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Enhancement of glucose isomerase activity by immobilizing on silica/chitosan hybrid microspheres

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ABSTRACT

Glucose isomerase (GI) plays a crucial role in the food industry as it serves as a catalyst for the conversion of glucose to fructose. Immobilized GI is often used due to increased stability as well as the expensive costs associated with free GI. In this study, GI was immobilized on silica/chitosan hybrid microspheres via simple process through in situ encapsulation. Enhanced rate of reaction was observed when the conversion of glucose to fructose was completed in 10 min catalyzed by immobilized GI because most GI was located on the shell of the support. Moreover, it was found that immobilized GI exhibited better pH, temperature, ions, storage and operation stability when compared to free GI. The relative enzyme activity was found to be above 90% with a wide pH range of 5.8–8.0, temperature range of 40–80 °C, storage range of 3 months and an increase in operation range of >15 times. Therefore, immobilized GI supported by silica/chitosan hybrid microspheres is an ideal candidate for biocatalysis.

1. Introduction

Glucose isomerase (GI), also known as D-xylose isomerase, is a very important water soluble enzyme commonly applied in drinks and various products in the food industry. GI is known to catalyze the conversion of many kinds of monosaccharides, such as D-glucose, D-xylose and D-ribose. Isomerization of glucose to fructose, is an indispensable process for the industrial production of high-fructose corn syrup (HFCS), main sweetener in many soft drinks and food [1,2]. The isomerization process should be performed in the pH range of 7.0–9.0 and temperature range of 70–80 °C in the presence of Mg^{2+} and Co^{2+} due to the reactive requirements of GI [3]. Additionally, the high price and difficulty in recycling of GI also lead to an increased cost associated with the production of HFCS. Comparatively, immobilized GI is usually used to increase the stability of GI against environmental changes and recycled to reduce the cost associated with the production process. Adsorption of enzyme on the carriers is usually the preferential immobilization method compared to other methods including embedding, co-valent binding and crosslinking mainly due to the simple process. However, some of the complications may arise with the method including a weak interaction between the carrier and the enzyme contributing to the loss of enzyme.

The stability of enzyme against environmental change is one of the most important properties for immobilized enzyme used in practical applications, resulting in more attention to improve or widen the stability range. So it is necessary to choose suitable carriers depending on the associated properties to make active sites of enzyme exposed and stabilized since it plays a crucial role in the catalytic performance and stability of immobilized enzyme. Various types of carrier materials have been reported and can mainly be divided into two kinds, polymeric materials (e.g., calcium alginate [4], GAMM [5], chitosan [6–8]) and inorganic materials (e.g., gold [9], TiO_2 [10], perovskite [11] and SiO_2 [12–14]). Even though the inorganic carriers are much more stable and of higher mechanical intensity, their interaction with enzyme through adsorption is weaker and usually results in the loss of enzyme. Chang et al. [14] prepared immobilized cellulase using mesoporous silica nanoparticles as carriers. Large loss percentage of cellulase from carriers was observed even though the yield of glucose was equal. Wu et al. [10] immobilized sucrose isomerase on mesoporous TiO_2 through adsorption to improve properties of enzyme. Better stability was obtained for immobilized sucrose isomerase compared to the free enzyme, however, the stability in reuse still needs to be improved. The immobilization of GI on the silica gel through adsorption has been previously studied in Song et al.'s work [15]. High catalytic activity was obtained in 45 min, however,

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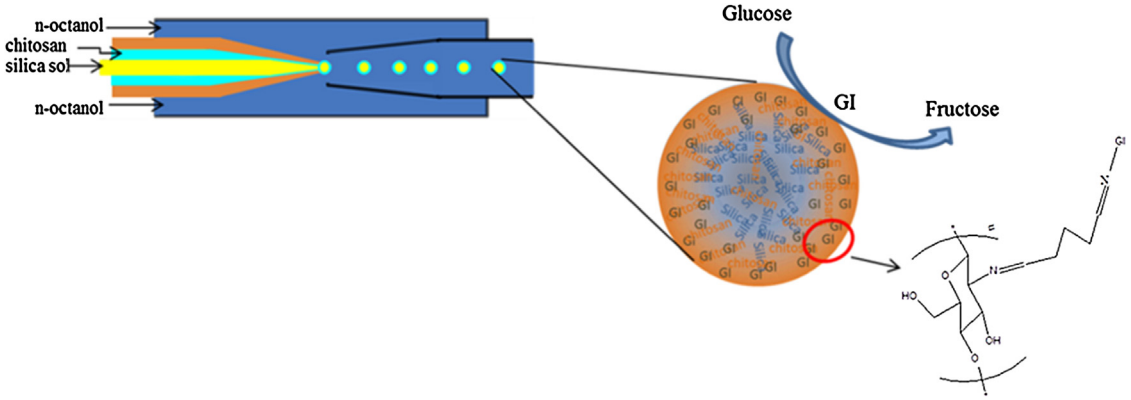

Scheme 1. The generation process of microspheres.

the catalytic activity was found to decrease largely in the recycle use. Another group of researchers were trying to address this problem by using surface-modified calcium alginate to adsorb GI to get much more stable recycle use, however, the stability range of pH and temperature was narrow [4]. High enzyme activity could be obtained only under the pH of 7.5 and temperature of 60 °C. Moreover, chitosan–polyacrylic acid hybrid microspheres were prepared as the GI carriers to improve the stability of pH, temperature and operation [16]. Although it was found that the immobilized GI had better operation stability due to crosslinking after GI adsorption, the process was complicated and dependent on the presence of ions with low enzyme activity. Therefore, it is necessary to develop a simple approach to prepare immobilized GI with higher enzyme activity and wider stability range against the environmental changes.

Silica/chitosan core–shell hybrid microspheres with good mechanical intensity and strong interaction with metal ions were prepared using microfluidic technology in our previous work [17]. The silica/chitosan supported catalyst can decrease mass-transfer resistance effectively while increasing reaction velocity of the mass-transfer limited reaction process due to most catalyst combined with chitosan was located on the shell of microspheres. Moreover, chitosan has outstanding properties of biocompatibility, adsorption and being environment-friendly, it is beneficial for the exposure of active sites of enzyme and interaction with enzyme strongly [18]. Hence, silica/chitosan core–shell hybrid microspheres can be an ideal carrier candidate to immobilize GI. Therefore, the immobilized GI was prepared simply by in situ encapsulation of GI in the silica/chitosan hybrid mic rospheres in this work. High yield was obtained in 10 min using immobilized GI as catalyst and the stability range of pH, temperature and storage was expanded to a large extent without dependence on metal ions. Furthermore, the immobilized GI can be recycled for many times with high catalytic activity.

2. Experimental

2.1. Materials and chemicals

Aqueous solution (10.0 g) of chitosan(0.20 g) with degree of deacelation below 95% (Sinopharm Chemical Reagent Co., Ltd., Beijing, PR China) dissolving in acetic acid (0.20 g, VAS Chemical Co., Ltd., Tianjin, PR China) was served as the middle fluid. Polymer aqueous solution (10.0 g) with tetraethoxysilane (TEOS) (0.20 g) dissolving in acetic acid (0.20 g) was used as the inner fluid whereas *n*-octanol (VAS Chemical Co., Ltd., Tianjin, PR China) as the continuous phase. *n*-Octane (10.0 g) with glutaraldehyde (0.040 g) and Span 80 (0.20 g, VAS Chemical Co., Ltd., Tianjin, PR China) was used as the solidification bath in which glutaraldehyde was served as the cross-linking reagent. Glucose isomerase from *Streptomyces rubiginosus* (Zhengzhou Zhongxin Chemical Reagent Co., Ltd.) and D-glucose (Beijing Chemical Works) were used as purchased and all of the reagents were analytically or chemically pure.

2.2. Preparation of silica/chitosan hybrid microspheres supported GI

The preparation procedure of silica/chitosan hybrid microspheres was described in detail in our previous work [17]. Dual co-axial microfluidic device was used, as shown in Scheme 1. 2.0 g of glucose isomerase powders were dissolved in 100 mL of deionized water and then centrifuged to get the supernatant as GI solution. 1.80 g of aqueous solution with 2.0 wt.% chitosan and 2.0 wt.% acetic acid was mixed with 0.20 g GI solution, which was used as middle fluid. Silica sol, the inner fluid, was obtained by stirring the aqueous solution with 2.0 wt.% acetic acid and 2.0 wt.% TEOS for 12 h at room temperature. They were dispersed into droplets by the shearing force of continuous fluid consisting of *n*-octane at the intersection of the microchannel. The droplets were collected out of the microchannel in the solidification bath consisting of *n*-octanol with 0.5 wt.% glutaraldehyde and 2.0 wt.% Span 80. The droplets were pre-solidified into microspheres through the Schiff's base reaction between —CHO from glutaraldehyde and $—NH_2$ from chitosan and GI and the extraction of water out of droplets by octanol. The microspheres were pre-solidified for a certain time (15–45 min) in the solidification bath and then washed with *n*-octane followed by a submersion for 24 h to gelate the silica sol. The silica/chitosan supported GI was then obtained after freeze-drying.

The immobilized GI prepared by adsorption of GI onto the microspheres were also prepared for comparison in this work. First, silica/chitosan hybrid microspheres were prepared according to the procedure mentioned above, with the exception that no GI solution was added in the middle fluid. Then silica/chitosan hybrid microspheres were incubated in the GI solution placed in a water-bath shaker under 25 °C, 130 rpm for 24 h for GI adsorption. The microspheres were then separated and washed with deionized water to remove the remnant GI followed by immersion in glutaraldehyde solution (0.2 wt.%) under 25 °C for 30 min enabling crosslinking between chitosan and GI for further immobilization. Lastly, the microspheres were washed with buffer solution to remove the residue glutaraldehyde and stored in fridge under 4 °C after freeze-drying.

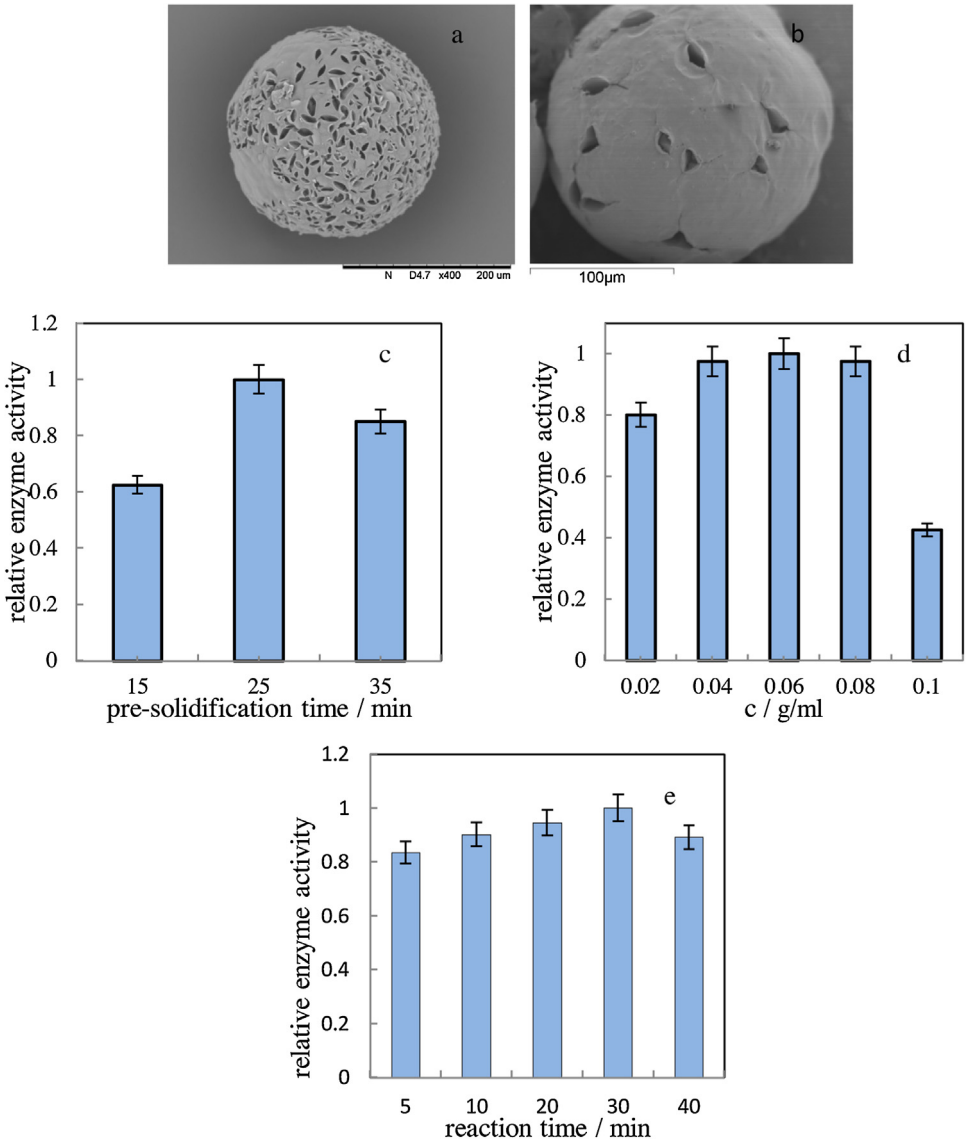

Fig. 1. SEM images and optimized reaction conditions (a) silica/chitosan hybrid microspheres without enzyme; (b) silica/chitosan hybrid microspheres with enzyme; (c) effect of pre-solidification time on enzyme activity; (d) effect of enzyme concentration on the relative enzyme activity; (e) effect of reaction time on the relative enzyme activity.

2.3. Enzyme activity assay

Enzyme activity of free and immobilized GI was examined through catalyzing isomerization of glucose to fructose in 10 min by stirring the substrate and GI in a three-neck flask under 70 °C and pH of 7.5. The temperature and pH will change in a certain range in the experiment of optimizing pH and temperature. The substrate was prepared by mixing 5 mL glucose with 4 mL phosphate buffer. 1 mL GI solution or 0.01 g of silica/chitosan hybrid microspheres supported GI was used as catalyst. In order to stabilize the structure of enzyme and increase its activity, 0.5 mL of $MgSO_4$ (0.2 mol/L) along with 0.5 mL of $CoCl_2$ (0.2 mol/L) were added into the substrate. Lastly, 5 mL of 0.5 mol/L $HClO_4$ was added to end the reaction.

Cysteine–carbazole method was used to analyze the concentration of fructose with a minor modification of Ryu's assay method [19]. 1.0 mL of the reaction solution mentioned above was taken out and diluted 100 times using phosphate buffer solution. Then 1.0 mL of the diluted solution was taken out and mixed with 0.2 mL of cysteine hydrochloride (1.5 wt.%), 6 mL of H_2SO_4(70%) and 0.2 mL of carbazole alcohol solution (0.12 wt.%) at 60 °C for 10 min to perform the color-developing reaction. The solution was then cooled down promptly using ice water to end the reaction. UV spectrophotometry was used to determine the concentration of fructose at the wavelength of 560 nm.

One unit of GI activity was defined as the amount of GI used in the production of 1 mmol of fructose per minute.

Relative enzyme activity was defined as the ratio of enzyme activity measured to the initial enzyme activity which was uniformly examined under temperature of 70 °C and pH of 7.5.

2.4. Recycle the immobilized GI

After reaction in one cycle, the immobilized GI was separated easily through sedimentation and washed with buffer solution. Then they could be used in another reaction cycle.

2.5. Characterization

An optical microscope (Type BX-61, Olympus, Japan) and an on-line CCD (Pixelink, Canada) were used to observe preparation process of droplets. Scanning electron microscopy (SEM,

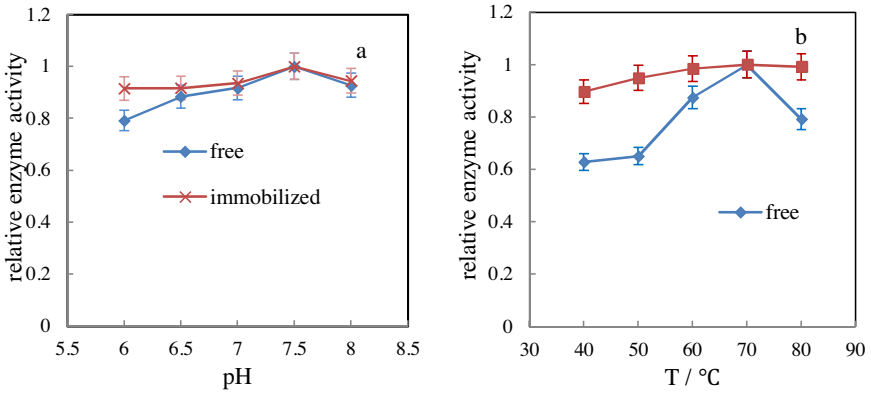

Fig. 2. Environmental stability of free and immobilized GI (a) pH stability; (b) temperature stability.

type TM3000 and F6301, Hitachi, Japan) was used to obtain the structure and element information of the carrier microspheres. UV spectrophotometry (SHIMADZU UV-2450, Japan) was used to determine the concentration of fructose at the wavelength of 560 nm.

3. Results and discussions

3.1. Formation of silica/chitosan hybrid microspheres supported GI

The inner and middle fluids were separated into droplets by the shearing force from continuous phase at the intersection in microchannel. In the solidification bath, the glutaraldehyde diffusing into the droplets crosslinked with chitosan and GI via the Schiff's base reaction. Water inside droplets was synchronously extracted out of the droplets by *n*-octanol. The chemical crosslinking and water extraction were combined to ensure the pre-solidification of microspheres. Then after being gelated in *n*-octane, the silica/chitosan hybrid microspheres supported GI could be obtained. Moreover, the immobilized GI with different carrier structures could be obtained by controlling their residence time in the solidification bath and *n*-octane. Because GI solution was added to chitosan solution directly, the immobilized GI was obtained through in situ encapsulation. Due to that most chitosan was distributed on the shell of the microspheres, most GI was found to be distributed on the shell. It was found that there was difference of the shell structure between microspheres pre-solidified for 25 min with and without enzyme as observed in Fig. 1a and b. The structure of microspheres depends on the pre-solidification time, as shown in Fig. S1. Pre-solidification of microspheres for 25 min was found to be the most optimal in Fig. 1c since a shorter or longer pre-solidification time would lead to a smaller specific surface area or larger mass-transfer resistance negatively effecting the exposure of active sites of GI. The amount of GI immobilized in the microspheres was also found to have a great effect on the enzyme activity. The reason for the optimal concentration at 0.06 g/mL as observed in Fig. 1d could be that enzyme activity increases with larger amount of enzyme in a certain range, however, after a certain concentration, agglomeration of enzyme occurred, inhibiting the exposure of active site. In previous researches, the reaction time of conversion glucose to fructose by GI was over 30 min. In this work, it was expected to shorten the reaction time because the immobilized GI had most GI on the shell of the carriers, as shown in Scheme 1. According to the results in Figs. 1 e and S2, reaction time of 30 min was best but the reaction was close to completion in 10 min. Therefore, much time was saved in this process and reaction time of 10 min was used in the following experiments.

3.2. pH and temperature stability of immobilized GI

pH and temperature are two of the important environmental factors affecting the enzyme activity greatly. The narrow stability range of free enzyme cannot meet the requirements in practical applications. Therefore, it is meaningful to enlarge the stability range of pH and temperature. In our present work, highest enzyme activity was obtained for both free and immobilized GI at pH of 7.5, as shown in Fig. 2a. The immobilized GI was found to maintain relative enzyme activity above 90% in a larger pH range of 5.8–8.0, while the relative enzyme activity of free GI was found to be lower

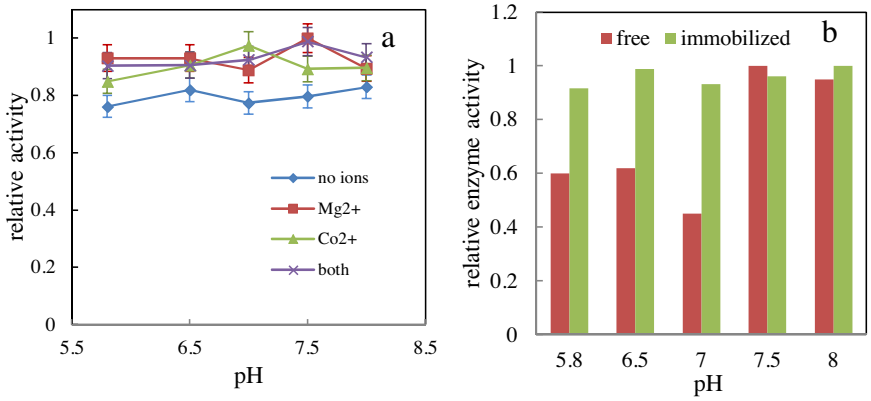

Fig. 3. Effect of metal ions on the pH stability (a) pH stability of immobilized GI with different metal ions; (b) comparison of pH stability between free and immobilized GI without metal ions.

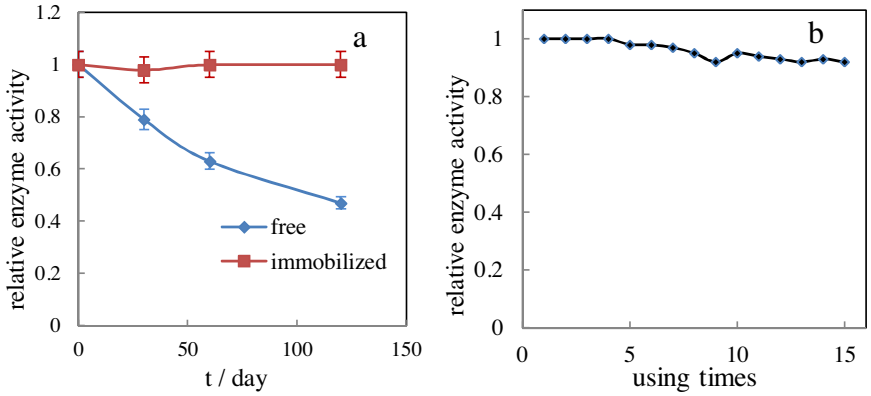

Fig. 4. Storage and operation stability. (a) Storage stability; (b) operation stability.

than 80% in weak acidic environment. The effect of temperature on enzyme activity for free and immobilized GI was then studied after fixing the pH at 7.5 as demonstrated in Fig. 2b. It could be found that the relative enzyme activity for free GI decreased largely when the temperature stayed away from the optimized temperature of 70 °C, whereas the immobilized GI was found to be more stable in a larger temperature range. It means that the silica/chitosan hybrid microspheres were beneficial for the exposure of active sites and could fix the conformation of enzyme effectively. Just as the results in Li et al.'s work [8], better pH and temperature stability of D-xylose isomerase and D-psicose 3-epimerase was also obtained by immobilizing them on the chitosan layer of spore wall.

GI, as a kind of metalloenzyme, will be affected largely in the presence of metal ions, especially Mg^{2+} and Co^{2+}. Mg^{2+} is the most important activator and Co^{2+} can stabilize the quarternary structure of GI. Therefore, the effect of the presence of Mg^{2+} and Co^{2+} on the pH stability of immobilized and free GI was studied, as shown in Fig. 3. It was found that the existence of either Co^{2+} or Mg^{2+} or a combination of them led to higher relative enzyme activity. Even though the enzyme activity of the immobilized GI was lower without ions, it had good pH stability with small fluctuation, as shown in Fig. 3a. This implied that the pH stability of immobilized GI depends little on the presence of metal ions, but Mg^{2+} and Co^{2+} could lead to activation of the enzyme and to higher enzyme activity. Comparatively, the pH stability of free GI was broken without Mg^{2+} and Co^{2+} and the relative enzyme activity was found to be as lower as around 50% under neutral and weak acidic conditions, as shown in Fig. 3b. Therefore, with the protection of carrier, the immobilized GI was less sensitive to the environmental changes and had better environmental stability, which could adapt to the practical conditions in industry.

3.3. Storage and operation stability of immobilized GI

The structure of enzyme is fragile and can be easily damaged in the process of storage leading to a decrease in enzymatic activity. Therefore, it is crucial to study the storage stability of the enzyme. To study the storage stability, the immobilized GI was kept at room temperature and the free GI solution was kept in the fridge with temperature of 4 °C. They were used after a certain period, as shown in Fig. 4a. Little change of enzyme activity had been observed for immobilized GI, but the enzyme activity for free enzyme had decreased over 50% in three months. Therefore, it could be concluded that the skeleton of microspheres provides good stability for the GI to avoid distortion effect caused by environment [20,21].

One of the most important advantages of immobilized GI is the possibility to be recycled. The results of operation stability of immobilized GI are shown in Fig. 4b. The relative enzyme activity was consistently found to be above 90% after being used for 15 times, demonstrating significant improvement and many possible industrial applications. The improvement in the operational stability mainly relied upon the in situ encapsulation of GI and crosslinking with chitosan by glutaraldehyde avoiding the GI loss effectively.

Table 1
Comparison of immobilized GI between in situ encapsulation and adsorption.

Enzyme	In situ	Adsorption
Apparent enzyme activity/U g^{-1} carrier	62.48491	44.20975

3.4. Comparison of immobilized GI prepared in different approaches

Most immobilized GI was prepared through adsorption of GI onto the microspheres in the past researches. In order to evaluate whether in situ encapsulation is better than adsorption for immobilizing GI, they were compared with each other and summarized in Table 1. Due to that apparent enzyme activity is most related to the practical application, therefore, it was used as the parameter for comparison. From the results it could be concluded that the apparent enzyme activity of immobilized GI prepared by in situ encapsulation was better than that of immobilized GI by adsorption. This was attributed to the greater loading capacity of GI dependent on the amount added to the chitosan solution. Furthermore, the properties of immobilized GI with different carriers are compared in Table 2. The stability range is defined as the period that enzyme activity is over 90%. It was shown that silica/chitosan hybrid microspheres supported GI could catalyze the reaction in 10 min and had wider stability range. It was because the core–shell

Table 2
Comparison of immobilized GI in different work.

Immobilized GI's carrier	pH stability range	Temperature stability range/°C	Operation stability/times	Reaction time/min
Silica gel [15]		50–70	4	30
Alginate gel [4]	7.5	60–65	9	10
Chitosan–poly(acrylic acid) [16]	6.0–7.5	60–80	11	30
Silica/chitosan in this work	5.8–8.0	40–80	15	10

structure of the carrier could reduce the mass transfer resistance effectively and chitosan could provide suitable microenvironment for GI due to its outstanding biocompatibility.

4. Conclusions

Silica/chitosan hybrid microspheres supported GI was prepared in an easy way through in situ encapsulation of GI in the microspheres to improve the effective utilization and stability against environmental changes. Under the optimized conditions, the conversion of glucose to fructose could be finished in 10 min under the catalysis of immobilized GI due to GI was located on the shell of carrier which could reduce mass transfer resistance effectively. Compared with free GI, the immobilized GI has better pH, temperature, ions, storage and operation stability. The relative enzyme activity were above 90% in the wide pH range of 5.8–8.0, temperature range of 40–80 °C, storage range of 3 months and operation range of >15 times. The high stability against environmental changes made the silica/chitosan hybrid microspheres supported GI an outstanding candidate to be applied in practical applications.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2016.01.013.

References

[1] M.B.R. Snehalata, H. Bhosale, Vasanti V. Deshpande, Am. Soc. Microbiol. 60 (1996) 280–300.
[2] Y. Zhang, K. Hidajat, A.K. Ray, Biochem. Eng. J. 21 (2004) 111–121.
[3] Asghar Molaei Dehkordi, Iman Safari, Ind. Eng. Chem. Res. 48 (2009) 3271–3278.
[4] Hayrettin Tumturk, Gokhan Demirel, Haydar Altinok, Serpil Aksoy, Nesrin Hasirci, J. Food Biochem. 32 (2008) 234–246.
[5] H. Yu, Y. Guo, D. Wu, W. Zhan, G. Lu, J. Mol. Catal. B: Enzym. 72 (2011) 73–76.
[6] J.A. Silva, G.P. Macedo, D.S. Rodrigues, R.L.C. Giordano, L.R.B. Gonçalves, Biochem. Eng. J. 60 (2012) 16–24.
[7] Q.X. Wu, S.J. Yao, Colloids Surf. B: Biointerfaces 109 (2013) 147–153.
[8] Z. Li, Y. Li, S. Duan, J. Liu, P. Yuan, H. Nakanishi, X.D. Gao, J. Ind. Microbiol. Biotechnol. 42 (2015) 1117–1128.
[9] K. Sawada, S. Sakai, M. Taya, Chem. Eng. J. 280 (2015) 558–563.
[10] L. Wu, Y. Liu, B. Chi, Z. Xu, X. Feng, S. Li, H. Xu, Food Chem. 187 (2015) 182–188.
[11] H. Li, K. Fu, P.P. Boix, L.H. Wong, A. Hagfeldt, M. Gratzel, S.G. Mhaisalkar, A.C. Grimsdale, ChemSusChem 7 (2014) 3420–3425.
[12] J. Huang, H. Liu, P. Zhang, P. Zhang, M. Li, L. Ding, Mater. Sci. Eng. C: Mater. Biol. Appl. 57 (2015) 31–37.
[13] Yi-Chun Lee, Ching-Tien Chen, Yu-Ting Chiu, Kevin C.-W. Wu, ChemCatChem 8 (2013) 2153–2157.
[14] R.H.-Y. Chang, J. Jang, K.C.W. Wu, Green Chem. 13 (2011) 2844–2850.
[15] Y.S. Song, J.E. Kim, C. Park, S.W. Kim, Korean J. Chem. Eng. 28 (2011) 1096–1100.
[16] J.H. Xu, X.M. Xu, H.C. Wu, G.S. Luo, RSC Adv. 4 (2014) 37142–37147.
[17] H. Zhao, J. Xu, T. Wang, Appl. Catal. A: Gen. 502 (2015) 188–194.
[18] J.H. Xu, H. Zhao, W.J. Lan, G.S. Luo, Adv. Healthc. Mater. 1 (2012) 106–111.
[19] S.H.C. D. Y. RYU, Biotech. Bioengi., (1977) 159–184.
[20] F. Sahin, G. Demirel, H. Tumturk, Int. J. Biol. Macromol. 37 (2005) 148–153, author reply 156–160.
[21] A. Yahsi, F. Sahin, G. Demirel, H. Tumturk, Int. J. Biol. Macromol. 36 (2005) 253–258.