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Determination of total sugars in lignocellulose hydrolysate by a mediated *Gluconobacter oxydans* biosensor

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Abstract

A microbial biosensor for sugar determination was prepared by surface modification of a graphite electrode using *Gluconobacter oxydans* cells. The sensitivity of amperometric detection was enhanced by using hexacyanoferrate(III) as a mediator. The *G. oxydans* cells contain membrane-bound aldose dehydrogenase, which catalyses the oxidation of wide range of sugars including all sugars present in lignocellulose hydrolysate. The substrate specificity of the biosensor, effect of pH, temperature, working potential, hexacyanoferrate(III) concentration as well as the physiological state of the cells for detection were carefully optimised. The upper value of the linear range of the optimised biosensor was in the range 1.1–2.2 g l^{-1} for determination of D-glucose, D-galactose, D-xylose, D-mannose and L-arabinose. The biosensor was used for total sugars determination during lignocellulose hydrolysate fermentation. A good correlation between total sugars determined in samples by the biosensor and by quantitative paper chromatography was obtained.

Keywords: Biosensor; Amperometric; *Gluconobacter oxydans*; Lignocellulose hydrolysate; Sugar determination

1. Introduction

Lignocellulose hydrolysate is the waste from the pulp and paper industry, so the use of hydrolysate as a source of sugars for microbial fermentations improves the economy of the process. Lignocellulose is a rich mixture of carbohydrate polymers (cellulose and hemicellulose), lignin, proteins and others compounds [1]. Processing of lignocellulose includes delignification to liberate cellulose and hemicellulose from their complexes with lignin and subsequent depolymerisation of macromolecular sugars into free sugars. Sulphite lignocellulose hydrolysate contains a mixture of pentoses and hexoses that can be used as a carbon source for biotechnological production. Actually lignocellulose hydrolysate is used mainly for the production of fuel ethanol using yeasts [2] or recombinant bacterial strains [3] and also for yeast biomass production [4]. The use of lignocellulose hydrolysate as a substrate is hampered by the presence of inhibitors, such as sulphite, acetic acid and carbohydrate-derived inhibitors, e.g. in birch wood hydrolysate 29 aromatic monomeric compounds, especially aromatic aldehydes, representing 0.4% of dry weight [5], were deter-

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mined. The presence of carbohydrate-derived inhibitors requires the use of very dilute hydrolysates [6].

Biosensors have some advantages over other analytical techniques, e.g. low requirements for sample pre-treatment, easy operation, and low price. Amperometric biosensors are most frequently used for sugar determination. This is mostly due to the commercial availability of many redox enzymes, oxidases and dehydrogenases, which can easily be coupled to an amperometric transducer [7]. Electron transfer between enzyme and electrode is achieved by using an electrochemical mediator instead of oxygen as an electron acceptor. Hexacyanoferrate(III) is a frequently used mediator because of its water solubility and high efficiency of electron transfer.

Gluconobacter oxydans has been identified as a prospective biocatalyst for saccharide biosensors because of the membrane localisation of oxidative enzymes [8]. D-Glucose oxidation by *Gluconobacter industris* with hexacyanoferrate(III), *p*-benzoquinone or 2,6-dichlorophenol indophenol as mediator was proposed for biosensor construction [9]. A biosensor with the same microorganism immobilised onto a carbon paste electrode surface with *p*-benzoquinone as mediator was used for detection of D-glucose, glycerol, D-fructose and ethanol [10].

Microbial biosensors have several advantages over enzyme biosensors: the enzyme does not need to be isolated, enzymes are usually more stable in their natural environment in the cell, and coenzymes and activators are already present in the system [11]. Especially favourable is the use of microbial biosensors for analysis of complex samples, containing many substrates, such as wastewater, starch hydrolysate and lignocellulose hydrolysate. In spite of these advantages microbial biosensors have some disadvantages: in many cases they have a low selectivity and longer response time.

Gluconobacter oxydans cells contain several quinoprotein membrane-bound dehydrogenases specific for D-glucose, D-fructose, D-sorbitol and D-mannitol [12]. Furthermore, use of non-specific aldose [13] and alcohol [14] dehydrogenases for oxidising substrates are expected to be crucial for biosensor construction. Aldose dehydrogenase is a non-specific enzyme which is able to catalyse the oxidation of monosaccharides like D-glucose, D-galactose, D-xylose, D-mannose, D-ribose and L-arabinose and disaccharides such as lactose, maltose and cellobiose [15]. Alcohol dehydrogenase acts on linear and branched monoalcohols up to C_4 to give the corresponding acids and ketones [16]. These enzymes are pyrroloquinoline quinone (PQQ)-dependent with non-covalent but tight bonding of the cofactor to the apoenzyme molecule. Several compounds could be used as electron acceptors such as 2,6-dichlorophenol indophenol, phenazine methosulphate, hexacyanoferrate(III), coenzyme Q or cytochrome c, but oxygen and NAD^+ cannot transfer electrons from the active redox centre of the enzymes. *Gluconobacter* or *Acetobacter* biosensors for lactate [17], ethanol [10], glycerol [18], BOD [19], D-xylose [20], D-fructose [10], mixture of D-glucose and ethanol [21], sucrose (co-immobilisation of *S. cerevisiae*) [22] and lactose (co-immobilisation of *Kluyveromyces marxianus*) [22] with an oxygen electrode as electrochemical detector have previously been reported.

Numerous methods for sugar analysis in complex food samples, mainly based on liquid chromatography (LC), have been developed [23]. Lignocellulose hydrolysate is a complex system and any analysis needs complicated pre-treatment and fractionation. Only one reliable system for sugar determination in lignocellulose hydrolysate has been presented [24]: the LC separation of the sugars on a ligand exchange column in the Pb^{2+} form with a set up of four coupled pre-columns. The use of enzymatic methods is limited by the presence of inhibitors. Methods based on reducing group determination suffer from interferences from other reducing compounds present in the hydrolysate.

The aim of the present study is to show an approach to overcome the above mentioned problems in sugar analysis in the lignocellulose hydrolysate. *G. oxydans* was chosen for this study as an appropriate biocatalyst because it oxidises all sugars present in the hydrolysate and is sufficiently resistant to inhibitors present in the hydrolysate. The principal aim of this study was to propose a method for routine analysis during lignocellulose hydrolysate fermentation.

2. Experimental

2.1. Gluconobacter oxydans cell cultivation

The strain *Gluconobacter oxydans* CCM 1783 (=ATCC 621) was maintained on the slant agar

containing ($g\,l^{-1}$) D-glucose, 100; yeast extract (Imuna, Šarisske Michalany, Slovak Republic), 10; calcium carbonate, 20; agar, 20; and transferred monthly. The cell biomass was prepared by aerobic cultivation at 28°C on a rotary shaker in 500 ml flasks filled with 100 ml of media. The growth medium contained ($g\,l^{-1}$): carbon source, 5; yeast extract, 5. As a sugar source was used D-glucose, glycerol, D-mannitol, D-sorbitol, galactitol (dulcitol), D-ribose, D-gluconate, L-arabinitol, L-sorbose and D-fructose. These carbon sources were purchased either from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

The culture inoculated from the slant agar was incubated until reaching the late exponential phase. Then the cells were collected by centrifugation, resuspended in cold 0.9% sodium chloride solution and this procedure was repeated three times to assure cells suspension without being a fermentation broth. The biomass concentration was expressed as the dry weight matter of cells determined by drying to a constant weight at 105°C.

2.2. Determination of dehydrogenase activity

Enzyme activities in intact cells were measured spectrophotometrically using 2,6-dichlorophenol indophenol (DCPIP) (Merck) as the artificial acceptor and phenazine methosulphate (PMS) (Sigma) as the mediator, at 600 nm [25].

2.3. Electrode preparation

Electrodes were prepared from graphite rods (diameter 5 mm). A detailed description is given in a previous study [26]. The electrode was covered with cell suspension in physiological solution. Each electrode contained 0.85 mg dry weight of bacterial cells. The suspension was dried using an electrical fan and subsequently covered with a dialysis membrane (cut off 12,000, Sigma) and held by an O-ring.

2.4. Biosensor measurements

The measurements were carried out using an amperometric detector ADLC2 and data were collected using a linear recorder TZ 4620 (both made by Laboratorní Přístroje, Prague). A saturated calomel electrode (SCE) was used as a reference electrode. All experiments were done in a vessel thermostated at 30°C in 20 ml of 0.066 M phosphate buffer, pH 5.5, at a working potential of +300 mV, if not mentioned otherwise. Samples of lignocellulose hydrolysate were pre-treated as follows: 3 ml of hydrolysate was quickly dried under hot air (100°C) and 3 ml of distilled water was then added.

2.5. Paper chromatographic analysis

Paper chromatography was performed by the descending method on Whatman No. 1 paper (Maidstone, Kent, UK) using 8:2:1 (v:v:v) ethyl acetate–pyridine–water as the mobile phase. Standard sugar solutions or samples previously diluted in water were applied to the chromatographic paper. After developing for 20 h at 25°C, the dry chromatograms were treated with the standard alkaline silver nitrate detection [27] and finally with an aqueous solution of sodium thiosulphate for stabilisation. The respective relative mobilities of D-mannose, D-galactose, D-xylose, and L-arabinose referred to that of D-glucose (R_{Glc}=1.00) were 1.31, 0.76, 2.04, and 1.61. Finally, the dry chromatograms were scanned and using a Scaner Jet 4p (Hewlett-Packard Co., Greeley, CO) and a software ScanPacK 3.0 (Biometra Biomedizinische Analytik GmbH, Göttingen, Germany) upgraded for paper chromatography.

2.6. Lignocellulose hydrolysate fermentation

Fermentation was carried out using *Candida lambica* CCY 29-97-11 (Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava) on an fermenter CF 2000 with controlled pH and temperature (CHEMAP A.G., Basel, Switzerland). The acid hydrolysate was prepared from spruce wood by Mg-bisulphite technology in BIOCEL, a.s. (Paskov, Czech Republic), where microbial biomass from lignocellulose hydrolysate is produced on an industrial scale. Before fermentation, excess of sulphite was removed by stripping and the hydrolysate was condensed to a dry weight of 140 $g\,l^{-1}$. During fermentation microbial biomass concentration changed from 8 to 22 $g\,l^{-1}$.

3. Results and discussion

3.1. Effect of carbon source on enzymatic activities inside G. oxydans cells

The carbon source used for cell growth substantially influences the dehydrogenase activities in *G. oxydans* cells [28]. A carbon source is preferred that stimulates oxidative activities towards sugars present in lignocellulose hydrolysate, i.e. D-glucose, D-galactose, D-xylose, D-mannose and L-arabinose. For that purpose 10 different carbon sources, viz. D-glucose, glycerol, D-mannitol, D-sorbitol, galactitol, D-ribose, D-gluconate, L-arabinitol, L-sorbose, and D-fructose, were used for growth. In general, the highest biosensor responses to D-glucose were obtained with all carbon sources. The cells cultivated on L-arabitol exhibited the most favourable activities for L-arabinose, D-galactose and D-mannose oxidation (Fig. 1) and were used later for biosensor preparation. Ratios between biosensor responses presented in Fig. 1 are very similar to that published for purified aldose dehydrogenase, measured by DCIP-PMS assay, except for L-arabinose [25]. The *G. oxydans* cells also exhibited high oxidative activities towards ethanol and propanol (Fig. 1) that may result in interference in real sample measurement. Further, we have investigated the oxidative activity of cells during growth using DCPIP-PMS assay. The maximum specific oxidative activity of 2.9×10^{-5} mol s^{-1} $g_{DW}{}^{-1}$ towards D-glucose was observed during exponential growth approximately at the 12th hour. Thus, the use of L-arabitol as a carbon source and a 12 h cultivation period was adopted as a procedure for cell preparation for further biosensor optimisation.

3.2. Biosensor performance and parameter optimisation

The biosensor response to D-glucose was used for further biosensor optimisation. The influence of pH was examined in 0.066 M phosphate buffer in the pH range 5.0–7.5 and a sharp optimum at pH 5.5 was found (Fig. 2). This value is exactly the same as the optimum for D-glucose oxidation by free cells and similar to the pH optimum obtained in a previous investigation (pH 6.0) on oxygen probe-based microbial biosensor [22].

The effect of temperature was investigated in the range 20–40°C (Fig. 3). The biosensor response significantly decreases above 30°C. Similar behaviour above 30°C was reported for D-fructose dehydrogenase purified from *Gluconobacter* sp. [29]. For further work 30°C was used.

The effect of mediator concentration was investigated with the aim of obtaining maximum biosensor sensitivity. The maximum biosensor response was found at 3 mM hexacyanoferrate(III) (Fig. 4) and this concentration was used further.

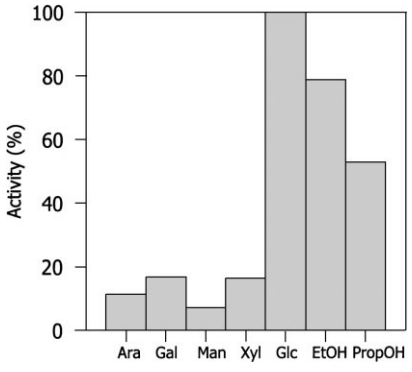

Fig. 1. Response of *Gluconobacter oxydans* microbial biosensor to various substrates. The cells were cultivated on L-arabitol as a sole carbon source at 28°C. Legend: 100% is the response to D-glucose. Ara: arabinose; Gal: galactose; Man: mannose; Xyl: xylose; Glc: glucose; EtOH: ethanol; PropOH: propanol.

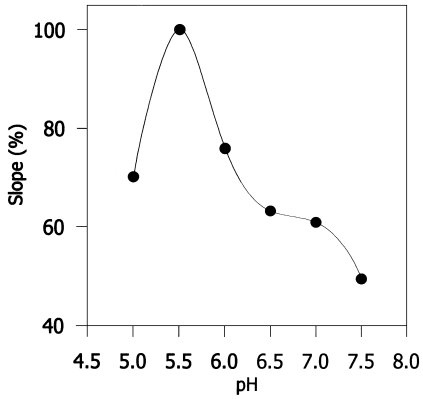

Fig. 2. Effect of pH on microbial biosensor response; 100% is the maximum response, as in the following figures. Measured in phosphate buffer at 30°C, at a working potential of +300 mV and with 3 mM hexacyanoferrate(III).

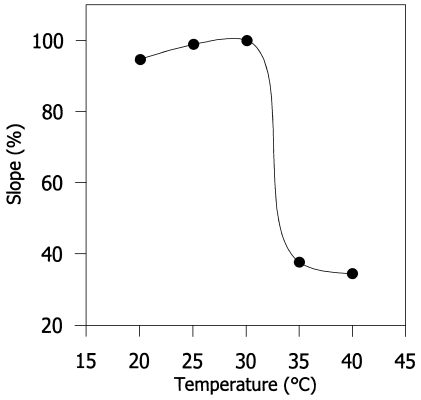

Fig. 3. Effect of temperature on biosensor response. Measurements were done in phosphate buffer at pH 5.5. Other conditions as in Fig. 2.

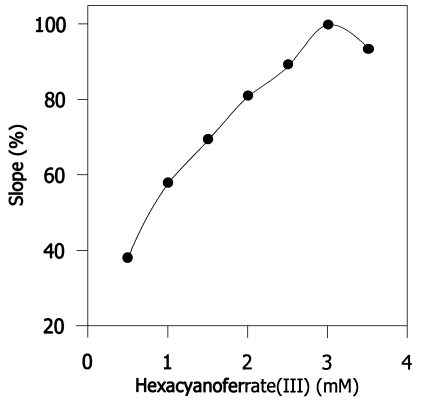

Fig. 4. Effect of mediator concentration on the biosensor response. For other conditions used see Fig. 2.

3.3. Analytical characteristics of the biosensor

Optimised conditions were used to characterise detection limits and linear ranges for determination of D-glucose, D-galactose, D-mannose, L-arabinose and D-xylose. The results obtained are summarised in Table 1. Response times (90% of steady-state) usually varied between 2 and 3 min. The achieved reproducibility of the biosensor measurement expressed as relative standard deviation of nine consequent D-glucose standard solution measurements (0.01 g l^{-1}) was 2.35%.

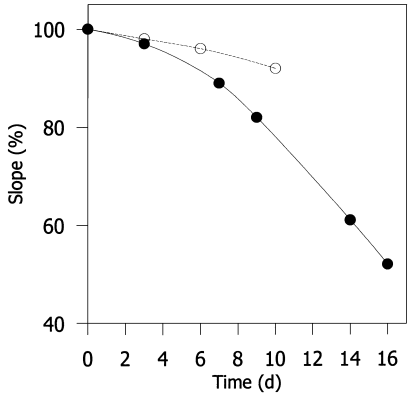

Fig. 5. Storage stability of the biosensor. Biosensors were stored at 4°C in the dry state (●), and in phosphate buffer pH 5.5 (□); 100% is the biosensor response determined on the first day.

3.4. Storage and operational stability

The storage stability of the biosensor was examined during storage in the dry state and in buffer, both at 4°C. Fig. 5 shows the decrease of biosensor response during a 16-day period. Storage stability was examined as a decrease of calibration slope for D-glucose during storage at 4°C. The electrode stored in the dry state was dried after every measurement using an electric fan. The use of trehalose resulted in an approximately two times prolonged half-life compared to the biosensor without a stabiliser (data not shown). Operational stability was investigated as a change of calibration slope, for five additions of D-glucose standard solution, with time. No significant decrease in sensitivity was observed over nine consequent measurements.

Table 1
Detection limits (signal to noise ratio=3) and linear ranges (R^2>0.995) for sugars determined by the microbial sensor with *Gluconobacter oxydans* cells

Sugar	Glucose	Galactose	Xylose	Mannose	Arabinose
Detection limit (mg l^{-1})	0.90	4.59	5.25	19.34	6.60
Linear range (g l^{-1})	0.002–2.2	0.009–1.3	0.011–1.2	0.039–1.3	0.013–1.1

3.5. Analysis of real lignocellulose hydrolysate samples

The developed biosensor was tested for measurement of samples of lignocellulose hydrolysate. Paper chromatography (PC) was used in this study as a reference sugar analysis. PC provides simple and reliable analysis with efficient resolution of all five saccharides present in the hydrolysate.

The microbial biosensor responds to all sugars present in the hydrolysate. For practical reasons, L-arabinose was chosen as a reference saccharide, i.e. the biosensor was calibrated using L-arabinose as a standard and the results of sample measurement were expressed in $g\,l^{-1}$ L-arabinose. 'L-Arabinose equivalent' is thus an arbitrary unit expressing the amount of all sugars present in the sample.

With the aim of eliminating the effect of inhibitors (present in real samples) all measurements were performed as five consequent additions of equivalent volumes of standard and sample in order: standard (1)–sample (2)–standard (3)–sample (4)–standard (5) (Fig. 6). The average of standard (3) and standard (5) was used to estimate the concentration in sample (4). A comparison of the biosensor analysis with the determination by PC is summarised in Table 2. The reproducibility of the biosensor sugar determination, expressed as 'L-arabinose equivalent', varied between 2.6 and 6.6% (n=3–4) depending on the sugar concentration.

The paper chromatographic analyses revealed that D-glucose was utilised first and its concentration reached zero in sample no. 2. The second sugar utilised was D-mannose (zero concentration in sample 3), followed by the third D-xylose (zero concentration in sample 4) and the last D-galactose (zero concentration in sample 5). L-arabinose was not utilised and it remained in samples no. 6–9 together with traces of other sugars. Concentrations of L-arabinose in all the samples varied between 0.8 and 2.6 $g\,l^{-1}$; this variation correspond to the accuracy of PC analysis. As can be seen from Table 2, the results of sugar content analysis were in a good agreement for the first three samples. During the last stage of fermentation (samples no. 6–9) the biosensor data were overvalued by 1–2 $g\,l^{-1}$. A considerable difference was observed

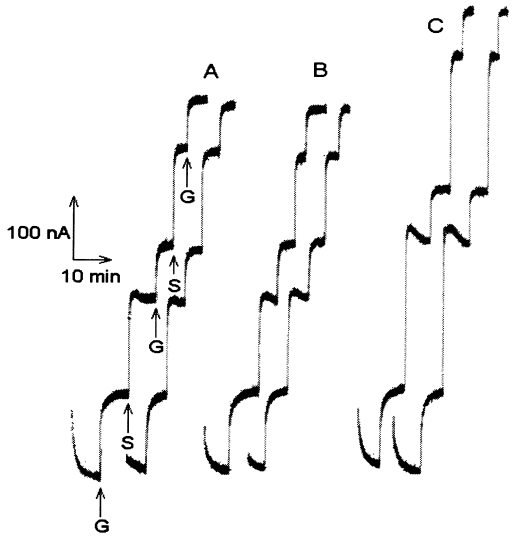

Fig. 6. Record of duplicate standard–sample–standard–sample–standard assays of three samples. A: sample 3 (50 μl); B: sample 4 (20 μl); and C: sample 5 (20 μl). Measurements were carried out before evaporation of ethanol and propanol, using 200 μl of a 1 $g\,l^{-1}$ glucose solution. G: standard glucose solution, S: sample of lignocellulose hydrolysate.

Table 2
Evaluation of paper chromatography and biosensor analysis of lignocellulose hydrolysate samples

Sample	Cultivation time (h)	Paper chromatography Σ sugars ($g\,l^{-1}$)	Biosensor arabinose equivalent ($g\,l^{-1}$)
1	0	28.28	27.46
2	0.5	16.99	17.98
3	1	9.74	10.29
4	2	3.36	8.79
5	3	1.75	5.25
6	3.5	2.56	4.05
7	4	1.11	3.57
8	4.5	0.83	3.02
9	5.5	1.37	2.09

in samples no. 4 and 5. This is apparently a consequence of changing the ratio between sugars during the fermentation. The response of the biosensor to present sugars is different (see Fig. 1) and moreover the biosensor responses to sugar mixtures are not additive (they do not even follow competitive kinetics — data not presented). The biosensor measurement expressed as 'L-arabinose equivalent' may serve as a routine method for empirical evaluation of lignocellulose hydrolysate during fermentation.

We tried to analyse lignocellulose hydrolysate from other kinds of wood and we observed different types of deviations from PC analysis. The application of the biosensor method, therefore, needs to be empirically adapted for any particular type of hydrolysate and fermentation process. Possible sources of interferences may be the content of alcohols and electrochemically active compounds in fermentation media. Gas chromatographic analysis of samples revealed the presence of small amounts of ethanol and propanol; they are produced by yeasts at low oxygen concentrations. This interference was eliminated by sample drying (see Section 2). Electroactive compounds, expected to be quinoid structures, cannot be efficiently eliminated but may be subtracted as a constant response specific to each particular type of hydrolysate.

4. Conclusion

The biosensor procedure may be a reliable method for sugar determination in lignocellulose hydrolysate. The correlation with PC is quite good and our tests confirmed that it is sufficient for routine fermentation process monitoring. One analysis takes ca. 25 min and the cost of consumables is negligible. Sample pre-treatment includes only sample drying and dilution.

Acknowledgements

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