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Short communication

Membrane fluidity sensoring microbial fuel cell

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Abstract

A study has been performed to examine the effect of temperature and ethanolic stresses on the coulombic efficiency of a microbial fuel cell. The conventional-type fuel cell containing Gram-negative bacteria, *Proteus vulgaris*, was investigated as a model system. From current output measurements, it was found that the coulombic yields were altered by environmental stresses such as temperature shock or ethanol treatment to the bacteria. While high-temperature or ethanolic shock led to a remarkable decrement in coulombic output, the low-temperature shock induced a slight increase in microbial fuel cell efficiency. These results indicate that the membrane fluidity is affected considerably by environmental stress, which in turn affects the electron transfer process through the bacterial cell membrane to and from the electrode. This interpretation was confirmed by the cyclic voltammetric study of a mediator on an electrode surface modified with the lipids extracted from the membrane of *P. vulgaris* under the given stress. Markedly different electrochemical behaviors were observed depending on the environmental stress. A reciprocal relationship between coulomb output and the ratio of saturation/unsaturation of fatty acids has been observed. This is the first report, to our knowledge, that the structural adaptation of membrane fatty acids in response to the environmental shock can regulate the coulombic efficiency of a microbial fuel cell.
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Keywords: Microbial fuel cell; Environmental shock; Cyclic voltammetry; Homeoviscous adaptation

1. Introduction

For many years, fuel cells have been a novelty in energy production. From a theoretical viewpoint, the thermodynamic efficiency of a fuel cell is not limited by the temperature restrictions of the various electromechanical cycles, but is simply dependent on the differences in Gibbs free energy between reactants and products [1]. Most inorganic fuel cells being used or developed are composed of hydrogen and oxygen as anodic and cathodic reactants, respectively. While extreme temperature and corrosive electrolyte requirements of inorganic fuel cells impose stringent restrictions on the types of materials that can be used for catalysts and for construction of these fuel cells [2], microorganisms offer advantages of much milder operation temperature with fewer corrosive electrolytes.

A microbial fuel cell is a biological device that converts chemical energy into electrical energy; in this process, anodic electrode potential develops when electrons produced from the oxidation of substrates by microorganisms are transferred to the anode [3–5]. In the development of a microbial fuel cell, especially notable is the work by Bennetto et al. since early 1980s. They not only tested a number of microorganisms and mediators in an effort to construct better fuel cells, but also demonstrated that an appreciable amount of energy could be available [6,7]. Among tested dyes such as phenoxazine, phenothiazine, phenazine, indophenol, and bipyridium derivatives, thionin was found to be very effective in maintaining relatively high cell voltage. The high performance was achieved in a combination of *Proteus vulgaris* as a microorganism, thionin as a mediator, and glucose as a substrate, where a coulombic yield of 30–60% was obtained in a phosphate buffer at pH 7 [6,8]. Kim et al. [9] found that the current output pattern changed dramatically when their initial culture condition was altered by various carbon sources. Recently, an interesting improvement was made with neutral red, a phenazine derivative, when it was coupled with NADH oxidation [10].

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Direct electron transfer from a microorganism to the anode is very inefficient because electrons cannot penetrate the cell wall and membrane, but the inclusion of redox mediators provides the electron transfer mechanism for efficient fuel cell operation [11,12]. Therefore, it is essential to find out a suitable mediator that can be applied to a specific microbial fuel cell system for high efficiency [7]. An ideal mediator is a species that undergoes reversible electron transfer reactions with large negative formal potential to give higher open circuit voltage. It should also be stable in both oxidized and reduced forms so as not to be decomposed during long-term redox cycling. The polarity of the mediator should be soluble in an aqueous solution and be able to reversibly pass through the bacterial cell [5]. Satisfying many of those properties, phenothiazine derivatives have been widely used as promising mediators.

In the present study, we concentrate on the mediator–membrane interactions. It is well known that microorganisms vary their fatty acyl chain compositions for the maintenance of optimal membrane fluidity against various growth conditions, which is the so-called the homeoviscous adaptation [13]. On the basis of this mechanism, we have demonstrated herewith that the mediator–bacterial membrane interaction is a decisive factor controlling coulombic efficiency in a microbial fuel cell.

2. Experimental

2.1. Preparation of microorganism

P. vulgaris (ATCC 6059) was obtained from the culture collection of the Korean Culture Center of Microorganisms (KCCM) and kept on a nutrient agar plate at 4 °C. Experimental cultures were grown aerobically at 37 °C in a nutrient broth containing 3 g of beef extract and 5 g of peptone/l. Various temperature (10, 25, 46 °C) shocks or different ethanol treatments (0.5%, 1.0%, 3.0%) were applied for 3 h at the mid-log phase of the microorganisms. After the shock treatments, each bacterial cell was harvested by centrifuging at $3000 \times g$ for 5 min and washed twice with 0.05 M phosphate buffer solution (pH 7.0). The washed microorganisms were resuspended in the same phosphate buffer solution to give 20 mg (dry wt.) ml^{-1} for the experiments.

2.2. Structural analysis of fatty acyl components of membrane lipids

Experiments were performed on harvested whole cells by treatment with methanolic HCl to prepare fatty acid methyl esters [14]. Cells suspended with 0.5 ml of chloroform and 1.5 ml of 5% methanolic HCl solution were sealed in a teflon-lined screw-capped vial and heated in a dry oven at 72 °C for 24 h. Chloroform (3 ml) was added every 8 h, followed by mild sonication for 5 min. After concentration to dryness under nitrogen gas, samples were partitioned between water and chloroform, and the aqueous layer was washed several times with chloroform or hexane. The combined solutions were filtered through the glass wool. Prepared fatty acid methyl esters were subjected to gas chromatography analysis on a 25 M J&W scientific DB1 column using nitrogen as the carrier gas. The relative proportions of lipid components were calculated from integrated peak areas. The fatty acid identification and molecular weight were determined by gas chromatography/mass spectrometry analysis using a Hewlett-Packard HP 5973 MSD spectrometer interfaced with an HP 6890 gas chromatography.

2.3. Fuel cell assembly

Each fuel cell unit was composed of anode and cathode compartments (internal dimension $45 \times 45 \times 15$ mm) and separated by a cation exchange membrane (Nafion, Aldrich). Reticulated vitreous carbon (RVC, $30 \times 30 \times 12$ mm) plate was used as an anode. RVC has a physical structure that allows easy access of organisms and mediators to the electrode surface through the open network and large surface area for the reaction. Microorganisms and mediators were added to the anodic compartment, and 0.05 M phosphate solution (pH 7.0) was used as an anolyte. A platinum plate ($30 \times 30 \times 0.5$ mm) was used as a cathode material, and 0.1 M ferricyanide solution as a catholyte. Thionin was used as an electron transfer mediator. Each compartment was sealed by a 1.5-mm-thick silicone rubber gasket and held in a frame that was gently bolted together [15]. During the experiments, nitrogen was flowed through the cell compartments. Operation temperature was maintained at 37 °C in a water bath.

2.4. Electrical measurements

The cell discharge was done by a 560 Ω external resistor between an anode and a cathode. The discharge curve was recorded only after the open circuit voltage was stabilized with nitrogen gas flowing through the cell. The cell voltage with time was then recorded with a personal computer equipped with an analogue-to-digital board (Computer Boards, Mansfield, MA, USA). An output current was simply calculated using the Ohmic law, $I = V_{cell}/R_{load}$. When the cell voltage dropped to the background level, the cell was charged with a carbon source (1 μmol of glucose) for another discharge measurement. Generally, the cell voltage increases rapidly upon injection of the glucose and reaches a plateau level as long as there are enough carbon sources to be consumed by microorganisms, and then the cell voltage begins to gradually decrease. Actually produced electricity can be calculated by integrating the discharge curve with time, $Q = \int I dt$.

2.5. Cyclic voltammetric analysis of thionin in a lipid film

Total lipid extracts were prepared by using a modified Bright-Dyer method [16] from the *P. vulgaris* cells, to which

different environmental shocks were applied. The purified lipids were diluted with chloroform to give 10 mg ml^{-1} for the experiment. 0.5-mM solution of thionin was prepared with 0.05 M phosphate buffer solution (pH 7.0) using 18 MΩ of deionized water. The cast layer of the lipid was formed by applying the measured volume of lipid solution onto the glassy carbon electrode, which was allowed to dry. The prepared electrode was transferred into the deaerated 30 ml of thionin solution immediately. Lipids used for the control experiments were extracted from *P. vulgaris* cultured at 37 °C without shock treatment. The conventional three-electrode system (Autolab PGSTAT 30 potentiostat, Eco Chemie, The Netherlands) was used to record voltammograms. A platinum wire and an $Ag|AgCl|KCl_{(sat)}$ electrode were used as the counter and the reference electrode, respectively. The glassy carbon electrode with an area of 0.8 cm^2 was used as a working electrode. All measurements were carried out at room temperature and atmospheric pressure. Experimental results were analyzed using General Purpose Electrochemical System (GPES) software.

3. Results and discussion

3.1. Effects of the environmental stresses on the bacterial membrane composition and coulombic output in the microbial fuel cell

Practical evaluation on the coulombic output change was carried out in order to ascertain how environmental stresses affect microbial fuel cell efficiency. Fig. 1A shows coulombic responses of the microbial fuel cell containing *P. vulgaris* against the temperature shocks. Upon thermal shock treatment, coulombic yield was significantly altered. When the bacteria were subjected to a cold shock at 10 and 25 °C, coulombic output was changed just slightly (+0.04 and +0.07 C, respectively). However, with a high-temperature shock at 46 °C, coulombic output drastically declined (−0.44 C). This observation might be explained as a result of the bacterial membrane fluidity, and not as being linked to a thermal damage to it. Because *P. vulgaris* can grow even at this high temperature [17] and some cellular thermal damages could be protected by the various heat shock proteins [18,19]. Temperature-induced alterations in fatty acid compositions are known to play a significant role in the thermal compensation of membrane fluidity. Temperature effects on the composition of the membrane fatty acids have been thoroughly studied for *Escherichia coli*. The configuration of unsaturated carbons (cis- or trans-) or the hydrocarbon chain length of fatty acids is known to be responsible for temperature-adaptive changes, because configurations of unsaturated carbons and increased chain length influence the packing density of membrane lipids and hence the membrane fluidity [20]. As another example, mitochondria from a liver of 7 °C-acclimated trout were equally or more permeable to some substrates at 5 °C than mitochondria

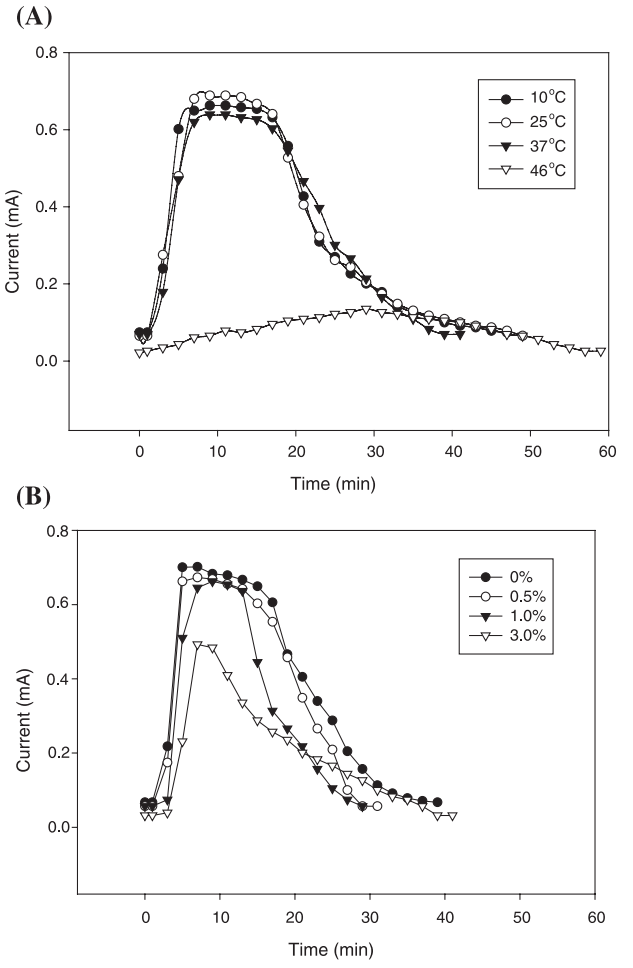

Fig. 1. Variation of current output with time through the 560 Ω external load for fuel cells containing *P. vulgaris* (A) with different temperature-shock treatment (B) with different ethanolic shock treatment. Organism concentration: 1 mg (dry wt.) ml^{-1}; 1 μmol of thionin; and 1 μmol of glucose were added.

from 20 °C acclimated trout assayed at 20 °C, indicating a nearly perfect compensation of barrier properties. Compensatory adjustments in water and nonelectrolyte permeability have also been reported [21]. There are numerous reports on the bacterial adaptations on the fatty acid compositions against alcoholic or thermal treatments [22–24]. Bacterial membrane is in many cases the primary site of alcohol (particularly ethanol) damage, although alcohol clearly affects the properties of all biological macromolecules to some degree. Ethanol-adaptive changes either strengthen the hydrophobic barrier or decrease the lipid sites available for passive leakage. The former includes the production of longer chain fatty acids and the latter includes a reduction of the lipid-to-protein ratio [23].

Effects of ethanolic shock on microbial fuel cell efficiency were also investigated in the same manner as temperature shock. The potency of ethanol as an inhibitor of a cellular function is directly related to its hydrophobicity as measured by the partitioning of ethanol between aqueous and hydrophobic environments, and the detrimental effects

of ethanol on bacterial cells appear to result from colligative effects of ethanol rather than from damage via a specific receptor [23]. Enteric bacteria such as *E. coli* are relatively sensitive and grow very little in ethanol concentrations above 6% by volume. However, at less than 4% concentration, their growth was not significantly inhibited by the ethanol treatment [24]. Concerning the lethal effect of high concentration ethanol treatment upon bacteria, we carried out the experiments below this upper limit value. Fig. 1B shows the current output pattern of *P. vulgaris* when the bacteria were treated with the ethanolic shock. These results were similar to the case of high-temperature treatments. The more ethanol molecules treated, the greater the number of observations of intense decrements in coulombic output.

Overall results are summarized in Table 1. Obvious decrements in coulombic output were observed when the high-temperature or ethanolic shock was applied. This information gives an explanation for the importance of the mediator–membrane lipid interaction in a microbial fuel cell operation. The alteration in fatty acyl chain composition is the most frequently observed response to various growth conditions in the microorganism. One of the most common responses of Gram-negative bacteria to high-temperature shock appears to be an increase in the degree of fatty acid saturation. Saturated fatty acids are packed more compactly due to their conformational property and show much higher melting points than their unsaturated homologs [20]. Ethanol molecules within the hydrophobic core of the membrane decrease the dielectric constant of the primary cell barrier, which in turn increase the ability of the membrane core to accommodate charged or polar molecules. The passage of molecules across the membrane is limited primarily by the energy barrier for their transfer from an aqueous environment to a hydrophobic environment. Ethanol decreases this energy barrier both by its effects on the aqueous milieu and by effects on the hydrophobic core, increasing the permeability of the membrane [25]. The adaptive response to ethanol treatment in the enteric bacteria is to increase the proportion of nonpolar lipids and average fatty acid chain length [23]. Therefore, the ratio of saturated fatty acids to unsaturated fatty acids can be used as a parameter for the bacterial membrane fluidity. Our experimental results illustrate cold shock treatment induces increasing of mediator-permeability in *P. vulgaris*, whereas the ethanolic or high-temperature shock treatments make the membrane more rigid so that it lowers the mediator-permeability through the membrane. Obvious reciprocal relationship between the saturated/unsaturated ratio and the coulombic output was found.

Table 1
Effects of thermal and ethanolic stress on the degree of saturation of the fatty acids and coulombic output variations in *P. vulgaris*

	Temperature shock				Ethanolic shock			
	10 °C	25 °C	37 °C	46 °C	0%	0.5%	1.0%	3.0%
S/U ratio[a]	1.84	1.75	2.27	2.60	2.28	1.94	2.59	2.88
Coulombic output	0.73	0.76	0.69	0.25	0.71	0.64	0.48	0.40

Relative amounts of unsaturated, saturated fatty acids were determined by the calculation of integrated peak areas on GC analysis at each condition.
[a] Saturated fatty acids/Unsaturated fatty acids.

3.2. Electrochemical behavior of mediator in the lipid film

The molecular penetration property of thionin to the lipid matrix was examined using cyclic voltammetry (CV) to prove the assumption that changes in the mediator–lipid interaction induce a change in the microbial fuel cell efficiency. Figs. 2 and 3 show the successive cyclic voltammograms of thionin depending on the stresses inside the lipid film, which is composed of total lipid extracts isolated from *P. vulgaris*. In the control experiment (Figs. 2A and 3A), thionin shows well-defined redox peaks corresponding to two-electron process. The gradual accumulation of thionin molecules into the lipid film is clearly observed from an increase of voltammetric peaks as the scan is repeated [26]. This phenomenon was explained by the hydrophobic interaction between the lipid film and thionin. The long alkyl chains of lipid molecules provide a hydrophobic environment for thionin to go into the film. Thionin inside the film is still electrochemically active, transferring electrons through the lipid layer. Highly charged species such as $Fe(CN)_6^{3-}$, in the mean time, are excluded by the lipid layer, giving very low current because of their hydrophilic nature [27]. Almost invariant voltammograms were obtained after heat treatment at 25 °C (Fig. 2B), showing the lipid structures were not affected. This explains why the discharging pattern of a fuel cell was almost identical (Fig. 1A). A dramatic change, however, has occurred after the temperature shock at 46 °C (Fig. 2C). Almost no thionin redox peaks were present, indicating that thionin molecules were excluded by the lipid layer. These results finely coincide with the very low efficiency of the fuel cell at this temperature (Fig. 1A).

Fig. 3 shows cyclic voltammograms of thionin without (A) and with 0.5% (B) and 3.0% (C) ethanolic shock. A 0.5% ethanol treatment led to a slightly less amount of incorporation of thionin, reflected in the slightly decreased fuel cell efficiency (Fig. 1B). A large change was observed after 3.0% ethanolic shock. Thionin did not easily penetrate the lipid layer, and thus the amount of incorporated thionin was quite limited, which explained the lower coulombic output of the fuel cell at this condition (Fig. 1B). In our experiments, the current density indeed dropped down after the shock (Fig. 1). Current in *y*-axis could also be interpreted as a current density because the same electrode was used. Actually, the coulombic output dropped quite a bit after the shock. If the mediator does not transfer electrons effectively because of the membrane fluidity change, most of residual electrons should be consumed by the bacteria themselves for their survival using their metabolic pools through many cellular redox enzymes (NAD/NADH, FMN/$FMNH_2$, FAD/$FADH_2$ dependent proteins) and electron transport chains for oxidative phosphorylation, which inevi-

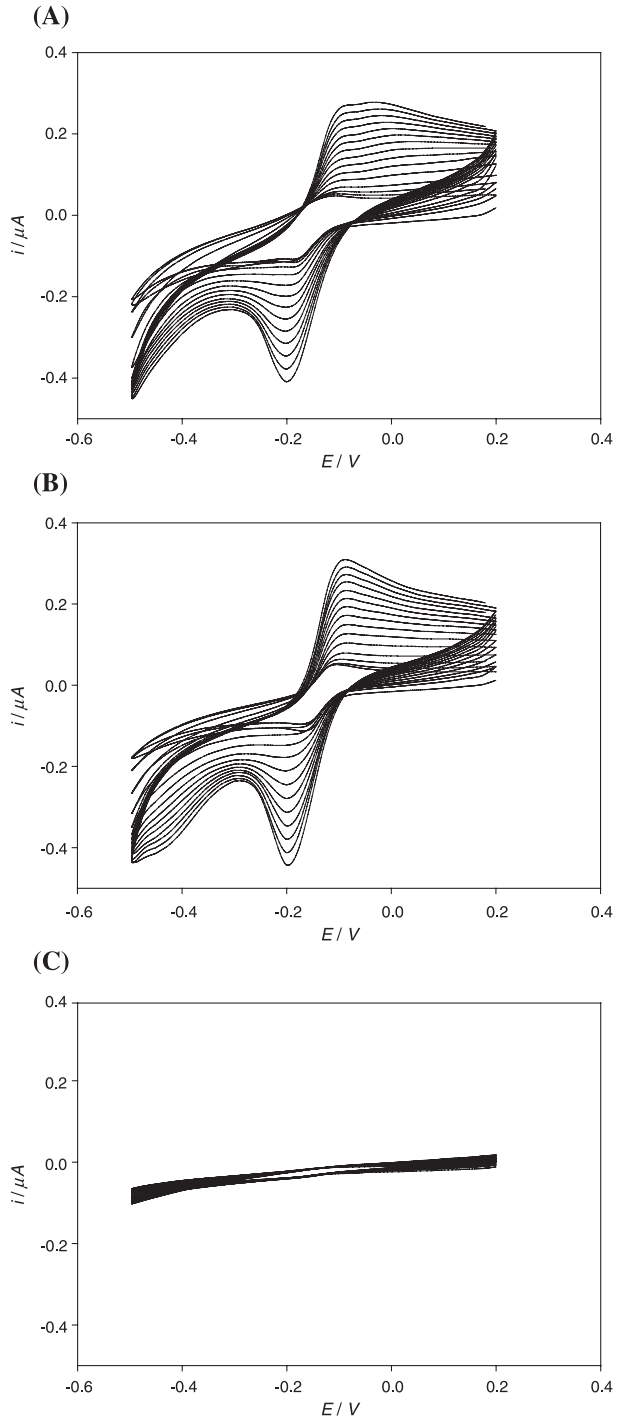

Fig. 2. Cyclic voltammograms of 0.5 mM thionin at a glassy carbon electrode coated with a total lipid film (4.3×10^{-7} mol/cm^2) of *P. vulgaris*. (A) Control, (B) 25, and (C) 46 °C temperature shock treated. Scan rate: 100 mV s^{-1}.

tably caused the electricity-producing ability of the fuel cell to be lower. Therefore, the lower columbic output could be observed. The action of electron transport mediator must be the primary factor for the operation of microbial fuel cell.

The effect of these environmental shocks on the fuel cell operation and electrochemical behavior of thionin could be explained by the compositional change of the fatty acids in response to the adaptive change of membrane fluidity. Heat or ethanolic shocks induced the high ratio of saturated per unsaturated fatty acids of bacterial membrane lipids, which caused thionin to be less permeable to the inside of the cell. Rigid lipid molecules also form a tightly packed layer on the

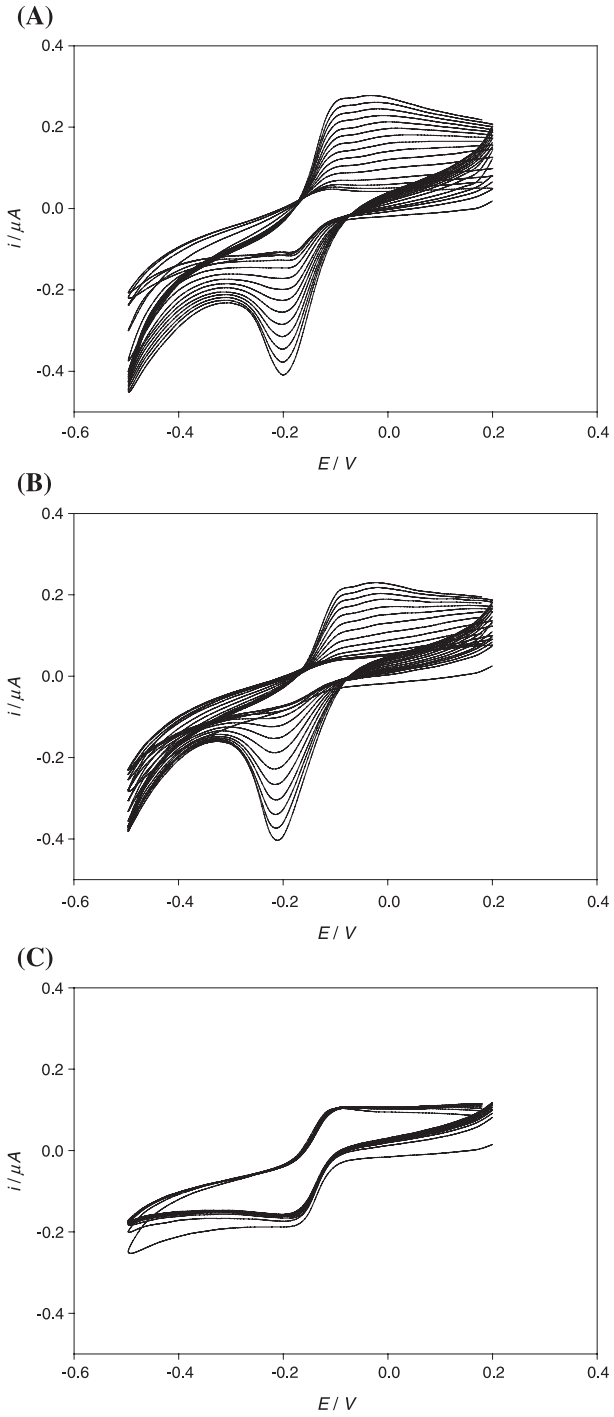

Fig. 3. Cyclic voltammograms of 0.5 mM thionin at a glassy carbon electrode coated with a total lipid film (4.3×10^{-7} mol/cm^2) of *P. vulgaris*. (A) Control, (B) 0.5%, and (C) 3.0% ethanolic shock treated. Scan rate: 100 mV s^{-1}.

electrode surface, through which thionin hardly penetrates. Abundant evidence of relevant correlation between membrane lipid properties and molecular diffusion has been discovered in previous works [28–30]. Tanaka and Tamamushi [31] studied the electron transfer reaction at an electrode covered with a layer of hydrophobic substance, and showed that they could be a simplified model of the electron transfer between microorganisms and mediators in microbial fuel cells. In an experiment using a ferrocene-containing synthetic lipid, there is a dramatic increase in redox current coming from the enhancement of the apparent diffusion coefficients. They have also demonstrated that the tuning of lipid bilayer rigidity regulates ferrocene-mediated electron transfer reactions of glucose oxidase embedded on the lipid cast films [32]. These changes in diffusional property were associated with the bilayer rigidity, which, in turn, was connected with the membrane phase transition temperature [33]. In a microbial fuel cell, the mediator should freely pass through the bacterial membrane to show high efficiency. The efficiency of microbial fuel cell is highly dependent on the action of electron transfer mediators [7,34]. However, the mediator is largely restricted in being incorporated into the lipid layer extracted from bacteria after high-temperature or ethanolic shock, causing a decrease in efficiency. These results suggest that mediator–membrane lipid interaction is an important factor for the efficient microbial fuel cell operation.

4. Conclusion

These studies have shown that the coulombic efficiency of the microbial fuel cell can be regulated by the change of bacterial membrane fluidity. The result was also confirmed with cyclic voltammetric measurements in terms of molecular penetration property of mediator to the cellular membrane lipids. In the microbial fuel cell, coulombic efficiency is highly dependent on the permeability of electron transfer mediator through the cellular membrane as well as the intrinsic metabolic characters of bacteria. The membrane lipids were adapted in response to changes in the environmental or physicochemical conditions, and thus, heat or ethanolic shocks induced an increase in the ratio of saturated per unsaturated fatty acids in the cellular membrane lipids. Then, this adaptive change allows the hydrophobic molecules like electron transfer mediators to be more impermeable into the membrane lipids. Further research on the bacterial adaptation of the membrane against various environmental shocks will provide valuable information for the operation and applications of microbial fuel cells.

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