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Influence of Electric Potential on Structure and Function of Biofilm in Wastewater Treatment Reactor: Bacterial Oxidation of Organic Carbons Coupled to Bacterial Denitrification

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Abstract Carbon electrode was applied to a wastewater treatment system as biofilm media. The spatial distribution of heterotrophic bacteria in aerobic wastewater biofilm grown on carbon electrode was investigated by scanning electron microscopy, atomic force microscopy, and biomass measurement. Five volts of electric oxidation and reduction potential were charged to the carbon anode and cathode of the bioelectrochemical system, respectively, but were not charged to electrodes of a conventional system. To correlate the biofilm architecture of bacterial populations with their activity, the bacterial treatment efficiency of organic carbons was measured in the bioelectrochemical system and compared with that in the conventional system. In the SEM image, the biofilm on the anodic medium of the bioelectrochemical system looked intact and active; however, that on the carbon medium of the conventional system appeared to be shrinking or damaging. In the AFM image, the thickness of biofilm formed on the carbon medium was about two times of those on the anodic medium. The bacterial treatment efficiency of organic carbons in the bioelectrochemical system was about 1.5 times higher than that in the conventional system. Some denitrifying bacteria can metabolically oxidize H_2 coupled to reduction of NO_3^- to N_2. H_2 was produced from the cathode in the bioelectrochemical system by electrolysis of water but was not so in the conventional system. The denitrification efficiency was less than 22% in the conventional system and more than 77% in the bioelectrochemical system. From these results, we found that the electrochemical coupling reactions between aerobic and anaerobic reactors may be a useful tool for improvement of wastewater treatment and denitrification efficiency, without special manipulations such as bacterial growth condition control, C/N ratio (the ratio of carbon to nitrogen) control, MLSS returning, or biofilm refreshing.

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Key words: Electrochemical oxidation potential, anodic biofilm media, cathodic biofilm media, bacterial denitrification, wastewater treatment, H_2-oxidizing bacteria

Various materials such as rubber, plastic, ceramic, and carbon have been used as a biofilm medium (matrix) in wastewater treatment systems. Wastewater biofilms are very complex multispecies of bacterial communities, displaying considerable heterogeneity with respect to both the microorganism present and their physicochemical microenvironments [1, 22]. Recent studies have demonstrated that the dynamics of biofilm formation are influenced by biotic and abiotic factors [4, 5, 17, 20, 34]. For example, the nature of the carbon source has been shown to control the pathways of biofilm development, species diversity, and also biofilm architectural heterogeneity [3, 8, 11]. In addition, the chemical nature of the biofilm substratum may also affect the success of cell attachment and the morphology of attached cells, as well as subsequent biofilm structure [6]. Based on the diverse metabolic processes carried out by different bacteria, the relative abundance of specific biofilm members would clearly be expected to play a role in regulating the overall metabolic potential of the biofilm system. It is consequently evident that numerous adaptive mechanisms are employed by microbial communities whose conditions are encountered at solid-liquid interfaces [12, 21]. Several researchers have reported that successive vertical zonations of bacterial communities were found in aerobic wastewater biofilms, and anaerobic bacterial communities such as sulfate-reducing bacteria and denitrification bacteria were found in substratum close to the media [7, 15, 17]. This property was due to the thickness of biofilms reaching to a few micrometers [22]. The control of biofilm thickness and density may be a solution for improvement of

treatment efficiency in aerobic wastewater treatment reactors.

Meanwhile, bacterial dentrification is a disimmilatory nitrate reduction, which is inhibited by O_2 or at higher redox potential than 0.0 volt (vs. NHE-natural hydrogen electrode). The redox potential around the bacterial community can be controlled in the electrochemical system [25, 30]. The dissimilatory nitrate reduction is influenced by the concentration of electron donor such as organic carbons, H_2 [29], or electrochemical energy [26]. H_2 can be a substitute electron donor for organic carbons in the wastewater treatment system for denitrification but is difficult to handle and potentially dangerous. Electric energy, therefore, has been of continuous interest as a substitute for H_2 in various bioreactors such as bacterial desulfurization of petroleum [23], succinate fermentation [24], and amino acid fermentation [9]. Bacterial oxidation of H_2 [28] is coupled to the reduction of NAD^+ [32], cytochromes [33], and quinones [13], which function as electron donors for sequential reduction of NO_3^- to N_2 via nitrite, nitric oxide, and nitrous oxide [14].

In the present study, we applied the electrochemical technique to aerobic biofilm media to determine the effect of oxidation potential on biofilm thickness, bacterial distribution on medium surface, and bacterial treatment efficiency, and to anaerobic biofilm media to appraise a possibility that the H_2 produced by electrolysis of H_2O may be a sole electron donor for bacterial denitrification.

Materials and Methods

Bioreactor

The bioelectrochemical system was composed of an aerobic bioreactor (anode compartment) and anaerobic bioreactor (cathode compartment), which were electrochemically coupled, and two different types of biofilm media (matrix) were placed in each bioreactor as shown in Fig. 1. The titanium plate coated with granular active carbons and sieve-type titanium plate were used as anode and cathode, respectively. Five sheets of anodes (150×250×15 mm) were placed in the aerobic bioreactor (ABR), and 0.3 volumes of gravels (mean diameter 15 mm) to reactant volume were irregularly piled on the cathode (150× 300 mm) located at the bottom of the anaerobic bioreactor (ANBR). The single-layered active carbons bound to the titanium plate function as anodic biofilm media (AB) and the gravels piled up titanium plate function as cathodic biofilm media (CtB). Active carbons were bound to titanium plate with graphite epoxy (Electrosynthesis, U.S.A.) and the resistance between titanium plate and active carbons was below 50 Ω, and the resistance between anode and cathode was below 300 Ω. DC 5 volts of electricity between anode and cathode were charged to

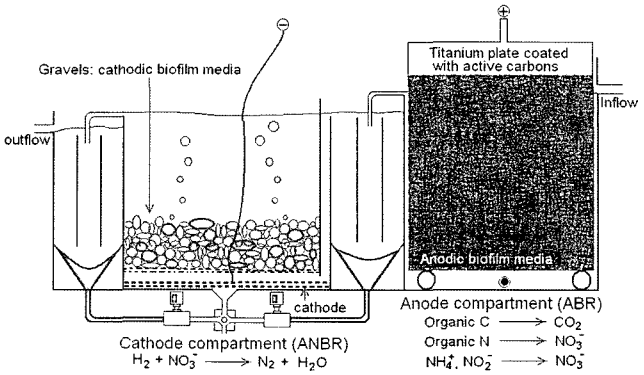

Fig. 1. Schematic structure of the bioelectrochemical system for bacterial oxidation of organic compounds coupled to bacterial reduction of nitrate to N_2.

The system was composed of an anode compartment (ABR: aerobic bioreactor) and cathode compartment (ANBR: anaerobic bioreactor). The anode was titanium plate coated with granular active carbons and the cathode was sieve-type titanium plate, which is located at the bottom of gravels. Irregular piles of gravels (5–15 mm diameter) and granular active carbons (5–8 mm diameter) were used as biofilm media. Active carbons were bound to the titanium plate with conductive epoxy and the resistance between titanium plate and active carbons was below 50 Ω. DC 5 volts between the anode and cathode were charged for production of electrochemical oxidation potential on the surface of the anodic biofilm media and production of H_2 from the cathode.

generate electrochemical oxidation potential on the surface of AB and to produce H_2 from the cathode. A completely identified system with the bioelectrochemical system but without application of electric charge was used as a control, which was named the conventional system. The biofilm media used in the ABR and ANBR of the conventional system were named as carbon biofilm media (CaB) and gravel biofilm media (GB), respectively, for separation from AB and CtB. The H_2 produced from the cathode may be temporarily stayed in interspaces among gravels or go up through the gravels, which functions as an electron donor for the denitrification bacterial biofilm growing on the surface of the gravels. Both volumes of ABR and ANBR were 20 l and inflow rate of wastewater (reactant) was adjusted to 30 ml/min. Before inoculation, the bioreactor was operated for 2 days to replace O_2 with H_2 in interspaces among the gravels and to induce oxidative potential on the surface of AB. Dissolved oxygen was adjusted to 3–5 mg/l by aeration in the ABR, and the reactant flow was adjusted to be upstream through interspaces among the gravels in the ANBR. Artificial wastewater was prepared with 2.0 g/l soluble starch, 5.0 g/l acetate, 200 mg/l NO_3^--N, 10 mM phosphate, 1.0 g/l yeast extract, 1.0 g/l peptone, and 5.0 g/l NaCl.

Scanning Electron Microscopy (SEM)

A piece (5×5×5 mm) of active carbon was cut from AB of the bioelectrochemical and CaB of the conventional systems at intervals of 3 weeks during operation for

3 months. The active carbon piece was fixed in 4% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4). Procedures for sample preparation were performed by the general method [18]. The prepared sample was examined under a JEOL JSM-6300 scanning electron microscope (Tokyo, Japan).

Atomic Force Microscopy (AFM)

AFM images were recorded in contact mode at room temperature using a Molecular Imaging Microscope (Digital Instrument, U.S.A.). Images were recorded in both height and deflection modes. Whereas height images provided quantitative information on sample surface topography, deflection images exhibited higher contrast of morphological profiles. Imaging forces were kept between 10 μm and 10 μm, and scan rates were between 4–5 Hz. All measurements were performed on the surface of the biofilm, which was bound to granular active carbon.

Comparison of Biomass in Biofilm

Substantially, the biomass of bacterial cell bound to biofilm media is difficult to be measured precisely. For comparison of biomass between AB and CaB, the concentration of cellular proteins was measured. The bacterial lysate was obtained from the AB and CaB by alkaline treatment at 100°C for 10 min with 1.0 N NaOH. The protein concentration of bacterial lysate was determined with Bradford reagent (Bio-Rad, Hercules, CA, U.S.A.).

Analysis

Chemical oxygen demand, total nitrogen, and nitrate concentration were analyzed according to standard methods [2].

Reproducibility of Results

All individual experiments were repeated three to five times with identical results in the range below 5.0% deviation.

RESULTS AND DISCUSSION

Architecture of Biofilms

To evaluate the effect of the electrochemical oxidation potential on the architecture of biofilms, SEM and AFM

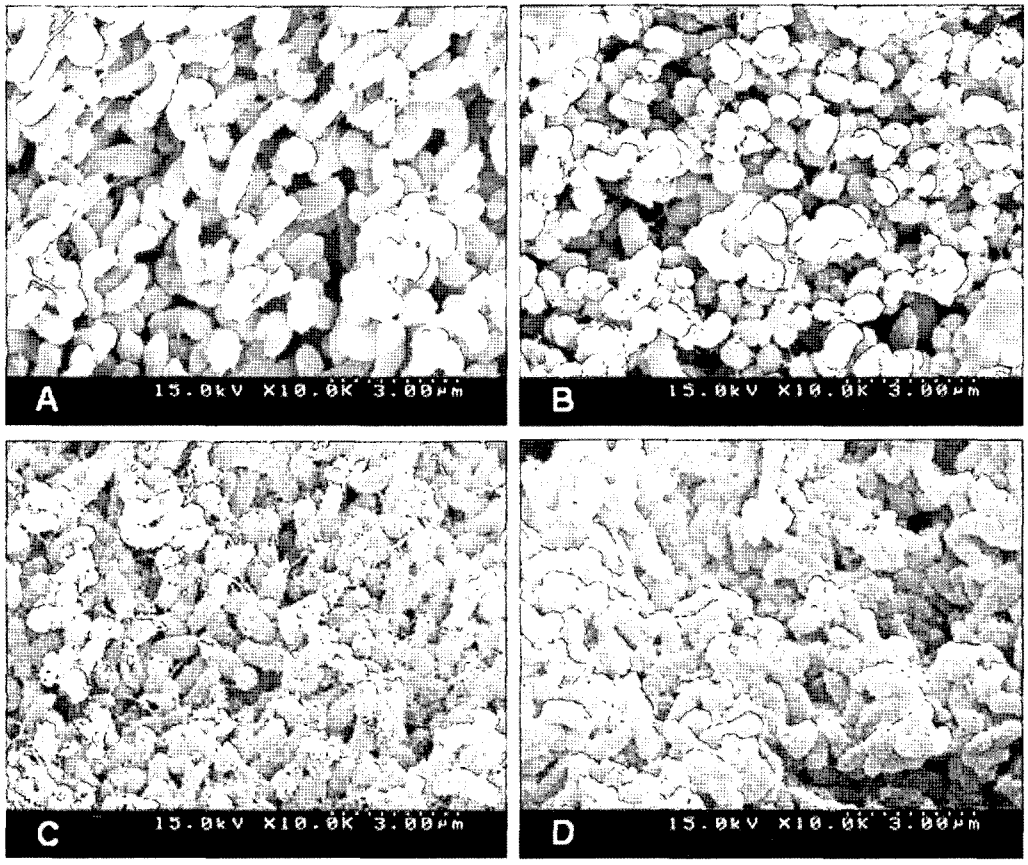

Fig. 2. SEM profiles of bacterial cells on CaB of the normal system after operation for 3 (A), 6 (B), 9 (C), and 12 weeks (D).
The bacterial cells were densely distributed and most of bacterial cells appeared to be shrinking or damaging. A piece (5×5×5 mm) of active carbon was cut from the biofilm media. The active carbon piece was fixed in 4% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4) for SEM preparation. CaB: carbon biofilm media to which electrochemical oxidation potential was not charged.

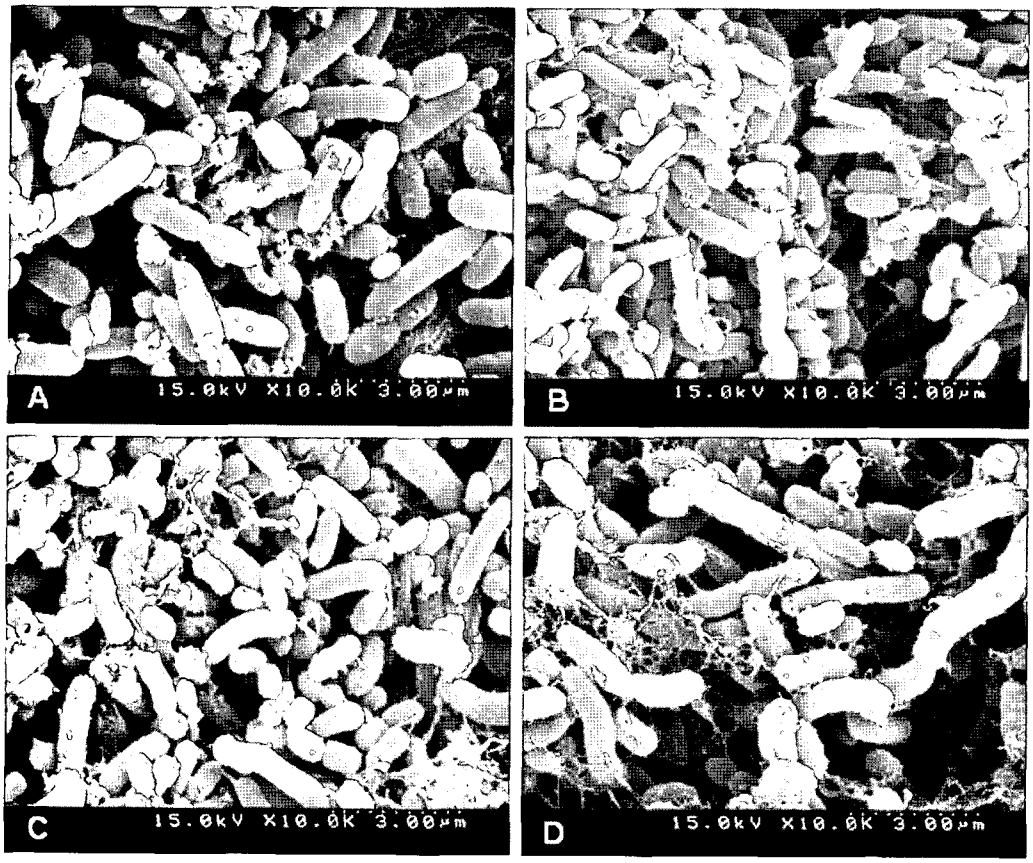

Fig. 3. SEM profiles of bacterial cells on AB of a normal system after operation for 3 (A), 6 (B), 9 (C), and 12 weeks (D).
The bacterial cells were loosely distributed and most of bacterial cells look intact or active. A piece (5×5×5 mm) of active carbon was cut from the biofilm media. The active carbon piece was fixed in 4% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4) for preparation of SEM. AB: anodic biofilm media to which electrochemical oxidation potential was not charged.

images were analyzed. As shown in Figs. 2 and 3, the SEM image of AB was significantly different from that of CaB. The bacterial cells on the AB were loosely distributed and looked intact or active; however, the bacterial cells on the CaB were densely distributed and appeared to be shrinking or damaging. Okabe *et al.* [22] reported that sulfate-reducing bacteria, one of the strict anaerobes, were spatially distributed in the substratum of thick biofilms operating in an aerobic wastewater treatment reactor. H_2S, ammonium, nitrite, and other reduced compounds have to be produced in the anaerobic environments, by which bacterial oxidation of organic carbons may be inhibited or limited [27]. From the SEM image of bacterial cells of AB and CaB, we suggest that the structural differences of the biofilms may be explained in terms of electrostatic influences between charged groups in the biofilm as shown in the model of Fig. 4. The dense biofilm may block diffusion of air from the liquid phase to the bacterial cells inside the biofilms or constitute an anaerobic environment around bacterial cells. Xu *et al.* [35] reported that 30 μM of the biofilm thickness limited diffusion of oxygen into the biofilm and inhibited alkaline phosphatase activity of bacterial cells growing in the substratum of the biofilm. If the aerobic bacteria were left in an anaerobic environment for a long time, bacterial metabolism and growth may become stopped and then bacterial cells may be ghost cell. Møller *et al.* [20] reported that the biofilms formed in neutral dextran media were thicker than the biofilms in polyanionic or polycationic dextran media. This supports a possibility that extracellular polymeric substances electrically charged might influence on the biofilm architecture and thickness. The electrochemical oxidation potential charged to AB may convert the extracellular polymers to cationic compounds. Stoodely *et al.* [31] reported that electrostatic interactions between negatively charged groups in the biofilm were a cause of biofilm expansion, but between negatively and positively charged groups in the biofilm were possible to be the cause of biofilm contraction. The AFM is a useful tool to understand the structure and measure the thickness of biofilms. As shown in Fig. 5, the AFM image of biofilm formed on CaB looked smooth, but that on AB looked rough and had many grooves, and the biofilm thickness formed on CaB was around two times of that on AB. From these results, we propose that the electrochemical potential

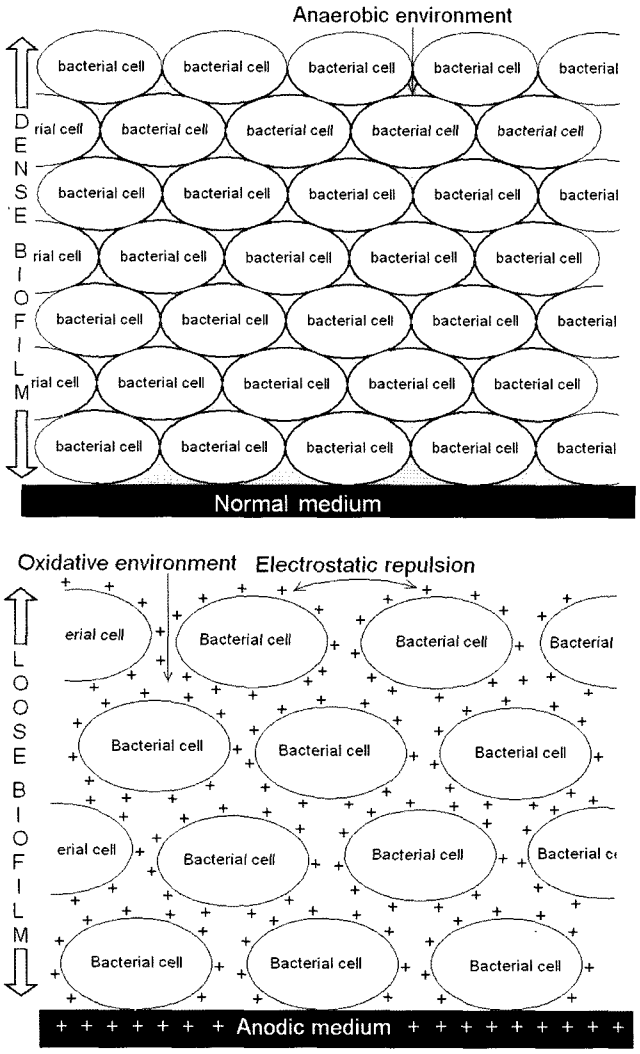

Fig. 4. Hypothetic model of carbon biofilm media (upper) and anodic biofilm media (lower), composed on the basis of SEM and AFM profiles.

In carbon biofilm media, interspaces between bacterial cells may be converted to an anaerobic environment, by which growth of aerobes may be inhibited or stopped. In anodic biofilm media, however, the oxidation potential charged to the active carbon surface generates an oxidative environment and repulsive force between bacterial cells, by which interspaces between bacterial cells may be expanded and the aerobic condition inside biofilms maintained.

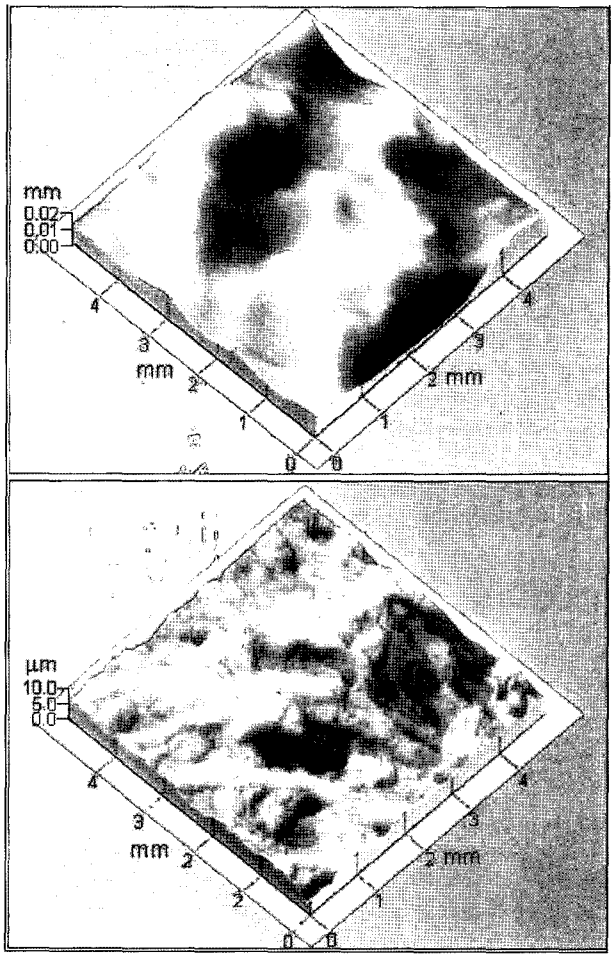

Fig. 5. AFM height image (5.0 mm by 5.0 mm; z range, 10 μm) in solid state of biofilms formed on active carbon biofilm media (upper) and anodic biofilm media (lower) after being operation for 3 months.

The biofilm thickness formed on carbon media and anodic media was around 20 μm and 10 μm, respectively. The biofilm formed on carbon media was smooth, which shows that bacterial cells may be densely distributed; however, the biofilm formed on anodic media was rough, which shows that bacterial cell may be loosely distributed. These images were obtained for comparison between biofilm profiles on carbon and anodic biofilm media, not for observation of bacterial morphologies.

charged to biofilm may induce the electrostatic force to the bacterial surface as shown in the models of Fig. 4. The electrostatic repulsive force formed between bacterial cells limits excessive accumulation of bacterial cells on the biofilm media, following which bacterial cell may be loosely distributed or the interspaces among bacterial cells may be expanded.

Biomass of Biofilms

Some bacterial cells in biofilms are growing but some of them are dying or becoming ghost cells. Dead or ghost cells have to lose their cell materials such as proteins, nucleic acid, or lipids. If the environment of the biofilm media was proper for bacterial growth, the biomass may be relatively higher than those in improper biofilm media. Theoretically, the viable cell number has to be measured for comparison of biomass; however, that is difficult because all bacterial cells cannot be completely separated from complex biofilm media. For solution of this problem, we obtained bacterial lysate from same-sized AB and CaB incubated in the same bioreactor and for the same period, and analyzed the protein concentration of the lysate. The total protein concentration of bacterial lysate obtained from AB and CaB was 740 mg±63 mg and 370 mg±25 mg,

respectively, which correspond to 0.82 mg±0.07 mg/cm^2 and 0.41 mg±0.03 mg/cm^2 of biofilm media. The biofilm thickness of CaB was two times of that of AB but the biomass of CaB was half of that of AB. These results show a critical clue that the oxidation potential charged to AB may inhibit excessive development of biofilm and help to maintain bacterial activity. The biomass is not related to the biofilm thickness, but may be related to the treatment efficiency.

Wastewater Treatment Efficiency

In the ABR, the treatment efficiency has to be proportional to the bacterial activity and growth rates. Theoretically, the proper environment for bacterial growth may be helpful for and influence on the treatment efficiency. As shown in comparison data of the SEM and AFM images, and biomass, the AB was expected to be better than CaB for bacterial growth. Generally, the bacterial oxidation efficiency of organic carbons in an ABR without biofilm is proportion to the biomass (MLSS: mixed liquor suspended solid) and proper oxygen concentration. If the MLSS is enough for wastewater treatment, dissolved oxygen is a critical factor determining treatment efficiency in an ABR without biofilm; however, the treatment efficiency in the ABR with biofilm media has to be influenced by various factors such as biofilm architecture, biofilm thickness, biomass, bacterial activity in the biofilm, oxygen concentration in the substratum of the biofilm, and diffusion rate of nutrient from the liquid phase into the substratum of the biofilm. All of these factors cannot be satisfied, but some of them are supposed to be solved by the electrochemical oxidation potential charged to biofilm media. As shown in Fig. 6, the treatment efficiency of COD was about 1.5 times higher in

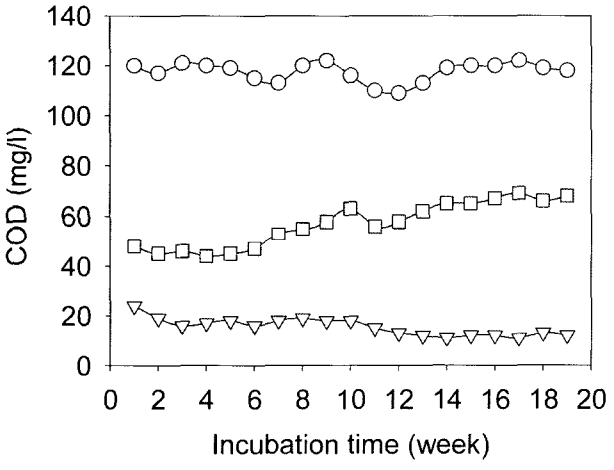

Fig. 6. COD variation of inflow wastewater (○), outflow from ABR-AB (▽), and ABR-CaB (□).
The treatment efficiency of COD in ABR-AB was about 2 times higher than that in ABR-CaB. COD variation was trending downward in ABR-AB but trending upward in ABR-CaB according to the treatment time. ABR-AB: aerobic bioreactor with anodic biofilm media; ABR-CaB: aerobic bioreactor with carbon biofilm media.

the ABR with anodic biofilm media (ABR-AB) than with carbon biofilm media (ABR-CaB). COD variation was trending downward in the bioreactor with AB, but that was trending upward in the bioreactor with CaB according to the treatment time. This is a better result than we expected, which may be caused by the oxidation potential charged to the extracellular polymer or outer wall of the bacterial cell, as shown in the model of Fig. 4. The bacterial respiration depends on the proton motive force coupled to the electron-driving force through the electron transport system, which is influenced by the oxygen concentration. The proton translocation from inside to outside the bacterial cell generates an electrochemical gradient of H^+ across the cell membrane or a potential difference between the inside and outside of a bacterial cell. Theoretically, the higher the positive (H^+) potential outside a bacterial cell is, the higher the free energy that can be produced [19].

Bacterial Denitrification

Bacterial denitrification is an anaerobic respiration with organic carbons as electron donor and nitrate as electron acceptor. Some denitrification bacteria can metabolically oxidize H_2 coupled to reduction of NO_3^- to N_2 [29]. We enriched the denitrification bacteria capable of oxidizing H_2 and applied the bacterial culture to an anaerobic bioreactor (ANBR) (Fig. 1). Before the H_2-oxidizing bacteria were applied to the ANBR, the bacteria were cultivated in an anaerobic medium with H_2 as a sole electron donor and nitrate as a sole electron acceptor by a continuous culture system for 7 days. During operation of the ANBR, the denitrification bacteria constructed biofilm on the surface of gravels. Some of the H_2 produced from the cathode may be dissolved into the reactant and some may be temporarily stayed in interspaces among the gravels. This H_2 can be metabolically oxidized by denitrification of the biofilm coupled to reduction of NO_3^- to N_2, as shown in Fig. 1. Meanwhile, denitrification bacteria have to oxidize organic carbons coupled to reduction of NO_3^- to N_2 in GB without H_2. After bacterial oxidation of organic carbons contained in wastewater in the ABR, the residual organic carbons are discharged to the ANBR. In the condition without H_2, bacterial cells have to oxidize residual organic carbons for production of reducing power (NADH/NAD$^+$). The bacterial denitrification efficiency has to be proportional to the ratio of NADH/NAD$^+$. As shown in Fig. 6, the residual organic carbons contained in wastewater discharged from ABR-AB and ABR-CaB were around 15 mg/l and 60 mg/l, respectively, as COD. These residual organic carbons are insufficient to produce reducing power (NADH/NAD$^+$) for biochemical reduction of NO_3^- to N_2. As shown in Fig. 7, the NO_3^--N concentration of inflow was around 180 mg/l, which is about 3 times as large as the COD discharged from ABR-CaB. Theoretically, the C/N ratio has to be more than 3 [10] but the C/N ratio of reactant discharged

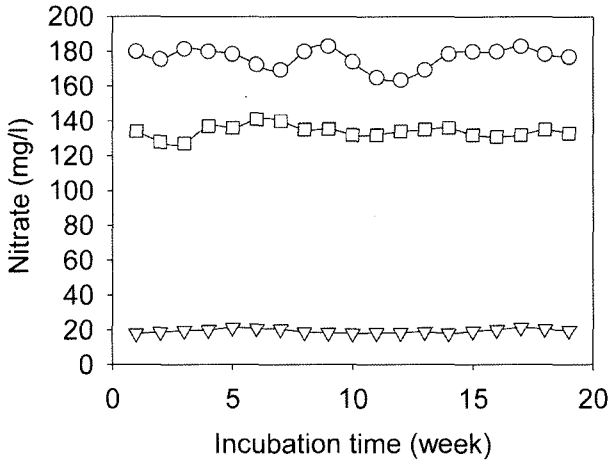

Fig. 7. Variation of nitrate contained in inflow wastewater (○), and outflow discharged from ANBR-CtB (▽) and ANBR-GB (□).
The treatment efficiency of nitrate in ANBR-CtB was about 7 times higher than that in ANBR-GB. ANBR-CtB: anaerobic bioreactor with cathodic biofilm media; ANBR-GB: anaerobic bioreactor with gravel biofilm media.

from ABR-CaB was less than 0.3. This is the reason why the denitrification efficiency was less than 20% in the ANBR-GB, whereas the denitrification efficiency was more than 90% in the ANBR with cathodic biofilm (ANBR-CtB). This indicates that H_2 is utilized as an electron donor by denitrification bacteria in the ANBR. The residual organic carbons (15 mg/l as COD) discharged from the ABR of the bioelectrochemical system may be utilized by bacterial cell as a carbon source in the ANBR.

In this research, we found that the electrochemical coupling reactions between aerobic and anaerobic reactors may be a useful tool for improvement of wastewater treatment and denitrification efficiency without dependence on special functions of bacteria, C/N ratio control, MLSS returning, or biofilm refreshing. We also found that a new electrode, the titanium plate coated with active carbons, is capable of functioning as both an electrode and a biofilm medium, which may be an essential factor for application of the electrochemical technique to any wastewater treatment reactor.

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