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Journal of Molecular Catalysis B: Enzymatic 42 (2006) 14–19
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Hollow silica nanotubes for immobilization of penicillin G acylase enzyme

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Received 15 February 2006; received in revised form 17 May 2006; accepted 31 May 2006
Available online 18 July 2006

Abstract

In this study, porous hollow silica nanotubes (PHSNTs) synthesized via a sol–gel route using nano-sized needle-like $CaCO_3$ the inorganic templates were employed as a support for immobilization of penicillin G acylase (PGA) biocatalyst. The produced PHSNTs were characterized by BET and transmission electron microscopy (TEM). Effect of various factors such as loading temperature and ratio of carries to free PGA (g/mL) on the catalytic activity of the immobilized PGA was also investigated by unitary factor testing method. The results show that under optimized conditions the relative loading amount and the total activity yield of immobilized enzyme (IME) amounts to 97.20% and 88.80%, respectively. Several advantages, i.e. the rapid immobilization of PGA onto PHSNTs, the high tolerability to the pH, the less sensitivity to the temperature and the improved storage stability render PHSNTs potential support materials for enzyme immobilization.

Keywords: Enzyme immobilization; Hollow silica nanotubes; Needle-like $CaCO_3$; Penicillin G acylase

1. Introduction

Penicillin G acylase (PGA) with molecular dimensions of 70 Å × 50 Å × 55 Å can catalyze the cleavage of the amide bond in the benzylpenicillin (penicillin G) side-chain specifically and release phenylacetic acid and 6-aminopenicillianic acid (6-APA), among which 6-APA is an important precursor for the synthesis of many semi-synthetic penicillin antibiotics [1–4]. Nevertheless, isolated enzymes in biocatalytic processes suffer from some drawbacks, i.e. weak enzyme stability under operational conditions as well as difficult recovery and reuse cycles [5–7]. Therefore, more efforts have been devoted to the development of carriers to immobilize this kind of industrially valuable biocatalyst [8,13].

To improve catalytic efficiency of PGA, various matrices such as inorganic and organic materials have been studied for the immobilization of the enzyme catalyst [8–11]. Singh et al. used agar-polyacrylamide resins to immobilize PGA from *E. coli* NCIM 2563 [12]. Bianchi et al. described a new procedure for the immobilization of an industrial PGA by covalent coupling on the poly (enthacrylic ester) resin [13]. Danica Mislovičová et al. utilized concanavalin A-bead cellulose to immobilize of PGA [14]. Compared with organic supports, inorganic supports are more stable and against from organic solvents and microbial attacks [15]. However, limited pore size of the conventional inorganic materials, for example, Al_2O_3, SiO_2, microporous zeolites, and mesoporous molecular sieves of the MCM-41s family, makes the bulky enzymes and substrates difficult to diffuse into the narrow channel of supports [16–18]. In this paper, we choose porous hollow silica nanotubes (PHSNTs) synthesized via a sol–gel route using needle-like $CaCO_3$ nanoparticles as a support to immobilize the PGA biocatalyst. The structure of PHSNTs and the loading kinetics of PGA onto PHSNTs were investigated in more detail. Also, the activity and stability of immobilized PGA were discussed.

2. Experimental

2.1. Materials

Needle-like $CaCO_3$ templates were prepared by a unique high gravity reactive precipitation (HGRP) technology accord-

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doi:10.1016/j.molcatb.2006.05.011

ing to the method previously reported by us [19–21]. PGA was purchased from Hiader Company, Zhejiang, China. All chemicals used in the experiments were obtained from commercial sources as analytical reagents without further purification. Distilled water was used throughout the study.

2.2. Preparation of PHSNTs

PHSNTs were synthesized via a sol–gel route as follows: 10 g of needle-like $CaCO_3$ nanoparticles were added to 200 mL of diluted ethanol solution ($V_{ethanol}/V = 3:2$). The resulting suspension solution was dispersed by a KQ-100 ultrasonic device with a power of 100 W. Fifty milliliters of ammonia solution (25%) and 2.2 g of cetyltrimethylammonium bromide were added into the $CaCO_3$ suspension solution at room temperature under vigorous stirring, followed by the addition of tetraethylorthosilicate. Afterwards, the slurry was further stirred, filtered, rinsed, dried and calcinated to obtain a core-shell structured composite containing $CaCO_3$ and SiO_2. The composite was then dissolved into HCl (10 wt.%) to remove the $CaCO_3$ template completely. The resulting gel was again filtered, rinsed and dried to obtain PHSNTs.

2.3. Enzyme immobilization

PGA was immobilized on the PHSNTs as follows: 1 mL of enzyme solution (800 U/mL) and a certain weight (0.1–1.0 g) of support were immersed in 50 mL of distilled water. The mixture of solution was stirred and rinsed with distilled water. The wet immobilized enzyme (IME) was in stock for subsequent enzyme activity assay.

2.4. Enzyme activity assay

Enzymatic catalytic activity of the immobilized biocatalysts was determined by titrating phenylacetic acid. The process of enzyme activity assay was as follows: 100 mL of 2% (w/v) solution of penicillin G potassium was kept at 28 °C in the thermostatic bath and titrated with 0.25 mol/L NaOH solution to pH 8.0. Then wet IME (or free enzyme) was immersed in the solution above. The mixture of solution was automatically titrated with 0.25 mol/L NaOH solution at constant pH 8.0. The specific activity of enzyme was measured according to the following equation: IME $A\ (U/g) = V_{NaOH}C_{NaOH} \times 10^3/Wt$, in which V_{NaOH} (mL) is the volume of NaOH solution consumed, C_{NaOH} (mol/L) is the concentration of NaOH solution, W (g) is the weight of immobilized PGA, t (min) is the reaction time and the unit of U is μmol/min. Unless otherwise noted, the total relative activity is the ratio of the activity of immobilized PGA on PHSNTs to the total activity of initial PGA solution.

2.5. Measurement of enzyme loading kinetics of PGA on the supports

The loading kinetics of PGA on the supports was measured as follows: 1 mL of PGA solution was first diluted by 49 mL of distilled water and then a certain weight of support was added in the diluted solution under vigorous stirring. The 200 μL of the supernatant liquid was taken out at the given time intervals and then subjected to Bradford assay [22,23]. Spectrophotometer operated at a wavelength of 595 nm was used to determine the concentration of enzyme from the supernatant liquid.

Transmission electron microscopy (TEM) was performed by a JEM-2010F. The concentration of PGA solution was detected by a Shimadzu UV 2501 spectrometer at a wavelength of 595 nm. An ASAP 2010 surface area analyzer was used to determine BET surface area (S_{BET}) and pore size (D_{pore}) distribution of the PHSNTs samples.

3. Results and discussion

3.1. Characterization of the PHSNTs

Fig. 1 shows TEM images of $CaCO_3$ templates with needle-like structure and the PHSNTs prepared by using $CaCO_3$ as

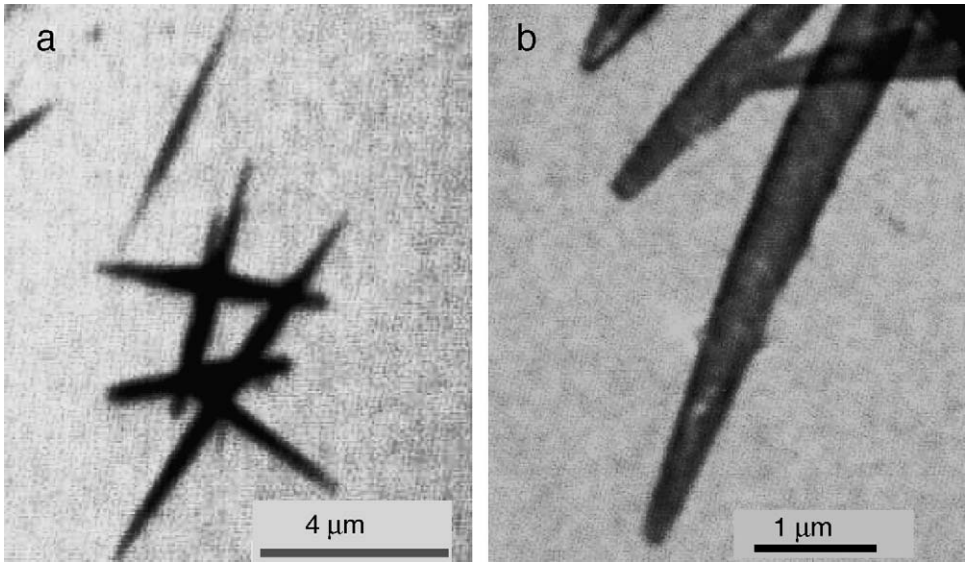

Fig. 1. TEM images of needle-like $CaCO_3$ templates (a) and porous hollow silica nanotubes (b).

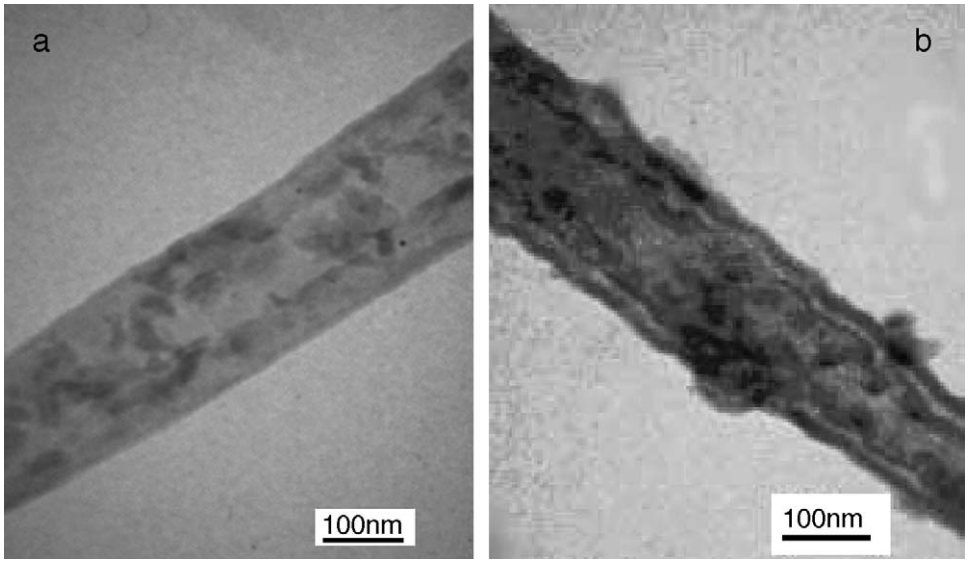

Fig. 2. TEM images of porous hollow silica nanotubes before (a) and after loading PGA (b).

templates. It can be seen that the as-synthesized PHSNTs possess hollow tubular structure with length of 2–5 μm and diameter of 120–200 nm, which is consistent with the report by our group [20]. The S_{BET} and D_{pore} distribution of the PHSNTs samples were determined by an ASAP 2010 surface area analyzer. The results from the N_2 adsorption–desorption isotherms indicate that the S_{BET}, D_{pore} of PHSNTs are 487.2 m^2/g and 107.9 Å, respectively.

Fig. 2 shows the TEM images of single porous hollow silica nanotube before (a) and after loading PGA (b). Obviously, the porous hollow silica nanotube before loading PGA was clearer and smoother, while the porous hollow silica nanotube after loading PGA became darker and rougher. This indicates the successful loading of PGA onto the supports.

3.2. Enzyme loading kinetics of PGA on the supports

The Bradford protein assay is one of several methods commonly used to determine the total protein concentration of a sample. The method is based on the proportional binding of the dye Coomassie to proteins. Coomassie absorbs at 595 nm. The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay. Though, determining the protein concentration of test samples the kinetics of PGA loading on the supports was shown in Fig. 3. From the figure, it can be seen that the adsorption equilibrium time of the enzyme on PHSNTs is *ca*. 120 min, which is far faster than those previously reported supports such as pure-silica SBA-15 with a pore size of about 90 Å (5 h) [23], mesoporous MCM-41 materials with a pore size of 40 Å (24 h) [24] and poly (vinyl acetate-co-diviyl benzene) (48 h) [25]. Possible reasons for the rapid adsorption of PGA on PHSNTs are a relatively facile diffusion process of enzyme PGA with a molecular size of 70 Å × 50 Å × 55 Å into the PHSNTs with a pore size of 107.9 Å, and unique big entrances (diameter of about 120–200 nm) at the two ends as well. The result shows that the initial concentration of enzyme is 25 mg/mL and that the balanceable concentration of enzyme is 0.7 mg/mL when the ratio of carries to enzyme is 0.50. Based on the data above, the percentage of PGA uptake on the supports amounted to 97.20% accordingly.

3.3. Effect of the various factors on the PGA loading process

3.3.1. Effect of the ratio of carries to free PGA

Fig. 4 shows the effect of the ratio of carries to enzyme on the PGA loading process. It was found that the total relative activity increased from 33.50% to 88.80% with increasing the amount of carries from 0.05 g to 0.55 g (volume of enzyme solution, 1 mL) and then kept unchanged with further increasing the amount of carriers, while the specific activity of IME (*U*/*g*) reduced towards an adverse trend with the increase of the amount of carriers.

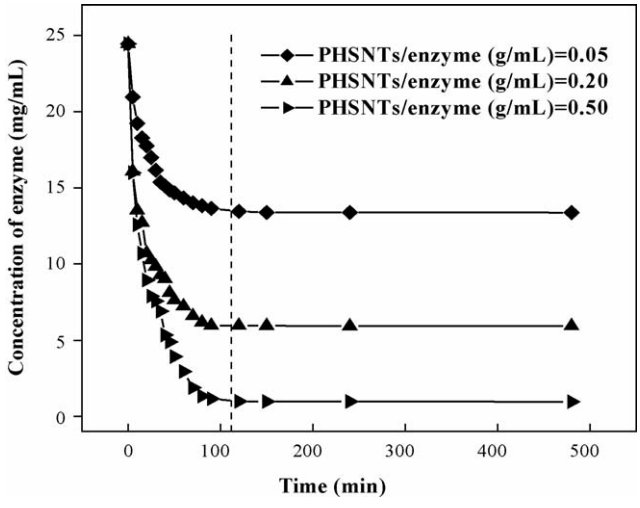

Fig. 3. The enzyme loading kinetics of PGA on the supports.

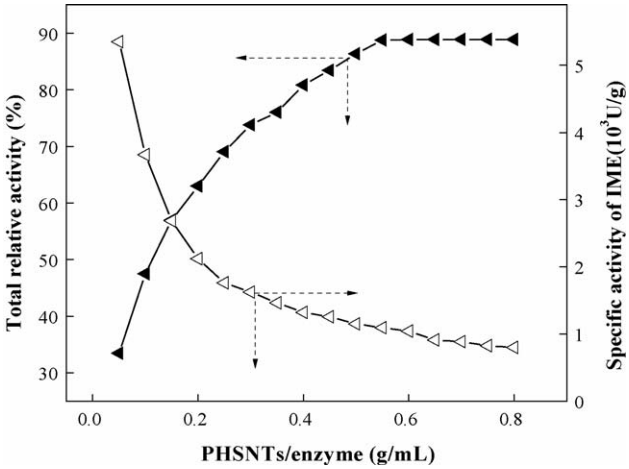

Fig. 4. The effect of the ratio of carries to enzyme on the PGA loading process.

From Fig. 4 the optimum ratio of carries to enzyme should be the point of intersection between the two curves: namely about 0.15, while taking a higher total relative activity into account here, a ratio of 0.5 was selected to prepare immobilized PGA and also used in subsequent experiments.

3.3.2. Effect of the loading temperature

Fig. 5 shows the effect of the different temperature on the PGA loading at pH 7.0 when the ratio of carries to the free PGA is 0.5. It was found that the total relative activity increased with the increase of loading temperature ranging from 4 °C to 12 °C, and then decreased with further increase of temperature ranging from 12 °C to 50 °C. The optimum temperature of the PGA loading on PHSNTs was about 12 °C. Possible reasons are that the adsorption amount of enzyme on PHSNTs at a lower temperature, e.g. 4 °C is poor and increases gradually with the increase of temperature ranging from 4 °C to 12 °C; while at higher temperature (over 12 °C upward) the enzyme activity loses partly or completely owing to the acceleration of the outspreading and the conformation change of enzyme molecules [26,27], hence leading to the decrease in the total relative activity.

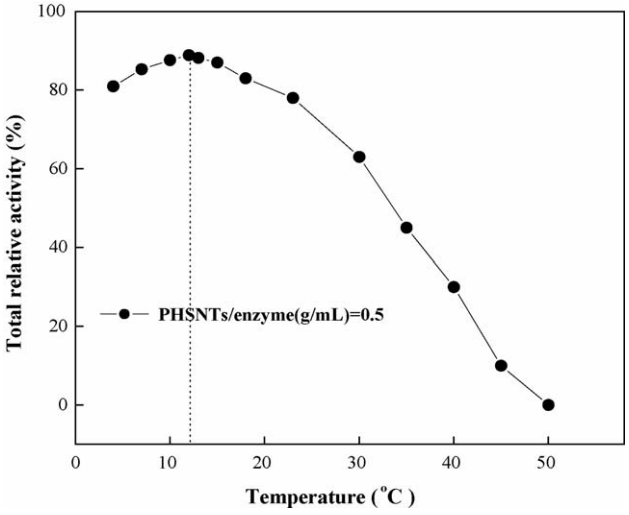

Fig. 5. The effect of the loading temperature on the PGA loading process.

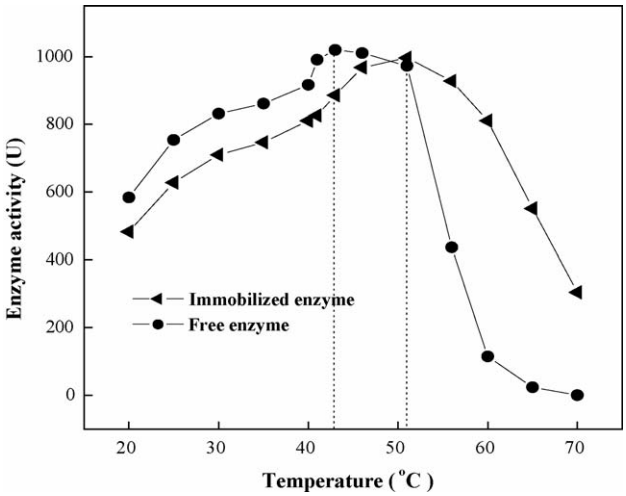

Fig. 6. The effect of temperature on the activity of immobilized PAG.

3.4. Biochemical characterization of immobilized PGA

3.4.1. Effect of temperature on the activity of immobilized PGA

The dependency of enzyme activity on the temperature is shown in Fig. 6. At low temperature level, the enzyme activity of both the free PGA and immobilized PGA increased with the increase of temperature and the enzyme activity of the free PGA was higher than that of the immobilized PGA. This may be explained by considering that the molecular diffusion resistance and the conformation change of enzyme immobilized on the supports cause the decrease of reaction rate of the immobilized PGA. At high temperature level, the enzyme activity of both the free PGA and immobilized PGA decreased sharply with the increase of temperature. Possible explanation is that with the increase of temperature the enzyme molecules outspreaded acutely and ultimately lost their biocatalytic activity. Also, it was observed that for the immobilized PGA the optimum activity temperature was 43 °C and for the free PGA the optimum activity temperature was 51 °C. This means that the immobilized enzyme has higher resistance to the change of temperature than the free enzyme.

3.4.2. Effect of pH on the activity of immobilized PGA

The activities of both free and immobilized PGA were determined at different solutions with pH ranging from 4 °C to 11 °C at 45 °C. The immobilized PGA had the highest activity at pH 8.5 and the maximum activity for the catalysis of free PGA was found at pH 9.0, which is close to those reported by the other investigators [25,26]. It is well known that the adsorption of enzyme on a support can cause significant changes in the catalytic behavior of the enzyme. The pH optimum value of an immobilized enzyme is shifted to a higher or lower pH, which depends on surface charges of the supports [28]. Thus, the enhancement of net positive charges on the surface of immobilized PGA particles may lead to the shift from alkaline pH to neutral pH. It can also be seen that the activity range of the immobilized PGA was broader than that of the free PGA (Fig. 7),

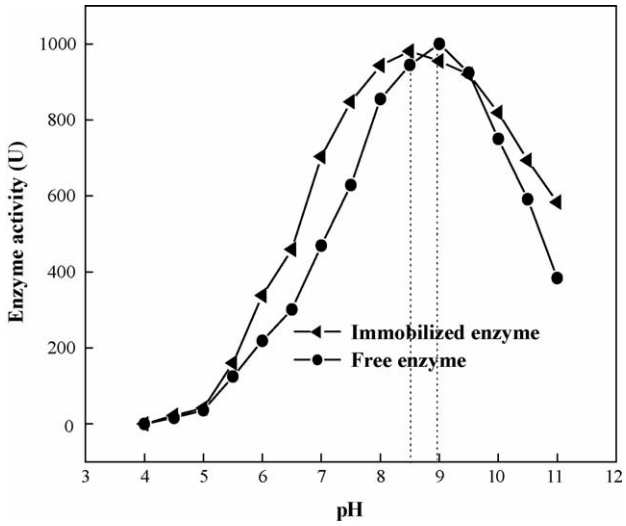

Fig. 7. The effect of pH on the activity of immobilized PGA.

indicating the increase of the enzyme tolerability to the pH variance in surrounding.

3.4.3. Thermal stability of immobilized PGA

The thermal stability of both free PGA and immobilized PGA was investigated by storing samples for 4 h at different temperatures, as shown in Fig. 8. The activity of free PGA decreased slowly with increasing storage temperature from 30 °C to 40 °C, and then decreased sharply with further increasing temperature up to 50 °C. In contrast, no significant decrease for the activity of the immobilized PGA was observed even if when the storage temperature reached 50 °C. Thus, it can be concluded that PGA immobilized on PHSNTs is more thermally stable than the free PGA.

3.4.4. Storage stability of immobilized PGA

Fig. 9 shows the storage stability of the free and immobilized PGA in the dark at 4 °C. Obviously, the activity of the immobilized PGA decreases slower than that of the free PGA. The immobilized PGA still retains about 82% of its original activity after 100 days, and the free PGA, however, can only maintain less than 66% of its original activity. Thus, the immobilized PGA exhibits higher storage stability than the free PGA.

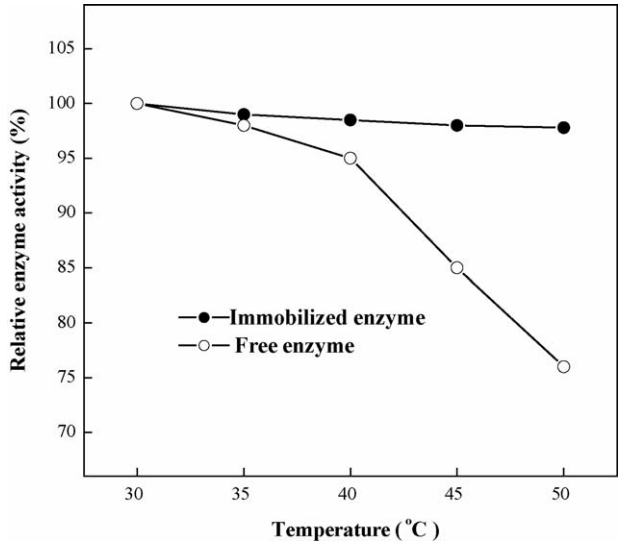

Fig. 8. The effect of storage temperature on the activity of immobilized PGA.

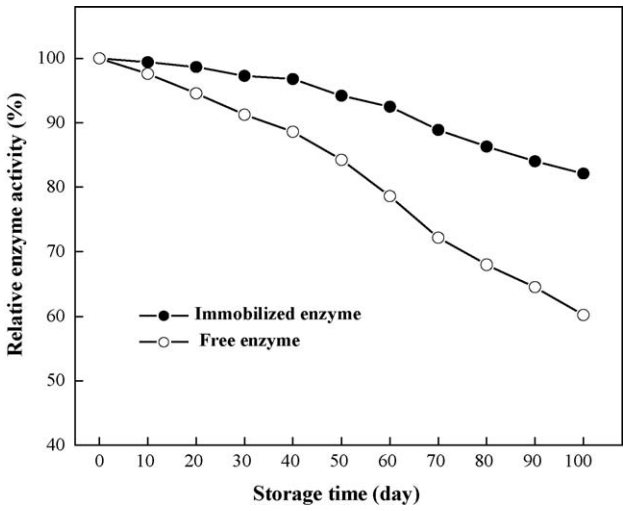

Fig. 9. The effect of storage time on the activity of immobilized PGA.

4. Conclusions

Porous hollow silica nanotubes have been successfully prepared by means of using needle-like $CaCO_3$ templates via a sol–gel route. The resulting PHSNTs can be as carriers to immobilize penicillin G acylase biocatalyst at a rapid rate. The immobilized PGA exhibits higher resistance to the change of temperature and stronger tolerability to the pH variance in surrounding. We expect the PHSNTs can be applicable as carriers for more versatile enzyme immobilization and macromolecule protection.

Acknowledgement

Financial support from NSF of China (no. 20325621) is acknowledged.

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