# Screening and Cultivation of Oligotrophic Aerobic Denitrifying Bacteria

**Haihan Zhang and Shilei Zhou**

**Abstract** During the past few decades, more and more people have poured nitrogen into the fresh water ecosystem, leading to serious environmental problems, such as eutrophication, algal bloom, and unsafe water, especially in drinking water reservoirs. Nitrogen removal in freshwater ecosystems is important for water utilization processes. Physical (air stripping) and chemical techniques (chemical precipitation) are widely used to remove nitrogen from wastewater, as the traditional biological method (nitrification by autotrophs under aerobic conditions and denitrification by heterotrophs under anaerobic conditions) is impractical. Conventional biological denitrification only occurs under anaerobic or anoxic conditions with the reduction from nitrate to nitrogen gas. Oxygen inhibits the reaction steps, which makes them impractical in natural waters, especially in reservoirs. However, few studies have focused on aerobic denitrifiers' characteristics for removing nitrogen from oligotrophic drinking water reservoirs. To end this, we isolated several strains using enrichment and screening processes. We found that some strains have perfect performance on nitrogen removal in aerobic conditions with low pollutant concentration. Therefore, the objectives of the present work were to determine the taxonomic status using the 16S rRNA method, and to determine nitrogen removal performance in nutrient medium. The results can be useful for applications of aerobic denitrifiers for micropollution reservoir bioremediation. There are two parts in this chapter: (1) Screening and isolation of oligotrophic aerobic denitrifying bacteria; (2) Denitrification performance in pure culture conditions. The results of this part demonstrated that oligotrophic aerobic denitrifying bacterial species had aerobic denitrification ability, and resist a low carbon to nitrogen ratio, therefore, provided the scientific evidence for micropolluted source water bioremediation processes in situ.

**Keywords** Aerobic denitrifiers • Aerobic denitrification • Nitrogen removal • Source water • Reservoir

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T. Huang (ed.), *Water Pollution and Water Quality Control of Selected Chinese Reservoir Basins*, The Handbook of Environmental Chemistry 38,
DOI 10.1007/978-3-319-20391-1_13

# 1 Screening and Isolation of Oligotrophic Aerobic Denitrifying Bacteria

## *1.1 Reservoir Sediment Sampling*

Reservoir sediment samples were collected from Zhoucun drinking water reservoir (#1, 34°56′38.85″N,117°40′27.42″E; #2, 34°57′9.84″N,117°39′34.17″E; #3, 34°57′21.50″N,117°39′48.67″E; #4, 34°57′3.03″N,117°40′4.78″E; #5, 34°57′19.59″N,117°40′51.69″E; #6, 34°56′35.16″N,117°41′04.09″E; #7, 34°56′31.41″N, 117°41′95.57″E). In June 2011, surface sediment samples were collected at a deep layer of 0–10 cm using a sterilized Petersen stainless steel grab sampler [1, 2]. The reservoir source water was also collected. The samples were stored in black plastic bags at 4 °C, and transferred to the Key Laboratory of Northwest Water Resource, Environment and Ecology, Xi'an University of Architecture and Technology (Fig. 1).

## *1.2 Enrichment Culturing and Isolation of the Aerobic Denitrifiers*

The 100-mL sludge sample was added into 700 mL of heterotrophic enrichment denitrification broth medium (HEDM) (in (g/L)) [1, 2]: $CH_3COONa$ 0.5; $NaNO_3$ 0.1; $K_2HPO_4 \cdot 3H_2O$ 0.1; $CaCl_2$ 0.05; $MgCl_2 \cdot 6H_2O$ 0.05; pH 7.0–7.5. Every three days, we threw out the liquid medium, reduced the concentration of the medium by

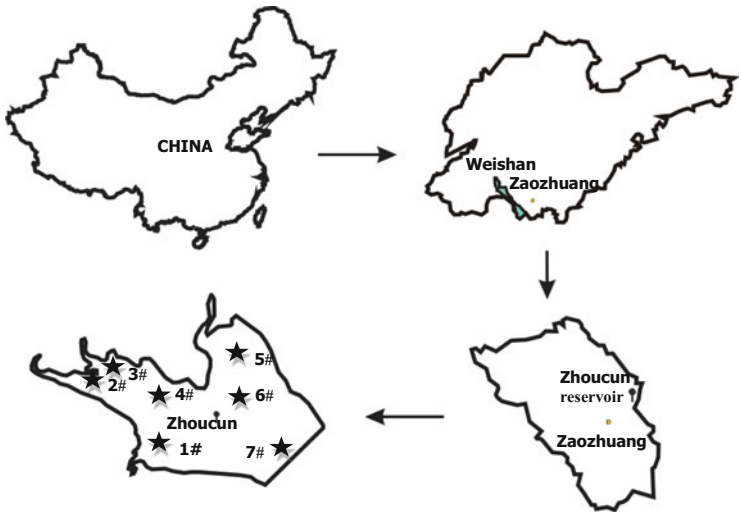


**Fig. 1** The location of the sediment sample collection


**Fig. 2** The enrichment and domestication of the aerobic denitrifiers

one-tenth, and put the new medium into the sludge sample, until the concentration of the HEDM became one-tenth of the first concentration (Fig. 2). The enrichment of aerobic denitrifiers lasted almost one month [3]. The temperature and the DO of the enrichment cultures were controlled to room temperature and nearly 5 mg/L, respectively. The enrichment sludge suspension was sampled via a series of gradient dilutions: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, and $10^{-7}$ dilutions. And the diluents were streaked on a screening medium (SM) [4–6] plate (g/L): $CH_3COONa$ 0.1; $NaNO_3$ 0.02; $K_2HPO_4 \cdot 3H_2O$ 0.02; $CaCl_2$ 0.01; $MgCl_2 \cdot 6H_2O$ 0.01; agar 20; pH 7.0–7.5, which was incubated at 30 °C. Prominent growing single colonies were harvested and cultivated in SM medium with $NaNO_3$ as the sole nitrogen source in order to detect the aerobic denitrifying bacteria performance. To this end, in our primary works, 196 strains were isolated using enrichment and screening processes, and 14 strains have perfect performances on the nitrogen removal process under low pollutant concentration and aerobic conditions. The isolated strains with high nitrogen removal efficiency were stored on SM slant medium at 4 °C and on SM Glycerin medium at −20 °C, respectively.

## *1.3 Morphological Characteristics of Aerobic Denitrifiers*

The 14 strains isolated from the oligotrophic reservoir have perfect performances on the nitrogen removal process under low pollutant concentration and aerobic conditions, and are named after ZHF2, ZHF3, ZHF5, ZHF6, ZHF8, ZMF2, ZMF5, ZMF6, N299, G107, 81Y, SF9, SF18, and SXF14. The morphological characteristics of aerobic denitrifiers were analyzed under a scanning electron microscope, and the results are shown in Fig. 3.

## *1.4 Analysis of 16S rRNA Gene Sequences*

The 16S rRNA sequences of the 14 strains were obtained via polymerase chain reaction (PCR) and sequencing. The PCR primers [7] were F27,

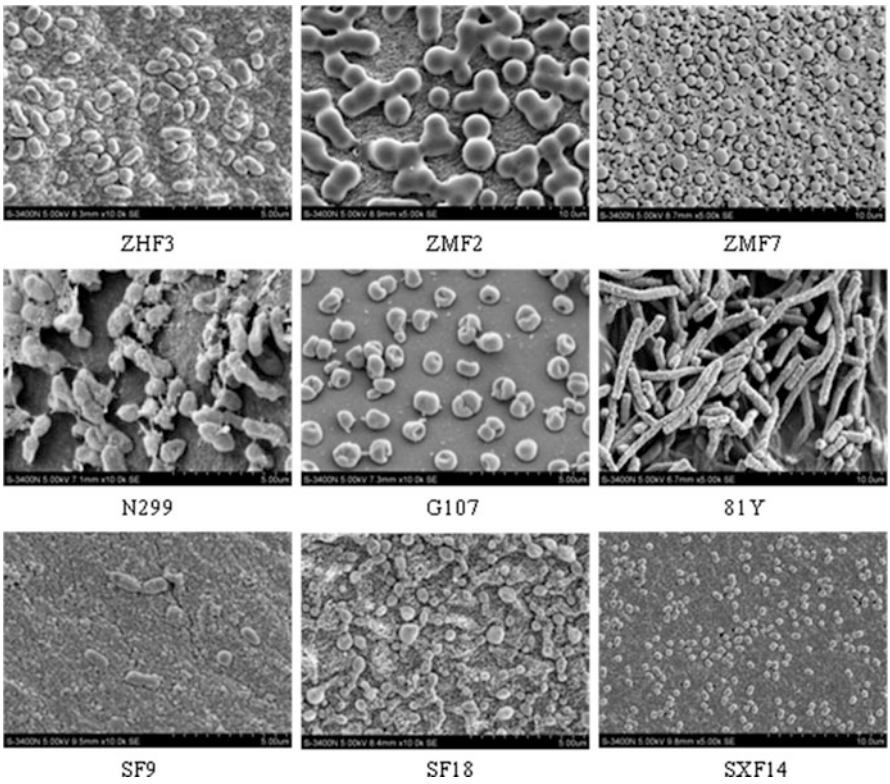


**Fig. 3** Scanning electron microscope (SEM) images of aerobic denitrifiers

5′-AGAGTTTGATCATGGCTCAG-3′, and R1492, 5-′-TACGGTTACCTTGTTACGACTT-3′. The PCR reaction mix consisted of the following reagents: 10× Taq buffer (2.0 μL); 2.5 mM dNTP mix (1.6 μL); 5p primer 1 (0.8 μL); 5p primer 2 (0.8 μL); template (0.5 μL); 5u Ex Taq (0.2 μL); with the sterile nuclease-free water to 20 μL. PCR was carried out as follows: 95 °C for 5 min for 1 cycle, then denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s, for 24 cycles. After a final extension at 72 °C for 10 min, reactions were held at 10 °C. Homologies searching of the sequences in GenBank by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were revealed. A neighbour-joining tree was constructed in the MEGA 5.0 program using the neighbor-joining (NJ) method with 1000 bootstrap replicates and the maximum composite likelihood model [2]. High similarity type culture strains to the genera are listed in Table 1.

An NJ tree based on the comparison of partial 16S rRNA gene sequences of 14 strains and other type culture strains sequences is shown in Fig. 4. The phylogenetic tree based on the 16S rRNA gene sequence of 14 strains with high nitrogen removal efficiency and other previously studied aerobic denitrifiers is shown in Fig. 5.

**Table 1** The sequence results of the aerobic denitrifiers

| Strains | GenBank no. | Sequence length | High similarity type culture strains | Similarity (%) |
|---|---|---|---|---|
| ZHF2 | KP717095 | 1394 | *Novosphingobium aromaticivorans* DSM 12444(T) | 98.06 |
| ZHF3 | KP717089 | 1379 | *Acinetobacter junii* CIP 64.5(T) | 99.64 |
| ZHF5 | KP717094 | 1447 | *Acinetobacter junii* CIP 64.5(T) | 97.64 |
| ZHF6 | KP717088 | 1393 | *Acinetobacter brisouii* CIP 110357(T) | 96.34 |
| ZHF8 | KP717087 | 1399 | *Novosphingobium aromaticivorans* DSM 12444(T) | 97.28 |
| ZMF2 | KP717086 | 1380 | *Acinetobacter junii* CIP 64.5(T) | 99.64 |
| ZMF5 | KP717085 | 1434 | *Aquabacterium citratiphilum* B4(T) | 98.01 |
| ZMF6 | KP717084 | 1400 | *Sphingomonas koreensis* JSS26(T) | 96.99 |
| N299 | KP717093 | 1361 | *Zoogloea caeni* EMB43(T) | 97.85 |
| G107 | KP717096 | 1392 | *Acinetobacter pittii* CIP 70.29(T) | 99.57 |
| 81Y | KP717097 | 1315 | *Acinetobacter pittii* CIP 70.29(T) | 99.92 |
| SF9 | KP717092 | 1396 | *Delftia lacustris* DSM 21246(T) | 100 |
| SF18 | KP717091 | 1227 | *Acinetobacter oryzae* B23(T) | 98.45 |
| SXF14 | KP717090 | 1362 | *Acinetobacter johnsonii* CIP 64.5(T) | 99.71 |

Based on 16S rRNA gene sequences, strains ZHF3, ZHF5, ZHF6, ZMF2, G107, 81Y, SF18, and SXF14 clustered with species from *Acinetobacter* sp., strains ZHF2 and ZHF8 clustered with species from *Novosphingobium* sp., strain ZMF5 clustered with species from *Aquabacterium* sp., strain ZMF6 clustered with species from *Sphingomonas* sp., strain N299 clustered with species from *Zoogloea* sp., and strain SF9 clustered with species from *Delftia* sp. Therefore, the aerobic denitrifiers were identified as *Acinetobacter* sp. ZHF3, *Acinetobacter* sp. ZHF5, *Acinetobacter* sp. ZHF6, *Acinetobacter* sp. ZMF2, *Acinetobacter* sp. G107, *Acinetobacter* sp. 81Y, *Acinetobacter* sp. SF18, *Acinetobacter* sp. SXF14, *Novosphingobium* sp. ZHF2, *Novosphingobium* sp. ZHF8, *Aquabacterium* sp. ZMF5, *Sphingomonas* sp. ZMF6, *Zoogloea* sp. N299, and *Delftia* sp. SF9.

An NJ phylogenetic tree based on the 16S rRNA gene sequences of 14 strains with high nitrogen removal efficiency and other previously studied aerobic denitrifiers was constructed in Fig. 5 of Chap. 1. The results showed that strains ZHF5, ZMF2, and SF18 were in the same group; strains ZHF2, ZHF6, ZHF8, and ZMF6 were in the same group; strain ZHF3 and *Acinetobacter* sp. N22 were in the same group; strains SXF14 and *Acinetobacter* sp. CGMCC 6052 were in the same group; strains G107, 81Y, and *Acinetobacter* sp. M9 were in the same group; strains SF9 *Delftia* sp. WXZ-15, *Delftia tsuruhatensis* strain WXZ-1, *Delftia tsuruhatensis* strain P18, and *Delftia tsuruhatensis* strain WYLW2–3 were in the same group; and strains N299, ZMF5, and *Comamonas testosteroni* strain GAD4 were in the same group.

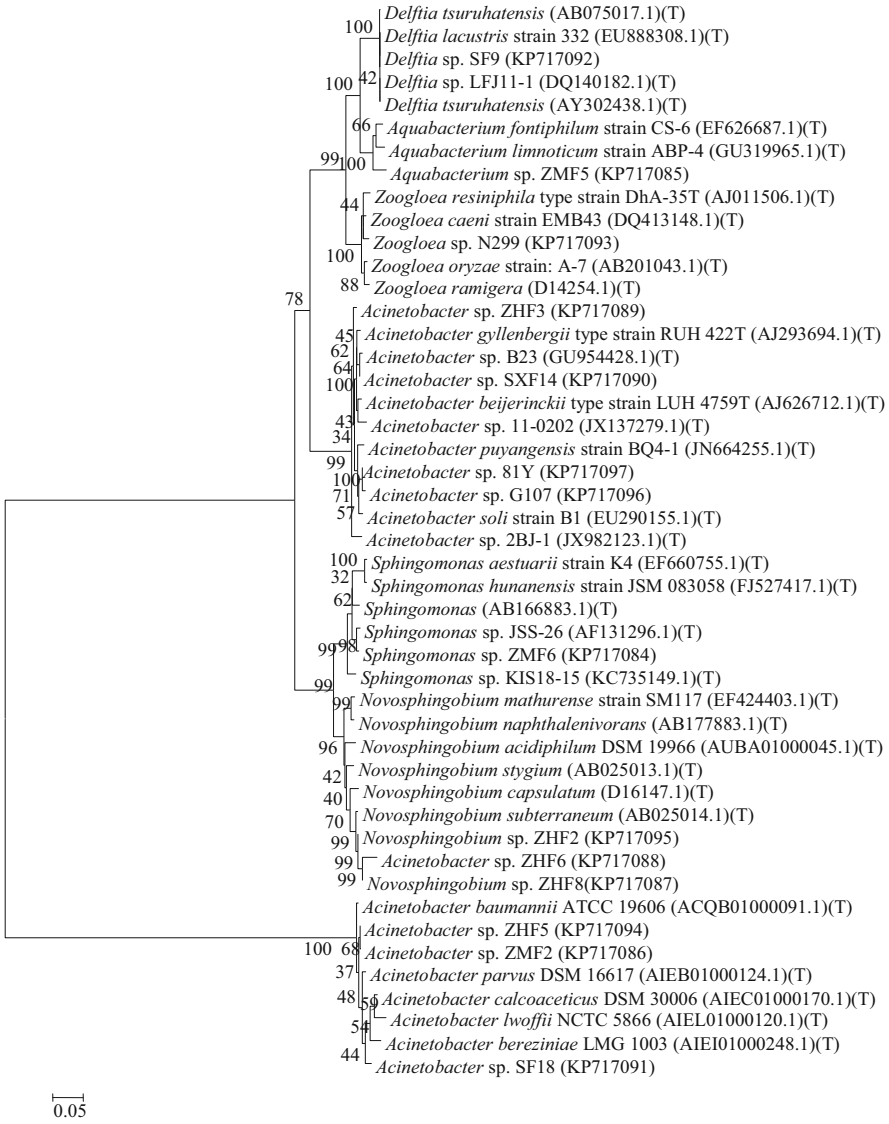


**Fig. 4** Phylogenetic tree based on the comparison of partial 16S rRNA gene sequences of 14 strains and other type culture strains sequences

## *1.5 Summary*

We isolated the aerobic denitrifiers from an oligotrophic reservoir water system and studied the taxonomic status. The oligotrophic aerobic denitrifiers were obtained, based on enrichment, domestication, and screening processes, and taxonomic

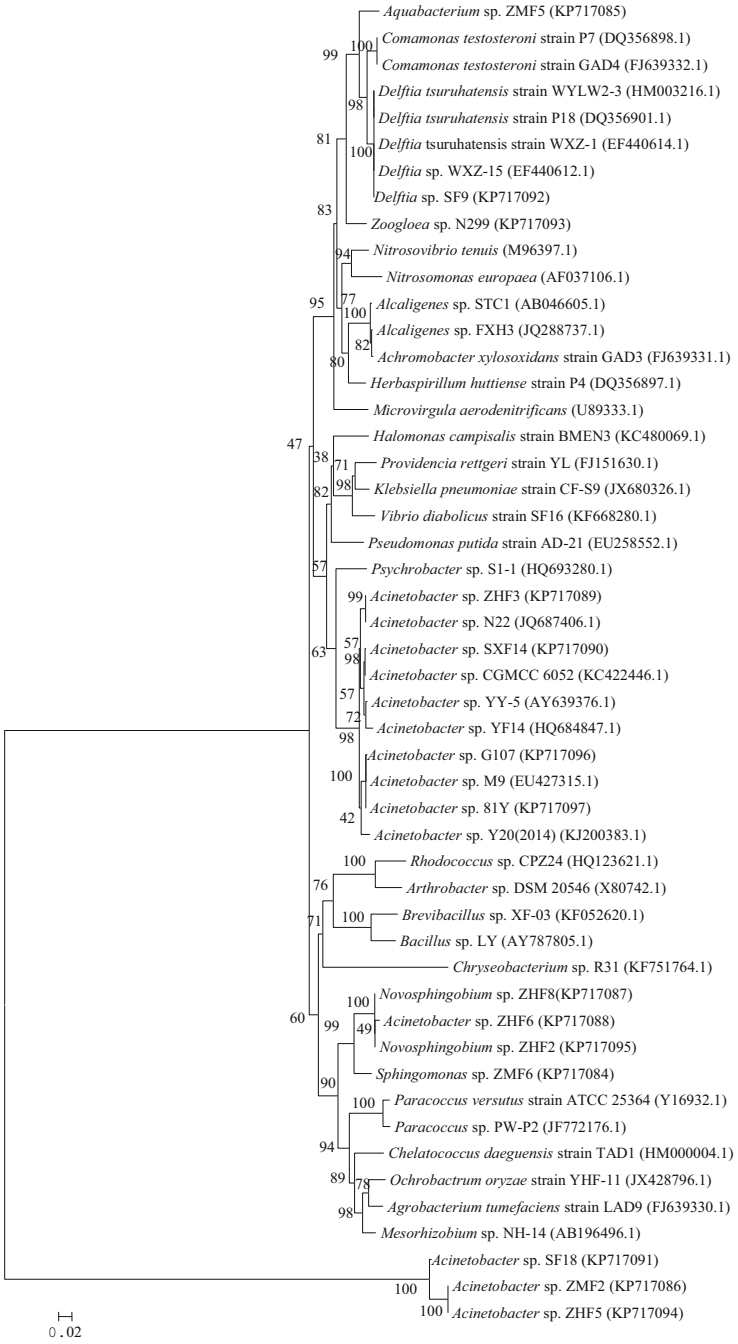


**Fig. 5** Phylogenetic tree based on the 16S rRNA gene sequences of 14 strains with high nitrogen removal efficiency and other previously studied aerobic denitrifiers

statuses were determined by 16S rRNA. In the preliminary experiment with enrichment culture isolation, 196 strains were isolated, and 14 strains (ZHF2, ZHF3, ZHF5, ZHF6, ZHF8, ZMF2, ZMF5, ZMF6, N299, G107, 81Y, SF9, SF18, and SXF14) demonstrated perfect nitrogen removal ability. Based on morphological and phylogenetic analyses, ZHF3, ZHF5, ZHF6, ZMF2, G107, 81Y, SF18, and SXF14 were identified as *Acinetobacter* sp.; ZHF2 and ZHF8 were identified as *Novosphingobium* sp.; ZMF5 was identified as *Aquabacterium* sp.; ZMF6 was identified as *Sphingomonas* sp.; N299 was identified as *Zoogloea* sp.; and SF9 was identified as *Delftia* sp. The isolation of oligotrophic aerobic denitrifiers enriched the species of aerobic denitrification bacteria, and the perfect nitrogen removal performances of oligotrophic aerobic denitrifiers provided a significant parameter to remediate the micropolluted reservoir water system.

## 2 Denitrification Performance in Pure Culture

During the past few decades, more and more nitrogen has been discarded into the fresh water environmental ecosystem, leading to many serious environmental problems [8–11], such as eutrophication, algal bloom, and lack of drinking water safety [12, 13], especially in the drinking water reservoirs [14, 15]. Nitrogen removal processes in freshwater ecosystems is a hot topic. Physical [16] and chemical techniques [4–6] have been widely used to removal nitrogen in wastewater, and the traditional biological method also has practical application [17]. Conventional biological denitrification only occurs under anaerobic or anoxic conditions with the reduction from nitrate to nitrogen gas [18]. Oxygen inhibits the reaction steps, which makes them impractical in natural waters, especially reservoirs [19].

The discovery of the first aerobic denitrification bacteria *Thiosphaera pantotropha* strain was done by Robertson and Kuenen [20] from a denitrifying, sulfide-oxidizing wastewater treatment plant. There is a new novel method of nitrogen removal, and it is not limited to oxygen [21, 22]. Aerobic denitrification has attractive advantages when compared to conventional denitrification under anaerobic conditions: (1) the nitrification and denitrification can occur in the same treatment system [23] and (2) the denitrification can produce alkalinity to balance the acid of nitrification [24]. There are recent reports of aerobic denitrification bacteria isolated from canals [25], ponds [26, 27], and soils [28], and the dominant species included *Thiosphaera pantotropha* [29], *Alcaligenes faecalis* [30], *Citrobacter diversus* [31], *Pseudomonas stutzeri* [32], and *Rhodococcus* sp. [33].

Compared with strains isolated in massive amounts from other environmental systems [25–28], aerobic denitrifiers are rarely isolated from reservoirs, and there is limited research to date on the use of aerobic denitrifiers to denitrify and bioremediate reservoir ecosystems [26, 27, 34]. Several studies have illustrated the difficulties in removing nitrogen from source water, owing to its low

concentration as a pollutant [35, 36]. Our research team has been researching the water quality of aerobic denitrifiers for a while, and the findings have been reported elsewhere [1, 2, 4–6, 37, 38]. However, fewer studies have focused on exploring the nitrogen removal characteristics of aerobic denitrifiers from oligotrophic drinking water reservoirs.

To end this, in our primary works, 196 strains were isolated using enrichment and screening processes, and 14 strains have perfect performance on the nitrogen removal process under low pollutant concentrations and aerobic conditions. The nitrogen removal performance of *Zoogloea* sp. N299, *Acinetobacter* sp. G107, and 81Y were explored. Therefore, the objectives of the present work were to: (1) determine the growth characteristics of oligotrophic aerobic denitrifiers, (2) investigate the denitrification performances in a pure culture medium system, and (3) examine the nitrification performances in a reservoir source water medium system.

## 2.1 *Growth Characteristics of* Zoogloea *sp. N299,* Acinetobacter *sp. G107, and* Acinetobacter *sp. 81Y*

To evaluate the growth characteristics of the isolated strain N299, G107, and 81Y, their growth conditions were investigated with a shake flask experiment. 400-mL of the liquid SM medium was placed in 1000-mL shake flasks, inoculated with 4 mL preculture of the strain, and then cultivated at 30 °C. During incubation, 3 mL of culture was removed periodically for the determination of the cell optical density. The aerobic denitrifying bacteria N299, G107, and 81Y were precultured for 24 h in 50 mL of liquid medium (without agar) in a 100-mL Erlenmeyer flask at 30 °C and120 rpm, in order to be activated [4–6]. Then, the N299, G107, and 81Y were inoculated at 1 % (v/v) into 400 mL of liquid SM medium ((in g/L): $CH_3COONa$ 0.1; $NaNO_3$ 0.02; $K_2HPO_4 \cdot 3H_2O$ 0.02; $CaCl_2$ 0.01; $MgCl_2 \cdot 6H_2O$ 0.01; pH 7.0–7.5.) in a 1000-mL Erlenmeyer flask in order to study the cell growth characteristics through measuring the $OD_{510}$.

Figure 6 shows the growth curve of the N299 strain as a sigmoid curve. The first 18 h comprised the lag phase, followed by a 16-h logarithmic growth phase, and the last 34 h was the stationary phase. During the growth period, the $OD_{510}$ of the strain N299 increased from 0.004 to 0.062. Figure 7 shows the growth curve of isolate G107 as a sigmoid curve. The first 6 h comprised the lag phase, followed by a 24-h logarithmic growth phase, and the last 38 h was the stationary phase. During the growth period, the $OD_{510}$ of the strain G107 increased from 0.009 to 0.052. Figure 8 shows the growth curve of isolate 81Y as a sigmoid curve. The first 15 h comprised the lag phase, followed by a 155-h logarithmic growth phase, and the last 50 h was the stationary phase. During the growth period, the $OD_{510}$ of the strain 81Y increased from 0.003 to 0.081.

The logistic growth equation [39], $y(t) = a/[1 + (a/c - 1)\exp(-bt)]$, describes the cell growth curve, where $t$ is time (h), $y$ is the bacterial cell growth rate ($h^{-1}$), and

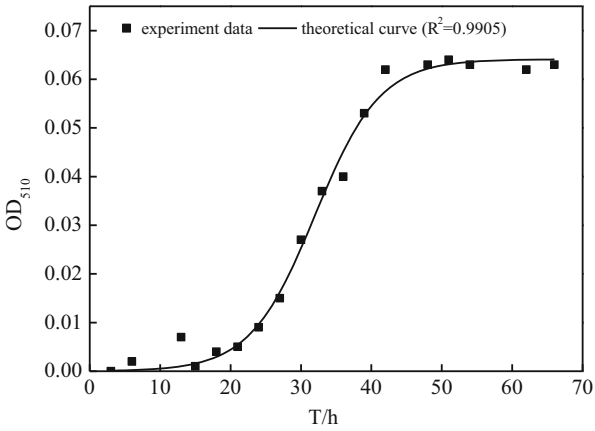


**Fig. 6** Growth curve of strain N299 in the liquid SM medium

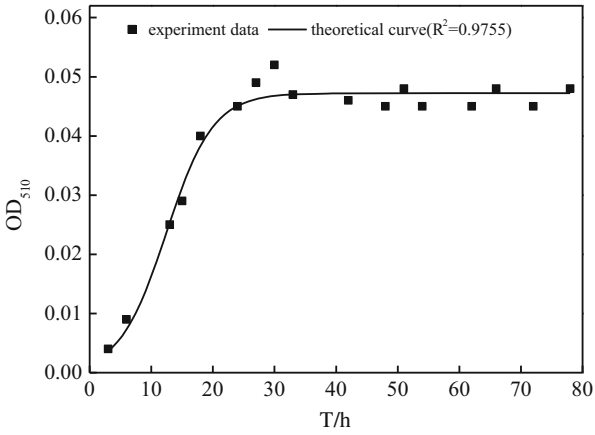


**Fig. 7** Growth curve of strain G107 in the liquid SM medium

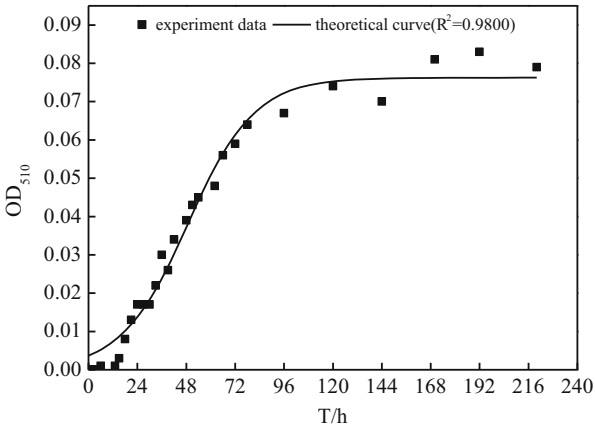


**Fig. 8** Growth curve of strain 81Y in the liquid SM medium

$a$ is the maximum bacterial cell density (OD). Correlation analysis of the strain N299 using OriginPro (Version 8.0) yielded $a = 0.064$, $c = 0.004$, and $b = 0.22\ h^{-1}$, with a correlation coefficient of 0.9905. The generation time for N299 was 3.15 h, and the generation time for G107 was 2.67 h ($b = 0.26\ h^{-1}$). T he generation time for the strain 81Y was 11.55 h ($b = 0.06\ h^{-1}$). The heterotrophic aerobic denitrification bacteria possessed higher growth rates and shorter growth cycles. The specific growths of N299, G107, and 81Y are 0.22, 0.26, and 0.06 $h^{-1}$, respectively. Comparing *N. europaea* (0.03–0.05 $h^{-1}$) [40], *Pseudomonas denitrificans* (0.19–0.23 $h^{-1}$), and *T. pantotropha* (0.28–0.45 $h^{-1}$) under different growth conditions [21, 22], and the specific growth rate of *A. faecalis* no. 4 was 0.2 $h^{-1}$ [18], because the aerobic denitrifiers N299, G107, and 81Y were cultured in the oligotrophic SM medium, then the results demonstrated the strong growth and substrate utilization abilities of the isolated aerobic denitrifiers in the present work.

## 2.2 *The Relationship Between OD and Colony Numbers of* Zoogloea *sp. N299,* Acinetobacter *sp. G107, and* Acinetobacter *sp. 81Y*

In order for the OD to represent the numbers of colonies in the medium, it is necessary to study the relationship between the OD and colony numbers. The aerobic denitrifying bacteria N299, G107, and 81Y were precultured. The N299, G107, and 81Y were inoculated at 2 %(v/v) into a 150-mL or 250-mL Erlenmeyer flask for 24 h. The cell pellet was prepared by centrifuging a 10-mL sample of broth culture at 5000 rpm for 10 min and then decanting the supernatant after washing twice with distilled water. Then, by adding the distilled water, we obtained a series of ODs. The numbers of colonies of every OD suspension was counted via gradient dilution.

From the data series, Fig. 9 shows the relationship between OD and the colony numbers of the N299, G107, and 81Y strains. We obtained the relationships between OD and colony as follows: strain *Zoogloea* sp. N299, $y(\lg(\text{colony})) = 5.23 + 38.51x(OD_{600})$, correlation coefficient $R^2 = 0.9497$; strain *Acinetobacter* sp. G107, $y(\lg(\text{colony})) = 6.63 + 44.17x(OD_{600})$, correlation coefficient $R^2 = 0.8247$; strain *Acinetobacter* sp. 81Y, $y(\lg(\text{colony})) = 6.46 + 43.53x\ (OD_{600})$, correlation coefficient $R^2 = 0.9266$. Therefore, we can obtain the number of colonies of the medium by measuring the $OD_{600}$.

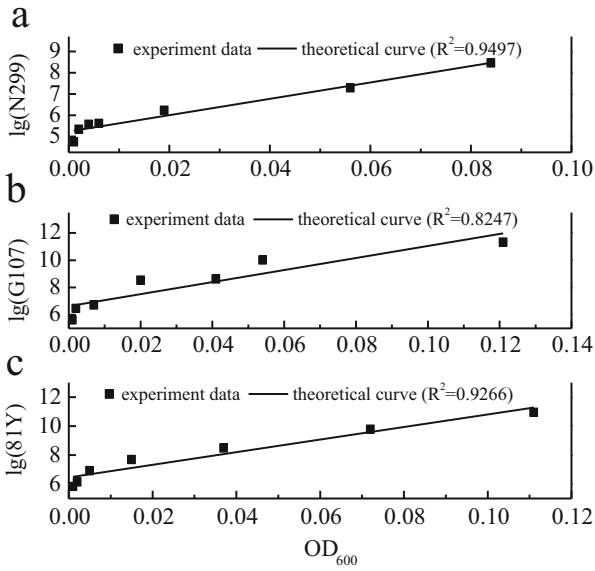


**Fig. 9** The relationship between $OD_{600}$ and colony numbers of *Zoogloea* sp. N299, *Acinetobacter* sp. G107, and 81Y

## 2.3 *The Denitrification Performances of* Zoogloea *sp. N299,* Acinetobacter *sp. G107, and* Acinetobacter *sp. 81Y in Nitrate Medium*

The precultured N299, G107, and 81Y were inoculated in 10 % (v/v) into 150 mL of liquid SM medium, in an Erlenmeyer flask at 30 °C and 120 rpm. The nitrate, nitrite, TN, TDN, and TOC concentrations, and cell optical density (OD) were measured to reflect the denitrification performances of strains N299, G107, and 81Y. All parameters were measured in triplicate ($n = 3$). SM medium (in g/L): $CH_3COONa$ 0.1; $NaNO_3$ 0.02; $K_2HPO_4 \cdot 3H_2O$ 0.02; $CaCl_2$ 0.01; $MgCl_2 \cdot 6H_2O$ 0.01; pH 7.0–7.5.

Under aerobic conditions (dissolved oxygen, DO = 7.0–8.0 mg/L), *Zoogloea* sp. N299, *Acinetobacter* sp. G107, and *Acinetobacter* sp. 81Y demonstrated clear denitrification performance. As shown in Figs. 10, 12, and 14, at 72 h, the nitrate concentrations of N299, G107, and 81Y decreased from $3.54 \pm 0.03$ mg/L to $0.87 \pm 0.06$ mg/L, $0.41 \pm 0.02$ mg/L, and $0.52 \pm 0.07$ mg/L, and the nitrite increased from 0 mg/L to $0.02 \pm 0.00$ mg/L, $0.08 \pm 0.01$ mg/L, and $0.08 \pm 0.01$ mg/L, with no nitrite accumulation. As shown in Figs. 11, 13, and 15, the TN (total nitrogen) and TDN (total dissolved nitrogen) concentrations decreased from $3.63 \pm 0.03$ mg/L to $1.93 \pm 0.01$ mg/L, $2.18 \pm 0.05$ mg/L, and $2.41 \pm 0.06$ mg/L; from $3.63 \pm 0.03$ mg/L to $1.00 \pm 0.02$ mg/L, $1.63 \pm 0.09$ mg/L, and $1.72 \pm 0.07$ mg/L at 120 h, respectively. The removal rate of TN and TDN reached $46.79 \pm 0.30$ %, $39.90 \pm 1.45$ %, and $33.72 \pm 1.78$ %; and $72.30 \pm 0.52$ %, $55.15 \pm 2.43$ %, and $52.71 \pm 1.97$ % at 120 h, respectively. The TOC of N299 and

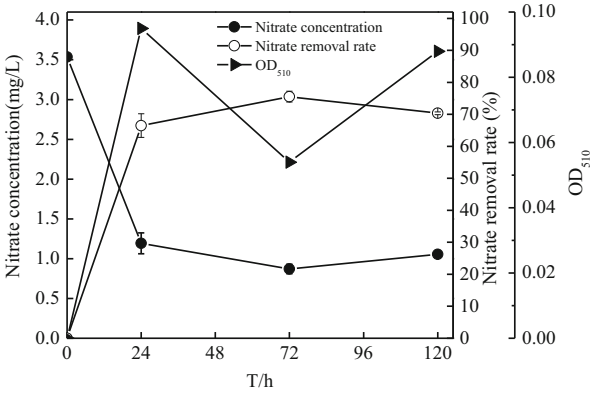


**Fig. 10** Changes in nitrate, nitrate removal rates, and $OD_{510}$ of strain N299 growth in nitrate nitrogen medium

G107 decreased to 1.62 mg/L and 2.48 mg/L in 24 h. The TOC of 81Y decreased from $28.38 \pm 0.69$ mg/L to $24.61 \pm 0.27$ mg/L at 0–24 h, and $2.00 \pm 0.03$ mg/L at 72 h. After that, the denitrification of 81Y became obvious, which was consistent with the characteristics of the isolate. It was suggested that the utilization of organic matter and the degradation of nitrate nitrogen took place simultaneously, indicating it was a true heterotrophic process. The denitrification of N299, G107, and 81Y became weak at low C/N. Because carbon is essential for cell growth and nitrate reduction processes, the optimal quantity of carbon is a key parameter in the denitrification process. However, Zhu et al. [26, 27] showed that the removal rate of nitrate and TN reached 31.7 and 45.0 % at low substrate levels (TOC 48 mg/L and nitrate 4 mg/L), respectively. At the same nitrate level, N299, G107, and 81Y demonstrated strong denitrification performance.

## *2.4 The Denitrification Performances of* Zoogloea *sp. N299,* Acinetobacter *sp. G107, and* Acinetobacter *sp. 81Y in Nitrite Medium*

The precultured N299, G107, and 81Y were inoculated at 10 % (v/v) into 150 mL of short SM medium in a 250-mL Erlenmeyer flask at 30 °C and 120 rpm. The nitrate, nitrite, TN, TP, and TOC concentrations, and cell optical density (OD) were measured to reflect the denitrification performance of the N299, G107, and 81Y strains. All parameters were measured in triplicate ($n = 3$). Short SM medium (in (g/L)): $CH_3COONa$ 0.1; $NaNO_2$ 0.018; $K_2HPO_4 \cdot 3H_2O$ 0.02; $CaCl_2$ 0.01; $MgCl_2 \cdot 6H_2O$ 0.01; pH 7.0–7.5 [3].

Few aerobic denitrifiers using nitrite as the sole nitrogen source were identified. Using nitrite as the sole nitrogen source, this experiment assessed the denitrification activity of N299, G107, and 81Y. Figures 16, 17, 18, 19, 20, and 21 show the time courses of the concentration, TN, nitrite, nitrate, TP, $OD_{510}$, and TOC levels at the

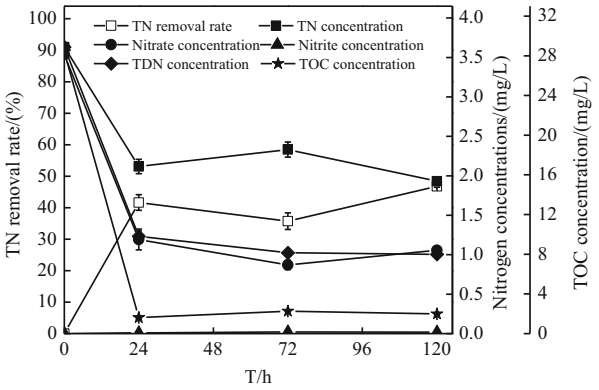


**Fig. 11** Changes in TN, TDN, nitrate, nitrite, and TOC concentrations of strain N299 in nitrate nitrogen medium

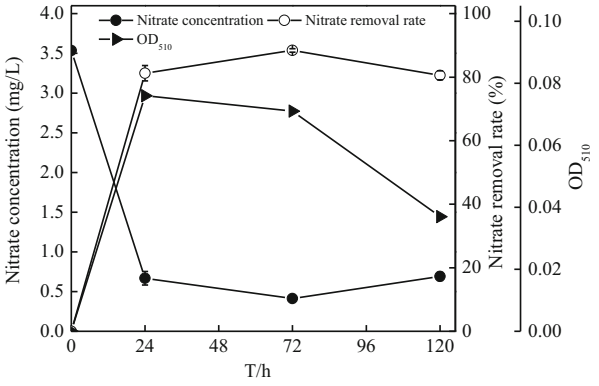


**Fig. 12** Changes in nitrate, nitrate removal rates, and $OD_{510}$ of strain G107 growth in nitrate nitrogen medium

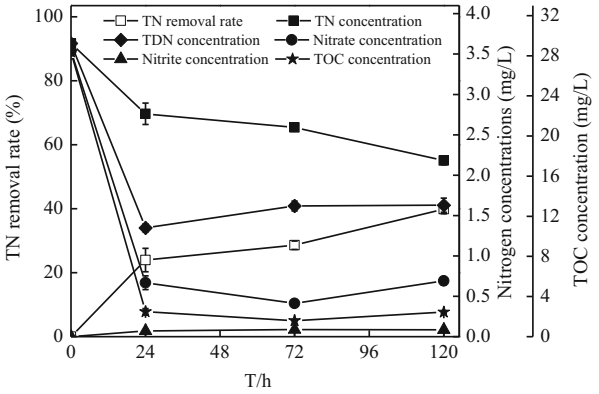


**Fig. 13** Changes in TN, TDN, nitrate, nitrite, and TOC concentrations of strain G107 in nitrate nitrogen medium

initial 3.76 mg/L of nitrite. The removal of nitrite and TOC correlated strongly with the growth rates of isolate N299, G107, and 81Y in Figs. 12, 14, and 16 of Chap. 2, with the fastest removal rates occurring during the log phase. The nitrite and TOC

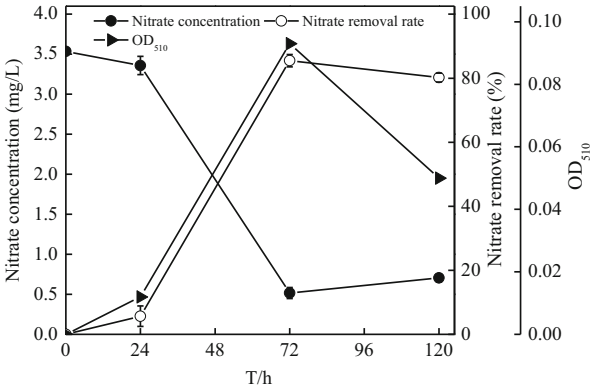


**Fig. 14** Changes in nitrate, nitrate removal rates, and $OD_{510}$ of strain 81Y growth in nitrate nitrogen medium

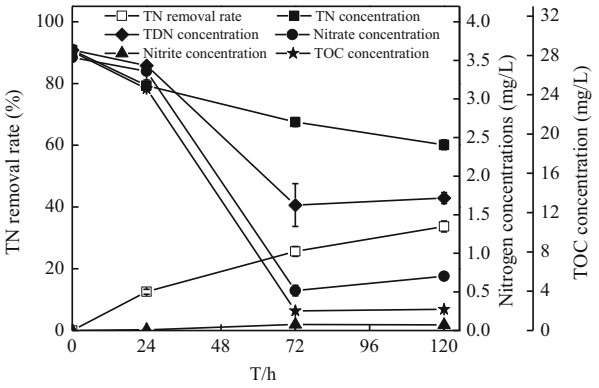


**Fig. 15** Changes in TN, TDN, nitrate, nitrite, and TOC concentrations of strain 81Y in nitrate nitrogen medium

were decreased from $3.76 \pm 0.08$ mg/L and $27.70 \pm 0.75$ mg/L to $1.56 \pm 0.01$ mg/L, $1.25 \pm 0.05$ mg/L, and $1.60 \pm 0.12$ mg/L; and 0 mg/L, $4.95 \pm 0.17$ mg/L, and $0.16 \pm 0.14$ mg/L at 120, 24, and 72 h, respectively. Meanwhile, with the strain's growth, the TN and TP were consumed, and the TN and TP removal rates reached $21.38 \pm 9.22$ %, $48.98 \pm 12.91$ %, and $45.37 \pm 4.66$ %; and $15.97 \pm 1.25$ %, $12.91 \pm 0.98$ %, and $17.42 \pm 3.76$ %, respectively. At the end of the experiment, the nitrate only was increased to $0.19 \pm 0.11$ mg/L, $0.08 \pm 0.04$ mg/L, and $0.35 \pm 0.04$ mg/L. From all of the results, N299, G107, and 81Y clearly demonstrated the denitrification of utilizing the nitrite as the sole nitrogen source.

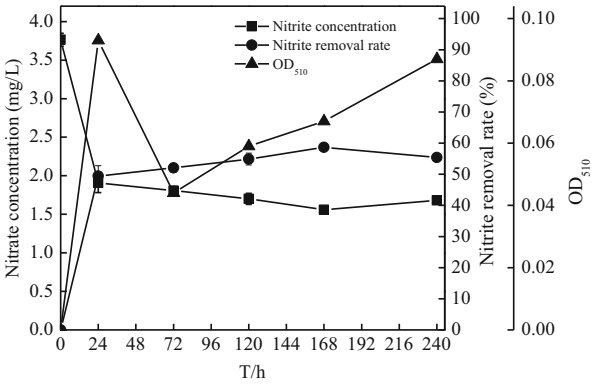


**Fig. 16** Changes in nitrite, nitrite removal rates, and $OD_{510}$ of strain N299 growth in nitrite nitrogen medium

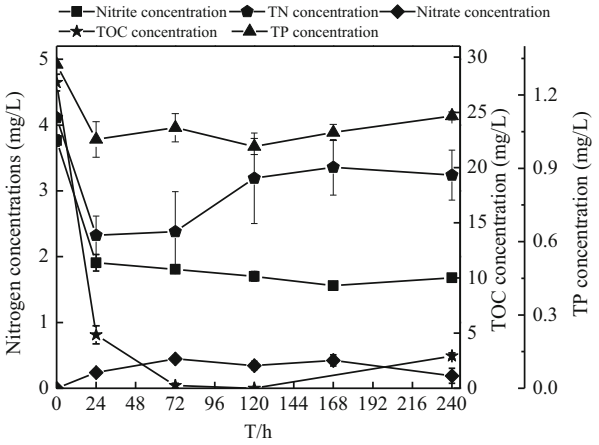


**Fig. 17** Changes in TN, nitrate, nitrite, TP, and TOC concentrations of strain N299 in nitrite nitrogen medium

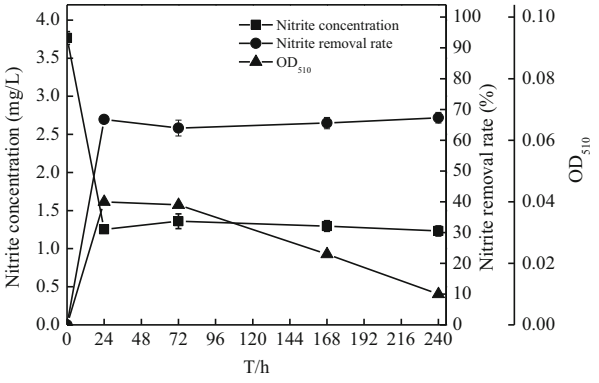


**Fig. 18** Changes in nitrite, nitrite removal rates, and $OD_{510}$ of strain G107 growth in nitrite nitrogen medium

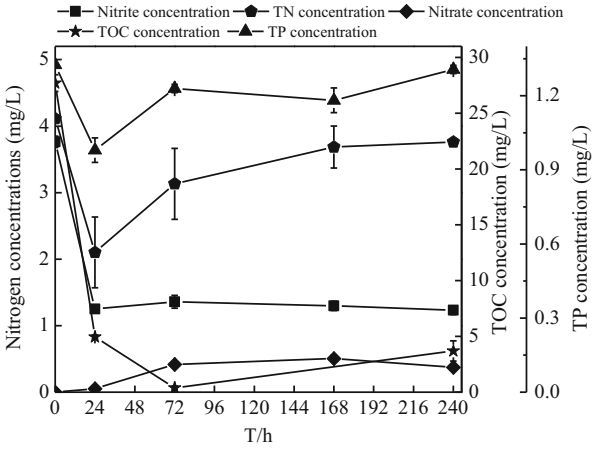


**Fig. 19** Changes in TN, nitrate, nitrite, TP, and TOC concentrations of strain G107 in nitrite nitrogen medium

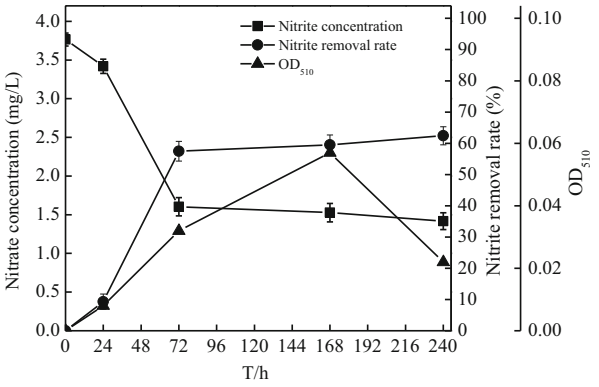


**Fig. 20** Changes in nitrite, nitrite removal rates, and $OD_{510}$ of strain 81Y growth in nitrite nitrogen medium

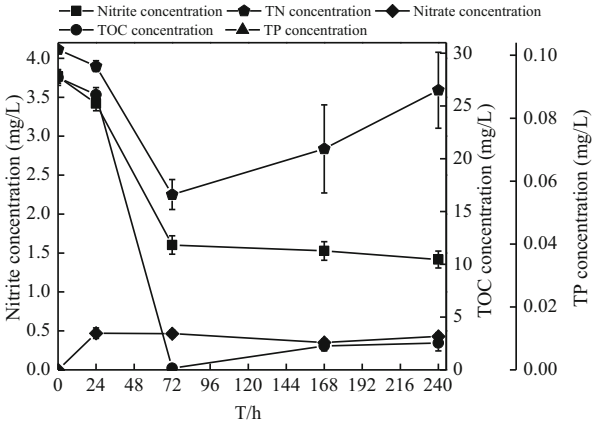


**Fig. 21** Changes in TN, nitrate, nitrite, TP, and TOC concentrations of strain 81Y in nitrite nitrogen medium

### 2.5 *The Nitrification Characteristics of* Zoogloea *sp. N299,* Acinetobacter *sp. G107, and* Acinetobacter *sp. 81Y in Ammonia Medium*

The precultured N299, G107, and 81Y were inoculated at10 % (v/v) into 150 mL liquid HNM in a 250-mL Erlenmeyer flask at 30 °C and 120 rpm. The nitrate, nitrite, TN, ammonia, TP, and TOC concentrations, and cell optical density (OD) were measured to reflect the denitrification performance of the N299, G107, and 81Y strains. All parameters were measured in triplicate ($n = 3$). Heterotrophic nitrification medium (HNM) (in (g/L)): $CH_3COONa$ 0.5; $NH_4Cl_4$ 0.1; $K_2HPO_4 \cdot 3H_2O$ 0.1; $CaCl_2$ 0.05; $MgCl_2 \cdot 6H_2O$ 0.05; pH 7.0–7.5.

The changes of various components in the flask culture are shown in Figs. 22, 23, 24, 25, 26, and 27. The concentration of ammonia decreased significantly, as did TN. The same trend could be seen in the removal of TOC. This indicates that active nitrification occurred largely with a decrease of TOC. At the same time, nitrate and nitrite began to accumulate by nitrification, and remained as denitrification occurred simultaneously, without any nitrate and nitrite accumulation. At the end of the experiment, the ammonia and TN of N299, G107, and 81Y decreased from $28.27 \pm 0.14$ mg/L and $30.68 \pm 0.06$ mg/L to $15.79 \pm 0.45$ mg/L, $18.57 \pm 0.99$ mg/L, $18.41 \pm 2.08$ mg/L; and $18.40 \pm 0.63$ mg/L, $19.41 \pm 1.45$ mg/L, $19.41 \pm 1.45$ mg/L, respectively. The removal rate of ammonia and TN reached $44.12 \pm 1.61$ %, $34.31 \pm 3.51$ %, $34.31 \pm 3.51$ %; and $40.05 \pm 2.04$ %, $36.75 \pm 4.73$ %, $26.85 \pm 7.18$ %, respectively. The TOC of N299, G107, and 81Y decreased from $146 \pm 0.04$ mg/L to $77.90 \pm 0.31$ mg/L, $24.21 \pm 0.68$ mg/L, and $24.12 \pm 0.93$ mg/L. The nitrate and nitrite reached $0.13 \pm 0.02$ mg/L, $0.15 \pm 0.02$ mg/L, $0.27 \pm 0.06$ mg/L; and $0.01 \pm 0.00$ mg/L, $0.01 \pm 0.00$ mg/L, respectively. With the growth of N299, G107, and 81Y, the removal rate of TP also reached $22.77 \pm 3.90$ %, $5.99 \pm 0.20$ %, and $6.38 \pm 1.03$ %. From all of the results, it can be seen that N299, G107, and 81Y showed significant nitrification performance. The ability of heterotrophic organisms to oxidize ammonium to nitrate has generally been linked to aerobic denitrification. Therefore, the utilization of ammonium by isolates was investigated. Previous reports found that the aerobic denitrification bacteria usually possessed nitrification ability [26, 27, 32]. Most of the aerobic denitrification bacteria possessed nitrification; however, *Pseudomonas stutzeri* C3 [41] could not exhibit ammonia. In this study, N299, G107, and 81Y could utilize the ammonia as the sole nitrogen source to grow, and, therefore, have heterotrophic nitrification ability. Like other previous aerobic denitrifiers [2], N299, G107, and 81Y demonstrated a good nitrification and no nitrate or nitrite accumulation.

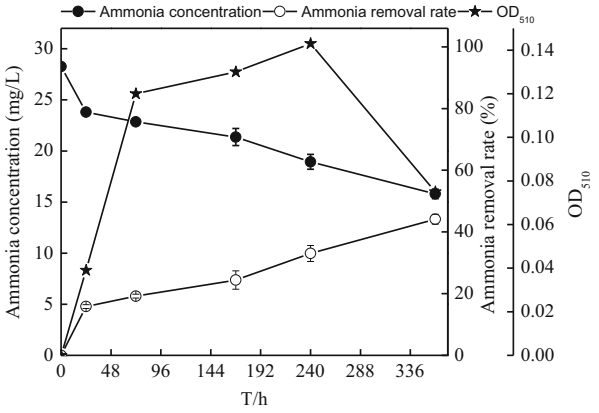


**Fig. 22** Changes in ammonia, ammonia removal rates, and $OD_{510}$ of strain N299 growth in ammonia nitrogen medium

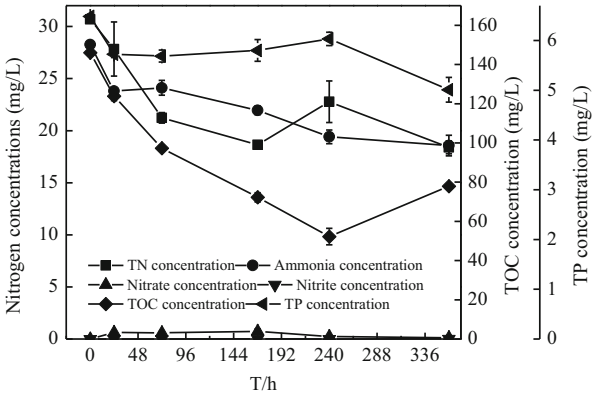


**Fig. 23** Changes in TN, ammonia, nitrate, nitrite, TP, and TOC concentrations of strain N299 in ammonia nitrogen medium

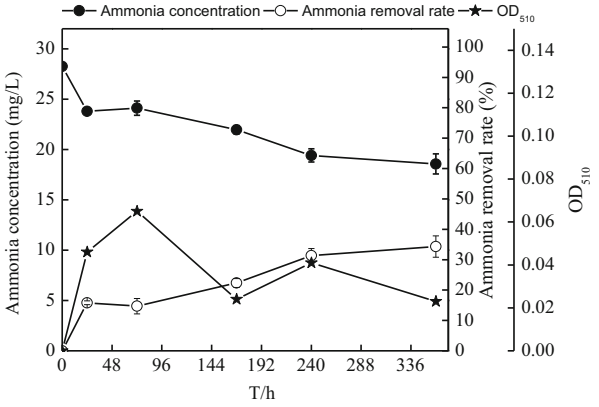


**Fig. 24** Changes in ammonia, ammonia removal rates, and $OD_{510}$ of strain N299 growth in ammonia nitrogen medium

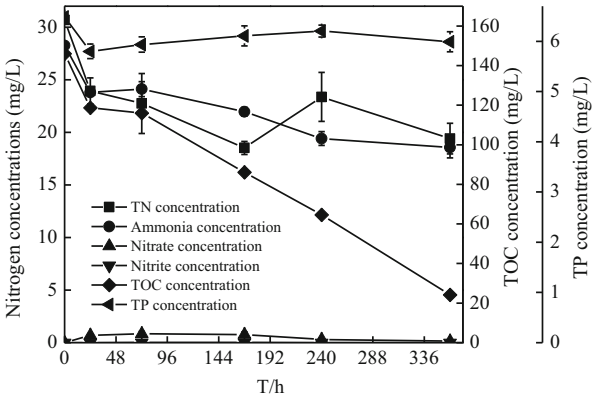


**Fig. 25** Changes in TN, ammonia, nitrate, nitrite, TP, and TOC concentrations of strain G107 in ammonia nitrogen medium

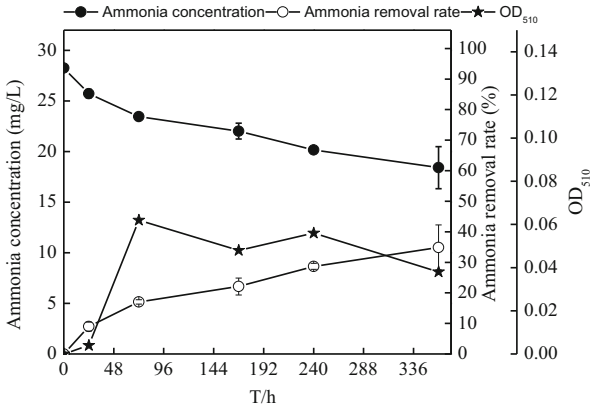


**Fig. 26** Changes in ammonia, ammonia removal rates, and $OD_{510}$ of strain 81Y growth in ammonia nitrogen medium

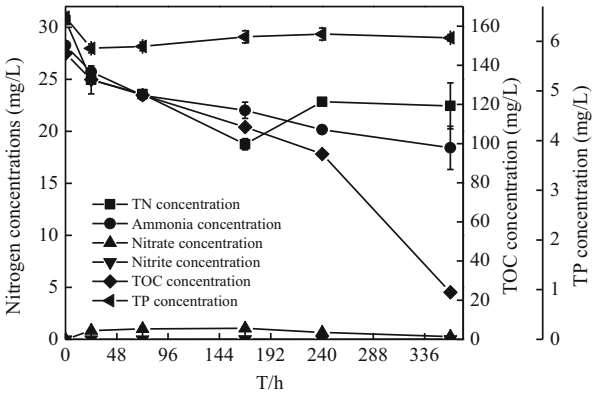


**Fig. 27** Changes in TN, ammonia, nitrate, nitrite, TP, and TOC concentrations of strain 81Y in ammonia nitrogen medium

## 2.6 Summary

The newly isolated indigenous aerobic denitrifiers, N299, G107, and 81Y, were named as *Zoogloea* sp. N299, *Acinetobacter* sp. G107, and *Acinetobacter* sp. 81Y. The specific growths of N299, G107, and 81Y are 0.22, 0.26, and 0.06 $h^{-1}$, respectively. We discovered the relationship between OD and colony: strain N299, $y(\lg(\text{colony})) = 5.23 + 38.51x(OD_{600})$, correlation coefficient $R^2 = 0.9497$; strain G107, $y(\lg(\text{colony})) = 6.63 + 44.17x(OD_{600})$, correlation coefficient $R^2 = 0.8247$; strain 81Y, $y(\lg(\text{colony})) = 6.46 + 43.53x(OD_{600})$, correlation coefficient $R^2 = 0.9266$. Therefore, we can obtain the number of colonies of the medium by measuring the $OD_{600}$. The strains showed the ability to utilize the nitrate and enabled nitrite nitrogen. The removal rates of nitrate of strains N299, G107, and 81Y reached 75.53, 88.4, and 85.31 %, respectively, with no nitrite accumulation. Comparing with the study conducted by Zhu et al. [26, 27], they showed that the removal rates of nitrate and TN reached 31.7 and 45.0 % at a low substrate level (with a TOC of 48 mg/L) under the same nitrate level (4 mg/L). The N299, G107, and 81Y showed further powerful advantages. The nitrite of strains N299, G107, and 81Y were decreased from $3.76 \pm 0.08$ mg/L to $1.56 \pm 0.01$ mg/L, $1.25 \pm 0.05$ mg/L, and $1.60 \pm 0.12$ mg/L, respectively. In this study, the strains N299, G107, and 81Y could utilize the ammonia as the sole nitrogen source, and they possessed heterotrophic nitrification ability.

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