

Immobilization of laccase on a novel ZnO/SiO₂ nano-composited support for dye decolorization

Wei-Xun Li, Huai-Yan Sun and Rui-Feng Zhang¹

Faculty of Materials Science and Chemical Engineering, Ningbo University, Ningbo, Zhejiang 315211, PR China

E-mail: zhangruifeng@nbu.edu.cn

Abstract. ZnO nanowires were introduced into macroporous SiO₂ by means of in situ hydrothermal growth. The obtained nano-composite was then used to immobilize laccase (secured from *Trametes versicolor*) through the process of static adsorption. The average loading amount was as high as 193.4 $\mu\text{mol}\cdot\text{g}^{-1}$. The immobilized laccase was proven to be an effective biocatalyst in the decolorization of two dyes: Remazol Brilliant Blue B, and Acid Blue 25. The decolorization percentage of Remazol Brilliant Blue B and Acid Blue 25 reached 93% and 82% respectively. The immobilized laccase exhibited enhanced thermal stability and pH adaptability compared to free laccase. After ten recycles, the immobilized laccase retained 42% decolorization catalytic activity.

1. Introduction

Every year, the textile industry generates large volumes of liquid effluent pollutants [1, 2]. Synthetic dyes are the largest of all textile dyestuffs produced, and their residuals left in wastewater have been a primary source of environmental contamination [3, 4]. Synthetic dyes contain azo bonds (-N=N-), aniline, phenol, or other aromatic compounds. Several physical and chemical approaches have been proposed to withdraw dyes from industrial wastewaters. Such examples are coagulation, adsorption, chemical oxidation, or photolysis [5-7].

The enzymatic treatment of pollutants is elegant process because of the mild reaction conditions they produce, without generating toxic sludge. Oxidative enzymes are one enzyme which maintains the capability for decolorizing dyes. Oxidative enzymes such as iron, which contains chloroperoxidase [8] and horseradish peroxidase [9], or manganese, which contains manganese peroxidase [10], have been applied to the decolorization of synthetic dyes. Laccases (EC 1.10.3.2) are copper-containing oxidases, and were first discovered in the Japanese lacquer tree, *Rhus vernicifera* [11, 12]. This enzyme has been applied to the decolorization of dyes, as stated in the previous report [13]. The reaction is easy to operate because it consumes O₂ at room temperature [14, 15]. Due to the susceptibility of enzymes to deactivate in the presence of other chemicals, and it is difficult to separate and recycle, which limits its application. So using immobilized laccase is separating expediently and recycling easily.

Generally, the nature of the support heavily influences the properties of the immobilized laccase.

¹ Address for correspondence: Rui-Feng Zhang, Faculty of Materials Science and Chemical Engineering, Ningbo University, Ningbo, Zhejiang 315211, PR China. Tel.: +86 13805843294; E-mail: zhangruifeng@nbu.edu.cn.



Polymer supports such as polyacrylamide [16] and alginate [17] are commonly used for enzyme immobilization, and are easy to be prepared, but short of thermal and chemical stability. Inorganic support maintains quality chemical stability, high mechanical strength, and low cost. Mesoporous materials particularly have large pore volume, tunable pore diameter, and a high specific surface area; these characteristics make them to be explored extensively as supports for immobilization of enzymes. The enzyme immobilization on different kinds of mesoporous materials: MCM [18, 19], SBA-15 [20, 21], CNS [22], MCF [23, 24], has been extensively researched. Macroporous materials such as diatomite [25], ceramic [26] have also been used to immobilize enzymes, however their low specific surface area creates a disadvantage in regards to the loading amount of enzymes.

A new kind of large-sized macroporous SiO_2 developed by our group recently. It offers enhanced mechanical stability, low density, and high porosity (93%) [27, 28]. To solve the issue of low specific surface area found in this macroporous material, a special *in situ* hydrothermal growth technology was established. The technology was created to introduce ZnO nanowires in the macroporous SiO_2 support [29]. The composite has a higher specific surface area compared to the pure macroporous silica, and the ZnO nanowires are usually positive-charged, which is very useful to immobilize negative-charged enzymes through electrostatic interaction. In our previous study, ZnO nanowires/macroporous SiO_2 composite support has been used to immobilize lipase through the method of static adsorption [30, 31]. In this study, the negative-charged laccase (IP is 3.7, working pH is about 5.5) was immobilized on ZnO nanowires/macroporous SiO_2 composite supports in an aqueous solution. Two dye substrates (Remazol Brilliant Blue B (RBBR) and Acid Blue 25, which is shown in figure 1) were decolorized under the catalysis of both free and immobilized laccases. The loading amount and stability of adsorption were measured by using a standard method. The reaction conditions such as pH and temperature were then investigated and optimized. Finally, the storage stability and reusability were tested.

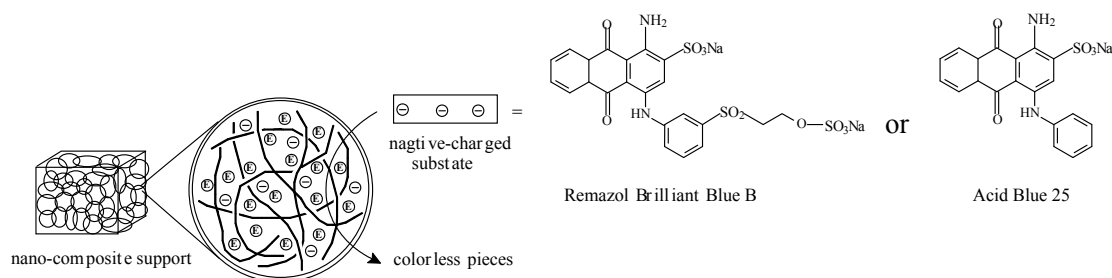


Figure 1. Scheme for composite support-laccase degradation two dyes.

2. Materials and methods

2.1. Materials

Laccase from *Trametes versicolor* was purchased from Aldrich, 2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), buffer salts, Remazol Brilliant Blue B and Acid Blue 25 are purchased from Aladdin (Shanghai).

2.2. Synthesis composite supports

A mixture of Epoxy resin (16.0 g) and polyethylene glycol (28.0 g of PEG1000 and 4.0 g of PEG2000) were heated and stirred to form a clear solution, 5.0 g of diethylenetriamine was then quickly added with strong stirring. The reaction system was kept at 70 °C for 3 hours. After curing the obtained the white product was immersed in pure water to completely remove the PEG resulting in a 3D skeletal polymer. Then, the white 3D skeletal polymer was naturally dried for 2 days and then immersed in pure ethyl silicate for 3 h. The hydrolysis of infiltrated ethyl silicate was carried out by exposing the samples in $\text{NH}_3 \cdot \text{H}_2\text{O}$ atmosphere at 40 °C for 12 h in a sealed container. After that the silica/polymer composites were dried at 60 °C for 2 h to remove the produced ethanol and the adsorbed $\text{NH}_3 \cdot \text{H}_2\text{O}$.

Calcinations of the composites were carried out at $5\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ in a muffle furnace up to a temperature of $820\text{ }^{\circ}\text{C}$ for 30 minutes. The white 3D silica has density of less than 0.2 g/mL [27].

Introduction of ZnO nanowires in macroporous silica was carried out by two steps. The first step was the formation of ZnO crystal seeds on the wall of silica support: A mixture of zinc acetate, PEG600 and water was used as the precursor solution, in which the SiO_2 supports were immersed 3 h and then dried at $100\text{ }^{\circ}\text{C}$ for 1 h to remove most of the water. Firstly the samples were heated in a muffle furnace at $200\text{ }^{\circ}\text{C}$ for 1 h, and then the temperature was raised to $650\text{ }^{\circ}\text{C}$ at a rate of $7.5\text{ }^{\circ}\text{C}/\text{min}$ and maintained for another 20 min. The second step was the *in-situ* growth of ZnO nanowires: zinc nitrate was dissolved in ammonia solution to form 1M solution, in this solution the seeds-contained supports were immersed for 6 h, and then the samples were placed in a sealed container equipped with ammonia-collecting material. The hydrothermal reaction was carried out in two stages: maintaining $90\text{ }^{\circ}\text{C}$ for 6 h in the first stage and $100\text{ }^{\circ}\text{C}$ for 12 h in the second stage. The obtained products were washed with pure water to completely remove the soluble inorganic salts and dried in vacuum at room temperature for 24 h [28-29].

2.3. Loading amount assay

Both of the supports are cut into small particles ($\sim 8\text{ mm}^3$). In the adsorption experiment, 0.3 g support is mixed with a 10 ml laccase solution in acetate buffer (pH 6.5), and the mixture continues to be shaken at 25°C for 6 hours. After that, the material was recovered by centrifugation for 5 minutes, then washed with an acetate buffer until no activity detected in the supernatant. The final enzyme preparation was kept in acetate buffer.

The amount of immobilized laccase (P_0) was determined according to the Bradford method [32], and the P_0 was calculated from the below equation:

$$P_0 = \frac{(C_1 - C_2)V_1 - C_3V_2}{m} \quad (1)$$

The leaching of laccase from the supports described in the previous [30]. The calculation equation was as follows:

$$P_t = P_0 - \frac{C_t V}{m} \quad (2)$$

2.4. Enzymatic dye decolorization

Reactions were carried out at room temperature in 1 mL reaction mixtures containing two dyes (0.1 mol/L) and an acetate buffer pH 3-8, then added free and immobilized enzymes into it respectively. For all reactions, the concentration of the two dyes was 0.1 mol/L, and the concentration of free and immobilized enzyme was maintained at 0.1 mmol/L. The decolorization efficiency was determined by monitoring the maximum absorbance (λ_{max}) of dyes at UV-Vis spectrophotometer, Remazol Brilliant Blue B at 550 nm and Acid Blue 25 at 600 nm respectively:

$$\text{Decolorization efficiency (\%)} = \frac{A_0 - A_t}{A_0} \times 100\% \quad (3)$$

2.5. Effect of pH, temperature and thermal stability on catalytic activity

The effect of pH and temperature on the catalytic activity of the free and immobilized laccase against the dyes was determined spectrophotometrically. This was done by measuring the changes in absorbance at the wavelength of maximum absorption of each dye at different pH balances and temperatures, keeping the constant temperature at 25°C and the pH=5.5.

The thermal stability of the free and immobilized laccases was determined by measuring the residual activity of the enzyme exposed to 80°C in acetate acid buffer pH 6.5 for 120 min. Every 20

min, a sample removed and assayed for enzymatic activity. The free and immobilized laccase activity was measured by monitoring the absorbance change of 2, 2-Azino-bis (3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt (ABTS) during its oxidation with a UV–visible spectrophotometer at 420 nm.

2.6. Storage stability

The immobilized laccase was kept at 25°C for different times (0-100 days), and was measured by monitoring the absorbance change of 2, 2-Azino-bis (3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt (ABTS) during its oxidation with a UV–visible spectrophotometer at 420 nm.

2.7. Reusability of an immobilized laccase

The reusability of the immobilized laccase was assayed in ten sequential cycles for the decolorization of RBBR. The immobilized laccase was recovered by performing centrifugation after each reaction cycle, and was placed in a fresh reaction media. The residual activity data was normalized to the first cycle assay, which was considered to be at 100% activity.

3. Results and discussion

3.1. Structural characterization of the supports

Figures 2 and 3 display the SEM images of the macroporous SiO₂ support and the composite with ZnO nanowires. The structural characterization of the supports is described in the previous literature [30].

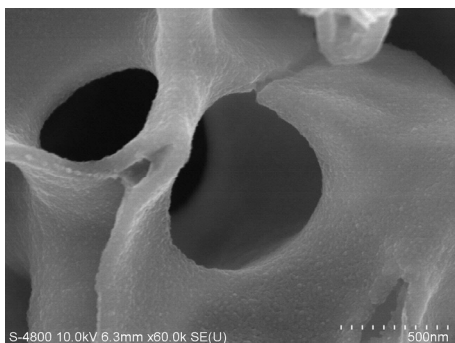


Figure 2. SEM image of macroporous SiO₂.

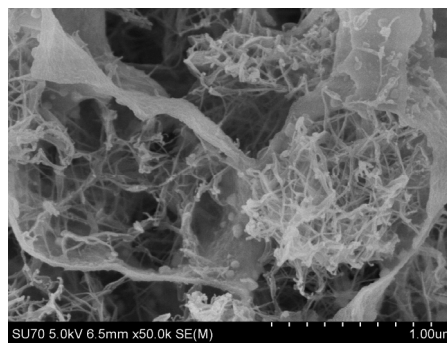


Figure 3. SEM image of the ZnO nanowires/macroporous SiO₂ composite.

3.2. Loading amount and adsorbing stability of the supports

The loading amount is described in table 1, and is about 193.4 nmol/g and pure macroporous SiO₂ is 43.7 nmol/g. It can be seen that the composite support is about 5 times the loading amount of macroporous SiO₂, and the loading amount of composite support is high than the silica-based mesoporous material [22]. The enhancement of adsorption and stability should be a result of the increase of surface area and the electrostatic interaction between positive-charged ZnO nanowires and a negatively-charged laccase. Therefore, the interaction of a laccase with composite support is much faster than that with pure macroporous SiO₂.

Table 1. The loading amount of laccase immobilized on different supports.

Soaking time/h	Composite support Loading/ $\mu\text{mol} \cdot \text{g}^{-1}$	Macroporous SiO ₂ Loading/ $\mu\text{mol} \cdot \text{g}^{-1}$
0	193.4	43.7
12	189.9	32.4
24	164.8	14.8
36	139.5	9.6
48	107.1	6.7

3.3. Effect of pH, temperature on the catalytic activity of free and immobilized laccases

3.3.1. pH. Figures 4 and 5 express the pH effect of immobilized and free enzymes on decolorization of the RBBR and Acid Blue 25 respectively. It is apparent that the decolorizations of both dyes under catalysis of immobilized and free enzymes are highly sensitive to the associated pH value, and that the optimum pH for free laccase is 5, and for the immobilized one is 5.5. Free laccase received the highest decolorization of 80% and 56% for RBBR and Acid Blue 25 respectively. The immobilized laccase provided the highest decolorization at 93% and 82% for RBBR and Acid Blue 25 respectively. The results indicate that the catalytic activity of laccase could be improved after immobilization in this way. Furthermore, the free laccase retained only 6% (RBBR) and 3% (Acid Blue 25) of decolorization activity at pH=8, while the immobilized laccase still has 32% (RBBR) and 25% (Acid Blue 25) of decolorization activity at the same pH value. It has been reported that supporting materials usually alter the effect of pH on the catalytic profile of some enzymes [33].

3.3.2. Temperature. The temperature of reaction is another important parameter affecting the activity of the enzyme. Figures 6 and 7 depict the temperature's effect on decolorization efficiency. The decolorization process was carried out under different temperatures varying from 20°C to 80°C. The results are illustrated in figures 6 and 7, both free and immobilized laccases maintained high activities as the temperature maintained at 20~30°C. As the temperature above 40°C, the free laccase showed a rapid decrease in activity as a result of the denaturation of enzyme at high temperature [34]. The immobilized laccase kept catalytic activities much higher than free laccase at all the range of temperature. For example, the immobilized laccase still have over 50% of catalytic activity at 60°C, 32% of catalytic activity at 80°C. The effect of supports protecting enzymes from deactivation at high temperature has also been found in other reports [35-37].

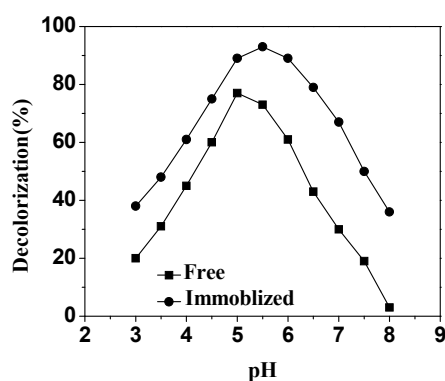


Figure 4. Effect of pH on the decolorization of RBBR by free and immobilized laccase.

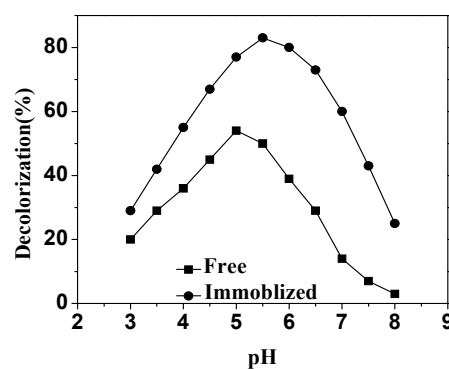


Figure 5. Effect of pH on the decolorization of Acid Blue 25 by free and immobilized laccase.

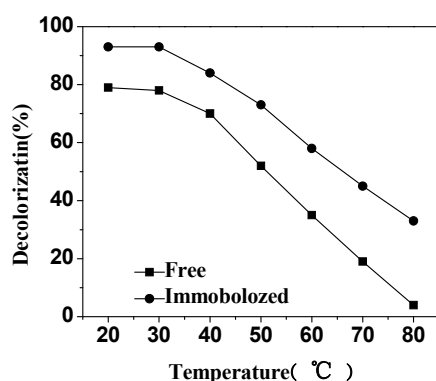


Figure 6. Effect of temperature on decolorization of RBBR by free and immobilized laccase.

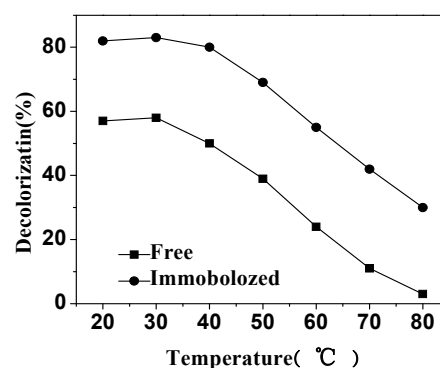


Figure 7. Effect of temperature on decolorization of Acid Blue 25 by free and immobilized laccase.

of RBBR by free and immobilized laccase.

decolorization of Acid Blue 25 by free and immobilized laccase.

3.3.3. Thermal stability. Figure 8 shows the heat inactivation curves for both the free and immobilized laccases. After an incubation period of 120 minutes at 80°C, the immobilized laccase retained 70% of its initial activity, while the free enzyme retained only 40%. Obviously, the nano-composite support is very effective at protecting laccase from inactivation at high temperatures. The electrostatic interaction improved the thermal stability of the immobilized enzyme, or, in other words, the immobilized laccase has an enhanced heat-resistance compared to the free laccase. The immobilized laccase could work in harsh environmental conditions with less activity loss as compared to the free laccase.

3.4. Storage stability

Figure 9 shows the decrease of relative activity in the immobilized laccase during incubation at 25°C for many days. The immobilization laccase retained approximately 80% of its initial activity after three months of storage. That means the laccase immobilized on the nano-composite support is a stable biocatalyst, which may have practical applications in the processes of dye-containing waste water.

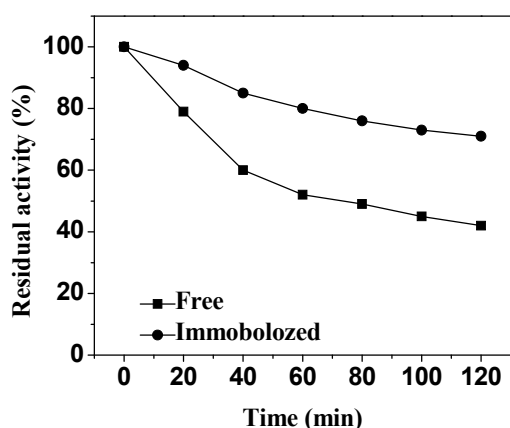


Figure 8. Thermal stability of the free and immobilized laccase at 80°C.

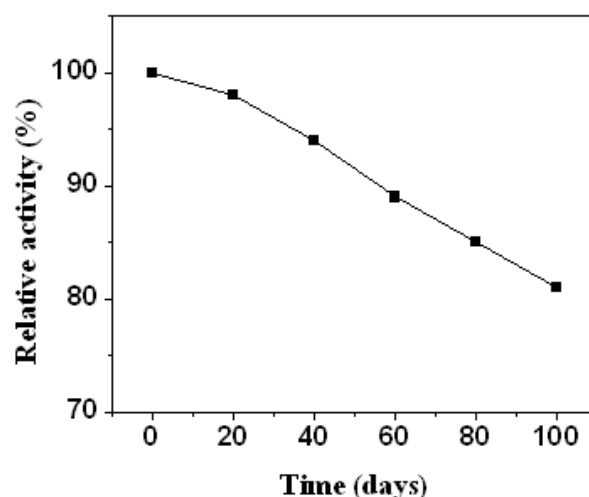


Figure 9. Storage stability of immobilized laccase at 25°C.

3.5. Reusability of the immobilized laccase

The reusable qualities of the immobilized laccase were tested by repeating catalysis of the decolorization of RBBR. The decolorization percentage was recorded as the relative activity of laccase in each cycle. As shown in figure 10, the relative activity showed a slight drop as the cycle number going from 1 to 10, 40% of initial activity maintained after ten times of cycle. It has never been reported that laccase immobilized on epoxy-activated carriers retained only 20% of initial catalytic activity after six cycles [37]. According to the comparison, it can be concluded that the laccase immobilized on the nano-composite support exhibits enhanced reusability.

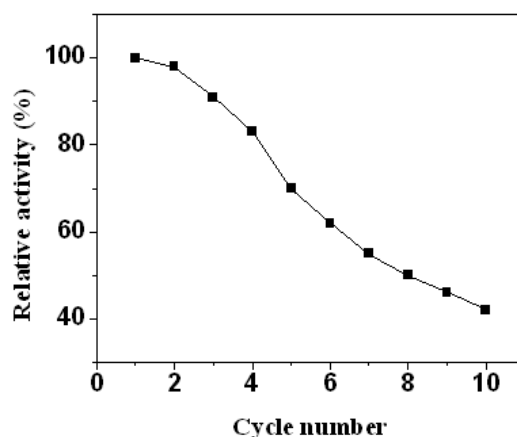


Figure 10. Reusability of the immobilized laccase for the decolorization of RBBR.

4. Conclusion

- The ZnO nanowires into macroporous SiO₂ resulted in a new composite support for the immobilization of laccase. The support contains 233 m²/g of specific surface area, and 193.4 μmol/g of adsorption capacity. The immobilized laccase exhibited excellent resistance to temperature and pH inactivation, high thermal stability, and reusability. After ten recycles, the immobilized laccase still maintained 40% of its initial activity.
- The optimal reaction conditions for 93% the decolorization of (RBBR) and 82% of the decolorization of Acid Blue 25 are as follows: molarity of two dyes (0.1 mol/L), molarity of free laccase and immobilized enzymes (0.1 mmol/L), reaction temperature (25°C), and pH (5.5); reaction time (1 h).

References

- [1] Sun J H, Shi S H, Lee Y F and Sun SP 2009 *Chem. Eng. J.* **155** 680
- [2] Bokare A D, Chikate R C and Paknikar K M 2008 *Appl. Catal. B: Environ.* **79** 270
- [3] Telke A, Kalyani D, Jadhav J and Govindwar S 2008 *Acta Chim. Slov.* **55** 320
- [4] Vandevivere P C, Bianchi R and Verstraete W 1998 *J. Chem. Technol. Biotechnol.* **72** 289
- [5] Colar L A, Ilinoiu E C, Manea F and Pode R 2012 *Environ Eng Manag J* **11** 61
- [6] Zucca P, Rescigno A, Olianias A, Maccioni S and Sollai F 2011 *J Mol Catal B Enzym.* **68** 216
- [7] Pakshirajan K and Jaiswal S 2011 *J Sci Ind Res* **70** 987
- [8] Zhang J, Feng M Y, Jiang Y C, Hu M C, Li S N and Zhai Q G 2012 *Chem. Eng. J.* **191** 236
- [9] Kim G Y, Lee K B, Cho S H, Shim J and Moon S H 2005 *J. Hazard. Mater.* **126** 183
- [10] Cheng X B, Jia R, Li P S, Tu S Q, Zhu Q, et al. 2007 *Enzyme Microb. Technol.* **41** 258
- [11] Giardina P, Faraco V, Pezzella C and Piscitelli A 2010 *Cell Mol Life Sci* **67** 369
- [12] Morozova O V, Shumakovich G P, et al. 2007 *Appl Biochem Microbiol* **43** 523
- [13] Rodríguez Couto S and Toca Herrera J L 2006 *Biotechnol Adv* **24** 500
- [14] Zamora P P, Pereira C M, Tiburtiusm E R L, et al. 2003 *Appl. Catal. B: Environ* **42** 131
- [15] Zille A, Tzanov T, Gübitz G M and Paulo A C 2003 *Biotechnol. Lett* **25** 1473
- [16] Dayaram P and Dasgupta D 2008 *J Environ Biol* **29** 831
- [17] Lu L, Zhao M and Wang Y 2007 *World J Microbiol Biotechnol* **23** 159
- [18] Wang Y, Zheng X and Zhao M 2008c *J Chem Eng Chin Univ* **22** 83
- [19] Xu X H, Lu P, Zhou Y M, Zhao Z Z and Guo M Q 2009 *Mater. Sci. Eng. C* **29** 2160
- [20] Salis A, Pisano M, Monduzzi V and Solinas E 2009 *J. of Mol. Catal. B: Enzym.* **58** 175
- [21] Bautista L F, Morales G R and Sanz R 2010 *Bioresour. Technol.* **101** 8541
- [22] Forde J, Tully E, Vakurov A, Gibson T D, et al. 2010 *Enzyme Microb. Technol.* **46** 430
- [23] Schmidt P, Lukens W W, Zhao D Y, Yang P D, et al. 1999 *J Am Chem Soc.* **121** 254
- [24] Xue P, Xu F and Xu L D 2008 *Appl Surf Sci.* **255** 1625
- [25] Yang Y X, Zhang J B, Yang W M, Wu J D and Chen R S 2003 *Appl Surf Sci.* **206** 20

- [26] Huang L and Cheng Z M 2008 *Chin J Catal* **29** 57
- [27] Zhang R F, Long N B and Zhang L L 2009 *Thin Solid Films* **517** 6677
- [28] Long N B and Zhang R F 2009 *Chin J Inorg Chem.* **25** 1153
- [29] Li X F and Zhang R F 2013 *Chin J Mater Res.* **27** 526
- [30] Shang C Y, Li W X and Zhang R F 2014 *Enzyme Microb. Technol.* **61** 28
- [31] Shang C Y, Li W X and Zhang R F 2015 *J. of Mol. Catal. B: Enzym.* **113** 9
- [32] Bradford M M 1976 *Analytical Biochemistr.* **72** 248
- [33] Wang F, Guo C, Yang L R and Liu C Z 2010 *Bioresour. Technol.* **101** 8931
- [34] Antczak S M, Kubiak A, Antczak T and Bielecki S 2009 *Renew Energ.* **34** 1185
- [35] Takahashi H, Li B, Sasaki T, Miyazaki C, et al. 2001 *Micropor Mesopor Mat.* **755** 44
- [36] Takahashi H, Li B, Sasaki T, Kajino T and Inagaki S 2000 *Chem. Mater.* **12** 3301
- [37] Kunamneni A, Ghazi I, Camarero S, Ballesteros A, et al, 2008 *Process Biochem.* **43** 169