Characterization of microbial community in nitrogen removal process of metallurgic wastewater by PCR-DGGE


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Abstract The metallurgic wastewater generated from the processes of recovering precious metals from industrial wastes contains high concentrations of nitrogen compounds such as ammonia and nitric acid and of salts such as sodium chloride and sodium sulfate. Biological nitrogen removal from this wastewater was attempted by a circulating bioreactor system equipped with an anoxic packed bed and an aerobic fluidized bed. The anoxic packed bed of this system was found to effectively remove nitrite and nitrate from the wastewater by denitrification at a removal ratio of 97%. As a result of denitrification activity tests at various NaCl concentrations, the sludge obtained from the anoxic packed bed exhibited accumulation of nitrite at 5.0 and 8.4% NaCl concentrations, suggesting that the reduction of nitrite is the key step in the denitrification pathway under hypersaline conditions. The microbial community analysis by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal DNA (rDNA) fragments revealed that the community diversity varied in accordance with water temperature, nitrate-loading rate and ionic strength. When particular major DGGE bands were excised, reamplified and directly sequenced, the dominant species in the anoxic packed bed were affiliated with the beta and gamma subclasses of the class Proteobacteria such as Alcaligenes defragrans and Pseudomonas spp., respectively.

Keywords Denitrification; hypersaline; metallurgic wastewater; microbial community; PCR-DGGE; 16S rDNA

Introduction It is important to recover precious metals such as gold, silver, platinum and palladium in order to efficiently utilize natural resources and reduce industrial wastes (Schuch et al., 2000). However, the metallurgic wastewater that is generated during the recovery of precious metals from industrial wastes contains a large amount of nitrogen as well as high salinity and various base metals. Therefore, to prevent eutrophication in lakes and inland seas, it is essential to develop a process of removing nitrogen from this hypersaline wastewaters. So far, Hirata et al. (2001) have performed biological treatment of such wastewater using an anaerobic-aerobic circulating process. However, the microbial community in this process has not been clarified. Analysis of the microbial community is important for domination and retention of microorganisms possessing denitrification abilities under hypersaline conditions.

Denaturing gradient gel electrophoresis (DGGE) has been used to resolve PCR-amplified regions of genes coding for 16S rDNA based on differences in the nucleotide sequence. This simple approach to obtaining profiles of microbial community can be used to identify temporal or spatial differences in response to various environmental conditions (Muyzer et al., 1993; Kowalchuk et al., 1997). It is also possible to infer the phylogeny of community members by DNA sequence analysis of re-amplified fragments after they are excised from the gel where bands corresponding to each microorganism have been separated through DGGE. In this study, we used the DGGE method to determine the relative genetic complexity of the microbial community mainly composed of halophilic denitrifying bacteria in metallurgic wastewater treatment systems. The technique was also used to monitor
population changes over time and their response to salinity. In addition, the taxonomic affiliation of some putative dominating members of the microbial community was determined by sequencing DGGE bands.

Methods
Metallurgic wastewater treatment system
Two series of anoxic-aerobic circulating bioreactors were used as metallurgic wastewater treatment systems. These systems consisted of an anoxic packed bed (2 or 50 l), an aerobic fluidized bed (1 or 50 l) and a sedimentation tank (Figure 1). One of the systems was of laboratory scale (volume of the anoxic packed bed was 1 l; Run 1) and the other was of pilot plant scale (volume of the anoxic packed bed was 50 l; Run 2). Treated wastewater in the sedimentation tank was circulated into the anoxic packed bed. The volume of the circulated wastewater was adjusted to four-fold that of the influent. The metallurgic wastewater used in this study was exhausted from a factory recovering precious metals from industrial wastes. Table 1 shows the water quality characteristics of the feeding wastewater to the system. To investigate temporal variations of microbial community by PCR-DGGE, sludge samples were collected from the anoxic packed bed of the two systems at different times (July, October and December 1999).

Denitrification activity test at various NaCl concentrations
Denitrification activity of the sludge in metallurgic wastewater treatment system (Run 2) at moderately halophilic conditions was examined. The sludge samples were inoculated into the solutions containing 41 mM NaNO₃, 11 mM KH₂PO₄, 7.1 mM NH₄HCO₃, 35 mM CH₃COOH, and incubated at 30°C with monitoring NO₂-N and NO₃-N concentrations. Sodium chloride concentrations of these solutions were prepared at 0.43%, 2.0%, 5.0% and 8.4%. After 8 days, the sludge samples were harvested and provided for the following DGGE analysis.

DNA extraction
DNA was extracted from a 0.5 g (wet weight) sludge pellet. The sludge was harvested by centrifugation at 10,000 g for 10 min. The harvested cells were sonicated for 30 s in a sucrose-lysis buffer (0.3 M sucrose, 0.7 M NaCl, 40 mM EDTA, 50 mM Tris-HCl), and then centrifuged at 2,000 g for 10 min. The supernatant was incubated at 55°C in the presence of sodium dodecyl sulfate, proteinase K and hexadecylmethyl ammonium bromide. DNA was extracted by applying phenol, chloroform and isoamyl alcohol, and precipitated by the addition of ethanol and sodium acetate.

PCR
The oligonucleotide primers 341f and 907r (Muyzer et al., 1998) were applied to selectively amplify 16S rDNA genes of all eubacteria. A 40-nucleotide GC-rich sequence

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Table 1 Characteristics of metallurgic wastewater fed to the treatment system

<table>
<thead>
<tr>
<th>Composition</th>
<th>Concentration (mg/l)</th>
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<tbody>
<tr>
<td>T-N</td>
<td>1,000–4,000</td>
</tr>
<tr>
<td>NO₃-N</td>
<td>1,000–4,000</td>
</tr>
<tr>
<td>NH₄-N</td>
<td>200–1,200</td>
</tr>
<tr>
<td>TOC</td>
<td>500</td>
</tr>
<tr>
<td>Salinity</td>
<td>20,000–50,000</td>
</tr>
</tbody>
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Figure 1 Metallurgic wastewater treatment system
was attached to the 5′ end of the primer 341f to improve the detection of sequence variation in amplified DNA fragments by subsequent DGGE (Myers et al., 1989). The mixtures used for PCR amplification of bacterial sequences contained 25 ng extracted DNA, 0.5 µM each primer, 200 µM each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 1.25 U of KOD DNA polymerase (Toyobo), 5 µl of 10× PCR buffer for KOD and sterile water to a final volume at 50 µl. PCR amplification was carried out with the following program: 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min; and a single final extension at 72°C for 2 min. The presence of PCR products was confirmed by analyzing 8 µl of product on 2% agarose gels stained with ethidium bromide.

DGGE
DGGE was performed with a D-CODE Multi-system (Bio-Rad). PCR samples were loaded onto 6% (wt/vol) polyacrylamide gels in 1.0× TAE (40 mM Tris, 40 mM acetate, 1 mM EDTA; pH 8.4). The polyacrylamide gels were made with denaturing gradient ranging from 30 to 50% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was run at 60°C, for 4 h at 130 V. After electrophoresis, the gels were soaked for 30 min in SYBR Green nucleic acid gel stain (1:10,000 dilution; Molecular Probes) and photographed on an UV transillumination table with a Kodak DC120 Zoom Digital Camera.

Sequencing of DGGE fragments and phylogenetic analysis
DNA fragments for nucleotide sequencing were punched from the gel with sterile tips and placed in sterile vials. DNA extraction from the gels was performed with a QIAEX II DNA extraction kit (QIAGEN). An extracted DNA was used as a template DNA in a PCR with the primers and conditions described above. After amplification, the PCR products were again analyzed by DGGE to confirm their electrophoretic mobility relative to the fragment from which they were excised. The PCR products were also used as template DNA in a cycle sequencing reaction with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in accordance with the manufacturer’s instructions. Sequencing of 16S rDNA fragments was carried out with an ABI PRISM 377 DNA Sequencing System (Applied Biosystems). Database searches were conducted by the sequencing match program with RDP II (Ribosomal Database Project II). Sequences determined in this study and those retrieved from the databases were aligned by Clustal W with DDBJ (DNA Data Bank of Japan). Neighbor-joining trees were constructed by Clustal W with DDBJ and Tree View.

Results and discussion
Denitrification performance in the anoxic packed bed of metallurgic wastewater treatment system
Figure 2 shows the time courses of NOₓ-N, i.e., NO₂-N+NO₃-N concentration in the influent and effluent of the anoxic packed beds of Runs 1 and 2. The nitrogen removal ratio of the anoxic packed bed was as high as 97%. Moreover, the main component of nitrogen compounds in the effluent of these systems was almost entirely ammonia nitrogen, which indicated that the denitrification ability of nitrate and nitrite in the anoxic packed bed was quite high.

Denitrification activity at various NaCl conditions
Figure 3 shows the time courses of NO₃-N and NO₂-N concentrations during denitrification activity test at various NaCl concentrations. After 8 days, nitrate and nitrite were completely denitrified at every NaCl concentration condition. However temporary accum-
mulation of nitrite was observed at 5.0% and 8.4% NaCl concentration conditions as shown in Figure 3(b), supposing that the reduction of nitrite is the rate-limiting step in the denitrification pathway under hypersaline conditions.

**Figure 3** Time courses of NO$_x$-N concentration during denitrification activity test at various NaCl concentration

DGGE band profile of sludge samples

Figure 4(a) shows DGGE band profiles of the PCR amplification products obtained from sludge samples in the anoxic packed bed. The microbial community in the sludge varied at the different sampling times. Differences in microbial community were hardly observed among July, October and December 1999 of Run 1. On the other hand, the samples of Run 2 showed temporal variations in microbial community from July 1999 to October 1999. Run 1 of laboratory scale was operated at a constant water temperature, whereas Run 2 of pilot plant scale was operated at the outside which caused water temperature change. It was supposed that the temporal variations in microbial community by DGGE analysis were caused by the temperature variations. Moreover, on comparing DGGE patterns of Runs 1 and 2, these patterns have acceptable similarity despite difference of scale. It was suggested that microbial community was affected by water temperature, salt concentration and composition of inlet wastewater rather than scale of systems.

In denitrifying activity tests, the band appearing at both 5.0% and 8.4% NaCl concentrations and bands appearing at all NaCl conditions were detected by DGGE (Figure 4(b)). It was suggested that there were two types of microbial community in the metallurgic wastewater treatment system. One of these microbial community was dominant at only 5.0–8.4% NaCl concentration conditions, and the other was able to grow at a wide range of salt concentrations. Considering that nitrogen removal efficiency was quite high at 2–5% salt concentration in the metallurgic wastewater treatment system, it was supposed that halophilic denitrification bacteria were dominant in this microbial community.
Sequencing of DGGE fragments and phylogenetic analysis
DGGE fragments were excised from gels and amplified by PCR, and the nucleotide sequences were determined. None of the sequences obtained perfectly matched those registered in the database, suggesting that previously uncharacterized populations might have been present in these samples. Figure 5 shows the phylogenetic relationships among DGGE fragments. One of the obtainable major groups showed high similarity to *Alcaligenes defragrans* affiliated with the beta subclass of the class *Proteobacteria*. Another major group showed high similarity to the *Pseudomonas* group affiliated with the gamma subclass of the class *Proteobacteria*. As a result, the analysis of the microbial community by PCR-DGGE (without cultivation) clarified that the microbial community in the metallurgic wastewater treatment system consisted mainly of beta and gamma subclasses of the class *Proteobacteria*. Nevertheless, it was still unclear what kind of bacteria was dominant and played a key role in denitrification activity in the metallurgic wastewater treatment system. Therefore, these bacteria are expected to be quantitatively estimated by another method such as FISH, and also in a further work, the microbial community should be analyzed based on functional genes like nitrite reductase (*nir*) as well as 16S rDNA.

Conclusions
High denitrification ability of nitrate and nitrite in the anoxic packed bed of metallurgic wastewater treatment systems was confirmed at both laboratory and pilot plant scale. As a result of denitrification activity test at various NaCl concentrations, it was suggested that halophilic denitrifying bacteria were present in these treatment systems and the reduction of nitrite is the key step in the denitrification pathway under hypersaline conditions.

DGGE analysis could detect the microbial community change caused by difference of water temperature. Moreover, it was suggested that microbial community varied in accordance with salt concentration. It was revealed that microbial community in the metallurgic wastewater treatment system consisted of mainly *Alcaligenes defragrans* and *Pseudomonas* groups.

Acknowledgements
This study was partly supported by Matsuda Sangyo Co., Ltd. We thank them for their assistance.
Figure 5 Neighbor-joining phylogenetic tree based upon partial 16S rDNA sequences derived from DGGE bands recovered from sludge in anoxic packed bed of metallurgic wastewater treatment system. The 16S rRNA sequence of *Aquifex pyrophilus* was used as an outgroup to root the tree. The bootstrap numbers indicate the value of 1,000 replicate trees supporting the branching order. Scale bar = 10% nucleotide substitution. “b” indicates sequences from DGGE of denitrification activity batch test at various salt concentrations, a: 0.43%, b: 2.0%, c: 5.0%, d: 8.4%. “c” indicates sequence from DGGE of metallurgic wastewater treatment system, R1: Run 1, 99J: July 1999, and so on

References


