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Highly efficient nitrogen removal of a coldness-resistant and low nutrient needed bacterium, *Janthinobacterium* sp. M-11

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ABSTRACT

A novel heterotrophic nitrification-aerobic denitrification bacterium, identified as *Janthinobacterium* sp. M-11, was isolated from the Songhua River. When the initial ammonium concentration was 5 mg·L^{-1}, 98% of ammonium was removed under cold condition (2 °C) with the C/N ratio of 5 at initial pH 7 and aerobic condition, which demonstrated the significant ammonium removal capacity of M-11 with low nutrient consumption at cold temperature. Denitrification processes under aerobic and anaerobic conditions were also investigated. 89% of nitrite and 89% of nitrate were removed under aerobic condition. Under anaerobic condition, 93% of nitrite and 98% of nitrate were removed. Interestingly, a high amount of nitrite accumulation was observed in the mid-stage of anaerobic denitrification for nitrate. This special phenomenon was probably because of the existence of *narG* gene amplified in the strain M-11, which would encode membrane-bound nitrate reductase and accelerate the nitrate conversion rate of M-11 under anaerobic condition.

1. Introduction

As the main reason for water eutrophication, nitrogen contamination of surface water has been considered as a worldwide challenge for surface water treatment. Traditionally, the most common approach for nitrogen removal is biological water treatment due to its high efficiency and economical cost (Li et al., 2017a, b). Nitrogen removal process commonly consists of nitrification and denitrification processes. Autotrophic nitrification for nitrogen removal is generally inefficient under low temperature condition (Huang et al., 2013; Chen et al., 2018). Recently, biological water treatment processes based on heterotrophic bacteria have attracted increasing attention due to its better performance than that of the autotrophs under extreme conditions, such as high salinity, heavy metal ions, strong alkali and so on (Duan et al., 2015; Sun et al., 2016; Wang et al., 2016). UP to now, intensive researches have been conducted on heterotrophic nitrification-aerobic denitrification bacteria under low temperature, such as *Acinetobacter* sp. HA2 (Yao et al., 2013b), *Acinetobacter* sp. HITLi 7^{T} (Qin et al., 2016), *Pseudomonas* sp. VNT and *Aeromonas* sp. VNT (Mohd Yasin et al., 2014). These heterotrophic bacteria exhibited efficient nitrogen removal capacity for high concentration nitrogen sewage in low temperature range.

However, in practical terms, most water sources are in a state of low nutrient condition, where the growth, proliferation and metabolism of general microorganism will be inhibited due to the lack of N source or C source (Worrich et al., 2017; Zhou et al., 2016). In addition, the lack of nutrient will exert restraint on the nitrification efficiency of coldness resistant bacteria and extend the time of nitrification process (Su et al., 2015). Reports about heterotrophic nitrification and aerobic denitrification bacterial species showed limited nitrogen removal efficiencies under low level of nutrient and low temperature conditions (Sun et al., 2017; Sun et al., 2015; Wan et al., 2017). Therefore, it is necessary to develop novel water treatment technologies to remove nitrogen efficiently at low temperature and low-nutrient conditions ($\leq$5 mg·L^{-1}) (Huang et al., 2013; Su et al., 2015).

In this paper, a new *Janthinobacterium* sp. strain M-11 was isolated from a pilot-scale bioreactor for the treatment of Songhua River in winter (Supplementary Data), which could significantly conduct the nitrogen removal under low nutrient and low temperature. The influence of temperature, carbon source, C/N ratio, initial pH and dissolved oxygen (DO) on ammonium removal capacity of *Janthinobacterium* sp. strain M-11 were investigated. Ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) were used as sole nitrogen source respectively to characterize heterotrophic nitrification and aerobic denitrification performance of M-11. Dissolved oxygen concentration would gradually decrease during its mass transfer process

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from the surface layer into the inner layer of biofilm, which may lead to an anaerobic environment inside of biofilm. For this reason, this study additionally examined the anaerobic denitrification performance of the strain, which provided a theoretical basis for practical application of this strain under anaerobic condition. The functional denitrification genes were also amplified to better understand the denitrification pathway of the strain M-11.

2. Materials and methods

2.1. Strain isolation

Fresh biological activated carbon (BAC) sample was collected from a pilot-scale bioreactor in December 2016. The sample was shaken in sterile water, and 100 μL supernatant liquor was spread on selection medium plates after centrifugation. The selection medium consisted of (per liter) $CH_3COOHNa$ 1 g, NH_4Cl 1 g, NaH_2PO_4 0.2 g, Na_2HPO_4 0.2 g, agar 18 g and 2 mL trace element solution, pH 7–7.2. Trace element solution (per liter) comprised the following reagents: $MnCl_2$ 0.5 g, $FeSO_4$ 0.5 g, $MgSO_4$ 1 g, $CaCl_2$ 1 g, $ZnSO_4$ 0.5 g and $CoCl_2$ 0.2 g. The selection plates were cultured at 2 °C for 10 days. To obtain the purified strain, colonies on the selection medium were separated and cultivated thrice on the selection medium under the same condition. The selected purified strains were enriched in the enrichment medium respectively (per liter): NH_4Cl 0.1 g, $CH_3COOHNa$ 1 g, Na_2HPO_4 0.2 g, KCl 0.1 g, 2 mL trace element solution, pH 7–7.2, at 2 °C for 3 days. 10 mL enrichment medium was centrifuged (5000r/min, 5 min). Sediment at the bottom of the centrifuge tube was washed by phosphate buffer saline (PBS) thrice and then was inoculated into nitrification medium (per liter): NH_4Cl 20 mg, $CH_3COOHNa$ 90 mg, Na_2HPO_4 20 mg, KCl 10 mg, 1 mL trace element solution. Rate and amount of NH_4^+-N removal in the nitrification medium were measured to select the strain with the highest nitrification ability.

2.2. Identification of bacterial strain Janthinobacterium sp. M-11

Biochemical and physiological experiments were tested to identify the strain (Dong and Cai, 2001). The micrograph of the strain was examined with a scanning electron microscopy (S-3400N, Hitachi Limited, Japan). Bacterial universal primers: forward primed F27 (5′-AGA GTTTGATCATGGCTCAG-3′) and reverse primer R1492 (5′-TACGGTT ACCTTGTTACGACTT-3′) were used to amplify the genomic DNA of the isolated strain. The experimental conditions were as follows: 95 °C for 5 min, 35 cycles of 0.5 min at 95 °C, 0.5 min at 58 °C, 1.5 min at 72 °C, and finally 7 min at 72 °C. The sequence of the 16S rDNA was compared with the gene sequences on the NCBI and Ezbiocloud (http://www.ezbiocloud.net/). Finally, a phylogenetic tree was constructed by the neighbor-joining method using MEGA software.

2.3. Analysis of nitrification process

2.3.1. Effect of different factors on NH_4^+-N removal

The purified strain with the highest efficiency of NH_4^+-N was cultured in the enriched medium for three days at 2 °C. After centrifugation, sediment at the bottom of the centrifuge tube was washed by PBS thrice and then was inoculated into nitrification medium. Effects of temperature, carbon source C/N ratio, initial pH and DO on the NH_4^+-N removal capacity of M-11 were investigated, respectively. The experiments were all tested with the same inoculum size (about 4×10^6 CFU/mL). The adaptability of M-11 to temperature was studied in the range of 2 °C–35 °C. Methyl alcohol, alcohol, glycerol, sodium citrate, glucose, sucrose and starch were used as sole carbon source respectively instead of sodium acetate with the fixed NH_4^+-N concentration of 5 mg·L^{-1}. C/N ratio was set at 1, 3, 5, 7 and 9 with the fixed NH_4^+-N concentration of 5 mg·L^{-1}. To detect the effect of the initial pH on the NH_4^+-N removal, the initial pH was adjusted to 5, 6, 7, 8, 9 and 10 by 2 mol/L HCl and 1 mol/L NaOH.

In our study, a closed reactor was used to investigate the influence of DO on NH_4^+-N removal during the nitrification process. In such case, DO would decrease gradually with the increase of biomass, leading to the aerobic, anoxic and anaerobic conditions sequentially. In order to consume the DO in the medium, initial concentration of NH_4^+-N was increased from 5 mg·L^{-1} to 17 mg·L^{-1} with the C/N ratio of 5. The variations of NH_4^+-N concentration, optical density at 600 nm (OD_{600}) and DO were measured over time, respectively. Initial and final concentrations of total organic carbon (TOC) were also determined. All experiments were carried out in triplicate.

2.3.2. Cell growth and NH_4^+-N conversion

To assess the cell growth and NH_4^+-N conversion of strain M-11 under low temperature and low nutrient conditions, the preculture strain was inoculated into the nitrification medium with an initial NH_4^+-N concentration of 5 mg·L^{-1} at 2 °C. The initial OD_{600} was controlled in the range of 0.02–0.03, which was real-time measured to estimate the cell growth. Medium without strain inoculated was used as control. Initial and final TN of the medium were measured. Samples were periodically taken from the medium and centrifuged. Their supernate was tested to determine the concentrations of NH_4^+-N, NO_2^--N, NO_3^--N and total nitrogen (TN). Ammonia monooxygenase (AMO) was the key enzyme which would catalyze the oxidation of NH_4^+-N to hydroxylamine. In this study, *amoA* gene was amplified to investigate the conversion way of NH_4^+-N for M-11. The amo-1F (5′-GGGGTTTC TACTGGTGGT) and the amo-2R (5′-CCCCTCKGSAAAGCCTTCTTC) were used as degenerate primers (Rotthauwe et al., 1997). The polymerase chain reaction (PCR) protocol of the *amoA* gene consisted of following conditions: denaturation step at 95 °C for 5 min; followed by 35 cycles of denaturing for 30 s at 95 °C; annealing for 30 s at 58 °C; extension step at 72 °C for 60 s and followed by a final extension for 7 min at 72 °C. The PCR mixtures were analyzed through electrophoresis on a 1% agarose gels and sequenced by the company of Sangon Biocompany (Shanghai, China).

2.4. Analysis of denitrification process

2.4.1. NO_3^--N and NO_2^--N removal under aerobic and anaerobic conditions

To investigate the denitrification performance of M-11, NO_2^--N and NO_3^--N were used as sole nitrogen source, respectively. Anaerobic and aerobic denitrification capacities of the strain M-11 were studied. Anaerobic medium was aerated with nitrogen gas to reduce the DO less than 0.2 mg·L^{-1}. Initial concentrations of NO_2^--N and NO_3^--N were adjusted to approximate 5 mg·L^{-1}. All samples were shaken at 150 rpm under 2 °C, pH was adjusted to 7 with the C/N = 5. The anaerobic denitrification experiment was conducted in an anaerobic reactor to ensure the anaerobic experiment. Concentrations of NO_2^--N and NO_3^--N were measured periodically to detect the denitrification capability of M-11. OD_{600} was monitored with the time changing. The average cell growth rate (μ^{250}) was calculated according to the equation as following (Hall et al., 2013):

$$\mu^{250} = (\ln X - \ln X_0)/(t - t_0)$$

X_0 is the number of cells at the starting point, X is the number of cells at the end, and μ^{250} is the slope of ln X versus t. In our study, μ^{250} was used as the slope of ln OD versus t. As the t was in days, the growth rate was μ^{250} d^{-1}. All experiments were conducted for three times.

2.4.2. Genes amplification and pathways analysis

Nitrate reductase gene is commonly used as the functional marker of heterotrophic nitrification-aerobic denitrification bacteria. A strain may include both periplasmic nitrate reductase gene (*napA*) and membrane-bound nitrate reductase gene (*narG*) which could encode nitrate reductase. NAP1 (5′-TCTGGACCATGGGCTTCAACCA-3′) and NAP2