PREPARATION AND CHARACTERIZATION OF TYROSINASE IMMOBILIZED GELATIN FILMS

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ABSTRACT

PREPARATION AND CHARACTERIZATION OF TYROSINASE IMMOBILIZED GELATIN FILMS

In this work, tyrosinase enyzme was immobilized on gelatin films by using entrapment and adsorption. In entrapment method, glutaraldehyde was used to crosslink the gelatin matrix. Fourier transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), contact angle were used for characterization of the films; UV spectrophotometer was used for measurement of the catalytic activity of the tyrosinase immobilized films in catechol oxidation reaction. The pH and temperature, enyzme and substrate concentration effects on the activity of enzyme were investigated. Immobilization of enyzme to gelatin support increases the pH, temperature and operational stability due to the appropriate flexibility and structural rigidity. Activity of immobilized enyzme has decreased compared to free enyzme activity due to the steric hinderance effect and mass transfer limitations. The reusability of the enzyme entrapped gelatin film was also investigated; there was almost 60% decrease in activity of the film after fifth usage. During activity measurement, the color of the gelatin films becomes brown showing that quinone produced with enyzmatic reaction was bound on the film.

ÖZET

TİROZİNAZ TUTTURULMUŞ JELATİN FİLMLERİN HAZIRLANMASI VE KARAKTERİZASYONU

Bu çalışmada, hapsetme ve adsorpsiyon yöntemleri kullanılarak tirosinaz enzimi tutturulmuş jelatin filmler elde edilmiştir. Hapsolma metodunda, jelatin yüzeyde çapraz bağlama yapmak için glutaraldehit kullanılmıştır. FTIR, SEM, temas açı ölçer cihazları ile filmler karakterize edilmiştir ve UV spektrofotometre yardımıyla katekol oksidasyon reaksiyonunda, tirosinaz immobilize olmuş filmlerin katalitik aktivitesi ölçülmüştür. pH, sıcaklık, enzim ve substrat konsantrasyonun enzim aktivitesi üzerindeki etkisi incelenmiştir. Enzimin jelatin yüzeye tutturulması yapısal sertlik ve uygun esneklik sağladığından enzimin pH, sıcaklık ve depolama kararlılığını serbest enzime göre arttırmıştır. İmmobilize edilen enzim aktivitesinde serbest enzime göre, sterik engelleme ve kütle transfer sınırlamaları yüzünden belirgin şekilde bir düşme olmuştur. Jelatin hapsolmuş filmlerin tekrar kullanılabilirliği de incelenmiştir ve beşinci kullanımdan sonra film aktivitesinde neredeyse % 60 lık bir kayıp olmaktadır. Enzimatik reaksiyon sonucunda jelatin filmler kahverengi olmuştur, bu da oluşan künon ürününün film üzerine tutunduğunu göstermektedir.

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CHAPTER 1

INTRODUCTION

Enzymes, protein that are used as catalyst in chemical reactions, takes place in many important biological reactions by lowering the activation energy of the reaction and helps obtaining product easily. Based on these, enzymes are very essential for human and all living organism. Thus, recently, there become a new research area about enzymes reusability The advantages and drawbacks of enzymes are given in Table 1.1

Table 1.1. Advantages and drawbacks of enzymes
(Source : Ilanes 2008)

Advantages	**Drawbacks**
High specificity	High production cost
Highly biodegradable	High molecular complexity
High activity	Intrinsic fragility
Generally natural products	

Enzymes are very sensitive compounds and their activity are effected easily from the conditions of reaction media such as pH, temperature, concentration of substrate and organic solvent. Immobilization of enzyme becomes a hot topic by researchers giving a chance for decrease in negative effect of reaction media and use enzyme repeatedly. Since, immobilization is a widely used technique in areas such as; reactions of organic synthesis, with an exqui-site selectivity, synthesis of pharmaceutical. Even the immobilization become a very popular methodology due to the many advantages , such as the reusability of enzyme, high operational stability, easy to separate the catalyst from reaction media and reduced cost of operation (Bayramoglu, Akbulut et al. 2013).

Tyrosinase is a binuclear copper enzyme and found in all organisms which catalyzes essential biological reactions (Asav et al,2008). This enzyme has a wide application area like; detection of phenol by biosensor preparation for removal of

phenols from waste water, melanin biosynthesis and synthesis of l-DOPA which is a medicine for use of Parkinson disease (Ates et al.,2007) due to the oxidation effect on monophenols. Lopez et al (2001) used tyrosinase to catalyze the o-hydroxylation of monophenol to o-diphenol.

There are several methods for immobilization of enzyme into suitable supports. Immobilization was achieved by binding enzyme into carriers or implication enzyme into polymeric matrices. Mainly, for immobilization; the covalent binding, crosslinking with bi functional reagents, adsorption onto insoluble materials, encapsulation and entrapment in polymeric gels methods have been used. (Munjal et al., 2001). There are many supports such as organic (agarose, starch, polyethylene, nylon) and inorganic materials (clays, zeolite) present for immobilization of enyzme.(Drauz and Waldmann 2002).

Gelatin easily available, nontoxic, hydrophilic, biodegradable, biocompatable compound ((Fu, Xu et al. 2012);Seo et al.,2003), have been used for immobilization of tyrosinase enzyme. In the study of Munjal et al. (2001) the tyrosinase entrapped gelatin gives the maximum activity when compared to alginate, polyacrylamide. The reusability of the enzyme was also examined; it preserved 30% of its initial activity even after 8 cycle reused. Aytar and Bakir (2008) immobilized tyrosinase using glutaraldehyde as crosslinking agent. They found out 100% activity recovery due to the enhanced thermal and storage stabilities of crosslinked enzyme aggregates CLEAs by immobilization. In the study of Dinçer et al. (2011), tyrosinase enzyme was immobilized into the crosslinked chitosan-clay composite beads using glutaraldehyde. These beads were used for phenol removal in waste water. Asav et al. (2008), developed tyrosinase immobilized gelatin films to investigate the inhibition effect of fluoride on tyrosinase activity. Tyrosinase can also be immobilized for use of electrochemical processes; Yildiz, Kiralp et al. (2005) immobilized tyrosinase in poly (ethyleneoxide) electrode via entrapment method in order to determine the phenolic compounds in red wines.

In this study tyrosinase immobilized gelatin films by using crosslinking agent, glutaraldehyde was prepared; the effect of pH, temperature, concentration of substrate on the films was investigated; storage and operational stabilities of the films were investigated.

CHAPTER 2

ENZYMES

Enzymes are protein. They are composed of amino acids bound by peptide bonds that accelerate the reactions. A peptide bond which is called also amide bond is a covalent bond formed between carbon atom of the carboxyl group (-COOH) of one amino acid and the nitrogen atom of the α-amino group(-NH_2) as indicated below.

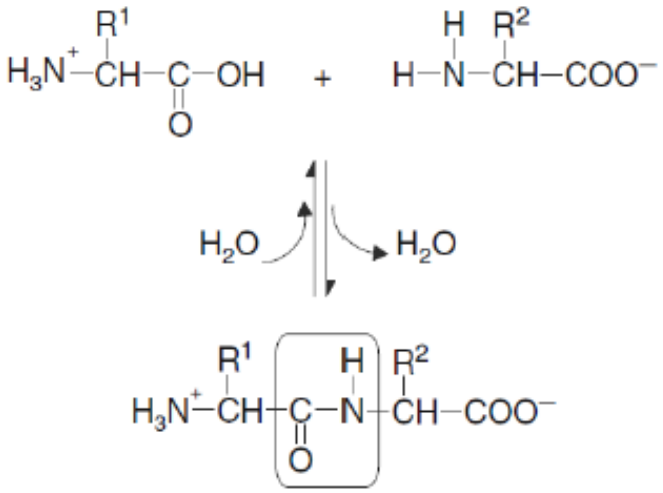
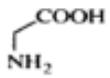
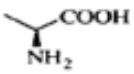
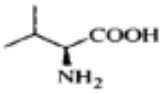
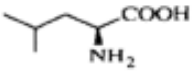
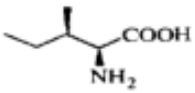
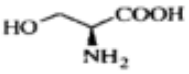
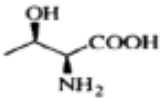
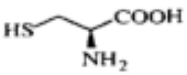
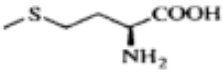
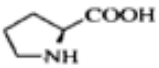
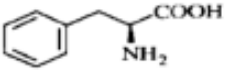
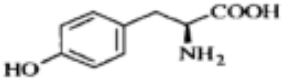
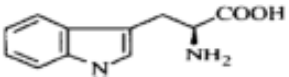
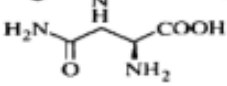


Figure 2.1.Formation of a peptide bond between two amino acid

Like other proteins, enzymes consist of millions amino acid residues (Table 2.1) that attached each other by chemical interactions. The arrangement of these amino acids determines the function of the enzyme.

The primary structure of an enzyme related with the amino acid sequences of protein. The secondary structure of enzyme is basic shape that chain of aminoacid takes on. The most common types are α- helix (regular coiled structure like spring) and β-sheets. The interaction between amino acids forces the enzyme into their three-dimensional structure, which is called tertiary structure of an enzyme. Figure 2. 2 shows the Hierarcy of protein structure.

Table 2.1. Structure of aminoacid side chains
(Source: Drauz and Waldmann 2002)

Name	Symbol	Structure	pKa of ionizing side chain
Glycine	Gly (G)		
Alanine	Aia (A)		
Valine	Val (V)		
Leucine	Leu (L)		
Isoleucine	Ile (I)		
Serine	Ser (S)		
Threonine	Thr (T)		
Cysteine	Cys (C)		9.1–9.5
Methionine	Met (M)		
Proline	Pro (P)		
Phenylalanine	Phe (F)		
Tyrosine	Tyr (Y)		9.7
Tryptophane	Trp (W)		
Asparagine	Asn (N)		

The main type of interactions that are responsible for the tertiary strucuture of enzyme are:

- Hydrogen bonds, especially in secondary structure of protein plays significant role in stabilization of tertiary structure
- Ionic interactions between positive and negative charges on aminoacids contribute stabilization of tertiary structure

- Disulphide bridges play important role in tertiary structure

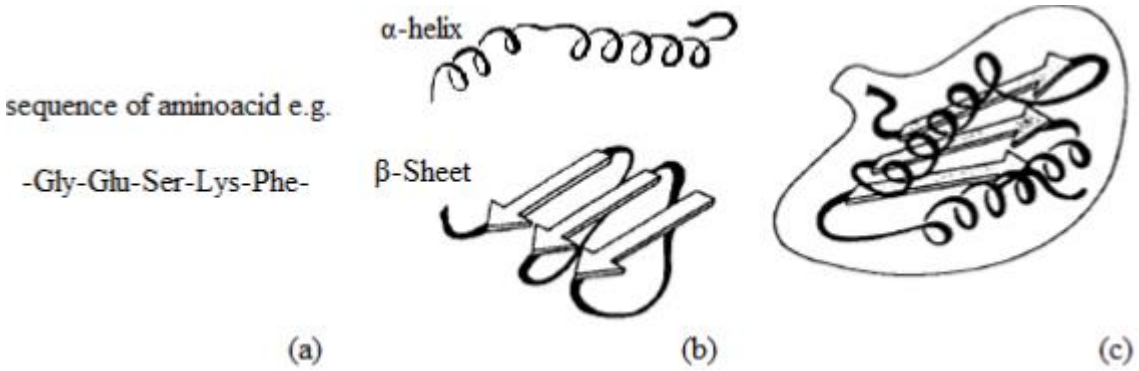


Figure 2.2.Hieararcy of protein structure; primary structure(a), secondary structure(b), tertiary structure(c).

Enzymes are also biological catalysts that speeds up all vital biological (or chemical) processes. They are large molecules and only a small part of enzyme which are called active sites participate in the reaction. Enzymes convert substrates to products by lowering the activating energy of the reaction (Figure 2.3).

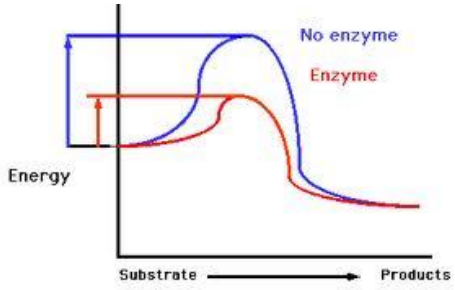


Figure 2.3. Enzymes lowering the activation energy of reaction.

Enzyme activity is defined as the capacity of enzyme to catalyze the reaction. Considering the complex nature of enzyme, enzyme activity can be influenced by:

- The concentration of enzyme and substrate
- pH

- Temperature
- Organic solvents

Amino acids as a building block of enzymes can be polar or nonpolar. The enzyme can be protonated or unprotonated, so that the active site of enzyme can be affected from pH of the media. Increase in temperature, an important factor that increases the reaction rate whereas decreases the enzyme activity. At high temperature, the enzyme activity will decrease sharply due to the denaturing of protein structure of enzyme. This temperature is too high to breakdown the weak ionic and hydrogen bond that stabilize the three dimensional structure of enzyme. Substrate and enzyme concentration is another important factor for enzyme activity. The increase in substrate concentration, increases the enzyme activity (rate of reaction). When enzyme is saturated; all active sites of molecule are occupied and the reaction will not more speed up even adding highly concentrated substrate.(Chaplin and Bucke 1990).

Consequently, the medium pH, temperature, substrate and enzyme concentration should be optimize to obtain high enzymatic activity in a reaction. Enzymes are mainly classified into six classes according to reaction catalyzed (Table 2.2).

Table 2.2. Classification of enzyme

Class	Type of reaction catalyzed
1.Oxidoreductases	Oxidation-reduction reactions (oxidases, peroxidases)
2.Transferases	Transfer of spesific group (kinases, transamines)
3.Hydrolases	Hydrolysis reaction (lipases, estrases, amylases)
4.Isomerases	Change of form of substrate
5.Lyases	Joining two molecules by new bonding
6.Ligases	Lead to formation of bonds between carbon and other atom

2.1.Tyrosinase Enzyme

Tyrosinase is member of oxidoreductase that has type-3 copper center and found in plants, fungi and bacteria. Tyrosinase catalyzes the monophenolic compunds to o-diphenols (monophenolose activity) , later oxidize the diphenols to o-quinones (diphenolase activity) using oxygen (Aytar and Bakir, 2008). Type-3 copper protein has two copper active sites that are active in catalytic reaction. (Zekiri F. et al.,2013).

Figure 2.4. shows the binuclear copper active site of tyrosinase enyzme. Tyrosinase contains two copper ions and each copper ion coordinated by three histidine residues that has six nitrogen donor atoms (Kitejima et al., 1989).

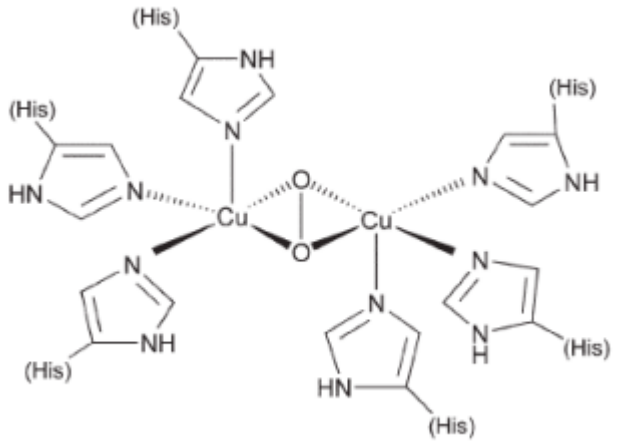


Figure 2.4. Schematic representation of binuclear copper site

The tyrosinase active site exists in three forms during catalytic reaction (Figure 2.5) : deoxystate, oxy state and met state. Deoxy state binds molecular oxygen immediately and result in form of oxy state. In oxy state molecular oxygen is bound to active site of enyzme and bring bridge between the copper atoms. Tyrosinase enyzme usually contains 85-90% met form that is resting state of copper site, where copper ions are bridged by a water molecule(Solomon et al.,1996). Figure 2.5 shows the basic reaction mechanism of tyrosinase on o-diphenols. First of all, the oxygens of *o*-diphenol are bound to copper of the met-tyrosinase to obtain Emet-D form. The oxidized form of D, is released as quinone which is the product. Then, enyzme become deoxy form. By using molecular oxygen the deoxy form is oxidized to oxy form in order to react again

o-diphenol(D) to produce Eoxy-D form. Then quinone was produced by oxidation of D and enyzme returns to its native met state.(Mohammadi et al.,2009)

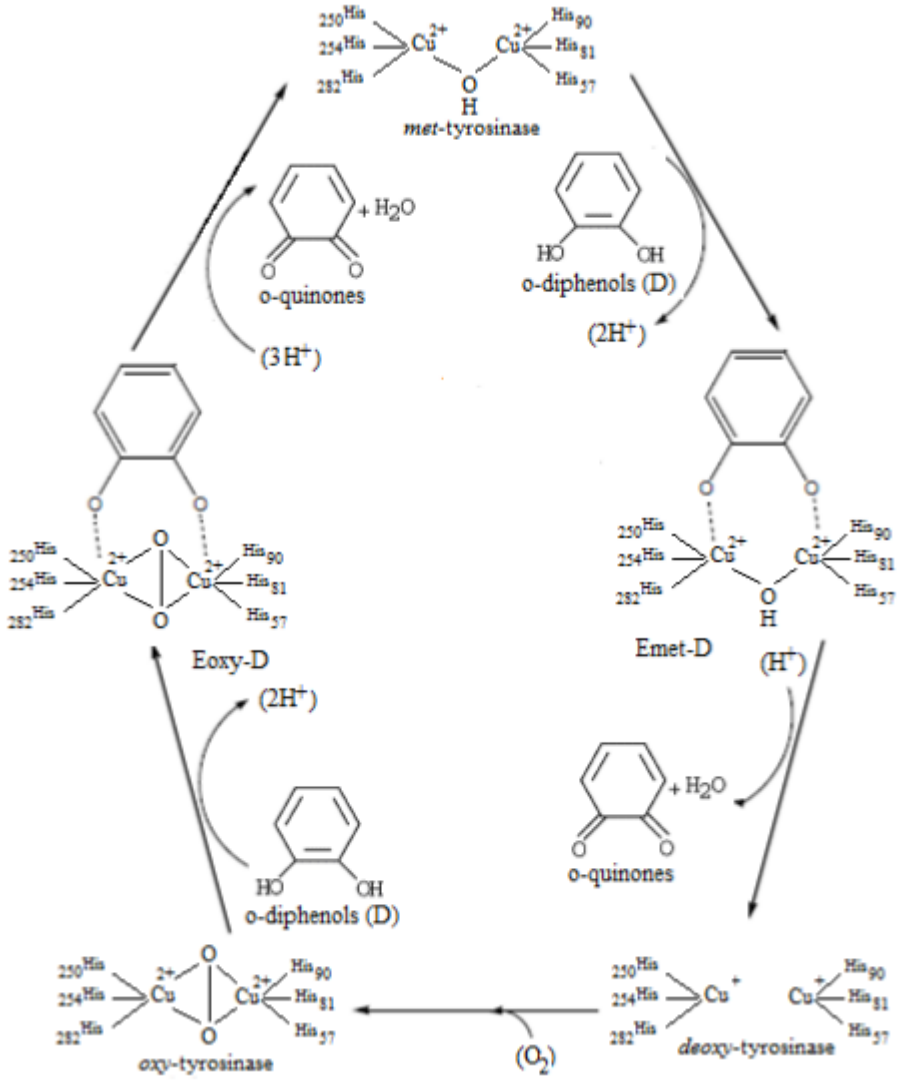


Figure 2.5.The reaction mechanism of tyrosinase to produce o-quinone.

Quinones are catalytically produced by using monophenols, diphenols and triphenols using tyrosinase enzyme (Munoz et all, 2008). However, tyrosinase enyzme shows much more affinity for diphenols. Tyrosinase enzyme has a wide application in much dephenolization technology. Tyrosinase was also used for treatment of phenolic compounds in waste waters, pesticides, paint and petrochemical industries. (Aytar and Bakir 2008, Dincer, Becerik et al. 2012). On the other hand, melanogenesis is process for production of dark macromolecular pigments by catalyzing tyrosinase (Te- Sheng , 2009).

Tyrosinase activity can be measured by many different methods (Munoz et al.,2008):

- Measuring the consumption of oxygen
- Measuring the disappearance of reducing agent such as ascorbic acid as a result of oxidation of o-quinone
- Directly measuring the production of o-quinone
- Using nucleophilic reagents that trap o-quinone and produce adducts

CHAPTER 3

IMMOBILIZATION

Immobilization is defined as leading the restricted mobility.(Wilhelm Tischer et al). Enyzme immobilization means that enzyme is attached in a support physically or chemically.

Enzyme can be used repeatedly; the product contamination with enzyme can be eliminated; resistance of enzyme to change in pH, temperature can be improved by immobilization. However, due to immobilization enzyme can be denatured and conformational changes can be occurred; the catalytic activity of enzyme is decreased resulting from steric hindrance and mass transfer limitations. Mass transfer limitations which is expressed as also diffusional restrictions has divided into two categories internal and external.(Drauz and Waldmann 2002)

One of the most important issue in immobilization is to increase the substrate accomodation with tyrosinase enyzme which depends on flexibility and structural rigidity. Therefore, the choise of method in immobilization is very important (Drauz and Waldmann 2002). The immobilization methods can be classified as chemical (covalent binding, covalent cross-linking) and physical (adsorption, entrapment, encapsulation) methods as shown in Figure 3.1.

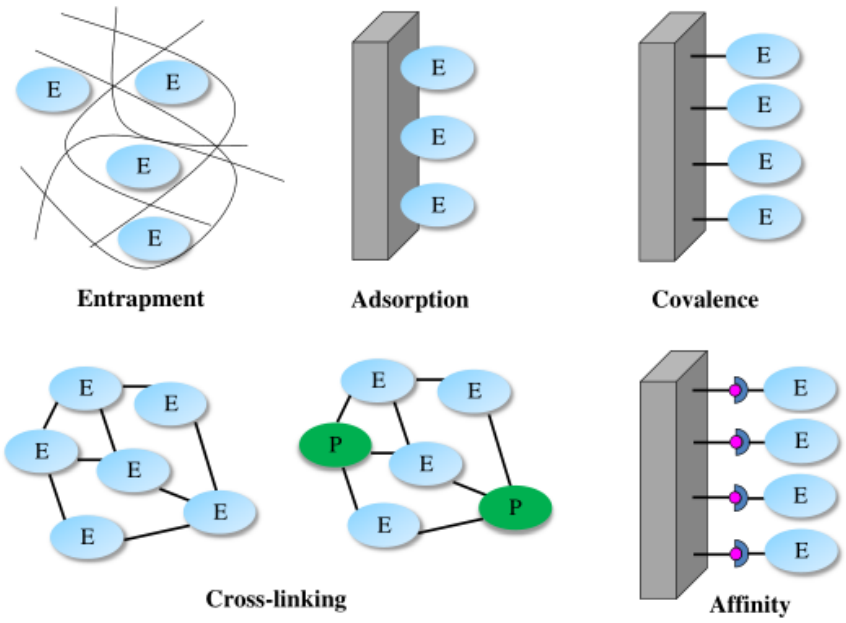


Figure 3.1. Classification of Immobilization Methods
(Source: Sassolas, Blum et al. 2012)

3.1.Chemical Methods

In this method the enzyme can be attached to support covalently. In some cases multifunctional reagents were used.

Covalent binding: The covalent binding occurs between functional group of support and enyzme like OH, COOH, NH_2 and SH. Multifunctional reagents such as glutaraldehyde and carboimide was used to activate the support for immobilization of enzyme following the removing of excess unbound molecules. In this technique the operational stability is high and enyzme release from support is minimal.

Cross-linking: Immobilization of enyzme by crosslinking is an another approach for immobilization of enyzme. Generally, glutaraldehyde and other bi-functional reagents such as glycoxal used as crosslinker. In this method, enyzme can be crosslinked with each other or inert protein. Cross-linking methos is attractive because of strong chemical binding between biomolecules and simplicity.

3.2.Physical Methods

Adsorption is the easiest pyhsical methods and non destructive for enzyme. The adsorption method is based on hydrophobic interaction, non covalent bonds like Van der Wals forces and ionic interactions. The main problem is that enyzme can easily get away from support surface which can be prevented by crosslinking (Illanes 2008; Drauz and Waldmann 2002). In *entrapment* method, enyzme is held by support such as silica gel, polyssaccaride, carbon paste and an electropolymerized film. There is no modification of biological element so that activity can be preserved. Using this method in biosensor applications the operational and storage stability of enyzme can be increased. However there will be some limitations of using entrapment method such as; leaching of components and some diffusion barriers (Audrey Sassolas et al.,2011). Enzymes can be *encapsulated* within the microscopic semi-permeable membranes or within the macroscopic hollow-fiber membranes. There is no covalent binding of enyzme to the medium. The main drawback is mass transfer limitations.

The advantage and drawbacks of immobilization methods are summarized in Table 3.1.

Table 3.1.Advantages and Drawbacks of Immobilization Methods
(Source: Sassolas, Blum et al. 2012)

	Advantages	Drawbacks
Adsorption	• Simple • No chemical modification of enzyme • inexpensive	• Weak Bonds • Leaching of enyzme • Desorption • Non- specific adsorption
Entrapment	• Simple • Activity is preserved • No chemical modification of enzyme • Operational and storage stability	• Diffusion barrier • Enyzme leakage
Cross-linking	• Simple • Strong chemical binding • No carrier required	• Chemical modification of enyzme
Covalent binding	• No mass transfer limitation • Stable • Short response time	• May limit mass transfer • Often expensive

3.3. Selection of Support Material

Support material should be have hydrophilicity, high affinity to proteins, available to strong stability, reactivity to functional groups and good film forming ability. In addition, insolubility in reaction media and flexilibility are other desired properties. Depending on the application area the material should be nontoxic, biocompatible, biodegredable and inexpensive (Pena et al.,2010; Dung et al.,2009). Although there are a lot of support material for immobilization of enzymes (Drauz and Waldmann 2002). Special care should be taken for choose of appropriate support material in immobilization. Chitosan and gelatin are the most widely used support materials in literature for tyrosinase enzyme immobilization.In Table 3.2 types of support materials are shown.

Table 3.2. Type of Support Material

Organic-synthetic polymer	**organic biopolymer**	**Inorganics**
Polyamides	**Polysaccharide**	**Minerals**
Nylon		
Polyalkelene	Cellulose	Sand
polyacrylates	Starch	Pumice
polyacrylamide	Agarose	clays
Polyethylene	Dextran	Diatomaceous earth
Polypropylene	Chitin	Metal oxides
Polyvinyl alcohol	Polyalginate	
Polyvinyl acetate	Carrageenan	
Polyvinyl chloride		
Polyethylene glycol	**Proteinaceous**	**Synthetic**
Polyester	Gelatin	Glass
Polycarbonate	Collagen	Zeolites
Polysiloxane	Silk	Silica
	Albumin	Sol-gel
	Bone	Alumina
		Metals

3.3.1.Gelatin as Support Materials

Gelatin is a protein that is derived from the chemical degredation of collagen. Gelatin is present in natural resources of animal origin and can be obtained inexpensively. It has high molecular weight from 65,000 to 300,000 g/mol. Gelatin contains mainly glycine, proline and 4-hydroxyproline (for pigskin gelatin 33%, 13% and 9%, respectively.(Shyni, Hema et al. 2014).

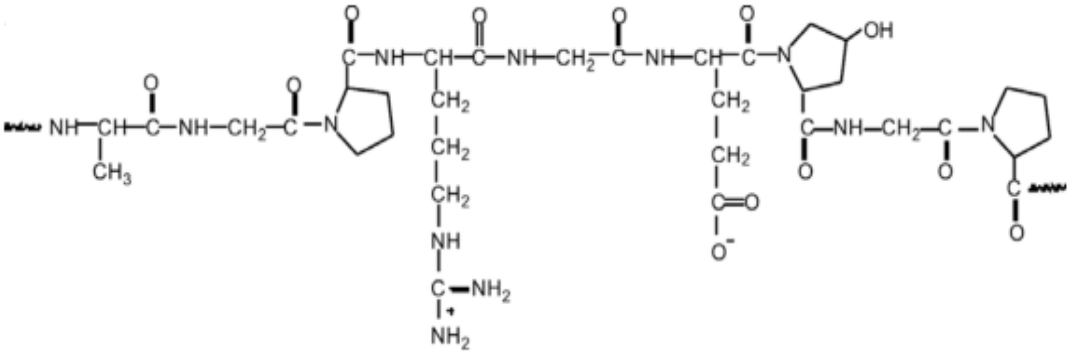


Figure 3.2. Chemical Structure of Gelatin

In the structure of gelatin there are hydrophilic and hydrophobic interactions. Gelatin is insoluble in organic solvents and soluble in hot water and acetic acid. The isoelectric point of gelatin ranges between 4.8 and 9.4. The gelatin is also odorless, brittle in dry and tasteless. Gelatin has wide application area such as; food, pharmacy, tissue engineering and photography industries.This material can be succsessfully used in forming films and further used for obtaining biosensors.(Pena et al. 2010 ; Shyni, Omri 2002; Martucci, Ruseckaite et al. 2006).

CHAPTER 4

EXPERIMENTAL

4.1Materials

In this study, tyrosinase from mushroom (T3824, Merck) with 25 KU, gelatin from porcine skin (Sigma), catechol (C9510, ≥99%, Merck), di-sodium hydrogen phosphate (Na_2HPO_4 98%, Merck), sodium-dihydrogen phosphate monohydrate ($NaH_2PO_4H_2O$, 98%, Merck) and glutaraldehyde (25%, Merck) were used. All aqueous solutions were prepared with ultra pure water (18.2 MΩ cm).

4.2.Methods

4.2.1.Preparations of the Solutions

Tyrosinase (activity: 25000U/7.99 mg) and catechol solutions were prepared by using phosphate buffer solution (50 mM), whereas gelatin stock solution (1%w/v) was prepared using pure water.

4.2.2.Preparation of Immobillized Enyzme Films

Gelatin films are prepared by coating the glass surface washed with ethanol then distilled water. Tyrosinase was immobilized to the film via adsorption or entrapment methods as represented below.

Immobilization via adsorption;

0.5 g of gelatin were taken and dissolved in 50 ml pure water at 45ºC in order to obtain the gelatin stock solution. From this gelatin solution 30 µl was taken and poured on the surface of glass (0.8 cm×1.5 cm) and left to dry at 25 ^{o}C for 2 hour. Then, 5µl

(25U) tyrosinase solution was poured on the dried gelatin glass rod. As a result of this, tyrosinase adsorbed film was prepared.

Immobilization via entrapment using crosslinker;

The gelatin solution (200 µl) and 50 µl tyrosinase (250U) were mixed with 10 µl glutaraldehyde (1,25%) in an eppendorf tube. 30 µl of the homogenous solution was obtained by using vortex (Wiseman VM-10) were poured onto the glass surface (0.8 cm×1.5 cm) and left to dry overnight at 4ºC so that the gelatin entrapped film using crosslinker was obtained.

Prior to the activity studies the tyrosinase immobilized gelatin films, were immersed into 3 ml PBS (phosphate buffer solution) to wash out the non-immobilized components from the surface of the film.

The effect of the pH, temperature and substrate concentration on catalytic activity of tyrosinase in the production of o-quinone from catechol (Figure 4.1) were investigated. For reaction, these films were immersed into the catechol solution in the quartz cell of UV-spectrophotometer.

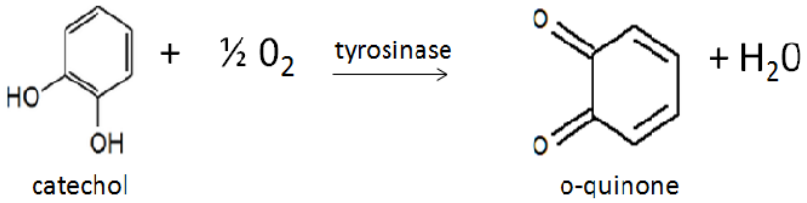


Figure 4.1: The production of o-quinone from catechol

The range of pH, temperature and substrate concentration were 5 and 7, 20°C and 40 °C and, 0.4 and 10µM, respectively.

4.2.3.Enzyme Assay

For enzyme assay measurement the UV-visible spectrophotometer (Perkin Elmer Lambda 45) was used. For determination of enzyme activity, the formation of o-quinone (wave length: 390 nm) was investigated. The experiments were performed at

30˚C during 1 min by stirring reaction media at 1000 rpm as in the study of Polatoglu (2012). Beer–Lambert equation was used in order to calculate the enzyme activity;

$$\frac{dp}{dt} = \frac{dA}{dt}\,\frac{1}{\varepsilon l}$$

where ε is extinction coefficient of o-quinone (ε_{390} = 1417 M^{-1} cm^{-1}) as stated by Aytar and Bakir (2008), *l* is path-length (1 cm) and dA/dt is the change in absorbance at 390 nm with 1min.

TEG and TAG are used to represent tyrosinase entrapped gelatin film and tyrosinase adsorbed gelatin films, respectively. The relative activity of enzyme (RA) was obtained from the comparison of activity with respect to maximum activity. For the determination of storage stability of free and immobilized enzyme, the relative activities were measured after a given time of storage and they were stored at 4 ^{o}C when not in use. For determination of reusability the activity of enzyme entrapped gelatin film was measured 5 times within new substrate (catechol) solution.

In characterization of the films, Scanning electron microscopy (SEM,Quanta 250), Fourier transform infrared spectroscopy (FTIR, Shimadzu 8400S) and Contact angle instruments were used. For FTIR analysis , KBr discs were prepared using the 2 mg sample and 28 mg KBr and recorded in the range of 4000–400 cm^{-1}. For contact angle measurement, a drop of sample was poured onto the glass surface and further the angle between surface and drop was determined.

CHAPTER 5

RESULTS AND DISCUSSION

In this part, the catalytic performance of tyrosinase in production of o-quinone from catechol was presented. Decrease in catalytic activity with immobilization, pH and temperature and substrate concentration was also presented.

5.1. Free Enzyme Studies

The effect of pH and tempereture were investigated in order to determine the optimum condition for free enyzme activity.

5.1.1. The Effect of pH and Temperature on Free Enzyme Activity

As shown in Figure 5.1, the maximum activity was observed at pH 6.0 for free enyzme. Towards acidic medium the activity decreases due to the protonation of the catalytic group that destroyes the strong binding of enzyme. On the other hand, toward basic medium the deactivation was observed due to the conformational change in the active site of enzyme as stated by Yoruk and Marshall(2003).

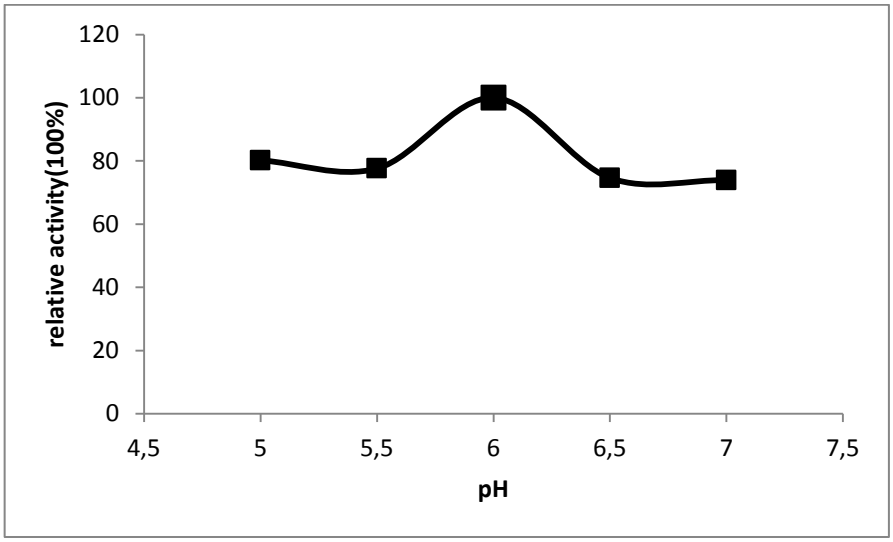


Figure 5.1. Change in free enzyme activity with pH at 30˚C (tyrosinase: 20U, catechol: 5mM)

As it can be seen from the Figure 5.2., the optimum temperature for free enyzme activity is 25˚C. The activity decreased at high temperature results from the denaturation of protein as stated by Suzuki et al (2010).

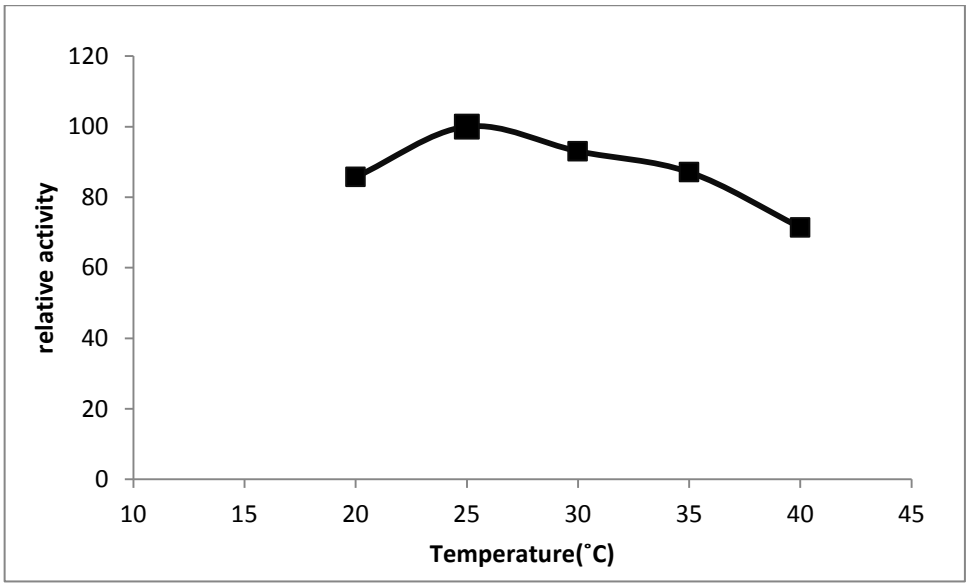


Figure 5.2. Change in free enyzme activity with temperature at pH 6.0.(tyrosinase amount: 20U, catechol: 5mM)

As a result of free enzyme studies, pH 6 and 25˚C was the optimum conditions which is compatible with literature (Akyilmaz et al. , 2010),where the optimum conditions are pH 7 and 30 ˚C.

5.1.2. The Effect of Enzyme Amount on Activity

The effect of enyzme amount on its catalytic activity was investigated at 25˚C and pH 6.0. As it can be seen from the Figure 5.3, the maximum catalytic activity was found out at 20 Unit enzyme amount due to the saturation of enyzme (Schnell S. et al. 2000) .

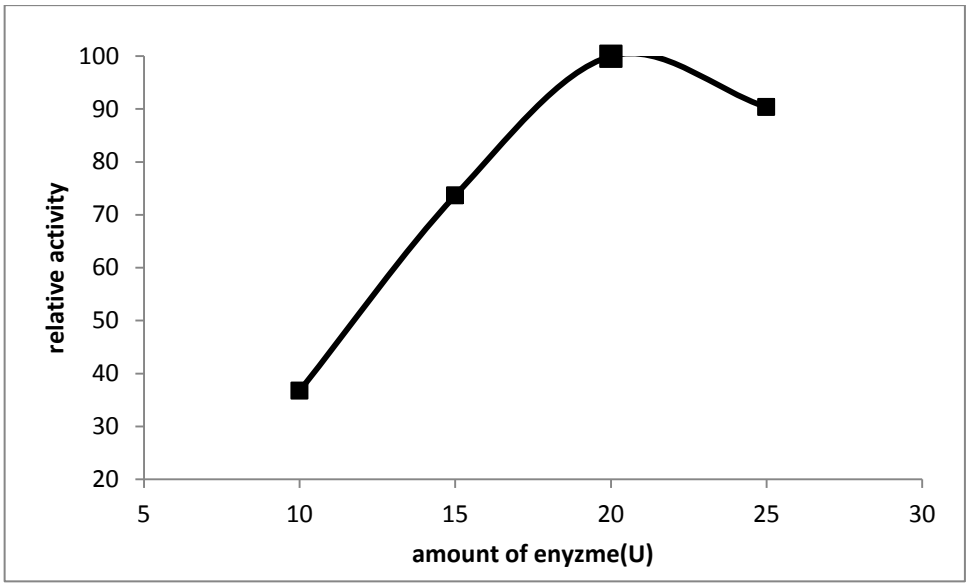


Figure 5.3.The effect of enzyme amount on catalytic activity (catechol:5mM)

5.1.3.The Effect of Catechol Concentration on Tyrosinase Enyzme

Even it is not clear the optimum concentration for maximum activity seems to be 5mM (Figure 5.4).

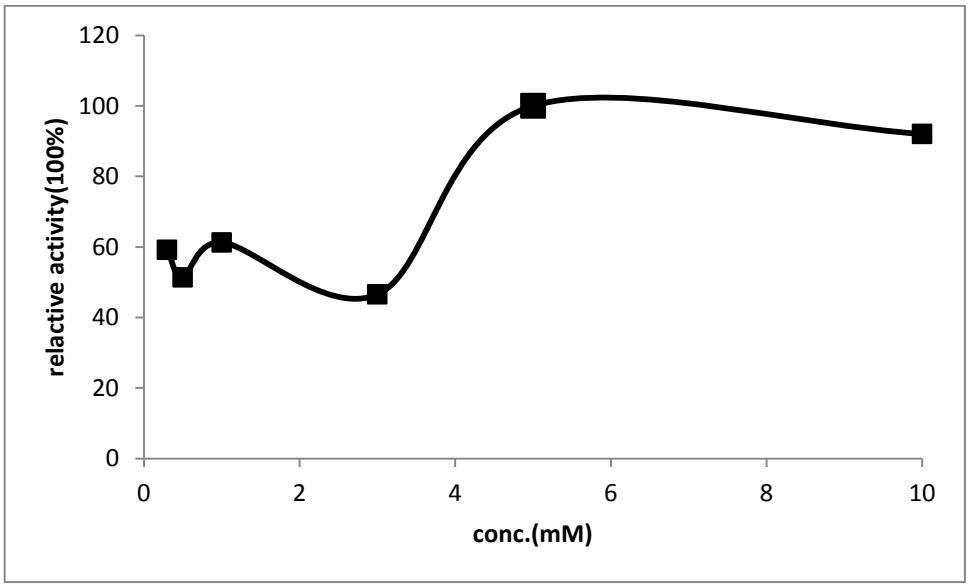


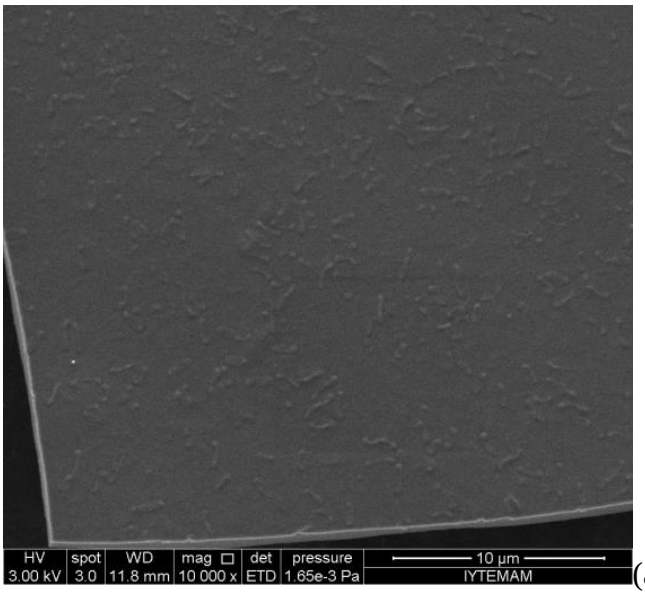
Figure 5.4. The effect of catechol concentration on free activity of tyrosinase enzyme (pH:6 T:25˚C U:20)

5.2. Immobilized Enzyme Studies: Tyrosinase Entrapped Gelatin Film

Tyrosinase enzyme was immobilized to gelatin support by using adsorption and entrapment methods. Then enzyme immobilized films were developed by taking into considerations some parameters such as; immobilized enzyme amount, reusability and operational stability.

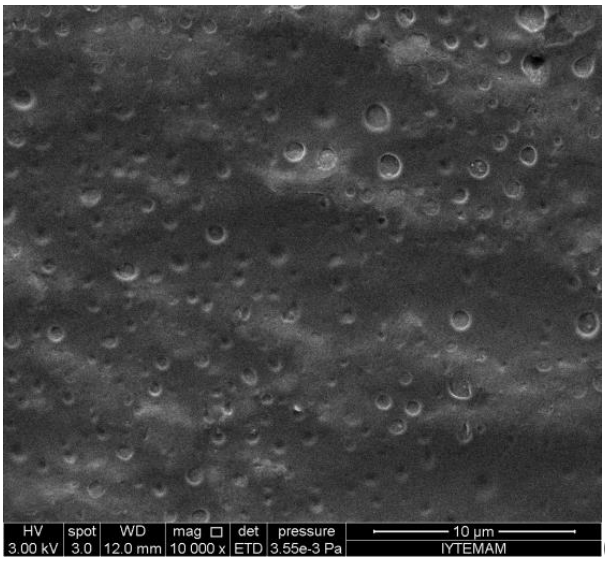
5.2.1. The Characterization of the Tyrosinase Entrapped Gelatin Films

In characterization of the films (SEM) scanning electron microscopy, FTIR and Contact angle measurement were used. The change in the morphology in preparation of tyrosinase immobilized gelatin film can be clearly seen from the images of SEM : the gelatin image displays uniform rod structure(Figure 5.5 a); glutaraldehyde create circles on the film (Figure 5.5 b) ; the enzyme entrapment enlarged the circles (Figure 5.5 c).

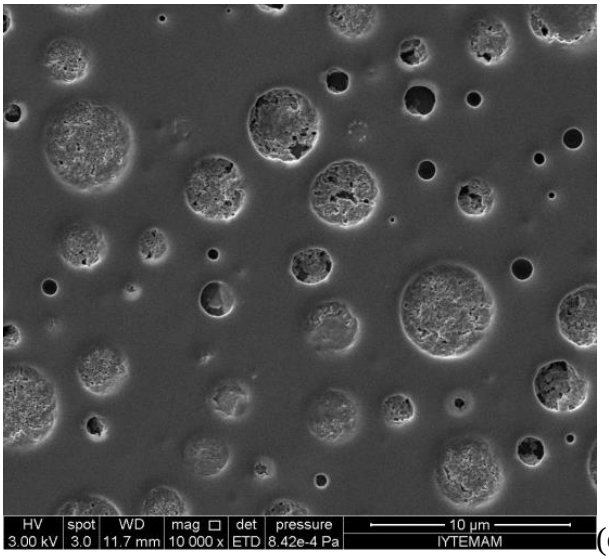


(a)

Figure 5.5. The images of gelatin(a), glutaraldehyde cross linked gelatin (b) and the enzyme entrapped gelatin(c) films.(**cont. on next page**)



(b)



(c)

Figure 5.5.(**Cont.**)

The spectrum of the films (Figure 5.6) were analyzed considering O-H and N-H, C-H stretching , Amide I (C=O and N-H bending) and symmetric NH_3^+ bending bands (Table 5.1)

Table 5.1: FTIR spectrum and assignments
(Source: Wang et al.2008, Kim et al.2006 and Kumar et al.1999)

Vibration (cm^{-1})	Assignment
3290-3450	O-H and N-H stretch
3100-3000	C-H stretch (aromatic)
1700-1600	Amide I (C=O)
1620-1500	Amide I (N-H bending)
1409	Symmetric NH_3^+ bending

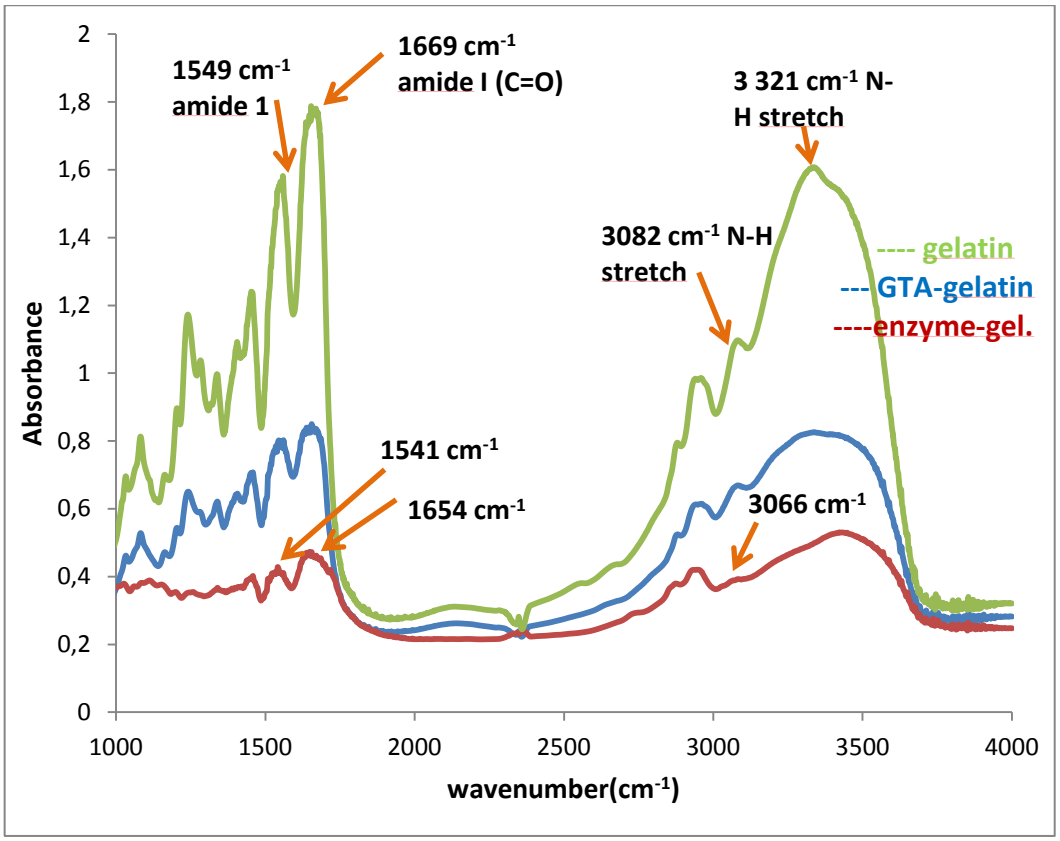


Figure 5.6. The FTIR Spectra of gelatin(----), glutaraldehyde cross linked gelatin (----) and the enzyme entrapped gelatin(----) films.

As seen as from the Figure 5.6, the vibrations of amide I (C=O), amide I (N-H bending and C-N stretching), symmetric NH_3 bending at 1669, 1549, 1409, respectively, weakened with enzyme entrapment with cross linker, glutaraldehyde. The N-H bending vibration observed in gelatin film shifted from 1549 cm^{-1} to 1545 and 1541 cm^{-1} for gelatin crosslinked with glutaraldehyde and enzyme entrapped gelatin, respectively.(Figure 5.7) This might be due to the amide groups may be involved in crosslinking reaction (Farris et al., 2010).

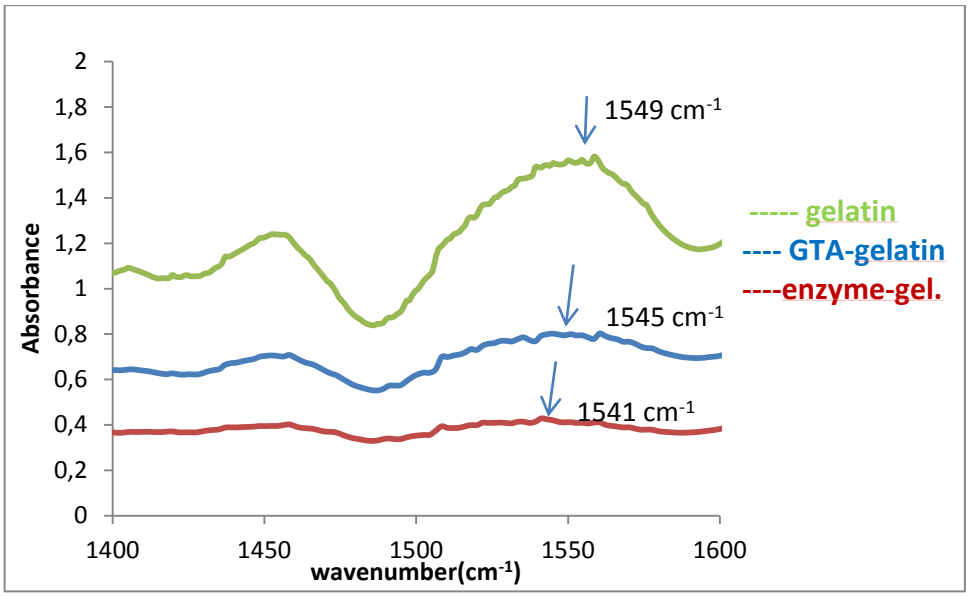


Figure 5.7.The N-H bending vibrations in gelatin(----), glutaraldehyde cross linked gelatin (----) and the enzyme entrapped gelatin(----) films.

The contact angle instrument was also used for investigate the wettability of gelatin solutions on glass surface.

Table 5.2. The contact angle of the solutions used in the study

Surface (solutions-thin plate)	Angle
water	20˚
Gelatin solution	22˚
Gelatin with GTA solution	28˚
Enzyme entrapped gelatin solution	26˚

According to the results that given in Table 5.2, it can be said that gelatin solutions have good wettability on the glass surface because contact angles obtained for gelatin solutions are similar to the contact angle of water.

5.2.2. Activity Measurement of the Tyrosinase Entrapped Gelatin Film

The Effect of Glutraldehyde (GTA) concentration

Since gelatin is soluble in aqueous solution, glutaraldehyde is used as crosslinker to prepare tyrosinase entrapped gelatin film. Figure 5.8 shows the possible reaction occurred between aldehyde and enzyme reactive group by crosslinking to improve thermal and mechanical properties. As it can be seen from the Figure 5.9, as glutaraldehyde amount increases, the activity of immobilized tyrosinase enzyme decreases because of glutaraldehyde is also toxic compound (Bigi et al., 2001). As a result of this, the amount of glutaraldehyde was chosen as 1.15 μL in solution for preparation of tyrosinase entrapped gelatin film (TEG).

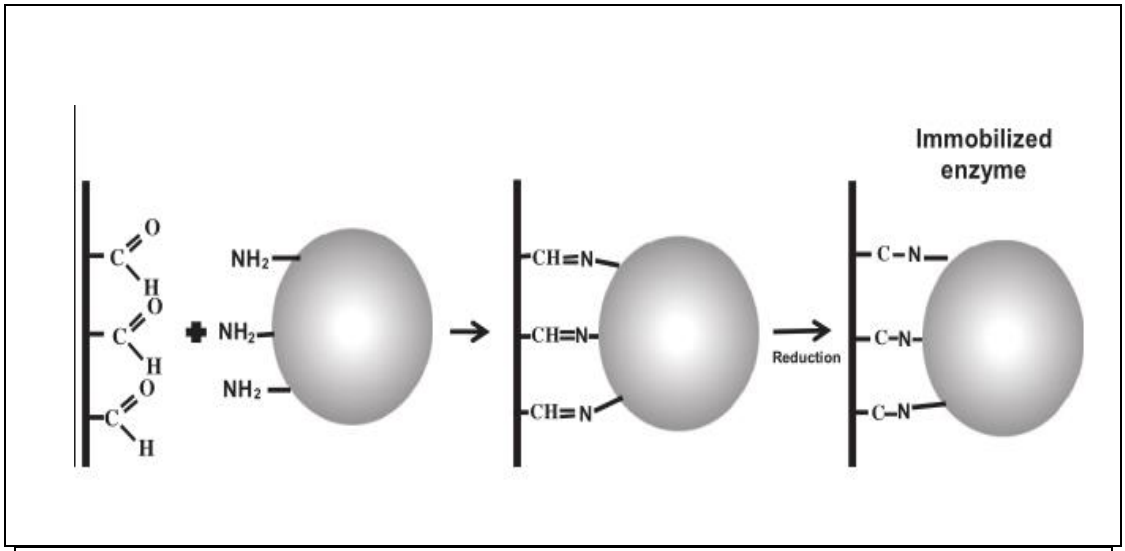


Figure 5.8. Schematic representation of immobilization of enzyme on gelatin support by covalent attachment.

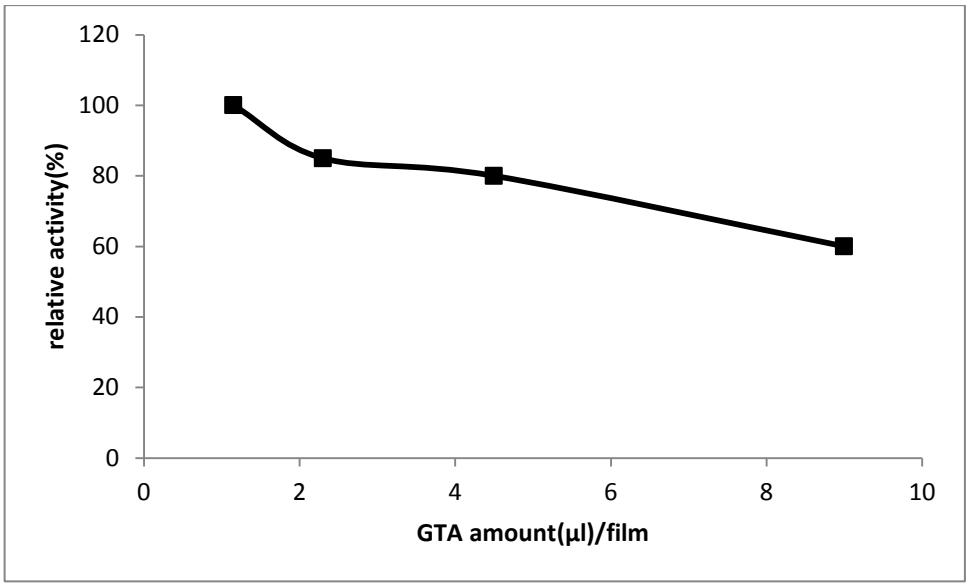


Figure 5.9.The effect of glutaraldehyde concentration on activity of TEG.

The effect of Enzyme Amount

As it can be seen from the Figure 5.10, the activity of gelatin entrapped film was increased as long as the enzyme amount increased and reached maximum value at 30 U enzyme (9.6µg) for each film. At this point, the maximum activity was found out as 0.02265 µmol/min. Further addition of enzyme to the film did not affect activity of film as observed by Hervas Perez, Sanchez-Paniagua Lopez et al.(2006).

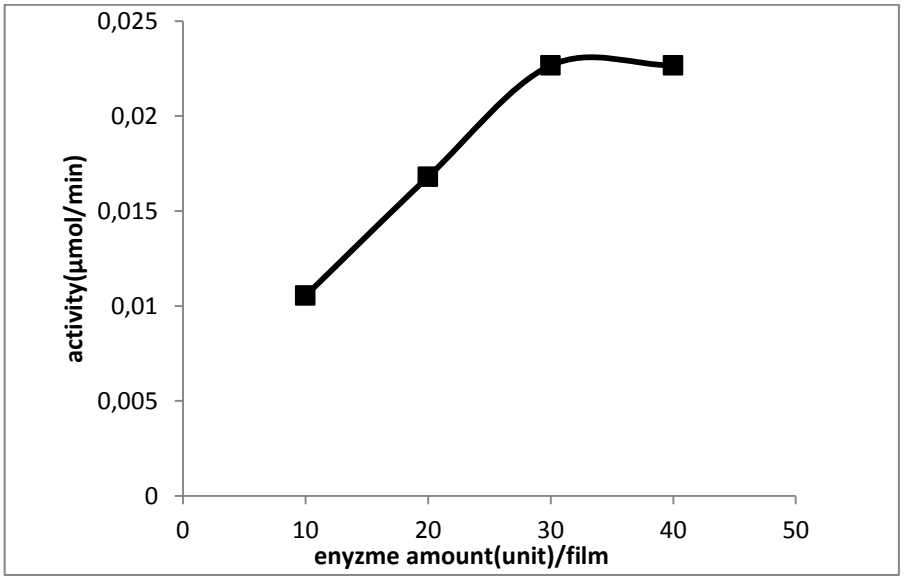


Figure 5.10. The effect of enzyme amount on the activity of the tyrosinase entrapped gelatin film.(pH:6 T:30 enzyme:20U)

The Effect of Catechol Concentration

The effect of catechol (substrate) on the tyrosinase activity was studied within the range of 0.4 to 10 mM. The activity of tyrosinase entrapped gelatin film was reached to maximum at 5mM substrate concentration similar to the free enzyme results. As concentration increases the activity is expected to increase.(Darnell et al, 1986). However after 5 mM concentration, the activity was decreased unexpectedly (Figure 5.11.)

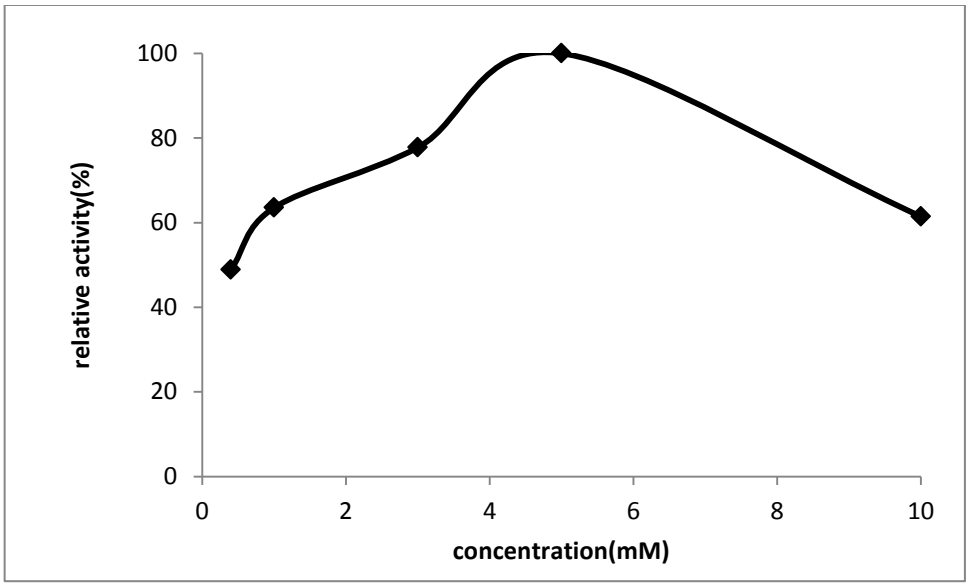


Figure 5.11.The effect of catechol concentration on activity of tyrosinase entrapped gelatin film.(pH:6 T:30 enzyme:20U)

The Effect of pH and Temperature

Comparison of the pH on free and gelatin immobilized enzyme shows that there is no change in maximum relative activity as observed by Aytar and Bakir (2008). The main reason of the decrease in activity will be the new interaction between enzyme and support material due to change in acidic and basic amino acid side chain.(Chaplin M., Bucke C.,1990)

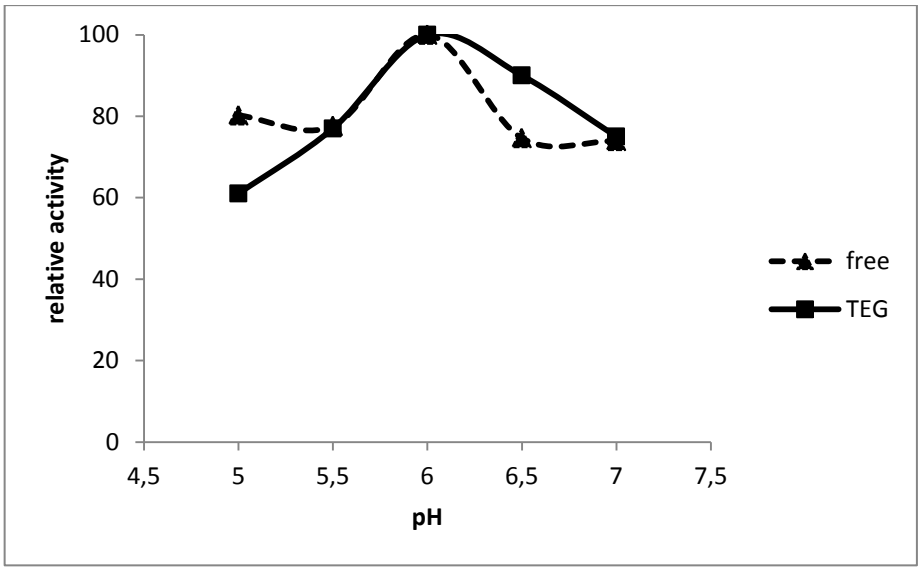


Figure 5.12. The effect of pH on the activity of free and tyrosinase entrapped gelatin film (T:30C enzyme:20U catechol: 5mM)

The impact of temperature on the immobilized enzyme was shown in Figure 5.12.

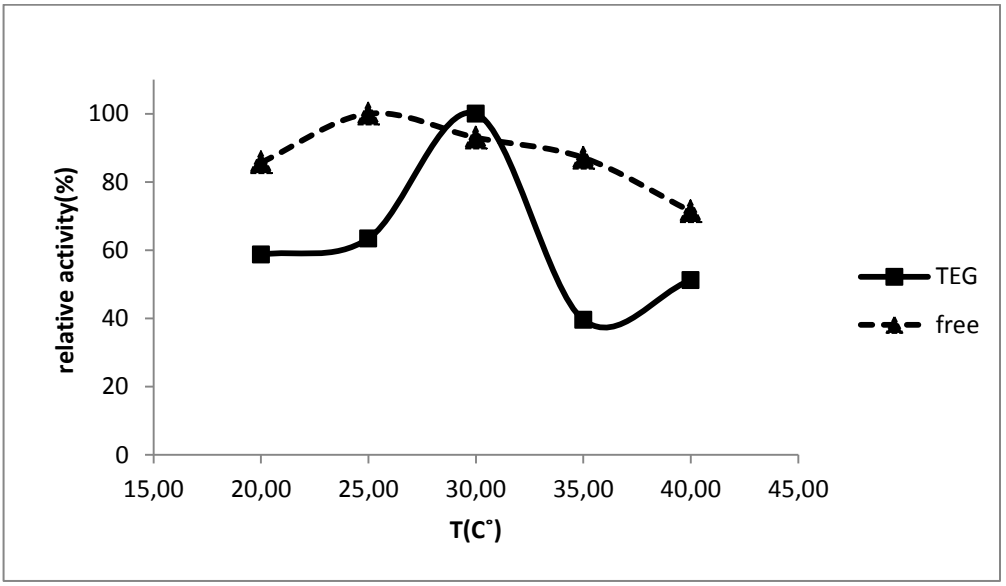


Figure 5.13. The effect of temperature on activity of free enzyme and TEG (pH 6.0, enzyme:20U catechol: 5mM).

As seen from Figure 5.13, there was a slight shift on optimum temperature from 25˚C to 30˚C on maximum when enzyme was immobilized. Decrease in activity at increased temperature value can be attributed to the denaturation of protein as in free enzyme.

5.2.3. Activity of Tyrosinase Adsorbed Film

The Effect of pH and Temperature

The influence of temperature and pH on activity of tyrosinase are compared in Figure 5. 14 and 5.15 , respectively. 30˚C is the optimum temperature for all immobilized films. However the optimum pH is 5.5 for tyrosinase adsorbed film but is 6 for tyrosinase entrapped film. In this thesis the entrapment of tyrosinase by using glutaraldehyde was chosen as the best method based on the loss in activity (Table 5.3).

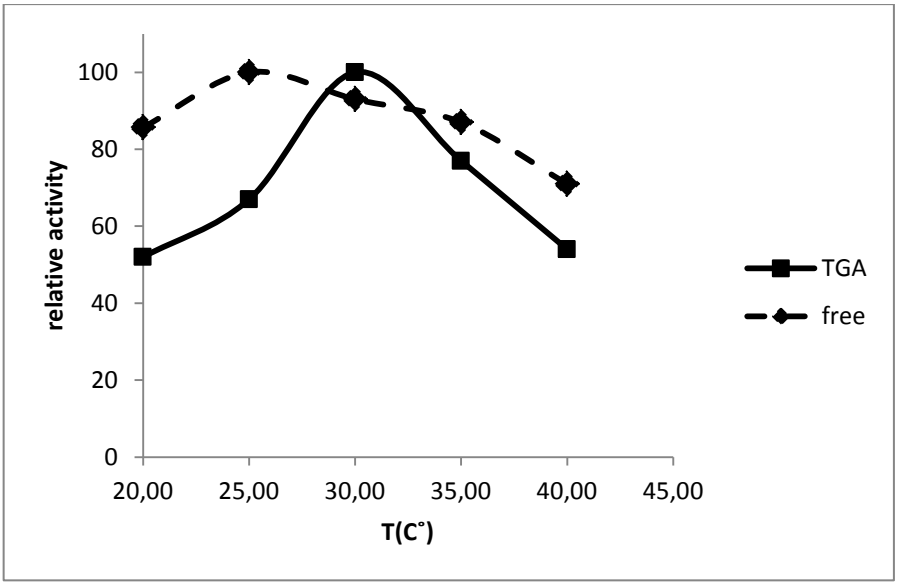


Figure 5.14. The effect of temperature on activity of tyrosinase adsorbed film (pH:6.0, enzyme:20U catechol: 5mM)

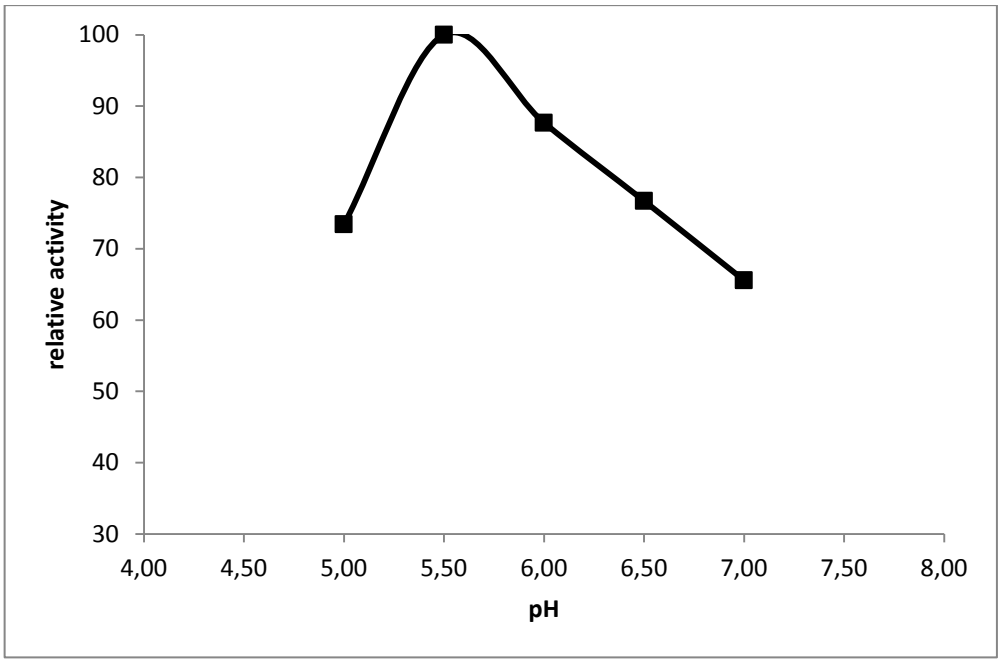


Figure 5.15.The pH effect on activity of tyrosinase adsorped gelatin film (pH:6.0, catechol: 5mM).

Table 5.3.The activities of the immobilized films and free enzyme(pH:6 [S]:5mM T:30˚C)

Film	Activity loss(100%)	Activity*	R^{2**}
Entrapment	56	0,066	0,989
Adsorption	70	0,045	0,978
Free enyzme	100	0,152	0,971

*Calculated from the slope of production rate of o-quinone within 1 minute
**correlation coefficient: determined from the slope of absorbance graph obtained from UV-spectrophotometer.

5.3. The Kinetic Studies

Michaelis-Menten model equation was used to analyze the production of o-quinone (P) . Kinetic parameters such as Michaelis-Menten constant (Km) and maximum velocity of production rate (dP/dt) with free and gelatin entrapped enyzme were evaluated by fitting the experimental data to the model. Michaelis–Menten constant (Km) defines the affinity of enzyme toward its substrate (S) .

Tyrosinase (E) catalyzed catechol (S) oxidation reaction can be expressed by Michaelis Menten model;

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$

And production rate is expressed by the equation;

$$\frac{dP}{dt} = \frac{Km+[S]}{[S]Vmax}$$

From the linearized form of the equation; Michaelis-Menten model

$$\frac{dP}{dt} = \frac{Km}{Vmax\ [S]} + \frac{1}{Vmax}$$

The Lineweaver-Burks plot for immobilized and free enzyme (Fig 5.16 and 5.17 respectively) and Vmax and Km were obtained (Table 5.3).

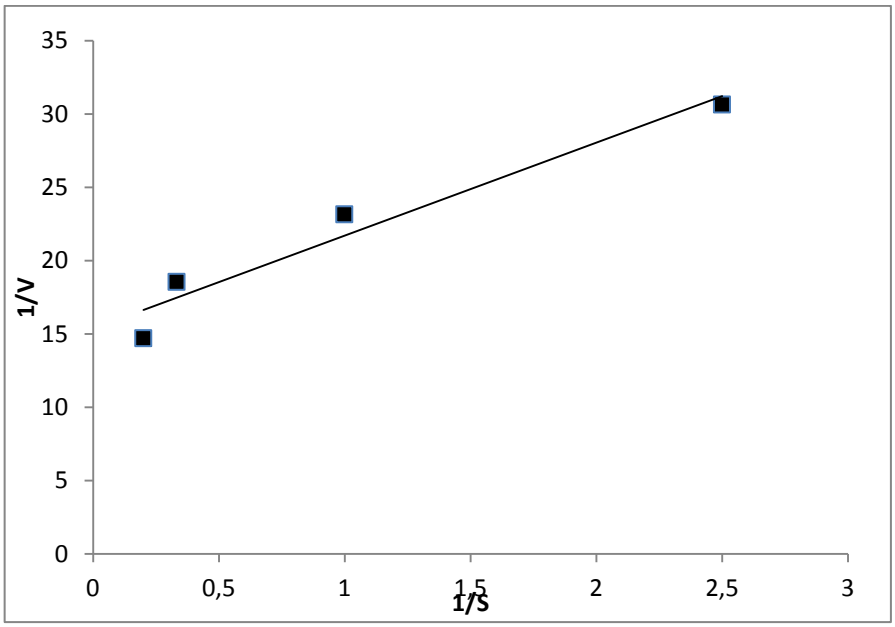


Figure 5.16.Lineweaver-Burks of TEG film.(pH:6 [E]:20U T:30˚C)

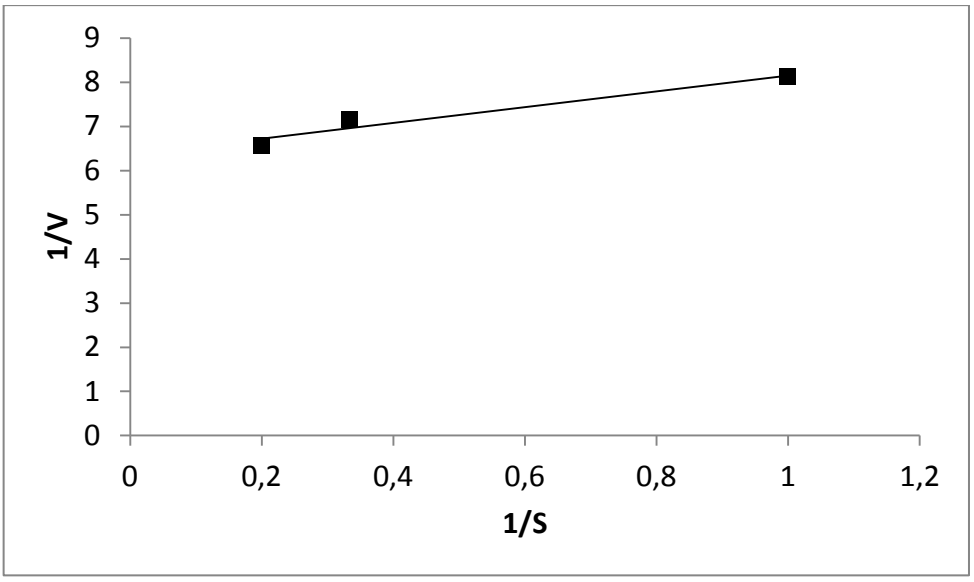


Figure 5.17. Lineweaver-Burk plot of free enzyme (pH:6 [E]:20U T:30˚C)

According to the results that shown in Table 5.4, the Km value of free tyrosinase enyzme is lower than the enyzme immobilized film. Therefore, it can be said that the affinity to substrate of free enyzme is higher than the enyzme immobilized film as observed by (Yildiz, Kiralp et al. 2005).Moreover, as expected, Vmax of free enzyme is higher than the immobilized enzyme.

Table 5.4. Michaelis-Menten model parameters for free and immobilized enzyme

Enyzme	Km	Vmax(µmol/min)	R^2
free	0.27	0.152	0.946
TEG	0.43	0.068	0.948

5.4.The Reusability of Tyrosinase Entrapped Gelatin Films

As it can be seen from the Figure 5.18, the relative activity was diminished to 40% of initial activity after 5^{th} use of the film.

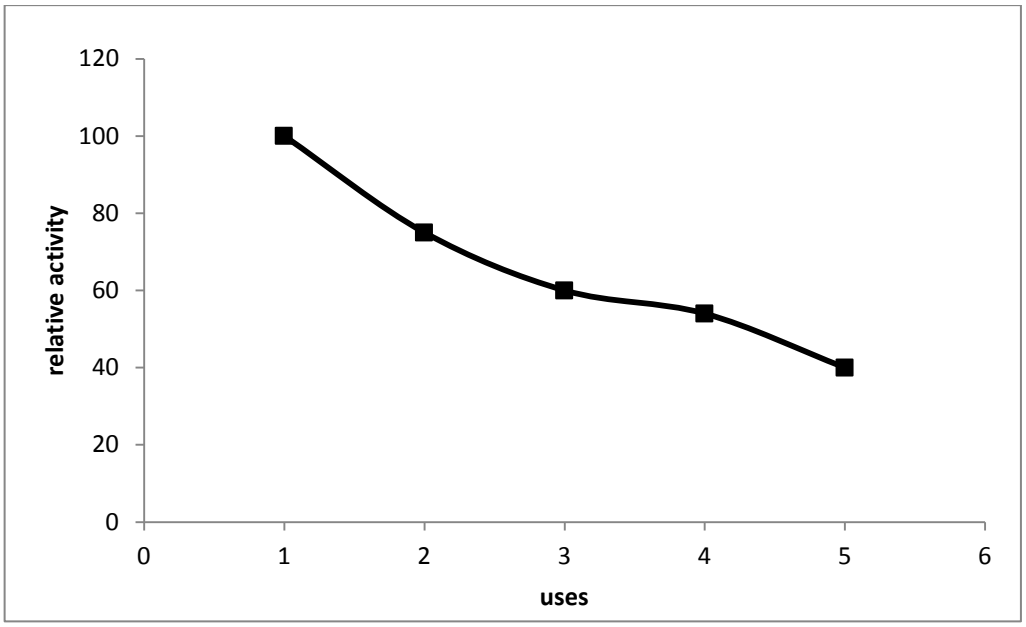


Figure 5.18.The reusability effect on activity of immobilized film.(pH:6 T:30C [S]:5mM)

5.5. The Storage Stability of Tyrosinase Entrapped Films

The storage stability of gelatin entrapped film was investigated during 30 days. As seen from the Figure 5.19, after 15 days the activity started to decrease obviously. At the end of the 30 day, the activity of enzyme was decreased to 58% and 38 % relative activity for immobilized and the free enzyme, respectively.

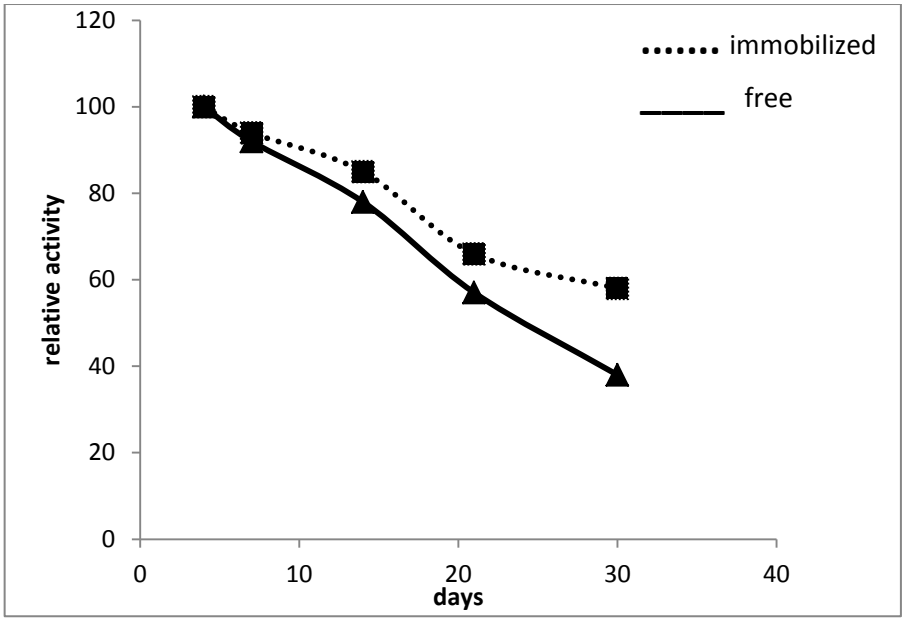


Figure 5.19. The storage stability on activity of free and immobilized enzyme

CHAPTER 6

CONCLUSION

In this study, tyrosinase enzyme entrapped gelatin films were prepared using glutaraldehyde as crosslinking agent. The optimum pH and temperature of the free and immobilized enzyme was found out as pH 6.0 and as 25^{o}C and 30^{o}C degrees, respectively.

The enzyme and glutaraldehyde amount were determined as 20U and 1.15 μl for preparation of the gelatin film using entrapment immobilization method. The concentration of substrate was determined as 5mM to measure the catalytic activity of immobilized enzyme. FTIR and SEM results show that the tyrosinase enzyme is entrapped successfully into the gelatin matrix.

Kinetic analysis of enzymatic reaction shows that the affinity to substrate for free enyzme is higher than the immobilized enzyme.

The immobilized tyrosinase retained about 40 % of its original activity after 5th uses. In addition, the immobilized tyrosinase exhibit higher stability than that of free enzyme after 30 days .

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APPENDIX A

FORMATION OF ORTHO-QUINONE

The formation of o-quinone was observed using UV spectrophotometer (Lambda 45) between the reaction of tyrosinase enyzme and catechol (5mM) as substrate. As it can be seen from the figure A.1, as time passed, the absorbances of substrate-catechol (275nm) was decreased and the absorbances of ortho-quinone was incereased. Ortho-quinone formation was observed at 390 nm.

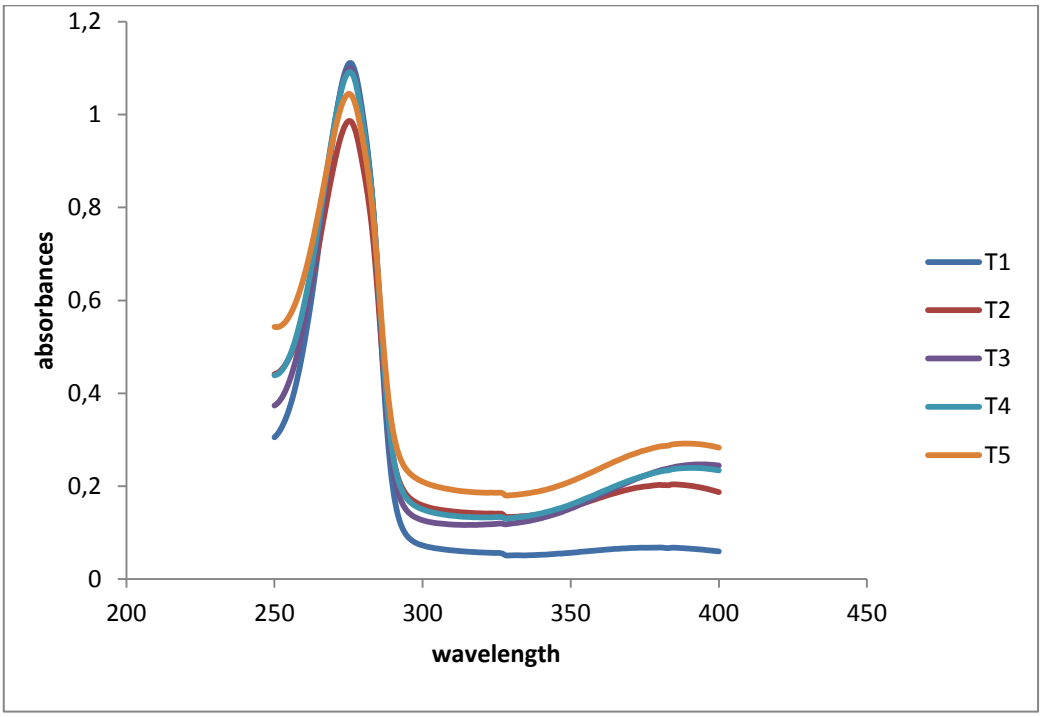

Figure A.1. The formation of ortho quinone with time