# ENZYME IMMOBILIZATION ONTO CARBON FIBER ELECTRODES BY ELECTROCHEMICAL POLYMERIZATION

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**BY**

**Hana B. Ashour ALSOUL**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR**
**THE DEGREE OF MASTER OF SCIENCE IN**
**DEPARTMENT OF**
**CHEMISTRY**

**March 2018**

I certify that in my opinion the thesis submitted by Hana B. Ashour ALSOUL titled "ENZYME IMMOBILIZATION ONTO CARBON FIBER ELECTRODES BY ELECTROCHEMICAL POLYMERIZATION" 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 a unanimous vote in the Department of Chemistry as a master thesis. March 23, 2018

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Hana B. Ashour ALSOUL

## ABSTRACT

**M. Sc. Thesis**

**ENZYME IMMOBILIZATION ONTO CARBON FIBER ELECTRODES BY ELECTROCHEMICAL POLYMERIZATION**

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In this study, a new electrode material was used and invertase enzyme electrodes were prepared by using carbon fiber as electrode material. Carbon fiber enzyme electrodes were obtained by immobilization of invertase in the conductive polypyrrole polymer matrix during electrochemical polymerization onto laboratory–made carbon fiber electrodes. Maximum reaction rate, ($V_{max}$) and substrate affinity, ($K_m$), of immobilized enzyme were determined. The influence of conditions on enzyme activity was reviewed. The optimum temperature, optimum pH value, linear working range and daily stability of immobilized enzyme were determined. The results were compared with the previous study in which invertase was placed in polypyrrole coated platinum electrodes.

**Key Words :** Electrochemical polymerization, polypyrrole, conducting polymer, enzyme immobilization, invertase, enzyme electrode, carbon fiber electrode.

**Science Code :** 201.1.041

# ÖZET

**Yüksek Lisans Tezi**

**ELEKTROKİMYASAL POLİMERİZASYONLA KARBON FİBER ELEKTROTLARA ENZİM TUTUKLAMASI**

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**Karabük Üniversitesi**
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**Tez Danışmanı:**
**Yrd. Doç. Dr. A. Elif BÖYÜKBAYRAM**
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Bu çalışmada yeni bir elektrot malzemesi kullanıldı ve invertaz enzim elektrotları elektrot malzemesi olarak karbon fiber kullanılarak hazırlandı. Karbon fiber enzim elektrotları laboratuvarda hazırlanan karbon fiber elektrotlar üzerine elektrokimyasal polipirol polimerizasyonu esnasında invertazın tutuklanmasıyla elde edildi. Tutuklanan enzimin kinetik parametreleri, $V_{max}$ (maksimum reaksiyon hızı) ve $K_m$ (substratın enzim ilgisi) elde edildi. Reaksiyon koşullarının enzim aktivitesine etkisi incelendi. Tutuklanmış enzimin optimum sıcaklığı ve pH'ı, doğrusal çalışma aralığı ve ölçümlerin ardışık stabilitesi saptandı. Sonuçlar daha önceden yapılmış çalışmalarda bulunan serbest enzim ve platin elektrotta ve polipirolde tutuklanmış enzim çalışmalarıyla karşılaştırıldı.

**Anahtar Kelimeler :** Elektrokimyasal polimerizasyon, polipirol, iletken polimer, enzim tutuklaması, invertaz, enzim elektrodu, karbon fiber elektrot.

**Bilim Kodu :** 201.1.041

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## ABBREVIATIONS

PPy : Polypyrrole

SDS : Sodium dodecylsulfate

SEM : Scanning Electrone Microscopy

CP : Conducting Polymer

$V_{max}$ : Maximum reaction rate

$K_m$ : Michaelis-Menten constant

CF : Carbon Fiber

# CHAPTER I

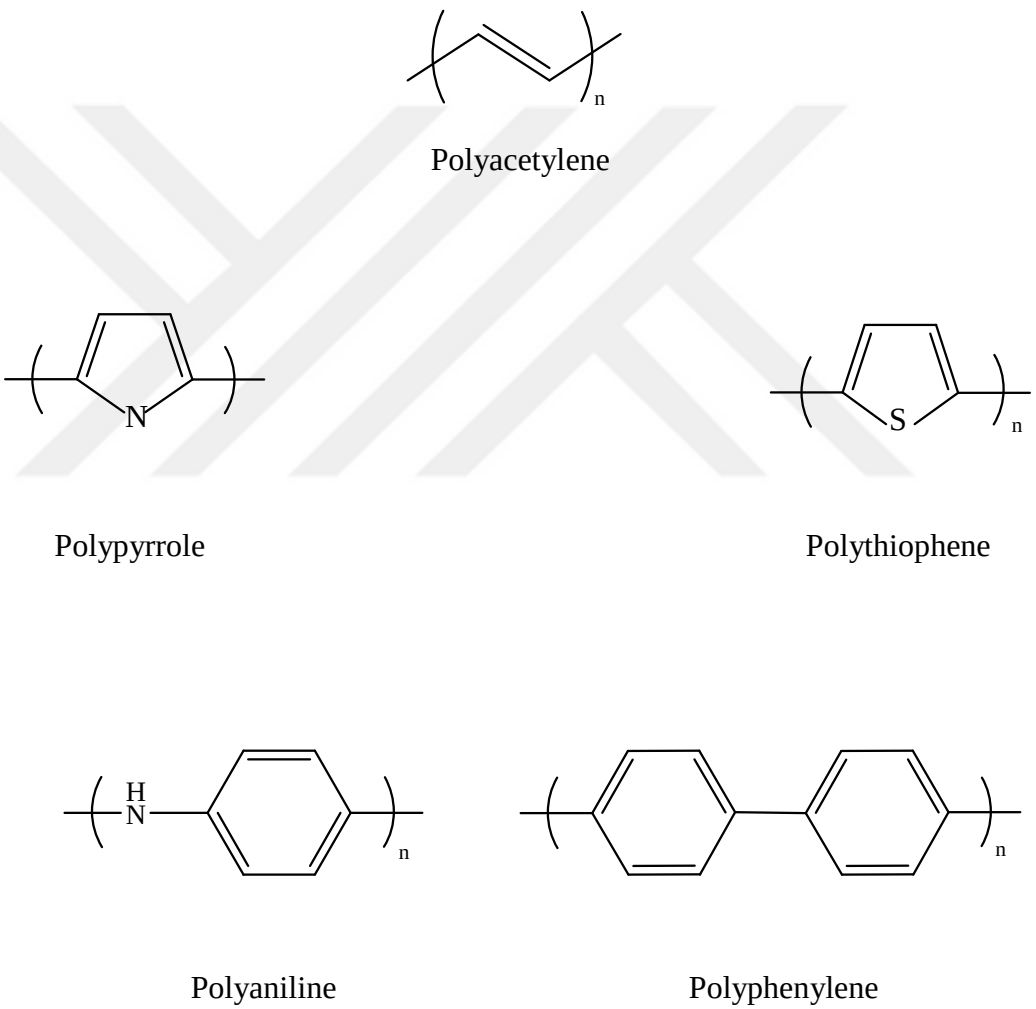
# INTRODUCTION AND LITERATURE SURVEY – SCOPE AND SUBJECT

## 1.1 Conductive Polymers

Non-conductive forms of polymers were popular before discovering other features of interest of polymers and their productivity [1]. The synthesized polyaniline by oxidation of aniline in the medium of sulphuric acid was the primary test for polymer conducted by H. Letheby in 1862 [2], then by Mohilner et al in 1962 [3]. Pyrrole black [4] had been known as a form of a conductive polymer through spontaneous polymerization in air in one side, and via pyrrole containers on other side as details and studies were given in 1916 of chemical polymerization [5]. Natta & his co-workers developed the system regarding polymerization of acetylene by using the Ziegler-type catalyst in 1958 that enabled Natta and Ziegler to win the Nobel Prize in Chemistry in 1966 [6]. In 1967 [7] the scholars conducted polymerization electrochemically by relying on furan, pyrrole and thiophene for electropolymerization as described by Dall Ollio in the early year of 1968 [8].

In spite of regular structure and highly crystalline form, their functionality was an air-sensitive a black, insoluble and infusible powder. So, this system had not been used correctly until 1970s when Shirakawa et al. incorporated polyacetylene as a film [9] when they were blowing the acetylene gas through the surface to get a catalyst solution. As a result, they came across on one hand that $10^{-3} - 10^{-2}\ s\,m^{-1}$ was value of conductivity of trans-polyacetylene, and on other hand $10^{-8} - 10^{-7}\ s\,m^{-1}$ was as well the value of cis-polyacetylene. They claimed also that the cis form may be converted to trans form by heating up to 170-200°C. Alan MacDiarmid & Alan Heeger carried out a study in 1975 by focusing on highly conductive polyacetylene to see the metallic properties in covalent inorganic polymer (SN)x. In cooperation with Shirakawa, the three scholars had discovered that the

oxidation with bromine, chlorine, and iodine vapors formed the film of polyacetylene 109 times more conducting than the original one that conducted to ‘plastic electronics [10]. For discovering and developing the electrically conductive system of polymers, they were awarded Nobel Prize in Chemistry at 2000. Today many studies have been performed for conducting polymers in order to improve their features. And they could be used contemporary in light emitting diodes, electrodes in transistors, and in electrochromic devices [11, 12, 13].




Polyacetylene

Polypyrrole

Polythiophene

Polyaniline

Polyphenylene

Figure 1.1. Some conductive polymers.

### 1.1.1 Conduction Mechanism of Conductive Polymers

To determine the electrical properties of a material is possible by investigating its electronic structure; this electronic and material structure can be explained by band theory. Orbits of each atom intersect in solid state with those adjacent atoms in all sides to get molecular orbits that put those particles together on the surface. Once these orbits come together, they get power ranges. The largest and highest one is called the conducting band, and the lowest one is called the valence band. The differentiation between the two ranges of energy called the power gap. Energy gap can be observed, and the conductivity range may be promoted via the electrons movement. In the nonconducting form, energy gap between the bands gets too large that it doesnt fit electrical conduction. Semiconductors have gaps narrow relatively. Metals have usually no band gap and two bands overlaps.

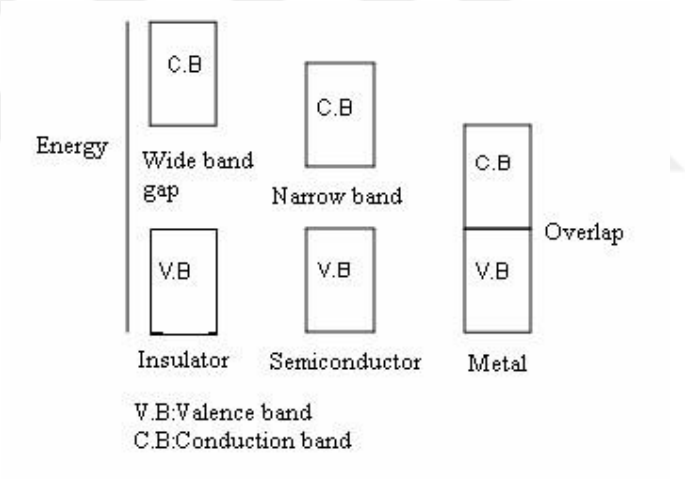



Figure 1.2. Simple band pictures of an insulator, a semiconductor, and a metal.

The electrical conduction of conducting polymers is difficult to explain by simple band theory because this theory is not detailed why the load carriers are not spins in polyacetylene and polypyrrole. In order to understand the electronic character of conducting polymers, polarons, solitons, and bipolar concepts were introduced in the 1980s [14]. Conductive polymers have poly-conjugated structures. They are insulators in the pristine state, and get conductive once they are put along with oxidizing or reducing agents. Hence, their conductivity can get better by doping.

Doping process is to add a polymer with a receptor molecule or with a donor, which was classified as n-type (reduction) and p-type (oxidation).

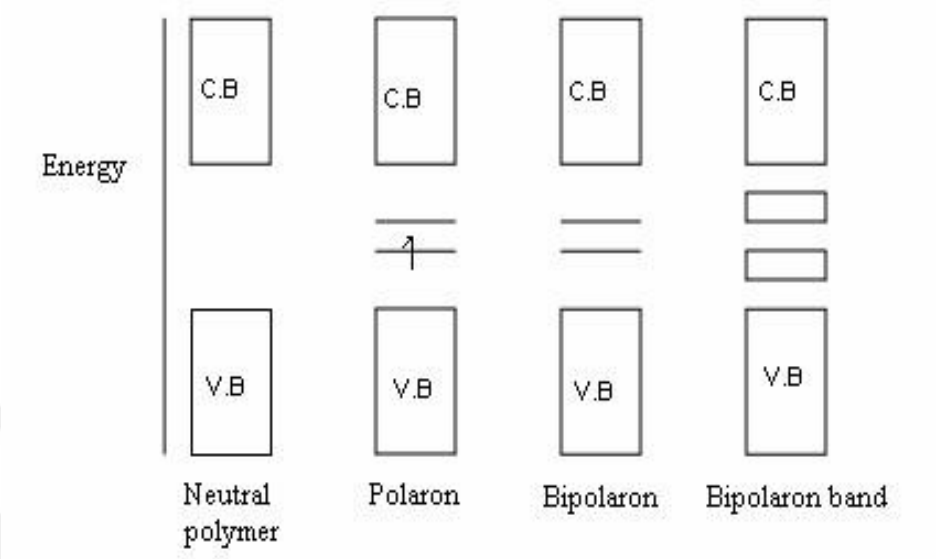



Figure 1.3. Band structures of neutral and doped polymer.

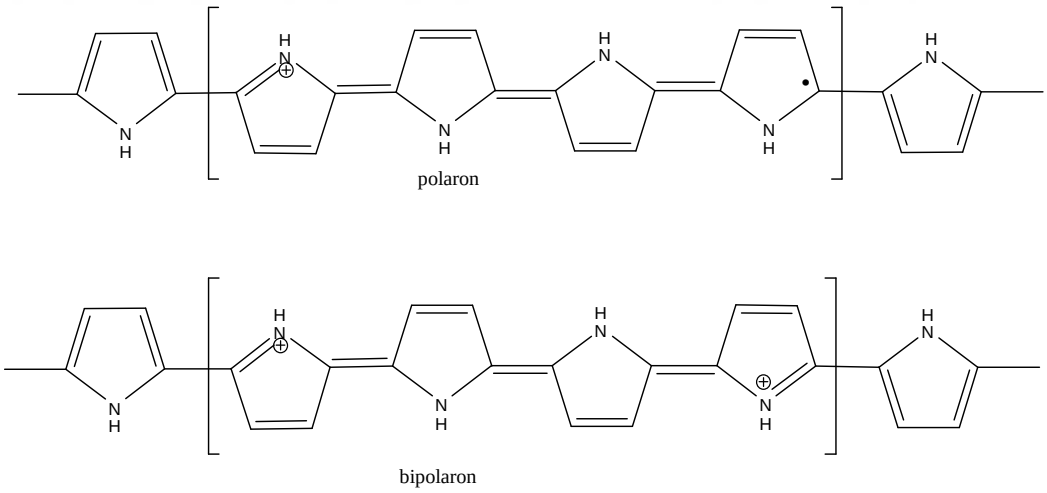



Figure 1.4. Structural schemes of polaron and bipolaron for polypyrrole

P-type doping is removing the electron from the valence band by the oxidizing agent thus the polymer has a positive charge. Unlike P-type doping, n-type doping is an electron attachment to the conducting band with a reducing agent. The first doping process for doping is to form an anion radical or a cation. While the second step for

electron transfer could be formed by putting dianion and dication. The higher doping levels produce bipolarons while the low doping levels lead to polarons. In the field of electricity, bipolarons and polarons may get toward the polymer chains by re-organising single and double bonds. The energy gap of the bipolar cells can be narrowed and overlapped bipolar bands in the band gap can be generated by increasing the level of doping (Figure 1.3). The higher conductivity of polymer is obtained with a higher level of doping. The structures of bipolaron and polaron for polypyrrole are given in detail in the following Figure 1.4.

### 1.1.2 Application Areas of Conductive Polymers

Studies on conducting polymers, has grown considerably in recent years. Conductive polymers used in combination with insulating polymers has found many applications in technology. Some of these are;

In rechargeable battery constructions: Conductive polymers are used as electrodes in rechargeable batteries due to reversible doping properties.

In pH sensors: The effect of pH on the conductivity of solutions of some conducting polymers has been investigated in a three-electrode system and it has been shown that this system can be used as a pH sensor.

In gas sensors: Since gases can exhibit strong reducing and oxidizing properties, they affect the conductivities of polymer films. Various properties of the conductive polymers have also been changed and gas sensor is made by using these properties.

In biosensors: Many of the sensors that use conductive polymers are both for chemical and biological purposes. Biological sensors are devices that are formed by interdisciplinary use of analytical chemistry, biochemistry and microelectronics science. These devices are useful for fuzzy biological fluids and have a simple appearance. In general, a biosensor is formed by the use of an appropriate energy delivery device with near contact to a biological component. The signals generated

by the analyzed solution and the biochemical reaction of the biological component are determined by converting it into an electrical signal at the detector [15].

In electronic devices: Electronic tools and devices such as diodes and transistors are also made using conductive polymers. In these devices, chemical signals that depend on the reduction and oxidation of the polymer can be read by converting it into an electrical signal.

In photo electrochemical cells: In recent years, solar energy conversion into chemical or electrical energy by photo electrochemical cells has been one of the interesting applications.

In electrochromic devices: Electrochromic devices are called reversible color-changing materials during electrochemical processing of charge and discharge. The oxidized structures of PAN (polyaniline) films, for example, are colored and have high conductivity. Conversely, the reduced structures are optically transparent and exhibit low conductivity.

In ion selective electrode fabrication: In addition to synthesizing conductive polymers on a variety of electrodes by electrochemical methods in various ways, they have been made into films on inert electrodes, allowing selective modification of various organic, inorganic and biological molecules and ions, making it possible to construct a large number of modified electrodes.

Corrosion inhibitor: Corrosion is an electrochemical phenomenon and is influenced by electric current. Corrosion can be reduced by providing a suitable environment when there is no current on the inner surfaces [16].

### 1.1.3 Electrochemical Polymerization

By the electrochemical oxidation, the obtained polymer of the interest as a stand-alone film on the electrode surface. Applying suitable potentials controls the oxidation of monomer and formation of cation radical. Two basic monomer cations

may be eliminated by coupling of the two protons, and they demoted a dimer in the step of proliferation. Since the potential for oxidation of a dimer is less than that of the monomer, polymerization through coupling of oxidized units of the monomer to the chain growing is promoted. Polymer formation on the electrode surface also increases the molecular weight. The electropolymerization of polypyrrole is as follows:

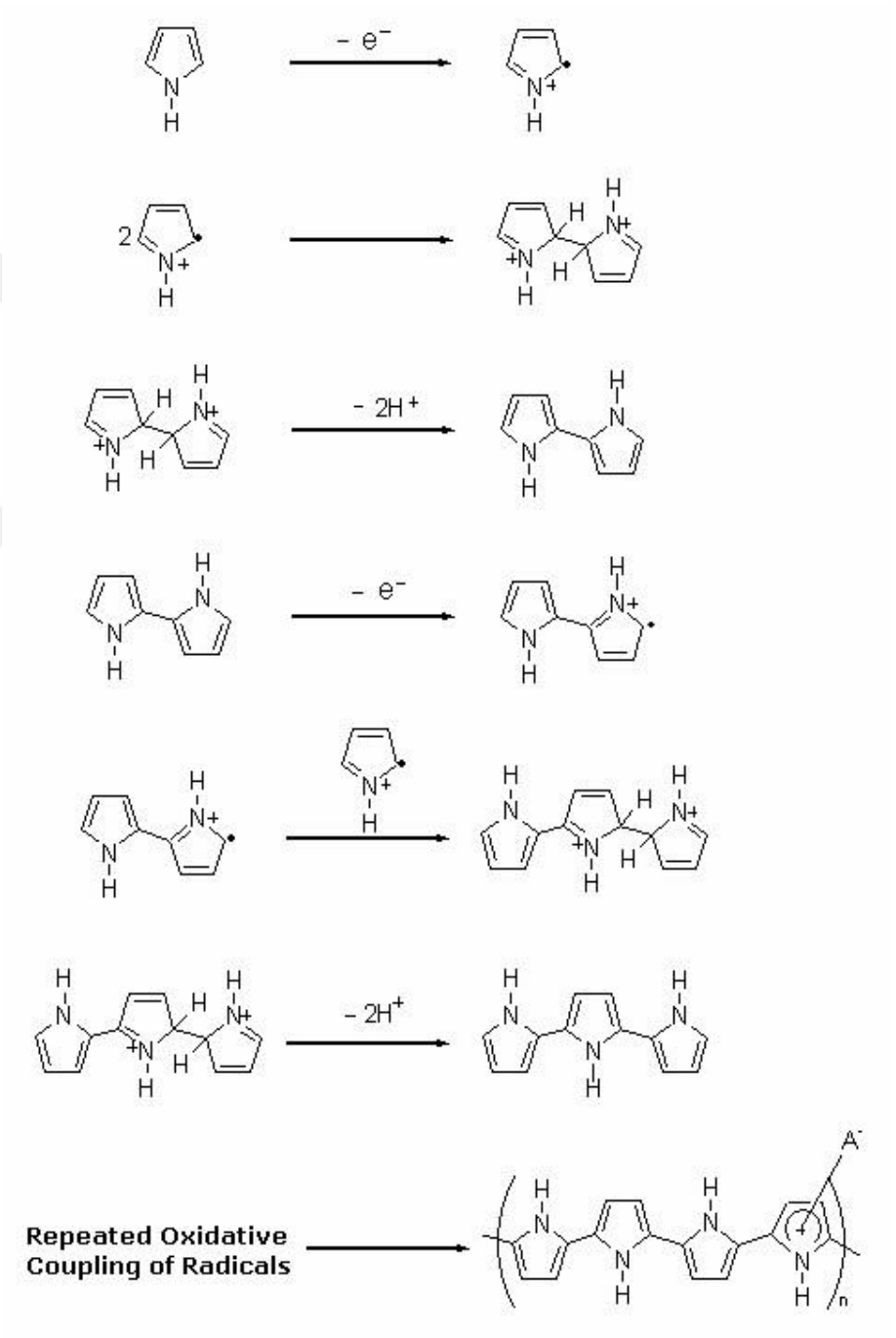



Figure 1.5. Electropolymerization of pyrrole.

The morphology of the resulting polymer is influenced by the conditions of electrochemical polymerization. The type of electrode material, solvent, and amount of ion may have effect on the dispersion. We must use the solvent with the constant pH buffer. We should not use nucleophilic solvent, because they inhibit the formation of the film by giving side reactions with the cations of the radical. Finally, the solvent shall be prepared on a large scale. We must choose to support the electricity system in its solubility character, and polypyrrole basically used as conducting polymer. The basic argument behind is its stability in the air, good electrical conductivity and environmental stability [17].

### 1.1.4 Advantages of Electrochemical Polymerization

By the technique of electrochemical polymerization, we can easily control the film thickness by changing current or the potential with time. The method is reproductive and simple. Control of molecular weight is possible. Occurring polymerization at room temperature is also possible to obtain polymers.

### 1.1.5 Polypyrrole

Polypyrrole as a conductive polymer was used frequently in commercial applications, for reason that optimal mechanical properties, forming photopolymers, and its long-term stability to get into. However, this material attracted little interest until it was set up in the form of thermoelectric stand-alone films. In recent years, investigations have been mainly focused on improving the physical characteristics of the polypyrrole, such as processing, stability, or mechanical safety [18]. This soluble polymer is available now and many of the applications is possible as a result of this processability, such as mixing for the formation of the composite that is improved in order to prepare what you want. Processability, as well as the control on the stability of polypyrole in air make these materials candidates for the use in technological applications.

### 1.1.6 Advantages of Electrochemical Synthesis of Polypyrrole

The electrochemical polymerization of the polypyrrole is preferred on the chemical polymerization because of its simplicity. The required electric capacity for the oxidation of monomer is much higher than the doped polymer formed. More over to obtain the polymer directly in the case of electrochemical polymerization is more favorable. Moreover, the thickness of the film can be easily followed by electrical charge used in the polymerization in addition to the implementation of reactions at room temperature. The importance of the incentivity of this method also comes from the possibility to use water as solvent to make the process more clean, fast and easy. Moreover, it is possible to obtain films of homogeneous polymers by means of the electrochemical method.

### 1.1.7 Mechanism of Electrochemical Synthesis of Polypyrrole

It is believed that the electrochemical polymerization of pyrrole starts with the formation of a cation radical of the monomer. Then it reacts with a second cation radical of the monomer. This reaction gives a dimer by the elimination of two protons. Since the oxidation of dimer or higher oligomers is possible at the necessary potential for oxidation of monomer, they are also oxidized, react further with the radical cation of the monomer and as a result, pyrrole chain forms. In order to stop the growth of polymer chain, either the radical cation end of the growing chain is made unreactive or most likely, the reactive end of the chain is sterically hindered from further reaction. The final polymer chain has one unit of charge for every three to four pyrrole rings. This charge is counterbalanced by the anion of electrolyte salt.

## 1.2 Enzymes

Enzyme is a catalyst that accelerates the reaction rate without incoming any permanent change [19]. The Swedish chemist Jon Jakob Berzelius had studied it in the early time of 1835 and chemical action of catalyst was found. The first enzyme was crystalized and isolated from the jack bean developed by James B. summer in 1926. He received the Nobel Prize in 1947 sharing with Wendell M. Stanley & John

H. Northrop. They found the procedure to isolate pepsin, which has been also used to crystallize several enzymes. Many studies have been done later on enzymes. Cech, in 1986, discovered RNA hydrolaz to serve as a catalyst for the reactions for hydrolysis of RNA where improvements of the rate can be achieved up to 1011 times [20]. Many of the enzymes is in the need of another element (cofactors) in order to stimulate the reaction. And most of the vitamins like niacin and thiamin helps an enzyme in the cells. Some other enzymes include nicotinamide adenin dinucleotid (NAD+) and flavin adenin dinucleotid (FAD) and coenzyme A. Metal ions such as $Cu^{+2}$, $Mg^{+2}$, $Zn^{+2}$, are bonded either to the apoenzyme or to the enzyme. It is usually a bonded metal ion that gets an enzyme as a catalyst. Enzymes are very specific materials that work on specific material or a certain type of material and motivate a particular response. Properties such as shape, water content and character of the hydrophobicity of the enzyme material is responsible for this specific response. For example, an enzyme invertase stimulates sucrose to the simple sugars glucose and fructose. This particularity can be interpreted as model key lock proposed by Emil Fisher in 1890. This model provides how the enzyme works. An enzyme has an active site where the substrate molecule binds and the reaction occurs. The substrate molecule (the key) fits into the special parts on the enzyme , which is called active site (the lock) to form a substrate enzyme complex. Active site is important for privacy because it works only for its substrate which gives the enzyme its specificity. While the substrate binds the enzyme, the new bonds are produced and products leave the enzyme. Figure 1.7 illustrates the key-lock model.

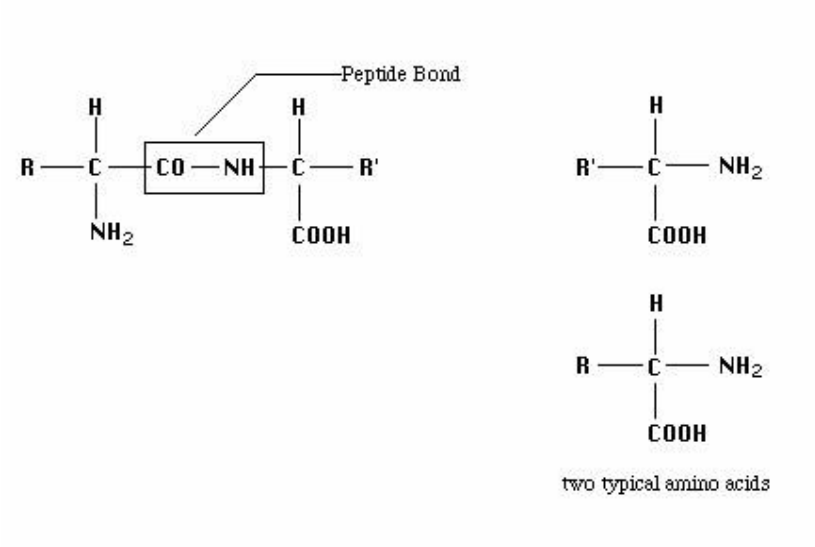



Figure 1.6. Structure of a peptide bond.

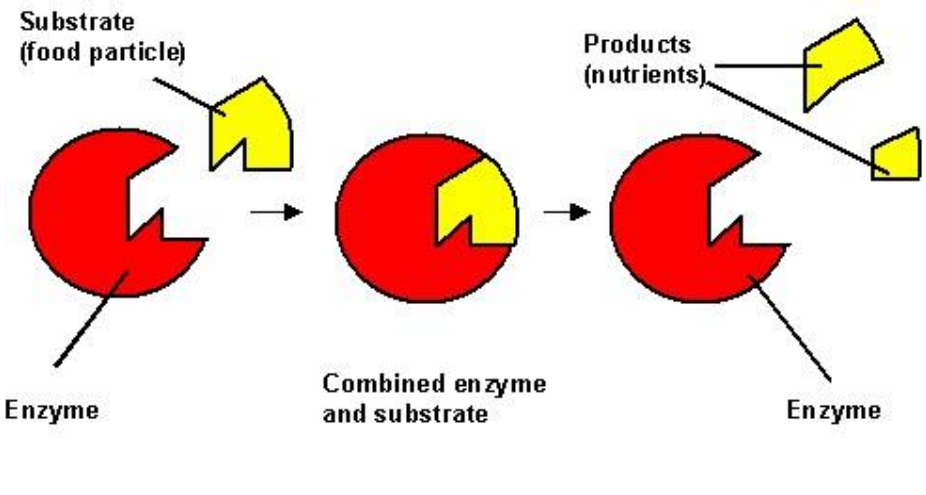



Figure 1.7. Enzyme specifity.

Enzymes like all catalysts reduce the activation energy of corresponding reaction which is then move forward faster. They increase the rate of reaction about 10-17 times fold. After completion of the reaction, they remain unchanged and therefore, they can be used several times. It does not affect the balance of the reaction because it does not change the relative energy between the products and reagents. Unlike other catalysts, the advantages of enzymes are specificity and chemosensitivity. The use of enzymes in food industry, pharmaceutical and chemical industry has been increased due to their catalytic property which is used in bread-making, alcoholic beverages, cheese-making and other medical applications.

### 1.2.1 Enzyme Classification

The discovery of different enzymes has come after purification of urease by Berzelius. Furthermore, the same enzyme that has gotten from various sources may have the different characteristics of the catalyst and different in term of physical properties. For example, alcohol dehydrogenase obtained from yeast, which contains four sub-modules and four atoms of $Zn^{2+}$ per molecule, has a molecular weight of 150,000. The same enzyme that is obtained from the liver of the horse is 70,000 in molecular weight, and contains two sub-units and four atoms of $Zn^{2+}$.

The classification of enzymes into six major categories by the Commission of the enzyme (EC) are:

a) Oxidoreductases
b) Transferases
c) Hydrolases
d) Lyases
e) Isomerases
f) Ligases

Enzymes that belong to the group of oxido reductase stimulate the reactions of oxidation-reduction. The enzyme oxidizes or reduces the substrate by the transfer of hydrogen or oxygen. Transfer of functional groups from the substrate molecules to the acceptor is catalyzed by transferase enzymes. Hydrolases are responsible for splitting a molecule into smaller molecules or more (reaction control) with the help of water. Lyases are the enzymes that increase the sets of pillars to mean non-hydrolysis. Isomerases are enzymes that facilitate the reactions among the isomers. Ligases catalyze a reaction mixture of two molecules with the expense of ATP. The Enzyme Committee gives code number to each enzyme (number EC) that contains four digits. The first one fixes the main enzyme group of the enzyme. The second and the third are for the reaction types, while fourth gives the identity of the enzyme by determining the substrate of the enzyme [21, 22].

### 1.2.2 Enzyme Kinetics

Kinetics of catalytic reaction of enzyme is important to obtain insight into the behaviour of enzyme. The first study was conducted by V. Henri on the enzyme kinetics in 1903, and later on, precisely in 1913, L.Michealis and Maud L. Menten conducted another study, which was depending on experimental data. It is believed that their studies are the basis of the movement of the enzyme. The mechanisms of enzymes kinetics are detailed in one-substrate as a simple model given by Michealis and Menten [23, 24] that is given below:

Enzyme(E) + Substrate(S) $\underset{K_2}{\overset{K_1}{\rightleftarrows}}$ Enzyme-Substrate Complex(ES)

Enzyme-Substrate Complex(ES) $\xrightarrow{K_3}$ Enzyme(E) + Product(P)

Where $k_1$, $k_2$ and $k_3$ represent the rate constants.

Here are stages to derivate the kinetic equation of Michaelis-Menten.

1) The rate of formation of ES is equal to that of the disharmony of ES. This can be obtained rapidly and it is known as steady-state equilibrium. Below the equations for the rate:

Rate of ES formation = $k_1$ [E][S]

Rate of ES disappearance = $k_2$[ES] + $k_3$[ES]

According to steady-state equilibrium,

$$k_1[E][S] = k_2[ES] + k_3[ES] \quad (1.1)$$

2) The total concentration of enzyme [$E_t$] is equal to the free enzyme [$E_f$] and the complex sum of the enzyme [ES].

$$[E_t] = [ES] + [E_f] \quad (1.2)$$

3) Initial speed of the reaction according to formation of product rate by

$ES \xrightarrow{K_3} E + P$ is given by,

$$V_o = k_3 [ES] \quad (1.3)$$

4) A maximum initial speed or velocity ($V_{max}$) is obtained when all enzymes are able to form complex with substrate. When

$[E_f] = 0$, $[ES]_{max} = [E_t]$ since $[E_t] = [ES] + [E_f]$.

Hence $V_{max}$ is straight proportional to $[E_t]$.

$$V_{max} = k_3[ES]_{max} = k_3[E_t] \quad (1.4)$$

By putting togather the equations 1.1, 1.2, 1.3, 1.4, the equation of velocity is obtained. From the equation 1.1, the equation for [ES] is also gotten where [E] is $[E_f]$.

$$[ES] = (k_1/k_2 + k_3)\,[E_f][S] \quad (1.5)$$

$(k_1/k_2 + k_3)^{-1}$ are known as the Michaelis – Menten constant ($K_m$). When $K_m$ are substituted into equation 1.5, the equation would be as follows:

$$[ES] = \frac{[E_f][S]}{K_m} \quad (1.6)$$

$[ES] = V_o/k_3$ from equation 1.3 is inserted into the equation 1.6

$$V_0 = \frac{[E_3][E_f][S]}{K_m} \quad (1.7)$$

$[E_f] = [E_t]-[ES]$ from the equation 1.2 is substituted for $[E_f]$ into equation 1.7 to obtain

$$V_0 = \frac{(K_3\ [E_t][S] - K_3\ [ES][S])}{K_m} \quad (1.8)$$

$V_o = k_3[ES]$ and $V_{max} = k_3[E_t]$ from equations 1.3 and 1.4 correspondingly are substituted into equation 1.8 to get

$$V_O = \frac{V_{max}\,[S] - V_O\,[S]}{K_m} \quad (1.9)$$

To arrange this equation for $V_o$ to obtain

$$V_O = \frac{V_{max}\ [S]}{K_m\ + [S]} \tag{1.10}$$

The equation (1.10) is called as the Michealis–Menten kinetic equation and its graphical representation is shown in the following Figure 1.8.

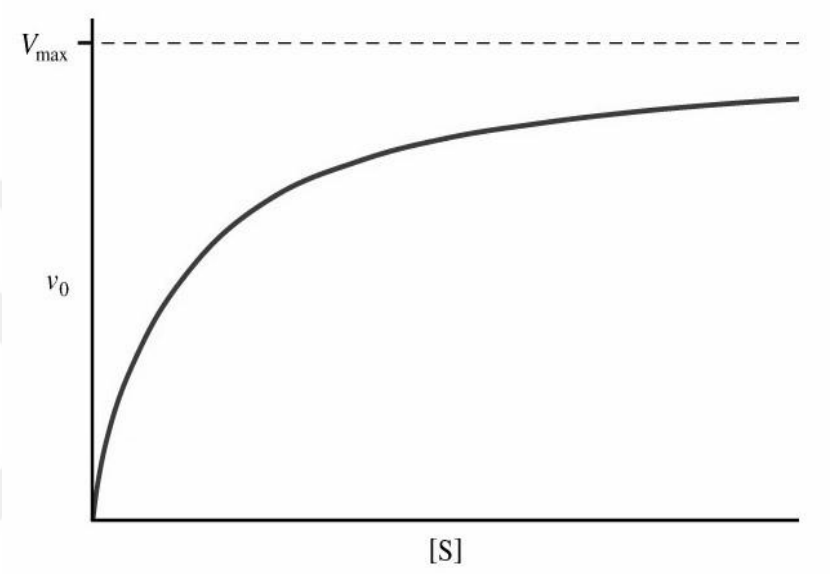



Figure 1.8. Graphical representation of Michaelis-Menten kinetic equation.

$V_{max}$ and $K_m$ are calculated straightly from Michaelis-Menten plot. $V_{max}$ calculation is driven from of the hyperbolic curve at the upper plateau region. $K_m$ = [S] where $V_O = \frac{V_{max}}{2}$. But, $V_{max}$ and $K_m$ are not obtained from Michaelis-Menten formula; for reason that amount of enzymes get a saturation curve. To avoid such inconveniency, Burk and Lineweaver reexamine the equation of Michaelis-Menten to calculate accurately the kinetic parameters as given below:

$V_O = \frac{V_{max}\ [S]}{K_m + [S]}$ is inverted to $\frac{1}{V_O} = \frac{K_m + [S]}{V_{max}\ [S]}$

$$\frac{1}{V_0} = \frac{K_m}{V_{max} + [S]} + \frac{1}{V_{max}} \tag{1.11}$$

This equation (1.11) is known as simple direct line y=mx+n when y=1/*V*o and x=1/[S]. If 1/*V*o is drawn as component of 1/[S], y intercept is $1/V_{max}$, the slope is $K_m/V_{max}$, and x intercept is $-1/K_m$. consequently, $K_m$ and $V_{max}$ may be accurately estimated by using Burk & Lineweaver [25] model as detailed in Figure 1.9.

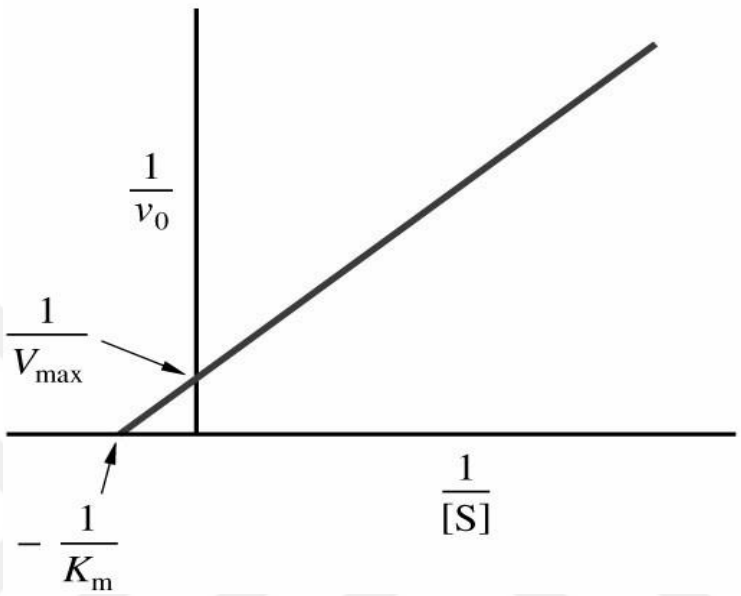



Figure 1.9. Lineweaver-Burk plot.

### 1.2.2.1 Effect of Factors on Enzyme Activity

Many factors affect the reaction rate and activity of enzymes, which are considered to be temperature, enzyme concentration, pH, and substrate concentration**.**

### 1.2.2.2 Effect of Substrate Concentration

The reaction velocity of the enzyme might be affected by substrate amount. In the case of enzyme concentration, when the substrate components are increased, it provokes an increase in the reaction rate until its maximum level before the enzyme is to be saturated. The detail is shown below in Figure 1.9.

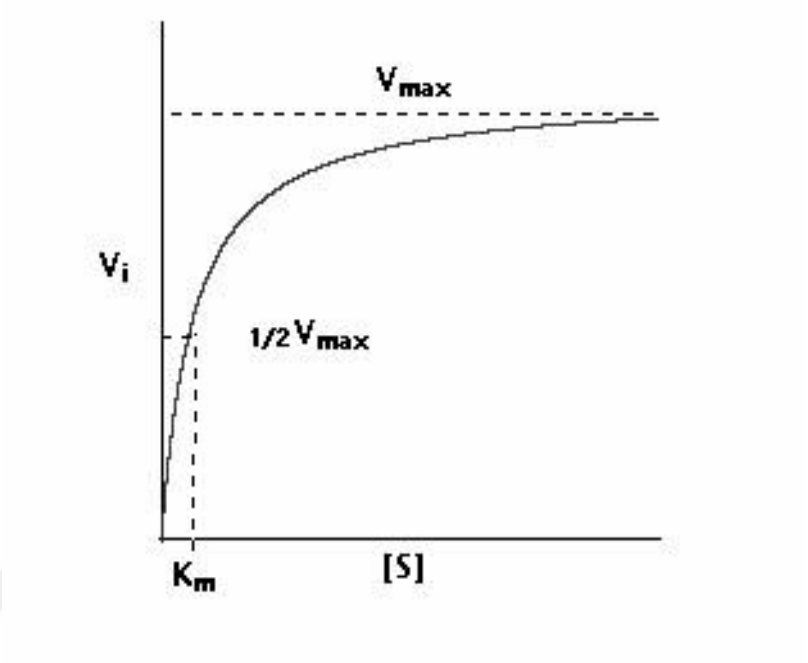



Figure 1.10. Effect of substrate amount.

### 1.2.2.3 Effect of Temperature

The speed of reaction can be increased as temperature increases. The complication comes out to the enzymatic reaction due to the nature of the enzymes. High temperature is basic cause to affect negatively many enzymes. The rate of enzymes reactions increases when temperature gets higher, then it decreases gradually as can be seen from the next Figure 1.10. As a result, many enzymes are quickly denaturized when they get to temperatures which is above 40 °C, so the determination of many enzymes are set somewhat near to that temperature. But, some enzymes activity can be lost when their temperature is even nearly to 0 ºC.

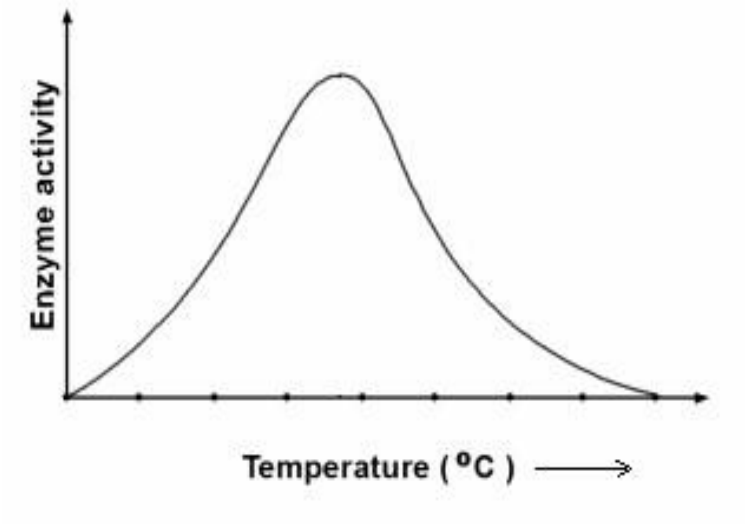



Figure 1.11. Effect of temperature.

### 1.2.2.4 Effect of pH

Activity of enzyme is also influenced with changing pH. Effect of pH is shown in Figure 1.11. Most enzymes lose their activities completely at extremely low or high pH values. Each enzyme has also an optimal pH stability region where they function effectively. The optimum pH value depends on the enzyme nature. Urease and invertase reveal maximum activity at pH 7.0 and pH 4.5.

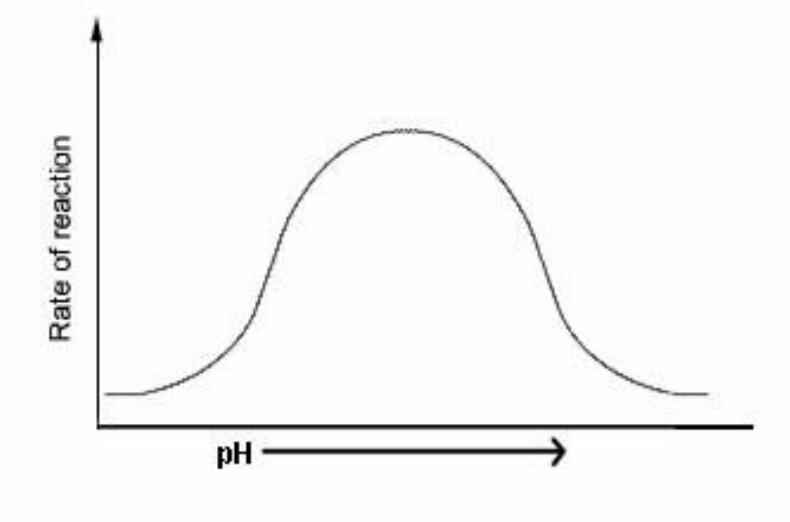



Figure 1.12. Effect of pH.

### 1.2.3 Enzyme Placement on a Matrix

The replenishment of the confined material or the localization of enzyme molecules during the process of motivational level with retention of its activity leads to the frequent and repetitive use of the material [26].

#### 1.2.3.1 Reason for Placement

There are a number of benefits to immobilize enzymes by attaching them on inert material.

- Multiple use of a single enzyme electrode
- The discharge is stopped quickly when the immobilized enzyme is removed from the solution.
- Enzyme does not contaminate the product and that is very useful for pharmaceutical and food industries.

#### 1.2.3.2 Methods of Placement

The enzymes may be immobilized through two main methods: by physical or by binding [27, 28]. In the binding type of the immobilization, a bond is formed between matrice and the enzyme. On the other hand, there is no such a composition of the bonds in entrapment through physical contact [29, 30, 31].

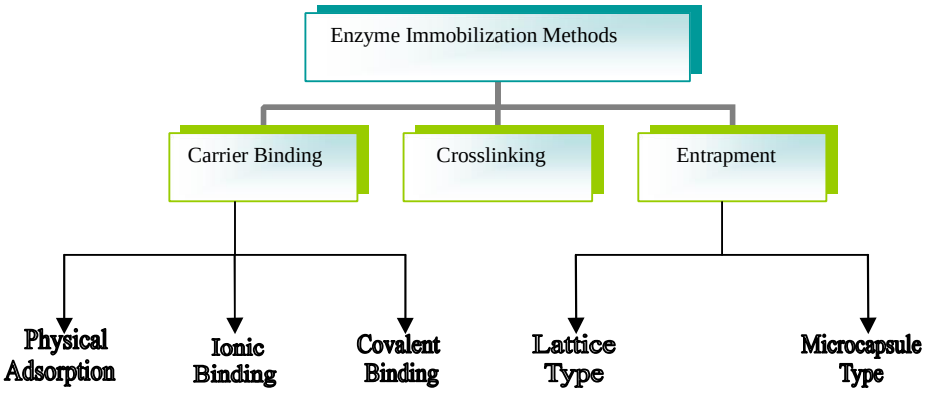



Figure 1.13. Immobilization methods.

Carrier Binding: In this type of immobilization, binding to the enzyme need to convert it non-soluble in water [32]. Some carrier examples used in this method are polyacrylamide gel, dextran and cellulose. The binding of ionic, adsorption, and covalent binding are sub-categories of this method [33, 34, 35]. Adsorption of the enzyme on the carrier insoluble in water by physical means is a very simple way. Interactions of hydrophobic and ionic parts are some of the factors. In this way of the immobilization, there is little or no destruction on the enzyme active site, so the activity is affected slightly [28]. The immobilization of invertase has been studied by Nelson and Griffin on active coal in 1916 [36]. The basic disadvantage of this model is the adsorption of the enzyme by weak forces.

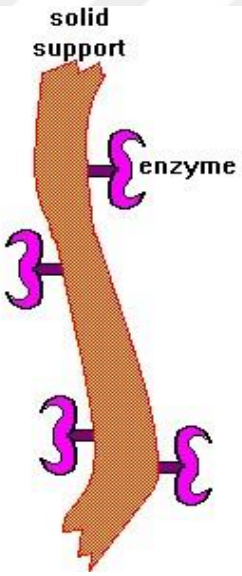



Figure 1.14. Carrier binding.

Ionic binding is formed when an interaction occurs between carrier ionic parts and the enzyme. Carriers usually used are sugars and synthetic polymers that have ionic centers. Metz was the first scholar who utilized the immobilization model in 1956 [37]. Covalent binding refers to technique of immobilization focused on the formation of linkages covalently between the matrix support and the enzyme [38, 39]. The reaction of binding, enzymatic activity should not be lost and conditions should be arranged that they do not cause loss of enzymatic activity. And the reagents which are used in the reaction medium should not affect the active site of the enzyme. Active site of enzyme and conformational structure can be affected by

conditional appropriate reaction. In addition, enzyme does not occur due to strong binding between the carrier and enzyme, if there is.

Crosslinking: Crosslinking refers to obtaining the immobilization by the entanglement between the protein molecules, either to the functional groups on the matrix support or protein molecules. This method is an expensive method for gathering materials since some protein material will serve as a support. This method has an important advantage that is the prevention of leakage or adsorption of the enzyme since the enzyme covalently joins to the matrix support.

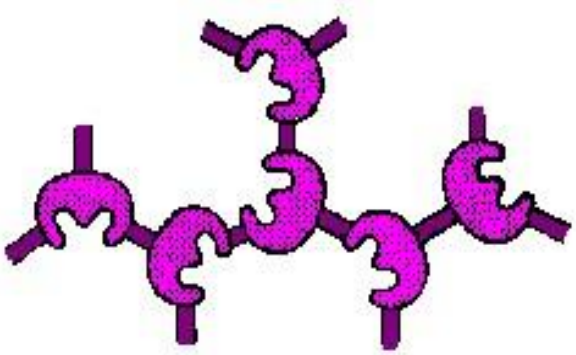


Figure 1.15. Crosslinking.

Entrapment: This method is the physical type of immobilization. Therefore, there is no variation in the enzyme active site and the activity of the enzyme is not lost. The immobilization of the enzyme is being frozen within the network of a matrix structure of a polymer [40-43] or membrane. In this way, penetration of the substrate is allowed through the membrane while penetration of the enzyme is not. Also the most reactions and condition of polymerization necessities various reaction, careful selection of conditions suitable to regenerate the enzyme is required to prevent loss of activity. The gel and fiber entrapments are examples for the entrapment matrix. In the past, many enzymes were entrapped in the gel which is a crosslinked with in water insoluble polymer. Enzyme molecules squeezed physically within the polymer matrix of interlocking cannot permeate the gel, while the substrate of the appropriate size of the particles of the product can be transferred over the gel.

Like more, the enzyme may be fit into fiber. The high surface area of the enzyme binding is one of the advantages of the entrapment of the fibers on the gel entrapment. Fiber is generally resistant to acids, weak alkalis and some organic solvents with ionic strength.

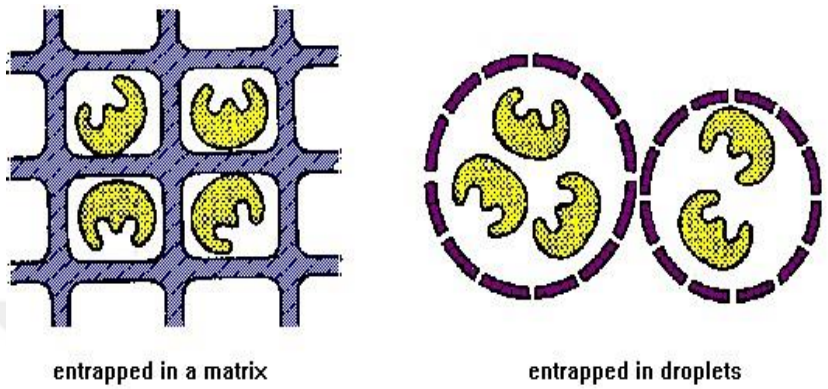



Figure 1.16. Entrapment.

Membrane Enclosure: In this method the enzyme is retained within the surface got in semipermeable membrane. This membrane is permeable to substrate and product, but impermeable to the enzyme. There is no chemical change on the enzyme and thus, enzyme molecules are free in membrane matrice.

### 1.2.3.3 Applications of Enzyme Placement

The techniques developed for immobilizing the enzyme makes easier the progression of the use of the enzyme in novel analytical and automated methods. In recent years the studies have been carrying out a great job on the development of the enzyme methods to examine glucose, amino acids, urea, alcohol, and lactic acid. These electrodes contain the ions or sensor of oxygen formed in enzymatical manner, i.e., glucose oxidase [44-47], the alcohol oxidase [48-50]. In the industry there are many parameters to use immobilized enzymes. High demand of the product, the movement of the reaction favorable, the price of production in the market, a catalyst of effective environmental process, pharmachemistry, special L-amino acid production (L-aminoacylase), and the production of derivatives of penicillin (penicillin AIS) or aspartic acid (L-aspartase) are examples of areas for using immobilized biocatalysts.

### 1.2.3.4 Electrochemical Placement

For the immobilization of an enzyme many systems and electrochemical techniques are available. Electrolytic cell contains an enzyme, electrodes, electrolytes, then with the applied current or a constant potential, polymerization and immobilization of enzyme is obtained. Electropolymerized pyrrole (PPy) provides the potential of immobilization and is attractive for many species. Electrochemical properties can be changed and the surfaces of the polymer can be modified by doping with non-organic anions which produce voltammetric sensor of carbonate and phosphate [51] while the Merge matrix of nitrotoluene Ppy puts selective response to aromatic compounds [52]. It turns out that mercury deposition and electrochemical electrodes are based on any signal analysis compared to the traditional solid [53].

### 1.2.3.5 Placement of Invertase

Sucrose is converted to glucose and fructose by the catalytic effect of invertase (β-fructofuranosidase) (EC 3.2.1.26) (Figure 1.16). This hydrolysis is hugely used to produce cream of nonpolycrystalline, to make artificial honey and jam. Invertase has the kind of less likelihood to achieve use in the market in an immobilized form, nevertheless, it is one of the most applicable and cheap model in all enzymes models. Invertase has been immobilized on several matrices by many different techniques [54, 55, 56]. It was linked covalently once collagen in adsorption statue then matrices based on petroleum, and resin phenol - formaldehyde [57] and poly (aminostyrene) [58]. Regularly, entrapment of the enzyme, invertase in a different conducting matrice of polymer was described by the group for electropolymerization in further detail [59].

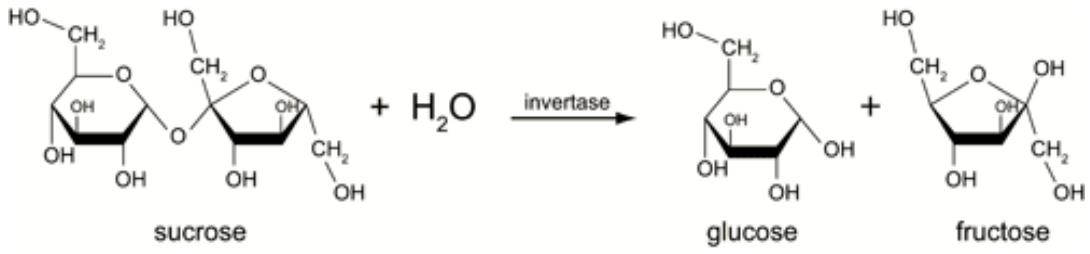



Figure 1.17. Conversion of sucrose to fructose and glucose.

### 1.2.4 Enzyme Sensors

In practical and commercial applications, the superiority of the enzyme catalysts is striking. For this reasons in biosensors enzymes are widely used as biocatalysts. Thousands of enzymes can be used directly or indirectly in the analysis of almost any material substance related to living systems.

In the most sense, one enzyme sensor consists of transmitter, a thin enzyme layer and other systems. The difference from other biosensors is the use of biomolecular enzymes in the bioactive layer [61, 62]. Figure 1.17 shows the structure of the enzyme sensor.

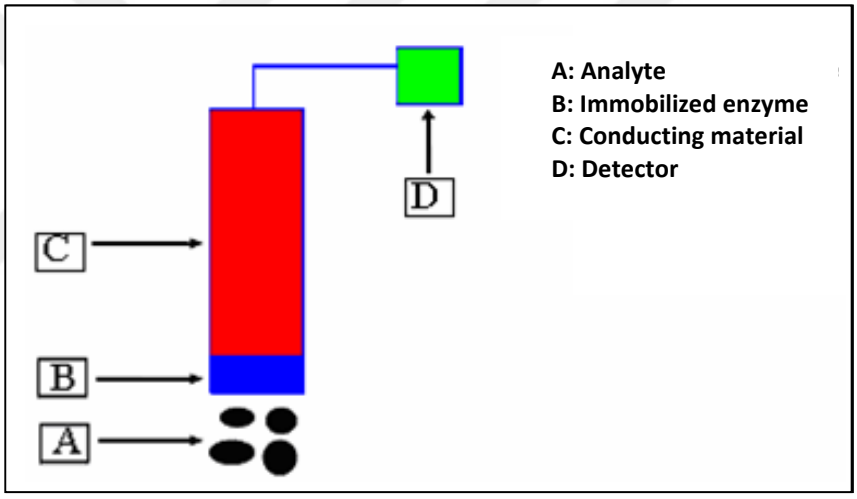



Figure 1.18. General scheme of an enzyme sensor.

### 1.2.5 Biosensors

Biosensors is a tool used to measure reversibly, continuously, fast and directly the changes in the concentration of the analyte. A biosensor consists of the binding of a piece to a sensitive area where chemical or biochemical reactions occur, which provide optical, electrical, thermal, or mass transfer [61].

Biosensors consist of a transducer system that can convert signals to electronic signals. It contains a “biomolecular / bioagents”, physical transformers, and turns

physicochemical signals to electronic signals. As biomaterial, mostly enzymes, tissues and yeasts, antibodies / antigens and antibodies are used [63]. Transducers are devices that can measure the biological reaction of receptors with the aid of a bioreactor, convert them into a physical signal, that is, convert one energy into another. It can be seen in Figure 1.18 the construction and working principle of the biosensors.

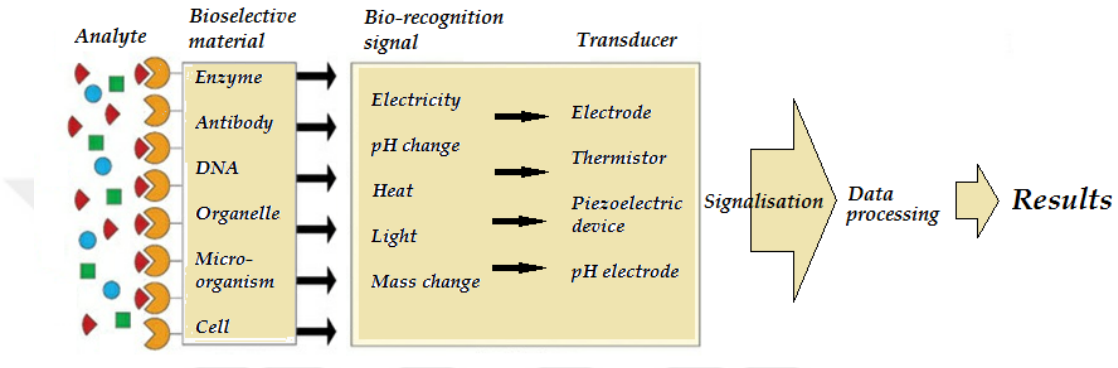



Figure 1.19. Structure and working principle of biosensors.

## 1.3 Carbon Fiber

Carbon fiber forms with the pyrolysis of appropriate fiber. Pyrolysis must be controlled in order to obtain the desired product. Carbon fiber is defined as fiber including carbon more than %90 according to *Textile Terms and Definitions*. If fiber has excess amount of carbon which is about %99, it is called "graphite fiber" [64]. Carbon fibers with several morphologies and several characteristics are produced from many different precursor fibers. Polyacrylonitrile (PAN), cellulosic fibers (viscose rayon, cotton), petroleum or coal tar pitch and certain phenolic fibers are the mostly used precursors [65].

Organic precursor fibers are exposed to the controlled pyrolysis in order to produce carbon fiber. Oxygen, nitrogen and hydrogen are eliminated from the precursor by applying heat treatment and as a result carbon fiber forms. It increases the crystallinity and orientation and decreases the defects of fiber in order to get better mechanical properties. For this purpose, precursor is chosen a highly oriented

starting fiber. It is necessary to keep this initial orientation during the steps of pyrolysis [64].

### 1.3.1 Polyacrylonitrile (PAN) Carbon Fibers

PAN precursor is converted to high-performance carbon fibers by three steps (Figure (1.20)).

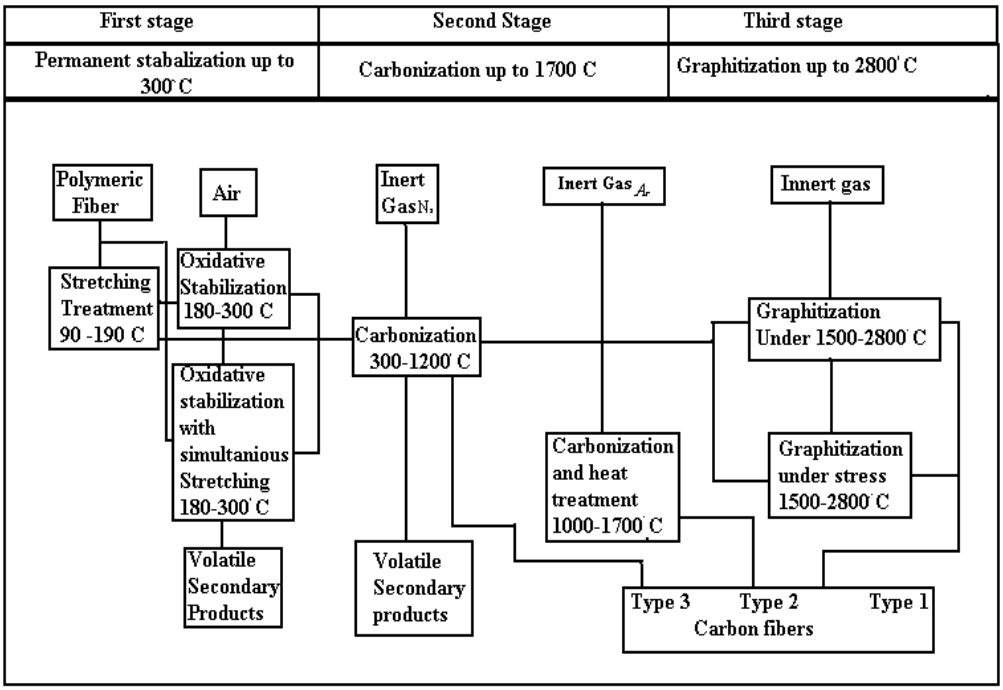



Figure 1.20. Schematic representation of carbon fiber preparation from PAN fibers.

Oxidative stabilization: It is the stretching and oxidation of polyacrylonitrile precursor with a heat treatment between 200-300 °C at the same time. Thermoplastic PAN is converted to a non-plastic fiber by this treatment.

Carbonization: Fibers are heated to 1000 °C in an inert atmosphere (normally nitrogen) without stretching. This step takes 1-3 hours and carbonization occurs by the loss of elements other than carbon during this treatment. With the removal of those elements, carbon fiber mass becomes about %50 of starting precursor.

Graphitization: Heat treatment of 1500-3000 °C is applied in this step. Temperature is selected according to the type of fiber desired. Fiber crystals order and orientation increases in this step along the fiber axis [64].

### 1.3.2 Applications

Carbon fiber is known with its strength and lightweight. It is the best material in the places where these properties needed like racing cars, motorbikes and powerboats especially racing-purpose type of those devices. They are expensive and huge amount of money has been put for safety and performance of these devices. Among the various materials and composites, carbon fiber stands out with its high tensile strength and torsional rigidity which provide protection in accident case. In addition, carbon fiber is resistive to flame and corrosion which are important properties in those devices.

In many sportive equipments, carbon fiber is used because of its physical properties; rigidity, strength and resistance to streching. Golf shaft, tennis racket, bicycles, yachts are some examples of these equipments.

Carbon fiber technology was originally developed for satellites and now planes and spacecrafts are two main areas where the carbon fiber is pioneered with the properties again lightweight, strength, resistance to fire and corrosion.

Like sports, carbon fiber finds a wide area of use in music. In many musical instruments, carbon fiber and other composite materials are used in order to improve the performance and acoustics of the instruments.

Carbon fiber also has several uses in science. One of its main applications is usage as electrode material. Carbon fiber sheet can be used as electrode and it should be not coated by resin. On the other hand, single fiber electrode has been used since 1970's. It is 7-8μm in diameter and placed in a glass capillary tube. Main use of these electrodes is in neuroscience; determination of a chemical by voltammetric

measurement of potentials upon action through the membranes of neuron cells [66-68]. This is widely reviewed in next section.

### 1.3.3 As Electrode Material, Carbon Fiber

Carbon fiber (CF) is a chemically inert material and is excellent base for electrochemical and biosensor electrodes on a micrometer and even nanometer scale. CF microelectrode is of good quality for extracellular recording similar to tungsten electrodes.

CF microelectrode is constructed by carbon filament and a glass or plastic sheathing for mechanical support. Individual fiber is glued to a metal wire with a conducting paint or epoxy glue. It is inserted into the sheathing which is a borosilicate glass capillary or a plastic tubing. Tip of the fiber protrudes from the sheathing tip with 10 – 30 μm and it provides conductive part of the electrode.

Surface of conducting carbon fiber can be used as bare electrode without any modification in determination of extracellular spike and measurement of biomolecules electrochemically by voltammetry or amperometry. But it can also be modified by variety of means such as covering with nafion for selectivity to biological species, covalent modifications (amine attachment, grafting of aryl or alkyl groups, attachment of polymers, electrochemical deposition of conducting polymers, electrochemical modifications), immobilization of carbon nanotubes to increase the interfacial area and thus sensitivity and modifications by biological materials [69].

Carbon fiber microelectrode, bare or nafion-coated, is a very good tool for in vivo extracellular determination of neuronal spike. This is measurement of electric current around neuron cells during neuronal action. Another application is in vivo voltammetry where three electrode-system is used. Voltage is applied and varied through the working carbon fiber electrode and the current produced by electroactive specie is measured. Again in vivo tracking of nitric oxide, NO, is pursued mostly by amperometry following the current upon oxidation of NO. In vivo determination of

cell oxygen is an important novel method and the signal here produced by the reduction of oxygen.

Immobilization of DNA on carbon fiber electrode is one of the major examples of biosensors where a biological agent is placed onto the electrode. In labelled DNA modification, a redox active specie binds to DNA and electrochemical signal changes. In label-free methods, either electrical characteristics of DNA–immobilized interface changes or DNA natural electroactivity becomes different. Besides, effective interface area increases by DNA modification. Other important properties that DNA-modified surface gains are abilities of adsorption and interchelating with bioactive molecules [69].

Enzyme–bound CF microelectrodes is another important application in the biosensing area and they are major components of an amperometric biosensor. A biochemical change occurs upon interaction between redox enzyme and its substrate and thus a biochemical signal is produced. This signal is converted to an electrical signal and can be detected amperometrically. Immobilized enzyme selection depends on the analyte to be determined. Enzyme layer on the electrode has an insulating effect and the conductivity between enzyme and electrode surface must be maintained. This is achieved by transforming insulating layer of enzyme to a charge-carrier matrix. For this purpose, several methods are applied such as placement of enzyme in conductive polymer, forming composites with metal complexes, carbon nanotubes and nanomaterials.

Enzyme–based biosensors are fabricated usually by a 7-30 μm diameter carbon fiber microelectrodes. Enzyme can be bound covalently to the electrode. It can be immobilized by a cross-linked polymer coating including enzyme inside. Gold or platinum nanoparticle deposition on CF electrode then enzyme immobilization is another method. Immobilization by electropolymerization of conductive polymer and immobilization of enzyme with implementation of carbon nanotubes are other methods [69].

### 1.3.4 Carbon Fiber Electrode in This Study

Here in this study, carbon fiber is used as an electrode material for immobilization of invertase enzyme. Some important properties of carbon fiber which make it possible are conductivity, porous structure of surface and chemical inertness. Immobilization is carried out by means of electropolymerization of conducting polymer, polypyrrole. It was performed in an electrolytic cell and carbon fiber was used in this assembly as working electrode. Porosity of the surface is another important parameter. Porous structure gives the carbon fiber high surface area. Since the enzyme is caught up by the polymer which cover the electrode surface, how much wider the surface area, so much polymer is coated and so much enzyme is caught up. As enzyme amount increases the signal of the electrode goes up and this gives an increase in sensitivity. And lastly, chemical inertness is a must property that is looked for all electrodes in order to prevent side interactions. Carbon fiber electrode is constructed in laboratory by hand.

## 1.4 Aim of the study

The main objective of this study was to use novel carbon fiber enzyme electrodes and to investigate the immobilization of enzymes in polypyrrole by electrochemical polymerization of the conductive polymer matrix.

This study investigated:

- The placement of invertase enzyme in PPy carbon fiber electrodes,
- The best conditions for fixing and determining enzyme activity,
- The kinetic parameters, daily stability, temperature and pH stability of the enzyme-mobilized electrodes.

# CHAPTER II

# EXPERIMENTAL, MATERIAL AND METHOD

## 2.1 Chemicals

Invertase (β-fructofuranosidase, EC 3.2.1.26) Type V and sodium dodecyle sulfate (SDS) were obtained from Sigma. Before use, pyrrole (Merck) was distilled and stored at 4°C. For the preparation of Nelson reagent, sodium sulfate (Merck), copper sulfate (Fluka), sodium carbonate (Riedel de Haen), sodiumbicarbonate (Merck), sodium potassium tartarate (Merck), and for the preparation of arsenomolibdate reagent, ammonium heptamolibdate (Merck) and sodium hydrogen arsenate (Riedel de Haen) were used as-received. Sulphuric acid and sodium hydroxide were also supplied from Merck. For the preparation of acetate buffer, acetic acid (Carlo Erba) and sodium acetate (Merck) were used as received.

### 2.1.1 Reagent of Nelson

Nelson's reagent A: 25 g anhydrous $Na_2CO_3$, 25 g sodium potassium tartarate, 20 g $Na_2HCO_3$, and 200 g anhydrous $Na_2SO_4$ are dissolved in 700 ml distilled water. After that, it is diluted to 1000 ml and stored at room temperature.

Nelson's reagent B: 15 g $CuSO_4$, to obtain 15% (w/v) solution, is dissolved in 100 ml distilled water and a few drops of concentrated $H_2SO_4$ was added.

Reagent A and B were mixed in 25 /1 (V /V) ratio just before each activity assay.

### 2.1.2 Arsenomolybdate Reagent

For the preparation of arsenomolybdate reagent, first $(NH_4)6\ MoO_{24}.4H_2O$, ammonium heptamolibdate tetrahydrate, in 450 ml distilled water 25 g is dissolved and 21 ml concentrated sulfuric acid is added. Then sodium arsenate dibasic-7-hydrate, ($Na_2HAsO_4\ .7H_2O$), 3 g in 25 ml distilled water is dissolved and added to the molybdate solution. Then the solution is left at 37 °C for 24 hours and it is in dark glass bottle stored.

In this method, reducing sugar glucose when heated with alkaline copper reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place, which is detectable at 540 nm.

## 2.2 Apparatus and Instruments

### 2.2.1 Potentiostat

In this study, the Potentiostat Wenking POS-88 was used. It has the potential kept constant between working and counter electrodes in a necessary value and compensate for the voltage drop in the electrolytic solution. The potential of the working electrode is related to the electric potential of the reference electrode and it is arranged to the necessary value by the help of the reference electrode.

### 2.2.2 Cell for Electrochemical Synthesis

Three-electrode system is used for constant potential electrolysis (CPE), in which the electrodes are working, counter and reference electrodes. Controlled potential electrolysis system cell is seen on Figure 2.1. The working electrode is carbon fiber, the counter electrode is platinum foil with an area of 2 $cm^2$. The reference electrode is $Ag^o/Ag^+$ and the total electropolymerization solution volume is 10 ml.

### 2.2.3 The UV–Visible Spectrophotometer

The absorption of enzyme-catalyzed transformation products was determined by the application of Nelson's method. For the determination of activities of immobilized enzyme, Shimadzu UV-1600 model spectrophotometer was used.

### 2.2.4 Carbon Fiber Electrodes

Textile material carbon fiber was purchased and used as working electrode. As shown on Figure 2.1, it was prepared with yellow type micropipette tips. A bunch of carbon fiber with diameter 50 μm was connected with a metal wire then carbon fiber was placed in the hole of the tip. End of tip was closed by heating. A glass tube was placed around metal wire.

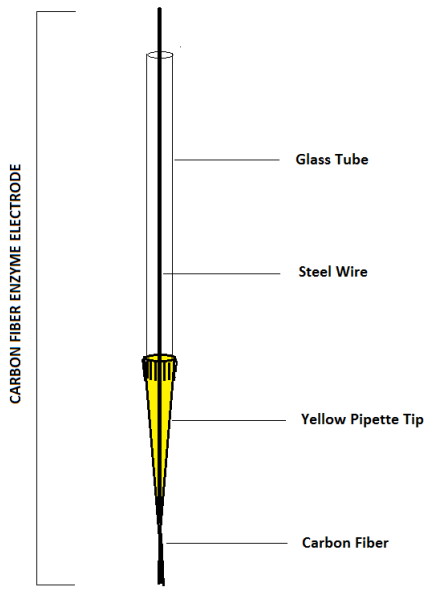



Figure 2.1. Carbon fiber electrode.

### 2.2.5 Scanning Electron Microscopy (SEM)

JEOL Scanning Microscope Model JSM-6400 was employed for the morphological studies on composite films. SEM photo of bare carbon fiber was taken without any pretreatment. After electropolymerization of polypyrrole, electrode was washed up

by distilled water and then left to dry. SEM photo of coated electrode was taken the day after.

### 2.3. Procedure

#### 2.3.1 Synthesis of PPy Polymer

Working electrode (carbon fiber), counter electrode (Pt, $2cm^2$) and reference electrode ($Ag/Ag^{+1}$) were soaked into electrolysis solution. Electrolysis were performed in this solution of 0,6 mg/ml sodium dodecyl sulphate (SDS) and 50 µl pyrrole in 10 ml water. 1.0 V was applied for 30 min.

#### 2.3.2. Placement of Invertase in PPy Matrix

Placement of invertase in polypyrrole matrix is achieved by electropolymerization which is conducted in a three-electrode system by electrolysis with constant potential at room temperature. Electrolysis solution contains 0,6 mg/ml invertase, 0,6 mg/ml sodium dodecyl sulfate (SDS) and 50 µl pyrrole in 10 ml 0,05 M acetate buffer (pH 5). Constant potential of +1.0 V is applied for 30 min for placement of invertase using platinum foil as the working electrode. Counter electrode is also platinum and reference electrode is a silver wire. Figure 2.2 shows electropolymerization and immobilization of enzyme on platinum electrodes instead of carbon fiber electrodes in order to see the process more clearly. After electrolysis in order to remove the unimmobilized enzyme residue and the excess supporting electrolyte, enzyme electrodes are cleaned with distilled water. Electrode is placed in acetate buffer (pH 5) at 4 °C when not in use.

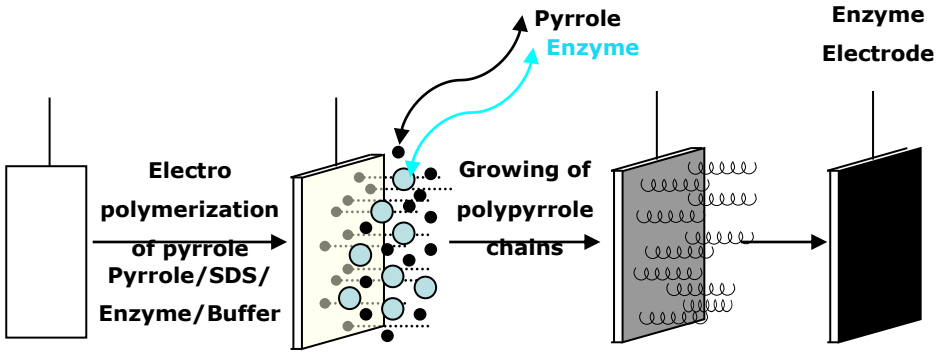



Figure 2.2. Electropolymerization and immobilization of enzyme on platinum electrode.

### 2.3.3. Invertase Activity Determination

The activity of immobilized invertase was investigated by the method of Nelson [70, 71] as shown on Figure 2.3. For the examination of immobilized activity of invertase, sucrose solutions of different concentration were put in the test tubes and then enzyme electrode (carbon fiber electrode) was soaked into the solution of sucrose, enzyme and substrate were allowed to combine and respond to a specific time (0, 2, 4, 6 minutes). After this specific reaction time, the electrode was replaced and 1ml Nelson reagent was put into the test tubes in order to terminate the reaction, which were incubated at 25 °C. Just after adding Nelson reagent and after each period, tubes were placed into a boiling water bath for 20 minutes. After 20 minutes, the test tubes of solutions were placed in a cold-water bath for 10 min. in order to get the solutions to be cooled, then 1 ml arsenomolibdate reagent and 7 ml distilled water were added for the color formation in the solution. Solution absorption measurements were performed at 540 nm.

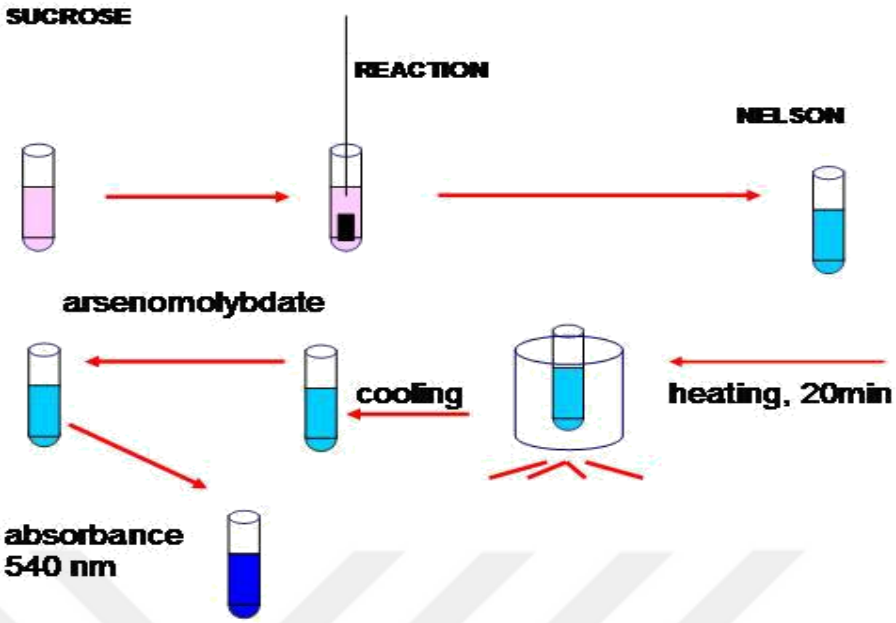



Figure 2.3. Nelson's method.

### 2.3.4. Kinetic Parameters Determination

Maximum rate of the reaction ($V_{max}$) and the interaction between substrate and enzyme namely Michaelis–Menten constant ($K_m$) were determined for invertase electrodes. For this purpose, different concentrations of sucrose were prepared and used in activity assay procedure.

### 2.3.5. Optimum Conditions Determination

To determine the optimum conditions for the maximum invertase activity, the parameters of temperature and pH during the activity measurements were changed and different values were used.

### 2.3.6. Optimum Temperature Determination

For determination of the optimum temperature of immobilized invertase, the matrix concentration was maintained constant (~5 $K_m$), and the incubation temperature was

changed between 10 °C to 80 °C. The remaining of the procedure was the same as activity assay.

### 2.3.7. Optimum pH Determination

The study on the pH stability of immobilized enzymes were performed in the pH range of 2.0-12.0 while the sucrose concentration is constant at ~5 $K_m$. The remaining of the procedure was the same as activity assay.

### 2.3.8. Daily Stability

Operational stability was investigated by 40 subsequent activity measurements in one day. Repeated enzyme activity was measured in order to investigate the stability of enzyme electrodes during one day. For this purpose, optimum conditions (optimum pH and optimum temperature) was maintained and constant concentration of sucrose was used.

# CHAPTER III

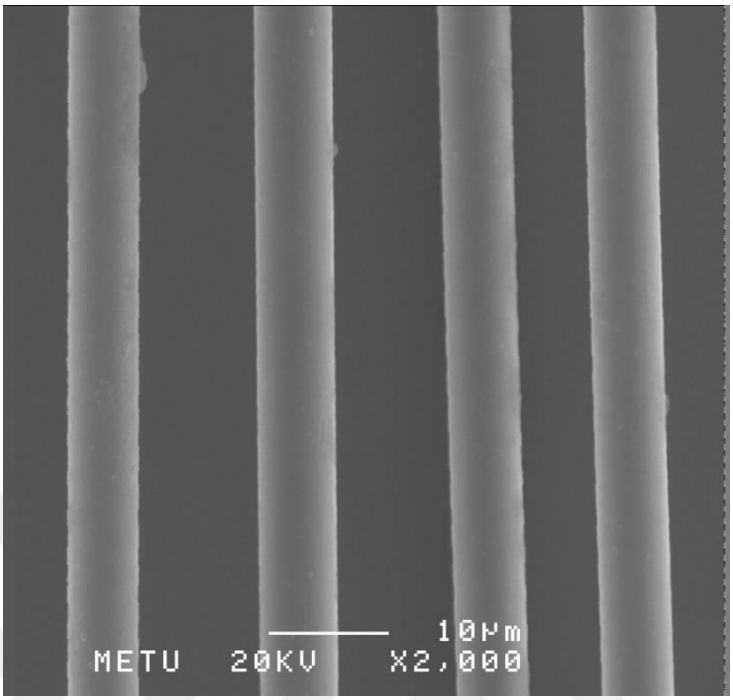
# RESULTS AND DISCUSSION

In this study, the placement of enzyme, invertase, was performed in polypyrrole matrix. The preparation and characterization of enzyme electrodes and the determination of optimum conditions were introduced.

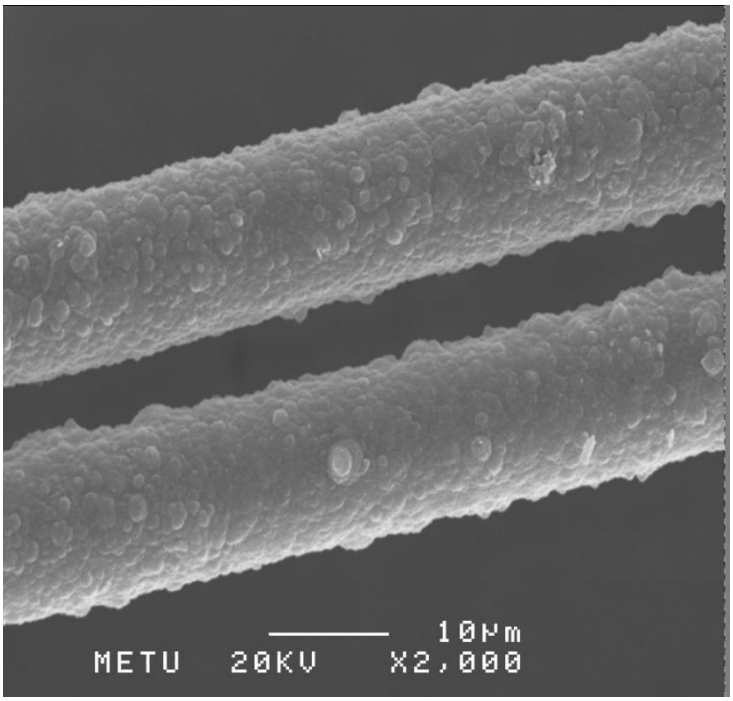
## 3.1 Morphology of Carbon Fiber Electrodes

In order to examine the morphology of carbon fiber electrodes, Scanning Electron Microscopy (SEM) was used and surface morphology of carbon fiber and polymer coated carbon fiber electrodes were studied. Electrodes were washed after electrolysis to remove unbound particles. It is seen on Figure 3.1 that coated carbon fiber has a thickness that is almost two times thicker than bare carbon fiber because of the coated polymer. After electropolymerization electrode surface exhibits standard cauliflower structure of polypyrrole.

**(a)**




**(b)**



**(c)**

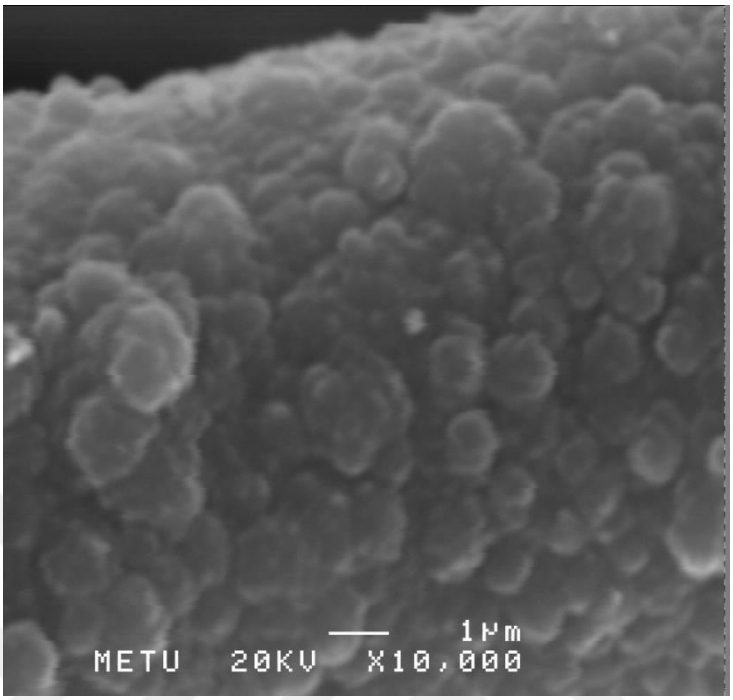



Figure 3.1. SEM photos of (a) bare carbon fiber, (b) polymer coated carbon fiber, (c) polymer coated carbon fiber in larger scale.

### 3.2 Carbon Fiber Electrode and Electropolymerization of Polypyrrole

Electropolymerization were performed in pH 5 buffer solution of 0,6 mg/ml sodium dodecyl sulphate (SDS) and 50 µl pyrrole in 10 ml water. It was scanned between +0,7 and -1.7 V for 14 cycles. As shown on Figure 3.2, the peak current increases in each cycle as the polymerization proceeds.

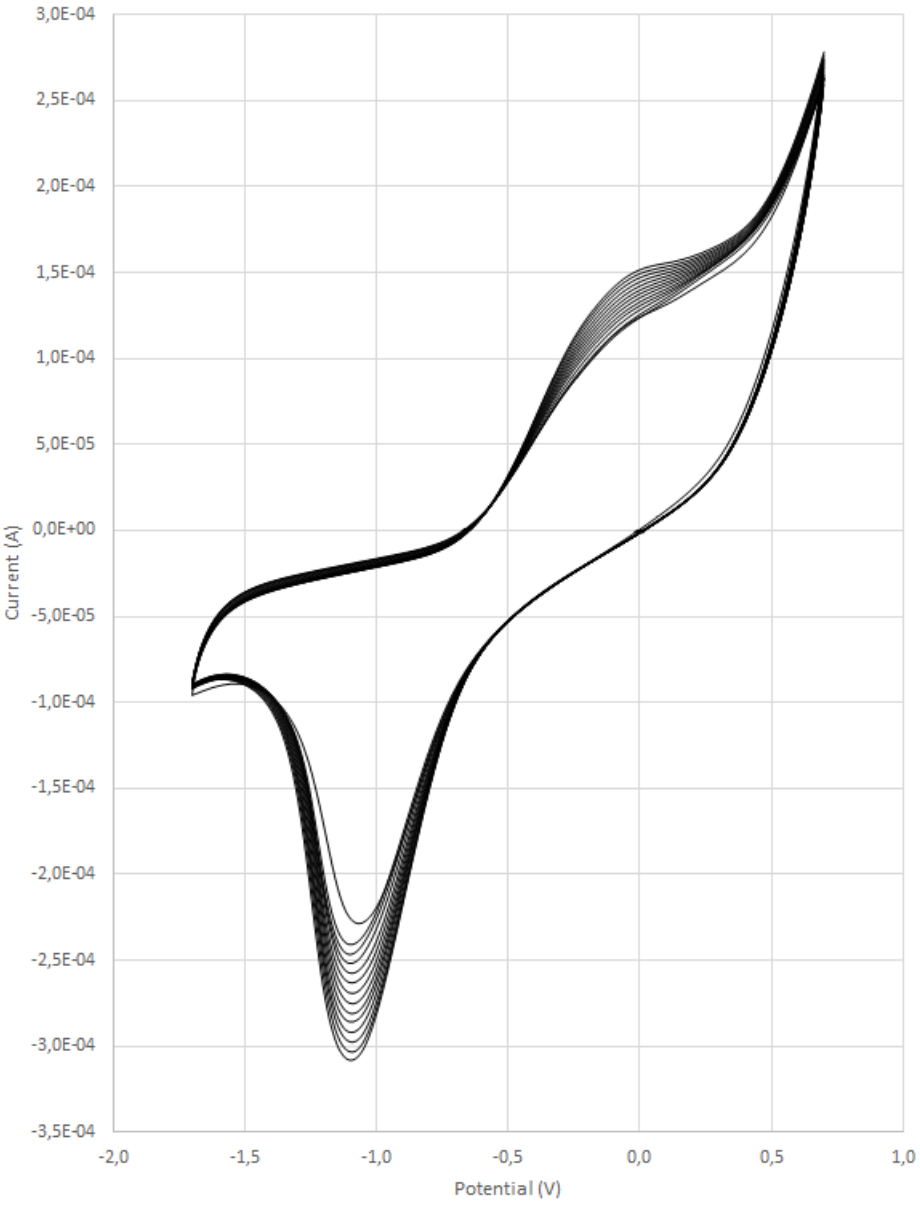



Figure 3.2. Cyclic voltammogram of polypyrrole.

### 3.3 Placement of Invertase

Placement of invertase was achieved via constant potential electrolysis at room temperature. Placement and polymerization was performed according to optimum reaction conditions and activity assay which were determined from previous studies [71]. Although its repeated and continuous utilization of immobilized form has no

economic advantage, it is used as a model enzyme for the immobilization of expensive and more applicable enzymes.

### 3.4 Enzyme Activity

A unit of Invertase Activity is described as the amount of enzyme which produces 1 µmole glucose from sucrose per minute, at a pH value of 5 and 25 °C.

The investigation of enzyme activity of immobilized invertase is performed according to the procedure described previously (2.3.3).

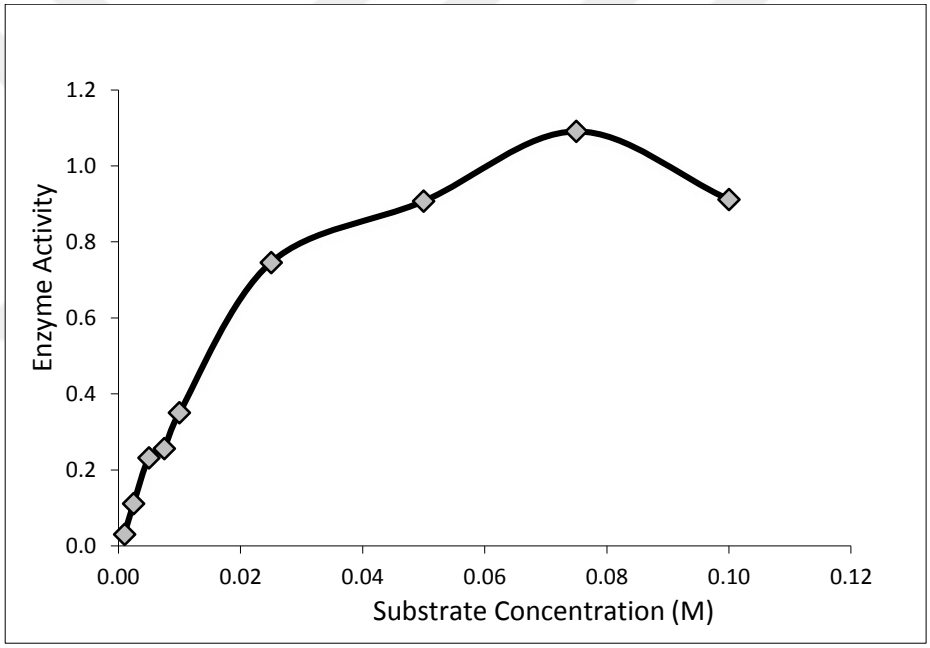



Figure 3.3. Enzyme activity changes by increasing substrate concentration.

Substrate concentration increases until a constant product formation rate is accomplished as shown on Figure 3.2. It means that maximum speed of enzyme reaction, $V_{max}$, is determined. Half of the maximum speed is utilized to get an idea about the specific value of substrate concentration referred as "$K_m$", which shows the interaction between enzyme and its substrate. There is a reverse relationship between affinity and $K_m$ value. Lower $K_m$ value means higher affinity.

From this calibration curve, working range is obtained by taking linear part of the curve. Upper limit of this range is the point at which the deviation starts. Lower point is the lowest detectable substrate concentration. Thus, working range is found as between 0,0025 and 0,0200 M.

### 3.5 Kinetic Parameters of Placed Invertase

Various substrate concentrations which is placed in the working range were used to perform the kinetic studies of invertase. $K_m$, the Michaelis-Menten constant, and $V_{max}$, maximum reaction rate, were determined from Lineweaver-Burk plots drawn in the working range. The kinetic parameters of immobilized invertase activity are illustrated and indicated in Figure 3.3.

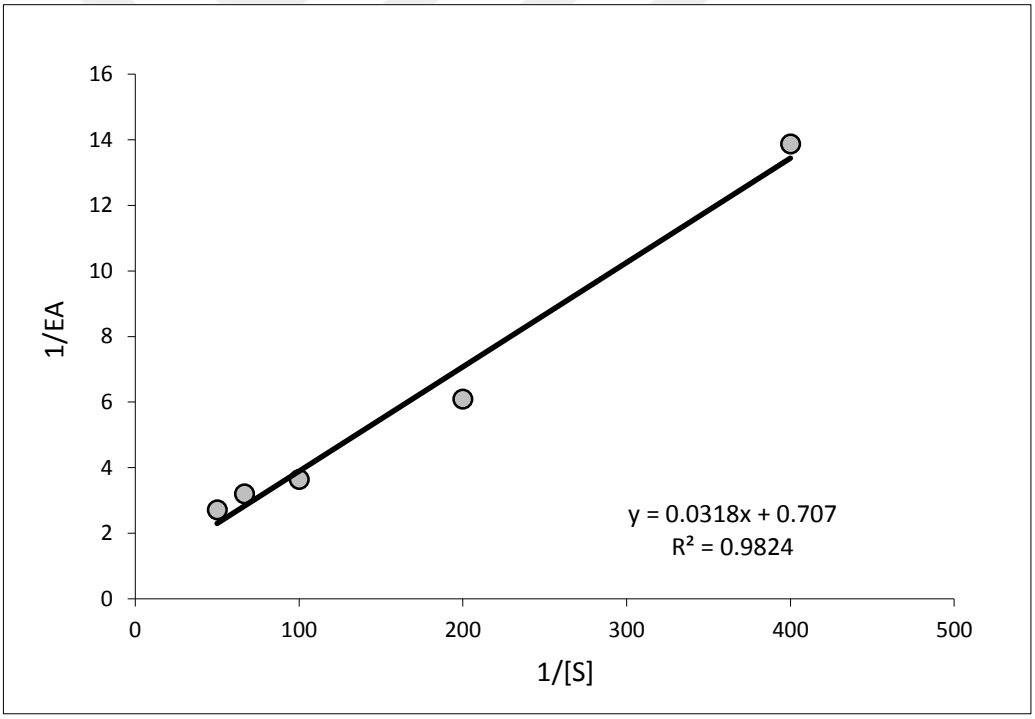



Figure 3.4. Lineweaver-Burk plot of invertase activity.

The outcome reveals that the $K_m$ value of the immobilized invertase on the carbon fiber electrode is very close to the value of the immobilized enzyme on the platinum electrode. The affinity between enzyme and substrate is almost same with two electrode types. Here, electrode material does not affect the affinity because the

environment where enzyme and substrate meet is the polymer. Polymer is same for both electrodes therefore affinity is almost same for two electrode types. On the other hand, reaction rate does not decrease too much compared to the immobilized invertase on platinum electrode. It shows that the amount of enzyme which is immobilized on carbon fiber is lower than that of platinum, which is an expected result. Although the reaction rate is slightly lower, main advantage of carbon fiber electrode is becoming a precursor for the micro and nano electrodes. Therefore, the kinetic parameters are quite acceptable for a micro-like electrode. The calculated values are listed in Table 3.1

Table 3.1. Kinetic parameters for free and immobilized invertase on Pt and CF electrodes.

| | $K_m$ (mM) | $V_{max}$ (µmol/min.electrode) |
|---|---|---|
| Free invertase* | 24,3 | 82,3 |
| Ppy/Pt/invertase* | 52,9 | 2,63 |
| Ppy/CF/invertase | 56,7 | 1,57 |

*Obtained from previous study [72].

### 3.6 pH and Invertase Activity

Enzymes are easily denaturized when they are taken-out from their natural environment since they are sensitive species. Hence, the pH value of medium is very fundamental and vital. In the process of immobilization on the conductive polymer matrix, the proton is released because of the reduction during electropolymerization. So as to avoid the change in pH and so degeneration of enzymes, buffer must be used.

The activity of the enzyme was supervised and examined between pH 2 and 12 (Figure 3.3). The maximum activity was found at the pH 3. At pH 2, enzyme is most probably denaturized due to the highly acidic medium. At pH 3, it shows the maximum activity. Between pH 3 and pH 9, there is a decline. It is more damaging to

work at pH 3. Therefore, in this study, pH 5 acetate buffer was used for practical reasons.

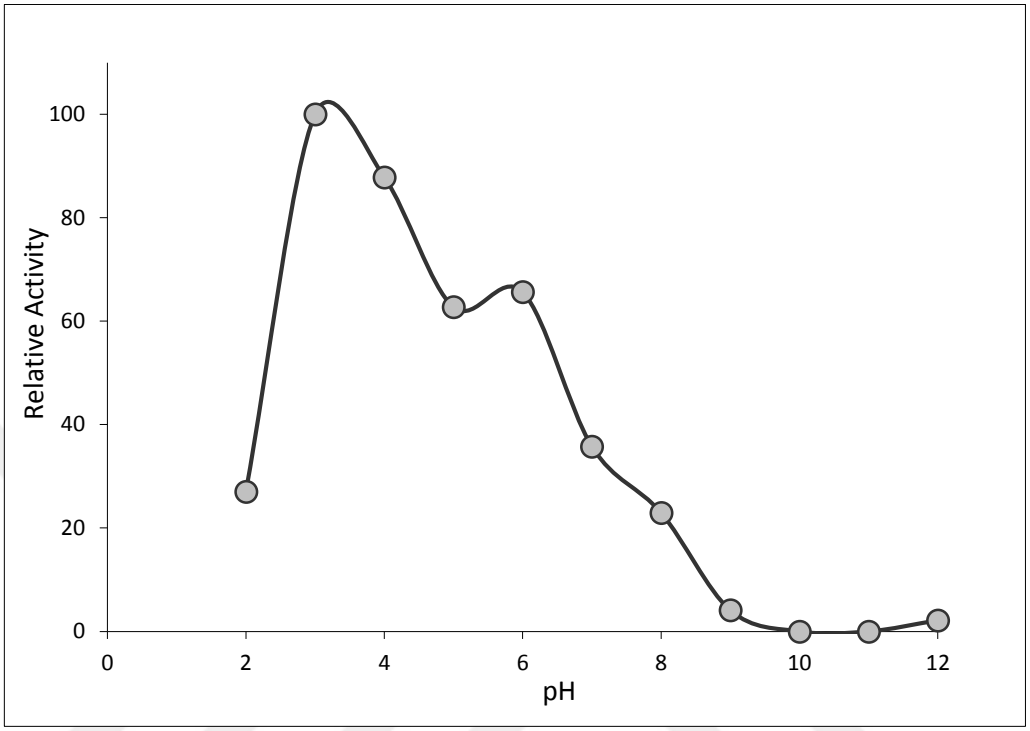



Figure 3.5. Effect of pH on enzyme activity.

### 3.7 Temperature and Invertase Activity

Changes in temperature has an influence on the enzymes which are sensitive molecules. The effect of the temperature on the activities of enzyme have been investigated and demonstrated in Fig 3.5.

The maximum enzyme activity was revealed to be at a level of 30 ºC. Enzyme activity shows a decline of denaturation after 30 ºC, however, the electrodes can be used for a wider temperature ranges between 20 ºC and 50 ºC with a 75% activity. There after 70 ºC, the activity of invertase in the polypyrrole matrix was all lost.

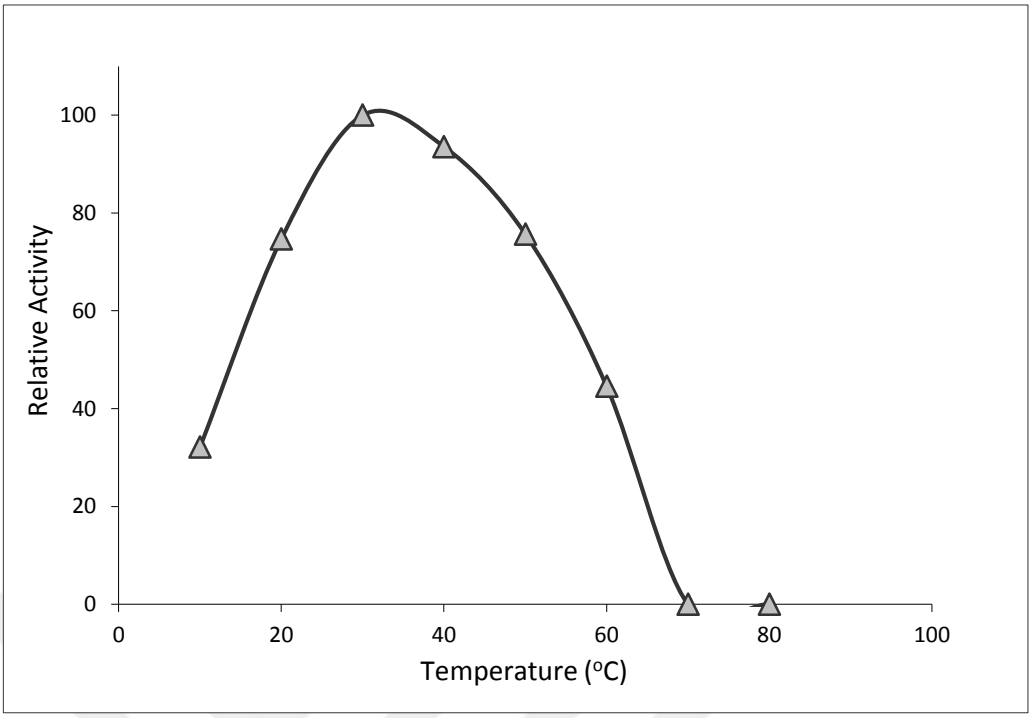



Figure 3.6. Effect of the incubation temperature on invertase activity.

### 3.8 Daily Stability of the Invertase Electrode

The daily stability study was performed by 40 consecutive measurements of invertase electrode on the same day. The enzyme activity after initial steps is about %70 and it keeps this activity until 40$^{th}$ measurement as shown in Figure 3.6.

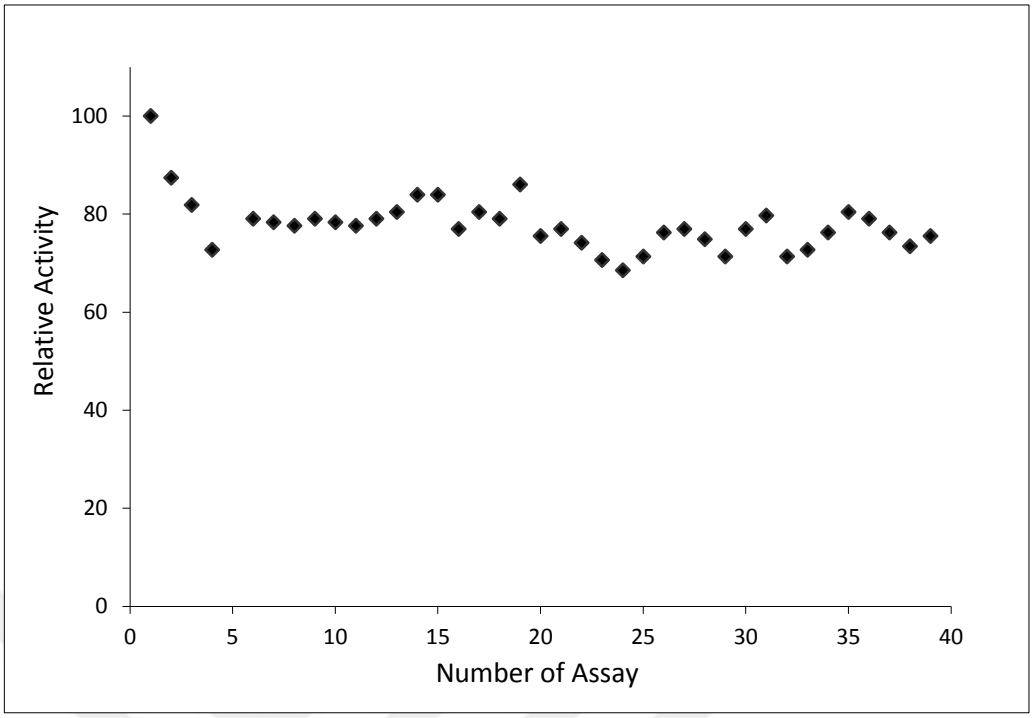



Figure 3.7. Operational stability of enzyme electrode upon consecutive measurements

# CHAPTER IV

## CONCLUSION AND SUGGESTIONS

Electrochemical technology is utilized and applied in the synthesis of conductive polymer. As an electrode material, carbon fiber was used. The proposed spectrophotometric biosensor is characterized and optimized. The kinetic parameters ($V_{max}$, $K_m$) and optimum parameters of the immobilized invertase electrode such as optimum pH and temperature value, linearity and repeatability were examined and determined.

Kinetic parameter was found as 1.57 μmol/min.electrode for maximum reaction rate and 56,7 mM for Michaelis–Menten constant.

In this study, micro level carbon fiber electrodes were examined as a precursor material for nano electrodes. Invertase enzyme was used as a model enzyme. It was found that this enzyme electrodes are promising and this study can be continued with the investigation of some other carbon fiber types and other enzymes.

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## RESUME

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