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INFLUENCE OF THE TYPE OF SUPPORT AND IMMOBILIZATION ON THE ACTIVITY AND STABILITY OF LACCASE ENZYME (*Trametes versicolor*)

INFLUENCIA DEL TIPO DE SOPORTE Y LA INMOVILIZACIÓN SOBRE LA ACTIVIDAD Y ESTABILIDAD DE LA ENZIMA LACASA (*Trametes versicolor*)

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Abstract

This work presents a comparative study of the influence of the nature of the substrate and the immobilization mechanism on laccase enzyme activity and stability. The supports employed were: hydrotalcite-like particles ($ZnAl_2$), amorphous silica crystals, and glassy carbon surface. Two immobilization mechanisms were applied: one physical, by adsorption, and the other chemical, with two versions of covalent bonding. In the first, using silanes and glutaraldehyde (GA), in the second, thiols were used as anchoring reagents. Hydrotalcite and silica supports were characterized before and after immobilizing the enzyme by X-ray diffraction analysis (XRD), while in the case of glassy carbon supports electrochemical characterization was performed. The catalytic properties K_m and K_{cat}/K_m of every enzymatic system were evaluated in a complementary fashion, as well as the free enzyme. The kinetic characterizations were done using ABTS (ammonium 2,2'azino-bis-(3-ethylbenzothiazoline-6-sulfonate acid)), as a reagent typical of laccase in 0.1 M acetate buffer solution pH 3.7. Efficiency of the enzyme adsorbed on each support it was demonstrated that the method of immobilization is decisive in the catalysis. Greater efficiency was observed in the system that employed glassy carbon with either immobilization mechanism and these results are analogous with the free enzyme.

Keywords: laccase, immobilization, thiol, glassy carbon, hydrotalcite.

Resumen

Este trabajo presenta un estudio comparativo de la influencia de la naturaleza del sustrato y del mecanismo de inmovilización en la actividad de la enzima y estabilidad. Los soportes empleados fueron: partículas de hidrotalcita ($ZnAl_2$), cristales amorfos de sílica y superficie de carbón vítreo. Se aplicaron dos mecanismos de inmovilización: uno físico por adsorción y el otro químico con dos versiones de enlace covalente. En el primero, utilizando silanos y glutaraldehído (GA) y en el segundo tioles. La hidrotalcita y la sílica se caracterizaron antes y después de la inmovilización de la enzima por análisis de difracción de rayos X, en el caso de carbono vítreo se realizó la caracterización electroquímica. Se evaluaron las propiedades catalíticas K_m y K_{cat}/K_m de cada sistema enzimático así como de la enzima libre. Se realizaron las caracterizaciones cinéticas usando ABTS (ácido 2,2' azino-bis-(3-etil benzotiazolin-6-sulfonato de amonio)) en 0.1 M buffer de acetatos pH 3.7. La eficiencia de la enzima adsorbida en cada soporte demostró que el método de inmovilización es decisivo en la catálisis, además hay mayor eficiencia en el sistema que emplea carbón vítreo con cualquier mecanismo de inmovilización; estos resultados son análogos a los datos obtenidos con la enzima libre.

Palabras clave: lacasa, inmovilización, tiol, carbón vítreo, hidrotalcita.

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1 Introduction

Laccase enzyme (1.10.3.2), also called polyphenol oxidase, was discovered in the exudates of lacquer tree *Rhus vernicifera* in Japan in 1883 (Mayer *et al*, 2002; Durán *et al*, 2002). The structure is a monomer organized in three sequenced arranged domains (T1, T2 and T3). Biochemistry overview is a globular glycoprotein containing 520-550 aminoacids and a varying amount of carbohydrates (10-45 %), with a molecular weight ranging between 50-130 kDa; it is produced by fungi and higher plants. The content and distribution of carbohydrates around the catalytic unit ensure conformational stability that protects the enzyme from proteolysis and inactivation. This enzyme is characterized by the sites, within the catalytic unit, with copper atoms distributed in three domains, each one of them with particular spectroscopic and catalytic properties (Mayer *et al*, 2002; Durán *et al*; 2002, Freyre *et al*, 2001; Piontek *et al*, 2002, Téllez-Téllez *et al*, 2012).

Laccase enzyme has received special attention of the investigators over past decades due to its catalytic properties and the capacity to oxidize phenolic substrates in the natural manner, and non-phenolic substrates, in the presence of mediators (Durán *et al*; 2002, Freyre *et al*, 2001; Piontek *et al*, 2002; Palmieri *et al*, 1994; Chiachierini *et al*, 2005, Solís-Oba M. *et al*, 2007), accompanied by simultaneous reduction of O_2 to H_2O. On the other hand, laccase is a biological tool useful and applicable in several biotechnological processes (Mayer *et al*, 2002; Durán *et al*; 2002, Freyre *et al*, 2001; Piontek *et al*, 2002; Palmieri *et al*, 1994), such as: elimination of recalcitrant soil contaminants, and detoxification of effluents from paper, textile (Solís-Oba M. *et al*, 2009) and petrochemical industry, chiefly.

In these processes, enzyme catalysis is usually performed by employing the free enzyme, which involves denaturation due to sudden changes of pH and temperature. The enzyme is only soluble in aqueous media and a new enzyme reload is required in every catalytic cycle. In consequence, the stability and activity of free laccase decrease markedly, and the sum of these factors limits the enzyme application due to the costly processes involved (Palmieri *et al*., 1994; Chiachierini *et al*., 2005).

In order to counteract these inconveniences, mechanisms of enzyme immobilization on different supports (montmorillonite, ceramic crystalls, silica, nanoparticles and electrodes (Chiachierini *et al*., 2005; Mateo *et al*., 2007; Zaborsk, 1974) have been investigated. These procedures are divided into physical (trapping, polymer matrices and adsorption) and chemical methods (covalent bonding), each with different characteristics and application as a function of the type of enzyme (Zaborsk, 1974). The following are advantages of immobilizing an enzyme: stability, reuse and resistance to pH changes, temperature or proteolytic degradation (Torres-Salas *et al*, 2011; Tavares *et al*, 2013).

The nature of the substrate and the immobilization mechanism are key factors in enzyme properties. This work presents a comparative study of the influence of these factors on laccase activity and stability by comparing them with the properties of the free enzyme. The evolution of the parameters that describe catalytic behavior of the free enzyme is generally analyzed using Michaelis-Menten model and pertinent modifications described in the area of enzymology. Immobilized enzymes, however, are shown as apparent kinetic values (K_{map}).

The *Trametes versicolor* laccase enzyme was immobilized on three different supports: hydrotalcite-like particles ($ZnAl_2$), amorphous silica crystals, and glassy carbon surface. Two immobilization mechanisms were applied: one physical, adsorption, and the other, chemical, with two different processes. In the first, surface was functionalized with silanes using glutaraldehyde (GA) as a bonding agent; in the second, thiols were used as anchoring reagents. These immobilizations systems provided information on the influence that the type of immobilization exerts on different surfaces, the way kinetic parameters get modified and their relationship to operation and storage stability of every system (surface-immobilization mechanism). All kinetic characterizations were made using ABTS (ammonium 2,2'azino-bis-(3-ethylbenzothiazoline-6-sulfonate acid)) as a reagent typical of laccase in 0.1 M acetate buffer solution pH 3.7.

2 Materials and methods

Remark: In this work the **substrate** is used to identify the chemical compound (ABTS) employed for the enzymatic catalysis and **support** is used to identify the surface employed for immobilized the enzyme.

2.1 Reagents

The ABTS (ammonium 2,2'azino-bis-(3-ethylbenzothiazoline-6-sulfonate acid)), APTES (3-

amino-propyl triethoxy silane), potassium chloride, acetic acid, glutaraldehyde 70%, anhydrous methanol, 2,2'dithiodipyridine and 5,5'dithiobis (2-nitro benzoic) acid 99% were obtained from Sigma-Aldrich, whereas sodium hydroxide and potassium ferrocyanide were obtained from J. T. Baker. The laccase enzyme (*Trametes versicolor*) was obtained from Sigma-Aldrich too. The conducting cement (Graphite conductive adhesive) and graphite bar 99.99 % were from Alfa Aesar. Hydrotalcite-like particles $\{[M(II)_{1-x}\ M(III)_x\ (OH)_2]\ (A^{n-})_{n/x}\cdot\ mH_2O$, where M(II)= Mg^{2+}, Zn^{2+} , M(III)= Al^{3+}, A=CO_3^2, were previously synthesized and characterized (López-Salinas *et al*, 2006).

2.2 *Surfaces employed for enzyme immobilization*

2.2.1 Hydrotalcite-like particles ($ZnAl_2$)

Hydrotalcite-like particles, synthetic compounds also called layered double hydroxides, were laboratory synthesized by maintaining a Zn^{2+}/Al^{3+} ratio 2 and using the corresponding nitrate salts in a reflux system at 90°C during 18 hours.

2.2.2 Silica crystals

Silica crystals were prepared by mixing the equal volumes (25 mL) of ethanol, distilled water and tetraethyl*orto*silicate with stirring. This solution was evaporated at room temperature during five to seven days, and silica crystals were cut into uniform-sized pieces (Ho *et al*, 1983).

2.2.3 Glassy carbon surface

The glassy carbon surface (Alfa Aaesar) was polished using different grade sandpaper; subsequently, it was treated with 0.5, 1 and 0.05 μm particle-sized alumina to a mirror-like finish. These treatments were carried out using a Buehler's MiniMet 1000 Grinder-Polisher.

2.2.4 Characterization of $ZnAl_2$ and silica with X-ray diffraction (XRD)

In order to perform XRD characterization, analyzed samples were ground and dried at 28°C during 24 h. The equipment consisted of Siemens D500 diffractometer copper lambda, secondary beam monochromator with diffract/T software. The following simples were analyzed:

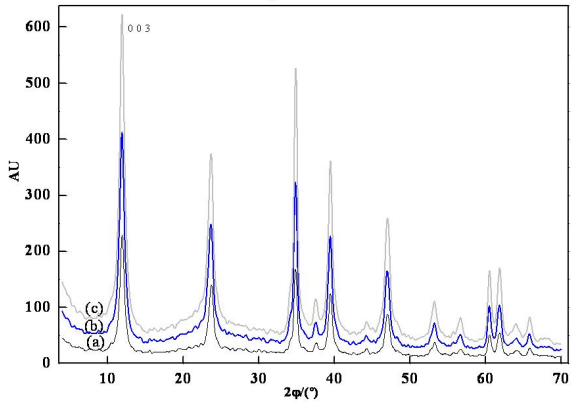


Fig. 1. Diffractograms of hydrotalcite-like materials $ZnAl_2$ on which laccase enzyme was immobilized (a) without enzyme (b) with enzyme (c) with enzyme after reaction with ABTS.

a) Hydrotalcite-like particles: $ZnAl_2$, $ZnAl_2$-laccase, $ZnAl_2$-laccase after reaction with ABTS.

b) Silica gel crystals, silica GA crystal -laccase, silica GA crystals-laccase after reaction with ABTS.

This characterization served to determine the support structure and the modification of diffraction pattern in the presence of the enzyme.

2.2.4.1 Hydrotalcite-like materials $ZnAl_2$

Figure 1a shows the XRD pattern of hydrotalcite-like material $ZnAl_2$, typical of particles without enzyme, which is similar to that reported by Ren *et al*. (2001, 2002). Fig. 1b shows the spectrum corresponding to the particles with enzyme, whereas Figure 1c exhibits the typical diffractogram of the particles with enzyme after their use in the reaction with ABTS. The fact that diffraction patterns of Figures 1a-c are practically equal (the plane (0,0,3) shows no displacement), proves that laccase does not adhere to the interlayer region, in contrast to what was reported by Ren *et al*. (2002), when they immobilized the enzyme of penicillin G acylase on hydrotalcites.

2.2.4 2 Hydrotalcite-like materials $ZnAl_2$ with GA and thiols

In this section no additional XRD results to those in Fig. 1a were obtained because during enzyme immobilization on hydrotalcites using the chemical mechanism with glutaraldehyde, a high-viscosity gel was formed which prevented us from obtaining the diffractograms.

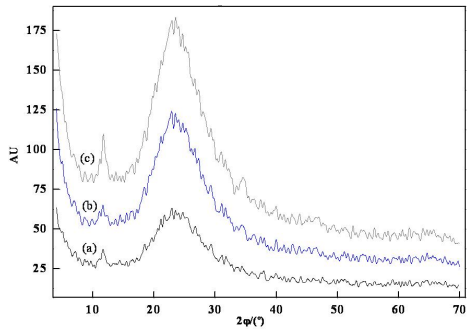


Fig. 2. Diffractograms of silica gel crystals on which the laccase enzyme was immobilized (a) without enzyme (b) with enzyme (C) with enzyme after reaction with ABTS.

The immobilization on hydrotalcites via thiols also generated a gel with particles immersed in it, thus making impossible to obtain XRD diffraction patterns.

Despite gelification of immobilization systems, the process was carried out until enzyme immobilization with the corresponding kinetic evaluation, whose results are shown in the corresponding sections.

2.2.5 Silica gel support with immobilization by adsorption

Figure 2a shows the silica gel diffraction pattern (XRD). The enzyme was immobilized on this support by physical adsorption with poor results.

2.2.5.1 Silica gel support with immobilization using GA

Fig. 2 shows the silica gel XRD patterns and its diffractograms are typical of this amorphous material. The patterns in figs. 2a and 2b are practically equal, which means that the immobilized enzyme probably did not penetrate the support structure forming bonds at the surface as a result of functionalization. The pattern 2c corresponds to the support with enzyme after reaction with ABTS, and no displacement is observed in its spectrum, which demonstrates that no ABTS adhered to the support.

2.2.5.2 Silica support with immobilization via thiols

This mechanism of immobilization is carrying out over the surface where the enzymatic immobilization takes place, so the diffraction pattern was not modified by immobilization via thiols.

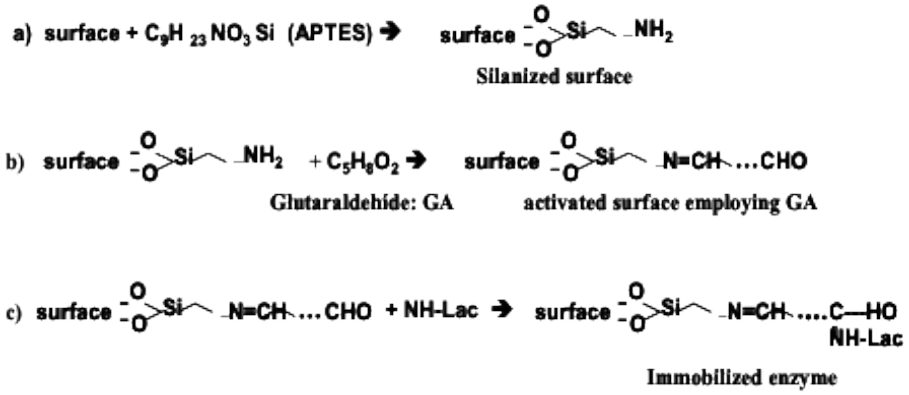
2.3 Laccase immobilization

2.3.1 Immobilization of laccase enzyme on $ZnAl_2$

To perform this immobilization, 200 mg of the support were initially weighed and activated with boiled distilled water during six hours with stirring; then, they were placed in 0.1 M acetate buffer solution pH 3.7 containing 5 mL of enzyme (0.5 mg mL^{-1}), at room temperature, for 24 hours with stirring. Enzymatic solution was recovered and the materials were rinsed with distilled water until reaction with ABTS was negative.

2.3.2. Immobilization of laccase enzyme on silica crystals

The crystals (5g) were placed in 50 mL 1 M NaOH for one hour, and rinsed with distilled water; afterwards, they were placed for three minutes in 50 mL of 1 M HNO_3 to get filtered so that they could later react with 10 mL of 50% aminopropyltriethoxysilane solution in ethanol during three hours. After this reaction, crystals were rinsed with distilled water and mixed with glutaraldehyde 10% for three hours, and finally rinsed with distilled water. The support was placed in 25 mL of enzyme solution (0.5 mg mL^{-1}) for 12 hours, after which enzyme solution was recovered and crystals were rinsed with distilled water until laccase-ABTS reaction was negative (Ho *et al*., 1983), the reactions of each stage in this immobilization mechanism are generally described as follows (Ho *et al*., 1983):



2.3.3 Enzyme Immobilization on the glassy carbon surface using thiols

Prior to enzyme immobilization, polished glassy carbon surfaces were prepared by an electrochemical treatment (cyclic voltammetry). The solution employed was 5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$ in 1 M KCl, while the cyclic voltammograms were obtained by performing potential scans in positive direction at a rate of 200 mV/s within a potential window of -200 to 600 mV. This method was performed using two thiol solutions [2, 2'dithiodipydine (thiol 1) and 5, 5'dithiobis (2-nitro benzoic) acid (thiol 2)], both at a concentration of 2 mM in methanol 50%. The procedure consisted of immersing the glassy carbon surfaces during 2 hours in thiol 1, after which they were rinsed with methanol 50%, and immediately immersed into thiol 2 for two hours. Subsequently, they were rinsed with methanol and introduced into a solution of 6 μM laccase in 0.1 M acetate buffer solution pH 3.7, for 24 hours. Finally, the glassy carbon surfaces were thoroughly rinsed with acetate buffer solution and kept at 4°C in the same buffer solution, the reactions of each stage in this immobilization mechanism are generally described as follow (Zaborsk, 1974; Ho *et al*, 1983):

a) Surface + $S_2C_6H_3(NO_2)CO_2H$ → Surface —$S_2C_6H_3(NO_2)CO_2H$

5,5'dithiobis(2-nitro) benzoic acid

b) Surface —$S_2C_6H_3(NO_2)CO_2H$ + $C_{10}H_8N_2S_2$ → Surface—$S_2C_6H_3(NO_2)CO_2H$-- $NHC_{10}H_7NS_2$

2,2' Dithiodipyridine

c) surface—$S_2C_6H_3(NO_2)CO_2H$-- $NHC_{10}H_7NS_2$ + Lac (Cu^I) →

Surface —$S_6C_6H_3(NO_2)CO_2H$ -- $NHC_{10}H_7NS$-NH-Lac (Cu^I)

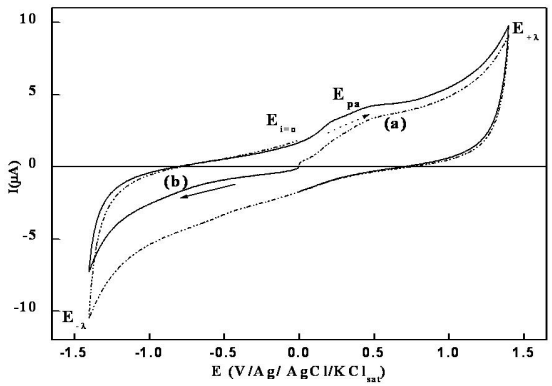


Fig. 3. Typical cyclic voltammograms (ν = 100 mV/s) of laccase immobilized on a glassy carbon surface by physical adsorption. The supporting electrolyte was 0.1 M acetate buffer solution pH 3.7; the scan was initiated in positive (a) and negative direction (b).

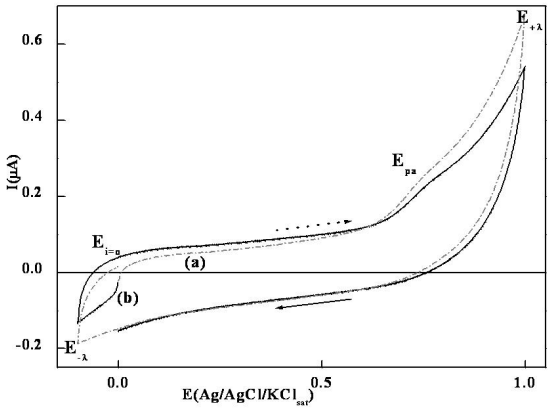


Fig. 4. Typical cyclic voltammograms (ν = 100 mV/s) of laccase immobilized on a glassy carbon surface by applying the GA immobilization process. The supporting electrolyte was 0.1 M acetate buffer solution pH 3.7, the scan was initiated in positive (a) and negative direction (b).

2.4 *Electrochemical characterization of glassy carbon surface modified with GA and thiols*

Electrochemical characterization of a modified surface was performed by cyclic voltammetry employing an acetate buffer solution 0.1 M at pH 3.7 as supporting electrolyte. The potential scans were performed in positive and negative direction at 100 mV/s using 10-mL cells with a three-electrode system: working (glassy carbon electrode), reference (Ag/AgCl/KCl) and auxiliary (graphite bar 99.99 % purity) electrode; the IR compensation was applied. The equipment used was Bas-Epsilon Ec-Ver 1.60.

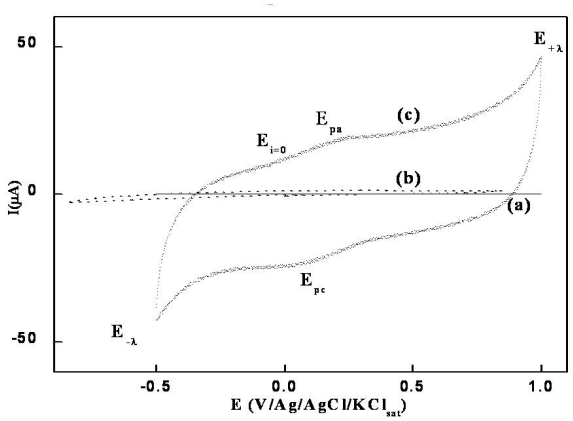


Fig. 5. Typical cyclic voltammograms (ν= 100 mV/s) of a) glassy carbon surface, b) glassy carbon surface modified with thiols c) glassy carbon modified with thiols and laccase. The supporting electrolyte was 0.1 M acetate buffer solution pH 3.7.

2.4.1 Laccase immobilization on the electroactive surface

2.4.1.1 Electrochemical characterization of glassy carbon using laccase immobilization by physical adsorption

Electrochemical characterization performed provided cyclic voltammograms in 0.1 M acetate buffer solution at pH 3.7 (Fig. 3). The potential scans were performed in both directions at 100 mV/s. Fig. 3 shows typical voltammograms of the enzyme adsorbed on the glassy carbon surface, scanned in positive direction initiated at zero current potential ($E_{i=0}$). In the $E_{i=0} - E_{+\lambda}$ interval (Fig. 3a), an oxidation signal was observed at 0.470 V/Ag/AgCl/KCl$_{sat}$, the scan was continued and no additional redox process was observed in the reverse direction $E_{-\lambda}$. Indeed, the scan in the negative direction (Fig. 3b) shows the similar behavior to that obtained in the positive direction.

These results suggest that there was conduction between the surface and redox site of the adsorbed enzyme; however, subsequent scans did not generate similar signals due to fast desorption of the enzyme, as indicated in several works (Sachdev *et al*, 2008; Onda *et al* 2008; Quan *et al*, 2002; Johnson *et al*, 2003, Solis-Oba *et al*, 2005); so, this immobilization mechanism was not capable of preserving the enzyme permanently adhered to the surface (Shleev *et al*, 2005; Ivnitski *et al*, 2007).

2.4.2 Electrochemical characterization of glassy carbon using the laccase immobilization with GA

Figure 4 shows the typical cyclic voltammetry of glassy carbon with laccase immobilized employing the chemical method. The scans were made at 100 mV/s, in anodic and cathodic direction. Fig. 4a shows the scan in positive direction started at zero current potential ($E_{i=0}$); the interval between $E_{i=0}$ and the switching potential $E_{+\lambda}$, is observed to present an oxidation signal at the potential (E_{pa}) of 0.742 V/Ag/AgCl/KCl$_{sat}$. After continuing the scan within the interval $E_{+\lambda}$ to $E_{-\lambda}$, no reduction peak is observed and upon scan completion at $E_{i=0}$, no other redox process is observed.

Figure 4b shows the scan in negative direction initiated at zero-current potential (Ei=0) towards $E_{-\lambda}$, where no reduction signals are identified; however, on continuing the scan within the interval $E_{-\lambda}$ to $E_{+\lambda}$, oxidation peak identified in figure 4a is observed with a peak potential of 0.742 V/Ag/AgCl/KCl$_{sat}$ which is associated with an irreversible electrochemical process. Similar results are obtained with the other four electrodes. On the other hand, the electrochemical behavior obtained within the potential range (0.4 to 0.8 V/Ag/AgCl/KCl$_{sat}$), is similar to those reported in several works (Piontek *et al*, 2002; Johnson *et al*, 2003; Shleev *et al*, 2005; Ivnitski *et al*, 2007), for oxidation peaks associated with laccase. A similar result was reported by Quan *et al*. (2002) who indicated that immobilization with GA forms an unstable layer easily separable from the surface.

2.4.3 Electrochemical characterization of glassy carbon using the immobilization with thiols

The search for a more efficient immobilization system in combination with the information available in the literature lead to the application of a new immobilization method based on the use of thiols as anchoring agents to fix the enzyme to the glassy carbon surface. Generally, this mechanism is applied in processes that use platinum (Pt) and gold (Au) electrodes as active surfaces, but in this case a glassy carbon active surface was employed. Five electrodes were used, and the active surface was made of glassy carbon with a 2 mm diameter and 5-6 mm height using nylamid as isolating material.

This result allows ensuring that within the potential range of -1.0 to 1.0 V vs. Ag/AgCl/KCl$_{sat}$, the thiol adhered to the surface shows no redox processes (Figure 5b) that could interfere with

electroactive properties of the enzyme when it is characterized by cyclic voltammetry.

The scans were made in both positive and negative directions at 100 mV/s (Figure 5c) employing acetate buffer solution 0.1 M pH 3.7. The electrochemical characterization of the immobilized enzyme confirms the enzyme presence on the active glassy carbon (modified electrode). This evidences the charge transfer between the enzyme active site and the active surface, which is likely to be governed by the mechanism of the external domain. On the other hand, the electrochemical characterization that used cyclic voltammetry at different pH produced similar results, which proves that the immobilized enzyme is stable with pH changes. Similar results were obtained with the other four electrodes with an average half wave potential ($E_{1/2}$= (Epa+Epc)/2) of -0.112 V/Ag/AgCl/KCl_{sat}.

The convenience of this process was verified by the procedure that determines the activity and kinetic parameters making use of spectroscopic characteristics of the ABTS oxidation. The results obtained from this kinetic characterization are shown in the following section and compared to the data obtained with free laccase.

2.5 *Kinetic characterization*

2.5.1 Kinetic characterization (activity and kinetic parameters)

Activity and kinetic parameters are properties that indicate catalytic capacity of enzymes. The activity (U/mg) of free laccase was determined with ABTS by measuring the change of absorbance as a function of time at 720 nm (ε = 14000 $M^{-1}cm^{-1}$). It has been shown that at this wavelength no interference between ABTS and $ABTS^{\bullet+}$ is present (Solís-Oba *et al*, 2005). The reaction mixture was 0.1 M ABTS and laccase in 0.1 M acetate buffer solution at pH 3.7. Kinetic characterization on the immobilized enzyme was performed using 200 mg of $ZnAl_2$-enzyme, 180 mg of silica-enzyme and glassy carbon surface; the experiments were made similarly to those for free enzyme characterization. The ABTS concentration used for kinetic characterization ranged between 0.12 and 20 mM in all cases.

A unit of activity (U) was defined as the amount of enzyme that oxidizes 1 μmol of ABTS per minute; the activity was shown in U/mg. In each one of the cases only the immobilized enzyme is considered.

All determinations were made in 1cm optical flow quartz cells, within the wavelength interval (λ) from 180 to 820 nm, using a Hewlett Packard diode array ultraviolet-visible spectrophotometer HP8452.

2.5.2 Operational and storage stability

Determination of operation stability on the three types of supports refers to the number of times a reaction is repeated. The stability of free and immobilized laccase, when preserved in refrigeration (4°C), was evaluated during 30 days. Each cycle consisted of putting the enzyme at room temperature, determine its activity and preserve it at 4°C for five days.

The processes carry out for these determinations as it is described in the section 2.5.1.

3 Results and discussion

The *Trametes versicolor* laccase was immobilized by applying different strategies of immobilization: adsorption and chemical linkage via glutaraldehyde and thiols. The three different supports were employed: hydrotalcite-like particles ($ZnAl_2$), whose application in biological systems is recent (Ren *et al*, 2002; Ren *et al*, 2001; Mousty *et al*, 2007; Sachdev *et al*, 2008; Onda *et al*, 2008), amorphous silica crystals, and glassy carbon surface (five surfaces used).

Adsorption is a fast method of immobilization, which does not modify the natural conformation of an enzyme. Immobilization with glutaraldehyde is a covalent bond; this kind of bonding between an enzyme and a support is, in theory, the most stable method of immobilization (Zaborsk, 1974; Quan *et al*, 2002). In this case, the surface was functionalized with silanes and glutaraldehyde was used as a bonding agent, another method of immobilization was that employs thiols as anchoring reagents (Zaborsk, 1974).

Hydrotalcite and silica supports were characterized before and after immobilizing the enzyme by X-ray diffraction analysis (XRD) (Figs. 1 and 2), while in the case of glassy carbon supports electrochemical characterization was performed (Figs. 3-5). On the other hand, we evaluated activity, kinetic parameters and stability of the enzyme immobilized by physical and chemical mechanisms. Results obtained on the three surfaces were contrasted with the data obtained for the free enzyme. The information collected is shown in the following sections.

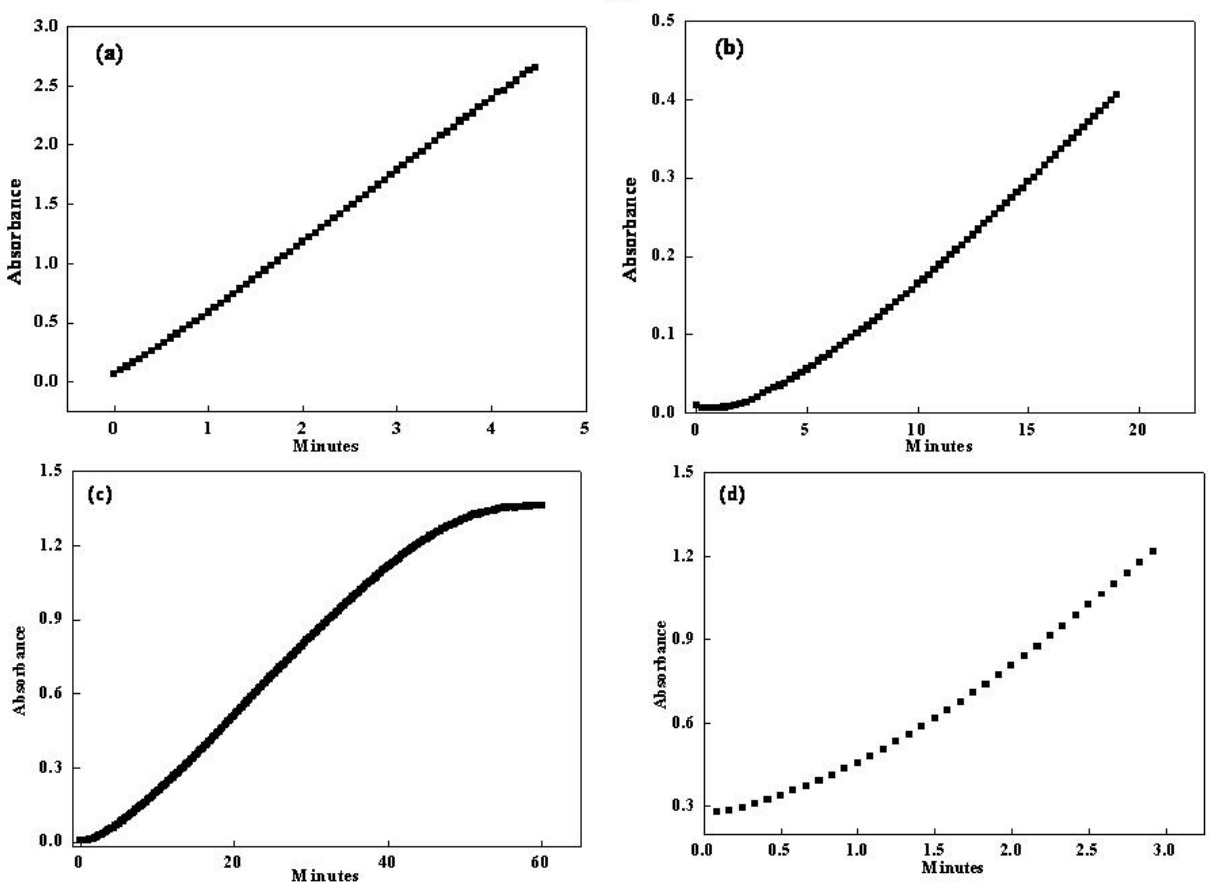


Fig. 6. Variation of Absorbance (λ=714 nm) vs. time of laccase immersion in aqueous solution 0.1 M ABTS in 0.1 M acetate buffer (pH=3.7). Laccase was physical immobilized: (a) free enzyme, (b) laccase-$ZnAl_2$, (c), laccase-silica and (d) laccase-GC.

3.1 Kinetic characterization

Kinetic characterization of immobilized enzyme was performed similarly to that for the free enzyme. The ABTS concentration used for kinetic characterization in all cases was 0.02 to 20 mM. In order to determine kinetic parameters K_m, V_{max} and K_{cat}, the analysis of graphs similar to those of figure 6 was performed. The free enzyme was observed to exhibit linear behavior (6a) showing the instantaneous nature of lac-ABTS reaction.

This behavior was not seen when the enzyme was immobilized on the three supports: laccase-$ZnAl_2$ (6b), laccase-silica (6c) and laccase-electrode (6d). For each one of them an "r stabilization or delay" time interval was indicated, possibly related to the substrate's coupling to the enzyme and associated with the mechanism of induced fitting (Voet *et al*, 1992). After this stage, the velocity was rapidly increased and the growing trend was similar to that shown for the free enzyme (Fig. 6a).

Experimental data of the free enzyme were analyzed using Michaelis-Menten method (MM) and Lineweaver-Burk modification:

$$1/V = k_m/V_{\max} * [1/S] + [1/V_{\max}] \qquad (1)$$

Where V and V_{max} are reaction and maxima velocity, respectively, S is substrate concentration and K_m is a Michaelis constant.

Linear behavior observed in Fig. 7a (to exemplify the results) was adequately fitted to the above equations.

However, in the graphs obtained for the enzyme immobilized on different supports (6b, 6c and 6d), it is quite evident that the delay phase could not be directly fitted by MM; so, it was necessary to include a term relative to this phase and formulate the fitting equation as follows:

$$dS/dt = \{K_{cat}\,(1 - \exp(-rt))\,E_0 S/(K_m + S)\} \qquad (2)$$

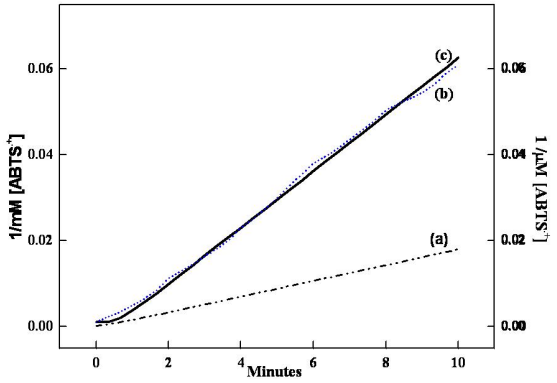


Fig. 7. Variation of [ABTS] vs. time of laccase immersion in an aqueous solution of 0.1 M ABTS in 0.1 M acetate buffer (pH 3.7). Laccase was chemical immobilized: (a) free; (b) immobilized on silica with GA. (b) Dots are experimental data and (c) line corresponds to fit performed using equations 1 and 2.

Where S is: substrate concentration, K_m: Michaelis constant, E_o: enzyme initial concentration, r: delay phase, t: time, K_{cat}: turnover number.

Experimental data acquired for the enzyme immobilized on different supports was fitted using equation 2 with non-linear regression and contrasting the data with simulation (sum of errors 3.32×10^{-11}) by Solver. Figures 7 b and c show an example of experimental and fit results, respectively.

The kinetics of laccase immobilized on different supports and using diverse immobilization mechanisms follow behaviors similar to those shown in figures 6b-d and 7b. Data regarding specific activity of the immobilized enzyme, obtained from the fitting data, are shown in Table 1; furthermore, free-enzyme data are shown to compare changes of activity. The variation of activity depends on the support and type of immobilization applied. As far as the physical adsorption of laccase is concerned, $ZnAl_2$-type particles retained only 1.24% (59 U mg^{-1}) of specific activity; on silica, 0.25% (12 U mg^{-1}) was preserved, and on glassy carbon 17% (770 U mg^{-1}) of specific activity was kept. In the case of the enzyme immobilized by chemical mechanism using GA, the following data came out: $ZnAl_2$ particles retained 0.074% (3.5 U mg^{-1}) of specific activity; silica crystals preserved 46.1% (2192 U mg^{-1}) and the glassy carbon surface retained 58.25% (2769 U mg^{-1}).

Finally, specific activity of the chemical immobilization performed using thiols produced the following results: for $ZnAl_2$ particles, the retained activity was 0.12% (5.7 U mg^{-1}), silica crystals preserved 53.4% (2537 U mg^{-1}) and the glassy carbon surface retained 77.6% (3690 U mg^{-1}) of specific activity in the three systems with respect to the free enzyme (4754 U mg^{-1}).

Table 1. Data on activity and K_{cat}/K_m (mM min)$^{-1}$ of the immobilized laccase on silica and glassy carbon electrode using ABTS as substrate and 0.1 M acetate buffering at pH 3.7.

Parameters / Enzyme	Activity (U/mg)	Efficiency K_{cat}/K_m (mM^{-1}min^{-1})	K_m (mM)
Free	4754	300	0.016
Physical adsorption			
Hydrotalcites ($ZnAl_2$)	59	3.32	1.54
Silica	12	1.8	1.7
Glassy carbon	770	11.2	1.24
Chemical immobilization with glutaraldehyde (GA)			
Hydrotalcites ($ZnAl_2$)	1.9	0.6	2.8
Silica	1682	94	0.9
Glassy carbon	2769	102	0.5
Chemical immobilization with thiols			
Hydrotalcites ($ZnAl_2$)	5.7	1.2	0.77
Silica	2137	114	0.45
Glassy carbon	3690	253	0.3

It is generally considered that the adsorption mechanism on layer surface type hydrotalcites is carried out by distributing the enzyme molecule between the layer spaces, however, the XRD patterns (Figure 1) indicate laccase does not distributed in the interlayer space (laccase molecular size, 35 x 45 x 55 Å, is greater than the available space of approximately,100 Å). Therefore, the enzyme adsorbed and distributed in irregular manner on the surface of the $ZnAl_2$ is easily desorbed producing low catalytic properties.

In contrast, the glassy carbon support produced a smaller loss in activity because its surface is regular, practically homogeneous, which is likely to favor a uniform distribution of the enzyme and thus allow having a more adequate exposure of active sites. This latter favored the interaction between ABTS and the enzyme, as shown by the values of specific activity with different immobilization mechanisms.

No comparison of enzymatic activity on modified electrodes with respect to the free enzyme was found in the references reviewed. Likewise, there can be a loss of activity due to causes attributable to: the modification and conformational changes suffered by the native structure of the enzyme when immobilized on a support (Durán *et al*, 2002; Palmieri *et al*, 1994; Chiacchierini *et al*, 2004; Mateo *et al*, 2007; Zaborsk, 1974), inactivation of some enzyme molecules, diffusion limitations and changes in affinity between the enzyme-substrate (K_m).

It was demonstrated that the method of immobilization is decisive in the catalysis. In the case of adsorption as a physical mechanism of immobilization, the process rapidly denaturalizes the enzyme, and the decrease in activity is faster when compared to the other methods as indicated Acunzo *et al* (2003); whereas D'annibale *et al* (1999) reported 45% preservation of the specific activity with respect to the free enzyme when immobilizing a laccase (*Lentinus edodes*), on chitosan/glutaraldehyde.

In the case of GA on GC, a possible secondary reaction with the typical enzyme substrate (ABTS) was observed, previously reported by Quan *et al*. (2002), who performed immobilization with GA on a platinum electrode and observed the tendency of ABTS to adhere to the surface after catalysis with the immobilized enzyme.

As for the immobilization with thiols, applied on gold and platinum electrodes, no information regarding ABTS/immobilized enzyme interaction was found (Shleev *et al* 2005; Ivnitski *et al*, 2007).

All the above information was confirmed by the values of K_m obtained for the enzyme immobilized on the three types of support, and the results achieved are shown in the following section.

3.1.1 Kinetic parameters

The values of K_m (Michaelis-Menten constant) are useful to demonstrate the capacity of an enzyme to bond to a substrate. In the case of immobilized enzyme, the orientation of the enzyme on the support is decisive (Shleev *et al*., 2005; Ivnitski *et al*., 2007; Acunzo *et al*., 2003) for this capacity. The values of K_m, when the enzyme was immobilized by adsorption were: 1.54 mM ($ZnAl_2$), 1.7 mM (silica) and 1.24 mM (glassy carbon). For chemical immobilization with GA, the K_m data obtained were: 2.8 mM ($ZnAl_2$), 0.9 mM (silica) and 0.5 mM (glassy carbon), whereas immobilization with thiols they were 0.77 mM ($ZnAl_2$), 0.45 mM (silica) and 0.3 mM (glassy carbon). The affinity free enzyme values revealed that the affinity of the enzyme immobilized on all supports with either immobilization mechanism was low in comparison with that of free enzyme (K_m=0.016 mM). In general, this variation indicates that enzyme's affinity for the substrate decreased, possibly due to the following factors:

a) Microenvironmental, where support particles are surrounded by an immovable solvent layer (Nernst layer) (Rodríguez *et al*, 2007; D'Annibale *et al* 1999), in which the substrate concentration is lower than in the bulk solution. Therefore, enzyme/substrate (Lac-ABTS) interaction is more difficult.

b) Internal diffusion effects can be excluded due to the non-porous nature of the support; however, the active site exposure to the substrate molecule is restricted because the flexibility of the enzyme molecule, when it is immobilized is lower than when the enzyme is free (Torres-Salas *et al*, 2011; Durán *et al*, 2002; Palmieri *et al*, 1994; Voet *et al*, 1992).

c) Favorable orientation of the enzyme to bond to the substrate and promote transition state formation in the enzyme-substrate (ES) complex and generate the product (Voet *et al*, 1992).

Kinetic parameters of the K_{cat}/K_m enzymes provide a catalytic efficiency measure relating the catalytic constant K_{cat} or enzyme turnover number, to the affinity or K_m (Voet *et al*, 1992). The difference between the values of the constants shows

that efficiencies demonstrated between supports and mechanisms applied were considerably lower with respect to the free enzyme, due to the above indicated restrictive factors. Greater efficiency was observed in the system that employed glassy carbon with either immobilization mechanism. The results showed that catalytic properties of laccase preserved more conveniently when it was immobilized on the regular surface of the glassy carbon electrode as compared to solid crystal surfaces, such as $ZnAl_2$ particles and silica crystals. Another advantage of employing an electrode is that it provided some redox properties of the enzyme. In addition to kinetic parameters and activity of the immobilized enzyme, it was important to establish the stability of both immobilization systems on the three supports and the corresponding results are exhibited in the following section.

3.2 Stability

3.2.1 Operation stability

Determination of operation stability on the three types of supports refers to the number of times a reaction is repeated. The following sections show results of residual activity, obtained after every 5 catalytic cycles with three repetitions for each evaluation, and standard deviation (SD) ≤ 3%.

3.2.2 Operation stability with physical adsorption

Figure 8 shows the activity of laccase immobilized by adsorption on the three supports after 30 catalytic cycles. The loss of activity of the enzyme adsorbed on $ZnAl_2$ was 60% (Fig. 8d), on silica crystals 100% (Figure 8c) and on glassy carbon 76.62% (Fig. 8b), these percentages refer to the activity of the enzyme immobilized at the beginning of the cycles (Fig. 8a). This result is not comparable to those of the free enzyme, which was resumed in every cycle since it was difficult to recover it from the reaction system. The loss of specific activity after 30 cycles was huge, perhaps because the enzyme only gets fixed on the surface and not quite efficiently, which confirms the reports from literature regarding physical adsorption of enzymes (Tavares *et al*, 2013; Rodríguez *et al*, 2007; D'Annibale *et al*, 1999).

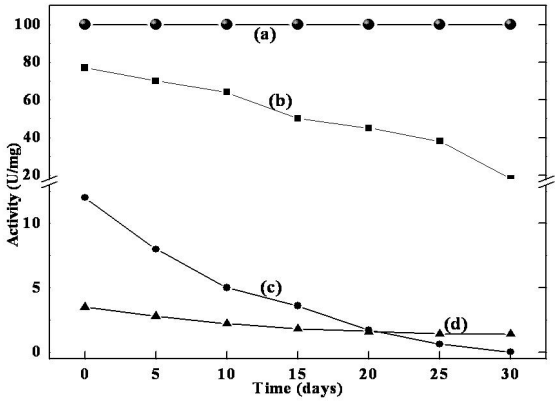


Fig. 8. Operation stability of laccase physical immobilized: (a) free laccase, (b) laccase-GC, (c) laccase-silica and (d) laccase $ZnAl_2$. The ABTS was used as substrate in acetate buffer solution 0.1 M pH 3.7.

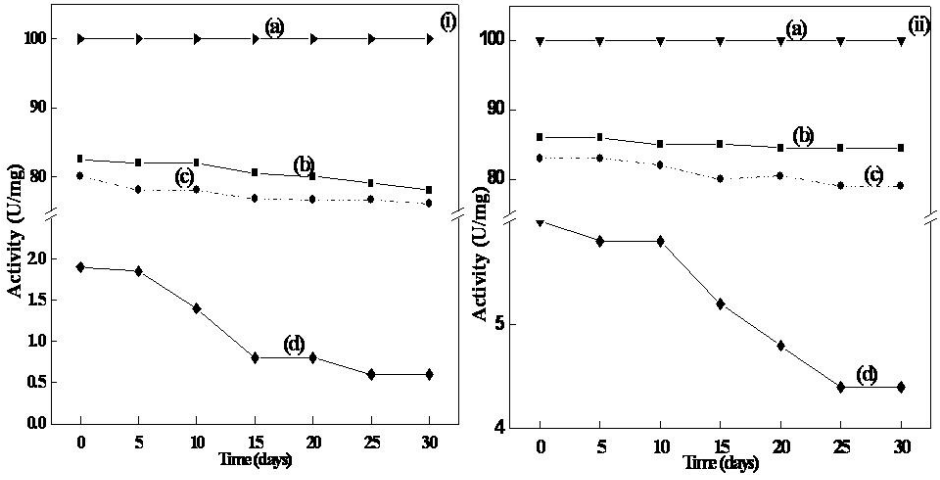


Fig. 9. Operation stability of laccase immobilized with (i) GA and (ii) thiols: (a) free laccase, (b) laccase-GC, (c) laccase-silica and (d) accase $ZnAl_2$, with ABTS used as substrate in acetate buffer solution 0.1 M pH 3.7.

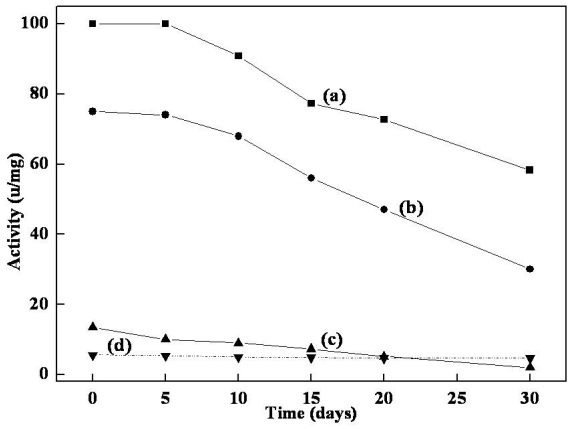


Fig. 10. Storage stability of laccase physical immobilized. Free laccase (a), laccase-glassy carbon (b), laccase-silica (c) and laccase $ZnAl_2$ (d). In all cases, ABTS was used as substrate in acetate buffer solution 0.1 M pH 3.7.

3.3.3 Operation stability using chemical immobilization (glutaraldehyde and thiols)

Operation stability of the chemically immobilized enzyme applying GA and thiols is shown in figs. 9i and 9ii, respectively. The loss of activity after applying immobilization with GA was 68.42% on $ZnAl_2$ (Fig. 9i, d), 5% on silica (Fig. 9i, c) and 5.45% on glassy carbon (Fig. 9i, d). However, the loss of specific activity of the enzyme immobilized with thiols was 26.67% on $ZnAl_2$ (Fig. 9ii, d), 4.82% on silica (Fig. 9ii, c) and 1.74% on glassy carbon (Fig. 9ii, b).

3.3.4 Storage stability

The stability of free and immobilized laccase, when preserved in refrigeration (4°C), was evaluated during 30 days. Each cycle consisted of putting the enzyme at room temperature, determine its activity and preserve it at 4°C for five days. Figure 10 exhibits the variation of laccase residual activity after each cycle of evaluation and storage at 4°C.

3.3.4.1 Storage stability of the enzyme immobilized by physical adsorption

The free enzyme activity was observed to diminish to 60% in 30 days (Fig. 10a); in contrast, the enzyme immobilized by adsorption on $ZnAl_2$ preserved 83.93% of activity (Fig. 10d), whereas on silica 14% (Figure 10c) and on glassy carbon 40% (Fig. 10b) were preserved.

In all cases, the reference was the immobilized enzyme at zero time; however, the activity was consistently preserved up to day 30, so the immobilization by adsorption favored stabilization of activity, which does not occur with the free enzyme because of its fast decrease.

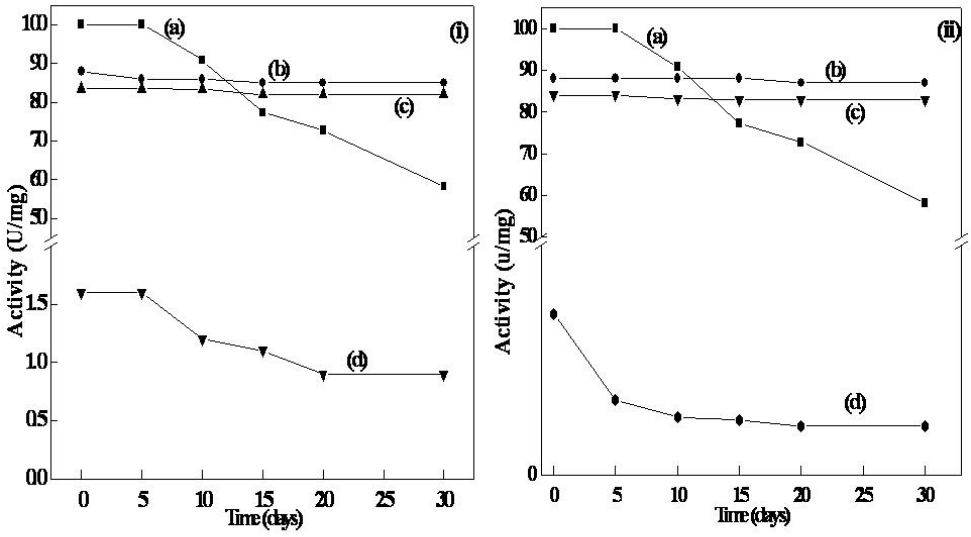


Fig. 11. Storage stability of laccase immobilized with GA (i) and thiols (ii): Free laccase (a), laccase-glassy carbon (b), laccase-silica (c) and laccase $ZnAl_2$ (d). In all cases, ABTS was used as substrate in acetate buffer solution 0.1 M pH 3.7.

3.4.1 Storage stability on chemically immobilized enzyme (glutaraldehyde and thiols)

Storage stability achieved with chemical immobilization mechanism using GA and thiols is shown in figures 11i and 11ii. The preservation of activity for GA was 56.25% on $ZnAl_2$ (Fig. 11i, d), 98.2% on silica (Fig. 11i, c) and 98.84% on glassy carbon (Fig. 11i, b). The immobilization with thiols produced the following preservation of activity: 65.38% on $ZnAl_2$ (Fig. 11ii, d), 98.81% on silica (Fig. 11ii, c) and 98.86% on glassy carbon (Fig. 11ii, b).

The retention indicated in all cases referred to the enzyme at zero time; however, the activity was consistently preserved up to day 30, so chemical immobilization favored stabilization of activity to a greater extent than immobilization by adsorption.

Although these results are lower as compared to those of the free enzyme, the data corroborated that immobilization on $ZnAl_2$, silica and glassy carbon, using both mechanisms, fulfilled its principal function: to preserve enzyme activity (operation and storage stability), which has a great advantage due to the possibility of carrying out continuous characterizations and reusing the supports during longer periods of time than with the free enzyme.

Conclusions

The results showed the modification of the catalytic properties of the enzyme immobilized on three different supports by means of two immobilization mechanisms, one physical and the other, chemical with GA and thiol. For chemical: the surface was functionalized with silanes using glutaraldehyde (GA) as a bonding agent, and in the second, the anchoring reagents were thiols.

In physical immobilization (on the different supports) and chemical immobilization on $ZnAl_2$, the activity was totally lost. As far as silica crystals are concerned, the loss of activity, by chemical adsorption was: 53.9% with Ga and 46% with thiols; finally, for GC, the decrease was: 41.75% in GA and 22.4% in thiols; in all cases, the loss of specific activity was reported with respect to the free enzyme.

In addition, the efficiency of every system was demonstrated as a function of the immobilization mechanism used and the properties of the support employed; the greatest efficiency was observed in the system that employed glassy carbon-Ga-laccase and these results are similar with those of free enzyme.

The evaluation of stability corroborated that the applied immobilization methods, even though different, preserve the enzyme activity (operation and storage stability) during longer periods than those achieved with the free enzyme.

Present results support the use of chemical immobilization by thiols on glassy carbon because it produced similar specific activity and catalytic efficiency as compared to free enzyme. Furthermore, activity stayed at 90 U/mg during 30 days of storage as compared to 60 U/mg residual activity of free enzyme, starting from 100 U/mg.

References

Acunzo F. and Galli C. (2003). First evidence of catalytic mediation by phenolic compounds in the lacase-induced oxidation of lignin models. *European Journal Biochemical 270*, 3634-3640.

Chiacchierini E, Restuccia D. and Vinci G. (2004). Bioremediation of food industry effluents: recent applications of free and immobilized polyphenoloxidases. *Food Science and Technological International 10*, 373-382.

D'Annibale A., Stazi S. R., Vinciguerra V., Mattia E. D. and Sermanni G. G. (1999). Characterization of immobilized laccase from *Lentinus edodes* and its use in olive-mill wastewater treatment. *Process Biochemical 34*, 697-706.

Durán N., Rosa M. A., D'annibale A. and Gianfreda L. (2002). Applications of laccase and tyrosinase (phenoloxidases) immobilized on different supports: a review. *Enzyme Microbiology Technology 31*, 907-931.

Fernández-Fernández M., Sanromán M. A. and Moldes D. (2013). Recent development and applications of immobilized laccase. *Biotechnology Advances*. Article in Press.

Freyre R. S., Duran N. and Kubota T. (2001). Effects of fungal laccase immobilization procedures for the development of a biosensor for phenol compounds. *Talanta 54*, 681-686.

Ho G. and Liao C. United States patent (1983) 4,384,045.

Ivnitski D. and Atanassov P. (2007). Electrochemical studies of intramolecular electron transfer in laccase from *Trametes versicolor*. *Electroanalysis 19*, 2307-2313.

Johnson D. L., Thompson J. L., Brin$K_m$ann S. M., Schuller K. A. and Martin L. L. (2003). Electrochemical characterization of purified *Rhus vernicifera* laccase: voltammetric evidence for a sequential four-electron transfer. *Bioelectrochemistry 42*, 10229-10237.

López-Salinas E. and Pedraza Archiva F. (2006). Hidrotalcitas: precursores de materiales adsorbentes de SOx. *IMP*, 91-96.

Mateo C., Palomo J. M., Fernández-Lorente G., Guisan J. M. and Fernández-Lafuente R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme Microbiology and Technology 40*, 1451-1463.

Mayer, A. M. and Staples R. C. (2002). Laccase: new functions for an old enzyme, review. *Phytochemistry 60*, 551-565.

Mousty C., Vieille L. and Cosnier S. (2007). Laccase immobilization in redox active layered double hydroxides: A reagentless amperometric biosensor. *Biosensors and Bioelectronics 22*, 1733-1738.

Onda A., Ochi T., Kajiyoski K. and Yanagisawa K. (2008). Lactic acid production from glucose over activated hydrotalcites as solid base catalysts in water. *Catalysis Communications 9*, 1050-1053.

Palmieri G., Giardina P., Desiderio B., Marzullo L., Giamberini M. and Sannia G. (1994). A new enzyme immobilization procedure using copper alginate gel: application to a fungal phenol oxidase. *Enzyme Microbiology and Technology 16*, 151-158.

Piontek K., Antorini M. and Choinowski T. (2002). Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *Biological Chemistry 277*, 37663-37669.

Quan D., Kim Y., Byung K. and Shin W. (2002). Assembly of laccase over platinum oxide surface and application as an amperometric biosensor. *Bulletin Korean Chemical Society 23*, 385-390.

Ren L., He J., Evans J. D., Duan X. and Ma R. (2001). Some factors affecting the immobilization of penicillin G acylase on calcined layered double hydroxides. *Journal Molecular Catalysis B: Enzyme 16*, 65-71.

Ren L., He J., Zhang S., Evans D. G. and Duan X. (2002). Immobilization of penicillin G acylase in layered double hydroxides pillared by glutamate ions. *Journal of Molecular Catalysis B: Enzyme 18*, 3-11.

Rodríguez Cuoto S., Osma J. F., Saravia V., Gübitz G. M. and Toca Herrera J. L. (2007). Coating of immobilized laccase for stability enhancement: A novel approach. *Applied Catalysis A: Chemical 329*, 156-160.

Sachdev D., Dubey A. and Mishra B. G. (2008). Enviromentally bening liquid phase oxidation of vanillin over copper containing ternary hydrotalcites. *Catalysis Communications 9*, 391-394.

Shleev S., Christenson A., Serezhenkov V., Burbaev D., Yaropolov A., Gordon L. and Ruzgas T. (2005). Electrochemical redox transformations of T1 and T2 copper sites in native Trametes hirsute laccase at gold electrode. *Biochemical Journal 385*, 745-754.

Solís-Oba M., Bárzana E., García-Garibay M. and Viniegra-González G. (2007). The $ABTS^{\bullet+}$ and oxidant agent of different chemical compounds and its recycling process between laccase and substrate. *Revista Mexicana de Ingeniería Química 6*, 275-281.

Solís-Oba M., Eloy-Juárez M., Teutli M., Nava J. L. and González I. (2009). Comparison of advanced techniques for the treatment of an indigo model solution: Electro incineration, chemical coagulation and enzymatic. *Revista Mexicana de Ingeniería Química 8*, 275-282.

Solís-Oba M., Ugalde V. M., González I. and Viniegra-González G. (2005). An electrochemical-spectrophotometrical study of the oxidized forms of the mediator 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) produced by immobilized laccase, *Journal of Electroanalysis Chemical 579*, 59-66.

Tavares P. M. A., Rodriguez O., Fernández-Fernández M., Domínguez A., Moldes D., Sanromán M. A. and Macedo A. (2013). Immobilization of laccase on modified silica:

stabilization, thermal inactivation and kinetic behavior in 1-ethyl-3-methylimidazolium ethyl sulfate ionic liquid. *Bioresource Technology 131*, 405-412.

Téllez-Téllez M., Sánchez C., Díaz R. and Díaz-Godínez G. (2012). Zymogram patterns of extracellular laccases of *Pleurotus* Species grown on non inducer agar medium. *Revista Mexicana de Ingeniería Química 11*, 383-388.

Torres-Salas P., Del Monte-Martínez A., Cutido-Avila B., Rodríguez-Colinas B., Alcalde M., Ballesteros O. A. and Plou J. F. (2011). Immobilized Biocatalysts: Novel approaches and tools for binding enzymes to supports. *Advanced Materials 23*, 5275-5282.

Voet D. and Voet J. G., (1992). *Bioquímica*, Ediciones Omega S. A. Barcelona España.

Zaborsk O. R. (1974). *Immobilized enzymes*. OH Cleveland, USA. CRC Press.