Tracking composition of microbial communities for simultaneous nitrification and denitrification in polyurethane foam

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ABSTRACT

The process of simultaneous nitrification and denitrification (SND) of immobilized microorganisms in polyurethane form is discussed. The effect of different positions within the polyurethane carrier on microbial community response for the SND process is investigated by a combination of denaturing gradient gel electrophoresis profiles of the 16S rRNA gene V3 region and scanning electron microscopy. Results show that polyurethane, which consists of a unique porous structure, is an ideal platform for biofilm stratification of aerobe, anaerobe and facultative microorganisms in regard to the SND process. The community structure diversity response to different positions was distinct. The distributions of various functional microbes, detected from the surface aerobic stratification to the interior anaerobic stratification of polyurethane, were mainly nitrifying and denitrifying bacteria. Meanwhile aerobic denitrifying bacteria such as *Paracoccus* sp., *Agrobacterium rubi* and *Ochrobactrum* sp. were also adhered to the interior and surface of polyurethane. The SND process occurring on polyurethane foam was carried out by two independent processes: nitrogen removal and aerobic denitrification.

Key words | immobilized microorganism, microbial community structure, polyurethane, simultaneous nitrification and denitrification

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INTRODUCTION

Over the past few decades, immobilized microorganisms have received increasing attention in the field of wastewater treatment (Yan & Viraraghavan 2001). Polyurethane has generated much interest (Ting & Sun 2000) among various immobilization carriers due to its high mechanical strength, excellent water absorption capacities, high porosity, resistance to attack from organic solvents and microbes, good regeneration ability and especially its very low cost (Oh *et al.* 2000; Zhou *et al.* 2010; Chu & Wang 2011). In fact, polyurethane was a light-weight carrier for immobilized microorganism technology in the 1990s, which absorbs and immobilizes microorganism by means of functional groups such as –OH, –COOH, $–CONH_2$ and $–NH_2$ (Basinka & Slomkowski 1995; Lu *et al.* 1995). Meanwhile, polyurethane foam provides a special porous structure: surface/interfacial stratification occurs during polyurethane film formation (Han & Urban 2001). Three-dimensional immobilized biological membranes can form when free microbes gather in internal pores. Further, the influence of microbial aggregation and growth depends on the porous structure and its entrapping capabilities. The control of surface hydrophilic/hydrophobic nature was necessary to prevent scouring by the shear force generated by the fluid (Pai *et al.* 1995; El-shahat & Moawed 2003).

The simultaneous nitrification and denitrification (SND) process has been frequently reported as an efficient process in both biofilm and suspended growth bioreactors for nitrogen removal in wastewater treatment (Kim *et al.* 2005; Canto *et al.* 2008; Daniel *et al.* 2009). Nitrogen removal through the SND process has been investigated in previous studies using various systems (Kotlar *et al.* 1996; Daniel *et al.* 2009; Chu & Wang 2011). A critical factor in the SND process was considered to be the need to provide a suitable environment for the growth of both aerobic and anaerobic bacteria. Previous researchers (Jun

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et al. 2000; Guo et al. 2010) have observed that dissolved oxygen (DO) concentration decreases deep inside polyurethane foams, indicating that the denitrification process could be possible within (Han et al. 2012). Therefore, the formation of aerobic and anoxic zones allowed nitrogen removal via SND. Many researchers have investigated the nitrogen removal performance of polyurethane foam through the SND process in wastewater treatment systems (Xia et al. 2008; Guo et al. 2010; Lim et al. 2011). Relatively little information has been reported on the effect of microbial community response to different polyurethane positions of immobilized microorganism in the SND process. Thus, the objectives of this paper are: (1) to reveal the relationship between microbial community diversity caused by the partitioned structure of the carrier and water purification performance; and (2) to track the SND process in polyurethane, and propose a theoretical basis for process optimization engineering.

MATERIALS AND METHODS

Reactor setup and chemical analysis

A laboratory-scale biological contact oxidation device made of organic glass with a total volume of 6 L was use (Figure 1). The immobilization carriers made of polyurethane foam with an average diameter of 12 mm were fixed at the top and bottom of the reactor. Influent and air was pumped from the bottom of the reactor and effluent was pumped from the top. The system was fed continuously with domestic sewage from Harbin Taiping wastewater treatment plant with operational parameters as follows: height 630 mm, carrier concentration 30% (volume ratio of cumulated carriers to reactor setup), hydraulic retention time 8 h, influent flow 0.75 m^3/h, water temperature 24 ± 1.2 °C, pH 7.1 ± 0.5. The domestic sewage contained chemical oxygen demand of 327 ± 5.6 mg/L, 53.6 ± 2.4 mg total nitrogen (TN)/L, 51.7 ± 2.1 mg ammonia nitrogen/L, 4.8 ± 0.6 mg total phosphorus/L, 105 ± 4.7 mg suspended solids/L and 3.2 ± 0.3 mg DO/L. The sewage purification performance of nitrogen compounds was analyzed according to standard methods (NEPA 1997) for 36 days.

Molecular identification of isolates

Before identification of the bacterial community by molecular methods comprising DNA extraction, polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE) separation, the immobilization samples were taken from the top and bottom of the reactor after different operation periods. After drying at 80 °C in the oven (Xiao et al. 2013), they were cut and surfaced on a clean bench by blade.

DNA extraction

Biofilm samples were obtained from the bulk and surface of polyurethane foams located at the top and bottom of the reactor, during the biofilm formation stage and early and late steady-state operation. Polyurethane carrier samples were placed into centrifuge tubes with sterile water and biofilms were detached by ultrasonication. The sonication was performed at 20 kHz with 10 s cycles for 10 cycles. The carriers were sonicated and washed three times to collect cells by centrifugation, and then 1 mL of call sap was washed three times using 1.0 mM phosphate buffer solution (pH = 7) in order to minimize impurity contamination. At the same time the sample DNA was extracted using a bacterial DNA extraction kit according to manufacturer's instructions (Cui et al. 2011).

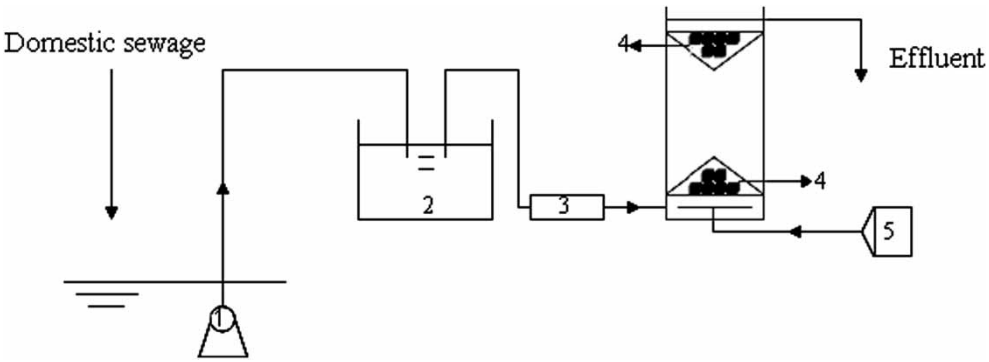

Figure 1 | Biological contact oxidation device. (1) Submersible sewage pump, (2) water tank, (3) water pump, (4) polyurethane carriers and (5) air pump.

PCR amplification

The primer pair F338 and R518 (Mobarry *et al.* 1996) were used for amplification of the V3 region of 16S rDNA genes to avoid nonspecific binding. PCR amplifications were conducted using an amplification kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. Each 50 μL PCR reaction contained 1 μL of template DNA (100 ng), 5 μL of Taq Flexi buffer (2.5 U), 4 μL of dNTP (0.3 mM), 1 μL of upstream primer (1 mM), 1 μL of downstream primer (1 mM), 0.5 μL of bovine serum albumin, 1 μL of *Taq* DNA polymerase (1 U) and nuclease-free water. The tubes were incubated in an automated gradient thermocycler (TGL-16G) that was programmed for initial denaturing at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 55 °C for 40 s, 72 °C for 40 s and final elongation at 72 °C for 8 min. The PCR products were separated by horizontal electrophoresis through a 0.8% (w/v) agarose gel solution. The gel was stained with ethidium bromide and the DNA separated pattern was observed in a UV light box.

DGGE separation and phylogenetic tree

DGGE was performed with the D-code System (Bio-Rad, USA) according to a standard method (Muyzer *et al.* 1993). Six microlitres of PCR products of the second round were loaded onto 8 and 6% polyacrylamide gels mixed with 6 μL of 1.0× TAE buffer. The polyacrylamide gels were made from a denaturing gradient ranging from 40 to 60% (100% of denaturant for 7 mol/L of urea and 40% of ion formamide mixture). The gels were run at 150 V for 7 h at 60 °C. After electrophoresis, the gels were soaked for 15 min in a fixation buffer (30% ethanol and 5% acetic acid) and then incubated in 0.2% silver nitrate solution for 20 min, twice washed in distilled water for 5 min, and kept in 2.5% sodium bicarbonate and 0.1% formaldehyde until bands appeared clearly. Finally the process was stopped with 10% acetic acid, and the glue pictures were immediately taken using a transmission scanner (Image Scanner III). Specific DGGE bands were excised manually from the gel, and used as a template that was re-amplified using the primers F338GC and R518 for sequencing.

To investigate the phylogenetic identities of some dominant DGGE bands, these nucleotide sequences were compared with known sequences in the NCBI database using the BLASTn search program. Phylogenetic evolutionary analyses were conducted with MEGA4.0 (Yuan 2011) and BIOEDIT version 5.0.9.1 based on a neighbor-joining method.

Scanning electron microscopy

After molecular identification, the immobilized bacteria obtained from inside and outside the polyurethane during steady operation was subjected to microbial morphological observation using scanning electron microscopy (SEM). Samples were fixed in a solution of glutaraldehyde (2.5% v/v) for 1.5 h at 4 °C (Badiei *et al.* 2012). After fixation, each sample was washed three times in 1.0 mM phosphate buffer solution (pH 7.0), 15 min for each wash, and dehydrated in a series of graded ethanol solutions (25, 50, 70, 90 and 99% ethanol) before being dried to its critical point. Prior to scanning, the samples were coated with a thin layer of gold to improve their electrical conductivity for a clear image. To take the micrographs, a Philips XL30 scanning microscope was used.

RESULTS AND DISCUSSION

Purification performance of nitrogen compounds

The purification performance of biological contact oxidation in polyurethane was remarkable compared with untreated water (Figure 2). After 12 days of reactor operation, the purification performance increased significantly and a brown biological membrane was attached on the surface of the polyurethane. When the concentrations of the influent TN and NH_4^+ were varied from 52 to 60 mg/L and 46 to 54 mg/L, respectively, the corresponding effluent maintained a high stability below 20 and 5 mg/L, respectively. The concentration of NH_4^+ reached the A standard level stipulated in GB18918-2002 (State Environmental Protection Administration 2002). The purification data showed that the SND process occurred in biological contact with polyurethane. Based on the fact that nitrification needs an aerobic environment and the denitrification occurs in anaerobic or facultative condition, the aerobic and anaerobic structures are partitioned within the carrier. The SND process involves the formation of NO_3^- from NH_4^+ via an NO_2^- intermediate by the significant quantity of nitrifiers that adhered to the aerobic polyurethane surface. The resulting NO_3^- is transferred along the pore to the internal anoxic zone where it is transformed by denitrifying bacteria into N_2 which escapes the water (Wei *et al.* 2011).

Structure of polyurethane foam

The internal structures of polyurethane foam with and without an attached biofilm were examined using SEM at a

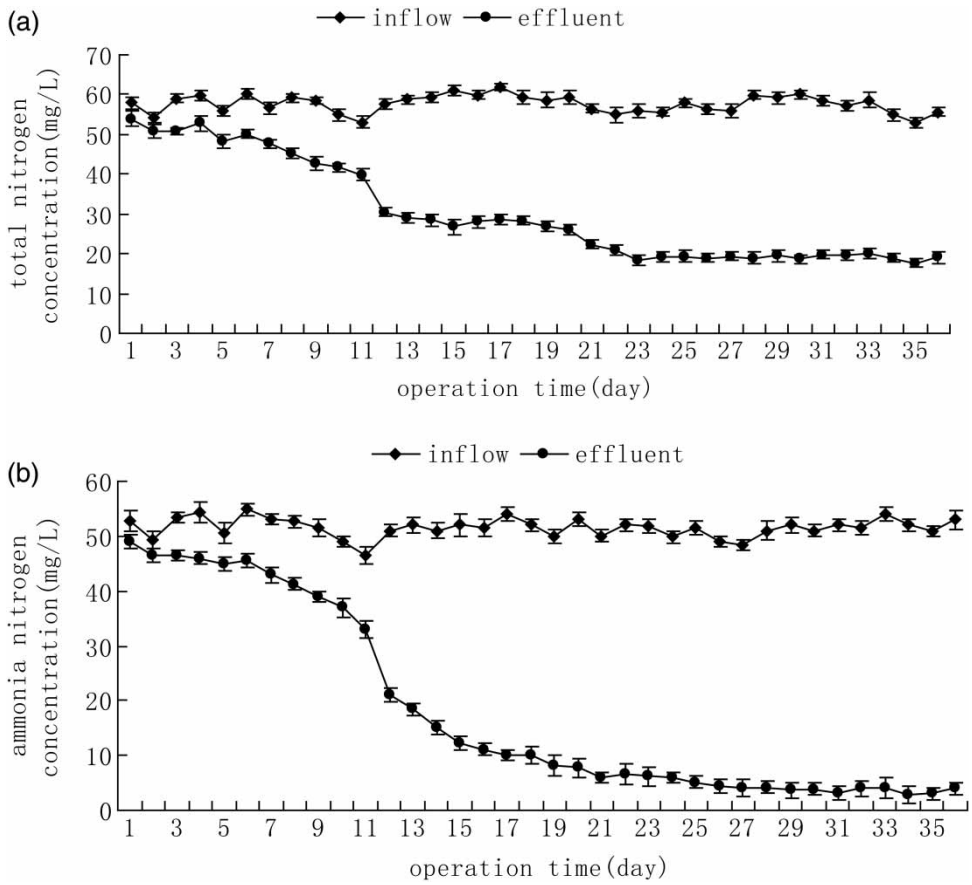

Figure 2 | (a) Purification performance of TN and (b) purification performance of NH_4^+.

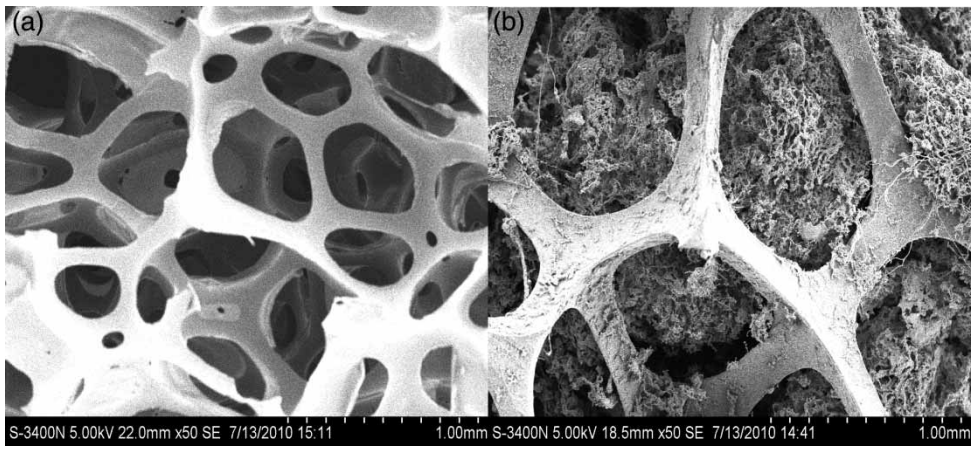

Figure 3 | SEM observation of polyurethane foam (a) with and (b) without a biofilm.

magnification of 50 (Figure 3). The structure was observed to have a porous structure and transport channel for substances and DO (Figure 3(a)). After acclimatization, the biofilm was closely attached to the bulk pores of polyurethane (Figure 3(b)), whereas free microorganisms gathered on the surface of polyurethane. This resulted in spatial heterogeneity between surface and bulk polyurethane, and the phenomenon of biological membrane stratification. In the first 12 days of reactor operation, a biological membrane formed on both the bulk and surface of

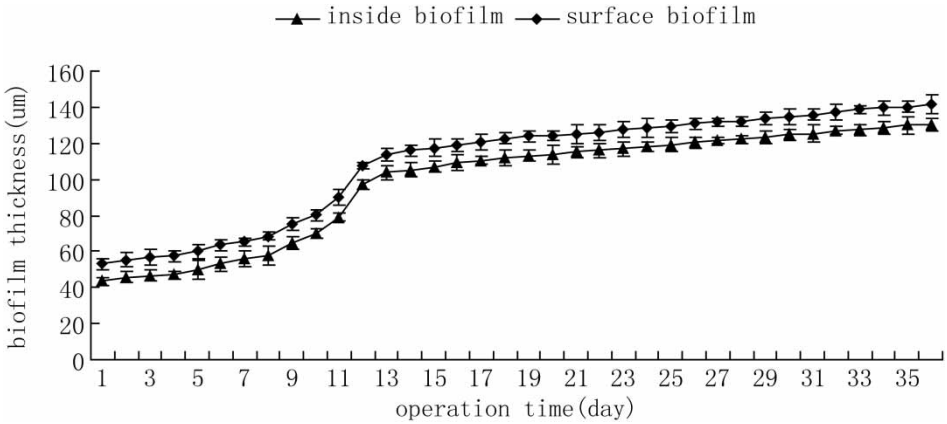

Figure 4 | Interior biofilm thickness and surface performance.

polyurethane. As the biofilm thickness increased (Figure 4) the surface biofilm was significantly thicker than that on the bulk. That is, the surface microbial community structure was more abundant. Compared to other carriers, the polyurethane carriers have obvious advantage in the film-forming time and the amount of pollution at the same temperature (Chen *et al*. 2007). The polyurethane partition structure resulted in the difference between the bulk and surface biofilm thickness and diversity of the microbial community structure.

Microbial community analysis at different positions of polyurethane

DGGE profiles of different positions and operation stages are shown in Figure 5. The sample sequences were obtained from polyurethane within the bulk, the top surface and bottom-bound foams. These samples were taken during the biofilm formation stage, and early and late steady-state operation. The DGGE profiles graphically exhibit distinct bacterial community structure of each position during different operation periods. Furthermore, every lane contained many bands and each was different at the same operational stage, which suggested that the microbial community structure of polyurethane was affected by its position within the reactor. The diversity of microbial composition was high and dominant species were distinct from successional species. As a result, the bioreactor exhibited an excellent capability for degrading organics. The observed differences in communities at different positions and operation times can be attributed to the formation of an anaerobic area which resulted from the concentration gradient of the organic substrate and DO; this was facilitated by the porous mesh structure of polyurethane (Lim *et al*. 2011).

A succession phenomenon was observed in several bands during different operation stages. Species adapted to changes in the biofilm environment and substrate concentration; the diversity from the sample of the top surface of polyurethane was the greatest, and the amount of bands was sequentially 17, 20 and 28. The species from the bottom were more abundant than the top during the period of biofilm formation. The DGGE profiles show that bands C6 and C9 were in each position at all operation stages, showing that representative dominant bacteria-uncultured *Nitrosospira* sp. and *Pseudomonas* sp. AY-2011-RS11 could play a key role in the degradation of pollutants.

The highest similar results based on the partial 16SrRNA gene sequences of 13 DGGE bands (Figure 5) are summarized in Table 1. The result of phylogenetic analysis of isolated bacteria, which was established with a neighbor-joining phylogenetic tree, is shown in Figure 6.

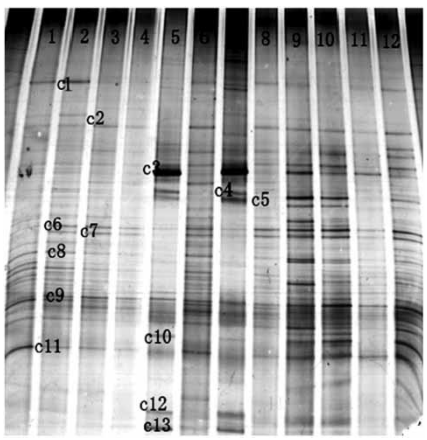

Figure 5 | Microbial community structure of different positions within the polyurethane at different operation stages.

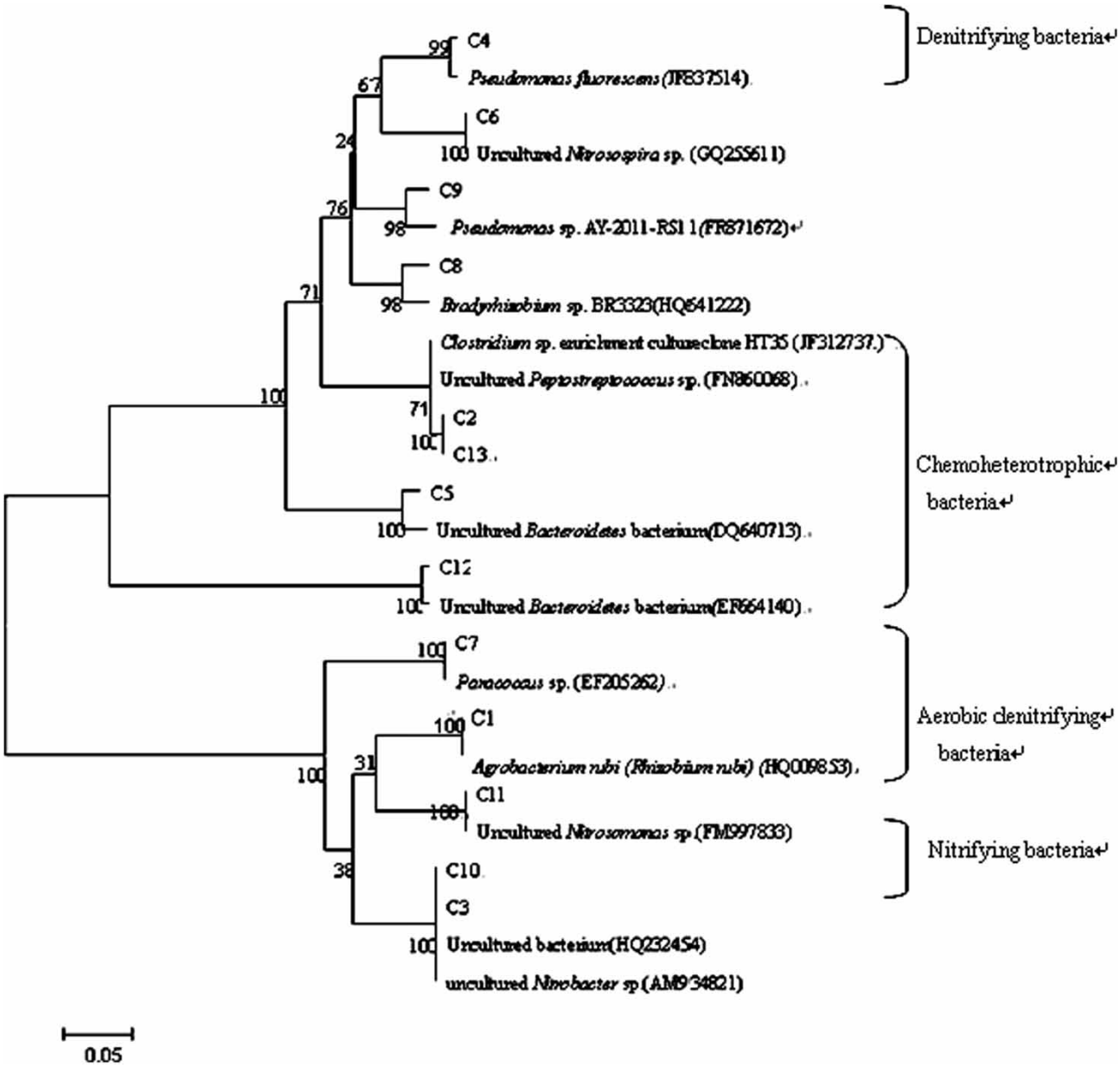

Figure 6 | Phylogenetic tree of isolated bacteria based on 16s rRNA V3 region sequences derived from DGGE bands.

In Huijie's study (Vareche *et al*. 1997), an uncultured bacterium (HQ232454) was identified by investigating glycerol assimilating denitrifying bacteria by using ^{13}C-DNA stable isotope probing. Band C3 was closely related to this uncultured bacterium (HQ232454) with 100% similarity. Biofilm was closely attached to the interior polyurethane during steady-state periods, resulting in the anaerobic conditions required for denitrification. Furthermore, SND could be seen from the wastewater purification performance, since the species in band C3 was a denitrifying bacteria. Band C4 was identified to be closely related to *Pseudomonas fluorescens* (JF837514) with 98% similarity. *Pseudomonas fluorescens* is a common denitrifying bacteria with a strong ability to adapt. Bands C3 and C4 comprised the functional bacteria in the anaerobic zone of the interior of polyurethane during early steady-state operation.

Chemoheterotrophic bacteria were within the biofilms. The similarity of band C2 with *Peptostreptococcus*, band C5 and C12 with *Bacteroidetes* and band C13 with *Clostridium* was up to or more than 97%. *Bacteroidetes* and *Clostridium* are both chemoheterotrophic and anaerobic bacteria, which provides sufficient evidence for a three-dimensional

Table 1 | Phylogenetic similarity to the closest relative of amplied 16s rRNA sequence using the BLAST program

Band	Accession number	Identified organism	BLAST similarity
C1	HQ009853	*Agrobacterium rubi* (*Rhizobium rubi*)	100
C2	FN860068	Uncultured *Peptostreptococcus* sp.	98
C3	HQ232454	Uncultured bacterium	100
C4	JF 837514	*Pseudomonas fluorescens*	98
C5	DQ640713	Uncultured *Bacteroidetes* bacterium	98
C6	GQ255611	Uncultured *Nitrosospira* sp.	99
C7	EF205262	*Paracoccus* sp.	97
C8	HQ641222	*Bradyrhizobium* sp. BR3323	96
C9	FR871672	*Pseudomonas* sp. AY-2011-RS11	100
C10	AM934821	Uncultured *Nitrobacter* sp.	100
C11	FM997833	Uncultured *Nitrosomonas* sp.	100
C12	EF664140	Uncultured *Bacteroidetes* bacterium	99
C13	JF312737	*Clostridium* sp. enrichment culture clone HT35	98

biofilm formed by the attached bacteria inside the porous polyurethane. The existence of denitrifying bacteria such as *Pseudomonas fluorescens* showed that the biofilm contained spatial heterogeneity, and simultaneously provided aerobic and anaerobic reaction zones.

Paracoccus – an aerobic denitrifying bacterium – was related to band C7 with 100% similarity. The reason for its occurrence inside and in the upper polyurethane sections during a great majority of the operation period was that *Paracoccus* species has both an aerobic and anaerobic denitrification function. Gram staining showed that the isolated bacteria was Gram negative and morphologically spherical. Colonies were round and white with flat edges and protrusions in the middle. The appropriate denitrification conditions were 30 °C and pH of 7.5; the species had a significant advantage of rapid degradation and could be denitrification with a specific enzyme system under both aerobic and anaerobic environments. The homology of C1 and the *Agrobacterium rubi* species, which also is generally an aerobic denitrifying bacterium, was up to 100%.

Microbial morphology of inside and surface polyurethane

Subsequent to molecular identification, the morphologies of the immobilized microorganism's community (attached to different positions of polyurethane during steady-state operation) were analyzed using SEM (Figure 7). The SEM analysis showed that a variety of forms of bacteria attached to polyurethane. For example, some were gathered at the surface or interior of polyurethane, while others were wrapped at the surface by filamentous bacteria in a similar manner to previous studies (Tommaso *et al*. 2002; Kartikch 2010).

The analysis confirmed the presence of spherical and short rod-shaped microbial morphologies (Figures 7(a) and 7(b)). Spherical bacteria could represent both the *Paracoccus* and *Peptostreptococcus* species which were detected in the DGGE-PCR profile. The short rod-shaped bacteria might also represent *Pseudomonas fluorescens* species, which is important in the degradation of nitrating compounds. The SEM image of the external polyurethane sample also revealed the presence of long rod-shaped and filamentous microbial morphologies (Figures 7(c) and 7(d)). The long rod shapes could represent both *Nitrobacter* and *Bradyrhizobium* species, which are used mainly to degrade ammonium nitrogen compounds. The results obtained by SEM further indicated a diverse bacterial community for different position sample from the bioreactor, compared to the PCR-DGGE profile. The difference in immobilized microorganism communities inside and at the surface resulted from a concentration gradient of nutrients and DO; these gave rise to the important environment for nitrifiers and denitrifying bacteria in the SND process.

CONCLUSIONS

Polyurethane foam was found to be a good carrier material for biological wastewater treatment. Its porous structure

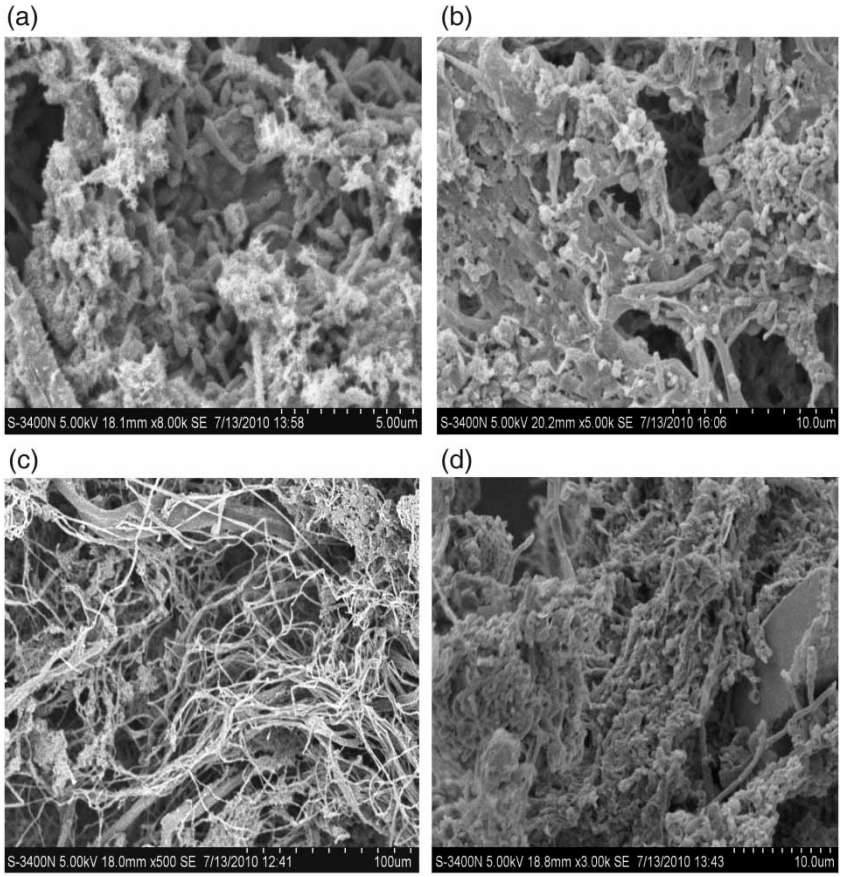

Figure 7 | Microbial morphologies at different positions on the polyurethane carrier during steady-state operation.

allows the formation of attached-growth biomass and the establishment of an anoxic zone. This enhances the nitrogen removal via SND: ammonia and nitrogen concentrations in biological contact with polyurethane were steady and below 20 and 5 mg/L, respectively.

The difference in community morphologies between the interior and surface confirmed the SND process was taking place. This was confirmed by a combination of DGGE profiles of the 16S rDNA V3 region and SEM, showing two independent simultaneous denitrification processes: the occurrence of aerobe, anaerobe and facultative bacteria (Han *et al.* 2012), which provided the important theoretical basis for the practical application of polyurethane in wastewater treatment. Nitrifiers, such as the species of *Nitrobacter hamburgensis* X14, *Nitrosomonas* sp., *Nitrosospira* sp. and *Bradyrhizobium* sp., were generally attached to surface of the polyurethane foam. These were responsible for transforming ammonia to nitrate and then nitrite; nitrite was transported to interior anoxic zones along the nutrient polyurethane pore system. Finally they were converted into N_2 in the water by the denitrifying species *Pseudomonas fluorescens* which was attached to interior anoxic zones. In addition, a lot of aerobic denitrifying bacteria existed both inside and outside the polyurethane. For example, *Paracoccus* sp. and *Agrobacterium rubi* were present, which made it possible for ammonia to be immediately transformed to gaseous N_2. This occurred because nitrogen and oxygen act as an electron acceptor, and then denitrification can be carried out in aerobic conditions, greatly improving the total nitrogen purification performance.

ACKNOWLEDGEMENTS

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