Development and application of a novel and effective screening method for aerobic denitrifying bacteria

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Abstract
Herein we describe a novel and effective screening method for aerobic denitrifying bacteria. For this procedure, we utilized KCN to inhibit the electron transfer from Cytaa3 to oxygen in the bacteria respiratory chain. We employed a 3-h aeration operation cycle and intermittent rotations. The resultant bacterial suspensions were plated on a KCN-screening medium and incubated aerobically. Single colonies were selected and incubated in an aerobic culture medium. Culture nitrate and nitrite levels were determined over time, and ultimately four bacterial strains that performed denitrifying under aerobic conditions were identified by this method. Of these, strain Y2-1-1 demonstrated the best aerobic denitrifying ability. In a 5-day test, the NO3-/C02-N of the aerobic culture medium was reduced from 282.0/68.3 mg L-1 to 149.2/17.1 mg L-1, with little nitrite or N2O production.

The morphological, physiological and biochemical characteristics and the 16S rRNA gene sequence homology comparison data for this strain were consistent with the classification of the genus Pseudomonas. We named this strain Pseudomonas sp. Y2-1-1.

Introduction
The removal of nitrogen from waste water has become one of the most important methods for water pollution control and remediation. For ammonium removal from waste water, biological nitrification and denitrifying processes are the most easily available. In general, nitrification requires an aerobic environment for successful oxidation of ammonium and nitrite. Conversely, denitrifying utilizes electron donors under anaerobic conditions. Generally, denitrifying bacteria are facultative and will, under aerobic and anaerobic conditions, utilize oxygen and nitrate molecules as electron acceptors, respectively. Therefore, traditional theories have presumed that denitrifying can only be achieved through aerobic nitrification and anaerobic denitrifying, which are costly and complicated operations. Since the 1950s, many reports describing aerobic denitrifying have been published (Marshall et al., 1953; Meschner & Wuhmann, 1963; Kruil, 1976). However, research in this field has been restricted due to a lack of effective screening methods and suitable model microorganisms (Chen et al., 2003; Yang et al., 2003; Shinoda et al., 2004). The purpose of this research is to develop a novel and effective method to screen aerobic denitrifying bacteria from environmental samples.

Materials and methods

Organisms
An aerobic denitrifying bacterium, strain Y2-1-1 of the genus Pseudomonas, isolated from the waste water drainage of a fertilizer yard, was used as a model microorganism in this work. The bacterium is preserved in the China General Microbiological Culture Collection Center (CGMCC; accession number CGMCC1155).

Media
We used a KCN screening medium, an aerobic culture medium and gas production analysis cultures in this study. The ingredients of the KCN screening medium were as follows (g L-1): two KNO3, five sodium citrate, one K2HPO4, one KH2PO4, 0.2 MgSO4 with 2 mL of a trace element solution (Vishniac & Santer, 1957) and 50 mM of KCN provided as a final concentration addition per medium liter. We added 20 g agarose to the KCN mixture as needed for plating. The ingredients of the aerobic culture medium were the same as the KCN screening medium, but without the KCN. The ingredients of the gas production analysis
culture were the same as those of the aerobic culture medium, but with the addition of 0.4 g L⁻¹ KNO₃ and 0.4 g L⁻¹ sodium nitrate.

**Screening and identification of aerobic denitrifiers**

**Screening of aerobic denitrifiers**

Samples from the Tianjin Fenghua fertilizer yard waste-water drainage, and the Tianjin Jizhuangzi waste-water treatment plant, were transferred to 300 mL of an aerobic culture medium in 1000-mL Erlenmeyer flasks and incubated at room temperature with intermittent aeration for 5 days. A 3-h aeration and intermittent operation cycle was utilized. A new aerobic culture medium was inoculated with 10 mL of culture solution and incubated under the same conditions. These procedures were repeated twice. The reduction of KNO₃ and the enrichment of NO₃⁻ were measured over time as described below.

After enrichment, we plated the resulting bacterial suspensions on the KCN screening medium plates and incubated them aerobically at 30 °C for 3 d. Soil samples were occasionally suspended in 0.9% NaCl and plated directly on the KCN screening medium plates. The resulting colonies were isolated and screened as follows: the bacteria colonies were transferred to 150 mL of liquid aerobic culture medium in 500-mL Erlenmeyer flasks; the flasks were sealed with gauze and rotary-shaken at 120 r.p.m. at 30 °C for 5 days. Those bacteria that decreased nitrate levels and produced negligible amounts of nitrite were selected for additional studies.

**Identification of aerobic denitrifiers**

The selected strains were identified by morphological observation, a survey of physiological characteristics and 16S rRNA sequence analysis (Bergey *et al.*, 1994; Dong & Cai, 2001). The primers used to amplify the 16S rRNA gene were: p1: 5'-CCGGATCCAGGTTTTTAGATCTGCAGAAACCAGAA CGCT-3' and p6: 5'-CCGGATCCAGGGGCTATTGTTTGTCAGAAACCAGAA CGCT-3' (BamHI sites are in bold). The PCR reaction mixture consisted of 2 μL of 100 mol L⁻¹ of each dNTP, 1 μL of 50 pmol of each primer, 5 μL of 10 × buffer (containing 25 mmol L⁻¹ MgCl₂), 1 μL of 2 U Taq polymerase, and 4 μL of approximately 1 mol L⁻¹ of template DNA in a total volume of 50 μL. The amplifications were performed with a GeneAmp PCR System 2400 Thermal Cycler (Perkin-Elmer Corp.) under the following conditions: an initial denaturation step at 94 °C for 60 s; followed by 30 cycles of 94 °C for 60 s, 52 °C for 60 s, and 72 °C for 60 s, and a final elongation cycle at 72 °C for 420 s.

**Amplification of napA gene**

The *napA* gene was amplified using the forward primer NAP1 (1188–1212 bp): 5'-CTCGGACCATTGGCTTTC AA CCA-3', and the reverse primer NAP2 (2064–2048 bp) 5'-ACGACGACC GGCCAGGCA CG-3' (*Bell et al.*, 1990; *Bell et al.*, 1993). The 50 μL PCR reaction mixture contained 4 μL of 100 pmol L⁻¹ of dNTP each, 1 μL of 50 pmol of each primers, 5 μL of 10 × buffer (containing 25 mmol L⁻¹ MgCl₂), 2 U Taq polymerase, and about 1 mol L⁻³ of template DNA. The PCR conditions were as follows: 94 °C for 5 min followed by 94 °C for 0.5 min, 59 °C for 0.5 min and 72 °C for 1 min for 35 cycles, and a final extension step at 72 °C for 5 min.

**Detection and sequencing of PCR products**

The PCR products were analyzed with agarose gel electrophoresis using 0.8% agarose gels stained with ethidium bromide. The amplified bands were visualized on a UV light box. The PCR products were purified with the QiAquick PCR purification kit (Qiagen, Hong Kong, China). The purified PCR products were transferred to TaKaRa Biotechnology (Dalian, China) Co., Ltd for sequencing.

**Phylogenetic analysis**

The 16S rRNA and *napA* gene sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) for homology. Nucleotide sequences were aligned using DNAMAN, and were used to produce a maximum-likelihood phylogenetic tree.

**Batch cultures in flasks**

The selected strains were batch-cultured in flasks as follows: seed cultures (1 mL) in 50-mL tubes were inoculated into 100 mL of a liquid aerobic culture medium in 250-mL Erlenmeyer flasks and were rotary-shaken at 30 °C for 5 days at 120 r.p.m. During this time, the DO was maintained at roughly 5.5 ± 0.5 mg L⁻¹. The nitrate and the nitrite concentrations were tested every 24 h, and the biomasses were determined according to Yang's methods (*Yang et al.*, 2003).

**Analytical methods**

We transferred 50 mL of the gas production analysis culture medium into 125-mL autoclaved crimp-sealed glass serum bottles. A bacterial suspension (1 mL) of the isolate was inoculated into the medium. These bottles were evacuated and the headspace of each bottle was pressurized with pure oxygen three times. The sealed bottles were rotary-shaken at 200 r.p.m. at 30 °C for 5 days. A non-inoculated sample that was otherwise treated the same served as the control. Oxygen and nitrogen levels were determined by gas chromatography with a thermal-conductivity detector (GC/TCD) (*Su et al.*, 2006).
The $\mathrm{N}_2\mathrm{O}$ levels were detected by gas chromatography with electron-capture detection (GC/ECD) (Remde & Conrad, 1990). $\mathrm{NH}_4^+\text{-N}$, total nitrogen (TN), $\mathrm{NO}_2^-\text{-N}$ and $\mathrm{NO}_3^-\text{-N}$ were analyzed colourimetrically using the standard methodology (APHA, 1995).

**Nucleotide sequence accession numbers**

The nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AY515308 for the 16S rRNA gene and AY515307 for the napA gene.

**Results**

**Isolation of aerobic denitrifiers**

After the intermittent aeration enrichment rotation, the resulting bacterial suspension representing the nitrate-reducing population was plated on the KCN screening medium and incubated aerobically at 30 °C for 3 days. We selected numerous single colonies as preliminary screening bacteria, inoculated them in the refreshed aerobic denitrifying culture medium and prepared them for the second screening (see Materials and methods for preparation details). Among these bacteria, four possessed superior denitrifying characteristics (Table 1).

The nitrate removal capacity of one strain, Y2-1-1, far surpassed that of the other three strains. Furthermore, the Y2-1-1 strain had the added benefit of producing negligible auxillary nitrite levels. For these reasons we selected Y2-1-1 as our model organism for aerobic denitrifying.

Strain Y2-1-1 is a gram-negative, rods and no spore-forming bacterium. Y2-1-1 cannot undergo glucose and alcohol fermentation; it is catalyze positive, oxidase negative, and capable of denitrifying and action. According to the Bergeys Manual of Determinative Bacteriology, Y2-1-1 is considered as a *Pseudomonas* species (*Pseudomonas* sp.). The genus designation was confirmed with VITEK identification kits.

The 16S rRNA Y2-1-1 gene analysis showed that this strain is 100% homologous with *Pseudomonas* sp. K50 and 99% homologous with *Pseudomonas mendocina* and *Pseudomonas pseudoalcaligenes*. Thus, we identified the strain as *Pseudomonas* sp. Y2-1-1 (Fig. 1).

**Batch cultures in flasks**

The *Pseudomonas* sp. Y2-1-1 cells were cultured and analyzed for nitrate and nitrite concentrations and biomass, as described above. After 5 d, the nitrate concentration had decreased from 282.0 ± 8.3 to 149.2 ± 17.1 mg L$^{-1}$, and the nitrite levels were below 0.8 mg L$^{-1}$ (Fig. 2). The biomass had increased by 0.03969 g, and the nitrate removal rate was 46.47 ng NO$_3^-\text{-N}$ mg cell$^{-1}$ min$^{-1}$. The nitrite concentration increased slightly in the control culture over the same time period, probably due to the evaporation of water from the bottles during the experiment.

**Detection of gas products during the nitrate reduction by Y2-1-1**

In 5 d, the nitrate concentration decreased from 42.0 ± 2.04 to 1.0 ± 0.02 mg L$^{-1}$. Meanwhile, 1.71 ± 0.08 ($\times 10^3$ p.p.m.) nitrogen was produced in the gas production. Simultaneously, 4.43 ± 0.17 ($\times 10^3$ p.p.m.) O$_2$ was consumed. We did not detect nitrite or nitrous oxide in any of the experimental Y2-1-1 groups (Table 2).

**Amplification of the napA gene**

We excised and sequenced an 876 bp DNA band representing napA from the agarose gel. Our comparison with other sequences showed the highest homology between PA01 and

<table>
<thead>
<tr>
<th>Strain</th>
<th>NO$_2^-$-N (mg L$^{-1}$)</th>
<th>NO$_3^-$-N (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>311.7 ± 7.8</td>
<td>0.1 ± 0.002</td>
</tr>
<tr>
<td>Y2-1-1</td>
<td>142.3 ± 5.1</td>
<td>0.2 ± 0.002</td>
</tr>
<tr>
<td>Y2-1-2</td>
<td>232.2 ± 5.7</td>
<td>2.7 ± 0.308</td>
</tr>
<tr>
<td>Y2-2-2</td>
<td>195.6 ± 2.6</td>
<td>5.2 ± 0.376</td>
</tr>
<tr>
<td>Y-1</td>
<td>253.6 ± 4.2</td>
<td>0.1 ± 0.001</td>
</tr>
</tbody>
</table>

**Fig. 1.** Phylogenetic tree of *Pseudomonas* sp.Y2-1-1 based on 16S rRNA gene sequence homology.
Y2-1-1 napA genes, at 89%. Figure 3 illustrates the homology analysis of Y2-1-1 and other related colonies.

**Discussion**

In general, the amount of aerobic denitrifying bacteria is much smaller than that of other heterotrophs in most ecosystems. Therefore, it is difficult to isolate or identify aerobic denitrifying strains using common methods. Robertson & Kuenen (1983) reported that intermittent aeration could help denitrifying bacteria to gain dominance in culture. In 2001, Li and coworkers domesticated the activated sludge sampled from sewage disposal and discovered the phenomena of aerobic denitrifying.

Theoretically, frequent changes between aerobic and anaerobic conditions have made it impossible for aerobic and anaerobic bacterial classes to grow normally, thus the potential of the aerobic denitrifying bacteria becoming dominant has increased greatly, but anaerobic denitrifying bacteria are facultative and will, in aerobic and anaerobic conditions, respectively, utilize oxygen molecules as electron acceptors and nitrates as electron acceptors. Therefore, this situation makes isolating the aerobic denitrifying bacteria difficult. There exist few protocols for effective aerobic denitrifying bacteria screening methods; the common procedure is both inefficient and effort-intensive. Bell and colleagues (Bell et al., 1990, 1993) reported that nitrate reductases (NAR) comprise two distinct categories. One is membrane-bound, active in anaerobic conditions and susceptible to the restrictions of oxygen molecules, while the other is periplasmic, not overly susceptible to the restrictions of oxygen molecules, and is probably connected to aerobic denitrifying (Bell et al., 1990; Bell et al., 1993). Robertson & Kuenen (1984) stated that the aerobic denitrifying bacteria can achieve transference of redundant electrons to nitrate through periplasmic NAR, thus reducing the nitrate to nitrite. Thus, we believe that if we can terminate the electron transfer to molecular oxygen in the respiratory chain factitiously under aerobic conditions, then we can easily screen out aerobic denitrifying bacteria.
because they can transfer electrons to nitrate through periplasmic NAR.

Aerobic denitrifying bacteria have nitrate and nitrite reductases, which do not possess oxygen limitations. Moreover, denitrifying bacteria NAR and NIR utilize quinone and Cyt bc as electron acceptors, respectively (Jetten et al., 1997). Thus, we hypothesize that aerobic denitrifying bacteria possess oxygen-resistant nitrate and nitrite reductases (orNAR and orNIR, respectively) that are free of oxygen restrictions, and can instead use quinone and Cyt bc 1 as electron acceptors, respectively.

Based on this model, we designed a method for screening the aerobic denitrifying bacteria that utilizes the respiration inhibitor KCN. The sequence of biological events is as follows: KCN, which terminates the electron transfer to molecular oxygen in the respiratory chain, is added into aerobic culture media in order to restrain normal aerobic bacterial respiration. Thus, neither normal aerobic bacteria nor anaerobic bacteria can grow on this medium. Meanwhile, under aeration conditions, the oxygen molecule will have a restrictive effect on the enzymes of the traditional facultative denitrifying bacteria, which are only effective under anaerobic conditions (such as NAR and NIR). Because the aerobic denitrifying bacteria, orNAR and orNIR, remain active in the aeration environment, they can transfer the quinone electron to a nitrate molecule, thus reducing it into nitrite or they can accept an electron from Cyt bc 1 (in the case of orNIR) and resolve it into NOx and complete the respiratory reaction (Fig. 4).

The present study demonstrated that aerobic denitrifying bacteria are easier to identify and to screen out under the conditions described above. We have tested this method and successfully selected several denitrifying bacteria with nitrate-reducing potential under aerobic conditions (Table 1). Among these, strain Y2-1-1 yielded the best denitrifying capacity, removing 97.62% and 73.2% of the nitrates and nitrites, respectively. Moreover, nitrogen gas was produced with low levels residual nitrites and little N2O production. All of these factors indicate that Y2-1-1 is an effective aerobic denitrifying bacterium, and confirm the benefits of our novel screening method.

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References

A screening method for aerobic denitrifying bacteria


