sits of PPO pass through transitions amongst methoxy, oxy which are associated with deoxy forms in a cyclical manner as presented in Figure 2.23 below.
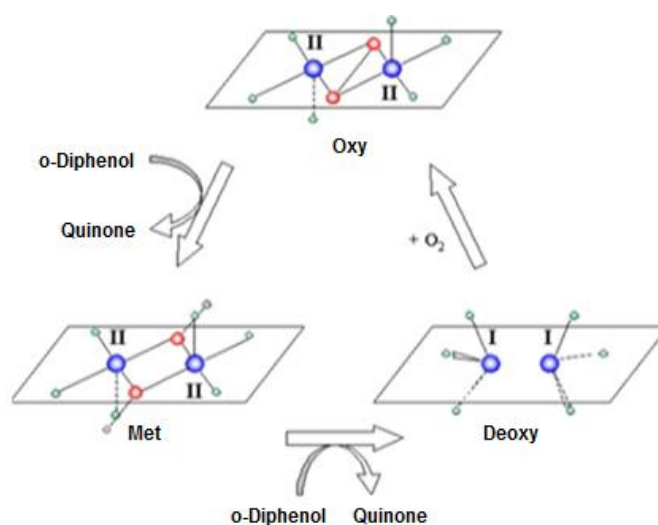


Figure 2. 23. Catalytic cycle and transitions of PPO's active site amongst methoxy, oxy and deoxy forms during catalysis. Blue, red and green circles represent copper, oxygen and histidine [43].

Two catechol molecules are oxidized in each cycle and one molecular oxygen is reduced to water and two quinone molecules are produced. The dioxygen $O_2$ is bonded to enzymes metal centre and it takes the place of water molecule which is bonded to

CuA in the reduced deoxy form of enzyme. $O_2$ is bonded first as a peroxide then catechol molecule binds. Deprotonation of one of the hydroxyl groups of catechol substrate occurs and it is bonded to CuB (oxy form). Then two electrons are transferred from catechol to peroxide which is followed by peroxide group protonation and O-O bond cleavage.

The second hydroxyl group that did not 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 sit 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 2.24). Cu(I)-Cu(I) form of the active site repeats catalytic cycle again [43,44].
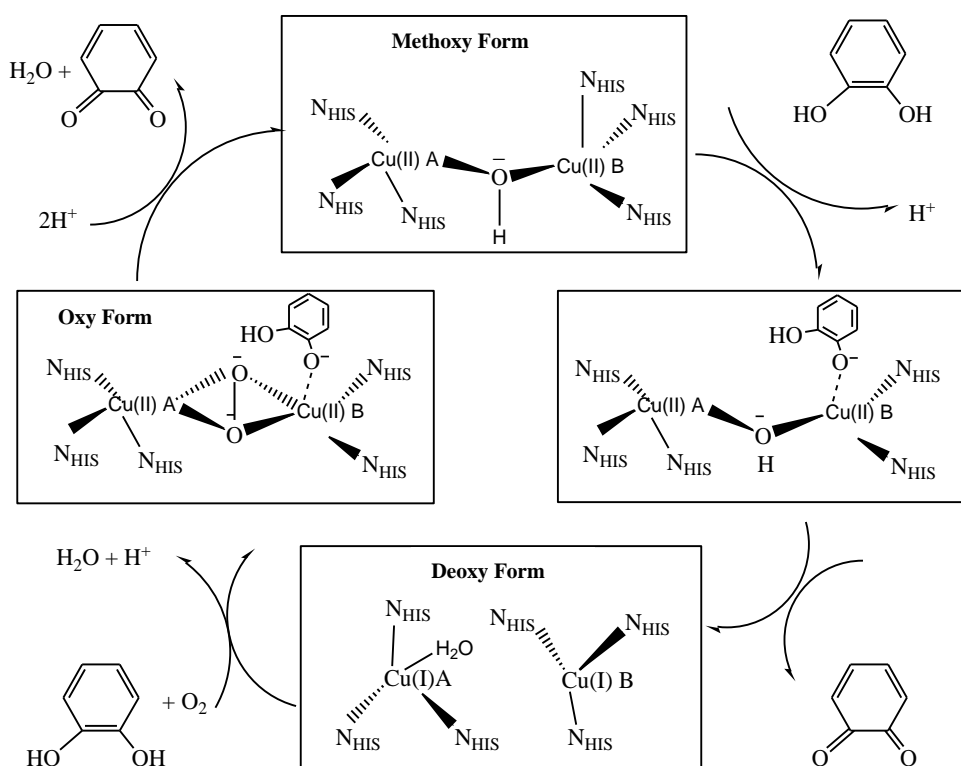


Figure 2.24. Reaction mechanism of PPO.

**2.2.5.2. Usage Areas of Polyphenol Oxidase**

Polyphenol oxidase has received significant attention from researchers working in the area of food, plant physiology, which is associated with cosmetics development, phenolic substances that contaminate environmental water with manufacturing and domestic waste creating pollution as well as damage for living species in water where tyrosinase is used for removing toxic phenolic compounds. 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 [45].

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 [43]. 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 [46,47].

Polyphenol oxidase is responsible for undesirable enzymatic browning in plant products, seafoods, and melanin formation in human skin. Inhibitors have been searched widely to prevent browning reactions. A variety of inhibitors of tyrosinase are recorded from natural sources, on the other hand, only a few are utilized as skin-whitening agents, mainly for the reason that various safety concerns. Linoleic acid, hinokitiol, kojic acid, arbutin, naturally occurring hydroquinones, as well as catechols, for example, have been documented to inhibit enzyme activity, on the other hand, have also had side effects (Maeda and Fukuda, 1991). For the reason that the critical role of tyrosinase in the process of melanogenesis and browning, several investigations have been documented on the identification of tyrosinase inhibitors from both natural, for instance, fungi, bacteria, plants as well as synthetic sources [42,48].

### 2.2.5.3. Use of Polyphenol Oxidase in Biosensors

The vegetable kingdom is a wide source of a diverse variety of enzymes with broad biotech domains. Furthermore, among the main classes of plant enzymes, the polyphenol oxidase, which convert phenolic compounds towards the related quinones, have been successfully utilized for biosensor development. The oxidation products from such enzymes can be electrochemically reduced, which is associated with the sensing is easily achieved via amperometric transducers. An approach, biosensors lead in this area usage of tyrosinase is that it exists as a biomaterial in biosensors that have been utilized in determining phenolic compounds. Additionally, biosensors that have sensitivity which is concerned with fast determining quality are obtained as an outcome of a combination of sensor frameworks with biological material. They remain utilized for analysis purposes in diverse areas like medicinal domain, defense, food industry and for environmental purposes. In enzymatic biosensors, the biological material remains enzymes, as well as the selective determination of polyphenols, can be made via tyrosinase based biosensors for the reason that the enzyme being a catalyzer specific to phenolics [49-53].

Recent publications have largely documented polyphenol oxidase-based biosensors built on the principles of electrochemistry. For the reason that their high sensitivity, usability, fast responsiveness and simple to miniaturize, these biosensors are widely documented in literature. Moreover, biosensors based on polyphenol oxidase have been applied to the clinical study of phenolic products, including acetaminophen and the study of acetyl salicylic acid. Correspondingly, it is utilized in determining phenolics in environmental water [54-56].

### 2.3. SAMPLE ANALYSIS

As presented in this study, total polyphenol material content of a peach sample was measured utilizing polyphenol oxidase enzyme electrodes. Furthermore, Folin-Ciocalteau approach has been utilized as a control technique towards validating the analysis via enzyme electrodes. Moreover, Folin-Ciocalteau technique that has been developed via Singleton et al is utilized towards measuring the total phenolic content.

Therefore, the technique is based on the fact that phenolic compound dissolved in water forms a colored compound with the Folin reactive in alkali environment [57,58] which is associated with measurement of absorbance according to this color intensity. Folin-Ciocalteau reactive (FCR) is considered which provide its name towards the approach remains a molibdophospotungstic heteropolyacid its assumed active center is Mo (VI) ($3H_2O.P_2O_5.13WO_3.5MoO_3.10H_2O$).

Mo (VI) (yellow) + $e^-$ (antioxidant) $\rightarrow$ Mo (V) (blue)
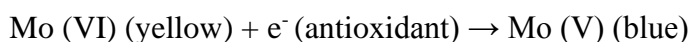
Phenolic compounds enter into reaction with FCR only under basic conditions and pH of the environment is set to ten via sodium carbonate solution. Furthermore, removal of a phenolic proton causes formation of a phenolate anion that has reducing capability of FCR and blue colored compounds are formed amongst phenolate as well as FCR. Moreover, FCR can be reduced via several non-phenolic compounds which is associated with it is not specific to phenolic compounds. Additionally, this approach measures the reduction capacity of a sample. In addition, it might interact with reductant materials in the environment, for instance, ascorbic acid [59]. In spite of these drawbacks, this approach remains utilized very frequently for the reason that its simplicity, repeatability which is associated with the correlation it presents with other approaches. In addition, approach outcomes is given as a standard phenolic material as mg gallic acid equivalent.

# PART 3

# MATERIALS AND METHODS

## 3.1. CHEMICAL MATERIALS

Tyrosinase was extracted from mushroom (EC 1.14.18.1). Polyvinylpyrrolidone and polyvinylpolypyrrolidone were purchased from Sigma-Aldrich and Acros. Triton X-100 and phenylmethanesulfonylfluoride were obtained from Merck and Sigma. Macherey-Nagel 617 filter paper was used in extraction. EDOT and Hydroxymethyl EDOT ((2,3-dihydrothieno[3,4-*b*][1,4]dioxin-2-yl)methanol) were purchased from Aldrich and TCI. SDS and $LiClO_4$ were obtained from Sigma and Fluka. Phosphate buffer components, $NaH_2PO_4$ and $Na_2HPO_4$ were from Merck. For MBTH solution, 3-methyl-2-benzothiazolinon 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. PEDOT and Hydroxymethyl PEDOT conducting polymers were coated over platinum electrode. 5000 times and 10000 times magnified pictures of surface of polymer coated electrode were taken with and without 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 ℃ 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 ℃ and 80 ℃ and activity measurements were made to determine the temperature at which enzyme electrode works best.

### 3.3. METHODS

### 3.3.1. Extraction and Preparation of Polyphenol Oxidase from Mushroom

Polyphenol oxidase enzyme extract was prepared by extraction of enzyme from Mushroom Sırtıgökçe (*Russula cyanoxantha* L.) (Figure 3.1). It was performed by cutting up 2.5 gram mushroom with blender (Philips) in extraction solution. Mushroom was stored in freezer at -18 ℃ and washed with distilled water before use. Extraction solution was 50 mL phosphate buffer (0.1 M pH 7.00) solution including 1% Triton X-100, 5% polyvinylpyrrolidone (PVP), 1mM phenylmethanesulfonylfluoride (PMSF). Mushroom and extraction solution were blended 4 times 10 seconds. This mixture was stirred 30 minutes and filtered through filter paper, then centrifuged at 7 ℃, 1 hour at 4500 RCF (relative centrifuge force). All steps till centrifuge were performed at 4 ℃ by using icebaths and cooling the solvents and chemicals in refrigerator before use. After centrifuge, upper liquid part was collected (enzyme extract) and used as enzyme source [60].

Figure 3.1. Mushroom Sırtıgökçe (*Russula cyanoxantha* L.).

### 3.3.2. Electropolymerization and Enzyme Immobilization
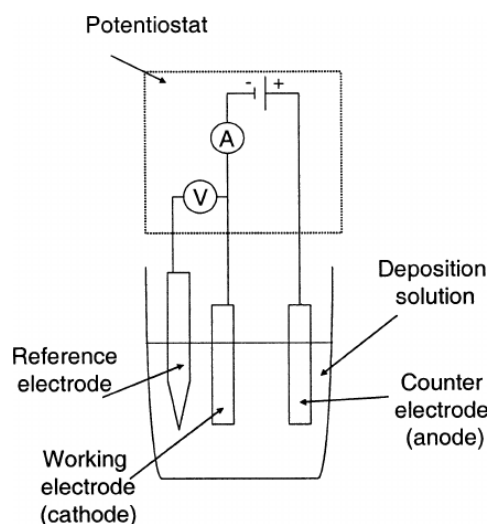


Figure 3.2. Three electrode system.

Electropolymerization cell was prepared by adding monomer and electrolyte to phosphate buffer (0.1 M pH 7.00) solution (deposition solution) (Figure 3.3). As electrolyte, 0.1 M lithium perchlorate/sodium perchlorate was used. Monomer concentration was taken as $10^{-4}$ M and polymerization window was determined by scanning with cyclic voltammetry using a 3-electrode system (Figure 3.2) in (-2) – (+2) V potential interval. Platinum was used as working and counter electrode and silver ($Ag/Ag^+$) electrode was used as reference electrode. Then by using determined window, polymerization of the monomer was maintained. In order to prepare enzyme electrodes, electropolymerization and enzyme deposition were performed in the above deposition solution with the addition of 0.2 mL enzyme extract. Poly(3,4-

ethylenedioxythiophene) (PEDOT) and hydroxymethyl PEDOT polymers were obtained having enzyme entrapped within the polymer matrix. After this operation, each enzyme electrode (Pt/PEDOT/PPO or Pt/MeOH-PEDOT/PPO) was washed with distilled water a few times to remove free supporting electrolyte remaining on the surface. When it is not used, it was kept at +4 °C in phosphate buffer (0.1 M pH 7.00) [61].
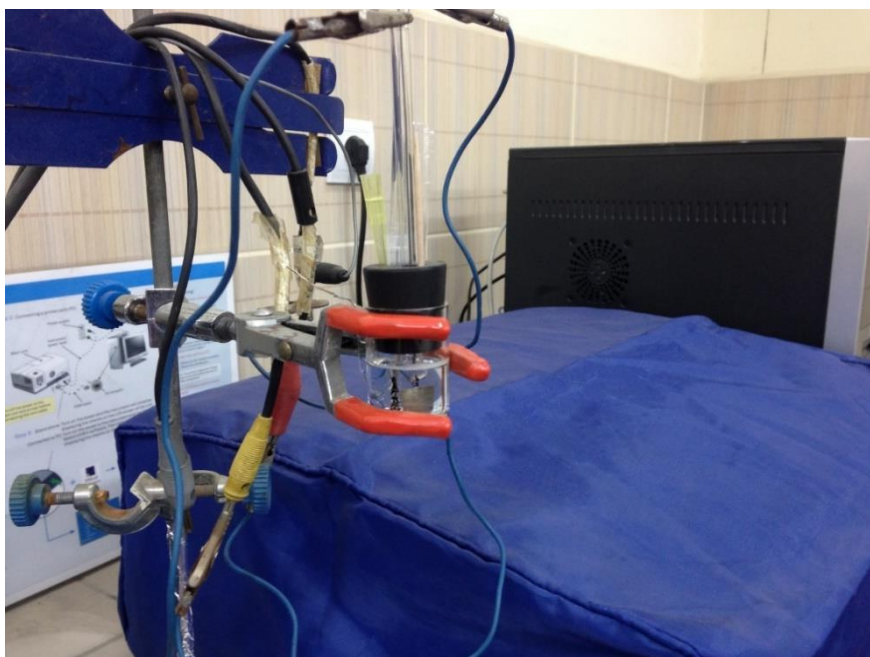


Figure 3.3. Electropolymerization cell.

Enzyme immobilization here, was made with entrapment 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. In cross-linking and covalent bonding, stability of the enzyme and therefore the electrode may increase. However, in this immobilization method, enzyme configuration may change upon covalent binding and activity losses is not a low possibility. In this respect, entrapment method is more advantageous than covalent binding. Enzyme in its free form, in the cavities of the polymer does not go to configuration change and not to activity losses. Entrapment is also superior to adsorption since it maintains the enzyme to remain stable for longer time.

### 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 [62]. Firstly, MBTH reactive is added to the solutions. Enzyme electrode is dipped for certain periods. After product formation, $H_2SO_4$ 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.).
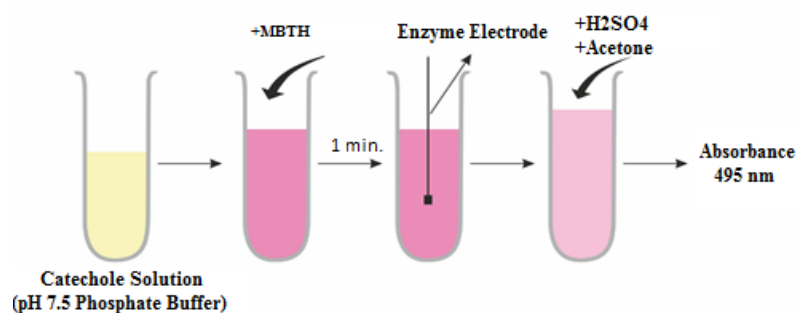


Figure 3.4. Besthorn's Hydrazone method.

MBTH solution used in this method was prepared as the concentration of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate ($C_8H_9N_3S.HCl.H_2O$) was 3.0 mg $mL^{1-}$ dissolving in ethyl alcohol.

### 3.3.4. Determination of the Enzyme Activity

In the reaction where polyphenol oxidase enzyme is the catalyst, 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).
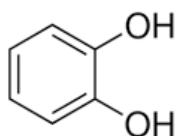


Figure 3.5. Pyrocatechol.

Solutions of pyrocatechol in different concentrations were prepared in phosphate buffer (0.1 M pH 7.00) 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.
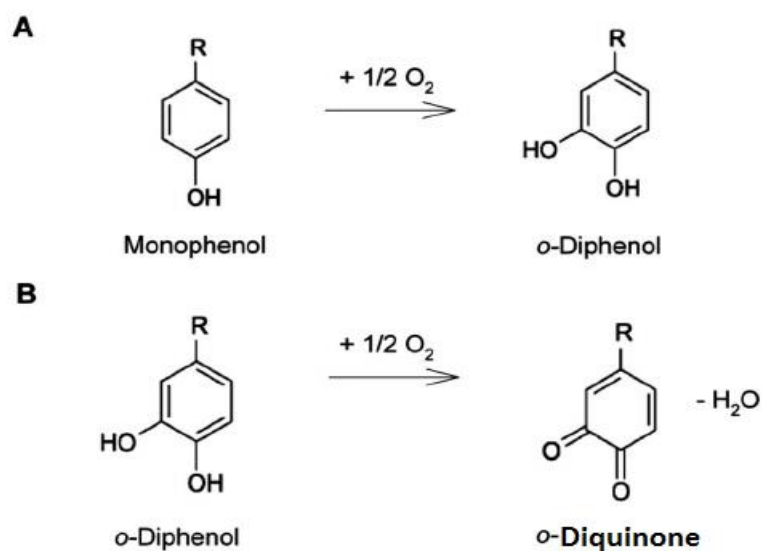


Figure 3.6. PPO's cresolase and catecholase activities: (A) monophenol's *o*-hydroxylation, (B) oxidation of *o*-diphenol to *o*-quinone.

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 [61].
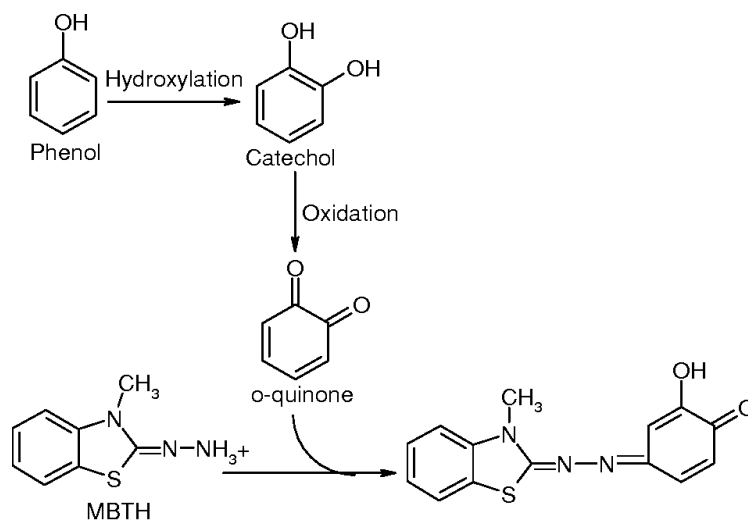
Figure 3.7. *o*-Quinone formed to establish colored compound with MBTH reactant [63].

### 3.3.5. Assessment of Kinetic Parameters

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. $1/[S]$) [64]. From Lineweaver-Burk graph, with Michaelis-Menten method [65], enzyme electrode's kinetic parameters, maximum enzyme activity ($V_{max}$) and Michaelis-Menten constant ($K_m$) were detected.

### 3.4. OPTIMIZATIONS

### 3.4.1. Determination of Best Dopant Ion

Dopants and dopant couples such as $10^{-3}$ M polyethyleneglycol (PEG), 0.01 M sodiumdodecylsulfate (SDS), 0.01 M SDS / 0.1 M LiClO$_4$ couple, 0.01 M SDS / 0.1 M KNO$_3$ couple were tried to find the best dopant. With each dopant system, enzyme electrode was prepared and enzyme activity measurements were performed with 0.1 M catechol solutions. With each electrode, 40 consecutive activity measurements were performed in order to obtain the stability of the electrodes.

### 3.4.2. Optimization of Immobilized Enzyme Amount

By changing the amount of enzyme added to electropolymerization solution, enzyme concentration that gives the best result was determined. For this purpose, 4 different amounts of enzyme were applied: 0.2, 0.5, 2.0, 3.0 mL enzyme extract were used and four different electrodes with different amount of immobilized enzyme were obtained using 0.1 M catechol solutions. Their enzyme activities were measured and optimum extract amount was determined.

### 3.4.3. Optimization of Polymerization Cycle Number

In electropolymerization by cyclic voltammetry, cycle number determines the extend of polymerization thus polymer thickness. Here, different number of polymerization cycles such as 30, 60, 120 and 180 were tried. Four electrodes were prepared with those polymerization cycle numbers. Enzyme activity measurements were performed with 0.1 M catechol solutions in order to determine the polymer thickness that gave the best result.

### 3.4.4. 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 using acetate, phosphate and TRIS buffer systems. 0.1 M catechol solutions were used and optimum pH value at the enzyme electrode that operates best was determined.

### 3.4.5. 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 ℃ and 80 ℃ using 0.1 M catechol solutions and the temperature at which enzyme electrode works best was determined.

### 3.4.6. Assessment of Stability

It was aimed to determine stability of enzyme electrodes with consecutive measurements. With activity measurements made one after another in the same day, it was checked whether activity changes or not when number of measurements increases. 0.1 M catechol solutions were used. Therefore, stability of the enzyme electrode was determined.

### 3.4.7. Determination of Shelf Life

For PPO electrode, activity measurements were made by 0.1 M catechol solutions once in a three-day to observe how the stability of enzyme electrode changes and shelf life was determined.

### 3.5. PHENOLIC SUBSTANCE DETERMINATION IN PEACH SAMPLE

Peach extract was obtained by blending 25 g peach sample with 50 mL phosphate buffer (0.1 M, pH 7.00) then by filtering the mixture. The solution obtained was analyzed for polyphenolic substances.

### 3.5.1. Analysis by Enzyme Electrode

Catechol standard solutions in phosphate buffer (0.1 M, pH 7.00) at different concentrations were prepared between $1.0 - 110.0$ mg mL$^{1-}$ and calibration curve was plotted with absorbance measurements of standards. First, enzyme electrode was dipped for 15 minutes to each solution and then Besthorn's Hydrazone method was applied and their absorbance was measured at 495 nm. Electrode was dipped for 15 minutes into the peach solution 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 µL were taken from standards and 3.16 mL water and 200 µL Folin-Ciocalteu reactive were added on them. It was mixed and after waiting 5 minutes, 600 µL 20% $Na_2CO_3$ solution was added and mixed. After waiting for 2 hours, absorbances of solutions were measured at 765 nm and calibration graph was plotted. Same operations were applied for samples also by taking 40 µL peach extract sample and absorbance values were measured and from the calibration curve, total polyphenol amount in sample was determined as mg catechol equivalent [57].
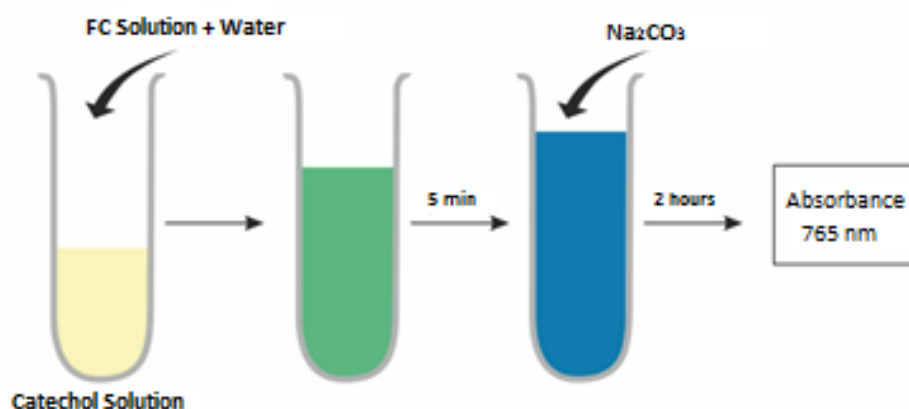
Figure 3.8. Process steps of Folin-Ciocalteu method.

## PART 4

## RESULTS AND DISCUSSION

In this study, formation of enzyme electrodes was performed by immobilization of polyphenol oxidase enzyme to conductive polymers PEDOT and hydroxymethyl-PEDOT. Optimum conditions for preparation of enzyme electrodes and also for activity measurements 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 peach extract sample.

### 4.1. ELECTROPOLYMERIZATION OF EDOT AND MeOH-EDOT

Synthesis of PEDOT and hydroxymethyl-PEDOT with cyclic voltametry were performed in phosphate buffer solution (0.1 M, pH 7.00) that includes 0.1 M SDS/LiClO$_4$ and $10^{-2}$ M monomer. Scans were made for 60 cycles between +0.200 and +1.500 V [66,67]. As it is seen in Figure 4.1, this interval is the scanning interval that oxidation and reduction peaks of the polymer take place.

**(a)**



**(b)**
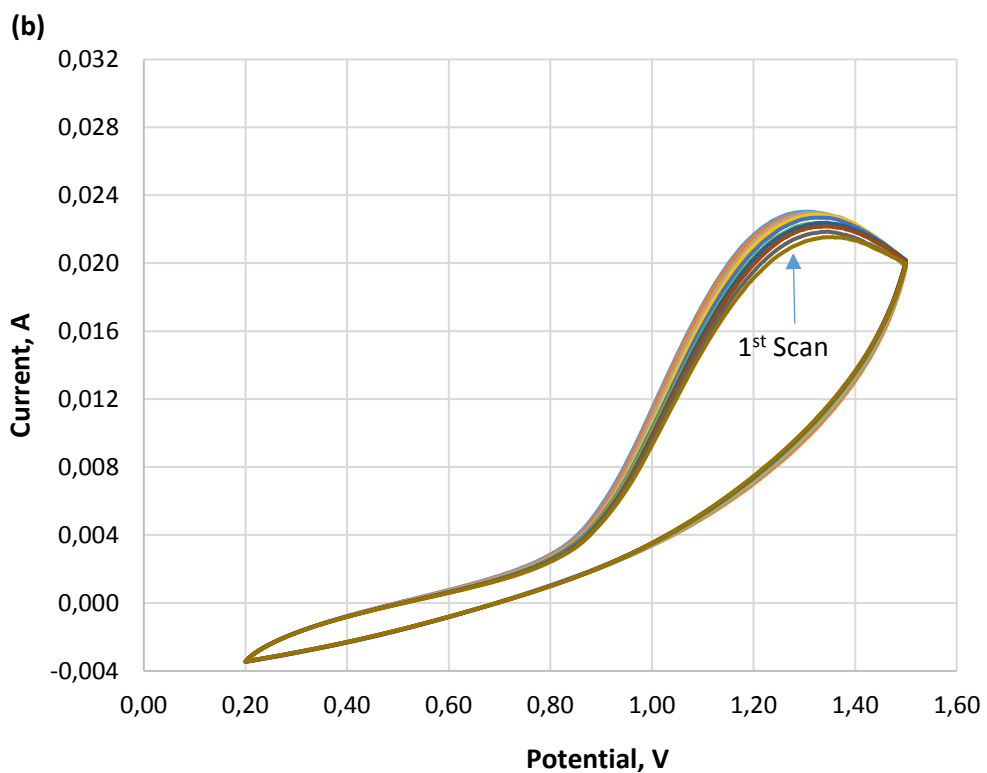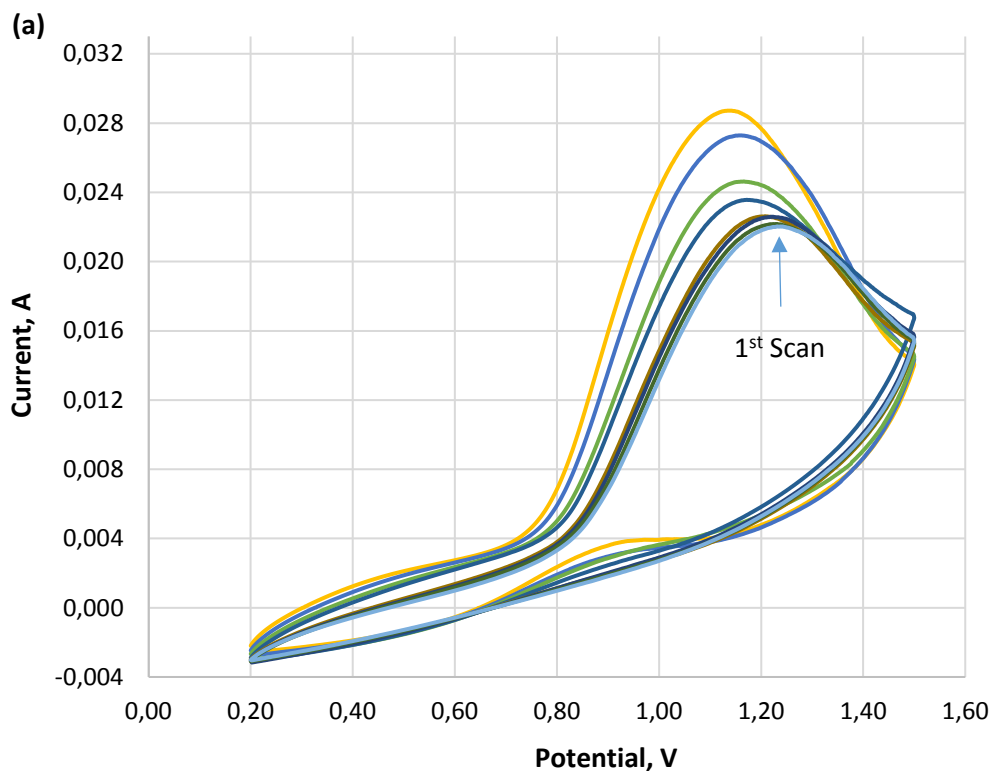


Figure 4.1. (a) Polymerization of EDOT monomer, (b) polymerization of hydroxymethyl-EDOT by graphite working electrode against Ag/AgCl with cyclic voltametry, pH = 7.0, 100 mV/min scan rate, first 8 scans of polymerization.

## 4.2. SEM ANALYSIS

Bare platinum electrode and electrodes covered by polymer with entrapped PPO enzyme were prepared. SEM images of the electrode surfaces were taken at 500 and 5000 magnification. As it is seen in Figure 4.2 (b), electrode surface covered with PEDOT/PPO has a uniform view of conducting polymer coating. Surface morphology shows that homogeneous immobilization of enzyme molecules is successfully carried out. (Figure 4.2 (c)). 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 vibrational motions under the effect of electron beam. Same process above was followed for also hydroxymethyl-PEDOT polymer and SEM images are shown on Figure 4.3.
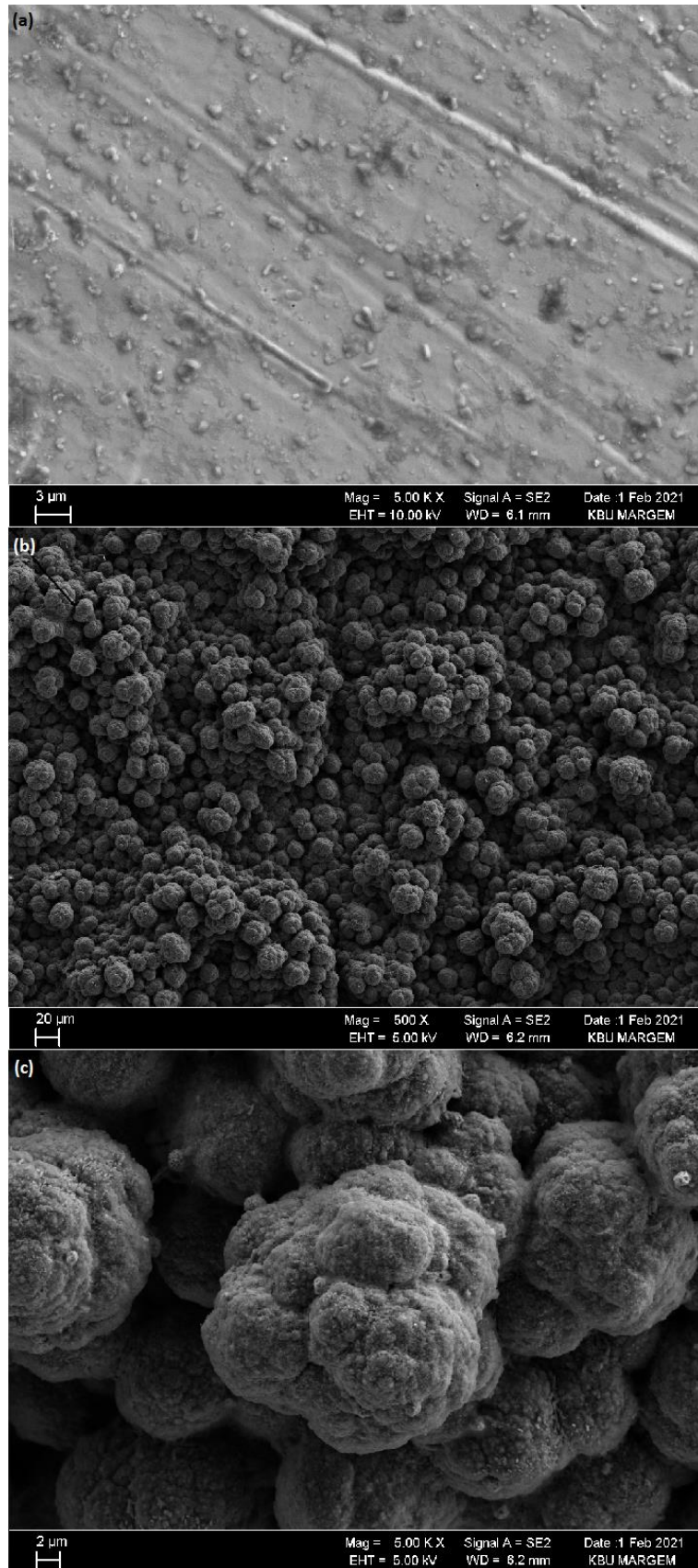
Figure 4.2. SEM images: a) Bare Pt (x5000), b) PEDOT/PPO (x500), c) PEDOT/PPO (x5000).
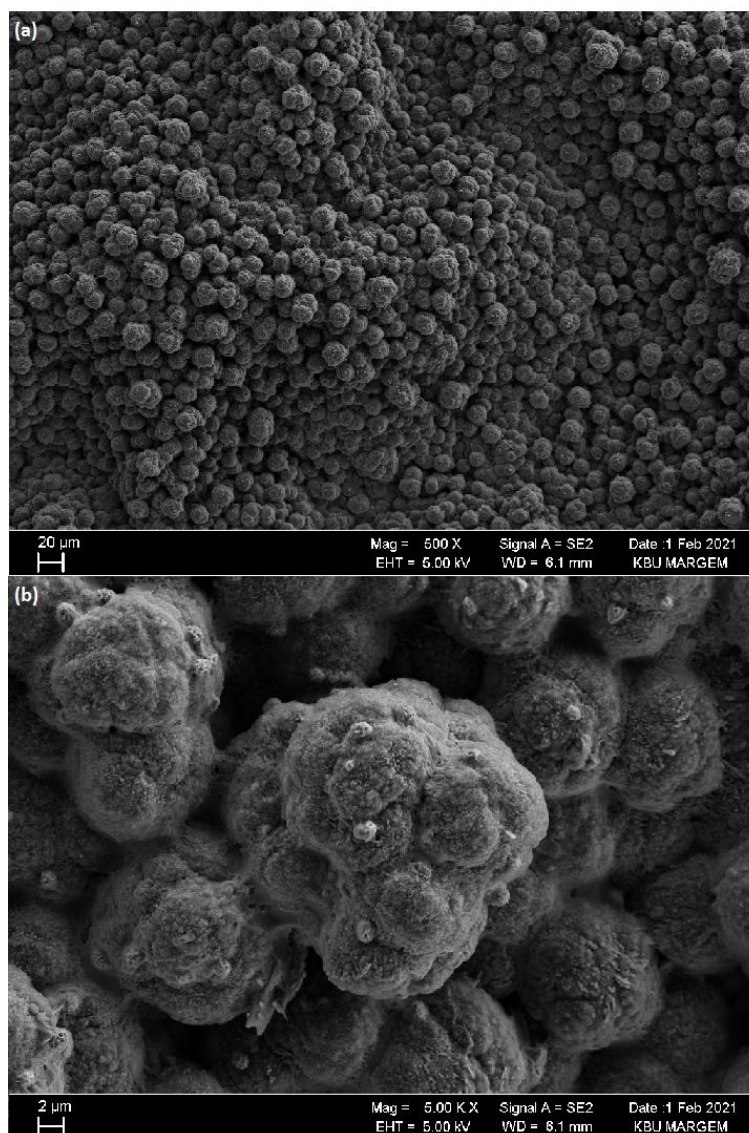
Figure 4.3. SEM images: a) MeOH-PEDOT/PPO (x500), b) MeOH-PEDOT/PPO (x5000).

## 4.3. OPTIMIZATION OF ENZYME ELECTRODES PREPARATION

Optimizations of Pt/PEDOT/PPO electrodes preparation step 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, reaction rate was obtained and enzyme activities were calculated. Results of optimizations of Pt/PEDOT/PPO were used for also Pt/MeOH-PEDOT/PPO electrodes.

**4.3.1. Effect of Dopant System**

Pt/PEDOT/PPO enzyme electrodes were prepared with each dopant system: 4 different electrodes for 4 dopant systems. 0.1 M catechol solutions in phosphate buffer (0.1 M, pH 7.00) were used. Stability evaluation was performed for each electrode and Figure 4.4 shows the graphics.
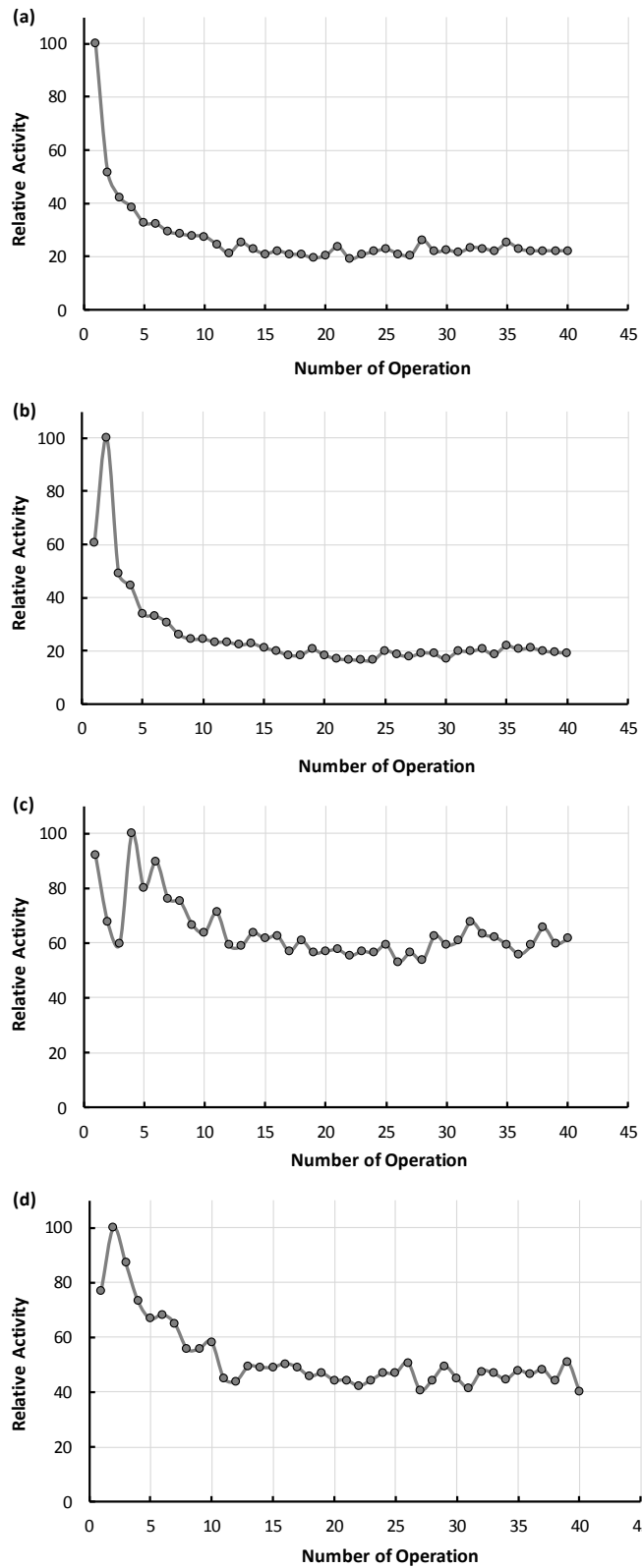
Figure 4.4. Stability graphics of Pt/PEDOT/PPO electrodes prepared with following dopant systems: (a) PEG (b) SDS (c) SDS / LiClO$_4$ (d) SDS / KNO$_3$.

SDS / LiClO$_4$ dopant system was selected since it shows higher signals than other dopants. This result shows that LiClO$_4$ makes the polymer to immobilize more enzyme relative to the other dopant systems. When this dopant used together with SDS during polymerization, the obtained polymer has most probably a more regular and packed structure which entraps more enzyme and makes the signal higher. Therefore, this dopant couple was used throughout the study.

### 4.3.2. Effect of Enzyme Extract Amount
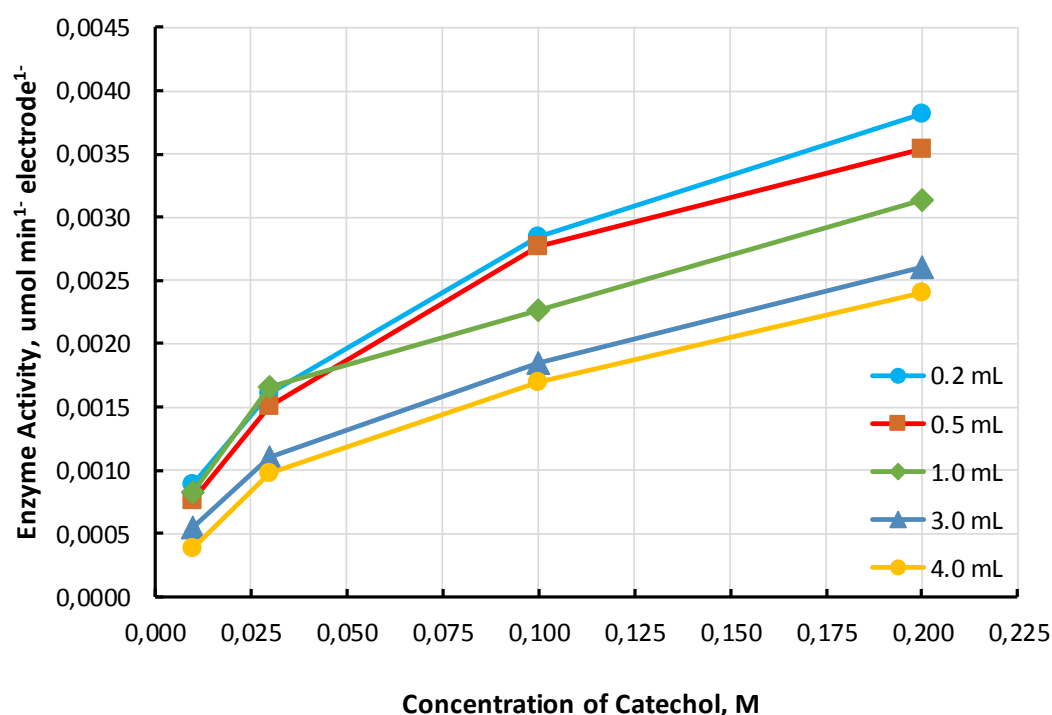


Figure 4.5. Effect of enzyme amount on Pt/PEDOT/PPO electrode activity.

Enzyme extract was prepared as explained in Section 3.3.1. Immobilization of PPO was performed with 5 different electrodes at 5 different amounts of enzyme extract and enzyme activities of electrodes were examined. 5 electrodes composed of polymer/PPO coated platinum plates were prepared by addition of 0.2, 0.5, 1.0, 3.0, 4.0 mL of enzyme extract to polymerization solution. Relative enzyme activities taken from electrodes are seen in Figure 4.5. Although it seems that maximum enzyme activity was observed in 1$^{st}$ electrode, the signal obtained from electrodes are actually quite close to each other. Therefore, 0.2 mL enzyme extract was selected and used in

other parts of this study. It was expected an increase in maximum enzyme activity while entrapped enzyme amount increases. This increase was actually not observed and activities of the electrodes were very close to each other. This situation indicates that the total amount of immobilized enzyme did not interact with substrate. Enzyme molecules placed at the surface performed the catalyzer function, however, enzyme remaining below the surface layer does not perform its function and substrate molecules can not reach inside. Although immobilized enzyme amount increases, it does not make any increase in enzyme activity.

### 4.3.3. Effect of Polymerization Cycle Number
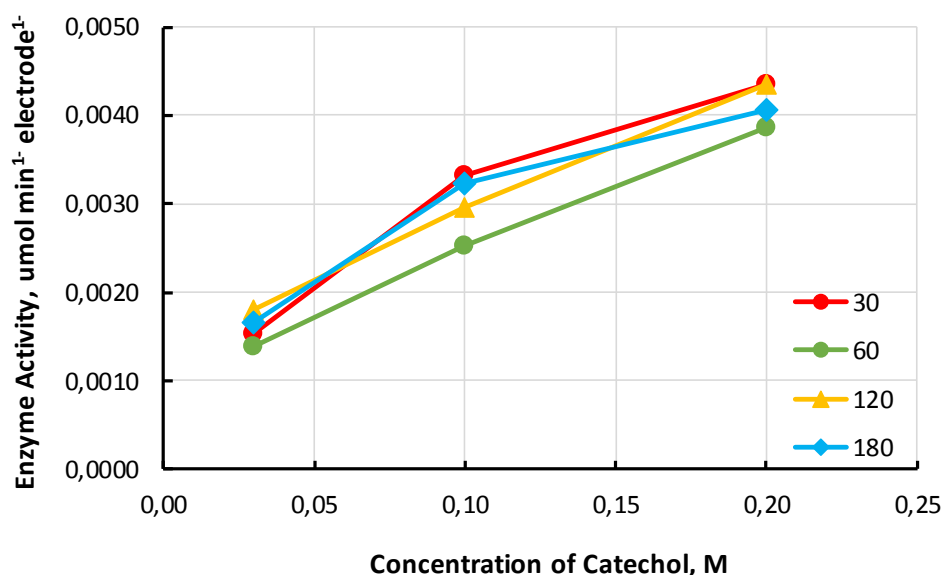


Figure 4.6. Effect of polymerization cycle number on Pt/PEDOT/PPO electrode activity.

The enzyme activities obtained from polymer/PPO electrodes produced with different number of cycle of polymerization are close to each other thus there is no significant difference between them (Figure 4.6). The discussion which is performed in Section 4.3.2 is also valid for this section. Although the polymerization thus enzyme immobilization takes more time with increasing number of polymerization cycle, the signal does not increase since only enzymes that exist on the electrode surface interact with the substrate molecules. However, the enzyme placed in a deeper position in the polymer matrix can not meet with its substrate.

## 4.4. OPTIMIZATION OF ACTIVITY MEASUREMENTS

### 4.4.1. Effect of pH on Enzyme Electrodes

Behaviour of immobilized polyphenol oxidase against pH change was examined in this study. Measurements were made with 1.0 M catechole solutions prepared in 0.1 M phosphate buffer at 25 ℃. Buffer pH was set to pH values between 3.0 and 10.0 and electrode activity were measured for each pH. In a previous study, optimum pH of free enzyme has been found as 5.0 [61]. In this study, the optimum pH for PPO immobilized to PEDOT polymer on platinum electrode was determined as 7.0. As it is seen in Figure 4.7, although maximum activity was observed at pH 7.0, the activity observed at pH 6.5, 7.0 and 7.5 are almost same and three pH's can be used. In case PEDOT and MeOH-PEDOT are used as matrices, it was observed that pH value at which the enzyme shows maximum activity increases compared 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, PEDOT and MeOH-PEDOT matrices contribute to protecting pH of the micro-environment that the enzyme is within up to a certain point. Thus pH 7.0 was used in optimizations, characterization, and sample analysis in this study. For the MeOH-PEDOT, however maximum enzyme activity was observed at pH 8.0, 7.5 and 9.0 can also be used since activity observed with these three pH's are very close to each other. MeOH-PEDOT matrix shows a higher optimum pH value than PEDOT which indicates that MeOH-PEDOT is a more protecting matrix for entrapped enzyme inside. It protects interior pH (pH 5.0) of microenvironment that enzyme was placed until the exterior pH changes to 8.0. In kinetic characterizations of this matrix pH 7.0 used although its optimum pH is 8.0, however, for kinetic parameters, corrections were made using the percent difference in enzyme activities at pH 7.0 and 8.0 which is 38%.
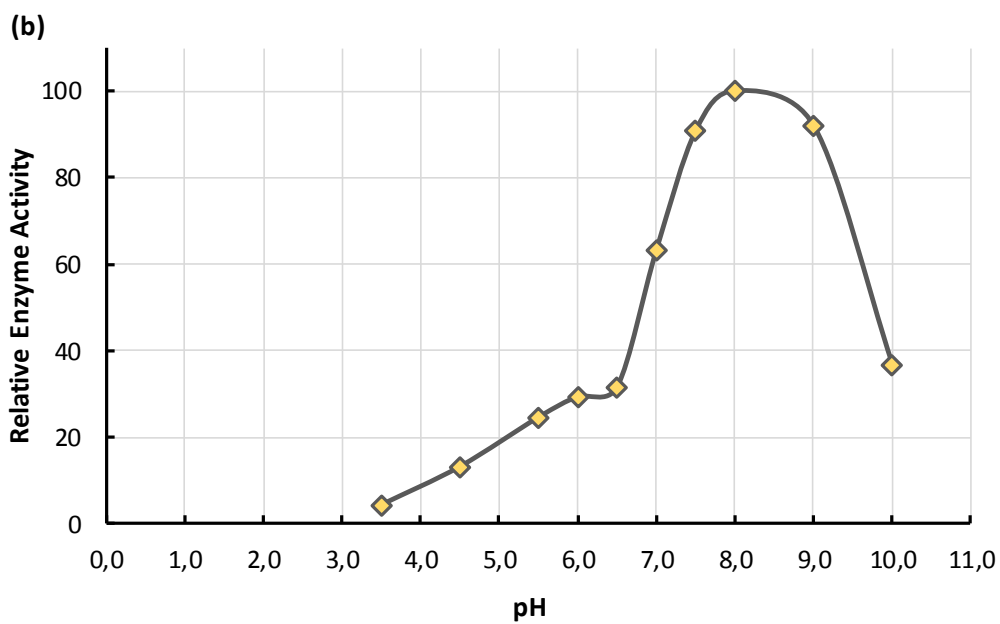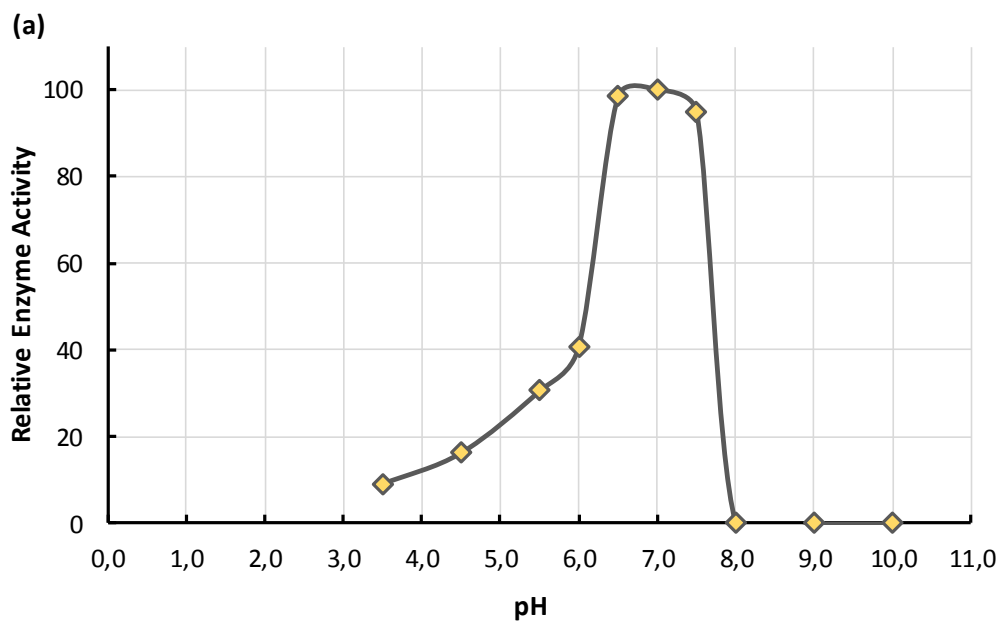
Figure 4.7. Effect of pH on (a) Pt/PEDOT/PPO electrode activity (b) Pt/MeOH-
PEDOT/PPO electrode activity.

## 4.4.2. Effect of Temperature on Enzyme Electrodes

The behaviour of enzyme that was immobilized to PEDOT and hydroxymethyl-
PEDOT polymer matrices was examined against temperature change in this study.
Measurements were made with 1.0 M catechole solutions prepared in pH 7.00
phosphate buffer. The temperature was set to values between 4 °C and 80 °C and

electrode activity measurements were made with 10 °C interval. The effect of temperature change over the activity of Pt/PEDOT/PPO and Pt/MeOH-PEDOT/PPO electrodes is given in Figure 4.8.
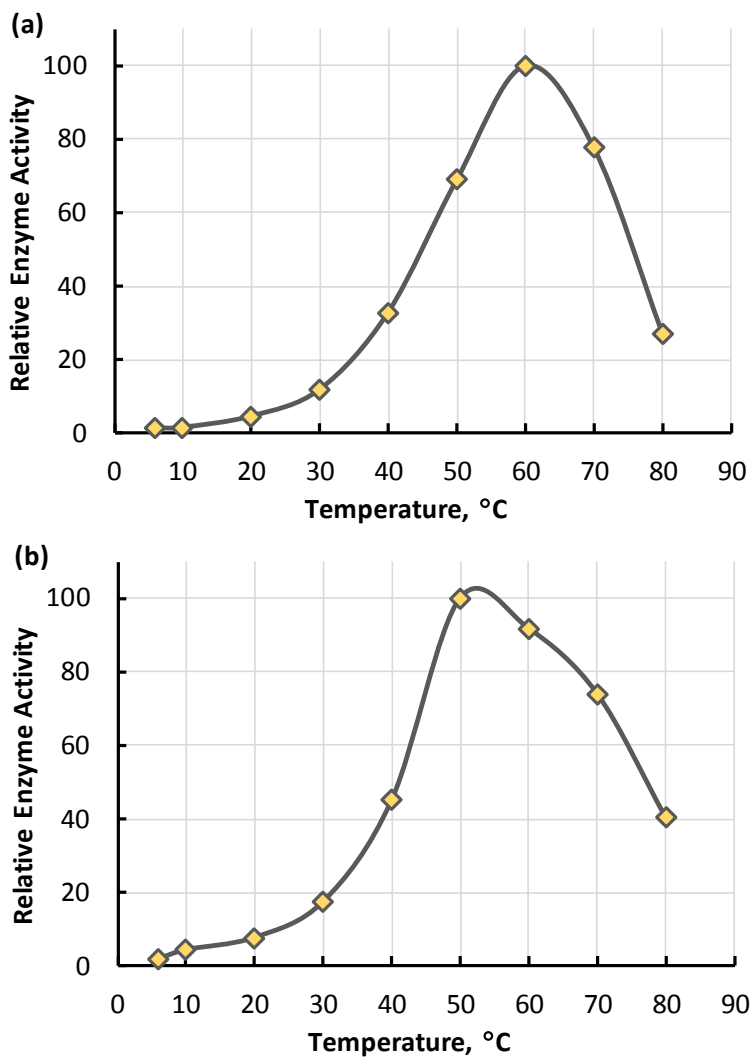


Figure 4.8. Effect of temperature on (a) Pt/PEDOT/PPO electrode activity (b) Pt/MeOH-PEDOT/PPO electrode activity.

Maximum activity of immobilized enzyme is at 60 °C and 50 °C for two polymers. Enzyme activities between 50 °C and 70 °C are close to each other and enzyme electrode can be used in this interval with around 70% activity or above. And for the free PPO, optimum temperature was indicated as 40 °C in previous studies [61]. 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. These changes may affect 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 4.8, PPO activity in the polymer matrix decreases after 60 ℃ 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 occuring in enzyme structure. At the temperatures higher than 60 ℃, 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.

## 4.5. DETERMINATION OF KINETIC PARAMETERS

Platinum electrodes were covered with PEDOT and hydroxymethyl-PEDOT 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 graphics were plotted for enzyme electrodes and from Michaelis-Menten graphics, Lineweaver-Burk plots were obtained (Figure 4.9 and 4.10). 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 calculated and shown on Table 4.1.
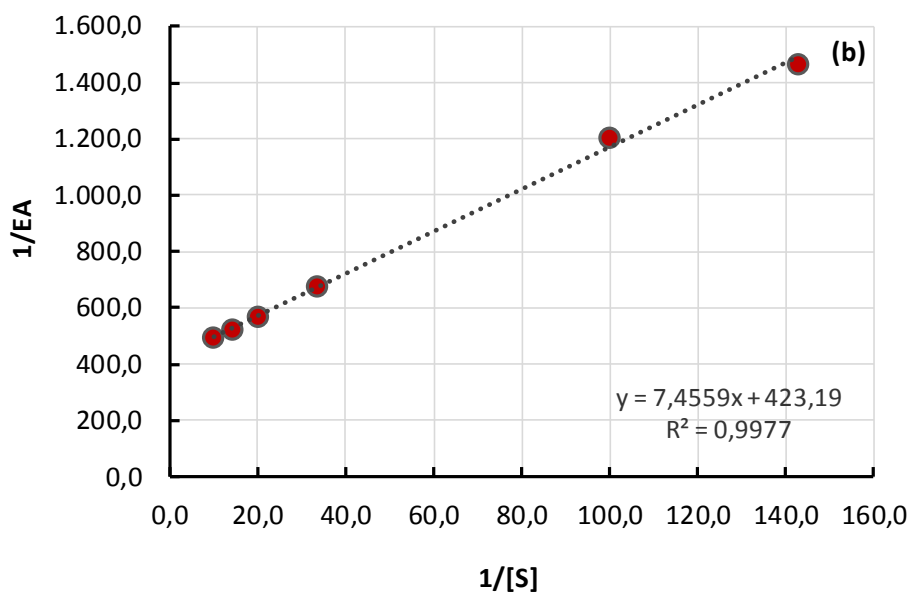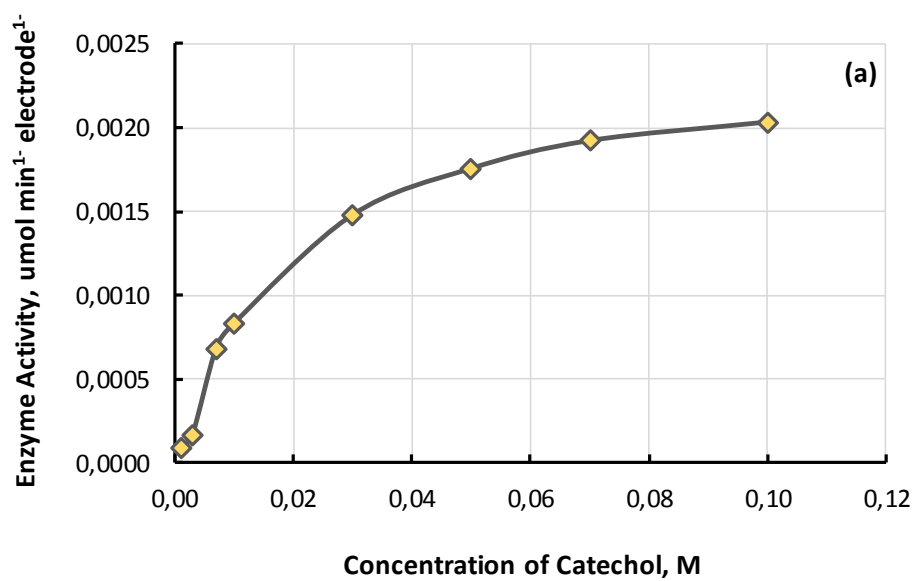
Figure 4.9. Pt/PEDOT/PPO electrode, (a) Michaelis-Menten graphic (b) Lineweaver-
Burk plot.
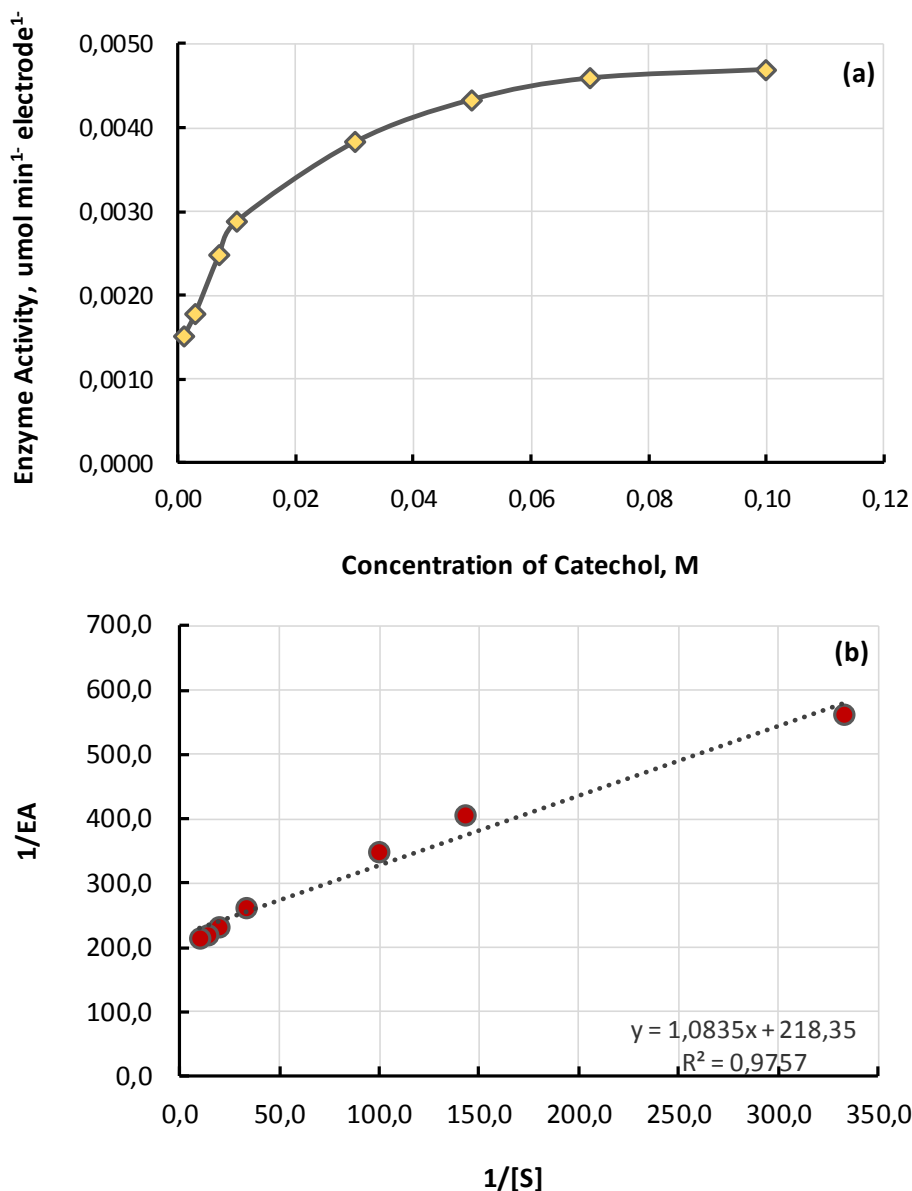
Figure 4.10. Pt/MeOH-PEDOT/PPO electrode, (a) Michaelis-Menten graphic (b) Lineweaver-Burk plot.

In Table 4.1, it is given the data for free PPO and kinetic parameters obtained in this study for the aim of comparison. As it is seen in the Table 4.1, free enzyme's $V_{max}$ value is considerably higher than immobilized enzyme's $V_{max}$ values and this finding is already 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 PEDOT and hydroxymethyl-PEDOT matrices are compared, it is seen that $V_{max}$ obtained from Pt/PEDOT/PPO electrode is almost double of $V_{max}$ obtained from Pt/MeOH-PEDOT/PPO. In these two matrices, enzymes show different activity which indicates that amount of enzyme entrapped in MeOH-PEDOT must be in double amount of enzyme entrapped in PEDOT. This might be a result of some extra attractive forces between MeOH-PEDOT and enzyme molecules since each unit of MeOH-PEDOT has an extra -OH group which can interact with enzyme molecules. Another parameter that can cause higher enzyme immobilization might be more porous structure of MeOH-PEDOT resulted from hydroxymethyl branches. Monomers possessing branching structure create a polymer in which the chains are separated from each other relative to more packed structure of PEDOT. Cavities resulting from this porosity provides more microenvironment for enzyme molecules to be immobilized.

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

|  | $V_{max}$ | $K_m$ |
|---|---|---|
| Free PPO [40] | 0.073 μmol min$^{1-}$ mL$^{1-}$ | 4 mM |
| Pt/PEDOT/PPO | 0.00236 μmol min$^{1-}$ electrode$^{1-}$ | 17.722 mM |
| Pt/MeOH-PEDOT/PPO | 0.00458 μmol min$^{1-}$ electrode$^{1-}$ | 4.962 mM |

$K_m$ value to be lower in Pt/MeOH-PEDOT/PPO electrode compared to Pt/PEDOT/PPO shows that in hydroxymethyl-PEDOT matrix environment, the affinity between PPO and its substrate is higher. This interest shows that PPO enzyme accepts the substrate easier in the microenvironment of the hydroxymethyl-PEDOT matrix and this points a higher interaction between hydroxymethyl-PEDOT and substrate which means probably some attractive forces between them. This interest is considerably less in PEDOT matrix and $K_m$ is much higher. PEDOT reduces enzyme-substrate interaction and this implies that there may be repelling forces between PEDOT and substrate.

## 4.6. STABILITY STUDIES

### 4.6.1. Operational Stability of Enzyme Electrodes

Operational stability of enzyme electrodes was determined by 40 measurements made one after another with Pt/PEDOT/PPO and Pt/MeOH-PEDOT/PPO electrodes prepared by immobilization of polyphenol oxidase using optimum enzyme amount. The measurements were made at 25 ℃, pH 7.00 and 1.0 M catechol concentration. After 40 measurements, it was seen that the remaining relative enzyme activity was around 60% and 75% for Pt/PEDOT/PPO and Pt/MeOH-PEDOT/PPO, respectively. Free enzyme can only be used one time but immobilized enzyme can be used multiple times as it is seen in Figure 4.11.
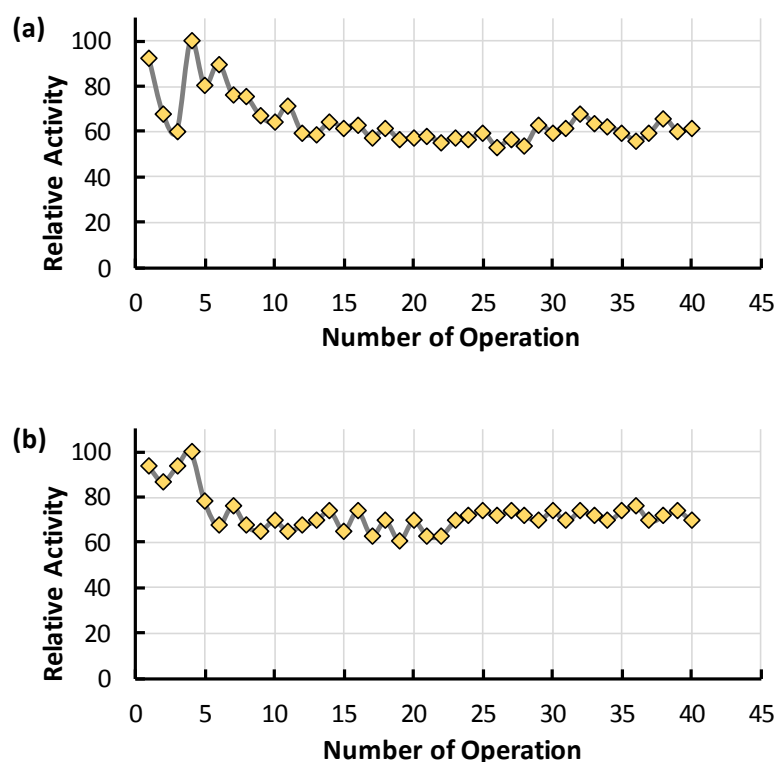


Figure 4.11. Operational stability (a) Pt/PEDOT/PPO (b) Pt/MeOH-PEDOT/PPO.

## 4.6.2. Shelf Life of Enzyme Electrodes

In order to determine how much of their activity of enzyme electrodes lost in how long a period, activity determination was made for a period of 50 days. The measurements were made at 25 ℃, pH 7.00 and 1.0 M catechol concentration.

**(a)**



**(b)**
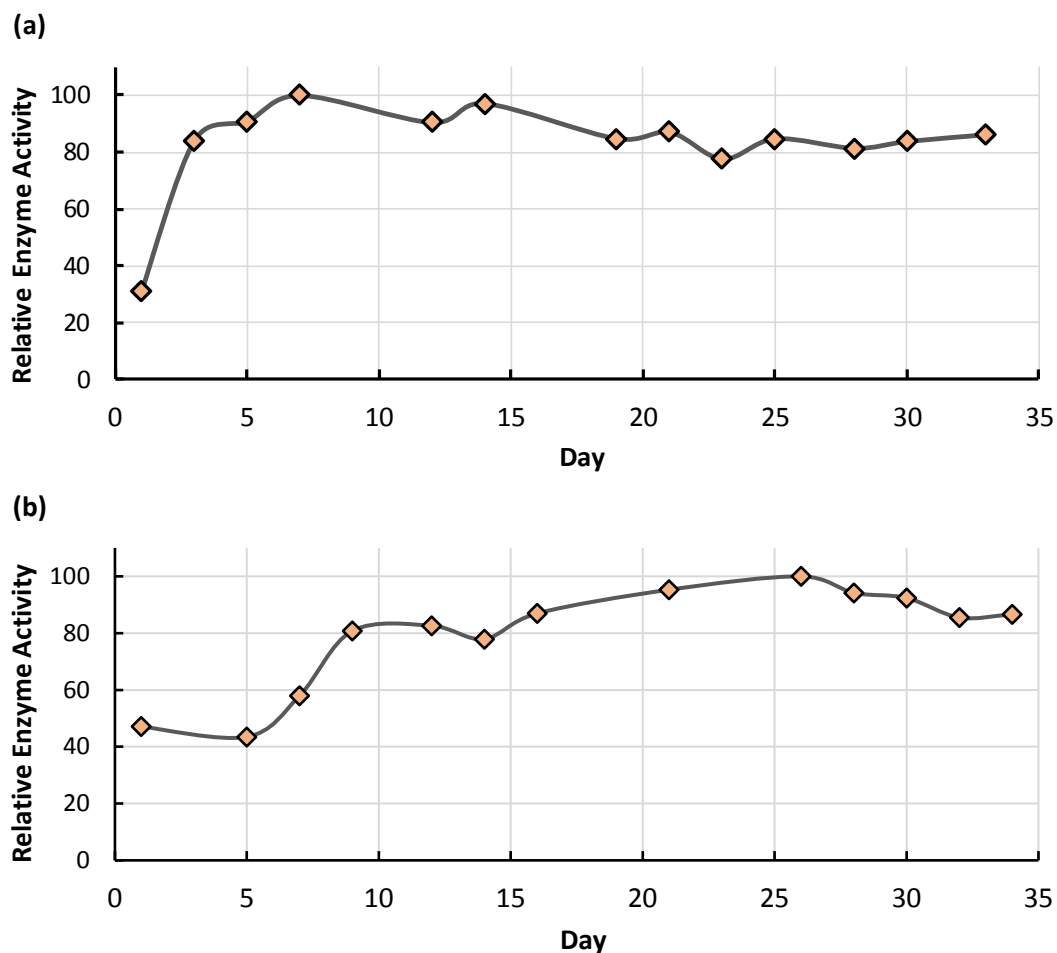


Figure 4.12. Shelf Life of  a) Pt/PEDOT/PPO b) Pt/MeOH-PEDOT/PPO.

Enzyme electrodes lost 30% of their initial activities at the end of 50 days and the remaining activity was determined as 70% as seen on Figure 4.12. This fall in enzyme activities may result from desorption of the enzyme in time. 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 at immobilized enzyme.

## 4.7. ANALYSIS OF PEACH EXTRACT SAMPLES

Polyphenolic material amount in peach extracts was analysed. Total polyphenol content that was determined by using Pt/PEDOT/PPO and Pt/MeOH-PEDOT/PPO enzyme electrodes and by Folin-Ciocalteau method is given in Table 4.2 as mg mL$^{1-}$ catechol equivalent.

### 4.7.1. Determination of Polyphenolics with Enzyme Electrodes

For the calibration curve, 6 catechol solutions between 1.0 and 110.0 mg mL$^{1-}$ were prepared in phosphate buffer (0.1 M, pH 7.00). The electrode was dipped to each solution for 15 minutes and the absorbance of solutions colored with Besthorn's Hydrazone method were measured and calibration curves seen in Figure 4.13 were obtained. 15 blank measurements were made and LOD's for PEDOT and MeOH-PEDOT were calculated as 1.241 mg mL$^{1-}$ and 1.038 mg mL$^{1-}$ by using standard deviation of 15 blank measurements ($\sigma$) and calibration curve slope (s) (LOD = 3.3$\sigma$/s).
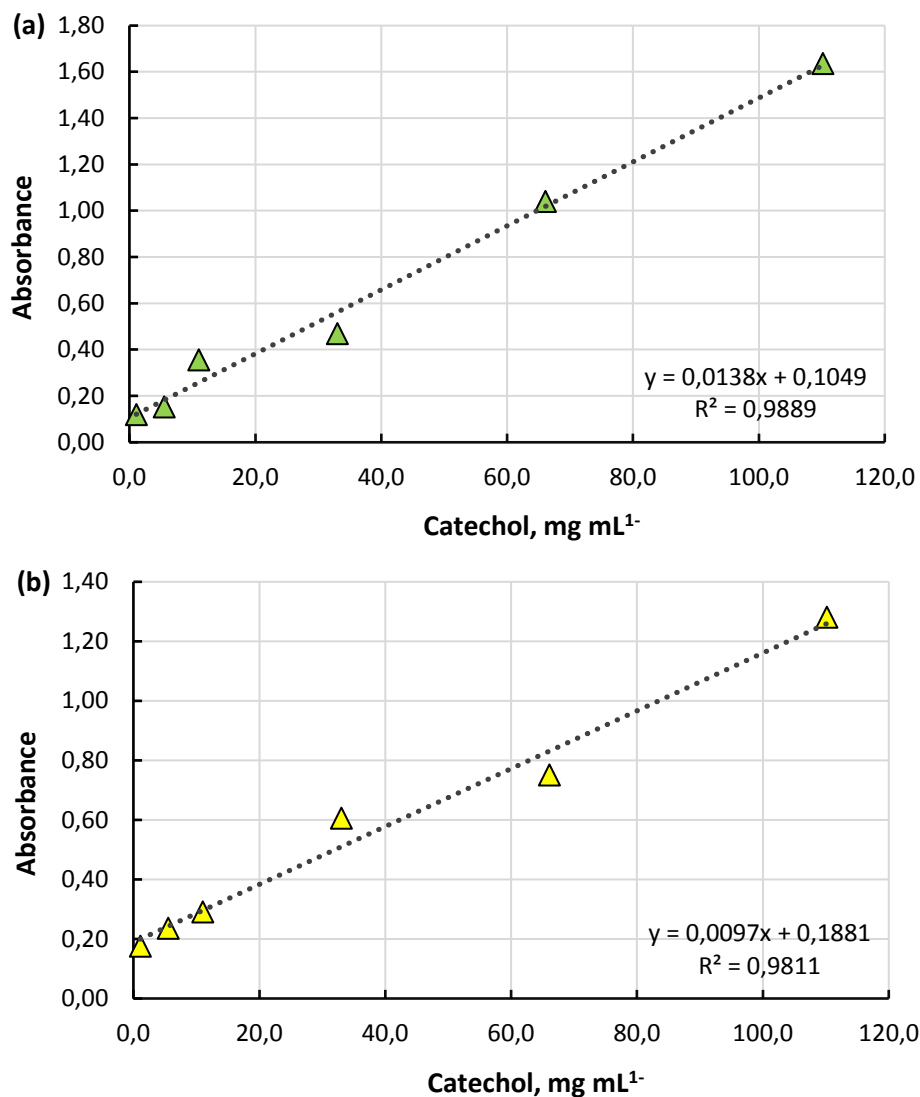
Figure 4.13. Calibration graph obtained by (a) Pt/PEDOT/PPO and (b) Pt/MeOH-PEDOT/PPO electrodes.

**4.7.2. Determination of Polyphenolics with Folin-Ciocalteau Method**

In order to confirm polyphenolic material determination made by enzyme electrodes, Folin-Ciocalteau analysis method was used as the control method. The calibration graph prepared by Folin-Ciocalteau method is seen in Figure 4.14.
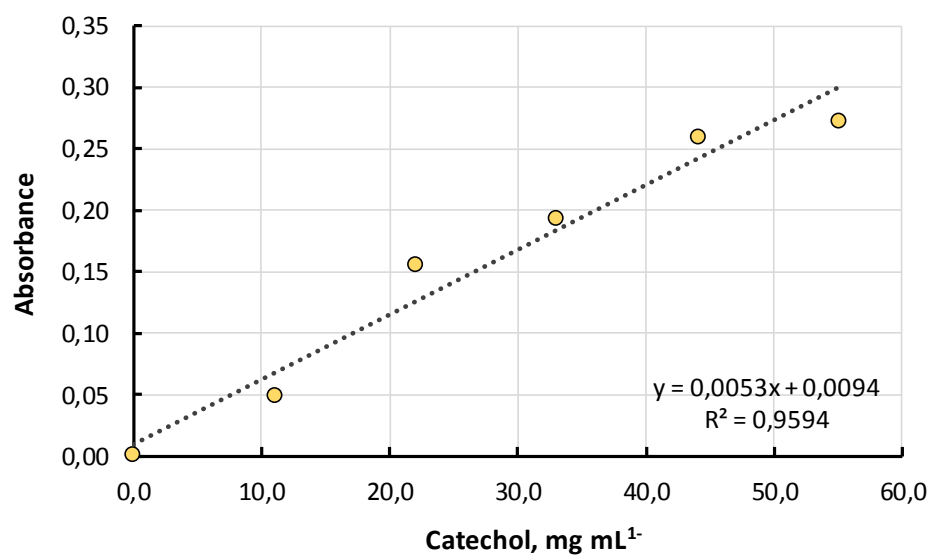
Figure 4.14. Calibration graph prepared by Folin-Ciocalteau method.

Table 4.2. Total polyphenolic material amounts determined as mg mL$^{1-}$ catechol equivalent in peach extract with Pt/MeOH-PEDOT/PPO and Pt/MeOH-PEDOT/PPO enzyme electrodes and Folin-Ciocalteau method.

| Method | Total Polyphenol Amount in Peach Extract (mg mL$^{1-}$) |
|---|---|
| Pt/PEDOT/PPO | $69.43 \pm 2.41$ |
| Pt/MeOH-PEDOT/PPO | $92.62 \pm 3.43$ |
| Folin-Ciocalteau | $84.45 \pm 5.21$ |

# PART 5

## CONCLUSION

PEDOT and hydroxymethyl-PEDOT polymers were coated over a platinum electrode and new enzyme electrodes were produced that are not found in literature. The formation of polymerization was shown by cyclic voltammetry. Kinetic parameters of immobilized polyphenol oxidase enzyme were determined. The effect of reaction conditions over enzyme activity were examined. Operational stability and shelf-life of electrodes were also determined. Calibration graph was plotted. LOD values and the working intervals were obtained. In total phenolic material analysis, the results found for peach extracts have been confirmed by Folin-Ciocalteau method.

Comparison the performances of two electrodes points that Pt/MeOH-PEDOT/PPO is superior to Pt/PEDOT/PPO with respect to kinetic parameters and limit of detection. $V_{max}$, maximum reaction rate of Pt/MeOH-PEDOT/PPO electrode has a value double of Pt/PEDOT/PPO electrode, which indicates that in MeOH-PEDOT environment, enzyme can increase the reaction rate more easily than PEDOT matrix. Moreover, $K_m$ is much smaller in Pt/MeOH-PEDOT/PPO electrode, which means that affinity between enzyme and substrate is much stronger in MeOH-PEDOT matrix. Furthermore, detection limit obtained from Pt/MeOH-PEDOT/PPO electrode is lower than Pt/PEDOT/PPO. However, optimizations exhibit that Pt/PEDOT/PPO electrode works better in milder pH's and temperatures.

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**RESUME**

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