BIOSENSOR BASED ON INTERPENETRATED POLYMER NETWORK OF ALGINIC ACID AND POLY (1-VINYLIMIDAZOLE)

A THESIS SUBMITTED TO
THE GRADUATED SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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

JANUARY 2008

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ABSTRACT

BIOSENSOR BASED ON INTERPENETRATED POLYMER NETWORK OF ALGINIC ACID AND POLY (1-VINYLIMIDAZOLE)

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January 2008, 63 pages

A new proton conductor polymer was prepared using alginic acid (AA) and poly (1-vinylimidazole) (PVI). The polymer network was obtained by mixing AA and PVI at various stoichiometric ratios, x (molar ratio of the monomer repeat units). The AA/PVI network was characterized by elemental analysis (EA) and FT-IR spectroscopy. Potential use of this network in enzyme immobilization was studied. Enzyme entrapped polymer networks (EEPN) were produced by immobilizing invertase and tyrosinase (PPO) in the AA/PVI network.

Additionally, the maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) were investigated for the immobilized invertase and enzymes. Also, temperature and pH optimization, operational stability and shelf life of the polymer network were examined.

Keywords: Alginic acid, Poly (1-vinylimidazole), invertase, tyrosinase, enzyme immobilization, proton conductivity.

ÖZ

ALGİNİK ASİT VE POLİ (1-VİNİLİMİDAZOL) İLE ELDE EDİLEN POLİMER AĞLARININ BİOSENSÖR OLARAK KULLANIMI

Kartal, Müjgan
Yüksek Lisans, Kimya Bölümü
Tez Yöneticisi: Prof. Dr. Levent Toppare

Ocak 2008, 63 sayfa

Bu çalışmada alginik asit ve poli(1-vinilimidazol) kullanılarak yeni bir proton iletken polimer sentezlendi. Bu polimer ağı farklı stokiometrik oranlarda, x (monomerdeki tekrarlanan kısmın molar oranı) alginik asit ve poli(1-vinilimidazol) 'un karıştırılarak elde edildi. AA/PVI ağı elemental analiz (EA) ve FT-IR spektroskopisi ile karakterize edildi. Ağın enzim tutuklaması işlemine uygunluğu incelendi. Enzim tutuklanmış polimer ağı (EEPN), invertaz ve tyrosinaz (PPO) enzimlerinin tutuklanması ile elde edildi.

Tutuklanmış invertaz ve tyrosinaz enzimlerinin maksimum reaksiyon hızı (V_{max}) ve Michaelis-Menten sabitleri (K_m) incelendi. Ayrıca uygun değer sıcaklık ve pH, kararlılıkları ve raf ömürleri tayin edildi.

Anahtar Kelimeler: Alginik asit, poli(1-vinilimidazol), invertaz, tyrosinaz, enzim tutuklanması, proton iletkenliği.

To Hakan Koray Dinç

ACKNOWLEDGMENTS

I would like to express appreciation to my supervisor Prof. Dr. Levent Toppare for his guidance and encouragement throughout this thesis.

I wish to express my sincere thanks to Dr. Senem Kıralp Kayahan and Dr. Ayhan Bozkurt for their valuable discussions and help throughout this study.

I also wish to express my endless thanks to Simge Tarkuç, Özlem Türkaslan for their patience, moral support and encouragement.

I would like to give my special thanks to Elif Yapar for her helps and moral support.

I also want to thank every member in our group for their friendship.

Finally I want to thank my family and Hakan Koray Dinç for their continuous support, understanding.

Appreciation is extended to colleagues at the Department of Chemistry, Middle East Technical University.

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LIST OF ABBREVIATIONS

AA	Alginic acid
PVI	Poly(1-vinylimidazole)
PPO	Polyphenol oxidase
PEM	Proton exchange membranes
HTPCs	High temperature proton conductors
EEPN	Enzyme entrapped polymer network
EA	Elemental analysis
EC	Enzyme commission
MBTH	3-methyl-2-benzothiozolinone
FT-IR	Fourier Transform Infrared Spectrophotometer
VIm	Vinylimidazole
PEO	Poly(ethylene oxide)
PVPA	Poly(vinylphosphonic acid)
P(4-VI)	Poly(4-vinylimidazole)
PEGMAP	Poly(ethyleneglycol methacrylate phosphate)
BnIm	Benzimidazole

CHAPTER I

INTRODUCTION

1.1 Conducting Polymers

In 2000 the Chemistry Nobel Prize was given to Alan Heeger, Alan McDiarmid and Hideki Shirakawa for their discovery polymers conduct electricity. Polyacetylene is the simplest conducting polymer. It was synthesized in 1977 [**1,2**]. Conducting polymers show unusual electrochemical properties like high electrical conductivity, low ionization potential, high electronic affinities, and optical properties. These properties are only because of conjugated π electron backbones in conducting polymers. There must be high degree of overlapping of the polymer molecular orbital, which permits the formation of a delocated molecular wave function and partial occupation of the molecular orbital if there is to be free movement of electrons throughout the lattice [**3**].

Conducting polymers exhibit intrinsic electronic conductivity ranging from about 10^{-14} to 10^{2} S cm^{-1} due to extension of the doped state [**4**]. In the neutral (undoped) state these materials are only semi-conducting and electronic conductivity only appears when the material is doped with small sized ions (e.g. when electrons or holes are injected).Doping is normally produced by chemical or electrochemical oxidation of the monomer, in which the polymer chains acquire positive charges and the electro-neutrality of the resulting material is preserved by the incorporation of small counter ions from the electrolyte solution. In recent years, there has been growing interest in conducting polymers because of their wide range of potential application in the areas such as rechargeable batteries [**5**], gas separation membranes [**6**], EMI shielding [**7**], electrochromic display devices [**8**].

1.2 Proton Conducting Polymer

Proton conducting materials are referred to substances in which protons can carry electrical current and contribute to the electrical conduction. Protons are either the necessary constituent of such a material or introduced into the material as an impurity. A variety of materials have been discovered showing proton conductivity, such as ice, inorganic salts with hydroxide groups, organic polymers, solid oxides, to name a few. These materials exhibit substantial proton conductivity at various temperature ranges, from room temperature to temperatures as high as ~ 800 °C [**9**]. Ion-conducting polymers have been the focus of much attention because of their application as separator membranes in high energy density batteries and fuel cells [**10**]. There is an increasing interest for such materials in hydrogen and methanol fuel cells as well as electrochromic displays and windows [**11**].

The proton is the only ion which has no electron shell of its own. Therefore, it strongly interacts with the electron density of its environment, which then takes on some H(1s) character. In metals this environment is the delocalized electron density of the conduction band, where the proton is considered to be a part of a hydrogen with some protonic or hydridic character depending on whether the energy of the H(1s) state is higher or lower than the Fermi energy of the pure metal. Only in metals may the "proton" (hydrogen) have a high coordination number, typically four or six on a tetrahedral or octahedral site. In nonmetallic compounds, however, the proton strongly interacts with the valence electron density of only one or two nearest neighbors.

In metals, hydrogen (proton) transfer reactions, usually taking place between interstitial octahedral or tetrahedral sites, result in long-range hydrogen (proton) transport, i.e. diffusion. In non-metallic environments, however, proton mobility requires not only proton transfer reactions within hydrogen bonds but also structural reorganizations, as illustrated schematically in Figure 1.1 **[12].**

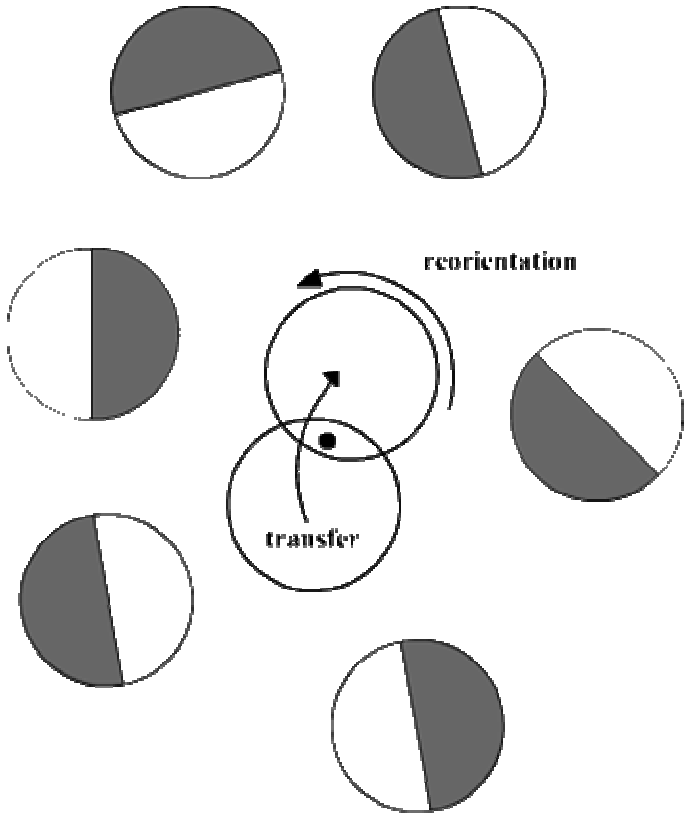


Figure 1.1 Schematic illustration of the two reaction steps involved in most long-range proton transport phenomena.

For a rigid array of host species this restriction allows some local motion (but no significant translational motion (diffusion) of protons) which may lead to proton conductivity. In the late 1960s, however, Fischer, Hofacker, and Rathner recognized that proton-phonon coupling may assist proton diffusivity, the dynamics of the proton environment is involved in proton conductivity [**13**].

The discovery of polymer-salt complexes as well as the recognition of their potential application as solid electrolytes resulted in the development of numerous polymer electrolytes, including those with a protonic type of conductivity. Entirely different classes of materials have attracted increasing attention as proton conductors including polymers, ceramics, and intercalation compounds [**14**].

Proton conductors are solid electrolytes in which protons or protonated molecular species are mobile in the host lattice. Interest in solid proton conducting polymer electrolytes started with the development of perfluorinated sulfonic

membranes in the 1960s [**10**]. In comparison with the usual liquid electrolytes, the polymer/ acid systems have obvious advantages in terms of reduced leakage and corrosion problems, and they represent less costly alternatives to perfluorinated polymers. The main advantage compared with conventional proton-conducting polymers is that it is relatively inexpensive [**14**].

Proton conductivity has been demonstrated in both non-hydrogen bonded and hydrogen bonded species. The discovery of a compound with high proton conductivity, low electrical conductivity and good chemical stability at moderately high temperatures could lead to totally new designs for hydrogen / oxygen fuel calls and water electrolyzers **[15].**

The ability to transport proton makes these materials promising for potential applications, such as (i) electrolyte for fuel cells, for example polymer proton exchange membrane-based fuel cells that operate between ~ 80 and 120 °C, and high temperature proton conductor-based solid oxide fuel cells operating between ~ 600 and 800 °C; (ii) a sensing element for gas sensors for the detection of gas species such as H_2, H_2O, NH_3, NO, NO_2, hydrocarbons etc.; (iii) permeation membranes for the separation or purification of H_2, and hydrogen isotope production; (iv) dehydrogenation / hydrogention of hydrocarbons; (v) synthesis of NH_3; (vi)CO_2 recycling, NO removal; and (vii) electrochromic devices such as switchable mirrors [**16,17**].

Research in the field of proton conductivity in the past few years has been driven by the goal of developing suitable proton-conducting materials for application in all solid-state electrochemical devices, such as batteries, smart windows, sensors, and most importantly, fuel cells [**18**]. Since their development in the 1960s, perfluorinated ionomers have emerged as standard materials for low-temperature fuel cell applications due to their high proton conductivity and their excellent chemical and thermal stability. For decades, no other type of material was found to be competitive, despite intense research and a number of severe limitations impeding an economical and wide-spread application of proton exchange membrane (PEM) fuel cells [**19**].

A proton-conducting material is discussed according to the range of temperature in which it can be used in technological applications. Proton conducting polymers can be divided into three types:

i) High temperatures proton conducting perovskite type oxides

ii) Low temperature proton conducting hydrated polymer membranes

iii) Intermediate temperatures proton conducting materials relying on proton transfer between nitrogen **acting** as proton donor as well as proton acceptor [**20**].

Two principal mechanisms describe proton diffusion in such a way that the proton remains shielded by some electron density along the entire diffusion path, i.e. not even the momentary existence of a free proton is required [**13**].

1.2.1 Proton Conduction Mechanisms

1.2.1.1 Grotthuss Mechanism

The proton transport in proton conductors is usually described in terms of the Grotthuss Mechanism where the protons move along on extended hydrogen bonded via a two step process. First is an intermolecular step by which a proton transfers from a proton donor to a proton acceptor. This step is sometimes described in terms of proton "hopping" from the donor to acceptor. It is more commonly assumed to involve quantum mechanical tunneling of the proton across a hydrogen bond. This step is than followed by an intramolecular reorientation of proton acceptor, making it available to take up another proton [**15**].

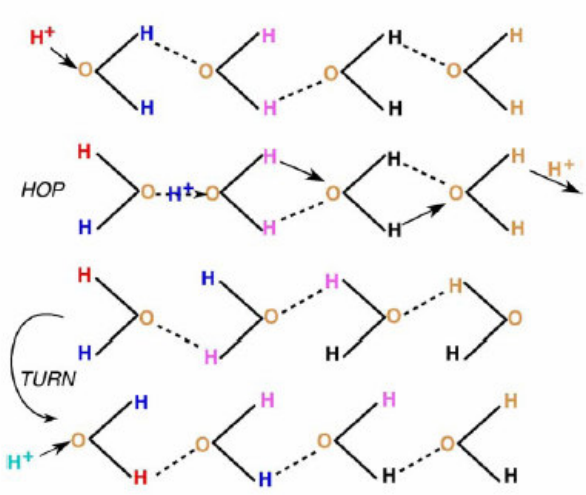


Figure 1.2 A highly schematic representation of a unidimensional chain of four water molecules interconnected via H-bonds (water or proton wire).

Figure 1.2 illustrates the basic ideas of a modern version of the Grothuss's mechanism. Consider four water molecules interconnected via H-bonds (water or proton wire). In Figure 1.2, there is an electrochemical gradient favoring the movement of H^+ from left to right. The approach of H^+ (first row 1 in Figure 1.2) to the O of the first water molecule in the chain will eventually lead to formation of a covalent OH bond. One of the protons that was originally covalently linked to the O of water 1 will now be shared between water molecules 1 and 2 forming a protonated water dimer (Zundel's cation, $(H_5O_2)^+$, 2nd row in Figure 1.2). This hopping step propagates along the water wire (2nd row in Figure 1.2). As the H^+ hops, the dipole moment of the water molecule donating the H^+ reverses. Once the H^+ leaves the last water molecule in the water chain of Figure 1.2 (2nd row), the total dipole moment of the chain is reversed (3rd row in Figure 1.2). If another H^+ is to be transferred in the same direction as before, the four water molecules need to rotate back (turn step) to their configurations (4th row, Figure 1.2). In other words, a 'red' H^+ enters and a 'green' H^+ leaves the water wire (as the hopping step described by Grotthuss himself) [**21**].

1.2.1.2 Vehicle Mechanism

Another model is "Vehicle Mechanism". It has been proposed for the interpretation of conductivity in proton conductors (Figure 1.3). According to this model the proton does not migrate as H^+. Instead, proton transport takes place with the aid of a vehicle such as H_2O or NH_3, so that the mobile species is actually the protonated complex ion, e.g. H_3O^+ or NH_4^+.

This model further requires that the unladen (non-protonated) vehicle move in opposite direction of the laden (protonated) vehicle during the conduction process. Note that unlike the Grotthuss mechanism, this model does not require the existence of extended hydrogen-bonded network for proton transport. Proton conductivity by this mechanism should depend on the ease by which the vehicle can translate though the lattice. In contrast, the conductivity for the Grotthuss Mechanism will depend on the ease by which the proton hops (tunnels) and acceptor reorients [**15**].

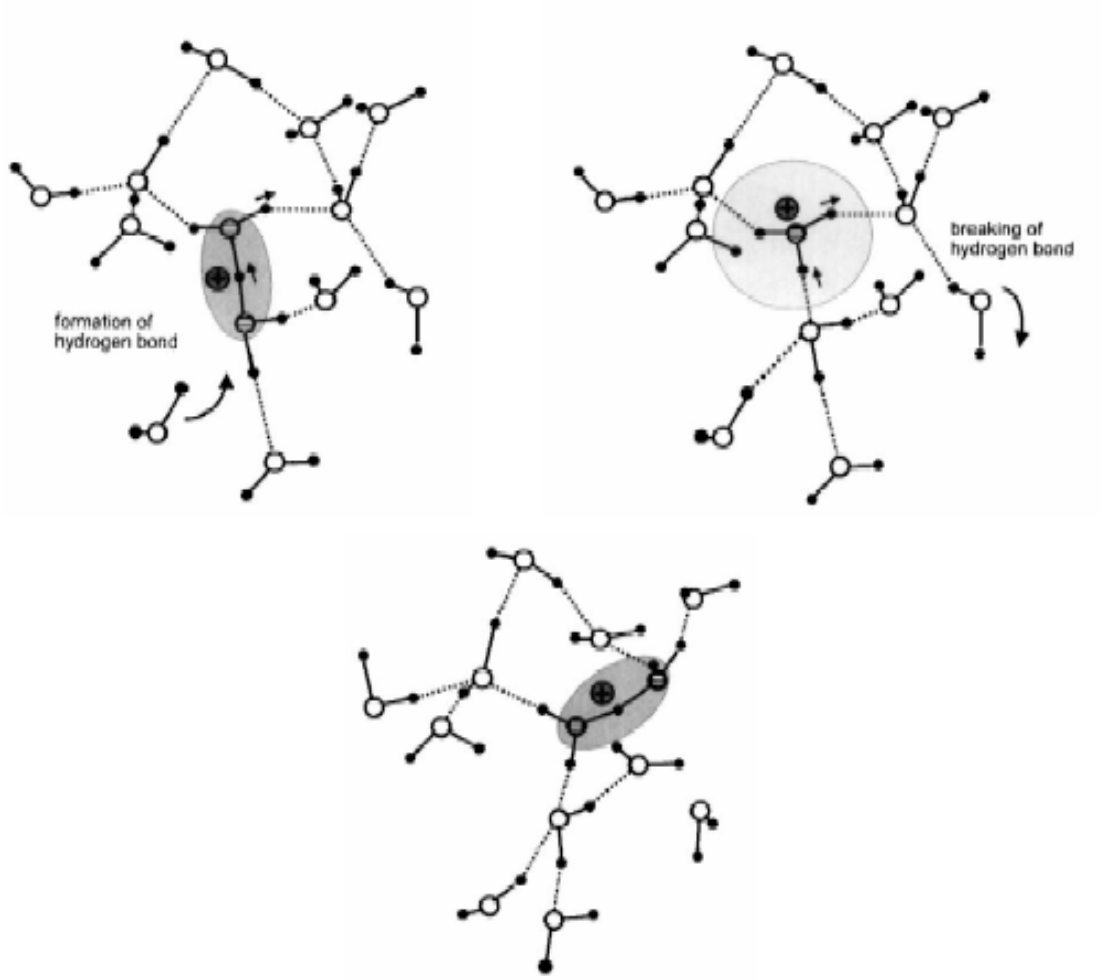


Figure 1.3 Vehicle Mechanism [**12**]

The transport properties of those membranes are largely depending on water content and can be useful below the dew point of water **[22].**

Heterocycles such as, imidazole have been reported to be promising in that respect. Their nitrogen sides act as strong proton acceptors, thus forming protonic charge carriers. Isometric geometry of molecules are advantageous for extended local dynamics and their protonated and unprotonated nitrogen functions may act as donors and acceptors in proton transfer reactions [**23**]. There is an increasing interest in anhydrous proton conducting polymer electrolytes due to their use as membranes in fuel cells at intermediate temperatures (T>100 °C) [**24**].

Recently, the synthesis of anhydrous proton conducting polymer electrolytes for high temperature operation (100–200°C) has been the focus of polymer research for their applications in various electrochemical devices. In this context, more

temperature tolerant polymer electrolyte membranes were obtained by doping the polymers bearing basic units such as amide, imine, ether with strong acids i.e. H_3PO_4 or H_2SO_4. Although these types of polymer blends have already been illustrated to have high protonic conductivity in the anhydrous state, self-condensation of acidic units may be problem at higher operation temperatures [**25**]. Recently, neutral (or basic) proton conducting polymer electrolytes have already been announced as they are likely to be more stable in the presence of electrode materials. In such membranes the basic dopant enhanced proton vacancy type conduction Heterocycles such as imidazole or benzimidazole have been reported to be promising in that respect. Their nitrogen sides act as strong proton acceptors thus forming protonic charge carriers [**26**].

1.2.2 Proton Conducting Perovskite Type Oxides

A technically important class of protonic conductors is those that are based on perovskite oxides, which are usually referred to as high temperature proton conductors (HTPCs). The pioneering work by Iwahara et al. has shown that many alkaline earth perovskites, when doped by rare earth oxides, are protonic conductors in H_2O containing atmosphere [**5**]. $BaCeO_3$ based materials have been found to exhibit the highest proton conductivities compared to other perovskites. An alkaline earth perovskite has the formula ABO_3, where A= Ba, Sr, or Ca; B= Ce, Zr, Ti or Th. The presence of vacancies leads to significant oxygen ion conductivities at elevated temperatures in a moisture-free oxygen gas atmosphere. When such oxygen-deficient materials are heated in a water-containing atmosphere, water can dissolve into the perovskite to fill up oxygen vacancies, thereby introducing protons into the structure. Figure 1.4 shows the structure of a typical perovskite oxide of composition of $CaTiO_3$.

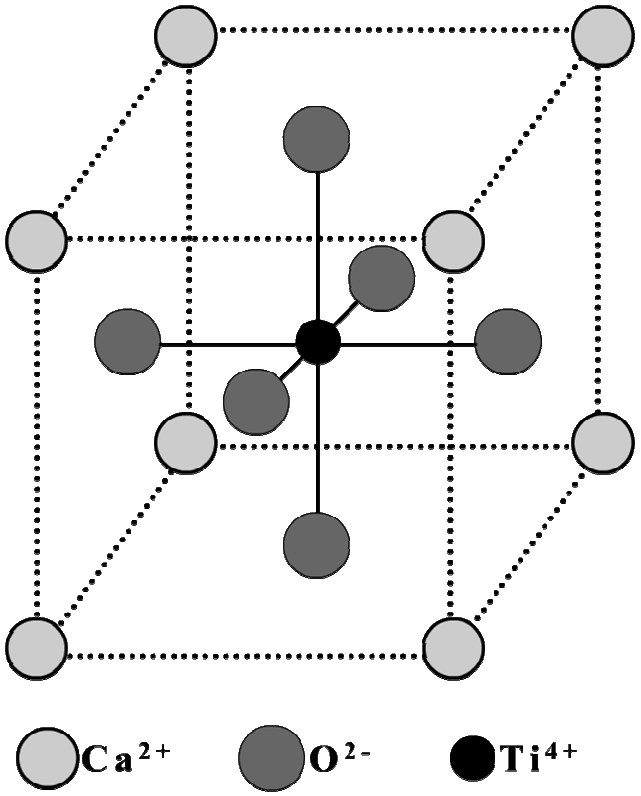


Figure1.4 Schematic of a perovskite crystal

All of the perovskite type oxides require the incorporation of H_2O into the structure for them to become proton conductors. The incorporation of H_2O can be given by the following reaction

$$H_2O(gas) + V_O^{\bullet\bullet} \leftrightarrow O_O^{x} + 2H^{\bullet}$$

$V_O^{\bullet\bullet}$ denotes an oxygen vacancy.

It is agreed that the transport of protons occurs through hopping between two neighboring oxygen ions (Grotthuss mechanism) and not by the movement of hydroxyl ions [**9**].

1.2.3 Low Temperature Proton Conducting Polymers

Interest in solid protonic polymers started with the development of perfluorinated sulfonic membranes [**27**]. Perfluorinated sulfonic acid membranes,

best known by the trade name Nafion (Figure 1.5), are the most commonly used conductors. They combine the high ability of proton conductivity in hydrated state with long term electrochemical stability [**28**].

Nafion®, the perfluorinated membrane from DuPont, or similar membranes commercialized by Dow and Asahi, have been intensively used for fuel cells. They show high proton conductivity and chemical stability [**29**].

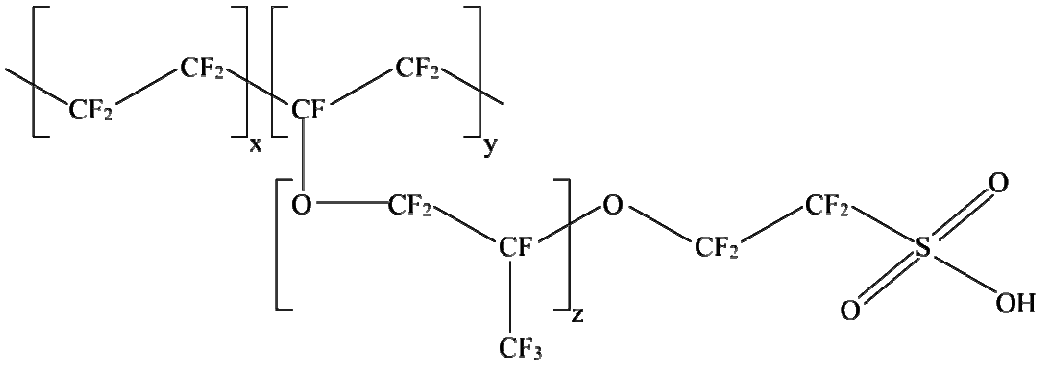


Figure 1.5 Chemical Structure of Nafion [**30**]

Nafion's perfluorinated carbon backbone provides membranes with good chemical, oxidative and thermal stability. The semicrystalline morphology of Nafion is important for its mechanical strength. To understand conduction mechanism of Nafion a cluster-network model was suggested. This model proposes inverted micelle structures, ionic clusters of perfluroalkylsulfonates, interconnected by narrow channels [**31**] (Figure 1.6).

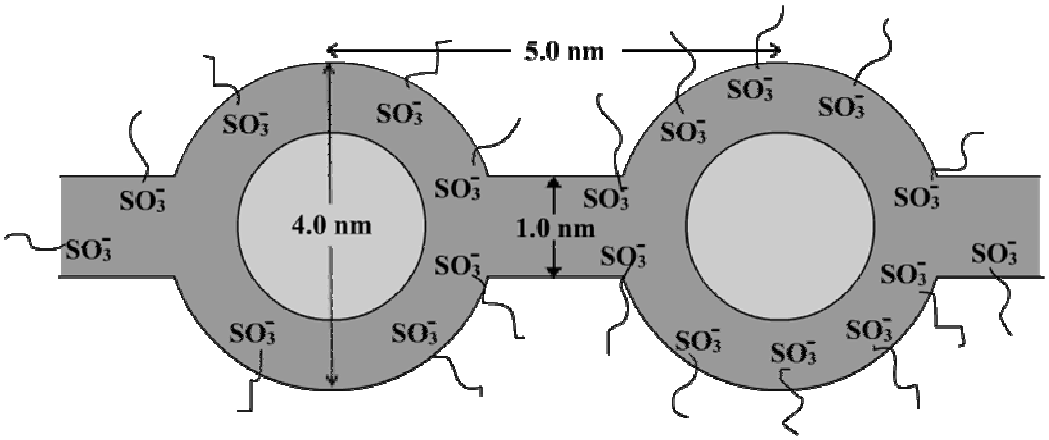


Figure 1.6 The cluster- network model

It combines the extreme hydrophobicity of the perfluorinated polymer backbone with the extreme hydrophilicity of the terminal sulfonic acid function. When hydrated, the high mobility of protons in the SO_3H groups at the end of side chains leads to highly conductive membranes. The water of hydration then acts as a plastisizer mobilizing the polymer backbone which leads to a further phase separation. Eventually, a stationary micro structure is formed which absorbs and desorbs water almost reversibly at moderate temperatures. One important feature of such micro structures is that their hydrophobic part provides relatively good mechanical stability even in the presence of water, while the hydrated hydrophilic domains provide very high proton conductivity [**20**] (Figure 1.7).

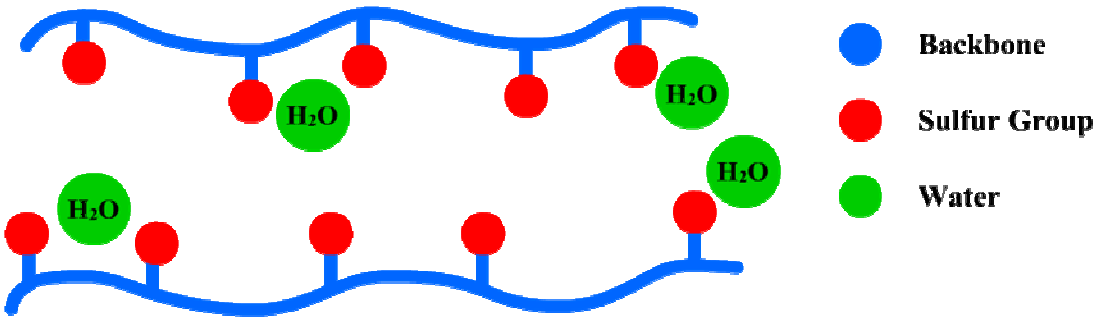


Figure 1.7. Channelles in Nafion

The disadvantages of these membranes are poor ionic conductivities at elevated temperatures or at low humidity conditions. For example, the conductivity of Nafion is as high as 10^{-2} S/cm in its fully hydrated state but the conductivity dramatically decreases at temperature above 100°C due to the loss of water absorbed in the membrane. The high cost of perfluorinated ionomer membrane is also a major limiting reason for its widespread application [**31**]. Safety concerns arise from evolution of toxic intermediates and corrosive gases liberated at temperatures above 150 ◦C. Decomposition products could be a concern during the manufacturing process or vehicle accidents and could limit fuel cell recycling options [**30**].

The proton conduction in these sulfonated polymers follows the vehicular mechanism, in which water content plays a vital role. The proton transport in Nafion membrane is due to the mobility of the water molecules in the membrane [**32**] and protons on the SO_3H (sulfonic acid) groups "hop" from one acid site to another.

1.2.4 Intermediate Temperature Proton Conducting Polymer

Although some proton conducting oxides and hydrated polymers are close to being technologically applied at high (>500^o C) or low temperatures (< 90^o C) , only few materials have been reported to show high proton conductivity at intermediate temperatures (100-400^o C) [**20**].

In order to support proton conductivity in this type of polymers, the formation of protonic defect and strong fluctuation proton donor to acceptor are necessary. Heterocycles such as imidazole which already have been shown to exhibit moderate proton conductivity as a pure material are promising in this respect. Their basic nitrogen sites act as a strong proton acceptors with respect to Bronsted acids such as sulfonic acid groups hence forming protonic charge carriers [**20].** Isometric geometry of molecules is advantageous for extended local dynamics and their protonated and unprotonated nitrogen functions may act as donors and acceptors in proton transfer reactions [**22**].

In these systems, polymers bearing basic sites interact with the acidic polymers or acids via hydrogen bonds and proton conduction occurs through structure diffusion, i.e., jump reorientation mechanism. The proton mobilities in imidazole, its derivatives and water show similar behavior when compared at respective melting temperatures. In water, proton migrates as the hydrated form such

as H_3O^+, $H_5O_2^+$ and the proton mobility in an environment of heterocycles completely relies on proton transfer between protonated and unprotonated molecules (Grotthuss mechanism) [**33**].

The proton transport under anhydrous (water-free) or low humidity conditions might be based on a non-vehicular mechanism, in which only protons are mobile from site to site without an assistance of diffusible vehicle molecules [**34**]. The melting points of heterocycles are higher than that of e.g. water; this makes them interesting candidates for supporting proton conductivity at medium temperatures. Their basicities provide another interesting feature with respect to the chemical compatibility with other compounds such as metal hydrates or hydrogen bronzes being present in certain applications [**20**].

1.3 Alginic Acid

Alginic acid, alginate, is a polysaccaride linear block copolymer composed of two monosaccarides D- mannuronic acid and L- guluronic acid (Figure 1.8). It is found in the cell walls of Brown algae from which it is usually isolated, providing mechanical strength and flexibility to the species. In the presence of divalent cations such as calcium, which can be bridge between two guluronic acid residues, cross-linked alginic acid is able to form gels in moist condition. Alginic acid gels have large pore size and high water absorbency. They provide an excellent moist covering to the surface of a wound, which in general facilitates normal healing [**35**]. Alginic acid has interesting applications. Due to the high viscosity of alginate solutions as well as their capacity to form gels, alginates are widely utilized industrially. In food industry, alginates are used as thickeners, stabilizers and emulsifying agents. Other applications include their use as tablet binders, suspending agents, antacid and antiulcer preperations in pharmaceuticals. Antitumor activity of alginates has been reported, as well as their use as cation-exchange polymers for the recovery of heavy metals such as cobalt, copper, zinc, cadmium and lanthanum. Recently, superswelling alginates gels with potential biomedical applications were reported. More recent applications of alginic acid are use as solid support for noncovalent immobilization or encapsulation of enzymes [**36**].

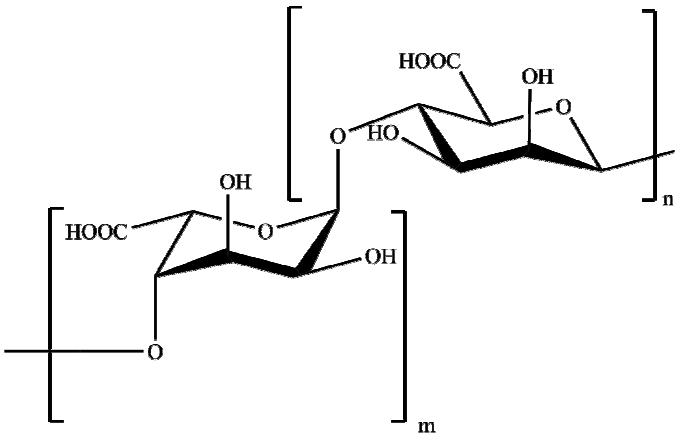


Figure 1.8 The structure of Alginic Acid

1.4 Enzymes

Enzymes are bioreactors that run all biochemical reactions of a living cell in a steady, controlled manner with extraordinary efficiency and specificity. *Efficiency* means high and widely ranging levels of rate acceleration; *specificity* means a specific molecular recognition between enzymes and their substrates [**37**]. Enzymes are sensitive substances. Their catalytic properties change mostly to disadvantage, when exposed to high temperature or to certain reagents or when introduced into on favorable milieu [**38**].

Enzymes have a number of distinct advantages over conventional chemical catalysts. Foremost amongst these are their specificity and selectivity not only for particular reactions but also in their discrimination between similar parts of molecules (regiospecificity) or optical isomers (stereospecificity). They catalyze only the reactions of very narrow ranges of reactants (substrates), which may consist of a small number of closely related classes of compounds (e.g. trypsin catalyzes the hydrolysis of some peptides and esters in addition to most proteins), a single class of compounds (e.g. hexokinase catalyzes the transfer of a phosphate group from ATP to several hexoses), or a single compound (e.g. glucose oxidase oxidises only glucose amongst the naturally occurring sugars). This means that the reaction chosen can be catalyzed to the exclusion of side-reactions, eliminating undesirable by-products. Thus, higher productivities may be achieved, reducing material costs. As a bonus, the product is generated in an uncontaminated state so reducing purification costs and

the downstream environmental burden. Often a smaller number of steps may be required to produce the desired end-product. In addition, certain stereospecific reactions (e.g. the conversion of glucose into fructose) cannot be achieved by classical chemical methods without a large expenditure of time and effort.

Enzymes work under generally mild processing conditions of temperature, pressure and pH. This decreases the energy requirements, reduces the capital costs due to corrosion-resistant process equipment and further reduces unwanted side-reactions. The high reaction velocities and straightforward catalytic regulation achieved in enzyme-catalysed reactions allow an increase in productivity with reduced manufacturing costs due to wages and overheads [**39**].

Most known enzymes are proteins. They are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds (Figure 1.9).

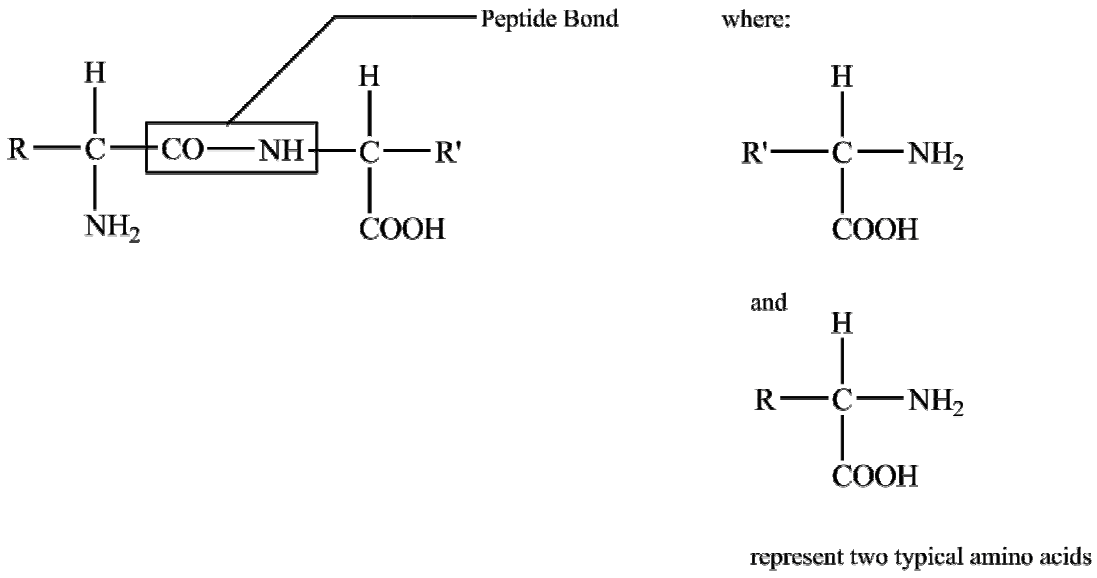


Figure 1.9 Typical Protein structure

1.4.1 Application Fields of Enzymes

- **Scientific research:** Enzymes are used as research tools for hydrolysis, synthesis, analysis, biotransformation, and affinity separations.
- **Cosmetic applications***:* Preparations for skin; denture cleansers.

- **Medical diagnostics and chemical analyses:** Blood glucose, urea, cholesterol; ELISA systems; enzyme electrodes and assay kits.
- **Therapeutic applications*:*** Antithrombosis agents, antitumor treatments, anti-inflammatory agents, digestive aids.
- **Industrial application:** Brewing and wine making; dairy processing; fruit, meat, and vegetable processing; starch modifications; leather processing; pulp and paper manufacture; sugar and confectionery processing; production of fructose; detergents and cleaning agents; synthesis of amino acids and bulk chemicals; waste water treatment; desizing of cotton.

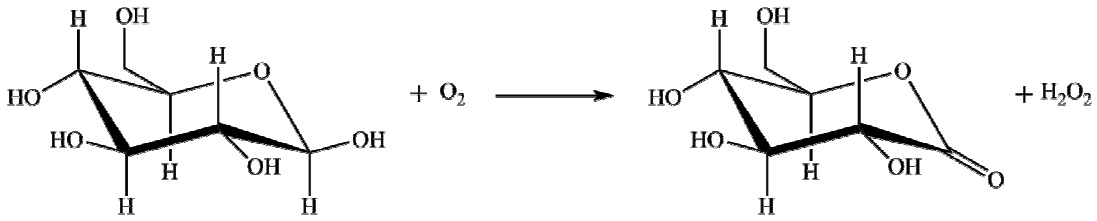
1.4.2 Enzyme Nomenclature

All enzymes contain a protein backbone. In some enzymes this is the only component in the structure. However, there are additional non-protein moieties usually present which may or may not participate in the catalytic activity of the enzyme. Covalently attached carbohydrate groups are commonly encountered structural features which often have no direct bearing on the catalytic activity, although they may well affect an enzyme's stability and solubility. Other factors often found are metal ions (**cofactors**) and low molecular weight organic molecules (**coenzymes**). These may be loosely or tightly bound by noncovalent or covalent forces. They are often important constituents contributing to both the activity and stability of the enzymes. This requirement for cofactors and coenzymes must be recognized if the enzymes are to be used efficiently and is particularly relevant in continuous processes where there may be a tendency for them to become separated from an enzyme's protein moiety.

Enzymes are classified according the report of a Nomenclature Committee appointed by the International Union of Biochemistry (1984). This enzyme commission assigned each enzyme a recommended name and a 4-digit distinguishing number [**40**]. The enzyme commissions (EC) numbers divide enzymes into six main groups according to the type of reaction catalyzed:

(1) **Oxidoreductases** catalyze redox reactions in which hydrogen or oxygen atoms or electrons are transferred between molecules (Figure 1.10) This extensive class includes the dehydrogenases (hydride transfer), oxidases (electron transfer to molecular oxygen), oxygenases (oxygen transfer from molecular oxygen) and

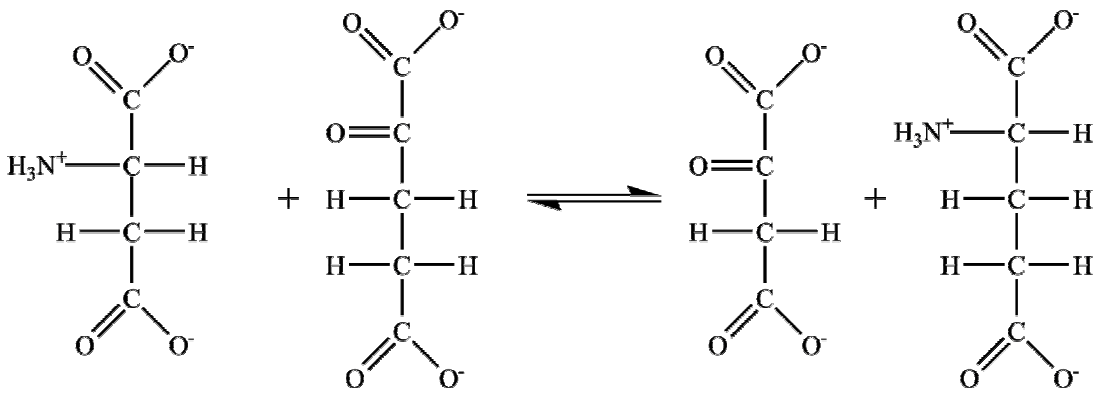
peroxidases (electron transfer to peroxide). For example: glucose oxidase (EC 1.1.3.4, systematic name, β-D-glucose: oxygen 1-oxidoreductase).



β -D-glucose + oxygen ⟶ D-glucono–1,5-lactone + hydrogen peroxide

Figure 1.10 Schematic representation of glucose oxidase activity

(2) **Transferases** catalyze the transfer of an atom or group of atoms (e.g. acyl-, alkyl- and glycosyl-), between two molecules, but excluding such transfers as are classified in the other groups (e.g. oxidoreductases and hydrolases) (Figure 1.11). For example: aspartate aminotransferase (EC 2.6.1.1, systematic name, L-aspartate:2-oxoglutarate aminotransferase; also called glutamic-oxaloacetic transaminase or simply GOT).



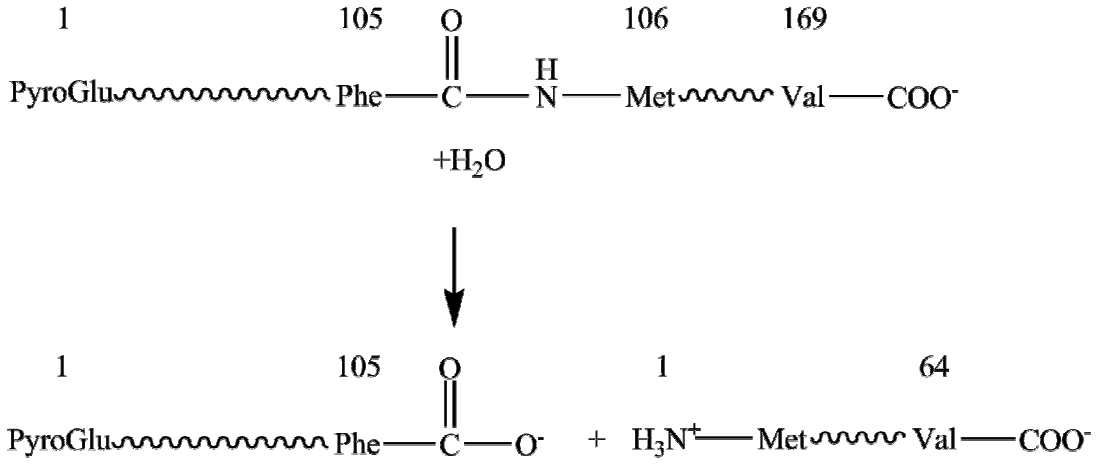
L-aspartate + 2-oxoglutarate ⇌ oxaloacetate + L-glutamate

Figure 1.11 Schematic representation of aspartate aminotransferase activity

(3) **Hydrolases** catalyze hydrolytic reactions and their reversal (Figure 1.12). This is presently the most commonly encountered class of enzymes within the field of enzyme technology and includes the esterases, glycosidases, lipases and proteases.

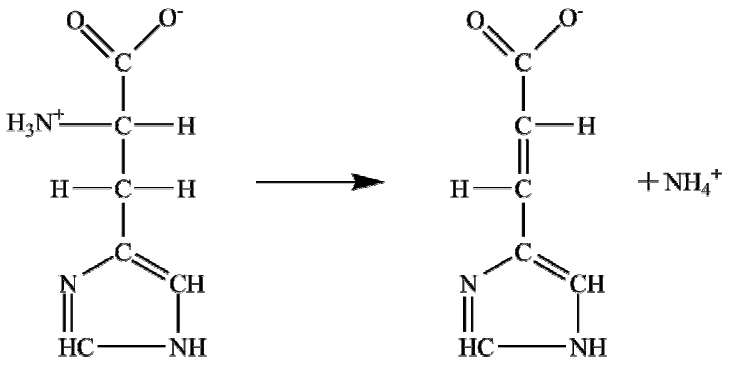
For example: chymosin (EC 3.4.23.4, no systematic name declared; also called rennin).

(4) **Lyases** catalyze elimination reactions in which a group of atoms is removed from the substrate (Figure 1.13). This includes the aldolases, decarboxylases, dehydratases and some pectinases but does not include hydrolases. For example: histidine ammonia-lyase (EC 4.3.1.3, systematic name, L-histidine ammonia-lyase; also called histidase).



κ-casein + water ⟶ *para*- κ -casein + caseino macropeptide

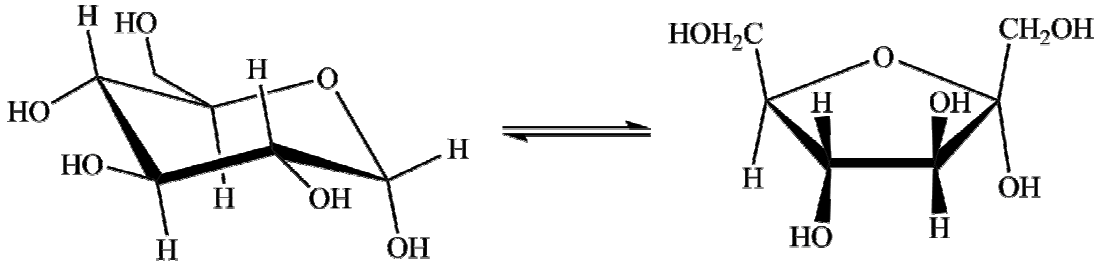
Figure 1.12 Schematic representation of chymosin activity



L-histidine ⟶ urocanate + ammonia

Figure 1.13 Schematic representation of histidine ammonia-lyase activity

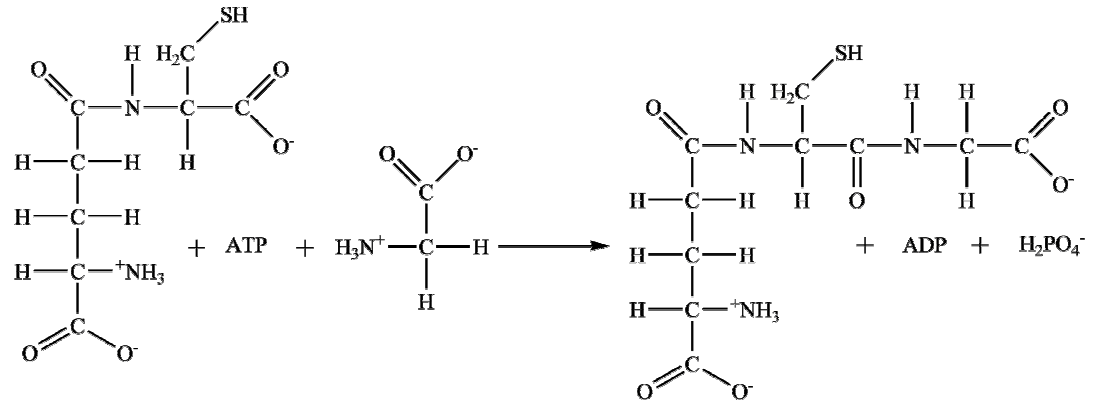
(5) **Isomerases** catalyze molecular isomerisations and include the epimerases, racemases and intramolecular transferases (Figure 1.14). For example: xylose isomerase (EC 5.3.1.5, systematic name, D-xylose ketol-isomerase; commonly called glucose isomerase).



α-D-glucopyranose ⇌ α -D-fructofuranose

Figure 1.14 Schematic representation of xylose isomerase activity

(6) **Ligases**, also known as synthetases, form a relatively small group of enzymes which catalyze the formation of a covalent bond joining two molecules together, coupled with the hydrolysis of a nucleoside triphosphate. For example: glutathione synthase (EC 6.3.2.3, systematic name, γ-L-glutamyl-L-cysteine: glycine ligase (ADP-forming); also called glutathione synthetase).



ATP + γ-L-glutamyl-L-cysteine + glycine ⟶ ADP + phosphate + glutathione

Figure 1.15 Schematic representation of glutathione synthase activity

1.4.3 Enzyme Kinetics

Enzymes are protein catalysts that, like all catalysts, speed up the rate of a chemical reaction without being used up in the process. They achieve their effect by temporarily binding to the substrate and, in doing so, lowering the activation energy needed to convert it to a product (Figure 1.16).

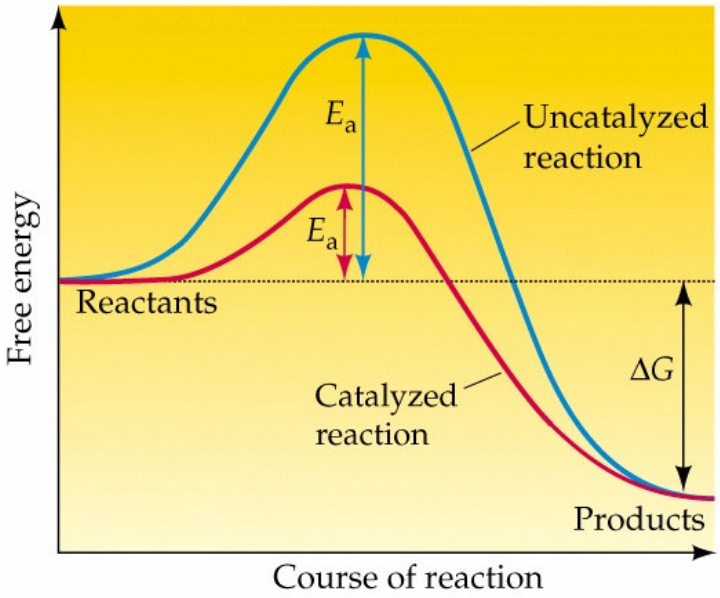


Figure 1.16 Activation energy diagram of enzyme catalyzed reaction

The rate at which an enzyme works is influenced by several factors. The concentration of substrate molecules, temperature the presence of inhibitors and pH are controlling factors of enzyme activity.

Enzymes are very specific, and it was suggested by Emil Fischer in 1894 that this is because both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another. This is often referred to as "the lock and key" model (Figure 1.17). However, while this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve. In 1958 Daniel Koshland suggested a modification to the lock and key model: since enzymes are rather flexible structures, the active site is continually reshaped by interactions with the substrate as the substrate interacts with the

enzyme. As a result, the substrate does not simply bind to a rigid active site; the amino acid side chains which make up the active site are moulded into the precise positions that enable the enzyme to perform its catalytic function. In some cases, such as glycosidases, the substrate molecule also changes shape slightly as it enters the active site. The active site continues to change until the substrate is completely bound, at which point the final shape and charge is determined.

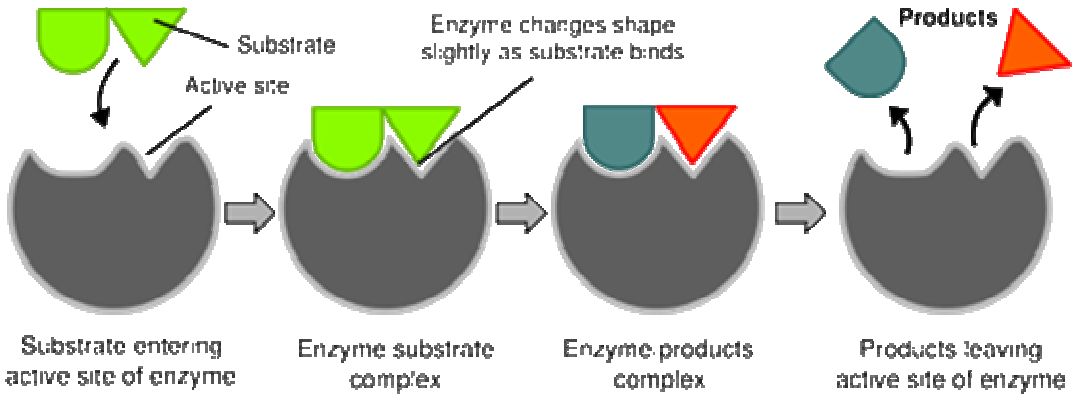


Figure 1.17 Lock and Key Model

In 1902 Victor Henri proposed a quantitative theory of enzyme kinetics, but his experimental data were not useful because the significance of the hydrogen ion concentration was not yet appreciated. After Peter Lauritz Sørensen had defined the logarithmic pH-scale and introduced the concept of buffering in 1909 , German chemist Leonor Michaelis and his Canadian postdoc Maud Leonora Menten repeated Henri's experiments and confirmed his equation which is referred as Henri-Michaelis-Menten kinetics (otherwise known as Michaelis-Menten kinetics) [**41**].

Using the mechanism based on that of Michaelis and Menten for a one-substrate reaction, by the reaction sequence:

Enzyme+Substrate $\rightleftharpoons$ (Enzyme-substrate complex) $\longrightarrow$ Enzyme +Product

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

where k_{+1}, k_{-1} and k_{+2} are the respective rate constants.

The rate of reaction (v) is the rate at which the product is formed.

$$v = \frac{d[P]}{dt} = k_{+2}[ES] \qquad (1.1)$$

The rate of change of the concentration of the enzyme-substrate complex equals the rate of its formation minus the rate of its breakdown, forwards to give product or backwards to regenerate substrate. Therefore:

$$\frac{d[ES]}{dt} = k_{+1}[E][S] - (k_{-1} + k_{+2})[ES] \qquad (1.2)$$

During the course of the reaction, the total enzyme at the beginning of the reaction ($[E]_0$, at zero time) is present either as the free enzyme ([E]) or the ES complex ([ES]).

i.e. $[E]_0 = [E] + [ES]$ (1.3)

therefore:

$$\frac{d[ES]}{dt} = k_{+1}([E]_0 - [ES])[S] - (k_{-1} + k_{+2})[ES] \qquad (1.4)$$

after arrangement:

$$\frac{d[ES]}{dt} = k_{+1}[E]_0[S] - k_{+1}[ES][S] - (k_{-1} + k_{+2})[ES]$$

$$\frac{d[ES]}{dt} = k_{+1}[E]_0[S] - (k_{+1}[S] + k_{-1} + k_{+2})[ES]$$

this gives:

$$\frac{\frac{d[ES]}{dt}}{k_{+1}[S]+k_{-1}+k_{+2}}+[ES]=\frac{k_{+1}[E]_0[S]}{k_{+1}[S]+k_{-1}+k_{+2}} \qquad (1.5)$$

The differential equation 1.5 is difficult to handle, but may be greatly simplified if it can be assumed that the left hand side is equal to [ES] alone. This assumption is valid under the sufficient but unnecessarily restrictive steady state approximation that the rate of formation of ES equals its rate of disappearance by product formation and reversion to substrate (i.e. d[ES]/dt is zero). It is additionally valid when the condition:

$$\frac{\frac{d[ES]}{dt}}{k_{+1}[S]+k_{-1}+k_{+2}}<<[ES] \qquad (1.6)$$

A single enzyme molecule can convert substrate into product multiple times during a given time period. To determine the maximum rate of an enzyme mediated reaction, the substrate concentration (*[S]*) is increased until a constant rate of product formation is achieved. This is the maximum velocity (V_{max}) of the enzyme. In this state enzyme active sites are saturated with substrate. Imagine that the concentration of substrate is so high that there is essentially no time when an enzyme molecule is not bound to substrate. That is, as soon as a bound substrate molecule reacts to form product, it leaves the enzyme and is instantly replaced by a fresh substrate molecule. Note that at the maximum velocity, the other factors that affect the rate of reaction (ie. pH, temperature, etc) are at optimal values. This is what we call a "saturating" concentration of substrate, since at this concentration, the active site is always filled (saturated) with substrate.

The Michaelis-Menten equation (Figure 1.18) is a quantitative description of the relationship among the rate of an enzyme-catalyzed reaction [v_1], the concentration of substrate [S] and two constants, V_{max} and K_m (which are set by the particular equation). The symbols used in the Michaelis-Menten equation refer to the reaction rate [v_1], maximum reaction rate (V_{max}), substrate concentration [S] and the

Michaelis-Menten constant (K_m) **[42].**

The Michaelis-Menten equation (below) is simply derived from equations 1.1 and 1.5, by substituting K_m for $\frac{k_{-1}+k_{+2}}{k_{+1}}$. K_m is known as the Michaelis constant with a value typically in the range 10^{-1} - 10^{-5} M. When $k_{+2} << k_{-1}$, K_m equals the dissociation constant (k_{-1}/k_{+1}) of the enzyme substrate complex [**39**].

$$v = k_{+2}[ES] = \frac{k_{+2}[E]_0[S]}{[S] + K_m} \tag{1.7}$$

or, more simply

$$v = \frac{V_{max}[S]}{[S] + K_m} \tag{1.8}$$

where V_{max} is the maximum rate of reaction, which occurs when the enzyme is completely saturated with substrate (i.e. when [S] is very much greater than K_m, V_{max} equals $k_{+2}[E]_0$, as the maximum value [ES] can have is $[E]_0$ when $[E]_0$ is less than $[S]_0$). Equation 1.8 may be rearranged to show the dependence of the rate of reaction on the ratio of [S] to K_m,

$$v = \frac{V_{max}}{1 + \frac{K_m}{[S]}} \tag{1.9}$$

and the rectangular hyperbolic nature of the relationship, having asymptotes at v = V_{max} and [S] = $-K_m$,

$$(V_{max}-v)(K_m+[S]) = V_{max}K_m \tag{1.10}$$

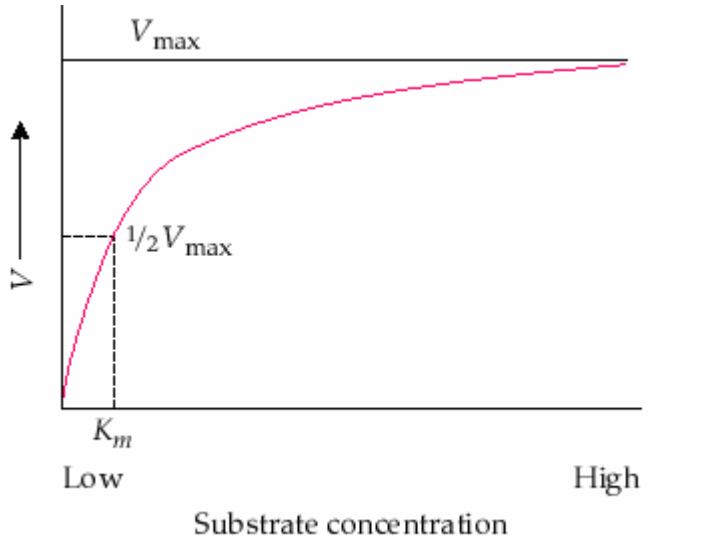


Figure 1.18 The variation of the rate of enzyme-catalyzed reaction as a function of substrate concentration

1.4.3.1 Lineweaver- Burk Plot

For determination of V_{max} and K_m, the graph of the Michaelis-Menten equation is not exactly satisfactory. The graph, being a curve, cannot be accurately extrapolated upwards from values of $[S_0]$ which are far from saturating. Therefore, in order to determine the kinetic parameters for an enzyme catalyzed reaction, a linear relation would be more useful. Lineweaver and Burk [**43**] overcame this problem without making any fresh assumptions. Lineweaver and Burk have preferred to rewrite the Michaelis and Menten equation in a form that permits the results to be plotted as a straight line,

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

The plot of 1/ v versus 1/[S] will give a straight line of Km/ Vmax and y intercept of 1/ Vmax (Figure 1.19)

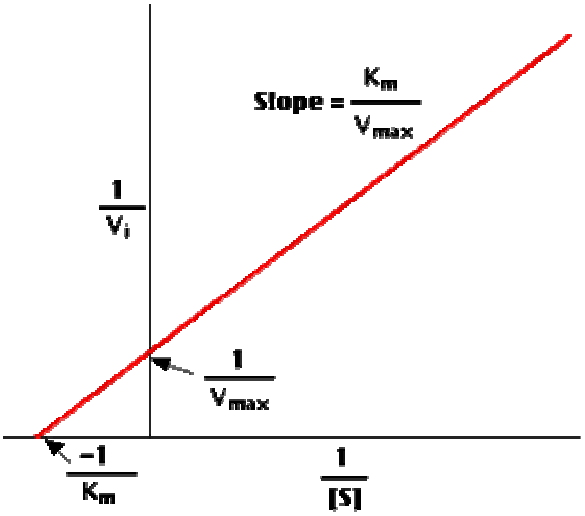


Figure 1.19 Lineweaver- Burk plot

1.4.4 Enzyme Immobilization

Enzymes are sensitive substances. Their catalytic properties change mostly when exposed to high temperature or to certain reagents or when introduced into an unfavorable milieu [**38**].

With the advance in the development of biosensors, several problems surfaced relating to functioning of enzyme system like loss of enzyme especially expensive enzymes, maintenance of enzyme stability and shelf life of the biosensors. In addition to this, there grew a need to reduce the time of enzymatic response and offer disposable devices, which can easily be used in stationary or in flow systems. In order to overcome these problems, several immobilization procedures have been developed [**30**].

When immobilizing an enzyme on a surface, it is most important to choose a method of attachment that will prevent loss of enzyme activity (by not changing the chemical nature or reactive groups in the binding site of the enzyme). In other words, attach the enzyme but do as little damage as possible. Considerable knowledge of the active site of the enzyme will prove helpful in achieving this task.
It is desired to avoid reaction with the essential binding site group of the enzyme. Alternatively, an active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity. In some

cases, this protective function can be fulfilled by a substrate or a competitive inhibitor of the enzyme.

The surface on which the enzyme is immobilized is responsible for retaining the structure in the enzyme through hydrogen bonding or the formation of electron transition complexes. These links will prevent vibration of the enzyme and thus increase thermal stability. The micro environment of surface and enzyme has a charged nature that can cause a shift in the optimum pH of the enzyme. This may be accompanied by a general broadening of the pH region in which the enzyme can work effectively, allowing enzymes that normally do not have similar pH regions to work together [**39**].

1.4.4.1 Benefits of Enzyme Immobilization

The term "immobilized" means unable to move or stationary. Immobilization is the conversion of enzymes from a water-soluble, mobile state to a water-insoluble, immobile state and an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product.

Immobilized enzyme has many operational advantages over free enzyme such as, multiple or repetitive use of a single batch of enzymes, enhanced stability, continuous operational mode, rapid termination of reaction, easy separation of biocatalyst from product, and reduced cost of operation.

1.4.4.2 Methods of Immobilization

1.4.4.2.1 Carrier Binding

This method is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier (Figure 1.20)

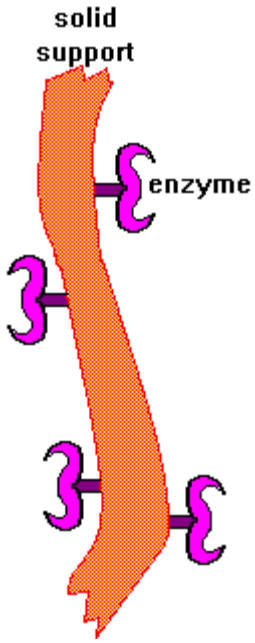


Figure 1.20 Schematic representation of Carrier-Binding type enzyme immobilization.

The selection of the carrier depends on the nature of the enzyme itself, as well as the:

- Particle size
- Surface area
- Molar ratio of hydrophilic to hydrophobic groups
- Chemical composition

In general, an increase in the ratio of hydrophilic groups in the concentration of bound enzymes, results in a higher activity of the immobilized enzymes. Some of the most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel.

- **Physical Adsorption**

The physical adsorption of an enzyme onto an insoluble support is the oldest technique of the enzyme immobilization. In the method, there is little or no conformational change of the enzyme or destruction of its active center. This method is very easy and cheap if a suitable carrier is found. The major disadvantage of this

method is that the adsorbed enzyme may leak from the carrier during use due to a weak binding force between the enzyme and the carrier [**44**].

Numerous surface-active materials including anion-cation exchange resins, activated charcoal, silica gel, alumina, controlled-porosity glasses, ceramics, etc, have been used in preparation of enzyme–adsorption complexes. Depending on the nature of surface, the enzyme binding may be the results of ionic interactions, physical adsorption, hydrophobic bonding, or van der Waals interactions [**45**].

- **Ionic Binding**

The ionic binding method depends on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues.

Polysaccharides and synthetic polymers having ion-exchange centers are usually used as carriers. The binding of an enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme. Therefore, this method yields immobilized enzymes with high activity in most cases. Leakage of enzymes from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH. This is because the binding forces between enzyme proteins and carriers are weaker than those in covalent binding.

The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages is much stronger for ionic binding although weaker than the one in covalent binding.

- **Covalent Binding**

Covalent attachment of enzyme molecules to functionalized support, based on the formation of a covalent bond between the enzyme molecules and support material is the most prevalent and experimentally easy to product [**46**]. When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics: (1) the binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and (2) the active site of the enzyme must be unaffected by the reagents used.

The conditions for immobilization by covalent binding are much more complicated and less mild than in the cases of physical adsorption and ionic binding. Therefore, covalent binding may alter the conformational structure and active center of the enzyme, resulting in major loss of activity and/or changes of the substrate. 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 [**39**].

1.4.4.2.2 Crosslinking

This type of immobilization is support-free and involves joining cells (or the enzymes) to each other to form a large, three-dimensional complex structure, and can be achieved by chemical or physical methods [**47**] (Figure 1.21). This method allows the preparation of membranous sheets of crosslinked enzyme with controlled pore size, by replacing enzyme onto membrane, then, introducing gluteraldehyde to crosslink the enzyme molecule. Cross-linking reactions are carried out under relatively severe conditions. 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 [**48**].

Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as the support. This will result in relatively low enzymatic activity. Generally, cross-linking is best used in conjunction with one of the other methods. It is used mostly as a means of stabilizing adsorbed enzymes and also for preventing leakage [**48**].

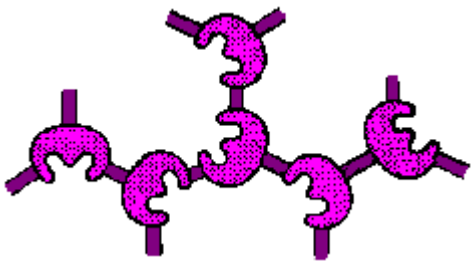


Figure 1.21 Schematic representation of Cross-Linking type enzyme immobilization.

These harsh conditions can change the conformation of active center of the enzyme and so may lead to significant loss of activity.

1.4.4.2.3 Entrapping Enzymes

The entrapment method is depending on the localization of enzyme the lattice of a polymer. It is done in such a way as to retain protein while allowing penetration of substrate. It can be classified into **lattice** and **micro capsule** types [**46**]. In this method the enzyme itself does not bind to a matrix or membrane. Enzyme molecules are free in solution, but entrapped by lattice or membrane (Figure 1.22). It can be achieved by using ionic interactions of enzyme and lattice structure.

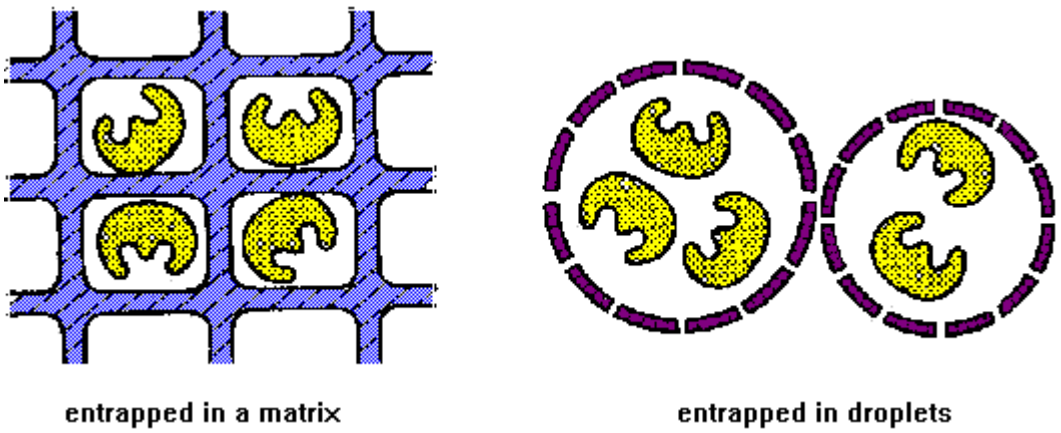


Figure 1.22 Schematic representation of entrapping enzyme

Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as polyarylamide, polyvinylalcohol, etc. and natural polymer (starch) have been used to immobilize enzymes using this technique.

Microcapsule-Type entrapping involves enclosing the enzymes within semi permeable polymer membranes [**39**].

1.4.5 Invertase

Invertase (E.C. 3.2.1.26) is a technologically important enzyme used for hydrolysis of sucrose to a mixture of glucose and fructose. β-fructofuranosidase (E.C. 3.2.1.26) catalysis the hydrolysis of sucrose to glucose and fructose which is known as the invert sugar (Figure 1.23). Sucrose crystallizes more readily than invert sugar, so the latter is widely used in the production of noncrstallizing creams, in making jam and artificial honey. Among several general methods of invertase immobilization the most popular techniques are covalent bonding, ionic adsorption, and entrapment [**31**].

Invertase (EC 3.2.1.26) is produced from suitable commercial yeast strains grown on molasses, and unlike bacterial and fungal exoenzymes, it must be released by distribution of the cell wall. Its use in confectionery ensures that the products remain fresh and soft even when kept for longer periods of time. Soluble invertase is used in the sweet industry in the production of artificial honey, and a small extent in the industrial production of liquid sugar [**49**].

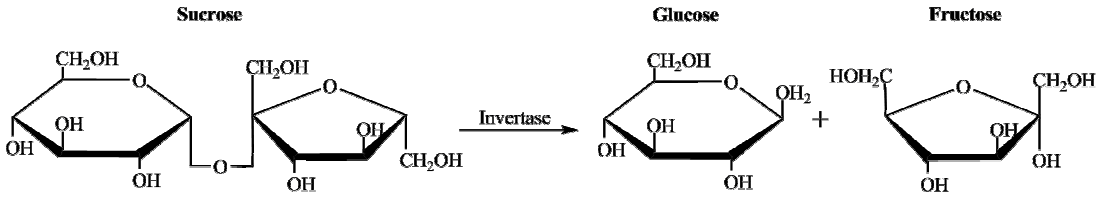


Figure 1.23 Hydrolysis of sucrose

Although, invertase has rather lower probability of achieving commercial use in immobilized form, it is one of the most studied of all enzymes because of being a model enzyme for experimental purposes.

1.4.6 Polyphenol Oxidase (Tyrosinase)

One of the most "versatile" enzymes in nature is tyrosinase (EC. 1.14.18.1). Tyrosinase was discovered by Bertnard and Bourquelot about 100 years ago. Polyphenol oxidases (EC 1.14.18.1) catalyze the oxidation of phenolic compounds

via hydroxylation with molecular oxygen to catechols and subsequent dehydrogenation to *o*-quinones. Quinones are electroactive species that can be reduced at low potentials. Tyrosinase-based biosensors have investigated for the low potential detection of phenols and catechols in foods, pharmaceuticals, and clinical and environmental samples [**50**].

In analyzing certain varieties of mushrooms, they 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 [**51**]. The enzyme is commonly found in yeast, mushrooms, grapes, bananas, apples, potatoes, frogs and mammals.

Its active site contains two copper atoms. Tyrosinase catalyzes two reactions via separate active sites:

(1) the orthohydroxylation of monophenols, commonly referred to as the cresolase activity and,

(2) the oxidoreduction of orthodiphenols to orthoquinones, commonly referred to as the catecholase activity.

Tyrosinase catalyzes the synthesis of melanin through the hydoxylation of tyrosine to dihydoxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to dopaquinone (Figure 1.24). The unstable dopaquinone will polymerize and precipitate into melanin. The cresolase activity of tyrosinase is of particular importance because it synthesizes DOPA. DOPA is a precursor of dopamine, an important neural message transmitter.

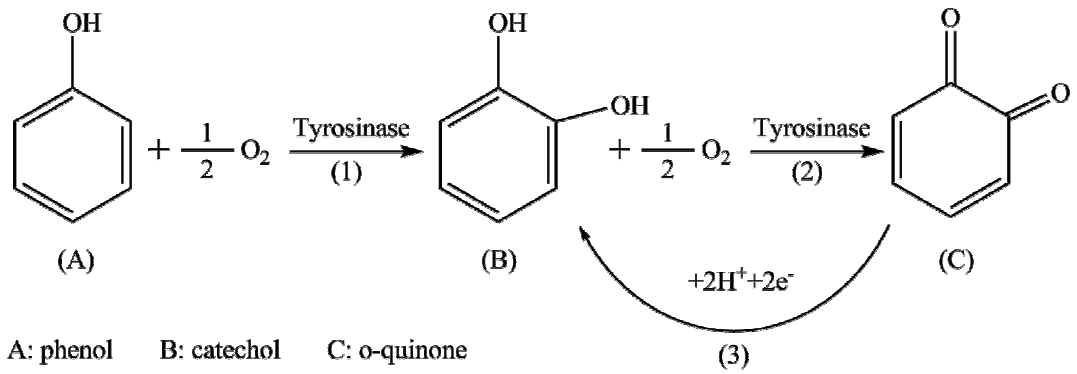


Figure 1. 24 Schematic representation of tyrosinase activity.

The catecholase activity is also important for analyzing phenols and its derivatives. Phenolic compounds are widely used chemicals and released into the environment by a large number of industries including industries of resins and plastics, wood preservation, petroleum refining, dyes, chemicals and textiles. They are a class of polluting chemicals, easily absorbed by animals and human through the skin and mucous membranes. Their toxicity is directed toward a great variety of organs and tissues, primarily lungs, livers, kidneys and genito-urinary systems. Therefore, the determination of phenolic compounds is very important due to their toxicity and persistency in the environment [**52**] .

Tyrosinase also has applications in the food industry, as it is responsible for the browning of fruits and vegetables. 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 [**53**].

1.5 Biosensors

A biosensor is a device that uses a combination of two steps: a recognition step and a transducer step (Figure 1.25). The recognition step involves a biological sensing element, or receptor, on the surface that can recognize biological or chemical analytes in solution or in the atmosphere. The receptor may be an antibody, enzyme, or a cell. This receptor is in close contact with a transducing element that converts the analyte-receptor reaction into a quantitative electrical or optical signal. The signal may be transduced by optical, thermal, electrical, or electronic elements. Lowe emphasizes that a transducer should be highly specific for the analyte of interest. Also, it should be able to respond in the appropriate concentration range and have a moderately fast response time (1–60 sec). The transducer also should be reliable, able to be miniaturized, and suitably designed for practical applications. [**54**].

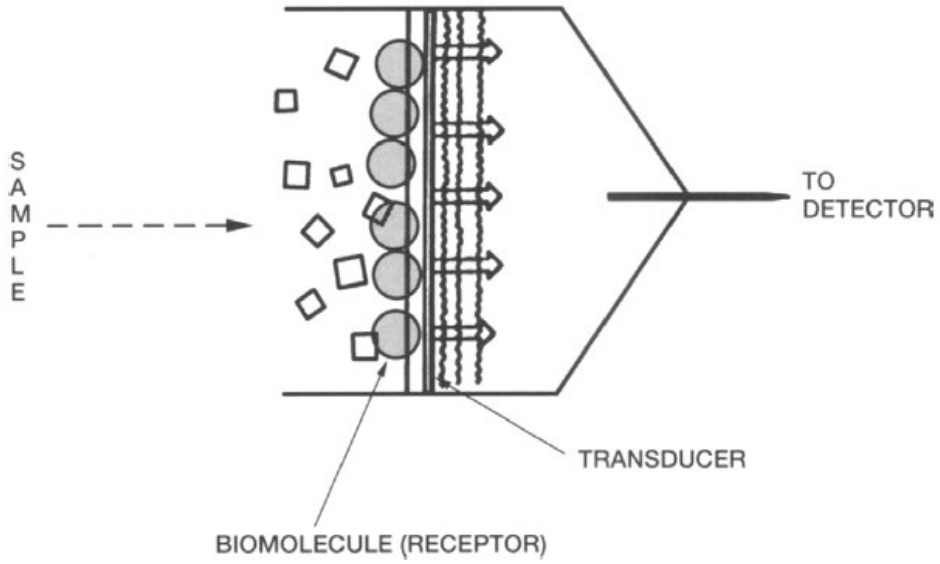


Figure 1.25 Biosensor Construction

1.6 Aim of the Study

(a) To achieve the synthesis of AA/ PVI network

(b) To determine the highest protonation of polymer network by mixing AA and PVI at several stoichiometric ratios, x (molar ratio of the monomer repeat units)

(c) To check the possibility of enzyme immobilization, invertase and tyrosinase, in polymer network via physical entrapment and to characterize the enzyme entrapped polymers.

(d) To determine the maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) for the immobilized invertase

(e) To determine the maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) for the immobilized tyrosinase

CHAPTER II

EXPERIMENTAL

2.1 Chemicals

Invertase (β-fructofuranoxidase) (E.C 3.2.1.26) (from bakers yeast, 53 units/ mg solid) and Tyrosinase (PPO) (E.C 1.14.18.1) were purchased from Sigma and used as received without any purification. The substrates, sucrose and catechol were also obtained from Sigma.

For the preparation of Nelson Reagent (used in the assay of invertase activity), sodium carbonate (Riedel de Haen), sodium potassium tartarate (Riedel de Haen), sodium bicarbonate (Merck), sodium sulfate (Merck), copper sulfate (Merck) were used as received. For the preparation of arsenomolibdate reagent, ammonium heptamolibdate (Merck), sodium hydrogen arsenate (Merck) were used as received. Acetate buffer (pH: 5.1) was prepared from sodium acetate (Sigma) and acetic acid. Citrate buffer (pH: 6.5) was prepared from citric acid and sodium citrate. Acetone, sulfuric acid and 3-methyl-2-benzothiozolinone (MBTH) used in spectrophotometric activity determination of PPO were also purchased from Sigma.

2.1.1 Nelson's Reagent

For reagent A; 2 g anhydrous Na_2CO_3, 25 g sodium potassium tartarate, 20 g $NaHCO_3$ and 200 g anhydrous Na_2SO_4 were dissolved in 700 ml distilled water, then diluted to 1 L. To prepare reagent B; 15 g $CuSO_4$ were dissolved in 100 ml distilled water and concentrated H_2SO_4 was added dropwise. Prior to activity assay, reagents A and B were mixed (25/1) (v/v)

2.1.2 Arsenomolybdate Reagent

25 g ammonium heptamolibdate tetrahydrate were dissolved in 450 ml distilled water and 21 ml concentrated acid were added, 3 g sodium arsenate dibasic-7- hydrate were dissolved in 25 ml distilled water and added to the molibdate solution. The final solution was incubated at 37 ^{o} C for 24-48 hours and stored in dark.

2.2 Instrumentation

2.2.1 Four Probe Conductivity Measurements

To measure the conductivity of pellets of Poly(AA-co-PVI), "the four probe technique" was used (Figure 2.1).

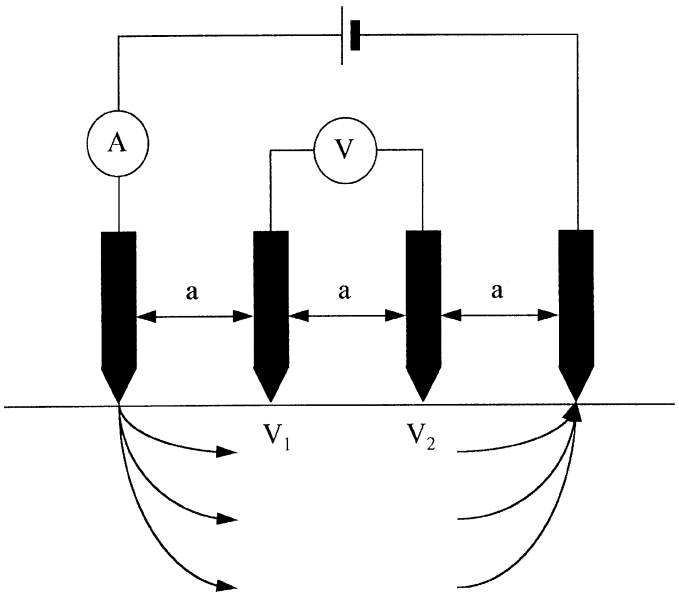


Figure 2.1 Four Probe Conductivity Measurement

The four point probe is preferable over the two point probe method since the contact and spreading resistances associated with the two point probe can be eliminated.

In four probe technique, current is applied to the outer contacts of a sample of known geometry, while the voltage drop is measured between the inner contacts. The

system involves a special probe head, which is composed of four equally spaced spring-loaded electrodes. The head is lowered onto the sample until all probes make good contact with the sample. To measure the resistance of the sample itself without that of the contacts and that of the wires leading to the voltmeter, the conductivity of the sample is calculated by the formula given below;

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

where σ is the conductivity, R is the resistance of the sample and t is the thickness of the film.

2.2.2 UV-Visible Spectrophotometer

A Shimadzu UV–1601 model spectrophotometer was employed in the determination of activities of immobilized enzyme.

2.2.3 Fourier Transform Infrared Spectrophotometer (FTIR)

The FTIR spectra of conducting polymer blends were measured as dispersed in KBr pellets, using Varian 1000 FTIR Spectrometer.

2.2.4 Elemental Analysis

Elemental analyses (C, H and N) of the composite electrolytes were performed in the METU Central Lab-Ankara by using LECO, CHNS-932. The contents of the composite electrolytes were examined through nitrogen analysis.

2.3 Experimental Procedures

2.3.1 Synthesis of AA/PVI Network and Entrapment of Invertase in a Polymer Electrolyte Matrix

To obtain the most suitable AA/PVI matrix, 0.10 grams of AA were mixed with different amounts of PVI. The aim of this work was to determine the maximum protonation. Actually, when the structure of AA was examined and compared with the other acidic biopolymers, the maximum protonation was expected at x=2 where x is the number of moles of 1-VIm per moles of –COOH group in AA. To check the maximum protonation, four samples were prepared with x between 1 and 4. The elemental analysis and FT-IR results showed that, the maximum protonation was obtained with x=1. Due to this reason, in this study the AA/PVI matrix was obtained

using x=1 (Figure 2.2).

Additionally, the maximum water absorbing capacity was checked. The maximum intake capacity was 1.6 ml in 2.0 ml of AA (0.10g/ml) and PVI (0.0556 g/ml).

The enzyme solution was prepared in pH 5.1 acetate buffer with an enzyme concentration of 4.0 mg/ml. Finally, AA/PVI matrix-enzyme solutions were stirred to obtain enzyme entrapped polymer network (EEPN) with a 80 % yield. As a result, 170 units enzyme was incorporated into the matrix. EEPN was used in activity determinations. The immobilization of invertase was achieved via physical entrapment during the complexation process.

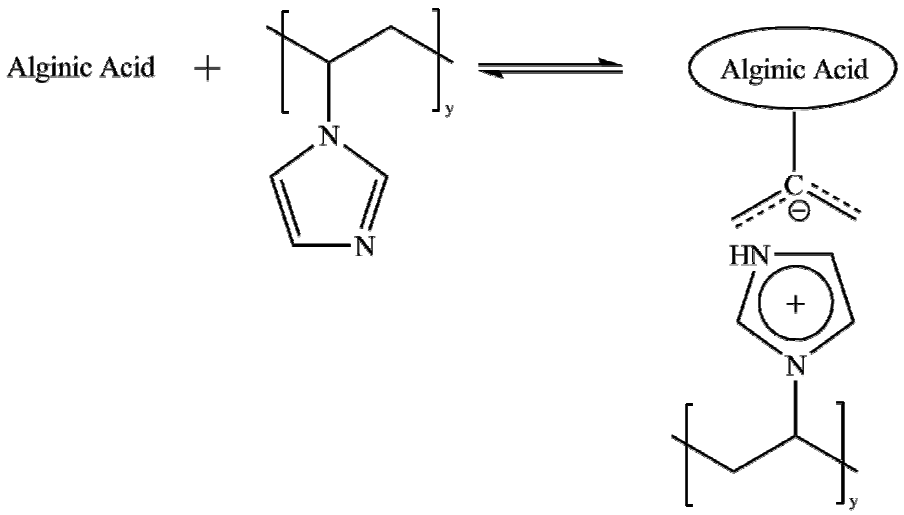


Figure 2.2 The structure of AA/PVI network

2.3.2 Determination of Invertase Activity

Nelson's method was used for activity determination**.** For this method different concentrations of sucrose solutions prepared in acetate buffer (pH 5.1) were kept in water bath at 25 °C for 5 min. Different incubation times (2, 4, and 6 min) were applied to allow enzyme to react with substrate in a total volume of 2.0 ml. After incubation time, 1 ml of this solution was drawn and added into 1 ml Nelson reagent. Test tubes were kept in boiling water for 20 min to terminate the enzymatic

activity completely, and then cooled to room temperature. Finally, 1.0 ml of an arsenomolybdate solution and 7.0 ml of distilled water were added. Absorbances were determined at 540 nm.

2.3.2.1 Determination of Kinetic Parameters

For determination of the maximum reaction rate (V_{max}) and the Michaelis-Menten constant (K_m), the activity assay was applied for different substrate (sucrose) concentrations. Sucrose solutions (5, 8, 10, 20, 30, 50 mM) were prepared in acetate buffer (pH 5.1) and kept in water bath at 25 °C for 5 min, and then the EEPN was added to the test tubes and shaken for incubation times of 2, 4, and 6 minutes.

2.3.2.2 Determination of Optimum Temperature and pH

The AA/PVI network was synthesized with a stoichiometric ratio of x=1. Optimum temperature was determined by changing incubation temperature between 10°C and 80°C while keeping the substrate concentration constant ($10K_m$). In addition to temperature optimization, pH optimization is also important for the improvement of the biosensor. In the present work, pH optimizations were carried out by changing the pH range between 2 and 11 at constant temperature (25 °C) and constant substrate concentration ($10K_m$).

2.3.2.3 Determination of Operational and Storage Stability for Immobilized Invertase

The operational stability of the enzyme biosensor was determined at optimum activity conditions by using electrodes in 40 activity assays per day. The shelf lives of the electrodes were investigated by performing activity measurements within 25 days.

2.3.3 Immobilization of Tyrosinase in AA/PVI Network Synthesis of AA/PVI Network and Entrapment of Tyrosinase in a Polymer Electrolyte Matrix

In this section, to synthesize AA/PVI network the same procedure was applied for the incorporation of tyrosinase. The enzyme solution was prepared in pH 6.5 citrate buffer with an enzyme concentration of 0.5 mg/ml. Finally, polymer-enzyme solutions were stirred to obtain the polymer electrolyte biosensor. Solutions

were converted into the polymer electrolyte matrix (80 % conversion). As a result, 0.4 mg enzyme was incorporated into the matrix. The immobilization of tyrosinase was achieved via physical entrapment during the complexation process.

2.3.3.1 Determination of Tyrosinase Activity

The activities of free and immobilized PPO were determined using Besthorn's Hydrazone Method. Besthorn's Hydrazone Method includes spectrophotometric measurements. In this method 3-methyl–2-benzothiozolinone (MBTH) interacts with the quinones produced by the enzyme to yield red products (instead of brown colored pigments in the absence of the color reagent)**.** The pathway proposed by Rodriguez et al is shown in Figure 2.3.

To determine activity of free PPO, different concentrations of catechol were prepared. Solutions contain 1.0 ml citrate buffer, 0.5 ml MBTH (0.3% in ethanol) and 0.5 ml catechol. 1 minute of reaction time was given after the addition of 0.5 ml enzyme solution (0.1mg/ml). 0.5 ml sulfuric acid (5% v/v) were added to stop the enzymatic reaction. Quinone reacts with MBTH to form a red color complex and this complex was dissolved by adding 3.0 ml acetone

To determine activity of immobilized PPO, different concentrations of catechol were prepared (3.0 ml) and put in water bath at 25°C. EEPN immersed in the solution and shaken for 3 minutes. After removing polymer, 1 ml sulfuric acid and 1 ml acetone were added for a total volume of 6 ml. After mixing, absorbances were measured at 495 nm **[55, 56].**

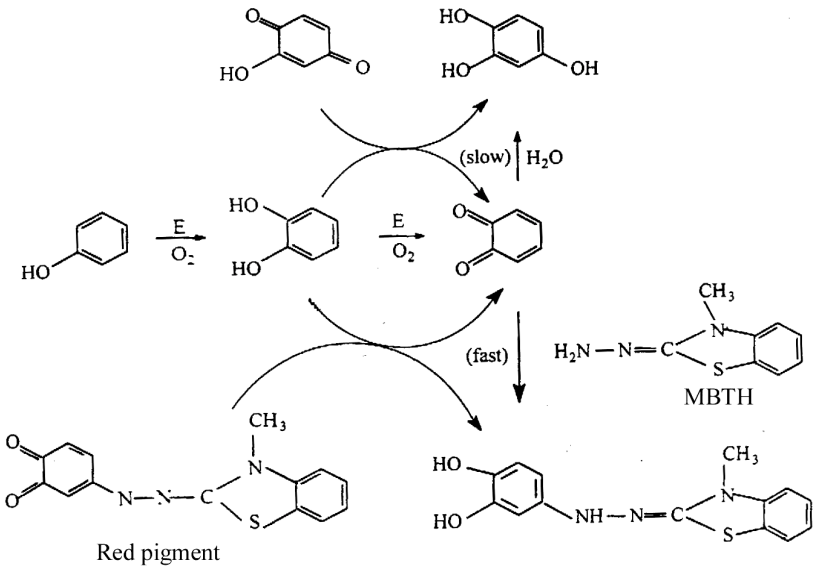


Figure 2.3 Schematic representation of Besthorn's Hydrazone Method

2.3.3.2 Determination of Kinetic Parameters

For determination of the maximum reaction rate (V_{max}) and the Michaelis-Menten constant (K_m), the activity assay was applied for different substrate (catechol) concentrations. Catechol solutions (8, 10, 30, 50, 80, 100, 300, 500, 800 µM) were prepared in citrate buffer (pH 6.5) and kept in a water bath at 25 °C for 5 min, and then the enzyme immobilized network was added to the test tubes and shaken for incubation times of 3, 6, and 9 minutes.

2.3.3.3 Determination of Optimum Temperature and pH

Optimum temperature was determined by changing temperature between 10 °C and 80 °C while keeping the substrate concentration constant ($10K_m$). In addition to temperature, pH optimization is also important for improvement of the biosensor. In the present work, pH optimizations were carried out by changing the pH between 2 and 11 at constant temperature (25 °C) and constant substrate concentration ($10K_m$).

2.3.3.4 Operational Stabilities and Shelf Life

The operational stability of the enzyme biosensor was determined at optimum activity conditions by using electrodes in 40 activity assays per day. The shelf life of the electrodes was investigated by performing activity measurements within 25 days.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Immobilization of Enzymes

3.1.1 Immobilization of Invertase

Immobilization of invertase was achieved in AA/PVI network by physical entrapment. The invertase is a small cost and a model enzyme for the immobilization.

3.1.1.1 Kinetic parameters

Kinetic parameters, Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) for free and immobilized enzymes were obtained at a constant pH and temperature and a different concentration of substrates.

In this matrix, 170 units (3.2 mg) invertase were incorporated. Kinetic parameters K_m and V_{max} were found using Lineweaver-Burk plots at constant temperature and pH while varying the substrate concentration **[57].** The calculated values were given in Table 3.1

Table 3.1 Kinetic parameters for free and immobilized invertase

	Km (mM)	Vmax (μmol/min.ml)
Free invertase	24.3	84.3
AA/PVI /invertase	80	3.33

The maximum rate for an enzymatic reaction is given by V_{max} .The Michaelis- Menten constant (K_m) of an enzyme is a measure of the affinity of the enzyme to its substrate. The value of K_m for a particular enzyme is defined as the substrate concentration at which half of the enzyme molecules are complexed with substrate. The results (Table 3.2) show that there is an increase in K_m values compared to that of the free enzyme **[58].** The high K_m value indicates lower enzyme–substrate affinity. In other words the observed increase in K_m value is due to the tendency of enzyme to leave substrate within a short time without giving a product. In this network, enzyme and substrate interaction and complex formation become more difficult hence, K_m value increases as V_{max} decreases.

3.1.1.2 Temperature Influence on Enzyme Entrapped Polymer

The temperature optimization process is very important in enzyme immobilization and biosensor construction. The effect of temperature on the enzyme activity is shown in Figure 3.1. At 50°C, the free invertase completely lost its activity **[59]**, however, the EEPN for x=1 showed maxima at 40 °C, and also EEPN did not lose enzymatic activity at 50 °C. Additionally this matrix show adequate activity in a low temperature range (10-40°C). EEPN provides a suitable immobilization medium for invertase and can be used as a biosensor for a wide range of temperature.

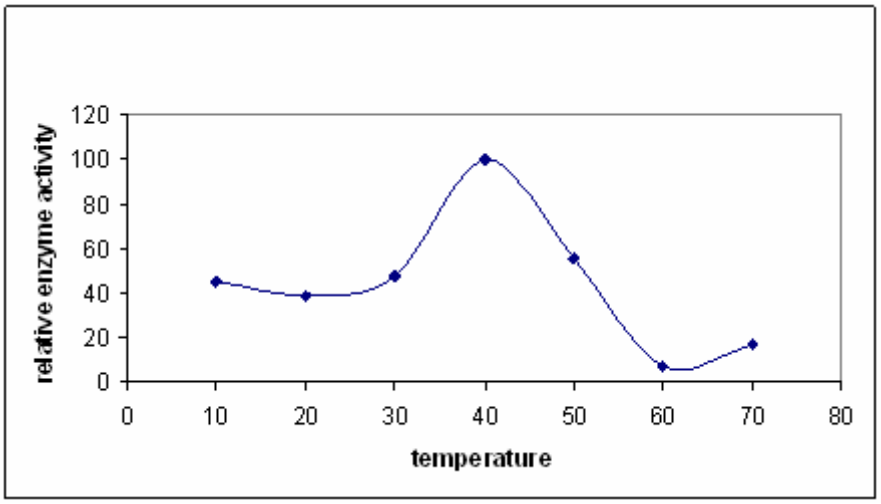


Figure 3.1 Optimum temperatures of enzyme biosensor

3.1.1.3 pH Influence on Enzyme Entrapped Polymer

The variation of invertase activity for a pH range from 2 to 11 was investigated for the AA/PVI matrix. In pH optimization experiments, the temperature of medium and concentration of the substrate were kept constant. The results are presented in Figure 3.2. The maximum activity of free invertase was observed at pH =5 **[16].** The immobilized invertase in the AA/PVI matrix showed a maximum activity at pH 10. There is a continuous increase in the enzyme activity from pH 2 to 6. In AA/PVI matrix, there is a protonated medium, for that matter the pH value in EEPN is different than that of bulk.

At pH= 7, there was a drastic decrease. Probably, the pH of microenvironment of enzyme is lower than 7 and close to the isoelectric point. The isoelectric point is the pH at which a molecule or surface carries no net electrical charge. The solubility of a molecule is often at a minimum when the pH is at the isoelectric point. Also, the enzymatic activity values were low.

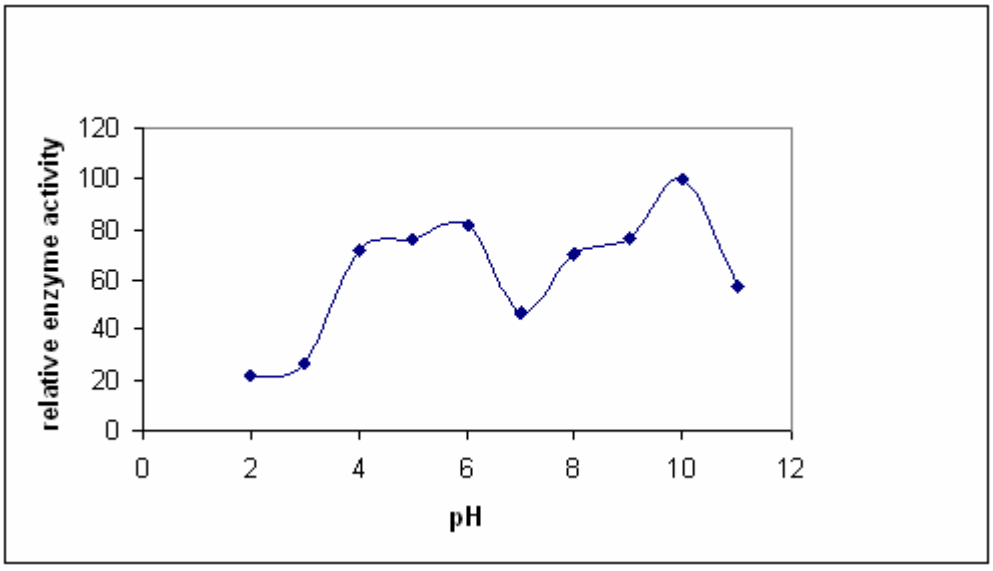


Figure 3.2 pH influence on enzyme biosensor

3.1.1.4 Operational Stability and Shelf Life of the Enzyme Biosensor

The operational stability was obtained by running 40 measurements in the same day at a constant temperature, pH and substrate concentration (Figure 3.3). An

activity loss of 20% was observed after the third use. At the end of 40 measurements, total activity loss of 30 % was observed. As a consequence, immobilized enzyme has an improved operational stability. The activity of EEPN was measured for every 5 days within consecutive 25 days to determine shelf life of immobilized enzyme. This enzyme lost 50% of its activity in the first 3 days, and completely lost its activity within the next 25 days (Figure 3.4). The enzymatic activity decreases during the storage period. It can be concluded that a biosensor constructed with this matrix on an enzyme shows good stability for a short time.

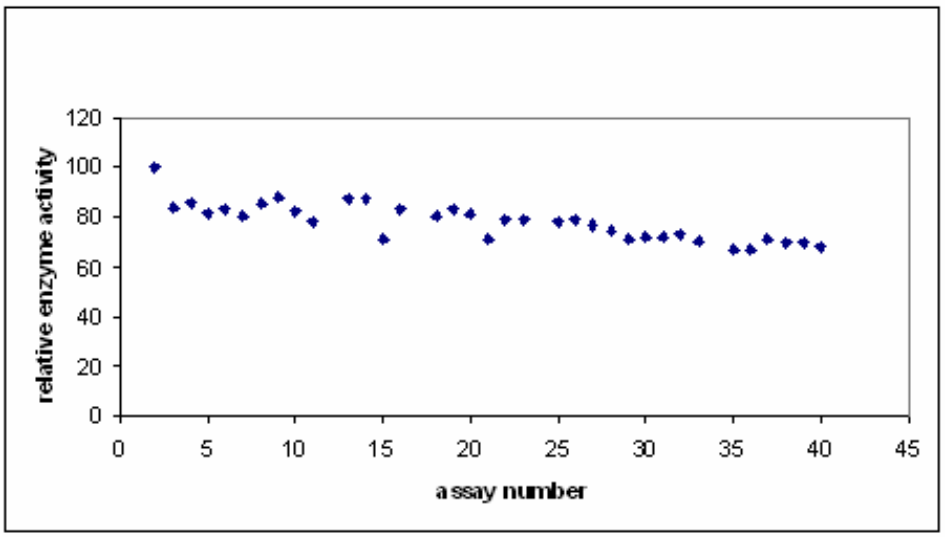


Figure 3.3 Operational stability of enzyme biosensor

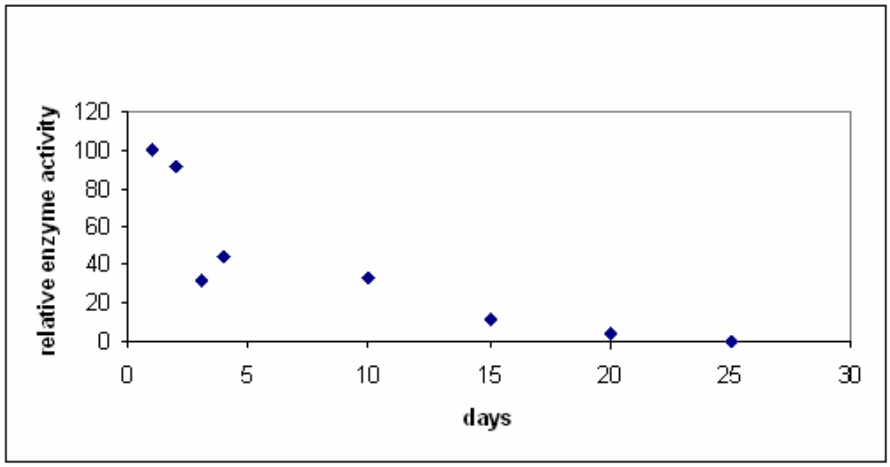


Figure 3.4 Shelf life of the biosensor

3.1.2Immobilization of PPO (Tyrosinase)

3.1.2.1 Kinetic Parameters

The enzyme assay is based on the measurement of *o*-quinone generated in enzymatic reaction **[60].**

For determination of kinetic parameters (K_m and V_{max}) of enzyme biosensor Lineweaver-Burk plots were used **[57]** at constant temperature and pH while changing the substrate concentrations. Free PPO has a maximum reaction rate of 11.2 µmol/min. mg protein and K_m of 4 mM **[61].** The Michaelis-Menten constant (K_m) of an enzyme is a measure of the affinity of the enzyme for its substrate. The kinetic parameters K_m and V_{max} of this matrix are $2.9x10^{-2}$ mM and 1.5 µmol/min.mg protein (0.585 µmol/min.mL) respectively. The tyrosinase enzyme is biocompatible with AA. For that reason; there is a drastic decrease in K_m values compared to that of the free enzyme. The decrease in K_m value means higher affinity. Since the microenvironment of immobilized enzyme is more biocompatible and it feels more comfortable than of free enzyme, as a result in this matrix the interaction of enzyme and substrate becomes easier than free tyrosinase. The decrease in V_{max} value when compared to the one for free enzyme is results from good enzyme substrate interaction. This means that enzyme and substrate prefer to come together, hence the amount of product decreases. The values were given in Table 3.2

Table 3.2 Kinetic parameters for free and immobilized tyrosinase

	K_m mM	V_{max} µmol/min.mg protein
Free tyrosinase	4	11.2
AA/PVI /tyrosinase	$2.9x10^{-2}$	1.5

3.1.2.2 Temperature Optimization of the Enzyme Biosensor.

The temperature optimization process is very important in enzyme immobilization and biosensor construction. The temperature is important parameter for the enzyme activity enzyme network. Free tyrosinase showed a maxima at

40°C. In the present matrix, the maximum activity was also observed at 40°C (Figure 3.5). Free PPO lost its activity completely at 50°C. This behavior resulted from the damaged structure of enzyme network. Due to that reason enzyme leaves network. Additionally, the polymer electrolyte can be decomposed to AA and PVI due to changes in polymer matrix hence, the enzyme leaks to the solution.

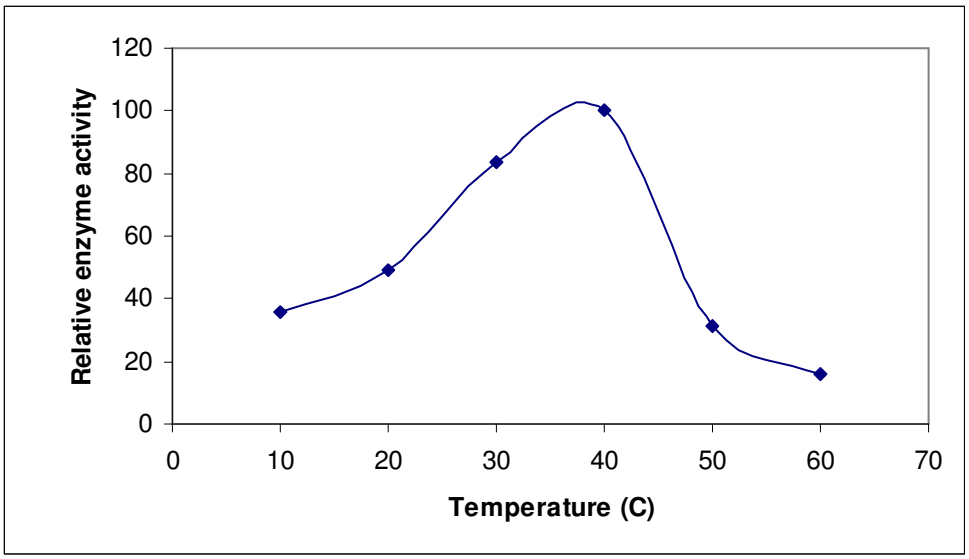


Figure 3.5 Optimum Temperature of Enzyme Biosensor

3.1.2.3 pH Optimization of the Enzyme Biosensor

In enzymatic assays pH optimization is important. Especially high and low pH medium can denature the enzyme. The variation of tyrosinase activity for a pH range of 2-11 was investigated for the AA/PVI matrix. The results are presented in Figure 3.6. The maximum activity of free tyrosinase was observed at pH 5, and immobilized tyrosinase in the AA/PVI polymer matrix showed its maximum activity at pH 9. For pH between 2 and 4, no activity was observed. Since in low pH medium the polymer electrolyte can be decomposed to AA and PVI , the enzyme enters the solution and gets denaturated in the acidic medium; as a result enzyme loses its activity. There is an increase in the enzyme activity from pH 5 to 9. The pH of maximum activity was shifted towards the alkaline side when compared to that of

free tyrosinase. The mobile protons entrapped into AA/ PVI network, protect the enzyme against high concentration of OH^- , in other words this matrix supplies high pH stability for the enzyme.

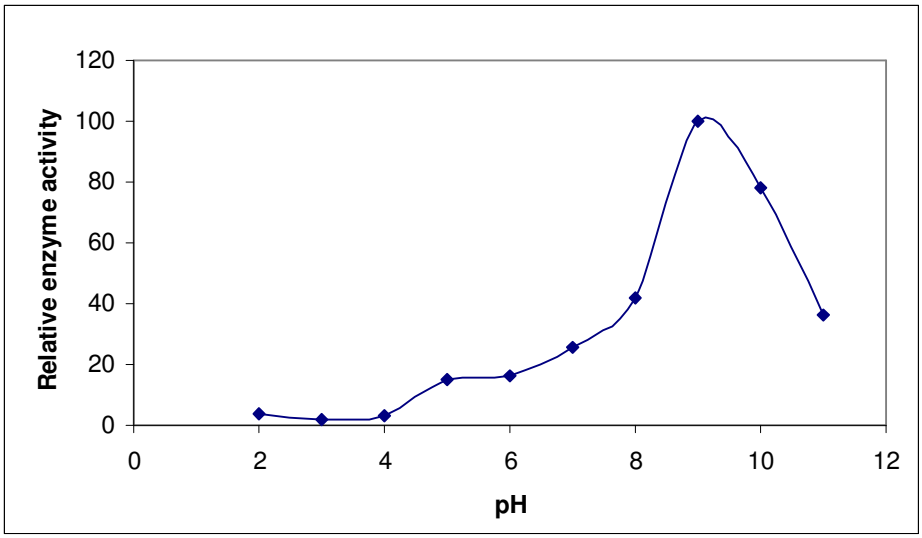


Figure 3.6 Optimum pH of Enzyme Biosensor

3.1.2.4 Operational Stability and Shelf Life of Enzyme Biosensor

One of the most important parameter is the operational stability. The operational stability was obtained by running 40 measurements in the same day at constant temperature, concentration and pH.

In this study, operational stability experiments were done in pH= 6.5 and 9 medium to check the pH effect on operational stability. At pH= 6.5 (Figure 3.7) the activity loss of 20% was observed after the fourth use.

At pH= 9 which is the highest enzymatic activity observed at constant temperature and substrate concentration (Figure 3.8), the activity loss of 20 % was observed after the fourth use. The decrease in activity values was observed at pH= 9. Both experiments showed that this behavior is related to the stability of tyrosinase.

The activity of AA/PVI/ tyrosinase sensor was measured every 5 days for 25 days. This matrix lost 50% of its activity in the first 2 days, 60 % within 5 days and

completely lost its activity within the next 20 days (Figure 3.9). The sharp decrease in enzyme activity results from diffusional effects and structural changes in the enzyme during the storage period. It can be concluded that a biosensor constructed with this matrix on an enzyme shows good stability for a short time.

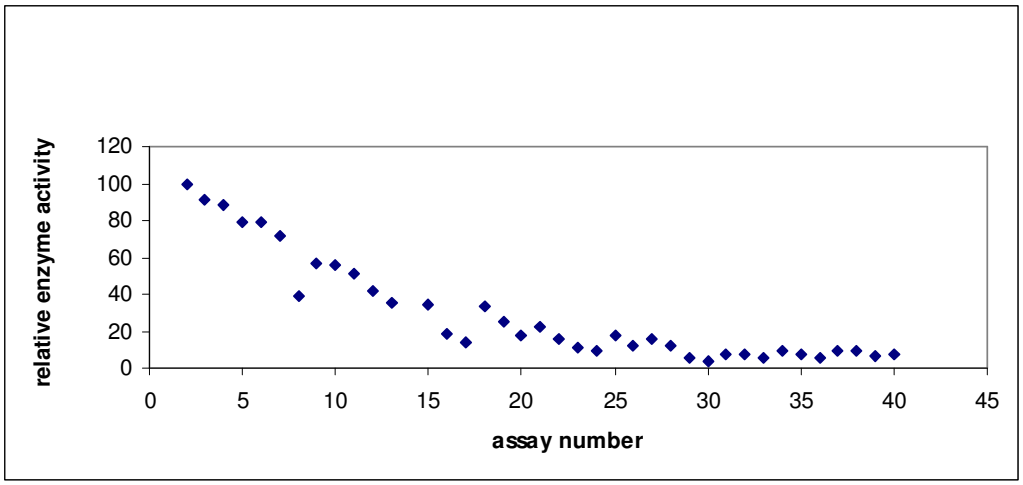


Figure 3.7 Operational Stability of Enzyme Biosensor (pH=6.5)

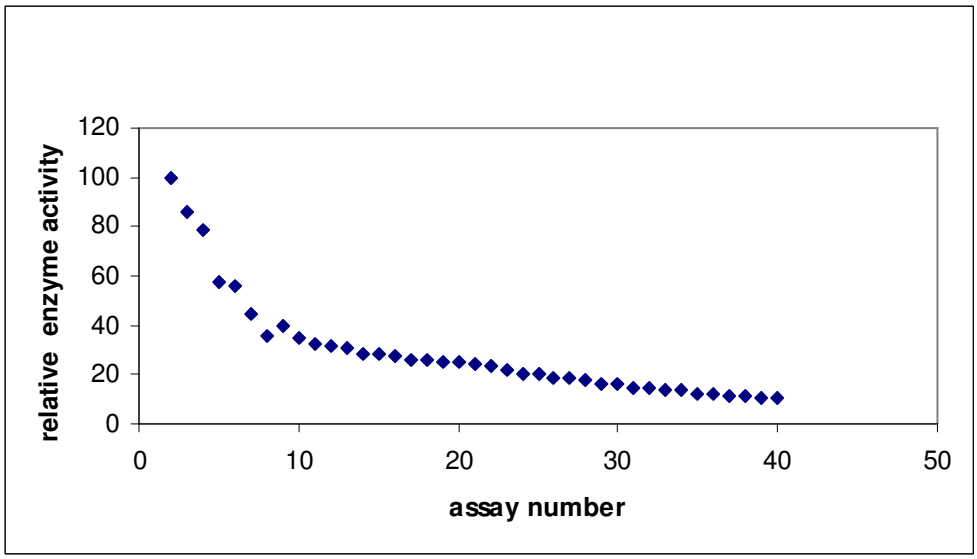


Figure 3.8 Operational Stability of Enzyme Biosensor (pH= 9)

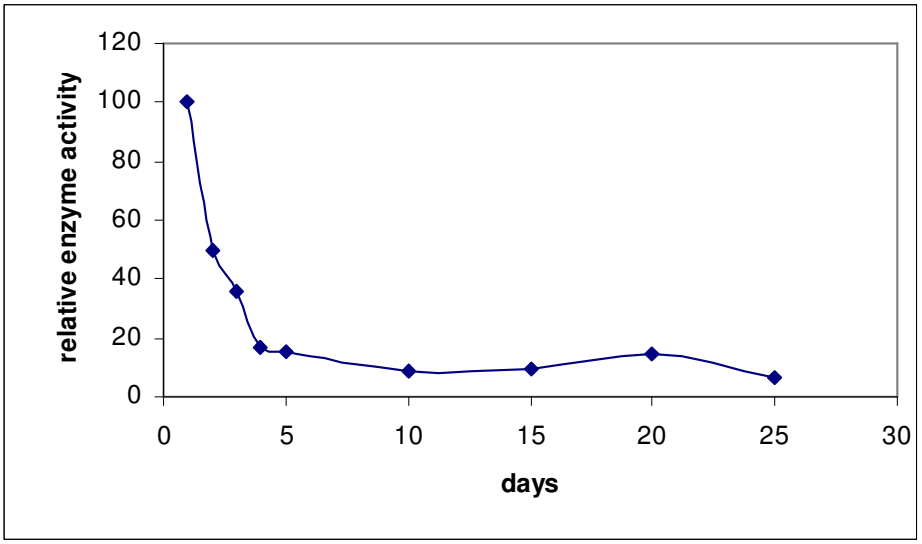


Figure 3.9 Shelf Life of Biosensor

3.2 FTIR Results

The FTIR spectra of the samples were obtained with a Varian 1000 FT-IR spectrophotometer.

A strong absorption appears at 1742 cm^{-1} that belongs to C=O stretching. 1247 cm^{-1} and 1419 cm^{-1} represent the C-O-H stretching of carboxylic acid groups of the AA (Figure 3.10). After obtaining polymer network by mixing AA and PVI in different ratios, a new peak was observed at 1604 cm^{-1}, and the intensity of the carbonyl stretching at 1742 cm^{-1} decreased due to the protonation of PVI with AA. These occur by the transfer of the acidic proton of carboxylic acid to the 'free' nitrogen side of PVI to form imidazolium ion. A broad peak centered at 3433 cm^{-1} was observed due to stretching vibration of the isolated non-hydrogen bonded NH group and a broad peak approximately located between 3050 and 3300 $cm^{-1}$was assigned to the stretching vibration of NH groups involved in hydrogen bonding of the protonated heterocycle **[62].** The percent protonation was evaluated for the peaks that are located at 1742 cm^{-1} and 1604 cm^{-1}.

Before immobilization of enzyme, the degree of protonation was evaluated. Presence of two acidic protons on Alginic Acid, the rise the expectations of highest protonation to with x=2 (molar ratio of the monomer repeat units). The percent

protonation of the complex electrolyte AA: PVI was 96 % for 1:1, 83 % for 1:2, 89% for 1:3 and 87% for 1:4. Clearly, the highest protonation with x=1. The reason can be attributed to the structure of AA. AA is a linear copolymer with homopolymeric blocks of (1-4)-linked ß-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively, covalently linked together in different sequences or blocks. The monomers can appear in homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M and G-residues (MG-blocks) or randomly organized blocks. Hence, we can conclude that due to the complicated structure, all carbonyl groups may not be involved in complexation reactions.

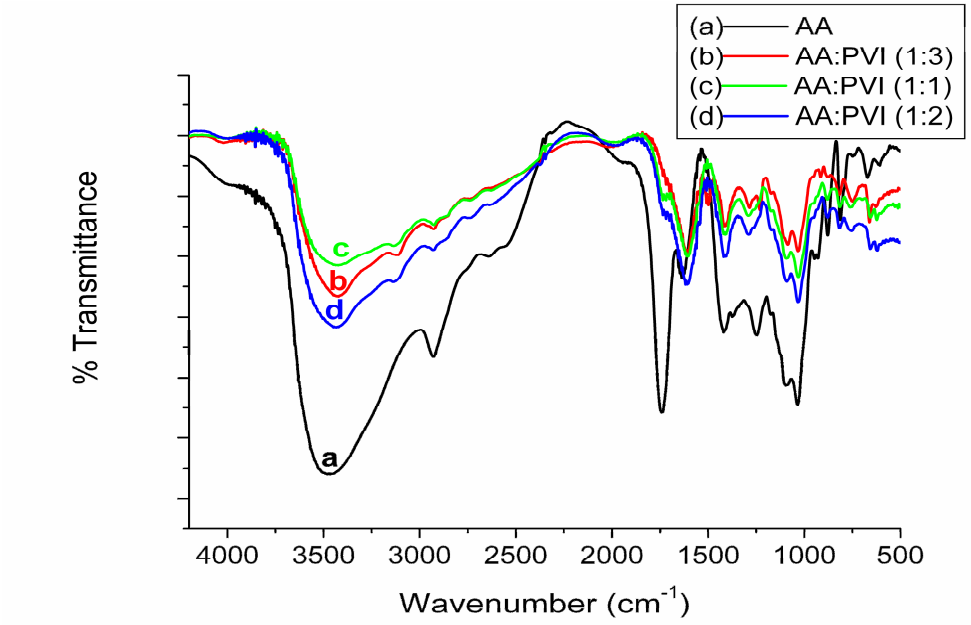


Figure 3.10 FT-IR results of AA and AA/ PVI networks at different x

3.3 Elemental Analysis

Table 3.3 and Table 3.4 show results of feed and the final compositions of the complex polymer electrolytes and elemental analysis. Stable gels were collected form the solution and dried under vacuum for EA analysis. While the PVI content in the feed was varied form 50 % to 80%, the PVI composition of the polymer

electrolyte was changed from 48% to 69% As the composition of the complex materials are known, they are successfully used for invertase and tyrosinase immobilization.

Table 3.3 Composition of sample in feed and actual complexes

Sample	**Composition AA/PVI in the feed**			**Actual composition of AA/PVI complexes**		
AA/PVI	%C	%H	%N	%C	%H	%N
1:1	51.36	6.66	10.89	46.18	5.49	11.7
1:2	54.69	6.61	15.94	47.07	5.55	13.25
1:3	56.62	6.56	18.86	48.94	5.93	16.95
1:4	57.87	6.54	20.77	50.35	5.91	17.92

Table 3.4 Results of elemental analysis

Sample (AA/PVI)	% VIm in the feed(mol)	% VIm in the complex (mol)
1:1	50	48
1:2	66	55
1:3	75	67
1:4	80	69

3.4 Conductivity Measurements

Standard four-probe technique was used for conductivity measurements. Conductivities AA/ PVI /enzyme networks were in the range of 10^{-4}-10^{-5} S/cm (Table 3.5).

Table 3.5 Conductivity values of AA/ PVI/ Enzyme

Polymer Network	**Conductivity (S/cm)**
Alginic acid / PVI / Invertase	$4.4x\ 10^{-4}$ (25 ° C)
Alginic acid / PVI /Tyrosinase	$7.0x10^{-5}$ (25 ° C)

In Table 3.6 the conductivities of some of the proton conducting polymers are summarized. It should be emphasized that PEO/H_3PO_4 and Nafion materials are thermally stable up to 100 ° C.

Proton conducting polymer that prepared from AA, one of the acidic biopolymers, and PVI, one of the basic heterocyclic molecules showed $1.7x10^{-6}$ proton conductivity at 25 ° C. Additionally these composite material is important for environmental safety and low cost polymer.

Table 3.6 A literature Survey on Conductivity of Proton Conducting Polymer

Polymer Network	**Working Temperature (^{o} C)**	**Conductivity (S/cm)**	**References**
Nafion	5	10^{-3} -10^{-2}	**[12]**
PEO/H_3PO_4	50	2.5x 10^{-4}	**[14]**
Poly(acrylic acid)/Imidazole	130	10^{-3}	**[22]**
PEGMAP/Imidazole	160	2×10^{-4}	**[19]**
Poly(adipic acid)/BnIm	130	$4x10^{-3}$	**[20]**
P(4-VI)/ H_3PO_4	25	10^{-4}	**[23]**
PVPA/ Imidazole	150	$7x10^{-3}$	**[26]**

CHAPTER IV

CONCLUSION

A new proton conducting polymer containing alginic acid (AA) and poly (1-vinylimidazole) (PVI) was synthesized. The polymer network was obtained by mixing AA and PVI with several stoichiometric ratios, x (molar ratio of the monomer repeat units). This polymer network was used as an enzyme immobilization matrice.

The polymer–heterocycle electrolytes were characterized by elemental analysis (EA) and FT-IR spectroscopy. The proton exchange reactions were studied by FT-IR spectroscopy where the maximum protonation was found to be with x=1. The composition of the materials was studied by elemental analysis. PVI was found to vary between 48 to 69 % depending on the feed composition. Proton conductivity occurs through diffusion between protonated and unprotonated PVI.

This study revealed that invertase and tyrosinase can be successfully immobilized in a proton conducting polymer. For both enzymes, AA/PVI matrix has supplies high temperature resistance. K_m value was found as 80 and 2.9x 10^{-2} mM and V_{max} 3.33 and 1.5 μmol/min.ml for AA/PVI/ invertase and AA/PVI/tyrosinase respectively. Optimum temperature was found as 40 ^{o} C for AA/PVI/ invertase and AA/PVI/tyrosinase. The maximum pH was found to be 10 for AA/PVI/ invertase and 9 for AA/PVI/ tyrosinase. Entrapped invertase exhibits high stability over a broad pH range compared to free enzyme.

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