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Effects of Electron Donors on Nitrate Removal by Nitrate and Nitrite Reductases

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Abstract Effects of artificial electron donors to deliver reducing power on enzymic denitrification were investigated using nitrate reductase and nitrite reductase obtained from *Ochrobactrum antropi*. The activity of nitrite reductase in the soluble portion was almost the same as that in the precipitated portion of the cell extract. Nitrate removal efficiency was higher with benzyl viologen than with methyl viologen or NADH as an artificial electron donor. The turn-over numbers of nitrate and nitrite reductase were 14.1 and 1.9 μmol of nitrogen reduced/min · mg cell extracts, respectively when benzyl viologen was used as an electron donor.

Keywords: denitrification, *Ochrobactrum antropi*, nitrate reductase, nitrite reductase, electron donor

INTRODUCTION

High nitrate concentration in drinking water present a potential risk to public health. Nitrate is relatively nontoxic to adults because it is quickly excreted by kidney. However, nitrate concentrations greater than 10 mg/L can be fatal to infants under six months of age, because, in infants NO_3^- is reduced to NO_2^-, which combines with hemoglobin in the blood to form methemoglobin, which in turn leads to a condition commonly known as 'blue baby syndrome'. The World Health Organization (WHO) has established a maximum acceptable concentration of 50 mg/L of NO_3^- and Korea has voluntarily restricted this to a maximum of 10 mg/L in drinking water [1].

Nitrate is stable and highly soluble in water, therefore, it is difficult to remove using conventional water treatment technologies, such as lime softening and filtration. More sophisticated technologies, such as ion exchange [2,3], reverse osmosis [4,5], electrodialysis [6] and catalytic denitrification [7] can be used to remove nitrates from drinking water [8]. Among these opinions, biological denitrification using microorganisms is both environment-friendly and inexpensive.

Denitrification is the process of forming nitrogen (N_2) and possibly the intermediate oxides of nitrogen, namely, nitric oxide (NO) and nitrous oxide (N_2O), from nitrate or nitrite [9]. Each step of the denitrification process is catalyzed by an enzyme. Biological reduction of nitrate to nitrite is catalyzed by membrane-bound nitrate reductase [10]. Nitrite reductase is both a membrane-bound and a cytoplasmatic enzyme [11]. It seems reasonable to believe that the enzyme is membrane-associated, but that it is readily solubilized, so that its activity is often found in the soluble fraction. Since oxygen inhibits the synthesis of nitrate reductase, the first enzyme involved in denitrification, the process is performed under strict anaerobic conditions [12]. Under such conditions, nitrate serves as an alternative for oxygen with respect to acting as an electron acceptor, and the nitrate is accordingly reduced to gaseous nitrogen. This process commonly occurs in various bacteria, such as *Pseudomonas, Achromobacter, Alcaligenes, Bacillus, Flavobacterium,* and *Thiobacillus* [1,13]. Various processes, such as fluidized or packed bed reactors have been studied for biological denitrification with immobilized microbial systems using alginate beads or other polymer matrices. The *in situ* denitrification of drinking water sources is a very attractive alternative due to simplicity, and its low investment and operational costs However, in the case of autotrophic denitrification there are many problems associated with the maintenance of a stable operation, achieving homogeneous substrate distribution, and a sufficiently high denitrification rate. The risk of clogging due to biomass accumulation and gas production is very high, and the denitrified water produced might require post treatment and disinfection, which in turn would increase the overall treatment costs. Biological denitrification can be easily disturbed when toxic chemicals are fed. Stable water quality is not guaranteed.

These problems could be resolved by using enzymes. Moreover, since denitrification *in vivo* is a respiratory process, an electron donor is needed as an energy source

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Table 1. Composition of the medium used to culture *Ochrobactrum anthropi* SY509

Component	Concentration (g/L)
Glucose	10.0
Yeast extracts	10.0
$CH_3COONa \cdot 3H_2O$*	5.0
KNO_3	7.0
$Na_2HPO_4 \cdot 12H_2O$	4.47
KH_2PO_4	1.7
$MgSO_4 \cdot 7H_2O$	0.3
NH_4Cl	2.0
$CaCl_2 \cdot 2H_2O$	0.03
$FeSO_4 \cdot 7H_2O$	0.05
$MnCl_2 \cdot 4H_2O$	0.0025
$ZnSO_4 \cdot 7H_2O$	0.0025
$CuSO_4 \cdot 5H_2O$	0.0025
$CrCl_3 \cdot 6H_2O$	0.0003
$NiCl_2 \cdot 6H_2O$	0.00015
$CoCl_2 \cdot 6H_2O$	0.00005
$Na_2MoO_4 \cdot 4H_2O$	0.012148
$Na_2 \cdot EDTA \cdot 2H_2O$	0.27985

* Removed in aerobic culture.

[1]. NAD(H) is known as the major intermediate electron donor in denitrification in living cell [14]. Thus, we reasoned that the addition of an artificial electron donor that provides an electron to nitrate reductase would facilitate the removal of nitrate enzymatically. In other paper, we reported that *Ochrobactrum anthropi* has denitrification activity and the extent of denitrification can be measured using fluorescence technique [15]. In this study, the effects of the electron donors including benzyl viologen, methyl viologen and NADH, on nitrate removal were investigated.

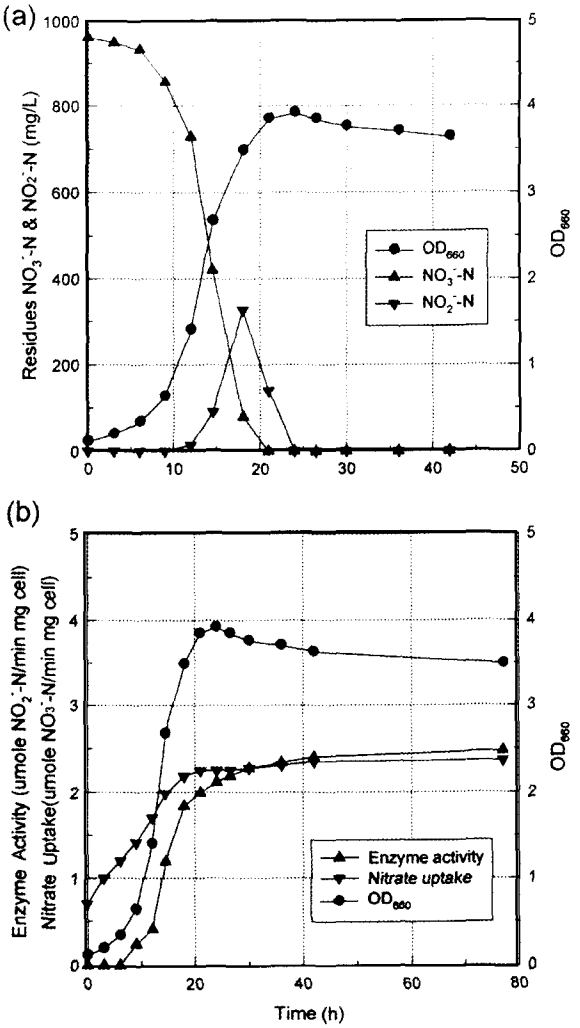

Fig. 1. Time courses of (a) the cell growth, the concentration of nitrate and nitrite, (b) nitrate removal and nitrate reductase activity.

MATERIALS AND METHODS

Medium Composition and Cell Culture

Ochrobactrum anthropi SY509 was screened from sludge obtained from the Kimpo landfill, Korea. The composition of the medium used for growth and maintenance of the bacterium is shown in Table 1.

Ochrobactrum anthropi SY509 was pre-cultured in a 250 mL flask (working volume: 100 mL) at 30°C and 200 rpm for 24 h. After the cells were harvested, they were inoculated (OD_{660} = 0.1) into a 100 mL flask (working volume: 90 mL). The cells were cultured for ca. 24 h at 30°C and 200 rpm, and separated by centrifugation at 40,000 rpm for 20 min. The soluble fraction was discarded and the cells were washed several times with distilled water and washed twice with 80 mM potassium phosphate buffer (pH 7.0). The separated and washed cells were stored at - 70°C until required.

Analysis

The enzyme assay was performed in a potassium phosphate buffer (80 mM, pH 7.0) containing benzyl viologen (1 mM) as an electron donor and dithionite as a reducing agent (10 mM). The addition of excess dithionite facilitated handling without the need for an anaerobic chamber. Cell extracts were added to a final optical density at 660 nm (OD_{660}) of 1.0, and the mixture was stirred at 30°C. The reaction was started by the addition of 10 mM of potassium nitrate. Nitrate reduction was stopped by vigorously vortexing the mixture to oxidize all of the dithionite and benzyl viologen. After removing the cell extracts by centrifugation, the nitrite concentrations were measured [16]. The cell extracts were obtained by sonicating the cells in potassium phosphate buffer for 5 min.

Under the conditions described one unit of nitrate reductase reduces and 1.0 μmole of nitrite-N per min with

Table 2. Location of nitrate reductase and nitrite reductase

	Nitrate reductase (unit)	Nitrite reductase (unit)
Precipitated portion	1.98	1.56
Soluble portion	0.28	1.63
Cell extract solution	2.74	2.29

OD of cell extracts: 1.0, reaction time: 5 min, benzyl viologen: 1 mM, sodium dithionite: 10 mM, initial nitrate/nitrite-N concentration: 140 ppm.

benzyl viologen at pH 7.0, 30°C. The nitrite reductase assay was performed as described for nitrate reductase, and nitrate and nitrite were analyzed by ion chromatography.

RESULTS AND DISCUSSION

The Characteristics of Nitrate Reductase and Nitrite Reductase

Fig. 1(a) shows the time courses of nitrate consumption, nitrate reductase activity, and of cell growth in anaerobic conditions. During the initial cell growth period, the concentration of nitrate slightly decreased. After about 10 h, nitrate was rapidly reduced so nitrite, and after the nitrate had been consumed, nitrite was reduced and removed completely at ca. 24 h. This confirms that the microorganisms selected nitrate as the terminal electron acceptor as an alternative to oxygen in anaerobic conditions. At the early stage under anaerobic conditions, the microorganisms could not use nitrate well. Thus, nitrate reductase should be induced to force the use of nitrate instead of oxygen. As shown in Fig. 1(b), the activity of nitrate reductase increased at *ca.* 10 h. The optical density of the cells at 660 nm and the activity of nitrate reductase were maximal at 20 h and these were maintained at the same level until 80 h though the OD of cells decreased slightly. Therefore, the cells were harvested after 24 h to obtain maximum activity of nitrate and nitrite reductases from the cell.

To determine the localization of the enzymes, enzymic activities were assayed in the soluble portion and the membrane portion, after centrifuging the cell extracts. It has previously been reported that nitrate reductase is a membrane-bound enzyme [11]. However, in the present study, the activity of nitrite reductase in the soluble portion was almost the same as that in the precipitated portion of the cell extracts as shown in Table 2. Our result is consistent with the findings of a previous study [17].

Effects of an Electron Donor on Nitrate and Nitrite Removal

In the case of enzymatic reduction, the role of the electron donor, which donates an electron to reductase, is very important. NADH is the natural electron donor

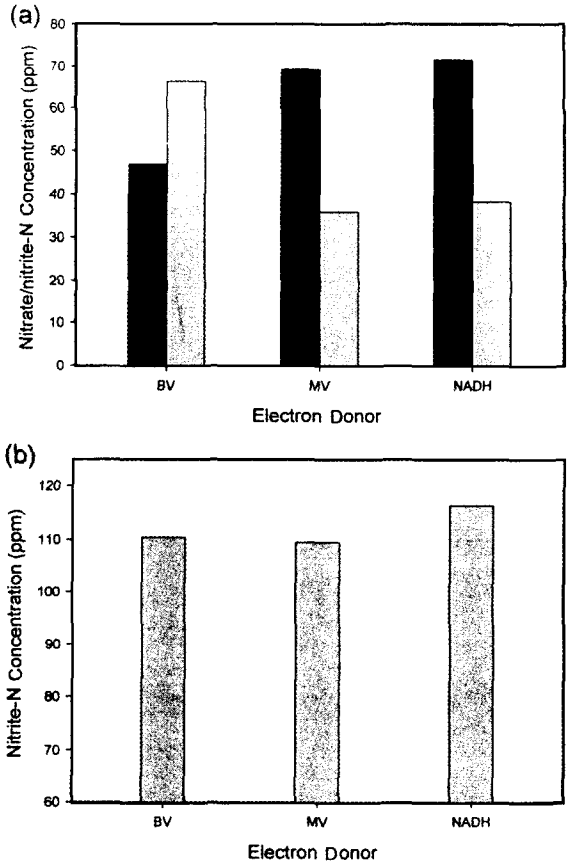

Fig. 2. The efficiency of nitrate and nitrite reduction versus electron donor, (a) nitrate reduction, (b) nitrite reduction. ■: nitrate-N, ▩ : nitrite-N, BV: benzyl viologen, MV: methyl viologen, OD_{660} of cell extracts: 1.0, reaction time: 5 min, electron donor: 1 mM, sodium dithionite: 10 mM, initial nitrate/nitrite-N concentration: 140 ppm.

in *Ochrobactrum anthropi* SY509. Plant nitrate reductases use pyridine nucleotides as the natural electron donor, also can accept electrons from artificial donors such as the vilogen dyes and bromophenol blue [10]. To determine which electron donor is most efficient at nitrate reduction, the reduction efficiencies of nitrate and nitrite was determined using benzyl viologen, methyl viologen, and NADH. As shown in Fig. 2, the efficiency of nitrate reduction using benzyl viologen was much higher than that obtained using methyl viologen or NADH. However, the effects of benzyl viologen and methyl viologen on nitrite reduction were almost the same. Dyes like benzyl viologen, and methyl viologen are electrically active, and therefore, have been used for mediating electron transfer between electrodes and oxidoreductase [18].

The effects of the benzyl viologen concentration on nitrate reduction and nitrite reduction were investigated. As shown in Table 3, the addition of benzyl viologen improved the reduction efficiency, though increasing its concentration from 1 to 2 mM did not af-

Table 3. Residual concentrations of nitrate and nitrite at various initial concentrations of benzyl viologen (BV)

	Nitrate reduction		Nitrite reduction
	NO_3^--N	NO_2^--N	NO_2^--N
0 mM BV	92.3	20.0	125.1
1 mM BV	54.0	51.7	81.3
2 mM BV	55.6	48.1	84.0

OD of cell extracts: 1.0, reaction time: 10 min, initial concentration of nitrate/nitrite-N: 140 ppm.

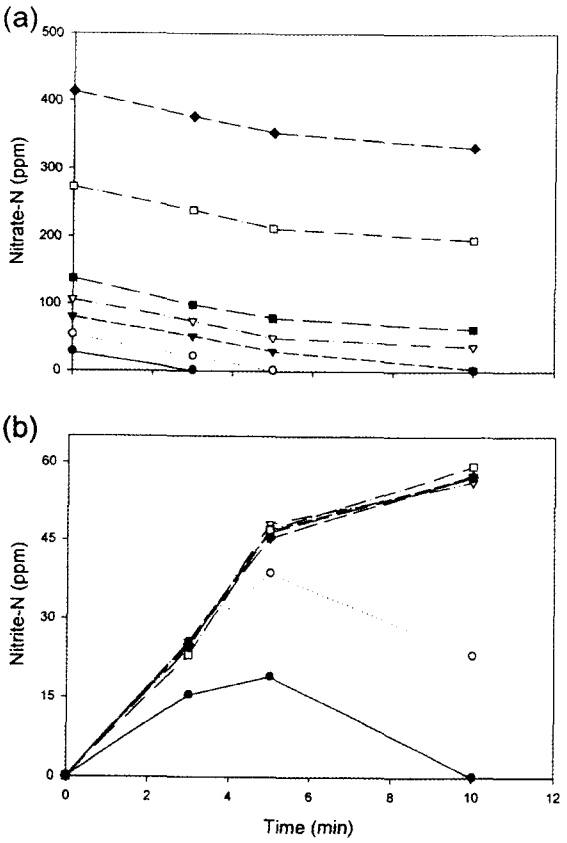

Fig. 3. The time courses of nitrate and nitrite concentrations at the various initial nitrate concentrations. (a) reduced nitrate, (b) produced nitrite. OD_{660} of cell extracts: 1.0, benzyl viologen: 1 mM, sodium dithionite: 10 mM.

fect the reduction efficiency.

Kinetics of Nitrate and Nitrite Reduction

To investigate the enzyme kinetics, the reductions were performed at various initial substrate (nitrate or nitrite) concentrations, from 28 to 420 ppm. Fig. 3 shows the time courses of the nitrate reduction when benzyl viologen was used as an electron donor. When initial nitrate-N concentration was 28 ppm, nitrate and nitrite were sequentially removed in 10 min. In the case of 56 ppm, the nitrate was completely removed in 5 min, and the nitrite remained in 10 min.

Table 4. Kinetic parameters of nitrate and nitrite reductases

	V_m (mM L^{-1} min^{-1})	K_m (mM)	k_2 (μmol/min·mg cell extracts)
Nitrate reductase	3.5	15.0	14.1
Nitrite reductase	0.5	1.2	1.9

The amounts of nitrite produced and nitrate eliminated were almost the same between 84 and 420 ppm, indicating the absence of substrate (nitrate) inhibition in this concentration range. The results on nitrite reduction at the various initial concentrations of nitrite were the same as those obtained for nitrate reduction. Therefore, it is also believed that nitrite had no inhibitory at these concentrations.

The rates of nitrate reduction and nitrite reduction followed Michaelis-Menten equation, without any inhibition of substrate, as shown by a previous study in *Pseudomonas fluorescence* [13]. The rate constants of the nitrate and nitrite reductions are shown in Table 4.

CONCLUSION

The effects of electron donors on denitrification were investigated using crude enzyme obtained from *Ochrobactrum antropi*. Nitrate removal efficiency was higher when using benzyl viologen as an artificial electron donor than methyl viologen or NADH. The reaction kinetics of nitrate and nitrite reduction followed the Michaelis-Menten relationship, and the turn-over numbers of nitrate and nitrite reductase were 14.1 and 1.9 μmol of nitrogen reduced/min · mg cell extracts, respectively when benzyl viologen was used as an electron donor.

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