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Off-line FIA monitoring of D-sorbitol consumption during L-sorbose production using a sorbitol biosensor

Jana Šefčovičová[a], Alica Vikartovská[a], Vladimír Pätoprstý[b], Peter Magdolen[c], Jaroslav Katrlík[a], Jan Tkac[a,*], Peter Gemeiner[a]

[a] Department of Glycobiotechnology, Center for Glycomics, Institute of Chemistry, Slovak Academy of Sciences, Dubravska cesta 9, SK-845 38 Bratislava, Slovakia
[b] Analytical Department, Center for Glycomics, Institute of Chemistry, Slovak Academy of Sciences, Dubravska cesta 9, SK-845 38 Bratislava, Slovakia
[c] Production Department, Center for Glycomics, Institute of Chemistry, Slovak Academy of Sciences, Dubravska cesta 9, SK-845 38 Bratislava, Slovakia

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ABSTRACT

A ferricyanide mediated amperometric biosensor system implementing D-sorbitol dehydrogenase together with diaphorase for sensitive detection of D-sorbitol was used. The biosensor system was successfully integrated into an off-line FIA system with a throughput of detection of 10 h^{-1}. The device exhibited limit of detection of 20 μM with an average relative standard deviation of analysis of samples of 2.2%. The signal of the biosensor was linear up to 1.1 mM for D-sorbitol with sensitivity of (72 ± 2) nA mM^{-1}, while a dynamic range was much wider up to 18 mM. The sorbitol biosensor gave reliable results even in the presence of a high molar excess of L-sorbose, a product of the biotransformation process, as judged from an excellent agreement with HPLC and GC.

1. Introduction

Biotechnology has enormous potential to produce a wide variety of natural products with high efficiency. Vast majority of natural products are so complex that it is almost impossible to produce them by a conventional chemical synthesis [1]. Production of vitamin C requires one biotransformation step (oxidation of D-sorbitol into L-sorbose) making the overall production process more efficient [2]. The annual production of 60,000 tons of L-sorbose is performed by cells of *Gluconobacter oxydans* with particular involvement of a membrane bound D-sorbitol dehydrogenase. *G. oxydans* is unsurpassed among microorganisms in the ability to produce wide range of natural products quickly and with high yield during fermentation/biotransformation processes [2–8].

Progress in the fermentation/biotransformation technology has to be accompanied with a tight control and regulation of the process in real time due to a complex and dynamic nature of the system. Flow injection analysis (FIA) system is frequently used for a real time bioprocess monitoring because of a short response time, high flexibility and inexpensive components [9,10]. Recently an off-line flow system was successfully used for monitoring of D-sorbitol during *Pichia pastoris* recombinant fermentation [11]. The detection method is based on a non-selective oxidation of polyols including D-sorbitol by periodate and L-sorbose can be chemically oxidized almost as efficiently as D-sorbitol [12]. Thus, the analytical procedure implementing a chemical oxidation step for detection of D-sorbitol will be compromised in the presence of equal concentration of L-sorbose. Therefore for robust monitoring of D-sorbitol in the presence of polyols (e.g. L-sorbose) an alternative and more selective analytical procedure has to be used.

Enzymatic biosensors offer high selectivity of detection in a complex matrix with flexibility to be implemented into a flow system for bioprocess monitoring [13,14]. These devices can be effectively used for on-line monitoring of the bioprocess and for feedback control of the substrate feeding strategy, as well [15,16]. Moreover, a highly sensitive biosensor device can be used for a simplified bioprocess monitoring without a need for a dialysis or a filtration probe [17]. Although, a sorbitol dehydrogenase of mammalian origin was used for development of sophisticated bioelectronic interfaces and for electroenzymatic synthesis [18–20], there are only two reports showing preparation of a biosensor device [21,22]. Only one such device was used for analysis of real samples (food) [21]. Here, a new application of using a biosensor device for monitoring of D-sorbitol during a biotransformation process is shown with extensive validation of the device by HPLC and GC.

* Corresponding author. Tel.: +421 2 5941 0263; fax: +421 2 5941 0222.
E-mail addresses: Jan.Tkac@savba.sk, jantkac@hotmail.com (J. Tkac).

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2. Experimental

2.1. Consumables

D-Sorbitol dehydrogenase (SDH, from sheep liver, 50 U mg^{-1}) was purchased from Sigma–Aldrich. Diaphorase (DP, from *Clostridium* sp., 59 U mg^{-1}) and β-nicotinamide adenine dinucleotide (NAD^+) were supplied from Sorachim (Paris, France). Both enzymes were used without further purification. D-Sorbitol and L-sorbose were obtained from Fluka (Steinheim, Germany). Agar and yeast extract used to maintain and cultivate *G. oxydans* were purchased from Oxoid (Basingstoke, UK). Potassium ferricyanide was supplied from Centralchem (Bratislava, Slovakia). Phosphate buffer (PB) was prepared from KH_2PO_4 and K_2HPO_4 (Mikrochem, Pezinok, Slovakia). Dialysis membrane Servapor® 44144 was supplied by Serva (Heidelberg, Germany).

2.2. Electrodes and biosensor preparation

All experiments were performed using a cylindrical glassy carbon electrode (d = 3 mm) with integrated Ag/AgCl reference electrode (d = 2 mm) in a Teflon casing (outer d = 9 mm) supplied from Elektrochemické detektory (ED, Turnov, Czech Republic). An electrode was polished with alumina suspension (0.05 μm, Struers A/S, Ballerup, Denmark) for 2 min and rinsed with distilled water (DW). Both enzymes were pipetted (5 μL each from 4% solution in 0.1 M PB pH 8) and mixed on the electrode, left to dry and covered by a dialysis membrane fixed by an O-ring. Finally, the enzyme modified electrode was integrated into the flow cell (dead volume of ~150 μL) of the FIA system. The cascade of reactions used for detection of D-sorbitol by the biosensor is summarized as follows:

$$\text{D-sorbitol} + NAD^+ \xrightarrow{SDH} \text{D-fructose} + NADH + H^+ \quad (1)$$

$$NADH + M_{ox} \xrightarrow{DP} NAD^+ + M_{red} + H^+ \quad (2)$$

$$M_{red} \xrightarrow{+300\ mV} M_{ox} + e^- \quad (3)$$

where M_{ox} is oxidized mediator (ferricyanide) and M_{red} is reduced mediator (ferrocyanide). D-sorbitol is oxidised by the SDH with production of a reduced cofactor ($NADH + H^+$) (Eq. (1)), which is enzymatically converted back into an oxidized form (NAD^+) by DP in the presence of ferricyanide (Eq. (2)). The latter one is converted into ferrocyanide as a result of action of DP (Eq. (2)) and the last step is an electrochemical recovery of the oxidized mediator from a reduced one on the electrode surface by an applied working potential of +300 mV (Eq. (3)) using a potentiostat PST-3 (FEI STU, Bratislava, Slovakia).

2.3. Instrumentation and FIA measurements

The FIA system supplied from FIAlab Instruments Inc. (Bellevue, USA) consists of peristaltic pumps (Watson-Marlow, Houston, USA) and sampling/injection valves (Vici, Houston, USA). Two streams consisting of 0.1 M PB pH 8 with 4 mM NAD^+; and 0.1 M PB pH 8 with 4 mM ferricyanide were mixed just before reaching the flow cell using T-shaped connector with the final flow rate of 0.38 mL min^{-1} (Fig. 1). An injection loop of 20 μL was used for sample/standard injections and an utility designed in Labview (National Instruments, Austin, USA) was used for control and data acquisition.

2.4. Batch mode of measurements

The batch mode of operation with the biosensor device was performed in a glass vessel in a 0.1 M PB pH 8 buffer containing 2 mM of NAD^+ and 2 mM of ferricyanide. A typical measurement was done in an initial volume of 10 mL by addition of 10 μL of 55 mM D-sorbitol stock solution and the solution was mixed using a stirrer (250 rpm).

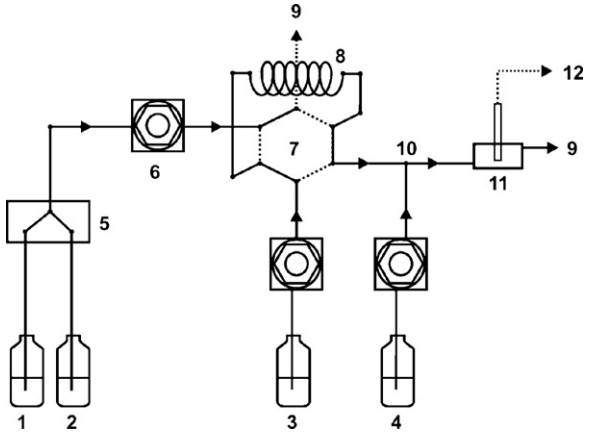

Fig. 1. A typical set-up of a flow-through biosensor system used. (1) Sample reservoir, (2) D-sorbitol standard, (3) 0.1 M PB pH 8 + 4 mM NAD^+, (4) 0.1 M PB pH 8 + 4 mM ferricyanide, (5) switch valve, (6) injection valve, (7) peristaltic pump, (8) sample loop, (9) waste, (10) T-shaped connector, (11) biosensor integrated in the FIA cell and (12) connection to PC.

2.5. High-performance liquid chromatography (HPLC)

D-Sorbitol and L-sorbose were determined by a Preparative Chromatography System WATERS Delta Prep 3000 (Milford, USA) using a Polymer IEX Pb^{2+} column (300 mm × 8 mm, Watrex, Prague, Czech Republic) and elution with DW (1 mL min^{-1} at 80 °C) was monitored by an RI detector (Philips PU 4026, Eindhoven, The Netherlands). Samples/standards were injected onto the column *via* the 100 μL loop with retention times of 22.4 min (D-sorbitol) and 9.6 min (L-sorbose), respectively.

2.6. Gas chromatography (GC)

Trifluoroacetic derivatives of samples [23] were analyzed by a Hewlett-Packard Model 5890 A chromatograph (Mount Holly, USA) using flame ionization detection and separated by a DB-1 column (60 m × 0.25 mm; Fison, UK). The following thermal cycle was used: 180 °C for 2 min, followed by an increase in temperature (6 °C min^{-1}) to 250 °C with retention time of 12.7 min for D-sorbitol. 5 μL of sucrose (300 g L^{-1}) was added to all samples as an internal standard.

2.7. Optical rotation (OR)

Optical rotation of L-sorbose measured using a Perkin-Elmer polarimeter Model 141 (Boston, USA) at 20 °C in a 0.5 mL cell was validated by a set of standards.

2.8. G. oxydans cultivation

G. oxydans BIODIOL 621 (Czech Collection of Microorganisms, Brno, Czech Republic) was maintained on an agar medium containing (g L^{-1}): D-sorbitol 100; yeast extract 10; calcium carbonate 20; agar 20; and transferred monthly. The cell biomass was prepared by aerobic cultivation at 30 °C on a rotary shaker (Shel Lab, Cornelius, USA) at 250 rpm in 250 mL Erlenmeyer flasks filled with 50 mL of medium. The growth medium contained (g L^{-1}): D-sorbitol 50; yeast extract 10; succinic acid 3; with the pH adjusted to 5.5–5.8 by 1 M HCl. Cells of *G. oxydans* grown in a growth medium for 24 h (A_{600} = 1.2, wet biomass of 8 g L^{-1} after centrifugation at 10,000 rpm for 3 min) were used for inoculation of the bioconversion medium.

2.9. L-Sorbose conversion reaction

Bioconversion of D-sorbitol into L-sorbose was done in the medium containing ($g\,L^{-1}$): D-sorbitol 70; sodium pyruvate 0.55; K_2HPO_4 0.13 g; NaH_2PO_4 0.35 and the pH was adjusted to 5.5–5.8 with 1 M HCl. 250 mL flasks containing 50 mL of sterilized medium were inoculated with 5% of seed culture broth. The conversion was performed at 30 °C on a rotary shaker at 200 rpm and samples (1.2 mL) for analytical determination were aseptically withdrawn every six hours during the biotransformation (48 h).

3. Results and discussion

Based on our previous work with dehydrogenase built biosensor systems a co-immobilisation of DP together with SDH to achieve a high sensitivity of measurements was chosen for this work [17,24]. The combination of dehydrogenase and diaphorase is a popular choice for sensitive amperometric detection of various substrates [25–27]. The concentration of both ferricyanide and NAD^+ in the running buffer was 2 mM what was optimal for efficient performance of the biosensor (data not shown). Two different modes of the assays by the biosensor were implemented e.g. batch and FIA mode and basic characteristics were evaluated.

3.1. Characteristics of a biosensor device in a batch mode

The biosensor response towards D-sorbitol was linear in the range 0.006–0.55 mM with a sensitivity of $950 \pm 20\,nA\,mM^{-1}$. A detection limit of the device in the batch mode was 3 μM, a calculation based on S/N = 3, comparable to previously published values (2 μM or 40 μM) obtained in a batch system [21,22]. Response time of the biosensor (a time needed to attain 95% of steady-state response) was 65 s, comparable to published values of 60 s [21] or 200–300 s [22].

3.2. Characteristics of a biosensor device in a FIA mode

Fig. 2 shows a calibration curve for D-sorbitol with a narrow linear range up to 1.1 mM and the slope of $72 \pm 2\,nA\,mM^{-1}$, while a dynamic range of the biosensor is much wider up to 18 mM, what is typical for dehydrogenase based biosensor systems [28]. Detection limit of the biosensor working in a FIA mode was 20 μM and a typical response time of 340 s translates into a throughput rate of ~10 h^{-1}, a value sufficient for a comfortable monitoring of substrate consumption during biotransformation processes [15–17]. Higher throughput can be achieved by an increased flow rate and/or by a smaller sample loop used for injection, but this can come on the expense of precision and sensitivity of the measurement. Alternatively an improvement of the sample throughput by integration of a flow cell with a smaller cell volume can be performed.

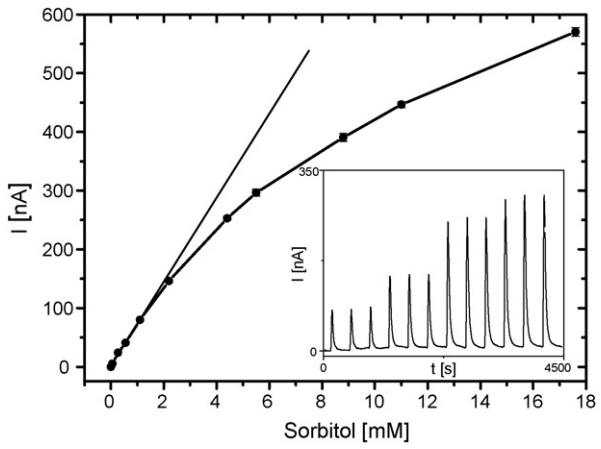

Fig. 2. A calibration curve of the biosensor with a linear range up to 1.1 mM and dynamic range up to 18 mM is shown. There is a real sensorgram for various D-sorbitol concentrations (1.1, 2.2, 4.4 and 5.5 mM) shown in the inset of the figure. Measurements were done under the flow rate of 0.38 mL min^{-1} with a sample loop of 20 μL.

Kinetic constants were calculated using two models (Lineweaver–Burke and Eadie–Hofstee model) with the former one providing more reliable results e.g. I_{max} of (917 ± 16) nA and K_m^{app} of (11.6 ± 0.2) mM. K_m^{app} is only slightly higher than values reported for the enzyme in the solution ranging from 1.1 to 9.8 mM [29–31].

Operational stability of the biosensor was investigated by repeated injections of 7 mM D-sorbitol over 46 h period of time with exponential decrease ($I = -190 + 947\,e^{(-t/33.8)}$, $R^2 = 0.998$) of the ini-

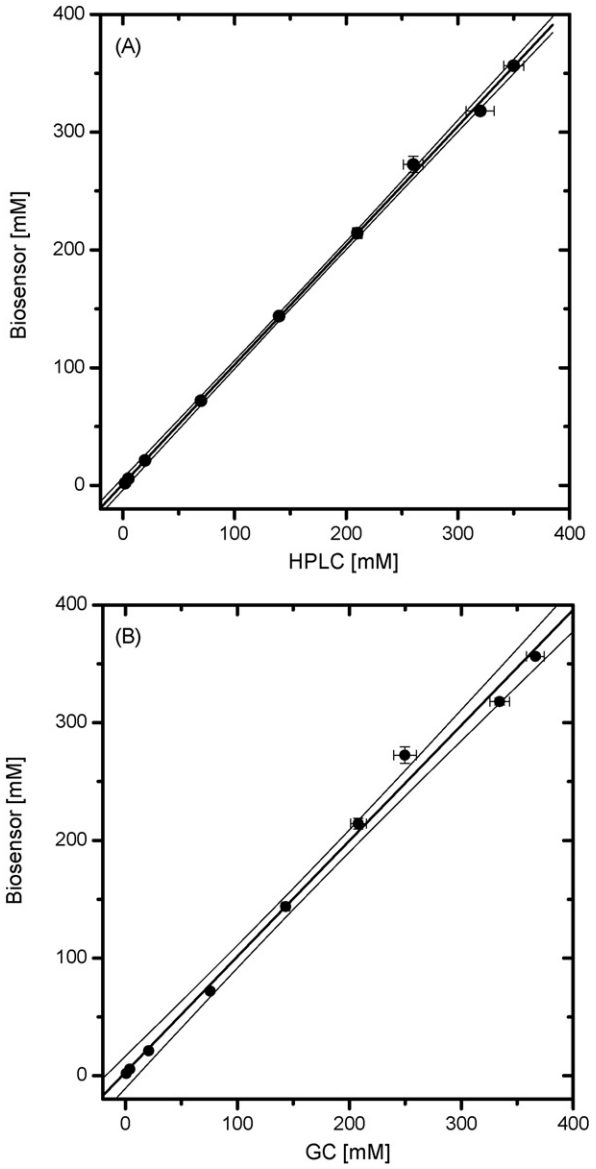

Fig. 3. D-Sorbitol concentration during biotransformation process as determined by three different analytical methods. (A) Correlation between biosensor and HPLC assays with the intercept of 0.4 ± 0.2 and the slope of 1.01 ± 0.01, $R^2 = 0.9998$ and (B) correlation between biosensor and GC assays with the intercept of 1.5 ± 0.3 and the slope of 0.97 ± 0.01, $R^2 = 0.9994$. Error bars shown for all three analytical methods are derived from determination of each sample at least in triplicate. Confidence intervals (95%) for both linear fits are included, as well.

tial current output in 46 h to 8%. This insufficient stability of the biosensor cannot be used for robust on-line monitoring of the bioconversion process. Thus, an off-line FIA system was used instead. Improvement of rather poor operational stability using polyelectrolytes [24] and hydrogel-like matrix of biopolymers (e.g. chitosan) [32,33] is under investigation.

3.3. Off-line monitoring of bioconversion

Samples were diluted (200× in the initial phase) *prior* use to a concentration falling into the linear range of the biosensor performance and measured with alternate injections of samples and standards, at least in triplicate. The concentration of D-sorbitol in the sample was calculated by linear interpolation of responses to D-sorbitol standards before and after sample injection. The D-sorbitol biosensor exhibited a very low average relative standard deviation (RSD) of 2.2% (RSD varied from 0.5% to 3.8%), when the RSD of 14.8% for the measurement of the last sample (2 mM) was not considered.

Biosensor provided reliable data as suggested from comparison with two reference analytical methods (HPLC and GC) (Fig. 3). Direct comparison biosensor *vs.* HPLC showed the slope of 1.01 (Fig. 3A), while biosensor *vs.* GC revealed the slope of 0.97 (Fig. 3B), both values close to the theoretical value of 1.00. Thus, the sorbitol biosensor performance was not affected by the presence of high amount of L-sorbose e.g. molar ratio of 60 or 180 in the last two samples.

The time needed for analysis of D-sorbitol by the biosensor was approximately 5.5 min, while 22 min was needed for HPLC measurement and 13 min required for GC analysis. The biosensor device has a clear advantage–a short time of analysis and possibility to be implemented in an on-line monitoring system. The initial D-sorbitol concentration in the medium before inoculation was 384 mM and the final concentration of L-sorbose produced was 348 mM (on average as determined by HPLC and OR analysis, data not shown), what gives an overall efficiency of the bioconversion of 91%. The bioconversion efficiency is really remarkable taking into account duration of the bioconversion of 48 h, when only 9% of the substrate was lost for bacterial growth and maintenance of physiological functions.

4. Conclusions

A ferricyanide mediated amperometric biosensor based on co-immobilisation of D-sorbitol dehydrogenase and diaphorase was used in a FIA mode for D-sorbitol detection with limit of detection of 20 μM with an average RSD of 2.2%. The FIA allowed sample throughput of 10 h^{-1}, what is sufficient for comfortable monitoring of D-sorbitol during its bioconversion into L-sorbose by *G. oxydans*. Operational stability was quite poor and only an off-line bioprocess monitoring was possible. Direct comparison of results obtained by the biosensor device to the results obtained by standard analytical techniques (HPLC and GC) is in an excellent agreement, suggesting selectivity of D-sorbitol detection in the presence of excessive L-sorbose concentration.

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