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Catalytic Oxidoreduction of Pyruvate/Lactate and Acetaldehyde/Ethanol Coupled to Electrochemical Oxidoreduction of NAD^+/NADH

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Abstract We deviced a new graphite-Mn(II) electrode and found that the modified electrode with Mn(II) can catalyze NADH oxidation and NAD^+ reduction coupled to electricity production and consumption as oxidizing agent and reducing power, respectively. In fuel cell with graphite-Mn(II) anode and graphite-Fe(III) cathode, the electricity of 1.5 coulomb (A x s) was produced from NADH which was electrochemically reduced by the graphite-Mn(II) electrode. When the initial concentrations of pyruvate and acetaldehyde were adjusted to 40 mM and 200 mM, respectively, about 25 mM lactate and 35 mM ethanol were produced from 40 mM pyruvate and 200 mM acetaldehyde, respectively, by catalysis of ADH and LDH in the electrochemical reactor with NAD^+ as cofactor and electricity as reducing power. By using this new electrode with catalytic function, the bioelectrocatalysts are engineered; namely, oxidoreductase (e.g., lactate dehydrogenase) and NAD^+ can function for biotransformation without electron mediator and second oxidoreductase for NAD^+/NADH recycling.

Key words: Electrochemical oxidoreduction, lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH), graphite-Mn(II) electrode, NADH fuel cell

One major limitation to the utilization of oxidoreductases in biochemical and chemical synthesis [15, 21] or in biochemical detection (i.e., biosensors) is the lack of a simple regeneration or recycling system for the electron transferring cofactors (NAD, Quinone, FAD, etc). All oxidoreductases are cofactor-dependent and the reducing or oxidizing equivalent is either supplied or taken by the cofactor. The most commonly occurring cofactors are NADH/NAD^+, NADPH/$NADP^+$, $FADH_2$/FAD, ATP/ADP, and PQQ. [3, 5, 9, 12, 27]. In industry, three applied approaches can mainly be found to solve this problem. When working with isolated enzymes, a second enzyme can be used. In the case of NADH, the best approach is to use formate dehydrogenase that utilizes formate and produces CO_2 or the cofactor can be regenerated by applying a second substrate [10, 11]. Another approach is the application of whole cells, with glucose as a C-source, for example; through this approach, the multicatalyst-system of the whole cell itself is used for the regeneration. There are also electrochemical cofactor regeneration methods known which have not yet made it to an industrial process, but have been tested in laboratory scale [1, 2, 8, 16]. Park and Zeikus [17-20] reported that neutral red was electrochemically oxidized and reduced on electrode surface, and that neutral red would undergo reversible chemical oxidoreduction with NAD^+ (i.e., electrochemically recycled NAD^+). The redox potentials of neutral red and NAD^+ are -0.325 volt and -0.32 volt, respectively, which are thermodynamically ideal as an oxidation-reduction couple. However, the most electron mediators, including neutral red, are soluble, a property that is disadvantageous to apply to a bioelectrochemical reactor, because the soluble electron mediators, cofactors, and soluble enzymes make the purification of the products difficult.

Since electron mediators and cofactors are quite expensive, an effective immobilization method of electron mediator, cofactor, and enzyme is absolutely required to design a cost-effictive process. It is possible that NAD^+ and enzymes can be immobilized to a carbon electrode by covalent bond; however, electron mediators are difficult, because functional residues such as amine, hydroxide, or carboxy for covalent bond are present in cofactors and enzyme molecule, but are not in most electron mediators [4, 7, 14]. Most transient metal ions are insoluble in water at neutral pH and can easily be immobilized to inorganic particles such as kaolin or clay by their opposite electrical charges [13, 23, 24, 25].

We developed a technique to immobilize Mn(II) and Fe(III) to graphite electrode by mixing metal ions with

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paste of kaolin, clay, and graphite powder and then baking the mixture at 1,000-1,200°C. We observed that the graphite-Mn(IV) electrode functioned to reciprocally catalyze electrochemical oxidoreduction of NAD^+/NADH. By using the graphite-Mn(IV) electrode in the electrochemical reactor, we also showed that: (1) the electricity could be produced from the biofuel cell with NADH which was electrochemically reduced as the sole electron donor and (2) the oxidoreductase could catalyze pyruvate and acetaldehyde reduction to lactate and ethanol, respectively, with NADH which was electrochemically reduced as the sole electron donor. In the present study, we selected lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) as model oxidoreductases capable of using NAD^+ as the electron carrier. Furthermore, we used crude LDH and ADH isolated from *Weissella kimchii* as well as purified preparation commercially obtained and compared them for NADH-dependent reduction reaction for industrial application.

MATERIALS AND METHODS

Enzymes

The cell-free extract of *W. kimchii*, which was isolated from kimchi and identified by 16s-rDNA sequencing, was used as a crude lactate dehydrogenase (LDH, specific activity: 6.49 mM mg protein^{-1}min^{-1}) and alcohol dehydrogenase (ADH, specific activity: 4.74 mM mg protein^{-1}min^{-1}), and other LDH (8.45 mM mg protein^{-1}min^{-1}) and ADH (5.23 mM mg protein^{-1}min^{-1}) preparations were purchased from Sigma (St. Louis, MO, U.S.A.). The specific activity of commercial enzymes from Sigma was experimentally measured by the same method as used for assay of crude enzymes isolated from *W. kimchii*. The bacterial cells of *W. kimchii* were cultivated in MRS broth at 30°C for 20 h, harvested by centrifugation at 4°C and 8,000 ×*g* for 30 min, and then washed twice with 50 mM Tris-Cl buffer (pH 7.5). The washed cells were disrupted by 400 watt of ultrasonication at 4°C for 20 min and the cell-free extract was prepared by centrifugation of the disrupted bacterial cell at 4°C and 15,000 ×*g* for 40 min.

Activity Staining of LDH and ADH

Nondenaturing gel electrophoresis was carried out for separation of the enzymes. Cell-free extract was resolved by nondenaturing polyacrylamide gel electrophoresis and the gel was soaked in 50mM Tris-HCl (pH 7.5) containing substrate (10 mg/ml of ethanol or lactate), NAD^+ (2 mg/ml), phenazine methosulfate (0.5 mg/ml), and methyl thiazolyl tetrazolium (1 mg/ml). Then, it was incubated at 25°C for 30 min. The NAD^+ is reduced to NADH by coupling with oxidation of the lactate or ethanol by the enzymes electrophoresed on the acrylamide gel, and phenazine methosulfate and methyl thiazolyl tetrazolium are converted to water-insoluble complex (formazan) coupled to oxidation of NADH to NAD^+. The water-insoluble formazan is fixed in acrylamide gel and appeared as a band with dark blue color, as shown in Fig. 1 [26].

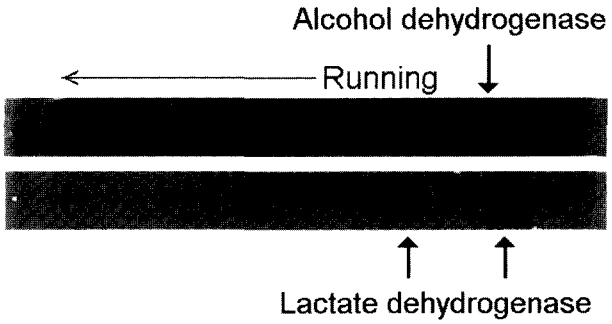

Fig. 1. Activity staining of lactate dehydrogenase and alcohol dehydrogenase separated from cell-free extract of *Weissella kimchii* by polyacrylamide native-gel electrophoresis.

Electrode Composition

A graphite-Fe(III) cathode was made from the mixture of 60% (w/w) fine graphite powder (mean particle size of 1-2 μm, Sigma-Aldrich, St. Louis, MO, 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) ferric ion. A graphite-Mn(IV) anode was made from the mixture of 60% (w/w) fine graphite powder, 37% (w/w) inorganic binder, and 3.0% (w/w) manganese ion. A normal graphite-cathode was made from the mixture of 60% (w/w) fine graphite powder and 40% (w/w) inorganic binder. The gaps among particles can be micro-pore with diameter less than 1 μm. Appropriate amount of distilled water was added to the mixture to make a graphite mixture paste, and the paste was configured to 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 solidifying by baking at 1,200°C for 12 h under anaerobic condition in an electric Kiln (Red Corona Model 50L, U.S.A.).

Porcelain Membrane

A porcelain membrane was made from the mixture of 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, and the paste was configured to 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 solidifying by baking at 1,200°C for 12 h in an electric Kiln (Red Corona Model 50L, U.S.A.). After baking, the porcelain membrane was confirmed to absorb water and not to leak water through the micro-pore.

Electrochemical Reactor

An electrochemical reactor was designed to be a two-compartment system whose cathode compartment was separated from the anode compartment by a porcelain membrane (5 mm thickness) and the working (reactant) volume of each compartment was adjusted to 25 ml. Fifty mM of headspace volume was filled with N_2, which was required for maintenance of gas volume. The cathode compartment was designed to seal with a butyl rubber stopper, but the anode compartment was not. The graphite-Mn(IV) electrode functions as both cathode and catalyst for reduction of NAD^+ to NADH, but the graphite-Fe(III) electrode functions as both anode and catalyst for oxidation of H_2O. As shown in Fig. 2, the electrochemical NAD^+ reduction to NADH by catalysis of the graphite-Mn(IV) cathode and enzymological reduction of pyruvate to lactate or reduction of acetaldehyde to ethanol coupled to oxidation of NADH which was electrochemically reduced were tested using a two-compartment electrochemical reactor. Fifty mM Tris-Cl buffer (pH 7.5) containing 40 mM pyruvate or 200 mM acetaldehyde and 1 mM NAD^+ was 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 was applied and N_2 was sparged into the reaction mixture to remove O_2 and reduce the reaction mixture for 20 min. The reaction was started by the addition of substrates to the reaction mixture in the cathode compartment. The enzymes used were 0.25 mg/reactor of commercial oxidoreductases and 0.3 mg/reactor of crude enzyme preparation. The reactor was completely sealed by butyl rubber stopper to prevent evaporation of acetaldehyde and ethanol as shown in Fig. 2B, and the headspace volume of the reactor was designed to be twice the reactant volume (working volume) for easy control of gas phase pressure.

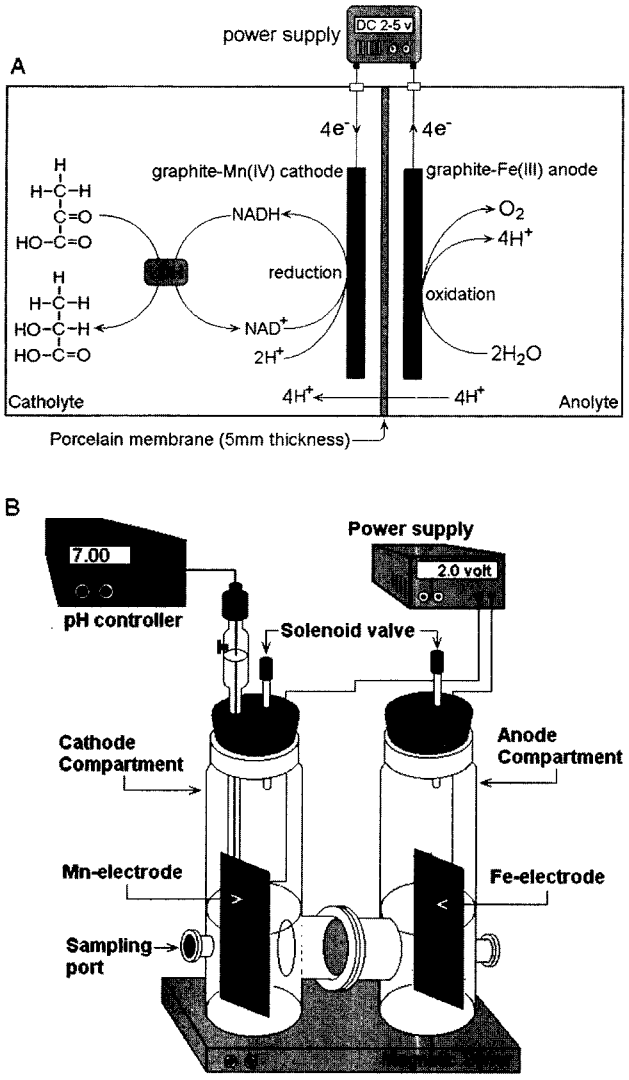

Fig. 2. Schematic (A) and real structure (B) of the bioelectrochemical system. Mechanism for NAD^+ reduction to NADH and pyruvate reduction to lactate coupled to electricity consumption by graphite-Mn(IV) cathode, which acts as a catalyst for electrochemical reduction of NAD^+ to NADH.
The electricity acts as a reducing power and NAD^+ acts like an electron mediator.

Biofuel Cell with NADH as Fuel

The electrochemically reduced NADH was used as a fuel, and the two-compartment electrochemical reactor (Fig. 2) was used as a biofuel cell [11]. For production of electricity from NADH in the biofuel cell, the graphite-Mn(IV) electrode, capable of reversible chemical NAD^+ oxidoreduction, was used as an anode, and the graphite-Fe(III) electrode with high affinity for O_2 was used as a cathode. Electricity produced from the biofuel cell was measured by ampere meter, which was close-circuited connected between the anode and cathode without external resistant. Initial concentration of NADH was 2 mM in 50 mM Tris-HCl buffer (pH 7.5), and the reaction was initiated by connecting to the ampere meter. The electricity production and consumption were converted to coulomb for calculation of total electricity used during operation of the reactor.

Analysis

Pyruvate, lactate, acetaldehyde, and ethanol 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.). The reaction mixture was centrifuged at 20,000 $\times g$ for 30 min and filtered through a membrane filter (pore sized, 0.22 mM), and the filtrate was used for analysis. The concentration was calculated using a standard calibration curve that was previously prepared. The NADH was spectrophotometrically analyzed under scanning mode.

RESULTS AND DISCUSSION

The NAD^+ is biochemically reduced by catalysis of oxidoreductase coupled to oxidation of substrates both *in vivo* [22] and *in vitro*, however, it is not electrochemically reduced to NADH unless an electron mediator such as neutral red is present [16, 17]. Electron mediators are not catalysts, but some of them can mediate electron transfer from electrode to cofactors [17, 23] or from cofactors to electrode [18, 19]. Hoogstraten *et al.* [6] reported that the Mn(II) ion is bound to a specific site within the framework of nucleotides (e.g., NAD^+ and ribonucleic acid) and activates reactions such as RNA splicing and aminoacyl-tRNA synthesis. Therefore, we proposed a possibility that the electrode modified with Mn(II) (graphite-Mn(II) electrode) might catalyze oxidoreduction of NAD^+/NADH coupled to electricity production and consumption as an oxidizing and reducing power, respectively, but without depending on enzyme catalysis or electron mediator. In the present study, we applied the graphite-Mn(II) electrode to electrochemical reactor and tested whether oxidoreduction of NAD^+/NADH could be catalyzed by the electrode. As shown in Fig. 3 and Fig. 4(A), the NAD^+ was electrochemically reduced to NADH which was coupled to electricity consumption by catalysis of graphite-Mn(II) cathode without electron mediator and enzyme catalysis. Figure 4(B) shows that the electricity produced was coupled to oxidation of NADH electrochemically reduced by the graphite-Mn(II) cathode during the process to obtain the results shown in Fig. 4(A), providing strong evidence that the graphite-Mn(IV) electrode can function as a catalyst for both reduction of NAD^+ and oxidation of NADH. The oxidoreduction reaction of NAD^+/NADH on the electrode surface without electron mediator or enzyme catalyst could be a tool to construction

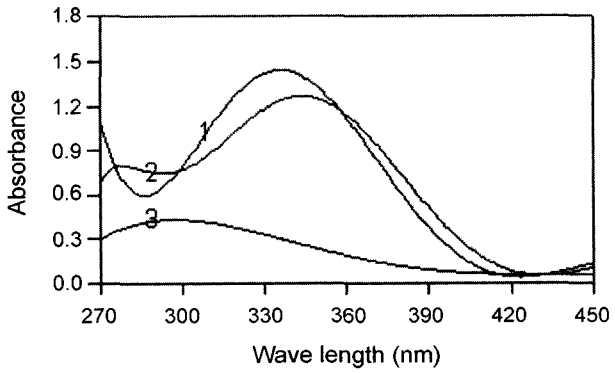

Fig. 3. Electrochemical reduction of NAD^+ to NADH using Mn(II)-graphite electrode as the cathode and Fe(III)-graphite as an anode. One mM NAD^+ solution in Tris-HCl buffer (100 mM, pH 7.0) was the catholyte and 200 mM KH_2PO_4 solution in DDW was the anolyte.
1. Spectrum of 0.5 mM NADH; 2. Spectrum of NADH reduced by Mn(II)-graphite electrode; 3. Spectrum of 1 mM NAD^+ that is not reduced by normal graphite felt electrode.

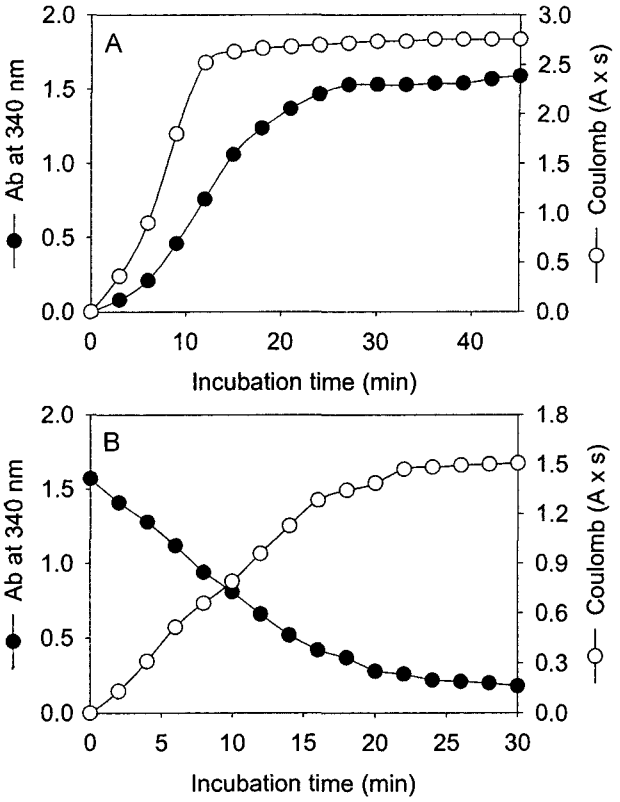

Fig. 4. Electrochemical NAD^+ reduction to NADH (A) coupled to electricity consumption in an electrochemical reactor with graphite-Mn(IV) cathode and graphite-Fe(III) anode, and electrochemical NADH oxidation to NAD^+ (B) coupled to electricity production in a two-compartment biofuel cell with graphite-Mn(IV) anode and graphite-Fe(III) cathode.
Initial concentration of NAD^+ was 2 mM and the NADH electrochemically reduced in test A was used as a fuel for test B.

of a biofuel cell or biosensor, however, the NADH reduced by electrochemical catalysis has yet to be proven to be a suitable cofactor for oxidoreductases such as LDH and ADH. We tested the biochemical function of NADH produced by electrochemical catalysis of the graphite-Mn(II) electrode. Two each of LDHs and ADHs, isolated from *W. kimchii* and commercially obtained, were compared for their cofactor-dependency. As shown in Fig. 5, ethanol was produced from acetaldehyde in a two-compartment electrochemical reactor with NAD^+ as a cofactor, alcohol dehydrogenase and 2 volt DC electricity as reducing power, but was not produced in the electrochemical reactor without electricity. In this test, the amount of ethanol production was not quantitatively balanced with aldehyde consumption, most possibly due to the lower boiling point (21°C) of acetaldehyde than the 78°C of ethanol and 30°C of the reactor, even though the reactor was completely sealed by a butyl rubber stopper for prevention of evaporation of acetaldehyde and ethanol, as shown in Fig. 2B. The headspace volume of the reactor was designed to

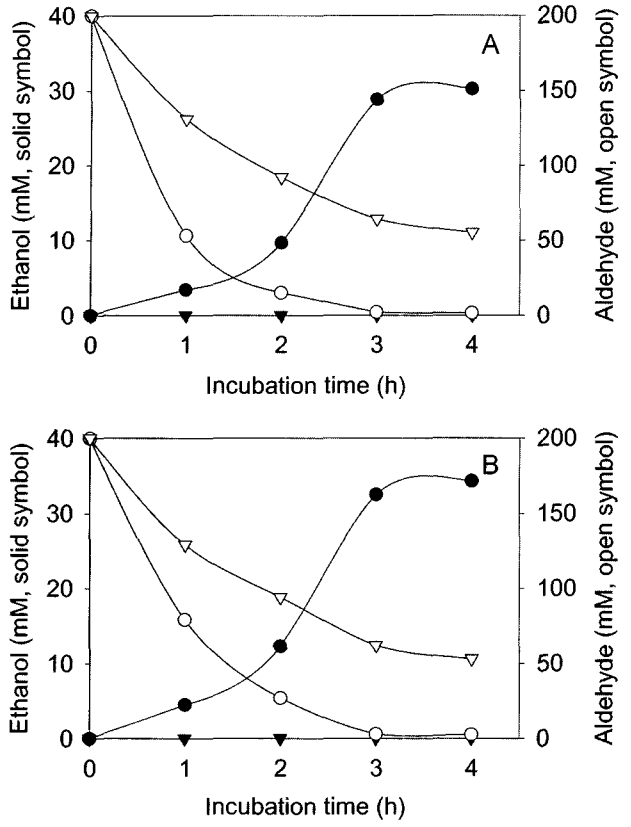

Fig. 5. Acetaldehyde reduction to ethanol by catalysis of alcohol dehydrogenase isolated from *Weissella kimchii* (A) and commercially purchased (B).
The co-enzyme for this reaction is NADH but NAD^+ was used instead. The reaction was performed in the electrochemical reactor with 2 volt electricity (circular symbols) as a reducing power for electrochemical NAD^+ reduction and without electricity (triangular symbols). The graphite-Mn(IV) and graphite-Fe(III) cathode were used as a cathode and anode, respectively.

be twice the reactant volume (working volume) for easy control of gas phase pressure. The acetaldehyde concentration in the headspace of the reactor might be equilibrated with that contained in the reactant, because the acetaldehyde was boiling in both the reactant and headspace inside the reactor, but ethanol was not. Theoretically, about 66% of the acetaldehyde may be located in the headspace and another 34% may be in the reactant. A fraction of 34% contained in the reactant might have been converted to ethanol but that in the headspace might not. Experimentally, about 50% (30-35 mM) of the acetaldehyde contained in the reactant was shown to be converted to ethanol (Fig. 5A and 5B). We tested another nonvolatile compound to prove that the concentration of the product was balanced with consumption of the substrate in the bioelectrochemical reactor with NAD^+ as a cofactor for enzyme-catalytic reduction. Pyruvate was chosen as a desirable model compound for test of an oxidoreduction reaction that is stable and not volatile. As shown in Fig. 6B, about 90% of the pyruvate consumed in the bioelectrochemical reactor with electricity as a reducing power was converted to lactate, but lactate was not produced from the pyruvate in the reactor without electricity. The 10% of the residual pyruvate was thought to be in the form of enzyme-substrate complex, because of product-increasing tendency in the time-course reaction. In comparison of Fig. 5 and Fig. 6, the time-dependent increasing tendency of lactate was different from that of ethanol: The ethanol production stopped after 3 h of reaction but pyruvate was continuously increased even after 4 h of reaction. This is most likely due to instability of acetaldehyde, which can be auto-oxidized to acetate by a reaction with trace oxygen or boiled at 21°C. The results obtained from the test with crude LDH and ADH were similar to those obtained from the test with purified LDH and ADH, thus demonstrating a potentially useful method for electrical recycling of NAD^+ with crude oxidoreductases in a biofuel cell and biotransformation. The purified enzymes are disadvantageous to be applied to a

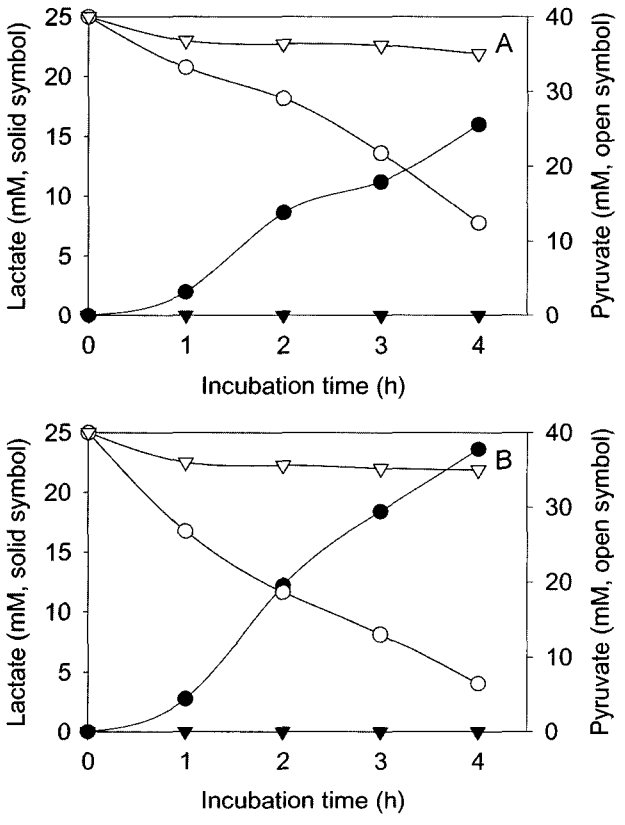

Fig. 6. Pyruvate reduction to lactate by catalysis of lactate dehydrogenase isolated from *Weissella kimchii* (A) and commercially purchased (B).
The co-enzyme for this reaction is NADH but NAD^+ was used instead. The reaction was performed in the electrochemical reactor with 2 volt electricity (circular symbols) as a reducing power for electrochemical NAD^+ reduction and without electricity (triangular symbols). The graphite-Mn(IV) and graphite-Fe(III) cathode were used as a cathode and anode, respectively.

bioelectrochemical reactor, because they are quite expensive and instable. However, the crude enzymes are much more suitable for application to an industrial reactor, because they can be extracted from bacterial cells, in which they are more stable than in frozen or lyophilized state. The instability or the expense of cofactor recycling and enzyme purification has limited the industrial use of oxidoreductases [1]. By the present method, bioelectrocatalysts are engineered: namely, oxidoreductase (e.g., lactate dehydrogenase) and NAD^+ can function for biotransformation without an electron mediator and second oxidoreductase for NAD^+/NADH recycling. Maximally, the lactate and ethanol production can not be more than 1 mM in the reactant containing 1 mM NADH as a reducing power, because the reduction of pyruvate to lactate or acetaldehyde to ethanol is coupled to oxidation of NADH in the biochemical reactor. In our system, however, the NAD^+ can be electrochemically reduced to NADH, which can be re-oxidized by coupling to reduction of pyruvate and acetaldehyde to lactate and ethanol, respectively. The product yield is proportional to the concentration of NADH in the biochemical reactor, but is proportional to the recycling number of NAD^+/NADH in the electrochemical reactor. We are currently in a process to bind both oxidoreductase and NAD^+ onto graphite-Mn(II) electrode and engineer the modified electrode with oxidoreductase and NAD^+ into bioelectrocatalysts. A few new methods for electrical recycling of cofactors are under development [1, 2, 8, 16]. Katz *et al.* [8] developed a novel fuel cell comprised of an anode with immobilized glucose oxidase, FAD, and pyroloquinoline; and a cathode with immobilized cytochrome c. Chen *et al.* [2] developed an enzyme fuel cell, using the modified anode with osmium salts, copolymer, and glucose oxidase, and the modified cathode with osmium salts copolymer and laccase. These new methods could perhaps be applied to further improve our bioelectrocatalyst engineering method.

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