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Aerobic denitrification and biomineralization by a novel heterotrophic bacterium, *Acinetobacter* sp. H36

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ABSTRACT

A novel aerobic denitrification and biomineralization strain H36 was isolated from the Qu Jiang artificial lake. Based on phylogenetic characteristics, the isolated strain was identified as *Acinetobacter* species. Strain H36 was confirmed to have the ability to perform simultaneous denitrification and biomineralization. Results showed the strain H36 had the capability to completely reduce 96.29% of NO_3^-–N and 78.59% of Ca^{2+} over 112 h under aerobic condition. Response surface methodology (RSM) analysis demonstrated the highest removal ratio of Ca^{2+} was 74.24% with hardness concentration of 350 mg/L, pH of 8.5, organic concentration of 0.75 g/L and inoculum size of 15%. The highest removal ratio of nitrate was 77.00% with hardness concentration of 350 mg/L, pH of 7.5, organic concentration of 0.75 g/L and inoculum size of 10%. Besides, X-ray diffraction (XRD) analysis showed calcium carbonate could be formed in the process of biomineralization.

1. Introduction

Nitrate contamination of groundwater is becoming more and more serious in many countries (Sahinkaya et al. 2015; Qu et al. 2015). Groundwater contamination by nitrate usually originates from anthropogenic sources, mainly as a result of wastewater discharges and the intensive application of fertilizers and animal manure to agricultural land (Qambrani et al. 2013). Nitrate can cause methemoglobinemia when ingested by infants (Winton et al. 1997; Sunger and Bose. 2009), and they can even cause carcinoma, malformation and mutation when transformed into nitrosoamines (Zhao et al. 2011).

Biological denitrification is the comparatively significant process removing nitrate in natural environments (Canziani and Bonomo. 1998). Biological denitrification process, which comprises autotrophic denitrification and heterotrophic denitrification, is considered as an efficient technology in the treatment of nitrate contaminated water. Heterotrophic denitrification is very efficient in nitrate removal if adequate amounts of organic carbon are available (Straub et al. 1996).

As one of the important water source for human, groundwater with a high concentration of hardness will have a certain effect on human health and everyday life. Hardness of water refers to the total concentration of calcium and magnesium. Groundwater with a high concentration of hardness will not have a good taste, and the water is easy to produce the white precipitation scale. Scaling on the surface of heat exchanger reduces boiler power output by 10–20%, decreases thermal efficiency by 10% (Pan et al. 2011; Zarga et al. 2013; Mavredaki et al. 2005; Nishida et al. 2009), plugs the pipeline (Zeng et al. 2007), and even makes more shut-downs (Koo et al. 2011). It was reported that >85% of boilers in the United States have hard water supplies (Straub et al. 1996).

At present, the water softening treatment technology was different. But with microorganism to soften the groundwater, it is still a blank. In the field of biomineralization, large numbers of research have carried out to explore the calcium carbonate deposition problem with a participation of microorganisms. Such as the research of mechanism of biomineralization by soil microorganisms mechanism (Monger et al. 1991). And the research of the formation of the nano-rod calcite by microorganisms and mineralization (Chen et al. 2005). Further evidence that microorganisms play a role in calcite precipitation is shown by ability of the bacteria to precipitate calcite in culture studies. Chahal et al. (2011) found that many strains of bacteria, including *Salmonella* spp., *Bacillus pumilus*, and *Pseudomonas aeruginosa*, could form calcite crystals in 1 to 20 days on a medium containing calcium acetate. The bacterium can be used to precipitate the calcium carbonate, so it can be used to the treatment of groundwater to soften the hardness.

The objective of this study was to isolate a novel heterotrophic denitrification and biomineralization bacterium, *Acinetobacter* sp. H36, from the Qu Jiang artificial lake. Factors affecting the performance of *Acinetobacter* sp. H36 were comprehensively evaluated based on response surface methodology (RSM) analysis. The purpose of the study

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was to determine the ability of bacterium H36 to remove nitrate and soften the water simultaneously. Meanwhile, find out the comparatively suitable environment on the removal of nitrate and hardness.

2. Methods

2.1. Sampling and culture medium preparation

The sediment samples were obtained from the surface of the rocks in Qu Jiang artificial lake (34°12′27.69″N, 108°58′33.73″E) and used to isolate simultaneous denitrification and biomineralization bacteria.

The ingredients of the enrichment medium (EM) in 1000 mL distilled water were as follows: 3.0 g of peptone, 0.1 g of $NaNO_3$, 0.1 g of KH_2PO_4, 0.05 g of $MgCl_2 \cdot 7H_2O$, 0.5 g of NaCl, 0.5 g of $CaCl_2$ and 2 mL of trace elements solution (Li and Qu. 2009). The final pH of EM was adjusted to 6.8–7.0. The basal medium (BM) used in this study was comprised of the following reagents per L: 1.3 g of $C_4H_4Na_2O_4 \cdot 6H_2O$, 0.2 g of $C_6H_{12}O_6$, 0.1 g of $NaNO_3$, 0.1 g of KH_2PO_4, 0.05 g of $MgCl_2 \cdot 7H_2O$, 0.5 g of NaCl, 0.5 g of $CaCl_2$ and 2 mL of trace elements solution. The final pH of BM was adjusted to 7.0 (Su et al. 2016a). The components of trace elements solution were as follows: 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L EDTA, 0.2 g/L $ZnSO_4$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.5 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L $CuSO_4 \cdot 5H_2O$ and 0.2 g/L $CoCl_2 \cdot 6H_2O$.

2.2. Enrichment and isolation of strains

The 100 mL sediment samples were added to 500 mL of EM and then incubated at 30 °C for 2 weeks under aerobic condition. After this, 10 mL of the enrichment suspension was transferred to 100 mL of fresh BM and then incubated under same conditions. Mature bacterial suspension was spread on the BM plates and incubated at 30 °C in stationary incubator under aerobic condition. Separate colonies were picked and purified by repeated streaking on the fresh BM plates. Finally, the isolates were cultivated in the fresh BM to test their ability of denitrification and biomineralization.

2.3. Bacterial strain identification

Strain H36 was identified by PCR amplification of the 16S rDNA gene of the isolate using bacterial universal primers 27f(50′-AGAGTTTGATCATGGCTCAG-30′) and 1492r(50′-TACGGTTACCTTGTTACGACTT-30′). The PCR product was sequenced by Sangon Biotech Co. Ltd. (Shanghai, China). The sequence was compared with available sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was then constructed using MEGA software (version 5.05) and the neighbor-joining algorithm.

2.4. The selection of the most appropriate organic matter

In this part, peptone, glucose, sodium acetate and sodium succinate, four kinds of organic matter were experimented to choose which was the most appropriate organic for the function of heterotrophic denitrification and biomineralization by stain H36. In this experiment, the initial nitrate-nitrogen concentration was 17.32 mg/L, the initial hardness concentration was 452.03 mg/L and the initial organic concentration was 1.5 g/L. The experiment was carried out in the constant temperature incubator at 30 °C with a rotate speed of 145 r/min, initial DO (dissolved oxygen) of the medium was about 5–6 mg/L. The medium was sampled after 4 days of reaction to determine nitrate, nitrite and hardness.

2.5. Treatment efficiency under the suitable conditions

450 mL medium was placed into 500 mL conical flask in triplicate and inoculated with 50 mL bacterial suspension of the isolate. These conical flasks were placed in the constant temperature incubator and cultivated at 30 °C with a rotate speed of 145 r/min.

The medium used in this experiment was comprised of the following reagents per L: 0.87 g of $C_4H_4Na_2O_4 \cdot 6H_2O$, 0.13 g of $C_6H_{12}O_6$, 0.1 g of $NaNO_3$, 0.1 g of KH_2PO_4, 0.05 g of $MgCl_2 \cdot 7H_2O$, 0.5 g of NaCl, 0.4 g of $CaCl_2$ and 2 mL of trace elements solution. The final pH of this medium was adjusted to 7.5.

The medium was sampled periodically to determine humus, nitrate, nitrite, pH and hardness.

2.6. Experimental design and statistical analysis

The response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for optimum condition of factors for desirable responses. This design is suitable for exploration of quadratic response surfaces and for construction of second order polynomial models, thus helping to optimize the process by using a small number of experimental runs. After the preliminary study, a four-factor central composite design (Box-Behnken) was obtained by using experimental design. It was employed to investigate the interactive effects of four variables, viz. Organic concentration, pH, inoculum size and Ca^{2+} concentration on denitrification and biomineralization. Design-Expert (version 8.06) software was used for the statistical design of experiments and data analysis.

The number of experiments (N) required for the development of BBD is defined as $N = 2k\ (k\text{-}1) + Co$ (where k is number of factors and Co is the number of central points). A line of experiments were carried out with different Organic concentration (0.5–1.0 g/L), pH (6.5–8.5), inoculum size (5%–15%) and Ca^{2+} concentration (200–500 mg/L).

2.7. X-ray diffraction test on the precipitation

X-ray Diffraction (XRD), a research technique which can achieve the material composition, internal information such as atomic or molecular structure or form by the analysis of the diffraction pattern. The precipitation system was conducted in the centrifuge 9000 rpm 5 min, then transfer out the liquid supernatant, retain the sediments. The sediments were carried out the operation of rinse-centrifugal repeatedly three times by deionized water to remove the residual medium components in precipitation. Then the sediments were tested with XRD after the dry processing.

3. Results and discussion

3.1. 16S rDNA gene sequencing and similarity analysis

A simultaneous denitrification and biomineralization strain H36, was isolated from the Qu Jiang artificial lake. A phylogenetic tree was reconstructed based on the 16S rDNA gene sequence of the isolate and some other phylogenetically related strains (Fig. 1). The results indicated that strain H36 was most closely related to *Acinetobacter* antiviralis strain KNF 2022 (NR 115739) (similarity 100%). Therefore, strain H36 is proposed to be an *Acinetobacter* species.

3.2. The selection of appropriate organic matter

The measurements of nitrate, nitrite and Ca^{2+} concentration were carried out after 4 days of reaction, the results (Fig. 2(a)) indicated that the highest removal ratio of hardness (63.65%) was obtained with sodium succinate as the organic matter, the lowest removal ratio was obtained with glucose as the organic matter (27.08%). However, nitrite accumulation was observed when using the sodium succinate. For the removal of nitrate, glucose was the most suitable organic matter with a removal ratio of 90%, without the accumulation of nitrite at the same time. In order to achieve the result that the removal of nitrate, the

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