Development of a biosensor for monitoring of glycerol during alcoholic fermentation

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Abstract

A biosensor for the measurement of glycerol in FIA was constructed using covalently immobilized glycerokinase and glycerol-3-phosphate oxidase in conjunction with a Pt based hydrogen peroxide probe. Different immobilization strategies have been studied including random and asymmetric immobilization onto a polymeric support and immobilization onto two different membranes. The latter resulted in the best configuration for batch measurement. The most effective configuration for measurement in FIA was the immobilization of glycerokinase in a glass beads reactor coupled with glycerol-3-phosphate oxidase on a preactivated Immobilon AV membrane kept at the electrode surface. Using a 250-μl injection loop, 3 mmol ATP(Mg²⁺) in 0.1 M borate buffer pH 8.5 and a flow rate of 0.5 ml/min, a linear response in the 2·10⁻⁶/10⁻³ mol/l range and a detection limit of 5·10⁻⁷ mol/l were obtained for glycerol. Lifetime of the glycerol-3-phosphate membrane was extended up to 1 month by storage in the working buffer containing 1% DEAE-dextran and 5% lactitil. More than 350 samples can be assayed with this system. The biosensor was used to monitor off-line glycerol production during alcoholic fermentations carried out at different pHs and temperatures. © 1998 Elsevier Science S.A. All rights reserved.

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1. Introduction

Glycerol is the most important secondary product of alcoholic fermentation and contributes to the smoothness and viscosity of a wine (Noble and Bursick, 1984) with a favourable effect on the taste (Ribèreau-Gayon et al., 1972). The amount of glycerol formed by the yeast during the fermentation process is from 1:10 to 1:15 of the alcohol formed (Ribèreau-Gayon et al., 1972) with final concentrations varying form 1 to 10 g/l (Moruno and Di Stefano, 1989). The amount of glycerol produced depends on the yeast strain (Radler and Schutz, 1982); however, the chemical composition of the medium and factors such as oxygen content, fermentation temperature and pH have been demonstrated to be relevant for the process (Gardner et al., 1993; Ough et al., 1972). Monitoring of glycerol during alcoholic fermentation would allow to detect and prevent unwanted metabolic changes affecting the quality of the final product. Moreover, more information about the parameters influencing the glycerol production could be obtained.

The use of biosensors and bioreactors for the determination of glycerol has been investigated by different authors. A glycerol dehydrogenase based reactor realised using a nylon tube (Hinsch and Sundaram, 1980) or controlled pore glass beads (Canizares and Luque de Castro, 1995) with spectrophotometric detection of NADH formed by the enzymatic reaction has been used for measurement in human serum and wine, respectively. Rank et al. (1995) used a glycerokinase (GK) thermistor to monitor glycerol during industrial fermentations with Saccharomyces cerevisiae, while a three-reactor system, consisting of glycerokinase (GK), glycerol-3-phosphate oxidase (GPO) and peroxidase (HRP) with spectrophotometric detection has been recently proposed by Kiranas et al. (1997). In the biosensors area, electrochemical detection still represents the most successful approach because of its sensitivity, reliability and cost effectiveness. Analysis of glycerol in human plasma with electro-
chemical detection has been attempted using GK and GPO enzymes physically retained at the electrode with a microdialysis probe (Murphy and Galley, 1994) or covalently immobilized onto a nylon membrane (Merchie et al., 1992). In this paper we report on the development of an amperometric FIA biosensing system for the determination of glycerol and its application for monitoring of glycerol during fermentation processes. The system consisted of a GK reactor and a GPO enzyme electrode coupled to a hydrogen peroxide probe.

2. Materials and methods

2.1. Reagents and apparatus

Glycerol-3-phosphate oxidase (EC 1.1.3.21; Aerococcus viridans, 96 U/mg), glycerokinase (2.7.1.30; Escherichia coli, 60 U/mg), glass aminopropyl (average pore size 700 Å, 80–120 mesh), ATP, DEAE-dextran, lactitol, glycerol-3-P, glycerol and borax were from Sigma Chemical Co. (USA); all other reagents were of analytical grade from Carlo Erba (Italy). Saccharomyces cerevisiae dry powder was from Chemical Research Company (Italy).

An amperometric biosensor detector, a platinum based hydrogen peroxide probe and a flow-through cell from Universal Sensors (USA) were used for amperometric measurements. Current was recorded using a model 868 AMEL (Italy) x-t recorder. A Gilson (France) model M 312 peristaltic pump and a model 5020 Teflon injection valve (Rheodyne, USA) were used for amperometric measurements. Current was recorded using a model 868 AMEL (Italy) x-t recorder. A Gilson (France) model M 312 peristaltic pump and a model 5020 Teflon injection valve (Rheodyne, USA) were also used for measurement in FIA.

Polycarbonate membranes of 0.1 μm pore size were from Nucleopore (USA), Biodyne immunoaffinity membranes from Pall (UK) and Immobilon AV membranes from Millipore (UK). Cut off cellulose acetate membranes of 100 Da molecular weight were prepared in our laboratory according to a published procedure (Mascini et al., 1987).

2.2. Procedures

The reaction scheme was as follows:

\[
\text{Glycerol} + \text{ATP(Mg}^2+\text{)} \rightarrow \text{Glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-phosphate} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Glycerone-3-phosphate} + \text{H}_2\text{O}_2
\]

The first reaction is catalysed by glycerokinase (GK), the second by glycerol-3-phosphate oxidase. A platinum based H₂O₂ probe polarised at + 650 mV was used as electrochemical transducer.

Immobilization onto preactivated membranes (Biodyne and Immobilon) was carried out as previously reported (Compagnone et al., 1993; Messia et al., 1996) using a 0.2 mg/20 μl enzyme(s) solution for 1 cm² of membrane. The enzyme solution was placed onto the membrane and allowed to react for 20 min; unreacted sites were blocked using 0.1 M glycine. The membranes were washed in 1 M NaCl before use. Asymmetric immobilization was obtained preparing the enzyme solutions separately (0.1 mg in 10 μl), pipetting the GPO solution onto one side of the membrane and (after 2 min) the GK solution onto the other side of the membrane. Reaction was allowed to go for 20 min at room temperature. Preliminary experiments indicated that the higher current output for glycerol was obtained using a 1:5 GPO:GK ratio for the random immobilization and 1:1 GPO:GK ratio for the asymmetric immobilization.

BSA-glutaraldehyde membranes were prepared by mixing 5 μl of 1% BSA solution, 0.5 mg of enzyme (10 μl) and 5 μl of a 0.25% solution of glutaraldehyde, allowing the mixture to dry at room temperature. A nylon net membrane was used to physically support the enzymatic membrane; unreacted glutaraldehyde was blocked by soaking the assembled probe with 0.1 M glycine for 2 min.

Covalent immobilization on nylon net was performed as reported by Mascini et al. (1983). Disks of 15 mm diameter were activated using dimethyl sulphate and anhydrous methanol, then reacted with lysine (used as spacer) for 2 h. The remaining free amino groups of lysine were treated with 12.5% glutaraldehyde for 45 min and washed overnight with 0.1 M phosphate buffer pH 7.0. 0.5 mg/cm² of GK was applied to the membranes and allowed to react for 2 h at room temperature. Before use the membranes were washed with 0.1 M glycine and 1 M NaCl.

Two-enzymatic membranes probes (Immobilon + BSA-glutaraldehyde and Immobilon + nylon net) were realized by assembling the GPO or GK membranes previously prepared. In the case of Immobilon + BSA-glutaraldehyde, a polycarbonate membrane was used as physical support for the GK-BSA mixture. This probe was then assembled with the “clean” polycarbonate side facing the solution and the GK membrane in contact with the Immobilon GPO membrane already prepared as described above.

In any of the configurations tested the membranes were placed on the jackets provided by the manufacturer with the Pt electrodes and held with a rubber O-ring. The enzyme electrodes were then assembled always with the cellulose acetate membrane in contact with the Pt surface, the polycarbonate as external membrane and the enzymatic membrane(s) sandwiched between the two.

Immobilization of GK onto aminopropyl glass was achieved by immersing 50 mg of beads in 2.5% glutaraldehyde for 1 h with continuous gentle magnetic stirring.
The beads were then extensively washed with deionised water and incubated for 3 h at 4°C with 1 mg of GK in 1 ml of 0.1 M phosphate buffer pH 7.0. The enzymatic reactor was realized by packing the beads into a 3-mm internal diameter Tygon tube. To retain the beads in the tube a nylon disk (mesh 80) was glued at one end, the glass beads were loaded using the peristaltic pump and a second nylon disk was glued at the distal end.

Calibration curves for glycerol were constructed by measurement of the steady-state (batch analysis) or peak height (FIA) current generated by injections of glycerol standard solutions after stabilization of the background signal. In the FIA manifold the GK reactor was placed between the injection loop and the electrochemical cell containing the GPO electrode; the working buffer and the cofactors were contained in a one-compartment reservoir and continuously pumped in the reactor-cell line.

Fermentation experiments were carried out as follows: must from Trebbiano grapes (Italy), stored at −20°C, was thawed and aliquoted (30 ml) in 50-ml glass tubes. The tubes were incubated for 15 min at 68°C for pasteurization (to inactivate “wild yeasts”). pH was then adjusted to the desired value using 0.1 M NaOH (original pH was 2.8). The fermentation process was started by adding 20 mg/ml of dry yeast (*Saccharomyces cerevisiae* 20.26 C, >3·10^10 cells/g), sealing the tubes with cotton wool and incubating at the selected temperature (18 or 25°C) in thermostated baths.

Samples of the fermentation medium were collected using the following procedure: the cotton wool was replaced with a rubber stopper and the medium was mixed for 30 s by inversion, as gently as possible. The tube was placed back in the original position for 1 min to allow a gross separation of the particulate matter (yeast) from the liquid medium. Three aliquots of 20 ml were taken (close to the liquid surface) and diluted as appropriate (1:100/1:500) with the working buffer. The inter- aliquots RSD was about 5–8%; samples giving RSD higher than 15% were not reported.

### 3. Results and discussion

#### 3.1. Enzyme immobilization

Different polymeric supports and immobilization procedures have been tested for the covalent immobilization of GPO and GK. Fig. 1 reports the calibration curves for glycerol-3-phosphate obtained after immobilization of GPO and GPO + GK. A better sensitivity and a wider linear range for glycerol-3-phosphate was obtained using the Immobilon AV membrane (curve a) with respect to the Biodyne (curve b) and the nylon net membrane (curve c). The Immobilon based GPO electrode exhibited linearity from 5×10^{-7} to 10^{-3} mol/l and a detection limit (twice the noise of the baseline) of 2×10^{-7} mol/l of glycerol-3-phosphate. Curves d and e of Fig. 1 were obtained with GPO and GK coimmobilized onto the preactivated Immobilon membrane. As expected, a decreased response was obtained due to the lower amount of GPO retained by the membrane; however, the enzyme electrode was still able to detect 4×10^{-6} mol/l of glycerol-3-phosphate when GPO and GK were symmetrically immobilised (curve d) and 10^{-5} mol/l in case of asymmetric immobilization (see below). Relative standard deviation for all the procedures tested was in the 4–6% range (10 measurements).

The response to glycerol was then tested for the GPO + GK Immobilon membranes and it is shown in Fig. 2 (curves d and e). As already reported for the development of a biosensor for the measurement of malate in wine with a pyruvate oxidase/malic enzyme electrode (Messia et al., 1996), the asymmetric immobilization carried out onto a preactivated membrane exhibited an improved sensitivity of the bienzyme electrode (curve d versus e) because of the better defined orientation of the enzymes (GPO towards the Pt electrode and GK towards the solution). However, a higher current output was recorded when GK and GPO were immobilized on separate membranes; in fact, as shown in Fig. 2, the best performances for measurement of glycerol were obtained with GPO immobilized onto the Immobilon membrane and GK in a BSA-glutaraldehyde membrane (curve b) or onto a nylon net support (curve a). The two glycerol enzyme electrodes exhibited similar sensitivity.
3. Measurement of glycerol in FIA

Our aim was to measure glycerol on-line during alcoholic fermentation, we thus adapted the system for measurement in FIA. The response of the Immobilon + nylon sensor to glycerol was about 60% of the response to glycerol-3-P (similar current outputs were obtained with or without the GK nylon membrane), indicating that there was not a complete conversion of glycerol to glycerol-3-P by the GK membrane. Moreover, considering that the lifetime of the immobilized GPO was considerably shorter than the lifetime of the immobilized GK (see next paragraph), it was decided to keep the enzymes in different compartments in the FIA system. GK was then immobilized on glass beads and packed to form a reactor while GPO was kept at the electrode surface immobilized onto an Immobilon membrane. Using this biosensing format, we obtained 100% conversion from glycerol to glycerol-3-P by the GK reactor, and an easier replacement of the GPO membrane, when needed, by substitution of the entire GPO electrode membranes set. The complete conversion of glycerol partially balanced the

3.2. Co-substrates and pH

The optimum pH for this enzyme electrode was studied using phosphate, borate and glycine buffer in the 7.0–10.0 range (data not shown). 0.1 M borate buffer pH 8.5 was selected for the analysis because of the good response to glycerol and the lowest background current signal. As with most of the kinase enzymes, GK catalyses the phosphorylation of the glycerol using ATP as cosubstrate; the catalytic efficiency of the reaction is improved in the presence of Mg$^{2+}$ because the enzyme is more active toward the ATP(Mg$^{2+}$) complex. The response of the Immobilon + nylon sensor versus cofactors concentrations is shown in Fig. 3 for a $10^{-4}$ mol/l glycerol solution. The maximum response to glycerol was found at 3 mmol/l ATP and Mg$^{2+}$, and these concentrations were selected for further experimental work. The difference in the signal/concentration profile for the two cofactors, shown in Fig. 3, was due to the activating effect that Mg$^{2+}$ ions had on GPO activity (40% increase in the glycerol-3-phosphate response for 3 mmol/l Mg$^{2+}$ in the working buffer).

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in the $10^{-6}/10^{-4}$ mol/l range, the linearity of the response of the nylon based electrode being extended up to $5 \times 10^{-4}$ mol/l of glycerol (not shown in the figure for clarity). The detection limit was $5 \times 10^{-7}$ mol/l of glycerol for both the electrodes. The improved performance obtained by orientation and separation of the enzymatic layers was confirmed by the calibration curve (curve c of Fig. 2) obtained using GPO and GK mixed in a BSA-glutaraldehyde membrane (with nylon net used only as a physical support). Even though the amount of GPO in the membrane was higher (0.5 versus $\approx 0.15$ mg retained by the Immobilon membrane) the resulting response was decreased.

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loss in sensitivity (about one order of magnitude) observed switching from batch analysis to FIA. Calibration curves for glycerol obtained at a flow rate of 0.5 ml/min and with injection loops of 100, 250 and 500 μl are shown in Fig. 4. Identical calibration curves were obtained by injection of glycerol-3-P, indicating 100% conversion of glycerol for all the loops used. The 250-μl injection loop was selected for further experimental work. A detection limit of 10⁻⁶ mol/l, linearity up to 10⁻³ mol/l of glycerol and RSD of 1–2% were obtained using 3 mmol/l ATP and Mg²⁺ in the working buffer.

A recovery study to ascertain the effect of the matrix (wine) on the biosensor performance was then carried out at different dilutions of the sample in the working buffer. The recovery values, reported in Table 1, ranged between 90 and 103%, indicating the suitability of the procedure for wine (and must) analysis.

### 3.4. Storage and operational lifetime

One of the major concerns in the development of biosensors is the lifetime of the biosensing elements in storage and operational conditions. During alcoholic fermentation there is a net production of glycerol in the first 7–10 days; it would therefore be desirable to have a system able to perform the analysis within this time frame. The lifetime of the coimmobilized enzyme membranes was very short: 1 day for dry storage of the membrane (calculated as the time necessary to reach 10% of the initial response) and 3 days for storage in the working buffer. The use of preservatives and reducing agents as NaN₃, glycerol, Kathon, benzoic acid, GSH, Na₂Si₅ in the storage solution did not result in a longer lifetime of the membrane. The rapid decay in the response was mainly due to the inactivation of GPO (decrease of the response to glycerol-3-phosphate). Separation of the enzymes improved the lifetime of the GPO on Immobilon membrane up to 6 days when stored in the working buffer. However, a significant increase of the lifetime of the immobilized GPO (up to 30 days) was obtained by storage in a 1% DEAE-dextran + 5% lactitol solution. This mixture has been already reported to have stabilization effects for GPO in solution (Gibson et al., 1992a) and for covalently immobilized enzymes (Gibson et al., 1992b). After 30 days the GK reactor was still able to convert 100% of a 10⁻³ mol/l glycerol standard solution to glycerol-3-phosphate. The storage solution for the GK reactor was the working buffer containing either DEAE-dextran/lactitol or benzoic acid (1%).

The operational lifetime was measured on must samples during alcoholic fermentation by running cycles of 6 samples + 1 standard injection. The response of the system to glycerol was reduced to 25% of the initial after 350 injections.

### 3.5. Glycerol production during alcoholic fermentation

The FIA system was tested off-line for measurement of glycerol during fermentation with the same substrate (Trebbiano grapes must) but varying the initial pH and the temperature of the process. The results, reported in Fig. 5 (triplicates), indicated that the influence of the initial pH within the 3.0–3.4 range was negligible for glycerol production. On the contrary, a difference in the temperature of the fermentation process (18 versus 25°C) influenced significantly both the kinetics of the production and the final content of glycerol.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Added (g/l)</th>
<th>Expected (g/l)</th>
<th>Found (g/l)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/100</td>
<td>0.90</td>
<td>8.30</td>
<td>8.15</td>
<td>98.0</td>
</tr>
<tr>
<td>1/200</td>
<td>0.10</td>
<td>7.50</td>
<td>7.13</td>
<td>95.0</td>
</tr>
<tr>
<td>1/500</td>
<td>0.50</td>
<td>7.90</td>
<td>8.12</td>
<td>103.0</td>
</tr>
<tr>
<td>1/1000</td>
<td>0.90</td>
<td>8.30</td>
<td>8.17</td>
<td>98.0</td>
</tr>
</tbody>
</table>

Fig. 4. Calibration curves for glycerol obtained with the FIA system using different injection loop volumes.
4. Conclusions

A FIA system for the amperometric determination of glycerol has been developed. The enzymes glycerol-3-phosphate oxidase and glycerokinase have been covalently immobilized and used in conjunction with a hydrogen peroxide electrode. The immobilization of the two enzymes in the form of a single probe demonstrated that better sensitivity is achieved by separate immobilization of the enzymes and subsequent assembling of the probe. A further improvement in sensitivity was obtained by immobilization of glycerokinase in a glass beads reactor for measurement in FIA. The system exhibited a linearity range of $2 \times 10^{-6} / 10^{-3}$ mol/l of glycerol with RSD of 1–2%. Glycerol production during alcoholic fermentation was monitored off-line with the FIA system. Preliminary data demonstrated that because of its sensitivity and stability the developed biosensor is suitable for online analysis of glycerol.

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References


