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ORIGINAL PAPER

PVA-alginate immobilized cells for anaerobic ammonium oxidation (anammox) process

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Abstract The feasibility of an anaerobic ammonium oxidation (anammox) process combined with a cell-immobilization technique for autotrophic nitrogen removal was investigated. Anammox biomass was cultivated from local activated sludge and achieved significant anammox activity in 6 months. The development of a mature anammox biomass was confirmed by fluorescence in situ hybridization (FISH) analysis and off-line activity measurements. The abundance fraction of the anammox bacteria determined by FISH analysis was estimated by software. The anaerobic ammonia oxidizers occupied almost half of the total cells. Additionally, the anammox biomass was granulated as spherical gel beads of 3–4 mm in diameter by using a cell-immobilization technique. The nitrogen removal activity was proved to be successfully retained in the beads, with about 80% of nitrogenous compounds (NH_4^+, NO_2^- and total nitrogen) removed after 48 h. These results offer a promising technique for the preservation of anammox microorganisms, the protection of them against the unfavorable surroundings, and the prevention of biomass washout towards the implementation of sustainable nitrogen elimination biotechnology. This is the first report on the immobilization of anammox biomass as gel beads.

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Introduction

In the past few years, several novel and cost-effective biological nitrogen removal processes have been developed. Compared with the conventional biological nitrogen removal (N-removal) process, the combination of partial nitrification and the subsequent anaerobic ammonium oxidation (anammox) process was considered as a sustainable biotechnology, economic and effective, leading to substantial savings of energy and resources in ammonium-rich wastewater treatment [1].

Anammox is a chemolithoautotrophic bioconversion process mediated by a group of Planctomycetes-like bacteria, with a highly unusual physiology, living by consuming ammonium in the absence of oxygen [21]. Anammox is also a distinctive process, involving the oxidation of ammonium with nitrite as the electron acceptor to yield N_2 and NO_3^- under anoxic condition. Strous et al. [20] reported that the stoichiometry of the anammox reaction based on mass balance over anammox enrichment culture was represented by the following equation.

$$NH_4^+ + 1.31NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

The main product of the anammox process is N_2, but about 10% of the fed nitrogen (NH_4^+ and NO_2^-) is converted to NO_3^-. The overall nitrogen balance gives a ratio of NH_4^+ to NO_2^- conversion of 1:1.31 ± 0.06.

Many challenges still remain in the optimization and application of anammox and its combination process in

pilot or full-scale plant. One of the main challenges is to decrease the long start-up time because of the extremely slow growth-rate of anammox bacteria, which will become a problem in practical use [23]. The accumulation of enough anammox biomass is required for fast start-up, but the mass enrichment still remains as a significant obstacle. Since the anammox bacteria are characterized by a long doubling time of 11 days [20], washout of slow-growing anammox biomass from the reactor should be prevented [11], therefore a reactor assured of complete biomass retention (such as immobilization or granulation process) is required to provide a more stable and efficient way for N-removal in a future wastewater treatment plant (WWTP). Taking into account these problems, immobilization of anammox sludge as gel beads was performed in this research.

Cell-immobilization techniques have been frequently applied to biological treatment processes, offering many advantages for promoting efficiency such as higher cell concentration in the reactor, easier solid–liquid separation in the settling tank, shorter lag period of biodegradation, and more stable than free-living cells [5, 12, 13]. There are many natural materials and synthetic polymers such as polyacrylamide, sodium alginate, agar and polyvinyl alcohol (PVA) have been applied extensively in cell immobilization [5, 7, 18]. Since PVA is a promising type of synthetic polymer and is nontoxic to microorganisms, it is therefore very suitable for entrapment of microbial cells in its polymeric matrixes. A new cell immobilization method has recently been developed in our laboratory and successfully applied to the immobilization of nitrifying and denitrifying sludge [4, 8, 9]. This immobilization technique by using PVA, Na-alginate and $NaNO_3$ can simultaneously prevent the agglomeration problem, eliminate the toxicity of saturated boric acid solution, and improve the gas permeability of PVA gel beads [4].

In this study, anammox biomass was cultivated from sludge of a local municipal WWTP. The cultivated anammox biomass was verified by FISH analysis and off-line activity measurements. Cell-immobilization technique was used to retain the anammox biomass in PVA-alginate gel beads. Moreover, the N-removal activity and stoichiometric characteristics of anammox reaction in the immobilized-cell system were also investigated.

Materials and methods

Synthetic wastewater

For culturing and the following experiments a mineral salt medium supplemented with different amounts of NH_4^+ and NO_2^- was used: $(NH_4)_2SO_4$ (0.12–0.48 g/L), $NaNO_2$ (0.163–0.65 g/L), $KHCO_3$ (1.25 g/L), Na_2HPO_4 (0.018 g/L), KH_2PO_4 (0.03 g/L), $MgSO_4$–$7H_2O$ (0.2 g/L) and a solution (1.25 ml/L) of trace elements according to Sliekers et al. [19]. The synthetic wastewater used in this study contained mainly ammonium and nitrite, no organic substrate was added, and the initial pH was adjusted to 7 ± 0.2.

Chemicals and seed sludge

The materials used in cell immobilization were as follows: PVA with a grade of 99.5% saponification and 1,600 degree of polymerization was obtained from Fluka Co., Na-alginate (with medium viscosity) and $CaCl_2$–$2H_2O$ were purchased from Sigma–Aldrich Co., $NaNO_3$ was supplied by Riedel-deHaen Co. All other chemicals used in this study were of analytical grade. The concentrated activated sludge collected from a local (Taipei city) municipal WWTP was used as seed sludge.

Cultivation of anammox biomass

Cultivation was performed in a 1,000 mL stoppered conical flask with 200 mL seed sludge inoculated in 500 mL of synthetic wastewater (with 25 mg/L NH_4^+–N, 25 mg/L NO_2^-–N, bicarbonate and other nutrients). The solution was purged with helium gas to reduce the dissolved oxygen (DO) concentration and immediately sealed with a rubber stopper and stirred gently at 35°C. Samples were directly collected from the flask with a syringe and filtered with a membrane filter of 0.22 μm; the remaining concentrations of nitrogenous compounds were measured by the IC system (Dionex 120). Gas samples withdrawn from the head space of the flask were analyzed by the GC system to identify the production of N_2 gas. From the results of our previous studies, the activated anammox biomass can be obtained after 6-month cultivation period. After the verification of anammox activity, the harvested anammox biomass was directly used for cell immobilization.

FISH analysis

Fluorescence in situ hybridization (FISH) is a recognized tool for the specific and sensitive identification of target organisms within complex microbial communities [2]. Visualization of FISH labeled cells in sludge samples can be carried out by some techniques, including fluorescence microscopy and confocal laser scanning microscopy (CLSM) [10, 22]. It is possible to identify the populations and quantify only the anammox bacteria by FISH analysis [16].

In this study, FISH analysis of sludge samples collected from the cultivation flask was done to verify the anammox

bacteria before cell immobilization. Sludge samples were mixed with 4% (w/v) paraformaldehyde solution and fixed for 30 min at 4°C, followed by FISH. Both fixation step and FISH were performed as described by Manz et al. [14]. For the hybridization, the oligonucleotide probes labeled at the 5′-end with Cy3 or Alexa Fluorescein 350 was used. EUB338 served as a positive control in specificity. All employed probes are listed with their sequences and specificities in Table 1. Appropriate probe sequences for the specific detection of each bacteria strain were retrieved from the listed literatures [3, 6, 15, 17]. The Amx368 probe was appropriate and specific for detection of all anammox bacteria [17]. Total cells were stained first with EUB338 (I–IV) labeled with Cy3, yielding bright fluorescence signals, followed by a second FISH with Amx368-alexa fluorescein 350 for the detection of anammox cells. After hybridization, a washing step and the following air-drying step were performed prior to microscopic examination. The FISH labeled cells in sludge samples were visualized using a fluorescence microscope. The models of microscope and digital camera used in this study are Olympus BX-41 and Sensovation EZ-140 LN, respectively. The abundance fraction of anammox cells to total cells was estimated from FISH data by use of the software Leica Application Suite-Advanced Fluorescence (LAS-AF).

Immobilization of anammox biomass

Cell immobilization by the PVA-sodium nitrate method was carried out as described by Chang et al. [4]. For experiments with immobilized anammox microorganisms, the alginate solution containing 16% w/v PVA and 1.2% w/v Na-alginate was earlier prepared by mixing and heating until dissolved. After cooling down to room temperature, 100 mL of cultivated anammox biomass was slowly added into 100 mL of mixture in a volume ratio of 1:1 with adequate mixing. The resulting mixture was dropped into a solidifying solution (50% w/v $NaNO_3$ and 2% w/v $CaCl_2$), gently stirred for about 30 min, and then immersed in the solidifying solution for 1 h to enforce the spherical gel beads. Using this method, it is possible to form porous beads that have uniform size and shape. These anammox beads were washed several times with large amount of distilled water to remove extra alginate or PVA on the surface, activated in synthetic wastewater containing nutrients, bicarbonate, 25 mg/L of NH_4^+–N and NO_2^-–N, then stored at 35°C until further use.

Evaluation of nitrogen removal activity

The immobilized gel beads with entrapped anammox biomass were used to remove NH_4^+ and NO_2^- in the synthetic wastewater of different initial nitrogen concentrations. The repeated batch experiments were conducted with a series of batch reactors (1,000-mL conical flask). Each reactor contained 100 mL of gel beads and 500 mL of synthetic wastewater (initial pH 7 ± 0.2, without adjustment). The batch reactors were then purged with helium gas The head space was also flushed and filled with helium gas to promote anoxic condition, after which, the flasks were sealed with rubber stoppers and gently stirred at 35°C to maintain homogeneity. Water samples were directly withdrawn from the reactors at intervals of 3 h and analyzed to determine levels of NH_4^+, NO_2^- and NO_3^-, in order to assess the nitrogen removal activity under various initial concentrations.

Results

Characterization of the cultivated anammox biomass

Although it was reported that the long-term enriched anammox cultures from activated sludge changed from brown to pinkish-red color [21], the macroscopic appearance of the cultivated biomass in this study remained a light brown color. As previously reported by Third et al. [22], the biomass also consisted visibly of small and fine aggregates rather than compact granules. Additionally the settling efficiency of the biomass was very remarkable. The bubbles emerged continuously from the cultivated sludge and accumulated in the head space of the stoppered flask.

Table 1 Characteristics of 16S rRNA-directed oligonucleotide probes used for FISH

Probe	Target	Sequence of probe (5′-3′)	Reference
EUB338 I	Most Bacteria	GCT GCC TCC CGT AGG AGT	Amann et al. [3]
EUB338 II	Planctomycetales	GCA GCC ACC CGT AGG TGT	Daims et al. [6]
EUB338 III	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	Daims et al. [6]
EUB338 IV	Bacterial lineages not covered by probes EUB338, EUB338II, and EUBIII	GCA GCC TCC CGT AGG AGT	Schmid et al. [15]
Amx368	All anammox microorganisms	CCT TTC GGG CAT TGC GAA	Schmid et al. [17]

The pH level increased significantly from 7 ± 0.2 to 8 ± 0.2 during the sludge cultivation period. The major component of the gas sample was identified as N_2 gas and there was no production of undesirable by-products like NO or N_2O. Furthermore, both NH_4^+ and NO_2^- concentrations decreased whereas the NO_3^- concentration increased (Fig. 1). In other words, the consumption of NH_4^+ and NO_2^- resulted in the production of N_2 and NO_3^-. The observed removal of NH_4^+ and NO_2^- could be attributed to a completely autotrophic process (i.e., the anammox reaction) rather than heterotrophic denitrification, because there was no addition of external organic carbon source in the medium.

Verification of anammox bacteria by FISH analysis

The presence of anammox bacteria in the enriched culture was verified by qualitative and quantitative FISH analysis. Positive hybridization with the probe Amx368 confirms the presence of anammox bacteria [17]. Figure 2 shows a distinct distribution of anammox bacteria within the aggregate, obviously a large part of the biomass consisted of anammox bacteria. The abundance fraction of anammox cells to total cells was estimated by software and indicated that the anaerobic ammonia oxidizers occupied almost half of the total cells. This is really a definite and strong evidence to explain why the immobilized gel beads performed as the typical anammox reaction.

Macroscopic appearance of the immobilized anammox beads

Figure 3 illustrates that the anammox beads were relatively regular in shape, approximately spherical with smooth

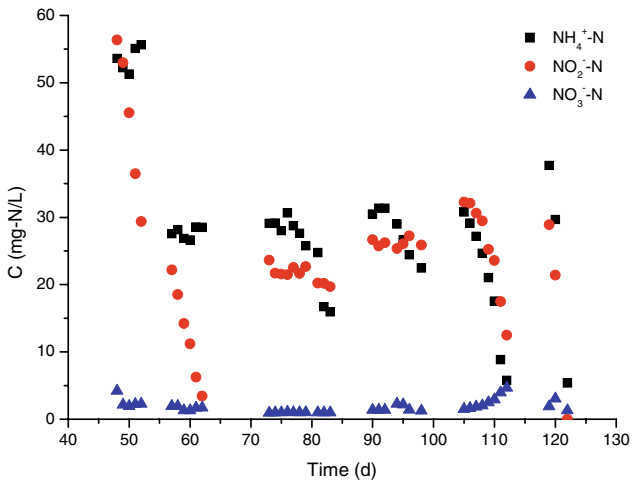

Fig. 1 Evolution of nitrogenous compounds concentrations during cultivation period

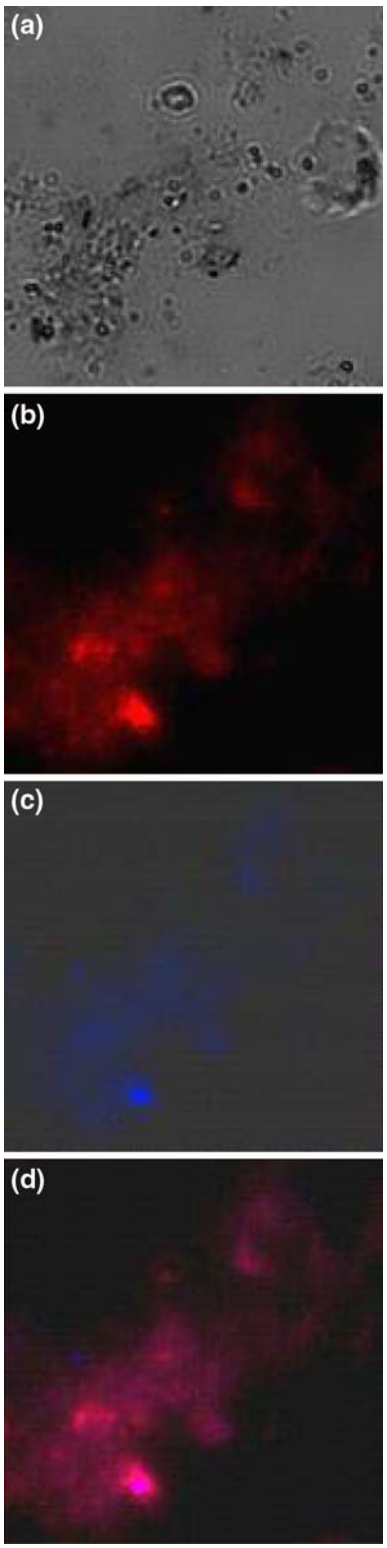

Fig. 2 Micrographs of analyzed biomass at 1000x magnification. Phase contrast image (**a**) and FISH images (**b–d**) of sludge samples from the cultivated culture. **b** Total cells were stained first with EUB 338(I–IV) (labeled with Cy3, *red*), **c** a second FISH with Amx368 (labeled with Alexa Fluorescein 350, *blue*); **d** combination of red and blue colors resulted in *purple color*

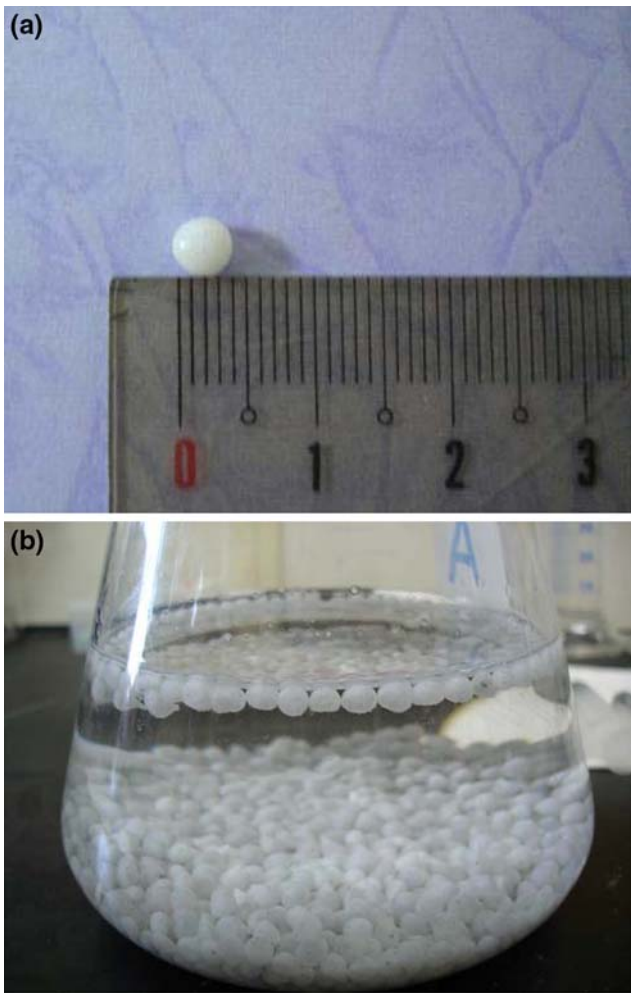

Fig. 3 Photographs of the PVA-alginate immobilized gel beads with entrapped anammox biomass. **a** Actual appearance of anammox beads; **b** anammox beads in batch experiments

surface and about 3–4 mm in diameter. During the activation process, some of these gel beads floated up to the solution surface. The floating effect of gel beads is a common phenomenon, which occurs especially in the activation stage when utilizing PVA-immobilized cells in a bioreactor [5]. Chang and Tseng [4] reported that the elasticity and mechanical strength of this type of PVA-alginate gel beads are adequate for the high shear stress encountered in an aerated tank or a fluidized bed reactor [4].

Nitrogen removal ability of the anammox beads

The removal of NH_4^+, NO_2^- and total nitrogen (T-N) at different initial concentrations is summarized in Fig. 4, showing that the concentration profile of nitrogenous compounds and the variations of concentrations of NH_4^+, NO_2^- and NO_3^- were very similar at various initial concentrations. NH_4^+ and NO_2^- concentrations decreased

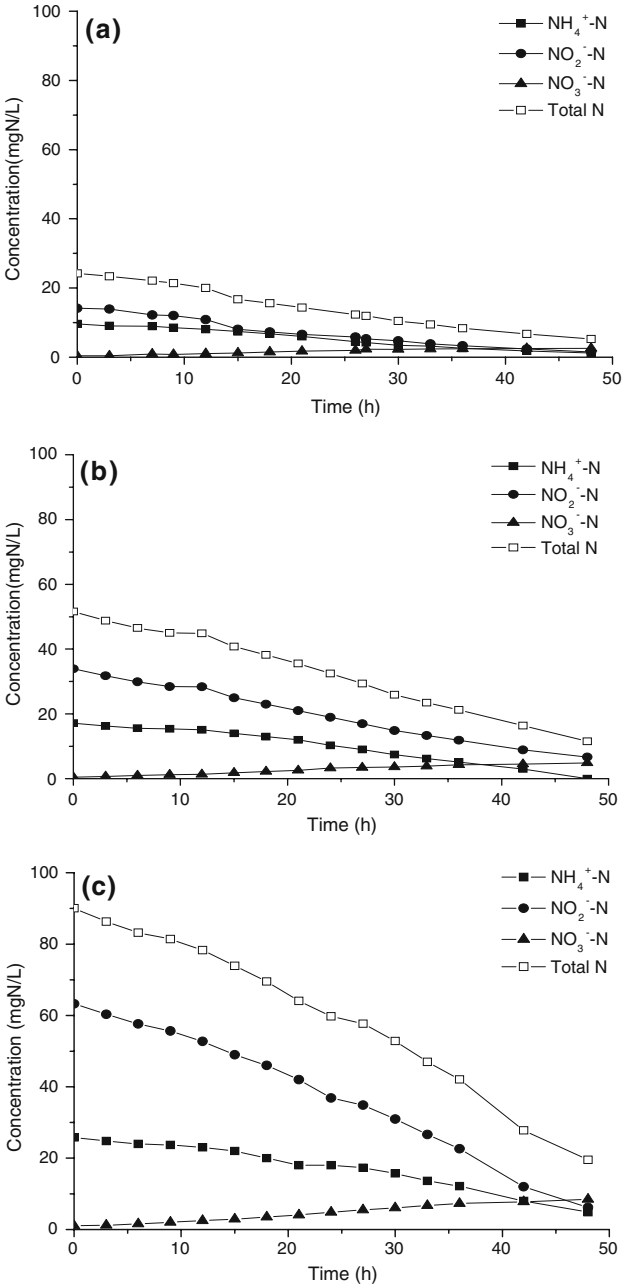

Fig. 4 Concentration profiles of NH_4^+(*filled square*), NO_2^- (*filled circle*), NO_3^- (*filled triangle*) and T-N (*open square*) during the experiments of different initial T-N concentrations at 35°C: **a** 25 mg N/L, **b** 50 mg N /L, **c** 100 mg N/L

obviously, meanwhile the NO_3^- concentration increased gradually. Both NH_4^+ and NO_2^- were simultaneously removed from the beginning and the removal rate appeared to be relatively steady till the end, moreover the NO_2^- removal rate was much faster than that of NH_4^+.

All these results revealed that the anammox beads still very active in N-removal, the PVA-sodium nitrate method is therefore a promising and economical technique for anammox cell immobilization. The gelation solution

(sodium nitrate) used in this method is of low toxicity and very cheap. Additionally, the immersion time for solidification is only 1 h and the N-removal activity of the beads is quite satisfactory.

Stoichiometric characteristics of the anammox reaction

Figure 5 presents the comparisons of NO_2^- removal and NO_3^- production to NH_4^+ removal, revealing that the transformation ratios are in agreement with the anammox reaction. The trend-line slopes appear to be increasing with the initial T-N concentration and can be directly compared to the molar stoichiometric coefficients of Eq. (1). In this study, the ratios of NO_2^- removal and NO_3^- production to NH_4^+ removal were 1:1.53–1:1.88 and 1: 0.29–1:0.41, respectively, bigger than suggested by the equation of the anammox reaction [21]. The results of these experiments accorded with Eq. (1), indicating that the anammox activity was successfully retained in the beads.

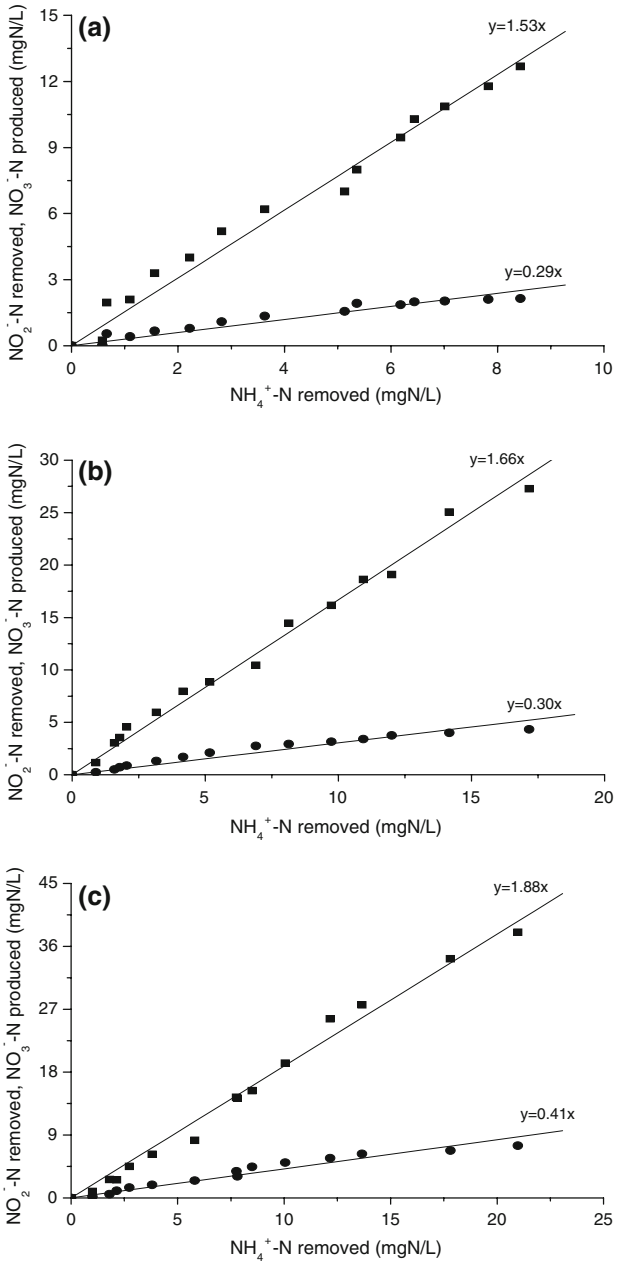

Fig. 5 NO_2^- removal and NO_3^- production as functions of NH_4^+ removal obtained during the experiments of different initial T-N concentrations: **a** 25 mg N/L, **b** 50 mg N/L, **c** 100 mg N/L. NO_2^- removal (*filled square*) and NO_3^- production (*filled circle*)

Discussion

The observed removal of ammonium and nitrite in the batch reactors could be attributed to the anammox reaction, implying that the cultivated sludge was characteristic of an anammox reaction after a 4-month cultivation period. The cultivated biomass showed significant and stable anammox activity in around 6 months, resulting in the anaerobic ammonia oxidizers occupied almost half of the total cells.

In our system, the immobilized beads were not disrupted and always remained in good shape with satisfactory N-removal efficiency during the repeated experiments, indicating that the beads had firm structure and the cultivated biomass had been successfully entrapped in the beads. To our knowledge, this is the first time to immobilize the anammox biomass as gel beads. The established cell-immobilization technique [4] used in this study offers a promising way to granulate the valuable anammox biomass, to protect these microorganisms against the unfavorable surroundings, and to efficiently retain their activity in the reactor. Therefore the problems encountered in conventional bioprocesses for nitrogen elimination such as solid–liquid separation and biomass washout could be solved simultaneously.

In environmental biotechnology, the combination of different groups of nitrogen elimination microorganisms and the process optimization will offer better solutions in N-removal. The anammox process combined with the preceding partial nitrification provides great promise for treatment of high-strength wastewater. Research to stimulate the activity of anammox microorganisms is required to achieve rapid accumulation of anammox biomass as the predominant species. In the future, full-scale introduction of autotrophic N-removal biotechnology combined with cell immobilization techniques will lead to substantial savings in energy and resources and better management of water environment [1]. Although the factors to speed up the growth rate of anammox biomass were not investigated, it must be emphasized that the aim of this experiment was to study the feasibility of the anammox process combined with a cell-immobilization technique. Further studies will

be done using a continuously stirred tank reactor (CSTR) with immobilized anammox beads to investigate practical use in wastewater nitrogen removal technology.

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