Denitrification characteristics of a newly isolated indigenous aerobic denitrifying bacterium under oligotrophic conditions

Jun-feng Su, Kai Zhang, Ting-lin Huang, Fang Ma, Lin Guo and Li-na Zhang

ABSTRACT

A novel indigenous bacterium, strain JM10, isolated from the oligotrophic Hei He reservoir was characterized and showed aerobic denitrification ability. JM10 was identified as Bacillus sp. by phylogenetic analysis of its 16S rRNA gene sequence. Strain JM10 displayed very high levels of activity in aerobic conditions, consuming over 94.3% NO3-N (approximately 3.06 mg L⁻¹) with a maximum reduction rate of 0.108 mg NO3-N L⁻¹ h⁻¹. Full-factorial Box–Behnken design and response surface methodology were employed to investigate the optimal nitrate degradation conditions. The optimum conditions for nitrate degradation, at a rate of 0.140 mg L⁻¹ h⁻¹, were found to be an inoculum size of 16.3% v/v, initial pH of 7.6, C/N ratio of 7.4, and temperature of 27.4 °C, and the C/N ratio and temperature had the largest effect on the nitrate degradation rate. Strain JM10 was added into the water samples from Hei He reservoir and the total nitrogen and nitrate removal rates of the strain reached 66.5% and 100%, respectively. Therefore, our results demonstrate that the strain JM10 favored the bioremediation of the oligotrophic reservoir.

Key words | aerobic denitrification, Bacillus sp. JM10, nitrogen removal, oligotrophic reservoir, response surface methodology

INTRODUCTION

Nitrogen pollution, which can lead to eutrophication of water bodies and make the aquatic plants and algae grow out of control, will rapidly deteriorate water quality and seriously threaten the survival of aquatic organisms (Zhang et al. 2012; Guo et al. 2013). Thus, efficient nitrogen removal in eutrophic water bodies is a stringent research requirement. Biological denitrification can occur under anaerobic or anoxic conditions (Kaspar 1982) with the reduction sequence of nitrate (NO3-N) → nitrite (NO2-N) → nitric oxide (NO) → nitrous oxide (N2O) → nitrogen gas (N2) (Adav et al. 2009, 2010; Waki et al. 2009). However, conventional nitrogen removal consists of two steps: nitrification by autotrophs under aerobic conditions and denitrification by heterotrophs under anaerobic conditions (Patureau et al. 2000a), and this type of system can be uneconomical and difficult to operate due to extremely slow nitrification and the necessity for separate nitrification and denitrification reactors (Joo et al. 2005).

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. These include Thiosphaera pantotropha (Paracoccus denitrificans) (Su et al. 2004), Alcaligenes faecalis (Joo et al. 2005), Citrobacter diversus (Huang & Tseng 2001), Pseudomonas stutzeri (Su et al. 2001), and Pseudomonas aeruginosa (Chen et al. 2006). Comparing with traditional denitrification that occurs only in an anoxic environment, this new type of aerobic denitrification has advantages as follows: (1) nitrification and denitrification could occur concurrently in one reactor, which will greatly reduce the reactor volumes and construction cost; (2) the alkali produced by aerobic denitrification could neutralize the acid produced by nitrification, which will reduce the extra addition of pH-adjusting chemical substances and decrease the treatment cost; and (3) aerobic denitrifying bacteria are easier to control (Huang & Tseng 2001).

Aerobic denitrifying bacteria often require high concentrations of nitrate and organic carbon, which are generally used in wastewater treatment. However, in this study, a strain, designated JM10, was isolated from Hei He oligotrophic reservoir, which is one of the main drinking water
resources for Xi’an citizens. Its aerobic denitrification characteristics were investigated and the purpose of the study is to determine the bacterial ability to remove nitrate under low nitrate conditions. Factors affecting the performance of Bacillus sp. JM10 were comprehensively evaluated based on the response surface methodology (RSM) analysis. Such a kind of indigenous aerobic denitrifying bacteria may play an important role in the nitrogen cycle in the low nutrition reservoir environment, and might be used in the nitrogen removal treatment of reservoirs under oligotrophic conditions.

**MATERIALS AND METHODS**

**Screening of aerobic denitrifying bacteria**

Enrichment of aerobic denitrifying organisms was carried out in the sediments from Hei He reservoir using basic screening medium (BSM). A total of 50 g of sediment was suspended in the medium to obtain a homogeneous suspension. The BSM used in this experiment contains (per liter): CH₃COONa of 0.1 g; NaNO₃ of 0.02 g; KH₂PO₄ of 0.02 g; MgSO₄·7H₂O of 0.01 g; CaCl₂ of 0.01 g, and trace element solution, 2 mL. The final pH of the medium was adjusted to 7.0. The components of the trace element solution (g L⁻¹) were as follows: ethylene diamine tetraacetic acid (EDTA) of 5; ZnSO₄ of 0.3; MnCl₂·4H₂O of 0.5; FeSO₄·7H₂O of 0.5; CuSO₄·5H₂O of 0.2; CoCl₂·6H₂O of 0.3. Sterilized glass beads were added and culture flasks (500 mL) were sealed with sterile culture vessel breathable sealing membranes, and then the flasks were shaken in a rotary shaker at 30 °C and 120 rpm for 7 days. After cultivation, bacterial suspensions were inoculated into triplicate 1,000 mL shaking flasks with 750 mL medium and was incubated aerobically at 30 °C with rotation speed 120 rpm and dissolved oxygen of about 6 mg L⁻¹ for about 76 hours for nitrate and 84 hours for nitrite. The liquid culture was sampled for measuring OD₆₀₀ (absorbance of the aqueous solution at 600 nm wavelength), pH and the concentrations of NO₂⁻ and NH₄⁺. The nitrite concentration was determined by colorimetry at a wavelength of 540 nm. The nitrate concentration was determined using a UV spectrophotometric screening method and by calculating the difference between OD₂₅₀ and 2 × OD₂₇₅. The ammonia concentration was then determined by Nessler assay at a wavelength of 420 nm.

**Nitrogen removal performance**

The medium used for performance evaluation contained 0.1 g CH₃COONa, 0.04 g KH₂PO₄, 0.01 g ZnSO₄·7H₂O, 0.01 g MgCl₂, and 0.01 g CaCl₂ per liter. Furthermore, 0.02 g L⁻¹ sodium nitrate was supplied as the sole nitrogen source when nitrate removal performance was investigated; similarly, 0.02 g L⁻¹ sodium nitrite was used as the sole nitrogen source for assessing the nitrite removal performance. A 75 mL cell suspension was inoculated into triplicate 1,000 mL shaking flasks with 750 mL medium and was incubated aerobically at 30 °C with rotation speed 120 rpm and dissolved oxygen of about 6 mg L⁻¹ for about 76 hours for nitrate and 84 hours for nitrite. The liquid culture was sampled for measuring OD₆₀₀ (absorbance of the aqueous solution at 600 nm wavelength), pH and the concentrations of NO₂⁻, NO₃⁻, and NH₄⁺. The nitrite concentration was determined by colorimetry at a wavelength of 540 nm. The nitrate concentration was determined using a UV spectrophotometric screening method and by calculating the difference between OD₂₅₀ and 2 × OD₂₇₅. The ammonia concentration was then determined by Nessler assay at a wavelength of 420 nm.

**Assessment of nitrogen removal in Hei He reservoir**

After a 48-hour incubation, strain JM10 was transferred into the water samples of Hei He reservoir at dosage of 2% v/v. Samples were collected for a week. Nitrogen concentrations (total nitrogen (TN), NO₃⁻, NO₂⁻, NH₄⁺) were then determined. The objective of the assessment is to evaluate the feasibility of improving the water quality of Hei He Reservoir above its current status (TN = 1.95–3.40 mg L⁻¹).
Grade V according to Chinese Surface Water Standard) to <1 mg L$^{-1}$ TN (Grade II).

**Box–Behnken design for optimizing the environmental factors**

The Box–Behnken experimental design was performed with four factors: inoculum size (2–18% v/v), initial pH (5–9), C/N ratio (3–9) and temperature (20–40°C). Strain JM10 was inoculated into nitrate reduction medium containing 0.02 g L$^{-1}$ sodium nitrate as the sole nitrogen source and the samples were collected every 24 hours for detecting the concentration of NO$_3$-N. The value of the dependent response was the mean of two replications. A three-variable Box–Behnken design containing 27 trials with five replicates at the center point was applied in this experiment.

**RESULTS AND DISCUSSION**

**Characterization of the isolated strain**

A BLAST search was performed against the available data in the GenBank/EMBL/DDBJ databases, and the results showed that strain JM10 bears high similarity (above 99%) to the *Bacillus mycoides* strain ATCC 6462 (Figure 1). Therefore, we designated this bacterium as *Bacillus* sp. JM10 and selected it for further studies. We carried out further taxonomic studies to conclusively identify this isolated bacterium. The genes encoding the periplasmic nitrate reductase and nitrite reductase enzymes were amplified using the genomic DNA of JM10. We successfully obtain an 876-bp fragment, *napA*, and an 890-bp fragment, *nirS*, which provided additional evidence that this strain had capabilities of nitrite and nitrate reduction. However, in this study, we failed to amplify the *nirK* gene in the strain JM10.

**Nitrate and nitrite reduction by strain JM10 under aerobic conditions**

Figure 2(a) illustrates cell growth and removal of nitrate by *Bacillus* sp. JM10 in a 1,000 mL flask system. In the initial period spanning over 32 hours, no remarkable change in nitrate levels could be seen; correspondingly, hardly any nitrite and ammonium accumulated in the medium. During the subsequent cultivation stage comprising 24 hours (32–56 hours), the amount of nitrate decreased rapidly to 0.58 mg L$^{-1}$ at an average removal rate of 0.108 mg NO$_3$-N L$^{-1}$ h$^{-1}$, corresponding to an increase in the growth rate from OD$_{600}$ 0.015 to OD$_{600}$ 0.041. This slight increase in OD$_{600}$ indicated that low substrate levels might inhibit the rapid growth of the strain JM10. Simultaneously, the amount of nitrite, which is usually considered the intermediate of aerobic denitrification (Third et al. 2005; Modin et al. 2007; Miyahara et al. 2010), peaked at 1.29 mg L$^{-1}$ at a time point t = 56 hours, and then gradually decreased. The concomitant increase in nitrite clearly indicated that the nitrate was reduced to nitrite; thus, nitrite replaced nitrate as the main electron acceptor. We clearly showed that 94.3% of external NO$_3$-N conversion took place with minor conversion of NO$_2$-N and NH$_4^+$-N, which were the additional
nitrogenous substances available in the solutions. As a consequence, strain JM10 was confirmed to possess the ability to perform aerobic denitrification, with nitrite as an intermediate product. For the entire process, including the batch tests, the pH values were maintained between 7.02 and 7.97 (Figure 2(a)), which provided mild conditions beneficial for the bacterial growth. The increase in pH during the assay can be attributed to the denitrification process (Moir et al. 1996; Patureau et al. 2000a, b). The whole process displayed pH changes that are consistent to those seen in conventional biological methods.

Figure 2(b) shows the growth rate and nitrite reduction of strain JM10 in a 1,000 mL flask. The increase of biomass was observed from 4 hours onwards and the strain started to reduce NO₂⁻N after 20 hours of stagnant time. Subsequently, the NO₂⁻N was reduced to 0.15 mg L⁻¹ after achieving a removal efficiency of 97%. However, when nitrite was added, 0.40 mg L⁻¹ NO₃⁻N was observed at the beginning of the nitrite removal process (Figure 2(b)). This phenomenon is consistent with that seen in the study conducted by Zhang et al. (2011). This phenomenon is caused by oxidation, originating from the brief exposure to air. Furthermore, negligible accumulation of low concentrations of NH₄⁺-N was also observed. The pH of the suspension increased gradually from 7.05 to 8.09, which can be attributed to the alkali production during denitrification (Feleke et al. 1998).

**Optimization of the key culture conditions for the nitrate degradation rate**

The design matrix and response of the dependent variable are shown in Table 1. Statistical analysis of the data was performed using the Design-Expert (8.0.6.1) program with the SAS software package. The following second-degree polynomial equation was obtained to explain the nitrate degradation rate by strain JM10:

\[
Y_{JM10} = 0.12 + 0.013X_1 - 0.0023X_2 + 0.009X_3 - 0.021X_4
+ 0.01X_1X_2 + 0.013X_1X_3 - 0.012X_1X_4 + 0.020X_2X_3
- 0.0078X_2X_4 - 0.008X_3X_4 - 0.0091X_3^2 - 0.021X_4^2
- 0.023X_5^2 - 0.064X_6^2,
\]

where \( Y_{JM10} \) is the predicted response rate of nitrate degradation, and \( X_1, X_2, X_3, \) and \( X_4 \) are the coded values for the inoculum size, initial pH, C/N ratio and temperature, respectively.

Model terms were considered to be significant at values of prob > F (\( P < 0.05 \)). The model was most significant (\( P < 0.0001 \)) with \( R^2 = 0.9474 \), adjusted \( R^2 = 0.8948 \). The linear terms of inoculum size (\( X_1 \)) and temperature (\( X_4 \)) for the nitrate degradation rate (\( \nu_N \)), \( P < 0.003 \), were more significant than the C/N ratio (\( X_3 \)), \( P < 0.05 \). The quadratic terms of the \( X_3^2 \), \( X_4^2 \), and \( X_6^2 \) had significant effects on the \( \nu_N \) (\( P < 0.0008 \)). In addition, the interaction between initial pH and C/N ratio (\( X_1X_3 \)) significantly affected the \( \nu_N \) (\( P < 0.006 \)). These results suggested that among the test variables, C/N ratio and temperature produced the largest effect on the nitrate degradation rate under low nutrient conditions.

In view of the main and interactive effects of the four factors evaluated, the optimal conditions for the nitrate degradation rate were determined to be an inoculum size of 16.5% v/v, an initial pH of 7.6, a C/N ratio of 7.4 and a temperature of 27.4 °C by ridge analysis using the SAS program (version 9.1.3; SAS). The maximum nitrate degradation rate that can be achieved, according to the model prediction.
under the optimal conditions, was 0.140 mg L⁻¹ h⁻¹. Validation experiments were then conducted in triplicate at this optimal condition (inoculum size 16.3% v/v, initial pH 7.6, C/N ratio 7.4 and temperature 27.4 °C). A degradation rate value of about 0.138 ± 0.05 mg L⁻¹ h⁻¹ was obtained in the validation tests, indicating that the model fit well with the experimental data.

The response surfaces, as shown in Figure 3(a), depicted the evolution of the νₙ according to the inoculum size (X₁) and C/N ratio (X₃). The nitrate degradation rate increased with increasing inoculum size as well as C/N ratio at a constant initial pH of 7 and temperature of 30 °C. This means that higher values of νₙ can be obtained by simultaneously increasing the inoculum size and C/N ratio. Furthermore, as shown in Figure 3(a), the maximum νₙ (0.133 mg L⁻¹ h⁻¹) occurred in the presence of a maximum inoculum size (18% v/v) and C/N ratio of 7.7, which is in accordance with the model predictions. The optimal C/N ratio of 7.7 conforms to the data reported in the literature, such as for Pseudomonas putida AD-21 (Kim et al. 2008), in which the optimal C/N ratio was 8, with a nitrate removal efficiency of 95.9%.

Figure 3(b) illustrates the effects of the interaction of initial pH (X₂) and C/N ratio (X₃) in the response process. As shown in Figure 3(b), the νₙ value increased with increasing pH and C/N to attain optimum conditions, and then decreased with a further increase. The increase of C/N ratio in the nitrate medium would facilitate nitrate removal by strain JM10. The νₙ value increased slightly when the C/N ratio was raised from 3.0 to 6.7, beyond which νₙ was slightly decreased. In addition, a maximum νₙ of 0.12 mg L⁻¹ h⁻¹ could be obtained at an initial pH range of 6.1–8.1. According to Zheng et al. (2011), the aerobic denitrifying bacterium Psychrobacter sp. strain S1-1 exhibited superior denitrifying ability between pH 6.0 and 7.5.

The combined effects of initial pH (X₂) and temperature (X₄) on the νₙ are shown in Figure 3(c). These results showed that strain JM10 displays high denitrifica-

<table>
<thead>
<tr>
<th>Box–Behken experimental design representing the response of nitrate degradation rate (νₙ) as affected by inoculum size, initial pH, C/N ratio and temperature</th>
<th>Run</th>
<th>Inoculum size (v/v, %)</th>
<th>Initial pH (X₂)</th>
<th>C/N ratio (X₃)</th>
<th>Temperature (C) (X₄)</th>
<th>Response NDR* (νₙ, mg L⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.00</td>
<td>7.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.1264</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18.00</td>
<td>7.00</td>
<td>3.00</td>
<td>40.00</td>
<td>0.0721</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.00</td>
<td>9.00</td>
<td>9.00</td>
<td>30.00</td>
<td>0.1170</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10.00</td>
<td>5.00</td>
<td>6.00</td>
<td>40.00</td>
<td>0.0401</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10.00</td>
<td>9.00</td>
<td>6.00</td>
<td>40.00</td>
<td>0.0023</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10.00</td>
<td>7.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.1270</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10.00</td>
<td>7.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.1255</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10.00</td>
<td>7.00</td>
<td>9.00</td>
<td>40.00</td>
<td>0.0053</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2.00</td>
<td>7.00</td>
<td>9.00</td>
<td>30.00</td>
<td>0.0860</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.00</td>
<td>5.00</td>
<td>9.00</td>
<td>30.00</td>
<td>0.0570</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2.00</td>
<td>7.00</td>
<td>3.00</td>
<td>40.00</td>
<td>0.0861</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.00</td>
<td>7.00</td>
<td>6.00</td>
<td>40.00</td>
<td>0.0362</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>18.00</td>
<td>9.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.1183</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>10.00</td>
<td>5.00</td>
<td>3.00</td>
<td>40.00</td>
<td>0.0831</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>10.00</td>
<td>9.00</td>
<td>3.00</td>
<td>40.00</td>
<td>0.0620</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2.00</td>
<td>7.00</td>
<td>6.00</td>
<td>20.00</td>
<td>0.0378</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2.00</td>
<td>9.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.0584</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>18.00</td>
<td>5.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.1091</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>2.00</td>
<td>5.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.0895</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>10.00</td>
<td>7.00</td>
<td>9.00</td>
<td>20.00</td>
<td>0.0840</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>10.00</td>
<td>7.00</td>
<td>3.00</td>
<td>20.00</td>
<td>0.0530</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>18.00</td>
<td>7.00</td>
<td>6.00</td>
<td>20.00</td>
<td>0.0890</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>10.00</td>
<td>7.00</td>
<td>3.00</td>
<td>40.00</td>
<td>0.0064</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>10.00</td>
<td>5.00</td>
<td>6.00</td>
<td>20.00</td>
<td>0.0606</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>10.00</td>
<td>7.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.1203</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>18.00</td>
<td>7.00</td>
<td>9.00</td>
<td>30.00</td>
<td>0.1228</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>18.00</td>
<td>7.00</td>
<td>6.00</td>
<td>40.00</td>
<td>0.0400</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>10.00</td>
<td>9.00</td>
<td>6.00</td>
<td>20.00</td>
<td>0.0540</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>10.00</td>
<td>7.00</td>
<td>6.00</td>
<td>30.00</td>
<td>0.1191</td>
<td></td>
</tr>
</tbody>
</table>

*Nitrate degradation rate.

Nitrogen removal in raw water from the Hei He reservoir by strain JM10

Nitrogen is a vital biogenic element in freshwater ecosystems (Guo et al. 2013). In this study, we have shown that through addition of Bacillus sp. strain JM10 to the water samples of Hei He reservoir, TN...
concentration decreased from nearly 2.81 to 0.94 mg L\(^{-1}\) (below 1 mg L\(^{-1}\)), and the TH removal rate was approximately 66.5%. It was noteworthy that the NO\(_3\)-N was reduced to zero.

**CONCLUSIONS**

In this study, a newly isolated indigenous strain was isolated from the oligotrophic Hei He reservoir and was identified as *Bacillus* sp. strain JM10 based on the analysis of its 16S rRNA gene sequence. Strain JM10 displays very high levels of activity in aerobic conditions, consuming over 94.3% NO\(_3\)-N (approximately 3.06 mg L\(^{-1}\)) with a maximum reduction rate of 0.108 mg NO\(_3\)-N L\(^{-1}\) h\(^{-1}\). RSM analysis demonstrated that \(v_N\) rate was predicted to be 0.140 mg L\(^{-1}\) h\(^{-1}\) under the conditions of an inoculum size of 16.3% v/v, initial pH of 7.6, C/N ratio of 7.4 and temperature of 27.4 °C, where C/N ratio and temperature produced the largest effect on the nitrate degradation rate.
ACKNOWLEDGEMENTS

This work is partly supported by the National Key Technology Research and Development Program of the Ministry of Science and Technology of China (2012BAC04B02), supported by the Open Project of State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology (QA201518) and the Key Laboratory of the Education Department of Shan Xi Province (12JS051).

REFERENCES


First received 19 January 2015; accepted in revised form 1 June 2015. Available online 17 June 2015