0273-2289/98/6901—0017$11.25

Immobilization of Glucose Isomerase and Its Application in Continuous Production of High Fructose Syrup

YUBIN GE,* HUI ZHOU, WEI KONG, YI TONG, SHUYAN WANG, AND WEI LI

Department of Molecular Biology, Jilin University, Changchun, 130023, P. R. China

Received October 21, 1996; Accepted July 25, 1997

ABSTRACT

Bilayer glucose isomerase was immobilized in porous *p*-trimethylamine-polystyrene (TMPS) beads through a molecular deposition technique. Some of the factors that influence the activity of immobilized glucose isomerase were optimized, with the enzyme concentration of 308 IU/mL, enzyme-to-matrix ratio of 924 IU/g wet carrier, and hexamethylene *bis*(trimethylammonium iodine) concentration of 15 mg/mL giving the maximum catalytic activity (2238 IU/g dry gel) of the immobilized bilayer glucose isomerase, retaining 68.5% of the initially added activity. The half-life of the immobilized bilayer glucose isomerase was approx 45 d at pH 8.5, 60°C, with 50% (w/v) glucose as substrate. The specific productivity of the immobilized bilayer glucose isomerase was 223 g dry D-glucose/g dry immobilized enzyme per d.

Index Entries: Glucose isomerase; enzyme immobilization; molecular deposition; bilayer enzyme; high fructose syrup.

INTRODUCTION

D-glucose/xylose isomerase (GI, EC 5.3.1.5), which converts D-xylose to D-xylulose and D-glucose to D-fructose, is one of the highest-value and highest-tonnage commercial enzymes. It has the largest market as an immobilized enzyme for production of high fructose syrup

*Author to whom all correspondence and reprint requests should be addressed.

(HFS) *(1)*. High fructose sweeteners are valuable, because they are 2–3 times as sweet as glucose and possess several other advantageous chemical and physical properties *(2)*. It is preferred by the food industry as a sweetener because it does not pose the problem of crystallization as sucrose does.

Enzyme immobilization is an important technique in biotechnology. The development of immobilized glucose isomerase (IGI) and its application for the production of HFS has been a subject of great interest *(3)*. Many methods have been reported for the immobilization of GI, but only a few have been used by enzyme suppliers to produce an economically feasible IGI preparation that has the properties necessary for the commercial production of HFS. In our previous work, a novel immobilization method was developed to immobilize GI, based on molecular deposition technique *(4,5)*, which forms multilayer ultrathin film by alternate deposition of bipolar amphophilic cationic and anionic layers *(6,7)*.

In the present work, bilayer GI is immobilized in the porous TMPS beads through molecular deposition technique. The immobilization conditions are optimized. The properties of soluble and immobilized enzyme are comparatively investigated. Particularly, the productivity of HFS using the bilayer IGI in a 26 × 4-cm packed-bed column is successfully conducted.

MATERIALS AND METHODS

Materials

GI from *streptomyces* with an activity of 3080 IU/mL was purchased from Genencor International (Rochester, New York). Beads of porous TMPS, mean pore diameter 46 nm, were provided by the Department of Chemistry of Jilin University. The hexamethylene *bis*(trimethylammonium iodine) was synthesized by ourselves. All other materials used were commercially available analytical grade.

Methods

Assay of GI Activity

The determination of GI activity was achieved by measuring the amount of fructose produced following Tomas' cysteine carbazole method *(8)*, using glucose as substrate. The standard catalytic reactions were carried out at pH 8.5, 60°C, using 2 *M* glucose as the substrate. To initiate a reaction, 3 mL of the substrate solution preheated to 60°C was added to a mixture of 1 mL 0.5 *M* Gly-NaOH buffer, pH 8.5, 1 mL 0.05 *M* $MgSO_4$, and 15 mg IGI, stirring at 3.8*g* for 15 min. The reaction was stopped by the addition of 5 mL 0.5 *M* $HClO_4$. Then 0.1 mL of the reaction mixture was sam-

pled for measuring the produced fructose. One unit of GI activity is defined as the amount of enzyme required to liberate 1 μmol fructose in 1 min under the reaction conditions.

Immobilization of GI

Before use, the TMPS beads were suspended into a pH 2.0–3.0 HCl solution for 2 h to transfer the exchange ion into Cl^-, then washed with distilled water until the wash pH became neutral. To immobilize GI, 1 g treated TMPS beads (wet wt) were suspended into 3 mL GI solution containing an activity of 308 IU/mL, at pH 7.0, 4°C for 2 h. After filtering and washing, the TMPS beads with the immobilized monolayer GI were obtained. Then the TMPS beads with monolayer GI were immersed in 3 mL hexamethylene *bis*(trimethylammonium iodine) solution under the conditions of pH 7.0, 4°C. Two h later, the TMPS beads were filtered and washed again with distilled water in 2 h, and subsequently reimmersed in 3 mL GI solution and incubated at pH 7.0, 4°C for 2 h to absorb the second layer of GI. After filtering and washing with distilled water, the bilayer IGI was obtained. In order to prevent the desorption of the adsorbed GI from the supports, the adsorbed enzyme molecules were crosslinked with 0.15% (v/v) glutaraldehyde at room temperature for 4 h.

Protein Measurements

The amount of bound enzyme was measured as follows: 2 mL of 6 *N* HCl and 1 mg phenol were added to a hydrolysis tube that contained 0.1 g immobilized enzyme (wet). The tube was sealed and the sample hydrolyzed for 24 h at 105°C. The hydrolyzate was dried using steam heat, then dissolved in 5 mL 0.002 *N* HCl, filtered through quantitative filter paper, and amino acid was measured using a Hitachi 835-50 amino acid analyzer (Hitachi, Tokyo, Japan).

Kinetic Parameters

Kinetic parameters (K_m and V_{max}) for soluble and immobilized GI were determined in 0.1 *M* Gly-NaOH (pH 8.5, containing 0.01 *M* Mg^{2+}) at 60°C. K_m and V_{max} values were calculated from Lineweaver-Burke plots.

Operational Stability of Bilayer IGI

One gram dry bilayer IGI was packed in a 6 × 1-cm column. The operating temperature was maintained at 60°C. The substrate solution containing 50% (w/v) glucose and 0.01 *M* Mg^{2+} was pumped through the column at a constant flow rate of 22.8 mL/h. The expressed activity of the packed bilayer IGI was determined by measuring the amount of resultant fructose.

Continuous Production of HFS by Bilayer IGI Column

Twenty-five g of dry bilayer IGI were packed in a 26 × 4-cm column. The packed bilayer IGI column was continuously operated at 60°C, using 50% (w/v) glucose solution (containing 0.01 *M* Mg^{2+} ions, pH 8.2–8.5) as substrate. The flow rate of the substrate solution pumped through the column was manipulated to maintain the isomerization rate at approx 42%.

RESULTS

Preparation of Bilayer IGI

There are many factors that influence the activity of immobilized enzyme. In order to obtain the maximum activity and relatively high activity recovery in the present work, the effects of enzyme concentration, enzyme-to-matrix ratio, and hexamethylene *bis*(trimethylammonium iodine) concentration on the activity of IGI were investigated.

Effect of Enzyme Concentration

The effect of enzyme concentration on the activity of monolayer IGI is shown in Fig. 1. The percent maximal activity increased with the increase in enzyme concentration, and reached a maximum at enzyme concentration of 308 IU/mL, but decreased with the further increase in enzyme concentration. Thereby, we select 308 IU/mL as the optimum enzyme concentration for preparing monolayer IGI.

Effect of Enzyme-to-Matrix Ratio

By inspection of Fig. 2, we can observe that in up to 800 IU/g wet gel, the activity of the monolayer IGI was proportional to the enzyme-to-matrix ratio. Throughout this range, the monolayer IGI retained between 75 and 95% of the initial activity. The maximum activity bound occurred at 924 IU/g wet gel, retaining 68.5% of the initial activity. In order to obtain the highest immobilized enzyme activity and relatively high activity recovery, the point of 924 IU/g wet gel was chosen as the most appropriate enzyme-to-matrix ratio for all the subsequent experiments.

Effect of Hexamethylene bis*(Trimethylammonium Iodine) Concentration*

Figure 3 shows the effect of hexamethylene *bis*(trimethylammonium iodine) concentration on the activity of bilayer IGI. The percent maximum activity sharply increased, and then decreased, which gave a maximum activity of 2238 IU/g dry gel at an optimum concentration of 15 mg/mL. The properties of GI immobilized on the TMPS beads and soluble GI are comparatively shown in Table 1.

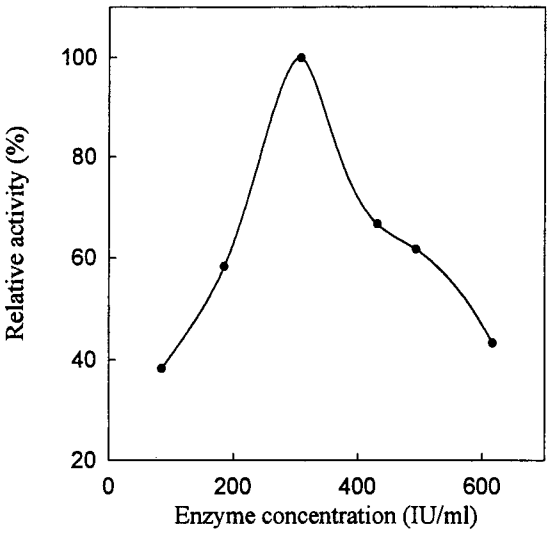

Fig. 1. Effect of enzyme concentration on the relative activity of immobilized monolayer glucose isomerase.

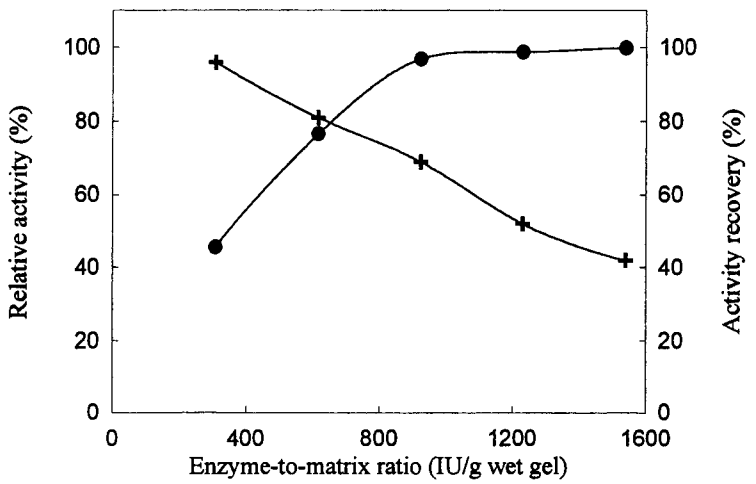

Fig. 2. Effect of enzyme-to-matrix ratio on the relative activity (•) and activity recovery (+) of immobilized monolayer glucose isomerase.

Properties of Bilayer IGI

Optimum pH

Bilayer IGI and soluble GI activities were assayed at pHs ranging from 7.5 to 9.5. The effect of pH on GI activity is illustrated in Fig. 4. The optimum pH values were found to be about 8.5 for both soluble GI and bilayer IGI.

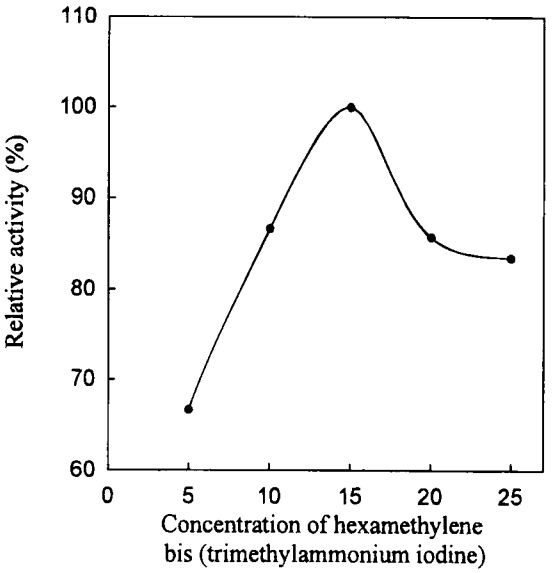

Fig. 3. Effect of concentration of hexamethylene *bis*(trimethylammonium iodine) on the activity of immobilized bilayer glucose isomerase.

Table 1
Properties of Soluble, Immobilized Monolayer and Bilayer Glucose Isomerase

Enzyme	Activity (IU/g dry gel)	Protein loading (mg/g dry gel)	Specific activity (IU/mg protein)
Soluble	370[a]	6.6[b]	56
Monolayer	1043	34.5	30
Bilayer	2238	70.3	29

[a]Activity of soluble enzyme in IU/mL.
[b]Protein content of soluble enzyme in mg/mL.

Optimum Temperature

The bilayer IGI and soluble GI were assayed at temperatures ranging from 50 to 90°C. The optimum temperature increased from 70°C for the soluble GI to 85°C for the bilayer IGI (Fig. 5).

Kinetic Parameters

Figure 6 shows the effect of immobilization on the kinetic parameters of GI with glucose as the substrate. The K_m for glucose of the monolayer IGI (0.30 *M*) and the bilayer IGI (0.33 *M*) increased 66.7% and

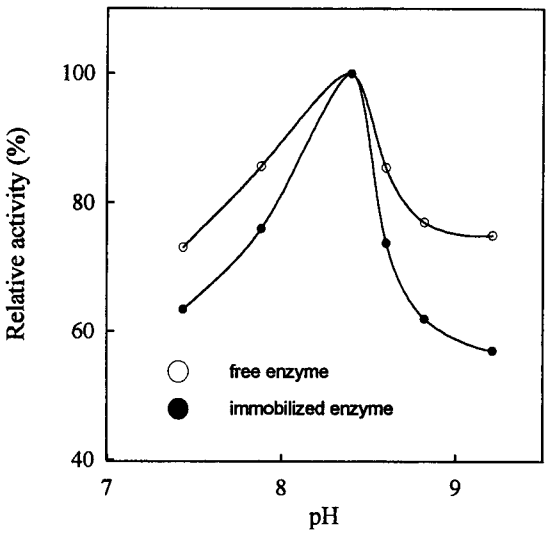

Fig. 4. Effect of pH on the activity of immobilized bilayer glucose isomerase. Enzyme assays were carried out under standard conditions except for the pH of the reaction mixture.

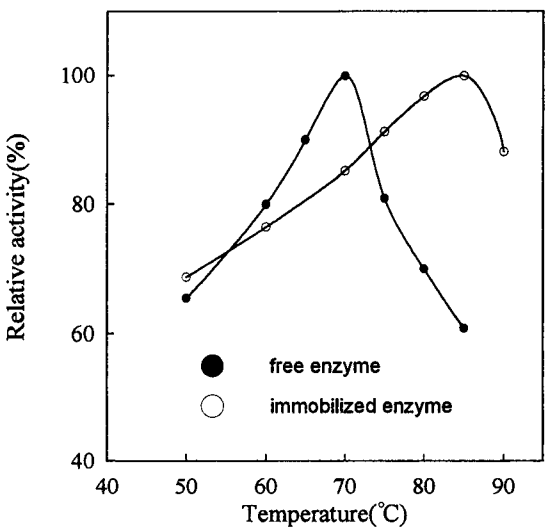

Fig. 5. Effect of temperature on the activity of immobilized bilayer glucose isomerase. Enzyme assays were carried out under standard conditions except for the temperature of the reaction mixture.

83.3%, respectively, compared to that of the soluble GI (0.18 *M*), upon immobilization. However, the V_{max} of monolayer IGI (298.3 μmol fructose/min/mL) and bilayer IGI (294.2 μmol fructose/min/mL) only decreased 4 and 5%, respectively, compared to that of free enzyme (310.3 μmol fructose/min/mL).

Table 2
Flow Rate Control of Continuous Production of HFS Using Immobilized Bilayer Glucose Isomerase Column

Time (d)	1–7	8–14	15–20	21–25	26–30	31–35	36–40	41–45
Flow Rate (mL/min)	17	15	13	11	9	7.5	6.5	5

Table 3
Specific Productivity of HFS in Continuous Production Using Immobilized Bilayer Glucose Isomerase Column

Operational time (d)	Specific productivity (g dry glucose/g dry bilayer IGI/d)	Average isomerization rate (% of initial)
5	338	42
10	350	44
15	330	42.7
25	285	42.4
35	254	42.3
45	223	42.2

Note: The total sugar concentration is 50% (w/v).

Operational Stability

The results for conversion of glucose by bilayer IGI in a 6 × 1-cm bed column at a constant flow rate showed that the bilayer IGI was more stable at higher pH. It can be seen that, at pH 7.5, when a 50% (w/v) substrate solution containing 0.01*M* Mg^{2+} was passed through the column, the activity decreased gradually to 50% of the initial activity after 21 d, but at pH 8.5, the activity of bilayer IGI still maintained 50% of the initial activity after 45 d.

Continuous Production of HFS by Bilayer IGI Column

The continuous production of HFS by bilayer IGI column was continuously operated at 60°C, pH 8.2–8.5, for 45 d, with the substrate solution containing 50% (w/v) glucose and 0.01 *M* Mg^{2+}. The flow rate was 17 mL/min at the beginning, then was adjusted according to the change

of the isomerization rate, to maintain the isomerization rate at approx 42%. The variation of flow rate and specific productivity of bilayer IGI are shown in Table 2 and Table 3, respectively.

It can be seen in Table 3 that the specific productivity of bilayer IGI, after an initial increase, dropped gradually with the operation time. It can be calculated that 1 g of dry bilayer IGI can convert 10 kg dry D-glucose to 42% HFS in 45 d.

DISCUSSION

The use of immobilized enzymes in industrial processes depends on many factors. The ability to produce, by simple means, a relatively low-cost immobilized enzyme product with high activity per weight of support, with adequate operational stability, and efficient use of that product, is an important technical consideration *(9)*. Here we have examined the preparation of bilayer IGI in inexpensive porous TMPS beads through molecular deposition technique. The preparation developed here provides several important advantages over the methods that have been previously outlined for the immobilization of GI *(3,10,11)*.

Immobilization of bilayer GI using porous TMPS beads can be achieved simply and without the use of harsh pH or temperature conditions. The TMPS beads have good mechanical and flow properties, and they are resistant to enzyme and microbial attack, unlike most previously used supports *(3,10,11)*. Moreover, bilayer enzyme can be successfully immobilized on the support surface by molecular deposition technique. Therefore, our method is superior to the traditional adsorption technique, which can only absorb one layer of enzyme molecules. In order to obtain bilayer IGI with maximum activity, the immobilization conditions should be optimized. There exist optimum values for enzyme concentration and enzyme-to-matrix ratio to give maximum activity of monolayer IGI (Figs. 1 and 2). Under the optimum conditions, the enzyme molecules may not be enough to saturate the supports, so the monolayer IGI activity increased with the enzyme concentration and enzyme-to-matrix ratio. The decrease of the monolayer IGI activity over the optimum value might be caused by the overcrowding of enzyme on the matrix, supported by the decrease in the specific activity of the immobilized enzyme (data not shown). The effect of hexamethylene *bis*(trimethylammonium iodine) concentration on the activity of bilayer IGI showed a peak plot. We consider 15 mg/mL the threshold concentration of hexamethylene *bis*(trimethyllammonium iodine) for absorbing the second layer of GI. Under this concentration, less amount of GI was absorbed because of the smaller amount of positive charge covering the first layer of GI provided by hexamethylene *bis*(trimethylammonium iodine) molecules. However,

over this concentration the excess hexamethylene *bis*(trimethylammonium iodine) molecules may shield the activity center of GI in the first layer. Under the optimized immobilizing conditions, about 68.5% of the enzyme added was successfully immobilized, giving the bilayer IGI an maximum activity of 2238 IU/g dry gel. The percentage of enzyme activity immobilized onto TMPS beads compares favorably with reports of GI binding to other supports: polyurethane foam 50% bound, and granular chicken bone 32% bound *(2,12)*. The maximum activity of bilayer IGI is higher than those reported for other IGI preparations, such as GI from *Streptomyces phaeochromogenes* bound to silanized-coated-porous glass (*13*) and GI from *Bacillus coagulans* bound to DEAE-cellulose (*9*).

The same optimum pH for both soluble GI and bilayer IGI was observed in our work (Fig. 4). This result is different from that of other IGI prepared through electrostatic adsorption, such as GI from *B. coagulans* bound to DEAE-cellulose, whose optimum pH shifted slightly from 7.2 to 6.8 *(9)*. Chibata *(14)* and others *(15–17)* have provided a thorough discussion, with many examples of the effects of charged supports on the pH optima or pH/activity profiles of ionically bound enzymes. In general, the apparent pH for optimal activity shifts toward the acid side, when an enzyme is bound to a polycationic support, and to the alkaline side, if bound to a polyanionic support. Shifts in pH optima may be minimized by carrying out the catalytic reactions at higher ionic strengths. In such cases, the charges on the carrier are neutralized by Na^+ and Cl^- rather than by H^+ and OH^-, with the result that the difference in the pH between the internal and external environments is lessened *(14)*. In the previous work conducted in our lab *(18)*, it was demonstrated that the pH optimum for GI immobilized on porous triethanolamine methyl polystyrene only shifted a little in higher-concentration buffer (higher than 0.016 *M*). The buffer concentration utilized in this paper was 0.1 *M*; the high ionic strength of the buffer shielded the charged groups on the supports, diminishing the pH differences between the interior of the support and the bulk solution.

The increase in the optimum temperature (Fig. 5) must be caused by the increased stability of GI because of immobilization. This result is similar to that of GI adsorbed onto Indion 48-R *(1)*, but different from that of GI from *Streptomyces flacogriseus* coupled to DEAE-cellulose *(19)*, whose optimum temperature did not change at all, compared to that of free enzyme. Although the bilayer IGI showed much higher activity at 85°C than 60°C, the temperature of 85°C was not preferred in industry because of glucose loss and color formation. Moreover, the half-life of IGI at 85°C was much shorter than 60°C. Thereby, the temperature of 60°C was chosen as the operating temperature in the production of HFS using packed bilayer IGI column.

The increased K_m and V_{max} values of GI after immobilization were observed in the present work (Fig. 6). The net result of these changes

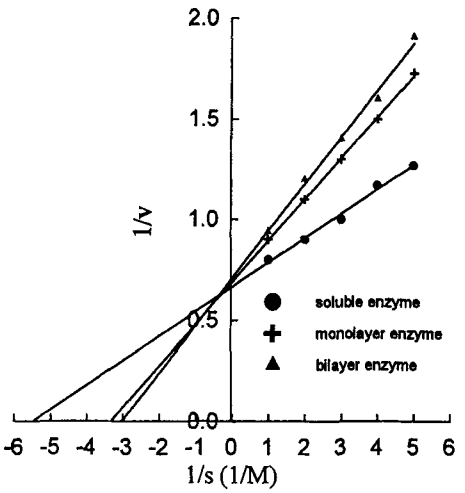

Fig. 6. Lineweaver-Burk plots of soluble, immobilized monolayer and bilayer glucose isomerase.

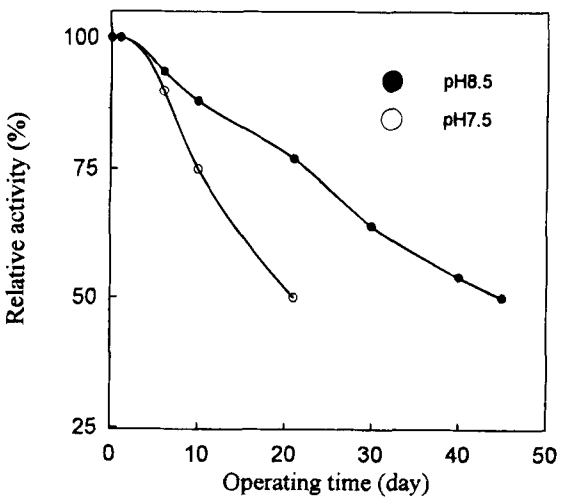

Fig. 7. Operational stability of immobilized bilayer glucose isomerase, with 50% D-glucose as substrate at pH 7.5 and pH 8.5.

resulted in the k_{cat} (V_{max}/K_m) values for monolayer IGI and bilayer IGI dropping by 1.2- and 1.3-fold, respectively (taking into account that only 68.5% of the enzyme was immobilized). Therefore, GI was adversely affected by the process of immobilization, but remained functional. Although the k_{cat} of bilayer IGI is little smaller than that of monolayer IGI, the activity per weight unit of bilayer IGI is much larger than that of monolayer IGI. This will increase the amount of enzyme loaded per unit of reactor column, thereby reducing energy and equipment costs. So the

preparation of bilayer IGI is economically more valuable than that of monolayer IGI.

Prolonged half-life (45 d) of bilayer IGI was observed at higher pH, which was two times longer than that at lower pH (Fig. 7). The activity loss of bilayer IGI at higher pH could be considered as the gradual denaturation of the enzyme. However, the sharp activity loss at lower pH may be mainly caused by the desorption of GI from the supports, and partially by the denaturation of the enzyme. The half-life of bilayer IGI at higher pH is longer than those obtained for several other IGI preparations *(1,9,11)*. The prolonged half-life of bilayer IGI is quite important for its application in industry.

Productivity of an immobilized enzyme is an important factor used to evaluate the immobilization preparation. It can be calculated from Table 3 that approx 10,000 g dry glucose were isomerized to 42% HFS in 45 d of the operating time by 1 g dry bilayer IGI. This result is higher than that of Miles, Clinton, and Novo IGI products, which were 8,000, 8,000–9,000, and 8,000 g glucose per gram dry IGI, respectively (Miles, Clinton, and Novo industry product information).

To prepare 1 kg dry bilayer IGI will cost $40.00 is our laboratory; these immobilized enzymes can isomerize 10 t glucose into 14 t 42% HFS (71% solid, w/v). So the cost attributed to immobilized enzyme of per ton HFS is only about $2.90. It is important to note that these cost estimates are based on lab-scale data and assume that the same level of productivity is possible at industrial scale. If we take into account the charge for capital costs, the resulting costs will be some degree larger than $2.90. Although these results are only estimates of operating costs, they are valuable for estimating the possibility of the application of bilayer IGI in industry.

CONCLUSIONS

In this study, bilayer GI was successfully immobilized onto porous TMPS beads through molecular deposition technique. The immobilization preparation is simple and mild, giving bilayer IGI with high activity per unit of weight of support (2238 IU/g dry gel) and relatively high activity recovery (68.5%). The support utilized in the present work is inexpensive, resistant to enzyme and microbial attack, and has good mechanical and flow properties. The bilayer IGI showed adequate half-life (45 d at 60°C) and high productivity (10,000 g dry glucose per g dry bilayer IGI in 45 d). All these results indicate that the bilayer IGI prepared according to this paper has tremendous potential for use in industry for HFS production.

ACKNOWLEDGMENTS

The authors wish to acknowledge Wei Liu for his critical reading of the manuscript and for useful suggestions. The authors also thank the Foundation of Natural Science of China.

REFERENCES

1. Gaikwad, S. M. and Deshpande, V. V. (1992), *Enzyme Microb. Technol.* **14,** 855–858.
2. Storey, K. B. and Chakrabartl, A. C. (1990), *Appl. Biochem. Biotechnol.* **23,** 139–154.
3. Verhoff, F. H., Boguslawski, G., Lantero, O. J., Schlager, S. T., and Jao, Y. C. (1985), in *Comprehensive Biotechnology*, Moo-Young, M., ed., Permagon, Oxford, UK, pp. 837–859.
4. Kong, W., Zhang, X., et al. (1994), *Macromol. Rapid Commun.* **15,** 405–409.
5. Kong, W., Wang, L. P., et al. (1994), *J. Chem. Soc. Chem. Commun.* **11,** 1297, 1298.
6. Decher, G. (1991), *Macromol. Chem. Symp.* **46,** 321–327.
7. Decher, G. (1991), *Ber. Bunsenges. Phys. Chem.* **95,** 1430–1434.
8. Ryu, D. Y. and Chung, S. H. (1977), *Biotechnol. Bioeng.* **19,** 159–184.
9. Huitron, C. and Limon-Lason, L. (1978), *Biotech. Bioeng.* **20,** 1377–1391.
10. Chen, W. P. (1980), *Process Biochemistry* (June–Sept.), 30–33.
11. Jensen, V. and Rugh, S. (1985), *Methods Enzymol.* **136,** 356–370.
12. Schafhauser, D. Y. and Storey, K. B. (1992), *Appl. Biochem. Biotechnol.* **32,** 79–87.
13. Havewala, N. B. and Pitcher, W. H., Jr., (1974), in *Enzyme Engineering*, Pye, E. K. and Wingard, L. B., eds., Plenum, New York, **2,** 315–319.
14. Chibata, I., ed. (1978), *Immobilized Enzymes*, Wiley, New York.
15. McLaren, A. D. (1957), *Science* **125,** 697–699.
16. McLaren, A. D. (1960), *Enzymologia* **21,** 356–360.
17. Chen, W. P. and Anderson, A. W. (1979), *Appl. Environ. Microbiol.* **38,** 1111–1119.
18. Hui, Z., Zhou, H., Kong, W., Cha, X., Li, W., and Shen, J. (1992), *J. Chem. Tech. Biotechnol.* **54,** 43–46.
19. McLaren, A. D. and Esterman, E. F. (1957), *Arch. Biochem. Biophys.* **68,** 157–160.