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Characteristics of heterotrophic nitrification and aerobic denitrification bacterium *Acinetobacter* sp. T1 and its application for pig farm wastewater treatment

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A novel heterotrophic bacterium was isolated from activated sludge of a pig farm wastewater treatment plant and identified as *Acinetobacter* sp. T1. It exhibited efficient heterotrophic nitrification and aerobic denitrification capability to utilize ammonium, nitrate or nitrite as the sole nitrogen source, and their removal rates were 12.08, 5.53 and 1.69 mg/L/h, respectively. Furthermore, the optimal conditions for the heterotrophic nitrification process were sodium citrate as the carbon source, C/N mass ratio of 10, pH of 8.5 and dissolved oxygen (DO) concentration of 5.1 mg/L. Only trace amounts of nitrate and nitrite were observed during the process. When the aerobic tank of the A^2O process of a pig farm wastewater treatment plant was inoculated with traditional activated sludge, the average removals of COD, NH_4^+ - N and TN in the effluent were 30%, 15% and 16%, respectively, which was much lower than that of inoculated with strain T1, the increase was statistically significant, indicating a great potential of strain T1 for full-scale applications.

[**Key words:** *Acinetobacter* sp. T1; Aerobic denitrification; Full scale plant; Heterotrophic nitrification; Nitrogen removal; Pig farm wastewater]

Biological nitrogen removal (BNR) processes have attracted more and more attention in recent years owing to their high efficiency, low cost and without causing secondary pollution (1,2). Conventional BNR typically starts from oxidizing ammonium (NH_4^+) to nitrate (NO_3^-) aerobically (nitrification) by autotrophic nitrifying bacteria and then reduce nitrate stepwisely to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and finally nitrogen (N_2) under anoxic condition (denitrification) by heterotrophic denitrifying bacteria (3,4). However, the conventional system nitrification/denitrification (N/DN) process tends to be time-consuming due to the slow nitrification rate and the large footage required for the aerobic and the anoxic tanks (5,6). Additionally, the denitrifiers are sensitive to oxygen and the nitrifiers are vulnerable to high loads of ammonium, and consequently, their applications for treating high-strength ammonium wastewater are limited (7). Recently, bacteria that have heterotrophic nitrification and aerobic denitrification capabilities, such as *Agrobacterium* sp., *Raoultella* sp., *Alcaligenes faecalis*, *Paracoccus versutus*, *Pseudomonas stutzeri* and *Pseudomonas tolaasii*, have been isolated and intensively studied as potential microorganisms in BNR systems (8–14). These bacteria exhibited higher growth rates than autotrophs, tolerated higher ammonium loadings, and performed N/DN simultaneously in the same reactor (13,15). These special bacteria could use various carbon sources and convert inorganic nitrogen (i.e., ammonium, nitrate, nitrite) into gaseous nitrogen products under aerobic conditions (14).

In this study, a novel bacterial strain was isolated from activated sludge of a pig farm wastewater treatment plant and identified as *Acinetobacter* sp. T1. Its heterotrophic nitrification and aerobic denitrification capabilities for using ammonia, nitrate or nitrite as the sole nitrogen source were studied. Furthermore, the impacts of different factors on ammonium removal were investigated. Finally, the nitrogen removal capacity of *Acinetobacter* sp. T1 at a full-scale pig farm wastewater treatment plant was examined.

MATERIALS AND METHODS

Microbial source Aerobic activated sludge sample was obtained from a pig farm wastewater treatment plant in Hubei, China and used to isolate the heterotrophic nitrification and aerobic denitrification bacteria. *Acinetobacter baumannii* ATCC 19606 from Shanghai Scitech Bioscientifc Co., Ltd.

Media The enrichment medium (EM) consisted of the following components: 5.0 g of sodium citrate, 2.0 g of KNO_3, 1.0 g of K_2HPO_4, 0.1 g of KH_2PO_4 and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water.

The basal medium (BM) used for strain T1 cultivation and heterotrophic nitrification study contained (per litter): 5.0 g of sodium citrate, 0.96 g of $(NH_4)_2SO_4$, 1.0 g of K_2HPO_4, 0.1 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $FeSO_4 \cdot 7H_2O$, and 2.0 mL of trace element solution. The trace element solution contained (per liter): 57.1 g of EDTA·2Na, 3.9 g of $ZnSO_4 \cdot 7H_2O$, 7.0 g of $CaCl_2 \cdot 2H_2O$, 5.1 g of $MnCl_2 \cdot 4H_2O$, 5.0 g of $FeSO_4 \cdot 7H_2O$, 1.1 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.6 g of $CuSO_4 \cdot 5H_2O$, and 1.6 g of $CoCl_2 \cdot 6H_2O$.

The Luria–Bertani (LB) agar plates contained (per liter) 10.0 g of tryptone, 5.0 g of yeast extract, 10.0 g of NaCl, and 15.0 g of agar.

The initial pH of all the media mentioned above were adjusted to 7.5.

Enrichment and isolation of bacterial strains A 10-mL activated sludge was added to 90 mL of the autoclaved EM medium in a conical flask and then incubated on a rotary shaker at 35 °C and 160 rpm to enrich for heterotrophic bacteria.

After 3 days of cultivation, 10 mL cell suspension was transferred to 90 mL fresh EM and incubated at 35 °C and 160 rpm for 3 days. Thereafter, 1 mL bacterial suspension was transferred to 100 mL fresh BM in a 250-mL flask for selective cultivation of bacterial cultures under condition of 35 °C and 160 rpm for 2 days. The above process was repeated 3 times. The enriched bacterial culture was gradient-diluted

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and the suspensions with different concentrations were spread onto LB agar plates using the dilution plate method and incubated at 35 °C 2 days. Separate colonies exhibiting different morphological features were selected and further purified. And then, pure isolates were picked and individually tested for their capability of nitrogen removal. The bacterium with the highest nitrification capability was obtained for subsequent tests.

DNA extraction, PCR amplification and 16S rDNA gene sequence analysis DNA was extracted from the T1 bacterial suspension by using the EZ Spin Column Bacterial Genomic DNA Isolation Kit (Sangon, Shanghai). 16S rDNA gene was amplified by PCR using the universal primers F27 (5′-AGAGTTTGATCMTGGCT CAG-3′) and R1492 (5′-TAC GGYTACCTTGTTACGACTT-3′). Partial sequencing of the 16S rDNA gene was performed by LC Sciences Corp. (Hangzhou, China). 16S rDNA sequence was compared with sequences in the GenBank database with online Basic Local Alignment Search Tool program (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). A phylogenetic tree was constructed using MEGA 4.0 by the neighbor-joining method.

Assessment of nitrogen removal capability of strain T1 To assess the performance of ammonium removal, 1% (v/v) of cell suspension was inoculated into BM (204.27 mg/L of NH_4^+-N) in 250-mL conical flasks. To evaluate the aerobic denitrification capability, nitrite (106.94 mg/L) or nitrate (170.17 mg/L) were used as the sole nitrogen source instead of ammonium. The flasks were incubated aerobically with constant shaking of 160 rpm at 35 °C. A medium without inoculation was used as the control. The samples were regularly collected to determine the concentration of ammonium, nitrite, nitrate and optical density at 600 nm (OD_{600}). For detection of gaseous nitrogen products during ammonium conversion, the isolate was cultivated at 35 °C in tightly-sealed flasks and aerated with pure oxygen. The gaseous nitrogen compounds from the headspace gas were analyzed by gas chromatograph (Agilent 4890, Agilent Technologies, Santa Clara, CA, USA).

Effects of different factors on heterotrophic ammonium removal The effects of the carbon source, C/N mass ratio, pH, and dissolved oxygen (DO) concentration on heterotrophic ammonium removal by strain T1 were investigated by single factor tests. Sodium citrate in the BM was substituted by sucrose (2.9 g/L), glucose (3.1 g/L), sodium acetate (6.9 g/L), and sodium potassium tartrate (7.2 g/L) to study the effects of carbon source on ammonium removal.

To evaluate the effects of pH on ammonium removal, different initial pH values (pH 6.5, 7.0, 7.5, 8.0 and 8.5) of BM were adjusted using 0.05M NaOH and/or H_2SO_4 solutions. The effects of C/N ratios were investigated using sodium citrate as the sole carbon source, with the C/N ratios of 3, 5, 10, 15, and 20 with a fixed amount of $(NH_4)_2SO_4$ (the N source) at 204.28 mg/L. The influences of DO concentrations (2.2, 3.4, 4.3, 5.1, 5.7 mg/L) were investigated at five different shaking speeds of 40, 80, 120, 160 and 200 rpm. All the experiments were conducted in triplicate, and the results were reported as mean with standard deviations.

Application of *Acinetobacter* sp. T1 for pig farm wastewater treatment The full-scale anaerobic-anoxic-aerobic (A^2O) plant is located in Huangshi, Hubei Province, China. The average flow rate was 100 m^3/d, and the hydraulic residence time for the hydrolytic acidification phase, anaerobic phase, anoxic phase, aerobic phase and lagoon were 2, 15, 1, 3, and 3 days, respectively. The DO concentrations of anaerobic, anoxic and aerobic tank were about 0.03, 0.15 and 4.8 mg/L, respectively. The full-scale system has two recycle flows: one is an internal recycle from the aerobic tank to the anoxic reactor, and the other is an external recycle from the secondary clarifier to the anaerobic tank. The internal recycle ratio was 200% and the external recycle ratio was 100%. To verify the nitrogen removal capability of the isolated strain in engineering applications, bioaugmentation was conducted in four steps as follows: (i) T1 microorganisms were co-cultivated in a 5 L reactor with a 3 L of working volume at 35 °C; (ii) The microbial consortium was dividedly transferred into 550 L of 2 tanks and high-densely cultivated for 3 periods through batch culture where new medium was supplied every period; (iii) Highly dense culture of 1 m^3 was then transferred into aeration tanks (total working volume is 300 m^3). The aeration tank contained 50 m^3 of pig farm wastewater. Augmentation was conducted under batch system for 12 days. (iv) Full-scale pig farm wastewater treatment processes were operated continuously at a flow rate of 100 m^3/d. The pH and temperature of the aerobic tank were about pH 7.98–8.15 and 30 °C. Samples were taken from the tank for measurements of ammonia, COD and TN levels in the influent and the effluent, the data presented in this study were obtained from steady-state 28 days after bioaugmentation.

Analytical methods OD_{600} was measured by a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). DO and pH were measured with a DO meter (JPBJ-608, Leici, Shanghai, China) and a pH meter (ZD-2, Leici). Culture samples were centrifuged at 10,000 rpm and then filtered through a 0.45 μm membrane filter. The filtrate was used for chemical analysis. The concentrations of ammonium, nitrate and nitrite were determined according to standard methods, i.e., ammonium by the method of Nessler's reagent spectrophotometry, nitrate by phenol disulfonic acid photometry method, nitrite by the *N*-(1-naphthalene)-diaminnoethane photometry method. Gas samples were analyzed following the method by Zhao et al. (16). Antibiotic susceptibility test was performed by Kirby–Bauer method as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines (17).

RESULTS AND DISCUSSION

Identification and characterization of strain T1 The colony of T1 on the agar plate was off-white, transparent, round, salient with a micro serrated edge. Strain T1 was gram-negative, nonspore-forming and in the shape of short rods. The BLAST results indicated that strain T1 was closely related to members of genus *Acinetobacter*, showing the highest similarity to *A. baumannii* strain ATCC 19606 (Fig. 1). A phylogenetic tree was constructed according to the neighbor-joining algorithm. The tree topology, supported by high bootstrap values, clearly showed that strain T1 was related to members of genus *Acinetobacter*. Furthermore, antibiotic susceptibility test of strain T1 and *A. baumannii* ATCC 19606 were compared (Table S1). The antibiotic susceptibility test showed that strain T1 was susceptibility to the antibiotics such as florfenicol, lincolmensin, cephalothin, gentamicin, amikacin, enrofloxacin, but was intermediate resistant to imipenem. While *A. baumannii* ATCC 19606 with different degrees resistant to florfenicol, lincolmensin, cephalothin, gentamicin, amikacin, enrofloxacin and imipenem except for colistin B, which could be recognized as multidrug-resistant strain, these results are consistent with the findings of the previous study (18). Therefore, we can conclude that strain T1 is

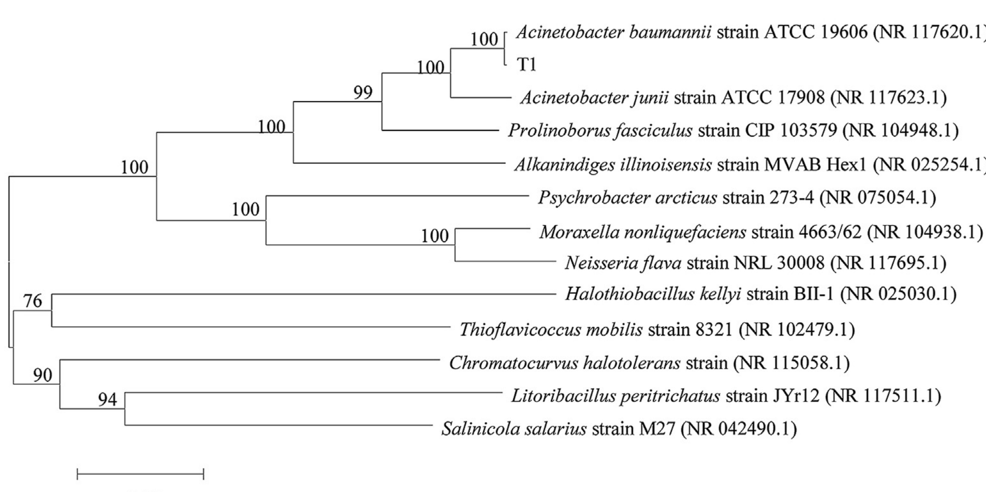

FIG. 1. Neighbor-joining phylogenetic tree based on 16S rDNA gene sequence of strain T1 and other reference sequences.

different from *A. baumannii* ATCC 19606. Combined with the above results, the present strain was identified to be an *Acinetobacter* species. *Acinetobacter* sp. being capable of heterotrophic nitrification have been studied (19). However, *Acinetobacter* sp. is capable of simultaneous heterotrophic nitrification and aerobic denitrification is seldom reported.

Assessment of ammonium nitrogen removal capacity of strain T1 To confirm the heterotrophic nitrification by strain T1, ammonium [$(NH_4)_2SO_4$] was used as the sole nitrogen source in the BM. The ammonium nitrogen removal characteristics of strain T1 and *A. baumannii* ATCC 19606 are shown in Fig. 2. As shown, the cell grew rapidly in the log growth phase during the first 12 h of inoculation, as the OD_{600} increased to 1.135. The maximum growth rate was reached between 6 and 12 h and calculated to be 0.101 h^{-1}. Meanwhile, the ammonium oxidation was coupled to cell growth, and the ammonium concentration (204.27 mg NH_4^+-N/L initially) descended significantly during the log growth phase and the removal efficiency reached 48% within 12 h with a maximum ammonium removal rate of 12.08 mg/L/h, which is higher than that of *P.tolaasii* Y-11 (2.04 mg/L/h) (14), *K.pneumoniae* CF-S9 (4.3 mg/L/h) (15), *Cupriavidus* sp. S1 (10.43 mg/L/h) (120), *Acinetobacter* sp. Y16 (0.092 mg/L/h) (21), *Rhodococcus* sp. CPZ24 (3.4 mg/L/h) (22), *Bacillus subtilis* A1 (3.52 mg/L/h) (23), and *Bacillus methylotrophicus* strain L7 (2.15 mg/L/h) (24). As shown in Fig. 2, we can also see that ammonium concentration had no obvious change within 48 h using *A. baumannii* strain ATCC 19606 as inoculum, indicating that ATCC 19606 does not have capability of heterotrophic nitrification.

Nitrification products, including NO_3^--N and NO_2^--N were not detected when *A. baumannii* strain ATCC 19606 was used as inoculum, whereas these products were detected when T1 was used as inoculum, and the concentrations of NO_3^--N and NO_2^--N after 12 h were 7.93 and 1.42 mg/L, respectively. The results indicated that some of ammonium nitrogen has been converted to gaseous nitrogen products during nitrification by strain T1, particularly during the log growth phase. Some researcher previously reported that heterotrophic nitrification and aerobic denitrification bacteria were able to oxidize ammonium to nitrite or nitrate and simultaneously denitrified these products to N_2O and/or N_2 (16,20,21). To further elucidate the gaseous nitrogen products during aerobic ammonium oxidation by strain T1, the changes of N_2 and N_2O concentration in the headspace were monitored by using gas chromatograph (Fig. S1). The N_2 concentration increased from 1.21 to 25.6 mg/L after 48 h, whereas N_2O was not detected. The production of nitrogen gas indicated the occurrence of aerobic denitrification by strain T1. Thus, these experiments confirmed that strain T1 has the capability of heterotrophic nitrification and aerobic denitrification.

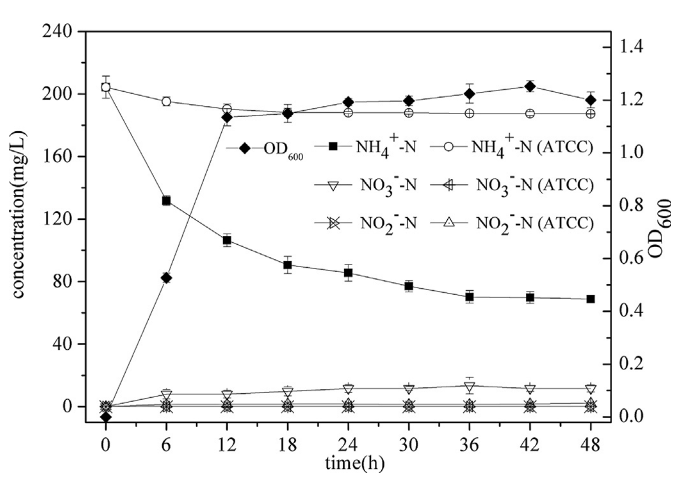

FIG. 2. The ammonium nitrogen removal characteristics of strain T1 and ATCC 19606.

Aerobic denitrification capability of strain T1 To further clarify the aerobic denitrification capability of strain T1, two nitrification intermediates, nitrate and nitrite, were separately used as the sole nitrogen source in the BM, and the results are shown in Fig. 3A and B, respectively.

In the period of 42 h, cells grew rapidly, the OD_{600} reached 1.084, but decreased to 1.059 at 48 h. The NO_3^--N concentration decreased from 170.17 to 125.92 mg/L, a 26% reduction within 12 h, and it correlated well with the cell growth rate. Up to 46% of NO_3^--N removed after 48h. The maximum removal rate of NO_3^--N was 5.53 mg/L/h, which is larger than that of *Rhodococcus* sp. CPZ24 (0.93 mg/L/h) (22) and *Enterobacter cloacae* HW-15 (2.73 mg/L/h) (25).

Meanwhile, nitrite was detected when nitrate was used as the sole nitrogen source, and the maximum concentration of nitrite was 29.77 mg/L after 36 h, and then gradually decreased to 19.52 mg/L at 48 h. However, NH_4^+-N concentration increased gradually to 2.18 mg/L at the end of incubation, which might be due to decomposition of death cells so that the ammonium contained in the cell and the organic nitrogen of death cell being converted to ammonium were released into the denitrification medium (14).

NO_2^--N has a well-documented toxicity to many species such as goldfish, anuran larvae, swiss albino mice, and even some bacteria (14). When nitrate was added as the nitrogen source, an obvious lag period was observed, the cell growth was slow at 6 h and OD_{600}

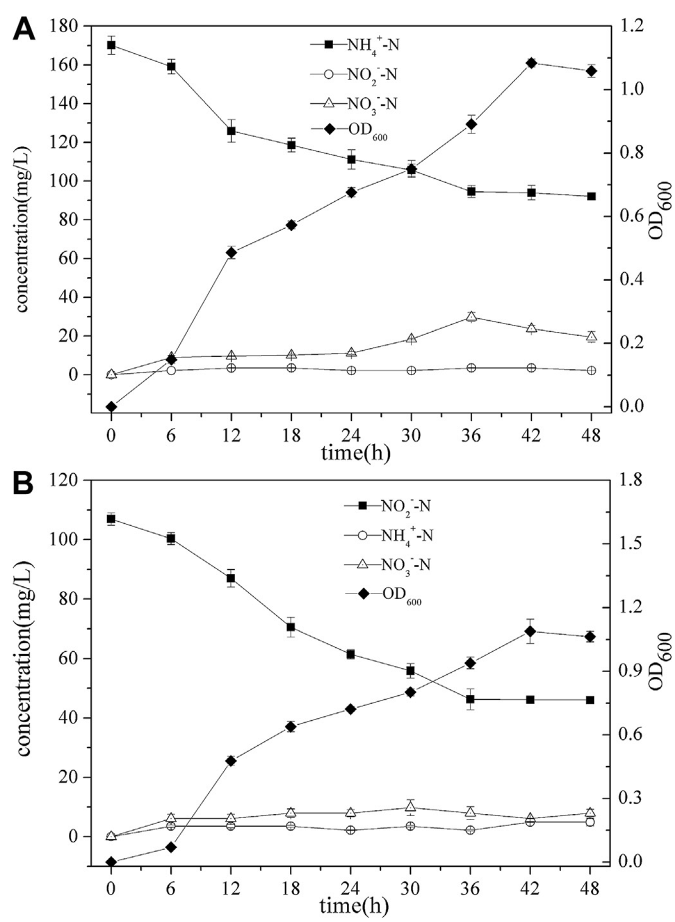

FIG. 3. The aerobic denitrification capability of T1 using (A) nitrate and (B) nitrite as sole nitrogen source.

reached only 0.07. However, after a few hours of acclimation, the OD_{600} increased rapidly from 0.07 to 1.088 between 6 h and 42 h, and then decreased to 1.063 at 48 h. Fig. 3B shows that NO_2^--N could be utilized by strain T1 under aerobic conditions, a significant decrease of nitrite concentration from 106.94 to 46.26 mg/L was observed at the end of 36 h, a 57% of removal, on the other hand, the average NO_2^--N removal rate was 1.69 mg/L/h, higher than those of other reported bacteria, such as *B. methylotrophicus* strain L7 (0.24 mg/L/h) (24) and *Pseudomonas* sp. yy7 (0.76 mg/L/h) (26).

Effects of different factors on heterotrophic ammonium removal carbon sources The effects of carbon source on cell growth and ammonium removal by strain T1 are summarized in Table S2.

It can be seen that cell growth and ammonium removal were significantly affected by the type of carbon sources. Sodium citrate appeared to be the most suitable carbon source for cell growth and ammonium removal in this study, an evidenced by the largest OD_{600} (1.30) and ammonium removal (66%) after 48 h. The plausible reason is that sodium citrate could be directly inserted into the metabolic process without modification, and it would make the medium more alkaline, which is better for nitrification (20). Accordingly, sodium citrate was employed as the carbon source in subsequent experiments.

C/N ratio Fig. 4 shows that the cell growth and ammonium removal exhibited a similar trend, and they both increased with increasing C/N mass ratio in the range of 3:1–10:1. It is mainly due to insufficient carbon supply that would impair both microbial growth and electron donor for denitrification (20,21). The maximal ammonium removal occurred at C/N mass ratio of 10:1, but further increase in C/N mass ratio yielded slight decreases in ammonium removal. These results are consistent with the findings of other denitrification bacteria, such as *Providencia rettgeri* YL (27), *A. faecalis* No. 4 (7) and *Vibrio diabolicus* SF16 (28).

Initial pH The influences of different pHs on cell growth and ammonium removal by strain T1 are shown in Fig. 5. As shown, the ammonium removal was in consistent with the cell growth, the optimal pH for ammonium removal and cell growth was 8.5, with the maximum removal efficiency and OD_{600} of 85% and 1.74, respectively. It appears that a slightly alkaline environment is favorable for the growth of strain T1. And the better ammonium removal under the slightly alkaline environment might be caused by more free ammonia available, which would be preferentially used by ammonia monooxygenase (5).

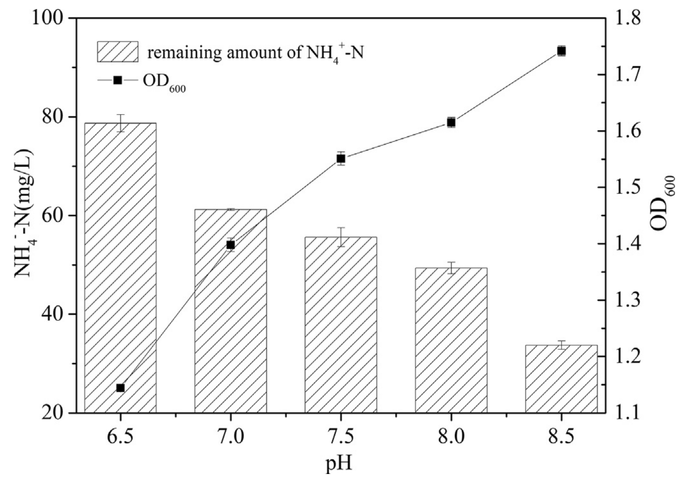

FIG. 5. Effects of initial pHs on cell growth and ammonium removal by strain T1: initial NH_4^+-N concentration of 204.27 mg/L, C/N of 10, and DO concentration of 5.1 mg/L.

Dissolved oxygen DO is one of the main process control parameters in aerobic denitrification (21). In this study, the DO concentration in the medium was adjusted by the speed of the shaker. Fig. 6 shows that higher DO concentrations significantly promoted the ammonium removal and cell growth. The removal efficiency increased with the DO concentration. The lowest ammonium removal (16%) was obtained at DO concentration of 2.2 mg/L after 48 h, compared to 50%, 76% and 83% at DO concentration of 3.4, 4.3 and 5.1, respectively. However, the ammonium removal decreased when the DO concentration exceeded 5.1 mg/L.

Application of *Acinetobacter* sp. T1 for pig farm wastewater treatment The COD, NH_4^+- N and total nitrogen (TN) concentrations in the influent and effluent of the aerobic tank inoculated with traditional activated sludge or strain T1 are shown in Fig S2. The COD, NH_4^+-N and TN concentrations in the effluent inoculated with traditional activated sludge were 630.72–654.89 mg/L, 425.14–439.08 mg/L, 541.93–560.15 mg/L, and the corresponding average removals of COD, NH_4^+-N and TN were 30%, 15% and 16%, respectively. In contrast, the COD, NH_4^+- N and TN concentrations in the effluent inoculated with strain T1 were reduced to 220.36–236.50 mg/L, 241.57–270.35 mg/L, 380.90–404.94 mg/L, which increased the average removals of COD, NH_4^+-N and TN to

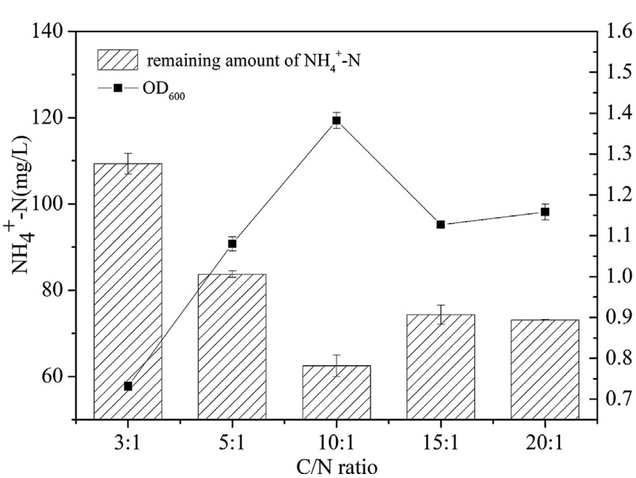

FIG. 4. Effects of C/N mass ratio on cell growth and ammonium removal by strain T1: initial NH_4^+-N concentration of 204.27 mg/L, pH of 7, and shaking speed of 160 rpm.

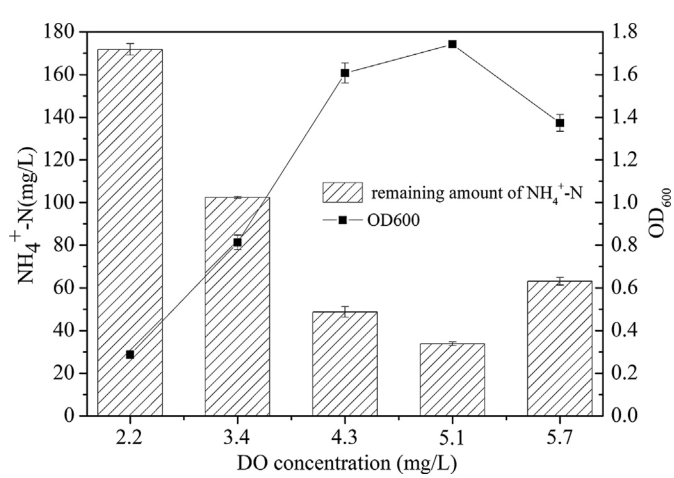

FIG. 6. Effects of DO concentrations on cell growth and ammonium removal by strain T1: initial NH_4^+-N concentration of 204.27 mg/L, C/N of 10, and pH of 8.5.

64%, 42% and 28%, respectively, and the increase was statistically significant (all $P < 0.01$). These results indicate that the use of isolated *Acinetobacter* sp. T1 would result in a significant improvement in BNR of pig farm wastewater.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.07.025.

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