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Effect of Oxidation-Reduction Potential on Denitrification by *Ochrobactrum anthropi* SY509

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Abstract The effect of oxidation-reduction potential (ORP) level on the denitrification by *Ochrobactrum anthropi* SY509 was investigated under nongrowing condition. The maximum ORP level of nitrate-containing buffer solution was -70~-80 mV under which the denitrification took place. By decreasing the initial ORP level, denitrifying enzyme activity was greatly enhanced, which led to higher denitrification efficiency.

Key words: Denitrification, nitrate reductase, nitrate removal, oxidation-reduction potential (ORP), wastewater treatment

Although nitrate in drinking water itself is relatively nontoxic, it can be microbially reduced to nitrite, which poses severe health threats to humans such as methemoblobinemia, liver damage, and cancer [15]. Also, eutrophication caused by nitrate with phosphorus is a great environmental concern [5, 7]. Nitrate can be removed by various methods such as ion exchange, reverse osmosis, biological denitrification, electrodialysis, and distillation. Among these, biological treatment is considered to be very attractive, in that it is economical and environmentally friendly [2]. In biological wastewater treatment, nitrate is converted into nitrogen gas by microbial denitrification. Biological denitrification is defined as microorganisms that use nitrate as a final electron acceptor instead of oxygen under anaerobic condition, or alternatively defined as the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O), which may then be further reduced to nitrogen and organic substrates needed for denitrification as an electron donor [6, 11]. Since denitrification occurs under oxygen limited condition, the synthesis and the activity of associated enzymes are inhibited in the presence of oxygen [4].

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One of the control variables for the denitrification process is the dissolved oxygen (DO) level [8]. However, since the denitrification process takes place at a low level of DO, it is almost impossible to monitor the DO level change during the denitrification using a commercial DO probe [16]. Therefore, an alternative tool for monitoring DO in the process is required. The measurement of ORP in a bulk liquid can be an alternative. The ORP is the tendency of a given system to forward donating electrons or accepting electrons [3]. Many researchers have recently applied ORP to monitor the extent of denitrification [12, 13]. In this report, the usage of ORP was greatly expanded, that is, a novel method associated with controlling the initial ORP level was suggested to enhance the efficiency of denitrification.

For this study, a microorganism with high denitrification efficiency was isolated from an activated sludge at Kimpo reclaimed land in Korea. The microorganism was identified and named *Ochrobactrum anthropi* SY509 [14]. A 3-l fermentor system (BioFlo II, New Brunswick Scientific Co., Inc.) with 2 l of working volume was used for batch cultures. The culture medium was optimized to increase the biosynthesis of denitrifying enzymes, the composition of which is described in Table 1. The initial optical density of cells at 660 nm was 0.2, corresponding to 0.06 g-dcw/l, and the culture temperature was 30°C. The dissolved oxygen (DO) level was measured using a steam-sterilizable galvanic type DO electrode (Cole-Parmer Instrument Company), which was calibrated from 0 to 100% with nitrogen and air before inoculation. The oxidation-reduction potential (ORP) was monitored with a steam-sterilizable glass type ORP electrode (Phoenix Electrode Co.).

The concentrations of nitrate and nitrite were measured using an ion-chromatography system (Waters 432). The mobile phase was composed of sodium borate/gluconate solution, n-butanol, and acetonitrile. Glucose concentration was measured by a glucose analyzer (YSI Model 2700, Yellow Springs Instrument Co., Inc.).

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

Components	Concentration (g/l)
Glucose	5
Yeast extracts	10
KNO_3	7.3
$Na_2HPO_4 \cdot 12H_2O$	4.5
KH_2PO_4	1.7
$MgSO_4 \cdot 7H_2O$	0.3
NH_4Cl	2
$CaCl_2 \cdot 2H_2O$	0.03
$FeSO_4 \cdot 7H_2O$	0.05
$Na_2EDTA \cdot 2H_2O$	0.28
$Na_2MoO_4 \cdot 4H_2O$	0.012
$CuSO_4 \cdot 7H_2O$	0.002

The activities of nitrate and nitrite reductase were assayed based on the reported methods [1, 9]. Cells were harvested by centrifugation and washed twice with potassium phosphate buffer (80 mM, pH 7.0). The cells were added to the assay mixture composed of potassium phosphate buffer (80 mM, pH 7.0), 1 mM benzyl viologen, and 10 mM sodium dithionite. The final optical density of the mixture at 660 nm was 1.0, and the mixture was stirred at 37°C for 10 min. Subsequently, the reaction was started by adding 20 mM KNO_3 or KNO_2. The reduction was stopped by vortexing the mixture vigorously in order to oxidize all dithionite and benzyl viologen, confirmed by complete decolorization of the mixture. After the cells were removed by centrifugation, the concentrations of nitrate and nitrite in the sample were measured by the ion-chromatography system.

For denitrification using the nongrowing cells, the cells were harvested by centrifugation at the end of the exponential growth phase under anaerobic condition and washed twice with potassium phosphate buffer (80 mM, pH 7.0). Glucose was added as a sole electron donor to the potassium phosphate buffer (80 mM, pH 7.0), and the initial optical density of the cells at 660 nm was 5.0.

Since *Ochrobactrum anthropi* SY509 uses nitrate as an electron acceptor under anaerobic condition, the ORP level which is dependent on oxidants (e.g., nitrate) changes during the denitrification due to the microorganism. To observe the changes of the ORP level, nitrogen gas was purged to ensure that the initial ORP level in the medium was low enough (ca., -250 mV). No air was allowed to enter the system, but gas in the fermentor was allowed to go out of the system during the experiments. When nitrate or nitrite was added to the phosphate buffer (80 mM, pH 7.0), the ORP level rapidly rose to the maximum values of -70~-80 mV, as shown in Fig. 1. The increase of ORP level was due to the increase in the level of oxidants. When nitrate or nitrite was completely reduced, ORP rapidly returned to the initial level. Figure 1 also shows that nitrate or nitrite had an intrinsic upper ORP value, irrespective of these concentrations, however, the duration of the maximum

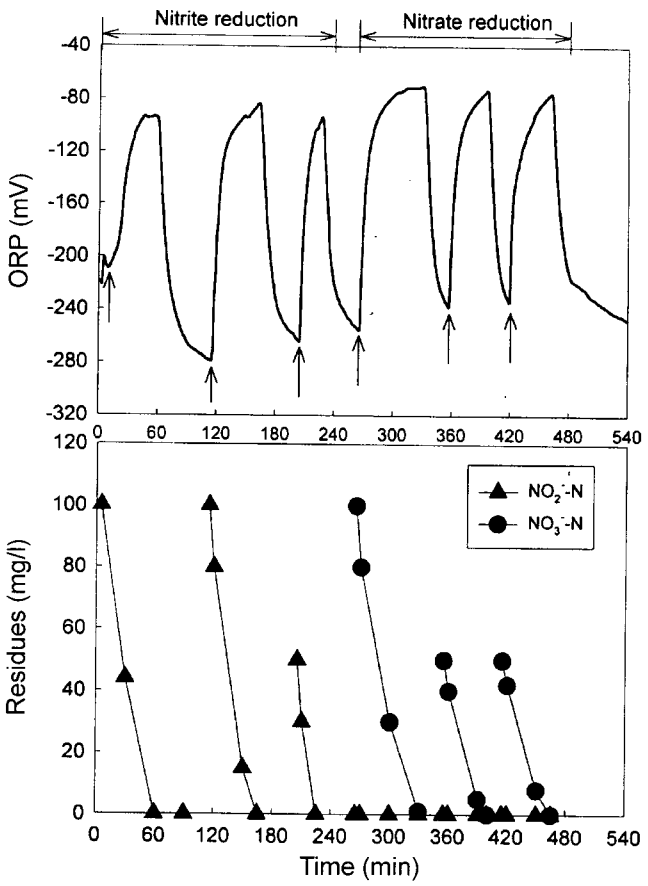

Fig. 1. The ORP profiles during nitrite and nitrate reduction by *Ochrobactrum anthropi* SY509 under nongrowing condition. Arrows represent the addition of nitrite or nitrate.

ORP value was dependent on the concentration of nitrite or nitrate (e.g., 62 min for 100 mg/l, and 37 min for 50 mg/l of nitrate-N).

The ORP changes can be also correlated to the bacterial metabolism. When an oxidant is added to the medium, the ORP level increases and the microbes begin to react for energy production using the oxidant. On the contrary, in the case of an oxidant-deficient condition, the microbes reduce all available components in the medium to get electron acceptors. Therefore, when all oxidants are completely consumed, the medium gains high reducing power, which makes the ORP level decrease sharply.

Since nitrate is sequentially transformed to nitrogen *via* nitrite, nitrate removal may take a longer time than nitrite removal. This hypothesis was confirmed in this study; it took about 65 min to reduce 100 mg/l of nitrate-N completely by the microorganisms, but about 40 min to reduce 100 mg/l of nitrite-N under the same conditions. Although nitrate was reduced *via* nitrite, nitrite was not accumulated in the medium, suggesting that nitrite was reduced immediately after nitrite was formed. The results may imply that the nitrate reduction is the rate-limiting step in the denitrification.

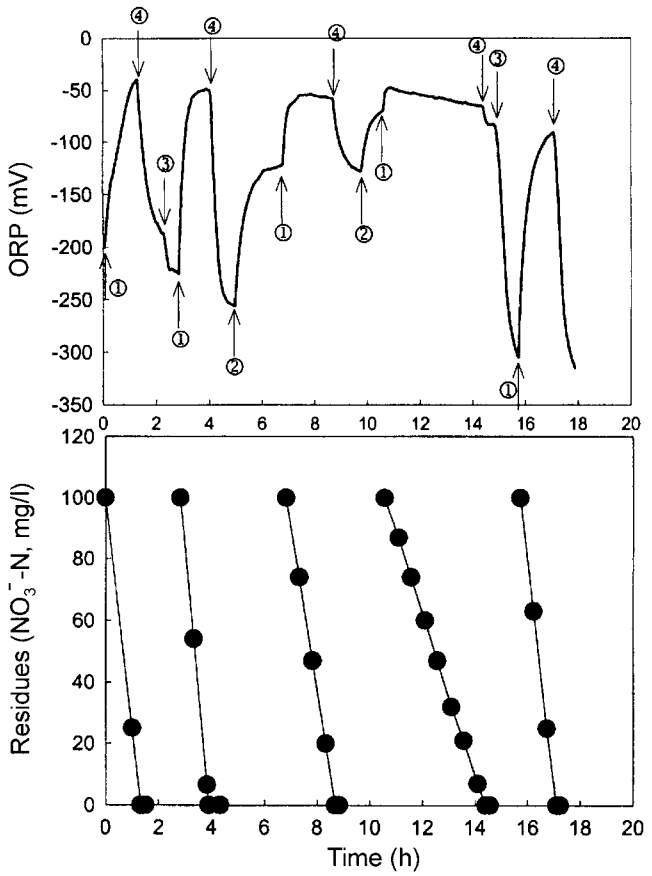

Fig. 2. The ORP profiles during nitrate reduction by *Ochrobactrum anthropi* SY509 under continuous varying conditions.
① nitrate addition, ② air purging, ③ nitrogen purging, ④ end-point of denitrification.

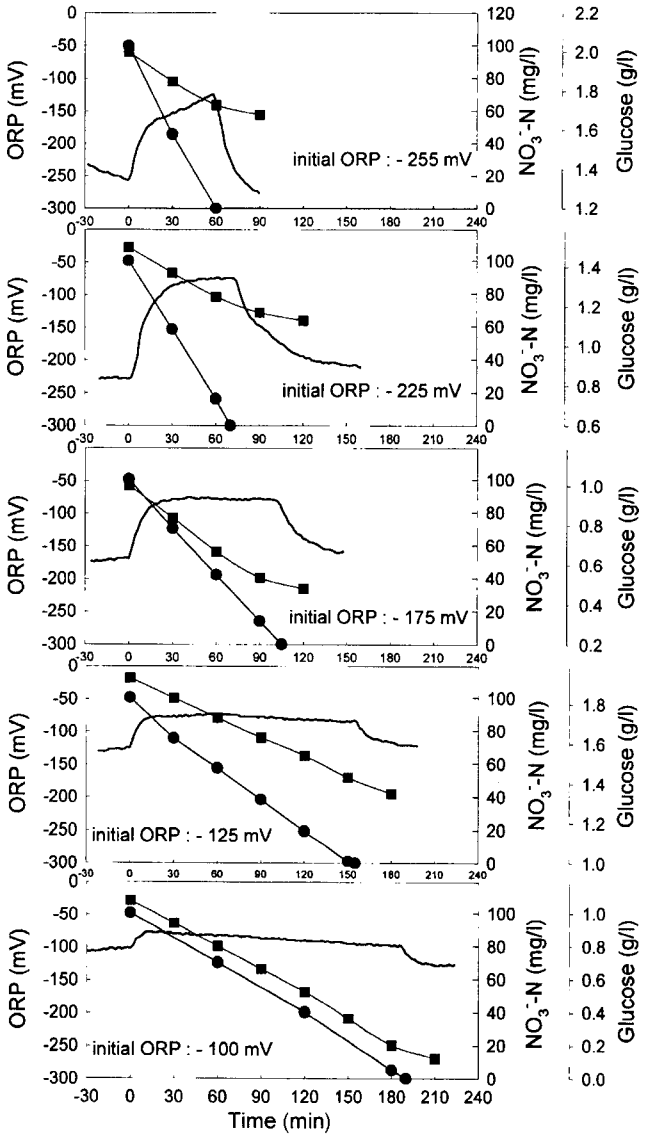

Fig. 3. Time courses of nitrate reduction, glucose consumption, and ORP change for various initial ORP levels.
——, ORP; ●, NO_3^--N; ■, glucose.

The ORP profile during nitrate reduction under various conditions was measured to reveal the relationship between the ORP level and denitrification. In Fig. 2, the sharp decreases under no nitrogen purging (④) indicate the complete reduction of nitrate to nitrogen *via* nitrite. After each reduction of 100 mg/l of nitrate-N, the ORP level increased with the increasing air flow rate (②) or was decreased by purging nitrogen (③) into the medium. It was found that the time taken for nitrate reduction became longer with the increase of initial ORP level. Also, after the initial ORP level was decreased by purging nitrogen, nitrate reduction time became shorter again (last peak in Fig. 2). The results implied that the denitrifying enzymes were not denatured even though they had undergone high ORP conditions.

As mentioned above, the initial ORP level significantly affected the denitrification process. To reveal the effect of initial ORP level on the denitrification, more elaborate batch experiments were conducted. The initial ORP level was controlled in the range between -270 to -70 mV by changing the agitator speed and air flow rate. As shown in Fig. 3, the change of DO level at below 0.05 mg/l was unnoticeable, while the ORP change was prominent throughout the experiments. Irrespective of the culture conditions, the maximum ORP level of nitrate was in the range of -70 to -80 mV in phosphate buffer. The denitrification process did not take place above the value, which suggests that there is an upper limit of ORP level for denitrification. The microorganism is known to produce more maintenance energy through denitrification process under low DO condition. Since low DO corresponded to a low ORP level, the time for nitrate reduction became shorter as the initial ORP level became lower. To find out if the denitrifying enzyme activity was inhibited under a high ORP level, the activity in each batch experiment was also measured. As shown

Table 2. Effect of initial ORP level on denitrification by *Ochrobactrum anthropi* SY509.

ORP (mV)	Nitrate reduction time (min)	Nitrate removal efficiency (nmol/min mg-cell)	Activity of nitrate reductase (unit/mg-cell)
-100	191.5	26.7	1.98
-125	156.2	32.7	2.12
-175	105.1	48.6	2.31
-225	69.9	73.1	2.49
-255	58.3	90.2	2.57

in Table 2, the denitrifying enzyme activity was decreased by a mere 30%, although nitrate removal efficiency at -100 mV of ORP was 3.3 times lower than that at -255 mV. The results showed that the denitrifying enzyme was not denatured even under a high ORP level, and that the denitrification efficiency was mostly affected by initial ORP level. The extrapolation with 2nd-order regression showed that the reduction time and denitrification efficiency could reach up to 45 min and 100 mmol/min mg-cell, respectively, at -280 mV of ORP.

Since nongrowing cells were used, most of the glucose served as an electron donor. The same amount of glucose (257.37 mg/l) is required to reduce 100 mg/l of nitrate, as in the following stoichiometric relationships [10], irrespective of initial ORP conditions,

$$2C_6H_{12}O_6+10NO_3^-\rightarrow 5N_2+12CO_2+6H_2O+12OH^-$$

When glucose was depleted, the denitrification did not continue any more. However, more glucose was consumed under a high ORP level, where glucose served not only as the sole electron donor for denitrification but also for the growth of the cells. Therefore, the longer denitrification time required more maintenance energy, such as glucose. Also, the C/N ratio was reduced as the initial ORP level was decreased. In this report, the C/N ratio was decreased to as low as 4.0 (w/w) at the lowest initial ORP level, which is beneficial for the real nitrate removal process.

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