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Characterization of *Thauera*-dominated hydrogen-oxidizing autotrophic denitrifying microbial communities by using high-throughput sequencing

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HIGHLIGHTS

- Firstly reported a *Thauera*-dominated hydrogenotrophic denitrifying consortium.
- *Thauera* were enriched into the dominant population from different seed sludges.
- Enriched denitrifying cultures were investigated by High-throughput sequencing.
- Enriched cultures achieved comparable nitrogen removal rates with *P. denitrificans*.

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ABSTRACT

The present study, for the first time, reported a *Thauera*-dominated hydrogen-oxidizing autotrophic denitrifying microbial community enriched from different seed sludges including activated sludge and anaerobic digestion sludge. After 244 days enrichment, nitrogen removal rates reached up to 0.2 mg N/mg VSS/d which were comparable to that of the model organism *Paracoccus denitrificans* under the same conditions. Furthermore, high-throughput sequencing was applied to characterize and compare the seed sludges and enriched cultures. Operational taxonomic units (OTU)-based analysis (97% similarity cutoff) of total 280,000 16S rRNA gene V6 region sequences from 7 sludge samples (40,000 sequences per sample) revealed that the microbial diversity decreased after the enrichment, indicated by OTU numbers drop of 55–60%. *Thauera* species in the class of β-*Proteobacteria* were enriched into the dominant populations with relative abundances of 47–62%, regardless of seed sludge sources.

1. Introduction

Hydrogen-oxidizing autotrophic denitrification is an effective biological process to remove nitrogen (N) from nitrate-contaminated organic-limited water systems such as groundwater and drinking water since it has unique advantages over heterotrophic denitrification, including less sludge generation and no external organic substrate requirement (Mansell and Schroeder, 2002; Smith et al., 2005; Sunger and Bose, 2009).

Autotrophic denitrification could be conducted by some bacteria through using hydrogen as the electron donor, inorganic carbon as the carbon source and nitrate/nitrite as the electron acceptor in the absence of oxygen (Mateju et al., 1992). Most of the reported hydrogen-oxidizing denitrifiers belong to the phylum of *Proteobacteria*, including *Paracoccus denitrificans* in the class of α-*Proteobacteria* (Szekeres et al., 2002; Vasiliadou et al., 2006b), *Hydrogenophaga* sp. (Zhang et al., 2009), *Rhodocyclus* sp. (Smith et al., 2005) and *Alcaligenes* sp. (Ho et al., 2001; Sunger and Bose, 2009) in β-*Proteobacteria*, and *Acinetobacter* sp. (Vasiliadou et al., 2006b), *Aeromonas* sp., *Pseudomonas* sp. and *Shewanella* sp. (Liessens et al., 1992) in γ-*Proteobacteria*.

So far the reported information about hydrogen-oxidizing autotrophic denitrifiers is still very limited. In order to enrich hydrogenotrophic denitrifiers, different types of seed sludges including activated sludge (AS) and anaerobic digestion sludge (ADS) from three sewage treatment plants (STPs) in Hong Kong were inoculated in different batch reactors respectively. Then the enriched cultures were compared to the autotrophic denitrification model microorganism (*P. denitrificans*) regarding to their denitrifying rates.

Advanced high-throughput sequencing (or next generation sequencing) can generate huge amounts of DNA sequences, and have been used for analysis of mixed cultures from sewage treatment plants (Zhang et al., 2011; Hu et al., 2012), nitrification reactors (Ye et al., 2011), cellulose degradation reactors (Xia et al., 2012), and etc. Applications of this technology revealed that the clone library used before was far from enough to reflect the whole

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profile of complicated microbial community even for simple lab-scale reactors.

In the present study, barcoded Illumina paired-end sequencing method targeting 16S V6 region was applied to characterize enriched microbial communities carrying out hydrogen-oxidizing autotrophic denitrification, and investigate the bacterial community change from seed sludge as well.

2. Methods

2.1. Enrichment process

Seed sludges for enriching hydrogen-oxidizing autotrophic denitrifiers came from three STPs in Hong Kong (Table 1). Among them, Sha-Tin STP treats saline sewage (1.1% salinity) due to seawater toilet flushing practice in Hong Kong; Shek-Wu-Hui STP treats sewage containing slaughterhouse wastewater because it locates next to a large-scale local slaughtering plant; and Stanley STP locates inside a cave treating municipal wastewater. All of them employ anoxic/aerobic (A/O) process for wastewater treatment. Besides reactors R2–R4, a control reactor R1 having no microorganism and two model reactors R5 and R6 inoculated by *P. denitrificans* 12449 purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen – German Collection of Microorganisms and Cell Cultures, Germany) were set during enrichment process. Moreover, to evaluate the effect of hydrogen on denitrification, another control reactor R7 of hydrogen additive free condition was inoculated by enriched sludge from R2 with initial [NO_3^--N] of ~30 mg/L. Headspace of R7 was purged with argon gas instead of hydrogen to keep hydrogen-free and anoxic environment.

Enrichment was performed in 550 mL serum bottles containing 300 mL medium and 250 mL headspaces filled by hydrogen gas under room temperature (21 ± 1 °C) (Supplementary Fig. S1). One liter medium contains 30 mg/L KNO_3-N, 500 mg/L $NaHCO_3$ and 1 mL trace element solution (Supplementary Table S1) (Till et al., 1998) with pH control to ~7.5 by phosphate buffer solution. The medium was replaced by centrifugation, and meanwhile hydrogen gas was re-filled twice per week.

2.2. Chemical analysis

Photocolorimetric method was used to analyze concentrations of nitrate, nitrite, ammonium, and total nitrogen according to the APHA standard methods (APHA, 1998). The collected water samples were filtered through 0.45 μm membrane filters, stored at 4 °C, and analyzed within 1 day.

Pressure in headspace and pH of liquid in serum bottles were tested by a manometer (Model 840080) and pH meter respectively. Total organic carbon, inorganic carbon and total carbon were determined by TOC-VCPH (SHIMADZU, Japan). Compositions of hydrogen, nitrogen, hydrogen sulfide, methane, carbon dioxide, and nitrous oxide in headspaces were measured by a gas chromatograph (GC) (Hewlett–Packard 5890II, USA) equipped with a thermal conductivity detector following the method published before (Xia et al., 2012).

2.3. N removal rate calculation

The autotrophic denitrification reactions using hydrogen could be expressed as follows:

$$NO_3^- + 1.13H_2 + 0.05HCO_3^- = 0.99NO_2^- + 0.01C_5H_7O_2N + 1.09H_2O + 0.06OH^- \quad (1)$$

$$NO_2^- + 0.122HCO_3^- + 1.78H_2 = 0.488N_2 + 0.0244C_5H_7O_2N + 1.19H_2O + 1.122OH^- \quad (2)$$

Summary of Eqs. (1) and (2),

$$NO_3^- + 2.892H_2 + 0.171HCO_3^- = 0.483N_2 + 0.034C_5H_7O_2N + 2.268H_2O + 1.171OH^- \quad (3)$$

According to Eq. (3), for each gram of NO_3^--N removed, approximately 0.27 g cells are generated, which is theoretically 48% lower than that for methanol-based heterotrophic denitrification. Meanwhile 3.57 g alkalinity (as $CaCO_3$) could be generated, and the pH will increase as protons were consumed during denitrification.

Assuming the insoluble nitrogen in batch reactor mainly from the nitrogen content of biomass, biomass concentration was calculated according to Eq. (4) below.

$$\text{Biomass (mg VSS/L)} = (\text{TN} - \text{soluble N})/0.12 \quad (4)$$

where TN – total nitrogen in solution, mg/L; soluble N – concentration of total soluble nitrogen ([NO_3^--N] + [NO_2^--N] + [NH_4^+-N]) in solution, mg/L; 0.12 – partial of nitrogen in empirical biomass formula $C_5H_7O_2N$.

N removal rate within the first 24 h was calculated according to Eqs. (5) and (6).

$$\text{N removal rate (mg N/mg VSS/d)} = \Delta N_{24h}/\text{Biomass} \quad (5)$$

Table 1
Different seed sludges for enriching hydrogen-oxidizing autotrophic denitrifiers.

Reactor	Seed sludge						Enrichment time (days)
	STP				Sludge type	Sampling tank	
	Code	Percentage (%) of municipal wastewater	Process	Flowrate (10^3 m^3/d)			
R1	–	–	–	–	–	–	244
R2[a]	ST	95	A/O	216	ADS	Sludge digester	244
	SWH	90	A/O	216	ADS	Sludge digester	
R3	ST	95	A/O	216	AS	Aeration tank	226
R4	SL	100	A/O	8	AS	Aeration tank	226
R5	–	–	–	–	*Paracoccus denitrificans* 12449	–	244
R6							244
R7[b]				Enriched sludge from R2			244

Abbreviations: STP, sewage treatment plant; ST, Sha-Tin; SWH, Shek-Wu-Hui; SL, Stanley; A/O, anoxic/aerobic; ADS, anaerobic digestion sludge; AS, activated sludge from aeration tank.

[a] Seed sludge of R2 was mixture of ADS from ST and SWH STPs according to ratio of 1:1.

[b] Headspace of R7 was purged with argon gas instead of hydrogen to keep hydrogen-free and anoxic environment.