Immobilization strategies for laccase from Trametes versicolor on mesostructured silica materials and the application to the degradation of naphthalene

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ABSTRACT

The oxidation of naphthalene by immobilized laccase from *Trametes versicolor* has been performed using diverse immobilization strategies on mesostructured silica materials. Laccase was immobilized by physical adsorption on several SBA-15 with different textural properties and by covalent attachment on functionalized SBA-15 prepared by co-condensation method (direct synthesis). The adsorption of laccase was partially reversible and showed some degree of lixiviation. However, covalently attached laccase to aminopropyl and aminobutyl functionalized SBA-15 exhibited important activity for the degradation of naphthalene with, respectively, 35% and 39%wt of removal in 5 hours. The aminopropyl biocatalyst retained higher activity after repeated uses than the corresponding aminobutyl.

Keywords: laccase; enzyme immobilization; degradation of aromatic compounds; phenol, naphthalene, mesostructured silica

1. Introduction

Xenobiotic pollutants are increasingly attracting the attention as a major environmental problem. Among them, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in nature and are found widespread in soils, waters and air. PAHs are part of the chemical composition of carbon and petroleum and are produced both from natural and anthropogenic sources by incomplete combustion of fossil fuels and biomass. These recalcitrant compounds show toxic, carcinogenic and mutagenic properties (Kim et al., 2001) and 16 of them are considered as priority pollutants by the US Environmental Protection Agency (USEPA) under the Clean Water Act (USEPA, 1972). Because of its relatively high volatility, naphthalene is also considered a hazardous air pollutant (USEPA, 1994; USEPA, 2004).

Lignolytic fungi related to the rot of the wood, especially white rot fungi such as *Trametes versicolor*, have been proved ability to degrade and mineralize PAHs (Pointing, 2001). Peroxidases (lignin peroxidases and manganese peroxidases) and oxidases (laccases) are the main constituents of the complex enzymatic system of these fungi (Mester and Tien, 2000).

Laccases (*p*-benzenediol:oxygen oxidireductase; E.C. 1.10.3.2) are extracellular enzymes that belong to the multicopper polyphenol oxidases. They catalyze the four 1-electron oxidation of electron-rich compounds with the simultaneous 4-electron reduction of molecular dioxygen to water (Wells et al., 2006). Besides their "natural" phenolic substrates, the lack of specificity lends them capacity to oxidize a wide range of substrates with higher redox potentials, including veratryl alcohol (Bourbonnais and Paice, 1990), chlorophenols (Bollag et al., 2003), dyes (Chhabra et al., 2009; Cristóvão et al., 2008; Rodriguez et al, 1999), or several PAHs (Majcherczyk

et al., 1998; Pozdnyakova et al., 2006), although in both reports naphthalene was not significantly degraded by laccase even with the aid of a mediator system. The extension of the catalytic activity of laccases towards non-phenolic substrates is usually carried out by the action of a mediator system acting as an electron shuttle between the enzyme and the substrate (Bourbonnais and Paice, 1990). N-hydroxybenzotriazole (HBT), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and siringaldehyde, among others, have been extensively reported as effective mediator compounds for laccase oxidations (Dodor et al., 2004).

It is known that enzyme immobilization on solid materials favours process design and development and allows the biocatalyst to be used at industrial scale. In that respect, laccases have been immobilized on numerous supports, such as porous and non-porous glass, agarose, amorphous silica, organic gels or kaolinite, and by different mechanisms, such as adsorption, entrapment or covalent attachment (see review by Durán et al., 2002; Hu et al., 2007). Mesoporous silica materials are very attractive as enzyme immobilization carriers due to their tuneable pore size, well-ordered porous structure, high surface area and improved hydrothermal resistance compared to sol-gel silica and other common supports (Lee et al., 2009; Ispas et al., 2009). Laccase from *T. versicolor* has been immobilized on magnetically separable mesoporous silica spheres for the oxidation of ABTS (Zhu et al., 2007), on kaolinite and SBA-15 by covalent coupling for the oxidation of anthracene and benzo[a]pyrene mediated by HBT and ABTS (Hu et al., 2009; Hu et al., 2007; Dodor et al., 2004). Among the different procedures typically used to incorporate organic moieties to a silica surface (Wight and Davis, 2002, Hoffmann et al, 2006), the covalent attachment of laccase has been solely performed by post-synthesis grafting of the corresponding organosilane containing the

selected functional group onto the surface of the silica carrier. To the best of our knowledge, immobilization of laccases has not been carried out on functionalized silica materials synthesized via co-condensation, where organosilanes and the silica source are added simultaneously during the synthesis, resulting in a more even distribution of the functional groups on the surface of the final support (Lee at al., 2009).

The aim of the present study was to assess different strategies for the immobilization of laccase from *T. versicolor* as a biocatalyst for the direct degradation of naphthalene, as a model PAH, without the addition of any oxidation mediator system. To achieve the above goal various "tailor-made" SBA-15 materials, both pure and functionalized by co-condensation (direct process) were synthesized showing different surface properties, pore sizes and functionalization.

2. Materials and Methods

2.1. Materials

Laccase from *Trametes versicolor* sp. was kindly provided by Amano Enzymes (Japan) under the commercial name of Laccase Daiwa Y120.

Pluronic 123 (EO_{20}-PO_{70}-EO_{20}) and Brij 76 ($C_{18}EO_{10}$) surfactant non-ionic polymers (Aldrich, Steinheim, Germany) were used as templates for the self-assembling synthesis of the mesoporous SBA-15-type materials. Tetraethylorthosilicate (TEOS) (Aldrich) was employed as silica source. Organosilane compounds used for the organic functionalization of the silica mesostructure were 3-aminopropyl-triethoxysilane (APTES, Aldrich) and 4-aminobutyl-trimethoxysilane (ABTMS, ABCR). glutaraldehyde (25% aqueous solution, Sigma-Aldrich) was used as bridging agent for the grafting of enzyme to the silica support. Sodium borohydride (98%, Sigma-Aldrich,

Saint Louis, MO, USA) was used as reducing agent in the imine moieties to amine.

Naphthalene, phenol (>99%, Aldrich) and 4-aminoantipyrine (98%, Fluka, Neu Ulm, Germany) were employed as substrate and co-substrate, respectively, for the enzymatic assays. Polyethylene glycol sorbitan monooleate, trademark Tween-80 (Sigma-Aldrich), was used as non-ionic surfactant to increase naphthalene solubility in aqueous solution.

2.2. Synthesis of mesostructured silica materials (SBA-15)

Siliceous SBA-15 was synthesized according to the procedure reported by Zhao et al (1998) using Pluronic 123 ($EO_{20}PO_{70}EO_{20}$) as micellar templating agent. Aging temperature was varied from 110ºC to 140ºC in order to obtain two otherwise similar mesostructured materials (SBA-I and SBA-II) with different mean pore sizes due to an expansion effect on the micellar system as a consequence of increasing temperature. The synthesis of an additional mesostructured material with lower mean pore size (designated as SBA-III) was achieved using Brij 76 ($C_{18}EO_{10}$) as templating agent. The molar composition of the mixture was similar to those used for the Pluronic 123. However, dissolving and TEOS hydrolysis conditions were changed. Thus, in order to obtain a clear micellar solution before adding the silica source, Brij 76 was stirred in 1.9M HCl for 3 h at 50ºC; and then temperature was also kept a 50ºC for the hydrolysis of TEOS (van Grieken et al, 2002).

2.3. Synthesis of mesostructured silica materials functionalized with alkyl-amino groups (NH_2-SBA-15)

Amino functionalized-materials were prepared following a co-condensation

method, wherein APTES and ABTMS were employed as amine precursors. The degree of organic functionalization was fixed at two levels: molar ratios of 1 and 10% referred to total silicon. In this way, four materials were prepared, two functionalized with aminopropyl moieties –AP1 and AP10– and two incorporating aminobutyl functions –AB1 and AB10. Synthetic procedure was similar to that described for pure-silica mesostructured materials except for the addition of the appropriate amount of the corresponding organosilane after a 45 min pre-hydrolysis step of TEOS. This step allows the formation of an incipient silica mesostructure that will allocate the amino organic moieties in an optimal fashion. In this case, aging temperature was 110ºC for all the samples, as the presence of the co-condensed organosilanes prevents the thermal expansion effect on the micelles (van Grieken et al, 2002).

2.4. Immobilization of laccase on different mesostructured materials

Immobilization of enzyme on the surface of the mesoporous materials was performed by two different methods: physical adsorption and covalent grafting. The first method consisted of an impregnation procedure where 0.5 g of solid support was suspended in 50 mL of an enzyme-containing aqueous solution for 3 hours under gentle stirring. The progress of the immobilization was monitored through the UV absorbance decrease at 280 nm in solution using a Cary-500 spectrophotometer (Varian, Palo Alto, CA, USA). On the other hand, the immobilization via covalent grafting of the enzyme to the silica surface needs the presence of grafted alkyl-amine moieties which act as anchoring sites for the enzyme.

Previous to the enzyme attachment, the NH_2-modified silica supports were further functionalized with reactive aldehyde groups. Thus, 1 g of amino-functionalized

mesostructured support (NH_2-SBA-15) was dispersed in phosphate buffer, pH 7.2, and then a 25% glutaraldehyde solution was added in excess. The resultant suspension was stirred at 25ºC for 3 hours in a temperature-controlled water bath. During this step, one of the -CHO moieties in the glutaraldehyde molecule reacts with the -NH_2 groups chemically attached to the silica surface. Then, the attachment of the enzyme was performed by adding the glutaraldehyde-modified support to an enzyme solution in phosphate buffer, pH 7.2, under gentle stirring. The free glutaraldehyde -CHO group in the silica material can react with an amino group from the enzyme in solution, thus forming a bridging molecular unit between the silica support and the enzyme. The reaction was monitored through the UV-Vis absorbance at 280 nm in solution using the above spectrophotometer. Finally, the resultant enzyme-silica biocatalyst was treated with 1 mg/mL of sodium borohydride for 1 hour under stirring to reduce the imine moieties into more stable amine groups.

2.5. Characterization of the mesostructured materials

In order to characterize the textural properties of the synthesized supports, nitrogen adsorption and desorption isotherms were measured at 77 K using a TRISTAR 3000 system. Data were analyzed using the BJH model and total pore volume was estimated at P/Po= 0.975 single point. Structural ordering was determined by means of X-ray powder diffraction (XRD) on a PHILIPS X`PERT diffractometer using Cu Kα radiation. Elemental analysis using a Vario EL III Elemental Analyzer and thermogravimetric analysis in a SDT 2960 simultaneous DSC–TGA from TA Instruments (5ºC·min^{-1} ramp until 700ºC in air) were used as analytical techniques for the determination and quantification of aminoalkyl moieties and final enzyme loadings

within the solid.

2.6. Evaluation of the enzymatic activity in the oxidation of phenol

The activity of mesostructured silica-supported laccase was determined using phenol and 4-aminoantipyrine (4-AAP) as substrates. Laccase-catalyzed oxidation of phenol in presence of oxygen and 4-AAP produces a colored quinine-imine complex whose formation can be monitored by spectrophotometric absorption at 505 nm.

The reaction mixture comprised 250 mM phenol and 9 mM 4-AAP in acetate buffer pH 4.5. The reaction was initiated by the addition of an appropriate amount of laccase, either soluble or immobilized in 10 mL of buffer solution. Reaction media was incubated at 25ºC for 20 min. The enzyme activity (*Y*) was expressed as polyphenol oxidase units per mass unit of laccase (POU/g) and was measured by the increase in the absorbance at 505 nm using the following equation (Daiwa Kasei's assay method for laccase activity (Ref.: P-4AA):

$$Y(POU/g) = \frac{300 \cdot (A_{40} - A_{10})}{(0.1 \cdot 10 \cdot W)},$$

where A_{10} and A_{40} are the absorbances at 505 nm after 10 and 40 seconds of reaction and *W* is the weight of enzyme, in g, in 1 mL of the sample.

2.7. Degradation of naphthalene

Likewise, in order to evaluate the enzymatic activity of the biocatalyst on the degradation of polycyclic aromatic hydrocarbons, the degradation of naphthalene from an aqueous solution was studied. In this case, catalytic tests were carried out by adding the immobilized enzyme to 2 mM naphthalene aqueous solution containing 5 wt% Tween-80 and acetate buffer pH 4.5 (reported pH for maximum activity of laccase). The

presence of this surfactant increased the solubility of naphthalene. Naphthalene degradation was then monitored by means of HPLC analysis on a reversed-phase C18 column (Luna C18(2) 7.5 cm x 4.6 mm I.D. 3 μm particle size; Phenomenex, Torrance, CA, USA) with the corresponding guard column. The HPLC system was a ProStar 230 apparatus (Varian, Palo Alto, CA, USA) equipped with a quaternary pump and photodiode array UV-Vis and fluorescence detectors. 10 μL of filtered solution containing naphthalene were injected and then eluted from the column at 0.8 mL/min flow rate using the following acetonitrile:water gradient program: isocratic 60:40 (0-2 min), gradient to 75:25 (2-14 min), isocratic 75:25 (14-15 min), gradient to 100:0 (15-16 min).

3. Results and discussion

3.1. Supports for the immobilization of laccase

Table 1 summarizes the physicochemical and textural properties of the different siliceous mesostructured materials used as supports for the immobilization of laccase by physical adsorption (materials SBA-I, SBA-II and SBA-III). Fig. 1A depicts the N_2 adsorption-desorption isotherms of these supports previous to the incorporation of the enzyme, whereas the X-ray diffraction patterns are shown in Fig. 1B. The diffractograms include the signals 100, 110 and 200, corresponding to hexagonally-ordered mesophases, with the unit cell parameters (d_{100}) shown in Table 1. On the other hand, N_2 adsorption-desorption isotherms clearly correspond to type-IV isotherms, according to IUPAC classification, which are characteristic of mesoporous solids. Likewise, a microporosity fraction can be deduced from the isotherms. This is typical for SBA-15-type materials when surfactant has been completely removed by calcination

(Van Grieken et al, 2003).

The textural and structural properties of these purely siliceous mesostructured materials indicate the success of the synthesis, as they display highly ordered mesostructures with tuned mean pore sizes (Table 1). Thus, the support SBA-II, subjected to a higher temperature during the aging step, shows a higher mean pore size as compared to the standard SBA-15 silica (SBA-I). However, this increase was accompanied by an important decrease of the BET surface area and the average wall thickness. Such a low wall thickness could jeopardize the stability of the mesostructure of this material when exposed to aggressive environments such as high temperatures in the presence of water, which could impair the integrity of the structure leading to a collapse of the pore system. Nevertheless, the use of this material as immobilization support for enzymes is not expected to be a demanding application in terms of thermal stability. On the other hand, the use of a smaller surfactant such as Brij76 provides a rather smaller micellar size than that achieved with Pluronic 123. As a consequence, SBA-III shows the lowest mean pore size (5 nm) and the highest surface area.

Table 2 shows the physicochemical, textural properties and quantities of alkyl-amino groups and enzyme immobilized into different functionalized mesoporous materials. Hexagonally-ordered mesostructure typical of SBA-15 was confirmed by XRD patterns, shown in Fig. 1D. The presence of low-angle diffractions corresponding to the directions 100, 110 and 200, typical of 2D-hexagonal symmetry, is clearly evidenced in the diffractograms. As alkylamino content was increased from 1 to 10 mol% in synthesis, BET surface areas and pore volumes evidenced a decrease (Fig. 1C). N_2 adsorption-desorption isotherms exhibited a broader H1-type hysteresis loop with the increase in the organic content, leading to wider pore size distributions centered at

lower mean pore sizes. On the other hand, the organic incorporation yield of amino species was high for the different materials, especially at the level of 1 mol% in synthesis. As expected, the samples prepared with 10 mol% amino content did not provide a total incorporation of organic species, but they still achieved prominent incorporation yields over 70%.

3.2. Immobilization of laccase on silica mesoporous supports

3.2.1 Physical adsorption method

The results of physically-adsorbed laccase on the purely siliceous mesostructured SBA supports are shown in Fig. 2. Different concentrations of enzyme in buffer solution have been used for the impregnation of the supports, ranging from 2 to 6 g/L. Final amount of incorporated enzyme (herein denoted as q) within the silica support has been deduced from the reduction of laccase concentration in solution as measured by means of UV absorbance. As can be observed, the increase in laccase concentration in solution globally resulted in an increase of q values. This indicates that, as a mass transfer-driven process, the incorporation of enzyme from solution onto the silica surface is clearly dependent on the laccase concentration in solution.

Although SBA-II showed the highest pore size (11.4 nm, Table 1) of all three silica supports and the accesibility for enzyme molecules is improved, SBA-I adsorbed higher laccase amounts than SBA-II and SBA-III, especially at higher laccase concentration in solution. However, SBA-II sample also has a reduced surface area – approximately half of that displayed by SBA-I– as a consequence of the higher aging temperature used during its synthesis. Thus, surface area is also an important factor to be taken into consideration. On the other hand, for a laccase concentration in solution of

6 mg/ml, SBA-III yielded lower q values than SBA-I despite of its slightly higher surface area (821 $m^2 \cdot g^{-1}$). In this case, the steric hindrances produced by the smaller mean pore size (5.0 nm) at high enzyme concentration seems to limit the incorporation of the enzyme. Therefore, SBA-I support can be considered as the most efficient material for the physical adsorption of laccase. It must be also noted that most likely the physical adsorption of laccase on mesoporous silica materials is a reversible process, and when these biocatalysts are suspended again on aqueous solution a partial leaching of enzyme from the solid to solution should be expected. This issue will be further discussed below in the assessment of the enzymatic activity.

3.2.2 Covalent grafting method

Table 3 summarizes the final enzyme loadings after immobilization via covalent grafting on the amino-functionalized mesoporous materials. The estimation of the actual amount of immobilized laccase was performed by means of thermogravimetric data, although nitrogen content from elemental analysis was also necessary to estimate the weight percent attributable to alkylamine- or glutaraldehyde-type moieties.

Fig. 3 includes the thermogravimetric analysis of the biocatalyst AB1/laccase (Fig. 3B) in comparison with that of AB1 (Fig. 3A), its parent support. For the latter, observed weight loss in the range 200-350ºC is attributed to thermal degradation of the aminobutyl moieties covalently incorporated on the pore walls during the co-condensation synthesis. Below that temperature –from 50 to 150ºC– the weight loss can be ascribed to labile molecules such as water or templating co-polymer; whereas higher temperatures lead to the thermal condensation of residual Si-OH groups into Si-O-Si

bonds, as usual in silica materials. Since the concentration of aminobutyl-trimethoxysilane (ABTMS) in synthesis for this sample was fixed at 1 mol% the amount of total organic content is relatively small. On the other hand, the weight loss curve for the biocatalyst –AB1/laccase– evidences an important increase in the organic content. Weight loss in the temperature range of 150 to 650ºC was 56.8 wt.% (Table 3), the highest value reached in the series of tested materials. This increase in organic content after the immobilization process is directly attributed to the incorporation of enzyme molecules via covalent grafting.

Using this calculation method based on TG analysis, enzyme loadings of the different amino-functionalized supports were estimated and are also incorporated in Table 3. It was observed that enzyme loading in AP1 and AB1 were higher that that in AP10 and AB10, respectively. Thus, considering the same amino-alkyl group, a higher amount of enzyme was fixed to the support with the lower amount of anchoring points, i.e. the lower concentration of amine functions on the pore surface gave the higher immobilization yield. This effect can be attributed to steric hindrances to the diffusion of the enzyme along the pores since laccase from *T. versicolo*r is a globular enzyme whose molecular dimensions are, approximately, 65 x 55 x 45 $Å^3$ (Piontek et al., 2002) and the average pore size of the functionalized silica supports are within the range 79-111 Å (Table 2), that is, about the same order of magnitude. In addition, when the surface density of the amino functional groups is high, is more likely for a laccase molecule to be covalently bound at the inlet of the pores, preventing another enzyme molecule to enter into the pore space.

With respect to the effect of the chain length of the amino functional group, the better immobilization of enzyme was obtained in butylamino-functionalized supports

over propylamino-functionalized materials. This can be attributed to the higher mobility of a longer alkyl-chain, which would allow a wider range of approximation angles of the enzyme to react with the anchoring point, thus facilitating the immobilization.

3.3. Assessment of enzymatic activity

Table 4 summarizes the analysis of the enzymatic activity through the phenol oxidation test described in the experimental section. The results are shown in terms of polyphenol oxidase units (POU) per gram of the different laccase-based mesoporous biocatalysts. In order to compare with an equivalent homogeneous system, the assay was also performed with laccase in solution.

As expected, the highest activity was achieved by the homogeneous system. On the other hand, the biocatalyst prepared by physical adsorption SBA-I/laccase was the heterogeneous biocatalyst with the higher activity despite the enzyme loading in this material is relatively low (6.3 g of laccase/g of material when prepared in an impregnation medium at a laccase concentration of 6 $g \cdot L^{-1}$, Fig. 2) as compared to the materials prepared by covalent grafting. However, as mentioned above, the immobilization of enzyme via physical adsorption is an equilibrium process where the adsorbed enzyme is prone to be desorbed when back in solution. Thus, a partial lixiviation of enzyme to the solution can be expected and the overall enzymatic activity would come from homogeneous as well as heterogeneous pathways.

In order to confirm this fact, an additional test was performed to check the extension of this enzyme leaching. A sample of SBA-I/laccase material was suspended in the reaction mixture in absence of phenol and the evolution of the system was monitored by means of UV. Leaching of enzyme was then confirmed by the appearance

of an increasing UV absorbance at 280 nm, indicative of the presence of laccase in solution. This test was also applied to the materials based on covalent-immobilization of laccase and no increase of UV absorbance at 280 nm was detected, confirming the irreversibility of the covalent binding between the enzyme and the mesoporous silica supports. For these materials, the activity is approximately proportional to the amount of enzyme incorporated in the material (Table 3).

Thus, AP1/laccase and AB1/laccase biocatalysts showed higher POU values than AP10/laccase and AB10/laccase. In the same way, the enzymatic activity was slightly higher with the biocatalysts functionalized with longer-chain alkyl groups (aminobutyl moieties). It must also be noted that, apart from the amount of enzyme, the higher mobility of a longer alkyl chain and the consequent reduced stiffness of the enzyme-support link is another positive factor for the enzyme to appropriately develop its biological activity. Additionally, Fig. 4 displays the kinetic curves for the degradation of phenol up to 1200 seconds of reaction time. The trends above-commented are confirmed, and the activity of the biocatalyst AB1/laccase is shown as the highest among the true heterogeneous catalysts also for long reaction times.

3.4. Degradation of naphthalene

To test naphthalene degradation was caused by the enzymatic activity, three replicates of a control experiment without biocatalyst were performed. After 24 h, the concentration of naphtlalene was 97.9 ± 0.1% of the initial concentration so that the naphthalene depletion shown in Fig. 5 is attributed to the laccase activity.

The results obtained in the enzymatic degradation of naphthalene are shown in Fig. 5. As observed, the decay of the naphthalene concentration was more pronounced

when using laccase in solution as biocatalyst (homogeneous catalysis), achieving after 300 min a conversion of naphthalene close to 70% (remaining naphthalene in solution about 30%). This not only indicates the higher biological activity of homogeneous laccase as compared with supported laccase, as expected, but also serves as a confirmation of the efficiency of this enzyme for the biodegradation of polycyclic aromatic compounds such as naphthalene.

On the other hand, the degradation rates using covalently-supported laccase are lower and follow the same trend observed in the above assay of phenol oxidation. Again, the most active mesoporous biocatalysts are those with the lowest surface density of alkyl-amino functional groups (AP1/laccase and AB1/laccase), as a consequence of its higher enzyme loading as discussed before. Furthermore, the immobilization via single-point linkages instead of multiple-point linkages contributes to favour the biological activity of the protein. Likewise, the butylamino-functionalized sample is slightly more active than the propylamino-functionalized sample, fact again attributed to its higher enzyme loading.

The statistical significance of the above results was confirmed through the replication (three times) of a degradation experiment using covalently immobilized laccase on functionalized SBA-15, showing an average error of ±2.1% for all measurements over the time course of the experiment.

Due to the practical interest of this application for soil bioremediation, deactivation of the biocatalysts was assessed by means of reutilization assays using mesoporous silica-immobilized laccase in three consecutive degradation cycles. Biocatalysts selected for this analysis were AP1/laccase and AB1/laccase, as the most active of the series. After each reaction cycle, biocatalysts were recovered by filtration

and washed with phosphate buffer, pH 7.2, before being placed back in reaction. Fig. 6 shows the results of the reutilization tests in terms of relative activity with respect to the activity of the fresh material.

AP1/laccase keeps its initial activity for a second catalytic run but shows a decrease over 30% in the third cycle (Fig. 6A). However, AB1/laccase evidences a dramatic decay of activity already in the second use, almost completely losing it in the third reutilization (Fig. 6B). As mentioned above, there are no evidences of enzyme leaching when using these materials, so that the loss of activity must be caused for a different deactivation mechanism. We ruled out the possibility of active sites poisoning by deposition of any substance because such a deactivation mechanism has not been reported before. Therefore, the most feasible deactivation cause is a change in the tertiary structure of the enzyme after a prolonged time at the reaction pH, 4.5. At this pH the stability of laccase can be compromised (Rancaño et al., 2003), especially considering that each reaction cycle takes 5 h at the above pH. An increase in the length of the surface functional group where the enzyme is grafted can led to a decrease in the stability of the biocatalyst since the higher mobility facilitates conformational changes in the tertiary structure of the enzyme (Cao, 2005). This can explain, at least partially, the higher degree of deactivation showed by AB1/laccase (aminobutyl ligand) than that of AP1/laccase (aminopropyl ligand).

4. Conclusions

Different strategies for the immobilization of laccase from *Trametes versicolor* on various mesostructured silica materials have been tested. Covalently immobilized laccase through an aminopropyl (AP1/laccase) and aminobutyl (AB1/laccase) functional

groups exhibited a significant and relevant activity for the degradation of naphthalene without the presence of a mediator system. AP1/laccase showed higher stability than AB1/laccase after repeated reaction runs. Furthermore, for each aminoalkyl functionalized silica matrix, both the highest enzyme loading and catalytic activity were attained for the biocatalyst with lower amino content (1 mol%) since a silica surface with larger (10 mol%) amino group coverage led to increasing steric hindrance limiting the enzyme entrance into the pores.

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Table 1. Physicochemical and textural properties of mesostructured silica supports.

Support	D_P [a] (Å)	d_{100} [b] (Å)	BET area ($m^2 \cdot g^{-1}$)	V_P [c] ($cm^3 \cdot g^{-1}$)	Wall thick [d] (Å)
SBA-I	90	101	732	1.16	27
SBA-II	114	103	376	1.01	5
SBA-III	50	56	821	1.17	15

[a] Mean pore size from the adsorption branch applying the BJH method.
[b] $d_{(100)}$ spacing, measured from small-angle powder X-ray diffraction.
[c] Total pore volume taken at $P/P_o = 0.975$ single point.
[d] Average wall thickness calculated by $a_o - D_P$ ($a = 2\ d_{100} / \sqrt{3}$).

Table 2. Physicochemical and textural properties of alkylamino-functionalized mesostructured silica supports.

Support	Alkylamino moiety		D_P [b]	d_{100} [c]	BET area	V_P [d]	Wall thick.[e]	Nitrogen [f]	Yield [g]
	Type	mol%[a]	(Å)	(Å)	(m^2/g)	(cm^3/g)	(Å)	(mmol N/g_{SiO2})	(%)
AP1	Propyl-NH_2	1	95	113	510	0.82	36	0.15	100
AP10	Propyl-NH_2	10	90	110	489	0.91	37	1.16	70
AB1	Butyl-NH_2	1	111	112	706	1.43	16	0.17	100
AB10	Butyl-NH_2	10	79	105	560	0.92	43	1.31	78

[a] Synthesis values of alkylaminosilane content as mol% based on total silicon atoms.
[b] Mean pore size from the adsorption branch applying the BJH method.
[c] $d_{(100)}$ spacing, measured from small-angle powder X-ray diffraction.
[d] Total pore volume taken at P/P_o=0.975 single point.
[e] Average wall thickness calculated by a_o - D_P ($a= 2\ d_{100} / \sqrt{3}$).
[f] Calculated from nitrogen wt% from elemental analysis and residual SiO_2 wt% from TG.
[g] Yield of incorporation of alkylamino moieties relative to synthesis values.

Table 3. Enzyme loading by covalent grafting through amino bonds.

Biocatalyst	Catalyst mass composition (wt%)				
	SiO_2 [a]	Organic [b]	Alkylamino [c]	Glutaraldehyde [d]	Enzyme [e]
AP1/laccase	77.1	19.3	1.1	2.6	15.6
AP10/laccase	78.9	18.2	6.5	4.8	6.9
AB1/laccase	41.9	56.8	1.5	1.4	53.9
AB10/laccase	52.8	42.6	7.1	7.4	28.1

[a] Residual SiO_2 wt% from TG at a final temperature of 650ºC.
[b] Total organic content as weight loss from 150ºC to 650ºC in thermogravimetric analysis.
[c] Weight percent of alkylamino moieties estimated by nitrogen content of the starting support and thermogravimetric analysis of the biocatalyst.
[d] Weight percent of glutaraldehyde linking functions assuming total conversion of grafted amine functions.
[e] Weight percent of laccase estimated as the difference: Total organic – (alkylamino + glutaraldehyde)

Table 4. Enzymatic activity of laccase in solution and supported on the different mesoporous materials as determined in the oxidation of phenol.

Biocatalysts	Activity ($POU \cdot g^{-1}$)
Laccase in solution	11550
SBA-I/laccase	7800
AP1/laccase	3473
AP10/laccase	2790
AB1/laccase	3798
AB10/laccase	2825

Caption of Figures

Fig. 1. N_2 adsorption-desorption isotherms (A) and X-ray diffraction patterns (B) of siliceous mesostructured materials (above) and N_2 adsorption-desorption isotherms (C) and X-ray diffraction patterns (D) of alkyl-amino-functionalized mesostructured materials.

Fig. 2. Laccase adsorption on pure silica mesostructured supports for different laccase concentrations in the solution used for the impregnation (SBA-I/laccase ▬**,** SBA-II/laccase ▨ and SBA-III/laccase ▭).

Fig. 3. TGA (continuous line) and DTG (dashed line) measurements corresponding to AB1 amino-functionalized mesoporous support (A) and AB1/laccase biocatalyst prepared via covalent grafting of enzyme on AB1 material (B).

Fig. 4. Catalytic activity of laccase in solution and immobilized on different mesoporous materials for the oxidation of phenol (free laccase ■, SBA-I/laccase ◆, AP1/laccase △, AP10/laccase ▲, AB1/laccase ○, AB10/laccase ●).

Fig. 5. Degradation of naphthalene with laccase in solution and supported on the different mesoporous materials (free laccase ■, AP1/laccase △, AP10/laccase ▲, AB1/laccase ○, AB10/laccase ●).

Fig. 6. Enzymatic activity of AP1/laccase (A) and AB1/laccase (B) in the degradation

of naphthalene after several consecutive reutilization cycles relative to the activity of the first run (Reaction time: 5 h).