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A new support for the immobilization of penicillin acylase

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Abstract

Mesoporous MCM-41 having well ordered long-range structure, large pore diameters, narrow pore-size distribution, high pore volume and specific surface area has been synthesized. The surface of MCM-41 has an abundance of weakly acidic hydroxyl groups. Assay results show that MCM-41 is a more effective support for the immobilization of Penicillin Acylase (PA) than many of other supports due to its structural and surface characteristics. PA can be immobilized on MCM-41 through either direct immobilization or covalent coupling. The former gives higher activity of IME than the later. In the direct immobilization, PA molecules are immobilized on MCM-41 through the hydrogen-bonded interaction between hydroxyl groups of MCM-41 and carbonyl or amino groups in the PA molecule.

Keywords: MCM-41; Penicillin acylase; Immobilization

1. Introduction

The most important industrial application of penicillin acylase (PA) is the catalysis of the hydrolysis of penicillin G to produce 6-aminopenicillanic acid (6-APA), which is a key raw material for the production of semi-synthetic penicillins. Efforts to immobilize PA have been prompted by its high cost, instability, and the difficulty in recovering the active enzyme for reuse. By immobilizing PA on a solid support, it can not only be used in a continuous process, but also be readily separated from the reaction mixture and reused. Furthermore, the properties of PA may be enhanced [1] or its useful lifetime extended because the immobilized PA is less susceptible to degradation, aggregation, or denaturation. PA has been immobilized on supports by several methods [2–5] including adsorption, crosslinking, adsorption followed by crosslinking, covalent attachment and physical entrapment. However, the solid supports used are almost always polymeric resins, natural polymeric derivatives, organic gels or fibers with limited capacity for reuse, therefore creating problems of disposal of the organic materials [6]. In addition, the surface structures of the organic sup-

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ports are also difficult to control. Inorganic carriers are more expensive than their organic commercial counterparts but have the advantages of being stable and reusable which in some circumstances can decrease the effective cost of the immobilization support.

MCM-41, one member of the M41S family of silicate/aluminosilicate mesoporous materials discovered by researchers at Mobil Corporation in 1992 [7], possesses a regular hexagonal array of uniform pore openings with a broad spectrum of possible pore diameters between 1.5 and 10 nm. Although the discovery of the M41S family of materials stimulated a great deal of research into their potential applications, the results have not lived up to expectations. This is mainly a result of the poor thermal and hydrothermal stability of the materials under most conditions of catalytic interest. In the case of enzyme catalyzed reactions, however, high thermal stability of the support is naturally not a prerequisite and we were therefore, interested to explore potential applications of MCM-41 materials as supports for enzyme immobilization. The structural characteristics of MCM-41 are expected to be suitable for the immobilization of PA. For example, the large pore size should allow the bulky enzyme molecules to diffuse into the pores, and the hydroxyl groups present on the surface as well as their modifiablity should enable the enzyme to be immobilized through an appropriate interaction between surface functional groups of MCM-41 and the enzyme molecules. For these reasons, MCM-41 has been chosen as the support for the immobilization of PA in this work.

2. Experimental

2.1. Chemicals

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

PA solution used here was from North China Pharmaceuticals Company and typically stored at 0–4°C. The activity of the used PA solution was 150 U min^{-1} ml^{-1}.Other reagents used in this work were all A.R.

2.2. Synthesis and characterization 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: $1.5Na_2O{:}4SiO_2{:}CTABr{:}250H_2O$. In the synthesis of Al-MCM-41, the Al source $NaAlO_2$ was added after the addition of silica source dropwise to the template. The as-synthesized Si-MCM-41 and Al-Si-MCM-41 were calcined in air at 500°C for 10 h or longer to remove the template.

The modification of Si-MCM-41 with a silylating agent was conducted as follows: 1 g of calcined MCM-41 was added to a solution consisting of 2 g of $(C_2H_5O)_3SiCH_2CH_2CH_2NH_2$ in 20 ml of inert solvent. After being stirred at 60°C for 3 h, the mixture was extensively washed with acetone and air-dried.

The long-range structures of the MCM-41 materials were characterized by XRD patterns collected on a Siemens D5005 diffractometer with Cu Kα radiation. The pore structures of MCM-41 were characterized by low temperature N_2 adsorption–desorption isotherms obtained at 77 K using an ASAP 2010 Micromeritics instrument.

The FT-IR spectra were obtained at ambient temperature using a Nicolet 5MX FT-IR spectrometer. The self-supported disk of a calcined MCM-41 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. To record the adsorbed pyridine-IR spectra, the disk was first outgassed at 200°C for 2 h and then exposed to a saturated vapor of pyridine for 5–20 min at ambient temperature. After the disk was outgassed to desorb physically adsorbed pyridine molecules, the IR spectra were recorded.

The amount of surface acid was determined by the method of temperature programmed desorption (TPD) of pyridine adsorbed on the MCM-41 support. Pyridine-TPD experiments were carried out in a custom-built TPD device with an SP3400 chromato-

graph as main part and a DS9202 data analyzer. The thermal conductivity detector (TCD) was operated at 230 and 136 mA. The flow rate of He carrier gas was 50 ml min^{-1} and the temperature programmed rate was 5°C min^{-1}.

2.3. Immobilization of penicillin acylase

The immobilization of PA on MCM-41 was carried out by two procedures:

In the first procedure (direct immobilization), 0.6 g of calcined MCM-41 support was mixed with 15 ml of penicillin acylase solution at a pH value of 6.3. The mixture of support and PA solution was stirred at 40°C for 24 h. Then the PA immobilized on MCM-41 was filtered and washed with 150 ml of deionized water for three times. The resulting wet filter-cakes of IME were for the subsequent activity assays. In addition, the resulting IME was also washed more times to investigate the changes of the activity with increasing the washed times. The amount of immobilized PA was about 23 wt.% calculated from the change of the support weights before and after immobilization, based on the dry weight of PA/MCM-41.

In the second procedure (Glutaraldehyde coupling), 0.6 g of silylated MCM-41 was first stirred at room temperature for 1 h with glutaraldehyde. The pH of glutaraldehyde was previously increased to about 7.0 by addition of phosphate buffer solution. After filtration and extensive washing with deionized water, the resulting MCM-41 was used to carry out the immobilization of PA following the same conditions as the first procedure. The PA immobilized on MCM-41 was filtered and washed thoroughly with deionized water and the resulting wet filter-cake of IME was for the subsequent activity assay. The pH value of 6.3 was used in both immobilization procedures because it is the most beneficial to the activity of IME [8].

2.4. Activity assays

2.4.1. Assay method

In the hydrolysis reaction (Fig. 1), phenylacetic acid (PAA) is produced as a side product, the production of which lowers the pH of the hydrolysis mixture. The activity of PA can be determined by titrating the phenylacetic acid by-product with NaOH

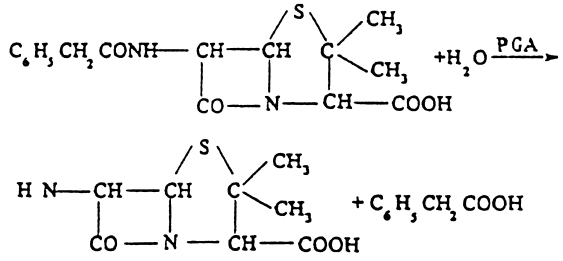

Fig. 1. Enzymatic hydrolysis of penicillin G.

to maintain constant pH. From the consumption of NaOH in the first 12 min, the amount of PAA produced is obtained from which the amount of 6-APA formed can be calculated.

2.4.2. Assay procedure

The activity of soluble PA was assayed as follows: 1 ml of soluble PA, 49 ml of deionized water and 1.25 ml of phosphate buffer (pH = 7.9) were mixed homogeneously and kept at 37°C in a thermostatic bath. Thereafter 50 ml of a 4% aqueous solution of penicillin G which was also maintained at 37°C was added. The mixture was titrated by 0.1 M NaOH solution to maintain the pH of titration mixture constant at 7.9. The volume of NaOH consumed in the first 12 min was recorded to calculate the activity of PA. The activity of immobilized PA was assayed by the same procedure except that immobilized PA was substituted for the soluble PA.

Before the activity of PA was assayed, a blank experiment was carried out. The blank experiment was done by the above assay procedure without adding soluble PA or immobilized PA to the mixture. The volume of NaOH solution consumed by blank titration was recorded. The results show that neither MCM-41 support nor penicillin G consume significant amounts of NaOH solution, indicating that assaying the activity of IME by titrating PAA with NaOH is a viable procedure.

3. Results and discussion

3.1. Structural characteristics of the MCM-41 support

3.1.1. Long-range and pore structures of MCM-41

Fig. 2 shows the XRD patterns of siliceous MCM-41. As illustrated in the figure, both the as-

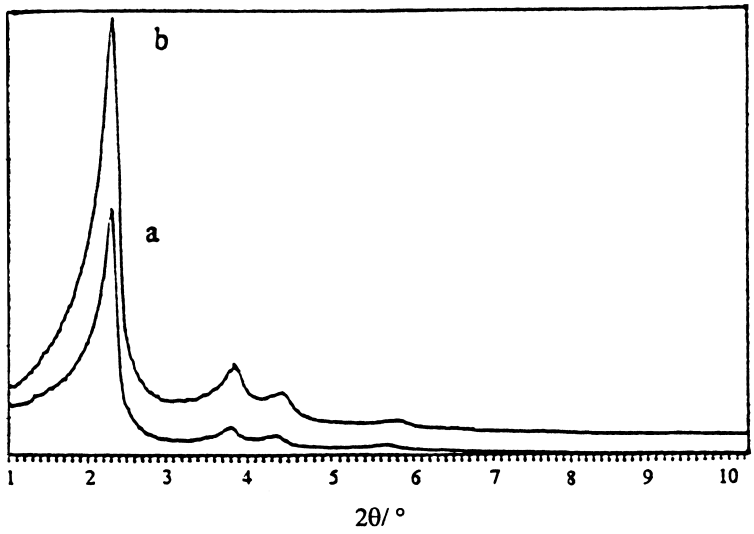

Fig. 2. XRD patterns of Si-MCM-41 (a) as-synthesized and (b) calcined.

synthesized and calcined material exhibit a very intense diffraction peak at $2\theta = 1–3°$ and three other weaker peaks at $2\theta < 6°$, which can be indexed to (100), (110), (200) and (300) planes, respectively, characteristic of the hexagonally-ordered long-range structure of MCM-41. By inserting aluminum into the framework of MCM-41, a silicon-aluminum MCM-41 is obtained. The XRD patterns illustrated in Fig. 3 indicate that Al-Si-MCM-41 also possesses a well-ordered structure even when the Si/Al ratio is as low as 25. From the TEM micrographs (Fig. 4) of the MCM-41 material, apparent one-dimensional channels in a hexagonal array can be observed.

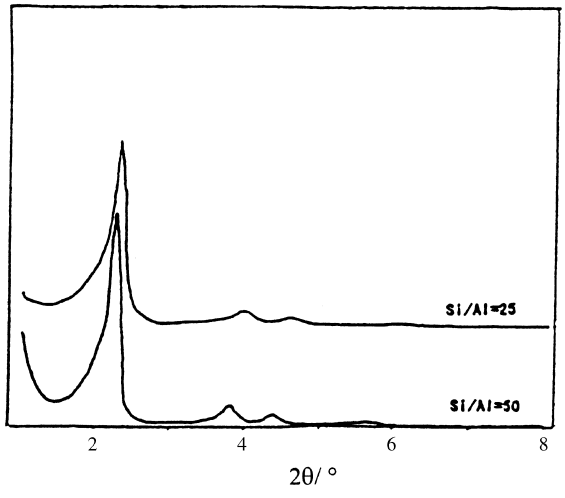

Fig. 3. XRD patterns of Al-MCM-41 with various Si/Al ratios.

The distribution of pore diameters for calcined MCM-41 has been obtained from N_2 adsorption–desorption isotherms at liquid nitrogen temperature. The distribution curve shown in Fig. 5 shows a narrow distribution of mesopores with a pore diameter of approximately 3.0 nm at the maximum. The specific pore volumes and specific surface areas (see Table 1) have been shown to be above 0.8 $cm^3\,g^{-1}$ and 800 $m^2\,g^{-1}$ respectively. All the pore structural characteristics of the MCM-41 support, such as large pore size, high pore volume and surface area, are likely to be benefical to the immobilization of PA, but also indicate MCM-41 may be superior to existing supports.

3.1.2. Surface properties of MCM-41

Fig. 6 shows the FT-IR spectra for the surface hydroxyl groups of Si-MCM-41 recorded after outgassing at different temperatures. Outgassed at 200°C, a sharp absorption band at 3740 cm^{-1} ascribed to free Si—OH groups [9–11] and another broad absorption band centered at 3554 cm^{-1} assigned to hydrogen-bonded Si—OH groups perturbed by physically adsorbed water [9,10] respectively are observed. With increasing outgassing temperature,

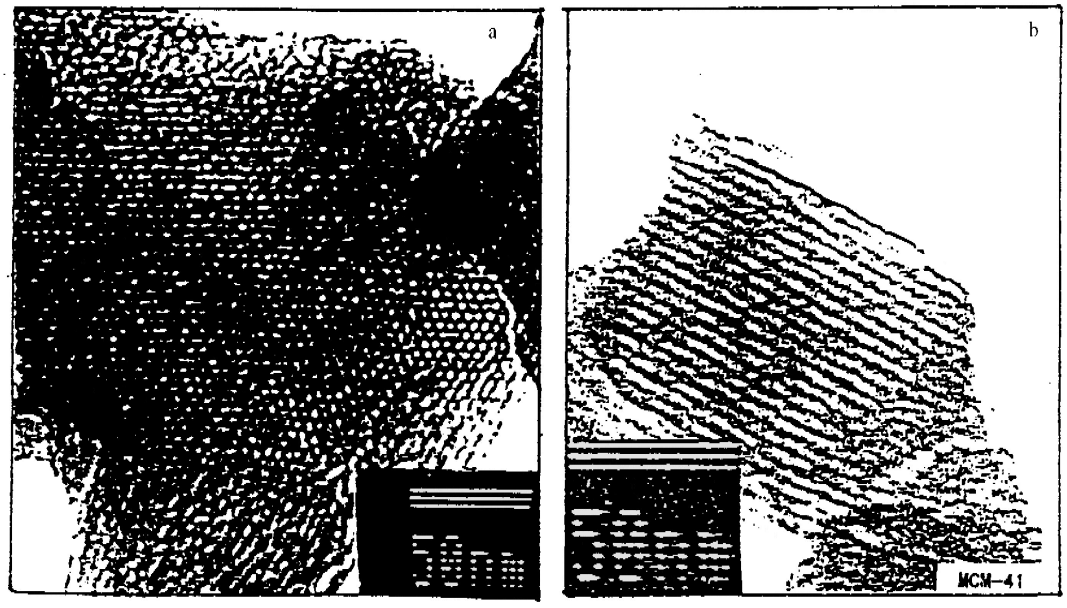

Fig. 4. TEM micrographs of MCM-41 (a) perpendicular; (b) parallel.

the intensity of the broad absorption band decreased because of dehydration and dehydroxylation. The presence of an intense absorption band at 3740 cm^{-1} indicates that the surface of Si-MCM-41 has an

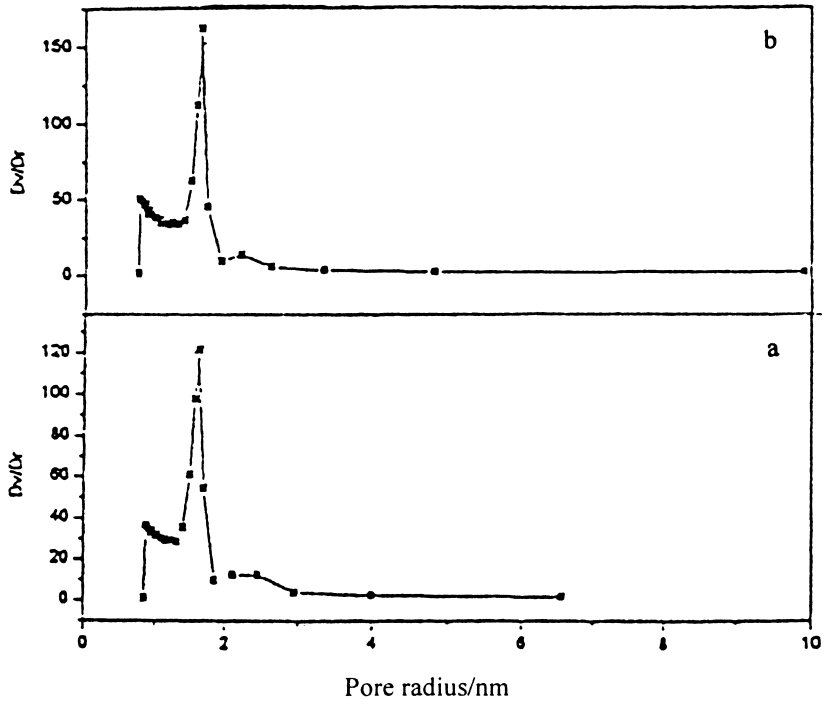

Fig. 5. pore size distribution of the MCM-41 support (a) Si/Al = 25; (b) Si/Al = 50.

Table 1
Pore structure parameters of the MCM-41 supports

Sample	Pore diameter (nm)	Pore volume ($cm^3 g^{-1}$)	Specific surface area ($m^2 g^{-1}$)
Si-MCM-41	3.32	0.83	798
Al-MCM-41 (Si/Al = 50)	3.26	0.98	1092
Al-MCM-41 (Si/Al = 25)	3.24	0.87	985

abundance of free Si—OH groups. After adsorption of pyridine, the intensity of the band at 3740 cm^{-1} decreased dramatically (as shown in Fig. 7 curve a), suggesting that the free Si—OH groups appear acidic and are able to interact with basic molecules or basic functional groups, which can possibly contribute to the immobilization of enzymes such as PA. The acidity of the hydroxyl groups of Si-MCM-41 is rather weak however, because the intensity of the 3740 cm^{-1} band can be restored by increasing the desorption temperature (Fig. 7 curve b). For Al-MCM-41 with different Si/Al ratios, the FT-IR spectra in the hydroxyl region (not shown) and their

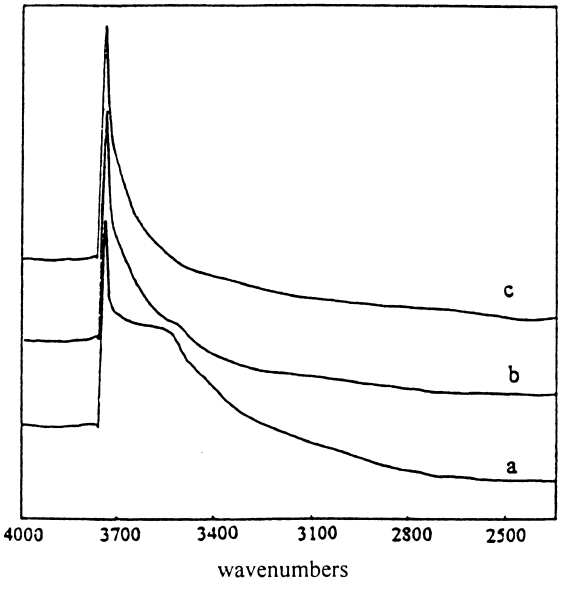

Fig. 6. FT-IR spectra of the hydroxyl region for Si-MCM-41 (a) outgassed at 200°C for 2 h (b) outgassed at 400°C for 1 h after (a) (c) outgassed at 500°C for 2 h after (b).

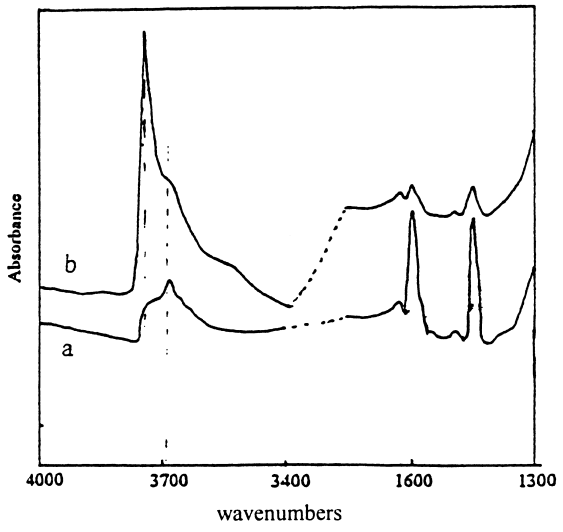

Fig. 7. FT-IR spectra of Si-MCM-41 (a) after adsorption of pyridine followed by desorption at ambient temperature (b) desorption at 150°C after (a).

changes with the adsorption or desorption of pyridine are the same as those for Si-MCM-41.

Fig. 8 shows the FT-IR spectrum of MCM-41 modified by silylation. The absorption band at 3740 cm^{-1} virtually disappears and a new band centered at 2940 cm^{-1} which can be assigned to Si–$(CH_2)_3NH_2$ groups appears after silylation. This indicates that the surface hydroxyl groups can react with the silylating agent to be silylated almost completely, and as a result the surface of MCM-41 becomes functionalized with amino groups which can be expected to be effective linking groups for the immobilization of PA.

The surface acidity of MCM-41 was characterized by pyridine-TPD. The TPD areas (A) of pyridine increase with the decreasing Si/Al ratio, as shown in Table 2, indicating that the number of acidic sites on the MCM-41 surface increase with increasing Al content.

3.2. Immobilization of penicillin acylase on MCM-41

As discussed above, there is an abundance of weakly acidic hydroxyl groups on the surface of the MCM-41 support, which can be modified through silylation allowing the surface to be thoroughly functionalized with amino groups. This suggests two

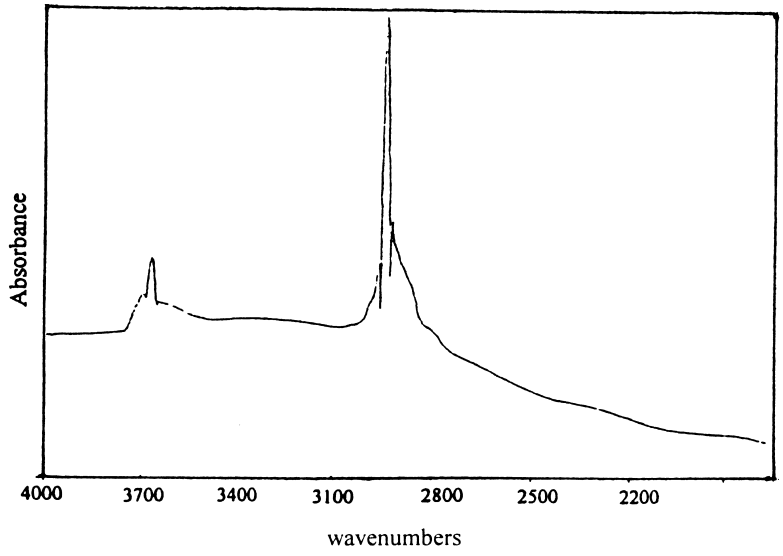

Fig. 8. FT-IR spectrum of silylated MCM-41.

ways by which PA could be immobilized on the MCM-41 support. PA could be directly immobilized on the parent MCM-41 support through interaction with the hydroxyl groups or be simply adsorbed, or could be immobilized on the amino-functionalized MCM-41 by covalent coupling with glutaraldehyde.

3.2.1. Direct immobilization

3.2.1.1. Activity. The activities of IME prepared by direct immobilization are shown in Table 3. It can be seen that the activity exhibits descendent with the operation number but remains constant at about 250 U min^{-1} g^{-1} after several operations. The activities of IME washed to different extents show almost no difference (as shown in Table 4), indicating that the activities result from IME instead of soluble PA and also suggesting that the PA molecules immobilized on MCM-41 can not be leached by washing.

3.2.1.2. Interaction between MCM-41 and PA. Considering the pore structure and surface properties of MCM-41 support, PA directly immobilized on MCM-41 may interact with the support surface or simply be adsorbed in the pores. The stability of IME activities upon washing indicates some interaction rather than adsorption. The activities of IME on MCM-41 supports with various Si/Al ratios (as shown in Table 5), show that the activity of IME increases steadily with increasing Al content of the

Table 2
TPD areas of pyridine desorbed from MCM-41 with different Si/Al ratios

Si/Al ratio	A (μVs)
25	3.2×10^6
50	1.4×10^6
α	0.33×10^6

Table 3
The activities and stability of IME immobilized on MCM-41(Si/Al = α)

Operation number	Activities (U min^{-1} g^{-1})
1	364
2	320
3	277
4	260
5	246
6	249

Table 4
The activities of IME washed for different times

Washed times	Activities (U min^{-1} g^{-1})
3	364
5	362
7	364

Table 5
The activity of IME on MCM-41 with various Si/Al ratios

MCM-41	Activity of IME ($U\,min^{-1}\,g^{-1}$)
Si/Al = ∝	364
Si/Al = 100	475
Si/Al = 50	491
Si/Al = 25	511

MCM-41 support. Comparing the activity of IME with the structure and properties of the MCM-41 support, it can be clearly seen that the change in activity of IME exhibits no direct correlation with the pore structure of MCM-41, but does show an apparent correlation with the number of surface acid sites of MCM-41. This suggests that PA is immobilized on MCM-41 through the acid sites which come mainly from the acidic hydroxyl groups on the MCM-41 surface. The interaction between PA and the support may be hydrogen bonding or chemical bonding between the amino or carboxyl groups in PA and the acidic hydroxyl groups on the support surface. The change of IME activities in the operation (as shown in Table 3) seems to suggest that such an interaction is not so strong as chemical bonding. In order to investigate the interaction between the MCM-41 surface and PA molecules in more detail, the effects of immobilizing PA on the hydroxyl groups of MCM-41 have been investigated by IR spectroscopy.

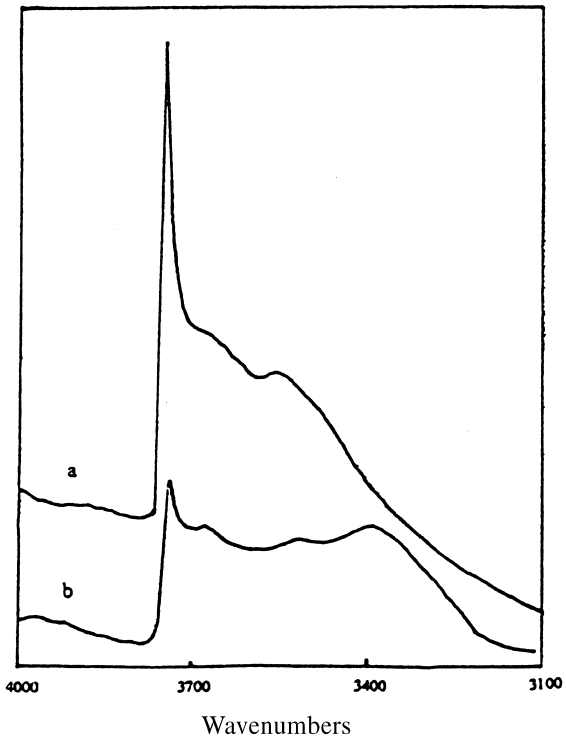

Fig. 9. FT-IR spectra of the hydroxyl region of (a) Si-MCM-41 and (b) PA/Si-MCM-41.

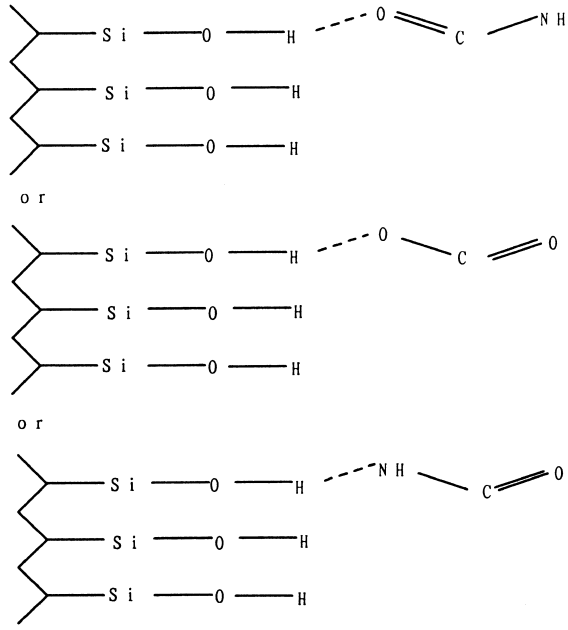

Fig. 10. schematic representation of the interaction between PA and MCM-41.

As shown in Fig. 9, the intensity of the IR absorption band at $3740\,cm^{-1}$ assigned to acidic Si—OH groups reduces dramatically after immobilization of PA and at the same time a new band centered at $3400\,cm^{-1}$ in the hydrogen bonding region appears. The decrease in the absorption intensity of the band at $3740\,cm^{-1}$ suggests that PA molecules may be immobilized on MCM-41 through

Table 6
The activity and stability of IME immobilized on MCM-41 (Si/Al = ∝)

Operation number	Activity ($U\,min^{-1}\,g^{-1}$)
1	90
2	61
3	48
4	47

Table 7
Pore structures of MCM-41 before and after silylated modification

	Pore diameter/nm	Pore volume ($cm^3 g^{-1}$)	Specific surface area ($m^2 g^{-1}$)
before silylation	3.32	0.83	798
after silylation	1.60	0.29	468

the interaction with weakly acidic Si—OH groups, and the appearance of the band at 3400 cm^{-1} indicates that the interaction of PA molecules and the hydroxyl groups on MCM-41 is essentially hydrogen bonding between hydroxyl groups and carbonyl or N–H groups in PA molecules as illustrated schematically in Fig. 10.

However, the absorption band assigned to Si—OH groups has not completely disappeared, presumably because PA molecules are sufficiently bulky such that not all hydroxyl groups are accessible to the guest species.

3.2.2. Covalent coupling through glutaraldehyde

Table 6 shows the activity of IME prepared through covalent coupling. The initial activity of IME prepared by this way is much lower than that by direct immobilization, which indicates that direct immobilization is probably preferable for MCM-41 support. Actually, this is reasonable and not surprising. First, the process of covalent coupling through glutaraldehyde is comprised of multisteps including silylation. The silylated modification decreases the pore diameter and pore volume as well as the specific surface area of the MCM-41 support as shown in Table 7, which consequently is adverse to immobilized amount of PA and the accessibility of active sites in IME. Second, the method of covalent coupling itself is adverse to the activity of IME [12].

4. Conclusion

From the above results, it is clear that mesoporous MCM-41 material possessing pore size of about 3.3 nm, pore volume above 0.8 $cm^3 g^{-1}$ and high specific surface area is an effective support for the immobilization of PA. PA can be immobilized on MCM-41 through either direct immobilization or covalent coupling. The former gives higher activity of IME than the later. In the direct immobilization, PA molecules are immobilized on MCM-41 through the hydrogen-bonded interaction between hydroxyl groups on the MCM-41 surface and carboxyl or N–H groups in PA molecules.

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