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Influence of Silica-Derived Nano-Supporters on Cellobiase After Immobilization

Peng Wang · Xiaoke Hu · Sean Cook · Huey-Min Hwang

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Abstract Core shell magnetite nanoparticle (CSMN) was successfully synthesized with diameter around 125 nm according to the determination with scanning electronic microscopy. SBA-15 with diameter around 31 nm was synthesized in our previous work as another supporter for immobilized degradation enzymes. The aim of this study was to investigate the influence of silica-derived nano-supporters on cellobiase after immobilization. With covalent method, glutaraldehyde was introduced to immobilize cellobiase. The immobilized enzyme efficiency, specific activity, and its characterization, including optimum pH, pH stability, optimum temperature for enzyme reaction, and enzyme thermal stability were investigated. Results show that the method of enzyme immobilization on both nano-supporters could improve cellobiase stability under low pH and high temperature conditions compared with the free enzyme. In the aspect of immobilization efficiency, SBA had higher amount of bounded protein than that of CSMN, but had lower specific enzyme activity than CSMN, assumably due to the change in silica surface properties caused by process of supporter synthesis.

Keywords Cellobiase · Immobilization · Core shell magnetite nanoparticle · SBA

Cellobiase (EC 3.2.1.21) is a critical part of the cellulase system [1] catalyzing the final step in cellulose hydrolysis to reducing sugar. For most cellulose bioconversion processing industries, cellulose was firstly hydrolyzed by the catalysis of endo-(1, 4)-β-D-glucan glucanhydrolases (EC 3.2.1.4) and exo-(1, 4)-β-D-glucan cellobiohydrolases (EC 3.2.1.91) to cellobiose, and then was ultimately hydrolyzed to reducing sugar by cellobiase as an economic way for ethanol production. During enzyme hydrolysis, the accumulation of cellobiose will cause severe feedback inhibition on both endoglucanase and exoglucanase activities. Therefore, addition of cellobiase in reaction system is crucial for both cellulose bioconversion and follow-up ethanol production [2]. In food industry, cellobiases are key enzymes and play an important role in the varietal aroma-enhancing process of fruit juices and wines by releasing odor aglycon from monoglucosidic complexes [3].

P. Wang · X. Hu · S. Cook · H.-M. Hwang (✉)
Biology Department, Jackson State University, Jackson, MS 39217, USA
e-mail: hwang@jsums.edu

The immobilized enzyme technique offers several advantages in industrial and biotechnological applications, including repeated use, ease of separating reaction products from the biocatalyst, and improvement of enzyme stability. There are many different strategies which have been proposed to immobilize enzymes. Examples include adsorption [4, 5], covalent binding [6], granulation [7], and entrapment in polymers [8]. Covalent immobilization has the advantage of forming strong and stable linkages between an enzyme and the carrier. Consequently, it eliminates the loss of activity caused by enzyme leakage from the support. Porous silica is the most commonly used supporter for the technique of immobilized enzyme [9]. However, the number of commercialized activated carriers for covalent immobilization is relatively small compared with available enzyme adsorbent materials.

According to our previous work, a nano-sized particle porous silica SBA-15 has been successfully used for immobilizing laccase [10]. In this paper, we introduced an innovated method, synthesis of core shell magnetite nanoparticles (CSMN), for immobilizing enzymes. The technique of synthesizing CSMN not only prevents the magnetite aggregation and the rapid biodegradation when they are directly exposed to biological system but also provides the convenience of collecting and recycling especially for the nano-scale supporters. We further investigated and characterized the immobilized enzymes in terms of their immobilization efficiency, thermal stability, pH stability, optimal pH, and temperature for biocatalysis.

Materials and Methods

Cellobiase (Novozym-188), tetramethylammonium hydroxide, tetraethyl orthosilicate (Si $(OC_2H_5)_4$, TEOS), glutaraldehyde, 3-aminopropyltrietoxysilane (APTES), and all other chemicals were obtained from Sigma Aldrich.

Synthesis of CSMN and its Size Determination

Aqueous dispersions of magnetite nanoparticles were prepared according to what was described in Correa-Duarte et al. [11]. Briefly, 20 ml of 1 M $FeCl_3$ and 5 ml of 2 M $FeSO_4$ in HCl (2 M) were added to 250 ml of 0.7 M NH_4OH under rapid mechanical stirring and the mixture was allowed to stir for 30 min. After centrifuging, the black solid product was redispersed in distilled water and tetramethylammonium hydroxide solution was added.

Silica-coated magnetite nanoparticles were synthesized by mixing 4.85 ml NH_4OH, H_2O, 58.8 ml EtOH, 6.22 ml of the previously washed magnetite nanoparticles, and 2 ml TEOS in aqueous solution with mechanical stirring for 4 h. The formed particles were centrifuged to eliminate excess reactants and redispersed in 50 ml of pure water [12].

The size of CSMN was characterized with scanning electron microscope (SEM, FEI Quanta 200), which was operated under a high vacuum condition. The CSMN was dispersed in 100% ethanol and deposited on pre-cleaned glass slide.

Immobilization of Cellobiase on SBA and CSMN

Immobilization of cellobiase was carried out by covalent coupling on silica spheres. Briefly, 2 g of SBA-15 or magnetite silica nanospheres prepared aforementioned, 2.5 ml H_2O, and 10 ml APTES were added into 250 ml methanol. The mixture was mixed with 150 ml glycerol and then transferred to a 500 ml three-necked flask equipped with a mechanical

stirrer. The temperature was kept at 85–90 °C with rapid stirring for 3 h. Deionized water and methanol were used to wash both nanospheres for several times. These washed nanospheres were immersed in 5% (*v*/*v*) glutaraldehyde solution with 0.1 M phosphate buffer, pH 7.4, for 6 h at room temperature and then washed with deionized water. Ten milliliters of phosphate buffer (pH 7.0) containing 50 mg of enzyme protein was added to the pellet to give a final cellobiase titer of 0.2 U mg^{-1} clay. The suspension was stirred at 5 °C for 24 h, centrifuged at 13,000 ×*g* for 10 min to remove the buffer, and washed several times with buffer until no enzyme activity was detected in the supernatant.

Determination of Enzyme Activity and Protein Concentration

A sample of enzyme solution (0.01 ml) was added to 0.5 ml of 0.1 M acetate buffer (pH 5.0) containing 2 mM 4-nitrophenyl β-D-glucopyranoside. The reaction mixture was incubated for 10 min at 30 °C. The absorbance was read at 405 nm upon addition of 0.5 ml Na_2CO_3 solution (1 M). One unit of enzyme activity is defined as the quantity of enzyme hydrolyzing 1 μmol of substrate under the conditions stated above. Protein concentration in the solution was determined by the method of Lowry et al. [13] using bovine serum albumin as the standard.

Stability and Catalytic Reaction Experiments

Resistance of immobilized enzymes to pH changes was conducted by measuring the activities after they were put in different buffer solutions (pH 2–8.5) for 24 h. Residual activity was measured and expressed as a percentage relative to the initial enzyme activity. Effect of pH on relative activity of cellobiase was investigated in the range of 2–8.5 for immobilized enzymes and free enzyme. The effect of temperature on enzyme activity was investigated in the range of 20–80 °C for both free and immobilized cellobiase. The estimation of the thermal stability of free and immobilized cellobiase preparations was carried out by measuring the residual activity of enzyme exposed to different temperatures (50, 60, and 70 °C) in acetate buffer at pH 5.0. Samples were taken at different time intervals (1, 3, 7, 15 h) during incubation.

Results and Discussion

Properties of SBA and CSMN

SBA-15 particles were synthesized and the related characterization was described in our previous work [10]. SBA-15 possesses highly ordered large mesopores with hexagonal structure. The average diameter of these particles is determined with atomic force microscope as 31 nm with a narrow size distribution. In this research, CSMN was successfully synthesized, and Fig. 1 shows the SEM micrograph of CSMN which was deposited onto a pre-cleaned glass. The CSMN had a spherical shape and was arranged in a continuous and homogeneous monolayer. Its average size is around 125 nm.

The surface of SBA and CSMN is biocompatible and can be functionalized with the silane coupling agent. APTES is a commercialized amino-functional silane coupling agent. They were covalently bound to the surface of silica spheres. The terminal amino groups are reactive and can be transferred to aldehyde groups by a glutaraldehyde method to facilitate the attachment of proteins or enzymes [14]. Table 1 is the comparison of immobilization

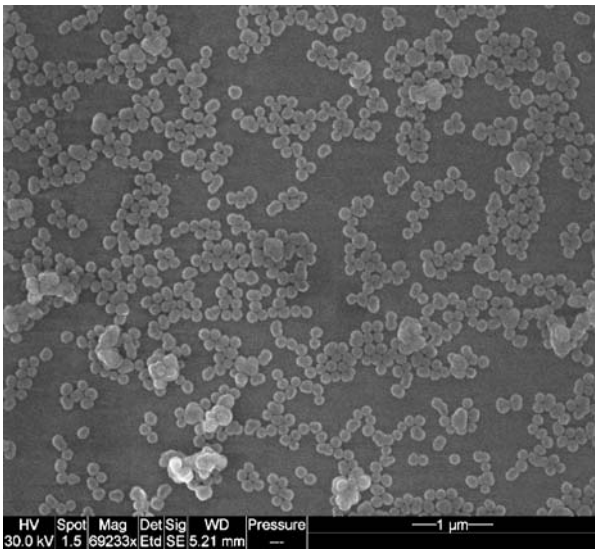

Fig. 1 SEM micrograph of CSMN

efficiency between the two supporters. The amount of protein bound on SBA surface is more than that of CSMN. Their surfaces are all made of silica. On the other hand, both supporters are APTES functionalized and activated with glutaraldehyde; consequently, the treatment decreased the pore diameters and pore volume. That is to say, the aminopropyl groups anchored at an entrance of the pores of support may block more enzymes to enter the channels of the support [15]. Obviously, the size of CSMN is about four times than that of SBA. Smaller particles have larger surface-to-volume ratios and larger capacity for binding more enzymes on their surface [16]. Therefore, we speculated the reason that caused the different immobilization efficiency is the ratio of surface area between SBA-15 and CSMN.

In the aspect of specific activity of the immobilized cellobiase, the group of SBA which has 21.5 U/g clay was lower than that of CSMN. Since their outside surfaces were all made of silica, there were no significant differences in their chemical properties. The only difference between them is the method with which they were synthesized. The pore size of CSMN surface synthesized by Stöber method was around 0.5 nm [17], while SBA holds highly ordered large mesopores with a diameter varying from 6 to 30 nm [18]. Although the method of APTES function prevents the enzyme from entering inside SBA, the size of cellobiase is less than 1 nm. Therefore, larger porous areas on supporter surface might increase the possibility of more enzyme catalytic region in SBA group taking part in covalent binding to supporters. Consequently, the final specific activity of cellobiase on CSMN was higher than that of SBA.

Table 1 Immobilized cellobiase on different supporters.

Supporters	Bound protein g/g clay	Enzyme activity U/g clay
SBA	0.37	21.5
CSMN	0.23	25.0

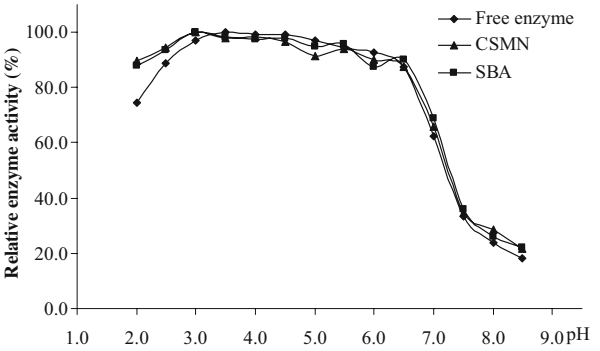

Fig. 2 pH stability of the immobilized cellobiase. Relative activity is expressed as percent of the highest enzymatic activity of each group

Effect of pH on the Stability of Immobilized Enzyme

Immobilized cellobiase was incubated in buffers with pH ranging from 2 to 8.5 at 4 °C for 24 h and then assayed for activity. Results (Fig. 2) show that, in the pH range studied, the relative activity of both immobilized enzymes is higher than that of free enzyme, when the pH is below 3.0. Therefore, the immobilized form is more resistant to low pH and more stable than that of free form [19]. Immobilized enzymes normally tend to show higher resistance to deactivation. The enzyme is confined in a region that is not exposed to the solution as in the case of the free enzyme. Because of this restriction, the microenvironment around the enzyme is not affected to the same extent as the surrounding solution with the changes in the parameters of the medium [20].

Effect of pH on Activity of the Immobilized Cellobiase

Activities of the immobilized cellobiase was compared with those of free enzyme in the pH range of 2.0–8.5. It can be seen in Fig. 3 that the optimum reaction pH for the group of free enzyme, CSMN, and SBA-15 is 4.0, 4.0, and 4.5, respectively. There is a slight shift towards the base region in the group of SBA, assumably due to the highly polyionic immobilized supports which could shift slightly in acidic or basic region relative to that of free enzyme [21]. A similar shift in the pH optimum for immobilized enzyme on different supporters was also reported in literatures [22, 23]. The reason that CSMN did not show this trend might be due to its large size that affects fewer enzymes on supporters relative to that of SBA. Furthermore, the cellobiase immobilized on SBA-15 and CSMN has higher

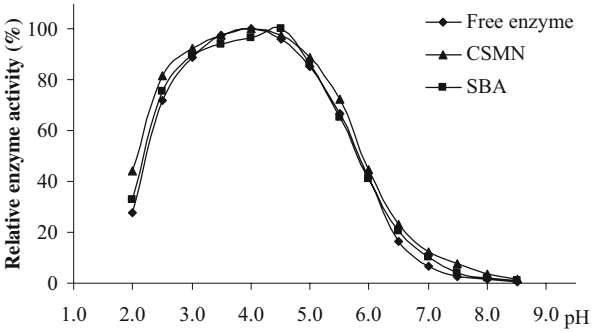

Fig. 3 Effect of pH on relative activity of the immobilized cellobiase

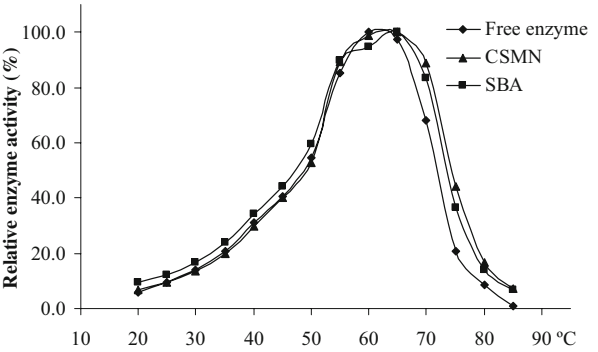

Fig. 4 Effect of temperature on relative activity of the immobilized cellobiase

activities across a broader pH range from 2.0 to 8.0. The relative activity of CSMN group is a little higher than that of SBA-15.

Effect of Temperature on Activity of the Immobilized Cellobiase

The effect of temperature on relative activity of the immobilized cellobiase is shown in Fig. 4. Immobilized enzymes and free enzyme showed a similar trend of temperature stability in the temperature range studied (25–80 °C). Cellobiase immobilized on SBA illustrated relatively higher enzyme activities at lower temperatures (20–50 °C) compared with the other two groups. The optimal temperature (i.e., the temperature that shows a maximum relative activity) is 60, 65, and 65 °C for free enzyme, SBA, and CSMN, respectively. The activity of all groups decreased sharply when temperatures continue to increase above that point. Above that point, SBA and CSMN showed better cellobiase activities and more stability. In order to investigate the effect of immobilization on the stability of cellobiase, we conducted the experiment of thermal inactivation of immobilized enzymes.

Thermal Inactivation of Immobilized Enzymes

Since all groups' optimum reaction temperatures were around 60–65 °C, we chose the temperature of 60, 65, and 70 °C, respectively to investigate thermal inactivation of the immobilized enzyme. Figure 5a–c shows time-course activities of cellobiase at different temperatures. It is evident that the immobilized cellobiase are more resistant to inactivation at higher temperatures. Thermal inactivation of cellobiase immobilized on different support shows the general trend CSMN>SBA>free enzyme at all test temperatures. In the case of 60 °C, free enzyme retained about half of the original enzyme activity at 180 min incubation. The relative enzyme activity of the immobilized groups was still around 80% of the original. At 65 °C, 180 min incubation almost inactivated free cellobiase, while the groups of SBA and CSMN retained 35% and 41% of the original activity, respectively. At 70 °C, activity of free enzyme holds 10% of the original enzyme activity at 60 min, while the same level of inhibition (i.e., 10% of the original activities) in groups of SBA and CSMN occurred at incubation time 120 min. Therefore, the immobilized cellobiase appeared to be more resistant to inactivation at the higher temperature. Both supporters generally show the protective effect at higher temperatures when inactivation of free enzymes occurred. The conformational flexibility of the enzyme is affected by immobilization. Immobilization of the enzyme causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denature while raising the temperature [24, 25].

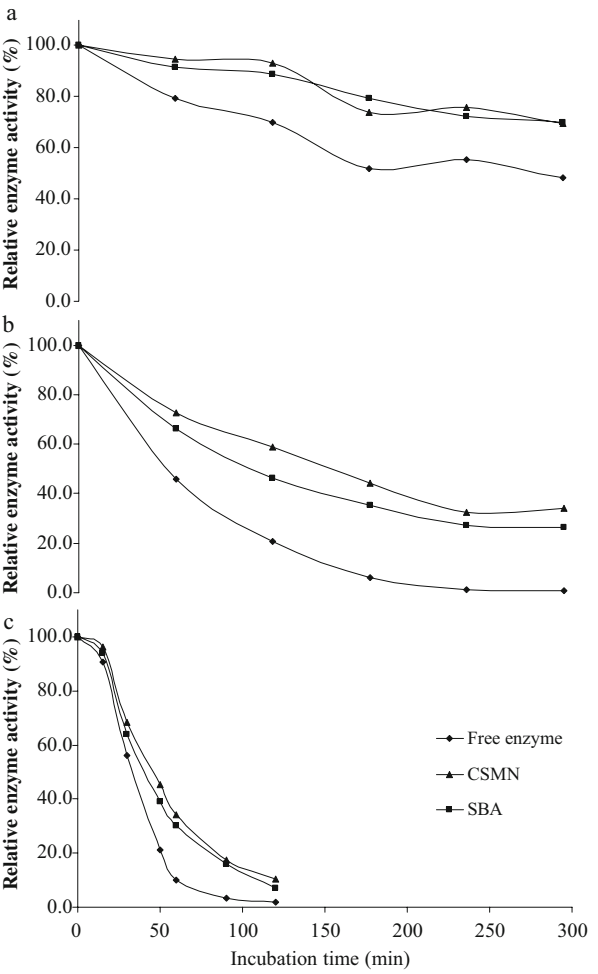

Fig. 5 Thermal inactivation of the immobilized cellobiase at 60 °C (**a**), 65 °C (**b**), and 70 °C (**c**). Relative activity is expressed as percent of the enzymatic activity at time zero

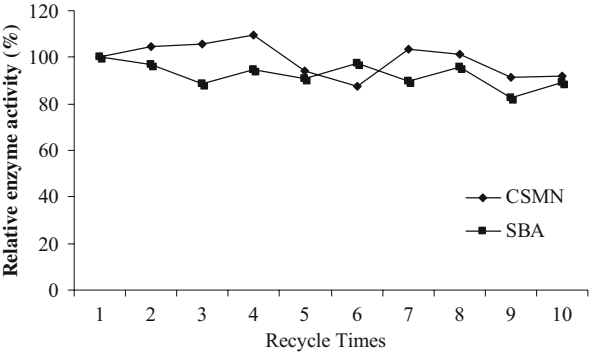

Fig. 6 Recycle assays of cellobiase immobilized on CSMN and SBA. Reaction of immobilized enzyme proceeded for 10 min with substrates at 30 °C for each cycle

Recycle Assays of Immobilized Cellobiase

Investigations were carried out to determine the durability of cellobiase immobilized on CSMN and SBA. The immobilized enzymes were recycled ten times and assayed the enzyme activity after each recycling. Figure 6 shows the results of remained enzyme activities of each recycle. Cellobiase immobilized on both supporters shows great reusability. Both supporters still hold the relative enzyme activities at a high level (≥80%). This result partly proved that the covalent method could prevent the loss of enzyme during reactions and improve the enzyme stability.

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References

1. Coughlan, M. P., & Ljungdahl, L. G. (1988). In J.-P. Aubert, & P. B. J. Millet (Eds.), *Biochemistry and genetics of cellulose degradation* pp. 11–30). Academic Press: London.
2. Lau, A. T. Y., & Wong, W. K. R. (2001). *Protein Expression and Purification*, *23*, 159–166. doi:10.1006/prep.2001.1486.
3. Palmeri, R., & Spagna, G. (2007). *Enzyme and Microbial Technology*, *40*, 382–389. doi:10.1016/j.enzmictec.2006.07.007.
4. Macario, A., Katovic, A., Giordano, G., Forni, L., Carloni, F., Filippini, A., et al. (2005). *Studies in Surface Science and Catalysis*, *155*, 381–394. doi:10.1016/S0167-2991(05)80166-1.
5. Reshmi, R., Sanjay, G., & Sugunan, S. (2007). *Catalysis Communications*, *8*, 393–399. doi:10.1016/j.catcom.2006.07.009.
6. Petri, A., Marconcini, P., & Salvadori, P. (2005). *Journal of Molecular Catalysis. B, Enzymatic*, *32*, 219–224. doi:10.1016/j.molcatb.2004.12.001.
7. Ferrer, M., Plou, F. J., Fuentes, G., Cruces, M. A., Andersen, L., Kirk, O., et al. (2002). *Biocatalysis and Biotransformation*, *20*, 63–71. doi:10.1080/10242420210153.
8. Pierre, A. C. (2004). *Biocatalysis and Biotransformation*, *22*, 145–170. doi:10.1080/10242420412331283314.
9. Stamatis, A., Malandrinos, G., Butler, I. S., Hadjiliadis, N., & Louloudi, M. (2007). *Journal of Molecular Catalysis A Chemical*, *267*, 120–128. doi:10.1016/j.molcata.2006.11.049.
10. Hu, X., Zhao, X., & Hwang, H. (2007). *Chemosphere*, *66*, 1618–1626. doi:10.1016/j.chemosphere.2006.08.004.
11. Correa-Duarte, M. A., Giersig, M., Kotov, N. A., & Liz-Marzan, L. M. (1998). *Langmuir, Chemical Physics Letters*, *14*, 6430–6435.
12. Salgueiriño-Maceira, V., Correa-Duarte, M. A., Spasova, M., Liz-Marzán, L. M., & Farle, M. (2006). *Advanced Functional Materials*, *16*, 509–514. doi:10.1002/adfm.200500565.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). *The Journal of Biological Chemistry*, *193*, 265–275.
14. Liu, X., Xing, J., Guan, Y., Shan, G., & Liu, H. (2004). *Colloids and Surfaces A*, *238*, 127–131. doi:10.1016/j.colsurfa.2004.03.004.
15. Lü, Y., Guo, Y., Wang, Y., Liu, X., Wang, Y., Guo, Y., et al. (2008). *Microporous and Mesoporous Materials*, *114*, 507–510. doi:10.1016/j.micromeso.2007.12.027.
16. Oh, J. T., & Kim, J. H. (2000). *Enzyme and Microbial Technology*, *27*, 356–361. doi:10.1016/S0141-0229(00)00232-5.
17. Balas, F., Rodriguez-Delgado, M., Otero-Arean, C., Conde, F., Matesanz, E., Esquivias, L., et al. (2007). *Solid State Sciences*, *9*, 351–356. doi:10.1016/j.solidstatesciences.2007.03.004.
18. Zhao, D., Feng, J., Huo, Q., Melosh, N., Fredrickson, G. H., Chmelka, B. F., et al. (1998). *Science*, *279*, 548–552. doi:10.1126/science.279.5350.548.
19. Eldin, M. S. M., Portaccio, M., Diano, N., Rossi, S., Bencivenga, U., D'Uva, A., et al. (1999). *Journal of Molecular Catalysis. B, Enzymatic*, *7*, 251–261. doi:10.1016/S1381-1177(99)00044-2.

20. Cano, À., Minguillón, C., & Palet, C. (2006). *Journal of Membrane Science*, *280*, 383–388. doi:10.1016/j.memsci.2006.01.041.
21. Goldstein, L., Levin, Y., & Katchalski, E. (1964). *Biochemistry*, *3*, 1913–1919. doi:10.1021/bi00900a022.
22. Huang, X. J., Ge, D., & Xu, Z. K. (2007). *European Polymer Journal*, *43*, 3710–3718. doi:10.1016/j.eurpolymj.2007.06.010.
23. Kim, M., Ham, H. O., Oh, S. D., Park, H. G., Chang, H. N., & Choi, S. H. (2006). *Journal of Molecular Catalysis. B, Enzymatic*, *39*, 62–68. doi:10.1016/j.molcatb.2006.01.028.
24. Abdel-Naby, M. A. (1993). *Applied Biochemistry and Biotechnology*, *38*, 69–81. doi:10.1007/BF02916413.
25. Chang, M. Y., & Juang, R. S. (2005). *Enzyme and Microbial Technology*, *36*, 75–82. doi:10.1016/j.enzmictec.2004.06.013.