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Bacterial community dynamics in a biodenitrification reactor packed with polylactic acid/poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) blend as the carbon source and biofilm carrier

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While heterotrophic denitrification has been widely used for treating such nitrogen-rich wastewater, it requires the use of additional carbon sources. With fluctuations in the nitrate concentration in the influent, controlling the C/N ratio to avoid carbon breakthrough becomes difficult. To overcome this obstacle, solid-phase denitrification (SPD) using biodegradable polymers has been used, where denitrification and carbon source biodegradation depend on microorganisms growing within the reactor. However, the microbial community dynamics in continuous-flow SPD reactors have not been fully elucidated yet. Here, we aimed to study bacterial community dynamics in a biodenitrification reactor packed with a polylactic acid/poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PLA/PHBV) blend as the carbon source and biofilm carrier. A lab-scale denitrifying reactor filled with a PLA/PHBV blend was used. With 85 mg/L of influent NO_3–N concentration and a hydraulic retention time (HRT) of 2.5 h, more than 92% of the nitrate was removed. The bacterial community of inoculated activated sludge had the highest species richness in all samples. Bacterial species diversity in the reactor first decreased and then increased to a stable level. *Diaphorobacter* species were predominant in the reactor after day 24. In total, 178 clones were retrieved from the 16S rRNA gene clone library constructed from the biofilm samples in the reactor at 62 days of operation, and 80.9% of the clones were affiliated with *Betaproteobacteria*. Of these, 97.2% were classified into phylotypes corresponding to *Diaphorobacter nitroreducens* strain NA10B with 99% sequence similarity. *Diaphorobacter*, *Rhizobium*, *Acidovorax*, *Rubrivivax*, *Azospira*, *Thermomonas*, and *Acidaminobacter* constituted the biofilm microflora in the stably running reactor.

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[**Key words:** Biodenitrification; Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate)/polylactic acid; Bacterial community; Denaturing gradient gel electrophoresis; 16S rRNA gene clone library]

Nitrogen, which is essential for protein synthesis, is a key limiting nutrient in most aquatic and terrestrial ecosystems. However, the excessive discharge of wastewater rich in nitrogen (in the form of nitrate or ammonia) results in eutrophication of lakes and rivers, lowering the quality of drinking water from these resources (1). The excessive use of nitrogen-containing fertilizers on crops in China has resulted in the groundwater being polluted by nitrate-nitrogen in vegetable-producing areas (2,3).

Heterotrophic denitrification, which is widely used for nitrate removal, results in biochemical nitrate reduction and oxidation of the carbon source simultaneously (4). However, additional carbon sources are required for treating the water and wastewater rich in nitrates at a low C/N ratio, for example nitrate-contaminated groundwater (5), aquaculture wastewater (6), semiconductor-produced wastewater, landfill leachate (7), and wastewater from power plants (8). The denitrification of nitrate-rich wastewater often requires the use of soluble carbon sources such as acetate, methanol, and ethanol (9). However, when the influent nitrate fluctuates heavily, controlling the C/N ratio to avoid organic carbon breakthrough in the effluent becomes difficult (10,11). To overcome these obstacles, novel solid-phase denitrification (SPD) processes have been developed to reduce the amount of nitrates in wastewater (12). Insoluble biodegradable polymers such as polyhydroxybutyrate (PHB) (13), poly (L-lactic acid) (14), PHBV (15), polycaprolactone (PCL) (16–18) and poly (butanediol succinate) (PBS) (19,20) are generally used as carbon sources and biofilm carriers in the SPD processes (12). The ongoing reduction in the cost of biodegradable polymers in addition to an increase in their production makes them an attractive alternative for use in SPD (17).

It was also found that, in many environments, polyhydroxyalkanoates (PHAs) could be degraded by bacteria adhering to a polymer film surface (21–23). In a SPD reactor, both the denitrification and the biodegradation rate of the solid carbon source depend on the microorganisms within the biofilms growing within the reactor. Consequently, the denitrification efficiency of SPD is closely related to the structure and dynamics of the microbial community in a reactor. The microbial diversity in different SPD reactors has been investigated using PCL (14–18), PBS (19,20) and a starch/PCL blend (24) as the carbon source and biofilm carrier,

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respectively. However, to the best of our knowledge, the previous studies focused only on the microbial community dynamics at the sequencing-batch SPD reactor with single polymer. Thus, the microbial community dynamics and their relationship with nitrate removal in the continued reactors with PLA/PHBV blends remain to be fully elucidated.

Here, for removing nitrate from nitrate-rich synthetic wastewater, we developed a fixed-bed bioreactor packed with a novel type of biodegradable PLA/PHBV blend as the carbon source and carrier. The objective of this study was to investigate the dynamic changes in the bacterial community in the biofilm that formed on the PLA/PHBV blend by using 16S rRNA gene cloning methods and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Our findings provide novel insights into our understanding of the microbiological characteristics of SPD.

MATERIALS AND METHODS

Operation of the denitrifying reactor A reactor filled with 350 g PLA/PHBV blends as the carbon source and carrier was established on a lab scale. The reactor comprised a Plexiglas column (6 cm in diameter and 50 cm in height), which was submerged and was operated in an up-flow mode (Fig. S1). The practical liquid volume in the reactor packed with 350 g of PLA/PHBV blends pellets was 0.98 L. The flow rate was regulated using a peristaltic pump. The PLA/PHBV blend was kindly provided by Shenzhen Esun Industrial Co., Ltd. (Shenzhen, China). The PLA/PHBV blend was in the form of pellets with a size of 2.5−3 mm, a specific surface area of 0.015 m^2/g, and a density of 1.23 kg/L. Average molecular weights for PLA and PHBV in blends are 80,000 and 330,000, respectively, and *co*-HV of PHBV is 2%.

Synthetic wastewater prepared by adding $NaNO_3$ and KH_2PO_4 to tap water at an N:P ratio (w/w) of 5:1 was used as the influent (24). Aluminum foils were used to prevent light from entering. The pH and dissolved oxygen concentration were not controlled during the tests.

The inoculated method of SPD reactor was as followed. Activated sludge (20 mL) mixed with the influent (2 L) containing 70 mg/L NO_3−N (final concentration of mixed suspended solids: 800 mg/L) collected from a sequencing batch reactor (SBR) treating wastewater was driven into the reactor. The mixed wastewater (the active sludge and synthetic wastewater) was recirculated in SPD reactor with the supplied nitrate each day at around 30°C with 4 h of hydraulic retention time (HRT) to inoculate the reactor with activated sludge in the first 3 days.

The continuous-flow reactor was then operated (day 1) with a 2.5 h HRT and a influent containing 70 mg/L NO_3−N, unless otherwise stated.

Sampling and analytical methods Each day, the water samples (50 mL) from the inlet and outlet were collected, riddled with 0.45 μm membrane, and analyzed immediately (less than 1 h after sampling). The concentrations of NO_3−N were determined with a UV-spectrophotometer (UV-6000PC, Metash, Shanghai, China) at 220 and 275 nm (25). The concentrations of NH_4−N, NO_2−N, and the chemical oxygen demand (COD) in the filtered samples were analyzed with a visible spectrophotometer (5B-3(B), Lian-hua Tech., Lanzhou, China) using the standard methods for examination of water and wastewater (25). Each measurement was carried out in duplicate. Organic acids (acetic acid, lactic acid and 3-hydroxybutyric acid) were determined by GC-6850 (Agilent, USA) with an FFAP column using nitrogen as carrier gas (J&W, USA) (26). About 2 g of the PLA/PHBV blend was taken from the reactor at 24, 48, and 62 days, and the biofilm attached to the PLA/PHBV blend was removed and collected using the method suggested by Chu and Wang (17). The biofilm samples were then maintained at −20°C.

Nitrate removal rate was calculated using the following equation:

$$D_r = \frac{C_i - C_e}{t} \quad (1)$$

where D_r is the denitrifying rate ($mg \cdot L^{-1} \cdot h^{-1}$), C_i and C_e are the NO_3−N concentrations (mg/L) in the input and output water, respectively, and t is the HRT (h).

DNA extraction The DNA from the biofilm samples was extracted using the PowerSoil DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the DNA were checked using a 1% (w/v) agarose gel containing GelRed (Biotium, Inc., Hayward, CA, USA).

PCR-DGGE 16S rRNA gene fragments were amplified using the primers PRBA338f (5′-CCTACGGGAGGCAGCAG-3′) (with a 40-bp GC clamp for DGGE) and PRUN518r (5′-ATTACCGCGGCTGCTGG-3′) (27). The PCR mixture comprised 1 μL of the DNA extract, each primer at 0.4 μmol/L, 200 μmol/L dNTPs, 5 μL 10× Ex Taq buffer, 3 U Ex Taq (Takara Biotechnology Co., Ltd., Dalian, China), and sterile deionized water made up to 50 μL. PCR was performed using the following conditions: 4 min at 94°C; 20 cycles of 1 min at 91°C, annealing for 1 min (the annealing temperature was decreased from 65°C to 55°C at intervals of 0.5°C per cycle), and 1 min at 72°C; 5 cycles of 1 min at 91°C, 1 min at 61°C, 1 min at 72°C; and a final step at 10 min at 72°C. The products of amplification were checked using 1% (w/v) agarose gels stained with GelRed (Biotium, Inc.) using an AlphaImager HP system (ProteinSimple, San Jose, CA, USA).

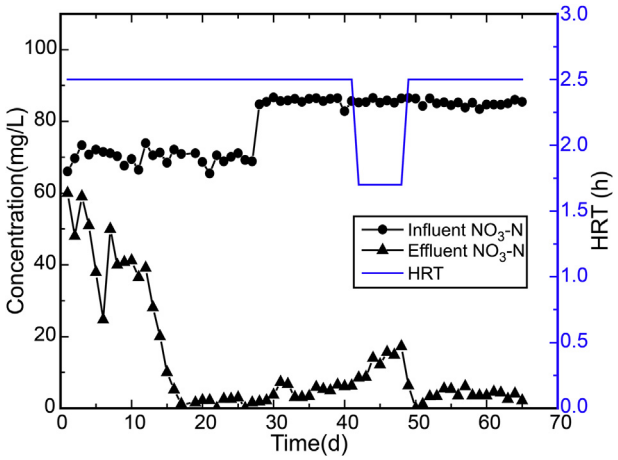

FIG. 1. Nitrate removal profile under different hydraulic retention times (HRT) and nitrate concentrations in the influent in a denitrification reactor packed with PLA/PHBV blend as the carbon source.

The DGGE analysis was based on the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). A 100% denaturant was defined as 40% formamide and 7 mol/L urea. A denaturing gradient ranging from 35% to 70% of the denaturant was used to prepare the polyacrylamide gels. In 0.5× Tris-acetate-EDTA (TAE) buffer, equal amounts of the PCR products were loaded, and DGGE was performed for 16 h at 130 V. The gels were stained using SYBR Green I, and the images were captured using the AlphaImager HP system.

Gel sections with the bands of interest were excised, washed once in distilled water, then broken using pipette tips in 20 μL distilled water, and placed at 4°C overnight. The eluted DNA was amplified using the primer pair PRBA338f and PRUN518r under the following cycling conditions: predenaturation at 94°C for 5 min; 30 cycles of 91°C for 40 s, 55°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. The reamplified products were cloned into competent *Escherichia coli* DH5α (Promega, Madison, WI, USA) using the pGEM-T Easy System I kit (Promega). The transformed clones were sequenced.

16S rRNA gene-based clone library analysis The universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) were used to amplify the 16S rRNA genes of the total bacteria in the biofilms on day 62. The amplification was performed using a Dongsheng EDC-810 thermal cycler with previously described cycling conditions (30). A gel extraction kit (Gragen Life Science Inc., USA) was used to purify the amplified products. The purified products were then subcloned using a pEASY-T1 Cloning Kit (Beijing TransGen Biotech Co., Ltd., Beijing, China). Transformed competent *E. coli* cells were cultured on LB agar plates and incubated overnight; the genes were then sequenced.

Sequencing and phylogenetic analysis 16S rRNA gene clones from DGGE and the clone library were sequenced by using an automatic sequencer system (ABI 3730, Applied Biosystems, Foster City, CA, USA). The sequences of 16S rRNA gene clone library were analyzed using the Bellerophon software program (28), and aligned with sequences in the RDP 11 database to identify the most closely related strains (29). All sequences were subjected to analysis using NCBI BLASTn and alignment using Clustal X (30). A neighbor-joining phylogenetic tree was then constructed using the Molecular Evolutionary Genetics Analysis software (MEGA) version 5.05 with the Jukes-Cantor model and Bootstrap phylogenetic test method (32−34).

Statistical analysis The DGGE band patterns were analyzed using the software Quantity One version 4.6 (Bio-Rad). The diversity of the microbial community was examined using Shannon index (H), which was calculated based on the trace quantity of each of the peaks representing different bacteria (bands). The Shannon index was calculated using the following equation:

$$H = -\sum_{i=1}^{S_b} \frac{n_i}{N} \ln \frac{n_i}{N} \quad (2)$$

where S_b is the number of bands; N is the sum of all peak trace quantities; n_i is the trace quantity of the peak.

The evenness (E) of the microbial community was calculated using the equation E is $H/\ln S$, where the richness (S) is equal to the amount of bands in each lane. The coverage of the clone library (C) was calculated as follows (35):

$$C = \left(1 - \frac{n}{N}\right) \times 100\% \quad (3)$$

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