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ARTICLE TYPE

Effects of Azide on Current Generation and Microbial Community in Air-cathode MFCs

Xiangtong Zhou[a], Youpeng Qu[a,c], Byung Hong Kim[a,b,d], Henan Li[a], Jia Liu[a], Yue Du[a], Da Li[a], Yue Dong[a], Nanqi Ren[a] ,Yujie Feng[a]***

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Azide is known to be a respiratory inhibitor, which can disrupt electron transfer in the process of aerobic respiration. It has been proposed to prevent the reduction of oxygen in anode compartment of MFC-based biosensors but also found functioning as an electron acceptor in recent research. Whereas, there are few reports about the effects of azide on microbial community structure and composition in air-cathode MFCs as well as the corresponding performance. Therefore, the current generation, electroactivity and community structure of anodic biofims were investigated using air-cathode MFCs acclimated with (1.5 mM) and without azide. The enrichment process was much slower in the presence of azide compared to the control. Biofilms enriched with and without azide were found producing similar voltammograms, but the difference lied in the current intensity of the predominant peaks. Pyrosequencing indicated that the distributions of microbes at the genus level were more uniform, with *Geobacter* and *Ignavibacterium* being the most dominant genera on both biofilms, although the community of the azide-enriched was less diverse than the control. These results demonstrate that the microbial community enriched with azide was not significantly altered compared to the control and the difference of the maximum current or peak current of （cyclic voltammograms）CVs was thought to be related to the amount of biomass.

1 Introduction

Organic contaminants in wastewater are potential sources of energy [1]. Microorganisms convert them to useful forms of energy through various biochemical processes such as methanogenesis. Among them, microbial fuel cells (MFCs) are attractive because they have functional and operational advantages[2], although much efforts are required to achieve practical applications. Besides electricity generation, MFCs are used to determine the biochemical oxygen demand (BOD)[3], since the coulombs obtained from MFCs are proportional to the concentration of fuels used[4]. These MFC-based biosensors, compared with conventional methods, have shown a long operational stability and very high substrate versatility[5]. Therefore, several types of MFC-based biosensor have been developed[6-8]. Generally, single-chamber MFCs with a membrane electrode assembly (MEA) show better performance than two-chamber systems[9].

During the process, oxygen is diffused through the membrane into the anode chamber, and nitrate is contained in the samples to be analyzed for BOD. The presence of these electron acceptors in the samples results in substantial electron losses, because oxygen and nitrate are preferred electron acceptors over the anode[10]. Consequently, their presence in anode compartment is a major reason for low columbic efficiency (CE). However, the CE should be improved to achieve better performance for MFC-based biosensors. Respiratory inhibitors such as azide can inhibit oxygen and nitrate reduction. Hence, adding azide into anode compartments has been considered to improve CE. Current increase was observed when azide was added into air-saturated or nitrate containing anode compartments[11], because current generation from an MFC was inhibited by NADH dehydrogenase, coenzyme Q and quinol-cytochrome *b* oxidoreductase inhibitors, but not by terminal oxidase inhibitors[12]. This contrasts with recent report that anodes enriched with azide from the beginning generated lower current than that without azide. For the latter, further analyses showed that azide added into anode compartment was used as an electron acceptor during the enrichment process[13]. Presumably, azide not only inhibited aerobic respiration, but also selected azide-respiring bacteria when azide was used from the beginning of enrichment process. Diverse electron donors and acceptors are used by prokaryotes employing different electron transport chains.

Previous researches have demonstrated that various factors, such as fuel type[14], external resistance[15] as well as anode potential[16], have an impact on the structure and composition of the microbial community, which in turn affects the current generation or power output.

The effect of azide on microbial community composition was observed in two-chamber systems, where electrochemically active bacteria (EAB) were enriched with and without azide from the beginning. Microbial communities on the anode of MFCs with azide were dominated by *Geobacter sulfurreducens* (> 94.5 % of all clones) and overall 98 % of the clones were

Deltaproteobacteria[13]. Unfortunately, the community analysis was not made for the control, because before that a similar test that use of acetate to enrich EAB, had been conducted in the same two-chamber reactors, where the population of the MFCs without azide consisted of *Deltaproteobacteria* (68.8 %), *Gammaproteobacteria* (17.3 %), *Alphaproteobacteria* (7.0 %), *Bacteroides* (3.4 %). Obviously, azide could narrow down the diversity of populations in the anode compartment of two-chamber MFCs[12]. Despite the availability of high percentage of *G. sulfurreducens* on the aizde-enriched biofilm, the peak current or CE (4.0 mA, 65.0 ± 5.0 %) in the presence of azide (0.2 mM) didn't relatively increase compared to that (4.5 mA, 70.0 ± 5.0 %) without azide. The difference in current generation was possible due to azide, which was believed consuming more electrons than oxygen in anode compartment of MFCs without azide[13].

Different from two-chamber MFCs, air-cathode single-chamber MFCs have a unique MEA configuration, which enables more oxygen diffusing from the cathode to the anode and reduces the system resistance by avoiding use of exchange membranes. It has been reported that the difference of dissolved oxygen[13, 17, 18] and system resistance[9] can affect the microbial community as well as the system performance. So the effect of azide on single-chamber MFCs can't be simply explained by the results from two-chamber ones. In addition, there are few reports about the effect of azide on microbial community structure and composition in air-cathode MFCs, as well as the corresponding performance. So far, cyclic voltammetry is thought the preferred method to bridge electrochemical behavior of biofilm and its community characteristics[19]. To investigate whether there exist specific bacterial species able to reduce azide, and how the microbial community or composition obtained from the azide-enriched biofilm influences the performance of MFCs, air-cathode MFCs were acclimated with and without azide from the beginning. Therefore, the electrochemical behavior of the biofilm and its community structure were examined for MFCs acclimated with and without azide.

2 Materials and Methods

2.1 Reactor construction and operation

The cube-shaped reactor with a cylindrical chamber (3 cm in diameter, 4 cm in length) was constructed as previously reported[20]. The anode (3 cm in diameter, 3 cm in length) was made of graphite fibers, wound into two twisted titanium wires and heat treated according to previous procedures[21]. The cathode was carbon cloth (B1B30WP 30 % wet proofing, E-Tek Division^{SM}), with one side as the catalyst layer (0.5 mg/cm^2 Pt, Hesen, Shanghai) and the other side as the diffusion layer (4 PTFE layers) [22]. Titanium contacts were used to connect the electrodes to the external circuit.

Four reactors (tested in duplicate) were initially inoculated (intervals of 2 days) with a 20:80 mixture of domestic wastewater and acetate medium. Eight days later, these reactors were divided into two groups, one as the control only fed with acetate medium, the others with acetate medium plus 1.5 mM azide. The acetate () medium (1.64 g/L, pH = 7.0, 7.8 mS/cm) contained the following (per liter): KCl, 0.13 g; $NaH_2PO_4 \cdot 2H_2O$, 3.32 g; $Na_2HPO_4 \cdot 12H_2O$, 10.32 g; NH_4Cl, 0.31 g; vitamin (5 mL); trace mineral (12.5 mL)[23]. During the operation, the reactors were placed in a temperature-controlled chamber (30 ℃) and were refilled with fresh medium when the voltage dropped below 50 mV.

2.2 Analyses and calculations

The voltage (U) across the external resistance (1000 Ω) was recorded every 30 min using a data acquisition system (PISO-813, ICP DAS Co., Ltd). Current (I) and power (P) were calculated based on Ohm′s law and normalized by cathode surface area (7 cm^2).

Cyclic voltammograms were performed for the anode biofilms and the cell-free effluents from the anode compartment using a potentiostat (PGSTAT128N, Utrecht, Netherlands). To prevent the interference of excreted redox mediators in the spent medium, CV test for the biofilm was conducted in a 50 mM PBS without acetate or azide. The anode was the working electrode, and the cathode was the counter electrode with an Ag/AgCl reference electrode (0.197 V vs. SHE). Scans started from -0.700 V and went up to 0.200 V and back at a scan rate of 5 mV/s. The spent medium was collected using a test vial and centrifuged (12,000 rpm, 4 min) for removing suspended solids. CV test for the supernatant was conducted in a three-electrode electrochemical system. A carbon cloth working electrode (B-1 designation B 30 % wet proofing, E-Tek, USA; 7 cm^2), an Ag/AgCl reference electrode and a platinum counter electrode were used in a 28-mL electrochemical cell. The potential was varied from -0.7 V to 0.2 V at a scan rate of 5 mV/s. Both the PBS and the supernatant were flushed (15 min) with nitrogen gas prior to analysis. These tests were performed using samples taken from 6 month old MFCs.

Samples taken from these reactors were immediately filtered through 0.22 μm pore diameter syringe filters before acetate was analyzed using HPLC equipped with an Aminex HPX 87H column (Bio-Rad Laboratories, Hercules, CA).

2.2 Bacterial community analysis

Bacterial communities were analyzed using samples taken from thirteen month old MFCs. Two representative biofilms, one taken from the controls (0 mM azide), and the others from the azide-acclimated (1.5 mM azide) reactors, were analyzed using a 454/Roche GS-FLX instrument to describe the community differences. The graphite fibers were cut from the anodes of the azide-acclimated MFCs and those of the controls. The total genomic DNA was extracted using a Bacteriag DNA Mini Kit (Watson Biotechnologies, Inc., Shanghai) according to the producer's instructions. DNA extracts were inspected by electrophoresis in 0.7 % agarose gels with ethidium bromide staining. The extracts were subjected to PCR to amplify bacterial 16S rDNA genes PCR and sequencing were performed using the universal bacterial primers: F (5′-CGTATCGCCTCCCTCGCGCCATCAG-3′) and R (5′-CTATGCGCCTTGCCAGCCCGCTCAG-3′). A mixture of amplicons was pyrosequenced using Roche 454 FLX instrument with Titanium reagents. Raw sequencing data were processed using QIIME (Quantitative Insights Into Microbial Ecology) pipeline. Sequences with length lower than 200 base pairs (bp) were removed according to standard protocols. Sequences with similarity at or above 97 % were clustered and defined as an

Operational Taxonomic Unit (OTU) for generating rarefaction curves and calculating the richness (Chao 1 and abundance-based coverage estimator) and diversity indexes. Representative sequences from each OTU were phylogenetically assigned to phylum, family and genus taxonomic groups using a RDP naïve Bayesian rDNA classifier with a confidence threshold of 80 % [24].

3 Results

3.1 Effect of azide on MFC performance

The control MFCs generated stable current of 0.533 ± 0.005 mA in repeated feeding cycles after 5 weeks of operation. But for the azide-acclimated MFCs the currents were much lower than the controls and increased during the feeding cycles from 0.115 mA to 0.511 mA (Fig.1). Correspondingly, the cycle time was almost stable (45.5 ± 1.5 h) for the controls and comparable to that (43.5 ± 1.4 h, except for 83 h obtained at the end of the second month) for the azide-acclimated reactors (Fig.1A). Upon the end of each cycle, the depleted medium was replaced with acetate medium with the same concentration. Therefore, the gradual increase of (maximum) current with time should be associated with the increase of biofilm mass. The longer cycle time at the end of the second month suggested that much slower substrate utilization rates of azide-acclimated MFCs as compared to that of the control. By contrast, the time in the following batch cycles of azide-acclimated MFCs were very close to and slightly shorter than that of the control (Fig. 1B,C,D), and acetate was below the detection limit at the end of the each cycle (data not shown). These further demonstrate that substrate utilization rates of azide-acclimated MFCs were not influenced by azide once these reactors were run more than a certain period of time.

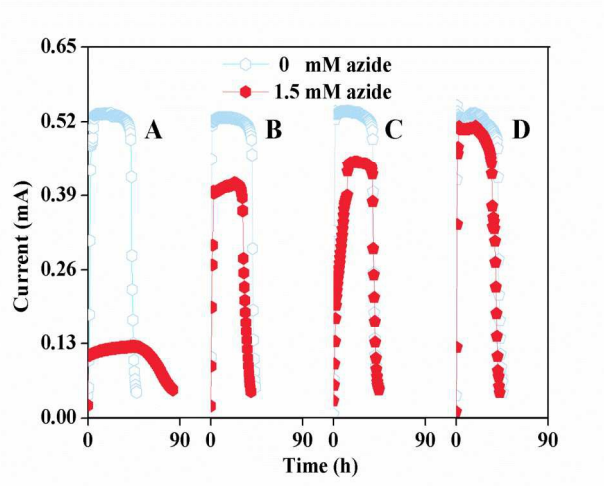

Fig.1 Current evolution over one batch cycle at the end of the second (A), fourth (B), sixth (C) and eighth (D) month for reactors added with and without azide.

In addition, acetate was not detected at the end of each cycle, which means that CO_2 was the end product and the released electrons (370 C) from acetate oxidation were diverted into other electron sinks. The recovered coulombs per cycle for the control was constant (79.2 ± 0.5 C), but increased from 32.3 C to 67.5 C for the azide-added reactors. This further indicates that the biomass of EAB increased during the enrichment process. The slow enrichment of EAB in the presene of azide might be due to molecular oxygen diffused through the cathode that is not consumed by aerobic respiratory organisms, since azide is a respiratory oxidase inhibitor [8].

3.2 CV analysis of anode biofilms

CVs were performed for studying the mechanism of electron transfer and the electroactivity of anode biofilms acclimated with and without azide in PBS (50 mM) containing no acetate. Lacking acetate could prevent current from acetate oxidation [25].

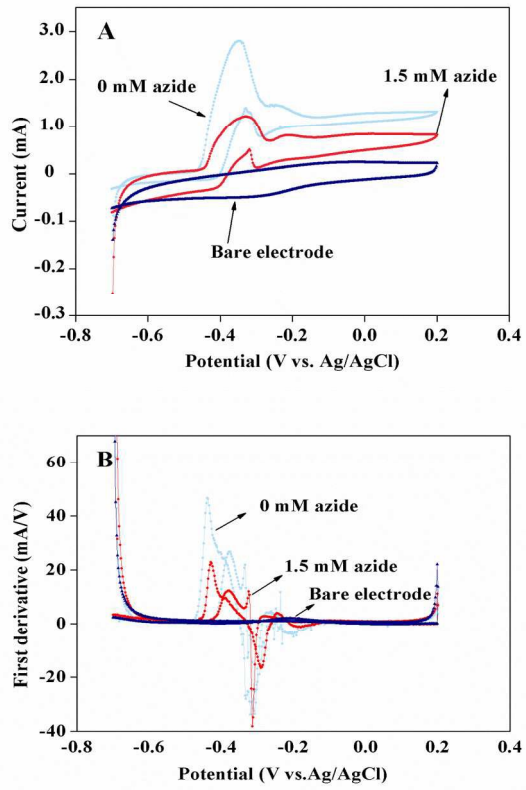

Fig. 2 (A) Cyclic voltammogram of the bacterial biofilms enriched with and without azide; (B) First derivatives of the voltammetric curve over the potential.

For the anode of the control, oxidation (positive) current with a threshold (～-0.53 V vs. Ag/AgCl) was observed during the forward scan, reflecting electron transfer to the anode. In the narrow region (between -0.53 and -0.47 V), the current was low and constant. Subsequently, the current increased rapidly until redox species under reduced state at the electrode surface were substantially oxidized, causing the current to peak (Fig. 2A). Thereafter, the current significantly decayed, which was possible due to the slow kinetics of electron transfer from adjacent cell layers by cell-cell interactions. In the wake of the first peak, a peak-like shape (-0.26 V vs. Ag/AgCl) appeared on the voltammogram (Fig. 2A). This observation may support the idea that electrons at the outer edges of biofilms can also be depleted with sufficiently positive potentials[26, 27]. This can give an

explanation of the ensuing limiting current produced in high potential region (from ~-0.16 to 2.00 V vs. Ag/AgCl). When the scan direction was switched to negative at 0.2 V for reverse scan (Fig. 2A), a reversible peak (-0.28 V) was observed but not evident on the voltammogram. This feature can be easily created by the rate limiting process, where oxidized redox proteins, not closely attached to the electrode surface, may not have time to accept more electrons from anode surface. In this study, biofilms enriched with and without azide were found producing similar voltammograms, but the difference lied in the current intensity of the predominant peaks: 2.80 mA (-0.34 V, 0 mM); 1.22 mA (-0.33 V, 1.5 mM). The biofilm enriched without azide showed the highest electroactivity, which was consistent with the maximum currents produced in the controls. The decay of peak current of azide-acclimated MFCs could be limited by the amount of EAB, because reactions occurring on the anodes were just related to the redox proteins.

To determine the midpoint potential in voltammograms of bioflims, the derivative of each voltammogram was plotted as a function of potential. Examination of the first derivative CV for biofilms enriched with and without azide revealed one predominant redox couple centered at -0.37 ± 0.01 V vs. Ag/AgCl (Fig. 2B), which was close to the midpoint potentials reported for periplasmic cytochrome c (PpcA, -0.37 V vs. Ag/AgCl) and OmcB (-0.39 V vs. Ag/AgCl) purified from *G. sulfurreducens* [19, 28]. Thus, the similarity in midpoint potential between anode biofilms enriched with and without azide suggests that the presence of azide didn't alter the extracellular electron transport pathways compared to that of the controls. Moreover, the bacteria dominant in the biofilms may have the genes encoding PpcA or OmcB, which functions as electron transfer carrier by direct contact with the anode.The CV scan of the spent medium, as well as the fresh medium without acetate, showed similar voltammograms (data not shown) without any apparent peaks, indicating that no self-produced mediators appeared in these reactors. This further demonstrated that the current generation was dependent on membrane-bound proteins in the biofilms.

3.3 Microbial diversity estimation and community distribution

The two samples yielded 14,819 (0 mM azide) and 18,327 (1.5 mM azide) qualified sequencing reads with an average length of 507 ± 3 bp. These reads were clustered to 5,882 and 3,972 operational taxonomic units (OTUs) with a distance limit of 0.03. To evaluate community diversity, rarefaction curves were generated based on the pyrosequencing data set. Additionally, estimators such as Shannon index, Chao1 as well as Good's coverage were also calculated for each sample (Table 1).

Table 1 Various estimators for evaluation of community diversity and richness.

Sample	Reads	OTU	Shannon	ACE	Chao1	Coverage
Azide-enriched	18327	3972	7.048188	16324.13	9892.806	0.865226
Azide-free	14819	5882	7.985024	27594.53	16324.43	0.730414

The shape of the rarefaction curves shows that new phylotypes would continue to merge even after 15,000 reads, as neither of the curves tends to reach a plateau (Fig.3). But the coverage value for each sample was more than 73 %, which indicated that the evaluation based on pyrosequencing could reflect the community compositions[29]. In the control, the Shannon index was 8.0, which was higher than that (7.0) of the azide-acclimated MFCs (Table 1), indicating higher microbial diversity present in the control reactor. Although the rarefaction-curve analysis for the biofilms showed that the community of the control was more diverse than that of the azide-acclimated, there were only a few genus bacteria dominating the anode biofilms enriched with and without azide.

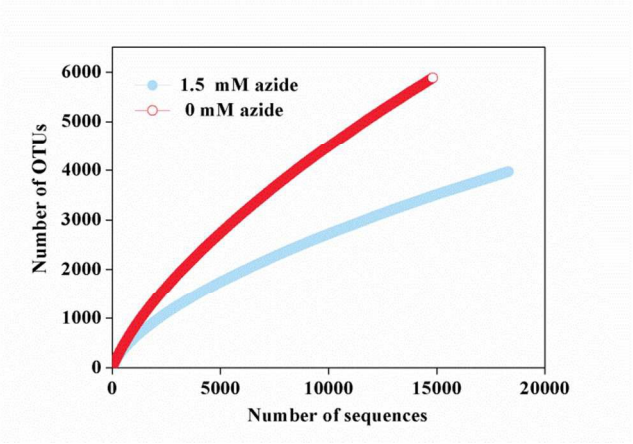

Fig.3 Rarefaction analysis of sequences for the anode samples with and without azide. OTUs, operational taxonomic units defined by clustering sequences with a 3% pairwise distance threshold.

To classify the bacterial community developed on the anodes, qualified reads were further assigned to known phyla, family and genera. Three dominant phyla (*Proteobacteria*, *Chlorobi* and *Bacteroidetes*) were identified on the anode of the control and the azide-acclimated reactors. *Proteobacteria* accounted for 85.2 % of the population in the control (Fig.4A), and 75.3 % in the azide-acclimated MFC (Fig. 4B). The other two phyla *Chlorobi* and *Bacteroidetes* present in the azide-acclimated reactors accounted for 9.1 % and 8.0 %, which contrasted with that (5.7 %, *Chlorobi*; 3.6 %, *Bacteroidetes*) in the control reactors. Furthermore, a certain number of sequences also contributed to the unclassified phyla. The relative abundance of each family on the anode was analyzed and compared in Fig. 4C. Results show that *Geobacteraceae* was abundantly detected in the controls (60.9 %) and the azide-acclimated reactors (69.8 %). But other major bacterial family such as *Ignavibacteriaceae* and *Comamonadaceae* present both in the control and the azide-acclimated reactors showed significant differences in relative abundance. *Comamonadaceae* made up 13.8 % of the community in the control, much higher than that (0.2 %) in the azide-acclimated reactor, while the family *Ignavibacteriaceae* present in the former was visibly lower than that in the latter (Fig. 4C). The *Comamonadaceae* are a family of the *Betaproteobacteria*, which have been confirmed capable of reducing oxygen in wastewater[30]. Thus, it seems that adding azide into the acetate medium might facilitate enrichment of *Ignavibacteriaceae*.

The classification of sequences at genus level could, from the perspective of bacterial function, gives description of biofilm communities[31]. It is clear that the anodes with and without azide were mainly colonized by *Geobacter*, with a relative abundance of 69.3 % for the azide-acclimated reactor, and 60.0 % for the control (Fig. 4D). *Geobacter* species are typically found in acetate-fed MFCs, which are used to enrich EAB with electrodes as electron acceptors[32]. Apart from the sequences identified as unclassified (30.0 %, 0 mM; 19.2 %, 1.5 mM), a certain number of sequences were affiliated with *Ignavibacterium* (5.5%, 0 mM; 8.9 %, 1.5 mM). As noted above, the microbial community in all samples mainly consisted of *Geobacter* and *Ignavibacterium,* as well as unclassified, with an average percentage of 64.7 ± 6.6 % for *Geobacter* and 7.2 ± 2.4 % for *Ignavibacterium.* The similarity in microbial community structure among the anode biofilms enriched with and without azide showed excellent agreement with that in the waveforms produced during CV test.

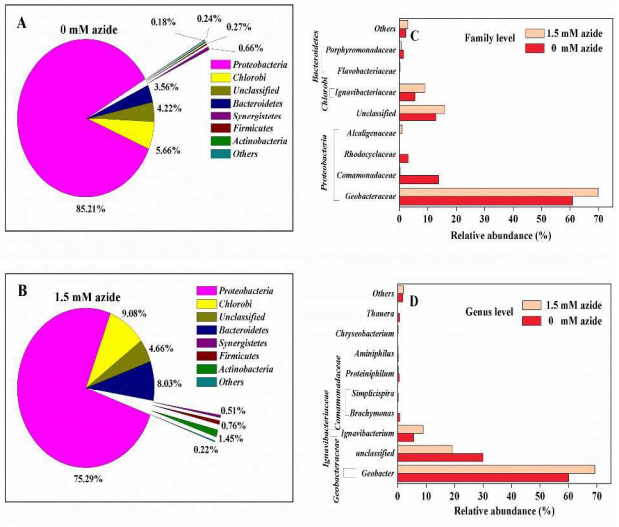

Fig.4 Microbial community distribution and relative abundance in different bioanodes enriched with and without azide.

4 Discussion

As noted above, the rarefaction curve analysis as well as the diversity index showed that the community of azide-enriched biofilms was much less diverse than the control. *The Comamonadaceae, for example, were detected on the control, making up 13.8 % of the population. Being aerobic, they were rarely detected on the azide-enriched anode.* Obviously, the presence of azide narrowed down the diversity by effectively eliminating aerobes. Even so, the community on both anodes was mainly dominated by microbes most similar to *Geobacter*. In either case, the distributions of microbes at the genus level were very similar, with *Geobacter* (60.0 %, 0 mM; 69.3 %, 1.5 mM) and *Ignavibacterium* (5.5 %, 0 mM; 8.9 %, 1.5 mM) (Figure 4D). The results conclusively demonstrate that the presence of azide in air-cathode MFCs did not significantly alter the structure of microbial community as compared to the controls. This is different from the communities in two-chamber MFCs continuously fed with and without azide, as the bacterial diversity or composition in azide-acclimated anode of two-chamber MFCs was much lower than in the controls[12, 13]. The factors that affect the microbial community between the single- and two-chamber reactors are more complicated, because reactor configuration and operating mode (single-chamber in fed batch mode and two-chamber in continuous flow mode) as well as external resistance (1000 Ω for the single-chamber and 10 Ω for the two-chamber) make it difficult to know which factors actually determine microbial community. The difference of composition of two-chamber MFCs with and without azide was possible due to the inoculum used during start-up period, because these tests were inoculated and operated at different times.

Previous studies have suggested that the members of *Geobacteraceae* are typically found in acetate-fed MFCs, with most sequences related to *Geobacter*. As two representative members of *Geobacteraceae, Geobacter sulfurreducens* and *Geobacter metallireducens* were initially known capable of reducing Fe(III) oxide before confirmed capable of directly transferring electrons to anode [33]. Furthermore, other alternative electron acceptors such as fumarate and azide are used for anaerobic respiration by *G.sulfurreducens*[13, 16]. In the case of *Geobacter* species, the available evidence indicates that direct electron transfer to the anode is mainly related to various outer membrane c-type cytochromes (e.g., OmcZ), whereas it's still unknown whether these cytochrmoes can react with soluble electron acceptors such as azide. In fact, an individual bacterium can use multiple electron transport chains, often simultaneously, because electrons can enter the chain at three levels (a dehydrogenase, the quinine pool and a mobile cytochrome carrier), which correspond to successively smaller Gibbs free energy changes for the overall redox reaction between electron donor and acceptor. Therefore, there exists possibility that azide reduction can be achieved via other chains instead of the c-type cytochromes. Together with the similar waveforms or peak currents of CVs (Fig. 2A), this assumption may account for the relatively uniform distribution of microbes despite the enrichment with azide or not.

Even so, the influence of azide on current generation was evident during the long-term operation, since current generation from the azide-acclimated reactors was found gradually increasing (Fig. 1). In MFCs, current generation depends on the response of EAB enriched on the anodes. In the case of anodes highly enriched with *Geobacter* species, the increasingly developed layers of cells are not causing a significant limitation in cell-cell transfer of electrons to the anode[27]. Several reports have shown that current generation by *Geobacter* species is positively correlated with biomass. Therefore, the gradual increase of current generation in azide-acclimated MFCs is thought due to the increase of biomass. Because the current generation from the azide-acclimated reactors was still lower than the controls even at the end of the experiment, the biomass on the azide-acclimated should be less than that on the control. This assumption was demonstrated in two-chamber MFCs, where similar trend in current generation was also observed, with 0.487 μg DNA /g electrode extracted from the control and 0.3856 μg DNA /g electrode from the azide-acclimated reactor at the end of the experiment (data not published). Theoretically, the energy required for microbial growth depends on the electron donor and

acceptor available in a biochemical system. According to the assumption that azide can enter the electron transport chain and accept electrons at low level (except for NADH dehydrogenase and the outer-surface cytochromes of the terminal complex), corresponding to low redox potentials, there will be less energy captured for bacterial growth in the presence of azide. This assumption was in agreement with the lower current generation or peak current of CVs compared to the controls. In addition, the enrichment process was slower in the presence of azide, also probably due to molecular oxygen diffused through the MEA, that might be removed by aerobic respiration in the control MFCs.

5 Conclusions

This study mainly investigated the effects of azide on current generation and microbial community in air-cathode MFCs.The results show that the enrichment process was much slower in the presence of azide when compared to the control. Correspondingly, the maximum current density gradually increased but was still below the control. Biofilms acclimated with and without azide were found producing similar voltammograms, but the difference lied in the current intensity of the predominant peaks. Pyrosequencing indicated biofilm communities enriched with and without azide were almost similar and dominated by bacteria most similar to *G. sulfurreducens*, although the community of the aizde-enriched biofilm showed less diversity. The microbial community structure was not significantly altered in the presence of azide compared to the controls. Differences in current generation or peak current of CVs were thought due to the biomass yields developed on the anodes.

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Notes and references

[a]*State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin, China*

[b]*Bioelectrochemistry Laboratory, Water Environment and Remediation Research Centre, Korea Institute of Science and Technology, Korea*

[c]*School of Life Science and Biotechnology, Harbin Institute of Technology, Harbin, China*

[d]*Fuel Cell Institute, National University of Malaysia, 43600 UKM, Bangi, Malaysia*

Corresponding author email address: yujief@hit.edu.cn; Fax: 86-451-86287017; Tel.: 86-451-86287017.

Co-corresponding author email address: pp198259@hit.edu.cn

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