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The pH as a Control Parameter for Oxidation-Reduction Potential on the Denitrification by *Ochrobactrum anthropi* SY509

KIM, SUNG HONG[1,3], SEUNG HOON SONG[2], AND YOUNG JE YOO[2,3]*

[1]*Bio-Pharmaceutical Research Team, SK Corporation, Daejeon 305-712, Korea*
[2]*Interdisciplinary Program for Biochemical Engineering and Biotechnology, and* [3]*School of Chemical Engineering, Seoul National University, Seoul 151-742, Korea*

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Abstract The pH as a control parameter for oxidation-reduction potential (ORP) was investigated through the denitrification by *Ochrobactrum anthropi* SY509 under non-growing condition. The optimal pH of nitrate reductase was 7.0, and the minimal ORP level was -250 mV for the denitrification under aerobic condition. In the case of anaerobic condition, the optimal pHs of nitrate and nitrite reductase were shifted to 10.0 and 9.0, respectively, and the minimal ORP levels of nitrate and nitrite reductase were decreased to -370 mV and -340 mV, respectively. In the case of alkaline pH and anaerobic condition, the denitrification efficiency of nitrate was increased up to about 2-fold over that of neutral pH and anaerobic condition. Therefore, the combined control of pH and ORP in the anaerobic condition is shown to be an important parameter in the biological denitrification process.

Key words: Denitrification, nitrate reductase, pH effect, oxidation-reduction potential, wastewater treatment

Extensive pollution of drinking water sources by nitrate is a serious environmental problem in the world [6]. Increased usage of nitrogenous fertilizers is one of the main causes for increasing levels of nitrate in domestic wastewater [11]. For this reason, standards have been set for nitrate in water for human consumption: the World Health Organization (WHO) recommended a Maximum Admissible Concentration and Guide Level for nitrate-N of 11.30 mg/l and 5.65 mg/l, respectively [19]. Besides the improvement of the water environment, the development of stable and efficient denitrification is an important solution and has been studied in order to remove nitrate in drinking water [8, 13]. Efficient treatment processes are required for lowering the concentration of nitrate to an acceptable level because conventional treatments of drinking water do not remove nitrate sufficiently. Many researchers have carried out the removal of nitrate and a number of processes have been developed such as chemical reduction, reverse osmosis, electrodialysis, ion exchange, and biological treatment [9, 18]. Among these, only ion exchange and biological denitrification are feasible on a large-scale process [2]. Furthermore, the biological process is the best choice for the environment, because nitrate is completely eliminated while the ion-exchange process generates a waste of highly concentrated nitrate and regenerating chemicals. The whole cost of biological treatment is usually lower than that of the ion-exchange process [14].

Biological denitrification consists of a series of enzymatic reactions leading to the evolution of gaseous nitrogen [5, 15]. Denitrifying microorganisms reduce nitrate and nitrite to nitrogen gas through the following processes:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

The enzymes associated with the denitrification are nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase [7]. Many microorganisms can use nitrate instead of oxygen as the terminal electron acceptor for the production of energy under anaerobic conditions. Since most microbes involved in this pathway are facultative anaerobes, they can use oxygen as the electron acceptor under aerobic conditions to obtain the energy for cell growth and maintenance, whereas they obtain energy through denitrification under anaerobic conditions [12]. In general, since the biosynthesis and activities of denitrification enzymes are inhibited in the presence of oxygen, denitrification proceeds under oxygen-limited conditions. However, recent studies have shown that denitrifying enzymes vary in their oxygen threshold for denitrification [20]. Some enzymes require completely anoxic conditions, while others can denitrify even under aerobic conditions.

*Corresponding author
Phone: 82-2-880-7411; Fax: 82-2-887-1659;
E-mail: yjyoo@snu.ac.kr

Dissolved oxygen (DO) level is one of the control variables for the denitrification process. However, an alternative tool is required to measure and control it at an extremely low DO level, because it is very difficult to monitor DO level during the denitrification using a commercial DO probe. Measurement of oxidation-reduction potential (ORP) level can be an alternative at low DO level [20]. In particular, many studies reveal that the control of ORP level is an important factor for the denitrification in wastewater treatment by microorganisms [1, 16]. It is also known that pH affects ORP, as shown in the Nernst equation for the relationship between ORP level and pH. The Nernst equation can be simplified to

$$ORP=ORP^0+(RT/nF)\times\ln[H^+]=ORP^0-2.3(RT/nF)\times pH \quad (2)$$

where R is the gas constant, T is the temperature in Kelvin, n is the mole number of electrons transferred between the species, and F is the Faraday constant [4]. In the case of activated sludge denitrification experiments, the denitrification is achieved at high pH values of 7.7, 8.5, and 9.0. As the pH rose, the specific rate of nitrate reduction increased [3].

In this study, the effects of pH and ORP level were investigated through the denitrification by *Ochrobactrum anthropi* SY509 under non-growing conditions.

Microorganism

A microorganism with high denitrification efficiency was isolated from activated sludge taken from Kimpo reclaimed land in Korea. The microorganism showed a higher denitrification rate than other microorganisms, as shown in reference [10]. The microorganism was identified through morphological, biochemical, and physiological methods, and named as *Ochrobactrum anthropi* SY509 [17].

Culture Conditions

The microorganism was cultured at 30°C, pH 7.0, in a 3-l jar fermentor (BioFloII, New Brunswick Scientific, Inc., Edison, NJ, U.S.A.). The culture medium (pH 7.0) was optimized to increase the biosynthesis of the denitrifying enzymes and the composition was described in a previous work [17]. The initial optical density of the cell mass was set at 0.2 at 660 nm, corresponding to 0.06 g-DCW/l. The cells were harvested by centrifugation at the end of the exponential growth phase under anaerobic condition in order to use non-growing cells for denitrification. The cells were washed twice with potassium phosphate buffer (80 mM, pH 7.0). Three-hundred-ml bottles were used with 250-ml volume in denitrification experiments. Glucose was added as the sole carbon source to the potassium phosphate buffer and the initial optical density of the cells at 660 nm was 5.0. The pH electrode was calibrated with a standard solution before autoclaving and the dissolved oxygen (DO) level was measured using a steam-sterilizable galvanic type oxygen electrode (Cole-Parmer Instrument Company, IL, U.S.A.), calibrated from 0 to 100% by purging nitrogen gas or air into the medium. The vent-gas of the fermentor was analyzed by an O_2-CO_2 analyzer (TOA Exhaust O_2-CO_2 meter, TOA Electronics, Kobe, Japan). Nitrogen gas was supplied to the fermentor in order to attain anaerobic condition, whereas air was passed for maintaining the initial non-growing condition.

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, Inc., Yellow Springs, OH, U.S.A.).

Effect of pH and ORP on the Denitrification

Many studies reveal that ORP level is an important factor for the biological denitrification in wastewater treatment. During the denitrification of the water, the reaction of nitrate reductase is the most important, and the rate-determining step in the whole process [3]. Also, the control of DO and pH are important control parameters in the denitrification process. In order to find the optimal pH for the denitrification process under aerobic condition without nitrogen purging, the pH was changed within the range of 6.0 to 10.0. The denitrification efficiencies of nitrate reductase and nitrite reductase under aerobic condition are shown in Table 1. The denitrification efficiencies of nitrate and nitrite reductases were 1.33 mg/min and 2.3 mg/min, respectively, at pH 7.0, but the denitrification efficiency of nitrite reductase decreased rapidly above pH 9.0 or below pH 7.0. The activity of nitrate reductase was less affected by pH change from 6.0 to 9.0, while the activity of nitrite reductase was decreased rapidly at non-neutral conditions. This means the denitrification efficiency of nitrate reductase is less affected by pH change than that of nitrite reductase within pH 6.0 to 9.0.

In the case of anaerobic condition, the denitrification efficiencies of nitrate reductase and nitrite reductase are shown in Table 2. The denitrification efficiency of nitrate

Table 1. The pH effect of nitrate and nitrite reductase on denitrification efficiency under aerobic condition.

pH	Denitrification efficiency (mg/min)	
	Nitrate reduction	Nitrite reduction
6.0	1.20	0.20
7.0	1.33	2.30
8.0	1.30	2.25
9.0	1.30	1.00
10.0	0.80	0.15

Table 2. The pH effect of nitrate and nitrite reductase on denitrification efficiency under anaerobic condition.

pH	Denitrification efficiency (mg/min)	
	Nitrate reduction	Nitrite reduction
6.0	1.50	2.01
7.0	1.89	3.79
8.0	1.95	4.31
9.0	2.10	4.98
9.5	2.15	5.01
10.0	2.17	4.92
11.0	2.00	4.51

reductase was 2.17 mg/min at pH 10.0, and the denitrification efficiencies of nitrate reductase were similar within pH 7.0 to 11.0. Otherwise, the denitrification efficiency of nitrite reductase was 5.0 mg/min at pH 9.0, and the denitrification efficiency of nitrite reductase decreased rapidly below pH 7.0. Therefore, the nitrate reductase was stable and the denitrification efficiency was similar within pH 7.0 to 11.0. But the nitrite reductase was unstable at acidic condition. This indicates that the denitrification efficiency of nitrate reductase is less affected by pH change within neutral pH than that of nitrite reductase in the case of anaerobic condition.

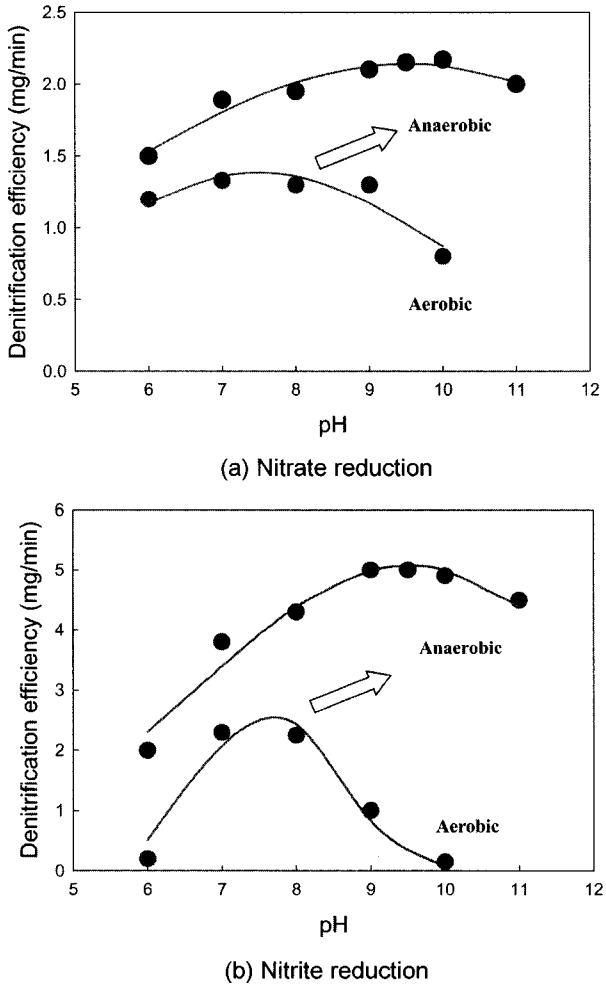

Fig. 1. The effect of pH on the denitrification efficiency during (a) nitrate reduction and (b) nitrite reduction by *Ochrobactrum anthropi* SY509 under aerobic and anaerobic conditions.

This study showed that the denitrification efficiency of nitrate reductase and nitrite reductase can be increased by shifting the pH from 7.0 to 10.0 under anaerobic condition using nitrogen purging as shown in Fig. 1. Otherwise, the optimal pH of nitrate reductase and nitrite reductase was 7.0, and the profile of denitrification efficiency was Bell-shape type in the case of aerobic condition. In addition, the denitrification efficiency of the anaerobic system by nitrogen purging was increased up to 2-fold over that of aerobic condition. The optimal pH is 7.0 under aerobic condition, but the optimal pH was shifted to alkaline in the case of anaerobic condition. Therefore, it is more beneficial to maintain anaerobic and higher pH conditions in order to increase the denitrification efficiency.

It seemed that the denitrification efficiency was dependent upon pH variation and oxygen concentration in the case of nitrate reductase and nitrite reductase. This assumption was supported by a previous study [16]. When oxygen was supplied into the reactor, ORP level was rapidly increased and the denitrification efficacy was decreased accordingly. Otherwise, if nitrogen was supplied into the reactor, the activity of the nitrate reductase was recovered as the ORP level was rapidly decreased. Even though the supply of oxygen and nitrogen changed repeatedly, the activity of the nitrate reductase was not seriously influenced by the change of ORP level, but the change of pH had an effect on the activity of nitrite reductase and the denitrification efficacy. Furthermore, the ORP level depended on pH variations. As the pH rose to 10.0, the minimal ORP level decreased to -380 mV as shown in Fig. 2. The minimal

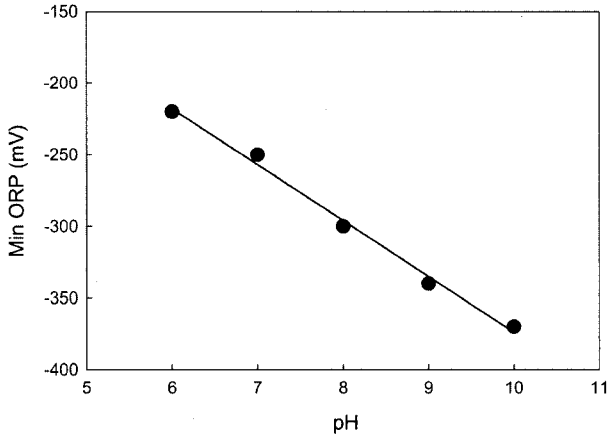

Fig. 2. The relationship of pH and ORP effect on nitrate and nitrite reduction under anaerobic condition.

ORP level was inversely proportional to pH and the experimental equation of the relationship between ORP level and pH was as follows: ORP=16.0-39.0×pH, where R^2=0.993. In order to increase the denitrification efficiency, the ORP level was lowered and that could be controlled by raising pH.

In this study, the pH effects on the denitrification by *Ochrobactrum anthropi* SY509 under aerobic or anaerobic conditions were investigated. As the pH was changed to alkaline condition, the ORP level was decreased and the denitrification efficiency could be increased. Therefore, the control of the pH at high level under anaerobic condition played an important role in increasing the biological denitrification efficiency. The combined control of pH and ORP is shown to be an important factor for increasing denitrification efficiency.

REFERENCES

1. Chen, K. C., C. Y. Chen, J. W. Peng, and J. Y. Houng. 2003. Real-time control of an immobilized-cell reactor for wastewater treatment using ORP. *Water Res.* **36:** 230-238.
2. Dahab, M. F and Y. E. Lee. 1988. Nitrate removal from water supplies using biological denitrification. *J. Water Pollut. Control Fed.* **60:** 1670-1674.
3. Glass, C. and J. Silverstein. 1998. Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Research* **32:** 831-839.
4. Hewitt, L. F. 1950. *Oxidation-Reduction Potentials in Bacteriology and Biochemistry.* 6th Ed, Livingstone, Edinburgh.
5. Hochstein, L. I. and G. A. Tomlinson. 1988. The enzymes associated with denitrification. *Annu. Rev. Microbiol.* **42:** 231-261.
6. Keith, L. H. and W. A. Telliard. 1979. Priority pollutants. 1. A Perspective view. *Environ. Sci. Technol.* **13:** 416-423.
7. Knowles, R. 1982. Denitrification. *Microbiol. Rev.* **46:** 43-70.
8. Lee, J. W., E. S. Choi, K. I. Gil, H. W. Lee, S. H. Lee, S. Y. Lee, and Y. K. Park. 2001. Removal behavior of biological nitrogen and phosphorus, and prediction of microbial community composition with its function, in an anaerobic-anoxic system from weak sewage. *J. Microbiol. Biotechnol.* **11:** 994-1001.
9. Lee, K. Y. and C. G. Lee. 2002. Nitrogen removal from wastewaters by microalgae without consuming organic carbon sources. *J. Microbiol. Biotechnol.* **12:** 979-985.
10. Lukow, T. and H. Diekmann. 1997. Aerobic denitrification by a newly isolated heterotrophic bacterium strain TL1. *Biotechnol. Lett.* **19:** 1157-1159.
11. Monteith, H. D. 1980. Industrial waste carbon source for biological denitrification. *Prog. Water Technol.* **12:** 127-141.
12. Park, E. J., J. K. Seo, J. K. Kim, K. H. Suh, and S. K. Kim. 2000. Denitrification characteristics and microorganism composition of acclimated denitrifier consortium. *J. Microbiol. Biotechnol.* **10:** 410-414.
13. Ra, C. S., K. V. Lo, J. S. Shin, J. S. Oh, and B. J. Hong. 2000. Biological nutrient removal with an internal organic carbon source in piggery wastewater treatment. *Water Res.* **34:** 965-973.
14. Sasaki, K., Y. Yamamoto, S. Ouchi, and Y. Mori. 1996. Development of 2-reactor intermittent aeration activated sludge process for simultaneous removal of nitrogen and phosphorous. *Water Sci. Technol.* **34:** 111-118.
15. Soares, M. I. M. 1985. Biological denitrification of groundwater. *Water Air Soil Pollut.* **123:** 183-193.
16. Song, S. H., S. H. Yeom, S. S. Choi, and Y. J. Yoo. 2003. Effect of oxidation-reduction potential on the denitrification by *Ochrobactrum anthropi* SY509. *J. Microbiol. Biotechnol.* **13:** 473-476.
17. Song, S. H., S. H. Yeom, S. S. Choi, and Y. J. Yoo. 2002. Effect of aeration on denitrification by *Ochrobactrum anthropi* SY509. *Biotechnol. Bioprocess Eng.* **7:** 352-356.
18. Teixeira, P. and R. Oliveira. 2000. Denitrification by *Alcaligenes denitrificans* in a closed rotating biological contactor. *Biotechnol. Lett.* **22:** 1789-1792.
19. World Health Organization. 1984. *Guidelines for Drinking Water Quality.* Geneva, Switzerland.
20. Zhao, H. W., D. S. Mavinic, W. K. Oldham, and F. A. Koch. 1999. Controlling factors for simultaneous nitrification and denitrification in a two-stage intermittent aeration process treating domestic sewage. *Water Res.* **33:** 961-970.