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Modified PMMA monosize microbeads for glucose oxidase immobilization

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Abstract

Glucose oxidase (GOD) was immobilized onto modified polymethylmethacrylate (PMMA) microspheres by covalent bonding. Monosize PMMA microbeads with 1.5 μm diameter were produced by dispersion polymerization of methylmethacrylate by using polyvinyl alcohol as a stabilizer. Hydroxyl groups on the microbeads were first converted to aldehyde groups by periodate oxidation. Three amino compounds, namely ammonium hydroxide, ethylene diamine and hexamethylene diamine were incorporated through the aldehyde groups. Then, GOD molecules were immobilized through the spacer-arms by using glutaraldehyde. The highest amount of immobilization and activity were obtained in which hexamethylene diamine was used as the spacer-arm with 14 atom length, and were 2.1 mg g^{-1} polymer and 129 IU g^{-1} polymer, respectively. The optimal conditions for GOD immobilization were obtained as follows: pH, 6.0; temperature, 30 °C; immobilization time, 60 min; and GOD initial concentration, 0.10 mg ml^{-1}. The optimal conditions for the GOD-immobilized PMMA microbeads were at pH 6.0 and at a temperature of 30 °C. The K_m and V_{max} values of the GOD-immobilized PMMA microbeads were, 13.79 mM and 26.31 mM min^{-1} calculated by non-linear regression, respectively.

Keywords: Modified PMMA microbeads; Glucose oxidase; Immobilization

1. Introduction

Enzymes are largely used as biocatalysts in chemical, pharmaceutical and food industries, and as specific ligands in clinical and chemical analysis [1–3]. Since the recovery and the reusability of the free enzyme are limited, immobilization of the enzyme has been proposed. Immobilized enzymes have the advantages that they can be used in batch and continuous systems, are removed easily from the reaction medium, and provide the facility of controlled production. However, the immobilized enzyme systems also have limitations mainly due to mass transfer problems [4,5]. Immobilization on the surface of a support material has been proposed to decrease mass transfer limitations. Support material, which plays an important role in the utility of an immobilized enzyme, should be readily available and non-toxic, and also should provide large surface area suitable for enzyme reactions, and substrate and product transport with the least diffusional restriction.

As support matrices, polymeric microbeads have attracted much attention because they may be produced easily in a wide variety of compositions, and can be modified for the immobilization systems by introducing a variety of activation methods. Non-porous or porous polymer microbeads with average diameters of usually more than 100 μm are generally used [6,7]. When non-porous large size microbeads are used, only the outer surface of the microspheres are available for the immobilization, which means low immobilization capacity due to the low surface area. Note also that these large size support particles reported in the literature always have a size distribution, narrow or wide, depending on the production process. The size distribution may even change from one batch to another in the same production process. Because of this, it is impossible to determine an exact surface area to define the immobilization capacity based on unit surface area.

In order to increase the surface area, porosity may be created within the microbeads. However, substrate and product transport problems due to the pore diffusion resistance are the main disadvantages of these type of support matrices.

In this study, monosize polymethylmethacrylate (PMMA) microbeads, 1.5 μm in diameter, carrying hydroxyl groups on their surfaces were produced by dispersion polymerization to use as a support matrix for enzyme immobilization. These polymer microbeads exhibit much higher outer surface area per unit weight, which is available for enzyme immobilization due to their size. It is obvious that this support matrix does not exhibit diffusion limitation that the porous sorbents do.

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The monodispersity of these microbeads enables exact calculation of the immobilization capacity based on unit surface area. Hydroxyl groups on the PMMA microspheres were converted to aldehyde groups by periodate oxidation, then, spacer-arms were bound to the microbeads through these groups. Glucose oxidase (GOD) was immobilized covalently onto these microbeads by using glutaraldehyde.

2. Experimental

2.1. Materials

The monomer, i.e. methylmethacrylate (MMA), was obtained from Rohm and Haas Ltd. (Germany), and washed with 10 wt.% aqueous sodium hydroxide (NaOH) (BDH Chemicals Co., UK) solution to remove the inhibitor, and stored in a refrigerator until used. The initiator was 2,2′-azobisiso-butyronitrile (AIBN) (BDH Chemicals Ltd., UK). The dispersion medium was prepared by mixing equal amounts of ethanol (Merck A.G., Germany) and distilled water (50/50 ml/ml). Polyvinyl alcohol (PVAL) (MW, 9000) was obtained from Fluka Chemicals Co. (Switzerland) and was used as a steric stabilizer. Sodium periodate ($NaIO_4$) from BDH Chemicals Co. (UK) and glutaraldehyde (50%) solution from BDH Chemicals Co. (UK) were used in the modification procedure. Ammonium hydroxide (NH_4OH), ethylene diamine ($(NH_2)_2C_2H_4$), hexamethylene diamine ($(NH_2)_2C_6H_{10}$) were obtained from BDH Chemicals Co. (UK) and were used as spacer-arms. Glucose oxidase (GOD) (EC 1.1.3.4, Type XS from *Aspergillus niger*), Peroxidase (POD) (EC 1.11.1.7, Type II from horseradish), and *o*-dianisidine were received from Sigma Chemical Co. (USA). D-glucose from BDH Chemicals Co. (UK) was used as the substrate. All other chemicals were commercially available products of reagent grade.

2.2. Preparation of modified PMMA microbeads

The PMMA microbeads, 1.5 μm in diameter, were prepared by dispersion polymerization as described in our previous paper, except using polyvinylalcohol as a steric stabilizer instead of polyvinyl pyrrolidine [8]. PVAL was included in the recipe in order to introduce the hydroxyl groups on the surfaces of the PMMA microbeads for further modification to immobilize GOD.

SEM micrographs of the PMMA microbeads were obtained using a scanning electron microscope (SEM, Leitz-AMR 1000 Raster Electronen Microscope, Frankfurt, Germany).

The hydroxyl groups on the PMMA microbeads were oxidized by $NaIO_4$ to yield aldehyde groups as described in the literature [9]. The oxidation was performed in pH 4.5 acetate buffer (10 ml) containing $NaIO_4$ of 1% (w/v) for 1 h at room temperature. The excess periodate was removed by washing with the same buffer solution. The oxidized microbeads were mixed with an excess of a neutral solution of sodium sulphite, and the number of hydroxyl groups converted to the aldehyde groups were then determined by titrating with standard sulphuric acid solution (0.05 M) using phenolphthalein as indicator [10].

At the next step, the spacer-arm was covalently bound to the microbeads through the aldehyde groups. Three amine compounds with different molecular sizes (i.e. NH_4OH, $(NH_2)_2C_2H_4$, $(NH_2)_2C_6H_{10}$) were used to study the effects of spacer-arm length on immobilization of GOD. The amine compound (3.5 mmol g^{-1} microbeads) was added to 10 ml of pH 11.5 borate buffer solution containing the aldehyde-modified PMMA microbeads [10]. Temperature and time for incubation were 80 °C and 1 h, respectively [9]. After incubation, excess amines were removed by washing with pH 11.5 and pH 8.2 borate buffer solutions.

The spacer-arm-incorporated PMMA microbeads were incubated with glutaraldehyde (1.25%, v/v) in 10 ml of pH 8.2 borate buffer solution [10]. The mixture was stirred for 2 h at room temperature. After the activation was completed, the resulting microbeads were washed with pH 6.0 phosphate buffer to remove the excess glutaraldehyde from the medium.

2.3. Immobilization of GOD

For immobilization, the modified PMMA microbeads (0.10 g) were incubated with 5 ml of buffer solutions (at selected pH and temperature values) containing different amounts of GOD (0.02–0.20 mg ml^{-1}) for different periods of time (30–180 min). At the end of the immobilization period, the GOD immobilized microbeads were separated from the supernatant by centrifugation, and washed twice with phosphate buffer (pH 6.0). The effects of pH and temperature were also studied in the ranges of 3.0–8.0 and 25–45 °C, respectively.

The amount of immobilized GOD was determined by measuring the concentration of the free enzyme in the supernatant with an UV spectrophotometer (Hitachi, Japan) at 219.7 nm, by using a calibration curve which was constructed with a series of GOD solutions (0.01–0.20 mg ml^{-1}).

Performances of the GOD-immobilized PMMA microbeads were studied at different pH values (3.0–8.0), at different temperatures (25–45 °C), and with different substrate concentrations (5–100 mM).

Activities of both free and immobilized GOD were obtained by measuring the amount of hydrogen peroxide formed from glucose conversion, spectrophotometrically [11]. 2.5 ml of a mixture containing POD (1.5 mg) and *o*-dianisidine (3.3 mg) was added in 50 ml of 0.1 mM phosphate buffer (pH 7.0), and incubated for 10 min at 25 °C. A 100 μl sample obtained by the oxidation of D-glucose by GOD, was added to the assay mixture. After 10 min, 1.5 ml of sulphuric acid solution (30%) was added to this mixture to stabilize the colour formed. The enzyme activity was measured spectrophotometrically at 525 nm.

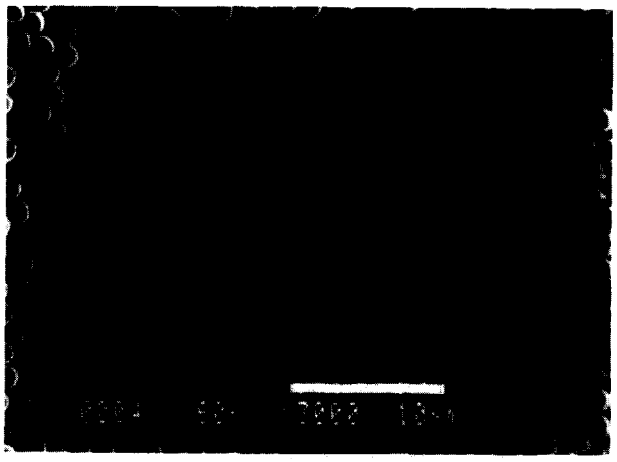

Fig. 1. SEM micrograph of PMMA microbeads.

One unit of glucose oxidase is defined as the amount of enzyme which oxidizes 1 μM of β-D-glucose to D-gluconic acid and hydrogen peroxide per min at 25 °C and at pH 7.0.

3. Results and discussions

The monosize PMMA microbeads, 1.5 μm in diameter, were produced by dispersion polymerization as previously described [8]. PVAL was used both as a steric stabilizer and to introduce hydroxyl groups onto the PMMA microbeads. Fig. 1 gives a SEM micrograph of PMMA microbeads, which clearly shows the monodispersity.

GOD immobilization was achieved by a four-step procedure, which is schematically shown in Fig. 2. The hydroxyl groups on the PMMA microbeads were oxidized by $NaIO_4$ to yield aldehyde groups at the first step. Then, spacer-arms were incorporated onto the aldehyde modified PMMA microbeads. Three amine compound with different atomic lengths, namely, ammonium hydroxide (NH_4OH), ethylene diamine ($(NH_2)_2C_2H_4$), hexamethylene diamine ($(NH_2)_2C_6H_{10}$) were studied to examine the effects of spacer-arm length on GOD immobilization. At the third step, glutaraldehyde molecules were allowed to react with the amine groups of spacer-arm incorporated PMMA microbeads for immobilization of GOD. Finally, GOD was immobilized onto the PMMA particles by covalent bonding via these functional aldehyde groups.

Effects of different parameters on GOD immobilization are discussed in the following subsections. The amount of GOD immobilized (i.e. "immobilized GOD") and activity of the immobilized GOD (i.e. "enzyme activity") were obtained by using the following expressions:

$$\text{Immobilized GOD (mg g}^{-1}\text{ polymer)} = \frac{C_o - C_d}{m} \times V$$

$$\text{GOD activity (IU g}^{-1}\text{ polymer)} = \frac{\text{Activity of immobilized GOD}}{m}$$

Here C_o and C_d are the initial and final concentrations of GOD (mg ml^{-1}); m is the amount of PMMA microbeads (g); and V is the total volume of the aqueous phase (ml).

3.1. Effects of spacer-arm on GOD immobilization

In this part of the experiments, we investigated the effect of spacer-arm length on the amount and activity of immobilized GOD. Fig. 3 gives the amount and activity of the immobilized GOD on both the aldehyde-modified PMMA and the spacer-arm-incorporated PMMA microbeads.

Both the amount of immobilized enzyme and enzyme activity increased with increasing the spacer-arm atomic length. There was a very low GOD immobilization (therefore activity) where there was no spacer-arm on the PMMA microbeads (i.e. the aldehyde-modified PMMA microbeads (Fig. 2)). The highest amount of GOD immobilized was

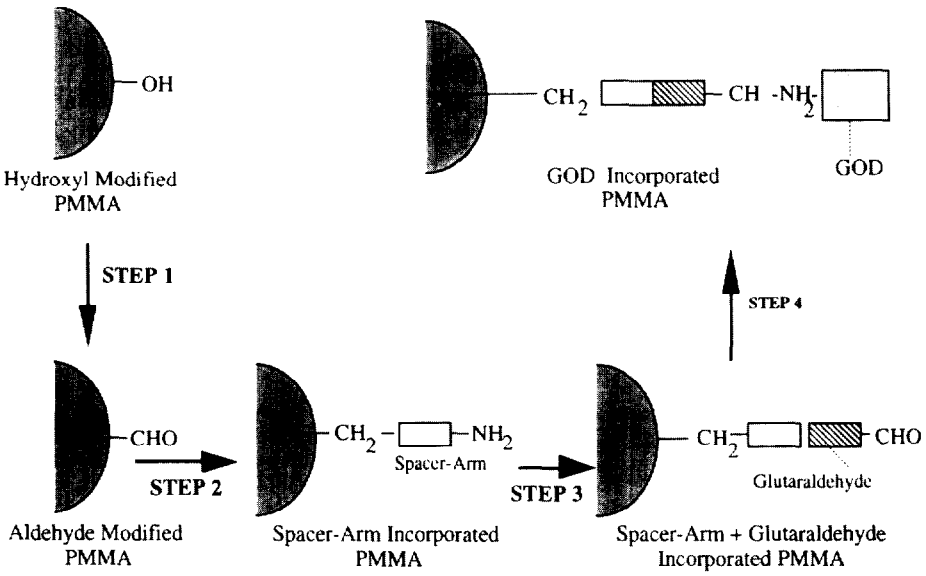

Fig. 2. Modification of PMMA microbeads and GOD immobilization.

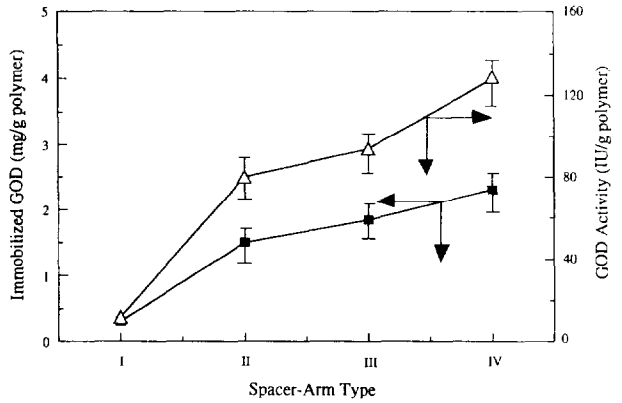

Fig. 3. Effects of spacer-arm length on GOD immobilization. I, without spacer-arm; II, ammonium hydroxide; III, ethylene diamine; and IV, hexamethylene diamine. Immobilization conditions: pH, 6.0; temp., 30 °C; time, 60 min; GOD initial conc., 0.10 mg ml^{-1}; aqueous phase, 5 ml of phosphate buffer; amount of microbeads, 0.10 g. Test conditions: pH, 6.0; temp., 25 °C; substrate conc., 20 mM.

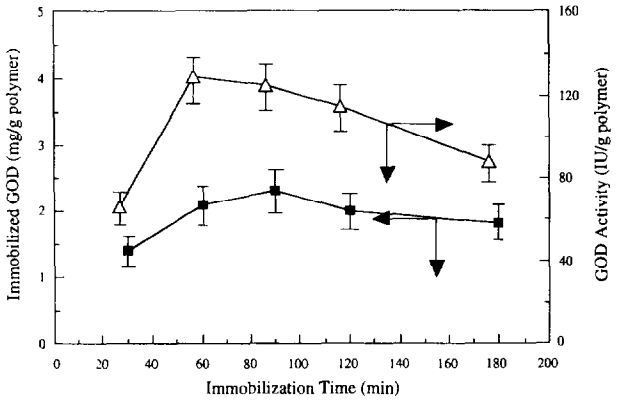

Fig. 4. Effects of immobilization time on GOD immobilization. Immobilization conditions: pH, 6.0; temp., 30 °C; GOD initial conc., 0.10 mg ml^{-1}; aqueous phase, 5 ml of phosphate buffer; amount of microbeads, 0.10 g. Test conditions: pH, 6.0; temp., 25 °C; substrate conc., 20 mM.

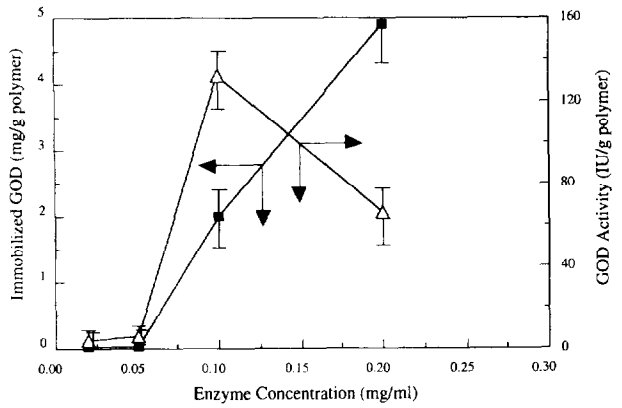

Fig. 5. Effects of GOD initial concentration on GOD immobilization. Immobilization conditions: pH, 6.0; temp., 30 °C; time, 60 min aqueous phase, 5 ml of phosphate buffer; amount of microbeads, 0.10 mg ml^{-1}. Test conditions: pH, 6.0; temp., 25 °C; substrate conc., 20 mM.

approximately 2.1 mg g^{-1} polymer, when hexamethylene diamine was used as the spacer-arm (14 atomic length). The activity of these microbeads was about 129 IU g^{-1} polymer which was again the highest GOD activity that we have observed at that specific immobilization and activity test conditions. This is a general behaviour which has been observed also by others in the immobilization of large molecules including enzymes [6,7,9]. Both undesirable interactions between the functional groups on the carrier surface and the enzyme molecules, and steric hindrance may be reduced by using spacer-arms, and therefore higher amounts of enzymes were immobilized with higher activities. Hexamethylene diamine was the best spacer-arm in our case, therefore we have used the PMMA microbeads modified with this compound in the other parts of the study.

3.2. *Effects of immobilization time on GOD immobilization*

GOD immobilizations were studied at different immobilization times between 30 and 180 min. Fig. 4 gives the changes in both the amount and activity of immobilized GOD with immobilization time. The amount of GOD immobilized first increased with immobilization time then slightly decreased, possibly because of conformational changes in the GOD molecules with time. Significant losses in the enzyme activity after 60 min may be considered as another indication of these conformational changes. According to these results we have concluded that 60 min is the optimum time for GOD immobilization at the conditions studied, and therefore in the other experiments this immobilization time was employed.

3.3. *Effects of GOD initial concentration on GOD immobilization*

In this group of experiments, immobilizations were repeated with four different initial GOD concentrations (0.02, 0.04, 0.10, 0.20 mg ml^{-1}). As illustrated in Fig. 5, the increase in the initial GOD concentration caused a significant increase in the amount of immobilized enzyme. However, there was a pronounced decrease in the enzyme activity using a GOD initial concentration greater than 0.10 mg ml^{-1}. These effects may be explained as follows: increasing the enzyme concentration resulted an aggregation of enzyme molecules on the polymer surface in addition to the covalent bonding through active points, and this led to blockage of the active sites on GOD molecules resulting in a drop in immobilized enzyme activity.

3.4. *Effects of pH on GOD immobilization*

The effects of pH on GOD immobilization were investigated in this group of experiments. The pH of the immobilization medium was changed between 3.0 and 8.0. As seen in Fig. 6, the pH of the immobilization medium significantly affected both the amount of immobilization and activity. The maximum amount of immobilization (4.2 mg g^{-1} polymer) was found at pH 4.0, which is very close to the isoelectric point of GOD. At this pH, GOD molecules have no net charge, which minimizes the electrostatic repulsion between

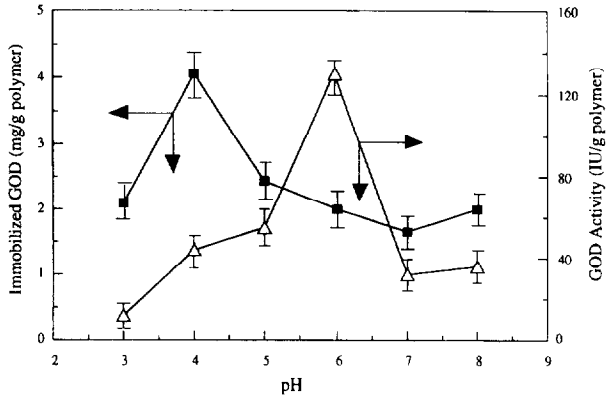

Fig. 6. Effects of immobilization pH on GOD immobilization. Immobilization conditions: temp., 30 °C; GOD initial conc., 0.10 mg ml^{-1}; time, 60 min; aqueous phase, 5 ml of phosphate buffer; amount of microbeads, 0.10 g. Test conditions: temp., 25 °C; substrate conc., 20 mM.

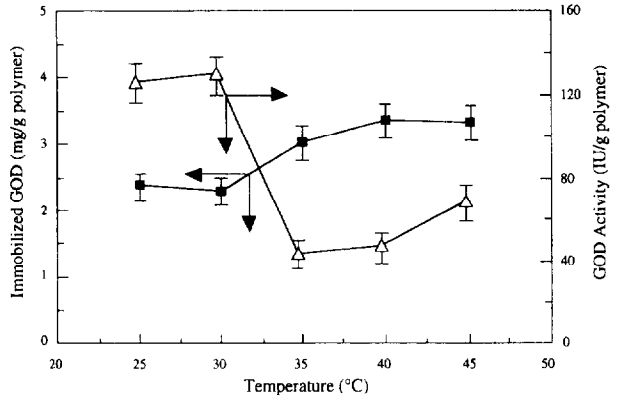

Fig. 7. Effects of immobilization temperature on GOD immobilization. Immobilization conditions: pH, 6.0; GOD initial conc., 0.10 mg ml^{-1}; aqueous phase, 5 ml of phosphate buffer; amount of microbeads, 0.10 g. Test conditions: pH, 6.0; substrate conc., 20 mM.

GOD molecules on the support material [12–15]. Fig. 6 clearly shows that higher amounts of immobilization of an enzyme on the support material does not always mean that they will exhibit higher activity. As in our case the maximum immobilized GOD activity (129 IU g^{-1} polymer) was at around pH 6.0. Therefore, in other parts of this study we conducted immobilizations at this pH.

3.5. *Effects of temperature on GOD immobilization*

In this group of experiments, the effect of temperature on GOD immobilization was studied in a temperature range of 25–45 °C. As seen in Fig. 7, the amount of immobilization increased with temperature, while the activity dropped significantly, especially above 30 °C. It is known that temperature is another important parameter which determines the three-dimensional structures or conformational states of enzymes, and therefore, as expected, changes both immobilization and activity [12,13]. Considering higher activities at lower temperatures, we selected an immobilization temperature of 30 °C as optimal in our conditions, and therefore, performed all immobilization at this specific temperature.

3.6. *Performance of GOD-immobilized PMMA microbeads*

We have also examined the performance of GOD-immobilized PMMA microbeads which were prepared under the following conditions: immobilization pH, 6.0; temperature, 30 °C; time, 60 min; initial GOD concentration, 0.10 mg ml^{-1}; aqueous phase, 5 ml of phosphate buffer; and amount of PMMA microbeads, 0.10 g.

In the first group of experiments, we changed only pH of the test medium between 3.0 and 8.0. The temperature and substrate concentration were 25 °C and 20 mM, respectively. For comparison we repeated these experiments with free enzyme. We used 0.50 mg free enzyme or 0.24 g of the GOD carrying PMMA microbeads in the respective experiments. Fig. 8 shows these results. The effects of pH on the mechanism of the glucose oxidation by glucose oxidase were

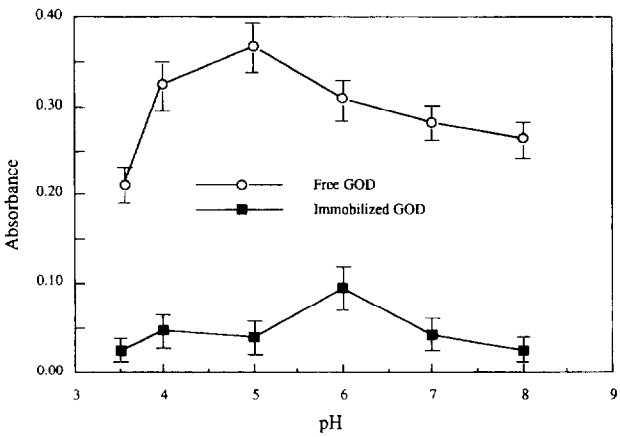

Fig. 8. Effects of pH on performance of free and immobilized GOD. Temp., 25 °C; substrate conc., 20 mM; 0.50 mg free enzyme or 0.24 g GOD carrying PMMA microbeads.

explained in detail by Wilson and Turner in their review article [15]. The free GOD molecules exhibited the maximum activity at around pH 5.0 [15–17], while the maximum activity was observed at around pH 6.0 in the case of immobilized GOD. Similar behaviour was also reported in the related literature [12,17–19].

In the second group of experiments, we changed the temperature of the test medium to between 25 and 45°C, but kept the pH and substrate concentration constant at 6.0 and 20 mM, respectively. We used 0.50 mg free enzyme or 0.24 g GOD carrying PMMA microbeads in the respective experiments. Both free and immobilized forms exhibited the maximum activity at the same temperature which was around the temperature of 30 °C.

In the final group of experiments, we studied the effects of substrate (i.e. glucose) concentration on the GOD-catalyzed reaction rate, with both free and immobilized GOD. These experiments were conducted at pH 6.0 and at a temperature of 25 °C. We used 0.50 mg free enzyme or 0.24 g GOD carrying PMMA microbeads in these experiments. The substrate (i.e. glucose) concentration was varied between 5 and

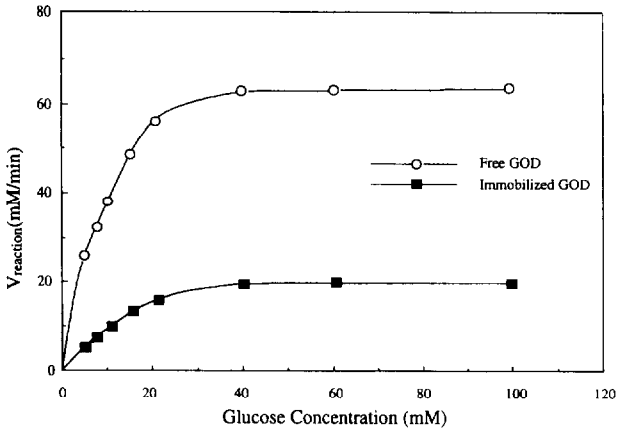

Fig. 9. Reaction rates of free and immobilized GOD at different glucose concentrations. pH, 6.0; temp., 25 °C; substrate conc., 20 mM; 0.50 mg free enzyme or 0.24 g GOD-immobilized PMMA microbeads.

Table 1
K_m and V_{max} values for free and immobilized GOD (pH 6, 30 °C)

	K_m (mM)	V_{max} (mM min^{-1})
Free GOD	6.65	61.18
Immobilized GOD	13.79	26.31

100 mM. The changes in reaction rates for free and immobilized GOD with glucose concentration are given in Fig. 9.

A similar bchaviour was observed with both free and immobilized GOD in the studied concentration range, as illustrated in this figure. The reaction rate increased linearly up to about 20 mM glucose concentration, then reached the plateau values [12].

The Michealis constant (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized GOD were calculated by using a non-linear regression computer program SYSTAT [20]. In general, apparent K_m values of immobilized enzymes are higher, and V_{max} values are lower than those for free enzymes, mainly due to diffusion limitations and steric hindrances in the immobilized form, which was also similar in our case as shown in Table 1 [12,21].

4. Conclusion

GOD was successfully immobilized onto the monosize modified PMMA microspheres (1.5 μm in diameter) by using hexamethylene diamine as a spacer-arm. Optimum immobilization yields and activities (2.1 mg g^{-1} polymer and 129 IU g^{-1} polymer, respectively) were achieved at pH 6.0, at a temperature of 30 °C with an immobilization time of 60 min and with a GOD initial concentration of 0.10 mg ml^{-1}. The GOD-immobilized PMMA microbeads were effectively operated at pH of 6.0 and at a temperature of 30 °C. The Michealis constant (K_m) of the immobilized GOD was lower than those of free GOD, while there was more pronounced difference in the maximum reaction velocities (V_{max}).

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