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Short communication

Enrichment of *Clostridia* during the operation of an external-powered bio-electrochemical denitrification system

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ABSTRACT

Bacterial community composition was investigated in a biocathode of an external-powered bio-electrochemical denitrifying system (BEDS), by analyzing 16S rRNA gene sequences determined from a high-throughput 454 pyrosequencing technology. The bacterial community of the inoculum sludge was mainly composed of *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria*. Over 45 days of the BEDS, *Bacteroidetes* and *Firmicutes* were enriched on the biocathode. Putative denitrifying *Clostridia* belonging to the *Firmicutes* were distinctively detected on the biocathode via phylogenetic analysis and appeared to be ubiquitous in various autotrophic, heterotrophic, and BEDSs. Elucidation of the bacterial community composition in the denitrification system deepens our knowledge about the bacteria involved in bio-electrochemical denitrification.

1. Introduction

Contamination of groundwater or surface water by nitrate ions (NO_3^-) can result in serious problems in human health and aquatic ecosystems; thus, proper treatment of nitrate is an important consideration [1–3]. Nitrate removal methods include ion exchange, reverse osmosis, activated carbon adsorption, and biological denitrification [4]. Biocathodic nitrate reduction is an alternative nitrate removal technology that uses the activity of denitrifying microorganisms [5]. The microorganisms, which are attached to a cathode electrode, are able to reduce nitrate into nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2), in sequential order, by using the electrons released from an anodic chamber. Biocathodic nitrate reduction can be carried out in two ways – via denitrifying microorganisms (microbial fuel cell (MFC) bio-electrochemical denitrification system (BEDS)) [6,7] or voltage induced electrolysis of water in an anodic chamber (external-powered BEDS) [4]. In both methods, the intensity of electrical current generated is an important consideration because it exhibited a linear relationship with the rate of denitrification [8]. Generation of a stable electrical current in MFC BEDS is limited because it relies solely on microbial activity [9]. In comparison, external-powered BEDS uses an external power source to regulate electrical current; thus, it is more flexible. This feature of external-powered BEDS can provide efficient and controllable denitrification [4].

In BEDS, the nitrate reduction rate is dependent on the activity of denitrifying microorganisms, as well as the applied (or generated) electric current. Therefore, it is necessary to investigate the denitrifying microorganisms formed on the biocathode to better understand BEDSs. Nevertheless, very little information is available on the microbial community compositions in BEDSs. The main objective of this study was to explore the microorganisms involved in the external-powered BEDS. We operated a laboratory-scale external-powered BEDS, and traced the bacterial community by analyzing 16S rRNA gene sequences to identify major phylotypes in the BEDS.

2. Materials and methods

2.1. Operation of an external-powered BEDS and chemical analysis

The external-powered BEDS used in this study was an H-type two chamber system with a working volume of 200 mL, similar to a previous study [10]. The anode and cathode chambers were separated by a pretreated proton-exchange membrane (diameter 15 mm, Nafion 117, Nara Cell-tech, Seoul, Korea). Membrane pretreatment was carried out by sequentially soaking it in boiled deionized water, H_2O_2 (30%, v/v), deionized water, 0.5 M H_2SO_4, and deionized water, with an incubation time of 1 h for each step [11]. The anode and cathode electrode (22.4 cm^2) were prepared using flat carbon paper (Toray, Tokyo, Japan). The electrical connections to the electrode were established using copper wire, and sealed by applying nonconductive

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epoxy resin (Hardex, Kuantan, Malaysia). Biofilm formation on the cathode electrode was initiated by inoculating anaerobic digester sludge collected from the Giheung Respia wastewater treatment plant (Yongin, Korea), with 2 g/L $NaHCO_3$. The cathode chamber was filled with 50 mg nitrate-N/L and 100 mM phosphate buffer solution (8.44 g/L $NaH_2PO_4 \cdot H_2O$, 5.5 g/L $Na_2HPO_4 \cdot H_2O$) by adjusting to pH 7.0 with 1 N NaOH. The anode chamber was maintained in abiotic condition using the same phosphate buffer. Both anode and cathode solutions were continuously stirred at 160 rpm using magnetic stirrers, and maintained at 30 ± 1 °C in a temperature-controlled incubator (Vision Scientific Inc., Daejeon, Korea). Both chambers were purged with argon gas (99.9% purity) before being sealed. An external cell voltage (0.7 V) was supplied to the BEDS from a DC power source. The solution of both chambers was replaced by fresh solution four times during 45 days of operation. For the evaluation of nitrate reduction of the BEDS, nitrite, nitrate, and ammonia measurements were taken every 48 h, following Standard Methods [12]. Coulombic efficiency (CE) of the BEDS was calculated by modifying a previous method for a MFC BEDS [10,13]. CE was determined as $CE = (C_R/C_A) \times 100\ (\%)$, where C_R is the total amount of coulombs over time for the reduced forms of nitrogens (i.e., N_2, NO_2^-, and NH_4^+) in the cathode chamber and C_A is the total coulombs of current applied to the BEDS.

2.2. Biomass sampling, DNA extraction, PCR, and pyrosequencing

To trace bacterial community changes in the external-powered BEDS, inoculum anaerobic digester sludge (0 day), cathode suspended biomass (45 day), and cathode biofilm (45 day) were collected. For the biofilm sample, a cathode sheet was rinsed in sterilized deionized water three times and then cut into ~20 pieces (total area = 1 cm^2) with sterilized scissors. The total DNA was extracted from the three biomass samples (with a duplicate of each) using the MoBio PowerSoil DNA extraction kit (Solana Beach, CA, USA) and following the manufacturer's protocol. 16S rRNA gene sequences were PCR-amplified from the six DNA samples (~20 ng) using the bacterial universal primer set 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′). The PCR condition and the purification method of PCR products were as described in a previous study [14]. Barcode 454 pyrosequencing was conducted at Macrogen (Seoul, South Korea), as previously described [14]. Briefly, after ligating different multiplex identifiers (14-bp long) to each sample's PCR product, six single-stranded DNA libraries were constructed. The DNA libraries were then attached to DNA capture beads, and followed emulsion-based clonal amplification. For the DNA sequences attached on the beads, pyrosequencing reactions were conducted in the Pico TiterPlate device, using the Genome Sequencer FLX Titanium Series (454 Life Science, Branford, USA).

2.3. Analyses of sequence data

A customized Perl script, the trimBarcode.pl (Macrogen), was used to sort the 16S rRNA gene sequences by sample, remove low quality (<Q20) and short (<250 bp long) sequences, and trim primer and multiplex identifier sequences. Potential chimeric sequences were further removed using chimera check algorithms in Mothur utility [15]. Taxonomic assignment of the sequences was conducted by the RDP's Classifier, and Chao1 richness estimator was calculated by Mothur utility. Detailed procedures for the sequence refinement, taxonomic assignment, and richness estimation were as described previously [14]. Phylogenetic association of the sequences was assessed by constructing a neighbour joining tree. Sequences obtained in this study were multiply aligned with reference sequences using the RDP's Pyrosequencing Aligner (http://pyro.cme.msu.edu/), and unaligned regions were trimmed. Evolutionary distances were calculated from the aligned sequences by Kimura's 2-parameter [16], and a neighbour joining tree was constructed based on distance calculation using the MEGA program [17].

3. Results and discussion

3.1. Performance of the external-powered BEDS

The applied voltage of 0.7 V of the external-powered BEDS was greater than the theoretical cell voltage for the reduction of nitrate to dinitrogen in conjunction with water oxidation to oxygen (−0.07 V at standard condition and neutral pH [17]). Yet, the voltage was less than that for reduction of proton to hydrogen in conjunction with water oxidation to oxygen (+1.23 V at standard condition and neutral pH [17]). The measured cathodic and anodic electrode potentials were −0.3 V and +0.4 V versus Ag/AgCl, respectively. After the third replenishing of the chambers with fresh solution, denitrification performance of the external-powered BEDS was evaluated from days 25 and 35. The BEDS clearly demonstrated the removal of nitrate (Fig. 1). During the operational period, nitrate reduced gradually from 50 to 9.5 mg NO_3^--N/L (81% removal), which corresponded to 0.175 mg NO_3^--N/cm^2 (cathode surface area) d or 4.05 g NO_3^--N/m^3 (cathode chamber volume) d.

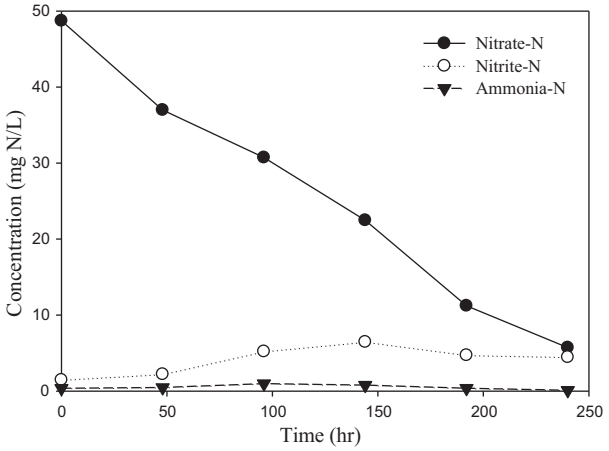

Fig. 1. Nitrate-, nitrite-, and ammonia-nitrogen profile observed in the external-powered BEDS from day 25 to day 35.

Concurrent with nitrate removal, nitrite and ammonia built up to 4.5 and 0.1 mg N/L, respectively. The rest of the nitrogen likely converted into dinitrogen, which was the primary gas compound in off-gas (data not shown). Mass balance calculation estimated 70% nitrate conversion into dinitrogen. The nitrate removal rate of the external-powered BEDS was comparable to that of a similar study. Park et al. [4] operated an external-powered BEDS with hydrogen producing conditions and obtained 0.17 NO_3^--N/cm^2 d. The nitrate removal rate was also compared with that of MFC BEDSs. Fang et al. [10] operated an MFC biocathodic nitrate reduction system for 168 h and observed only 22% nitrate removal and a 0.0436 NO_3^--N/cm^2 d nitrate removal rate. Jia et al. [18] operated a similar MFC system for 192 h and obtained 58% nitrate removal. The University of Queensland [9] and the Ghent University research groups [19] reported much higher rates; however, these rates were expressed in volumetric nitrate removal rate (410–500 g NO_3^--N/m^3 d). In terms of electron transfer efficiency, the external-powered BEDS had 75% CE. Considering the CE of MFC ranged from 8 to 82% [20], the CE of the external-powered BEDS was relatively high. On the other hand, there is a possibility that the nitrate reduction was partly achieved electrochemically because nitrate can be reduced without a biofilm or suspended microorganisms in the external-powered BEDS [21].

3.2. Bacterial community structure of the external-powered BEDS

From the external-powered BEDS, the bacterial community was traced by analyzing 16S rRNA gene sequences determined from a high-throughput 454 pyrosequencing. Using the sequencing method, a total of 4657 good quality sequences were obtained from the six samples (duplicates of inoculum sludge, cathode suspended biomass, and cathode biofilm). Changes in bacterial community were noticeable during the operation of the BEDS (Fig. 2 and Table 1). The bacterial community of the inoculum sludge (0 day) was mainly composed of *Proteobacteria* (27.7%), *Bacteroidetes* (21.1%), *Acidobacteria* (18.1%), *Chlorobi* (6.0%), and *Nitrospira* (4.6%) at a 50% bootstrap cutoff. The bacterial community composition of the inoculum sludge was that of typical anaerobic digester sludge [14,22]. During 45 days of operation, the bacterial community had been changed into *Bacteroidetes* (52.8%), *Firmicutes* (30.1%), and *Proteobacteria* (12.2%) in the cathode biofilm, and *Bacteroidetes* (70.1%), *Proteobacteria* (19.1%), and *Firmicutes* (5.9%) in the cathode suspended biomass. *Proteobacteria* decreased its proportion both in the cathode biofilm (27.7% → 12.2%) and in the cathode suspended biomass (27.7% → 19.1%). The proportion of *Bacteroidetes* and *Firmicutes* increased in the cathode biofilm (21.1% → 52.8%) and