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Isolation and characterization of *Acinetobacter* sp. JQ1004 and evaluation of its inhibitory kinetics by free ammonia

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ABSTRACT

A strain with high tolerance to high-strength ammonia, which grew and metabolized at an ammonia concentration of 2,000 mg/L, was isolated from activated sludge. The strain was identified as *Acinetobacter* sp. by morphological and phylogenetic 16S rDNA analyses. Based on the high removal efficiency of ammonia/nitrate and enzyme assays, the strain was confirmed to exhibit heterotrophic nitrification and aerobic denitrification abilities with limited accumulation of intermediates. The inhibitory effect of free ammonia (FA) on *Acinetobacter* sp. was evaluated in batch experiments. Three biokinetic models (Haldane, Aiba, and Yano) were used to describe the experimental data with adjusted R^2 values of 0.995, 0.981, and 0.994, respectively. The K_s and K_i values for the Haldane kinetic model were 2.997 ± 0.041 and 64.736 ± 0.023 mg/L, respectively. The maximum specific degradation rate of ammonium (q_{max}) occurred at the substrate (FA) concentration of S_{max} = 13.94 mg/L with a value of 3.946 ± 0.021 g/(g DCW d)$^{-1}$. The specific degradation rate of ammonia decreased by about 50% (IC_{50}) at an FA concentration of 76.31 mg NH_3–N/L, calculated using a logistic dose-response model. The real-time polymerase chain reaction results revealed that FA inhibited nitrogen removal and growth of the bacterial strain during heterotrophic nitrification by inhibiting expression of the *amoA* gene.

Keywords: amoA gene; Biokinetics; Free ammonia inhibition; Heterotrophic nitrification and aerobic denitrification; Real-time PCR

1. Introduction

A large volume of high-nitrogen organic wastewater is being discharged into the environment with the rapid development of the economy and industry, which poses a serious threat to the water environment. The ammonium concentrations in these ammonium-rich wastewaters are even high over 1,000 mg/L, such as leachate, petrochemical effluent, and coking wastewater [1]. Strong inhibition of free ammonia (FA; NH_3) easily occurs under such high ammonium concentrations. Until now, the remedy for high-strength ammonium wastewaters was usually dependent on traditional biological treatments, such as partial nitritation [2], simultaneous nitrification and denitrification [3], and other combined processes. However, most of these traditional biological processes depend on autotrophic aerobic/anaerobic bacteria (autotrophic nitrifying bacteria and anaerobic AOB (ANAMMOX) bacteria), which have a longer generation time and are more vulnerable to inhibitory substrates (such as FA) and other environmental factors. Therefore, resisting or weakening the inhibition of FA has become a focus for treating high-ammonium wastewater.

In contrast to autotrophic nitrifiers, heterotrophic nitrifying bacteria have a shorter generation time, higher cell yield, and stronger environmental adaptability. Until now, numerous heterotrophic nitrification-aerobic denitrifying bacteria, such as *Enterobacter cloacae* CF-S27 [4],

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Pseudomonas tolaasii Y-11 [5], *Acinetobacter* sp. Y16 [6], and *Bacillus methylotrophicus* strain L7 [7], have been isolated from nature. As reported previously, most of these heterotrophic nitrifiers can be potentially used to treat high-strength ammonium-polluted water. The heterotrophic nitrifier *Alcaligenes faecalis* strain No. 4 [8] has been employed to treat piggery wastewater containing high initial concentrations of ammonium ranging from 1,050 to 1,950 mg/L under aerobic conditions; about 2,000 mg/L NH_4^+–N and 12,000 COD_{cr} mg/L were finally removed. Phatak et al. [9] reported applying *Thiosphaera pantotropha* in sequence batch reactors by bioaugmentation, which achieved a nitrogen removal rate of about 50%–60%, at the highest nitrogen concentration of 1,000 mg/L.

FA, produced at high ammonium concentrations and a high pH, is a more common and important substrate inhibitor in high-strength ammonia wastewater. The inhibitory effect of FA on autotrophic nitrifiers has been widely studied over the recent decades. Several findings related to inhibition of FA on ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are listed in Table 4. Anthonisen et al. [10] demonstrated that *Nitrobacter* is inhibited by FA at about 0.1–1.0 mg NH_3/L, whereas the threshold of *Nitrosomonas* is about 10–150 mg NH_3/L. Li et al. [11] indicated that the Haldane model described the inhibitory effect of FA on the nitritation process and obtained the adjusted R^2 value of 0.982. Nevertheless, studies on the growth and degradation kinetic parameters of heterotrophic nitrifiers under different initial FA concentrations are rarely reported. In our study, strain JQ1004, which was isolated from activated sludge, grew and metabolized at the highest ammonia concentration of 2,000 mg/L. Controlled batch experiments were conducted under aerobic conditions to examine the growth pattern and biokinetic parameters for degradation by the bacterial strain under varying initial FA concentrations. This study provides a theoretical basis for using strains to treat high-strength ammonium wastewater.

2. Materials and methods

2.1. Isolation and identification of the strain

JQ1004 was originally isolated from activated sludge in a pilot-scale rotating biological contactor located at the Gaobeidian sewage treatment plant (Beijing, China). The sludge samples were thoroughly suspended in sterilized water, diluted stepwise, and ultimately streaked on enrichment agar media. The plates were incubated at 30°C for 2–3 d. Single colonies were picked up and individually evaluated for nitrogen degradation rate in nitrification media (NMs).

Strain JQ1004 was characterized and identified by morphological and phylogenetic analyses. The morphological characteristics were observed by scanning electron microscopy (Hitachi S-4300; Tokyo, Japan). The Ezup Column Bacterial Genomic DNA Isolation Kit (Sangon, Shanghai, China) was used to extract DNA. 16S rDNA was amplified by polymerase chain reaction (PCR) using the universal bacterial primers 27 f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') [12]. The reaction conditions consisted of the following steps: 95°C for 1.5 min; 5 cycles at 95°C for 0.5 min, annealing at 60°C for 0.5 min, and 72°C for 2 min; 5 cycles at 95°C for 0.5 min, annealing at 55°C for 0.5 min, and 72°C for 2 min; 15 cycles of 95°C for 0.5 min, annealing at 50°C for 0.5 min, and 72°C for 2 min; and a final extension at 60°C for 10 min. After sequencing, the BLAST online program was applied to align the sequence data with published sequences from the NCBI database. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 5.0 software based on an algorithm with 1,000 bootstrap replicates.

2.2. Medium

The NM (per liter) consisted of 0.47 g $(NH_4)_2SO_4$, 4.219 g $(CH_2COONa)_2{\cdot}6H_2O$, and 50 mL of trace element solution. The trace element solution (per liter) consisted of 6.55 g $K_2HPO_4{\cdot}3H_2O$, 2.5 g $MgSO_4{\cdot}7H_2O$, 2.5 g NaCl, 0.038 g $MnSO_4{\cdot}H_2O$, and 0.05 g $FeSO_4{\cdot}7H_2O$. The denitrification medium (DM; per liter) was composed of 0.72 g KNO_3, 4.219 g $(CH_2COONa)_2{\cdot}6H_2O$, 7.9 g $Na_2HPO_4{\cdot}7H_2O$, 1.5 g KH_2PO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, and 2 mL trace element solution. The trace element solution (per liter) contained 50.0 g Na_2EDTA, 2.2 g $ZnSO_4{\cdot}7H_2O$, 5.5 g $CaCl_2$, 5.06 g $MnCl_2{\cdot}4H_2O$, 5.0 g $FeSO_4$, 1.57 g $CuSO_4{\cdot}5H_2O$, and 1.60 g $CoCl_2{\cdot}6H_2O$ [12]. The LB enrichment medium (per liter) was composed of 10 g peptone, 5 g yeast extract, and 10 g NaCl. Na_2HPO_4 and NaH_2PO_4 buffer solutions were used to adjust pH. Solid medium was added with 1.5%–2% agar. All media were sterilized in an autoclave for 15 min at 121°C before use.

2.3. Assessment of heterotrophic nitrification and aerobic denitrification

The cells were collected by centrifugation (8,000× g, 10 min, 4°C) after a 12-h culture under the following conditions: 30°C, pH 7.2, and 160 rpm in NM. The cells were suspended in phosphate buffer (pH 7.4) three times. Furthermore, the homogeneous bacterial suspension (optical density at 600 nm (OD_{600}) = 0.5–0.6) was inoculated into 100 mL sterile NM and DM in 250-mL Erlenmeyer flasks with an inoculum size of 2% (v/v) and incubated aerobically at 30°C and 160 rpm. All experiments used control groups without inoculum and were performed in triplicate. Samples were taken periodically to determine the OD_{600}, pH, and concentrations of NH_4^+–N, NO_3^-–N, NO_2^-–N, and total nitrogen (TN).

2.4. Enzyme assays

To elucidate the possible degradation pathway of nitrogen by strain JQ1004, the key enzymes [ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO), nitrate reductase (NR), and nitrite reductase (NIR)] involved in heterotrophic nitrification-aerobic denitrification were determined and analyzed. Cells were harvested by centrifugation (10,000 rpm, 10 min, 4°C) after an overnight incubation for 12 h. The pellets were suspended in phosphate buffer three times and then sonicated with lysis buffer to determine enzyme activities. AMO was detected by spectrophotometry using NADH as the electron acceptor, while HAO was examined by spectrophotometry using

cytochrome *c* as the electron acceptor [13,14]. Moreover, NR activity was evaluated by reducing nitrate in the presence of the crude enzyme in a reaction mixture, whereas NIR was measured by monitoring the reduction of nitrite in the presence of the crude enzyme [15].

2.5. Inhibitory kinetics of FA during aerobic growth of the bacterial strain

To assess the inhibitory effect of FA on cell growth and nitrogen removal efficiency, batch experiments were conducted in flasks using NM (per liter) supplied with different quantities of ammonium sulfate to control the initial FA concentration. In the batch experiments, NH_4^+–N was initially added at varying concentrations of 50, 100, 300, 500, 700, 1,000, 1,500, 2,000, or 2,500 mg/L (the corresponding FA concentrations of 4.535, 9.07, 27.21, 45.35, 63.49, 90.7, 136.05, 181.4, or 226.75 mg/L), with a C/N ratio of 7.5 and pH = 8.0. The inoculum was added to 100 mL sterile media in 250 mL flasks with an inoculum size of 2% (v/v) and incubated aerobically at 30°C and 160 rpm. The cultures were periodically sampled during the 48 h treatment to determine OD_{600} and the concentration of NH_4^+–N. The FA concentration was computed as follows [10]:

$$\mathrm{FA}=\frac{17}{14}\times\frac{\rho_{\left[\mathrm{NH_4^+-N}\right]}\times 10^{\mathrm{pH}}}{e^{\left[\frac{6{,}344}{(273+T)}\right]}+10^{\mathrm{pH}}} \tag{1}$$

where ρ[NH_4^+–N] is the concentration of NH_4^+–N (mg/L) and T is the temperature (°C). The specific nitrogen removal rate (q_{FA}) of the isolate for each experiment was calculated as follows [16]:

$$q_{\mathrm{FA}}=-\frac{\left(\left(\mathrm{NH_4^+}\right)_i-\frac{\left(\mathrm{NH_4^+}\right)_f}{\left(t_0-t\right)}\right)}{X_0} \tag{2}$$

where X is the final cell concentration of the logarithmic growth, X_0 is the initial cell concentration of logarithmic growth, t is the final time, t_0 is the initial time, $(NH_4^+)_i$ is the initial ammonia concentration of logarithmic growth, and $(NH_4^+)_f$ is the final ammonia concentration of logarithmic growth. The specific growth rate (μ, h^{-1}) of the isolate was estimated as follows [17]:

$$\left(\frac{dX}{dt}\right)=\mu X-K_dX \tag{3}$$

where X is cell concentration (mg/L) and K_d is the endogenous attenuation coefficient (h^{-1}). K_d can be disregarded during the logarithmic growth period. Thus, the equation was changed to

$$\mu=\frac{1}{X}\times\frac{dX}{dt}=\frac{d\ln X}{dt}=\frac{\ln X-\ln X_0}{t-t_0} \tag{4}$$

The degree of inhibition was calculated as follows:

$$\mathrm{Inhibition},\%=\frac{q_{\max}-q_{\mathrm{FA}}}{q_{\max}} \tag{5}$$

where q_{max} is the maximum growth rate of degrading different concentrations of NH_4^+–N and q_{FA} is the specific growth rate for each experiment.

To reveal the dose-response relationship between FA and nitrogen degradation of the bacterial strain, the raw data were fitted to a four-parameter logistic model [18] based on a sigmoidal dose-response curve. The inhibitory degree of FA (IC_{50}) was calculated in OriginPro 8.0 (Origin Software Inc., North Hampton, MA, USA).

2.6. Expression of the amoA gene by real-time PCR

To further understand the inhibitory mechanism of FA on the bacterial strain, expression of the *amoA* gene, which is involved in heterotrophic nitrification, was analyzed by real-time PCR. Cells in NM with different initial FA concentrations were collected by centrifugation (8,000 g, 10 min, 4°C) and washed three times in phosphate buffer (pH 7.4). Total mRNA was obtained from the bacterial suspensions using the Trizol method [19]. First-strand cDNA was synthesized using the kit according to the manufacturer's instructions. RT-PCR was carried out using SYBR Green master mix (Roche, Basel, Switzerland) to detect the different transcript levels of the *amoA* gene during the nitrogen removal process by strain JQ1004 under different initial FA concentrations. The DNA and protein data from *Acinetobacter schindleri*, *Acinetobacter pittii*, *Acinetobacter towneri*, and *Acinetobacter gyllenbergii* published in the GenBank database were applied to design the primers for the *amoA* gene of strain JQ1004. The relative expression levels of the *amoA* gene in the different experimental groups were compared and analyzed using 16S rRNA as the reference gene. The 16S rRNA primers used for RT-PCR were 16S-1F (5′-GCTAGGTAGCAACCCTTT GTAC-3′) and 16S-2R (5′-CAGCTCGTGTCGTGAGATGT-3′). Another pair of primers for the *amoA* gene was amoA-1F (5′-AAGTCGTTGCTGCCCATAG-3′) and amoA-2R (5′-TACCC ACAAACAGAGCCAGAC-3′). The reaction conditions for the PCR were as follows: 3 min of denaturation at 95°C, 45 cycles of 5 s at 95°C, 10 s at 55°C, and 15 s at 72°C, 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. The relative transcript levels of the *amoA* gene in the different experimental groups were analyzed by the comparative threshold cycle method ($2^{-\Delta\Delta CT}$ method) [20]. The experiments were all carried out with three replicates under the same experimental conditions.

2.7. Analytical methods

The concentrations of NH_4^+–N, NO_2^-–N, NO_3^-–N, and COD_{cr} were analyzed in accordance with the standard methods [21]. TN was estimated by alkaline persulfate oxidation with a UV spectrophotometer. Temperature, dissolved oxygen, and pH were determined using a WTW/Multi 3420 multiparameter device. Cell density of the strain was measured using a spectrophotometer at a wavelength of 600 nm. The biomass concentrations were determined based on the dry weight

of the strain [22]. The figures and dynamic model fitting in the study were completed using the Excel 2016 program and OriginPro 8.0 software.

3. Results and discussion

3.1. Identification of JQ1004

The isolated colony was off-white, round, and had a smooth surface with a neat edge on the agar plate (Fig. 1(a)). Bacterial strain JQ1004 appeared as short rods measuring approximately 0.8–0.9 μm under a scanning electron microscope (Fig. 1(b)). The 16S rDNA gene sequence of the isolate obtained by PCR was a continuous stretch of 1,400 bp, which was deposited in the GenBank database (Accession number MF033517.1). The homology sequence analysis using BLAST in GenBank indicated that strain JQ1004 was more similar (98% similarity) to *Acinetobacter* sp. An17. A neighbor-joining phylogenetic tree was constructed based on the 16S rDNA gene sequence of the strain, other phylogenetically related strains, and other reported strains with heterotrophic nitrification and aerobic denitrification capabilities (Fig. 1(c)).

3.2. Heterotrophic nitrification and aerobic denitrification abilities of strain JQ1004

The data in Fig. 2(a) present the heterotrophic nitrification capability of bacterial strain JQ1004 under aerobic conditions. After 3 h lag period, the bacteria began to grow, and the OD_{600} value increased rapidly. NH_4^+–N decreased markedly from 101.19 to 2.77 mg/L, and the corresponding

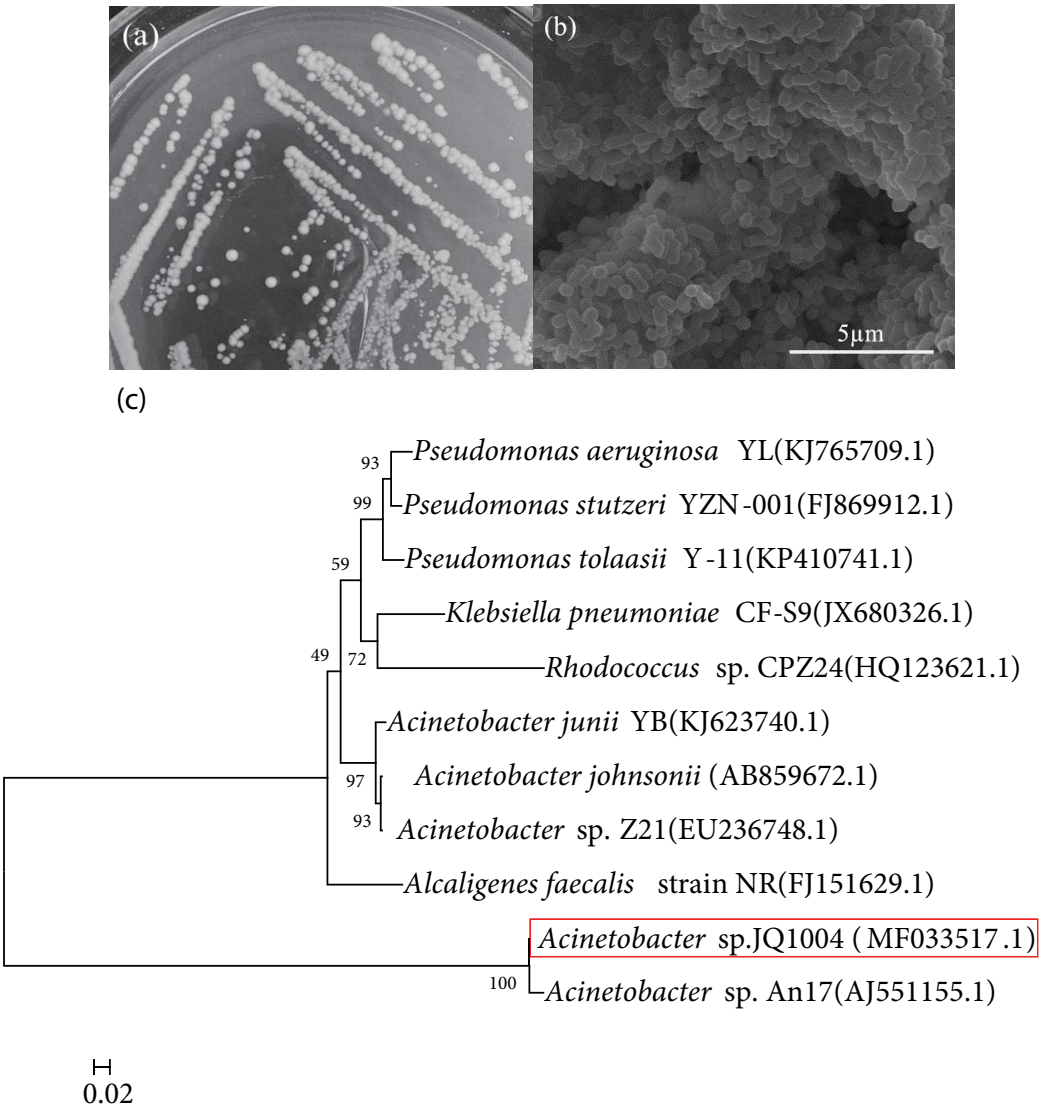

Fig. 1. (a) The colonial morphology of strain JQ1004 in the plate, (b) the characteristics of strain JQ1004 observed under scanning electron microscopy (×20,000), and (c) the neighbor-joining phylogenetic tree based on 16S rDNA gene sequences showed the position of strain JQ1004 (GenBank ID: MF033517.1) among its closely related organisms and other strains with heterotrophic nitrification and aerobic denitrification capabilities.

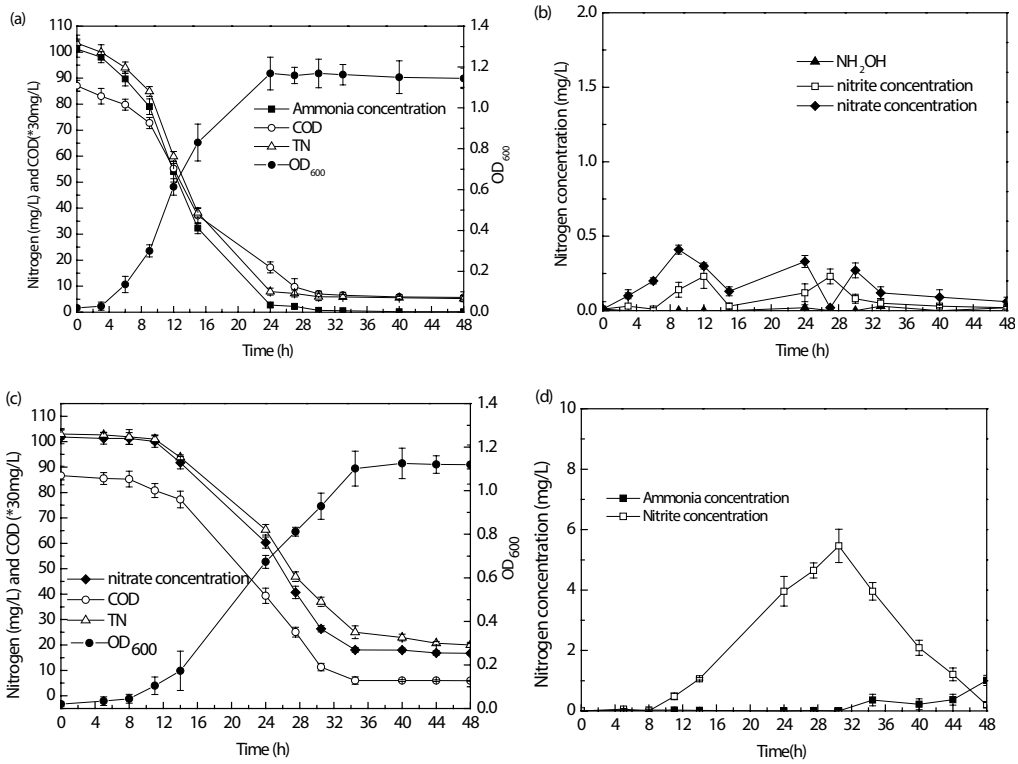

Fig. 2. Nitrogen removal characteristic of the strain JQ1004. (a) and (b): Ammonium was used as sole nitrogen source; (c) and (d): nitrate was used as sole nitrogen source. (Data are the means for three independent experiments and presented as the means ± standard error.)

removal rate was 4.10 mg/(L h) over 24 h. About 92% of TN was removed during the 24 h incubation, indicating that part of the ammonium nitrogen was converted to gaseous nitrogen during the nitrification process. As shown in Fig. 2(b), no remarkable accumulation of intermediates and only slight concentrations of NO_2^-–N and NO_3^-–N could be tracked below 0.5 mg/L. The maximum specific growth rate from Eq. (4) was estimated to be 0.386 h^{-1}, which was much higher than the rate of the autotrophic nitrifier *Nitrosomonas europaea* (0.03–0.05 h^{-1}) [23]. The specific growth rate of strain JQ1004 was comparable with those of reported heterotrophic nitrifiers, such as *Acinetobacter junii* YB (0.24–0.31 h^{-1}) [12], *Thiosphaera pantotropha* (0.28–0.45 h^{-1}) [24], and *Alcaligenes faecalis* No. 4 (0.2 h^{-1}) [25]. In addition, the COD_{cr} concentration decreased rapidly with bacterial growth, and approximately 80.23% of the initial COD_{cr} was removed within 24 h. These results demonstrate that *Acinetobacter* sp. JQ1004 utilizes sodium succinate as an organic carbon source for heterotrophic growth. In contrast to autotrophic nitrification, ammonium removal and cellular growth were significantly promoted by the organic carbon of heterotrophic nitrification [26,27]. The utilization of organic matter and degradation of NH_4^+–N, which occurred simultaneously, demonstrated the heterotrophic nitrification ability of the strain.

DMs were applied to estimate the aerobic denitrification ability of strain JQ1004. Fig. 3(c) depicts the growth and nitrate reduction of strain JQ1004 in a 250-mL flask under aerobic conditions. After a lag period of 5 h, the amount of nitrate decreased by the isolate was dramatic, and

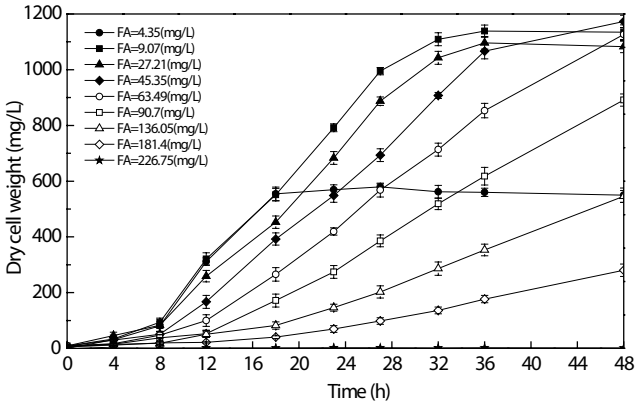

Fig. 3. Growth pattern of *Acinetobacter* sp. JQ1004 on different initial concentrations of FA. (Data are the means for three independent experiments and presented as the means ± standard error.)

approximately 82.28% of the nitrate was removed in 34.5 h under aerobic conditions, which was an average removal rate of 2.43 mg/(L h). The OD_{600} reached 1.10 at 34.5 h, and the corresponding TN and COD_{cr} removal rates were 75.71% and 93.05%, respectively. Simultaneously, the concentration of nitrite, usually referred to as an intermediate of aerobic denitrification [13,28], increased to 5.46 mg/L as the peak at 30.5 h and then decreased gradually. The maximum specific growth rate of the isolate with nitrate as the sole nitrogen

was 0.283 h^{-1}, which was slower than that of the strain using ammonium. This finding indicates that ammonium was more suitable as a nitrogen source. Moreover, in contrast to that of *Pseudomonas stutzeri* YG-24 in the study by Chune Li [29], removal efficiency of the two different nitrogen compounds was in the order: NH_4^+–N > NO_3^-–N, which might be attributable to their diverse metabolic pathways.

3.3. Enzyme activities

The activities of four key enzymes related to controlling nitrogen utilization for cell growth during the heterotrophic nitrification-aerobic denitrification process were estimated to further investigate the metabolic pathways of the strain. As shown in Table 1, the specific activities of the four functional enzymes, i.e., AMO, HAO, NR, and NIR, were 0.0216 ± 0.0081, 0.0469 ± 0.0031, 0.0237 ± 0.0015, and 0.2538 ± 0.0047 (U/mg protein), respectively. The order of the enzyme activities was inconsistent with that of *Paracoccus versutus* LYM [13] in which NR exhibited the highest activity (0.4677 U/mg protein). Based on this result, all four enzymes were successfully expressed in the JQ1004 isolate, indicating that the heterotrophic nitrification pathway was as follows: NH_4^+–N→NH_2OH→NO_2^-–N→NO_3^-–N. The high activities of HAO, NR, and NIR also explained the limited accumulation of intermediate products (nitrate and nitrite) evaluated in our study.

3.4. Effect of FA on aerobic growth and ammonia oxidation of the strain

FA reportedly inhibits autotrophic nitrification [10], and the tolerance of the heterotrophic strain JQ1004 to FA was discussed in the present study. This study indicated that the strain grew and metabolized within a broad range of ammonia concentrations from 0 to 2,000 mg/L (corresponding to FA concentrations of 0–181.40 mg/L). The strain *Pseudomonas fluorescens* WSW-1001 reportedly grows and degrades ammonia at initial ammonia concentrations of 5–1,000 mg/L [30] and *Bacillus subtilis* A1 [31] removes 36.3%, 19.5%, 6.5%, and 2.3% ammonium at the initial concentrations of 105.58, 257.23, 536.21, and 1,014.17 mg/L, respectively. The pattern of biomass accumulation was apparently related to the FA concentration (Fig. 3). Large amounts of biomass were accumulated at lower FA concentrations but decreased steadily with increasing concentration. The growth curve showed a change in the lag time of bacterial growth. The lag phase was observed within 4 h at low FA concentrations of 4.54 and 9.07 mg/L, whereas the lag phase was extended to the end of 24 h at an FA concentration of 90.7 mg/L. The strain required more time to acclimatize to the environment at higher FA concentrations which was more toxic than lower concentrations. As shown in Table 2, the maximum specific ammonium degradation rate occurred at 9.07 mg/L with 2.743 g/(g DCW d)$^{-1}$, which was higher than that of *Rhodobacter sphaeroides* ADZ101 [1 × 10^{-3} g/(g DCW d)$^{-1}$] with an ammonia concentration of 170 mg N/L [32]. High FA concentrations inhibited cell growth and the ability to degrade ammonia. As described in Fig. 4, the bacterial strain exhibited a stronger ability to remove nitrogen at low FA concentrations (<27.21 mg/L). For example, the strain completely degraded ammonia in 48 h at FA concentrations of 4.54 and 9.07 mg/L, and the corresponding TN removal rates were 96.2% and 96.11%, respectively. Subsequently, the ammonium and TN removal efficiency markedly decreased with an increase in FA concentration from 27.21 to 181.4 mg/L. At FA concentrations of 27.21 and 45.35 mg/L, 57.96% and 33.15% of the ammonium was removed within 48 h, and the corresponding TN removal rates were 55.30% and 30.42%, respectively. As the FA concentrations increased to 63.49 and 90.70 mg/L, 22.83% and 15.08% of the ammonia was degraded, respectively, after a 48-h culture. The percentages decreased to 9.04% and 3.86% when 136.05 and 181.4 mg/L of FA concentrations, respectively, were used. The same trend in the FA effect has been confirmed in autotrophic bacteria, such as autotrophic nitrifiers [33] and ANAMMOX bacteria [18]. This result might be attributable to the direct inhibitory effect of FA on AMO, an enzyme related to electron transport and proton translocation. Previous studies have indicated that a lower concentration of FA positively stimulates AMO activity [34], whereas an excessive FA concentration inhibits bacterial strains.

Table 1
Specific activities for key enzymes of strain JQ1004

Enzymes	Activity of enzymes (U/mg proteins)
Ammonia monooxygenase (AMO)	0.0216 ± 0.0081
Hydroxylamine oxidoreductase (HAO)	0.0469 ± 0.0031
Nitrate reductase (NR)	0.0237 ± 0.0015
Nitrite reductase (NIR)	0.2538 ± 0.0047

3.5. Inhibition kinetic characteristics of FA on ammonia oxidation

Three substrate inhibition models (Haldane, Aiba, and Yano) were used to describe the inhibitory effect of FA on the nitrogen degradation activities of strain JQ1004 in Origin8.0 software. The results of fitting these three kinetic models were evaluated by three statistical parameters, such as residual sum of squares (RSS), root-mean-square error (RMSE), and the adjusted correlation coefficient. As depicted in Table 3 and Fig. 5, the Haldane, Yano, and Aiba models predicted the experimental data fairly accurately, obtaining adjusted R^2 values of 0.995 > 0.994 > 0.981, respectively. The RMSE values of the Haldane, Yano, and Aiba models were 0.05927 < 0.06493 < 0.11792, while the order of the RSS values from low to high was Haldane = Yano < Aiba. Liu et al. [35] stated that the closer the adjusted R^2 values approximates 1 or the RMSE and RSS approximate 0, the closer the experimental value is to the predicted value. Thus, the Haldane model was employed for further discussion owing to its mathematical simplicity and wide applicability in previous literature. The maximum specific degradation rate of 3.946 ± 0.021 g/(g DCW d)$^{-1}$ (Table 3) was observed at $S_{\max} = \sqrt{K_s K_i} = \sqrt{2.997 \times 64.74} = 13.94 \text{mg / L}$. Subsequently, the q_{FA} value decreased as the FA concentration increased once the FA concentration exceeded 13.94 mg NH_3–N/L. As presented in Table 4, *Nitrospira* strain ND1 was initially inhibited by a low concentration of FA (0.85 mg NH_3/L), and

Table 2
Specific ammonium degradation rate for each initial free ammonia concentration

Initial FA concentration (mg/L)	Initial ammonia concentration (mg/L)	Initial			Final			q_{FA} [g/(g DCW d)$^{-1}$]
		Incubation time t_0 (h)	Ammonia concentration S_0 (mg/L)	Cell concentration X_0 (mg/L)	t (h)	Ammonia concentration S (mg/L)	Cell concentration X (mg/L)	
4.54	50	4th	48.22 ± 0.45	31.29 ± 4.11	18th	7.21 ± 1.01	311.02 ± 2.13	2.247 ± 0.021
9.07	100	4th	97.35 ± 0.37	34.01 ± 3.89	27th	7.92 ± 1.22	994.93 ± 1.98	2.743 ± 0.018
27.21	300	4th	296.11 ± 1.19	45.88 ± 2.19	32nd	161.19 ± 3.19	1,042.74 ± 3.02	2.520 ± 0.032
45.35	500	8th	492.73 ± 2.09	50.22 ± 1.21	36th	358.84 ± 4.01	1,066.57 ± 2.11	2.285 ± 0.017
63.49	700	8th	688.57 ± 5.11	47.25 ± 1.89	48th	540.17 ± 3.29	1,126.65 ± 4.73	1.784 ± 0.011
90.7	1,000	12nd	980.65 ± 3.19	52.70 ± 2.91	48th	849.23 ± 4.10	891.12 ± 4.19	1.662 ± 0.039
136.05	1,500	12nd	1,488.39 ± 4.89	50.86 ± 1.79	72nd	1,333.56 ± 4.27	1,031.35 ± 5.01	1.218 ± 0.023
181.4	2,000	18th	1,980.22 ± 3.94	40.27 ± 3.09	72nd	1,881.73 ± 5.01	612.26 ± 3.12	1.087 ± 0.014
226.75	2,500	/	/	/	/	/	/	/

Temperature is 30°C, pH is 8.0.

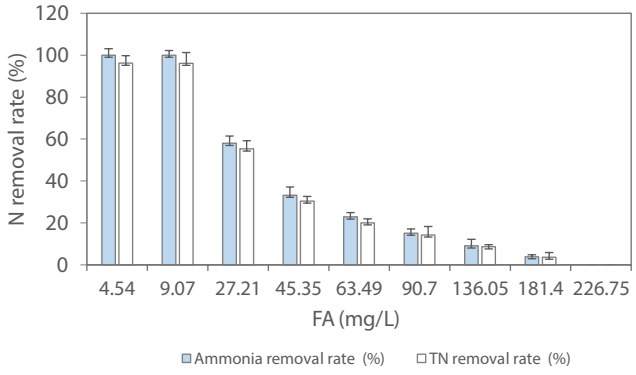

Fig. 4. Effect of varying initial free ammonia on NH_4^+–N removal rate. (Data are the means for three independent experiments and presented as the means ± standard error.)

the inhibitory threshold of the *Nitrospira japonica* strain NJ1 is 4.3 mg NH_3/L [36]. Vadivelu et al. [37] revealed no inhibitory effect on either the catabolic or anabolic processes of a *Nitrosomonas* culture when the concentration of FA was 16 mg/L. However, the specific removal rate of ammonia by strain JQ1004 compared with autotrophic nitrifiers was still up to 1.087 g/(g DCW d)$^{-1}$ at an FA concentration of 181.4 mg/L and was completely suppressed at 226.75 mg/L, the highest concentration used in our study (Table 2). The apparent maximum specific degradation rate (q_{ap}) was calculated using the formula $q_{ap} = \frac{q_{max}}{1+2\sqrt{\frac{K_s}{K_i}}} = \frac{3.946}{1+2\sqrt{\frac{2.997}{64.74}}} = 2.758 \text{g}/\left(\text{gDCW d}\right)^{-1}$, which was below the q_{max} of 3.946 g/(g DCW d)$^{-1}$ as a result of the inhibitory effect of FA. The inhibition coefficient (K_i) expresses the sensitivity of the strain to environmental inhibitors. A smaller K_i value indicates that the culture

Table 3
The values of parameters for various inhibition kinetic models

Model	Equation	q_{max} [g/(g DCW d)$^{-1}$]	K_s (mg/L)	K_i (mg/L)	K (mg/L)	Adj. R-square	RMSE	RSS	Degrees of freedom
Haldane	$\mu = \frac{\mu_{max} S_0}{K_s + S_0 + \left(\frac{S_0^2}{K_i}\right)}$	3.946 ± 0.021	2.997 ± 0.041	64.736 ± 0.023	/	0.995	0.05927	0.02108	6
Aiba	$\mu = \frac{\mu_{max} S_0}{K_s + S_0} \exp\left(\frac{-S_0}{K_s}\right)$	3.171 ± 0.030	1.423 ± 0.025	149.111 ± 0.056	/	0.981	0.11792	0.08343	6
Yano	$\mu = \frac{\mu_{max} S_0}{K_s + S_0 + \left(\frac{S_0^2}{K_i}\right)\left[1+\left(\frac{S_0}{K}\right)\right]}$	3.946 ± 0.038	2.997 ± 0.011	64.736 ± 0.019	9.27E + 23	0.994	0.06493	0.02108	5

was more sensitive to substrate inhibition. In the present study, the K_i value of the *Acinetobacter* sp. JQ1004 strain was 64.736 mg/L. This value was higher than the values for *Nitrospira* strain ND1 (31 ± 2.0 mg/L) and *Nitrospira japonica* strain NJ1 (42 ± 6.1 mg/L) [36] for pure culture (Table 3) and less than that of *Nitrosomonas* cultured sludge (376 ± 45 mg/L) [38]. Thus, a comparison of the inhibitory constants demonstrated that *Acinetobacter* sp. JQ1004 was more tolerant to FA than *Nitrospira* strain ND1 and *Nitrospira japonica* strain NJ1 (NOB); however, it was more sensitive than *Nitrosomonas* cultured sludge (AOB). Additionally, Fig. 6 shows that the four-parameter logistic model efficiently simulated the inhibitory effect of FA on strain JQ1004 with an adjusted correlation coefficient (R^2) of 0.991. The concentration causing 50% inhibition (IC_{50}) is a commonly used tool for measuring the degree of toxicity. According to the results of fitting, the specific degradation rate of ammonium by the isolate was reduced by 50% (IC_{50}) at an FA concentration of 76.31 mg NH_3–N/L, which directly indicated the high tolerance of strain JQ1004 to FA.

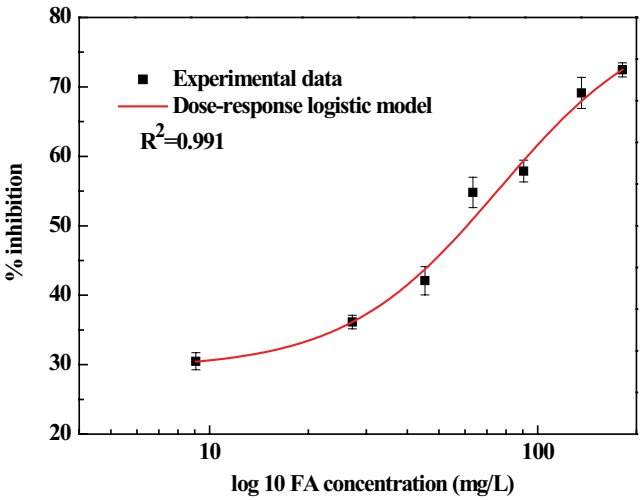

Fig. 6. Estimation of median inhibitory concentrations of free ammonia. (Data are the means for three independent experiments and presented as the means ± standard error.)

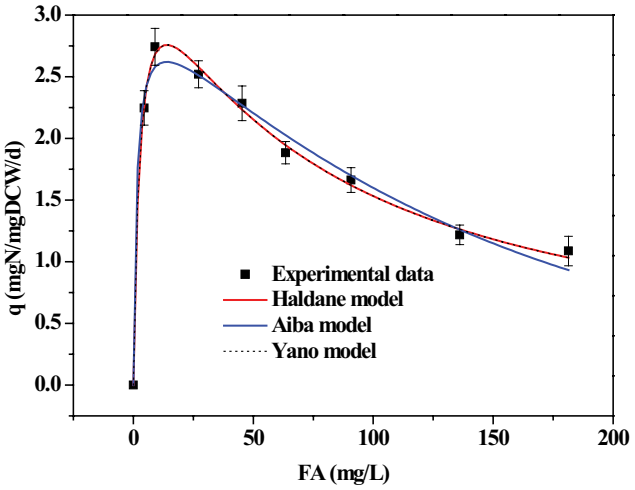

Fig. 5. Kinetic curves for the ammonia nitrogen degradation by strain JQ1004 under varying FA concentrations (at 30°C, 160 rpm, pH 8.0 and data are the means for three independent experiments and presented as the means ± standard error).

3.6. Effect of FA on the amoA gene transcriptional level

To investigate whether transcription of the *amoA* gene was affected by FA, *Acinetobacter* sp. JQ1004 was grown in NM with different initial FA concentrations at 30°C for 24 h. Then, total RNA was extracted and used for real-time RT-PCR to analyze the relative *amoA* mRNA level. As shown in Fig. 7, there was only 0.06 fold observed at a concentration of 4.54 mg/L. From the growth curve shown in Fig. 3, ammonium was completely degraded by strain JQ1004 at t = 24 h, due to the low initial concentration of substrate ($[NH_4^+–N]$ = 50 mg/L), at which the strain was in the stable growth stage. Due to insufficient nutrition, the growth of the strains became slower or even stopped, resulting in a low transcriptional level of the *amoA* gene. As shown in Fig. 7, a 1.49 fold in the *amoA* mRNA transcript level was observed in the experimental group with an FA concentration of 9.07 mg/L, indicating rapid production of AMO in the log phase. The expression levels of the *amoA* gene were 1.38, 1.15, 1.07, 0.75, 0.51, and 0.31 fold, respectively, with the initial concentration of FA at 27.21, 45.35, 63.49, 90.70, 136.05, and 181.40 mg/L. These results reveal that FA inhibited nitrogen removal and growth of the strain during heterotrophic nitrification

Table 4
FA biokinetic parameters of autotrophic nitrifiers in the published literature

	Bacteria	Inhibition thresholds of free ammonia (mg/L)	Kinetic parameters		References
			K_s (mg/L)	K_i (mg/L)	
AOB	Enriched *Nitrosomonas* culture	Up to 16 mg NH_3/L not inhibiting the bacteria	0.36 ± 0.02	/	[37]
	Nitrosomonas cultured sludge		0.28 ± 0.04	376 ± 45	[38]
NOB	*Nitrospira* strain ND1	0.85	/	31 ± 2.0	[36]
	Nitrospira japonica strain NJ1	4.3	/	42 ± 6.1	[36]
	Enriched *Nitrobacter* culture	50	1.2–1.3	/	[39]
	Enriched *Nitrospira* culture	0.04–0.08	0.9–1.1	/	[39]

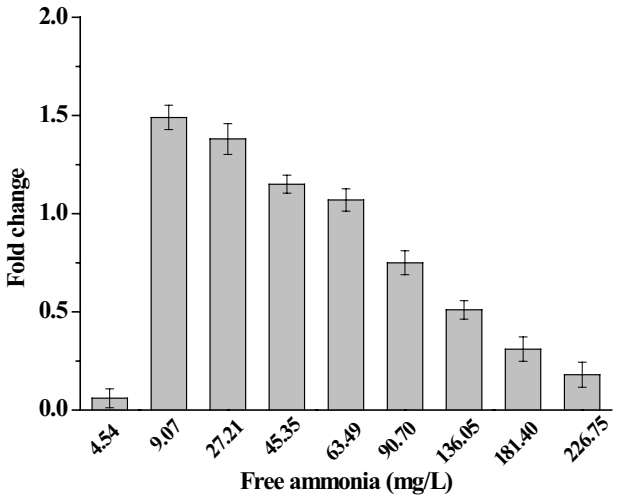

Fig. 7. The mRNA level of the *amoA* gene under different initial FA. (The mRNA level of *amoA* gene was normalized to that of 16S rRNA, and all data are presented in terms of relative mRNA. Data are the means for three independent experiments and presented as the means ± standard error.).

by inhibiting expression of the *amoA* gene. Notably, the expression level of *amoA* gene was still observed at 0.18 fold when the strain was treated with 226.75 mg/L FA for 24 h. This result further indicates the excellent ability of strain JQ1004 to resist high FA concentrations at the gene level.

4. Conclusions

The bacterium *Acinetobacter* sp. JQ1004 exhibited heterotrophic nitrification and aerobic denitrification abilities with limited accumulation of intermediate products (nitrite or nitrate). The strain showed a strong tolerance to high FA concentrations. Lower concentrations of FA stimulated growth and metabolism of the bacterial strain, whereas higher FA concentrations caused a decrease in the specific removal rate. The three kinetic models (Haldane, Aiba, and Yano) applied in this study simulated the effect of FA on degradation of strain JQ1004, obtaining the adjusted R^2 values of 0.995, 0.981, and 0.994. The Haldane model was employed to further evaluate the influence of FA; q_{max}, K_s, and K_i were 3.946 ± 0.021 g/(g DCW d)$^{-1}$, 2.997 ± 0.041 mg/L, and 64.736 ± 0.023 mg/L, respectively. In addition, the FA concentration of 76.31 mg NH_3–N/L, calculated using the four-parameter logistic model, resulted in 50% inhibition of strain JQ1004 for a pure culture. The RT-PCR results indicated that FA inhibited transcription of the *amoA* gene during heterotrophic nitrification.

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