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Electricity-Assisted Biological Hydrogen Production from Acetate by *Geobacter sulfurreducens*

JEANINE S. GEELHOED*,† AND ALFONS J. M. STAMS

Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands

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Geobacter sulfurreducens is a well-known current-producing microorganism in microbial fuel cells, and is able to use acetate and hydrogen as electron donor. We studied the functionality of *G. sulfurreducens* as biocatalyst for hydrogen formation at the cathode of a microbial electrolysis cell (MEC). *Geobacter sulfurreducens* was grown in the bioelectrode compartment of a MFC with acetate as the substrate and reduction of complexed Fe(III) at the counter electrode. After depletion of the acetate the electrode potential of the bioelectrode was decreased stepwise to −1.0 V vs Ag/AgCl reference. Production of negative current was observed, which increased in time, indicating that the bioelectrode was now acting as biocathode. Headspace analyses carried out at electrode potentials ranging from −0.8 to −1.0 V showed that hydrogen was produced, with higher rates at more negative cathode potentials. Subsequently, the metabolic properties of *G. sulfurreducens* for acetate oxidation at the anode and hydrogen production at the cathode were combined in one-compartment membraneless MECs operated at applied voltages of 0.8 and 0.65 V. After two days, current densities were 0.44 A m^{-2} at 0.8 V applied voltage and 0.22 A m^{-2} at 0.65 V, using flat-surface carbon electrodes for both anode and cathode. The cathodic hydrogen recovery ranged from 23% at 0.5 V applied voltage to 43% at 0.9 V.

Introduction

Microbial electrochemical systems have recently been developed for the generation of electrical energy and hydrogen from organic matter (*1, 2*). Electrochemically active microorganisms oxidize organic compounds and transfer the generated electrons to the anode. In a microbial fuel cell (MFC), a terminal electron acceptor (often oxygen) is reduced at the cathode, and electrical current is produced (*2*). In a microbial electrolysis cell (MEC), no oxygen or other alternative electron acceptors are present, resulting in the reduction of protons to hydrogen gas at the cathode. This process is supported by the input of an additional voltage to render an exergonic reaction (*1, 3*). The production of hydrogen requires a good catalyst, and for this platinum, nickel, and stainless steel have been investigated (*3, 4*). In addition, microorganisms in combination with a (noncatalytic) electrode that serves as the current source may be employed as catalysts. The functionality of a hydrogen-producing biocathode was shown in a system with a chemical anode reaction (oxidation of hexacyanoferrate(II)) (*5*) and recently also in a MEC (*6*).

Studies on electrochemically active microorganisms have largely focused on electron transfer to the anode of MFCs. The mechanisms involved in electron transfer are either direct, utilizing specific membrane proteins and cell appendages, or indirect by utilization of electron shuttle compounds, for example, flavins, phenazines, or sulfur compounds [refs *7, 8* and references therein]. One of the most well-studied electrochemically active bacteria is *Geobacter sulfurreducens* which uses *c*-type cytochromes and multicopper proteins for extracellular electron transfer (*9*−*11*). Analyses of the anodic microbial community upon application of a voltage in a MEC compared to MFC operation have shown the dominance of *Beta-* and *Deltaproteobacteria* (including *Geobacter* sp.) (*12, 13*). Recently, the activity of *Geobacter* sp. in a MEC anode was demonstrated (*14*).

In the experiments with a hydrogen-producing biocathode, the microbial community of the biocathodes was not analyzed (*5, 6*). However, a number of general criteria should be met by microorganisms in order to have an active role in hydrogen formation at the cathode (*7*). They should be able to utilize the cathode surface as electron donor, possibly by using similar mechanisms, but in opposite direction, as extracellular electron transfer to the anode, and application of a voltage should not be inhibitory. In addition, hydrogenase activity is required to catalyze the conversion of electrons and protons to hydrogen. Based on its physiological characteristics, we reasoned that *G. sulfurreducens* may be a good candidate for biocatalytic hydrogen formation at the cathode. *G. sulfurreducens* is electrochemically active at the anode of the MFC and MEC where it uses the electrode as electron acceptor (*14, 15*). In addition, *G. sulfurreducens* was found to be electrochemically active at the cathode of MFCs, where electrons donated by the cathode were used to anaerobically respire fumarate (*16, 17*). In these experiments also the production of small amounts of hydrogen was noted (*17*). In the genome of *G. sulfurreducens* four hydrogenases are encoded, of which two are predicted to be present in the cytoplasm and two are periplasmically oriented membrane-bound hydrogenases. One of the latter is responsible for hydrogen uptake by *G. sulfurreducens* (*18, 19*).

Here, we describe the hydrogen-producing activity of *G. sulfurreducens* at a graphite cathode in a microbial half-cell with oxidation of hexacyanoferrate(II) as the anode reaction. Hydrogen production was also assessed in a single chamber membraneless MEC with a graphite anode and cathode. This system was inoculated with *G. sulfurreducens* as the biocatalyst for both the anodic and cathodic reaction.

Materials and Methods

Strain and Cultivation. *Geobacter sulfurreducens* strain PCA (DSM 12127^T) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). It was cultured with acetate as the electron donor and fumarate as the electron acceptor in anaerobic medium in closed bottles with a N_2/CO_2 (80%:20%) headspace. The medium consisted of (mM) NaCl 5.1, $MgCl_2$ 0.5, $CaCl_2$ 0.75, NH_4Cl 5.6, Na_2SO_4 0.35, KH_2PO_4 3, Na_2HPO_4 3, $NaHCO_3$ 50, supplemented with trace elements (*20*), selenite/tungstate solution (1 mL/L (*21*)), vitamins (2 mL/L (*22*)) and resazurin (0.5 mg/L).

* Corresponding author phone: +49 421 2028823; fax: +49 421 2028690; e-mail: jgeelhoe@mpi-bremen.de.

† Present address: Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, 28359 Bremen, Germany.

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***G. sulfurreducens* Bioanode Inverted to Biocathode.** A microbial half-cell was setup, consisting of polycarbonate anode and cathode chambers (volume 33 mL each, width perpendicular to the electrode 15 mm) separated by a cation exchange membrane (Fumasep FKS, Fumatech, St. Ingbert, Germany) (Supporting Information (SI) Figure S1). Nonporous flat-surface graphite (MR200, gastight impregnated, Müller & Rössner, Troisdorf, Germany; surface area in contact with solution 22 cm^2) was used for both anode and cathode. For the chemical half-reaction, 1.5 L anoxic 50 mM K-hexacyanoferrate(II) + 50 mM K-hexacyanoferrate(III) in 10 mM phosphate buffer at pH 7, was cycled through one of the chambers and a stirred vessel, at a rate of 0.85 L h^{-1}. The chamber was filled with graphite felt (National Electrical Carbon B.V., Hoorn, The Netherlands) that was in contact with the graphite electrode, to facilitate the chemical reaction. Medium (1 L) was cycled through the other chamber and a stirred vessel (headspace 0.45 L), also at a rate of 0.85 L h^{-1}. Both anolyte and catholyte solutions were purged with N_2 unless stated otherwise. The system was kept at 33−35 °C. The basal medium as above was used, except without resazurin and with 10 mM phosphate buffer at pH 7 instead of $NaHCO_3/CO_2$ as the buffer. Acetate was added at a concentration of about 2 mM. During the experiment, the medium pH was kept at 7 by dosing 1 M HCl or 1 M NaOH. Initially, the system was operated as MFC with a resistor, and cell voltage and anode potential were recorded every 5 min. The anode potential was measured against a Ag/AgCl/3 M KCl reference electrode (SSE) that was in contact with the medium. The cathode potential (also vs SSE) was measured occasionally. During potentiostatic operation, potentials and current production were recorded every minute, and every 10 s during 30 min voltammetric tests. Cell voltage was measured using a multimeter (Fluke 70, Fluke Corporation, Everett, WA, USA).

Forty mL of *G. sulfurreducens* batch culture grown to mid-to-end log phase was filtered over a 0.2 μm filter under anaerobic conditions and the cells resuspended in anaerobic phosphate buffer (pH 7). The biological compartment was inoculated with this suspension, and during the experiment the purity of the culture was checked by microscopy. At the initial MFC stage, an external resistor of 1 kΩ was applied. After current production commenced and the anode potential had decreased to below −0.36 V (SSE), a potentiostat (Bank Electronik, Pohlheim, Germany) was used to maintain the electrode potential at −0.3 V (SSE). Two independent experiments (I and II) were carried out using this setup. At t = 8.5 h (exp I) or 9.6 h (exp II) after depletion of the acetate, the potential of the electrode was lowered stepwise to −1.0 V (in 0.1 V steps for 30 min per step) and the corresponding current recorded. Experiment II was continued at a controlled electrode potential of −0.9 V for 9.2 days, when a second potential step voltammetry run was carried out. After another 2.8 days at −0.9 V, N_2 purging was stopped and hydrogen accumulation was measured at an electrode potential of −0.8 V for 40 h, −0.9 V for 19 h, and −1.0 V for 7.5 h.

***G. sulfurreducens* One-Compartment Membraneless MEC.** One polycarbonate chamber (as above) was mounted between two graphite electrodes. One L of medium was cycled through the chamber and a stirred recirculation vessel (headspace 0.45 L). The medium was purged with N_2, unless otherwise indicated, and the pH maintained at 7. Forty mL of culture was filtered, the cells resuspended and used for inoculation. Five mM acetate was added. Two experiments were carried out: in experiment III the system was initially operated at an applied voltage of 0.8 V and in experiment IV at 0.65 V. During subsequent operation, the applied voltage was varied (see Results and Discussion and SI Figures S2 and S3). Anode potential and current output were recorded every min. The cathode potential was measured occasionally, but could also be calculated from the applied cell voltage and the anode potential.

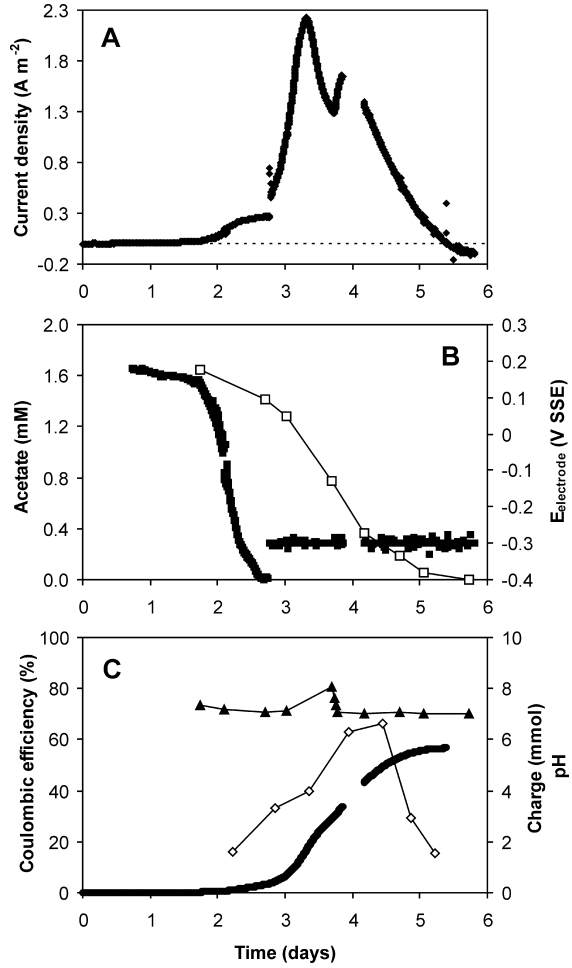

FIGURE 1. Start-up of the bioanode with *G. sulfurreducens* and inversion to biocathode. (A) Observed current density (◆) during operation as a MFC with an external resistor of 1 kΩ (up to 2.8 days), and using an electrode potential controlled at −0.3 V. (B) Acetate concentration in the inoculated compartment (□) and observed (until 2.8 days) or controlled potential of the bioanode/biocathode (■). (C) Coulombic efficiency during MFC operation (◇), total transferred charge (●), and pH in the bioelectrode compartment (▲).

Chemical Analyses. Acetate was analyzed by HPLC separation on a Varian Metacarb 67H 300 mm column and quantification using refractive index (RI-150, Thermo Electron Corporation). Hydrogen was analyzed by gas chromatography (Hitachi GC 14B equipped with a thermal conductivity detector and molecular sieve column).

Results and Discussion

Start-Up of *G. sulfurreducens* Bioanode and Inversion to Biocathode. Inoculation of the biological anode compartment of the MFC resulted in the consumption of acetate and a gradual increase in the cell voltage to approximately 0.6 V with a current density of 0.28 A m^{-2} electrode area (Figure 1). Previous research has shown that increased current production in a MFC with *G. sulfurreducens* could be attributed to biomass accumulation on the electrode (*23*). During the first 2.8 days of our experiments, the anode potential decreased from +0.22 to −0.39 V SSE (Figure 1B). At this point the current production was restricted by the external load that was applied on the MFC. Subsequent potentiostatic operation of the MFC at a controlled anode potential of −0.3 V SSE resulted in a steep increase in the

current production to 2.2 A m^{-2} (Figure 1A). The decline in the current density at $t = 3.4-3.9$ days was attributed to a gradual but temporary increase in the pH from 7 to 8 (Figure 1C), which resulted from erroneous NaOH dosing by the automatic pH stat system. From day 4 onward, a lowering of the current production was observed coinciding with the decrease in the acetate concentration over time. The Coulombic efficiency (CE) of current production from the oxidation of acetate ranged from 16 to 66% during the period of MFC operation (Figure 1C). At the start of the experiment, when current densities are low, the presence of traces of oxygen that may be used as alternative electron acceptor will have a marked influence on the CE. After 3.5 days, higher current densities were achieved with a CE of approximately 65%, which likely reflects the investment of acetate for biomass production. During acetate limitation, low values for CE were observed.

After 5.4 days of operation, reversal of current flow was observed. This suggests that acetate was depleted, which was confirmed by analysis of the acetate concentration at $t = 5.7$ days. Reversal of current flow can be interpreted by a change in the reaction at the bioelectrode from anodic to cathodic. Instead of acetate oxidation to bicarbonate, protons, and electrons, an electron acceptor is reduced at the cathode. Cathodic reactions that may occur are the reduction of oxygen to water, or at lower potentials, the combination of protons and electrons to form hydrogen. At a low hydrogen concentration in a N_2-purged system, the midpoint equilibrium potential for hydrogen formation from protons and electrons is −0.49 V vs SSE (pH 7, H_2 10 Pa).

The current density in response to the cathode potential was assessed for both systems starting 8.5 or 9.6 h after current reversal. Compared to an uninoculated system, current production in the system with *G. sulfurreducens* was approximately 0.1 to 0.25 A m^{-2} larger, that is, yielding more negative current density values (Figure 2A). During a subsequent 9.2 days of operation of the biocathode in experiment II at −0.9 V, the current density increased from −0.43 to −1.2 A m^{-2}. After a total of 15 days of operation at a cathode potential of −0.8 to −1.0 V, the current density response to cathode potential was evaluated again. The current density was larger (more negative values) with lower cathode potential and ranged from −0.7 to −2.4 A m^{-2} for cathode potentials from −0.5 to −1.0 V. Particularly high current densities were reached at cathode potentials of −0.9 and −1.0 V (Figure 2A).

***G. sulfurreducens* Catalyzes Hydrogen Formation at the Cathode.** Hydrogen production in the biocathode was analyzed for cathode potentials of −0.8, −0.9, and −1.0 V in the time period of 12−15 days after reversal of current flow (Figure 2B). The production of hydrogen increased with lower cathode potential. Also, much more hydrogen was produced by the biocathode compared to an uninoculated control at a cathode potential of −0.9 V. The shape of the hydrogen accumulation curves shows that some hydrogen was lost from the cathodic compartment, which is probably due to transport of hydrogen over the membrane to the anodic compartment (*3*). Therefore, only the first two data points per curve were used to calculate the rate of H_2 production and the cathodic hydrogen recovery, which is defined as the hydrogen produced relative to the possible production based on the measured current. Hydrogen production amounted to 4.1 μmol h^{-1} at −0.8 V, 13 at −0.9 V and 24 μmol h^{-1} at −1.0 V cathode potential. These cathode potentials corresponded to cell voltages of 1.07−1.27 V. The cathodic hydrogen recovery of the biocathode increased with lower cathode potential and was 32% at a cathode potential of −0.8 V, 53% at −0.9 V and 56% at −1.0 V.

Hydrogen production in the chamber that was inoculated with *G. sulfurreducens* indicates that the system was successfully inverted from a bioanode for the oxidation of acetate to a biocathode in which hydrogen is produced from protons

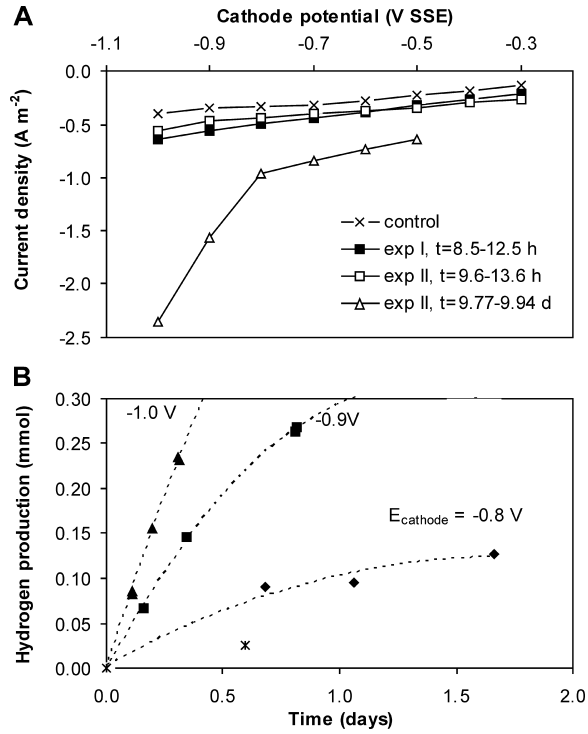

FIGURE 2. Performance of the *Geobacter sulfurreducens* biocathode. (A) Cathodic current production at a cathode potential of −0.3 to −1.0 V measured 8−10 h after charge reversal (experiments I and II) and after a subsequent period of 9.2 days at a cathode potential of −0.9 V (experiment II) (B) Hydrogen production by the biocathode at a cathode potential of −0.8 to −1.0 V (experiment II, determined in period day 12−15). The asterisk indicates hydrogen production of the uninoculated control at a cathode potential of −0.9 V.

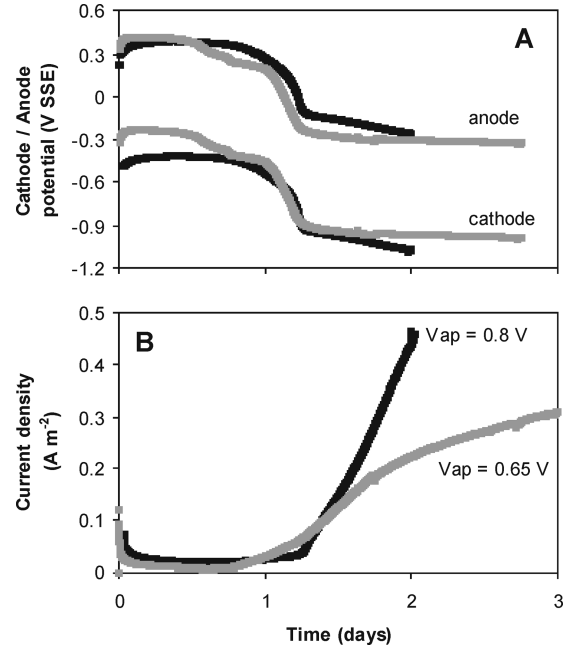

FIGURE 3. Performance of the one-compartment membraneless MEC inoculated with *G. sulfurreducens*. The MECs were fed with acetate and run at an applied voltage of 0.8 V (experiment III, black markers) or 0.65 V (experiment IV, gray markers). (A) Potential of the anode and of the cathode (vs SSE), (B) current density.

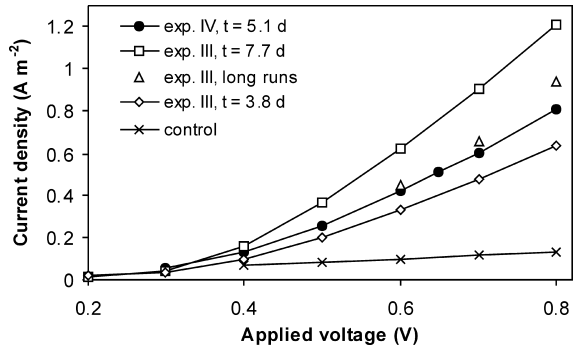

FIGURE 4. Current production of the one-compartment membraneless MEC at a range of applied voltages. Data are for 30 min runs of the uninoculated control, for experiment III at t = 3.8 and 7.7 days, and experiment IV at t = 5.1 days. Also shown are the current densities measured for experiment III during 15−24 h runs at 0.6, 0.7, and 0.8 V in the time period 5.0−7.6 days.

and electrons. The chemical reaction at the counter electrode changed from reduction of Fe(III) complexed as hexacyanoferrate to the donation of electrons from complexed Fe(II). Inversion of such a bioelectrochemical system has been observed previously for mixed microbial communities (*5*), but it has not been shown before for a single microbial species.

One-Compartment Membraneless MEC with *G. sulfurreducens*. The abilities of *G. sulfurreducens* to oxidize acetate at the anode and to catalyze hydrogen formation at the cathode, were combined in a single-compartment membraneless MEC. In separate experiments, voltages of 0.8 (experiment III) and 0.65 V (experiment IV) were applied to the system. Within one day of inoculation, the anode potential started to decrease and a small amount of current was produced (Figure 3). The anode potentials were similar compared to the MFC phase of experiments I and II (Figure 1A and B) and indicate utilization of acetate. After 1.2 days, the anode potential in experiment III had decreased to −0.11 V and in experiment IV to −0.26 V. In both cases the cathode potential had decreased to approximately −0.9 V. At this point, a strong increase in current production was observed, in particular for experiment III (Figure 3). The biocathode experiments with hexacyanoferrate(II) as electron donor showed high hydrogen evolution and current production at a cathode potential of −0.9 V or lower (Figure 2). This suggests that in the fully biocatalysed MEC, hydrogen formation and hence current production could also increase quickly at cathode potentials ≤ −0.9 V. In experiment III, a current density of 0.44 A m^{-2} was reached after 2 days (applied voltage 0.8 V) and in experiment IV, 0.36 A m^{-2} was reached after 5 days (applied voltage 0.65 V) (Figure 3B).

Experiment III was subsequently run at an applied voltage of 0.5 V, resulting in a current production at this voltage of 0.12 A m^{-2}, which increased to 0.21 A m^{-2} over the course of 1.7 days. The ensuing potential step voltammetry test (carried out at t = 3.8 days) showed larger current production with greater voltage applied (Figure 4). The largest applied voltage tested was 0.8 V and resulted in a current density of 0.64 A m^{-2}. The anode potential ranged from −0.48 to −0.36 V for cell voltages from 0.2 to 0.8 V (Table 1). Overpotentials were computed as the difference between the observed electrode potentials and theoretical midpoint potentials calculated for the oxidation of acetate at the anode and formation of hydrogen at the cathode. Larger cell voltages resulted in larger anode overpotential, ranging from close to zero to approximately 0.15 V. Cathodic overpotential exceeded the overpotential at the anode and ranged from around −0.05 at 0.2 V to approximately −0.6 V at an applied cell voltage of 0.8 V (Table 1). Following the potential step test, the system was run at applied voltages of 0.5−0.8 V (SI Figure S2) during which also hydrogen production was evaluated (see below). A second voltammetry test at 7.7 days showed that current production had about doubled compared to 4 days earlier, indicating an increase in the number of microbial cells and/or their activity during the experiment. At an applied voltage of 0.8 V, the 30 min run showed a current density of 1.21 A m^{-2}. For experiment IV, current density response to applied voltage was evaluated after 5.8 days, and the obtained values (e.g., 0.81 A m^{-2} at 0.8 V) are in accordance with experiment III when the time of incubation is considered.

Hydrogen production by the one-compartment membraneless MEC of experiment III was evaluated between 4.6 and 5.0 days of operation. Hydrogen accumulated linearly during the measurement periods that lasted up to 2.7 h. The production of hydrogen was greater with larger applied voltage and increased from 2.0 μmol H_2 h^{-1} at 0.5 V to 17 μmol H_2 h^{-1} at 0.8 V (Table 1).

Conversion Efficiency of *G. sulfurreducens* in a MEC. The cathodic hydrogen recovery increased with larger applied voltage; from 23% at 0.5 V to 43% at 0.8 V. These recoveries include the investment of energy for microbial metabolism and maintenance processes. Part of the produced hydrogen may also have been consumed by *G. sulfurreducens* at the anode. However, this effect is likely to be small, since *G. sulfurreducens* and *G. metallireducens*, which does not respire hydrogen, showed very similar hydrogen recoveries in single-chamber MECs with a platinum catalyst containing cathode (*14*). For mixed culture biocathodes, cathodic hydrogen recoveries of 17−49% have been reported (*5, 6*), whereas for platinum cathodes recoveries of 57−96% were achieved (*3, 4, 24*). In mixed culture MECs, hydrogen may also be lost due to activity of hydrogenotrophic methanogens and acetogens.

The observed CE of current production from acetate, averaged for the whole duration of MEC operation, was 34% for both experiments (SI Figures S2 and S3). Somewhat higher values of approximately 42% were obtained in the second half of the experiments. As indicated for experiments I and II, the low CE values can be explained by leakage of oxygen and by investment of acetate as C-source for growth. The CE is likely to be higher with a larger applied cell voltage, since this results in a higher anode potential (Table 1) and hence

TABLE 1. Hydrogen Production and Cathodic Hydrogen Recovery in the One-Compartment Membraneless MEC[a]

applied voltage (V)	anode potential (V vs SSE)	cathode potential (V vs SSE)	H_2 production (μmol H_2 h^{-1})	H_2 production (m^3 H_2 m^{-3} day^{-1})[b]	cathodic hydrogen recovery (%)
0.5	−0.44	−0.94	2.0	0.036	23
0.6	−0.40	−1.00	5.4	0.10	29
0.7	−0.36	−1.06	10.8	0.20	36
0.8	−0.33	−1.13	17.2	0.31	43

[a] Data for experiment III, determined from t = 4.6−5.0 days. Measuring errors in anode and cathode potential were <2% and for hydrogen production <5%. [b] Calculated per volume liquid in the chamber.

more available energy for microbial metabolism (*25*). Previous studies for MEC anodes have reported CE values >80%, however, those studies were performed with very high cell densities (CE = 81−89% (*14*)) or grown biofilms (CE = 92 ± 6% (*3*)). The use of a small amount of inoculum (4%) in our experiments implies growth was required for MEC performance.

The observed increase in cathodic current production in time in the biocatalysed half-cell of experiment II (Figure 2A) suggests an increase in cell density and/or hydrogenase expression and activity. A putative mechanism for energy conservation from hydrogen formation at a cathode is the activity of an energy-conserving hydrogenase or a cytoplasmic hydrogenase together with a membrane-bound ATPase (*7*). The genome does not show the presence of an energy-conserving hydrogenase, but two cytoplasmic hydrogenases have been identified (*18*). Conversion of protons by such hydrogenases results in a proton gradient across the cytoplasmic membrane, which potentially can be harnassed by a membrane-integrated ATPase. The cathodic overpotential in the one-compartment MEC ranged from approximately −0.3 to −0.6 V at an applied voltage of 0.5−0.8 V (Table 1). Assuming a loss of −0.1 V as concentration overpotential (*26*) leaves an energy available for hydrogen formation, microbial growth and maintenance of about −40 to −95 kJ/mol H_2. Clearly, further research on the growth rate, growth yield, and mechanism of energy conservation under MEC cathode conditions is necessary. Moreover, the extracellular electron transfer mechanisms involved in the utilization of a cathode as electron donor also need to be elucidated (*27*).

The production of current in the one-compartment membraneless MEC was 1.2 A m^{-2} at a cell voltage of 0.8 V after approximately 8 days operating time (Figure 4). In comparison, during MFC operation, the current density reached a maximum of 2.2 A m^{-2} after 3.2 days only (Figure 1A), indicating that the rate of hydrogen formation was limiting current production in the MEC. In the biocathode experiments with hexacyanoferrate(II) as the electron donor, larger current densities were achieved than in the MEC (up to 2.4 A m^{-2}, Figure 2). However, at the same cathode potential, the cell voltage was much larger ($E_{cathode} = -0.9$ V, $E_{cell} = 1.17$ V compared to 0.5 V in the MEC (Table 1)), indicating that a direct comparison is not possible.

Our study shows that *G. sulfurreducens* can be used as biocatalyst for hydrogen production at the cathode of a MEC. Electrical energy supports the overall conversion of acetate into bicarbonate and hydrogen by a pure culture of *G. sulfurreducens*. This conversion could be regarded as a special case of extracellular electron transfer, in which the electrical circuit functions as the electron carrier. The system could be started up in a relatively short time (<2 days), and the large increase in current production indicated that growth occurred in the system. Because a one-compartment membraneless layout was used, it is possible that cells were growing at the anode and, after detachment, started functioning at the cathode. However, based on thermodynamic data and known physiology of *G. sulfurreducens*, growth at the cathode could be feasible.

Acknowledgments

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Supporting Information Available

Schematic representation of the electrochemical cell used for experiments I and II (Figure S1) and Figures showing applied voltage, observed current densities, acetate consumption and calculated transferred charge for experiments III and IV (Figures S2 and S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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