Pol. J. Environ. Stud. Vol. 26, No. 2 (2017), 861-869
DOI: *10.15244/pjoes/65838*

Original Research

Nitrogen Removal from Piggery Wastewater within a Sequencing Batch Reactor Using *Pseudomonas putida*

Yunlong Yang, Feixue Chen, Linxiang Xie, Jun Xiong, Kaihui Hu*

College of life science, Fujian Agriculture and Forestry University, Fuzhou 350002, China

Received: 18 August 2016
Accepted: 15 October 2016

Abstract

This work evaluates the efficiency of ammonium removal from piggery wastewater using a sequencing batch reactor (SBR) inoculated with a newly isolated strain of *Pseudomonas putida* HJH1. The strain HJH1 not only could survive and remove up to 72.4 mg/L NO_2^--N under aerobic conditions, but it also has good performance for simultaneous nitrification and denitrification (SND) with no nitrite accumulation. The SBR system was able to consistently remove: 1) 76.4-100% ammonium nitrogen and 71.4-100% COD from artificial wastewater in stage 1, and 2) 89.2-99.1% ammonium nitrogen and 82.4-100% COD from piggery wastewater in stage 2. During the whole operation, the strain HJH1 predominated in the SBR all the time to function together with other bacteria. Results indicated that the SBR system inoculated with *Pseudomonas putida* HJH1 can efficiently remove ammonium nitrogen from piggery wastewater, thereby having potential applications for future nitrogen removal.

Keywords: nitrogen removal, piggery wastewater, *Pseudomonas putida*, sequencing batch reactor, simultaneous nitrification and denitrification

Introduction

Due to increasingly serious eutrophication, removal of nitrogen from discharged wastewater has been required in many wastewater treatment plants, and a variety of methods have been set up, among which simultaneous nitrification and denitrification (SND) has become an attractive technology for nitrogen removal. SND not only means that nitrification and denitrification occur concurrently in the same reaction vessel under identical operating conditions, but also implies that nitrite and ammonium could be removed simultaneously, both of which are important factors affecting the environment.

Nitrite plays a critical role in the nitrogen-cycle of the ecosystem. However, its accumulation in the water becomes increasingly intense as a result of rapid development in the fields of aquaculture, agriculture, and industry in recent years, which makes nitrite become a matter of great concern for the environment. For example, the toxicity of nitrite could cause a fish disease outbreak that leads to huge economic losses [1-2]. Therefore, it becomes extremely necessary to decrease the accumulation of nitrite in the water. In general, there are three methods involved in nitrite removal: physical [3], chemical [4], and biological [5]. Of them, the best is the biological method without secondary pollution and residues. According to the traditional denitrification

*e-mail: 474585312@qq.com

theory, the denitrifying process is limited to anaerobic conditions, but aerobic denitrification has attracted more and more attention due to some advantages [6]. The first aerobic denitrifier was reported to be the *Thiosphaera pantotropha* strain [7] (now reclassified as *Paracoccus denitrifican*) in 1984. Following this pioneering work, some other new aerobic denitrifiers belonging to genera such as *Pseudomonas, Alcaligenes,* and *Bacillus* were also isolated [8-10], and more and more isolates were applied to the nitrogen removal system.

Ammonium is also an important factor contributing to eutrophication. In view of containing a high concentration of ammonium, effective treatment of piggery wastewater will improve the local environment – especially in China and many other developing countries. Statistically, the recorded piggery wastewater discharged to the environment was more than 1.1×10^7 tons in 2014 [11]. Since these pollutants become heavy burdens to the environment, various biological methods have been developed, such as anaerobic bioreactors [12], membrane bioreactors [13], and upflow microaerobic sludge reactors [14]. Moreover, the sequencing batch reactor (SBR), owing to its characteristics of easy operation and high efficiency, is also widely used in the treatment of piggery wastewater [15-17]. However, the SBR in which a functional microorganism is inoculated to remove ammonium from piggery wastewater was rarely reported [18].

Some studies have revealed that strains capable of heterotrophic nitrification and aerobic denitrification existed, such as *Acinetobacter calcoaceticus* [19], *Pseudomonas stutzeri* [8], *Halomonas campisalis* [20], *Chelatococcus daeguensis* [21], and *Bacillus* [10]. These strains can oxidize ammonium to nitrite and, simultaneously, reduce nitrite to N_2 by way of the aerobic denitrification function, meaning that high efficiency of treating piggery wastewater by inoculating these strains into bioreactors to strengthen the SND process could be obtained. Although the aerobic denitrification by *Pseudomonas putida* [22-23] was well documented, its SND potential for ammonium removal from piggery wastewater has not yet been explored.

Therefore, in this study a bacterium strain for SND was isolated and identified. Its nitrogen removal performance was investigated step by step, including: 1) nitrite removal capability from denitrification medium in shaking flasks and a bioreactor, and 2) ammonium removal capability from piggery wastewater in an SBR, which aims to determine whether the augmentation of *Pseudomonas putida* could successfully improve SND performance to enhance nitrogen removal in an SBR system for piggery wastewater treatment.

Materials and Methods

Media

The screening medium (SCM) consisted of (g/L): $NaNO_2$, 0.1; KH_2PO_4, 0.5; $FeCl_2\cdot6H_2O$, 0.05; $CaCl_2$, 0.02; $MgSO_4\cdot7H_2O$, 0.5; sodium citrate, 0.3; and 1 ml of trace element solution. The trace element solution was composed of (g/L): $FeSO_4\cdot7H_2O$, 3; H_3BO_3, 0.01; $Na_2MoO_4\cdot2H_2O$, 0.01; $MnSO_4\cdot H_2O$, 0.02; $CuSO_4\cdot5H_2O$, 0.01; $ZnSO_4$, 0.01; and ethylene diamine tetraacetic acid (EDTA), 0.5. The screening medium plate (SMP) was the same as SM besides containing 20 g/L agar. The seed medium (SEM) modified from Merk medium was as follows (g/L): peptone, 8.6; NaCl, 6.4; sodium citrate, 1.5; and potassium nitrate, 1.5. The aerobic denitrification medium (DM) comprised (g/L): sodium citrate, 4; KH_2PO_4, 0.5; $Na_2HPO_4\cdot7H_2O$, 1; $FeSO_4\cdot7H_2O$, 0.1; $MgSO_4$, 0.2; trace element solution, 2 mL; and an appropriate amount of sodium nitrite. The SND medium (SNDM) was almost the same as DM except that sodium nitrite was replaced with ammonium chloride when used. All media had pH maintained at 7.0-7.5 and were autoclaved at 115ºC for 20 min.

Experimental Wastewater Characteristics

The raw piggery wastewater was collected from a local pig farm in Fuzhou, China. Since piggery wastewater is a typical high-strength ammonium (NH_4^+-N) wastewater, artificial wastewater was used and injected into the SBR prior to piggery wastewater with the aim of making *Pseudomonas putida* adapt to the ammonium environment in the SBR gradually. On day 19 we used a six-fold dilution of piggery wastewater. On days 20 and 21 we injected a two-fold dilution of piggery wastewater into the SBR. Raw piggery wastewater was used from day 22 on. The experimental wastewater characteristics are shown in Table 1.

Screening of Aerobic Denitrifiers and its Identification

Aerobic denitrifying organisms were enriched from Guanyin Lake (sometimes eutrophication is high) in Fujian Agriculture and Forestry University, Fu Zhou, China. The water samples were transferred to 100 mL SCM in 200-mL flasks with eight layers of gauze. The flasks were incubated in a shaking incubator at 180 rpm and 30ºC for three days. Then a 5% (volume ratio) of bacteria suspension was

Table 1. Qualities of experimental wastewater characteristics.

Item (mg/L)	Artificial wastewater	Piggery wastewater
COD	424-576	1,200-1,528
NH_4^+-N	29.1-38.7	227.3-274.5
TN	29.5-39.2	297.6-349.7
NO_2^--N	ND	ND
NO_3^--N	ND	ND
pH	7-8	6-9

ND: not detected

added to freshly autoclaved SCM and cultured at 30ºC. After 24 h, the suspension was diluted via gradient and the diluent was streaked on the SMP and incubated at 30ºC for three days. Prominent growing single colonies were then inoculated to 20 mL of SEM and incubated at 180 rpm for 16 h. The resultant cellular suspension (2.5 mL) was inoculated to 50 mL SCM and cultured for another 48h. Through measuring the concentration of NO_2^--N, we selected isolates with removal efficiency of more than 80%. This screening process was repeated three times. Only one strain was finally isolated, which was named HJH1.

The HJH1 isolate obtained was maintained at 4ºC in a plate containing SEM for short-term use and stored at -20ºC in 30% glycerol for long-term use.

Identification of the isolated strain was determined by 16S rRNA gene sequences. The genomic DNA of HJH1 was extracted using a DNA isolation kit (Biotech, Beijing, China). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with universal bacterial primers (27F: AGAGTTTGATCATGGCTCAG and 1492R: GGTACCTTGTTACGACTT) on a Mastercycler gradient thermocycler (Eppendorf, Germany). 20 µl of PCR reaction mixture was used: 2 µl of 10×PCR buffer, 1.6 µl of dNTP mixture, 0.4µl of each primer, 0.5 ng of DNA template, 0.5 U of Tag DNA polymerase (Takara, Dalian, China), and 14 µl double-distilled water. The amplification protocol was as follows: predenaturation at 94ºC for three min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 45 sec and elongation at 72ºC for two min, with a final extension at 72ºC for 10 min. The PCR product was sequenced by Sangon Biotech (Shanghai, China). The resultant sequence was examined in the Genebank database through the BLAST program to search for the closet matching sequences. Then the phylogenetic tree was constructed with MEGA 4 using the maximum parsimony algorithm.

Preliminary Experiments for HJH1 Nitrogen Removal

The effects of pH, carbon sources, temperature, and initial NO_2^--N concentrations on HJH1 denitrification characteristics were conducted in flasks. A single colony in a plate was inoculated into 30 mL SEM in a 100-mL flask cultured at 30ºC for 12h in a shaker at 180 rpm. Then 5% (volume ratio) of cellular culture was transferred to 100 ml DM in a 250-mL flask incubated at 30ºC for 24h in a shaker at 180 rpm. Samples were periodically taken out and stored at -20ºC.

Denitrification performance of HJH1 under optimal conditions was carried out in a bioreactor (Peiqing, Shanhai, China). This column bioreactor (with a maximum volume of 5 L) was equipped with an aerator, a dissolved oxygen (DO) monitor (the unit of DO is percentage, which can be transferred to mg/L), and a pH and temperature control system. The seed culture (150 mL) was inoculated into 3L DM in the bioreactor and incubated at an aeration rate of 0.8 L/min and stirring rate of 200 rpm. The initial NO_2^--N concentration, pH, and temperature were selected on the basis of the above experiments. Samples were periodically taken out and stored at -20ºC.

SND performance of HJH1 was conducted in shaking flasks. A single colony on a plate was inoculated into 30 mL SEM in a 100-mL flask cultured at 30ºC for 12h in a shaker at 180 rpm. Then, a 5% (volume ratio) of cellular culture was transferred to 100 ml SNDM in a 250-mL flask incubated at 30ºC for 24h in a shaker at 180 rpm. Samples were periodically taken out and stored at -20ºC.

SBR Device and Operation

A device with a working volume of 1.5L was used as the sequencing batch reactor, which is illustrated in Fig. 1. The SBR temperature was controlled at 30ºC by a temperature control heater, and the airflow rate was controlled by a gas flow meter through which gas was diffused into the reactor using an aerator installed at the bottom of the SBR. The device was operated at a circle time of 6h, consisting of a 2-min feeding, a 240-min aerobic reaction, and a 30-min settling, followed by a 3-min decanting and an 85-min idle period.

The whole operation period was composed of stages 1 and 2. In stage 1, the volumetric exchange ratio was 100%. In order to make *P. putida* predominant in this stage, the pure *P. putida* culture of 5% (volume ratio) was inoculated into the artificial wastewater in the reactor. After settling, all cultures were centrifuged at 5,000 rpm for 10 min and then pallets were all transferred to the reactor to start the next circle. In stage 2, piggery wastewater was injected into the SBR, and volumetric exchange ratio was maintained at 30%. The dissolved oxygen concentration during all stages was controlled at 2.5-4.0 mg/L.

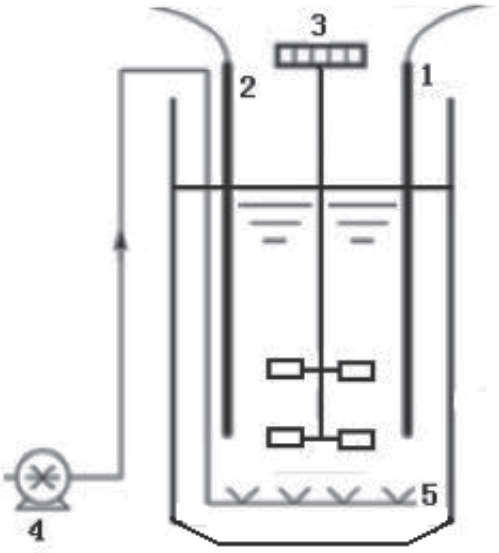

Fig. 1. Schematic diagram of the sequencing batch reactor: 1. pH meter, 2. temperature control heater, 3. stirrer, 4. air pump, and 5. aerator.

Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis

Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) was conducted on the cultures in SBR. The genomic DNA, extracted as described at section 2.3, was used as a template to amplify 16sRNA genes. PCR was performed in a Mastercycler gradient (Eppendorf 5331, Germany) using the following primers: GC341F (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G CCT ACG GG A GGC AGC AG-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′). DGGE was carried out in a Universal Mutation Detection System (BIO-RAD DCodeTM, USA). The gel contained a gradient of denaturant ranging from 30% to 60% (100% denaturant is 7 M urea and 40% deionized formamide). DGGE was run at 200 V for 5 h at 60ºC. After electrophoresis, the gel was stained with GoldView II (Solarbio, Beijing, China) for 30 min and viewed with a UV transilluminator (BIO-RAD, Italy).

Analytical Methods

Total nitrogen (TN), ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N), and COD were analyzed using Standard Methods [24]. DO and pH were determined online. All samples were filtered except for TN before analysis.

Results and Discussion

Phylogenetic Analysis of Aerobic Denitrifier HJH1

Based on maximum parsimony, a phylogenetic tree prepared using the HJH1 gene fragment and Genebank database sequences was displayed in Fig. 2. The isolate HJH1 showed the most similarity (99%) with *Pseudomonas putida*, which had been characterized before as an aerobic denitrifier with an ability to remove nitric oxide [22] and nitrate [23, 25].

Effects of Different Factors on HJH1 Nitrite Removal

Fig. 3 showed the effects of different factors on HJH1 nitrite removal and cell growth. At 30°C NO_2^--N concentration of 55.8 mg/L was reduced completely in 21 h, and at 37ºC about 51 mg/L NO_2^--N was removed in 24h (Fig. 3a). However, both cell growth and nitrite removal were poor at 20ºC. At 20, 30, and 37ºC, maximum cell density (OD_{600}) achieved 0.3, 0.52, and 0.47, and corresponding nitrite removal efficiency was 40.8%, 100%, and 92.2%, respectively. Undoubtedly, the optimum temperature for HJH1 denitrification is 30ºC.

Clearly, there was almost no change in both cell growth and nitrite removal at pH 5 and 6 (Fig. 3b). Although nitrite was consumed completely in 24h at pH 8, 9, and 10, their cell growths all had a long lag phase (about 12 h), which was mainly attributed to the adaptability of the cell to high pH conditions. At pH 7, cell density and nitrite removal efficiency reached 0.41 and 100% in 24 h, respectively, and therefore the optimum pH for HJH1 denitrification is 7.

The selected carbon sources were sodium citrate, sodium succinate, sodium acetate, and glucose. Out of them, sodium citrate contributed to the best cell density and nitrite removal efficiency, which got up to 0.6 and 100%, respectively (Fig. 3c). By contrast, glucose was bad for the strain HJH1, because both cell density and nitrite removal did not have significant change. Although it has been confirmed that carbon sources exert an important influence on denitrification, these influences vary with

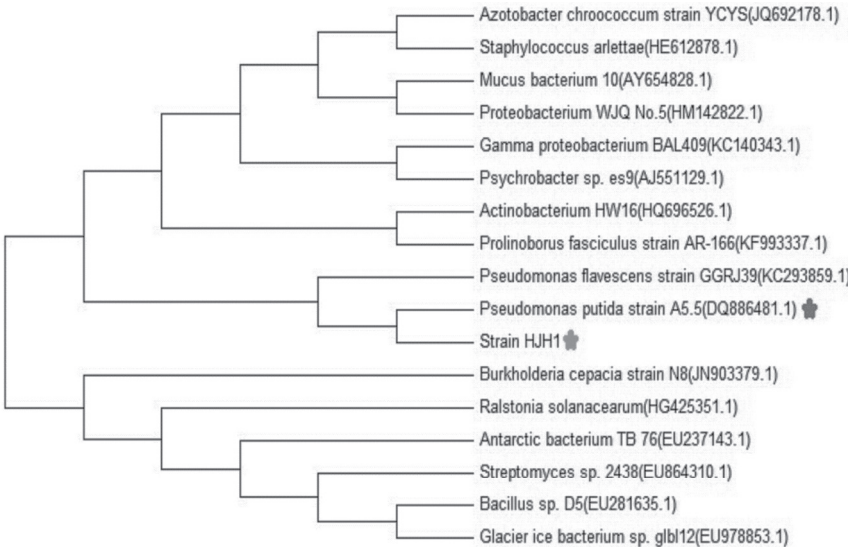

Fig. 2. Phylogenetic tree for aerobic denitrifier HJH1. Identification of the isolated strain was determined by 16S rRNA gene sequences and the phylogenetic tree was constructed with MEGA 4 using the maximum parsimony algorithm.

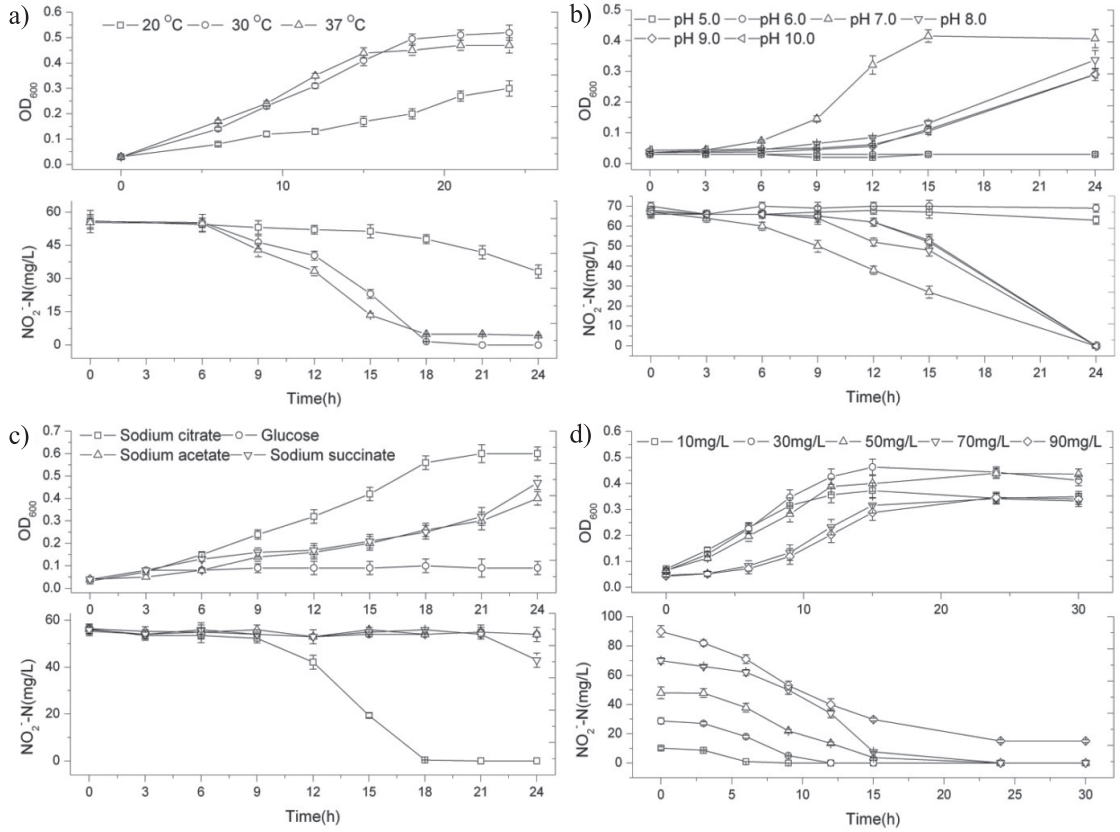

Fig. 3. Effects of different factors on HJH1 characteristics of nitrite removal in flasks: a) Temperature, b) pH, b) Carbon sources, and d) Initial nitrite-N concentration.

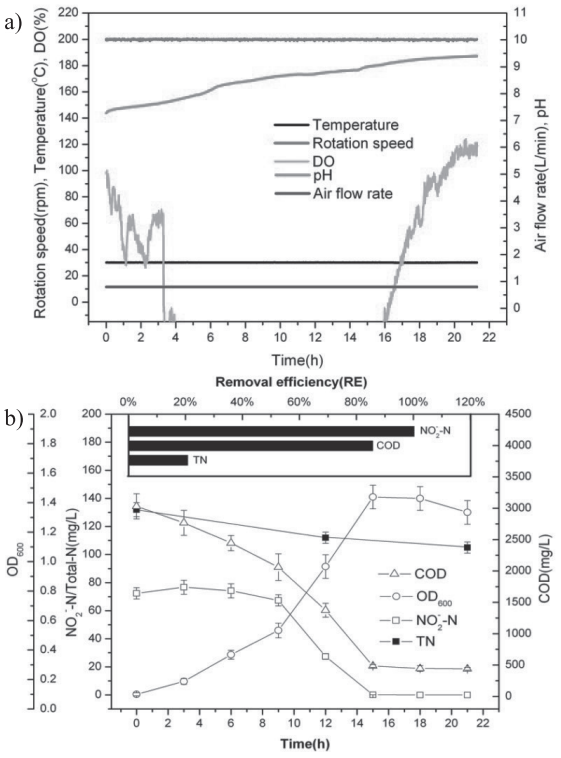

Fig. 4. Nitrite removal by HJH1 in the bioreactor: a) Flow chart including DO (%), pH, rotation speed, temperature, and airflow rate; and b) Time courses including COD, OD_{600}, nitrite, and total nitrogen concentration.

conditions tested. It was reported that nitrate removal efficiency was in the order: acetate > glucose > methanol > ethanol, out of which acetate-fed biofilm reduced nearly 100% nitrate with a negligible amount of nitrite accumulation [26], suggesting that acetate was most favorable for nitrite reductase (NiR). However, a distinct result could be found that the capacity of carbon sources on enhancing NiR enzyme activity follows: methanol > acetate > ethanol on molar basis [27]. In the present study, acetate and glucose seemed to be adverse regarding nitrite removal in HJH1.

At an initial NO_2^--N concentration of less than 70 mg/L, nitrite decreased to zero (not detected) in 24h and the corresponding nitrite removal efficiency was 100%, whereas it was still 15 mg/L in 30 h at an initial NO_2^--N concentration of 90 mg/L and the corresponding

Table 2. Comparison of denitrification rates in *Pseudomonas* strains.

Strains	Denitrification rates	References
Pseudomonas sp.	18.2 mg NO_2^--N l^{-1} d^{-1}	[29]
Pseudomonas putida	16.2 mg NO_3^--N l^{-1} d^{-1}	[36]
Pseudomonas stutzeri	18.14 mg NO_3^--N l^{-1} d^{-1}	[28]
Pseudomonas putida	72.4 mg NO_2^--N l^{-1} d^{-1}	This study

Table 3. Nitrogen balance for nitrite removal[a] (units: mg/L).

Initial TN	Final Nitrate-N	Final Nitrite-N	Final Ammonium-N	Final Organic N[b]	N in biomass[c]	% N lost[d]
72.4±1.8	ND	ND	ND	0.8±0.1	57.1±0.3	19.6

ND: not detected
[a]Values represent mean ± S.D. of triplicates
[b]Calculated value
[c]Biomass composition was assumed to be C5H7O2N
[d]% N lost = 100× {(initial TN)-(final Nitrate-N)-(final Nitrite-N)-(final ammonium-N)-(final Organic-N)-(N in biomass)}/(initial TN)

nitrite removal efficiency was about 83% (Fig. 3d). With regard to cell density, 30 mg/L NO_2^--N was best for cell growth with a maximum OD_{600} of 0.463. Generally, the high nitrite concentration has a negative effect on the microorganism, and so far there have been no bacteria reported that could endure such high nitrite concentrations as in this study.

Nitrite Removal by HJH1 in a Bioreactor

Nitrite removal by HJH1 was carried out in a bioreactor. A flow chart including DO (%), pH, rotation speed, temperature, and airflow rate was shown in Fig. 4a, and time courses including COD, OD_{600}, nitrite, and total nitrogen concentration are shown in Fig. 4b. Meanwhile, a detailed nitrogen balance for nitrite removal was carried out (Table 3). Obviously, removal rates for nitrite and COD correlated with the growth rate of stain HJH1, because the fastest removal rates occurred during the log phase. COD concentration decreased from 3,031 to 440 mg/L and the corresponding removal efficiency was 85.5%. The NO_2^--N concentration decreased from 72.4 to 0 mg/L, and the corresponding denitrification rate was 72.4 mg NO_2^--N l^{-1} d^{-1}, which was much higher than those reported by previous studies (Table 2). It is worth noting that NO_2^--N, as the sole nitrogen source, was consumed completely in 15h, which was insufficient for further cell growth, thus ending all biological reactions. Considering that cell density reached a maximum value of 1.44 and that approximately 20% of total nitrogen (Table 3) was converted to nitrogen gas, the intracellular assimilation and extracellular reduction should occur simultaneously. This is in good agreement with the co-respiration of O_2 and NO_2^--N supported in some reports [7, 28, 29].

The pH in the suspension elevated gradually from 7.2 to 9.4, which is consistent with the fact that denitrification is a process with alkaline production [30]. The DO concentration (%) decreased rapidly from 100% to 0% in the first 4h, and maintained that level for 12h, after which it increased to 100% by 20h. This phenomenon should be attributed to the fast cell growth that respired oxygen rapidly during the log phase. From above, nitrite was also removed primarily during this phase, so it is possible that nitrite respiration was enhanced when DO was not enough, thereby confirming the existence of co-respiration of O_2 and NO_2^--N.

Simultaneous Nitrification and Denitrification (SND) by HJH1

As displayed above, the strain of aerobic denitrification could have heterotrophic nitrification ability. Accordingly, the SND batch experiment was conducted in shaking flasks, and results are shown in Table 4. Obviously, the strain HJH1 has good performance for SND, because ammonium nitrogen decreased rapidly from 80 mg/l at 0h to 31.8 mg/l at 12 h, and was consumed completely (not detected) at 24 h with the removal efficiency (RE) being 100%. Meanwhile, the nitrite nitrogen accumulated to 2.55 mg/l at 9h, but then it was not detected at 24 h.

Table 4. Simultaneous nitrification and denitrification by HJH1 in flasks.

Time(h)	Concentration (mg/L)				
	NH_4^+-N	TN	COD	NO_2^--N	NO_3^--N
0	80±0.5	80±4	4021±227	ND	ND
6	62±0.8	77±2.1	3011±127	0.51±0.012	ND
9	44±0.7	74±1.6	2311±113	2.55±0.02	ND
12	31.8±0.42	70±3	869±84.9	0.61±0.014	ND
18	6.8±0.2	68±4.2	386±18.7	0.10±0.013	ND
24	ND	67±3.2	384±28.3	ND	ND
RE (%)	100	17.5	90.4		

ND: not detected

Table 5. Nitrogen balance for ammonium-N removal[a] (units: mg/L).

Initial TN	Final Nitrate-N	Final Nitrite-N	Final Ammonium-N	Final Organic N[b]	N in biomass[c]	% N lost[d]
80.0±4.0	ND	ND	ND	3.2±0.19	59.3±0.24	17.5

ND: not detected
[a]Values represent mean ± S.D. of triplicates
[b]Calculated value
[c]Biomass composition was assumed to be C5H7O2N
[d]%N lost = 100×{(initial TN)-(final Nitrate-N)-(final Nitrite-N)-(final ammonium-N)-(final Organic-N)-(N in biomass)}/(initial TN)

Thus total nitrogen (TN) RE was calculated to be 17.5%, which was in good agreement with the nitrogen balance for ammonium-N removal (Table 5).

The data in Table 4 and 5 indicated that strain HJH1 could utilize ammonium to produce nitrite, which is a dominant product generated by ammonia monooxygenase [31] and hydroxylamine oxidoreductase [32]. When the strain HJH1 was cultivated in SNDM, only a trace of nitrite was observed and then not detected, showing that HJH1 might have a high activity of denitrifying enzymes. This phenomenon seems to be in conformance with the data displayed in Fig. 4. All these results confirmed that HJH1 could remove ammonium nitrogen through SND without any nitrite accumulation.

Performance of the SBR

It is well known that piggery wastewater usually contains high amounts of organic mater and ammonium nitrogen. In order to make HJH1 accustomed to ammonium surroundings step by step, artificial wastewater was used in stage 1, after piggery wastewater was steadily fed to the SBR in stage 2.

In stage 1, the average influent concentration of COD was about 550 mg/L (Fig. 5). On the whole, COD removal increased gradually during this stage, ranging from 71.4% on day 1 to 100% on day 10. Although the COD RE dropped slightly in the following eight days, the average RE achieved 88.6%. From day 19 on, piggery wastewater was fed in stage 2, and the COD removal sharply decreased to 51.4%, but this phenomenon existed for only a short time. Afterward, the COD RE resumed to normal, and finally to 100% on days 31 and 32. In stage 2, the average influent COD was about 1,500 mg/l, which was obviously much higher than in stage 1. However, COD removal averaging 92.2% was maintained in the last eight days.

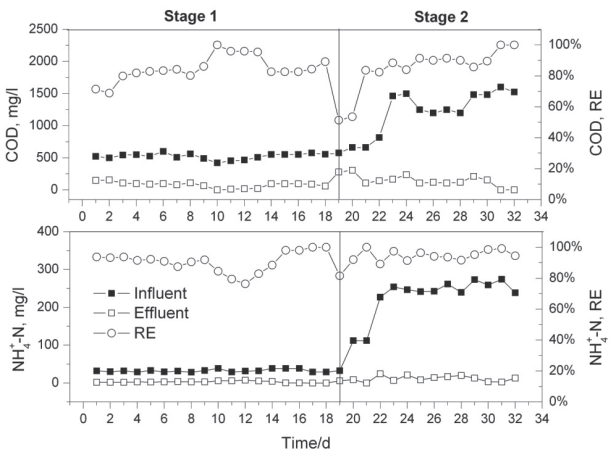

Fig. 5. Pollutant removal in the SBR. The whole operation was composed of two stages: 1) artificial wastewater was fed, and 2) piggery wastewater was fed.

As far as NH_4^+-N was concerned, a relatively steady RE average of 93.9% was maintained (except for days 11 to 14 in stage 1), which was different from COD. Although the average influent NH_4^+-N was only about 30 mg/L during this stage, NH_4^+-N RE decreased from 91.9% on day 9 to 76.4% on day 12, and then increased to 98% on day 15. Subsequently, stage 2 started when piggery wastewater was injected into the SBR on day 19 and, similar to COD, the RE of ammonium nitrogen went down to 81.5%, but this trend also lasted for only a short time. In stage 2, the average influent NH_4^+-N was about 240 mg/L, which did not exert any influences on its RE. On the contrary, from day 24, a relatively high RE maintained above 91.4% and even achieved 99.1% on day 31. Moreover, the cyclic profile for pollutant removal on 31d was conducted, where NO_2^--N accumulated 1.08 mg/L at 1.5 h and then was not detected at 3.5 h, and pollutants were all removed completely within a few hours (Table 6), suggesting that SND might play an important role in treating piggery wastewater in this SBR.

As mentioned previously, both COD and NH_4^+-N REs presented a remarkable fluctuation during days 10 to 14. This result probably was ascribed to the following facts. Initially, the pure *P. putida* was inoculated to the SBR, and all pollutants were entirely degraded by *P. putida*. However, with time elapsing, some bacteria appeared in the SBR (see section 3.6) and functioned together with *P. putida* to resume REs back to a steady state, which is similar to our previous study [33]. As illustrated in Fig. 5, although the piggery wastewater fed to the reactor was diluted on day 19, REs dropped abruptly due to a totally different loading, and recovered rapidly when faced to the raw piggery wastewater. This was mainly because *P. putida* might have a good adaptability to piggery wastewater. Most importantly, both nitrite and nitrate were not detected in the effluent (Fig. 5), which was in good agreement with the above results that *P. putida* possesses

Table 6. Cyclic profile for pollutant removal on 31d.

Time (h)	Item (mg/L)			
	NH_4^+-N	NO_2^--N	NO_3^--N	COD
0	274.5±2.1	ND	ND	1,604±80.2
1.5	29.5±2.6	1.08	ND	225.3±17.1
2.5	5.3±0.92	0.014	ND	61.4±2.0
3.5	2.55±0.06	ND	ND	ND
4.5	2.60±0.08	ND	ND	ND

ND: not detected

a good performance of aerobic denitrification (Fig. 4) and SND (Table 4) without any nitrite accumulation. Nitrite is critical for nitrogen removal because a significant amount of nitrite is the premise for the subsequent denitrification, so the stability of nitrite accumulation was investigated in a sequencing batch reactor [16]. In addition, it has been reported that some factors such as pH [17], carbon source [15], and temperature [34] affected nitrite accumulation in the treatment of piggery wastewater, which seems to be contradictory to the results in the current study. However, considering that *P. putida* could reduce nitrite rapidly (Fig. 4), it would be reasonable if this strain predominates in the SBR.

Stability of HJH1 in the SBR

For the purpose of analyzing the stability of *P. putida* HJH1 during the whole operation, PCR-DGGE was carried out on active sludge of 8d, 16d, and 32d, and results are displayed in Fig. 6. Clearly, HJH1 predominated in the SBR all the time, especially in the lane of 8d. Though some other strains existed on day 16, the nitrogen removal was conducted mainly by strains B, E, and HJH1. Compared with lane 16d, some new stains A, C, and D appeared in lane 32d due to piggery wastewater. The strain *P. putida* HJH1, however, still inhabited the reactor to function together with other bacteria.

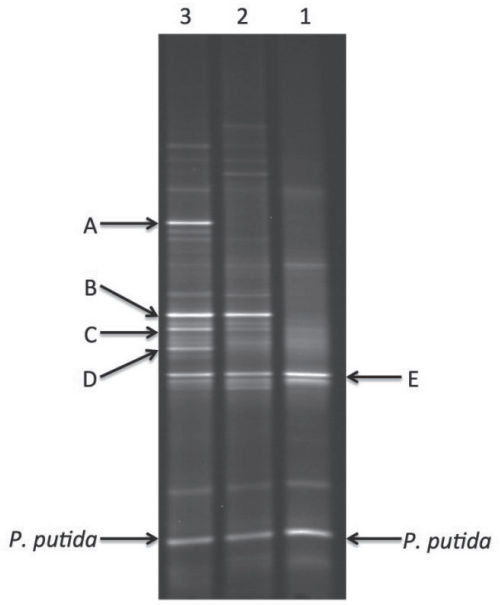

Fig. 6. DGGE profile of PCR-amplified 16S rDNA fragment. Fragments were obtained with bacterial primer set and DNA isolated from active sludge. Lane 1: active sludge of 8d, Lane 2: active sludge of 16d, and Lane 3: active sludge of 32d.

It is very common to use the SBR to treat piggery wastewater, and results revealed that some important functional microorganisms such as ammonium oxidation bacteria (AOB), nitrite oxidation bacteria (NOB), and denitrifiers are required. Meng and coworkers treated piggery wastewater with an upflow microaerobic sludge reactor (UMSR) and found that ammonium oxidation bacteria, heterotrophic denitrifiers, autotrophic denitrifiers, and phosphate-accumulating organisms coexisted perfectly in the system [11]. Furthermore, some other unknown and as yet uncultured microorganisms [12] and algae [35] were significant for the nitrogen removal from piggery wastewater. In the present study, as exhibited in Fig. 6, some microorganisms were also detected, but *P. putida* HJH1 predominated in both stage 1 and 2, which undoubtedly consolidated the results in section 3.5.

Conclusions

The general results of the present study demonstrate that the ammonium nitrogen of piggery wastewater could be effectively removed by SBR inoculated with *Pseudomonas putida* HJH1. The SBR system was able to remove 89.2-99.1% ammonium nitrogen and 82.4-100% COD from piggery wastewater. Although many bacteria existed, *Pseudomonas putida* HJH1 predominated and cooperated with those strains to remove ammonium nitrogen and COD in the reactor. Overall, the results presented and discussed herein obviously indicate that using the SBR system inoculated with *Pseudomonas putida* HJH1 to treat piggery wastewater is practically feasible.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China (grant No. 21407024) and the Fujian Science and Technolgy Department (grant No. 2015J01594).

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