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RESEARCH ARTICLE

Preparation and characterization of a novel microorganism embedding material for simultaneous nitrification and denitrification

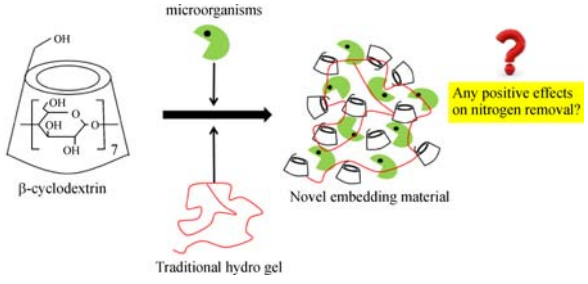
Ming Zeng[1], Ping Li[1], Nan Wu[2], Xiaofang Li[1], Chang Wang (✉)[1]

1 College of Marine and Environmental Science, Tianjin University of Science &Technology, Tianjin 300457, China
2 College of Engineering and Technology, Tianjin Agricultural University, Tianjin 300384, China

HIGHLIGHTS

- CD enhances the hydrophilic property of traditional PVA-SA gel solution.
- CD increases the density of embedded microorganism and micro porosity of bead.
- CD makes the maximum endogenous respiration rate being high.
- 30-1.7-CD contributes the highest total inorganic nitrogen removal efficiency.
- *Comamonas* sp. mainly realize the simultaneous nitrification and denitrification.

GRAPHIC ABSTRACT



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ABSTRACT

A novel microorganism embedding material was prepared to enhance the biological nitrogen removal through simultaneous nitrification and denitrification. Polyvinyl alcohol (PVA), sodium alginate (SA) and cyclodextrin (CD) were used to compose gel bead with embedded activated sludge. The effects of temperature, CD addition and concentrations of PVA and SA on nitrogen removal were evaluated. Results show that the gel bead with CD addition at 30°C contributed to the highest nitrogen removal efficiency and nitrogen removal rate of 85.4% and 2.08 $mg\cdot(L\cdot h)^{-1}$, respectively. Meanwhile, negligible NO_3^- and NO_2^- were observed, proving the occurrence of simultaneous nitrification and denitrification. The High-Throughput Sequencing confirms that the microbial community mainly contained *Comamonadaceae* in the proportion of 61.3%. Overall, CD increased gel bead's porosity and resulted in the high specific endogenous respiration rate and high nitrogen removal efficiency, which is a favorable additional agent to the traditional embedding material.

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1 Introduction

The human activities release excessive nitrogen in global environment, causing several potential toxic effects on aquatic life like the eutrophication phenomenon in the water environment. Thus it is quite urgent to eliminate the excessive nitrogen in the aquatic environment in order to restore global nitrogen balance. Comparing with other techniques, biological technique is of great efficiency to control the nitrogen pollutant [1]. Generally, the traditional A^2/O process is one of the most widely used nitrogen removal processes although it has disadvantages of large energy consumption and big required building volume [2]. Recently, simultaneous nitrification and denitrification has attracted great attentions, and both traditional suspended activated sludge and biofilm can realize this process in different bioreactors [3,4].

Among above alternatives, the immobilization technology has been reported to efficiently enhance simultaneous nitrification and denitrification [5,6]. As one kind of immobilization technology, the embedding technology is

✉ Corresponding author
E-mail: wangc88@163.com

to prepare the gel carrier by cross-linking the objective microorganism with the embedding agents. It has several advantages of high cell density, long cell retention time and easy separation of solid and liquid phases [7,8]. The common used embedding agents include polyvinyl alcohol (PVA), sodium alginate (SA), polyethylene glycol (PEG) and polyurethane (PU). For the moment, immobilization technique has received increasing attention in the single stage nitrogen removal process. Duan [9] immobilized nitrifying bacteria and Anammox bacteria in 15% PVA and 2% SA gel solution to prepare the gel bead. After 362 days' operation, total nitrogen removal efficiency was kept above 85% with highest nitrogen removal rate of 1.85 kgN $\cdot m^{-3} \cdot d^{-1}$. However, the challenge is to guaranty the enough stable structure and strong hydrophilic property of gel bead. For instance, Hu [10] used immobilized *Micrococcus* sp. to degrade phthalic acid esters, but they found that 20% immobilized particles were expanded after 30 days' operation. It can be noticed that PVA and SA are both straight chain polymer and the formed network structure has poor stability. Thus, the author supposes that adjusting certain modified agent of cyclic structure could increase the stability of gel bead as well as the activity of immobilized biomass.

Cyclodextrin (CD) is a kind of oligosaccharide composed by several D-glucopyranose units under ring arrangement. The molecules are in a hollow cylinder with a narrow width and the inner cavity is relatively hydrophobic, while all hydrophilic groups stay outside the cylinder. This chemical compound can form clathrate compound with other subject substances by embedding them in the inner cavity, like different contaminants. Due to the low producing cost and appropriate inner diameter, β-CD is most widely used. Nowadays, CD polymers have been applied in the field of environmental protection, especially detoxification from heavy metals and organic contaminants [11]. For example, β-CD polymer was found to be capable to remove dissolved estrogens over a wide range of contaminant concentrations in sewage effluent [12]. In this study, β-CD is first introduced to the PVA and SA embedding agents to increase the activity of microorganism in the gel carrier and nitrogen removal efficiency.

The aim is to prepare a novel microorganism embedding material to enhance the simultaneous nitrification and denitrification. The prepared gel bead was characterized by the Scanning Electron Microscope (SEM) and Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR). The orthogonal experiments were designed to evaluate the effects of temperature, addition of β-CD and concentrations of embedding agents on the nitrogen removal efficiency. The biological respiration rate was measured to assess the microbial activity between different treatments. Finally, the High-Throughput Sequencing technique was conducted to reveal the microbial community inside the gel bead.

2 Materials and methods

2.1 Immobilization technique

The gel bead of PVA and SA (abbreviated as PVA-SA) was prepared with PVA-HC (hydrolyzed 97 + %) and SA. Besides, the gel bead of PVA, SA and CD (in the abbreviation of PVA-SA-CD) was prepared by adding CD to the gel of PVA-SA. All above chemicals were purchased from Guangfu Fine Chemical Research Institute, Tianjin, China. The mixture of these chemical compounds and water was heated with water bath at 90°C to allow their completely melting and mixing. The gel solution was then cooled to 35°C and concentrated sludge that was centrifuged under 3000 $r \cdot min^{-1}$ for 10 min was completely dispersed in gel solution at w/w ratio of 2:1 (VSS: gel). Then the gel solution was slowly pumped into the solidifying solution (saturated boric acid and 2% (w/v) $CaCl_2$) by a peristaltic pump (BT100-2J Longerpump Co., Ltd, Baoding, China) to make spherical beads. The pH of solidifying solution was controlled to be 7 by adding 4 mol $\cdot L^{-1}$ NaOH. Then the prepared gel beads were still immersed in the solidifying solution at 4°C for 24 h. At last, the gel beads were washed with distilled water and cultured by the nutrient solution (section 2.2).

2.2 Batch test of nitrogen removal

The orthogonal experiments were designed to investigate the effects of temperature, CD addition and concentrations of embedding agents on nitrogen removal performance. Due to two levels for each factor, there are four experimental treatments: 1.7% (w/v) PVA and 1.7% (w/v) SA with 1.0% (w/v) CD at 30°C abbreviated as 30-1.7-CD, 1.2% (w/v) PVA and 1.2% (w/v) SA without CD at 30°C abbreviated as 30-1.2, 1.2% (w/v) PVA and 1.2% (w/v) SA with 1.0% (w/v) CD at 25°C abbreviated as 25-1.2-CD, 1.7% (w/v) PVA and 1.7% (w/v) SA without CD at 25°C abbreviated as 25-1.7. For each treatment, 200 mL embedding solution was used to prepare gel bead in order to ensure the similar number of gel bead produced and biomass embedded.

The batch tests of nitrogen removal were conducted in the conical flasks inside a thermostatic oscillator (SHY-2A, Guo Yu Co., Ltd, Jiangsu, China). Three replications were set for each treatment, so there are total 12 flasks reactors inside the thermostatic oscillator. A synthetic medium prepared to feed the microorganism contained 100 $mg \cdot L^{-1}$ NH_4Cl–N, 100 $mg \cdot L^{-1}$ sucrose, 27 $mg \cdot L^{-1}$ KH_2PO_4, 500 $mg \cdot L^{-1}$ $NaHCO_3$, 180 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 300 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$. And 1 mL trace element was added into per liter synthetic medium. Trace element solution contained 625 $mg \cdot L^{-1}$ EDTA, 190 $mg \cdot L^{-1}$ $NiCl_2 \cdot 6H_2O$, 430 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 220 $mg \cdot L^{-1}$ $NaMoO_4 \cdot 2H_2O$, 240 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$, 990 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 250 $mg \cdot L^{-1}$

$CuSO_4 \cdot 5H_2O$. The synthetic medium supplied nitrogen nutrient and organic carbon for the growth of autotrophic and heterotrophic bacteria. The temperature of feeding solution was kept at 25°C and 30°C by a thermostatic oscillator. The oxygen for the growth of bacteria was supplied by an aerator to control dissolved oxygen being 1.5 $mg \cdot L^{-1}$. Due to the addition of $NaHCO_3$, pH of solution was around 8. The feeding medium was renewed each 4 days.

After filtered through 0.45 μm membrane (Millipore, USA), the feeding solution was sampled along the feeding period to immediately analyze the concentrations of NH_4^+-N and NO_2^--N by the colorimetric method, and NO_3^--N concentration by the cadmium reduction method, according to Standard Methods [13].

2.3 Specific surface area measurement

The specific surface area was measured according to the method of Bai et al. [14]. The dried gel bead was immersed in 100 mL methylene blue (MB) of 20, 40, 60, 80 and 100 $mg \cdot L^{-1}$. The adsorption process of methylene blue was kept at 25°C in the thermostatic oscillator for one hour. Then the concentration of methylene blue in the solution was detected to calculate the specific surface area of gel bead by multiplying maximum adsorbed mass of methylene blue (Q_{max}) with 2.45, because the specific surface area of methylene blue is 2.45 $m^2 \cdot mg\ MB^{-1}$.

Q_{max} with the unit of g MB·g carrier^{-1} can be calculated by fitting Eq. (1).

$$\frac{Ce}{Q} = \frac{Ce}{Q_{max}} + \frac{1}{b \times Q_{max}}, \quad (1)$$

where Ce is the concentration of methylene blue under equilibrium adsorption (g $MB \cdot L^{-1}$); Q is the mass of methylene blue under equilibrium adsorption, g MB·g carrier^{-1}; b is the adsorption coefficient ($L \cdot g^{-1}$).

The mass of methylene blue under equilibrium adsorption (Q) is calculated by Eq. (2).

$$Q = (C_0 - C_t)\frac{V}{W}, \quad (2)$$

where C_0 is the initial concentration of methylene blue ($g \cdot L^{-1}$); C_t is the concentration of methylene blue at t time ($g \cdot L^{-1}$); V is the volume of solution (L); W is the mass of carrier (g).

2.4 SEM observation

The SEM images were taken by using a Scanning Electron Microscopy (SU1510, Hitachi, Japan). The gel bead with embedded microorganism were first washed with the phosphate buffer saline solution (PBS, Solarbio Science & Technology Co., Ltd, Beijing, China), and was then dewatered successively in 50%, 80%, 100% alcohol solution. Finally the gel bead was hemisected and completely dried at room temperature in a silica gel dryer. The internal and surface of gel bead were coated with platinum and the observation was done in the N_2 medium.

2.5 ATR-FTIR analysis

The gel solution of PVA, SA and CD was coated on the surface of PVDF membrane (RGE membrane Co., Ltd, Nanjing, China) and then was solidified with saturated boric acid and $CaCl_2$. After completely dried in the silica gel dryer, samples were analyzed by an ATR-FTIR analyzer (Tensor 27, Bruker, Germany).

2.6 High-Throughput Sequencing analysis

The sampled gel bead with embedded microorganism was washed with PBS solution, and then freezed at –20°C. The High-Throughput Sequencing analysis of microbial community was conducted by the Biozeron Co., Ltd, Shanghai, China. Online similarity searching was performed using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/).

2.7 Respirometric test

The endogenous and exogenous respiration rates were measured to evaluate the activity of embedded microorganism. The gel bead was first put into the 250 mL glass bottle with distilled water to calculate the endogenous respiration rate by a DO meter (Oxi3210, WTW, Germany). The temperature was controlled at 25°C or 30°C with water bath. After the stable endogenous respiration rate was obtained, the distilled water was replaced by the feeding medium (section 2.2) to calculate the exogenous respiration rate.

3 Results and discussion

3.1 Physical characteristics

Table 1 shows the physical characteristics of prepared gel bead. The diameter of bead ranged from 0.33 to 0.37 cm. The moisture of bead ranged from 54.51% to 60.69%, which means that less than half of bead volume was available for the solid phase, like gel agents and embedded microorganism. When the gel bead was filled into a column as fixed bed, the porosity of bed reached 25%. Generally, the embedding materials were prepared in the form of cube or bead. Cao et al. [15] used 10% PVA and 2% sodium alginate to form gel beads about 3 mm by the injector that were used to immobilize the nitrifying and denitrifying bacteria. However, the peristaltic pump was

Table 1 Summary of physical properties of different gel beads

	30-1.7-CD [a]	30-1.2 [b]	25-1.2-CD [c]	25-1.7 [d]
diameter /cm	0.34±0.024	0.33±0.016	0.37±0.026	0.37±0.26
moisture/%	55.98±0.98	54.51±0.48	60.96±1.28	59.82±1.36
porosity of fixed bed %	25.0	25.2	24.9	24.8

Notes: [a] 1.7% PVA + 1.7% SA + 1.0% CD at 30°C; [b] 1.2% PVA + 1.2% SA without CD at 30°C; [c] 1.2% PVA + 1.2% SA + 1.0% CD at 25°C; [d] 1.7% PVA + 1.7% SA without CD at 25°C

used in this study to make the process being man-cost and faster than the traditional injector. Isaka et al. [16] cut polyethylene glycol (PEG) gel carriers into 3 mm cubes containing entrapped heterotrophic denitrifying bacteria.

Figure 1 shows the adsorption curves of methylene blue for the calculation of specific surface area. By fitting the curves, the specific surface area of gel bead arrived at 24.52 $m^2 \cdot (g\ dry\ carrier)^{-1}$ for the bead with CD and 29.10 $m^2 \cdot (g\ dry\ carrier)^{-1}$ for the bead without CD. The addition of CD was found to slightly decrease the specific surface area. In the study of Duan [9], the specific surface area of PVA-SA gel bead was reported to be 57.32 $m^2 \cdot (g\ dry\ carrier)^{-1}$, which can be attributed by the high PVA concentration of 7.5%. This suggests that the higher concentration of PVA results in the larger specific surface area of carrier.

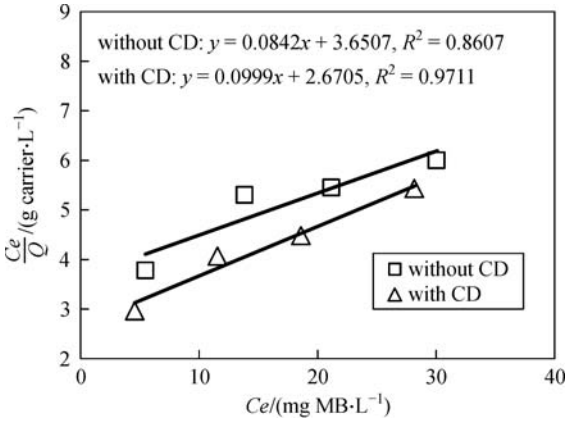


Fig. 1 Adsorption curves of methylene blue for gel beads with and without CD

3.2 ATR-FTIR spectra

To obtain the information about the blend composition and interaction between different polymers, FTIR spectra of PVA, SA, PVA-SA, and PVA-SA-CD were summarized in Fig. 2. A medium peak occurred at 2900-3000 cm^{-1} for the sample of PVA that diminished in the mixed samples of PVA-SA and PVA-SA-CD, which is caused by the hydrogen bonded OH groups and CH stretching. For the samples of SA, the strong absorption at 1590 cm^{-1} was the characteristic peak of the ionic bonded carboxylic group of alginate caused by the C = O stretching [17]. Correspondingly, this strong absorption was still observed in the mixed

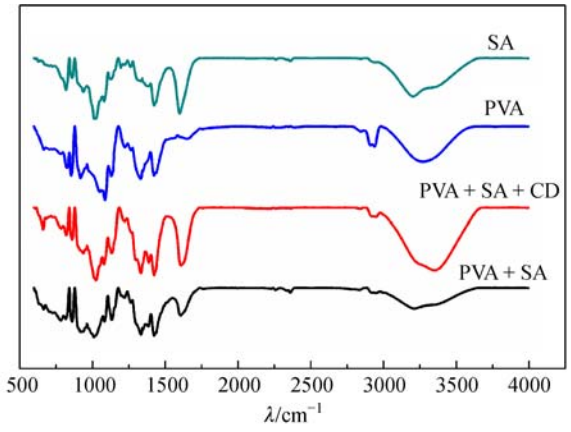


Fig. 2 Fourier transforms infrared spectra of different compounds

samples of PVA-SA and PVA-SA-CD, which suggests the successful cross linking of SA with calcium in the mixed samples.

It is shown that a broad band appeared at 3000 to 3600 cm^{-1} expressing the –OH stretching vibrations. Interestingly, this peak was sharper in the sample of PVA-SA-CD than that in the sample of PVA-SA. Although the difference could be caused by the membrane thickness of embedding agents, it can be still assumed that the difference is attributed by the addition of CD introducing large amounts of –OH. Because the difference between characteristic peak of SA at 1590 cm^{-1} for PVA-SA-CD and PVA-SA should be similar to the difference between the –OH stretching vibrations of PVA-SA-CD and PVA-SA, if the difference was caused by the membrane thickness. However, the latter difference was obviously quite larger than the former difference. Thus, the fact is verified that the sample of PVA-SA-CD has a strong hydrophilic property, which is favorable for the embedding of microorganism. In one word, the FTIR spectra confirm that the cross-linking process of PVA and SA was successful, and the addition of CD greatly increased the hydrophilic property of gel bead.

3.3 SEM micrograph

SEM micrograph directly presents the distribution of microorganism and physical structure inside and outside the gel bead. Compared with the internal of gel bead, there were fewer microorganisms on the surface of bead (Figs. 3A and 3C), while a large amount of microorganisms could

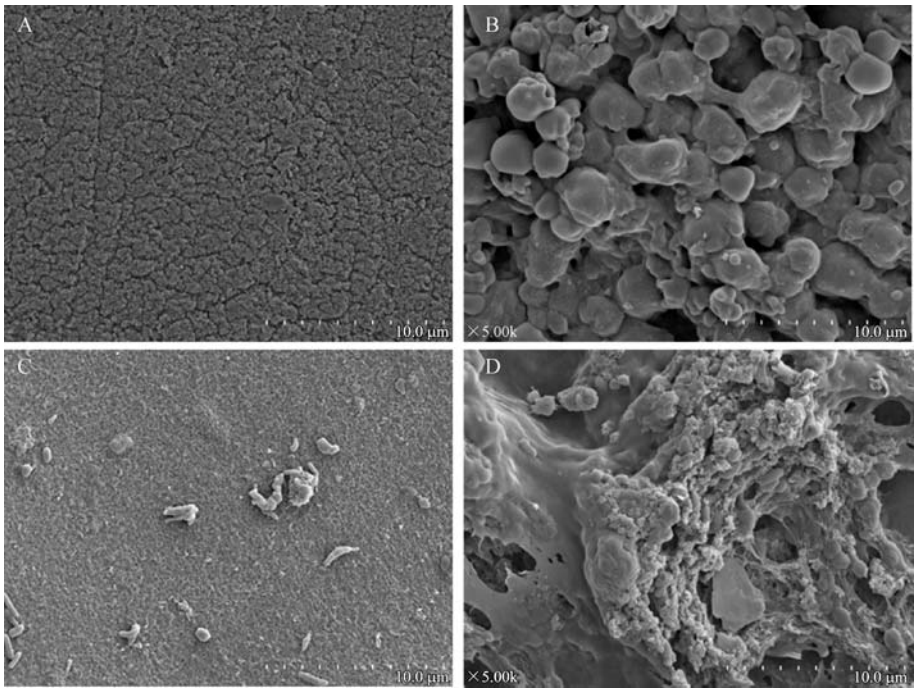


Fig. 3 The SEM micrograph of gel bead prepared by PVA and SA concentrations of both 1.2% with embedding microorganisms (A: external of bead with CD; B: internal of bead with CD; C: external of bead without CD; D: internal of bead without CD)

be seen inside the gel bead (Figs. 3B and 3D). Particularly, Fig. 3A shows that the crack on the surface of bead with CD was remarkable and wider than that on the surface of bead without CD, which is benefit for the penetration of oxygen and nutrient into the internal of bead. Zhu et al. [8] also found that the PVA-SA immobilized beads' surface microstructures were loose and finely porous, which facilitated the penetration of nutrient into the inside of bead. In brief, CD is assumed to further enhance the nutrient transfer into the PVA-SA gel bead according to the SEM micrographs.

By comparing Fig. 3B with Fig. 3D, the shape of embedded microorganism for the gel bead with CD was found to be significantly larger than that for the gel bead without CD. The diameter of gel sphere arrived at around 2 μm and the density of bacteria was high for the sample with CD (Fig. 3B), while the few sphere and bacillus bacteria with small diameter were observed in the sample without CD (Fig. 3D). This phenomenon for the sample without CD is similar to the results from the study of Zhu et al. [8], where the diameter of spherical bacteria observed inside the PVA-SA gel bead was less than 10 μm. Besides, the porosity between bacteria can be clearly observed in the gel bead with CD.

The similar micrograph occurred in the gel bead with high PVA and SA concentrations of both 1.7% (Supplementary material Fig. S1). The surface of bead was rough with cracks and the number of microorganism inside the bead for the sample with CD was comparatively more than that for the sample without CD. Due to the high concentrations of PVA and SA, the dense cracks appeared on the surface of gel bead, and the diameter of microorganism inside the bead was small, less than 2 μm (Supplementary material Fig. S1B and S1D). In general, the addition of CD is assumed to increase the density of embedded microorganism and the micro porosity on the surface of bead as well.

3.4 Respirometric test

Table 2 shows the endogenous and exogenous respiration rates of suspended sludge and embedded microorganism. In terms of specific endogenous respiration rate, the gel bead with CD was found to have high value no matter what temperature, arriving at 9.37 and 8.52 $mgO_2 \cdot (g\ MLVSS \cdot h)^{-1}$ for 30-1.7-CD and 25-1.2-CD, respectively, whereas the specific endogenous respiration rate arrived at 3.25 and 4.56 $mgO_2 \cdot (g\ MLVSS \cdot h)^{-1}$ for 30-1.2 and 25-1.7, respectively. Thus, the positive effect of CD on the activity of embedded microorganism was highlighted, especially under the adverse environment, like the starving condition. Moreover, regarding with the specific exogenous respiration rate, the value of embedded microorganism in the gel bead was nearly two times as many as that of suspended sludge, which proves that embedding technique can enhance the microbial activity. However, Duan [9] reported that the specific oxygen uptake rate of suspended sludge was higher than that of embedded sludge inside the PVA-SA gel carrier. Their reasons refer to the mass transfer resistance of gel carrier and the toxicity of boric acid. In

Table 2 Respiration rates of gel beads with embedded microorganism of four treatments and suspended sludge

	30-1.7-CD [a]	30-1.2 [b]	25-1.2-CD [c]	25-1.7 [d]	SS[3]
specific [1]endo. respiration rate ($mgO_2 \cdot (g\ MLVSS \cdot h)^{-1}$)	9.37	3.25	8.52	4.56	-
Max. [2]exo. respiration rate ($mgO_2 \cdot (L \cdot h)^{-1}$)	19.44	11.16	17.28	17.31	10.97
MLVSS of embedded sludge ($g\ MLVSS \cdot L^{-1}$)	1.42	0.78	1.10	1.10	1.42
Max. specific exo. respiration rate ($mgO_2 \cdot (g\ MLVSS \cdot h)^{-1}$)	13.67	14.39	15.73	15.75	7.73

Notes : [1]endo. is endogenous; [2]exo. is exogenous; [3]suspended sludge
[a] 1.7% PVA + 1.7% SA + 1.0% CD at 30°C; [b] 1.2% PVA + 1.2% SA without CD at 30°C; [c] 1.2% PVA + 1.2% SA + 1.0% CD at 25°C; [d] 1.7% PVA + 1.7% SA without CD at 25°C

this study, low PVA concentration, different sludge source and its feeding condition might explain the opposite findings in present study.

In addition, the specific exogenous respiration rate did not have a large variation that ranged from 13.67 to 15.75 $mgO_2 \cdot (g\ MLVSS \cdot h)^{-1}$ for four treatments. The reason can be the similar strong activity of heterotrophic bacteria in each type of gel beads. Due to the sucrose in feeding solution, most consumed dissolved oxygen at the beginning of respirometric test was caused by the activity of heterotrophic bacteria rather than autotrophic bacteria. Thus the values of maximum exogenous respiration rates for different treatments could be identical that was aroused by the heterotrophic bacteria. Spanjers & Vanrolleghem [18] indicated that the maximum oxygen uptake rate was mainly contributed by heterotrophic bacteria under the addition of acetate and ammonium, arriving at 24 $mgO_2 \cdot (L \cdot h)^{-1}$ during first 15 min. After that, the oxygen uptake rate slowly decreased from 6 $mgO_2 \cdot (L.h)^{-1}$ to the endogenous value due to the nitrification of autotrophic bacteria.

3.5 Nitrogen removal performance

The sum of ammonium, nitrite and nitrate was defined as Total Inorganic Nitrogen (TIN) in this study. Figure 4A shows the TIN removal efficiencies of four treatments: 30-1.7-CD, 30-1.2, 25-1.2-CD, 25-1.7. The treatment 30-1.7-CD had the highest TIN removal efficiency of 85.4% after 75 h as well as the fast growing rate at the beginning. Reasons could be the high temperature, large density of microorganism and strong microbial activity in the gel bead with CD (Supplementary material Fig. S1). Comparing the treatment 30-1.2 with 25-1.2-CD, their TIN removal efficiencies were close to 57.0%, even though the temperature was comparatively lower for the treatment 25-1.2-CD. This demonstrates that the CD can help to increase the TIN removal efficiency regardless of the low temperature. Furthermore, the treatment of 25-1.7 had the lowest TIN removal efficiency of 46.3% because of the lowest temperature and lack of CD added, although the high concentrations of PVA and SA were used to prepare the gel bead.

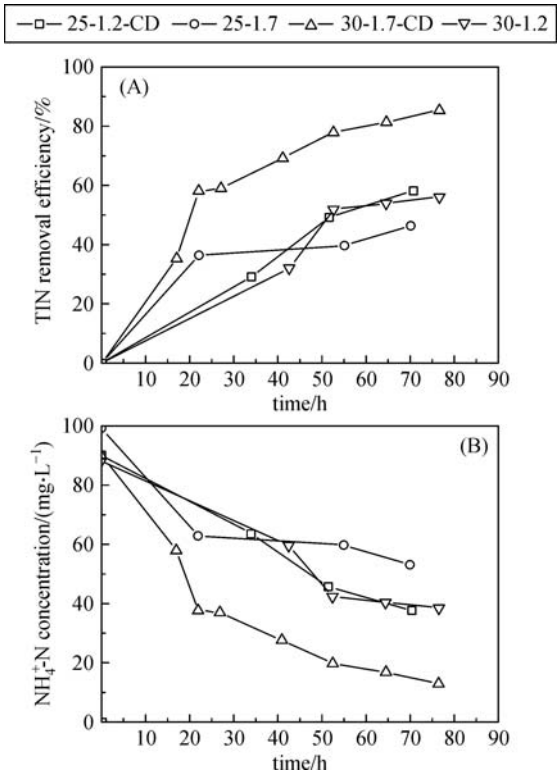


Fig. 4 The comparison of (A) TIN removal efficiencies and (B) ammonium concentrations of four treatments during the batch test (30-1.7-CD: 1.7% PVA + 1.7% SA + 1.0% CD at 30°C; 30-1.2: 1.2% PVA + 1.2% SA without CD at 30°C; 25-1.2-CD: 1.2% PVA + 1.2% SA + 1.0% CD at 25°C; 25-1.7: 1.7% PVA + 1.7% SA without CD at 25°C)

In terms of ammonium, nitrite and nitrate concentrations, Fig. 4B shows that the NH_4^+-N concentration decreased during the time and the treatment 30-1.7-CD owned the highest reducing value. However, the concentrations of NO_3^--N and NO_2^--N were negligible during all time of batch test, and the highest values of produced NO_3^--N and NO_2^--N only arrived at 3 $mg \cdot L^{-1}$ (seen in Supplementary material Fig. A2). This phenomenon suggests that the expected simultaneous nitrification and denitrification was taken place inside gel bead. The TIN

removal rate varied from 0.67 to 2.08 $mg \cdot (L \cdot h)^{-1}$ for these four treatments and the treatment 30-1.7-CD contributed to the highest value. Cao et al. [15] also found the quite low NO_3^--N and NO_2^--N concentrations in PVA-SA gel particles, whose nitrogen removal rate changed between 2.34 and 4.5 $mg \cdot (L \cdot h)^{-1}$ depending on the organic carbon source.

Thus, it can be concluded that materials of gel agents greatly influence the nitrogen removal performance, and the difference between TIN removal efficiencies of four treatments ought to be attributed by the beneficial effects of CD. In particular, the addition of CD into the traditional gel agents of PVA and SA affects the inner microorganism activity and mass transfer of nutrient and oxygen. Based on the fact that PVA and SA are recognized to strength interior structure and to accelerate mass transfer of gel material, respectively [19], CD was employed to maintain the viability of microorganisms as microbial inoculants in SA gel [20]. Also, Li et al. [21] used β-CD to prepare a new kind of polyurethane embedding material to remove organic matter and ammonium in wastewater. They found that the addition of β-CD significantly enhanced the hydrophilicity and biocompatibility of carrier, and increased the amount of embedded microorganism as well.

The results of orthogonal experiments are shown in Table 3, where the order of *R* values was successively β-CD, temperature and gel concentration. Results indicate that the CD addition was the most important one of these three factors, and the effect of temperature was secondary. The concentration of PVA and SA had the least effect on the nitrogen removal efficiency. Consequently, the optimal operating conditions for preparing immobilized gel beads are: PVA concentration of 1.7% (w/v), SA concentration of 1.7% (w/v), CD concentration of 1.0% (w/v), temperature of 30°C. According to the *F* value, no significance was observed among four treatments under the confidence level of 95%. However, the *F* value of CD addition being 1.498 was close to the critical *F* value, followed by the *F* value of temperature being 1.227. The concentrations of PVA and SA had the weakest impact on the nitrogen removal performance.

3.6 Microbial community

The microbial community inside the gel beads was analyzed by the High-Throughput Sequencing technique. Results show that unclassified *Comamonadacease* and unclassified *Comamonas* were two main bacteria inside the gel bead, accounting for 50.0% and 11.3% of total microbial community, respectively (Fig. 5). Both of them belong to the family of *Comamonadacease.* The similarity searching was performed according to Quast et al. [22] (Release 119 http://www.arb-silva.de), Cole et al. [23] (Release 11.1 http://rdp.cme.msu.edu/) and Desantis et al. [24] (Release 13.5 http://greengenes.secondgenome.com/). Moreover, BLAST searches of the GenBank database confirm that the sequenced clones of unclassified *Comamonadacease* matched *Comamonas* sp. clone PII2B with sequence identities of 100% and the sequenced clones of unclassified *Comamonas* matched *Comamonas* denitrificans with sequence identities of 98%. Probably the low dissolved oxygen condition inside the gel bead caused by the DO value of 1.5 $mg \cdot L^{-1}$ in the feeding solution allows *Comamonadaceae* being dominate microbial species. Similarly, in a study of treating wastewater from food-processing factory, the major bacterial population changed from *Anaerolinaeceae* (15.6%) to *Comamonadaceae* (52.3%) when the dissolved oxygen concentration decreased to less than 1 $mg \cdot L^{-1}$ [25]. Importantly, *Comamonadaceae* was recognized to be common in the sludge of nitrification and denitrification with good nitrogen removal ability [26]. Li et al. [27] also found *Comamonadaceae* (6.1%) in aerobic granular sludge by the construction of a 16S rDNA clone library. Besides, *Comamonas* is assumed to be one of the most abundant microorganisms in biofilm communities, especially in nitrate removing microbial communities [28,29]. Moreover, even under bulk aerobic conditions, *Comamonas testosteroni* in mature biofilms can well reduce nitrate, maintaining high viability of biofilm [30]. In addition to heterotrophic denitrification, *Comamonas* sp. was detected in heterotrophic nitrification-aerobic denitrification system from the landfill leachate treatment that simultaneously

Table 3 Results of orthogonal experiments

	temperature/°C	β-CD	concentrations of PVA and SA/%	TIN removal efficiency /%
1	25	with	1.2	58.1
2	25	without	1.7	46.3
3	30	with	1.7	85.4
4	30	without	1.2	56.1
K1	52.18	71.72	57.07	
K2	70.74	51.21	65.85	
R	18.56	20.51	8.78	
F value[1]	1.227	1.498	0.275	

Note: [1]significance is 0.05

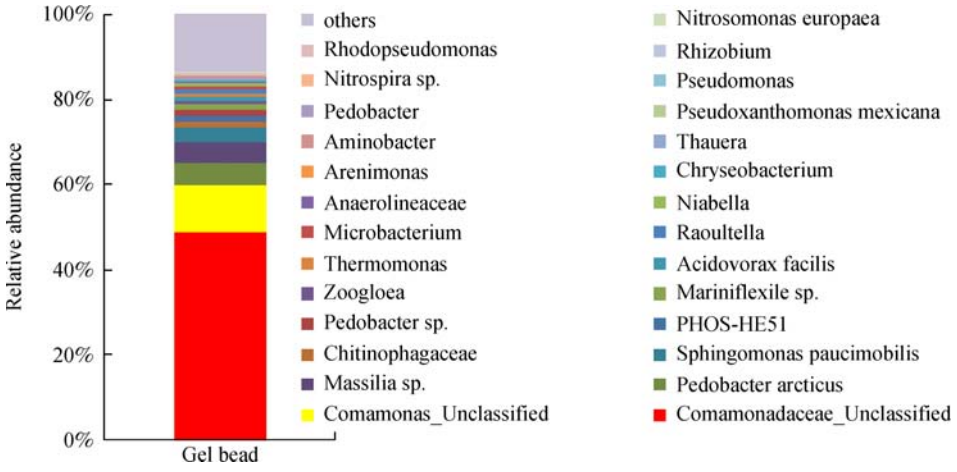


Fig. 5 The barplot of relative abundance of mcrobial communities inside gel bead

removed carbon and nitrogen [31]. Therefore, the large amount of *Comamonadaceae* and *Comamonas* (61.3% of total microbial community) probably can explain why there is simultaneous nitrification and denitrification inside the gel bead.

However, the lack of nitrifying microbial inside the gel bead needs to be indicated. The proportion of *Nitrospira* sp. and *Nitrosomonas europaea* only arrived at 0.17% and 0.15%, respectively. This could explain the reason of low nitrogen removal rate and long reaction time that more than 80 h were required to reach the high nitrogen removal efficiency. Interestingly, the role of *N. europaea* is remarkable that could nitrify and denitrify simultaneously under oxygen limitation condition by using nitrite in addition to O_2 as electron acceptor when ammonia and suitable organic compounds exist [32]. Apart from these two common nitrifying species, *Thauera* and *Pseudomonas* species were found in the gel bead, arriving at 0.24% and 0.22% of total communities, respectively. Like *Nitrosomonas*, *Thauera* is regarded to be closely correlated with exopolysaccharide (EPS) excretion and nitrogen removal [33], which was seen to be aerobic denitrifiers in the study of Calli et al. [34]. While *Pseudomonas stutzeri* T13 is recognized to be a heterotrophic nitrifying-aerobic denitrifying bacterium [35].

4 Conclusions

Simultaneous nitrification and denitrification was successfully achieved, and the gel bead with PVA and SA of both 1.7% (w/v) and with CD addition at 30°C contributed to the highest TIN removal efficiency of 85.4%. Besides the positive effect on the nitrogen removal, the addition of CD increased micro porosity on the bead's surface and gave rise to the high specific endogenous respiration rate. The microbial community mainly contained *Comamonadaceae* bacteria in the proportion of 61.3% to guarantee the good nitrogen removal ability. Overall, CD is assumed to be a favorable additional agent to the traditional PVA-SA embedding material.

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