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ORIGINAL RESEARCH PAPER

Analysis of ethanol in fermentation samples by a robust nanocomposite-based microbial biosensor

Jana Šefčovičová · Jaroslav Filip · Vladimír Mastihuba · Peter Gemeiner · Jan Tkac

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Abstract A robust microbial biosensor was constructed from a bionanocomposite prepared by a direct mixing of bacterial cells of *Gluconobacter oxydans* and carbon nanotubes with ferricyanide employed as a mediator for enhanced sensitivity of ethanol oxidation. A successful integration of the device into flow injection analysis mode of operation provided a high sensitivity of detection of (74 ± 2.7) $\mu A\ mM^{-1}\ cm^{-2}$, a low detection limit of 5 μM and a linear range from 10 μM up to 1 mM. A short response time of the biosensor allowed a sample throughput of 67 h^{-1} at 0.3 ml min^{-1}. The biosensor exhibited high operational stability with a decrease in the biosensor response of 1.7% during 43 h of continuous operation. The device was used to analyse ethanol in fermentation samples with a good agreement with a HPLC method.

Keywords Carbon nanotubes · Ethanol · *Gluconobacter oxydans* · Microbial biosensor

J. Šefčovičová · J. Filip · V. Mastihuba · P. Gemeiner · J. Tkac (✉)
Department of Glycobiotechnology, Center for Glycomics, Institute of Chemistry, Slovak Academy of Sciences, Dúbravská Cesta 9, 845 38 Bratislava, SK, Slovakia
e-mail: Jan.Tkac@savba.sk

Introduction

Whole cells are promising biorecognition elements for preparation of biocomponent-based functional devices compared to enzyme-based ones because of advantages such as higher stability of the bioelements, presence of coenzymes/activators and a possibility to utilise a cascade of few enzymes with an overall positive economic benefit of their use (Lei et al. 2006; Svitel et al. 2006; Su et al. 2011).

A low selectivity of analyte detection and sluggish response time of microbial biosensors for single analyte detection were overcome using various sophisticated protocols (Riedel 1991; Kuroda and Ueda 2011). Another direction of whole cell use is construction of biosensors to read biological O_2 demand value, presence of inhibitors, toxic compounds, utilisable organic matter and for generation of electric energy in the biofuel cells (Moon et al. 2004; Lei et al. 2006; Lovley 2006; Logan 2008; Tkac et al. 2009; Su et al. 2011; Juang et al. 2011). There is still a main limitation for practical application of microbial biosensors such as a low sensitivity of oxidation of a particular analyte limiting their overall performance. Some microbial species including *Gluconobacter* sp. have enzymes localised either in the periplasmatic space or in the cytoplasmatic membrane with the active sites exposed to the periplasm. Thus, enzymatic machinery can be easily reached by a soluble redox shuttle with quick charge transfer kinetics comparable to the one of isolated enzymes (Ikeda and Kano 2001;

Svitel et al. 2006; Tkac et al. 2009). An increase in the sensitivity of the enzyme biosensor devices has been achieved by preparation of a 3D matrix with an enhanced loading of the biocatalyst (Cooney et al. 2008; Xiong et al. 2010) and this approach is an interesting tool how to further increase sensitivity of the microbial biosensors.

Recently, we have described an efficient enhancement of the sensitivity of the microbial biosensor by construction of a novel 3D carbon nanotube (CNT) composite matrix with *Gluconobacter oxydans* integrated within. The biosensor device developed offered the highest sensitivity to ethanol among microbial biosensors published so far (Šefcovicová et al. 2011). The biosensor was extensively optimised and its basic electrochemical properties were characterised in the batch analytical system. Here, the 3D bionanocomposite has been integrated into a flow injection analysis (FIA) system for preparation of the biosensor device allowing quick assay format with basic characteristics of the biosensor revealed. Moreover, the biosensor was successfully applied to analysis of ethanol in fermentation samples.

Materials and methods

Chemicals and material

Single walled CNT (outer diam. = 1.1 nm, L = 0.5–100 μm, >90% purity), chitosan (degree of deacetylation of 85%), potassium ferricyanide were purchased from Sigma. Buffer components (e.g. 100 mM citrate buffer pH 6.5 containing 2 mM $CaCl_2$) and all other chemicals were of analytical grade. *G. oxydans* CCM 1783 was obtained from Czech collection of microorganisms (Brno, Czech Republic), *Saccharomyces cerevisiae* 28–23 was provided from collection of yeasts (Institute of Chemistry, Bratislava, Slovakia), yeast extract and agar were supplied from Oxoid Ltd. (Basingstoke, UK).

Gluconobacter oxydans cultivation

Gluconobacter oxydans was grown at 28°C on a rotary shaker in 500 ml flasks with 100 ml medium (Tkac et al. 2003). The medium contained (g l^{-1}): glycerol 5, yeast extract 5. Cells were harvested in their late growth phase, 5 ml medium was centrifuged (5 min, 8000×g) and the cells washed with cold, sterile 0.9% NaCl + 2 mM $CaCl_2$.

Saccharomyces cerevisiae cultivation

The yeast was grown in 100 ml medium [(g l^{-1}): D-glucose 30, yeast extract 5 and peptone 10] on a rotary shaker at 28°C overnight. Fermentation was carried out by inoculating 5 into 100 ml medium containing (g l^{-1}): D-glucose 200, yeast extract 5, peptone 10, $(NH_4)_2SO_4$ 10, KH_2PO_4 10, $MgSO_4 \cdot 7H_2O$ 10, and $CaCl_2$ 0.2. Batch ethanol fermentation was carried out at 28°C under anaerobic conditions. Samples were taken at late growth phase to be analysed by the biosensor and by HPLC.

Instrumentation and analytical methods

The FIA system (FIAlab Instruments Inc., Bellevue, USA) consisting of peristaltic pumps, sampling/injection valves and a flow cell (7 μl), was run with an injection loop of 20 μl at 0.3 ml min^{-1}. An utility designed in Labview (National Instruments, Austin, USA) was used for control and data acquisition (Sefcovicova et al. 2009).

For electrochemical measurements a potentiostat Autolab PGSTAT 128 N (Ecochemie, Utrecht, Netherland) was used with glassy carbon electrode (GCE, d = 1 mm) as a working one and an Ag/AgCl reference electrode (both provided by Cypress system). GCE were polished to a mirror-lustre on Microcloth (Buehler, Lake Bluff, IL, USA) with an aqueous alumina suspension (0.3 μm, Struers, Copenhagen, Denmark), rinsed with distilled water and sonicated for 30 s to remove adsorbed particles from an alumina suspension. All measurements were carried out at an applied potential of +300 mV versus Ag/AgCl electrode.

HPLC measurements were performed with RID detection at 35°C and a 20 μl injection loop. Glucose and ethanol were assayed on Watrex Polymer IEX H^+ column (300 × 8 mm) eluted with 9 mM H_2SO_4 (1 ml min^{-1}) at 30°C. Samples were diluted 50× in eluent and filtered through 0.22 μm filter prior injection. Typical elution times of glucose and ethanol were ~6 and ~14 min, respectively.

Biosensor preparation

Dispersion of CNTs was prepared at 5 g l^{-1} in distilled water using an ultrasonication bath (Bandelin

DT 102 H, Bandelin electronics, Berlin, Germany) at 25°C for 15 min. Then, 80 µl suspension of *G. oxydans* (5 g_{DW} l^{-1}) was mixed with 40 µl CNT dispersion and sonicated for a total time of 5 min (repeating steps consisted of 30 s of sonication and 30 s of mixing). Then, 5 µl of this mixture was deposited on the GCE surface and allowed to dry at an ambient temperature. Next, 5 µl of 0.1% chitosan in 0.3% acetic acid was deposited on the modified GCE electrode and left to dry prior further investigation. In order to stabilise the outer chitosan layer, 2.5 µl 5% (w/v) glutardialdehyde was applied for 5 min as the final step to crosslink highly abundant $–NH_2$ groups in the chitosan layer to prevent chitosan dissolution. All measurements were performed in 100 mM citrate buffer pH 6.5 containing 2 mM $CaCl_2$ and 1.5 mM ferricyanide.

Results and discussion

Substrate specificity

Investigation of the substrate specificity of *G. oxydans* biosensor revealed ethanol is the best substrate followed by propanol; 1,3-propanediol; glucose and xylose (Fig. 1). In our recent study, we suggested 1,3-propanediol is oxidised by PQQ-alcohol dehydrogenase (Katrlík et al. 2007). This means that ethanol, propanol and 1,3-propanediol are all oxidised by alcohol dehydrogenase. On the other hand, glucose and xylose are oxidised by PQQ-dependent glucose (aldose) dehydrogenase (Tkac et al. 2003; Reshetilov et al. 2001). Thus, under the cultivation conditions used for growth of the bacterium (e.g. glycerol used as a sole carbon source), mainly alcohol dehydrogenase within the cells is expressed. The advantage of using microbial cells is the tailoring of their specificity by a choice of the carbon substrate used for growth (Lee et al. 2002; Tkac et al. 2003; Voronova et al. 2008). Thus, *G. oxydans* biosensor can be prepared in advance with enzymatic activities adjusted for oxidation of a particular analyte by properly selected cultivation conditions.

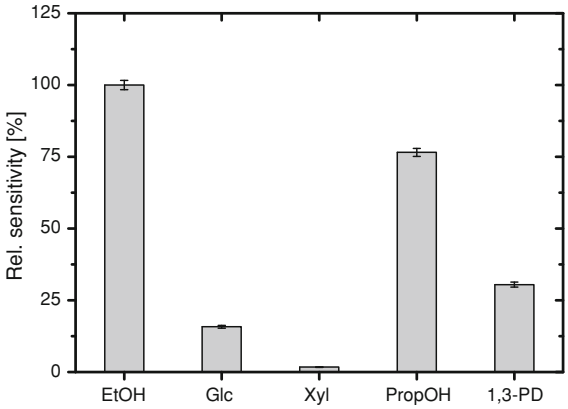

Fig. 1 Substrate specificity of the microbial biosensor using ethanol (EtOH), glucose (Glc), xylose (Xyl), propanol (PropOH) and 1,3-propanediol (1,3-PD) as the substrate. Measurements were performed in 10 ml of 100 mM citrate buffer pH 6.5 containing 2 mM $CaCl_2$ and 1.5 mM ferricyanide

Biosensor characterisation

The biosensor performance was tested in a FIA mode of operation what is suitable for highly automated and high-throughput assay formats (Ruzicka and Hansen 2008). In this case, with an increased flow rate, the current output decreased exponentially while the throughput of assay had its maximal value of 82 h^{-1}, with a reasonably high current density output at 0.5 ml min^{-1} with 0.75 mM ethanol as substrate. Finally a flow rate of 0.3 ml min^{-1} was chosen as a compromise between the sensitivity (e.g. 74 ± 2.7 µA mM^{-1} cm^{-2}) of the biosensor and its sample throughput (67 h^{-1}) (Fig. 2a). When a whole calibration curve was fitted a maximal current density of 212 ± 29.6 µA cm^{-2} was observed. An apparent Michaelis–Menten constant K_m^{app} was 2.2 ± 0.5 mM (Fig. 2b), comparable to a previously published value of 1.24 mM in a batch mode of operation (Šefcovicová et al. 2011). The biosensor exhibited linear range from 0.01 up to 1 mM with a detection limit calculated as 5 µM (S/N = 3).

Operational stability was initially investigated with outer chitosan layer without any further treatment and a monotonic increase of the biosensor response during continuous operation was observed with the final relative response of 135% after 21 h (Fig. 3). This could be ascribed to the dissolution of the outer chitosan layer with lowering a permeability resistance towards the substrate with a permanent increase of the response. In order to investigate real operational stability of the microbial biosensor, glutardialdehyde treatment of chitosan layer was performed. Such modification of the biosensor resulted in very high

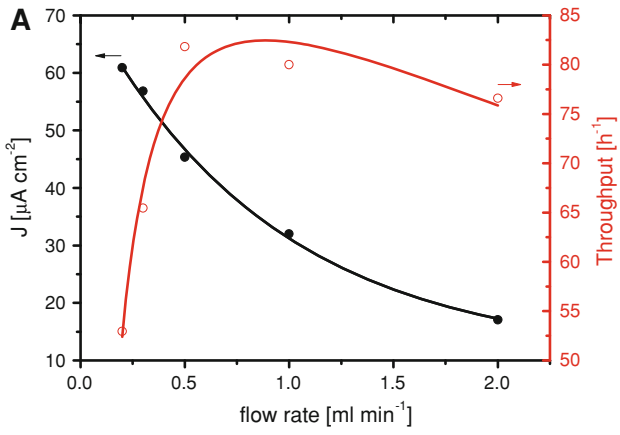

Fig. 2 **a** Optimisation of the flow rate on the biosensor response and sample throughput, when inserted in a FIA system. **b** A calibration curve in the FIA mode of operation at 0.3 ml min^{-1} used. A *dashed line* determines a linear range of the biosensor, while in the figure *inset* a real sensorgram is shown

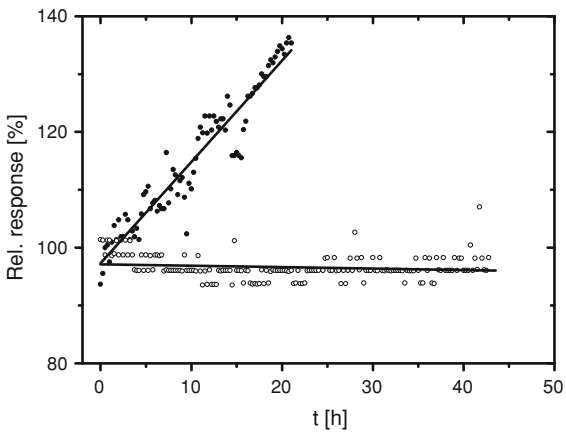

Fig. 3 An operational stability of the microbial biosensor tested in the FIA mode of operation with (*white circle*) or without (*black circle*) glutardialdehyde (GDA) used as a chitosan crosslinking agent

operational stability of the device with 98.3% of the initial biosensor sensitivity observed after 43 h of continuous operation (Fig. 3).

Overall robustness of the microbial biosensor performance for detection of ethanol was checked by comparing to basic characteristics of the best ethanol biosensors published to date working in the FIA mode (Table 1). This extensive comparison proves the microbial biosensor presented here outperforms ethanol biosensors based on microbial cells or various enzymes. Since the main aim of the work was to show beneficial effect of CNTs on the biosensor performance, it is worth comparing this microbial biosensor to other CNTs-based biosensors. In a recent review dealing with integration of CNTs with an electrochemical platform of detection for construction of sensors and biosensors, detection limits and sensitivity of such devices were summarised (Vashist et al. 2011). This survey showed detection limit for enzyme biosensors is mainly in the low micromolar or submicromolar concentrations comparable to the value in the present study. Moreover, the sensitivity of detection by CNT-enzymatic biosensors is only in occasional case exceeding the one provided by the *G. oxydans* based microbial biosensor working in FIA mode (Vashist et al. 2011). The characteristics of the biosensors were acquired in a batch system which is more sensitive compared to FIA e.g. 162 versus 74 μA mM^{-1} cm^{-2} for current microbial biosensor (Šefcovicová et al. 2011). In the lower part of Table 1, characteristics of other microbial biosensors with CNTs-modified interfaces published to date are summarised. The main analyte investigated by these biosensors was glucose with a sensitivity of detection from 0.1 to 2.1 μA mM^{-1} cm^{-2}. In order to compare published data with current approach the sensitivity of the present microbial biosensor for glucose was calculated as 12 μA mM^{-1} cm^{-2} (calculation done based on data in Table 1) for FIA mode of operation or as 26 μA mM^{-1} cm^{-2} for the batch mode of operation. This means the sensitivity of the present CNT-based microbial biosensor is at least one order of magnitude more sensitive than previously published approaches. Moreover, the operational stability of the biosensor is much better in comparison to previously developed microbial biosensors (Table 1).

Table 1 Comparison of the *G. oxydans* based microbial biosensor to other EtOH biosensors or microbial CNTs-based microbial biosensors

Biocomponents	Sensitivity ($\mu A\ mM^{-1}\ cm^{-2}$)	DL (μM)	RT or throughput	Operational stability	Reference
ADH	14	nd	30 s	90% after 24 h	Dominguez et al. (1993)
ADH	7	nd	8 h^{-1}	50% after 24 h	Lobo et al. (1996)
ADH[a]	nd	100	50–240 s	80% after 48 h	Svensson et al. (2005)
PQQ-ADH	38	nd	<5 min	85% after 9 days	Hikuma et al. (1995)
PQQ-ADH	10	nd	2–3 min	55% after 50 h[b]	Razumiene et al. (2001)
PQQ-ADH	179	1	30 h^{-1}	85% after 5 h	Razumiene et al. (2002)
AOD and HRP	27	15	30 h^{-1}	87% after 8 h	Vijayakumar et al. (1996)
AOD	4	2.3	30 h^{-1}	95% after 6 h	Carelli et al. (2006)
AOD[c]	17	9.9	69 s	86% after 18 h	Hämmerle et al. (2011)
Gluconobacter oxydans	5	3.3	3 min	0.32% h^{-1}[d]	Valach et al. (2009)
Gluconobacter oxydans	≈15	nd	nd	50% after 51 h	Vostiar et al. (2004)
Gluconobacter oxydans and CNTs	74	5	67 h^{-1}	98% after 43 h	This work
Pseudomonas fluorescens and CNTs[e]	0.1 for Glc	nd	nd	89% after 4 h	Demirkol and Timur (2011)
Gluconobacter oxydans and CNTs[e]	1.2 for Glc	nd	40 s	nd	Odaci et al. (2009)
P. fluorescens or *P. putida* and CNTs[e]	1.3 for Man	nd	nd	89% after 4 h	Odaci et al. (2008)
Pseudomonas putida and CNTs[e]	0.1 for Glc	nd	35 s	80% after 6.5 h	Timur et al. (2007)
Pseudomonas fluorescens and CNTs[e]	2.1 for Glc	nd	100 s	87% after 4 h	Kirgoz et al. (2007)

DL detection limit, *RT* response time, *ADH* NAD^+-dependent alcohol dehydrogenase, *PQQ-ADH* PQQ-dependent alcohol dehydrogenase from *G. oxydans*, *AOD* alcohol oxidase, *HRP* horseradish peroxidase, *Glc* glucose, *Man* mannose

[a] Commercially available from Chemel AB (Biotech IgG)

[b] Only slight decrease of the response after 133 h

[c] FIA until steady state current response achieved

[d] Increase of the response to about 120% of the initial response during 10 h, followed by a slow decrease of the response in time of 0.32% h^{-1}

[e] Batch mode of operation

Analysis of fermentation samples

Finally, the robust microbial biosensor was applied in analysis of fermentation samples taken from ethanol fermentation at late growth phase. Analysis of fermentation samples was highly reproducible with relative standard deviation for sample assay from 0.4 to 3.1% (average error of assay of 1.5%). Results suggest the biosensor offered analytical performance in a good agreement with HPLC assay with an average error of measurement of 2.5% even though glucose was still present at high concentration (90–290 mM) (Table 2). The microbial biosensor provided higher sensitivity of detection compared to HPLC, what can be judged from dilution ratio used for ethanol assays of 2000× or 50×, respectively. Since the biosensor was extremely stable, it was not necessary to recalibrate the biosensor during assays of fermentation samples. Thus, it was possible to analyse 12 samples within 1 h, while with HPLC it was possible to analyse three samples in 1 h.

Table 2 Analysis of fermentation samples by the *G. oxydans* biosensor (Bs) and by HPLC

Sample no.	Glc–HPLC (mM)	EtOH–HPLC (mM)	EtOH–Bs (mM)	(Bs–HPLC)/HPLC (%)
1	290	1,265	1,208	−4.49
2	240	1,353	1,352	−0.05
3	200	1,445	1,481	2.49
4	140	1,572	1,525	−3
5	90	1,710	1,669	−2.43

Glc glucose, *EtOH* ethanol

Conclusions

The work presented here addressed a practical outcome of the bionanotechnology, an emerging and interdisciplinary scientific field, by designing and subsequent utilisation of a robust microbial biosensor based on 3D bionanocomposite. The concept shown here can have a direct impact on construction of high-performance biofuel cells, as well. The beauty of using microbial cells is a possibility to tailor their substrate specificity by a choice of the carbon source used for cells growth to high extend or cells can tune activity towards a particular biofuel/analyte during operation of microbial fuel cell or a biosensor device. Integration of microbial cells with CNTs offers a robust EtOH biosensing outperforming many enzyme-based CNTs biosensor in a very cost-effective way and the concept can be easily applied for detection of other analytes by integration of other types of cells.

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