Scientific paper

Candida antarctica Lipase B Immobilization Onto Hybrid Organic-inorganic Mesoporous Materials

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Abstract

Based on MCM-41 mesoporous silica, three different hybrid organic-inorganic mesoporous materials were synthesized. Functionalization and hydrophobization of MCM-41 with 3-aminopropyltriethoxysilane (APTES), chlorotrimethylsilane (CTMS), and propyltrimethoxysilane (PTMS) were carried out by post-synthesis method after the removal of hexadecyltrimethylammonium bromide used as template. The effect of grafted organosilanes on the mesostructural properties of the resulted hybrid materials was systematically analyzed by different techniques such as X-ray diffraction (XRD), N_2 adsorption isotherms, FT-IR and ^{29}Si MAS NMR. Functionalized MCM-41 samples were used as support for *Candida antarctica* lipase B (CALB) immobilization. The activity of the new biocatalysts was evaluated in the alcoholysis of ethyl acetate with two alcohols (1-hexanol and 1-butanol, respectively) and compared with that of the free enzyme. All reactions were performed in organic media using n-hexane as solvent.

Keywords: MCM-41, functionalization, immobilization, *Candida antarctica* lipase B, alcoholysis

1. Introduction

In the last years, the mesoporous molecular sieves (e.g., MCM-41, FSM-16, SBA-15 and others) were a real challenge for enzyme immobilization on the inorganic supports. Nowadays, literature refers to the immobilization of enzymes such as subtilisine,[1] tripsine,[2] cytcrom *c*,[3] chloroperoxidase,[4] lipase[5] on such supports. Due to their high surface area and large pore diameter (2–50 nm), the mesoporous materials are more appropriate hosts for these macromolecules than the microporous sieves where the immobilization process occurs at surface only (pores diameter smaller than 2 nm). However, the efficiency of immobilization depends on the enzyme size, as well. For instance, immobilization of tripsine (diameter = 38 Å) on MCM-48 (24 Å) and SBA-15 (56 Å) led to good results for the last porous solid only because, as expected, the immobilization on MCM-48 was completed only at external surface.[2] Simultaneous immobilization onto the outer surface and into the pores has an advantage in comparison to that onto the surface only since the amount of immobilized enzyme increased hence the increased productivity per unit mass of biocatalyst. In addition, the pore diameter does not allow to accommodate more than one macromolecule, fact which avoids enzyme agglomerates.[6] Furthermore, the increasing the pore diameter could solve other problems related to diffusion phenomenon and pore blocking.[2] Nevertheless, not only textural characteristics of the support (such as surface area, or pore size) are the key factors for a successful immobilization but chemical properties (i.e., the presence of amino groups on the surface after grafting), as well. Therefore, there are attempts to improve them. One of these consists in the support functionalization with organic reagents such as silane derivatives leading to hybrid organic-inorganic nanoporous materials with ordered mesostructure.[7] The new hybrid materials play a special role not only in enzyme immobilization but also in other technological area such as the developing of new catalysts, adorbents, nanomaterials, etc.

Lipase immobilization on these kinds of supports could improve the properties of biocatalyst. Usually, lipases tend to form aggregates or to adsorb as multilayers on support surface hindering the access of the substrate molecules to the active site and thus, the catalytic performance of lipase is decreased. Consequently, the support functionalization with organosilanes will keep the lipase ma-

cromolecules far away each other so that more active sites will be able to perform the catalytic process.[8] The strength of interactions between enzyme and support is increased so that the enzyme leaching (as it is obviously happen for physisorbed enzymes) from support is avoided.[9]

On the other hand, hydrophobic interactions play a particular role in lipase immobilization.[5] Many authors have proven that a higher hydrophobicity affects positively both the stability and the activity of the most lipases. The majority of the functionalization compounds are hydrophobic organic molecules, which will determine an increase of the hydrophobicity of support surface.[10]

The present study is focused on lipase immobilization on organic modified MCM-41 molecular sieve. Though the lipase diameter is greater than those of the pores of MCM-41, we have chosen this support to have only immobilization upon the external surface of support for better evidence of the influence of functionalization on the biocatalyst properties (avoiding thus the influence of diffusion phenomenon from pores). The influence of the support on the lipase activity was investigated by testing the enzyme preparations in the alcoholysis reactions of ethyl acetate with two alcohols, 1-hexanol and 1-butanol, respectively. For comparison, experiments with free enzyme in solution were performed.

2. Experimental

2. 1. Chemicals and Materials

Candida antarctica lipase B (CALB) (CHYRAZIME LYO-2) was provided by Roche (Germany) as lyophilized powder. Pure form was obtained by a method already published in the literature.[11]

Preparation of the pure siliceous MCM-41 was carried out using sodium silicate (Na_2O 14%, SiO_2 27%) purchased from Carlo Erba and hexadecyltrimethylammonium bromide (CTABr) from Aldrich, respectively.

The functionalization compounds, namely: 3-aminopropyltrietoxysilane (APTES), propyltrimetoxysilane (PTMS) and chlorotrimethylsilane (CTMS) were purchased from Aldrich.

The chemical reactants used in the alcoholysis reactions were ethyl acetate (EA, 99%, Chimopar, Romania), n-hexanol (1–HxOH, normal puriss, Loba-Chemie, Austria), and n-butanol (1–BuOH, Reactivul, Romania). The both alcoholysis reactions were carried out in n-hexane (95%, Aldrich) as solvent.

2. 2. Synthesis of the MCM-41 Support

The hydrothermal synthesis of siliceous MCM-41 material was carried out using hexadecyltrimethylammonium bromide as template and sodium silicate as silicon source. The molar composition of the gel was: $1SiO_2$: $0.4Na_2O$: 0.2CTMABr : $135H_2O$. A typical synthesis was performed in a polypropylene beaker as follows. 6.89 g of CTABr were mixed with 140 ml of water at 35 °C and stirred until a clear solution is formed (solution A). Solution B was prepared by adding the corresponding amount of sodium silicate to water at 35 °C. Then, solution B was added drop wise to solution A at room temperature and the precipitation took place. The precipitate was left under stirring for a while. After that, the pH was adjusted from 12.5 to around 8.5 with 1M hydrochloric acid solution. After 2 h of stirring, the resulting gel was introduced into Teflon-lined stainless-steel autoclave, and heated at 100 °C for 24 h. After cooling the autoclave in cold water, the sample was filtered, and the recovered solid was washed thoroughly with deionized water and dried at 60 °C overnight. The template was removed by calcination in air at 500 °C for 7 h with a heating rate of 2.5 °C/min.

2. 3. Post – Synthesis Functionalization of Pure Silica MCM-41

The pure silica MCM-41 was further submitted to post–synthesis functionalization based on a method previously described in the literature.[12] The functionalization was performed as follows. A 1:4 (v/v) solution of liquid silane derivative (APTES, CTMS, or PTMS) and toluene was mixed with the calcined MCM-41 in a 100 ml round-bottom flask (0.01 mole of reagent per 1g of support). The resulting slurry was allowed to stir at ambient temperature for a period of 2 h and then under reflux for an additional period of 6 h during which the temperature was gradually increased up to 100 °C (~15 °/h). The purification of solid involved repetitive washing with toluene (four times) and benzene (twice), alternated with vacuum filtration. The new material was finally dried at ambient temperature in a vacuum desiccator for a period of 24 h. The new supports were denoted as MCM41UNH1, MCM41UM3, and PrMCM41, respectively.

2. 4. Characterization of Mesoporous Materials

All supports, namely MCM–41 and functionalized MCM-41, were characterized by various techniques.

X-ray diffraction (XRD) was performed in order to estimate the structural organization of all samples. The XRD patterns were collected on a Philips X'Pert diffractometer using Cu–Kα radiation, with steps of 0.01° and 4s per step, in the range 2θ from 0.0 to 10°.

Nitrogen adsorption-desorption isotherms were recorded at –196 °C on a Micromeritics ASAP 2010 system. The specific surface areas were determined by the BET method. The pores size distributions and the pore volume were estimated from the BJH equation.

Infrared transmission measurements were carried out at room temperature on a Digilab spectrometer with a spectral resolution of 4 cm^{-1} averaging. The samples were prepared by using the standard KBr disk method.

Solid-state NMR spectra were recorded on a fully digital Bruker Avance 400 NMR spectrometer equipped with a 4 mm MAS probe head. The ^{29}Si MAS NMR spectra were recorded at a frequency of 400 MHz and a spinning rate of 4 kHz. The chemical shifts were referenced to tetramethylsilane (TMS).

2. 5. Immobilization of Enzyme

2. 5. 1. Physical Adsorption of Lipase on Hydrophobic Supports

Immobilization of lipase on the two hydrophobic supports (MCM41UM3 and PrMCM41, respectively) was performed following a procedure described elsewhere,[5] but with some improvements. Typically, 2 ml of lipase solution in 0.1M phosphate buffer, of pH 7.0 (4.38 mg enzyme/ml), were added to 200 mg of support and gently stirred (in order to protect the enzyme molecules to denaturation) for 4 h at room temperature. The resulting enzyme solid preparation was recovered by centrifugation and washed with the same phosphate buffer solution until no coloration appeared in the xantoproteic reaction of the washing solutions. The new biocatalyst was dried under vacuum for 45 min and then in an oven at 30 °C for 20 h. An additional step was required when lipase was adsorbed on PrMCM41. In this case, the support was firstly "wetted" with absolute ethanol, after that the immobilization process was carried out identically as in the first case.

2. 5. 2. Chemical Bonding of Lipase on Activated Support

The covalent immobilization of CALB involved two steps. Firstly, the functionalized support (MCM41UNH1) was activated and then, on the new active support, CALB was chemically bonded. The procedure was based on a method described elsewhere,[13] and it was adapted to our system.

Support activation: 200 mg of functionalized support was treated with 20 ml 2.5% glutaradialdehyde in 0.1 M sodium phosphate buffer of pH 7.0 during 4 h at 25 °C under stirring. The activated support was centrifuged and washed three times with phosphate buffer solution.

Immobilization step: 2 ml of CALB solution (4.38 mg/ml) in 0.1M phosphate buffer, pH 7.0, were added to the activated support and gently stirred for 4 h at room temperature in a 25 ml glass beaker. The immobilized enzyme was collected by filtration through a sintered glass funnel, and washed with phosphate buffer solution. The enzyme preparation was dried under vacuum for 45 min and then in an oven at 30 °C for 20 h. The new immobilized preparation was denoted as CALB-GA.

Loading capacity for all the immobilized (CALB-GA, PrMCM41-CALB1 and MCM41UM3-CALB samples, respectively) was expressed as percent immobilization and it was calculated by mass balance of the activity of initial solution protein, on the side and the activity of the protein in the supernatant and washing solutions, on the other side. For this aim, tributyrin assay was applied as previously reported.[14] From this analysis, the following percents of immobilization were obtained: 54.57 % (CALB-GA), 64.61 % (PrMCM41-CALB1) and 68.72 % (MCM41UM3-CALB), respectively.

2. 6. Catalytic Measurements

The catalytic reactions were performed in a four-neck glass reactor equipped with reflux condenser, termometer and stirrer, and placed in a water thermostated bath. A typical reaction mixture consisted of immobilized lipase (50 mg), 5.42 mmole ethyl acetate and 1.64 mmole alcohol (1–BuOH and 1–HxOH, respectively) and 33.25 mmole n-hexane. The reactions were conducted at constant temperature (40 °C) and stirring. 0.2 ml of clear liquid sample was periodically withdrawn from the reaction mixture and analyzed by GC (CARBOWAX 20M column, flame ionization detector).

3. Results and Discussion

3. 1. Synthesis and Characterization of Supports

MCM-41 has uniform cylindrical pores of 2.5 – 10 nm diameter in hexagonal arrangement and high specific surface area. Tailoring of these materials for specific applications requires both structural and compositional control. This can be achieved by surface chemical modification using two general strategies, namely post–synthesis functionalization (grafting), or direct synthesis (co-condensation).[15]

Post-synthesis functionalization supposes the reaction between an organosilane and surface silanol groups of the support by using a suitable solvent and under reflux (Figure 1). The template is removed before functionaliza-

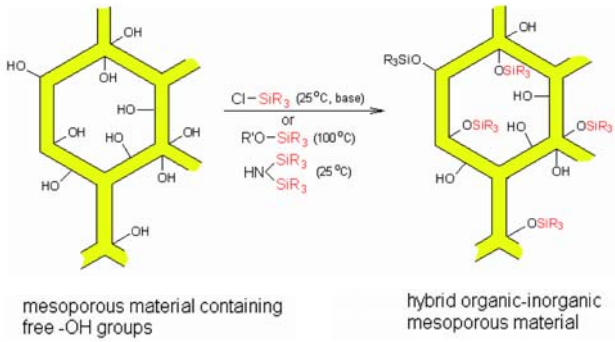


Figure 1. Mesoporous silicates functionalization by post – synthesis method.

tion either by calcination or by proper extraction methods. During functionalization, the porous structure of the support does not suffer change, and this is the greatest benefit of this method. In addition, the wetting stability and resistance to the mechanical compression are improved. The supplementary adding of the siloxane groups has the effect to protect the mesoporous walls of the support even after the alkyl groups are removed by calcination. The great shortcoming of the method is the decrease of pores size.[6]

By comparing to the first method, the second method (i.e., the co – condensation, Figure 2) consists in the condensation between tetraalkoxysilanes and organosilanes offering both a better distribution of the functional groups and a better control of the surface properties of the new materials.[6]

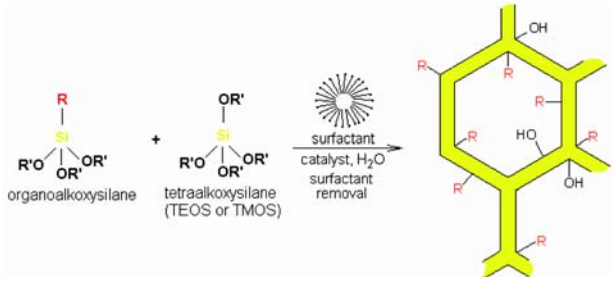


Figure 2. Mesoporous silicates functionalization by co – condensation method.

Our study is focused on the ability of some derivates of MCM-41 mesoporous material, obtained by post-synthesis functionalization, for lipase immobilization as supports. The new functionalized hybrids are MCM41 UM3 (MCM-41 functionalized with CTMS), PrMCM41 (MCM-41 functionalized with PTMS), and MCM41 UNH1 (MCM-41 functionalized with APTES), respectively. The physico-chemical characteristics of supports are collected in Table 1.

Table 1. Textural aspects of studied supports

Sample	S_{BET} ($m^2\ g^{-1}$)	Pore diameter (Å)	Pore volume ($cm^3\ g^{-1}$)
MCM-41	801	35	0.57
MCM41UM3	709	24	0.44
PrMCM41	401	23	0.22
MCM41UNH1	499	30	0.37

As a result of the surface modification, the surface area decreased from 801 $m^2\ g^{-1}$ for calcined MCM-41 to 709 $m^2\ g^{-1}$ for MCM41UM3, 401 $m^2\ g^{-1}$ for PrMCM41, and 499 $m^2\ g^{-1}$ for MCM41UNH1, respectively. As expected, when the size of the functional groups increases, the diminishing of both the pore diameter and the pore volume are noticed. This is a first proof of the attachment of the functional groups inside the pores. Generally, the IV type of adsorption isotherms characteristic to the MCM-41 materials was preserved upon post-synthesis modification but the capillary nitrogen condensation was shifted gradually to lower relative pressures, and thus indicating the diminishing of pores size.

Powder X-ray diffraction patterns for all the samples are collected in Figure 3.

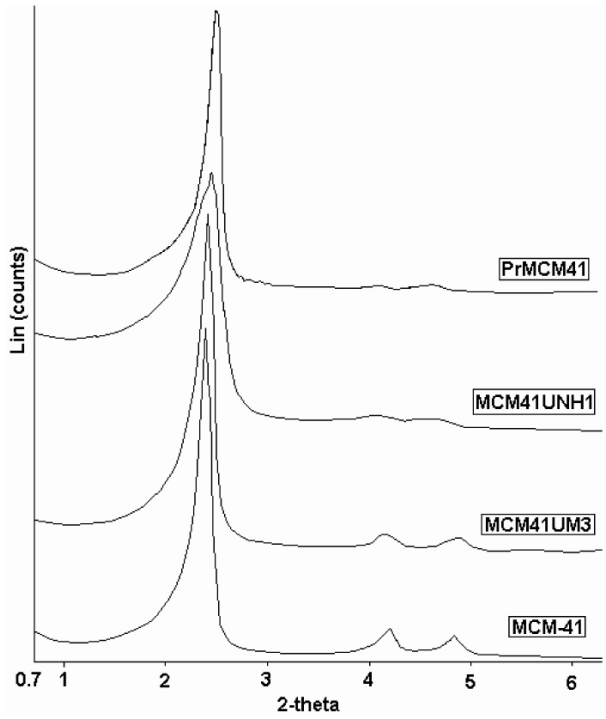


Figure 3. X-ray diffraction patterns of MCM-41, MCM41UM3, PrMCM41, MCM41UNH1.

The pattern of calcined MCM-41 shows an intense peak corresponding to the d_{100} reflection, which is accompanied by weaker reflection at 2θ close to 4.2 and 4.8. These last values are related to the d_{110} and d_{200} spacing of the hexagonal symmetry *P*6mm of MCM-41. Silylated sample with CTMS shows almost unmodified X-ray diffractogram. Surface functionalized materials exhibit XRD patterns at the low-angle region, as well. Generally, post-synthesis surface functionalization does not drastically affect their initial structural ordering.[16] As Lim and Stein also observed (cited by Sayari[16]), the overall intensity of the XRD peaks decreased after this treatment. Nevertheless, in our study, little structural changing was observed by post-synthesis treatment. Thus, sample functionalized with APTES shows larger peaks reflecting a smaller ordered pore structure, while the d_{200} reflection disappears from XRD pattern upon grafting with propyl groups.

The incorporation of organosilanes into mesostructured MCM-41 was also investigated by ^{29}Si MAS NMR and the spectra are shown in Figure 4.

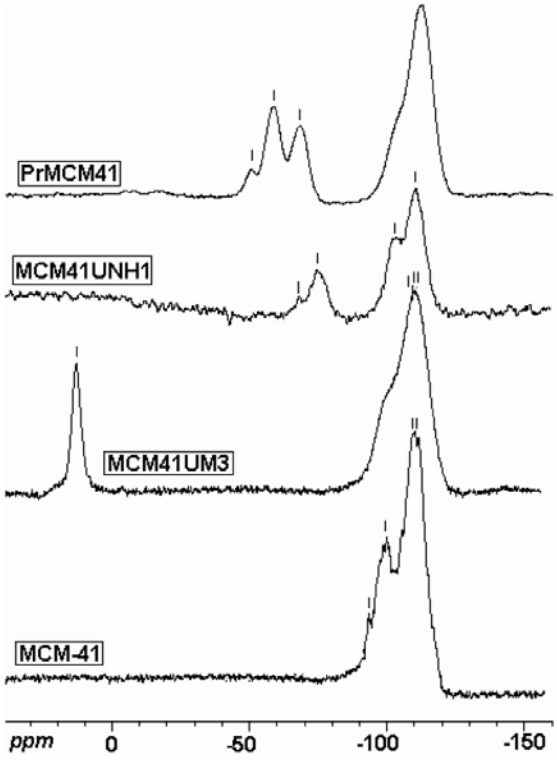


Figure 4. ^{29}Si MAS NMR spectra for calcined MCM-41 and functionalized MCM-41.

Different resonances were observed for siloxane (Q^n = $Si(OSi)_n$–$(OH)_{4-n}$, n = 2–4) and organosiloxane (T^m = $RSi(OSi)_m$–$(OH)_{3-m}$, m = 1–3) (Table 2).

Table 2. ^{29}Si chemical shifts

Species	Chemical shifts
$Si(O–)_4$	**–110**
$Si(OH)(O–)_3$	**–109**
$Si(OH)_2(O–)_2$	**–99**
$SiR(–O)_3$	–66.57
$SiR(OR')(–O)_2$	–57.18
$SiR(OR')_2(–O)$	–49.62
$Si(CH_3)_3(O–)$	14

R = $–CH_2–CH_2–CH_3$; $–CH_2–CH_2–CH_2–$ H_2N; R' = $–OC_2H_5$; $–OCH_3$

The spectrum of calcined MCM-41 shows three overlapping broad resonances at –110 *ppm*, –109 *ppm*, and –99 *ppm*, respectively corresponding to Q^4 ($Si(OSi)_4$), Q^3 ($HO–Si(OSi)_3$) and Q^2 ($(HO)_2–Si(OSi)_2$) silicon sites, which are in agreement to the data already published.[12,17,18] A sharp peak at +14 *ppm*, which was ascribed to trimethylsilyl groups attached to silicon sites ($Si(CH_3)_3(O–)$), is observed for the sample MCM41UM3. This single peak confirms that CTMS could react only monofunctionally with the surface hydroxyls as it is illustrated in the scheme 1.[19]

Disappearance of Q^2 silicone resonance in this spectrum demonstrates that geminal silanols are not hydrogen-bonded and are active sites for silylation as well as the isolated SiOH groups. The main by-product, HCl, does not interact with silica surface and it can be easily removed.

Functionalization with alkoxysilane (in our case, APTES and PTMS) leads to alcohols as by-products that besides water (introduced intentionally or as contaminant, could also result in the hydrolysis or additional condensation of non-reacted functional groups, which are present in the initial bonded phase) might interact with silica surface.[12] The spectrum of PrMCM41 shows the most intense peak at –57.18 *ppm* and it is assigned to $Si(CH_2CH_2CH_3)(OCH_3)(–O)_2$. Disappearance of Q^3 and Q^2 resonances reveals a good attachment of propyl groups on silica surface. Amino-functionalization was easily succeeded, as well. Only Q^3 resonance comple-

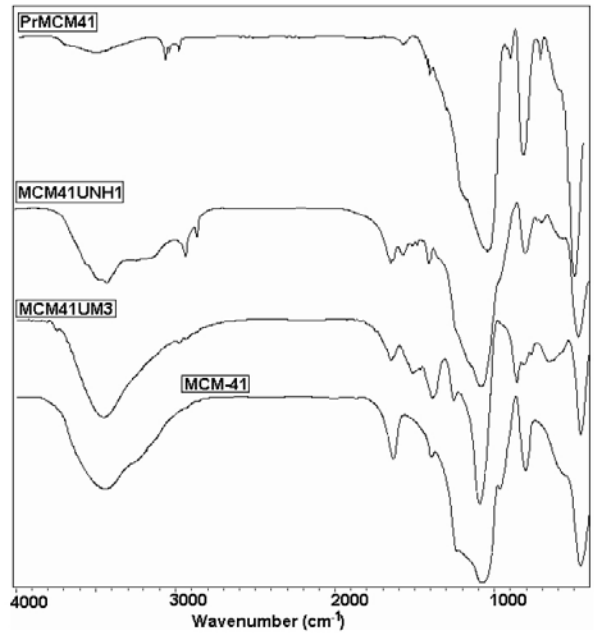


Figure 5. FT-IR spectra of calcined MCM-41, MCM41UM3 and MCM41UNH1.

Scheme 1. Silica functionalization by trimethylsilyl groups

tely disappeared. The resonance at –57.18 *ppm,* which was better represented before, it is now almost imperceptible. Here, the most intense peak is at –66.57 *ppm* reflecting that isolated silanols allowed a better functionalization than geminal ones. ^{29}Si NMR results are in good agreement with XRD results showed above. The decreasing in intensity of the Q^4 resonance indicates small architectural chancing of the new organic-inorganic hybrids.

Figure 5 displays FT-IR spectra of calcined and functionalized MCM-41, respectively.

These spectra could be divided in three main frequency regions according to Ma et al. and Lenza et al., respectively.[18,20] The interval between 4000–3000 cm^{-1} (the first interval) corresponds to the vibrations of silanols and H_2O molecules. Organic residues absorb in the range 3000–1350 cm^{-1} (the second interval). The absorptions at 3000 cm^{-1} and 2900 cm^{-1} are associated with C–H stretching from methyl and methylene groups and their presence in the spectrum of functionalized MCM-41 also confirms the results obtained by ^{29}Si MAS NMR. The band at around 1460 cm^{-1}, which is observed in both the MCM41UM3 and PrMCM41 spectra, characterizes C–H deformation. As the functionalization was performed on a template free MCM-41, it is a piece of evidence that the incorporation of organic moieties was successful. The existence of amino groups in MCM41UNH1 is confirmed by absorptions at 1471 cm^{-1} and 1620 cm^{-1}, respectively. The third interval (1300–400 cm^{-1}) belongs to the vibrations of silica network.

3. 2. CALB Immobilization

Mesoporous molecular sieves, which have large surface area, variable pore diameters, hydrophobic/hydrophilic behaviour and electrostatic interactions hold great promise for their use as supports to immobilize enzymes.[21] CALB is a globular protein with approximate molecular dimensions of 30 Å × 40 Å × 50 Å, and relative mass of 33 kDa.[22] Depending on the surface properties of support, the immobilization of CALB could occur by either physical or chemical adsorption. *Physical adsorption* supposes that enzyme is simply retained onto the support (only by physical forces like electrostatic and van der Waals forces), which is an advantage because the functional conformation of lipase is not affected by immobilization. The main shortcoming is the easy leaching of enzyme during exploitation, especially if the activity measurements conditions are changed (i.e., pH, ionic strength). These could be overcame when the activity measurements are performed in organic solvents, which could allow the "immobilization", as well. *Chemical immobilization* through reactive groups (such as glutaraldehyde, cyanogen bromide, etc.) leads to very stable enzyme-support bonds under mild immobilization conditions (e.g., neutral pH values).[23]

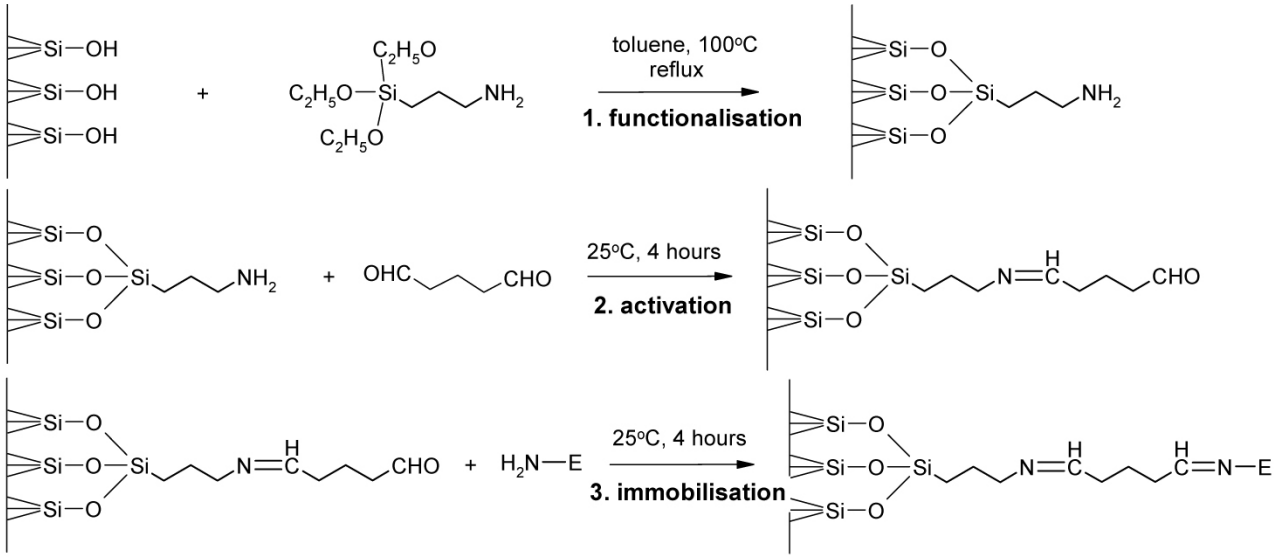
3. 2. 1. Immobilization Onto Hydrophobic Supports

The results already published in the literature indicated poor adsorption of the enzyme onto simply siliceous surface of the mesoporous sieves. This could be explained either by the weak interactions between enzyme macromolecules and siliceous surface or by the high tendency of lipases to form inactive aggregates because the accurate distribution of the enzyme in a monolayer does not always happen. The use of functionalized surface was introduced to eliminate these limitations. The functionalization increases the interaction between the enzyme molecules and support. The supports for which the hydrophobic nature is obtained by alkylsilane or alkylalkoxysilane compounds are the most frequently used. This method has proven to be very useful to achieve hyperactivation of the most lipases: the hydrophobic surface could induce the conformational change of lipase necessary to make free access of the substrates to its active site. Moreover, such a surface could direct enzyme molecules towards a particular orientation, which seems to be the only possibility to preserve its active configuration.[8] It has been established that increasing hydrophobicity exponentially, the rate of lipase-catalyzed reactions increases. Indeed, a recent study performed by Bhandarkar and Neau[24] has point out that lipase performance increases with hydrophobicity but only up to a limit after which the effectiveness of lipase can not be improved. In their study, these authors pointed out that the activity of *Candida rugosa* lipase in the esterification of fluorbiprofen with n-butanol was evaluated in solvents with gradually increasing in hydrophobicity such as toluene (log P = 2.5), n-heptane (log P = 4.0) and isooctane (log P = 4.5), and n-nonane (log P = 5.1). Their preliminary studies showed that the reaction proceeded slowly in toluene and faster in isooctane than in n-heptane. The *Candida rugosa* lipase catalyzed reaction in n-nonane proceeded at about the same rate as in n-heptane. It would seem that a limit of the solvent hydrophobicity has been reached, but some factors other than hydrophobicity may affect the reaction in isooctane, taking into account that this solvent is the only branched alkane of this series. Extending this finding to hydrophobic surface of the support, for this study it was considered as usefully to choose a molecule with short alkane branch for functionalization in order to avoid limiting processes.[24] Our study can confirm the existence of a limit of hydrophobicity. Although both the methyl and propyl groups are hydrophobic, it seems that there is an influence of the chain length on the catalytic activities of the biocatalysts. Thus, the biocatalyst obtained by enzyme immobilization on the hybrid material having propyl groups on the surface led to lower conversions than the corresponding biocatalysts with methyl groups (Figure 6).

3. 2. 2. Immobilization by Covalent Bonding

When an enzyme is used in aqueous media, physical adsorption is not the most suitable method as we pointed out before. In such conditions, a strong immobilization is required and this could be achieved by covalent bonding of enzyme onto the support surface. For this end, after functionalization of the silica surface with a suitable organosilane derivative, subsequent activation of the support was performed. In our experiments, MCM41UNH1 containing $-NH_2$ reactive groups was firstly activated with glutardialdehyde. The chemical modification of MCM-41 surface is illustrated in scheme 2 (first two steps).

In the first step, the reaction between the silanol groups of the MCM-41 and APTES was carried out and the resulted product was then treated with glutardialdehyde. The activated support was mixed with lipase and the superficial amino groups of the enzyme interacted with the free aldehyde groups of the support leading to a strong enzyme-support bond.



Scheme 2. Main steps of CALB immobilization by covalent bonds

3. 3. Catalytic Measurements

Figure 6 shows the conversion of ethyl acetate by the alcoholysis with two different alcohols in the presence of free and immobilized CALB onto the hybrid organic-inorganic mesoporous materials.

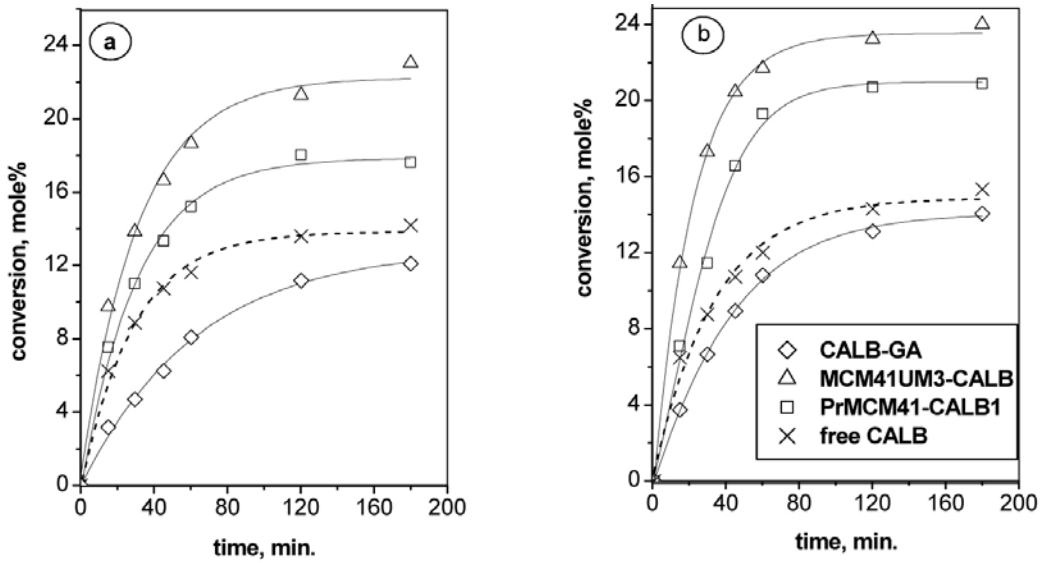


Figure 6. Conversion vs. time in the following reactions: EA + 1–BuOH (a) and EA + 1–HxOH; reaction conditions: 40 °C, 50 mg biocatalyst; EA:alcohol = 3.3:1 (molar ratio); solvent: hexane.

Catalytic activity during these two reactions (alcoholysis of ethyl acetate with alcohols (1-butanol (Figure 6a) and 1-hexanol (Figure 6b), respectively) was depending on the state of the enzyme (free or immobilized) as well as on the characteristics of the supports surface (hydrophobized or functionalized). When the behaviour of the biocatalysts during the first alcoholysis reaction (EA + 1–BuOH) is taken into account, the smallest efficiency was observed for the enzyme covalently immobilized and the greater one for the CALB immobilized on trimethylsilyl functionalized MCM-41. The conversion in the presence of free CALB is placed between conversions obtained for covalent immobilized CALB and physical adsorbed CALB on PrMCM41. The smallest conversion for covalent immobilization could be explained by the changing of the functional conformation of enzyme, which probably affected its catalytic performance. It is possible that some active sites to be covered by the rest of the lipase macromolecule making them inaccessible to the substrate molecules or the active sites could be affected by the chemical condensation with glutaraldehyde residues. The positive effect of the hydrophobization of the support surface over catalytic activity is also confirmed by these experimental results. It is clear that hydrophobic interaction between enzyme and the support makes enzyme more efficient probably by the facility the adoption of a more favourable to the catalytic act. Analogous behaviour was reported for the alcoholysis of ethyl acetate with 1–HxOH on free and immobilized CALB onto hybrid organic-inorganic mesoporous materials. Table 3 shows the maximum conversions obtained for CALB immobilized by two methods (adsorption: PrMCM41-CALB1, and chemical bonding: CALB-GA), and the conversions ratio for the two alcoholysis reactions.

Table 3. Comparison between conversions obtained on hydrophobized biocatalyst (PrMCM41-CALB1) and a functionalized biocatalyst (CALB-GA).

Reaction	Biocatalyst	Conversion	Conversions ratio
EA + 1–BuOH	PrMCM41-CALB1	11.82	1.40
	CALB-GA	8.43	
EA + 1–HxOH	PrMCM41-CALB1	13.70	1.89
	CALB-GA	7.26	

Higher values obtained for the first biocatalyst (PrMCM41-CALB1) during the both catalytic reactions indicate the hydrophobicity as a key factor of lipase activity as it was already pointed out above. Secondly, the hypothesis made before is not excluded, namely during covalent immobilization (i.e., CALB-GA) the catalytic performance of enzyme is affected, which results in a lower conversion. Nevertheless, it is evident from Figure 6 that immobilization of lipase, especially onto hydrophobized supports, increases the catalytic efficiency of lipase. Similar results were obtained by Dumitriu et al. for CALB immobilized on MCM-36 micro-mesoporous material.[5] The activity of the immobilized enzyme was better than that for the free CALB. It can be observed in Table 3 that the hydrophobicity of the surface affects the reactivity of substrate, as well. This finding is in agreement with our previous work made only for free CALB.[25] In that work we have shown that the alkyl moiety of the alcohol influenced the lipase activity. Conversions as well as specific activities were enhanced for alcoholysis of ethyl acetate with 1–HxOH than for the alcoholysis with 1–BuOH. It seems that enzyme exhibited more affinity for hexanol than for butanol. This finding can be considered as normal behaviour since the hexanol is more hydrophobic than butanol. By this study, it was also confirmed that the hydrophobic support is able to improve the enzyme activity.

Although the performance of enzyme covalently anchored on the support was poor, its stability and activity was maintained for 3 catalytic cycles, while for the other biocatalysts (e.g., PrMCM41-CALB1), the catalytic performance was loosed after the first catalytic cycle. This behaviour could be explained either by a leaching process (which occurs obviously in aqueous medium, but it is less probable in an organic solvent) or by a mechanical denaturation/removal of adsorbed enzyme of the leaching phenomenon inevitable during physical adsorption (Figure 7).

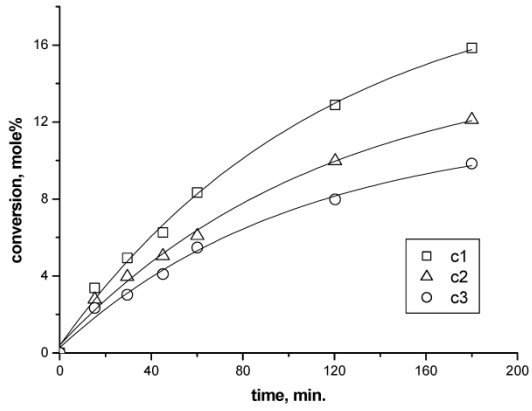


Figure 7. Conversion vs. time for PrMCM41-CALB1 catalysed EA + 1–BuOH alcoholysis after 3 catalytic cycles; c_i (i = 1– 3) is number of catalytic cycles. Reaction conditions: 40 °C, 50 mg biocatalyst; EA : alcohol = 3.3 : 1 (molar ratio); solvent: hexane.

4. Conclusions

Hybrid organic–inorganic mesoporous MCM41 UNH1, MCM41UM3, and PrMCM41 were synthesized by the method of post–synthesis functionalization of the calcined MCM-41 molecular sieve. PrMCM41 and MCM41UM3 have hydrophobic surfaces while MCM41

UNH1 presents amino groups allowing the supplementary functionalization with glutardialdehyde for the covalent binding of enzyme molecules.

Lipase CALB was physically immobilized on the hydrophobic supports and covalent bonded on the glutardialdehyde activated one, respectively. The activity of the new biocatalysts was checked in the alcoholysis of ethyl acetate with n-butanol and n-hexanol. This study pointed out that both, the method and supports influence the activity of the resulted biocatalysts in the alcoholysis reactions. The higher activities were obtained for CALB immobilized on the hydrophobized supports (MCM41UM3, PrMCM41) in comparison to CALB covalently retained on MCM41UNH1. Nevertheless, the stability of the enzymatic preparations was improved by covalent immobilization; although lower after the first catalytic cycle, the biocatalyst still exhibited significant activity after the third cycle.

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Povzetek

Iz mezoporoznega silikatnega materiala MCM-41 smo sintetizirali tri različne vrste hibridnih organskih-anorganskih mezoporoznih nosilcev in jih uporabili za imobilizacijo encima. Funkcionalizacijo in hidrofobizacijo MCM-41 smo izvedli z aminopropiltrietoksisilanom (APTES), klorotrimetilsilanom (CMTS) in propiltrimetoksilanom (PMTS) po odstranitvi templata (strukturnega usmerjevalca) heksadeciltrimetilamonijevega bromide iz por MCM-41. Vpliv organosilanov na mezostrukturne lastnosti hibridnih materialov smo spremljali z rentgensko difrakcijo, N_2 adsorpcijskimi izotermami, FT-IR in ^{29}Si MAS NMR. Funkcionalizirane MCM-41 materiale smo uporabili kot nosilce za imobilizacijo encima *Candida antarctica* lipase B (CALB). Aktivnost novih biokatalizatorjev smo testirali z alkoholizo etil acetata z dvema alkoholoma (1-heksanol in 1-butanol) in jo primerjali z aktivnostjo prostega encima. Vse reakcije smo izvedli v n-heksanu kot topilu.