J. Microbiol. Biotechnol. (2009), **19**(8), 836–844
doi: 10.4014/jmb.0810.569
First published online 7 February 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY
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Electrochemical Activation of Nitrate Reduction to Nitrogen by *Ochrobactrum* sp. G3-1 Using a Noncompartmented Electrochemical Bioreactor

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Received: October 13, 2008 / Reveised: November 17, 2008 / Accepted: December 6, 2008

A denitrification bacterium was isolated from riverbed soil and identified as *Ochrobactrum* sp., whose specific enzymes for denitrification metabolism were biochemically assayed or confirmed with specific coding genes. The denitrification activity of strain G3-1 was proportional to glucose/nitrate balance, which was consistent with the theoretical balance (0.5). The modified graphite felt cathode with neutral red, which functions as a solid electron mediator, enhanced the electron transfer from electrode to bacterial cell. The porous carbon anode was coated with a ceramic membrane and cellulose acetate film in order to permit the penetration of water molecules from the catholyte to the outside through anode, which functions as an air anode. A non-compartmented electrochemical bioreactor (NCEB) comprised of a solid electron mediator and an air anode was employed for cultivation of G3-1 cells. The intact G3-1 cells were immobilized in the solid electron mediator, by which denitrification activity was greatly increased at the lower glucose/nitrate balance than the theoretical balance (0.5). Metabolic stability of the intact G3-1 cells immobilized in the solid electron mediator was extended to 20 days, even at a glucose/nitrate balance of 0.1.

Keywords: *Ochrobactrum* sp., solid electron mediator, air anode, denitrification

Bacterial denitrification ($2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2$) is an anaerobic respiration based on the dissimilatory reduction of nitrate to nitrogen in coupling with the metabolic oxidation of organic carbons [5, 12, 26]. Theoretically, 10 electrons (5 NADH) are required to reduce $2NO_3^-$ to N_2 in the bacterial denitrification metabolism, and 10 NADH and 2 $FADH_2$ are regenerated in the glucose oxidation metabolism, from which the glucose/nitrate balance for denitrification metabolism can be calculated [31]. The theoretical glucose/nitrate balance for dissimilatory denitrification calculated without the balance for maintenance and biosynthesis is about 0.5 [27]. Accordingly, the dissimilatory nitrate reduction must be dependent upon the electron donor and acceptor concentration, since the ratio of carbon to nitrogen (C/N ratio) has been adjusted to be more than 3 in the wastewater treatment system [10]. The addition of organic carbons for control of the C/N ratio can be a cause to increase the process cost, and the residual carbon source can be a cause for the secondary pollutant [11]. In order to minimize the C/N ratio, a substitute reducing power capable of regenerating NADH is required [1, 9, 28, 29].

Park and Zeikus [15] found that the electrochemical reduced neutral red could catalyze NADH regeneration without enzyme catalysis. Dissolved neutral red in bacterial cultures or enzyme reactants can transfer electrons from electrodes to biocatalysts; however, the transfer efficiency may be lower than the immobilized one [19, 20].

Generally, a two-compartmented electrochemical bioreactor composed of an anode and a cathode part is separated by an ion-selective nafion membrane (Electrosynthesis, U.S.A.) [23]. The concentration difference between anolyte and catholyte separated by nafion membrane can induce osmotic action. Osmotic action may cause water molecules, gases dissolved in electrolytes, and protons to be transferred through the nafion membrane, by which the culture condition may be disturbed and the bacterial culture volume may be changed.

In this study, we employed a noncompartmented electrochemical bioreactor (NCEB) composed of a solid electron mediator and an air anode to control bacterial metabolism under stable condition. The denitrifying bacterium, *Ochrobactrum* sp. G3-1, was cultivated in the NCEB to enhance conversion of the electrochemical reducing power to the biochemical reducing power, as well as to maintain

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the stable reaction condition without the osmotic action, by which the denitrification efficiency of G3-1 was greatly increased at C/N ratio lower than 0.5.

MATERIALS AND METHODS

Chemicals

All chemicals and coenzymes utilized in this research were purchased from the Korean branch of the Sigma-Aldrich Co. (Yongin, Korea), with the exception of the medium ingredients and the Bradford reagent (Bio-Rad, U.S.A.).

Isolation of Denitrification Bacterium

Soil was sampled at a depth of 50–100 mm from a riverbed at the bottom of a water stream in Junrang-cheon (Seoul, Korea) in April 2008. The soil sample diluted with saline was spread on an agar plate containing a glucose-nitrate medium. The glucose-nitrate medium was comprised of 2 g/l of yeast extract, 2 g/l of KH_2PO_4, 500 mM KNO_3, 146 mM glucose, and 2 ml/l of trace mineral stock solution. The pH of the medium was adjusted to 7.0±0.1 prior to autoclaving. The trace mineral stock solution contains 0.01 g/l of $MnSO_4$, 0.01 g/l of $MgSO_4$, 0.01 g/l of $CaCl_2$, 0.002 g/l of $NiCl_2$, 0.002 g/l of $CoCl_2$, 0.002 g/l of $SeSO_4$, 0.002 g/l of WSO_4, 0.002 g/l of $ZnSO_4$, 0.002 g/l of $Al_2(SO_4)_3$, 0.0001 g/l of $TiCl_3$, 0.002 g/l of $MoSO_4$, and 10 mM EDTA. The agar plates were incubated for 72 h at 30°C under an anoxic argon atmosphere, and some of the single colonies grown on the agar plates were transferred to glucose-nitrate broth. During cultivation in the electrochemical bioreactor, nitrates and nitrites in the bacterial culture were periodically analyzed to select the denitrifying bacterium. A bacterium was selected based on the denitrifying activity in the electrochemical bioreactor.

Identification of Strain G3-1

The best nitrate-consuming bacterium was strain G3-1, which was identified *via* 16S rDNA sequencing. 16S ribosomal DNA was amplified *via* direct PCR using the following universal primers: forward 5'-GAGTTGGATCCTGGCTCAG-3' and reverse 5'-AAGGAGGGGATCCAGCC-3'. The PCR reaction mixture (50 µl) consisted of 2.5 U *Taq* polymerase, 250 µM of each dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 100 ng of template, 50 pM primer, and 1.5 mM $MgCl_2$. Amplification was conducted for 30 cycles of 1 min at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C using a PCR machine (T Gradient model, Biometera, Germany). The PCR products were directly sequenced with an ABI Prism 3700 genetic analyzer upon request to a professional company (Macrogen Inc., Korea). The 16S rDNA sequences were analyzed using the GenBank database, and identification was performed on the basis of 16S rDNA sequence homology.

Assay of NADH-Dependent Nitrate or Nitrite Reduction

Cell-free extract was used as a crude enzyme. The nitrate and nitrite reductases were biochemically assayed base on NADH oxidation in coupling with nitrate and nitrite reduction [13, 7, 22]. Variation of NADH concentration was measured using a spectrophotometer at 340 nm. Nitrate and nitrite concentrations were analyzed using ion chromatography. The protein concentration of crude enzyme was adjusted to 2.0 mg/ml, and 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM NADH and 10 mM nitrate or nitrite was utilized as a substrate. The reaction was started by addition of crude enzyme to the substrate mixture. The reaction mixture was incubated in a water bath at 30°C for 5 min and then the NADH concentration difference between before and after reaction was measured by a spectrophotometer at 340 nm. The specific activity is defined as µM NADH consumed coupled to the reduction of nitrate or nitrite per minute and mg protein. NADH concentration was calculated with the millimolar extinction coefficient of NADH ($\varepsilon340=6.23\ mM^{-1}cm^{-1}$) and the Beer-Lambert equation ($Ab=\varepsilon lc$).

Denitrification Enzyme-Coding Genes

A specific DNA fragment was amplified *via* direct PCR with the chromosomal DNA of strain G3-1 as the template and the primers designed based on the denitrifying enzyme-coding genes, as shown in Table 1. Amplification was conducted *via* the same protocols and procedures utilized for 16S rDNA amplification, with the exception of the annealing temperature. The PCR products were sequenced by a professional company (Macrogen Inc., Korea), and the sequence homology was assessed using the GenBank database.

Noncompartmented Electrochemical Bioreactor

An NCEB (working volume, 300 ml) was newly designed to induce an electrochemical cathodic (reduction) reaction without anolyte and anode compartments, as shown in Fig. 1. A three-layered electrode composed of cellulose acetate film (35 µm thickness; Electron Microscopy Science, U.S.A.), porous ceramic membrane (2 mm thickness), and porous carbon plate (20 mm thickness) was substituted for the anolyte and anode compartment; it functioned here as an air anode. The porous ceramic membrane and porous carbon plate were constructed based on the method developed by Park and Zeikus [16, 17]. A modified graphite felt (100 mm×440 mm, thickness 10 mm; Electrosynthesis, U.S.A.) with neutral red was employed as a cathode [16, 17]. Neutral red can catalyze the

Table 1. Primers for PCR of nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos), which were extracted from the GenBank database.

Gene	Primer	Sequence (5'→3')	Organisms (GenBank Accession No).	Ref.
*nir*K	K15-F	GGC ATG GTA CCT TGG CAC GTA ACC TCG GGC	*Alcaligenes faecalis* (D13155)	[14]
	K16-R	CAT TAG ATC GTC GTT CCA ATC ACC		
*nor*B	cnorB-F	GAR TTY CTN GAR CAR CC'	*Paracoccus denitrificans* (U28078)	[2]
	cnorBR	GAA NCC CCA NAC NCC NGC-3'		
nosZ	nosZ-F	CGY TGT TCM TCG ACA GCC AG	*Paracoccus denitrificans* (398932)	[8]
	nosZ-R	CAT GTG CAG MGC RTG GCA GAA		

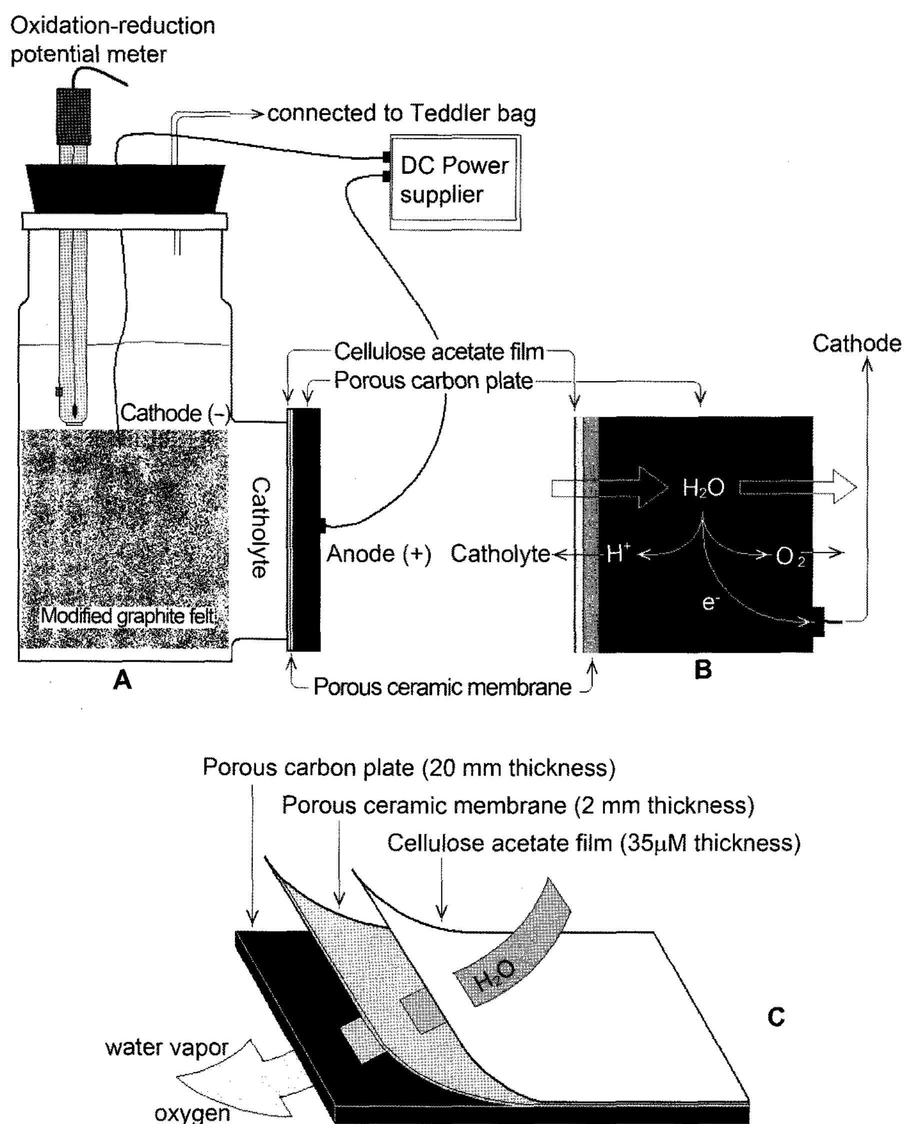

Fig. 1. Schematic structure of the noncompartmented electrochemical bioreactor (NCEB) (**A**), mechanism of anodic reaction (**B**), and three-layered anode (**C**).

Water, transferred from the cathode compartment to the porous carbon anode through the cellulose acetate film, may be electrolyzed to protons, electrons, and oxygen. The cellulose acetate film can selectively allow penetration of water or gas but not solutes.

electrochemical reduction of NAD^+ to NADH without enzyme catalysis [21]. A very small amount of water (4–5 ml/day/300 ml) contained in the reactant (catholyte) can be transferred to the porous carbon plate (anode) through the semipermeable cellulose acetate film, and then electrolyzed into protons, electrons, and oxygen inside the anode, as shown in Fig. 1B. The electricity (2 volt) charged between the anode and cathode electrolyzes water molecules and functions as a driving force to transfer electrons to the cathode, and protons to the catholyte. The modified graphite felt (100 mm×40 mm, thickness 10 mm; Electrosynthesis, U.S.A.) with neutral red was utilized as a cathode [18, 19]. Neutral red was immobilized to the graphite felt by covalent bonds between neutral red and polyvinyl alcohol (mean molecular weight, 80,000; Sigma) in accordance with the procedures. The graphite felt (10×100×40 mm) was soaked into 1% (w/v) polyvinyl alcohol solution (100 ml) at 60°C under vacuum for 3 h, and then dried for 12 h at 100°C. The completely dried graphite felt was then soaked into 100% chloroform containing 10% thionylchloride and 0.1% neutral red to induce a covalent bond between -OH of polyvinyl alcohol and $-NH_2$ of neutral red for 6 h, after which the graphite felt was washed with methanol to remove the unbound neutral red. In this reaction, the water-soluble polyvinyl alcohol was converted to a water-insoluble polyvinyl neutral red, and graphite felt was changed to a hardened one, like plastic sponge. The polyvinyl neutral red–graphite felt complex was very stable, and remained unchanged after more than 10 times of autoclaving; it functioned here as a solid electron mediator.

Preparation of Biocatalysts

The cells incubated in the glucose-nitrate medium for 24 h were harvested by centrifugation at 5,000 ×*g* and 4°C for 30 min. The

harvested cells were washed twice with 25 mM phosphate buffer (pH 7.0). Some of the washed cells were used as the resting cells and some were disrupted by 5-second-pulsed ultrasonic treatment (400W) at 4°C for 20 min. The cell-free extract was prepared from disrupted cells by centrifugation at 4°C and 15,000 ×*g* for 50 min, and utilized as a crude enzyme. The protein concentration was determined using Bradford reagent (BioRad, U.S.A.).

Metabolic Balance of Glucose/Nitrate

The G3-1 strain was cultivated on modified glucose-nitrate medium containing different levels of glucose, yeast extract, and nitrates. The medium volume was adjusted to 100 ml and glucose concentration was adjusted to 1, 2, 3, 4, or 5 mM. The nitrate concentration was adjusted to 10 mM (138.6 mg/l as nitrate-N) for the estimation of the experimental carbon/nitrogen mole balance. The yeast extract was reduced to 0.5 g/l, allowing for the reduction of glucose concentration. No glucose was used in the control test. Five % (v/v) of bacterial culture previously cultivated for 24 h was inoculated into the different culture media containing various concentrations of glucose from 1 to 5 mM. The bacterial culture was incubated at 30°C in a 200 rpm shaking incubator. Nitrate contained in the bacterial culture was analyzed at initial, 12 h, and 24 h of incubation time, but glucose was analyzed only at 24 h of incubation time.

Electrochemical Analysis of Denitrification Metabolism

Cyclic voltammetry was employed to analyze electrochemical redox reactions between the solid electron mediator and electron acceptors (nitrate or nitrite). Cyclic voltammetry was conducted using a voltammetric potentiostat (BAS model CV50W, U.S.A.) linked to a data acquisition system. A modified glassy carbon electrode (5 mm diameter) with polyvinyl alcohol–neutral red complex, a platinum wire, and an Ag/AgCl electrode were utilized as a working electrode, counter-electrode, and reference electrode, respectively. The reaction mixture was composed of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM NAD^+, 5 mM NaCl, and cell extract (12 mg/ml protein) or intact cells (OD_{660}=5.0). Prior to use, the electrodes were cleaned with ultrasonic cleaner, and the dissolved oxygen in the reaction mixture was purged by argon (99.99%) gassing. The scanning rate was 25 mV/s over a range of 0.0 to −1.0 volts. In order to observe the electrochemical reaction for the nitrate or nitrite reduction of intact cells or cell extracts, 10 mM nitrate or nitrite was injected into the reactor with a microsyringe at the second of cycle conversion from oxidation to reduction scanning as the scanning progressed. After that, the variations in upper peak (reduction reaction) and lower peak (oxidation reaction) height were saved in a PC using a data acquisition system.

Denitrification Under Electrochemical Reduction Condition

Strain G3-1 was immobilized in a solid electron mediator (modified graphite felt with neutral red) or native graphite felt electrode by using calcium alginate coagulation [4]. The immobilized cell mass per electrode (40×100×6 mm) was adjusted to 1.2 g as the dry cell mass. An electric potential (reduction potential) of −2.0 V was charged to the cathodes to induce an electrochemical reduction reaction, but not to that for control tests. The glucose and nitrate concentrations contained in 25 mM phosphate buffer (pH 7.0) were adjusted to 20 mM and 200 mM (2,772 mg/l as nitrate-N), respectively, based on the glucose/nitrate balance of 0.1. Nitrate consumption and nitrogen production were quantified to estimate the denitrification metabolism of G3-1.

Metabolic Stability Under Electrochemical Reduction Condition

Strain G3-1 immobilized in the solid electron mediator was utilized as a biocatalyst to assay metabolic stability under conventional and electrochemical reduction conditions. Glucose and nitrate contained in 25 mM phosphate buffer (pH 7.0) were adjusted to 25 and 250 mM (3,465 mg/l as nitrate-N), respectively, to control the glucose/nitrate mole balance at 0.1. The volume of the bioreactor was adjusted to 300 ml, and the hydraulic retention time was adjusted to 3 days.

Analysis

Nitrate and nitrite were analyzed using an ion chromatograph (Dionex DX-500, U.S.A.) equipped with an anion column (IonPac, Dionex AS14A). The column temperature and flow rate were adjusted to 35°C and 1.0 ml/min, respectively. Glucose conversion from electrochemical reducing power to a biochemical reducing power was detected *via* HPLC with an Aminex HPX-87H ion-exchange column (Bio-Rad, CA, U.S.A.) and a refractive index detector. The column and detector temperatures were adjusted to 35°C. Sulfuric acid (0.008N) was utilized as a mobile phase at a flow rate of 0.6 ml/min. The nitrogen was analyzed using a GC (Acme 6000, Young Lin, Korea) equipped with a thermal conductivity detector and a Carboxen 1000 column (Supelco, U.S.A.). Column temperature and carrier gas (He) flow rates were adjusted to 30 ml and 35°C, respectively. The concentration was calculated based on the peak area obtained with standard materials or gas.

RESULTS

Identification of Isolate

The 16S rDNA sequence of strain G3-1 was 99% identified with *Ochrobactrum* sp. and registered in the GenBank database system, from which the accession number EU569294 was obtained.

Biochemical Assay of Nitrate and Nitrite Reductases

The biochemical reduction of nitrate and nitrite by the crude enzymes of strain G3-1 was analyzed base on NADH oxidation rates. As shown in Table 2, 4.37 mM and 2.48 mM NADH were oxidized in coupling with nitrate and nitrite reduction, respectively, per mg protein and per minute, 0.68 mM nitrite was generated by nitrate reductase, and

Table 2. Specific activity of crude enzyme extracted from strain G3-1 measured based on the NADH oxidation in coupling with nitrate or nitrite reduction.

Substrates	Specific activity (mM/min/mg protein)	Reaction results
Nitrate	4.37	0.68 mM nitrite production/min
Nitrite	2.48	1.52 mM nitrite consumption/min

1.52 mM nitrite was consumed by nitrite reductase in coupling with NADH oxidation for 30 min.

Denitrification Enzyme-Coding Genes

The nitrifying enzyme-coding genes of strain G3-1 were homologous with a variety of bacterial strains at a level of more than 96%, as shown in Table 3. The DNA sequences were registered in the GenBank database system, from which accession numbers for the nitrite reductase-coding gene (EU983104), nitric oxide reductase-coding gene (FJ422142), and nitrous oxide reductase-coding gene (FJ422143) were obtained.

Metabolic Balance of Glucose/Nitrate

Theoretically, 5 moles of NADH are oxidized in coupling with the reduction of 2 moles of nitrate (NO_3^-) to 1 mole of nitrogen (N_2) as follows: $2HNO_3+10H$ (5NADH)$\rightarrow 6H_2O+N_2$. Accordingly, the glucose/nitrate balance may be approximately 0.5 based on the denitrification metabolism. As shown in Fig. 2, the experimental balance based on nitrate and glucose consumption was consistent with the theoretical balance. After 24 h of incubation, glucose was completely consumed except in the control test, and nitrate was consumed in proportion to glucose contained in medium. Accordingly, the minimal glucose/nitrate balance for bacterial denitrification may be at least 0.5 under conventional condition.

Electrochemical Analysis of Denitrification Metabolism

The metabolic or biochemical redox reaction of strain G3-1 in coupling with nitrate or nitrite reduction was analyzed using cyclic voltammetry (CV). As shown in Fig. 3, the upper peak height of CV for both intact cells and cell-free extract (crude enzyme) was increased as the result of the addition of nitrate. This phenomenon can occur when electrons are unidirectionally transferred from the electrode to nitrate in coupling with the redox reaction of neutral red. Nitrite addition did not alter the upper peak height of the CV for the intact cells, but altered that for the cell-free extract. This result indicates that nitrite cannot be an electron acceptor for intact cells, but may be a substrate for the crude enzyme.

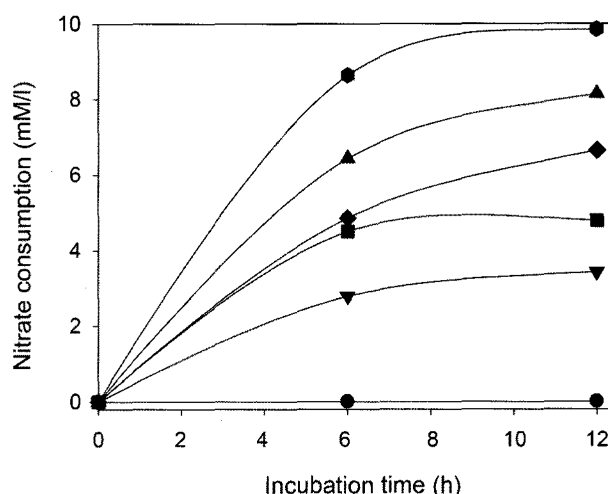

Fig. 2. Effect of glucose concentration on nitrate consumption by growing cells of strain G3-1. Initial concentrations of glucose were adjusted to 0 (●), 1 (▼), 2 (■), 3 (◆), 4 (▲), and 5 mM (⬢), and the initial concentration of nitrate was commonly adjusted to 10 mM under all reaction conditions.

Denitrification Under Electrochemical Reduction Condition

In the previous test (Fig. 2), the metabolic nitrate consumption was limited in proportion to the glucose/nitrate balance, and the solid electron mediator induced the redox reaction between the electrode and nitrate under the condition without glucose (Fig. 3). The denitrification by the immobilized cells in the native graphite felt electrode was proportional to the glucose/nitrate balance under both conventional and electrochemical reduction conditions (Fig. 4, circular and reverse triangular symbols). Meanwhile, the denitrification by the immobilized cells in the solid electron mediator was a little increased under conventional condition (Fig. 4, square symbol), which is a very peculiar phenomenon, but greatly activated under electrochemical reduction condition (Fig. 4,

Table 3. DNA sequence homology of PCR product obtained with specific primers for the nitrite reductase-coding gene, nitric oxide reductase-coding gene, and nitrous oxide reductase-coding gene.

Gene	Primer Sources	Homologous strain and (homology)	Accession No.
Nitrite reductase (*nir*K)	*Alcaligenes faecalis* (D13155)	*Alcaligenes faecalis nir* (99%) Uncultured bacterium *nir* (99%) *Paracoccus* sp. *nir* (99%)	D131551 AB162314.1 AM230830.1
Nitric oxide reductase (*nor*B)	*Paracoccus denitrificans* (U28078)	*Ochrobactrum* sp. R-24638 *nor* (99%) *Ochrobactrum* sp. R-28410 *nor* (99%) *Alcaligenes faecalis nor* (98%)	AM284369.1 AM284385.1 AB031072.1
Nitrous oxide reductase (*nos*Z)	*Paracoccus denitrificans* (398932)	*Ochrobactrum anthropi* ATCC49188 *nos* (97%) *Ochrobactrum anthropi nos* (98%) Uncultured temperate forest soil *nos* (96%)	CP00759.1 AY072229.1 AY072236.1

Chromosomal DNA of *Ochrobactrum* sp. strain G3-1 was employed as the template.

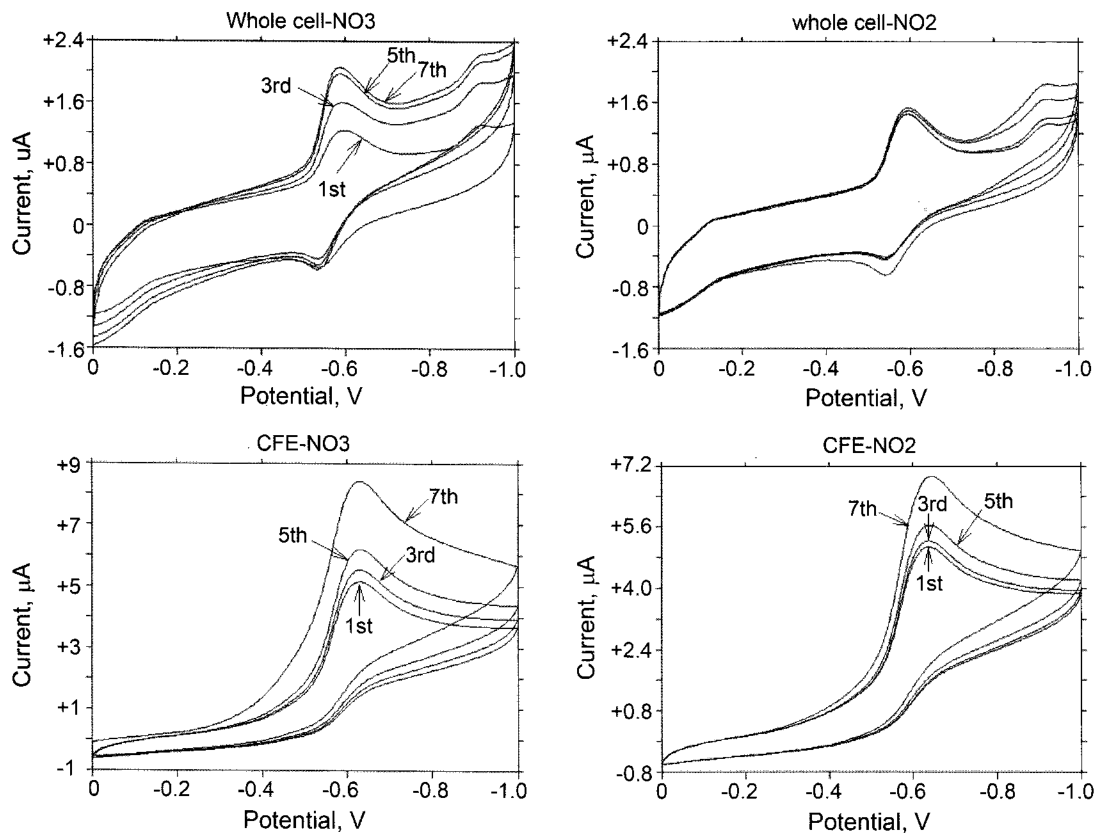

Fig. 3. Cyclic voltammograms obtained from whole cells (intact cell) and cell-free extract (CFE) of G3-1 modified with neutral red during reaction with nitrate and nitrite.

At the start point of the 3rd cycle, 1.0 mM nitrate or nitrite was added to the reactant to induce the metabolic reaction of intact cells or CFE. Upper graphs and lower graphs indicate odd-numbered and even-numbered cycles, respectively.

diamond symbol). The nitrogen production and nitrate consumption according to the incubation time showed a very similar tendency, but the quantitative balance between nitrogen production and nitrate consumption was not consistent with the theoretical balance. Theoretically, 170 mM nitrate can be metabolically converted to 85 mM nitrogen, which corresponds to 85 ml/l of nitrogen under standard condition.

Metabolic Stability Under Electrochemical Reduction Condition

In the previous test (Fig. 4), the nitrate consumption and nitrogen production were greatly activated by the immobilized G3-1 cells in the solid electron mediator under electrochemical reduction condition. On the basis of these results, the metabolic stability for denitrification was estimated with the continuous culture system at the glucose/nitrate balance of 0.1. As shown in Fig. 5, the nitrate and glucose consumption by the immobilized strain G3-1 cells in the solid electron mediator reached a maximal value and the metabolic stability for denitrification was maintained for 15 days under electrochemical reduction condition. Meanwhile, the denitrification activity was approximately 60% of the maximum value, and the metabolic stability for denitrification was not observed under the conventional condition. These results constitute a clue that the electrochemical reducing power can be converted to the biochemical reducing power for denitrification metabolism. In particular, the same tendencies of nitrate and glucose consumption under both the conventional and electrochemical reduction conditions demonstrate that the biochemical energy for the biosynthetic metabolism of the G3-1 can be regenerated by the electrochemical reducing power.

DISCUSSION

Commonly, denitrification bacteria reduce 2 NO_3^- to 1 N_2 *via* anaerobic respiratory metabolism [25]. Nitrate and nitrite reductases could be biochemically assayed with the redox reaction of nitrate/NADH and nitrite/NADH [13]. The presence of nitric oxide (NO) and nitrous oxide (N_2O) reductases was estimated with the enzyme-coding genes based on the DNA sequence homology, because it is very difficult to conduct biochemical assays [3]. The NADH/ nitrate mole balance is the critical factor in the denitrification

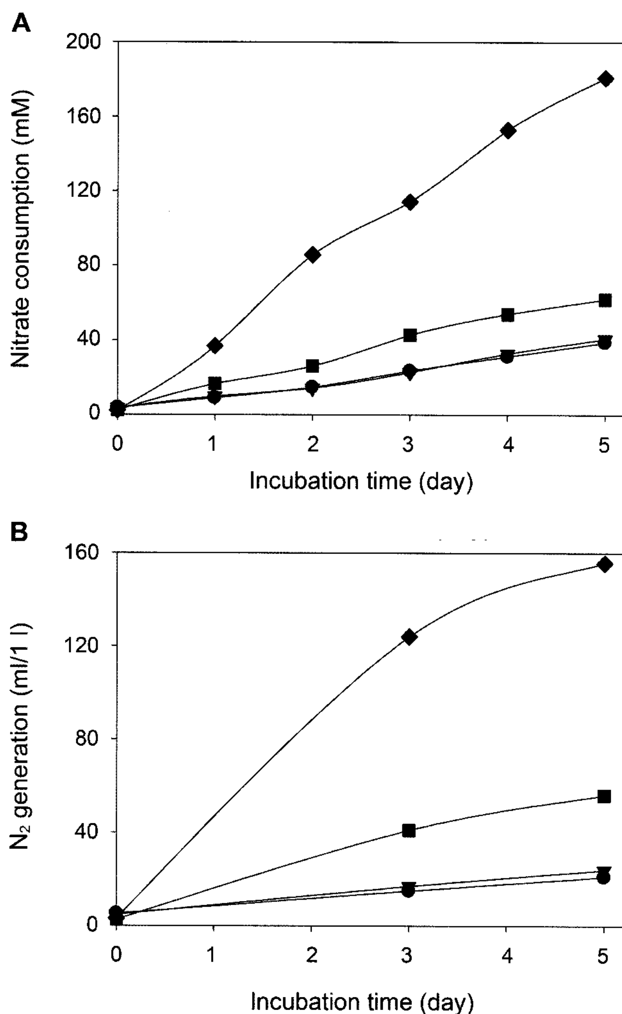

Fig. 4. Nitrate consumption (**A**) and nitrogen generation (**B**) by the immobilized cells in the native graphite felt electrode under conventional condition (●) and electrochemical reduction condition (▼), and that in the solid electron mediator under conventional condition (■) and under electrochemical reduction condition (◆).

Initial concentration of glucose and nitrate was adjusted to 20 and 200 mM, respectively.

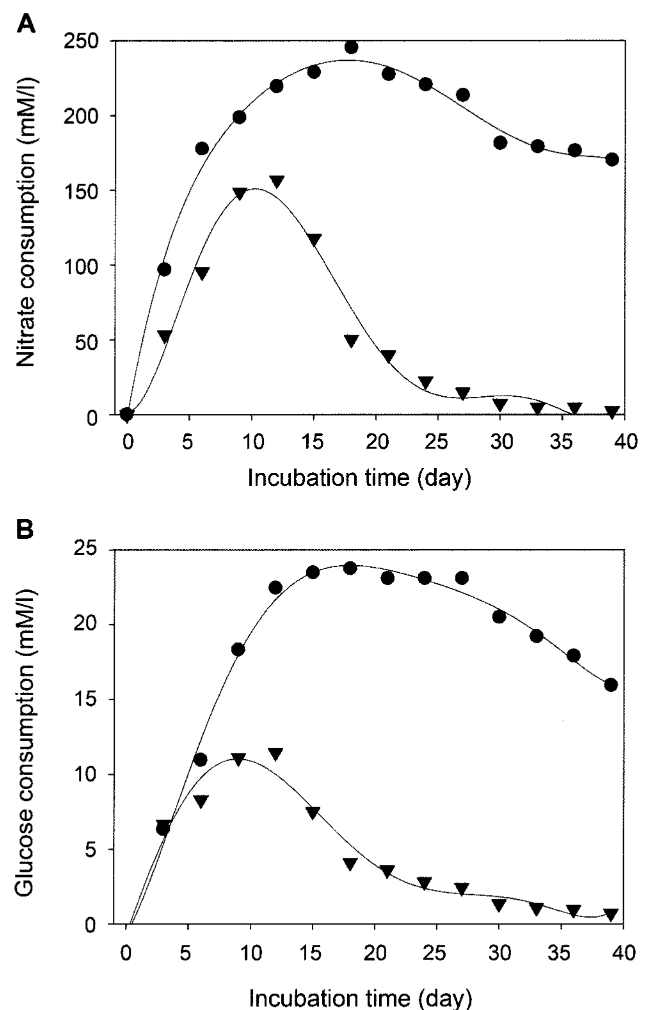

Fig. 5. Nitrate consumption (**A**) in coupling with glucose consumption (**B**) by the resting cells of strain G3-1 immobilized in the solid electron mediator electrochemically reduced (●) or not (▼).

The medium containing 250 mM of nitrate and 25 mM glucose flowed continuously into the bioreactor at the speed of 100 ml per day.

metabolism, for which the glucose/nitrate mole balance must be at least 0.5 [6, 26]. Practically, a carbon source/ nitrate balance must be higher than the theoretical one in the wastewater treatment reactor, because various species of bacterial cells have to compete for substrates [12]. A suitable substituted reducing power for carbon sources is required for lowering of carbon source/nitrate balance. Hydrogen may be the substitute reducing power for bacterial denitrification but is difficult to apply to a wastewater treatment reactor because of both difficulty of dissolution into water and explosive [24]. Electric energy is also possible as a substitute reducing power, but depends absolutely upon the electron mediator being capable of catalyzing the conversion of electric energy to biochemical reducing power, as well as a special bioreactor for the maintenance of stable culture conditions [11, 15, 19, 20, 23]. The cyclic voltammograms (Fig. 3) demonstrated that the solid electron mediator functions as an electron donor in coupling with the metabolic nitrate reduction of the G3-1 cells. The cathodic (reduction) reaction may be disturbed by the osmotic action generated by the concentration difference between the anolyte and catholyte in the two-compartmented electrochemical bioreactor [23]. However, the osmotic action cannot be generated in the NCEB (Fig. 1) because the air anode functions as the anolyte and a counter part of the catholyte is air. Accordingly, the G3-1 cells immobilized in the solid electron mediator may be the catalyst for activation of the metabolic reduction of nitrate to nitrogen lower C/N balance, and the NCEB is a solution for maintenance of stable cathodic reaction [4, 20].

The biochemical denitrification efficiency of the G3-1 cells was substantially increased in the NCEB system, even at the glucose/nitrate balance of 0.1 (Fig. 4). The G3-1 strain consumed approximately 170 mM nitrate and generated 60 ml/l nitrogen but consumed 20 mM glucose, which corresponds to a glucose/nitrate mole balance. The immobilized cells in the solid electron mediator may be metabolically activated by the biochemical reducing power regenerated in coupling with the electrochemical reduction reaction [29], by which ATP regeneration may be activated in coupling with nitrate respiration, as shown in the proposed mechanism of Fig. 6. Theoretically, maintenance of $NADH/NAD^+$ and ATP/ADP balance is the critical factor for maintaining the physiological function and structure of bacterial cells [30].

Kim *et al.* [11] reported that *Ochrobactrum anthropi* SY509 immobilized in a modified electrode with neutral red could remove nitrate under electrochemical reduction, but carbon source-absent conditions. This is a practical example that the electrochemical reducing power can be converted to biochemical reducing power for denitrification. However, the *Ochrobactrum anthropi* SY509 used for denitrification experiment was not an intact cell, which functioned like a enzyme and is difficult to employ in the wastewater treatment reactor. Accordingly, intact or growing cells have to be employed in the wastewater treatment reactor, for which minimal carbon sources have to be required to make up the building blocks for biosynthesis.

In our experimental results, we are unable to state confidently that the optimal glucose/nitrate balance for maximal denitrification is 0.1 under electrochemical reduction conditions. However, we can presume that the minimal balance for the effective denitrification of strain G3-1 may be 0.1, based on the data that the denitrification efficiency was close to maximum (Fig. 4) and nitrogen production was nearly proportional to the nitrate consumption (Fig. 5) at a glucose/nitrate mole balance of 0.1.

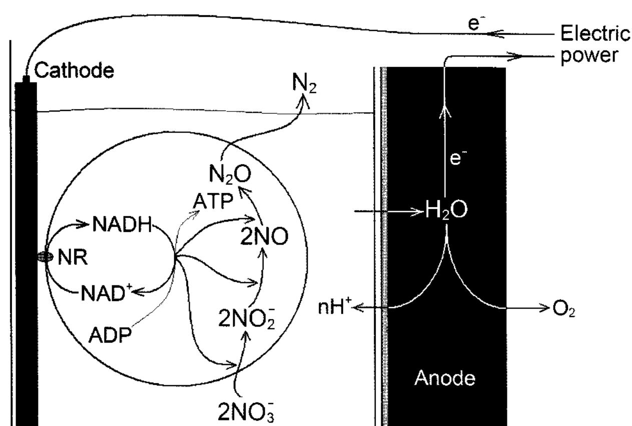

Fig. 6. Proposed scheme for electrochemical denitrification in NCEB composed of solid electron mediator (cathode) and air anode (anode).

ATP may be regenerated by the respiratory metabolism in coupling with NADH oxidation [23].

In future experiments, we will examine the peculiar phenomenon that the denitrification efficiency was slightly increased by the immobilized G3-1 cells in the solid electron mediator under the conventional condition without electrochemical reducing power, which showed the same tendency in the five times-repeated tests.

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