Bioresource Technology 143 (2013) 439–446

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Microbial community and N removal of aerobic granular sludge at high COD and N loading rates

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HIGHLIGHTS

- *Thauera* strain TN9 was the most dominant microorganism in the granular sludge.
- *Thauera* strain TN9 is important to granular formation, stability and N removal.
- Heterotrophic and autotrophic nitrification coexist in the aerobic granular sludge.
- No AOA *amoA* or anammox bacterium *hzo* gene was detected in the granules.
- Heterotrophic nitrification might contribute more to N removal at high C, N loads.

ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form 4 June 2013
Accepted 8 June 2013
Available online 14 June 2013

Keywords:
Aerobic granular sludge
Microbial community
N removal
High COD and N loading rates
Heterotrophic nitrification

ABSTRACT

An aerobic granular sludge, cultivated with modified piggery wastewater, was capable of simultaneously removing COD and N at high COD and N loading rates. Confirmed to be identical with the DGGE band B9, isolate *Thauera* strain TN9 was the most dominant microorganism in the granular sludge. *NirS* and *NosZ* gene were amplified and sequenced from strain TN9 suggested it is crucial to N removal. Some other dominant DGGE bands belonged to *Zoogloea* and TM7, might play important roles in the formation and the stabilization of the granules. Meanwhile, no AOA *amoA* or anammox bacterium *hzo* gene was detected in the granules. All *amoA* clone libraries of AOB were clustered to *Nitrosomonas*. Yet those AOB were not present in DGGE dominant bands. Therefore, the heterotrophic nitrification and autotrophic nitrification coexist in the granules, the heterotrophic nitrification might contribute more to the N removal at high COD and N loading rates.

1. Introduction

Aerobic granulation, having developed since the last two decades, draws increasing interest in the quest for innovative techniques in biological wastewater treatment. Compared to conventional activated sludge flocs, aerobic granulation has several advantages, such as excellent settleability (Yang et al., 2003), ability to withstand high organic load (Adav et al., 2010), dense and strong microbial structure, and tolerance to toxicity (Adav et al., 2007).

Simultaneous COD, N and P removal may be realized by aerobic granular sludge in a sequencing batch reactor (SBR) (de Kreuk et al., 2005). But little is known about the N removal mechanism at high COD and N loading rates in the heterotrophic aerobic granular sludge. In the aerobic nitrifying granular sludge (Jang et al., 2003), the N removal mechanism of simultaneous nitrification and denitrification (SND) was explained based on the special granular structure of sludge. This structure forms an oxygen concentration gradient, with the autotrophic ammonia oxidizing bacteria (AOB) in the surface where oxygen is rich, and the anoxic denitrifiers inside the granule where oxygen is rare. Consequently, SND occurs due to these two types of bacteria (Beun et al., 2001; de Kreuk et al., 2005). However, in the heterotrophic aerobic granular sludge cultured with additional organic carbon sources, such as glucose and acetate, heterotrophic microorganisms appear and coexist with the autotrophic ones. The relative abundance of heterotrophic microorganisms increases considerably with the raise of influent COD/N, while AOB and nitrite oxidizing bacteria (NOB) decrease remarkably (Yang et al., 2004). The SND mechanism depends on the function of autotrophic nitrifiers and anoxic denitrifiers is not adequate to explain the N removal when heterotrophic microorganisms are dominant. Therefore, explorations of

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http://dx.doi.org/10.1016/j.biortech.2013.06.020

the N removal mechanism need to be done at a high COD and N loading rates in the heterotrophic aerobic granular sludge.

Heterotrophic nitrification provides a type of nitrogen removal pathway different from the autotrophic nitrification (Castignetti and Hollocher, 1984). Some heterotrophic nitrifiers are also shown to have capability of aerobic denitrification (Robertson et al., 1989). By using organic carbon source, they convert ammonia into gaseous nitrogen, and then discharge the gas out of the bioreactor. In this way, COD and N are removed simultaneously. In a recent study, aerobic granular sludge was cultured with heterotrophic nitrifiers as an inoculum (Gou and Huang, 2009). It suggests that heterotrophic nitrifiers of a large quantity may exist in aerobic granular sludge at high COD and N loading rates. On the other hand, it is largely unknown whether other kinds of N removal microorganisms exist in aerobic granular sludge, such as anaerobic ammonium oxidizing (anammox) bacteria (Mulder et al., 1995) and ammonia oxiding archaea (AOA) (Könneke et al., 2005).

This study aims to demonstrate the evolution of the dominant microbial populations in aerobic granular sludge during granulation and load impact process by using PCR-DGGE; to isolate and identify the dominant bacteria from the sludge; to investigate the existence of AOB, AOA and anammox bacteria. The ultimate goal is to provide a theoretical and practical basis for revealing N removal mechanism and for developing a new wastewater treatment process with the aerobic granular sludge.

2. Methods

2.1. Characteristics and performance of aerobic granular sludge

The aerobic granular sludge was cultured in a 4 L reactor, which was fed with modified piggery wastewater (MPW). The raw piggery wastewater (RPW): COD 4100–5200 mg L^{-1}, NH_4^+–N 470–580 mg L^{-1}, NO_2^-–N <0.02 mg L^{-1}, NO_3^-–N <2.0 mg L^{-1}, pH 7.0–8.5. The MPW came from series diluted RPW with glucose and ammonium sulfate as the additional carbon and nitrogen sources, respectively. The MPW was COD 1000–16,000 mg L^{-1}, NH_4^+–N 50–750 mg L^{-1}, pH 7.5–8.5. The reaction was performed under controlled conditions, that is, progressively increasing COD and NH_4^+–N loadings, and decreasing settlement time. The mature aerobic granular sludge was brown–yellow, and formed an irregularly spherical shape with a diameter of 0.5–3.5 mm. Table 1 shows the stepwise influent loading rates and the granule performance. No obvious accumulations of nitrite and nitrate appeared in the whole process (Zhao et al., 2011).

2.2. Nitrogen balance test of aerobic granular sludge

The MPW (about COD 5000 mg L^{-1}, NH_4^+–N 200 mg L^{-1}) was used as the medium for nitrogen balance test (a batch experiment). The test was carried out in a 150 mL triangular flask with 40 mL medium and 10 mL aerobic granular sludge (in triplicate). The reactions were conducted at 24 °C, 180 rpm for 12 h. The TN of the precipitate (sludge), and the TN, NH_4^+–N, NO_2^-–N, NO_3^-–N and COD concentrations of the supernate (centrifuged at 6000 rpm for 10 min, Centrifuge 5804 R, Eppendorf, Germany) were measured at the initial and final of the test.

NH_4^+–N, NO_3^-–N, NO_2^-–N, TN and COD were detected by the Spectroquant Analysis System PhotoLab 6100 (WTW, Germany) with the matching reagents (Merck, Germany). The digestion of sample was conducted in Thermoreaktor CR 3200 (WTW, Germany).

2.3. DNA extraction and PCR

Sludge samples were harvested from the middle of the reactor, genomic DNA was extracted immediately by the E.Z.N.A.™ Soil DNA Kit (Omega, U.S.). The DNA concentration was measured using TU-1800 Series Spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., PR, China). The extracted DNA samples were stored in a −20 °C freezer before use.

Details of the primers and thermal programs are listed in Table 2. The PCR amplifications were performed with a TProfessional thermocycler (Biometra, Germany), with a mixture of total volume 50 μL, containing 2 U of *Taq* DNA polymerase (Takara, Japan), 5 μL of 10× *Taq* buffer, 3 μL of 25 mM $MgCl_2$, 4 μL of 10 mM dNTPs mixture, 1 μL of 10 μM each primer, and 2 μL of the DNA extract (about 1 ng/μL isolated strain DNA, or about 100 ng/μL of sludge DNA). The amplification products were examined by standard agarose gel electrophoresis (1% agarose, 0.5 × TAE). The gels were then stained with ethidium bromide (0.5 mg/L) for 10 min, and visualised on a GDS-8000 system (UVP, U.S.) to confirm the product size and purity.

2.4. Isolation and identification of microorganisms from the aerobic granular sludge

Granules were collected and aseptically broken in sterilized tubes that contained the MPW. Samples were serially diluted (10^{-1}–10^{-7}). A volume of 0.1 ml from each 10^{-4} to 10^{-7} dilution was spread onto an agar plate containing the MPW (COD 10,000 mg L^{-1}, NH_4^+–N 500 mg L^{-1}). The plate was then placed in an incubator at 30 °C. The morphologically identified strains were isolated via several cycles by replacing on an agar plate containing the MPW (COD 2000 mg L^{-1}, NH_4^+–N 100 mg L^{-1}). The COD and NH_4^+–N removal ability of the isolates was investigated (in triplicate) with the MPW (COD 1000 mg L^{-1}, NH_4^+–N 50 mg L^{-1}) at 30 °C, 180 rpm for 48 h.

The PCR-amplified 16S rRNA gene (Primer: 27f-1492R) of the isolated strains was partially (670 bp) sequenced by the forward primer 27f (Table 2). The PCR-amplified *NirS* and *NosZ* gene of TN9 were purified with the Agarose Gel DNA Purification Kit Ver.2.0 (Takara, Japan), then ligated into the pMD18-T vector (Takara, Japan), and transformed in *Escherichia coli* JM109. Each PCR product was sequenced in duplicate by the Sangon Biotech (Shanghai) Co., Ltd. (China). The sequences were compared with the 16S rRNA gene sequences obtained via the BLAST searches of the National Center for Biotechnology Information Database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments

Table 1
Influent loading rate, pollutant removal efficiency and modality of the sludge.

Running time (d)	0–5	6–17	18–23	24–40	41–56	57–61	62–65
COD loading rate (kg m^{-3} d^{-1})	1.04	1.59	3.00–4.00	4.80–6.90	7.80–12.60	12.60–15.68	5.50
NH_4^+–N loading rate (kg m^{-3} d^{-1})	0.06	0.07–0.09	0.13–0.14	0.22–0.26	0.30–0.50	0.50–0.72	0.24
COD removal efficiency (%)	–	94.1–94.8	93.5–95.5	94.1–97.2	96.4–97.2	96.1–97.3	94.5–95.4
NH_4^+–N removal efficiency (%)	–	44.5–76.6	94.8–99.9	98.2–99.9	99.8–99.9	81.6–100.0	98.9–99.2
Modality of sludge	Small floc	Bigger floc	Small granule	Spherical granular sludge			
Diameter (mm^{-1})	–	–	<0.5	0.5–2.5	0.5–3.5		