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Aerobic Denitrification of *Pseudomonas putida* AD-21 at Different C/N Ratios

Mia Kim,[1] Seong-Yun Jeong,[2] Su Jeong Yoon,[1] Sun Ja Cho,[1] Young Hun Kim,[2] Min Ju Kim,[1] Eun Yoen Ryu,[1] and Sang-Joon Lee[1]*

Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Korea[1] *and Korea BIO-IT Foundry Center, Pusan National University, Busan 609-735, Korea*[2]

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An aerobic denitrifier was newly isolated from soil and its denitrification activity under different C/N (carbon/nitrogen) ratios was investigated. The isolate was identified as *Pseudomonas putida* AD-21 by biochemical studies and 16s rDNA sequencing analysis. *P. putida* AD-21 tolerated oxygen levels of 5.0–6.0 mg/*l*. An $^{15}NH_4/^{14}NO_3$ analysis indicated that the nitrogen of NH_4 was preferentially assimilated into the cell mass and that the nitrate removed could be considered an indication of bacterial denitrification efficiency. Increasing the C/N ratio increased the nitrate removal rates, whereas nitrogen assimilation into the cell mass was not affected. The optimal C/N ratio was 8 with a maximum nitrate removal rate of 254.6 mg/l/h and a nitrate removal efficiency of 95.9%. The results suggest that *P. putida* AD-21 may be a good candidate for aerobic wastewater treatment.

[**Key words:** aerobic denitrification, C/N ratio, $^{15}N/^{14}N$ analysis, *Pseudomonas putida* AD-21]

Denitrification is the biological removal of nitrogen from nitrate by its conversion to nitrogen gas. Denitrification gene expression occurs under anoxic conditions and requires the presence of an N-oxide (1). Many denitrifying bacteria have been isolated from soil (2) and activated sludge including (3–5), *Achromobacter*, *Aerobacter*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Lactobacillus*, *Micrococcus*, *Proteus*, *Pseudomonas*, and *Spirillum*. Conventional nitrogen removal systems in wastewater treatment plants consist of nitrification ($NH_4 \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$) by autotrophs, such as *Nitrosomonas* spp. and *Nitrobacter* spp., under aerobic conditions and denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) by heterotrophs under anaerobic condition (6, 7). However, this type of system can be uneconomical and difficult to operate due to extremely slow nitrification and the necessity for separate nitrification and denitrification reactors (7).

There are recent reports of aerobic denitrifying species isolated from canals, ponds, soils, and activated sludge that can simultaneously utilize oxygen and nitrate as electron acceptors. These include *Thiosphaera pantotropha* (*Paracoccus denitrificans*) (5), *Alcaligenes faecalis* (7), *Citrobacter diversus* (4), *Pseudomonas stutzeri* (5), and *Pseudomonas aeruginosa* (8). Using these aerobic denitrifiers, a simultaneous nitrification/denitrification process occurs in one reactor (9, 10). Aerobic denitrification has attractive advantages when compared to conventional denitrification under anaerobic conditions: (i) aerobic denitrifiers can be easily controlled during the operation and (ii) it can accelerate nitrification and denitrification by directly utilizing nitrite-N and nitrate-N, as the denitrifying reactant (4, 11). The rate-limiting parameters in aerobic denitrification are the carbon/nitrogen (C/N) load ratio, temperature, pH, water activity, dissolved oxygen (DO) concentration, and the microbial population (4, 12). Because the C/N ratio can affect denitrification efficiency and operating cost, it is one of the most important factors.

In this study, an aerobic denitrifier was newly isolated from soil and its denitrification activity was investigated using different C/N ratios and compared to *P. stutzeri* KCTC 2760, which is a well-known aerobic denitrifier.

MATERIALS AND METHODS

Bacterial strain and media We used the media as described by Takaya *et al.* (13). To screen the aerobic denitrifiers, we used bromothylmol blue (BTB) medium (L-asparagine, 1 g/*l*; KNO_3, 1 g/*l*; KH_2PO_4, 1 g/*l*; $FeCl_2$–P6H_2O, 0.05 g/*l*; $CaCl_2$–2H_2O, 0.2 g/*l*; $MgSO_4$–7H_2O, 1 g/*l*; BTB reagent, 1 ml/*l* [1% in ethanol]; and agar, 20 g/*l*; pH 7.0–7.3) and modified screening medium (SM) (sodium citrate, 9.63 g/*l*; $NaNO_3$, 10 mM; KH_2PO_4, 1.36 g/*l*; $(NH_4)_2SO_4$, 0.27 g/*l*; yeast extract, 1 g/*l*; $MgSO_4$–7H_2O, 0.19 g/*l*; and a trace element solution, 1 ml/*l*). The components of the trace element solution were EDTA, 50.0 g/*l*; $ZnSO_4$, 2.2 g/*l*; $CaCl_2$, 5.5 g/*l*; $MnCl_2$–4H_2O, 5.06 g/*l*; $FeSO_4$–7H_2O, 5.0 g/*l*; $(NH_4)_6Mo_7O_{24}$–4H_2O, 1.1 g/*l*; $CuSO_4$–5H_2O, 1.57 g/*l*; and $CoCl_2$–6H_2O, 1.61 g/*l*; pH 7.2 (14). To test denitrification activity, we used a modified denitrification medium (DM) (sodium citrate, 9.63 g/*l*; $NaNO_3$, 1 g/*l*; CH_3COONH_4, 0.5 g/*l*; KH_2PO_4, 1.5 g/*l*; Na_2HPO_4, 0.42 g/*l*; NH_4Cl, 0.6 g/*l*; $MgSO_4$–7H_2O, 1.0 g/*l*; and trace element solution, 2 ml/*l*) and LB medium (tryptone, 10 g/*l*; yeast extract, 5 g/*l*; NaCl, 5 g/*l*). *Pseudomonas*

* Corresponding author. e-mail: sangjoon@pusan.ac.kr
phone: +82-51-510-2193 fax: +82-51-514-1778
The first two authors contributed equally to this work.

stutzeri KCTC 2760 was obtained from the Korea Collection for Type Culture (KCTC).

Screening of aerobic denitrifiers The soil samples for the isolation of aerobic denitrifiers were collected from the O horizon of humus soil (15) at five different sites in Busan, Korea and within a depth of 10 cm from the surface. The soil samples were transferred to SM broth and incubated in a shaking incubator (200 rpm) at 30°C for 2 d. Five milliliters of the culture was added to fresh SM broth and incubated under the same conditions. This was repeated three times. The resulting bacterial suspension was streaked onto BTB medium plates containing sodium citrate (9.63 g/*l*) and incubated at 30°C for 1–3 d. Blue colonies were selected from the BTB agar plates, transferred to DM broth containing $NaNO_3$ (1 g/*l*), and then incubated under aerobic conditions at 30°C.

Identification of the isolated bacterium The isolated bacterium that showed the best nitrate reduction was grown at 30°C on LB agar. Standard physiological and biochemical characteristics were examined according to previously described methods (16). Additional biochemical tests were performed using API kits (API 20NE, BioMerieux, SA, France). PCR amplification of the 16S rRNA genes was performed on a Takara PCR Thermocycler. Genomic DNA was prepared using the AccuPrep^R Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). PCR was conducted using genomic DNA (0.1 μg) as the template and the commercially synthesized (Bioneer, Daejon, Korea) universal primers 27F (AG AGTTTGATCMTGGCTCAG) and 1492R (GATTACCTTGTTAC GACTT). The PCR conditions were 30 cycles at 94°C for 20 s, 55°C for 1 min, 72°C for 1 min, and extension at 72°C for 10 min. The 1.5 kb PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI), sequenced using a Termination Sequencing Ready Reaction kit (Perkin Elmer, Wellesley, MA, USA), and analyzed using an ABI 377 genetic analyzer (Perkin Elmer, Wellesley, MA, USA). The near-complete 16S rDNA sequence (1498 bp) was aligned using CLUSTAL W software ver. 1.7 (17). The 16S rDNA sequences used for the phylogenetic analysis were derived and compared with those of other bacterial 16S rDNA sequences available in the DDBJ/EMBL/GenBank database. Kimura's two-parameter model (18) was applied for the calculation of evolutionary distance. A phylogenetic tree was constructed by the neighbor-joining method (19). Bootstrap analyses of 1000 replicates were carried out using MEGA ver. 2.0 (20).

Aerobic denitrification by the isolated bacterium

Electron donor specificity The isolated bacterium was transferred to 100 ml of DM containing $NaNO_3$ (1.0 g/*l*) and precultured under aerobic conditions at 30°C. Glucose, citrate, methanol, ethanol, glycerol, acetate, and succinate were added to the DM as carbon sources. All tests were performed in triplicate.

Nitrogen removal rate The cultures were harvested when they reached the logarithmic growth phase. The cell density was adjusted to a constant 0.8 g/*l* under continuous culture (Fig. 1). The C/N ratio was controlled at 10 and the DM medium flowed continuously into the culture system at a dilution rate of 0.25–0.35/h. Oxygen was supplied continuously to the culture by pumping air through a 0.20 μm glass filter (Millipore, Bedford, MA, USA) and the DO concentration was adjusted to 5.0–6.0 mg/*l* (4). The nitrogen concentration was adjusted to 373 mg/*l* using $NaNO_3$. The amount of nitrogen was calculated by measuring cellular nitrogen (biomass) and the nitrogen in the medium (the concentrations of nitrate and nitrite) and converting it to nitrogen atoms. All of the experiments were performed in triplicate.

Effect of C/N ratios Sodium acetate and sodium nitrate were used as the carbon and nitrogen sources, respectively. The C/N ratios in the medium were adjusted to 2, 4, 6, 8, and 10 by changing the amount of the carbon source and by maintaining a constant nitrogen concentration. The precultured isolated bacterium (0.5% v/v) was inoculated into the DM and incubated at 30°C under aerobic conditions. For the batch-culture experiments, the medium was incubated in a shaking incubator at 30°C for 24 h. For the continuous-culture experiments, the cultures were harvested in the logarithmic growth phase, and the cell density was adjusted to 0.8 g/*l* by resuspending the cells in fresh DM. The cultures were incubated at 30°C under aerobic conditions. The medium was continuously passed into the aerobic reactor at a dilution rate of 0.25–0.35/h. The concentrations of ammonium and nitrate in the culture broth were analyzed.

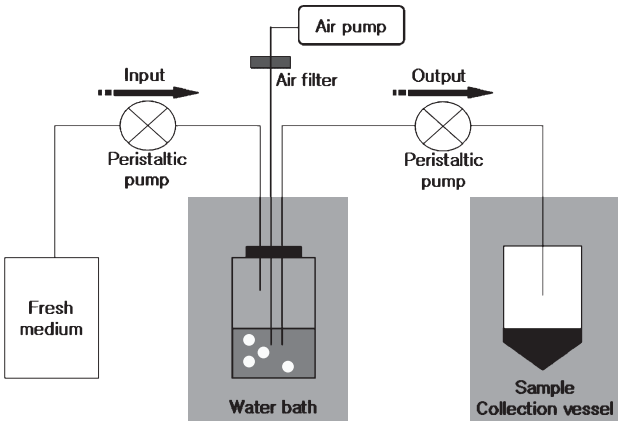

FIG. 1. Schematic diagram of the continuous culture apparatus under aerobic conditions. The cell density was adjusted to a constant 0.8 g/*l*. The medium was passed into the culture system at a dilution rate of 0.25–0.35/h. Oxygen was continuously supplied to the culture using an air pump and the DO was adjusted to 5.0–6.0 mg/*l*.

Analytical methods The cell density was measured using a spectrophotometer (UV-1200; Shimadzu, Kyoto) at an absorbance of 660 nm. The intracellular nitrogen content was calculated by measuring the $^{15}N/^{14}N$ ratios in the cell mass and the amount of $^{15}NH_4^+$ removed from the medium. The cells were incubated in a continuous culture in medium containing $CH_3COO^{15}NH_4$ and $Na^{14}NO_3$ as the nitrogen sources. The cells were harvested and washed twice with phosphate buffer (NaCl, 8 g/*l*; KCl, 0.2 g/*l*; Na_2HPO_4, 1.44 g/*l*; KH_2PO_4, 0.24 g/*l*; pH 7.4) to remove the ^{14}N remaining in the medium. The dry cells were processed using a vacuum freeze dry system (SFDSM06; Samwon, Busan, Korea). The cells were homogenized and analyzed using a stable isotope mass spectrometer (GV Instrument Isoprime and EuroVector EA, GV Instrument Ltd., Manchester, UK). All treatments were performed in duplicate. The supernatant was centrifuged (10,000×*g*), filtered through a 0.22 μm membrane (Millipore), and the ammonium and nitrate concentrations were measured using an AutoAnalyzer (Bran and Luebbe, Germany). The sequence data of the isolated strains have been submitted to the DDBJ/EMBL/GenBank databases under accession no. EU258552.

RESULTS AND DISCUSSION

Isolation and identification of the aerobic denitrifying bacterium AD-21 Aerobic denitrifying bacteria were isolated from the soil. Twenty-four strains were selected from the first screening and they formed blue colonies and/or halos on the BTB medium due to an increase of pH. After the second screen, the nitrate-removal rate was examined in DM broth under aerobic conditions. The AD-21 isolate had a 99.4% nitrate removal rate and was selected for further characterization. The AD-21 isolate was a gram-negative

TABLE 1. The morphological, physiological, and biochemical characteristics of *P. putida* AD-21

Characteristics	Results	Characteristics	Results
Gram staining	−	Assimilation of	
Cell form	Rod	D-Glucose	+
Catalase test	+	Arabinose	−
Oxidase test	+	Mannose	−
Indole production	−	Mannitol	−
Glucose fermentation	−	*N*-Acetyl-D-glucosamine	−
Arginine dehydrolase test	+	D-Maltose	−
Urease test	−	Potassium gluconate	+
Gelatin protease test	−	Capric acid	+
β-Glucosidase test	−	Adipic acid	−
β-Galactosidase test	−	Malic acid	+
		Trisodium citrate	+
		Phenylacetic acid	+

TABLE 2. The concentration of nitrate removed by *P. putida* AD-21 using different carbon sources under aerobic conditions at 30°C

Carbon source	Removed nitrate concentration (mg/*l*)
Glucose	211.5±8.56
Citrate	198.0±21.35
Methanol	178.9±2.19
Ethanol	150.1±10.75
Glycerol	208.6±8.49
Acetate	237.1±0.54
Succinate	216.0±7.71

C/N ratio was controlled at 10.
Initial concentration of nitrate was 672 mg/*l*.

rod with catalase and oxidase activity (Table 1). It contained arginine dehydrolase, but did not hydrolyze gelatin. It assimilated D-glucose, potassium gluconate, capric and malic acid, trisodium citrate, and phenylacetic acid. These characteristics indicated that the AD-21 isolate is a member of the genus *Pseudomonas* of the β *subclass-Proteobacteria*.

A BLAST search of available data in the DDBJ/EMBL/GenBank database showed a high similarity (99.0%) with *Pseudomonas putida* IAM 1236^T (Fig. 2). Thus, we designated this bacterium as *Pseudomonas putida* AD-21 and selected it for further study. We will conduct further taxonomic studies to conclusively identify this isolated bacterium.

Characteristics of the aerobic denitrifier

Electron donor specificity The denitrification electron-donor specificity of *P. putida* AD-21 was investigated during the early stationary phase under aerobic conditions. The use of different carbon sources as electron donors affected the denitrification activity of this bacterium (Table 2). Of the carbon sources tested, *P. putida* AD-21 efficiently removed nitrate from acetate, succinate, and glucose. However, slow growth and a long lag phase indicated that acetate was not a suitable carbon source for *P. putida* AD-21. There was low nitrate removal efficiency when methanol and ethanol were used as carbon sources.

Nitrogen removal rate Under aerobic conditions (dissolved oxygen, DO = 5.0–6.0 mg/*l*), *P. putida* AD-21 was more effective in removing nitrate than the well-known aerobic denitrifier *P. stutzeri* KCTC 2760. The nitrogen removal rate by *P. putida* AD-21 was 77.2±4.43 mg-N atom/*l*/h, whereas it was 60.3±2.44 mg-N atom/*l*/h for *P. stutzeri* KCTC 2760. Most aerobic denitrifying bacteria are able to denitrify at a DO less than 3 mg/*l* (4), and the nitrate reductase of *Pseudomonas* sp., which can tolerate a low DO, is inhibited above 4 mg/*l* (4). These results show that *P. putida* AD-21 has a better tolerance to oxygen compared to other aerobic denitrifiers.

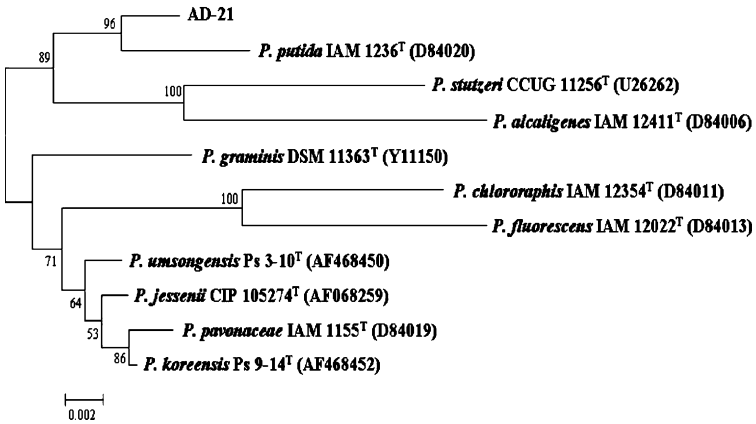

FIG. 2. Phylogenetic tree based on a comparison of the 16S rDNA gene sequence. The phylogenetic tree was generated using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Bar shows two nucleotide substitutions per 1000 nucleotides.

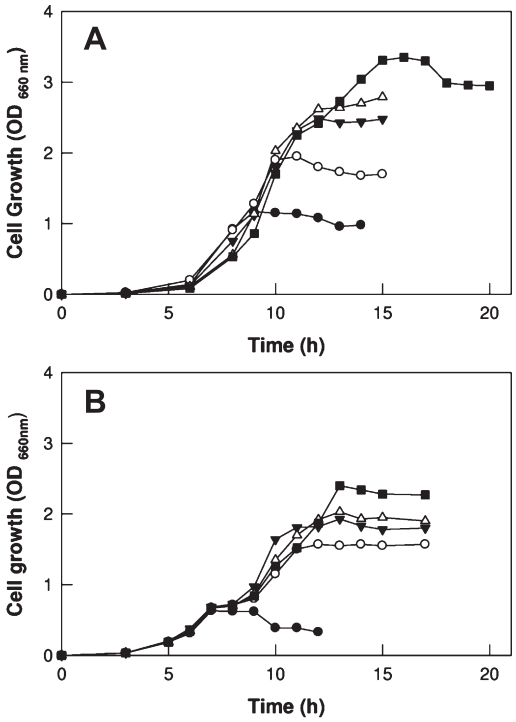

FIG. 3. Growth curves for *P. putida* AD-21 (A) and *P. stutzeri* KCTC 2760 (B) at different C/N ratios in batch cultures. Closed circles, C/N=2; open circles, C/N=4; closed triangles, C/N=6; open triangles, C/N=8; closed squares, C/N=10.

Effect of C/N ratio on cell growth and denitrification

Cell growth under aerobic condition in batch culture mode The growth profiles of *P. putida* AD-21 and *P. stutzeri* under various C/N ratios indicated that cell growth increased as more carbon was added to the system (Fig. 3). At an insufficient carbon concentration, the electron flow is too low to provide enough energy for cell growth (4). *P. putida* AD-21 grew faster than *P. stutzeri* KCTC 2760 under all of the C/N ratios. After a 15-h culture with a C/N ratio of 10, the maximum growth rates of *P. putida* AD-21 and *P. stutzeri* KCTC 2760 were 3.4 (dry cell weight 2.72 g/*l*) and 2.4 (dry cell weight 1.92 g/*l*), respectively. At a C/N ratio of 8, up to 70.0% of the nitrates in the medium were removed by *P. putida* AD-21. However, batch culture made it difficult to measure the exact denitrification efficiency at various C/N ratios. It appeared that nitrate was converted to cell mass once the ammonium was exhausted from the medium. To obtain more precise denitrification efficiency, we maintained the bacteria in a continuous culture mode for the remaining experiments.

Method for measuring denitrification during continuous growth Prior to examining denitrification, we established a method to measure intracellular nitrogen that did not include the detection of N_2 gas. Joo *et al.* (7) used a ^{15}N tracer to measure N_2 production and verify bacterial denitrification. In this study, we attempted to determine bacterial denitrification efficiency by measuring intracellular nitrogen using $^{15}N/^{14}N$ analysis. The conversion of ammonium ions into the cell mass can be determined by analyzing the $^{15}N/^{14}N$ ratio using a stable-isotope mass spectrometer ($CH_3COO^{15}NH_4$ and $Na^{14}NO_3$ were used as ^{15}N and ^{14}N sources, respectively). At all of the C/N ratios, the intracellular nitrogen included over 99.98% of the ^{15}N atoms (data not shown). This result implies that N from ammonium-N may have been preferentially assimilated into the cell mass rather than other types of inorganic nitrogen, such as nitrate-N. All types of nitrogen must be converted to glutamate through NH_4^+ to be assimilated into the cell mass (Fig. 4). When nitrate and ammonium coexist in the medium, ammonium-N is assimilated into the cell mass, whereas nitrate-N is denitrified. Therefore, the concentration of nitrate removed from the medium could be considered as an indication of the denitrification efficiency.

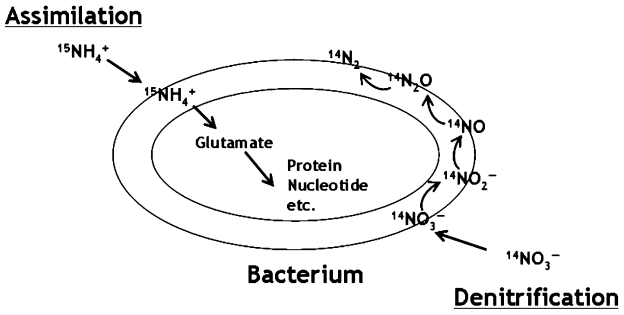

FIG. 4. Schematic diagram of bacterial assimilation and denitrification. The diagram represents NH_4^+-N assimilation into the cell mass and the denitrification process of nitrate to N_2; NO_2^-, NO, and N_2O in and on both sides of the cytoplasmic membrane of the bacterium (1) when nitrate and ammonium coexist in the medium.

Denitrification at different C/N ratios under aerobic conditions in continuous culture mode Because the bacterial densities were constantly maintained during the experiment, varying the C/N ratio did not change the rates of ammonium removal by either strain (Fig. 5). The ammonium removal could be converted to cell mass by the ^{15}N analysis. This result suggested that the amount of nitrogen assimilated into the cell mass was similar between the two strains and was not affected by the C/N ratio. However, the nitrate removal rates changed in relation to the C/N ratio, and there were different removal patterns between the two strains. When the C/N ratio was increased to 8, the nitrate removal rate by *P. putida* AD-21 quickly increased to a maximum of 254.6 mg/l/h. In contrast, at a C/N ratio of 10, the nitrate removal rate by *P. stutzeri* KCTC 2760 increased at a slower rate than that of *P. putida* AD-21 and reached a maximum of 169.2 mg/l/h. *P. putida* AD-21 removed up to 95.9% of the nitrate in a DO of 5.0–6.0 mg/*l*, whereas *P. stutzeri* KCTC 2760 removed up to 63.7% of the nitrate under the same conditions. The nitrate-N removal efficiencies by aerobic denitrifiers isolated from sequencing batch reactors are 30.5% to 60.5% (11). We are currently conducting additional C/N experiments to identify denitrification mechanisms in *P. putida* AD-21. Because carbon is essential for cell growth and nitrate reduction processes, the optimal quantity of carbon is a key parameter in the denitrification process (21). Denitrification efficiency decreases under extremely low or high carbon concentrations; denitrifiers grow slowly in suboptimal carbon concentrations, due to insufficient energy, whereas their growth is inhibited in an extremely high car-

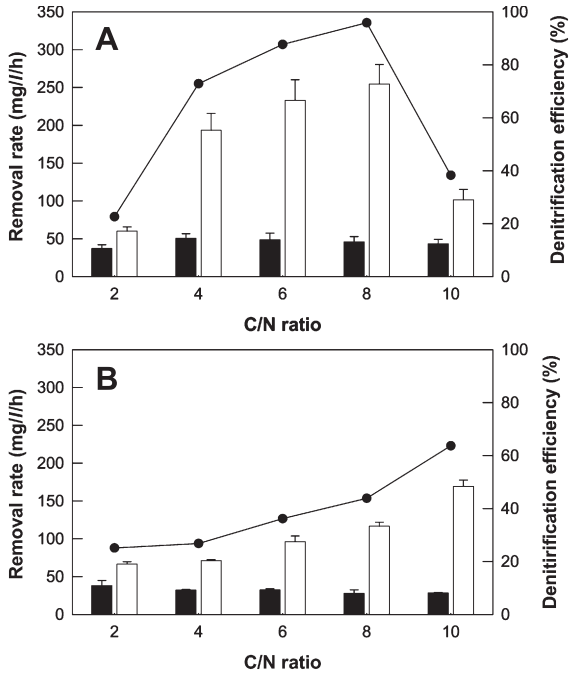

FIG. 5. The effect of the C/N ratio on the nitrate and ammonium removal rates by *P. putida* AD-21 (A) and *P. stutzeri* KCTC 2760 (B) under continuous aerobic conditions. Bacterial yields were controlled at a constant 0.8 g/*l*. Nitrate and ammonium were adjusted to 759 and 122 mg/*l*, respectively, for all of the C/N ratios. Closed squares, ammonium concentration; open squares, nitrate concentration; closed circles, denitrification efficiency.

bon concentration (4). Therefore, it is important to optimize the C/N ratio for each denitrifier. In this study, the optimal C/N ratios of *P. putida* AD-21 and *P. stutzeri* KCTC 2760 were 8 and 10, respectively. *P. putida* AD-21 had higher denitrification efficiency, especially at lower C/N ratios, compared with *P. stutzeri* KCTC 2760. These results should be useful information for operating aerobic denitrification process in wastewater treatment plants.

REFERENCES

1. **Zumft, W. G.:** Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev., **61**, 533–616 (1997).
2. **Chèneby, D., Philippot, L., Hartmann, A., Hénault, C., and Germon, J. C.:** 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. FEMS Microbiol. Ecol., **34**, 121–128 (2000).
3. **Frette, L., Gejlsbjerg, B., and Westermann, P.:** Aerobic denitrifiers isolated from an alternating activated sludge system. FEMS Microbiol. Ecol., **24**, 363–370 (1997).
4. **Huang, H. K. and Tseng, S. K.:** Nitrate reduction by *Citrobacter diversus* under aerobic environment. Appl. Microbiol. Biotechnol., **55**, 90–94 (2001).
5. **Su, J. J., Liu, B. Y., and Liu, C. Y.:** Comparison of aerobic denitrification under high oxygen atmosphere by *Thiosphaera pantotropha* ATCC 35512 and *Pseudomonas stutzeri* SU2 newly isolated from the activated sludge of a piggery wastewater treatment system. J. Appl. Microbiol., **90**, 457–462 (2001).
6. **Tchobanoglous, G. and Burton, F. L.:** Wastewater engineering: treatment, disposal and reuse, 3rd ed., p. 429–433. McGrew-Hill, NY (1991).
7. **Joo, H. S., Hirai, M., and Shoda, M.:** Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* no. 4. J. Biosci. Bioeng., **100**, 184–191 (2005).
8. **Chen, F., Xia, Q., and Ju, L. K.:** Competition between oxygen and nitrate respirations in continuous culture of *Pseudomonas aeruginosa* performing aerobic denitrification. Biotechnol. Bioeng., **93**, 1069–1078 (2006).
9. **Kshirsagar, M., Gupta, A. B., and Gupta, S. K.:** Aerobic denitrification studies on activated sludge mixed with *Thiosphaera pantotropha*. Environ. Technol., **16**, 35–43 (1995).
10. **Helmer, C. and Kunst, S.:** Simultaneous nitrification/denitrification in an aerobic biofilm system. Water Sci. Technol., **37**, 183–187 (1998).
11. **Wang, P., Li, X., Xiang, M., and Zhai, Q.:** Characterization of efficient aerobic denitrifiers isolated from two different sequencing batch reactors by 16S-rRNA analysis. J. Biosci. Bioeng., **103**, 563–567 (2007).
12. **Lloyd, D.:** Aerobic denitrification in soils and sediments: from fallacies to facts. Trends Ecol. Evol., **8**, 352–356 (1993).
13. **Takaya, N., Catalan-Sakairi, M. A. B., Sakaguchi, Y., Kato, I., Zhou, Z., and Shoun, H.:** Aerobic denitrifying bacteria that produce low levels of nitrous oxide. Appl. Environ. Microbiol., **69**, 3152–3157 (2003).
14. **Robertson, L. A., van Niel, E. W. J., Torremans, R. A. M., and Kuenen, J. G.:** Simultaneous nitrification and denitrification in aerobic chemostat cultures of *Thiosphaera pantotropha*. Appl. Environ. Microbiol., **54**, 2812–2818 (1988).
15. **Pipkin, B. W. and Trent, D. D.:** Geology and the environment, 3rd ed., p. 177–178. Brooks/cole, CA (2001).
16. **MacFaddin, T. F.:** Biochemical tests for identification of medical bacteria, p. 36–308. Williams & Wilkins, Baltimore, MD (1984).
17. **Thompson, J. D., Higgins, D. G., and Gibson, T. J.:** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., **22**, 4673–4680 (1994).
18. **Kimura, M. A.:** Simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol., **16**, 111–120 (1980).
19. **Saitou, N. and Nei, M.:** The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., **24**, 189–204 (1987).
20. **Kumar, S., Tamura, K., Jakobsen, I. B., and Nei, M.:** MEGA2: molecular evolutionary genetics analysis software. Bioinformatics, **17**, 1244–1245 (2001).
21. **Patureau, D., Bernet, N., Delgenès, J. P., and Moletta, R.:** Effect of dissolved oxygen and carbon-nitrogen loads on denitrification by an aerobic consortium. Appl. Microbiol. Biotechnol., **54**, 535–542 (2000).