IMMOBILIZATION OF INVERTASE, POLYPHENOL OXIDASE AND GLUCOSE OXIDASE IN CONDUCTING COPOLYMERS OF THIOPHENE-CAPPED POLYTETRAHYDROFURAN AND PYRROLE

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Approval of the Graduate School of Natural and Applied Sciences.

Prof. Dr. Canan Özgen
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Hüseyin İşçi
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Levent Toppare
Supervisor

Examining Committee Members

Prof. Dr. Nesrin Hasırcı (METU,CHEM) _____

Prof. Dr. Levent Toppare (METU,CHEM) _____

Prof. Dr. Mustafa Güllü (ANKARA UNIV,CHEM) _____

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Name, Last name : **A. Elif Böyükbayram**

Signature :

ABSTRACT

IMMOBILIZATION OF INVERTASE, POLYPHENOL OXIDASE AND GLUCOSE OXIDASE IN CONDUCTING COPOLYMERS OF THIOPHENE-CAPPED POLYTETRAHYDROFURAN AND PYRROLE

Böyükbayram, Ayşe Elif

Ph.D., Department of Chemistry

Supervisor: Prof. Dr. Levent Toppare

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Immobilization of invertase, polyphenol oxidase (PPO) and glucose oxidase (GOD) enzymes were performed in electrochemically synthesized two types of conducting copolymers. One end and two end thiophene-capped polytetrahydrofuran (TPTHF-1 and TPTHF-2) were copolymerized with pyrrole under conditions of constant potential electrolysis. The copolymers were characterized by thermal, spectroscopic and scanning electron microscopy analyses.

Immobilization was carried out via entrapment of enzymes in two types of matrices during the copolymerization of pyrrole with the insulating polymers in the presence of sodium dodecyl sulphate (SDS). Kinetic parameters: Maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) were determined for the enzyme electrodes. Temperature optimization, pH optimization, operational stability and shelf-life of the enzyme electrodes were investigated.

Enzyme electrodes of polyphenol oxidase and glucose oxidase were used to determine the amount of their substrates in samples. Polyphenol oxidase converts mono and diphenols to quinone. Amount of phenolic compounds in two kinds of wines were determined by analyzing the quinone amount. Glucose oxidase converts β-D-glucose to D-glucono-1,5-lactone. Glucose amount was determined in two kind of factory-produced orange juices by analyzing D-glucono-1,5-lactone.

Keywords: Electrochemical polymerization, conducting copolymer, immobilization of enzymes, invertase, polyphenol oxidase, glucose oxidase.

ÖZ

İNVERTAZ, POLİFENOL OKSİDAZ VE GLUKOZ OKSİDAZ ENZİMLERİNİN TİYOFEN İLE SONLANDIRILMIŞ POLİTETRAHİDROFURAN İLE PİROLÜN İLETKEN KOPOLİMERLERİNDE TUTUKLANMASI

Böyükbayram, Ayşe Elif
Doktora, Kimya Bölümü
Tez Yöneticisi: Prof. Dr. Levent Toppare

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Invertaz, polifenol oksidaz (PPO) ve glukoz oksidaz (GOD) enzimleri elektrokimyasal yolla sentezlenen iki iletken polimerde tutuklandı. Tek ucu ve iki ucu tiyofenle sonlandırılmış politetrahidrofuran sabit gerilim elektroliz ortamında pirolle kopolimerleştirildi. Elde edilen kopolimerler termal, spektroskopik ve taramalı elektron mikroskobu analizleriyle karakterize edildi.

Tutuklama pirolün yalıtkan polimere kopolimerleşmesi sırasında sodyum dodesil sülfat varlığında gerçekleştirildi. Enzim elektrotlarının kinetik parametreleri: maksimum tepkime hızı (V_{maks}) ve Michaelis-Menten sabitleri (K_m) saptandı. Enzim elektrotlarının optimum sıcaklık ve pH değerleri ve kararlılıkları incelendi.

Polifenol oksidaz ve glukoz oksidaz enzim elektrotları numunelerde bulunan sübstratlarının miktar tayininde kullanıldı. Polifenol oksidaz mono ve difenolleri kinona çevirir. Kinon miktarı tayin edilerek iki kırmızı şarapta fenolik yapıların miktar tayini yapıldı. Glukoz oksidaz β-D-glukozu D-glukono-1,5-laktona çevirir. D-glukono-1,5-lakton miktarı tayin edilerek iki portakal suyu çeşidindeki glukoz miktarları saptandı.

Anahtar Kelimeler: Elektrokimyasal polimerleşme, iletken kopolimer, enzim tutuklaması, invertaz, polifenol oksidaz, glukoz oksidaz.

To the dear memory of my father

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ABREVIATIONS

CB	Conduction Band
DSC	Differential scanning calorimetry
EE	Enzyme electrode
FAD	Flanine adenine dinucleotide
FTIR	Fourier infrared spectrometry
GOD	Glucose oxidase
HOMO	Highest occupied molecular orbital
INV	Invertase
LUMO	Lowest unoccupied molecular orbital
MBTH	3-methyl-2-benzothiozolinone
PPO	Polyphenol oxidase
Ppy	Polypyrrole
PTHF	Polytetrahydrofuran
Py	Pyrrole
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TGA	Thermal gravimetry analysis
TPTHF1	One end thiophene capped polytetrahydrofuran
TPTHF2	Two end thiophene capped polytetrahydrofuran
VB	Valence Band

CHAPTER I

INTRODUCTION

1.1 Conducting Polymers

Conducting polymers have attracted a great deal of attention in the last twenty years because of their unusual electronic properties. They exhibit conducting properties of metals, attractive mechanical properties and processing advantages of polymers.

1.1.1 History of Conducting Polymer

Chemical oxidative polymerization of aniline was described by Letheby in 1862, and studied in more detail by Mohilner et al. in 1962 [1]. Pyrrole was known to form a conductive "pyrrole black" [2] via spontaneous polymerization in air on the sides of pyrrole containers, and its chemical polymerization studied in detail in 1916 [3]. Since 1957, studies of electrochemical oxidation of aromatic monomers,

now widely used as one of the method of synthesis of conducting polymers, have been reported under various descriptions such as electro-organic preparations and electro-oxidations [4,5]. More recently, in 1967, electrically conducting polymers from pyrrole, thiophene and furan were characterized [6] and the electrical conductivity of polyanilines noted [7]. As early as 1968, dall'Ollio [8] described electropolymerization of polypyrrole. Poly (*p*-phenylene sulfide), PPS, has been commercially produced for thermoplastics applications since the early 1970's, and well-defined syntheses of polyacetylene have been reported since 1971 [8].

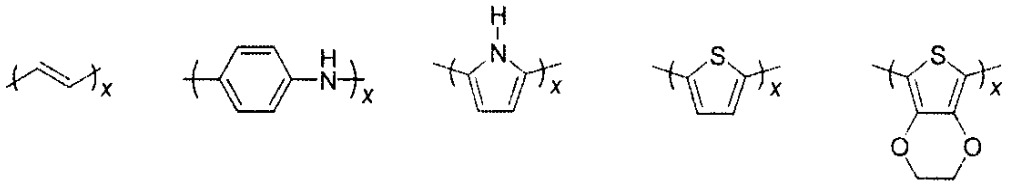
A key discovery in the development of conducting polymers was the finding in 1973 that the inorganic polymer sulfur nitride, $(SN)_x$, showed metallic property [9]. The room temperature conductivity of $(SN)_x$ is of the order of 10^3 S/cm, to be compared with 1×10^6 S/cm for copper, 10^{-14} S/cm for polyethylene. Although its other physical properties, such as its explosive nature, prevented it from becoming commercially important, $(SN)_x$ provided the field with the insight that eventually led to the discovery of an entirely new class of conducting polymers.

MacDiarmid and Shirakawa in 1977 discovered that the simplest conjugated polymer, conducting polyacetylene show very high and well characterized conductivities for an organic material when oxidized by suitable reagents namely vapors of the halogens. This resulted that the field of conducting polymers has developed

and expanded at a rapid pace [10]. Largely in recognition of the important role that these materials are expected to play in the near term technology, the Nobel Prize in chemistry was awarded in 2000 to the discoverers of conducting polyacetylene; Shirakawa, MacDiarmid and Heeger.

Further impetus to the field was given by the repetition and refinement, by the Diaz group [11] of the electrochemical polymerization of pyrrole originally ascribed to dall'Ollio, with a much better characterized polymer produced.

The concepts of conductivity and electroactivity of conjugated polymers were quickly broadened from polyacetylene to include a number of conjugated hydrocarbon and/or aromatic heterocyclic polymers such as poly (p-phenylene) [12], polypyrrole [13,14] and polythiophene [15]. Structures of repeating units of several conjugated polymers; polyacetylene (PA), polyaniline (PANI), polypyrrole (Ppy), polytyhiophene (PTh), poly(3,4-ethylenedioxythiophene) (PEDOT) are shown in Figure 1.1.



(PA) (PANI) (Ppy) (PTh) (PEDOT)

Figure 1.1. Common conducting polymers.

Although the conducting polymers have some advantages such as low density and easy processibility, they do not have good environmental and thermal stability. Recently the researches focused mainly to improve their properties [15-17].

1.1.2 Conductivity Mechanism in Conducting Polymers

1.1.2.1 Conductivity and Band Theory

Electronic conduction in general is the transport of charge carriers such as electrons, holes, polarons, solitons, through a medium, under the influence of electric field. Thus, the conductivity displayed by any medium is characterized by the number of charge carriers available and their ability to move through the medium.

Conducting polymers are materials consisting of polymeric molecules with polyconjugation in which the conduction depends on the deficiency or excess π-electrons exist in the polyconjugated chain. This can be achieved by doping (*p-type* or *n-type*) which refers to insertion of holes or electrons [18] and their conductivity can be increased over about ten orders of magnitude in the same material. Figure 1.2 shows conductivities of various conductors.

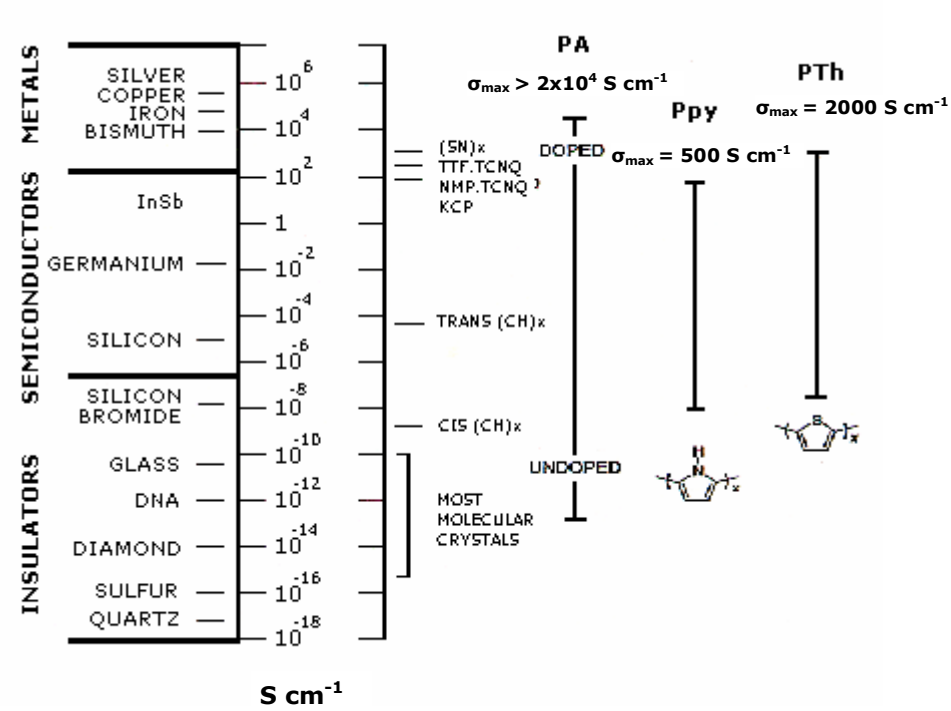


Figure 1.2. Conductivity ranges.

The removal and insertion of electrons in conjugated polymers as a result of doping process can be explained by band theory. According to band theory, atomic orbitals combine to form a spatially extended, delocalized energy band and the conductivity then depends on the relative population of each band and the energy difference between bands. Charge transport can occur only in partially occupied bands. Figure 1.3 illustrates the generic band structure for a metal, semiconductor and insulator. The gap between valance band (VB) and conduction band (CB), better defined as the *band gap*, E_g, differentiates those three materials [19].

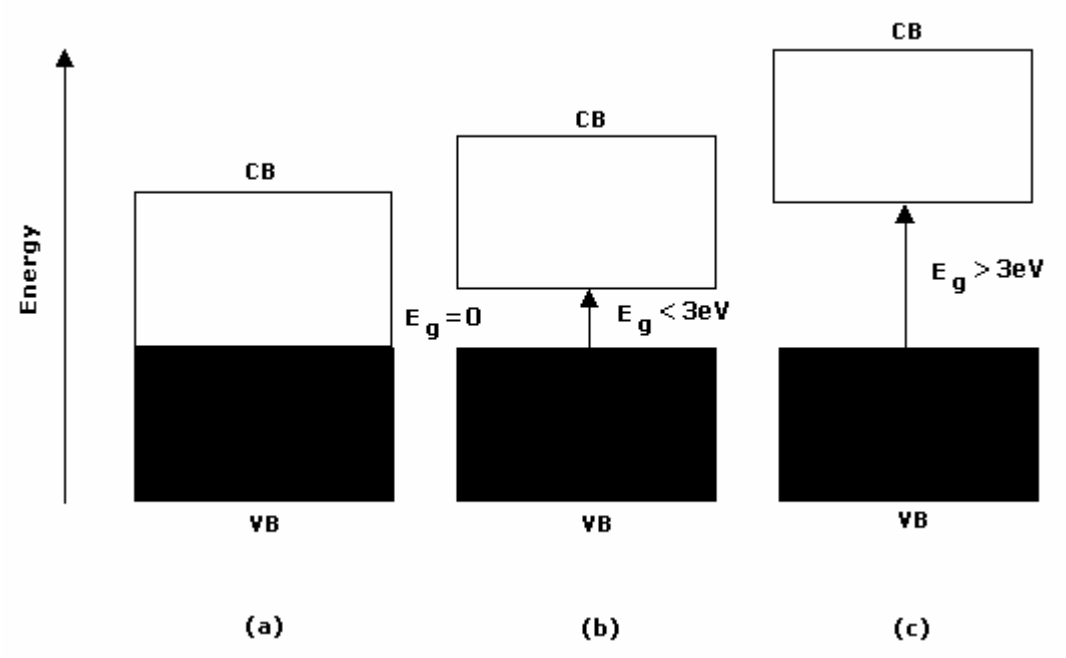


Figure 1.3. Band structure **(a)** metal, **(b)** semiconductor, **(c)** insulator.

Metals are the most familiar conducting materials and characterized by a zero band gap originated from partially filled bands, where movement of charge carriers can occur freely and conduction occurs. Therefore, even at low temperatures, they show high conductivity.

In semiconductors, there is a filled valence band and an empty conduction band, separated by a band gap (E_g < 3eV), thereby, making conduction slightly more difficult. Introduction of dopants into semiconductor materials increases conductivity by producing states which lie close to either the conduction or valence bands. The conduction band can be populated at the expense of the valence band by exciting electrons across the band gap either thermally or

photochemically. In semiconductors, doping increases their conductivity. Degree of increase in conductivity depends on the type of dopant used either holes (*p-type*) or electrons (*n-type*) as the charge carrier [19].

Insulators have a band structure similar to semiconductors except they have very large band gaps (E_g > 3eV) that are inaccessible under normal environmental conditions. Insulators exhibit a much higher band gap over resulting in poor conductivities.

Neutral conjugated polymers are usually treated as semiconductors and band theory can be used to describe their electronic energy levels. As shown in Figure 1.4, the energy difference between the highest occupied molecular orbital (HOMO) and the lowest occupied molecular orbital (LUMO) decreases as the conjugation length increases along with an increase in number of energy levels. When referring to discrete π conjugated molecules, the energy between the HOMO and the LUMO is a π to π* transition in UV-VIS spectrum. The band gap of a polymer refers to the onset of absorption of π to π* transition. Conjugated polymers, semiconductor in its neutral form, show interband transitions between VB and CB lowering the effective band gap upon the oxidation or reduction. This results in the formation of charge carriers along the polymer backbone.

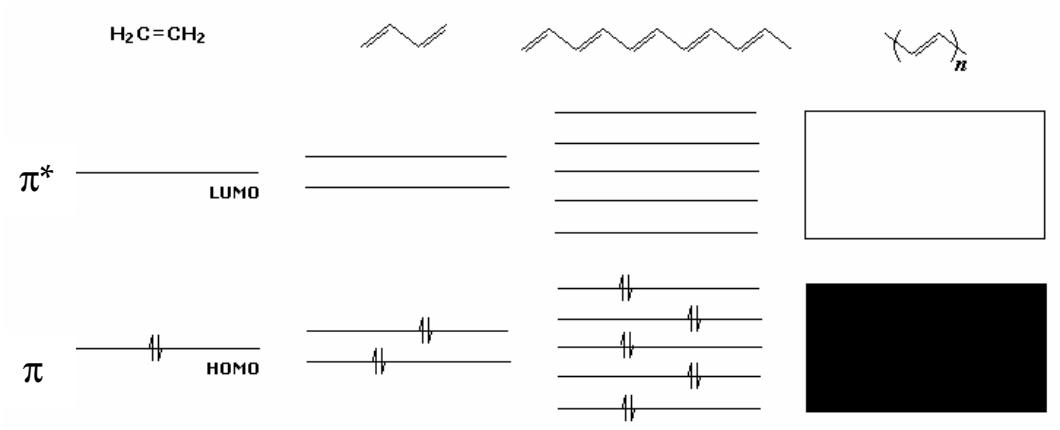


Figure 1.4. Energy levels in conjugated polyenes with increasing conjugation length.

1.1.2.2 Charge Carriers

Doping is the creation of defects in the structure of polymer without destroying the chain. These defects in the polymer, which can be radicals, anions, cations or combinations of these, are the charge carriers. They are called solitons and polarons. Conductivity by movement of charge carriers is explained by intra- and inter-chain hopping. The behavior of a charge carrier is "hopping" along the chain and between the chains respectively. Although intra-chain model is suggested at low doping levels, inter-chain hopping is also used as the doping level increases [8].

PA is unique in that both resonance forms of the neutral polymer are degenerate, leading to the formation of solitons. PA

chains with odd number of carbon atoms have an unpaired electron (*a neutral soliton*). Upon oxidation or reduction, a radical cation (*positive soliton*) or anion (*negative soliton*) is generated which moves along a polymer chain by the mechanism shown in Figure 1.5.

Figure 1.5. Charge carrier soliton in conducting polymer PA.

The localized electronic state associated with the soliton is a nonbonding state at an energy lying at the middle of the π-π* gap, between the bonding and antibonding levels of the perfect chain. Soliton model as shown in Figure 1.6, was first proposed for degenerated conducting polymers (PA in particular) and it was noted for its extremely one dimensional character, each soliton being confined to one polymer chain. Thus, there was no conduction via interchain hopping [20] and the conduction by soliton can be attributed to intrachain hopping.

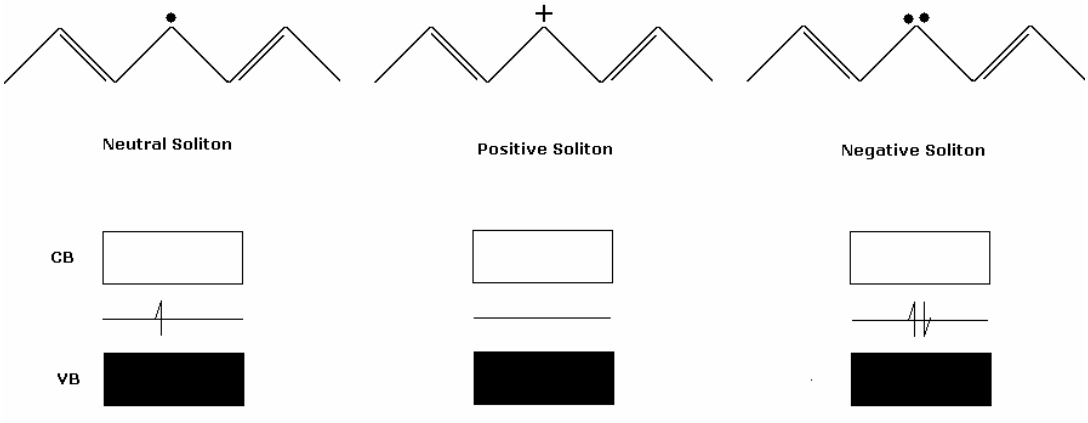


Figure 1.6. Soliton models and associated band diagrams in PA.

All conducting polymers other than PA have non-degenerate states and a different proposal exists for those aromatic polymers. In poly (heterocycles) there is an aromatic state and a quinoid state of higher energy. Oxidation of a poly (heterocycle) creates a radical cation called *polaron*. Polaron has a spin number of ½ as a radical cation. Further oxidation causes more and more polarons to form and eventually the unpaired electron of the polaron is removed, or two polarons can be combined to form spinless dications or *bipolarons*. Unlike the solitons in polyacetylene, the two positive charges of the bipolarons stay associated and act as one charge carrier dispersed over many rings. The length of the bipolaron unit is thought to be 5 to 8 rings, although this is an arbitrary number that can be different depending on the specific polymer system [21]. Experimental results from electron paramagnetic resonance spectroscopy (EPR) support this mechanism showing neutral and heavily doped polymers have no unpaired electrons, but moderately doped polymers do exhibit an EPR signal and are paramagnetic

[22,23]. The mechanism of charge carrier generation in these systems has been studied extensively, and the most widely accepted mechanism is shown in Figure 1.7.

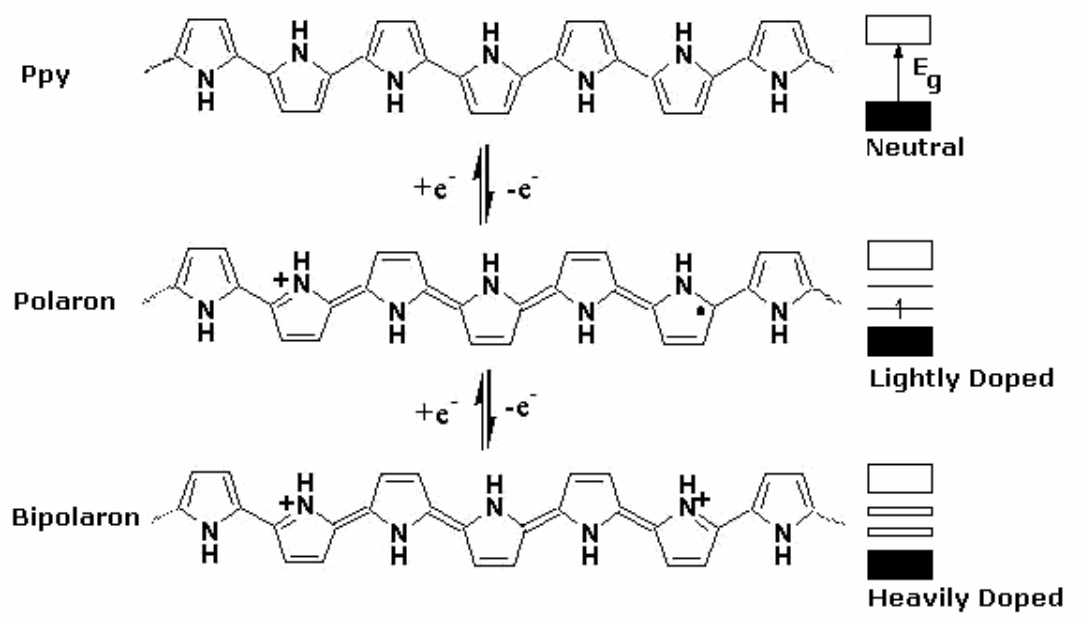


Figure 1.7. Structural schemes of polaron and bipolaron formed by oxidative doping of Ppy and associated band structures.

In fact, conduction mechanism of conducting polymers has been found to be a very complex phenomenon while many models, such as ionic conduction, band-type conduction, hopping conduction, have been proposed, and to date no single model is comprehensively accurate. In varying degrees, the various models are able to account for conduction behavior within a specific temperature range or doping range or dopant type or other conducting copolymers, but then fail for other range of types, or for other conducting copolymers.

1.1.3 Synthesis of Conducting Polymers

1.1.3.1 Chemical Polymerization

Among the chemical synthesis, oxidative polymerization of conducting polymers represents the least expensive and most widely used means [24] in which a stoichiometric amount of oxidizing reagent is used to form polymer that is in its doped or conducting form. Poly(heterocycles) are usually polymerized with $FeCl_3$ as the chemical oxidant [25,26]. Isolation of neutral polymer is accomplished by addition of a strong base such as ammonium hydroxide or hydrazine. Chemical oxidative polymerizations suffer from several disadvantages that often result in poor quality polymers such as being more rigid and decomposition by overoxidation. The last one can be overcome by electrochemical oxidative polymerization method, because the potential here can be finely controlled. Furthermore, there is also an abundance of side reactions occurring during chemical oxidation polymerization of heterocycles including formation of coupling defects along the backbone [26].

1.1.3.2 Electrochemical Polymerization

Electropolymerization is an efficient method for preparing electrically conducting conjugated polymers as smooth films on conductive substrates. It involves the oxidation of a monomer

dissolved in a supporting electrolyte solution by applying an external potential which is the monomer oxidation potential to form reactive radical cations and allows for easy probing of electrical properties. Because of the oxidative nature of electropolymerizations, the deposited polymer is typically in its oxidized state, thus necessitating the presence of a supporting electrolyte anion to compensate the positive charges along the polymer backbone. The efficiency of the polymerization is dictated by the ease with which electrons can be removed from the monomer and by the stability of the resultant radical cation through the resonance stabilization. Electrochemical polymerization mechanism for polypyrrole is show in Figure 1.8. Electron-rich monomers, such as thiophene and pyrrole, are able to lose an electron easily, and are also able to stabilize the resultant radical cation through resonance across the p-electron system better than relatively electron-poor compounds. Upon electropolymerization, stable, electroactive polymers can be derived from electron-rich heterocycles.

Electrochemical polymerization presents several advantages which make it a unique tool for electrochemical studies as well as for some specific electrochemically oriented technological applications of conjugated polymers [27]. Rapidity is the most immediate feature of electropolymerization. The growth of a polymer film of a few hundred nanometers thickness requires only a few seconds, compared to several hours and tedious work-up required by chemical methods.

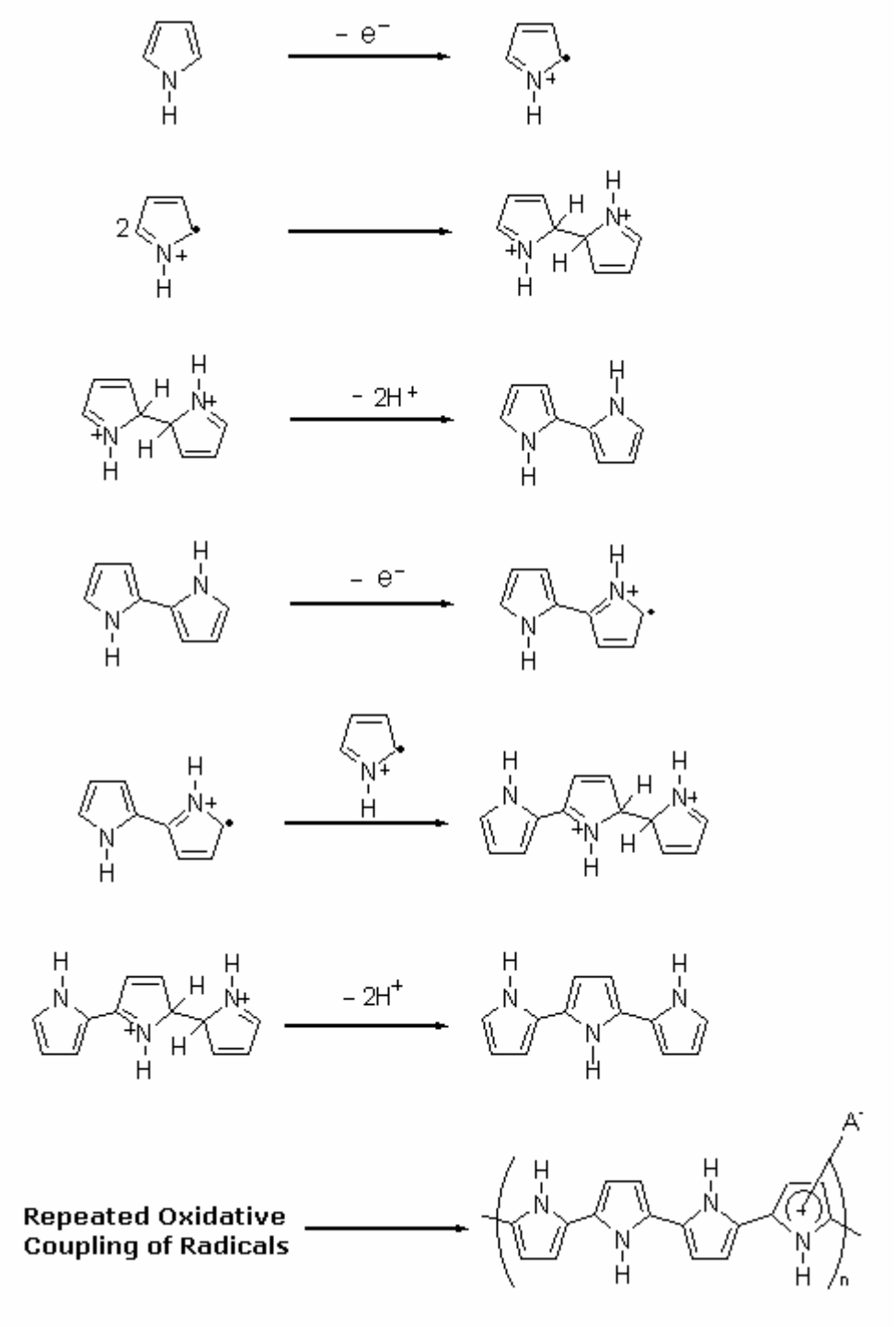


Figure 1.8. Resonance stabilization and electrochemical polymerization mechanism for poly (heterocycle).

Simplicity is another evident advantage that conducting polymers are directly obtained in their oxidized (doped) conducting form. In addition to further time saving, this specific one-step process leads to more heavily and more homogenously doped materials than post polymerization doped chemically synthesized polymers.

Despite the facile nature of electrochemical polymerization, this method possesses the major limitation of yielding insoluble materials, precluding the analysis of primary structure by traditional analytical techniques. Because of this limitation, chemical polymerization methods have gained popularity for synthesizing novel soluble conjugated polymers [27].

1.1.4 Conducting Copolymers

Copolymerization with other polymers to form block or graft copolymers leads to ability to be processed into a conducting polymer. Conducting polymers are promising for commercial applications because of their facile synthesis, good environmental stability, and long term stability of electrical conductivity. However, several drawbacks restrict their processing and applications for practical use. These films are insoluble, hard and brittle and have a variety of conductivity values depending on the electrolysis conditions. Copolymerization approach allows these limitations to be overcome [28]. A blend of a conducting polymer with an

insulating polymer also has considerable utility for improving the processing of the conducting polymer. Electrochemical synthesis of copolymer films of pyrrole and substituted pyrroles [28] pyrrole and thiophene [29,30] and other combination of aromatic compounds [31] have been reported.

For the preparation of conducting composites and copolymers, electropolymerization of the conducting component is carried out on electrode previously coated with the insulating polymer. Several groups have reported that the electrochemical polymerization of pyrrole and thiophene may occur on an ordinary insulating polymer film [32,33]. In these cases, monomer and solvent molecules and electrolyte anions swell the polymer film as they diffuse into the polymer coating and polymerization starts in the interface between the electrode surface and the insulating polymer film. Constant potential electrolysis (CPE) is carried out in a three electrode system (working, counter and reference electrodes). In this method the potential of the working electrode with respect to a reference electrode is adjusted to a desired value and kept constant by a potentiostat. The current is allowed to alternate while the potential is constant.

1.1.5 Applications of Conducting Polymers

Conducting polymers have been investigated for a very wide variety of applications mostly for rechargeable batteries [34,35],

owing to their attractive features of light weight, low cost, presumed having ability being processed into odd shapes and sizes, less corrosive nature and compatibility with organic liquid and solid electrolytes. Present and potentially future applications of conducting polymers also include EMI shielding, electrostatic materials, LED devices [36], transistors, anti-corrosion coatings, electrochromic displays [37], semiconductor devices like molecular electronics, aerospace applications, textile materials, biosensors [38-46], artificial nerves, membranes [47-50].

1.2 Enzymes

Life depends on a complex network of chemical reactions carried out by biological catalysts named as enzymes which increase the rate of chemical reactions taking place within the living cells [51]. Because of their role in maintaining life processes, the assay and pharmacological regulation of enzymes have become key elements in clinical diagnosis and therapeutics. Enzymes consist of protein chains of amino acids held together by peptide bonds. These protein chains fold to form a compact macromolecule with a precisely defined tertiary and quaternary structure.

The concept of enzyme specifity and close steric relationship of an enzyme to its substrate can be described by lock-and-key analogy of enzyme-substrate interaction. A necessary consequence of this close stereospecific fit between enzyme and substrate at a

specific region of the protein called active site is that each enzyme acts on a limited number of substances. Absorbing the particular substrate molecule onto the complementary surface of the enzyme and catalyzing the conversion of this substrate to a product that is released back to solution leave the enzyme catalyst unaltered [51]. Enzymes are catalysts different from their inorganic counterparts in possessing high activity, high degree of specificity of action and the ability of operating very mild circumstances (pH, solvent, temperature and pressure). Moreover, enzymes, being a natural product, are susceptible to degradation even at low temperature [52].

In the past several decades an enormous number of enzymatic reactions have been discovered. Individual enzymes have been isolated from bacteria, molds, yeast, plants and animals.

1.2.1 Enzyme Nomenclature

A systematic list based on a numerical notation, the EC number, has been laid down by the *Commission on Enzymes of the International Union of Biochemistry*. The systematic name of the enzyme consists of two parts: The first part describes the substrates and the second part defines the type of reaction catalyzed [53].

1.2.2 Enzyme Classification

The increasing number of isolation and characterization of enzymes has made it necessary for a systematic nomenclature to be introduced, so that any given enzyme can be precisely identified. Enzymes are divided into six main classes, on the basis of the total reaction catalyzed [51]. Some enzymes exhibit the ability to catalyze a single unique reaction. More frequently, however, enzymes exhibit group specificity, catalyzing a number of reactions involving a common functional group.

According to this approach, all enzymes catalyze one of six types of reactions [53]:

1. Oxidoreductases catalyze redox reactions.
2. Transferases catalyze transfer of groups such as methylenes or phosphates.
3. Hydrolases catalyze hydrolysis.
4. Lyases catalyze the addition of groups to double bonds or vice versa.
5. Isomerases catalyze molecular isomerization.
6. Ligases catalyze synthesis reaction involving condensation of two molecules with the hydrolysis of a pyrophosphate bond.

Each enzyme was assigned a code number, the EC number, consisting of four elements, separated by dots. The first digit shows the main class the enzyme belongs. The second and third digits in

the code further describe the kind of reaction being catalyzed. Enzyme catalyzing very similar but non-identical reactions, e.g. the hydrolysis of carboxylic acid esters, will have the same first three digits in their code. The fourth digit distinguishes between them by defining the actual substrate. An enzyme code allocated only once, so that it provides an unambiguous and clear definition of the enzyme concerned.

1.2.3 Enzyme Activity

With most enzymes the amount of change produced by the enzyme is only proportional to the substrate concentration in the early part of the reaction [54]. The method employed should yield results which are independent of the concentration of the enzyme solutions. Activity of the enzyme is determined at its optimum with respect to pH, temperature, concentration of substrate, presence of activators, etc.

Enzyme activity power is expressed in units. The amount of change or destruction of a given quantity of a substrate under certain definite conditions is expressed as unit activity. The number of units is defined as μmoles of substrate transformed to product per minute per mg of total enzyme-polymer conjugate. Another way of expressing the activity of an enzyme is by specific activity, the number of μmoles of substrate transformed to product per minute

per mg of protein, under certain specified conditions of temperature, pH, etc.

1.2.4 Enzyme Kinetics

Catalytic function efficiency of the enzyme is usually determined by measuring their effect on the rate of chemical reaction. The rate of enzyme catalyzed reactions was first studied in late nineteenth century. In 1913, Michaelis and Menten [54] measured initial rates of the invertase catalyzed hydrolysis of sucrose by keeping all experimental parameters including enzyme concentration, pH and temperature while substrate concentration varied between wide limits. It was found that an enzyme substrate complex was formed and the reaction rate increases hyperbolically toward a maximum rate as the substrate concentration increases. Michaelis and Menten derived the rate equation for the enzyme catalyzed reaction involving single substrate, single intermediate and equilibrium between the free enzyme and the enzyme-substrate complex. The following reaction mechanism was proposed:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P$$

where E is the free enzyme, S is the substrate, ES is the enzyme-substrate complex and P is the reaction product. It was assumed that

the rate of breakdown of the ES to product was much slower than the dissociation of ES into enzyme and substrate.

Michaelis and Menten derived the rate equation based on the assumption that the rate of breakdown of the ES to product was much slower than the dissociation of ES into enzyme and substrate therefore,

$$k_{+2} \ll k_{-1}$$

However, the formulation of Michaelis and Menten, which treats the first step of enzyme catalysis as on equilibrium, makes unnecessary and unwarranted assumptions about the rate constants. Thus, a more valid derivation was proposed by Briggs and Haldane [55] based on the initial rate of reaction. It was assumed that almost immediately after the reaction starts a steady state is achieved in which the concentration of the intermediate (enzyme-substrate complex) is constant.

$$d[ES]/dt = k_{+1}[E][S] - k_{-1}[ES] - k_{+2}[ES] = 0 \quad (1)$$

Total enzyme concentration, $[E]_o$ can be written as the sum of [E] and [ES],

$$d[ES]/dt = k_{+1}[E]_o[S] - (k_{+1}[S] + k_{-1} + k_{+2})[ES] = 0 \quad (2)$$

then by dividing (2) by k_{+1} and solving for [ES],

$$[ES] = \frac{[E]_o [S]}{(k_{-1} + k_{+2}) / k_{+1} + [S]}$$

the following rate equation is obtained;

$$V = k_{+2} [ES] = \frac{k_{+2} [E]_o [S]}{(k_{-1} + k_{+2}) / k_{+1} + [S]}$$

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

K_m is known as *the Michaelis constant*, the equilibrium constant for dissociation of enzyme-substrate complex and is inversely related to the affinity of the enzyme for its substrate. The V_{max} is the *maximum velocity of reaction* which is attained when the entire enzyme is in the form of enzyme substrate complex.

It was shown that this theory and equation could account accurately for their results with invertase, and because of the definitive nature of their experiments, which have served as a standard for almost all subsequent enzyme kinetic measurements. Today, Michaelis and Menten are regarded as the founders of modern enzymology and equation 1 is generally known as the *Michaelis-Menten equation*.

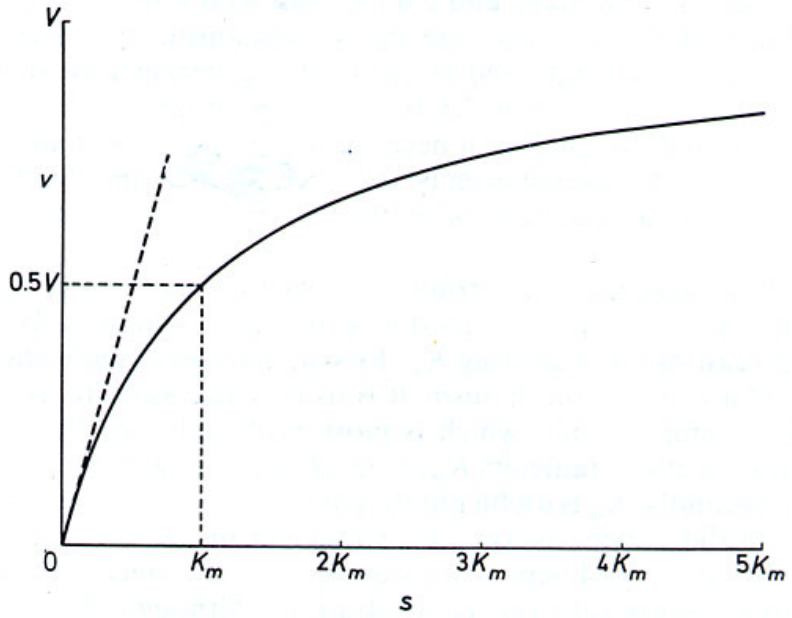


Figure 1.9. Plot of initial velocity against substrate concentration for enzyme catalyzed reaction.

The initial rate of reaction obeying Michaelis-Menten equation as a function of the substrate concentration at constant enzyme concentration is given in Figure 1.9. At very low substrate concentrations, velocity is directly proportional to [S] so that reaction is apparently first order in S. At very high substrate concentrations, velocity will approach to V_{max} and the reaction is apparently zero order in S. Under these conditions, the enzyme is said to be saturated with substrate.

Graphical representation of Michaelis and Menten equation is desirable if a series of initial velocities at different substrate concentrations is measured, so that the kinetic parameters, K_m and V_{max} can be determined. However, the plot of V against S, generating

a rectangular hyperbola which is unsatisfactory in practice due to difficulty in drawing hyperbolas accurately and then estimating asymptotes. Therefore, in order to determine these kinetic parameters, for an enzyme catalyzed reaction, a linear relation would be more useful.

Lineweaver and Burk [56] have preferred to rewrite the Michaelis and Menten equation in a form that permits the results to be plotted as a straight line,

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

The plot of 1/V versus 1/[S] will give a straight line of slope K_m/V_{max} and y intercept of $1/V_{max}$ (Figure 1.10).

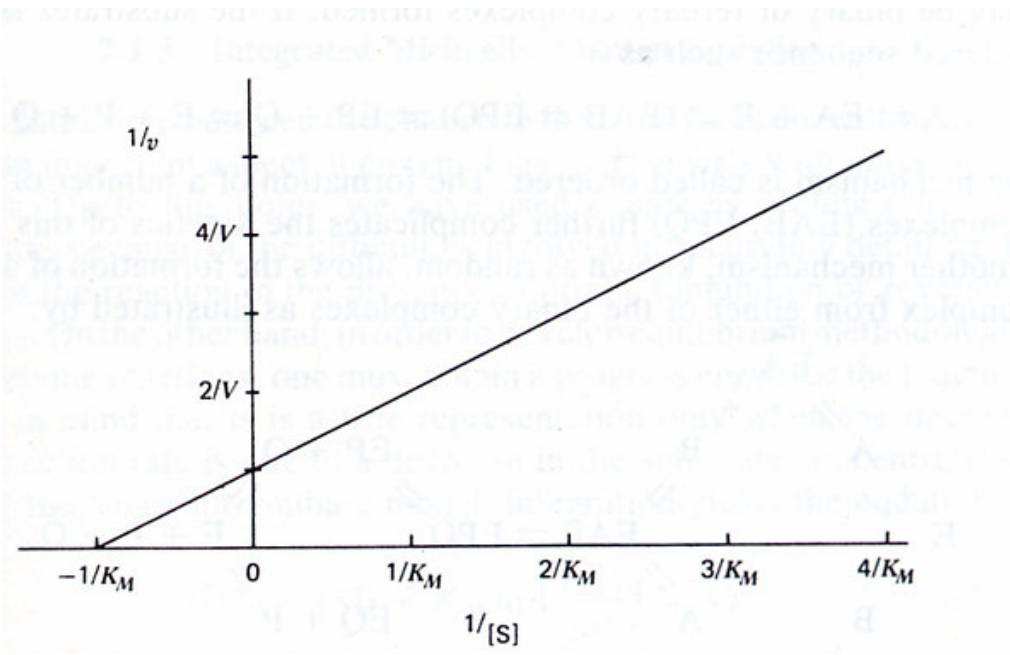


Figure 1.10. Lineweaver- Burk plot.

1.3 Enzyme Immobilization

A biocatalyst is termed as *immobilized* if its mobility has been restricted by chemical or physical means, while retaining the catalytic activity. In this process, enzyme molecules are in distinct phase which allows exchange with, but is separated from, the bulk phase in which substrate molecules are dispersed and monitored.

The environments where the enzymes are present affect the enzyme in several ways:

1) Partitioning effect: Presence of ionic solute in the microenvironment around the enzyme at a concentration different to that in the bulk phase because of inhomogeneous distribution may occur when polyionic polymer support is used.

2) Diffusion limitation: It depends on the physical characteristics of support. If it has no proper porous structure, enzymes may not be able to react with the substrates.

3) Conformational changes: There are two factors making the enzymes specific catalysts; one is its being in correct conformation and the other is ability to change its conformation during reaction. For example covalent attachment may distort the conformational changes irreversibly, which causes decrease in activity of enzyme.

4) Steric restrictions: Substrate-enzyme interactions may be prevented by polymeric cage after immobilization.

5) Inactivation: Inactivation of immobilized enzymes may be caused by the reaction conditions, which are able to damage, such as high pH, presence of free radicals, oxidizing agents. Therefore, the entrapped enzyme activity is less then the free one [57].

1.3.1 Reasons for Enzyme Immobilization

Immobilized enzymes offer a considerable operational advantage over free enzymes. Immobilization makes it possible to achieve and maintain a high enzymatic activity in a small volume. Therefore, there is a reduction in the reaction time. Reusability, enhanced stability, possibility of batch or continuous operational modes, rapid termination of reactions, controlled product formation, possible greater efficiency in consecutive multistep reactions are advantages of enzyme immobilization. Furthermore, efficient separation of the biocatalyst from the products is possible via immobilization, which is very advantageous in industrial point of view. The use of enzyme repeatedly and continuously can be achieved by means of immobilization. Another aspect of immobilization process is being highly practical for automated analysis.

In solution, soluble enzyme molecules behave as any other solute in that they are readily dispersed in the solution and have complete freedom of movement. Enzyme activity is being lost or destroyed in an arbitrary fashion due to the contamination of the product with active proteins which often require removal or deactivation when the soluble enzymes are used. In contrast, immobilized enzymes and cells can be exploited for their full lifetime under ideal conditions without loss or unnecessary deactivation. Therefore, immobilization provides the maximum use of catalyst, particularly important factor for expensive enzymes and cells.

Immobilized catalyst environment is different significantly from the soluble enzyme molecules. Surface charge or hydrophobic regions on the matrix will interact specifically with the catalyst, which can substantially increase the stability of the enzyme. However, the measured activity is usually lost by the binding of the catalyst to the support. Chemical or physical interactions between the delicate biochemical structure and a relatively robust support, damage the enzyme activity. Besides the lack of specificity, binding may result in some coupling which occludes the active sites or binds the enzymes in regions of the matrix inaccessible to substrate molecules [57].

Immobilized enzymes are always in competition with native ones. In the case of very cheap, soluble, highly active enzymes, immobilization is usually of little importance. The nature of the

substrate is also a factor in determining whether the enzyme should be used as it is or be immobilized.

1.3.2 Methods of Immobilization

A classification based on the method of immobilization covers the most important known form of immobilized biocatalysts. Immobilization methods are divided into two main categories: binding and physical retention methods [57,58]:

Binding

- Binding to carriers
  - Adsorption
  - Ionic Binding
  - Covalent Binding
- Crosslinking
  - Co-crosslinking

Physical Retention

- Matrix Entrapment
  - Gel Entrapment
  - Fiber Entrapment
- Membrane Enclosure
  - Microencapsulation
  - Liposome Technique
  - Membrane Reactors

Main types of enzyme immobilization are shown in Figure 1.11. However, more recent developments involve combination of types which do not fit any of the method shown in this figure. In order to avoid disadvantages of each method and exploit every advantage, the basic immobilization methods mentioned above are

combined, yielding the greatest possible specific activity and selectivity [57,58].

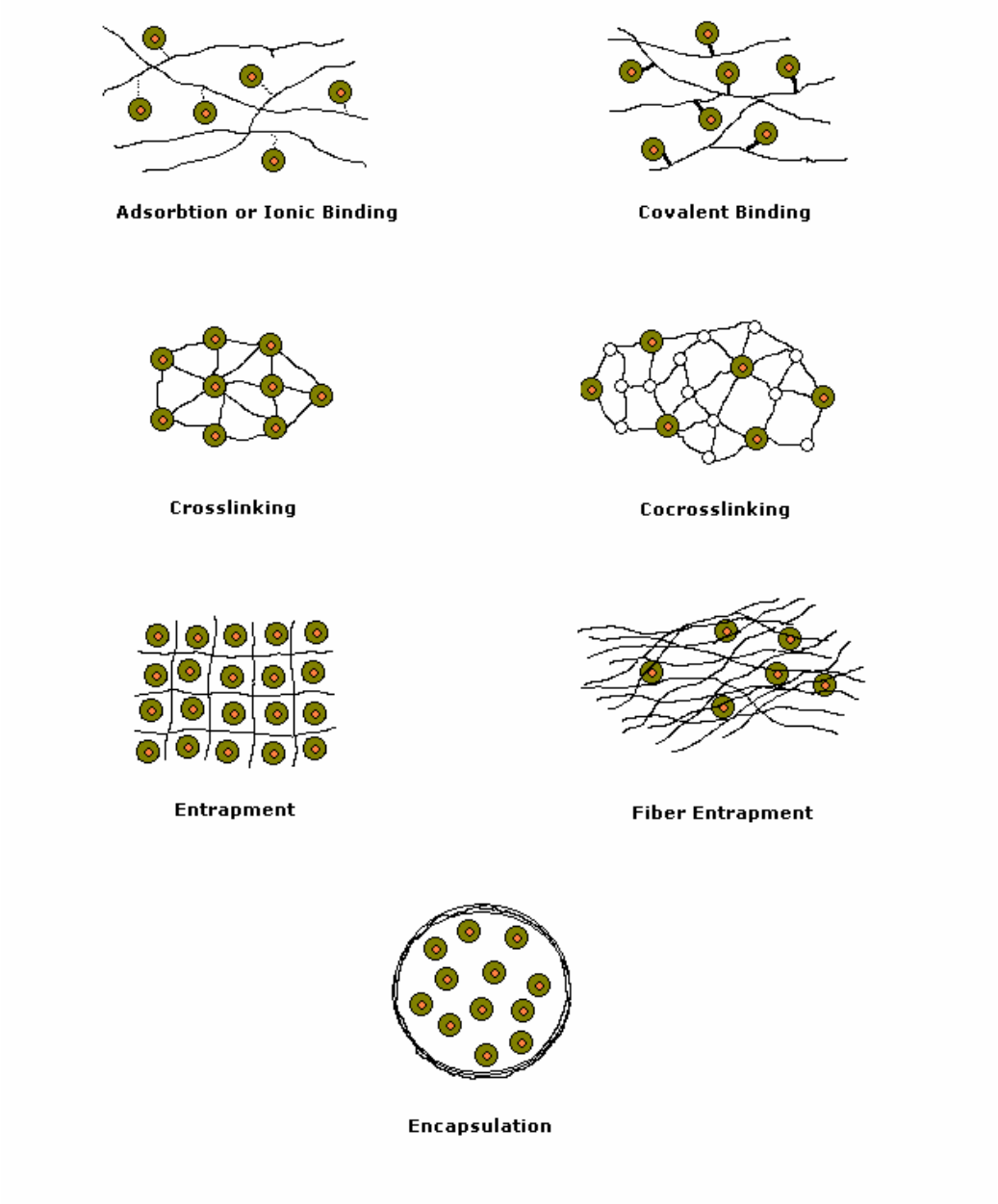


Figure 1.11. Main types of enzyme immobilization.

1.3.2.1 Binding Methods

Adsorption is the simplest and oldest immobilization technique, in which a water-insoluble carrier is used. In 1916, Nelson and Griffin [59] observed that invertase adsorbed on active charcoal retained its sucrose-splitting activity. Numerous surface-active materials including anion-cation exchange resins, activated charcoal, silica gel, alumina, controlled-porosity glasses, ceramics, etc, have been used in the preparation of enzyme-adsorption complexes [57]. This method has been applied to a large number of enzymes and whole cells. In adsorption, the enzyme is held to the surface of the carrier by physical adsorption, ionic interactions, H-bonding, hydrophobic bonding, or van der Waals forces depending on the nature of the carrier. Those adsorptive binding forces are relatively weak and the enzyme can be easily desorbed by change in experimental conditions such as pH, temperature, ionic strength, type of solvent. However, this method relatively simple and almost no influence observed on the enzyme conformation and activity [57].

Ionic Binding is an immobilization method based on the electrostatic attraction between oppositely charged groups of the carrier and the enzyme. Commonly used carriers are polysaccharide based synthetic resins and ion-exchangers. Mitz first utilized this technique, by the immobilization of catalase in cellulose in 1956 [60]. Although it is as simple as adsorption, one disadvantage of the method is the interference from other ions present in the medium.

Because it depends on ionic activity, ionic strength and pH must be carefully controlled to prevent detachment of enzyme.

Covalent Binding is binding of enzyme and carrier by covalent bond. A frequently encountered problem with this technique is the great stress that is placed on enzymes, which usually causes conformational structural changes and therefore, loss of enzymatic activity. It is important that the amino acids essential to the catalytic activity of the enzyme are not involved in the covalent linkage to the support. This may be difficult to achieve, and enzymes immobilized in this fashion generally lose activity upon immobilization. This problem may be prevented if the enzyme is immobilized in the presence of its substrate- a step in procedure which would tend to have a protective effect on the catalytic site of the enzyme during immobilization [58]. However, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs; even in the presence of substrate or solution of high ionic strength, consequently, the derivatized enzyme may exhibit superior chemical and physical properties relative to its soluble counterpart. [58].

Crosslinking method allows the preparation of membranous sheets of crosslinked enzyme with controlled pore size, by placing enzyme onto membrane, then, introducing gluteraldehyde to crosslink the enzyme molecule. One part of the crosslinking agent is used to introduce an appropriate functionality into the preformed

polymer and the other part is used to bind the enzyme. Crosslinking agent produces a two dimensional skin over the supports to provide the increase the average molecular weight of the enzyme, dramatically reducing solubility and increasing average binding points per molecule [53]. The bifunctional aldehyde, glutaraldehyde, the most widely used crosslinking agent that holds a unique position in being cheap, relatively in volatile, of low toxicity and extremely effective. The stability of its conjugates with enzyme has been attributed to imine groups, as a crosslinking agent. Other crosslinking agents such as formaldehyde, diethylpyrocarbonate, dimethyl adipate have been used at mildly alkaline pHs whereas in general have no marked advantage over glutaraldehyde [53].

The stability of the enzyme is increased by crosslinking procedures whereas that may inactivate the enzyme. The major drawback of this method is that the crosslinking agent may preferentially attack the active site of enzyme. Furthermore, it is possible the molecules to react with amino acid residues only of the same enzyme.

1.3.2.2 Physical Retention Methods

Matrix Entrapment is based on the localization of enzyme in the lattice of the support material like a natural or synthetic polymer matrice, a gel like structure, natural or polymer fiber and does not contain a direct link between the matrice and the enzyme. This

method prevents the release of the enzyme and at the same time allows the substrate and product traverse the matrice. Based on the application, the external form of the matrice-entrapped biocatalyst can be varied such as spherical, cylindrical, fiber-like or sheet-like. This method is extremely popular for whole cell immobilization, in which usually natural polymers are employed due to their nontoxicity. Since the pores of the matrice must be small enough to entrape the enzyme, these matrices are not suitable for enzyme immobilization because of their large mesh sizes. To be an effective barrier a matrice must have pores within a limited size range, being sufficiently small to retain the catalyst while not impeding the free diffusion of substrate and product. Enzyme molecules are physically entrapped within the polymer lattice and permeate out of the matrice; however, appropriately sized substrate and product molecules can transfer across and within network of polymer to insure a continuous transformation [57].

In the gel entrapment method, enzyme molecules are physically entrapped within the highly crosslinked water-insoluble polymer matrice and can not permeate out of this gel, but favorable sized substrate and product molecules can transfer across and within this network to ensure a continuous transformation. Fiber entrapment of enzymes has several advantages over gel entrapment. High surface area for enzyme binding can be obtained by using very fine fibers which are resistant to weak acids and alkalis, high ionic strengths and some organic solvents.

Membrane Enclosure method includes microencapsulation, liposome technique and utilization of membrane reactors. The enzyme is entrapped in membrane, which is semi-permeable, where only the substrate diffuses in and the products out.

Microencapsulation differs from other immobilization methods in that it is mainly based on maintaining a solution environment around the enzyme rather than involving physical or chemical forces. In this process, the original solution containing the enzyme is wholly immobilized instead of selectively immobilizing enzyme molecule. Enzyme molecules being larger than the mean pore diameter of the sphere retained within the encapsulated sphere and cannot diffuse out while small substrate and product molecules can be readily diffuse into the capsule [57].

Compared to classical entrapment method, the major advantage including the possibility of formation of high surface area per unit enzyme immobilized (high concentrations of enzymes in the original solution) that provides an extremely large surface area for contact of substrate and enzyme. In this method, no changes in the inherent properties of the enzyme are expected, since no chemical modification is required, and the enzyme molecules are free in solution. However, a great deal of technology is needed to make uniform spheres and provide high activity retention [58].

Liposome Technique is the immobilization of enzyme within a membrane consisting of a lipid double layer. In contrast to microcapsules, soft, deformable and almost liquid lipid membranes are formed. Liposomes can be obtained by sonification of suitable lipids which have a hydrophilic head and a hydrophobic tail, so they tend to arrange themselves with their hydrophobic tails end to end, forming a lipid double layer with a hydrophobic interior. The double layers have the tendency to merge with one another at their edges to form closed vesicles called liposomes. Enzymes encapsulated in liposomes are obviously unsuited for harsh industrial conditions because of the sensitivity of the double membrane structure. However, they could be useful tools in medical therapy and in modeling natural systems for basic research.

Membrane reactors include the enzymes retained in a reactor in hollow-fiber membranes or in sheet-like ultrafilter membranes. In this way they are continuously available over longer periods of time. Reaction products can pass through the membrane and thus be removed continuously while the biocatalysts are held back by the membrane. The two great advantages of this method are that commercially available and relatively cheap ultrafilter devices can be used and that the biocatalysts are not exposed to inactivating steps.

1.3.3 Enzyme Immobilization by Electropolymerization

Immobilization of enzymes by electrochemical methods has been considered as efficient methods for biosensor construction. This method consists of the application of an appropriate potential to the working electrode soaked in an aqueous solution containing both enzyme and monomer molecules. Conducting polymers such as polythiophene, polyaniline, polyindole, polypyrrole, can be grown electrochemically on the electrode surface. Enzymes present in the immediate vicinity of the electrode surface are thus incorporated in the growing polymer [61,62].

Electropolymerization could be demonstrated as an easy and attractive approach for the immobilization of enzymes through its being rapid one-step procedure (Figure 1.12), enabling the control of the thickness of the polymer layer and possibility of precisely electrogenerating a polymer coating over a small electrode surface of complex geometry. Effectively, the electrochemical polymerization of controlled thickness constitutes a reproducible and nonmanual procedure of biosensor fabrication. Therefore, stable immobilization of enzymes on conductive microsurfaces with complete retention of their biological recognition properties has been gaining importance against the conventional methods which suffer from low reproducibility and poor controlled deposition. Moreover, the recent design of electrochemical micro cell allows the electrogeneration of

polymer films from small volumes electrolyte reducing consequently the amount of enzyme required for its entrapment [63-68].

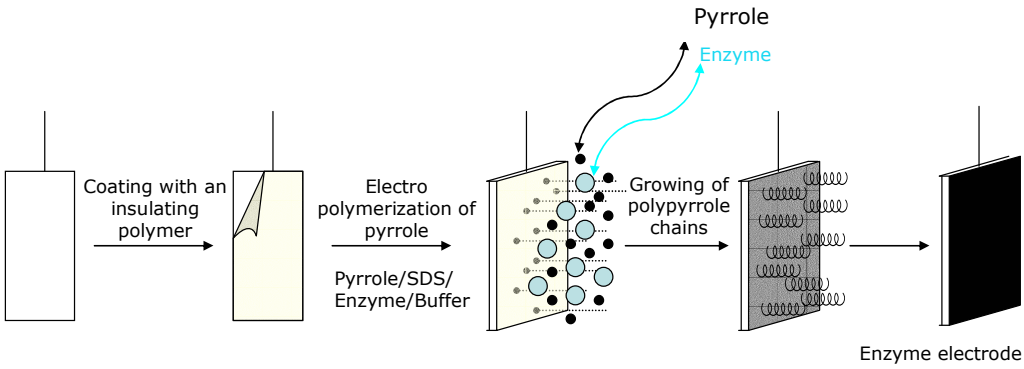


Figure 1.12. Enzyme immobilization by electropolymerization.

Another electrochemical method involves electropolymerization of functionalized monomers. The entrapment of enzymes into the polymer is obtained by chemical grafting or by affinity of the enzyme at the functional group [65,66].

1.3.4 Applications of Enzyme Immobilization

The techniques developed in enzyme immobilization facilitated the progress of enzyme electrodes and novel enzyme based automated analytical methods. Considerable work has been carried out in recent years on the development of enzyme electrodes for the assay of glucose, urea, amino acids, alcohol, and lactic acid.

These electrodes contain a sensor for oxygen or the ions formed in an enzymatic way and the appropriate immobilized enzymes, i.e., glucose oxidase [69-71], urease [67-69].

In industry there are several parameters for the use of immobilized enzymes, i.e., great demand for the product, favorable reaction kinetics, a high market price for the product, native biocatalysts not already use etc. L-amino acid production (L-aminoacylase), production of derivatives of penicillins (penicilin amilase) or aspartic acid (L-aspartase) are the examples of areas where the immobilized biocatalysts are used.

1.3.5 Invertase Immobilization

In 1860 Berthelot discovered an enzyme in yeast which changed the direction of optical rotation of sucrose solutions and named subsequently as *invertase*. β-fructofuranosidase (EC No.3.2.1.26) commercially named as invertase, catalyses the hydrolysis of sucrose to glucose and fructose which is known as invert sugar. The reaction is shown in Figure 1.13. Invertase has an isoelectric point of 4.5. It occurs in the small intestine of mammals and in the tissues of certain animals and plants. It may be obtained in a relatively pure state from yeast, which is a very good source.

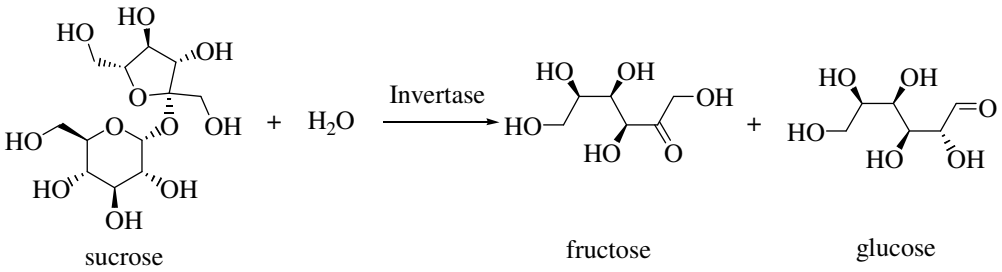


Figure 1.13. The reaction catalyzed by invertase.

In food industry, since the sucrose crystallizes more readily than invert sugar, invertase is used to obtain invert sugar which is widely used in the production of noncrystallizing sugary products such as creams, jam, artificial honey.

Invertase has a rather lower probability of achieving commercial use in immobilized form because of its cheap and easy extraction. However, it is one of the most studied of all enzymes because of being a model enzyme for experimental purposes. Immobilization of invertase to a wide range of carriers by a large number of techniques has been studied. It has been immobilized in fibers of cellulose triacetate [72], poly (acrylamide) gel and other acrylic gels [73], polyethylene [74], polyaniline [75]. It has also been widely studied by electrochemical immobilization [76,77], and nonpolymeric immobilization techniques [78,79].

1.3.6 Polyphenol Oxidase Immobilization

Tyrosinase (EC No. 1.14.18.1) which is commonly found in yeast, mushrooms, grapes, bananas, apples, potatoes, frogs and mammals, was discovered by Bertnard and Bourquelot about 100 years ago. Tyrosinase weights about 130,000 Daltons and its active site contains two copper atoms which exist in three states; met, oxy, and deoxy. In analyzing certain varieties of mushrooms, Bertnard and Bourquelot observed that as oxidation progressed, the mushrooms changed color and finally become dark brown or black. Subsequently studies showed that this new oxidase catalyzed the aerobic oxidation of mono-phenols, and the final product of tyrosine oxidation was melanin [80].

Tyrosinase catalyzes two reactions shown in Figure 1.14 via separate active sites:

I. orthohydroxylation of monophenols, commonly referred to as the cresolase activity and

II. oxidation of orthodiphenols to orthoquinones, commonly referred to as the catecholase activity.

Tyrosinase catalyzes the synthesis of melanin through the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to dopaquinone. The unstable dopaquinone will polymerize and precipitate into melanin. However, in the presence of a reductor, the reaction will stop at the diphenol level [81]. The cresolase activity of tyrosinase is of

particular importance because it synthesizes DOPA. DOPA is a precursor of dopamine, an important neural message transmitter. Patients who suffer from Parkinson's disease show a significant decrease in the concentration of dopamine found in the substantia nigra of the brain [82].

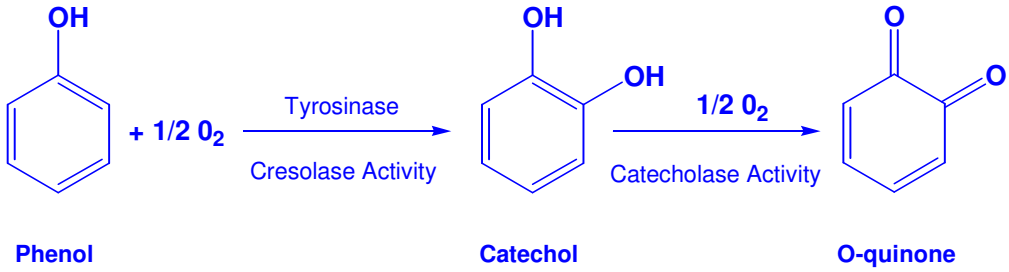


Figure 1.14. Schematic representation of tyrosinase activity.

The catecholase activity also has important applications, since this activity can be used in the analysis of phenols and its derivatives. Phenolic pollutants are frequently found in surface waters and in the effluent of industrial discharge sources. Some of the industrial sources of phenol discharge include oil rafineries, coke and coal conversion plants, plastics and petrochemical companies, dyes, textiles, timber, mining, and the pulp and paper industries. Virtually all phenols are toxic. Moreover, they have a high oxygen demand and can deplete the oxygen in the body of water [83]. As a consequence, this may affect the ecosystem of water sources where phenols are discharged.

A successful application of soluble tyrosinase in the "cleansing" of polluted waters have reported by Atlow et al. [84]. Tyrosinase causes the precipitation of phenols, which can then be filtered out from surface waters and industrial discharge sources. The enzyme has also been used as a sensor to detect the concentration of phenols in waste water [85-86]. The detection of phenols is of importance also in the medical field. Tyrosinase has been used as part of an enzyme-electrode system to detect catechols and assess catecholamines in the urine of patients with neural crest tumors [87]. Because tyrosinase is responsible for the browning of fruits and vegetables it has also applications in the food industry. Interest in the enzyme has been demonstrated by tannin oil companies due to the role that it plays in melanogenesis. Also, it has been considered for use in melanin-related disorders, such as albinism, vitiligo, and melanoma [88].

Although tyrosinase has widespread applications, its use is limited by its inherent instability and rapid inactivation. By using enzyme immobilization technology, good operational stability and long-term stability can be achieved for tyrosinase.

Red and white wine contain many phenolic substances which have a number of important functions in wine. They affect the tastes of bitterness and astringency, especially in red wines, the color of red wine is caused by phenolics, they are also bactericidal agents and

impart antioxidant properties, being especially found in the skin and seeds of the grapes.

There are two types of phenols in wines. “flavonoids” and “non-flavonoids”. The flavonoids are composed of three benzene rings and react readily, binding to other molecules and there are between 6,000 and 8,000 species of flavonoids. A group of flavonoids, called the flavon-3-ols, have been well characterized in wine. Flavon-3-ols are usually concentrated in grape seeds, stems and skin. When these parts of the grape are left in for as long as possible during the wine-making process, more flavon-3-ols end up in the resulting wine than if the seeds, stems and skin are removed earlier [89].

The non-flavonoids in wine comprise many classes of chemicals including hydroxycinnamates, benzoates, and stilbenes. The health benefits of a particular kind of stilbene, called "resveratrol," are known widely since it is unique to grapes and is not found in other fruits or vegetables [90].

Amount of phenolics vary from one brand and type of wine to another since the chemical composition of a wine is influenced by the climatic and atmospheric conditions, soil type, vine cultivation and the treatment to which it is subjected. Process difference cause the red wines to contain almost ten times higher amount of phenolics than the white wine.

The typical methods for the determination of phenolic compounds are gas and liquid chromatography [91,92]. These methods involve complex sample pre-treatment procedures and are unsuitable for on site or field based analyses. A biosensing approach with advantages of high specificity, high sensitivity and rapid detection mechanism may provide a solution [93-97]. In this study immobilization of polyphenol oxidase enzyme in a conducting polymer electrode was studied as an alternative method for the determination of phenolic compounds[98-99].

1.3.7 Glucose Oxidase Immobilization

Glucose oxidase enzyme (EC No. 1.1.3.4) has a molecular weight of 150,000 dalton. It catalyzes the oxidation of β-D-glucose to D-glucono-1,5-lactone in the presence of molecular oxygen as the electron acceptor. The reaction is given in Figure 1.15. The enzyme contains 2 mol of FAD (flavin adenine dinucleotide) per mole of enzyme, and has an isoelectric point near pH 4.2.

Glucose oxidase has rather high specificity for β-D-glucopyronose. There is an absolute requirement for a hydroxyl group C(1) and the activity is about 160 times higher if the hydroxyl group is in the β-position. Changes in the substrate C(2) through C(6), except for L-glucose and 2-o-methyl-D-glucose, do not completely prevent the compounds from serving as substrates, but activity is much reduced.

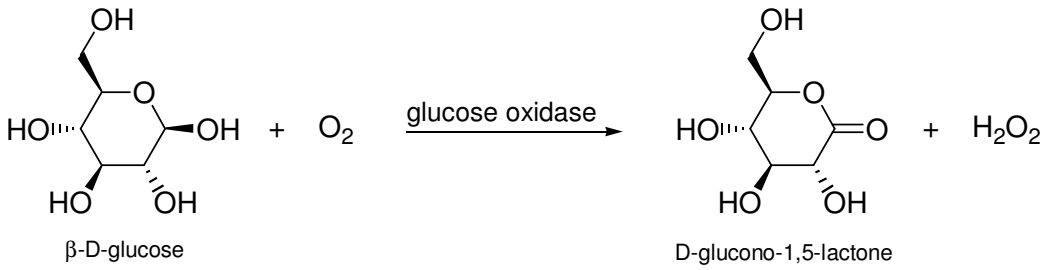


Figure 1.15. The reaction catalyzed by glucose oxidase.

It was known that glucose oxidase does not oxidize β-D-glucose by direct combination of molecular oxygen with the β-D-glucose. There is a direct correlation between riboflavin content and enzymatic activity. The highly purified enzyme is yellow and contains two molecules of FAD per enzyme molecule. Removal of FAD leads to complete loss of activity, which can be restored by the addition of FAD. Glucose oxidase does not catalyze the complete reaction under anaerobic conditions and the reaction rate is dependent on O_2 concentration.

Glucose oxidase is widely used for the determination of glucose in body fluids and in removing residual glucose and oxygen from beverages and foodstuffs. Furthermore, glucose oxidase producing moulds such as *Aspergillus* and *Penicillum* species are used for the biological production of gluconic acid.

Enzyme electrodes have been produced by a number of different methods of immobilization or confinement of the enzymes and with different methods of detection. In literature, many studies were performed involving the immobilization of glucose oxidase in different matrices including covalent attachment, chemical crosslinking besides the electrochemical immobilization of enzymes and amperometric biosensor construction which is the mostly studied method of glucose oxidase. Oxidoreductase type enzyme, such as glucose oxidase, can be electrically "wired" to electrodes by electrostatic complexing or by covalent binding of redox polymers so that the electrons flow from the enzyme, through the polymer, to the electrode [100,101]. Covalent attachment of glucose oxidase to polypyrrole and its application as a glucose sensor have been reported [102,103]. Immobilization in gel-like layer or keeping in place by a membrane, covalent binding, direct adsorption to platinum or graphite surface, [104] have studied for the preparation and properties of a glucose sensor made by adsorption of the glucose oxidase on non-porous carbon electrode. The hydrogen peroxide which was produced by the enzymatic reaction was oxidized at the electrode and detected amperometrically. Immobilization of glucose oxidase in p-benzoquinone-carbon paste electrode [105], in a nonwoven fabrics with *Bombyx mori* silk fibroin gel, [106] and in polymer membranes [107-110] have been reported. Polymer membranes widely used for the immobilization of enzymes. They possess some specific characteristics, such as exact chemical composition and physical structure, fixed porosity and hydrophilic-

hydrophobic balance, which provide a possibility to carry out quantitatively defined immobilization of enzymes. The additional modification of polymer membranes increases their suitability as enzyme carriers.

Electrochemical polymerization of phenol and its derivatives and their use to immobilize glucose oxidase at a platinum electrode surface have been reported [111-115]. The responses of the immobilized enzyme electrodes toward glucose have been analyzed.

Electrochemical immobilization of glucose oxidase in polypyrrole and poly(amphiphlic pyrrole) films [116-118], optimization of the conditions and preparation parameters of the electrode was studied.

1.4 Aim of the Study

I. To achieve the electrochemical synthesis of conducting copolymers of thiophene capped polytetrahidrofuran with pyrrole in the presence of sodium dodecyl sulphate and their characterization.

II. To investigate the thermal, morphological and conducting properties of resultant conducting copolymers.

III. To construct enzyme electrodes by immobilization of INV, PPO and GOD enzymes in TPTHF copolymers via their electrochemical polymerization.

IV. To characterize the enzyme electrodes by investigation of kinetic parameters and storage stabilities of electrodes.

V. To apply PPO and GOD immobilized electrodes to the analysis of polyphenolic compounds in red wine and the analysis of glucose in orange juice, respectively.

CHAPTER II

EXPERIMENTAL

2.1. Materials

The monomer, pyrrole (Merck), was purified by conventional distillation procedure prior to use and stored at 4 °C. The solvent, dichloromethane (Merck) in which the macromonomer is dissolved, was used without further purification. The supporting electrolyte, sodium dodecyl sulfate (Sigma) was used as purchased. Sulfuric acid was supplied by Merck.

The enzymes, invertase (β-fructofuranosidase, EC No. 3.2.1.26) Type V (from yeast), polyphenol oxidase, glucose oxidase, Type II-S, (GOD, EC: 1.1.3.4), peroxidase, Type II, (POD, EC: 1.11.1.7), o-dianisidine were purchased from Sigma and the substrates, sucrose, catechol and glucose obtained from Merck, were used as received. For the preparation of *Nelson`s reagent*, sodium carbonate (Riedel de Haen), sodium potassium tartarate (Riedel de Haen), sodium

bicarbonate (Merck), sodium sulfate (Merck), copper sulfate (Merck) and for the preparation of *Arsenomolibdate reagent,* ammonium heptamolibdate (Merck), sodium hydrogen arsenate (Merck) were used as received. Bovine Serum Albumin and Folin & Ciocalteu's Phenol Reagent from Sigma were used as purchased.

2.2 Experimental Methods

2.2.1 Electrolysis

Constant potential method was used for the synthesis of conducting polymers. Potentiostat is an instrument which is used to maintain the potential difference between working and counter electrodes at the required value. It eliminates the IR drop in the electrolysis medium by continuous comparison of working electrode potential to reference electrode potential. In this work, Wenking POS-73 model potentiostat was used.

The synthesis of copolymers and enzyme immobilization processes were performed in a typical H-shaped electrolysis cell containing platinum (Pt) flag electrodes as the working and counter electrodes and a silver (Ag) wire (pseudo reference) reference electrode were used. Anode and cathode parts of the cell are divided by a sintered glass disc with medium porosity. H-shaped electrolysis cell is presented in Figure 2.1.

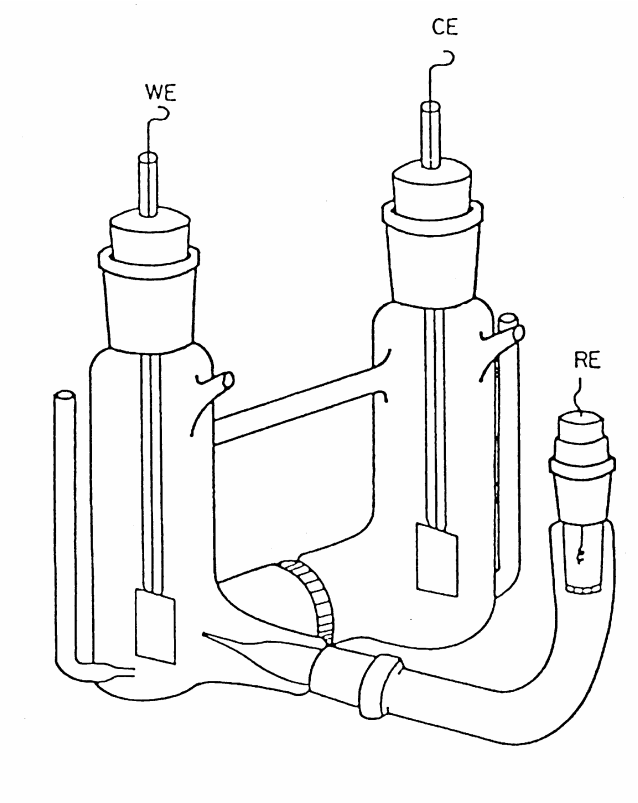


Figure 2.1. H-shaped electrolysis cell.

2.2.2 Four Probe Conductivity Measurements

Four probe technique allows a number of conductivity measurements over a broad range of applied currents, usually varying between 1 μA and 1 mA. In this device, four equally spaced points on a line make electrical contact with a polymer film on an insulating surface. Between the outer pair of these points, a constant current is applied while the potential difference arising from this current is measured at the two inner contacts. By applying a constant current across the two outer leads, the voltage drop across the two

inner leads can be measured allowing for the calculation of resistance using Ohm's law. Figure 2.2 shows the simplest form of the device. A known current I is injected at the electrode 1 and collected at the electrode 4, while the potential difference between contacts 2 and 3 is measured. Conductivity is calculated from the following equation:

$\sigma = \ln 2 / (\pi R t)$

where R is the resistance of the sample, t is the thickness.

To measure the conductivities, electrochemically prepared polymers films at sufficient thickness were removed from the electrolysis medium, followed by rinsing, drying and removal of the film by peeling from the electrode surface. Their conductivities were determined by four probe technique.

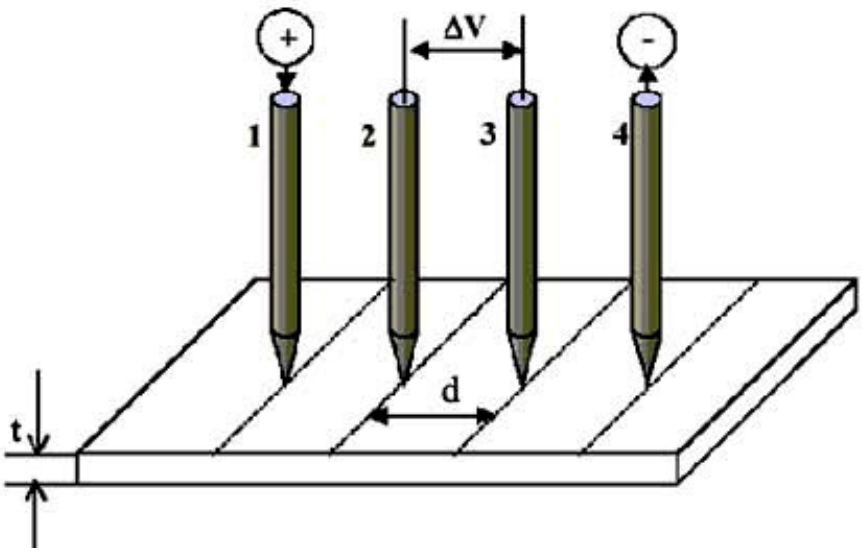


Figure 2.2. Four- probe conductivity measurement.

2.2.3 Fourier Transform Infrared Spectrophotometry (FTIR)

FTIR is a useful method for the characterization of conducting polymers because it does not require polymers to be soluble. It is primarily used for the detection of functional groups, but analysis of spectra in the lower frequency finger print region can give evidence of degree of polymerization, the effect of substituents on the electronic properties of the polymer backbone. In this work, FTIR spectra of the polymers were recorded on a Nicolet 510 FT-spectrophotometer.

2.2.4 Scanning Electron Microscope (SEM)

SEM is a surface analytical technique which is employed to study the morphology of conducting polymer film surfaces and provides valuable information on the structure of the monomer, the nature of dopant and the thickness of the film. SEM images a sample which is placed in a vacuum and scanned by an electron beam over the surface of the film. Electrons are displaced from the surface of the film and are collected by a detector to eventually form an image of the surface.

SEM films of copolymers were performed using a JEOL model JSM-6400 scanning electron microscope. For preparation of the sample, polymer films were peeled back from Pt electrode and glued

to copper holder, and then coated by sputtering with a thin gold film.

2.2.5 Thermal Analysis

Thermal characterization of polymers was carried out using a DuPont modular thermal analyzer system in conjunction with 951 thermal gravimetric analyzer and 910 differential scanning calorimeter. Thermal gravimetry analysis (TGA) experiments were performed under a dry nitrogen purge. A constant heating rate of 10 °C/min was used during differential scanning calorimetry (DSC) experiments.

2.2.6 UV-Visible Spectrophotometer

To determine the enzyme activity, spectroscopic methods were used. Absorbance measurements reflecting the concentration of products formed by the enzymatic reaction were obtained by the help of a Shimadzu UV-1601 model spectrophotometer.

2.3 Experimental Procedures

2.3.1 Synthesis of Thiophene-capped Polytetrahydrofuran

2.3.1.1 Synthesis of Two End Thiophene-capped Polytetrahydrofuran

Polymerization flask equipped with nitrogen inlet and a magnetic stirrer was flushed with dry nitrogen. Then 10 ml of freshly distilled THF was introduced with a syringe. The polymerization was started at room temperature by injecting triflic anhydride (0.097ml, 8.9×10^{-4} mol) as the initiator. After 30 minutes, an aliquot was taken out from reaction vessel and terminated by methanol. The rest was terminated with excess sodium thiophene methonate (8.9×10^{-3} mol), and the solution was stirred for additional one night. Polymerization mixture was precipitated in methanol and cooled. The precipitated polymer was filtered off and dried in vacuum. Figure 2.3 shows the route for the synthesis of TPTHF2 [119].

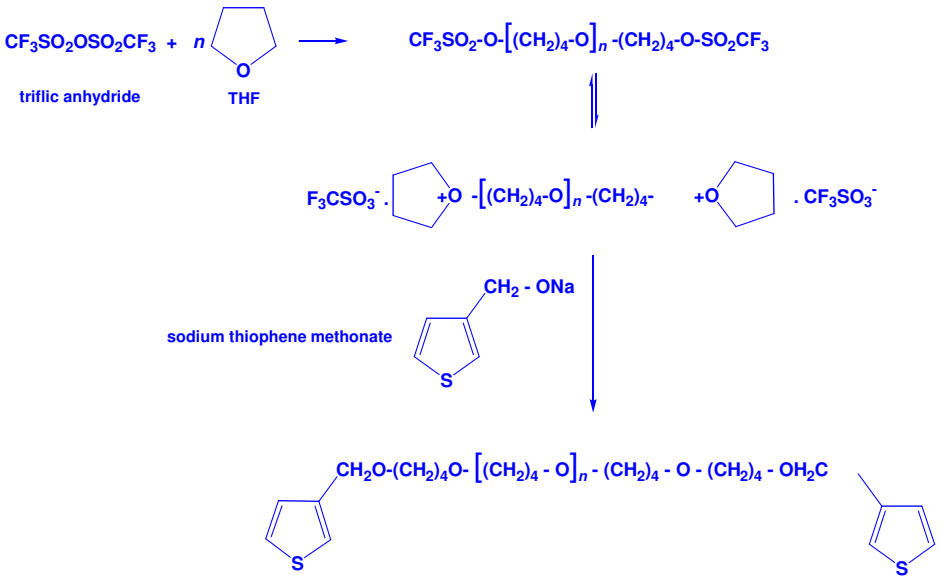


Figure 2.3. Synthesis of TPTHF2.

2.3.1.2 Synthesis of One End Thiophene-capped Polytetrahydrofuran

Synthesis route same with the one in the synthesis of TPTHF2. As an initiator methyl triflate is used instead of triflic anhydride in the synthesis of TPTHF1. One end living polymer of PTHF is terminated by the same reagent, thiophene methonate [120].

The resulting polymers were used as immobilization matrices by grafting polypyrrole onto them in the presence of dissolved enzyme molecules.

2.3.2 Synthesis of Graft Copolymers of Thiophene-capped Polytetrahydrofuran with Pyrrole

Electrochemical polymerization of graft copolymers of thiophene-capped polytetrahydrofuran with pyrrole was carried out in water-SDS media in the presence of 20 mM pyrrole, after deposition of TPTHF1 or 2 (1%, w/v solution in CH_2Cl_2) onto Pt electrode which was used as the working electrode. Polymerization of pyrrole was performed on the working electrode in the presence of 0.05 M supporting electrolyte, sodium dodecyl sulfate (SDS) by applying a constant potential of +1.0 V, oxidation potential of pyrrole. The electrolysis was allowed to proceed 30 min, until sufficiently thick films were obtained. After electrolysis, the working electrode was removed from the cell, washed several times with

distilled water to remove supporting electrolyte adhered on the surface of polymer film and allowed to stand in CH_2Cl_2 for several hours to remove the ungrafted precursor polymer (Figure 2.4).

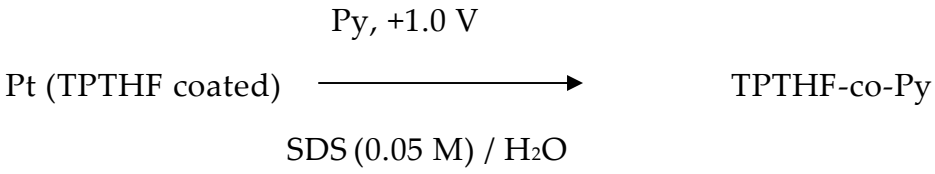


Figure 2.4. Electrochemical route for copolymerization of TPTHF with pyrrole.

2.3.3 Immobilization of Enzymes

2.3.3.1 Immobilization Procedure

Immobilization of enzymes was achieved by electrochemical polymerization of pyrrole either on bare or previously TPTHF coated Pt electrodes of 1.5 cm^2. Constant potential electrolysis was performed 30min in the 3 electrode cell applying +1.0 V at room temperature. Electrolysis solution was prepared as 10 ml buffer solution containing supporting electrolyte, 10 mM pyrrole and enzyme. Based on previous studies, SDS was chosen as supporting electrolyte and used in the electrochemical polymerization of pyrrole on host-polymer-coated Pt electrodes, in the absence or presence of enzyme. In invertase studies, acetate buffer at pH 4.8 containing 0.4 mg/mL SDS and 0.4 mg/mL for invertase were used. In polyphenol

oxidase studies, citrate buffer at pH 6.5 containing 1 mg/mL SDS and 0.3 mg/mL PPO were used. In glucose oxidase studies acetate buffer at pH 5.1 containing 0.6 mg/mL SDS and 2.0 mg/mL glucose oxidase were used. All buffers were in 0.05 M.

After immobilization, enzyme entrapped electrode was removed and washed several times with buffer solution to remove the supporting electrolyte on the surface. Next, the electrode was placed in buffer for 10 minutes and solution was examined for the enzyme activity due to unbound enzyme. This procedure was repeated for several times with the buffer until no activity was observed. Electrodes were stored in buffer at 4 °C when not in use.

2.3.3.2 Invertase Immobilization

2.3.3.2.1 Preparation of Assay Reagents

Nelson's Reagent: Composed of 2 solutions, namely, reagent A and reagent B, for the preparation of *Nelson reagent A*, sodium carbonate (25 g), sodium potassium tartarate (25 g), sodium bicarbonate (20 g), sodium sulfate (200 g) were dissolved in distilled water and diluted to 1000 mL. *Nelson reagent B* was prepared by dissolving copper sulfate (15 g) in 100 mL distilled water. Reagent A and reagent B were mixed in 25:1 (v/v) prior to activity assay [40].

Arsenomolibdate Reagent: Prepared by dissolving ammonium heptamolibdate (25 g), in 450 mL distilled water and by adding 21 mL concentrated sulfuric acid. Another solution, prepared by dissolving sodium hydrogen arsenate (3 g) in distilled water, was added to the above solution. After incubation of resultant solution for 24-48 hours at 37 °C, it was stored in dark [40].

2.3.3.2.2 Determination of Invertase Activity

The activities of immobilized invertase were determined by Nelson method [121]. According to this assay, different concentrations of sucrose solution were prepared and placed into water bath at 25 °C for 10 min. Enzyme entrapped electrode (EE) was immersed in the test tube containing sucrose solution, and at the end of 2, 4 and 6 minutes, 1 mL of sample was drawn and 1 mL Nelson reagent was added. After 20 minutes of incubation in boiling water bath, solutions were allowed to cool for 3 minutes and 1 mL arsenomolibdate reagent was added and solutions were allowed to stand for 5 minutes for color development, then they were diluted with 7 mL distilled water. Finally, the absorbances for the blank and the solutions were determined at 540 nm with a double beam spectrophotometer. One unit of invertase activity was defined as the amount of enzyme required to release 1 μmol glucose equivalents per minute under the assay conditions of pH at 4.8 and temperature at 25 °C.

2.3.3.2.3 Kinetic Parameters of Immobilized Invertase

In order to obtain kinetic parameters of V_{max} and K_m, Lineweaver-Burk plot was drawn by the reaction rate data obtained from the kinetic studies of the reaction performed at varying concentrations of sucrose by keeping assay conditions such as pH at 4.8 and temperature at 25 °C constant.

2.3.3.2.4 Temperature Optimization

For optimum temperature determination, incubation temperature was changed between 10 °C and 70 °C at the optimum pH of the immobilized invertase by keeping sucrose concentration (10 K_m) constant. For the activity measurement, the procedure described above was applied.

2.3.3.2.5 pH Optimization

The optimum pH for immobilized invertase was determined in the pH range of 2.0 - 11.0 by keeping sucrose concentration (10 K_m) and temperature at 25 °C constant. Then, the activities were determined as described above.

2.3.3.2.6 Operational Stability

In order to determine the operational stability of enzyme entrapped electrodes, their activities were measured at 40 repetitive uses of electrode.

2.3.3.2.7 Shelf-life Determination

Shelf-life of enzyme entrapped electrodes which were preserved in buffer solution at 4 °C was obtained by measuring their activities for a 40 days period.

2.3.3.3 Polyphenol Oxidase Immobilization

2.3.3.3.1 Determination of PPO Activity and Kinetic Parameters of Immobilized PPO

The activities of immobilized PPO were determined by using Besthorn's Hydrazone Method [122]. which is based on the interaction of 3-methyl-2-benzothiozolinone hydrazone (MBTH) with the quinones produced by the enzyme to yield red products instead of brown colored pigments in the absence of the coloring agent [123]. The pathway proposed by Rodriguez et al is shown in Figure 2.5 [124].

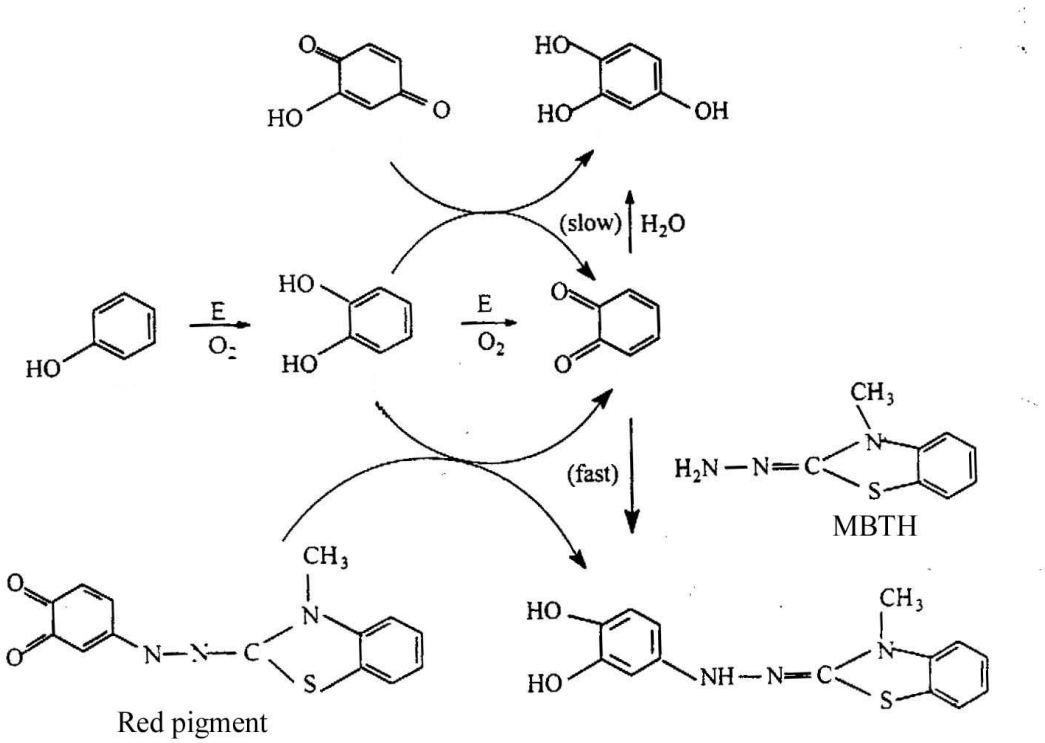


Figure 2.5 Assay reactions of polyphenol oxidase.

In this assay, several concentrations of catechol solutions were prepared and placed into water bath at 25 °C for 10 min. 1 ml of 3% MBTH solution in ethanol was added to the test tube containing 3 mL catechol solution and 1 minute of reaction time was given. EE was immersed into the solution. After shaken for 5 minutes, 1 ml of 5% sulfuric acid and 1 ml acetone were added for a total volume of 6 ml. After mixing, absorbances were measured at 495 nm.

Kinetic studies of the enzymatic reaction for immobilized PPO were performed at varying concentrations of catechol according to the activity assay explained above.

2.3.3.3.2 Temperature and pH Optimization

Optimum temperature and pH were determined by changing incubation temperature and pH between 10°C – 80°C and 2-11, respectively. The rest of the procedure was the same as the determination of PPO activity.

Operational stability and shelf-life determination studies were performed as described in sections 2.3.3.2.6 and 2.3.3.2.7.

2.3.3.3.3 Determination of Phenolic Compounds in Red Wine

Immobilized enzyme electrodes were used to determine phenolic compounds in two kinds of Turkish red wines, Brand K and Brand D. Total phenolic compounds in wines produced in Turkey were reported as 2000-3000 mg/L [125-127]. Red wines are diluted with citrate buffer of pH 6.5 to a 1:3 volume and activity assay was applied as described in section 2.3.3.3.1.

2.3.3.4 Glucose Oxidase Immobilization

2.3.3.4.1 Determination of GOD Activity and Kinetic Parameters of Immobilized GOD

The activity determination of immobilized enzyme was performed by using a modified version of Sigma Bulletin [128]. According to this assay, different concentrations of glucose solution were prepared and placed in water bath at 25 °C at 10 min.

Enzyme entrapped electrode (EE) was immersed in the test tube containing glucose solution. At the end of 2 min, 0.5 mL of sample was drawn and then, as shown in Figure 2.6, 0.1 ml POD (60 U/ml) was added to catalyze the reaction of hydrogen peroxide. Then the coloring agent, o-dianisidine was added as 2.4 ml (0.21 mM). The reaction was terminated with the addition of 0.5 ml 2.5 M sulfuric acid. Spectrophotometric measurements were performed at 530 nm. H_2O_2 standard calibration curve was used in order to define enzyme activity.

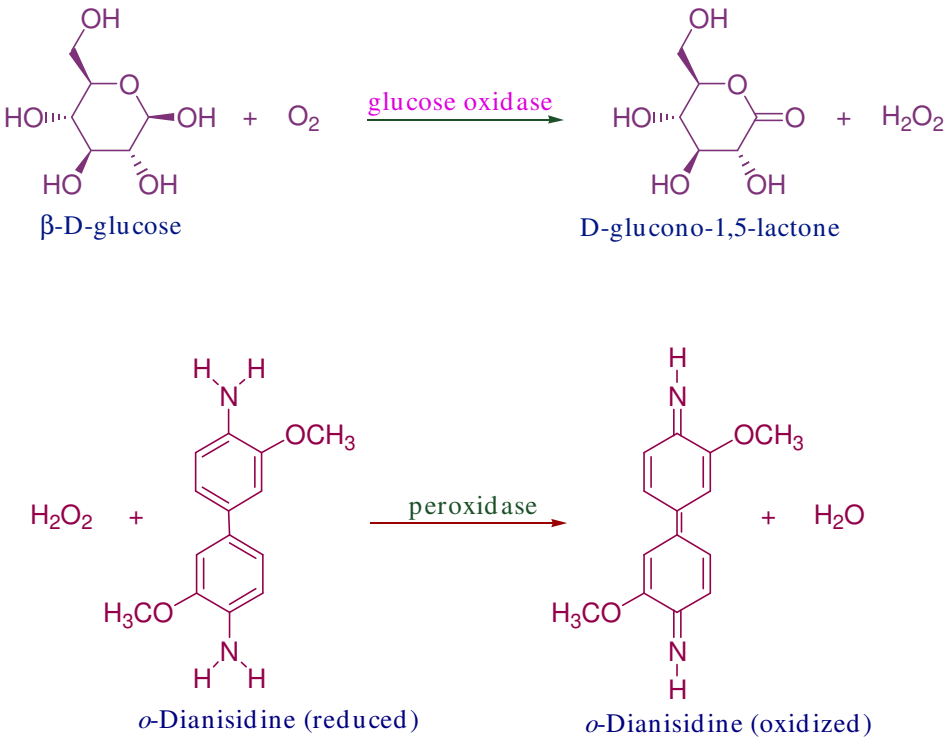


Figure 2.6 Assay reactions of glucose oxidase.

One unit of glucose oxidase activity was defined as the amount of enzyme required to produce 1 µmol of D-gluconic acid and H_2O_2 per minute at pH 5 at 25 °C.

2.3.3.4.2 Temperature and pH Optimization

Optimum temperature and pH were determined by changing incubation temperature and pH between 10°C – 80°C and 4–11 respectively. The rest of the procedure was the same as the determination of GOD activity.

Operational stability and shelf-life studies were performed as described in sections 2.3.3.2.6 and 2.3.3.2.7.

2.3.3.4.3 Determination of Glucose in Juices

Constructed glucose oxidase electrodes were used to determine glucose amount in two kind of Turkish orange juices. Orange juice was diluted in a proportion of 1:25 with buffer. Activity assay described for glucose oxidase was applied for the juice solutions in the same way.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of Copolymers of TPTHF and Pyrrole

3.1.1 Synthesis of Copolymers of TPTHF with Pyrrole

Electrochemical polymerization of graft copolymer of thiophene capped polytetrahydrofuran coded as TPTHF1 and TPTHF2 respectively was achieved in the presence of pyrrole by constant potential electrolysis (Figure 3.1).

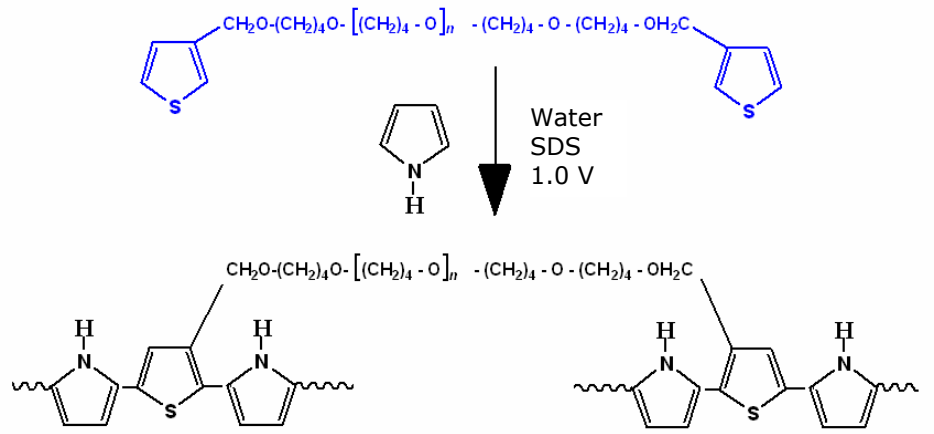


Figure 3.1. Electrochemical synthesis route for copolymerization.

3.1.2 Characterization of Copolymers of TPTHF with Pyrrole

3.1.2.1 FTIR Studies

FTIR spectrum of insulating polymer TPTHF exhibits characteristic C-O-C asymmetric stretching peak at 1111 cm^{-1}. Aliphatic CH_2 vibrations show three peaks between 2750 cm^{-1} and 3000 cm^{-1}. Electrochemically synthesized copolymers, TPTHF1-co-Py and TPTHF2-co-Py exhibited a characteristic band of Py, N-H wagging at about 900 cm^{-1}, the band at about 780 cm^{-1} which is the proof of 2,5 disubstitution on the thiophene ring and a broad band in the range of 1000-1200 cm^{-1} which belongs to SDS dopant anion and C-O-C group. Because of this broad band, the characteristic peak of C-O-C at 1111 cm^{-1} can not be observed. In order to see this peak, copolymer was reduced. It was observed that ether peak appeared as the dopant ion removed from the copolymer.

3.1.2.2 Conductivities of the films

Standard four-probe technique was used for conductivity measurements. Films were produced in water-SDS media as explained in section 2.3.2. Conductivities of graft copolymers TPTHF1-co-Py and TPTHF2-co-Py were shown on Table 3.1. For the copolymers no significant difference was observed. The conductivities of both electrode and solution sides were also in the same order of magnitude, which reveal the homogeneity of the films.

Table 3.1. Conductivities of the films.

Matrice	Conductivity(S/cm)
Ppy (in SDS)	18.1
TPTHF1-co-Py (in SDS)	1.2
TPTHF2-co-Py (in SDS)	1.0

Difference between conductivities of polypyrrole and copolymer films shows that copolymerization of pristine polymer and pyrrole was achieved and an intermediate value of conductivity which is lower than conductivity of pyrrole, 18.2 S/cm but a relatively high conductivity was obtained.

3.1.2.3 Thermal Properties

Thermal behavior of the samples was investigated by using a Du Pont 2000 Thermal Gravimetry Analyser and Differential Scanning Calorimetry. Pristine polymers, TPTHF1 and TPTHF2, showed thermal stability against heating up to 230 °C. TGA thermogram of these macromonomers showed one weight loss at 363.6 °C, (Figure 3.2 (A)), and at 383.9 °C, (Figure 3.2 (B)), respectively. They lose weight rapidly after 380.0 °C with a continuous degradation. The char residue of both TPTHF1 and TPTHF2 is only 1%.

Copolymers of pristine polymers and pyrrole lose weight at lower temperatures. A two-weight-loss pattern was observed for both copolymers. Thermogram of TPTHF1-co-Py copolymer revealed

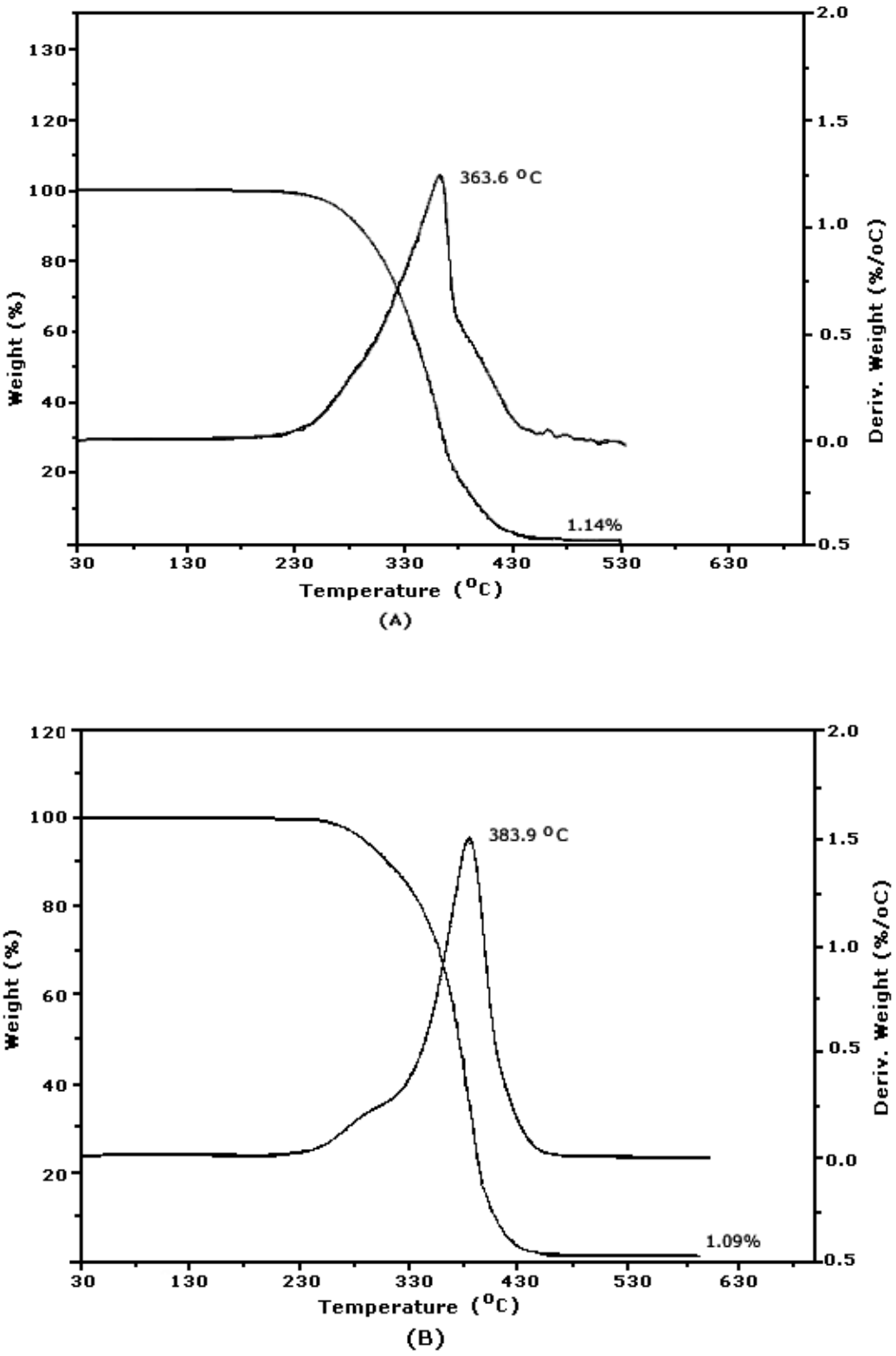
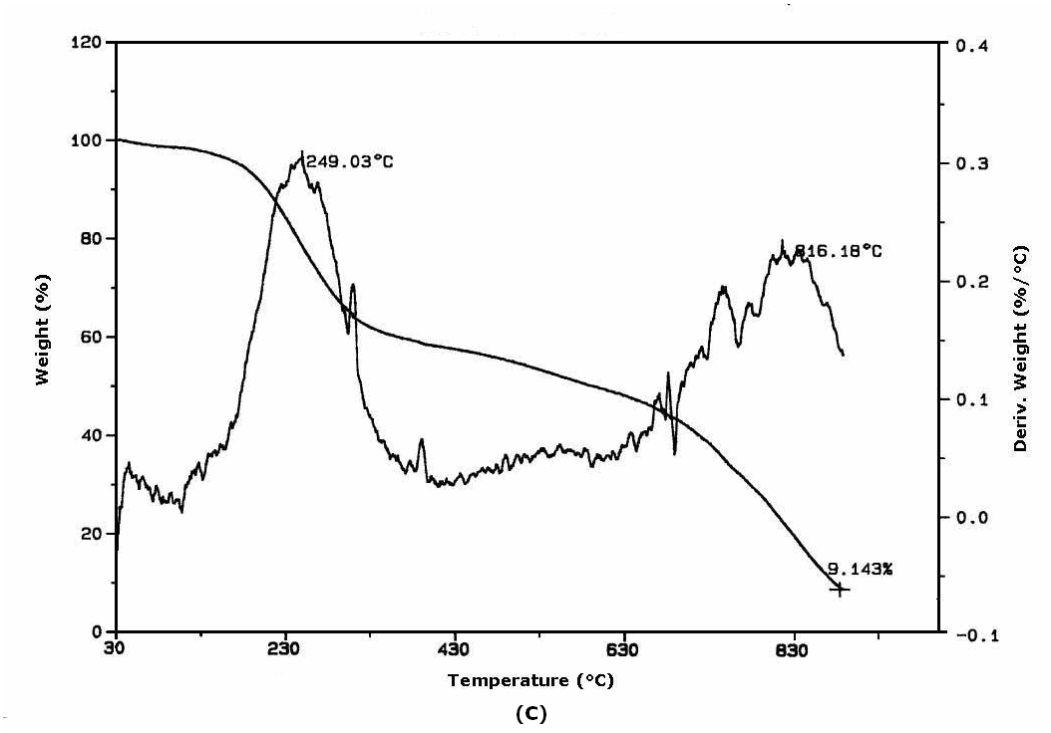


Figure 3.2. TGA thermograms for (A) TPTHF1 (B) TPTHF2.

two transitions at 249.0 °C which is due to the removal of dopant ion and at 816.2 °C with a 9% residue (Figure 3.2 (C)). For TPTHF2-co-Py first thermal transition at 237.2 °C indicating removal of dopant ion and second transition at about 800.0 °C with a gradual degradation after 600.0 °C were observed with a 22% residue (Figure 3.2 (D)). Ppy thermogram shows one weight loss at 255.1 °C due to the dopant loss. Weight loss behaviors of copolymers are somewhat similar to that of polypyrrole because of the pyrrole content in the copolymers and pyrrole content proves a higher thermal stability to the copolymer. Both copolymers show a higher thermal resistivity at the high temperatures. About 50% of the copolymer was remained until about 600.0 °C whereas the remaining macromonomer percentage is only 1% at that temperature.



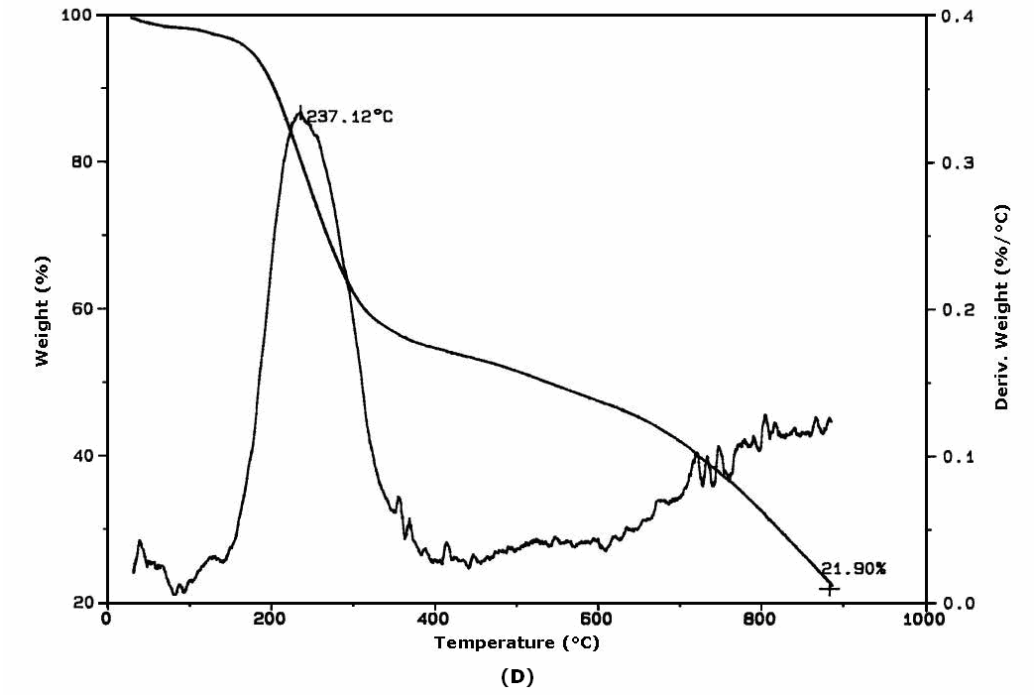


Figure 3.2.(Continued) TGA thermograms for (C) TPTHF1-co-Py (D) TPTHF2-co-Py.

DSC analyses were carried out under N_2 atmosphere by a Dupont 2000 instrument with a heating rate of 10 $K.min^{-1}$. DSC thermograms of both precursor polymer and copolymers were examined in the range 30 °C to 450 °C. DSC thermograms of TPTHF1 and TPTHF2 show a sharp melting point at 45.3 °C and 43.4 °C and it is stable up to 405.3 °C as shown in Figures 3.3 (A) and (B). Pure Ppy yields two endotherms at 66.2 and 247.1 °C due to the loss of solvent and removal of dopant ion SDS. TPTHF1-co-Py thermogram shows three broad transitions at 63.6 °C, 201.7 °C and 401.5 °C (Figure 3.3 (C)). TPTHF2-co-Py shows two broad transitions at 89.9 °C and 263.2 °C (Figure 3.3 (D)). First and second transitions in the copolymer thermograms may be attributed to the extraction of solvent and dopant ion removal respectively. These transitions observed in the thermograms of copolymers are different than the pure cases and prove the formation of the copolymers. Differences between the thermograms for Ppy and copolymers reveal the fact that copolymers are more heat resistant than the macromonomers.

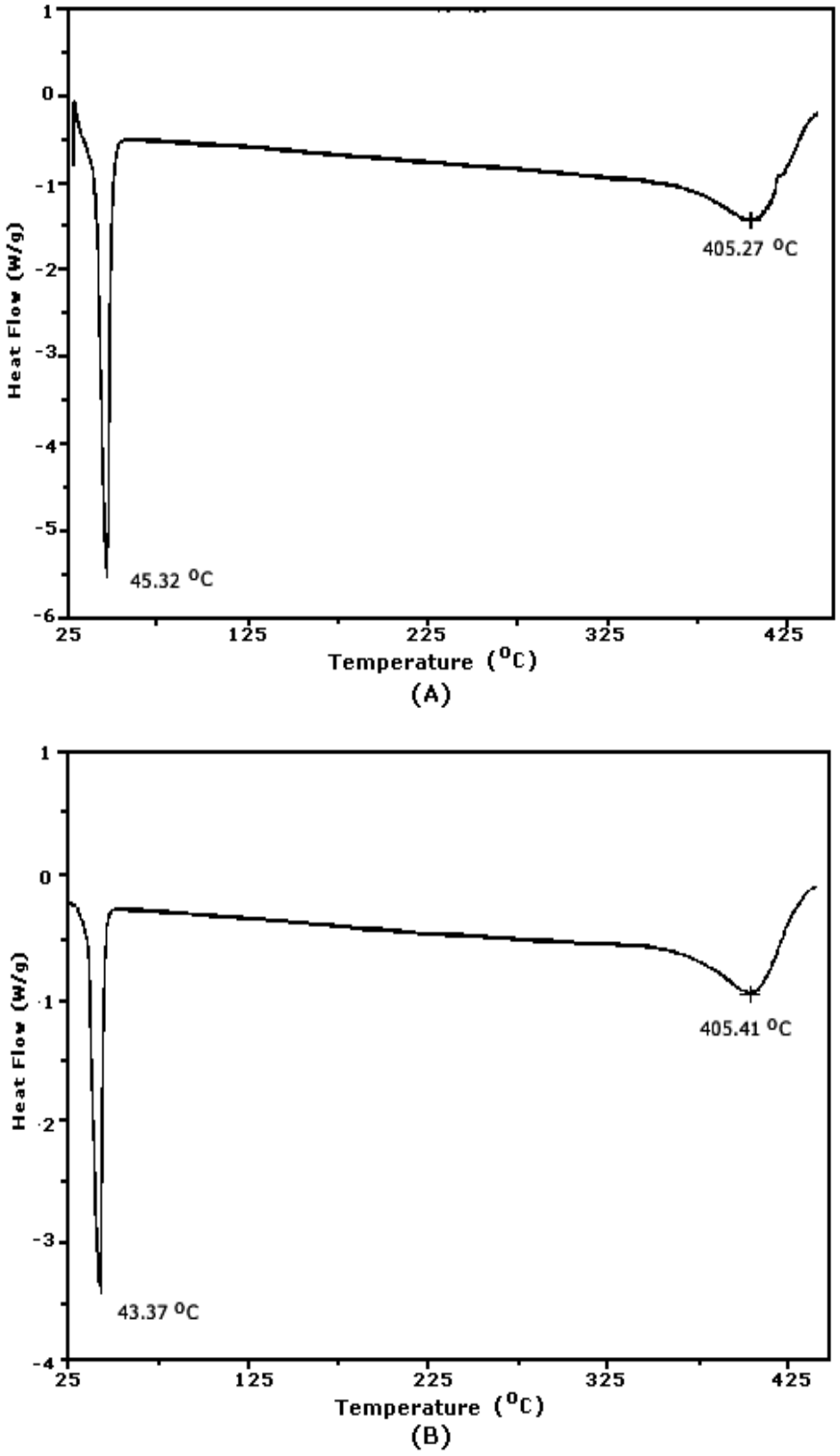
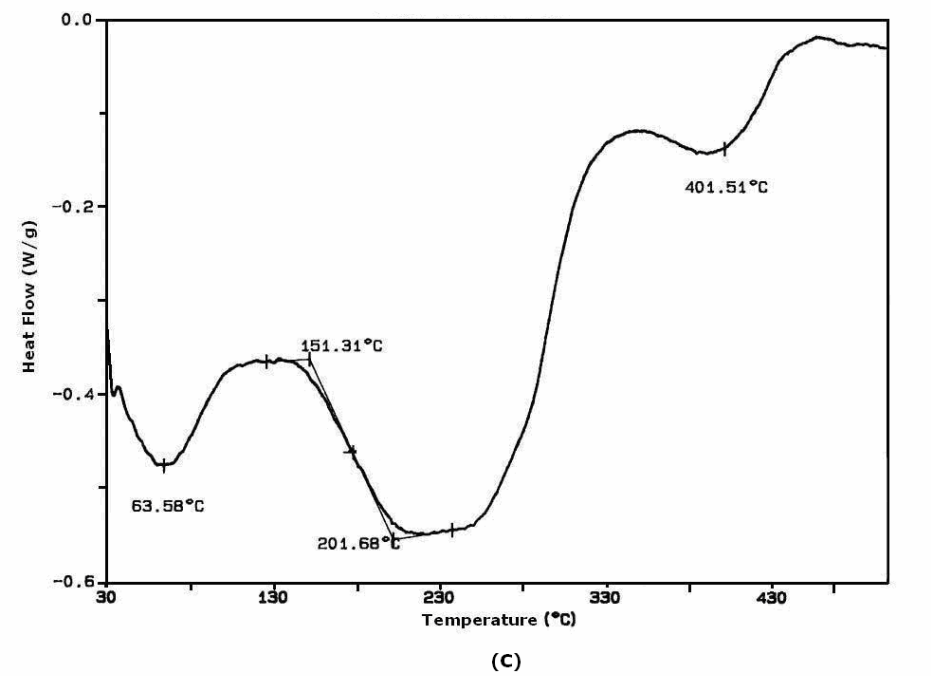
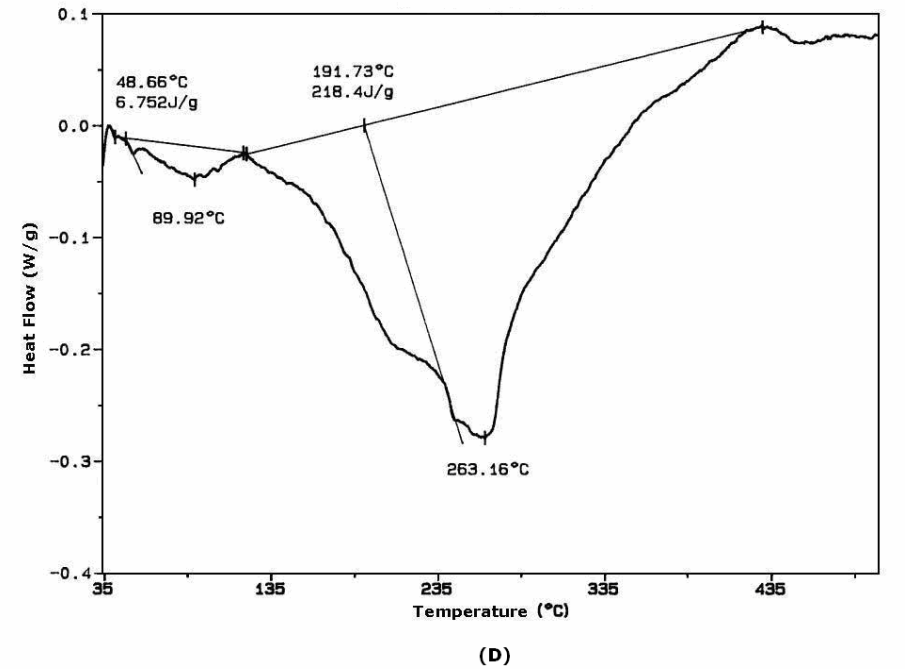


Figure 3.3. DSC thermograms for (A) TPTHF1 (B) TPTHF2.



(C)



(D)

Figure 3.3.(Continued) DSC thermograms for (C) TPTHF1-co-Py (D) TPTHF2-co-Py.

3.1.2.4 Morphologies of the Copolymer Films

The morphologies of the copolymer films were investigated by Scanning Electron Microscopy (SEM) studies (JEOL JSM-6400). Figure 3.4 shows the SEM photographs of Ppy, TPTHF1-co-Py and TPTHF2-co-Py. Typical cauliflower-like structure was observed for the solution side of SDS doped Ppy film. However, morphology of the solution sides of washed TPTHF1-co-Py and TPTHF2-co-Py copolymers exhibited a little different surface properties. They show more compact structure but no significant difference was observed between the two copolymers films.

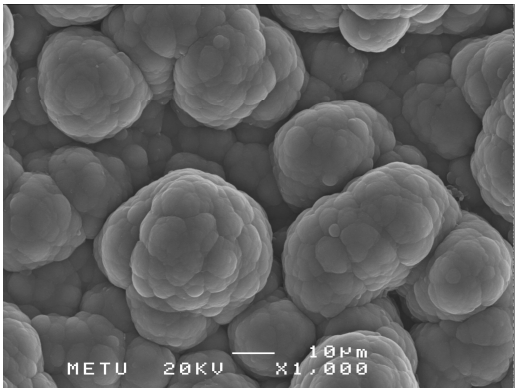
3.2 Immobilization of Enzymes

3.2.1 Invertase Immobilization

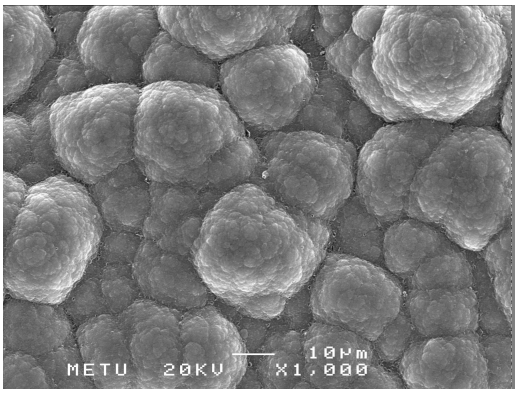
3.2.1.1 Kinetic parameters of Immobilized Invertase

Invertase enzyme is available at a small cost and it serves as a model enzyme for the immobilization of other expensive and more applicable enzymes. 1 unit of invertase activity (EU) was defined as the amount of enzyme required to release 1 μmol glucose from sucrose per minute under the assay conditions.

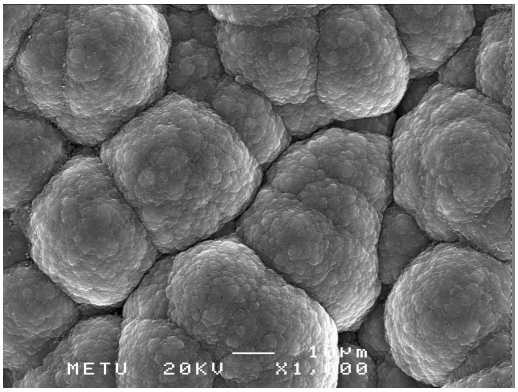
Activity assay for immobilized invertase was carried out according to previously described procedure (Sec. 2.3.3.2.2). For the calculation of invertase activity, a standard calibration curve which correlates invertase concentration and absorbance was obtained by



(A)



(B)



(C)

Figure 3.4. SEM micrographs of
(A) Electrode side of washed Ppy film
(B) Electrode side of washed TPTHF1-co-Py film
(C) Electrode side of washed TPTHF2-co-Py film

performing activity assay at constant temperature and pH. Activities were determined by using the slopes of calibration, and the reaction rate curves. Reaction rate curve correlates the reaction time and absorbance at constant substrate concentration and gives the initial velocity at this substrate concentration. By the calculation of initial velocities at various concentrations of the substrate, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrode activities were obtained with respect to substrate concentrations, as shown in Figures 3.5 and 3.7 respectively. When calculating the activity of enzyme, total reaction volume was also taken into account. Corresponding Lineweaver-Burk plots are shown in Figures 3.6 and 3.8.

The kinetic parameters of the reaction catalyzed by immobilized invertase, namely the apparent Michaelis constant, K_m, and maximum velocity, V_{max}, were obtained from Lineweaver-Burk plots. The calculated values of the kinetic parameters, the Michaelis constants and the maximum reaction rates are listed in Table 3.2.

Table 3.2. Kinetic parameters of invertase.

Electrode Matrice	**V_{max} (μmole/min.mL)**	**K_m (mM)**
Ppy	2.9*	24.4*
TPTHF1-co-Py	1.9	27.0
TPTHF2-co-Py	2.0	22.2

*[129].

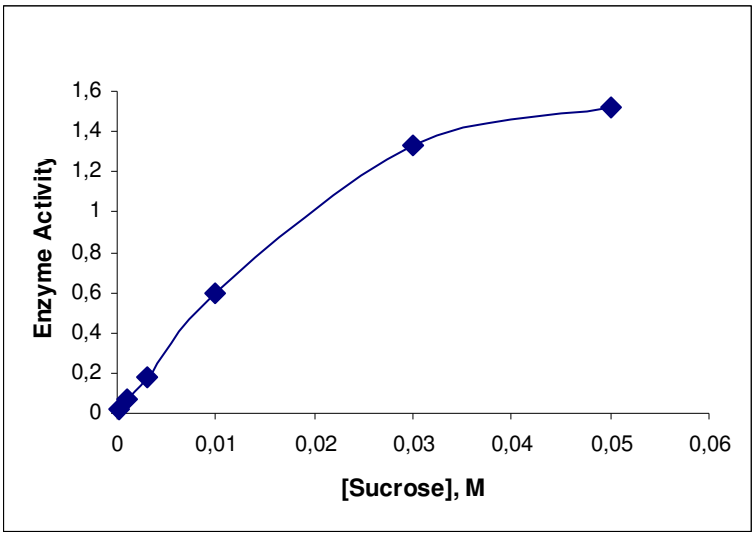


Figure 3.5. Invertase activity versus substrate concentration for immobilized invertase in TPTHF1-co-Py matrice.

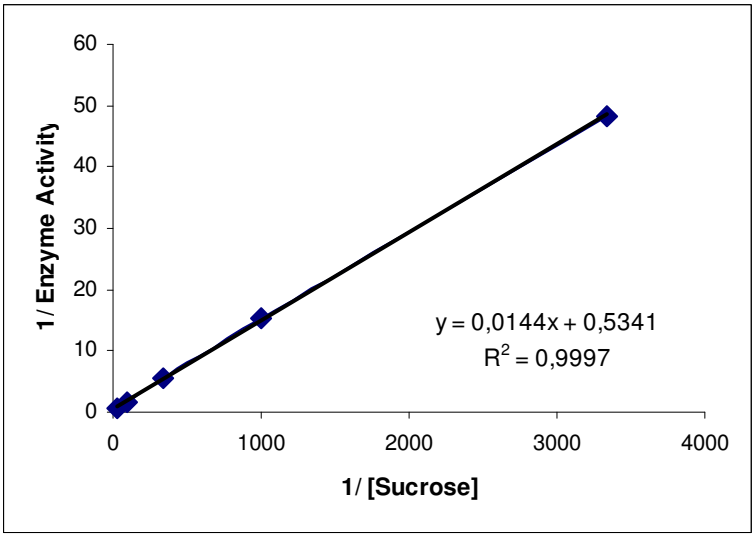


Figure 3.6. Lineweaver-Burk plot for invertase immobilized in TPTHF1-co-Py matrice.

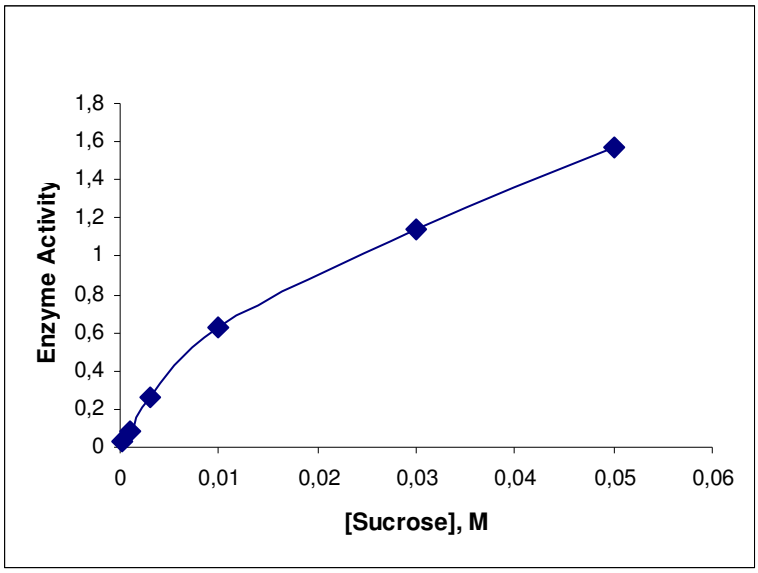


Figure 3.7. Invertase activity versus substrate concentration for immobilized invertase in TPTHF2-co-Py matrice.

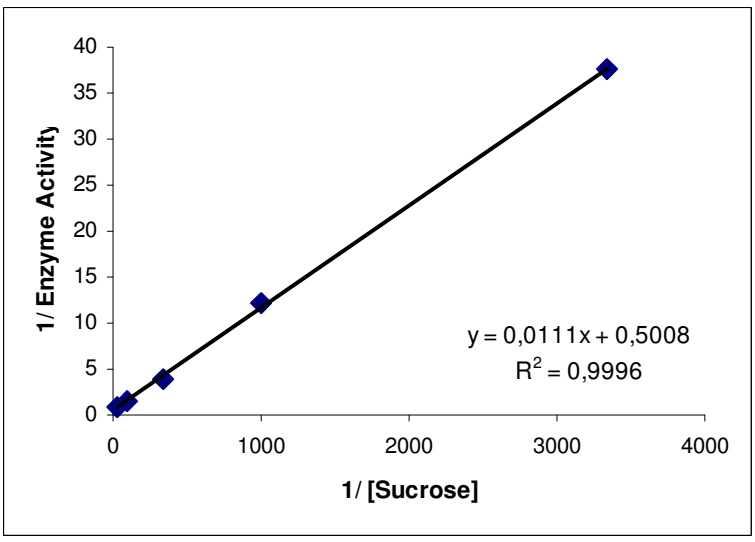
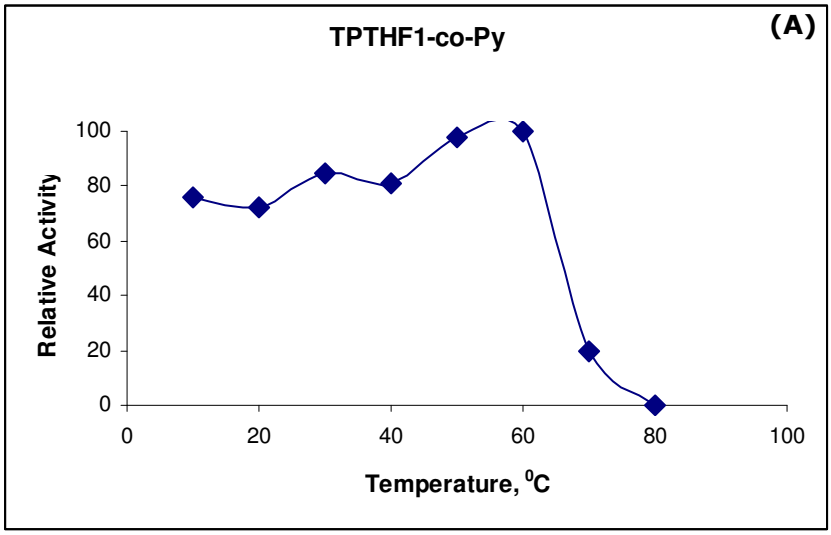


Figure 3.8. Lineweaver-Burk plot for invertase immobilized in TPTHF2-co-Py matrice.

For free invertase, V_{max} and K_m values are 82.3 μmol/min.mL and 24.3 mM respectively [129]. V_{max} and K_m values for Ppy and copolymer matrices are significantly different from those of free invertase. Ppy and copolymer matrices show similar K_m but lower V_{max} values than free invertase, indicating that these matrices provide a microenvironment which does not prevent enzyme and substrate to come together, yet slow down the reaction rate of the enzyme catalyzed reaction.

3.2.1.2 Effect of Temperature on Invertase Activity

The effect of incubation temperature on the relative enzyme activities was investigated and illustrated in Figure 3.9. The maximum activity for free enzyme is at about 50 °C. Ppy electrode reveals a temperature of 50 °C where invertase shows the maximum activity, whereas TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes reveal a stability range between 10°C – 60°C with an activity about 90% of its original.



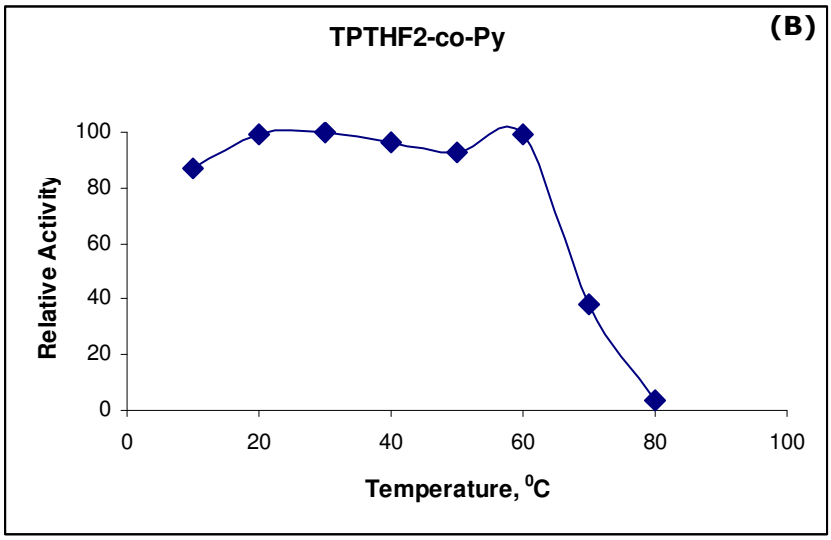
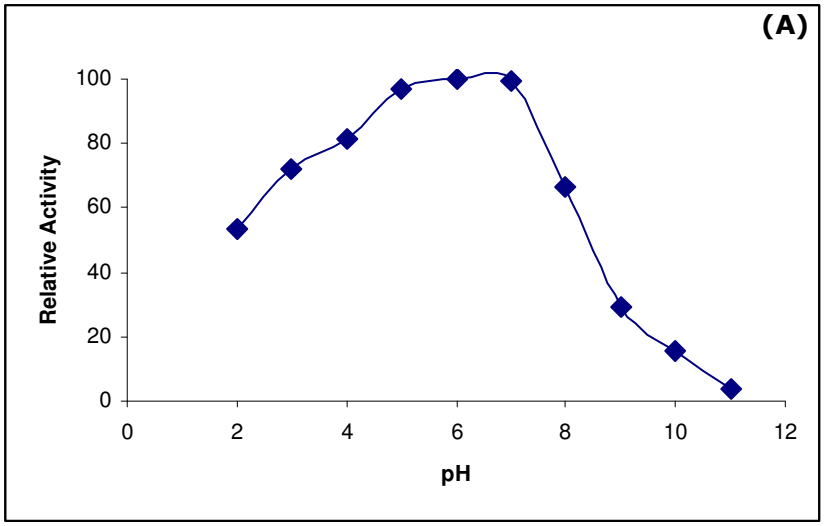


Figure 3.9. Effect of temperature on invertase enzyme activity immobilized in (A) TPTHF1-co-Py (B) TPTHF2-co-Py enzyme electrodes.

3.2.1.3 Effect of pH on Invertase Activity

The pH effect on the activity of the immobilized invertase was studied at a constant concentration of sucrose (10 K_m) and with an incubation temperature of 25 °C. Figure 3.10 shows the plot of enzyme activity of immobilized invertase at different pH values. Free enzyme showed an optimum pH range between 4 and 5.8 [129]. Relative activities were above 60% between that pH ranges. After pH 5.8, relative activities were decreased. Optimum pH values were 4.6 for free invertase, 5.0 for Ppy matrice, and 7.0 for both TPTHF1-co-Py and TPTHF2-co-Py matrices (Figures 3.10 (A) and (B)). The optimum pH resulting in maximum response was more alkaline than the isoelectric point of the enzyme. The optimum pH was shifted toward the alkaline side, that is a result probably due to attraction of H-ions in solution by negatively charged groups of matrice, creating a microenvironment for the bound enzyme that has higher hydrogen ion concentration (lower pH) than the surrounding solution where the pH was actually measured. Copolymer matrices provide a different working condition with the optimum pH of 7.0 which is different than the optimum pH for Ppy matrice.



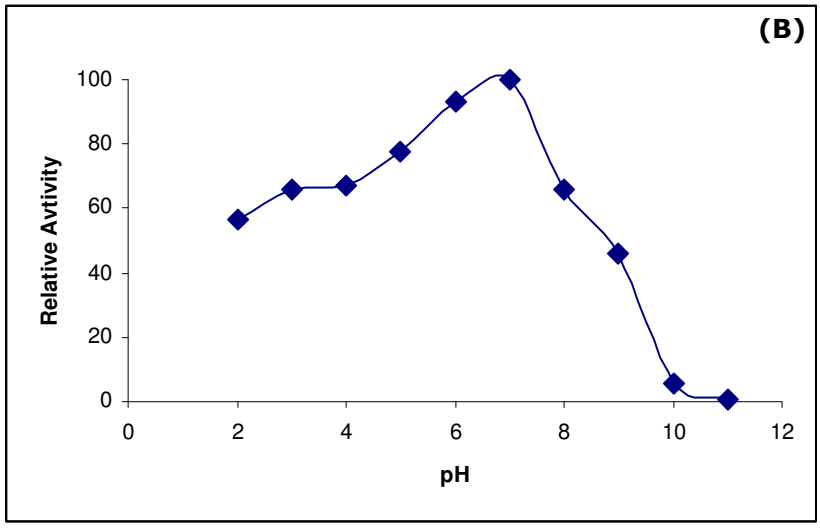


Figure 3.10. Effect of pH on invertase enzyme activity immobilized in (A) TPTHF1-co-Py (B) TPTHF2-co-Py enzyme electrodes.

3.2.1.4 Storage Stability

Both TPTHF1-co-Py and TPTHF2-co-Py electrodes showed similar responses compared to Ppy matrice which reveals a constant activity about 80%. However, the relative activities of the copolymer electrodes were about 90% which is better than that of Ppy and remained constant for 40 repetitive uses as shown in Figure 3.11.

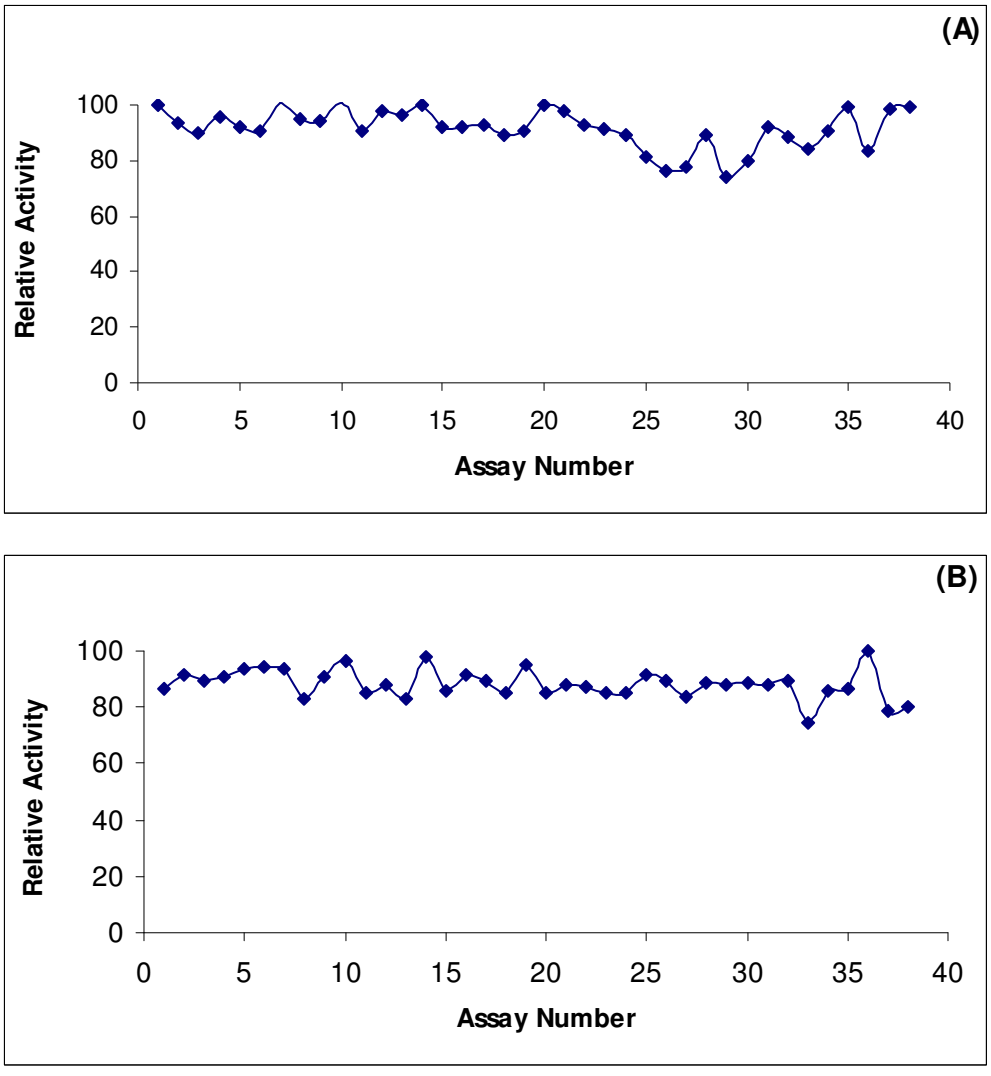


Figure 3.11. Effect of repetitive use on the activity of invertase immobilized in (A) TPTHF1-co-Py (B) TPTHF2-co-Py enzyme electrodes.

In order to determine the storage stability namely, shelf-life of immobilized invertase, electrodes were kept in buffer solution at 4 °C and the activity of invertase was measured periodically at 25 °C on the first, second, third, fourth, and the following days of a month. The results of the storage stability of immobilized invertase are shown in Figure 3.12. The relative activity of the enzyme electrodes stayed about 90% for the first 15 days then decreased sharply and completely lost at the end of 30 days.

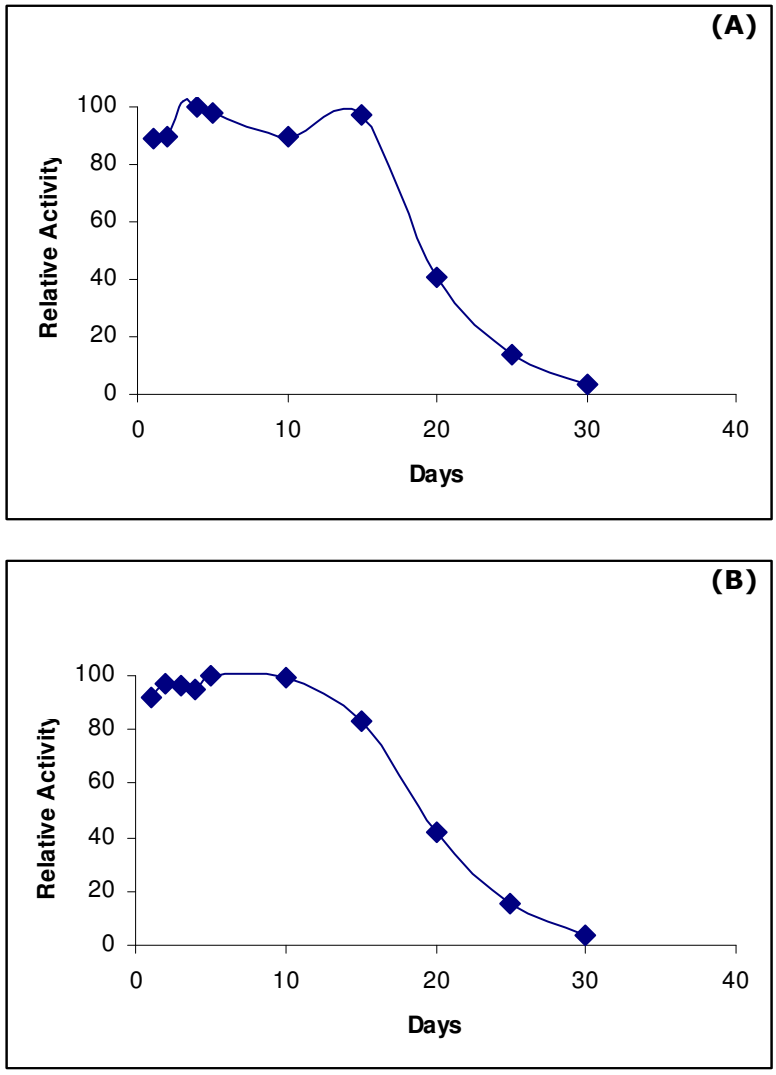


Figure 3.12. Shelf-life of immobilized invertase in (A) TPTHF1-co-Py (B) TPTHF2-co-Py enzyme electrodes.

3.2.2 Polyphenol Oxidase Immobilization

3.2.2.1 Kinetic parameters of Immobilized PPO

Kinetic parameters, V_{max} and K_m were obtained according to the enzyme assay based on the measurement of *o*-quinone generated in enzymatic reaction. The method described in section 2.3.3.3.1 is based on the spectrophotometric measurements of absorbance of the compound produced when MBTH interacts with the quinones produced by the enzyme to yield colored products. The maximum reaction rate, V_{max}, and Michaelis-Menten constant, K_m, for the immobilized PPO were obtained from Lineweaver-Burk plots as explained in section 3.2.1.1. TPTHF1-co-Py and TPTHF2-co-Py enzyme electrode activity curves are shown in Figures 3.13 and 3.15 respectively. Corresponding Lineweaver-Burk plots are shown in Figures 3.14 and 3.16. The maximum reaction rate, V_{max}, and Michaelis-Menten constant, K_m, for the immobilized PPO are shown in Table 3.3 together with those of Ppy and free PPO.

Table 3.3. Kinetic parameters of PPO.

	V_{max}		**K_m (mM)**
Free PPO	0.073	(μmol/min.mL)	4
Ppy	0.031	(μmol/min.electrode)	96
TPTHF1-co-Py	0.021	(μmol/min.electrode)	25
TPTHF2-co-Py	0.022	(μmol/min.electrode)	20

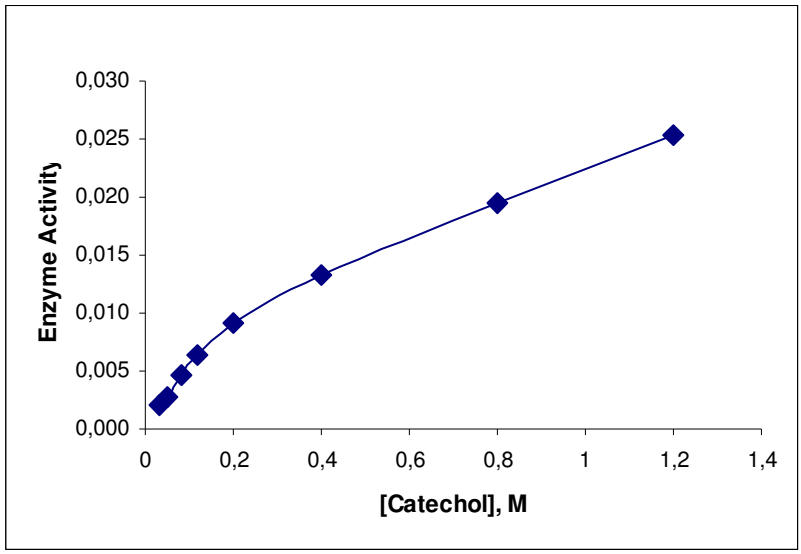


Figure 3.13. PPO activity versus substrate concentration for immobilized PPO in TPTHF1-co-Py matrice.

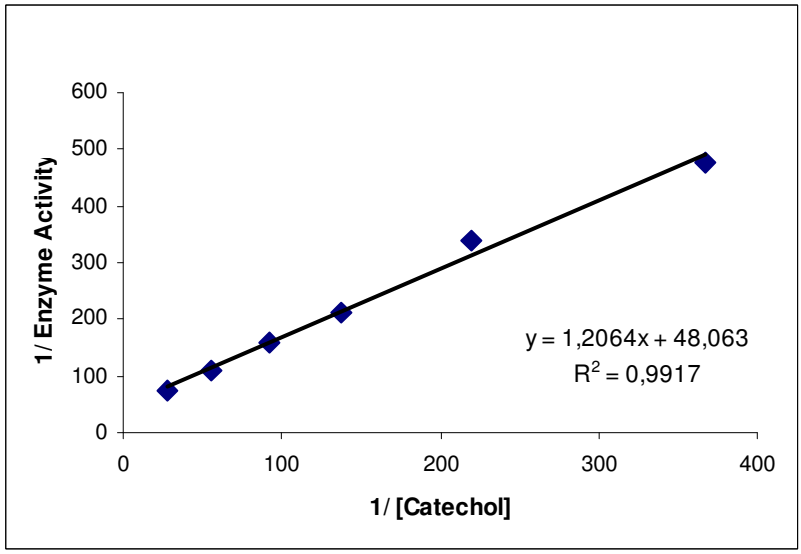


Figure 3.14. Lineweaver-Burk plot for PPO immobilized in TPTHF1-co-Py matrice.

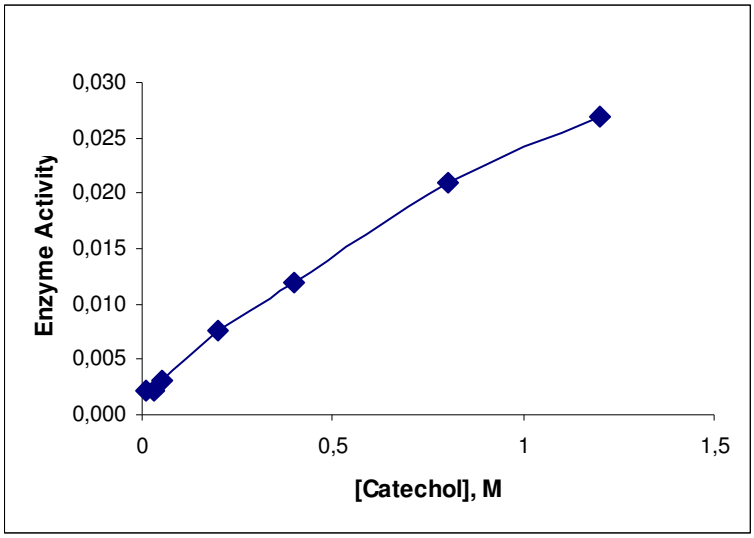


Figure 3.15. PPO activity versus substrate concentration for immobilized PPO in TPTHF2-co-Py matrice.

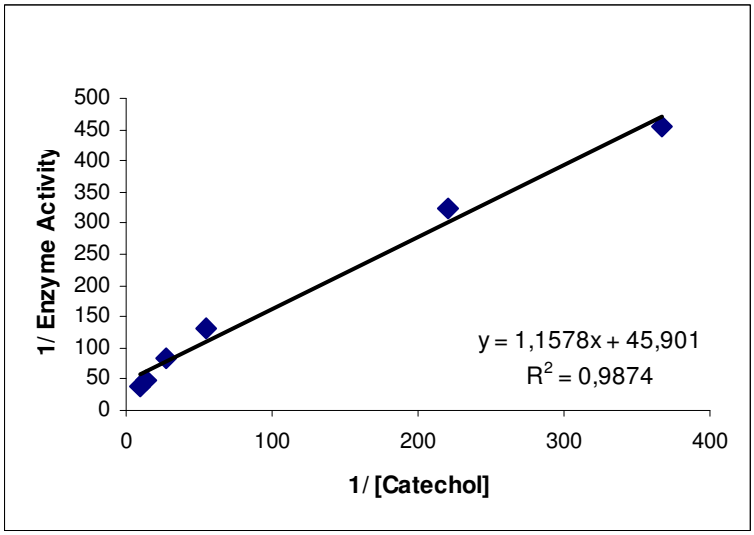


Figure 3.16. Lineweaver-Burk plot for PPO immobilized in TPTHF2-co-Py matrice.

Rate of reaction decreases due to immobilization as expected. The diffusion of substrate from the bulk solution to the micro-environment of an immobilized enzyme can limit the rate of the enzyme reaction. Thus, Ppy and copolymer entrapped enzymes have comparable reaction rates but lower than that of the free enzyme. However, K_m values of immobilized enzyme in both Ppy and copolymer matrices are higher than that of free enzyme thus, showing a lower affinity between the substrate and immobilized enzyme compare to the one between substrate and free enzyme. This might be an outcome resulted from the electrostatic effects of matrice on the enzyme and substrate. Entrapment by the matrice might result in developing similar charges on the enzyme and substrate and hence a repulsion between enzyme and substrate. Similarly, this charge on the matrice and substrate may induce a repulsion between substrate and matrice and make difficult for enzyme and substrate stay together. On the other hand, copolymers have much better affinity constants than Ppy. It means that enzyme entrapped in copolymer has much better affinity towards its substrate where copolymers have slightly more opposite charges to the substrate than that of Ppy.

3.2.2.2 Effect of Temperature on PPO Activity

The effect of temperature on the activity of free and immobilized enzyme was investigated and is given in Figure 3.17. Both free and immobilized polyphenol oxidase in Ppy showed an optimal temperature of 40°C. Free PPO loses its activity completely at 50 °C but PPO immobilized in Ppy electrode only 40% of original activity at this temperature. However, optimum temperature shifts to 50 °C for immobilized PPO in TPTHF1-co-Py and TPTHF2-co-Py electrodes. Copolymer matrices protect the enzyme against higher temperatures.

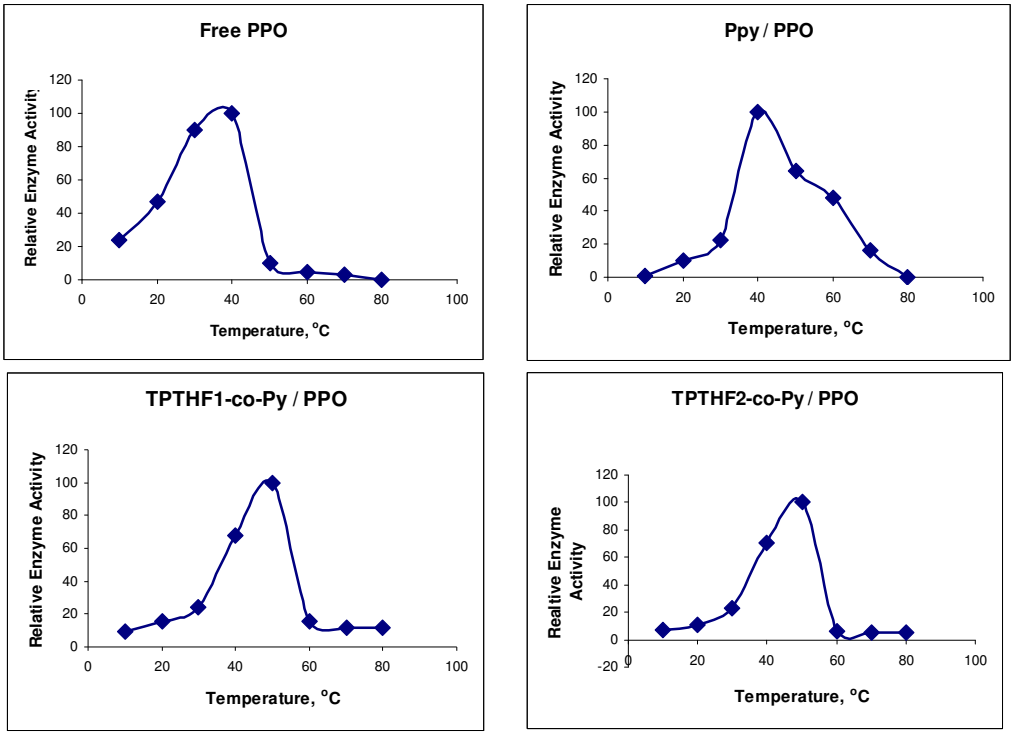


Figure 3.17. Effect of temperature on PPO enzyme activity of free and immobilized enzyme in Ppy, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

3.2.2.3 Effect of pH on PPO Activity

The effect of pH on enzyme activity was investigated and given in Figure 3.18. Free PPO had an optimum pH of 5. Immobilized PPO revealed an optimal pH for Ppy/PPO as 7 and thus, pH stability is increased upon immobilization in Ppy. The optimum pH values for both TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes were shifted towards the alkaline side compared to that of the free enzyme. This reveals a high degree of stability against pH. This might be explained by the partitioning of protons. The negative groups of the matrice are concentrated around the enzymes and attract the protons in the solution. This tendency makes

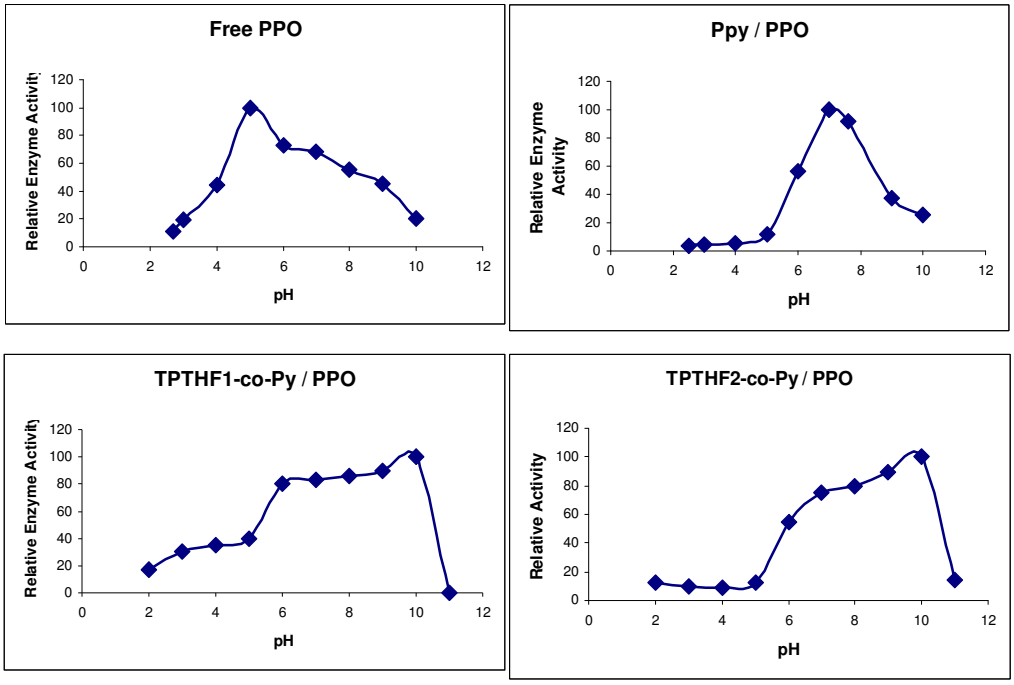


Figure 3.18. Effect of pH on PPO enzyme activity of free and immobilized enzyme in Ppy, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

the pH around enzyme lower than that of the bulk. Higher the tendency of matrice to concentrate protons within, higher the pH stability of enzyme in the matrice.

3.2.2.4 Storage Stability of PPO Electrodes

Operational stability is an important consideration for immobilized enzymes. To determine this parameter, activities of the enzyme electrodes were studied for 40 successive measurements. Figure 3.19 shows the operational stability of Ppy and copolymer electrodes respectively The rate of loss of activity of the immobilized enzyme is high for the first 10 uses retaining 60% of the initial activity, since

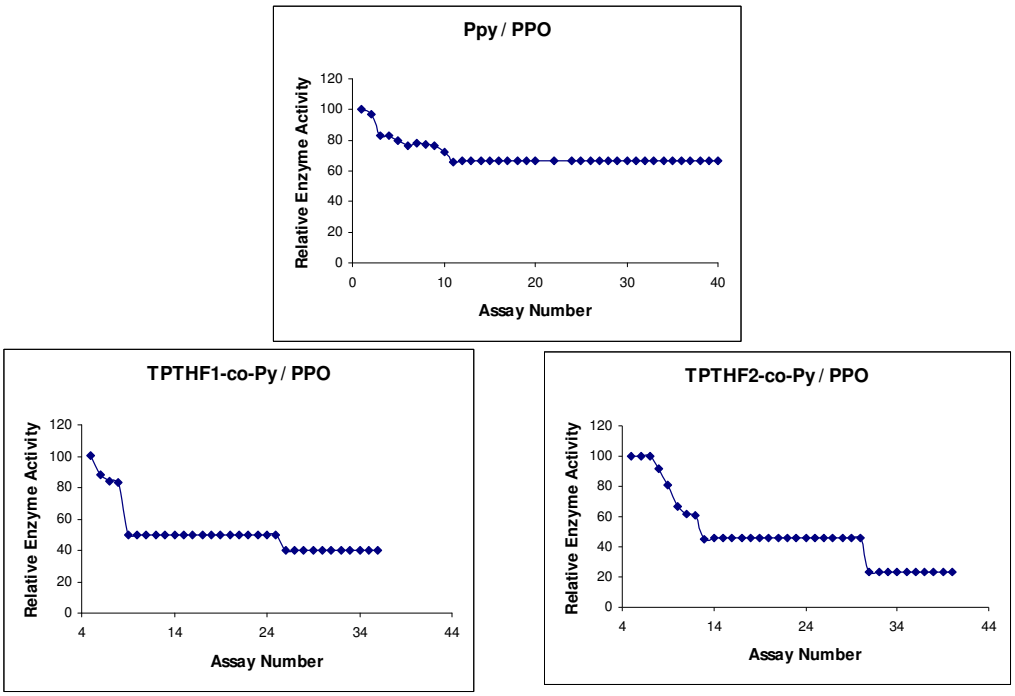


Figure 3.19. Effect of repetitive use on PPO enzyme activity immobilized in Ppy, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

most of the loosely bound enzyme was leached during early stages of the well-entrapped enzyme in the films. The PPO immobilized in copolymer matrices retained 50% of its initial activity after 10 use.

Since enzymes can easily lose their catalytic activity and get denatured, stability of electrodes as a function of time over a period of 40 days was also investigated. Before performing the enzyme assays, the electrodes were washed by buffer solution to remove any loosely bound enzyme that might have diffused to the surface of the film in time. Figure 3.20 shows the effect of time on the activity of the immobilized PPO. The rate of loss of activity of the Ppy immobilized enzyme was high for the first days, retaining 60% of the initial activity after 10 days. However, PPO immobilized in copolymer matrices retained 100% activity during 20 days.

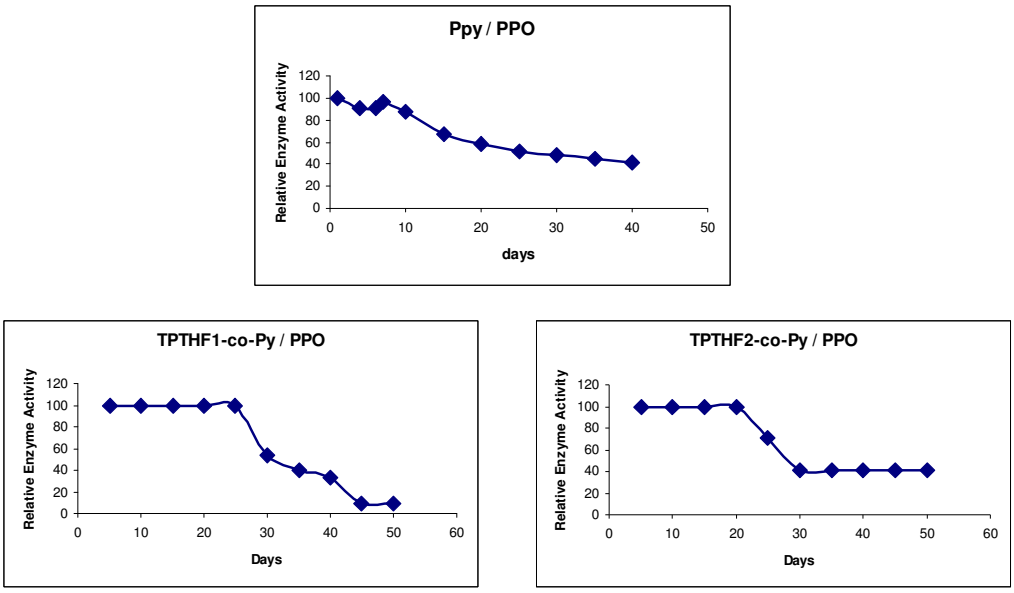


Figure 3.20. Shelf-life of PPO immobilized in TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

3.2.2.5 Phenolic Compounds in Red Wine

TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes were used to determine total phenolic compounds in wines. Total phenolic compounds in Turkish wines were reported as 2000-3000 mg/L [125-127]. Polyphenol oxidase enzyme act on –OH groups on phenolic compounds. Total amount of –OH groups in red wines was obtained through activity determination of enzyme electrodes. Two Turkish red wines (Brand K and Brand D) were analyzed for their concentration of phenolic compounds.

Results for phenolic determination by using free PPO enzyme give misleading values when compared to immobilized enzyme electrodes as shown in Table 3.4 due to the inhibitors present in the wine. As it is known from the literature, benzoates act as inhibitors for free PPO and they are found naturally in wines. However, PPO protected via matrix entrapment was not affected by the inhibitors and the results are found to be comparable to the literature values of phenolic compounds in Turkish red wines. Ppy enzyme electrode gave 4000mg/L phenolics in Brand K. However, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes gave lower values the same brand. This shows that as to the determination of phenolics, copolymer matrices do not function as properly as Ppy matrice does.

Brand K contains twice the amount of phenolics compared to that of Brand D. This result was confirmed by all enzyme electrodes. High amount of phenolics in Brand K is responsible for the bitter taste of the wine. Results are reported in Gallic Acid Equivalent (GAE) [124].

Table 3.4. Phenolic compounds in Turkish red wines.

	Brand K	**Brand D**
Free PPO*	0.004M –OH 220mg/L	0.005M –OH 270mg/L
Ppy/PPO*	0.072M -OH 4000mg/L	0.04M -OH 2200mg/L
TPTHF1-co-Py / PPO	0.048M -OH 2670mg/L	0.027M -OH 1460mg/L
TPTHF2-co-Py / PPO	0.040M -OH 2200mg/L	0.022M -OH 1200mg/L

*[98]

3.2.3 Glucose Oxidase Immobilization

3.2.3.1 Kinetic parameters of Immobilized GOD

The maximum reaction rate, V_{max}, and Michaelis-Menten constant, K_m, for the immobilized GOD were obtained from Lineweaver-Burk plots as explained in section 3.2.1.1. TPTHF1-co-Py and TPTHF2-co-Py enzyme electrode activity curves are shown on Figures 3.21 and 3.23 respectively. Corresponding Lineweaver-Burk plots are shown in Figures 3.22 and 3.24.

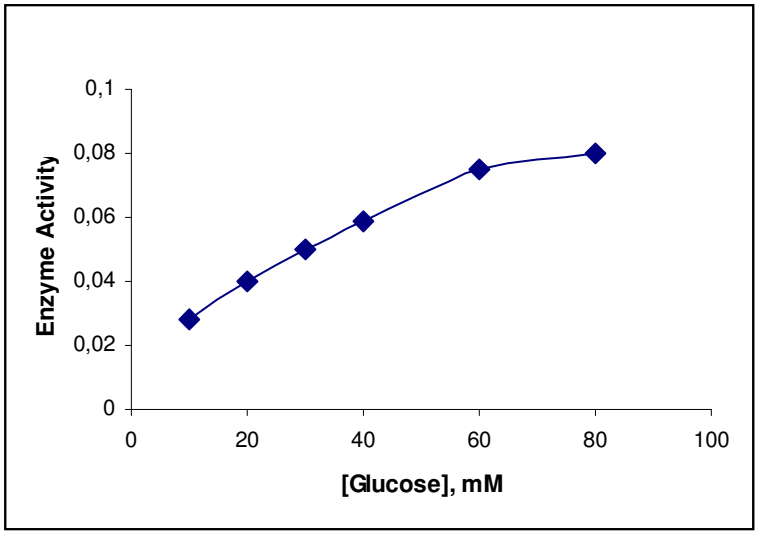


Figure 3.21. GOD activity versus substrate concentration for immobilized PPO in TPTHF1-co-Py matrice.

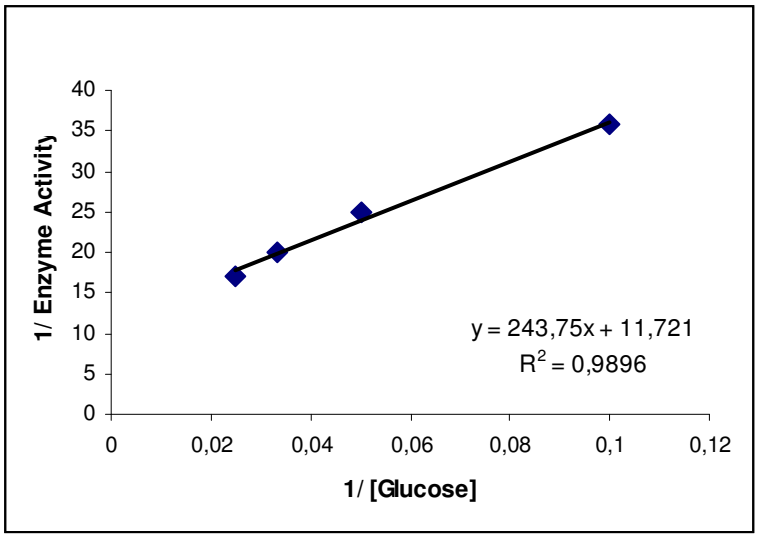


Figure 3.22. Lineweaver-Burk plot for GOD immobilized in TPTHF1-co-Py matrice.

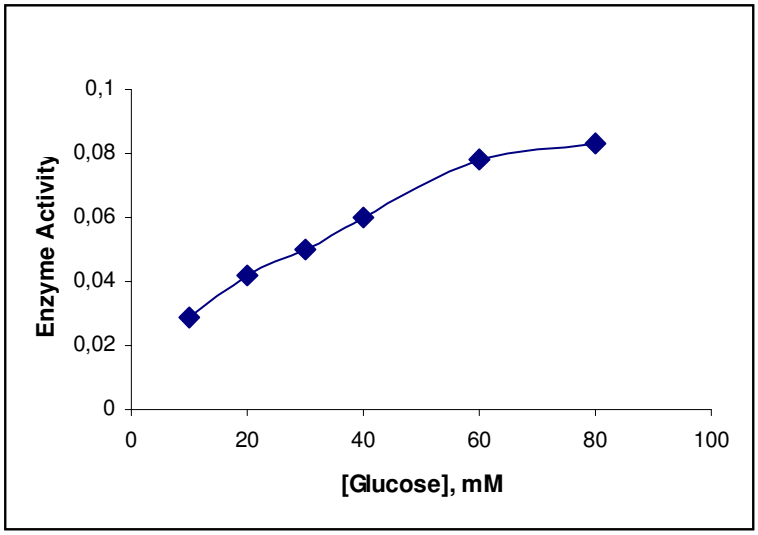


Figure 3.23. GOD activity versus substrate concentration for immobilized PPO in TPTHF2-co-Py matrice.

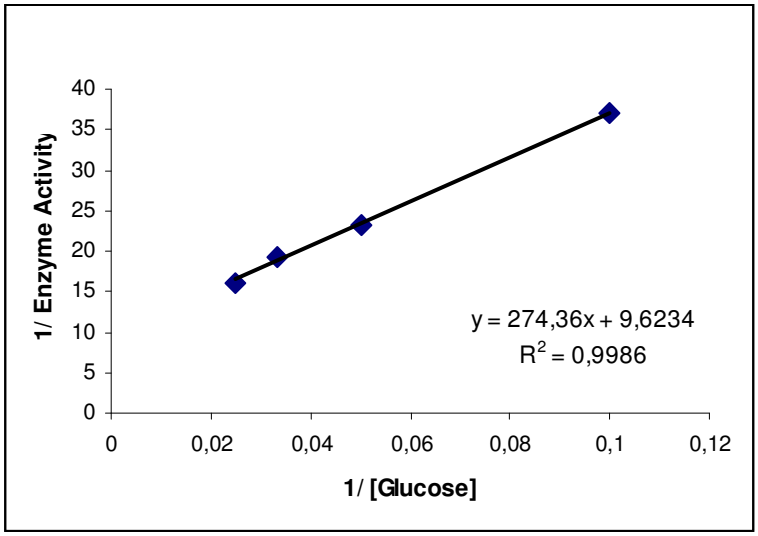


Figure 3.24. Lineweaver-Burk plot for GOD immobilized in TPTHF2-co-Py matrice.

The comparison between the kinetic parameters of free and immobilized GOD showed that, the maximum reaction rate decreases for immobilized GOD, as shown in Table 3.5, which is attributed to the restricted diffusion of substrate to the cavities of polymer support. GOD enzyme immobilized in Ppy matrice shows higher reaction rate than the enzyme immobilized in copolymer matrices, which might be a result of porosity differences between Ppy and copolymer matrices.

K_m values of immobilized GOD are higher than that of the free counterpart. The increase in K_m values upon immobilization clearly indicates a lower affinity of immobilized enzyme to the substrate as compared to that of free enzyme. This behavior may be attributed to changes in enzyme structure induced by the interaction of enzyme molecule with the support. In addition, the increase in diffusional resistance encountered by the substrate towards the catalytic sites of immobilized enzymes resulted in the same behavior. However, the Michaelis constant was lower for TPTHF1-co-Py and TPTHF2-co-Py

Table 3.5. Kinetic parameters of GOD.

	V_{max}		**K_m (mM)**
Free GOD	10.3	(μmol/min.mL)	9.4
Ppy / GOD	2.10	(μmol/min.electrode)	36.6
TPTHF1-co-Py / GOD	0.09	(μmol/min.electrode)	21.7
TPTHF2-co-Py / GOD	0.11	(μmol/min.electrode)	28.4

electrodes than for Ppy matrice. This result shows that copolymer matrices provide a more convenient microenvironment to enzyme and substrate.

3.2.3.2 Effect of Temperature on GOD Activity

The activity of free GOD is about 100% of its original activity for a range between 10 and 40 °C while immobilized enzyme in Ppy has an optimum of 30 °C. After the optimum, as the temperature increases, the structure of the enzyme becomes altered and its catalytic properties are reduced and eventually destroyed. Effect of temperature on enzyme electrodes is given in Figure 3.25. Maximum activity was found in the range 20 to 40 °C for enzyme electrodes after which activity decreases and is completely lost at 70 °C.

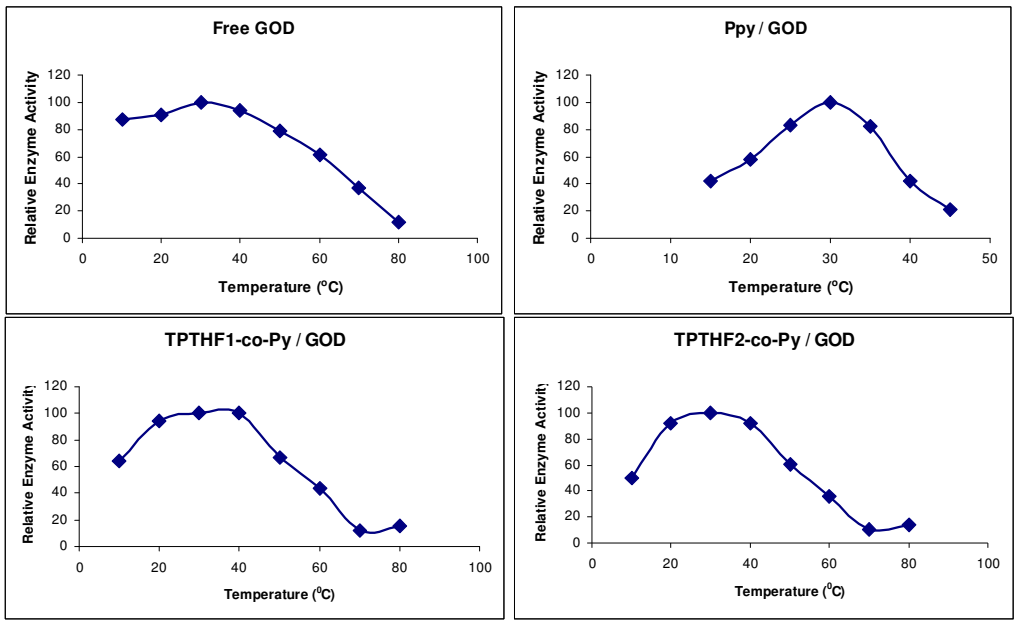


Figure 3.25. Effect of temperature on GOD enzyme activity of free and immobilized enzyme in Ppy, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

3.2.3.3 Effect of pH on GOD Activity

Free GOD has an optimum pH of 5.5 which is a value near to isoelectric point. Ppy immobilized enzyme exhibits an optimum pH at 6 which is close to that of free enzyme. TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes show an optimum pH of 5 after which the activity decreases rapidly. Figure 3.26 exhibits the effect of pH on the activity of GOD enzyme.

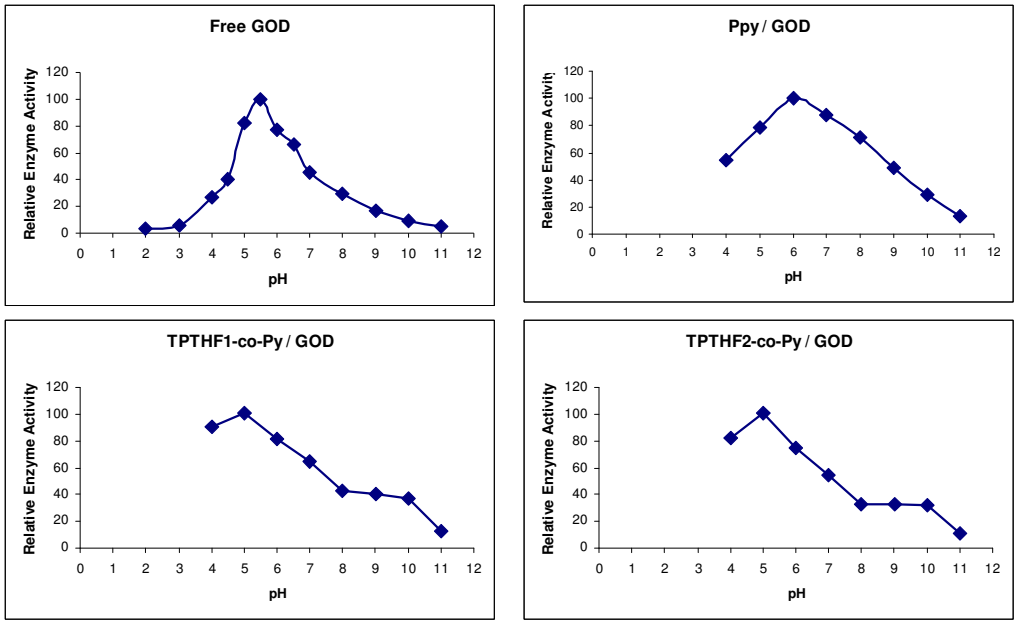


Figure 3.26. Effect of pH on GOD enzyme activity of free and immobilized enzyme in Ppy, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

3.2.3.4 Storage Stability of GOD Electrodes

TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes exhibit good stability against the repetitive uses as presented in Figure 3.27. Response of the copolymer electrodes is similar to that of Ppy enzyme electrode where there is a gradual decrease in the enzyme activity in 10 uses, after which the electrodes retain 80% of their original activity.

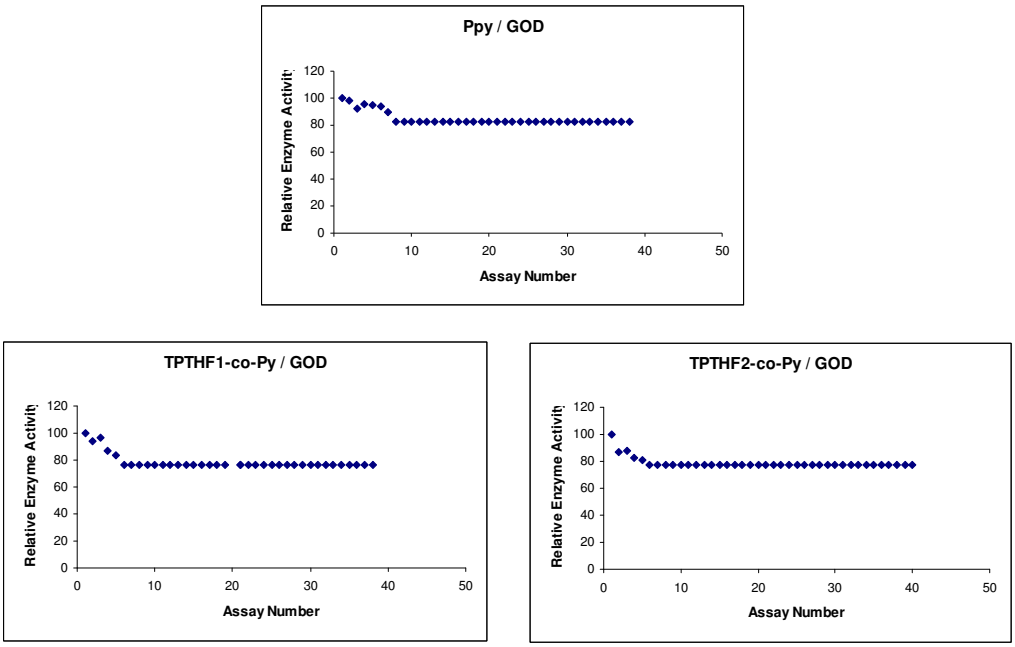


Figure 3.27. Operational stability of GOD immobilized in Ppy, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

Shelf-life behaviors of GOD enzyme immobilized in TPTHF1-co-Py and TPTHF2-co-Py are given in Figure 3.28. Both enzyme electrodes show a high enzyme activity for the first ten days. Then the activities decrease and stay constant at about 40% of its initial activity.

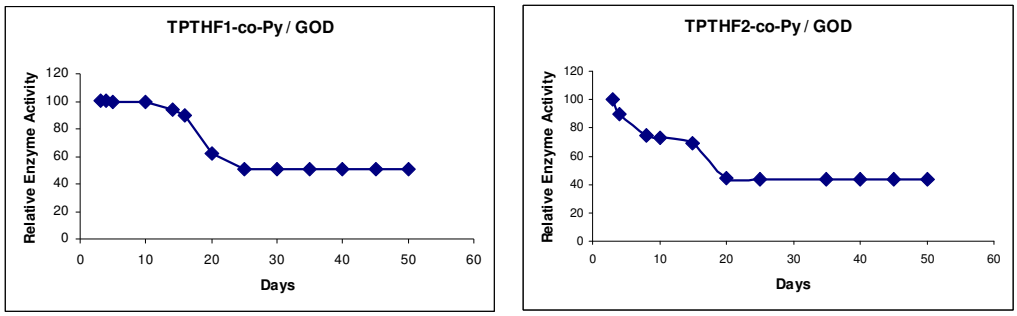


Figure 3.28. Shelf-life of GOD immobilized TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes.

3.2.3.5 Glucose in Orange Juices

This study was performed to determine glucose concentration in orange juices by using the enzyme electrodes constructed. Two Turkish market orange juices (Brand D and Brand M) were analyzed for their glucose content using TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes. Lane-Eynon analysis of glucose in the same juices was performed. As seen in Table 3.6, comparable results were obtained for both methods. Brand M was found to have a slightly higher glucose content than Brand D.

Table 3.6. Glucose amount in two kinds of orange juices.

	Brand D (g/100ml)	**Brand M (g/100ml)**
Lane-Eynon Method	1.50	2.15
Ppy	1.45	1.44
TPTHF1-co-Py	1.69	1.82
TPTHF2-co-Py	1.83	2.25

CHAPTER IV

CONCLUSIONS

The syntheses of graft copolymers of TPTHF1 and TPTHF2 and pyrrole were achieved via constant potential electropolymerization. Thermally stable and electrically conducting polymer films were obtained.

Immobilizations of invertase, PPO and GOD enzymes were carried out in conductive matrices. Kinetic parameters for enzyme activity, V_{max} and $K_{m,}$ temperature and pH effect on the enzyme electrodes and storage stability were investigated. In terms of the enzyme activity and the stability of the enzyme electrodes, good and comparable results were obtained with the conducting copolymer matrices.

PPO enzyme electrodes exhibited better V_{max} and K_m values. Although they have limited storage capacities, these enzyme

electrodes are very useful as their high kinetic parameters point. In addition to that, they exhibit a wide pH working range. Their application for analysis of phenolic compounds in red wine as an alternative method to the conventional methods is reasonably possible.

GOD enzyme electrodes show better operational stability with reasonable shelf-life and comparable kinetic parameters to the ones obtained with the other electrodes constructed. They were utilized successfully for the determination of glucose in orange juices. The results were approved by the conventional analysis methods.

Two copolymer matrices; TPTHF1-co-Py and TPTHF2-co-Py show comparable results since they have no superior performance over one another as enzyme electrodes.

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VITA

A. Elif Böyükbayram was born on November 17, 1965 in İzmir. She graduated from Middle East Technical University (METU), Department of Chemistry in 1995. She began her MS studies at Chemistry Department of METU where she became a research assistant. During this time, she worked on trace metal determination by instrumental techniques under the supervision of Prof. Dr. Mürvet Volkan. She began doctorial studies in 1999. She has worked with Prof. Dr. Levent Toppare in the area of electrochemical polymerization, conducting polymers and enzyme immobilization.

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