

A mediator-less microbial fuel cell using a metal reducing bacterium, *Shewanella putrefaciens*

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Received 11 April 2001; received in revised form 27 July 2001; accepted 15 August 2001

Abstract

Direct electron transfer from different *Shewanella putrefaciens* strains to an electrode was examined using cyclic voltammetry and a fuel cell type electrochemical cell. Both methods determine the electrochemical activity of the bacterium without any electrochemical mediators. In the cyclic voltammetric studies, anaerobically grown cells of *Shewanella putrefaciens* MR-1, IR-1, and SR-21 showed electrochemical activities, but no activities were observed in aerobically grown *Shewanella putrefaciens* cells nor in aerobically and anaerobically grown *E. coli* cell suspensions. The electrochemical activities measured by the cyclic voltammetric method were closely related to the electric potential and current generation capacities in the microbial fuel cell system. Cytochromes localized to the outer membrane are believed to facilitate the direct electron transfer to the electrode from the intact bacterial cells. The concentration of the electron donor in the anode compartment determined the current generation capacity and potential development in the microbial fuel cell. When the high concentration of the bacteria (0.47 g dry cell weight/liter) and an electrode that has large surface area (apparent area: 50 cm²) were used, relatively high Coulombic yield (over 3 C for 12 h) was obtained from the bacteria. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Mediator-less microbial fuel cell; *Shewanella putrefaciens*; Metal-reducing bacteria

1. Introduction

Over last two decades, a number of bacteria have been isolated and characterized based on their ability to use ferric iron [Fe(III)] as their electron acceptor [5,7,18,22]. It is recognized that the Fe(III) reducing bacteria play important roles in the cycling of iron and organic matters in sediments and other anaerobic environments [8,18,21,24]. Studies have been conducted to understand the mechanisms of Fe(III) reduction by bacteria such as *Shewanella putrefaciens* and *Geobacter metallireducens* [7,20,22]. Since Fe(III) is essentially insoluble in water at neutral pH [30], physical and direct contact between the bacterial cell and Fe(III) minerals is required for the anaerobic respiration [7,9,19,22]. It was reported that approximately 80% of membrane-bound cytochromes of anaerobically grown *S. putrefaciens* are localized in its outer membrane (OM)

[2,22,23]. Four distinct cytochromes have been identified in the OM fraction of the bacterium, and the content of an 83 kDa *c*-type cytochrome was lower in the cells grown under aerobic conditions than that of anaerobically grown cells. Through transposon insertion metagenesis, an Fe(III) reduction deficient mutant of *S. putrefaciens* SR-21, has been isolated [3]. The mutant lacked MtrB which is required for Fe(III) reduction in vivo of ferric reductase. Electrochemical techniques, therefore, could be the method of choice to study the electron transfer reactions in the Fe(III)-reducing bacterium complementing these biochemical studies.

Several electrochemical techniques have been used to characterize redox proteins including cytochromes [11,12,13]. Large numbers of redox proteins are electrochemically active. However direct electron exchange between a redox protein and an electrode is usually hindered by the peptide chain adjoining the active redox center of the protein. The electrochemical reaction of a protein is generally enhanced by the modification of the electrode for the efficient electron transfer or the protein to direct the active center of the protein toward the electrode surface [29]. Additionally, bac-

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terial cell itself is electrochemically inactive because the active electron carriers are enclosed by an electrically non-conductive cell wall and other surface structures. For these reasons, various electrochemical mediators (i.e. electron shuttles) have been used in the electrochemical studies of intact bacterial cells [14,16,17,27,28] and to construct of microbial fuel cells [1,26,31].

A microbial fuel cell is an electrochemical device which converts the chemical energy of fuel to electrical energy by the catalytic actions of microorganisms [4]. Several types of biofuel cells including microbial fuel cell and enzymatic biofuel cell have been well documented in the literature [32]. Various microbial or biochemical fuel cells have been developed using *Desulfovibrio desulfuricans*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas* species and redox enzymes as biocatalysts [32]. In a typical microbial fuel cell, an anodic electrode potential is generated when the electrons from the oxidation of the substrate by microorganisms are available to the electrode. Though the direct transfer of electrons from microbial cells to electrodes has been demonstrated, this process is inefficient both in terms of the proportion of electron transferred (i.e. the Coulombic yield) and the rate of electron transfer (i.e. the current generation) [4,26,31,32]. For this reason it has been recognized that electrochemical mediators are essential for the microbial fuel construction. These mediators include thionine, methyl viologen, 2-hydroxy-1, 4-naphthoquinone, neutral red among others [25,32]. Mediators shuttle electrons between the bacterial cells and the electrode. A microbial fuel cell system can be used for various purposes including bacterial activity monitoring, electricity generation in local area, wastewater treatment processes. Microbial fuel cells have not been commercialized yet, and this may be due to difficulties in using mediators at a commercial scale [4,32].

Recently, we have shown that anaerobically-grown cells of the iron-reducing bacterium, *S. putrefaciens* IR-1, are electrochemically active using cyclic voltammetry [13], and that the bacterium can be cultivated in an electrochemical cell without additions of any terminal electron acceptors such as oxygen or Fe(III) or electrochemical mediator [12, 15]. In this study we describe the development of a mediator-less microbial fuel cell using *S. putrefaciens* IR-1.

2. Materials and methods

2.1. Microbial strains, medium and cultivation

Bacterial strains listed in Table 1 were grown on LB agar at 25 (*S. putrefaciens*) or 37°C (*E. coli*). For aerobic growth, cultures of 200 ml in 1000 ml flasks were shaken continuously on a rotary shaker (160 rpm). After 36 h of growth, the cells were harvested by centrifugation (13,000× g, 15 min, 4°C) and washed three times in a buffer (50 mM Na-phosphate buffer, pH 7.0, containing 0.1 M NaCl). For

Table 1
Bacterial strains used in this study

Strain	Source or reference
<i>S. putrefaciens</i> IR-1	(10)
<i>S. putrefaciens</i> MR-1 ^a	(22)
<i>S. putrefaciens</i> SR-21 ^{a,c}	(3)
<i>E. coli</i> NCIB 10772 ^b	

^a Strains were kindly supplied by D. Saffarini (Department of Biological Sciences, Univ. of Wisconsin, Milwaukee, WI, USA).

^b The strain has been maintained in the laboratory for a number of years.

^c A transposon mutant of *S. putrefaciens* MR-1.

anaerobic growth, approximately 10 liter of anaerobically prepared LB broth in a 13 L Carboy (Wheaton Scientific Co., Millville, NJ, USA) was inoculated with 100 ml of an aerobically grown overnight culture and incubated without agitation. Strict anaerobic culture techniques were employed for the anaerobic cultivation and buffer preparation [33]. After 96 h of growth, the cells were harvested under anaerobic conditions by a continuous centrifugation system (KSB-R, Kendro Laboratory Product, Newtown, CT, USA) at 13,000× g, 4°C. The cell paste was then washed three times in a buffer (anaerobically prepared, 50 mM Na-phosphate buffer, pH 7.0, containing 0.1 M NaCl).

The washed cells were re-suspended in the buffer to the desired cell concentration, estimated by reading optical density, which was converted to cell concentration using a pre-established calibration curve (dry cell weight, g vs. OD at 660 nm) for each strain.

2.2. Cyclic voltammetry

Direct electrode reaction of bacterial cells was examined by cyclic voltammetry using a conventional three-electrode electrochemical cell of 5 ml capacity [12]. A cyclic voltammogram (CV) of the bacterial cell suspension (0.36 ± 0.02 g dry cell weight/liter) was obtained using a potentiostat (CV-50W, BAS, West Lafayette, IN, USA). A glassy carbon working electrode (MF-2012, BAS), a platinum counter electrode (MW-4130, BAS) and a silver/silver chloride reference electrode (RE-5B, BAS) were used in the electrochemical cell [12]. Measurements were carried out at 25°C under anaerobic conditions at the scanning rate of 0.1 Vs⁻¹. All experiments were repeated more than five times.

2.3. Electrodes for microbial fuel cell

Both anode and cathode of the microbial fuel cell were graphite felt (50 × 50 × 3 mm in dimension, GF series, Electrosynthesis, E. Amherst, NY, USA). Platinum wire contacts (0.5 × 70 mm) were bonded to the electrodes with a conducting epoxy resin (EPOX-4, Electrosynthesis) which was cured at 150°C for 6 h. To aid initial wetting the electrode were boiled in deionized water. Electrodes were cleaned and stored in HCl (0.1 M) and thoroughly rinsed in deionized water prior to use [1].

2.4. Microbial fuel cell

The fuel cells were constructed using transparent polyacrylic material and were similar to those used previously [1,6]. The fuel cells had electrode compartments of approximately 20 ml capacity measuring $50 \times 57 \times 7$ mm. Each cell compartment had three ports at the top, for electrode wire, the addition and sampling of solutions, and gassing. The two compartments of each cell were separated by a cation-permeable ion exchange membrane (model 55165, BDH Lab supplies, Dorset, UK) sealed between 2 mm thick silicon rubber gaskets. The anode compartment was loaded with freshly prepared bacterial suspension and the cathode compartment was loaded with 50 mM Na-phosphate buffer (pH 7.0) containing 0.1 M NaCl [1,6,15]. Nitrogen and air were continuously purged through anode and cathode compartments to maintain anaerobic or aerobic conditions, respectively (flow rate: approximately 25 ml per min). The microbial fuel cell was immersed in a water bath to maintain temperature (25°C). In some experiment, 25 mM (final concentration) of methylene blue (BDH Lab supplies, Dorset, UK) was used as a mediator.

2.5. Potential and current measurements of the microbial fuel cell

Potential (V, volt) and current (I, ampere) of the fuel cells were measured using a voltmeter (Model 2000, Keithley, MA, USA) linked to a multichannel scanner (Model 2000-SCAN, Keithley). Data were recorded digitally on an IBM compatible personal computer via IEEE488 input/output system (Model KPC-488.2AT, Keithley) and a cable (Model CTMGPIB-1, Keithley). The voltmeter and the scanner were controlled using a control software (TestPoint[®], Keithley). This software was used to calculate the current and Coulombic yield (current (I) \times time (t)) produced from the fuel cells.

When the fuel cell was initially set-up, the electrodes were connected to the voltmeter to start the measurement of the potential. When the potential from the fuel cell had stabilized, lactate was added to the anode compartment to give the final concentration of 10 mM as the fuel and the potential development from the fuel cell at open circuit condition was monitored. To measure the current at a close circuit condition, the fuel cell was connected to its load resistance (R_{load}), and the resultant current (I) was measured as $I = V/R_{load}$, where V is the potential drop across R_{load} . A resistor (1 k Ω) was routinely used as the load resistor. The measured values were given as an on-line graphic display which showed values made every 2 min or as required.

2.6. High performance liquid chromatography

Organic acids were quantified by HPLC [12]. An HPLC (Model M910, Young-In Science, Korea) equipped with a sulfonated divinyl benzene-styrene copolymer column

(300×7.8 mm, Aminex HPX-87H, BioRad, USA) and a photometric detector (216 nm) was used. H_2SO_4 (0.01N) solution was used as the mobile phase at the flow rate of 0.6 ml per min.

3. Results

3.1. Cyclic voltammograms of the bacterial cell suspension

Cell suspensions (0.36 ± 0.02 g dry cell weight/liter) were prepared from cultures made under anaerobic and aerobic conditions to determine their electrochemical activities. Fig. 1 shows the cyclic voltammogram (CV) of whole cell suspensions of the bacterial strains used. Anaerobically grown cell suspensions of all *S. putrefaciens* strains used were electrochemically active. The wild-type strain, *S. putrefaciens* MR-1, showed the strongest activity, and the Fe(III)-reduction defective mutant *S. putrefaciens* SR-21 showed reduced but significant electrochemical activities. The CVs show that the bacterial cell suspensions have a redox potential of around -0.2 V against the Ag/AgCl reference electrode [34]. It is plausible that the electrochemical activity observed in this study is due to the cytochromes on the cell surface, with the orientation of the electrochemically active heme group toward the cell surface [13]. Electrochemical activity was not observed in either suspensions of *S. putrefaciens* strains grown under aerobic condition, nor the *E. coli* cell suspension.

3.2. Potential development from the microbial fuel cell systems

The anode compartment of the microbial fuel cells were loaded with freshly prepared *S. putrefaciens* IR-1 suspension (0.2 ± 0.02 g dry cell weight/liter) or *E. coli* NCBI 10772 (0.2 ± 0.02 g dry cell weight/liter) to observe potential development under open circuit conditions. Fig. 2 shows the change in the potential of the fuel cells. Before the fuel (carbon source) was added, approximately 0.21 volt and 0.03 volt were observed from the microbial fuel cell containing suspensions of *S. putrefaciens* IR-1 and *E. coli* NCBI 10772, respectively. The addition of lactate as the fuel to the cell containing *S. putrefaciens* IR-1 resulted in a rapid rise in potential up to 0.5 V. The microbial fuel cell containing *E. coli* NCBI 10772 did not show an increase in potential following the addition of glucose as fuel. However, when an electrochemical mediator (methylene blue) was added to the fuel cell containing *E. coli* NCBI 10772, an increase in potential up to 0.52 V was observed. The potential development from the fuel cell containing *E. coli* showed different pattern from the fuel cell containing *S. putrefaciens*. This may be due to difficulties in diffusion of the added mediator through the filamentous and compressed electrode structure.

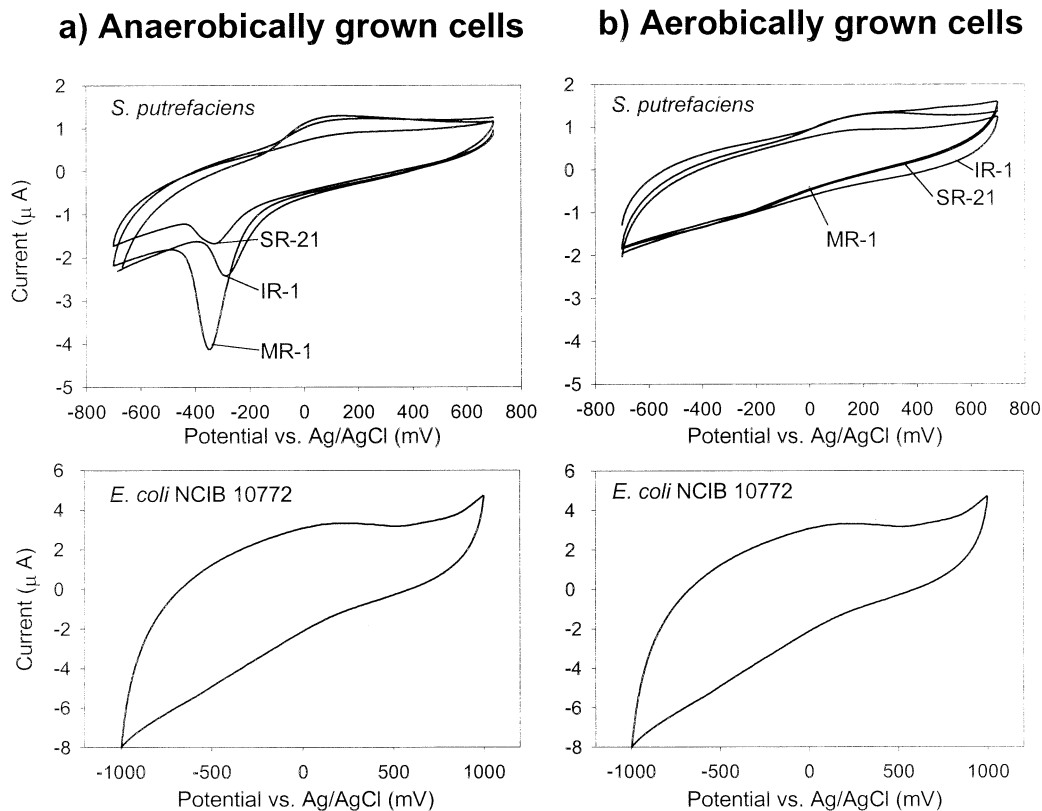


Fig. 1. Cyclic voltammograms of the experimental strains grown aerobically and anaerobically. The cell suspensions (0.36 ± 0.02 g dry cell weight/liter) were prepared under anaerobic conditions.

Similar experiments were carried out using the cell suspensions of the wild type and mutant strains of *S. putrefaciens* (cell concentration: 0.2 ± 0.02 g dry cell weight/liter). As shown in Fig. 3, before the addition of fuel, about 0.2 V of potential was observed. However when the fuel was added to fuel cell, rapid increase of potential was observed.

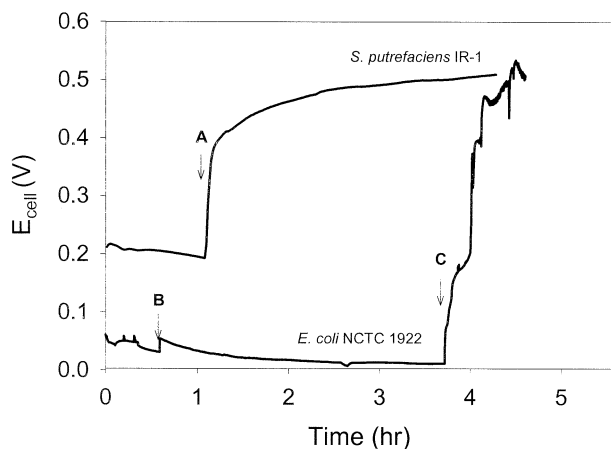


Fig. 2. Potential development from the fuel cell containing *S. putrefaciens* IR-1 (0.2 ± 0.02 g dry cell weight/liter) and *E. coli* NCTC 1922 (0.2 ± 0.02 g dry cell weight/liter). A: addition of lactate (10 mM); B: addition of glucose (10 mM); C: addition of methylene blue (25 mM). Apparent electrode surface area: 50 cm^2 .

This increase in potential is mainly due to the increase the ratio of reduced/oxidized cytochromes. The potential rise to around 0.44 V was observed in all fuel cells including mutant SR-21 upon the addition of the fuel. Strain MR-1 showed a similar increase in the potential as the other *S. putrefaciens* strains, IR-1 and SR-21. Fuel cells loaded only with lactate or glucose did not develop potential without the bacterial cell suspensions (data not shown).

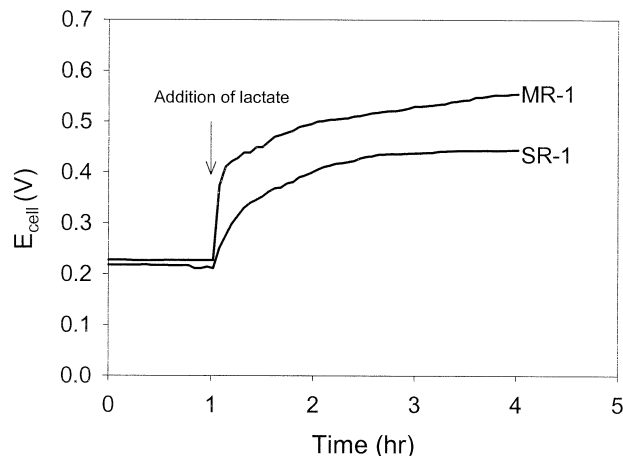


Fig. 3. Potential development from the fuel cell containing *S. putrefaciens* MR-1 and SR-21 (0.2 ± 0.02 g dry cell weight/liter, respectively) with addition of lactate (10 mM). Apparent electrode surface area: 50 cm^2 .

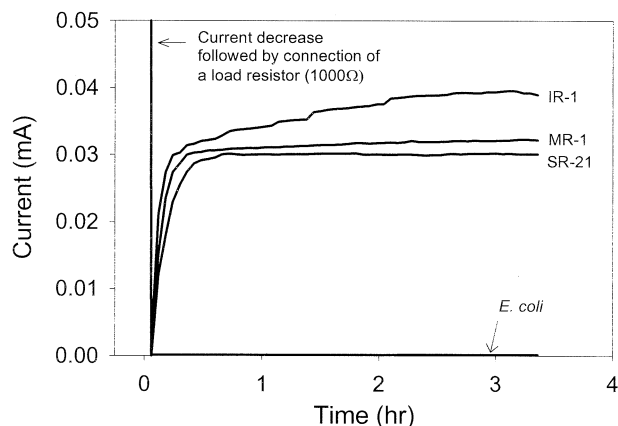


Fig. 4. Current generation from the microbial fuel cells using the experimental strains (0.2 ± 0.02 g dry cell weight/liter in each strain with 10 mM lactate). A 1000Ω of resistor was applied at the initial stage of the experiment. Apparent electrode surface area: 50 cm^2 .

3.3. Current generation from the mediator-less microbial fuel cell

The mediator-less microbial fuel cells were prepared using *S. putrefaciens* strains (0.2 ± 0.02 g dry cell weight/liter), and fed with 10 mM of lactate. When the potential reached a plateau, the fuel cells were discharged through an external resistance of $1 \text{ k}\Omega$, and changes in current at a close circuit condition were monitored. Fig. 4 shows the current generation patterns of the fuel cells using different *S. putrefaciens* strains. When the microbial fuel cell containing the strain IR-1 was loaded with the resistor, there was a rapid decrease in current output in the initial stage of the discharge. Following this stage, the current slightly rose and reached a plateau. Similar patterns were observed in the fuel cells containing other strains, but little differences were observed in current generation. The fuel cell containing *S. putrefaciens* IR-1 produced the highest current ($\cong 0.04$ mA). The strain MR-1 produced lower currents than *S. putrefaciens* IR-1, and the mutant strain SR-21 produced the lowest current ($\cong 0.031$ mA). The microbial fuel cell using *E. coli* in the absence of the mediator produced negligible amount of current (below 0.001 mA). The experiments were repeated at least 3 times. The results were highly reproducible in terms of the measured values from each trial. Further experiments were carried out using the strain IR-1 as a model strain that generated the highest potential and current in the mediator-less microbial fuel cell.

3.4. Sequential-batch operation of the mediator-less microbial fuel cell

A microbial fuel cell was set-up using *S. putrefaciens* IR-1 at 0.2 ± 0.02 g dry cell weight/liter with 10 mM lactate as the fuel. When the potential was reached a plateau, the cell was loaded with an external resistance of 1000Ω , and the change in current was recorded. When the current

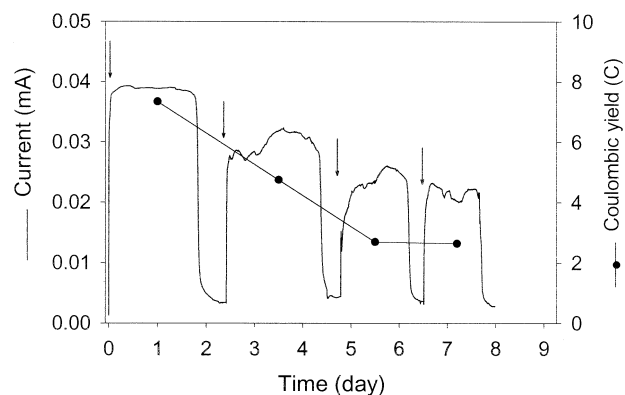


Fig. 5. Changes in current, and Coulombic yield from the microbial fuel cell containing *S. putrefaciens* IR-1 with a 1000Ω resistor (0.2 ± 0.02 g dry cell weight/liter). Arrows indicate addition of lactate (10 mM). Apparent electrode surface area: 50 cm^2 .

reached a background level below 0.005 mA, a small volume of sample (0.7 ml) was taken from both anode and cathode compartments. Then a volume of a concentrated lactate solution was added again to the anode compartment to obtain the final lactate concentration of 10 mM for a sequential-batch operation of the fuel cell (Fig. 5). The current vs. time profile from the microbial fuel cell was similar to the previous experiment. Lactate concentration was less than 0.01 mM in the anode compartment of the fuel cell when the current had reached the background value. Lactate was not detected in the cathode compartment that was separated from the anode compartment by an ion exchange membrane. These results showed that lactate was consumed in the anode compartment coupled to current generation and repeated lactate additions were coupled to current generation. However, a gradual decrease in both Coulombic yield and maximum current value were observed during the sequential-batch operation of the microbial fuel cell. This might be due to nutrient limitation, where Na-phosphate buffer with NaCl was used as the electrolyte. Another possibility for the decrease is that lactate and dead cells were metabolized and utilized by contaminants under the nutrient limited conditions rather than the *Shewanella* strains.

3.5. Effect of bacterial concentration

Microbial fuel cells were prepared using different cell concentration of *S. putrefaciens* IR-1. The potential development from the fuel cells were recorded separately before and after the addition of fuel (Fig. 6). The initial potential was proportional to the added bacterial cell concentration. This result implies that the potential development is directly related to the concentration of electrochemically active components on the bacterial cell surface. Theoretically, the maximum potential difference (i.e. potential between anode and cathode compartments in the microbial fuel cell containing *S. putrefaciens* IR-1) should be around 0.8 V, be-

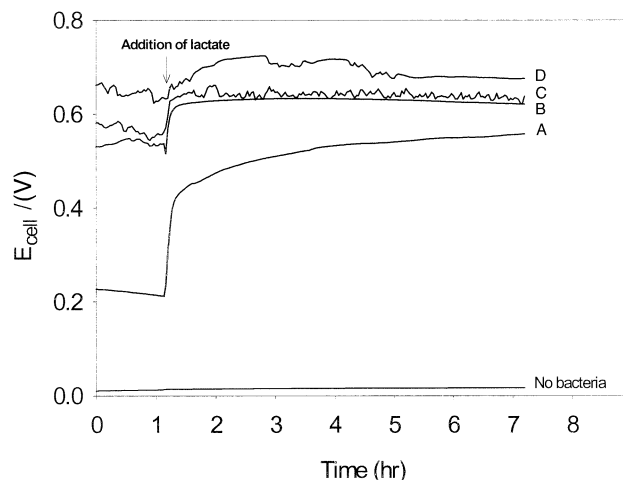


Fig. 6. Potential developments from the microbial fuel cells containing different concentrations of *S. putrefaciens* IR-1. The bacterial cell concentration in the microbial fuel cell; A: 0.2 ± 0.02 g dry cell weight/liter; B: 0.47 ± 0.02 g dry cell weight/liter; C: 1.06 ± 0.02 g dry cell weight/liter; D: 1.72 ± 0.02 g dry cell weight/liter. Apparent electrode surface area: 50 cm^2 .

cause the bacterial cell suspension showed a redox potential of about -0.2 V against Ag/AgCl electrode (approximately 0.0 V against the natural hydrogen electrode) by the cyclic voltammetry (Fig. 1) [13,34]. In this experiment, however, only 0.6 V was obtained. This low potential development could be explained by the poor operational performance of the fuel cell [1,32].

Using the same fuel cell configuration, additional experiments were carried out to explore the relationship between the bacterial concentration and Coulombic yield. When the potential was reached to the steady state after the addition of the fuel, the current through the $1 \text{ k}\Omega$ resistor was monitored for 12 h, and was converted to Coulombic yield (Fig. 7). Without the bacterial cells, no significant current generation was observed in the fuel cell. Calculation of Coulombic

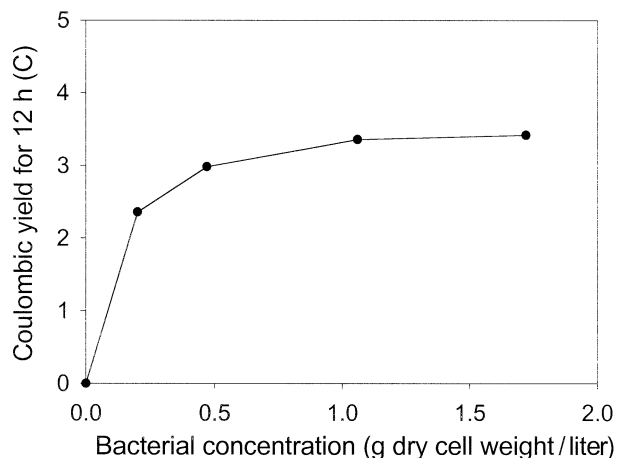


Fig. 7. Coulombic yields from the microbial fuel cells containing different concentrations of *S. putrefaciens* IR-1 for 12 h. Coulombic yield was measured with a 1000Ω resistor. Apparent electrode surface area: 50 cm^2 .

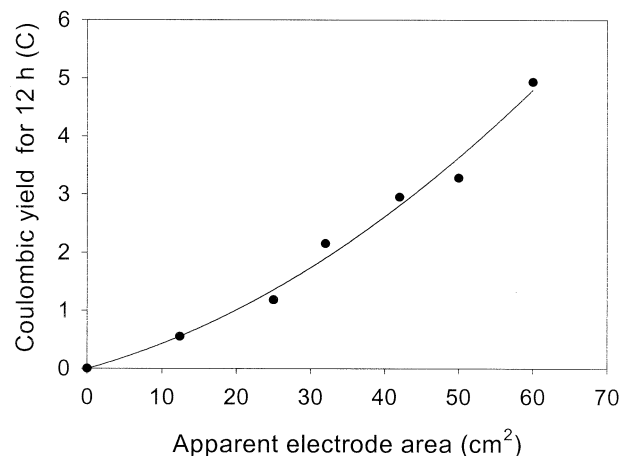


Fig. 8. Relationship between electrode surface area and Coulombic yield in a microbial fuel cell using *S. putrefaciens* IR-1. The bacterial cell concentration was 0.47 ± 0.02 g dry cell weight/liter and the Coulomb was measured under a 1000Ω resistor with 10 mM lactate.

yield showed a similar trend with the initial open circuit potential. Low Coulombic yield was obtained from the microbial fuel cell with the bacterial cell concentration lower than 0.5 g/liter . Over the range of 0.5 g/liter of bacterial concentration, the Coulombic yield reached an approximate equilibrium value (3.2 C for 12 h). A possible reason for this equilibrium could be attributed to a saturation of electrode surface by the bacterial cells.

3.6. Effect of the electrode surface area

A series of fuel cells with different anode surface areas up to 60 cm^2 were operated for 12 h using bacterial suspension ($0.47 \text{ g dry cell weight/liter}$) to measure Coulombic yield with the $1 \text{ k}\Omega$ resistor. As shown in Fig. 8, the Coulombic yield was increased proportionally with the increase in surface area of the electrodes. This result indicates that further increases in Coulombic yield might be possible with the incorporation of a larger electrode in the fuel cell construction.

4. Discussion

Several types of biofuel cells including microbial fuel cell and enzymatic biofuel cell have been well documented in the literature [32]. However, they need electrochemical mediator for efficient electron transfer of electron from microbial cells to an electrode [4,25,26]. In this study, we show evidence for direct electron flow from an iron-reducing bacterium to the electrode using the cyclic voltammetry technique and the fuel cell. Cyclic voltammetry showed that anaerobically grown cell suspensions of the Fe(III) -reducing bacterium, *S. putrefaciens*, are electrochemically active, while cell suspensions made under aerobic conditions shows no electrochemical activity. Potential was developed

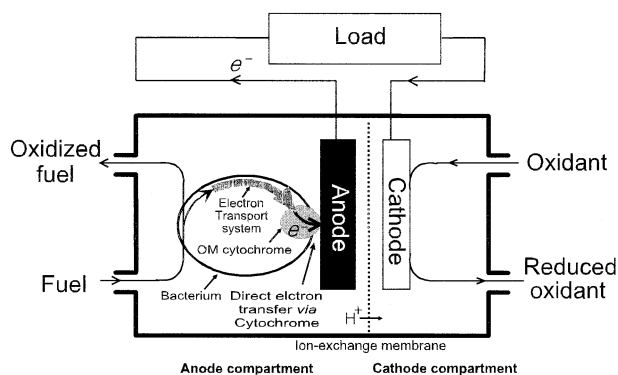


Fig. 9. A proposed schematic diagram of the mediator-less microbial fuel cell.

between the two electrodes of the open-circuited microbial fuel cell using *S. putrefaciens*, and current was generated when the fuel cell was loaded with a resistor. These results, therefore, suggests that current generation from the metal-reducing bacterium may depend on their electrochemical activity, principally oxidation of fuel (i.e. lactate) by the bacterial metabolism, and the direct electron transfer capacity from the bacterial surface to the electrode. In addition, the fuel cell current response of the strain IR-1 species is larger than that of MR-1. In the contrary, the CV current response of MR-1 is slightly larger than that of IR-1. The reason for this difference is not clear, but is tentatively attributed to the difference in affinity of the organisms toward the specific electrode material [9,29]. The sequential-batch operation of the microbial fuel cell showed that the current generation is fuel (lactate) dependent. These results show that *S. putrefaciens* can transfer electrons to the electrode without the aid of a mediator. A proposed schematic diagram of the electron flow in the mediator-less microbial fuel cell system is presented in Fig. 9.

The electrochemical activity is believed to be due to the direct electron transfer from the electron carriers located on the cell surface, possibly outer membrane cytochromes, to the electrode. The ferric reductase negative mutant, *S. putrefaciens* SR-21 developed potential differences between the anode and the cathode of the fuel cell at the open circuit condition and generated current when the two electrodes were connected through a resistor. The potential development and current generation by the mutant was lower than those by the wild strains, *S. putrefaciens* MR-1 and IR-1. These results suggest that the electrochemical activity of the bacterium is associated with the OM cytochromes rather than ferric reductase.

The current generation by the microbial fuel cell was dependent on the bacterial cell concentration and on the electrode surface area (Fig. 7 and 8). These results suggest that the electron transfer from the bacterial cells to the electrode also depends on physical contact between the cells and the electrode [12,13,15].

Electron donors used by *S. putrefaciens* under anaerobic conditions are limited to few organic acids including lactate

and pyruvate [10]. For this reason the microbial fuel cell using *S. putrefaciens* can not be used to convert complex organic fuels into electricity, though this microbial fuel cell can be used as a lactate biosensor with low specificity [15]. For a general use of the microbial fuel cell electrochemically active microbes with the ability to use wide range of electron donors are needed in pure cultures or in consortia. Microbial consortia are widely used in the field of biotechnology. We believe that more useful mediator-less microbial fuel cell system would be obtained by modifying and improving the fuel cell format and electrode. Studies are also being done to obtain microbial cultures which metabolize complex organic contaminants in the microbial fuel cell.

Acknowledgments

A part of this study was supported by a grant from the Ministry of Science and Technology, Korea (97-N1-01-01-A-03). H. J. Kim was a recipient of post-doctoral fellowship from the Korea Science and Engineering Foundation.

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