Aerated *Shewanella oneidensis* in Continuously Fed Bioelectrochemical Systems for Power and Hydrogen Production

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ABSTRACT: We studied the effects of aeration of *Shewanella oneidensis* on potentiostatic current production, hydrogen production in a microbial electrolysis cell, and electric power generation in a microbial fuel cell (MFC). The potentiostatic performance of aerated *S. oneidensis* was considerably enhanced to a maximum current density of $0.45\,A/m^2$ or $80.3\,A/m^3$ (mean: $0.34\,A/m^2$, $57.2\,A/m^3$) compared to anaerobically grown cultures. Biocatalyzed hydrogen production rates with aerated *S. oneidensis* were studied within the applied potential range of 0.3–0.9 V and were highest at 0.9 V with $0.3\,m^3\,H_2/m^3$ day, which has been reported for mixed cultures, but is ~10 times higher than reported for an anaerobic culture of *S. oneidensis*. Aerated MFC experiments produced a maximum power density of $3.56\,W/m^3$ at a 200-Ω external resistor. The main reasons for enhanced electrochemical performance are higher levels of active biomass and more efficient substrate utilization under aerobic conditions. Coulombic efficiencies, however, were greatly reduced due to losses of reducing equivalents to aerobic respiration in the anode chamber. The next challenge will be to optimize the aeration rate of the bacterial culture to balance between maximization of bacterial activation and minimization of aerobic respiration in the culture.
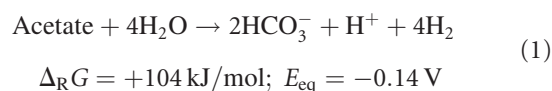
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KEYWORDS: *Shewanella oneidensis*; microbial electrolysis cell; microbial fuel cell; lactate; hydrogen

Introduction

Shewanella oneidensis MR-1 is a model microbe to study electron transfer processes in the anode compartment of bioelectrochemical systems (BESs) because it is capable of direct and indirect electron transfer to solid electron acceptors (i.e., metal oxides and electrodes) with L-lactate as the electron donor. During direct electron transfer, outer membrane cytochrome *c* proteins facilitate the microbe's respiration with, for example, electrodes (Kim et al., 1999). *S. oneidensis* can also transfer electrons indirectly with the help of (ribo)flavins as electron mediators (Marsili et al., 2008; von Canstein et al., 2008).

In recent years, investigations of BESs have broadened the application range from power generation with microbial fuel cells (MFCs) to the production of valuable chemicals at the cathode in microbial electrolysis cells (MECs). The latter systems were first described for the production of hydrogen at the cathode with acetate as the electron donor, a process which is also referred to as biocatalyzed electrolysis (Liu et al., 2005; Rozendal and Buisman, 2005). When acetate is provided as an electron donor in the anode, the microbial reduction power itself is not sufficient to allow hydrogen evolution to occur at the cathode (Formula 1), and therefore externally applied potentials are necessary to offset the overpotential (i.e., offset potential; at least +0.14 V for acetate). However, the favored electron donor of the studied organism *S. oneidensis* is L-lactate, which—at least thermodynamically—is favorable for hydrogen production at the cathode (Formula 2 shows a negative $\Delta_R G$ and a positive equilibrium potential E_{eq}).
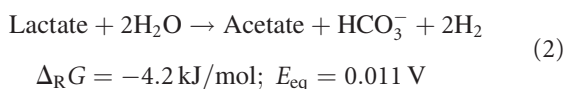
$$\text{Acetate} + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2$$
$$\Delta_R G = +104\,kJ/mol; \quad E_{eq} = -0.14\,V \tag{1}$$

$$\text{Lactate} + 2H_2O \rightarrow \text{Acetate} + HCO_3^- + 2H_2$$

$$\Delta_R G = -4.2 \, \text{kJ/mol}; \; E_{eq} = 0.011 \, \text{V} \qquad (2)$$

Since this reaction is not occurring spontaneously, but instead an activation overpotential needs to be overcome (a biocatalyst is necessary for the substrate oxidation), we wanted to verify if this thermodynamical advantage of lactate oxidation compared to acetate oxidation can be translated into an advantage of an MEC with lactate as electron donor.

While traditional BES anodes are operated anaerobically to prevent the loss of microbial reducing power to oxygen instead of transferring electrons to the anode, Biffinger et al. (2008) reported that power generation with *S. oneidensis* is possible under aerobic conditions with various substrates (Biffinger et al., 2009b; Ringeisen et al., 2007). These were unexpected and exciting results, since maintaining anaerobic conditions in the anode and preventing oxygen crossover from cathode to anode are major challenges of BES technology. However, all aerated experiments were performed either in miniature MFCs with an anode liquid volume of $\sim$1 cm^3 (Ringeisen et al., 2007), or in high throughput assaying systems of 0.5 cm^3 volume (Biffinger et al., 2009a). While it is known for BESs, that *S. oneidensis* only oxidizes lactate to acetate and four electrons (Formula 2), other literature reports on *S. oneidensis* aerobic physiology confirmed a more complete lactate oxidation under oxygenated versus oxygen-limited conditions (Tang et al., 2007). Therefore, we decided to test the applicability of aerated *S. oneidensis* in macroscopic continuous-flow BESs (both in power-producing MFC and hydrogen-producing MEC mode). To gain more insight in the effects of aeration on anaerobic respiration of *S. oneidensis*, we also performed liquid culture tests with soluble iron (III) citrate as electron acceptor. We are aware that the biochemical pathways for electrode respiration (at the outer membrane) and soluble iron (III) reduction (in the periplasm) only partly overlap, and therefore results from these liquid tests cannot directly be related to electrode physiology. However, these tests can give a general impression of oxygen effects on anaerobic respiration processes for *S. oneidensis*.

Materials and Methods

Chemicals and Media

S. oneidensis MR-1 (a gift from Tim Gardner, Boston University, Boston, MA, USA) was grown in LB medium for strain maintenance and in defined growth medium for all experiments. The anode defined medium was prepared according to Myers and Nealson (1988) and was modified by adding 1.27 mM K_2HPO_4, 0.73 mM KH_2PO_4, 125 mM NaCl, 5 mM HEPES, 0.5 g/L yeast extract, and 0.5 g/L tryptone (no addition of amino acids). After autoclaving, sodium L-lactate and K_2HPO_4 were each added to final

concentrations of 20 mM. For the planktonic culture tests, the same defined growth medium composition was used, except that 50 mM iron(III)–citrate and either 10 mM L-lactate *or* acetate were added. The catholyte was a 100 mM potassium phosphate buffer. All chemicals were ACS grade.

Planktonic Culture Tests

For planktonic culture tests of *S. oneidensis* in iron(III) defined medium, triplicate cultures, without carbon source or with either 10 mM lactate or acetate, were grown for 22 h aerobically (10 mL medium in 150 mL flasks, shaken (100 rpm) at 30°C under room atmosphere), microaerobically (10 mL medium in 15 mL tubes, shaken (100 rpm) at 30°C under room atmosphere, while shaking did not allow for complete mixing and aeration of the medium), or anaerobically (10 mL medium in 15 mL tubes, shaken (100 rpm) at 30°C in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) with a 5% H_2/20% CO_2/ 75% N_2 atmosphere). Subsequently the cultures were analyzed for optical density, pH, iron(III) reduction and metabolite formation (see the Analysis Section).

Reactor Set-Up

The H-type electrochemical reactor was made of glass with an anode and cathode liquid chamber volume of 220 mL each. The anode and cathode chambers were separated by a glass bridge with an anion exchange membrane (19.6 cm^2, AMI-7001 Membranes International, Glen Rock, NJ). The anode chamber was temperature controlled at 30°C with a water jacket, stirred, aerated at 30 mL/min, continuously fed with defined growth medium at an hydraulic retention time (HRT) of 5–10 h, and was equipped with a carbon-fiber-fabric anode (15 × 15 cm, Panex 30–SW08, Zoltek, St. Louis, MO). The carbon-fiber-fabric anode was bound to a graphite rod (Poco Graphite, Inc., Decatur, TX) with carbon cement (CCC Carbon Adhesive, EMS, Hatfield, PA). We used Ag/AgCl (saturated KCl) as the reference electrode to control (potentiostatic experiments) or measure the anode potential. The cathode chamber was operated in batch and had a gas inlet for air or nitrogen sparging, a liquid sampling port, a gas outlet connected to a Milligascounter (Ritter, Bochum, Germany), and a graphite block electrode (3 × 9 × 1 cm^3, PocoGraphite, Decatur, TX), which was modified with 5% platinum on activated carbon powder (3 mg/cm^2; Alfa Aesar, Ward Hill, MA). The entire assembled set-up, including three 10-L feeding tanks, was autoclaved before the experiment. In preliminary experiments, *S. oneidensis* was grown anaerobically in the same reactor set-up and under the same experimental conditions, except that the medium tanks and the reactors were kept under a positively pressured 20% CO_2/80% N_2 atmosphere and that a carbon paper electrode (4 × 6.25 cm^2, AvCarb P50, The Fuel Cell Store, San Diego, CA) was used as the working electrode.

Experimental Sequence

The experimental run was divided in the following four phases (Table I): *Phase I—potentiostatic start-up*: a steady state *S. oneidensis* biofilm was pregrown under potentiostatic conditions in a three-electrode set-up at 0.4 V (all potentials refer to standard hydrogen electrode, SHE) (VSP potentiostat, BioLogic, Knoxville, TN); *Phase II—hydrogen production in MEC-mode*: once steady state was reached, the cathode was flushed with nitrogen for 5 h and the cell was switched into MEC mode. The applied potentials (in a two-electrode set-up) were increased in 0.1-V increments from 0.3 to 0.9 V. Each potential was maintained for two days. Catholyte pH was adjusted to pH 2 at the beginning of every potential step after which the pH change was monitored (because of continuous operation and sufficient buffer capacity, the anolyte pH was not influenced by this pH adjustment); *Phase III—intermittent potentiostatic control*: after the last MEC potential step, the cell was switched back into potentiostatic mode (three-electrode set-up). Cyclic voltammetry tests (-0.3–0.7 V, $v = 1$ mV/s) were performed regularly during both potentiostatic phases; and *Phase IV— power production in MFC-mode*: the cell was transitioned into MFC mode at a constant external resistor of 200 Ω. Cell and anode potentials were recorded with a digital multimeter (Keithley Instruments, Inc., Cleveland, OH). Triplicate polarization tests (with 2 days wait time in between) were performed by changing resistors in step periods of 30 min after keeping the cell at open circuit over night. Current, power, and coulombic efficiency were calculated following Logan et al. (2006), whereby the coulombic efficiency was based on net substrate conversion calculated from the metabolite analysis data: ([electrochemically harvested e$^-$]/ [e$^-$ in consumed substrate $-$ e$^-$ in metabolic products]).

This experimental series was repeated three times with similar performance but with different length of operating periods. Only the data for the last run was reported here and efficiencies were calculated for time windows of stable performance.

Analysis

Anode effluent samples were taken every other day to determine HRT, effluent pH, L-lactate (Accutrend Lactate Analyzer, Roche Diagnostics, Indianapolis, IN), and other soluble metabolites. Filtered samples (0.2-μm nitrocellulose filter, Millipore, Billerica, MA) were analyzed for sugars, organic acids, and ethanol using a SpectraSYSTEM liquid chromatography system equipped with a refractive index detector (Thermo Fisher Scientific, Inc.) and with an organic acids column (Aminex HPX-87H Column, Bio-Rad Laboratories, Inc., Hercules, CA). Samples were run at 65°C and eluted at 0.6 mL/min with 5 mM sulfuric acid. During the hydrogen production phase (phase II), replicate gas samples were taken from the cathode headspace and analyzed for molecular hydrogen with a gas chromatograph (model 310 SRI Instruments, Torrance, CA; with a 3 ft Molsieve 5A column, a TCD detector, and nitrogen carrier gas at 5 psi). Optical density of the planktonic culture tests was measured in triplicate at 600 nm with a 96-well plate reader (Synergy4, BioTek Instruments, Inc., Winooski, VT). For determining iron(III) reduction, iron(II) was quantified with a Ferrozine-assay modified after Ruebush et al. (2006). To assay, 100 μL of sample were mixed with 33 μL of 2 N hydrochloric acid. After centrifugation, 50 μL of the acidified sample were combined with 950 μL Ferrozine dye (1 g/L Ferrozine in 100 mM HEPES, pH 7), mixed, and absorbance was measured in triplicate at 562 nm with a plate reader (as above) and compared to a freshly prepared iron(II) standard curve that was processed in the same way as the samples. For SEM imaging, 5×5 mm^2 pieces of the BES carbon paper electrode were stored over night in 2.5% glutaraldehyde (with Complete mini Protease Inhibitor, Roche Diagnostics); then, washed in DI water; stored in 1% osmium tetroxide (in DI water), washed in DI water; and dehydrated in a series of 50%, 70%, 90%, 100% ethanol (20 min incubation for at each concentration), followed by critical point drying and gold coating. Imaging was done with a Hitachi S-450 scanning electron microscope at 20 kV accelerating voltage.

Table I. Performance summary of MEC and MFC experiments with aerobic *S. oneidensis*.

Experimental mode (at E vs. SHE)	Time period (day)	$j_{\text{A-max}}$ (A m^{-2})[a]	$j_{\text{A-avg}}$ (A m^{-2})[b]	$j_{\text{V-max}}$ (A m^{-3})[a]	$j_{\text{V-avg}}$ (A m^{-3})[b]	CE$_{\text{max}}$ (%)[c]	CE$_{\text{avg}}$ (%)[c,d]	Lactate removal (%)[a]
Potentiostatic at 400 mV	7	0.451	0.341 ± 0.042	80.3	57.2 ± 7.5	29.43	22.19 ± n.d.	63
MEC at 300 mV	2	0.082	0.070 ± 0.009	14.6	12.6 ± 1.6	3.14	2.70 ± n.d.	48
MEC at 400 mV	2	0.122	0.095 ± 0.015	21.8	16.7 ± 2.8	5.72	4.45 ± n.d.	53
MEC at 500 mV	2	0.149	0.124 ± 0.014	26.5	21.9 ± 2.5	6.01	5.01 ± n.d.	45
MEC at 600 mV	2	0.177	0.142 ± 0.018	31.4	25.3 ± 3.3	6.86	5.52 ± n.d.	41
MEC at 700 mV	2	0.198	0.150 ± 0.025	35.2	27.6 ± 4.4	8.35	6.33 ± n.d.	35
MEC at 800 mV	2	0.215	0.165 ± 0.024	38.3	29.8 ± 4.4	17.26	13.21 ± n.d.	36
MEC at 900 mV	2	0.225	0.170 ± 0.025	40.0	30.0 ± 4.5	14.39	10.88 ± n.d.	25
Potentiostatic at 400 mV	9	0.398	0.162 ± 0.042	70.8	31.1 ± 7.4	24.90	10.19 ± n.d.	32
MFC	9	0.047[e]	0.041 ± 0.004	8.3[e]	7.8 ± 0.8	4.08	2.72 ± n.d.	41

[a]Maximum during respective experimental mode.
[b]Average during respective experimental mode.
[c]CE is based on maximum or average current and the actual substrate consumption and metabolite formation.
[d]A realistic statistical calculation of standard deviations for CE was not possible, n.d. not determined.
[e]Current density at power maximum.

Results

Preliminary Potentiostatic Tests With and Without Oxygen

Preliminary potentiostatic experiments showed that the current production of anaerobically grown *S. oneidensis* was significantly enhanced during oxygen (air) exposure (Fig. 1). When the medium storage tank was aerated on day 21, the current almost tripled from 0.2 to 0.58 A/m^2 (or 4.4–12.9 A/m^3). Even after the air influx was stopped after a few hours, and the medium tank was switched back to a CO$_2$/N$_2$ atmosphere, the enhanced current production lasted over several days. Rather than an immediate decline, we observed a gradual decline of current production after restoring anaerobic conditions.

Performance of Aerobic *S. oneidensis* for Hydrogen and Power Production

S. oneidensis was grown in a controlled, continuous-flow H-type BES under sterile aeration. The continuous experimental run lasted over 40 days and was structured in four phases (Table I):

(I) *Potentiostatic start-up*: To obtain a stable and active *S. oneidensis* biofilm, the electrochemical cell was started up under potentiostatically controlled conditions (anode at 0.4 V vs. SHE). Within 12 h after inoculation, the culture produced a current with an overall maximum of 0.45 A/m^2 of electrode surface area (mean: 0.34 ± 0.042 A/m^2) or 80.3 A/m^3 of anode

liquid volume (mean: 57.2 ± 7.5 A/m^3). After 2 days, the HRT was lowered from 16 to 5 h to match the *S. oneidensis* lactate consumption. While the current output was increased with an aerated culture of *S. oneidensis* (as shown in the preliminary test), the coulombic efficiency was low (mean: 22%). Compared to the preliminary experiments with *S. oneidensis* under anaerobic conditions (Fig. 1), the mean surface related current density was 1.7-fold higher, and the mean volumetric current density was 13-fold higher in the aerated experiment. The increase in surface related current density can be accounted for by the increased current production of the aerated culture of *S. oneidensis*. On the other hand, the higher volumetric current density is primarily a result of the high-surface carbon-fiber-fabric electrode that was used instead of the carbon paper electrode (ratio of electrode surface to reactor liquid volume: 22 and 178 m^2/m^3 for the carbon paper and the carbon-fiber-fabric, respectively).

(II) *Biocatalyzed electrolysis of lactate*: The BES was switched into electrolysis mode on day 8 by applying a small voltage to the cell to offset the activation overpotential for hydrogen production. Slow potential scans (1 mV/s) showed cathodic reduction currents starting at 0.1 V (data not shown). Yet, detectable (and visible) hydrogen production only started at 0.3 V. Every 48 h, the potential was increased in 0.1-V steps from 0.3 to 0.9 V, resulting in higher hydrogen production with each step. The hydrogen production rates at the different applied potentials were highest at 0.9 V with 0.3 m^3/m^3 day (calculated from the current flow) (Fig. 2). The coulombic efficiency for the
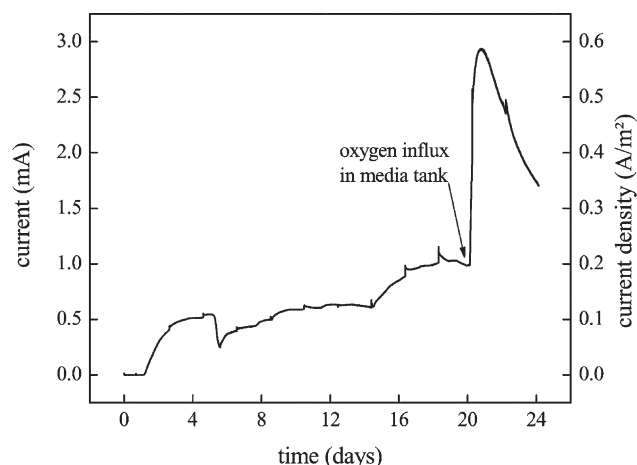

Figure 1. Chronoamperometric curve of a *S. oneidensis* pure culture, potentiostatically poised at 0.4 V, with oxygen influx into the medium storage tank as indicated by an arrow. Defined growth medium with 20 mM L-lactate; carbon paper electrode (surface area 50 cm^2); all other information can be found in the Materials and Methods Section of the text.
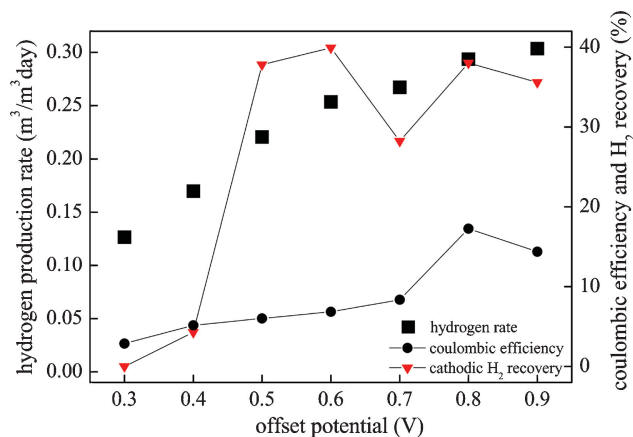

Figure 2. Performance of biocatalyzed hydrogen production from lactate with aerated *S. oneidensis*: The *left axis* shows the volumetric hydrogen production derived from the recorded current of the MEC, and the *right axis* shows the coulombic efficiency and the hydrogen recovery (how much of the generated hydrogen could actually be collected), all as a function of the externally applied potential. Further details are given in the Experimental Sequence Section. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

biocatalyzed hydrogen production was ~7% at 0.5 V and ~14% at 0.9 V (assuming 100% of current derived from biocatalyzed hydrogen production). The hydrogen recovery yield—the portion of the produced hydrogen gas that can actually be collected—was 30–40% for potentials >0.5 V (Fig. 1, right axis), and it was below 5% at voltages <0.5 V.

(III) *Intermittent potentiostatic phase*: Following the MEC mode, the electrochemical cell was switched back under potentiostatic anode control on day 22. Growth of *S. oneidensis* in the effluent lines slowed down the flow rate and increased the HRT to ~10 h. The flow rate was adjusted to tune the HRT back to 5 h, and the system restabilized at a performance level that was lower than during the start-up phase with a new mean current density of $0.162 \pm 0.042 \, A/m^2$ (or $31.1 \pm 7.4 \, A/m^3$).

(IV) *Microbial fuel cell operation*: After stable performance was re-established, the BES was switched into MFC mode at an external resistor of $500 \, \Omega$. From a first polarization test, the internal resistance was determined to be $213 \, \Omega$ (calculated as the slope of the potential-current function) after which the MFC was operated with an external resistor of $200 \, \Omega$ except during polarization tests. The polarization test was performed three times with a 2-day steady-state operating period between runs. The mean volumetric power maximum was $3.56 \pm 0.15 \, W/m^3$ at a mean volumetric current density of $8.17 \pm 0.17 \, A/m^3$ (or $0.047 \, A/m^2$) (Fig. 3). The coulombic efficiencies at the maximum power point (MPP) and during constant resistor operation were 4.08% and 2.72%, respectively. At the MPP the cell potential was 0.367 V and the anode potential -0.05 V. Therefore, the cathode potential was 0.317 V (0.367–0.05 V). The fairly electropositive anode potential was limiting the BES performance.
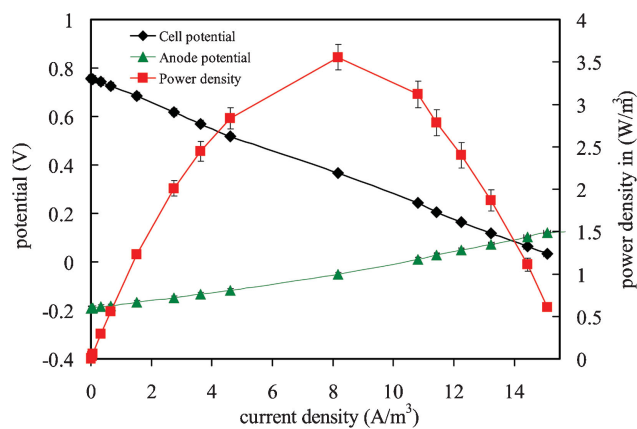

Figure 3. Polarization and power plot of a microbial fuel cell with aerated *S. oneidensis*. The *left axis* shows the cell potential and the anode potential, and the *right axis* shows the volumetric power density (based on the anode liquid volume) as a function of the volumetric current density. All curves represent the mean of triplicate experiments; both horizontal and vertical error bars are shown. Experimental details are given in the text. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Planktonic Culture Tests With Iron(III) Citrate as Terminal Electron Acceptor

We also examined the capability of *S. oneidensis* to reduce soluble iron(III) with either lactate or acetate as electron donor under aerobic, microaerobic, and anaerobic conditions (Table II). All data have been corrected for negative control cultures, which were grown in medium containing the additives yeast extract and tryptone, but without lactate or acetate as energy sources. Growth with lactate was comparable under aerobic and microaerobic conditions, but much lower under anaerobic atmosphere (OD 0.239, 0.252, and 0.117 after 22 h for aerobic, microaerobic, and anaerobic conditions, respectively). Significant growth with

Table II. Planktonic growth of *S. oneidensis* with iron(III)-citrate under aerobic, microaerobic, and anaerobic conditions.

	Carbon source (10 mM)					
	Aerobic		Microaerobic		Anaerobic	
	Lactate	Acetate	Lactate	Acetate	Lactate	Acetate
OD (600 nm)[a]	0.239 ± 0.044	0.160 ± 0.041	0.252 ± 0.002	-0.033 ± 0.012	0.117 ± 0.006	-0.100 ± 0.007
Fe(II) in mM[a,b]	0.097 ± 0.011	0.046 ± 0.022	23.611 ± 1.750	1.001 ± 0.679	33.885 ± 1.779	0.179 ± 0.646
Fe(II)/OD[c]	0.4	0.3	93.7	n.a.	289.6	n.a.
End-lactate in mM[a,d]	-6.66 ± 0.55	2.27 ± 0.08	-8.44 ± 0.11	n.d.	-9.88 ± 0.77	n.d.
End-acetate in mM[a,d]	2.44 ± 2.22	-6.08 ± 0.12	10.60 ± 0.35	$-.50 \pm 1.26$	10.05 ± 4.69	3.55 ± 2.01
End-formate in mM[a,d]	0.36 ± 2.50	3.80 ± 0.15	-2.46 ± 0.12	-3.19 ± 0.82	-2.17 ± 2.77	2.10 ± 0.82

n.a., not available; n.d., not detected.

[a]All data were corrected for activity of *S. oneidensis* in the background medium (containing 0.5 g/L yeast extract and 0.5 g/L tryptone, but no lactate or acetate).

[b]Experiments were started with 50 mM Fe(III), thus a maximum of 50 mM Fe(II) could be produced.

[c]To normalize the iron reduction activity for the bacterial growth, it was divided by the OD.

[d]End-metabolite concentrations at the end of each experiment (from initially 10 mM substrate fed as indicated in table head). A negative sign stands for total net uptake, no sign for total net production of metabolites.

acetate was only observed under aerobic conditions ($OD_{aerobic} = 0.160$), while the subtraction of control growth data without acetate in the medium resulted in slightly negative optical densities; and thus *no* net growth for microaerobic and anaerobic conditions. Based on iron(II) measurements, iron(III) reduction was highest for lactate under anaerobic conditions, followed by microaerobic and aerobic conditions (68%, 47%, and 0.2% of total iron(III) input, respectively). In addition, specific iron(III) reduction (related to optical density of the culture) under aerobic conditions was very low for both lactate and acetate as the electron donor (0.4 and 0.3 mM/OD, respectively) compared to 289.6 mM/OD for lactate under anaerobic conditions. It should be noted, though, that the iron(III) reduction under aerobic conditions was underestimated because iron(III) reduction was measured by quantifying iron(II) accumulation in the medium. Iron(II) is not stable under aerobic conditions and is easily back oxidized to iron(III). Similar experiments performed in aerobic medium without iron(III) showed lower optical densities for acetate (OD = 0.092), indicating that iron(III) indeed is involved in growth under aerobic conditions, even though oxygen is the preferred electron acceptor.

The metabolite analysis showed a perfect coherence between anaerobic oxidation of lactate and acetate product formation (ratio 1:1). Under aerobic conditions, only 0.37 moles of acetate and 0.05 moles of formate were produced per mol of lactate, representing a much more complete oxidation of the carbon and energy source, and explaining why the growth (i.e., OD) is higher despite lower absolute carbon source consumption (only 67% of the total lactate provided was consumed). With acetate, about 60% of the supplied substrate was metabolized under aerobic conditions, with most of it being oxidized by oxygen; however as we have shown also iron reduction was involved. Under anaerobic conditions no acetate was metabolized; instead, additional acetate was formed from medium additives like yeast extract or tryptone (initial acetate concentration was 10 mM, end concentration was 13 mM). Under microaerobic conditions, 84% of the fed lactate was consumed and acetate was produced at a ratio of 1:1.25 (lactate consumption/acetate formation). The accumulation of acetate indicates that microaerobic conditions were not sufficient for a more complete oxidation of lactate. However, despite the incomplete metabolic reactions, the microaerobic growth rate was comparable to aerobic conditions (OD 0.252 and OD 0.239 after 22 h for microaerobic and aerobic growth, respectively).

Discussion

Reaction Rates Are Increased in BES With Aerated *S. oneidensis* at the Anode

In both our current generation and hydrogen production experiments the performance of aerated *S. oneidensis* was significantly enhanced compared to measured or reported anaerobic data. In potentiostatic mode during our preliminary experiments, the current production almost tripled upon aeration of the culture (from 0.2 to 0.58 A/m²), and our subsequent continuously aerated experiments showed in average 70% higher current production (0.34 A/m²). The achieved current density in potentiostatic mode was about 2.5 times higher than has been published for *S. oneidensis* MR-1 under anaerobic conditions at a 10Ω external resistor (0.138 A/m²) (Bretschger et al., 2007). Note that the Bretschger et al. study was performed with cells in batch with a lactate/buffer (PIPES) system, while our study was with complete medium under continuous conditions (we are not aware of any continuous anaerobic and potentiostatically controlled experiments with *S. oneidensis*). Our hydrogen production rates (0.25 m³/m³ day at 0.6 V and 0.3 m³/m³ day at 0.9 V) compare well to reported rates for undefined mixed cultures (0.01–3 m³/m³ day (Logan et al., 2008)), despite the fact that pure bacterial cultures often perform worse than mixed culture systems (Zuo et al., 2008). Thus far, only one research team has published biocatalyzed hydrogen production with a pure culture of *S. oneidensis* MR-1 (Hu et al., 2008). In that study, hydrogen production in an anaerobic semi-continuous system was evaluated at an applied potential of 0.6 V. Hydrogen production rates were measured to be 0.025 m³/m³ day, and therefore were 10 times lower than we observed with a continuous, aerated culture. The difference in performance probably is not solely explainable by using an aerobic versus an anaerobic culture of *S. oneidensis* MR-1, since the entire experimental set-up of the two studies was very different (e.g., continuous vs. semi-batch operation, different electrode materials, membrane reactor vs. membrane less reactor). However, in the same study, with only a slightly different experimental set-up than was used in the *S. oneidensis* experiments, mixed culture MECs produced hydrogen at a rate of up to 0.69 m³/m³ day, indicating that the nature and activity of the microbial culture is more significant for efficient hydrogen production than the experimental parameters (Hu et al., 2008).

Two potential reasons for the enhanced current and hydrogen production activity, reflecting an increase in the rate of bioelectrochemical reaction, include:

(i) The presence of oxygen led to more biomass formation, and therefore to an increase in the number of electrochemically active cells on the electrode. Indeed, a thick *S. oneidensis* electrode biofilm was visible under aerobic conditions, whereas under anaerobic conditions a monolayer biofilm could only be observed with the help of SEM imaging (Fig. 4).

(ii) The presence of oxygen changed metabolic reaction patterns, enhancing the electron flux to the electrode. Under anaerobic conditions lactate is oxidized to acetate, and only $4 e^-$ per mol lactate (one third of the stored electrons) can be recovered for current production, cell maintenance, and/or biomass formation. Biffinger et al. (2009a) found that acetate can be used as
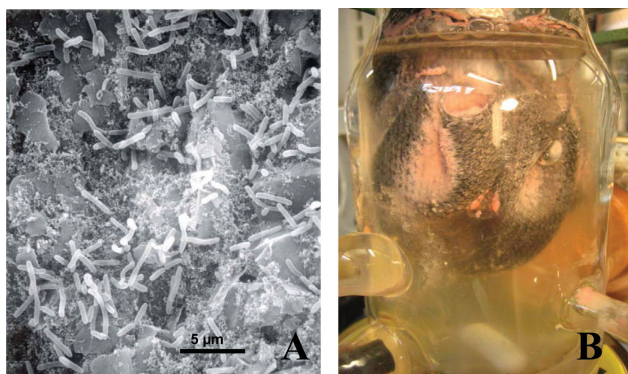
Figure 4. SEM image (at 2,000×) of anaerobically grown *S. oneidensis* on a carbon paper anode (A) and photograph of aerobically grown *S. oneidensis* on a carbon-fiber-fabric anode (B). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

electron donor for current generation under aerobic conditions and thus theoretically up to $12\,e^-$ can be generated from lactate. This hypothesis is also supported by our planktonic culture tests for iron(III) reduction under different aeration conditions with lactate and acetate as electron donor. Although, iron(III) reduction is difficult to quantify under aerobic conditions, control experiments with acetate and oxygen but without iron(III) as electron acceptor indicated that growth with acetate can be coupled to iron(III) reduction under aerobic conditions even though oxygen is the preferred electron acceptor. Thus, it is possible that the influx of oxygen in the bacterial medium opened new metabolic possibilities for a more complete utilization of lactate (oxidation does not stop at the stage of acetate), and therefore the transformation of waste acetate into a substrate for electricity generation. While iron(III) reduction under aerobic conditions cannot be experimentally verified due to back oxidation of its product iron(II), we have shown with our BES that the electrode is an ideal experimental tool to study partitioned respiration activity in the presence of oxygen.

To further evaluate the relative influence of increased biomass and more complete substrate oxidation on the current production, future experiments should be directly related to the biomass yield on the electrode (e.g., per cell dry weight or per gram of protein). This will allow us to evaluate if the same amount of biomass would produce the same current under anaerobic and aerobic conditions, or if metabolic changes are predominant to enhanced current output.

Yields Are Decreased in BES With Aerated *S. oneidensis* at the Anode

While current output and hydrogen gas production are increased with an aerated culture of *S. oneidensis*, the

coulombic efficiencies of current or hydrogen production (how much of the stored energy in the substrate is converted to current or hydrogen; see Table I) are very low compared to conventional anaerobic BESs where coulombic efficiencies typically range from 50% to nearly 100% (Chae et al., 2008; Liu et al., 2005; Rozendal et al., 2006). Thus far, no efficiency data are known for an anaerobic pure culture of *S. oneidensis* (Hu et al. did not report coulombic efficiencies). The low efficiencies mainly result from losses to aerobic respiration processes in the anode chamber, where reducing equivalents are preferentially transferred to oxygen as the terminal electron acceptor instead of being recovered as electrons at the anode. Another possibility would be the loss of reducing equivalents to the production of other metabolites under aerobic conditions. Our analysis showed, that no pyruvate, slightly lower amounts of acetate, and higher amounts of formate (2–5 mM vs. <1.5 mM under anaerobic conditions) were produced in the aerated BES compared to anaerobic operation. Since formate is a fairly oxidized metabolite (oxidation state +II), its production cannot explain major electron losses to metabolites, and losses to aerobic respiration represent the main reason for the low coulombic efficiencies. The hydrogen yield (the amount of hydrogen produced per substrate fed) was 0.3 mol H_2/mol lactate, compared to a theoretical maximum of 2 mol H_2/mol lactate for anaerobic conditions. Thus, the yield of the process is low despite the fact that 10 times more hydrogen can be produced with aerobic compared to anaerobic *S. oneidensis* (see previous section). Consequently, we observed that with availability of oxygen as competing electron acceptor the overall biocatalytic activity of *S. oneidensis* is enhanced, resulting in increased current or hydrogen production. This was observed even though the majority of reducing equivalents were lost to oxygen reduction and could not be recovered at the electrode.

Biocatalyzed Hydrogen Production From Lactate Is Not More Favorable Than With Acetate

With *S. oneidensis*, lactate is oxidized under anaerobic conditions to acetate and 4 electrons (or 2 mol of hydrogen) ($\Delta G^{\circ\prime}_r = -4.2$ KJ/mol (Thauer et al., 1977)). This negative $\Delta G^{\circ\prime}_r$ and the resulting theoretical equilibrium potential ($E_{eq,\ lactate} = +0.011$ V) indicate that hydrogen production is thermodynamically favorable and could occur spontaneously when lactate is the electron donor in the anode. However, as for many electrochemical processes, we observed an activation overpotential. Therefore, hydrogen production only started at applied offset potentials of 0.1 V, which means that the activation overpotential for our experimental set-up was 0.111 V ($E_{eq,\ lactate} + E_{offset} = 0.011 + 0.1$ V $= 0.111$ V). Significant collectable amounts of hydrogen were produced from lactate at offset potentials >0.3 V. In experiments with acetate as the electron donor, the minimum offset potential for biocatalyzed electrolysis to

occur is theoretically as low as the inverse of the equilibrium potential ($E_{\text{eq. acetate}} = -0.14$ V) (Logan et al., 2008). It was reported that >0.2 V applied offset potential were required to produce some hydrogen from acetate at the cathode, while significant hydrogen production starts at potentials ≥ 0.6 V (Rozendal et al., 2007). Thus, the activation overpotential for hydrogen production from acetate in experiments was ~ 0.06 V ($E_{\text{eq, acetate}} + E_{\text{offset}} = -0.14 + 0.2$ V $= 0.06$ V), which is lower than for hydrogen production from lactate in our experiments. However, a direct comparison of the activation overpotentials for biocatalyzed electrolysis of lactate and acetate is not possible with the available data because the overpotentials were dependent on the experimental conditions (e.g., electrode materials, microbial kinetics). Significant hydrogen production for both electron donors was achieved with offset potentials ≥ 0.6 V. Thus, there may be no practical advantage of having a more positive equilibrium potential for different electron donors ($E_{\text{eq. donor}}$) in MECs.

System Parameters Limit Rate and Yield

High Internal Resistance

Besides losses to oxygen reduction in our aerobic BES anode, the relatively high internal cell resistance of 213 Ω, as measured in the MFC tests, plays a major role in limiting the power and hydrogen generation, and thus the coulombic efficiency. The continuous-flow glass reactors were designed for pure culture studies to accommodate sterilization and controlled culture growth, and not power optimization. Our engineered systems for power optimization (Fornero et al., 2008; He et al., 2005, 2006), on the other hand, are typically made of plastic materials and are not autoclavable, a prerequisite for continuous pure culture studies. It is clear, therefore, that the measured MFC performance of our *S. oneidensis* pure culture is below its possible capacity. A cathode potential of 0.317 V at the MPP shows that our BES was not cathode limited and that most of the potential loss was derived from the anodic reaction ($E_{\text{anode, MMP}} = -0.05$ V). The low anode potential (absolute value) is a result of the aeration of the anode chamber and it represents a mixed potential of the microbial electron transfer processes at electronegative potentials and electropositive oxygen reduction. Since the potentiostatic performance of the *S. oneidensis* culture was decreased after adjusting for reactor flow problems (phase III, new mean current density 16 A/m^2 from 34 A/m^2 before), we anticipate that the MFC performance was negatively impacted by general activity losses of the microbial culture.

Hydrogen Recovery

Even though we used a glass reactor and sealed all fittings with Teflon tape and clamps, we observed a big divergence between electrochemically produced hydrogen and volumetrically collected hydrogen gas (Fig. 2). Hydrogen might have escaped by diffusion into the anode compartment and/or diffusion from the reactor through connecting parts or tubing. The high diffusivity of molecular hydrogen gas is a problem that has also been encountered by other researchers when bench-scale size reactors were used (Rozendal et al., 2006). It is important to note, therefore, how difficult the efficient recovery of biocatalyzed hydrogen from practical reactor systems will be.

Does Aeration of the Anode Chamber of BES Make Sense?

Biffinger, Ringeisen, and their colleagues showed unexpected positive effects of aeration in their studies with *S. oneidensis* in miniature MFCs (Biffinger et al., 2008, 2009a; Ringeisen et al., 2007). Their small reactor size (1.2 cm^3) resulted in a very close anode to cathode geometry and a high electrode surface area to reactor volume ratio. Thus, these miniature MFCs were optimized for power output, and very high power densities of 330 W/ m^3 for aerobic *S. oneidensis* have been observed. Here, we showed with a bench-scale BES that aerated anodes indeed improved current and hydrogen production. We attribute this enhanced performance to an improved energetic state of *S. oneidensis* because of increased biomass formation and extended substrate oxidation. For many applications of BES (e.g., for biosensors) this increased reaction rate (current = output signal) will be of great benefit. Thus, it should be considered and investigated whether other pure or mixed culture BES would profit from anode aeration. On the other hand, reaction yields (i.e., the coulombic efficiency) were greatly diminished in aerated BES because reducing equivalents are partially dissipated by the supplied oxygen. This represents a disadvantage for BES as power or fuel generating devices, since the primary fuel efficiency is negatively impacted. Thus, for these power or hydrogen generating systems aeration of the anode has to be carefully considered and optimized.

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