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Catalytic efficiency of immobilized glucose isomerase in isomerization of glucose to fructose

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ABSTRACT

Glucose isomerase (GI) from *Streptomyces rubiginosus* was immobilized covalently onto Eupergit C 250 L made by copolymerization of *N*,*N*-methylene-*bis*-methacrylamide, glycidyl methacrylate, allyl glycidyl ether and methacrylamide. The catalytic efficiency of immobilized GI in isomerization of glucose to fructose was found as three fold higher than that of free GI. The residual activity of immobilized GI after 18 reuses in a batch type stirred reactor was about 85% of its initial activity. The thermal stability of immobilized GI was almost same with that of the free GI at 60 °C for 18 h preincubation time. The residual activities of immobilized GI when stored at 5 °C and 25 °C for four weeks were 72% and 69% of the initial activity, respectively. However, free GI retained 88% and 78% of its initial activity at 5 °C and 25 °C upon four weeks storage, respectively. Thus, the use of Eupergit C 250 L immobilized GI instead of free GI is suggested in enzymatic isomerization of glucose to fructose.

1. Introduction

Glucose isomerase (GI) (EC 5.3.1.5) catalyzes the reversible isomerization of D-glucose to D-fructose (Tsumura & Sato, 1965). The enzymatic conversion of D-glucose to D-fructose is an important industrial process, especially in the production of high fructose corn syrup (HFCS) (Bhosale, Rao, & Desphande, 1996). HFCS is used as sweetener in soft drinks and other food products where it replaces beet and/or cane sugar. It has the advantage that at an equal sweetener level, it is some 10–20% cheaper than sucrose and is less caloric because of the lower resorption of fructose. A technical advantage of HFCS is the better solubility of glucose and fructose compared to sucrose and therefore the lesser tendency to crystallize in a wide range of food products. This had led to their application in confectionery jam and jellies, ice-cream, canned products, baking, pickles, sauces, meal products etc., (Misset et al., 1991). In view of the fact that there is a high consumption of HFCS in beverages, many nutrition researchers have focused on the possible relationship between HFCS sweetened beverage consumption and obesity, diabetes or other related chronic diseases (Basciano, Federico, & Adeli, 2005; Bray, Nielsen, & Popkin, 2004). Recently, Lo et al. (2008) reported that HFCS in carbonated beverages was the major source of reactive α-dicarbonyl compounds (glyoxal, methylglyoxal and 3-deoxyglucosone) with the potential genotoxic and carcinogenic properties. In the same paper it was also reported that the concentrations of reactive α-dicarbonyl compounds vary significantly from different sources of HFCS in terms of the production and storage conditions. Conversely, some of the studies showed that HFCS sweetened beverage consumption was not a significant contributor to the obesity occurrence (Janket, Manson, Sesso, Buring, & Liu, 2003; Sun & Empie, 2007). Besides, GI has received increased attention by industries for its potential application in the production of ethanol from hemicelluloses (Bhosale et al., 1996; Petrovska, Winkelhausen, & Kuzmanova, 2000).

Immobilization of the glucose isomerase can offer several advantages for industrial and biotechnological applications, including repeated use, ease of separation of reaction products from the biocatalyst, improvement of enzyme stability, continuous operation in a packed-bed reactor and the alteration of the properties of the enzyme. GI obtained from different sources such as *Flavobacterium*, *Bacillus* and some *Streptomyces* and *Arthrabacter* species have been immobilized on different support materials such as DEAE-cellulose (Chen & Anderson, 1979; Huitron & Limon-Lason, 1978), polyacrylamide gel (Demirel et al., 2006; Strandberg & Smiley, 1971), alginate beads (Rhimi, Messaud, Borgi, Khadra, & Bejar, 2007).

In this study, Eupergit C 250 L was chosen as support material for GI. This support consists of macroporous beads with a diameter of 250 μm (oxirane density:300 μmol g^{-1} dry beads) made by copolymerization of *N*,*N*-methylene-*bis*-methacrylamide, glycidyl methacrylate, allyl glycidyl ether and methacrylamide. Because of its structure, Eupergit is stable, both chemically and mechanically, over a pH range from 0 to 14, and does not swell or shrink even upon drastic pH changes in this range. Thus, it has been described as a suitable carrier for enzymes in enzyme immobilization

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(Boller, Meier, & Menzler, 2002; Katchalski-Katzir & Kraemer, 2000).

The aim of this study was to investigate the use of Eupergit C 250 L as support for covalent immobilization of GI for the first time and the catalytic efficiency of immobilized GI in isomerization of glucose to fructose. Some of the characteristics of free and immobilized GI has been evaluated by determining the following parameters such as the intrinsic activity, reusability, thermal stability and storage stability.

2. Materials and methods

2.1. Materials

GENSWEET Soluble Glucose Isomerase (SGI) was a kind gift from Genencor International (Rochester, NY, USA). Its typical characteristics such as activity, appearance, solubility and specific gravity were given as 3000 GIU/g(minimum), brown liquid, completely miscible in water and 1.11–1.30 g/mL, respectively. The activity of GENSWEET SGI enzyme is expressed in Glucose Isomerase Units (GIU) per gram of enzyme preparation. One GIU is defined as the amount of enzyme that will convert one micromole of glucose to fructose in one minute under the conditions of the assay. Eupergit C 250 L, bovine serum albumin (BSA) and all other reagents and solvents were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Immobilization of glucose isomerase onto Eupergit C 250 L

The immobilization procedure has been described earlier by Mateo, Fernandez-Lorente, Abian, Fernandez-Lafuente, & Guisan, 2000. Briefly, 1 g of Eupergit C 250 L was mixed with 9 mL of GI solution (1 mg/mL) prepared in 1 M phosphate buffer (pH 7.0). This amount was predetermined as optimum amount since the maximum bound protein value (92% of initial amount of protein). The reaction was allowed to continue for 24 h at 25 °C. The mixture was shaken gently during immobilization period in a shaken water bath. After that, the resulting immobilized GI was washed exhaustively with distilled water until no protein was detected in the filtrate and dried in an incubator, then stored at 5 °C. The protein contents of the solutions were determined by the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951). BSA was used as a standard. The amount of immobilized enzyme protein was estimated by subtracting the amount of protein determined in the filtrate from the total amount of protein used in immobilization procedure.

2.3. Assay of glucose isomerase activity

The GI activity was determined spectrophotometrically by measuring the amount of fructose produced by GI under the described assay condition. Briefly, the enzyme reaction mixture contained 1 mL of 2.6 M glucose solution (in 200 mM phosphate buffer at pH 7.0, 20 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CoCl_2$) and 0.25 mL of enzyme solution (10 mg GI/mL) or 5 mg of immobilized GI. The mixture was incubated at 60 °C for 30 min and the reaction was stopped by adding 0.25 mL of 20% (v/v) HCl. After the reaction was stopped, the amounts of fructose were determined in an aliquot of reaction mixture. Assay of fructose was based on the HCl-resorcinol method in which resorcinol forms a red colored complex with fructose. The absorbance of the complex was measured spectrophotometrically at 540 nm (Mcrary & Slattery, 1945). One unit GI activity was defined as the amount of the enzyme that produced 1 μmol of D-fructose per min under the assay conditions described.

2.4. Characterization of glucose isomerase

2.4.1. Effect of pH

The effect of pH on the activities of free and immobilized GIs was assayed using a 2.6 M glucose solution in 200 mM acetate buffer (pH 5.0), citrate buffer (pH 6.0), phosphate buffer (pH 7.0, 8.0), and borate buffer (pH 9.0).

2.4.2. Effect of buffer concentration

The effect of buffer concentration on the activities of free and immobilized GI preparations was determined by using a 2.6 M glucose solution in 100, 200, 300, 400, 500 mM at pH 7.0, phosphate buffer.

2.4.3. Effect of temperature

The effect of temperature on the activities of free and immobilized GIs was investigated at 40, 50, 60, 70 and 80 °C.

2.4.4. Kinetic parameters

Activities of free and immobilized GIs were determined by using the classical Michaelis–Menten kinetics. In the graphical evaluation of Michaelis–Menten constants and maximum activities, Lineweaver–Burk plots obtained by plotting experimental values of $\frac{1}{V}$ vs. $\frac{1}{[S]}$ were used. In the Lineweaver–Burk equation, $\frac{1}{V}$ and $\frac{1}{[S]}$ are the reciprocals of initial avtivity (V) and initial substrate concentration ($[S]$), respectively.

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad \text{(Lineweaver—Burk equation)}$$

V_{max} is the maximum activity attained at infinite initial substrate concentration and K_m is the Michaelis–Menten constant.

To determine V_{max} and the K_m, the activity assay was applied for different glucose concentrations (100, 500, 750, 1000 and 1500 mM). Activities of free and immobilized GIs were determined at predetermined optimum conditions. The catalytic efficiencies of both free and immobilized GI were calculated.

2.5. Reusability of immobilized glucose isomerase

Reusability of immobilized GI was investigated by using a batch-type stirred column reactor (diameter 1.25 cm and length 5.5 cm). Five milligrams immobilized GI were loaded into the reactor and 1 mL of 2.6 M GI solution was added into column and the reaction was allowed to continue for 30 min. The reaction solution was removed immediately from the column and fructose amount was determined. The same experiment using the same immobilized GI sample was repeated 18 times in every 30 min.

2.6. Thermal stability

Stock solution of free GI and immobilized GI were kept at 5, 25, 40 and 60 °C and residual activities were determined after 1, 3, 7 and 18 h incubation times.

2.7. Storage stability

Stock solution of free GI and immobilized GI were stored at 5 and 25 °C and their residual activities were measured periodically for four weeks.

2.8. Statistics

All measurements were performed at least five times and the results were expressed as means ± SD. Statistical analysis of data was carried out using SPSS statistical package programs. One-way ANOVA was used the compare variables among free and

immobilized GI and a p value of <0.001 was considered statistically significant.

3. Results and discussion

The effect of pH of the medium on the activities of free and immobilized GIs were studied in buffer solutions of pHs from 5.0 to 9.0. The results were shown in Fig. 1. Free and immobilized GI both showed their maximum activities at the same pH value as 7.0. Immobilized GI protected 81% of its maximum activity at pH 5.0 and 83% of its maximum activity at pH 9.0. However, activity of free GI at pH 5.0 was determined as 51% of its maximum activity and activity of free GI at pH 9.0 was determined as 59% of its maximum activity. Chen & Anderson, 1979 were reported the optimal pH value as 7.5 for immobilized *Streptomyces flavogriseus* GI onto benzyl DEAE-cellulose, TEAE-cellulose and DEAE-cellulose. Demirel et al., 2006 found optimum pH as 7.5 for free *Streptomyces rubiginosus* GI and that immobilized GI entrapped in various gels. Rhimi et al. (2007) co-expressed the thermostable L-arabinose isomerase of *Bacillus stearothermophilus* US100 (L-AI US100) and the mutant D-glucose isomerase obtained from that of *Streptomyces* SK (SKGI-A103G) in *Escherichia coli* HB101 strain. The immobilized recombinant cells in alginate beads were optimally active at pH 7.5 for D-glucose isomerization.

Free and immobilized GIs activities were determined in buffer solutions of different concentrations (100–500 mM) at pH 7.0. Immobilized GI activity was maximum at 400 mM buffer concentration. However, free GI activity increased almost proportionally with the increase in buffer concentration from 100 to 500 mM (Fig. 2).

The activities of free and immobilized GIs were investigated at temperature range of 40–80 °C and the results were shown in Fig. 3. The optimum temperature for immobilized GI was found as 60 °C. Immobilized GI showed about 80% of its maximum activity at 40 °C and 90% of its maximum activity at 80 °C. However, the activity of free GI increased while increasing the temperature to 80 °C. The optimum temperature of *Streptomyces phaeochromogenes* GI was reported as 80 °C by Strandberg and Smiley (1971). Hodge (1953) and Bucke (1983) indicated that degradation of ketoses occurs at high temperatures, characterized by a pronounced discoloration of an aqueous sugar solution. Thus, 70 °C was chosen by Chou, Ladisch, and Tsao (1976) to isomerize glucose even though the optimum temperature was 80 °C.

The kinetic parameters were determined in 200 mM phosphate buffer, pH 7.0 at 60 °C for free GI, and 400 mM phosphate buffer, pH 7.0 at 60 °C for immobilized GI. The kinetic parameters of free and immobilized GI were investigated and K_m values of free and immobilized GI were found as 423 and 2602 mM, respectively. The maximum activity of free glucose isomerase was determined as 5000 U/g prot. and maximum activity of immobilized glucose isomerase was determined as 98092 U/g prot. The catalytic efficiency of immobilized glucose isomerase (108 s^{-1} M^{-1}) was about 3-fold higher than that of free glucose isomerase (34 s^{-1} M^{-1}). Strandberg & Smiley, 1971 reported K_m value of polyacrylamide-entrapped *S. phaeochromogenes* GI as 250 mM. Demirel et al., 2006 entrapped *S. rubiginosus* GI into polyacrylamide, semi-interpenetrating polyacrylamide/κ-carrageenan and polyacrylamide/alginate polymer networks and they reported K_m values of free and entrapped GIs as 18.87, 1.22, 2.78, and 4.54 mg/mL, respectively, while the V_{max} values as 2.51, 0.63, 0.72, and 0.82 mg/mL min, respectively. They reported that the decrease in K_m values is due to electrostatic interactions between the substrate and matrix and also diffusional effects.

The reuse numbers of immobilized enzymes are one of the most important aspects for industrial application. An increased stability could make the immobilized enzyme more advantageous than its free form. As shown Fig. 4, the immobilized GI was used repeatedly 18 times and the residual activity was about 85% its initial activity. The repeated use of immobilized *E. Coli* HB101/pMR20 cells in alginate beads were reported and remaining activity of immobilized GI was found as 70% of its initial activity after eight cycle of use (Rhimi et al., 2007). On the other hand, the entrapped *S. rubiginosus*

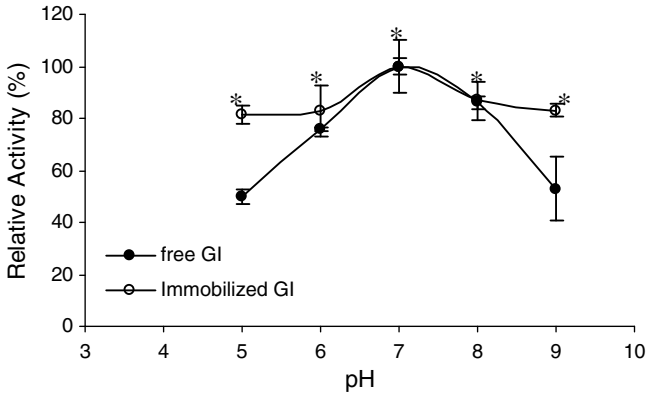

Fig. 1. The effect of pH on the activities of free and immobilized GIs. P value indicate the results of one-way ANOVA while asterisks indicate significant ($p < 0.001$) differences resulted from the Duncan tests between free and immobilized GI.

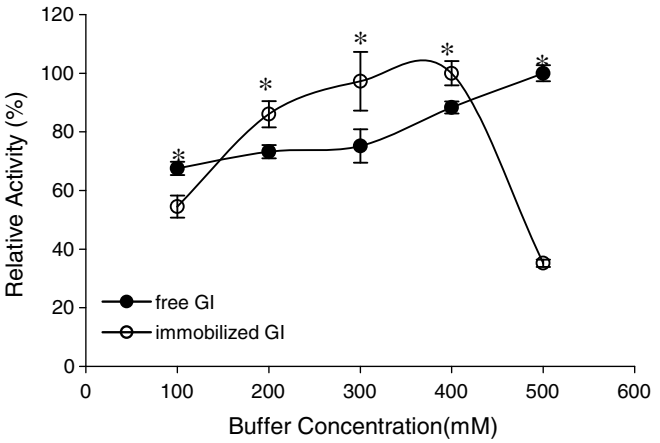

Fig. 2. The effect of buffer concentration on the activities of free and immobilized GIs. See Fig. 1 for detail.

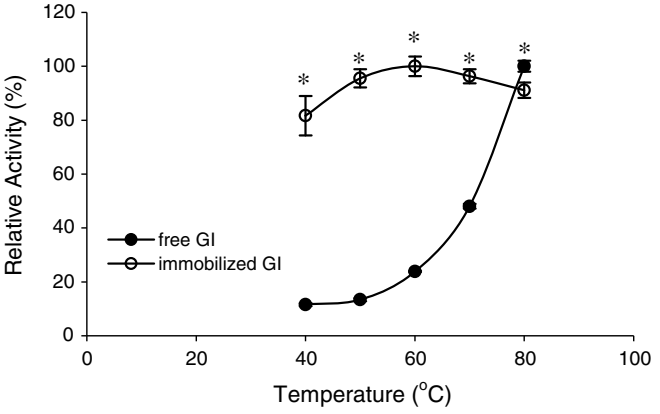

Fig. 3. The effect of temperature on the activities of free and immobilized GIs. See Fig. 1 for detail.

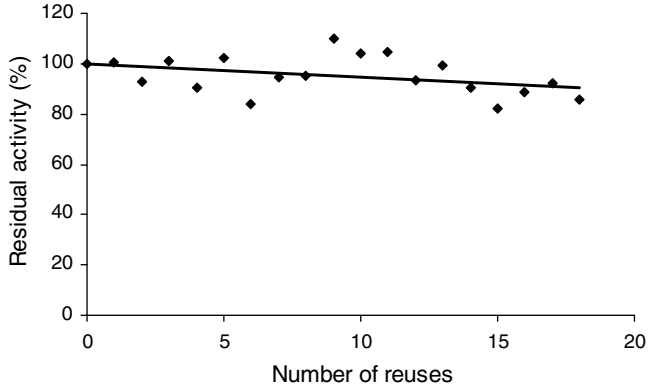

Fig. 4. The operational stability of immobilized GI.

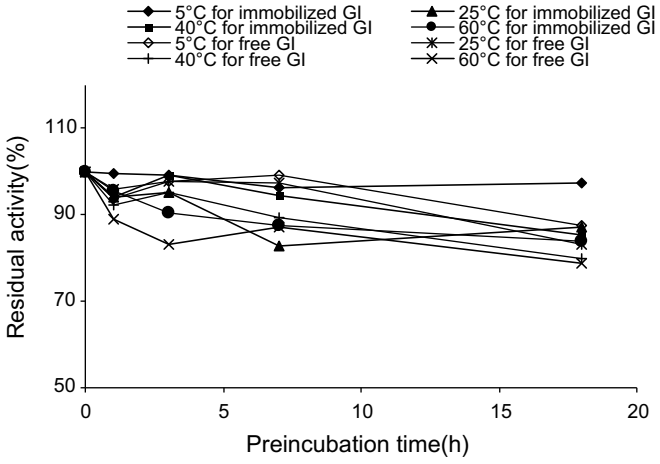

Fig. 5. The thermal stabilities of free and immobilized GIs.

GI in polyacriylamide, semi-interpenetrating polyacrylamide/κ-carriageenan and polyacrylamide/alginate were retained, 98%, 71% and 72% of their initial activities, respectively at the end of 25th use (Demirel et al., 2006).

Thermal stabilities of free and immobilized GIs were investigated and the results were shown in Fig. 5. Free and immobilized GI preparations were preincubated at 5, 25, 40 and 60 °C for 18 h and residual activities were calculated for 1, 3, 7 and 18 h intervals. The residual activities for free and immobilized GIs 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. In a previous report (Chen & Anderson, 1979), it was reported that almost no difference existed in thermal stability between the soluble and immobilized GI from *S. flavogriseus* onto benzyl DEAE-cellulose, TEAE-cellulose and DEAE-cellulose.

Immobilized GI samples as in the form of solid particles and free GI samples as stock solution were stored at 5 and 25 °C during four weeks and the residual activities were weekly determined. The immobilized GI stored at 5 and 25 °C retained 72% and 69% of its initial activity at the end of four weeks, respectively. However, the storage stability of free GI protected 88% and 78% of its original activity at 5 and 25 °C at the end of four weeks storage, respectively (Fig. 6). The storage stabilities of entrapped *Streptomyces rubiginosus* GI in different hydrogels at 4 °C found as 81%, 33% and 32% of their initial activities in polyacrylamide, polyacrylamide/κ-carrageenan and polyacrylamide/alginate at the end of 42 days, respectively (Demirel et al., 2006).

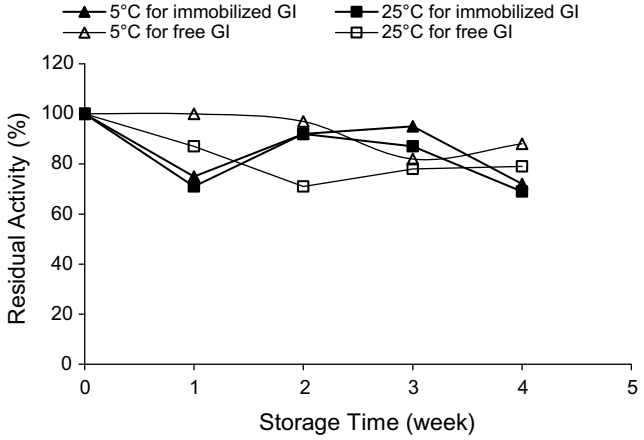

Fig. 6. The storage stabilities of free and immobilized GIs.

4. Conclusions

In this study, *Streptomyces rubiginosus* GI immobilized onto Eupergit C 250 L and kinetics of free and immobilized GIs were investigated at predetermined conditions. Immobilization of glucose isomerase onto Eupergit C 250 L highly improved both the catalytic efficiency and reusability of glucose isomerase. Immobilization had little effects on the thermal and storage stabilities of GI. Therefore, it may be suggested to use the Eupergit C 250 L immobilized GI instead of free GI in glucose isomerization.

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