Treatment Capability and Microbial Community of Anammox Process Using PVA Gel Beads as Biomass Carriers

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Abstract

The start-up and treatment ability of a stirred tank anammox reactor (STAR) using PVA gel as biomass carrier were systematically investigated. PVA gels, as a new kind of biomass carrier, exhibited satisfactory treatment ability. Experimental results showed that a maximum nitrogen removal rate of 4.3kg/m³/d was achieved after 305 days of operation. Different operational strategy indicated that there was limited effect of influent concentration on the treatment ability of the anammox sludge, while the change of HRT has a positive effect on the treatment ability of the anammox sludge. The effluent SS level was relatively low, with an average of about 10mg/L, which indicated the slight loss of anammox sludge. Stereo microscope and SEM observation showed that anammox sludge gradually and firmly attached on the surface of the PVA gels, showing the effectiveness of PVA gel as the biomass carrier of anammox sludge. DNA analysis showed anammox bacterium KSU-1 was dominant in the anammox reactor, further confirming the effectiveness of using PVA gel bead to culture anammox bacteria.

Key words: anammox; PVA gel; nitrogen removal; stirred tank anammox reactor (STAR)

INTRODUCTION

The anaerobic ammonium oxidation (anammox) is a novel and promising nitrogen removal process which has received considerable attention.¹⁾ It involves the oxidation of ammonium under anoxic conditions with nitrite as the electron acceptor and nitrogen gas as the main product. This autotrophic process allows over 50% of reduction in oxygen supply and no organic carbon supplementation.

However, the application of anammox process is limited by its long start-up periods due to slow growth rates and low biomass yields.²⁾ Another typical drawback is the flotation of anammox granular sludge under high loading rates, which was observed by

Strous *et al.*³⁾ and many other researchers. As a result, present studies mainly focus on the development and application of different kinds of biomass carriers. Researchers have shown that biomass carriers are effective in enabling the growth and maintenance of slow-growing micro-organisms and reducing bacterial loss.

In the literature, different types of carrier materials were studied, such as polyester non-woven carriers used by Furukawa, *et al.*⁴⁾, malt ceramics used by Tran *et al.*⁵⁾, polyethylene sponge strips used by Zhang *et al.*⁶⁾, zeolite particles used by Fernández *et al.*⁷⁾, glass beads and sand used by Strous *et al.*⁸⁾ and PEG gel carriers used by Isaka *et al.*⁹⁾. Previously, PVA gel has also been studied as a carrier in Stirred reactor¹⁰⁾,

packed-bed reactor¹¹⁾ and fluidized-bed reactor¹²⁾. It is reported that PVA gel beads have a porous microstructure that allows for microorganisms to penetrate and colonize throughout the gel material, thus providing favorable conditions for retention and cultivation of slowly growing anaerobic microorganisms.¹⁰⁾ This study focused on nitrogen removal capacity of stirred tank reactor using PVA gel as a biomass carrier. Results showed that highly concentrated anammox bacteria were attached to the PVA carriers and that stable nitrogen removal rate was achieved.

MATERIALS AND METHODS

Properties and pretreatment of PVA gels PVA gels supplied by Kuraray Co. (Osaka, Japan) were used in this study as biomass carriers. The PVA gels have a diameter of 4 mm with a solids content of about 10% and a specific gravity of 1.025, making them easy to suspend in water. The PVA gels are hydrophilic in nature and have a porous structure with a continuum of passages 10 to 20 μm in throughout each bead.¹¹⁾ Before installing them into the reactor, the PVA gels were exposed in the effluent from an upflow anammox column reactor using nonwoven as biomass carrier in our laboratory¹³⁾ for about two months, so that anammox bacteria might attach on the PVA gels.

Experimental setup A stirred tank anammox reactor (STAR) was used and the configuration of the reactor is depicted schematically in Fig. 1. The reaction zone was 1.4 l and the stirring speed was kept between 70 ~ 120rpm. The reactor was covered with black cloth to avoid light

inhibition and operated under the temperature of 33-35°C, thermostatically controlled by a water jacket, and influent pH of 7.5 ~ 8. Influent DO concentration was maintained under 0.5mg/l by flushing the tank with nitrogen gas¹⁴⁾. The total volume of PVA gels supplied to the reactor was 0.42l, accounting for 30% of the total volume of the reactor.

The reactor was fed with synthetic wastewater consisting of: $(\text{NH}_4)_2\text{SO}_4$ (75-380 mg/l), NaNO_2 (75-380 mg/l), KHCO_3 (125 mg/l), KH_2PO_4 (54 mg/l), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (9 mg/l), $\text{EDTA} \cdot 2\text{Na}$ (5mg/l) and a solution (2ml/l) of mineral salt¹⁾. The ammonium to nitrite molar ratio in the feeding media was fixed at 1.0.

Analytical methods NO_2^- -N, NO_3^- -N and NH_4^+ -N were measured according to standard methods, as set out by the American Public Health Association.¹⁵⁾ NO_2^- -N and NH_4^+ -N were measured by using the different colorimetric method and NO_3^- -N was analyzed by using the UV spectrophotometric method. pH and DO were measured by standard electrode method. The concentrations of solids, determined as total suspended solids (TSS) and the fraction corresponding to the biomass as volatile suspended solids (MLVSS) of the sludge were determined according to Standard Methods.

SEM observation The anammox granular sludge was observed using a scanning electron microscope (SEM). Specimen preparation was as follows. Firstly, it was fixed by 2.5% glutaraldehyde fixative solution and 1% osmic acid solution, and then washed with phosphate buffer. Secondly, the specimen was exposed to sequential ethanol dehydration (including 10%, 30%, 50%, 70%, 90%, 95% and 99.5%). After dehydration, it was dried at critical point. Finally, the specimen was sputtered with gold and then examined under a scanning electron microscope (JEOL, JAPAN).

DNA extraction and PCR amplification A sample for 16S rRNA gene analysis was taken from the reactor at day 291. 10 PVA gel beads were first ground with a pestle under liquid nitrogen. Meta-genomic DNA was extracted using an ISOIL kit (Wako, Osaka, Japan) according to the manufacturer's

Fig.1. Configuration of reactor used for continuous anammox treatment.

instructions. The amplification of 16S rRNA gene was performed with Phusion High-Fidelity DNA polymerase (FINNZYMES Finland) using conserved eubacterial primers 6F (forward primer: 5'-GGAGAGTTAGATCTT GGCTCAG-3') and 1492r (reverse primer: 5'-GGTTACCTTG TTACGACT-3')^{16,17}. PCR was carried out according to the following thermocycling parameters: 30 s initial denaturation at 98°C, 25 cycles of 10 s at 98°C, 20 s at 51°C, 35 s at 72°C, and 5 min final elongation at 72°C. The amplified products were electrophoresed on a 1% agarose gel and the excised fragments were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, USA).

Cloning and sequencing of 16S rRNA gene The purified fragments were ligated into the EcoRV site of pBluescript II KS+ (Stratagene, USA), and *Escherichia coli* DH10B was transformed using the constructed plasmids. White colonies including the insert were randomly chosen and the plasmids were extracted by the alkaline method. The nucleotide sequences were determined with a 3130xl genetic analyzer and BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequences determined in this study were compared with the sequences in the nr-database using the basic local alignment search tool (BLAST) program available on the NCBI web site.

RESULTS AND DISCUSSION

STAR performance According to the nitrogen removal performance of the STAR, the operation could be divided into three phases.

During phase 1 (0-195 days), the reactor was started up by inoculating it with cultivated PVA gels, which accounted for 30% of the reactor volume. The influent T-N concentration was set to 100mg/l, (Fig. 2) The ratio of influent $\text{NH}_4^+\text{-N}$ to $\text{NO}_2^-\text{-N}$ ratio was set to 1:1. HRT was set at 10.5h. The initial NLR set at 0.3kg/m³/d. (Fig. 3) The reactor shows stable and satisfactory treatment performance even when the influent T-N concentration was increased to 160mg/l. The removal efficiencies of T-N, $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ were 86%, 98% and 95%,

respectively. (Fig. 4) Therefore, after four days of operation, HRT was reduced to 7h. NLR increased to 0.5kg/m³/d spontaneously. Then, the nitrogen removal rate was gradually increased by increasing the influent T-N concentration with HRT of 10.5h. When the nitrogen loading rate reached to 0.8 kg/m³/d, the operation was inhibited. However, the reactor performance was restored very quickly within a week by reducing the influent T-N concentration. Till day 57, the nitrogen loading rate was restored to 0.8 kg/m³/d. It could be concluded that reactor has been successfully started up. Hereafter, NLR was increased stepwise and reached 3.49 kg/m³/d on day 185, with the influent T-N concentration of 400 mg/l and HRT of 2.7h. The removal efficiencies of T-N, $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ were 80%, 84% and 92%, respectively.

It should be noted that, no anammox sludge was added to the reactor in the start-up phase. However, stereo microscope and SEM observation done on day 41 showed clearly the attachment of anammox biomass on the surface of the PVA gels. (Fig. 6e, 6f and 7b) This may indicate that anammox biomass could attach on PVA gel carriers in a relatively short time. In previous study, Strous *et al.* reported that anammox sludge easily adheres to any solid surface such as the inner surface of a reactor and had used sand particles as carrier material to investigate the growth of anammox sludge. In his study, it took 2-3months to attach anammox biomass onto the surface of the carrier, although a large amount of seed sludge was inoculated.⁹⁾ Isaka *et al.* used the immobilized anammox gel using PEG and reported that the nitrogen removal activity increased gradually to 3.4kg/m³/d on day 67. The start-up process was faster than this study, but it may be due to the fact that certain amount of seed sludge was entrapped in the carriers. He also reported that the start-up period clearly decreased as the concentration of entrapped anammox sludge was increased.¹⁸⁾

However, the high NLR obtained in phase 1 was maintained only for ten days. This situation last for about one month and the treatment ability of the reactor failed to be restored to its former level. Therefore, in

phase 2 (195-265 days), due to the inhibition caused by the quick increase of influent NO_2^- -N concentration in phase 1, NLR was reduced to $0.5\text{kg/m}^3/\text{d}$ by extending HRT. The influent T-N concentration was decreased to 200mg/l and was increased step by step with HRT remaining unchanged. Until day 265, the influent T-N concentration increased to the maximum 650mg/l . (Fig. 2) The removal efficiencies of T-N, NH_4^+ -N and NO_2^- -N were 75%, 75% and 91%, respectively. (Fig. 4) But NLR was only $1.49\text{kg/m}^3/\text{d}$ and increased very slowly. (Fig. 3)

Fig.2. Time courses of influent nitrogen concentrations. Symbols: Closed diamonds, influent NH_4^+ -N; Closed squares, influent NO_2^- -N; Open diamonds, effluent NH_4^+ -N; Open squares, effluent NO_2^- -N; Open circles, effluent NO_3^- -N.

Fig.3. Time courses of HRT, NLR and RR. Symbols: Closed diamonds, NLR; Closed squares, RR; Closed triangles, HRT.

Fig.4. Profile of T-N, NO_2^- -N and NH_4^+ -N removal efficiencies during the whole operation period. Symbols: Closed diamonds, T-N; Closed squares, NO_2^- -N; Closed triangles, NH_4^+ -N.

This phase showed that it was difficult to enhance the treatment ability of anammox sludge only by increasing the influent T-N concentration. This may indicate that there is limited effect of influent T-N concentration on the treatment ability of the anammox sludge. The result conforms to the research done by Tsushima *et al.* They reported that there was a limitation to enhance the nitrogen removal rate by only increasing the influent nitrogen concentration at a fixed HRT.⁵⁾

From phase 3 (265-305days), the influent TN concentration was fixed to 300mg/l and NLR was increased stepwise by reducing HRT. After about a month's operation, NLR reached the maximum of $5.5\text{kg/m}^3/\text{d}$ on day 295 and NRR was $4.3\text{kg/m}^3/\text{d}$. (Fig. 3) The removal efficiencies of TN, NH_4^+ -N and NO_2^- -N were 79%, 79% and 91%, respectively. (Fig. 4) NLR could increase very quickly by reducing HRT in phase3. This may indicate that change of HRT has a positive effect on the treatment capacity of anammox sludge. This also confirmed the result reported by Tsushima *et al.* They reported that the higher nitrogen removal rate was obtained at the shorter HRT.⁵⁾

Variations of effluent SS During the operation, the effluent SS concentrations were relatively stable and were maintained at a low level. (Fig. 5) In phase 1, the effluent SS was below 10mg/l . In phase 2 and phase 3, there were slight increases in effluent SS concentration, the highest level being 30mg/l , but the average level was still about 10mg/l . The low effluent SS indicated that most of the anammox sludge was attached firmly on the surface of the PVA gels and the loss of anammox sludge was

Fig.5. Variations of effluent SS. Symbols: Closed triangles, effluent SS.

insignificant. It can also be concluded that anammox sludge growing on PVA gels bead could be more easily maintained in the reactor, thus ensuring stable performance of the reactor.

Average reaction ratios of NH_4^+ -N removal rate, NO_2^- -N removal rate and NO_3^- -N production rate The reaction ratio of total NH_4^+ -N consumption, NO_2^- -N consumption and NO_3^- -N production in this study was 1.0:1.13:0.21, which was similar to the previously reported value for the anammox reaction ($1:1.31 \pm 0.06:0.22 \pm 0.02$)²². The slightly lower NO_2^- -N consumption could be attributed to partial nitrification (i.e., conversion of NH_4^+ -N to NO_2^- -N) in the reactor²¹.

Comparison of anammox activities using PVA gels as carriers It can be seen from Table 1 that most previous studies using PVA gels were inoculated by PVA gels as well as cultivated anammox sludge. Therefore it is difficult to use their studies for comparison. Tran *et al.* reported that a maximum nitrogen removal of 3.0kg/m³/d was obtained in a fluidized bed reactor using PVA gel beads without adding anammox sludge to the reactor. However, it took more than one year for the anammox biomass to attach to the surface of the beads, much longer than this study (two-month pre-treatment and about one-month operation), and it took more than 600 days to obtain the maximum nitrogen removal rate of 3.0kg/m³/d, while in this study, a maximum nitrogen removal rate of 4.3kg/m³/d was

obtained after 295 days' continuous operation.¹⁹

In previous reports, anammox enriched sludge with different carriers, such as PVA gel beads, glass beads and PEG gel cubes, was used to start up the anammox reactor. Table 1 lists the immobilization methods and nitrogen removal performance in different anammox reactors.

Tran *et al.* showed that a successful nitrogen removal maximum of 3kg was obtained in a fluidized bed reactor using PVA gel beads. However, it took more than one year to attach the anammox biomass to the surface of PVA gel beads in a packed-bed reactor and consequently, it took 500 days to reach this high removal rate in the fluidized bed reactor. On the other hand, Isaka *et al.* used PEG gels for whole cell entrapment of anammox sludge. they reported that it took 67 days and 25days to obtain a removal rate of 3.7 with an initial anammox sludge amount of 0.72 and 4.7g dry SS. According to another report, Hsia *et al.* used the PVA/alginate immobilization method but only conducted a batch experiment in their study for comparison.

However, the aim of this study and theirs might be different. In this study, a high and stable performance was investigated but the potential maximum nitrogen removal rate after the shortest operational time was their goal. If we operated anammox reactor with their strategy, the same removal performance could be obtained. Besides, a maximum removal rate of 8.2kg-N was confirmed after

Table 1. Comparison of the anammox activities using PVA gels as carriers.

Reactor	Carrier	Influent and temperature (°C)	Initial sludge concentration (g/l)	Operation time (day)	NRR (kg/m ³ /d)	Reference
Agitation type	PVA gels	Synthetic wastewater, 30	0.5	168	5.5	Ge <i>et al.</i> ¹⁰⁾
STR	PEG gel entrapments	Synthetic wastewater, 36	0.72g dry-SS	67	3.7	Isaka <i>et al.</i> ¹⁸⁾
STR	PEG gel entrapments	Synthetic wastewater, 36	4.7g dry-SS	25	3.4	Isaka <i>et al.</i> ¹⁸⁾
FBR	PVA gels	Synthetic wastewater, 33-35	no	617	3.0	Tran <i>et al.</i> ¹⁹⁾
STR	PVA gel entrapments	Synthetic wastewater, 33-35	5.0g dry-SS	250	8.2	Quan <i>et al.</i> ²⁰⁾
STAR	PVA gels	Synthetic wastewater, 33-35	no	295	4.3	This study

FBR: fluidized bed reactor

Fig.6. Changes of the PVA gels observed by stereo microscope. a, c, e, g, and i showed the sections of a PVA gel and b, d, f, h, and j showed the surfaces of a PVA gel. a and b showed the original gel; c and d showed the gel after two-month pretreatment; e and f showed the gel on day 41; g and h showed the gel on day 155; i and j showed the gel on day 280.

250 days of operation in this study. This result demonstrates that whole cell entrapment by PVA gel is one of the most effective techniques for immobilizing anammox bacteria.

Taking into account the above mentioned results, it may be concluded that it is practical to use pre-inoculated PVA gels for getting stable anammox reaction within short periods with PVA gels only and a stable operation result could be obtained.

Macroscopic observation of the PVA gels Fig. 6 illustrated the changes of the PVA gels observed by stereo microscope. Fig. 6a and 6b showed the original gels. They were totally white. After about two months of pre-treatment, changes of the gel surface could be observed but the color changes at this period were not very distinct. (Fig. 6c and 6d) However, after 41 days of continuous operation, the surface of the gels apparently became brown, indicating the attachment of anammox sludge on the gel surface. (Fig. 6e and 6f) Pictures taken on day 155 showed more clearly the red anammox sludge growing on the surface of the PVA gels. (Fig. 6g and 6h) The gels became extremely red color till day 280, and the surface of the gels was smooth and bright, indicating the dense growth of the anammox sludge on the gel surface. (Fig. 6i and 6j) The changes of the thickness of the anammox sludge growing on the surface of the gel were also very striking. The anammox sludge became thicker and thicker with the passage of time.

SEM observation Fig.7 shows the

Fig.7. Scanning electron microscope (SEM) images of the PVA gel. a showed the surface of a brand new PVA gel; b showed the PVA gel after two-month pretreatment; c showed the PVA gel on day 41; d showed the PVA gel on day 280.

growth of anammox sludge on the gel surface observed by scanning electron microscope (SEM). The new PVA gels are hydrophilic in nature and have a porous structure with a continuum of passages 10 to 20 μm in throughout each bead. The passage is also observable on the surface of the PVA gels. (Fig.7a) After 2 months' pre-treatment, the passages are no longer observed. (Fig.7b) After 41 days' stable operation, the growth of anammox sludge could be easily observed. (Fig.7c) Photo taken on day 280 (Fig.7d) showed that a very thick layer of anammox sludge grew on the surface of the gel and the surface was very smooth with clusters of

Table 2. Homology search results for 16S rRNA gene sequences of the main bacterial members in PVA gel beads.

OTU	Taxon	Accession	Identity	Number of clones
1	Planctomycete KSU-1	AB057453	100	8
2	Uncultured bacterium clone: Answdl_02	AB594204	99	7
	Uncultured Planctomycetales bacterium clone DTU1-13	FJ710639	99	
	<i>Candidatus</i> Brocadia anammoxidans	AF375994	94	
3	Uncultured anoxic sludge bacterium KU2	AB054007	100	1
	<i>Candidatus</i> Kuenenia stuttgartiensis	CT573071	100	
4	Uncultured bacterium clone T32	HM991635	98	5
	Ignavibacterium album	AB478415	97	
5	Uncultured bacterium clone Dok23	FJ710742	99	1
	Uncultured bacterium clone B18	EU888817	99	
	Uncultured Chlorobi bacterium clone T12	HM991629	95	
6	Uncultured bacterium clone: Answdl_03	AB594205	98	3
	Uncultured bacterium clone: A4	AB462405	98	
	Uncultured Chloroflexi bacterium clone QEDQ2AB09	CU923588	94	
7	Uncultured bacterium clone: AnDHS-6	AB430337	99	1
	Uncultured bacterium clone S-73	EF522844	99	
	Uncultured bacterium clone: AnSal-10	AB434262	99	

Operational taxonomic units (OTUs) were defined by a 1% distance level in nucleotide sequences

anammox sludge.

Bacteria community analysis In this study, the seed sludge used in the reactor was inoculated from the anammox reactor. The bacterial community was analyzed by using universal eubacterial primers after 291 days of cultivation with synthetic wastewater. Table 2 shows the main results of homology search for 16S rRNA gene sequences in the community existing in PVA gel beads. OUT 1, OUT 2 and OTU 3 had 100%, 94% and 100% sequence identities with Planctomycete KSU-1, *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis*, which were detected in the anammox reactors and known to be anammox bacterium. This result shows that the PVA gel bead is useful for culturing these anammox bacteria. Additionally, OUT 6 and OUT 7 had 98% and 99% sequence identities with Uncultured bacterium clone: Answdl_03 and Uncultured bacterium clone: AnSal-10, which were also detected in our previous studies^{23,24}. Although the operation strategies of these anammox reactors were quite different, these microbial communities appear to have some similarities. Future studies are needed to investigate the function of the coexistent bacteria.

CONCLUSIONS

In this study, the STAR reactor was successfully started up by using PVA gels only and a stable operation was obtained within a relatively short period of time. The experimental results showed that the operational strategy was practical and that the PVA gel carrier, as a novel carrier, have great application potential to anammox reactor. Anammox sludge could firmly attach to the surface of the PVA gels and the loss of anammox sludge was insignificant. DNA analysis also confirmed that the PVA gel bead is effective in culturing anammox bacteria with anammox bacteria Planctomycete KSU-1, *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis* dominating in the bacterial community.

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