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Effect of Aeration on Denitrification by *Ochrobactrum anthropi* SY509

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Abstract Aeration was found to affect the biological denitrification by *Ochrobactrum anthropi* SY509. Although cell growth was vigorous under 1 vvm of aeration and an agitation speed of 400 rpm in a 3-L jar fermentor, almost no nitrate was removed. Yet under low agitation speeds (100, 200, and 300 rpm), denitrification occurred when the dissolved oxygen was exhausted shortly after the inoculation of the microorganism. *Ochrobactrum anthropi* SY509 was found to express highly active denitrifying enzymes under anaerobic conditions. The microorganism also synthesized denitrifying enzymes under aerobic conditions (1 vvm and 400 rpm), yet their activity was only 60% of the maximum level under anaerobic conditions and the nitrate removal efficiency was merely 15%. However, although the activities of the denitrifying enzymes were inhibited in the presence of oxygen, they were fully recovered when the conditions were switched to anaerobic conditions.

Keywords: aeration, denitrification, nitrate reductase, nitrate removal, *Ochrobactrum anthropi*, wastewater treatment

INTRODUCTION

Recently, concern for our water environment has continued to increase. Nitrate, ammonia, and phosphorus stimulate eutrophication, resulting in fatal pollution due to high algal growth in the water system, as they are the limiting nutrients for biosynthetic purposes [1, 2]. Currently, a maximum contamination level of 10 mg/L nitrate-N has been set by the U.S. Environmental Protection Agency due to the health effects associated with the ingestion of nitrate (Safe Drinking Water Act of 1974 and its amendments of 1986), for example, infant methemoglobinemia (blue baby syndrome) [3] and the possible formation of nitrosoamines, which are known carcinogens [4].

Nitrate can be removed by various methods, including ion exchange, reverse osmosis, biological denitrification, electrodialysis, and distillation. Among them, biological treatment is more economical and environmentally friendly, and involves the conversion of nitrate into nitrogen gas through denitrification by microorganisms [5]. The enzymes associated with denitrification are nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, plus an electron donor, such as NADH, is needed for these enzymatic reactions [6,7].

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Many microorganisms use nitrate instead of oxygen as the final electron acceptor for the production of energy under anaerobic conditions. Since most microbes involved in this pathway are facultative anaerobes, they use oxygen as the electron acceptor under aerobic conditions to obtain the energy for cell growth and maintenance, whereas under anaerobic conditions they obtain energy through denitrification [5,8]. In general, since the biosynthesis and activities of denitrifying enzymes are inhibited in the presence of oxygen, denitrification only proceeds under oxygen-limited conditions [9]. However, recent denitrification studies including the measurement of dissolved oxygen (DO) have shown denitrifiers vary in their oxygen threshold for denitrification. Some require completely anoxic conditions [10], while others can denitrify even under fully aerated conditions [11].

Accordingly, the current study investigated the effect of aeration on denitrification by *Ochrobactrum anthropi* SY509. By observing the relationship between the DO and the activities of denitrifying enzymes, a novel method for obtaining a high level of cell mass with a high denitrifying enzyme activity is also suggested.

MATERIALS AND METHODS

Microorganism and Culture Conditions

A microorganism with a high denitrification efficiency

Table 1. Comparison with other microorganisms on denitrification

Microorganisms	Initial concentration (NO_3^--N mg/L)	Time required for nitrate reduction (h)	References
Paracoccus denitrificans	250	12	[12]
Pseudomonas fluorescens	320	85	[13]
Pseudomonas CW4	143	15	[14]
This study	250	9	-

was isolated from activated sludge taken from Kimpo reclaimed land in Korea. The microorganism showed higher denitrification rate than other organisms as shown in Table 1. The microorganism was identified through morphological, biochemical and physiological methods and named as *Ochrobactrum anthropi* SY509.

A 3-L jar fermentor (BioFlo II, New Brunswick Scientific Co., Inc.) with 2 L working volume was used for the main batch cultures. The culture medium (pH 7.0) was elaborately formulated to increase the biosynthesis of the denitrifying enzymes and the composition is described in Table 2. The initial optical density of the cell mass was 0.2 at 660 nm, corresponding to 0.06 g-DCW/L, and the culture temperature was 30°C. The pH electrode was calibrated with a standard solution before autoclaving and the dissolved oxygen (DO) level measured using a steam-sterilizable galvanic type DO electrode (Cole-Parmer Instrument Company), calibrated from 0 to 100% by purging nitrogen gas or air into the medium. Fermentation vent-gases were analyzed by an O_2-CO_2 analyzer (TOA Exhaust O_2-CO_2 meter, TOA Electronics).

Analytical Methods

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

Enzyme Assay

The nitrate and nitrite reductase activity was assayed as follows [15,16]. The cells were harvested by centrifugation and washed twice with a potassium phosphate buffer (80 mM, pH 7). The cells were then added to an assay mixture composed of a potassium phosphate buffer (80 mM, pH 7), 1 mM of benzyl viologen, and 10 mM of sodium dithionite. The benzyl viologen along with sodium dithionite generates free electrons and give them to the denitrifying enzymes. The final optical density of the mixture at 660 nm was 1.0 and the mixture was stirred at 37°C for 10 min. Thereafter, the reaction was started with the addition of 20 mM of KNO_3 or KNO_2. The reduction was then stopped by vortexing the mixture vigorously to oxidize all the dithionite and benzyl viologen, that is, these chemicals were able to supply electrons any more, which was confirmed by the decolorization of the mixture. After the cells were removed by centrifugation, the concentrations of nitrate and nitrite in the sample were measured using an ion-chromatography system.

Table 2. Medium composition for culture 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

RESULTS AND DISCUSSION

Microbial denitrification is generally known to take place under anaerobic conditions. However, some microbes can carry out denitrification under low DO conditions using nitrate as a final electron acceptor [11].

The effect of agitation speed, which practically controls the DO level in a fermentor, on cell growth and nitrate removal was investigated. The air flow rate was fixed at 1.0 vvm and the agitation speed (rpm, rotation per minute) was 100, 200, 300 or 400. As shown in Fig. 1, the final cell mass became higher as the agitation speed increased, for example, the final cell mass at 400 rpm was 60% higher than that at 100 rpm, as shown in Fig. 1(a). For nitrate removal, it took about 12 h to completely remove 800 mg/L of nitrate-N at 100, 200, and 300 rpm, yet almost no nitrate was removed at 400 rpm, as shown in Fig. 1(b).

Microbes produce a higher energy from a substance with a high redox potential as the final electron acceptor. Therefore, under aerobic conditions, facultative anaerobes capable of denitrifying prefer oxygen to nitrate, because oxygen has a higher redox potential than nitrate [17]. As shown in Fig. 2, at 300 rpm, the DO decreased to zero shortly after the cell inoculation, then nitrate was used as the final electron acceptor. However, at 400 rpm, the DO level was maintained above 1.8 mg/L and no denitrification took place, indicating that the microorganism used oxygen instead of nitrate as the final electron acceptor for cell growth and energy maintenance. In other to clarify that the microorganism used oxygen not nitrate at 400 rpm, the OUR(oxygen uptake rate) was measured at different agitation speeds. As

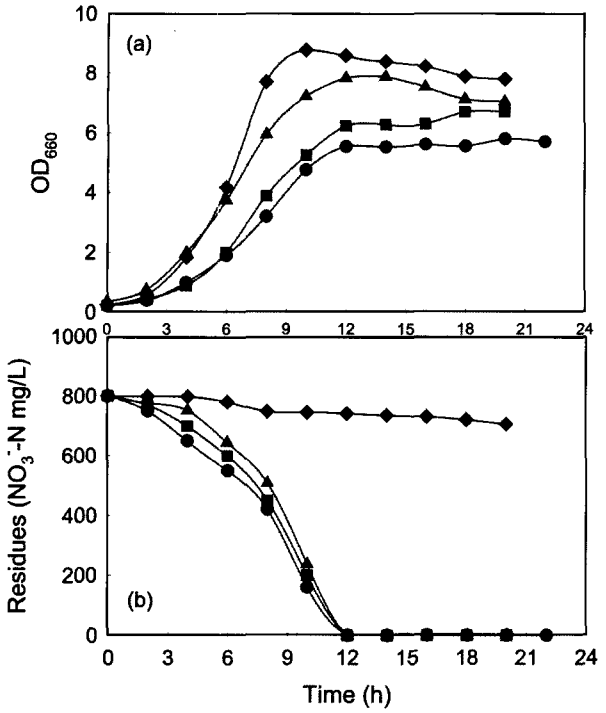

Fig. 1. Time course of cell growth and denitrification of *Ochrobactrum anthropi* SY509 at various agitation speeds (●, 100 rpm; ■, 200 rpm; ▲, 300 rpm; ◆, 400 rpm).

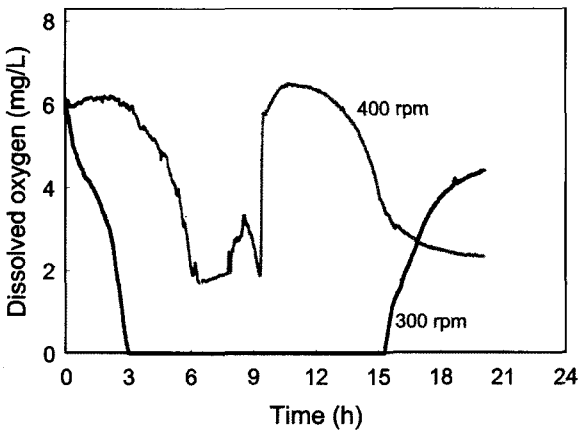

Fig. 2. DO profiles during microbial culture at various agitation speeds.

shown in Fig. 3, the OUR at 400 rpm was much higher than those at other agitation speeds. The experimental results are summarized in Table 3. As mentioned above, since there was an insufficient amount of available oxygen at the low agitation speeds, the microbes used nitrate instead of oxygen as the electron acceptor. Yet the maximum cell mass was relatively low, as the energy efficiency from nitrate reduction was lower than that from oxygen.

When comparing the microbial growth (Fig. 1(a)) with the DO (Fig. 2), the DO level was found to increase during the death phase at 300 and 400 rpm. Hewitt [18] previously reported that the DO increases to some extent after the logarithmic phase of growth due to peroxide formation, yet does not reach the high levels. However, in the case of 100 and 200 rpm, since the stationary phase was maintained for a long time and the microorganism continuously consumed oxygen, the DO level remained at a certain fixed level.

Table 3. Nitrate removal efficiency and maximum cell mass at various agitation speeds

Agitation speed (rpm)	Maximum cell mass (OD_{660})	Removal efficiency (μmol/h mg-cell)
100	5.54	2.87
200	6.24	2.54
300	7.84	2.03
400	8.60	0.14

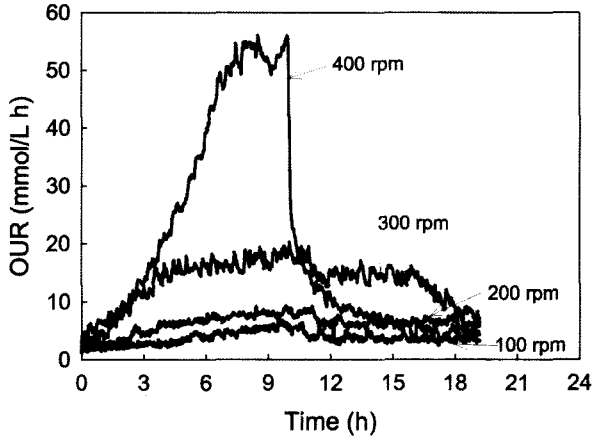

Fig. 3. OUR profiles during microbial culture at various agitation speeds.

As no nitrite was accumulated in the medium during denitrification, it would appear that the nitrite formed from nitrate was immediately reduced by nitrite reductase. The enzyme activities of nitrate reductase and nitrite reductase were assayed at 300 and 400 rpm to investigate the effect of oxygen on the activities of these enzymes. Both enzyme activities were lower at 400 rpm than at 300 rpm, as shown in Fig. 4, suggesting that the activities of the denitrifying enzymes were inhibited by oxygen at a high agitation speed and that nitrite reductase was more vulnerable to oxygen than nitrate reductase. In addition, the activity levels of nitrite reductase and nitrate reductase were found to be the same, as shown in Fig. 4.

Although it has been generally reported that denitrifying enzymes are not induced under aerobic conditions [9], there have been reports of the induction of nitrate reductase by *Thiosphaera pantotropha* [19] and *Paracoccus denitrificans* [20] even under aerobic conditions. In the current study, *Ochrobactrum anthropi* SY509 was found

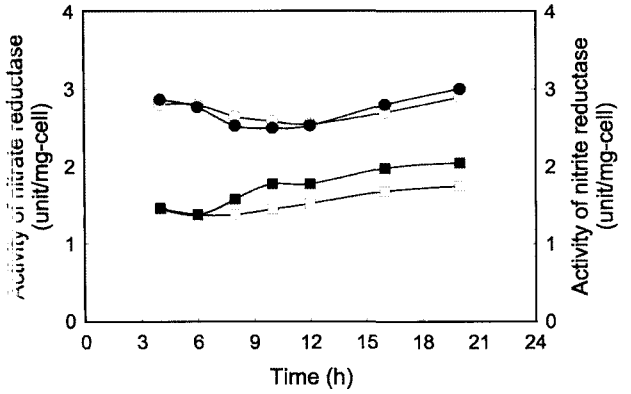

Fig. 4. Effect of agitation speed on activities of nitrate and nitrite reductases (○, nitrite reductase at 300 rpm; ●, nitrate reductase at 300 rpm; □, nitrite reductase at 400 rpm; ■, nitrate reductase at 400 rpm).

to biosynthesize denitrifying enzymes under aerobic conditions, yet their activity was only 60% of the maximum level under anaerobic conditions (Fig. 4) and the denitrification efficiency merely 15% (Table 3).

Consequently, as mentioned above, *Ochrobactrum anthropi* SY509 pre-cultured under anaerobic conditions was able to synthesize denitrifying enzymes under aerobic conditions, although their enzyme activity was much lower than under anaerobic conditions. As shown in Fig. 5, the cell growth under aerobic conditions was vigorous, yet only a very small amount of nitrate was consumed because the microorganism preferred oxygen to nitrate as the final electron acceptor. The minimum DO level was 2 mg/L under the aerobic conditions, however, when the conditions were switched to anaerobic conditions, the DO level sharply decreased. As a result, the cell growth stopped and the concentration of nitrate in the broth rapidly decreased. The enzyme activity during this period was higher than that during the aerobic culture. Only 200 mg/L of nitrate-N was consumed in the first 12 h under aerobic conditions, mostly for cell components, while 750 mg/L of nitrate-N was consumed for denitrification within 2 h under the subsequent anaerobic conditions.

Therefore, once the denitrifying enzymes were synthesized, the enzymes appeared to remain in the microorganism even when it was transferred to aerobic conditions. As such, although the activities of the enzymes were inhibited by oxygen under aerobic conditions, the activities were recovered and denitrification resumed when the aerobic conditions were switched to anaerobic conditions.

To investigate more clearly the relationship between the culture conditions and the biosynthesis of the denitrifying enzymes, the culture conditions were changed as follows. The microorganism was initially cultured under anaerobic conditions (200 rpm, 1st batch), then some of the broth was taken in the early stationary phase and transferred to aerobic conditions (400 rpm, 2nd batch). Some of the 2nd batch was then taken in early stationary phase and transferred to anaerobic conditions (200 rpm, 3rd batch). The characteristics of each batch are summarized in Table 4. Nitrate was rapidly consumed as an electron acceptor in the 1st batch. However, almost no nitrate was consumed in the 2nd batch, even though the microorganism still contained denitrifying enzymes, although their activities were lower than those in the 1st batch. When the microor-

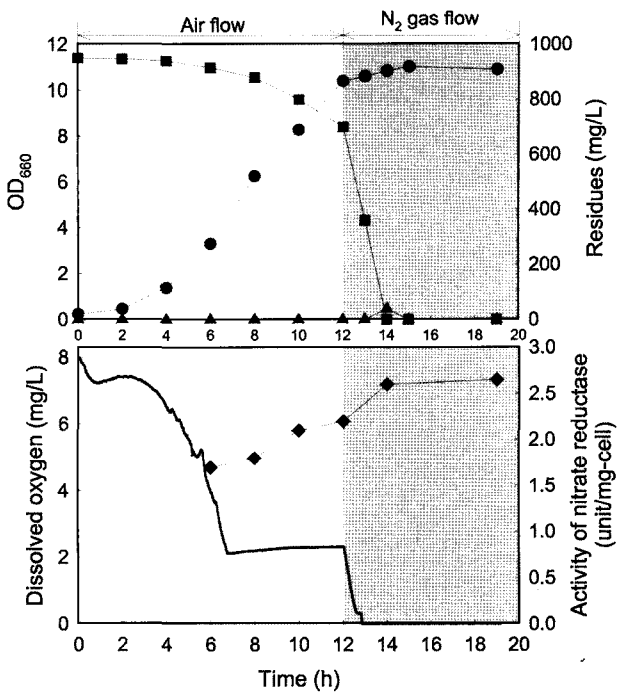

Fig. 5. Effect of changes in culture conditions on cell growth and denitrifying enzyme activity (Aerobic conditions were established with 1 vvm air flow at 400 rpm, while anaerobic conditions were established with 1 vvm of nitrogen gas at 400 rpm; ■, NO_3^--N; ▲, NO_2^--N; ●, OD_{660}; ◆, activity of nitrate reductase).

Table 4. Effect of changes in culture conditions on denitrification

Batch	1st	2nd	3rd
Condition (rpm)	Anaerobic (200)	Aerobic (400)	Anaerobic (200)
Maximum cell mass (OD_{660})	5.8	8.5	6.2
Nitrate removal efficiency (μmol/h mg-cell)	2.53	0.38	2.38
Maximum activity of nitrate reductase (unit/mg-cell)	2.80	1.75	2.86

ganism was transferred to anaerobic conditions again (3rd batch), the values for the maximum cell mass, nitrate removal efficiency, and enzyme activities were almost the same as those for the 1st batch. Consequently, it would appear that no denitrification occurred under aerobic conditions, even though the microorganism had denitrifying enzymes, however, the enzymes were reactivated and denitrification rapidly resumed when the conditions were switched to anaerobic conditions.

In this study, we showed that the denitrification efficiency could be controlled by agitation speed. Also, it should be noted that the denitrifying enzymes induced under anaerobic condition maintained about 60% of its maximum activity even when the culture condition was changed to aerobic state but when the condition was changed to anaerobic state by purging the nitrogen its activities were fully recovered their activities. In the further study, it will be show that a high level of cell mass bearing a high enzyme activity can be obtained by consecutively switching other conditions (aerobic and anaerobic state) and that the microorganism can be applied to the actual wastewater treatment system.

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