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An Effective Cellulose-to-Glucose-to-Fructose Conversion Sequence by Using Enzyme Immobilized Fe_3O_4-Loaded Mesoporous Silica Nanoparticles as Recyclable Biocatalysts

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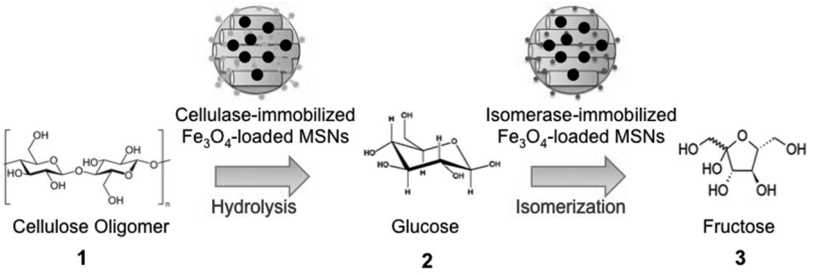
Fructose, an isomer of glucose, is widely used as a sweetener or as high-fructose corn syrup (HFCS) in the food industry, because fructose is the sweetest sugar in nature and its sweetening capacity is twice that of sucrose.[1] Recently, other than as a sweetener, fructose has been considered as a renewable energy resource derived from lignocellulosic biomass.[2] Fructose can be converted easily into 5-hydroxyfurfural (HMF),[3] which is a versatile platform for biofuels such as 2,5-dimethylfuran (DMF).[4]

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 the physical and chemical processes have shown their efficacy to a certain extent, these processes are energy consuming and produce many byproducts.[2a,5] On the contrary, enzyme-based biological processes are performed under mild conditions with high specificity for one product. Therefore, enzyme-assisted cellulosic conversion is an alternative green approach that reduces experimental costs, inhibits unwanted byproducts, and elevates reaction efficiency and specificity.[6]

The maintenance of enzyme activity upon reaction and the recyclability of the enzyme after the reaction are two big issues in enzymatic reactions. 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 immobilization can offer several advantages, including repeated use, ease of separation from the product, alteration of the properties of the enzyme, improved stability of the enzyme, and easy storage.[7] For example, several solid materials, such as amorphous silica or agarose gel, have been used as host materials for the immobilization of enzymes.[8] In addition, mesoporous silica materials have also been used as potential host materials for the immobilization of enzymes owing to their large surface areas, adjustable pore sizes, and diverse surface functionalities.[7a,9]

We previously synthesized mesoporous silica nanoparticles (MSNs) and used them to immobilize cellulase through physical adsorption and chemical binding for cellulose-to-glucose conversion.[6c] The cellulase-immobilized MSNs showed great potential as a green biocatalyst with high working efficacy and enhanced stability. However, as with our previous report, most of the enzyme-related papers report single-step reactions with the use of a single enzyme-immobilized catalyst. For multistep reactions such as cellulose-to-fructose conversion, it is necessary to use more than one enzyme. In this study, we demonstrate the synthesis and application of different enzyme-immobilized MSNs for multistep cellulose-to-fructose conversion in an aqueous solution, as shown in Scheme 1. Because each enzyme has its own optimal reaction conditions, we separately immobilized cellulase and isomerase into MSNs instead of their simultaneous immobilization to achieve the maximum yield of fructose. In addition, to recycle the MSN catalysts, we also loaded iron oxide (Fe_3O_4) nanoparticles into the MSNs during their synthesis.



Scheme 1. An illustration expressing a continuous cellulose-to-glucose and glucose-to-fructose conversion sequence by using cellulase and isomerase separately immobilized onto Fe_3O_4-loaded MSN catalysts.

In this work, we first optimized the reaction conditions, including reaction temperature, reaction time, the amount of free enzyme, and pH values, for isomerase. Then, we used cellulase-immobilized and isomerase-immobilized Fe_3O_4-loaded MSNs as catalysts for continuous cellulose-to-glucose and glucose-to-fructose conversion, respectively. The best yield of fructose was approximately 51%, which is almost the maximum yield obtained in industry for the production of fructose from glucose. In addition, and in contrast to nonporous silica hosts, we also demonstrated that the enzyme-immobilized Fe_3O_4-

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loaded MSN catalysts are stable and recyclable over at least five cycles without an obvious decrease in the product yield.

The reaction conditions for cellulase were optimized (i.e., 50 °C, 24 h, 4.5 mg cellulase/50 mg cellulose in a citric buffer with pH 4.8) in our previous report.[6c] Here, the reaction conditions, including reaction temperature, time, the amount of isomerase, and pH value, for glucose-to-fructose isomerization were optimized, and the results are shown in Figure 1. Glucose (15 mg) was added to a phosphate buffer solution (pH 7.5) containing glucose isomerase (4 mg), and the conversion was carried out at different temperatures for 24 h. As shown in Figure 1a, the yield of fructose increased from approximately 43 to 60% when the reaction temperature was increased from room temperature to 70 °C, which is indicative of the enhanced efficacy of isomerase at higher temperatures. However, like most enzymes, the working efficiency decreased if the temperature was over a threshold (i.e., 70 °C in this case). Consequently, we chose 70 °C as the optimal reaction temperature. Next, we used the same reaction conditions but varied the reaction time. The results shown in Figure 1b indicate that the yield of fructose increased from 45 to 70% when the reaction time was increased from 3 to 24 h. The yield decreased when the time period was further increased to 48 h, and this suggests that isomerase is not stable for such a long time and that the fructose produced further degrades to other byproducts. After optimization of the reaction temperature and time, we studied the effects of the amount of isomerase from an economic viewpoint so that a minimum amount of isomerase could be used to achieve a maximum yield of fructose. Various amounts of isomerase ranging from 0.033 to 9.9 mg were used for glucose-to-fructose conversion at 70 °C for 24 h. Figure 1c shows that the optimal amount of isomerase was 3.3 mg (i.e., 97.5 unit). The fructose yield decreased if the amount of isomerase was less than 3.3 mg, but it remained at a similar value (≈65%) if the amount exceeded 3.3 mg. The effect of pH on the working efficacy of isomerase was also studied. As shown in Figure 1d, the optimal pH value for isomerase is obviously 7.5. Higher or lower pH values resulted in lower fructose yields.

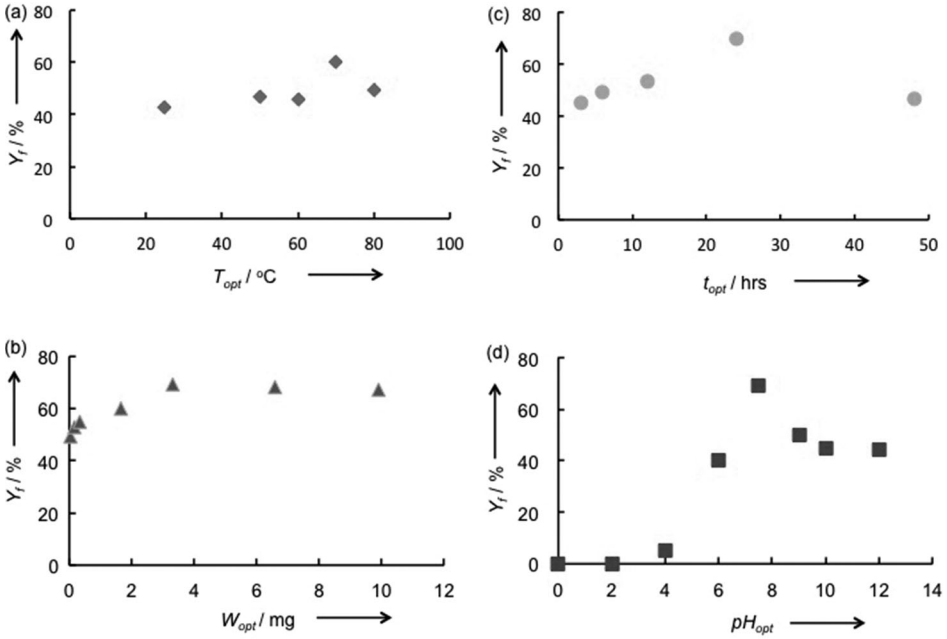

Figure 1. Optimization of the reaction conditions for isomerase-catalyzed glucose-to-fructose conversion. Optimization of a) reaction temperature, b) reaction time, c) isomerase amount, and d) pH value.

The synthesis of Fe_3O_4 nanoparticles and MSNs was modified from previous papers, and the experimental details are described in the Experimental Section. The synthesized Fe_3O_4-MSNs were characterized with TEM and nitrogen adsorption–desorption isotherms, as shown in Figure 2. The TEM image in Figure 2a shows that the Fe_3O_4 nanoparticles are embedded in the MSNs to form a Fe_3O_4–MSN nanocomposite with a worm-like porous structure. As shown in Figure 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 $m^2 g^{-1}$ and 3.7 nm, respectively.

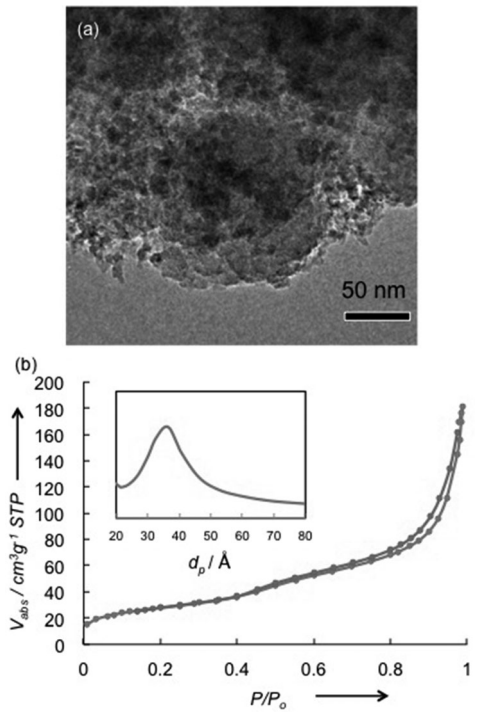

Figure 2. Characterization of Fe_3O_4-loaded MSNs. a) TEM observation and b) N_2 adsorption–desorption isotherm (inset: pore-size distribution).

After the synthesis of Fe_3O_4-loaded MSNs, cellulase and isomerase were separately immobilized onto the surface of the Fe_3O_4-loaded MSNs by physical adsorption. For 50 mg of Fe_3O_4-loaded MSNs, the amounts of immobilized cellulase and isomerase were 7.3 and 0.65 mg, respectively, which were quantitatively measured by UV/Vis spectroscopy (data not shown). To compare the conversion efficiencies of the free enzymes with those of the immobilized enzymes, the yields of the corresponding converted products are shown in Figure 3a. The results indicate that the immobilized enzymes mostly kept the activity of the free enzymes if the amount of the enzyme

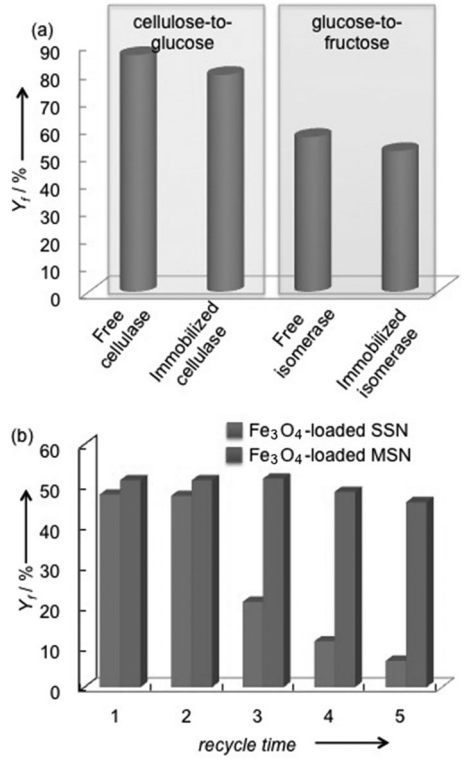

Figure 3. a) Yield of glucose and fructose from cellulose and glucose, respectively, with the corresponding free and immobilized enzyme (i.e., cellulase and isomerase, respectively). b) Fructose yields for reactions catalyzed by Fe_3O_4-loaded SSNs and Fe_3O_4-loaded MSNs at different recycle times.

used was the same. 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 activity of the enzyme. In addition, the leaching problem of the immobilized enzyme (i.e., cellulase and isomerase) during recycling was examined. As shown in Figures S1 and S2 (Supporting Information), the conversion yields and the amount of immobilized enzyme remained almost the same, even after eight cycles. These results indicate that the enzyme could be immobilized strongly onto the Fe_3O_4-loaded MSNs without any severe leaching and that it could retain the excellent activity of the free enzyme. Blank MSNs (i.e., non-enzyme-immobilized MSNs) did not show any catalytic ability, and this indicates that the catalytic conversion was the result of the immobilized enzyme only.

After confirmation of the activity of the immobilized enzyme, we further performed the continuous cellulose-to-glucose and glucose-to-fructose conversion sequence by using cellulase-immobilized Fe_3O_4/MSNs and isomerase-immobilized Fe_3O_4/MSNs, respectively, as catalysts. Typically, ionic-liquid pretreated cellulose (0.015 g) was added to a phosphate buffer (pH 4.8, 1 mL) containing cellulase-immobilized Fe_3O_4/MSNs (0.05 g). After reaction at 50 °C for 24 h, the cellulase-immobilized Fe_3O_4/MSNs catalysts were separated by a magnetic field, and the residue was transferred into another vial that contained isomerase-immobilized Fe_3O_4/MSNs (0.05 g). We then added an aqueous solution of sodium hydroxide (1.0 M) to increase the pH value of the solution to 7.5. The mixture was then heated to 70 °C for another 24 h. Notably, we previously confirmed the effects of buffer and pH value on the working efficiency of cellulase and isomerase (see Table 1), and the results showed that cellulase is only sensitive to pH but that isomerase is affected by both pH and the type of buffer. Therefore, to achieve a continuous cellulose-to-glucose and glucose-to-fructose reaction sequence, we chose phosphate buffer as the reaction media and adjusted the pH to 4.8 for the cellulose-to-glucose reaction and to 7.5 for the glucose-to-fructose reaction.

Table 1. Working efficacy of cellulase and isomerase in different buffers and at different pH values.

Enzyme	Reaction	Buffer conditions	Product yield [%]
cellulase	cellulose-to-glucose	citric buffer, pH 7.5	0
cellulase	cellulose-to-glucose	phosphate buffer, pH 4.8	85
isomerase	glucose-to-fructose	citric buffer, pH 7.5	35
isomerase	glucose-to-fructose	phosphate buffer, pH 4.8	0

One of the advantages of enzyme immobilization is that the enzyme can be easily separated from the substrate after the reaction; thus, it is possible to recycle and reuse the enzyme-immobilized catalysts. Because we found that it was difficult to separate the enzyme-immobilized solid catalyst from the residue of converted cellulose by filtration, we loaded Fe_3O_4 into the MSN materials so that we could easily recycle the Fe_3O_4-loaded MSN catalysts by passing them through a magnetic field. As a proof of principle, recycling tests for both the cellulase- and isomerase-immobilized Fe_3O_4-loaded MSN catalysts for cellulose-to-fructose conversion were conducted. As shown in Figure 3b, the final yield of fructose was kept in the range of 46–50% even after recycling five times. The above results indicate that our enzyme-immobilized Fe_3O_4-loaded MSN catalysts exhibit excellent recyclability and stability.

To demonstrate the advantages 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 recyclability of the MSNs to those of the SSNs. As shown in Figure 3b, although enzyme-immobilized Fe_3O_4-SSNs exhibited final fructose yields similar to those exhibited by enzyme-immobilized

Fe_3O_4-MSNs for the first and second cycles, the fructose yield decreased from 47 to 6% after the fifth cycle. Such a gradual decrease in the fructose yield in the case of enzyme-immobilized Fe_3O_4-SSNs is indicative of a gradual loss of the enzyme as the catalyst is recycled, and this could be due to the loose adsorption of the enzyme onto the external surfaces of the SSNs. Because the SSNs lack mesopores, a shielding effect for enzyme immobilization would not be provided.

In conclusion, we disclose a sequential enzymatic concept for the direct conversion of cellulose into fructose in aqueous solution. First, the reaction conditions of glucose isomerase were optimized as 70 °C, 24 h, 3.3 mg (per 15 mg of glucose), and phosphate buffer with pH 7.5 for the maximum production of fructose. The enzyme was then immobilized successfully into Fe_3O_4-loaded MSNs without losing its activity. We also demonstrated that such enzyme-immobilized Fe_3O_4-loaded MSNs could catalyze a continuous cellulose-to-glucose and glucose-to-fructose conversion sequence and could achieve a high fructose yield up to 50%, which is the same yield obtained when using the free enzyme. In addition, the utilization of Fe_3O_4-loaded MSNs as enzyme hosts provides both excellent recyclability and stability. The results obtained in this study indicate that enzyme-immobilized Fe_3O_4-loaded MSNs would be effective, green, recyclable, and stable biocatalysts for various enzymatic applications.

Experimental Section

Chemicals

Poly(oxyethylene) oleyl ether (Brij-97, C18H35EO10), ammonia hydroxide (37%), hydrochloride acid (37%), iron(II) chloride tetrahydrate, 3-aminopropyltrimethoxysilane (APTMS, 97%), dimethyl phthalate (DOP, >99%), tetraethoxysilane (TEOS), ethanol (99.8%), cellulase (*Trichoderma reesei* ATCC 26921), 1-butyl-3-methylimidazolium chloride (BMIM), cellulose (powder, ca.20 micron), D-(+)-glucose (>99.5%), D-(−)-fructose (>99%), sodium phosphate tribasic, magnesium sulfate, and sodium chloride were purchased from Sigma–Aldrich. Citric acid (anhydrous, powder), sodium hydroxide (NaOH), and acetonitrile were purchased from J. T. Baker. Iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) was purchased from Alfa Aesar. Methyl alcohol was purchased from Mallinckrodt Chemical. Glucose isomerase (purified from *Streptomyces rubiginosus*) was purchased from Hampton Research.

Synthesis of magnetite (Fe_3O_4)

$FeCl_3$ (hexahydrate, 1.349 g) and $FeCl_2$ (tetrahydrate, 0.781 g) were dissolved in deionized water (600 mL) with stirring. Then, ammonia hydroxide (1.5 M) was added to the iron-containing aqueous solution until the pH value of the solution increased to 9. The iron oxides (i.e., Fe_3O_4) were then collected by magnetic force and washed with deionized water and ethanol several times. The result sample was redispersed to deionized water (600 mL) for further use.

Synthesis of Fe_3O_4-loaded MSNs and Fe_3O_4-loaded nonporous silica nanoparticles

The Fe_3O_4-loaded MSNs were synthesized by a co-condensation method as follows: Brij-97 (6.92 mL) was added to an aqueous solution (180 mL) of magnetite with stirring at room temperature. After complete dissolution of Brij-97, APTMS (0.3 mL) and DOP (0.8 mL) were added to the mixture with stirring. After stirring for 30 min, TEOS (6.7 mL) was introduced, and the mixture was stirred at room temperature for 1 d followed by heating at reflux at 100 °C for another 24 h. Finally, the precipitate was collected by filtration, washed with methanol several times to remove the surfactant, and dried in a lyophilizer. The resulting sample was Fe_3O_4-loaded MSNs.

Fe_3O_4-loaded nonporous silica nanoparticles were synthesized by using the same procedure but without the addition of the surfactant.

Enzyme immobilization

For immobilization of cellulase, Fe_3O_4-loaded MSNs (50 mg) were suspended in a citric buffer (10 mM, 2 mL, pH 4.8). The cellulase solution (1 mL) was added to the citric buffer mixture, and the resulting mixture was stirred at 4 °C for 1 d.

For immobilization of isomerase, the same amount of Fe_3O_4-loaded MSNs was suspended in a phosphate buffer (20 mM sodium phosphate/0.15 M sodium chloride/5 mM magnesium sulfate, 2.5 mL, pH 7.5). Then, the isomerase solution (0.5 mL) was added to the phosphate buffer, and the mixture was stirred at 4 °C for 1 d.

Finally, the enzyme-immobilized Fe_3O_4-loaded MSNs were collected with a magnet. The enzyme remaining in the supernatant was considered the nonadsorbed enzyme, and its amount was measured by UV/Vis spectrometry at a wavelength of 280 nm. Therefore, the amount of immobilized enzyme could be calculated from the initial amount of the enzyme minus the amount remaining in the supernatant. The final catalysts were washed with citric or phosphate buffer several times and redispersed into citric or phosphate buffer (1 mL) for further use.

Characterization

The morphology of the Fe_3O_4-loaded MSNs was observed with TEM. The porous properties were analyzed with nitrogen adsorption–desorption isotherms with a Micromeritics ASAP 2000 instrument. The specific surface area and pore size were calculated by using BET and BJH methods, respectively.

Pretreatment of cellulose with ionic liquids

Cellulose (50 mg) was added to [BMIM]Cl (0.95 mL), and the mixture was stirred at 120 °C for 1 h. Methanol (3 mL) was added to the mixture to quench the reaction. Then, the resulting oligomer cellulose was separated from the ionic liquid by centrifugation, washed with methanol and water several times, and dried in a lyophilizer.

Enzymatic reactions

For cellulose-to-glucose conversion, pretreated cellulose (0.015 g) was added to citric buffer (1 mL) containing free cellulase or cellu-

lase-immobilized Fe_3O_4/MSNs, and the mixture was heated at 50 °C for 1 d.

For glucose-to-fructose conversion, glucose (0.015 g) was added to phosphate buffer (1 mL) containing free isomerase or isomerase-immobilized Fe_3O_4-loaded MSNs, and the mixture was heated at 70 °C for 1 d.

For cellulose-to-fructose conversion, the sequent cellulose-to-glucose and glucose-to-fructose reaction was catalyzed by two kinds of solid catalysts (i.e., cellulase-immobilized Fe_3O_4/MSNs for the first step and isomerase-immobilized Fe_3O_4/MSNs for the second step) in phosphate buffer. In this case, cellulase-immobilized Fe_3O_4/MSNs (0.015 g) were added to phosphate buffer (pH 4.8, 1 mL) containing pretreated cellulose (0.015 g) at 50 °C. After reaction for 1 d, the cellulase-immobilized Fe_3O_4/MSNs were collected with a magnet, and the supernatant was transferred to another vial that contained isomerase-immobilized Fe_3O_4/MSNs. Sodium hydroxide was then added to adjust the pH value of the mixture to 7.5, and the reaction was conducted for 1 d at 70 °C.

HPLC analysis

Upon completion of the reaction, we removed the catalysts or impurities by syringe filter and analyzed the composition of the solution by using HPLC (ASI500 system) with a Shodex NH2P50 4E column. Possible products such as cellobiose, glucose, and fructose were previously identified, and their calibration curves were measured (data not shown).

Optimization of buffer conditions

The working conditions of cellulase are very different from those of isomerase. For example, a citric buffer with pH 4.8 is usually used for cellulase, whereas a phosphate buffer with pH 7.5 is suitable for isomerase. To achieve the cellulose-to-fructose reaction, we first needed to optimize the reaction conditions, including the optimal buffers and their pH values. We used four kinds of buffers, that is, two citric buffers with pH 4.8 and 7.5 and two phosphate buffers with pH 4.8 and 7.5 for both cellulose-to-glucose and glucose-to-fructose reactions. As shown in Table 1, there were no yields for the cellulose-to-glucose and glucose-to-fructose reactions if cellulase in the critic buffer (pH 7.5) was used and if isomerase in the phosphate buffer (pH 4.8) was used, respectively (Table 1, Rows 1 and 4). From these results, we know that the pH of the buffer is very important for both enzymes. In contrast, if we changed the type of buffer but kept the same pH value, cellulase exhibited almost the same yield in the phosphate buffer (pH 4.8) as that in the citric buffer (pH 4.8) (85 vs. 90%; Table 1, Row 2) but isomerase exhibited much lower yield in the citric buffer (pH 7.5) than in the phosphate buffer (pH 7.5) (35 vs. 50%; Table 1, Row 3). These results indicate that cellulase could bear the difference of buffers once the pH of the buffer was 4.8. Therefore, we used phosphate buffer with pH 4.8 for the first cellulose-to-glucose reaction and then changed the pH value of the phosphate buffer to 7.5 for the subsequent glucose-to-fructose reaction.

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