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Enrichment of Anaerobic Ammonium Oxidation (Anammox) Bacteria for Biological Nitrogen Removal of Wastewater

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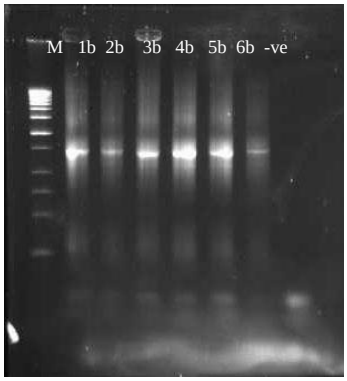
Graphical abstract



Abstract

Anaerobic ammonium oxidation (anammox) bacteria enrichment was explored for the potential application of ammonium rich wastewater removal. Samples of sludge from mature and young landfill leachate treatment plants were screened and used as inocula for anammox enrichment cultures. Enrichments were monitored for N-NH3, N-NO2- and N-NO3- to detect anammox potential activity. Six of the twelve enrichment cultures showed anammox activity after more than five months of enrichment period. All enrichment cultures that gave positive results were obtained from bottom part of sequencing batch reactor (SBR) lagoon indicating localization of anammox bacteria in anaerobic condition. Polymerase Chain Reaction (PCR) with specific primers targeting anammox and planctomycete were able to amplify the 16S rRNA sequence for anammox bacteria under PCR optimum condition. However, only three of six positive samples were successfully sequenced. DNA sequence analysis using NCBI (BLAST) and RDP showed that the anammox bacterial sequences of the investigated samples were identified as Candidatus Kuenenia stuttgartiensis with similarity of 100% (NCBI) and 99.3% (RDP).

Keywords: Anammox; biological nitrogen removal

Abstrak

Pengayaan bakteria anaerobic ammonium oxidation (anammox) dikaji untuk potensi aplikasi bagi rawatan air buangan yang mempunyai kandungan ammonium yang tinggi. Sampel enapcemar daripada loji rawatan cecair larut resap tapak kambusan sisa pepejal disaring dan digunakan sebagai inokulum untuk kultur pengayaan. Pemantauan N-NH3, N-NO2- and N-NO3- semasa pengayaan dilakukan untuk mengesan potensi aktiviti anammox. Enam daripada dua belas kultur pengayaan menunjukkan aktiviti anammox selepas lebih lima bulan tempoh pengayaan. Semua kultur pengayaan yang menunjukkan hasil yang positif adalah diperolehi dari bahagian bawah sequencing batch reactor (SBR) menggambarkan penempatan bakteria anammox di dalam keadaan anaerobik. Polymerase Chain Reaction (PCR) menggunakan primer spesifik mensasarkan bakteria anammox dan planctomycete mampu menggandakan jujukan 16S rRNA di bawah keadaan optima PCR. Walaubagaimanapun, hanya tiga daripada enam sampel yang positif berjaya dijujukkan. Analisis jujukan DNA menggunakan NCBI (BLAST) dan RDP menunjukkan jujukan bakteria anammox bagi sampel yang dikaji dikenalpasti sebagai Candidatus Kuenenia stuttgartiensis dengan 100% persamaan (NCBI) dan 99.3% (RDP).

Kata kunci: Anammox; penyingkiran nitrogen biologi

1.0 INTRODUCTION

Ammonium can be removed from waste/wastewater through biological, chemical and physical approaches. Among these methods, biological nitrogen removal is much preferred from economic and environmental point of view. The biological nitrogen removal commonly practiced at wastewater treatment requires carbon source as the electron donor.[2]

Among the drawbacks of biological nitrogen removal via nitrification and denitrification are they require energy for aeration and carbon sources as electron donor. As a consequence, in recent years, many studies have been focusing on more economic and efficient ways in removing ammonium from plant is nitrification and denitrification.[1] Nitrification is a two-step biological conversion of ammonia to nitrite and subsequently to nitrate which requires at least two moles of oxygen. The nitrate and/or nitrite produced are then reduced to nitrogen through a process known as denitrification. This process is anoxic and

wastewater. Among the potential technology that could be applied for biological nitrogen removal is anaerobic ammonium oxidation (anammox). This technology has a great impact on microbiology and engineering fields as the anammox bacteria is capable to convert ammonium to nitrogen with the presence of nitrite as the electron acceptor. The anammox might benefit the biological nitrogen removal system through the following; (i) suitable for removal of high-strength ammonium wastewater; (ii) energy saving as less oxygen is consumed and (iii) suitable for removal of ammonium wastewater that contain limited carbon source.

The bacterium responsible for anammox is a chemoautolithothroph and found to be planctomycete-like microorganisms. The anammox bacteria using CO_2 as the sole carbon source and its growth rate in enrichment cultures is extremely low with a doubling time of about three weeks.[3] Therefore, the main difficulty in developing anammox technology is the long period required to start up the anammox reactor due to the extremely slow growth rate of this microorganism. In this study, we attempt to enrich anammox bacteria from landfill leachate sludge. To date, very limited information on anammox bacteria enrichment has been reported for Malaysia. The presence of anammox bacteria was assessed using both chemical and molecular analyses. This finding would be of prominence important and served as a basis for application of anammox in ammonium rich wastewater treatment such as landfill leachate.

2.0 EXPERIMENTAL

2.1 Seed Sludge

The seed sludge for anammox enrichment was obtained from Air Hitam, Puchong and Jeram landfills operating an old and young landfill leachate, respectively. Sludge samples at Jeram Landfill namely 1a, 1b, 2a, 2b, 3a, 3b were collected from different points at the upper and bottom parts of the sequencing batch reactor (SBR). Whereas, the sludge samples obtained from Air Hitam landfill (4a, 4b, 5a, 5b, 6a, 6b) were collected from upper and lower parts of the SBR that received treated leachate from an ammonia stripping reactor. The sludge samples were washed several times with sterilized distilled water by centrifugation at 5000 rpm for 10-15 minutes until all N compounds remained low. The initial number of heterotrophs measured in all samples was about 1.2-2.3 x 10^5 CFU/ml. The characteristics of the sludge samples are summarized in Table 1.

2.2 Enrichment Medium And Condition

The anammox medium used for enrichment consisted of (in mg/L) 1050 $NaHCO_3$, 25 KH_2PO_4, 300 $CaCl_2$. $2H_2O$, 165 $MgCl_2.6H_2O$, 12 $FeSO_4.7H_2O$ and 7 EDTA. $2H_2O$ (pH 7.2-7.6) with 1.25 mL of trace element solution per litre.[4] The trace element solution contained (in g/L) of 15 EDTA, 0.43 $ZnSO_4.7H_2O$, 0.24 $CoCl_2.6H_2O$, 0.99 $MnCl_2.4H_2O$, 0.25 $CuSO_4.5H_2O$, 0.22 $NaMoO_4.2H_2O$, 0.19 $NiCl_2.2H_2O$, 0.21$NaSeO_4.10H_2O$, 0.014 H_3BO_4 and 0.050 $NaWO_4.2H_2O$.[5] The enrichment medium and the inoculums were prepared in Scott bottles with two exits, one with 0.25 µm filter for N_2 input and another for N_2 release. Prior to be used, the serum bottles, enrichment medium and inoculums were sparged with nitrogen gas for about 15 minutes to remove oxygen. The medium was once again sparged with nitrogen gas after sterilization to maintain anaerobic condition. Inoculation of sludge samples to the medium was carried out strictly under anaerobic condition in an anaerobic chamber. The enrichment medium was added into 120 ml serum bottle and prepared in triplicate for each sample with about 25% (v/v) of sludge samples. Three serum bottles containing medium were prepared without addition of sludge samples and served as control. All samples were placed in a shaking incubator in the dark at 37ºC. Since anammox bacteria are slow growers, sampling was performed once a week. Samples were subjected to analysis of ammonium (N-NH_3), nitrite (N-NO_2) and nitrate (N-NO_3^-). $NH_4Cl/NaNO_2$ was supplied when ammonium or nitrite was consumed to maintain anammox activity. During the initial steps of the enrichment procedure, nitrate ($NaNO_3$) was also added to a final concentration of 20 mg/L in order to favor the elimination of degradable biomass by denitrifying bacteria and to avoid the production of H_2S by sulfate reducing bacteria.

2.3 Nitrogen Compound Analysis

N-NH_3 and N-NO_2^- were measured colorimetrically using Nessler and diazotization methods, respectively. N-NO_3^- was analyzed at 200 nm and 220 nm of wavelength using UV spectrophotometer. All analyses were performed according to standard method.[6]

2.4 Nucleic Acid Extraction And PCR Amplification

DNA extraction was carried out by using PowerSoil DNA Kit (Cat # 12800-50; Mo Bio Laboratory Inc., USA). Partial 16S rDNA fragments of the entire bacterial community were amplified from the extracted genomic DNA by PCR using the primer 27F (AGA GTT TGA TCC TGG CTC AG) with 1492R (ACG GTT ACC TTG TTA CGA CTT). To amplify partial 16S rDNA fragments from only anammox bacteria, PCR was performed using a planctomycete-specific primer, Pla46F, (GGA TTA GGC ATG CAA GTC) coupled with the anammox specific primer Amx368R, (CCT TTC GGG CAT TGC GAA).[4] The PCR ingredients (total volume, 25 µL, Fermentas ExTaq DNA polymerase) consisted of 2.5 µL 10× PCR buffer, 0.2 µL ExTaq DNA polymerase, 0.5 µL 5 mM dNTP, 2.5 µL $MgCl_2$ (25 mM), 0.5 µL of each primer, and 1.0 µL of the above template and an adequate amount of sterile distilled water was added to reach the desired volume. The PCR program consisted of an initial 4 min denaturation step at 95ºC followed by 35 cycles of repeated denaturation at 95ºC for 45 sec, annealing at 56ºC for 45 sec, and extension at 70ºC for 1 min followed by a final extension at 72ºC for 3 min. The samples were amplified using a PCR Thermal Cycler Dice, Takara, Japan.

2.5 PCR Purification And Sequencing

PCR purification was performed using Agencourt Ampure Xp, Beckman Coulter Genomics. 40 µL of PCR product was added with agencourt onto SPRI Plate 96R for 10 mins. The supernatant was then washed with 70% absolute ethanol before dried at room temperature. The purified PCR product was viewed by electrophoresis and sent to First BASE Laboratories for sequencing. Primer 750R for soil and environmental sample was used in sequencing. Sequence similarity searches were conducted using the BLAST (Basic Local Alignment Search Tool) network service of the GenBank database through the website (http://www.ncbi. nlm.nih.gov/) and Ribosomal Database Project II (RDP) through the website (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) to identify the nearest relatives of the partially sequenced 16S rRNA genes.

Table 1 Source of seed sludge and its characteristics

Enrichment culture	Sampling point	N-NH_4^+ (mg/L)	N-NO_3^- (mg/L)	N-NO_2^- (mg/L)	VSS (mg/L)	Heterotrophic count (CFU/ml)
1a, 2a, 3a	Upper part of SBR, Jeram landfill	5.98 -7.50	163 -180	27.5-30.3	2.2-2.7 × 10^4	2.3 x 10^5
1b, 2b, 3b	Lower part of SBR, Jeram landfill	5.66-7.83	150-188	25.0-26.3	2.9-3.3 x 10^5	1.7 x 10^5
4a, 5a, 6a	Upper part of SBR, Air Hitam landfill	8.13-9.1	4.9-5.6	Below detection limit	1.4-1.6 x 10^4	1.2 x 10^5
4b, 5b, 6b	Lower part of SBR, Air Hitam landfill	7.81-8.7	4.3-4.8	Below detection limit	1.8-2.3 x 10^5	1.5 x 10^5

3.0 RESULTS AND DISCUSSION

3.1 Monitoring of Anammox Activity in Enrichment Cultures

Sludge samples from different environments were used as inoculum for anammox enrichment cultures. The changes of N-compounds particularly N-NH_3, N-NO_2^- and N-NO_3^- were monitored throughout the enrichment study and to be considered as indicative for anammox activity (Table 2). The N-compounds consumption was detected for the potential activity of anammox based on the simultaneous decrease of N-NO_2^- and N-NH_3 and a slight increase of N-NO_3^- concentration in the medium. About 1:1.32 ratio of and NH_4^+ and NO_2^- is consumed and 0.26 NO_3^- produced in anammox reaction according to the following stoichiometry:[7]

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 0.066CH_2O_{0.5}N_{0.15} + 1.02N_2 + 0.26NO_3^- + 2.03H_2O$$

Enrichment of anammox cultures were performed for more than five months. The long period required for enrichment of anammox bacteria were commonly reported due to its slow growth rate.[3,7] Six out of twelve enrichment cultures showed potential anammox activity determined by decreasing amount of N-NH_3 and N-NO_2^- and a slight increase of N-NO_3^- concentrations (Table 2). In all successful anammox enrichment cultures, denitrification activity was also detected at least for the first four months. This might be due to the presence of heterotrophic bacteria or denitrifiers that convert nitrate and organic carbon from the sludge sample to nitrogen molecule. All samples with anammox activity were originated from bottom part of both Jeram and Air Hitam landfill leachate treatment plants (1b, 2b, 3b, 4b, 5b, 6b). Jeram landfill leachate treatment plant is operating young leachate characterized by high organic and ammonium concentrations. Meanwhile, Air Hitam landfill leachate contained low organic and high ammonium concentrations. Except for Air Hitam landfill, all sludge samples were obtained from a single biological treatment plant and SBR lagoon. At Air Hitam landfill, the high strength ammonium in leachate was treated by a combination of chemical and biological means using ammonia stripping before further removal in the SBR lagoon. In the SBR lagoon the biological oxidation was carried out through submersible aeration system indicating that the bottom part of lagoon might be more anaerobic as compared with the upper part.

Table 2 Metabolic activities detected in the enrichments from changes in N-compounds

Enrichment	Ammonium (N-NH_4^+)	Nitrite (N-NO_2^-)	Nitrate (N-NO_3^-)	Metabolism
1a	No changes	Decrease	No changes	Denitrification
2a	No changes	Decrease	No changes	Denitrification
3a	No changes	Decrease	No changes	Denitrification
4a	No changes	Decrease	Decrease	Denitrification
5a	No changes	Decrease	Decrease	Denitrification
6a	No changes	Decrease	Decrease	Denitrification
1b	Decrease	Decrease	Increase	Anammox
2b	Decrease	Decrease	Increase	Anammox
3b	Decrease	Decrease	Increase	Anammox
4b	Decrease	Decrease	Increase	Anammox
5b	Decrease	Decrease	Increase	Anammox
6b	Decrease	Decrease	Increase	Anammox
Control	No changes	No changes	No changes	No nitrogen removal activity

Enrichments 1a, 2a, 3a, 4a, 5a and 6a did not demonstrate any potential anammox activity. Throughout the enrichment period, ammonium consumption was not observed in these enrichment cultures. However, consumption of either nitrite or nitrate or both of them represent the occurrence of denitrification activity. Detection of anammox activity in enrichment cultures with sludge sample from bottom part of the SBR lagoon might indicate that localization of anammox bacteria in wastewater treatment plant (WTP) was influenced by dissolved oxygen concentration.

3.2 Identification of Anammox Bacteria from Enrichment Cultures

In addition to activity measurement, anammox bacteria were also detected and identified using molecular analysis based on PCR. Total DNA of all samples was successfully extracted using PowerSoil DNA Kit. The initial detection by PCR was performed using universal primer 27F together with 1492R. Partial 16S rDNA fragments of the entire bacterial community were successfully amplified from the extracted genomic DNA by PCR using the universal primers. About 1.25 kb and 1.3 kb of rDNA were detected from enrichment cultures 1a, 2a, 3a, 4a, 5a, 6a (upper part of SBR) and 1b, 2b, 3b, 4b, 5b, 6b (lower part of SBR), respectively at optimum PCR condition. Following the PCR amplification using bacterial universal primer, amplification of 16S rDNA fragments from only anammox bacteria using primers Pla46F and Amx368R targeting planctomycete and anammox bacteria, respectively was carried out. PCR amplification with specific primers for planctomycete and anammox bacteria for enrichment cultures 1a, 2a, 3a, 4a, 5a, 6a were tested to different annealing temperature gradient (53°C to 61°C). However, none of these PCR conditions show any PCR band as viewed through electrophoresis. DNA extracted from enrichment cultures 1b, 2b, 3b, 4b, 5b and 6b were also subjected to PCR amplification for anammox bacteria detection. Under optimum PCR condition as described in methodology, the amplification produced libraries of partial anammox bacteria 16S rRNA genes for all enrichment cultures (1b, 2b, 3b, 4b, 5b, 6b), with size of about 1300 bp (Figure 1) similar to the size of anammox bacteria reported elsewhere.[8] The PCR products were further purified and sequenced for identification of the anammox bacteria.

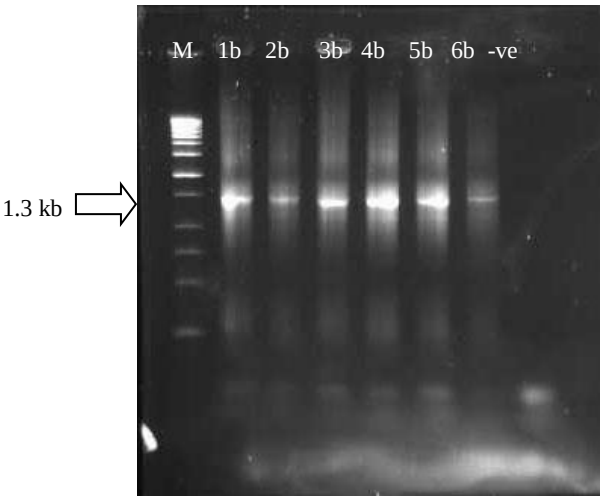


Figure 1 PCR amplification with specific primer for anammox bacteria Pla46 and Amx368R at annealing temperature of 56°C shows band with size of 1.3 kB

DNA sequence analysis using NCBI (BLAST) and RDP showed that the anammox bacterial sequences of the investigated samples (4b, 5b and 6b) were all closely related to the *Candidatus Kuenenia stuttgartiensis* (Table 3) with similarity of 100% (NCBI) and 99.3% (RDP) (GenBank accession no HM769654.1). However, another three samples (1b, 2b and 3b) were not able to be sequenced. *Candidatus Kuenenia stuttgartiensis* was initially found in biofilm reactors in Stuttgart, Germany and have also been identified from other wastewater treatment plant.[9,10] Results of this study show that the anammox bacteria might be detected in the samples obtained from bottom part of the SBR as compared with the samples from the upper part of the SBR lagoon. It was believed that anaerobic condition at the bottom part of SBR lagoon provides favorable condition for anammox bacteria to grow. In addition, anammox bacteria might play an important role in ammonium removal and has syntrophic association with other nitrogen bacterial community such as nitrifiers and denitrifiers in the aerated lagoon.

Table 3 Sequencing results using NCBI (BLAST) and/or Ribosomal Database Project (RDP)

Sample	Accession	Description	Source	Similarity (%)
4b	HM769654.1 (NCBI)	*Candidatus Kuenenia sp.* enrichment culture clone RAS-SJ-1	Environmental sample	100
5b	HM769654.1 (NCBI)	*Candidatus Kuenenia sp.* enrichment culture clone RAS-SJ-1	Environmental sample	100
6b	HM769654.1 (NCBI)	*Candidatus Kuenenia sp.* enrichment culture clone RAS-SJ-1	Environmental sample	100
4b	CT573071 (RDP)	*Candidatus Kuenenia stuttgartiensis*	Environmental sample	99.3
5b	CT573071 (RDP)	*Candidatus Kuenenia stuttgartiensis*	Environmental sample	99.3
6b	CT573071 (RDP)	*Candidatus Kuenenia stuttgartiensis*	Environmental sample	99.3

4.0 CONCLUSION

The anammox bacteria were successfully enriched in six out of twelve of the enrichment cultures. Sequence analyses results of the 16S rDNA revealed high genetic similarity (100%) between the three enrichment cultures obtained from the bottom part of Air Hitam landfill leachate treatment plant with *Candidatus Kuenenia stuttgartiensis*. This finding is of significant importance for future application of anammox bacteria in ammonium rich wastewater treatment such as landfill leachate.

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