

Screening and identification of aerobic denitrifiers

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Abstract. With the standards of the effluent quality more stringent, it becomes a quite serious problem for municipalities and industries to remove nitrogen from wastewater. Bioremediation is a potential method for the removal of nitrogen and other pollutants because of its high efficiency and low cost. Seven predominant aerobic denitrifiers were screened and characterized from the activated sludge in the CAST unit. Some of these strains removed 87% nitrate nitrogen at least. Based on their phenotypic and phylogenetic characteristics, the isolates were identified as the genera of *Ralstonia*, *Achromobacter*, *Aeromonas* and *Enterobacter*.

1. Introduction

For a long time, removing COD (chemical oxygen demand) and suspended solids was the main goal for the treatment of wastewater, while nitrogenous compounds removal was not taken into full consideration. In recent years, the pollution of nitrogenous compounds has been seriously increasing, because of the fast development of economy and urbanization, which have endangered the safety of environmental water in China, particularly in drinking water. For nitrogen removal from wastewater, nitrification and denitrification processes by microbes are the most easily available on account of their low maintenance cost, as well as the effective contaminant removal performance [1][2]. However, nitrification demands an aerobic condition for successful oxidation of ammonium and nitrite, and denitrification uses electron donors in the anoxic environment conversely. Thus conventional theories have assumed that nitrification and denitrification can only be achieved in different levels of oxygen, which were complicated operations.

In 1984, aerobic denitrification was first proposed by Robertson and Kuenen [3]. Since then, many reports describing aerobic denitrification have been published [4][5][6]. Aerobic denitrification by microbes offers a new way for biological degradation of nitrogen. That is to say, aerobic denitrification, which is conducted by aerobic denitrifiers, may provide the potential to eliminate the requirement for two separate tanks and reduce sludge yield[7]. The alkalinity consumption during the nitrification process can be partly compensated by the accumulation of the alkalinity in denitrification, so that pH of



the biosystem keeps relatively stable. In addition, some aerobic denitrifiers are able to conduct combined heterotrophic nitrification and aerobic denitrification [8][9][10].

The purpose of this writing is to screen aerobic denitrifiers from activated sludge in CAST unit, and evaluate the characterization of these denitrifiers.

2. Materials and methods

2.1. Sample sites

The sample was obtained from the CAST unit in the Liaohe petrochemical sewage treatment plant and then stored in a sealed bottle. The sample was resuspended in the enrichment medium to obtain a homogeneous suspension.

2.2. Media

We used a denitrification medium (DM), a R₂A medium, a screening medium and denitrifying performance detecting cultures in this research.

The constituents of the DM were as follows: 7.9 gL⁻¹ Na₂HPO₄·7H₂O; 1.5 gL⁻¹ KH₂PO₄; 0.3 gL⁻¹ (NH₄)₂SO₄; 0.5 gL⁻¹ MgSO₄·7H₂O; 4.7 gL⁻¹ sodium succinate; 1.5 gL⁻¹ KNO₃, and 2mL trace element solution. Adjusting the pH of the cultures to 7.5 is necessary [11]. The components of trace element solution were as follows: 50 gL⁻¹ EDTA; 2.2 gL⁻¹ ZnSO₄; 5.5 gL⁻¹ CaCl₂; 5.1 gL⁻¹ MnCl₂·4H₂O; 1.1 gL⁻¹ (NH₄)₆Mo₇O₂·4H₂O; 5.0 gL⁻¹ FeSO₄·7H₂O; 1.6 gL⁻¹ CuSO₄·5H₂O; 1.6 gL⁻¹ CoCl₂·6H₂O.

The constituents of the R₂A medium were as follows: 0.5 gL⁻¹ yeast extract; 0.3 gL⁻¹ K₂HPO₄; 0.5 gL⁻¹ peptone-D; 0.5 gL⁻¹ casamino acid; 0.5 gL⁻¹ D-glucose; 0.5 gL⁻¹ starch soluble; 0.05 gL⁻¹ MgSO₄·7H₂O; 0.3 gL⁻¹ C₃H₃NaO₃. 15 g agarose was added to the R₂A mixture as needed for plating.

The screening medium was comprised of following reagents: 1 gL⁻¹ KNO₃; 0.2 gL⁻¹ CaCl₂; 1 gL⁻¹ KH₂PO₄; 0.5 gL⁻¹ FeCl₃·6H₂O; 1 gL⁻¹ MgSO₄·7H₂O; 8.6 gL⁻¹ sodium succinate; 21 gL⁻¹ agarose; 1mL bromothymol blue. The final pH of the medium was adjusted to 7.0±0.2.

Denitrifying performance detecting cultures were comprised of following reagents: 1.31 gL⁻¹ C₆H₅Na₃O₂·2H₂O; 1.10 gL⁻¹ CH₃COONa; 0.361 gL⁻¹ KNO₃; 0.2 gL⁻¹ MgSO₄·7H₂O; 1.0 gL⁻¹ KH₂PO₄; 5.0 gL⁻¹ K₂HPO₄; 0.5 gL⁻¹ NaCl; and 1mL trace element solution. Adjusting the pH of the cultures to 7.4 is necessary.

2.3. Accumulation culture and screening of aerobic denitrifiers

To cultivate the aerobic denitrifiers, 10mL of the sample was transferred into the Erlenmeyer flask with 250 mL of the denitrification medium. The Erlenmeyer flask was sealed with a breathable sealing membrane, and cultivated in a shaker at 30°C and 175 rpm for two days. After enrichment, the resultant bacterial suspensions with different concentration gradient were plated on screening agar media and incubated aerobically. Colonies with different apparent characteristics covered the agar plates, and each colony was selected to fresh screening mediums until purified isolates were obtained. In order to detect nitrogen removal performance, isolates were incubated in the denitrifying performance detecting cultures, using KNO₃ as the sole nitrogen source. In this study, seven single strains owning high nitrogen removal efficiency were selected, and the strains were stored on R₂A medium in the fridge at 4°C for further use.

2.4. Identification of the aerobic denitrifiers

The experiments to examine the physiological and biochemical characteristics of the strains were conducted as previous description in *Bergey's manual of determination bacteriology and identification and guidance of bacteria* [12]. To put it simply, to measure the oxidase activity, the oxidation of tetramethyl-p-phenylenediamine reacting on filter paper was monitored. The activity of catalase was determined via examining the bubble formation of the fresh colonies that had been added in 3% hydrogen peroxide (V/V). Moreover, the microscopic characteristics of the isolates were obtained by Gram staining and agar-stab culture.

Genomic DNA of the single isolate was extracted from bacterial suspension using the Bacterial DNA Kit (50) (Omega Bio-TEK), which was following the manufacturer's instructions. Strains were identified by PCR (polymerase chain reaction amplification) of the 16S rRNA gene of the isolated strain by bacterial universal primers, 27f (AGAGTTTGATCATGGCTCAG), and 1492r (TACGGTTACCTTGTTACGACTT) [13]. The PCR was conducted under the following conditions: 94°C for 5 min; 35 cycles at 94°C for 30s; 55°C for 30s; 72°C for 1 min, and extension at 72°C for 7 min. The PCR products were analyzed with agarose gel electrophoresis using 0.8% agarose gels which were stained with ethidium bromide. Then, the amplified bands were visualized on a UV light box (Molecular Imager Gel Doc EX System by Newbio industry). The PCR products were then sequenced by Sangon Biotech Co. Ltd. (Shanghai, China). Finally, there were multiple alignments for the sequences, in comparison with available sequences in the GenBank database, using the Basic Local Alignment Search Tool[14]. A phylogenetic tree figure was then built by MEGA software (version 7.0) as well as the neighbor-joining algorithm.

2.5. Nitrogen removal performance of the aerobic denitrifiers

The isolates were pre-cultured in R₂A medium at 30°C and incubated in a shaker with a speed of 175 rpm and centrifuged at 4°C and 10000 rpm for 10 minutes during their logarithmic phase. Bacteria were washed by buffer solution three times, and then inoculated into denitrifying performance detecting cultures (5% inoculum). After seven days' cultivation, the supernatants which were used to detect the content of nitrite (NO²⁻), nitrate (NO³⁻), total nitrogen (TN) and chemical oxygen demand (COD) were obtained by centrifuging the cultures.

2.6. Analytical methods

The OD₆₀₀ value was tested at a wavelength of 600 nm by a spectrophotometric assay (SHIMADZU UVmini⁻¹240). NO²⁻, NO³⁻, TN and COD were tested by procedures described in the standard methods [15]. In a brief, NO²⁻ was examined by the N-(1-naphthalene)-diaminoethane photometry method. TN and nitrate were analyzed using the hydrochloric acid photometry method. COD was tested through the potassium dichromate method (CHEMetrics). All indexes were tested in triplicate.

3. Results and discussion

3.1. Isolation of the aerobic denitrifiers

In the initial screening work, a total of thirteen strains were isolated from the activated sludge sample from Liaohu petrochemical sewage treatment plant under aerobic conditions, and then seven strains that could remove nitrate nitrogen were selected according to their BTB plate's reaction.

3.2. Identification of the aerobic denitrifiers

According to the morphological observation, colonies of strain 1 and strain 4 were gray white, moist and transparent; strain 6 and strain 7 were cream white, smooth and non-transparent. The colonies of strain 2, strain 3 and strain 5 showed light yellow with moist and transparent surface. Gram staining indicated that all the isolates belonged to rod-shaped, or short rod-shaped, gram-negative bacteria. Strain 2 and strain 3 were oxidase-negative and catalase-positive, and other strains showed positive characteristics in both catalase and oxidase activities.

The high similarities to the genera were listed in table 1 and BLAST was used in the homology searching of the sequences in GenBank. Through partial 16S rRNA sequences analysis in comparison with each other, or with some of the reported bacteria with nitrogen removal performance, a phylogenetic tree figure of the isolates using neighbor-joining method was built by MEGA7.0 shown in figure 1, and several formerly recorded[16] representative aerobic denitrifiers were also included like *Achromobacter* sp.

Table 1. Similarities of 16SrRNA sequence between the strains and related affiliations.

Isolate No.	Affiliation	GenBank No.	Similarity %
1	<i>Ralstonia</i> sp.	KM823929.1	100
2	<i>Achromobacter</i> sp.	HM246520.1	100
3	<i>Enterobacter cloacae</i>	JX188069.1	100
4	<i>Aeromonas hydrophila</i>	KC252599.1	100
5	<i>Aeromonas aquariorum</i>	KC202271.1	100
6	<i>Enterobacter cloacae</i>	KP313043.1	100
7	<i>Aeromonas</i> sp.	KJ817212.1	99

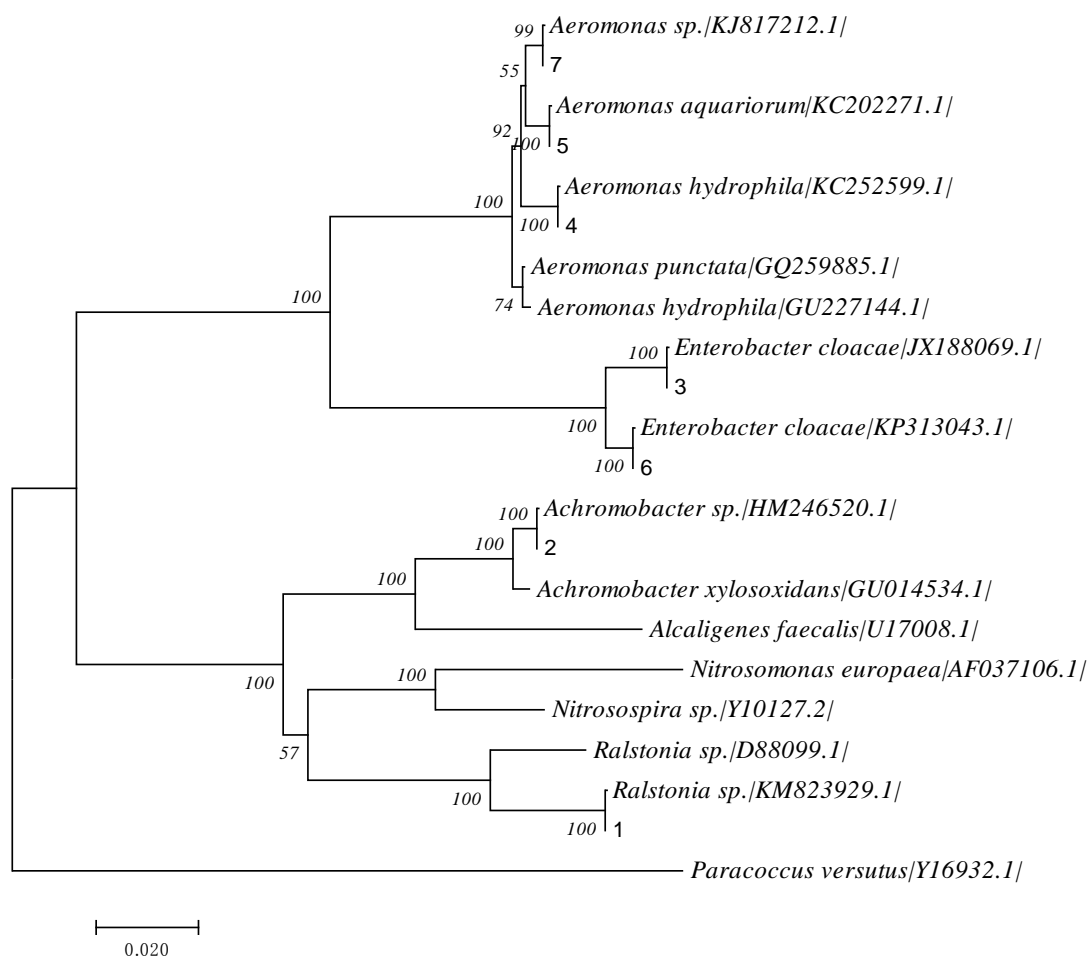


Figure 1. The phylogenetic tree figure according to partial 16S rRNA sequences revealing phylogenetic relationships between the isolates and some other typical nitrogen-removing aerobic denitrifiers from other groups.

3.3. Nitrogen removal performance of the aerobic denitrifiers

The nitrate-removal rate of each isolate in denitrifying performance detecting cultures under aerobic conditions was shown in figure 2. It revealed that strain 2, strain 3 and strain 7 tended to be good at degrading nitrate nitrogen, while the removal rate of strain 5 was unsatisfactory.

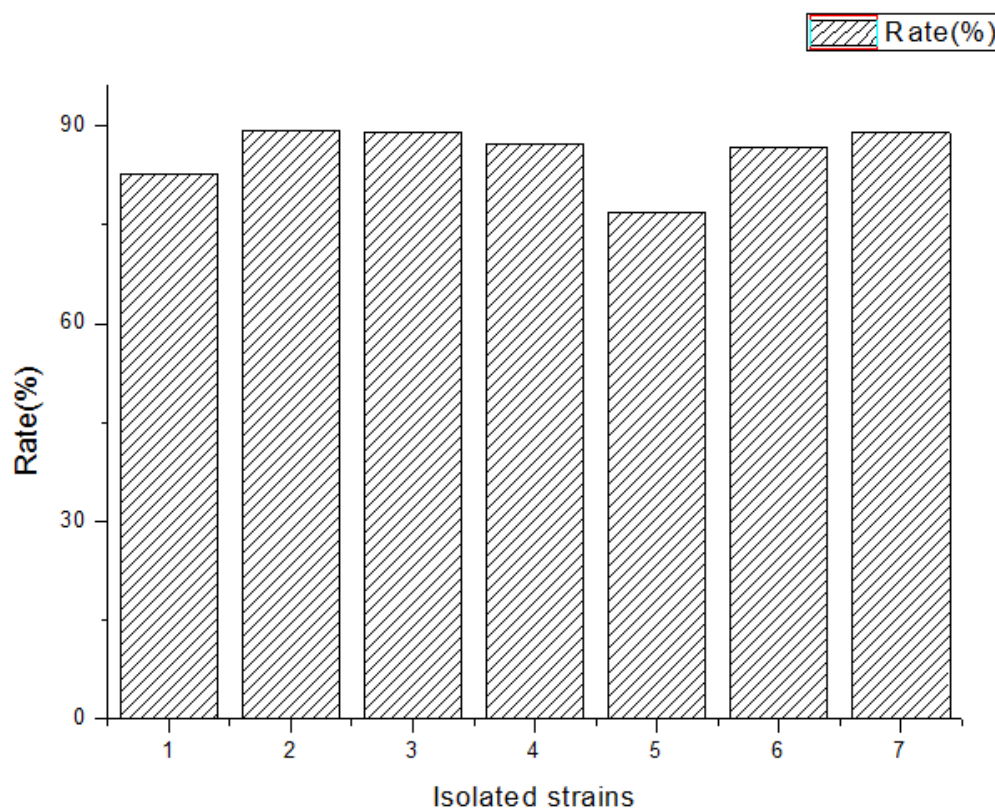


Figure 2. Nitrate nitrogen removal rate of the isolates.

4. Conclusion

In this paper, 7 predominant aerobic denitrifiers were screened and characterized from the activated sludge in the CAST unit in Liaohe petrochemical sewage treatment plant, and identified as the genera of *Ralstonia*, *Achromobacter*, *Aeromonas* and *Enterobacter*. Five of them can remove more than 86% nitrate nitrogen.

The aerobic denitrification process rebuts the traditional nitrogen removal theory, which believes that denitrification is proceeded by heterotrophs only in anaerobic environments. Discovery of aerobic denitrifiers made it possible for nitrification and denitrification processes to be conducted simultaneously, which would greatly diminish operating costs. Therefore, aerobic denitrifiers have an outstanding potential in the application of contaminated source water bioremediation.

In my succeeding work, I will optimize the conditions of the culture (including DO concentration, C/N load ratio, carbon source, temperature and pH) for the isolated strains, discuss the nitrogen removal efficiency of the combination of the isolated strains, and try to make a practical application in industrial effluent.

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