On-Line Monitoring of Monosaccharides and Ethanol During a Fermentation by Microdialysis Sampling, Liquid Chromatography and Two Amperometric Biosensors


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Summary
Two amperometric biosensors were used as detection units in liquid chromatography for on-line monitoring of the production of fuel ethanol during a fermentation. Sampling was performed with a microdialysis probe, and the dialysate was introduced into a liquid chromatographic system providing separation of the substrates and the product: three monosaccharides and ethanol. The analytes were detected by two carbon paste electrodes based on pyranose oxidase and alcohol oxidase, respectively, co-immobilised with horseradish peroxidase, operating in parallel at -50 mV vs. Ag/AgCl. The measured linear ranges of the biosensors by direct injection into the LC system were for glucose 0.3-2.5 g L⁻¹, xylose 0.5-6.7 g L⁻¹, galactose 0.5-6.7 g L⁻¹, and ethanol 1.6-11.7 g L⁻¹ and the detection limits were 0.1, 0.2, 0.4, and 0.2 g L⁻¹, respectively. Injection of standard solutions were required to monitor the stability of the biosensors. Due to the strict selectivity of the biosensors, no interference from other compounds in the broth was encountered in the quantification. The fermentation process was monitored for 16 h. The on-line results were compared with off-line measurements.

Introduction
In our time of environmental awareness, ethanol provides a good alternative to fossil fuels [1]. Hexoses and pentoses available in energy wood and in waste water from the pulp and paper industry can be fermented into fuel ethanol. The production process must, however, be at a reasonable cost in order for fuel ethanol to compete with existing fuels. Rapid process monitoring and control are therefore essential both in the development stage and in the production process to keep costs low. It has been found, however, that using traditional detection methods such as refractive index (RI) or UV in conjunction with liquid chromatography (LC) in the analysis, matrix components in the complex fermentation broth may interfere in the analysis leading to unreliable results [2].

Sensitive and more selective detection can be obtained with sensors based on the biological recognition of enzymes. Bioprocess monitoring systems with enzyme mediated detection in flow injection analysis have been based on immobilised enzyme reactors [3, 4], enzyme thermistors [5, 6], and microstructured thin-layer biosensors [7]. Further, in systems utilising sequential injection analysis, membrane immobilised enzymes have been used [8]. Another approach is to couple enzyme based detection systems to LC. Then group selective detection can be obtained and at the same time interferences from co-eluting compounds can be avoided. Enzyme modified carbon paste electrodes have been used as detectors in LC for the analysis of alcohols in ethanol fermentations [9, 10]. However, when considering online monitoring of fermentation processes, not only the product formed in the fermentation but also the depletion of substrates is of great importance to monitor. The possibility of coupling several biosensors to the LC system would provide the selective detection of both substrates and products. The use of two biosensors as detectors in an LC system has been reported earlier for the

A number of papers have reported on the co-immobilisation of hydrogen peroxide producing oxidases with various peroxidases in carbon paste [10, 14–18]. Carbon paste electrodes based on alcohol oxidase (AOD, EC 1.1.3.13) and horseradish peroxidase (HRP, EC 1.1.3.1) have been used for the determination of alcohols in bioreactors [9, 10] and in biological fluids [19] after LC separation. Pyranose oxidase (P20, EC 1.1.3.10) [20, 21] co-immobilised with HRP in carbon paste has shown specificity towards a number of monosaccharides [22], all of which can be found in lignocellulose hydrolysates. This group selective sensor has also been used for the detection of monosaccharides in LC [22].

This contribution presents a new system for on-line monitoring of both substrates and product during a fermentation with Pichia stipitis as the fermenting organism. The system integrates microdialysis sampling, liquid chromatography separation, and post column amperometric detection of carbohydrate carbon source and ethanol using two different enzyme based biosensors.

Experimental

Chemicals

The chemicals used in this study were of highest analytical quality. Ethanol (99.5 %) was obtained from Kemetyl (Stockholm, Sweden) and all carbohydrates were purchased from Sigma Chem. Comp. (St. Louis, MA, USA). Hydrogen peroxide, acetic acid, and acetaldehyde were obtained from Merck (Darmstadt, Germany). Standard solutions were prepared using water from a Milli-Q purification system (Millipore, Bedford, MA, USA).

Organism and Cultivation

The yeast Pichia stipitis CBS 6054 was maintained at 4 °C on agar plates containing (per litre) 10 g of yeast extract, 20 g of Bactopeptone, 18 g of Bacto-agar, and 20 g of xylose. The cellmass was produced in a medium containing (per litre) 10 g of yeast extract, 10 g of malt extract broth, 20 g of Bactopeptone, 10 g of glucose, 20 g of xylose and 20 g of galactose. Sugars were added to the media after a separate sterilisation by autoclaving. One loopful of yeast was suspended in 100 mL of media in a one-litre baffled shake-flask. The flask was incubated in a water rotary shaker at 30 °C and 130 rpm, overnight. 20 mL of the inoculum were transferred to 250 mL media in a one-litre baffled shake-flask and the flask was incubated as above. The cellmass was harvested by centrifugation at 6400g at 4 °C for 15 min.

The fermentation was performed in a BiofloIII fermenter (New Brunswick Scientific CO. Inc., Edison, NJ, USA) with a working volume of 2.8 L. The fermentation temperature was 30 °C, pH was kept at 5.5 by automatic addition of 1 M KOH and the stirrer speed was 400 rpm. Oxygen was added to the culture as air. The overall gas flow into the culture was maintained at approximately 1 L min⁻¹. The dissolved-oxygen concentration in the culture was monitored with an Ingold polarographic oxygen electrode (Ingold Messtechnik AG, Urdorf, Switzerland) and was kept at 0 % during the whole fermentation. The fermentation medium contained (per litre): 5 g each of yeast extract, malt extract, and Bactopeptone, 10 g of glucose, and 20 g each of xylose and galactose, 5 g of MgSO₄ · 7H₂O, 1.16 g of Na₂HPO₄, 12.9 g of NaH₂PO₄. An initial cell dry weight of approximately 10 g L⁻¹ was used.

Biosensor Preparation

The carbohydrate sensor was based on the co-immobilisation of pyranose oxidase (P20) and horse-radish peroxidase (HRP) in carbon paste according to the procedure rendering the best sensor as described previously [22]. P20 was purified from wet mycelial mass of the basidiomycete Phanerochaete chrysosporium [22–24]. P20 (110 U) and HRP (576 U, Sigma Chem. Comp.) were covalently bound to graphite powder together with the additives polyethyleneimine [14] and lactitol [25]. After drying, the enzyme modified graphite was mixed with 40 μL of paraffin oil and the paste was packed into the tip of a 1 mL plastic syringe filled with unmodified carbon paste and a silver wire providing electrical contact. The electrode surface was gently wiped on a soft napkin giving a final surface area of 0.053 cm².

The amperometric alcohol biosensor contained carbon paste with co-immobilised alcohol oxidase (AOD) and HRP, as described previously [19]. HRP (Boehringer Mannheim) and AOD (from Hansenula polymorpha, obtained as a gift from Dr. Tim Gibson, University of Leeds, Leeds, UK) were adsorbed onto the graphite powder with lactitol solution as an additive. The remaining procedure for preparing the carbon paste and the electrodes was the same as described for the carbohydrate sensor.

Protective membranes were applied to the electrode surfaces. First, o-phenylenediamine was electropolymrised onto the electrode surface [10]. Two and six layers of Eastman AQ 29D, a cationic exchanger, were then applied to the monosaccharide and alcohol sensor, respectively, providing a charge exclusion membrane [26].

On-Line Analysis

The set-up for on-line analysis is shown in Figure 1. Sampling was performed using a microdialysis probe (CMA/10, CMA/Microdialysis AB, Stockholm, Sweden) equipped with a polycarbonate membrane (membrane length 5 mm, o.d. 0.5 mm, probe shaft length 100 mm). The probe was supplied sterilised by ethylene oxide, and inserted into the fermenter through a port equipped with a rubber membrane. The rubber membrane was made aseptic with 70 % ethanol before inser-