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Research article

Enrichment and characterization of acid-tolerant nitrifying sludge

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ABSTRACT

Nitrification is an acidifying process that requires the addition of external alkalinity because of the alkaliphilic nature of the most ammonia-oxidizing bacteria. In this study, aerobic activated sludge was used as inoculum in an internal loop air-lift reactor, which resulted in successful enrichment of acid-tolerant nitrifying (ACIN) sludge at pH 6.0 by sequential addition of tea orchard soil suspension. The results showed that ACIN sludge had a remarkable acid tolerant capability with a volumetric ammonia conversion rate of 1.13 kg N m^{-3} day^{-1}. ACIN sludge showed a higher maximum specific ammonia conversion rate (0.29 g N g^{-1} VSS day^{-1}) than neutrophilic nitrifying sludge (0.14 g N g^{-1} VSS day^{-1}) at pH 6.0 and had good resistance against pH fluctuations, with a maximum specific ammonia conversion rate (0.584 g N g^{-1} VSS day^{-1}) at pH 7.5. Microbial community analysis indicated that the higher abundance of acid tolerant *Nitrosospira* and ammonia-oxidizing archaea laid a solid foundation for the remarkable acid-tolerant capability of ACIN sludge.

1. Introduction

Nitrification includes ammonia oxidation ($NH_4^+ \rightarrow NO_2^-$) and nitrite oxidation ($NO_2^- \rightarrow NO_3^-$), and ammonia oxidation is the key and rate-limiting step (Jin et al., 2010; Wett and Rauch, 2003). The generation of nitrite and nitrate results in the acidification of nitrification (Wett and Rauch, 2003). However, ammonia-oxidizing bacteria (AOB), the dominant functional microorganisms of ammonia oxidation are alkaliphiles, which are highly sensitive to pH and show optimum growth at pH 7.5–8.0 (Gieseke et al., 2006; Metcalf et al., 2002; Morris, 2011; Tarre and Green, 2004). Acidification of the nitrification system severely inhibited the activity of AOB and reduced the nitrification rate. For example, the activity of *Nitrosomonas* was reduced to 20% of the optimum activity when the environmental pH decreased from 8.1 to 6.7 (Grunditz and Dalhammar, 2001), and the ammonia oxidizing reaction stopped when the pH fell to 6.5 (Burton and Prosser, 2001; Tarre and Green, 2004). Therefore, the amount of external alkalinity added to the nitrification system was vital and decisive. With this background, it is worth enriching the acid-tolerant nitrifying (ACIN) sludge and reducing the environmental pH of nitrification systems from 7.5 to 6.0 to reduce external alkalinity consumption, thereby reducing chemical costs. The previous studies showed that the alkalinity consumption of nitrification was high up to 7.07 g-$CaCO_3$ g^{-1}-NH_4^+-N, and if we can reduce the pH of nitrification system from 7.5 to 6.0, its alkalinity consumption could save 1.7 g-$CaCO_3$ g^{-1}-NH_4^+-N at least (Wang, 2014). And it would save almost 5.1 dollars per kilogram NH_4^+-N.

Acid tolerant nitrification and its microorganisms were intensively studied in acid soils in the 1990s. However, few studies have documented the nitrification process in wastewater treatment systems (Tarre and Green, 2004). Recently, the discovery of ammonia-oxidizing archaea (AOA) increased our knowledge of the global nitrogen cycle (Leininger et al., 2006) and put forward a new perspective regarding the development of acid tolerant nitrification processes. Published studies indicated that nitrification in acid soils were mainly driven by AOA (Gubry-Rangin et al., 2010; Yao et al., 2011). Recently, Lehtovirta et al. (2011) cultivated the first chemolithotrophic acidophilic AOA, *Nitrosotalea devanaterra*, which shows optimum growth in the pH range of 4–5 and at extremely low ammonia concentrations of 0.18 nM. These particular characteristics are beneficial for growing chemolithotrophic acidophilic AOA

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in an acidic environment and enriching acid tolerant nitrifying flocs.

The main objective of this study was to enrich acid-tolerant nitrifying sludge and investigate its nitrifying activity, aggregate characteristics and microbial community structure.

2. Materials and methods

2.1. Experimental system

Two laboratory air-lift reactors, composed of a 2 L effective volume and a 2 L settling tank was used to enrich ACIN sludge and neutrophilic nitrifying (NEUN) sludge, respectively. The reactor structure and its operation were provided by Wang et al. (2016). The synthetic wastewaters were used in the experiments (Table 1) and their pH values of ACIN and NEUN reactors were controlled at 6.0 ± 0.2 and 7.5 ± 0.2, respectively. The temperature and the dissolved oxygen (DO) concentration were regulated at 30 ± 1 °C and 0.3–3.0 mg L^{-1}, respectively. The ACIN and NEUN reactors were run continuously 140 days and influent and effluent samples from every reactor were taken every day for measuring the concentrations of ammonium, nitrite and nitrate.

2.2. Enrichment method

For ACIN and NEUN reactors, fixed pH values of 6.0 ± 0.2 and 7.5 ± 0.2 were set to enrich ACIN sludge and NEUN sludge, respectively. Two automatic pH controllers were used to control the pH values, which used pumps to regulate pH with 2 M sodium hydroxide and 2 M hydrochloric acid. A volume of 0.5 L aerobic activated sludge was taken from the aerobic tank of a municipal sewage treatment plant in Hangzhou, China for using as inoculum for the ACIN and NEUN reactors, respectively (Wang et al., 2016). The pH value of the aerobic tank was 7.0–8.5. The influent ammonium concentration was 130.8 ± 20.5 mg N L^{-1}. A volume of 0.2 L tea orchard soil suspension was added to the influent of the ACIN reactor and NEUN reactor every day to enrich acid-tolerant AOA.

2.3. Analytical methods

Concentrations of ammonium, nitrite and nitrate were daily measured using UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) according to standard procedures (Sliekers et al., 2002). The DO concentration and pH were detected using SevenGo pro SG9 meter (Mettler Toledo, Greisensee, Switzerland) and Leici pHS-3C meter (Leici Equipment Factory, Shanghai, China).

The images of ACIN and NEUN sludge were taken using a Discovery V8 stereoscope (ZEISS). The particle size distributions of ACIN and NEUN sludge were analyzed using the QJCPIC system (Sympatec).

2.4. Batch activity tests

The maximum specific aerobic ammonia-oxidizing activities of ACIN and NEUN sludge were measured by batch experiments. The residual ammonium, nitrite and nitrate in sludge were washed out first using phosphate buffer. A 3 g washed sludge was put into an Erlenmeyer flask with a volume of 150 mL. During the experiments, the pH was controlled at 7.5 or 6.0 using 0.5 M phosphate buffer. The culture solution was the same as the synthetic wastewater of NEUN reactor and the total volume of each flask was 100 mL. Every flask was incubated on a shaker under the same conditions (temperature: 30 ± 0.5 °C, DO concentration: higher than 3.0 mg L^{-1}, speed: 180 rpm). Liquor samples were taken every 1–5 h for the analysis of NH_4^+, NO_2^- and NO_3^- and every test was performed in triplicate.

2.5. PCR-DGGE test

Genomic DNA was extracted from the Inoculum (sample 1), ACIN sludge (sample 2) and NEUN sludge (sample 3) using the Fast DNA SPIN kit for soil (MP Biochemical, Carlsbad, USA), following the manufacturer's instructions. PCR and DGGE (denaturing gradient gel electrophoresis) were conducted following the recently developed protocol of Wang et al. (2016) but with some modifications. Briefly, the primer sets *amoA*-1F/*amoA*-2R (Wu et al., 2011) and 19F/616R (Pester et al., 2012) with a 40-bp GC clamp attached to the 5′ end of *amoA*-1F and 19F were used to amplify AOB and AOA *amoA* gene fragments. The PCR conditions were as described in the researches by Wu et al. (2011) and Pester et al. (2012). The PCR products were purified and determined by 1% agarose gels. The DGGE of purified PCR products were carried out using 8% (w/v) polyacrylamide gel (20%–50% and 30%–55% gradient for AOB and AOA *amoA* gene fragments, respectively) with the DCode™ Universal Mutation Detection system (Bio-Rad, Hercules, CA, USA). Electrophoresis and silver staining were conducted as described by Xing et al. (2013).

The DGGE bands named DGGE AOB-1 to DGGE AOB-39 and DGGE AOA-1 to DGGE AOA-10) was amplified again using primer sets without GC clamp. The cloning and sequencing of amplification products was performed according to the method as described by Hu et al. (2012). The sequences were aligned with their closest matches obtained by BLAST analysis of GenBank using MEGA5.1. The phylogenetic tree was constructed using the MEGA5.1 program by the neighbor-joining methods (Tamura et al., 2007). The sequences were deposited into GenBank under accession numbers: KX087184-KX087212.

Table 1
Composition of synthetic wastewaters and trace nutrient solutions.

Synthetic wastewaters	Concentration/(g L^{-1})		Trace nutrient solutions	Concentration/(g L^{-1})	Vitamin solution	Concentration/(mg L^{-1})
	ACIN	NEUN				
NH_4Cl	0.5	0.5	I: Na_2-EDTA	6.25	vitamin A	5000 IU
$NaHCO_3$	1	2	$FeSO_4$	6.25	vitamin D	400 IU
NaCl	0.5	0	II: $ZnSO_47H_2O$	0.144	vitamin E	10
$MgSO_4 \cdot 7H_2O$	0.3	0.3	$MnCl_24H_2O$	0.1	vitamin B_1	5
$CaCl_2 \cdot 2H_2O$	0.1	0.004	H_3BO_3	0.03	vitamin B_2	5
KH_2PO_4	0.1	0.01	$CoCl_2 \cdot 6H_2O$	0.19	vitamin B_6	0.5
KCl	0.5	0	$CuCl_2 \cdot 2H_2O$	0.0022	vitamin B_{12}	0.1
Vitamin solution	1 mL L^{-1}	1 mL L^{-1}	$NiCl_2 \cdot 6H_2O$	0.024	vitamin C	50
Traces solution I	1 mL L^{-1}	1 mL L^{-1}	$Na_2MoO_42H_2O$	0.036	niacinamide	15
Traces solution II	1 mL L^{-1}	1 mL L^{-1}			calcium pantothenate	5
pH	6.0 ± 0.2	7.5 ± 0.2				