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Marinobacter strain NNA5, a newly isolated and highly efficient aerobic denitrifier with zero N_2O emission

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HIGHLIGHTS

- A new aerobic denitrifier, named NNA5, was isolated, identified as *Marinobacter* sp.
- Strain NNA5 could perform only aerobic denitrification but not nitrification.
- Aerobic denitrification was efficient with NO_3^--N removal rate of 112.8 mg/L/d.
- The aerobic denitrifying product of strain NNA5 is N_2 while no N_2O emission.

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ABSTRACT

An efficient aerobic denitrification bacterium, strain NNA5, was isolated and identified as *Marinobacter* sp. NNA5. NNA5 did not perform heterotrophic nitrification. GC/IRMS analysis revealed that $^{15}N_2$ was produced from $Na^{15}NO_2$ and $K^{15}NO_3$. GC/MS and quantitative analyses showed that no N_2O emission occurred when nitrite or nitrate was used as substrate. Single factor experiments indicated that optimal conditions for aerobic denitrification were: sodium succinate or sodium pyruvate as carbon source, temperature 35 °C, NaCl concentration 2–4%, C/N ratio 6–8, pH 7.5, rotation speed 150 rpm (giving dissolved oxygen concentration 6.08 mg/L), NO_3^--N concentration ranging from 140 to 700 mg/L. NNA5 displayed highly efficient aerobic denitrifying ability, with maximal NO_3^--N removal rate 112.8 mg/L/d. In view of its ability to perform aerobic denitrification with zero N_2O emission, NNA5 has great potential for future application in aerobic denitrification processes in industrial and aquaculture wastewater treatment systems.

1. Introduction

Biological denitrification is a microbially facilitated process consisting of sequential conversion of nitrate (NO_3^-) → nitrite (NO_2^-) → nitric oxide (NO) → nitrous oxide (N_2O) → nitrogen gas (N_2) (Alvarez et al., 2014; Robertson et al., 1988). For many decades, biological denitrification was considered to occur only under anaerobic or anoxic conditions. Aerobic denitrification was first reported in 1984, for the bacterium *Thiosphaera pantotropha* (Robertson and Kuenen, 1984). Further studies showed that *T. pantotropha* is not only an aerobic denitrifier, but also a heterotrophic nitrifier (Robertson et al., 1988). *Alcaligenes faecalis* DSM 30030 was reported in 1989 to also be capable of growth with simultaneous heterotrophic nitrification and aerobic denitrification (Papen et al., 1989). Many other aerobic denitrifiers have subsequently been isolated from a variety of environments, and the coupling of heterotrophic nitrification with aerobic denitrification has been shown to occur in many bacterial species, including *Pseudomonas stutzeri* (Takaya et al., 2003), *Paracoccus versutus* LYM (Shi et al., 2013), *Bacillus methylotrophicus* (Zhang et al., 2012), *Acinetobacter* sp. (Yao et al., 2013), *Halomonas campisalis* (Guo et al., 2013), *Microvirgula aerodenitrificans*, *Comamonas testosterone*, (Patureau et al., 2000; Wan et al., 2011), and *Marinobacter* sp. F6 (Zheng et al., 2012).

Many studies have focused on enhancement of heterotrophic nitrification and aerobic denitrification for increased nitrogen removal efficiency during biological wastewater treatment, or on simultaneous nitrification and denitrification (SND) processes. A much smaller number of studies to date have addressed emission of gaseous nitrogen products during aerobic denitrification, and have shown that the major emitted gas is usually N_2O. N_2O-emitting aerobic denitrifying bacteria have limited applicability,

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because N_2O is a well-known "greenhouse gas" that has harmful environmental effects (Davidson and Kingerlee, 1997).

A novel aerobic denitrifying bacterium, termed strain NNA5, was isolated from a recirculating aquaculture system (RAS), and identified as *Marinobacter* sp. Aerobic denitrification by NNA5 using either nitrite nor nitrate as substrate resulted in zero N_2O emission. Heterotrophic nitrification by NNA5 using ammonia as substrate was not occurred resulted in zero gaseous nitrogen products and no TN reduction. The aerobic denitrifying performance of NNA5 was analyzed and described. NNA5 is a promising new microbial resource for nitrogen removal treatment of wastewater, and for aerobic denitrification of wastewater in industry and aquaculture.

2. Methods

2.1. Culture media

Enrichment denitrifying medium (EDM) (per liter artificial seawater): tryptone 5.00 g, yeast extract 2.50 g, nitrate 1.00 g. For preparation of solid EDM, 1.5% agar was added. Artificial seawater was composed of (per liter) NaCl 23.60 g, KCl 0.64 g, $MgCl_2{\cdot}6H_2O$ 4.53 g, $MgSO_4{\cdot}7H_2O$ 5.94 g, $CaCl_2{\cdot}2H_2O$ 1.30 g.

Denitrification medium (DM) (per liter distilled water): KNO_3 1.00 g, sodium succinate 4.68 g, $MgSO_4{\cdot}7H_2O$ 0.20 g, $CaCl_2$ 0.01 g, EDTA 0.07 g, KH_2PO_4 0.50 g, Na_2HPO_4 0.50 g, $FeSO_4$ 0.01 g, NaCl 30.00 g, trace element solution 2.00 mL, pH 7.5.

Nitrite denitrification medium (NDM) (per liter distilled water): $NaNO_2$ 0.28 g, sodium succinate 3.16 g, $MgSO_4{\cdot}7H_2O$ 0.20 g, $CaCl_2$ 0.01 g, EDTA 0.07 g, KH_2PO_4 0.50 g, Na_2HPO_4 0.50 g, $FeSO_4$ 0.01 g, trace element solution 2.00 mL, pH 7.5.

Heterotrophic nitrification medium (HNM) (per liter distilled water): $(NH_4)_2SO_4$ 0.66 g, sodium succinate 4.72 g, KH_2PO_4 0.50 g, Na_2HPO_4 0.50 g, $MgSO_4{\cdot}7H_2O$ 0.20 g, NaCl 30.00 g, trace element solution 2.00 mL, pH 7.5.

Trace element solution (Joo et al., 2005) (per liter): EDTA·2Na 57.10 g, $ZnSO_4{\cdot}7H_2O$ 3.90 g, $CaCl_2{\cdot}2H_2O$ 7.00 g, $MnCl_2{\cdot}4H_2O$ 1.00 g, $FeSO_4{\cdot}7H_2O$ 5.00 g, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 1.10 g, $CuSO_4{\cdot}5H_2O$ 1.60 g, $CoCl_2{\cdot}6H_2O$ 1.60 g, pH 6.0.

2.2. Isolation and identification of aerobic denitrifying bacteria

Strain NNA5 was isolated from the biofilm of a biological aerated filter used for treatment of recirculating water of an RAS in Shandong Oriental Sea Polytron Technologies, Yantai, China. The sample was incubated in EDM on a rotary shaker (120 rpm) at 30 °C for 3 days. The resulting bacterial suspension was streaked on solid EDM. Single colonies were tested individually for nitrifying and denitrifying activity in basal media (HNM, DM, and NDM). Standard culture was performed using Difco Marine Agar 2216, with storage as glycerol stocks (15%, w/v) at −80 °C.

16S rRNA gene was amplified by PCR using bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGA-CTT-3′), and sequenced by SinoGenoma Corp. (Beijing, China). PCR conditions: 94 °C for 5 min; 35 cycles at 94 °C for 1 min; 55 °C for 1 min; 72 °C for 1½ min; final extension at 72 °C for 10 min. 16S rRNA gene sequences were compared with available sequences in GenBank databases using the BLAST program (Altschul et al., 1990) on NCBI (http://www.ncbi.nlm.nih.gov), and also on the EzTaxon-e server (www.ezbiocloud.net/eztaxon) using identity analysis (Kim et al., 2012). Phylogenetic trees were constructed using neighbor-joining methods in the MEGA5 software program (Tamura et al., 2011). Robustness of tree topologies was assessed by bootstrap analysis based on 1000 replications.

2.3. Analytical methods

Bacterial growth was monitored by measuring OD_{600} (spectrophotometer model UV-7200, UNICO, Shanghai, China). Concentrations of ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and total oxidized nitrogen (TON) were determined by standard methods using an Aquakem 600 Analyzer (Thermo Scientific). Ammonia was reacted with hypochlorite ions generated by alkaline hydrolysis of sodium dichloroisocyanurate to form monochloramine. Nitrite was determined by diazotizing with sulfanilamide and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye. For TON analysis, nitrate was reduced to nitrite by hydrazine under alkaline conditions, and nitrate was calculated based on nitrite and total nitrite. Total nitrogen (TN_b) was determined using a TOC/TN_b analyzer (Elementar Vario TOC cube, Germany). Results are presented as mean ± SD from three independent experiments.

2.4. Detection of gaseous nitrogen products

Gaseous nitrogen products of denitrification were analyzed by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) and gas chromatography/mass spectrometry (GC/MS) (Ai et al., 2011). Strain NNA5 was incubated in 8 mL medium in a 25-mL anaerobic tube at 30 °C on a rotary shaker (150 rpm). Gases from the headspace were detected following hermetic incubation for one week. The GC/IRMS and GC/MS devices were both equipped with GS-Carbon Plot (30 m × 0.32 mm × 3.0 μm, Agilent, USA).

Gaseous nitrogen products of N_2 were analyzed by GC/IRMS (Thermo Fisher Scientific Trace GC ultra/DELTA V Advantage Isotope Ratio MS, Agilent). NNA5 was incubated in DM, NDM, and HNM media containing 50% $K^{15}NO_3$, 50% $Na^{15}NO_2$, or 50% $^{15}NH_4Cl$ (by atomic fraction, Spectra Corp., USA), respectively. The headspace gas product was analyzed from 5 μL upper gas using a 10-μL gas-tight syringe. Conditions: sample injection port and detector temperatures 120 °C; temperature programmed from 35 to 140 °C at 30 °C/min (maintained at 35 °C and 140 °C for 6 min and 1 min, respectively); gas flow = carrier He at 1.0 mL/min; splitting ratio 30:1.

Gaseous nitrogen products of N_2O were analyzed by GC/MS (model 7890A/5975C, Agilent). NNA5 was incubated in three basal media as above. The headspace gas product was analyzed from 50 μL upper gas using a 100-μL gas-tight syringe. Conditions: sample injection port and detector temperatures 120 °C; temperature programmed from 35 to 120 °C at 25 °C/min (maintained at 35 °C and 120 °C for 5 and 3 min, respectively); gas flow = carrier He 1.2 mL/min; quantity added 600 μL.

Quantitative measurement of N_2O concentration was performed by the Environmental Testing Center of The Institute of Geographic Sciences and Natural Resources Research (Chinese Academy of Sciences, Beijing). A blank control and a positive control (aerobic denitrifier *A. faecalis* CGMCC 1.1837) were run in parallel.

2.5. Single-factor experiments

To evaluate the effects of various culture conditions (carbon source, temperature, salinity, C/N, pH, dissolved oxygen) on aerobic denitrification efficiency of strain NNA5, single-factor experiments were performed in flasks using DM medium. Sole carbon sources tested were sodium succinate, sodium acetate, sodium pyruvate, potassium sodium tartrate, glucose, methanol, and ethanol. Other experimental conditions were: initial nitrogen concentration 140 mg/L-N (using KNO_3), C/N ratio 6, initial pH 7.5, NaCl concentration 30 g/L, culture temperature 30 °C, shaking speed 150 rpm. For temperature experiments, sodium succinate was