Journal of Molecular Catalysis B: Enzymatic 97 (2013) 303–310

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Immobilization of β-glucosidase on mercaptopropyl-functionalized mesoporous titanium dioxide☆

Ce Wei[a,b], Qingshan Lu[a], Jia Ouyang[a], Qiang Yong[a], Shiyuan Yu[a,*]

[a] Key Laboratory of Forest Genetics and Biotechnology, Ministry of Education, Nanjing Forestry University, Nanjing 210037, China
[b] College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 211816, China

ARTICLE INFO

Article history:
Received 27 January 2013
Received in revised form 21 May 2013
Accepted 8 July 2013
Available online 15 July 2013

Keywords:
β-Glucosidase
Immobilization
Mesoporous titanium dioxide
Mercaptopropyl group
Enzymatic hydrolysis

ABSTRACT

Mesoporous titanium dioxide (M-TiO_2) and mercaptopropyl-functionalized M-TiO_2 (SH-M-TiO_2) were prepared as carriers for immobilization of β-glucosidase (BG). Analyses suggested that in SH-M-TiO_2 the grafted mercaptopropyl groups attributed to about 3.5% of the total weight of the matrices. For the immobilization of BG, SH-M-TiO_2 or M-TiO_2 powder was added with free BG solution and stirred at 4 °C for 8 h, followed by centrifugation and washing. BG was effectively immobilized on SH-M-TiO_2 with an enzyme activity of 10.9 U/g, which was 92.8% of the total enzyme activity in the starting solution of free BG. BG immobilized on SH-M-TiO_2 (BG-SH-M-TiO_2) was more stable in pH, thermal, and storage tests than BG on M-TiO_2 or free BG in solution. The K_m and v_{max} of BG-SH-M-TiO_2 were similar to values for free BG in solution. Batch hydrolysis of cellobiose-containing substrate by BG-SH-M-TiO_2 was conducted. Conversion rates of cellobiose in 10 batches were consistently around 90%. Although the residual enzyme activity of BG-SH-M-TiO_2 decreased gradually, only a small amount of BG activity was detected in supernatants. This indicated effective adsorption of enzyme molecules to SH-M-TiO_2 matrices.

1. Introduction

The enzyme β-glucosidase (BG) is ubiquitous across a broad range of eukaryotic and prokaryotic species [1,2]. It exhibits wide substrate specificity and is capable of cleaving the β-glucosidic linkages of conjugated glucosides and some disaccharides [3]. The substrates of BG include oligosaccharides such as cellotetraose and cellopentaose [4] as well as cellooligosaccharides. BG also releases glucose from the disaccharide cellobiose [5] and hydrolyze anthocyanins, the main coloring agents in vegetables [6,7]. BG is used in the food industry to improve the organoleptic qualities of wine and fruit juices [8].

Extensive production of renewable biofuels and high-value organic solvents through the enzymatic hydrolysis of lignocellulosic wastes is possible. The hydrolysis of lignocellulose requires the cooperative action of exocellulase, endocellulase and BG, ultimately producing the fermentable monosaccharide glucose, which is the major substrate for the production of fuel ethanol, isopropanol and acetone [9]. However, BG is inhibited through a competitive feedback mechanism by high concentrations of the disaccharide cellobiose and glucose [10]. This substrate inhibition can be alleviated by elevating the levels of BG in bioreactors [11].

Titanium dioxide (TiO_2) is an inexpensive, safe and highly stable nanocrystalline material that is resistant to corrosion in oxidizing solutions. This material can be used as photo-catalyst, anti-viral agent, deodorant, and self-cleaning glass [12,13]. TiO_2 nanotubes or nanoparticles are mesoporous, with pores diameter of 10 to 100 nm. The morphology and porosity of TiO_2 materials, specifically the density, size and distribution of the micropores, can be controlled during fabrication [14,15]. Mesoporous TiO_2 (M-TiO_2) forms chemically and thermally robust, highly crystalline particulate materials with a high surface-to-volume ratio. These properties make M-TiO_2 an ideal matrix for the immobilization or attachment of enzymes in nanoscale enzymatic bioreactors. Enzyme immobilization on M-TiO_2 is thought to occur via simple adsorption of proteins to the material surface or via covalent attachment, electrostatic binding or encapsulation [16,17]. The immobilization of enzymes on M-TiO_2 has the potential to improve stability, storage and reuse of enzymes in a number of bioreactor applications [18,19]. M-TiO_2 is a useful support matrix for attachment of BG enzymes because the oxide layer has a negative charge at physiological pH, allowing quick and stable attachment of BG [20] without compromising activity or reaction specificity. Most mesoporous silica materials, such as SBAs, MCM-41 and HMS, were prepared by "template-growth" process, which is cost prohibitive and is unlikely to be utilized on an industrial scale [21]. In contrast,

* Corresponding author. Tel.: +86 25 85427623; fax: +86 25 85424121.
E-mail address: syu@njfu.edu.cn (S. Yu).

1381-1177/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2013.07.005

M-TiO_2 in this work was prepared through a simple synthesis route at considerably lower cost that might be suitable for commercial enzyme immobilization. [22].

Mesoporous materials are often modified with functional groups that enhance the capacity for stable attachment of large amounts of enzyme with activity and specificity that is similar to or better than soluble enzyme. Bai *et al.* [23] reported the immobilization of lipase on mesoporous silica nanotubes and aminopropyl-grafted mesoporous silica nanotubes. The hydrolysis activity of the lipase immobilized on aminopropyl-grafted mesoporous silica nanotubes was almost twice of the activity of the enzyme on mesoporous silica nanotubes. Yiu *et al.* [24] modified SBA-15 with thiol, chloride, amine, and carboxylic acid. Trypsin on thiol-functionalized SBA-15 was found to be the most promising. However, no information on the use of functionalized M-TiO_2 for enzyme immobilization could be retrieved.

This work investigated M-TiO_2 functionalized with mercaptopropyl groups by post-synthesis grafting. The functionalized product SH-M-TiO_2 was used as a carrier for BG immobilization. The structural properties of SH-M-TiO_2 were characterized by several methods. BG immobilized on SH-M-TiO_2 was evaluated for optimum reaction pH and temperature; thermal, pH, and storage stability; reusability; kinetics; and repeated enzymatic hydrolysis.

2. Materials and methods

2.1. Enzyme and carriers

β-Glucosidase (Novozyme® 188, from *Aspergillus niger*) was from Sigma-Aldrich and stored at 2–8 °C.

M-TiO_2 was provided by Lu [25,26] and prepared as follows: A mixture with a TiO_2/K_2O molar value of 1.9 was prepared by adding reagent grade K_2CO_3 to $TiO_2 \cdot nH_2O$ and sintering at 810 °C for 2 h. The sintered product was wet-ground and dried at 60 °C and 10 g was soaked in 7 ml of distilled water at ambient temperature in a closed container for 7 days, during which the potassium-rich nanophase formed. When the product was completely transformed to amorphous phase, it was suspended in 100 ml of vigorously stirred 0.1 M HCl solution to remove K^+ ions. The product was separated by filtration and washed with distilled water, followed by desiccation at 60 °C under vacuum. Calcinations of the dried titanium sample were in a muffle oven at elevated temperature for 2 h. M-TiO_2 with an average pore size of about 20 nm was obtained by adjusting calcination temperature.

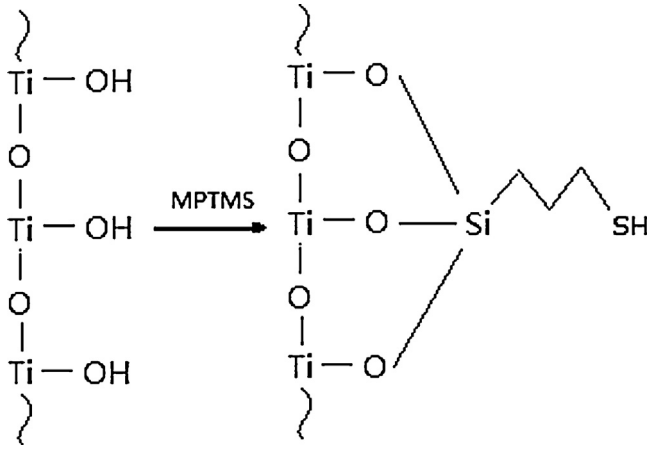

Fig. 1. Formation of mercaptopropyl-functionalized M-TiO_2 (SH-M-TiO_2). MPTMS, 3-mercaptopropyl trimethoxysilane.

SH-M-TiO_2 was obtained as shown in Fig. 1. M-TiO_2 powder (1 g) was dispersed in 50 ml toluene, then 5 ml (3-mercaptopropyl) trimethoxysilane (MPTMS, 175617, Sigma-Aldrich) was added. The mixture was heated under reflux at 110 °C for 8 h. After filtration, the powder was washed with ethanol and dried.

2.2. Structural characteristics of M-TiO_2 and SH-M-TiO_2

Nitrogen absorption and desorption isotherms of M-TiO_2 and SH-M-TiO_2 were obtained (ASAP 2020M Micromeritics, Atlanta, USA) using liquid nitrogen. Specific surface areas were calculated by the Brunauer–Emmett–Teller (BET) method with data in a relative pressure range of $P/P_0 = 0.1–1.0$. Pore size distributions were determined by analyzing absorption branches with the Barrett–Joyner–Halenda (BJH) method [26,27].

M-TiO_2 and SH-M-TiO_2 were tested by scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM/EDS, S-4800, Hitachi, Japan).

Thermal analysis was with a thermo gravimetric analyzer (STA409PC, NETZSCH, Germany) with temperature programming of 10 °C/min in flowing N_2.

2.3. Immobilization of β-glucosidase

SH-M-TiO_2 (or M-TiO_2) powder (10 mg) was added to 0.2 ml of BG solution (0.261 to 1.19 U/ml) in citric acid-Na_2HPO_4 buffer (50 mM, pH 4.8). The mixture was stirred at 150 r/min at 4 °C for 8 h to establish adsorption equilibrium. BG immobilized on SH-M-TiO_2 (BG-SH-M-TiO_2) or BG immobilized on M-TiO_2 (BG-M-TiO_2) was collected by centrifugation at 4000 × *g* for 10 min, and washed with citric acid-Na_2HPO_4 buffer until no BG activity was detected in the supernatant. All supernatants were collected for BG assays.

2.4. Determination of BG activity

BG activity was measured as p-nitrophenol (pNP) released by enzymatic degradation of p-nitrophenyl β-D-glucopyranoside (pNPG). BG solution (0.1 ml), BG-M-TiO_2 powder (1–10 mg) or BG-SH-M-TiO_2 powder (1–10 mg), which had approximately the same BG activity, were mixed with 0.9 ml of citric acid-Na_2HPO_4 buffer (50 mM, pH 4.8) and 5 mM of pNPG (Sigma) and incubated at 50 °C for 10 min with stirring. Enzymolysis was terminated with 2 ml of Na_2CO_3 (1 M). The mixture was centrifuged at 10,621 × *g* for 30 min at 4 °C to remove the powder and the supernatant was used in reactions. Product pNP in the supernatant was measured at 400 nm using an UV spectrophotometer (752PC UV–VIS spectrophotometer, Shanghai Shunyu Hengping, China). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of pNP per minute [27].

2.5. Optimum reaction pH and temperature

The optimum reaction pH was determined for free BG in solution, BG-M-TiO_2 or BG-SH-M-TiO_2 by incubating with 5 mM pNPG in 50 mM citric acid-Na_2HPO_4 buffer from pH 3.0 to 7.2 at 50 °C for 10 min. The amount of pNP was estimated as described in 2.4.

The optimum reaction temperature was determined with free BG in solution, BG-M-TiO_2 or BG-SH-M-TiO_2 by incubating with 5 mM pNPG in 50 mM citric acid-Na_2HPO_4 buffer at 25–70 °C for 10 min. Product pNP was estimated as described in 2.4.

2.6. Stability

Stability under different pH conditions was determined for free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2 by measuring

residual BG activity after incubation in 50 mM citric acid-Na_2HPO_4 buffer at pH 3.0 to 7.2, at 25 °C for 24 h.

Thermal stability was tested for free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2 by measuring residual BG activity after incubation in 50 mM, pH 4.8 citric acid-Na_2HPO_4 buffer at 25 to 70 °C for 4 h.

Storage stability was determined for free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2 by measuring residual BG activity after 5 months in citric acid-Na_2HPO_4 buffer at 50 mM, pH 4.8. Residual activity was tested once per month.

Reusability was tested for BG-M-TiO_2 and BG-SH-M-TiO_2 by BG assays as described in 2.4. BG-M-TiO_2 and BG-SH-M-TiO_2 were recovered by centrifugation and washing three times with 50 mM, pH 4.8 citric acid-Na_2HPO_4 buffer.

2.7. Determination of kinetic constants

Michaelis constant (K_m) and maximum reaction velocity (v_{max}) of free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2 were determined by measuring initial reaction rate with pNPG (0.5–8 mmol/l) in citric acid-Na_2HPO_4 buffer, 50 mM, pH 4.8 at 50 °C for 10 min. K_m and v_{max} were obtained by Lineweaver–Burk double reciprocal plots using Michaelis–Menten kinetic equations.

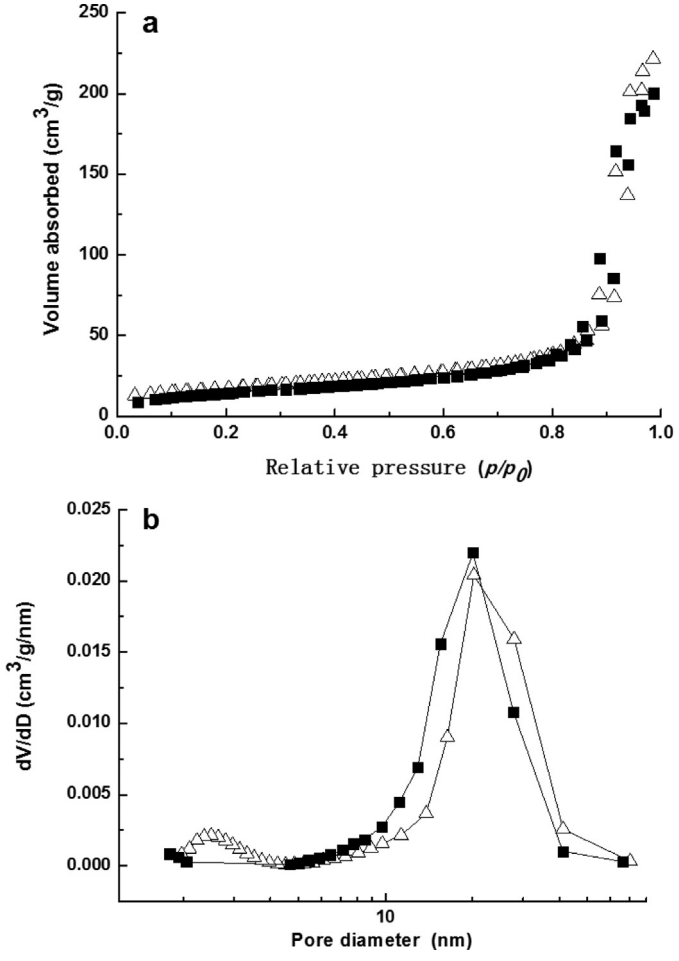

Fig. 2. N_2 absorption and desorption isotherms (a) and pore diameter distribution profiles (b) of M-TiO_2 and SH-M-TiO_2. M-TiO_2, mesoporous TiO_2, △; SH-M-TiO_2, mercaptopropyl-functionalized M-TiO_2, ■.

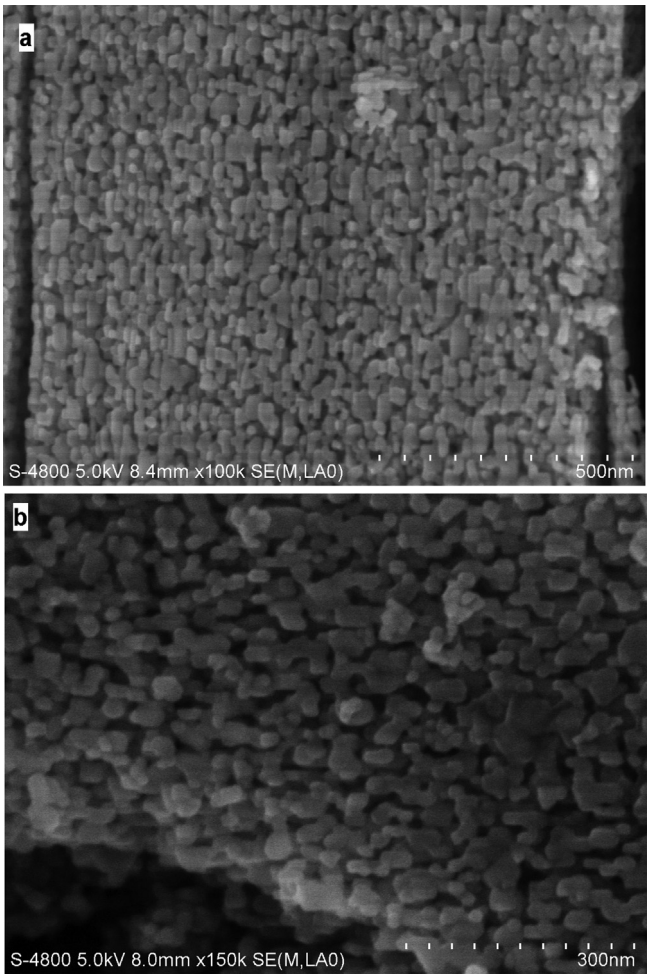

Fig. 3. Representative SEM images of (a) M-TiO_2, mesoporous TiO_2; (b) SH-M-TiO_2, mercaptopropyl-functionalized M-TiO_2.

3. Results and discussion

3.1. Structural characteristics of mesoporous TiO_2 and mercaptopropyl-functionalized M-TiO_2

3.1.1. Analysis of N_2 absorption and desorption isotherms

N_2 absorption and desorption isotherms of M-TiO_2 and SH-M-TiO_2 are in Fig. 2a. Although the absorption isotherm curves of M-TiO_2 and SH-M-TiO_2 are similar, less N_2 absorption was observed for SH-M-TiO_2. After mercaptopropyl functionalization, the mesoporous structure of M-TiO_2 changed slightly. Fig. 2b indicates that the pore size distribution profile of SH-M-TiO_2 shifted to the left compared to M-TiO_2. The number of pores with diameters in the range of 20–30 nm decreased after M-TiO_2 functionalized, probably because the mercaptopropyl groups occupied pore space. Table 1 shows that the surface area decreased from 62.04 to 53.50 m^2/g and the total pore volume decreased from 0.37 to 0.31 cm^3/g after M-TiO_2 was functionalized. Both Bai *et al.* [23] and Zhang *et al.* [28]

Table 1
Textural property of M-TiO_2 and SH-M-TiO_2[a].

	Surface area (m^2/g)	Pore diameter (nm)	Total pore volume (cm^3/g)
M-TiO_2	62.04	20.3	0.37
SH-M-TiO_2	53.50	18.2	0.31

[a] M-TiO_2, mesoporous TiO_2; SH-M-TiO_2, mercaptopropyl-functionalized M-TiO_2.

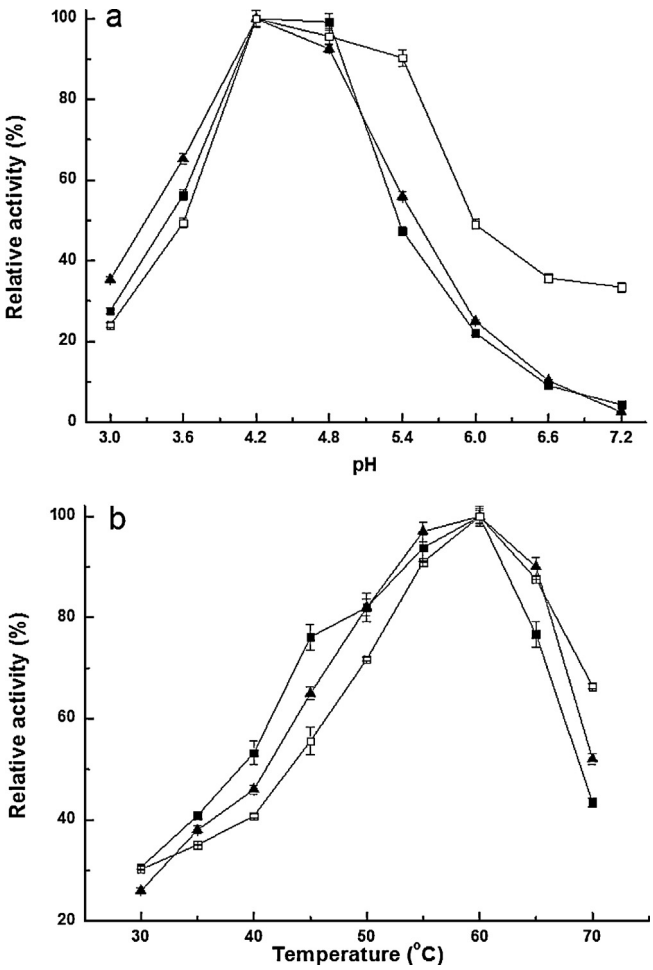

Fig. 4. EDS spectrums of (a) M-TiO_2, mesoporous TiO_2; (b) SH-M-TiO_2, mercaptopropyl-functionalized M-TiO_2.

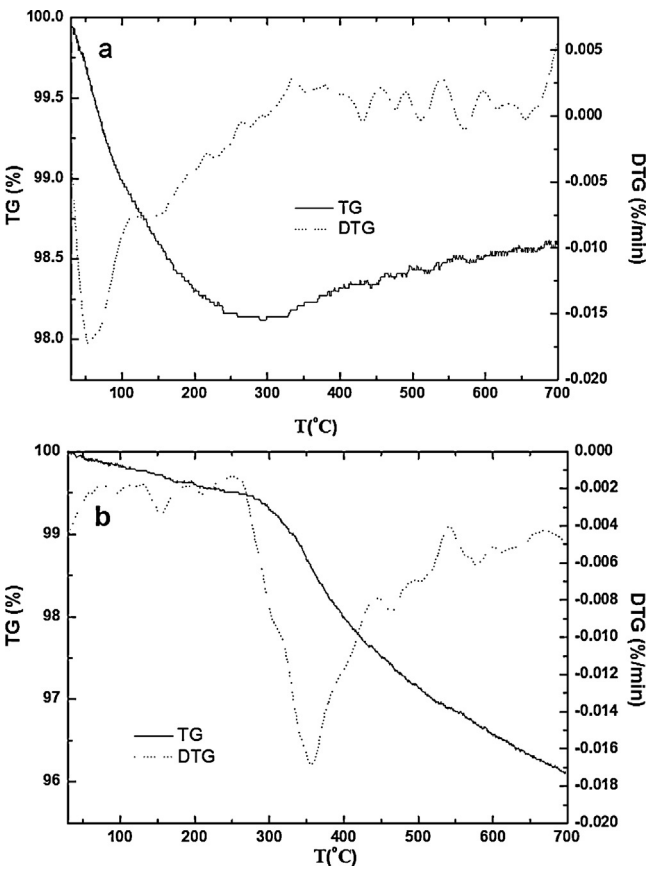

Fig. 5. Thermal gravimetric and differential thermal gravimetric (TG/DTG) curves of (a) M-TiO_2, mesoporous TiO_2; (b) SH-M-TiO_2, mercaptopropyl-functionalized M-TiO_2.

reported that surface area and total pore volume decreased after carriers were functionalized.

3.1.2. Scanning electron microscopy/energy dispersive X-ray spectroscopy analysis

Scanning electron microscopy (SEM) images of M-TiO_2 and SH-M-TiO_2 are in Fig. 3. Both M-TiO_2 and SH-M-TiO_2 had highly porous structures with similar distributions. Because of the porous structure and large surface area, the two carriers might be good matrices for immobilization of enzyme molecules. Comparisons of the energy dispersive X-ray spectroscopy (EDS) spectra of M-TiO_2 (Fig. 4a) and SH-M-TiO_2 (Fig. 4b), shows Si and S elements in SH-M-TiO_2. Analysis of the data presented in the insets of Fig. 4a and Fig. 4b indicated that, after M-TiO_2 was functionalized, the grafted mercaptopropyl groups attributed to about 3.5% of the total weight of the matrices.

3.1.3. Thermal analysis

Thermal gravimetric and differential thermal gravimetric (TG/DTG) curves of M-TiO_2 and SH-M-TiO_2 are in Fig. 5. A clear exothermic DTG peak at 355 °C with an accompanying weight loss could be seen for SH-M-TiO_2 in Fig. 5b but was not observed for M-TiO_2 (Fig. 5a). The exothermic peak was attributed to the thermal decomposition of mercaptopropyl groups, consistent with another report [28]. The results of TG analysis furthermore demonstrated that about 3.5% mercaptopropyl groups grafted on the total weight of matrices after M-TiO_2 was functionalized.

3.2. Immobilization of β-glucosidase

BG was immobilized on M-TiO_2 or SH-M-TiO_2 at initial concentrations of 0.261 to 1.19 U/ml free BG in solutions. BG-M-TiO_2 and BG-SH-M-TiO_2 were analyzed for enzyme activity. The percentage of immobilized BG activity to initial BG activity in the solution was defined as activity recovery. BG activity in the supernatant after immobilization was also determined.

As shown in Table 2, as initial BG activity in free BG solution increased, immobilized BG activity on M-TiO_2 or SH-M-TiO_2 increased. SH-M-TiO_2, the mercaptopropyl-functionalized carrier, showed higher immobilization efficiency than M-TiO_2. When initial free BG activity in solution was 1.19 U/ml, SH-M-TiO_2 immobilized a BG activity of 15.2 U/g, twice as much as M-TiO_2 at 7.67 U/g. Immobilized enzyme attained the most effective activity balance when the highest activity recovery was obtained. The highest activity recovery of BG-M-TiO_2 was 64.1% as initial BG activity in solution was 0.364 U/ml. By contrast, the highest activity recovery of BG-SH-M-TiO_2 was 92.8% as initial BG activity in solution was 0.592 U/ml, with an immobilized BG activity of 10.9 U/g. These results indicated that SH-M-TiO_2 had a better capacity than M-TiO_2 to load BG molecules.

3.3. Optimum reaction pH and temperature

Free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2 were incubated at 50 °C in 5 mM pNPG solution at pH 3.0 to 7.2 for 10 min. The effects of reaction pH for the three enzyme preparations were similar, as shown in Fig. 6a. The optimum reaction pH for the three enzyme preparations was 4.2. BG-SH-M-TiO_2 exhibited more stable

Table 2
Efficiency of β-glucosidase immobilization on M-TiO_2 and SH-M-TiO_2 [a].

Initial BG activity in the free BG solution (U/ml)	BG-M-TiO_2			BG-SH-M-TiO_2		
	Activity (U/g)	Activity recovery (%)	Activity in the supernatant (%)	Activity (U/g)	Activity recovery (%)	Activity in the supernatant (%)
0.261	3.51 ± 0.01	63.0 ± 0.4	28.0 ± 1.8	3.79 ± 0.13	72.5 ± 0.5	26.8 ± 1.6
0.364	4.68 ± 0.03	64.1 ± 0.5	27.5 ± 1.6	5.36 ± 0.06	73.6 ± 0.2	26.1 ± 1.5
0.474	5.25 ± 0.02	56.3 ± 0.4	31.2 ± 1.7	7.90 ± 0.26	83.3 ± 3.3	13.7 ± 0.7
0.592	6.40 ± 0.06	51.1 ± 1.1	34.2 ± 1.9	10.9 ± 0.1	92.8 ± 0.6	6.88 ± 0.35
0.677	6.53 ± 0.06	48.2 ± 1.1	38.4 ± 2.2	12.0 ± 0.3	88.7 ± 1.9	7.34 ± 0.35
0.790	7.04 ± 0.08	44.6 ± 0.7	45.8 ± 2.4	13.2 ± 0.3	83.3 ± 1.7	12.9 ± 0.5
0.948	7.59 ± 0.13	41.8 ± 0.6	49.4 ± 2.2	14.8 ± 0.4	78.2 ± 0.4	17.5 ± 0.7
1.19	7.67 ± 0.09	8.45 ± 0.25	86.0 ± 4.2	15.2 ± 0.5	63.7 ± 1.0	27.3 ± 1.0

[a] BG-M-TiO_2, β-glucosidase immobilized on mesoporous TiO_2; BG-SH-M-TiO_2, β-glucosidase immobilized on mercaptopropyl-functionalized M-TiO_2. Each value is an average of three parallel replicates and is represented as mean standard deviation.

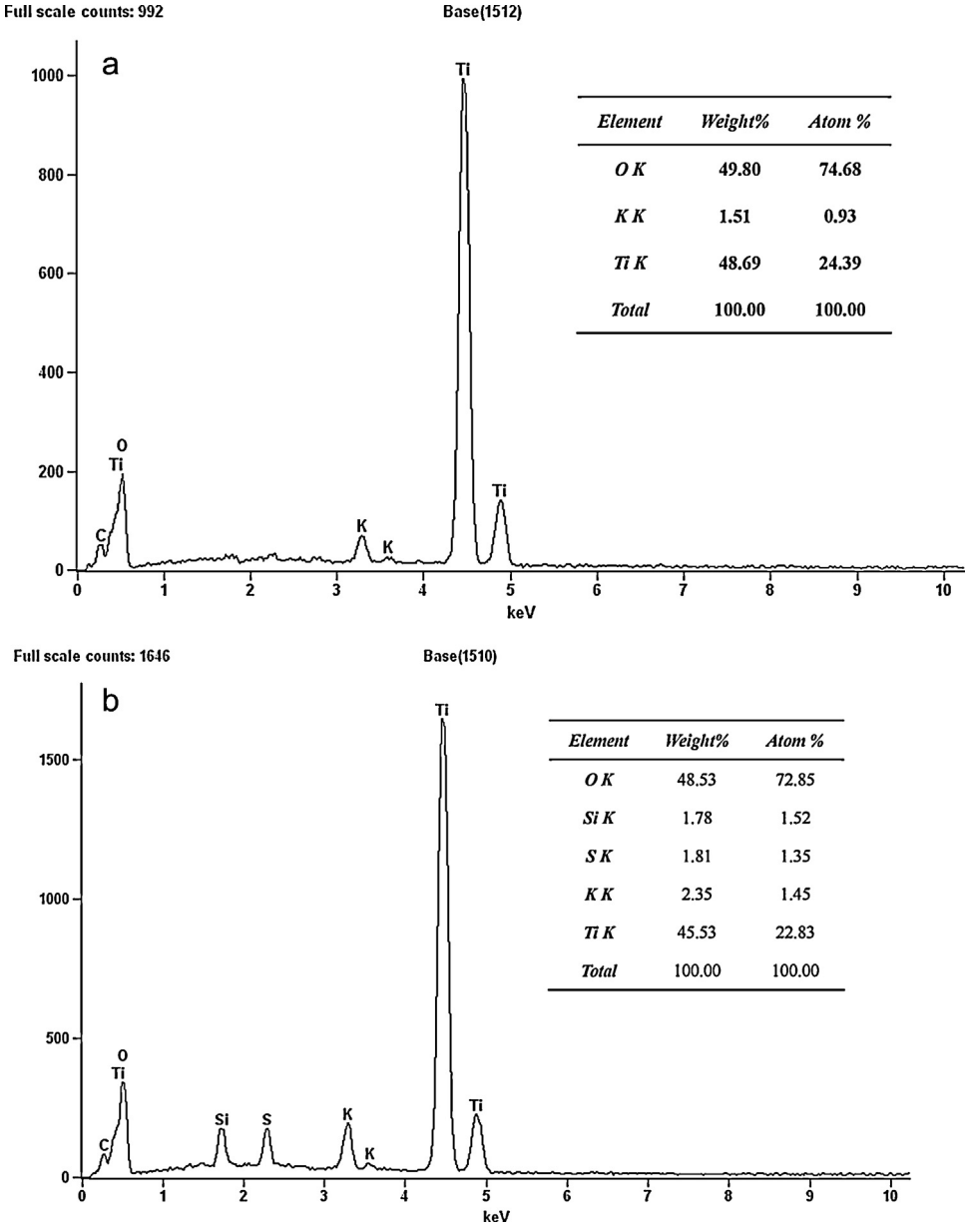

Fig. 6. Effect of reaction pH (a) at 25 °C and temperature at pH 4.2 (b) on relative activity of free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2. Free β-glucosidase in solution, ▲; BG-M-TiO_2, β-glucosidase immobilized on mesoporous TiO_2, ■; BG-SH-M-TiO_2, β-glucosidase immobilized on mercaptopropyl-functionalized M-TiO_2, □. Values are averages from duplicate experiments.

Table 3
Apparent kinetic parameters of the three enzyme preparations[a].

	K_m (mmol/l)	V_{max} (mmol/(l min))
Free BG	0.89 ± 0.03	0.41 ± 0.01
BG-M-TiO_2	2.66 ± 0.15	0.39 ± 0.02
BG-SH-M-TiO_2	1.0 ± 0.04	0.42 ± 0.03

[a] Free BG, free β-glucosidase in solution; BG-M-TiO_2, β-glucosidase immobilized on mesoporous TiO_2; BG-SH-M-TiO_2, β-glucosidase immobilized on mercaptopropyl-functionalized M-TiO_2. Each value is an average of three parallel replicates and is represented as mean standard deviation.

reaction activity between pH 4.2 and pH 5.4 than free BG in solution or BG-M-TiO_2. Free BG, BG-M-TiO_2 and BG-SH-M-TiO_2 were incubated at 25–70 °C for 10 min in 5 mM pNPG solution at pH 4.2. Fig. 6b shows that the optimum reaction temperature of the three enzyme preparations was at 60 °C.

3.4. Stability

Stability at different pH values was determined with free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2 by measuring residual BG activity after incubation at pH 3.0 to 7.2 for 24 h. As shown in Fig. 7a, free BG in solution and BG-M-TiO_2 were stable, maintaining more than 90% activity at pH 4.2–4.8 while BG-SH-M-TiO_2 retained 90% activity at pH 4.2–5.4. BG-SH-M-TiO_2 was more stable than both free BG and BG-M-TiO_2 at pH less than 4 and above 5. Therefore, immobilization of BG on mercaptopropyl-functionalized M-TiO_2 maintained BG activity in a wider pH range.

Thermal stability was tested for free BG, BG-M-TiO_2 and BG-SH-M-TiO_2 by measuring residual activity after incubation in pH 4.8 at 25–70 °C for 4 h. As shown in Fig. 7b, the three enzyme preparations showed similar activity at 20 to 50 °C. However, at incubation temperatures over 50 °C, the thermal stability of BG-SH-M-TiO_2 was better than free BG or BG-M-TiO_2.

Storage stability was studied with free BG, BG-M-TiO_2 and BG-SH-M-TiO_2 by measuring residual BG activity after storage at 4 °C for 5 months. Fig. 7c shows that after 3 months, the residual activity of free BG and BG-M-TiO_2 declined to below 20% of initial BG activity, whereas residual activity of BG-SH-M-TiO_2 was higher than 95% at 5 months. These results indicated that immobilization of BG on SH-M-TiO_2 effectively preserved BG activity.

The operational stability of BG-M-TiO_2 and BG-SH-M-TiO_2 is important for applications. This stability was tested by repeated BG assays. Fig. 7d shows that after 6 rounds of BG assays, residual activity of BG-M-TiO_2 was below 50% of initial activity but residual activity of BG-SH-M-TiO_2 was above 80%. After 30 rounds of BG assays, the residual activity of BG-SH-M-TiO_2 remained at about 50%.

These results showed that BG-SH-M-TiO_2 offered better stability than free BG in solution or BG-M-TiO_2 at different pH, temperature, storage and operational conditions. Comparison of BG-M-TiO_2 and BG-SH-M-TiO_2 for storage and operational stability showed weak adsorption interaction between M-TiO_2 and BG. Interaction between SH-M-TiO_2 and BG molecules was relatively stronger, which could be attributed to the covalent attachment of disulfide bonds between the surface of the enzyme and the SH-M-TiO_2 mercaptopropyl groups. The resulting BG-SH-M-TiO_2 could be used repeatedly, similar to the results of Yiu *et al.* [24], who showed that trypsin supported on thiol-functionalized SBA-15 was recyclable.

3.5. Kinetics of enzyme preparations

K_m and v_{max} of free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2 were calculated using Lineweaver–Burk plots with pNPG as substrate. Table 3 shows that K_m of free BG in solution was the lowest of the three enzyme preparations, indicating that free BG

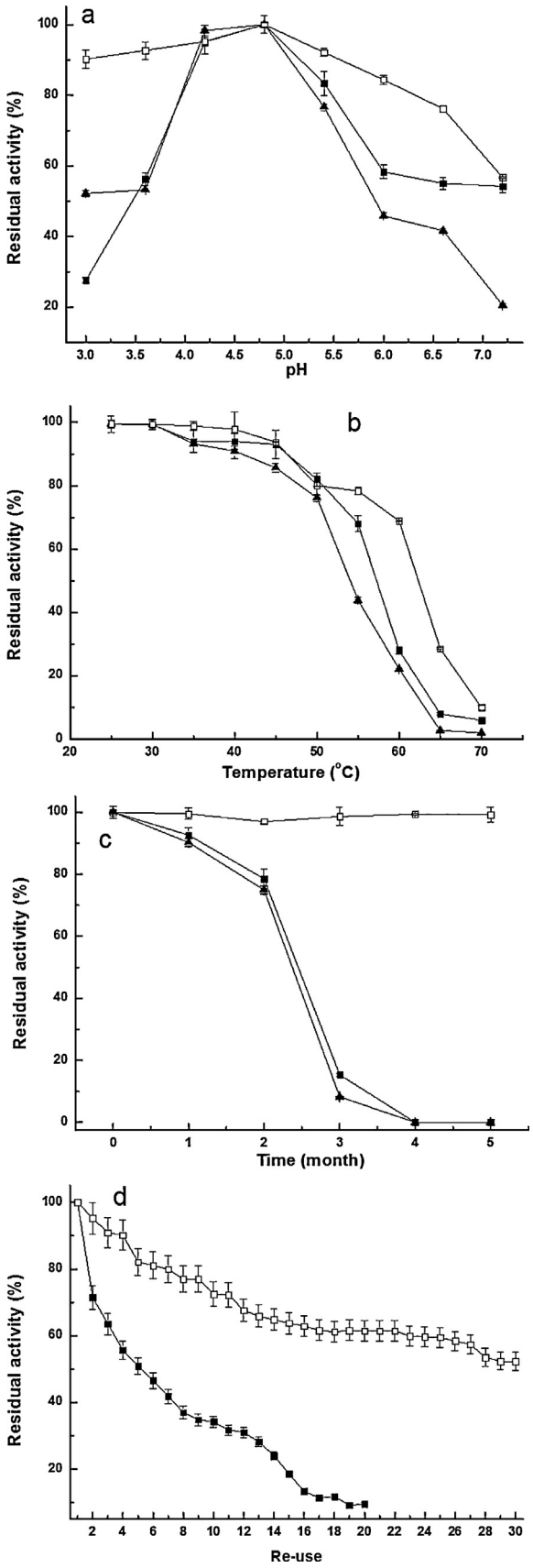

Fig. 7. Stability under different pH (a), temperature (b), storage (c), and re-use (d) conditions of free BG in solution, BG-M-TiO_2 and BG-SH-M-TiO_2. Free β-glucosidase, ▲; BG-M-TiO_2, β-glucosidase immobilized on mesoporous TiO_2, ■; BG-SH-M-TiO_2, β-glucosidase immobilized on mercaptopropyl-functionalized M-TiO_2, □. Values are averages from duplicate experiments.

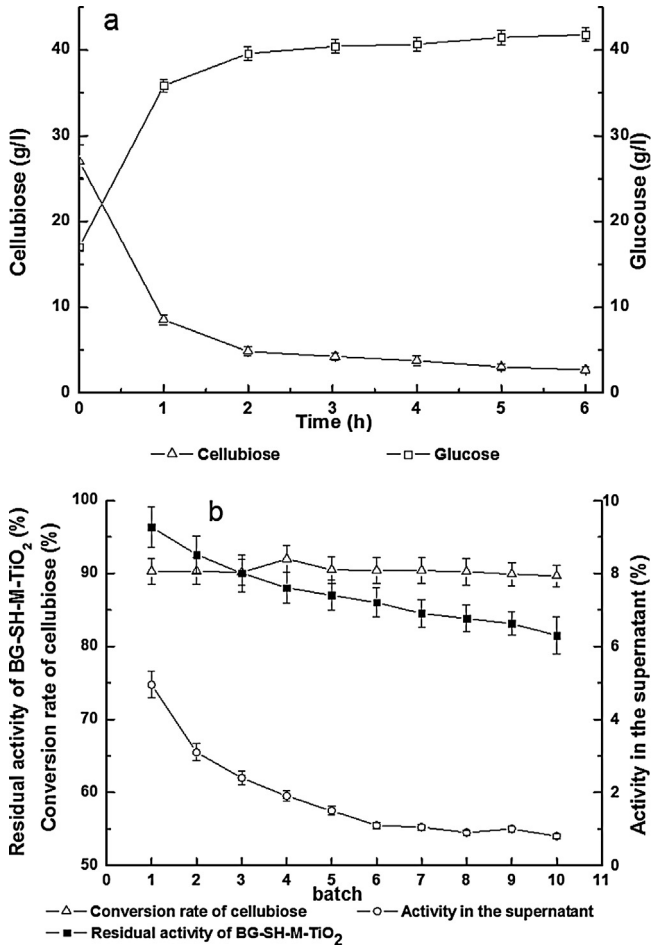

Fig. 8. Enzymatic hydrolysis of cellobiose-containing substrate by BG-SH-M-TiO_2. (a): Course of batch hydrolysis; (b): repeated hydrolysis of 10 batches. Substrate contained 26.93 g/l cellobiose and 16.88 g/l glucose. BG-SH-M-TiO_2, β-glucosidase immobilized on mercaptopropyl-functionalized mesoporous TiO_2, was added at a total BG activity of 20 U/g sugar. Batch hydrolysis was conducted at pH4.8 and 60 °C for 6 h. Values are averages from duplicate experiments.

had the highest affinity for the substrate pNPG. V_{max} of BG-M-TiO_2 was slightly lower than for free BG, probably due to diffusion limitation and steric hindrance [29]. Free BG and BG-SH-M-TiO_2 had similar values for K_m and v_{max}. Therefore, BG-SH-M-TiO_2 also had good affinity for the substrate pNPG.

3.6. *Repeated enzymatic hydrolysis of cellobiose-containing substrate by BG-SH-M-TiO_2*

Previously, we proposed a three-stage enzymatic hydrolysis model for steam-exploded corn stover with a solid content of 30% [30]. A high yield of saccharification took only 30 h and the resulting sugar solution contained a substantial portion of cellobiose. Since the sugar solution is separated from solid residues, further conversion of cellobiose to glucose is easily achieved by repeated batch mode or a continuous process with immobilized BG.

A repeated batch hydrolysis was designed to test the practical efficiency of BG-SH-M-TiO_2. A sugar solution containing 26.93 g/l cellobiose and 16.88 g/l glucose was used as the substrate. BG-SH-M-TiO_2 with a total BG activity of 20 U/g sugar was applied in batch hydrolysis at pH 4.8 and 60 °C for 6 h. Residual immobilized BG activity was determined and the supernatant was checked for sugar concentration and lost enzyme activity. BG-SH-M-TiO_2 was reused for another batch of hydrolysis.

Fig. 8a shows a batch hydrolysis course by BG-SH-M-TiO_2. The hydrolysis velocity of cellobiose was high for the first hour, then declined gradually because of product inhibition. After 6 h, the conversion rate of cellobiose was 90%, which is acceptable in practice.

Fig. 8b presents the results of 10 batches of hydrolysis using the same BG-SH-M-TiO_2. The conversion rates of cellobiose were around 90% for all 10 batches, indicating a stable performance by the immobilized enzyme. BG activity in the supernatant represents the degree of desorption of immobilized enzyme. After the first batch of hydrolysis, 5% of the initially immobilized BG activity was lost; thereafter, enzyme desorption gradually slowed. The residual BG activity of BG-SH-M-TiO_2 after each batch also decreased slowly; after 10 batches of hydrolysis, 81% of the initially immobilized BG activity remained. After repeated hydrolysis with immobilized enzyme at 60 °C, such as in this experiment, proteins are expected to irreversibly deactivate. The loss of residual BG activity from the BG-SH-M-TiO_2 could not be attributed mainly to desorption of enzyme molecules. Instead, BG activity in the later supernatants was low. Thus, the results in Fig. 8b show effective adsorption of enzyme molecules to mercaptopropyl-functionalized mesoporous TiO_2. Since enzyme deactivation at high temperature is inevitable, the addition of fresh immobilized enzyme is necessary during enzymatic hydrolysis.

4. Conclusion

For immobilization of BG on an inorganic material, M-TiO_2, a nanostructured crystalline TiO_2, was determined as the carrier since it is inexpensive, safe, highly stable, and easily prepared at large scale. To enhance the loading and stability of enzyme, M-TiO_2 was functionalized with mercaptopropyl group by post-synthesis grafting; our analysis confirmed the success of this modification. The resulting SH-M-TiO_2 immobilized BG effectively and BG-SH-M-TiO_2 offered better stability in pH, temperature, and storage tests than BG-M-TiO_2 or free BG in solution. When BG-SH-M-TiO_2 was used in repeated batches of enzymatic hydrolysis, the conversion rate of cellobiose was high, while desorption of enzyme activity was minor. Although the mechanisms of enzyme attachment to SH-M-TiO_2 require further investigation, effective adsorption of enzyme molecules to SH-M-TiO_2 matrices is proposed. For large-scale tests, however, M-TiO_2 should be fabricated to a specific size and shape to construct a practical enzymatic bioreactor. During fabrication, the morphology and porosity of M-TiO_2 might differ from the powder used in this study. More work on the fabrication of M-TiO_2 particles or tubes and immobilization of BG is required.

Acknowledgments

We thank Professor Xiaohua, Lu and Dr. Wei Zhuang for M-TiO_2. This research was supported by the China Governmental Public Industry Research Special Funds (Grant No. 201004001) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

[1] M.C. Hsieh, T.L. Graham, Phytochemistry 58 (2001) 995–1005.
[2] Q.F. Wang, C. Qian, X.Z. Zhang, N. Liu, X. Yan, Z.H. Zhou, Enzyme Microb. Technol. 51 (2012) 319–324.
[3] M.P. Dale, W.P. Kopfler, I. Chait, L.D. Byers, Biochemistry 25 (1986) 2522–2529.
[4] A.G. Day, S.G. Withers, Biochem. Cell Biol. 64 (1986) 914–922.
[5] S.M. Pitson, R.J. Seviour, B.M. McDougall, Enzyme Microb. Technol. 21 (1997) 182–190.
[6] H.T. Hang, J. Agric. Food Chem. 3 (1995) 141–146.
[7] A. Martino, P.G. Pifferi, G. Spagna, J. Chem. Technol. Biotechnol. 60 (1994) 247–252.
[8] R.S.R. Leite, H.F. Alves-Prado, H. Cabral, F.C. Pagnocca, E. Gomes, R. Da-Silva, Enzyme Microb. Technol. 43 (2008) 391–395.

[9] S.H. Baek, S. Kim, K. Lee, J.K. Lee, J.S. Hahn, Enzyme Microb. Technol. 51 (2012) 366–372.
[10] G.D. Haki, S.K. Rakshit, Bioresour. Technol. 89 (2003) 17–34.
[11] Z.P. Guo, L. Zhang, Z.Y. Ding, Z.H. Gu, G.Y. Shi, Enzyme Microb. Technol. 49 (2012) 105–112.
[12] S. Oh, C. Daraio, L.H. Chen, T.R. Pisanic, R.R. Finones, S. Jin, J. Biomed. Mater. Res. Part A 78A (2006) 97–103.
[13] H. Tang, F. Yan, Q. Tai, H.L.W. Chan, Biosens. Bioelectron. 25 (2010) 1646–1651.
[14] J. Lee, M.C. Orilall, S.C. Warren, M. Kamperman, F.J. Disalvo, U. Wiesner, Nat. Mater. 7 (2008) 222–228.
[15] P.D. Yang, D.Y. Zhao, D.I. Margolese, B.F. Chmelka, G.D. Stucky, Chem. Mater. 11 (1999) 2813–2826.
[16] J. Kim, J.W. Grate, P. Wang, Chem. Eng. Sci. 61 (2006) 1017–1026.
[17] G. Cisani, P.E. Varaldo, A. Ingianni, R. Pompei, G. Satta, Curr. Microbiol. 10 (1984) 35–40.
[18] J.M. Moreno, J.V. Sinisterra, J. Mol. Catal. 93 (1994) 357–369.
[19] R. Darias, R. Villalonga, J. Chem. Technol. Biotechnol. 76 (2001) 489–493.
[20] J.A. Lori, A.J. Nok, Bio-Med. Mater. Eng. 14 (2004) 557–563.
[21] H. Tang, F. Yan, Q.D. Tai, H.L.W. Chan, Biosens. Bioelectron. 25 (2010) 1646–1651.
[22] W. Li, Y. Bai, C. Liu, Z.H. Yang, X. Feng, X.H. Lu, Environ. Sci. Technol. 43 (2009) 5423–5428.
[23] W. Bai, Y.J. Yang, X. Tao, J.F. Chen, T.W. Tan, J. Mol. Catal. B: Enzym. 6 (2012) 82–88.
[24] H.H.P. Yiu, P.A. Wright, N.P. Botting, J. Mol. Catal. B: Enzym. 15 (2001) 81–92.
[25] M. He, X.H. Lu, X. Feng, L. Yu, Z.H. Yang, Chem. Commun. 19 (2004) 2202–2203.
[26] T. Anri, S. Toru, I. Keita, T. Tatsuo, K. Akiko, M. Fujio, S. Kengo, Mesoporous Mater. 116 (2008) 601–606.
[27] M. Matsuva, J. Sasaki, S. Murao, Biotechnol. Biochem. 59 (1995) 1623–1627.
[28] C. Zhang, W. Zhou, P. Lu, S.X. Liu, Chemistry of China. 7 (2006) 529–531.
[29] N.N. Tochukwu, A. Hideki, N.O. Bartholomew, Y. Shigeki, J. Mol. Catal. B: Enzym. 78 (2012) 1–8.
[30] J. Yang, X. Zhang, Q. Yong, S.Y. Yu, Bioresour. Technol. 102 (2011) 4905–4908.