

TITLE

Temperature effect on nitrogen removal performance and bacterial community in culture of marine anammox bacteria derived from sea-based waste disposal site

RUNNING TITLE

Temperature effect on marine anammox bacterial culture

KEY WORDS

anaerobic ammonium oxidation, anammox, marine anammox bacteria, nitrogen removal, temperature, bacterial community

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ABSTRACT

Anaerobic ammonium oxidation (anammox) bacteria have been detected in variety of marine environment in recent years, however, there have been only a few studies on their characteristics in the culture. The aim of this study is to reveal the effect of temperature on nitrogen removal ability and bacterial community in a culture of marine anammox bacteria (MAAOB). The MAAOB was cultured from the sediment of a sea-based waste disposal site at the North Port of Osaka Bay in Japan. The maximum nitrogen removal rate (NRR) was observed at 25°C in the MAAOB culture, and it decreased both at below 20°C and over 33°C. The activation energy of the MAAOB culture was calculated to be 54.6 kJ mol⁻¹ in the 5 to 30°C range. No significant change in bacterial community according with temperature (5-37°C) was confirmed in the results of polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE). A number of sulfur-metabolizing bacteria were confirmed in the MAAOB culture, thus some kind of relationship between MAAOB and sulfur-metabolizing bacteria was speculated.

INTRODUCTION

Anaerobic ammonium oxidation (anammox) has been recognized as a latest nitrogen pathway in nitrogen cycle of nature (1-3). Recent studies have suggested that anammox reaction may account for 30-50% of the total N_2 production in a marine environment (1,4-6). Many studies concerning anammox and the bacteria performing anammox reaction have been conducted in recent years (3,7-14). The anammox bacteria have unique properties that NH_4^+ is oxidized with NO_2^- to N_2 gas in anoxic condition and grow autotrophically without any organic carbon sources (11, 15). Therefore, application of anammox bacteria to nitrogen removal technique in wastewater treatment have been studied so far (11,15-19).

Most of the studies on wastewater treatments utilizing anammox reaction have been conducted by using enrichment cultures of anammox bacteria. However, if the anammox bacterial culture is damaged by unexpected accidents, appreciable period would be required for recovery because of quite low growth rate of anammox bacteria (doubling time is 11 days) (20). Thus, further improvements of robustness and flexibility of anammox culture to environmental conditions must be desired for application of anammox reaction to real wastewater treatment.

Temperature is one of the important conditions in wastewater treatment. Optimal temperatures for anammox culture have been reported to be around 37°C for the anammox bacteria of terrestrial (freshwater) environment origin (20, 21). As more than 30°C is common temperature in wastewater treatment, anammox activity may be well maintained without strict temperature control. Nevertheless, if anammox activity would be maintained in a good state at even lower temperature, the applications of anammox reaction to real wastewater treatment would become more practical in colder areas and during winter season even in other areas, for the reason, the adaptation of anammox reactor, in which the optimal temperature is 37°C, to low temperature (below 20°C) has been attempted (21).

The relatively lower temperatures are reported to be suitable for anammox bacteria derived from marine environment than freshwater-derived anammox bacteria (22), and particularly low temperatures such as below 15°C were optimal for anammox activity in Arctic marine sediments (23, 24). These studies suggest that the anammox bacteria derived from marine environment could be useful for wastewater treatment at relatively low temperature although the above studies were performed not with cultural experiments but with batch test using natural sediment samples. On the other hand, there have been a few successful cases with establishment of MAAOB culture (22,25-27) and little information about the effect of temperature on the cultures of marine anammox bacteria (MAAOB). The aim of this study is an establishment of continuous culture system of MAAOB by using a fixed bed reactor for application to wastewater treatment and reveal effects of temperature on anammox activity and bacterial community constituting the MAAOB culture.

MATERIALS AND METHODS

Inoculum source and medium.

As we have previously established a MAAOB culture system by using marine sediment which was collected from a sea-based waste disposal site at the North Port of Osaka Bay in Japan (28). The same sediment sample was used as inoculum source in this study. A synthetic seawater medium for MAAOB culture containing 40-170 mg-N l^{-1} ($(NH_4)_2SO_4$ and $Na(NO_2)$) was prepared; (30g NaCl, 5g $MgSO_4 \cdot 7H_2O$, 6g $MgCl_2 \cdot 6H_2O$, 1g $CaCl_2 \cdot 2H_2O$, 0.7g KCl, 0.1g NaBr, 20mg $SrCl_2 \cdot 6H_2O$, 20mg H_3BO_3 , 2mg NaF, 0.08mg KI,

500mg KHCO₃, 54mg K₂HPO₄, and 0.5ml of Micro Fe/EDTA solution (29) (18g FeSO₄·7H₂O and 10 g EDTA·2Na in 1000ml pure water) and 1ml of Micro nutrient solution (29) (250 mg CuSO₄·5H₂O, 430mg ZnSO₄·7 H₂O, 240mg CoCl₂·6H₂O, 990mg MnCl₂·4H₂O, 220mg Na₂MoO₄·2H₂O, 190mg NiCl₂·6H₂O, 110mg Na₂SeO₄, 14mg H₃BO₃, 15g EDTA·2Na), and predefined concentration of (NH₄)₂SO₄ and Na(NO₂) in 1000ml sterilized pure water, pH 7.5). The medium was always autoclaved for 20 minutes and deoxidized by purging of Argon gas before use.

Procedure for MAAOB culture and experimental condition.

One litter of cylindrical column reactor was used for MAAOB culture in which 8 strips of the porous polyester nonwoven fabric (3×18 cm strips, 0.7 cm material thickness, 0.5 mm mean pore-size) was placed as a biofilm carrier. The sediment sample weighting 5 g (wet weight) was put through a 0.5 mm-mesh strainer and daubed evenly on the nonwoven fabric. The medium and the inoculated nonwoven fabric were placed in the reactor and sequential batch cultivation was conducted in order to set the inoculated bacteria in place. The medium was circulated with upward flow at 5 times per day between the reactor and the 1000 ml glass bottle filled with the medium using a peristaltic pump. The reactor was placed in an incubator with other apparatuses together and controlled temperature at 30°C during a period of sequential batch cultivation. In continuous cultivation, the medium was continuously fed into and drawn from the reactor by a pump at hydraulic retention time of 24 h, and nitrogen concentration in the influent was gradually increased. The operating temperature was controlled by the incubator, and effect of temperature on the MAAOB culture was investigated by determining the variations of anammox activity and bacterial community with temperature.

Analysis

The nitrogen concentrations of NH₄⁺, NO₂⁻ and NO₃⁻ were measured by colorimetric method according to the method standardized by American public health association (APHA) (30). Nitrogen loading rate (NLR; kg-N m⁻³ d⁻¹) in the continuous culture was calculated by dividing total nitrogen concentration (kg-N m⁻³) in the influent (medium) by hydraulic retention time (HRT; d). Nitrogen removal ability in the MAAOB culture was evaluated by nitrogen removal rate (NRR; kg-N m⁻³ d⁻¹) and nitrogen removal efficiency (NRE; %), which were calculated by means of the following equations.

$$\text{NRR} = \frac{(C_{in} - C_{ef})}{\text{HRT}} \quad (1)$$

$$\text{NRE} = \frac{(C_{in} - C_{ef}) \cdot 100}{C_{in}} \quad (2)$$

where C_{in} and C_{ef} are the nitrogen concentrations in influent and effluent (kg-N m⁻³), respectively.

Bacterial community analysis was conducted by denaturing gradient gel electrophoresis (DGGE) following to polymerase chain reaction (PCR) targeting the region of v.3 to v.5 in 16S rRNA. Biofilm samples which attached on the nonwoven fabric at the bottom of the reactor were collected at each temperature condition to extract a DNA. DNA extraction and amplification were conducted according to a previously reported method (31). In PCR reaction, two kinds of primer sets were used for detecting of MAAOB (UKf1:5'-CCTACGGGAGGCTGCAG-3' and UKr1: 5'-CCCCGTCAATTCTTTGAGTTT-3') (26) and other eubacteria (GM5f :5'-

CCTACGGGAGGCAGCAG-3' and DS907r :5'-CCCCGTCAATTCCTTTGAGTTT-3') and GC-clamp (32) was connected to each forward primer. DGGE was performed at 200V, 60°C for 5h using a gel which generated denaturant-gradient in the 20% to 80%. DNA bands were stained with SYBR Green I (TAKARA Bio Inc., Japan) and excised from the gel on a transilluminator to determine the base sequence. The base sequence of each DNA band was compared homology with DNA sequence database stored in DDBJ using the BLAST program (33). phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (34).

RESULTS

Establishment of MAAOB culture system

Figure 1 shows NH_4^+ and NO_2^- concentrations at varying times in every batch culture of MAAOB. Total 4 times batch cultures were conducted at 30°C. The concentrations of both NH_4^+ and NO_2^- were prepared at 20 mg-N l^{-1} at the beginning of culture. In the first batch, both the nitrogen concentrations decreased within 5 days although NH_4^+ consumption percentage remained at only 20%. In the second batch, simultaneous decrease of NH_4^+ and NO_2^- was observed within 10 days, thus the nitrogen concentration in the medium was set at 40 mg-N l^{-1} in the third batch. In further batches, since both the decreases of NH_4^+ and NO_2^- suggesting the contribution of anammox reaction on nitrogen removal were observed, the reactor was switched to continuous operation to promote enrichment of MAAOB.

Nitrogen loading rate was increased to 0.35 kg-N $\text{m}^{-3} \text{d}^{-1}$ prior to the start of the study about temperature effect on the MAAOB culture because that stable nitrogen removal ability had been maintained at NLR of less than 0.4 kg $\text{m}^{-3} \text{d}^{-1}$ in the MAAOB culture in our previous study (28). Figure 2 shows the variation in each nitrogen concentration of NH_4^+ , NO_2^- and NO_3^- in the effluent with time during 0 to 120 days (Fig. 2(a)) and 0 to 360 days (Fig. 2(b)). As shown in Fig. 2(a), only a little decrease of nitrogen concentrations in the effluent was observed and no distinct NO_3^- production was confirmed for first 50 days, however, gradual NO_2^- and NH_4^+ decreases and NO_3^- production were observed after the operating temperature was changed to 25°C. After that, NLR was gradually increased to 0.35 kg-N $\text{m}^{-3} \text{d}^{-1}$ over almost one year to establish a continuous culture system (Fig. 2(b)). Nitrogen removal ability was stably-maintained during this period and the MAAOB growth was ascertained by PCR-DGGE analysis as described hereinafter. The mol-reaction ratio of nitrogen (MRRN; consumption rate of NH_4^+ : consumption rate of NO_2^- : production rate of NO_3^-) throughout the startup period was estimated at 1.0:1.15:0.15 (shown in Fig. 3).

Temperature effect on nitrogen removal performance in MAAOB culture system

We gradually changed the reactor temperature and determined the effects of temperature on nitrogen removal ability and microbial community in the MAAOB culture. Figure 4 shows the change in the nitrogen concentrations of NH_4^+ , NO_2^- and NO_3^- , and MRRN with the temperature shift. Nitrogen removal was well maintained and NRE was around 75% at 25°C. When the temperature was turned down to 20°C, both the nitrogen concentrations of NH_4^+ and NO_2^- in effluent increased slowly and NRE dropped to 55%. The same behavior in nitrogen removal was observed in the 15°C to 5°C range, and NREs were 45% (at 15°C), 30% (at 10°C) and 12% (at 5.0°C), however, when the temperature was changed back to 25°C stepwise, NRE was rapidly recovered and became stable. A slight change in MRRN with temperature shift was observed during this period. The

average values of MRRN were 1:1.18:0.16 at 25-20°C and 1:1.50:0.18 at 15-10°C.

When temperature was increased to 30°C, no remarkable change in nitrogen removal rate was observed. However, both concentrations of NH_4^+ and NO_2^- in effluent moderately increased at 33°C, and then they drastically increased and NRE dropped to 18% at 37°C. When the temperature was raised, an increase of residual NH_4^+ concentration with the decrease of NRR was observed as well as at low temperature. The MRRN value also changed with the increase of temperature. In particular, an average value of MRRN was 1.0:2.4:0.11 at 37°C due to increase of NH_4^+ concentration. When temperature was finally returned to 25°C, simultaneous decrease of NH_4^+ and NO_2^- concentrations was confirmed after lag time of about 10 days. From these results, the optimal temperature range for the MAAOB culture is estimated to be 25-30°C and the activation energy was estimated at 54.6 kJ mol⁻¹ from the relationship between temperature and NRR in the 5 °C to 30°C range (shown in Fig. 5).

Temperature effect on bacterial community in MAAOB culture system

Figure 6 shows DGGE results using the MAAOB specific primer set (Fig. 6(A)) and bacterial universal primer set (Fig. 6(B)), respectively. The one dense DNA band (band A) and another thin band (band B) were confirmed on the DGGE gel in all samples. The band A showed 100% sequence similarity to planctomycete UKU-1 (AB433172) which we have previously reported as MAAOB (26), indicating that the bacteria bearing this sequence would contribute to the anammox activity in the reactor. On the other hand, no fine DNA-sequence information was obtained as for the band B. These two DNA band were observed in the same manner in all samples taken at every temperature condition, which suggests that temporal temperature change would not have significantly effect on the presence of MAAOB.

Total 7 base sequences of DNA bands on DGGE gel using bacterial universal primer set were determined. The DNA bands were divided into 4 broad groups as shown in Fig. 7. The DNA sequence of US1107a showed the highest similarity (91%) to *Candidatus Aquirestis calciphila* (AJ786331) and was affiliated with the bacteria of Family Saprospiraceae which commonly known as filamentous bacteria. The suspended granules in the bottom of the reactor were collected for DGGE analysis, thus these bacteria may contribute to formation of the bacterial granules. In addition, as it has been previously known that a use of biofilm-carrier was effective to culture anammox bacteria (28), there is a possibility that these filamentous bacteria have an important role in the biofilm formation and adherence to the carrier. The US1107c and US1107d were affiliated with Class Gamma-proteobacteria and also showed high similarity to sulfur-oxidizing bacteria having denitrifying ability. On the other hand, the US1107e, US1107f and US1107h were affiliated with Phylum Firmicutes, and the US1107e and US1107f particularly showed high similarity to the bacteria possessing sulfate-reducing ability which isolated from a nitrogen-removing biofilm. Remarkable change in a detection pattern of DNA bands on the gel was not confirmed with temperature shift in the DGGE analysis using bacterial universal primer set.

DISCUSSION

Although MAAOB inhabiting or anammox activity have been identified in some seawater-environment (1-4), there are only a few reports on the culture of MAAOB and little knowledge about their characteristics. In this study, we established a MAAOB culture system using the sediment samples of the sea-based waste disposal site and revealed the

effects of temperature on anammox activity and bacterial community in the MAAOB culture.

A few studies on the cultivation of MAAOB have been conducted under 25°C so far (22, 25). This is probably because MAAOB habitats in ocean environment are assumed to be under low temperature. On the other hand, we first tried a startup of MAAOB culture system at 30°C in this study because that the MAAOB have been cultured even at over 30°C in our previous study (28). However, only a little decreases of NH_4^+ and NO_2^- were observed for 50 days after beginning of culture as shown in Fig. 2, thus we tried to change the temperature from 30 to 25°C to promote enrichment of MAAOB. The both NH_4^+ and NO_2^- showed distinct decreases after the 60th day, suggesting lower than 30°C would be suitable for MAAOB growth. We therefore regarded 25°C as default temperature and conducted following studies.

The NO_2^- consumption on the 80th day and 115th day were 5 mg-N l⁻¹ and 19 mg-N l⁻¹, respectively as shown in Fig. 2, thus the net specific growth rate of MAAOB was estimated on the supposing that NO_2^- consumption (amount) would be proportional to the biomass amount. As a result, the net specific rate and doubling time of the MAAOB were calculated to be 0.039 d⁻¹ and 18 d. Although further study on the characteristics of MAAOB would be required, these values were roughly comparable with those of other anammox bacteria previously reported (20).

The MRRN value at startup period of the culture system was estimated at 1.0:1.15:0.15. This value is a little lower than that (1.0:1.32:0.26) reported by Strous et al. (15) which is frequently quoted as a criteria, but comparable with the value obtained in our previous study on MAAOB culture (1.0:1.1:0.1) (26). There may be some reasons for this difference, for example, that elementary composition of anammox biomass was different, endogenous or autotrophic denitrification by symbiotic bacteria was simultaneously occurred and so on. Whatever the reason, a discussion on the difference of MRRN value will require more detail information about enrichment-degree of MAAOB in the culture and more purified MAAOB biomass because that elemental composition biomass is needed to calculate more reliable MRRN value. Interestingly, change in MRRN value was observed with temperature shift whenever temperature was decreased or increased. In particular, average of MRRN was 1.0:2.4:0.11 at 37°C. The change of MRRN value with temperature shift was due to the increase of residual NH_4^+ concentration, which may suggest that denitrifying bacteria contributed to NO_2^- removal at higher temperature than 25°C. The optimal temperature for the MAAOB was estimated at 25-30°C in this study. This value is a little different from common values (more than 30°C) for anammox bacterial culture derived from a freshwater environment (21, 35). On the other hand, the optimal temperature for anammox reaction in the sediment sample taken from the coasts of Greenland is reported to be 12°C (24), thus the optimal temperature for MAAOB culture in this study would be regarded as an intermediary value of these. The activation energy was calculated to be 54.6 kJ mol⁻¹ from the results shown in Fig. 5. The values of activation energies in anammox culture have so far been reported in 33 to 94 kJ mol⁻¹ (21, 24) range. The value obtained in this study was almost comparable to the activation energy for arctic marine sediments (51 kJ mol⁻¹) (24). There have so far been some challenges of acclimation of terrestrial anammox bacteria to low temperature condition (21), however, they require higher activation energy at low temperature range. On the other hand, the MAAOB culture have low activation energy and moderately optimal temperature, therefore it would be useful to expand the application of anammox reaction to wastewater treatment under low temperature condition.

From the results of PCR-DGGE analysis on bacterial community analysis by PCR-DGGE, it was suggested that the temperature change would not affect on the bacterial community in the MAAOB culture. However, more detail and long-term investigation may be needed in order to reach a conclusion because that there is possibility that bacterial community change would require more time. In the meanwhile, a number of sulfur-related bacteria including sulfur oxidizing bacteria and sulfate reducing bacteria. In addition, we found sulfur reducing bacteria in MAAOB biomass in our previous study (28). Growth of sulfate-metabolizing bacteria is not strange because that the inoculum was seawater-sediment and sulfate concentration in the medium was higher than that of the culture for freshwater-anammox bacteria. However, some sulfate-reducing bacteria indentified from PCR-DGGE are suggested to be nitrate reducers (e.g., *Thiopfundum lithotrophica*). From these results, some kind of relationship between MAAOB and sulfur-metabolizing bacteria is speculated in the MAAOB culture.

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FIGURE LEGENDS

- Fig. 1. Change in nitrogen concentrations of NH_4^+ and NO_2^- in batch cultivation for fixation of marine anammox bacteria and reactor startup
- Fig. 2. Variation in nitrogen concentrations of NH_4^+ , NO_2^- and NO_3^- in the effluent with time. The result during preceding period (0 to 120 days) is shown in (a) and entire period (0 to 360 days) is shown in (b)
- Fig. 3. Relationship of NH_4^+ consumption rate and NO_3^- production rate to NH_4^+ consumption rate. Approximate curve (line) was obtained by the method of least squares. MRRN (mol-reaction ratio of nitrogen; consumption rate of NH_4^+ : consumption rate of NO_2^- : production rate of NO_3^-) was estimated at 1:1.15:0.15 from the slope of the approximate curve.
- Fig. 4. Change in the nitrogen concentrations of NH_4^+ , NO_2^- and NO_3^- , and the ratios of NH_4^+ consumption and NO_3^- production rates to NH_4^+ consumption rate with the temperature shift in the 5°C to 37°C range. Dot line and numbers indicate setting temperature and solid line indicates measured values.
- Fig. 5. Relationship between actual measured temperature and nitrogen removal rate.
- Fig. 6. Denaturing gradient gel electrophoresis (DGGE) result using (A) specific primer set for marine anammox bacteria targeting 16S rRNA sequence and (B) universal primer set for eubacteria targeting 16S rRNA sequence.
- Fig. 7. Phylogenetic tree showing the affiliation of 16S rRNA gene sequences of symbiotic bacterial clones derived from MAAOB culture. Clones are depicted in bold and each DNA band (a-h) on the DGGE gel are renamed as “US1107a” to “US1107h” in the tree. The tree was built based on neighbor-joining and confirmed by maximum parsimony analysis. The numbers on nodes indicate bootstrap values higher than 50%. The bar indicates 5% sequence divergence.