Pol. J. Environ. Stud. Vol. 28, No. 2 (2019), 693-700
DOI: *10.15244/pjoes/81700* ONLINE PUBLICATION DATE: 2018-10-08

Original Research

Effects of Using Anode Biofilm and Cathode Biofilm Bacteria as Inoculum on the Start-up, Electricity Generation, and Microbial Community of Air-Cathode Single-Chamber Microbial Fuel Cells

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Received: 22 November 2017
Accepted: 2 January 2018

Abstract

Inoculum is critical for the start-up and performance of microbial fuel cells (MFCs). The effluent of mature MFCs is usually used as inoculum for the start-up of immature MFCs. However, the effluent of mature MFCs contains bacteria both from anode biofilm (ASB) and cathode biofilm (CSB). Here, ASB and CSB and their mixtures were used as inoculum in the start-up of MFCs in order to gain more insight into the influence of CSB on the start-up of MFCs. Compared to anode inoculum-enriched MFCs, using cathode inoculum reduced start-up time from 5 d to 3 d. The time needed for scavenging oxygen was reduced from 900 min to 600 min, maximum power density was 19% lower (691 mW/m^2 vs 823 mW/m^2), and the charge transfer resistance increased from 29.0 Ω to 48.3 Ω. The decreased start-up time and power generation of cathode inoculum-enriched MFCs was attributed to the increasing abundance of *Azospirillum* (80.02% vs. 12.68%) and the decreasing abundance of *Geobacter* (9.08% vs. 61.25%). This research suggested that CSB in the effluent of mature MFCs, when used as inoculum, has a side-effect on the start-up of MFCs.

Keywords: microbial fuel cells, inoculum, anode biofilm, cathode biofilm, microbial community

Introduction

Microbial fuel cells (MFCs) have been considered a promising technology for wastewater treatment and energy recovery [1-2]. Unlike traditional fuel cells, MFCs use bacteria as bio-catalysts to oxidize organic matter and convert chemical energy to electrical energy. These bacteria include anaerobic bacteria such as several *Geobacteraceae* strains [3], facultative bacteria such as *Shewanella* [4], and aerobic bacteria such as *Pseudomonads* [5]. Usually these bacteria can be inoculated from various alternative inoculum sources such as wastewater [6-9], heat-treated soil [10], garden

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compost [11], river water [12], and so on. Though microbial communities of the inoculums are abundant and diverse, the MFCs show strong selective effects for selecting electrochemical active bacteria and forming stable anodic microbial communities [8]. Normally it will take 10 to 15 days for the MFCs to reach the first maximum power production when using sludge or wastewater as inoculum [8]. For the fast start-up of MFCs, the effluent of mature MFCs is widely used as inoculum for the start-up of immature MFCs. The start-up time could be reduced to 3 to 5 days. It is also shown that in a two-chambered microbial electrolysis cell (MEC), the anode biofilm reformation has little influence on main functional groups of bacteria [9].

Air-cathode single-chamber microbial fuel cells have been considered an ideal configuration for practical application. In air-cathode single-chamber MFCs, cathode biofilm forms inevitably with the growth of anode biofilm. Previous research showed that cathode biofilm or oxygen-reducing biocathodes could form in 60-100 h [13-14], which is more rapid than the formation of the anode biofilm. Due to direct contact with the air-cathode, aerobic and facultative anaerobic bacteria are more enriched on cathode biofilm, while the microbial population is maintained similarly to anode biofilm [15]. Therefore, bacteria form cathode biofilm also contains exoelectrogens as alternative inoculum. The effluent of mature MFCs contains bacteria both from anode biofilm (ASB) and cathode biofilm (CSB). When the effluent of mature MFCs was used as inoculum, it is necessary to know the effects of CSB on the start-up of MFCs. In this work, bacteria from anode biofilm and cathode biofilm were used to investigate the effects of relative abundance of facultative and aerobic bacteria on the performance of MFCs. Acclimation time, polarization tests, and electrochemical impedance spectroscopy (EIS) were used to evaluate MFC performance. The bacterial community was analyzed by the MiSeq Illumina sequencing technology.

Material and Methods

MFC Configuration

Air cathode cubic-shaped MFCs with a cylindrical chamber (working volume 10 mL, electrode spacing 2 cm) were constructed as previously described [16]. Anodes were made of graphite felt (Beijing Sanye Carbon Co., Ltd, China). Raw graphite felt was soaked into 0.1 mol/L NaOH and HCl solution successively, rinsed with deionized water until its pH value equaled 7, and then dried and cut into circles of 4 cm in diameter (working area 7 cm^2). The air cathodes were made of nickel foam containing an activated carbon catalyst [17]. Glass fiber was used to cover the water-side surface of the air-cathode to reduce the effect of oxygen on the anode biofilm.

MFC Setup and Operation

Anode biofilm and cathode biofilm were scraped form the anode and cathode of an MFC reactor that was inoculated with the primary clarifier overflow of the local wastewater treatment plant and that had operated for more than one year. The biofilms were swirled, resuspended in 50 mmol phosphate-buffered solution (PBS, 2.45 g/L $NaH_2PO_4 \cdot H_2O$, 4.58 g/L Na_2HPO_4, 0.31 g/L NH_4Cl, 0.13 g/L KCl), and diluted to the same cell density (based OD_{600} = 0.07). The anodic suspended bacteria, cathodic suspended bacteria, and their mixture (1:1) were referred to as ASB, CSB, and MSB, respectively. These three inocula were mixed with 50 mmol PBS containing 2 g/L acetate, 25 ml/L metal salts, and 10 ml/L vitamins [18] in a proportion of 1 to 1 and then used to inoculate MFCs (cycle 1). The corresponding MFCs were referred to as ASB-MFC, CSB-MFC, and MSB-MFC. Starting from the second cycle, 50% of each MFC effluent was mixed with the same volume of 50 mmol PBS containing 2 g/L acetate, 25 ml/L metal salts, and 10 ml/L vitamins, and then used to refill each MFC. This solution was replaced until a similar output voltage was produced over two consecutive cycles (1000 Ω external resistance). The solution was then switched to 50 mmol PBS containing 1 g/L acetate, 12.5 ml/L metal salts, and 5 ml/L vitamins. The anode solution was replaced every 24 h, forming one complete cycle of operation. All tests were conducted in a 30ºC temperature-controlled room.

Analysis

Cell voltage across an external resistor was recorded every 20 mins using a multimeter with a data acquisition system (34970A, Agilent, U.S.). Electrochemical tests were conducted in cycles 11 and 30, showing consistent results. The polarization and power density curves were obtained by varying the external resistance from 1000 Ω to 80 Ω, with MFCs running for 20 min at each resistance. Electrochemical impedance spectroscopy was conducted on an electrochemical analyzer (Bio-Logic, Claix, France). A standard three-electrode configuration was used, with the anode serving as the working electrode, the cathode as the counter electrode, and an Ag/AgCl electrode (0.201 mV vs SHE) as the reference electrode. The Ag/AgCl reference was placed in close proximity to the anode. EIS tests were conducted at the circuit voltage under 1000 Ω external resistance over a frequency range of 10^5-0.01 Hz with sinusoidal perturbation of 10 mV amplitude.

The mixed culture biofilm was analyzed for the bacterial community by the MiSeq Illumina sequencing technology. DNA was extracted, amplified, and purified using a DNA isolation kit (PowerSoil DNA Isolation Kit, American). The paired primers in the variable regions V3-V4 (F: 5'-ACTCCTACGGGAGGCAGCAG-3', R: 5'-GGACTACHVGGGTWTCTAAT-3') were used for PCR amplification. The MiSeq Illumina sequencing

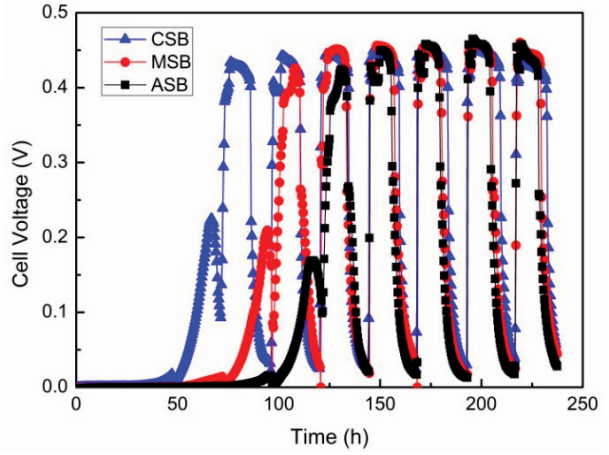

Fig. 1. Time-potential relationship in MFCs inoculated with ▲ ASB, the anodic suspended bacteria; ● MSB, their mixture (1:1); and ■ CSB, cathodic suspended bacteria.

was conducted and analyzed as described previously [19].

Results

Electricity Production and Oxygen Consumption of MFCs during Start-up

Inoculating MFCs with CSB required 50 h before a rapid increase of cell potential (Fig. 1). The reactors needed another 30 h to reach the first maximum power production and refuel three cycles before the cell voltages became reproducible in terms of maximum voltages. Using the MSB and ASB inocula, the time needed for rapid increase of cell potential was increased to 70 h and 100 h, respectively, with a first maximum power cycle and reproducible cycle of voltage production requiring a number of cycles similar to that obtained with CSB.

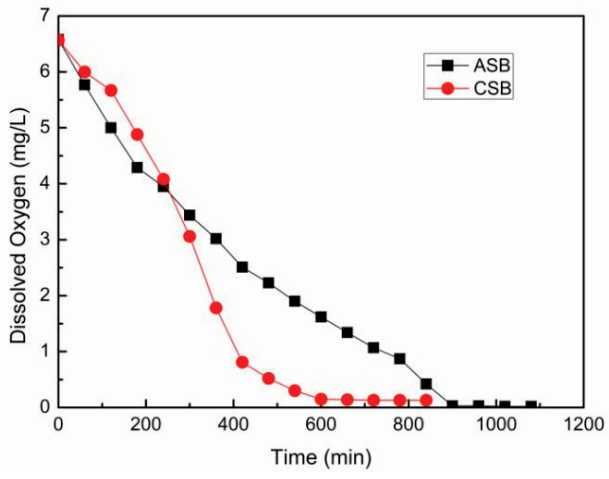

Fig. 2. Typical profiles of DO vs. time in a cycle during the start-up period.

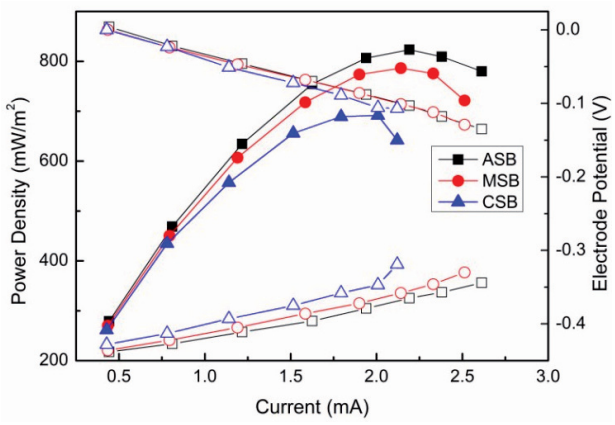

Fig. 3. Polarization curves of the MFCs inoculated with ASB, MSB, and CSB; the hollow points represent the anode (down) and cathode (up) potential.

During the start-up stage, the typical profiles of dissolved oxygen (DO) vs. time in a cycle is shown in Fig. 2. Inoculating MFCs with ASB required 900 min before the DO reached 0 mg/L. The oxygen consumption rate appeared to be independent of DO, with a rate of 0.007 mg DO/L/min. Using the CSB inoculum, the time needed for scavenging DO was reduced to 600 min. The oxygen consumption rate first increased and then decreased when DO fell below 1 mg/L

Electricity Properties of MFCs

The power density curve and polarization curves are shown in Fig. 3. The maximum power density of ASB-MFC was 823 mW/m^2, which was 5% and 19% higher than MSB-MFC (786 mW/m^2) and CSB-MFC (691 mW/m^2), respectively. Electrode polarization curves showed that the increased power density was attributed to improved anode performance rather than cathode performance. When the cell current increased to more than 2 mA, the anode potential of CSB-MFC rapidly increased 30 mV, while the anode potential of ASB-MFC and MSB-MFC increased 8 mV and 12 mV. Further reducing the external resistor (increasing current), "power overshoot" was observed for CSB-MFC (data not shown), indicating severe electrode polarization of the CSB-MFC anode. Therefore, the maximum current obtained by CSB-MFC was 2.1 mA, which was 19% and 24% lower than MSB-MFC (2.5 mA) and ASB-MFC (2.6 mA), respectively.

Anode EIS curves are shown in Fig. 4. An equivalent circuit of R_1 (R_2Q) (R_3Q) was used for estimating anode resistance, in which R_1 represents ohmic resistance, R_2 represents charge transfer resistance, and R_3 and Q in parallel represent finite diffusion [20]. The total anode resistance of ASB-MFC was 52.1 Ω, which was 14% and 26% lower than MSB-MFC (60.6 Ω) and CSB-MFC (70.2 Ω), respectively. The solution resistance R_1 and diffusion resistance R_3 of all MFCs were ~15 Ω and ~7 Ω, respectively. Therefore, the reduced charge transfer

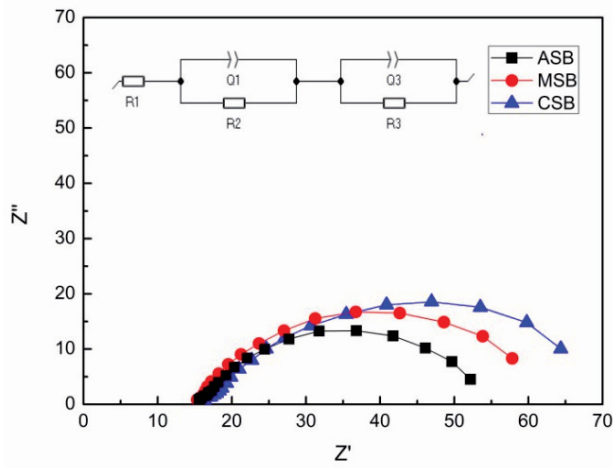

Fig. 4. Nyquist plots of EIS spectra of anode biofilm enriched with ASB, MSB, and CSB.

resistance R_2 of ASB-MFC was credited for the improved anode performance. The charge transfer resistance R_2 values for ASB-MFC, MSB-MFC, and CSB-MFC were 29.0 Ω, 39.2 Ω, and 48.3 Ω, respectively, indicating better electrochemical kinetics from ASB-MFC.

Microbial Community of Inocula and MFCs

Composition and relative abundances of bacterial classes of ASB, CSB, ASB-MFC, and CSB-MFC are shown in Fig. 5. ASB and CSB were similar in bacterial populations, but vary from each other in the relative abundance of microbial communities. The dominating classes in ASB were *Betaproteobacteria* (relative abundances, 10.59%), *Deltaproteobacteria* (13.02%), *Bacteroidia* (27.02%), *Synergistia* (14.35%), and *Actinobacteria* (7.98%). Meanwhile, the dominating classes in CSB were *Betaproteobacteria* (37.86%), *Alphaproteobacteria* (22.21%), and *Flavobacteria* (11.61%). The compositions and dominating classes became more similar to each other in anode biofilms. The dominating classes in ASB-MFC and CSB-MFC were *Betaproteobacteria*, *Deltaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidia*. The main difference between ASB-MFC and CSB-MFC was the relative abundance of *Deltaproteobacteria* (44.02% vs. 7.69%) and *Alphaproteobacteria* (11.33% vs. 63.57%).

Table 1 shows the compositions and relative abundances of the bacterial genera of ASB, CSB, ASB-MFC, and CSB-MFC. The dominating genera of ASB were *Geobacter* (19.08%), *Azoarcus* (9.19%), *Blvii28* (37.83%), and *HA73* (11.69%). The dominating genera of CSB were *Pseudomonas* (3.93%), *Azoarcus* (64.01%), *Comamonas* (5.23%), and *Ignavibacterium* (3.09%). The dominating genera in ASB-MFC and CSB-MFC were *Geobacter*, *Azospirillum*, *Blvii28*, and *Dechloromonas*. The most dominating genera, *Geobacter* and *Azospirillum*, comprised 70% to 90% of the relative abundances of anode biofilms.

Discussion

ASB was dominated by anaerobic bacteria. *Geobacter* is a typical anaerobic exoelectrogen. *Blvii28* and *HA73* were found to be anaerobic fermentation bacteria [21] and dominated in an anaerobic reactor [22]. *Blvii28* tend to use complicated substrates such as peptone, yeast extract, maltose, and glucose, while some simple organic matters such as formic acid, acetate, and ethyl alcohol are not exploitable [21]. Thus, as the substrate provided was acetate, the high relative abundance of *Blvii28* and *HA7* might be caused by long-term operation in an anaerobic environment, in which they might be responsible for the degradation of metabolites and dead microorganisms. CSB was dominated by facultative and aerobic bacteria, which was consistent with Matteo Daghio's results [15] due to the micro-aerobic environment around the cathode. *Azoarcus*, which are facultative bacteria,

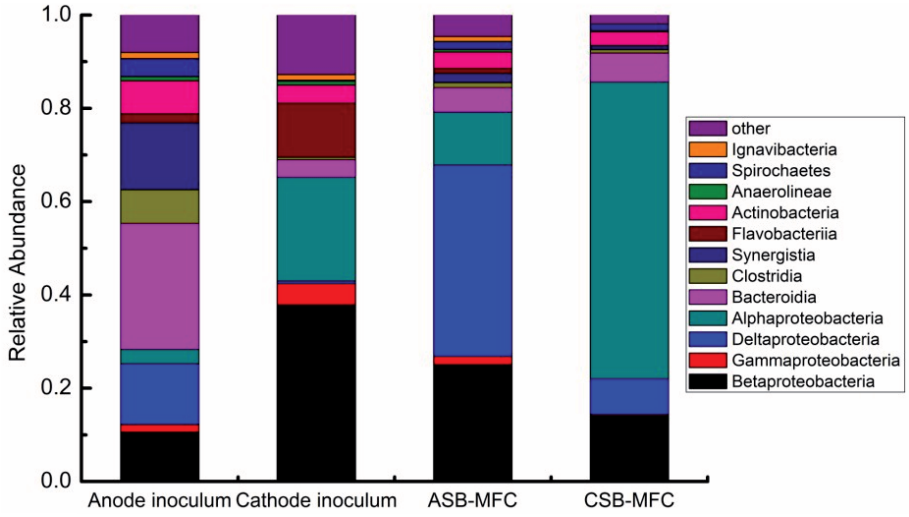

Fig. 5. Composition and relative abundances of bacterial classes in ASB, CSB, ASB-MFC, and CSB-MFC.

Table 1. Relative abundances of bacterial OTUs based on the V3-V5 primer set (>0.5% of total population) in ASB, CSB, ASB-MFC, and CSB-MFC.

Classification	ASB /%	CSB /%	ASB-MFC /%	CSB-MFC /%
Geobacter	19.08	0.90	61.24	8.98
Pseudomonas	0.21	3.92	0.22	0.02
Azospirillum	0	0.56	12.68	80.02
Azoarcus	9.19	64.01	2.46	0.77
Acinetobacter	0.02	0.68	0.10	0.05
Arcobacter	0.93	0.40	0.06	0.02
Comamonas	2.48	5.23	2.81	0.34
Blvii28	37.83	0.31	2.96	3.35
HA73	11.69	0	1.96	0.30
Fusibacter	0.78	1.14	0.13	0.02
Dechloromonas	0.80	1.95	5.98	2.66
Desulfovibrio	0.95	0.15	0.13	0.07
Aminiphilus	0.80	0.03	0.22	0.38
Fluviicola	0.61	0	0.56	0.02
Anaerovorax	1.59	0.15	0.52	0.22
Agrobacterium	1.59	0.15	0.52	0.22
Mycobacterium	0	0.19	1.89	1.48
Sphaerochaeta	0.42	0	0.75	0.59
PSB-M-3	1.48	0.09	0.11	0.02
Thauera	0.13	4.67	0.11	0.13
Sterolibacterium	0.04	0	3.02	0.01
Candidatus	0	0.56	0.09	0.01
Clostridium	1.02	0	0.17	0.04
Treponema	2.14	0	0.15	0.06
Corynebacterium	0.83	0	0.01	0
B-42	0.47	1.36	0.09	0.02
Propionicimonas	0.57	0	0	0.02
Paracoccus	0.08	2.23	0	0
Ignavibacterium	0.25	3.09	0.06	0.01
Devosia	0.30	0.68	0.07	0.01
Parvibaculum	0	1.48	0.16	0.01
Clavibacter	0	3.59	0	0
Leptonema	0.02	0.59	0.17	0.07
Hyphomonas	0.21	1.08	0.01	0
Gemmatimonas	0	0.80	0.01	0
Anaerolinea	0.19	0	0.53	0.02
SJA-88	1.63	0	0.03	0.05
Methylosinus	0.93	0	0.01	0
Dehalobacterium	0.70	0	0	0

have been reported in river sediment-inoculated MFCs and ethyl alcohol-fed MFCs [23-24]. *Pseudomonas* are aerobic bacteria, and they could use mediator for extracellular electron transfer [5]. *Comamonas* (5.23%) [25] and *Thauera* (4.67%) [26] are aerobic bacteria and facultative bacteria, respectively.

The time required for the rapid increase of cell potential with CSB-MFC was 50 h shorter than ASB-MFC, indicating that inoculating with CSB could accelerate the growth and adsorption of microorganisms to form an anode biofilm. This might be attributed to the fast growth of aerobic and facultative bacteria, as the oxygen consumption rates in CSB-MFC were two-times faster than in ASB-MFC during the start-up stage. When the anode biofilms formed, the relative abundance of *Azospirillum* sequencing in CSB-MFC was 80.02%, while in ASB-MFC it was only 12.68%. *Azospirillum* were considered to be facultative bacteria and had been reported in other MFC systems [27-28], and was suspected to have the ability to extracellularly transfer electrons [29]. Although the mechanism of extracellular electron transfer by *Azospirillum* has not been reported, the high relative abundance of *Azospirillum* in CSB-MFC and ASB-MFC indicated that *Azospirillum* might be able to transfer electrons to the anode. The reduced acclimation time of CSB-MFC might be attributed to the fast growth of *Azospirillum* in the start-up stage.

The maximum power density of ASB-MFC was 823 mW/m^2 based on the anode surface area, comparable to other MFCs using a similar configuration [16, 30]. The improved performance of ASB-MFC anode, relative to CSB-MFC, was attributed to the enhanced activity and number of redox proteins in the anodic biofilm, as shown by EIS results. The dominating bacterial genera in ASB-MFC and CSB-MFC were similar, while the main difference lay in the relative abundances of *Geobacter* (61.25% vs. 8.98%), *Azospirillum* (12.68% vs. 80.02%), *Azoarcus* (2.45% vs. 0.77%), *Comamonas* (2.81% vs. 0.34%), *HA73* (1.96% vs. 0.30%), *Dechloromonas* (5.98% vs. 2.66%), and *Sterolibacterium* (3.02% vs. 0.01%). *Geobacter* is famous for its excellent electricity generation and long-range extracellular electron transfer [31] and used to be the dominating species in the anode biofilms of bioelectrochemical systems fed with acetate [19, 25, 32]. *Comamonas* is able to generate electricity with acetate as an electron donor in MFCs [25]. *Dechloromonas* has been widely found in MFC systems [33-34], known to be an electrochemically active microorganism [35]. *Sterolibacterium* is a genus of gram-negative bacteria from the family of *Rhodocyclaceae*, which belongs to the class of *Betaproteobacteria* and usually dominates in the anode biofilms of bioelectrochemical systems, and it shows an ability to extracellularly transfer electrons [36]. In general, the dominating genera in the anode biofilm of ASB-MFC were all known as electrochemically active microorganisms supporting the construction of high-efficiency electrogenic biofilms. Considering that the maximum power density of CSB-MFC was only 19% lower than ASB-MFC, while the relative abundances of known exoelectrogens was 60% less than ASB-MFC, there may have been other bacteria – perhaps *Azospirillum* – contributing to the electricity generation.

Although the relative abundance of the dominating bacterial communities of CSB and ASB varied from each other, the population of anode biofilms inoculated with CSB and ASB were similar. This might be attributed to the fact that CSB and ASB are both enriched in the same acetate-fed systems and well adapt to the environment. However, it also led to the fierce community competitions when use the mixture of CSB and ASB as inoculum. Although using CSB as inoculum will decrease the power density of MFCs, it may help for the construction of functional anode biofilms. For example, *Azospirillum* is a nitrogen-fixing bacterium that can potentially be applied in a nitrogen-fixing bioelectrochemical system [37].

Conclusions

ASB was dominated by anaerobic bacteria while CSB was dominated by facultative and aerobic bacteria. The time required for the rapid increase of cell potential with CSB-MFC was 50 h shorter than with ASB-MFC. The maximum power density of CSB-MFC was 19% lower than ASB-MFC (691 mW/m^2 vs. 823 mW/m^2). The reduced performance of the CSB-MFC anode was attributed to the decreased activity and number of redox proteins in anodic biofilm, as shown by EIS results. Community analysis of the anode biofilm of ASB-MFC and CSB-MFC showed that dominating genera in ASB-MFC and CSB-MFC were *Geobacter*, *Azospirillum*, *Blvii28*, *Comamonas*, and *Dechloromonas*. ASB-MFC possessed higher abundances of *Geobacter, Comamonas*, and *Dechloromonas,* known as exoelectrogens, whereas CSB-MFC was abundant in *Azospirillum*, demonstrating that using anode inoculum performed better for the construction of high-efficiency electrogenic biofilm. This research suggested that CSB in the effluent of mature MFCs, when used as inoculum, has a side-effect on the start-up of MFCs. And *Azospirillum* species in the anodic biofilm might be exoelectrogen playing a role in electricity production.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.51478414) and the National Key Research and Development Plan (2016YFB0600505).

Conflict of Interest

The authors declare no conflict of interest.

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