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Monitoring of ethanol during fermentation using a microbial biosensor with enhanced selectivity

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Abstract

The present study is concerning the construction of ferricyanide-mediated *Gluconobacter oxydans* cell ethanol biosensor. The size exclusion effect of a cellulose acetate membrane was used for elimination of glucose interferences during ethanol assays in real samples. A typical response time of the biosensor was 13 s with a high sensitivity of 3.5 μA mM^{-1}. The microbial biosensor exhibits a very low detection limit of 0.85 μM and a wide linear range from 2 to 270 μM. The operational stability was excellent. During 8.5 h of repetitive ethanol assays, no decrease in the sensor sensitivity was observed. The biosensor was successfully used in the off-line monitoring of ethanol fermentation with a good agreement with HPLC measurements ($R^2 = 0.998$). © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Amperometric biosensor; *Gluconobacter oxydans*; Ethanol fermentation; Cellulose acetate membrane

1. Introduction

The determination of ethanol is very important for the bioprocess control in fermentation industry. Biosensors are very promising for this purpose, providing necessary informations that allows feedback control. For the construction of ethanol biosensors mainly two enzymes are used. Alcohol oxidase is an expensive and unstable enzyme that is able to oxidise also methanol with oxygen as a co-substrate. NAD-dependent alcohol dehydrogenase needs soluble cofactor (NAD) for active site regeneration and the enzyme kinetics has an unfavourable equilibrium.

The application of PQQ-dependent dehydrogenases (PQQ = pyrroloquinoline quinone) is exploited to overcome the disadvantages related to the use of oxidases and NAD-dependent dehydrogenases [1]. These enzymes contain a cofactor (PQQ) that is tightly but not covalently bound to the holoenzyme molecule. Moreover, the oxidation of ethanol by PQQ-dependent alcohol dehydrogenase is irreversible [2] and the enzyme is unable to oxidise methanol [3]. The main drawback of PQQ-dependent dehydrogenases is the low specific activity and stability after enzyme purification.

Microbial biosensors have several advantages over enzyme biosensors: the enzyme does not need to be isolated; the enzymes are usually more stable in their natural cell environment; and the coenzymes and activators are already present in the system [4]. On the other hand, microbial biosensors are less specific compared to the enzyme biosensors.

Recently, a method for the analysis of samples containing both ethanol and glucose was developed. This system consists of the non-specific *Gluconobacter oxydans* sensor, which detects both glucose and ethanol, and the selective glucose sensor which detects only glucose. The concentration of ethanol was subtracted from these two signals [5].

A simplified procedure for determination of ethanol selectively in the presence of glucose by mediated *G. oxydans* biosensor using the size exclusion effect of a cellulose acetate membrane is presented here.

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2. Experimental

2.1. Cultivation of G. oxydans

The strain of *G. oxydans* CCM 1783 (ATCC 621) was cultivated using glycerol as a carbon source according to a procedure described in our study [4]. The cell biomass was prepared by aerobic cultivation at 28 °C. Then, the cells were collected by centrifugation after reaching the late exponential phase and washed twice with 0.9% sodium chloride solution containing 2 mM $CaCl_2$. The biomass concentration was expressed as the weight matter determined by drying to a constant weight at 105 °C.

2.2. Apparatus

Biosensor measurements were carried out on Amperometric Detector ADLC2 (Laboratorní Přístroje, Prague, Czech Republic) using a glassy carbon electrode (GCE) as a working electrode ($d = 6$ mm) and saturated calomel electrode (SCE) as a reference electrode.

2.3. Procedures

A cellulose acetate membrane was prepared by dissolving 1 g of cellulose acetate (approximately 40% of acetyl, Sigma, St. Louis, USA) in a mixture of 55 ml of acetone and 45 ml of cyclohexanone [6].

The glassy carbon electrode was covered with a suspension of *G. oxydans* (0.01 mg_{DW}) in McIlvaine buffer pH 6.0 containing 2 mM $CaCl_2$ together with a ferricyanide solution (10 nmol). After water evaporation, 25 μl of 1% cellulose acetate solution was spread on the surface of the modified glassy carbon electrode. The sensor was left at ambient temperature until solvents evaporation.

Ethanol was assayed by HPLC as a reference method (Waters, Milford, USA) with a column packed by Ostion LGKS 0800 in H^+ cycle (TESSEK, Prague, Czech Republic) with differential refractometric detection using 1.25 mM H_2SO_4 as a mobile phase.

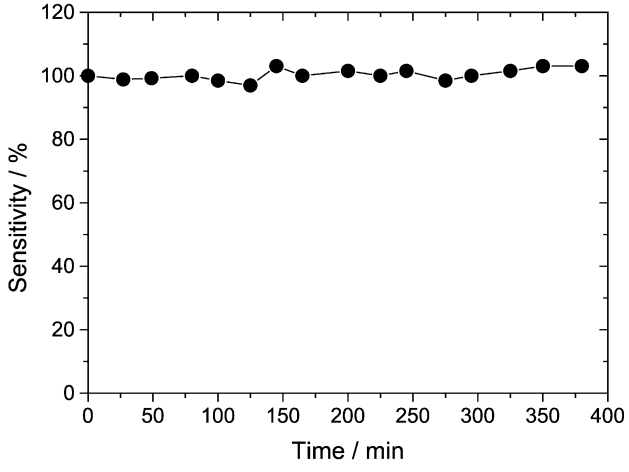

Fig. 1. Operational stability of the *G. oxydans* cell-based ethanol biosensor. Measurement was carried out at 28 °C at a working potential of 300 mV in McIlvine buffer pH 6.0 containing 10 mM of ferricyanide and 2 mM $CaCl_2$.

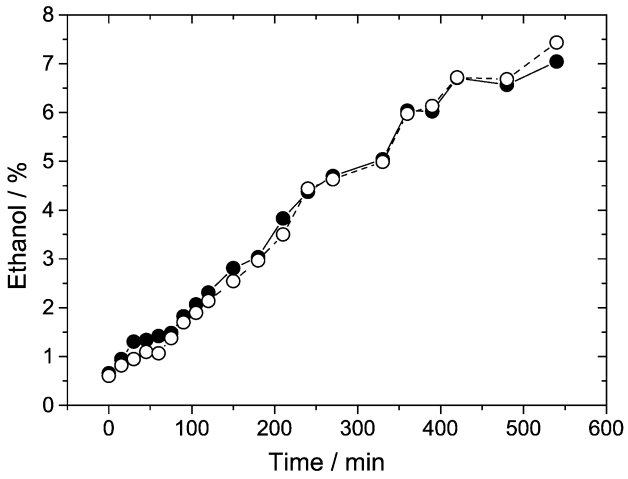

Fig. 2. The concentration profile of ethanol during the alcoholic fermentation with immobilised *S. cerevisiae*. Biosensor assay (●) and reference HPLC assay (○).

2.4. Ethanol fermentation

The distillery strain of *Saccharomyces cerevisiae* GY2 (Collection of Microorganisms of the Department of Biochemical Technology, Faculty of Chemical and Food Technology, Bratislava, Slovak republic) was propagated and immobilised in alginate beads according to the literature [7]. Batch ethanol fermentation was carried out at 30 °C and the process was monitored for 9 h.

3. Results and discussion

3.1. Optimization of measurement conditions

It is well known that *G. oxydans* oxidises ethanol very effectively. To enhance the sensitivity of detection, ferricyanide was chosen as a mediator [8]. Furthermore, the optimization of several parameters affecting sensor response was done. The effect of a working potential on the sensor sensitivity was carried out from 20 to 340 mV with an optimum at 300 mV. The sensor performance was not affected by pH in the range studied (5.0–7.0). Further, McIlvaine buffer pH 6.0 with 2 mM $CaCl_2$ was chosen because this value was optimal for the biosensor with cells immobilised behind a dialysis membrane.

3.2. The effect of the cellulose acetate membrane on substrates diffusion

It was confirmed that even a cellulose acetate membrane spread on a *G. oxydans* layer hampers the diffusion of

glucose through this membrane, whereas ethanol diffusion was possible. The ethanol/glucose sensitivity ratio increased from 9.3 (a dialysis membrane application) to 541.1 when a cellulose acetate membrane was used. The cellulose acetate membrane also had an effect on glycerol diffusion when the ethanol/glycerol sensitivity ratio increased from 183 to 1367 under the same conditions.

3.3. The basic biosensor parameters

The sensor exhibits excellent operational stability when the signal was stable during 8.5 h of continual operation (Fig. 1). The sensitivity of the sensor was very high (3.5 μA mM^{-1}) resulted in a very low detection limit (0.85 μM, S/N = 3) and wide linear range (2–270 μM). The response time (13 s, 90% of the steady state) was comparable or better than in the case of enzyme biosensors. However, the sensitivity of the biosensor towards propanol and butanol was at the same order of magnitude compared to ethanol, the biosensor performance was not disturbed by the presence of methanol.

3.4. Analysis of real samples

The microbial biosensor with enhanced selectivity was successfully applied in an off-line monitoring of the batch fermentation with *S. cerevisiae*, immobilised in alginate beads (Fig. 2), even in the presence of 200 g/l of glucose in the initial stage. The results obtained by the biosensor were in very good agreement with the HPLC measurements ($R^2 = 0.998$).

Acknowledgements

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