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Physicochemical characteristics and microbial community of cultivated sludge for nitrate-dependent anaerobic ferrous-oxidizing (NAFO) process

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ABSTRACT

The nitrate-dependent anaerobic ferrous oxidizing (NAFO) is a newly discovered bioprocess, which is valuable to develop autotrophic denitrifying technology. In this work, the physicochemical characteristics and microbial community of the cultivated sludge (NAFO sludge) from a lab-scale reactor operating for over 200 days were investigated. A comparison between the seeding sludge and NAFO sludge was also carried out. The results showed that the NAFO sludge possessed distinctly high settleability, and the average settling velocity was 390.1 ± 161.3 m/h. The increasing particle size and density, with average values of 1469 ± 65 μm (surface area-weighed mean diameter, SMD)/2389 ± 132 μm (volumetric-weighed mean diameter, VMD) and 1580 ± 80 kg/m^3, were revealed to the main factors causing the profile of excellent settleability. High content of iron with peak value of 87.5% (wt) was detected in NAFO sludge, which was mainly in the form of oxide compounds, such as hematite and magnetite, endowing unique morphology characteristics of NAFO sludge. After the long-term operation, a microbial succession was supposed to occur in the granular sludge, and the dominant bacterial communities in the NAFO sludge were *Gamma-proteobacteria*, *Thermoleophilia*, *Clostridia* and *Alpha-proteobacteria* classes. The present work will benefit the development of NAFO technology, and also the separation and iron-recycling processes of the NAFO sludge.

1. Introduction

Nitrate pollution is a worldwide environmental issue, which could lead to eutrophication, infant methemoglobinemia, and potential threat to human or animal health [1,2]. Biological technology is a popular approach to treat nitrate-containing groundwater and wastewater, and it could be classified into heterotrophic and autotrophic processes depending on carbon source [3]. Autotrophic technology is gradually attracting more attention for obvious advantages, such as less operation cost and lower sludge production. What's more, it offers an effective method to treat wastewaters with high nitrogen content and low carbon content, i.e. low C/N molar ratios, which is known as a bottleneck for the heterotrophic technology [4,5].

Nitrate-dependent anaerobic ferrous oxidation (NAFO) is an autotrophic biological process, which could take ferrous ion as electron donor to convert nitrate into nitrogen gas. NAFO was reported for the first time in the mid of 1990s by Straub et al., and since then great strides were made in the fields of microbiology and geology in the past 20 years [6]. Most recently, an autotrophic denitrification technology based on NAFO process was developed to treat nitrate-containing wastewater [7,8]. The potential nitrate removal rate (NRR) could be as high as 4.42 kg-N/(m^3 d), which was considerable compared with other autotrophic denitrification processes (shown in Table S1 in supplementary material). In addition, the NAFO process has potential to remove nitrate and phosphorus simultaneously [8,9]. Hence, the NAFO process deserves to be developed further to reap the benefits of the technology.

Active sludge serves as a biocatalyst and plays a significant role in NAFO process. Characterizing the profiles of the cultivated sludge for NAFO is vital for the development of NAFO technology.

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So far, however, scarce information is available on the characteristics of NAFO sludge, especially on the microbial community and physicochemical aspects.

In the present work, the cultivated sludge from an UASB reactor operating for over 200 days were taken to examine the physicochemical characteristics and microbial community. The density, particle size, settleability, morphology, elemental content and compounds composition were chosen to demonstrate the physicochemical characteristics of the sludge. The Illumina sequencing was used to investigate the microbial community in the sludge. A comparison between the seeding sludge and cultivated sludge was carried out to illustrate the unique characteristics of the NAFO sludge.

2. Materials and methods

2.1. Seeding sludge and NAFO sludge

The seeding sludge was anaerobic granular sludge, taken from a full-scale internal circulation (IC) reactor treating wastewater in a paper mill. The total solids (TS) and volatile solids (VS) of the seeding sludge were 138.5 ± 0.9 g/L and 59.80 ± 1.2 g/L, respectively.

The NAFO sludge investigated in this work was taken from a lab-scale UASB reactor under steady state. The synthetic wastewater and configuration of the reactor were described in previous study [8]. The initial Fe/N molar ratio was 5.0. The initial pH (6.5 or so) was regulated by NaOH using a concentration of 2.0 mol/L. The temperature was controlled at 28 ± 1 °C during the experiment. The reactor was particularly designed for NAFO process and it remained in operation for over 200 days. The nitrate removal rate of the NAFO reactor was 115.0 ± 7.7 g m^{-3} d^{-1}, and the nitrate and ferrous removal efficiencies were 62.30% and 46.76%, respectively. The average consumed Fe/N molar ratio was 2.00 (Fig. S1 in supplementary material).

2.2. Particle size distribution and shape factors analysis

The seeding sludge and NAFO sludge were taken to examine the particle size distribution. Before the experiments, the sludge was washed three times with 0.9% NaCl solution. For each test, 5 mL sludge was taken randomly to analyze the particle size and shape factors, including aspect ratio, convexity and sphericity. The parameters related to particle size include surface area-weighed mean diameter (SMD) and volumetric-weighed mean diameter (VMD) [10]. All the parameters were examined using the QICPIC system (Sympatec, Germany) [11]. All the samples were run in triplicates.

2.3. Density and settleability measurement

The seeding sludge and NAFO sludge were taken to measure the density and settleability. The density was measured according to the method reported previously [12]. The settling velocity of granular sludge was measured using a glass-column, which was 50 cm in height so as to obtain the terminal settling velocity and 10.0 cm in internal diameter so as to minimize the wall effect on granule settlement. In addition, the procedures reported by Lu et al. were followed in the settling experiment [13].

2.4. Morphological analysis

The seeding sludge and NAFO sludge were taken to perform the morphological analysis. The morphological characteristics of sludge samples were observed with a stereoscope Discovery V8 (ZEISS, Germany) [11]. The NAFO sludge was further examined using a scanning electron microscopy (SEM) and Transmission electron microscope (TEM) for detailed analysis. The procedures for SEM and TEM were followed as reported by Tang et al. [14]. The SEM and TEM images were performed with the model SIRON (FEI, Holland) and JEM-1200EX (NEC, Japan).

2.5. Elements and compounds analysis

The NAFO sludge was taken to determine the elemental contents and compounds composition. The treatment of the sludge sample was the same as referred to for the SEM sample. Then, the element contents of the samples were analyzed using energy-dispersive X-ray spectroscopy (EDS) (FEG650 FEI, Holland). The chemical components in the samples were further determined using X-ray diffraction (XRD) (X'Pert PRO, PANalytical, Holland). The experimental data was analyzed using the software Jade 6.5.

2.6. Microbial community analysis

The seeding sludge and NAFO sludge were taken to analyze the microbial community. The DNA extraction was performed according to the instruction for users, and then stored at −20 °C for further PCR amplification. The primer pairs of 520F (5′-AYTGGGYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′) is used to amplify the bacterial 16S rRNA genes. DNA from each sample was amplified in a 25 μL reaction system, which consisted of 8.75 μL deionized and distilled water (DDW), 5.0 μL 5 × Q5 reaction buffering solution (Takara, Japan), 5.0 μL 5 × Q5 GC high enhancer, 2.0 μL dNTPs mixtures (2.5 mM)(Takara, Japan), 2 μL extracted DNA, 1 μL each of the primers (10 μmol/L)(Takara, Japan), 0.25 μL Q5 Polymerase (5U/μL)(Takara, Japan). The PCR used the following protocol: 98 °C for 5 min, 27 cycles at 98 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amplified products were checked on 2% (m/v) agarose, and purified with an SanPrep DNA purification kit (Sangon biotech., China). The purified PCR products were bidirectionally sequenced on the Illuminca Miseq pyrosequencing platform according to the manufacturer's protocol (Illumina, San Diego, CA, USA).

A bioinformatic analysis was performed using the Mothur software package (http://www.mothur.org) following the standard procedure [15]. The raw sequences obtained were initially screened by the barcodes and primers. Then, the sequences with the length less than 150 bp were excluded. The chimera sequences were removed by using the chimera.uchime command in the Mothur package [15,16]. After denoising and chimera inspection, the high-quality reads were obtained, and further used to calculate the operational taxonomic units (OTUs) using a 97% sequence-identity threshold. The high-quality reads were then aligned against the bacterial SILVA database, and each sequence was taxonomically classified. Each OTU was assigned by using the classifying OTU command in Mothur package. In addition, the diversity indices, such as Chao, ACE, Shannon and Simpson indexes of the samples were calculated as described in the manual of MOTHUR software. The raw sequence data generated in the present work using Illumina Miseq platform was deposited in GenBank under the Accession Number PRJNA282182.

3. Results

3.1. Settleability, density and particle size of the granular sludge

The setting velocity was chosen as an index of the settleability of the granular sludge, and the results were shown in Fig. 1. The velocity values for the seeding sludge and NAFO sludge were