J. Microbiol. Biotechnol. (2001), **11**(5), 863–869

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Optimization of the Performance of Microbial Fuel Cells Containing Alkalophilic *Bacillus* sp.

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Received: May 24, 2001
Accepted: July 30, 2001

Abstract A systematic study of microbial fuel cells comprised of alkalophilic *Bacillus* sp. B-31 has been carried out under various operating conditions. A significant amount of electricity was generated when redox mediators were used. Among the phenothiazine-type redox dyes tested, azure A was found to be the most effective both in maintaining a high cell voltage and for the long-term operation. The maximum efficiency was obtained at ca. 50°C, giving an open circuit voltage of 0.7 V. A small change in temperature did not significantly affect the cell performance, but a rapid decrease in performance was observed below 20°C and above 70°C. It was noticeable that fuel cell efficiency and discharge pattern depended strongly on the carbon source used in the initial culture medium. Regardless of the initial carbon sources, only glucose and trehalose were utilized as substrates. Galactose, however, was not substantially utilized except when galactose was used in the initial medium. Glucose, in particular, showed 87% coulombic efficiency, which was the highest value ever reported, when *Bacillus* sp. was cultured in a maltose-containing medium. This study demonstrates that highly efficient microbial fuel cells can be constructed with alkalophilic microorganisms by fine-tuning the operating conditions and by carefully selecting carbon sources in the initial culture medium.

Key words: Microbial fuel cell, alkalophile, *Bacillus* sp., initial culture condition

As a clean energy source and an alternative to the conventional energy production device, microbial fuel cells have drawn much attention, in which intact microorganisms are utilized as catalysts for converting chemical energy into electrical energy [1-3, 7, 9, 23-25]. Electrons are initially trapped as a form of reduced intermediates following the degradation of substrate, and transferred to the anode. Redox mediators as well as microorganisms constitute an essential part of the microbial fuel cell, in that a fuel cell operation could be largely improved by selecting a redox mediator that can act best as an electron shuttle between an anode and a microorganism [4, 5, 7]. An ideal mediator is a species that undergoes reversible electron transfer reactions with a large negative formal potential to give a high open circuit voltage. It should also be stable in both oxidized and reduced forms and not be decomposed during a long-term redox cycling. The polarity of the mediator should be such that it makes the molecule have enough solubility in an aqueous solution and reversibly pass through the bacterial cell membrane [2]. Having satisfied many of these properties, phenothiazine-derivatives have been widely used as a promising mediator, although an interesting improvement has recently been made with neutral red, a phenazine-derivative, in which it was coupled with NADH oxidation [16, 17].

Among many kinds of microorganisms, *Proteus vulgaris* is one of the well known and intensively studied microbial catalysts. Reduction rates of various organic compounds by *P. vulgaris* have been measured, and a coulombic efficiency of 50–65% has been reported with a thionin-glucose system [2, 7, 22]. The use of *Anabaena variabilis* or *Synechococcus* sp. should also be noted, in which light energy was utilized instead of carbon sources [18, 26], as well as *Shewanella putrefaciens*, where microbial fuel cells were constructed without electron transfer mediators [10].

While most studies have been performed under neutral pH conditions, operations under alkaline conditions may be advantageous for cathodic reactions, where oxygen reduction is usually employed, and whose reaction rate is high under a strong acidic and alkaline condition with suitable catalysts, thereby resulting in a better fuel cell operation. Generally, alkalophilic microorganisms are widely distributed in the high pH ecosystem in nature, therefore developing their own physiology and characteristic membrane structures. For this reason, alkalophiles have been employed

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in various industrial areas such as the tannery industry, silver recovery, medical uses, detergent additives, food industry, waste treatment, and chemical industry. Many alkalophile enzymes, such as cellulases, proteases, β-lactamases, α-amylases, and xylanases, have also been used as biocatalysts under various extreme environments [14, 15].

Despite of all these advantages, fuel cell study with alkalophilic organisms is very rare. Akiba *et al.* [1] was the only group to report preliminary results by using strains of alkalophilic *Bacillus* organisms. To the best of our knowledge, no systematic work to apply alkalophiles to the microbial fuel cell has been done. In this work, for a systematic study, microbial fuel cells were constructed from alkalophilic *Bacillus* sp. B-31 in order to find optimum operating conditions. Also, the effect of the initial culture conditions on the fuel cell efficiency was thoroughly examined, as demonstrated in our previous work with *P. vulgaris* [11].

MATERIALS AND METHODS

Chemicals

Azure A (Fluka, Switzerland), methylene blue (Aldrich, U.S.A.), and thionin (Janssen Chimica, Geel, Belgium) were used without further purification. Other reagents were of the reagent grade or better.

Preparation of Microorganisms

Bacillus sp. B-31 (KCTC 1754) was obtained from the culture collection of KCTC (Korean Collection for Type Cultures) and grown aerobically at 30°C in a LB broth medium containing 0.5% of Na_2CO_3 (pH 9.2). To test initial carbon source effects on the fuel cell efficiency, the microorganism was cultured in a medium that contained 10 g of NaCl, 10 g of trypton, 5 g of Na_2CO_3, and 5 g of a carbon source per liter: Galactose, glucose, mannose, maltose, sucrose, or trehalose were used as an initial carbon source and each was added to the medium after sterilization. Each culture containing a carbon source was subcultured daily with 5% inoculums for three days. Cells at their early stationary phase were harvested by centrifugation at 3,000 ×g for 10 min and washed three times with a 0.05 M sodium bicarbonate buffer of pH 9.2 at 4°C. The washed microorganisms were resuspended in the buffer to give 20 mg (dry wt) per ml for the experiments.

Fuel Cell Assembly

Each cell unit is composed of anode and cathode compartments (internal dimensions of 45×45×15 mm) and separated by a cation-exchange membrane (Nafion, Aldrich, U.S.A.) [12]. A reticulated vitreous carbon (RVC, 30×30×12 mm) plate was used as an anode. The RVC had a physical structure that allowed easy access of organisms and mediators to the electrode surface through the open network and provided a high surface area for the reaction. The anolyte and catholyte were composed of 0.05 M sodium bicarbonate buffer and 0.1 M ferricyanide solutions, respectively. Microorganism and mediators were added to the anodic compartment. A platinum plate (30×30×0.2 mm) was used as a cathode. Each compartment was sealed by 1.5-mm-thick silicon rubber gaskets, and held in a frame that was lightly bolted together. During the experiments, nitrogen flowed through the cell compartments to keep oxygen from entering the cell and for effectively mixing the solution. The operation temperature was maintained constant in a water bath.

Electrical Measurements

The discharge curve was recorded only after the open circuit voltage was stabilized with nitrogen gas flowing through the cell. Discharging was done by connecting an external resistor of various values between the anode and the cathode to obtain a polarization curve. The cell voltage with time was then recorded with a personal computer, equipped with an analogue-to-digital board (Computer Boards, Mansfield, MA, U.S.A.). A current was simply calculated by using the Ohm's law, $I=V_{cell}/R_{load}$. When the cell voltage dropped to the background level, the cell was charged with a carbon source for another discharge measurement. Generally, the cell voltage increases rapidly upon injection of carbohydrates and reaches a plateau level as long as there are enough carbohydrates to be consumed by microorganisms, and then the cell voltage begins to decrease gradually. An electricity actually produced can be calculated by integrating the discharge curve with time, $Q=\int Idt$. The coulombic efficiency is calculated as the ratio of the output charge obtained from the fuel cell to the theoretical value calculated from the complete oxidation of a substrate.

RESULTS

Operation Temperature Effect

The effect of operation temperature on the polarization curve was investigated in the range of 20 to 60°C (Fig. 1). Improving as temperature rose, the optimal operation was observed at 50°C. This might be due to the increased rate of electron transfer of a mediator. However, a rapid deterioration of the fuel cell performance was observed at above 70°C, thus indicating that the operation efficiency depended on the metabolic status within microorganisms. Azure A showed the best performance among the mediators tested.

Effect of Initial Carbon Source

Figure 2 shows discharging characteristics with time through the external load (560 Ω) for the cells containing azure A

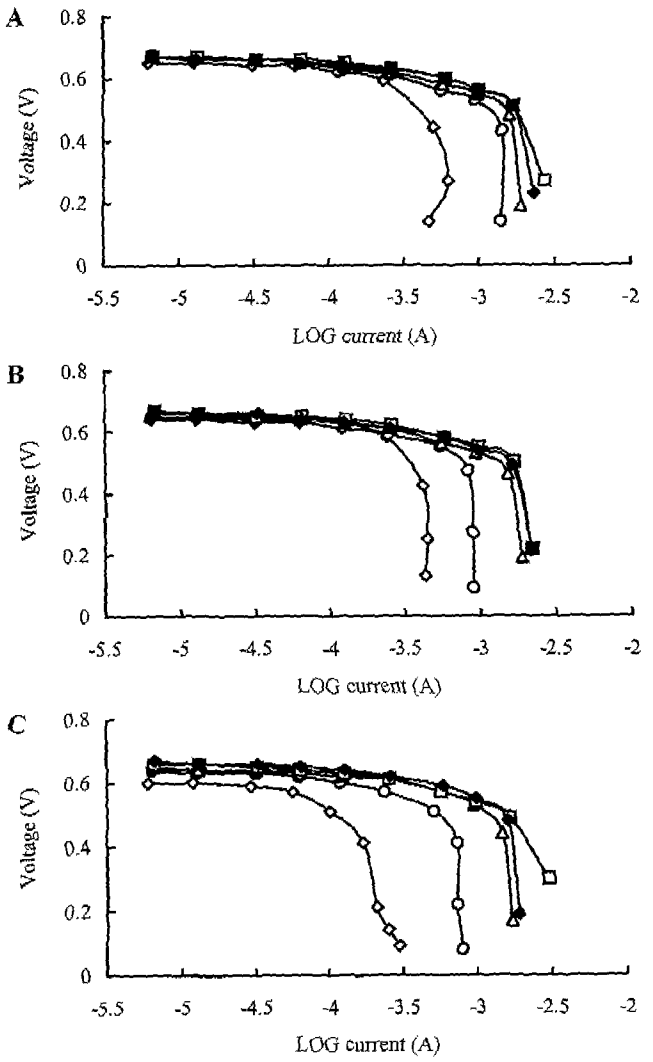

Fig. 1. Current-voltage curves for fuel cells containing *Bacillus* sp. B-31
At 20°C (◇), 30°C (○), 40°C (△), 50°C (□), and 60°C (◆) with azure A (A), methylene blue (B), and thionin (C) as a mediator. Organism concentration: 2 mg (dry wt) ml^{-1}; 1 μmol of mediator and 100 μmol of glucose were added initially.

together with various carbon sources. Alkalophilic *Bacillus* sp. was grown at different initial culture conditions. The cell was fully discharged before each carbon source was added. We denoted Glu-ini, for example, to describe the condition where glucose was initially added to culture as a carbon source. Likewise, Gal-ini, Man-ini, Mal-ini, Suc-ini, and Tre-ini represent that galactose, mannose, maltose, sucrose, and trehalose were initially used as a carbon source, respectively. The amount of monosaccharides was fixed at 1 μmol and disaccharides at 0.5 μmol for the direct charge comparison, because twice more charge was required to fully oxidize disaccharides than monosaccharides. Current output did not drop to zero even after a long-time discharge but rather reached a constant value of ca. 0.1 mA in all cases. Coulombic efficiency was calculated by measuring charge over this constant level, which was regarded as a background.

Among the carbon sources tested, only glucose and trehalose were significantly utilized regardless of initial culture conditions. Utilization of other carbon sources was strongly dependent on the initial conditions. Galactose, for example, did not show any measurable current output except for the Gal-ini condition, but only 0.3 C or equivalently 12.7% of coulombic efficiency was obtained even in this case. All the carbon sources were utilized under only Gal-ini condition where glucose showed a highest coulombic output of 1.7 C (72.9%) and other sources were less utilized, ranging from 0.13 C for maltose to 0.55 C for sucrose (Fig. 2A). Gal-ini cells may be metabolically adapted for the glucose utilization, inducing more general carbohydrate transporters in the membrane of alkalophilic *Bacillus* sp. On the other hand, with the Glu-ini condition (Fig. 2B), glucose and trehalose showed a similar discharge pattern and coulombic outputs (0.93 C and 0.89 C, respectively). Maltose and other carbon sources, however, showed small or negligible outputs. With the Man-ini condition (Fig. 2C), glucose showed the highest coulombic output of 1.3 C (57.2%), and mannose and trehalose were somewhat utilized. Interestingly, the current output from mannose was low, but it was maintained for a long period of time, showing 0.7 C (33.0%). With the Mal-ini condition (Fig. 2D), both glucose and sucrose were the best-utilized, showing more than 86% coulombic efficiency that amounted to ca. 2.0 C.

Utilization of maltose and trehalose under different initial culture conditions was noteworthy. While trehalose was somewhat utilized regardless of the culture conditions, maltose showed a negligible utilization except for the Mal-ini condition. This strongly indicated that efficiency was dependent on the linkage pattern of two glucose units in disaccharides, where trehalose is connected by an $\alpha1\rightarrow\alpha1$ linkage and maltose by an $\alpha1\rightarrow\alpha4$ linkage, suggesting that the linkage pattern was an important factor in a fuel cell operation. With the Suc-ini condition (Fig. 2E), glucose, sucrose, and trehalose showed similar coulombic outputs of ca. 1.0 C. Other carbon sources did not show any significant output. It was noted that the discharge pattern for glucose was rather different from other carbon sources, in that current output at ca. 0.2 mA was maintained for a long time. With the Tre-ini condition (Fig. 2F), trehalose showed the highest coulombic output of 1.0 C (45.2%) and only a limited amount of mannose was utilized.

Table 1 summarizes coulombic efficiencies calculated from coulombic outputs for each initial culture condition. From 1.0 μmol of a monosaccharide and 0.5 μmol of a disaccharide, a theoretical value of 2.316 C should be produced, since 24 and 48 electrons were generated by complete oxidation of a mono- and disaccharide, respectively, thus the coulombic output directly reflected the coulombic

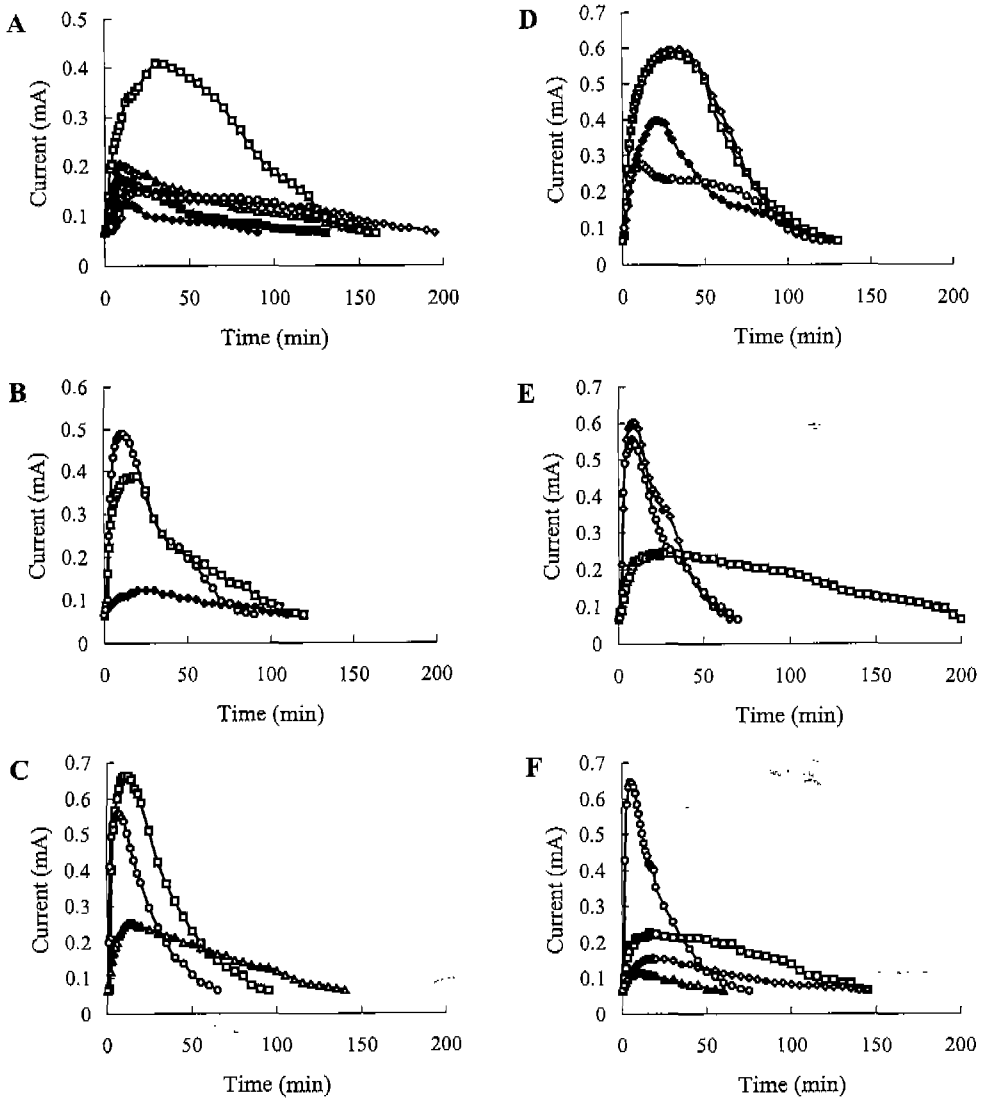

Fig. 2. Variation of current output with time through the 560 Ω external load for fuel cells containing *Bacillus* sp. B-31.
Galactose (■), glucose (□), mannose (△), maltose (◆), sucrose (◇), and trehalose (○) were added. Cells were grown on galactose (A), glucose (B), mannose (C), maltose (D), sucrose (E), and trehalose (F)-containing media. Organism concentration: 1 mg (dry wt) ml^{-1}; 1 μmol of azure A and 1 μmol of different carbohydrates were added.

efficiency. The best efficiency of 86.7% was obtained when glucose was used for the maltose-adapted cells. These phenomena could be explained by the fact that the different metabolic adaptations induced by the initial carbon sources

Table 1. Coulombic efficiency for various carbon source conditions.

Initial carbon source conditions	Utilized carbon sources (%)					
	Galactose	Glucose	Mannose	Maltose	Sucrose	Trehalose
Galactose	12.7	72.9	23.8	5.8	24.0	21.0
Glucose	–[a]	40.3	–	8.7	–	38.4
Mannose	–	57.2	33.0	–	–	32.4
Maltose	–	86.7	–	43.6	86.4	37.4
Sucrose	–	44.6	–	–	38.8	33.9
Trehalose	–	40.1	5.1	–	17.2	45.2

[a]Below 0.1 C of coulombic output was obtained.

had directly or indirectly influenced some of the redox reactions among the many metabolic reactions within the alkalophilic *Bacillus* sp. Therefore, the induced redox reactions might have changed the coulombic efficiency via the mediators; however, its exact mechanism needs to be explored.

Charging and Discharging Characteristics

Repetitive fuel cell operations were tested with various carbon sources. The constant amount of a carbon source was added after the current output reached a background level. As expected from the initial carbon source experiments, only glucose and trehalose showed a significant current output when the microbial culture was carried out under the LB broth medium. This result assumes that the enzymatic systems related with glucose or trehalose were expressed constitutively, but other carbon sources were utilized after the induction took place. Although glucose showed a better performance than trehalose, the output current gradually decreased and eventually reached a background current level after five or six charging cycles (Fig. 3).

Effect of Different Mediators

The effect of three phenothiazine mediators on the fuel cell performance was tested with the system containing 50 μmol of glucose for a long-term operation (Fig. 4). The current output gradually decreased and reached a background level after 1 day. The decreasing pattern and rate strongly depended on the type of mediators used. Azure A showed the best performance, and methylene blue gave the similar discharge behavior as with azure A at the initial stage but the current output rapidly decreased to a certain extent. Thionin did not give a high current output even at the initial stage of discharging. These results show that the choice of a proper mediator is a decisive factor for a better fuel cell operation.

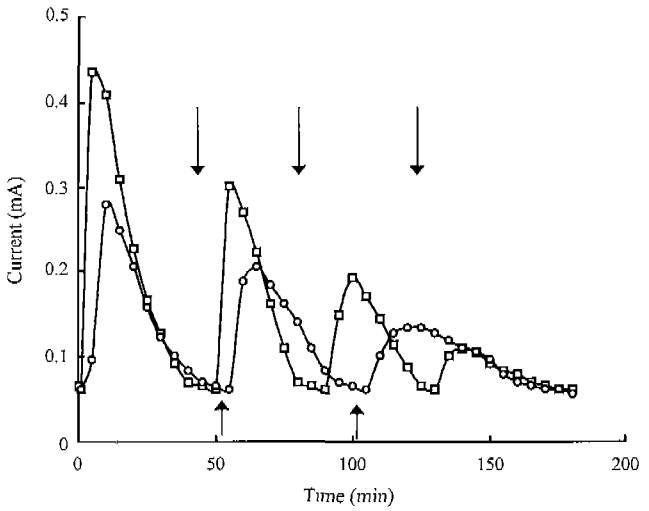

Fig. 3. Variation of current output with time through the 560 Ω external load for fuel cells.
Bacillus sp. B-31 and different substrates on the repetitive addition of 0.5 μmol of glucose (□) and trehalose (○) are marked by the arrows. Organism concentration: 1 mg (dry wt) ml^{-1}; azure A: 1 μmol.

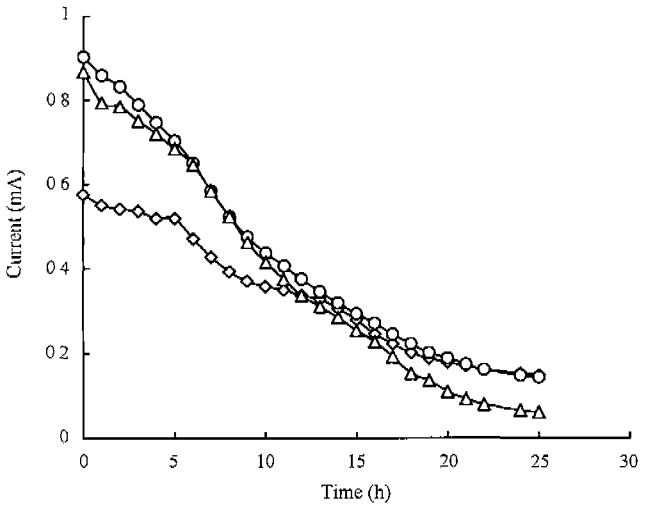

Fig. 4. Variation of current output with time through the 560 Ω external load for fuel cells.
Bacillus sp. B-31 when azure A (○), methylene blue (△), and thionin (◇) were used. Organism concentration: 3 mg (dry wt) ml^{-1}; 1 μmol of mediators and 50 μmol of glucose were added initially.

DISCUSSION

Previous study conducted by Akiba *et al.* [1] has established the potential importance of alkalophilic microorganisms as catalysts for a microbial fuel cell. They showed that the ability of alkalophilic *Bacillus* strains to reduce redox mediators could be applied to the construction of microbial fuel cells under alkaline conditions. An appreciable amount of current was drawn when the cell was operated at the optimum growth pH of the microorganisms, with glucose as a substrate, although outputs were about an order of magnitude less than those obtained by using neutralophilic organisms. Systematic studies with alkalophilic microorganisms were not carried out.

In the present study, we carried out a systematic study on the fuel cell performance made of alkalophilic *Bacillus* sp. B-31. Various mediators and substrates were investigated under various operation temperatures and initial culture conditions. Among the *Bacillus* strains screened, *Bacillus* sp. B-31 was selected as a potential microorganism, since it gave the highest current output. We tested three phenothiazine dyes (azure A, methylene blue, and thionin) for a representing redox mediator. Azure A showed the highest performance in our system, even though it showed a poor electron transfer in other fuel cell systems containing different microorganisms [4]. Moreover, azure A functioned well through a wide range of temperatures. This might be

due to the fact that *Bacillus* sp. has a high pH adaptability and unique metabolic systems or membrane lipid compositions for the penetration of azure A. In order to survive under different circumstances, microorganism needs to have some kind of metabolic systems and adaptability for various substrates. The current output pattern changed dramatically when their initial culture condition was altered by various carbon sources. This indicated that the fuel cell performance could easily be manipulated by a simple change in culture conditions. Kim *et al.* [11] suggested that these phenomena would be induced by different metabolic adaptation states resulting from initial carbon sources, which directly and/or indirectly influences some of the redox reactions within the microorganism. We found that the maximum fuel cell efficiency was obtainable with glucose as a substrate and maltose as an initial carbon source. In contrast to the effective electron generation ability of *Bacillus* sp., some problems were found in the repetitive fuel cell operation. When substrates were supplied after their complete consumption, the coulombic output was gradually decreased and eventually reached a background level. At this point, an additional supply of microorganism was needed for the continuous fuel cell operation. However, this problem was found to be insignificant in the previous reports made on a microbial fuel cell produced from *P. vulgaris* [7]. Much of the interests on the alkalophiles are concerned with the expected alkalostability of their metabolic cycle and the cell integrity. Several properties are shared by all of the extreme alkalophiles studied so far. These include the composition of membrane lipids and the ratio of membrane lipid/protein, very high levels of respiratory-chain components in the membrane, a generally more acidic amino acid composition of proteins, which are excreted into the external milieu, and a Na^+ cycle that facilitates solute uptake and pH homeostasis. Any or all of these properties could be prerequisites of alkalophilicity. The alkalophiles were suggested by the apparent requirement for proton addition or the operation of natural proton pumps at a very high pH level [19]. The model accommodates the finding that most extreme aerobic alkalophiles possess very high concentrations of the respiratory-chain components. In one model, the proton-pumping elements of the respiratory-chain polypeptides can directly interact with the proton-binding elements of the F_0-ATPase within the membrane, so that the proton is sometimes transferred directly before being able to escape into the bulk. The ATPase and a proton-pumping respiratory-chain component might be trapped together in transient membrane domains that arise from specific properties of the particular coupling membrane. In addition, the frequency of localized proton transfer might be envisioned as being similar to the multicollisional events described by Hackenbrock and colleagues to account for the rates of mitochondrial electron transport [6, 8]. The very high concentration of respiratory-chain components may provide the mechanism in which the alkalophiles maximize productive proton-transferring collisions between respiratory-chain components and the ATPase. Moreover, the alkalophiles possess somewhat high membrane lipid/membrane protein ratios, and low obstructive proteins. The alkalophile membrane also consists of high cardiolipin concentrations and a fatty acid composition that is consistent with a fluid membrane [20, 21]. These properties might be needed to maximize diffusion-based collisions. However, such a leaky membrane property might allow various kinds of electron transfer mediators to pass freely through the bacterial membrane [13]. In fact, various mediators function with *Bacillus* sp. B-31 despite their structural diversity. This postulation may be supported by the fact that only a few mediators could be used in the case of the *P. vulgaris* fuel cell. In addition, a high concentration of respiratory-chain components may increase the efficiency of mediators, which is connected to a total fuel cell efficiency. For the reasons stated above, this *Bacillus* sp. B-31 system probably showed high coulombic yields and availability for various mediators. It is suggested that bacterial membrane fluidity could be related to mediator actions. Detailed analysis of this possibility is in progress.

Acknowledgment

This work was supported by the Korea Research Foundation (Grant no. 1999-015-DP0258) SDG.

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