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Magnetic Nanoparticles Functionalized And Modified With Cross-Linking To Improve The Invertase Immobilization

Nanopartículas magnéticas funcionalizadas y modificadas con entrecruzamiento para mejorar la inmovilización de la invertasa

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Abstract

Procedures of immobilization invertase have been developed using different supports. However, disadvantages such as use of small particles for invertase immobilizations in packed-bed reactors are being solved using magnetic particles. In this study, composites containing Fe_3O_4 were prepared by incorporation of a polysiloxane layer required for the physical adsorption of the invertase. Besides, the functionalized magnetite was activated with glutaraldehyde and polyethylenimine (PEI) with the aim of performing a covalent immobilization. The effect of different conditions such as enzyme: support ratio, pH, and temperature were analyzed in the preservation of invertase. The results demonstrated that the optimum enzyme:support ratio is higher for covalent bonding than for physical adsorption. The ideal pH for the immobilized enzyme is 5.0, and the enzymatic activity is retained until 70 °C. The values of k_m are similar in both immobilization methods. The analysis of the effect of pH and thermostability showed that the catalytic activity of invertase is not affected in comparison with the free enzyme. The covalent immobilization displays higher efficiency in the immobilization process ($F\varepsilon$), less inhibition and twice as much stability. The enzymes immobilized by physical and covalent methods can be reused for up to four cycles and can be removed from the reaction medium by applying an external magnetic field.

***Keywords*:** invertase, Fe_3O_4, immobilization, composites

Resumen

Se han desarrollado procedimientos de inmovilización con invertasa utilizando diferentes soportes. Sin embargo, las desventajas como el uso de partículas pequeñas para inmovilizaciones de invertasa en reactores de lecho compacto se están resolviendo utilizando partículas magnéticas. En este estudio, los compuestos que contienen Fe_3O_4 se prepararon mediante la incorporación de una capa de polisiloxano necesaria para la adsorción física de la invertasa. Además, la magnetita funcionalizada se activó con glutaraldehído y polietilenimina (PEI) con el objetivo de realizar una inmovilización covalente. Se analizó el efecto de diferentes condiciones como la relación enzima: soporte, pH y temperatura en la conservación de la invertasa. Los resultados demostraron que la relación enzima: soporte óptima es mayor para la unión covalente que para la adsorción física. El pH ideal para la enzima inmovilizada es 5,0 y la actividad enzimática se mantiene hasta 70 °C. Los valores de k_m son similares en ambos métodos de inmovilización. El análisis del efecto del pH y la termoestabilidad mostró que la actividad catalítica de la invertasa no se ve afectada en comparación con la enzima libre. La inmovilización covalente muestra una mayor eficacia en el proceso de inmovilización ($F\varepsilon$), menos inhibición y el doble de estabilidad. Las enzimas inmovilizadas por métodos físicos y covalentes se pueden reutilizar hasta por cuatro ciclos y se pueden eliminar del medio de reacción aplicando un campo magnético externo.

***Palabras clave*:** invertasa, Fe_3O_4, inmovilización, compuestos

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1 Introduction

The enzymatic method is the most widely used to obtain inverted syrup with invertase (EC.3.2.1.26) from *Saccharomyces cerevisiae* strains [1]. However, the lifetime of invertase is short, and the bioactive species are inestable and less selective. The immobilization of invertase extends its lifetime, and the stability of bioactive species is enhanced due to the protection of the support. These characteristics are useful in bioreactors because the separation of the products is easier, and the enzymatic recovery is efficient [2]. So, different ways to immobilize invertase have been developed. Invertase has been immobilized on supports such as montmorillonite micropores [3], polyacrylonitrile composite fibers [4], functionalized silicon pores [5], nylon microspheres [6], chitosan nanoparticles [7], calcium alginate [8][9] and polyaniline-modified surfaces [10], hydrogel comprising of methacrylic acid (MAAc) and N-vinyl pyrrolidone (N-VP) [11], among others. As a result, these studies reported that immobilized invertase is most selective allowing the formation of bioproducts derived from glucose and fructose cyclization with temperatures ranging from 30 to 40 °C. However, in packed-bed reactors when small particles are used for invertase immobilization high pressure drops occur, a disadvantage that can be solved by using magnetic particles [12].

Invertase immobilization by covalent bonding on magnetic particles has been performed using nanocomposites of magnetic polyvinyl alcohol microspheres [12], or chitosan- coated γ-Fe_2O_3 [13], and in magnetic diatomaceous earth nanoparticles [14], Recently,with the aim of increase the enzyme adsorption Bayramoglu et al.,[15] studied the covalent immobilization of invertase by modifying magnetic nanoparticles of Fe_3O_4@SiO_2 with glycidyl methacrylate (GMA). Uzun et al. [16] immobilized invertase on polyamidoamine (PAMAM)-magnetite nanoparticles functionalized with aminopropyltrimethoxysilane (APTMS), which were then used for successive step-by-step addition of methylacrylate and ethylenediamine to form the dendritic structure of PAMAM on the superparamagnetic nanoparticles.

Others methods involved the use of silica-coated magnetic nanoparticles providing many silanol groups on the surface which can be used in the subsequent surface functionalization with organosilanes. However, lowest retained enzyme is obtained when Fe_3O_4 nanoparticles are directly modified. In this sense, a step of cross-linking agent is necessary to obtain a better immobilization and to avoid the leaching of the enzyme. Besides, a subsequent activation with glutaraldehyde as cross-linking agent favors enzymatic stability due to the multipoint covalent attachment [17] . Fe_3O_4 covered by SiO_2 prevents the loss of the magnetic properties and increase the amount of immobilized enzyme [18]. Previous results reported by our group using X-ray photoelectron spectra demonstrated the absence of signals associated with Fe 2 $p_{3/2}$ and $p_{1/2}$ [19]. This suggested that the Fe_3O_4 particles were totally covered when the particles were previously covered with SiO_2 and again treated with polyethyleneimine (PEI) and glutaraldehyde. Besides, with Fe_3O_4-SiO_2 a larger amount of aminopropylethoxysilane (APTES) could be anchored increasing the amount of enzyme adsorbed. In this study, we studied magnetic nanoparticles functionalized with APTES and posteriorly modified with a cross-linking agent to improve the invertase retention.

2 Experimental

2.1 Synthesis of Fe_3O_4-SiO_2-NH_2magnetic nanoparticles

The magnetic nanoparticles were synthesized by the alkaline co-precipitation method proposed by Kang *et al*. [20] using a molar ratio of Fe(II)/Fe(III) = 0.5; then, these nanoparticles were encapsulated with SiO_2 following the Stöber method [21]. A ratio 1:1 of magnetic particles and the silica source was employed in the synthesis of Fe_3O_4 -SiO_2. The functionalization these particles with APTES (99%) was performed following the protocol described by Diez et al. [22] using APTES as a source of amino groups. These Fe_3O_4-NH_2 SiO_2- particles were used as support for immobilization by physical adsorption.

2.2 Synthesis of activated Fe_3O_4-SiO_2-NH_2 magnetic nanoparticles

For immobilization by covalent bonding, Fe_3O_4-SiO_2-NH_2 nanoparticles were activated following a protocol previously established by our group [19]. The procedure was based on the suspension of the support in functionalizing agents to provide carboxyl and secondary amino groups. To do this, the particles were suspended in a glutaraldehyde solution and vigorously stirred. Deionized water was used to filter and wash the solution, and once again resuspended in a solution of polyethylenimine (PEI) 5% (w/v) sodium tetraborate buffer. This solution was incubated at 25 °C for 3 h.

Subsequently, the support was resuspended in 15 mL of a solution of 1 % glutaraldehyde in 0.05M acetate buffer for reactivating the functional groups bonded to the substrate; this solution was incubated at 25° C for 15 min. The activated support was filtered and washed with sufficient deionized water to remove the excess of glutaraldehyde. Finally, the support was conditioned with 0.05M acetate buffer at pH 7.0. These particles were denominated activated Fe_3O_4-SiO_2-NH_2.

2.3 Invertase immobilization

Invertase (β-D-fructofuranosidase) from *Saccharomyces cerevisiae* (Fluka Chemie) was immobilized by physical adsorption on Fe_3O_4-SiO_2-NH_2 and covalent immobilization on activated Fe_3O_4-SiO_2-NH_2 supports. The enzyme was dissolved in acetate buffer (0.05 M, pH = 5.5) using an enzyme:support ratio of 1:2, and the solution was mechanically stirred for 24 h at 100 rpm at 4?C. Then, the support impregnated with the enzyme was collected, washed, and resuspended in acetate buffer for further use and stored at 4 ?C for further enzymatic activity ssays. The amount of protein bound to the support was determined by difference between the initial and final amount of protein using the Lowry method using Follin-Ciocalteau reagent at 600 nm [23].

Miller method (DNS) [24] was used to determine the reducing sugars released from the hydrolysis of sucrose by the free and immobilized enzyme. The enzyme was incubated at 50°C in 0.3 M sucrose solution in 0.05M acetate buffer, pH 5.5. For this study an international activity unit (IU) was defined as 1 μmol of sucrose hydrolyzed per minute under the test conditions. Specific activity was expressed as IU per mg protein.

The effectiveness factor ($F\varepsilon$) was determined as the retained activity after the immobilization process and calculated from the following equation expressed as:

$$F\varepsilon = \frac{Ai - Ae}{Ao} \tag{1}$$

where A_i is the activity of the immobilized enzyme, A_o is the activity of the free enzyme, and A_e is the enzymatic activity remaining in the solution after immobilization.

The relative activity is the ratio between the enzymatic activity of the immobilized enzyme and the enzymatic activity of the free enzyme, and it was expressed as:

$$\%\ Relative\ activity = \frac{Ai}{Ao} \times 100 \tag{2}$$

The efficiency factor (η) determines the effect of diffusional limitations or alterations in mass transfer with respect to the reaction speed: it was calculated from the maximum reaction speed of the immobilized enzyme divided by the reaction speed of the free enzyme:

$$\eta = \frac{V_{\max}\ immobilized\ enzyme}{V_{\max}\ Free\ enzyme} \tag{3}$$

2.4 Variable optimization

The immobilization time was assessed within a range of 0 to 24 h, and the support-enzyme ratio (0 to 5000 mg enzyme /g support) at pH 5.5. To carry out the optimization of enzyme immobilization conditions, in each experiment only one variable was modified, all other variables kept steady.

2.5 Effect of pH and optimum temperature on the immobilized enzyme

The optimum pH was evaluated in a range from 4.0 to 7.0 at room temperature. The incubation temperature was evaluated in a range from 40 to 70 °C.

2.6 Determination of kinetic parameters (V_{max}, k_m, and k_i)

The V_{max} and k_m kinetic constants were assigned as response variables and determined under optimum conditions for both immobilized and free enzymes. This parameter was calculated from the Lineweaver-Burk plot using different substrate concentrations in a range from 0.025 to 2.0M. The inhibition constant (k_i) was calculated with the substrate Dickensheets model.

2.7 Stability studies and reusability

Stability was evaluated in a batch of immobilized invertase for 90 days at a storage temperature of 4 °C using an aliquot of 100 μL of immobilized invertase.

The reuse of immobilized enzyme was evaluated using the same amount of enzyme immobilized by several cycles while a constant concentration of substrate (sucrose) was maintained. The immobilized enzyme was evaluated until reaching enzymatic activity loss or constant. After each cycle, the excess dye (DNS) was removed washing the support with distilled water.

3 Results and discussion

Effectiveness factor (Fε) obtained for invertase immobilized by physical adsorption is relatively low (Fε = 0.26) compared with that of covalent immobilization (Fε = 0.74). The Fε value relates to the porosity of the support used, and porous supports cause some diffusional effects decreasing this value. In fact, the activated Fe_3O_4-SiO_2-NH_2 particles have lower size and pore volume values, as has been described previously [25]. Besides, electrostatic interactions are obtained when the enzyme is only immobilized in the magnetic composites modified with APTES. To assure covalent interactions, we used the functionalization of the support to provide carboxyl and secondary amino groups. The PEI assures an amount sufficient of amine groups, and the glutaraldehyde allows to obtain carboxylic groups. This density of -NH_2 and C = O groups is necessary to anchor the enzyme and to assure more porosity of the support.

Figure 1a shows the relative activity of invertase immobilized by physical adsorption or immobilized covalently. In the physical adsorption of invertase, when the enzyme concentration increases, the relative activity decreases as a result of leaching processes. On the contrary, this phenomenon is not obvious until an enzyme/support ratio of 1500 mg/g in invertase immobilized covalently. That is, in physical adsorption more enzyme molecules are adsorbed than in covalent immobilization because there are more amino groups available to bind with the enzyme in comparison with groups available in a reticulated support (activated Fe_3O_4-SiO_2-NH_2). However, a saturation surface of enzymes promotes diffusional limitations due to the blockage of the active sites by vicinal proteins [6, 7]. The optimum enzyme:support ratio is higher for covalent bonding than for physical adsorption, being 1500 and 1000 mg/g of enzyme/support, respectively (Figure 1b).

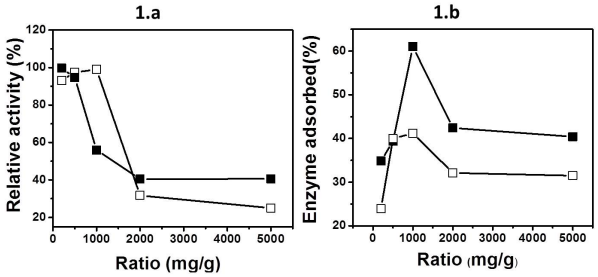

Figure 1. Effect of the support:enzyme ratio on the relative activity (Figure 1.a) and enzyme adsorbed (Figure 1.b) by physical adsorption (■) or covalently bonding (□).

Figure 2a and b displays the optimum time for invertase immobilization in both methods, which occurs at 24 h. Consequently, the protein values obtained are near 60% for physical adsorption and 50% for covalent bonding, i.e., in the physical adsorption a smaller amount of enzyme is immobilized than in covalent bonding, a higher enzyme:support ratio being required in this type of immobilization (Figure 2a). Interestingly, the enzyme immobilized covalently has a higher activity compared with the enzyme physically retained as can be seen in the Figure 2b. This result is attributed to the fact that the reticulation of the enzyme avoids its anchor on active sites, which is probably occurring in the physical adsorption.

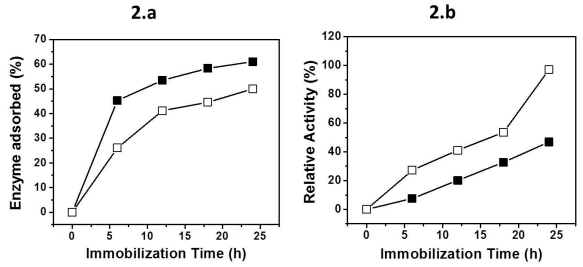

Figure 2. Effect of immobilization time on the enzyme adsorbed (Figure 2a) and relative activity (Figure 2b) by physical adsorption (■) or covalently bonding (□).

The results of enzymatic activity of the immobilized enzyme at different pH values are shown in Figure 3. It has been reported that depending on the support used, the pH range varies between 4.5 and 5.5 [5-6,25]. In our study the ideal pH for the free enzyme is 5.5, whereas in the enzyme immobilized by both methods the pH is 5.0. However, it is important to note that the relative activity at pH 7.0 is higher for the invertase immobilized by covalent bonding. The displacement of 0.5 pH units to the acidic region can be explained by secondary interactions between the enzyme and the support. Considering that the support was modified with PEI and glutaraldehyde to increase the interaction of the enzyme on the reticulation process, probably not all the -NH_2 groups providing PEI interact with enzyme. This excess of groups can act as buffer in changes of pH in the medium favoring the enzyme functioning in a broad range of pH, i.e. the reticulation not affects the active sites of the enzyme.

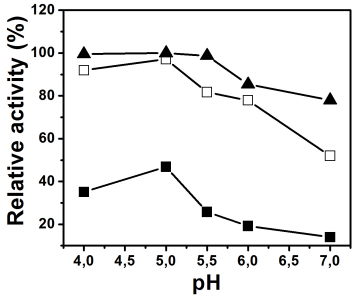

Figure 3. Effect of pH on the relative activity of invertase free (▲) and immobilized by physical adsorption (■) or covalently bonding (□).

Figure 4 shows profiles of relative activity of free and immobilized enzymes with respect to temperature. The enzymatic activity of free and immobilized invertase was near 90% and was maintained until 70 °C. At higher temperatures, the activity decreased. However, in the enzyme adsorbed physically the best temperature was 60 °C, but the relative activity was only of 75%, because of a lower binding force between the enzyme and the support [4]. It is also remarkable that the immobilization process allowed greater retention of relative activity above the critical temperature (80 °C). While the free enzyme retained only 20% of its relative activity, in covalent and physical immobilization this value was 40% and 30%, respectively.

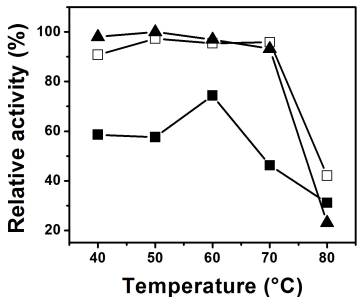

Figure 4. Effect of temperature on the relative activity of invertase free (▲) and immobilized by physical adsorption (■) or covalently bonding (□).

Table 1 shows the kinetic parameters determined using the Excel solver software with a nonlinear regression method (Lineal-Newton). The obtained values are consistent with the Michaelis-Menten model and the kinetic equation of substrate inhibition. The V_{max} of the enzyme immobilized by physical adsorption is half of that obtained for the free enzyme due the presence of noncompetitive inhibitors, which is evidenced by the higher value for k_i (1.21 for free enzyme, 2,95 for physical adsorption). In contrast, the enzyme immobilized by covalent bonding has a higher V_{max} value than the free enzyme, which is attributed to possible changes in the structure of the enzyme allowing further decoupling of the product/enzyme complex. Besides, the similar values of k_i (1.75) indicate that the covalent immobilization method provides greater stability to the enzyme, preventing its inhibition [5, 13], in agreement with the efficiency factor (η) obtained.

The values of k_m were similar in both immobilization methods and 6-fold higher compared to the free

enzyme. Conformational changes in the enzyme hinder the access of sucrose molecules to the active sites of the invertase and therefore the formation of the enzyme-substrate complex is more difficult, leading to a decrease in substrate affinity and a high k_m value [3-4].

Table 1. Kinetic parameters for free and immobilized invertase and comparison with other studies on different supports

Support	**Method**	V_{max} **(UI/mg enzyme)**	k_m **(mM.ml^{-1})**	**Efficiency factor** (η)	**Ref.**
Free enzyme	–	1.86	0.0087	–	This paper
Fe_3O_4-SiO_2-NH_2	P.A.	0,96	0,053	0,51	This paper
Activated Fe_3O_4-SiO_2-NH_2	C.I.	2.70	0.054	1.45	This paper
Montmorillonite micropores	P.A.	0,000514	0,72	0,28	[3]
Polyacylonitrile composite fibers	P.A.	0,95	0,031	0,64	[4]
Montmorillonite micropores	C.I.	0.000657	0.39	0.36	[3]
Functionalized porous silicon	C.I.	1.243	0.037	1.31	[5]
Magnetic polyvinyl alcohol microspheres	C.I.	0.97	0.55	0.80	[12]
Nylon-6 microbeads	C.I.	1.06	0.029	1.29	[6]
Chitosan nanoparticles	C.I.	1.83	0.20	1.09	[7]
Surface-modified polyaniline	C.I.	0.052	0.67	0.71	[10]

A comparison of kinetic parameters with those of other studies reported in the literature also is observed in the table 1; however, in the physical adsorption is only an estimate to observe the increase or decrease of the values of V_{max}, k_m and η. A direct comparison was not possible due to the different conditions employed in each study. Although, few supports have been reported by the physical adsorption of invertase, our results do not differ of those reported by other authors using supports with high efficiency factors (η) and high value of V_{max}. In this work, values of efficiency factor (η), V_{max}, and k_m obtained with invertase immobilized on different supports by covalent bonding were like those obtained by Azodia et al with invertase immobilized on functionalized porous silicon using glutaraldehyde as a functionalization agent. They showed that the lowest values of k_m reflect a decreasing of enzyme affinity versus the substrate upon immobilization mainly due to eventual conformational changes. Also, highest values in V_{max} were attributed to several phenomena such as favorable change of enzyme immobilizated structure and enhanced dissociation of the product from the product/enzyme complex. In this study, we can describe similar reasons in our results. In all case, the affinity of the enzyme decreased upon immobilization (increase of k_m), the value Vmax increased significantly, and a higher efficiency factor (η) provided greater stability of the enzyme over time.

The stability of the invertase physical and covalently immobilized was also studied for 90 days; the results are summarized in Figure 5. For the invertase immobilized by physical adsorption a loss of activity of approximately 25% occurred during the first 16 days, mainly due to enzyme denaturation processes. However, in the enzyme immobilized by the covalent method 50% of activity was observed at 30 days, which is similar to the stability obtained by Azodi *et al*. [5] using functionalized silicon pores as support. Glutaraldehyde activated supports have been reported to provide multipoint covalent enzyme immobilization improving enzymatic rigidity, and, thus, enzyme stability[17]. Because the reticulation maintains an intense multipoint interaction with the enzyme, the relative positions of all groups involved in the immobilization cannot alter the relative positions of the enzyme.

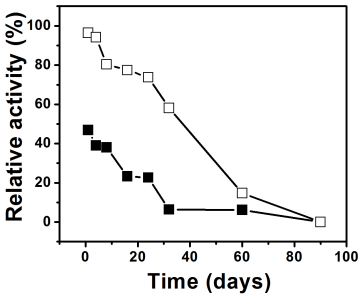

Figure 5. Stability of the invertase immobilized physical (■) and covalently (□).

The immobilized enzyme reusability was studied five times (Figure 6) using an external magnetic field. A 50% and 60% loss of activity occurred in the first reuse for the enzyme physically or covalently immobilized. The same behavior was observed in subsequent reuses, mainly due to leaching of the enzyme to the magnetic support, which was verified by monitoring the first residue, showing that the enzymatic activity was maintained. Other authors such as Bayramoğlu *et al*. [4] reported a 76% loss of activity due to leaching.

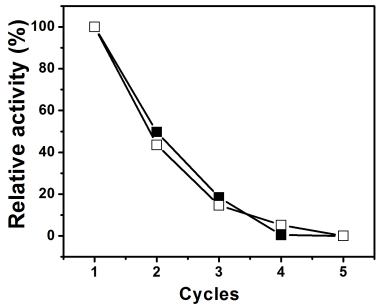

Figure 6. Reuse of invertase immobilized by physical adsorption (■) and covalent bonding (□) in terms of relative activity (%).

Table 2 summarizes the parameters evaluated in the immobilization of invertase using the physical adsorption and the covalent bonding method. Covalent immobilization of invertase on composites of Fe_3O_4-SiO_2-NH_2 displays higher efficiency in the immobilization process (Fε), higher catalytic efficiency, less inhibition and twice as much stability. In fact the stability is increased two fold when is used the covalent bonding method. Furthermore, immobilized invertase by physical adsorption or covalent immobilization can be reused for up to four cycles, and using an external magnetic field can be removed from the reaction medium.

4 Conclusions

Invertase was immobilized on magnetic composites by physical adsorption or covalent immobilization in magnetic composites. The support activated with glutaraldehyde improved the retention, stability and catalytic efficiency of the invertase, besides the enzyme showed less inhibition. The catalytic activity of the enzyme immobilized by both methods was not affected by different pH and temperatures in comparison with the free enzyme. The enzyme immobilized in the magnetic composites studied can be reused for up to four cycles and can be removed from the reaction medium using an external magnetic field.

Table 2. Parameters evaluated in the immobilization of enzymes in Fe_3O_4-SiO_2-NH_2 and activated Fe_3O_4-SiO_2-NH_2

Parameters	**Invertase immobilized**	
	Fe_3O_4-SiO_2-NH_2	**Activated Fe_3O_4-SiO_2-NH_2**
Effectiveness factor (Fε)	0.26	0.74
Efficiency factor (η)	0.51	1.45
Catalytic efficiency	17.89	50
V_{max}	0.96	2.70
k_m	0.053	0.054
k_i	2.95	1.75
pH	5.0	5.0
Thermostability	60 °C	50 °C
Stability	30 days	60 days
Reusability	4 cycles	4 cycles

Acknowledgements

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