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Electrochemical Regeneration of FAD by Catalytic Electrode Without Electron Mediator and Biochemical Reducing Power

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Abstract We created a new graphite-Cu(II) electrode and found that the electrode could catalyze $FADH_2$ oxidation and FAD reduction coupled to electricity production and consumption, respectively. In a fuel cell with graphite-Cu(II) anode and graphite-Fe(III) cathode, the electricity was produced by coupling to the spontaneous oxidation of $FADH_2$. Fumarate and xylose were not produced from the enzymatic oxidation of succinate and xylitol without FAD, respectively, but produced with FAD. The production of fumarate and xylose in the reactor with FAD electrochemically regenerated was maximally 2–5 times higher than that in the reactor with FAD. By using this new electrode with catalytic function, a bioelectrocatalysts can be engineered; namely, oxidoreductase (e.g., lactate dehydrogenase) and FAD can function for biotransformation without an electron mediator and second oxidoreductase for cofactors recycling.

Key words: Electrochemical oxidoreduction of $FADH_2$ and FAD, graphite-Cu(II) electrode, succinate oxidation, xylitol oxidation, $FADH_2$ fuel cell

All biochemical reactions catalyzed by the oxidoreductases are cofactor dependent [17]. The reducing or oxidizing equivalent required for the biochemical reaction is either supplied or taken up by the cofactors. The most commonly needed cofactors are $NADH/NAD^+$, $NADPH/NADP^+$, $FADH_2$/FAD, ATP/ADP, and PQQ [3, 5, 10, 23]. For example, the reduction of fumarate to succinate is coupled to oxidation of NADH to NAD^+, and the oxidation of succinate to fumarate is coupled to reduction of FAD to $FADH_2$. Since these cofactors are quite expensive, an effective cofactor regeneration system is required to design a cost-effective process. In laboratory research and in industry, three applied approaches can mainly be found to solve this problem. If working with the isolated enzymes, either a second enzyme can be used or the cofactor can be regenerated by application of a second substrate, in which the second substrate is absolutely required as the electron donor for the regeneration of the cofactor. In the case of NADH, the best approach to produce the reducing equivalent is to use formate dehydrogenase that oxidizes formate to CO_2, coupled to reduction of NAD^+ to NADH. Another approach, for example, is an application of whole cells with glucose as a C-source. Through this approach, the multicatalyst-system of the whole cell itself is used for the regeneration. Electrochemical regeneration methods are also known, however, they are not enough to be applied to the bioreactor in the laboratory or industrial process [16]. The electrochemical regeneration method of cofactors is dependent on the artificial electron mediators such as neutral red [15], methyl viologen [14], phenazine methosulfate [13], and benzoquinone [1], and redox potential has to be lower or higher than that of cofactors for the reduction or oxidation reaction, respectively. In the electrochemical bioreactor using the soluble electron mediator, cofactor, and oxidoreductase, the electron mediator electrochemically reduced is oxidized in couple with reduction of cofactor, and the reduced cofactor is then re-oxidized in couple with reduction of substrate. In this serial electron transfer pathway, the efficiency for electron transfer from electrode to substrate has to be proportional to the contact frequency between electrode and electron mediator, electron mediator and cofactor, and cofactor and enzyme-substrate complex. However, it is possible that the electron mediator may interfere with the right contact between cofactor and enzyme-substrate complex, or enzyme-substrate complex may interfere with the right contact between electron mediator and electrode. In order to solve these problems, electron mediator has to be eliminated from the electrochemical bioreactor. In the bioreactor with a catalytic electrode

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instead of electron mediator, the cofactor has to selectively contact with the catalytic electrode or the enzyme-substrate complex, because the enzyme-substrate complex can selectively react with one of the oxidized or reduced cofactors and the cofactor can selectively be oxidized or reduced on the electrode surface [18].

FAD acts as a cofactor for fatty acyl-CoA dehydrogenase in the β-oxidation of lipid [8], hydroxylase in the bacterial oxidation pathway of aromatic compounds [4, 6, 20, 24], alcohol dehydrogenase in polyethylene glycol degradation [7], and alkane oxidase in nitropropane degradation [19]. FAD oxidized in couple with reduction of metabolic intermediates can be regenerated by coupling to oxidation of NADH to NAD^+ in bacterial metabolism, however, the FAD or $FADH_2$ cannot be regenerated without another reducing power and the second oxidoreductase *in vitro*. In the present research, we developed a catalytic electrode for the regeneration of $FADH_2$ and FAD, and tested the function of the electrode by using cyclovoltammetry, biofuel cell, and bioreactor system.

MATERIALS AND METHODS

Crude Enzymes

The crude enzymes for oxidation of succinate to fumarate and xylitol to xylose were isolated from *Escherichia coli* K12 and *Saccharomyces cerevisiae*, respectively. *S. cerevisiae* has a catalytic function to reduce xylose to xylitol, coupled to oxidation of NADH produced from the oxidative metabolism of glucose; however, it is not known to have the enzyme to oxidize xylitol to xylose. *E. coli* K12 and *S. cerevisiae* were cultivated in LB broth and YM broth at 35°C and 30°C for 20 h, respectively. The cultivated cells were harvested by centrifugation at 8,000 ×*g* and 4°C for 30 min and then washed twice with 50 mM Tris-HCl buffer (pH 7.5). The washed cells of *E. coli* and *S. cerevisiae* were disrupted by ultrasonication at 4°C and 400 watt for 20 min and French Press at 20,000 PSI in the cold chamber, respectively. The cell-free extract was prepared from disrupted bacterial cell by centrifugation at 15,000 ×*g* and 4°C for 40 min. The protein concentration was determined with Bradford reagent according to the user's manual.

Electrode Composition

A graphite-Cu(II) electrode was composed of a mixture of 60% (w/w) fine graphite powder (mean particle size of 1-2 μm, Sigma-Aldrich, St. Louis, Missouri, U.S.A.), 37% (w/w) inorganic binder (white clay mainly composed of Kaolin whose mean particle size was 1-2 μm), and 3.0% (w/w) copper ion. A graphite-Fe(III) electrode was composed of a mixture of 60% (w/w) fine graphite powder, 37% (w/w) inorganic binder, and 3.0% (w/w) ferric ion. Appropriate amount of distilled water was added to the mixture for making a graphite mixture paste. The paste was configured to a square-shaped plate (20 cm×20 cm×1 cm thickness) by pressing at 44 kg/cm^2, drying on air for two weeks at room temperature, and solidified by baking at 1,200°C for 12 h under anaerobic condition, using an electric Kiln (Red Corona Model 50L, U.S.A.).

Porcelain Membrane

A porcelain membrane contained 100% (w/w) white clay powder mainly composed of Kaolin whose mean particle size was 1-2 μm. Appropriate amount of distilled water was added to the white clay powder to make a clay paste. The paste was configured to a square-shaped plate (20 cm×20 cm×5 mm thickness) by pressing at 44 kg/cm^2, drying on air for two weeks at room temperature, and solidified by baking at 1,200°C for 12 h using an electric Kiln (Red Corona Model 50L, U.S.A.). After baking, the porcelain membrane was confirmed to absorb water, but not to leak water through the micropore.

Cyclic Voltammetry

The cyclic voltammograms were obtained using the transformed graphite-Cu(II) into rod type (diameter 5 mm, length 4 cm) as a working electrode, platinum wire as a counter electrode, and Ag/AgCl as a reference electrode in 50 mM Tris-HCl buffer (pH 7.5). Cyclic voltammetry was performed using a cyclic voltammetric potentiostat (model CV50W, BAS, U.S.A.) linked to an IBM personal computer

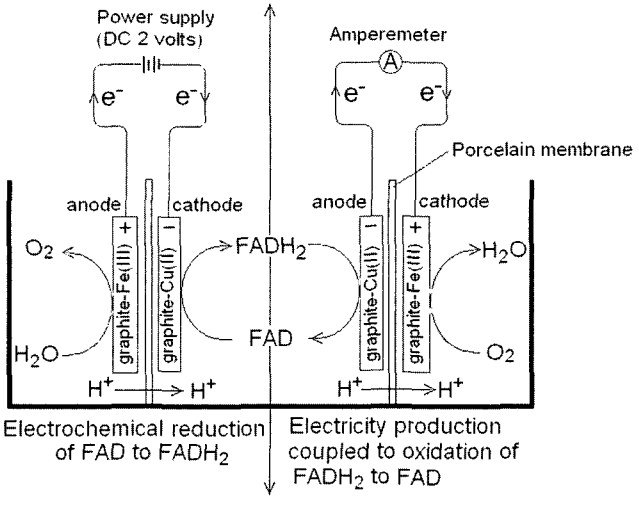

Fig. 1. A schematic description of an electrochemical system for the reduction of FAD to $FADH_2$ (left side) and oxidation of $FADH_2$ to FAD coupled to electricity production (right side).
We drew the integrated system for easy showing, but the two systems were separated. The $FADH_2$ electrochemically reduced in the system at left side was spontaneously re-oxidized in the system at right side coupled to electricity production by the catalytic function of graphite-Cu(II) electrode without electron mediator. In this system, the graphite-Cu(II) anode and graphite-Cu(II) cathode act as an oxidizing agent for $FADH_2$ to FAD and reducing power for FAD reduction to $FADH_2$, respectively.

data acquisition system. Prior to use, the electrodes were cleaned with an ultrasonic cleaner. The scanning rate was 50 mVs^{-1} over the range of +2.0 volt to -2.0 volt. To observe the reaction between the graphite-Cu(II) electrode and FAD, 100 μM FAD was added to the reactor after 2 scanning cycles.

Biofuel Cell with $FADH_2$ as Fuel

As shown in Fig. 1, the electrochemically reduced $FADH_2$ was used as a fuel and the two-compartment electrochemical reactor was used as a biofuel cell. For production of electricity from $FADH_2$ in the biofuel cell, a graphite-Cu(II) electrode, which was capable of reversible chemical oxidoreduction of $FADH_2$ and FAD, was used as an anode, and a graphite-Fe(III) electrode with high affinity for O_2 was used as a cathode. Electricity produced from the biofuel cell was measured by an amperemeter, which was close-circuited between anode and cathode without external resistance.

Electrochemical Reactor

We designed an electrochemical reactor as a two-compartment system whose cathode compartment was separated from the anode compartment by a porcelain membrane (5 mm thickness) and the working volume of each compartment was adjusted to 25 ml: The graphite-Cu(II) electrode functions as a catalyst for the oxidation-reduction of $FADH_2$ and FAD without the electron mediators, and the graphite-Fe(III) electrode functions as a catalyst for the oxidation-reduction of H_2O/O_2. As shown in Fig. 2, the enzymatic oxidation of succinate to fumarate or xylitol to xylose coupled to the reduction of FAD to $FADH_2$, which is electrochemically re-oxidized by catalysis of the graphite-Cu(II) cathode. In the above, 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM succinate or 10 mM xylitol and 1 mM FAD were used as a basal reaction mixture (catholyte), and 200 mM potassium phosphate buffer (pH 7.0) was used as an anolyte. Before starting the reaction, 2 volt of DC electricity were supplied and N_2 was sparged into the reaction mixture for 20 min to remove the interference of O_2. The reaction was started by addition of the crude enzymes (final concentration was adjusted to 50 mg/ml on the basis of protein).

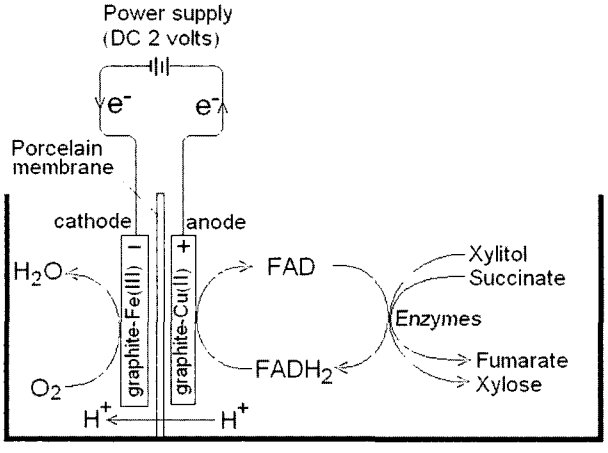

Fig. 2. A schematic description of an electrochemical system for the electrochemical oxidation of $FADH_2$ to FAD coupled to oxidation of succinate to fumarate and xylitol to xylose by the catalytic function of graphite-Cu(II) electrode without electron mediator.

The enzymes for the oxidation of succinate and xylitol were isolated from *E. coli* and *S. cerevisiae*, respectively.

Analysis

Succinate, fumarate, xylitol, and xylose were analyzed by using HPLC (Waters model, Milford, U.S.A.) equipped with a RI detector and Aminex HPX-87H ion-exchange column (Bio-Rad, Burlington, U.S.A.) [9]. Reaction mixture was centrifuged at 20,000 ×*g* for 30 min and filtered through

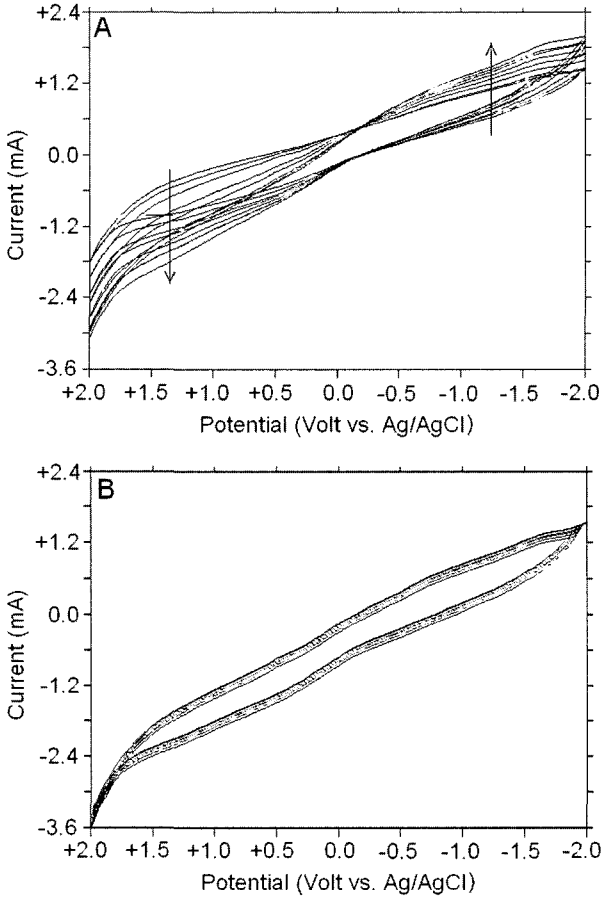

Fig. 3. Cyclic voltammograms measured with a graphite-Cu(II) electrode during successive cycles, following the introduction of the electrode into Tris-HCl buffer (50 mM, pH 7.5) with 100 μM FAD (A) or without FAD (B).

The scan rates were 50 mV s^{-1}, the reference electrode was Ag/AgCl, and the counter electrode was platinum wire. The downward arrow mark in Figure A indicates that $FADH_2$ may be oxidized to FAD, whereas the upward arrow mark indicates that FAD may be reduced to $FADH_2$.

membrane filter (pore sized, 0.22 μM), which was used for analysis. The concentration was calculated using a standard calibration curve that was prepared previously. The FAD was spectrophotometrically analyzed under scanning mode.

RESULTS AND DISCUSSION

The electrochemical regeneration of cofactors has been shown to be dependent on an electron mediator [1, 2, 12]. Most of the electron mediators developed have mainly been applied to the system for the regeneration of NAD^+ and NADH [21]. On the other hand, FAD has been studied relatively less than NAD^+ as the useful cofactor for the industrial oxidoreductase. However, FAD is absolutely required for some specific oxidoreduction reactions such as the oxygenation of aromatic compounds and alcohol dehydrogenation. We designed a new electrode with catalytic activity for oxidoreduction of FAD and tested the function by cyclic voltammetry. As shown in Fig. 3, the current in the cyclic voltammogram for the graphite-Cu(II) electrode was increased by the addition of FAD, as the arrow marks indicate, showing that the graphite-Cu(II) electrode can donate electron to FAD (upward arrow mark) or accept from $FADH_2$ (downward arrow mark). As shown in Fig. 1, on the basis of this result, we designed the electrochemical system capable of examining the electrochemical reduction and oxidation of FAD and $FADH_2$. In the electrochemical system with the graphite-Cu(II) electrode for the oxidoreduction of $FADH_2$ and FAD, we achieved the electrochemical reduction of FAD and re-oxidation of $FADH_2$ without electron mediator. During the course of the experiment, the reaction mixture was periodically sampled and spectrophotometrically measured. As seen in Fig. 4, the specific spectrum of FAD was proportionally decreased to the reaction time. However, the spectrum of $FADH_2$ could not be measured, because the specific spectrum can not be detected in the wavelength from the 900 nm to 300 nm range. The result strongly suggests the possibility that the FAD was electrochemically reduced to $FADH_2$, however, we cannot exclude the possibility that the FAD might have electrochemically been degraded or damaged on the surface of the graphite-Cu(II) electrode. Therefore to confirm the electrochemical re-oxidation of $FADH_2$, we designed a biofuel cell using $FADH_2$ as the fuel, which was electrochemically reduced, measured the electric current produced in coupling with oxidation of $FADH_2$ to

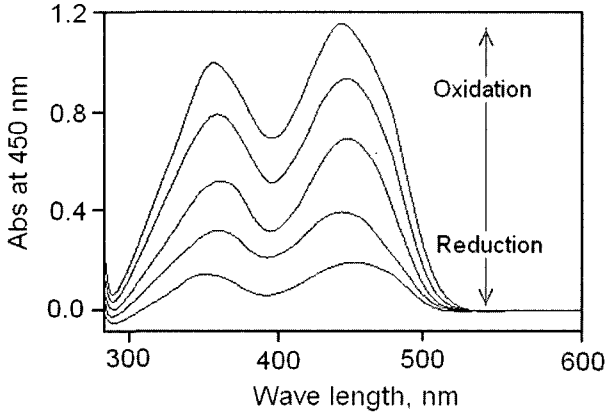

Fig. 4. Electrochemical reduction of FAD to $FADH_2$ by the graphite-Cu(II) cathode and graphite-Fe(III) electrode used as an anode.

One mM FAD solution in Tris-HCl buffer (50 mM, pH 7.5) was catholyte, and 200 mM KH_2PO_4 solution in DDW was anolyte. Absorbance of FAD at 450 nm was continuously decreased in proportion to the reaction time, measured at interval of 20 min. No specific absorbance of $FADH_2$ is in the wavelength range from 300 to 900 nm. The continuous decrease of peak height of the FAD spectrum means that FAD is being reduced to $FADH_2$.

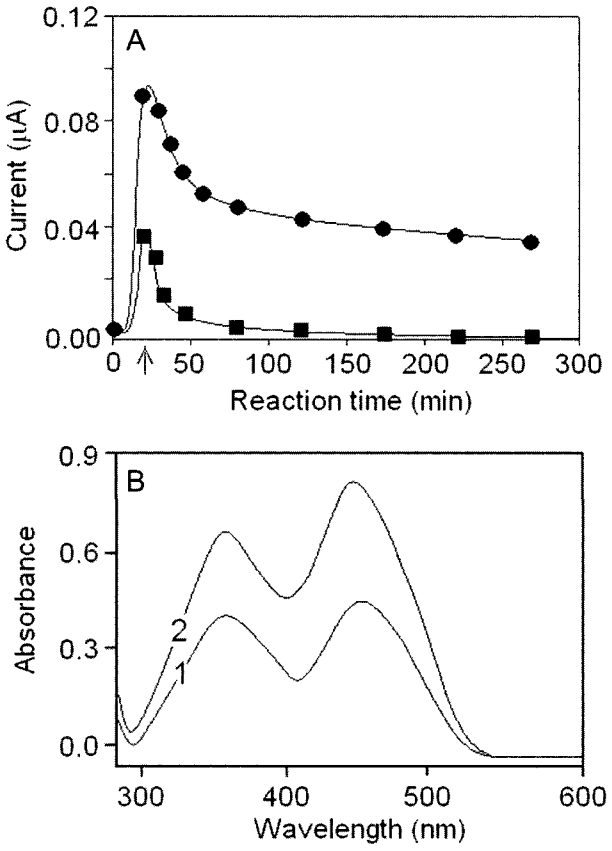

Fig. 5. Electricity production (A) coupled to spontaneous oxidation of $FADH_2$ to FAD (B) in a biofuel cell system with graphite-Cu(II) anode and graphite-Fe(III) cathode, but without catalytic enzyme.

Current was produced in the biofuel cell system with $FADH_2$ (●) but not without $FADH_2$ (■), and the specific peak height of FAD spectrum after reaction (2) was increased 2 times of that before the reaction (1). The $FADH_2$ electrochemically reduced, as shown in Figure 1, was used as the fuel. The current production was automatically measured with the data acquisition system connected to an IBM computer. The arrow mark in Figure A indicates the time when the circuit between anode and cathode was closed.

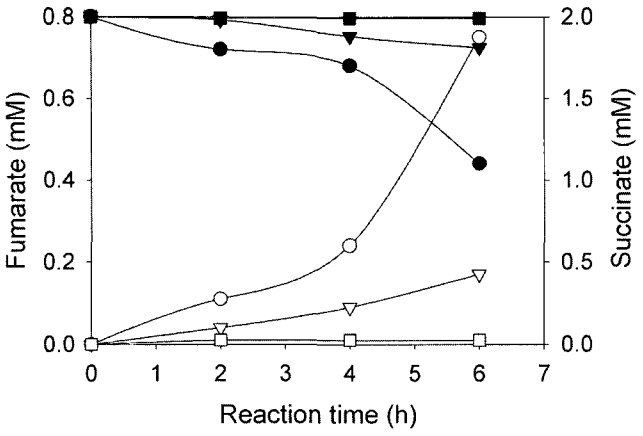

Fig. 6. Electrochemical oxidation of succinate (closed symbols) to fumarate (open symbols) in the electrochemical system with graphite-Cu(II) anode and graphite-Fe(III) cathode under the reaction condition with both FAD and electric energy (○, ●), with FAD but without electric energy (▽, ▼), and without FAD but with electric energy (□, ■).
The electric volt between anode and cathode was adjusted to DC 2.0 volt, and the initial concentration of succinate and FAD was adjusted to 2 mM and 1 mM, respectively. The crude enzyme for succinate oxidation was isolated from *E. coli* K12.

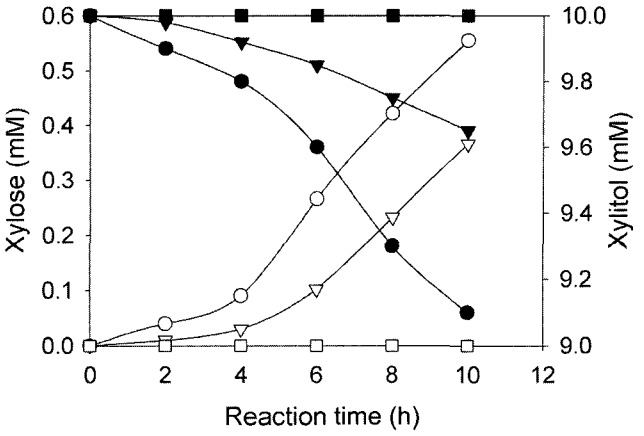

Fig. 7. Electrochemical oxidation of xylitol (closed symbols) to xylose (open symbols) in the electrochemical system with graphite-Cu(II) anode and graphite-Fe(III) cathode under the reaction condition with both FAD and electric energy (○, ●), with FAD but without electric energy (▽, ▼), and without FAD but with electric energy (□, ■).
The electric volt between anode and cathode was adjusted to DC 2.0 volt, and the initial concentration of xylitol and FAD was adjusted to 10 mM and 1 mM, respectively. The crude enzyme for xylitol oxidation was isolated from *S. cerevisiae*.

FAD, and analyzed the spectrum of the reactant before and after reactions, as shown in Fig. 5. The regeneration of the FAD spectrum from the reactant indicates that the $FADH_2$ was spontaneously oxidized to FAD coupled to the electricity production. The $FADH_2$ electrochemically reduced was electrochemically re-oxidized by the same electrode, however, we could not be certain whether the FAD functioned as the cofactor for the biochemical oxidation. In order to examine the biochemical function of electrochemically oxidized FAD, succinate and xylitol were used as substrates, and cell extracts of *E. coli* and *S. cerevisiae* were used as the biocatalyst for oxidation reaction coupled to reduction of FAD to $FADH_2$ (as shown in Fig. 2) [11, 22]. As shown in Fig. 6, fumarate was not produced from succinate without FAD, but was produced when coupled to biochemical reduction of FAD, and more fumarate was produced with the electrochemically regenerated FAD. This is a clear evidence that the $FADH_2$ biochemically reduced, when coupled to oxidation of succinate to fumarate, can be electrochemically regenerated by a graphite-Cu(II) electrode, by which the ratio of FAD/$FADH_2$ in the reactant may be constant or at least higher than 1.0. When xylitol was applied to the bioreactor, we obtained the same results as shown in Fig. 7. The result suggested a new enzyme capable of catalyzing the re-oxidation of xylitol to xylose when coupled to reduction of FAD to $FADH_2$.

The electrochemical regeneration of cofactors by using electrode is easier and simpler than the system with electron mediator, because the electron mediator has to be continuously added to the reactor and constitutes a contaminant for the bioreactor after the reaction. The cofactors can possibly be immobilized to the functional electrode by peptide bond between the carboxyl of electrode and the amine of cofactor, such as NAD^+ and FAD. We are in a process to examine the immobilization of the cofactor to the graphite-Cu(II) electrode, and to design a continuous flow reaction without product purification process by using membrane incapable of passing enzymes through.

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