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Temperature dependence of nitrogen removal activity by anammox bacteria enriched at low temperatures

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The anaerobic ammonium oxidation (anammox) process, which is applicable at ambient temperature, is necessary to develop more versatile nitrogen removal technologies. In this study, two anammox reactors, Low-R1 and Low-R2 inoculated with activated sludge respectively in Kumamoto and Hokkaido, Japan, achieved nitrogen removal rates (NRRs) of 1.5 kg-N/m^3/day at 20°C. The specific anammox activity (SAA) of the Low-R1 and Low-R2 sludge samples had peaks, respectively, of 2.8 ± 0.3 mg-N/g-VSS/h at 25°C and 4.2 ± 0.3 mg-N/g-VSS/h at 30°C and dropped over the optimum temperature. Moreover, the SAA values of the Low-R1 and Low-R2 were higher at 10–25°C and 10–35°C, respectively, than that of an anammox reactor inoculated with activated sludge in Kumamoto operated at 35°C (Mod-R). The apparent activation energy for anammox of Low-R1, Low-R2, and Mod-R were 108 kJ/mol (10–25°C), 73 kJ/mol (10–30°C), and 89 kJ/mol (10–35°C), respectively. *Candidatus* Kuenenia stuttgartiensis dominated in the Mod-R sludge. The Low-R1 sludge was comprised of *Ca.* K. stuttgartiensis, *Ca.* Brocadia caroliniensis and *Ca.* B. fulgida and uncultured anammox-like or planctomycete-like bacteria. The Low-R2 sludge was comprised of various uncultured anammox-like or planctomycete-like bacteria. As Low-R2 was constructed, enrichment of freshwater anammox bacteria at low temperature with seed sludge collected from cold regions is expected to be an effective strategy for anammox applications under a wide temperature range.

[**Key words:** Anaerobic ammonium oxidation; Ambient temperature; Temperature dependence; Apparent activation energy; Phylogenetic analysis]

Anaerobic ammonium oxidation (anammox) is a biologically mediated reaction in which ammonium (NH_4–N) is oxidized to nitrogen gas using nitrite (NO_2–N) as the electron acceptor. Nitrogen removal from wastewater using anammox can provide important benefits compared to conventional processes: less oxygen demand, external carbon supply, and excess sludge production (1,2). Recently, the anammox process has been implemented for treating wastewater produced by some industries (3). However, the anammox process generally requires an additional heating system to maintain 30–37°C temperatures for efficient nitrogen removal (4–8), thereby lowering its energy efficiency.

Therefore, application of the anammox process at lower temperatures (≤25°C) is anticipated as a promising means of extending nitrogen removal processes. Several trials have been undertaken to start-up anammox reactors directly under ambient or rather lower temperatures (9–13). Our research group also conducted start-up of three lab-scale anammox reactors at 20°C, which were inoculated with activated sludge and groundwater samples in October 2009. Among the three anammox reactors, two reactors inoculated with activated sludge achieved high nitrogen removal rates (NRRs) of 0.64 kg-N/m^3/day after 800 days (14). Results of the earlier reported study showed that the ubiquitous presence of anammox bacteria and activated sludge was a better seed for the starting up of anammox reactors under ambient temperature. However, little is known about anammox reactions under ambient temperature compared with those under mesophilic conditions. Additional studies of their characteristics must be conducted to establish rational design and operational strategies of anammox reactors.

This study investigated the temperature dependence of the anammox activity of the sludge in anammox reactors operated at 20°C in comparison with anammox sludge enriched at a moderate temperature of 35°C (15). In addition, bacterial communities in the anammox sludge were characterized phylogenetically based on the 16S rRNA gene sequences.

MATERIALS AND METHODS

Restart of anammox reactors at 20°C Acrylic column reactors (500 mL each) packed with nonwoven polyester fabric material (Japan Vilene Co. Ltd.) were used to enrich anammox sludge at 20°C (Low-R1 and Low-R2) in this study, as depicted in Fig. 1. Seed sludge samples for Low-R1 and Low-R2 were originally collected respectively from a domestic wastewater treatment plant (WWTP) in Kumamoto City and from a livestock WWTP in Hokkaido Prefecture (14). After investigation in an earlier study (14), those reactors were kept operating at low NLRs without chemical analysis. The sludge in Low-R1 and Low-R2 darkened, suggesting decreased anammox activity. Therefore, reactor operations were conducted carefully to restore the anammox activity.

Synthetic inorganic wastewater contained 54 mg KH_2PO_4, 125 mg $NaHCO_3$, 0.5 mL of Trace element solution I ($FeSO_4$ 18 g/L, EDTA 10 g/L), and 1 mL of Trace element solution II (EDTA 15 μg/L, $ZnSO_4 \cdot 7H_2O$ 0.430 μg/L, $CuSO_4 \cdot 5H_2O$ 0.250 μg/L, $NiCl_2 \cdot 6H_2O$ 0.190 μg/L, $NaMoO_4 \cdot 2H_2O$ 0.22 μg/L, and H_3BO_4 0.014 μg/L) (1) in 1 L of tap water. Synthetic wastewater for Low-R1 and Low-R2 also contained

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FIG. 1. Configurations of up-flow reactor packed with nonwoven fabric material in a thermostatic incubator set to 20°C: Low-R1 (left) and Low-R2 (right) on day 949. The nitrogen removal performance of both reactors was 1.5 kg-N/m^3/day as NRR with 1.7 kg-N/m^3/day as NLR.

$(NH_4)_2SO_4$ 30–100 mg-N/L and $NaNO_2$ 30–120 mg-N/L. The synthetic wastewater was purged with 99% of N_2 gas to achieve dissolved oxygen (DO) concentration lower than 0.5 mg/L. The NLR was decided by adjustment of the hydraulic retention time (HRT).

Anammox sludge Anammox granular sludge detached from the nonwoven material was collected from the bottom of Low-R1 and Low-R2. Anammox granular sludge was also collected from a 2-L reactor operated at 35°C (Mod-R). Mod-R was fed with synthetic wastewater containing ammonium and nitrite at NLRs of 1.2 kg-N/m^3/day with 0.5 kg-N/m^3/day as NRRs at 35°C (15,16). The seed anammox sludge of Mod-R was derived from activated sludge of the WWTP of Kumamoto City like Low-R1.

Batch assay for measuring specific anammox activity Batch assays were performed to determine the specific anammox activity (SAA) of the sludge sampled from the three reactors. Anammox granular sludge collected from each reactor was washed three times with a minimal inorganic medium (17). The sludge sample was inoculated to 20 mL solution containing 50 mg-N/L ammonium and 50 mg-N/L nitrite at the volatile suspended solid (VSS) concentration of 1400–1600 mg/L in a 30-mL vial. The vial was sealed with a butyl rubber stopper and an aluminum cap and purged with 99.9% N_2 gas for 30 min. The pH value after gas purging was 7.2. The vials were anaerobically incubated on a reciprocal shaker (70 rpm) in a water bath at 10–35°C. Water samples were taken from the vials periodically for chemical analysis.

The SAA was calculated from the sum of the ammonium and nitrite removal rates per g-VSS of sludge (expressed as mg-N/g-VSS/h). The apparent activation energy (E_a, kJ mol^{-1}) of anammox was calculated based on the Arrhenius plot of temperature dependence of the SAA (8).

$$\ln(k) = \ln(A) + \left(\frac{-E_a}{R} \cdot \frac{1}{T}\right) \quad (1)$$

In that equation, k stands for the SAA, A represents the Arrhenius constant, R signifies the gas constant (8.31 J K^{-1} mol^{-1}), and T denotes the absolute temperature (K).

The temperature coefficient (Q_{10}), which is the factor of the reaction rate change when the temperature is increased by 10°C was calculated as shown below.

$$Q_{10} = exp\left[\frac{E_a \cdot 10}{RT(T+10)}\right] \quad (2)$$

Chemical analysis The NH_4–N, NO_2–N and nitrate (NO_3–N) concentrations were measured using an ion-chromatography system (HIC-SP system; Shimadzu Corp., Kyoto, Japan). An AS4A-SC column (Dionex Corp., CA, USA) was used to measure the NO_2–N and NO_3–N concentrations. The mobile phase was a 3 mM Na_2CO_3 solution. A column (Shim-pack IC-C4; Shimadzu Corp., Kyoto, Japan) was used for determination of the NH_4–N concentration. The mobile phase was a 3.0 mM $(COOH)_2$ solution.

Clone library analysis The anammox biomass was harvested on day 304 for the clone library analysis when the reactors demonstrated stable nitrogen removal. Total DNA was extracted from the anammox sludge using ISOIL for a Beads Beating DNA extraction kit (Nippon Gene Co. Ltd., Toyama, Japan) according to the standard instrument. Amplification of 16S rRNA gene fragments was performed using Ex-Taq DNA polymerase (Takara Bio Inc., Shiga, Japan) using conserved eubacterial primers 6F (forward: 5′-GGAGAGTTAGATCTTGGCTCAG-3′) (18) and 1492R (reverse: 5′-GGTTACCTTGTTACGACT-3′) (19) followed by 10 min at 98°C, 30 cycle of (10 s at 98°C, 30 s at 56°C, 90 s at 72°C) and 10 min at 72°C. The purified PCR productions were ligated using the pGEM-T Easy Vector System (Promega Corp., WI, USA) according to standard instructions with *Escherichia coli* DH5α competent cells (Takara Bio Inc.). Then, colonies were selected randomly with needles and were transferred to Super broth (consist of tryptone 32 g, yeast extract 20 g, NaCl 5 g, 1N–NaOH 5 ml per 1 L of autoclaved-ultra pure water). Nucleotide sequence determination of the clones was entrusted to Hokkaido System Science Co. Ltd. (Hokkaido, Japan).

The obtained sequences were compared to those in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) from the DNA Data Bank of Japan (DDBJ). The sequenced 16S rRNA genes were regarded as the same operational taxonomic unit (OTU) when sequences had identity higher than 99%. The phylogenetic tree was constructed using software MEGA 6.0 (20) with a neighbor joining tree.

The GenBank/DDBJ accession numbers of this study are published for the 16S rRNA gene of clone sequences used for the phylogenetic tree LC192349 to LC192426.

RESULTS

Nitrogen removal performance of Low-R1 and Low-R2 Until the reactors re-started, they were just kept at low NLRs without chemical analysis for influent and effluent nitrogen concentrations.

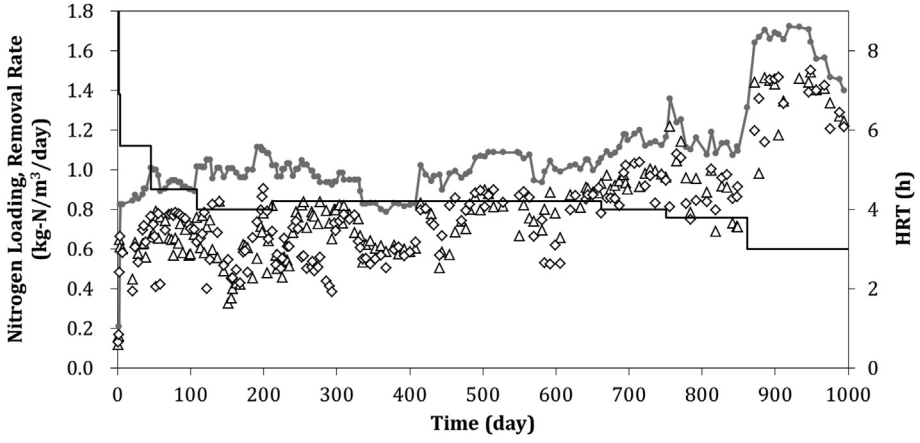

FIG. 2. Time courses of HRT, NLR, and NRR of Low-R1 and Low-R2 operating at 20°C: circles, NLR; triangles, NRR of Low-R1; diamonds, NRR of Low-R2; the solid line represents HRT.

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