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ENRICHMENT AND MOLECULAR DIVERSITY OF ANAMMOX BACTERIA IN UASB REACTOR

Anaerobic ammonium-oxidizing bacteria were successfully enriched in an upflow anaerobic sludge blanket bioreactor. In this balanceable ecosystem, the proportion between the conversion of ammonium and nitrite and the production of nitrate was found to be 1: 1.30: 0.29, and the removal efficiency of TN reached 90.35%. The microbial community and its diversity in enrichment cultures have been characterized using microscopy and molecular biotechnology. Based on 16S rRNA and phylogenetic analysis, we found four strains in the amplified DNA fragments. Three new species of anammox bacteria were found in this ecosystem.

1. INTRODUCTION

Anaerobic ammonium oxidation (anammox) is a promising method for removing nitrogen in wastewater treatment. It also plays a key role in the biological cycle of nitrogen in the oceans. It was estimated that the anammox reaction contributed to the more than 50% removal of fixed nitrogen from the oceans (ARRIGO [1], THAMDRUP and DALSGAARD [27]). It is a remarkable microbial pathway that allows anammox bacteria to use $N\text{-}NO_2^-$ as the electron acceptor, and $N\text{-}NH_4^+$ as the electron donor to yield the dinitrogen gas. The discovery of anammox bacteria took place in the late 1970s (BRODA [2]). After they were for the first time found in a pilot plant treating wastewater from a yeast-producing company in Delft, The Netherlands (MULDER et al. [17]), they attracted particular attention because of their unique biochemical features.

Anammox bacteria have not yet been purified using conventional microbiological techniques due to their very slow growing rate (FUJII et al. [7], QIN and ZHOU

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[20]). The recent development of molecular biology techniques enables scientists to overcome the limitations of conventional techniques of cultivation. So far only one anammox species, i.e. *Canditatus* "Scalindua sorokinii", was detected in natural saline ecosystems (KUYPERS et al. [14]). The other five species, which are named temporarily *Candidatus* "Kuenenia stuttgartiensis", *Candidatus* "Brocadia anammoxidans", *Candidatus* "Scalindua wagneri", *Candidatus* "Scalindua brodae" and *Candidatus* "Anammoxoglobus propionicus", are known to exist in freshwater ecosystems (KARTAL et al. [11], [12], NAKAJIMA et al. [18], SCHMID et al. [22], STROUS et al. [25]).

In this study, we cultivated and studied the anammox bacteria in a UASB reactor because this reactor could provide high biomass concentration and a stable configuration that prevented substrate concentration shock (BUZZINI et al. [3], JIN et al. [10]). The bioreactors had been operating continuously for more than one year in the lab of South China University of Technology. The anaerobic sludge and its biodiversity in nitrogen-rich wastewater were studied, which was beneficial for the practical use of the process.

2. METHODS

2.1. SYSTEM CONFIGURATION AND EXPERIMENTAL CONDITIONS

The UASB reactor was used for anammox enrichment. Each reactor contained a total volume of 3.2 dm^3, providing a reaction zone of 2.28 dm^3 and a settling zone of 0.92 dm^3 in the upside (figure 1). The reaction temperature ranged from 32 to 34 °C and the reaction took place in dark due to black plastics. The activated sludge that was introduced into the UASB reactor came from a landfill leachate wastewater treatment plant and the UASB reactor. The volume of the inoculum was 0.75 times that of reactor. The influent was a synthetic solution of the following mineral composition (g/dm^3): KH_2PO_4, 0.027; $MgSO_4 \cdot H_2O$, 0.3; $CaCl_2$, 0.136; $NaHCO_3$, 0.5; NH_4Cl, 0.153–0.497; and $NaNO_2$, 0.197–0.641; the microelements (mg/dm^3): EDTA, 5; $FeSO_4$, 5; $ZnSO_4 \cdot 7H_2O$, 0.43; $CuSO_4 \cdot 5H_2O$, 0.25; $MnCl_2 \cdot 4H_2O$, 0.99; $NiCl_2 \cdot 6H_2O$, 0.19; $CoCl_2 \cdot 6H_2O$, 0.24; and H_3BO_4, 0.014. The pH value was adjusted to 7.5–7.8 with $NaHCO_3$. Dissolved oxygen(DO) was not removed from a synthetic solution before its introduction into the reactor, because anammox bacteria can grow in a limited or anoxic condition (LIU et al. [15]).

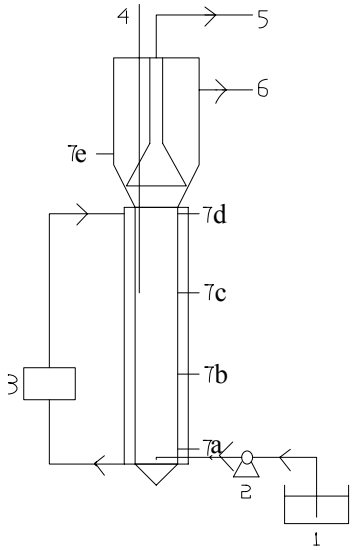

Fig. 1. Schematic structure of the UASB reactor:
1 – influent tank; 2 – influent pump, 3 – thermostat-waterbath, 4 – thermometer,
5 – off-gas; 6 – effluent, 7 – sampling point

2.2. ANALYSES OF AMMONIA, NITRITE, AND NITRATE CONCENTRATIONS

Total ammonia (NH_3 and NH_4^+) was determined based on the hypochlorite oxidation reaction; nitrite concentrations were measured using the sulfanilamide reaction; nitrate contents were analyzed with UV spectrophotometry (State Environmental Protection Administration of China, 2002).

2.3. ANALYSIS OF ANAMMOX ACTIVATED SLUDGE USING OPTICAL AND ELECTRON MICROSCOPY

The structure of the activated sludge was studied based on optical and electron microscopy. The morphology of the bacteria was examined using high resolution scanning electron microscope (SEM) (FEI-XL30, Philips, Holland) and transmission electron microscope (TEM) (FEI-Tecnai, Philips, Holland). Electron microscopical analysis was carried out in the Laboratory for Electron Microscopy, Testing Facility Center of South China Agriculture University, Guangzhou. The samples for SEM and TEM analyses were prepared according to Zhang's method (ZHANG et al. [28]). The samples for the latter analysis were cut to 50–70nm slices (Leica UCT, Leica, Germany).

2.4. DNA EXTRACTION, PCR AMPLIFICATION AND CLONING OF 16S rRNA

10-cm^3 sludge samples for DNA extraction were collected from a sampling point (figure 1, 7c) located in the middle of the UASB reactor. The nucleic acids were extracted according to LOGEMANN et al. [16]. Amplifications of 16S rRNA from chromosomal DNA were carried out in a DNA thermal cycler model 2720 (Singapore) using two pairs of anammox primers. One primer pair is Pla46F (*Planctomyctales* primer, 3′-GGA TTA GGC ATG CAA GTC-5′) and AMX368R (the primer of all anammox organisms, 5′- CCT TTC GGG CAT TGC GAA-3′) (NEEF et al. [19], SCHMID et al. [23]). The other pair of primers are Pla46F and AMX820R (*Candidatus* "Kuenenia stuttgartiensis" and *Candidatus* "Brocadia anammoxidans" primer, 5′-AAA ACC CCT CTA CTT AGT GCC C-3′) (SCHMID et al. [22]). The two pairs of primers were designed to amplify the part of 16S rRNA genes of the anammox cluster containing 323 base pairs and 775 base pairs, respectively. The PCR amplification program consisted of an initial 5-min denaturation step at 94 °C followed by 30 cycles at 94 °C for 40 s, 52 °C for 40 s and 72 °C for 90 s; and a final 7-min extension step at 72 °C. The PCR-amplified DNA was ligated into a Pmd18-T Vector (Taka Ra, Japan) and transformed into *Escherichia coli* cells. The presence of cloned inserts was verified by PCR amplification. 100 replicated white colonies were randomly selected and sent to the Shanghai Biological Company for sequencing. The 16S rRNA Sequences obtained in this study were available from the GenBank sequence database under accession numbers.

2.5. PHYLOGENETIC ANALYSIS

Sequences were submitted to the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information. Phylogenetic tree and molecular evolutionary analyses were conducted using MEGA version 4.0 (TAMURA et al. [26]).

3. RESULTS

3.1. ENRICHMENT OF ANAMMOX BACTERIA

The growth of the population of anammox bacteria exhibited a high correlation with the rate of nitrogen conversion (ISAKA et al. [8]). The higher the removal efficiencies of ammonium and nitrite, the higher the concentration of anammox cells. So the anammox bacteria enrichment in the reactor was determined by the removal efficiency of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$. An enrichment culture was eventually prepared by

a continuous supplying the reactor with the mineral medium containing NH_4Cl and $NaNO_2$ at increasing concentrations for more than one year. Figure 2 shows the concentrations of nitrogenous compounds in the influent and effluent of the UASB reactor during the operation. The respective concentrations of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ were 40 mg/dm^3 and 52 mg/dm^3 at the beginning and 130 mg/dm^3 and 169 mg/dm^3 at the end of the reactor operation. The enrichment process of the anammox bacteria can be divided into three phases: operation (from the 1st to the 46th day), propagation (from the 47th to the 59th day) and cultivation (after the 60th day) (figure 2). At the operation stage, the organic compound of the sludge was decomposed by microorganisms, and the concentration of NH_4^+ in the reactor was higher than that of the influent. At first,

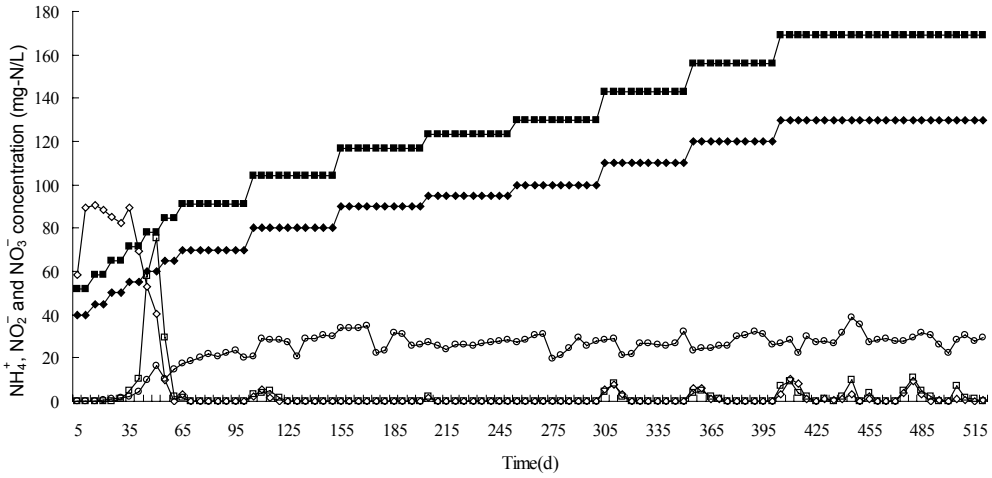

Fig. 2. Changes in concentrations of $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$. Influent $N\text{-}NH_4^+$ (♦), effluent $N\text{-}NH_4^+$ (◇), influent $N\text{-}NO_2^-$ (■), effluent $N\text{-}NO_2^-$ (□); effluent $N\text{-}NO_3^-$(○)

nitrification and denitrification were the main reactions because the original sludge contained a large number of nitrifying and denitrifying bacteria. The new inorganic and anaerobic conditions benefited the anammox bacteria propagation. The anammox bacteria propagated rapidly in the propagation stage. On the 60th day, the removal efficiencies of the $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ were 99.85% and 96.77%, respectively, and the volumetric conversion rate of TN was 0.198 kg/m^3/d. This demonstrated that the anammox process had started up successfully. Then the anammox bacteria entered the cultivation stage. The effluent concentration of $N\text{-}NO_3^-$ was a slightly higher than that of the influent (figure 2). After startup for 400 days, the volumetric conversion rates of $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and TN in the reactor were 0.192 kg/m^3/d, 0.249 kg/m^3/d and 0.398 kg/m^3/d, respectively, and the removal efficiency of TN was 91.82%. In

this balanceable ecosystem, the proportion between the conversion of ammonium and nitrite and the production of nitrate was found to be 1: 1.30: 0.29, which was similar to that reported previously (1: 1.32: 0.26) (STROUS et al. [25]). Thereafter, the conditions and the components of the influent were invariable, hence we dealt with a balanced ecosystem in the reactor. The bioreactor has started up for a long period and its operation took place in the darkness and in the presence of inorganics, so the number of photolithotroph and heterotrophs became less and less. The new population of chemoautotrophs propagated and became the dominant population.

3.2. OPTICAL AND ELECTRONICAL MICROSCOPY ANALYSES OF SLUDGE AND BACTERIA

The sludge sample from the UASB reactor which had run up for 400 days was salmon pink. The sludge was very loose. Many bacteria were present on the granule surface. According to FANG [6] the microbe distribution on a granule depends on the pathway of the biodegradation they carry out. Consequently, ammonium and nitrite played an important role in establishing the predominating microorganisms. The predominant bacteria of the enrichment cultures were observed in SEM. Most of the cells were elliptical (figure 3a). The biomass in the community was dominated by morphologically conspicuous bacteria. The morphologically conspicuous bacteria had the same shape as that reported by other scientists (CHAMCHOI and NITISORAVUT [4], EGLI et al. [5]). The size of most cells was (0.6–0.8) μm × (0.9–1.2) μm. Thin sections of enrichment samples were also analyzed by TEM. There are six cells in figure 3b whose cell wall, plasma and membrane can be observed.

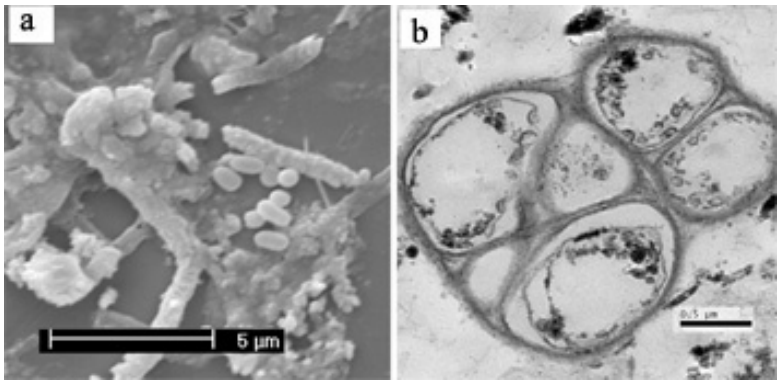

Fig. 3. Micrographs of a biomass aggregate and activated sludge from anammox enrichment cultures. (a) Scan electron micrograph of the anammox sludge. The cluster bacteria on an arrowhead place. The scale bar indicates a size of 5 μm. (b) Transmission electron micrograph of thin-sectioned anammox microcolony, showing close apposition of cells with each other. The scale bar indicates a size of 0.5 μm

3.3. IDENTIFICATION AND PHYLOGENETIC ANALYSIS

We determined DNA sequences of the inserts of 100 recombinant plasmids among several hundred transformants using Pla46F and AMX368R primers, and found four different sequences corresponding to anaerobic ammonium-oxidizing planctomycetes (figure 4), i.e., CloneA238 (accession no. Eu301731), CloneA224 (accession no. Eu301732), CloneA240 (accession no. Eu301734) and CloneA125 (accession no. Eu301737). Their sequence identity was between 82.5% and 89.4%, so they belong to different anammox species. CloneA238 (72 clones, 72%) was the same species as Planctomycete KSU-1 gene (AB057453) (100% similarity) which was obtained from a continuous up-flow column reactor filled with non-woven carriers (FUJII et al. [7]). The partial sequences of CloneA240 (9 clones, 9%) and CloneA125 (4 clone, 4%), being 90% and 90.8%, respectively, were similar to that of *Candidatus* "Brocadia anammoxidans" (AF375994) which was found in the anammox sludge of a SBR reactor (KUENEN and JETTEN [13]). CloneA224 (15 clones, 15%) was closely related to *Candidatus* "Kuenenia stuttgartiensis" (AF375995) (97.7% similarity) which had been found in a biofilm reactor in Stuttgart and in a rotation biological contractor treating ammonium-rich leachate (EGLI et al. [5], SCHMID et al. [22]).

There was only one species cloneA03 (the accession no. EU301738) examined based on DNA sequences of the inserts of 100 recombinant plasmids among several hundred transformants using Pla46F and AMX820R primers. Partial sequences of CloneA03 and Clone A238 have 100% identity, so we can conclude they are the same species or have common ancestor.

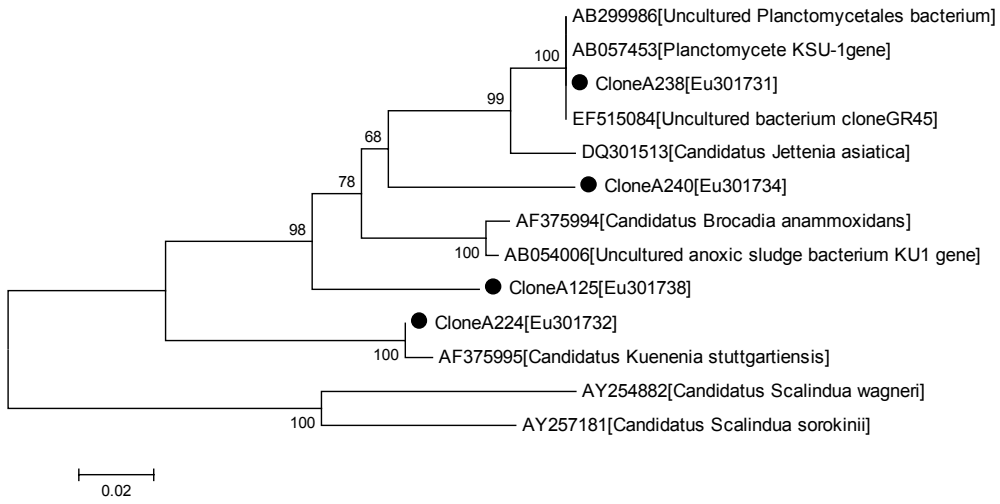

Fig. 4. Phylogenetic tree of the 16S rRNA sequences from clone libraries.
The bar below indicates the genetic distance between sequences

Based on the phylogenetic tree and the sequences of the clones, we found that the anammox bacteria in the UASB reactor were to some extent related to the known anammox bacteria. Only one kind of dominant anammox bacteria (CloneA238) was found, and the other three (CloneA240, CloneA125 and CloneA224) were new species which had not been reported before (figure 4). One ecosystem has one genus of dominant anammox bacterium. The biodiversity of anammox bacteria was not high, which corroborated our previous research (QIN & ZHOU [21]).

4. CONCLUSION

In this study, the start-up time was as short as only about 2 months in a UASB reactor, and the removal efficiency of nitrogen was very high. Our results show that the reactor, an optimal seeding strategy and optimal conditions are the solutions to the problem of the slow start-up of the anammox process. We can select the activated sludge similar to inocula. This similar activated sludge is abundant in anaerobes and comes from treating the wastewater rich in nitrogen. The optimal conditions are pH 7.5–7.8 at 32–34 °C.

The morphologically conspicuous bacteria were observed on micrographs. We cannot be certain that their key function is the anammox process, because there are no pure cells. This species adapts itself to dark, anaerobic and inorganic conditions, and we can conclude it represents chemoautotrophic bacteria. The shape of morphologically conspicuous cells allows us to compare this species with bacteria used in conventional treatment methods. We used two pairs of primes to clone anammox bacteria, and four kinds of anammox species were found.

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