Immobilization of Glucose Oxidase on Meso-Porous Glass-Ceramic with the Skeleton of $CaTi_4(PO_4)_6$

M. Ahmadi[a], M. Rezvani[a], SH. SHoa Bargh[b], M. S. SHakeri[c,*], A. Karimi[b]

[a] *Department of Mechanical Engineering, school of material Engineering, University of Tabriz, Tabriz, Iran.*
[b] *Department of Chemistry, University of Tabriz, Tabriz, Iran.*
[c] *Department of Ceramics, Materials and Energy Research Center, Karaj, Iran.*

ARTICLE INFO

Article history:

Received 23 Nov 2013
Accepted 23 Dec 2013
Available online 25 Dec. 2013

Keywords:

Microporous glass ceramic
Glucose oxidase
Immobilized enzyme
$CaTi_4(PO_4)_6$

ABSTRACT

Microporous glass ceramic with skeleton of $CaTi_4(PO_4)_6$with average pore size of 12.7 nm has been synthesized and used as a carrier of glucose oxidase. The glass ceramic was prepared by controlled heat treatment of glass samples, which causes phase separation in their structure and creates $CaTi_4(PO_4)_6$ and β-$Ca_3(PO_4)_2$ phases. The β-$Ca_3(PO_4)_2$ phase was dissolved by soaking the glass ceramics in HCl and $CaTi_4(PO_4)_6$ built the skeleton of microporous glass ceramic. Analysis of the ability of the carrier for immobilization of glucose oxidase (GOx) was undertaken. Average amount of immobilized enzyme and percentage of enzyme activity on thecarrier were 27 gr GOx/gr carrier and 60.15%, respectively. Effect of pH and temperature variations on the enzyme activity was studied and results demonstrated that maximum activity for both free and immobilized enzyme was at T=40˚C and pH=7.0. Due to the same value of maximum activity, no serious conformational change of enzyme occurred through immobilization. However, immobilization of GOx on CTP caused considerable increase of enzyme stability under different environmental conditions.

1. Introduction

Enzymes are biological catalysts that promote the transformation of chemical species in living systems [1]. The ability of enzymes to catalyze reactions has made them indispensable to science for decades [2].The effective catalytic properties of enzymes have already promoted their introduction into several industrial products and processes. The immobilization of enzymes has proven particularly valuable, because it has allowed the enzyme to be reused multiple times for continuous operations with longer half-life and less denaturation and has provided a straightforward method of controlling reaction rate as well as reaction start and stop time. It has also helped to prevent the contamination of the product with enzyme/protein or other compounds, which decreases purification costs. These benefits of immobilized enzyme have made it highly applicable to a range of evolving biotechnologies [3]. Recently, the issue of designing biocompatible materials and interfacial structures which allow stable attachment of enzyme while maintaining its

Corresponding author:
E-mail address: ms.shakeri@merc.ac.ir (Mohammad Sadegh Shakeri).

activity and function as close as possible to their native state has become controversial. Porous silica glass is one of these materials, found to pick up large quantity of enzyme under investigation of Chibata *et al.* [4]. Since SiO_2 is soluble near neutrality and above, its derivative was unstable and lost activity rapidly [5]. Microporous glass-ceramics with skeleton of the $CaTi_4(PO_4)_6$ crystal (CTP), which has developed by Hosono *et al.*, reached high and excellent chemical durability in large pH range in comparison to Controlled Pore Glass (CPG) and polymer carriers. The latter result is because of high content of TiO_2. This system is too bioactive under investigation by Ribeiro *et al.* [6]. Its properties such as ion exchange capacity, chemical adsorption and ability to act as an immobilization matrix for several enzymes suggest that it can successfully be used in different fields such as biomedical.

Recent trend is to use nanostructured materials for the purpose of enhancing enzyme retention while providing high biocatalytic activity and efficiency [7]. Besides the mentioned advantages, microporous CTP has high accessible surface area which greatly enhances the stability and the amount of enzyme immobilization. In addition, its nanostructure reduces mass transfer limitation [8]. Glucose oxidase (GOx) was selected as a model enzyme to study the features of CTP carrier as a biocarrier due to its being a useful reagent for selective determination of glucose and an analyst of clinical as well as of industrial interest, especially with considering its price per unit activity [9]. Pore size is the most important characteristic of the porous carrier that influences the enzyme immobilization and its activity. The first objective of this paper is preparation of CTP carriers with controlled pore sizes. To our best knowledge, GOx immobilization on CTP particles hasn't been studied. So, investigatingCTP particle's application as a carrier for immobilization of GOx is the second worthy aim of this paper. However, analysis of the carrier's ability to maintain GOx activity under different operation conditions such as pH, temperature and reaction duration was undertaken.

2. Experimental

2. 1. Materials

The raw materials used to make the glass ceramic carrier were reagent grade $CaCO_3$ (Merck 102069), TiO_2 (Merck 100808), P_2O_5 (Merck 100540) and Na_2CO_3 (Merck 106398). Glucose oxidase (GOx, EC 1.1.3.4, from Aspergillusniger), β-D-glucose, KI (99.99%), NaOH (99.99%), potassium hydrogen phetalate (KHP) and ammonium molybdatetetrahydrate (Mo(VI)) obtained from Sigma Aldrich and Hydrogen peroxide (30%v/v , Merck AG) were used to investigate immobilization of glucose oxidase enzyme on the carrier.

2. 2. Preparation of the carrier

The chemical composition of the base glass is $45CaO\text{-}25TiO_2\text{-}30P_2O_5\text{+}2Na_2O$ (mol%). The base glass was prepared using the Hosono method [10]. In order to melt the glass, sample was put in an alumina crucible and placed in an electric furnace where it was heated for 1 hour at 1350˚C and then cast onto a steel mold that was preheated at 600˚C.

To investigate the nucleation temperature, differential thermal analysis (DTA) was performed on glass specimens (Shimadzu-DTG60AH). The reference material in this experiment was $\alpha\text{-}Al_2O_3$ and the heating rate was $10\ ^{\circ}C.\min^{-1}$. Glass specimens were heat treated at T_n (nucleation temperature), chosen at the middle of the T_g-T_d interval (T_g is glass transition temperature and T_d is dilatation softening temperature). Also, to determine the nucleation time, samples were nucleated at T_n for different lengths of time and were then examined with DTA.

Suitable crystallization temperature was found by first subjecting specimens to the aforementioned 'nucleation' treatment, and second soaking them for 3 h at different crystallization temperatures between T_c and (T_c-30) where T_c is the DTA exothermic peak onset temperature. Finally, the optimum crystallization time was determined by nucleating them and subsequently soaking them for different lengths of time at determined crystallization temperature. The resulted specimens were analyzed by X-ray diffraction (Philips-XRD).

Heat treatment was performed on the basis of the pervious analysis and glass ceramic specimens were obtained. To reach high percent of immobilization, increasing interaction of the enzyme and specimen was needed. The latter was achieved by grounding and milling specimens. Glass ceramic specimens were soaked in a 1 M HCl (Merck-100314 with density of 37%) solution for 3 days in ambient temperature in order to dissolve away the more soluble β-$Ca_3(PO_4)_2$ phase and to produce a porous skeleton of $CaTi_4(PO_4)_6$. The specific surface area and average pore size were examined by BET (Belsorp mini II). Microstructural examinations were carried out using Scanning Electron Microscopy (SEM). SEM studies were performed on glass-ceramic samples both before and after leaching using Hitachi-S-4800, Japan.

2. 3. Enzyme immobilization

GOx was chosen as a model enzyme to study the potential of CTP as an enzyme support. The batch experiments of GOx immobilization were conducted in clean Erlenmeyer flasks in incubator to control the temperature and shaking speed (rpm). Enzyme solution was obtained by dissolving the enzyme in phosphate buffer (PBS, 0.1 M, pH=6.0). As reported by Piskin *et al*. [11], the highest immobilization of GOx with higher activity occurs when the solution has a concentration of 0.1 mg GOx/ml. However, this concentration was kept by our prepared solution. 50mg of clean CTP microspheres were incubated in 2 ml enzyme solution (with activity of 220 U/ml) at 25 ℃ for 2 h to allow immobilization equilibrium. Thereafter, carriers were separated from the solution and washed with phosphate buffer twice to remove free enzymes completely. The separated solution (supernatant) and carriers were stored at $4^\circ C$ for further testing.

2. 4. Determination of GOx activity

The activity of GOx was assessed by colorimetric method. The reactions could be expressed as follows:

$$C_6H_{12}O_6 + O_2 + H_2O \xrightarrow{GOx} H_2O_2 + C_6H_{12}O_7$$

$$H_2O_2 + 2I^- \xrightarrow{Mo(VI)} I_2 + H_2O$$

$$I_2 + I^- \rightarrow I_3^-$$

The principle of this method is that, in the presence of oxygen, GOx as a catalyst oxidizes β-D-glucose to β-D-glucono-δ-lactone and hydrogen peroxide. The production of H_2O_2 was measured by I_3^- method [12] in an aqueous solution containing phosphate buffer 0.1 M and glucose 20 mM. The measurement of H_2O_2 at concentrations as low as $1\,\mu M$ can conveniently be done by this method. Ammonium molybdatetetrahydrate was employed as a catalyst. Also, peroxidase enzyme which has the advantage of higher efficiency can be used [13]. Reaction between I_2 and I^- forms a product that is measured by taking UV−vis spectra of reaction mixture at wavelength of 351 nm. The concentration of hydrogen peroxide could be determined by a standard calibration curve. Theoretically, immobilization percentage of enzyme was calculated by Eq. 1. One unit of catalyst activity (U) is defined as the amount of GOx required for production of $1\,\mu mol\ H_2O_2$ in one minute at $25^\circ C$.

$$\text{Immobilization percent of Enzyme} = (1 - \frac{\text{GOx concentration in supernatant}}{\text{GOx concentration in solution}}) \times 100 \quad [1]$$

3. Results and Discussion

3. 1. Characterization of microporous CTP

Fig. 1 shows the DTA result of the base glass. According to previous investigations [14], a suitable temperature for the nucleation process is the temperature between the glass transition (T_g) and the dilatometric softening temperatures (T_d). The mentioned temperature is determined using DTA curve and $\frac{T_g + T_d}{2}$ has been chosen as nucleation temperature which in this study is 690˚C.

In order to determine the suitable nucleation period at 690˚C, the specimens were nucleated for 8, 16, 24, 32 and 48 h. Then DTA analysis was undertaken. Fig. 2 shows the DTA traces for the nucleated samples. As suggested by Ray & Day [15], the best condition for nucleation is related to traces that have the sharpest

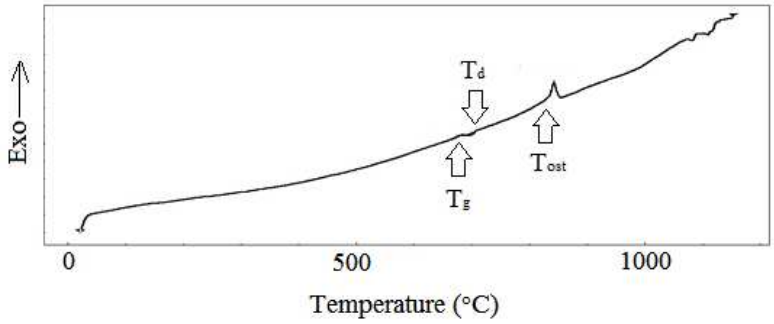

Fig. 1. DTA trace for base glass specimen

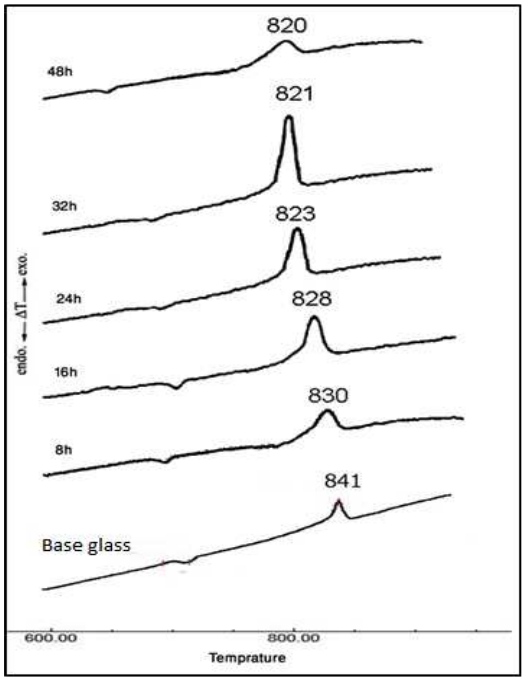

Fig. 2. DTA traces for specimens nucleated for different lengths of time at 690˚C

crystallization peak. It can be seen that till 32 h, with increase of the nucleation time, T_p decreased and became sharper. Therefore, the effectiveness of the nucleation process was proven. After 32 h nucleation, T_p became wider. It means that nucleation times more than 32 h causes nucleate growth in the glassy matrix which isn't a suitable procedure for production of meso-porous materials. Thus, we conclude that a 32-h nucleation period is suitable.

The DTA exothermic peaks usually correspond to crystallization in glass specimens. If the crystallization process happens in the mentioned exothermic peak, subsequent to the nucleation treatment, it develops a very coarse microstructure and the pore size of the final glass ceramic exceeds above the nanometer range. In order to get a fine and desirable microstructure, a lower temperature should be chosen. According to Fig. 1, crystallization starts at around 770˚C. It is the start temperature point of crystallization peak. It is also called the "Onset Temperature". In order to find the suitable crystallization temperature, the samples were first subjected to the aforementioned 'nucleation' treatment, and then soaked for 3 h at 765, 775, 785, and 795˚C. Fig. 3 depicts the XRD patterns of the specimens heat treated according to the above procedure. The microstructure of glass ceramics controls the pore sizes of final products. To keep the pore diameters as small as possible, the lowest temperature shows the preferred crystallization according to XRD patterns. It is concluded from the semi-quantitative results. Accordingly, 775 ˚C was chosen as the 'crystallization' temperature.

To determine the optimum crystallization time for the specimens, they were first nucleated for 32 h at 690˚C and subsequently soaked for different lengths of time at 775˚C. Fig. 4 shows the XRD patterns for the above specimens. The main phases in all the specimens are CP and CTP. After leaching CP phase will be corroded and CTP will build the structure of the final product. Therefore, CP dictates the amount of the pores. In order to reach a structure with maximum amount of pore, the ratio of CP/CTP should be maximum value. Basically, 24 h was chosen as the suitable crystallization period.

The crystallized specimens were soaked in 1 M HCl solution for 3 days in ambient temperature. Then, they were leached with distilled water. BET analysis shows that the average pore size of the specimens and specific area of them were 12.7nm and 24m^2/g, respectively. It should be

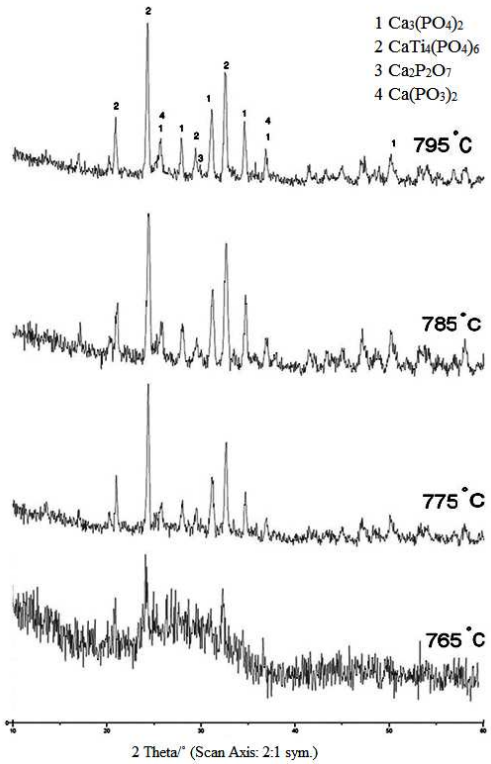

Fig. 3. XRD patterns for specimens nucleated at 690˚C for 32 h and soaked for 3 h at different temperatures

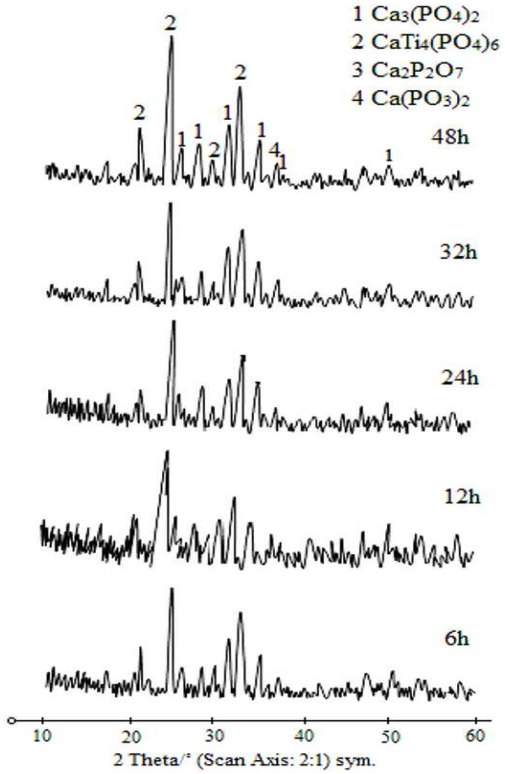

Fig. 4. XRD patterns for specimens nucleated for 32 h at 690˚C and soaked for different lengths time at 775˚C

noted that only BET analysis of the optimum sample was analyzed in this investigation.

Fig. 5 shows the microstructure of glass-ceramic both before and after leaching in 1 M HCl for 3 days. The nano-porous structure of glass-ceramic after leaching has been shown in fig. 5-b. As it is illustrated, the SEM results confirm the results of BET analysis.

3. 2. GOx immobilization onmicroporous CTP Glass-ceramic

The activity of immobilized enzyme depends on the available surface area, porosity, hydrophilic character, reaction conditions and the method chosen for the immobilization [16]. Various strategies to immobilize enzymes have been employed such as physical adsorption, covalent binding, physical entrapment, etc. Generally, physical adsorption is the easiest, inexpensive and the least denaturing immobilization method. GOx was immobilized on microporous CTP glass ceramic by physical absorption. After completion of GOx immobilization on CTP, the activity percent of carrier was measured by measuring activity of immobilized enzyme in carrier directly. Immobilization percent and activity percent of carrier are shown in Table 1. Since properties of immobilized GOx on carriers are affected by the operating conditions, data of activity percent was measured at optimum condition (pH=7.0 and T=40˚C). Bautista *et al.* studies on immobilization of GOx on amorphous $AlPO_4$ support revealed that 54.95% immobilization had occurred [17]. However, 27.85% immobilization of GOx on CCR support and 50.12% of immobilization of enzyme on LMCCR reported by Donge *et al.* while activity percent were 64.87% and 82.65%, respectively [18].

Fig. 6 shows FT-IR traces for GOx, microporous CTP glass ceramic and immobilized GOx on CTP. In the case of GOx, the most important features are the amide bands [19]. The characteristic band of amide I caused by C=O stretching vibrations of peptide linkages in the GOx backbone was visible at 1642.88 cm^{-1} in GOx-CTP spectra. Other important peaks in the FT-IR spectra of GOx-CTP were observed at

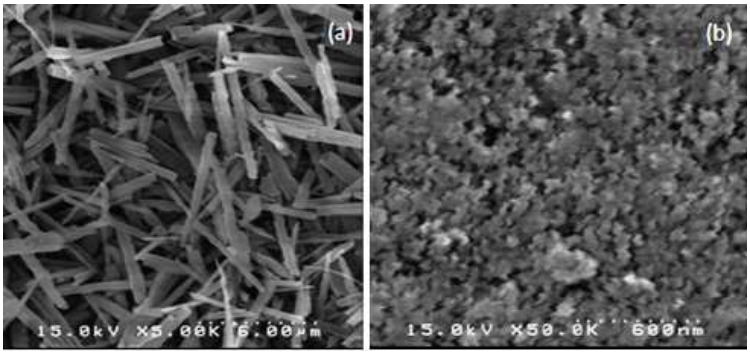

Fig. 5. SEM micrograph of glass-ceramics (a) before leaching, and (b) after leaching in 1 M HCl for 3 days.

Table 1. Physical properties of the carrier and enzyme immobilization percentage on it

Carrier	Surface area(m^2/g)	Mean pore size(nm)	Immobilization Percent	Activity percent	mg GOx/ g CTP
CTP microporous glass ceramic	24	12.7	81.46%	60.15%	27

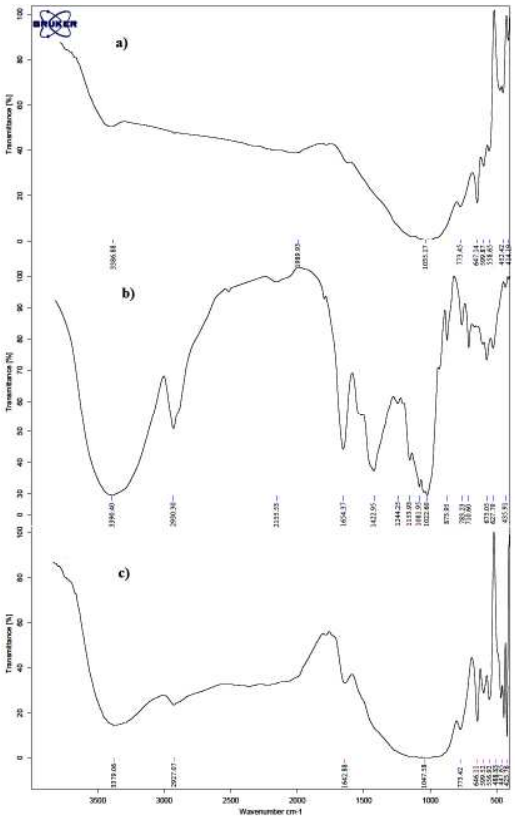

Fig. 6. FT-IR traces for (a) glucose oxidase, (b) microporous CTP glass ceramic before immobilization process, and (c)) microporous CTP glass ceramic after immobilization process

3379.06 and 2927.07 cm^{-1} correspond to N-H stretch of amides, C-Hvibrations, respectively. The characteristic peaks were found in spectra of GOx and GOx-CTP whereas being not visible in the spectra of CTP, indicating enzyme attachment onto particles.

3. 2. 1. PH values effects on enzymatic activity

The effect of pH values of the substrate solution on the enzyme activity was investigated at different pH values ranging from 4.0 to 8.0 at constant temperature of 25°C. The results are shown in Fig. 7. By changing pH values, the immobilized enzyme would be affected less than free enzyme. Enhancement of the enzyme stability against the changing pH values is the most important aim of enzyme immobilization. The highest activity (U) of free and immobilized enzyme was seen at pH value of 7.0 which had an activity percent of 61% for immobilized one. As can be seen from this figure, in large range of pH values, immobilized enzyme has similar relative activity. For example, at pH=6 the relative activity is 99.4%, so near to optimal pH value. One of the problems of enzyme immobilization is conformational change of enzyme. After immobilization, the optimum pH value of immobilized enzyme changes due to the latter reason [20]. However, since optimum pH value for free GOx and immobilized GOxon CTP are the same, it seems reasonable to assume that no serious conformational change of enzyme had taken place through immobilization.

3. 2. 2. Temperature effects on enzymatic activity

The effect of temperature on the activity of

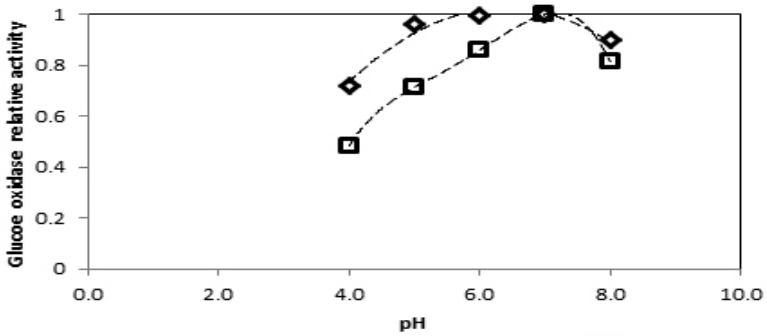

Fig. 7. Relative activity of free (⊟) and immobilized GOx (◇) versus pH at T=25˚C

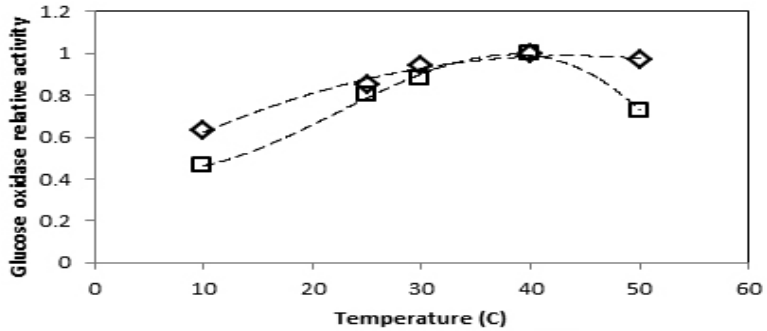

Fig. 8. Relative activity of free (⊟) and immobilized GOx (◇) versus temperature at pH=7.0

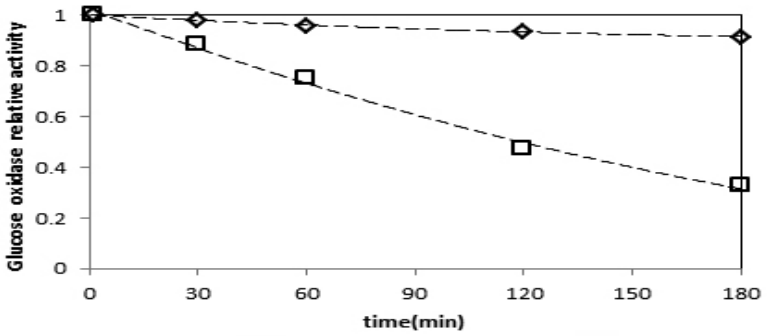

Fig. 9. Relative activity of free (⊟) and immobilized GOx (◇) at pH=7.0 and T=40˚C

enzyme was measured by varying temperature from 10 to 50℃ at optimal pH value. Fig. 8 represents two profiles of free and immobilized enzyme relative activity versus temperature. By increasing temperature, activity of both free and immobilized enzyme increased. However, activity of free enzyme grows with sharper slope compared to the immobilized one. In fact, after immobilization, enzymes become more rigid and stable to heat and denaturing agent. They can function in a broader range of temperature. The highest activities of both types of enzyme were observed at 40 ˚C . The deviation from optimum temperature of enzyme leads to significant decrease of enzyme activity which is due to denaturing. Also, immobilized enzyme kept its activity in a broad range of 20 up to 50℃ and may be 60 ˚C . After 70 ˚C a rapid fall is expected due to nearly whole denaturation [21].

3. 2. 3. Enzymatic activity at different reaction time lengths

In order to investigate the stability of enzyme activity at reaction condition, activity of both free and immobilized enzyme was measured at pH=7.0 and T=40˚C for different lengths of reaction time. Activity of enzyme at different reaction times at constant pH and temperature is shown in Fig. 9. As depicted in this figure,

free enzyme activity has rapidly diminished and during 3 h its relative activity reached to 0.3, while the relative activity of immobilized enzyme after this period of time has become 0.92. This result shows the efficiency of the enzyme immobilization on the carrier. By continuing the linear figure the half-life of the immobilized enzyme was calculated 14 h that is approximately 7 times more than free enzyme half-life.

4. Conclusions

1. Microporous phosphate glass-ceramic with the skeleton of $CaTi_4(PO_4)_6$, is a practical biocarrier for GOx. due to having fine size of pores, high specific area and chemical stability in different pH

2. Immobilized GOx amount (81.46%) and high enzyme activity after immobilization (60.15%) revealed CTP's role as a suitable biocarrier. This amount of immobilization is much more than the previously reported works.

Application of GOx at different industrial conditions can possibly be acheived by application of highly stable CTP particles.

3. Same value of the optimum condition for both free and immobilized enzyme implied that, immobilization causes no serious conformational change of enzyme.

4. Immobilization increases enzyme half-life 7 times. This feature allows using the immobilized enzyme for long time with relatively high activity.

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