1 **Microbiosensor for the detection of acetate in**
2 **electrode-respiring biofilms**

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Abstract

The goal of this work was to develop a microbiosensor to measure acetate concentration profiles inside biofilms *in situ*. The working principle of the microbiosensor was based on the correlation between the acetate concentration and the current generated during acetate oxidation by *Geobacter sulfurreducens*. The microbiosensor consisted of a 30- μm carbon microelectrode with an open tip as a working electrode, with *G. sulfurreducens* biofilm on the tip and a pseudo Ag/AgCl reference electrode, all enclosed in a glass outer case with a 30- μm tip diameter. The microbiosensor showed a linear response in the 0-1.6 mM acetate concentration range with a $79 \pm 8 \mu\text{M}$ limit of detection (S/N=2). We quantified the stirring effect and found it negligible. However, the interfering effect of alternative electron donors (lactate, formate, pyruvate, or hydrogen) was found to be significant. The usefulness of the acetate microbiosensor was demonstrated by measuring acetate concentration depth profiles within a *G. sulfurreducens* biofilm. The acetate concentration remained at bulk values throughout the biofilm when no current was passed, but it decreased from the bulk values to below the detection limit within 200 μm when current was allowed to pass. The zero acetate concentration at the bottom of the biofilm showed that the biofilm was acetate-limited.

Keywords: Microbiosensor, acetate, biofilm, *Geobacter*, electron transfer, microelectrode.

1. Introduction

Needle-type microelectrodes are among the most used and valuable tools in biofilm research. With tip dimensions on the order of tens of microns, they can be used to monitor the microscale gradients in biofilms without damaging biofilm structure. Direct measurement of these gradients provides critical information on the local physicochemical environment in which the cells within biofilms respire and therefore yields important mechanistic information. Typically, the physicochemical parameters measured using microelectrodes include pH, dissolved oxygen concentration, and redox potential (Lee et al. 2007; Lewandowski et al. 1989; VanHoudt et al. 1992). This list has expanded significantly to include H₂S, CO₂, NO₂⁻, and H₂O₂ microelectrode measurements (Beyenal et al. 2004; De Beer et al. 1997b; Jeroschewski et al. 1996; Liu et al. 1998). In addition, microbiosensors have been constructed with immobilized microorganisms, e.g. glucose, methane, nitrate, nitrous oxide, and galactose microbiosensors (Cronenberg and van den Heuvel 1991; Damgaard and Revsbech 1997; Larsen et al. 1996; Lewandowski and Beyenal 2003; McLamore et al. 2011; Peteu et al. 1996). Because of challenges in their construction and operation, the number of available microbiosensors is limited. New microbiosensors are needed to quantify electron donor concentrations in biofilms (Renslow et al. 2013).

Acetate is one of the electron donors used for biofilms growing and transferring electrons to solid electrodes in microbial fuel cells or in bioelectrochemical systems (Hamelers et al. 2010; Logan 2009). This type of biofilms is called electrochemically active biofilms, and they are able to transfer electrons out of the cell to electrodes via extracellular electron transfer (Du et al. 2007; Logan et al. 2006; Lovley 2006). Electrochemically active biofilms are able to transfer electrons out of the cell and to the electrode surface directly using their outer membrane proteins or using electron transfer mediators (Logan and Rabaey 2012). Many bacterial species are capable of extracellular electron transfer. However, only a few are able to respire at extreme rates because of their conductive nature (Reguera et al. 2005). In particular, *Geobacter sulfurreducens* biofilms are able to respire on acetate using a solid electrode as the terminal electron acceptor (Bond and Lovley 2003). *G. sulfurreducens* biofilms form thick, stable biofilms on electrodes that tend to remain on the electrode surfaces even in the absence of acetate. These properties of *G. sulfurreducens* biofilms make it an excellent candidate for converting acetate to electricity on electrodes. It is not clear whether current generation by *G. sulfurreducens* biofilms is limited by acetate diffusion in biofilms. To address this question, Renslow *et al.* recently used an electrochemical nuclear magnetic resonance (EC-NMR) micro-imaging system to measure acetate concentration depth profiles *in situ* in biofilms (Renslow et al. 2013). The acetate concentration measurement limit was ~1 mM, and measurements were averaged over a 20 μm × 4 mm × 2 mm volume. It was found that a bulk acetate concentration above 2.3 mM does not limit the current from *G. sulfurreducens* (Tront et al. 2008). Therefore, a technology which can measure acetate concentration sensitively below mM concentrations is needed to understand acetate limitations in *G. sulfurreducens* biofilms. This is especially critical when a biofilm is thicker than hundreds of microns. Thus, a microelectrode capable of measuring acetate directly within biofilms in the micromolar range would prove useful in linking acetate concentration to local metabolisms in biofilms and identifying whether acetate completely limits biofilm growth. Since *G. sulfurreducens* biofilms can use electrodes as their sole electron acceptor under anaerobic conditions, a microelectrode in a needle-type microbiosensor can be used to detect acetate concentrations amperometrically. To the best of our knowledge, there is currently no microbiosensor which can measure μM-level acetate concentrations *in situ* within biofilms.

The goal of this work was to develop a microbiosensor based on *G. sulfurreducens* to measure local acetate concentrations *in situ* within biofilms. The developed acetate microbiosensor was based on the extracellular electrons generated during acetate consumption by *G. sulfurreducens* biofilm growing on the tip of a carbon microelectrode (this is referred to as a microelectrode-biofilm). Though the microelectrode-biofilm oxidizes acetate efficiently over other possible metabolites, we also tested the microbiosensor response to various alternative electron donors. Once we had developed and calibrated the microbiosensor, we tested it to measure acetate concentration depth profiles inside a *G. sulfurreducens* biofilm grown on a large glassy carbon electrode (macro-biofilm) when no current was passed and when current was passed.

2. Materials and Methods

2.1. Acetate microbiosensor construction

The construction of the acetate microbiosensor is detailed in the Supplementary Information (SI). Briefly, the microbiosensor was constructed by placing a carbon microelectrode and a bare Ag/AgCl reference electrode into a glass outer case as shown in Figure 1a. A photograph of the assembled microbiosensor is also shown in the SI. The carbon wire used to construct microbiosensors was a 30- μ m-diameter electrochemically activated carbon fiber (World Precision Instruments, Sarasota, FL, USA, catalog #C3005). The construction of the glass capillary—sealing the carbon wire, covering the carbon wire with glass, making the glass outer case out of a pulled Pasteur pipette, and making the silver/silver chloride reference electrode—has been detailed in the SI and previously (Nguyen et al. 2012). Following the construction of the microbiosensor parts, the microelectrode tip (diameter: 30 μ m) and the glass outer case tip (diameter: 30 μ m) were first assembled under 40 $\times$ microscope magnification using micromanipulators. The carbon microelectrode was inserted into the outer case, with its tip positioned ~200 μ m behind the outer case tip (Figure 1a). We should note that the carbon wire was completely covered by glass except for the tip, where the biofilm and carbon wire were electronically connected. The microelectrode was then glued to the outer case using five-minute epoxy. At least one hour was allotted to ensure that the drying process was completed. Finally, a Ag/AgCl reference electrode made of silver wire (Sigma-Aldrich, St. Louis, MO, USA, 0.25-mm diameter, $\geq 99.99\%$ pure, catalog #327034) was inserted and glued in place using five-minute epoxy. The *G. sulfurreducens* microelectrode-biofilm shown in Figure 1 was grown later on a carbon microelectrode after the microbiosensor parts were assembled.

2.2. Sensing mechanisms of the proposed acetate microbiosensor

The transducer is the microelectrode-biofilm inside the outer case (Figure 1). The medium containing acetate diffuses through the open tip with a ~30- μ m diameter. Then, acetate is oxidized by a pre-grown *G. sulfurreducens* biofilm on the carbon-wire microelectrode (Figure 1b) according to the following reaction (He and Angenent 2006):

Equation (1)

The electrons generated from this reaction are passed to the counter electrode (CE), which also acts as a reference electrode (RE). The reaction on the CE/RE is given below (Lewandowski and Beyenal 2014).

Equation (2)

We should note that since the current passed between the working electrode and RE is on the order of nanoamperes, we successfully used a pseudo Ag/AgCl RE. At this level of current, the RE was not damaged by the current. We also compared our measurements using a three-electrode system with a graphite counter electrode and the response was identical to that of a two-electrode system (results not shown). Therefore, we used a two-electrode microbiosensor in which the RE also served as the CE. As detailed later in this paper, the current generated from acetate oxidation (Equation 1) is correlated with the acetate concentration.

2.3. Bioelectrochemical reactor

G. sulfurreducens biofilms were grown on the carbon microelectrode tips within a temperature-controlled electrochemical cell (Gamry Instruments #990-00249) nearly identical to the setup used in (Babauta and Beyenal 2014b) but with the following modifications. The cell was operated in batch mode with 150 mL of growth medium. Microelectrodes replaced the macroelectrodes. The counter electrode was a graphite rod (Sigma-Aldrich #496545), and the reference electrode was a custom-made Ag/AgCl electrode filled with saturated KCl. A mixture of N₂/CO₂ (80%/20%) gas was sparged using a 0.2-μm filter to maintain anaerobic conditions. The complete electrochemical cell setup except for the microelectrodes and the reference electrode was autoclaved for 20 min at 121 °C. The growth medium was autoclaved separately in a 2-L autoclavable glass bottle (Corning #1395-2L) for 20 min at 121 °C. Once the growth medium cooled, 150 mL of it was aseptically added to the electrochemical cell. The reference electrode and the microelectrodes were sterilized, first using 70% v/v ethanol, then drying under UV exposure for 45 min prior to insertion into the electrochemical cell. Since the tip of the outer case was open, the tip was partially filled with sterilized growth medium, up to ~1 mm from the tip after insertion. The temperature was controlled using a Fisher Scientific Model 910 water bath at 30 °C circulated using the built-in glass jacket.

Figure 1. a) The acetate microbiosensor consists of a carbon microelectrode with a tip diameter of 30 μm , a Ag/AgCl reference electrode, and an outer case. b) Photo of the carbon microelectrode with *G. sulfurreducens* microelectrode-biofilm grown on the tip. Figure not drawn to scale.

2.4. Growth medium

The growth medium used to grow *G. sulfurreducens* strain PCA (ATCC 51573) biofilms was prepared according to the following recipe (Babauta et al. 2012): potassium chloride, 0.38 g/L; ammonium chloride, 0.2 g/L; sodium phosphate monobasic, 0.069 g/L; calcium chloride, 0.04 g/L; magnesium sulfate heptahydrate, 0.2 g/L; sodium bicarbonate, 2g/L; Wolfe's vitamin solution, 10 mL/L; modified Wolfe's mineral solution, 10 mL/L. Acetate was provided (20 mM). There was no fumarate or other soluble electron acceptor in the growth medium.

2.5. Growing microelectrode-biofilms

Prior to inoculation, the electrochemical cell was sparged with a gas mixture of N_2/CO_2 (80%/20%) for at least 24 h to remove oxygen from the system. Carbon microelectrodes were polarized continuously at +300 mV_{Ag/AgCl} using a Gamry Interface 1000 (Gamry Instruments, Warminster, PA) to measure a background current. Fifteen mL of *G. sulfurreducens* inoculum was then added, which was prepared according to a previously published procedure (Babauta et al. 2012). Within 24 h, a current response was observed. Figure 2a shows the microelectrode-biofilm current response for ~120 hours of growth after inoculation. Based upon prior work (Babauta and Beyenal 2014a), the current was expected to be ~40 nA, although longer growth times could result in current up to 60 nA. Cyclic voltammograms (CVs) were taken for each microelectrode at t=24 h and t=160 h. A representative microelectrode-biofilm CV is shown in Figure 2b. At t=24 h before the inoculation, the current response was flat, while a CV taken after the growth (at t=160 h) is characteristic of *G. sulfurreducens* biofilm. The limiting current was

observed after $-200\text{ mV}_{\text{Ag/AgCl}}$ (Fricke et al. 2008). A photo of the microelectrode-biofilm is shown in Figure 1d.

Figure 2. a) Current response of the *G. sulfurreducens* microelectrode-biofilm growth on the microelectrode tips. b) Cyclic voltammograms of *G. sulfurreducens* microelectrode-biofilm grown on a carbon microelectrode tip.

2.6. Calibration of the acetate microbiosensor

A calibration cell identical to the biofilm growth cell was used for the calibration. However, the medium was replaced with deoxygenated acetate-free medium. The microbiosensor was removed from the growth cell and $\sim 1\text{ mL}$ of deoxygenated acetate-free (non-turnover) medium was used to purge the outer case of medium containing acetate. After two rinses, the acetate microbiosensor was inserted into the calibration cell and was polarized at $+300\text{ mV}_{\text{Ag/AgCl}}$. The potential of $+300\text{ mV}_{\text{Ag/AgCl}}$, in the limiting current region (Figure 2b), was selected as the operation potential of the acetate microbiosensor. The microbiosensor current was allowed to reach a steady state before calibration. Aliquots of acetate solution from 1 M sterilized deoxygenated stock was added to make 50 , 100 , 200 , 400 , 800 , and $1600\text{ }\mu\text{M}$ acetate concentrations.

2.7. Testing for interference of electron donors and stirring effect

Interference from lactate, formate, and pyruvate was tested by calibrating the microbiosensor in 1 mM solutions of these compounds. Interference from hydrogen was tested by equilibrating the calibration solution with a gas mixture of He/H_2 ($92\%/8\%$). Based on Henry's Law, the dissolved hydrogen concentration was calculated to be $64\text{ }\mu\text{M}$ at $30\text{ }^\circ\text{C}$ (Sander 2015). The interference responses observed for alternative electron donors were normalized with the acetate response to the same concentration of alternative electron donors and are reported as percentages of the response to acetate.

Since we used an open tip filled with liquid medium, it was necessary to determine whether this membraneless tip is immune to the stirring effect. To test this, a magnetic stirrer (IKA, catalog #2669923) and a stir bar with a 2-cm diameter were used to agitate the calibration solution at 50 , 100 , 150 , and 200 rpm . The rpm values refer to the rotation rate of the stir bar at

the base of the calibration chamber. Under these conditions, calibration curves were obtained and their slopes compared.

2.8. Preparing electrode-respiring *G. sulfurreducens* biofilms to test the microbiosensor

The utility of the acetate microbiosensor was tested by measuring acetate concentration profiles within macroscale *G. sulfurreducens* biofilms as described in Babauta *et al.* (Babauta *et al.* 2012). The biofilms were grown on a glassy carbon working electrode (SPI-GlasTM grade 11; 25 mm × 25 mm × 2 mm) placed in a three-electrode bioreactor. A flat plate reactor which allowed us to perform microbiosensor measurements was used. The counter electrode was graphite (ground finish isomolded graphite plates, Glassmate grade GM-10, 25 mm × 25 mm × 3 mm, Poco Graphite, Inc., Decatur, TX), and the reference electrode was a Ag/AgCl wire in 4 M KCl saturated with AgCl. All protocols were followed as in (Babauta *et al.* 2012) except that the reactor was operated in fed-batch with a 2-mL/min recirculation rate. The medium was replaced with fresh medium when the acetate was depleted in the medium. For acetate concentration depth profile measurements, the acetate concentration in the reactor was set to 1.5 mM by considering the linear calibration range of the developed microbiosensor. The current generation of the *G. sulfurreducens* macro-biofilm during depth profile measurements at +300 mV_{Ag/AgCl} was $865 \pm 38 \mu\text{A}$, and the open circuit potential (OCP) during OCP depth profile measurements was $-441 \pm 3 \text{ mV}_{\text{Ag/AgCl}}$.

2.9. Measuring acetate concentration depth profiles in *G. sulfurreducens* biofilm

First, a custom-built microprofiling system was set up around the biofilm reactor (Babauta *et al.* 2012). A three-axis micromanipulator with both a coarse (vertical) range of 10 cm and a motorized precision linear actuator (PI M-230.10S, Physik Instrumente, Auburn, MA, USA) controlled using custom-made LabVIEW software (National Instruments, Austin, TX, USA) was used to position the microbiosensor tip. The vertical range of the motorized actuator was 8 mm with a submicron step resolution. An adjacent stereomicroscope (Zeiss Stemi 2000) was used to locate the tip of the microbiosensor held by the micromanipulator. After the microprofiling system was set up, the microbiosensor was placed within the micromanipulator arm. Subsequently, the biofilm reactor was opened by removing the rubber stopper above the biofilm electrode. Using the coarse knobs on the micromanipulator, the microbiosensor tip was moved above the open port, lowered into the reactor volume, and positioned to approximately 100 μm from the macro-biofilm surface. Custom-developed microprofiler software was then used to automate the measurement of acetate by depth from 100 μm above the macro-biofilm surface to the electrode surface in 10- μm increments. The software automatically recorded more than 20 data points for 100 secs for each location and reported the average and the standard deviation.

3. Results and Discussion

3.1. Calibration of the microbiosensor

The acetate microbiosensor is based on the conversion of acetate to electricity by a pregrown *G. sulfurreducens* microelectrode-biofilm. Initially the microelectrode-biofilm was kept in acetate-free solution within the outer case. Under this condition, the microelectrode-biofilm was dormant but capable of responding to acetate. Figure 3a shows the response of the microbiosensor to acetate additions starting from a baseline of zero acetate. The response time is defined as the time needed to reach 90% of the final response (de Beer *et al.* 1997a; Suzuki *et al.*

1999). The average response time of the acetate microbiosensor was 0.86 ± 0.59 min within the 0–1.6 mM calibration range, and the response time was concentration-dependent above 0.4 mM of acetate. While the response time was 0.54 ± 0.11 min for the 0.05, 0.1, 0.2 and 0.4 mM acetate concentrations, it was 1 min and 2 min for the 0.8 mM and 1.6 mM acetate concentrations, respectively. This information provides the time that elapses before the microbiosensor is ready to measure another sample. Figure 3b shows the correlation between the bulk acetate concentration and current generation. The inset extends the data points up to 6.4 mM acetate to show that acetate saturation occurred at 3.2 mM. This is consistent with previous work (Tront et al. 2008), in which 2.3 mM acetate provided the maximum current output and no increase in current was observed when the acetate concentration was above 2.3 mM. In addition, this type of nonlinear response is expected for a biosensor (Lewandowski and Beyenal 2014). The microbiosensor showed a linear response between 0.05 mM and 1.6 mM acetate concentrations with a 79 ± 8 μ M limit of detection (S/N = 2) obtained from three independent calibrations.

We constructed multiple microbiosensors and calibrated them. We note that each microbiosensor had a unique calibration curve based on the mass of pre-grown biofilm on the microelectrode tip. We found that the calibration range was consistent but the variation in the slopes was 1.98 ± 0.95 nA/mM (n=3). The background current varied between 0 and 15 nA. The background current could be reduced to zero nA if we waited long enough. However, the background current did not cause any impact on the calibration.

We should note that the current density of the microelectrode-biofilm was 56.6 A/m² at the end of 160 hours of growth. In replicated experiments, the current density varied between 42.4 and 70.7 A/m². This current density was extremely high compared to that of *G. sulfurreducens* biofilms growing on larger electrodes (Babauta et al. 2012; Nevin et al. 2008). For example, in our system, for the biofilms growing on the larger electrode (where we measured acetate concentration depth profiles) the maximum current density was 3.88 A/m². In addition, Nevin *et al.* reported a current density of 3.1 A/m² on graphite anodes (Nevin et al. 2008). The reasons for these high current densities for smaller electrodes are not known and are still in debate (Beyenal and Babauta 2015; Call and Logan 2011). In our case, the high current density generated by microelectrode biofilms and their small size allow them to be more sensitive and have a quicker response time. The superiority of microbiosensors over their larger-size equivalents is already well known in the literature (Bard and Faulkner 2000; Lewandowski and Beyenal 2014).

Figure 3. a) Acetate microbiosensor responses to acetate additions marked with arrows. Each addition corresponds to a concentration shown in b). b) Calibration between 0 and 1.6 mM acetate concentrations. The inset figure shows the variation of current with acetate concentration for 0–6.4 mM acetate concentrations.

3.2. Interfering effects of alternative electron donors

G. sulfurreducens biofilms are expected to use alternative electron donors. Lactate, formate, pyruvate, and hydrogen could illicit current responses that would interfere with acetate measurement (Speers and Reguera 2012). Therefore, we tested the current responses of the acetate microbiosensor to these alternative electron donors at their equivalent concentrations of acetate. Figure 4 shows that the presence of lactate, formate, pyruvate, or hydrogen induced an increase in current of $11\% \pm 1.7\%$, $39\% \pm 6.2\%$, $15\% \pm 3.0\%$, or $19\% \pm 3.7\%$, respectively. The microelectrode-biofilm is most sensitive towards formate, indicating that variation in formate concentration should be accounted for. However, when the measurements were performed without these alternative electron donors, the acetate microbiosensor provided reliable results.

Figure 4. Response of the acetate microbiosensor to a range of substrates measured as a percentage of the acetate response.

3.3. Interfering effect of convection on the microbiosensor response

The acetate microbiosensor employs a 200- μm -long cylindrical open diffusion barrier that separates the microelectrode-biofilm from the external environment. Because it is open to the external environment, however, we had to ensure that convection did not alter the microbiosensor response. Figure 5 shows the effect of stirring on the acetate microbiosensor calibration from 0 mM to 1.6 mM bulk acetate concentration. The rpm value refers to the rotation rate of the stir bar at the base of the calibration chamber, and the Reynolds numbers (Re) calculated for 50, 100, 150 and 200 rpm stirring rates are 333, 667, 1000, and 1333, respectively. It has been reported that $\text{Re} = 100$ is the transition from laminar flow to turbulent flow and $\text{Re} = 1000$ is highly turbulent flow (Nauman 2008). The tests at a wide range of Re confirmed that we tested microbiosensors in laminar and turbulent zones. From 50 rpm to 200 rpm, there was no detectable effect on the calibration slope. This proves that the diffusion barrier adequately separated the internal environment of the acetate microbiosensor from the external environment and verified that the diffusion barrier acted as a membrane.

Figure 5. Effect of stirring on the acetate microbiosensor calibration slope at stirring rates of 50, 100, 150, and 200 rpm. There is no detectable effect on the calibration slopes.

3.4. Acetate concentration depth profile measurements within a *G. sulfurreducens* biofilm

Figure 6 shows the acetate concentration depth profiles measured within *G. sulfurreducens* biofilm when 1) no current was passed at the open circuit potential and 2) current was passed at a polarization potential of +300 mV_{Ag/AgCl}. At the open circuit potential, the acetate concentration remained at bulk values throughout the macro-biofilm regardless of location. When current was allowed to pass, a decrease in acetate was observed from the bulk value to below the detection limit at the base of the biofilm. A similar result for acetate utilization in substantially thicker *G. sulfurreducens* biofilms was observed using EC-NMR (Renslow et al. 2013). However, the minimum detection limit of the acetate microbiosensor described here was substantially improved, from mM to μM . The depth profile demonstrates that the biofilm consisted of two layers: a top layer oxidizing acetate and a bottom without acetate. The bottom of the biofilm served as a scaffold for the acetate-oxidizing layer to transfer electrons to the electrode. This finding agrees with the previous publication demonstrating a two-layered *G. sulfurreducens* biofilm with a metabolically active top and an acetate-limited bottom (Renslow et al. 2013).

Figure 6. Acetate concentration depth profiles in *G. sulfurreducens* biofilms in OCP mode and at +300 mV_{Ag/AgCl} polarization. The photo on the right shows the biofilm and the acetate microbiosensor entering the biofilm. In some cases the error bar is smaller than the average and is not visible.

3.5. Potential applications of the acetate microbiosensor

Although our primary goal in developing the acetate microbiosensor was to understand the acetate limitations in electrode-respiring biofilms, the use of the developed microbiosensor can be extended to various applications in which acetate is utilized as a substrate. The developed microbiosensor can be used to measure acetate concentrations either in bulk solutions or inside biofilms *in situ*. For example, acetate is consumed or produced during anaerobic conversion in wastewater treatment and in anaerobic digestion processes (Estevez-Canales et al. 2015; Henze 2008). In addition, there is a need to measure acetate concentrations in the polyphosphate-accumulating biofilms that use acetate as the main electron donor in biological phosphorus removal processes (Meyer et al. 2002; Sudiana et al. 1999). Overall, the developed microbiosensor can be used to measure acetate concentration and its variation by depth in many anaerobic biofilms in μM resolution. It is also advantageous to use a microbiosensor rather than a macroscale sensor to monitor acetate concentrations in bulk solutions because the small size of the electrode tip provides faster response times, lower detection limits and higher resolutions than a large electrode (Beyenal and Babauta 2014). The developed microbiosensor can be modified for electron acceptors such as nitrate and fumarate since *G. sulfurreducens* biofilms can reduce nitrate and fumarate when a solid electrode is used as an electron donor (Choi and Sang 2016; Gregory et al. 2004; Pous et al. 2016). To achieve this, the operation of the microbiosensor should be switched to a cathodic mode by setting an appropriate potential. Therefore, the developed membraneless acetate microbiosensor can provide a platform for developing microbiosensors that detect organic compounds other than acetate.

4. Conclusions

In this work, we developed and tested an acetate microbiosensor based on *G. sulfurreducens*. The microbiosensor consisted of a 30- μm -thick carbon microelectrode as the working electrode with *G. sulfurreducens* biofilm on the tip and a Ag/AgCl reference electrode, all enclosed in a glass outer case. The microbiosensor was operated at +300 mV_{Ag/AgCl}. The microbiosensor was calibrated between 0 and 6.4 mM acetate concentrations and showed a linear response between 0 and 1.6 mM acetate concentrations with a $79 \pm 8 \mu\text{M}$ limit of detection (S/N=2). The microbiosensor was immune to the stirring effect. Using the acetate microbiosensor, we successfully measured acetate concentrations inside *G. sulfurreducens* biofilm grown on a glassy carbon electrode. The measurements revealed that the acetate concentration was at the bulk concentration throughout the biofilm at the open circuit potential, when the biofilm was not respiring, but that the acetate concentration decreased below the detection limit within the 200- μm thickness of the biofilm when the electrode was polarized at +300 mV_{Ag/AgCl}. These measurements demonstrate that the biofilm consisted of two layers: a top oxidizing acetate and a bottom without acetate. The bottom of the biofilm served as a scaffold for the acetate-oxidizing layer to transfer electrons to the electrode.

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References

- Babauta, J.T., Beyenal, H., 2014a. *J. Electrochem. Soc.* 161, H3070-H3075.
- Babauta, J.T., Beyenal, H., 2014b. *Biotechnol. Bioeng.* 111, 285-294.
- Babauta, J.T., Nguyen, H.D., Harrington, T.D., Renslow, R., Beyenal, H., 2012. *Biotechnol. Bioeng.* 109, 2651-2662.
- Bard, A.J., Faulkner, L.R., 2000. *Electrochemical Methods: Fundamentals and Applications*. Wiley.
- Beyenal, H., Babauta, J., 2014. Microsensors and Microscale Gradients in Biofilms, in: Muffler, K., Ulber, R. (Eds.), *Productive Biofilms*. Springer International Publishing, pp. 235-256.
- Beyenal, H., Babauta, J.T., 2015. *Biofilms in Bioelectrochemical Systems: From Laboratory Practice to Data Interpretation*. Wiley.
- Beyenal, H., Davis, C.C., Lewandowski, Z., 2004. *Sensor. Actuat. B-Chem.* 97, 202-210.
- Bond, D.R., Lovley, D.R., 2003. *Appl. Environ. Microbiol.* 69, 1548-1555.
- Call, D.F., Logan, B.E., 2011. *Biosens. Bioelectron.* 26, 4526-4531.
- Choi, O., Sang, B.-I., 2016. *Biotechnol. Biofuels* 9, 1-14.
- Cronenberg, C.C.H., van den Heuvel, J.C., 1991. *Biosens. Bioelectron.* 6, 255-262.
- Damgaard, L.R., Revsbech, N.P., 1997. *Anal. Chem.* 69, 2262-2267.
- de Beer, D., Glud, A., Epping, E., Kùhl, M., 1997a. *Limnol. Oceanogr.* 42, 1590-1600.
- De Beer, D., Schramm, A., Santegoeds, C.M., Kuhl, M., 1997b. *Appl. Environ. Microbiol.* 63, 973-977.
- Du, Z., Li, H., Gu, T., 2007. *Biotechnol. Adv.* 25, 464-482.
- Estevez-Canales, M., Berná, A., Borjas, Z., Esteve-Núñez, A., 2015. *Energies* 8, 12366.
- Fricke, K., Harnisch, F., Schroder, U., 2008. *Energy Environ. Sci.* 1, 144-147.
- Gregory, K.B., Bond, D.R., Lovley, D.R., 2004. *Environ. Microbiol.* 6, 596-604.
- Hamelers, H.M., Ter Heijne, A., Sleutels, T.J.A., Jeremiasse, A., Strik, D.B.T.B., Buisman, C.N., 2010. *Appl. Microbiol. Biotechnol.* 85, 1673-1685.
- He, Z., Angenent, L.T., 2006. *Electroanal.* 18, 2009-2015.
- Henze, M., 2008. *Biological Wastewater Treatment: Principles, Modelling and Design*. IWA Pub.
- Jeroschewski, P., Steuckart, C., Kùhl, M., 1996. *Anal. Chem.* 68, 4351-4357.
- Larsen, L.H., Revsbech, N.P., Binnerup, S.J., 1996. *Appl. Environ. Microbiol.* 62, 1248-1251.
- Lee, J.-H., Seo, Y., Lim, T.-S., Bishop, P.L., Papautsky, I., 2007. *Environ. Sci. Technol.* 41, 7857-7863.
- Lewandowski, Z., Beyenal, H., 2003. Use of microsensors to study biofilms, in: Lens, P., O'Flaherty, V., Moran, A., Stoodley, P. and Mahony, T. (Eds.), *Biofilms in Medicine, Industry and Environmental Biotechnology: Characteristics, Analysis and Control*. IWA Publishing, London, pp. 375-412.
- Lewandowski, Z., Beyenal, H., 2014. *Fundamentals of Biofilm Research*, Second Edition. Taylor & Francis.
- Lewandowski, Z., Lee, W.C., Characklis, W.G., Little, B., 1989. *Corrosion* 45, 92-98.
- Liu, X., Roe, F., Jesaitis, A., Lewandowski, Z., 1998. *Biotechnol. Bioeng.* 59, 156-162.
- Logan, B.E., 2009. *Nat. Rev. Microbiol.* 7, 375-381.
- Logan, B.E., Hamelers, B., Rozendal, R., Schröder, U., Keller, J., Freguia, S., Aelterman, P., Verstraete, W., Rabaey, K., 2006. *Environ. Sci. Technol.* 40, 5181-5192.
- Logan, B.E., Rabaey, K., 2012. *Science* 337, 686-690.
- Lovley, D.R., 2006. *Curr. Opin. Biotechnol.* 17, 327-332.

457 McLamore, E.S., Shi, J., Jaroch, D., Claussen, J.C., Uchida, A., Jiang, Y., Zhang, W., Donkin,
 458 S.S., Banks, M.K., Buhman, K.K., Teegarden, D., Rickus, J.L., Porterfield, D.M., 2011.
 459 Biosens. Bioelectron. 26, 2237-2245.
 460 Meyer, R.L., Larsen, L.H., Revsbech, N.P., 2002. Appl. Environ. Microbiol. 68, 1204-1210.
 461 Nauman, E.B., 2008. Chemical Reactor Design, Optimization, and Scaleup. Wiley.
 462 Nevin, K.P., Richter, H., Covalla, S.F., Johnson, J.P., Woodard, T.L., Orloff, A.L., Jia, H.,
 463 Zhang, M., Lovley, D.R., 2008. Environ. Microbiol. 10, 2505-2514.
 464 Nguyen, H.D., Renslow, R., Babauta, J., Ahmed, B., Beyenal, H., 2012. Sensor. Actuat. B-
 465 Chem. 161, 929-937.
 466 Peteu, S.F., Emerson, D., Mark Worden, R., 1996. Biosens. Bioelectron. 11, 1059-1071.
 467 Pous, N., Carmona-Martínez, A.A., Vilajeliu-Pons, A., Fiset, E., Bañeras, L., Trably, E.,
 468 Balaguer, M.D., Colprim, J., Bernet, N., Puig, S., 2016. Biosens. Bioelectron. 75, 352-358.
 469 Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., Lovley, D.R., 2005.
 470 Nature 435, 1098-1101.
 471 Renslow, R.S., Babauta, J.T., Dohnalkova, A.C., Boyanov, M.I., Kemner, K.M., Majors, P.D.,
 472 Fredrickson, J.K., Beyenal, H., 2013. Energy Environ. Sci. 6, 1827-1836.
 473 Sander, R., 2015. Atmos. Chem. Phys. 15, 4399-4981.
 474 Speers, A.M., Reguera, G., 2012. Appl. Environ. Microbiol. 78, 437-444.
 475 Sudiana, I.M., Mino, T., Satoh, H., Nakamura, K., Matsuo, T., 1999. Water Sci. Technol. 39,
 476 29-35.
 477 Suzuki, H., Arakawa, H., Sasaki, S., Karube, I., 1999. Anal. Chem. 71, 1737-1743.
 478 Tront, J.M., Fortner, J.D., Plötze, M., Hughes, J.B., Puzrin, A.M., 2008. Biosens. Bioelectron.
 479 24, 586-590.
 480 VanHoudt, P., Lewandowski, Z., Little, B., 1992. Biotechnol. Bioeng. 40, 601-608.

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