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Heterotrophic nitrogen removal by *Acinetobacter* sp. Y1 isolated from coke plant wastewater

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A strain of *Acinetobacter* sp. Y1, which exhibited an amazing ability to remove ammonium, nitrite and nitrate, was isolated from the activated sludge of a coking wastewater treatment plant. The aim of this work was to study the ability, influence factors and possible pathway of nitrogen removal by *Acinetobacter* sp. Y1. Results showed that maximum removal rate of NH_4^+-N by the strain was 10.28 mg-N/L/h. Carbon source had significant influence on the growth and ammonium removal efficiencies of strain Y1. Pyruvate, citrate and acetate were favourable carbon sources for the strain. Temperature, pH value and shaking speed could affect the growth and nitrogen removal ability. Nitrate or nitrite could be used as a sole nitrogen source for the growth and removed efficiently by the strain. N_2 levels increased to 53.74%, 50.21% and 55.13% within 36 h when 100 mg/L NH_4^+-N, NO_2^--N or NO_3^--N was used as sole nitrogen source in the gas detection experiment. The activities of hydroxylamine oxidoreductase (HAO), nitrate reductase (NR) and nitrite reductase (NiR), which are key enzymes in heterotrophic nitrification and aerobic denitrification, were all detectable in the strain. Consequently, a possible pathway for ammonium removal by the strain was also suggested.

[**Key words:** *Acinetobacter*; Heterotrophic nitrification; Aerobic denitrification; Nitrogen removal; Coke plant wastewater]

The traditional ammonium removal used in wastewater treatment involves two separate steps: aerobic nitrification by autotrophs and anaerobic denitrification by heterotrophs (1). Nitrification and denitrification are generally carried out by adopting individual bioreactors or different aeration intervals because of the different demands for oxygen and organic matter (2,3). Furthermore, autotrophic nitrifiers are sensitive to high loads of ammonium and organic matter (4), and their slow growth rate generally make the nitrification become the rate-limiting step (2,5). All these inevitably increase the operating cost and the difficulty in treatment condition control. Recently, bacteria which are both heterotrophic nitrifier and aerobic denitrifier have attracted increasing attention. Under aerobic conditions, ammonium compounds can be converted to gaseous products by the pure cultures of these heterotrophic microorganisms with concomitant removal of carbon compounds (2).

Up to now, certain groups of heterotrophic nitrifying-aerobic denitrifying microorganisms have been isolated and reported, such as *Alcaligenes faecalis* (6), *Acinetobacter calcoaceticus* (7) and *Bacillus subtilis* (8). They have different characteristics in the intermediates accumulation, nitrogen removal ability and removal pathway (9). It is difficult to generalize the biochemical mechanisms of heterotrophic nitrification and aerobic denitrification according to these limited species. Therefore, further investigation based on a large amount of species is necessary.

In this study, a heterotrophic nitrifying–aerobic denitrifying bacterium, which showed the high-strength nitrogen removal ability, was isolated in our laboratory from the activated sludge of a coking wastewater treatment plant. The aim of the present study is to determine the capability of nitrogen removal under different conditions and aerobic nitrogenous gas production by the strain. In addition, the assay of the key enzymes in nitrification and denitrification was also conducted to propose the possible pathway of nitrogen metabolism in the strain.

MATERIALS AND METHODS

Culture media Beef extract peptone, used as enrichment medium, contained the following components (per liter): 5 g of meat extract, 10 g of peptone and 5 g of NaCl. The ingredients of the basic medium (BM) were as follows (per liter): 4.90 g of trisodium citrate, 0.47 g of $(NH_4)_2SO_4$, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.20 g of K_2HPO_4, 0.12 g of NaCl, 0.01 g of $MnSO_4 \cdot 4H_2O$, 0.01 g of $FeSO_4$. Unless mentioned otherwise, the pH of the media was adjusted to 7.0.

Isolation of bacteria An activated sludge sample was taken from the aeration tank of the coking wastewater treatment plant located in Shanxi Province, North China. Sample of 20 mL sludge was aseptically added to 180 mL of enrichment medium in a conical flask and then incubated at 30°C in a rotary shaker at 120 rpm. After 7 days of cultivation, the sample was serially diluted (10^{-1}–10^{-10}). The dilution (0.1 mL) from each 10^{-3} to 10^{-10} was spread on the BM agar plates. The agar plates were cultivated at 30°C to obtain colonies. Different colonies were selected and transferred into sterile BM broth. Cultures were sampled periodically and tested for their ability to remove ammonium, form nitrite and nitrate using the Nessler's reagent, the Griess-Ilosvay method and the phenol-disulphonic method, respectively (10). The culture broth showing significant ammonium removal ability was selected and purified several times until pure single colony was obtained.

Identification of the isolated bacterium The elementary identification was performed according to the morphological and biochemical characteristics with

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reference to Bergey et al. (11). The further identification was confirmed using 16S rRNA gene sequence analysis. DNA extraction, PCR amplification and 16S rRNA gene sequence analysis were entrusted to TaKaRa Biotechnology Co., Ltd., Dalian, China. The sequence was compared with other 16S rRNA gene sequences by way of BLAST in NCBI. The related sequences were selected and aligned by the Clustal X program (12). Phylogenetic tree was constructed by MEGA 4.1 (13).

Measurement of heterotrophic nitrification One milliliter inoculum of the strain grown in the BM broth about 20 h was inoculated into 250 mL conical flasks with 100 mL fresh BM. The flasks were cultivated at 30°C in a rotary shaker at 120 rpm for 32 h. During incubation, the cultures were sampled periodically to determine the growth of the strain, the pH values and the levels of NH_4^+-N, NO_2^--N, NO_3^--N, NH_2OH-N and total nitrogen (TN).

Factors affecting heterotrophic nitrification To determine the impact of carbon sources, sodium acetate, sodium pyruvate, sucrose, sodium carbonate, glucose and sodium citrate were used as the sole carbon source in the BM. The NH_4^+-N concentration was maintained at 100 mg/L in the culture medium. The amount of each carbon source was adjusted to maintain the C/N ratio of 14.

The role of pH value, cultivation temperature and shaking speed on the nitrifying capacity of the isolated bacterium was assessed in the BM. The initial pH values of the media were successively adjusted to 5, 6, 7, 8 and 9. Cultivation temperatures were set at 20°C, 25°C, 30°C, 35°C, 40°C and 45°C. To observe the effect of dissolved oxygen (DO) concentration on ammonium removal, the shaking speed was adjusted to 40 rpm, 80 rpm, 120 rpm and 150 rpm, respectively. Flasks without inoculation were employed as controls.

One milliliter culture grown in the BM about 20 h was inoculated into 250 mL conical flasks containing 100 mL fresh BM. Unless otherwise noted, the initial pH of the media was adjusted to 7, and the flasks were incubated at 30°C in a rotary shaker at 120 rpm.

Consumption of nitrogen sources To elucidate the denitrification mechanism, hydroxylamine, nitrite and nitrate were respectively used instead of ammonium as the sole nitrogen source in the BM. NH_2OH-N, NO_2^--N and NO_3^--N were adjusted to 100 mg/L, respectively. All these media were placed in 250 mL conical flask, and 1 mL culture grown in the BM about 20 h was inoculated. The cultivation conditions were the same as described above.

Gas detection experiment The BM (100 mL), in which ammonium, nitrite and nitrate were used as the sole nitrogen resource, was inoculated with 1 mL of the bacterial suspension and sealed in 250 mL serum bottle. The serum bottles were aerated with pure oxygen at constant pressure (0.6 MPa) for 5–6 min. Then the bottles were cultivated at 30°C and 120 rpm. The gas samples were collected periodically by gas tight syringes and detected the changes of N_2 using gas chromatography (SP-2100, Beifen-Ruili, China). The serum bottles without inoculation served as controls.

Enzyme assay The bacteria cultivated in the BM broth about 16 h were harvested by centrifugation (4°C, 10,000 rpm, 15 min). Then the bacterial cells were suspended in a 0.01 M potassium phosphate buffer (pH 7.4) and disrupted by ultrasonication. The whole cells and cell debris were removed by centrifugation at 15,000 rpm and 4°C for 30 min. The supernatant was immediately used for the enzyme assay.

Hydroxylamine oxidoreductase (HAO) activity was determined by the reduction of potassium ferricyanide as described by Otte et al. (14). The activities of nitrate reductase (NR) and nitrite reductase (NiR) were determined by the reduction of NADH according to Zhao et al. (7). Protein concentration in the crude extract was detected by the Bradford Reagent Kit (Sangon, Shanghai). One enzyme unit (U) was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of substrate per minute. The specific activity was defined in terms of enzyme units per mg protein (U/mg). The tests were started by adding substrate. Potassium phosphate buffer solution (vide supra) instead of supernatant in the reaction system was used as control.

Analytical methods The growth of the strain was determined by measuring the optical density of the culture broth using a spectrophotometer at 600 nm (OD_{600nm}). The culture samples were centrifuged at 12,000 rpm for 20 min and filtered through a membrane filter, and then the filtrate was used for the following chemical analysis. The concentration of NH_4^+-N was analyzed by Nessler's reagent photometry. NO_2^--N concentration was determined by *N*-(1-naphthalene)-diaminoethane photometry method. NO_3^--N concentration was measured by ion chromatography (Dionex ICS-90). The concentration of hydroxylamine was determined according to the method of Frear and Burrell (15). TN was analyzed using a TOC/TN analyzer (Shimadzu TNM-1). The tests were conducted in triplicate.

RESULTS

Isolation and identification of the bacterium The isolated bacterium, which exhibited the high-strength nitrogen removal characteristic, was named as strain Y1. Colonies of strain Y1 on the BM agar plate were off-white, glossy and round. The diameter of the colony was about 4–4.5 mm. The strain was gram-negative, short rod-shaped (approximately 0.8–1.1 μm × 0.5 μm). The results of oxidase, catalase, nitrate reduction were positive, while indole test, methyl red test, hydrolysis of gelatin were negative.

A fragment of 1452 16S rRNA gene was obtained from PCR and sequencing. A BLAST search of available data from the GenBank database showed that strain Y1 had a high level of sequence similarity (99.0%) to *Acinetobacter* sp. YC-X2 (HM629335.1) (Fig. 1). Therefore, the bacterium was named *Acinetobacter* sp. Y1 and selected for further study. Ultimately, the 16S rRNA gene sequence of strain Y1 was submitted to GenBank under the accession number JX867369.

Heterotrophic growth and ammonium removal by strain Y1 Fig. 2 shows the growth and nitrogen removal during the heterotrophic nitrification by *Acinetobacter* sp. Y1. After a lag phase of 4 h, strain Y1 began to enter into the exponential phase that lasted for about 20 h. During the exponential growth phase, NH_4^+-N decreased rapidly from 108.8 mg/L to 0.96 mg/L. The removal ratio of NH_4^+-N reached 99% in 20 h, and the maximum removal rate was 10.28 mg-N/L/h. Small amounts of

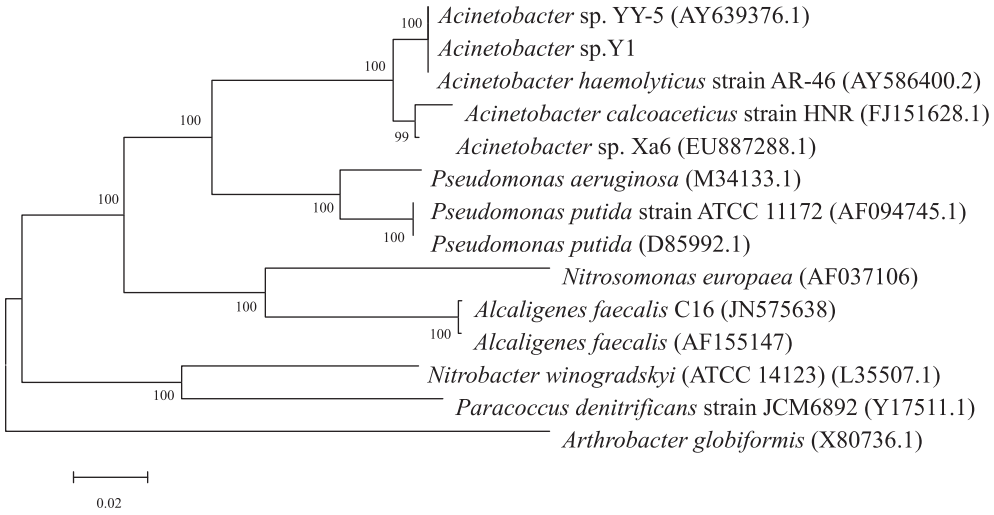

FIG. 1. Phylogenetic tree of *Acinetobacter* sp. Y1 and other correlative species based on 16S rRNA gene sequences comparisons. Bootstrap values obtained with 1000 repetitions are indicated as percentages at all branches. The bar represents 2 nucleotide substitution per 100 nucleotides.