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BIOCATALYSTS OBTAINED BY ENZYME IMMOBILIZATION ON FUNCTIONALIZED MESOPOROUS SILICA SUPPORTS

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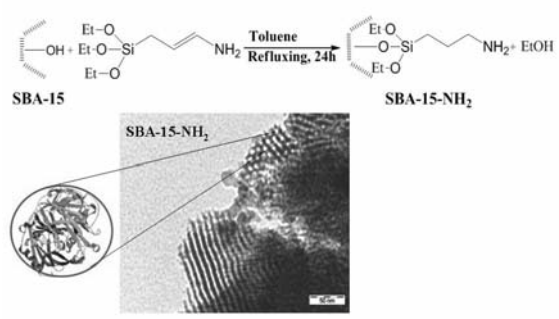
Three mesoporous silica (MCM-41, MCM-48 and SBA-15), with different surface properties, pore size and porous structure, were synthesized and used as supports for laccase immobilization. The supports were functionalized with 3-aminopropyl-triethoxysilane (APTES) and 3-glycidyloxypropyltrimethoxy-silane (GPTMS). Laccase immobilization was carried out through physical adsorption and covalent binding on functionalized mesoporous silica with different textural properties. The obtained materials were characterized by X-ray diffraction at small angle, SEM and TEM microscopies, thermal analysis, N_2 adsorption-desorption, UV-Vis and FTIR spectroscopies. Immobilization yield and relative activity of laccase varied with pore size, functionalization and support pretreatment. The best values for these parameters were obtained for SBA-15 support functionalized with GPTMS after its pretreatment with glutaraldehyde. The influence of pretreatment time was also evidenced. The obtained biocatalysts were tested in oxidation of anisole and phenol with H_2O_2. The obtained results sustain the effect of support and method of immobilization on catalytic performances and biocatalysts stability.



INTRODUCTION

Enzymes have high catalytic activity and they can catalyze a variety of reactions in mild conditions with a very high degree of substrate specificity. Nevertheless, they present a number of disadvantages such as high costs for commercial use, are often very fragile and have a low stability, the recovery is difficult or impossible. These inconveniences were removed through the immobilization on solid supports. Moreover, immobilization allows enzyme recovering by simple filtration and its reuse for several reaction cycles.[1] Different methods for enzyme immobilization on appropriate supports are available and the selection of the right support and immobilization method determines the biocatalyst efficiency and influences the properties of the biocatalyst that improves the enzyme activity. The methods used for immobilization are mainly based on physical and chemical mechanisms.[2] A lot of materials with well-developed porous structure are

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available as enzymatic supports. These possess affinity for the enzymes providing maximum enzyme activity, are thermostable, chemically durable, resistant to contamination, and available at a reasonable cost.[3] Ceramic monoliths, membranes, hydrogels, and beads of natural and synthetic mineral and polymers have been used as supporting material for enzyme immobilization.[4-9] Carbon-based mesoporous magnetic composites prepared from different lignocellulosic wastes with suitable porosity are attractive enzyme supports because of their higher water stability and lower cost.[9] Immobilization of laccase and protease substrates on activated silica gels demonstrate that such preparations can be used for applications ranging from medical (diagnosis of wound infection) to environmental (biomarkers, airborne protease) fields where soluble substrates are often not suitable.[10] Furthermore, ordered mesoporous materials, such as SBA-15 or MCM-41 are receiving great attention because of their tuneable pore size, well-ordered porous structure and high values of surface area and pore volume.[11] All of these factors make mesoporous silica an ideal support for enzymes immobilization.[12, 13]

The aim of the present study was to assess different strategies for the immobilization of laccase from *Trametes versicolor* as a biocatalyst for anisole and phenol oxidation with H_2O_2, in the presence of water and acetonitrile as co-solvent. To achieve the above goal MCM-41, MCM-48 and SBA-15 supports with different pore size were used, either as-synthesized or functionalized with 3-aminopropyl and 3-glycidyloxypropyl functional groups.

Laccase (polyphenoloxidase; EC 1.10.3.2) is a member of the blue multi-copper-oxidase family, containing four copper atoms per monomer distributed in three redox sites (T1, T2 and T3).[14-16] Laccase catalyses the oxidation of polyphenols, polyamines and lignins by using oxygen or hydrogen peroxide as an electron acceptor.[16] The mechanism of laccase starts by substrate oxidation near the T1 followed by the internal electron transfer from the reduced T1 to the T2 and T3 copper sites. This mechanism was supported by numerous studies on applications as biocatalytic glucose oxidation processes.[17-21]

RESULTS AND DISCUSSION

X-ray diffraction patterns were collected for the functionalized materials (Fig. 1). All the XRD patterns suggest that the ordered mesostructures were perfectly retained after introducing the organic groups and evidenced a slight decrease of the well resolved peak intensity in the 0.5-6° range which indicates a low variation of the inorganic wall structure after the functionalization.

SEM images evidenced typical morphologies for these materials. As example, we selected SEM image of SBA-15-NH_2 (Fig. 2a) which highlight a rod-like morphology, the same as for the SBA-15 support.

TEM microscopy images highlighted the hexagonal ordered structure for MCM-41 and SBA-15 samples and a cubic structure for MCM-48 materials. These images reveals that the functionalized materials preserved the hexagonal array of uniform channels (Figs. 2b, 2c, 2d).

The modification of SBA-15 after functionalization was monitored through FTIR spectroscopy and the spectra are presented in Fig. 3. The spectrum of the unmodified SBA-15 has a broad band around 3450 cm^{-1} that corresponds to molecular water hydrogens bonded to each other and to Si-OH groups and another one at about 980 cm^{-1} that could be due to stretching vibrations of free silanol (Si-OH) groups on the surface of the amorphous solid samples (Fig. 3).

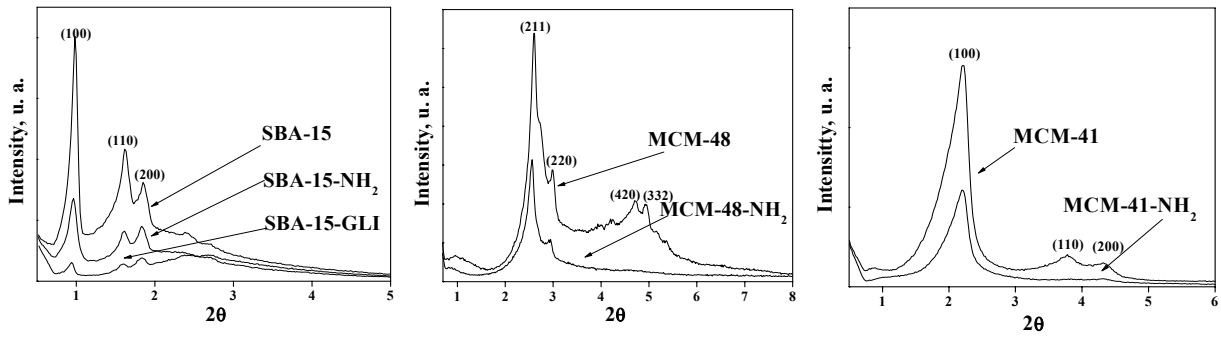


Fig. 1 – XRD patterns of the different functionalized silica supports.

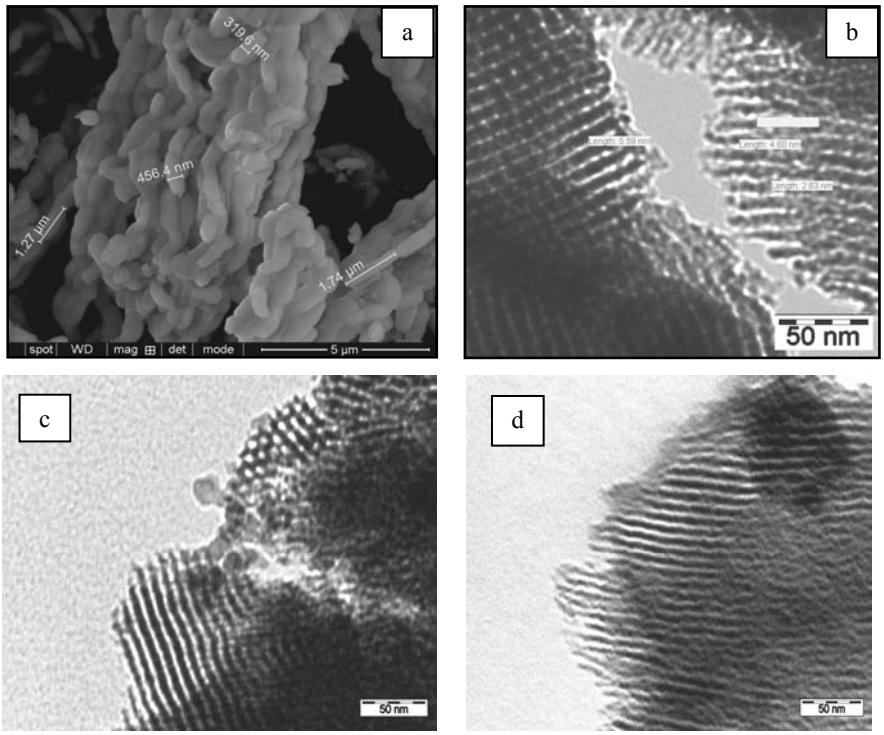


Fig. 2 – SEM image of SBA-15-NH_2 (a) and TEM images of SBA-15(b), SBA-15-NH_2 (c) and SBA-15-GLI (d).

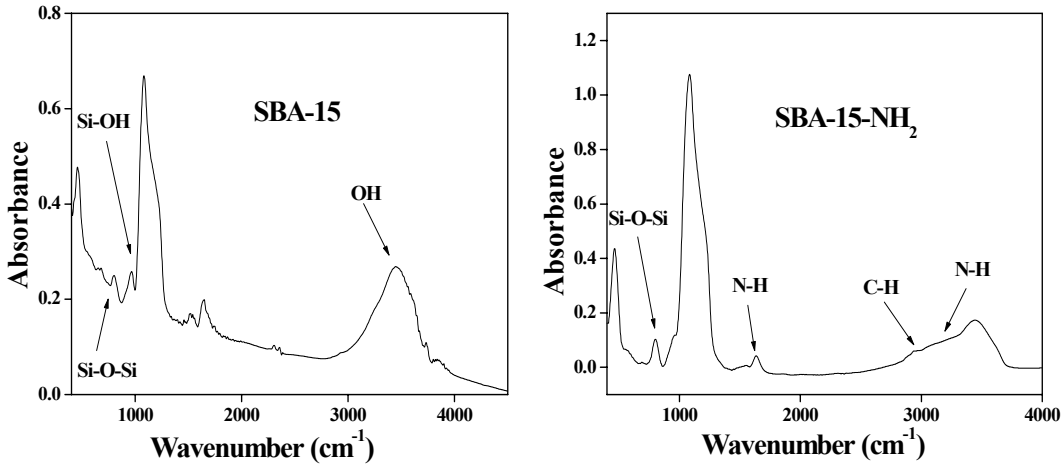


Fig. 3 – FTIR spectra of SBA-15 and SBA-15-NH_2.

After surface modification with APTS, a new band due to N–H stretching appears at 3150cm^{-1}, which is not very intense because it is overlapped with the O–H band (3450 cm^{-1}) and is less intense in comparison with that of SBA-15. Other evidences of the SBA-15 functionalization are the disappearance of the band at 980cm^{-1} due to Si–OH stretching and the appearance of a band at about 2925 cm^{-1} due to the asymmetric and symmetric vibration of $–CH_2$- from APTES functionalization agent.[22] Table 1 summarizes the physico-chemical and textural properties of the supports as compared with some of the obtained biocatalysts (Table 1). The amount of immobilized laccase was determined as the organic mass loss from the TG curves, after substracting the amount of grafted functional groups (APTS or GPTMS) and gluraldehyde, respectively. We mention that the amount of glutaraldehyde used as a cross-linking agent for the covalent grafting was determined by assuming total conversion of grafted amine or glicydoxy functional groups and subtracted from the total mass loss in order to obtain the amount of immobilized laccase. Mass loss, obtained by thermal analysis (TGA) sustains the immobilization of organic groups and laccase on mesoporous silica

supports. The variation of organic mass loss was significantly influenced by the surface area of the support. High immobilization yield of laccase was obtained for the majority of the supports. The effect of functionalization and support pretreatment on immobilization yield values is presented in Figs. 4 and 5.

High immobilization yields were obtained for samples functionalized with 3-glycidyloxy-propyltrimethoxysilane (GLI) and pretreated with glutaraldehyde. The time of pretreatment also increases the immobilization yield (Fig. 4).

Similar effects were observed for MCM-48 (Fig.5). However, the immobilization yield is lower for these support even though the surface area is higher. These results are explained by the difference between the pore size of this support and the enzyme dimmensions.

Comparing the weight loss obtained by thermal analysis for the two carriers used to immobilize laccase we can see a higher amount of laccase immobilized onto the SBA-15 support, that is explained by the higher value of the pore diameter. Enzyme immobilization yield values are also higher for SBA-15 support even if MCM-48 has a larger surface area. The immobilization yield obtained for the samples were the enzyme was chemically linked is superior to the physical adsorption. The relative activity of laccase, obtained from UV-Vis data with syringaldazine, showed a small decrease for immobilized enzyme comparing with free enzyme activity (Fig. 6).

Table 1

Physico-chemical and textural properties of the samples

Sample	**S_{BET} (m^2/g)**	**V_P (cm^3/g)**	**d_p (nm)**	**Organic mass loss (TGA) (mg/g solid)**
MCM-48	1605.1	0.82	2.23	-
MCM-48-NH_2-A-L	378.34	0.67	2.65	80.51
MCM-48-NH_2-C-L	424.80	0.50	3.90	47.09
SBA-15	893.90	1.27	6.58	-
SBA-15-NH_2-A–L	290.85	0.53	5.60	145.80
SBA-15-NH_2-C–L	293.81	0.44	4.69	276.06
SBA-15-GLI-A-L	453.74	0.25	5.47	143.04

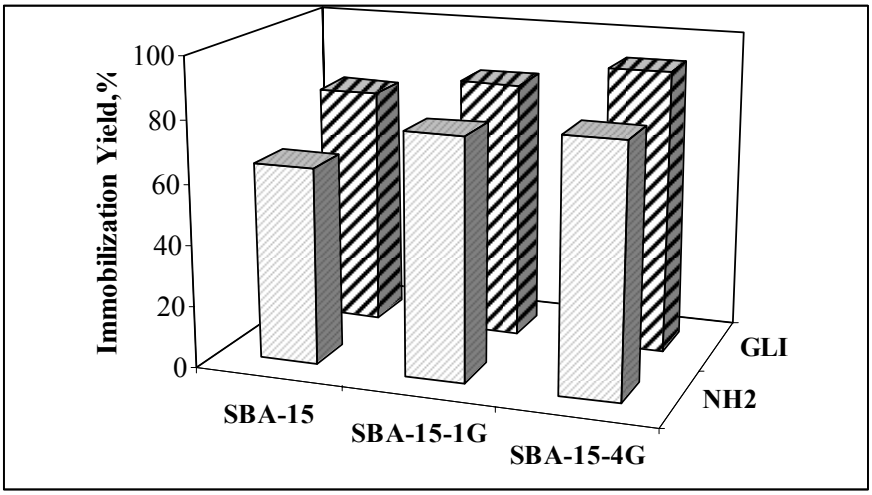


Fig. 4 – Effect of functionalization and pretreatment with glutaraldehyde on immobilization yield of laccase on SBA-15 support.

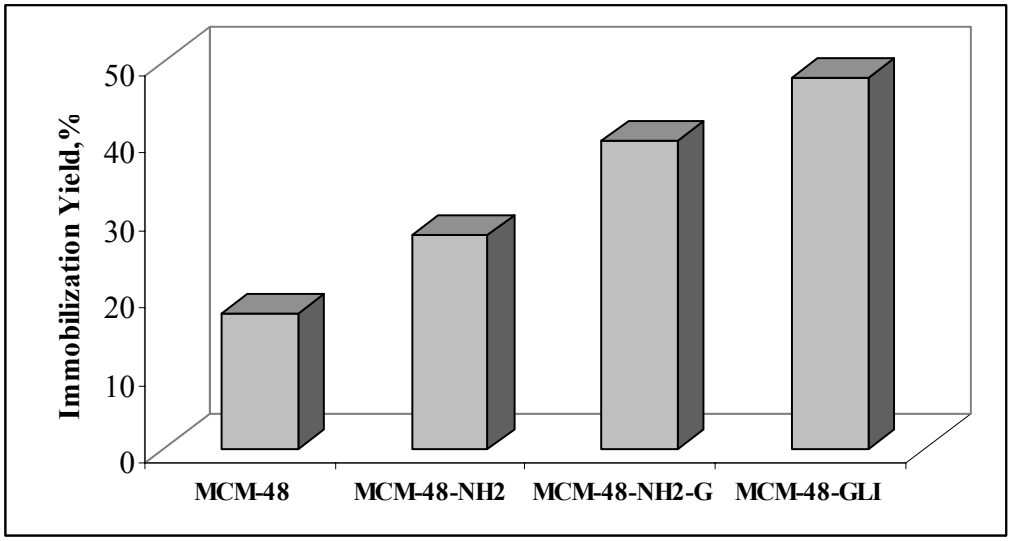


Fig. 5 – Effect of functionalization and pretreatment with glutaraldehyde on immobilization yield of laccase on MCM-48 support.

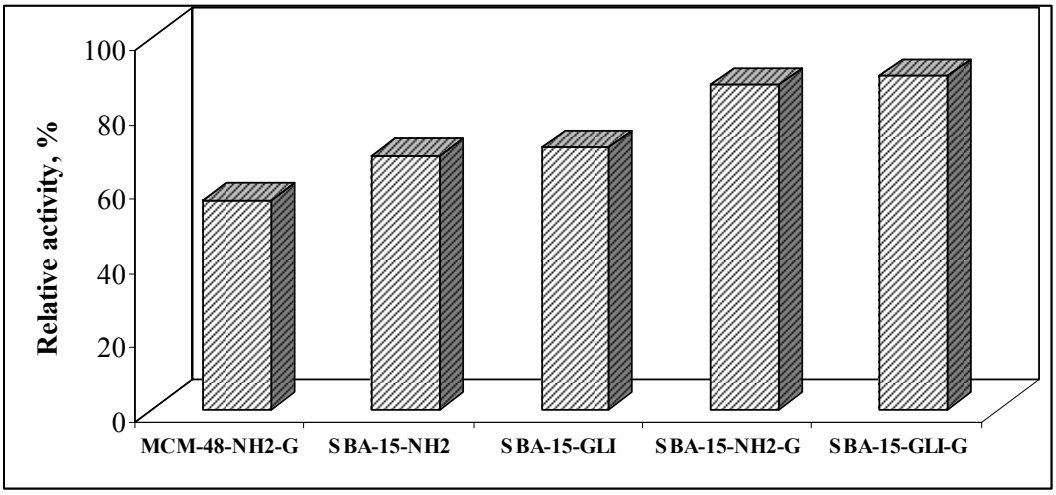


Fig. 6 – Variation of enzymatic activity with support functionalization and pretreatment.

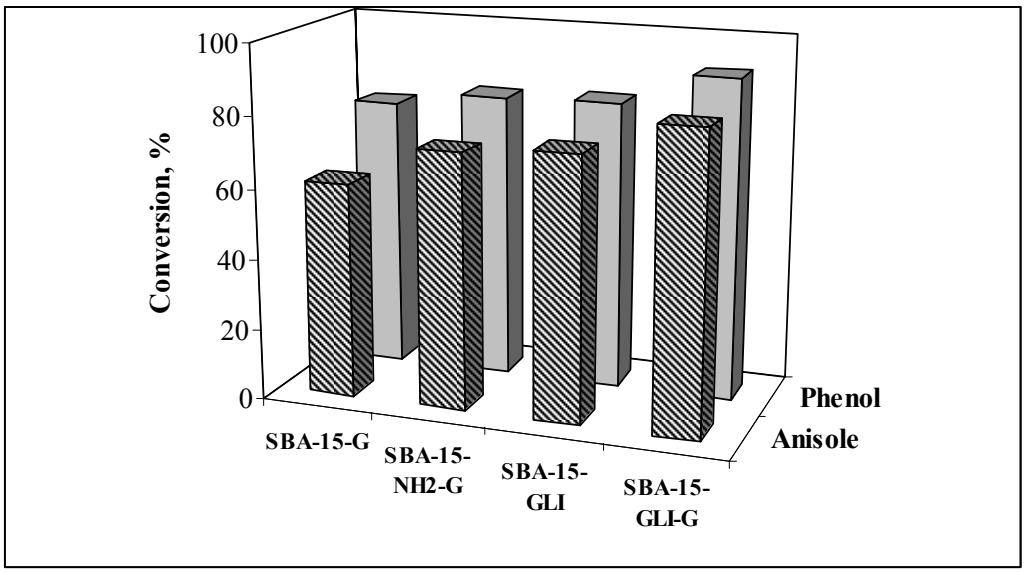


Fig. 7 – Effect of functionalization and pretreatment of SBA-15 support on laccase activity.

The obtained biocatalysts were tested in oxidation of aromatic compounds (anisole, phenol) with H_2O_2. The obtained results (Fig. 7) evidence a high catalytic activity in oxidation of aromatic compounds. The activity of about all the samples obtained by covalent immobilization of laccase using glutaraldehyde was higher in all the tested reactions. The lowest conversion of phenol was obtained for the supports with lowest degree of laccase immobilization. A high conversion was obtained also in the oxidation of anisole with the immobilized enzyme. These higher conversions of both aromatic substrates obtained for the immobilized laccase by covalent grafting, especially after the first cycle of reaction, confirms the nature of enzyme binding with the support and a positive effect of the functional groups grafted on the support surface on enzyme catalytic properties.

EXPERIMENTAL

Synthesis of the functionalized supports

MCM-41, MCM-48 and SBA-15 materials were synthesized as described in literature.[23-25] The organic-inorganic hybrid materials were obtained by a post-grafting procedure with 3-aminopropyl-triethoxysilane (APTES) and 3-glycidyloxypropyltrimethoxysilane (GPTMS) according to a previously described procedure[26] and were denoted and MCM-41-NH_2, and MCM-41-GLI, MCM-48-NH_2, MCM-48-GLI, SBA-15-NH_2 and SBA-15-GLI, respectively (Scheme 1).

Laccase immobilisation on functionalised mesoporous silica

Laccase (L) from *Trametes versicolor* obtained from Sigma Aldrich was immobilized on the surface of the mesoporous materials by physical adsorption (A) and covalent grafting (C). The first method consisted of an impregnation of 200 mg of solid support with a 5mg/mL enzime solution in 10 mL of sodium phosphate buffer (0.1 M and pH 7.0). The obtained mixture was treated with acetone and slowly rotated at 278K for 30 minutes. Then it was centrifuged at 3000 rpm, washed with phosphate buffer and dried in the desiccator. The dried powder was stored at 4°C protected from light, for further application.

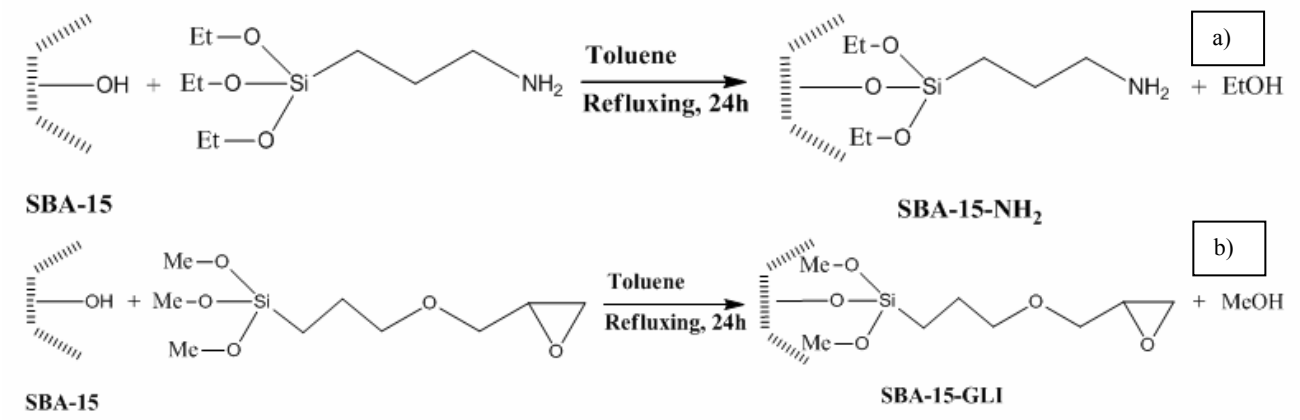
Previous to the enzyme immobilization by covalent grafting method, the NH_2- or Gli- modified silica support was further cross-linked with reactive aldehyde groups. Thus, 200 mg of amino- or glicidoxy-functionalized mesostructured support (MCM-41-NH_2, MCM-48-NH_2, SBA-15-NH_2,) were treated with a 5% aqueous glutaraldehyde solution for 1 h or 4 h at room temperature, washed with phosphate buffer (0,1M; pH 7) and dried at 60^0C for 1 h. Then, the supports were immersed in a laccase solution in phosphate buffer, at room temperature. The next steps were as for the adsorption method.

The activity of free and immobilized enzymes was spectrophotometrically determined at room temperature by oxidation with air of 4-syringaldazine in phosphate buffer and a suitable amount of free or immobilized enzyme. That amount was chosen so that the absorbance of the reaction product at 530 nm was within the linear range of the calibration curve. The amount of the imobilized enzyme was determined as a difference between initial and residual enzyme concentrations (immobilization yield).

The obtained biocatalysts were tested in oxidation of the organic compounds (anisole, phenol). The oxidation reactions with H_2O_2 were carried out in glass mini reactors (5 mL) at 25°C for 8 hours, using 0.05 g catalyst and a mixture of water as solvent and acetonitrile as co-solvent.

Characterization of materials

The ordered mesoporous structure of silica supports was chracterized by small-angle X-ray diffraction using a Rigaku Ultima IV diffractometer using Cu Kα (λ = 0.15406 nm), N_2 adsorption/desorption by Micromeritics ASAP 2020 automated gas sorption system. Thermogravimetric analysis was carried out with Setaram TG-DSC 111 thermal analyzer with a rate of 2°C/min up to 650 °C. The morphology and dimension of the particle samples and their porous structure were examined using a scanning electron microscope (SEM, FEI Quanta 3D FEG scanning microscope) and a transmission electron microscope (TEM, Tecnai 10 G2-F30). Fourier-transform infrared (FT-IR) spectra were measured at room temperature on an Jasco FT/IR-4100 spectrometer using the KBr disk technique. The analysis of oxidation products was performed using a GC-MS analysis (Thermo Scientific chromatograph) equipped with MS DSQ II Thermo.



Scheme 1 – Functionalization of SBA-15 mesoporous support by post-synthesis grafting with APTS (a) and GPTMS (b).

CONCLUSIONS

New biocatalysts were obtained by MCM-41, MCM-48 and SBA-15 functionalization with APTS and GPTMS and immobilization of laccase. High immobilization yields were obtained for samples supported onto SBA-15 mesoporous silica, with higher pore size. Biocatalysts with high activity in oxidation with H_2O_2 of anisole and phenol were obtained.

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