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Simultaneous Enzymatic Synthesis of Gluconic Acid and Sorbitol

Continuous Process Development Using Glucose-Fructose Oxidoreductase from *Zymomonas mobilis*

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ABSTRACT

The production of sorbitol and gluconic acid by isolated glucose-fructose oxidoreductase (GFOR) from *Zymomonas mobilis* has been studied in a convective, 100–mL loop reactor with tangential ultrafiltration. Using a dilution rate of 0.04/h and 5 kU/L GFOR, substrate conversion (3 *M* sugar) in a single stage was >85%, and productivities of 126 g sorbitol/(L·d) were obtained. At a constant recycle rate (3/min) and a membrane area of 50 cm^2, the dilution rates (and thus productivities) were however limited by a more than 30-fold reduction of the permeate flow in the presence of high sugar and protein concentrations (5 g/L). Protein was added, together with 10 m*M* dithiothreitol, to improve the stability of GFOR during substrate turnover and crossflow filtration, thus leading to a stable operation of the enzyme reactor for at least 5 d.

Index Entries: Glucose-fructose oxireductase; sorbitol; gluconic acid; *zymomonas mobilis*.

INTRODUCTION

Glucose-fructose oxidoreductase (GFOR) from *Zymomonas mobilis (1)* converts fructose to sorbitol and, simultaneously, glucose to glucono-δ-

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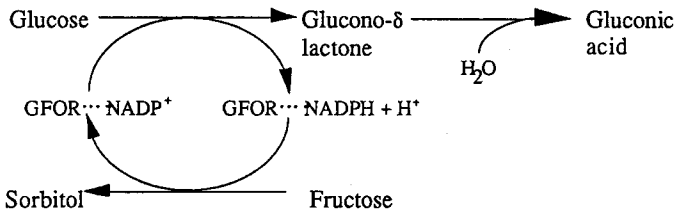

Fig. 1. Simultaneous synthesis of glucono-lactone and sorbitol by glucose-fructose oxidoreductase from *Zymomonas mobilis*. The enzyme contains tightly bound NADP(H). Glucono-lactone hydrolyzes spontaneously to gluconic acid.

lactone (Fig. 1). The enzyme has a high specific activity (250–300 U/mg) but a very low affinity for fructose (K_m approx 400–450 m*M*) *(2–4)*. GFOR catalyzes two half reactions, the reduction of fructose and the oxidation of glucose, and NADP(H) serves as the nondissociable coenzyme *(1–4)*. The glucono-δ-lactone product is degraded rapidly and irreversibly to gluconic acid, so that the reaction equilibrium lies completely on the product side *(2–4)*.

Sorbitol and gluconic acid have various food and nonfood applications. Hence, the conversion of mixtures of glucose and fructose by GFOR is thought to be of a significant technical relevance. Cells of *Zymomonas* can be made permeable to substrates and products, whereas proteins like GFOR are retained in the cellular matrix *(5,6)*. Such systems have been used for the continuous production of sorbitol and gluconic acid and are characterized by good operational stabilities at temperatures of up to 39°C *(5–11)* that lead to rapid inactivation of the isolated enzyme *(12–14)*. The degrees of substrate conversion reported in these studies are in a range of 50–95% *(5–11)*. The high K_m for fructose is clearly responsible for the difficulty to achieve complete substrate conversion in a single reactor, and a two-stage cascade was shown to improve not only the amount of substrate converted but also the productivities *(5)*.

Use of isolated, soluble GFOR in high concentrations would, in principle, offer the opportunity to increase the productivities of such a biocatalytic reactor and could make a significant contribution to the production of gluconic acid and sorbitol. Unfortunately, GFOR is not very stable in the cell-free form, and even at 25–30°C stabilization of the enzyme is necessary during substrate conversion *(12,13)*. Given that the low operational stability of soluble GFOR can be significantly improved *(12,13)*, a continuous enzyme reactor with GFOR retained by ultrafiltration membranes could be an interesting production system. Results obtained in a single-stage laboratory enzyme reactor with dead-end ultrafiltration (flat-membrane configuration) indicated that the half-life of soluble GFOR can be extended beyond 500 h reaction time, and, even at a reaction temperature of 25°C, good productivities can be obtained *(13)*. The aim of the work presented in this communication was to study the performance of GFOR in a reactor

with a technically more realistic recycle or loop configuration using an external tangential flow membrane module to separate the biocatalyst from products and substrates. Hence, transport data for process development and scale up can be obtained together with information concerning the stability and kinetic properties of GFOR.

MATERIALS AND METHODS

Enzymes

For the production of GFOR, *Zymomonas mobilis* DSM 473 was used *(12,14)*, and the crude cell extract with a specific GFOR activity of approx 2 U/mg was employed in all experiments without further purification. Gluconolactonase was partially purified from cell extracts of *Rhodotorula rubra* DSM 70403 by ammonium sulfate precipitation (50% saturation) followed by Sephadex-G 25 gel filtration *(14)*.

Assays/Analytical

GFOR activity was measured by a reported assay *(2,12)* using excess (10 U/mL) of gluconolactonase from *R. rubra*. One unit of activity refers to 1 μmol gluconic acid formed per min by the action of GFOR together with gluconolactonase. Sorbitol, gluconic acid, fructose, and glucose were quantitated by high-performance liquid chromatography (HPLC) *(13)*.

Continuous Conversions

Reactions were carried out at 30°C in a total volume of 100 mL, including the volume of the ultrafiltration loop (Fig. 2). The pH was constant at 6.2, and 2 *M* Tris or Na_2CO_3 were used for automatic pH control. Conductivity was recorded. An ultrafiltration membrane cassette with a 30-kDa cut-off and a membrane area of 50 cm^2 (mini-ultrasette; Filtron, Nortborough, MA) was employed. The recycle flow was constant at 300 mL/min (Watson Marlow, Falmouth, UK, 505 S pump), equivalent to a recirculation rate of 3/min, and the corresponding flux of permeate was measured. Substrate (1.5 *M* glucose, 1.5 *M* fructose) was fed continuously (Pharmacia, Uppsala, Sweden, P-500 pump), and the flow from the substrate tank together with the titrated alkaline component was adjusted to a variation of the permeate flux with reaction time. The substrate solution contained 10 m*M* dithiothreitol and 0.05% sodium azide (by weight) as a biocide. The activities of GFOR in the reactor were 5 kU/L, and the total protein concentration was adjusted to 5 g/L with bovine serum albumine (BSA). Samples (0.5 mL) from the permeate were taken in regular intervals and used for sugar analysis *(13)*. GFOR activities were measured in gelfiltered samples (NAP5 columns, Pharmacia) taken from the retentate. Substrate conversion (%) was calculated from the concentrations of sorbitol and fructose as: $100 \times [\text{sorbitol}]/\{[\text{sorbitol}] + [\text{fructose}]\}$.

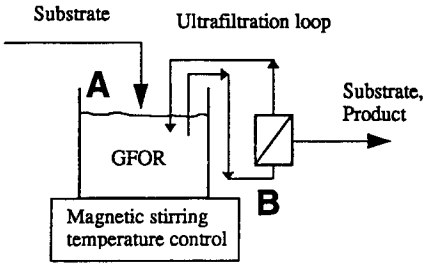

Fig. 2. Schematic representation of the recycle reactor used in this study, equipped with an external ultrafiltration loop. **(A)** Batch reactor. **(B)** Ultrafiltration loop.

RESULTS AND DISCUSSION

In recent work, the authors have been studying the process properties of isolated GFOR in a continuous 50-mL enzyme reactor that had a flat membrane configuration and thus operated with dead-end filtration *(12,13)*. Whereas this system is well-suited to investigate characteristic properties of the enzymatic conversion such as kinetics and stability of the biocatalyst, transport data for scale-up cannot be obtained. A small 100-mL recycle reactor (Fig. 2) was therefore used here to gather information more directly relevant to process development. To avoid inactivation of GFOR during the course of substrate turnover *(12–14)*, the following conditions were chosen.

1. Use of Tris (2 *M*) *(13)* or sodium carbonate (2–3 *M*) for the neutralization of gluconic acid.
2. Addition of dithiothreitol (10 m*M*) to prevent thiol oxidations, and BSA, to give a total protein concentration of 5 g/L, that is thought to protect against aggregation of GFOR during substrate conversion *(15)*.

Tangential Ultrafiltration

Using a constant recycle flow of 300 mL/min at 30°C, the corresponding permeate flow was measured as a function of the composition of the reaction medium. Compared to water, the reduction in permeate flow was approx 12-fold when a solution of 3 *M* sugar was employed. In the presence of 5 g/L protein, another threefold decrease in permeate flow was observed (Fig. 3). Hence, limitations to permeate flow have to be considered as factors potentially limiting the range of applicable substrate flow rates at high concentrations of sugar and protein.

Continuous Conversions

The results of a typical conversion reaction using isolated GFOR in the recycle reactor are shown in Fig. 4. Initially, a permeate flow of

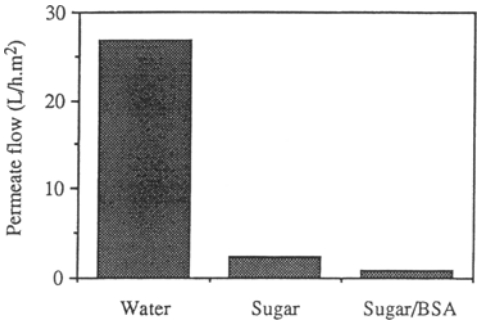

Fig. 3. Permeate flow as a function of the solute composition. The recycle flow was 300 mL/min and the corresponding permeate flow (at 30°C) was measured. The sugar concentration was 3 *M* (1.5 *M* glucose, 1.5 fructose), the total protein concentration was 5 g/L.

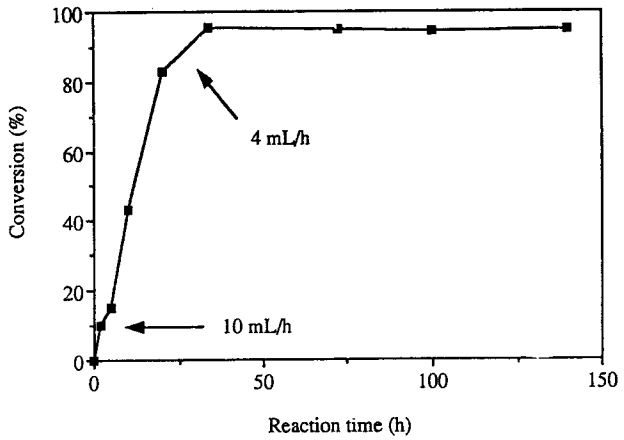

Fig. 4. Continuous conversion of glucose and fructose in the recycle membrane reactor. The substrate solution contained 3 *M* sugar, 10 m*M* dithiothreitol, and 20 m*M* sodium phosphate, pH 6.2. The reactor was operated at 30°C and contained 5 kU/L GFOR and a total protein concentration of 5 g/L. The pH was constant at 6.2, titration was carried out with 3 *M* sodium carbonate. The recycle flow was 300 mL/min. The permeate flow is indicated by arrows. The substrate flow is approximately half of the permeate flow because of the addition of alkali.

10 mL/h was obtained, so that the substrate feed was adjusted (5–6 mL/h) to give an average residence time of approx 10 h, taking into consideration the dosage of alkali. The permeate flow was not constant during the reaction and decreased to a lower limit of 4 mL/h after approx 20 h reaction time. Accordingly, the substrate feed was reduced because the recycle flow was set to a constant value in these experiments (300 mL/min). At an average residence time of 25 h (dilution rate of 0.04), the degree of substrate conversion was approx 85%, and the reactor performance was stable for

at least 5 d (not fully shown in Fig. 4). The resulting productivity is 126 g sorbitol/(L·d), a value that is certainly limited by the permeate flow that in turn is expected to increase significantly when a larger membrane area is used and eventually higher recycle flow rates are employed. It is important to notice that the alkaline component used for the neutralization of gluconic acid was sodium carbonate. The advantages of sodium carbonate, compared to Tris *(13)*, are lower costs and a presumably easier separation of the number of components present in the final product mixture. In the enzyme reactor with a flat membrane configuration, carbonate was difficult to use because the formation of carbon dioxide seemed to hamper the dead-end ultrafiltration. In case of the recycle reactor (Fig. 2), the results obtained with Tris or sodium carbonate as alkaline component were identical with regard to enzyme stability, degrees of substrate conversion, and productivity. Current activities are focused on the efficient separation of a typical product mixture, obtained from a conversion experiment like shown in Fig. 4.

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