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Immobilization of lipase in a mesoporous reactor based on MCM-41

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Abstract

An immobilized enzyme has been prepared by incorporation of porcine pancreatic lipase (PPL) in the channels of MCM-41 by virtue of the hydrogen bonding interactions between the abundant weakly acidic hydroxyl groups of the support and the enzyme. The activity of the immobilized enzyme falls off rapidly when reused, however, because the weakly held enzyme is leached out from the pores. When the immobilized enzyme is treated with vinyltrimethoxysilane, the N_2 adsorption–desorption plot of the product shows a type I isotherm with a steep region in the desorption branch of the hysteresis loop, suggesting that most of the organic groups have been coupled to the walls at the pore openings, which partly shrink the pore opening. The ^{29}Si MAS-NMR spectrum shows three peaks between −60 and −80 ppm consistent with the presence of siloxane groups grafted at the pore openings, resulting in a decrease in their size. After the grafting process is complete, pores with narrow necks and wide bodies (so-called 'ink bottle' pores) are formed. When the modified immobilized enzyme was used to hydrolyze triacetylglycerol in a batch process, the activity remained constant over five cycles of reuse. This confirms that reaction with vinyltrimethoxysilane has led to the enzyme being immobilized inside a 'mesoporous reactor' from which leaching of the enzyme is prevented without inhibiting access to the substrate and release of the products.

Keywords: Porcine pancreatic lipase; MCM-41; Vinyltrimethoxysilane; Silylated; Mesoporous reactor

1. Introduction

The immobilization of enzymes on insoluble supports has been the subject of considerable research for over 40 years and consequently many different methodologies have been suggested, including crosslinking, covalent attachment, physical entrapment and physical adsorption [1–4]. Of these methods, physical adsorption and covalent attachment are most widely used. These two methods have their own virtues and drawbacks. Physical adsorption results in minimal disruption of the structure of the immobilized enzymes but loss of the enzyme immobilized on the support is unavoidable due to the weak bonds between the enzyme and the support. Covalent attachment involves bonding of some residues of the enzyme to the support and although this should prevent leaching of the enzyme, it may give rise to a decrease in activity of the immobilized enzyme compared with the native enzyme.

MCM-41, one number of the M41S family of silicate mesoporous materials discovered by researchers of Mobil Corporation in 1992 [5], possesses a regular hexagonal array of uniform pore openings with a broad spectrum of possible pore diameters between 1.5 and 10 nm, depending on the template used during synthesis. The structural characteristics of MCM-41 are highly suitable for the immobilization of enzymes [6]. For example, the large pore size should allow the bulky enzyme molecules to diffuse into the pore; the terminal silanol groups present on the surface of MCM-41 should facilitate immobilization of enzymes via hydrogen bonding; enclosure of the protein in a well-defined space may also help prevent denaturing of the protein and enhance enzyme stability. Diaz and Balkus[7] have reported the immobilization of cytochrome C, papain and trypsin in MCM-41, Wright and co-workers [8] have described the immobilization of trypsin in MCM-41 and other mesoporous molecular sieves and we [9] have re-

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ported the immobilization of penicillin acylase in the same material.

Lipases are enzymes which catalyze the hydrolysis of triglycerides to give fatty acids and glycerol, both essential chemicals in the oleochemical industry. Lipases have been immobilized on a variety of supports including zeolite Y [10], polyacrylamide beads possessing carboxylic functional groups [11] and in calcium alginate gel [12]. In this paper, we describe a method whereby a lipase enzyme, porcine pancreatic lipase (PPL), is first physically adsorbed in the channels of an MCM-41 support, and the size of the mouth of the channels is subsequently reduced by chemical modification by covalent coupling with an organic siloxane. This produces pores with narrow necks and wide bodies (so-called 'ink bottle' pores) [13] which prevent leaching of the weakly bound immobilized enzyme, but do not restrict access of the substrate to the enzyme. PPL is a small globular protein with a molecular mass of 45,000–50,000 [14] and dimensions of approximately, 4.6 nm × 2.6 nm × 1.1 nm [15] and is therefore a suitable candidate for immobilization on MCM-41 in this way.

2. Experimental

2.1. Chemicals and materials

The silica gel used to prepare a sodium silicate (Na/Si = 0.75) solution as the silica source for the synthesis of MCM-41, was technical grade containing 25–26 wt.% silica solid.

PPL was from Sigma Chemical and was stored at 0–4 °C. The activity of the PPL was 170 U g^{-1}. Other reagents used in this work were all A.R.

2.2. Synthesis of the MCM-41 support

Siliceous MCM-41 was synthesized using a solution of sodium silicate (Na/Si = 0.75) as silica source and cetyltrimethylammonium bromide (CTABr) (25% in water) as template. The final gel composition was: $1.5Na_2O{:}4SiO_2{:}CTABr{:}250H_2O$. The synthesis mixture was heated at 100 °C for 4 days during which the pH was adjusted to 10–11 at 24 h intervals. The as-synthesized Si-MCM-41 was calcined in air at 500 °C for 10 h to remove the template.

2.3. Preparation of enzyme immobilized in mesoporous reactor

Step 1 (Direct immobilization): PPL (0.25 g), pH 7.5 phosphate buffer (1.25 ml) and deionized water (49 ml) were added to 0.5 g of calcined MCM-41 support. The mixture was stirred at 30 °C for 12 h. The supernatant was separated from the solid material by centrifugation. The amount of enzyme remaining in the filtrate was assayed, from which it was estimated that the immobilization yield of the enzyme was 76%. The MCM-41 immobilized PPL (IME-MCM) was washed with phosphate buffer (pH = 7.5) solution and then washed with cold acetone until the mixture was completely dry.

Step 2 (Modification of pore openings of IME-MCM): IME-MCM (0.5 g) was added to a solution of vinyltrimethoxysilane (VS) (2.0 g) in toluene (20 ml). After being stirred at 35 °C for 3 h, the mixture was filtered and the solid (IME-MCM-VS) extensively washed with cool acetone and dried.

In addition, a blank experiment without added enzyme was carried by the above assay procedure. MCM-41 was directly added to a solution of vinyltrimethoxysilane (VS) in toluene. After being stirred at 35 °C for 3 h, the mixture was filtered and the solid (MCM-VS) extensively washed with cold acetone and dried.

2.4. Characterization

Powder XRD patterns were obtained using an XRD-6000 diffractometer with Cu Kα radiation (40 kV, 40 mA), a step size of 0.02°, and 4 s per step scan. The low-temperature N_2 adsorption–desorption experiments were carried out using a Quantachrome Autosorb-1 system. The pore diameter distribution was calculated using the BJH and Horvath–Kawazoe methods based on the desorption isotherm, and the surface area was calculated using the BET method based on the adsorption isotherm.

FT-IR spectra were generally obtained at ambient temperature using a Bruker Vector 22 spectrometer. The samples were prepared using the standard KBr disk method.

FT-IR spectra of the hydroxyl region were obtained using the same instrument. The self supporting disk containing the sample was placed in the IR absorption cell and outgassed at an appropriate temperature (at least 200 °C) to remove the adsorbed water before the IR spectra of the hydroxyl region were recorded.

The NMR spectroscopic investigations were performed on a Bruker AV300 NMR spectrometer at resonance frequencies of 75.47 MHz for ^{13}C MAS-NMR and 59.6 MHz for ^{29}Si MAS-NMR. Chemical shifts were referenced relative to TMS for ^{13}C and ^{29}Si.

2.5. Activity assays

Assay method: In the hydrolysis reaction of triacetylglycerol, acetic acid is produced as a by-product, which lowers the pH of the hydrolysis mixture. The activity of the immobilized enzyme can be determined by titrating the acetic acid produced (with NaOH) to maintain constant pH. From the consumption of NaOH in the first 15 min, the amount of acetic acid produced is obtained, from which the activity of the immobilized enzyme can be calculated.

Assay procedure: A mixture of triacetin (2.0 g), deionized water (49 ml) and pH 7.5 phosphate buffer (25 ml) were

stirred for about 20 min to prepare a triacetin emulsion [16]. The stirring speed was set to such a value that a further increase in the stirrer speed did not lead to an increase in the base consumption per minute. When the pH stabilized, 0.25 g PPL was added. The mixture was continuously titrated with 0.1 M sodium hydroxide solution for 15 min to maintain a constant pH value. The volume of sodium hydroxide consumed was recorded and the activity of PPL calculated in the standard way [16].

The activity of IME-MCM and IME-MCM-VS were assayed by the same method. Blank experiments were carried out by repeating the above assay procedure adding MCM-41 to the mixture and in the absence of MCM-41. The volume of NaOH solution consumed by the blank titrations was recorded. The results show that, neither MCM-41 support nor substrate consume significant amounts of NaOH solution, indicating that assaying the activity of IME-MCM and IME-MCM-VS by titration with NaOH is a viable procedure.

3. Results and discussion

3.1. Structure of the mesoporous reactor

As shown in Fig. 1a, the calcined MCM-41 exhibits a well resolved XRD pattern with an intense diffraction peak at $2\theta = 1$–3° and three weaker peaks at $2\theta < 6^\circ$, which can be indexed to (1 0 0), (1 1 0), (2 0 0) and (2 1 0) reflections respectively, characteristic [5] of the hexagonally ordered long-range structure of MCM-41 with $d_{100} = 4.09$ nm. After immobilization of PPL, the intense diffraction peak at low angle shifts to higher angle (corresponding to $d_{100} = 3.77$ nm) and becomes weaker and broader (Fig. 1b), indicating that PPL has entered into the channels of MCM-41 and affected the long-range structural order. After treatment with vinyltrimethoxysilane, there is a further slight shift of the low angle diffraction peak to higher angle (corresponding to $d_{100} = 3.52$ nm).

Table 1
Pore structure parameters of the MCM-41, IME-MCM and IME-MCM-VS

Sample	Surface area (m^2/g)	Pore volume (cm^3/g)	Pore diameter (nm)
MCM-41	1265	1.45	4.50
IME-MCM	938	0.80	3.40
IME-MCM-VS	403	0.26	1.40

The N_2 adsorption isotherm of MCM-41 is typical of type IV as illustrated in Fig. 2a, in which there is a steep increase in the p/p_0 range of 0.25–0.55, indicating that the MCM-41 has a narrow distribution of pore size. The pore size distribution shown in Fig. 2b shows a modal pore diameter of approximately 4.5 nm. These results are consistent with the literature [5]. The pore structure parameters calculated from the N_2 adsorption isotherms are given in Table 1. The data indicate that the parent MCM-41 sample possesses a large pore volume and specific surface area. When PPL is immobilized in the MCM-41, the N_2 adsorption isotherm of the IME-MCM product is also typical of type IV (as shown in Fig. 3), but the specific surface area, the pore volume and pore diameter all decrease markedly (see Table 1), indicating that the PPL has entered into the pores of MCM-41.

When the IME-MCM is treated with vinyltrimethoxysilane, the N_2 adsorption–desorption plot of the resulting IME-MCM-VS exhibits a type I isotherm with a small hysteresis loop as shown in Fig. 4, in contrast to the type IV isotherms of the parent MCM-41 and IME-MCM. The surface area and pore volume are reduced significantly

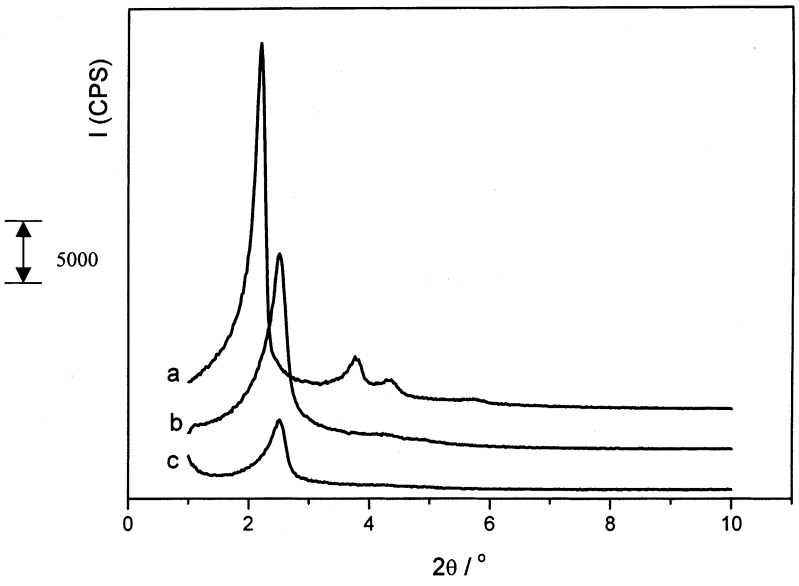

Fig. 1. XRD patterns of (a) MCM-41, (b) IME-MCM and (c) IME-MCM-VS.

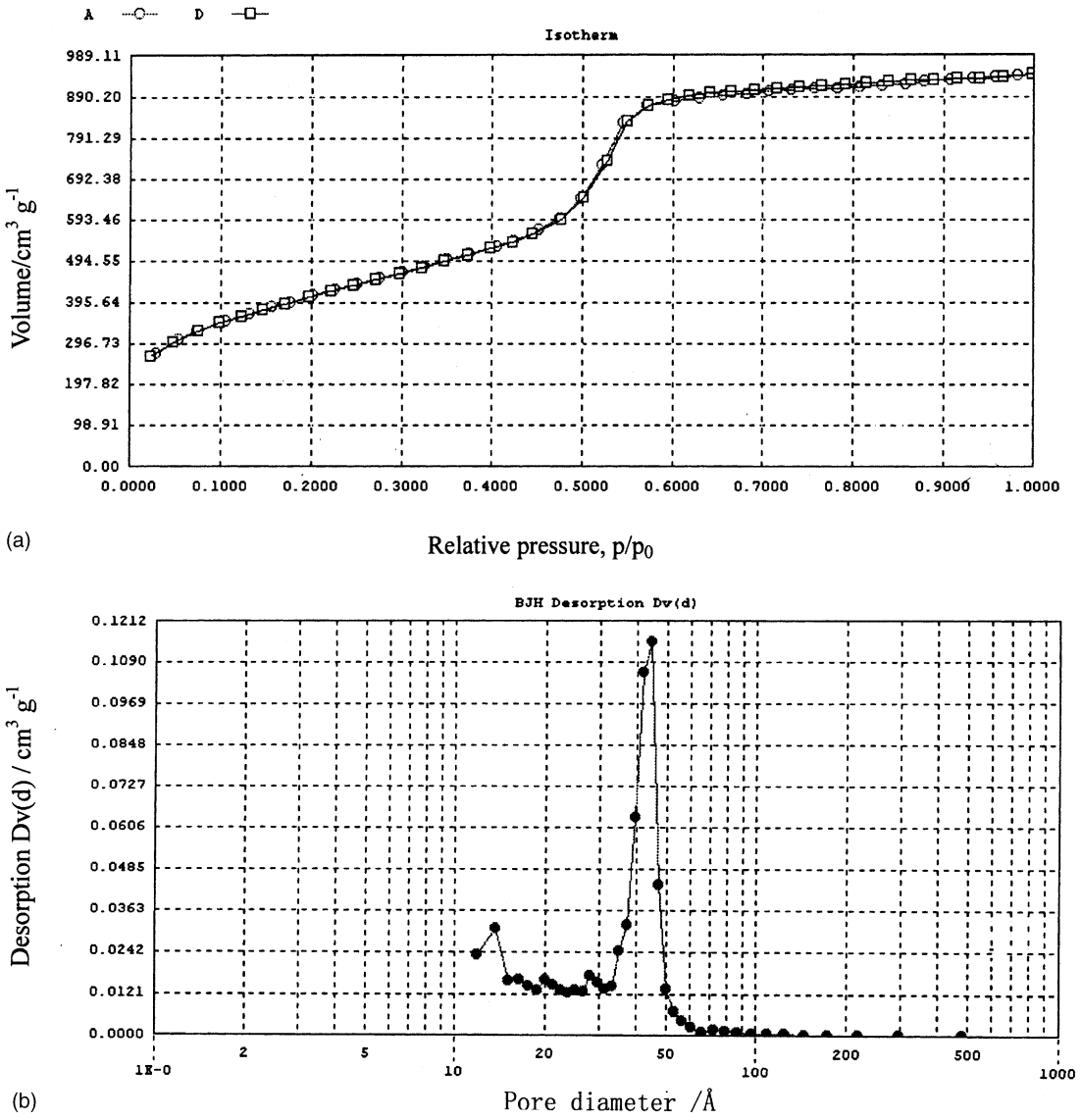

Fig. 2. (a) N_2 adsorption–desorption isotherms of MCM-41. The type IV isotherm is typical of MCM-41. (b) Pore size distribution of MCM-41.

compared with IME-MCM. A type I isotherm indicates that a microporous structure has been formed and the steep region in the desorption branch of the hysteresis loop suggests that most of the organic groups have been coupled to the walls at the pore openings resulting in pores having narrow necks and wide bodies (so-called 'ink bottle' pores) [13]. From the N_2 adsorption data, the diameter of the pore openings in the silylated material was calculated to be around 1.4 nm according to the Horvath–Kawazoe pore size distribution, which is smaller than that of PPL. In grafting reactions, it has been found that the external surface is more easily accessible and is functionalized predominantly over the internal mesopore suface [17]. Whilst the reduction in specific surface area and pore volume may indicate that some of the organic groups have entered into the pores of MCM-41 and have been grafted on the inner walls, the large decrease in pore diameter indicates that the majority of the vinylsiloxy groups have indeed been grafted onto the mesoporous opening and thus, shrunk the pore opening.

Fig. 5 shows the ^{13}C MAS-NMR spectrum of MCM-VS, in which the signals at 136 and 128 ppm are consistent with the presence of vinyl groups grafted on the surface of MCM-41 [18]. The absence of peaks below 40 ppm confirms that no polymerization of the vinyl groups has occurred during the silylation process. The peak at 49 ppm in Fig. 5 is associated with residual CH_3O groups of the organosiloxane.

Fig. 6 shows the ^{29}Si MAS-NMR spectrum of MCM-VS, in which three peaks at −63, −72 and −79 ppm are characteristic [19] of $R(R'O)_2Si–O–$, $R(R'O)Si(–O–)_2$, and $RSi(–O–)_3$ groups, respectively, grafted to the silica surfaces, where (–O–) can represent either oxygen atoms of the silica surface or those of adjacent grafted centres. Re-

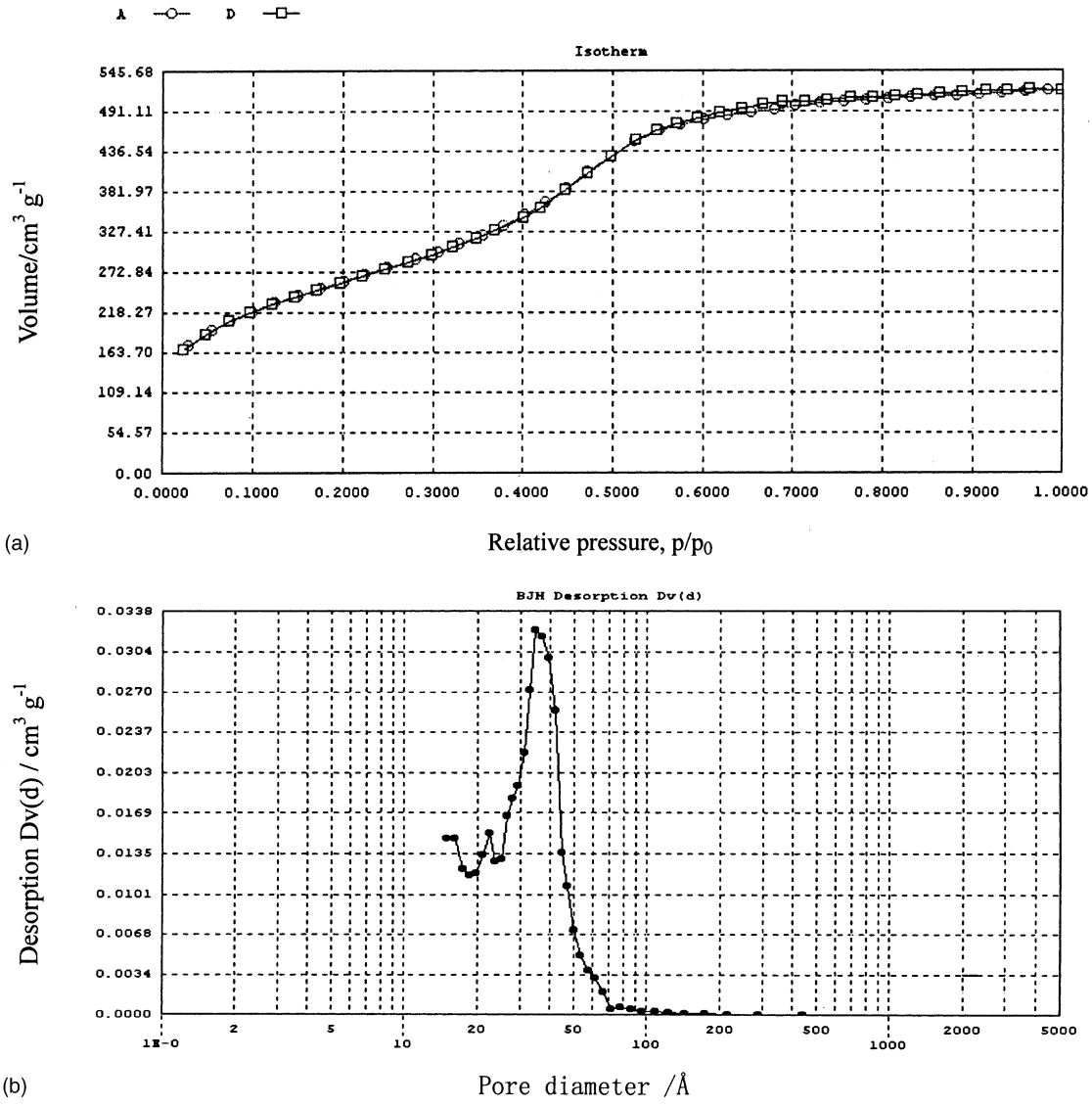

Fig. 3. (a) N_2 adsorption–desorption isotherms of IME-MCM. (b) Pore size distribution of IME-MCM.

action of some of the $R(R'O)_2Si–O–$ and $R(R'O)Si(–O–)_2$ groups giving oligomerized $RSi(–O–)_3$ groups of the type (I) or (II) leads to a further shrinkage of the pore openings and leads to the formation of so-called 'ink bottle' pores. The Q_4 peak at −108 ppm is associated with the bulk MCM-41.

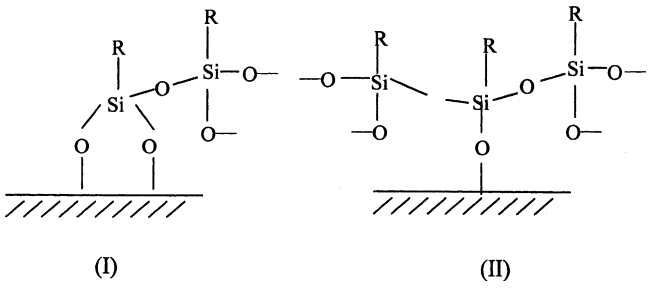

Fig. 7 shows the FT-IR spectra of MCM-41, PPL and IME-MCM. Absorption bands associated with C–H stretching (between 2900 and 3000 cm^{-1}), C = O stretching (1638 cm^{-1}) and C–H deformations (around 1450 cm^{-1}) are observed in the spectra of PPL and IME-MCM but not in the parent MCM-41, confirming that the enzyme has been incorporated in the host.

Fig. 8 shows the high-frequency region of the FT-IR spectra of MCM-41, IME-MCM and IME-MCM-VS obtained after degassing the samples. The presence of an intense absorption band at 3742 cm^{-1} indicates that the surface of MCM-41 has an abundance of free Si-OH groups. After adsorption of PPL, the intensity of this band decreased dramatically (as shown in Fig. 8b), suggesting a hydrogen bonding interaction between free Si–OH groups and the N–H groups of PPL. After reaction with

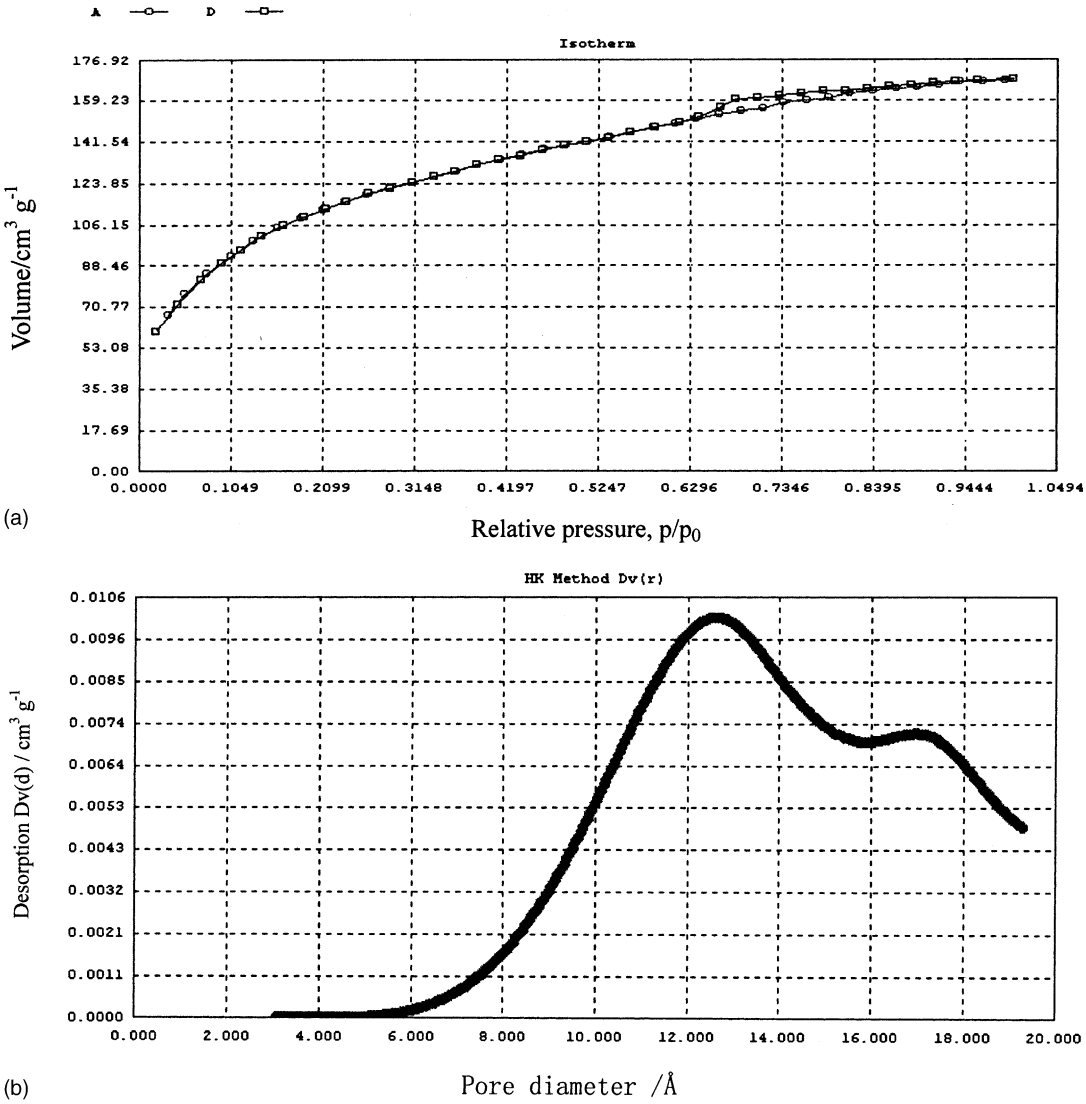

Fig. 4. (a) N_2 adsorption–desorption isotherms of IME-MCM-VS. (b) Pore size distribution of IME-MCM-VS.

VS, the intensity of the band at 3742 cm^{-1} decreased further (Fig. 8c), indicating that the free Si–OH groups had been silylated to a considerable extent. In addition, a band around 2950 cm^{-1}, which can be assigned to saturated C–H stretching vibrations, confirms that the enzyme remains after silylation. An additional band at 3078 cm^{-1} in Fig. 8c, not present in Fig. 8b, can be assigned to an unsaturated C–H stretching vibration, confirming that the vinyl group has been coupled to the surface of MCM-41, consistent with the ^{29}Si MAS-NMR data reported in Fig. 6.

These results confirm that, the PPL enzyme has been immobilized in the channels of the MCM-41 by hydrogen bonding with the hydroxyl groups of the host. Coupling of vinyltrimethoxysilane with silanol groups at the pore openings of the IME-MCM host have led to a reduction in the size of the pore mouth. The structure of the modified immobilized enzyme IME-MCM-VS is shown schematically in Fig. 9.

3.2. Activity of PPL immobilized in the mesoporous reactor

The activity of the IME-MCM was assayed by following the hydrolysis of acetin. The expressed activity (34 U g^{-1}) corresponds to just over 50% of the activity of the same amount of free PPL as that calculated to be adsorbed on the support. This suggests that a part of the PPL has been denatured during the immobilization process or is inaccessible to the substrate. In order to confirm that the PPL is immobilized in the pores of the MCM-41 and not merely adsorbed on the external surfaces, the adsorption of PPL was repeated with as-synthesized MCM-41 from which the template had not been removed by calcination. The resulting solid had a negligible enzyme activity, showing that the PPL is indeed incorporated inside the pores of the MCM-41 in IME-MCM.

When the IME-MCM is separated from the assay mixture and reused, the activity decreases dramatically as shown in Fig. 10. The activity in the fifth cycle is only around 55%

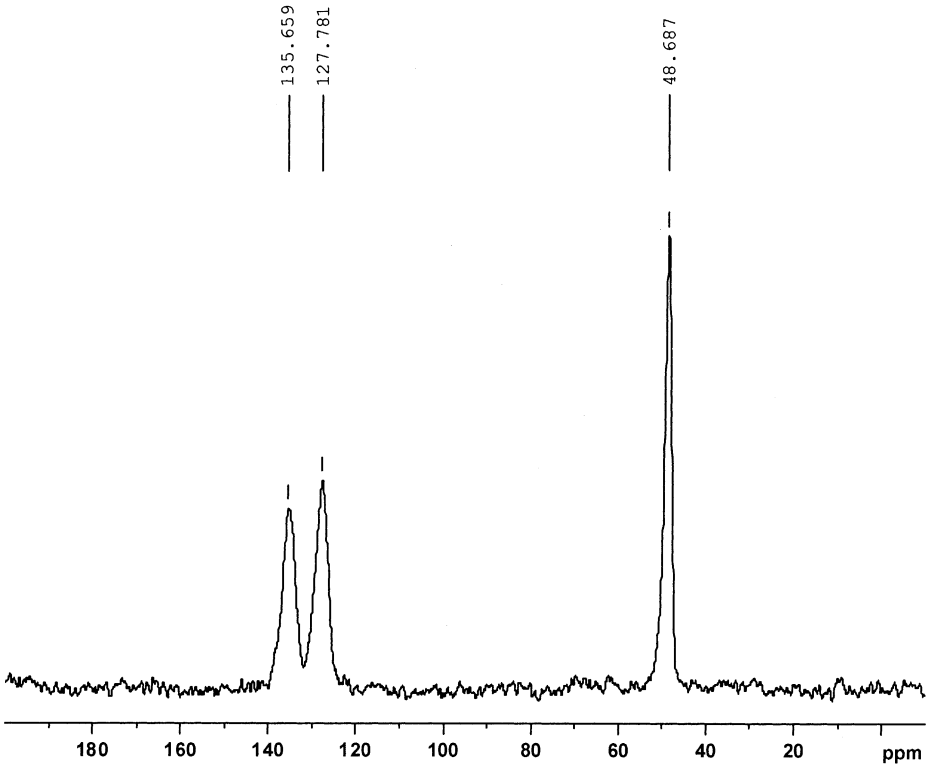

Fig. 5. ^{13}C MAS-NMR spectrum of MCM-VS.

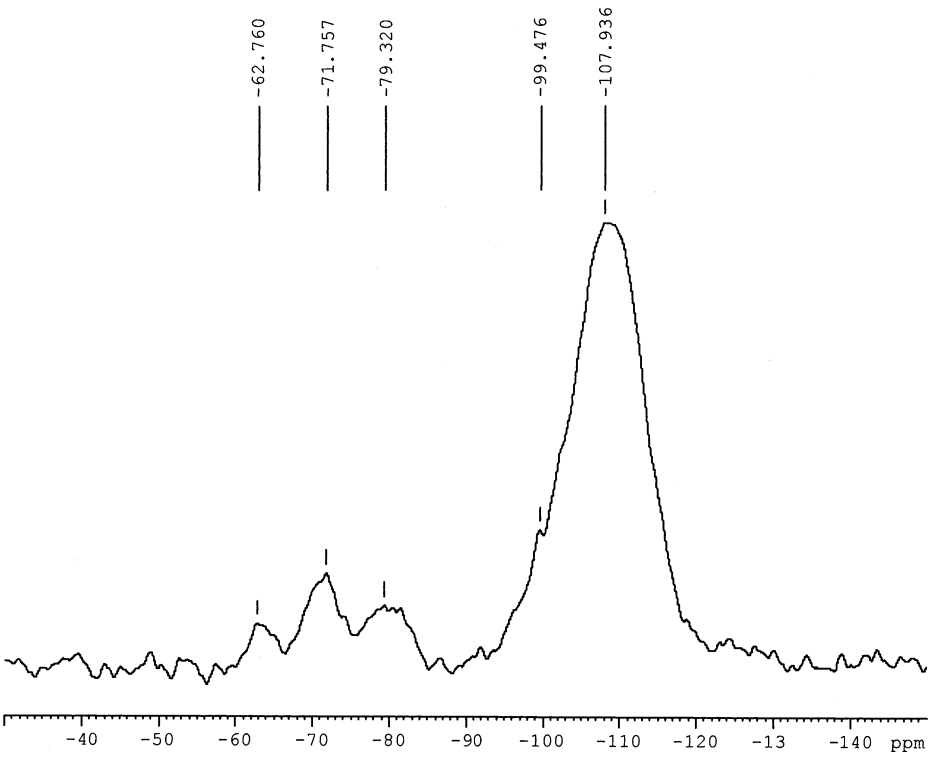

Fig. 6. ^{29}Si MAS-NMR spectrum of MCM-VS.

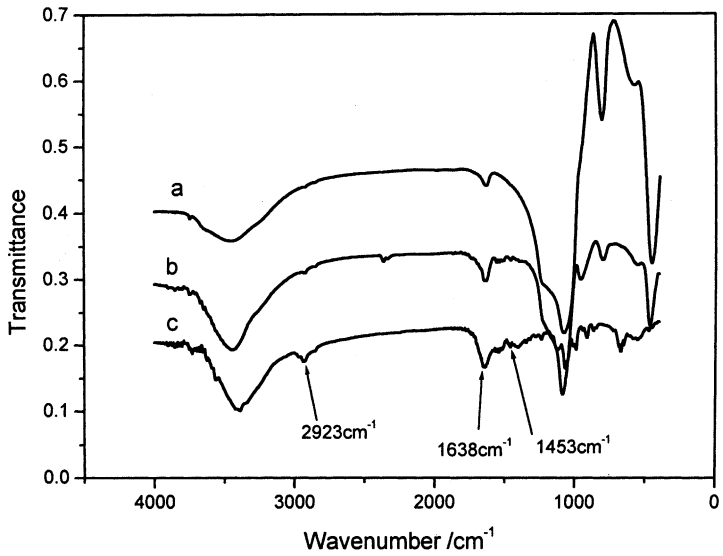

Fig. 7. FT-IR spectra of (a) MCM-41, (b) IME-MCM and (c) free PPL.

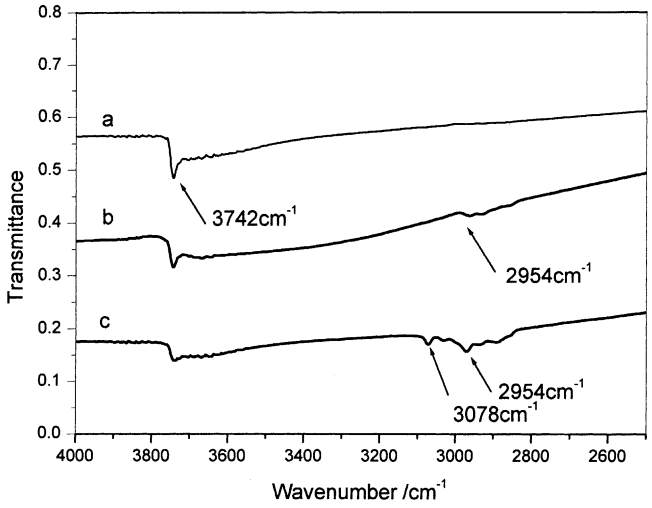

Fig. 8. FT-IR spectra of (a) MCM-41, (b) IME-MCM and (c) IME-MCM-VS obtained after degassing the samples.

of that of the first cycle. This suggests that the PPL is being leached out of the support during the reaction because it is only attached by relatively weak hydrogen bonding with the silanol groups of the support.

After treatment with vinyltrimethoxysilane, the initial expressed activity of the immobilized enzyme is reduced from 50% to around 40%. This suggests that some of the immobilized enzyme has been displaced or denatured during the silylation reaction or that some of the pores have been sealed, or almost sealed, rendering the enzyme inaccessible to the substrate. In contrast to IME-MCM however, the activity of IME-MCM-VS remains essentially constant when reused and after five cycles the activity of the latter is considerably higher (Fig. 10). This indicates that silylation of the material has indeed led to a reduction in the size of the pore mouth, thus preventing leaching of the immobilized enzyme from material.

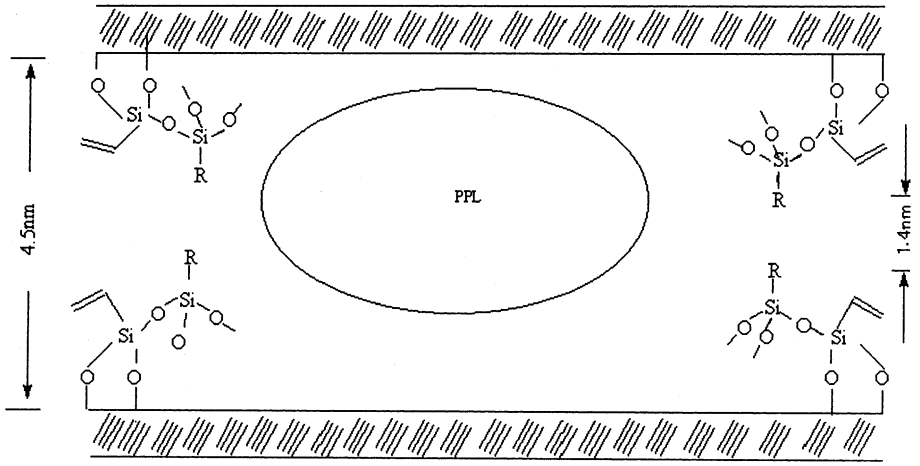

Fig. 9. Schematic representation of the structure of the mesoporous reactor.

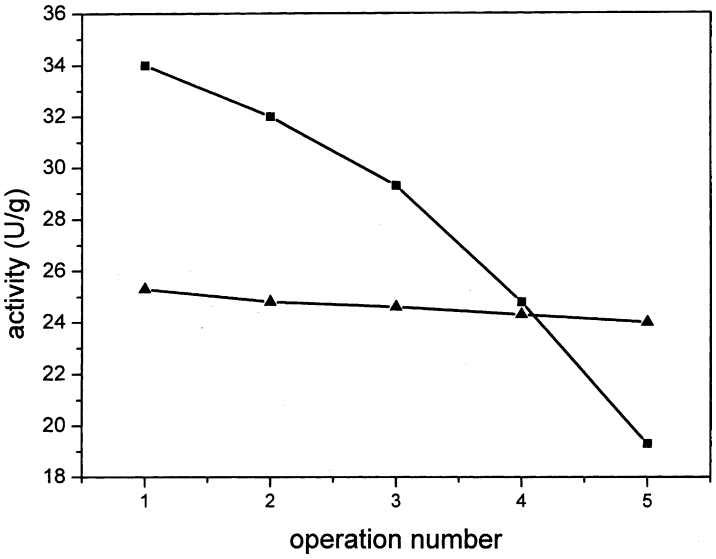

Fig. 10. Activity of IME-MCM (■) and IME-MCM-VS (▲) upon repeated use.

4. Conclusions

A mesoporous reactor for the immobilization of an enzyme has been constructed by modifying the pores of the MCM-immobilized enzyme by silylation with vinyltrimethoxysilane. This has the effect of reducing the diameter of the pore mouth which prevents loss of enzyme without inhibiting access of the substrate to the enzyme.

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