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STABILIZATION OF HEMICELLULASE ENZYMES WITH NANO-LAYER

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ABSTRACT. In order to increase their stability, the conjugation of hemicellulase enzyme molecules with a polymer nano-layer is reported. This enzyme nanobiocomposites as single enzyme nanoparticles (SENs) have a good stability under extreme conditions. Thus, after 6 hours at 80 ^{o}C, SENs have 40% of its original activity but the natural enzymes lost their activity after a half an hour.

Keywords: *single enzyme nanoparticles, enzyme stability, nano-bio-composites, hemicellulase enzymes*

INTRODUCTION

An *extremophile* is an organism that thrives in and may even require physically or geochemically extreme conditions that are detrimental to most life on Earth. A *thermophile* is an organism — a type of extremophile — that thrives at relatively high temperatures, between 45 and 80 °C. Cellular components of thermophilic organisms, enzymes, proteins and nucleic acids, are also thermostable. Thermostable enzymes are highly specific hence they have considerable potential for many industrial application [1].

Thermobifida spp. are gram-positive, compost- and soil-inhabiting bacteria with broad degradative activity on plant cell wall constituents. *Thermobifida fusca*, the most extensively studied species of this genus, is the model organism of thermophilic, aerobic cellulolytic bacteria. *Thermobifida fusca* produces multiple extracellular enzymes including cellulases that are responsible for the decomposition of cellulose and lignocellulose residues, which make up the bulk of agricultural and urban wastes. While there are ample data on the cellulolytic system of *T. fusca,* the hemicellulolytic enzyme system of this species is still poorly characterized [1] (Figure 1).

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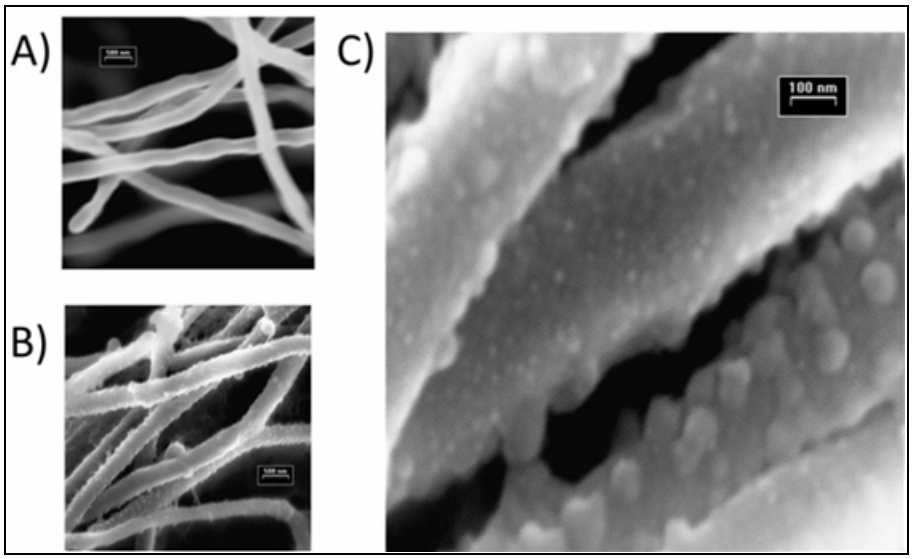

Figure 1. Hyphal surface of *Thermobifida fusca* TM51 A) Smooth surface of mycelium grown on glucose B) Cellulosome-like structures emerged upon induction by cellulose C) Cellulosome-like structures at higher magnification

Hemicelluloses act as linkers between lignin and cellulose. The high percentage of hemicellulose fraction in the cell wall of higher plants makes this material the second most abundant biopolymer in nature (Figure 2).

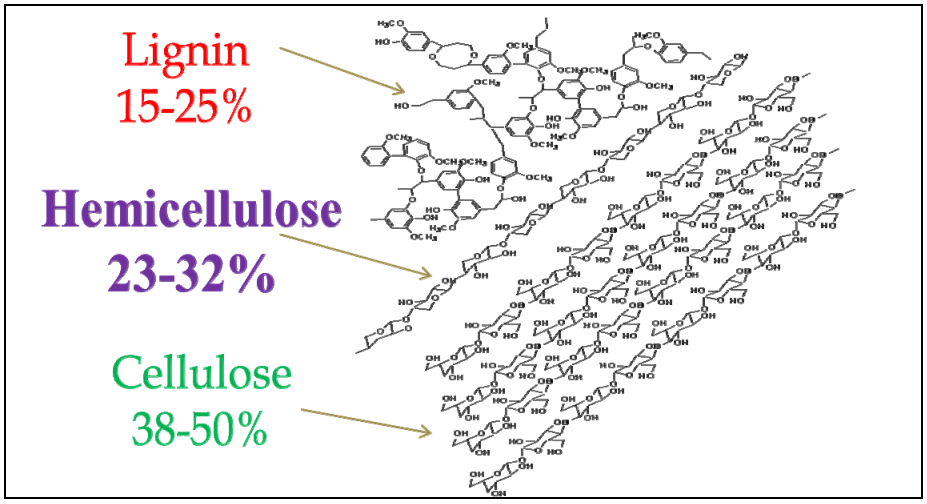

Figure 2. Major components in lignocellulosic biomass

Single enzyme nanoparticles (SENs) represent a new approach in industrial enzyme research [2, 3] (Figure 3). Classical techniques for improving the stability of enzymes are enzyme immobilization to the surface or inner cavity of the carrier, modification of the surface of the enzyme, protein engineering, reaction medium engineering and cross-linked enzyme crystals. The new approach in the industrial enzyme stabilization is the reducing the size of the enzyme carriers. Nano-carriers provide minimum diffusional limitation, maximum surface area per unit mass, and high enzyme loading. Nano-carriers are 1) a) metal [4], or b) magnetic nanoparticles [5], 2) Enzyme molecules could be encapsulated into a) hyperbranched polymers [6] b) dendrimers [7, 8]. 3) Single enzyme nanoparticles means single enzyme molecules encapsulated with a) polymer network (nanogel) [9] b) inorganic layer. The inorganic layer could be α) hollowed metal or silica nano-sphere [10, 11] β) mezoporous silica [12] or γ) magnetic nanolayer [13] (Figure 3).

The form of SEN means that each enzyme molecule is surrounded with a nanometer scale polymer matrix layer, resulting in stabilization of enzyme activity without any serious limitation for the substrate transfer from solution to the active site of the enzyme. The synthesis of SEN particles is needed more or less simple laboratorial technique.

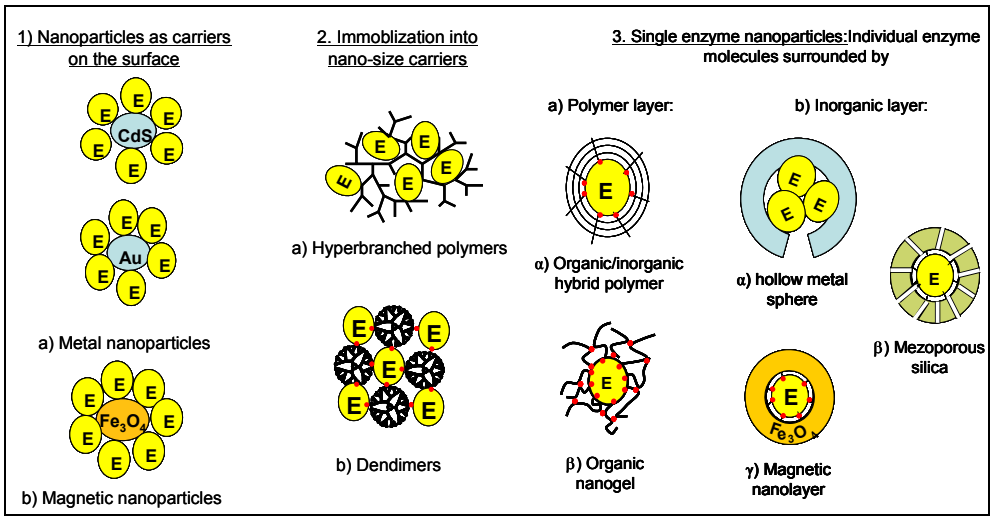

Figure 3. Nanotechnological methods for enzyme stabilization.

The utilization of single enzyme nanoparticles is possible industrial and scientific areas. This technique can be used for stabilization of enzymes in industrial enzyme research. Thus, using mesoporous silica gels single enzyme nanoparticles can fixed into the inner wall of the mezopores and it means that nanoreactors can be fabricated (Figure 4) [2].

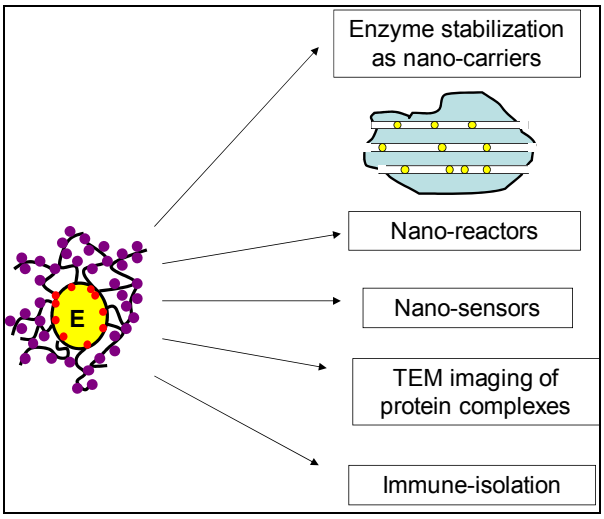

Figure 4. Utilization of single enzyme nanoparticles

The polymer layer of the single enzyme nanoparticles is electrodense so transmission electron microscopic detection of the three-dimensional structures of the enzyme complexes can be realized easily. Polymers composed with MAPS monomers have a good biocompatibility and no toxicity [14]. It means that the technique of the preparation of single enzyme nanoparticles can be used in biological areas.

Previously we have decided to apply this technique for industrial bioethanol synthesis able to comply with the requirements of green chemistry. We would like to investigate that how can the SEN-enzymes digest higher-size substrates. We investigated the chemical stability of different enzymes and enzyme complexes in the form of SEN.

RESULTS AND DISCUSSION

The preparation process of single enzyme nanoparticles (SEN) has three steps (Figure 5). The detailed description of the procedure was described earlier [2, 3]. The first step is a modification of CK enzyme complex and its solubilization in a hydrophobic medium. The second step is the polymerization of the vinyl group in hexane, and the final (third) step is hydrolysis and condensation of the trimethoxysilyl functional group (TMS). Julaba F12 cryostate was used to keep the reaction mixture at 0 ^{o}C. For the polymerization step, the enzyme should be dissolved in a hydrophobic medium (n-hexane).

The surfaces of the enzymes have hydrophobic and hydrophilic regions. If hydrophilic parts of the surface of the enzymes are covered by surfactant molecules, the enzyme lost its hydrophilic characteristic and became hydrophobic, so the enzyme can not solve in water but can solve in hydrophobic solvents, e.g. hexane. During the hydrophobic ion pairing ion-pairs are bounded between the ionic part of the surfactant and the ionic part on the surface of the enzyme.

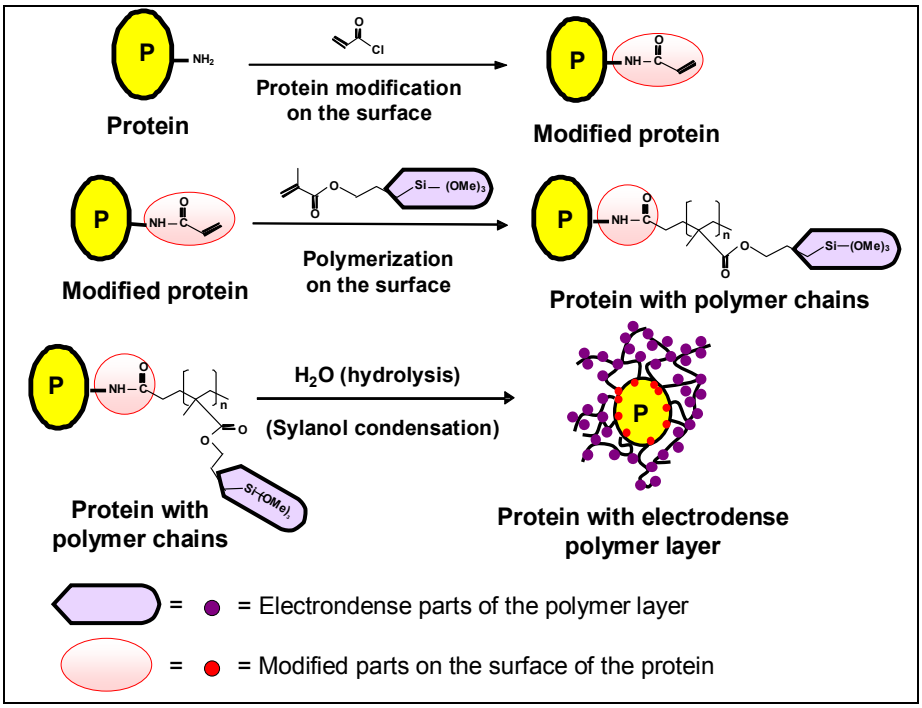

Figure 5. Thee-step method to synthesize single enzyme nanoparticles

Polymerization on the surface of the enzyme can only be achieved if there is direct contact between the enzyme surface and the hexane medium. For this reason, the specific solubilization method of hydrophobic ion pairing may be used (Figure 6). In this process, vinyl groups on the enzyme surface (synthesized in the first step) are well exposed to the organic solvent (and reagents).

We measured the activities of natural enzymes (enzymes without polymer layer) and compared the activities of the SEN-products. The concentrations of the resulting SEN-products were measured at the second step (in hexane media) by the absorbance at 280 nm. Relative activities was calculated, the activities of the SEN-enzymes were divided by the activities of the natural enzymes. The

activity of SEN-mannosidase is 47.8% of the natural mannosidase enzyme. The activity of SEN-xylosidase enzyme is 55.3% of the natural one and the activity of SEN-endomannanase is 63.0% of the natural endomannanase.

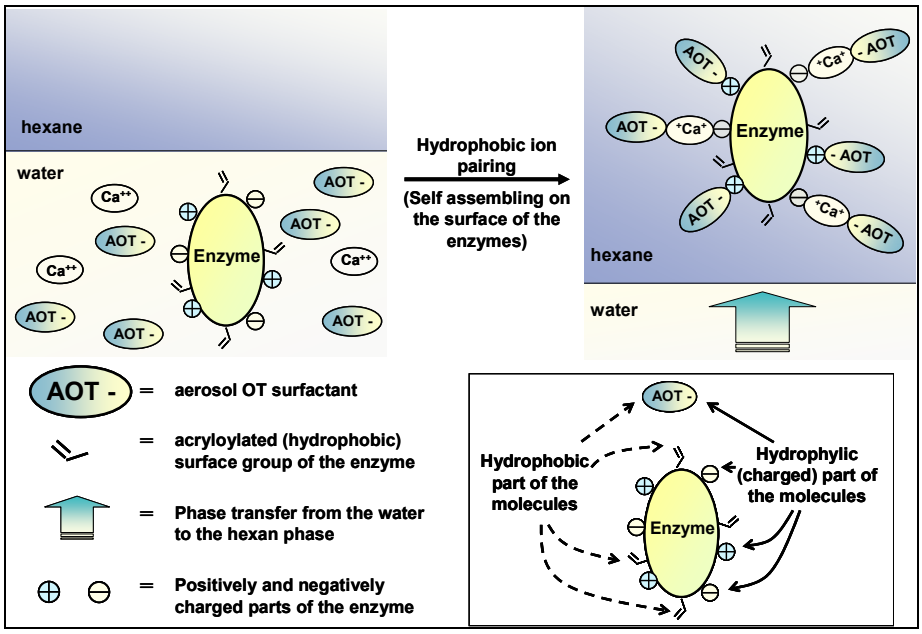

Figure 6. Mechanism of hydrophobic ion pairing

The size distribution of the endomannanase enzyme during the preparation was investigated (Figure 7). The peak of the size distribution of the natural endomannanase enzyme (EM/water) is about 6 nm, but the peak of the size distribution of the SEN-endomannanase (SEN-EM/water) is about 15 nm. SEN-mannosidase and SEN-xylosidase have higher peak of their size distribution (about 60 nm).

The results show that the range of the size of the enzyme nanoparticles on the hexane phase after the hydrophobic ion pairing process are between 2-10 nm (EM/hexane, see above, Figure 7). We measured three different enzymes (EM = endomannanase, MM = mutant mannosidase, MN = β-mannosidase, XI = β-xylosidase enzyme).

At +4 ^{o}C single enzyme nanoparticles from β-xylosidase enzymes have 40% of its original activity, while the natural β-xylosidase enzymes lost its activity after about 40 days (Figure 8). At 80 ^{o}C after 6 hours SENs of β-mannosidase enzymes have 40% of its original activity but the natural enzymes lost their activity after a half an hour (Figure 9).

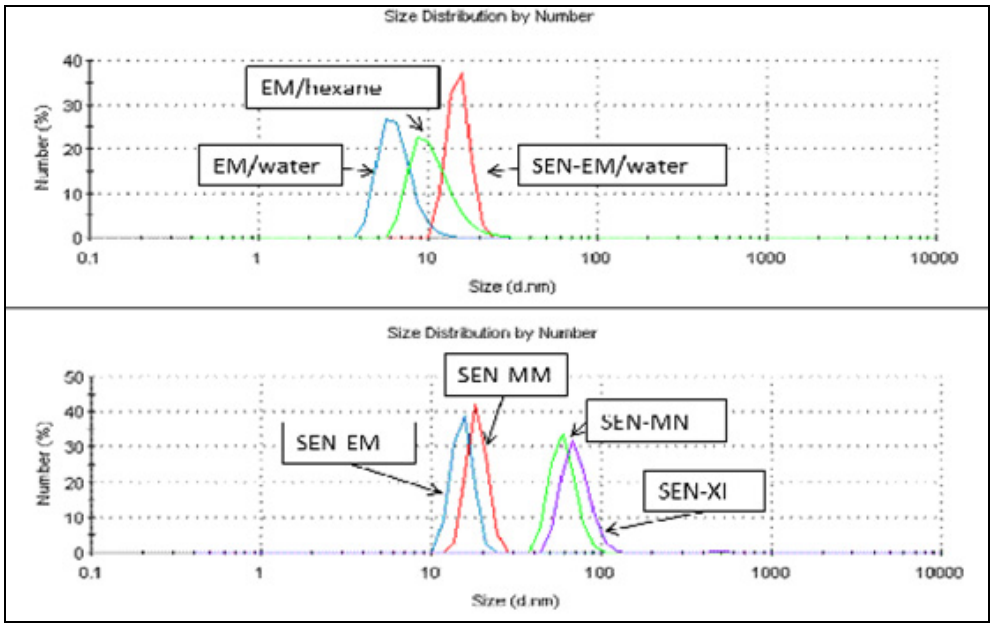

Figure 7. Size distribution of different type of single enzyme nanoparticles after the solvatation in hexane using hydrophobic ion pairing (EM = endomannanase, MM = mutant mannosidase, MN = β-mannosidase, XI = β-xylosidase enzyme)

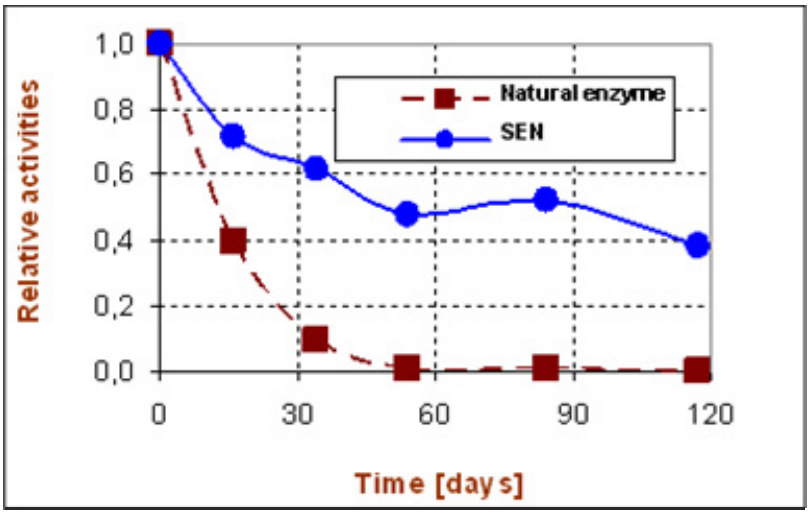

Figure 8. Stability of SEN β-xylosidase enzymes and control β-xylosidase enzymes at +4 °C

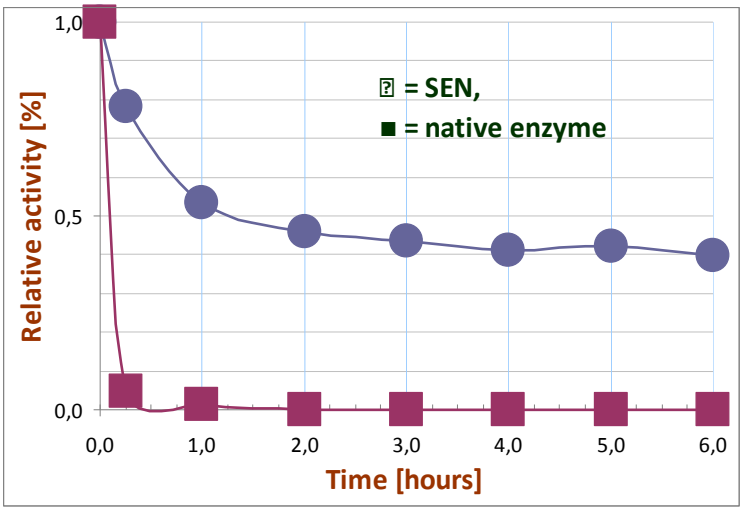

Figure 9. Stability of SEN β-mannosidase enzymes and natural β-mannosidase enzymes at 80 °C

CONCLUSIONS

Single enzyme nanoparticles (SENs) were fabricated using thermostable hemicellulase enzyme molecules from Thermobifida fusca (β-D mannosidase, β-D-xylosidase, endomannanase and mutant β-D mannosidase enzymes). Each individual enzyme molecule were covered with a polymer nano-layer in order to increase their stability. the stabilization of thermophyl enzymes seemed successfully This enzyme nanobiocomposites have a good stability under extreme conditions. Even after 6 hours at 80 °C, SENs have 40% of its original activity but the natural enzymes lost their activity after a half an hour.

EXPERIMENTAL SECTION

Enzymes: endomananase (EM), β-xylosidase (XI) and β-mannosidase (MN) from Thermobifida fusca and mutant β-mannosidase (MM). Enzymes were isolated from Thermobifida fusca species in Szent István University, Faculty of Agriculture and Environmental Sciences, Gödöllő and were purified in University of Debrecen, Faculty of Science and Technology, Research Institute of Genetics and Applied Microbiology, Debrecen.

Chemicals: acryloyl chloride, 1,3-bis[tris(hydroxymethyl)methylamino] propane or Bis-Tris propane (Sigma®), sodium bis(2-ethylhexyl) sulphosuccinate or aerosol OT (AOT) (Fluka®), disodium hydrogen phosphate, potassium dihydrogen phosphate, calcium chloride, 2-propanol, n-hexane (Spektrum-3d®, Scharlau®), methacryloxypropyltrimetoxysilane (MAPS), 2,2-azobis(2,4-dimethyl-valeronitrile) (Fluka®), 3,5-dinitro-salicylic acid (Sigma®).

Cryostate was used to keep the reaction mixture at 0 ^{o}C during the first step of the preparation of SEN. A gas chromatographic syringe (volume 5 μl) was used for the addition of a few microlitres of acryloyl chloride to the enzyme solution. The polymerization step in the synthesis of single enzyme nanoparticles (SEN) was carried out in a double-walled stirring vessel. The solution was irradiated by a UV-lamp made by Vilber Lourmat®. Filtration of the surface-polymerized enzymes was carried out with a syringe filter (pore size 0.1 μm) made by Millipore®.

UV-spectra were recorded and enzyme activity measurements carried out by means of a Biochrom 4060 spectrophotometer made by Pharmacia®. A New Brunswick Scientific G24 incubator shaker was used for the stability measurements. For the detection of the size distribution of the enzyme nanoparticles Malvern Zeta-sizer® was used.

ABBREVATIONS

AOT:	sodium bis(2-ethylhexyl) sulfosuccinate (aerosol OT)
EM	endomannanase
MM	mutant mannosidase
SEN:	single enzyme nanoparticle
MAPS:	methacryloxypropyltrimethoxysilane
MN	β-mannosidase
TEM:	transmission electron microscope
XI	β-xylosidase

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REFERENCES

1. J. Kukolya, I. Nagy, M. Láday, O. Oravecz, K. Máraligeti, L. Hornok, *Int. J. Evol. Microbiol.*, **2002**, *52*, 1193-1199.
2. J. Kim, J.W. Grate, P. Wang, *Chemical Engineering Science*, **2006**, *61* (3), 1017-1026.
3. I. Hegedűs, E. Nagy, *Chemical Engineering Science*, **2009**, *64*,1053-1060.
4. R. Hong, T. Emrick, V.M. Rotello, *Journal of American Chemical Society*, **2004**, *126* (42), 13572-13572.
5. J. Hong, D. Xu, P. Gong, H. Ma, L. Dong, S. Yao, *Journal of Chromatography B*, **2007**, *850*, 1-2, 499-506.

6. Y. Ge, Y. Ming, D. Lu, M. Zhang, Z. Liu, *Biochemical Engineering Journal*, **2007**, *36*, 93-99.

7. Y-L. Zeng, H-W. Huang, J-H. Jiang, M-N. Tian, Ch-X. Li, G-L. Shen, R-Q. Yu, *Analytica Chimica Acta*, **2007**, *604*,170-176.

8. K. Yao, Y. Zhu, X. Yang, C. Li, *Materials Science and Engineering: C* , **2008**, *28* (8), 1236-1241.

9. Y. Ming, Y Ge, Z. Liu, P. K. Ouyang, *Journal of American Chemical Society*, **2006**, *128*, 11008-11009.

10. R. Kumar, A.N. Maitra, P.K. Patanjali, P. Sharma, *Biomaterials*, **2005**, *26*, 6743–6753.

11. R.S. Kumar, S. Das, A. Maitra, *Journal of Colloid and Interface Science*, **2005**, *284*, 358–361.

12. R.R. Naik, M.M. Tomczak, H.R. Luckarift, J.C. Spain, M.O. Stonea, *Chemical Communications,* **2004**, 1684–1685.

13. Z. Yang, S. Shihui, Z. Chunjing, *Biochemical and Biophysical Research Communications*, **2008**, *367*, 169-175.

14. A. Mori, C. Ohtsuki, T. Miyazaki, A. Sugino, M. Tanihara, K. Kuramoto, A. Osaka, *Journal of Materials Science: Materials in Medicine* **2005**, *16* 713–718.