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Use of bamboo charcoal reduced the cultivated anammox seed sludge dosage during the start-up period

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ABSTRACT

Bamboo charcoal (BC) was used in an anaerobic ammonium oxidation (anammox) process used for synthetic wastewater treatment with the aim of evaluating its use as a biocarrier to reduce the cultivated anammox seed sludge (CASS) dosage. The CASS dosage was reduced by more than 50% after BC was added to the anammox reactor. Furthermore, the time required for the start-up of the anammox reactor under the experimental conditions was reduced about six weeks after the addition of BC. A genetic analysis of the organisms in the granular bed revealed an abundance of *Kuenenia stuttgartiensis* and uncultured bacterium clone KIST-JJY001 enriched in the reactor.

Keywords: Anammox; Bamboo charcoal; Granulation; Seed sludge

1. Introduction

Use of the anaerobic ammonium oxidation (anammox) reactor has been increasing owing to its high efficiency, low running cost, and reasonable footprint compared with conventional biological nitrogen removal processes [1–3]. The start-up is a key issue in the application of an anammox reactor; the growth of anammox bacteria during reactor start-up is a slow process, and in some cases, the anammox reactor fails to start up.

Van der Star et al. reported [4] that 3.5 years were required to start up their first full-scale plant. Subsequently, the use of cultivated anammox seed sludge (CASS) as inoculum was considered, leading to a relatively rapid start-up compared with that observed with kinds of seed sludge [5]. However, CASS availability is limited; thus, the amount of CASS required for start-up is a concern with respect to economical feasibility. Moreover, drawing a considerable amount of CASS from a running anammox reactor could affect the nitrogen removal performance substantially and even cause a collapse of the running system owing to the accumulation of nitrite, which could inhibit the activity of anammox bacteria [6–8]. Wenjie et al. [9] have suggested a strategy that involves in removing smaller amounts (below 10% of the whole sludge) of CASS more frequently and maintaining them under refrigerated conditions; however, the extra cost of storage might affect the capital cost of the anammox

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process. Consequently, reducing CASS dosage was crucial to the application of the anammox process. Previous studies [10–14] have invested more efforts in searching for the appropriate biocarrier for CASS to ensure sufficient retention of CASS in the reactor. However, CASS was still required in large quantities during the start-up period. Bamboo charcoal (BC), which has been investigated and documented previously [15], has been considered to play an important role in significantly increasing the number of anammox bacteria, thereby enhancing the start-up period with uncultivated sludge. Thus far, few reports have described its effect on reducing CASS dosage during the start-up period of the anammox reactor [16].

In this study, BC was used at a reduced size to further evaluate its effectiveness in reducing CASS dosage in an anammox reactor. BC has a porous microstructure that is suitable for the retention of bacteria and a specific gravity slightly higher than that of water; thus, it was thought to be a suitable candidate for successful use as a biocarrier. The reactor performance of the BC-filled reactor seeded with a reduced CASS dosage was evaluated by comparing it with a parallel reactor seeded with a normal CASS dosage without the addition of BC. Genetic analysis using the 16S rRNA gene was employed to characterize the microbial population of the BC-based anammox granules; thus, the overall function of BC was discussed.

2. Materials and methods

2.1. Anammox reactor and substrate

The reactor had an inner diameter of 14 cm with a total liquid volume of 10 L including a reaction zone of 8 L and a settling zone of 2 L. The reactor was made of acrylic resin and had a water jacket for temperature control. Sampling ports were located at heights of 3, 17, 20, and 25 cm above the reactor bottom. Part of the effluent was collected in a 5-L container (with mixer and heater) for use as recycle (Fig. 1). The pH was adjusted by an online pH controller (TPH/T-10, Tengine, China) using 0.5 mol/L H_2SO_4. The reactor was enclosed in a black-vinyl sheet to inhibit the growth of photosynthetic bacteria and algae.

The reactor was operated in up-flow mode, with influent introduced at the bottom using a peristaltic pump (BT100-2J, LongerPump, China). A recirculation pump (BT600-2J, LongerPump, China) was used to dilute the influent (Fig. 1) with the treated wastewater in the 5-L recycle container.

The CASS used in the reactor was cultivated in one 50-L reactor for 3 years [9]. The CASS was in natural granules form (diameter, 1–3 mm). The initial seeding concentration (mass of mixed liquor suspended solids (MLSS) per liter) was set at 1 g MLSS/L with BC and 2 g MLSS/L without BC.

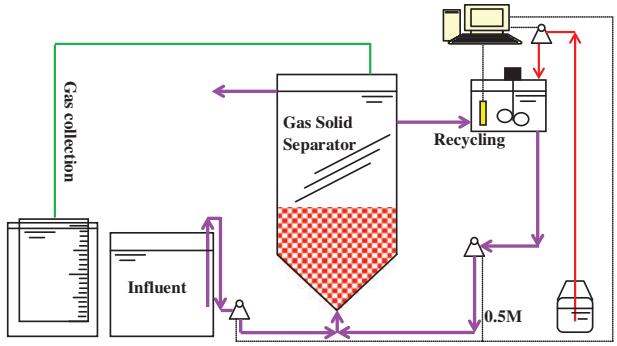

Fig. 1. Schematic view of the anammox reactor system. Symbols: GSS, gas solid separator.

The original BC (Ruicheng, China) had an average diameter of 2–3 mm, apparent specific gravity of 1.45, and a porous microstructure suitable for retention of bacteria. The BC bed was easily expanded, being only slightly heavier than water. Prior to use, the BC was black in color and had an average settling velocity of 150 m/h. In total, 2 L of BC was added to the reactor in this study.

The reactor was fed with synthetic wastewater with a nitrite to ammonium molar ratio of 1.0–1.2. The composition of synthetic wastewater was shown in Table 1.

2.2. Analytical methods

NO_2-N and NH_4-N were measured by the colorimetric method according to standard methods [17]. Total nitrogen (TN) was determined by the persulfate method [17] using the UV spectrophotometric screening method [17] for quantification of TN as NO_3-N (the oxidization product of the persulfate digestion). NO_3-N (of the original sample) was determined by calculation of the difference of TN and the sum of NO_2-N and NH_4-N. The pH was measured using a pH meter (9010, Jenco, USA), and dissolved oxygen (DO) was measured by using a DO meter (6010, Jenco, USA).

The suspended solids content was determined according to standard methods [17]. The total sludge content was estimated as mixed liquor suspended solids (MLSS). For determination of MLSS, a sludge sample of known volume was washed twice by centrifuging at 1,000×*g* for 15 min with decanting and re-suspending in deionized water and then dried to a constant weight at 105°C, with cooling to room temperature under desiccation prior to weighing.

Table 1
Composition of substrate

Composition	Concentration (mg/L)
$(NH_4)_2SO_4$, $NaNO_2$ (as mg N/L)	200–1,000
$KHCO_3$	1,000
KH_2PO_4	50
$CaCl_2{\cdot}2H_2O$	100
$MgSO_4{\cdot}7H_2O$	200
$Na_2S_2O_3$	24.81
Trace element solution 1 (g/L): $FeSO_4{\cdot}7H_2O$ 10, $C_{10}H_{14}N_2Na_2O_3$ 5.6	0.5 mL/L
Trace element solution 2 (g/L): $MnCl_2{\cdot}4H_2O$ 0.352, $CoCl_2{\cdot}6H_2O$ 0.096, $NiCl_2{\cdot}6H_2O$ 0.08, $CuSO_4{\cdot}5H_2O$ 0.1, $ZnSO_4{\cdot}7H_2O$ 0.172, $NaSeO_4{\cdot}10H_2O$ 0.105, $NaMoO_4{\cdot}2H_2O$ 0.11, $C_{10}H_{14}N_2Na_2O_3$ 5.0	1 mL/L

2.3. Scanning electron microscopy (SEM)

Samples were first washed in a 0.1-M phosphate buffer solution (pH 7.4) for 5 min. Then, samples were hardened for 90 min in a 2.5% glutaraldehyde solution prepared with the buffer solution. Next, samples were washed in the buffer solution three times for 10 min each and then fixed for 90 min in a 1.0% OsO_4 solution prepared with the buffer solution. After washing samples three times for 10 min each in the buffer solution, they were dewatered for 10 min each in serially graded solutions of ethanol at concentrations of 10, 30, 50, 70, 90, and 95%. Scanning electron microscopy (SEM) observations were conducted using a scanning electron microscope (JSM-6380LV, JEOL, Tokyo).

2.4. DNA extraction and high-throughput 16s rRNA gene pyrosequencing

After 30 d of operation, the BC granules and natural granules (both reactors, with BC and without BC) were taken out from the Anammox reactor. A granular sludge sample was first ground with a pestle under liquid nitrogen. Meta-genomic DNA was extracted using the E.Z.N.A. Soil DNA Kit (OMEGA Biotec. D5625-01, USA) according to the manufacturer's instructions. Amplification of the 16S rRNA gene was performed using primers 27F (forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (reverse primer: 5′-TTACCGCGGCTGCTGGCAC-3′). PCR was carried out according to the following thermocycling parameters: 120 s initial denaturation at 95℃, 25 cycles of 30 s at 95℃, 30 s at 55℃, 30 s at 72℃, 5 min final elongation at 72℃, then held at 10℃ until halted by user. Duplicate PCR products were pooled and purified using the AXYGEN gel extraction kit (Axygen, USA) [18].

Pyrosequencing was carried out by a 454 Life Sciences Genome Sequencer FLX Titanium instrument (Roche). Sequences were clustered into operational taxonomic units (OTUs) by setting a 0.03 distance limit (equivalent to 97% similarity) using the MOTHUR program.

3. Results

3.1. Reactor performance

A nitrogen loading rate (NLR) of 0.5 kg $N/m^3/d$ was used at the start-up of the anammox reactor, which was gradually increased as the anammox bacterial population was enriched. The levels of nitrogenous compounds and the TN removal efficiency over the course of the study are shown in Fig. 2. In this study, an NLR of 1 kg $N/m^3/d$ was considered as the achievable goal for the start-up of the anammox reactor. When effluent NO_2-N was less than 20 mg/L, the NLR was increased by shortening the HRT or increasing the influent TN.

The start-up periods with BC and without BC were considered to be from day 0 to day 51 (Fig. 2(A)) and from day 0 to day 8 (Fig. 2(B)), respectively, during which time the NLRs were increased from 0.5 to 1 kg $N/m^3/d$. Start-up was accomplished in 8 d using BC as a biocarrier and 1 g/L seed sludge, while, even with 2 g/L seed sludge, the start-up time was about six weeks longer in the reactor without BC. Thus, BC could largely reduce the dosage of CASS and shorten the start-up time for the anammox reactor.

Throughout the study, the temperature in the reactor was maintained at 33 ± 1℃, and the DO concentration was held below 0.5 mg/L. Furthermore, the effluent pH (7.6–8.2) was notably higher than the influent pH (6.9–7.2), which is due to the consumption of acidity in the anammox reaction [19].

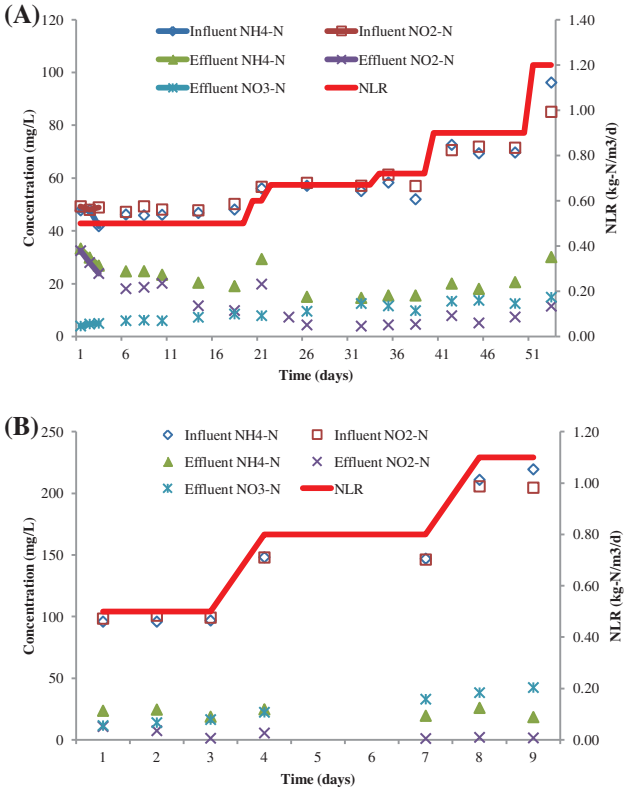

Fig. 2. Reactor performance during the study. Symbols: NLR, nitrogen loading rate; NRR, nitrogen removal rate.

3.2. BC granules

At first, BC was black in color and CASS was red. Within 1 month, the BC had turned red due to the growth of anammox bacteria in the form of biofilm on the BC surface. The matured, red BC-seeded granules had an average settling velocity of 200 m/h (5 cm/s), compared to the typical values of 73–88 m/h for biomass granules [20], while the settling velocity of sludge without BC remained same as that of the seed sludge.

The anammox biomass granules seeded with BC were similar in appearance to granules reported by others [21]. SEM micrographs (Fig. 3) indicate that a relatively large number of bacteria were attached to the BC nuclei. The spaghetti theory suggests that filamentous microorganisms form a frame or a mesh in which other bacteria such as cocci are trapped and retained, thereby leading to granule growth, as observed in this study.

3.3. Bacterial community analysis

In this study, the seed sludge used in the anammox reactor was drawn from a running pilot-scale reactor.

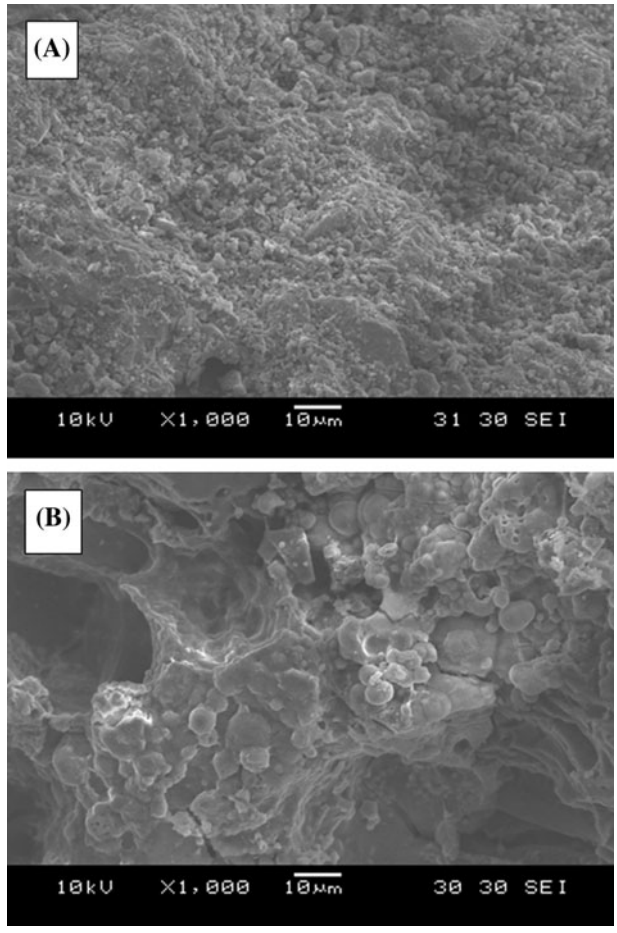

Fig. 3. SEM microphotograph of BC: (A) blank and (B) day 30.

After 30 d of cultivation with synthetic wastewater, *Kuenenia stuttgartiensis* and *Uncultured bacterium clone KIST-JJY001* appeared and soon became dominant in the reactor with BC, while, in terms of the microbial community, the sludge of the reactor without BC and natural granules with BC remained same with the initial sludge [9]. The results of the BC granule sequence analysis of major DGGE bands are summarized in Table 2. From the 16S rRNA analysis, *Uncultured bacterium clone KIST-JJY024*, *Uncultured bacterium cloneFN-11*, *Uncultured bacterium clone KIST-JJY012*, and *Uncultured bacterium clone: AnSal-09*, which were often detected as common coexistent anammox bacteria, prevailed as the majority of the clones (61%). The function of coexistent anammox bacteria is still largely unknown. *Uncultured bacterium clone KIST-JJY024* and *Uncultured bacterium cloneFN-11* are well known to form net-like structures, which could contribute to the aggregation process or biofilm formation. Therefore, the BC added in this study could have served to first promote the growth of the coexistent bacteria, thus enhancing

Table 2
Homology search results for 16S rRNA gene sequences of the main bacterial members in the community

Phylum (class)	Taxon	Identity (%)	Number of clones	Accession
Proteobacteria (β)	Uncultured bacterium clone KIST-JJY030	97	1	EF654699
				AB194898
	Uncultured bacterium clone: A	97		AY118150
Planctomycetes	*Kuenenia stuttgartiensis*	98–99	4	CT573071
	Uncultured bacterium clone KIST-JJY001	96–100	2	EF515083
	Uncultured bacterium clone Dok53	99	1	FJ710771
Chloroflexi	Uncultured bacterium clone KIST-JJY024	99	5	EF594056
		99		FJ710774
	Uncultured bacterium clone Dok55	95–96		CU923478
	Uncultured chloroflexi bacterium clone QEDQ2AD10			
	Uncultured bacterium cloneFN-11	91	1	FJ917566
	Uncultured chloroflexi bacterium clone QEDR3BB09	85		CU921946
Chlorobi	Uncultured bacterium clone KIST-JJY012	99–100	7	EF584532
		99–100		FJ710742
	Uncultured bacterium clone Dok23	95–97		FJ484824
	Uncultured chlorobi bacterium clone Z4MB25			
Candidate division TM7	Uncultured bacterium clone delph2B11	96	1	FM209162
	Uncultured candidate division TM7 bacterium clone EMP2	96		AM936584
Candidate division OP11	Uncultured bacterium clone: AnSal-09	99	1	AB434261
	Uncultured candidate division OP11 bacterium clone 062DZ46	89		DQ329875

the granulation process and providing a niche for the enrichment of anammox bacteria.

4. Discussions

Biocarriers have often been employed to enhance the sludge retention time in anammox reactors. Zhang et al. [22] studied the feasibility of starting up an anammox process using polyethylene sponge strips as biocarriers; by this method, start-up was achieved in approximately 8 weeks. Compared to the original full-scale UASB reactor, the start-up period was greatly reduced; however, it was still considered prohibitively long. Comparatively, in this study, start-up was achieved within 1 week using BC as the biocarrier. In addition, the seed sludge dosage was only a quarter of that required when using polyethylene sponge strips as biocarriers.

Although the functions of coexisting bacteria in anammox consortia are largely unknown, the *Uncultured bacterium clone KIST-JJY024* and *Uncultured bacterium clone FN-11* (Table 2) are considered to be key organisms in the granulation process. In this study, BC is thought to have provided the necessary environmental characteristics for the growth of anammox bacteria, such as protective niches with growth-supportive surfaces. Nonetheless, the coexisting organisms soon adapted to the experimental conditions employed and achieved predominance in the consortium.

The predominant bacteria in this study are *Uncultured bacterium clone KIST-JJY024*, *Uncultured bacterium cloneFN-11*, *Uncultured bacterium clone KIST-JJY012*, and *Uncultured bacterium clone: AnSal-09*; this population balance was clearly different from that observed in other cases, which shows that the BC seed acts well not only as a biofilm carrier by replacing the structural function of filamentous growth, but also by evidently stimulating the growth of *Uncultured bacterium clone KIST-JJY024* and *Uncultured bacterium clone FN-11*, which are well known as key components of anammox granulation and may have been involved in extending the granulation process beyond BC. Thus, it is considered that the added BC seed used in this study can function effectively as nuclear particles that enhance the formation of granules can be readily enhanced.

5. Conclusions

Using BC as the biocarrier of CASS, start-up was achieved within an 8-d period. The seed sludge dosage required was only half of that required in a reactor without BC. The red, matured granules demonstrated excellent retention characteristics with an average settling velocity of 200 m/h (5 cm/s). Thus, BC shows great potential in providing a medium that is well suited for the growth of coexisting bacteria, which in turn enhances granule formation.

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