

## Enrichment of Electrochemically Active Bacteria Using a Three-Electrode Electrochemical Cell

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**Abstract** Electrochemically active bacteria were successfully enriched in an electrochemical cell using a positively poised working electrode. The positively poised working electrode (+0.7 V vs. Ag/AgCl) was used as an electron acceptor for enrichment and growth of electrochemically active bacteria. When activated sludge and synthetic wastewater were fed to the electrochemical cell, a gradual increase in amperometric current was observed. After a period of time in which the amperometric current was stabilized (generally 8 days), linear correlations between the amperometric signals from the electrochemical cell and added BOD (biochemical oxygen demand) concentrations were established. Cyclic voltammetry of the enriched electrode also showed prominent electrochemical activity. When the enriched electrodes were examined with electron microscopy and confocal scanning laser microscopy, a biofilm on the enriched electrode surface and bacterium-like particles were observed. These experimental results indicate that the electrochemical system in this study is a useful tool for the enrichment of an electrochemically active bacterial consortium and could be used as a novel microbial biosensor.

**Key words:** Electrochemical enrichment, electrochemically active bacteria, three-electrode electrochemical cell

In recent years, increasing efforts have been made to characterize the metal-reducing bacteria, which use water-insoluble terminal electron acceptors, and to develop novel bioelectrochemical devices including mediator-less microbial fuel cells (MLMFCs) and microbial biosensors using the metal-reducing bacteria that are electrochemically active [4, 7, 11, 12, 15–21, 24, 27]. Currently, a number of bacteria

have been isolated and characterized based on their ability to catalyze the reduction of Fe(III) as their terminal electron acceptor [3, 8, 22, 23, 25, 26]. Since Fe(III) is essentially insoluble in water at neutral pH, physical and direct contact between the bacterial cell and Fe(III) minerals is required for anaerobic respiration [8, 9, 22, 23, 25, 28].

Several studies also showed that a water-insoluble terminal electron acceptor for the anaerobic respiration of bacteria could be replaced by an anode of a microbial fuel cell format or a poised electrode of an electrochemical cell [7, 14, 27]. For example, it has been demonstrated that the metal-reducing bacterium *Shewanella putrefaciens* can be grown in an electrochemical cell with a poised working electrode [14, 20]. Mediator less microbial fuel cells (MLMFCs) using Fe(III)-reducing bacteria such as *Shewanella putrefaciens*, *Geobacter sulfurreducens*, *Desulfuromonas propionicus*, and *Rhodospirillum rubrum* have also been developed in recent years [4, 18, 19]. In a MLMFC, current generated from the fuel cell is based on direct electrochemical contact between the bacteria and the anode of the fuel cell. At this stage, the anode of the fuel cell acts as an electron acceptor for bacterial anaerobic respiration [7, 14, 20]. Therefore, these microorganisms have been currently recognized as anodophiles [11] and/or electrochemically active bacteria (EAB) [4, 18, 27].

Several studies showed that EAB can be successfully enriched in a microbial fuel cell format [15, 18, 21, 24, 27]. It has been proposed that the nature of both the inoculation source and the fuel used in MLMFC is crucial to the types of EAB that eventually populate at the anode. Currently, EAB-enriched MLMFC is relevant to a variety of emerging application fields. It has been demonstrated that MLMFCs using enriched EAB are applicable in various types of biosensors including BOD (biochemical oxygen demand) sensors and toxicity monitoring sensors [13, 16,

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20, 21]. In an MLMFC, it should be noted that the cathode potential is directly linked with the anode potential via an outer load circuit and ion-selective membrane [1, 14]. Therefore, a drawback associated with this method is the unstable cell potential of electrodes and for the step of stabilization of the cathode with addition of oxidizer [21, 24]. Although this method has the potential of application of a high degree of automation and precision, the instrument requires a relatively long enrichment time (2 to 4 weeks) with careful maintenance [6, 13, 15, 17].

Using a conventional three-electrode arrangement and a potentiostat, the present work attempted to develop an EAB enrichment system without using an MLMFC format. In this three-electrode EAB enrichment system, a positively poised working electrode (*vs.* Ag/AgCl) was used as an alternative electron acceptor for the enriched EAB. In the enrichment system, stable and constant poised potential via a potentiostat was applied to the EAB via a working electrode. When the enrichment had been completed, bioelectrochemical reaction of the enriched electrode with different concentrations of BOD was monitored. Preliminary microbial, electrochemical, and microscopic studies of the enriched EAB were also undertaken, with the aim of elucidating the mechanistic aspects underlying the methodology. To the authors' knowledge, this is the first report on the enrichment of EAB using a three-electrode system.

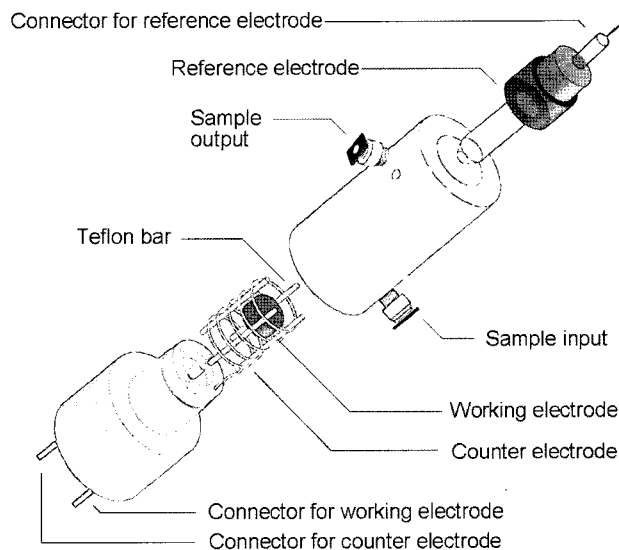
## MATERIALS AND METHODS

### Wastewater and Sludge

Glucose and glutamic acid test solutions for BOD were modified and used as synthetic wastewater throughout the study [5, 24]. The synthetic wastewater was autoclaved at 121°C for 15 min before being added with filter-sterilized glucose and glutamic acid solutions to the final BOD values of 100–300 mg/l. Activated sludge was obtained from the Jung-Nang Sewage Treatment Plant (Seoul, Korea) for the enrichment and adaptation of the EAB in the electrochemical cell format. In order to prevent clogging of the electrochemical cell, the sludge suspension was partially filtered through a filter paper (Grade 4, particle retention diameter: 20–25 µm; Whatman, U.S.A.), and the filtrate was then used as the microbial source (*i.e.*, inoculum) for EAB enrichment in the electrochemical cell. The remaining filtrates were stored at –20°C for inoculation in subsequent experiments.

### Three-Electrode Electrochemical Cell

Although most previous electrochemically active bacterial studies have employed a simple microbial fuel cell format [1, 13, 16, 21], there are advantages in using a potentiostated working electrode as an electron acceptor. The principal advantage is that this approach allows for stable poisoning of



**Fig. 1.** Schematic diagram of a three-electrode electrochemical cell for the enrichment of electrochemically active bacteria.

the working electrode and the capability of application to a high-resistance enrichment environment through adjustment of the poisoning potential and autocompensation circuits [4, 15, 18]. A representation of the electrochemical cell (working vol. 60 ml) assembly and instrumentation is shown in Fig. 1. A conventional three-electrode arrangement using a potentiostat (WMPG 1000 X10328, IBM PC (compatible) controlled, Wonatech Co., Korea) was used, which consisted of a working electrode poised at a pre-set potential (15×15×5 mm, Graphite felt, GF series, Electro-synthesis Co., U.S.A.), a reference electrode (Ag/AgCl, Phoenix, U.S.A.), and a counter electrode (50×2 mm, Pt).

### Enrichment Procedure

The three-electrode electrochemical cell was used to enrich EAB, using synthetic wastewater as the electron donor and sludge as a bacterial source. The wastewater was used to fill an electrochemical cell containing 20 ml of the sludge preparation and 40 ml of the synthetic wastewater before the potential was applied [6, 15, 19]. For the initial set-up of the microbial fuel cell, the electrodes were connected to the potentiostat to start the potential application (+0.7 V *vs.* Ag/AgCl). When the potential from the electrochemical cell had stabilized, the synthetic wastewater (BOD 100 mg/l) was added to the cell (flow rate: 2 ml/min for 1 h with every 4-h intervals) using a programmable peristaltic pump (ISM597A, Ismatec, Switzerland), and the increase in the amperometric signal was recorded. The enrichment was performed in a temperature-controlled chamber at 35°C. In the case of the control experiment, the enrichment system was operated without the sludge inoculation. Schematic diagrams of the experimental set-up are presented in Fig. 2.

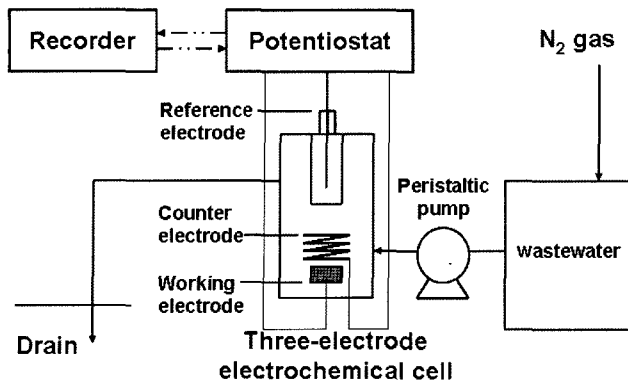


Fig. 2. Schematic diagram of the EAB enrichment system.

### Cyclic Voltammetry

Electrochemical activity and direct electrode reaction of the EAB-enriched electrode were examined using cyclic voltammetry [14–16, 27]. The cyclic voltammograms of the working electrode were obtained using a potentiostat (CHI 600B, CH Instruments Inc., U.S.A.) interfaced to an IBM-compatible PC with software supplied by the manufacturer. After enrichment, a section of the graphite electrode (4×4×5 mm) in the electrochemical cell used in the enrichment was connected to a platinum wire and placed in an electrochemical cell for cyclic voltammetry. A platinum counter electrode (MW-4130, BAS, U.S.A.) and an Ag/AgCl reference electrode (MF-2052, BAS, U.S.A.) were used in an electrochemical cell with a working volume of 5 ml. The electrochemical cell was filled with synthetic wastewater (5 ml), and oxygen-free nitrogen was gassed through the cell for 10 min before measurements. A scan rate of 100 mV/sec was employed over a range from +0.5 V to -0.6 V.

### Microscopy

For direct observation of the enriched bacteria, the electrodes were examined by scanning electron microscopy (SEM) (JSM-6380, JEOL, U.S.A.). Electron micrographs were taken after the enriched electrode samples were prepared according to the standard method [2, 10]. To observe Gram-staining bacteria, the electrodes were examined by confocal laser scanning microscopy (CLSM) (LSM-410, Carl Zeiss Ltd., Germany). The samples were stained with the LIVE BacLight bacterial Gram-stain kit (L-7005, Molecular Probes, U.S.A.) [15, 18].

## RESULTS AND DISCUSSION

### Enrichment of EAB

The activated sludge preparation was inoculated to the three-electrode electrochemical cell for the enrichment of

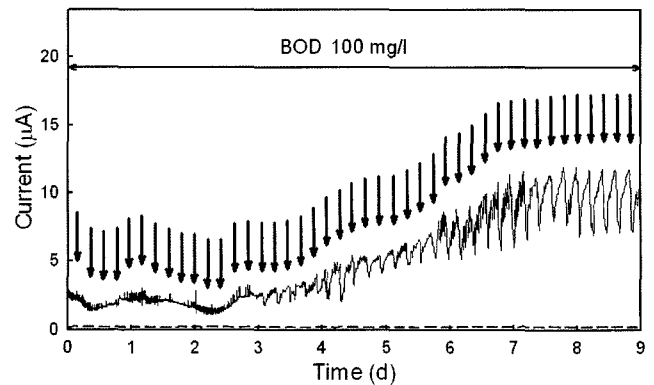
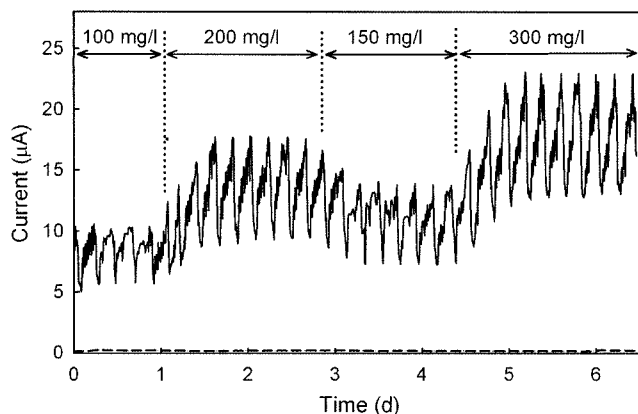


Fig. 3. Amperometric signal generation during the EAB-enrichment procedure of the three-electrode electrochemical cell with inoculation of sludge (solid line) and without sludge (dashed line).

The poised potential of the working electrode and feeding rate of synthetic wastewater were +0.7 V (*vs.* Ag/AgCl) and 2 ml/min (for 1 h with every 4-h intervals), respectively. Arrows indicate the feeding of wastewater.

EAB. The working electrode was then poised at a fixed potential by a potentiostat, generally at +0.7 V (*vs.* Ag/AgCl), and the amperometric current was monitored [15]. The enrichment of EAB with the poised working electrode was maintained for 7 days using synthetic wastewater with a BOD of 100 mg/l as an electron donor. At the initial stage of operation (*i.e.*, enrichment for 0–4 days), no significant amperometric signal was observed. After 7 days of enrichment, however, a stable current in a range of 6–12 µA was observed. Typical traces of the current change due to the action of the electrochemically active bacteria in the electrochemical cell with the addition of the synthetic wastewater are shown in Fig. 3. The amperometric response to addition of synthetic wastewater (*i.e.*, nutrient) was typically a rise in current as a function of time. The increase in current with the addition of wastewater to the cell is likely due to a direct electron transfer activity of the enriched electrochemically active bacteria at the working electrode [7, 13–19, 21, 27]. The sudden addition of wastewater (at every 5 h) to the electrochemical cell might induce a decrease in the current. In the case of the control experiment (*i.e.*, without the sludge inoculation), no change in amperometric response with sequential addition of wastewater was recorded, apart from a minor perturbation arising from re-equilibration at the electrodes [14]. This did not change with the addition of further quantities of wastewater (*i.e.*, over 7 days of the wastewater feeding) to the cell. Previous experimental results show that activated sludge contains a large amount of EAB and the sludge could be used as a source for EAB inoculation [15, 21, 27, 29]. Therefore, the failure of the control experiment to generate any amperometric signal is clearly attributed to its absence of EAB inoculation.



**Fig. 4.** Amperometric signal generation from the electrochemical cell with different BOD concentrations of synthetic wastewater. The solid line indicates the amperometric signal from the electrochemical cell with the sludge inoculation. The dashed line indicates the control experiment (without sludge inoculation).

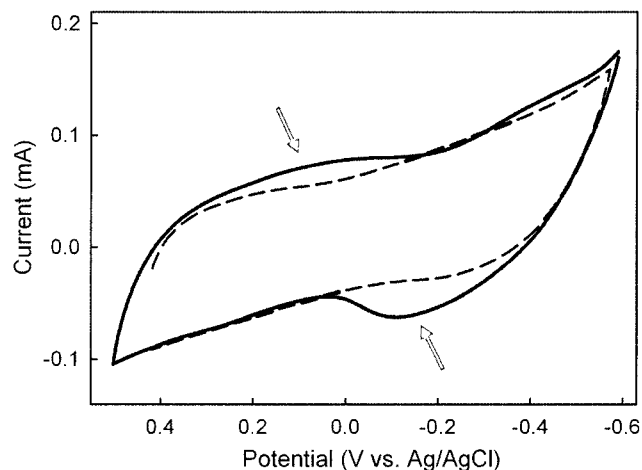
#### Variation of Amperometric Signal with Different BODs of the Synthetic Wastewater

A series of synthetic wastewater samples with different concentrations ranging from 100 to 300 mg/l as the BOD (synthetic wastewater) was prepared and fed into the EAB-enriched electrochemical cell to monitor the effect of nutrient concentration on the amperometric signal generation. As shown in Fig. 4, the amperometric signal in this experiment was variable with the variation in BOD concentration. In the control experiment without inoculation, however, no change in current was observed. This indicates that variation of the amperometric signals from the electrochemical cell can be attributed to the metabolic action of the enriched EAB, with oxidation of nutrients and subsequent direct electron transfer activity of EAB to the working electrode [13, 16, 19, 21, 27].

Previous experimental results using the EAB and MLMFC format show that generally more than 14 days are required for the enrichment of EAB [15, 21]. However, in this experiment, only 6–7 days were needed for the enrichment. The main reason for the delay in enrichment in the MLMFC format is tentatively attributed to the unstable fuel cell potential, which arises from variation of oxidants concentration (*i.e.*, oxygen) in the cathode. In addition, it should be noted that a change in the humidity of the supplied oxidant also results in a change in the potential [12]. Therefore, the rapid enrichment of EAB might be the result of the constitutional stability of the enrichment system using the three-electrode electrochemical cell.

#### Cyclic Voltammogram

After the enrichment, the working electrodes used in the electrochemical cell were examined using cyclic voltammetry to determine their electrochemical activities. Cyclic voltammograms (CVs) of the electrodes are shown



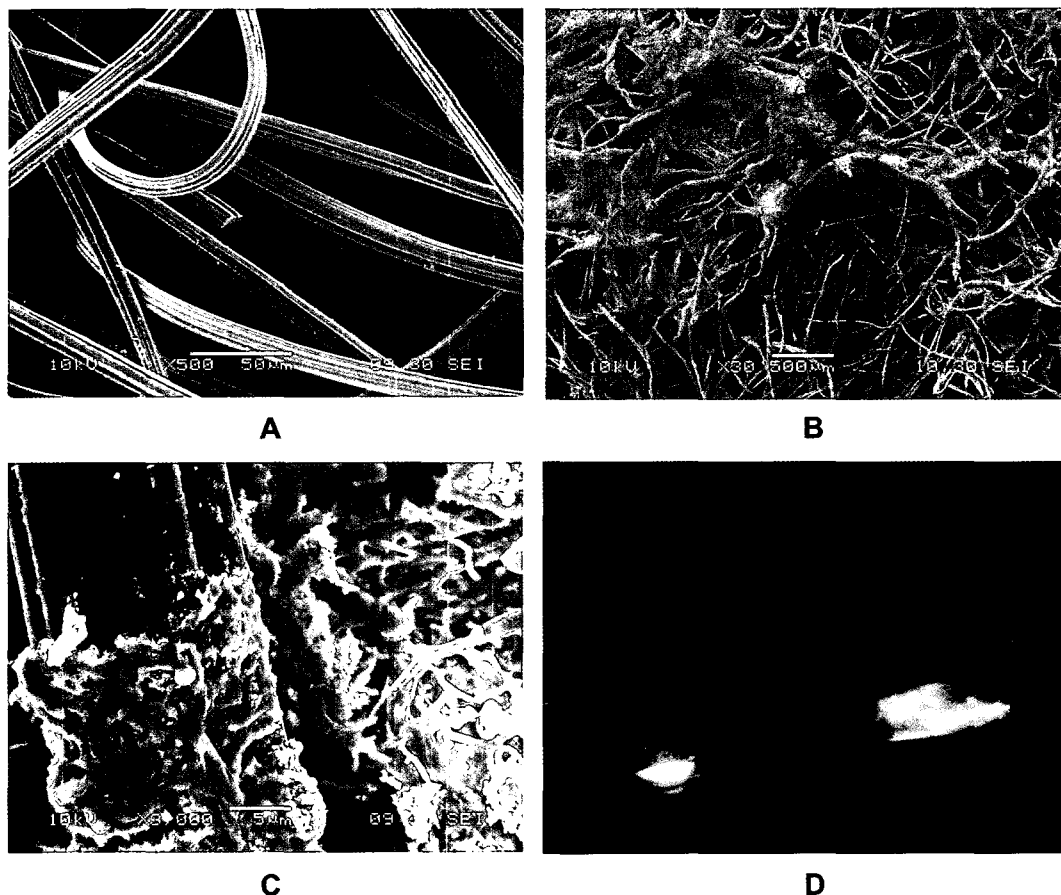
**Fig. 5.** Cyclic voltammograms of the electrodes used in this study.

The dashed line indicates the electrode from the control experiment. The solid line indicates the enriched electrode with sludge inoculation. Arrows indicate redox peaks of the EAB.

in Fig. 5. No peaks were observed in the CV of the electrode that was used in the control experiment (*i.e.*, the electrode without sludge inoculation). However, the electrode with inoculation and poised potential showed clear electrochemical activity compared with the control electrode in the CV. At approximately  $-0.15$  V (*vs.* Ag/AgCl electrode), prominent electrochemical peaks were observed. Many previous studies have shown that EAB including *Shewanella* sp. and *Clostridium* sp. can be conveniently measured by the use of cyclic voltammetry with an electrochemical cell [14–17, 27]. Most previous cyclic voltammetric analyses of EAB have employed cultured bacterial suspensions isolated from the electrode rather than an intact enriched electrode. In this study, the EAB-enriched intact electrodes produced clear electrochemical activity. It is plausible that the electrochemical activity is due to the enriched EAB at the electrode and their direct electron transfer capability to the positively poised working electrode [4, 17, 18].

#### Microscopy

At the end of the experiment, heavy growth of bacterium-like particles on the surface of the working electrode was observed by SEM (Fig. 6). Similar samples were also used for confocal laser scanning microscopy (CLSM) after staining with the fluorophors to distinguish the Gram reactions of the bacteria. The average particle size was  $0.5 \times 5$   $\mu\text{m}$ . CLSM showed that the enriched biomass on the electrode surface consisted of live bacterial cells, and both Gram-positive and Gram-negative bacteria formed microcolonies throughout the electrode surface. These results suggest that the majority of the bacteria are present in the biofilm, and the bacterial clumps were alive. Previous



**Fig. 6.** Scanning electron micrographs and a confocal scanning laser micrograph of the working electrode used in this study. **A.** Electron micrograph of the electrode from the control experiment. **B, C.** Electron micrographs of the enriched electrode. **D.** Confocal scanning laser micrograph (magnification  $\times 400$ ) of the enriched electrode [Red: G(+); green: G(-)].

investigations with MLMFC have shown that EAB were enriched on the surface of an anode of an MLMFC, and the EAB were verified with microscopic techniques [4, 14, 15, 18, 27]. In the present study, therefore, a series of SEM and fluorescence microscopy photographs revealed bacterium-like particles, which are likely enriched EAB.

From the results in this study, a three-electrode electrochemical cell with a poised electrode with EAB inoculation was found to be useful for developing novel bioelectrochemical devices including a BOD sensor, as it can easily enrich EAB species without the complicated microbial fuel cell formats including oxidant supplies. In addition, the electrochemical activity of the enriched bacteria in the electrode can be simply monitored using cyclic voltammetry. It should be noted that the previous EAB enrichment procedures were performed in a microbial fuel cell format. However, in this study, a simple electrochemical cell with a poised electrode was firstly used for the enrichment of EAB. Identification of bacterium-like particles and applicability of this method to the development of useful biosensors for a water environment will be explored in future work.

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