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# Properties of a glucose oxidase covalently immobilized on amorphous $AlPO_4$ support

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**Abstract**

Glucose oxidase (GOD) was covalently immobilized on amorphous $AlPO_4$ as well as on an $AlPO_4$/clay mineral Sepiolite system. Immobilization of the enzyme was carried out through the ε-amino group of lysine residues through an aromatic Schiff's-base. Activation of the support was obtained after reaction of appropriate molecules with support surface –OH groups. The enzymatic activities of native, and different immobilized GOD systems and filtrates, were followed by the amount of liberated D-gluconic acid obtained in the enzymatic β-D-glucose oxidation with the aid of an automatic titrator. The kinetic properties of native and immobilized GOD were obtained for glucose concentrations in the range of physiological conditions and at different working conditions such as reaction temperature, reaction pH, and enzyme concentration.

The binding percentage of enzymes was in the 50–80% range, with residual and specific activities in the 65–80% and 90–150% ranges, respectively. No change in the pH optimum and only slight changes in the $V_{max}$ and $K_M$ kinetic parameters with respect to native GOD were observed, so that not only was little deactivation of enzyme obtained throughout the immobilization process but also that the stability of the covalently bound enzyme in the two supports appeared to have increased with respect to the soluble enzyme. GOD immobilization also increased its efficiency and operational stability in repeated uses on increasing the amount of immobilized enzyme. 

*Keywords:* Covalent immobilization; Immobilized glucose oxidase; Inorganic support; Michaelis–Menten equation; Amorphous $AlPO_4$; Glucose oxidase; Immobilized enzyme

## 1. Introduction

Glucose oxidase (GOD) is very widely used in clinical and chemical analysis as well as in foodstuffs industries [1,2]. In these procedures, enzyme immobilization is a useful tool to achieve technological advantages and to meet cost savings [6]. Some immobilized enzyme devices form a major category of what have been termed biosensors, including from test strips [3] to enzyme electrodes, [4,5], analytical devices containing GOD as well as some conducting material used as electrode.

Covalent binding to an insoluble support is the most interesting enzyme immobilization methodology because it combines the high selectivity of enzy-

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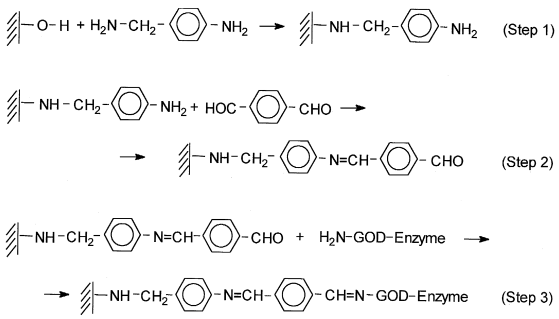


Fig. 1. General scheme for covalent immobilization of the enzyme through the ε-amino group of lysine residues. Activation of $AlPO_4$ support by microwave heating with 4-aminobenzylamine (step 1), and terephthaldicarboxaldehyde (step 2) before covalent immobilization of the enzyme through the lysine residues (step 3).

matic reactions with the chemical and mechanical properties of the support [3,7]. Covalent immobilization on the external surface of a support material has also been proposed to decrease mass transfer limitations associated with several immobilization techniques, such as entrapment or adsorption in gels. In this respect, inorganic matrices have a number of advantages over organic ones: no swelling and no porosity changes occur with pH; there is excellent storage stability of enzymes and they are not subject to microbial attack [3,6–8].

However, many references can be obtained in the literature about immobilized GOD used as a biosensor where polymer membranes are in most cases used as GOD carriers [9–14]. Comparatively, references to immobilized GOD on inorganic supports are always very reduced. In these cases, silica gel is the most common support where immobilization is carried out by sol–gel entrapment [15–17], by using some polymer-attaching technique [18] or glutaraldehyde as the covalent linker [19]. Thus, some difficulties arise when trying to obtain an inorganic solid, which has surface organic molecules able to attach themselves to some biomolecules [20]. Silica gel has received great attention due to its ability to immobilize organic functional groups on its surface by using some organosilane-coupling agent, which reacts with the silanols on the solid surface [21].

In this study, the kinetic properties of GOD have been obtained when covalently immobilized on amorphous $AlPO_4$, as well as on an $AlPO_4$/clay mineral Sepiolite system as inorganic supports. Enzymatic activities of native and immobilized GOD were determined for glucose concentrations in the range of physiological conditions and different working conditions such as reaction temperature, reaction pH, and enzyme concentration. In this regard, we have previously reported the use of amorphous $AlPO_4$ and a clay mineral Sepiolite, as metal supports [22–25] as well as heterogeneous catalysts in the field of selective organic synthesis [26–32]. This amorphous material, tailored by a controlled sol–gel method that allows us to obtain a high surface area as well as a high number of surface –OH groups, is a very adequate support component for the covalent attachment of enzymes, according to the results obtained in the immobilization of lipases, phosphatase and GOD enzymes [33–36].

According to the general scheme in Fig. 1, covalent immobilization of the enzyme is carried out through the ε-amino group of lysine residues (Step 3). Due to the increased electrophilic character of benzaldehyde, the aromatic Schiff's-base obtained [33–36] seems to be more stable than those usually obtained by using glutaraldehyde [10,37] or other aliphatic aldehydes like gycidol [38]. Functionalization of the support surfaces (Steps 1 and 2) is developed by anchoring a functionalized linker with pendant benzaldehyde through the reaction of appropriate molecules with the surface –OH groups of inorganic solids.

## 2. Experimental

### 2.1. Support synthesis and support functionalization

Amorphous $AlPO_4$ was prepared according to a sol–gel method previously described [22–36]. This

Table 1
Surface area, $S_{BET}$ ($m^2\ g^{-1}$) and acid–base properties of inorganic supports vs. irreversibly adsorbed ($\mu mol\ g^{-1}$) pyridine (PY, $pK_a = 5.25$), 2,6-dimethylpyridine (DMPY, $pK_a = 6.99$) and benzoic acid (BA, $pK_a = 4.19$)

| Support | $S_{BET}$ | Acidity | | Basicity |
|---|---|---|---|---|
| | | PY | DMPY | BA |
| $AlPO_4$ | 211 | 200 | 249 | 352 |
| Sepiolite | 233 | 10 | 13 | 115 |
| $AlPO_4$/Sepiolite | 139 | 198 | 128 | 393 |

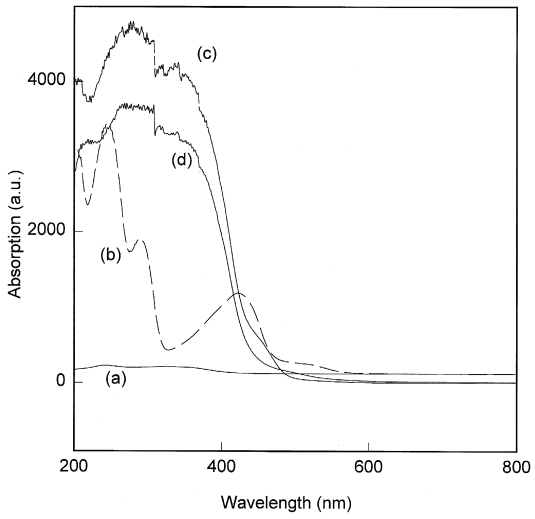


Fig. 2. Visible-Ultraviolet/Diffuse Reflectance spectra of different samples obtained along different steps in Fig. 1. (a) $AlPO_4$ support, (b) 4-aminobenzylamine on support after microwave heating (step 1); (c) terephthaldicarboxaldehyde reaction (step 2) and (d) immobilized GOD through the ε-amino group of lysine residues (step 3).

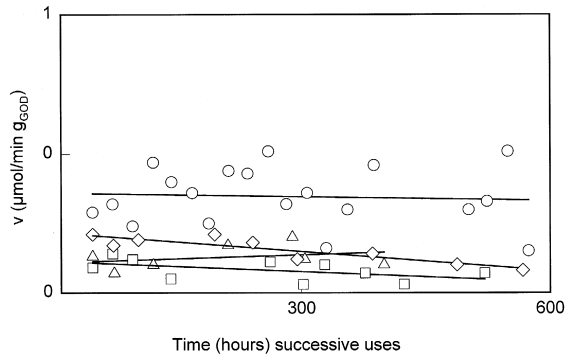


Fig. 4. Influence of re-uses on the catalytic activity of 0.02 g of GOD immobilized on 4 g of $AlPO_4$ support, when it is operated in the same batch reaction by successive addition of 0.25 $10^{-3}$ g of glucose, under standard experimental conditions, at 30°C and different reaction pH. (△) pH = 6.0; (○) pH = 7.0; (□) pH = 8 and (◇) pH = 9.

support was obtained by precipitation from aqueous solutions of $AlCl_3 \cdot 6H_2O$ and $H_3PO_4$ (85 wt.%) at pH = 6.1 by addition of ammonium hydroxide. The solid obtained after filtration was then washed with isopropyl alcohol and dried at 120°C for 24 h. In the present case, the resulting powder was calcined by heating at 350°C for 3 h and then screened to a particle size $< 0.149$ mm (100 mesh size). An $AlPO_4$/Sepiolite (20–80 wt.%) system was obtained by adding sepiolite to a reaction medium where the precipitation of $AlPO_4$ was initiated as indicated above. The support $AlPO_4$/Sepiolite was obtained after undergoing the identical treatments undergone by pure $AlPO_4$ support. Natural sepiolite, a hydrous magnesium silicate: $Si_{12}Mg_8O_{32} \cdot nH_2O$, exhibiting a nominal chemical composition of $SiO_2$ 62.0, MgO 23.0, $Al_2O_3$ 1.7, $Fe_2O_3$ 0.5, CaO 0.5, $K_2O$ 0.6 and $Na_2O$ 0.3, is an inexpensive clay mineral from Vallecas (Madrid) supplied by Tolsa. The procedure for the textural and acid–base properties of these solids has been published elsewhere [22–29]. The surface areas, $S_{BET}$ in $m^2\ g^{-1}$ determined by nitrogen adsorption from the B.E.T. method, are summarized in Table 1, where the surface acidity and basicity of

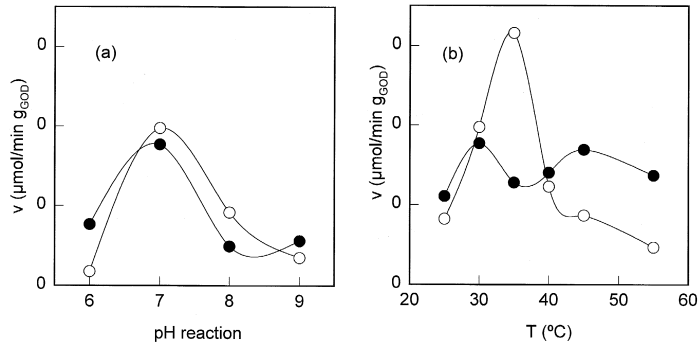


Fig. 3. Influence of the reaction pH and temperature on the catalytic activity of native (○) and immobilized enzyme (●) under standard experimental conditions, with 0.25 $10^{-3}$ g of glucose substrate weight and at: (a) 30°C and different reaction pH and (b) pH = 7 and different reaction temperatures.

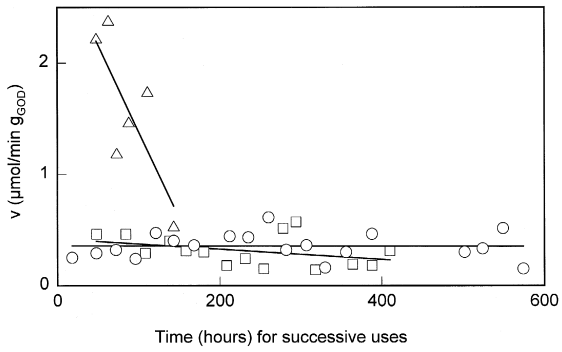


Fig. 5. Influence of re-uses on the catalytic activity of different enzymatic systems when it is operated in the same batch reaction by successive addition of 0.25 $10^{-3}$ g of glucose, under standard experimental conditions, at 30°C and reaction pH = 7. These systems are obtained when different amounts of GOD are immobilized on 4 g of $AlPO_4$: (△) 0.005 g of GOD; (□) 0.010 g of GOD and (○) 0.020 g of GOD.

supports are also collected. These values were determined by a spectrophotometric method that allows titration of the amount (in μmol $g^{-1}$) of irreversible adsorbed pyridine (PY, $pK_a = 5.25$), 2,6-dimethylpyridine (DMPY, $pK_a = 6.99$), and benzoic acid (BA, $pK_a = 4.19$), employed as titrant molecules of acid and basic sites, respectively.

Activation of the $AlPO_4$ support surface was initiated by anchoring a functionalized linker through the reaction of 4-aminobenzylamine with support surface –OH groups [33–36], according to step 1 in Fig. 1. The linker was obtained by a microwave heating reaction (15 min at 380 W) of the support (20 g) and 4-aminobenzylamine (4 g) and, after that, the composite is made to react with terephthaldicarboxaldehyde (step 2) by microwave heating (5 min at 380 W). As a consequence of the high conjugation of the molecule, a yellow solid was obtained.

### 2.2. God immobilization and enzymatic activity

Immobilization of GOD was carried out by thoroughly mixing from time to time, for 48 h at 4°C, the activated solid (4 g) and GOD (Sigma Type X-S, 0.001 g) (β-D(+)-Glucose oxygen 1-oxidoreductase, EC 1134) from *Aspergillus niger* in 25 ml of phosphate buffer ($KH_2$ $PO_4$, 0.03 M, pH 5.00). Finally, the immobilized GOD was collected by centrifugation and the resulting filtrate was separated to obtain the activity of supernatant GOD. Before the first use of immobilized GOD, the system was "equilibrated" at room temperature for 24 h with 50 ml of phosphate buffer at the prefixed reaction pH constant value. Efficiency of Steps 1–3, in Fig. 1, are confirmed by Visible-Ultraviolet/Diffuse Reflectance experiments shown in Fig. 2. Spectra were recorded on a UV-Visible Diffuse Reflectance Spectrophotometer Varian Carey 1E.

Enzymatic activities of different soluble and immobilized GOD systems were obtained in the oxidation of β-D(+)-Glucose to D-gluconic acid, carried out under stirring by a magnetic bar in a batch

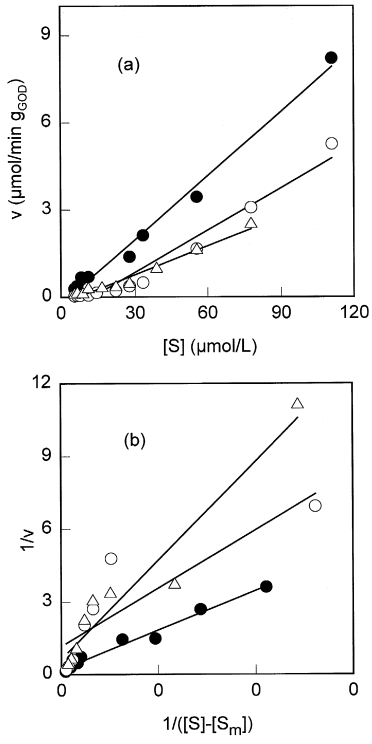


Fig. 6. Influence of the substrate concentration on the catalytic activity of native (●) and immobilized enzyme on $AlPO_4$ support (○) as well as on $AlPO_4$/Sep support (△), using low substrate concentration, under standard experimental conditions at 30°C and reaction pH = 7. (a) Relationships between initial specific velocities, *V*, and initial substrate concentrations, [*S*], and (b) the corresponding double reciprocal plot of Eq. (8), including the [$S_m$] values in Table 4. In both immobilized GOD systems, the immobilized enzyme used was always the same throughout the experiment, so that in every new reaction there is also, in fact, a new re-use.

Table 2
Percentage of immobilized enzyme, $E_{imm}$, residual activity, $E_{res}$, and specific activity of immobilized enzyme, $E_{spe}$, determined from the corresponding values of the catalytic activity of the native enzyme, $r_{nat}$, the activity of the filtrate in the immobilization process, $r_{fil}$, and the activities of immobilized enzyme, $r_{imm}$, obtained with different amounts of immobilized GOD on 4 g of $AlPO_4$ support, under standard operating conditions

| GOD (g) | $r_{nat}\ 10^3$ (μmol/min) | $r_{fil}\ 10^3$ (μmol/min) | $r_{imm}\ 10^3$ (μmol/min) | $E_{imm}$ (%) | $E_{res}$ (%) | $E_{spe}$ (%) |
|---|---|---|---|---|---|---|
| 0.005 | 7.68 | 3.46 | 6.08 | 54.95 | 79.17 | 144.08 |
| 0.010 | 5.01 | 2.45 | 3.76 | 51.10 | 75.05 | 146.88 |
| 0.020 | 7.90 | 1.86 | 5.42 | 76.46 | 68.61 | 89.73 |
| 0.020[a] | 7.19 | 2.80 | 5.06 | 61.05 | 70.38 | 115.27 |

[a] $AlPO_4$/Sepiolite as support.

reactor system (100 ml). With the aid of an automatic titrator, Crison, mod. micro TT 2050 TP, enzymatic activities were followed by the amount of NaOH 0.1 M necessary to neutralize liberated D-gluconic acid obtained from enzymatic glucose oxidation at the prefixed glucose concentration, reaction temperature and pH constant value. Thus, according to Mosbach et al. [39], enzymatic activity of GOD can be nicely measured by the pH change produced by the gluconic acid at low buffer capacity.

The selected amount of GOD was dissolved in 10 ml of phosphate buffer ($KH_2PO_4$, 0.03 M, pH 7.00) and added to 40 ml of phosphate buffer containing glucose (usually $0.25\ 10^{-3}$ g) to obtain reaction rates of native enzymes, $r_{nat}$. Identical experimental conditions were used with filtrates of supernatant GOD, $r_{fil}$, and with immobilized GOD systems, $r_{imm}$. Reaction rates, $r$, (in μmol min) were determined by taking the slope of the linear plot of NaOH consumption vs. time. Diffusional limitations in $r_{imm}$ values were minimized by the sufficiently small particle size of the supports ($< 0.149$ mm). Taking into account that GOD is mostly used for measuring glucose concentrations in the range of physiological conditions, the kinetic behavior was determined for glucose concentrations in the range 5–115 μmol/l. In addition, some kinetic properties of native and immobilized GOD were determined at different working conditions such as reaction temperature, reaction pH, and enzyme concentration. Results obtained under these different experimental conditions are shown in Figs. 3–6 and Tables 2 and 3.

## 3. Results and discussion

The properties of covalently immobilized enzyme at different working conditions such as enzyme concentration and reaction pH, were easily determined from experimental results in Tables 2 and 3, [40]. Thus, the percentage of immobilized enzyme, $E_{imm}$, was determined by the difference between the catalytic activity of the native enzyme and the activity

Table 3
Influence of the reaction pH on the catalytic activity of 0.020 g of native and immobilized GOD on 4 g of $AlPO_4$ support, under standard reaction conditions

| pH | $r_{nat}\ 10^3$ (μmol/min) | $r_{fil}\ 10^3$ (μmol/min) | $r_{imm}\ 10^3$ (μmol/min) | $E_{imm}$ (%) | $E_{res}$ (%) | $E_{spe}$ (%) |
|---|---|---|---|---|---|---|
| 6.0 | 0.71 | 0.36 | 1.51 | 49.29 | 212.68 | 431.43 |
| 7.0 | 7.90 | 1.86 | 5.42 | 76.46 | 68.61 | 89.73 |
| 8.0 | 3.66 | 1.39 | 1.22 | 62.02 | 33.33 | 53.74 |
| 9.0 | 1.39 | 0.92 | 0.76 | 33.81 | 54.68 | 161.70 |

of the filtrate in the different immobilization processes:

$$E_{imm} = \frac{r_{nat} - r_{fil}}{r_{nat}} \times 100. \quad (1)$$

The residual activity upon immobilization, obtained from the percentage of catalytically active immobilized enzyme, $E_{res}$, was obtained from the relation between the activities of immobilized and native enzymes:

$$E_{res} = \frac{r_{imm}}{r_{nat}} \times 100. \quad (2)$$

For Michaelis–Menten-based enzyme kinetics, this relation between the activities of immobilized and native enzymes also indicates the extent of mass transfer control in the enzymatic reaction [3], that is usually expressed by the efficiency coefficient or effectiveness factor, $\eta$, which may be practically considered like the quotient $E_{res}/100$.

The specific activity, $E_{spe}$, indicating the efficiency of the immobilized enzyme, with respect to the native one, was obtained from the relation:

$$E_{spe} = \frac{E_{res}}{E_{imm}} \times 100 = \frac{r_{imm}}{r_{nat} - r_{fil}} \times 100. \quad (3)$$

The different values obtained are also shown in Tables 2 and 3. In Table 2, we can see that, in the different immobilization processes, the percentages of immobilized GOD obtained, $E_{imm}$, were in the 51–76% range. These values must correspond to the yield of step 3, in Fig. 1. With respect to this, in Fig. 2, UV-Visible experiments enable us to follow the changes obtained in the support surface by the effects of different reactions carried out in every step of Fig. 1, including GOD attachment by formation of aromatic Schiff's-bases with the lysine enzyme residues. The existence of a high number of –OH surface groups in $AlPO_4$ may be deduced from the great and very close amounts of acid sites titrated with DMPY with respect to those titrated with PY. It is known that DMPY is selectively adsorbed on Brönsted acid sites, but not on Lewis acid sites because of a steric hindrance of two methyl groups, whereas sterically non-hindered PY is adsorbed on both Brönsted and Lewis acid sites. Thus, the present results indicate that, in fact, all surface acid sites in $AlPO_4$ titrated with both amines are Brönsted sites. Thus, in Table 2, the higher values of $E_{imm}$ obtained with amorphous $AlPO_4$ with respect to the $AlPO_4$/Sepiolite system, can be explained from the higher values in DMPY obtained in the former support, according to the results shown in Table 1. At the same time, here we can see the important changes developed on the Sepiolite surface after the treatment to obtain the $AlPO_4$/Sepiolite system. While the external surface area, $S_{BET}$, is clearly reduced, the number of –OH surface groups, titrated with DMPY, increased 10-fold. Consequently, the addition of only 20 wt.% of $AlPO_4$ to Sepiolite achieves an important increase in the external surface –OH groups of the support system. Thus, any inorganic solid with high enough surface area could be used as a support after activation with $AlPO_4$ under the conditions here studied.

An extensive immobilization is not enough to obtain an appropriate behavior of the enzyme because, after immobilization, the enzyme sometimes becomes inactive [36]. Among other possible causes, this can be due to the fact that the active sites of the enzyme may be involved in its attachment to the support surface. The high values of $E_{res}$, also shown in Table 2, indicate little deactivation of GOD throughout the immobilization process as well as low influence of mass transfer effects on reaction rates in immobilized GOD systems [3].

On the other hand, the high values of $E_{spe}$, point out that GOD immobilization increases its efficiency to a varied extent with respect to the soluble enzyme, whatever experimental conditions are used. However, some changes can be obtained for different supports and different amounts of immobilized enzyme. Thus, instead of the very close values of $E_{res}$ obtained by the two supports studied, we have different $E_{spe}$ values. Besides, on increasing the amount of immobilized enzyme (from 0.005 to 0.020 g), $E_{imm}$ increased while both $E_{res}$ as well as $E_{spe}$ decreased.

Results obtained at different pH reaction conditions in Table 3 as well as in Fig. 3a, indicate that the highest activity of native and immobilized enzyme was obtained at pH = 7. However, results shown in Fig. 3b, indicate that temperature promotes different behavior in the native enzyme than in the immobilized one. Thus, native enzyme clearly exhibits a maximum at 35°C while the temperature

influence on reaction rate is not so clear in the immobilized GOD. Sometimes, immobilized enzymes are less temperature-dependent because reaction rates are diffusion controlled [3], but this behavior cannot be directly associated to low values of the efficiency factor, $\eta$, according to the reasonably high values of $E_{res}$, in Table 2. Actually, in both cases, immobilization increases its efficiency with respect to the soluble enzyme, this efficiency increasing as the experimental conditions used became more extreme in pH and temperature.

On the other hand, operational stability in the repeated uses of immobilized GOD was also high at different pH studied for the system with 0.02 g of GOD on 4 g of support $AlPO_4$. Thus, to determine the stability of immobilized enzyme, one set of experiments was developed by operating continuously under batch conditions by successive addition of the same amount of glucose ($0.25\ 10^{-3}$ g) under standard experimental conditions, at 30°C and the different pH reaction values also studied in Table 3. Results shown in Fig. 4 indicate that after a high number of consecutive cycles for 3 weeks, residual activity was always near 90%, independently of the reaction rate obtained under the different pH conditions studied. Furthermore, when soluble GOD was used in the same way, its catalytic activity was negligible after 24 h.

Enzyme immobilization also increased its efficiency and operational stability in repeated uses on increasing the amount of immobilized enzyme. Thus, in Fig. 5, we can see the influence of re-uses on the catalytic activity of the different enzymatic systems studied in Table 2, when it was operated in the same batch reaction by successive addition of $0.25\ 10^{-3}$ g of glucose, under standard experimental conditions, at 30°C and reaction pH = 7. The use of the specific enzymatic activity values, $V$, (i.e. enzyme activity units/g of GOD, in $\mu$mol/min $g_{GOD}$) permits a better comprehension of the results in Table 2. Immobilization of lower amounts of GOD gave higher efficiency values (higher $E_{spe}$ values) but, at the same time, operational stability was comparatively much lower. Thus, from slopes and intercepts, in Fig. 5, we find that, while in the system arranged with 0.02 g of GOD, the mean residual activity was 97% (after re-use in 19 consecutive cycles for 4 weeks), in the system obtained with 0.01 g of GOD mean residual activity was 63% (after re-use in 15 consecutive cycles for 2 weeks) and with the system prepared with 0.005 g of enzyme, mean residual activity was only 11% (after re-use in six consecutive cycles for a week).

This behavior could be explained by the low proportion of glucose substrate concentration with respect to the available number of GOD active sites, under the selected experimental conditions. Thus, the Michaelis–Menten model assumes that for low enough values of substrate concentrations, the observed reaction rate is proportional to the substrate concentration, and only depends on how efficiently the enzyme can bind the substrate at that concentration, under the other selected experimental parameters like pH, temperature, or ionic strength [2,6,41,42]. Here, we have $[S] \ll K_M$, where $[S]$ is the substrate concentration, $K_M$ the Michaelis constant for the enzyme and the reaction rate is first order in the $[S]$ following:

$$V = \frac{k_{cat}[E_0][S]}{K_M + [S]} = \frac{V_{max}[S]}{K_M} \quad (4)$$

where $[E_0]$ is the total amount of enzyme and $k_{cat}$ the first order rate constant for the reaction that represents the number of micromoles of substrate converted per unit weight of enzyme per minute. $V_{max}$ is the maximum rate at which the reaction can take place when running under zero order reaction conditions with no diffusional constraints.

In this way, when $[S]$ is low enough respect to $[E_0]$, only a minor portion of enzyme is active during operation. The other unused portion may, in simple terms, replace the enzyme as it is being inactivated in the normal course of the reaction. Constant values of $V$ will be obtained for a so long time as being available in some enzyme in reserve. Thus, in Fig. 5, we can see that the higher amounts of immobilized GOD determines the slower decay in $V$ along the time. The progressive GOD deactivation during its use must not be noted by a change in $V$ until the increased proportion of glucose substrate, with respect to the immobilized GOD still active, determines a change in the kinetic behavior, that is, when the reaction becomes zero order in glucose concentration following the rate expression:

$$V = k_{cat}[E_0] = V_{max}. \quad (5)$$

From the Lineweaver-Burk double reciprocal linear form [2,5,41,42], the kinetic parameters $K_M$, $k_{cat}$ and $V_{max}$ can be obtained:

$$\frac{1}{V} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}. \quad (6)$$

Thus, the influence of the substrate concentration on the catalytic activity of native and immobilized enzymes on $AlPO_4$ as well as on $AlPO_4$/Sepiolite supports, was determined for glucose concentrations in the range 5–115 μmol/l, at 30°C and pH = 7, under standard experimental conditions. In both immobilized GOD systems, the enzymatic catalyst used was always the same during the experiment, so that in every new reaction with different $[S]$, there was also, in fact, a new re-use. The kinetic parameters $K_M$, $k_{cat}$ and $V_{max}$ could be obtained from Eqs. (4) and (6) by plotting the relationships between initial specific activity of GOD, $V$, and initial substrate concentrations, $[S]$, and the corresponding double reciprocal linear form. In Fig. 6, the corresponding results are plotted for enzyme amounts $[E_0] = 1$ g, so that, according to Eq. (5), the corresponding values of $k_{cat}$, and $V_{max}$, are coincidental.

However, we can see in Fig. 6a that negative intercepts were obtained. Consequently, Eq. (4) and (6) could not be used to obtain these kinetic parameters. This behavior is sometimes produced at very low substrate concentrations [2] and can be ascribed to the development of the Michaelis–Menten equation itself (Eq. 4), where no provision is made for any reverse reaction [2,6]. More realistic models have been proposed in the development of the Michaelis–Menten equation including the thermodynamic driving forces due to substrate and product concentration [6]. According to this, the results obtained could be explained if we take into account that a threshold concentration of glucose would be needed to obtain some GOD enzymatic activity. This minimal amount of substrate $[S_m]$ represents the $[S]$ value where $V = 0$ in every GOD system in Fig. 6a. It could be easily obtained from slopes and intercept values in Fig. 6a, considering a new expression for Eq. (4) where the postulated $[S_m]$ is incorporated:

$$V = \frac{k_{cat}[E_0]([S] - [S_m])}{K_M + ([S] - [S_m])}$$

$$= \frac{V_{max}}{K_M}([S] - [S_m])$$

$$= \frac{V_{max}}{K_M}[S] - \frac{V_{max}}{K_M}[S_m]. \quad (7)$$

The results obtained from slopes ($V_{max}/K_M$) and intercepts ($V_{max}[S_m]/K_M$) as well as $[S_m]$ values are collected in Table 4. Similarly, the kinetic parameters $K_M$ and $V_{max}$ can now be obtained by inverting both sides of Eq. (7) to give a new Lineweaver-Burk double reciprocal linear form also including $[S_m]$:

$$\frac{1}{V} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S] - [S_m]} + \frac{1}{V_{max}}. \quad (8)$$

Thus, Table 5 collects the values of $V_{max}$ and $K_M$ that were obtained according to Eq. (8), from slopes ($K_M/V_{max}$) and intercepts ($1/V_{max}$) obtained by plotting $1/V$ vs. $1/([S] - [S_m])$ in Fig. 6b where the corresponding $[S_m]$ values in Table 4 are used.

Taking into account that the corresponding values of all kinetic parameters obtained are not very different, according to the results in Tables 4 and 5, we can again conclude that little deactivation was obtained throughout the immobilization process on ei-

Table 4
Experimental values of $[S_{min}]$ obtained by using Eq. (7) from the results plotted in Fig. 6a for native and immobilized enzymes on $AlPO_4$ support as well as on $AlPO_4$/Sep support. Uncertainties are determined up to a confidence limit of 95%. Significance levels from the correlation obtained were always higher than 99%

| GOD enzyme system | $V_{max}/K_M$ ($min^{-1}$) | $V_{max}[S_m]/K_M$ (μmol/min $g_{GOD}$) | $[S_m]$ (μmol/l) |
|---|---|---|---|
| Native | $0.074 \pm 0.003$ | $-0.23 \pm 0.01$ | $3.2 \pm 0.2$ |
| $AlPO_4$ | $0.048 \pm 0.003$ | $-0.61 \pm 0.04$ | $12.5 \pm 1.1$ |
| $AlPO_4$/Sepiolite | $0.033 \pm 0.002$ | $-0.23 \pm 0.01$ | $6.8 \pm 0.5$ |

Table 5
Experimental values of $V_{max}$ ($\mu$mol/min $g_{GOD}$) and $K_M$ (in $\mu$mol/l) obtained by using Eq. (8) from the results plotted in Fig. 6b for native and immobilized enzyme on $AlPO_4$ support as well as on $AlPO_4$/Sep support. Uncertainties are determined up to a confidence limit of 95%. Significance levels from the correlation obtained were always higher than 99%

| GOD enzyme system | $K_M/V_{max}$ (min) | $1/V_{max}$ (min $g_{GOD}/\mu$mol) | $V_{max}$ ($\mu$mol/min $g_{GOD}$) | $K_M$ ($\mu$mol/1) |
|---|---|---|---|---|
| Native | $8.2 \pm 0.5$ | $0.20 \pm 0.01$ | $5.1 \pm 0.3$ | $41.4 \pm 3.5$ |
| $AlPO_4$ | $12.0 \pm 3.1$ | $1.16 \pm 0.29$ | $0.9 \pm 0.2$ | $10.4 \pm 2.6$ |
| $AlPO_4$/Sepiolite | $20.4 \pm 2.4$ | $0.62 \pm 0.07$ | $1.6 \pm 0.2$ | $32.7 \pm 5.1$ |

ther of the two supports studied. However, the best results were always obtained with native enzyme, all GOD systems studied, both native and immobilized, could be used as an analytical devise to securely determine glucose concentration, by a kinetic measurement of the amount of NaOH necessary to neutralize liberated D-gluconic acid obtained from enzymatic glucose oxidation, at the physiological concentration range studied, when operated at $[S] \geq [S_m]$ values shown in Table 4.

## 4. Conclusions

The results obtained here, as compared to those described when GOD was covalently immobilized by Schiff's-base formation on NiO [43], as well as on controlled pore silica [44] or glass beads [45] as supports, through a silane coupling agent, allow us to conclude that GOD covalently immobilized on amorphous $AlPO_4$ or $AlPO_4$/Sepiolite supports could be advantageously used in most previously described applications [9–19]. Thus, no change in the pH optimum and only slight changes in the kinetic parameters $V_{max}$ and $K_M$ were observed with respect to soluble enzyme. Immobilized enzyme, $E_{imm}$, in the 50–80% range with high residual and specific activities, $E_{res}$, always near 75%, and $E_{spe}$ in the 90–150% range, clearly indicate that not only little deactivation of enzyme along the immobilization process was obtained, but also that the stability of the covalently bounded enzyme studied in the two supports appears to have increased over the soluble native enzyme.

In this respect, we can conclude that due to their textural and acid–base properties, amorphous $AlPO_4$ may not only be a very adequate support component in self, but also could be used as an activating agent of other inorganic supports towards the covalent attaching of organic chains to give new hybridized composites [46,47]. Thus, adding only 20 wt.% of $AlPO_4$ to the inexpensive and inactive Sepiolite, an important increase in the external surface –OH groups of the support system was obtained. Not only Sepiolite, but any inorganic solid with a high enough surface area could be similarly used as a support, after activation with $AlPO_4$ under the conditions here studied.

At the present time, to obtain a covalent attachment of some organic functionality on the surface of an inorganic support, a previous treatment of surface activation by reactions with some functional organosilane is the most commonly used procedure [21]. Subsequent reactions can expand the organic covalent chain to attach the desired complex-forming group to the main chain or to a side chain [48]. However, we could use amorphous $AlPO_4$ instead of the corresponding organosilane to obtain the activation of the inorganic support. Thus, we can directly obtain the functionalization of $AlPO_4$ surfaces, by the reaction of Brönsted acid sites (surface –OH groups) with suitable functionalized molecules such as 4-aminobenzylamine [33–36] (or *p*-hydroxy-benzaldehyde [33,34,36]) under microwave heating. Within the same general schemes currently used with silanized silica, these functionalized molecules attached on $AlPO_4$ surfaces can be modified by some reactions such as diazotization [36] or aromatic Schiff's-base condensation to give a new "spacer" of higher length [33–35].

In this way, due to the easy activation of surface –OH groups, the supports here studied open many possibilities associated with the facility for obtaining organic "linkers" of variable length, attached to the

$AlPO_4$ surface, within the general schemes for covalent immobilization of enzymes on silanized inorganic supports [20,21,49]. Thus, tailor-made hybridized organic/$AlPO_4$-inorganic composites, could be appropriately functionalized not only by linking some aliphatic or aromatic aldehyde or by diazotization but also, through several activation reactions with cyanuric chloride, carbodiimides, thiophosgene, thionyl chloride, or *N*-hydrosuccinimides [50], which may incorporate a variety of organic macromolecules, including biomolecules and enzymes, like GOD, with great efficiency and yield.

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