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Whole-cell *Gluconobacter oxydans* biosensor for 2-phenylethanol biooxidation monitoring

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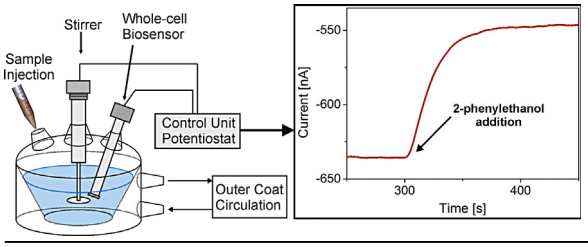
HIGHLIGHTS

- The first described biosensor for 2-phenylethanol measurement.
- Simple construction based on a Clark electrode and immobilized *Gluconobacter oxydans* cells.
- Fast, reliable and environmentally friendly system for monitoring of biotech processes.

GRAPHICAL ABSTRACT



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ABSTRACT

A microbial biosensor for 2-phenylethanol (2-PE) based on the bacteria *Gluconobacter oxydans* was developed and applied in monitoring of a biotechnological process. The cells of *G. oxydans* were immobilized within a disposable polyelectrolyte complex gel membrane consisting of sodium alginate, cellulose sulphate and poly(methylene-co-guanidine) attached onto a miniaturized Clark oxygen electrode, forming whole cell amperometric biosensor. Measured changes in oxygen concentration were proportional to changes in 2-PE concentration. The biosensor sensitivity was 864 nA mM^{-1} (RSD = 6%), a detection limit of 1 μM, and the biosensor response towards 2-PE was linear in the range 0.02–0.70 mM. The biosensor preserved 93% of its initial sensitivity after 7 h of continuous operation and exhibited excellent storage stability with loss of only 6% of initial sensitivity within two months, when stored at 4 °C. The developed system was designed and successfully used for an off-line monitoring of whole course of 2-PE biooxidation process producing phenylacetic acid (PA) as industrially valuable aromatic compound. The biosensor measurement did not require the use of hazardous organic solvent. The biosensor response to 2-PE was not affected by interferences from PA and phenylacetaldehyde at concentrations present in real samples during the biotransformation and the results were in a very good agreement with those obtained *via* gas chromatography.

1. Introduction

White biotechnology, defined as industrial biotechnology using microorganisms and enzymes to produce chemical products, belongs to a sustainable green chemistry allowing production of many chemicals from renewable feedstock and minimizing the use and generation of hazardous chemicals. Biotechnological production of aromatic compounds allows defining and labeling them as “natural” (Regulation (EC) No. 1334/2008 of the European Parliament and of the Council of 16 December 2008). Use of such products as additives in food, beverages or perfumery instead of “non-natural” can bring not

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only environmental benefit but also for example marketing advantage. These products are usually sophisticated, commercially interesting commodities with a high added value. For example, 1 kg of natural phenylacetic acid (PA) is selling for almost 2000 euro (Sigma–Aldrich). PA, having a honey-like odor at low concentrations, is used as a flavor in cosmetics and food industry. It is also a by-product during enzymatic production of 6-aminopenicillanic acid. The industrial production of PA is based on hydrolysis of benzyl cyanide using strong acids which both are toxic and hazardous compounds. The alternative process is bioproduction of PA starting with transformation of amino acid L-phenylalanine *via* Ehrlich pathway of yeasts to 2-phenylethanol (2-PE) [1], followed by biooxidation of 2-PE to PA using whole cells of acetic acid bacteria [2]. In the biooxidation step two enzymes, alcohol dehydrogenase and aldehyde dehydrogenase, are involved. These enzymes are both membrane-bound containing subunits, therefore having limited stability when isolated [3] and their isolation is expensive, as well. For these reasons, use of whole cells of *Acetobacter* sp. or *Gluconobacter oxydans* is preferred.

The periplasmic location of PQQ-dependent enzymes or periplasmic orientation of active sites of membrane-bound enzymes in bacteria [4] enables their easy accessibility for the substrates, what leads to their wide use not only in bioprocesses, but also in biosensors and biofuel cells. The membrane-bound enzymes are able to oxidize a wide number of biotechnologically important compounds efficiently resulting in a rapid response of biosensors based on them. The benefits of microbial biosensors are numerous and their application potential in the fields as biotechnology, environmental monitoring and clinical medicine is broad [5–8]. The main advantage is that enzymes do not have to be isolated and purified. They are comfort and stable in their natural environment, where all cofactors and activators are present. The main problem to be solved is low selectivity of cell based biosensors [9]. *G. oxydans* as biorecognition element is frequently used in electrochemical biosensors for bioanalysis of *e.g.*, triacylglycerols, disaccharides, aldoses, ketoses, mono- and poly-alcohols [10,11]. *G. oxydans* based sensors ("gluconosensors") were used also in a flow injection analysis (FIA) system for analysis of beverages [12] and to monitor real biotechnological processes [9,13]. The amperometric transducer of "gluconosensors" can be based on either oxygen measurement using Clark electrode or use of electrochemical mediators [14].

In this work we focused on development of a whole-cell *G. oxydans* biosensor for 2-PE biooxidation monitoring. The progress of 2-PE biotransformation is usually controlled *via* gas chromatography (GC), since, it is generally simpler to use and has a higher efficiency compared to high performance liquid chromatography (HPLC). On the other hand, sample processing by extraction into hazardous organic solvent is needed. Further, in case of PA analysis *via* GC, esterification must be made for compound volatilization. These pretreatments may distort actual concentration values. Our aim was to develop a simple whole-cell biosensor without the need of any sample pretreatment applicable for a rapid monitoring of the biotechnological process. We decided to apply previously described approach used for Baeyer–Villiger biooxidation monitoring, considering good analytical characteristics, reproducibility and stability of the biosensor based on the miniaturized oxygen electrode with bacterial cells immobilized within disposable polyelectrolyte complex gel membrane [15]. The immobilization matrix in the form of polyelectrolyte complex capsules made of sodium alginate, cellulose sulphate and poly(methylene-co-guanidine) was originally designed to encapsulate pancreatic cells aiming to cure diabetes [16] and was proved to be biocompatible for preserving bacterial cell viability and stability [17]. To our best knowledge, this is the first described biosensor for 2-PE monitoring.

2. Material and methods

2.1. Chemicals and material

2-PE (≥99.0%), used as substrate for the biotransformation as well as the standard for GC and biosensor analyses, PA (≥99%) and phenylacetaldehyde (PAA ≥ 90%, Kosher) were purchased from Sigma–Aldrich. Dichloromethane (Suprasolv® for GC, Merck, Germany) was used as an organic solvent. Methylbenzoate (≥99.5%, Fluka, Germany) was used as an internal standard in GC analysis. Agar medium consisted of ($g\,L^{-1}$): glucose 20; yeast extract 10; agar 15. Propagation medium consisted of ($g\,L^{-1}$): glycerol 25; yeast extract 10. Buffer consisting of ($g\,L^{-1}$): K_2HPO_4 0.125; NaH_2PO_4 0.35, pH 6.8 was used as a biotransformation medium and medium for biosensor measurements. High viscosity sodium alginate from ISP Alginates (Girvan, Ayrshire, UK) and cellulose sulphate, sodium salt, from Acros Organics (NJ, USA) were used as received. Poly(methylene-co-guanidine) hydrochloride from Scientific Polymer Products Inc. (Ontario, NY, USA) supplied as 35% (w/v) aqueous solution was lyophilized prior to use.

2.2. Cultivation of G. oxydans

G. oxydans NCIMB 8035 (NCIMB, Aberdeen, Scotland, UK) was stored on an agar Petri dish in refrigerator at 4 °C. To prepare vegetative inoculum, a colony was inoculated into 20 mL of propagation medium and let grown 24 h at 28 °C, 150 rpm. Inoculum was pipetted into required volume of propagation medium (5% v/v) and let grown 24 h at 28 °C, 150 rpm. Biomass was centrifuged at 4 °C with 4000 rpm for 10 min and used for the biotransformation experiment or the biosensor preparation.

2.3. Biotransformation of 2-PE

80 mg of wet biomass (15% dry cell weight) was suspended in 100 mL of the biotransformation medium within 250 mL Erlenmeyer flask. Reaction was started by addition of 2-PE to final concentration of 2.9 mM and it was carried out at 28 °C, 150 rpm. Samples taken from the biotransformation medium were deprived of biomass by centrifugation and filtration through AZ Filters PTFE, 13 mm, 0.2 μm (AZ Chrom, Slovakia).

2.4. Biosensor construction

The biosensor was prepared analogically to a previously reported amperometric biosensor [15] using the same commercial miniaturized oxygen electrode, potentiostat, reaction chamber and software Bioanalyzer from BVT Technologies (Brno, Czech Republic). Wet biomass of *G. oxydans* (10% w/v) was suspended in a polyanion solution prepared from sodium alginate (0.9% w/v) and cellulose sulphate (0.9% w/v) in NaCl (0.9% w/v). Disk membranes were prepared by pipetting 5 μL of this mixture on a Petri dish where an 8 min gelling process in a polycation solution took place. Polycation was made of poly(methylene-co-guanidine) (1.8% w/v) and $CaCl_2$ (1% w/v) in NaCl (0.9% w/v). Final treatment with 50 mM citrate in NaCl solution (0.9% w/v) took 5 min. Membranes were stored in a buffer solution at 4 °C. Before measurement, one membrane was attached to the surface of oxygen electrode, fastened by elastic porous polyamide membrane and an O-ring.

2.5. Biosensor measurement

Measurements were performed in 10 mL of buffer solution within the stirred reaction chamber at 25 °C and 950 rpm. To obtain a calibration curve, 5 × 10 μL and then 50 μL aliquots of 2-PE standard solution (20 mM) were added until electrode signal

reached ~0 nA (*i.e.*, oxygen was completely depleted from the bioreceptive layer of the biosensor). Measurement of real samples from the biotransformation was performed by addition of 100 μL of the sample without further pretreatment into 10 mL of buffer solution using the method of standard addition.

2.6. Gas chromatography

Samples from biotransformation were extracted by dichloromethane containing methylbenzoate (0.5 g L^{-1}) as an internal standard. Organic layer was analyzed *via* Agilent Technologies 7890A GC System with H_2 as a carrier gas (flow of 22 mL min^{-1}). Injector operated at 250 °C in a split mode with split ratio 5:1 and split flow of 16 mL min^{-1} injecting 1 μL of the sample. Optima® delta-6-0.25 μm column (30 m, 0.25 mm ID) was used with an oven program set as follows: 130 °C for 2 min, then 20 °C min^{-1} to 210 °C. FID detector operated at 300 °C.

3. Results and discussion

3.1. Biosensor preparation and characteristics

The performance of whole-cell biosensors is strongly dependent on many factors such as pH, temperature and the amount of immobilized cells. *G. oxydans* uses for the oxidation of 2-PE, the same enzyme (alcohol dehydrogenase) as for the oxidation of ethanol [2]. In the case of the whole-cell biosensor with *G. oxydans*, the response to ethanol is not significantly affected by pH of phosphate buffer up to pH 7.0 [18,19]. The pH profile of prepared *G. oxydans* biosensor for 2-PE determination showed similar characteristics, and the biosensor response was affected by pH in the measured pH range pH 5.3–7.8 only moderately with the maximum sensitivity at pH 6.3 (Fig. S1 in Supplement material). The temperature has considerable influence on *G. oxydans* biosensor response with rapid decrease at temperature above 50 °C due to thermal destruction of the microorganism [20]. In the case of prepared *G. oxydans* biosensor for 2-PE, the sensitivity increased up to 40 °C. At temperature of 45 °C, the decrease of sensitivity was observed with a subsequent fall to zero at 50 °C (Fig. S2 in Supplement material). Therefore, all further experiments were performed at pH 6.8 ensuring sufficient capacity of phosphate buffer and at 25 °C to avoid both the prolongation of the initial biosensor signal stabilization and the use of energy consuming additional heating. Three different concentrations, 5, 10 and 20% (w/v) of wet biomass within the biosensor membrane were tested to obtain sensitivity and linear range of the calibration curve of the biosensors based on them. The biosensor with membrane containing 5% (w/v) of wet biomass had the lowest sensitivity (307 nA mM^{-1}, RSD = 6%) and with 20% (w/v) had the lowest effective current range (500 nA), resulting in a short linear range of the calibration curve (0.02–0.5 mM). Thus, all further experiments were carried out using the biosensor with membrane containing 10% (w/v) of wet biomass suspended in a polyanion solution. This membrane composition ensured both sufficient sensitivity and effective current range (Fig. S3 in Supplement material). The analytical characteristics of the developed biosensor were as follows: sensitivity of 864 nA mM^{-1} (RSD = 6%), detection limit of 1 μM, noise < 1 nA and a linear range of the calibration curve 0.02–0.7 mM for 2-PE (Fig. 1). A response time of the biosensor was about 100 s. This is the first described biosensor for 2-PE determination. The method based on genetically engineered whole cells of *Pseudomonas* sp. [21] for detection of styrene, responding also to 2-PE and other compounds based on an increased induction of β-galactosidase in presence of toxic compounds *via ex situ* measurements, is completely different compared to a direct approach of 2-PE analysis presented here. Moreover, since no relevant analytical characteristics were therein reported [21], the comparison with biosensor described in present work is not possible. The other amperometric whole-cell *G. oxydans* biosensors with their analytical characteristics and application are summarized in Table 1. The parameters of the biosensor described here are comparable with other *G. oxydans* whole-cell amperometric biosensors utilizing endogenous alcohol dehydrogenase activity.

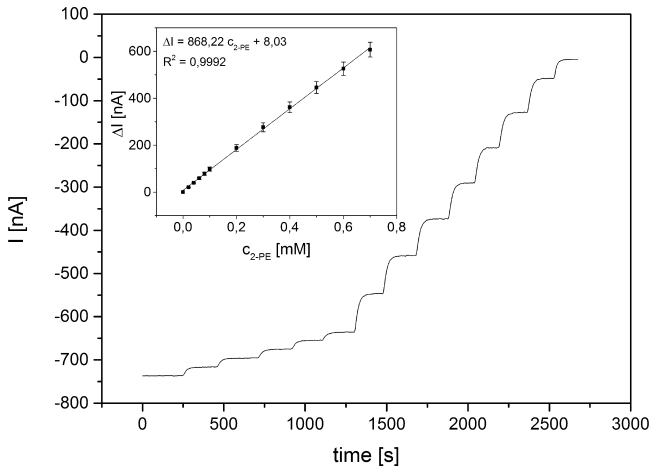

Fig. 1. A response of whole-cell *Gluconobacter oxydans* biosensor to 2-PE. The calibration curve with the intercept of 8.0 nA, a slope of 868 nA mM^{-1} and $R^2 = 0.9992$ is in the inset graph.

3.2. Biosensor stability

Operational stability of the prepared biosensor was tested. Set of aliquots of 2-PE standard solution were added once in an hour into the reaction chamber, the calibration curve was obtained and the sensitivity calculated. After each set of measurements (once per hour) the reaction chamber was washed 3 times with distilled water and new buffer was added to permit stabilization of the biosensor before another measurement. The decrease of initial sensitivity of the biosensor was only about 7% within 7.2 h of continuous operation (Fig. S4 in Supplement material) and the sensitivity of the biosensor reached about 92% of its initial sensitivity after subsequent overnight running in the stirred buffer

Table 1
Comparison of *Gluconobacter oxydans* whole-cell amperometric biosensors utilizing endogenous alcohol dehydrogenase activity.

Analyte	Concentration range (mM)	Detection limit (μM)	Response time (min)	Operational stability (h)	Use of mediator	Reference
Ethanol	0.01–1.5	3.3	3	72	Yes	[13]
Ethanol	0.05–10	50	nd	12	No	[20]
Ethanol	0.01–1	5	1	43	Yes	[22]
Ethanol	0.002–0.27	0.85	0.2	8.5	Yes	[23]
1,3-Propanediol	0.003–15	2	4	140	Yes	[9]
2-PE	0.02–0.70	1	1.7	7.2	No	This work

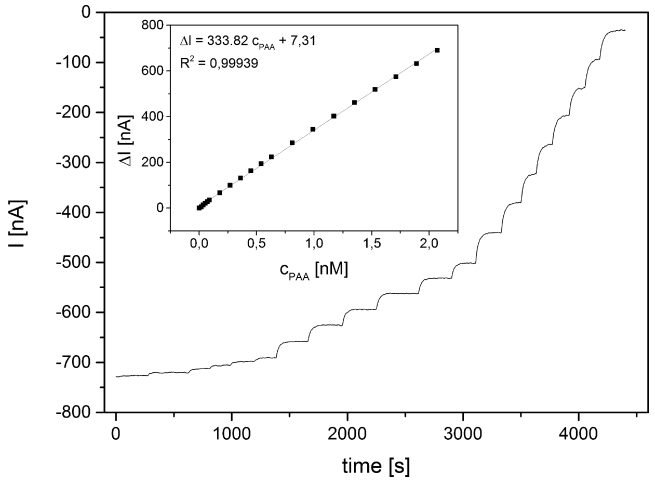

Fig. 2. A response of whole-cell *Gluconobacter oxydans* biosensor to PAA. A calibration curve with the intercept of 7.3 nA, a slope of 334 nA mM^{-1} and R^2 = 0.99939 is in the inset graph.

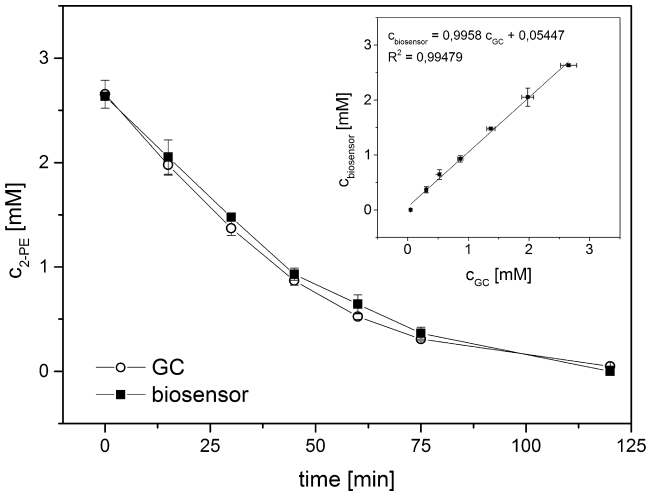

Fig. 3. Real sample analyses. Concentration of 2-PE in real samples from biotransformation were measured both *via* whole-cell *Gluconobacter oxydans* biosensor and GC in triplicates. Correlation between biosensor and GC assays with R^2 = 0.99479 is in the inset graph.

solution. Since the biotransformation of 2-PE was terminated after 2 h, the developed biosensor could be used for on-line monitoring as well.

Storage stability of the biosensor was tested as well. Prepared membranes were stored in a Petri dish filled with buffer at 4 °C. Before the measurement, one membrane was attached onto the oxygen electrode and the sensitivity of the biosensor was measured. After the measurement, used membrane was discarded. The storage stability was very good, maintaining 94% of the initial biosensor sensitivity after 57 days and still 55% after 127 days of storage. Increasing of the sensitivity during initial phase of storage (days 11, 13 and 22) was observed (Fig. S5 in Supplement material). This behavior was already reported in case of the biosensor with glucose dehydrogenase immobilized in polycarbamoyl sulfonate hydrogel and was explained by wetting and probably reorganizing of the hydrogel [24].

3.3. Real samples measurement

PAA and PA were tested as the only possible interferences in measured real samples, since, any other components which can either act as *G. oxydans* substrates (sugars, alcohols) or influence the biosensor response by other means are not present in biotransformation media nor in a measurement buffer. The interferences were tested by addition of 100 μL of the sample into 10 mL of a buffer solution. There was no biosensor response to addition of sample with concentration of PA of 18 mM, what is 6 times higher concentration than the initial concentration of the 2-PE during the biotransformation. However, the prepared biosensor was sensitive to PAA. The biosensor calibration curve for PAA provided a sensitivity of 334 nA mM^{-1}, while the signal noise was 0.2 nA with the detection limit of 1.8 μM of PAA (Fig. 2). According to GC analysis, PAA was found only at the beginning of the biotransformation at maximal concentration of 0.04 mM. While adding 100 μL of the sample containing 0.04 mM PAA to 10 mL of reaction buffer, PAA concentration within reaction chamber is 0.4 μM, what is lower concentration than the detection limit of the biosensor for PAA determination (1.8 μM). Since PAA as an intermediate of 2-PE oxidation to PA during the oxidative metabolism of acetic acid bacteria at normal condition is not accumulated, the use of developed biosensor for monitoring of this biotransformation is not limited by interference.

The biotransformation started with 2-PE concentration of about 3 mM. Higher initial concentrations of 2-PE might be toxic for the *G. oxydans* used for biotransformation and can limit use of whole cells for continuous or repeated conversions using immobilized cells. The samples taken during biotransformation were deprived from the biomass and then measured without further treatment. Amount of 100 μL of the sample was used for each biosensor measurement. The samples were analyzed in triplicates, each time with a different membrane. RSD for these triplicate sets of measurements were in the range of 1–24% thus showing reproducibility of the biosensor preparation and measurement. Biosensor measurements were correlated with GC as reference analytical method and very good agreement was achieved (Fig. 3).

4. Conclusions

Whole-cell bacterial amperometric biosensor based on *G. oxydans* immobilized in polyelectrolyte membrane onto a miniaturized oxygen electrode was prepared and successfully used for an off-line analysis of real samples from 2-PE biooxidation. This is the first reported biosensor for 2-PE determination and for monitoring of a process of PA production from 2-PE. The developed biosensor exhibited sufficient sensitivity and a fast response. The main advantages of presented biosensor in comparison with other *G. oxydans* based amperometric biosensors utilizing alcohol dehydrogenase are simplicity of the system (no mediator needed) and low detection limit. The prepared biosensor was sensitive also to PAA. However, considering the composition of real samples, the concentration of PAA does not exceed the detection limit and the monitoring of the biotransformation was not influenced by presence of PAA and PA. The reported biosensor excellently correlated the reference GC analysis and its operational stability was significantly longer than the biotransformation reaction itself. Moreover, the biosensor measurement does not need unlike GC analysis, the sample processing step by extraction into the organic solvent. The developed biosensor is a fast, cheap and environmentally friendly alternative for the 2-PE biooxidation process monitoring, after adaptation even in an on-line configuration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.11.012.

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