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Effects of carbon source on denitrification efficiency and microbial community structure in a saline wastewater treatment process

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ABSTRACT

Two different denitrifying reactors were monitored in order to evaluate the effects of carbon source on denitrification efficiency and microbial community structure under various saline conditions. Nitrogen removal performances were determined when salinity concentrations increase gradually in acetate- or methanol-fed denitrifying reactor. As a result, acetate-fed process attained high nitrate removal at 0–10% NaCl, while methanol was proven beneficial electron donors at 0–3% NaCl. A parallel analysis of T-RFLP and cloning in the acetate-fed sludge showed that a specialized microbial population (i.e. the genera *Halomonas* and *Marinobacter*) adapted to a high saline environment. Meanwhile, there were no major changes of bacterial populations in the methanol-fed reactor at 4% NaCl, although the relative abundances of the genera *Azoarcus* and *Methylophaga* increased when salinity concentration was at 1–3% NaCl, indicating that methanol-utilizing populations in activated sludge was unable to adapt to a high saline environments (>4% NaCl).

1. Introduction

The biological nitrate removal from wastewaters is achieved by bacterial denitrification, which involves the reduction of nitrate, via nitrite and nitric oxide, to nitrous oxide or dinitrogen gas (Zumft, 1997). With industrial diversification, the biological nitrogen removal technology is required to apply to the various industrial wastewaters. One of them, metal refinery wastewater, that contains large amounts of nitrate and saline, is difficult to be treated, because high salinity causes the inactivation of bacteria responsible for denitrification (Hirata et al., 2001; Diner and Kargi, 1999). On the other hand, our recent studies have demonstrated that denitrification efficiency was greatly improved at a high salinity by use of the sludge acclimated to a saline wastewater (i.e. metal refinery wastewater) supplemented with acetate as a carbon source for a prolonged period (Yoshie et al., 2001, 2006a,b).

Some industrial wastewater lacks an organic carbon source as an electron donor for denitrification. Thus, it is necessary to add an external carbon source (e.g. acetate, ethanol, methanol) to achieve denitrification. In wastewater treatment plants, methanol has been often chosen because of its relatively low cost and the small amounts of sludge production compared with other organic carbon sources (Nyberg et al., 1992). On the other hand, it has been demonstrated that only some bacterial populations can utilize methanol as a carbon source under denitrifying conditions (Hallin et al., 1996, 2006; Hallin and Pell, 1998; Labbe et al., 2003; Ginige et al., 2004; Osaka et al., 2006). Therefore, the external carbon source for denitrification needs to be selected depending on the characteristic of wastewater (Lee and Welander, 1996). However, there are few investigations about the carbon source added to denitrification system treated saline industrial wastewater.

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Some PCR-based genetic fingerprinting techniques have so far been developed for exploring of microbial diversity or microbial community succession under various conditions or environments (Marsh, 1999; Muyzer, 1999). Terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al., 1997) has been focused as a high throughput fingerprinting technology by the development of automated capillary electrophoresis systems that provided digital output. A combination of cloning and T-RFLP analysis provides information about the population dynamics in complex ecosystems.

In this study, we characterized the microbial community structure in the denitrifying reactor using acetate or methanol as an external carbon source with the increase of salinity concentrations. Microbial community structure of each saline condition was monitored by T-RFLP and cloning analysis to identify the bacteria playing important role in high saline denitrifying process.

2. Materials and methods

2.1. Reactor operation

The denitrification system used in this study consisted of a 0.5 l continuously stirred tank reactor (CSTR) under anoxic conditions and sedimentation tank. The hydraulic retention time was 2 days. All of the settling sludge in the sedimentation tank was circulated to the CSTR once a day. The characteristics of the synthetic wastewater were as follows: total organic carbon, 2,250 mg l^{-1}; nitrate-nitrogen (NO_3-N), 1500 mg l^{-1}; and total phosphorus, 20 mg l^{-1}. Acetate or methanol was used as an organic carbon source for denitrification. The sludge used in this study was acclimated with each organic carbon source under salinity-free denitrifying conditions for 4 months. The initial concentration of mixed liquor volatile suspend solids (MLVSS) was 5000 mg l^{-1}. In this study, the saline concentration was steadily increased by 1% salinity with NaCl from 0% in the acetate- or methanol-fed denitrifying reactor. The water temperature was 25 ± 2 °C. These experiments were carried out without controlling the pH because denitrification from high salinity wastewater favors high pH levels (Glass and Silverstein, 1999; Hwang et al., 2006; Peyton et al., 2001; Van der Hoek et al., 1987). All the samples obtained from the acetate- and methanol-fed reactors were filtered with a glass fiber filter (GF/C, Whatman, UK) and used in water quality measurement. Both NO_2-N and NO_3-N concentrations were measured by HPLC with a UV detector (column: IC-Anion-PW, Tosoh Corp., Tokyo, Japan). Total organic carbon (TOC) was measured using a TOC analyzer (TOC-5000A, Shimadzu Corp., Kyoto, Japan). MLVSS was measured according to the standard method (APHA et al., 1992).

2.2. DNA extraction and PCR amplification

For the characterization of bacterial communities, sludge samples were collected from the acetate- and methanol-fed reactors operated more than two weeks in each saline condition and stored at −80 °C. DNA extraction from 0.15 g (wet weight) sludge samples was performed using ISOPLANT (Nippon Gene Inc., Toyama, Japan) according to the manufacturer's instructions. DNA was precipitated by adding ethanol and sodium acetate, resuspended in 50 μl TE buffer (pH 8.0) and stored at −20 °C.

The following primer sets were used for PCR amplification: forward primer 8f (Weisburg et al., 1991) and reverse primer 926r (Liu et al., 1997). The PCR mixture contained 10 ng of extracted DNA, 0.2 μM concentrations of each primer, 200 μM concentrations of dNTP, 2 mM concentrations of $MgCl_2$, 1 U of TaKaRa Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), and 5 μl of 10× PCR buffer for TaKaRa Ex Taq. The PCR amplifications were performed in a total volume of 50 μl in 0.2 ml reaction tubes by using a model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following programs: 3 min at 94 °C, 25 cycles (30 s at 94 °C, 40 s at 56 °C, 60 s at 72 °C), and 3 min at 72 °C. The presence of PCR products was confirmed by 2% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide.

2.3. Cloning, sequencing, and phylogenetic analysis

PCR products were purified by using a Wizard SV gel and a PCR clean-up system (Promega, Madison, WI, USA). The PCR amplicons were cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions. Then, colonies were randomly picked up with a needle and transferred to Insert Check Ready Solution (Toyobo, Osaka, Japan). Clones were sequenced by an ABI PRISM 3100-*Avant* DNA sequencing system (Applied Biosystems) using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. The 16S rRNA gene sequences with more than 97% identity were considered to belong to the same operational taxonomic unit (OTU). A database search was conducted using BLAST from the DDBJ (DNA Data Bank of Japan). Sequences determined in this study and those retrieved from the database were aligned using Clustal W (Thompson et al., 1994). Phylogenetic trees were constructed using a neighbor-joining algorithm (Saitou and Nei, 1987). The 16S rRNA gene sequences of clones obtained in this study were analyzed *in silico* with respect to *Hha*I restriction site for predicting the theoretical length of T-RF of each clone using GENETYX-MAC software (Genetyx, Tokyo, Japan).

2.4. Terminal restriction fragment length polymorphism (T-RFLP) analyses

T-RFLP analysis of eubacterial 16S rRNA gene was carried out using the forward primer 8f labeled at the 5′ end with the dye 6-carboxy-fluorescein and the reverse primer 926r. After purification of PCR products with a Wizard SV gel and a PCR clean-up system (Promega), 4 μl of the PCR-products were digested with 10 U of the restriction enzyme *Hha*I [GCG′C] (TaKaRa) in the manufacturer's recommended reaction buffers for 4 h at 37 °C. The enzyme was subsequently inactivated by incubation at 65 °C for 20 min. Aliquots of the digested amplicons were desalted by ethanol precipitation. Desalted digests were suspended in 15 μl of Hi-Di formamide (Applied Biosystems) containing GeneScan-1000 size standard ROX (Applied Biosystems), denatured (5 min at 94 °C), cooled on ice, and