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Immobilization of glucose isomerase onto GAMM support for isomerization of glucose to fructose

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ABSTRACT

Glucose isomerase (GI) from *Streptomyces rubiginosus* was immobilized covalently onto GAMM support prepared by our patented inverse suspension polymerization with glycidyl methacrylate (GMA), ally glycidyl ether (AGE), N,N′-methylene-bis(acrylamide) (MBAA), and acrylamide (MAA), and used for isomerization of glucose to fructose. Effects of immobilization conditions and reaction conditions on the activity of immobilized GI, the kinetic parameters, the operational stability, thermal stability and storage stability of immobilized GI were investigated. The optimum immobilization conditions were GI addition amount of 0.3 ml GI/g support, immobilization time of 24 h, and immobilization temperature of 25 °C. The optimum reaction conditions were pH value of reaction solution of 7.5 and reaction temperature of 65 °C. The activity of immobilized GI was 450 U/g (wet). K_m and V_{max} values of immobilized GI were 1.16 mol/L and 1.07×10^{-3} mol/L min, respectively. Immobilized GI onto GAMM support has better operational stability, thermal stability and storage stability, in which it retained 91% of its initial activity after recycled for 18 times and retained 97% of its initial activity after stored at 4 °C for six weeks. Therefore immobilized GI onto GAMM support was an excellent catalyst for isomerization of glucose to fructose.

1. Introduction

The isomerization of glucose to fructose catalyzed by glucose isomerase (GI) is one of the most important processes in the food industry due to the unique dietetic properties of fructose. Fructose can improve the sweetening, color, viscosity, and hygroscopic characteristics. Moreover, fructose is much sweeter than sucrose, is absorbed more slowly than glucose, and is metabolized without the intervention of insulin. This isomerization process is widely studied over free or immobilized GIs or GI-contained cell [1–7]. The isomerization of glucose to fructose is the most crucial step in the production of high fructose corn syrup (HFCS), which is generally carried out over the immobilized GI in the packed bed reactor [8]. Moreover, GI has received increasing attention for its potential application in the production of ethanol from hemicelluloses [9].

Some supports for immobilization of GI were reported, such as DEAE-cellulose [10], alginate beads [11], carbon-containing foamed ceramics [12], silica xerogel [13] and Eupergit C 250L [14]. Among the above mentioned supports, Eupergit C 250L with epoxy groups, a kind of acrylic resin, is a better support for covalent immobilization of GI. The epoxy groups of Eupergit C 250L support can react with the amino groups of GI under mild conditions, which makes the immobilization process simpler and increases the operational stability of immobilized enzyme.

The GAMM support with epoxy groups, similar to Eupergit C 250L, is synthesized by our patented inverse suspension polymerization [15,16] using Span-60 and Tween-20 as the complex dispersants, N,N′-methylene-bis(acrylamide) as the crosslinking reagent, and glycidyl methacrylate and allyl glycidyl ether as reactive monomers. GAMM is an excellent support for covalent immobilization of penicillin G acylase, in which the activity of immobilized penicillin G acylase achieved 353 U/g [16]. In this paper, GI was immobilized covalently onto GAMM support, which was used for isomerization of glucose to fructose. Effects of immobilization conditions and reaction conditions on the activity of immobilized GI, the kinetic parameters, and the operational performance of immobilized GI (operational stability, thermal stability and storage stability) were investigated.

2. Experimental

2.1. Chemicals

Glucose isomerase from *Streptomyces rubiginosus*, purchased from Wuhan Xinhuayuan Bio-technology Co. Ltd., was brown liquid and completely miscible in water with the activity of 1615 U/mg GI and the specific gravity of 1.17 g/mL. Other chemical reagents

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were purchased from Sinopharm Chemical Reagent Co. Ltd. and then used without any further purification.

2.2. Synthesis of GAMM support

GAMM was synthesized by our patented inverse suspension polymerization technique [15,16]. Typically, 120 mL mixture solvent of n-heptane and tetrachloroethylene (3:1, volume ratio) and 1 g the complex dispersants containing Span-60 and Tween-20 (7:3, mass ratio) were mixed homogeneously in a glass flask, then 25 g monomer mixtures including glycidyl methacrylate (GMA), ally glycidyl ether (AGE), N,N′-methylene-bis(acrylamide) (MBAA), acrylamide (MAA) and carboxamide porogen (1:1:3:0.5:13, mass ratio) were added under vigorous stirring with 0.7 g azobisisobutyronitrile (AIBN) as initiator. The polymerization reaction was carried out under nitrogen atmosphere for 4 h at 55 °C. The product was filtered to get ivory polymer beads, washed with ethanol for 3 times, soaked in ethanol for 48 h, then soaked in n-heptane for 48 h. After drying at 60 °C under vacuum to the constant weight, microspherical GAMM support with epoxy groups was obtained.

2.3. Immobilization of glucose isomerase

One gram GAMM was immersed in 0.3 mL GI diluted by phosphate buffer (0.2 mol/L, pH = 7.0) to 5.0 mL. Then the mixture was shaken for 24 h at the rotational speed of 170 rpm and in the water bath at 25 °C. The resultant immobilized GI was washed thoroughly with the distilled water until no protein was detected in the filtrate, dried in an incubator, and then stored at 4 °C.

2.4. Determination of enzyme activity

The GI activity was determined spectrophotometrically by measuring the amount of fructose produced by GI. The isomerization of glucose to fructose was carried out in a three-neck flask at the stirring rate of 150 rpm, in which 0.2 g immobilized GI or 0.1 mL GI solution (6 mg GI/mL) was added into 10 mL reaction mixtures composed of 5 mL glucose solution of 70% (w/v), 4 mL phosphate buffer of 0.2 mol/L at pH = 7.5, 0.5 mL $MgSO_4$ of 0.5 mol/L, and 0.5 mL $CoCl_2$ of 0.01 mol/L. The mixture was incubated at 65 °C or 70 °C for 30 min, then the reaction was stopped by adding 5.0 mL $HClO_4$ solution of 0.5 mol/L. The GI activity was determined by the amount of fructose isomerized from glucose according to Tomas's cysteine–carbazole method [17]. 0.1 mL sample was taken from the reaction solution and diluted by 100 times to the fructose concentration of 10–50 μg/mL. 1 mL diluted fructose solution and 0.2 mL L-cysteine hydrochloride solution (1.5%, w/v) and 6.0 mL H_2SO_4 solution (70%, v/v) were added in the water bath at 60 °C for 10 min. Then, 0.2 mL carbazole/ethanol solution (0.12%, w/v) was added, and the color-developing reaction was performed at 60 °C for 10 min. The mixture was kept in the ice bath for 1.5 min then at room temperature.

The absorbance was measured spectrophotometrically at 560 nm, in which the fructose concentration was determined by the fructose calibration curve. One unit GI activity was defined as the amount of the enzyme which produced 1 μmol of fructose per min.

3. Results and discussion

3.1. Effect of immobilization conditions

One gram GAMM was immersed in 0.1–0.6 mL GI diluted by phosphate buffer (0.2 mol/L, pH = 7.0) to 5 mL, then GI was immobilized for 24 h at 25 °C. Effect of GI amount on the activity of immobilized GI is shown in Fig. 1, in which the optimum GI amount of GAMM support was 0.3 ml GI/g.

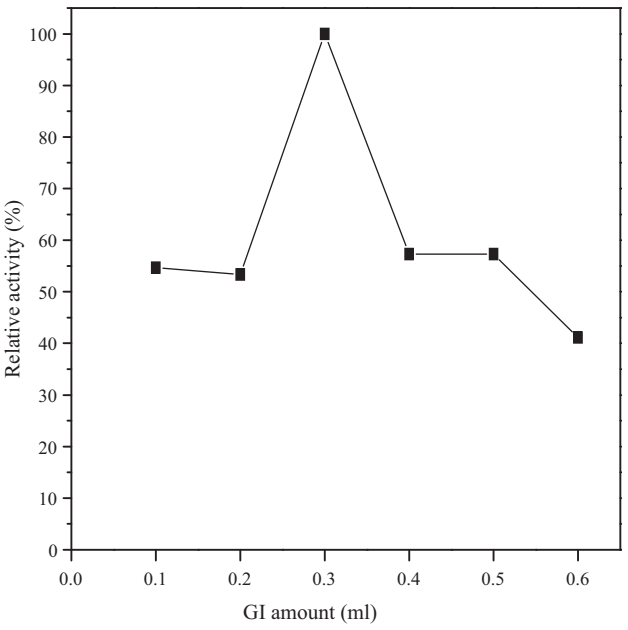

Fig. 1. Effect of GI amount on the activity of immobilized GI.

One gram GAMM was immersed in 0.3 mL GI diluted by phosphate buffer (0.2 mol/L, pH = 7.0) to 5 mL, then GI was immobilized for 4–44 h at 25 °C. Effect of immobilization time on the activity of immobilized GI is shown in Fig. 2, in which the optimum immobilization time was 24 h. During the immobilization process, firstly GI was physically adsorbed on the surface of GAMM support, and then was immobilized covalently by reaction of the epoxy groups of GAMM support with the amino groups of GI [18]. With an increase in immobilization time, more GI molecules were immobilized covalently and thus the enzyme activity increased, however, when immobilization time was greater than 24 h, the enzyme activ-

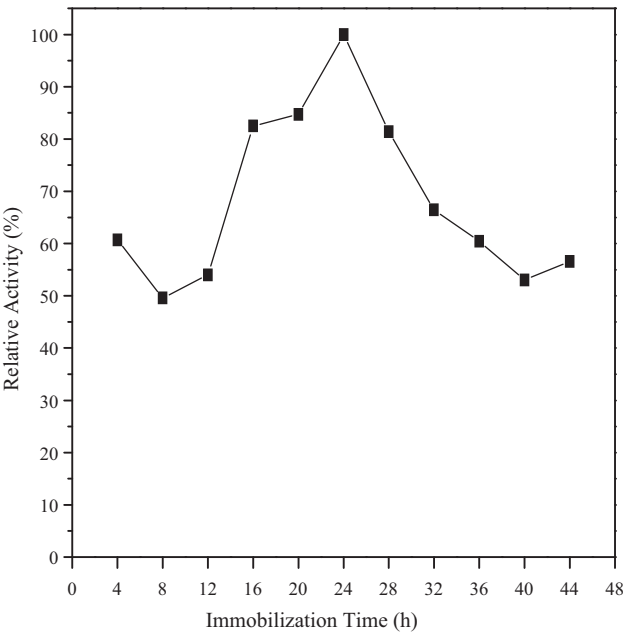

Fig. 2. Effect of immobilization time on the activity of immobilized GI.

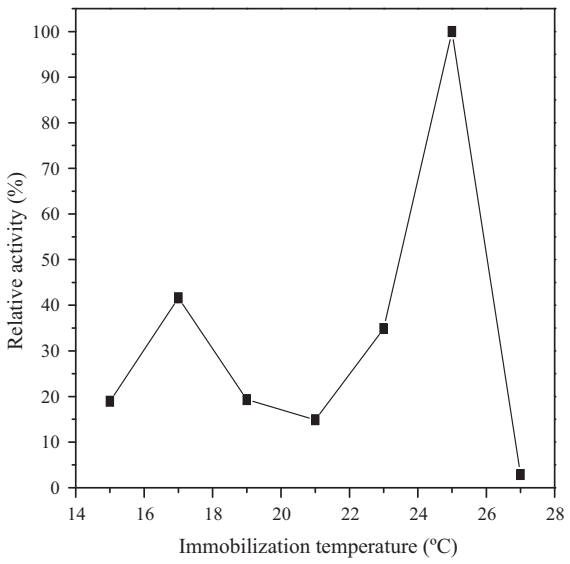

Fig. 3. Effect of immobilization temperature on the activity of immobilized GI.

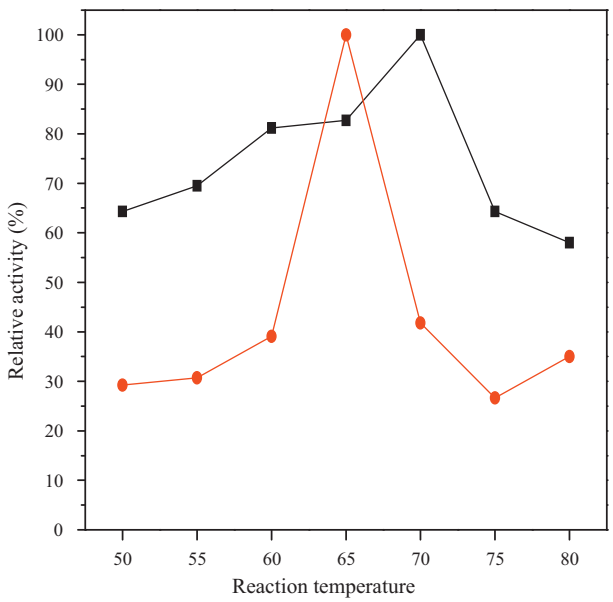

Fig. 4. Effects of reaction temperature on the activities of free and immobilized GIs (■, free GI; ●, immobilized GI).

ity decreased significantly due to multipoint attachment and then change of the steric configuration of GI molecules.

One gram GAMM was immersed in 0.3 mL GI diluted by phosphate buffer (0.2 mol/L, pH = 7.0) to 5 mL, then GI was immobilized for 24 h at 15–27 °C. Effect of immobilization temperature on the activity of immobilized GI is shown in Fig. 3, in which the optimum immobilization temperature was 25 °C. High immobilization temperature was beneficial to thermal motion and diffusion of GI molecules into the pores of GAMM support, but too high temperature made thermal motion of GI molecules too intense to be adsorbed on the support.

3.2. Effects of reaction conditions

Effects of pH value of reaction solution on the activities of free and immobilized GIs were investigated, in which the optimum pH values of reaction solution for free and immobilized GIs were both 7.5. Immobilized GI had wider application range of pH value of reaction solution, in which it retained 92% of its maximum activity at pH= 6.0 and 88% of its maximum activity at pH = 8.5. However, free GI only retained 60% of its maximum activity at pH = 6.0 and 70% of its maximum activity at pH = 8.5. The optimum pH values were 7.5 for free and immobilized GIs entrapped in various hydrogels such as poly(acrylamide), semi-interpenetrating poly(acrylamide)/κ-carrageenan, and poly(acrylamide)/alginate polymer networks [19], and the optimal pH value was also 7.5 for immobilized *Streptomyces flavogriseus* GI onto benzyl DEAE-cellulose, TEAE-cellulose and DEAE-cellulose [10]. However, the optimum pH values were 7.0 for free GI from *Streptomyces rubiginosus* and immobilized GI onto Eupergit C 250L [9].

Effects of reaction temperature on the activities of free and immobilized GIs are shown in Fig. 4, in which the optimum reaction temperature for immobilized GI was 65 °C and the optimum reaction temperature for free GI was 70 °C. Free GI had wider application range of reaction temperature, in which it retained 64% of its maximum activity at 50 °C and 58% of its maximum activity at 80 °C. However, immobilized GI only retained 29% of its maximum activity at 50 °C and 35% of its maximum activity at 80 °C. The optimum temperatures were 60 °C for free and immobilized GIs entrapped in various hydrogels [19], and the optimum temperature was also 60 °C for immobilized GI onto Eupergit C 250L [9]. However, the optimum temperatures were 70 °C for free and immobilized GIs onto benzyl DEAE-cellulose, TEAE-cellulose and DEAE-cellulose [10].

3.3. Kinetic parameters

Michaelis–Menten constant (K_m) and the maximum reaction rate (V_{max}) were determined from the classical Michaelis–Menten kinetics and Lineweaver–Burk plots by measuring the initial reaction rates with different glucose concentration solutions (0.1, 0.5, 0.75, 1.0 and 1.5 mol/L), in which 0.1 mL free GI or 0.2 g immobilized GI were used. The kinetic parameters were determined at pH = 7.5 and 70 °C for free GI and at pH = 7.5 and 65 °C for immobilized GI. K_m values of free and immobilized GIs were 0.36 and 1.16 mol/L, respectively. V_{max} values of free and immobilized GIs were 1.27×10^{-3} and 1.07×10^{-3} mol/L min, respectively.

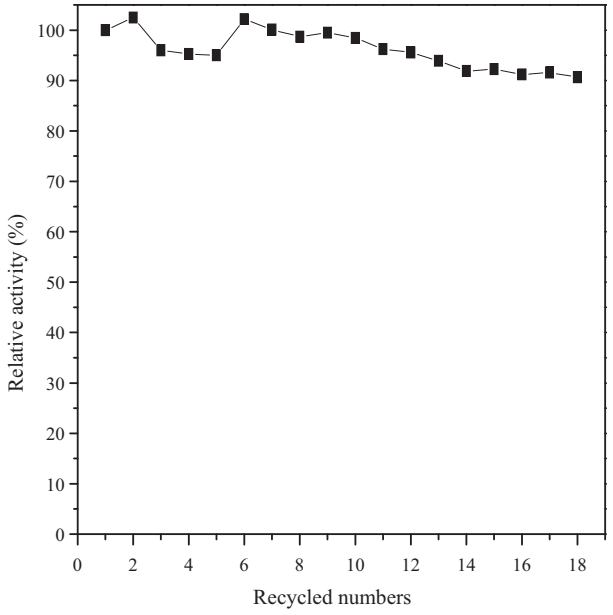

Fig. 5. The operational stability of immobilized GI.

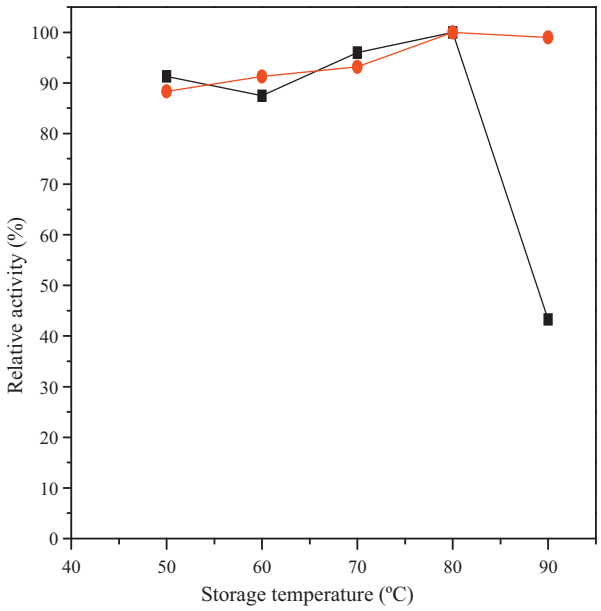

Fig. 6. The thermal stabilities of free and immobilized GIs (■, free GI; ●, immobilized GI).

3.4. Operational performance of immobilized GI

The operational stability is one of the most important aspects for industrial application of immobilized GI. After the isomerization reaction, the immobilized GI was filtered immediately, and the fructose amount in the filtrate was determined. The initial activity of immobilized GI was determined as 450 U/g (wet). The operational stability of immobilized GI is shown in Fig. 5, in which after recycled for 18 times, immobilized GI retained 91% of its initial activity. After 25th use in 5 days, the retained activities for GIs entrapped in poly(acrylamide), semi-interpenetrating poly(acrylamide)/κ-carrageenan, and poly(acrylamide)/alginate polymer networks were 98%, 71% and 72%, respectively [19]. Immobilized GI onto Eupergit C 250L was recycled for 18 times and the residual activity was about 85% of its initial activity [9]. Therefore immobilized GI onto GAMM support has better operational stability.

The thermal stabilities of free and immobilized GIs are shown in Fig. 6, in which 0.2 g immobilized GI or 0.1 mL free GI was placed in 5 mL phosphate buffer (0.2 mol/L, pH = 7.5) and kept at 50–90 °C for 1 h. With an increase in storage temperature, the enzyme activities of free and immobilized GIs increased to a maximum at 80 °C and then decreased significantly. Immobilized GI had better thermal stability than free GI. When kept at 50–80 °C for 1 h, immobilized GI had similar thermal stability to free GI, however, immobilized GI retained 99% of its maximum activity and free GI only retained 43% of its maximum activity after kept at 90 °C for 1 h. There were almost no difference existed in the thermal stability between free and immobilized GIs onto benzyl DEAE-cellulose, TEAE-cellulose and DEAE-cellulose [10]. The residual activities for free and immobilized GIs onto Eupergit C 250L were correspondingly calculated as 89% and 79% of their initial activities at 5 °C and 97% and 84% of their initial activities at 60 °C at the end of 18 h preincubation time [9]. Therefore immobilized GI onto GAMM support has better thermal stability.

The storage stability of immobilized GI was investigated, and its activity was measured every week for six weeks. Results showed that immobilized GI retained 97% of its initial activity after stored at 4 °C for six weeks. However, the storage stabilities of immobilized GIs entrapped in poly(acrylamide), semi-interpenetrating poly(acrylamide)/κ-carrageenan, and poly(acrylamide)/alginate polymer networks systems were observed as 81%, 33% and 32% of their initial activities, respectively, after stored at 4 °C for six weeks [19]. Immobilized GI onto Eupergit C 250L retained 72% and 69% of its initial activity after stored at 5 and 25 °C for four weeks, respectively [9]. Therefore immobilized GI onto GAMM support has better storage stability.

4. Conclusions

Glucose isomerase was immobilized covalently onto GAMM support, by reaction of the amino groups of GI with the epoxy groups of GAMM support under mild conditions, for isomerization of glucose to fructose. Optimum parameters for immobilization and reaction, the kinetic parameters and the operational performance of immobilized GI were investigated. K_m values of free and immobilized GIs were 0.36 and 1.18 mol/L, respectively. V_{max} values of free and immobilized GIs were 1.27×10^{-3} and 1.07×10^{-3} mol/L min, respectively. Immobilized GI onto GAMM support, which activity was 450 U/g (wet), has better operational stability, thermal stability and storage stability. Therefore immobilized GI onto GAMM support was an excellent catalyst for glucose isomerization.

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