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A novel support of MCM-48 molecular sieve for immobilization of penicillin G acylase

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Abstract

As a novel support of immobilizing penicillin G acylase (PGA), MCM-48 and Co-MCM-48 molecular sieves were synthesized and characterized by XRD, N_2 adsorption, NH_3-TPD, FT-IR and so on. The studies show that MCM-48 and Co-MCM-48 has well ordered long-range structure, narrow pore size distribution, larger surface area and higher concentration of the weakly acidic silanol groups on their surface. Penicillin G acylase was immobilized on MCM-48 or Co-MCM-48 by interacting silanol groups on the surface. The presence of cobalt in the framework of MCM-48 increases the amount of the weak acid sites. For the hydrolysis of penicillin G catalyzed by PGA/Co-MCM-48 (Co/Si = 0.01), its specific activity reaches 1682 U/g. After used for six cycles, PGA/MCM-48(0.01) can keep 1375 U/g of the specific activity. If MCM-41 was used as the support, the activity of immobilized PGA is only 402 U/g.

Keywords: MCM-48; Co-MCM-48; Penicillin G acylase; Immobilization; Hydrolysis

1. Introduction

Immobilized penicillin G acylase, an enzyme catalyzing hydrolysis of linear amide bond in the penicillin G molecules, is being used industrially for the production of 6-amino penicillanic acid (6-APA) [1,2]. The immobilization of penicillin G acylase is an important step in whole process of producing 6-APA. As a solid enzyme catalyst, PGA immobilized on a solid support can not only be used in a continuous process, but also be separated readily from the reaction mixture and reused. The various matrices, including inorganic and organic materials, have been explored to use as the supports for an immobilization of the enzyme catalysts to improve its catalytic efficiency and reduce the cost of using enzyme catalyst [3–5]. Compared with the organic support, the inorganic support can be regenerated easily and reused in case of the deactivation of enzyme immobilized, which can reduce the cost of support; in addition, the surface properties and structures of the inorganic support are controlled more easily and more stable. However, the limited pore size of the conventional inorganic materials, such as SiO_2, Al_2O_3 and microporous zeolites, makes the bulky enzymes and substrates diffuse difficultly into the narrow channel of the support. The mesoporous molecular sieves of the M41S family are recently developed and immediately attract many attentions, because of its large surface area, uniform pores with 2–10 nm [6]. The recent studies show that the mesoporous MCM-41 molecular sieve contains an abundance of silanol groups on the surface of channels, therefore an enzyme can be immobilized on the surface by an interaction with the silanol groups [7]. Among the M41S family, MCM-48 with three-dimensional channel system (3D) [8] has several advantages over MCM-41 with one-dimensional pore system (1D) when applied to immobilization of enzyme. Three-dimensional channel system of MCM-48 can be more resistant to blockage of enzyme than the one-dimensional pore system of MCM-41. Besides, the reaction substrate and products can transfer more easily and fleetly inside three-dimensional channel than inside the one-dimensional pores.

In this paper, penicillin G acylase immobilized on mesoporous MCM-48 and Co-MCM-48 are reported for the first time. The structure and surface acidity of Co-MCM-48 and

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the activity of the immobilized enzyme were investigated by means of XRD, NH_3-TPD, pyridine-IR and so on. Moreover, the effects of the pore structures of supports on the catalytic performance of immobilized penicillin G acylase are discussed.

2. Experimental

2.1. Preparation and characterization of Co-MCM-48

The Co-MCM-48 molecular sieve was prepared by the hydrothermal method as follows: tetraethyl-orthosilicate (TEOS) and $Co(NO_3)_2{\cdot}6H_2O$ were added to the mixture solution of cetyltrimethylammonium bromide ($C_{16}H_{33}Me_3NBr$, CTAB), NaOH and de-ionized H_2O at room temperature under stirring. The molar composition of this mixture solution was $1.0SiO_2$:0.01–0.02CoO:0.48CTAB:0.48NaOH:$50H_2O$. After stirred at 70 °C for 1 h, the matrix solution was sealed into a Teflon-lined steel autoclave and kept at 100 °C for 72 h. The solid products were filtrated, washed and dried in air, then calcined to remove the template at 260 °C for 3 h and at 550 °C for 10 h. The cobalt including samples were denoted as Co-MCM-48(0.01) (Co/Si = 0.01, molar ratio) and Co-MCM-48(0.02). If the cobalt compound was not added to the synthesis solution, the Co-free MCM-48 molecular sieve was prepared. The MCM-41 molecular sieve was synthesized as described in Ref. [9].

X-ray powder diffraction (XRD) data were recorded by Rigaku D/MAX-2550/PC diffractometer (Japan) with Ni filter and Cu Kα radiation at 40 kV and 30 mA. The BET surface area, pore volume and pore size distribution were obtained by N_2 adsorption isotherms measured at liquid-N_2 temperature, using Micromeritcs ASAP 2010.

The NH_3-TPD experiments were done in the conventional flow apparatus equipped with a thermal conductivity detector. The sample (150 mg) was pretreated at 500 °C for 1 h under helium stream (25 ml/min). After cooling to 50 °C, the sample was heated programmedly to 500 °C at 8 °C/min to record the TPD spectra of fresh-sample. After the temperature declined again from 500 to 50 °C, the sample was pretreated with a mixture of NH_3–He for 1 h and with helium stream for 1 h at 50 °C. Then the sample was heated programmedly at 8 °C/min. The TPD curve of the ammonia-adsorbed sample was corrected by subtracting the TPD curve of fresh-sample.

The FT-IR spectra were obtained on the Nicolet Nexus 470 FT-IR spectrometer. The self-supported disk (9.8–10.0 mg, φ 20 mm) of sample was placed in the IR adsorption cell and outgassed at 300 °C for 4 h to remove the adsorbed water. After cooling to ambient temperature, the background IR spectrum of sample was recorded. Then the disk outgassed was exposed to a saturated vapor of pyridine for 15 min at ambient temperature. After the disk cell was outgassed at the given temperature, the IR spectrum of pyridine adsorbed on sample was recorded.

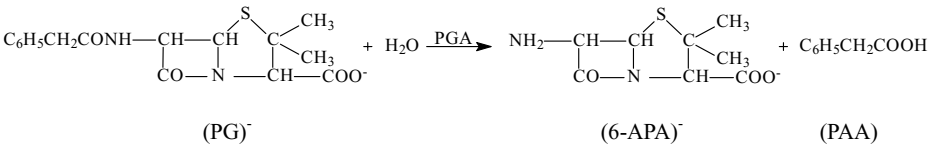
2.2. Enzyme immobilization

Penicillin G acylase (PGA, 804 U/ml) was from the Shanghai Institute of Biochemistry, Academia Sinica. PGA was immobilized on the mesoporous support as follows: 0.1 g support was immersed in 4 ml enzyme solution that was prepared by diluting five times the original enzyme solution with 0.1 mol/l phosphate buffer pH 7.0. The mixture solution above was gently stirred for 12 h at 30 °C. Then the immobilized PGA was filtered and washed with de-ionized water and buffer. The wet immobilized enzyme (IME) was ready for the subsequent activity testing.

2.3. Activity testing

The processes of hydrolyzing penicillin G potassium salt (Huabei Pharmaceutical Group, China) can be described in Fig. 1, in which phenylacetic acid (PAA) is a by-product. The presence of PAA lowers the pH value of the hydrolysis mixture. The activity of PGA can be determined by titrating phenylacetic acid produced with NaOH solution to an initial pH value. Based on the volume of NaOH consumed in the first 10 min, the amount of PAA produced is calculated, which equals the molar amount of 6-APA formed.

The procedure of testing was as follows: the wet IME (or free enzyme), 8 ml de-ionized water and 2.00 ml 0.1 mol/l phosphate buffer (pH 7.8) were mixed homogeneously and kept at 37 °C in the thermostatic bath, and then 10 ml 4% (w/v) aqueous solution of penicillin G potassium salt kept at 37 °C was added. The mixture solution was automatically titrated with 0.0878 mol/l NaOH solution to pH 7.8. The volume of NaOH consumed in the first 10 min was measured. The specific activity of IME $A\,(\mathrm{U/g}) = V_{\mathrm{NaOH}}C_{\mathrm{NaOH}}/10^{-3} \times mt$, in which V_{NaOH} (ml) is the volume of NaOH solution consumed, C_{NaOH} (mol/l) the concentration of NaOH solution, m (g) the dry weight of support and t (min) the reaction time. The coupled yield $Y\,(\%) = (A_0 - A_1)/A_0$, and relative activity $R\,(\%) = A/(A_0 - A_1)$, in which A_0 (U) is the total activity of initial PGA solution,



(PG)$^-$ (6-APA)$^-$ (PAA)

Fig. 1. Hydrolysis of penicillin G potassium salt catalyzed by PGA.

A_1 (U) the total activity remained in solution (including supernatant and washing solutions) and A (U) the activity of immobilized enzyme.

3. Results and discussion

3.1. Characterization of Co-MCM-48 synthesized

The XRD patterns of the MCM-48 and Co-substituted samples (Fig. 2) show sharp diffraction peaks that is a characteristic of mesoporous molecular sieve MCM-48 [8]. The d_{211} values, cubic unit cell parameter (a_0), BET surface area and average pore volume of the samples are listed in Table 1. It shows that the a_0 value of Co-MCM-48 is greater than a_0 of MCM-48, which suggests that some Co^{2+} ions have been incorporated in the framework of molecular sieve and caused an expansion of the unit cell. The curves of pore size distribution in Fig. 3 indicate there are single pores of 2.57–2.61 nm for all the samples. Compared with the MCM-48 sample, the surface area of the Co-MCM-48 sample is smaller, and its pore size minishes slightly.

The FT-IR spectra of the MCM-48 and Co-MCM-48 samples are showed in Figs. 4 and 5. After the MCM-48 and Co-MCM-48 samples were outgassed at 300 °C for 4 h, there are the absorption peaks at 3743 cm^{-1} assigned to Si–OH groups [10], which shows there are a great number of the hydroxyl groups on the surface of MCM-48 and Co-MCM-48.

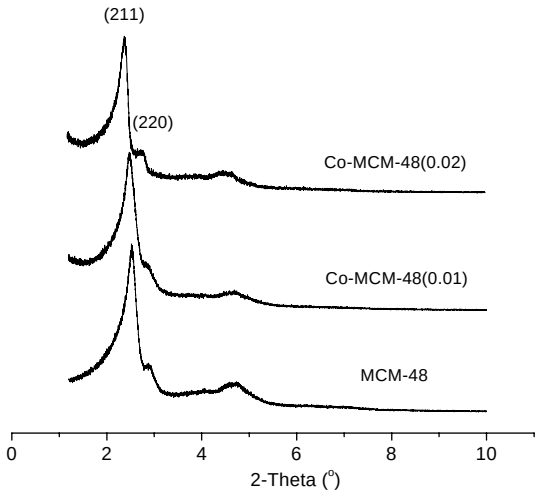


Fig. 2. XRD patterns of MCM-48 and Co-MCM-48.

Table 1
Structural parameters of MCM-48 and Co-MCM-48

Sample	d_{211} (nm)	a_0[a] (nm)	S_{BET} (m^2/g)	Pore volume (cm^3/g)
MCM-48	3.45	8.45	1189	1.40
Co-MCM-48(0.01)	3.56	8.72	1161	1.66
Co-MCM-48(0.02)	3.60	8.82	1002	1.72

[a] $a_0 = d_{hkl}(h^2 + k^2 + l^2)^{1/2}$.

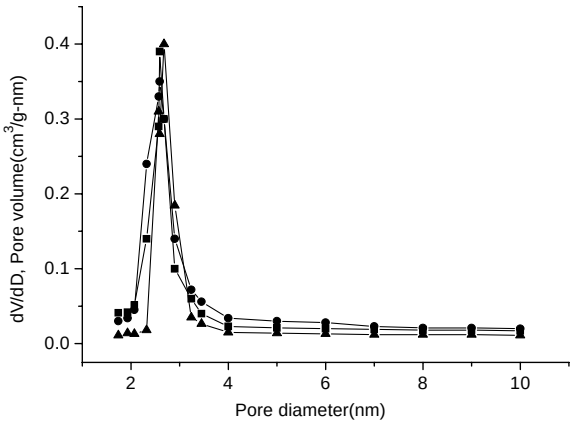


Fig. 3. Pore size distributions of MCM-48 (▲), Co-MCM-48(0.01) (■) and Co-MCM-48(0.02) (●).

After adsorption of pyridine, an intensity of the peak at 3743 cm^{-1} decreased dramatically. With increasing the temperature of degasification, an intensity of peak at 3743 cm^{-1} increases due to part desorption of the pyridine absorbed. This suggests that the Si–OH groups have weak acidity and are able to interact with basic molecules such as pyridine. In the FT-IR spectra of pyridine adsorbed on MCM-48 (Fig. 4), there is one peak at about 1446 cm^{-1}, which corresponds to pyridine interacting with weak Lewis acid sites (weakly acidic Si–OH groups) [11]. The absorption bands at 1544 and 1455 cm^{-1}, assigned to pyridine adsorbed on Brönsted acid sites and strong Lewis acid sites, respectively, do not present in Fig. 4. The results above show that the MCM-48 sample possesses a large amount of weak Lewis acid sites. As the outgassing temperature increased from 100 to 150 °C, an intensity of peak at 1446 cm^{-1} decreased sharply, and as outgassing at 200 °C, this peak disappeared.

The FT-IR spectra of pyridine adsorbed on the Co-MCM-48 samples are shown in Fig. 5. Like the situation of MCM-48, the absorption bands corresponding to Brönsted acid sites and strong Lewis acid sites are not detected on Co-MCM-48. As the outgassing temperature increased from 100 to 150 °C, the absorption peaks are reduced obviously. In the IR spectrum of pyridine-Co-MCM-48 (0.02) (Fig. 5(b)), there is one evident absorption peak at ∼1488 cm^{-1}, that is larger than one of Co-MCM-48 (0.01) and minishes after outgassed at 150 °C. This absorption peak is not found in MCM-48 (Fig. 4) and may be due to an adsorption of pyridine on Co^{2+} or CoO, but this adsorption is weaker.

The peak areas (a.u.) at ∼1446 cm^{-1} in Figs. 4 and 5 are listed in Table 2. The peak areas of Co-MCM-48 are bigger than that of MCM-48, whatever outgassing at 100 or 150 °C. As the molar ratio of Co/Si increases from 0.01 to 0.02, the peak area at ∼1446 cm^{-1} after outgas at 100 °C is almost unchanged, but its peak area after outgas at 150 °C is increased. The strength of acid sites of MCM-48 and Co-MCM-48 is so weak, that the peaks at ∼1446 cm^{-1} of pyridine adsorbed on the acid sites disappear entirely as the ougassing temperature is above 200 °C.

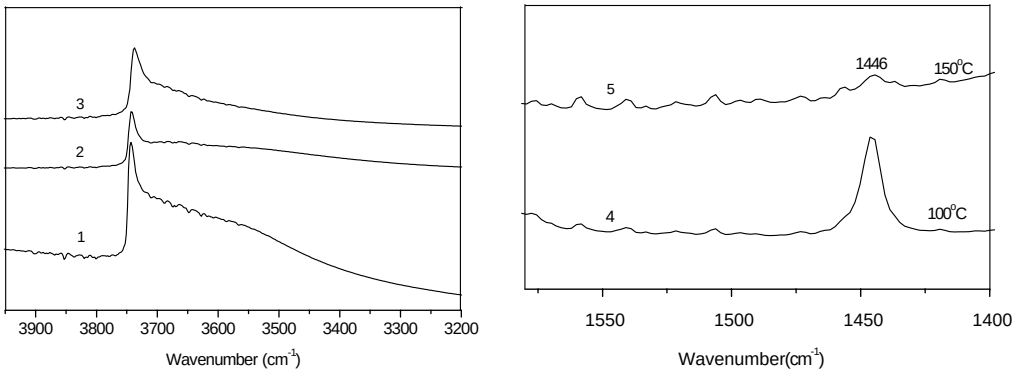


Fig. 4. FT-IR spectra of MCM-48 pretreated by: (1) outgassing at 300 °C for 4 h; (2) adsorbing pyridine and then outgassing at 100 °C for 5 min; (3) after run (2), outgassing at 200 °C for 5 min; (4) adsorbing pyridine and then outgassing and taking spectrum at 100 °C; (5) adsorbing pyridine and then outgassing and taking spectrum at 150 °C.

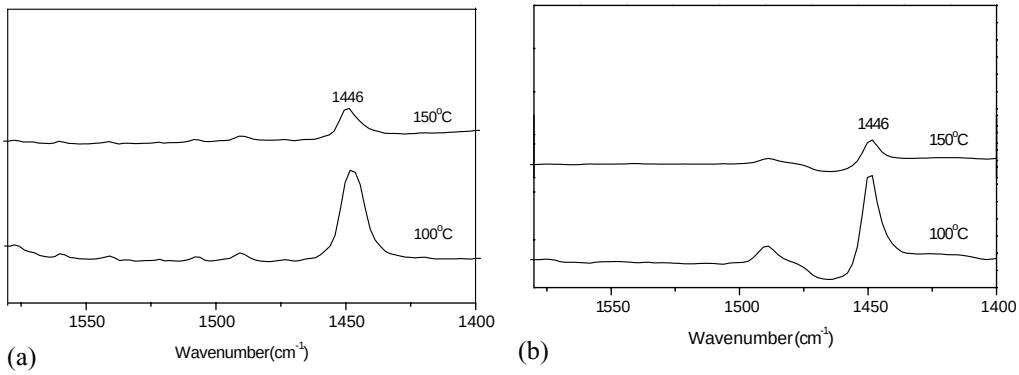


Fig. 5. FT-IR spectra of pyridine adsorbed on Co-MCM-48(0.01) (a) and Co-MCM-48(0.02) (b) after outgassed at 100 or 150 °C.

The acidic properties of MCM-48 and Co-MCM-48 were further investigated using the technique of NH_3-TPD. The TPD profiles of ammonia adsorbed on MCM-48 and Co-MCM-48 are depicted in Fig. 6. The top temperature of desorption peak of NH_3 on Co-MCM-48 is the same as that of MCM-48. But the peak area of NH_3-TPD (the amount of ammonia desorbed) increases visibly when Co^{2+} is incorporated in MCM-48 (Co/Si = 0.01), that is to say, the number of weak acid sites increases. As the ratio of Co/Si in Co-MCM-48 increases from 0.01 to 0.02, the peak area of NH_3-TPD decreases from 273 to 202 (a.u.) (Table 3), corresponding to reducing the number of acid sites. One reason of reduction of the acid sites on Co-MCM-48(0.02) may be relative to its lower specific surface area (Table 1), and another may be there are some CoO in the extra-framework of Co-MCM-48 as the ratio of Co/Si reaches 0.02.

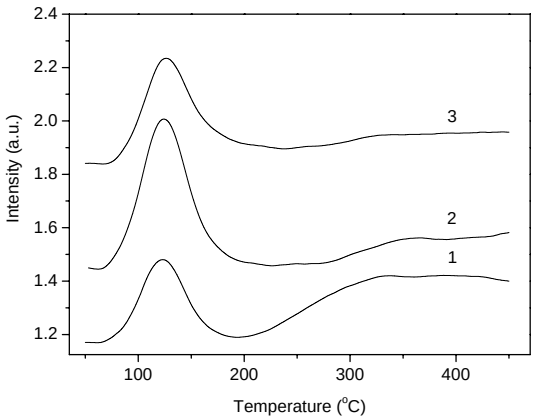


Fig. 6. NH_3-TPD profiles of MCM-48 (1), Co-MCM-48(0.01) (2) and Co-MCM-48(0.02) (3).

Table 2
Peak area at ~1446 cm^{-1}

Sample	Peak area (a.u./g)	
	100 °C[a]	150 °C
MCM-48	105	5
Co-MCM-48(0.01)	120	38
Co-MCM-48(0.02)	117	47

[a] The outgassing temperature.

Table 3
Areas of desorption peak of NH_3-TPD

Sample	*A* (a.u./g)	Temperature of peak (°C)
MCM-48	175	123
Co-MCM-48(0.01)	273	125
Co-MCM-48(0.02)	202	126

Table 4
Influence of PGA concentration on the activity of IME[a]

PGA concentration (V_{enzyme}/V_{buffer})	Coupled yield, Y (%)	Specific activity, A (U/g)	Relative activity, R (%)
1/8	56.9	1245	61.2
1/4	35.9	1509	65.3
1/2	25.0	1545	57.8

[a] Immobilization conditions: 0.1 g dry support, 4 ml enzyme solution (0.1 mol/l phosphate buffer at pH 7.0), at 30 °C for 12 h.

The results above show that the presence of Co^{2+} can help to increase greatly the number of weak Lewis acid sites (Si–OH groups) on MCM-48, and promote ratherish the strength of these acid sites.

3.2. Study of immobilization condition of PGA/MCM-48

The concentration effects of PGA solution on the specific activity of IME are shown in Table 4. With an increase of the PGA concentration (V_{enzyme}/V_{buffer}) in the immobilization solution, the coupled yield decreased and the specific activity of IME rose, and the relative activity reached the maximum at $V_{enzyme}/V_{buffer} = 1/4$.

The influence of immobilization time on the activity of IME is shown in Table 5. With increasing the immobilization time, the coupled yield and specific activity of IME increased gradually and reached the maximal values at nearly 12 h. For the porous support, the rate of enzyme immobilization is relative to the diffusion rate of PGA inside the pores of support. Due to large size of enzyme molecule, a longer immobilization time is in favor of the diffusion of enzyme into the mesoporous pores of MCM-48.

The effect of pH value of medium on the activity of IME is shown in Table 6. The results show that the optimal pH value for an immobilization of PGA on MCM-48 is 6.5–7.0. As its pH value is higher (e.g. >8.0), the specific activity and coupled yield of IME descends rapidly. The studies of pyridine-IR and NH_3-TPD indicate there are an abundance of weakly acidic hydroxyl groups (weak acid sites) on the surface of MCM-48. In an immobilization of PGA, the alkalic medium may supply a large amount of OH^- ions to interact with the weak acid sites on the support surface, leading to the decrease of the weak acid sites. So the alkalic medium is not available for an immobilization of IME on the MCM-48 support.

Table 5
Influence of immobilization time on the activity of IME[a]

Time (h)	Coupled yield, Y (%)	Specific activity, A (U/g)	Relative activity, R (%)
3	16.3	923	88.3
6	27.7	1254	70.3
9	32.6	1409	67.1
12	35.9	1509	65.3
24	36.6	1507	64.1

[a] Immobilization conditions: 0.1 g dry support, 4 ml enzyme solution (0.1 mol/l phosphate buffer at pH 7.0), $V_{enzyme}/V_{buffer} = 1/4$, at 30 °C.

Table 6
Influence of pH value of medium on the activity of IME[a]

pH	Coupled yield, Y (%)	Specific activity, A (U/g)	Relative activity, R (%)
6.0	36.4	1498	64.0
6.6	36.3	1505	64.5
7.0	35.9	1509	65.3
7.4	34.7	1495	67.0
8.0	30.2	1320	68.0
8.5	23.4	1054	70.1
9.1	15.1	753	77.4

[a] Immobilization conditions: 0.1 g dry support, 4 ml enzyme solution (0.1 mol/l phosphate buffer), $V_{enzyme}/V_{buffer} = 1/4$, at 30 °C for 12 h.

3.3. Activity and stability of PGA/Co-MCM-48

The activities of PGA immobilized on Co-MCM-48 (PGA/Co-MCM-48) for hydrolysis of penicillin G to 6-APA at 37 °C are shown in Fig. 7. The presence of Co in MCM-48 can vary the specific activity of IME, but the activity change of IME is not direct correlation with the surface area and pore size of the supports, e.g. the surface area of MCM-48 is the highest, but its activity is low. The results in Fig. 7 show that, with a change of the ratio of Co/Si in Co-MCM-48, the specific activity of IME changes in the same changing trend of the area of NH_3 desorbed. Co-MCM-48(0.01) has a maximum amount of weak acid sites and PGA immobilized has the highest specific activity (1682 U/g). This suggests that PGA is immobilized on the support by PGA interacting with the weakly acidic hydroxyl groups on the MCM-48 surface, and the activity of IME is relative with the amount of weak acid sites on the surface of support.

In order to investigate an interaction between hydroxyl groups on the surface of MCM-48 and PGA molecules, the IR spectra of MCM-48 and PGA/MCM-48 after outgassing at 300 °C have been measured. As shown in Fig. 8, an intensity of the absorption peak at 3743 cm^{-1}, that is attributed to weakly acidic Si–OH groups decreased largely after immobilization of PGA. The PGA molecules are immobilized on the surface of MCM-48 through the amino groups of enzyme interacting with the weak acidic Si–OH groups. Based on the fact that the absorption peak at 3743 cm^{-1} has not completely disappeared after immobilizing PGA (Fig. 8(2)), that is, not all hydroxyl groups bond with the PGA enzymes. Because of the space obstacle, some of hydroxyl groups inside pores are not accessed by PGA.

The high activity of PGA/MCM-48 shows that the weakly acidic sites of MCM-48 can offer the suitable microenviron-

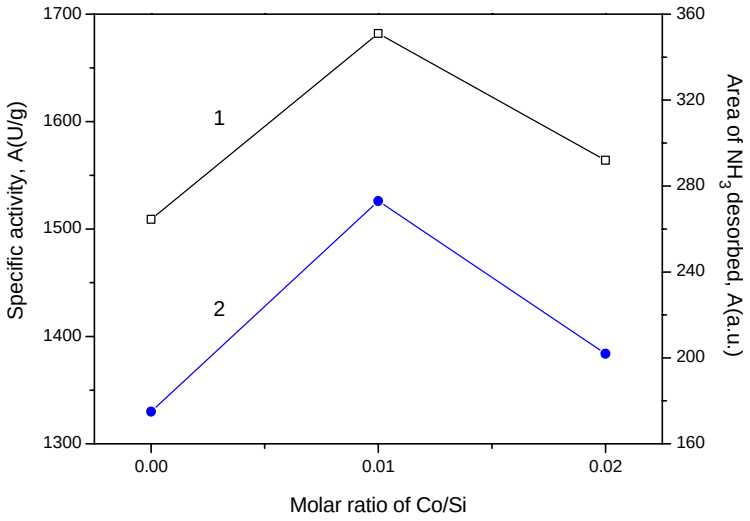


Fig. 7. The specific activity (1) and area of NH_3 desorbed (2) of PGA immobilized on Co-MCM-48 vs. the molar ratio of Co/Si.

ment for an immobilization of penicillin G acylase. Suitable amount of Co^{2+} incorporated into the framework instead of Si^{4+} can create charge defects that can be converted to acid sites [12], leading to the increase of the acid sites.

In order to understand the effect of Co^{2+} in framework of MCM-48, CoO/MCM-48 (0.01) was prepared by impregnation, and then PGA was immobilized on CoO/MCM-48 (0.01). The studies show that the performance of PGA/Co-MCM-48 (0.01) (1682 U/g) for the hydrolysis is higher than PGA/CoO/MCM-48 (0.01) (1502 U/g), which is similar to the performance of PGA/MCM-48 (1509 U/g). This result shows that the role of framework Co^{2+} in Co-MCM-48 is different from that of extra-framework Co^{2+} in CoO/MCM-48. The CoO_4 tetrahedron in MCM-48 has probably suitably electric and geometric structures to enhance the amount of hydroxyl groups and the activity of IME on MCM-48.

The operation stability of IME on Co-MCM-48 for the hydrolysis of penicillin G was tested in 100 ml reactor at 37 °C. 0.1 g IME (dry weight of support) and 4% (w/v) concentration of penicillin G potassium salt were used. IME used in the hydrolysis was washed with deionized water and then used repeatedly. The results in Fig. 9 show that the activity of IME on Co-MCM-48 (0.01) reduced from 1682 to 1375 U/g (about 81.7% its initial activity) after used for six cycles. For IME on Co-MCM-48(0.02) or MCM-48, the similar results were obtained, their activities kept 84.4 and 83.5% of the initial activity after used for six cycles, respectively.

The effect of usage time on the activity of IME is shown in Fig. 9. The activities of IME on MCM-48 or Co-MCM-48 are higher than that of PGA/MCM-41. The initial activity of PGA/MCM-48 is about 3.7 times more than that of PGA/MCM-41. The initial activity of PGA/MCM-41 prepared and PGA/MCM-41 reported [7] is 402 and 364 U/g, and after used for six cycles their activities decrease to 222 and 249 U/g, respectively. The specific surface areas of the MCM-41 samples are 953 and 798 m^2/g [7], respectively, which are slightly smaller than that of MCM-48. The pore size of MCM-41 prepared is approximately the same

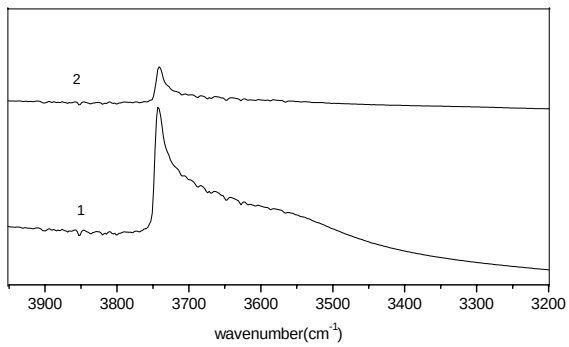


Fig. 8. IR spectra of MCM-48 (1) and PGA/MCM-48 (2) after outgassing at 300 °C.

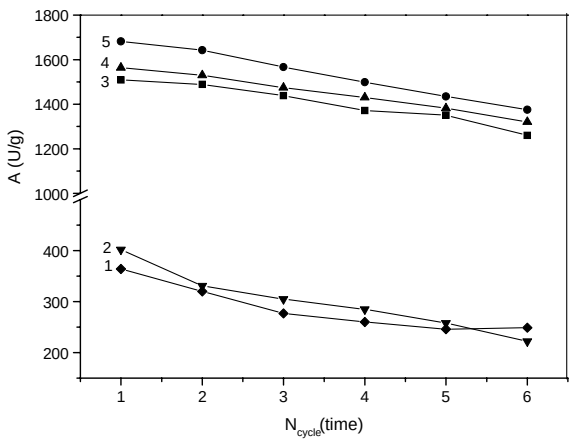


Fig. 9. The activity of IME on MCM-41 [7] (1), MCM-41 (2), MCM-48(3), Co-MCM-48(0.02) (4) and Co-MCM-48(0.01) (5) vs. the time of run repeatedly.

(2.61–2.73 nm) as that of MCM-48. Apparently, the structural parameters of support, such as the specific surface area and pore size, can explain hardly the great difference in the activity and operation stability between IME on MCM-48 and MCM-41.

Different from hexahedral MCM-41 with one-dimensional pore system, the cubic MCM-48 having three-dimensional pore systems can be accessible easily from all sides and is available for transmission of enzyme or reactants. As a support of penicillin G acylase, MCM-48 is more suitable than MCM-41 and in favor of the PGA enzyme loading and the substrate more rapidly accessing to the enzyme immobilized on the internal surface, which increases the reaction rate. So the higher activity of IME on MCM-48 should be attributed to the 3D pore system. On the other hand, during the enzymatic hydrolysis of penicillin G in addition to the product 6-APA the phenyl acetic acid has released concomitantly, which leads to an appearance of the pH gradient between inside pores and the reaction bulk. For the PGA/MCM-41 catalysis system, the concentration of the phenyl acetic acid may be very high in a surrounding microenvironment of an immobilized enzyme, thanks to the poor diffusivity of one-dimensional pore system, which induces deactivation of part immobilized enzyme or a diminish of the rate of reaction catalyzed by IME. This is a main reason that the MCM-48 molecular sieve is superior to MCM-41 as a novel support for the immobilization of penicillin G acylase.

4. Conclusions

Mesoporous MCM-48 and Co-MCM-48 molecular sieves possessing three-dimensional pore system were synthesized. It was found that MCM-48 molecular sieve is an effective support for the immobilization of penicillin G acylase. The PGA enzyme molecule can be immobilized on the surface of the MCM-48 molecular sieve by interaction with the weakly acidic silanol groups on the surface. The PGA immobilized on MCM-48 has the high catalytic activity and operation stability for the hydrolysis of penicillin G and its activity was further improved by cobalt incorporating into the silica framework of MCM-48. When PGA immobilized on Co-MCM-48 (Co/Si = 0.01), its specific activity can reach 1682 U/g. The enzyme immobilized on the MCM-48 support shows much higher activity than that on MCM-41. The reason is attributed to the 3D pore system in MCM-48, which is favor of transmission of the enzyme, substrate and products inside the pore channels.

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