An Effective Sequent Cellulose-to-Glucose and Glucose-to-Fructose Conversion Using Cellulase and Isomerase Separately Immobilized, Fe_3O_4-Loaded Mesoporous Silica Nanoparticles as Recyclable Biocatalysts

Yi-Chun Lee, Ching-Tien Chen and Kevin C.-W. Wu*

Department of Chemical Engineering, National Taiwan University

E-mail: kevinwu@ntu.edu.tw

Abstract

Cellulase and isomerase were separately immobilized into Fe_3O_4-loaded mesoporous silica nanoparticles (Fe_3O_4-loaded MSN) for a sequent cellulose-to-glucose and glucose-to-fructose conversion, respectively, in aqueous solutions with a high yield of fructose (ca. 51%) and excellent stability and recyclability, which demonstrates their potential applications in high-value enzymatic biorefinery.

Keywords : immobilization, cellulase, isomerase, mesoporous silica nanoparticle

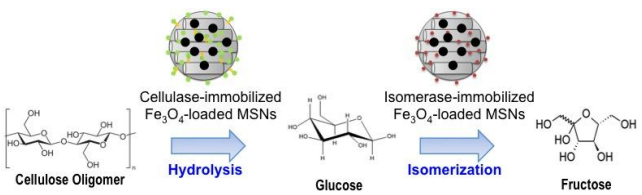
Introduction

Fructose, an isomer of glucose, is widely used as a sweetener or high-fructose corn syrup (HFCS) in food industry because fructose is the sweetest sugar in nature and its sweetening capacity is twice than sucrose.[1-6] Moreover, fructose is more suitable than other sweeteners for diabetics because the absorption of fructose by the stomach is slow and such adsorption does not influence the glucose level in blood. Recently, other than as a sweetener, fructose has been considered as a renewable energy resource derived from lignocellulosic biomass.[2] Fructose can be converted easily to 5-hydroxyfurfural (HMF)[7-11] which is a versatile platform for biofuels such as 2,5-dimethylfuran (DMF).[12-14]

Three major approaches have been widely used in cellulosic conversion and they are physical (e.g., high temperature and pressure), chemical (e.g., strong acid treatment) and biological procedures. Although physical and chemical processes have shown their efficacy to a certain extent, these processes are usually energy-consumption and produce many by-products.[15, 16] On the contrary, enzyme-based biological processes are performed at mild conditions with high specificity for one product.[17] Consequently, enzyme-assisted cellulosic conversion is an alternative green approach to reduce experimental cost, to inhibit un-wanted by-products, and to elevate reaction efficiency and specificity.[18, 19]

The maintenance of enzyme activity upon reaction and the recyclability of the enzyme after reaction are two big issues in enzymatic reaction. To overcome these problems while keeping the advantages of enzymes, immobilization of enzymes on a suitable host material has been considered as a good solution because such immobilization could offer several advantages, including repeated use, ease of separation from product, alteration of properties of enzyme, improved stability of enzyme and easy to storage.[20, 21] For examples, several solid materials, such as amorphous silica or agarose gel have been used as host materials for immobilization of enzyme.[22, 23] Recently, mesoporous silica materials have also been used as potential host materials for immobilization of enzymes owing to their large surface areas, adjustable pore sizes and diverse surface functionalities.[23-28]

We have previously synthesized mesoporous silica nanoparticles (MSNs) and used them to immobilize cellulase through physical adsorption and chemical binding for cellulose-to-glucose conversion. The cellulase-immobilized MSNs showed a great potential as a green biocatalyst with high working efficacy and enhanced stability. Since our previous results demonstrated that cellulase could effectively convert cellulose to glucose, one can consequently image to use another enzyme to convert glucose to another product. Here we immobilize glucose isomerase into MSNs to convert glucose to fructose. Because each enzyme has its own optimal reaction conditions, we separately immobilize cellulase and isomerase into MSNs instead of simultaneous immobilization in order to achieve the maximum yield of fructose, as shown in **Scheme 1**. In addition, in order to recycle the MSN catalysts, we also load iron oxides (Fe_3O_4) nanoparticles into MSNs in advance upon the synthesis of MSNs.[29]



Scheme 1. An illustration expressing a sequent cellulose-to-glucose and glucose-to-fructose conversion with the presence of cellulase and isomerase separately immobilized, Fe_3O_4-loaded MSN catalysts.

In this work, we first optimize reaction conditions, including reaction temperature, reaction time and the amounts of free enzymes. Then, we use cellulase-immobilized or isomerase-immobilized, Fe_3O_4-loaded MSNs as efficient and recyclable biocatalysts for a sequent

cellulose-to-glucose and glucose-to-fructose conversion, respectively. The results reveal that the best yield of fructose is approximately 51%. In addition, we also demonstrate that the enzyme-immobilized, Fe_3O_4-loaded MSNs catalysts are stable and recyclable at least for 5 times without an obvious decrease of fructose yield.

Results and discussion

The reaction conditions for cellulase have been optimized in our previous report (i.e., 50 ℃, 24 hrs, 4.5 mg in a citric buffer with pH=4.8). Here, the optimal reaction temperature, time, and the amounts of isomerase for the glucose-to-fructose isomerization were studied, and the results were shown in **Fig. 1**. Glucose (15 mg) was added into a glucose isomerase (4 mg)-containing phosphate buffer solution (pH 7.5), and the conversion was carried out at different temperature for 24 hours. As shown in **Fig. 1a**, the yield of fructose increased from around 43% to 60% when the reaction temperature was increased from room temperature to 70 ℃, respectively, indicating an enhanced efficiacy of isomerase at higher temperature. However, like most enzymes, the working efficiency decreases when the temperature is over a threshold (i.e., 70 ℃ in this case). Consequently, we chose 70 ℃ as the optimal reaction temperature. Next, we used the same reaction conditions but varied the reaction time. The results shown in **Fig. 1b** indicate that the yield of fructose increased from 45% to 70% when the reaction time was increased from 3 hr to 24 hr, respectively. The yield was decreased when the time period further increased to 48 hr, suggesting isomerase is not stable for such a long time or the fructose product further degrades to other by-products. After optimization of reaction temperature and time, we studied the effect of the isomerase amount in order to use the minimum amount of isomerase while maximizing the fructose yield for an economic viewpoint. Various amounts of isomerase, ranging from 0.033 to 9.9 mg, were used for the glucose-to-fructose conversion at 70 ℃ for 24 hrs. **Fig. 1c** shows that the optimal amount of isomerase was 3.3 mg (i.e., 97.5 unit). More or less than this amount resulted in lower fructose yields.

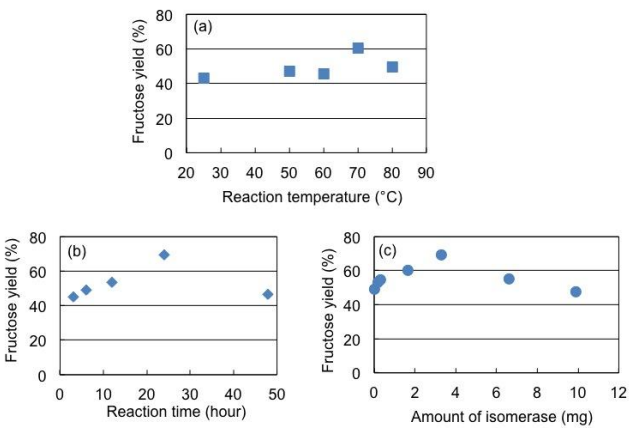


Fig 1. The optimization of reaction conditions for isomerase-catalysed glucose-to-fructose conversion. (a) Optimization of reaction temperature. (b) Optimization of reaction time. (c) Optimization of isomerase amount.

The synthesis of Fe_3O_4 nanoparticles and MSNs was modified from previous papers, and the experimental details are described in **ESI†**. The synthesized Fe_3O_4-MSNs were characterized with TEM and nitrogen adsorption/desorption isotherms, as shown in **Fig 2**. The TEM image in Fig. 2a shows that the Fe_3O_4 nanoparticles are embedded in the MSN, forming a Fe_3O_4/MSN nanocomposite with a worm-like porous structure. As shown in **Fig. 2b**, the Fe_3O_4-loaded MSNs exhibit a type III nitrogen adsorption-desorption isotherm with a narrow pore size distribution. The BET specific surface area and pore size are 100.9 m^2/g and 3.7 nm, respectively.

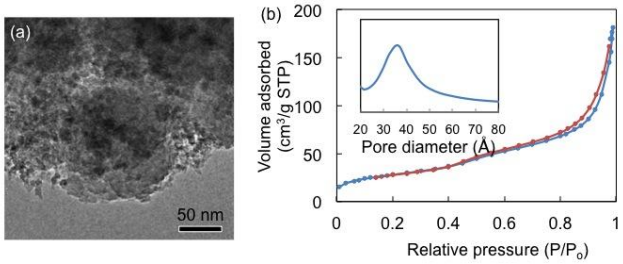


Fig 2. Characterization of Fe3O4-loaded MSNs. (a) TEM observation and (b) N_2 adsorption-desorption isotherm (inset: pore size distribution).

After the synthesis of Fe_3O_4-loaded MSNs, cellulase and isomerase are separately immobilized onto the surface of Fe_3O_4-loaded MSNs by physical adsorption (**ESI†**). For 50 mg of Fe_3O_4-loaded MSNs, the amounts of the immobilized cellulase and isomerase were 7.3 and 0.65 mg, respectively, that were quantitatively measured by UV-Vis spectroscopy (**ESI†**). To compare the conversion efficiencies between free enzymes and immobilized enzymes, the yields of the corresponding converted products are shown in **Fig. 3a**. The results indicated that the immobilized enzyme mostly kept their activity as well as free enzyme when the amout of immobilized enzyme was the same as free enzyme. This result clearly demonstrates that the immobilization of cellulase or isomerase into Fe_3O_4-loaded MSNs in this study did not greatly alter the enzyme activity.

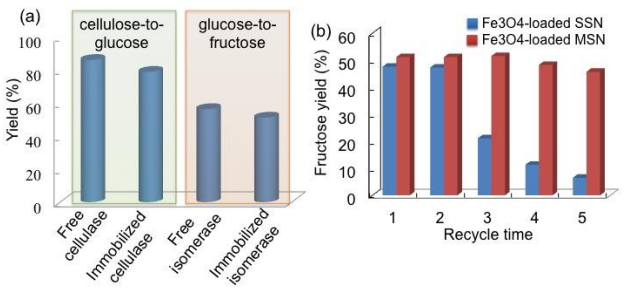


Fig 3. (a) Yields of glucose and fructose from cellulose and glucose, respectively, with the corresponding free and immobilized enzyme (i.e., cellulase and isomerase, respectively). (b) Fructose yields catalysed by Fe_3O_4-loaded SSN and MSN at different recycle times.

After confirmation of the activity of the immobilized enzyme, we further performed the sequent cellulose-to-glucose and glucose-to-fructose conversion by using cellulase and isomerase separately immobilized, Fe_3O_4-loaded MSNs, respectively, as catalysts. Typically, 0.015 g of ionic liquid-pretreated cellulose was added to 1 mL citric buffer (pH 4.8) containing cellulase-immobilized, Fe_3O_4-loaded MSNs (0.05 g). After reaction at 50 ℃ for 24 hrs, the cellulase-immobilized, Fe_3O_4-loaded MSNs were separated by magetic field, and the residue was placed into another vial which contained isomerase-immobilized, Fe_3O_4-loaded MSNs (0.05 g). We then added a sodium hydroxide aqueous solution (1.0 M) to increase the solution pH value to 7.5. The mixture was then heated to 50 ℃ for another 24 hrs. It is worth of noting that we have previsouly confirmed that isomerase can work at the "citrate" buffer with pH = 7.5 but cellulase cannot work at the "phosphate" buffer with pH = 4.8, indicating that isomerase is only sensitive to pH value but cellulase is sensitive to both pH value and the kind of buffer. As shown in **Fig 3b**, through a sequent cellulose-to-glucose and glucose-to-fructose conversion with the presence of our enzyme-immobilized, Fe_3O_4-loaded MSNs, a high yield of fructose up to 51% could be achieved. Since the yield of glucose from immobilized cellulase was higher than 51% (i.e., around 80%), we proposed that the final fructose yield was determined by the working efficiency of the immobilized isomerase.

One of the advantages for enzyme immobilization is to easily separate enzymes from substrates after reaction, thus it is possible to recycle and reuse the enzyme-immobilized catalysts. Because we have found that the enzyme-immobilized solid catalysts was difficult to be separated from the residue of converted cellulose through filtration, we loaded Fe_3O_4 into the MSN materials so that we can easily recycle the Fe_3O_4-loaded MSN catalysts by magnetic field. As a proof of principle, the recycle test for both cellulase and isomerase-immobilized, Fe_3O_4-loaded MSN catalysts were conducted. As shown in **Fig. 3b and ESI**, the final fructose yield was kept in the range of 46-50% even after recycle for five times. The results above indicate that our enzyme-immobilized Fe_3O_4-loaded MSN catalysts exhibit an excellent recycliability and stability.

To demonstrate the advantage of MSNs, we synthesized Fe_3O_4-loaded silica solid nanoparticles without mesopores (namely, Fe_3O_4-loaded SSNs) and used them to immobilize enzymes. We then compared the reaction efficiency and recycliability between enzyme-immobilized, Fe_3O_4-loaded MSNs and Fe_3O_4-loaded SSNs. As shown in **Fig. 3b**, although the case of enzyme-immobilized, Fe_3O_4-SSNs exhibited similar final fructose yields as the case of enzyme-immobilized, Fe_3O_4-MSNs for the first and second recycle times, the fructose yield decreased from 47% to 6% after the fifth recycle time, in contrast to a steady fructose yield in the case of enzyme-immobilized. Such gradual decrease of fructose yield in the case of enzyme-immobilized, Fe_3O_4-SSNs indicated a gradual lose of enzyme as increasing recycle time, which was due to the loose adsorption of the enzyme onto the merely external surface of SSNs. The lack of mesopores in SSNs would not be able to provide a shielding effect for enzyme immobilization.

Conclusion

In conclusion, we have optimized the reaction conditions of glucose isomerase as 70 ℃, 24 hrs, and 3.3 mg for the maximum production of fructose. The enzyme was then immobilized successfully into Fe_3O_4-loaded MSNs exhibiting high surface area, large pore size, and magnetic property without losing activity. We have also demonstrated that such enzyme-immobilized, Fe_3O_4-loaded MSNs could catalyze a sequent cellulose-to-glucose and glucose-to-fructose conversion and could achieve a high fructose yield around 50%. In addition, these catalysts exhibited excellent recycliability and stability. The results obtained in this study indicate that the enzyme-immobilized, Fe_3O_4-loaded MSNs would be effective, green, recyciable and stable biocatalysts for various enzymatic applications.

Acknowledgements

Department of Chemical Engineering, National Taiwan University. No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan. Fax: 886 2 2362 3040; Tel: 886 2 3366 3064; E-mail: kevinwu@ntu.edu.tw

† Electronic Supplementary Information (ESI) available: Synthesis and characterization of Fe3O4-loaded MSNs, immobilization of enzyme, recycle test. See DOI: 10.1039/b000000x/

References

1. Yu, H. T.; Guo, Y. L.; Wu, D. L.; Zhan, W. C.; Lu, G. Z., *J. Mol. Catal. B-Enzym.* **2011,** *72* (1-2), 73-76.
2. Yu, D. H.; Wu, H.; Zhang, A. J.; Tian, L.; Liu, L. D.; Wang, C. M.; Fang, X. X., *Process Biochem.* **2011,** *46* (2), 599-603.
3. Tukel, S. S.; Alagoz, D., *Food Chem.* **2008,** *111* (3), 658-662.
4. Moller, M.; Harnisch, F.; Schroder, U., *Biomass Bioenerg.* **2012,** *39*, 389-398.
5. Kaneko, T.; Takahashi, S.; Saito, K., *Biosci. Biotechnol. Biochem.* **2000,** *64* (5), 940-947.
6. Mostafazadeh, A. K.; Sarshar, M.; Javadian, S.; Zarefard, M. R.; Haghighi, Z. A., *Sep. Purif. Technol.* **2011,** *79* (1), 72-78.
7. Jadhav, A. H.; Kim, H.; Hwang, I. T., *Catal. Commun.* **2012,** *21*, 96-103.
8. Zhang, Z. H.; Liu, B.; Zhao, Z. B., *Carbohydr. Polym.* **2012,** *88* (3), 891-895.
9. Liu, J. T.; Tang, Y.; Wu, K. G.; Bi, C. F.; Cui, Q., *Carbohydr. Res.* **2012,** *350*, 20-24.
10. Musau, R. M.; Munavu, R. M., *Biomass* **1987,** *13* (1), 67-74.
11. Shi, C. Y.; Zhao, Y. L.; Xin, J. Y.; Wang, J. Q.; Lu, X. M.; Zhang, X. P.; Zhang, S. J., *Chem. Commun.* **2012,** *48* (34), 4103-4105.

12. Kazi, F. K.; Patel, A. D.; Serrano-Ruiz, J. C.; Dumesic, J. A.; Anex, R. P., *Chem. Eng. J.* **2011,** *169* (1-3), 329-338.
13. Hu, L.; Sun, Y.; Lin, L., *Prog. Chem.* **2011,** *23* (10), 2079-2084.
14. Simmie, J. M.; Metcalfe, W. K., *J. Phys. Chem. A* **2011,** *115* (32), 8877-8888.
15. Fitzpatrick, S. W., The Biofine technology A "bio-refinery" concept based on thermochemical conversion of cellulosic biomass. In *Feedstocks for the Future: Renewables for the Production of Chemicals and Materials*, Bozell, J. J.; Patel, M. K., Eds. Amer Chemical Soc: Washington, 2006; Vol. 921, pp 271-287.
16. Amarasekara, A. S.; Wiredu, B., *Appl. Catal. A-Gen.* **2012,** *417*, 259-262.
17. Chen, K. C.; Lee, S. L.; Wei, G. T.; Hsieh, H. C.; Yu, C. Y., *J. Biosci. Bioeng.* **2009,** *108*, S112-S112.
18. Gusakov, A. V.; Salanovich, T. N.; Antonov, A. I.; Ustinov, B. B.; Okunev, O. N.; Burlingame, R.; Emalfarb, M.; Baez, M.; Sinitsyn, A. P., *Biotechnol. Bioeng.* **2007,** *97* (5), 1028-1038.
19. Demirbas, M. F.; Balat, M., *Energy Conv. Manag.* **2006,** *47* (15-16), 2371-2381.
20. Tran, D. N.; Balkus, K. J., *ACS Catal.* **2011,** *1* (8), 956-968.
21. Hirsh, S. L.; Bilek, M. M. M.; Nosworthy, N. J.; Kondyurin, A.; dos Remedios, C. G.; McKenzie, D. R., *Langmuir* **2010,** *26* (17), 14380-14388.
22. Kovalenko, G. A.; Perminova, L. V.; Chuenko, T. V.; Sapunova, L. I.; Shlyakhotko, E. A.; Lobanok, A. G., *Appl. Biochem. Microbiol.* **2011,** *47* (2), 151-157.
23. Weetall, H. H., *Appl. Biochem. Biotechnol.* **1993,** *41* (3), 157-188.
24. Popat, A.; Hartono, S. B.; Stahr, F.; Liu, J.; Qiao, S. Z.; Lu, G. Q., *Nanoscale* **2011,** *3* (7), 2801-2818.
25. Rosenholm, J. M.; Sahlgren, C.; Linden, M., *Curr. Drug Targets* **2011,** *12* (8), 1166-1186.
26. Tang, F. Q.; Li, L. L.; Chen, D., *Adv. Mater.* **2012,** *24* (12), 1504-1534.
27. Zhu, Y. F.; Ikoma, T.; Hanagata, N.; Kaskel, S., *Small* **2010,** *6* (3), 471-478.
28. Rahman, N. A.; Hussain, M. A.; Hasan, M.; Jahim, J. M., *Afr. J. Biotechnol.* **2011,** *10* (14), 2766-2779.
29. Liu, J.; Qiao, S. Z.; Hu, Q. H.; Lu, G. Q., *Small* **2011,** *7* (4), 425-443.

