# ENRICHMENT OF ANAEROBIC AMMONIUM OXIDATION BACTERIA USING ANAEROBIC UP-FLOW BIOFILM COLUMN REACTOR

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## ABSTRACT

This research aims to enrich anaerobic ammonium oxidation (anammox) culture which capable of oxidizing ammonium to dinitrogen gas in a laboratory-scale anaerobic up-flow biofilm column reactor. A 16S rDNA gene analysis targeting planctomycetes-anammox bacteria was performed to screen anammox microorganism from sludge samples obtained from five sources of wastewater around Perak and Kuala Selangor. Anammox enrichment was carried out for 180 days in 1.0 L anaerobic up-flow biofilm column reactors with three different mode of feeding; (i) batch, (ii) fed-batch and (iii) continuous. The $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ concentrations were monitored throughout the enrichment period. The enriched anammox was identified using 16S rDNA and fluorescence *in situ* hybridization (FISH) techniques. After amplification using primer pair Pla46F-Amx368R, it was found that the genomic DNA from sludge of Jeram sanitary landfill showed a similarity in 16S rDNA gene to uncultured anammox bacteria clone AMX-MB05-10 and been used in the following enrichment study. Changes in $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ concentrations indicated the feasibility of anammox culture enrichment in all reactors. Reactor with continuous feeding mode showed an effective consumption of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ with the highest specific $N\text{-}NH_4^+$ removal of 0.34 g $N\text{-}NH_4^+$g/VSS/d at NLR of 0.33 kg $N/m^3$/d. The enriched anammox culture showed closed similarity to "*Candidatus* Kuenenia sp." and *"Candidatus* Jettenia sp." genus. FISH analysis with rhodamine-stained oligonucleotide probes targeting 16S rDNA gene of anammox bacteria further confirmed the existence of anammox population in the enriched culture for all feeding mode. In conclusion, the anammox culture was successfully enriched in anaerobic up-flow biofilm column reactor by appropriate selection of seeding sludge. Reactor with continuous feeding mode is the most effective for anammox enrichment. The implication of this study is a strategic way to enrich the anammox culture for application in biological nitrogen removal of wastewater.

# PENGAYAAN BAKTERIA PENGOKSIDAAN AMMONIUM SECARA ANAEROBIK MENGGUNAKAN REAKTOR *ANAEROBIC UP-FLOW BIOFILM COLUMN*

## ABSTRAK

Kajian ini bertujuan untuk memperkaya kultur pengoksidaan ammonium secara anaerobik (*anammox*) yang mampu mengoksida ammonium kepada gas dinitrogen di dalam reaktor *anaerobic up-flow biofilm column* berskala makmal. Analisis gen 16S rDNA yang menyasarkan bakteria *planctomycetes-anammox* telah dijalankan bagi menyaring mikroorganisma *anammox* daripada enapcemar yang diperoleh dari lima sumber air buangan sekitar Perak dan Kuala Selangor. Pengayaan *anammox* telah dijalankan selama 180 hari di dalam reaktor *anaerobic up-flow biofilm column* 1.0 L dengan tiga mod suapan yang berbeza; (i) kelompok, (ii) sesekelompok dan (iii) selanjar. Kepekatan $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ telah dipantau sepanjang tempoh pengayaan. Bakteria *anammox* yang diperkaya telah dikenalpasti menggunakan analisis 16S rDNA dan teknik *fluorescence in-situ hybridization (FISH).* Selepas amplifikasi menggunakan pasangan primer Pla46F-Amx368R, DNA genomik daripada enapcemar tapak pelupusan sanitari Jeram menunjukkan persamaan kepada klon tidak kultur bakteria anammox AMX-MB05-10 dan telah digunakan dalam kajian pengayaan seterusnya. Perubahan pada kepekatan $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ menunjukkan keberkesanan pengayaan kultur *anammox* di dalam semua reaktor. Reaktor dengan mod suapan selanjar menunjukkan penggunaan $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ yang berkesan dengan penyingkiran $N\text{-}NH_4^+$ spesifik tertinggi sebanyak 0.34 g $N\text{-}NH_4^+$/gVSS/d pada NLR 0.33 kg $N/m^3$/d. Kultur *anammox* yang telah diperkaya menunjukkan persamaan rapat dengan genus "*Candidatus* Kuenenia sp." dan "*Candidatus* Jettenia sp.". Analisis *FISH* menggunakan prob oligonukleotida diwarna-*rhodamine* yang menyasarkan gen 16S rDNA bakteria *anammox*, seterusnya membuktikan kehadiran populasi *anammox* di dalam kultur untuk kesemua mod suapan. Kesimpulannya, kultur *anammox* telah berjaya diperkaya menggunakan reaktor *anaerobic up-flow biofilm column* melalui pemilihan enapcemar yang sesuai. Reaktor dengan mod suapan selanjar adalah paling efektif bagi pengayaan bakteria *anammox*. Implikasi kajian ini adalah satu kaedah strategik untuk memperkaya kultur *anammox* bagi penggunaan dalam penyingkiran nitrogen dari air buangan secara biologi.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AnMBR | Anaerobic membrane bioreactor |
| AOB | Ammonia-oxidizing bacteria |
| BLAST | Basic local alignment search tool |
| BNR | Biological nitrogen removal |
| CANON | Completely autotrophic nitrogen removal over nitrite |
| COD | Chemical oxygen demand |
| DGGE | Denaturing gradient gel electrophoresis |
| DHS | Down flow hanging sponge |
| DNA | Deoxyribonucleic acid |
| DO | Dissolved oxygen |
| EGSB | Expanded granular sludge bed |
| FBR | Fluidized bed reactor |
| FISH | Fluorescence in situ hybridization |
| HDPE | High density polyethylene |
| HRT | Hydraulic retention time |
| LDPE | Low density polyethylene |
| MBR | Membrane bioreactor |
| MSBR | Membrane sequencing batch reactor |
| MSW | Municipal solid waste |
| NCBI | National center for biotechnology information |
| NLR | Nitrogen loading rate |

| | |
|---|---|
| NOB | Nitrite-oxidizing bacteria |
| NRR | Nitrogen removal rate |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| POME | Palm oil mill effluent |
| PVDF | Polyvinylidene fluoride |
| RBC | Rotating biological contactor |
| SBR | Sequencing batch reactor |
| SEM | Scanning electron microscopy |
| SHARON | Single reactor system for high rate ammonium removal over nitrite |
| TEM | Transmission electron microscope |
| TN | Total nitrogen |
| TSS | Total suspended solid |
| UASB | Up-flow anaerobic sludge blanket |
| UBF | Up-flow biofilter |
| VSS | Volatile suspended solid |
| WWTP | Wastewater treatment plant |

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# CHAPTER 1

# INTRODUCTION



## 1.1 Background of study

Municipal solid waste (MSW) landfills and industrial activities have contributed to the gradual increase of ammoniacal nitrogen rich wastewater which is a toxic pollutant to the aquatic life. Excessive nitrogen level in water promotes excessive growth of green algae and cyanobacteria. Disposal of the highly nitrogenous wastewater into surface water bodies may lead to serious environmental problems such as eutrophication and oxygen depletion (Banihani, Hadadin, & Jamrah, 2012). These problems have become a major threat towards aquatic organisms. Therefore the removal of the wastewater constituents is a major concern in environmental engineering and ecosystem protection.

Wastewater constituents from treatment plants can be removed either by chemical, biological or physical approaches. Biological nitrogen removal (BNR) process is more favoured for nitrogen removal due to its advantages from economic and environmental point of view. The commonly practiced BNR system for wastewater treatment involves sequential autotrophic nitrification and heterotrophic denitrification that requires separate aerobic and anaerobic units for treatment. This process is highly dependent on the supply of organic carbon source. The conventional nitrification-denitrification process is suitable in treating wastewaters with high concentration of biodegradable carbon, however it is uneconomical in treating wastewaters with low ratios of carbon and nitrogen, for example in anaerobic sludge digestion effluent.

Nitrification is a two steps biological process that involves the oxidation of ammonium to nitrite followed by the oxidation of nitrite to nitrate in aerobic condition (Equations 1 and 2). This aerobic process was aided by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Meanwhile, denitrification is a nitrate reduction process which is facilitated by heterotrophic anaerobic (denitrifying) bacteria to produce dinitrogen gas (Equation 3). This heterotrophic process requires organic carbon sources as electron donor (Koeve & Kähler, 2010).

$$NH_4^+ + 1.5O_2 \xrightarrow{AOB} NO_2^- + 2H^+ + H_2O \quad (1)$$

$$NO_2^- + 0.5O_2 \xrightarrow{NOB} NO_3^- \quad (2)$$

$$6NO_3 + 5CH_3OH + H_2CO_3 \xrightarrow{denitrifiers} 3N_2 + 8H_2O + 6HCO_3^- \quad (3)$$

Anaerobic ammonium oxidation (anammox) process was first discovered in a denitrifying fluidized bed reactor in the early 21st century in Netherlands (Mulder, Van de Graaf, Robertson, & Kuenen, 1995). It is a novel process of BNR from wastewater which offers a more sustainable, promising and economical alternative compared to the conventional nitrification-denitrification system (Mulder et al., 1995; Van de Graaf et al., 1995). Anammox process is performed by autotrophic planctomycetes bacteria which uses ammonium as electron donor (energy source) and nitrite as electron acceptor in the process of ammonium oxidation to produce dinitrogen gas in anaerobic condition (Equation 4).

$$NH_4^+ + NO_2^- \xrightarrow{Anammox} N_2 + NO_3^- \quad (4)$$



The process is capable of removing nitrite and ammonium simultaneously from wastewater without the presence of organic carbon supplementation and aeration system. In fact, low yield of the anammox bacteria due to its long doubling time (10-12 days) (Ibrahim, Yusof, Mohd Yusoff, & Hassan, 2016) has contributed to lower sludge production compared to conventional nitrification-denitrification system and hence contributes to the substantially lower operational costs of the system (Banihani et al., 2012).

Anammox has been applied in laboratory scale as well as full scale anammox reactor to treat various kind of wastewater such as landfill leachate (Scaglione, Ruscalleda, Ficara, Balaguer, & Colprim, 2012), digester liquor (Furukawa et al., 2009), pig manure effluents (Molinuevo, García, Karakashev, & Angelidaki, 2009), turtle breeding wastewater (Chen, Huang, Lei, Zhang, & Wu, 2013) and pharmaceutical wastewater

(Tang et al., 2011). Most recent data indicated that there are more than 100 full-scale anammox reactors which have been successfully implemented in the early 2015 worldwide (Ali et al., 2015; Lackner et al., 2014).

Anammox application has been reported for a super high-rate anammox performance with nitrogen removal rate of up to 76.7 kg/$m^3$/d in an up-flow anaerobic sludge bed (UASB) reactors (Tang et al., 2011). Despite the advantages, long start-up time of anammox reaction due to its extremely slow growth rate of the bacteria (0.072/days at 32 ºC) has contributed to the difficulty in application of the system in wastewater treatment (Anjali & Sabumon, 2014). Thus the eminent challenge is to deal with the slow-growing bacteria.



Many efforts have been made by researchers to accelerate the start-up time of anammox reaction during anammox enrichment. It is believed that proper selection of inoculum, appropriate reactor configuration system and optimal operational conditions are capable to enhance the anammox reaction therefore shorten the start-up time of the anammox activity (Li, Zhou, Ma, Huang, & Xu, 2012). Enrichment of anammox bacteria was carried out by monitoring the chemical nitrogen transformations (Dapena-Mora, 2004) and by studying the microbial eco-physiology through molecular biology techniques (Penton, Devol, & Tedje, 2006).

The present study practiced anammox enrichment in anaerobic up-flow biofilm reactor with non-woven fabric as biomass carrier by applying three different feeding

modes; batch, fed-batch and continuous. These feeding modes were studied in order to shorten the start-up duration of anammox activity. The experimental design comprises of three main stages of the experiment: (i) selection of seeding sludge for anammox enrichment, (ii) monitoring the feasibility of anammox enrichment in anaerobic up-flow biofilm reactor, and (iii) identification of the enriched anammox bacteria.

## 1.2 Problem statement

### 1.2.1 Nitrification-denitrification system requires high cost of operation

Conventional nitrification-denitrification system can be costly and complicated especially when dealing with high strength nitrogenous wastewater. High-strength ammonium wastewater is believed could inhibit the nitrification process since high concentration of free ammonia ($N\text{-}NH_3$) potentially inhibits nitrification. Municipal landfill leachate typically contain extremely high ammonium concentration that may reach up to ~1000 mg/l $N\text{-}NH_4^+$ (Kim, Lee, & Keller, 2006). AOB and NOB which aids the conversion of ammonia and nitrite to nitrate and nitrogen in nitrogen removal process is altered when exposed to high ammonia concentration. NOB was reported to be more sensitive to high concentration of free ammonia in comparison to AOB. $N\text{-}NH_3$ concentration of 0.1-1.0 mg/l can possibly inhibit the NOB whereas 10-150 mg/l of $N\text{-}NH_3$ can negatively affect the AOB (Kim et al., 2006).

Consequently, the inhibition of the AOB and NOB activity leads to ammonium and nitrite accumulation in nitrification-denitrification system and thus promotes an inefficient operating system. This problem limits the application of nitrification-denitrification when dealing with strong wastewater with high ammonium concentration. For that reason, ammonia concentration should be maintained at low levels in this system in order to avoid the inhibition effect and optimization of the nitrification process. Moreover, nitrification and denitrification process requires two separate subunits since the process demands for a different operational conditions. Nitrification requires oxygen while denitrification process take place in anaerobic condition.

Apart from that, among the shortcomings of the conventional nitrification-denitrification system include the requirement of oxygen supply for nitrification and supplementation of organic carbon source for the subsequent denitrification step. High power of energy is also required for aeration in nitrification step, meanwhile the denitrification step depends on supplementation of readily biodegradable organic carbon. Supplementation of organic carbon is an important factor for a successful denitrification process especially when dealing with high strength nitrogenous wastewater with low carbon to nitrogen (C/N) ratio in which only small amounts of biologically-degradable carbon compounds are available (Chamchoi, Nitisoravut, & Schmidt, 2008; Kim et al., 2006).

The conventional BNR system demands a high operational cost to comply with the requirement of nitrification-denitrification process. Anaerobic process is an alternative

solution to replace the conventional nitrification-denitrification system as it is more promising, cost-effective and sustainable. According to an energy balance comparison study for a treatment of high-strength wastewater at 20 ºC, 1.9 x $10^6$ kJ/d of electrical energy is required for aerobic process (Metcalf & Eddy, 2003; Chen et al., 2011) whereas the anaerobic process produces a total energy of 12.5 x $10^6$ kJ/d. Anaerobic process itself is a net energy producer instead of energy consumer, as in the case of aerobic process (Metcalf & Eddy, 2003).

### 1.2.2 Anammox process requires long start up period

Recently, research on anammox system has been developing rapidly due to its advantages. Despite of its arising popularity, the extremely slow growth rate of the anammox bacteria has contributed to the difficulty in its enrichment process. Anammox enrichment process takes a very long start-up time for anammox activity to take place which may take between 105 to 420 days to occur due to the long doubling time of the anammox bacteria (10-12 days) even at its optimal condition (Ibrahim et al., 2016; Zhou & Yao, 2010). This limitation may be a frustrating effort for researchers especially for those dealing with it for the first time.

Moreover, anammox enrichment application is practically limited due to sludge wash-out problem. A continuous bioreactor system with improper reactor configuration and without the application of supporting material for biomass retaining process will lead to the loss of anammox bacteria in sludge together with the effluent. Loss of sludge fraction