The Preparation and Papain Immobilization of Novel Mesoporous SiO_2/Fe_3O_4 Microspheres with Size-tunable Hollow Cavity

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Abstract. The SiO_2 microspheres with different diameters were prepared by a seeding technique through adjusting the mass fraction ratio of TEOS and silica sol in the reaction solution. The mesoporous SiO_2/Fe_3O_4 microspheres with size-tunable hollow cavity were prepared by using the SiO_2 microspheres of different diameters as templet. The magnetic mesoporous SiO_2/Fe_3O_4 hollow microspheres were used as the carriers to immobilize papain, and the effect of hollow cavity on papain immobilization was researched in detail. The results show that the amount of papain immobilization increased with the size of hollow cavity increasing. The amount of papain immobilization washigh(296 $mg \cdot g^{-1}$), when the hollow cavity average size was about 330nm. It was indicated that the hollow cavity is a natural warehouse storing papain molecules effectively. When the temperature was below 60℃ and the pH was from 2 to 8, the residual activity of immobilized papain still remained more than 60%, much higher than that of free papain.

Introduction

Papain has been widely used in medicine, food, cosmetic and wine industry[1]. However, the enzyme is easy to beinactivated due to environmental factors, such as pH and temperature in the catalytic process. Moreover, it is difficult that the enzyme molecules are separated from the mixed reactant, resultingin the difficulty in recycling. Therefore, these problems largely restrict the application of the enzyme molecules. However, the technology of enzyme immobilization[2, 4] can solve the above problems well.

In recent years, mesoporous SiO_2 materials are considered as the most promising inorganic immobilized carriers[5, 8]. It is mainly due to that it has desirable biological compatibility, easy functionalization, adjustable mesoporous channel for different sizes enzyme molecules, high specific surface area[9, 11] and other significant advantages. Mesoporous silica materials as enzyme carriers can immobilize enzyme well, but it is difficult that the mesoporous carriers are separated and reused from reaction solution. To overcome this disadvantage, several research groups devote themselves to some new magnetic mesoporous composite materials, which could be separated under the added magnetic field. Deng and co-workers [12] have investigated the magnetic mesoporous silica microspheres by hydrothermal synthesis method. Furthermore, The magnetic mesoporous silica microspheres as immobilized enzyme carriers can be efficientlyseparated from the catalytic reaction solution. Our reaearch group[13, 16] have developed a novel magnetic mesoporous SiO_2/ Fe_3O_4 hollow microsphere through coating mesoporous silica on the magnetic Fe_3O_4 hollow microspheres constructed with Fe_3O_4 nanorods. And these novel microspheres have lots of advantages, such as independently controllable magnetic layer and mesoporous layer, and the size-tunablehollow cavity. It is interesting that the hollow cavity is supposed to be as the natural warehouse for enzyme storage, explaining the large amount of papain immobilization in our previous work. However, the effect of the size of hollow cavity on papain immobilization has rarely been reported in detail. In this paper, the magnetic mesoporous SiO_2/Fe_3O_4 microspheres with different diameters of hollow cavity were prepared by adjusting the diameter of silica microspheres as templet. The effect of hollow cavity on papain immobilization was investigated detailedly.

Experimental

Chemical

Tetraethoxysilane (TEOS, 99%, Aldrich),Ferrous chloride ($FeCl_2$•$4H_2O$, ≥99.7%), ethanol, sodiumhydroxide(NaOH,>97%), Dibasic sodium phosphate (Na_2HPO_4·$12H_2O$，≥99.0%), Sodium dihydrogen phosphate (NaH_2PO_4·$2H_2O$, ≥99.0%), Sodium citrate ($C_6H_8O_7$, ≥99.5%), hydrofluoricacid (HF, 40wt%)and ammonia solution (NH_3•H_2O, 25wt%)were purchased from Beijing Chemical Co..Cetyltrimethylammonium bromide (CTAB, >99%) and 1,3,5-triisoporpylbenzene (TMB, ≥99.6%) were purchased from Tianjin Fuchen Chemical Co.. Papain (powder, 2500units/mg), Casein($C_{47}H_{48}N_3NaO_7S_2$, SIGMA). Deionized water was used in all experiments. All reagents were used as-received without further purification.

Preparation of Samples

The Preparation of SiO_2 Microspheres with Different Diameter

Firstly, 0.01g silic sol, 14g NH_3·H_2O and 21.60g H_2O was taken into 200ml volumetric flask, and used ethanol as dispersingagent. Then the mixture was transferred into the 500ml flat bottom flask, and preheated for 10min at 35℃. The TEOS with different mass concentration was added into the 500ml flat bottom flask and reacted for two hours. After that it was continued to stir for 2h. Finally, the SiO_2microspheres were washed with alcohol and deionized water twice. The as-prepared samples were named as A_1, A_2 and A_3, when the molar ratios of TEOS and silica sol is 3:1, 5:1 and 7:1, respectively.

Preparation of Mesoporous SiO_2/Fe_3O_4 Microspheres with Size-tunable Hollow Cavity

Briefly, hydrofluoricacid was added into ferrous chloride (Fe^{2+}) solution (0.1M) to make the ratio of Fe^{2+} to F^- is 3:1, adjusted the solution pH to 4.60 by dropping an ammonia solution. Then the as-prepared monodisperse SiO_2 microspheres with different diameter were added into the solution, and the mixture was stirred at 333 K in a water bath for 2 h. After repeating the above procedure twice, the SiO_2cores was removed through the NaOH solution (5wt%) and kept in a water bath at 313 K for two hours.Then the as-synthesized β-FeOOH hollow microspheres were dispersed in aqueous solution containing NaOH (0.07g), CTAB (0.268g) and TMB(0.176g). The TEOS (1.26g) was added into stirring suspension at a constant rate.The mixture was stirred for 0.5 h and then was settled for 2h. The microspheres were washed with ethanol and deionized water twice and separated by centrifugation.The final mesoporous SiO_2/Fe_3O_4 microspheres were obtained by calcination at 773 K in air and then being reduced in a flowing gas mixture of H_2 (5%) and Ar (95%) at 623K.The as-prepared samples were named asM_1, M_2 and M_3 with different diameter monodisperse silica microspheres (A_1, A_2, A_3) as templet.

Immobilization of Papain on Mesoporous Supports and Activity Stabilities

The mesoporous SiO_2/Fe_3O_4 hollow microspheres (10mg) were first stirred at room temperature for different time (0.5~12 h) in 10mL of sodium phosphate buffer (pH=7.0) containing a certain amount of papain (10mg).The supernatant was separated from solid materials by centrifugation. The amount of papain was monitored by a ultraviolet-visible spectrophotometry (UV-vis), that is, by measuring the papain absorbance at 285 nm before and after adsorption. The standard curve of papain can be obtained by measuring different concentrations of papain solution (0.1, 0.25, 0.4, 0.5, 1.0mg/ml).And then the loading of papain can be calculated by using the following equation:

$$q_t = \frac{(c_0 - c_t)V}{m} \quad (1)$$

Where c_0 is the initial concentration, c_t is apparent concentration at time t, V is the total volume of dissolution medium, and m is the mass of carrier materials.

The activity of free and immobilized papain was determined in a reaction medium containing

2mL casein aqueous solution (1mg•mL^{-1}) as substrate in phosphate buffer (pH=7.0) at 50℃ in the absorbance at 285 nm. Papain was added into the substrate solution and stirred immediately for 4h. The absorbance of the supernatant was determined by a UV-vis spectrophotometer through centrifuging the reaction solution.The free and immobilized papain were put in different buffer solutions (pH=2.0~8.0) at room temperature for 24h to detect the pH stabilities. The thermal stabilities of free and immobilized papain activity were determined in different temperature (30~80℃) for 2h kept in the buffer solution (pH=7.0). Here the papain activity was expressed in relative units (%), and the maximal activity value of free and immobilized papain measuredat pH=7.0 and 50℃.

Results and Discussion

The Structure Formation of the Samples with Different Sizehollow Cavity

Fig.1 shows the TEM images of the monodispersed SiO_2 microspheres and the mesoporous SiO_2/Fe_3O_4 microspheres with different diameters. From Fig.1A, 1B and 1C, the SiO_2 microspheres have desirable monodispersity. The average diameter of SiO_2 microspheres (sample A_1,A_2,A_3) was about 230nm,280nm and 330nm, respectively. From Fig.1D, E and F, it could be seen that the magnetichollow microspheres were constructed with Fe_3O_4nanorod, andthe SiO_2 core is removed without causing the collapse of the hollow Fe_3O_4layer. And the hollow SiO_2/Fe_3O_4 microspheres was easily separated from the reaction solution due to the magnetism of the Fe_3O_4nanorod. But the magnetism of the hollow Fe_3O_4nanorod layer has been researched in our previous work[13,15]. The mesoporous SiO_2/Fe_3O_4 microspheres with size-tunable hollow cavity have been prepared successfully, on the basis of the SiO_2microspheres with the different diameters.The averagesize of hollow cavity (sample M_1, M_2, M_3) is about 230nm, 280nm and 330nm, respectively. From Fig.1D, E and F, it can clearly be seen that themesoporous silica has been coated into the interspace of the Fe_3O_4layer.

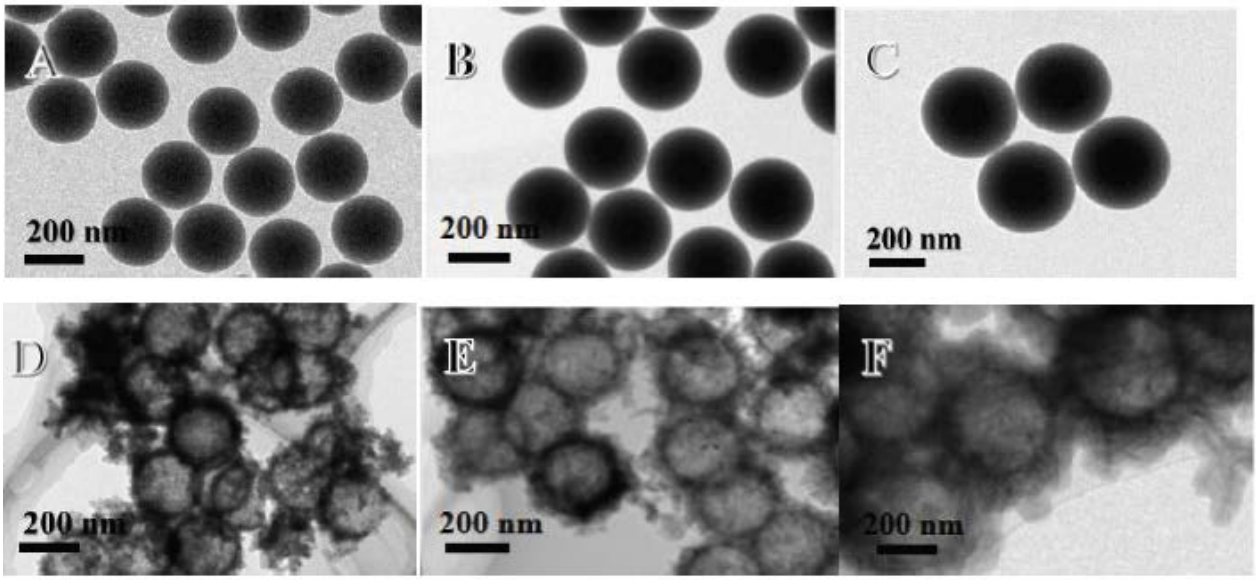

Fig.1 TEM images of mesoporous SiO_2/Fe_3O_4 microspheres with different sizes in hollow cavity (A)the sample A_1, (B) the sampleA_2, (C) the sampleA_3,(D) the sampleM_1,(E) the sampleM_2, (F) the sampleM_3

The Mesopore Structure of Mesoporous SiO_2/Fe_3O_4 Hollow Microspheres and Papain-mesoporous SiO_2/Fe_3O_4 Hollow Microspheres

The N_2 adsorption-desorption isotherms and the corresponding pore size distributions of the sample $M_2$and the sample P-$M_2$are shown in Fig.2. From Fig.2A, it can be seen that the mesoporous SiO_2

/Fe_3O_4 hollow magnetic microspheres exhibits type Ⅳ adsorption-desorption isotherms, suggesting that the as-prepared SiO_2/Fe_3O_4 hollow composite microspheresare mesoporous materials. Before adsorpting papain, the composite microspheres have obvious hysteresis loop at p/p_0=0.4~0.5, demonstrating that the mesoporous microspheres have the mesopore and a narrow pore size distribution. From Table 1, it can be seen that the surface area of the composite microspheres is 739 m^2/g, the pore volume is 0.84 cm^3/g, and the peak pore size is 3.12nm. The hysteresis loop of mesoporous SiO_2 /Fe_3O_4 hollow microspheres almost disappeared after immobilizing papain, suggesting that the mesoporous channel of composite microspheres is reduced and the BET surface area and pore volume decreased significantly. The surface area and the pore volume of the papain-mesoporous SiO_2 /Fe_3O_4 hollow microspheres is only 116 m^2/g and 0.41 cm^3/g, respectively.From Fig.2B, it could be seen that the pore size distribution of the papain-mesoporous SiO_2 /Fe_3O_4 hollow microspheres is very wide and almost all of them are big mesopore due to the papain molecules occupied the pore channel. This shows that the papain molecules can be adsorbed on mesoporous SiO_2 /Fe_3O_4 hollow magnetic microspheres effectively.

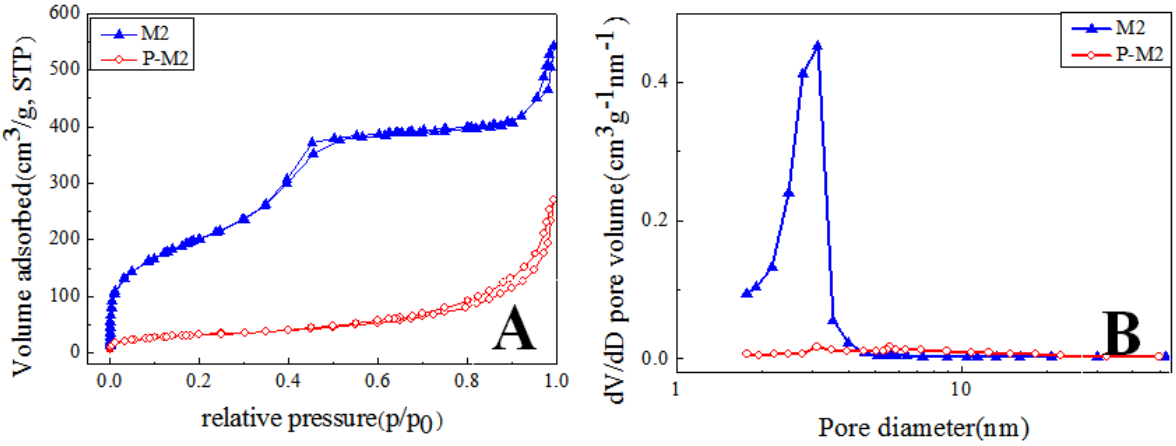

Fig.2 N_2 adsorption-desorption isotherms(A) and pore size distributions(B) of mesoporous SiO_2 /Fe_3O_4 hollow microspheres(sampleM_2) and papain-mesoporous SiO_2 /Fe_3O_4 hollow microspheres(sample P-M_2)

Table1 Structure parameters of mesoporous SiO_2/Fe_3O_4 hollow microspheres(sampleM_2) and Papain-mesoporous SiO_2/Fe_3O_4 hollow microspheres(sample P-M_2)

sample	Surface area(m^2/g)	Pore volume (cm^3/g)	The peak pore size(nm)
M_2	739	0.84	3.12
P-M_2	116	0.41	3.10

The Papain Adsorption Kinetics on Mesoporous SiO_2 /Fe_3O_4 Hollow Microspheres

To further explore the internal relation between the hollow cavity structure of mesoporous composite microspheres and the performance of papain immobilization, the researchgroup have investigated the effect of the novel mesoporous SiO_2/Fe_3O_4 microspheres with size-tunable hollow cavity on the papain immobilization behavior. Papain adsorption curves(A) and its pseudo-second-order kinetic curves(B) of the mesoporous SiO_2/Fe_3O_4hollow microspheres (sample M_1, M_2, M_3)with different sizes are shown on Fig.3. From Fig.3A, it could be seen that the adsorption rate of composite microspheres for papain is rapid and the adsorption amount is increased obviously within one hour. The adsorption rate increased slowly at 1~4h and the adsorption amount was basicly saturated at 4h. The adsorption amount of sample M_1, M_2, M_3 reached 242mg·g^{-1}, 266mg·g^{-1} and 296mg·g^{-1} at 4h, respectively. This shows that the size of hollow cavity has a great influence on the amount of papain immobilization. And the amount of papain

immobilization increased with the size of hollow cavity increasing. From Fig.3B, it could be seen that the fitting lines of all samples (M1, M2, M3) have a desirable linear correlation, and the correlation coefficient R_2 are 0.9996, 0.9987 and 0.9999, respectively. The fitting line formulas of all samples(M_1, M_2, M_3) are y=0.00166+0.00399x, y=0.00216+0.00357x and y=0.00216+0.00357x, respectively. According to the slope formulas, it can be calculated that the maximum adsorption amounts of sample M_1, M_2, M_3 reached 250mg·g^{-1}, 280mg·g^{-1} and 301mg·g^{-1}, respectively. Therefore, this almost reached the experimental saturated adsorption amount, and the adsorption amounts of sample M_1, M_2, M_3 for papain are suitablefor pseudo-second-order kinetic dynamic model. Meanwhile, the adsorption rate of sample M_1, M_2, M_3 are 9.638×10^{-3}, 5.905×10^{-3} and 0.0225. The adsorption rate of sample M_3 is obviously bigger than that of the sample M_1 and M_2, suggesting that the adsorption rate of sample M_3 is much faster. This is mainly due to that the peak pore size of sample M_3 is much biggest, and therefore, the adsorption amount of sample M_3 is also increased.

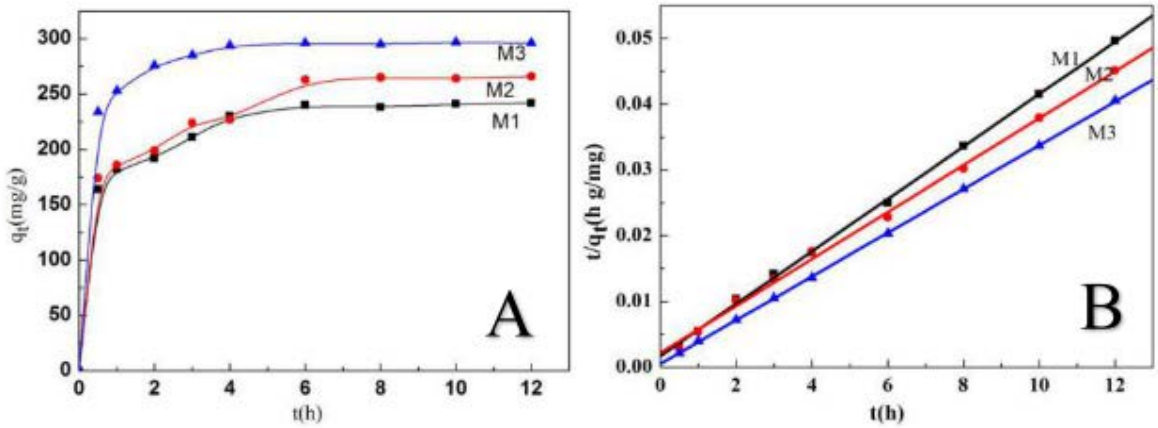

Fig.3 Papain adsorption curves(A) and its pseudo-second-order kinetic curves(B) of the mesoporous SiO_2/Fe_3O_4hollow microspheres with different sizes

The Temperature Stabilities and pH Stabilities

The enzyme molecules are easily affected by environmental factors, such as temperature and the pH of buffer solution. Temperature stabilities and pH stabilities of free papain and immobilized papain with mesoporous SiO_2/Fe_3O_4 hollow microspheres of different sizes are shown as Fig.4. Compared free papain with immobilized papain, it conld be seen that the residual activity of immobilized papain was much higher than free papain. Meanwhile, it is also seen that, the residual activity of free and immobilized papain were all first increased and then decreased in 30℃~80℃, and its optimum temperature is 50℃. When the temperature was above 60℃, the residual activities of free and immobilized papain were both reduced quickly. Moreover, it is due to that that the mesopore wall of the composite microspheres has played a protective role on the papain molecules. This could reduce the temperature effect on the activity of papain and further improve the temperature stability. Fig.4B shows the residual activity of free and immobilized papain in phosphate buffer solution with different pH (2.0~8.0). From Fig.4B, it could be seen that the optimum pH of free and immobilized papain were both 7.0. The residual activities of free papain increased faster than immobilized papain from pH 2 to pH 7. This is mainly due to that the carrier of composite microspheres has played an important isolation role for papain molecules to avoid its deactivation. Thus, it improve the pH stability.

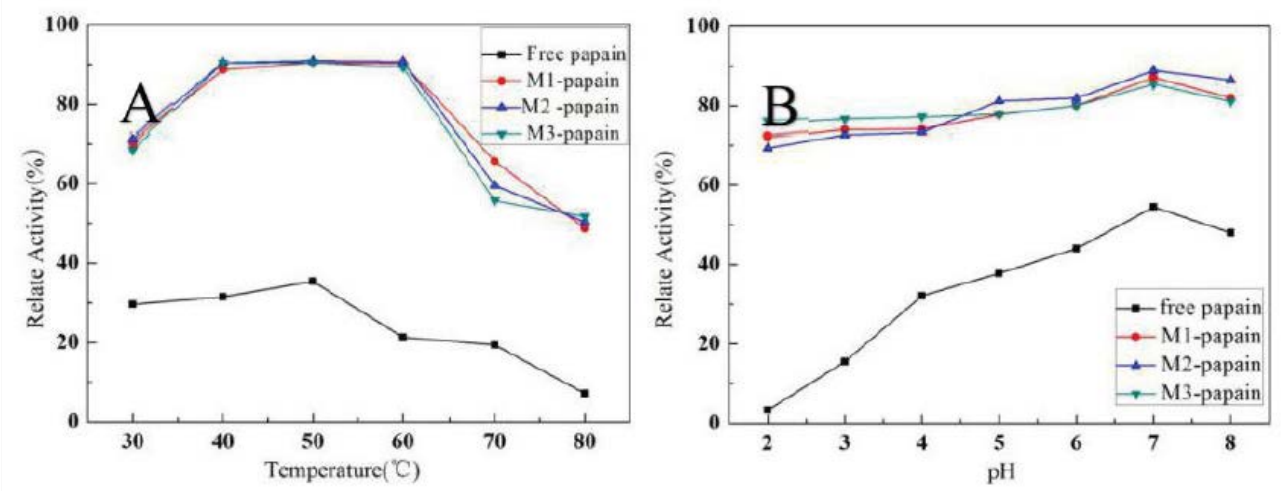

Fig.4 Temperature stabilities (A) and pH stabilities (B) of free papain and immobilized papain with mesoporous SiO_2/Fe_3O_4 hollow microspheres of different sizes

Conclusion

In this paper, magnetic mesoporous SiO_2/Fe_3O_4composite hollow microspheres have been successfully prepared via a sol-gel and hydrothermal synthesis process by using monodisperse silica spheres as templat. And the size of hollow cavity could be effectivelycontrolledby adjusting the size of monodisperse silica microspheres with average diameters of 230nm, 280nm and 330nm, respectively. The amount of immobilized papain of composite microspheres with large hollow cavity of 330nm reached high immobilized amount ($296mg \cdot g^{-1}$).And the amount of papain immobilization increased with the size of hollowcavity increasing. It is due to that the hollow cavity could be a natural warehouse for enzyme storage. Meanwhile, the temperature stability and pH stability of the immobilized papain are both more desirablethan that of free papain.

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References

[1] S. Nitsawang, H.K. Rajni,P. Kanasawud, Purification of papain from carica papay alatex :Aqueous two-phase extraction versus two-step salt precipitation, Enzyme Microb Techn.39(2006)1103.

[2] H. Ma,J. He,D.G. Evans,Immobilization of Lipase in a Mesoporous Reactor Based on MCM-41, J. Mol. Catal. B: Enzym. 30(2004) 209-217.

[3] M. Park, S.S. Park, M. Selvaraj, D.Y. Zhao, Hydrophobic mesoporous materials for immobilization of enzymes, Micropor. Mesopor. Mater.124(2009)76-83.

[4] E. Serra, A. Mayoral, Y. Sakamoto, Immobilization of lipase in ordered mesoporous materials: Effect of textural and structural parameters, Micropor. Mesopor. Mater.114(2008)201-213.

[5] X. Xu, P. Lu, Y. Zhou, Laccase Immobilized on Methylene Blue Modified Mesoporous Silica MCM-41/PVA, Mater. Sci. Eng. C. 29(2009)2160-2164.

[6] A. Salis, M. Bhattacharyya, M. Monduzzi, Role of the Support Surface on the Loading and the Activity of Pseudomonas Fluorescens LipaseUsed for Biodiesel Synthesis, J. Mol. Catal. B- Enzym. 57(2009)262-269.

[7] J. Foede, E. Tully, A. Vakurov, Chemical Modification and Immobilisation of Laccase from Trametes Hirsuta and from Myceliophthora Thermophila, Enz. Mic. Tec. 46(2010)430-437.

[8] H. Qi, J. Han, X.L. Jiang, Preparation and Hydrothermal Stability of Organic- inorganic Hybrid Silica Membrane, Int. J. Inorg. Mater. 25(2010)758-764.

[9] M.Arruebo, M.Galán, N. Navascués,Development of Magnetic Nanostructure Silica-Based Materials as Potential Vectors for Drug-Delivery Applications, Chem. Mater. 18(2006): 1911-1920.

[10]N. K. Mal, M. Fujiwara, Y. Tanaka, Photocontrolled Reversible Release of Guest Molecules from Coumarin-Modified Mesoporous Silica, Nature. 421(2003) 350-359.

[11]Y. F. Zhu, J. Shi, W. H. Shen,Stimuli-Responsive Controlled Drug Release From a Hollow Mesoporous Silica Sphere/Polyelectrolyte Multilayer Core-Shell Structure, Angew. Chem. Int. Ed. 32(2005)5083-5090.

[12]Y.H. Deng, D.W. Qi, C.H. Deng, Superparamagnetic High-Magnetization Microspheres with an Fe_3O_4 @ SiO_2 Core and Perpendicularly Aligned Mesoporous SiO_2 Shell for Removal of Microcystin, J. Am. Chem. Soc. 130(2008)28-29.

[13]M.M. Wang, Q.Y. Li, Q. Wei, Studies on Laccase Immobilization of Mesoporous SiO_2 /Fe_3O_4 Hollow Microspheres, Chem J Chin U. 2(2013)299-305.

[14]X.H. Li, Q.Y. Li, Q.Q. Zhu, Study on Pore Size Regulation and Laccase Immobilization of Mesoporous SiO_2 /Fe_3O_4 Hollow Microspheres, Journal of Synthetic Crystals. 43(2014)2958-2965.

[15]Q.Y. Li, Q.Q. Zhu, Q. Wei, Pore Controllable Regulation and Laccase Immobilization of Hollow Mesoporous SiO_2 /Fe_3O_4 Microspheres, Advanced Materials Research. 833(2014)93-98.

[16]M.M. Wang, Q.Y. Li, Q. Wei, Monodisperse Mesoporous SiO_2 /Fe_3O4/SiO_2 Microspheres: Preparation and Laccase Immobilization, Chemical Reaction Engineering and Technology. 28(2012)123-128.