Identification of removal principles and involved bacteria in microbial fuel cells for sulfide removal and electricity generation

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Abstract

Simultaneous sulfide and organics removals with electricity generation can be achieved in microbial fuel cells (MFCs). In present research, principles of sulfide removal as well as the involved bacteria in the MFCs with sulfide and glucose as the complex substrate are investigated. Results indicated that electrochemical and biological oxidations are the main effects for sulfide removal. Community analysis shows a great diversity of bacteria on the anode surface, including the exoelectrogenic bacteria and sulfur-related bacteria. They are present in greater abundance than those in the MFCs fed with only sulfide and responsible for the effective electricity generation and sulfide oxidation in our proposed MFCs. The results are conducive to reveal the interactions between the pollutants and microbes in aspects of pollutants removals and energy recovery in the MFCs for sulfide removal.

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1. Introduction

Dissolved sulfide and hydrogen sulfide are common pollutants found in petrochemical processing wastewater, hydrocarbon wastewater and digester effluent [1,2]. Several problems are related to sulfide buildup, including release of obnoxious odors, corrosion of concrete sewer pipes, toxicity to plants and animals, safety hazards to sewer workers due to the volatilization of sulfide gas and negative influences on the post wastewater treatment [3], thus it should be removed from the water streams.

Of the available means for sulfide removal, biological oxidation is the most commonly used method due to its low cost and easy operation [4,5]. Microbial fuel cells (MFCs), devices that use bacteria as catalysts to oxidize organic or inorganic matters with current generation and convert chemical energy to electricity in mere one step [6-9], is demonstrated to be effective to oxidize sulfide to elemental sulfur by Rabaey et al. [10]. Additionally, their group also finds that sulfide could also be oxidized spontaneously in abiotic fuel cell (AFC) systems but with lower removal efficiency and less energy recovery [11]. These indicate that bacteria play an important role in sulfide removal and electricity generation, while little attention has been paid to these aspects in previous research [12-15]. Moreover, sulfide and its co-substrates such as degradable organics and nitrate have also been considered to be treated in MFCs by our research group and other researchers [16-18], while the mechanisms of sulfide removal and electricity generation should be further investigated.

In present research, principles of sulfide removals in MFCs were investigated. The removal

effects were quantitatively determined. The involved bacteria as the most important key factor of electricity generation and pollutant removal in this system was also monitored and analyzed. The results were helpful to reveal the interactions between the pollutants and microbes in aspects of pollutants removals and energy recovery in the MFCs for sulfide removal.

2. Materials and methods

2.1 Construction of the MFCs and the electrolyte conditions

Eight double-chamber MFCs built in cylindrical geometry chamber were constructed using plane Plexiglas. Four of them had been employed in our previous research [16]. The anode and cathode compartments were separated by a proton exchange membrane (Nafion117#. Dupont, USA), with the volume of electrolyte in each compartment of 250 mL. Both anode and cathode were 16 cm^2-carbon fiber felt with specific surface area of 1050 m^2/g (3 mm thickness, 4 cm length and width. Beijing Evergrow Resources CO., LTD, China). The two electrodes were connected to a data acquisition system (Measurement Inc, USA) through copper wires with the external resistance of 1000 Ω to record voltage at an interval of 5 min. The anode solution contained the following components (per L): 0.812 g of $C_6H_{12}O_6$; 4.97 g of $NaH_2PO_4 \cdot H_2O$; 2.75 g of $Na_2HPO_4 \cdot H_2O$; 0.31 g of NH_4Cl; 0.13 g of KCl; 1.25 mL of vitamin solution; and 12.5 mL of trace mineral element solution [16]. Sulfide (100 mg/L) was added to the anode solution of the MFCs in the form of $Na_2S \cdot 9H_2O$. Vanadium (V) with concentration of 500 mg/L and pH of 2 was employed as the electron acceptors in the cathode compartment as it had been demonstrated to be promising electron acceptors in previous studies [16]. Two of the MFCs were inoculated with 25 mL anaerobic granular sludge obtained from an up-flow anaerobic sludge blanket (UASB) reactor

treating high sulfate wastewater (Dept. of Environmental Engineering, Peking University). After they were well developed, the suspended sludge was removed from the anode compartment. Other six MFCs without inoculation (abiotic controls) were divided into three groups which served as control sets under respective conditions.

2.2 Operation of the MFCs and the analytical methods

The experiments were carried out in 72 h fed-batch mode as most sulfide was removed within that time. Samples were taken at 12 h intervals to measure the sulfide concentration. Two of the control sets were first filled with the anolyte and catholyte mentioned above but with no electrodes to examine the influence of sulfide volatilization during the experiment (Control 1). Then another two control sets were also filled with fresh electrolyte and equipped with new anodic electrode to study the effects of volatilization and anode adsorption for sulfide removal (Control 2). After that, the last two abiotic controls without inoculation but with fresh electrodes were operated in the closed circuit mode to evaluate the spontaneously electrochemical oxidation as well as volatilization and anode adsorption for sulfide removal in AFC systems (Control 3). Afterwards, two well developed MFCs used in previous research were operated to investigate the multiple effects of volatilization, adsorption, electrochemical oxidation and biological oxidation for sulfide removal [16]. At last, microbes on the two anodic electrodes in these two well developed MFCs were tested and analyzed, compared with the initially inoculated sludge.

Sulfide was determined according to the methylene blue method (n = 665 nm) [19]. The indication of "sulfide" described all species (H_2S, HS^-, and S^{2-}). Sulfate was measured by standard barium chromate colorimetry (n = 420 nm). Measurement of COD was based on digestion with potassium dichromate in concentrated sulfuric acid for 2 h at 150 ºC [15]. pH was measured by a

pH-201 meter (Hanna, Italy). Polarization curves were employed to obtain the maximum power density by varying external resistances from 5000 Ω to 10 Ω using a resistor box and were run at least twice under each resistance to ensure the repeatability of power outputs. Current (I) was calculated at a resistance (R) from the voltage (V) by $I = V/R$. Power (P) was calculated by $P = I \times V$ and normalized by the cathode area. Coulombic efficiency (CE) was calculated as reported previously [9].

2.3 The microbiological analysis

Molecular biology analysis was carried out to acquire characteristics of microbial population in the proposed MFC systems. The polymerase chain reaction (PCR) of 16S rDNA gene fragments of bacteria on the anode surface of the MFCs was performed with Applied Biosystem Gene Amp PCR system 9700, after the MFCs being operated for about half a year. Part of the anode electrode (3 cm × 1cm) was cut off by sterile scissors, and oscillated for 20 min by ultrasonic to collect the bacteria attached to the anode (Sample M). The extracted DNA was amplified by the universal 27F (50-AGA GTT TGA TCM TGG CTC AG-30, M=C or A) and 1492R (50-TAC GGY TAC CTT GTT ACG ACT T-30, Y=C or T) and the amplified products were then quantified by Nanodrop (Thermo, USA) and ligated into the pEASY-T1 easy vector (Promega, USA). The resulting plasmids were transformed into E. coli DH5 a cells following the manufacturer's instructions [20]. Microbes in the anaerobic sludge for initial inoculation were also analyzed in the same way as described above (Sample S).

The phylogenetic analysis was performed as follows: the clone sequences obtained were checked foe chimeric artifacts by the check-chimera program of the Ribosomal Database Project (RDP). And then were compared with the 16S rDNA gene sequences that deposited in public

database GenBank using the BLAST search program. The sequences obtained from the GenBank database were aligned with the new ones by BioEdit 7.0. Phylogenetic trees were constructed by the neighbor-joining method with robustness of 1,000 bootstrapping value in MEGA 4.0. Sequences reported in this paper have been submitted to GenBank with accession numbers from KC481401 to KC481511.

3 Results and Discussion

3.1 Effects of sulfide removals in proposed MFCs

The voltage outputs of the MFCs were 250 - 800 mV during the total operating cycle (Fig. 1), with the external resistance of 1000 Ω, when the MFCs were initially filled with anolyte containing 100 mg/L of sulfide and 800 mg/L of glucose and catholyte with 500 mg/L of V(V), respectively. The highest power output obtained from the polarization curves was 572.4 ± 18.2 mW/m^2 at the current density of 1094.0 ± 50.6 mA/m^2, showing advantage to air cathode MFCs only for organics removal [16,21]. At the end of the operating cycle (72 h), the sulfide and COD removal efficiencies reached 84.7 ± 2.8% and 54.0 ± 1.9%, respectively, with a CE of 12.4 ± 1.0%, demonstrating that MFC was promising for sulfide wastewater treatment [16].

In the anode compartment, sulfide removal mainly depended on three effects, including physical, chemical actions and biological oxidation. The process related to sulfide removal included volatilization, anode electrode adsorption, chemical oxidation and biological oxidation. This study majored in the effect of the four processes in sulfide removal through experiment designing.

The anolyte containing 100 mg/L of sulfide and 800 mg/L of glucose was added to the Control 1. It was found that the concentration of sulfide declined with time (Fig. 2), due to the generated

hydrogen sulfide from sulfide vitalization because of the dissolving and ionizing balance of sulfide in the aqueous solution and gas phase (Equ. 1 and 2) [10]. The removal efficiency of sulfide approached 9.4% after 72 h due to sulfide volatilization from aqueous solution based on Equ. 1 and 2.

$$S^{2-} + H_2O \rightarrow HS^- + OH^- \quad (1)$$

$$HS^- \rightarrow H^+ + H_2S \quad (2)$$

Then the fresh anode electrode without microbes and fresh anolyte was added to the Control 2 and the sulfide concentration gradually decreased with time (Fig. 2). The observed sulfide removal efficiency approached 21.8% after 72 h operation. Omitting the volatilization effect mentioned above (9.4%), the effect of anode electrode adsorption on sulfide removal approached 12.4%, due to the high specific surface area (1050 m^2/g) and strong adsorption ability of the electrode material [22]. Sulfide might be removed mainly by anode electrode adsorption first, and then chemical and biological oxidation.

After that, fresh electrode and electrolyte were added into the Control 3 and they were operated in the closed circuit. 64.3% of sulfide was removed after 72 h, with a relatively obvious decreasing trend (Fig. 2), which depended on the co-effects of volatilization, anode electrode adsorption and electrochemical oxidation. The electrochemical oxidation was realized because of the natural electric potential difference between electrolytes in the anode and cathode compartments, respectively, which had been reported before [11] and verified in our previous research [16]. According to the above analysis, the contribution of electrochemical oxidation for sulfide removal was 42.5%.

Depending on the above analysis of the other three effects, combined with the gradual removal of sulfide (Fig. 2) and the total removal efficiency in the present MFC system (84.7%), the effect

of biological oxidation for sulfide removal was 20.4% within the 72 h operation.

In conclusion, 84.7% of sulfide was removed by the improved system in the total operating cycle. The contribution of volatilization was 9.4%, and 12.4% of sulfide was removed through the anode electrode adsorption, while 42.5% of sulfide was removed by electrochemical oxidation, and 20.4% of sulfide was removed through biological oxidation. The contribution proportion of these four effects for sulfide removal was shown in Fig. 3. It could be seen that electrochemical oxidation and biological oxidation were the main factors affecting sulfide removal. These two effects were combined in MFCs, resulting in a promising sulfide containing wastewater treatment method.

It should be mentioned that the above mechanisms studies were carried out by subtracting the contribution of the former effects from the following controls. These effects for sulfide removal were also evaluated when they were simultaneously present. In the former test where total removal efficiency of 84.7% was obtained during the 72 h operation, the concentration of H_2S in the gas phase was measured by collecting it with airbag and dissolving it into the water to evaluate the volatilization, while the extent of adsorption effect was estimated through measuring the concentration of sulfide on the anode by cleaning the electrode after the test. Results showed that the contributions of volatilization and adsorption were 8.7% and 10.4% respectively under this condition, which were lower than those obtained in the control experiments, with relatively small differences. These implied that physical effects were suppressed when oxidation effects worked together, again demonstrating that electrochemical and biological oxidations were the main effects for sulfide removal in the proposed system.

In another aspect, sulfide was first oxidized to sulfur electrochemically and then sulfur was

further oxidized to sulfate quickly due to the sufficient electrons from electrode during the electrochemical oxidation process, while elemental sulfur was the main product for sulfide removal when the biological oxidation was performed in MFCs, due to ([11,16]). Sulfur granules could be easily removed through separation methods. Moreover, sulfide is the most toxic among all the species of sulfur compounds, thus it is indicated that sulfide containing wastewater can be successfully treated along with simultaneous energy recovery based on MFC technology.

3.2 Identification of the involved microbes

3.2.1 Acquirement of the microbes information

Microbes in the anode compartment, especially on the anode surface, play an important role in sulfide removal and electricity generation in MFCs, thus they were examined attentively in present study. With operation, the electricity output was enhanced through the microbial enrichment, suggesting that an active bacterial consortium capable of electricity production from sulfide and glucose oxidation was established in the MFCs, thus PCR and 16S rDNA gene sequences analysis were performed to obtain the strains information and their effects on sulfide removal and energy recovery. In this study, 150 and 120 white colonies with inserted small-subunit ribosomal genes were randomly chosen to construct bacterial libraries, respectively. The results of the in situ PCR indicated that there were 56 genotypes covered in Sample S (the anaerobic sludge for inoculation) and 40 in Sample M (the microbes attached on anode). The 16S rDNA gene sequences of the Sample S bacterial library fell into mainly nine phylogenetic divisions (Fig. 4a): Firmicutes (occupying 43% of total bacterial clones), Chlorobi (27%), Alphaproteobacteria (1%), Betaproteobacteria (1%), Gammaproteobacteria (3%), Deltaproteobacteria (4%),

Epsilonproteobacteria (2%), Bacteroidetes (11%) and uncultured (5%). There were ten phylogenetic divisions of the Sample M bacterial library, while its colony structure had obviously altered over domestication time (Fig. 4b). Firstly, the Firmicutes which comprised the largest portion of the Sample S gene library had decreased to 35%. Secondly, the Chlorobi with few bacteria reported with electrogenesis activity, the second predominant bacteria in Sample S, had disappeared after six months domestication. In the meantime, two kinds of newly electricigens, namely Lentisphaerae (10%) and Armatimonadetes (2%) appeared on the anode of the MFCs [23,24]. Moreover, with the MFCs' functions of electricity generation and sulfide removal enhancing, plenty of bacteria with electrochemical activity were domesticated, such as the *Geobacter sulfurreducens* and *Bacteroides* sp. which are classified as the Deltaproteobacteria and Bacteroidetes. Besides, microbes involved in sulfide removal also multiplied, namely the *Rhodobacter* sp. and *Pseudomonas* sp. in Alphaproteobacteria and Gammaproteobacteria. These indicated that structures of the bacteria community had evolved as adapting to the new conditions during the operation of the MFCs. Some specific species might be responsible for the high performance of the MFCs and thus should be further investigated.

3.2.2 Deduction of the microbes effects

To know more about how those bacteria played specific roles in generating electricity and removing sulfide in the MFCs, a neighbor-joining tree was constructed with these and related sequences from the GenBank database (Fig. 5). Some critical species responsible for the electricity generation and sulfide removal were discovered, which also exhibited specific characteristics in our proposed MFCs. Moreover, most of these bacteria related to electricity generation and sulfide removal were not found in Sample S, signaling the great alteration in the abundance of microbial

biodiversity due to the domestication of bacteria in the developed MFCs in this study

Electrochemically activated bacteria were conducive to sustainable electricity generation in MFCs. In our system, Bacteroidetes was the most frequently founded in the anode biofilms (11%), involving in generating electricity in the MFCs, which had also been indicated in early MFCs studies [25,26]. Besides, *Pseudomonas* sp. which was found in the employed MFCs and belonged to Gammaproteobacteria (11%), had also been widely studied in MFCs [27], owing to its fast electron collection efficiencies in reaction processes. Moreover, *Geobacter* species, one of the observed Deltaproteobacteria, were often the predominant organisms when extracellular electron transfer was an important bioremediation process in various environments. Typically, *Geobacter sulfurreducens* was one of the most widely studied electricigens in MFCs. For it could attach to the electrode and remain viable for long periods of time as to completely oxidize organic substrates with quantitative transfer of electrons to the electrode. In addition, Lentisphaerae（10%） and Armatimonadetes (2%) were new electricigens appeared on the anode, demonstrating more electrochemically activated bacteria in our system than those reported by Sun et al. [14], probably due to the complex substrate (sulfide and glucose) employed in our study.

Sulfur-oxidizing and sulfate-reducing bacteria were two groups of bacteria participating in the global sulfur cycle. Sulfur-oxidizing bacteria could oxidize hydrogen sulfide, sulfur, sulfite, thiosulfate, and various polythionates under acidic, neutral or alkaline conditions. After the domestication in present research, the abundance of Alphaproteobacteria had increased to 12% of the 16S rDNA gene library, owing to the rapid growth of electricigens and sulfide related microbes, such as *Rhodobacter* sp. and *Rhodopseudomonas palustris* [28,29]. Clone type M-80 shared a high gene sequence identity (100%) with *Rhodobacter* sp.. *Rhodobacter* was a genus of the

Rhodobacteraceae in taxonomy, a widely studied bacterium in MFCs oxidating S^{2-} to S^0, thus helping to get sulfide removed and elemental sulfur generated in MFCs [30]. Furthermore, the observed Gammaproteobacteria comprised several most widely studied groups of bacteria in MFCs, such as the Enterobacteriaceae, Vibrionaceae and Pseudomonadaceae. Members of *Citrobacter* sp. (99% similarity with M-5 and M-10) belonged to Chromatium were photosynthetic and oxidized hydrogen sulfide instead of water, producing sulfur as excrement, which was the desired way to remove sulfide and recover sulfur [31]. The Deltaproteobacteria comprised a branch of predominantly aerobic genera, which contained most of the known sulfate (*Desulfovibrio*, *Desulfobacter*, etc) and sulfur (*Desulfuromonas* spp.) reducing bacteria alongside several others with different physiology. *Desulfovibrio* sp. (in 100% similarity) was not restricted to sulfate reduction and many species of them could also produce sulfide by reduction of sulfite and thiosulfate [12]. Besides, M-100 showed as high as 100% similarity value with the *Thiobacillus* sp., obligate autotrophic organisms that required inorganic molecules as an electron donor and inorganic carbon as a source. Therefore, it was able to oxidize a variety of sulfur compounds, namely sulfide, sulfur, thiosulfate, sulfite, dithionite with oxygen as the electron acceptor [32]. As to Epsilonproteobacteria in our study, they mainly contained *Sulfurospirillum cavolei* and *Sulfurospirillum deleyianum*, which were able to utilize elemental sulfur, thiosulfate, sulfite, dithionite and etc as electron acceptors with organics as the electron donors as well as the energy and carbon source [33,34].

In another aspect, the Firmicutes group accounted for the largest portion (26%) of the Sample M 16S rDNA gene libarary, mainly due to its domestication of the inoculated anaerobic sludge (Sample S). Many Firmicutes produce endospores, which were resistant to desiccation and help

them survive in various conditions. Meanwhile, those who could not adapt in the MFCs died out finally, and others had mutated to ones with electricigensis activity to involve in electricity generations. Meanwhile, this group was not observed in Sun et al. [14], which employed sulfide as the sole electron donor. Firmicutes took the greatest proportion in Sample S due to high concentration of organic matters in anaerobic sludge. In present MFCs, glucose was also added, thus this group still appeared. Specially, the sequence type M-2 showed 100% similarity value with *Clostridium* sp., which was able to reduce sulfate to lower valencies [35].

4 Conclusions

It was found that electrochemical oxidation and biological oxidation were the main effects for sulfide removal in MFCs with sulfide and glucose as the complex substrate. Community analysis showed a great diversity of bacteria on the anode surface, including the exoelectrogenic bacteria and sulfur-related bacteria, responsible for the effective electricity generation and sulfide oxidation in the MFCs. Moreover, the exoelectrogenic bacteria and sulfur-related bacteria were present in greater abundance than those in the MFCs fed with only sulfide. The results were helpful to reveal the interactions between the pollutants and microbes in the MFCs.

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Captions for Figures

Figure 1. Voltage outputs of the MFCs with 1000 Ω external resistance during four operating cycles (arrows indicating the replacement of the electrolytes).

Figure 2. Variations of sulfide concentration with time in proposed MFCs during the four different processes.

Figure 3. Proportions of different effects for sulfide removal in the proposed MFCs during the 72 h operation.

Figure 4. Proportions of each phylotype in Sample S (a) and Sample M (b) clone library.

Figure 5. Phylogenetic tree of bacteria in Sample S libraries based on 16S rDNA sequences data (Numbers in parentheses are the GenBank accession numbers; The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 re-sampled datasets).

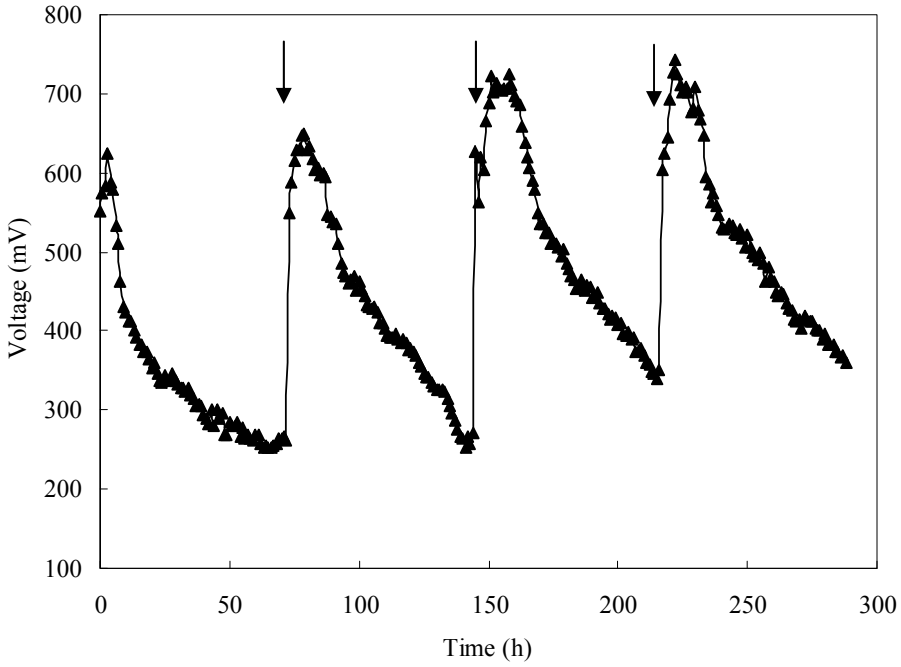
800
700
600
500
400
300
200
100
Voltage (mV)
0
50
100
150
200
250
300
Time (h)

Figure 1

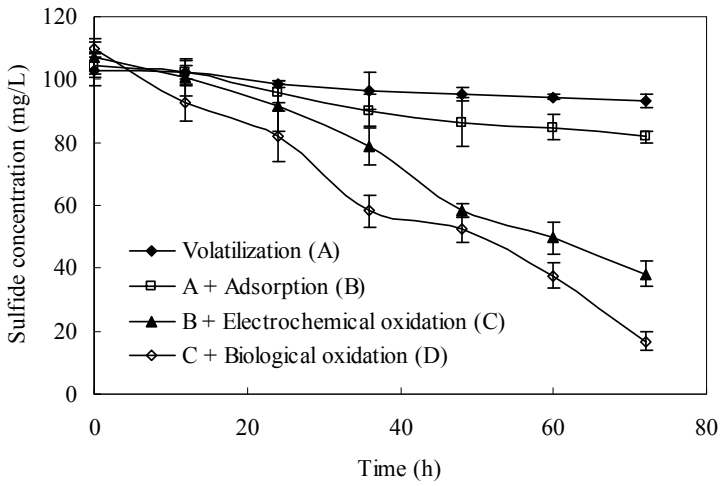
120
100
80
60
40
20
0
Sulfide concentration (mg/L)
0
20
40
60
80
Time (h)
Volatilization (A)
A + Adsorption (B)
B + Electrochemical oxidation (C)
C + Biological oxidation (D)

Figure 2

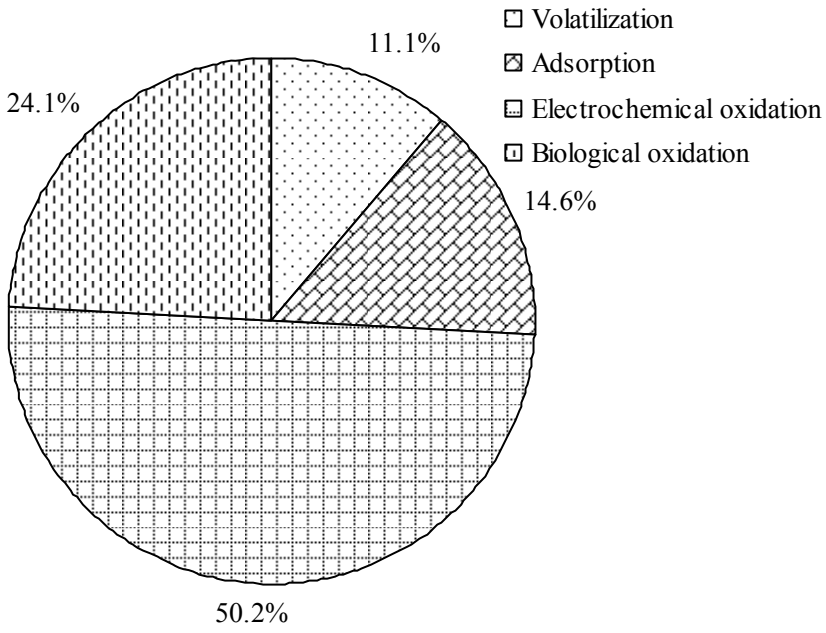
Volatilization
Adsorption
Electrochemical oxidation
Biological oxidation
11.1%
14.6%
50.2%
24.1%

Figure 3

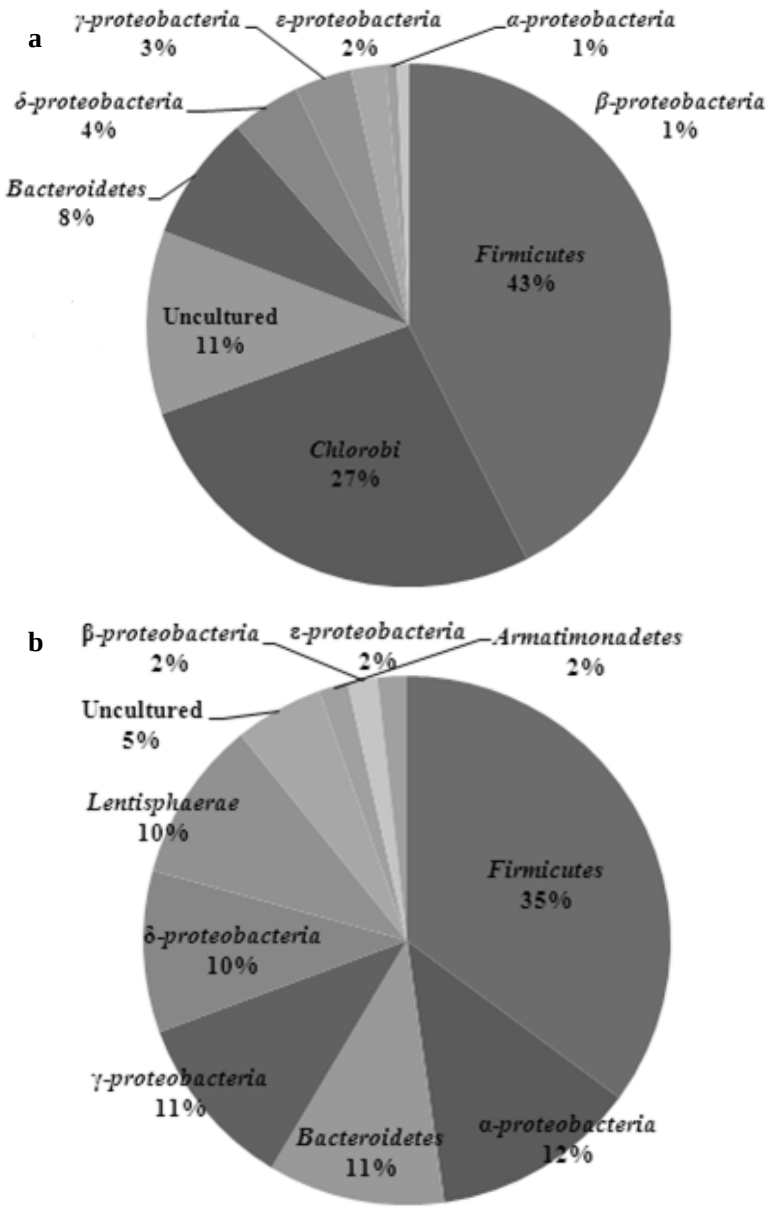
a
γ-proteobacteria
3%
ε-proteobacteria
2%
α-proteobacteria
1%
δ-proteobacteria
4%
β-proteobacteria
1%
Bacteroidetes
8%
Firmicutes
43%
Uncultured
11%
Chlorobi
27%
b
β-proteobacteria
2%
ε-proteobacteria
2%
Armatimonadetes
2%
Uncultured
5%
Lentisphaerae
10%
Firmicutes
35%
δ-proteobacteria
10%
γ-proteobacteria
11%
Bacteroidetes
11%
α-proteobacteria
12%

Figure 4

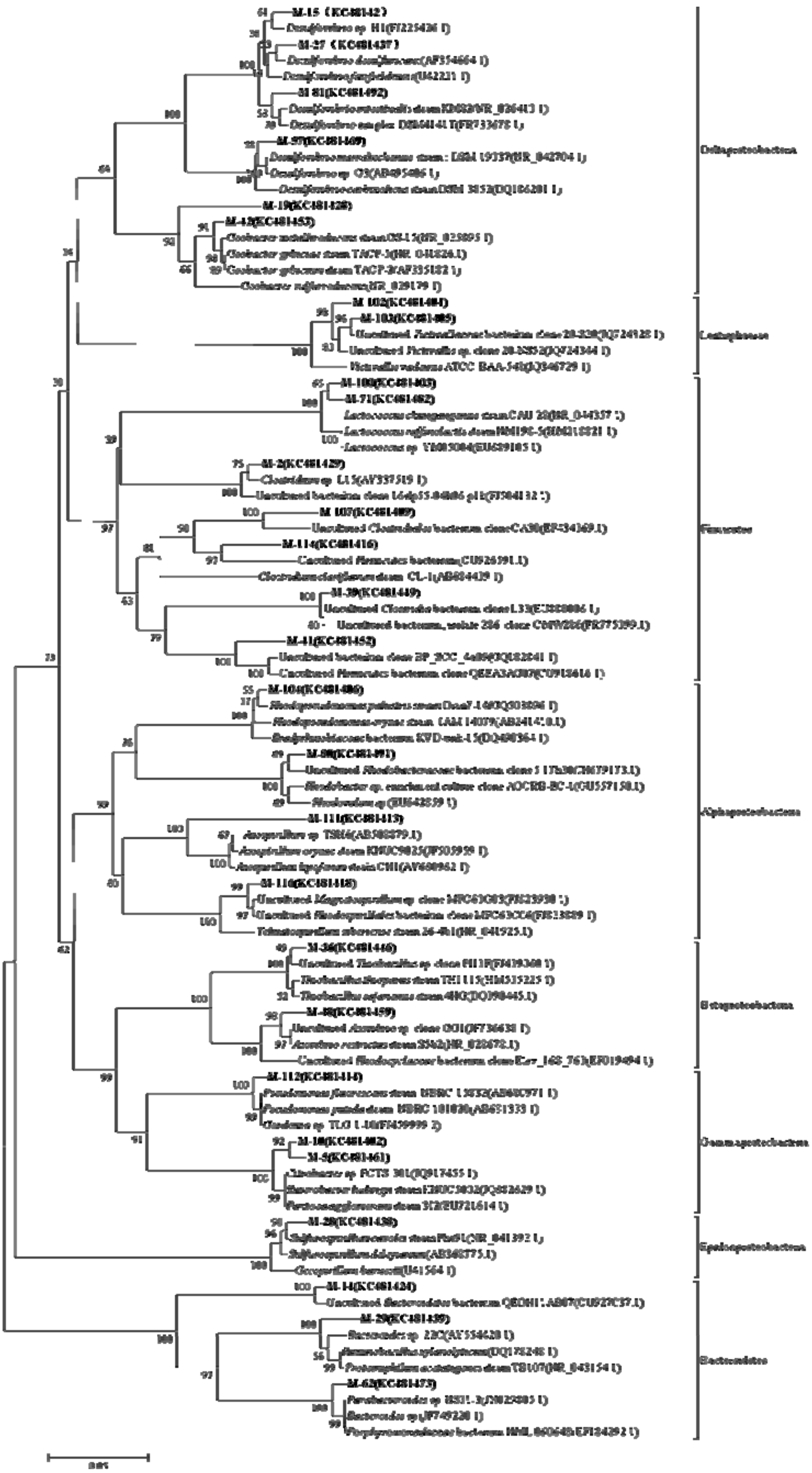

Figure 5