Direct measurement of glucose profiles in immobilized yeast gels with a pH-insensitive micro-electrode under anaerobic conditions

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DIRECT MEASUREMENT OF GLUCOSE PROFILES IN IMMOBILIZED YEAST GELS WITH A pH-INSENSITIVE MICRO-ELECTRODE UNDER ANAEROBIC CONDITIONS.

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SUMMARY: A 10 μm glucose sensor was developed based on a glucose oxidase coated Pt-electrode inserted in a capillary shaft. The internal buffer medium effected in a glucose response that was insensitive for the external pH. The sensor was successfully utilized at pH 4 under anaerobic conditions in gel particles containing homogeneously dispersed immobilized yeast cells.

INTRODUCTION

The combined action of mass transfer and reaction complicates the description of conversion rates in biofilms. A continuous exchange of reactants and products takes place between active biofilms and the surrounding bulk liquid. In combination with mass transfer resistances, this results in the development of concentration profiles. Since biological activity and concentration profiles influence each other mutually, modelling of processes inside natural biofilms is complex, and *in situ* measurements are required. For this purpose, needle-type microsensors are increasingly popular because they offer a sufficiently high spatial resolution.

Up to now, publications on this subject where mainly restricted to concentration measurements of inorganic substances like oxygen and several ions. A glucose microsensor based on the enzyme glucose oxidase has been described before (Cronenberg & Van den Heuvel, 1991), however, applications were limited by the dependency of the enzyme activity on the dissolved oxygen concentration and the pH value. Here we report on the development and use of a glucose oxidase based microsensor, which is relatively insensitive for its co-substrate and the local pH. The principle of operation under anaerobic conditions has been published previously (Cronenberg et al., 1991); the sensor described here contained an internal buffer solution to operate in a broad pH-range.

The use of this new sensor was demonstrated in a model biofilm with well defined properties. The model system consisted of *Saccharomyces cerevisiae* immobilized in spherical gels (Furusaki & Seki, 1985) under conditions of a low pH and the absence of oxygen. With the conventional type of glucose microsensor (Kim & Lee, 1988), measurements under these conditions would not have been possible.

EXPERIMENTAL

The glucose microsensor: A pencil-shaped glass capillary with a tipsize of 10 μm was dipped into a 2 % (w/v) agar solution (extra pure; Merck) at 50 °C for 1 minute. As a result, the ultimate 1 - 2 mm of the tip was filled with gel. Subsequently, the capillary was filled with a filtered and aerated solution containing 0.05 M KCl in a 0.1 M acetate buffer at pH 5.2. The relatively strong buffer is necessary for a pH insensitive operation. A glucose oxidase coated platinum micro-electrode with a tipsize of 10 μm and a high specific activity was prepared as described previously (Cronenberg et al., 1991). It was inserted into the fluid-filled capillary and into the agar layer until a distance of 0.1 mm from the tip opening was reached. Finally, a 0.1 mm Ag/AgCl reference electrode was inserted in the capillary at 5 mm from the tip opening. Except for the tipsection (see Fig.1), the sensor was shielded with a metal tube.

During the measurements a polarization voltage of + 0.7 V was applied between the enzyme coated Pt-electrode and the Ag/AgCl reference. Hydrogenperoxide, produced by the enzymatic oxidation of glucose in the coating, is detected at the Pt-surface and the current was measured with a high performance electrometer (Keithly 617). The sensor response was investigated in a 20 cm^3 well-stirred cell under anaerobic conditions at 30°C.

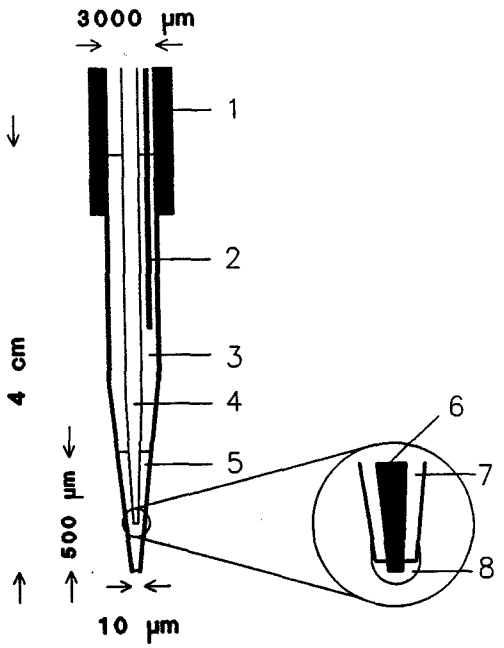

Figure 1. *Schematic representation of the glucose microsensor. 1: brass tube; 2: Ag/AgCl wire; 3: buffer solution; 4: electrode; 5; agar gel; 6: platinum; 7: glass; 8: enzyme.*

The immobilized yeast system: Immobilized yeast gels were prepared according to Cronenberg & Van den Heuvel (1991) from a mixture containing 1.5 % w/v agar (extra pure, Merck) at 38°C and 0.3 % w/v dried baker's yeast (*Saccharomyces cerevisiae*, type II, Sigma). The average diameter of the spherical gels was 3.62 mm.

In a well-stirred reactor, 100 cm^3 of gels were incubated in 500 cm^3 mineral medium (Evans et al. 1970) at 30°C. The pH of the medium was continuously measured and adjusted to 4. After an equilibration time of 1 hour, glucose was added up to a concentration of 10 mol/m^3.

Macroscale measurements: Every 15 minutes a 0.5 cm^3 sample of medium was taken for the determination of glucose with HPLC (Animex HPX-87H column), to obtain the glucose concentration as a function of time. From this relationship, the total amount of beads, and the total bead surface area in the reactor, the average surface flux of a single bead was calculated as a function of time.

The maximal volumetric reaction rate of the immobilized yeast cells was estimated as 5.5 x 10^{-3} $mol/m^3.s$ by measuring the glucose consumption rate of the gels at the higher glucose concentrations. The Monod constant of the reaction was estimated from separate batch experiments with suspended yeast cells and amounted to 2.2 mol/m^3. With these kinetic parameters concentration profiles inside the well defined gel beads could be calculated.

Microscale measurements: For each microprofile measurement a fresh gel particle was taken from the fermenter and fixed in the centre of a flow cell by entomological-specimen needles. In order to maintain the same physical and chemical bulk conditions in the flow cell and the reactor, medium was recirculated from the fermenter over the flow cell at an average flow rate of 1.4 cm^3/s (see Fig.2).

A temperature of 30°C was maintained in the flow cell by an electrical heating element. The micro-electrode set-up was placed in a Faraday cage to reduce electro-magnetic interference. The fluid-flow was electrically separated from the outside by tricklers. The micro-electrode was introduced into the biofilm with a motordriven micromanipulator. The position of the electrode tip could be examined with a stereo microscope. Before and after each measurement the calibration of the glucose electrode was checked for drift.

When the bulk glucose concentration had reached a level of 6, 4, 3 and 1.6 mol/m^3 respectively, a profile measurement was carried out. According to Fick's law, the slopes of the profiles are proportional to the glucose flux.

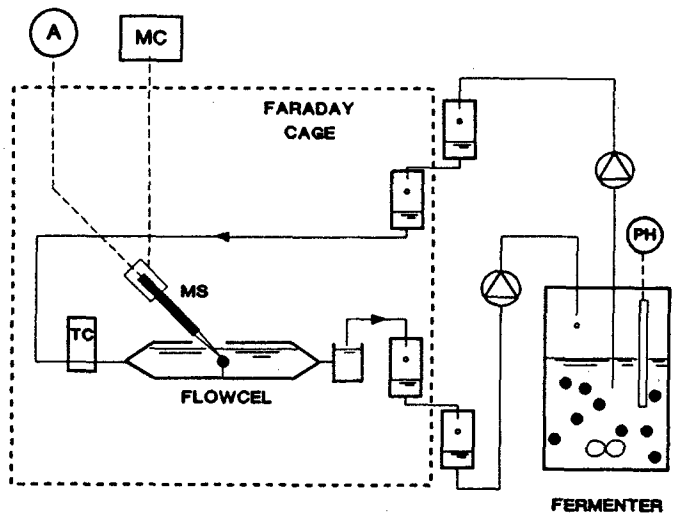

Figure 2. *Experimental set-up. A: electrometer; MC: micromanipulator control; MS: microsensor; TC: temperature control; PH: pH control.*

RESULTS AND DISCUSSION

Sensor response: The buffer-filled glucose sensor showed somewhat different characteristics compared to the previously described sensor without buffer (see Table 1). Interestingly, the noise was reduced, indicating the internal buffer solution possibly acted as an electrical shield, or exhibited capacitive damping properties.

Table 1: *Influence of the internal buffer on the properties of the glucose sensor.*

property	air	buffer
useful pH range	6 - 8	2 - 11
measuring range:		
aerobic	0.05 - 20 mol/m^3	0.05 - 20 mol/m^3
anaerobic	0.05 - 12 mol/m^3	0.05 - 7 mol/m^3
typical response time	< 60 s	100 s
drift	< 5% per hour	< 10% per hour
electrical noise	considerable	relatively small
life time	1 month	1 week

In Fig. 3 the response of the sensor is given at completely different pH conditions. Although the enzyme activity strongly depends on the pH, the response of the sensor itself was linear and insensitive for the pH of the media. Therefore, it was concluded that the internal buffer was able to neutralize the pH of the media investigated.

For immobilized glucose oxidase a pH optimum of 5.8 to 8.0 is reported (Guilbault, 1984). The introduction of a separate micro-environment in the sensor shielded the enzyme from the bulk environment by a diffusional barrier, and made the enzymatic conversion appear to be insensitive for the external pH.

The response time of the sensor was 1 to 2 minutes, while the drift of the electrode signal was less than 5 % per hour for glucose concentrations below 5 mol/m^3. At higher glucose concentrations the sensor response showed a pronounced drift. When the sensor was taken out of such a concentrated solution into air, it took a long time to yield a stable response again (Fig 4).

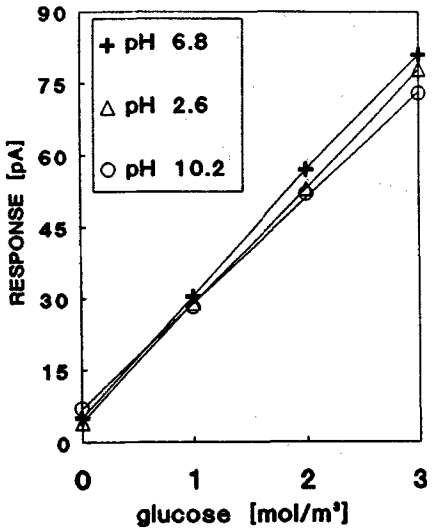

Figure 3. *Calibration curves of the glucose microsensor at different pH values in several media. pH 6.8: 0.1 M phosphate buffer; pH 2.6: HCl solution; pH 10.2: NaOH solution.*

In the latter situation, the hydrogen peroxide and glucose that have accumulated in the tip of capillary cannot diffuse into the bulk liquid, but is removed only by reaction or diffusion into the buffer medium in the shaft of the sensor. It should be noticed that the liquid volume in the shaft is very large compared to the volume in the tip.

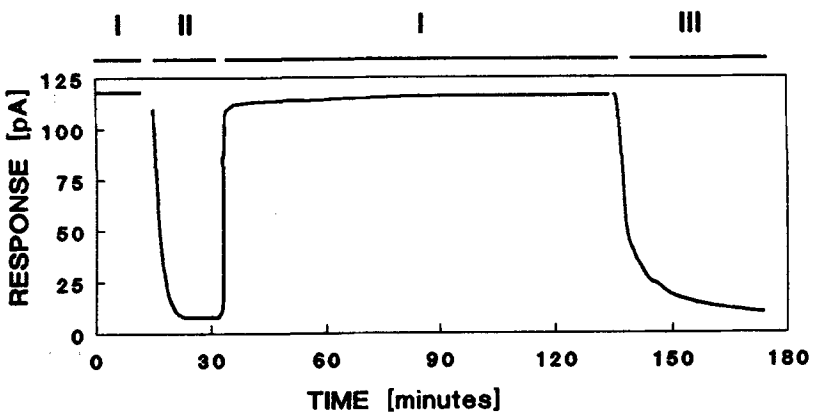

Figure 4. *Time-response curve of the microsensor after glucose concentration steps. I: 5 mol/m^3; II: 0 mol/m^3; III: sensor placed out of the solution.*

A good sensitivity as well as a good selectivity is achieved if a low glucose concentration in the bulk medium causes a relatively high hydrogen peroxide concentration around the Pt-tip, *i.e.* the total enzyme activity should be large compared to the Pt-surface area. For our needle-type sensor with a small tip diameter this implies the application of a thick enzyme layer on a long electrode.

However, high levels of hydrogen peroxide negatively affect the response time and the electrode drift due to possible hydrogen peroxide accumulation in the shaft of the sensor. Furtheremore, the rate of enzyme inactivation will increase, resulting in a short electrode lifetime.

A smaller total enzyme activity compared to the Pt-surface, however, has disadvantages too. Firstly, the selectivity of the sensor will be less, since the hydrogen peroxide concentration decreases compared to interfering oxidizable substances in the medium. Secondly, if the total enzyme activity is too low, the signal drift will increase due to the accumulation of non converted glucose. Therefore, it can be concluded that the enzyme loading and Pt-surface area are critical parameters for the design and operation of the sensor.

Measurements in immobilized yeast particles: About half an hour after the start of the fermentation, a steady decrease of the glucose concentration was observed. The first microsensor measurement was performed after 115 minutes, when the glucose concentration had reached a level of 6 mol/m^3.

The glucose microsensor measurements in and around the immobilized yeast gels are given in Fig. 5. It typically took 10 minutes to penetrate the bead from the surface to the centre. Bulk concentrations measured with the microsensor and those determined with HPLC showed a good correspondence. Therefore, it can be concluded that the sensor gave reliable readings in the anaerobic fermentation medium at the pH of 4.

A diffusive boundary layer of approximately 100 μm can be noticed around the gelbeads in the flowcell. This information can be used to characterize both the hydrodynamic regimen and the diffusion coefficient in the medium.

In Fig. 5 the measured profiles inside the gels are compared with calculated profiles. For these calculations a simple model was used, assuming (i) homogeneous gelbeads, (ii) a pseudo-steady state, (iii) the absence of external mass transfer resistances, and (iv) Monod kinetics.

The parameters needed for the model were determined with macroscopic methods, independently from the microelectrode measurements. The same parameters were used for all profiles presented, and growth was not taken into account.

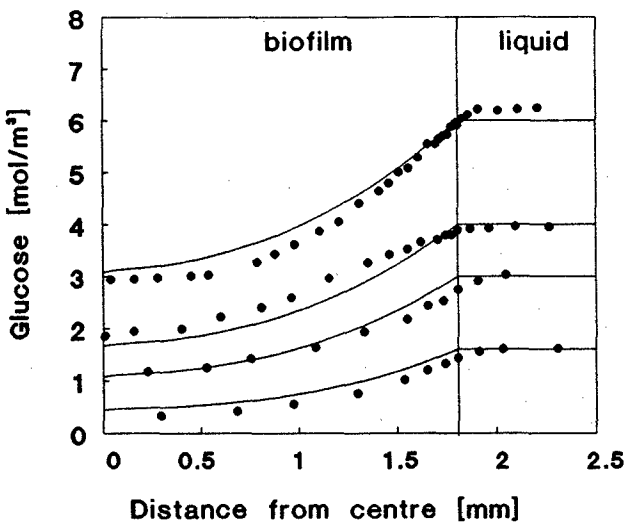

Figure 5. *Glucose micro-profiles in yeast gels. Solid lines in liquid: HPLC measurement. Solid curves in biofilm: calculated profiles. Dots: microsensor measurements.*

The glucose fluxes into the gelbeads determined on macro- and microscale are given in Table 2. The fluxes based on the microsensor measurements were obtained by multiplying the interfacial slope of the measured concentration profiles with an effective diffusion coefficient of 6.7 x 10^{-10} m^2/s (Cronenberg & Van den Heuvel, 1991). The calculated fluxes were taken from the modelled profiles mentioned above.

Table 2: *Comparison of fluxes through the interface of a yeast gel.*
(a) macroscopic measurement, (b) microscopic measurement, (c) model calculation.

glucose (mol/m^3)	time (minutes)	Interfacial fluxes (10^{-6} mol/m^2s)		
		(a)	(b)	(c)
6.0	115	2.2	2.6	2.24
4.0	155	2.2	1.0	1.88
3.0	175	2.1	1.4	1.61
1.6	210	0.8	1.0	1.06

In spite of all model simplifications a reasonable agreement can be seen between the measured and calculated profiles and fluxes. Deviations are likely due to the fact that the cell distribution inside the gels was not completely uniform. It should be stressed that the measured profiles represent the glucose distribution of an *individual* gelbead. In contrast to macroscale measurements, the variation between different gelbeads, as well as inhomogeneities inside a single gelbead can be examined with microelectrodes.

It is concluded, that this glucose microsensor is a useful analytical tool for mechanistic biofilm studies on a microscale, even when the pH is not optimal for the enzyme, *i.e.* lower than 6 or higher than 8.

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