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# Laccase from *Pleurotus sajor-caju* on functionalised SBA-15 mesoporous silica: Immobilisation and use for the oxidation of phenolic compounds

Andrea Salis[a,*], Marco Pisano[a], Maura Monduzzi[a], Vincenzo Solinas[a], Enrico Sanjust[b]

[a] *Laboratorio di Biocatalisi, Dipartimento di Scienze Chimiche, Università di Cagliari–CSGI, Cittadella Universitaria, S.S. 554 bivio Sestu, 09042 Monserrato (CA), Italy*
[b] *Laboratorio di Enzimologia Applicata, Dipartimento di Scienze e Tecnologie Biomediche, Università di Cagliari–CSGI, Cittadella Universitaria, S.S. 554 bivio Sestu, 09042 Monserrato (CA), Italy*

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ABSTRACT

Laccase from *Pleurotus sajor-caju* was immobilised on functionalised SBA-15 mesoporous silica. The immobilisation process reached the equilibrium after about 100 min. In order to study the effect of loading ($L$) on activity of the immobilised laccase, the adsorption isotherm was built and the activity of the corresponding immobilised biocatalysts was determined. The activity of the immobilised preparations reached a maximum at $L = 217\ \mathrm{kU\ g_{SBA\text{-}15}^{-1}}$, whereas higher loadings gave rise to a less-efficient biocatalyst. The immobilised laccase was used for the oxidation of a mixture of four phenolic compounds (protocatechuic acid, ferulic acid, sinapic acid and caffeic acid) chosen among those present in olive mill wastewaters (OMWs). These compounds determine the phytotoxicity of OMWs. Different reaction rates were observed for the oxidation of the examined phenolic compounds. The biocatalyst was recycled and a conversion of 84 mol% at the 10th reuse and of about 60 mol% after the 14th reuse was obtained. In conclusion, the laccase immobilised on SBA-15 is a potential biocatalyst for bioremediation of OMWs, which is an important environmental problem in the regions around the Mediterranean Sea.



## 1. Introduction

Laccases (E.C. 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase) belong to the so-called blue-copper family of oxidases and, whether pure, display a characteristic blue colour. They are present in nature mainly in fungi, several plant species and some bacteria [1]. This enzyme has a high biotechnological interest as demonstrated by several studies reporting its use for green processes [2], such as wood pulp delignification, dye decolorization in the textile industry [3,4], ethanol production [5], wine processing [6], treatment of polycyclic aromatic hydrocarbons [7,8], bioremediation [9] and for the realisation of biosensors [10]. Structurally, laccases are monomeric or multimeric glycoproteins, with different content of carbohydrates and Cu atoms. For example, laccases from fungi generally contain four Cu(II) atoms per protein molecule having different spectroscopic features (type 1, 2 and 3 Cu) [11]. The characteristic blue colour is due to the paramagnetic type 1 Cu atom, whereas the other three Cu atoms do not contribute to the colour. Nevertheless, they are involved in the catalytic mechanism. Laccases display a wide substrate specificity and oxidation is coupled with the reduction of an oxygen molecule to water. Although laccases act mainly towards phenolic compounds, their action spectrum is wide and moreover the range of oxidable substrates depends on the specific laccase used. Laccases can oxidise *o*- and *p*-diphenols, aminophenols, methoxy-susbstituted phenols, polyphenols, aromatic polyamines [12]. Their activity can be inhibited by several agents such as $CN^-$, $F^-$, fatty acids, which are able to bind to Cu ions thus not allowing the internal electron transfer, and $Hg^{2+}$ ions that can induce protein conformational changes [7].

Although enzymes are highly active catalysts, they are not always useful for biotechnological applications because of their low stability. This can be increased through the immobilisation on solid supports. Moreover, immobilisation allows enzyme recovering by simple filtration and its reuse for several reaction cycles. Ordered mesoporous materials (OMMs), such as SBA-15 [13], are receiving great attention because of their unique properties of highly controlled and uniform pore size and high values of surface area and pore volume [14]. These properties confer them a high applicative potential in different fields, such as enzyme immobilisation [15,16]. With respect to zeolites and other microporous materials, OMMs can be used as supports, since their pore size is larger than the diameter of many enzymes. Several enzymes, namely, cytochrome c [17–19], xylanase [17], lysozyme [18,20], catalase [18], horseradish peroxidase [21], subtilisin [21], chloroperoxidase [22,23], trypsin [24], lipase [25], etc., have been immobilised on OMMs. Most papers

* Corresponding author. Tel.: +39 070 6754362; fax: +39 070 6754388.
*E-mail address:* asalis@unica.it (A. Salis).

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focused on the process of immobilisation, and only few deal with biocatalytic applications [26].

The olive culture is one of the most important crops in the countries of the Mediterranean region. A by-product of the manufacturing process of olive oil is olive mill wastewaters (OMWs). The high level of polluting substances makes OMWs one of the major agent of pollution in the Mediterranean region. The large concentration of highly toxic phenolic compounds limits the biological treatment of this effluent. In some countries, OMWs can be shed in the soil, although several studies have evidenced that this practice may cause environmental pollution due to the phytotoxicity of phenolic compounds [27]. The presence of phenolic compounds in OMWs represents a significant environmental hazard and, therefore, the development of methods for their removal and transformation have received great attention in recent years [9].

The present paper reports the immobilisation of a laccase (from *Pleurotus sajor-caju*) on SBA-15 mesoporous silica. In order to bind the laccase on the support via chemical adsorption, the SBA-15 surface was modified and the modification was checked through FTIR spectroscopy. The immobilised laccase was used for the oxidation of some phenolic compounds chosen among those present in OMWs. Biocatalyst reuse for several reaction cycles was also examined.

## 2. Experimental

### 2.1. Chemicals

Laccase from *P. sajor-caju* was extracted and purified at the Biochemistry laboratory of the Biomedical Science and Technology Department of Cagliari University. Syringaldazine was from Sigma (Milan, Italy). Protocatechuic acid (3,4-dihydroxybenzoic acid, >97.0%), sinapic acid ((*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid, >97.0%), ferulic acid ((*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid, >98.0%), caffeic acid ((*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid, >95.0%), disodium hydrogen phosphate (> 99.5%), sodium dihydrogen phosphate (> 99.0%), citric acid monohydrate (minimum 99.5%) were from Fluka (Milan, Italy). Sodium chloride 99.5%, hexane (> 98%) were from Merck (Darmstadt, Germany). Tetraethylorthosilicate (98%), Pluronic copolymer 123 ($EO_{20}PO_{70}EO_{20}$), 3-aminopropyltrimethoxysilane (97%) and glutaraldehyde (50% aqueous solution w/v), sodium citrate dihydrate (minimum 99%) and 3,4,5-trimethoxybenzoic acid (99%) were purchased from Aldrich. Acetonitrile (99.5%) and 2-propanol were from J.T. Baker.

### 2.2. Synthesis and characterisation of SBA-15

A mass of 4 g of Pluronic copolymer 123 was dissolved in 20 mL of 37 wt% HCl and 120 mL of distilled water; the resulting mixture was stirred at 35 °C for 16 h. Then, 8.5 g of tetraethylorthosilicate (TEOS) were added and the final solution was stirred at this temperature for 24 h. Finally, the mixture was aged into a Teflon-lined autoclave at 100 °C for 24 h. After filtration and washing, the solid was dried at 40 °C and then heated at 550 °C for 5 h. $N_2$ adsorption/desorption isotherms at 77 K were determined through a Thermoquest-Sorptomatic 1990. Before analysis, pure silica samples were heated up to 250 °C at a rate of 1 °C $min^{-1}$ under vacuum. The specific surface area, the total pore volume and the pore size distribution were assessed by the *Brunauer–Emmett–Teller* (BET) and the *Barret–Joyner–Halenda* (BJH) methods, respectively [28,29]. Transmission electron microscopy (TEM) images were obtained on a JEOL 200CX microscope equipped with a tungsten cathode operating at 200 kV. Finely ground samples were dispersed in *n*-octane by sonication and dropped and dried on a carbon-coated copper grid for observations.

### 2.3. Functionalisation of SBA-15 mesoporous silica

The modification of mesoporous silica SBA-15 was carried out as follows. A volume of 1 mL of 3-aminopropyltrimethoxysilane (APTS) was added to a suspension of 1 g of SBA-15 in 30 mL dry toluene. The mixture was heated under reflux for 15 h. The aminosilylated SBA-15 (SBA-15-APTS) was collected by filtration, washed with acetone and dried overnight at room temperature under vacuum. Glutaraldehyde-activated SBA-15 (SBA-15-GA) was prepared by soaking 125 mg aminosilylated mesoporous silica in a mixture of 100 μL 50% aqueous glutaraldehyde and 2.75 mL 0.1 M phosphate buffer solution (pH = 7.5) for 1 h. The carrier was washed twice with 5 mL of the same buffer for 30 min under stirring, centrifuged and the washing liquors removed. The wet solid was immediately used for laccase immobilisation. Upon modification with glutaraldehyde, the colour of the support changed from white to red. SBA-15 functionalisation was checked through FTIR spectroscopy through a FTIR Brucker Equinox 55 spectrometer operating in transmission mode.

### 2.4. Laccase activity assay

Laccase activity was measured through the syringaldazine assay [30]. The assay mixture contained 40 μL of 1 mM syringaldazine (dissolved in ethanol), 1450 μL of citrate buffer (pH 6.3, 0.5 M) and 10 μL of laccase solution. Oxidation of syringaldazine was checked by measuring absorbance increase at 525 nm ($\varepsilon_{525}$ = 65,000 $dm^3$ $mol^{-1}$ $cm^{-1}$) at a temperature of 25 °C using a Varian Cary 50 UV–Vis spectrophotometer. One unit (U) of laccase activity is the amount of enzyme that oxidises 1 μmol of syringaldazine per minute.

### 2.5. Laccase immobilisation on the functionalised SBA-15

A laccase solution in sodium phosphate buffer (100 mM and pH 8.0) was added to the tube containing the modified SBA-15 sample. The activity of the laccase solution was measured before mixing with the support powder through the syringaldazine assay. The flask was slowly rotated overnight at 25 °C and the solution was removed from the flask with a pipette and checked with the syringaldazine assay to measure the residual activity. The support particles were washed with fresh buffer until the residual water did not exhibit any enzymatic activity. The adsorbed laccase preparation was kept wet with a film of liquid, since drying causes the inactivation of the enzyme. The enzyme loading, *L*, was calculated according the following equation:

$$L = \frac{A_i - A_r - A_w}{m_s}$$

where $A_i$ is the initial enzyme activity, $A_r$ is residual enzyme activity, $A_w$ is enzyme activity detected in the washing solutions, $m_s$ is the mass of support (g). Hence, the loading is expressed as U $g_{support}^{-1}$. The dynamic evolution of the immobilisation process was followed by withdrawing, at established times, 10 μL samples from the vessel containing the enzymatic solution and the support suspension. The samples were analysed by the syringaldazine assay. The adsorption isotherm was determined by operating in the same way as described above, but using seven different laccase concentrations and always the same mass of the functionalised SBA-15 support.

### 2.6. Biocatalytic oxidation of phenolic compounds

A typical substrate solution was obtained by dissolving 0.050 g of protocatechuic acid in 50 mL of a 90% phosphate buffer (0.5 M, pH = 6.3) and 10% 2-propanol mixture. Blank runs were carried out

**Table 1**
Characterisation data of the laccase from *Pleurotus sajor-caju* and SBA-15 mesoporous silica support.

| Enzyme: laccase from *Pleurotus sajor-caju* | | | | | | Support: SBA-15 mesoporous silica | | | |
|---|---|---|---|---|---|---|---|---|---|
| [a]$K_M$ (μM) | [a]$V_{MAX}$ (μM s$^{-1}$) | [a]pH activity maximum | [a]pH stability range | [a]Temperature stability range (°C) | Molecular weight (kDa) | Pore size (nm) | Wall thickness (nm) | $S_{BET}$ (m$^2$ g$^{-1}$) | $V_P$ (cm$^3$ g$^{-1}$) |
| 35.7 ± 0.6 | 0.33 ± 0.04 | 5.5–6.5 | 7–11 | 5–30 | 65 | 6.7 | 3 | 766 | 2.14 |

[a] Determined through syringaldazine assay (see Section 2.4).

in order to determine the amount of acid adsorbed by the functionalized SBA-15 support. The reaction was started by adding to the immobilized biocatalyst (50 mg) the substrate solution (5 mL) in a Teflon-lined screw-capped vial. Reaction vials were shaken through a horizontal shaking water bath (80 oscillations per minute) at 30 °C. Samples (25 μL) were withdrawn at different times and 25 μL of the internal standard solution (3,4,5-trimethoxybenzoic acid 0.792 mM) were added. The resulting mixtures were diluted to the final volume of 150 μL with mixture A (see next paragraph) and analysed by HPLC. A solution of four phenolic compounds (protocatechuic acid, caffeic acid, sinapic acid and ferulic acid) was obtained by dissolving 0.25 g of each compound in 25 mL of a 90% phosphate buffer (0.5 M, pH = 6.3) and 10% 2-propanol mixture. The enzymatic oxidation of the four phenolic compounds was carried out similarly to what is reported above for protocatechuic acid. The study of immobilised laccase recycling was performed with the same amounts of catalyst and substrate as in previous experiments. At the end of a reaction cycle, the mixture was centrifuged and the liquid phase removed. The solution was analysed by HPLC for the determination of substrate conversion. A new cycle was started by adding 5 mL of fresh protocatechuic acid solution without any catalyst washing. All reactions were performed in triplicate.

### 2.7. HPLC analysis

HPLC analysis was performed through a VWR-Hitachi LaChrom ELITE HPLC system constituted by a L-2130 pump, a Lichrospher 100 RP-18 end capped, 5 μm, column (Merck, Darmstadt, Germany) and monitored by an UV–Vis detector L-2400 set at 260 nm. Analysis was carried at constant flow of 1 mL min$^{-1}$ with the following isocratic solvent mixture: 70% mixture A (0.5% formic acid in water) and 30% mixture B (5% mixture A in acetonitrile). A good resolution was obtained for phenolic compounds. Protocatechuic acid (4.69 min), caffeic acid (7.74 min), sinapic acid (10.51 min) and ferulic acid (10.88 min). Phenolic compounds conversion was calculated according to calibration curves obtained with the internal standard method (3,4,5-trimethoxybenzoic acid, 0.792 mM). All analyses were performed in triplicate with reproducibility always within 3%.

## 3. Results and discussion

### 3.1. Enzyme and support characterisation

The enzyme used in the present work is a laccase, induced in *P. sajor-caju* by ferulic acid, extraction and purification of which have been recently described [31]. Some data regarding its characterisation—such as the molecular weight (65 kDa), the kinetic parameters $K_M$ (35.7 μM) and $V_{MAX}$ (0.33 μM s$^{-1}$), the pH range of activity (5.5–6.5) and the pH range of stability (7–11) determined through the syringaldazine assay—are summarised in Table 1.

Fig. 1a shows the TEM image of the SBA-15 sample; the typical hexagonal pattern and pores of uniform size are shown. Fig. 1b reports the $N_2$ isotherm adsorption/desorption curve of the SBA-15 sample. It is a type IV isotherm with a $H_1$ hysteresis typical of mesoporous materials having cylindrical parallel channels, as confirmed by the narrow pore size distribution displayed in Fig. 1c. The data of these characterisations are reported in Table 1. The surface area of SBA-15 mesoporous silica is 766 m$^2$ g$^{-1}$, the total pore volume is 2.14 cm$^3$ g$^{-1}$. Pore size distribution has a maximum at 6.9 nm as calculated by the desorption branch through the BJH method [29].

### 3.2. Chemical modification of SBA-15 surface

Depending on the functional groups available on the support surface, either physical or chemical adsorption (covalent binding) can occur. Although physical adsorption is an easy immobilisation method, covalent immobilisation warrants a more stable catalyst for aqueous media. For this purpose, the surface of the SBA-15 sample was modified in order to introduce the suitable functional groups that allow enzyme binding. The –$NH_2$ group is introduced on the SBA-15 surface through reaction with aminopropyltrimethoxysilane (APTS), then the –CHO function is introduced through reaction with glutaraldehyde. Laccase immobilisation occurs since the –$NH_2$ groups of lysine residues on the laccase surface reacts with the modified SBA-15 surface.

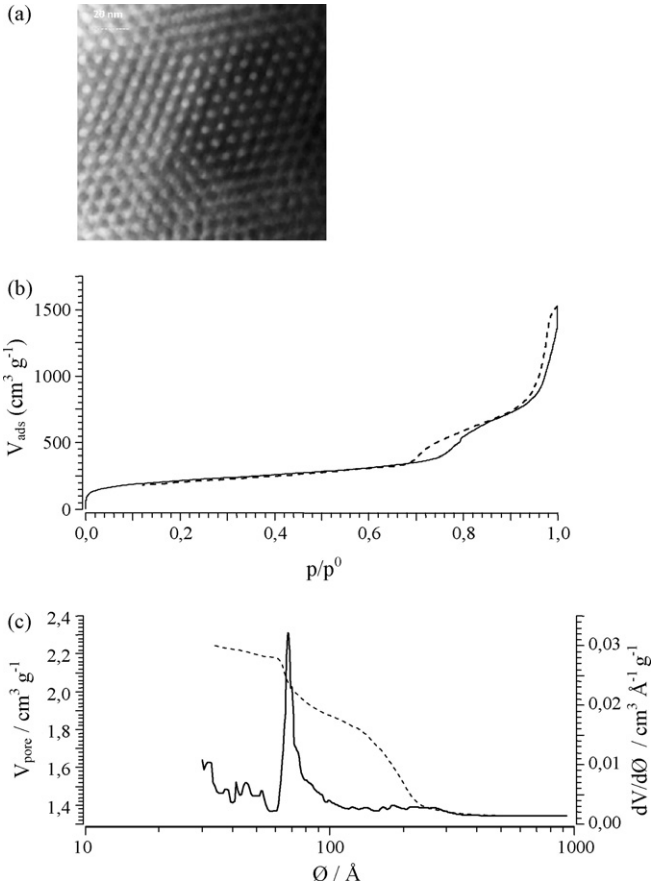


**Fig. 1.** Characterization of SBA-15 mesoporous silica. (a) TEM micrograph; (b) $N_2$ adsorption/desorption isotherm; (c) pore size distribution calculated by the desorption branch through BJH method.

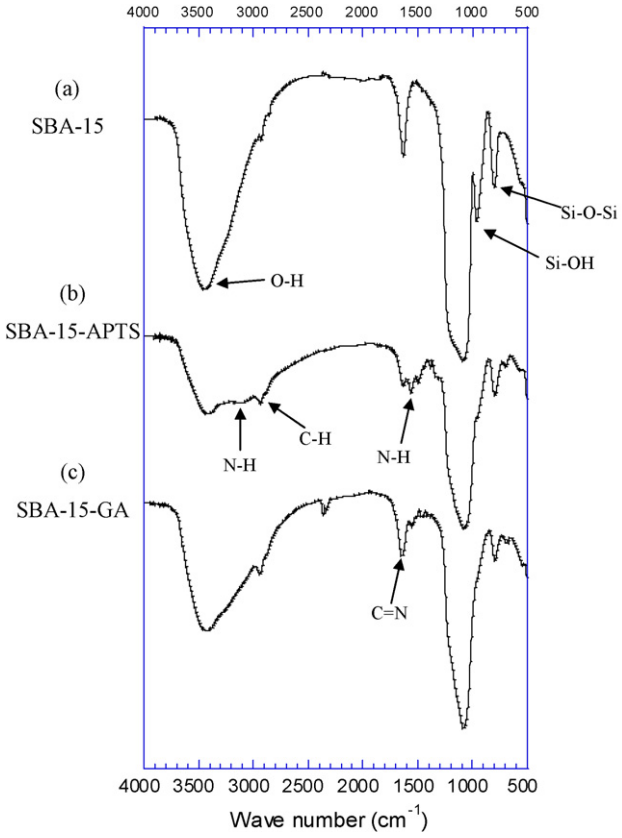


**Fig. 2.** FTIR spectra of SBA-15 functionalisation steps.

The modification of SBA-15 was monitored through FTIR spectroscopy. The IR spectra of original SBA-15 and modified SBA-15 are reported in Fig. 2. The spectrum of the unmodified SBA-15 has a broad band with a maximum at about 3450 $cm^{-1}$ due to O–H stretching, one at about 960 $cm^{-1}$ due to Si–OH stretching and one at 795 $cm^{-1}$ due to Si–O–Si stretching (Fig. 3a). After surface modification with APTS, a band at 3100 $cm^{-1}$, due to N–H stretching, appears. The band is not very intense also because it is overlapped with the O–H band (3400 $cm^{-1}$), which, in any case, is less intense in comparison with that of SBA-15 sample [32]. Other evidences of the SBA-15 modification are the disappearance of the band at 960 $cm^{-1}$ due to Si–OH stretching and the appearance of a small band at 690 $cm^{-1}$ due to N–H bending [32]. Moreover, a band at about 2930 $cm^{-1}$, due to C–H stretching of APTS, appears [33]. Finally, two small bands at 1490 and 1560 $cm^{-1}$, due to N–H bonds vibration, occurs. All these evidences confirm that silanol groups reacted with APTS. The successive step involved the modification with glutaraldehyde. A band at 1647 $cm^{-1}$ (–C = N– bonds), due to the reaction between the $–NH_2$ of APTS-modified SBA-15 and –CHO of glutaraldehyde, appears [33]. In addition, the bands at 1500 and 1550 $cm^{-1}$, due to N–H vibration, decrease their intensity.

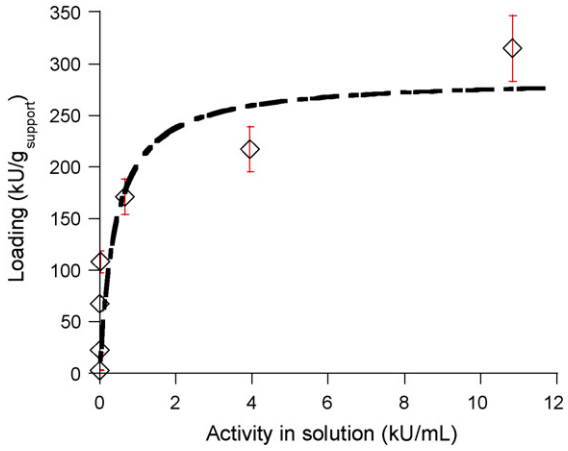


**Fig. 4.** Adsorption isotherm ($t$ = 25 °C) of the laccase on SBA-15.

### 3.3. Laccase immobilisation on SBA-15

The laccase was then immobilised on the chemically modified SBA-15 support. Fig. 3 shows the time course of the enzyme immobilisation ($t$ = 25 °C). The process is quite fast as demonstrated by the rapid decrease of enzyme activity in the immobilising solution within the first minutes. The equilibrium of the process was reached after about 100 min when about 98% of the initial enzyme amount was immobilised.

In a previous study, the immobilisation of laccase from *Trametes versicolor* on different supports (chemically modified silica, amberlite IRA-400, glass–ceramic and montmorillonite) was reported [3]. Different immobilisation rates for the different supports were found. Indeed, the immobilisation process reached the equilibrium after about 30 min for amberlite IRA-400 and after 120 min for

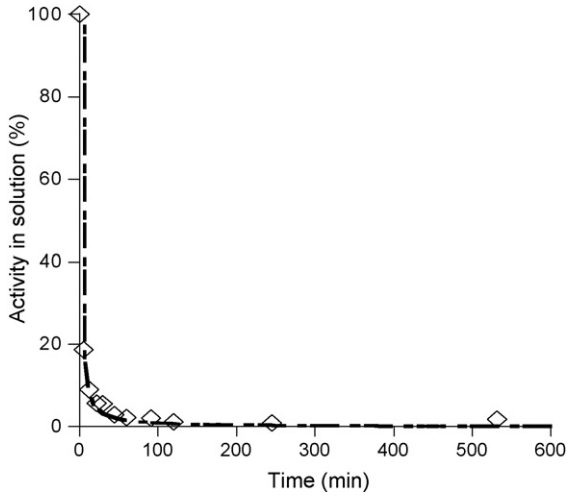


**Fig. 3.** Time course of the laccase on SBA-15 immobilisation process ($t$ = 25 °C).

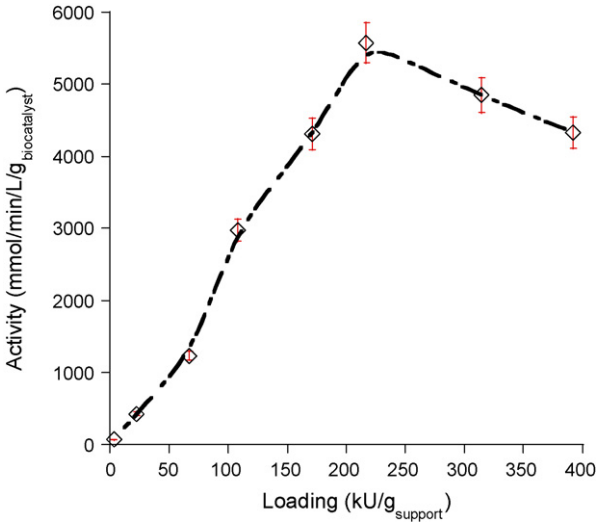


**Fig. 5.** Effect of loading on laccase activity ($t$ = 25 °C).

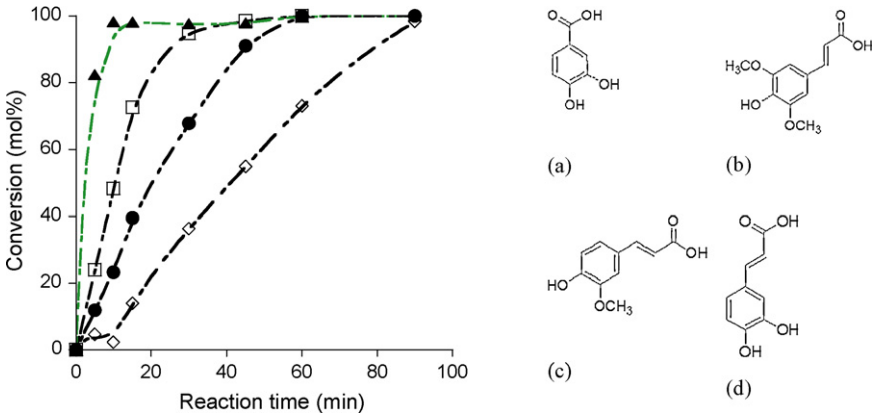


**Fig. 6.** Time course of the enzymatic oxidation of an aqueous solution of a mixture of phenolic compounds ($t$ = 30 °C). (a) Protocatechuic acid (◊); (b) sinapic acid (▲); (c) ferulic acid (●); and (d) caffeic acid (□).

montmorillonite. These so different trends can be explained taking into account that the immobilisation of a protein macromolecule on a porous support is a complex phenomenon that involves different steps (i. protein diffusion from the aqueous solution to the support surface; ii. protein diffusion inside the pores; iii. protein adsorption). Hence, the observed rate depends on the rate of each single step, which, in turn, depends on the particular enzyme, the surface (extension and chemical nature) and the structure of the adsorbing support.

### 3.4. Effect of loading on laccase activity

In order to study the effect of loading on enzyme activity, the adsorption isotherm at 25 °C—a graph of the loading versus the enzyme concentration in the immobilising solution—was determined. According to what is described in Section 2.5, both loading and enzyme concentration were quantified in terms of enzymatic activity by mean of the syringaldazine assay. As shown in Fig. 4, the isotherm reaches a saturation value according to the Langmuir model. The different immobilised biocatalysts at different loadings were tested toward the oxidation of protocatechuic acid. Fig. 5 shows that enzymatic activity increases linearly with the loading up to a maximum (217 kU $g_{SBA\text{-}15}{}^{-1}$). Higher loadings resulted in a decrease of laccase activity, so that there was no need to further increase the loading. This result can be explained in terms of limitations due to the diffusion of the substrates inside the support pores before reaching the enzyme active site. This phenomenon was previously observed also for other immobilised enzymes at high loadings [34]. The optimal loading was then used in the successive steps of the work.

### 3.5. Phenolic compounds oxidation

A possible application of the immobilised laccase could be the oxidation of the phenolic compounds contained in several wastewaters. Gianfreda et al. reported the oxidation of a mixture of four phenolic compounds (catechol, methylcatechol, *m*-tyrosol and hydroxytyrosol), simulating a wastewater derived from an olive oil factory, by a laccase from *Rhus vernicifera* [35]. Ghosh et al. studied the potential use of laccase (SP-504) in an oxidation-based treatment to remove 2,4-dimethylphenol from water with and without the additive, polyethylene glycol [36]. Here, a solution containing a mixture of four phenolic compounds—namely, protocatechuic acid, ferulic acid, caffeic acid and sinapic acid—chosen among those most abundant in OMWs [37], was prepared. The chemical structures of these compounds are reported in Fig. 6. It also reports the oxidation curves (conversion) of each phenolic compound, as a function of reaction time, due to the action of the laccase immobilised on SBA-15. On the basis of a preliminary screening (data not shown), the temperature of 30 °C was found to be the best choice to optimise enzyme activity and stability. Blank runs, carried out in order to determine the amount of acid adsorbed by the functionalised SBA-15 support, showed that this was negligible with respect to the oxidation process. Indeed, all the compounds were fully oxidised, since all the curves reached 100 mol% of conversion, even though with different reaction rates. Sinapic acid disappeared after only 10 min, caffeic acid after 45 min, ferulic acid after 60 min and protocatechuic acid was fully oxidised after 90 min. This result is similar to what is reported by Koschorreck et al., who studied the dimerisation of sinapic acid, caffeic acid and ferulic acid catalysed by a laccase (cloned from *Bacillus licheniformis* gene and expressed in *Escherichia coli*) [38]. They observed that the laccase displayed its highest activity towards sinapic acid. Analogously, results achieved by Gianfreda et al. confirmed that laccase-mediated transformation of phenols depends on several parameters such as the nature and the initial concentration of the involved phenol, the time course of the reaction and, mainly, the complexity of the incubated phenolic mixture. Indeed, they found that the four phenols had a completely different response to enzyme action both in terms of quantitative and kinetic transformation [35]. In this work, a different laccase and phenolic compounds were used; nevertheless, the laccase immobilised on SBA-15 was able to quantitatively remove all the components of the mixture in 90 min.

### 3.6. Biocatalyst recycling

One of the most important advantages of enzyme immobilisation is the possibility of its reuse for several reaction cycles, since

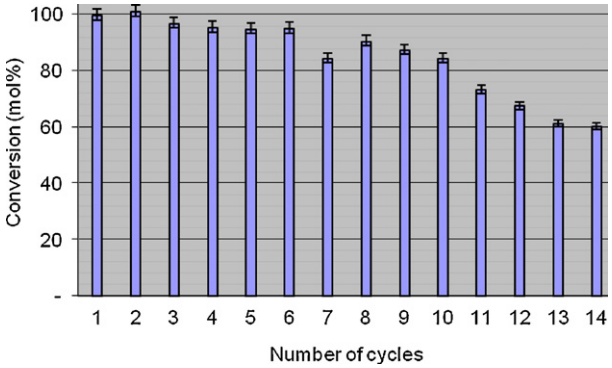


**Fig. 7.** Recycling of the immobilised laccase on SBA-15 ($t$ = 30 °C).

it can be easily separated by the reaction medium and added to a fresh substrate solution. Recently, Kunamneni et al. reported the immobilisation of the laccase from *Myceliophthora thermophila* on Sepabeads EC-EP-3 and its use for decolorisation of synthetic dyes [4]. Their biocatalyst showed a good operational stability, maintaining 84% of its initial activity after 17 cycles of oxidation of ABTS. Zhu et al. reported that the laccase from *T. versicolor*, immobilised on magnetic mesoporous silica spheres, was used for 10 cycles retaining 70% of activity [39].

The number of biocatalyst reuses was determined for *P. sajor-caju* laccase immobilised on SBA-15. Protocatechuic acid was used as the substrate since it was the most resistant to laccase oxidation. Fig. 7 reports the results of this study that was carried out at t = 30 °C. Starting from a full conversion at the first use, conversion slightly decreased being still considerable after the 10$^{th}$ reaction cycle (84 mol%) and reached about 60 mol% after the 14$^{th}$ reaction cycle.

## 4. Conclusions

The potential use of laccases for several biotechnological applications is under study at the present time [2]. The present work has shown that the laccase from *P. sajor-caju* can be successfully immobilised through chemical bonding on an ordered mesoporous support. The obtained biocatalyst retains its activity that has an optimal value at $\sim$220 U $g_{SBA\text{-}15}{}^{-1}$ when protocatechuic acid is used as the substrate. The obtained biocatalyst is stable for more than 10 reaction cycles, reaching a conversion of 84 mol%. An aqueous solution of four phenolic compounds simulating OMWs was successfully treated by the immobilised laccase. Hence, this immobilised biocatalyst has a potential interest in the field of bioremediation of wastewaters coming from the food industry.

The most remarkable finding of this investigation is related to the use of highly ordered inorganic mesoporous supports such as SBA-15. Several benefits can be emphasised: i) the reproducibility of the material that through a standardised synthesis procedure assures the same surface area, pore size and pore volume; ii) the chemical modification of the surface that allows enzyme chemisorption; and iii) the high stability of the chemically immobilised enzyme that allows for reuse of the biocatalyst.

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