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% 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}. 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

Jun-feng Su
Kai Zhang
Ting-lin Huang
Lin Guo
Li-na Zhang
School of Environmental and Municipal Engineering,
Xi'an University of Architecture and Technology,
Xi'an 710055,
China

Jun-feng Su
Fang Ma (corresponding author)
State Key Laboratory of Urban Water Resource and Environment, School of Municipal and Environmental Engineering,
Harbin Institute of Technology,
Harbin 150090,
China
E-mail: *mafang@hit.edu.cn*

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 (NO_3^--N) → nitrite (NO_2^--N) → nitric oxide (NO) → nitrous oxide (N_2O) → nitrogen gas (N_2) (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.* 2001), *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

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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): CH_3COONa of 0.1 g; $NaNO_3$ of 0.02 g; KH_2PO_4 of 0.02 g; $MgSO_4 \cdot 7H_2O$ of 0.01 g; $CaCl_2$ 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; $ZnSO_4$ of 0.3; $MnCl_2 \cdot 4H_2O$ of 0.5; $FeSO_4 \cdot 7H_20$ of 0.5; $CuSO_4 \cdot 5H_2O$ of 0.2; $CoCl_2 \cdot 6H_2O$ 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 fresh medium (10% inoculum). Subculturing was repeated two times, and nitrogen conversion rate in the upper supernatant was determined. After 10-fold serial dilution, 0.5 mL of each bacterial suspension was blended with BSM agar plate and incubated at 30 °C. To detect the denitrifying performance, isolates were cultivated in the BSM. Strain JM10 with high nitrogen removal efficiency was obtained.

Bacterial identification and denitrification gene amplification

The 16S rRNA gene of the isolate was amplified by polymerase chain reaction using bacterial universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TTG GYTACCTTGTTACGACT-3′). The amplified products were purified and sequenced by the TaKaRa Biotechnology Co. Ltd (Dalian, China). Sequence alignment was performed using the Basic Local Alignment Search Tool (BLAST) program. A neighbor-joining tree was constructed using the MEGA 5.1 program.

The *napA* gene encoding periplasmic nitrate reductase, which reduces nitrate to nitrite, and *nirK* or *nirS* gene encoding nitrite reductase, which reduces nitrite to nitric oxide, were amplified for confirming the aerobic denitrification. Primers NAP1/NAP2 were used for *napA* amplification using the conditions described by Kong *et al.* (2006); primers nirK1F/nirK5R and nirS1F/nirS6R were used for *nirK* and *nirS* amplification respectively, conducted as described by Braker *et al.* (2000). The length of amplicon was confirmed using 0.8% agarose gel electrophoresis and then staining with ethidium bromide for visualization.

Nitrogen removal performance

The medium used for performance evaluation contained 0.1 g CH_3COONa, 0.04 g KH_2PO_4, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $MgCl_2$, and 0.01 g $CaCl_2$ 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 OD_{600} (absorbance of the aqueous solution at 600 nm wavelength), pH and the concentrations of NO_2^--N, NO_3^--N and NH_4^+-N. 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 OD_{220} and $2 \times OD_{275}$. 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), NO_3^--N, NO_2^--N, NH_4^+-N) 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 29 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

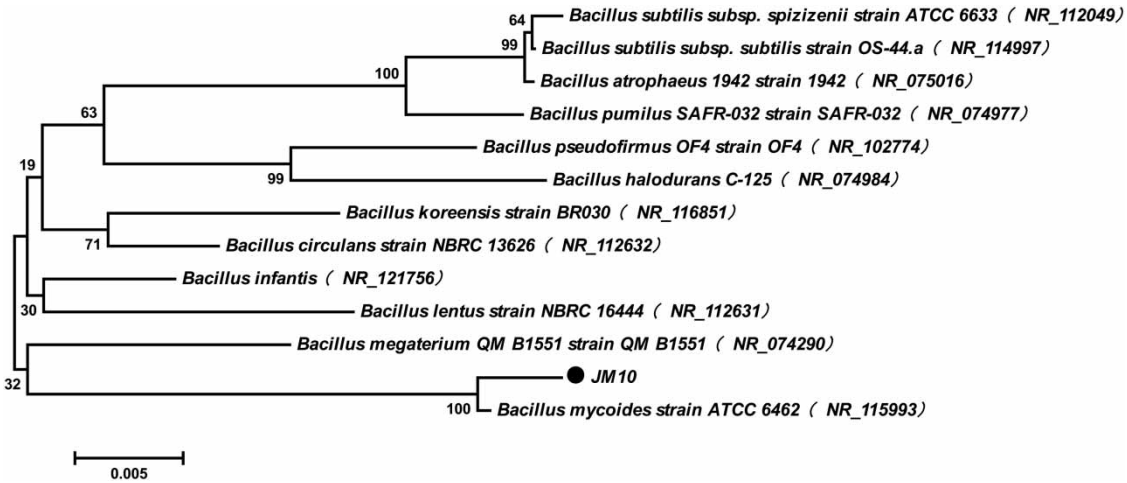

Figure 1 | Neighbor-joining tree based on a comparison of the 16S rRNA gene sequence showing the phylogenetic position of strain JM10 and representatives of some other related taxa. Bootstrap values, expressed as percentages of 1,000 replications, are given at branching points. Bar shows two nucleotide substitutions per 1,000 nucleotides.

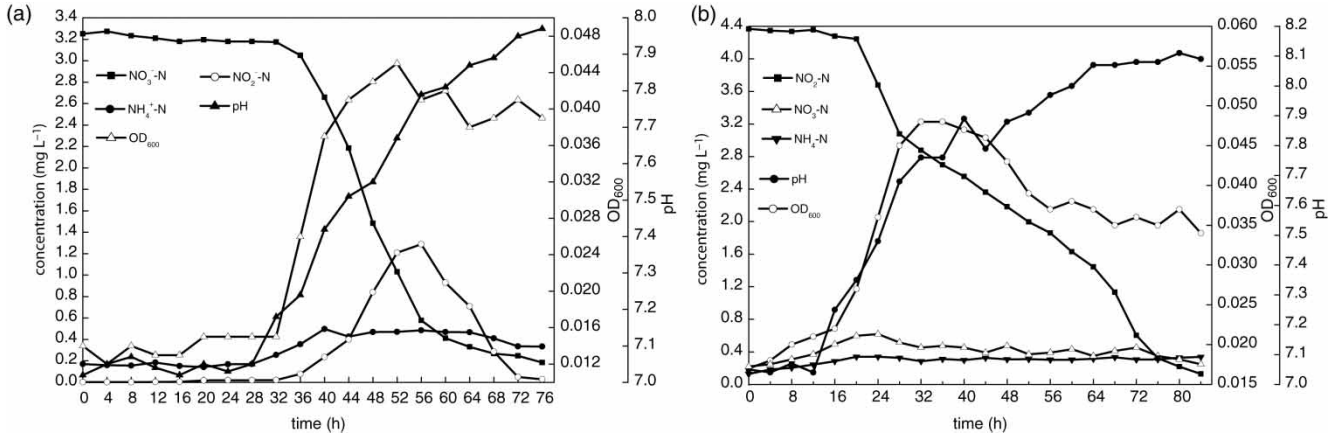

Figure 2 | Changes in the nitrogen conversion, pH and OD_{600} by *Bacillus* sp. JM10 in (a) sodium nitrate medium under aerobic conditions and (b) sodium nitrite medium under aerobic conditions.

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 NO_2^--N after 20 hours of stagnant time. Subsequently, the NO_2^--N was reduced to 0.13 mg L^{-1}, after achieving a removal efficiency of 97%. However, when nitrite was added, 0.40 mg L^{-1} NO_3^--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 NH_4^+-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:

$$\begin{aligned} Y_{JM10} = {} & 0.12 + 0.013X_1 - 0.0023X_2 + 0.009X_3 - 0.021X_4 \\ & + 0.01X_1X_2 + 0.013X_1X_3 - 0.012X_1X_4 + 0.020X_2X_3 \\ & - 0.0078X_2X_4 - 0.008X_3X_4 - 0.0091X_1^2 - 0.021X_2^2 \\ & - 0.023X_3^2 - 0.064X_4^2, \end{aligned} \quad (1)$$

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 (v_N), $P < 0.003$, were more significant than the C/N ratio (X_3), $P < 0.05$. The quadratic terms of the X_2^2, X_3^2, and X_4^2 had significant effects on the v_N ($P < 0.0008$). In addition, the interaction between initial pH and C/N ratio (X_2X_3) significantly affected the v_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 16.3% 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

Table 1 | Box–Behken experimental design representing the response of nitrate degradation rate (v_N) as affected by inoculum size, initial pH, C/N ratio and temperature

Run	Inoculum size (v/v, %) (X_1)	Initial pH (X_2)	C/N ratio (X_3)	Temperature (°C) (X_4)	Response NDR[a] (v_N, mg L^{-1} h^{-1})
1	10.00	7.00	6.00	30.00	0.1264
2	18.00	7.00	3.00	30.00	0.0721
3	10.00	9.00	9.00	30.00	0.1170
4	10.00	5.00	6.00	40.00	0.0401
5	10.00	9.00	6.00	40.00	0.0023
6	10.00	7.00	6.00	30.00	0.1270
7	10.00	7.00	6.00	30.00	0.1255
8	10.00	7.00	9.00	40.00	0.0053
9	2.00	7.00	9.00	30.00	0.0860
10	10.00	5.00	9.00	30.00	0.0570
11	2.00	7.00	3.00	30.00	0.0861
12	2.00	7.00	6.00	40.00	0.0362
13	18.00	9.00	6.00	30.00	0.1183
14	10.00	5.00	3.00	30.00	0.0831
15	10.00	9.00	3.00	30.00	0.0620
16	2.00	7.00	6.00	20.00	0.0378
17	2.00	9.00	6.00	30.00	0.0584
18	18.00	5.00	6.00	30.00	0.1091
19	2.00	5.00	6.00	30.00	0.0895
20	10.00	7.00	9.00	20.00	0.0840
21	10.00	7.00	3.00	20.00	0.0530
22	18.00	7.00	6.00	20.00	0.0890
23	10.00	7.00	3.00	40.00	0.0064
24	10.00	5.00	6.00	20.00	0.0606
25	10.00	7.00	6.00	30.00	0.1203
26	18.00	7.00	9.00	30.00	0.1228
27	18.00	7.00	6.00	40.00	0.0400
28	10.00	9.00	6.00	20.00	0.0540
29	10.00	7.00	6.00	30.00	0.1191

[a]Nitrate degradation rate.

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 v_N according to the inoculum size (X_1) and C/N ratio (X_3). 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 v_N can be obtained by simultaneously increasing the inoculum size and C/N ratio. Furthermore, as shown in Figure 3(a), the maximum v_N (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 (X_2) and C/N ratio (X_3) in the response process. As shown in Figure 3(b), the v_N 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 v_N value increased slightly when the C/N ratio was raised from 3.0 to 6.7, beyond which v_N was slightly decreased. In addition, a maximum v_N 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 (X_2) and temperature (X_4) on the v_N are shown in Figure 3(c). These results showed that strain JM10 displays high denitrification efficiency between pH 6.0 and 7.9 and a temperature from 26.1 to 31.0 °C, with a peak nitrate degradation rate of 0.12 mg L^{-1} h^{-1} at pH 7.04 and 28.6 °C. This strain exhibited inferior activity compared to most bacterial strains at moderate temperature, such as *Rhodococcus* sp. CPZ24 (Chen *et al.* 2012), which shows a nitrate removal rate of 0.93 mg L^{-1} h^{-1} at an experimental temperature of 30 °C. It is likely that the bacterial growth might be restricted under the low nutrient conditions, which resulted in the low nitrate degradation rate observed.

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

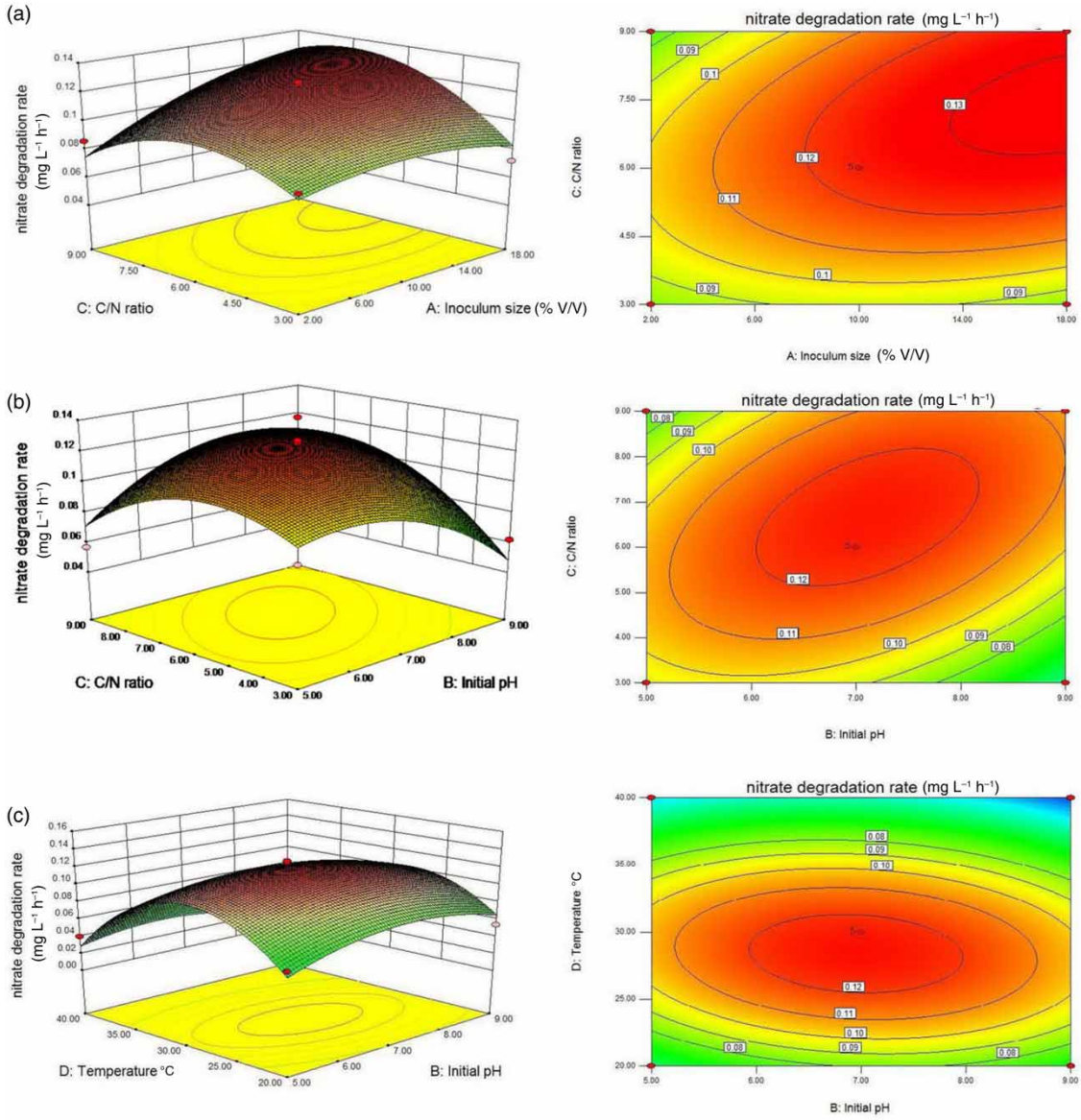

Figure 3 | Two- and three-dimensional contour plots for the nitrate degradation rate. RSM plots were generated using the data shown in Table 1. (a) Nitrate degradation rate as a function of inoculum size and C/N ratio. (b) Nitrate degradation rate as a function of initial pH and C/N ratio. (c) Nitrate degradation rate as a function of initial pH and temperature.

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.

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