Introduction

Nitrogen compounds such as NOx are released into the natural environment in vast quantities by chemical nitrogen fixation and industrial activities. Currently, strong regulations are in place for the removal of nitrogen compounds, especially ammonium, from wastewater discharge. Ammonium causes eutrophication in freshwater environments and is toxic to saltwater organisms. The removal of ammonium in wastewater treatment plants is performed primarily by the combination of aerobic nitrification catalyzed by autotrophic microbes and anaerobic denitrification by heterotrophic bacteria and fungi (Lin et al., 2005; Matsuzaka et al., 2003).

The denitrification reaction usually occurs in a few strains of fungi and many bacterial species, but is rarely seen in thermophilic bacteria (Knowles, 1982; Payne, 1973). Bacterial denitrification involves four reduction steps in which nitrate (NO3⁻) is sequentially transformed into dinitrogen (N2) via nitrite (NO2⁻), nitric oxide (NO), and nitrous oxide (N2O). Four terminal oxidases are necessary to achieve this stepwise reaction (Ye et al., 1994), and until now, denitrification studies have been carried out using normal temperature bacteria (5–20°C).

An enormous amount of energy is unnecessarily released into natural environments through thermal power generation. The application of co-generation systems to use the waste energy is very useful for en-
ergy saving. Conditions in wastewater treatment favor the growth of nitrite oxidizers, resulting in the complete oxidation of ammonia to nitrate. It seems very promising to apply thermophiles to wastewater treatment at high temperature. By using thermophilic bacteria to improve the conditions for denitrification, the capacity of treatment plants for denitrification could increase and treatment time would shorten. Although the application of thermophiles to denitrification by using waste energy may be an effective way to denitrify and improve environmental quality, thermophiles are currently not being used in co-generation systems. To establish an effective denitrification system utilizing thermophilic bacteria, we screened field soil, mud, and spa samples to isolate thermophiles, and characterized the isolated bacteria and the denitrification conditions of the isolates. We also compared the conditions under which the isolates could denitrify urban wastewater relative to those of _Pseudomonas stutzeri_ JCM 5965^T_, which can denitrify and grow at 30°C (Stanier et al., 1966).

**Materials and Methods**

**Chemicals.** All chemicals were analytical grade and of the highest available purity.

**Medium and culture conditions.** For this study, we used a basal medium (BM; 5 mM NH_4Cl, 10 mM KH_2PO_4, 20 mM Na_2HPO_4, 20 mM KNO_3) containing nitrate (BM/NO_3^-) supplemented with MgSO_4 solution (PS-1), vitamin solution, and trace element solution (VS salt solution; Su et al., 2001). The BM/NO_3^- ingredients were added to 20 or 60 mM of potassium nitrate (KNO_3). Disodium succinate hexahydrate (Na-succinate) was used with nitrate as the sole carbon source and the terminal electron acceptor for anaerobic respiration. One hundred millimolar Na-succinate was added to the BM before sterilization. The air phase of sealed glass vials (ø 30 mm × 50 mm) containing 15 ml BM/NO_3^- was replaced by He gas (0.1 MPa, 30 s) before cultivation at 60°C for 2 days.

**Enrichment culture.** For screening thermophilic bacterial strains, we used 150 soil, mud, and spa samples collected from several parts of Japan. For screening thermophilic denitrification bacteria, small amounts of field or spa soil and mud were placed into sealed glass vials (ø 40 mm × 120 mm) containing 70 ml of BM/NO_3^- with potassium nitrate, replacing the air phase with He gas (0.1 MPa, 30 s). Standing cultivation was carried out at 60°C for 4 days.

**Isolation of bacterial strains.** Samples of water, soil, and mud were collected from hot springs in Yamanashi, Tokyo, and Saitama prefectures. Spring water samples were transported without temperature control and analyzed within 24 h of collection. Aliquots of untreated soil samples (1 g) and water samples (1 ml) were mixed directly into 70 ml of BM/NO_3^- in shield glass vials (ø 40 mm × 120 mm). The vial air was subsequently exchanged with He gas and the vials were then sealed with butyl rubber caps and aluminum caps. After 2 days of cultivation, 1 ml of the culture broth was transferred into fresh BM/NO_3^- at 60°C. Similar enrichment subcultivations were performed three times, and the production of gas and reduction of NO_3^- and NO_2^- were evaluated using Griess-Romijn reagent according to the manufacturer’s instructions. This was performed by mixing with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid). Absorbance was measured at 540 nm after incubation for 10 min. Subcultures were spread onto 0.7% gerangum plates and cultivated at 60°C.

**Determination of the growth range.** The growth-supporting temperature range of TDN01 was measured by varying the temperature from 37 to 65°C in sealed glass vials (ø 30 mm × 50 mm) containing 15 ml of BM/NO_3^- . The tolerated pH range was measured in sealed glass vials (ø 30 mm × 50 mm) containing 15 ml of BM/NO_3^- by changing the pH from 5.0 to 10. The nitrate concentration was varied from 5 to 80 mM, and the phosphate concentration from 0 to 80 mmol of phosphate. The pH range and the concentrations of NO_3^- and phosphate suitable for growth were determined by the turbidity (at 660 nm) of liquid cultures grown at 60°C. The different pH values were obtained using biological buffers.

**Conditions for continuous culture.** Continuous culture of TDN01 with a dilution rate (Dr) of 0.166/h was performed at 60°C in a 10-L jar fermentor containing 3 L of BM/NO_3^-.

**Analysis.** Optical density was determined by spectrophotometry (Amersham Biosciences, Piscataway, NJ, USA) at 660 nm (OD_660). The amounts of NO_3^- and NO_2^- were determined by ion-chromatography (CH-9101; Metrohm, Ltd., Herisau, Switzerland) using a Shodex IC SI-90 4E column (Shoko Co., Ltd., Tokyo, Japan). For determining the amount of N_2 and N_2O, 20-μl samples (injection volume) were taken from the
Denitrification of thermophilic strain TDN01 character

headspace of the vials using a pressure-lock syringe to analyze with a gas chromatography/thermal conductivity detector (GC/TCD; GC-14B; Shimadzu, Kyoto, Japan) procedure. The carrier gas was He at a flow rate of 40 ml min\(^{-1}\). The column used was a P/N ZT-12 (GC stainless column 4.0 mm × 3.0 mm ID). The column and detector temperatures were 180°C. The amounts of both N\(_2\) and N\(_2\)O were calculated by applying the ideal gas law.

**DNA manipulation.** DNA isolation, restriction enzyme digestion, cloning, and agarose gel electrophoresis were performed following standard protocols (Sambrook et al., 1989). DNA fragments were extracted from agarose gel using glass powder for DNA recovery EASYTRAP version 2 (TaKaRa Shuzo Co., Ltd.), according to the manufacturer’s instructions. For amplifying the 16S rDNA gene from *Geobacillus* sp. strain TDN01 using PCR, the 10F (5’-AGAGTTTGATCCTGCTCAG-3’) and 1500R (5’-GGTTACCTTGTTACGCTT-3’) primers corresponding to the *Escherichia coli* 16S rDNA gene sequence were used (Weisburg et al., 1991). The fragment generated was purified by agarose gel electrophoresis for sequencing analysis.

**Nucleotide sequencing analysis.** The nucleotide sequences were determined using the chain termination method with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. A homology search was performed using the DDBJ/EMBL/GenBank DNA databases in the BLAST program (ver. 2.0.10; Altschul et al., 1997).

**RFLP analysis.** Purified DNAs of the bacteria (concentration 60 ng/µl) were reacted with 3 types of restriction enzymes (*Hae*III, *Afa*I and *Alu*I) at 37°C for 3 h. The reaction solution consists of 5 µl of purified DNA, 3.5 µl of dH\(_2\)O, 1 µl of 10× Buffer and 0.5 µl of restriction enzyme. Reaction solution was then run by electrophoresis in 2% of agarose gel (50 V, 70 min) and visualized by UV. The classification groups of the strains were determined by the differences of restriction fragment lengths.

**Results and Discussion**

**Isolation and identification**

Eight thermophilic strains and one symbiotic (data not shown) were isolated from the field soil, mud, and spa samples, and the phenotypic and physiological characterizations were performed under standard conditions. Thermophile TDN01 was a rod-shaped facultative anaerobic gram-positive motile and spore-forming bacterium. The thermophile showed good growth in aerobic on Na-succinate, pyruvate, acetate, glycerol, glucose, sucrose, and cellobiose. The physiological properties of thermophile TDN01 were as follows: catalase-positive, oxidase-positive, O/F-negative, and denitrification-positive.

The PCR products of each strains showed about 1,500 bp and the sequence analysis of readable length for 16S rDNA of 972 bp, 1,406 bp, 697 bp and 704 bp from TDN01, TDN05, TDN06 and TDN18, respectively; these sequences showed 98% identity to *Geobacillus* using the BLAST program against the DDBJ/GenBank/EMBL nucleotide sequence databases. The alignment of the 16S rDNA of TDN01, TDN05, TDN06 and TDN18 showed very high similarity (>98%) to *Geobacillus* LH8. The RFLP of the other strains showed patterns similar to that of TDN01.

**Characterization of the isolates**

The gases, i.e., N\(_2\)O and N\(_2\), produced by isolated bacteria were analyzed by gas chromatography (GC). Nos. TDN05 and TDN06 produced N\(_2\)O and N\(_2\), and the other bacteria produced N\(_2\), as shown in Table 1. *E. coli* for negative control didn’t produce N\(_2\) (data not shown). Strain TDN01 was chosen because it had the best denitrification activity and good growth.

The growth range of TDN01 was determined by varying the temperature, pH, and nitrate and phosphate concentrations. The temperature analysis showed that isolate TDN01 was therophilic and able to grow at 60°C. The growth range of temperature was 50–60°C, pH was 6.6–8.0, concentration of nitrate was 10–60 mM and concentration of phosphate was 0.1–50 mM.

**Table 1. Generated gas and growth of denitrification bacteria.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Generated gas</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N(_2)</td>
<td>NO(_2)</td>
</tr>
<tr>
<td>TDN01</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>TDN05</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>TDN06</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>TDN18</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>TDN1G</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>TDN23G</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>TDN15N</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>TDN23N</td>
<td>○</td>
<td>×</td>
</tr>
</tbody>
</table>
Comparison of denitrification capability among thermo-
philic Geobacillus

Thermophilic Geobacillus TDN01 grown at 60°C and
P. stutzeri JCM 5965T grown at 30°C were monitored
for OD and gas composition, respectively, over time.
The seed cultures of both strains were cultivated in
500-ml flasks with 300 ml of BM/NO3 for 2 days under
aerobic conditions and these cultures were used for
the main cultivations.

The main cultivations were performed under three
conditions with a concentration of 60 mM nitrate. One
of the cultivations was carried out in a flask containing
100 mM Na-succinate, and the second cultivation was
performed in a flask containing 50 mM glucose as the
carbon source. The third cultivation was cultured in a
20-L jar fermentor using Na-succinate as the carbon
source, and the pH was kept at 7.5. In each case, the
gas phase was replaced by He to maintain anaerobic
conditions, and the flasks were cultivated at 60°C. The
amount of nitrate decreased with the growth of ther-
mophilic TDN01 on Na-succinate, and the nitrite con-
centration remained at 15 mM even after the consump-
tion of nitrite cultivating of over 30 h, as shown in Fig.
1A. When the thermophilic TDN01 was grown on glu-
cose, nitrate was consumed in proportion with the
viable cells of TDN01; nitrite was formed but eventually
disappeared, as shown in Fig. 1B. In this case, pH was
not greatly influenced by the growth of TDN01. The
gas phase of the fermentor was replaced by N2 gas.
The pH maintained at 7.5 in a 20 L jar fermentor con-
taining 2 L medium with Na-succinate as the carbon
source demonstrated the consumption of nitrate and
the formation of nitrite with bacterial growth, and nitrite
was eventually completely eliminated, as shown in
Fig. 1C. This result indicates that pH was the important
factor for maintaining viable cells of TDN01 and
affected the total reduction of nitrite. P. stutzeri JCM
5965T grown at 30°C on Na-succinate also showed

Fig. 1. (A) Time course of nitrate and nitrite reduction by TDN01 grown on Na-succinate. (B) Time course of ni-
trate and nitrite reduction by TDN01 grown on glucose. (C) Time course of nitrate and nitrite reduction by TDN01
grown on Na-succinate, adjusting pH at 7.5. (D) Time course of nitrate and nitrite reduction by P. stutzeri JCM 5965T
grown on Na-succinate.

Growth (open rectangle), pH (closed rectangle), nitrate concentration (closed circle), and nitrite concentration
(open circle) in cultures of TDN01. Samples were removed for analysis at the times shown. (A), (B), (D) were run
three times with flasks and (C) was performed one time using a jar fermentor.
decreased nitrite levels and did not form nitrite, as shown in Fig. 1D.

**Capability of denitrification**

The capability of viable cells under each condition to denitrify was measured, as shown in Table 2. The nitrate and nitrite consumption rates of the thermophilic TDN01 were $44\times75$ times and $9\times41$ times greater, respectively, than those of *P. stutzeri* JCM 5965T. To demonstrate the possibility of applying a continuous culture system, a preliminary continuous culture was carried out. The OD$_{660}$ of the continuous culture stabilized at 0.28 after 20 h; conditions for dissolved oxygen (DO), pH and oxidation reduction potential (ORP) value were stabilized at 0 ppm, pH 7.5, and $-100$ mV, respectively. In that time, nitrate and nitrite removal value were both $3.33$ mM/h and the rate of nitrate and nitrite removal was $99.85\%$ with $2.7\times10^5$/ml of viable cells, as shown in Fig. 2. The level of denitrification per viable cell count was $12.2$ pmol/h, and an excellent value. These results indicate that the use of thermophiles in a continuous culture system for denitrification is a promising strategy for reducing the amount of nitrogen compounds released into the environment.

**Conclusions**

This study described the utility of denitrification by thermophilic bacteria. We obtained nine thermophilic denitrification bacteria, which were isolated from field soil, mud, and spa samples. One of them, *Geobacillus* sp. strain TDN01, had NO$_3^-$ and NO$_2^-$ removal ratios $44$–75 and $9$–$41$ times higher, respectively, than that of *P. stutzeri*. With the controlled pH at 7.5, TDN01 stably denitrified without accumulating NO$_2^-$. In addition, continuous culture of TDN01 resulted in removal of NO$_3^-$ and NO$_2^-$ at $3.33$ mM/h, a value stronger than usual for water processing facilities (Bilanovic et al., 1999; Constantin and Fick, 1997; Nyberg et al., 1996; Timmermans and Van Haute, 1983).

**References**


