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Direct electron transfer of *Cellulomonas fimi* and microbial fuel cells fueled by cellulose

Wichean Khawdas,[1] Keigo Watanabe,[1] Hajime Karatani,[2,3] Yuji Aso,[1] Tomonari Tanaka,[1] and Hitomi Ohara[1,*]

Department of Biobased Materials Science, Kyoto Institute of Technology, 1 Hashigami-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan,[1] Department of Biomolecular Engineering, Graduate School of Science and Technology, Kyoto Institute of Technology, 1 Hashigami-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan,[2] and Kyoto Luminous Science Laboratory, Keihanna Plaza, Laboratory Wing, 1-7 Hikaridai, Seika-cho, Souraku-gum, Kyoto 619-0237, Japan[3]

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The strain of *Cellulomonas fimi* NBRC 15513 can generate electricity with cellulose as fuel without mediator using a single chamber type microbial fuel cell (MFC) which had 100 mL of chamber and 50 cm^2 of the air cathode. The MFCs were operated over five days and showed the maximum current density of 10.0 ± 1.8 mA/m^2, the maximum power density of 0.74 ± 0.07 mW/m^2 and the ohmic resistance of 6.9 kΩ. According to the results of cyclic voltammetry, the appearance of the oxidation or reduction peak was not observed from the cell removed solution. The fact is that *C. fimi* does not secrete mediator-like compounds, while the oxidation peak was observed at +0.68 V from the phosphate buffer containing the washed cell. The peak appearance was caused by the electron transfer activity of which corresponds to cytochrome *c*, and disappeared after adding antimycin A which inhibits the electron transfer activity. The cell was alive throughout the experiment as the result of a colony forming unit on Luria–Bertani agar plates. This was thought that cytochrome *c* was on the membrane surface of the living cell and played a role in the direct electron transfer between the cells and anode.

[**Key words:** Direct electron transfer; *Cellulomonas fimi*; Microbial fuel cell; Cellulose; Cytochrome *c*; Antimycin A]

Nowadays mankind is facing serious energy issue, which is why technologies with high efficiency of energy conversion are needed. Microbial fuel cell (MFC) is one of the important technologies for a sustainable society and can convert the chemical energy in biomass into electricity by microbial metabolism. Cellulosic biomass is one of the most abundant renewable sources of energy on the earth. A large amount of agricultural cellulosic waste products is discarded in the environment, and definitely wastewater contains a large amount of cellulose (1). If the chemical energy of cellulose in water can be converted to electric energy, this will help contribute to reducing the amount of petroleum usage and to realizing sustainable society. MFCs are a possible option for evaluating the electrical performances of cellulose materials; however, cellulose-fed MFCs particularly need to overcome the following three problems: the low efficiency of electric generation, suitable microorganisms for cellulose decomposition, and the addition of a mediator to facilitate bacterial electron transfer, which is rather expensive and harmful to the microorganisms. Previously, we reported that the addition of excess anthraquinone-2,6-disulfonate in the *Cellulomonas fimi*-based MFC resulted in the decrease of current density (2).

MFCs using *Shewanella oneidensis* require no mediator because the strain secretes flavins to the outside of the cell (3), and has outer membrane cytochromes which transfer electron from the cell to the electrode (4). This microorganism produces electrically conductive networks of pilus-like appendages called bacterial nanowires (5). This strain cannot use cellulose, whereas it mainly uses lactic acid as fuel for the MFCs (6).

The self-electron transferable microorganism has been extensively investigated because of the fact that it does not use external electron shuttles which are expensive and may affect the bacterial growth as mentioned previously (2). Some microorganisms also inherently transfer the electron using self-provided electron shuttles such as flavins, coenzyme A, cytochrome, and NADH (7–9). However, to the best of our knowledge, the cellulose-fed mediator-less MFC with *C. fimi* has not yet been reported.

Mediator-less double chamber MFC using *Pseudomonas aeruginosa* was reported (10) This microorganism is known as cytoplasmic membrane-embedded respiratory chain (11), which plays a role in the electron transfer. Similarly, mediator-less MFC with *Hansenula anomala* using glucose as fuel has been reported with the analysis of the isolated membrane containing redox enzymes such as lactate dehydrogenase, NADH-ferricyanide reductase, NADPH-ferricyanide reductase, and cytochrome b5 (12). Ren et al. (13) analyzed electricity generation and the microbial ecology of cellulose-fed MFCs of a two-chambered type by using the defined co-culture of *Clostridium cellulolyticum* and *Geobacter sulfurreducens*. Ishii et al. (14) inoculated the soil from rice paddy fields using cellulose as a carbon and energy source for a two-chambered MFC with *Clostridiales*, *Chloroflexi*, *Rhizobiales*, and *Methanobacterium*. In these two reports, the fuel solution contained vitamins,

* Corresponding author. Tel.: +81 75 724 7689; fax: +81 75 724 7690.
E-mail address: ohara@kit.ac.jp (H. Ohara).

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consequently it is difficult to consider that cell directly transfers electron or secretes mediator because vitamins can perform as mediator. Rezaei et al. (15) reported a sediment-based system of chitin and cellulose used as substrate. The sediment used in their study was the anaerobic sediment obtained from the Delaware Bay, and it contained several types of microorganism. Inoue et al. (16) reported the MFC using lignocellulosic biomass in cattle manure used as fuel, and explained the bacterial community of *Bacteroides* and *Clostridium* performing as electric generators. In these two types of research, microbial species have not been clarified, therefore there is a possibility that the fuel itself contains substances which function as mediators. Rismani-Yazdi et al. (17) examined the electricity generation using rumen microorganisms as biocatalysts and cellulose as the electron donor in two-compartment MFCs. While the microorganisms in rumen had not also been clarified in this research, the possibility that components of rumen fluid perform as mediator cannot be denied.

To the best of our knowledge, no study concerning MFCs using cellulose as fuel with direct electron transfer has been examined. We have reported the MFC using *Cellulomonas* spp. with cellulose as fuel in the previous report (2). In the report, *C. fimi* generated current approximately 0.9 mA (180 mA/m^2) in Luria–Bertani (LB) medium with 0.4 mM of anthraquinone-2,6-disulfonic acid disodium salt (AQDS), using cellulose as fuel. In this research, we have investigated the mediator-less MFC using cellulose in water and clarified the electron transfer mechanism from the cell to electrode.

MATERIALS AND METHODS

Bacterial strain and fuel solution *C. fimi* NBRC 15513 was used in this study. Luria–Bertani (LB) medium was prepared by 1 g of Tryptone (Nacalai Tesque Inc., Kyoto, Japan), 0.5 g of yeast extract (Nacalai Tesque), and 1 g of NaCl, dissolved in 100 mL of distilled water. The cellulose fuel solution was prepared by 0.5 g of cellulose powder (38 μm: through 400 mesh, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1.0 g of NaCl, dissolved in 100 mL of phosphate buffer (0.1 M, pH 7.0). A seed culture was prepared with LB medium cultivated at 30°C for 48 h, shaken at 120 rpm. The whole cell was centrifuged (MX-301, Tomy Digital Biology Co., Tokyo, Japan) at 2500 ×*g* for 5 min, at 4°C. Then the precipitate was washed with physiological saline for 3 times to remove the components of LB medium.

The chamber configuration of MFC and MFC operation The MFC chamber used in this study is a single chamber MFC which is the same as the previous report (2) (Fig. S1). The following is the points: The anode is a carbon felt (LFP-210, Osaka Gas Chemicals Co., Osaka, Japan). The air cathode has the effective area of 50 cm^2 with three layers, namely a catalyst layer consisting of Pt-supported carbon (IFPC40-III, Ishifuku Metal Industry Co., Tokyo, Japan) with perfluorinated resin (Nafion 510211, Sigma–Aldrich, St. Louis, MO, USA), a carbon-paper layer (TGP-120, Toray Co., Tokyo, Japan), and a polytetrafluoroethylene layer (PTFE, 60% dispersion, 31-JR, Du Pont–Mitsui Fluorochemicals Co., Tokyo, Japan) and the MFC chamber volume is 100 mL. Before assembling, the chamber components were sterilized by irradiating UV light on the detached components in a clean bench. The MFC was filled with 100 mL of cellulose culture containing the washed cell at an optical density (OD_{600}) of 0.2, and then nitrogen gas (N_2) was bubbled (100 mL/min for 3 min) into the culture in the MFC. As the reference experiment, same conditions without cell were operated. To support our idea regarding the electron transfer mechanism, the reference experiments in the *C. fimi*-free system were carried as described above. After preparation, the MFC was incubated for 5 days at 30°C. The experiment without the addition of the cell was performed as a reference. The current measurement was started at this time. The 1 mL of fuel solution was sampled to measure colony forming unit (CFU) and optical density (OD_{600}) every day. After sampling, the fuel solution in the MFC was purged with N_2 again.

Measurement of electric generation The circuit for measuring the generated current of the MFC was the same as our previous report (2) (Fig. S2). The following is the points: The electric current generated by the MFC was monitored using a digital multimeter (KEW 1062, Kyoritsu Electrical Instruments, Tokyo, Japan) and was automatically recorded by application software (model 8241, Kyoritsu Electrical Instruments). Polarization and power density curves were obtained using a rheostat at various external resistances (0–30 kΩ). The current had steadily risen to the maximum point when the electric current and voltage of each step were measured. The potential was recorded after it had stabilized approximately 1 min. The maximum power density and ohmic resistance of the MFC was acquired by the polarization curves.

Cyclic voltammetry The electron transfer of the cellulose fuel solution was confirmed whether it contains mediator-like compounds or not, the cellulose fuel solution was filtered with a polytetrafluoroethylene membrane filter (pore size = 0.22 μm, As One Co., Osaka, Japan) to remove the precipitates of cellulose for the cyclic voltammetry (CV) measurement. The direct electron transfer from cell surface of *C. fimi* to electrode was also studied, two samples were prepared by adding 0.5 g of the washed cell into 5.0 mL of phosphate buffer (0.1 M, pH 7.0). One sample was filtered to remove the cell before the CV measurement but another sample still containing the cell was measured without filtration. An effect on inhibition of antimycin A was carried out, more samples were prepared as mentioned above and then each sample was added antimycin A (from *Streptomyces* sp, Sigma–Aldrich) of 1 μg, 10 μg and 1 mg to 5.0 mL of phosphate buffer (0.1 M, pH 7.0) containing 0.5 g of washed cell. All the samples were kept at 30°C for 24 h under anaerobic condition and then CVs were carried out.

Each 5 mL of sample was poured into the CV test vessel, then N_2 was bubbled (100 mL/min for 3 min) in the sample not only before keeping the sample for 24 h but also before the CV measurement. To clarify the effect of remaining oxygen in the fuel solution, the cyclic voltammograms of the filtrated cellulose fuel solution of without bubbling N_2 at the start of operation and after the 5-day operated MFC were measured as an experimental reference.

The condition of the CV measurement was as follows: A glassy carbon (diameter: 3 mm), platinum wire, and Ag/AgCl (1 M KCl) (+0.20 V vs. NHE) were used as a working electrode, a counter electrode, and a reference electrode, respectively. The glassy carbon electrode was polished using 0.5-μm alumina powder (Wako Pure Chemical Industries), then sonicated to remove the remaining powder and washed again with distilled water in an ultrasonic bath (US-102, SND Ltd., Nagano, Japan) before the measurement, potential scan started at +0.5 V toward positively at 5 mV/s of the scan rate at room temperature. Furthermore, the alive cell before and after keeping the cell solution for 24 h was observed by the CFU on LB agar plates.

RESULTS AND DISCUSSION

MFC operation Fig. 1 shows the current generation of the MFC incubated over five days with and without *C. fimi*. The current density of the MFC inoculated with *C. fimi* increased gradually and reached a maximum of 10.0 ± 1.8 mA/m^2. However, the current density without *C. fimi* was 1.6 ± 0.8 mA/m^2. This fact indicates that *C. fimi* generates electricity from cellulose. Moreover, the inoculated cell of pre-cultured medium was washed with physiological saline to remove the component of LB medium consisted of yeast extract which contains riboflavin and other type of flavin compounds performing as mediator (18,19). Therefore, the electric generation of the MFC

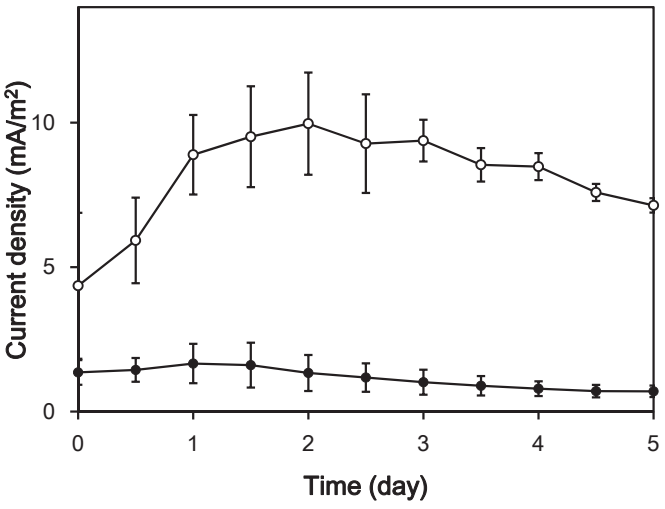

FIG. 1. Current generation of MFC with cellulose fuel solution containing 0.5 g of cellulose powder and 1.0 g of NaCl in 100 mL of phosphate buffer (0.1 M, pH 7.0). Open cycles indicate inoculated OD_{600} = 0.2 of washed cells, while closed cycles indicate no cells. MFCs are incubated at 30°C. Error bars indicate the standard deviations of the three-time experiments.

demonstrates that metabolite of *C. fimi* causes electron transfer or *C. fimi* secretes mediator-like compounds to the outer electron transfer system (20).

As mentioned above, the electrical performances of the cellulose-fed MFC using *C. fimi* with anthraquinone-2,6-disulfonate as an electron shuttle have been reported. Even though the current generation was relatively higher than that observed in the present study, this study has revealed that the cellulose-fed MFC using *C. fimi* can generate electricity without the requirement of an additional mediator.

Polarization A polarization curve of the MFC is consisted of three characteristic regions located at different current ranges: The charge transfer overpotential is in the region of low current density; the ohmic overpotential (ohmic resistance) is in a region of intermediate current density; lastly, the mass transfer overpotential is in a region of relatively high current density. The ohmic resistance is caused by ionic resistance in electrolyte, membrane, and by electronic resistance in the electrodes, current collectors, interconnects, and the electronic components (21). The ohmic resistance of the MFC can be calculated from the linear portion of the slope in the region of the straight line of the voltage versus current density of the MFC and this ohmic resistance is used as an evaluation parameter. The polarization curve of the MFC is shown in Fig. 2. The maximum power density was 0.74 ± 0.07 mW/m^2 and the ohmic resistance was 6.9 kΩ estimated from the linear portion (0.015–0.039 mA).

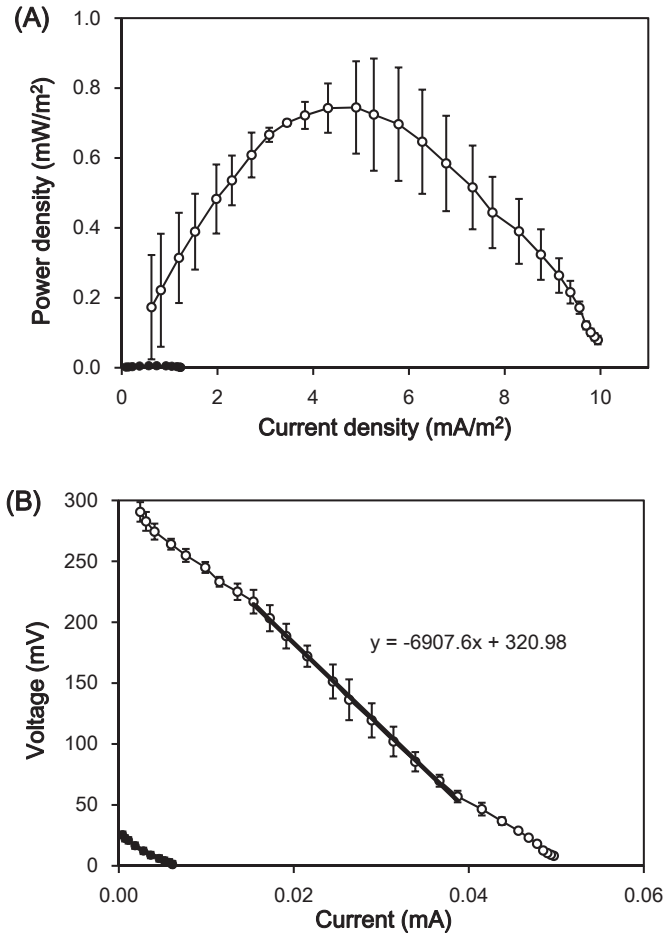

FIG. 2. Performance of the cellulose-fueled mediator-less MFC. (A) Potential of MFC and (B) polarization curves of MFC. Ohmic resistance is estimated from the linear portion of the curve (0.015–0.039 mA). Error bars indicate the standard deviations of the three-time experiments. Open symbols indicate containing cells (OD_{660} = 0.2) and closed indicate without cells.

Cyclic voltammetry The electron transfer classification of microorganisms can be classified into three types; Firstly, artificial mediators such as anthraquinone-2,6-disulfonate, potassium ferricyanide and neutral red; secondly, the bacteria-itself secreted mediators such as quinone and flavin derivatives; and finally, direct electron transfer from the cell surface to an anode (22).

Cyclic voltammograms of cellulose fuel solution without N_2 bubbling at the start of MFC operation (Fig. 3A) and after the 5-day operation (Fig. 3B) are shown. The reduction peak of oxygen at −0.60 V vs. Ag/AgCl (−0.40 V vs. NHE) was observed from both of CV measurements (23). Therefore, oxygen could perform as the electron acceptor in the MFC, since the oxygen still remains in the cellulose fuel solution. Owing to the result, the fuel solutions were then bubbled by N_2 later in this study.

No peak was observed in the cyclic voltammogram of the cellulose fuel solution (Fig. 4A). Therefore, this demonstrates that there is no electron transferring compound in the cellulose culture. Similarly, no peak appearance was observed from the filtrated phosphate buffer containing the washed cell (Fig. 4B). Therefore,

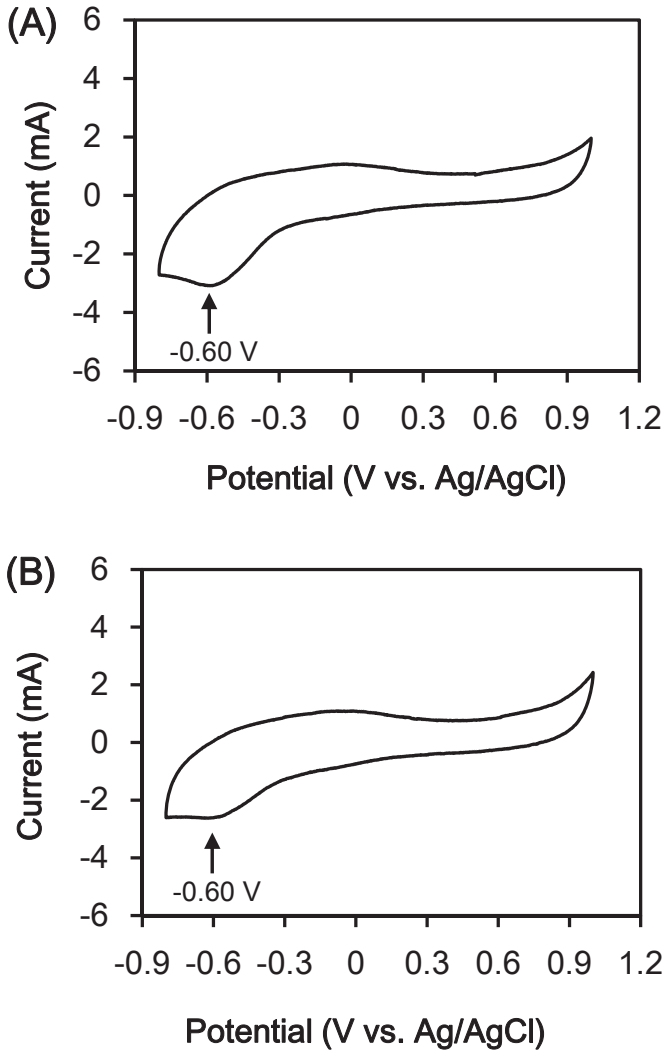

FIG. 3. Cyclic voltammograms of the cellulose fuel cell solution without N_2 bubbling. (A) Filtrated at the start of operation, (B) filtrate obtained after 5 days MFC operation. The reduction peak of oxygen at −0.60 V vs. Ag/AgCl was observed from both measurements.

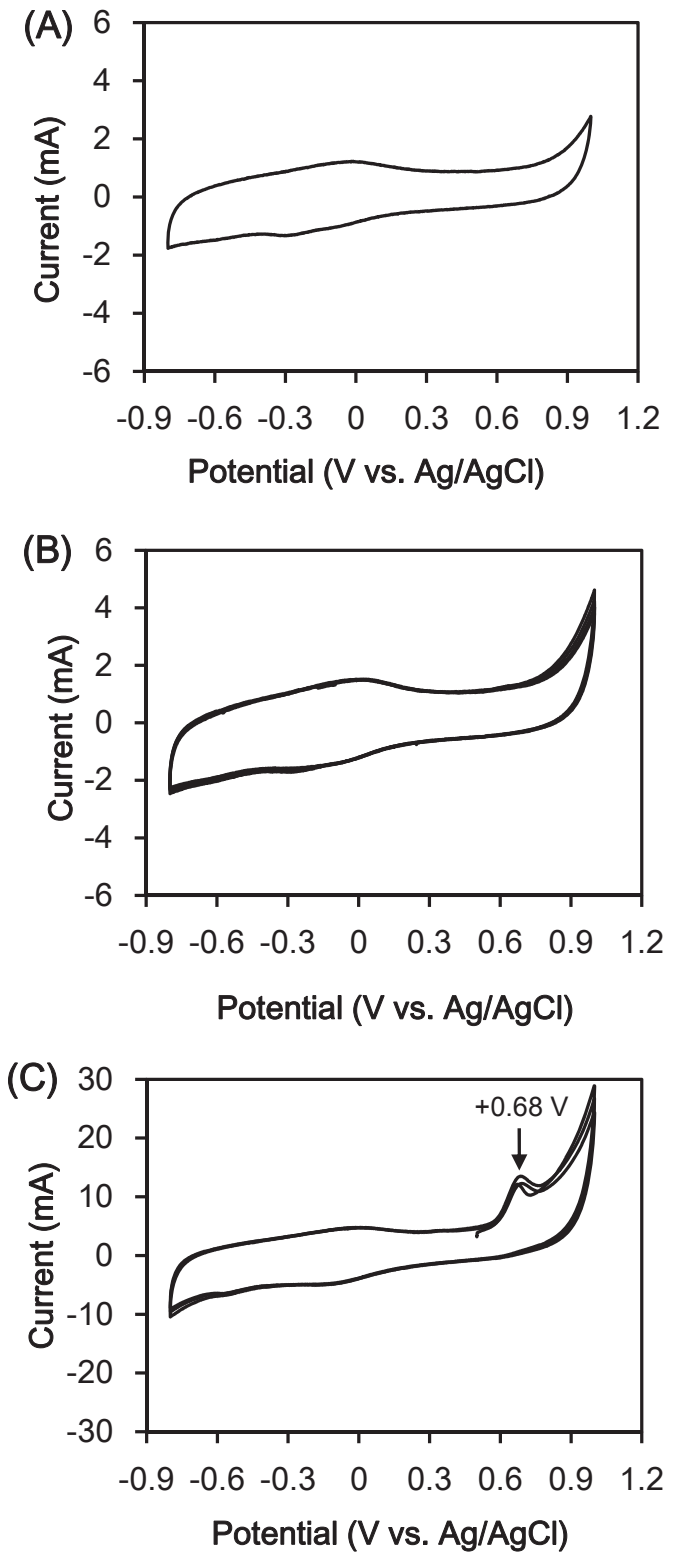

FIG. 4. Cyclic voltammograms of the cellulose fuel solution without N_2 bubbling at room temperature. (A) Filtrated cellulose fuel solution. (B) Washed cell was added in phosphate buffer (0.1 M, pH 7.0) and then filtrated. (C) Washed cell was added in phosphate buffer. Each CV was performed in three cycles. Potential scan started at +0.5 V in the positive direction at a potential scan rate of 5 mV/s. Oxidation peaks gradually decreased at +0.68 V vs. Ag/AgCl.

C. fimi does not secrete mediator-like compound. On the other hand, the washed cell containing in phosphate buffer provided oxidation peak at the potential of +0.68 V vs. Ag/AgCl (+0.88 V vs. NHE), but reduction peak was not observed (Fig. 4C). The peak observed at +0.68 V is due to the oxidation of the reduction form of cytochrome *c* (Fe^{2+}) which is usually present in the reduced form in an anaerobic condition as this experiment. No peak observation during the negative scan indicated that this reaction is almost irreversible and that this is similar to the irreversible oxidation of ascorbic acid at the electrode on which the reduction of dehydroascorbic acid never proceeds (24,25). This may be due to the low reduction of the oxidized cytochrome *c* (Fe^{3+}) at the electrode without any mediator. In the three cycles, the oxidation peaks gradually decreased except not adding the cell (Fig. 4A), this is probably caused by the adhesion of the cell on the electrode surface. In this measurement, the cell was still alive during the CV measurement (Fig. 5). These facts demonstrate that the alive cell donates electron to the electrode and oxidation reaction occurred during the metabolism of *C. fimi*. Similarly, it has revealed that the gram-positive organisms in the same phylum as *C. fimi* can transfer electrons from the inner membrane to the cell wall or membrane via periplasmic proteins, which involves the extensive scattering of soluble cytochrome *c* on the inner membrane (26,27); therefore, *C. fimi* has the possible ability of direct electron transfer from the cell surface to the anode. In other words, electro-active enzymes in the membrane can mediate the transfer of electrons generated during metabolism to the electrode directly without external mediator.

Antibiotics effect on electron transfer A previous study has reported that cytochrome *c* can enable direct electrical contact with an electrode (28). For instance, *C. fimi* was assumed to possess cytochrome *c* on its outer membrane surface, which enables direct transfer of electrons in the MFCs. Moreover, cells in *G. sulfurreducens* was disrupted to demonstrate the existence of

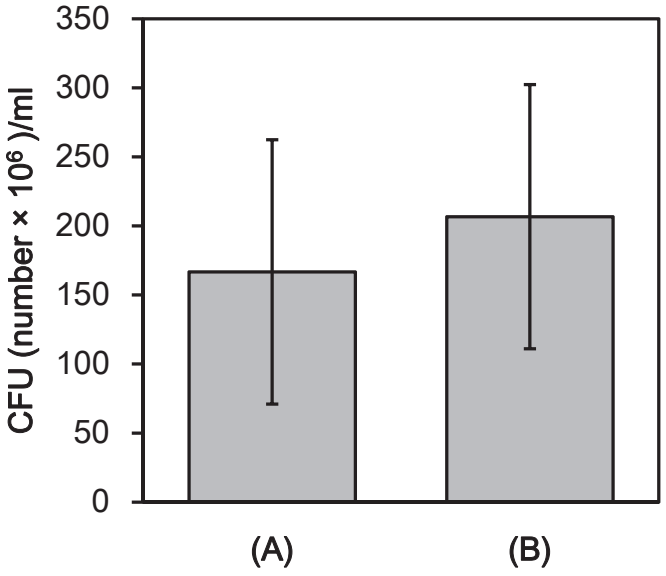

FIG. 5. Colony-forming unit of living cell on LB agar plate. (A) Immediately after suspended in phosphate buffer (0.1 M, pH 7.0), and (B) keeping 24 h just before measuring by cyclic voltammetry. Error bars indicate standard deviations of the three-time experiments.

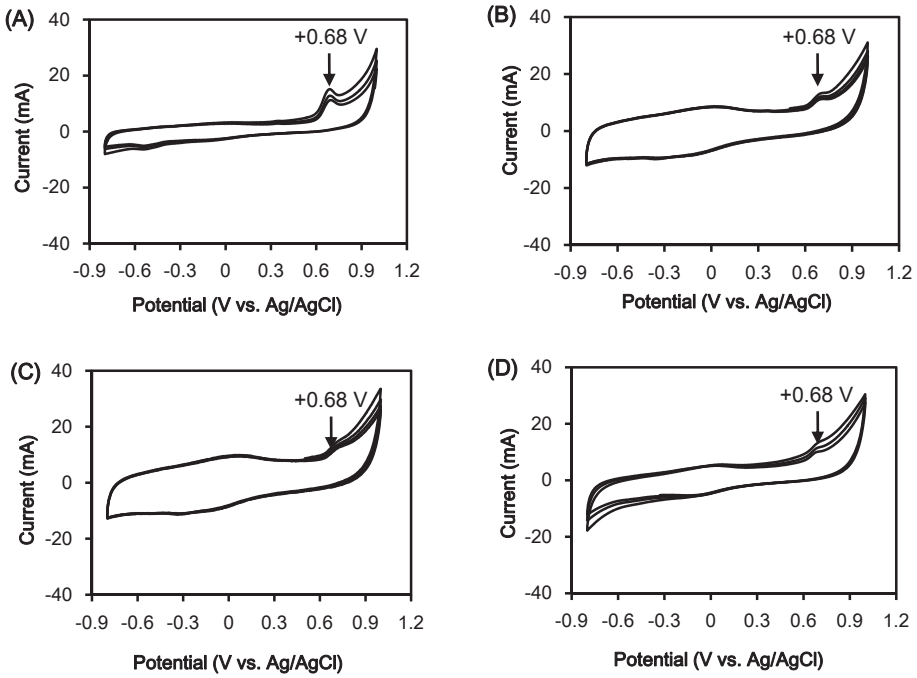

FIG. 6. Cyclic voltammograms of 5.0 mL of phosphate buffer (0.1 M, pH 7.0) containing 0.5 g of washed cells without antimycin A (A), with 1 μg of antimycin A (B), with 10 μg of antimycin A (C), and with 1 mg of antimycin A (D). Cyclic potential scan is repeated three times in each system. Potential scan started at +0.5 V in the positive direction at a potential scan rate of 5 mV/s. CV measurements are performed at room temperature. Oxidation peaks at +0.68 V vs. Ag/AgCl gradually decreased on each cyclic voltammogram.

cytochrome *c* (28). However, such method described in the previous study could not measure cytochrome *c* on the cell membrane surface of an alive cell. The measurement of cytochrome *c* from alive cell was assumed to be more efficient than dead cell or bacterial cell membranes in the present study because of its efficient performance in current generation. Notably, detection of cytochrome *c* from an alive cell was suggested via bacterial biofilm with spectroscopic analyses (29,30). Herein, an alternative technique was proposed to study the presence of cytochrome *c* from an alive cell. Antimycin A binds to the quinone reduction site of the cytochrome bc_1 complex and inhibits the electron transfer (31). The sample without adding antimycin A shows the peak position at around +0.68 V vs. Ag/AgCl. The oxidation peaks are supposed to be the electrochemical response of cytochromes of *C. fimi*. After adding into the sample, the peaks decreased obviously even in the addition of 1 μg of antimycin A (Fig. 6). It is thought that the electron transfer activity of cytochrome *c* in the respiratory chain is inhibited by antimycin A, and cytochrome *c* is located on the surface of the cell. As well as Fig. 4, the oxidation peaks gradually decreased as shown in the 3 time-scanned voltammograms because of the cell adhesion onto the glassy carbon electrode. As aforementioned, the much cell adhesion becomes resistance of electron transfer, thus electron may not be transferred to the entire cell surface of the whole *C. fimi* in the CV sample. The cell survival after adding antimycin A into the samples was measured by the CFU and the result showed that the cell was alive (Fig. 7).

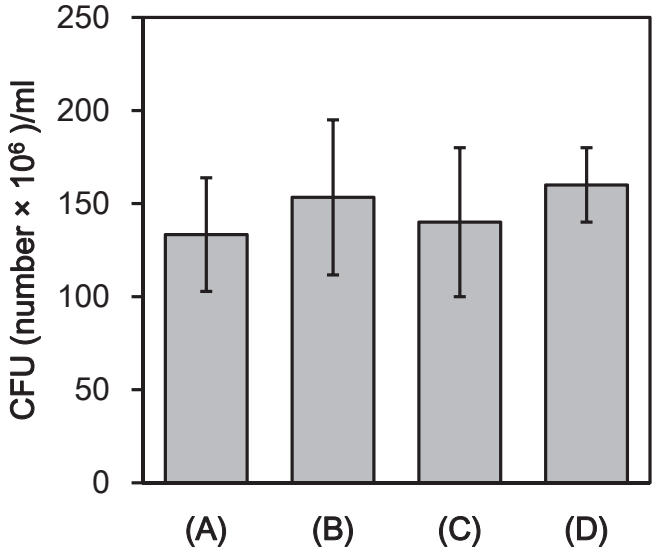

FIG. 7. Colony-forming unit of living cell on LB agar plate of 5.0 mL of phosphate buffer (0.1 M, pH 7.0) containing 0.5 g of washed cells without antimycin A (A), with 1 μg of antimycin A (B), with 10 μg of antimycin A (C), and with 1 mg of antimycin A (D). Error bars indicate the standard deviations of the three-time experiments.

In this report, it is demonstrated that *C. fimi* can generate electricity using cellulose as fuel without any mediator, and cytochrome *c* is strongly involved in the electron transfer from the cell surface to the electrode. In comparision with our previous research using the same MFC chamber and the strain except adding 0.4 mM AQDS (2), the ohmic resistance was 99.3 Ω and the maximum power density was 37.8 mW/m^2. Therefore, mediator was very effective to increase the maximum power of the MFC. However, the low efficiency of electric generation of MFC remains unresolved in this study. Possible solutions may incorporate the expression of cytochrome *c* or cellulase via genetic engineering, MFCs ware stacking, or increasing cell density.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.05.001.

ACKNOWLEDGMENTS

The authors declare no conflict of interest.

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