

## Electricity production and microbial biofilm characterization in cellulose-fed microbial fuel cells

Z. Ren, L. M. Steinberg and J. M. Regan

### ABSTRACT

Converting biodegradable materials into electricity, microbial fuel cells (MFCs) present a promising technology for renewable energy production in specific applications. Unlike typical soluble substrates that have been used as electron donors in MFC studies, cellulose is unique because it requires a microbial consortium that can metabolize both an insoluble electron donor (cellulose) and electron acceptor (electrode). In this study, electricity generation and the microbial ecology of cellulose-fed MFCs were analyzed using a defined co-culture of *Clostridium cellulolyticum* and *Geobacter sulfurreducens*. Fluorescent *in situ* hybridization and quantitative PCR showed that when particulate MN301 cellulose was used as sole substrate, most *Clostridium* cells were found adhered to cellulose particles in suspension, while most *Geobacter* cells were attached to the electrode. By comparison, both bacteria resided in suspension and biofilm samples when soluble carboxymethyl cellulose was used. This distinct function-related distribution of the bacteria suggests an opportunity to optimize reactor operation by settling cellulose and decanting supernatant to extend cellulose hydrolysis and improve cellulose-electricity conversion.

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### INTRODUCTION

The finite resource of fossil fuels and environmental pollution derived from their use are driving the search for renewable and clean energy alternatives. This replacement of fossil fuels will require the use of myriad energy sources and energy carriers suited to meet different end uses. As the most abundant biopolymer on earth, cellulose is a promising renewable resource for sustainable energy production. In the United States alone, besides 65–90 billion dry tons of standing cellulosic vegetation, cellulose is also a significant component in the annual production of 250 million tons municipal solid wastes and 40 billion cubic metres wastewater (Perlack *et al.* 2002). Cellulose can be converted to a variety of energy carriers such as ethanol (Mielenz 2001), biodiesel (Powlson *et al.* 2005), and hydrogen (Ren *et al.* 2007a), but the treatment and energy recovery from

cellulose in wastewater is very difficult because of its recalcitrance to biological treatment and low energy density.

Representing an alternative method of renewable energy recovery, the direct conversion of organic waste to electricity using a microbial fuel cell (MFC) or hydrogen using a microbial electrolysis cell (MEC) offers the potential of clean and distributed energy production (Logan & Regan 2006). MFCs use electrochemically active microorganisms as biocatalysts and have tremendous electron donor versatility, using virtually any biodegradable resource as substrates. Only recently has this versatility been extended to cellulose (Rezaei *et al.* 2007; Rismani-Yazdi *et al.* 2007; Ren *et al.* 2007b; Ishii *et al.* 2008). Because there are no known microorganisms that can both use cellulose as an electron

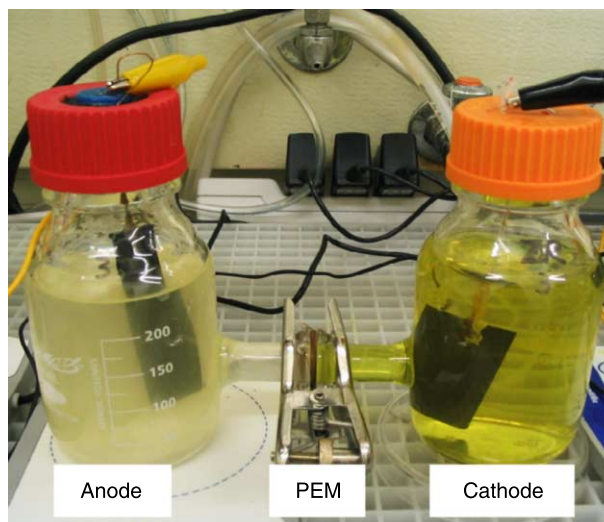
donor and transfer electrons to the anode as an electron acceptor, the conversion of cellulosic wastes to electricity requires a syntrophic consortium. Ren *et al.* (2007b) employed a defined co-culture of polymer-degrading, fermentative *Clostridium cellulolyticum* and fermentation product-utilizing, electrochemically active *Geobacter sulfurreducens* to synergistically convert cellulose into electricity in an MFC. The maximum power density using a two-chamber MFC with a ferricyanide catholyte was 143 mW/m<sup>2</sup> from soluble carboxymethyl cellulose (CMC) and 59.2 mW/m<sup>2</sup> from insoluble MN301 cellulose.

The coupling of insoluble electron donor and acceptor substrates, combined with discrete community members that can metabolize each substrate, exerts a unique ecological pressure presumably with the cellulose degrader adhering to cellulose particles and the anode reducer forming a biofilm on the electrode surface. Further analyses of bacterial distribution and operational implications are needed to improve the performance of cellulose-fed MFCs. In this study, the same binary culture of *C. cellulolyticum* and *G. sulfurreducens* was used in cellulose-fed, two-chamber MFCs, and the bacterial distribution and anode biofilm architecture were investigated.

## METHODS

### MFC inoculum, construction, and operation

Two-chamber MFCs as previously described were used in this study (Ren *et al.* 2007b). Two medium bottles (310 ml capacity, VWR Inc.) were separated by a proton exchange membrane (Nafion™ 117, Dupont Co.) (Figure 1). The anode chamber was sealed with a stopper and bottle cap to keep it free of oxygen. *G. sulfurreducens* (ATCC 51573) and *C. cellulolyticum* (ATCC 35319) cultures were inoculated into the anode chambers with medium containing (per liter) 1.05 g NH<sub>4</sub>Cl, 0.1 g KCl, 4.90 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 9.15 g Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g yeast extract, 2.5 g NaHCO<sub>3</sub>, 10 ml trace mineral mix, and 10 ml vitamin mix (Cheng *et al.* 2006). For electron donor, 1 g/L sodium CMC or MN301 was used in different reactors. Graphite plates (16 cm<sup>2</sup> surface area, equally fluted into 4 divisions) were used as anodes. Cathode chambers were filled with 100 mM K<sub>3</sub>Fe(CN)<sub>6</sub>



**Figure 1** | Two-chamber microbial fuel cells used in this study. Anode chamber (left) was filled with growth medium, cathode chamber (right) was filled with buffered ferricyanide solution.

solution in 100 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.0) and exposed to air. Plain carbon paper (same projected surface area as anodes) was used for cathodes. The electrodes were connected through an external circuit by a copper wire. All exposed surfaces of the wire were coated with nonconductive epoxy. MFCs were operated in fed-batch mode in a 30°C room, and medium and K<sub>3</sub>Fe(CN)<sub>6</sub> were replaced when the voltage dropped below ~ 40 mV.

### Analyses

MFC voltage (V) was continuously monitored using a data acquisition system (ADC22, Pico Technology, Ltd.) under a fixed load of 1,000 Ω. Polarization measurements were conducted during the stable power production stage of each batch. Power (*P*) was calculated as  $P = V^2/R$  (where *R* is the external circuit resistance) and normalized by the surface area of the anode to give power density (Cheng *et al.* 2006).

### FISH procedure

Suspension and anode samples were collected for fluorescent *in situ* hybridization (FISH) analyses after attaining stable power generation (3 to 4 batches of operation). MFCs were disassembled in an anaerobic

chamber, and samples were fixed in 4% paraformaldehyde for 8 hours at 4°C. After fixation, sections of graphite anode were mounted onto glass slides using silicon adhesive and surrounded by an equal height of support to allow a coverslip to rest above the biofilm. Suspension samples were concentrated by centrifuge, resuspended in PBS, and spotted onto Teflon-coated slides (Park *et al.* 2002; Regan *et al.* 2003). Following the dehydration of samples in a gradient of 50, 80, and 95% ethanol for 3 min each, hybridizations with oligonucleotide probes were performed in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% SDS, 30% formamide, pH 7.2) at 46°C for 2 hours. After hybridization, the samples were washed (71 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, pH 7.2) at 48°C for 20 min, rinsed with deionized ice-cold water, air dried, and mounted in DABCO to reduce photo-bleaching. Two oligonucleotide probes were used: modified SRB385 (5'-CGGCGTYGCTGCGTCAGG-3') labeled with Cy3 to target *G. sulfurreducens*, and EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') labeled with FITC (Ito *et al.* 2002) to target both members of the binary community. After the FISH procedure, some samples were counter-stained using 4'-6-diamidino-2-phenylindole (DAPI) or SYTO-9. In some MN301 samples, a 1% solution of Congo red was added to the fixed samples on the slides for 5 min before the dehydration step to stain cellulose particles (Amann *et al.* 1990; Burrell *et al.* 2004). Visualization of fluorescently stained cells was performed on a Zeiss Axiophot epifluorescent microscope equipped with a mercury lamp and an Olympus Fluoview 1000 Confocal Laser Scanning Microscope (CLSM; Olympus America Inc., Melville, NY) equipped with 3 lasers (peaks at 488, 543, and 633 nm). The three-dimensional anode biofilm architecture was scanned and displayed as ortho view. Images were analyzed by Photoshop and FV10-ASW software.

### Real-time PCR

Primers were designed to quantify *C. cellulolyticum* and *G. sulfurreducens* abundance in MFC suspensions and anode biofilms. The primer pair targeting *C. cellulolyticum* and other members of *Clostridium* Group III consisted of forward primer Clos956f (5'-CCTTCTGTGCCGGAGTTAACA-3') and reverse primer Clos1428r (5'-CCCACMATCTGA-ACTGGGACTAT-3'), which generated a fragment of

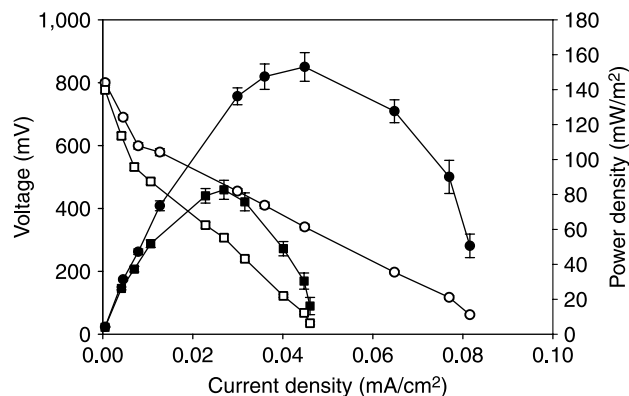
approximately 470 base pairs (bp). The primer pair targeting *G. sulfurreducens* and other closely related *Geobacter* consisted of forward primer Geo587f (5'-GGGAGGGAA-GAAATGATTG-3') and reverse primer Geo978r (5'-GGG-CTCAATACCCGCCAACA-3'), which generated a fragment of approximately 400 bp. Primer sequences were checked for specificity using the Probe Match function of the Ribosomal Database Project II release 9 (<http://rdp.cme.msu.edu/>) and the BLAST function of GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>), and they were checked for appropriate melting temperature and possible secondary structures using NetPrimer (<http://www.premierbiosoft.com/netprimer/index.html>). Experimental primer verification was performed with DNA extracted from cultures of *C. cellulolyticum* and *G. sulfurreducens*. Polymerase chain reaction (PCR) conditions consisted of an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 30 s, with a final extension at 72°C for 7 min. PCR products were run on a 1% agarose gel at 15 V/cm for 25 min to confirm appropriate size and no non-specific amplification. Fragments from this PCR were ligated into vector pCR2.1 and cloned into *E. coli* Top10 cells using a TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Blue-white screening was used to determine clones containing the PCR fragments. Positive clones for each PCR product were grown overnight at 37°C in LB broth containing 100 µg/mL ampicillin. Plasmids containing the PCR fragment insert were purified using a QIAprep Miniprep Kit (QIAGEN, Valencia, CA). Plasmid was quantified at 260 nm on a spectrophotometer, and stock solutions of 10 ng/µl were made in 10 mM Tris-HCl, pH 8.5. Standard curves for real-time quantitative PCR (qPCR) were prepared in 10 mM Tris-HCl, pH 8.5 as 10-fold dilutions from 1 ng/µl to 0.002 pg/µl. Quantitative PCR was performed on a Bio-Rad iCycler using 96-well polypropylene plates and optical sealing tape (Bio-Rad, Hercules, CA). Standards were run in duplicate and samples from the MFCs were run in triplicate. Total reaction volumes of 20 µl contained final concentrations of 1 × PCR buffer and 0.03 U/µl Taq polymerase (USB, Cleveland, OH), 2.5 mM MgCl<sub>2</sub>, 0.25 µM each primer, 0.5 M Betaine, 10 nM fluorescein (Bio-Rad, Hercules, CA), a 150,000 × dilution of SYBR Green (Molecular Probes, Invitrogen, Carlsbad, CA),

and 2  $\mu\text{l}$  of template DNA. Reaction conditions were the same as already described except that 40 cycles of PCR were run and each cycle contained a 10 s step at 85°C inserted after extension for image capture. This step was necessary to eliminate fluorescence caused by the formation of primer dimers during the PCR.

## RESULT AND DISCUSSION

### Power production

Cultures of *G. sulfurreducens* and *C. cellulolyticum* were added concurrently to an MFC. Previous data showed that pre-acclimation of a *G. sulfurreducens* anode biofilm reduced the lag time relative to a concurrently inoculated system, but eventually both inoculation strategies produced comparable maximum power densities after reaching stable operation (Ren *et al.* 2007b). After four batches of operation, the voltage of the CMC-fed reactor was stable at approximately 470 mV (1,000  $\Omega$  resistance), while the voltage of the MN301-fed reactor was stable at approximately 350 mV. Power density curves showed maximum power densities of 153 mW/m<sup>2</sup> for CMC and 83 mW/m<sup>2</sup> for MN301, respectively (Figure 2). These maxima were slightly higher than reported for previous experiments with this binary culture because a higher buffer capacity was used in this study to maintain a stable pH. Lower electricity generation was still observed from MN301 relative to CMC, presumably due to the lower rate of MN301 hydrolysis by *C. cellulolyticum*.

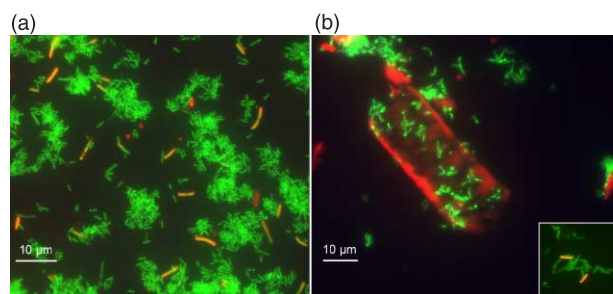


**Figure 2** | Voltage (hollow symbols) and power density (solid symbols) vs. current density obtained by varying the external resistance (50–50,000  $\Omega$ ), 1 g/L CMC (● and ◯) and 1 g/L MN301 (■ and □).

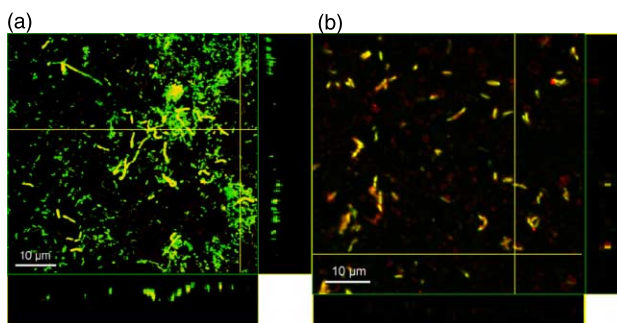
Refer to Ren *et al.* (2007b) for end-product variation and cellulose consumption data for similarly operated reactors.

### FISH analysis of suspension and biofilm samples

Two fluorescently labeled oligonucleotide probes were applied to the samples, modified SRB385 to specifically target *G. sulfurreducens* and EUB338 targeting both bacteria. FISH images of MFC suspension samples showed *C. cellulolyticum* cells were the dominant community member in suspension in both CMC- and MN301-fed reactors (Figure 3). In the CMC-fed reactor, *Clostridium* aggregates were observed in suspension, with individual *Geobacter* cells at a much lower cell density. In the MN301-fed reactor, many of the *Clostridium* cells were attached to the surface of cellulose particles, and very few *Geobacter* cells could be found in suspension. CLSM was used to analyze the architecture of the anode biofilms. It was found that both *Clostridium* and *Geobacter* resided on CMC-fed anode biofilms and they tended to form aggregates instead of evenly colonizing the whole anode area (Figure 4). In areas of biofilm coverage, the thickness of the biofilm was approximately 25  $\mu\text{m}$ , with no clear stratification of the two bacteria in the thin biofilm with soluble cellulose. Only *Geobacter* cells were found in the biofilm of MN301-fed reactors, and the cells were quite scarce on the electrode compared to the CMC biofilm. Counterstaining of cells with DAPI or SYTO-9 showed a correspondence with the EUB338 probe fluorescence, indicating successful hybridization.



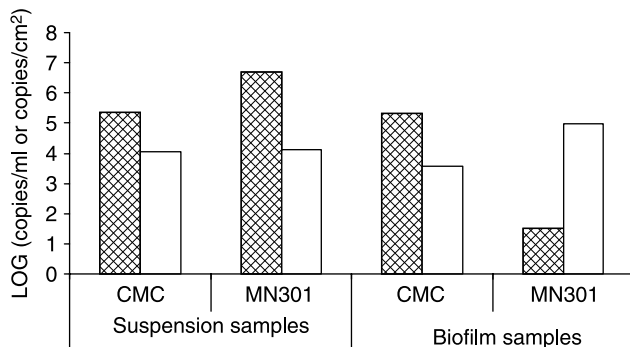
**Figure 3** | FISH images from suspended samples: (A) CMC-fed MFC, and (B) MN301-fed MFC. Samples were hybridized with Cy3-labeled SRB385 probe targeting *Geobacter* and FITC-labeled EUB338 probe targeting both bacteria, resulting in yellow to orange *Geobacter* cells and green *Clostridium* cells. MN301 cellulose particles were stained with Congo red. Inset in panel B shows the fluorescent response of *Geobacter* cells. Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from <http://www.iwaponline.com/wst>.



**Figure 4** | Confocal FISH images of biofilm growing on MFC anodes: (A) CMC-fed MFC, and (B) MN301-fed MFC. Anode biofilms were hybridized with Cy3-labeled SRB385 probe targeting *Geobacter* and FITC-labeled EUB338 probe targeting both *Clostridium* and *Geobacter*, resulting in yellow-orange *Geobacter* cells and green *Clostridium* cells. Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from <http://www.iwaponline.com/wst>.

### Real-time PCR analyses

The FISH results were complemented with quantitative PCR analyses to separately quantify *C. cellulolyticum* and *G. sulfurreducens* abundance. The results showed cell concentrations of *Clostridium* in suspension were 21 and 370 times higher than *Geobacter* in CMC and MN301 reactors, respectively (Figure 5). This is consistent with the qualitative FISH observations, in which *G. sulfurreducens* cells were rarely visualized in the MN301 suspension samples. Additionally, there was a five thousand times higher *Clostridium* density in CMC biofilm than in MN301 biofilm, while 25 times more *Geobacter* were found attached on the anode in the MN301 reactor than in the CMC reactor. These quantitative data were consistent with FISH observations, suggesting a distinct function-related distribution of the two bacteria. When soluble substrate was used, both bacteria resided in bulk solution as well as biofilms, but when



**Figure 5** | Comparison of gene copy numbers in four different conditions using real-time PCR. *C. cellulolyticum* (▨); *G. sulfurreducens* (□).

particulate cellulose was used, much more *Clostridium* cells were found attached on cellulose in suspension than in biofilm, while more *Geobacter* were adhered on the biofilm than in bulk solution. Based on standard curves, the linear dynamic range for qPCR was seven orders of magnitude, from  $4.15 \times 10^8$  to 415 target gene copies/reaction. The reaction efficiency ranged between 90–100%, with a  $y$ -intercept of 38–40 and  $R^2$  of greater than 0.99 for all standard curves. No-template controls did not show amplification.

*C. cellulolyticum* does not need the anode for cellulose fermentation, but adhesion of these cells to cellulose is required for rapid and efficient cellulose hydrolysis and soluble cellodextrin utilization (Desvaux 2005; Ren et al. 2007a). Therefore, it is understandable that most *Clostridium* were found adhered to MN301 cellulose particles and very few cells were found in the anode biofilm, presumably growing on the soluble MN301 hydrolysis products generated by suspended cells. Contrarily, *C. cellulolyticum* would be expected to metabolize CMC both in suspension and on the surface of electrode, since this is already a soluble form of cellulose. For *G. sulfurreducens*, which used the electrode as the sole electron acceptor in these systems, cellular attachment to the anode was apparently preferential for electron transfer. That almost all *Geobacter* cells were found attached to the electrode is consistent with the findings of other studies (Bond & Lovley 2003). The limited number of *Geobacter* found in suspension may have been due to the detachment of biofilm-associated *Geobacter* by continuous stirring or sample collection. There was no clear stratification of the two bacteria found within the anode biofilm. The distinct roles of the two bacteria in this unique system with insoluble electron donor and acceptor led to a function-related distribution, with *Clostridium* hydrolyzing and fermenting cellulose primarily in suspension and *Geobacter* preferentially attaching to the anode to transfer electrons. Coupled with the consideration that cellulose hydrolysis is the rate-limiting factor for power generation from cellulose, an improvement in reactor performance might be realized in a system with settling and decanting.

### CONCLUSIONS

Using two-chamber MFCs, cellulose was directly converted into electricity by a co-culture of *C. cellulolyticum* and

*G. sulfurreducens*. FISH and quantitative PCR analyses showed a consistent function-related bacterial distribution, with most *Geobacter* attached to the anode surface and most *Clostridium* adhered to MN301 cellulose particles. When soluble cellulose (i.e., CMC) was used, a more even distribution was observed. This study revealed a unique ecology system in MFCs, and further study is needed to correlate biocatalyst density with reactor performance for system optimization and to explore the effectiveness of settling and decant at retaining a higher cellulolytic cell density for enhanced kinetics and extent of cellulose conversion.

## ACKNOWLEDGEMENTS

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