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Removal of ammonium-N from ammonium-rich sewage using an immobilized *Bacillus subtilis* AYC bioreactor system

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Abstract

A self-design bioreactor system employing a fixed bed operation process with immobilized *Bacillus subtilis* AYC beads for NH_4^+-N removal from slightly polluted water was proposed. Polyvinyl alcohol and Na-alginate were used as a gel matrix to entrap *Bacillus subtilis* AYC to form the immobilized beads. The NH_4^+-N removal process was studied in a intermittent operation mode to examine the start-up and steady state behaviors of the immobilized AYC in the reactor. The results indicated that the reactor was in the start-up state during the first week. NH_4^+-N began to be steadily removal since the second week, and the nitrogen removal rate was between 84.61% and 96.19% when the hydraulic retention time (HRT) was 30 min. To apply *Bacillus subtilis* AYC to develop a practical nitrogen removal system and further understand its nitrogen removal ability, the bioreactor was continuously operated under different experimental perameters. The results showed that under the optimum conditions of an HRT of 20 min and DO of 3.77–5.80 mg/L, the NH_4^+-N removal rate reached 99.55%. The NH_4^+-N removal rate increased as the C/N ratio increased. However, a high C/N may cause a high residual carbon level in the effluent, therefore, the most suitable C/N ratio was 10. In addition, the results showed that the bioreactor system could remove many types of nitrogen such as NH_4^+-N, NO_3^--N and organic-N, and had a good performance for inorganic nitrogen removal from sewage.

Key words: immobilized *Bacillus subtilis* AYC beads; biologic nitrogen removal; bioreactor; effect

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Introduction

Rapid expansion of the economic activities and urbanization imposes tremendous pressure on enclosed water bodies. The quality of water resources becomes worse and worse in response to eutrophication, which is primarily caused by increasing nutrient wastes from many scattered small-scale point sources of pollution such as domestic households, factories and agricultural non-point pollution. NH_4^+-N is one type of pollution that leads to eutrophication of aquatic systems (Mallick, 2002).

Because ammonium can be assimilated or transformed to nitrite by bacteria (Bastian, 1992), numerous types of bioreactors such as fixed bed or fluidized bed systems are utilized to remove nitrogenous wastes from water (Tam and Wang, 2000; de-Bashan et al., 2002).

Several studies have found that autotrophic nitrifiers and heterotrophic denitrifiers can convert nitrogenous compounds to nitrogen gas (Andrade et al., 2007; Khardenavis et al., 2007). Autotrophs are known to be sensitive to carbon concentrations (Joo et al., 2005), therefore the application of autotrophs' nitrification was restricted. Nitrification process of autrophic bacteria should be separated in two tanks with the denitrification process (Khin and Annachhatre, 2004). Recent studies have showed that some bacteria such as *Pseudomonas stutzeri* and *Alcaligenes faecalis* not only performed heterotrophic nitrification but also denitrified their nitrification products under aerobic conditions, and nitrification and denitrification have been found to occur simultaneously in a single reactor (Su et al., 2006; Takayuki et al., 1998).

Immobilization in support gel is one method of maintaining high cell density, enhancing the efficiency and preventing microbe loss due to water flow. Many different gel matrices have been proposed as carriers, including natural biopolymers (polysaccharides, alginate, carrageenan and agar), and synthetic polymers (polyethylene, polyurethanes and polyethers) (Lozinsky and Plieva, 1998). Additionally, studies have consistently demonstrated efficient and rapid nitrogen removal from wastewater by immobilized microbes (Zhang et al., 2008; Cao et al., 2002; Chen et al., 1998).

In this study, a high density of heterotrophic *Bacillus*

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subtilis AYC was immobilized using polyvinyl alcohol (PVA) and Na-alginate gels to remove NH_4^+-N from slightly polluted water. The efficiency of immobilized *Bacillus subtilis* AYC in removing NH_4^+-N was then examined. The factors affecting NH_4^+-N removal such as HRT, C/N ratio, DO and initial nitrogen concentration were also investigated.

1 Materials and methods

1.1 Synthetic sewage

The composition of synthetic sewage used in the system is shown in Table 1. The synthetic sewage was made from water, a nutrient mixture and other chemicals for pH buffering, as well as the trace metals to maintain the bacterial growth. Sucrose was used as a carbon source.

The NH_4^+-N concentration of sewage #1 was the same as that of Grade V water (2 mg/L) according to the environmental quality standards for surface water (GB3838-2002). NH_4^+-N concentration of sewage #2 was the same as that of the inferior Grade V water (4 mg/L). The nitrogen concentration of sewage #3 depended on the average monitoring data of the Xiaozhigao River's long-term water quality in Chao Lake of Anhui Province, China.

1.2 Culture of bacterium removing NH_4^+-N

A heterotrophic bacterium AYC capable of removing NH_4^+-N was isolated from the activated sludge of a sewage treatment plant and identified as *Bacillus subtilis* AYC (GenBank accession number HQ 263248). The enriching medium consisted of 5 g/L beef exact, 10 g/L peptone and 5 g/L NaCl (pH 7.0, sterilization 121°C, 20 min). The cultures were grown in 250 mL flasks containing 100 mL of sterile medium, and the cells of AYC were incubated in enriching medium on a shaker at 200 r/min and 28°C for 24 hr. The cell suspensions of AYC were then centrifuged at 10,000 r/min for 5 min using a high-speed centrifuge, after which they were washed with normal saline and centrifuged three times, and stored at 4°C until used in the experiments.

1.3 Immobilization of microbe

Twenty-four gram wet cell suspensions were absorbed by activated carbon (2%, *W*/*V*) for 10 min. Na-alginate (0.25%, *W*/*V*) solution was mixed with PVA (12%, *W*/*V*), after which the activated carbon with the concentrated cells were added to the mixture and stirred to the uniformity. The mixed solution was then dropped into a 4% $CaCl_2$ and 4% H_3BO_3 solution to form spherical beads and then incubated at 4°C. The beads were subsequently washed with normal saline solution three times.

1.4 Operation of fixed-batch bioreactor

The reactor was made using organic glass with a column (1 m in length, internal diameter 148 mm). The schematic diagram of the reactor setup is shown in Fig. 1. In the fixed bed reactor, there were four parallel clapboards with pores that allowed water to flow out while retaining the beads. This design also results in the air bubbles being equally

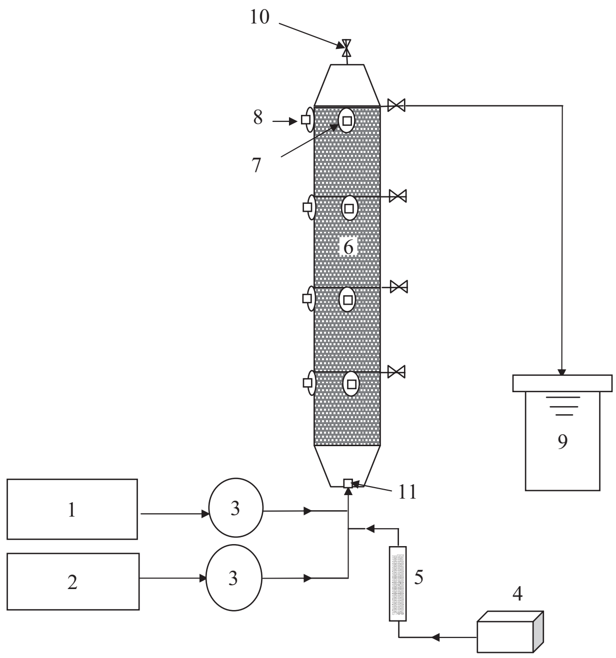

Fig. 1 Schematic diagram of fixed bed reactor. (1) feed tank; (2) carbon source solution tank; (3) peristaltic pump; (4) air compressor; (5) air meter; (6) reaction tank; (7) DO meter; (8) pH meter; (9) effluent tank; (10) exhaust opening; (11) spray head.

Table 1 Composition of synthetic sewage

Type of sewage	Chemical	Concentration (mg/L)	Chemical	Concentration (mg/L)
Acclimation Sewage	$(NH_4)_2SO_4$	75.20	$KHPO_4$	30
	Sucrose	1140.8	$MnSO_4·H_2O$	7.5
	NaH_2PO_4	10	$MgSO_4·H_2O$	30
Sewage #1	$(NH_4)_2SO_4$	9.43	$KHPO_4$	30
	Sucrose	142.6	$MnSO_4·H_2O$	7.5
	NaH_2PO_4	10	$MgSO_4·H_2O$	30
Sewage #2	$(NH_4)_2SO_4$	18.80	$KHPO_4$	30
	Sucrose	285.2	$MnSO_4·H_2O$	7.5
	NaH_2PO_4	10	$MgSO_4·H_2O$	30
Sewage #3	$(NH_4)_2SO_4$	10.61	$CaCl_2$	23
	KNO_3	25.25	KH_2PO_4	23
	Peptone	14.89	$MgSO_4·H_2O$	23
	Sucrose	312.87	$NaHCO_3$	65
	KCl	63	Trace element ($FeSO_4$, $MnSO_4$, $CuSO_4$)	0.2

dispersed and prevents extrusion among the immobilized cell beads due to gravity leading to baffling of the mass transfer of liquid and gas. A quarter of the reactor was used, which had a capacity of 4.3 L. The filling rate of the immobilized AYC beads was 53.47%. Synthetic sewage was fed from the bottom of the bioreactor by a peristaltic pump. At the same time, air was supplied to the reactor through a sparger in the bottom of reactor. During the experiment, the reaction temperature was controlled at room temperature.

1.4.1 Start-up and stable state operation of the bioreactor operated in the intermittent mode

Synthetic wastewater was fed into the bioreactor, and the intermittent operation mode experiment was started. During the experiment, the DO concentration was controlled at 3–5 mg/L by adjusting the air flow. Prior to the experiment, the co-immobilized cell beads were cultivated for a period of time in the synthetic sewage. Samples from the effluent water of the reactor were taken to determine the NH_4^+-N concentration at intervals. When the NH_4^+-N concentration of the effluent was lower than 2 mg/L and stable, the reactor system was assumed to be operating at stable state. All sewage flowed out after each round of treatment and was replaced with fresh sewage.

1.4.2 Continuous operation of bioreactor

Sewage #2 was used as the influent for continuous operation. During continuous operation, the sewage was pumped continuously into the reactors with a certain speed. The composition of the influent is shown in Table 1. After a certain hydraulic retention time (HRT) was attained, the effluent overflowed continuously from the top of the reactors. The flow rate of the effluent was controlled to be the same as that of the influent. The NH_4^+-N concentration of the effluent was measured. HRT of 40, 30, 20, 15 and 10 min were evaluated to test the effects of HRT on NH_4^+-N removal. The effects of DO on NH_4^+-N removal were observed by adjusting the air flow. The effect of C/N on NH_4^+-N removal was examined by adjusting the ratio to 0, 10, 20, and 30. Sewage #1, #2 and #3 were respectively evaluated to observe the effect of different initial ammonium nitrogen levels on ammonium nitrogen removal.

1.5 Analytical methods

All samples from the reactor influent and effluent were analyzed in accordance with the analysis methods described by the Chinese National Environmental Protection Agency (2002). Samples were filtered using 0.45 μm membrane disc filters prior to analysis. TN was measured by the alkaline potassium persulfate digestion-UV spectrophotometric method (GB11894-89). NH_4^+-N, NO_3^--N and NO_2^--N concentrations were measured using a HACH DR 2800 spectrophotometer with HACH Chemicals Ammonia Cyanurate, Ammonia Salicylate Reagent, Nitraver 5, and Nitriver 3, respectively. Dissolved oxygen (DO) was measured using a FG4 DO meter (Mettler-Toledo, Switzerland), and pH was analyzed using a FE20 pH meter (Mettler-Toledo, Switzerland). The reactor was operated at room temperature.

2 Results and discussion

Previous studies indicated that *Bacillus subtilis* could be aerobic denitrifiers (Kim et al., 2005), and the heterotrophic nitrifier *Bacillus* sp. LY had the ability to remove nitrogen (He et al., 2007). In the present study, *Bacillus subtilis* AYC, which efficiently removed NH_4^+-N, was isolated and immobilized in PVA and Na-alginate to form beads with a diameter of 3 mm.

2.1 NH_4^+-N removal by intermittent operation of the reactor system

To investigate the feasibility of NH_4^+-N removal in the reactor, the start-up and steady state behaviors of the immobilized AYC in the reactor during operation in the intermittent mode were examined. The reactor was initially operated with sewage containing a high NH_4^+-N concentration (16 or 8 mg/L). The influent and effluent water qualities during cultivation are illustrated in Fig. 2.

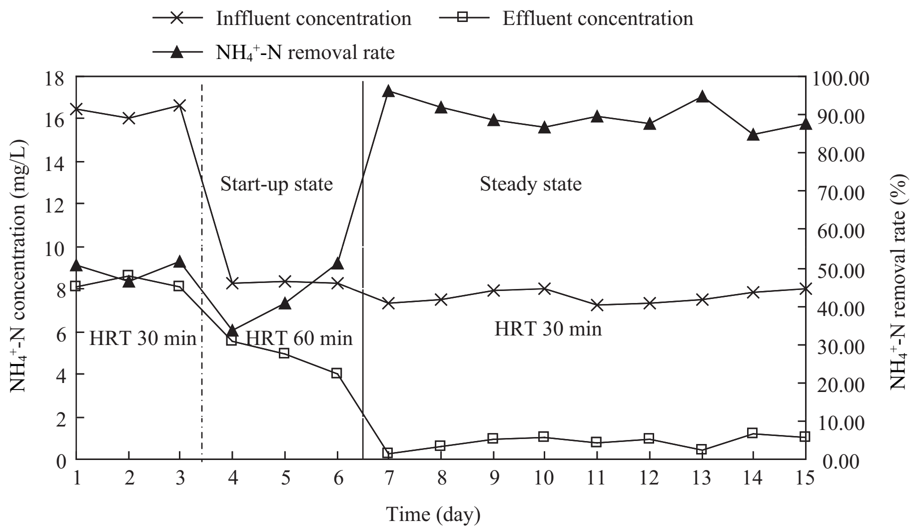

Fig. 2 Start-up and steady state behaviors of the reactor system operated in intermittent mode.

During the first week, the reactor was in the start-up state. From the second week, influent with approximately 8 mg/L nitrogen was fed into the reactor. After treatment with HRT 30 min, NH_4^+-N was removed steadily. The NH_4^+-N concentration of effluent was low, and the nitrogen removal rate was greater than 80%. Thus, the reactor system was considered to be in the steady state.

The time course of nitrogen concentrations in the effluent were examined during the nitrogen removal process under steady state conditions (Fig. 3). During the initial 10 min, the NH_4^+-N concentration decreased quickly. After 30 min, the NH_4^+-N concentration was low and maintained to steadiness. Thus, steady state was examined at a HRT of 30 min. In addition, no accumulation of nitrite and nitrate in the effluent was observed.

2.2 Effect of HRT on NH_4^+-N removal during continuous operation of the reactor system

For the continuous bioreactor containing immobilized AYC beads, sewage #2 was used as the influent water. Figure 4 shows the results of continuous operation using immobilized AYC beads at HRT ranging from 40 to 10 min. The results showed that HRT had a significantly effect on nitrogen removal.

When HRT decreased from 40 to 20 min, the NH_4^+-N concentration of the effluent water decreased raging from 0.28 to 0.11 mg/L. The average effluent water quality of the NH_4^+-N reached Grade I or II according to the surface water environment quality standard (GB3838-2002), and the removal rate was between 93.33% and 97.37%. The NH_4^+-N removal rate of the system operated at HRT of 15 min was greater than 91.41%, and water quality achieved Grade II. When HRT was 10 min, the effluent water achieved Grade III, and the rate of nitrogen removal decreased to 84%.

In addition to the nitrogen removal rate, the nitrogen removal efficiency (Eff) was also evaluated according to the following equation:

$$\text{Eff} = \frac{(N_{\text{in}} - N_{\text{out}}) \times V_1}{V_2 \times t}$$

where, N_{in} (mg/L) is the nitrogen concentration of the influent, N_{out} (mg/L) is the nitrogen concentration of the effluent, V_1 (L) is the volume of sewage, V_2 (L) is the total apparent bead volume in the experiment, and t (hr) is the time of the sewage treatment.

As shown in Fig. 5, NH_4^+-N removal efficiency was higher when the HRT was shorter. Specifically, when HRT was 40, 30, 20, 15 and 10 min, the NH_4^+-N removal efficiencies (Eff) increased (3.154, 4.940, 7.253, 7.497 and 10.190 mg/(L·hr), respectively).

The long HRT was beneficial for the treatment because it enabled full contact between the contaminants and microbes, which resulted in a high removal rate. However, there were some disadvantages to HRT that was too long, such as NH_4^+-N removal efficiency decreasing and increasing operating costs. Therefore, 20 min was selected

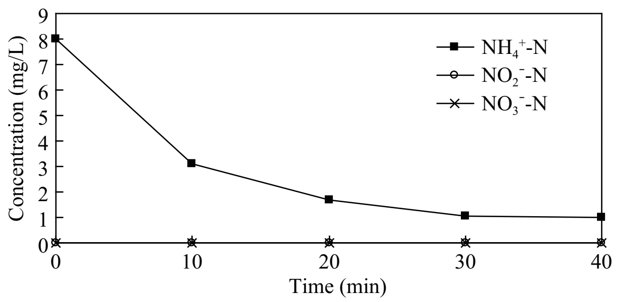

Fig. 3 Time course of effluent nitrogen concentrations during intermittent operation mode.

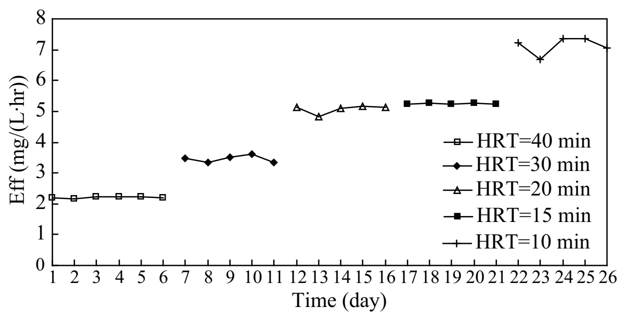

Fig. 5 NH_4^+-N removal efficiencies (Eff) under different HRTs.

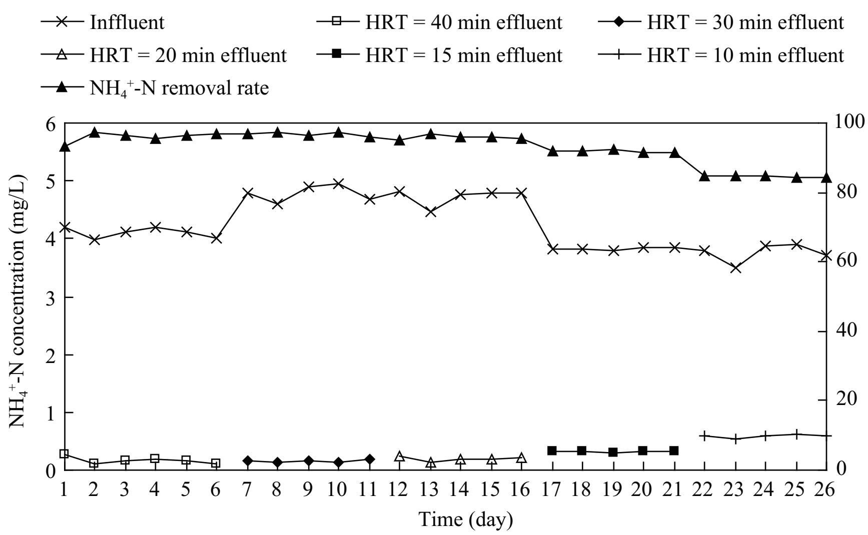

Fig. 4 Effect of HRT on nitrogen removal during continuous operation mode

as the optimal HRT, where the NH_4^+-N removal rate was greater than 93.33%, and the NH_4^+-N concentration of the effluent water was below 0.3 mg/L, reaching Grade I or II according to GB3838-2002.

2.3 Effect of DO on NH_4^+-N removal by the continuous reactor system

Factors such as DO, C/N and the concentration of NH_4^+-N affecting nitrogen removal by immobilized cells have previously been studied (Cao et al., 2002). The nitrification process of microbes demands oxygen. However, providing more DO results in increased costs. Accordingly, the effect of DO in the reactor was investigated. The results are shown in Fig. 6.

When a low volume of air was provided (DO in the effluent was 0.19–0.39 mg/L) and HRT was 20 min, the NH_4^+-N water quality in the effluent reached Grade II standard according to GB3838-2002, and the nitrogen removal rate was 95.98%. While a high volume of air was provided (DO in the effluent was 3.77–5.80 mg/L), the quality of the effluent reached Grade I and the nitrogen removal rate reached 99.55%.

When the HRT was 10 min under low DO conditions, the effluent water quality reached Grade II or close to Grade III, and the nitrogen removal rate was only 88.94%. The nitrogen removal rate increased obviously following the increase in DO. Under high DO conditions, the quality of the effluent achieved Grade I, and the nitrogen removal rate was 98.64%. At the moment, the NH_4^+-N removal efficiency was increased to 13.89 mg/(L·hr).

It was previously reported that the removal rate of ammonium by CMC anammox-immobilized beads was 100% after 48 hr (Zhu et al., 2009). *Scendesmus* sp. entrapped in calcium alginate as algal sheets was employed to remove inorganic NH_4^+-N, which resulted in a removal efficiency of 99.1% after 105 min (Zhang et al., 2008). In the present experiment, an HRT of 20 or 10 min was required to achieve nitrogen removal rates of 99.55% and 98.64%, respectively. These findings indicate that the time required to remove nitrogen in this study was obviously less than that of previous studies, and that the nitrogen removal rate was still higher. Thus, the reactor removed the nitrogen from the slightly polluted sewage well.

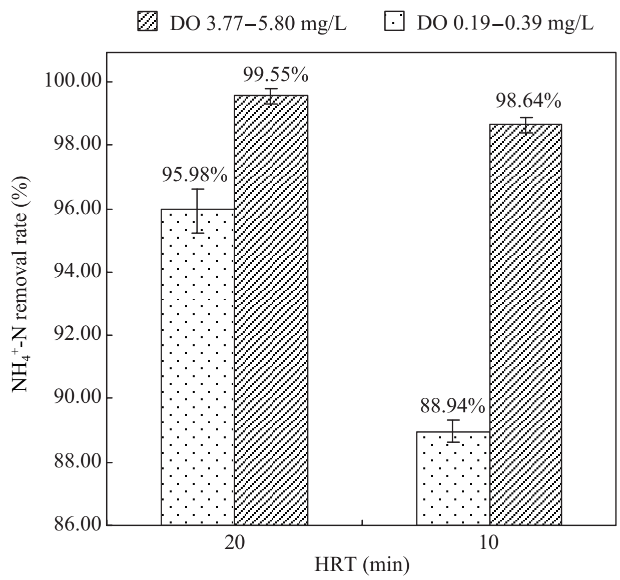

Fig. 6 Effect of DO on nitrogen removal during continuous operation mode.

2.4 Effect of C/N on NH_4^+-N removal by the continuous reactor system

The NH_4^+-N removal rate for heterotrophic bacterium AYC could be related to C/N ratio, therefore, the effect of the C/N ratio on NH_4^+-N removal was examined in the reactor (Fig. 7). At C/N ratios of 10, 20 and 30, the NH_4^+-N removal rate reached greater than 93.55%. When there was no carbon source in the sewage, the NH_4^+-N removal rate was 81.94%. Therefore, the carbon source was beneficial to the removal of the NH_4^+-N, and a high C/N led to an increased NH_4^+-N removal rate. Moreover, the results showed that a low supply of carbon results in reduced ammonium nitrogen removal ability, while a high C/N may cause a high residual carbon level in the sewage. Overall, the most suitable C/N ratio was 10 in the present study.

2.5 Effect of initial nitrogen concentration on nitrogen removal by the continuous reactor system

Treatment of sewage with different initial NH_4^+-N concentrations (#1, #2) and simulated river water (#3) was examined.

As shown in Fig. 8, NH_4^+-N and NO_3^--N was removed. In addition, NO_2^--N did not accumulate in the experiment. With the treatment at HRT of 20 min by immobilized AYC beads in the fixed bed, the NH_4^+-N concentration in the effluent of sewage #1 was 0.12 mg/L, which reached Grade I according to the standard (GB3838-2002). The NH_4^+-N in the effluent of sewage #2 was 0.28 mg/L, which reached Grade II. The NH_4^+-N removal rates were 93.00% and 94.00%, respectively. So the results had little discrepancy. There was organic-N, NH_4^+-N and NO_3^--N in the sewage #3. After treatment in a reactor with HRT of 20 min, NH_4^+-N, NO_3^--N and TN concentration was reduced. Specifically, the NH_4^+-N concentration was reduced from 2.25 to 0.41 mg/L, nitrate-N was reduced from 3.5 to 0.5 mg/L, and TN was reduced from 7.76 to 2.42 mg/L. These findings indicated that the reactor system had good

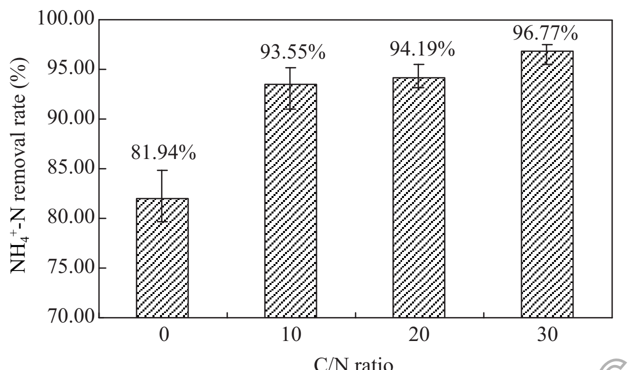

Fig. 7 Effect of C/N on nitrogen removal during continuous operation mode.

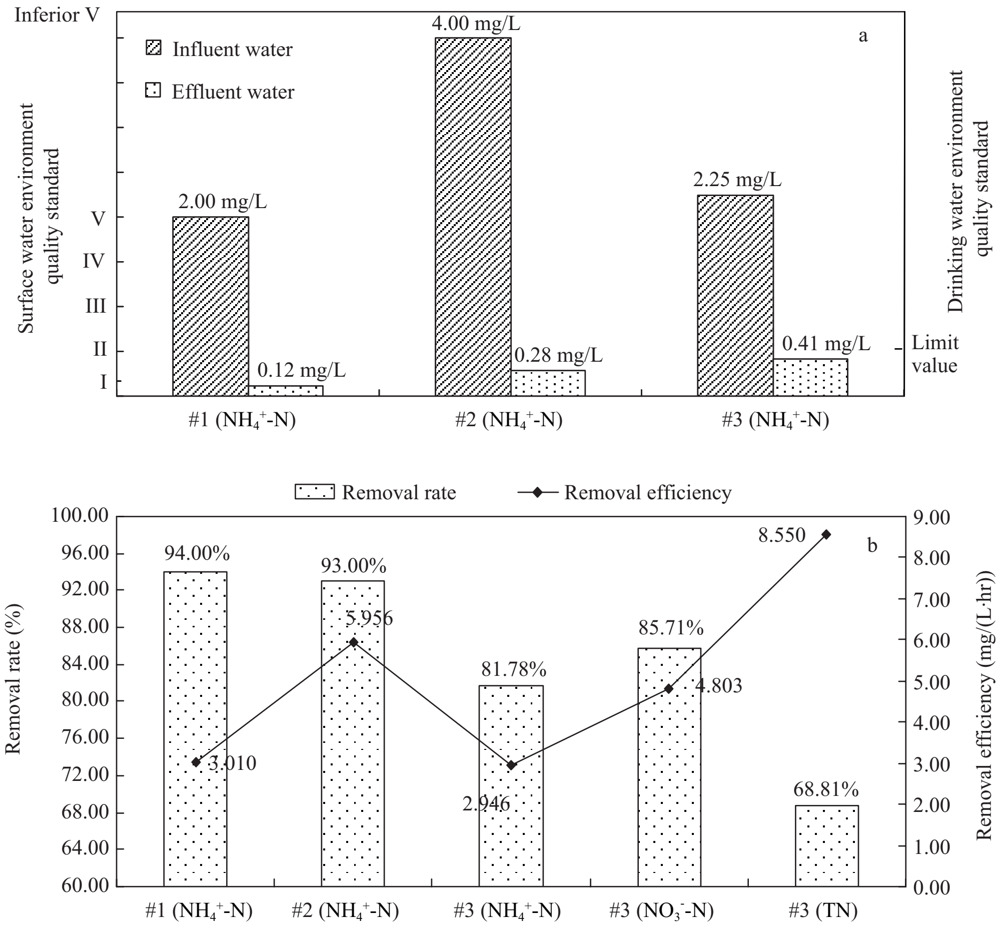

Fig. 8 Treatment of sewage #1, #2 and #3 in the continuous reactor: (a) the nitrogen concentration of the influent and effluent, and comparison with Surface/drinking water environmental quality standard (GB3838-2002, GB5749-2006); (b) the nitrogen removal rate and efficiency when different sewage was treated.

effect on treatment of sewage containing inorganic forms of nitrogen such as NH_4^+-N and NO_3^--N (Fig. 8b).

2.6 Proposed route of nitrogen removal

It has been proposed that during treatment, ammonium is initially oxidized to nitrite followed by nitrate which is then reduced to nitrite and ultimately nitrogen gas. Therefore we assumed the nitrogen removal process of *Bacillus subtilis* AYC was $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow N_2$. In this study, little nitrite and nitrate were observed. These findings could have been due to the following reasons: (1) there have been reports that some heterotrophic nitrifiers do not accumulate nitrite or nitrate, which would result in low amounts of nitrite and nitrate being detected (Robertson et al., 1989; van Niel et al., 1992). (2) Wang et al. (2007) reported that the transfer to nitrite and nitrate may be too rapid to allow adequate detection. In addition, it has been suggested that N_2 production which was a possible pathway for ammonium nitrogen removal by AYC, should be investigated.

3 Conclusions

In summary, the strain *Bacillus subtilis* AYC has the ability to remove NH_4^+-N. The immobilized AYC beads in the reactor were examined, and the results showed a high efficiency for nitrogen removal. Furthermore, the ability to remove nitrogen was not inhibited by higher DO, C/N and initial concentration and occurred best at a DO of 3.77–5.80 mg/L, C/N of 10, and initial concentration of 2 or 4 mg/L. In addition, HRT of 20 min was most suitable. The NH_4^+ removal rate reached 99.55%. During the NH_4^+-N removal process, low levels of the intermediate products nitrite and nitrate were detected. The reactor system treated sewage containing inorganic nitrogen well. In light of these results, a possible pathway for ammonium removal was suggested as ammonium to nitrite or nitrate, followed by transformation of nitrate to nitrite and eventually to nitrogen gas. However, to confirm these findings the further research is necessary to determine the precise ammonium removal pathway of AYC.

Acknowledgments

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