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To cite this article: Xiaoming Zhu *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **300** 052009

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Characteristics of an aerobic denitrifier isolated from unconfined aquifer

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Abstract. An aerobic denitrifier strain named X11 was isolated from the shallow unconfined aquifer samples in Suzhou city, China. According to the physiological biochemical identification and the 16S rDNA gene analysis, the strain X11 was generally identified as Pseudomonadales Pseudomonadaceae. Single factor experiments were used to investigate the influence of temperature, pH and carbon sources on its growth and denitrification characteristics. The results showed that under aerobic conditions the total nitrogen loss and nitrate reduction efficiency within 60 hours were up to 70.3% and 95.1%, respectively, with initial nitrate concentration of 276.25 mg/L. The nitrate removal rates reached 18.24 mg/L·h within 12 hours. It was found that strain X11 can convert nitrate into nitrogen gas without obvious accumulation of nitrite under aerobic conditions. The pH increased and ORP decreased by activated denitrifier in liquid medium. the optimum conditions were obtained as follows; temperature value of 30°C, pH value of 7. The results indicate that the isolated aerobic denitrifier X11 has high nitrogen removal efficiency, it may be a good candidate for in-situ bioremediation of nitrogen-contaminated shallow groundwater or aerobic wastewater treatment.

1. Introduction

Due to extensive use of nitrogen fertilizers in intensive agriculture and discharge of domestic and industrial wastes, the nitrate in groundwater aquifers and surface water has steadily been increasing in recent years [1, 2]. The presence of large amounts of nitrate in water is detrimental to the health of humans and animals and is damaging to the environment. To remove nitrate, several physical, chemical and biological processes have been proposed, and biodenitrification is considered as the most effective process in wastewater treatment and groundwater remediation [3].

It has been widely reported that the denitrification process occurs only under anaerobic conditions, during which nitrate is successively reduced to nitrite, nitric oxide, nitrous oxide and nitrogen gas [4]. However, since the first report about aerobic denitrifiers was published by Robertson in 1988 [5], more and more strains were found to be capable of denitrifying under both aerobic and anaerobic conditions. There are recent reports of aerobic denitrifying species isolated from canals, ponds, soils, and activated



sludge that can simultaneously utilize oxygen and nitrate as electron acceptors [6-9]. However, studies on aerobic denitrifiers isolated from groundwater, unconfined aquifer or confined aquifer were rarely reported so far.

The objectives of this study were: (1) to detect and isolate aerobic denitrifying bacteria from unconfined shallow aquifer samples; (2) to identify the isolated strain and study its biological denitrification activities using morphological, biochemical/biophysical and 16S rRNA gene analyses; (3) to investigate the influence of temperature, pH and carbon sources on its growth and denitrification characteristics; (4) to evaluate nitrogen removing efficiency of the denitrifier under aerobic conditions.

2. Materials and methods

2.1. Sample sites and sample collection

Fourteen samples of porous media were collected from two unconfined shallow aquifer profiles (2.6 m and 6.5 m beneath the surface soil) in Suzhou city, eastern China. The fresh aquifer samples were stored in a sealed bottle and immediately used for bacterial isolation. The denitrifying bacteria isolation were carried out by adding 10 g samples to 90 ml of 0.9% NaCl solution and incubated at room temperature for 30 min with 120 rpm shaking.

2.2. Media

To screen and isolate the aerobic denitrifiers, we used bromothymol blue (BTB) medium and modified screening medium (SM) as described by Takaya et al [10]. To test denitrification activity, we used a modified denitrification medium (DM) base of the following composition (in grams per liter): KNO₃, 0.72; sodium citrate, 5; KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.2; pH, 7.0-7.5; trace element solution, 2ml. The components of the trace element solution were EDTA·2Na, 57.1; ZnSO₄·7H₂O, 3.9; CaCl₂·2H₂O, 7.0; MnCl₂·4H₂O, 5.1; FeSO₄·7H₂O, 5.0; (NH₄)₆Mo₇O₂₄·4H₂O, 1.1; CuSO₄·5H₂O, 1.6; CoCl₂·6H₂O, 1.6; pH=7.0.

2.3. Screening and Isolation of aerobic denitrifiers

To cultivate the aerobic denitrifiers, the extraction solution was serially transferred to SM broth and incubated in a shaker (120 rpm) at 30°C for 3 d. Five milliliters of the culture was added to fresh SM broth and incubated under the same conditions. This was repeated three times. The resulting bacterial suspension was streaked onto BTB medium plates containing sodium citrate (5.0 g/l) and incubated at 30°C for 3 d. Blue colonies were selected from the BTB agar plates, transferred to DM broth containing KNO₃ (0.72 g/l), and then incubated under aerobic conditions at 30°C. Single colonies were restreaked several times to obtain pure isolates. In this study, sixty-two strains owning high nitrogen removal efficiency were selected, and the strains were stored on 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 [11]. 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. PCR amplification of the 16S rRNA genes was performed on a Takara PCR Thermocycler. PCR was conducted using genomic DNA as the template and the commercially synthesized universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GATTACCTTGTTACGACTT). The PCR was conducted under the following conditions: 95°C for 5 min; 30 cycles at 94°C for 30s; 53°C for 30s; 72°C for 1 min, and extension at 72°C for 10min. The PCR products were analyzed with agarose gel electrophoresis using 1.0% agarose gels which were stained with ethidium bromide. Then, the amplified bands were visualized on a UV light box. The 1.5 kb PCR product was cloned into the pGEM-T easy vector, sequenced using a Termination Sequencing Ready Reaction kit, and analyzed by BGI Co. Ltd. (Nanjing, China). The 16S rDNA

sequences used for the phylogenetic analysis were derived and compared with available sequences in the GenBank database. Kimura-Parameter model was applied for the calculation of evolutionary distance. A phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analyses of 1000 replicates were carried out using MEGA software (version 3.0).

2.5. Nitrogen removal performance of the aerobic denitrifiers

The influence of temperature and pH on the growth and denitrification characteristics of isolated bacteria were firstly studied at different temperatures, i.e. 10, 20, 25, 30, 35, 40°C to find out the optimum cell growth and nitrate removal temperature. Each 250mL flask contained 100mL culture media (nitrate, 50mg/L; C: N=10) with cell suspensions (2mL) containing 10^7 cells/mL. After sealing with cotton stoppers, the flasks were shaken at 120 rpm and samples were taken at appropriate time to observe nitrogen removal. Control experiments were also performed where necessary. Similar experiments were performed to find the effect of pH (4, 5, 6, 7, 8, and 9) on nitrogen removal at 28°C.

Nitrogen removal performance were then studied with initial nitrate concentration of 276.25 mg/L under aerobic conditions (DO=6.9-7.8 mg/L), the initial DOC concentration was 1400 mg/L with C: N=5, the flasks were shaken at 120 rpm and samples were taken at appropriate time to observe nitrogen removal at 28°C. After 60h cultivation, the supernatants which were used to detect the content of nitrate, nitrite, total nitrogen (TN) and dissolved oxygen carbon (DOC) were obtained by centrifuging the cultures.

2.6. Analytical methods

Temperature, pH and ORP values were monitored by a pH meter (Model UB-7, DENVER, CHINA). DO concentration was monitored by DO meter (Model HQ40d, HACH, USA). Cell concentration was determined by measuring the optical density (OD) at an absorbance of 600 nm using a Rayleigh UV–Visible Spectrophotometer UV-2100. For all liquid samples, total nitrogen, nitrate, ammonia, nitrite was measured standard methods issued by National Environmental Protection Administration of China [12]. The Dissolved oxygen carbon (DOC) was analyzed using a TOC analyzer (Model IL530, HACH, USA). All indexes were tested in triplicate.

3. Results and discussion

3.1. Isolation of the aerobic denitrifiers

In the initial first screening work, a total of sixty-two strains that can utilize nitrate as source of nitrogen were isolated from aquifer samples under aerobic conditions, and then five strains that could remove nitrate nitrogen were selected according to their BTB plate's reaction in second screening period. X11 was particularly studied due to its better nitrogen removal effect in the following study.

3.2. Identification of the aerobic denitrifiers

The detailed morphological, physiological, and biochemical characteristics of the isolated X11 were given in Table 1. As can be seen, strain X11 was light yellow with moist and transparent surface, gram-negative bacteria with positive characteristics in both catalase and oxidase activities.

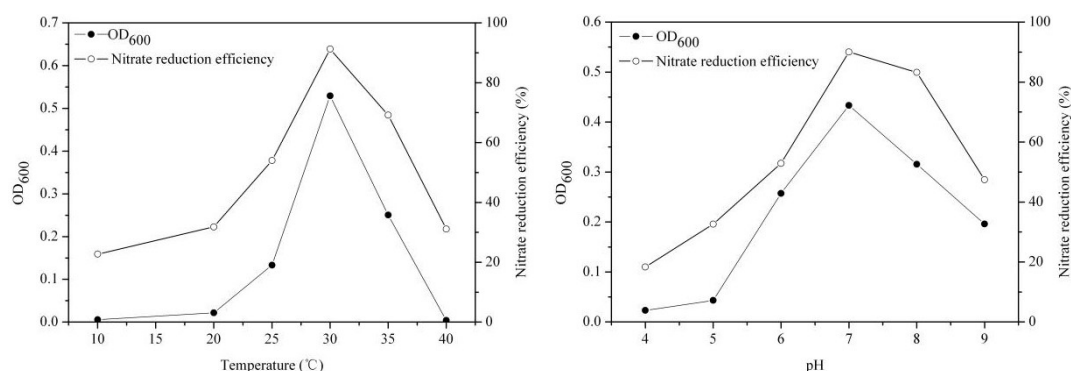
Upon comparison of the partial 16S rDNA gene sequence obtained from strain X11 with sequences from the Genbank Database, the highest degree of identity was obtained with the 16S rDNA gene sequence of a Pseudomonadales Pseudomonadaceae AMD4. Analysis of the 16S rDNA gene sequence and phenotypic analysis (Table 1) suggested that this strain (X11) was a Pseudomonadales Pseudomonadaceae one.

Table 1. Morphological and taxonomic characteristics of bacterial isolate strain X11.

Test item	Result	Test item	Result
Colony color	Light yellow	Voges-Proskauer test (V. P.)	-
Gram staining	Negative	Nitrate, nitrite	+
Growth at 4-35°C	+	Glucose fermentation test	-
Catalase reaction	+	Sucrose fermentation test	-
Oxidase reaction	+	Gelatin liquefaction	+
Methyl red test (M. R.)	+	Cellulose decomposition	-

3.3. Influence of temperature and pH on growth and nitrogen removal

Figure 1 shows the growth rate of X11 and nitrate removal rate with different temperatures (10-40°C) and pH (4-9). The optimum growth temperature of *Pseudomonadales Pseudomonadaceae* X11 was around 30°C by utilizing 50 mg/L of nitrate as sole nitrogen substrate. Figure 1(a) shows that growth inhibition of X11 occurred at 10 and 40°C, just 20-30% of nitrate was removed after the cultivation experiment, and about 93% of nitrate was removed at 30°C. Figure 1(b) shows that the optimum pH for nitrate removal was between 6 and 9, X11 was also observed to degrade nitrate maximally at pH 7.0, showing that a neutral to slightly alkaline pH may be required for nitrate removal.

**Figure 1.** Effect of temperature (a) and pH (b) on cell growth and nitrate removal

3.4. Nitrogen removal performance of the aerobic denitrifier X11

The changes of pH and ORP throughout the period of cultivation under aerobic condition were shown in figure 2. The ORP of the culture solution was about -5 mv with the initial culture conditions (temperature 28°C and pH 7.3). It revealed that with the increasing of culture time, pH value increased gradually after 6 hours, and increased to 9.0-9.3 at 18-24 hours, indicating that bacterial alkali production existed during the process of denitrification and reduction of nitrate (as shown in formula 1 and 2). At the same time, after 24 hours, the ORP of the culture solution increased from -5 mv down to -100 mv.



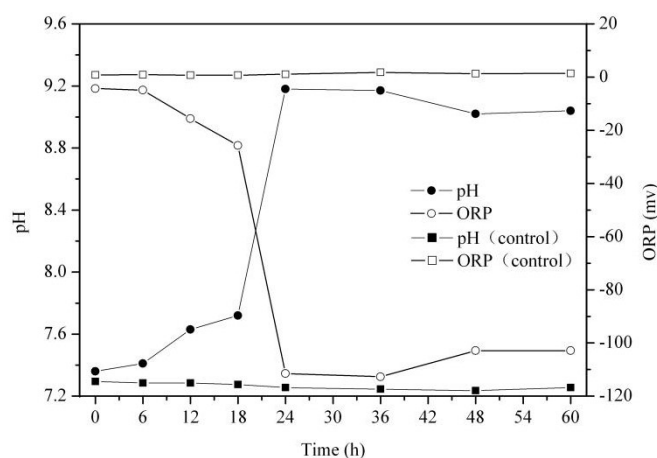


Figure 2. Changes of pH and ORP throughout the period of cultivation of strain X11

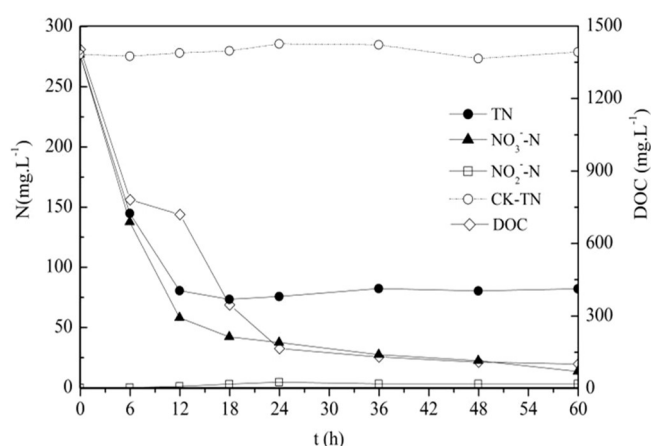


Figure 3. Changes of nitrogen and DOC throughout the period of cultivation of strain X11

The denitrification characteristics and results of the strain X11 under aerobic conditions were shown in figure 3. Concentrations of total nitrogen, nitrate, nitrite, dissolved oxygen carbon (DOC) were measured. The result demonstrated that the strain X11 could operate denitrification reaction rapidly under aerobic conditions with initial nitrate nitrogen concentration of 276.25 mg/L. After 12 hours, the total nitrogen content in the system decreased significantly, and the total nitrogen reduction rate reached 70.9%, and the denitrification rate reached 18.24 mg/L. h. At the same time, the nitrate concentration in the culture solution decreased to about 50 mg/L and the nitrite concentration was only 3 mg/L, respectively. After 60 hours of cultivation, the nitrate utilization rate reached above 95.1%, the total nitrogen reduction rate reached 70.3%, and there was no obvious nitrite accumulation during the culture period. The screened strain X11 can achieve efficient denitrification rate in a short time under aerobic or anaerobic conditions.

4. Conclusion

In this paper, a strain of predominant aerobic denitrifier X11 was screened, isolated and characterized from shallow unconfined aquifer samples, and identified as *Pseudomonadales Pseudomonadaceae* by physiological biochemical identification and the 16S rDNA gene analysis. the optimum conditions of temperature and pH were 30°C and 7, respectively. The results showed that under aerobic conditions the total nitrogen loss and nitrate reduction efficiency within 60 hours were up to 70.3% and 95.1%, respectively, with initial nitrate concentration of 276.25 mg/L. The nitrate removal

rates reached 18.24 mg/L·h within 12 hours. The results indicate that the isolated XK11 has high nitrogen removal efficiency, it may be a good candidate for in-situ bioremediation of nitrogen-contaminated shallow groundwater or aerobic wastewater treatment.

Acknowledgments

This research was financially supported by the Major Science and Technology Program for Water Pollution Control and Treatment (2012ZX07204-003), and the National Natural Science Foundation of China (41230640, 41807187).

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