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Effective NO_3^- and NO_2^- removal at both of 10℃ and 20℃ by *Arthrobacter* sp. N6 harboring *nirS* from activated sludge treating municipal wastewater

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1. Introduction

Nitrite reductases encoded by nitrite reductase genes (*nirK* and *nirS*) are the key enzymes in the bacterial dissimilatory denitrification process. Because of widespreadedness of *nirS* gene among denitrifiers in environment, using *nirS* gene as a molecular marker is convenient and adequate to select useful denitrifiers from environment (1). On the other hand, denitrification in biological wastewater treatment system is still difficult in the condition of low temperature and low C/N ratio. It is known that the activity of denirifiers is seriously hampered by low temperature. This is a major problem of biological denitrification process at low temperature. In this study, it was tried to select effective denitrifiers from activated sludge treating municipal wastewater and to investigate the temperature independency of them.

2. Materials and Methods

2.1 Activated sludge, municipal wastewater, and denitrifiers

Around 50 culturable bacteria were isolated on Nutrient and R_2A Agar (Difco) media from activated sludge treating municipal wastewater (Sung-Nam sewage treatment plant, Korea) by serial dilution plating. Three *nirS*-harboring denitrifiers were selected (Fig. 1) from around 50 isolates by PCR with the *nirS* primers, S1F and S6R (1). *Pseudomonas aeruginosa* ATCC 10145 was chosen as the control denitrifier harboring *nirS* gene (1). Only one of the three selected *nirS*-harboring denitrifiers, *Arthrobacter* sp. N6 (Table 1), could grow at 10℃. The characteristics of municipal wastewater are described in Table 2.

2.2 Identification of selected denitrifiers

For the 16S ribosomal DNA analysis, chromosomal DNAs of selected denitrifier was extracted by modified CTAB method. And PCR was performed with 16S rDNA primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3'). The annealing temperature was 57℃. For the putative *nirS* DNA analysis, approximately 890 base pairs, PCR was performed with *nirS* gene primers, S1F (5'-CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T-3') and S6R (5'-CGTTGAACTT(A/G)CCGGT-3') (1). The annealing temperature was 45℃ (Fig. 1). The identification and confirmation of *nirS* of selected denitrifier were carried out by homology search based on partial 16S rDNA sequences and putative *nirS* gene sequences of selected denitrifier in the Basic Local Alignment Search Tool (BLAST) program (Table 1). Accession number is AF335919 in GenBank.

2.3 Condition of batch culture and characteristics of municipal wastewater

The control and selected denitrifiers used in this study were adapted to 500 mg NO_3-N/l in LB broth before the experiment and centrifuged at 4,500 rpm. Then each precipitated cells was inoculated into each batch reactor containing the filtered municipal wastewater supplemented with KNO_3. Conditions of batch culture are described in Table 2. In each batch experiment, the samples were incubated for 30 hours at 20℃ or for 48 hours at 10℃.

2.4 Analytical method

Sample was centrifuged at 4,500 rpm and the precipitate was used for VSS measurement, and the supernatant for COD (APHA, 1995). The 10-fold diluted supernatant was used to measure the concentration of NO_3^- and NO_2^- as N by ion chromatography. The specific denitrification rate (SDNR) of batch culture was calculated as follows:

• SDNR = mg (ΔNO_3-N or ΔNO_2-N)/ (g VSS·h)

where $SDNR_{NO3}$ is SDNR for NO_3^- as N and $SDNR_{NO2}$ is SDNR for NO_2^- as N. The ratio of COD consumed to reduced nitrate ($\Delta COD/\Delta NO_3$-N) was calculated as C requirement for NO_3^- removal.

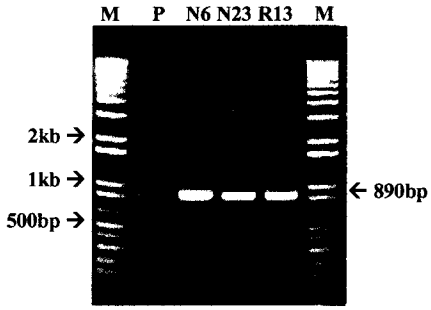

Fig. 1. Gel electrophoretic pattern of *nirS* gene from selected denitrifiers. M, 1kb ladder DNA marker; P, *P. aeruginosa* ATCC 10145; N6, N23, and R13, selected denitrifiers harboring *nirS*.

Table 1. Homology search based on 16S rDNA and *nirS* gene sequences of selected denitrifiers in BLAST

Selected strains	Partial 16S rDNA gene sequences: Closest match	Compared bps	% identity	Sequenced bps
N6	*Arthrobacter oxydans* 16S rRNA gene	407	95	557bp AF335919
	A. polychromogenes 16S rDNA gene	407	95	*bps; base pairs.
Selected Strains	**Partial *nirS* gene sequences: Closest match**	**Compared bps**	**% identity**	**Sequenced bps**
N6	*Pseudomonas stutzeri nirS, nirT, nirB, nirM* genes	322	83	458bp

Table 2. Conditions of batch culture and characteristics of municipal wastewater

Batch culture		Municipal wastewater			
Constituents	**Average**	**Constituents**	**Average**	**Constituents**	**Averag**
Initial total COD (mg/l)	350 – 550	*Total COD (mg/l)	300	TKN (mg/l)	44.0
Initial concentration of NO_3-N (mg/l)	100 – 160	*Soluble COD (mg/l)	100	NH_4-N (mg/l)	25.0
VSS of initial inoculum (mg/l)	50	Total BOD (mg/l)	80	TP (mg/l)	6.3
VSS of bacterial mass of culture (mg/l)	100 – 200	Soluble BOD (mg/l)	30	PO_4-P (mg/l)	5.4
Temperature (℃)	10, 20	SS (mg/l)	130	VFA (mg/l)	15.0
Working volume (l)	2	VSS (mg/l)	110	pH	7.2
Agitating speed of magnetic bar (rpm)	100	Alk. (mg/l)	190		

*The values were included in the initial total COD of batch culture.

3. Results and Discussion

The results of the batch experiments are summarized in Table 3 and in Fig. 2. *A.* sp. N6 showed over 10-fold $SDNR_{NO3}$ of the average $SDNR_{NO3}$ (3.5~5 mg NO_3-N/(g VSS·h)) of general wastewater treatment plants (WWTPs) at 20℃. The $SDNR_{NO3}$ of *A.* sp. N6 was over 15 fold of the average $SDNR_{NO3}$ (0.1~1.5 mg NO_2-N /(g VSS·h); 2) of general WWTPs in Korea at 10℃. The activated sludge could not reduce most of NO_3^- at 10℃ and 20℃. As for the C requirements for NO_3^- removal of *A.* sp. N6 at 10℃ and 20℃, it was practically lower than those (5~10 g COD/g NO_3-N; 2) in wastewater treatment whereas those of control denitrifier and activated sludge were still high.

The reduction of SDNRs of *A.* sp. N6 at low temperature was believed due to less volatile fatty acid (VFA) contents in influent wastewater and slower bacterial reaction itself.

4. Conclusion

From these results, it might be concluded that *Arthrobacter* sp. N6 could be employed for effective biological denitrification in wastewater treatment system rarely being influenced by the problems of low temperature and low C/N ratio. Further studies of the temperature effects on denitrification of other denitrifiers from activated sludge are needed for the improvement of biological wastewater treatment system.

Table 3. The SDNRs of the cultures in different temperature

Temp.	Strains	ΔCOD[a]	VSS[b]	ΔNO_3-N[c]	ΔNO_2-N[d]	$SDNR_{NO3}$[e]	$SDNR_{NO2}$[f]	ΔCOD/ΔNO_3-N[g]
At 20℃	Activated sludge	- 63	175	- 9.6	- 0.3	4.6	NC	6.6
	P. aeruginosa	- 324	144	- 50.4	- 3.9	29.2	NC	6.4
	A. sp. N6	- 317	168	- 103.1	- 61.0	51.1	30.3	3.1
At 10℃	Activated sludge	NC	NC	NC	NC	NC	NC	NC
	P. aeruginosa	- 462	111	- 77.0	- 20.9	28.8	23.5	6.0
	A. sp. N6	- 337	126	- 87.7	- 20.7	28.9	20.5	3.8

*All values were averaged from three measurements. The units were [a]mg/l, [b]mg/l, [c]mg/l for 12 hours at 20℃; 24 hours at 10℃, [d]mg/l for 10 hours at 20℃; 8 hours at 10℃, [e]mg NO_3-N/(g VSS·h), [f]mg NO_2-N/(g VSS·h), and [g]g COD/g NO_3-N. NC, not calculated; -, consumed or removed.

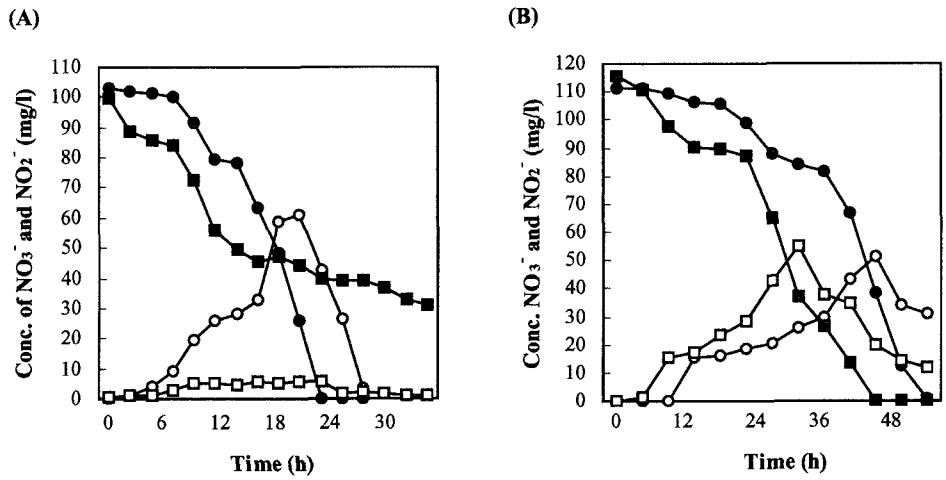

Fig. 2. NO_3^- and NO_2^- removal by batch cultures of the selected strains. NO_3^- (close symbol), NO_2^- (open symbol), *Peudomonas aeruginosa* ATCC 10145 (■, □), *Arthrobacter* sp. N6 (●, ○). At 20℃ (A) and at 10℃ (B).

5. References

1. **Braker, G., A. Fesefeldt, and K.-P. Witzel.** (1998) Development of PCR primer systems for amplification of nitrite reductase gene (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl. Environ. Microbiol.* **64:** 3769-3775.
2. **Choi, E., D. Rhu, Z. Yun, and E. Lee.** (1998) Temperature effects on biological nutrient removal system with weak municipal wastewater. *Wat. Sci. Technol.* **37(9):** 219-226.