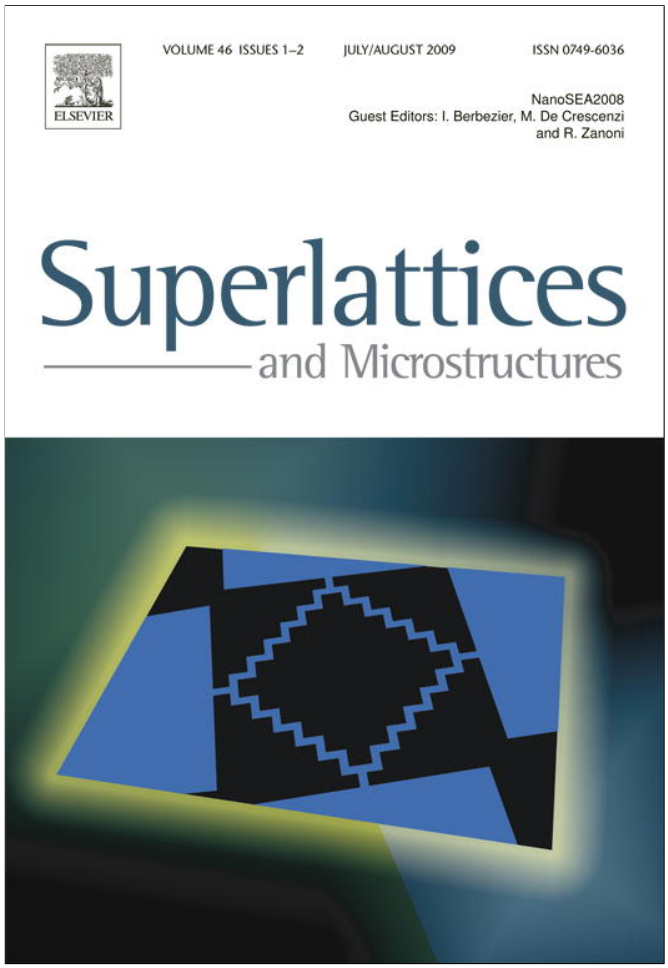

Superlattices and Microstructures 46 (2009) 77–83

Contents lists available at ScienceDirect

Superlattices and Microstructures

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

Improvement of the alkaline protease properties via immobilization on the TiO_2 nanoparticles supported by mesoporous MCM-41

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ARTICLE INFO

Article history:
Available online 30 November 2008

Keywords:
Titanium oxide nanoparticles
Mesoporous MCM-41
Alkaline Protease immobilization
Biocatalytic materials

ABSTRACT

Enzymes are able to catalyze the most complex chemical processes under the mildest experimental and environmental conditions. However, in general, enzymes do not fulfill the requirements for industry. Therefore, most enzymes have to be greatly improved before industrial implementation. They may be immobilized by using very different protocols. Nanoparticles have been significantly used for coupling with biomolecules. They are excellent biocompatible surfaces for the immobilization of enzymes. In this work, we demonstrated that the alkaline protease enzyme immobilized on the TiO_2 nanoparticles assembled on the porous MCM-41 (Mobile Crystalline Material No. 41) particles could provide an active biocatalyst, stable at different pH and temperature. Absorption of the TiO_2 nanoparticles on the MCM-41 was monitored by UV–visible spectroscopy. Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and dispersive analysis of X-RAY (EDAX) were used to characterize the size and morphology of the TiO_2 nanoparticles on the MCM-41.

1. Introduction

Biotechnology is undergoing impressive advances in the synthesis of biocompatible surfaces for the immobilization of a range of biomolecules, with important applications in biosensing and medicine [1, 2]. Insofar as enzymes are concerned, advantages of immobilized enzymes over their counterparts in solution, include enhanced temperature and stability of the biocatalyst and ease of separation from the reaction medium, enabling multiple reuses [3–10]. In the last decade, nanosized materials have been widely used as support for immobilization. Among these materials, gold and titanium oxide

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doi:10.1016/j.spmi.2008.10.022

nanoparticles are very popular when used in conjunction with biological materials including proteins, peptides, enzymes antibodies and nucleic acids, because of their unique characteristic properties. Although we are still at the beginning of exploring the use of these materials for biocatalysis, various nanostructures have been examined as hosts for enzyme immobilization via approaches including enzyme absorption, covalent attachment and enzyme encapsulation. A number of templates which have been used for enzyme immobilization are silica nanotubes, phospholipid bilayers, self-assembled monolayers, Langmuir–Blodgett films, polymer matrices, galleries of a-zirconium phosphate, mesoporous silicates such as MCM-41, silica nanoparticles, and thermally evaporated lipid films, each with its characteristic advantages and disadvantages [11–16].

In this investigation, we demonstrated that the assembly of TiO_2 nanoparticles supported by porous MCM-41 (Mobile Crystalline Material No. 41) particles is an appropriate template for immobilization of the alkaline protease in providing an excellent nano biocatalyst with a good PH and temperature stability and high biocatalytic activity. The alkaline protease, immobilized on titanium oxide nanoprticles/MCM-41, could be easily removed from the reaction medium by simple sedimentation, and exhibits excellent reuse characteristics.

2. Experiments

Titanium tetra-butoxide ($Ti(OBut)_4$), ethanol, hydrochlorid acid, trichloroacetic acid from Merck, alkaline protease and casein from sigma were used as the starting materials.

To prepare transparent nano particles supported by MCM-41, a TiO_2 solution was prepared by slowly instilling the $Ti(OC_4H_9)_4$ and ethanol mixture into diluted HCl aqueous solution under continuous stirring for 3 h at room temperature. Then, 1 g of MCM-41 was added to 3 mL of the above prepared TiO_2 solution under continuous stirring. After 14 h of stirring, the white color TiO2 nanoparticles supported by MCM-41 were obtained. The product was heated in an oven at 400 °C and air-dried for further use.

To immobilize the alkaline protease enzyme, fifteen milligrams of the above prepared titanium oxide nanoparticles/MCM-41 powder was dispersed in 2 mL of tris–HCl buffer (0.05 M, pH 8) and to this solution, 100 μL of a stock solution consisting of 50 mg/mL of alkaline protease in Tris–HCl buffer (0.05 M, pH 8) was added under vigorous stirring for 2 h. At this stage, the alkaline protease/nano titanium oxide/MCM-41 bioconjugate material was separated by centrifugation. The powder obtained was rinsed several times with tris–HCl buffer (0.05 M, pH 8) solution and re-suspended in the buffer solution (pH 8) and stored at 4 °C for further experimentation.

Scanning electron microscopy SEM (Philips) equipped with a Dispersive analysis of X-RAY (EDAX) attachment, and Transmission Electron Microscopy, TEM (Philips EM 208 100KV) were used to characterize the size and the morphology of the TiO_2 nano particles on the MCM-41. The transmittance measurement was carried out by UV–visible spectrometer (CARRY) respectively.

3. Results and discussion

3.1. UV–Vis spectroscopy studies

Fig. 1 shows UV–vis spectrum recorded for as prepared titanium oxide nano particles solution (curve 1) and the supernatant titanium oxide solution obtained after stirring with MCM-41 for 12 h and filtration (curve 2). Fig. 1 clearly indicates the binding of titanium oxide nanoparticles to the MCM-41 particles has occurred.

3.2. TEM micrographs

Fig. 2a shows TEM micrograph of the prepared MCM-41 and TiO_2 nanoparticle/MCM-41 particles. The titanium oxide nanoparticles (dark spots) bound on the surface of the MCM-41 particles are fairly high density and spherical shapes. Fig. 2b shows the TEM image of the titanium oxide particles/MCM-41 nano particles support, after immobilization of the alkaline protease. The size of TiO_2 nano particles loaded on MCM-41 can be estimated was about 3–5 nm.

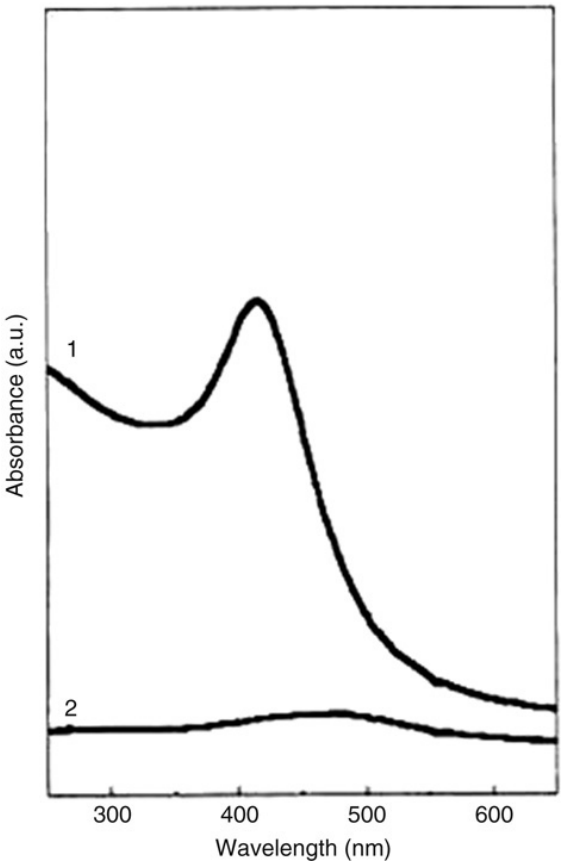

Fig. 1. UV–Vis spectrum recorded from the as-prepared titanium oxide nano particle solution (curve 1) and titanium oxide solution after stirring with MCM-41 for 12 h and centrifugation (curve 2, see text for details).

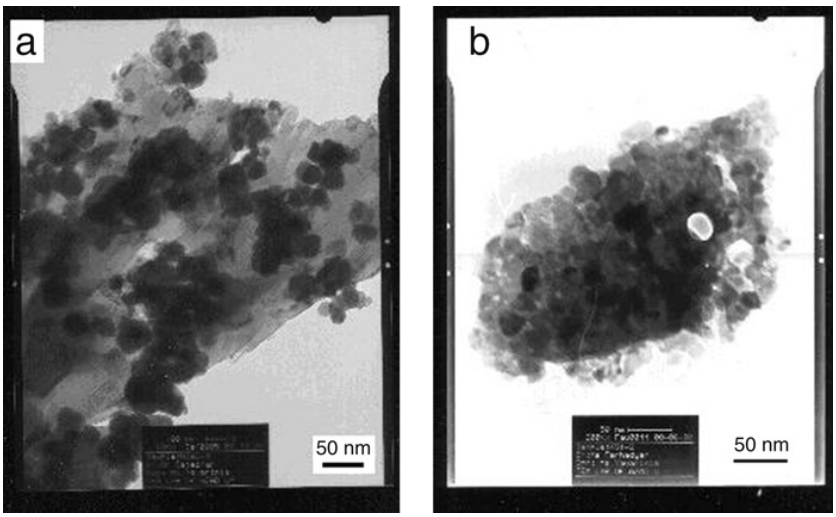

Fig. 2. (a) Representative TEM images of TiO_2/MCM-41 prepared on the carbon-coated TEM grid. (b) TEM micrograph of titanium oxide nanoparticles (dark spots) bound to the surface of the MCM-41 particles after immobilization of the alkaline protease.

3.3. SEM micrographs and Dispersive X-RAY analysis (EDAX)

Fig. 3 shows SEM micrographs of the prepared samples. Fig. 3a shows the hexagonal image of MCM-41 particles with slightly regular edges. Fig. 3b shows SEM image of the TiO_2 nanoparticles bound to the surface of MCM-41 at fairly high density. Fig. 3c shows SEM image of the alkaline protease adsorbed on TiO_2 nanoparticles supported by MCM-41. X-RAY dispersive analysis (EDAX)

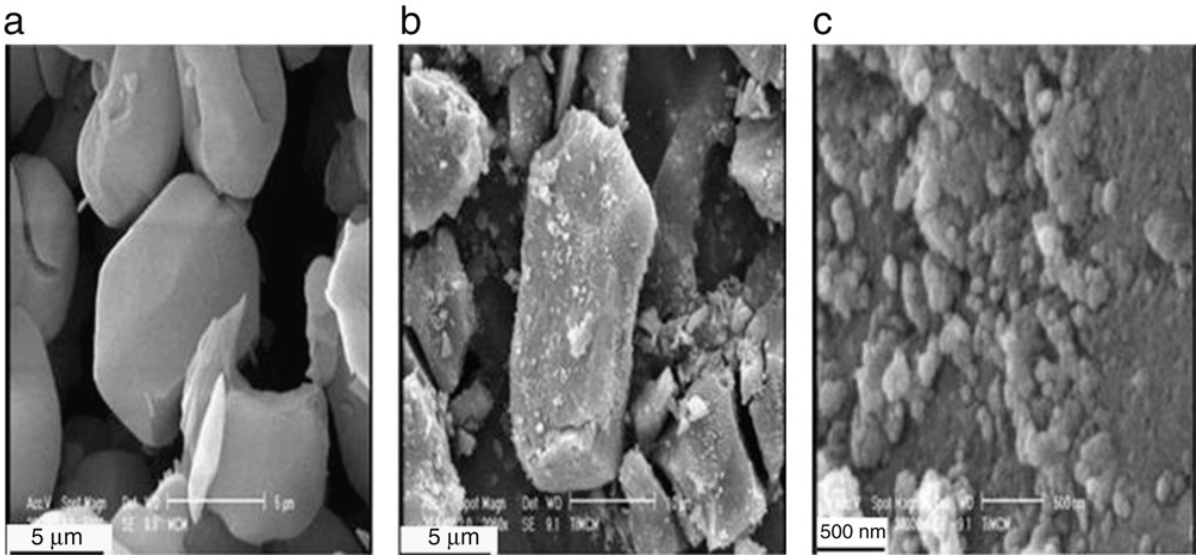

Fig. 3. Representative SEM images of: (a) MCM-41; (b) TiO_2/MCM-41and (c) alkaline protease/TiO_2 nanoparticles bound to the surface of the MCM-41, on gold-coated SEM grid.

Table 1
EDAX ZAF quantification (Standardless) of the element normalized.

Element	MCM-41/wt%	Ti-MCM-41/wt%	MCM-41/at.%	Ti-MCM-41/at.%
Al	1.95	1.48	2.03	1.84
Si	98.05	58.92	97.97	70.41
Ti	0	39.60	0	27.74
Total	100	100	100	100

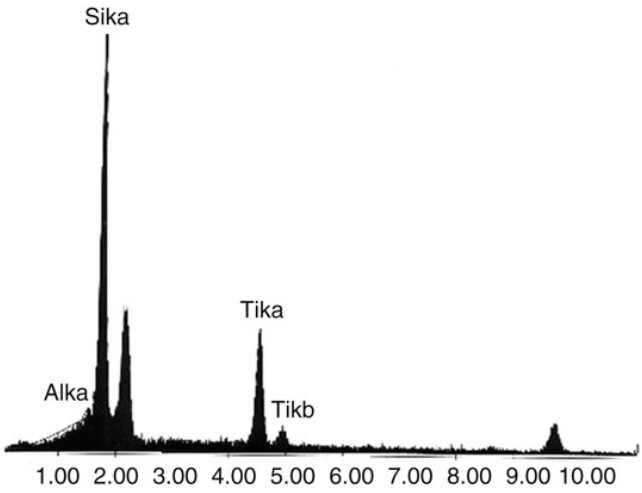

Fig. 4. Dispersive analysis of X-RAY (EDAX) for the sample of TiO_2/MCM-41.

of the samples shown in (Table 1) gives the percentage ratio of Ti and Si in the MCM-41 and TiO_2 nano particle on MCM-41. Dispersive analysis of the X-RAY (EDAX) for TiO2/MCM-41 is shown in Fig. 4.

3.4. Biocatalytic activity measurements

Biocatalytic activity of the alkaline protease immobilized on TiO_2/MCM-41 was determined in Tris–HCl buffer (0.05 M, pH 8) by the reaction of alkaline protease with 0.5% casein at 55 °C for 30 min. Control experiments of the biocatalytic activity of the free alkaline-protease was performed under

Table 2
Biocatalytic activities of the alkaline protease immobilized on TiO_2/MCM-41 bioconjugate materials.

No. of cycles	Biocatalytic activity of alkaline protease immobilized on TiO_2/MCM-41 (U/mg)
1	75
2	39
3	24
4	18

identical conditions. The biocatalytic activity of the free alkaline protease in solution was 64 U/mg (Units per milligram) whereas that of the enzyme assembled in the system was 75 U/mg.

3.4.1. Retention

The most important aspect of this study concerns retention of the biocatalytic activity of alkaline protease enzyme after absorption of the enzyme into TiO_2/MCM-41 surface. For recycling studies, the alkaline protease/TiO_2/MCM-41 material was separated from the reaction medium by centrifugation (14 000 rpm). In the typical experiments, to estimate the biocatalytic activity of the materials, a carefully measured amount of the alkaline protease/TiO_2/MCM-41 in buffer was incubated with 1 mL of 0.5% casein solution at 55 °C for 30 min. The amount of casein in the alkaline protease bound to the biocatalyst material was estimated quite accurately from the UV–Vis spectroscopy measurements. So, it will be possible to compare the biocatalytic activity of the immobilized enzyme and also the free enzyme in solution under identical assay conditions.

The new and used biocatalytic materials was easily separated from the reaction medium by sedimentation or mild centrifugation and exhibits excellent reuse characteristics over four successive cycles (Table 2). Table 2 shows the results of the bioactivities calculated over 4 sequential reuse cycles. This data confirmed major advantageous in our study.

3.4.2. Temperature stability

Temperature stability of the alkaline-protease/TiO_2/MCM-41 was checked by preincubating the biocatalyst for 1 h at different temperatures in the range 35–55 °C, and was compared with an identical amount of free enzyme in the—Tris–HCl buffer (0.05 M, pH 8) under similar conditions. All reactions were carried out after preincubation for 1 h at the different temperatures and measuring the biocatalytic activity at pH 8 and 55°C, as described earlier. Three separate measurements were done to check the reproducibility of the assay (Fig. 5). At lower temperatures, dramatic differences were observed in biocatalytic activity of the enzyme in the two cases. At 40 °C, the free enzyme retained 29% of the starting biocatalytic activity, whereas the alkaline – protease TiO_2 nanoparticle – MCM-41 bioconjugate retained was 12% of the 40 °C biocatalytic activity. This remarkable trend continued to higher temperatures as well, with retention of 31% of initial biocatalytic activity for the immobilized enzyme at 45 °C, whereas the free enzyme molecules in solution retained only 21% of their biocatalytic activity. The increase in the thermal stability of the enzyme in the bioconjugate may arise from the conformational integrity of the enzyme structures after assembling on the TiO_2 nanoparticles/MCM-41.

3.5. The pH stability

The pH-dependent variations in biocatalytic activity of the bioconjugate materials and free enzyme were studied at five different pH values (pH 6, 6.5, sodium phosphate buffer; pH 8 and 10, glycine–NaOH buffer) by preincubating materials for 1 h at 55 °C. All reactions were carried out after preincubation for 1 h at the different pH levels and measuring the biocatalytic activity at pH 55 °C, as described earlier (Fig. 6). It has been seen that optimum biocatalytic activity in both the cases was at pH 8, with a marginal loss in biocatalytic activity at pH 5. At pH 6, however, free enzyme molecules in solution retained only 7% of the biocatalytic activity recorded at pH 8, whereas the alkaline protease molecules immobilized on theTiO_2 nanoparticle/zeolite template retained as much as 47% of the catalytic activity recorded at pH 8. Even at pH 8, alkaline protease in the bioconjugate material showed significant catalytic activity.

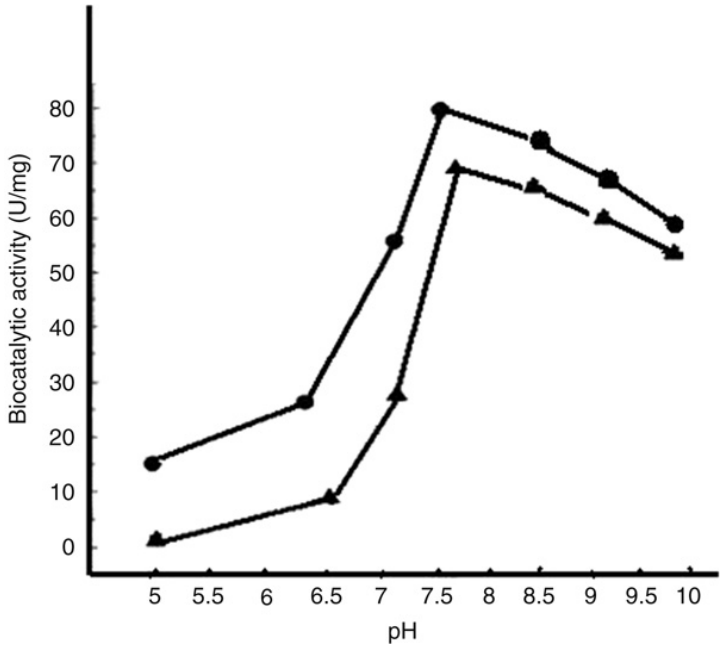

Fig. 5. pH-dependent biocatalytic activity of alkaline – protease in solution (triangles) and alkaline – protease in the nano bioconjugate material (circles) preincubating for 1 h at different pH.

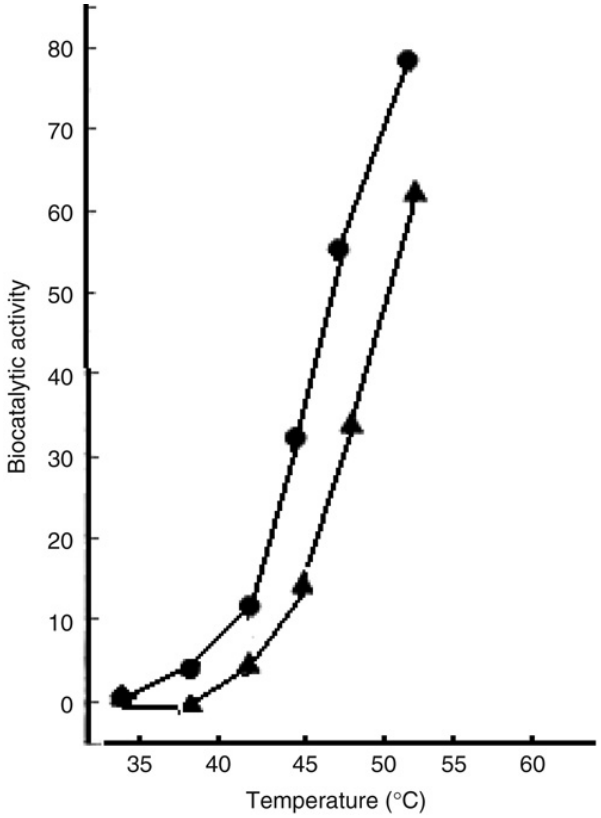

Fig. 6. Temperature dependent biocatalytic activity of free alkaline – protease in solution (triangles) and alkaline – protease in TiO_2/MCM-41 bioconjugate material (circles) preincubating for 1 h at different temperature.

4. Conclusions

In this study, we demonstrated the assembly of TiO_2 nanoparticles on the porous MCM-41 particles acts as a template for immobolization of the alkaline protease enzyme. Binding of the titanium

oxide nanoparticles on MCM-41 particles was shown by the UV–visible analysis. The size of TiO_2 nanoparticles loaded on MCM-41 was estimated from the TEM image to be about 5–12 nm. X-RAY dispersive analysis (EDAX) of the samples shown in this work gives the elemental percentage ratio of TiO_2 nanoparticle/MCM-41. Very low reactivity of the TiO_2 nanoparticles towards biomolecules allowed us to use it as a support for immobilization alkaline protease. Immobilization of the alkaline protease on TiO_2 nanoparticles/MCM-41 can be figured on the TEM image. Enhanced stability toward pH and temperature has been observed for this immobilized enzyme. The new biocatalyst material, separated from the reaction medium by sedimentation or mild centrifugation, exhibits reuse characteristics over four successive cycles. The optimum temperature for the alkaline protease in the assembly system was observed to be higher than that of the free enzyme in a solution.

Acknowledgment

This work was financially supported by Research Vice presidency of Science and Research Campus, Islamic Azad University, Tehran Iran.

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