The bacterial communities of bioelectrochemical systems associated with the sulfate removal under different pHs

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Abstract

Sulfate contamination in ecosystems has been a serious problem. Among various technologies, bioelectrochemical systems (BESs) show the advantage of no-pollution and low-cost for removing sulfate. In order to further expound the biological process of sulfate removal in BESs, 454 pyrosequencing was applied to analyze the bacterial communities under different pH conditions. The bacterial community profiles were analyzed from three aspects: (a) the α-diversity and β-diversity of bacterial communities, (b) the distribution of bacterial phylotypes, and (c) the characterizations of dominant operational taxonomic units (OTUs). We demonstrated that the indexes of phylotype richness and phylogenetic diversity were positively correlated across the pH gradient in the BESs. Among the dominant OTUs, the OTUs which were highly similar to Desulfiturhabdium butyrovorans, Desulfovibrio marrakechensis and Desulfomicrobium sp. might participate in removing sulfate. Standing on genus level, Desulfomicrobium and Sulfuricurvum play conducing and adverse roles for sulfate removal in alkaline condition, respectively. Desulfovibrio contributed to removing sulfate in the neutral and acidic conditions, while Thiomonas mainly weakened the performance of sulfate removal in neutral pH condition. These results further clarified how pH condition directly affected the bacterial communities, which consequently affected the performance of sulfate pollutant treatment using BESs.

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1. Introduction

Wastewater from animal husbandry, mining and food processing is usually highly polluted with sulfate, and high concentration sulfate-based compounds raise serious health risks and warnings of environmental deterioration [1]. Therefore, various technologies such as reverse osmosis [2], electrochemical method [3] and biotechnology [4–6] have been applied to control sulfate pollutants.

Bioelectrochemical system (BES), as a kind of effective method for sulfate removal, has outstanding advantages in simultaneously transforming toxic sulfate into value-added elemental sulfur and producing electric power. Most of previous studies [7–9] show that the BESs suitably remove sulfate, however they mainly focus on the electrochemical process of BESs. The pH condition has been considered a significant environmental factor on the overall process performance of BES technology, which coupled electrochemical process and biological process [10]. During biological process, the microbial community of electrode biofilm plays a key role in BESs. The electrode biofilm, an extremely complex community, consists of eukaryotes, bacteria, archaea and viruses, in which bacteria account for dominant population [11]. Nowadays, comprehensive understanding of the microbial community is impeded by the low sequencing depth of traditional techniques, such as denaturing gradient gel electrophoresis [12] and terminal restriction fragment length polymorphism [13]. The traditional techniques only detect a snapshot of the dominant species and can hardly represent the taxa with low abundances [14]. If the bacterial communities of the BESs under different pH condition could be clarified in depth, more supporting basis will catalyze the development of the BESs for sulfate removal.

In this study, the biofilm of sulfate removal BESs was explored using high-throughput pyrosequencing, which provides enough sequencing depth to sufficiently reflect the vast genetic diversity. With the aid of open source softwares and scripts written by ourselves, the pyrosequencing data of bacterial communities
were analyzed from three aspects: (a) comparing different samples using the α-diversity indexes and the UniFrac β-diversity matrix, (b) discussing the distribution of bacteria phylotypes, and (c) exploring the identifications and relative abundances of dominant operational taxonomic units (OTUs). With the above analyses, we revealed how pH condition directly affected the bacterial communities of the BESSs, which indirectly affected the performance of sulfate pollutant treatment.

2. Materials and methods

2.1. BESS construction and DNA extraction

The BESSs were built for simultaneously removing sulfates and generating electricity with organics consumption. The BESSs consisted of a working electrode (activated carbon cloth, 10 × 10 cm), a counter electrode (activated carbon cloth, 10 × 10 cm) and an Ag/AgCl reference electrode (+197 mV vs. standard hydrogen electrode, CH111, Chenhua, China) in one chamber. The simulated wastewater contained the following components (per liter of deionized water): 1.0 g Na2SO4, 0.06 g MgSO4·7H2O, 0.5 g KH2PO4, 1.0 g NH4Cl, 0.03 g CaCl2, 0.3 g sodium citrate, 0.1 g ascorbic acid and 1 ml absolute ethyl alcohol (24.58 mmol). The potential of the BESSs was held at +0.2 V (vs. Ag/AgCl). A series 4300 Battery Tested System (Maccor Inc., USA) was used to monitor the current of the BESSs. After 20 days of stable operation, microbial samples were taken from the top, middle, and bottom sections of the working electrode. After evenly mixing, the samples were flushed gently with 50 mM phosphate buffer (pH 7) and centrifuged at 6000 × g to collect bacteria. In order to make the taking of samples representative for the BESSs under different pH, two samples were taken from the BESSs under each pH as replications. For each sample, genomic DNA was extracted and purified using a previously reported protocol [15]. The purified genomic DNA was quantified by Micro-Ultraviolet Spectrophotometer ( Nanodrop Inc., USA) and stored at −20 °C for further use.

2.2. Bacterial 16S rRNA amplification and high-throughput pyrosequencing

Before pyrosequencing, the purified DNA was PCR amplified with a set of primers targeting the V1–V3 hypervariable regions of bacterial 16S rRNAs. The forward primer was 5′-AGAGTTTGATCCTGGCTCAG-3′ (27F) with the Roche 454 ‘B’ adapter, and the reverse primer was 5′-TACCAGGGCCTTCGGGAC-3′ (533R) containing the Roche 454 ‘A’ adapter and specific 10 bp barcode. The Roche 454 ‘A’/‘B’ adapter was located on the 5′-end of each primer, respectively. Each sample was amplified with 20 μl PCR reaction system following a previously reported study [16]. The PCR products were quantitated by Quantifast™ SYBR Green PCR Kit (Qiagen) and then mixed for pyrosequencing. The high-throughput pyrosequencing was processed on Roche GS FLX PLUS System.

2.3. Processing of pyrosequencing data

Pyrosequencing data 16S rRNA genes was processed using the Quantitative Insights Into Microbial Ecology (QIME, version 1.6) pipeline [17]. Before the statistical analysis of data, QIIME was used to (a) check the completeness of the barcodes and the primer sequencing, (b) remove reads shorter than 200 bp and quality score under 25, and (c) remove reads comprising chimera. Next, the sequences of different samples were exactly assigned using the unique 10 bp barcodes, and then the barcodes were removed. Only the 97% identity of the effective sequences were divided into OTUs for further analysis, and the most abundant sequence from each OTU was selected as the representative sequence. After the representative sequences were assigned by PyNAST [18], they were used for the classification of taxonomic according to the Greengenes database. In order to control error as much as possible, we randomly selected 9000 sequences per sample to explore the α-diversity in each sample and compare the β-diversity between samples. To reflect the diversity and structure of bacterial community in each sample, Chao1 index and phylogenetic diversity index were calculated from each sample. In light of the UniFrac matrix, we performed principal coordinate analysis and cluster analysis in R v2.15.0. The pyrosequencing sequences have been deposited into the NCBI with accession number SRP028827.

2.4. Statistical analysis

Based on the information of bacteria related to sulfate metabolism [19], we constructed a functional database containing the sulfate metabolism bacteria. By comparing the data of OTUs with the functional database, a group of sulfate metabolism bacteria were selected from samples. In order to explore the function of electricity production, all OTUs were aligned with the known electrochemically active bacteria [20] by Bioedit version 7.2.0 [21].

Thirty dominant OTUs, as the representative research cells, were selected, which covered the top 10 abundant OTUs in all samples and the top 12 abundant OTUs of each sample. Then, the representative sequences of the 30 OTUs were aligned with sequences listed in the GenBank database by Basic Local Alignment Search Tool (BLAST). A phylogenetic tree of 30 OTUs was constructed using MEGA 4.0 program with the neighbor-joining method. The representative sequences of 30 OTUs were deposited in GenBank under accession numbers KF264507-KF264536.

3. Results and discussion

3.1. Enhance sulfate removal using BESSs

The BESSs were built for simultaneously removing sulfates and generating electricity with organics consumption. Considering the capital cost, treatment effect and secondary pollution, pH 4.5 was the optimum condition for sulfate removal from wastewater (Fig. 1). The performance of sulfate removal declined with pH, but the electrogenesis capacity show a opposite trend [22]. The samples and corresponding reduplications under three pH were marked as BES4.5–1, BES4.5–2 (pH 4.5), BES6.5–1, BES6.5–2 (pH 6.5), BES8.5–1, BES8.5–2 (pH 8.5).

3.2. Overall pyrosequencing information

Overall pyrosequencing information about six samples is exhibited in Table 1. In this study, 79,374 valid reads were produced by Roche GS FLX PLUS System. The optimization procedure of data generated a total of 60,589 high-quality sequences with quality score above 25, length over 200 bp and without chimeras. The average length of sequences was 477.2 bp which was enough to perform the identification of bacterial 16S rRNA genes. Six 16S rRNA gene libraries were constructed from BES4.5–1, BES4.5–2, BES6.5–1, BES6.5–2, BES8.5–1 and BES8.5–2. As shown in Fig. S1, the number of OTUs of BES4.5 and BES6.5 began to reach a plateau at 9000

![Fig. 1](image-url) The configuration and performance of bioelectrochemical systems for sulfate removal. (The reference electrode denotes to Ag/AgCl: the two bars in Fig. 1b represents parallel test under different pHs).
sequences, while new bacterial phylotypes continued to emerge in BES8.5. To ensure the fair comparison of different samples at same sequencing depth, 9000 sequences was extracted from each sample, randomly. The good's coverages were 97.2% (BES4.5-1), 97.3% (BES4.5-2), 93.5% (BES6.5-1), 93.3% (BES6.5-2), 79.0% (BES8.5-1) and 79.8% (BES8.5-2) using random selections of 9000 sequences per sample, suggesting that the 9000 sequences was enough to reflect the characteristic of the bacterial communities.

### 3.3. The α-diversity and β-diversity of bacterial community

Chao1 index and phylogenetic diversity were calculated using random selections of 9000 sequences per sample (Table 1). The two α-diversity indexes were used for estimating and comparing the richness and diversity of the bacterial communities. In addition, the rarefaction curves of Chao1 index and phylogenetic diversity were constructed at different pHs (Fig. S2). Phylotype richness, measured as Chao1 index, was positively correlated with the pH condition ($r = 0.9477$, $P = 0.0040$). The result was consistent with phylogenetic diversity which also increased across the pH gradient ($r = 0.9632$, $P = 0.0020$). The observation of α-diversity indicated that the sample with higher pH represented greater richness and diversity of bacterial community in this study.

Principal coordinated analysis (PCoA) was used to evaluate the difference and similarity of bacterial communities by two different forms of un-weighted PCoA and weighted PCoA (Fig. S3).

Firstly, un-weighted PCoA analysis reflected the relationship of community diversity. The contribution rates of un-weighted principal components 1 and 2 accounted for 44.39% and 25.60% of the total community variations, respectively. Secondly, the structure of bacterial community, including community diversity and richness, was reflected by weighted PCoA. There were relatively few variences in the distribution of samples between un-weighted PCoA and weighted PCoA, while weighted principal component 1 soared to 94.17% and weighted principal component 2 shifted to 4.76%. The result indicated that weighted principal component 1 was more influential to community structure than community diversity. As pH was the dominated environment variable, pH might be represented by weighted principal components 1. Considering the values of principal components, the distance between BES4.5 and BES6.5 was the shortest in weighted PCoA, suggesting that the community structure of BES4.5 and BES6.5 exhibited the highest similarity. This result was also supported by the cluster analysis (Fig. 2) where BES4.5 and BES6.5 were separated from BES8.5.

### 3.4. The distribution of bacteria phylotypes

In order to compare the difference of bacterial distribution among BES4.5, BES6.5 and BES8.5, the most abundant 500 OTUs (3% distance) could be classified into three groups (I, II, III) based on the cluster analysis of OTUs (Fig. 2). Three OTU groups I, II, III represented relatively high abundance in BES8.5, BES6.5

![Fig. 2. Cluster analysis of bacterial communities of the bioelectrochemical systems. The 500 most abundant OTUs (3% distance) were clustered in two forms: OTUs and samples. The relative abundance of OTUs is reflected by the color of scale. Three OTU groups I, II, III represent relatively high abundance in BES8.5, BES6.5 and BES4.5, respectively. The three OTU groups were assigned to taxonomies at phylum and class level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

**Table 1**

Overall pyrosequencing information from the six samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Reads Raw valid reads</th>
<th>Reads Effective reads</th>
<th>Chimeras</th>
<th>Effective sequences</th>
<th>Per 9000 sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>BES4.5-1</td>
<td>14,114</td>
<td>11,262</td>
<td>353</td>
<td>10,909</td>
<td>873</td>
</tr>
<tr>
<td>BES4.5-2</td>
<td>13,767</td>
<td>10,627</td>
<td>363</td>
<td>10,264</td>
<td>864</td>
</tr>
<tr>
<td>BES6.5-1</td>
<td>13,703</td>
<td>10,603</td>
<td>341</td>
<td>10,262</td>
<td>2038</td>
</tr>
<tr>
<td>BES6.5-2</td>
<td>11,703</td>
<td>9557</td>
<td>249</td>
<td>9308</td>
<td>2166</td>
</tr>
<tr>
<td>BES8.5-1</td>
<td>14,093</td>
<td>10,891</td>
<td>462</td>
<td>10,429</td>
<td>7544</td>
</tr>
<tr>
<td>BES8.5-2</td>
<td>11,994</td>
<td>9784</td>
<td>367</td>
<td>9417</td>
<td>7134</td>
</tr>
</tbody>
</table>
and BES4.5, respectively. These OTUs were assigned to known taxonomies at phylum and class level, and there were 6 main phyla and 8 main classes (the relative abundances were more than 1%). The sum of proportion of Proteobacteria, Chloroflexi and Bacteroidetes accounted for 72.62% in group I, 88.19% in group II and 85.45% in group III.

The relative abundance of Proteobacteria was significantly correlated across the pH gradient, although in the opposite direction. At least 33 species of electrochemically active bacteria were confined to the Proteobacteria [20]. Proteobacteria, a major phylum of bacteria, included not only a number of electrochemically active bacteria but also a wide variety of bacteria involving in sulfur metabolism. The Proteobacteria are divided into five sections, referred to Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria and Epsilonproteobacteria. The five classes also mainly existed in the bacterial communities of our BESs.

The filamentous Chloroflexi bacteria have been detected growing specifically on BES anodes [23], and a previous study revealed the robust coexistence of sulfate-reducing bacteria and Chloroflexi in the sediment [24]. In these sulfate removal BESs, Chlororflexi abounded in group I, but it rarely existed in groups II and III.

In this study, ethanol was selected as the sole carbon source in the BESs, because ethanol was a preferred electronic donor for sulfur-reducing bacteria [25]. Similar with our system, a previous study constructed the thermophilic BESs for distillery wastewater treatment, which achieved high efficiency for electricity generation and also reduced sulfate along with oxidizing complex organic substrates [22]. These studies all demonstrated that Bacteroidetes bacteria were abundant in the sulfate-ethanol BESs.

Pyrosequencing detected 128 bacterial genera in all samples, and the genera related to sulfate metabolism were selected. There were mainly four types of bacteria which were involved in sulfate metabolism: sulfate-reducing bacteria, sulfur-reducing bacteria, sulfur-oxidizing bacteria and the disproportionation bacteria. Fig. 3a was drawn according to how the bacteria contributed to removing sulfate.

Sulfate-reducing bacteria played a conducing role in the sulfate metabolism; on the contrary, sulfur-reducing bacteria exerted an adverse influence on the generation of sulfur, and sulfur-oxidizing bacteria weakened on the accumulation of sulfur under the condition of constant voltage +0.2 V (vs. Ag/AgCl); disproportionation bacteria was like a double-edged sword in the sulfate metabolism. Since Fig. S4 showed good reproducibility between the samples and corresponding reduplications, we averaged the relative abundance of the samples and the corresponding reduplications for convenient comparison. As illustrated in Fig. 3b, BES4.5 and BES6.5 exhibited similar bacterial community composition and both highly abounded with the genus of Desulfovibrio and Thiomonas. Desulfovibrio, as a sulfate-reducing bacterium, was more easily detected in BES4.5 (2.14%) and BES6.5 (2.16%) than in BES8.5 (0.43%), suggesting that Desulfovibrio mainly contributed to removing sulfate in the neutral and acidic conditions. As a sulfur-oxidizing bacterium, the Thiomonas in BES6.5 was about 1.69 times more than that in BES4.5, reflected in the fact that Thiomonas exerted greater adverse influence on removing sulfate in the neutral pH system. Unlike BES4.5 and BES6.5, BES8.5 was enriched with Desulfomicrobium (2.55%) and Sulfuricurvum (8.24%), which could be respectively classified as sulfate-reducing bacteria and sulfur-oxidizing bacteria. There were 2, 6 and 7 genera belonging to sulfur-oxidizing bacteria in BES4.5, BES6.5 and BES8.5, respectively, indicating that the diversities of sulfur-oxidizing bacteria in BES6.5 and BES8.5 were greater than that in BES4.5. Based on the above analysis and the performance of sulfate removal, the higher diversity of sulfur-oxidizing bacteria, the performance of sulfate removal more likely was weakened. Within the sulfur-reducing bacteria, Geobacter was the abundant populations in BES8.5 which was widely reported for producing electricity. Geobacter metallireducens, Geobacter sulfurreducens and Geobacter brevis were isolated from the anode of BESs [26–28]. Besides, G. metallireducens, G. sulfurreducens and Geobacter lovleyi were also detected in the cathode of BESs [29,30]. Geobacter might play an important role in producing electricity at pH 8.5. In addition, disproportionation bacteria were only detected in BES8.5.

3.5. The characteristics of dominant OTUs

The α-diversity and β-diversity of bacterial community had been analyzed from a macroscopic perspective. The relative identifications and abundances of the dominant OTUs were microscopically illustrated in Fig. 4. By pyrosequencing, 6071 different OTUs were detected, while 30 dominant OTUs abundantly existed in the samples. The relative abundances of the 30 OTUs summed up to 63.62% in all samples, suggesting that the 30 OTUs covered the majority of effective sequences. According to the results of BLAST and references, the identifications of 30 OTUs were displayed in Fig. 4 for discussing their possible function. There were 12 GenBank matches belonging to cultured bacteria, and the others were uncultured bacteria. Considering the function of the
BESs, we discussed the possible function of OTUs from the two perspectives of sulfate removal and power generation. As illustrated in the phylogenetic tree (Fig. S5), the 30 OTUs mainly fell into Alphaproteobacteria (13.33%), Anaerolineae (16.67%), Bacteroidia (10.00%), Betaproteobacteria (26.67%), Deltaproteobacteria (16.67%) and Gammaproteobacteria (6.67%) at class level.

Among the 30 dominant OTUs, OTU-2921 and OTU-6798 showed salient abundance. OTU-2921, as the most abundant OTU, was the dominant population in BES4.5 (38.02%) and BES6.5 (44.38%), but rarely appeared in BES8.5 (0.01%). The results indicated that OTU-2921 had higher adaptability in the neutral and acidic conditions. OTU-2921 was phylogenetically related (97% identity) to Paludibacter spp. Previous studies demonstrated that Paludibacter spp., as a fermentative bacteria, had appeared in enrichments of sulfate reduction systems in acidic condition [31], and Paludibacter spp. was often accompanied by the sulfate-reducing bacteria in sulfate reduction systems [32–34]. However, there was no valid evidence to prove that Paludibacter spp. can directly reduce sulfate. OTU-6798 was the second most OTU. OTU-6798 had a similarity of 99% with Thiomonas sp., which was a sulfur oxidizer [35]. OTU-6798 showed relatively more enrichment in BES6.5 (18.34%) than that in BES4.5 (8.44%), and the proportion of OTU-6798 in BES8.5 was only 0.76%. The results suggested that the function of sulfate removal was weakened by OTU6798 in BES8.5 at a large extent, but the situation could be better in BES4.5 and BES6.5. The result contributed to clarifying the reason why the performance of sulfate removal in BES4.5 was better than that in BES8.5.

Except for the above OTU-2921, there were 8 other OTUs (OTU-5839, OTU-230 [36], OTU-312, OTU-3813, OTU-7213, OTU-2761 [25], OTU-6555, OTU-4254) which had been detected in sulfate reduction systems, three of which could be identified as sulfate-reducing bacteria. OTU-5839, OTU-3813 and OTU-6555 had high homology (over 97%) with Desulfatirhabdium butyrativorans [37], Desulfovibrio marxrekakensis [38] and Desulfovibrio sp. [39], respectively. All of them might make a contribution to removing sulfate or might be involved in sulfate metabolism. There were another two OTUs (OTU-2332, OTU-5876) belonging to sulfur-oxidizing bacteria except for the above OTU-6798. They could reduce part of sulfur compounds (such as sulfide, elemental sulfur and thiosulfate) to form sulfate, which undermined the removal efficiency of sulfate in the BESs.

Electrochemically active bacteria were an indispensable portion of the BESs we constructed. Among 30 dominant OTUs, OTU-2238, OTU-676 and OTU-5199 [40], showed similarities of 99% to uncultured species detected in microbial fuel cells. These OTUs might make a contribution to the electricity production. However, part of electrochemically active bacteria might represent relatively less abundance in the BESs. Accordingly aligned with the known electrochemically active bacteria, 56 OTUs showed relative high homology (over 96%) with the known electrochemically active bacteria (Fig. 5). There existed eight kinds of electrochemically active bacteria, which were Burkholderia cepacia [41], Pseudomonas aeruginosa [42], Acinetobacter calcoaceticus [43], Brevundimonas diminuta [41], Rhodopseudomonas palustris [44], G. lovleyi [30], G. sulfurreducens [27] and Desulfovibulbus propionicus [45]. Among 56 OTUs, 21 OTUs (37.5%) were identified as B. cepacia, and were primarily detected in BES4.5. The other OTUs abounded in BES8.5, which were defined as R. palustris, G. lovleyi, G. sulfurreducens and D. propionicus. As previous study reported [45], D. propionicus was not only an electrochemically active bacterium but also a sulfate-reducing bacterium.

In this study, the bacterial community was analyzed from α, β-diversity, phyotypes and dominant OTUs. Though part of OTU's function remained unknown and required further study for confirmation, community analysis demonstrated a range of
interesting phylotypes and OTUs. For example, *Paludibacter* spp. was accompanied by the sulfate-reducing bacteria. Consequently, it is helpful to purposefully chose study subjects for further physiological and biochemical research. Based on the information, the microorganisms agent, such as inoculant of *Desulfovibrio* bacteria, could be produced to enhance the performance of sulfate removal under acidic environment.

4. Conclusions

We comprehensively explored the bacterial communities of bioelectrochemical systems under three pHs using 454 pyrosequencing. Our study demonstrated that the indexes of phylotype richness and phylogenetic diversity were positively correlated across the pH gradient in the BESs. Among dominant OTUs, the OTUs which were highly similar to *D. butyrovorans*, *Desulfovibrio marrackehensis* and *Desulfomicrobium* sp. might participate in removing sulfate. Based on genus level, *Desulfomicrobium* and *Sulfuricurvum* play conducing and adverse roles for sulfate removal in alkaline condition, respectively. *Desulfovibrio* contributed to removing sulfate in the neutral and acidic condition, while *Thiomonas* mainly weakened the performance of sulfate removal in neutral pH condition. These results further clarified how pH condition directly affected the bacterial communities, which consequently affected the performance of sulfate pollutant treatment.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.procbio.2014.04.019.

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