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Characterization of microbial communities during anode biofilm reformation in a two-chambered microbial electrolysis cell (MEC)

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ABSTRACT

GeoChip (II) and single strand conformation polymorphism (SSCP) were used to characterize anode microbial communities of a microbial electrolysis cell (MEC). Biofilm communities, enriched in a two-chamber MEC (R1, 0.6 V applied) having a coulombic efficiency (CE) of 35 ± 4% and a hydrogen yield (Y_{H_2}) of 31 ± 3%, were used as the inoculum for a new reactor (R2). After three months R2 achieved stable performance with CE = 38 ± 4% and Y_{H_2} = 31 ± 7%. Few changes in the predominant populations were observed from R1 to R2. Unlike sludge inoculation process in R1 in the beginning, little further elimination was aroused by community competitions in anode biofilm reformation in R2. Functional genes detection of biofilm indicated that *cytochrome* genes enriched soon in new reactor R2, and four genera (*Desulfovibrio*, *Rhodopseudomonas*, *Shewanella* and *Geobacter*) were likely to contribute to exoelectrogenic activity. This work also implied that symbiosis of microbial communities (exoelectrogens and others) contribute to system performance and stability.

1. Introduction

The development of microbial electrolysis cells (MECs) is a promising technology to overcome the "fermentation barrier" by further recovering hydrogen from waste biomass (Liu et al., 2005; Logan et al., 2008). Since 2005, different MEC designs have focused on improvement of process efficiency and energy recovery. MECs can now achieve over 90% electron recovery as current (coulombic efficiency) and 86% hydrogen recovery using single chamber reactors, which are values typically much higher than recoveries in microbial fuel cells (MFCs) (Cheng and Logan, 2007; Call and Logan, 2008). However, there are many unknown aspects regarding the functional bacterial groups (exoelectrogens) involved in the electron transfer in MECs. It is important to gain a better understanding of how exoelectrogens work together, and to better define the role of other communities in the anode biofilm.

Exoelectrogens that are typically enriched in MFCs (Jung and Regan, 2007; Kim et al., 2007; Chae et al., 2009) include *Geobacter* sp. and *Shewanella* sp., which are known to have outer-membrane cytochromes and conductive pili (Reguera et al., 2005), and *Pseudomonas* sp. (Pham et al., 2008), that has used self-produced mediators to shuttle electrons outside the cell or between cells. Pure cultures have been used to better understand the electron transfer mechanisms and improve power output, but power production by pure cultures of *Shewanella* sp., *Geobacter* sp. and *Pseudomonas* sp. is often less than those of the mixed cultures (Rabaey et al., 2004; Ishii et al., 2008; Watson and Logan, 2010). It has been found that interaction between two microbes can improve system performance and energy recovery efficiency when combining *Brevibacillus* sp. with *Pseudomonas* sp. (Rabaey et al., 2007). However, synergistic interactions and the dynamics of community changes have been insufficiently examined in mixed cultures reactors (Logan, 2009).

The process of anodic biofilm community change was examined in a two-chamber MEC reactor over long-term operations at a fixed external voltage. In this study we examined changes of microbial communities and functions in anodic biofilms. Understanding these relationships will help to find the key for a high-efficiency bioelectrochemical system for bio-energy conversion.

2. Materials and methods

2.1. Setup of two-chamber MEC reactors

Two-chamber MECs were "H" type reactors as previously described (Liu et al., 2008). The anode was plain carbon cloth (128 cm^2, without wet proofing; E-Tek) and the cathode was carbon paper with 0.35 mg cm^{-2} Pt (9 cm^2, E-Tek). A voltage of 0.6 V was applied using a direct current power source (PS-B202D, Yizhan Electronic Instrument Co., Ltd.). Current was measured

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by multimeters in the circuit for the efficiency calculation. The coulombic efficiency was calculated by $CE = Q_c/Q_T \times 100\%$, where Q_c was current coulombs calculated by the equation $Q = I \cdot t$ and Q_T was coulombs of used acetate. The hydrogen yield was calculated using the equation $Y_{H_2} = Q_H/Q_T \times 100\%$. The Q_H presents the electron coulomb used to produce hydrogen according to $Q_H = 2nF$, where F is Faraday's constant (96485 C mol^{-1}) and n is the moles of hydrogen produced, calculated as $n = PV/(RT)$, where P is the atmospheric pressure (101325 Pa), V is the hydrogen volume (m^3), R is the gas constant (8.314 J mol^{-1} K^{-1}), and T is the temperature (K).

A two-chamber MEC reactor (R1) was set up and inoculated using sewage sludge with acetate as the fuel. Anode medium was prepared as follows: 0.1 g of KCl, 0.2 g of NH_4Cl, 0.6 g of NaH_2PO_4, 2.5 g of $NaHCO_3$, 10.0 mL of Wolfe's vitamin solution, 10 mL of Wolfe's mineral solution, and 10 mmol of sodium acetate as sole carbon source, 1000 mL, pH 7.0. The sludge was collected from raw sludge (combination of primary and secondary sludge) at a local waste water treatment plant in Harbin, China. The sludge was kept under anaerobic conditions for 24 h before mixing with the anode medium (50/50, v/v). The reactor was operated in fed-batch mode, with the medium replaced when the current decreased to <0.2 mA. The solutions were sparged with nitrogen gas (99.99%) for 20 min each time the medium was replaced. After R1 running three months, a second reactor (R2) was then started up by inoculating R1 biofilm communities and operated all the same conditions as R1. Reactor R2 keep running three months for taking community samples. Phosphate buffer solution (10 mM PBS, pH 7.0, 10 mS/cm) was sterilized and used for the cathode. All experiments were performed at room temperature (25 °C).

2.2. Community sampling and single strand conformation polymorphism (SSCP)

Samples were taken at the end of batch cycle at different time points as listed in Table 1. Each sample was randomly collected from three different positions on the anode surface, using a sterile platinum loop, and then combined into a single biofilm sample. The treatment of communities samples, including storage, pretreatment, DNA extraction, purification, and amplification were done as we described in details before (Liu et al., 2008). Following descriptions before, a polyacrylamide gel was made based on MDE Gel Solution (Cambrex Bio science). The SSCP profile was scanned using UMAX Powerlook 2000. Specific bands were cut for DNA recovery and further sequencing. Quantity One (Bio-Rad Laboratories, US) was used to define lanes and bands in the SSCP profile. Sequence similarity and classication were completed with sequence matching software developed by the Ribosomal Database Project and the BLAST program on the website of the National Center for Biotechnology Information (NCBI).

2.3. Functional gene analysis by GeoChip (II)

Further community structure changes were analyzed at two time points during biofilm reformation, using GeoChip (II), based on established methods described previously (Wu et al., 2004). Sample S1 was collected the second day (30 h) after reactor setup. Sample S2 was taken after stable hydrogen generation was observed (~three months). The gene categories examined were those involved in the main metabolic functions for environmental microorganisms (He et al., 2007). Spots of GeoChips with signal-to-noise ratios (SNR) < 2, outliers (standard deviation, SD > 3), and poor spots were removed. Data normalization was based on the method of mean signal intensity across all genes (Wu et al., 2008), and any gene with more than 1/3 of the probe spots hybridized was considered positive (He et al., 2007).

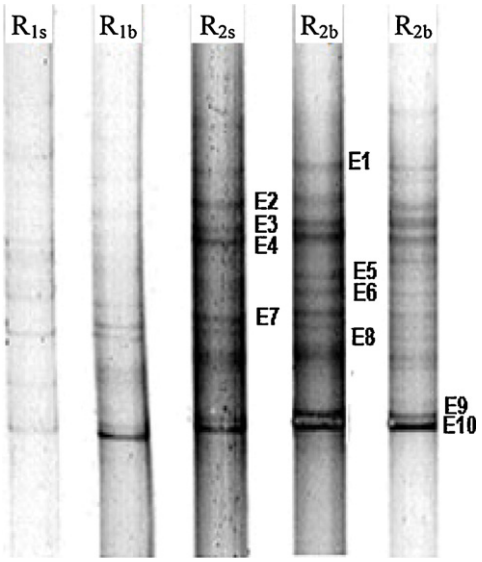

Fig. 1. Analysis of microbial communities and defining lanes/bands by SSCP.

3. Results

3.1. Reactor performance over the operations

The reactor inoculated with sludge (R1) achieved a coulombic efficiency of CE = 35 ± 4% and hydrogen yield of $Y_{H_2} = 31 \pm 3\%$, based on acetate consumption, after three months of operation (Table 1). The R2 reactor, inoculated using biofilm from R1, generated current in batch cycles after 30 h of operation, with a CE = 30 ± 5% and $Y_{H_2} = 20 \pm 4\%$ after one week. After three months, the reactor performance increased CE from 32 ± 4% to 38 ± 4%, Y_{H_2} from 29 ± 4% to 31 ± 7% on average. COD removal was consistent, averaging 82 ± 4% (range of 80–83%) over three months. The consistency of the reactor performance suggests that there was a stable microbial community in the anode biofilm for electron generation and the consortium could attach to new anode and work together in a short time.

3.2. Overall community structures and changes over the operations

Fig. 1 shows the results of microbial community analysis during different operational periods using SSCP technology. A total of 10 bands were observed over the operational period representing 12 genera based on similar sequences (Table 2). Generally, microorganisms detected were related to four functional groups: complex organic degradation organisms, including *Stenotrophomonas* (γ-Proteobacteria, G-) and *Lactobacillus* (Bacilli, G+); soil organisms, including *Curtobacterium* (Actinobacteria, G+), *Agrobacterium* (α-Proteobacteria, G-), *Flavobacterium* (Bacteroidetes, G-) and *Bradyrhizobium* (α-Proteobacteria, G-); known exoelectrogens, including *Pseudomonas* (γ-Proteobacteria), *Desulfovibrio* (δ-Proteobacteria) and *Shewanella* (γ-Proteobacteria); and other organisms including *Desulfonauticus*, *Xenohaliotis* and *Marinicola*. By SSCP analysis, some microorganisms detected both in R1 and new reactor R2 were *Stenotrophomonas* (band 1), *Desulfovibrio* (band 2), *Pseudomonas* (band 3), *Agrobacterium* (band 4), *Bradyrhizobium* (band 8) and *Shewanella* (band 10). Other microorganisms, characterized by the remaining bands, were not shared in all samples.

Table 1
Reactor performance at time points of sampling.

Time points	MEC-R1	MEC-R2 30 h	MEC-R2 1 week	MEC-R2 1 month	MEC-R2 3 months
Coulombic efficiency %	35 ± 4%	na	30 ± 5%	32 ± 4%	38 ± 4%
COD removal %	86 ± 5%	21 ± 8%	72 ± 6%	80 ± 4%	83 ± 5%
Hydrogen yield %	31 ± 3%	na	20 ± 4%	29 ± 4%	31 ± 7%

Data were averaged at least three cycles, including the time point.
"na" not detected or not calculated.

Table 2
Similarity analysis of 12 OTUs shown in Fig. 1 based on BLASTn and sequence matches.

OTU	Band no.	GenBank No.	Most similar sequence, similarity	Most similar genus
1	E1-1 E1-4	EU374944.1	*Stenotrophomonas* sp. APH3 100%	*Stenotrophomonas*
2	E1-3	EF411134.1	*Curtobacterium* sp. C01 100%	*Curtobacterium*
3	E2-2	EF520765.1	Uncultured bacterium clone b9-3 100%	*Desulfovibrio*
4	E3-1 E3-2	AM710608.1	*Pseudomonas* sp. 129(43zx) 100%	*Pseudomonas*
5	E4-1	EU072019.1	*Agrobacterium* sp. X9 99%	*Agrobacterium*
6	E5-2	AB066236.1	*Agrobacterium* sp. X9 98%	*Flavobacterium*
7	E6-3	EF520765.1	Uncultured bacterium clone b9-3 100%	*Desulfonauticus*
8	E7-2	AB362652.1	Lactobacillus plantarum 95%	*Lactobacillus*
9	E8-1	AY547290.1	*Bradyrhizobium* sp. CCBAU 95%	*Bradyrhizobium*
10	E9-1 E9-2	DQ898723.1	*Sistotrema biggsiae* isolate FCUG 782	*Xenohaliotis*
11	E9-3	DQ443995.1	Uncultured bacterium clone ET10-39 99%	*Marinicola*
12	E10-1	AF005250.1	*Shewanella alga* 92%	*Shewanella*

Table 3
The detected genes and the represented genera.

Samples	Gene detected (unique genes[a])	Genera represented by unique genes	Cytochrome gene (unique genes)	Genera represented by cytochrome gene
B1	173 (67)	65 (34)	11 (5)	4 (4)
B2	377 (271)	110 (92)	16 (10)	4 (3)
Common no. detected	106	61	6	4

Sample B1 was collected from anode on 2nd day after new reactor R2 inoculated; B2 was collected on 3rd month of R2.
[a] The unique genes were only found in one sample; and common genes were detected on both samples.

3.3. Characterization of functional genes in anode biofilm communities

The relative abundance of functional genes represented in GeoChip (II) was analyzed on two time points from reactor R2. Gene number detected in sample B1 (biofilm of R2, 2nd day) was 173 and increased highly to 377 in B2 (biofilm of R2, 3rd month) over three months operations (Table 3), with 106 genes shared each other. Unique genes, only detected in one sample, were 67 in B1 and increased to 271 in B2. Genera involved all functional genes were 110 in B2, which was much higher than the 65 from B1. Almost all communities detected in the initial biofilm (B1) were also detected in biofilm (B2) later, with up to 61 common genera, suggesting an efficient attaching process in a short time when inoculated by anode biofilm of a long term running MEC (R1).

Based on averaged signal intensity of genes detected (Fig. 2), functional gene structures were similar on gene categories over three months. Carbon cycling genes accounted for 18.2–18.4%, with 10.1–10.5% of carbon degradation (CDEG), 7.9–8.1% of carbon fixation (CFIX), 9.8–14.8% of methane related genes. Nitrogen cycling genes accounted for approximately 33.1–38.4% over all genes in the two samples, with 9.6–19.6% of nitrogen fixation (NFIX), 10.3–10.9% of nitrification (NIT), 8.5–12.6% of nitrogen reduction (NRED). There were 10.8–11.1% of the genes involved in dissimilatory sulfate reduction (DSR). Metal related genes (MET) were ranged 11.5–11.7%, including ~1% of metal resistance genes (arsenic, cadmium, chromium and tellurium) and ~11% of metal reduction genes (mercury, and other metal). In GeoChip (II), metal reduction genes were specific to mercury reduction (*merA*) and other metal reduction (*cytochrome*), the later taken as an indicator of electron transport (He et al., 2007).

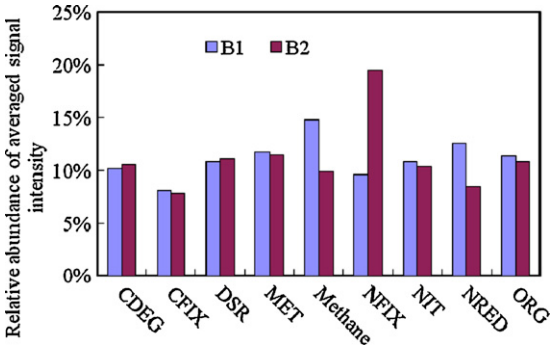

Fig. 2. Gene categories detected based on over all functional genes.

3.4. Functional groups involved in electron transport

Cytochrome genes were specifically examined in greater detail due to the fact that they are regarded as indices during extracellular electron transport. A total of 21 *cytochrome* genes were detected: 11 genes in the initial biofilm (B1), increasing to 16 genes in later biofilm (B2), with 6 genes detected in both samples (Table 3). A total of 4 genera were involved in cytochrome gene: *Geobacter*, *Desulfovibrio*, *Rhodopseudomonas* and *Shewanella*, and all of them were increased over three months after new biofilm formed in R2 based on average normalized signal intensity of genes detected (Fig. 3). Reactor performances on electron transport and hydrogen production were closely contributed by their increasing.

Consistent with SSCP results, these four communities above were the first exoelectrogenic members attaching to the biofilm

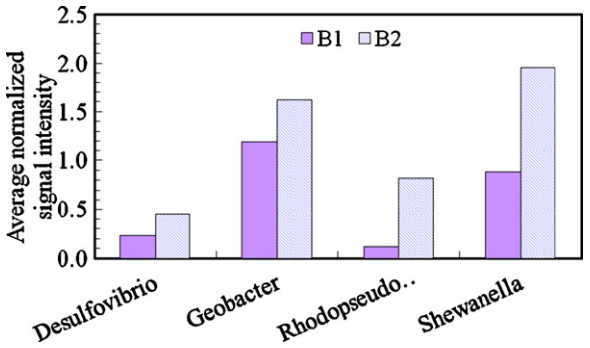

Fig. 3. *Cytochrome* genes and related exoelectrogens detected in GeoChip.

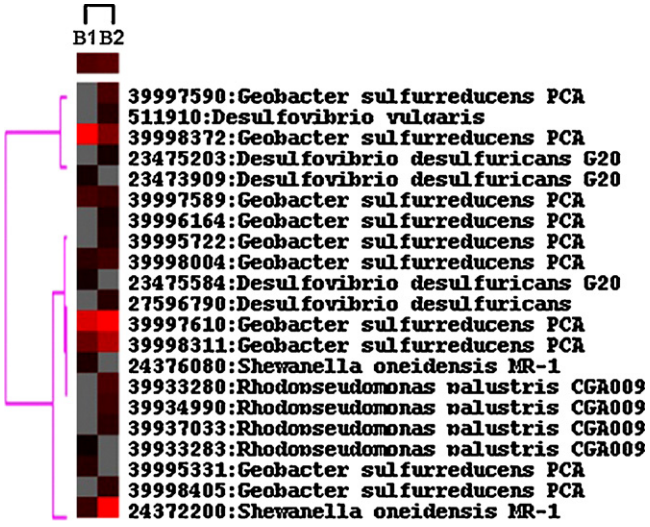

Fig. 4. Hierarchical cluster analysis of genes related to *cytochrome* gene, based on average signal intensity. The figure was generated using CLUSTER and visualized with TREEVIEW. Black represents no hybridization above background level and red represents positive hybridization. The color intensity indicates differences in hybridization patters.

(30 h) and became dominant after three months. Throughout the biofilm growth, total of 11*cytochrome* genes detected in B1 and increased to 16 in B2, and the increased genes related to *Geobacter*, *Desulfovibrio* and *Rhodopseudomonas* (Fig. 4). There are 6 shared *cytochrome* genes only from *Geobacter* and *Shewanella* and all of them increased signal intensities over time. And there were two eliminated *cytochrome* genes (Gene ID: 23473909, 23475584) from *Desulfovibrio* over operations. Totally, communities related to electron transporting increased based on *cytochrome* genes both on detected numbers and signal intensity from the initial biofilm to the long-term running biofilm.

4. Discussions

In this study, reactor performances were not excellent. Coulombic efficiency was almost 50% lower than other studies of two-chamber MECs, and hydrogen production was about 1.2 mol/mol acetate, which was less than 2.1–2.2 mol/mol acetate in their results (Rozendal et al., 2006; Tartakovsky et al., 2009). But the performance was better than previous study which was inoculated from waste water using the same type of reactor (Liu et al., 2008). One of considerable reasons for difference of coulombic efficiencies was that the reactor had huge internal resistance (Wang et al., 2010). Regardless of reasons from reactor architecture, it was noticed that COD removal was over 80%, similar to other reported studies, suggesting that there was competition on substrate among exoelectogens and other microbial communities in the system. Negatively, methanogens (Fig. 2) were detected by GeoChip in the study, and their existing is one of reasons for hydrogen production loss.

Pseudomonas, *Desulfovibrio* and *Shewanella* were detected both in SSCP and GeoChip. It is reasonable that *Pseudomonas* could help other microbes to increase power (Rabaey et al., 2005, 2007; Pham et al., 2008). Similarly, *Desulfovibrio* has been earlier studied in co-culture with *G. sulfurreducens* (Cord-Ruwisch et al., 1998) and has recently reported to be involved in electricity generation (Borole et al., 2009). In our results, detected functional genes from *Pseudomonas* and *Desulfovibrio* almost all related to organic degradation and metal resistance in the system but not cytochrome (Lovley, 2008; Hartshorne et al., 2009). These two species were likely to perform little function of direct electron transport in our system but assisted to electron transport.

A part of microbes were not enriched but disappeared in the startup process in bioelectrochemical systems (Kim et al., 2004; Liu et al., 2008) when a new reactor was started from waste water or sludge. However, there was no further elimination when inoculated by a formed biofilm to another new reactor in this study. Based on SSCP results, some other functional microbes, which have not been reported yet related to directly bioelectrochemical process, also grew as dominant community in the biofilm, though it is not clear what functions they exactly played in bioelctrochemcial system. For example, *Desulfonauticus*, isolated from deep-sea environments, could use acetate to reduce sulfate or thiosulfate when no other carbon sources were present (Audiffrin et al., 2003); *Agrobacterium* and *Bradyrhizobium*, capable of denitrification or nitrogen fixation, have been seldom mentioned on the function of electron transfer in MFC or MEC. But lately they have been mentioned in some specific bioelectrochemical systems. *Agrobacterium*, detected in the anode of a MFC for sulfide oxidation (Sun et al., 2010), and *Bradyrhizobium*, dominant in an oligotrophic microbial fuel cell (Phung et al., 2004), their functions were likely related to anaerobic respiration and reduction of complex compounds. They grew well with exoelectrogens in anode biofilm again in new reactor, but few of them were eliminated or depressed, suggesting that they could play a positive role and contribute to establish a stable microbial structure.

5. Conclusion

Community structures of MEC biofilm were detected on their members and some specific functional genes when starting up a new reactor by inoculating anode biofilm communities of a long-term running two-chamber MEC reactor. All exoelectrogens and most coexisting bacteria made up new biofilm and performed electron transport in short time. Fewer communities eliminated for function competitions (e.g. substrate competition) compared to community changes from waste water or sludge as inocula. The microbial communities, combined by exoelectrogens and other functional members, grew together again for a similar stable system. And a part of other functional communities potentially contributed to electron transport.

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