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Bioelectrochemical Denitrification Using Permeabilized *Ochrobactrum anthropi* SY509

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Abstract To remove nitrate from wastewater, a novel bioelectrochemical denitrification system is introduced. In this proposed system, biological reactions are coupled with reactions on the electrode, whereby the electrons are transferred to the bacterial enzymes via a mediator as an electron carrier. The denitrification reaction was achieved with permeabilized *Ochrobactrum anthropi* SY509 containing denitrifying enzymes, such as nitrate reductase, nitrite reductase, and nitrous oxide reductase, and methyl viologen was used as the mediator. The electron transfer from the electrode to the enzymes in the bacterial cells was confirmed using cyclic voltammetry. A high removal efficiency of nitrate was achieved when the bioelectrochemical system was used with the permeabilized cells. Furthermore, when the permeabilized cells were immobilized to a graphite felt electrode using a calcium alginate matrix containing graphite powder, a high removal efficiency was achieved (4.38 nmol/min mg cell) that was comparable to the result when using the free permeabilized cells.

Key words: Bioelectrochemical denitrification, nitrate removal, nitrate reductase, wastewater treatment

Bacterial denitrification is a process of producing nitrogen (N_2) from nitrate or nitrite through an intermediate nitric oxide (NO) or nitrous oxide (N_2O) [6]. Each step of the process is catalyzed by an enzyme system composed of nitrate reductase, nitrite reductase, and nitrous oxide reductase in the periplasmic and/or inner membrane [8, 13]. Dissimilatory nitrate reduction also requires an electron donor, such as NADH, which can only be generated in the presence of carbon sources. Therefore, the aim of this study was to develop a novel method for substituting a continuous supply of the carbon sources during biological denitrification.

Many oxidoreductases containing denitrifying enzymes utilize cofactors (e.g., NAD^+, $NADP^+$, NADH, NADPH) when products are formed [8]. Yet, such cofactors are very costly, so they need to be regenerated [19]. Various methods already exist for cofactor regeneration, including an enzymatic method involving the use of a second enzyme and second substrate [19], photochemical method using a water-soluble photosensitizer or semiconductors to transform light energy into chemical energy [16], and the use of electric energy as a substitute for the electron donor. Among these methods, the electrochemical method is the most promising in terms of cost and simplicity, since it does not require any other substrate addition. Several studies have already been conducted on the use of electric energy as the electron donor in succinate fermentation [10], the transformation of nicotinic acid into 6-hydroxynicotinic acid [18], and the production of 6-bromo-2-tetralol [14]. In these systems, the enzymatic reactions are coupled with electrode reactions, so the electrons from the electrode are transferred to the enzymes via a mediator as the electron carrier [5, 12, 15].

Accordingly, this study presents an electrochemical cofactor regeneration system for denitrification, as summarized in Fig. 1. The proposed system is distinct in several ways from existing denitrification methods. First, the biological reaction is controlled using electricity. Second, the use of a mediator decreases the overpotential required to transfer the electrons to the denitrifying enzymes. In addition, the mediator can be regenerated while the reducing power is supplied from the electrode. Third, the denitrification reaction is attained using permeabilized *Ochrobactrum anthropi* SY509 containing denitrifying enzymes. In the case of permeabilized cells, the permeability barrier of the cell membrane is lowered, thereby avoiding energy

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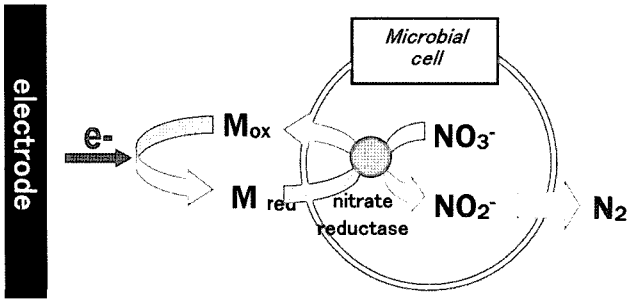

Fig. 1. Schematic diagram of bioelectrochemical denitrification. M_{ox}, oxidized from mediator; M_{red}, reduced from mediator.

consumption for cell growth [1, 2]. Furthermore, since the proposed bioelectrochemical method with an immobilized biocatalyst can be continuously operated, an electrochemical bioreactor containing cofactor-dependent enzymes is feasible.

MATERIALS AND METHODS

Preparation of Permeabilized Cells

Ochrobactrum anthropi SY509 was used as the model microorganism and isolated from activated sludge taken from reclaimed land at Kimpo, Korea. The cells were grown and harvested using the same method as described previously [17]. The washed cell pellet was resuspended in a potassium phosphate buffer (80 mM, pH 7.0), and then 0.5% (v/v) chloroform (Sigma-Aldrich Co.) was added to the cell suspension (100 g DCW/l) to permeabilize the cells. After incubating the suspended cells at 4°C for 15 min [3], the permeabilized cells were recovered by centrifugation at 16,000 ×*g* for 20 min and washed twice with a potassium phosphate buffer before being used in the denitrification reaction.

Cyclic Voltammetry

The electrochemical system was composed of three electrodes: a glassy carbon electrode (BAS) as the working electrode, plus a platinum wire counter electrode (BAS) and Ag/AgCl reference electrode (BAS). The cyclic voltammetry was performed with a scanning potentiostat (362 model, Princeton Applied Research). The current was measured at potentials between 0.5 and −1.25 V, and the scan rate was 10 mV/s. The electrochemical cell was closed with a teflon cover, and N_2 gas bubbling conducted for 10 min to remove the oxygen. The measurements were carried out in a potassium phosphate buffer (80 mM, pH 7.0), containing 1 mM of the mediator (Sigma-Aldrich Co.) and 20 mM potassium nitrate with or without the whole cells.

Denitrification Reaction Using Electricity

The working electrodes used to remove the nitrate were graphite felt electrodes, and since these electrodes have an entangled fiber structure, the actual surface area was much larger than the external area. Before initiating the denitrification reaction, the reactor was purged using nitrogen gas to create an anaerobic environment. After 10 min of nitrogen gas purging, a constant potential (−0.8 V) was imposed on the reaction system as determined by the cyclic voltammetry, representing a more negative value than the reduction potential of the methyl viologen and sufficient reducing power for the denitrification reaction. The nitrate and nitrite were analyzed using an ion-chromatography system (Waters 432) with an IonPac Anion HR column.

Cell Immobilization

After dissolving sodium alginate (Sigma-Aldrich Co.) in distilled water, the cell suspension and graphite powder (Sigma-Aldrich Co.) were added and mixed with the sodium alginate solution. This solution was then well absorbed by the graphite felt electrode, owing to its entangled fiber structure. Thereafter, the electrode was soaked in a calcium chloride solution (0.2 M) for 20 h, and then washed and used in the bioelectrochemical denitrification reaction.

RESULTS AND DISCUSSION

Electron Transfer from Electrode to Bacterial Cell

The mediated electron transfer from the electrode to the enzymes was investigated using cyclic voltammetry, and Fig. 2 shows a cyclic voltammogram of the methyl viologen and *Ochrobactrum anthropi* SY509, where the reduction peak represents the methyl viologen reduced by the transfer of electrons from the electrode, and the oxidation peak represents the methyl viologen oxidized and electrons transferred to the electrode [11]. In Fig. 2, the height of the oxidation peak changed when the whole cells took part in

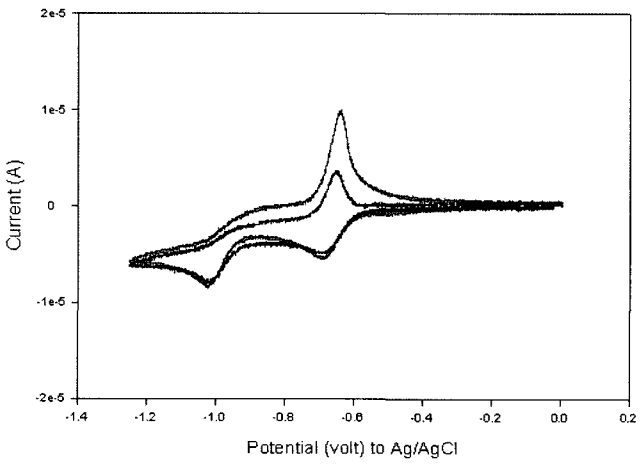

Fig. 2. Cyclic voltammogram of methyl viologen in the presence (dark line) and absence (light line) of *Ochrobactrum anthropi* SY509.

the reaction, indicating that the reduced methyl viologen was enzymatically oxidized in proportion to the difference in the oxidation peaks. Accordingly, electrons were clearly transferred from the electrode to the enzymes via the methyl viologen, suggesting that the nitrate reductase of *Ochrobactrum anthropi* SY509 is capable of catalyzing the reduction of nitrate using electrical energy. In addition, the redox potential of the methyl viologen was also determined using cyclic voltammetry. This potential (versus Ag/AgCl) was located between −0.7 and −0.8 V. Thus, a constant potential (−0.8 V) was applied to the reaction system to supply the electricity required for the bioelectrochemical denitrification.

Evaluation of Permeabilized Cells in the Bioelectrochemical System

To confirm the bioelectrocatalytic activity of the permeabilized cells, the nitrate removal was investigated according to time courses with and without the electrical energy supply. We have reported that permeabilized cells exhibit a higher enzyme activity than whole cells in the denitrification process [1]. Here, the denitrification reaction occurred under the following conditions; the reaction mixture was composed of the mediator (10 mM), potassium nitrate (2 mM), and cell suspension (14.7 g/l), and the reaction initiated after 10 min of nitrogen gas purging.

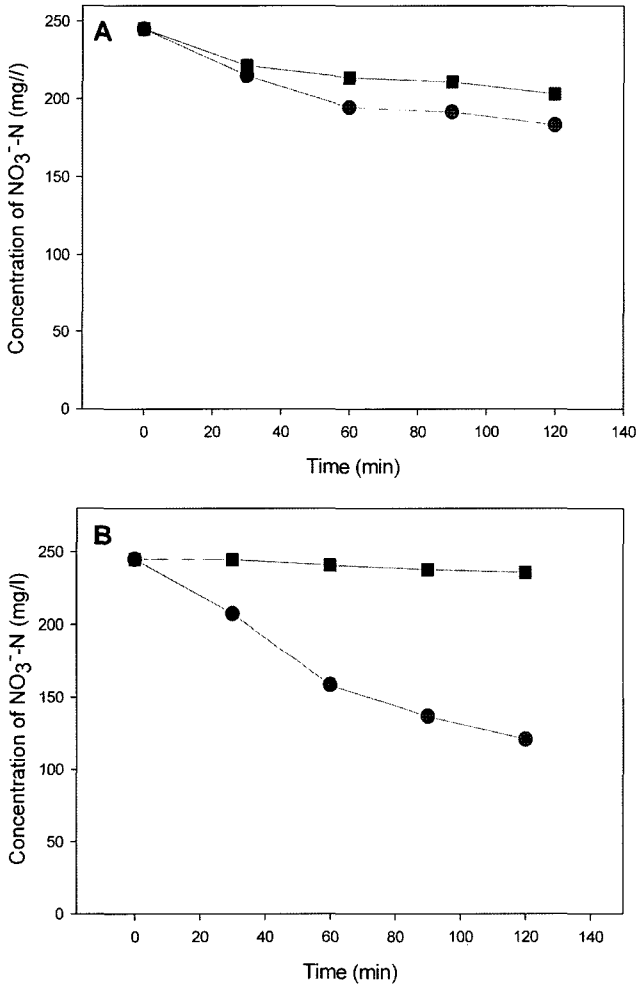

Fig. 3. Amount of residual nitrate with and without electricity when using (**A**) intact cells and (**B**) permeabilized cells as biocatalyst: ■, no electricity; ●, electricity (−0.8 V).

The nitrate removal results when using the intact cells are shown in Fig. 3**A**. In the case of no electricity, only a small amount of nitrate was reduced, which was due to the absence of nutrients, such as glucose, and the lack of NADH, which functions as an electron donor to nitrate reductase. When electrons were released from the electrode, an insufficient amount was transferred to the active site because of the cell wall barrier, and the small number of electrons that did pass through the cell membrane was mainly used up by cell growth. Therefore, only a small amount of nitrate was removed by the electricity.

In contrast, the permeabilized cells produced a better denitrification performance when the electricity was supplied. In the absence of electricity, no nitrate was removed, as the permeabilized cells were in a dead state. However, a dramatic decrease in the residual nitrate was found when supplying the electricity. Approximately 30 mg/l of nitrate was removed during each reaction of 30 min (Fig. 3**B**). This result was due to the lower permeability barrier of the permeabilized cells, which facilitated an easy transfer of the electrons from the electrode to the nitrate reductase and the concomitant reduction of nitrate. Moreover, less energy was consumed during the cell mass synthesis, indicating that most of the electrons from the electrode were used for the enzyme reaction.

Consequently, the combination of these observations confirmed the occurrence of mediated bioelectrocatalysis based on methyl viologen. In addition, the permeabilized cells exhibited a better performance in the bioelectrochemical denitrification than the intact cells.

Immobilization of the Permeabilized Cells to the Electrode

Cell immobilization is required for a continuous process and repeated use of the biocatalyst. Among the various immobilization methods [4, 7, 9], this study selected an entrapment method using a gel matrix.

As such, graphite powder and a calcium alginate matrix were used in the permeabilized cell immobilization, as illustrated in Fig. 4. The use of the calcium alginate matrix to entrap the whole cell and graphite powder resulted in an increased nitrate removal efficiency. Thus, to further improve the nitrate removal, the cell immobilization method was optimized. First, different concentrations of graphite powder were tested, while fixing the sodium

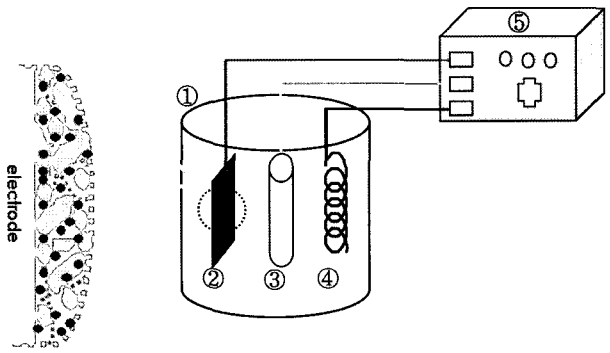

Fig. 4. Schematic diagram of bioelectrochemical reactor: ①, reactor; ②, working electrode; ③, reference electrode; ④, counter electrode; ⑤, potentiostat; ●, graphite powder; ○, permeabilized cell; ▫▫▫▫, calcium alginate matrix.

alginate concentration at 3% (w/v). The amount of removed nitrate was then investigated after 3 h of reaction, and the results are shown in Table 1. The amount of nitrate removed increased in proportion to the amount of graphite powder up to a concentration of 1% (w/v), implying that the electron transfer rate was due to the expanded electrode surface area. However, the nitrate removal decreased with a graphite powder concentration above 2% (w/v), possibly due to inhibited substrate access resulting from the surplus graphite powder. Thus, the optimal graphite powder content was found to be 1% (w/v).

Thereafter, the effect of the sodium alginate concentrations was tested based on the optimal graphite powder content. In this case, 3% (w/v) of sodium alginate demonstrated the highest efficiency (Table 2) after 3 h of the denitrification reaction. Since the concentration of sodium alginate influences the density of the entrapment matrix, high sodium alginate concentrations affected the diffusional restrictions, whereas low concentrations caused leakage of the permeabilized cells from the matrix. Therefore, the optimal concentration of sodium alginate was determined as 3% (w/v).

Removal of Nitrate Using Immobilized Biocatalyst

In addition to optimizing the immobilization method, the nitrate removal was examined according to time courses.

Table 1. Effect of graphite powder content on nitrate removal by immobilized *Ochrobactrum anthropi* SY509.

Graphite powder (%, w/v)	Removed nitrate (mg/l)
0	31.93
0.5	41.20
1	55.66
2	51.19
3	32.21

*Cell suspension: 5 g/l.
*Sodium alginate: 3% (w/v).

Table 2. Effect of sodium alginate concentration on nitrate removal by immobilized *Ochrobactrum anthropi* SY509.

Sodium alginate (%, w/v)	Removed nitrate (mg/l)
1	36.24
2	44.35
3	55.66
4	31.97

*Cell suspension: 5 g/l.
*Graphite powder: 1% (w/v).

In this experiment, the electrodes were constructed with 0.02 g of permeabilized cells per electrode and an immobilization matrix containing 3% (w/v) sodium alginate and 1% (w/v) graphite powder. Except for the working electrode, the same reaction mixture and conditions were used as in the previous experiment so as to confirm the bioelectrocatalytic activity of the permeabilized cells. As shown in Fig. 5, the nitrate removal occurred constantly; and approximately 50 mg/l of nitrate was removed after 2 h.

In general, the cell immobilization results remained stable at the expense of activity. When the cells were immobilized to the electrode using a typical calcium alginate matrix, the efficiency of the nitrate removal was lower compared with that when using the free permeabilized cells. However, when the permeabilized cells were immobilized to the graphite felt electrode using the graphite powder and modified calcium alginate matrix, a high removal efficiency was achieved (4.38 nmol/min mg cell), which was comparable to the efficiency obtained with the free permeabilized cells (4.40 nmol/min mg cell) (Fig. 6). Therefore, the novel immobilization method using graphite powder would appear to counter-balance the activity loss by increasing the electron transfer rate.

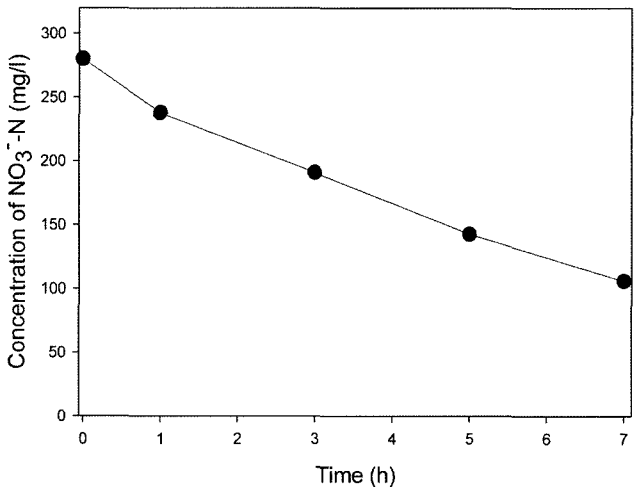

Fig. 5. Removal of nitrate using immobilized biocatalyst.

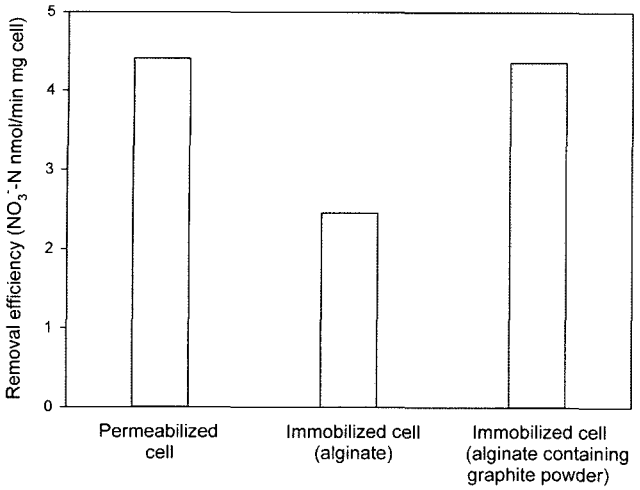

Fig. 6. Efficiency of novel immobilization method.

The nitrate removal using the immobilized biocatalyst was approximately 50% of the removal obtained when using free cells with a supply of glucose. However, the efficiency can be improved when increasing the surface area of the electrode, as verified by a separate experiment where the nitrate removal was increased up to 160% when increasing the surface area of the electrode two-fold under the same conditions (data not shown). Furthermore, if the mediator is integrated into the proposed system, this would facilitate continuous bioelectrochemical denitrification, although this requires further study.

This study performed bioelectrochemical denitrification using permeabilized cell immobilized to the electrode. *Ochrobactrum anthropi* SY509 was permeabilized based on treatment with chloroform. When using a mediator, instead of NADH, as the electron carrier, electrons can be regenerated without the addition of carbon sources while the reducing power is supplied from the electrode. A high removal of nitrate was achieved that is comparable with the efficiency obtained with free permeabilized cells, when using the proposed immobilization method involving a matrix containing graphite powder. This bioelectrochemical system can also be applied to other biotransformation reactions containing cofactor-dependent enzymes.

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