Microbial Community Composition Is Unaffected by Anode Potential

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Supporting Information

ABSTRACT: There is great controversy on how different set anode potentials affect the performance of a bioelectrochemical system (BES). It is often reported that more positive potentials improve acclimation and performance of exoelectrogenic biofilms, and alter microbial community structure, while in other studies relatively more negative potentials were needed to achieve higher current densities. To address this issue, the biomass, electroactivity, and community structure of anodic biofilms were examined over a wide range of set anode potentials (−0.25, −0.09, 0.21, 0.51, and 0.81 V vs a standard hydrogen electrode, SHE) in single-chamber microbial electrolysis cells. Maximum currents produced using a wastewater inoculum increased with anode potentials in the range of −0.25 to 0.21 V, but decreased at 0.51 and 0.81 V. The maximum currents were positively correlated with increasing biofilm biomass. Pyrosequencing indicated biofilm communities were all similar and dominated by bacteria most similar to Geobacter sulfurreducens. Differences in anode performance with various set potentials suggest that the exoelectrogenic communities self-regulate their exocellular electron transfer pathways to adapt to different anode potentials.

INTRODUCTION

Various bioelectrochemical systems (BESs), such as microbial fuel cells (MFCs) and microbial electrolysis cells (MECs), have been developed to extract energy from wastewaters in different forms including electricity and hydrogen.1–4 Microorganisms on the anode oxidize organics to CO₂, releasing protons into solution and electrons to the circuit. In MFCs, oxygen is reduced at the cathode to water, consuming protons and electrons (e.g., O₂ + 4H⁺ + 4e⁻ ⇌ 2H₂O, E° = 1.229 V vs SHE) and producing electrical power.5,6 By application of additional voltage of 0.3–1 V to that produced by the anode, hydrogen can be generated in MECs at the cathode (e.g., H⁺ + 2e⁻ ⇌ H₂, E° = 0 V vs SHE) and producing electrical power.5,6 This voltage can be produced by an external power source, or through insertion of a reverse electrodialysis stack between the electrodes.5,7

The anode potential can influence the performance of BESs.9 Theoretically, microorganisms can gain more energy for growth at higher anode potentials if metabolic pathways are available to capture this energy, as shown by 9–11

\[ \Delta G^{\circ'} = -nF(E_{\text{anode}} - E_{\text{substrate}}) \]  (1)

where \( \Delta G^{\circ'} \) (J/mol) is the Gibbs free energy at standard biological conditions (\( T = 25 \) °C, pH = 7), \( n \) is the number of electrons transferred, \( F \) is the Faradays constant (96,485 C/mol e⁻), and \( E_{\text{anode}} \) (V) and \( E_{\text{substrate}} \) (V) are the anode potential and the standard biological redox potential of the substrate. Despite the availability of greater energy with higher anode potentials, a review of the literature on the effects of set anode potentials on BESs performance revealed no clear effect of anode potential on performance.9 Several studies have shown that improved performance (higher current) was obtained at more positive potentials, in the potential ranges of −0.09–0.41,12 0.15–0.83 V,13 or 0.30–0.80 V13 (vs SHE). In other studies, current increased with anode potential in a lower (more negative) potential range, but it did not further increase when potentials were increased to 010 or 0.21 V.15 In some cases, current decreased at potentials higher than 0 or 0.41 V.17 Two studies have shown the opposite trend in current and set potential, as the highest current densities were produced at the lowest anodes potentials of −0.15 (compared to −0.09, 0.02, and 0.37 V)18 and 0.54 V (relative to 0.74 and 0.94 V).19

The lack of agreement in studies with set potentials could be due to a combination of factors, but the most important factor is the predominance of specific microorganisms in the anode community. High power densities are usually associated with...
communities dominated by various Geobacter species. Several studies have shown that current generation by Geobacter sulfurreducens was positively correlated with biomass. It has also been shown using cyclic voltammetry (CV) that the expressed cytochromes, based on predominant peaks in the CVs, are altered with set potentials, suggesting that G. sulfurreducens self-regulates their extracellular electron transport pathways to adapt to different anode potentials. While the influence of anode potential on the current and power generation has been well studied in BESs using mixed cultures, there are few comprehensive investigations on how biofilm community structure and biomass on the anode are influenced by different set potentials. In one of the most comprehensive examinations of set potentials in MECs, Torres et al. showed that low set potentials of −0.15, −0.09, and 0.02 V selectively enriched G. sulfurreducens (92–99% of clones based on 16S rDNA clone library) with the highest current generation at higher potential (0.02 V) is surprising given that G. sulfurreducens was positively correlated with biomass. It has been shown to produce higher current densities at potentials of 0 V relative to −0.16 V by others.

One unusual aspect of the study by Torres et al. was that a single reactor was inoculated with four electrodes all set at different potentials in the same anode chamber. Placement of electrodes next to each other in the same vessel will result in “crosstalk” between these electrodes. Electric field gradients would exist between these electrodes and influence the migration of charged ions, substrate, and bacteria. Mediators produced on one electrode could be used by biofilms growing on other electrodes. Thus, the effect of set anode potential might not have been adequately addressed in their study.

To more comprehensively examine the effects of set anode potential, MECs (in duplicate) were inoculated and operated at different set potentials of −0.25, −0.09, 0.21, 0.51, and 0.81 V vs SHE using a potentiostat. When current generation became stable and the reactors were operated for more than one month, cyclic voltammetry (CV) was performed to characterize the electroactivity of the biofilms, the microbial community was analyzed by pyrosequencing, and total biomass was measured to determine the extent of bacterial growth at each set potential.

**MATERIALS AND METHODS**

**Reactor Configuration and Operation.** Single-chamber MECs were constructed using 5 mL clear glass serum bottles. Graphite plates 1 cm × 1.5 cm (GM-10, 0.32 cm thick) were used as anodes. Prior to use, they were polished using sandpaper (grit type 400), sonicated to remove debris, cleaned by soaking in 1 M HCl overnight, and rinsed three times with Milli-Q water. Stainless steel (SS) mesh (Type 304, mesh size 90 × 90) with the same size was used as the cathode. The distance between the anode and the cathode was ~1 cm, and Ag/AgCl reference electrodes (+210 mV vs SHE) were inserted between the two electrodes. Bottles were sealed using thick butyl rubber stoppers (20 mm diameter) and aluminum crimp caps. The electrode wires for the anodes (Ti) and cathodes (SS) as well as the reference electrode were inserted through the rubber stopper. All potentials were reported here versus SHE for comparisons to other studies.

The reactors (duplicates) were sparged with CO2/N2 (20%:80%) gas, and then inoculated using 1 mL of primary clarifier effluent collected from the Pennsylvania State University wastewater treatment plant, and 4 mL of growth medium sparged and maintained under a CO2/N2 (20%:80%) atmosphere. The growth medium contained (per L): 0.82 g of sodium acetate, 0.6 g of NaH2PO4, 1.5 g of NH4Cl, 0.1 g of KCl, 2.5 g of NaHCO3, 10 mL of minerals, and 10 mL of vitamins (pH 6.8). The MECs were incubated at five different anode potentials (−0.25, −0.09, 0.21, 0.51, and 0.81 V) set using a potentiostat (Uniscan PG580RM) in a temperature controlled room (30 °C). Solution in the MECs was replaced with 5 mL of fresh growth medium every cycle by piercing the butyl stopper with needles and syringes.

**Biomass Measurement.** Biomass on the anode was measured using a bicinchoninic acid protein assay kit (Sigma). Protein was extracted from the electrodes with 0.2 N NaOH. The graphite anode was removed from the MECs, placed in a test tube containing 2 mL of 0.2 N NaOH at 4 °C for 1 h, and vortexed every 15 min for 10 s. The extracted solution was collected, and the electrode was further rinsed with 2 mL of deionized water. The liquids were mixed together (final concentration of 0.1 N NaOH), frozen at −20 °C, and thawed at 90 °C. This freeze−thaw cycle was conducted three times. Then, 0.1 mL of the sample was used for protein analysis.

**Cyclic Voltammetry.** Cyclic voltammetry (CV) was conducted on the anode of MECs with the cathode as the counter electrode, and an Ag/AgCl reference electrode, in fresh growth medium. Scans ranged from −0.39 V to +0.91 V at a rate of 1 mV/s with only the fourth stable cycle shown. Nonturnover CVs were performed in depleted sodium acetate growth medium (when the current in MECs decreased to almost 0 mA).

**Microbial Community Analysis.** The anodic biofilms were removed from the graphite blocks with sterile pipet tips. The DNA was extracted using the PowerSoil DNA kit (MoBio, CA) according to manufacturer’s instructions. DNA samples were sent to Research and Testing Laboratory (www.researchandtesting.com) for pyrosequencing. Samples were amplified for pyrosequencing using a forward primer constructed with (5′−3′) Roche A linker, an 8−10 bp barcode, and the 28F primer (GAGTTTGATCNTGGCTCAG). The reverse primer was constructed with (5′−3′) with a biotin molecule, the Roche B linker, and the 519R primer (GTNTTACNGCGGCKGCTG). Amplifications were performed in 25 μL reactions under the following thermal profile: 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 54 °C for 40 s, 72 °C for 1 min, followed by one cycle of 72 °C for 10 min and then held at 4 °C. Amplification products were visualized, cleaned, and size selected following Roche 454 protocols (454 Life Sciences, Branford, Connecticut). DNA was hybridized to Dynabeads M-270 (Life Technologies) to create single stranded DNA following Roche 454. Single-stranded DNA was diluted, used in emPCR reactions, and sequenced following established manufacturer protocols (454 Life Sciences).

Sequences were processed for quality using the Mothur (version 1.3) software package. Briefly, sequences were denoised, screened for barcode and primer mismatches, homopolymeric runs, and sequence length. Chimeric sequences were detected and removed using the Uchime Chimera Detection Tool. Approximately 6000 (1100 per sample) sequences passed through quality control steps and were used for further study. High quality sequences were aligned, grouped into OTUs (97% similarity), and classified to the phylum and
genus level using the Silva 108 nonredundant database as the reference taxonomy. Dominant OTUs were further classified at the species level using the BLAST database. Sequences were deposited into the NCBI SRA under accession numbers SRP031498. The Jaccard similarity index was used to calculate the similarity between the bacterial communities.

**RESULTS**

**Current Generation.** The currents produced in MECs with different anode potentials were examined when repeatable cycles appeared after one month using fresh medium (Figure 1A). The maximum current and cycle time varied for anodes with different potentials, but the recovered Coulombs per cycle were similar (35–39°C) as shown by high Coulombic efficiencies of 90–100% based on the Coulombs contained in added sodium acetate (39°C). The longer cycle times for anodes set at potentials of −0.25 and 0.81 V indicated much slower substrate utilization rates of these anodic biofilms compared to biofilms acclimated at other anode potentials (−0.09, 0.21, and 0.51 V). The maximum current first increased with anode potential over the range of −0.25–0.21 V, but then decreased slightly at 0.51 V and more at 0.81 V (Figure 1B), with values of 0.44 ± 0.05 (−0.25 V), 1.73 ± 0.34 (−0.09 V), 2.47 ± 0.07 (0.21 V), 2.22 ± 0.14 (0.51 V), and 0.53 ± 0.21 mA (0.81 V). The increase in maximum current with set potentials up to 0.21 V suggested that the microorganisms benefitted from the greater differences between the anode potential and the standard biological redox potential of the substrate, that is, a more negative $\Delta G^\circ$ (eq 1). A decrease in maximum current at more positive potentials of 0.51 and 0.81 V might indicate the use of less effective metabolic pathways, changes in the biofilm community, or the induction of chemical reactions harmful to the bacteria. Additionally, the bell shaped response (Figure 1B) might be an example of the “inverted region” predicted by Marcus theory. According to this theory, the reaction rates usually become higher with increasing exergonicity of the reaction (more negative $\Delta G^\circ$), whereas electron transfer should become slower in the very negative $\Delta G^\circ$ domain because of increasing activation energy.

Biomass measurements were consistent with maximum currents as higher currents were associated with greater biofilm masses (Figure 2). The mass of biofilms (μg of protein) measured on the anodes at different potentials were 6 ± 1 (−0.25 V), 294 ± 20 (−0.09 V), 737 ± 70 (0.21 V), 528 ± 42 (0.51 V), and 41 ± 1 μg/cm² (0.81 V). According to previous measurements, a monolayer of G. sulfurreducens contained about 20 μg/cm² of protein. This suggests that the biofilm on the anode set at −0.25 V was sparse and did not completely cover the anode. For the other cases, there could have been complete coverage with two layers for the biofilm developed at 0.81 V. Much thicker biofilms would have been present on the other anodes. If we assume a minimum thickness of 1 μm per layer (the approximate diameter of a cell), the biofilms would have been 15 (−0.09 V), 37 (0.21 V), and 26 μm (0.51 V) on these anodes, which are similar to those previously reported. The ratios of current:protein were 48 (−0.25 V), 4 (−0.09 V), 2 (0.21 V), 3 (0.51 V), and 9 μA/μg (0.81 V). These ratios suggest that the electron transfer rate by cells was faster in thinner biofilms, especially for monolayer. It is reasonable that some cells in the biofilm did not facilitate efficient electron transfer especially in the thicker biofilms. Biomass increased with anode potentials in the range of −0.25 to 0.21 V and resulted in greater current, indicating more energy was gained for bacterial growth as anode potentials were increased over this range. However, biomass decreased when the potential was increased from 0.21 to 0.81 V, indicating less favorable conditions for biofilm growth at these high potentials.

**Electrochemical Analysis.** CVs were performed to examine the electroactivity of biofilms acclimated at different anode potentials in fresh growth medium (Figure 3A). All biofilms started producing currents at −0.3 to −0.2 V, and then currents rose with increasing potentials and became stable in high potential region. The stabilized peak currents varied for biofilms acclimated at different potentials, with values of 0.29 ±

**Figure 1.** (A) Current evolution of MECs developed at different anode potentials over time for a single cycle, and (B) the maximum current of MECs acclimated at different anode potentials (−0.25, −0.09, 0.21, 0.51, and 0.81 V vs SHE) after stable operation for over one month. Growth medium: 0.82 g/L sodium acetate in bicarbonate buffer with pH of 6.8.

**Figure 2.** The relationship between the maximum currents of MECs developed at different anode potentials (−0.25, −0.09, 0.21, 0.51, and 0.81 V vs SHE) and the biomasses of anodic biofilms after stable operation for over one month. Growth medium: 0.82 g/L sodium acetate in bicarbonate buffer with pH of 6.8.
There is no diffusion of current decay in these CVs, indicating that sodium acetate is unlikely depleted when added at a concentration of 0.82 g/L, and therefore the different steady-state currents likely reflect transport of redox species through these biofilms.

To examine the exocellular electron transfer components (EETCs) in biofilms with these different set potentials, nonturnover CVs were performed in depleted sodium acetate growth medium (current decreased to nearly 0 mA) (Figure 3B). Four obvious oxidation peaks were observed at −0.18 ± 0.02 (P1), −0.11 ± 0.02 (P2), 0.12 ± 0.01 (P3), and 0.77 ± 0.02 (P4). The potential of P1 was close to the midpoint potentials reported for multiheme cytochrome OmcZ (−0.22 V), OmcB (−0.19 V), and the periplasmic cytochrome C (PpcA, −0.17 V) purified from *G. sulfurreducens*. These nonturnover CV results showed that different predominant EETCs were expressed when the biofilms were acclimated at different potentials. For the biofilms acclimated at low potentials (−0.25 and −0.09 V), peaks P1 and P2 were predominant (Figure 3B), which was consistent with the fast current increase of these biofilms in the potential range of −0.3 to −0.1 V in fresh growth medium (Figure 3A). An additional peak of P3 was observed for the biofilm incubated at 0.21 V (Figure 3B), resulting in a great increase in current around a potential of 0.1 V for this biofilm in the presence of substrate (Figure 3A). When anode potential further increased to 0.51 V, the peak P3 disappeared and P4 appeared (Figure 3B), consistent with the slow current increase of this biofilm in the potential range of 0.1 to 0.7 V in fresh growth medium (Figure 3A). For the biofilm acclimated at 0.81 V, peaks P1 and P2 also disappeared and P4 became apparent (Figure 3B), resulting in a very slow current increase over the whole potential range of −0.3 to 0.7 V with fresh growth medium (Figure 3A). The changes of the EETCs predominance for the biofilms acclimated at different potentials demonstrated either bacteria altered their extracellular electron transfer pathways to adapt to different anode potentials, or that different bacteria became predominant in the biofilm that expressed different EETCs.

**Microbial Community Analysis.** The microbial communities of biofilms developed at different anode potentials were 79 ± 8% similar based on OTUs (defined as >97% sequence similarity) shared between samples, with high percentages of the sequences indicating dominance of *Proteobacteria*: 85% (−0.25 V), 78% (−0.09 V), 78% (0.21 V), 67% (0.51 V), and 80% (0.81 V) (Figure 4A). The *Proteobacteria* in each reactor were all most similar to *G. sulfurreducens* (94%–99% of Proteobacterial sequences, with a 97% or higher similarity) (Figure 4B). *G. sulfurreducens* was dominant in all samples with an average percentage of 75 ± 6%. The lack of a change in bacterial community composition suggests that the changes in the EETCs noted in CVs were not due to changes in community. This conclusion is supported by pure culture studies using *G. sulfurreducens* which shows that different EETCs, at potentials similar to those here, similarly evolve with changes in anode potentials.
DISCUSSION

These results conclusively demonstrate that set anode potential does not alter the structure of the microbial community when these potentials are set in separate, single-chamber MECs. The communities were predominantly Proteobacteria at all the different anode potentials, with sequences most similar to G. sulfurreducens. This similarity of the communities is somewhat different than that reported by Torres et al.18 in their tests over a smaller range of potentials. Although Torres et al. similarly reported that lower anode potentials of −0.15, −0.09, and 0.02 V produced communities that were most similar (>97% similarity for 92% of clones) to G. sulfurreducens, a higher anode potential of 0.37 V produced a very diverse community with a predominance of the phyla Bacteroidetes, Actinobacteria, Firmicutes, and Proteobacteria. We suggest that their results may have been affected by placing the four electrodes set at different potentials in the same chamber which induced “crosstalk” among these electrodes. Electric fields surrounding these electrodes would influence the preferential flux of negatively charge species (substrate and bacteria), and consequently the colonization and growth of bacteria on these electrodes. Biofilms growing on one electrode could produce mediators that affect growth and current production of biofilms on other electrodes. Here, we used separate reactors at each potential so that there was no interaction between the different anodes.

The use of multiple electrodes in the same chamber also will affect the anode potential that will produce the highest current. Torres et al.18 found that the most negative anode potential (−0.15 V compared to −0.09, 0.02, and 0.37 V) produced the highest current. Parot et al.19 also placed three electrodes in a single chamber using different potentials (0.54, 0.74, and 0.97 V) and found higher currents were obtained at lower positive potentials. These results are different from that obtained here, as well as in many other studie., where higher current was obtained at more positive potentials when electrodes were placed in separate reactors.10,13,14

Another important variable that can affect microbial community development is the use of single- or two-chamber reactors. Kumar et al.12 compared the performance of biofilms grown at different set anode potentials (−0.09, 0.01, and 0.41 V), and found the maximum current density was obtained at 0.41 V in the single-chamber reactor, but at 0.01 V with the two-chamber system. The biofilm community showed >76% similarity among all the different set potentials for samples from anodes in single-chamber reactors, and >65% similarity for samples from anodes in two-chamber reactors, but the anode communities were different from each other in these two systems. Proteobacteria dominated on the anodes in single-chamber reactors, while Tenericutes was the most dominant phylum for the two-chamber reactors. The reasons for the differences were not clear, and it may be that community development was affected by hydrogen gas production and accumulation in the anode chamber of the single-chamber system. G. sulfurreducens can use hydrogen gas as an electron donor, and experiments in single-chamber systems with this microbe showed use of hydrogen gas based on Coulombic efficiencies larger than 100% due to hydrogen cycling from the cathode to anode.6,37 In addition, anode communities fed acetic acid decreased in species richness and diversity, and increased in numbers of G. sulfurreducens, when reactors were shifted from single-chamber MFCs (little or no H2 evolution) to MECs (H2 generated).38 Acetate utilization by G. sulfurreducens is inhibited by high concentrations of H2, and thus biomass production could be adversely affected as this species cannot fix CO2.39 However, it appears that the net result of hydrogen gas evolution in a single-chamber system is to favor the growth of G. sulfurreducens and other hydrogen oxidizing exoelectrogens.40 In both single- and two-chamber systems, growth and current generation of G. sulfurreducens were improved at anode potentials more positive than 0 V compared to more negative potentials.10,15 Thus, while the chamber configuration is a factor in community development with mixed cultures, the growth of G. sulfurreducens was favored in both configurations at more positive potentials. The presence of hydrogen gas in our experiments, versus that conducted with multiple anodes in two-chamber systems,18 is another important factor to account for the differences in optimal potentials and community evolution, and should be examined further.

The responses of mixed cultures obtained here to the different anode potentials is not the same as that previously obtained using pure cultures of G. sulfurreducens (Supporting Information Figure S1). Previous tests15 in our laboratory using G. sulfurreducens at the same anode potentials and with the same reactors used here showed the maximum current increased with anode potentials up to 0.21 V, and then became constant at the higher anode potentials (Supporting Information Figure S1). This observation on the effect of anode potential was consistent with another study10 on G. sulfurreducens in a two-chamber MEC, where current increased at potentials of −0.16 to 0 V, with no further increase at 0.4 V. The different optimum potentials found in our study (0.21 V) and the other study (0 V) could be due to the use of the single-chamber design in our study, versus two-chamber design in the other study. The different responses of mixed culture (predominantly G. sulfurreducens) and pure cultures of G. sulfurreducens indicated that the presence of other bacteria can impact current generation at these relatively high set potentials.

Community development in both MECs and MFCs is complex, and clearly affected by a number of factors in addition to anode potentials. For example, in MFCs anode community development and power production can adversely be affected by oxygen transfer from the cathode,42 or the resistance used to acclimate the reactors.42 A comparison of set potentials compared to fixed resistances used in MFCs with anodes close to the cathodes, with a separator used to reduce oxygen transfer, showed that a set anode potential of 0 V worked better for biofilm acclimation than 0.2 V or a fixed resistance (1000 Ω).43 However, multiple tests at −0.2 V often produced erratic results in startup. The most consistent method to produce good performance in the MFCs was to use a transfer of solution from an existing MFC.43,44 Such transfers to inoculate MFCs and MECs are commonly reported in many studies.43−46 Microbial community development is also affected by the substrate used,47−49 although it appears that the community that produces good current densities is inevitably associated with the predominance of various Geobacter species when acetate is used.20,50

ASSOCIATED CONTENT

Supporting Information

Maximum currents of MECs with mixed culture and G. sulfurreducens developed at potentials of −0.25, −0.09, 0.21, 0.51, and 0.81 V vs SHE. This material is available free of charge via the Internet at http://pubs.acs.org.
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