Anodic ammonia oxidation to nitrogen gas catalyzed by mixed biofilms in bioelectrochemical systems

Guoqiang Zhan, Lixia Zhang, Yong Tao, Yujian Wang, Xiaoyu Zhu, Daping Li

A R T I C L E   I N F O

Article history:
Received 4 March 2014
Received in revised form 9 May 2014
Accepted 9 May 2014
Available online 20 May 2014

Keywords:
Microbial anode electrochemical activity Ammonia oxidation nitrogen removal

A B S T R A C T

In this paper we report ammonia oxidation to nitrogen gas using microbes as biocatalyst on the anode, with polarized electrode (+600 mV vs. Ag/AgCl) as electron acceptor. In batch experiments, the maximal rate of ammonia-N oxidation by the mixed culture was ~ 60 mg L⁻¹ d⁻¹, and nitrogen gas was the main products in anode compartment. Cyclic voltammetry for testing the electroactivity of the anodic biofilms revealed that an oxidation peak appeared at +600 mV (vs. Ag/AgCl), whereas the electrode without biofilms didn’t appear oxidation peak, indicating that the bioanode had good electroactivities for ammonia oxidation. Microbial community analysis of 16S rRNA genes based on high throughput sequencing indicated that the combination of the dominant genera of Nitrosonomas, Comamonas and Paracoccus could be important for the electron transfer from ammonia oxidation to anode.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Ammonia-N pollution, which can cause eutrophication and be toxic to aquatic species, is usually converted into nitrogen gas via anaerobic ammonium oxidation (ANAMMOX) or nitrification/denitrification [1]. However, additional nitrite-N is necessary for anammox reaction and the cultivation of the anammox bacteria is difficult [2]. Besides, the nitrification/denitrification process need two stages starting with nitrification, which is the aerobic oxidation of ammonia-N to nitrite-N or nitrate-N, followed by heterotrophic denitrification under anaerobic conditions [3].

Although the electrochemical method has been shown effective for ammonia removal from wastewater [4], the catalytic oxide materials are confined to alloy electrode. Different anodic materials such as Pt [5], boron-doped diamond (BDD) [6] and dimensionally stable anode (DSA) [7,8], have been extensively assessed. What’s more, the rigorous reaction conditions and the expensive electrode materials limit its application in wastewater treatment. Therefore, it is necessary to develop a low-cost electrochemical system that could removal nutrients under mild conditions.

Recently, bioelectrochemical systems (BESs) have been proposed as a promising alternative for contamination removal, energy generation and biosynthesis because of sustainable bacterial metabolic reactions [9]. In the device, the bioanode is the terminal electron acceptor for substrates oxidation including glucose [10], acetate [11] and sulfur [12]. However, to date, there is no report on ammonia oxidation to nitrogen gas with microbial anode as electron acceptor. In fact, the Gibbs free energy of ammonia is high than that of the nitrogen molecule, so ammonia has the potential to be oxidized to nitrogen gas [13]. Furthermore, recent researches have pointed out that potential control is an effective method to domesticate electroactive biofilms [14] and control substrates oxidation [15,16]. Therefore, the study on microbially catalyzed anode ammonia oxidation controlled by potential is feasible and indispensable.

In this study, the characteristic of ammonia oxidation was investigated in a BES with polarized microbial anode (+600 mV vs. Ag/AgCl) as electron acceptor. The electroactivity of the biofilms was determined by cyclic voltammetry test. In addition, the microbial community of the bioanode was analyzed through 16S rRNA genes analysis based on high throughput sequencing, which was responsible for ammonia oxidation to nitrogen gas with anode as electron acceptor.

2. Experimental

2.1. BESs setup and operation

The BES used in this study consisted of two compartment (made of polymethyl methacrylate), physically separated by an anion exchange membrane (AEM, Zhejiang Qianqiu Co., Ltd, anion
exchange capacity 1.9 mmol g⁻¹). Gas vent was connected on the top of each chamber (5 cm × 5 cm × 6 cm) for collecting gas. Both anode (working electrode) and cathode (counter electrode) electrodes were carbon felt of 4.0 cm × 4.0 cm. Both chambers were filled with 130 ml medium containing the following components (per liter of distilled water): 0.14 g K₂HPO₄, 3.2 g Na₂HPO₄·12H₂O, 2.0 g NaHCO₃, 0.1 g MgSO₄·7H₂O, 0.01 g CaCl₂ and 1.0 ml of trace mineral mix [17]. In addition, the anode compartment was added 10–30 mM NH₄Cl (140–420 mg L⁻¹ ammonia-N). Both chambers were flushed with argon gas for 20 min to strip out the dissolved air, and then connected to a potentiotstat. Ag/AgCl (sat. KCl, 0.197 V vs. SHE) electrode was used as reference electrode. The pH of the two chambers was maintained at 7.7 ± 0.2 by adding NaHCO₃ and 1 mM HCl. The abiotic control experiments were also performed under closed circuit conditions without microbial culture while the biotic control experiments were carried out under open circuit conditions.

The mixed culture inoculated in the anode chamber was originated from a wastewater treatment plant of Chengdu, China. In order to produce hydrogen at cathode, the mixed biofilms were acclimated with anode potential set to +600 mV (vs. Ag/AgCl). The domestication of biofilms was monitored by measuring the change of ammonia-N in the anode compartment. The performance of nitrogen removal was repeated several times at 28 ± 2 °C until obvious and consistent ammonia removal was determined.

2.2. Analytical techniques

NH₄⁺-N, NO₂⁻-N and NO₃⁻-N were measured regularly according to the standard methods for the examination of water and wastewater [18]. N₂ and O₂ were analyzed using gas chromatography with thermal conductivity detector (GC-1690), with a chromatographic column of 2 m length × 3 mm filled with 13X molecular sieves as a separating column. Loading gas: high-purity helium; Flow rate of loading gas: 50 ml/min; Column temperature: 30 °C; Inlet temperature: 60 °C; Detector temperature: 80 °C.

The current of working electrode was collected every 50 s by CHI 1000 C with a Power Laboratory 85P unit connected to a computer. Cyclic voltammetry measurements were performed with an electrochemical working station (ESC550, China) at a scan rate of 10 mV s⁻¹ in the potential range from 0.8 V to -0.6 V. Micrographs were taken by the scanning electron microscope (S-4800, Japan) as our previous described [14]. The Coulombic efficiency (CE) was calculated to address the recovery of electron as electrical current from the oxidation of ammonia to nitrogen as described in our previous study [19].

2.3. DNA extraction and MiSeq sequencing of 16S rRNA gene amplicons

DNA density and quality were checked using a NanoDrop Spectrophotometer. Extracted DNA was diluted to the concentration of 10 ng µl⁻¹ and stored at -40 °C for concentration use. Universal primers 515F (5′-GTGCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCA-3′) with Illumina barcodes were used to amplify the V4 hypervariable regions of 16S rRNA genes for pyrosequencing using MiSeq sequencer [20,21]. The PCR mixture (50 µl) contained 1× PCR buffer, 1.5 mM MgCl₂, each deoxynucleoside triphosphate at 0.4 µM, each primer at 1.0 µM and 1 U of TransStart Fast Pfu DNA Polymerase (TransGen, China) and 10 ng genomic DNA. The PCR amplification program included initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 40 s, 56 °C for 60 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were subjected to electrophoresis using 1.0% agarose gel. The band with a correct size was excised and purified using Gel Extraction Kit (Omega Bio-tek, USA) and quantified with Nanodrop. The sequencing samples were prepared using TruSeq DNA kit according to manufacturer’s instruction. The purified library was diluted, denatured, re-diluted, mixed with PhiX (equal to 30% of final DNA amount) as described in the Illumina library preparation protocols, and then applied to an Illumina MiSeq system for sequencing with the Reagent Kit v2 2 × 250 bp as described in the manufacturer manual.

2.4. Data analysis

The sequence data were processed using QIIME Pipeline—Version 1.7.0 (http://qiime.org/tutorials/tutorial.html). All sequence reads were trimmed and assigned to each sample based on their barcodes. Multiple steps were required to trim the sequences, such as removal of sequences < 150 bp and average base quality score Q > 30. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier at a confidence level of 80% (http://pyro.cme.msu.edu/).

3. Results and Discussion

3.1. Ammonia oxidation catalyzed by the anode biofilms

Fig. 1 showed the characteristic of ammonia oxidation at anode with or without microbes as catalyst. Specifically, ammonia was oxidized into nitrogen gas without nitrite and nitrate accumulation in anode compartment with the presence of bacteria (Table 1 and Fig. 1a), and the nitrogen gas content increased from 34.1 ± 0.2% to 95.2 ± 0.5% when the concentration of ammonia-N increased from 140 mg L⁻¹ to 420 mg L⁻¹ (Fig. 1b). The maximal rate of ammonia-N oxidation by the mixed culture was ∼ 60 mg L⁻¹ d⁻¹, and the ammonia-N removal efficiency reached 41.3 ± 3.2%, 55.9 ± 5.5% and 47.7 ± 3.0% with different initial ammonia-N concentrations at 140 mg L⁻¹, 280 mg L⁻¹ and 420 mg L⁻¹, respectively (Table 1). In contrast, there was almost no ammonia removal and no nitrogen gas generation (Fig. 1a and b) in the absence of microbes (abiotic experiments). During the experiments, ammonia was not transferred into cathode because of the presence of anion exchange membrane. In addition, there were also no ammonia, nitrite and nitrate detection in the cathode (data not show). What’s more, oxygen was not detected in gas composition in all the experiments including the abiotic contrast experiments (Table 1). Although the theory oxygen evolution potential shifts to 0.57 V (vs. Ag/AgCl) at pH 7.7, which indicates that oxygen could have been generated at the potential examined here (0.6 V vs. Ag/AgCl) and have immediately been scavenged by the biofilms, the generated oxygen (in the form of dissolved oxygen) is insufficient to consume the metabolic ammonia. Here, we assume the saturated dissolved oxygen is 7.9 mg L⁻¹ at 28 °C. In the abiotic experiments, there is no oxygen detection in the gas phase, so the maximum oxygen production may be 7.9 mg L⁻¹ (0.25 mM) in the solution, which only oxidizes 4.62 mg L⁻¹ ammonia-N (0.33 mM) to nitrogen gas, 2.38 mg L⁻¹ ammonia-N (0.17 mM) to nitrite and 1.75 mg L⁻¹ ammonia-N (0.125 mM) to nitrate. However, according to Table 1, in this study the total amount of removal ammonia-N reached 57.8 ± 4.5 mg L⁻¹, 156.5 ± 15.4 mg L⁻¹ and 200.3 ± 12.6 mg L⁻¹, and the efficiency of ammonia-N transformation into N₂ reached 85%, 82% and 69% with different initial ammonia-N concentrations at 140 mg L⁻¹, 280 mg L⁻¹ and 420 mg L⁻¹, respectively. Therefore, ammonia oxidation mainly happened with the anode as the electron acceptor catalyzed by microorganisms for nitrogen gas production.

In order to further determine ammonia oxidation with anode as electron acceptor, the generating current was evaluated at different
concentrations of ammonia. The maximal current increased from 0.51 mA to 1.35 mA with the initial ammonia-N concentration range from 140 mg L\(^{-1}\) to 420 mg L\(^{-1}\) (Fig. 2). However, the current was fairly low without ammonia addition. These results demonstrate that ammonia can be served as an alternative electron donor for electricity production, which is consistent with the results of our previous report [19] and He et al. [22], but in this study, we firstly report nitrogen gas production in the process of anodic ammonia oxidation catalyzed by microbes.

The CE is an effective parameter for evaluating the ability of substrate oxidation with electrode as electron acceptor. During 5 days, the CE reached 79.6 ± 2.0%, 43.5 ± 1.5% and 53.4 ± 3.6% with initial ammonia-N concentration at 140 mg L\(^{-1}\), 280 mg L\(^{-1}\) and 420 mg L\(^{-1}\), respectively (Table 1), indicating that the electron released from ammonia oxidation can be retrieved as electrical current. However, there are maybe other pathways for anodic ammonia removal because the CE decreases when ammonia concentration increases, which will be explained in the following section of microbial community analysis.

### 3.2. Electrochemical activities of the mixed biofilms

The electrochemical activities of the mixed biofilms for ammonia oxidation were investigated by cyclic voltammetry (CV). An oxidation peak of anode with biofilms presented at 0.6 V (vs. Ag/AgCl) in medium containing 280 mg L\(^{-1}\) ammonia-N while the abiotic anode with 280 mg L\(^{-1}\) ammonia had no oxidation peak (Fig. 3a). In addition, the oxidation peak of bioanode became inconspicuous when there was no ammonia addition (Fig. 3a). Furthermore, the CV with different ammonia concentrations was also performed to identify the electrochemical activities of the mixed biofilms. Fig. 3b showed that the peak currents increased from 2 mA to 5.5 mA when ammonia-N concentration increased from 140 mg L\(^{-1}\) to 420 mg L\(^{-1}\). The changes of current indicate that the substrate concentrations have obvious effect on microbial metabolism. The microbial metabolism includes at least two processes [23]: (1) biochemical oxidation of a substrate, which occurs inside the microorganism and yields the products and the released electrons; (2) the heterogeneous electron transfer to the electrode, which occurs via a redox component from the microorganism to

#### Table 1

<table>
<thead>
<tr>
<th>NH(_4^+)-N/mg L(^{-1})</th>
<th>NO(_2^+)-N/mg L(^{-1})</th>
<th>NO(_3^+)-N/mg L(^{-1})</th>
<th>N removal/%</th>
<th>CE/%</th>
<th>(\Delta)NH(_4^+)-N/mg L(^{-1})</th>
<th>N(_2)/mL</th>
<th>O(_2)/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>280(^{a})</td>
<td>N.D</td>
<td>N.D</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>0(^{b})</td>
<td>N.D</td>
<td>N.D</td>
<td>0</td>
<td>0</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>140(^{a})</td>
<td>N.D</td>
<td>N.D</td>
<td>41.3 ± 3.2</td>
<td>79.6 ± 2.0</td>
<td>57.8 ± 4.5</td>
<td>5.1 ± 0.2</td>
<td>N.D</td>
</tr>
<tr>
<td>280(^{a})</td>
<td>N.D</td>
<td>N.D</td>
<td>45.9 ± 5.5</td>
<td>43.5 ± 1.5</td>
<td>156.5 ± 15.4</td>
<td>13.3 ± 0.4</td>
<td>N.D</td>
</tr>
<tr>
<td>420(^{a})</td>
<td>N.D</td>
<td>N.D</td>
<td>47.7 ± 3.0</td>
<td>53.4 ± 3.6</td>
<td>200.3 ± 12.6</td>
<td>143.4 ± 0.5</td>
<td>N.D</td>
</tr>
<tr>
<td>280(^{a,b})</td>
<td>N.D</td>
<td>N.D</td>
<td>8.6 ± 2.2</td>
<td>-</td>
<td>240.0 ± 6.2</td>
<td>N.D</td>
<td>N.D</td>
</tr>
</tbody>
</table>

\(^a\) Abiotic experiments without biofilms. \(^b\) Biotic experiments with biofilms.

\(^a\) Closed circuit. \(^b\) Open circuit. N.D: no detection.
from Fig. 3, the oxidation-reduction peaks appeared at about 0.6 and 0.3 V (vs. Ag/AgCl), respectively and the peak potentials were similar to that of Geobacter sulfurreducens which was also polarized at 0.6 V (vs. Ag/AgCl) [25]. The electrochemical detection of redox signals in Fig. 3 clearly showed that some elements of the cell surface were close enough to the electrode to undergo the electron transfer. It is possible that the electrochemical activities for anode extracellular electron transfer from biofilms to electrode are mediated by the cell surface cytochromes [26]. In order to determine the biofilms on the anode, a piece of carbon felt in the anode was sampled and analyzed by scanning electron microscope (SEM) after batch experiments. Compared with aseptic experiment (Fig. 4b), the bioanode was covered with bacillus (Fig. 4a), which further demonstrated the formation of biofilms on the anode. In this study, a well-defined anodic peak appears at 0.6 V (vs. Ag/AgCl) which is lower than that on BDD electrode at 1.16 V (vs. Ag/AgCl) [6], showing that the bioanode has good electroactivities for ammonia oxidation.

3.3. Microbial community structure of the anode biofilms

Microbial community analysis of 16S rRNA genes based on high throughput sequencing showed the bacterial diversity of the anode biofilms at initial acclimation stage (acclimation) and stable ammonia removal stage (stable phase) (Fig. 5). At the early stage the majority of dominant populations were affiliated with Thermomonas (33.86%) and Fontibacter (6.46%). In addition, some minority populations such as Nitrosomonas (1.54%), Paracoccus (1.17%) and Comamonas (1.14%) were also involved in the anode biofilms. However, the majority of dominant populations belonged to Stenotrophomonas (13.07%), Nitrosomonas (12.89%), Comamonas (10.79%) and Paracoccus (10.56%) at the stable stage of ammonia oxidation (3 month later than the early acclimation). No evidence for the presence of anammox bacteria was seen at the biofilms. Generally, biologic ammonia oxidation into nitrogen gas need two-stage nitrification and denitrification except one-stage anammox reaction. Here, we achieved a novel nitrogen removal coupling to electricity production in a bio-electrochemistry system without anammox bacteria. In this study, the genus Nitrosomonas may play an important role in oxidizing ammonia and releasing electron to the anode. Schmid and Bock [27,28] have demonstrated that Nitrosomonas europaea is able to anaerobically oxidize ammonia using nitrite as the acceptor, which suggests that oxygen is not indispensable for ammonia oxidation. He et al. [22] have pointed out that Nitrosomonas europaea can transfer electrons to the anode. Besides, Qu et al. [29] have also pointed out that the genus Nitrosomonas are highly enriched on the anode, as well as the genus Empedobacter which enable the genus Nitrosomonas to achieve extracellular electron transfer for ammonia oxidation with electrode as the electron
accepotor. Here, the genus *Nitrosomonas* was highly enriched and dominated the microbial community on the anode, as well as denitrifying bacteria. Thus, nitrogen removal and ammonia oxidation without nitrite or nitrate accumulation could be elucidated by the ammonia-oxidizing bacteria (*Nitrosomonas*) and denitrifying bacteria (*Comamonas* and *Paracoccus*). Unfortunately, the CE over a period of 5 days was not high according to Table 1. The recovery of electron is affected by many factors, such as biomass growth, substrate lost and mixed culture. Pure culture tests have reported CE as high as 98.6% [30]. Therefore, the use of selected bacteria may increase electron recovery where such systems can be attained under sterile conditions. Qu et al. [29] have demonstrated that the reduced CE may be due to the soluble microbial products which are produced by nitrifiers to support heterotrophic growth. Here, the most possible reasons for the reduced CE may be that the ammonia-assimilating microorganism (*Stenotrophomonas* sp.) assimilated part of the ammonia, which reduced the amount of ammonia for releasing electrons to the electrode. Table 1 showed that the amount of ammonia removal from solution reached 24.0 ± 6.2 mg l⁻¹ under open circuit conditions, and the removal efficiency of ammonia-N reached 8.6 ± 2.2%. Although *Stenotrophomonas* sp. has been reported to assimilate ammonia from cattie manure composting [31], the competitive mechanism of ammonia-oxidizing bacteria and ammonia-assimilating microbes is unclear in the presence of electric field. Therefore, further research should be carried out for improving the function of ammonia-oxidizing bacteria and enhancing CE by adjusting the electrode potential. Moreover, the specific extracellular electron transfer mechanisms involved in anodic ammonia oxidation catalyzed by mixed culture also need to be identified.

4. Conclusions

To our knowledge, this study firstly reports the ammonia oxidation to nitrogen gas at anode catalyzed by microorganism. The tests of CV reveal that a well-defined anodic peak appears at +600 mV (vs. Ag/AgCl), indicating that some elements of the cell surface are close enough to the electrode to undergo the electron transfer. Microbial community analysis of 16S rRNA genes based on high throughput sequencing demonstrates that the combination of the dominant genera of *Nitrosomonas*, *Comamonas* and *Paracoccus* could be of importance for nitrogen removal and the electron transfer from ammonia oxidation to anode. The findings provide a novel insight into the pathway of biologic ammonia oxidation, as well as a new method for ammonia-N removal from wastewater.

**Acknowledgements**

This work was supported by the National Natural Science Foundation of China (No. 31270166), Western Light Talent Culture Project (No. Y3CS031), and China Postdoctoral Science Foundation (No. 2013MS42299), as well as Key Laboratory of Environmental and Applied Microbiology, Chengdu Institute of Biology, Chinese Academy of Sciences (No. KLCAs201203).

**References**


