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Removal of D-glucose from a mixture with D-mannose using immobilized glucose oxidase

D. Mislovičová*, J. Turjan, A. Vikartovská, V. Pätoprstý

Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dúbravska cesta 9, SK-845 38, Bratislava, Slovakia

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ABSTRACT

Glucose oxidase (GOD) was immobilized on bead cellulose (BC) or Eupergit (Eup) to produce three types of GOD preparations: GOD directly immobilized on BC, GOD biospecifically immobilized on BC through Concanavalin A and GOD immobilized on Eup. These samples were used to remove D-glucose from a D-glucose–D-mannose solution obtained via the epimerization of glucose. The removal of D-glucose by immobilized GOD was markedly improved by the incorporation of an immobilized catalase, which converts hydrogen peroxide (an enzyme inactivator) produced by GOD to molecular oxygen and water. GOD covalently immobilized on bead cellulose (GOD-TBC) showed the best operational stability of the three types of immobilized GOD tested. It was found that GOD-TBC, supplemented with immobilized catalase, could be used up to 8 times without lowering its ability to exhaustively eliminate residual glucose. The removal of gluconic acid generated during the biotransformation of D-glucose was performed by precipitation with $CaCl_2$. The final product contained D-mannose that was free of D-glucose, as confirmed by GC and HPLC tests.

1. Introduction

D-Mannose (Man) is a naturally occurring monosaccharide that is used in many medical therapies. Oral mannose therapy is a safe, practical treatment for urinary tract infections [1] and can be successfully administered to patients with congenital glycosylation disorders [2]. D-Glucose (Glc) epimerization is often used to prepare Man [3]. One drawback of this method is that 0.5–2% residual Glc remains in the product. Glc and Man are compounds with similar properties and are competitive substrates in many biochemical reactions [4,5]. Thus it is very important to use only one component in such cases. For example, glucose should be absent from Man as dietary supplement used to treat CDGS syndrome [6] and from a Man preparation used to stabilize blood samples from patients suffering from diabetes mellitus [7]. Recrystallization of product can reduce the glucose content to 1%. Further purification and desalting of Man using the strong anion exchanger in OH^- groups often results in its isomerization to Glc [8]. A more efficient method for the removal of residual Glc from Man prepared for medical purposes is therefore highly desirable. One protocol for the removal of Glc that is widely used in beverage and foodstuff production [9–12] is the enzymatic conversion of Glc to an easily removed product via the action of glucose oxidase.

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase) (GOD), a flavoenzyme, catalyzes the oxidation of Glc to δ-gluconolactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. The product δ-gluconolactone spontaneously hydrolyzes to gluconic acid [13]. The GOD molecule consists of two identical subunits of 80 kDa each. It also contains one mole of cofactor FAD [14] that works as the initial electron acceptor and is reduced to $FADH_2$.

GOD has become an important tool in several industries, with uses ranging from a Glc biosensor for the control of diabetes [15–17] to a food preservative and colour stabilizer [9]. It is widely used to determine Glc levels in body fluids [15] and for the biological production of gluconic acid [18,19]. GOD from *Aspergillus niger* is a glycoprotein with a mannose-type carbohydrate content of approximately 10–16%, and is known to be highly selective for Glc, which is thought to be its primary substrate. Other sugars can also be oxidized by GOD, but their oxidation rates are negligible compared with that of Glc [19,20].

To enhance enzyme properties such as reusability, operational stability, recovery and shelf life, GOD has been immobilized on different supports using various immobilization methods. Enzyme immobilization is a strategy to improve the enzyme's stability, thereby optimizing its operational performance in the industrial process. Immobilization inside a porous structure prevents aggregation and proteolysis by proteases. Moreover, the immobilized enzymes are not in contact with air bubbles, which may inactivate soluble proteins, but cannot inactivate the immobilized enzyme.

* Corresponding author. Tel.: +421 2 59410263; fax: +421 2 59410222.
E-mail address: chemmisl@savba.sk (D. Mislovičová).

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Multipoint covalent attachment of enzymes via short spacer arms reduces any conformational changes involved in enzyme inactivation and greatly increases enzyme stability [21–23].

GOD has previously been immobilized on supports such as poly-(L-lysine)-modified polycarbonate membrane [24], calcium alginate gel [12], chitosan-SiO_2 [25], cellulose-polymethyl methacrylate membrane [26], bead cellulose and Eupergit [27], which are used for a variety of applications. Eupergit is a synthetic support containing reactive oxirane groups that react with the amino groups of enzyme molecules to form covalent bounds stable within a pH range from 1 to 12 [28,29]. Chlorotriazine bead cellulose (CHTBC) is a support consisting of macroporous hydrophilic cellulose derivatized with cyanurchloride [30]. CHTBC react with an enzyme's amino groups to form stable covalent bounds [31]. Due to the presence of mannose-type oligosaccharides in the GOD glycoprotein, biospecific binding to Concanavalin A (Con A) has also been used to immobilize GOD on Con A-TBC [27,32].

During the oxidation of Glc by GOD, the resulting hydrogen peroxide can attack residues of GOD and decrease its catalytic efficiency [33]. Therefore, it would be advantageous to remove hydrogen peroxide, for example with the enzyme catalase (CAT), which converts hydrogen peroxide to water and molecular oxygen. Commercial GOD preparations often contain CAT in concentrations sufficient for this purpose [9,11]. The GOD–CAT system is able to scavenge reactive oxygen species and can thus protect foods and beverages against detrimental oxidation and browning [34]. This system also has the potential to completely remove Glc and hydrogen peroxide from reaction mixtures.

The aim of this work was to develop a method for the complete removal of residual Glc from a Man solution that was prepared by the epimerization of Glc. This procedure is based on the significant difference in oxidation rates of GOD for Glc and Man. GOD was prepared in our laboratory in three different immobilized forms: GOD-triazine bead cellulose (GOD-TBC), GOD-Con A-triazine bead cellulose (GOD-Con A-TBC) and GOD-Eupergit (GOD-Eup) [27], which were used in biotransformation procedure. An effective method to remove gluconic acid generated during Glc biotransformation was then developed.

2. Experimental

2.1. Materials

Glucose oxidase GO 3B2 (EC 1.1.3.4) from *A. niger* and catalase (EC 1.11.1.6) from bovine liver CAT 60640 were obtained from Biozyme Laboratories (Gwent, UK) and from Fluka (Buchs, Switzerland) respectively. Cellulose MT 100 beads were obtained from Iontosorb (Ústí nad Labem, Czech Republic). Eupergit® C was supplied by Degussa Röhm GmbH&Co. KG, Pharma Polymers (Darmstadt, Germany). Concanavalin A was purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Immobilization of GOD on bead cellulose

2.2.1.1. Preparation of chlorotriazine bead cellulose (CHTBC). A 1.8 g sample of cyanurchloride was dissolved in 24 ml of acetone reacted with 10 g of bead cellulose (alkalized previously with 3 M NaOH) for 30 min at 4 °C to obtain CHTBC. The product was washed and used in immobilization reactions. The procedure was carried out as described [31].

2.2.1.2. Preparation of GOD-Con A-TBC and GOD-TBC. The procedures for immobilization of GOD on bead cellulose linked either covalently through triazine reactive groups or biospecifically through Con A have been described [27]. Con A-TBC was prepared via the procedure used for immobilization of proteins and modified as described in a previous work [31]. Briefly, 5 g of CHTBC was reacted with 25 mg Con A in a 50 mM sodium acetate buffer (pH 5.8) in the presence of 0.2% (w/v) D-mannose and 0.1 mM $MgCl_2$ for 2 h. A support containing 5 mg of Con A per 1 g of wet cellulose was used for the biospecific immobilization of GOD. Then, 10 mg of GOD in 50 ml of 20 mM sodium phosphate buffer (pH 7) containing 100 mM NaCl, 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+} was gently stirred with 10 g of wet Con A-TBC for 2 h at ambient temperature. The obtained biocatalyst was then separated by filtration and washed with 20 mM sodium phosphate buffer.

A modified procedure was used for the covalent immobilization of GOD on bead cellulose. 10 g of wet CHTBC was added to 10 mg of GOD dissolved in 25 ml of 50 mM sodium acetate buffer (pH 5.8). The suspension was stirred for 3 h, after which the prepared GOD-TBC was filtered and washed several times with acetate buffer.

2.2.2. Separate immobilization of GOD and CAT on Eupergit support

A suspension of 15 mg of GOD and 4 g of dry Eupergit C in 20 ml of 0.8 M sodium phosphate buffer (pH 7) was shaken for 1 h and stored for 72 h at ambient temperature with occasional shaking. Finally, 2 ml of 1% (v/v) formaldehyde in the same buffer was added and stored for 72 h. The obtained biocatalyst was filtered and washed with water and then with 100 mM sodium phosphate buffer.

CAT-Eupergit was prepared using the following procedure: 1 g of dry Eupergit C, 0.5 ml of CAT and 19.5 ml of 0.7 M sodium phosphate buffer (pH 6) were intermittently shaken at room temperature over a period of 48 h. Subsequently, the product was filtered and washed with 50 mM sodium phosphate buffer (pH 7).

2.2.3. Determination of activity of immobilized GOD and CAT

The activity of immobilized GOD was determined at 25 °C via an assay based on the horseradish peroxidase-catalysed oxidation of o-dianisidine by hydrogen peroxide that has been described previously [27]. CAT activity was determined according the method described by Beers and Sizer [35]. The modified assay for immobilized CAT utilized a suspension consisting of 15 ml of 50 mM sodium phosphate buffer (pH 7) containing 59 mM hydrogen peroxide and 10 mg of wet immobilized CAT. One unit of CAT activity corresponds to the decomposition of 1 μmol of hydrogen peroxide, measured as a change in absorbance at 240 nm per minute at 25 °C, under the specified conditions.

2.2.4. Determination of kinetic parameters of Glc and Man oxidation

Kinetic parameters for Glc or Man as substrates were measured at 30 °C using the same procedure by which the activity of immobilized GOD was determined. The initial reaction rate was measured within a range of saccharide concentrations from 10 to 400 mM and an oxygen concentration of 20 mg/l.

2.2.5. Biotransformation of Glc to D-gluconic acid in Man solution

Our model solution contained 0.96% of Man and 0.04% of Glc, which is a higher amount of Glc than is present in Man preparations obtained by epimerization. A 10 ml aliquot of this solution in 100 mM potassium phosphate buffer (pH 6) was mixed with 33–100 mg of immobilized GOD and continuously aerated with oxygen at 30 °C. One hundred microliter samples were taken at intervals to determine the Glc content. Biotransformation was carried out by adding immobilized CAT to the reaction mixture to accelerate the removal of Glc. The GOD-support was mixed with CAT-Eupergit (CAT-Eup) at ratios of 2:1 or 1:1. Glc content was determined using a Glucose test kit (BioSystems S.A., Barcelona, Spain) based on the spectrophotometric quantification of hydrogen peroxide generated during the enzymatic oxidation of Glc.

Table 1
Results of GOD loading on supports.

Immobilized GOD	Loaded GOD		Bound GOD			Activity immob. GOD
	[$mg_{prot}/g_{wet\ support}$]	[$U/g_{wet\ support}$]	[$mg_{prot}/g_{wet\ biocat.}$]	[$U/g_{wet\ biocat.}$]	[%]	[$U/g_{wet\ biocat.}$]
GOD-TBC	0.85	210	0.72	179	85	35.4
GOD-Con A-TBC	0.82	202	0.82	202	100	52.0
GOD-Eup	0.39	256	0.18	115	45	24.5

To remove hydrogen peroxide generated during the previous Glc biotransformation, 10 μL of CAT solution (9 U) was added to each sample and left to react for 5 min. Afterwards, the samples were heated for 10 min at 50 °C to inactivate CAT. Because Man acts as a secondary substrate of GOD, spectrophotometric quantification of Glc at 460 nm was corrected for using a control sample containing the same concentration of Man as that in the experimental sample. Glc elimination from each solution was additionally assayed by paper chromatography on Whatman paper (No. 1) that was then developed in a mixture of ethyl acetate:pyridine:water (8:2:1) (v/v/v). Chromatograms were detected using $AgNO_3$. The initial and final concentrations of Man after 2 h reaction were determined by GC (as described in Section 2.2.7). All biotransformation experiments were repeated 3 times.

2.2.6. Operational stability of mixtures of immobilized GOD with CAT-Eup

To test the stability of the mixture, 300 mg of GOD-sorbent and 200 mg of CAT-Eup were suspended in 30 ml of Man–Glc solution (0.96% Man and 0.04% Glc in 100 mM potassium phosphate buffer (pH 6)) and continuously aerated with oxygen at room temperature for 2 h. The sorbent was then separated by suction and used in further biotransformation cycles after the addition of new Man–Glc solution. This procedure was repeated 10 times. Filtrates were analyzed for Glc content by glucose test kits and checked by paper chromatography. Operational stability experiments were repeated 3 times.

2.2.7. Removal of gluconic acid after biotransformation

Gluconic acid generated in reaction mixtures was precipitated by adding an equal volume of 100 mM $CaCl_2$. Sedimented calcium gluconate was separated and the filtrate was desalted by ion exchange chromatography on Dowex 100 (H^+) and Amberlite 405 (HCO_3^-) columns (5 cm × 1 cm) connected in series. Desalted fractions were pooled and dried.

2.2.7.1. High-performance liquid chromatography. Glc and Man content was determined by a WATERS Delta Prep 3000 Preparative Chromatography System (Milford, MA, USA) using a Polymer IEX Pb column (300 mm × 8 mm, Watrex, Prague, Czech Republic). Elution with water was performed at a flow rate of 1 ml min^{-1} at 80 °C and monitored with an RI detector (Waters R 403).

2.2.7.2. Gas chromatography. Trimethylsilyl derivatives prepared from the resulting Man samples were analyzed by a Hewlett-Packard Model 5890 A chromatograph using flame ionization detection and separation by a DB-1 column (60 m × 0.25 mm; Fison, Loughborough, UK). The following thermal cycle was used: 80 °C for 2 min, followed by stepwise increases in temperature (6 °C/min) to 250 °C.

3. Results and discussion

3.1. Kinetic studies of enzymatic oxidation of Glc and Man

Three immobilized GOD preparations on various supports were prepared and tested for their ability to remove residual Glc from a Man–Glc mixture. The products of different immobilization procedures are summarized in Table 1.

Glucose was the preferred substrate of the immobilized enzyme. With all three supports its oxidation follows Michaelis–Menten kinetics (Fig. 1). Fig. 1 shows that the oxidation rate of saccharides with GOD-Con A-TBC is substantially higher than with the other two immobilized GOD preparations. In addition to Glc, oxidation of Man was also observed. However, the Man served as a very poor substrate. In the concentration range used the reaction velocity increased linearly with the monosaccharide concentration. This allowed us to calculate the ratios k_{cat}/K_m for GOD-TBC (0.73 min^{-1} mM^{-1}), for GOD-Con A-TBC (1.4 min^{-1} mM^{-1}) and for GOD-Eup (0.79 min^{-1} mM^{-1}), but not the individual kinetic constants. Comparison of the catalytic efficiencies (k_{cat}/K_m) of Man and Glc using all three immobilized GODs (Glc oxidation by GOD-TBC: 36 min^{-1} mM^{-1}, by GOD-Con A-TBC: 893 min^{-1} mM^{-1} and by GOD-Eup: 28.5 min^{-1} mM^{-1}) suggests that Man is a 36–638 times worse substrate than Glc.

3.2. Glc elimination by biotransformation with immobilized GOD

The operational and storage stability of each immobilized GOD preparation was estimated by flow calorimetry as described in our previous work focusing on the removal of Glc from dextran mixtures [27]. Biotransformation experiments performed in that study revealed that Glc removal from dextran mixtures was incomplete. This drawback was solved in the present work by adding immobilized CAT to the reaction mixture. The co-immobilized CAT is known to improve the catalytic efficiency of GOD by protecting it from hydrogen peroxide-mediated inactivation [36,37]. We investigated the influence of CAT on the enhancement of GOD catalytic activity in biotransformation experiments by adding CAT immobilized on Eupergit (1000 U/g of wet support) to reaction mixtures.

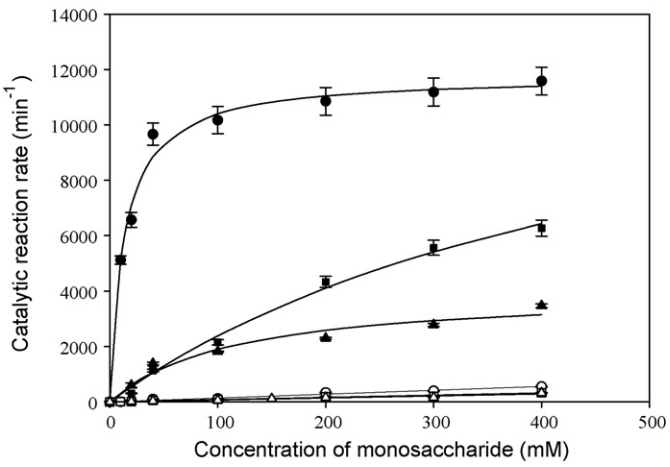

Fig. 1. Catalytic reaction rate of Glc or Man oxidation by immobilized GODs. –●– Glc, –○– Man on GOD-Con A-TBC; –■– Glc, –□– Man on GOD-Eup; –▲– Glc, –△– Man on GOD-TBC. Conditions: 0.05 M sodium phosphate buffer (pH 6) temperature 30 °C, oxygen concentration 20 mg/l.

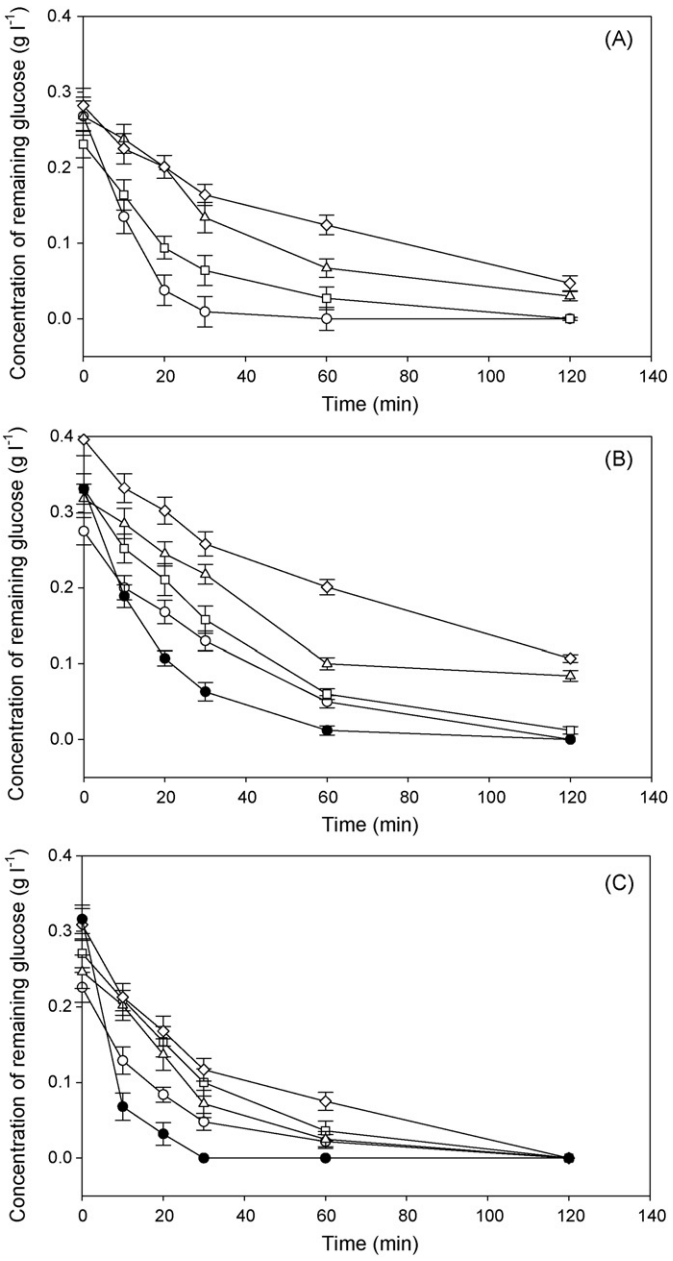

Fig. 2. Bioconversion of Glc in a Man solution using GOD-Con A-TBC. Batch experiments in 10 ml 50 mM phosphate buffer (pH 6) with 0.96% Man and 0.04% Glc (w/v) under continuous aeration at 30 °C with: –○– 100 mg, –□– 66 mg, –△– 50 mg and –◊– 33 mg GOD-Con A-TBC. (A) No CAT-Eup (CAT solution added); (B) CAT-Eup (no CAT solution); (C) CAT-Eup (CAT solution added); with GOD-Con A-TBC and CAT-Eup in a 2:1 ratio; –●– 100 mg of GOD-Con A-TBC with CAT-Eup 1:1 were used. 10 μL of CAT solution was added to 100 μL sample to remove hydrogen peroxide before Glc determination.

Fig. 2 shows the effects of added CAT-Eup or soluble CAT on Glc elimination by GOD immobilized on Con A-TBC. Complete removal of Glc was confirmed by paper chromatography, HPLC and GC–MS. We obtained the best results when CAT-Eup was added to the reaction mixture containing GOD-support at a ratio of 1:2 and when samples (taken out at time intervals) were treated with soluble CAT before determination of Glc. A comparison of the three sets of experiments (Fig. 2) indicates that Glc elimination was most effective with the addition of both CAT-Eup and CAT solution. Increasing the amount of CAT-Eup in the reaction mixture up to a 1:1 ratio with GOD-Con A-TBC (Fig. 2B and C filled symbols) had a positive effect on Glc elimination.

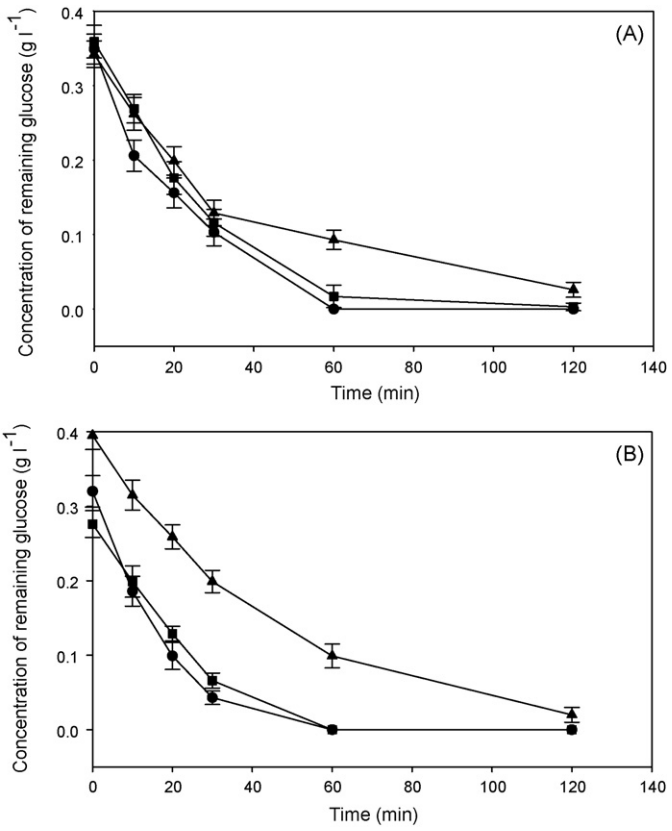

Fig. 3. Bioconversion of glucose by (A) GOD-Eup and (B) GOD-TBC with CAT-Eup and CAT solution added. Batch experiments in 10 ml 50 mM phosphate buffer (pH 6) with 0.96% Man and 0.04% Glc (w/v) under continuous aeration at 30 °C with: –●– 100 mg, –■– 66 mg, –▲– 40 mg of immobilized GOD with CAT-Eup in a 2:1 ratio, with 10 μL of CAT solution added to each 100 μL sample.

Two other sorbents, GOD-TBC and GOD-Eup, were tested in the presence of CAT-Eup with CAT solution added at Glc determination. Both showed similar time courses to that of GOD-Con A-TBC for Glc elimination (Fig. 3). These results indicate that 100 mg of immobilized GOD biocatalyst and 50 mg of CAT-Eup are required for the complete elimination of 0.04% Glc from a 0.96% Man solution within 1 h.

The GC analyses after 2 h of biotransformation show an undetectable decrease in Man concentration.

3.3. *Operational stability of GOD-biocatalysts*

The potential of immobilized GOD to completely remove residual Glc from a mixture after repeated use is an essential test of its reusability [33]. The results of multiple uses of immobilized GOD with added CAT-Eup (3:2 ratio of immobilized GOD:CAT-Eup) for Glc elimination are shown in Fig. 4. GOD-TBC exhibited the best operational stability of the three immobilized GOD types examined. It was able to reduce Glc to non-detectable levels in eight repeated biotransformation cycles. The two other types of immobilized GOD displayed slightly lower bioconversion stability after repeated use. The stabilizing effect of immobilized CAT on GOD can be seen by comparing the operational stability of immobilized GOD-TBC without CAT-Eup to the operational stability of GOD-TBC with CAT-Eup (Fig. 4). The ability of immobilized GOD alone to remove the low amount of Glc from Man solution decreased after repeated use. This was confirmed by reduction of GOD-TBC activity from 35.4 to 14.8 U/g after 10 repeated use.

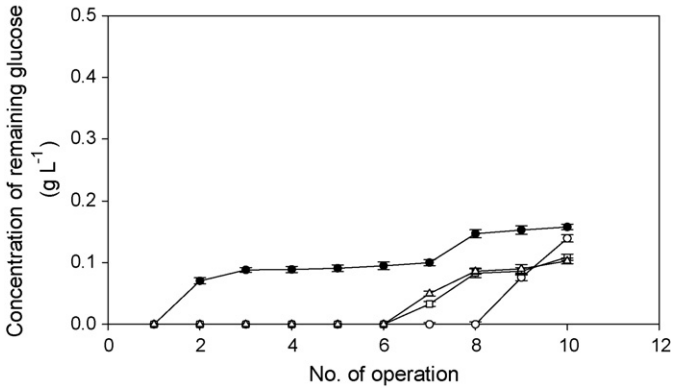

Fig. 4. Operational stability of immobilized GOD. Batch experiments in 30 ml 0.05 M phosphate buffer (pH 6) with 0.96% Man and 0.04% Glc (w/v) under continuous aeration at room temperature for 2 h with: 300 mg of immobilized GOD without CAT-Eup: –●– GOD-TBC, and 300 mg of immobilized GOD with 200 mg CAT-Eup: –○– GOD-TBC, –□– GOD-Con A-TBC, –△– GOD-Eup.

3.4. Removal of gluconic acid

To obtain Man of high purity, we found it necessary to remove the δ-gluconolactone and gluconic acid that were generated during the biotransformation. Sodium hydroxide, a strong base that can be used to neutralize gluconic acid was unsuitable in this case because it can isomerize Man back to Glc [8]. For this reason, a solution of $CaCl_2$ was chosen as a precipitation agent. To shift the chemical equilibrium from δ-gluconolactone, the primary product of GOD, to gluconic acid, the precipitation was performed overnight at ambient temperature. After removing the calcium gluconate precipitate and desalting by ion exchange-chromatography, we obtained pure Man and confirmed the total removal of gluconic acid via HPLC and GC.

4. Conclusion

Glc is often used as a starting material in the preparation of other saccharides, and is therefore a frequent contaminant in prepared carbohydrates [38]. However, medical therapies require pure Man that is free of trace Glc [39]. In this work, we have described a protocol for the removal of small amounts of Glc from mixtures with Man. A model solution mixture of Glc:Man (w/v), which contains a higher percentage of Glc than is found in common preparations of Man after epimerization protocols, was successfully treated with immobilized GOD. Thus our method has the potential to remove higher contents of residual Glc from mixtures with other saccharides.

Three different forms of immobilized GOD were tested in batch experiments to determine the optimal conditions for complete Glc elimination. We demonstrated that 0.04% Glc could be entirely removed from a 0.96% solution of Man using all three types of immobilized GOD in combination with immobilized CAT, (with specific activities ranging from 24.5 to 52 U/g of wet sorbent), within 1 h under aeration with oxygen at 30 °C. Although the rate of Glc oxidation with GOD-Con A-TBC was much higher than with the other two immobilized GODs, GOD-TBC showed the best results in biotransformation experiments for Glc elimination and also demonstrated the best operational stability. The disadvantage of using GOD-Con A-TBC is that it is more expensive preparation than GOD-TBC. A mixture of GOD-TBC and CAT-Eup (3:2) resulted in the complete and specific oxidation of Glc from Glc–Man mixtures after eight cycles. Supplementing with immobilized catalase (to eliminate hydrogen peroxide) improved Glc removal and increased the operational stability of immobilized GOD during repeated application. No decrease in the Man content after 2 h of biotransformation was observed. This result is consistent with the fact that Glc is a 50-times better substrate of GOD-TBC than Man. The complete removal of gluconic acid was achieved by precipitation with $CaCl_2$. Product desalting by ion exchange chromatography resulted in pure Man.

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