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Rapid cultivation of anammox sludge based on Ca-alginate cell beads

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ABSTRACT

Current gel entrapment technology has certain advantages for the enrichment of anammox sludge. In this study, the optimal preparation conditions and cultivation equipment of Ca-alginate cell beads for the culturing anammox sludge were proposed. The preparation parameters of the Ca-alginate cell beads were as follows: 3% sodium alginate, 4% $CaCl_2$, V_{SA}:V_{cell} = 1:1, a drop height of 9 cm, stirring speed of 300 rpm, and cross-linking time of 24 h. The prepared cell beads were regular spheres with a uniform size and hard texture. Throughout the 9 days of cultivation, the number of anammox bacteria in the Ca-alginate cell beads was 4.3 times that of the initial sludge, and the color of the cell beads changed from yellowish-brown to reddish-brown. Scanning electron microscopy (SEM) analysis showed that the SA gel beads had a good microporous structure. The fluorescence *in situ* hybridization (FISH) results illustrated that the bacteria were mostly dispersed inside the Ca-alginate cell beads. Additionally, the qPCR results implied that only a relatively small amount of anammox biomass (2.74×10^6 copies/gel-bead) was required to quickly start the anammox process. The anammox bacteria in the Ca-alginate cell beads grew with a fast growth rate in a short period and exhibited high activity due to diffusion limitations. In addition, the anammox bacteria cultivated in the Ca-alginate cell beads could adapt to the increase in substrate concentration in a short period. The optimal incubation time of this gel entrapment method for anammox sludge was no more than 17 days under the experimental conditions of this work. Therefore, this simple and practicable gel entrapment method may serve as a suitable pre-culture means for the rapid enrichment of anammox bacteria.

Key words: anammox bacteria, Ca-alginate cell beads, enrichment culture, gel entrapment, nitrogen removal

HIGHLIGHTS

- The optimal preparation conditions and cultivation equipment for Ca-alginate cell beads were proposed.
- Anammox bacteria cultivated in Ca-alginate cell beads could adapt to substrate concentration disturbance in a short time.
- This gel entrapment method only required few anammox cells to start the anammox process.
- The optimum incubation time of this gel entrapment method for anammox bacteria was no more than 17 days.

INTRODUCTION

Anaerobic ammonia oxidation is an autotrophic nitrogen removal process that directly converts ammonium and nitrite into nitrogen in one step, achieving both ammonium and nitrogen removal. The advantages of this biological process include a low oxygen demand, no need for an organic carbon source, low sludge production, and reduction of N_2O emission (Ali *et al.* 2015b). However, as functional microorganisms in the anammox process, anammox bacteria restrict the development of this process. Additionally, anammox bacteria have two limiting factors. First, they have a slow growth with a low cell yield, where the doubling time of anammox bacteria can be as long as 10–30 days (Manonmani & Joseph 2018); thus, anammox bacteria require a long incubation time to obtain sufficiently high activity of the anammox sludge. Second, certain environmental conditions in the water treatment system have a significant influence on the metabolic activity of anammox bacteria, such as substrates, dissolved oxygen, organic matter, pH, and temperature (Yan & Hu 2009).

The first successful enrichment of suspended anammox bacteria *Candidatus* Kuenenia stuttgartiensis utilized a membrane-assisted bioreactor (MBR) (van der Star *et al.* 2008). Currently, MBRs have been used to enrich different types of anammox

bacteria to study their physiological and kinetic characteristics, such as *Candidatus* Jettenia (Ali *et al.* 2015a), *Candidatus* Brocadia (Lotti *et al.* 2014), and *Candidatus* Scalindua (Awata *et al.* 2013). However, these studies used suspended cell cultures, which require a long period, typically about 100 days (Lotti *et al.* 2014). One efficient method that can prevent biomass from being washed out and allow for hyper-concentrated culture is cell immobilization technology, which can be used to overcome the problems of anammox bacteria (Zhu *et al.* 2014). Moreover, this technology offers a sufficient supply of substrates and no hydraulic shear stress; thus, nascent daughter cells can be fully retained. Immobilized cell typically refers to the microorganism that can be obtained through physical and chemical means, and reproduce in a certain carrier space (Shin *et al.* 2019). One of the most common techniques for artificial immobilization is gel entrapment, which allows for the cultivation of slow-growing bacteria (Date *et al.* 2008).

At present, gel entrapment technology has been successfully used for anammox cells (Isaka *et al.* 2013; Ali *et al.* 2014, 2015b). The synthetic polymer polyvinyl alcohol (PVA) has been used as a regular entrapment matrix to form PVA/sodium alginate (SA) gel beads (Chen *et al.* 2020). Because PVA gel beads easily clog, boric acid used to solidify the PVA gel beads is toxic to the entrapped microbes (Ge *et al.* 2009). Currently, PVA/SA gel beads have been used to rapidly cultivate free-living planktonic anammox cells (Zhang & Okabe 2017). However, the long-term use of PVA/SA gel beads for anammox cell cultivation has always faced issues of expansion and bursting, due to the dense surface (Choi *et al.* 2017). Therefore, to improve the mass transfer efficiency of PVA/SA gel beads, researchers controlled the thickness of the outer PVA/SA gel bead layer or added $NaHCO_3$ as a foaming agent (Bae *et al.* 2017; Choi *et al.* 2017). However, the use of a simpler SA entrapment matrix with better mass transfer efficiency for the preparation of gel beads and the rapid cultivation of anammox cells has rarely been reported. Some researchers selected the four best entrapment supports including sodium carboxymethylcellulose (CMC), SA, PVA/SA, and chitosan (CTS) for entrapping the nitrifying biomass to compare the partial nitrification rate and adsorption efficiency (Yan & Hu 2009). The SA and PVA/SA entrapment supports were both cross-linked using the same concentration of calcium chloride solution to form gel beads, and the order of partial nitrification efficiency for the four regular entrapment supports was SA>PVA/SA>CTS>CMC. SA was selected as the best entrapment support after comparing the partial nitrification rate and adsorption efficiency. Furthermore, the activity of the anammox sludge in the SA gel beads was higher than that in the PVA/SA gel beads (Zhu *et al.* 2009). However, the enrichment of anammox sludge in SA gel beads has not been reported.

In this work, an SA entrapment matrix cross-linked with calcium chloride solution to form gel beads for rapid enrichment of anammox bacteria was studied. Optimal preparation conditions for the SA gel beads were determined, and the morphological, physical, and chemical properties of the Ca-alginate cell beads were characterized. Subsequently, the substrate removal and cell growth of the embedded anammox sludge were explored, and the effect of substrate concentration disturbance on anammox bacteria activity in the Ca-alginate cell beads was investigated. The anammox bacteria enrichment performance of the Ca-alginate cell beads was also discussed.

MATERIALS AND METHODS

Preparation conditions optimization of Ca-alginate cell beads

The SA entrapping agent and anhydrous cross-linking agent calcium chloride ($CaCl_2$) were the analytical reagents (Sinopharm Chemical Reagent Co., Ltd, China). Ultrapure water was produced by a Direct-Q 5 water purification system (Merck Millipore, USA). The SA and $CaCl_2$ solutions were prepared with certain concentrations. First, a 1 mL syringe (inner diameter of 2 mm) was used to add the SA solution dropwise to the $CaCl_2$ solution, to prepare the SA gel beads. The beaker containing $CaCl_2$ solution was placed on a magnetic stirrer (DF-101S, Gongyi Yuhua Instrument Co., Ltd, China). Then, we examined different SA concentrations (0.5, 1, 2, 3, and 4%), $CaCl_2$ concentrations (1, 2, 3, 4, and 6%), drop heights of the beads (6, 9, 12, and 15 cm), and rotating speeds (100, 200, 300, 400, and 500 rpm) under the conditions of gel bead formation to select the best scheme for preparing the SA gel beads.

The anammox seed sludge was removed from the A^2/O process of Jingkou Sewage Treatment Plant (Zhenjiang, Jiangsu, China) and its mixed liquor suspended solid (MLSS) was 4,850 mg/L. To prepare the SA solution with an optimal concentration, an appropriate amount of anammox mixed culture was mixed with the SA solution. Then, this mixture was added dropwise to an optimal concentration of $CaCl_2$ solution to obtain the Ca-alginate cell beads. By utilizing different ratios of SA solution, mixed culture (V_{SA}: V_{sludge}, 1:0.5, 1:1, 1:1.5, 1:2), and cross-linking time (6, 12, 18, and 24 h), we could optimize the preparation of the Ca-alginate cell beads.

Enrichment culture of the embedded anammox sludge

Figure 1 shows the experimental device diagram. An anaerobic culture medium reserve bottle (500 mL) was heated to 35 °C in a water bath (DCJ-4D, Jintan Economic Development Zone Xiangbo Instrument Factory, China), which was connected to a nitrogen cylinder and maintained the dissolved oxygen (DO) concentration of the medium below 0.5 mg/L. The culture medium contained the following: 2 mg/L of KH_2PO_4, 110 mg/L of $KHCO_3$, 20 mg/L of $MgSO_4{\cdot}7H_2O$, 20 mg/L of $CaCl_2{\cdot}2H_2O$, and 1.5 mL/L of trace element I and trace element II. The composition of trace element I was 5 g/L of EDTA and 5 g/L of $FeSO_4$, while the composition of trace element II was 15 g/L of EDTA, 0.25 g/L of $CuSO_4{\cdot}5H_2O$, 0.99 g/L of $MnCl_2{\cdot}4H_2O$, 0.43 g/L of $ZnSO_4{\cdot}7H_2O$, 0.24 g/L of $CoCl_2{\cdot}6H_2O$, 0.19 g/L of $NiCl_2{\cdot}6H_2O$, and 0.22 g/L of $NaMoO_4{\cdot}2H_2O$. NH_4^+-N and NO_2^--N were provided by NH_4Cl and $NaNO_2$.

The prepared Ca-alginate cell beads were washed three times with sterilized water and then inoculated into three anaerobic reaction bottles (200 mL) with a filling rate of 60% (v/v). The three anaerobic reaction bottles were placed in a water bath (DCJ-4D, Jintan Economic Development Zone Xiangbo Instrument Factory, China) at 35 °C, and at the same time, the culture medium was quickly pumped into the three anaerobic reaction bottles to maintain an anaerobic state at all times. Then, the three anaerobic reaction bottles were wrapped in tin foil and incubated in a constant temperature shaker (SHZ-82A, Jintan City Baita Xinbao Instrument Factory, China) at a shaking speed of 60–80 rpm. The cultivation parameters were temperature of 35 °C and pH of 7.2 ± 0.3, and the hydraulic retention time (HRT) was 20 h.

Scanning electron microscopy

The sample pretreatment method was referred to a previous report (Liu *et al.* 2015). The SA gel beads and Ca-alginate cell beads were fixed with 2.5% of glutaraldehyde at 4 °C for 4 h and then washed three times with phosphate buffer solution (PBS). The fixed beads were treated by ethanol gradient dehydration and then dried in a vacuum freeze dryer (FD-1A-50, Shanghai Bilang Instrument Manufacturing Co., Ltd, China). Finally, the dry beads were observed under a scanning electron microscope (JSM-7800F, JEOL, Japan).

Specific surface area and pore size determination

The specific surface areas and pore sizes of the SA gel beads and Ca-alginate cell beads were determined by a high-performance automatic mercury porosimeter (AutoPore IV 9510, Micromeritics Instrument Corporation, USA). The details were referred to a previous report (Zhang *et al.* 2017).

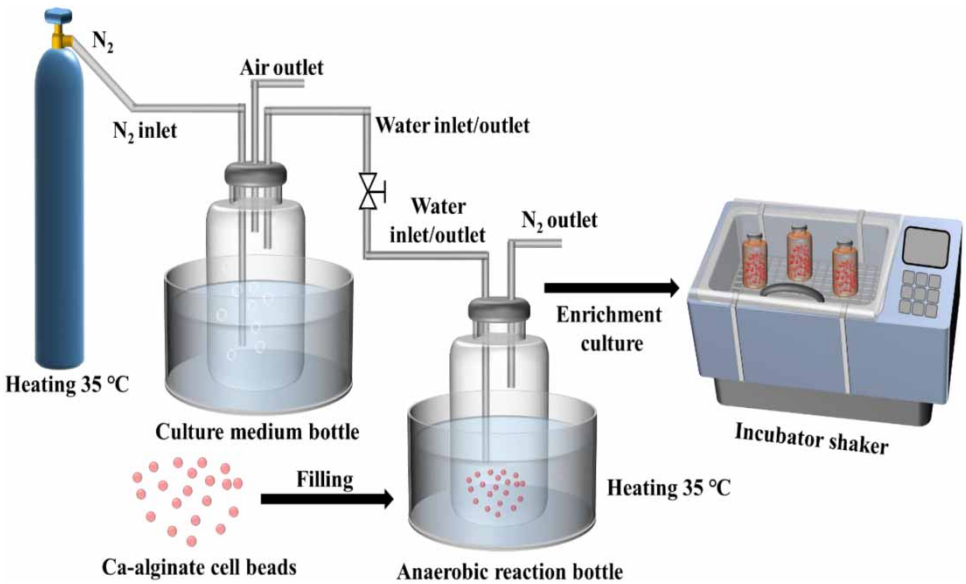

Figure 1 | The experimental schematic diagram of Ca-alginate cell bead cultivation.

Zeta potential and mechanical strength measurements

The zeta potential measurements were conducted according to a previously published study (Yan & Hu 2009). First, a beaker containing 100 mL of deionized water was filled with 2 g of SA gel beads or Ca-alginate cell beads and then stirred by a mechanical stirrer at a high speed. Then the suspension was stored in a biochemical incubator (SPX-150B-Z, Shanghai Boxun Industry & Commerce Co., Ltd, Medical Equipment Factory, China) at 30 °C, and the zeta potential measurements were obtained by a micro-electrophoresis apparatus (JS94 K, Shanghai Zhongchen Digital Technic Apparatus Co., Ltd, China).

The mechanical strength measurements were conducted according to a previous report (Zhang *et al.* 2011). A filter paper was used to trap SA gel beads and Ca-alginate cell beads. The excess water on the surface of SA gel beads and Ca-alginate cell beads was dried in a drying oven (DHG-9140A, Shanghai Yiheng Scientific Instrument Co., Ltd, Shanghai, China) at 60 °C. Then, the weight of SA gel beads or Ca-alginate cell beads was weighted by an electronic microbalance (ME104E, Mettler Toledo Instrument (Shanghai) Co., Ltd, China). SA gel beads or Ca-alginate cell beads were placed on the slide A. Slide B was placed on the top of the beads, and weights were gradually put on slides B to observe the cracking of the beads. The mechanical strength calculation formula is as follows:

$$\text{Mechanical strength}\left(\frac{\mathbf{kg}}{\mathbf{g}}\right)=\frac{\mathbf{Mw}}{\mathbf{Mb}}$$

M_w is the weight of the weights and slide B, M_b is the weight of the beads.

Chemical analyses

An UV-visible spectrophotometer (L8, INESA, China) was used to analyze the concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N. NH_4^+-N was assayed by Nessler's reagent spectrophotometry at a wavelength of 420 nm, NO_2^--N was determined by the N-1-naphthyl-1,2-diaminoethane dihydrochloride spectrophotometric method at a wavelength of 540 nm, and NO_3^--N was measured by ultraviolet spectrophotometry at wavelengths of 220 and 275 nm.

Fluorescence *in situ* hybridization tests

Fluorescence *in situ* hybridization (FISH) technology uses fluorescence-labeled specific oligonucleotide fragments as probes hybridized with DNA or RNA molecules from the microbial genome. Thus, the specific microbial community could be observed under a fluorescence microscope at certain excitation and emission wavelengths. Six Ca-alginate cell beads were collected from each of the three anaerobic reaction flasks on days 0, 9, and 17 for FISH testing. The samples were treated by a series of operations such as smear, fixation, dehydration, pre-hybridization, hybridization, washing, and 4,6-diamidino-2-phenylindole (DAPI, Solarbio, China) nucleus counterstaining. The fluorescein isothiocyanate (FITC)-labeled (green) EUB338 probe (5′-GCTGCCTCCCGTAGGAGT-3′, ordered from HIPPOBIO, HuZhou, China) was used to target the entire bacterial community at an excitation wavelength of 465–495 nm and emission wavelength of 515–555 nm. The Cy3-labeled (red) AMX820 probe (5′-AAAACCCCTCTACTTAGTGCCC-3′, ordered from HIPPOBIO, HuZhou, China) was also used to mark the anammox sludge at an excitation wavelength of 510–560 nm and emission wavelength of 590 nm (Zhang & Okabe 2017). Then, the stained samples were sliced and observed under an upright fluorescence microscope (Eclipse ci, Nikon, Japan).

Quantitative polymerase chain reaction assays

Six Ca-alginate cell beads were collected from each of the three anaerobic reaction flasks on days 0, 9, and 17 for quantitative polymerase chain reaction (qPCR) assays. The DNA samples were extracted by a DNA rapid extraction kit (B518233, BBI, China). Then, the extracted DNA samples were dissolved in sterilized deionized water and stored at −20 °C. The qPCR reaction system (20 μL) consisted of ddH_2O (7.2 μL), Sybr Green qPCR Master Mix (10 μL, 2X SG Fast qPCR Master Mix (High Rox, B639273, BBI, China), DNA template (2 μL, 1–10 ng), primer hzsA_F (5′-CACGTGACCGGTAATTCTCT-3′, 0.4 μL, and 10 μmol/L), and primer hzsA_R (5′-GGTATTTTGAAGGAGACTGG-3′, 0.4 μL, and 10 μmol/L). QPCR was performed by a real-time fluorescence quantitative PCR instrument (StepOne Plus, ABI, USA), and the operating conditions were as follows. Pre-denaturation was conducted at 95 °C for 3 min, followed by cycling according to the following operations of denaturing at 95 °C for 15 s, annealing at 57 °C for 20 s, and extension at 72 °C for 30 s, which was conducted 45 times. Each sample was run three times, and the DNA concentration in each sample was determined by a micro ultraviolet spectrophotometer

(SMA4000, Merinton, China). QPCR amplification efficiency was 106.463%. The correlation coefficient of the standard curve was 0.998, and the y-intercept was 36.797.

RESULTS AND DISCUSSION

Optimization of the embedding conditions

The concentrations of the embedding agent SA and cross-linking agent $CaCl_2$, the drop height of the beads, and the stirring speed all affected bead formation. The gel beads with uniform size, regular spherical shape, and certain hardness were selected. Table 1 shows the optimization results for each variable.

As shown in Table 1, if the SA concentration was too low (0.5–1%), the gel beads were soft and could not form. When the concentration of SA was too high (≥4%), the gel beads experienced the drag tail phenomenon. The cross-linking agent $CaCl_2$ had little effect on the bead size and shape, and mainly affected the texture of the gel beads. If the $CaCl_2$ concentration was too low (1–3%), the gel beads were mostly oval, soft, and easily broken. While the $CaCl_2$ concentration was too high (≥6%), the gel beads were too hard and fragile. At a slow stirring speed (100 rpm), the gel beads were irregular in shape, and at a fast stirring speed (≥400 rpm), the gel beads easily deformed. In addition, when the drop height was low (6 cm), the prepared gel beads had different shapes and were mostly ellipsoidal. When the drop height was high (≥12 cm), it caused the gel beads to be uneven in shape and was prone to tailing. Therefore, the ideal SA gel beads were prepared when the SA concentration was 3%, the $CaCl_2$ concentration was 4%, the stirring speed was 300 rpm, and the drop height was 9 cm.

In addition, the effects of the ratio of SA and anammox mixed culture, and the cross-linking time on the activity of the prepared Ca-alginate cell beads were also investigated. The preparation conditions were further optimized by measuring the removal efficiency of NH_4^+-N by the Ca-alginate cell beads within 20 h. As shown in Figure 2(a), the substrate NH_4^+-N and NO_2^--N concentrations were 50 and 66 mg/L, respectively, and the cross-linking time was 6 h. The NH_4^+-N removal efficiency of the Ca-alginate cell beads prepared with different mixture ratios of SA and anammox mixed culture followed 1:1.5>1:1>1:2>1:0.5. The hardness of the Ca-alginate cell beads increased with an increase in V_{SA}/V_{sludge}, and when the ratios of V_{SA}/V_{sludge} were 1:1 and 1:1.5, there was little difference in the removal efficiency of NH_4^+-N. Following comprehensive consideration, the V_{SA}/V_{sludge} ratio of 1:1 was chosen. The NH_4^+-N removal efficiency of Ca-alginate cell beads with different

Table 1 | SA gel bead optimization experiment

Fixed parameters	SA concentration (%)	Molding effect
4% $CaCl_2$, 250 rpm, 9 cm	0.5	Unable to molding.
	1	Basic molding, irregular shape, and uneven size.
	2	Regular spherical shape, 1–2 mm in diameter, and hard texture.
	3	Regular spherical shape, 2–3 mm in diameter, and hard texture.
	4	Irregular spherical shape, trailing, and uneven size.
Fixed parameters	**$CaCl_2$ concentration (%)**	**Molding effect**
3% SA, 250 rpm, 9 cm	1	Irregular ellipsoid.
	2	Irregular ellipsoid.
	3	Regular spherical shape, and slightly different in size.
	4	Regular spherical shape, uniform size, and hard texture.
	6	Regular spherical shape, uniform size, and solid texture.
Fixed parameters	**Stirring rate/rpm**	**Molding effect**
3% SA, 4% $CaCl_2$, 9 cm	100	Irregular shape.
	200	Regular spherical shape, and slightly different in size.
	300	Regular spherical shape, and uniform size.
	400	Irregular ellipsoid, and containing bubbles.
	500	Irregular shape, and trailing.
Fixed parameters	**Drop height/cm**	**Molding effect**
3% SA, 4% $CaCl_2$, 300 rpm	6	Irregular ellipsoid.
	9	Regular spherical shape, and uniform size.
	12	Slightly irregular shape.
	15	Irregular shape, and trailing.

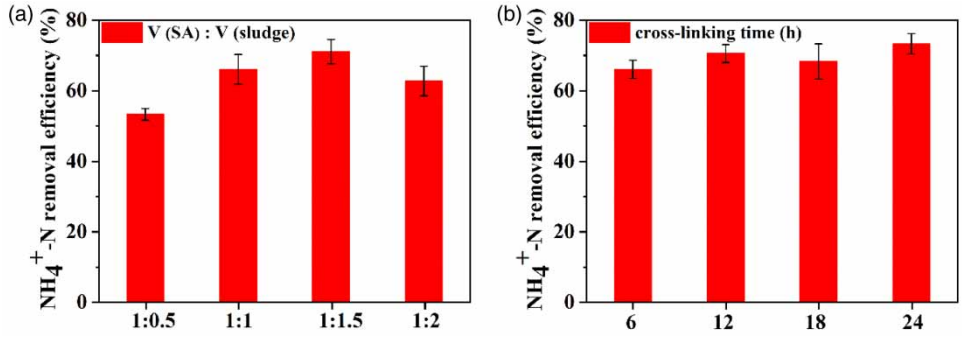

Figure 2 | NH_4^+-N removal efficiency of Ca-alginate cell beads prepared under different variables. (a) V_{SA}/V_{sludge}, and (b) cross-linking time.

cross-linking times showed no differences (Figure 2(b)); however, extended cross-linking time was beneficial to enhance the hardness of the beads. Thus, the chosen cross-linking time was 24 h, and the preparation method of the Ca-alginate cell beads was as follows. First, 3% SA and anammox sludge were mixed uniformly at a volume ratio of 1:1. Then, a 1 mL syringe (inner diameter of 2 mm) was used to transfer the mixed colloid, which was dropped into the 4% $CaCl_2$ solution from a height of 9 cm to form the cell beads. During this operation, the 4% $CaCl_2$ solution was stirred continuously at 300 rpm. Finally, the prepared cell beads were equilibrated in $CaCl_2$ solution for 24 h to improve their physical strength.

The nitrogen removal performance of Ca-alginate cell beads

The removal efficiencies of NH_4^+-N, NO_2^--N, and TN, and the changes in the removal ratios of NO_2^--N and NH_4^+-N by the Ca-alginate cell beads on different days are shown in Figure 3. During the initial stage (0–10 days), the NH_4^+-N influent concentration was fixed at 50 mg/L, while the NO_2^--N influent concentration was gradually increased to ensure that the NO_2^--N substrate was sufficient for the anammox reaction, as denitrification would remove a portion of the NO_2^--N substrate. Especially during the first few days, the removal efficiency of NO_2^--N was close to 100%, with a low removal efficiency of NH_4^+-N. This indicated that when the level of anammox biomass in the initial sludge was low, denitrification occurred with organic sources from endogenous decay (Shaw *et al.* 2020), causing NO_2^--N to become the limiting substrate in the anammox reaction. As the influent concentration of NO_2^--N gradually increased, the NH_4^+-N removal efficiency increased from 50% to over 95%, while the NO_2^--N removal efficiency decreased, especially after the NO_2^--N influent concentration increased to 90 mg/L. This indicated that increasing the NO_2^--N influent concentration inhibited denitrification and increased the activity of the anammox sludge; however, higher NO_2^--N concentrations would inhibit the anammox activity. Subsequently, the NO_2^--N influent concentration was adjusted to 75 mg/L with the removal matrix ratio of NO_2^--N and NH_4^+-N, which gradually decreased from initial 2.2 to 1.47. At the same time, the accumulation of NO_3^--N gradually increased, and the removal efficiency of total nitrogen increased from an initial 80.8% to 90.6%. This indicated that the anammox bacteria gradually surpassed the denitrifying bacteria to become the dominant species in the Ca-alginate cell beads.

During the middle stage (10–14 days), the influent concentrations of NH_4^+-N and NO_2^--N increased to 100 and 150 mg/L, respectively. The removal efficiencies of NH_4^+-N, NO_2^--N, and TN all rebounded after abruptly decreasing, and finally stabilized at around 91, 95, and 89%, respectively (Figure 3). The removal efficiencies of TN was consistent with 89%=(1+1.32 - 0.26)/(1+1.32) of the theoretical value of the anammox reaction (Zhang *et al.* 2019). Therefore, we considered that the reaction achieved the steady stage condition after the substrate concentration was increased. This illustrated that the anammox sludge cultivated in the Ca-alginate cell beads could adapt to the high concentrations of NH_4^+-N and NO_2^--N in a short amount of time.

During the late stage (14–17 days), the influent concentration of NH_4^+-N, NO_2^--N and the total nitrogen loading rate (TNLR) increased to 130, 195, and 0.39 kg/(cm^3·d) respectively. The removal efficiencies of NH_4^+-N, NO_2^--N, and TN all decreased and finally stabilized at around 89, 92, and 83%, respectively (Figure 3). At this time, the cellular metabolic residues as organic carbon sources might revive denitrification. Thus, the removal matrix ratio of NO_2^--N and NH_4^+-N increased from 1.47 to 1.62, and the accumulation of NO_3^--N also decreased. This indicated that the interior of the Ca-alginate cell beads was not suitable for the continued growth of the anammox bacteria at this time. Additionally, the culture period of the Ca-alginate cell beads was about 17 days, which was similar to that of the PVA/SA gel beads (18 days) reported in the literature (Zhang & Okabe 2017).

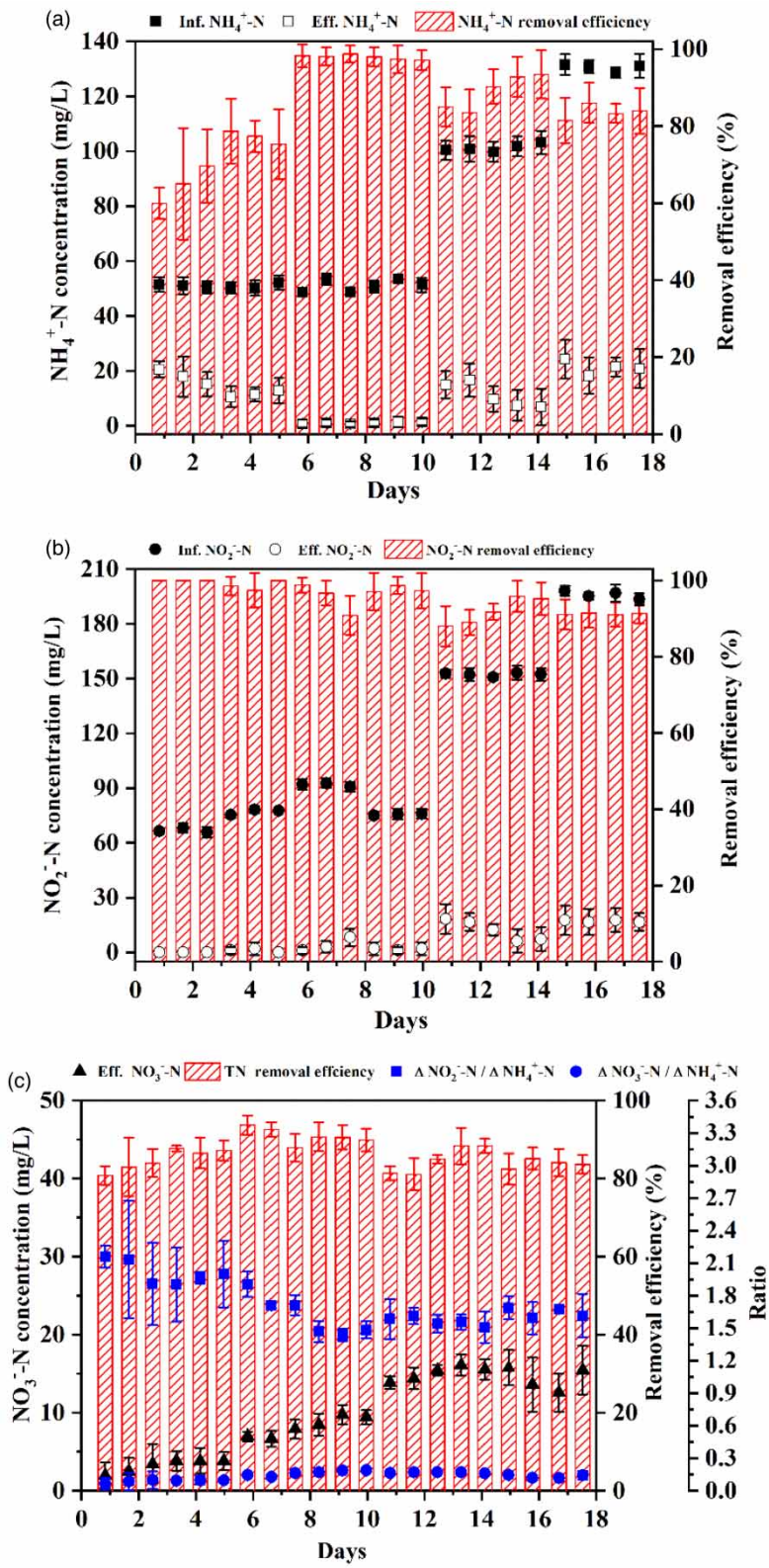

Figure 3 | Nitrogen removal of Ca-alginate cell beads in the cultivation process. (a) Ammonia nitrogen, (b) nitrite nitrogen, and (c) nitrate nitrogen, total nitrogen, $NO^-_{2\,removal}/NH^+_{4\,removal}$, and $NO^-_{3\,produced}/NH^+_{4\,removal}$.

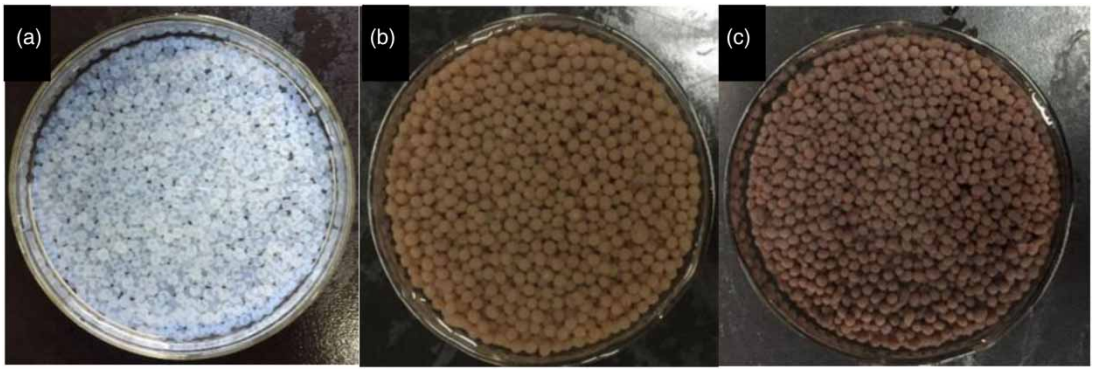

Figure 4 | Appearance photos of (a) SA gel beads, and Ca-alginate cell beads on (b) day 0 and (c) day 17.

Morphological characteristics of the Ca-alginate cell beads

The morphologies of the Ca-alginate cell beads were recorded by a camera. As shown in Figure 4(a), SA gel beads consisted of spheres that were firm and flexible in texture, with uniform size and no bubbles in the interior. The initial sludge-embedded Ca-alginate cell beads were also spheres with uniform size and no bubbles. However, the difference was that the particle size was significantly larger than SA gel beads (Table 2) and the color was yellow-brown (Figure 4(b)). The average diameter of the Ca-alginate cell beads was 2.28 mm, while the SA gel beads were 1.7 mm in diameter. In addition, the Ca-alginate cell beads had a better settleability due to the embedded anammox sludge. After 17 days of cultivation, the color of the Ca-alginate cell beads changed to brown-red, and some became ellipsoidal (Figure 4(c)). The observed color change in the Ca-alginate cell beads from days 0 to 17 indicated that the proportion of anammox bacteria in the sludge increased significantly. Because anammox enrichment cultures were the typical blood red color (Kartal & Keltjens 2016).

At the same time, a small number of cell beads swelled, floated, broke, and disintegrated with clearly visible bubbles inside. This phenomenon was related to the effects of interior space and mass transfer during the late stage of the cell beads. The rapid growth of the anammox bacteria and rapid apoptosis of the other strains occurred simultaneously inside the Ca-alginate cell beads, causing the internal growth space and physical strength of the cell beads to gradually decrease. However, the accumulation of biomass inside the cell beads hindered the exchange of substances inside and outside; thus, the external substrates could not be used very well by the internal bacteria, and simultaneously, the N_2 and residues generated inside the cell beads could not be transported to the external medium in time. Reports have shown that anammox cells cultivated in PVA/SA gel beads have always faced problems of expansion and bursting during long-term use (Choi *et al.* 2017). While the diameter of the PVA/SA gel beads (1–2 mm) (Zhang & Okabe 2017) was smaller than the Ca-alginate cell beads (2.28 ± 0.19 mm), the surface of the PVA/SA gel beads became denser (Choi *et al.* 2017); thus, the bacterial growth space and mass transfer was not as effective as in the Ca-alginate cell beads. For all this, the gel entrapment technology has shown to be limited by the volume and aperture of the gel beads, and can only be used for small-batch and short-term cell culture.

Figure 5 shows the micromorphology of the Ca-alginate cell beads as observed by SEM. The surface and cross-section of the Ca-alginate cell beads showed a loose porous structure that was beneficial to substance transmission inside and outside the cell beads; thus promoting the growth of bacteria inside the cell beads. It has been reported that PVA/SA gel beads provided a

Table 2 | Physical and chemical properties of Ca-alginate beads

Parameters	SA gel beads	Ca-alginate cell beads
Particle size (mm)	1.7 ± 0.16	2.28 ± 0.19
Specific surface area (m^2/g)	10.56 ± 1.47	12.64 ± 1.62
Aperture (nm)	25.3 ± 1.04	56.48 ± 1.07
Zeta potential (mV)	-38.14 ± 0.90	-31.83 ± 0.78
Mechanical strength (kg/g)	1.02 ± 0.14	1.68 ± 0.20

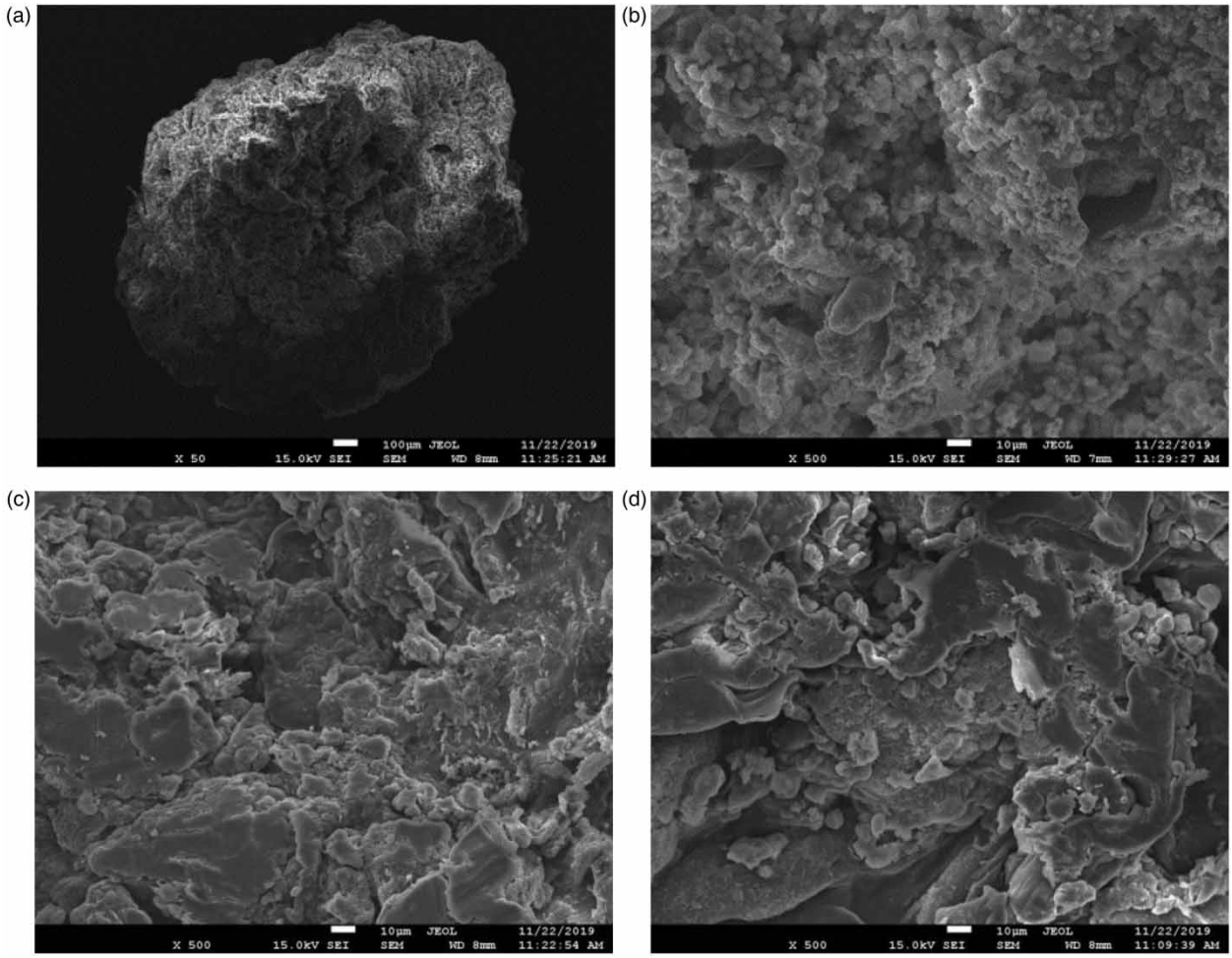

Figure 5 | SEM images of Ca-alginate cell bead (a) appearance, (b) surface, and interior on (c) day 0 and (d) day 9.

porous matrix, which allowed for substrate diffusion into the internal pores and facilitated efficient microbial growth (Cho *et al.* 2017). In addition, some bacteria adhered to the surfaces of the Ca-alginate cell beads, allowing them to grow. These observations suggested that the Ca-alginate cell beads had a good microporous structure.

Physical and chemical characteristics of Ca-alginate beads

Studies have shown that the mass transfer was affected by the specific surface area of the gel beads (Bae *et al.* 2015). Table 2 shows that the specific surface area of the Ca-alginate cell beads (12.64 m^2/g) was larger than the SA gel beads (10.56 m^2/g). This indicated that the anammox mixed cultures in the Ca-alginate cell beads had sufficient contact area with the substrate in the external medium. Moreover, the pore diameter of the Ca-alginate cell beads was 56.48 nm, which was about 2.23 times that of the SA gel beads (25.3 nm). Therefore, matrix transport channels were possibly present within the Ca-alginate cell beads, which were beneficial for the rapid cultivation of the anammox mixtures using sufficient substrates.

According to the literature, the surface of anammox sludge contains few electronegative functional groups (Hou *et al.* 2015). In this work, the zeta potentials of the Ca-alginate cell beads (−31.83 mV) and the SA gel beads (−38.14 mV) were all higher than the anammox mixtures (−16.32 mV). This feature was favorable for the adsorption of positively charged NH_4^+-N in the matrix to the cell beads; thus, promoting substrate removal and growth of the anammox mixtures in the Ca-alginate cell beads.

Mechanical strength is an important parameter in characterizing the gel beads, which could indicate whether the gel beads are potentially useful as polymeric carriers (Zhang *et al.* 2011). As shown in Table 2, the average mechanical strength of the Ca-alginate cell beads (1.68 kg/g) was better than that of the SA gel beads (1.02 kg/g). This suggests that embedding the

anammox mixture into the SA gel beads improved the mechanical strength of the Ca-alginate cell beads without making them more vulnerable to fragmentation.

Identification of the anammox bacteria

The anammox bacteria in the Ca-alginate cell beads were identified by FISH experimentation and observed under a fluorescence microscope. The results are shown in Figure 6, in which the anammox bacteria are shown in red, all bacteria are shown in green, and the overlapping region of the two is shown in orange. With increased cultivation time, the fluorescence intensity of bacteria was enhanced significantly, indicating that the bacterial content inside the Ca-alginate cell beads increased, especially the proportion of anammox bacteria. Although the increase in bacterial density was accompanied by the decrease of growth space inside the cell beads, even on day 17 when the cell density was the highest, the bacteria were still mostly dispersed inside the cell beads. These results showed that the Ca-alginate cell beads could rapidly enrich the suspended anammox bacteria during the 17 days of cultivation.

Cell growth of embedded anammox bacteria

QPCR was used to test the biomass of the anammox bacteria in the Ca-alginate cell beads. Figure 7 shows that there was minimal content of anammox bacteria in the original sludge (2.74×10^6 copies/gel-bead). With increased incubation time, the biomass of the anammox bacteria increased significantly to 1.1×10^7 copies/gel-bead on day 9, which was 4.3 times that of the initial sludge. This indicated that the anammox bacteria could rapidly proliferate in the Ca-alginate cell beads

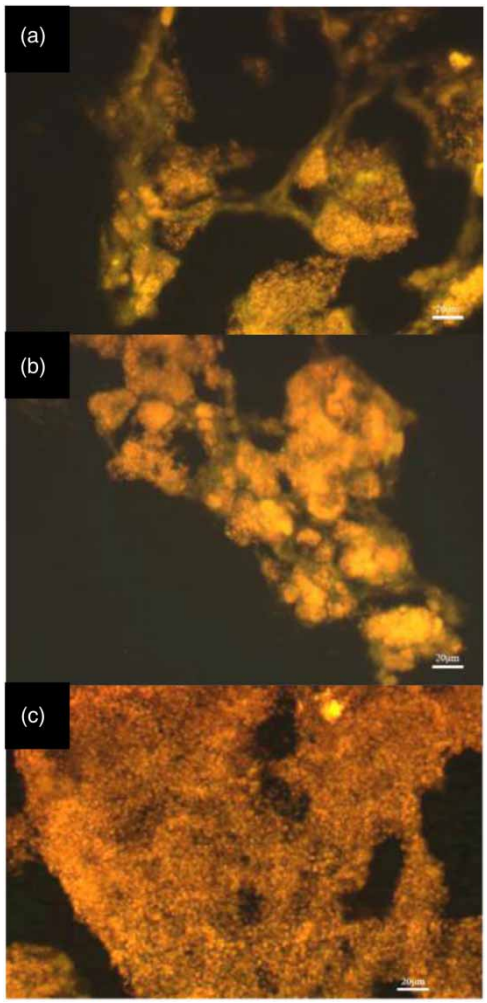

Figure 6 | FISH images of bacteria cultivated in Ca-alginate cell beads on (a) day 0, (b) day 9, and (c) day 17.

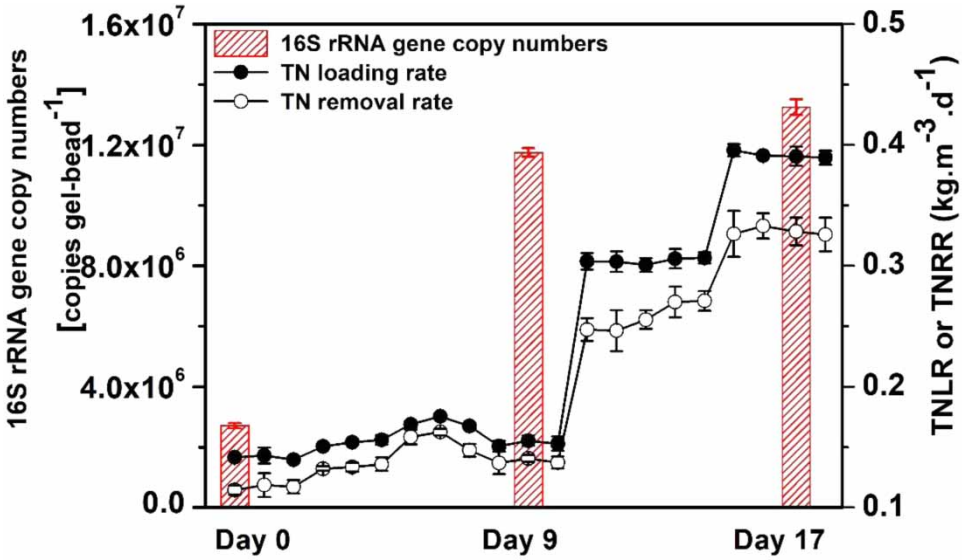

Figure 7 | 16S rRNA gene copy number of anammox bacteria in Ca-alginate cell beads in different cultivation periods.

in a short time. After 9 days of operation, the total nitrogen loading rate (TNLR) increased from 0.14 kg/(cm^3·d) to 0.39 kg/(cm^3·d), and metabolism and activity of the anammox bacteria were inhibited (Figure 3). Simultaneously, the various performance factors of the cell beads deteriorated, which served as a bottleneck for the long-term operation of gel entrapment technology. Therefore, during the middle and late stages, the growth of the anammox bacteria in the cell beads was restricted and the growth rate slowed down, which was consistent with the growth of anammox bacteria in PVA/SA gel beads reported in the literature (Zhang & Okabe 2017). On day 17, the 16S rRNA gene copy number of anammox bacteria was 1.32×10^7 copies/gel-bead, which was not a significant increase compared to day 9. Another reason might be that qPCR copies increased exponentially during the first 9 days and stopped due to the exponential phase ended. The above results demonstrated that the rapid enrichment of anammox bacteria in the Ca-alginate cell beads required only a relatively small number of anammox cells. In this work, the initial biomass of anammox bacteria was much lower than the initial inoculation concentration of anammox bacteria cultured in PVA/SA gel beads, as reported in the literature ($1–3\times10^8$ copies/gel-bead) (Zhang & Okabe 2017). However, the proliferation rate of anammox bacteria in the Ca-alginate cell beads was comparable to the anammox bacteria cultured in the PVA/SA gel beads.

Ca-alginate cell beads could achieve rapid culturing of the anammox sludge because they could overcome the unfavorable conditions of anammox bacteria growth. (1) The anammox bacteria did not take on the activity until the cell concentration was sufficiently high (Ding *et al.* 2013). The aggregation behavior and cell density of the anammox bacteria were possibly regulated by quorum sensing (QS, a means of bacterial communication), and many physiological characteristics of the anammox bacteria were confirmed to be associated with QS, including the specific anammox activity, growth rate, and production of extracellular polymeric substances (EPS) (Zhang *et al.* 2021). The Ca-alginate cell beads provided specific carrier space for the anammox bacteria to facilitate the transmission of signal molecules, thereby accelerating cell growth. (2) Anammox bacteria were sensitive to environmental conditions. The Ca-alginate cell beads provided a barrier for the anammox bacteria from various environmental conditions and hydraulic shear forces, and the cell beads maintained an environment conducive to the growth of anammox bacteria. Therefore, the Ca-alginate cell beads showed great potential for the rapid enrichment of anammox bacteria.

CONCLUSIONS

In this work, preparation conditions for the Ca-alginate cell beads were optimized. The nitrogen removal performance, physical and chemical properties, microscopic morphology, and anammox cell proliferation of the Ca-alginate cell beads were investigated to explore the enrichment effect of the Ca-alginate gel entrapment method. The effect of substrate concentration disturbance on the activity of the anammox bacteria in the Ca-alginate cell beads was investigated by increasing the substrate

concentration for a short period. The results showed that the Ca-alginate cell beads exhibited good short-term and rapid enrichment performance for the anammox bacteria. First, the anammox bacteria cultivated in the Ca-alginate cell beads could adapt to the increase of substrate concentration in a short period. Second, only a relatively small amount of anammox biomass (2.74×10^6 copies/gel-bead in this work) was required to quickly start the anammox process. Third, the anammox bacteria grew in suspension in the Ca-alginate cell beads with a large growth rate in a short period and exhibited high activity due to the limitation of diffusion. Fourth, under the experimental conditions of this work, the optimal incubation time of this gel entrapment method for the anammox sludge did not exceed 17 days, as the various performance properties of the cell beads declined after 17 days. As such, the Ca-alginate gel entrapment method greatly shortened the incubation time of the anammox sludge and was suitable for small-batch and short-term cell pre-culturing.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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