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Identification and denitrification characterization of a novel hypothermia and aerobic nitrite-denitrifying bacterium, *Arthrobacter arilaitensis* strain Y-10

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ABSTRACT

Based on morphology, phospholipid fatty acid and 16S rRNA gene sequence analyses, a novel hypothermia and aerobic nitrite-denitrifying bacterium, named to be Y-10, has been identified as *Arthrobacter arilaitensis*, which was further tested for its efficiency in disposing of wastewater in alkaline or/and low-temperature circumstances. This is the first report showing that *A. arilaitensis* can act as hypothermia and aerobic nitrite-denitrifying bacteria. Then, various factors that potentially affect the aerobic denitrification of strain Y-10 are comprehensively investigated in this study. The optimal temperature for denitrification is 15°C, where the nitrite and total nitrogen removal efficiencies reach up to 100 and 52.8%, respectively. In addition, it has been found that the nitrogen removal efficiency decreases sharply when temperatures are below 10°C or beyond 30°C. Alkaline conditions are beneficial for the aerobic denitrification of strain Y-10, and the highest removal efficiencies correspond to pH 9.0 (15°C) with nitrite and total nitrogen removal efficiencies of 100 and 84.0%, respectively. The optimal conditions of other factors such as shaking speed, incubation quantity, carbon source, and nitrite nitrogen content on the aerobic denitrification have also been determined; e.g. for sodium acetate (one of the carbon sources presently considered), 150 r/min and 1.5×10^6 CFU/mL are the most favorable. In addition, strain Y-10 can perform denitrification over a wide range of nitrite loadings, implying the great potential to treat high concentrated nitrite nitrogen.

Keywords: Hypothermia; *Arthrobacter arilaitensis*; Denitrification characteristics; Nitrogen removal; Alkali tolerance

1. Introduction

Nitrite, which is known to be a natural component of nitrogen cycle, and an intermediate product in bio-nitrification and bio-denitrification processes, has also been considered as one of the nitrogen-resulting pollutants around the world [1,2]. Nitrite can cause problems when concentrated in animal bodies, and its toxicity to animals, such as goldfish, anuran larvae, swiss albino mice, and even human beings, has been well documented [2–5]. Among them, aquatic animals are more sensitive to nitrite intoxication, because of the gradual accumulation to very high concentrations in their body fluids [6]. Therefore, it is necessary to find an effective method to control the nitrite nitrogen

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concentrations in water. Previous reports demonstrated that bioremediation is high efficient, low-costing and easy to implement compared with physical and chemical methods for removal of nitrogenous compounds from wastewater [7,8]. Biodegradation can be divided into bio-nitrification and bio-denitrification. To the best of our knowledge, bio-denitification is acknowledged as the most effective process to convert nitrogen compounds such as nitrate and nitrite nitrogen sequentially from polluted water [9,10]. Up to date, many efficient denitrifying bacteria have been discovered from soil, marine, or activated sludge, such as *Alcaligenes*, *Bacillus* [11,12], *Pseudomonas* [13–15], *Achromobacter*, *Acinetobacter* [16], *Azospirillum*, *Ochrobactrum* [17], *Paracoccus,* and *Arthrobacter* [18–20]. Most of the aerobic denitrification bacteria including those afore mentioned are mesophilic and the nitrate or ammonium are common, as nitrogen source for them to conduct biodegradation. Few reports were given that aerobic nitrite denitrifiers can perform denitrification effectively using nitrite nitrogen as the sole nitrogen at temperatures below 15˚C.

In this work, a hypothermia and aerobic nitrite-denitrifying bacterium, named as Y-10, was identified by using morphology, phospholipid fatty acid (PLFA), and 16S rRNA sequence analysis. Its growth characteristics was investigated in nitrite-denitrifying broth medium and the effects of various culturing conditions such as temperature, shaking speed, pH, incubation quantity, carbon source, and nitrite content on its aerobic denitrification were carefully examined. The results indicated that strain Y-10 was identified as *Arthrobacter arilaitensis*, and it can efficiently remove nitrite and total nitrogen in alkaline conditions with sodium acetate as the sole carbon source at 15˚C. To the best of our knowledge, this is the first report to show that the *A. arilaitensis* is capable of aerobic denitrification at ambient temperatures. Such kind of hypothermia and aerobic nitrite-denitrifying bacterium may play an important role in the nitrogen-polluted alkaline wastewater, especially at low temperatures.

2. Methods

2.1. Bacterium and media

Source of hypothermia and aerobic nitrite-denitrifying bacterium *A. arilaitensis* strain Y-10 was isolated in our laboratory. The bromothylmol blue medium (BTB) [21], denitrification broth medium (DM), and Luria-Bertani medium (LB) were used to observe the colonial morphology, detect the denitrification characteristics, and enlarge the cloned cells, respectively. The BTB solid medium per liter (pH 7.2) comprised $NaNO_2$ 1 g, KH_2PO_4 1 g, $FeCl_2{\cdot}6H_2O$ 0.5 g, $MgSO_4{\cdot}7H_2O$ 1 g, $CaCl_2{\cdot}7H_2O$ 0.2 mg, sodium succinate 8.5 g, BTB reagent 1 mL [1.5% in ethanol], and agar 20 g. The DM medium per liter [13] (pH 7.2) contained: sodium acetate 4.72 g, $NaNO_2$ 0.05 g, KH_2PO_4 1.5 g, Na_2HPO_4 0.42 g, and $MgSO_4{\cdot}7H_2O$ 0.2 g. LB medium (pH 7.0; per liter) included tryptone 10.0 g, yeast extract 5.0 g, and NaCl 10 g. For the preparation of LB solid plates, 2% (w/v) agar was added.

2.2. Identification of the hypothermia and aerobic nitrite-denitrifying bacterium strain Y-10

Colony morphologies of strain Y-10 were monitored on the BTB medium plates after incubating at 15˚Cfor 3 d. Cell morphologies were observed under the HITACHI S-3000N scanning electron microscope. Gram staining was examined using colonies on the BTB plates.

PLFAs were extracted using about 40 mg pure culture of strain Y-10. Each type of PLFAs was analyzed by Agilent 6850.

The nearly full-length 16S rRNA gene sequence was amplified using DNA as template which was extracted by genomic DNA purification kit (Thermo scientific). The universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) for polymerase chain reaction (PCR) amplification were synthesized by Invitrogen. The PCR amplification was conducted in the 50 mL volume containing 2 μL of DNA, 2 μL of primer, 25 μL of 2 × Taq PCR Master Mix, and 19 μL of sterile water. The PCR conditions were denaturation for 5 min at 94˚C; 30 cycles of 1 min at 94˚C, 30 s at 55.5˚C, 1 min at 72˚C; and extension for 10 min at 72˚C. The 1.5 kb product was separated on a 1.5% agarose gel and purified by BioSpin gel extraction kit (BioFlux). The purified product cloned into pMD®20-T vector (Takara) and then sequenced by Invitrogen company. Then, the 16S rRNA sequence of strain Y-10 was submitted to NCBI for accession number. Sequence alignment and multiple alignment were performed using NCBI Search Tool program (BLAST: http://www.ncbi.nlm.-nih.gov/blast/Blast.cgi) and CLUSTAL W. And, the phylogenetic tree was constructed using MEGA 6.0 software by neighbor-joining distance method and bootstrap analysis of 1,000 replicates.

2.3. Growth characteristics

The growth characteristics of strain Y-10 was investigated in a 250-mL conical flask containing 100 mL of DM broth medium that was incubated at 15˚C with 150 r/min shaking speed. The OD_{600} values were

determined every 12 h using BECKMAN COULTER (DU800) at the wavelength of 600 nm.

2.4. Effects of culturing conditions on the denitrification ability of strain Y-10

Effects of six cultivation conditions including temperature (5, 10, 15, 20, 25, 30, and 35°C), shaking speed (0, 50, 100, 150, and 200 r/min), pH (5.0, 6.0, 7.0, 8.0, and 9.0), inoculation quantity (0.5×10^8, 1.0×10^8, 1.5×10^8, 2.0×10^8, and 2.5×10^8 CFU within 100 mL DM), carbon source (sodium citrate, sodium succinate, sodium acetate, sucrose, and glucose), and nitrite concentrations (10, 50, 100, 150, and 200 mg/L) on denitrification performance of strain Y-10 were determined by the single-factor tests. About 1.0×10^8 CFU precultured bacterial suspension was inoculated into a 250-mL sterilized conical flask which contained 100 mL of DM broth medium for testing, except for inoculation quantity which will be specified latter. After incubating for 48 h, nitrite and total nitrogen were then determined for these samples, in order to analyze the influences of the various factors indicated above. The nitrite and total nitrogen removal efficiencies were calculated by the equation: $Rv = (T_1 - T_2)/T_1 \times 100\%$ to assess the denitrification ability of strain Y-10. Note that Rv, T_1, and T_2 represent nitrite (or total nitrogen) removal efficiency, the initial concentration of nitrite (or total nitrogen) in DM broth medium, and the final concentration of nitrite (or total nitrogen) in DM broth medium after incubation for 48 h, respectively. All experiments were conducted in triplicate.

2.5. Analytical methods

Nitrite nitrogen was determined at the wavelength of 540 nm after adding 1 mL of chromogenic reagent including 0.1 mL of phosphoric acid, 0.002 g of N-(1-naphthyl)-1,2-diaminoethane dihydrochloride, and 0.04 g of sulfanilamide [22]. Total nitrogen was calculated by the absorbance value at 220 nm subtracting the two times background absorbance value at 275 nm using alkaline potassium persulfate digestion chromatography [23].

2.6. Statistical analysis and graphical work

Statistical analysis and graphical work were carried out using Excel, SPSS Statistics, MEGA6.0, and Origin8.6. The results were presented as means ± SD (standard deviation of means).

3. Results and discussions

3.1. Bacterial identification and growth characterization

Colony morphologies of strain Y-10 are white circular, viscous, and smooth with dry surfaces when it grows on the BTB solid plate after incubation at 15°C for 3 d (Fig. 1(A)). Cells are Gram-positive, short rod, non-spore, and without flagellum (Fig. 1(B) and (C)) that are in accordance with the results of *A. arilaitensis* reported by Carter et al. [24] and Irlinger et al. [25].

As a key component of cell membrane, PLFAs are an important indicator for identifying bacteria and fungi, which have been proven to be relatively simple, fast, inexpensive, and yet sensitive to analyze by gas chromatography [26,27]. The PLFAs of strain Y-10 that are determinate by the Sherlock® Microbial identification system with version 6.0 show a 0.619 similarity index with *Arthrobacter mysorens* (Fig. 2). However, we cannot locate the species of *A. arilaitensis* from the library of RTSBA6, RCLIN6, and MI7H10 of the Sherlock® Microbial identification system. The PLFAs of strain Y-10 shows a higher similarity index with *A. arilaitensis*.

Approximately 1,465 bp 16S rRNA gene sequences of strain Y-10 are obtained via the PCR and sequencing. The 16S rRNA gene sequences exhibit 99% similarity with *A. arilaitensis*. MEGA 6.0 software is used for constructing the phylogenetic tree and also indicates that a clear evolutionary divergence of strain Y-10 is closely related to *A. arilaitensis* instead of *A. mysorens* (Fig. 3). Strain Y-10 is further corroborated as *A. arilaitensis* by the combination of morphology, PLFA analyses, and 16S rRNA gene sequence, which offers the taxonomic assignment with higher confidence. The 16S rRNA gene sequence of strain Y-10 has been submitted to GenBank nucleotide sequence databases under accession number KP410739. Up to date, few members of *Arthrobacter genus* have been reported to be able to denitrify at low temperatures. Additionally, strainY-10 is deposited in China Center for Type Culture Collection with the collection number of CGMCC No. 10536.

According to strain Y-10 growth characteristics in DM broth medium, the growth curve is created for 180 h (Fig. 4). The result shows that strain Y-10 with single colony is inoculated into the DM medium up to logarithmic phase within 60 h and possess a long platform stage for 108 h at 15°C, indicating strain Y-10 can maintain the denitrifying activity for rather a long time in the nitrite-polluted wastewater.

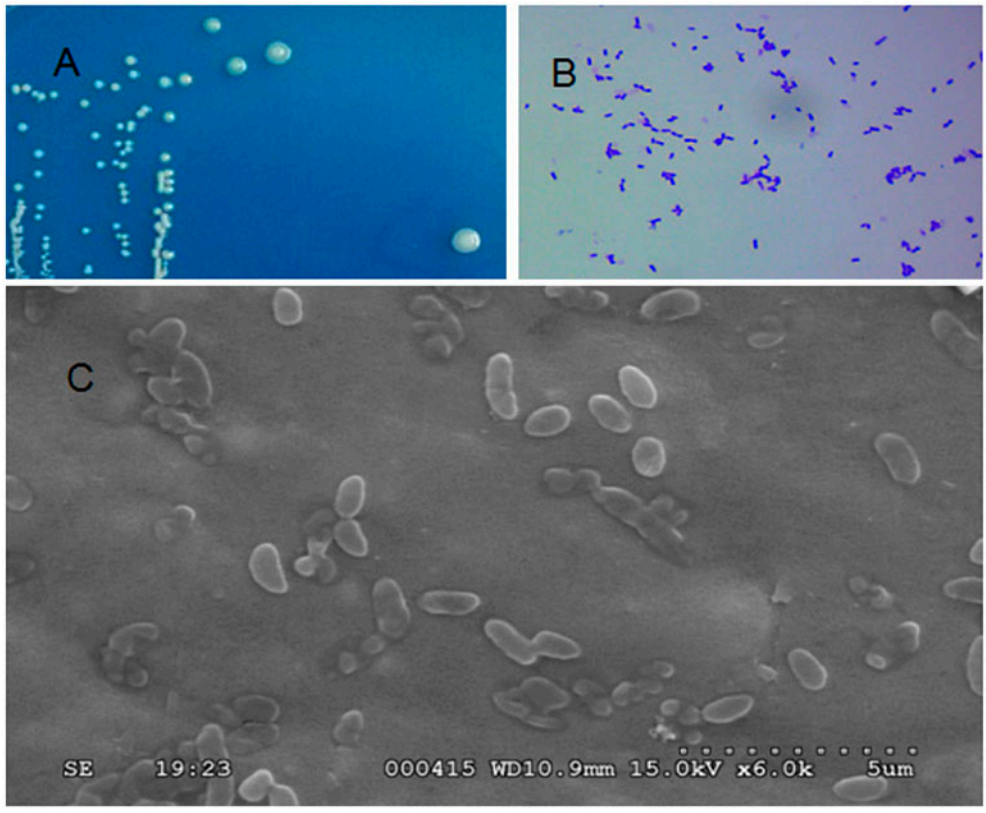

Fig. 1. The morphologies of the strain Y-10. Colony on BTB plates (A), unicell morphology of strain Y-12 (10 × 100) (B), and cells under the scanning electron microscope (6,000×) (C).

Matches:

Library	Sim Index	Entry Name
RTSBA6 6.21	0.619	Arthrobacter-mysorens
	0.498	Bacillus-megaterium-GC subgroup B
RCLIN6 6.20	0.387	Micrococcus-luteus-GC subgroup A
	0.303	Arthrobacter-pascens
	0.266	Bacillus-circulans-GC subgroup A
	0.246	Micrococcus-lylae-GC subgroup A
MI7H10 3.80		(No Match)

Fig. 2. The result of the specific PLFAs identification.

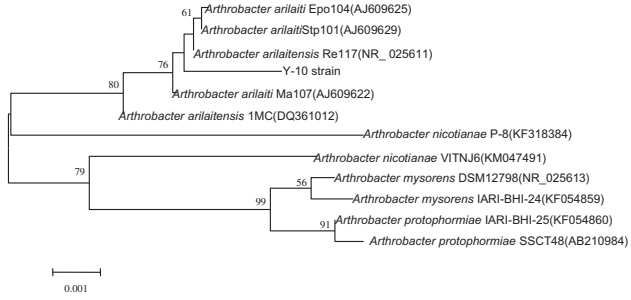

Fig. 3. Phylogenetic tree of the Y-10 strain. Numbers in parentheses represent the sequences' in GenBank. The number at each branch points is percentage supported by bootstrap (1,000 re-samplings). Bar: 0.1% sequence divergence.

3.2. *Effect of temperature on nitrogen removal*

Temperature is a key parameter for affecting the nitrogen removal process. Previous studies report that functions of nitrification and denitrification will be strongly inhibited at temperatures below 10℃ [28,29]. According to the previous works [30], the optimal temperature of most aerobic denitrifiers range from 25 to 40℃. Few reports are about the performances of nitrite aerobic denitrifiers at low temperatures. The nitrogen removal performances of strain Y-10 at different temperatures are evaluated by the nitrite and total nitrogen removal efficiencies in shake flask experiments. The results in Fig. 5(A) show that the nitrite and total nitrogen removal efficiencies increase with temperature (from 5 to 15℃) and nitrogen removal efficiency decreases significantly when the temperature further increases up to 30℃. It also shows that the nitrite and total nitrogen removals are strongly inhibited below 10℃. However, strain Y-10 can survive between 5 and 35℃. In combination with

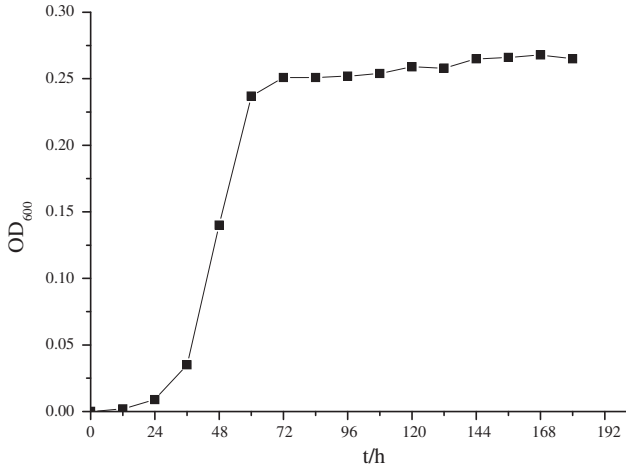

Fig. 4. The growth curve of the Y-10 in the denitrification broth medium.

the maximal nitrite and total nitrogen removal efficiencies, it is concluded that the optimal temperature is 15℃ for the aerobic denitrifying activity of strain Y-10. The nitrite and total nitrogen removal efficiencies, respectively, reach the peak values of 100 and 52.8% at 15℃ (48 h), which improve significantly than those of psychrophilic aerobic denitrifying bacterium of S1-1 strain with the nitrite and total nitrogen removal efficiencies of 63.50 and 30.30% under 160 r/min rotation speed at 20℃ (5 d) [31]. As reported previously [32–34], temperature does not only affect the biological growth, but also denitrify the activity. The optimal temperature for some of denitrifiers is more than 30℃ such as *Acinetobacter junii* YB (37℃) [35], *Bacillus licheniformis* (32.5℃) [36], *Alcaligenes faecalis* strain No. 4 (30℃) [37], and *Acinetobacter* sp. C-4(30℃) [38]. Few nitrifiers or denitrifiers can tolerate the low-temperature conditions. However, the optimal temperature for the cell growth and its aerobic denitrifying activity of *A. arilaitensis* strain Y-10 is as low as 15℃. This clearly demonstrates that strain Y-10 is a psychrophilic aerobic denitrifying bacterium.

3.3. Effects of shaking speed on nitrogen removal

In the nitrite aerobic denitrification process, both oxygen and nitrite act as electron acceptors, but oxygen can also inhibit the nitrous oxide reductase which may enhance the production of N_2O [13]. In order to clarify the effect of DO concentration on the denitrification of strain Y-10, five different DO concentrations that are represented by shaking speeds are presently used. As depicted in Fig. 5(B), nitrite nitrogen is almost fully removed by strain Y-10 when the shaking speed reaches 50 r/min, and the removal efficiency has no observable changes when the shaking speed increases from 100 to 200 r/min. This is in contrast to the previous report that nitrite reductase is extremely sensitive to DO concentration ranging from 1 to 3.6 mg/L [39]. It has been indicated that the oxygen level with 120 r/min shaking speed is equivalent to 7.0 ± 0.2 mg/L [40]. Obviously, strain Y-10 can perform denitrification when DO concentration is beyond 3.6 mg/L. The results are in accordance with the previous work that the *Pseudomonas stutzeri* can tolerate high DO concentrations [41]. The peaks of the nitrite and total nitrogen removal efficiencies at 15℃ are 100 and 51.0% with 150 r/min shaking speed. However, the total nitrogen removal efficiency is inhibited only slightly at the shaking speed of 200 r/min, probably due to that the nitrite reductase is sensitive to high concentration of oxygen inhibition [42]. Accordingly, it has clearly demonstrated that strain Y-10 can tolerate high DO concentrations.

3.4. Effect of pH on nitrogen removal

The bacterium growth and nirS gene are important for denitrification, which can be affected significantly by pH [43]. Effect of different pH on the aerobic denitrification characteristics of strain Y-10 is illustrated in Fig. 5(C). The nitrite and total nitrogen removal efficiencies increase with the increase in pH values from 5.0 to 9.0, and it has been found that the nitrite removal performance does not change obviously when pH is higher than 7.0, but the total nitrogen removal efficiency continues to increase remarkably. These results demonstrate that the nitrite and total nitrogen removal rates are higher under alkaline conditions (pH 8.0–9.0) than acidic conditions (pH 5.0–6.0). The highest removal efficiency (15℃) is detected at pH 9.0 after 48 h cultivation, with the corresponding nitrite and total nitrogen removal efficiencies of 100 and 84.0%. These are contrary to some previous results that the optimal pH range from 6.5 to 7.0, such as *Psychrobacter* sp. (6.5) [31], *Pseudomonas mendocina* 3–7 (7) [29], and *Ochrobactrum* sp. (6.5–7.0) [44]. Additionally, an aerobic denitrifying bacterium is found that it has the highest denitrification efficiency at pH 7.0, and it will lose the denitrification ability at pH below 5.5 and above 9.0 [45]. Apparently, the denitrification performance of strain Y-10 is strongly inhibited under acidic conditions, and the nitrite and total nitrogen removal efficiencies are merely 1.76–6.0% and 1.42–4.19% at pH 5.0 and 6.0, respectively. This indicates that the nitrite reductase of strain Y-10 is sensitive to acidic conditions, but tolerant to alkalinity during the denitrification process. That is, strain Y-10 is an alkali-tolerant denitrifying bacterium.

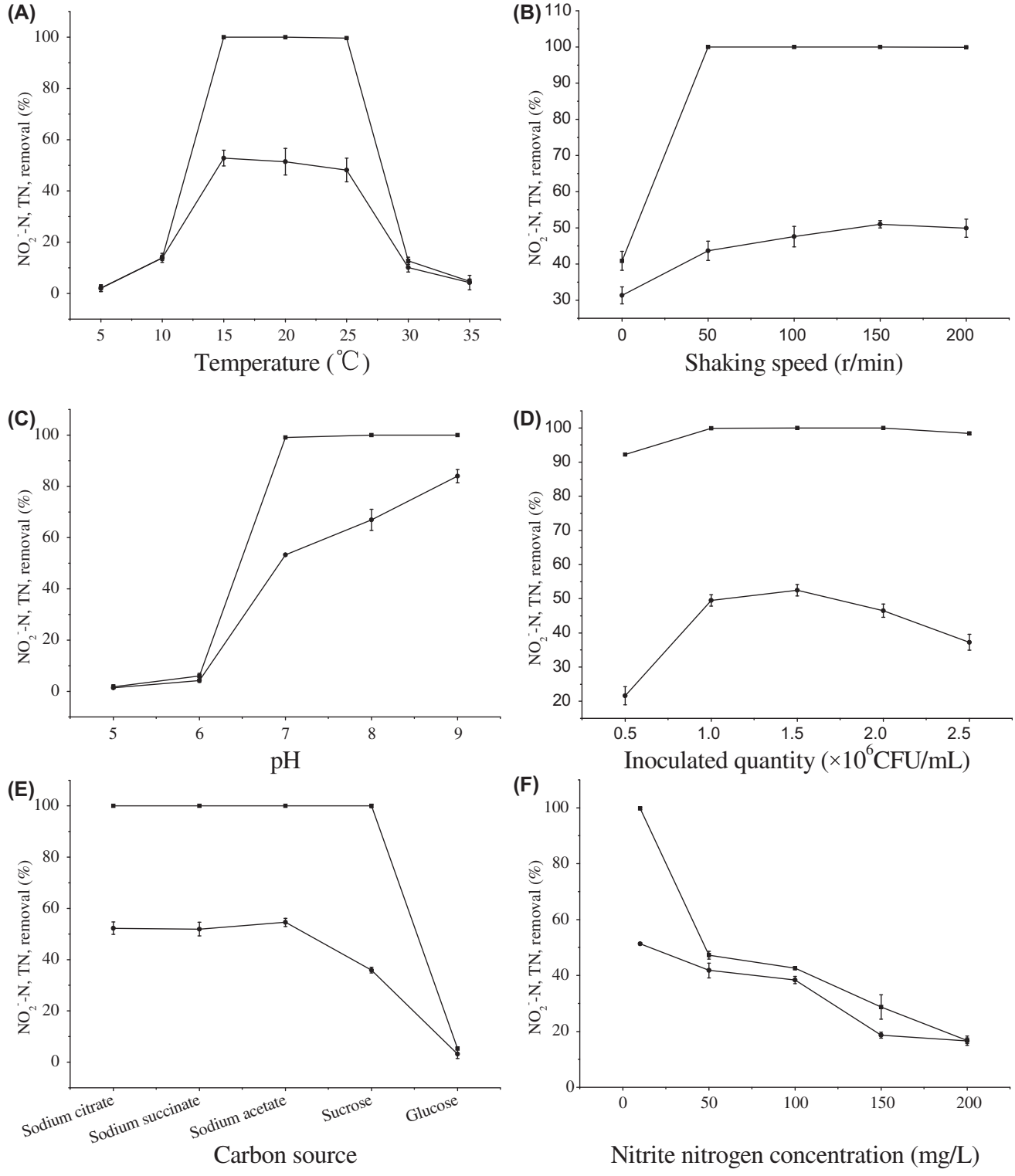

Fig. 5. Effect of factors on denitrification ability of strain Y-10. Temperature (A), shaking speed (B), pH (C), inoculated quantity (D), carbon source (E), and nitrite nitrogen concentration (F). (■) removal percentage of NO_2^--N; (●) removal percentage of total nitrogen. Values are means ± SD (errors bars) for three replicates.

3.5. Effect of inoculated quantity on nitrogen removal

Inoculated quantity is one of the most important factors to affect the aerobic denitrification. Shortage of inoculated quantity results in the ineffective removal of nitrogen, whereas inoculated amount too much will cause biological competition for nutrients which may lead to the death of bacteria. Thus, a proper inoculum is helpful to improve the nitrogen removal efficiency of denitrification. The effect of inoculums quantity on nitrite and total nitrogen removal characteristics of strain Y-10 is illustrated in Fig. 5(D). The results show that the nitrite and total nitrogen removal efficiencies increase with the increase in bacterial density from 0.5×10^6 to 1.5×10^6 CFU/mL. When the bacterial density equals to 0.5×10^6 CFU/mL, the nitrite and total nitrogen removal efficiencies are only 92.18 and 21.6%, while when the bacterial density reaches 1.0×10^6 CFU/mL, the removal efficiencies amount to 99.89 and 49.48%, respectively (Fig. 5(D)). The nitrite and total nitrogen removal efficiencies ascend to their respective peaks (100 and 52.5%) at the bacterial density of 1.5×10^6 CFU/mL. The total nitrogen removal efficiency decreases sharply from 46.5 to 32.27% as the bacterial density further increases from 2.0×10^6 to 2.5×10^6 CFU/mL.

3.6. Effect of carbon source on nitrogen removal

Different carbon sources with distinct structure and molecules are usually used as electron donors to reduce nitrite to N_2 progressively, which can affect the efficiency of the aerobic denitrification [46]. In addition, the sufficient carbon source can provide more energy for bacterium growth. The influence of different carbon sources on the aerobic denitrification of strain Y-10 is shown in Fig. 5(E). The results indicate that strain Y-10 can conduct denitrification by utilizing most types of carbon sources including sodium citrate, sodium succinate, sodium acetate, sucrose, and glucose. However, the difference between the hypothermia and aerobic denitrfication abilities is significantly affected by the different carbon sources. The removal efficiencies of nitrite nitrogen with sodium citrate, sodium succinate, sodium acetate, or sucrose used as carbon sources are significantly higher than that with the use of glucose. The carbon sources except glucose can finely support the nitrite removal, with nitrite nitrogen removal efficiency of 100%. The total nitrogen removal efficiency of strain Y-10 is by far more effective when using sodium citrate, sodium succinate, and sodium acetate as the sole carbon source rather than using sucrose or glucose. These results imply that glucose is not beneficial for nitrogen removal of strain Y-10 at 15°C. With consideration of the total nitrogen removal efficiency, the optimal carbon source for the aerobic denitrification of strain Y-10 is sodium acetate, in contrast to that the sodium succinate, is the best carbon source for the previously reported strain *Pseudomonas* sp. [47]. The total nitrogen removal efficiency of strain Y-10 is up to 54.55% in 48 h at 15°C with sodium acetate as the sole carbon source. It can be concluded here that strain Y-10 can use the carbon source of sucrose to survive and remove nitrite nitrogen significantly but not to remove total nitrogen efficiently.

3.7. Effect of nitrite nitrogen concentration on nitrogen removal

Nitrite nitrogen can act as electron acceptors and nitrogen source for denitrifier during the denitrification process. Nitrite is also a well-documented toxic substance for aquatic and terrestrial animals [1,6]. However, few studies have been reported regarding to whether the nitrite nitrogen is toxic to the denitrification bacterium. Effects of different nitrite nitrogen concentrations on the hypothermia and aerobic nitrite-denitrifying performances of strain Y-10 are shown in Fig. 5(F). The nitrite and total nitrogen removal efficiencies of strain Y-10 decrease continuously with the increase of nitrite nitrogen contents from 10 to 200 mg/L. The highest nitrite and total nitrogen removal efficiencies (15°C, 48 h) are 99.75 and 51.37% at the nitrite nitrogen concentration of 10 mg/L, and then, decrease significantly to 16.69 and 16.63%, respectively, when the concentration of nitrite nitrogen increases up to 200 mg/L. This suggests that *A. arilaitensis* strain Y-10 can survive and perform the nitrogen removal in spite of the concentration of nitrite nitrogen is as high as 200 mg/L, different from the opinions that nitrite harms the cell growth so that inhibits the denitrifying process [48] and some of denitrifiers cannot remove nitrite when nitrite is the sole nitrogen source, such as *Paracoccus versutus* LYM [49]. From the above results, it can be concluded that strain Y-10 has great application potential at different concentrations of nitrite nitrogen and may play an important role in eutrophic wastewater, especially at low-temperature circumstances.

4. Conclusion

This study is the first report on *A. arilaitensis*, named Y-10, as a hypothermia and aerobic nitrite-denitrifying bacterium. Strain Y-10 exhibits the highest aerobic denitrification ability at pH 9.0, where the

nitrite and total nitrogen removal efficiencies are up to 100 and 84.0%, respectively. In addition, strain Y-10 can perform denitrification at a wide range of nitrite loadings, implying that it can tolerate rather high concentrations of nitrite nitrogen. Accordingly, this strain can be potentially used as aerobic denitrifier for disposing of alkaline wastewater, especially at low-temperature circumstances.

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