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Synthesis of bi-metallic Au–Ag nanoparticles loaded on functionalized MCM-41 for immobilization of alkaline protease and study of its biocatalytic activity

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ABSTRACT

In this work, Au–Ag nanoparticles (Au–Ag-bi-MNPs) have been prepared on amine functionalized Si-MCM-41 (NH_2–Si-MCM -41) particles through a reduction of $AgNO_3$ and $HAuCl_4$ by $NaBH_4$ at ambient conditions. Au–Ag-bi-MNPs loaded on the NH2–Si-MCM-41, provide a good biocompatible surface for immobilization of the enzyme alkaline protease. This immobilization, presumably due to bonding between core shell nanoparticles and OH in serine 183 in alkaline protease seems to be of an ionic exchange nature. We found that the alkaline protease immobilized on the Au–Ag-bi-MNPs/Si-MCM-41 is an active biocatalyst, stable at different pH and temperature. The bio catalytic activity of free alkaline protease in solution was 64 U/mg (Units per milligram), whereas that of the alkaline protease immobilized on Au–Ag-bi-MNPs/Si-MCM-41 was 75 U/mg. This improvement of the biocatalytic activity may be due to a really increased activity per molecule of immobilized enzyme or to a purification of the enzyme. The alkaline protease molecules immobilized on the (Au–Ag)/NH_2-MCM-41 surface retained as much as 80% of the catalytic activity recorded at pH = 8, and showed significant catalytic activity of alkaline protease in the bioconjugate material. The biocatalytic materials were easily separated from the reaction medium by mild centrifugation and exhibits excellent reuse and stability characteristics over four successive cycles. The optimum temperature ranged from 35 °C–55 °C and pH = 8 for bioactivity of the alkaline protease in the assembly system was observed to be higher than that

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of the free enzyme in solution. The enzyme biocatalytic activity 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 prepared materials.

1. Introduction

Proteases execute a large variety of functions and have important biotechnological applications. They represent one of the three largest groups of industrial enzymes and find applications in detergents, leather, food, pharmaceutical industries and bioremediation processes. Probably the largest application of proteases is in laundry detergents, where they help in removing protein based stains from clothing. The enzyme should be stable and active in the presence of typical detergent ingredients for use in detergents.

Immobilization and stabilization of enzymes has received much attention in recent years. Stabilization against thermal inactivation can be performed in several ways such as cross-linking to a water insoluble carrier with a bifunctional reagent or covalent coupling to natural and synthetic polymers and entrapment in gels. Chemical modification with low molecular weight monofunctional reagents cross-linking with bifunctional reagent polymer attachment has been reported [1,2]. Development of the immobilization enzyme is a major challenge in industrial bio-catalysis and it was of great interest for many years.

But immobilization and stability of the enzymes have been revolutionized with the development of nano materials that can provide large surface areas and porous size. Since then, nanosized materials have been widely used as a support for immobilization of enzymes. This approach has been successfully applied to a wide range of enzymes and has been developed for the production of large quantities of products. Among these materials, gold and gold nanoparticles are very popular when used in conjunction with biological materials including proteins, peptides, enzymes antibodies and nucleic acids, because of their unique properties [3,4]. However, separation of the gold nanoparticle–enzyme bioconjugate material from the reaction medium is often difficult. Insofar as enzymes are concerned, advantages of immobilized enzymes over their counterparts in solution include enhanced temperature and temporal stability of the biocatalyst and ease of separation from the reaction medium, enabling multiple reuse [5–8].

In addition to gold metal, bimetallic nanoparticles either in the form of alloys or core–shell nanostructures are being increasingly investigated because of their unusual electronic/optical and catalytic properties [9–11]. Core–shell nanoparticles in particular, have attracted significant topical interest since addition of the second metal in the form of a shell provides control over the physical and chemical properties of the nanoparticles [11–16].Various groups have demonstrated that properties such as surface plasmon resonance (SPR) and surface enhanced Raman scattering (SERS) associated with gold and silver nanoparticles can be tailored by synthesizing these nanoparticles in the core–shell configuration [17–23].

Note that, the reuse characteristics of the bioconjugate materials were poor. Indeed, separation of the biocatalyst material from the reaction medium often could not be achieved, even by ultracentrifugation. This is clearly a major disadvantage in the use of otherwise biocompatible core shell nanoparticles in the immobilization of enzymes. This problem may be overcome if the core shell nanoparticles could be tethered at high density to a more massive surface, such as that provided by micron-sized particles.

Nowadays, a number of templates has been used as a support for enzyme immobilization, such as silica nanotubes, phospholipid bilayers, self-assembled monolayers, Langmuir–Blodgett films, polymer matrices, galleries of α-zirconium phosphate, mesoporous silicates such as MCM-41, silica nanoparticles, and thermally evaporated lipid films, each with its characteristic advantages and disadvantages [23–29].

In this work, we report the synthesis of nanometer-sized Ag–Au particles co-existing together inside the channels of amine functionalized MCM-41 and its use as an efficient heterogeneous catalyst. Amine functionalization prevents the agglomeration of nanoparticles and gives well-defined and stabilized nanoparticles. By this method we demonstrate enhanced temperature, pH, stability and the reuse characteristics of the bioconjugate (Au–Ag) nanoparticles when loaded on amine-functionalized MCM-41 particles.

2. Experiments

Silver nitrate ($AgNO_3$) and chloroauric acid ($HAuCl_4$) were supplied by Merck and alkaline protease was purchased from Sigma.

For Preparation of Ag colloids, all glassware used in the following procedures were cleaned in a bath of fresh prepared HCl: HNO_3 solution and rinsed thoroughly with distilled water before experiments. Silver (Ag) nanoparticles were prepared by $NaBH_4$ reduction of $AgNO_3$. One hundred milliliters of an aqueous solution containing 8 mg $AgNO_3$ were added to 2ml of 1% sodium borohydride ($NaBH_4$) solution and the mixture was kept for 1 h at least. The color of Ag nanoparticles thus prepared was gray.

*Preparation of Au nanoparticles:*In a typical experiment, 100 mL of 10^{-4} M concentrated aqueous solution of chloroauric acid ($HAuCl_4$) was reduced by 0.01 g of $NaBH_4$ at room temperature to yield a ruby-red solution containing Au nanoparticles. The color of solutions rapidly changed from yellow to red in a few minutes depending on $HAuCl_4$ concentration.

For Preparation of Ag–Au nanoparticles solution: In this step Au nanoparticle solution was added to an Ag nanoparticle solution. The color of the solution changed rapidly.

For Formation of Ag–Au-MCM-41 Composites: 15 mg of the amine-functionalized MCM-41 was dispersed in 50 mL of the prepared (Au–Ag) solution under continuous stirring. After 14 h of stirring, the originally white colored MCM-41 attained a gray hue. The MCM-41 particles capped with Au–Ag-bi-MNPs were separated by mild centrifugation, washed with double distilled water, and air-dried for further use.

For formation of Protease/ Au–Ag-bi-MNPs/*MCM-41 Bioconjugate:* Fifteen milligrams of the Au_Ag-bi-MNPs/*MCM-41*powder was dispersed in 2mL of tris–HCl buffer (0.05 M, pH8). To this solution, 100 μL of a stock solution consisting of 50 mg/mL of alkaline protease in Tris–HCl buffer (0.05 M, pH8) was added under vigorous stirring. After 2 h of stirring, the alkaline protease/(Au–Ag)/MCM-41 bioconjugate material was separated by centrifugation. For recycling studies, the alkaline protease/(Au–Ag)/NH_2–MCM-41 used material was separated from the reaction medium by centrifugation (14000 rpm).

For Biocatalytic activity measurements:

In a typical experiment, to estimate the biocatalytic activity of materials, a carefully measured amount of the alkaline protease/(Au–Ag)/MCM-41/MCM-41 in buffer was incubated with 1 mL of 0.5% casein solution at the range of 35 °C–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 used biocatalytic materials were easily separated from the reaction medium by sedimentation or mild centrifugation and exhibit excellent reuse characteristics over four successive cycles.

For temperature and pH stability measurement: The temperature stability of the alkaline-protease immobilized on (Au–Ag)/NH_2-MCM-41 surface was checked by preincubating the bioconjugate for 1 h at different temperatures in the range of 35 to 55 °C, and was compared with an identical amount of free enzyme in the– Tris–HCl buffer (0.05 M, pH8) under similar conditions. All reactions were carried out after preincubation for 1 h at different temperatures and the biocatalytic activity measured at pH 8 and 35 °C–55°C, as described earlier. Three separate measurements were done to check the reproducibility of the assay.

The Powder X-ray diffraction (XRD) pattern was recorded on a Seisert Argon 3003 PTC using nickel-filtered XD-3a CuKα radiation ($\lambda = 1.5418$ Å). A Philips EM208 and microscope operated at 100 kV Transmission Electron Microscope (TEM) was used to observe the morphology and size

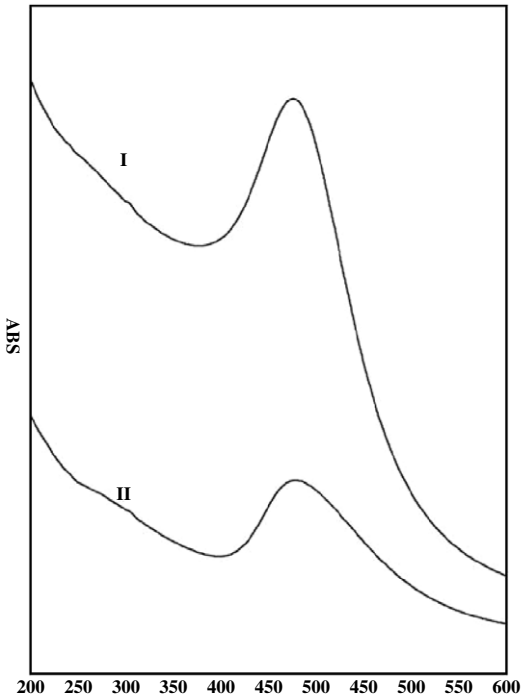

Fig. 1. UV–Vis spectrum recorded from the as prepared from as prepared Au–Ag-bi-MNPs solution (curve 1) and Au–Ag-bi-MNPs solution after stirring with amine-functionalized Si-MCM-41 for 12 h and centrifugation (curve 2, see text for details).

distribution of the Ag nanoparticles. The samples were prepared by carbon-coated copper grids. Scanning Electron Microscopy (SEM) images of the prepared silver nanocomposites were obtained using a Philips electron microscope equipped with a Dispersive analysis of X-RAY (EDAX). The samples were rinsed with distilled water, dried and coated with a thin layer of gold by evaporation at vacuum to form a conducting film. The absorption measurement was carried out by UV-visible spectrometer (CARRY).

3. Results and discussion

3.1. UV-Vis spectroscopy studies

Fig. 1 shows the UV-Vis spectra recorded from the as prepared Au–Ag-bi-MNPs solution (curve 1) and the supernatant solution obtained after stirring with MCM-41 for 12 h and filtration (curve 2). Only one surface plasmon resonance band can be seen clearly at ca.410 nm and it was concluded that gold and silver atoms are homogeneously mixed in the alloy nanoparticles. After stirring the Au–Ag-bi-MNPs solution with the MCM-41 for 12 h, there was a loss in intensity of the surface plasmon resonance due to a decrease in concentration of Au–Ag-bi-MNPs in the aqueous solution (curve 2).

3.2. TEM micrographs

Fig. 2 shows TEM image of the as prepared Au–Ag-bi-MNPs capped mesoporous MCM-41. The particles were fairly spherical, with slightly irregular edges. The Au–Ag-bi-MNPs core–shell surrounded the surface of the amine-functionalized MCM-41 particles can be seen clearly in the image. As mentioned elsewhere, the Au–Ag-bi-MNPs bind to the NH_2-MCM-41 template via the primary amine groups. The size of nano particle that loaded on MCM-41 was estimated at 3–5 nm.

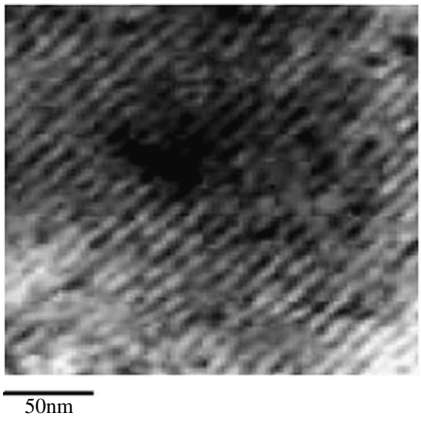

Fig. 2. Representative TEM images of the Au–Ag-bi-MNPs on the carbon- coated TEM grid. TEM micrograph of the gold nanoparticles (dark spots) bound to the surface of the MCM-41 particles-alkaline protease.

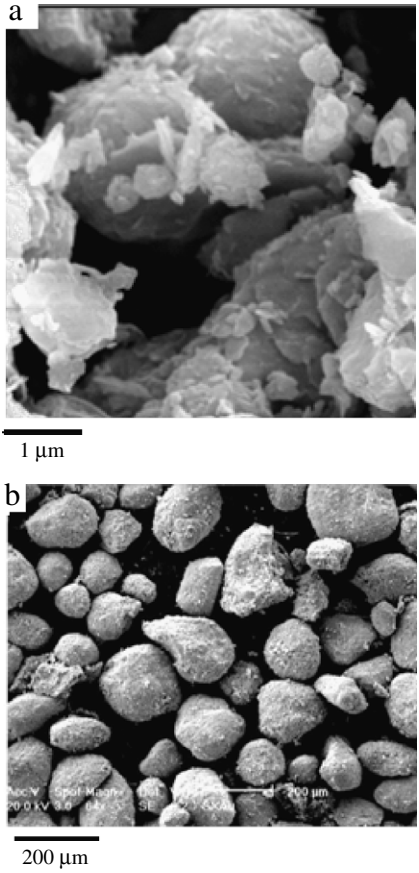

Fig. 3. (a) SEM image of Au–Ag-bi-MNPs./MCM-41 (b) Alkaline protease immobilized Au–Ag-bi-MNPs./MCM-41 bioconjugate material. All films were cast onto Si(111) wafers.

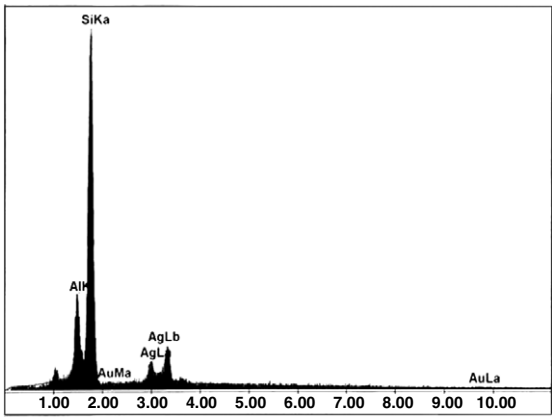

Fig. 4. Dispersive analysis of X-RAY (EDAX) result for Au_Ag-bi-MNPs capped in MCM-41.

Table 1
EDAX ZAF quantification (standardless) element normalized.

Element	MCM-41/ Wt%	Au–Ag/MCM-41/ Wt%	MCM-41/ At%	Au–Ag/MCM-41/ At%
Al	1.95	1.54	2.03	1.91
Si	98.05	76.29	97.97	92.24
AgL	0	13.78	0	4.30
AuL	0	8.39	0	1.43
Total	100	100	100	100

Table 2
Biocatalytic activities of the alkaline protease immobilized on Au–Ag/NH_2MCM-41 bioconjugate materials.

No. of cycles	U/mg Biocatalytic activity of alkaline protease immobilized on TiO2/MCM-41(U/mg)
1	50
2	32
3	11
4	2

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

Fig. 3(a, b) show SEM micrographs of drop-cast films of as prepared Au–Ag-bi-MNPs/MCM-41 and alkaline protease immobilized on this bioconjugate material. The surface texture of the MCM-41 particles capped with (Au–Ag) bi-MNPs was quite homogeneous with some regular orientation of Au–Ag-bi-MNPs. The SEM instrument was clearly unable to resolve the (Au–Ag) bi-MNPs bound to the surface of the MCM-41. After conjugation of the (Au–Ag) bi-MNPs/MCM-41 with alkaline protease, thin sheets of presumably aggregated enzyme can be seen together with smooth (Au–Ag)/MCM-41 spheres (Fig. 3b). The X-RAY dispersive analysis (EDAX) of the samples shown in Fig. 4 and Table 1, gives the percentage ratio of Au and Ag, in Au–Ag-bi-MNPs capped MCM-41 (Table 1).

3.4. Biocatalytic activity

The comparative biocatalytic activity of the free alkaline protease in solution and alkaline protease immobilized on (Au–Ag)/MCM-41 bioconjugate was estimated from the UV-vis spectroscopy measurements. The results show that 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 75U/mg. This increased enzyme catalytic activity may be related to the catalytic activity improvement in immobilized enzyme or to the purification of the enzyme.

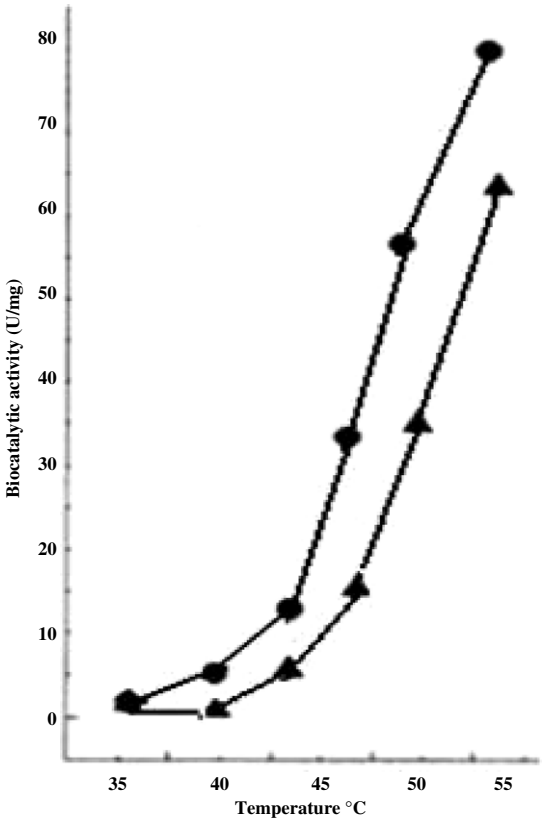

Fig. 5. The pH-dependent of biocatalytic activity of the free alkaline protease in solution (triangles) and alkaline–protease immobilized on Au–Ag-bi-MNPs/ NH_2-MCM-41(circles) pre- incubated for 1 h at different pH.

3.4.1. Retention

The most important aspect of this study concerns the retention of biocatalytic activity for alkaline protease enzyme after immobilization of the enzyme onto (Au–Ag)/ MCM-41 surface. The result of the bioactivities calculated over 4 sequential reuse cycles is shown in Table 2. This data confirmed that the alkaline protease molecules immobilized on Au–Ag-bi-MNPs/Si-MCM-41surface retained as much as 80% of the catalytic activity (recorded at pH 8) and showed significant catalytic activity of alkaline protease in the bioconjugate material. The biocatalytic materials was easily separated from the reaction medium by mild centrifugation and exhibits excellent reuse and stability characteristics over four successive cycles.

3.4.2. Temperature stability

Variation in biocatalytic activity of free alkaline protease in solution (triangles) and immobilized alkaline protease on the (Au–Ag)/MCM-41 template (circles) as a function of temperature stability is shown in Fig. 5. As it can be seen from this fig., the optimum temperature ranged from 35 °C–55 °C at pH = 8 for bioactivity of the alkaline protease in the assembly system which was observed to be higher than that of the free enzyme in solution.

As noted early this reaction was carried out after preincubation for 1 h at the different temperatures and measuring the biocatalytic activity at pH = 8, 35–55 °C (Table 2).

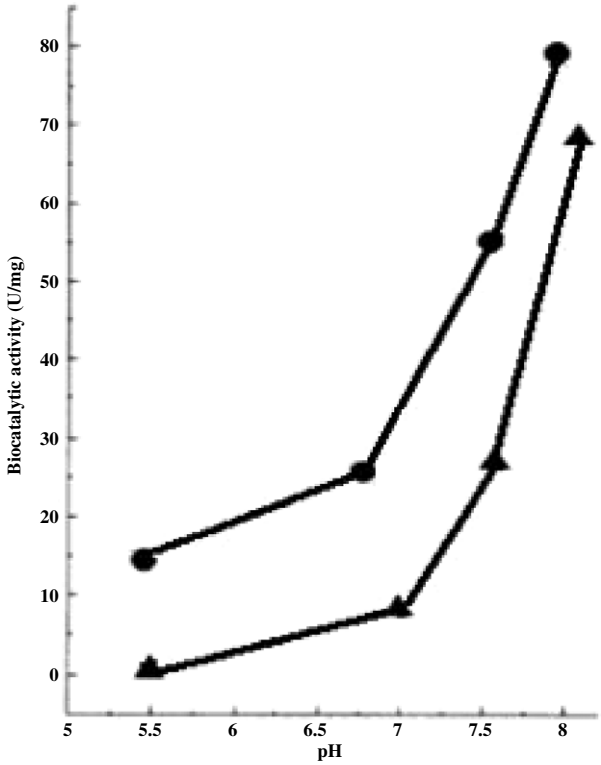

Fig. 6. Temperature dependence of biocatalytic activity of the free alkaline–protease (triangles) and alkaline protease immobilized/Au–Ag-bi-MNPs (Au–Ag)–NH_2-MCM-41 bioconjugate material (circles) preincubated for 1 h at different temperature. The solid lines in all cases are visual aids and have no physical significance.

3.4.3. pH stability

pH value is one of the most influential parameters altering enzyme activity in an aqueous medium. Fig. 6. Shows the pH-dependent variations in biocatalytic activity of the bioconjugate and free enzyme at five different pH values (pH 6, 6.5, sodium phosphate buffer; pH = 8 and 10, glycine–NaOH buffer) by preincubation for 1 h at 27 °C. It has been seen that optimum biocatalytic activity in both 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 (Au–Ag)/NH_2-MCM-41 surface retained as much as 80% of the catalytic activity recorded at pH 8. Even at pH 8, alkaline protease in the bioconjugate material showed significant catalytic activity.

4. Conclusion

In this study, we demonstrated that the assembly of Au–Ag-bi-MNPs with NH_2-MCM-41 particles provides a good biocompatible surface for immobilization of the alkaline protease enzyme. This method of immobilization of enzyme on the nanoparticle seems to be advantageous because we can use any other support with good mechanical properties and there is possibility of co-immobilization of different nanoparticles carrying different enzymes. The immobilization presumably due to bonding between core shell nanoparticles and OH in serine 183 in alkaline protease seems to be of an ionic exchange nature. Binding of the nanoparticles on functionalized MCM-41 was shown by the UV-visible analysis. The size of the Au–Ag nanoparticles loaded on NH_2-MCM-41was estimated from the TEM image to be about 3–5 nm. X-RAY dispersive analysis (EDAX) of the samples shown in this

work gives the elemental percentage ratio of Au and Ag nanoparticles on a NH_2-MCM-41support. Immobilization of the alkaline protease on the Au–Ag-bi-MNPs/NH_2-MCM-41 was shown on the SEM image. Enhanced stability toward pH and temperature has been observed for this immobilized enzyme. The optimum temperature for the alkaline protease activity in the assembly system was observed to be higher than that of the free enzyme in solution. The improved enzyme biocatalytic specific activity estimated from the UV-Vis spectroscopy measurement may be due to a greatly increased activity per molecule of enzyme or to the purification of the enzyme. The new biocatalyst material, separated from the reaction medium by sedimentation or mild centrifugation, exhibits reuse characteristics over four successive cycles.

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References

[1] M. Lum, P. Metcalf, S.C. Harrison, D.C. Wiley, J. Appl. Crystallogr. 20 (1987) 235–242.
[2] W. Bode, F.X. Gomis-Ruth, R. Huber, R. Zwilling, W. Stocker, Nature 358 (1992) 164–167.
[3] G. Chumanov, K. Sokolov, T.M. Cotton, J. Phys. Chem. 100 (1996) 5166.
[4] D.G. Georganopoulou, D.E. Williams, C.M. Pereira, F. Silva, T.J. Su, J.R. Lu, Langmuir 19 (2003) 4977.
[5] E.M. Oliveira, S. Beyer, J. Heinze, Bioelechem. 71 (2007) 186.
[6] D. Brambley, B. Martin, P.D. Prewett, Adv. Mater. Opt. Electron. 4 (1994) 55.
[7] R. Jin, Y. Cao, C.A. Mirkin, K.L. Kelly, G.C. Schatz, J.G. Zheng, Science 294 (2001) 1901.
[8] T. Huang, R.W. Murray, J. Phys. Chem. B 105 (2001) 12498.
[9] T.S. Ahmadi, Z.L. Wang, T.C. Green, A. Henglein, M.A. El-Sayed, Science 272 (1996) 1924.
[10] S.C. Tsang, C.H. Yu, X. Gao, K. Tam, J. Phys. Chem. B 110 (2006) 16914–16922.
[11] M.S. Sadjadi, N. Farhadyar, K. Zare, J. Nanosci. Nanotechnol. 9 (2009) 1365–1368.
[12] L. Zeng, K.K. Luo, Y.F. Gong, J. Mol. Catal. B: Enzym. 38 (2006) 24–30.
[13] R.A. Sheldon, Adv. Synth. Catal. 349 (2007) 1289.
[14] M. Sastry, Trends Biotechnol. 20 (2002) 185.
[15] M. Sastry, A. Kumar, P. Mukherjee, Coll. Surf. A. 181 (2001) 255.
[16] M. Sastry, M. Rao, K.N. Ganesh, Acc. Chem. Res. 35 (2002) 847.
[17] C.M., Quantitation of protein, Meth. Enzymol. 182 (1990) 50.
[18] R. Amadelli, A. Molinari, I. Vitali, L. Samiolo, G. Maria Mura, A. Maldotti, Catal. Today 101 (2005) 397.
[19] E.M. Oliveira, S. Beyer, J. Heinze, Bioelectrochemistry 71 (2007) 186.
[20] C. Mateo, O. Abian, M. Bernedo, E. Cuenca, M. Fuentes, G. Fernandez-Lorente, et al., Enzyme Microb. Technol. 37 (2005) 456–462.
[21] K.M. Koeller, C.H. Wong, Nature 409 (2001) 232–240.
[22] E. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[23] V. Iyer Padma, A. Laxmi, Process Biochemistry 43 (2008) 1019–1032.
[24] L. Betancor, F. Lopez-Gallego, A. Hidalgo, N. Alonso, G. Dellamora-Ortiz, J.M. Guisan, et al., J. Biotechnol. 121 (2006) 284–289.
[25] L. Wilson, J.M. Palomo, G. Fernandez-Lorente, A. Illanes, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 38 (2006) 975–980.
[26] A. Hidalgo, L. Betancor, F. Lopez-Gallego, R. Morenob, J. Berenguer, R. Fernandez-Lafuente, et al., Enzyme Microb. Technol. 33 (2003) 278–285.
[27] G. Irazoqui, C. Giacomini, F. Batista-Viera, B.M. Brena, J. Mol. Catal. B Enzymatic. 46 (2007) 43–51.
[28] E. Kalogeris, Y. Sanakis, D. Mamma, P. Christakopoulos, D. Kekos, H. Stamatis, Enzyme Microb. Technol. 39 (2006) 1113–1121.
[29] M. Yoshimoto, H. Sakamoto, N. Yoshimoto, R. Kuboi, K. Nakao, Enzyme Microb. Technol. 41 (2007) 849–858.