Biodegradation (2011) 22:1155–1167
DOI 10.1007/s10532-011-9471-3

ORIGINAL PAPER

Reject water treatment by improvement of whole cell anammox entrapment using polyvinyl alcohol/alginate gel

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Received: 24 August 2010 / Accepted: 21 March 2011 / Published online: 1 April 2011

Abstract Reject water treatment performance was investigated by whole cell anammox sludge entrapped polyvinyl alcohol/sodium alginate gel in the stirred tank reactor (STR). The whole experiment was conducted through Phase 1 and Phase 2 in which synthetic wastewater and modified reject water were used as feeding medium, respectively. The anammox reactor demonstrated quick start-up after 22 days as well as stable and relatively high nitrogen removal rate of more than 8.0 kg-N m^{-3} day^{-1} during the two both phases even under moderately low temperature of 25 ± 0.5°C during the last 2 months of Phase 2. The matured brownish red PVA beads had good characteristics with buoyant density of 1.10 g cm^{-3}, settling velocity of 141 m h^{-1} and diameter of 4 mm. The bacterial community was identified by 16S rDNA analysis revealing the concurrent existence of KSU-1 and new kind anammox bacterium Kumadai-I after changing influent from synthetic wastewater to reject water. It was speculated that Kumadai-I might play a role as "promotion" factor together with KSU-1 on high nitrogen removal rate. These results demonstrate the potential application of whole cell anammox entrapment by PVA/alginate gel for achieving stable and high-rate nitrogen removal from high ammonium with low C/N ratio contained wastewaters, such as reject water, digester liquor or landfill leachate.

Keywords Anammox · PVA/alginate · Reject water · Whole cell entrapment

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Introduction

The nitrogenous pollutants causing a eutrophication have attracted increasing attention recently. Conventionally, ammonium nitrogen removal from wastewater by biological processes involves well-known aerobic nitrification (ammonium NH_4^+-N as the electron donor while oxygen as the electron acceptor) followed by anoxic denitrification (organic matter as carbon source and the electron donor and nitrate NO_3^--N as electron acceptor). Biological nitrogen removal is considered to be common because of its low cost and high efficiency compared to physical and chemical treatments (Van Dongen et al. 2001a, b). However, the application of conventional biological processes to wastewaters containing high ammonium and low carbon content, such as reject water,

digester liquor or landfill leachate seems to be limited. Because the available biodegradable carbon in these wastewaters is insufficient for the heterotrophic denitrification, an external carbon sources such as acetate, glucose, ethanol and methanol must be added.

Recently, the new biological approach, anaerobic ammonium oxidation—anammox, which bypasses the formation of NO_3^--N and converts NO_2^--N to dinitrogen (N_2) gas with NH_4^+-N as the electron donor and NO_2^--N as the electron acceptor under anaerobic condition is recommended to remove ammonia from wastewater without addition of biodegradable carbon source. The stoichiometric conversion of NO_2^--N and NH_4^+-N to N_2 gas with negligible production of cell material and nitrate is shown below (Strous et al. 1998):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

However, the maintenance of a sufficient amount of anammox bacteria in the reactor, which is very important during the start-up, is not easy due to its extremely slow growth rate, low biomass yield and vulnerability to being washed out from the reactor by intensive N_2 gas bubble production. Because the anammox process produces large amounts of N_2 gas under high-rate nitrogen removal, gas bubbles become trapped in the anammox biomass, causing it to float.

Immobilization of microbial cells has received increasing interest in the field of wastewater treatment. It offers a promising potential for the improvement of bioprocess efficiency. Compared to free cell, immobilized cell has significant advantages as follow: (1) it can increase the biodegradation rate through a higher cell density; (2) the continuous process can be carried out under high loading rate without washing out of cell because it is easier to release gas bubble under mixing condition; and (3) it is easy to separate liquid and solid phase in the reactor, leading to simple operation and maintenance.

Whole cell entrapment in polymeric matrixes is widely used for cell immobilization. Various natural and synthetic polymers have been used (Wang and Liu 1996; Wang and Shi 1998; Wang et al. 2000); however, each has its drawbacks. Natural polymers (agar, agarose, alginate, kappa-carragenan) are not toxic to microorganisms but they possess poor mechanical strength and durability. Conversely, synthetic polymers have strong mechanical strength but are toxic to microorganisms (Zhang et al. 2007). Recently, a promising type of synthetic polymer, polyvinyl alcohol (PVA), which is cheap and non-toxic to microorganisms, has been used for cell immobilization (Hashimoto and Furukawa 1987; Amanda and Wisecarver 1992; Asano et al. 1992; Vogelsang et al. 1997; Chang and Tseng 1998; Long et al. 2004). A simple and economical technique of cell immobilization with PVA is PVA-boric acid method. Two potential problems with this technique, however, are the agglomeration of PVA gel beads and the toxicity of saturated boric acid to microorganisms (Lozinsky and Plieve 1998). Another simple technique is freezing-thawing method, but it has some drawbacks such as: high energy cost for freezing at extremely low temperature of −20°C and negative effect of low temperature to microorganisms activity.

In this study, an improved immobilization technique using the complex of PVA and sodium alginate solution solidified by solution of $NaNO_3$ and $CaCl_2$ was applied. Previous report (Amanda and Wisecarver 1992) suggested that concentration of sodium alginate below 0.4% (w/v) was used for preventing the agglomeration of PVA gel beads. It was presumed that there was nothing to do with the improvement of PVA network structure. In our work, the concentration of sodium alginate was enhanced to 1.0% (w/v) which cannot only avoid the agglomeration of PVA gel beads but also can efficiently improve and control PVA network structure (Wang et al. 2006).

To our knowledge, it was the first time that continuous experiment of whole cell anammox entrapment using PVA/alginate gel has been investigated. The objectives of this study were to: (1) improve the whole cell entrapment technique using PVA/alginate gel; (2) evaluate sludge retaining capability of PVA gel bead and its characteristics; (3) investigate the start-up period and treatment capacity of immobilized anammox sludge through the continuous nitrogen removal experiment; and (4) investigate the function of bacterial community on anammox performance.

Materials and methods

Seed anammox sludge

Anammox sludge which had been cultivated in our laboratory was used as the seed sludge for anammox pellets. This enrichment of anammox sludge was carried out using 50 l of up flow fixed-bed reactor filled with a polyester non-woven fabric carrier at 36°C (Furukawa et al. 2005). The enriched anammox sludge collected from the bottom of reactor was suspended into the effluent from the anammox reactor and was then concentrated by centrifuge. The concentrated anammox sludge characteristics were 33.3 g-SS (suspended solid) l^{-1} and 25.2 g-VSS (volatile suspended solids) l^{-1}, respectively.

Immobilization technique

PVA/sodium alginate was prepared with PVA-HC (100% saponification, Kuraray Co. Ltd, Osaka, Japan) at the concentration of 15% (w/v) and sodium alginate (Wako Pure Chemical Industries Ltd., Osaka, Japan) at the concentration of 2% (w/v). This mixture was heated by autoclave at 120°C for 20 min until dissolved. The mixture was then cooled to room temperature and 150 ml of concentrated anammox sludge, equivalent to 5 g dry SS, was added slowly into the PVA solution to a final volumetric ratio of 1:1. The final concentration of PVA, sodium alginate and anammox sludge were 7.5, 1 and 1.67% (w/v), respectively. The mixture of PVA/sodium alginate and anammox sludge was dropped slowly into solidifying solution (50% w/v $NaNO_3$ and 2% w/v $CaCl_2$) by syringe to make spherical beads. PVA gel beads were then immersed in a solidifying solution for 12 h at room temperature to increase their mechanical strength ("harden" the beads). The PVA gel beads were then washed with a large amount of distilled water and used for continuous experiments.

Feeding media

A synthetic medium and reject water taken from Kumamoto East Wastewater Treatment Plant (Kumamoto, Japan) were used for Phase 1 and Phase 2, respectively. The synthetic medium contained (per liter): NH_4^+-N, 0.05–0.55 g; NO_2^--N, 0.050–0.55 g; $KHCO_3$, 1.0 g; KH_2PO_4, 0.027 g; $MgSO_4$, 0.3 g; $CaCl_2$, 0.18 g; and 1 ml trace element solutions 1 and 2. Trace element solution 1 contained (per liter): ethylenediamine tetraacetic acid (EDTA), 5 g and $FeSO_4$, 5 g. Trace element solution 2 contained (per liter): EDTA, 15 g; $ZnSO_4 \cdot 7H_2O$, 0.43 g; $CoCl_2 \cdot 6H_2O$, 0.24 g; $MnCl_2 \cdot 4H_2O$, 0.99 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $NaMoO_4 \cdot 2H_2O$, 0.22 g; $NiCl_2 \cdot 6H_2O$, 0.19 g; $NaSeO_4 \cdot 10H_2O$, 0.21 g and H_3BO_4, 0.014 g.

The compositions of reject water contained (per liter): NH_4^+-N, 0.8–0.85 g; NO_2^--N and NO_3^--N, not detectable; BOD_5, 0.1–0.2 g; SS, 0.02–0.1 g; pH, 7.6–9.1. Tap water was used for dilution; the concentrated NO_2^--N aqueous solution was artificially added to make the suitable NH_4^+-N/NO_2^--N ratio for anammox of 1:1. This modified reject water had the similar characteristics with effluent from partial nitritation reactor using reject water as influent (Zhang et al. 2010b). The influent pH for anammox reactor was adjusted around 7.0 by adding 1 N hydrochloric acid.

Reactor and experimental setup

The stirred tank reactor (STR) with a total volume of 1.2 l and reaction volume of 1.0 l was used in continuous experiments (Fig. 1).

300 ml of gel carriers containing anammox sludge concentration of 1.67% w/v were placed inside the reactor. Considering the reaction volume was 1.0 l and the packing ratio of gel carriers was 30%,

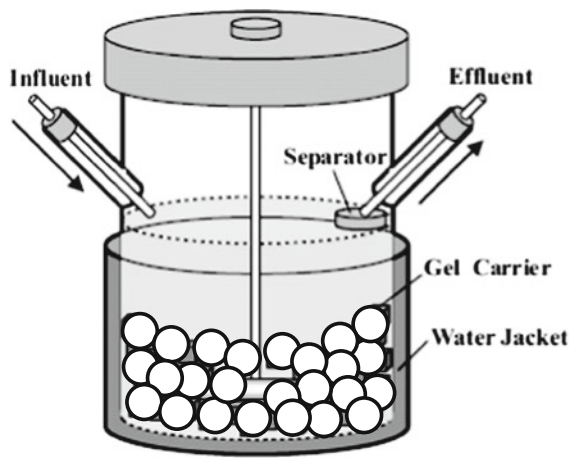

Fig. 1 Schematic of immobilized anammox reactor used for continuous treatment

inoculated anammox sludge concentration was 5 g-SS l^{-1} reactor. The reactor temperature was maintained at 33°C, controlled thermostatically with a water jacket. The reactor pH was not controlled. The gel beads were stirred continuously at 100 rpm. Stirring was essentially required for mixing the influent and removing nitrogen gas bubbles which formed on the surface of the gel beads. Purging with nitrogen gas was used to keep the dissolved oxygen (DO) level in the influent below 0.5 mg l^{-1}. In addition, small polystyrene balls were placed in the feed storage tank to retard oxygen transfer to the influent wastewater. Light is known to have a negative effect (30–50% reduction) on ammonium removal rate (Van de Graaf et al. 1996), so darkness was maintained using black vinyl sheet enclosures.

Experimental periods for anammox reactor

Experiments with various sets of operational conditions as shown in Table 1 were carried out. The whole experiment was divided into 2 phases with 10 periods.

Chemical analyses

Nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) were determined colorimetric method and UV cadmium reduction method, respectively. Ammonium was quantified based on the indophenol reaction with ortho-phenylphenol (OPP) (Kanda 1995). For the determination of MLSS, a sludge sample was washed twice by centrifuging at 1,000×*g* for 15 min, decanted and resuspended in deionized water and then dried at 105°C to a constant weight (with cooling under desiccation). Absorbance, pH, and DO were measured using a spectrophotometer (U-1900, Hitachi High Technologies Corporation, Tokyo, Japan), a pH meter (F-55, Horiba Ltd, Kyoto, Japan) and a DO meter (D-55, Horiba Ltd), respectively.

Scanning electron microscope observation of porous structures of PVA gel beads

A PVA gel was cut into 1–2 mm pieces and washed by 0.1 M phosphate buffer (pH 7.4) twice for 5 min each. The PVA gel pieces were then fixed by 2.5% glutaraldehyde solution prepared with 0.1 M phosphate buffer for 1–2 h and washed by 0.1 M phosphate buffer three times for 10 min each. The samples were then fixed by 1.0% OsO_4 solution prepared with 0.1 M phosphate buffer and washed again by 0.1 M phosphate buffer three times for 10 min each. Subsequently, the samples were dehydrated in serially graded ethanol solution at concentrations of 10, 30, 50, 70, 90 and 95% for 5–15 min each, and at a concentration of 99.5% twice for 30 min each. The samples were frozen in a freezer then dried by a freeze-drying device (JEOL JFD-300) and sputter-coated with gold for 100 s by an ion sputtering device (JEOL JFC-1100E). Finally, the samples were observed by scanning electron microscope (SEM) (JEOL JSM 6390LV).

Table 1 Experimental periods for anammox reactor

Phase	Period (term)	Feeding conditions				
		NH_4^+-N (mg l^{-1})	NO_2^--N (mg l^{-1})	HRT (h)	Temperature (°C)	Influent pH
Phase 1 (synthetic medium)	1 (0–13)	42–103	52–110	12	33	7.0–7.2
	2 (14–22)	107–203	112–221	8	33	7.1–7.3
	3 (23–46)	195–425	209–432	6	33	6.8–7.2
	4 (47–65)	417–546	431–544	4.8	33	6.9–7.0
	5 (66–83)	509–557	525–540	4.0	33	6.8–7.0
	6 (84–95)	528–555	530–552	3.4–3.0	33	6.8–7.1
	7 (96–119)	526–558	538–547	2.7	33	6.8–6.9
Phase 2 (modified reject water)	8 (120–140)	163–368	164–382	2.4–2.2	33	6.9–7.1
	9 (141–168)	326–337	375–398	2.0	33	6.9–7.1
	10 (169–230)	337–410	363–467	2.0	23–25	6.9–7.2

DNA extraction and PCR amplification

The sludge sample was taken from the reactor on day 119 and day 200. The sludge sample was 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 instructions. The amplification of 16S rRNA gene was performed with Phusion High-Fidelity DNA polymerase (FINNZYMES, Finland) using conversed eubacterial primers 6F (forward primer: 5′-GGAG AGTTAGATCTTGGCTCAG-3′) (Tchelet et al. 1999) and 1492r (reverse primer: 5′-GGTTACCTTG TTACGACT-3′) (Lane 1991). PCR was carried out according to the following thermocycling parameters: 30 s initial denaturation at 98°C, 25 cycles of 10 s each at 98°C, 20 s each at 51°C, 35 s each at 72°C, and 5 min final elongation at 72°C. The amplified products were purified using a Wizard SV Gel and PCR Clean-up System (Promega, USA).

Cloning and sequencing of 16S rDNA

The purified fragments were ligated into the EcoRV site of pBluescript II KS+ (Stratagene, USA) and *E. coli* DH 5 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 on the NCBI website.

Results and discussion

Anammox reactor operation

For the enrichment of anammox sludge, the synthetic medium was first used as the influent during Phase 1 of experiment. After successful achievement of high rate nitrogen removal, the influent wastewater was changed to modified reject water.

The STR was started-up fed with a nitrogen loading rate (NLR) of 0.2 kg-N m^{-3} day^{-1} corresponding to 100 mg l^{-1} T-N concentration. The time courses of influent and effluent nitrogen concentrations during operation are shown in Fig. 2.

In Phase 1 (from day 0 to day 119), the NLR was increased by adjusting the influent nitrogen concentration as well as the HRT. For the first 13 days, the NLR was increased by an increase in influent T-N concentration from 100 to 200 mg l^{-1} while the HRT was kept constant at 12 h. Immobilized anammox sludge quickly adapted to the increase in NLR and a maximum nitrogen removal rate (NRR) of 0.34 kg-N m^{-3} day^{-1} was obtained. Consequently, the HRT was decreased to 8 h and T-N concentration was increased from 200 to 400 mg l^{-1} corresponding to maximum NLR of 1.2 kg-N m^{-3} day^{-1}. Anammox performance showed the satisfactory results with the maximum NRR of 1.0 kg-N m^{-3} day^{-1}. In this study, an ammonium removal rate of 0.5 kg NH_4-N m^{-3} day^{-1} was considered as standard criteria for the start-up of anammox process which surpassed the upper limit for nitrification/denitrification process, i.e. 0.3–0.5 kg NH_4-N m^{-3} day^{-1} (Zhang et al. 2010a). Strous et al. (1997) reported that it took 115 and 84 days for starting up the fixed-bed and fluidized-bed reactor with NRR achieving at 1.1 and 1.8 kg-N m^{-3} day^{-1}, respectively. Zhang et al. (2010a) showed that the NRR reached 1.0 kg-N m^{-3} day^{-1} after 56 days of operation in an up-flow reactor. Trigo et al. (2006) demonstrated that the breakage of the granules due to an excess of agitation of 75 rpm was considered to be one of the reasons of the loss of system activity during the first 80 days, leading to the fail of quick start-up. Moreover, the activity still diminished in spite of diminishing the stirring speed in order to reduce the inhibition of shear stress on the biomass. In our study, the start-up with NRR reaching at 1.0 kg-N m^{-3} day^{-1} was successfully achieved within 22 days and there was no breakage of PVA beads observed even under stirring speed of 100 rpm. These indicate that the start-up in this study, from our best knowledge, was the shortest compared to the others and PVA beads themselves should be considered to be the perfect granules and to protect anammox sludge against the effect of shear stress, showing that the whole cell entrapment of anammox sludge was ideal to start up a new reactor. The explanation for the quick start-up may be attributed to (1) mild immobilization; (2) a high concentration of the initial anammox sludge of 5 g-MLSS l^{-1} in this reactor; and (3) high sludge

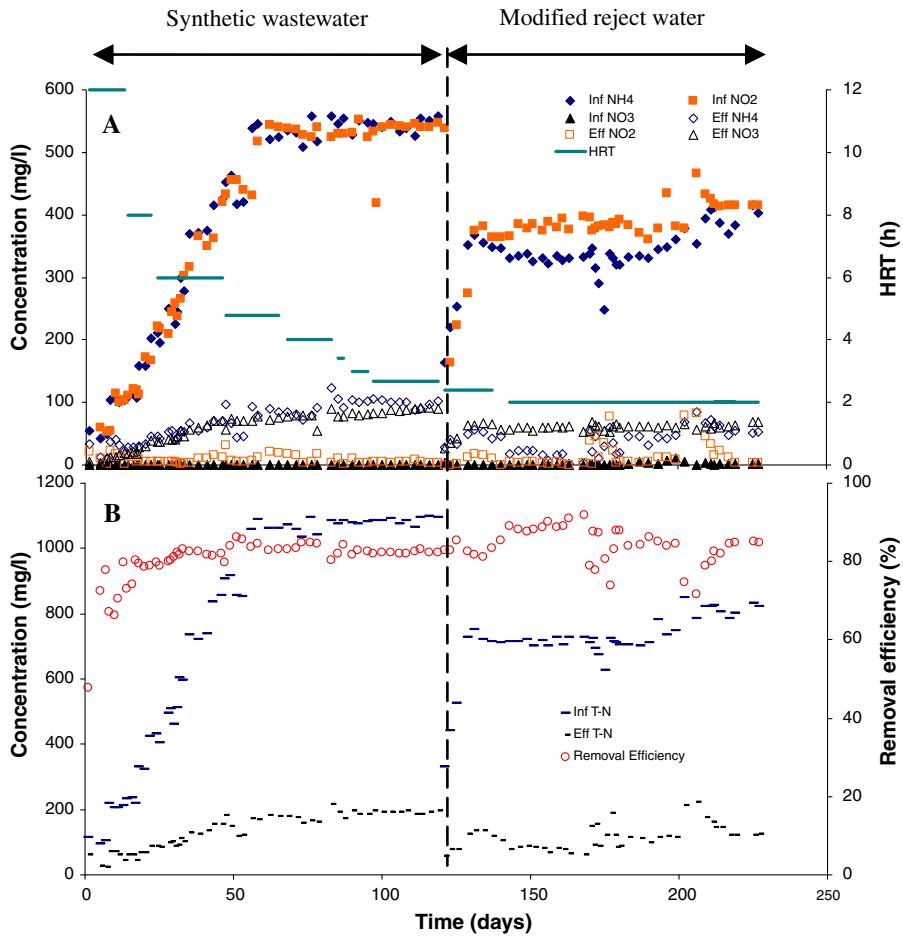

Fig. 2 Time courses of influent and effluent concentrations of nitrogenous compounds (**a**) and total nitrogen (**b**)

retaining capability of whole cell entrapment technology indicated by the effluent SS as almost zero during these periods (data not shown).

After successful start-up, the NLR was increased rapidly by the means of increasing influent T-N concentration and decreasing HRT. From day 23 to the end of Phase 1 (day 119), the influent concentration of NH_4^+-N and NO_2^--N was increased step wisely from 195 to 550 mg l^{-1} and from 205 to 550 mg l^{-1}, respectively. The stable and high removal efficiencies of NH_4^+-N and NO_2^--N was shown as 83 and 98%, respectively. The maximum NRR of 8.2 kg-N m^{-3} day^{-1} was achieved at maximum NLR of 9.9 kg-N m^{-3} day^{-1} while the average effluent NO_2^--N concentrations were always below 10 mg l^{-1}. The performance of anammox reactor during Phase 1 demonstrated that stable and relatively high-rate nitrogen removal was successfully established with synthetic wastewater as feeding medium.

From day 120, the reject water from Kumamoto East Wastewater Treatment Plant supplemented with artificial NO_2^--N was fed to anammox reactor in order to investigate the treatability of reject water and the reactor performance with the presence of organic matter. The reactor was re-started fed with an influent T-N concentration of 330 mg l^{-1} (5 times dilution of reject water) at HRT of 2.4 h corresponding to NLR of 3.3 kg-N m^{-3} day^{-1}. During period 8 (Table 1), NLR was increased to 7.8 kg-N m^{-3} day^{-1} with decrease in reject water dilution factor, however, anammox bacteria showed the quick response to the change of NLR. At the end of this period (day 140), nitrogen removal efficiency of around 85% was obtained. Consequently, NLR was kept constant at 8.8 kg-N m^{-3} day^{-1} during the next 28 days (period 9) to evaluate the stability of our immobilized anammox reactor. Stable anammox treatment with high nitrogen removal efficiency of 91% was obtained.

In period 10 (from day 169 to day 230), the reaction temperature was kept constant at moderately low of 25 ± 0.2°C while NLRs were changed in the range of 8.4–10.2 kg-N m^{-3} day^{-1} to investigate the treatment capacity of immobilized anammox sludge under low temperature and high nitrogen loading rate (the dilution factor decreased to two). The same trend of nitrogen removal performance as seen in previous periods was observed except that effluent NO_2^--N concentration increased to 78 mg l^{-1} on day 177 due to sudden decrease in temperature to 20°C. However, the treatment performance quickly recovered right after the temperature was increased to 25°C. At the end of this period, the maximum NRR of 8.5 kg-N m^{-3} day^{-1} was successfully achieved. These results demonstrated that there was no negative effect of organic matter and moderately low temperature of 25°C on anammox activity. However, further studies should be required to make clear the effect of higher organic matter and lower temperature. The daily changes in nitrogen loading and removal rates during the whole experiment are shown in Fig. 3 and Table 2.

Effect of influent nitrite concentration

Some previous researchers reported that the anammox activity decrease by a half when influent NO_2^--N concentration was above 350 mg-N l^{-1} (Dapena et al. 2007) or 37% under influent NO_2^--N concentration of 430 mg-N l^{-1} (Kimura et al. 2010). The results achieved from our study were different from their results. Through whole experiment, no obvious inhibitory effect of maximum influent NO_2^--N concentration of 550 mg-N l^{-1} was observed. The functional anammox species (as shown in the next section), the characteristics of biomass carrier and the structure of anammox reactor should be considered as possible reasons. From 16S rDNA analysis, the anammox bacterium KSU-1 was identified as the dominant species in the consortium. It was assumed that KSU-1 enables to adapt to the high influent NO_2^--N concentrations. On the other hand, whole cell immobilization can prevent bacteria activity from toxic compounds and strengthen the sludge retention which enhanced the nitrogen removal performance of the reactor. Furthermore, the structure of anammox reactor could play an integral role. The nitrite concentration usually accumulated at the bottom of fixed-bed or packed-bed reactor, leading to the inhibition of high nitrite concentration. Conversely, stirred tank reactor used in this study provides complete mixing condition which gives uniform substrate concentrations, avoiding the nitrite accumulation in the reactor.

Organic matter removal

The reject water used in this study was low C/N ratio with BOD_5 concentration of 100–210 mg l^{-1}. During

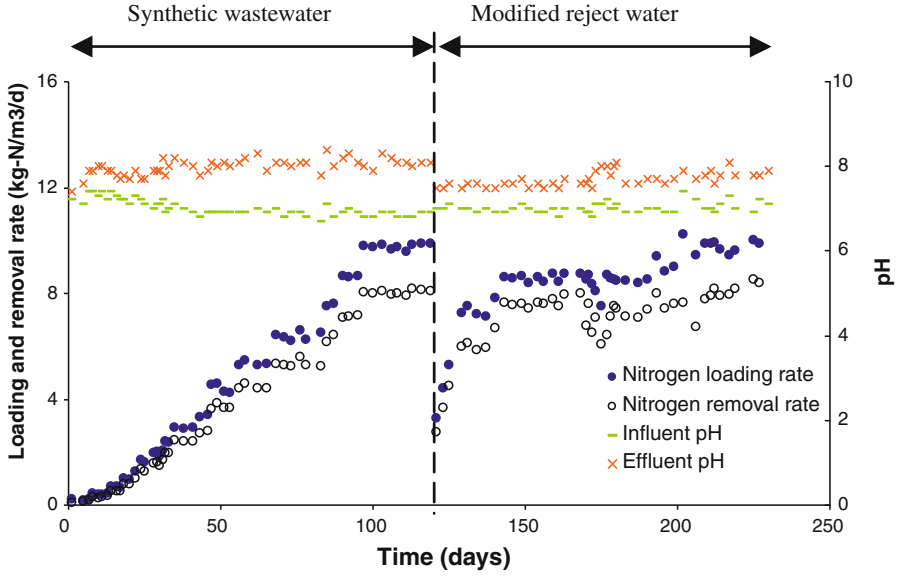

Fig. 3 Daily changes in nitrogen loading, removal rates, influent and effluent pH

Table 2 Performance of anammox reactor during different periods

Phase	Period (term)	NLR (kg-N m^{-3} day^{-1})	NRR (kg-N m^{-3} day^{-1})	Influent BOD_5 (mg l^{-1})	Effluent pH	Removal efficiency (%)
Phase 1 (synthetic medium)	1 (0–13)	0.19–0.43	0.11–0.34	–	7.4–8.0	48–80
	2 (14–22)	0.66–1.27	0.51–1.01	–	7.7–7.9	73–80
	3 (23–46)	1.62–3.43	1.28–2.81	–	7.7–8.2	79–83
	4 (47–65)	4.26–5.45	3.63–4.61	–	7.9–8.3	80–86
	5 (66–83)	6.20–6.58	5.23–5.59	–	7.8–8.1	80–85
	6 (84–95)	7.53–8.66	6.17–7.16	–	8.0–8.3	82–84
	7 (96–119)	9.59–9.88	7.93–8.18	–	7.9–8.3	82–83
Phase 2 (modified reject water)	8 (120–140)	3.30–7.83	2.74–6.69	60–70	7.5–7.6	81–85
	9 (141–168)	8.41–8.74	7.42–8.02	60–70	7.5–7.7	88–92
	10 (169–230)	7.54–10.21	6.08–8.53	60–70	7.5–8.1	79–88

steady-state period (days 169–230), the influent BOD_5 concentrations were measured as 60–70 mg l^{-1} and the removal efficiencies were around 20% (data not shown). These relatively low organic matter removal efficiencies could be attributed to the presence of non-biodegradable or slowly biodegradable organic matter in the test reject water (Ruscalleda et al. 2008). Consequently, denitrifiers which coexist with anammox bacteria could not consume such organic matters for heterotrophic denitrification. However, there was no negative effect caused by remaining organic matter on anammox reactor performance during Phase 2. This result might give the conclusion that anammox bacterial community can show the proper treatment capability under low organic matter concentration from reject water.

Effect of pH and inhibition of free ammonia

In anammox reactors, pH plays a very important role. The optimal pH range of anammox bacteria is within 7.7–8.2 (Strous et al. 1997). Various researchers have reported an increase in effluent pH along with increase in anammox activity (Tang et al. 2009; Liu et al. 2008; Szatkowska et al. 2007). On the other hand, Strous et al. (1997) showed that the specific anammox activity at pH of 9 was only 1/5 of that at pH of 8. Treatment performance failure may occur under long-term operation of anammox reactor at high pH range. Correspondingly, high pH was accompanied by a high free ammonia (FA) concentration (Anthonisen et al. 1976):

$$\mathrm{FA}\ (\mathrm{mg\ l^{-1}}) = \frac{17}{14} \times \frac{\mathrm{TAN} \times 10^{\mathrm{pH}}}{[\exp(6344/(273+T)) + 10^{\mathrm{pH}}]} \quad (2)$$

where TAN is the total ammonium as N (mg l^{-1}) and T is the reactor's temperature (°C).

Free ammonia was toxic to the anabolic and catabolic processes of microorganisms (Vadivelu et al. 2006). Waki et al. (2007) and Zheng and Huo (2001) have presumed that anammox activity was inhibited at FA concentration of 13–90 and 75.6 mg l^{-1}, respectively. Tang et al. (2009) observed very high effluent pH in the range of 8.7–9.1 resulting to the inhibition of anammox activity due to FA concentrations in the range of 64–73 mg l^{-1} from their research. However, they attributed their observation to lower buffering capacity of medium. In order to increase the buffering capacity and to reduce the inhibition caused by high pH and FA concentration, they increased the influent alkalinity by dosing $KHCO_3$ from 0.5 to 1.25 g l^{-1}. Another research (Liao et al. 2008) also reported that the anammox activity doubled as the influent bicarbonate concentration increased from 1.0 to 1.5 g l^{-1}. In the present study, $KHCO_3$ dose was maintained at 1.0 g l^{-1} which imparted 550–600 mg $CaCO_3$ l^{-1} of alkalinity to the synthetic medium. In addition, the alkalinity concentration of modified reject water was measured in the range of 500–1,000 mg $CaCO_3$ l^{-1} depending on dilution factor. The buffering capacity of medium (both synthetic and modified reject water) due to high alkalinity concentration could be considered as one of

the reasons for preventing increase in effluent pH and thereby toxicity to anammox activity due to FA concentration at higher pH. Through whole experiment, the average FA concentrations were lower than 15 and 5 mg l^{-1} during Phase 1 and Phase 2, respectively, with the highest FA concentration was calculated to be 21.3 mg l^{-1} on day 103.

Assessed stoichiometric characteristics of the anammox reaction

The removal ratios of NO_2-N to NH_4-N and production ratios of NO_3-N to NH_4-N for anammox reaction were reported to be 1.32 and 0.26, respectively (Strous et al. 1998). In this study, the synthetic medium was used as influent (Phase 1) giving ratios of NO_2-N to NH_4-N and NO_3-N to NH_4-N of 1.22 and 0.2, respectively. Furukawa et al. (2003) suggested that the ratio of NH_4^+-N:NO_2^--N:NO_3^--N was 1:1.17:0.21 obtained from anammox reactor with a non-woven biomass carrier and fed by synthetic wastewater. This ratio was likely to the observed one in Phase 1 of this study. The reaction ratio slightly changed when switching influent to modified reject water, corresponding to 1:1.24:0.18. The relatively lower nitrate/ammonium ratio might be related to heterotrophic denitrification. However, Ruscalleda et al. (2008) considered that denitrifiers could coexist with anammox bacteria and play an important role on treating low C/N ratio wastewaters with high quantities of slowly-biodegradable organic carbon, such as reject water, digester liquor and landfill leachate. In treating such wastewaters, conventional heterotrophic denitrification is limited due to the low availability of easily biodegradable organic matter. Consequently, slight proliferation of denitrifiers would not have a severe impact on anammox reaction, giving a negligible change on reaction ratio between the two phases.

PVA gel beads characteristics

The PVA beads were ivory white color at the start of continuous experiment due to a small amount of entrapped anammox sludge. The variation in appearance was recorded using stereomicroscope after 200 days of operation (shown as below). On day 200, the PVA beads had become brownish red due to the growth of anammox bacteria on the outer layer of PVA beads.

Typical reported buoyant densities of granules are 1.03–1.08 g cm^{-3} (Andras et al. 1989). Reported settling velocities for granular sludge are in the range of 18–100 m h^{-1} (Fukuzaki et al. 1991). In this study, the matured brownish red PVA beads had an average settling velocity of 141 m h^{-1} (3.9 cm s^{-1}) based on the method of Ghangrekar et al. (2005), buoyant density of 1.10 g cm^{-3} and average diameter of 4 mm. An appropriate diameter of PVA beads and high settling velocity resulted in the complete retention of PVA gel beads (no wash-out of PVA gel beads) during the experiment. In addition, the PVA gel beads showed the excellent mechanical strength under stirring condition during whole experiment.

Stereomicroscopic and SEM observation of the porous structure of PVA gel beads

Figure 4 shows the immobilized anammox sludge in PVA gel beads. Compared with the original appearance of PVA beads, there seems to be an increase in size and abundance of the colonies towards the outer part of the beads while they are less near the core based on purely visual. The obviously different amount of anammox sludge after long-term operation could be speculated that anammox sludge successfully retained by PVA gel beads and probably grew at inner part of the beads. This resulted in the quick start-up and high nitrogen removal rate as mentioned above.

Scanning electron microscopic was also carried out to observe the outer and inner structure of PVA gel beads. When the complex PVA/alginate hydrogels were dripped into $CaCl_2$ solution, Ca^{2+} ions in solution replaced Na^+ to form calcium alginate which would harden the complex beads. Because of the inter-solubility between sodium alginate and PVA, the calcium alginate gels could restrict the fluidity of PVA molecules, enhancing the agglomeration between PVA molecules and, to some extent, enhancing the density of crosslinking sites and controlling the pore size of PVA gels. Cell immobilization requires a fundamentally important point that is the favorable microenvironment conditions for the immobilized cells particularly when using living cells which require an adequate supply of substrates and rejection of metabolites. Lozinsky and Plieve (1998) reported that an "ideal" gel carrier should possess a highly porous (preferably macroporous) structure to

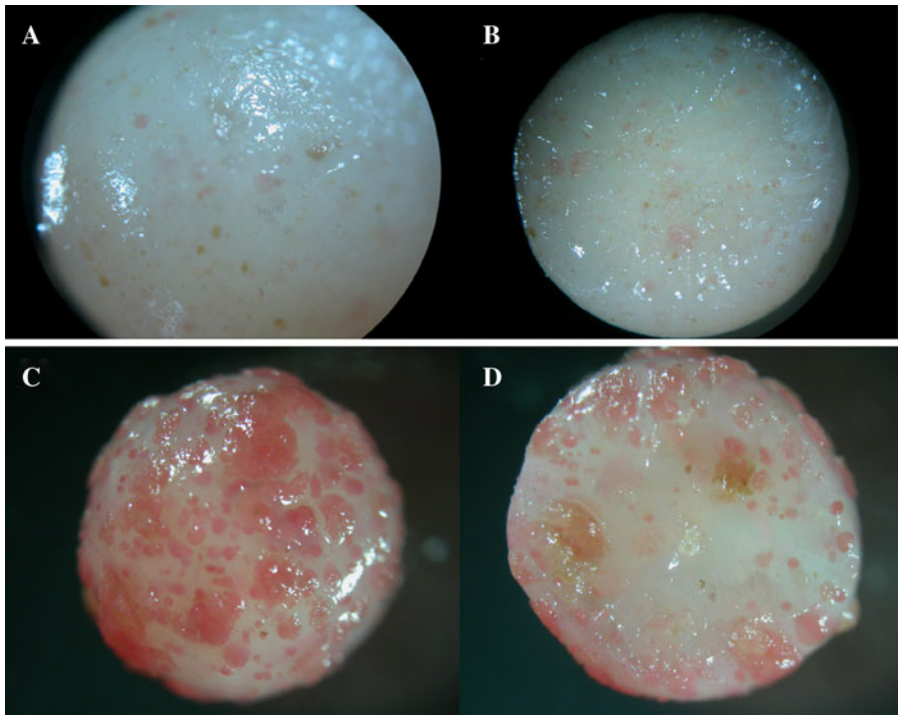

Fig. 4 Stereomicroscopic observation of surface and cross-section of PVA gel bead at the beginning of experiment (**a**, **b**) and on day 200 (**c**, **d**)

facilitate the nonhindered diffusion of solutes and dissolve gas. They also demonstrated that macropores of the order 0.1–1.0 μm commonly exist in the gel materials. In our study, the macropores size of 1.0–3.0 μm, which is bigger than that reported by Lozinsky, were mainly observed (Fig. 5). In addition, Wang et al. (2006) demonstrated that the micropore frame formed accompanied with the reproduction of bacteria would provide an excellent inter-space and conditions for the transfer of substrates and for the growth of cells. From these results, we could assume that three-dimension network structure with macropore of PVA gel beads could facilitate the transfer of both substrates and metabolic products.

Genomic characterization of the bacterial community

The change of feeding medium from synthetic water (SW) to modified reject water (MRW) could lead to a shift in the microbial community. Observation of this shift is helpful to understand the treatment performance of anammox reactor.

DNA extracted from the biomass sample of anammox reactor was amplified and 35 and 34 clones were obtained on days 119 and 200, respectively when cloning this amplified DNA fragment. The results of homology search using NCBI BLAST for these clones are given in Table 3.

On day 119, the anammox bacteria KSU-1 (Accession No. AB057453, Fujii et al. 2002) was identified as the dominant species, accounting for 94% (33/35) which is in agreement with previous research in our laboratory (Rouse et al. 2005). After switching influent medium to modified reject water, the new kind anammox bacteria Kumadai-I (Accession No. J.B.B 110 72-78 (2010)) and *Candidatus Brocadia anammoxidans* (Accession No. AF375994) were detected, accounting for 26% (9/34); simultaneously, the anammox bacteria KSU-1 substantially decreased to 62% (21/34) in the consortium. However, the dominant bacterial species were still KSU-1.

It is very difficult to clearly explain the existence of new kind bacteria; however, possible that it is due to the mode of enrichment, presence of organic matter, reaction temperature. The species of anammox

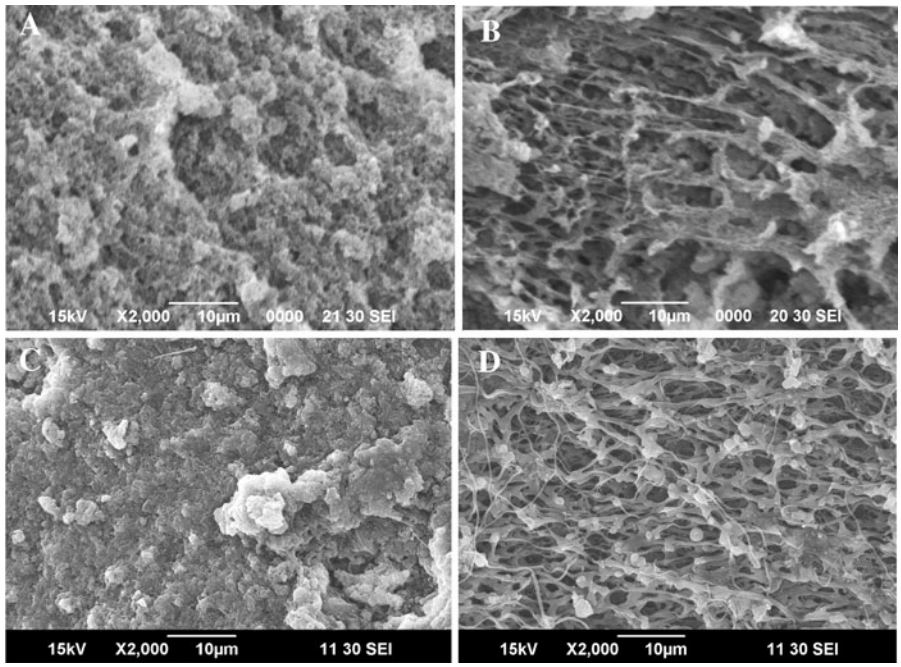

Fig. 5 SEM observation of outer and inner part of PVA bead at the beginning of experiment (**a**, **b**) and after 200 days of operation (**c**, **d**)

Table 3 Results of bacterial community analysis during different phases in the anammox reactor

OTU	Taxon	Accession	Identity	Day 119 (SW)	Day 200 (MRW)
1	Planctomycete KSU-1	AB057453	100–99	33 (33/35)	21 (21/34)
2	Kumadai-I	J.B.B 110 72–78 (2010)	100–99	0 (0/35)	9 (9/34)
	Candidatus Brocadia anammoxidans	AF375994	95–94		
3	Uncultured bacterium clone C65	EU234228	99	1 (1/35)	0 (0/34)
	Comamonas granuli strain: Ko03	AB187586	99		
	Variovorax paradoxus isolate TG27	AF508103	99		
4	*Flavobacteria bacterium* HMD1051	GQ148880	98	1 (1/35)	0 (0/34)
	Flavobacteriales bacterium CF-1	AY145539	96		
	Uncultured bacterium clone SINN925	HM128881	95		
5	Bacterium enrichment culture clone 1.21	GQ162347	100	0 (0/35)	2 (2/34)
	Uncultured bacterium clone: A4	AB462405	99		
	Uncultured bacterium clone KIST-JJY024	EF594056	98		
6	Uncultured bacterium clone Dok23	FJ710742	99	0 (0/35)	1 (1/34)
	Uncultured bacterium clone B18	EU888817	99		
	Uncultured bacterium clone: UASB6	AB329640	99		
7	Uncultured bacterium clone P1H01	FJ416446	97	0 (0/35)	1 (1/34)
	Uncultured bacterium clone M1-14	EU015107	97		
	Uncultured Bacteroidetes bacterium clone bf2-32	GU257873	97		

bacteria were responsible for the adaptation of reactor to modified reject water under moderately low temperature. Considering to the fact that both sludge samples were taken under the same high nitrogen loading rate of 10 kg-N m^{-3} day^{-1} but only KSU-1 strain existed when synthetic wastewater was used as

influent; while Kumadai-I concurrently appeared when introducing modified reject water as influent under moderately low temperature. There appear to be two plausible explanations of our results obtained. One is that anammox bacteria in the freshwater (KSU-1) were partly replaced by other organic matter-tolerant anammox strain (Kumadai-I). The other is that Kumadai-I originally existed in the inoculums and could survive and grow under existence of organic matter. Despite that the co-existing heterotrophic denitrifiers might consume organic matter for denitrification and played an important role on treatment of low C/N contained wastewaters (Ruscalleda et al. 2008), it could still be speculated that Kumadai-I might play a role as "promotion" factor together with KSU-1 on high-rate nitrogen removal from such wastewaters. In addition, the detection of Kumadai-I under moderately low temperature also demonstrated the potential application of these bacteria for energy reduction in anammox process. However, further studies on Kumadai-I should be investigated to make clear the mechanism of its appearance.

Conclusions

The potential application of whole cell anammox entrapment by PVA/alginate gel on relatively high-rate nitrogen removal from synthetic and modified reject water was successfully achieved in this study. Anammox reactor showed quick start-up after 22 days as well as stable and high nitrogen removal rates of more than 8.0 kg-N m^{-3} day^{-1}, which are the highest values compared to other anammox reactors using whole cell immobilization technique. The matured brownish red PVA beads had good characteristics with the buoyant density of 1.10 g cm^3, settling velocity of 141 m h^{-1} and diameter of 4 mm, leading to complete PVA beads retention whole experiment. In addition, the macroporous structure of beads could facilitate the transfer of both substrates and metabolic products. The switching from synthetic wastewater to modified reject water as influent led to the concurrent existence of new kind anammox bacteria Kumadai-I and KSU-1 in the consortium. It was speculated that Kumadai-I might play a role as "promotion" factor together with KSU-1 on high-rate nitrogen removal from low C/N contained wastewaters.

Acknowledgment We would like to thank Kuraray Co. Ltd (Osaka, Japan) for their PVA material supply.

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