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Ethanol *Gluconobacter* biosensor designed for flow injection analysis Application in ethanol fermentation off-line monitoring

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ABSTRACT

A microbial amperometric biosensor for the measurement of ethanol in flow injection analysis (FIA) system was constructed. The system used bacteria *Gluconobacter oxydans* attached on the surface of combined (glassy carbon vs. Ag/AgCl) electrode mounted into the flow cell. The parallel colorimetric glucose determination was used to eliminate non-specific glucose response of microbial biosensor to samples from monitored ethanol fermentation. The current response of microbial biosensor to samples containing both ethanol and glucose consisted of two current portions (ethanol and glucose) whereas the ethanol portion was not influenced by various glucose concentrations. The value of ethanol portion was calculated as the difference of total current signal of microbial biosensor and the portion of signal belonging to glucose obtained from parallel glucose colorimetric determination and glucose calibration curve of microbial biosensor. The response of microbial biosensor to ethanol was linear in the range from 10 μM to 1.5 mM, the response time of the biosensor was up to 3 min. No decrease of response to ethanol was observed during 72 h of continuous measurement with microbial biosensor. Described biosensor FIA system was used for off-line monitoring of ethanol production during alcoholic fermentation. Obtained ethanol concentrations were in a good correlation with a gas chromatography (GC) reference method.

1. Introduction

Ethanol assay is very important and necessary in numerous industries and biotechnological processes such as production of wine, beer, foodstuffs, cosmetics and pharmaceutical products. Chemical methods, for example redox titrations, colorimetric techniques, and refractive index measurements, chromatographic and spectroscopic assays have been conventionally used for ethanol detection. Enzyme-catalyzed reactions followed by spectroscopic or electrochemical detection are offered as alternatives for simple, specific and practical ethanol detection. Various biosensing systems based on NAD^+ dependent alcohol dehydrogenase (NAD-ADH) and alcohol oxidase (AOX) have also been developed as alternatives to the classical analytical methods for ethanol analysis [1,2].

Whole cells are used for biosensor preparation because of the enzymes does not need to be isolated, they are usually more stable in their natural environment in the cell, and coenzymes and activators are already present in the system [3,4]. The main disadvantage of microbial biosensors is their lower selectivity that might be overcome using different approaches as a coupling of enzymes with microbial cells to form hybrid sensors, or the inhibition or suppression of undesired transport mechanisms and/or metabolic pathways [5], genetic engineering [6] and selective membranes [7]. The bacterial genera *Gluconobacter* have been frequently used for preparation of microbial biosensors [8,9] because of high efficiency of wide variety substrates oxidation due to content of many periplasmatic membrane-bound dehydrogenases. These enzymes contain a cofactor pyrroloquinoline quinone (PQQ). One member of the PQQ enzymes family founding in *Gluconobacter* is PQQ-dependent alcohol dehydrogenase (PQQ-ADH) having better selectivity to ethanol than NAD-ADH or AOX [10]. However, PQQ-ADH is not commercially available enzyme in purified form, and purified PQQ enzymes have a low stability [11]. The alternative way for use of this enzyme for biosensor preparation with satisfactory stability is use of whole cells *Gluconobacter oxydans*. *Gluconobacter* contains as well as PQQ-dependent glucose dehydrogenase (PQQ-GDH) [11] so this approach requires overcoming of ethanol and glucose cross selectivity during measurement of samples containing both ethanol and glucose. For this purpose we have used parallel colorimetric glucose determination and subsequent subtraction of glucose increment from the biosensor response.

Amperometric biosensors operate at fixed potential with respect to a reference electrode and involve the detection of the current

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generated by redox processes joined with transport of electrons at the surface of the electrode. Natural/physiological electron acceptors of PQQ enzymes are cytochrome *c*, cytochrome *b* or ubiquinone. However, there are also various synthetic electron acceptors (mediators) exploitable to oxidize reduced enzymes [12]. Moreover, PQQ-ADH is able to transfer electrons directly to solid surfaces [13] or to conducting polymers [14]. Another choice is application of the water soluble mediator ferricyanide in combination with biosensors based on NAD^+ and PQQ dependent enzymes (free or involved in cells). This approach is frequent because of (i) solubility of the mediator and possibility of use in buffer, (ii) decrease of working potential of biosensor to about of +300 mV vs. reference electrode, (iii) reasonable price and availability, and (iv) non-toxicity.

We have chosen the mediator ferricyanide for electron transfer to/from whole cells *G. oxydans* containing membrane-bounded PQQ-ADH for the construction of the flow biosensing system as well. The aim of this work was to develop microbial ethanol biosensor designed for use in flow injection analysis (FIA) system enabling off-line monitoring of ethanol fermentation with wider linear range in comparison with previous published microbial ethanol biosensors.

2. Experimental

2.1. Chemicals and materials

G. oxydans CCM 1783 was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic), yeast extract and agar were from Oxoid Ltd. (Basingstoke, UK). Dialysis membrane Servapor® 44144 was supplied by Serva (Heidelberg, Germany). Potassium ferricyanide was supplied by Centralchem (Bratislava, Slovakia). All other chemicals used were provided by Mikrochem (Pezinok, Slovakia).

2.2. G. oxydans cultivation and biosensor preparation

Details of the cultivation conditions and the isolation procedure of strain *G. oxydans* CCM 1783 were reported previously [15].

A tailor-made combined cylindrical glassy carbon (GC) electrode (diameter 3 mm) with an integrated Ag/AgCl (0.1 M KCl) reference electrode (diameter 2 mm) embedded in a Teflon casing with the outer diameter of 9 mm (Elektrochemicke detektory, Turnov, Czech Republic) was used for biosensor preparation (Fig. 1). 5 μL of *G. oxydans* suspension (5 g_{DW}/l) were dropped on the GC surface of the combined electrode. After drying of suspension at 25 °C using an air stream the electrode surface was covered with a dialysis membrane presoaked in water. The membrane was fixed tightly with a silicone rubber O-ring. The biosensor was integrated into a flow-through system.

2.3. Amperometric measurement and flow injection analysis (FIA) set-up

All amperometric measurements were performed in a two electrode system connected to a potentiostat PST-3 (FEI STU, Bratislava, Slovakia) with applied potential of +300 mV vs. Ag/AgCl (0.1 M KCl). The FIA system (Fig. 2) consisted of peristaltic pumps 403U/L2 (Watson–Marlow, Wilmington, USA), an injection, and a selection valves (Rheodyne, Rohnert Park, USA). The first peristaltic pump maintained constant almost pulse-free flow of the buffer (McIlvaine buffer, pH 6.0, 0.1 M KCl, 2 mM $CaCl_2$, and 2 mM ferricyanide) through the system at flow rate of 0.5 ml/min. Samples were injected into a buffer stream by the second pump through a computer controlled injection valve with the injection loop of 12 μl. The biosensor was mounted into a flow cell (dead volume approximately 150 μl) of the flow injection system. The whole FIA configuration was supplied by FIAlab Instruments Inc. (Bellevue, USA). The operation of the FIA system was controlled and data were collected by PC using control software designed in LabVIEW (National Instruments, Austin, USA).

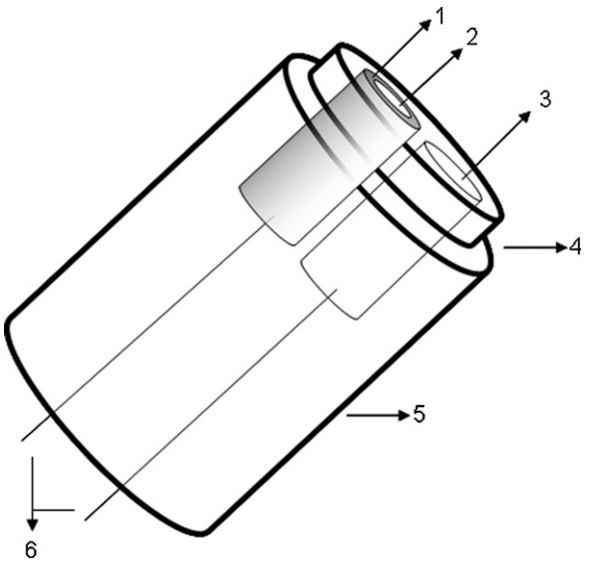

Fig. 1. The illustration of prepared microbial biosensor based on the combined electrode system: (1) working glassy carbon electrode, (2) active thin layer of *G. oxydans* cells, (3) reference Ag/AgCl electrode, (4) groove shape for sealing ring, (5) teflon body, (6) electrical contacts. In addition, the active surface of combined electrode was covered by dialysis membrane.

2.4. Analysis of real samples

Real samples were taken from continual ethanol fermentation process using yeasts *Saccharomyces cerevisiae* C11–3 immobilized in calcium pectate gel beads. All conditions of the fermentation process were reported previously [16]. The samples were collected from fermenter in regular intervals, after centrifugation diluted in the buffer used as mobile phase to achieve a maximum ethanol concentration of 1.0 mM, and then injected into the FIA system. Ethanol standard with concentration of 0.61 mM was used to determine the ethanol contents in measured samples, glucose standard of 0.67 mM was used to determine sensitivity of the biosensor to glucose. Both ethanol and glucose standard concentrations were chosen to be approximately in the middle of measured ethanol and glucose concentration ranges. The order of injections was sample – ethanol standard – glucose standard.

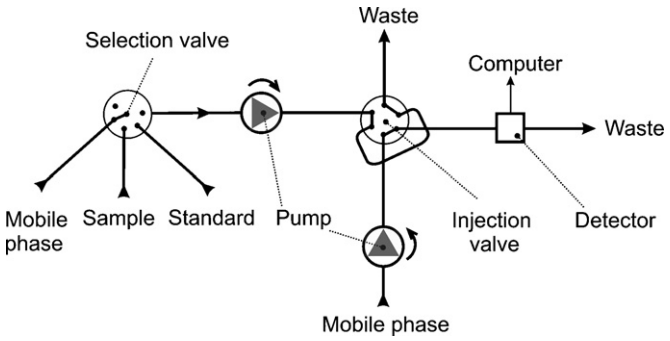

Fig. 2. The scheme of particular components set-up used in experimental FIA arrangement. The flow cell with the microbial biosensor was applied in the role of detector.