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Investigation Biocatalysts of Immobilized Enzyme on New Supports with Ferri and Ferro Nuclei

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Abstract: Ferri and ferro coordination polymers in sphere structure were synthesized. Scanning Electron Microscopy (SEM) Energy Dispersive X-Ray Spectroscopy (EDX), Gel Permeation Chromatography (GPC), elemental analysis, and Fourier Transform Infrared Spectroscopy (FT-IR) were performed for chemical and structural characterization of the coordination polymers. Glucose oxidase (GOD) enzyme immobilized to compare of kinetic parameters deal with glucano-1,5 lacton formation. Analyses results illustrate that structures coordination of ions Fe^{2+} and Fe^{3+} are different to the same support. It was seen that 2 mol of Fe^{2+} ion ((PS-N-($[Fe(CN)_4L]K_3)_2$) was bound per unit structure while 1 mol of Fe^{3+} ion (PS-N-($[Fe(CN)_2L]K$)) is attaching. *Km* values of were found as 15.32 and 10.93 for (PS-N-Fe^{2+})-GOD and (PS-N-Fe^{3+})-GOD, respectively. *Km* value for (PS-N-Fe^{3+})-GOD was found to be 0.5 times higher, possible reason of such a case is the larger reduction potential of Fe^{3+}. As the charge on the coordination structure increased, the enzyme's affinity for the substrate increased. After 20 repeated measurements, GOD immobilized on (PS-N-Fe^{3+}) polymer retained 45.47% activity, while GOD immobilized on (PS-N-Fe^{2+}) polymer retained 57.86% activity.

Ferri ve Ferro çekirdekli Yeni Destekler Üzerinde İmmobilize edilen Enzimin Biyokatalizör Özelliğinin Araştırılması

Anahtar Kelimeler
Ferrisiyanür,
Ferrosiyanür,
Enzim immobilizasyonu,
Koordinasyon polimerleri,
Glukoz oksidaz,
SEM-EDS

Öz: Küre yapısındaki ferri ve ferro koordinasyon polimerleri sentezlendi. Koordinasyon polimerlerinin kimyasal ve yapısal karakterizasyonunda Taramalı Elektron Mikroskopisi (SEM), Enerji Dağılımlı X-Işını Spektroskopisi (EDX), Jel Geçirgenlik Kromatografisi (GPC), elemental analiz ve Fourier Dönüşümlü Kızılötesi Spektroskopisi (FT-IR) kullanılmıştır. Glukano-1,5 lakton oluşumu ile ilgili kinetik parametrelerin karşılaştırılmak için glukoz oksidaz (GOD) enzimi koordinasyon polimerlerine immobilize edilmiştir. Analiz sonuçları, Fe^{2+} ve Fe^{3+} iyonlarının aynı desteğe farklı yapılarda koordinasyon gösterdiğini ortaya koymuştur. Birim yapı başına 2 mol Fe^{2+} iyonu ((PS-N-($[Fe(CN)_4L]K_3)_2$) bağlanırken, 1 mol Fe^{3+} iyonunun (PS-N-($[Fe(CN)2L]K$)) bağlandığı görülmüştür. K_m değerleri (PS-N-Fe^{2+})-GOD ve (PS-N-Fe^{3+})-GOD için sırasıyla 15,32 ve 10,93 olarak bulunmuştur. (PS-N-Fe^{3+})-GOD için Km değeri 0,5 kat daha yüksek bulunmuştur, böyle bir durumun olası nedeni Fe^{3+}'ün daha büyük indirgenme potansiyelidir. Tekrarlanan 20 ölçümden sonra, (PS-N-Fe^{3+}) polimeri üzerine immobilize edilen GOD'un %45,47 aktivitesi korunurken, (PS-N-Fe^{2+}) polimeri üzerine immobilize edilen GOD'un %57,86 aktivitesi korunmuştur.

1. Introduction

Iron is an essential element for various metabolisms. The electron exchange capability of iron help oxygen transfer, besides is used in DNA and RNA synthesis also. Iron is found in the structure of many different enzymes which are used in the development and continuation of neurological functions [1,2]. Transition metals copper, manganese and iron have important positions in the functioning of enzymes by taking part in the redox-active part of the enzyme. Iron those cations has several vital functions in the body. It

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serves as a carrier of oxygen (O_2) to the tissues and transport of electrons within cells in various tissues [3]. Therefore, it appears that ferro / ferri complexes are being investigated as DNA binding agents [4]. There are many studies on the antigen and antibody properties of iron ions, as well as extensive research on enzyme immobilization [5-7].

Iron ions is also used in different industrial implications [8, 9]. For example, potassium ferricyanide is used in wine industry and in the production of citric acid [10], while potassium ferrocyanide is commonly used in photography (Cyanotype process). It is seen that potassium ferrocyanide is used as a buffer in some enzymatic reactions (Beta-galactosidase) [11].

Thanks to the electrochemical properties of various structures containing iron ions, they are used in sensor production [12], improving mechanical properties and in rocket propeller as fuel [13, 14]. It is used in materials science with its high thermal stability and reversible redox behavior.

Enzymes are special biomolecules in protein form which catalyse the chemical reactions (increase the reaction velocity) [15]. Enzyme catalysed reactions are up to 10^3-10^8 times faster than regular reactions. Moreover, enzymes are 10^6-10^{16} times more effective than chemical catalysers [16]. It is important that enzymes, which have a very high economic cost, can be recovered from the reaction medium and reused. Immobilization of enzymes enable users to reuse the enzyme. Moreover, immobilization of the enzyme can increase the enzyme stability, control the enzymatic reaction, and help enzyme to remain pure (uncontaminated) [17, 18].

Generally, enzymes are less stable and expensive compared to conventional chemical catalysts. Losing the performance of such a costly product is something unwanted. Immobilization of enzymes on an insoluble solid enable users to overcome these obstacles. Up to know, different polymers and inorganic materials, which called solid supports, were reported in the literature which were used in the immobilization of enzymes [19, 20].

Glucose oxidase (GOD) (β-D-glucose:oxygen l-oxidoreductase, *E.C. 1.1.3.4*) is an enzyme which catalyse the reaction where glucose molecules are oxidised to glucono-δ-lactone (glucono-1,5 lactone) and produce hydrogen peroxide (H_2O_2). Lactones oxidizes to gluconic acid with via hydrolysis where no enzyme was needed [21-23]. GOD is used to extract remanent glucose and oxygen for extending the shelf life of food and beverages [24]. GOD was used in sensor applications to detect the glucose concentration in blood which may result in hypoglycaemia or hyperglycaemia [25]. Moreover, glucose oxidase was used as antimicrobial agent in mouth sanitation liquids and pastes [26]. To control the glucose concentration in subcutaneous tissues and determine the glucose level in the blood, the production of sensitive, selective, reliable and low-cost glucose sensors is increasing.

Enzyme support materials were expected to have mechanic stability with pH resistant characteristics and support materials should not be soluble in water. To prepare support materials in such characteristics, potassium ferricyanide, potassium ferrocyanide, amino polystyrene and salicylaldehyde are used where two new coordination polymers are prepared in our study. The reason of preparing coordination polymers using potassium ferricyanide and potassium ferrocyanide is the idea of producing stable support structure using -CN groups. The immobilization studies took place after a detailed characterization study. Furthermore, very few studies in the literature compare the results of immobilized enzymes on potassium ferricyanide and potassium ferrocyanide coordinated supports. Our study is presented to the attention of the health industry.

2. Material and Method

2.1. Preparation of ferricyanide/ferrocyanide attached Schiff base containing polymers (General method)

Ferricyanide/Ferrocyanide containing polymers were synthesized at 50°C using condenser in N_2(g) atmosphere. 1g N-{2-[Bis(2-aminoethyl)amino]ethyl} aminomethyl-polystyrene was dissolved in DMF and added to the condenser connected flask. While boiling and mixing under reflux, the dripper tap was opened, the salicylaldehyde (7 mmol) solution dissolved in DMF was dropped on the polymer solution in the flask for a period of about 5 min. After two hours, the mixture taken into a clean beaker was precipitated with acetone. Unreacted reagents were removed by washing with acetone, and the product was dried in an oven.

2.2. Preparation of ferricyanide containing polymer support (PS-N-Fe^{3+})

1 g N-{2-[Bis(2-aminoethyl)amino]ethyl}amino methyl-polystyrene was put into a flask and dissolved with 10 mL DMF. 6 mmol (0.1070 g) potassium ferricyanide solution dissolved in 10 mL of DMF (23 °C) was placed in the dropping funnel, and it was added to the reaction medium according to the general method in section 2.1. The synthesized (PS-N-Fe^{3+}) polymer was dried in a drying oven.

2.3. Preparation of ferrocyanide containing polymer support (PS-N-Fe^{2+})

1 g N-{2-[Bis(2-aminoethyl)amino]ethyl}amino methyl-polystyrene was put into a flask and dissolved with 10 mL DMF. 6 mmol (0.1070 g) potassium

ferrocyanide solution dissolved in 10 mL of DMF (23 ^{o}C) was placed in the dropping funnel, and it was added to the reaction medium according to the general method in section general method. The synthesized (PS-N-Fe^{2+}) polymer was dried in a drying oven.

2.4. Assay for enzyme activity

To prepare the stock GOD enzyme solution; 20 mg of the enzyme was dissolved in DMF: deionized water solution (100 mL, 9:6). This solution was stored in the refrigerator at +4°C. To prepare the stock glucose solution, 0.45 g glucose was dissolved in 50 mL of optimum pH (pH 6 for PS-N-Fe^{2+}-GOD and pH 7 for PS-N-Fe^{3+}-GOD). Trinder's colorimetric method was used for enzyme activity measurement [20]. By this method, the GOD enzyme reacts with the substrate glucose. Glucose is oxidized to gluconic acid and hydrogen peroxide in the presence of glucose oxidase.

$$\text{4-AAP} + H_2O_2 + \text{Phenol} \xrightarrow[\text{Pink } (504\ nm)]{\text{peroxidase enzyme}} \text{Quinonemine} + H_2O \quad (1)$$

H_2O_2 reacts with 4-amino antipyrine (4-AAP) and phenol to form a pink-colored quinone imine dye. The maximum absorbance of quinone imine is observed at 504 nm.

2.5. Immobilization of GOD enzyme on synthesized support polymer

To immobilize GOD on (PS-N-Fe^{2+}) and (PS-N-Fe^{3+}) polymers, 0.5 g working polymers were dissolved in 5 mL DMF. Polymer solutions were heated at 40°C for 30 min under reflux. After 30 min, the room temperature was continued to reflux for another 10 min. 15 mL of the stock enzyme solution prepared as in Section 2.4 was added and stirring was continued at 24°C under reflux for 24 *h*. At the end of the reaction, the solids were washed with acetone, the non-immobilized enzyme was removed. Subsequently, immobilized enzymes were filtered through a filter paper and dried room temperature. İmmobilized support polymers were stored in refrigerator at +4°C.

2.6. Effect of pH on the activity of GOD enzyme

Before examining the effect of pH on enzyme activity, pH buffers were prepared as follows. The pH 3 and 4 (NaH_2PO_4/H_3PO_4) buffer: 6.24 g (40 mmol) $NaH_2PO_4.2H_2O$ was dissolved in 250 mL water. Drops of the H_3PO_4 85% solution were added to the mixture until reaching both pH value of 3 and 4. The pH 5 ($CH_3COO\text{-}Na^+/CH_3COOH$) buffer: Drops of the 0.25 M sodium acetate solution onto 100 mL 0.25 M acetic acid dropwise until pH 5. The pH 6, 7 and 8 ($NaHPO_4/NaH_2PO_4$) buffer: It is added dropwise from 0.25 M NaH_2PO_4 to 100 mL 0.25 M Na_2HPO_4 until pH 6, 7 and 8.

For effects of pH; 20 mg of immobilized polymer was transferred to 6 flasks. 4 mL of each pH buffer (pH=3-9) and 20 mg of the glucose were added to the flasks and the mixtures were stirred for 15 min. in an ultrasonic water bath at 25°C. After stirring, 10 mg 4-AAP, 20 mg phenol and 0.5 mg peroxidase enzyme were added and stirring continued for another 30 min. And their maximum absorbance values were determined at 504 nm using UV-Vis spectrophotometry. The absorbance values were plotted and optimum pH was determined.

2.7. Heat effect on the activity of immobilized GOD enzyme

To examine the effect of temperature on the activity of GOD enzyme immobilized on the synthesized polymers, the optimum pH was studied in the range of 30°C-90°C. The maximum absorbance value of the product at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C was recorded at 504 nm, using the operating method specified in section 2.6.

2.8. Effect substrate concentration on the activity of immobilized GOD enzyme

Stock glucose solution was added in varying concentrations between 50-0.5 mM to 20 mg immobilized enzyme and the volume made up to 5 mL with buffer solution at optimum pH. 4-AAP, phenol and peroxidase enzyme were added to the solution as mentioned in section 2.6 and the same protocol was applied.

2.9. Reusability of immobilized GOD enzyme

20 mg immobilized enzyme was put in to a tube and 4 mL of buffer solution was added to the tube (pH 6 and pH 7 buffers for (PS-N-Fe^{2+})-GOD and (PS-N-Fe^{3+})-GOD; respectively). 0.016g glucose was added to the heterogeneous solution; the mixture was stirred at 24°C for 15 min 4-AAP, phenol and peroxidase enzyme were added to the solution regarding the protocol as mentioned in section 2.6. The solution was stirred for 15 min at shaker bath at optimum temperature. The residue was transferred to a quartz cell and UV-Vis spectra was obtained at absorbance mode at 504 nm.

2.10. Storage stability of immobilized GOD enzyme

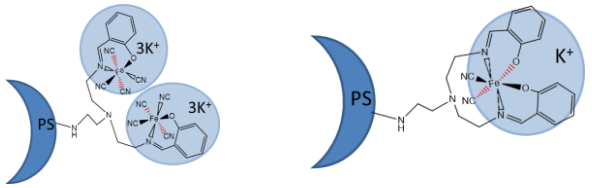
20 mg immobilized enzyme was added to a tube; 4-AAP, phenol and peroxidase enzyme were added, respectively. The protocol as mentioned in section 2.6 was performed. 5 min later, the residue was transferred to quartz cell and UV-Vis spectra was obtained at absorbance mode at 504 nm. The method was repeated for 9 months.

3. Results

3.1. Chemical characterization of support coordination polymers

GPC, FT-IR and elemental analysis results were presented in Table 1. Possible structures are given in

Figure 1. Regarding GPC results of (PS-N-Fe^{2+}) and (PS-N-Fe^{3+}) polymers, M*w* (average molecular weight) / Mn (average molecular number) values were found to be 930 /840 and 850/826 for (PS-N-Fe^{2+}) and (PS-N-Fe^{3+}); respectively. *Mw* regarding the element analysis results and and *Mw** results obtained from GPC are found to be coherent. These results show that our suggestions are correct. Moreover, PDI values observed in GPC analysis, which are close to 1, confirms that the chain lengths of synthesized polymers are close to each other [27].



PS-N-($[Fe(CN)_4L]K_3)_2$ (a)

PS-N-($[Fe(CN)_2L]K$) (b)

Figure 1. The structures proposed for the modified supports; (a) PS-N-Fe^{2+} and (b) PS-N-Fe^{3+}

FT-IR results of polymers illustrates apparent peaks at 1633 and 1635 cm^{-1} which indicate -HC=N- vibrations; peaks observed at 2115, 2074 and 2043 cm^{-1} were attributed to -C≡N- related vibrations. IR bands at *ca.* 3015-3020, 2928-2847 and 1479-1471 cm^{-1} are characteristic of $\nu(CH)_{arom}$, $\nu(CH)_{aliph}$, and $\nu(C\text{-}C)_{arom.}$ ring, respectively [28]. Mid-range 510-511 cm^{-1} peaks were attributed to Fe-C≡N related $\upsilon_{Fe\text{-}C}$ vibrations [29].

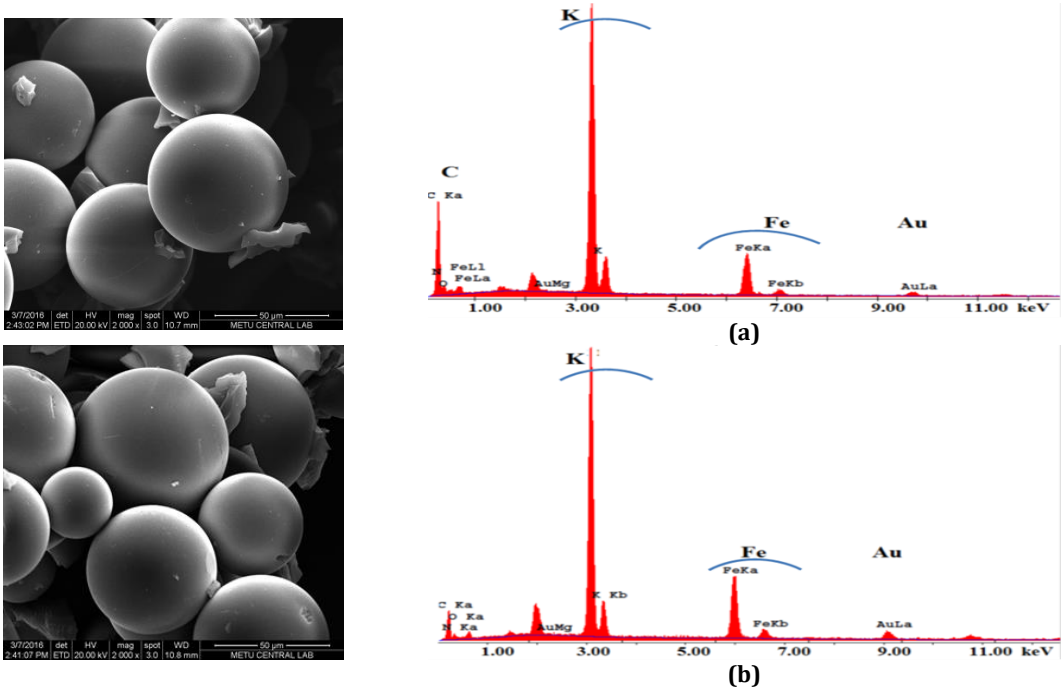
SEM-EDX results were presented in the Figure 2. Elemental analysis distributions and image were evaluated according to SEM-EDX. Images of two different supports were found to be similar. However, EDX results were found to be different. First of all, the observation of Fe peaks (~ 6.25 kev) in the EDX analysis of the two supports supported the coordinated covalent bonding of iron ions to the structure. EDX ZAF Quantification

(Standardless)/Element Normalized values were presented in Figure 2. SEM-EDX results show that the

Table 1. GPC, Elemental analysis and important IR vibration frequencies (cm^{-1}) of the modified spheres

Symbol Suggested unit Mw, *Mw, Mn, (PDI)	Elemental analysis Found (Calcd.) (%)				IR vibration frequencies		
	C	H	N	Fe	ν-CH=N ν-CH / -C=C(arom)	ν-C≡N /ν Fe-C≡N	νFe-N ν Fe-O
(PS-N-Fe^{2+}) $C_{46}H_{50}N_2$-($[Fe(CN)_4L]K_3)_2$ L:-C_7H_4NO 1342, *930, 840, (1.11)	55.60 (54.54)	5.52 (4.02)	6.79 (7.30)	7.86 (8.35)	1633 3020 /1479	2115, 2074, 2043 /510	504 no
(PS-N-Fe^{3+}) $C_{46}H_{50}N_2$-($[Fe(CN)_2L]K$) L:-C_7H_4NO 817, *850, 826, (1.02)	80.51 (80.78)	6.83 (6.61)	7.23 (8.56)	6.18 (6.85)	1635 3015 / 1471	2113, 2074, 2043 /511	502 no

M_w* : Weight average molar mass according to GPC analysis result, no: non observed



Element (%)

C: 44.19
N: 17.77
O: 3.55
K: 22.27
Fe: 8.07

C: 23.42
N: 13.57
O: 1.55
K: 32.72
Fe: 16.52

Figure 2. SEM-EDX results of supports; (a) (PS-N-Fe^{3+}) and (b) (PS-N-Fe^{2+})

coordination of Fe^{2+} ions and Fe^{3+} ions are different for the same support. According to EDX data, the percentage of iron in structure PS-N-$([Fe(CN)_4L]K_3)_2$ is higher than in structure PS-N-$([Fe(CN)_2L]K)$. This result is consistent with the elemental analysis in Table 1. It was seen that while 2 mol of Fe^{2+} ions was attaching per unit structure, 1 mol of Fe^{3+} ions is attached. As far as we could see from the sources, such a result is the first.

The reason for the different binding of Fe^{2+} ions and Fe^{3+} ions may be the difference in charge density. Fe^{3+} ions have a greater charge density than Fe^{2+} ions. This may have led to the formation of a tetradentate chelate structure with nitrogen (N) and oxygen (O) donor atoms in the structure of the support. (Figure 1b). Since the charge density of Fe^{2+} ions is smaller, N and O donor atoms in the same support have caused the formation of a bidentate structure. Thus, we can say that it is possible to bind 2 moles Fe^{2+} ions in the unit structure.

3.2. pH and temperature effect on the activity of free and immobilized GOD enzyme

To determine the effect of pH and temperature of the immobilized GOD enzyme, the method in Section 2.4. have been applied. The optimum activity values (pH and temperature) found are shown in Figure 3. In immobilization studies, since it will be easier to comparing research results, PS-N-$([Fe(CN)_4L]K_3)_2$ and PS-N-$([Fe(CN)_2L]K$ have defined as (PS-N-Fe^{2+})-GOD and (PS-N-Fe^{3+})-GOD; respectively. Optimum pH values of immobilized enzymes were found to be 6 and 7 for (PS-N-Fe^{2+})-GOD and (PS-N-Fe^{3+})-GOD, respectively.

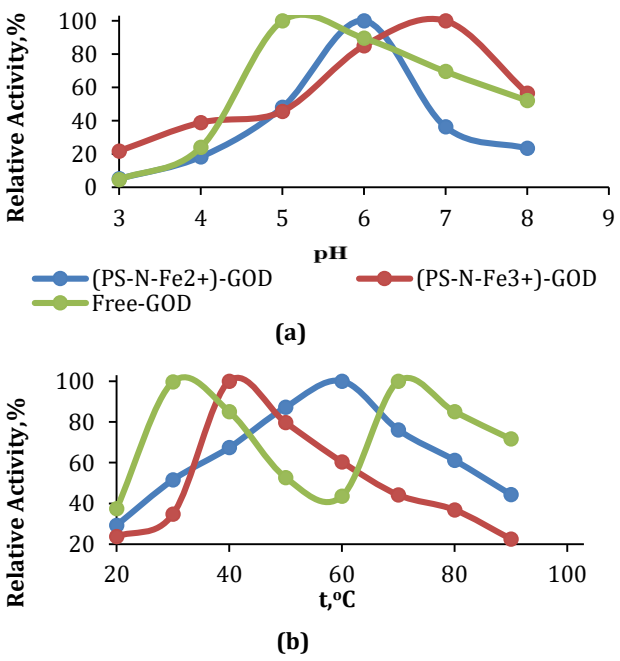


Figure 3. (a) Optimum pH and (b) Temperature for (PS-N-Fe^{2+})-GOD, (PS-N-Fe^{3+})-GOD and Free GOD

Optimum temperature values of immobilized enzymes were found to be 60°C for (PS-N-Fe^{2+})-GOD and 40°C for (PS-N-Fe^{3+})-GOD. As seen in Figure 3, while two different optimum temperatures were determined for the free enzyme, one optimum temperature was determined for (PS-N-Fe^{2+})-GOD and (PS-N-Fe^{3+})-GOD. Among immobilized enzymes, the lowest activity was seen in (PS-N-Fe^{3+})- GOD (90°C, 44.38%).

3.3. The substrate concentration effect, reusability and storage effect on the activity of free and immobilized GOD

In this study, two *Km* values were calculated since GOD has two optimum temperatures. The *Km* value at pH 7, 40 °C is 10.42 mM; *Km* value at pH 6, 60 °C is 4.40 mM. *Km* values of were were found as 15.32 and 10.93 for (PS-N-Fe^{2+}) and (PS-N-Fe^{3+}) polymer immobilized enzymes, respectively (Figure 4). According to the *Km* values, it may be said that the enzyme immobilized to the (PS-N-Fe^{3+}) coded polymer has more interest to the substrate. This result is better than our previous work [18,20,30]. Vmax value at pH 7, 40 °C is 2.33 mM/min while it was found to be 4.15 mM/min at pH 6, 60 ^{0}C for free enzyme. *Vmax* values was found to 10.72 mM/min and 5.53 mM/min for GOD immobilized to (PS-N-Fe^{2+}) and (PS-N-Fe^{3+}) respectively. As the load on the coordination structure increased, the enzyme's interest in the substrate increased. This interest is very close to that of free enzyme. When *Vmax* values were compared with the free enzyme, it was observed that there was no significant change.

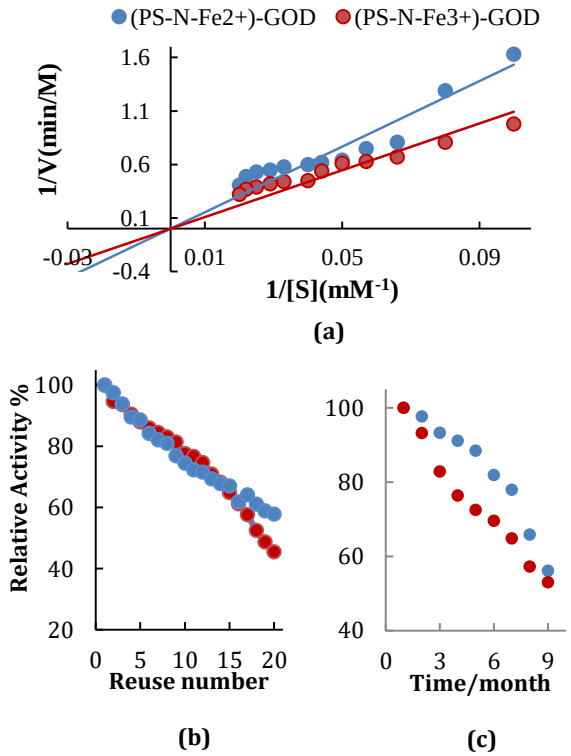


Figure 4. *Lineweaver-Burk* plot, reuse number and storage effect data for immobilized supports

Table 2. Optimum conditions and kinetic parameters for free and immobilized GOD.

Optimum conditions	Enzyme	*Km* (mM)	*Vmax* (mM/min)	Storage Stability (9th month)	Reusability (20 times)
pH 6.60 °C	Free-GOD	4.40	4.15	-	-
	(PS-N-Fe^{2+})-GOD	15.32	10.72	56.13%	57.86%
pH 7.40 °C	Free-GOD	10.42	2.33	-	-
	(PS-N-Fe^{3+})-GOD	10.93	5.53	52.95%	45.47%

The results of the absorbance measurements at 504 nm, the % relative activity data against time are given in Figure 4 for reuse number and storage effect data. As a result of 20 repetitions, it showed that while GOD immobilized to (PS-N-Fe^{3+}) preserved 45.47% of its activity, GOD immobilized to (PS-N-Fe^{2+}) preserved 57.86% of its activity (Figure 4). These results are better than our previous studies [18, 30-34].

To be able to store an enzyme at room temperature without losing its activity is important. So storage effect has been investigated on the activity of immobilized GOD. When the immobilized and free enzyme have been stored for 9 month in a refrigerator at +4 °C.

When the relative activities at the end of the 9*th* month are compared, the relative activities of (PS-N-Fe^{3+})-GOD and (PS-N-Fe^{2+})-GOD were found to be 52.95% and 56.13%, respectively. There is no obvious difference between them. However, the reason for the high relative activity of (PS-N-Fe^{2+}) may be due to 2 moles iron ions in the structure. The storage stability of immobilized enzymes is higher than free enzymes.

4. Discussion and Conclusion

In summary, different optimum pH values were observed for immobilized enzyme. Different amino acid residues on active side of enzyme may play a role for each polymer; therefore, different optimum pH values may be observed. Increase in the charge of coordination structure result in decreased *Km* values where no considerable change was seen in the *Vmax* values. The important result of our research is that the *Km* value (PS-N-Fe^{3+})-GOD is close to the free enzyme. The *Km* value of (PS-N-Fe^{3+})-GOD is 50% greater than the *Km* value of (PS-N-Fe^{2+})-GOD. As seen in the following potentials, possible reason of such a case is the larger reduction potential of Fe^{3+}.

$$Fe_{(aq)}^{3+} + e^- \rightarrow Fe_{(aq)}^{2+} \quad E^o = 0.77\ v \qquad (2)$$

$$Fe_{(aq)}^{2+} + e^- \rightarrow Fe_{(s)} \quad E^o = -0.44\ v \qquad (3)$$

It can be thought that the electron in the (PS-N-Fe^{3+})-GOD contributes to the following reaction.

$$\beta\text{-}D\text{-Glucose} + O_2 \xrightarrow{\text{(PS-N-Fe}^{3+}\text{)-Enzyme}} D\text{-glucono-}\delta\text{-lactone} + H_2O \qquad (4)$$

Fe^{3+} ions in (PS-N-Fe^{3+})-GOD may have had an accelerating effect on the separation of two electrons during the oxidation of glucose. This may have caused the enzyme substrate relationship to increase. Because Glucose oxidase (GOD) catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone by co-reducing the enzyme-bound flavin in the flavin adenine dinucleotide (FAD).

-CN^- ligands may also play a role in this mechanism. With the back-bonding of the -CN^- ligands, the transition of electrons in the d orbital to the empty π^* orbitals of the ligand is possible [35]. This transition may also contribute to *Km* values.

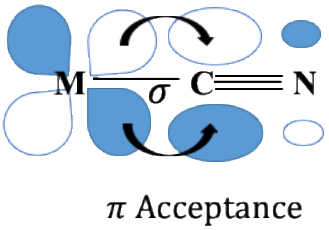


Figure 5. Back-bonding of -CN ligand and the transfer of electrons from the d orbital to the empty π* orbitals of the ligand.

Acknowledgment

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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